EDITORS
STEFANO BETTATI
ANDREA MOZZARELLI

Chemistry and Biochemistry of Oxygen Therapeutics

From Transfusion to Artificial Blood

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From Transfusion to Artificial Blood

Edited by

ANDREA MOZZARELLI AND STEFANO BETTATI

Department of Biochemistry and Molecular Biology, University of Parma, Parma, Italy

and

National Institute of Biostructures and Biosystems, Rome, Italy

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Contents

List of Contributors

Seetharama A. Acharya, Departments of Medicine and of Physiology and Biophysics, Albert Einstein College of Medicine, Bronx, NY, USA

Kulal Ananda, Departments of Medicine and of Physiology and Biophysics, Albert Einstein College of Medicine, Bronx, NY, USA

Véronique Baudin-Creuza, Inserm U779, CHU Bicêtre, Paris, France

Stefano Bettati, Department of Biochemistry and Molecular Biology, University of Parma, Parma, Italy and National Institute of Biostructures and Biosystems, Rome, Italy

Joseph Bonaventura, The Nicholas School of the Environment Marine Laboratory, Beaufort, NC, USA, and The Bonaventis Research Laboratory, Mayaguez, PR, USA

Carlo Brugnara, Departments of Pathology and Laboratory Medicine, Children's Hospital, Harvard Medical School, Boston, MA, USA

Stefano Bruno, Department of Biochemistry and Molecular Biology, University of Parma, Parma, Italy

Enrico Bucci, University of Maryland Medical School, Department of Biochemistry and Molecular Biology, Baltimore, MD, USA

Paul W. Buehler, Laboratory of Biochemistry and Vascular Biology, Division of Hematology, Center for Biologics Evaluation and Research (CBER), Food and Drug Administration (FDA), MD, USA

Yu-Feng Yvonne Chan, Department of Emergency Medicine, The Mount Sinai Medical Center, New York, NY, USA

Rosa Chianese, Transfusion Medicine and Haematology Service, Hospital of Ivrea (Torino), Italy

Anna Curley, Neonatal Unit, Cambridge University Hospitals NHS Trust, Cambridge, UK

Felice D'Agnillo, Laboratory of Biochemistry and Vascular Biology, Division of Hematology, Center for Biologics Evaluation and Research (CBER), Food and Drug Administration (FDA), MD, USA

Angelo D'Alessandro, Department of Environmental Sciences, Tuscia University, Viterbo, Italy

Robertson Davenport, University of Michigan Medical School, Ann Arbor, MI, USA

Lucia De Franceschi, Department of Medicine, University of Verona, Policlinico GB Rossi, Verona, Italy

Linzy Doherty, David Geffen School of Medicine at UCLA, Ronald Reagan UCLA Medical Center, Los Angeles, CA, USA

Serena Faggiano, MRC National Institute for Medical Research, London, UK

Giuliano Grazzini, Italian National Blood Centre, Istituto Superiore di Sanità, Rome, Italy

John R. Hess, University of Maryland School of Medicine, Baltimore, MD, USA

Chien Ho, Department of Biological Sciences, Carnegie Mellon University, Pittsburgh, PA, USA

Hirohisa Horinouchi, Department of Surgery, School of Medicine, Keio University, Shinjuku, Tokyo, Japan

Marcos Intaglietta, Department of Bioengineering, University of California, San Diego, La Jolla, CA, USA

Jonathan S. Jahr, David Geffen School of Medicine at UCLA, Ronald Reagan UCLA Medical Center, Los Angeles, CA, USA

Hae Won Kim, Department of Molecular Pharmacology, Physiology and Biotechnology, Brown University, Providence, RI, USA

Harvey G. Klein, Department of Transfusion Medicine, Clinical Center, National Institutes of Health, Bethesda, MD, USA

Koichi Kobayashi, Department of Surgery, School of Medicine, Keio University, Shinjuku, Tokyo, Japan

Raymond C. Koehler, Department of Anesthesiology and Critical Care Medicine, The Johns Hopkins University, Baltimore, MD, USA

Sibylle A. Kozek-Langenecker, Department of Anesthesia and Intensive Care, Evangelical Hospital Vienna, Vienna, Austria

Alvin Li, College of Letters and Science, UCLA, Los Angeles, CA, USA

Michael C. Marden, Inserm U779, CHU Bicêtre, Paris, France

Tim J. McMahon, Department of Medicine, Duke University and Durham VA Medical Centers, Durham, NC, USA

Fantao Meng, Departments of Medicine and of Physiology and Biophysics, Albert Einstein College of Medicine, Bronx, NY, USA

Anna Rita Migliaccio, Tisch Cancer Institute, Mount Sinai School of Medicine, New York, NY, USA

Giovanni Migliaccio, Department of Cell Biology and Neurosciences, Istituto Superiore Sanità, Rome, Italy

Hooman Mirzakhani, Department of Anesthesia, Critical Care and Pain Medicine, Massachusetts General Hospital, Harvard Medical School, Boston, MA, USA

Ari Moskowitz, Department of Emergency Medicine, The Mount Sinai Medical Center, New York, NY, USA

Andrea Mozzarelli, Department of Biochemistry and Molecular Biology, University of Parma, Parma, Italy and National Institute of Biostructures and Biosystems, Rome, Italy

Priya Muthukumar, Neonatal Unit, Cambridge University Hospitals NHS Trust, Cambridge, UK

Lena M. Napolitano, Department of Surgery, University of Michigan, Ann Arbor, MI, USA

Kenneth E. Nollet, Department of Blood Transfusion and Transplantation Immunology, Fukushima Medical University, Fukushima, Japan

Ala Nozari, Department of Anesthesia, Critical Care and Pain Medicine, Massachusetts General Hospital, Harvard Medical School, Boston, MA, USA

Hitoshi Ohto, Department of Blood Transfusion and Transplantation Immunology, Fukushima Medical University, Fukushima, Japan

Kenneth W. Olsen, Department of Chemistry, Loyola University, Chicago, IL, USA

Rakesh P. Patel, Department of Pathology and Center for Free Radical Biology, University of Alabama at Birmingham, AL, USA

Luca Ronda, Department of Biochemistry and Molecular Biology, University of Parma, Parma, Italy

Umberto Rossi, European School of Transfusion Medicine (ESTM), Italy

Arezou Sadighi, David Geffen School of Medicine at UCLA, Ronald Reagan UCLA Medical Center, Los Angeles, CA, USA

Martin K. Safo, Department of Medicinal Chemistry, School of Pharmacy and Institute for Structural Biology and Drug Discovery, Virginia Commonwealth University, Richmond, VA, USA

Hiromi Sakai, Waseda Bioscience Research Institute in Singapore, Singapore, Republic of Singapore and Research Institute for Science and Engineering, Waseda University, Shinjuku, Tokyo, Japan

Aneesh B. Singhal, Department of Neurology, Massachusetts General Hospital, Boston, MA, USA

Ester Spagnolli, Department of Anesthesia and Critical Care, Massachusetts General Hospital, Harvard Medical School, Boston, MA, USA

Simon Stanworth, NHSBT Oxford Centre, John Radcliffe Hospital, Oxford, UK

Ryan Stapley, Department of Pathology and Center for Free Radical Biology, University of Alabama at Birmingham, AL, USA

Eugene Tarasov, Department of Chemistry, Loyola University, Chicago, IL, USA

Amy G. Tsai, Department of Bioengineering, University of California, San Diego, La Jolla, CA, USA

Eishun Tsuchida, Research Institute for Science and Engineering, Waseda University, Shinjuku, Tokyo, Japan

Vidheya Venkatesh, Neonatal Unit, Cambridge University Hospitals NHS Trust, Cambridge, UK

Dario A. Vitturi, Department of Pathology and Center for Free Radical Biology, University of Alabama at Birmingham, AL, USA

Richard B. Weiskopf, University of California, San Francisco, CA, USA

Carolyn Whitsett, Tisch Cancer Institute, Mount Sinai School of Medicine, New York, NY, USA

Chisa Yamada, University of Michigan Medical School, Ann Arbor, MI, USA

Warren M. Zapol, Department of Anesthesia and Critical Care, Massachusetts General Hospital, Harvard Medical School, Boston, MA, USA

Lello Zolla, Department of Environmental Sciences, Tuscia University, Viterbo, Italy

Preface

We are oxygen-dependent. Consequently, it is not surprising that researchers have intensively addressed the issue of oxygen homeostasis, and clinicians have developed therapies devoted to keeping the level of oxygen supply in the body over a critical threshold. In the latter case, blood transfusion is the safest and most frequently used procedure. However, very strict rules must be followed for blood collection, conservation and transfusion. Moreover, there is an emerging concern about the relationship between blood aging and adverse effects, leading to "bad" blood. Somewhat surprisingly, blood transfusions have never been challenged via controlled clinical trials. Only recently have extensive clinical trials been undertaken, and these are presently ongoing. Moreover, there is a general alarm at the World Health Organization (http://www.who.int/bloodsafety/en/ and documents here reported) about the constant supply of safe blood, because the number of donors is not increasing at the same rate as population aging, which implies an expanding requirement of blood. The blood shortage has begun to hit many Western countries, calling health services and hospitals to a very careful and wiser use of blood and blood products, as well as to a strong effort in the recruitment of voluntary blood donors, especially in the younger generations. In Eastern Europe and even more dramatically in Third World countries, the impairment of blood requirements and supply often makes blood transfusion outside clinical possibility, causing thousands of deaths, especially in women during hemorrhagic events accompanying childbirth and in children due to malaria. These unmet clinical needs, as well as several other pathological situations, including ischemic patients, patients that are immunoreactive to all blood types, hemorrhagic events in combat areas, natural disasters and car accidents in remote areas, have prompted and are still prompting many investigations for the development of novel therapeutic agents aimed at delivering oxygen to patients. Universal red cells and red cells generated from stem cells are just some of the strategies that have been undertaken to try and develop safe alternatives to donor blood. To this end, one of the most contradictory fields is that of hemoglobin-based oxygen carriers (HBOCs), which exploit genetically and/or chemically modified hemoglobin (polymerized, cross-linked, polyethylene glycol-conjugated, vesicle-encapsulated and capsule-adsorbed). Unfortunately, the products developed so far have not proved safer or more effective than blood transfusions. The negative outcome of many years of investigations, partially due to the rush for FDA approval, calls for a deeper understanding of the chemistry, biochemistry and physiology underlying the mechanism of action and toxicity of oxygen therapeutics. Furthermore, there is an increasing awareness that a fundamental switch is required in the design of new HBOCs. HBOCs should not be regarded as "all-purpose competitors"

of donor blood, but as valuable alternatives tailored to specific clinical conditions or situations where safe (and correctly stored) blood is not immediately available.

Some of the ideas and issues reported in this book emerged during the XII International Symposium on Blood Substitutes that we organized in Parma in 2009. However, the scope of the present book is intended to be broader, as we feel that only a multidisciplinary approach can lead to a safe alternative to allogeneic blood and to a better use of transfusion and all other therapies in treating hypo-oxygenation pathologies. We feel that chemistry and biochemistry represent the basis for the understanding of: (i) the physiological events associated with oxygen and nitric oxide homeostasis and their complex interlinked regulations; (ii) the many distinct clinical situations characterized by hypo-oxygenation; and (iii) the different therapeutic actions aimed at providing enough oxygen to tissues for their survival.

Although aware of the limitations of a single book, we hope that many students and postdocs, as well as senior scientists and clinicians, will find this volume a useful instrument for their work, attracting at least some of them to the challenging field of oxygen therapeutics.

Finally, we are in debt to all the authors who have enthusiastically accepted the invitation to contribute and have prepared rigorous, still synthetic chapters. A special thank is due to Fondazione Cariparma, Parma, Italy, for supporting our research towards the development of a platform for the safety assessment of blood alternatives.

Andrea Mozzarelli and Stefano Bettati
Editors

1
Introduction

Richard B. Weiskopf
University of California, San Francisco, CA, USA

The red blood cell (RBC) is a magnificently engineered apparatus. Manifestations of a deficiency or genetic error of red cells or their principal constituent, hemoglobin (Hb), are varied and may be severe or fatal. Recognition of the need for augmentation of native red cells from an external source initiated and propelled the fields of blood banking and transfusion medicine, and contributed substantially to the development of hematology and immunology.

Initial transfusion of blood from animal and human sources was performed more than three centuries ago [1]. The subsequent path has been tortuous and at times tortured. Discovery of human blood types by Landsteiner [2, 3] and subsequent development of knowledge of immunology and the science and technology of blood and blood component storage has allowed for the current relative safety of transfusion, while at the same time permitting the advancement of several areas of clinical medicine such as surgery, anesthesiology, and hematology.

However, regulatory criteria for blood or RBC approval do not definitively address efficacy or safety. The US Food and Drug Administration (FDA) requires that blood or RBC units for transfusion have a mean unit RBC recovery of $\geq$75% at 24 hours after transfusion, with a standard deviation of $\leq$9%, and hemolysis of $<$1.0%. These types of criteria were developed in the 1970s, and although they have slightly been modified since then, they cannot be regarded classically as specifying efficacy and safety. Thus, it is not surprising that there is conflicting evidence for both of these issues.

Red-cell efficacy has never been a condition for regulatory approval. The decrease of 2,3-DPG [4, 5] and increase of Hb affinity for oxygen (decreased p50) with storage

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duration (see Chapters 17 and 18, and [5]) led many to hypothesize that stored transfused red cells could not offload oxygen until 2,3-DPG was regenerated, a process that takes many hours [6, 7]. An experiment in normal healthy humans found that RBCs stored for three weeks are as efficacious as fresh RBCs (stored for $\leq$3 hours) in reversing anemia-induced cognitive deficits [8]. However, there are no large clinical prospective randomized trials examining *in vivo* efficacy of stored RBCs.

Similar to the issue of efficacy, safety has never been a condition for FDA approval for unmodified blood or red cells (aside from donor screening and testing for markers of selected infectious diseases). Red-cell transfusion is not without risks, including disease transmission (viral, bacterial, parasitic), transfusion-associated lung injury (TRALI), hemolytic transfusion reactions, graft versus host disease, immunomodulation, circulatory overload, and perhaps risks associated with the age of stored red cells (see Chapters 13 and 15). The risk of transfusion-transmitted infectious disease is at an all-time low in many countries [9, 10], but is substantially higher in many others. Furthermore, there is a continual possibility of emergence of new pathogens or mutation of existing vectors, or of introduction of an existing pathogen into a geographically naive population or blood supply owing to increased international travel. Current blood-banking procedures and processes center on donor selection, testing of donor blood, and quality control. However, these measures do not address storage lesions or potential resultant clinical outcomes.

Many RBC changes that occur with storage likely do not have important clinical safety implications. However, laboratory studies have shown that lysophosphatidyl cholines accumulate with blood storage [11], and when added to perfusates of isolated rat lungs [12] or injected in rats, produce pulmonary injury, as do plasma [12] and lipid extracts of plasma of blood stored for 42 days [12].

Numerous publications, including original reports, reviews [13], and commentaries [14], have sought to examine a potential association of storage duration of transfused RBCs with adverse outcomes, with both positive and negative findings. The original reports are largely retrospective examinations of databases of varying sizes and scopes, with disparate results. For example, Koch *et al*. noted that transfusion of blood stored for more than 14 days compared to blood stored for a lesser period was associated with a substantial increased risk of mortality, renal failure, and sepsis or septicemia in their retrospective analysis of a single-center database of 2872 patients who underwent cardiac surgery [15]. On the other hand, more recently Edgren *et al*. examined the Danish and Swedish database of nearly 400 000 transfused patients and found no association between duration of storage of red cells and mortality within seven days of transfusion, and a 5% risk for long-term follow-up, which they attributed to confounding factors [16]. Retrospective database analyses are subject to many potential confounders, including severity of illness [16] and number of units transfused [17]. Some retrospective analyses, when corrected for either or both of these, find that a potential association no longer pertains [18]. Most importantly, retrospective analyses cannot account for the clinical reason for the transfusion: frequently blood is transfused in an attempt to obviate complications in a more sick population or to treat complications that tend to occur in more sick patients. In as much as there are no prospective randomized controlled trials adequately examining such a putative association, four such trials (two in Canada and two in the USA) have been initiated.

Issues surrounding transfusion have prompted alternative approaches. The rationale and the science and clinical application(s) for synthetic or semisynthetic oxygen carriers have undergone substantial development over the many years of investigation. There is little question that the various molecules that have been developed carry and offload oxygen. A wide variety of laboratory experiments attest to that, and need not be described here. There are several potential clinical applications for the use of these biologics: (i) in preference to transfusion of RBCs to prevent or treat ischemia, while avoiding the potential adverse effects of transfusion (see Chapter 10); (ii) for delivery of oxygen to tissues that may be inaccessible to red cells (e.g. to provide oxygen to tissues beyond a tight arteriolar fixed stenosis); (iii) when compatible or any red cells are not available (e.g. pre-hospital trauma; mass disaster; recipient rare red-cell type); (iv) in cases of recipient refusal of transfusion (e.g. religious reasons) (see Chapter 14); (v) for tumor therapy; and (vi) as a "place holder" to conserve red cells in cases of substantial hemorrhage and transfusion. These potential applications are all discussed in this volume and do not require elaboration here. Other therapies that increase Hb and red-cell mass, such as erythropoietin or iron therapy (oral or intravenous), do not act sufficiently rapidly to satisfy these indications for an acute need. Hence the need for development of oxygen carriers capable of providing oxygen rapidly to tissues/organs with these acute needs.

The first infusions of free Hb in humans are attributed to Sellards and Minot, who used a preparation of unmodified hemolyzed RBCs [19]. Substantial development with partial purification of Hb by Amberson *et al*. followed in the 1940s [20]. Further experimentation was delayed owing to the observed deleterious renal effects [20]. In the 1960s and 1970s Rabiner [21, 22] and Savitsky [23] infused somewhat purified Hb solutions and although these investigators named their preparations "stromal-free", stroma removal was incomplete (more than 1% of stroma remained) [23] and the Hb molecule was unmodified, allowing for dissociation to dimers and monomers. These preparations did not eliminate renal function impairment or hemoglobinuria. Chemical crosslinking and/or polymerization of native human or bovine Hbs [24], or crosslinking of recombinant human Hb at the genetic level [25–27], and improved purification techniques resolved the renal toxicity of at least some [28], but not all [29, 30], of these preparations.

The central difficulty for the clinical development of non-red-cell oxygen carriers has been real and perceived issues of safety and adverse effects. The first phase III clinical trial conducted under the aegis of 21CFR50.24 [31], exception from informed consent, in the USA resulted in increased mortality in those patients receiving the hemoglobin-based oxygen carrier (HBOC) [32], casting a pall over further clinical development. Several HBOCs have undergone phase II and phase III clinical trials and while at times they have shown potentially promising efficacy, they simultaneously produced a variety of adverse events, including myocardial, vascular, renal, and gastrointestinal disturbances. The thought that these might be a class effect in part led to an FDA/National Institutes of Health (NIH)-sponsored conference in 2008. Publicly available information regarding the safety profile of human exposure was reviewed, largely confirming the increased incidence of these adverse events [29, 30]. More recently, another phase III clinical trial in pre-hospital trauma failed to meet its end point of mortality non-inferiority [33]. The incidence of adverse events and failed clinical trials poses substantial challenges to further development of HBOCs.

Many at the 2008 FDA/NIH conference suggested that further understanding of the mechanism of the noted adverse events should precede clinical development. Many believe that Hb scavenging of nitric oxide [34] can explain all of the noted adverse findings [29, 30]. Included in these effects could be: limitation of vascular dilation with resultant hypertension; inability to respond to tissue/organ hypoxia, perhaps causing the myocardial and renal injury; constriction of the sphincter of Oddi, resulting in symptoms in unmedicated conscious humans [28] and perhaps the elevated pancreatic and hepatic enzymes; and, as has been suggested [29, 30, 35], the interaction of NO with platelets [36, 37] and the inflammatory response (see also Chapters 4, 5 and 17).

As always, regulatory approval and clinical use of any drug, biologic, or device, requires judgment as to the balance of the risk and benefits of a proposed therapy, drug, or biologic and the alternative. Thus, adverse effects of HBOCs should not be examined in isolation, but rather in juxtaposition with the alternatives: red-cell transfusion or lack of transfusion – that is, acute anemia. Some of the risks of RBC transfusion have been highlighted above.

Not transfusing RBCs when Hb concentration requires augmentation (acute anemia) has the risk of inadequate tissue oxygenation. Normal humans demonstrate reversible (by red-cell transfusion [8, 38] or breathing oxygen [39]) inadequate cerebral oxygenation at Hb concentrations of 5 g/dL and 6 g/dL [8, 38–40]. Retrospective database analyses have found an association of mortality with anemia [41–44]. Full examination of the risks of transfusion or acute anemia (not transfusing) is beyond the scope of this introduction or this volume, but has been reviewed succinctly recently [45].

The NIH/FDA conference and the symposium that prompted this book contained forward-looking elements. The former suggested that populations be studied where there is a favorable balance of benefits and risks. However, risks for acute anemia, transfusion, and likely HBOCs are not uniform across all populations. Formal analysis of HBOC risk by patient age, disease processes, and other important risk factors might identify populations of improved benefit : risk. The symposium, as delineated in this book, focused on current research intended to improve the balance of benefits and risks. The reader will find approaches that include development of encapsulation of Hb (see Chapter 27), further molecular modifications (see Chapters 24, 25 and 26), large naturally occurring Hbs, and red-cell "pharming" (see Chapter 19). Only time will tell whether any of these will succeed. In addition to the issue of resolution of HBOC-induced adverse events, other questions remain. While the pharmacodynamics of HBOCs has received much attention, the pharmacokinetics has not. What should be the ideal pharmacokinetic profile remains an unresolved issue. Clinical development of HBOCs must also focus on indications that have valid end points for which practical and feasible clinical trials with reasonable cost can be implemented and completed. This area of endeavor has vigor and hope, but can retain that only insofar as these questions can be resolved.

One symposium or volume cannot cover this entire field of research and development. This book emanates in large measure from the XXII Symposium on Blood Substitutes held in Parma, Italy in 2009. The intention of that symposium and this book is to relate and discuss the current state of the art. A perusal of the chapter titles and their authors should quickly impress the reader that the meeting organizers and editors have gathered the experts on these subjects, who have succeeded admirably in that endeavor.

References

[1] Klein, H.G. and Anstee, D.J. Chapter 2. Transfusion of blood, blood components and plasma alternatives in oligaemia, in *Mollison's Blood Transfusion in Clinical Medicine*, *11th Edition*, Blackwell Publishing Ltd, Oxford, UK (2005).

[2] Landsteiner, K. Zur Kenntnis antifermentativen, lytischen und agglutinierenden Wirkungen des Blutserums und der Lymphe, *Zbl. Bakt*., **27**, 357–362 (1900).

[3] Landsteiner, K. Uber Agglutinationserscheinungen normalen menschlichen Blutes, *Klin. Wschr*., **14**, 1132–1134 (1901).

[4] Beutler, E., Meul, A. and Wood, L. Depletion and regeneration of 2,3-diphosphoglyceric acid in stored red blood cells, *Transfusion*, **9**, 109–115 (1969).

[5] Chanutin, A. and Curnish, R. Effect of organic and inorganic phosphates on the oxygen equilibrium of human erythrocytes, *Arch. Biochem. Biophys*., **121**, 96–102 (1967).

[6] Beutler, E. and Wood, L. The *in vivo* regeneration of red cell 2,3 diphosphoglyceric acid (DPG) after transfusion of stored blood, *J. Lab. Clin. Med*., **74**, 300–304 (1969).

[7] Heaton, A., Keegan, T. and Holme, S. *In vivo* regeneration of red cell 2,3-diphosphoglycerate following transfusion of DPG-depleted AS-1, AS-3 and CPDA-1 red cells, *Br. J. Haematol*., **71**, 131–136 (1989).

[8] Weiskopf, R.B., Feiner, J., Hopf, H.W. *et al*. Fresh blood and aged stored blood are equally efficacious in immediately reversing anemia-induced brain oxygenation deficits in humans, *Anesthesiology*, **104**, 911–920 (2006).

[9] Zou, S., Dorsey, K.A., Notari, E.P. *et al*. Prevalence, incidence, and residual risk of human immunodeficiency virus and hepatitis C virus infections among United States blood donors since the introduction of nucleic acid testing, *Transfusion*, **50**, 1495–1504 (2010).

[10] Zou, S., Stramer, S.L., Notari, E.P. *et al*. Current incidence and residual risk of hepatitis B infection among blood donors in the United States, *Transfusion*, **49**, 1609–1620 (2009).

[11] Silliman, C.C., Clay, K.L., Thurman, G.W. *et al*. Partial characterization of lipids that develop during the routine storage of blood and prime the neutrophil NADPH oxidase, *J. Lab. Clin. Med*., **124**, 684–694 (1994).

[12] Silliman, C., Voelkel, N., Allard, J.D. *et al*. Plasma and lipids from stored packed red blood cells cause acute lung injury in an animal model, *J. Clin. Invest*., **101**, 1458–1467 (1998).

[13] van de Watering, L. Red cell storage and prognosis, *Vox Sang*., **100**, 36–45 (2011).

[14] Liumbruno, G.M. and Aubuchon, J.P. Old blood, new blood or better stored blood? *Blood Transfus*., **8**, 217–219 (2010).

[15] Koch, C.G., Li, L., Sessler, D.I., Figueroa, P. *et al*. Duration of red-cell storage and complications after cardiac surgery, *N. Engl. J. Med*., **358**, 1229–1239 (2008).

[16] Edgren, G., Kamper-Jorgensen, M., Eloranta, S. *et al*. Duration of red blood cell storage and survival of transfused patients (CME), *Transfusion*, **50**, 1185–1195 (2010).

[17] van de Watering, L., Lorinser, J., Versteegh, M. *et al*. Effects of storage time of red blood cell transfusions on the prognosis of coronary artery bypass graft patients, *Transfusion*, **46**, 1712–1718 (2006).
[18] Vamvakas, E.C. and Carven, J.H. Length of storage of transfused red cells and postoperative morbidity in patients undergoing coronary artery bypass graft surgery, *Transfusion*, **40**, 101–109 (2000).
[19] Sellards, A. and Minot, G. Injection of hemoglobin in man and its relation to blood destruction, with especial reference to the anemias, *J. Med. Res*., **34**, 469–494 (1916).
[20] Amberson, W., Jennings, J. and Rhode, C. Clinical experience with hemoglobin-saline solutions, *J. Appl. Physiol*., **1**, 469–489 (1949).
[21] Rabiner, S., Helbert, J., Lopas, H. and Friedman, L. Evaluation of a stroma-free hemoglobin solution for use as a plasma expander, *J. Exp. Med*., **126**, 1127–1142 (1967).
[22] Rabiner, S.F., O'Brien, K., Peskin, G.W. and Friedman, L.H. Further studies with stroma-free hemoglobin solution, *Ann. Surg*., **171**, 615–622 (1970).
[23] Savitsky, J.P., Doczi, J., Black, J. and Arnold, J.D. A clinical safety trial of stroma-free hemoglobin, *Clin. Pharmacol. Ther*., **23**, 73–80 (1978).
[24] Olsen, K.W., Zhang, Q.Y., Huang, H. *et al*. Stabilities and properties of multilinked hemoglobins, *Biomater. Artif. Cells Immobil. Biotechnol*., **20**, 283–285 (1992).
[25] Fronticelli, C., Brinigar, W.S., Olson, J.S. *et al*. Recombinant human hemoglobin: modification of the polarity of the beta-heme pocket by a valine67(E11) → threonine mutation, *Biochemistry*, **32**, 1235–1242 (1993).
[26] Hoffman, S.J., Looker, D.L., Roehrich, J.M. *et al*. Expression of fully functional tetrameric human hemoglobin *Escherichia coli*, *Proc. Natl. Acad. Sci. U S A*, **87**, 8521–8525 (1990).
[27] Looker, D., Abbott-Brown, D., Cozart, P. *et al*. A human recombinant haemoglobin designed for use as a blood substitute, *Nature*, **356**, 258–260 (1992).
[28] Viele, M., Weiskopf, R. and Fisher, D. Renal effects and pharmacokinetics of recombinant human hemoglobin, *Anesthesiology*, **86**, 848–858 (1997).
[29] Silverman, T.A. and Weiskopf, R.B. Hemoglobin based oxygen carriers: current status and future directions, *Anesthesiology*, **111**, 946–963 (2009).
[30] Silverman, T.A. and Weiskopf, R.B. Hemoglobin based oxygen carriers: current status and future directions, *Transfusion*, **49**, 2495–2515 (2009).
[31] Food and Drug Administration. Protection of human subjects: informed consent. Exception from informed consent requirements for emergency research. 21CFR50.24 (2007).
[32] Sloan, E.P., Koenigsberg, M., Gens, D. *et al*. Diaspirin cross-linked hemoglobin (DCLHb) in the treatment of severe traumatic hemorrhagic shock: a randomized controlled efficacy trial, *JAMA*, **282**, 1857–1864 (1999).
[33] Moore, E.E., Moore, F.A., Fabian, T.C. *et al*. Human polymerized hemoglobin for the treatment of hemorrhagic shock when blood is unavailable: the USA multicenter trial, *J. Am. Coll. Surg*., **208**, 1–13 (2009).
[34] Eich, R.F., Li, T., Lemon, D.D. *et al*. Mechanism of NO-induced oxidation of myoglobin and hemoglobin, *Biochemistry*, **35**, 6976–6983 (1996).

[35] Weiskopf, R.B. Hemoglobin-based oxygen carriers: compassionate use and compassionate clinical trials, *Anesth. Analg*., **110**, 659–662 (2010).
[36] Villagra, J., Shiva, S., Hunter, L.A. *et al*. Platelet activation in patients with sickle disease, hemolysis-associated pulmonary hypertension, and nitric oxide scavenging by cell-free hemoglobin, *Blood*, **110**, 2166–2172 (2007).
[37] Olsen, S.B., Tang, D.B., Jackson, M.R. *et al*. Enhancement of platelet deposition by cross-linked hemoglobin in a rat carotid endarterectomy model, *Circulation*, **93**, 327–332 (1996).
[38] Weiskopf, R.B., Kramer, J.H., Viele, M. *et al*. Acute severe isovolemic anemia impairs cognitive function and memory in humans, *Anesthesiology*, **92**, 1646–1652 (2000).
[39] Weiskopf, R.B., Feiner, J., Hopf, H.W. *et al*. Oxygen reverses deficits of cognitive function and memory and increased heart rate induced by acute severe isovolemic anemia, *Anesthesiology*, **96**, 871–877 (2002).
[40] Weiskopf, R.B., Toy, P., Hopf, H.W. *et al*. Acute isovolemic anemia impairs central processing as determined by P300 latency, *Clin. Neurophysiol*., **116**, 1028–1032 (2005).
[41] Carson, J.L., Poses, R.M., Spence, R.K. and Bonavita, G. Severity of anaemia and operative mortality and morbidity, *Lancet*, **i**, 727–729 (1988).
[42] Carson, J.L., Noveck, H., Berlin, J.A. and Gould, S.A. Mortality and morbidity in patients with very low postoperative Hb levels who decline blood transfusion, *Transfusion*, **42**, 812–818 (2002).
[43] Carson, J.L., Duff, A., Poses, R.M. *et al*. Effect of anaemia and cardiovascular disease on surgical mortality and morbidity, *Lancet*, **348**, 1055–1060 (1996).
[44] Gould, S.A., Moore, E.E., Hoyt, D.B. *et al*. The life-sustaining capacity of human polymerized hemoglobin when red cells might be unavailable, *J. Am. Coll. Surg*., **195**, 445–452; disc. 452–455 (2002).
[45] Weiskopf, R.B. Emergency transfusion for acute severe anemia: a calculated risk, *Anesth. Analg*., **111**, 1088–1092 (2010).

Part I

Oxygen: Chemistry, Biochemistry, Physiology and Toxicity

2

Hemoglobin Reactivity and Regulation

Stefano Bettati and Andrea Mozzarelli

Department of Biochemistry and Molecular Biology, University of Parma, Parma, Italy

and

National Institute of Biostructures and Biosystems, Rome, Italy

2.1 Introduction

Oxygen serves key roles in living organisms, including the generation of ATP in the mitochondrial oxidative phosphorylation carried out by respiratory-chain enzymes. It is intriguing that, on one hand, oxygen is a vital molecule that sustains life, and, on the other, it is a toxic molecule because it can generate radical species, the so-called reactive oxygen species (ROS), causing devastating cellular damage. This oxygen Janus face calls for a tight regulation of oxygen homeostasis, which is achieved by the interplay of many molecular, cellular and physiological actors. These mechanisms involve several proteins, among which hemoglobin (Hb) is the most relevant and most investigated.

2.2 Oxygen Loading and Transport

The oxygen concentration in air is about 160 torr, corresponding to 21% of the dry air gas content, with nitrogen accounting for 78% and other trace gases for about 1%. In the alveola, the air enters in contact with the blood circulation and gas exchange takes place. Carbon dioxide is released and oxygen is loaded on Hb contained within red cells. Within the lung, the oxygen pressure is about 100 torr, as a result of the diminished molar

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fraction (14%) due to the gas exchange with venous blood, and the increased contribution of water vapor to the total air pressure (up to 47 torr at body temperature). Hb acts as an efficient molecular transducer that is able to balance the loading and unloading of oxygen depending on a large variety of physiological conditions. Part of the bound oxygen is unloaded from Hb to tissues for the generation of ATP, and part is transferred to myoglobin (Mb) localized in muscle tissues for storage and further use.

Hb function and regulation are intimately related to protein structural arrangement [1]. Hb is a tetrameric protein consisting of two α and two β chains forming tight αβ dimers. The tetramer–dimer dissociation equilibrium depends on the oxygenation state of Hb. Oxy-Hb tetramers exhibit a dissociation constant of about 10^{-6} M, whereas deoxygenated Hb tetramers are much more stable [2]. Within the red cell, Hb tetramer concentration is about 5 mM and Hb dimer concentration is extremely low. On the other hand, Hb released from red cells or injected free in the plasma is mostly present as dimers. In the plasma, dimers bind with high affinity to the glycoprotein haptoglobin, forming a complex that is presented to the macrophage scavenger receptor CD163, triggering endocytosis [3]. Given the relatively low concentration of haptoglobin, 90 mg/100 ml of plasma, dimeric Hb is filtered in the kidney glomerula and excreted in urine in case of (i) pathological conditions leading to high levels of hemolysis, such as sickle cell disease or malaria (see Chapter 12); (ii) transfusions with aged blood, causing up to 30% of hemolysis of transfused red cells (see Chapter 15 and [4]); and (iii) injection of free Hb in the plasma, as carried out in the early attempts of transfusions (see Chapters 15 and 22). Therefore, an excess of Hb dimers causes severe nephrotoxicity [5, 6].

About 70% of Hb α and β globin chains are arranged in a helical conformation, leading to the so-called globin fold, consisting of a three-over-three helix motif (Figure 2.1a). Helices are labeled as A, B, C,... and the amino acids are labeled as AAA1, AAB3, AAE8, for example, indicating the first amino acid of helix A, the third amino acid of helix B and the eighth amino acid of helix E. Each chain binds a heme group: the ferrous protoporphirin IX derivative (Figure 2.1b). The iron atom is coordinated with the nitrogens of the four pyrroles and HisF8 (the "proximal histidine"). The sixth iron

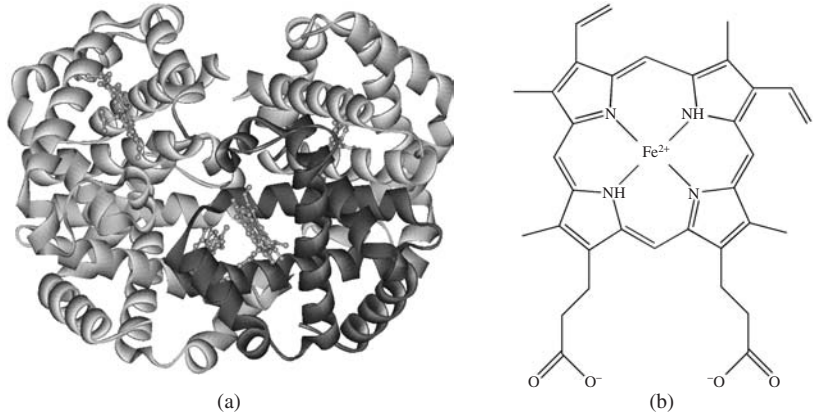

Figure 2.1 *(a) Human deoxy-Hb structure (PDB code 2DN2). The three-over-three helix motif is emphasized in the front subunit (dark grey). (b) Chemical structure of protoporphyrin IX.*

coordination position is the site of oxygen binding. A "distal histidine", HisE7, plays a role in stabilizing the bound oxygen via an H-bond. The microenvironment of the heme site is apolar, leading to the stabilization of the ferrous state even in the presence of oxygen; that is, the electrochemical potential of the Fe^{3+}/Fe^{2+} is more positive than in aqueous solution, where the ferrous species in the presence of oxygen is readily converted to the ferric species. Alteration of the active-site geometry and polarity due to amino-acid mutations can lead to pathological concentrations of oxidixed Hb (met-Hb), accompanied by methemoglobinemia and methemoglobinuria.

The fully liganded Hb, oxy-Hb, binds four oxygen molecules (Figure 2.2). In the lung, about 97% of Hb is saturated with oxygen. Oxygen-saturation Y is defined as the concentration of oxy-Hb over the sum of the concentrations of deoxygenated Hb (deoxy-Hb) and oxy-Hb. Hb oxygen affinity is defined as p50: the oxygen pressure at which 50% of Hb hemes are saturated with oxygen. The physiological p50 (pH 7.4, 37 °C, in the chemical environment found inside the erythrocyte) is 26 ± 1 torr. The p50 depends on the concentration of allosteric effectors, molecules that bind to Hb, affecting function through the regulation of conformational equilibria. The physiological allosteric effectors of Hb are protons, 2,3-diphosphoglycerate (DPG), chloride, inorganic phosphate and carbon dioxide. The concentration of protons and carbon dioxide varies within the circulation, thus affecting the actual p50. For example, the pH is higher in the lung and lower in active tissues such as muscles, leading to a lower and higher p50, respectively. This proton concentration-dependent p50 shift is called the Bohr effect and is physiologically relevant because it favors the loading of oxygen in the lung and

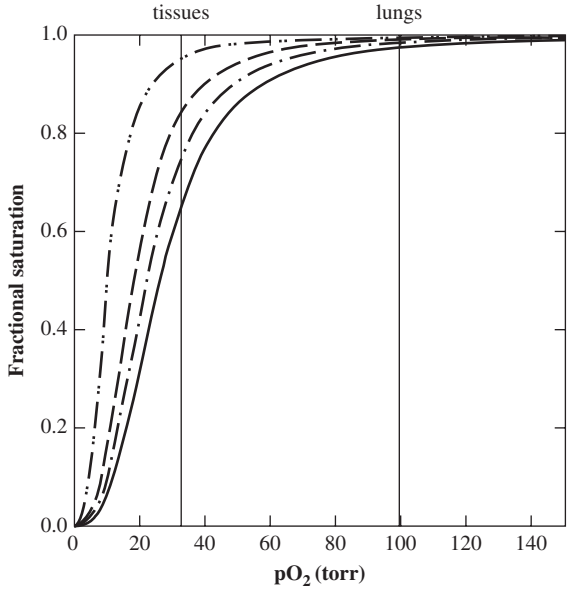

Figure 2.2 *Oxygen-binding curves of human Hb in the absence of allosteric effectors (dash-dot-dotted line), in the presence of CO_2 (dashed line) and DPG (dash-dotted line), and in whole blood (solid line).*

unloading in the tissues. Similarly, a higher carbon dioxide concentration present in the peripheral tissues increases p50, favoring oxygen unloading. The resulting oxygen-binding curve (OBC) is right-shifted (Figure 2.2). The most relevant allosteric effector is DPG, which binds to the Hb central cavity and has a concentration of about 5 mM inside the red cells, a value close to the concentration of Hb tetramers. This indicates that one DPG molecule is bound per Hb tetramer. In the absence of DPG, the p50 is about 10 torr. The variation in DPG concentration is responsible for the physiological adaptation to changes in altitude and to anemic conditions. The higher the altitude, the higher the DPG concentration and the p50. Similarly, the lower the Hb concentration, the higher the DPG concentration and the p50. The right shift of the OBC causes a slightly lower saturation of Hb in the lung but ensures a larger amount of oxygen unloading in the microcirculation, since the steeper part of the sigmoidal OBC is in the range of oxygen pressures of the lung and veins. Pathological conditions are associated with deficiencies of the glycolytic enzymes hexokinase and pyruvate kinase, causing abnormally low and high concentrations of DPG, respectively, and thus left and right shifting of the OBC, respectively [7]. Molecules that mimic the action of negative and positive allosteric effectors (left- and right-shifters) have been developed to treat sickle cell disease and ischemia, respectively (see Chapter 21). The sites of binding of many of these artificial allosteric effectors do not overlap with the natural allosteric effectors, evidencing the existence on Hb of other allosteric sites (see Chapter 21). It was also demonstrated that different allosteric effectors can elicit opposite effects upon binding to the same sites [8].

Hb oxygen loading and unloading is favored by the sigmoidal shape of OBC. A protein exhibiting a hyperbolic binding curve cannot serve the role of efficient physiological oxygen carrier. In fact, a protein endowed with a lower affinity will load a lower amount of oxygen in the lung and a protein with higher oxygen affinity will unload a lower amount of oxygen in the tissues. The sigmoidal shape of oxygen binding is indicative of a cooperative behavior of Hb; that is, oxygen binding favors further oxygen binding, while oxygen unloading favors further oxygen unloading. Several models have been proposed to explain the cooperative behavior of Hb, serving as a paradigm of allosteric proteins. The Monod, Wyman and Changeux (MWC) model [9] postulates the presence of an equilibrium between two quaternary structures of Hb – the T and R states – endowed by low and high oxygen affinity, respectively. In the absence of oxygen, the equilibrium favors T over R; thus the first oxygen molecule binds with low affinity to the T state. As oxygen binds, the equilibrium is altered, favoring the concerted transition of T-state Hb to R-state Hb. Thus, the sigmoidal OBC results from the linear combination of binding to low-affinity T-state Hb molecules and high-affinity R-state Hb molecules, with their relative concentration changing as a function of oxygen saturation. Within the frame of the MWC model, allosteric effectors stabilize either the R or the T state, with all physiological effectors belonging to the latter class. Alternative models have been proposed. These include Koshland, Nemethy and Filmer's sequential model [10], Minton and Imai's three states model [11], Ackers' symmetry code [12], Brunori's cooperon model [13] and Yonetany's global allosteric model [14] and dynamic allostery model [15]. More recently, the MWC model has been expanded to the tertiary two-state (TTS) model [16] to account for the effect of allosteric effectors on the intrinsic oxygen affinity of the T and R states (Figure 2.3). The TTS model is based on the existence of tertiary *t* and *r* states, in both quaternary T and R conformations. These conformations

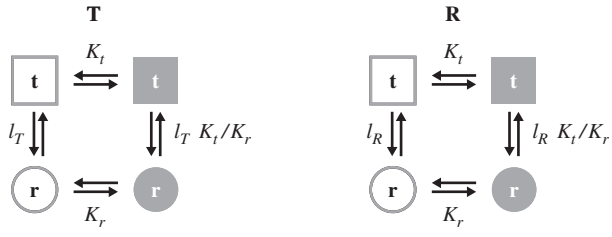

Figure 2.3 *Schematic representation of the TTS model [16]. t and r are tertiary conformations endowed by low and high oxygen affinity defined by K_t and K_r, respectively. l_T and l_R are the equilibrium constants of the t to r transition within the quaternary T and R state, respectively. Empty and filled squares and circles refer to unliganded and liganded subunits, respectively.*

are endowed with low and high ligand affinity and reactivity, respectively, independent of the quaternary state. The quaternary state only affects the distribution between *t* and *r*; that is, the T state biases the distribution towards *t* and the R state towards *r*. Negative allosteric effectors act by favoring *t* rather than shifting the quaternary equilibrium. This model explains a wealth of solution data and is supported by oxygen-binding studies carried out on Hb in crystal and in silica gels [17–21], the CO rebinding kinetics of Hb in silica gels [22, 23], and CD [24] and Raman-detected [25] structural perturbations. Despite the fact that Hb allosteric regulation is often regarded as a consolidated textbook matter, the new TTS model provides a framework in which to accommodate and interpret many observations of the effect of chemical and genetic perturbations to Hb structural and functional properties, and to direct further Hb engineering.

2.3 NO Reactivity with Hb

Nitric oxide (NO) binds to Hb through three distinct mechanisms, as reported below and further explained in Chapters 4 and 5.

(i) Deoxy-Hb reacts with NO to produce nitroso-Hb. The reaction of deoxy-Hb with NO is extremely fast, of the order of 60 μM/s [26]. NO binds to the iron and causes the breakage of HisF8-iron coordination. As a result, in spite of the ligation state, HbNO is in the T state because no tertiary conformational changes destabilizing the T state are communicated to the $\alpha_1\beta_2$ interface.

(ii) Oxy-Hb reacts with NO to produce nitrate and met-Hb [26–28]. This reaction takes place at a rate in the order of 70 μM/s under physiological conditions. The occurrence of this reaction may have physiological relevance as it decreases the amount of available NO, given the low concentration of NO (less than 1 μM) and the high Hb concentration within the red cells. However, NO produced by the endothelial cells of the vessel should penetrate red cells, that are known not to approach the vessel walls, in the so-called unstirred-layer region. This diffusion barrier limits the rate of the reaction of NO with Hb in red cells. In contrast, free Hb in the plasma can diffuse at the site of NO synthesis, scavenging NO with 1000-fold higher rate and thus causing hypertension and increasing blood flow [29].

(iii) Oxy-Hb reacts with NO to produce a thiol-derivative, nitrosyl-βCys93 Hb. βCys93 is 70-fold more reactive with NO in the R state than in the T state. Upon deoxygenation, NO is released from nitrosyl-Cys Hb within the red cells and can migrate outside, controlling vessel tension [30]. The physiological relevance of these events for NO homeostasis and the control of vessel tension is currently under discussion (see Chapters 4 and 17).

2.4 Hb Oxidation

Hb spontaneously autoxidizes, forming met-Hb and oxygen superoxide. Within the red cells, methemoglobin reductase keeps the level of met-Hb very low, whereas in the plasma a very limited reducing power is available, mostly accounted for by ascorbic acid. In humans and guinea pigs, as opposed to mice and rats, ascorbic acid cannot be synthesized endogenously, causing a rapid depletion of the plasma reducing power under oxidative stresses as in the case of a high degree of hemolysis [31]. The series of redox reactions that can occur, generating ROS and reactive nitrogen species (RNS), is as follows:

$$\mathrm{HbFe[II]\text{-}O_2 \rightarrow HbFe[III] + O_2\bullet}$$
$$\mathrm{HbFe[II]\text{-}O_2 + O_2\bullet + 2H^+ \rightarrow HbFe[III] + H_2O_2 + O_2}$$
$$\mathrm{HbFe[II]\text{-}O_2 + H_2O_2 \rightarrow HbFe[III] + OH\bullet + OH^- + O_2}$$
$$\mathrm{HbFe[II]\text{-}O_2 + OH\bullet \rightarrow HbFe[III] + OH^- + O_2}$$
$$\mathrm{HbFe[III] + H_2O_2 \rightarrow \bullet HbFe[IV] = O + H_2O}$$
$$\mathrm{HbFe[II]\text{-}O_2 + \bullet HbFe[IV] = O \rightarrow 2\ HbFe[III] + 3/2\ O_2}$$

In the red blood cells, superoxide dismutase transforms the oxygen superoxide in hydrogen peroxide, which is then either reduced to water by glutathione peroxidase or dismutated to water and oxygen by catalase. These protective enzymatic systems are not present in the plasma, and ROS generated by free Hb cause oxidative damage. In particular, hydrogen peroxide can react with free oxy-Hb to yield met-Hb, or with met-Hb to yield oxoferryl-Hb.

The superoxide radicals react with NO to produce the highly oxidant peroxinitrite [27]:

$$\mathrm{O_2^{\bullet -} + NO \rightarrow ONOO^-}$$

ROS and RNS react with lipids and proteins to form other radical species which, in turn, generate a cascade of oxidative events, leading to the deleterious effects associated with free plasma Hb [32].

2.5 Nitrite Reactivity with Hb

In recent years, considerable attention has been devoted to the reaction of nitrite with Hb and its physiological relevance (see Chapter 5 and [33, 34]). Nitrite has been proposed

to be a less reactive storage species of NO. In fact, nitrite reacts with Hb according to the following equation:

$$\text{HbFe[II]} + \text{NO}_2^- + \text{H}^+ \rightarrow \text{HbFe[III]} + \text{NO} + \text{OH}^-$$
$$\text{HbFe[II]} + \text{NO} \rightarrow \text{HbFe[II]} - \text{NO}$$

The time course of nitrite reaction with Hb is sigmoidal, indicating an autocatalytic process [35]. This behavior has been explained as being due to a change in the T to R equilibrium as the reaction proceeds. In fact, the bimolecular rate constants of nitrite reaction with R- and T-state Hb are 6 and 0.03 M/s, respectively. During the reaction, the formation of met-Hb and nitrosyl-Hb is responsible for a shift in the quaternary equilibrium, progressively increasing the concentration of the R state [34].

2.6 Amino-acid Determinants of Hb Reactivity: Natural and Engineered Hbs

The α and β subunits contain 141 and 146 amino acids, respectively. The role of each amino acid in determining Hb reactivity has been investigated by characterizing either naturally occurring or genetically produced Hb variants. Many mutations have been associated with diseases [36], the most well known being βGlu6Val, which causes sickle cell anemia (see Chapter 12). Recent reviews and papers provide a comprehensive view of the wealth of information accumulated over the years on the role of many amino acids in Hb function and on the effects of mutations [36–40]. Some examples are provided to emphasize the fine tuning of Hb reactivity by single or multiple mutations. Other examples are reported in Chapter 26, on the generation of Hb polymers.

2.6.1 Modulation of Oxygen Affinity and Cooperativity

At least three situations have been observed: (i) stabilization of either the T or R state; (ii) alteration of the interactions between oxygen and protein residues within the heme pocket; and (iii) modification of the accessibility of the heme site.

(i) Classic examples are βAsn102Thr (Hb Kansas) and βAsn108Lys (Hb Presbyterian) mutants, which are characterized by a low affinity and low cooperativity as a result of the destabilization of the R state. This effect is due to the loss of an interaction between βAsn102 and αAsp94 in the R state. Similarly, αAsp94Asn (Hb Titusville) and αAsp94Ala mutants exhibit low oxygen affinity and cooperativity. On the other hand, Hb carrying the αVal96Trp mutation at the $\alpha_1\beta_2$ interface exhibits low oxygen affinity and conserved cooperativity, due to the water-mediated H-bond with βGlu101, which stabilizes the T state without affecting the R state. An increase in oxygen affinity is obtained by mutations that prevent the formation of either salt bridges stabilizing the T state, such as αArg141His (Hb Suresnes), αArg141Leu (Hb Legnano) and βHis146Leu (Hb Cowtown), or of $\alpha_1\beta_2$ interactions stabilizing the T state, such as in βTrp37Arg (Hb Rothschild).

(ii) Oxygen binding to the heme pocket is stabilized by the interaction with His E7 (α58 and β63). Mutations of this residue affect oxygen affinity, depending on

whether interactions with oxygen are increased, such as in βHis63Arg (Hb Zurich), or decreased.

(iii) In Mb, the oxygen affinity can easily be tuned by single mutations at Leu29 [41]. A 38-fold decrease in oxygen affinity was obtained by replacing the wild-type Leu29 (B10), protruding into the heme pocket, with Trp. The same strategy was adopted for Hb. Bulky, hydrophobic residues at B10 (Leu) and at E11 (His) cause an increase in the steric hindrance of the oxygen entrance channel to the heme pocket, decreasing oxygen affinity [42].

2.6.2 NO Reactivity and Oxidation

The B10 and E11 residues have been demonstrated to strongly affect the rate of NO-induced oxidation of Hb as well as auto-oxidation rates [28, 43]. Substitution with apolar and bulky residues, such as Trp and Phe, decreases the rate of oxidation and of NO-induced oxidation.

Engineered Hb mutants might be exploited as artificial oxygen carriers free in the plasma [44], acting as Hb-based blood substitutes (see Chapters 22 and 26). Many single and multiple mutants have been prepared in order to generate an Hb molecule characterized by a low oxygen affinity, high cooperativity and low rate of oxidation and NO reaction [44]. Moreover, molecular biology has been used to obtain stable tetramers via either alpha–alpha [45] or alpha–alpha and beta–beta linking [46]. Indeed, one of these products containing the alpha–alpha linking and the βAsn108Lys mutation was evaluated in clinical trials [47]. Engineered Hbs have been obtained to generate Hb polymers (see Chapter 26 and [48]). Presently, the main limitations to the use of recombinant Hbs in preclinical and clinical trials are the high costs, purity issues and relatively low expression yields in *E. coli* [44]. Efforts to improve yields have aimed at stabilizing the tetramer by: (i) favoring globin folding versus degradation/aggregation; (ii) increasing heme synthesis via heme precursor supplementation and heme availability via heme transport systems [40]; and (iii) specific mutations [49]. Since several grams of modified Hb must be provided to patients to be effective as a blood substitute, it is difficult at the moment to envision an industrial application.

As an alternative to genetic modification of Hb, chemical modifications have been exploited for the stabilization of the tetramer via crosslinking (see Chapter 24) and/or conjugation with bulky and non-immunogenic polyethylene molecules (see Chapter 25). In both genetic and chemical approaches, the goal is to prevent dimer formation and increase the size of the Hb circulating in the plasma in order to avoid extravasation and NO scavenging, as well as nephrotoxicity. To date, the goal of obtaining an Hb-based oxygen carrier that is safer and more effective than allogeneic blood transfusions has not been achieved [50].

2.7 Conclusion

In spite of more than a century of intense investigation, Hb function and regulation and oxygen binding-associated events are not fully understood. The recent discovery of Hb enzymatic function in the reaction with NO and nitrite, the adverse effects caused

by red-cell aging, the toxicity of genetically and chemically modified Hb derivatives developed for operating free in the plasma, and the many still not-well-understood processes controlling oxygen and nitric oxide homeostasis all call for a renewed scientific effort. These investigations are even more necessary in order to properly and safely treat the many pathologies that have as their outcome reduced levels of tissue oxygenation. In fact, none of the traditional therapies, such as red-cell transfusions and normobaric/hyperbaric oxygen treatment, or the more innovative potential therapeutic approaches, such as "blood substitutes", NO-delivering compounds, red cells from stem cells and universal red cells, seem to provide safe and effective clinical options for the entire range of hypo-oxygenation pathologies.

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References

[1] Perutz, M.F. Stereochemistry of cooperative effects in haemoglobin, *Nature*, **228**, 726–739 (1970).
[2] Antonini, E. and Brunori, M. *Hemoglobin and Myoglobin in their Reactions with Ligands*, Noth-Holland Publishing Company, Amsterdam, The Netherlands (1971).
[3] Alayash, A.I. Old protein with new function, *Clin. Chim. Acta*, in press.
[4] Lee, J.S. and Gladwin, M.T. Bad blood: the risks of red cell storage, *Nat. Med*., **16**, 381–382 (2010).
[5] Sellards, A.W. and Minot, G.R. Injection of hemoglobin in man and its relation to blood destruction, with especial reference to the anemias, *J. Med. Res*., **34**, 469–494 (1916).
[6] Somers, M., Piqueras, A.I., Strange, K. *et al*. Interactions of ultrapure bovine hemoglobin with renal epithelial cells *in vivo* and *in vitro*, *Am. J. Physiol*., **273**, F38–F52 (1997).
[7] Delivoria-Papadopoulos, M., Oski, F.A. and Gottlieb, A.J. Oxygen-hemoglobulin dissociation curves: effect of inherited enzyme defects of the red cell, *Science*, **165**, 601–602 (1969).
[8] Abraham, D.J., Safo, M.K., Boyiri, T. *et al*. How allosteric effectors can bind to the same protein residue and produce opposite shifts in the allosteric equilibrium, *Biochemistry*, **34**, 15006–15020 (1995).
[9] Monod, J., Wyman, J. and Changeux, J.P. On the nature of allosteric transitions: a plausible model, *J. Mol. Biol*., **12**, 88–118 (1965).
[10] Koshland, D.E. Jr, Nemethy, G. and Filmer, D. Comparison of experimental binding data and theoretical models in proteins containing subunits, *Biochemistry*, **5**, 365–385 (1966).
[11] Imai, K. *Allosteric Effects in Haemoglobin*, Cambridge University Press, Cambridge, UK (1982).
[12] Ackers, G.K. and Holt, J.M. Asymmetric cooperativity in a symmetric tetramer: human hemoglobin, *J. Biol. Chem*., **281**, 11441–11443 (2006).

[13] Brunori, M., Coletta, M. and Di Cera, E. A cooperative model for ligand binding to biological macromolecules as applied to oxygen carriers, *Biophys. Chem*., **23**, 215–222 (1986).
[14] Yonetani, T., Park, S.I., Tsuneshige, A. *et al*. Global allostery model of hemoglobin: modulation of O(2) affinity, cooperativity, and Bohr effect by heterotropic allosteric effectors, *J. Biol. Chem*., **277**, 34508–34520 (2002).
[15] Yonetani, T. and Laberge, M. Protein dynamics explain the allosteric behaviors of hemoglobin, *Biochim. Biophys. Acta*, **1784**, 1146–1158 (2008).
[16] Henry, E.R., Bettati, S., Hofrichter, J. and Eaton, W.A. A tertiary two-state allosteric model for hemoglobin, *Biophys. Chem*., **98**, 149–164 (2002).
[17] Mozzarelli, A., Rivetti, C., Rossi, G.L. *et al*. Crystals of haemoglobin with the T quaternary structure bind oxygen noncooperatively with no Bohr effect, *Nature*, **351**, 416–419 (1991).
[18] Rivetti, C., Mozzarelli, A., Rossi, G.L. *et al*. Oxygen binding by single crystals of hemoglobin, *Biochemistry*, **32**, 2888–2906 (1993).
[19] Eaton, W.A., Henry, E.R., Hofrichter, J. and Mozzarelli, A. Is cooperative oxygen binding by hemoglobin really understood? *Nat. Struct. Biol*., **6**, 351–358 (1999).
[20] Bettati, S. and Mozzarelli, A. T state hemoglobin binds oxygen noncooperatively with allosteric effects of protons, inositol hexaphosphate, and chloride, *J. Biol. Chem*., **272**, 32050–32055 (1997).
[21] Eaton, W.A., Henry, E.R., Hofrichter, J. *et al*. Evolution of allosteric models for hemoglobin, *IUBMB Life*, **59**, 586–599 (2007).
[22] Viappiani, C., Bettati, S., Bruno, S. *et al*. New insights into allosteric mechanisms from trapping unstable protein conformations in silica gels, *Proc. Natl. Acad. Sci. U S A*, **101**, 14414–14419 (2004).
[23] Ronda, L., Abbruzzetti, S., Bruno, S. *et al*. Ligand-induced tertiary relaxations during the T-to-R quaternary transition in hemoglobin, *J. Phys. Chem. B*, **112**, 12790–12794 (2008).
[24] Ronda, L., Bruno, S., Viappiani, C. *et al*. Circular dichroism spectroscopy of tertiary and quaternary conformations of human hemoglobin entrapped in wet silica gels, *Protein Sci*., **15**, 1961–1967 (2006).
[25] Samuni, U., Dantsker, D., Juszczak, L.J. *et al*. Spectroscopic and functional characterization of T state hemoglobin conformations encapsulated in silica gels, *Biochemistry*, **43**, 13674–13682 (2004).
[26] Olson, J.S., Foley, E.W., Rogge, C. *et al*. NO scavenging and the hypertensive effect of hemoglobin-based blood substitutes, *Free Radic. Biol. Med*., **36**, 685–697 (2004).
[27] Herold, S. and Rock, G. Mechanistic studies of the oxygen-mediated oxidation of nitrosylhemoglobin, *Biochemistry*, **44**, 6223–6231 (2005).
[28] Eich, R.F., Li, T., Lemon, D.D. *et al*. Mechanism of NO-induced oxidation of myoglobin and hemoglobin, *Biochemistry*, **35**, 6976–6983 (1996).
[29] Han, T.H., Hyduke, D.R., Vaughn, M.W. *et al*. Nitric oxide reaction with red blood cells and hemoglobin under heterogeneous conditions, *Proc. Natl. Acad. Sci. U S A*, **99**, 7763–7768 (2002).
[30] Stamler, J.S., Jia, L., Eu, J.P. *et al*. Blood flow regulation by S-nitrosohemoglobin in the physiological oxygen gradient, *Science*, **276**, 2034–2037 (1997).

[31] Buehler, P.W., D'Agnillo, F., Hoffman, V. and Alayash, A.I. Effects of endogenous ascorbate on oxidation, oxygenation, and toxicokinetics of cell-free modified hemoglobin after exchange transfusion in rat and guinea pig, *J. Pharmacol. Exp. Ther*., **323**, 49–60 (2007).

[32] Jia, Y., Buehler, P.W., Boykins, R.A. *et al*. Structural basis of peroxide-mediated changes in human hemoglobin: a novel oxidative pathway, *J. Biol. Chem*., **282**, 4894–4907 (2007).

[33] Isbell, T.S., Gladwin, M.T. and Patel, R.P. Hemoglobin oxygen fractional saturation regulates nitrite-dependent vasodilation of aortic ring bioassays, *Am. J. Physiol*., **293**, H2565–H2572 (2007).

[34] Gladwin, M.T., Grubina, R. and Doyle, M.P. The new chemical biology of nitrite reactions with hemoglobin: R-state catalysis, oxidative denitrosylation, and nitrite reductase/anhydrase, *Acc. Chem. Res*., **42**, 157–167 (2009).

[35] Grubina, R., Huang, Z., Shiva, S. *et al*. Concerted nitric oxide formation and release from the simultaneous reactions of nitrite with deoxy- and oxyhemoglobin, *J. Biol. Chem*., **282**, 12916–12927 (2007).

[36] Bunn, F.H. and Forget, B.G. *Hemoglobin: Molecular, Genetic and Clinical Aspects*, W.B. Saunders Company, Philadephia (1986).

[37] Bellelli, A., Brunori, M., Miele, A.E. *et al*. The allosteric properties of hemoglobin: insights from natural and site directed mutants, *Curr. Protein Pept. Sci*., **7**, 17–45 (2006).

[38] Ronda, L., Bruno, S., Abbruzzetti, S. *et al*. Ligand reactivity and allosteric regulation of hemoglobin-based oxygen carriers, *Biochim. Biophys. Acta*, **1784**, 1365–1377 (2008).

[39] Maillett, D.H., Simplaceanu, V., Shen, T.J. *et al*. Interfacial and distal-heme pocket mutations exhibit additive effects on the structure and function of hemoglobin, *Biochemistry*, **47**, 10551–10563 (2008).

[40] Graves, P.E., Henderson, D.P., Horstman, M.J. *et al*. Enhancing stability and expression of recombinant human hemoglobin in E. coli: Progress in the development of a recombinant HBOC source, *Biochim. Biophys. Acta*, **1784**, 1471–1479 (2008).

[41] Dou, Y., Maillett, D.H., Eich, R.F. and Olson, J.S. Myoglobin as a model system for designing heme protein based blood substitutes, *Biophys. Chem*., **98**, 127–148 (2002).

[42] Wiltrout, M.E., Giovannelli, J.L., Simplaceanu, V. *et al*. A biophysical investigation of recombinant hemoglobins with aromatic B10 mutations in the distal heme pockets, *Biochemistry*, **44**, 7207–7217 (2005).

[43] Miele, A.E., Santanche, S., Travaglini-Allocatelli, C. *et al*. Modulation of ligand binding in engineered human hemoglobin distal pocket, *J. Mol. Biol*., **290**, 515–524 (1999).

[44] Olson, J.S. and Maillett, D.H. Designing recombinant hemoglobin for use as a blood substitute, in *Blood Substitutes* (ed. R.M. Winslow), Academic Press, Amsterdam, The Netherlands, pp. 354–374 (2006).

[45] Looker, D., Abbott-Brown, D., Cozart, P. *et al*. A human recombinant haemoglobin designed for use as a blood substitute, *Nature*, **356**, 258–260 (1992).

[46] Panetta, G., Arcovito, A., Morea, V. *et al*. Hb(alphalpha,betabeta): a novel fusion construct for a dimeric, four-domain hemoglobin, *Biochim. Biophys. Acta*, **1784**, 1462–1470 (2008).

[47] Shoemaker, S.A., Gerber, M.J., Evans, G.L. *et al*. Initial clinical experience with a rationally designed, genetically engineered recombinant human hemoglobin, *Artif. Cells Blood Substit. Immobil. Biotechnol*., **22**, 457–465 (1994).

[48] Fronticelli, C. and Koehler, R.C. Design of recombinant hemoglobins for use in transfusion fluids, *Crit. Care Clin*., **25**, 357–371 (2009).

[49] Weickert, M.J., Pagratis, M., Glascock, C.B. and Blackmore, R. A mutation that improves soluble recombinant hemoglobin accumulation in *Escherichia coli* in heme excess, *Appl. Environ. Microbiol*., **65**, 640–647 (1999).

[50] Natanson, C., Kern, S.J., Lurie, P. *et al*. Cell-free hemoglobin-based blood substitutes and risk of myocardial infarction and death: a meta-analysis, *JAMA*, **299**, 2304–2312 (2008).

3

The Major Physiological Control Mechanisms of Blood Flow and Oxygen Delivery

Raymond C. Koehler

Department of Anesthesiology and Critical Care Medicine, The Johns Hopkins University, Baltimore, MD, USA

3.1 Introduction

Perfusion of the microcirculation depends on the arterio-venous pressure gradient, rheological properties of the blood, and vasomotor tone. Vasomotor tone, in turn, depends on the intrinsic myogenic contractile state of the vascular smooth muscle, signaling molecules released from parenchymal cells, endothelium, and autonomic nerves and circulating hormones. The relative importance of each of these factors varies with the individual tissue. In this chapter, an overview of how each of these factors influences the regulation of blood flow will be presented, and the influence of cell-free hemoglobin (Hb) in the plasma on blood flow regulation will be discussed.

3.2 Autoregulation of Blood Flow to Changes in Perfusion Pressure

An increase in arterial pressure will increase the pressure gradient across a vascular bed and will thereby act to increase blood flow. In a nonregulating vascular bed with a constant vascular resistance, the increase in the pressure gradient will produce a proportional increase in blood flow (Figure 3.1). Moreover, blood vessels are distensible, and

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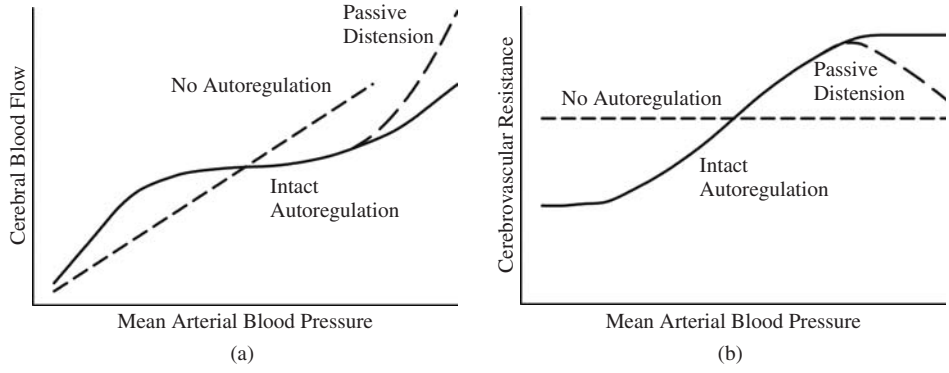

Figure 3.1 *(a) Idealized relationship of blood flow to mean arterial blood pressure for brain with intact autoregulation (solid line). (b) Blood flow is relatively constant over a broad pressure range because of active changes in vascular resistance. With very low pressure, arterioles cannot dilate further, and blood flow falls. With very high pressure, constriction is maximal and flow increases. In some cases, rapid increases in pressure can overcome the myogenic response and passively distend arterioles, leading to a decrease in resistance and a disproportionate increase in flow (long-dashed line). For the case in which there is no active change in arteriolar diameter over the entire arterial pressure range, vascular resistance is constant and blood flow is linearly related to arterial pressure (short-dashed line).*

an increase in transmural pressure could act to passively dilate blood vessels, decrease vascular resistance, and produce a blood flow increase that is disproportional to the increase in the pressure gradient. However, vascular smooth muscle responds to an increase in stretch, produced by an increased distending pressure, with an increase in active wall tension that either prevents passive dilation or results in an actual decrease in vascular diameter. This intrinsic response is called the myogenic response and serves to prevent overperfusion of the tissue when arterial pressure increases. Indeed, in some organs the myogenic response is sufficiently strong to produce vasoconstriction that offsets the increase in perfusion pressure and results in relatively constant blood flow over a pressure range of 50–150 mm Hg (Figure 3.1). This property of the vascular bed to actively change its vascular resistance in the face of increases or decreases in perfusion pressure and thereby help maintain blood flow constant is defined as autoregulation of blood flow.

The primary mechanism of the myogenic response is thought to be stimulation of stretch-activated channels, such as those in the transient receptor potential (TRP) family (Figure 3.2). Stretch-receptor channels permit cation influx and promote depolarization. Smooth muscle stretch is also thought to stimulate phospholipase C (PLC), which results in release of Ca^{2+} from intracellular stores. Protein kinase C (PKC) is also activated and results in increased contractile sensitivity to Ca^{2+}. In addition to stimulating contraction, Ca^{2+} can elicit opening of Ca^{2+}-activated K^+ (K_{Ca}) channels in the plasma membrane, cellular hyperpolarization, smooth muscle relaxation, and arteriolar dilation that ordinarily would act to limit autoregulatory constriction. However, a family of cytochrome P450 enzymes possessing ω-hydroxylase activity converts arachidonic acid to 20-hydroxyeicosatetraenoic acid (20-HETE), which closes K_{Ca} channels and

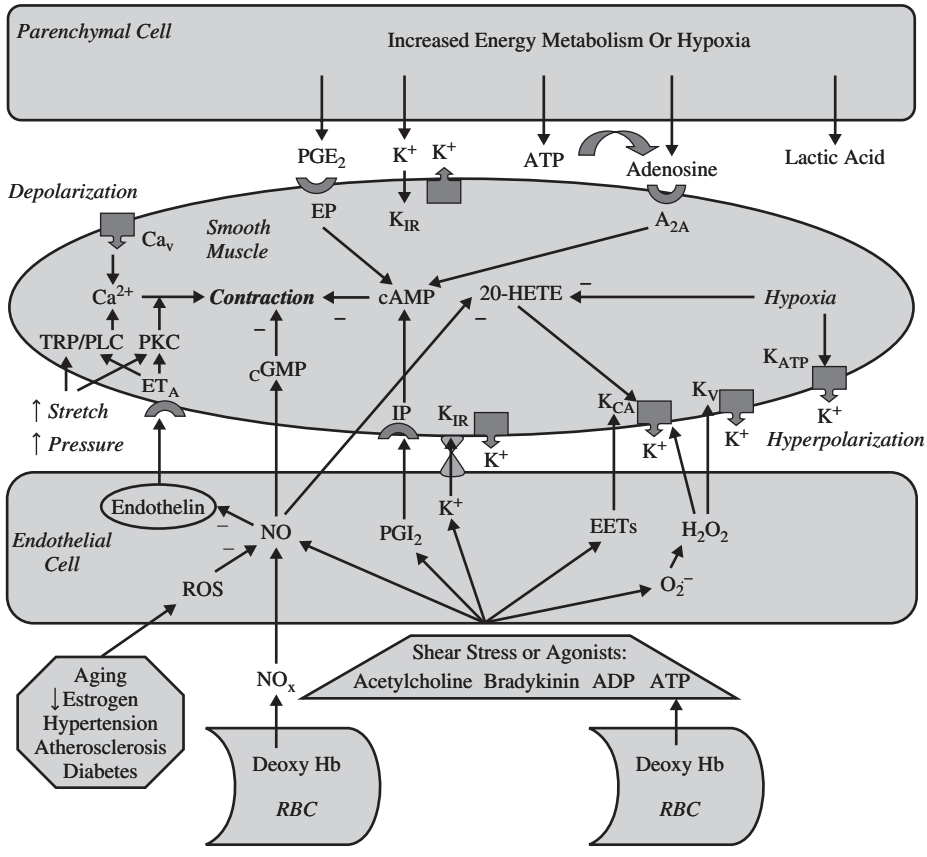

Figure 3.2 *Schematic diagram of the main factors in parenchymal cells, endothelial cells, RBCs, and vascular smooth muscle cells controlling the contractile state of vascular smooth muscle. The diagram illustrates generalized relationships among various factors; the relative importance of individual factors released from parenchymal cells and endothelial cells varies in different vascular beds, in small and large vessels, in males and females, and with different endothelial-dependent agonists. Factors released by parenchymal cells during increased energy metabolism are not necessarily the same as those released during hypoxia. Factors exerting a negative effect are denoted with a minus sign next to the arrow.* Ca_V*, voltage-dependent* Ca^{2+} *channel;* PGE_2*, prostaglandin* E_2*; EP,* PGE_2 *receptor;* K_{IR}*, inward-rectifier* K^+ *channel;* A_{2A}*, adenosine* A_{2A} *receptor; TRP, transient receptor potential channel; PLC, phospholipase C; PKC, protein kinase C;* ET_A*, endothelin receptor A; 20-HETE, 20-hydroxyeicosatetraenoic acid;* PGI_2*, prostaglandin* I_2*; IP,* PGI_2 *receptor;* K_{Ca}*, calcium-activated* K^+ *channel;* K_V*, voltage-dependent* K^+ *channel;* K_{ATP}*, ATP-sensitive* K^+ *channel; ROS, reactive oxygen species; EETs, epoxyeicosatrienoic acids;* O_2^-*, superoxide anion; NO, nitric oxide;* NO_x*, stable storage form of NO; deoxy-Hb, deoxygenated hemoglobin.*

permits membrane depolarization [1, 2]. Cell depolarization causes voltage-dependent Ca^{2+} channels to open and permit extracellular Ca^{2+} entry [3]. The increase in Ca^{2+} influx, together with the release of Ca^{2+} from intracellular stores, produces an increase in contractile force. Furthermore, other signaling mechanisms are thought to participate in integrating the myogenic response, and our understanding of this response remains incomplete.

With mild decreases in arterial pressure, a decrease in myogenic tone produces arterial dilation that prevents or minimizes a decrease in blood flow. With greater decreases in arterial pressure, release of vasodilator agents from parenchymal cells is believed to be responsible for further dilation. These agents are thought to include adenosine, lactic acid, and arachidonic acid metabolites. In the case of severe arterial hypotension, vasodilation is no longer adequate to preserve blood flow, and hypoperfusion or frank ischemia occurs.

Thus, autoregulation of blood flow to increases and decreases in perfusion pressure is a fundamental characteristic of vasoregulation. The degree of autoregulatory capacity varies among organs and is particularly strong in brain and kidney. Autoregulation is present in the coronary circulation. However, changes in arterial pressure also produce changes in left ventricular afterload, which alters myocardial energy needs. Release of metabolic mediators from myocardial cells can override intrinsic myogenic reactivity to maintain an appropriate level of O_2 transport. Indeed, regulation of vascular tone by mediators released from parenchymal and endothelial cells can be viewed, in general, as modulating the intrinsic myogenic tone of the smooth muscle cell. No evidence to date indicates that the presence of chemically stabilized Hb in the plasma interferes with autoregulation of blood flow to changes in perfusion pressure.

3.3 Metabolic Regulation of Blood Flow

In general, tissues with high basal energy metabolism have high basal blood flow. Moreover, tissues with a broad dynamic range of metabolic rates, such as skeletal muscle, cardiac muscle, and the gastrointestinal tract, have a broad dynamic range of blood flow that helps to match dynamic changes in energy metabolism. Within brain, regions with high basal glucose consumption have high blood flow, and dynamic increases in neuronal activity produce dynamic increases in blood flow that are proportional to the dynamic increases in glucose consumption. However, in some tissue, blood flow exceeds the needs of energy metabolism. For example, blood flow to skin is primarily under local and central thermoregulatory control. Endocrine tissue, such as pituitary and adrenal gland, has a relatively high blood flow that helps ensure rapid systemic delivery of hormones. Kidney has a high metabolic rate, but blood flow to renal cortex is disproportionately high and helps to maintain a high glomerular filtration rate. Thus, the relationship of blood flow to energy metabolism is not strictly uniform among organs or within organs. Measurements of cardiac output and whole-body O_2 consumption may not necessarily reflect regionally disparate changes in metabolic rate.

The signaling mechanisms coupling dynamic increases in blood flow with dynamic increases in energy metabolism are multifactorial and vary with the type of tissue. In many tissues, adenosine has long been considered a major mediator of metabolic

vasodilation. Release of adenosine does not necessarily require tissue hypoxia. Rather, secretion of ATP can lead to increases in extracellular adenosine produced by ectonucleotidase enzymes. Adenosine can then act locally on smooth muscle adenosine receptors to produce vasodilation. Blood vessels also possess purinergic receptors that are sensitive to ATP and ADP and which generally produce vasodilation. Other mediators of vasodilation include acidosis, increased extracellular K^+, nitric oxide (NO), prostaglandins, and other arachidonic acid metabolites. However, these mediators probably do not fully account for metabolic vasodilation. Signaling mechanisms involving reactive oxygen species (ROS), reactive nitrogen species (RNS), and H_2O_2 are being investigated [4]. The effect of cell-free Hb on the blood flow response to increased energy demand has not been well studied. During graded exercise in humans, the work load and O_2 consumption at the anaerobic threshold was found to be unaltered by exchange transfusion of polymeric Hb compared to exchange transfusion with autologous blood [5].

3.4 O_2 Transport

Bulk O_2 transport to the microcirculation is defined as the product of arterial O_2 content and blood flow. In considering the regulation of O_2 transport, fractional O_2 extraction is a useful parameter because it is a straightforward measurement that reflects the relationship between O_2 transport and O_2 consumption. If CaO_2 equals arterial O_2 content (Hb-bound O_2 + dissolved O_2), CvO_2 equals venous O_2 content, and Q equals blood flow to a particular tissue then the ratio of O_2 consumption : O_2 transport $= Q \times (CaO_2 - CvO_2)/Q \times CaO_2 = (CaO_2 - CvO_2)/CaO_2 =$ fractional O_2 extraction. Thus, a high fractional O_2 extraction implies that the tissue is underperfused relative to O_2 consumption and a low fractional O_2 extraction implies that the tissue is overperfused relative to O_2 consumption. Graded exercise produces graded increases in fractional O_2 extraction which indicate that the increase in skeletal-muscle blood flow is not strictly proportional to the increase in energy demand. In the coronary circulation, fractional O_2 extraction is ordinarily in the range of 0.6–0.7. Because of the limited O_2 extraction reserve, increases in myocardial work must be matched by nearly proportional increases in coronary blood flow and thus require a high gain control of the microcirculation to avoid ischemia. Human brain has a fractional O_2 extraction of approximately 0.35–0.40 and can sustain nearly 50% reductions in blood flow before grossly impairing O_2 consumption, although smaller reductions in flow for prolonged periods are likely to impair neuronal function and other aspects of metabolism. In the kidney, the high cortical blood flow results in a relatively low fractional O_2 extraction.

3.5 O_2 Delivery

The terms "O_2 transport" and "O_2 delivery" are often used interchangeably. However, it is preferable to use "O_2 transport" to refer to the bulk transport of O_2 into the microcirculation and to restrict the term "O_2 delivery" to the O_2 that is actually delivered to the surrounding parenchymal cells. Thus, O_2 delivery equals the flux of O_2 across the vascular wall of arterioles and capillaries minus any flux of O_2 back into the venous system

arising from the proximity of venules and arterioles (diffusional shunt). In the steady state, O_2 delivery will equal O_2 consumption. However, dynamic differences between O_2 delivery and O_2 consumption will produce dynamic changes in tissue pO_2, which can then influence vascular smooth muscle tone by feedback mechanisms.

The flux of O_2 across the vascular wall depends on the pO_2 gradient between the blood and mitochondrial consumption sites, the temperature-dependent diffusion constant for O_2 in aqueous and lipid phases, and the vascular surface area. The intraluminal pO_2 that sets up the diffusion gradient for O_2 delivery into the tissue has a longitudinal (axial) pO_2 gradient from the arterial to the venous end of the microcirculation. This longitudinal pO_2 gradient depends on tissue O_2 consumption, blood flow, arterial pO_2, Hb concentration, and the shape of the O_2-dissociation curve of the Hb. Measurements of Hb saturation, intravascular pO_2, and perivascular pO_2 all indicate that a significant amount of O_2 diffuses into the tissue before blood arrives at the capillary [6]. Nevertheless, most of the O_2 is usually exchanged at the level of the capillary. Relative to arteries and arterioles, capillaries within a vascular bed have a greater total surface area for diffusion and a slower velocity of red blood cells (RBCs).

In vitro experimental evidence indicates that adding acellular Hb to erythrocyte solutions perfusing a capillary tube increases the efficiency of O_2 delivery, and that the increase is half-maximal when only 10% of the Hb is acellular [7]. The presence of an acellular O_2 carrier in the plasma can affect O_2 delivery in a variety of ways, such as facilitating O_2 delivery from the RBCs, increasing the effective surface area for O_2 diffusion, and altering the heterogeneity of O_2 flux among capillaries.

Although the diffusion of O_2 from the RBCs to the endothelium is rapid, a significant limiting factor is the low solubility of O_2 in the plasma [8]. In arteries and arterioles, a RBC-free zone near the endothelium produces a finite resistance to O_2 diffusion. A radial O_2 diffusion gradient has been detected between the centerline and the luminal wall of arterioles [9]. Small amounts of cell-free Hb in the plasma will increase the amount of O_2 that can be carried in the plasma several-fold over that normally present. This Hb can exchange O_2 with the endothelium with which it comes into contact, then pick up O_2 from RBCs and thereby facilitate the exchange of O_2 between the RBCs and the endothelium. This facilitation would be expected to increase pO_2 in the perivascular tissue around arteries and arterioles and may contribute to a counterregulatory constriction that reduces blood flow.

To overcome counterregulatory constriction, some cell-free Hbs with a low p50 (pO_2 at 50% Hb saturation) have been designed to limit precapillary O_2 loss [10]. Perfusion of capillary tubes with acellular Hb solutions without RBCs supports a significant role of the p50 of the acellular Hb in the axial desaturation rate [11]. However, model simulations of mixtures of RBCs and acellular Hb suggest that the axial desaturation profile is relatively insensitive to the p50 of the acellular Hb in vessels of arteriolar size [12], but becomes more sensitive to acellular Hb p50 in vessels of capillary size [13]. A computational model of the more complex three-dimensional microvascular geometry found in skeletal muscle indicates that acellular Hb with a high p50 increases tissue pO_2 but also increases diffusional shunting of O_2 from arterioles to venules [14].

Resistance to the diffusion of O_2 in capillaries differs from that in arteries and arterioles in that RBCs deform into an elongated shape as they pass through capillaries in a single file. Here, the gap between RBCs and the endothelial glycocalyx is small.

Consequently, the radial resistance to O_2 diffusion between the RBCs and the endothelium presented by the intervening plasma is much less than in arteries [15]. On the other hand, axial gaps between RBCs are filled with plasma. Thus, a snapshot of a capillary bed reveals discreet sources of O_2 because of the particulate nature of RBC passage through capillaries. These gaps increase the resistance to O_2 transfer [8, 15] and result in transient fluctuations in intracapillary pO_2 at a specific locus, especially when the oxygenation state is low [16, 17]. The presence of acellular Hb in the axial gaps between erythrocytes will act to increase the effective surface area for O_2 diffusion across the endothelium at any instant in time and to buffer transient fluctuations in capillary pO_2 at a given locus. Moreover, with low RBC velocity occurring during hemorrhagic hypotension or ischemia, the influence of increasing the effective surface area for diffusion at any instant will be expected to have a disproportionately greater effect on improving O_2 delivery to the tissue. The O_2 delivered by the plasma-based Hb will depend on its O_2 affinity relative to the tissue pO_2. Tissue pO_2 would be expected to be boosted by cell-free Hb possessing either a low or a high p50 when tissue pO_2 is low, whereas tissue pO_2 would be expected to be boosted less so by a low-p50 Hb when tissue pO_2 is already high. Thus, a low-p50 Hb may improve O_2 delivery selectively in underperfused vascular beds.

The delivery of O_2 occurs in microvascular networks with complex three-dimensional geometry [14]. RBC spacing and velocity typically are not uniform among individual capillaries [18]. Heterogeneity of RBC flux contributes to the heterogeneity of tissue pO_2. Dilation of feeding arterioles not only increases RBC velocity, but can also lead to increases in the density of RBCs in capillaries and the effective surface area for O_2 diffusion. However, RBC flux can still remain heterogeneous in the vasodilated state. Even at normal flow, the movement of RBCs in individual capillaries can be intermittent while plasma flow is continuous [19]. The presence of Hb in the plasma space will act to increase the surface area for diffusion, and the improvement in O_2 delivery would be expected to be greatest in capillaries with low RBC flux. Thus, the perfusion of acellular Hb through capillaries with low RBC flux would be expected to make O_2 delivery more uniform and avoid pockets of tissue hypoxia during low-flow states [14].

The aforementioned effects of acellular Hb on O_2 delivery assume that the acellular Hb does not change arteriolar tone. If the acellular Hb does cause upstream constriction, the benefits of increased effective surface area for O_2 diffusion and homogeneity of O_2 flux may be negated by an overall decrease in RBC flux and preferential distribution of RBCs to selective capillaries or to arteriovenous thoroughfares [10, 20]. Therefore, it is important to understand how a particular acellular Hb influences vasomotor control of arterioles.

3.6 Endothelial Control of Vasomotor Tone

The endothelial lining of the circulation contains transporters that regulate the exchange of nutrients between the blood and tissue. The endothelium also plays a role in vascular smooth muscle regulation, thrombosis/hemostasis, inflammation, angiogenesis, and regulating the extracellular matrix. Critical signaling molecules, such as endothelin, von Willebrand factor, interleukin-8, P-selectin, angiopoietin-2, and tissue plasminogen

activator are released from Weibel–Palade bodies and small granules in endothelial cells. In addition, muscarinic receptors, purinergic receptors, and mechanosensitive channels are coupled to type 3 nitric oxide synthase (NOS) and cyclooxygenase-1, both of which produce molecules that regulate vascular smooth muscle tone, hemostasis, and inflammation. Acetylcholine acting on endothelial muscarinic receptors stimulates NOS via Ca^{2+}/calmodulin signaling to generate NO from arginine. ATP released from RBCs when Hb becomes deoxygenated can produce vasodilation by acting on endothelial purinergic receptors to stimulate NO production [21, 22]. ADP released from platelets acts on distinct endothelial purinergic receptors to stimulate formation of NO and prostaglandin I_2 (PGI_2), both of which produce vasorelaxation and inhibit platelet aggregation. Shear stress exerted by blood moving along the endothelial wall activates mechanosensitive receptors that can also stimulate NO, PGI_2, and ROS formation, possibly via Ca^{2+} entry through TRP vallinoid 4 (TRPV4) channels and by cytoskeletal signaling to mitochondria and protein complexes that generate ROS [23, 24]. Thus, the endothelium serves to integrate vasoactive signaling from a variety of blood elements (Figure 3.2).

PGI_2 released from the endothelium acts on IP receptors on vascular smooth muscle and stimulates production of cyclic adenosine monophosphate (cAMP), which produces vasorelaxation. Release of NO from the endothelium activates soluble guanylyl cyclase in vascular smooth muscle and produces cyclic guanosine monophosphate (cGMP), which also produces vasorelaxation. In addition to diffusion of dissolved NO, NO may be stored, transported, and exchanged via thiol-containing compounds. NO can also bind to heme-containing proteins. One example is NO inhibition via ω-hydroxylase activity of cytochrome P450 enzymes, which produce 20-HETE from arachidonic acid. As discussed above, 20-HETE permits autoregulatory constriction in response to arterial hypertension by inhibiting K_{Ca} channel opening. Decreasing NO bioavailability can produce vasoconstriction by disinhibiting 20-HETE formation in addition to the well-established effect of inhibiting cGMP formation [25, 26].

Vasorelaxation induced by increases in cAMP and cGMP does not require a change in the membrane potential of vascular smooth muscle. However, activation of the endothelium has been observed to produce smooth muscle hyperpolarization independent of NO and PGI_2 formation, and this effect is most prominent in small arterioles [23]. The endothelium produces several hyperpolarizing factors that lead to smooth muscle relaxation. One postulated hyperpolarizing factor is K^+. In some vascular beds, K^+ is thought to be transported from the endothelium to the smooth muscle via myo-endothelial gap junctions, where increased K^+ activates inward-rectifier K^+ (K_{IR}) channels and produces smooth muscle K^+ efflux. Another likely hyperpolarizing factor is epoxyeicosatrienoic acids (EETs). EETs are produced in the endothelium of some vascular beds by a set of cytochrome P450 enzymes with epoxygenase activity, which form EETs from arachidonic acid [2, 27]. EETs can act to open the large-conductance K_{Ca} channels on the vascular smooth muscle membrane and produce hyperpolarization and vasorelaxation [28]. EETs may also play a role in the longitudinal conduction of vasodilation mediated by intercellular communication between adjacent endothelial cells [29]. A third hyperpolarizing factor is H_2O_2. Bradykinin produces vasorelaxation of human coronary arteries via endothelial NADPH-dependent production of H_2O_2, which can inhibit EET production and thereby supplant EETs as an endothelial-derived hyperpolarizing factor [30, 31].

In addition to molecules that cause vasodilation, the endothelium can release molecules that produce vasoconstriction. For example, the endothelium can release endothelin, which acts on vascular smooth muscle endothelin receptors to produce vasoconstriction. The mechanism of release of granules containing endothelin is not fully understood, but decreases in NO bioavailability appear to promote endothelin release. Therefore, the endothelium releases a variety of factors that regulate smooth muscle tone (Figure 3.2).

The profile of releasing factors varies with the size of the blood vessel and the particular tissue. Moreover, the profile is influenced by sex hormones, aging, and disease states such as hypertension, atherosclerosis, diabetes, and the metabolic syndrome. Many of these disease states decrease NO bioavailability by decreasing NOS activity or by increasing production of superoxide, which rapidly combines with NO and forms highly reactive peroxynitrite. In addition to Ca^{2+}/calmodulin, the activity of NOS depends on subcellular localization, serine phosphorylation, and bioavailability of the cofactor tetrahydrobiopterin [32]. Furthermore, local availability of the substrate arginine can be limited by the activity of arginase, which can also be upregulated in disease states [33]. Uncoupled NOS activity generates superoxide instead of NO and leads to toxic peroxynitrite formation. Another major source of superoxide is NADPH oxidase, which is upregulated in many vascular disease states. Chronic reductions in NO bioavailability and increased vascular oxidative stress promote an inflammatory response and lead to remodeling of the arterial wall. Endothelial-dependent hyperpolarizing factors take on increasing importance when NO bioavailability is reduced during aging or in vascular disease states. When considering the effects of transfusion of cell-free Hb solutions in a large patient population, one needs to take into account the diversity of endothelial disease in that population and the effect of that disease on the background level of NO, PGI_2, endothelial-derived hyperpolarizing factors, and endothelial-derived constricting factors.

3.7 Effect of Cell-free Hb on Endothelial Function

Based on the knowledge that heme moieties in Hb have a higher affinity for NO than O_2, the identification of NO as an endothelial-derived relaxing factor was supported by evidence showing that submicromolar concentrations of Hb surrounding an artery decreased smooth muscle relaxation in response to endothelial stimulation. Hb entering the interstitial space between the endothelium and smooth muscle scavenges NO and interferes with endothelial signaling to the smooth muscle. The endothelial lining of the blood vessel restricts the movement of macromolecules such as albumin and Hb out of the plasma. However, pores between adjacent endothelial cells permit small molecules and, in some cases, macromolecules to exchange between the plasma and interstitial space. These pores are actively regulated and can vary considerably in size among organs. Pores can be quite large in liver, spleen, intestine, renal peritubular capillaries, and endocrine glands, whereas they are essentially absent in brain endothelium. Cardiac and skeletal muscle has intermediate size pores. Moreover, the size of pores in tissues such as the lung and intestine can increase in pathological states. Consequently, the effect of transfusion of cell-free Hb on NO signaling between the endothelium and vascular smooth muscle depends on the size of the Hb molecule, the particular organ, and any underlying pathological state affecting pore size.

Hb can be crosslinked to stabilize the tetramer with a 64 kDa molecular weight and to prevent formation of 32 kDa dimers that are readily filtered by renal glomeruli. Transfusion of crosslinked Hb produces an increase in arterial blood pressure and decreases in blood flow selectively to tissues that have large endothelial pores (kidney, intestines, pituitary and adrenal gland) [34]. Interestingly, transfused crosslinked Hb appears in the renal lymph that drains from peritubular capillaries but not in urine [35]. This observation is consistent with the concept of a larger pore size in peritubular capillaries than glomerular capillaries. Administration of an inhibitor of NOS decreases blood flow to the kidney and blocks any further reduction in blood flow by transfusion of crosslinked tetrameric Hb [36]. Thus, the vasoconstriction produced by transfusion of crosslinked tetrameric Hb appears to be largely related to extravasation of Hb and resultant scavenging of NO. Furthermore, NO normally inhibits endothelin release, and endothelin antagonists can blunt the decrease in regional blood flow after transfusion of crosslinked Hb [37]. Therefore, the vasoconstriction seen with crosslinked Hb transfusion may not only be related to loss of the vasodilator NO, but also to the release of the vasoconstrictor endothelin.

Vasoconstriction induced by the scavenging of NO by extravasated Hb counteracts the benefit provided by cell-free Hb in improving O_2 delivery. To reduce extravasation, efforts have been directed at increasing the molecular radius of Hb. These efforts include the generation of large polymers of Hb tetramers and conjugation of polyethylene glycol chains on to the surface of the tetramer [5, 38] (see also Chapters 22, 24, 25 and 26). Transfusion of these larger Hb molecules results in less vasoconstriction and smaller changes in arterial blood pressure as long as residual smaller Hb molecules are removed from the final product [35]. Transfusion of a solution of large purified polymers avoids the appearance of Hb in renal lymph [35]. Hence, limiting Hb extravasation is a critical component of designing clinically useful transfusion fluids.

Normally, the abundant amount of Hb in RBCs keeps the intravascular concentration of NO extremely low. In capillaries where deformable RBCs are in contact with the endothelial glycocalyx, the radial concentration gradient for NO is extremely steep between the endothelial luminal wall and the blood. Addition of Hb to the plasma is predicted to have very little effect on this NO gradient and the consequent loss of NO from the endothelium to the blood. However, in arteries and arterioles, the situation may be different because the moving RBCs tend to come into contact with the endothelium much less frequently. Thus, arterioles and arteries have a finite boundary layer of red-cell-poor plasma in contact with the endothelium. Consequently, the radial gradient for NO from the endothelium into the blood is somewhat less steep in arteries and arterioles compared to capillaries [39]. Mathematical modeling of this process indicates that adding cell-free Hb to the plasma produces a steeper radial NO gradient inward from the endothelial wall, a greater loss of NO across the luminal wall of the endothelium, a lower concentration of NO on the abluminal side of the thin endothelium, and a lower concentration of NO in vascular smooth muscle [39]. Whether the predicted decrease in vascular smooth muscle NO is sufficient to produce observable vasoconstriction after transfusion of non-extravasating Hb has not been resolved experimentally. However, with agonist-induced stimulation of endothelial NOS, dilation of brain pial arterioles possessing tight endothelial junctions remains normal and NO-dependent after transfusion of either crosslinked tetrameric Hb or polymeric Hb in healthy animals [40–42]. The concentration of Hb in the plasma is over three orders of magnitude greater than the

abluminal concentration required to impair the endothelial-dependent response. Although the presence of Hb in the plasma space might reduce NO bioavailability in vascular smooth muscle, this effect does not appear to be sufficient to scavenge all of the released NO or to limit the evoked endothelial-dependent response. Nevertheless, in diseased vessels in which endothelial-dependent dilation is already impaired, the possibility remains that plasma-based Hb might scavenge much of the remaining NO and reduce NO in smooth muscle to critically low levels. Because of this concern of intravascular scavenging of NO by acellular Hb, efforts have been directed at designing recombinant Hbs with reduced NO affinity [43, 44], using S-nitrosylated Hb to act as an NO donor [45, 46], and coadministering nitrite or other compounds with exchangeable NO stores that are protected from scavenging by Hb [47, 48] (see Chapter 5).

In addition to regulating arteriolar vascular tone, the endothelium plays an important role in regulating transport of other nutrients. In the intestines, transfusion of crosslinked tetrameric Hb was found to cause degranulation of mast cells, disrupt endothelial junctions, and increase vascular permeability [49, 50]. These effects were present to a lesser extent after transfusion of polymeric or PEGylated Hb. It is unclear how much of the residual effect of the larger Hb solutions was due to remaining amounts of tetrameric Hb in the solutions. The precise mechanisms of the increase in vascular permeability remain uncertain, but reduction of NO bioavailability, formation of prooxidant met-Hb, and release of iron are likely possibilities [51–54]. The gastrointestinal symptoms occurring acutely after transfusion of crosslinked tetrameric Hb [55] may be the result of intestinal endothelial damage. Whether similar endothelial damage occurs in other vascular beds has not been well explored.

3.8 Hypoxic Hypoxia

Changes in arteriolar diameter regulate O_2 transport to match changes in metabolic needs. The transport of O_2 is determined by blood flow and CaO_2. Decreases in CaO_2 can occur from a decrease in arterial pO_2 (hypoxic hypoxia), a decrease in RBC concentration (anemia), or a decrease in the ability of Hb to carry O_2 (e.g. carbon monoxide, methemoglobinemia). A decrease in CaO_2 produced by any of these mechanisms will act to decrease pO_2 in parenchymal cells. However, the decrease in oxygenation is sensed by a variety of pathways that signal vascular smooth muscle and produce vasodilation (Figure 3.2).

The mechanisms involved in sensing changes in tissue pO_2 are incompletely understood [4], but signaling mechanisms have been described in parenchymal cells, blood elements, endothelium, and vascular smooth muscle. Hypoxic parenchymal cells release adenosine and ATP, which can be broken down to adenosine. Adenosine acts on adenosine A_{2A} receptors on vascular smooth muscle to increase cAMP and produce vasodilation. RBCs can also serve as O_2 sensors. Deoxygenated Hb causes RBCs to release ATP, which acts on the purinergic receptors on endothelial cells to stimulate NO and PGI_2 [21]. Deoxygenated Hb also causes release of NO bound at cysteine 93 of Hb [56, 57]. The NO generated from RBCs is thought to be transported to vascular smooth muscle by stable chemical intermediaries to prevent autoscavenging of NO by other erythrocytes [58–60].

Hypoxia can also act directly on isolated arteries to produce dilation, through mechanisms that are not fully understood. As discussed above, 20-HETE is synthesized

from arachidonic acid by a cytochrome P450 enzyme in vascular smooth muscle, and 20-HETE permits myogenic constriction in response to an increase in transmural pressure by inhibiting K_{Ca} channels. Interestingly, the synthesis of 20-HETE is sensitive to pO_2 over the physiological range of 10–100 mm Hg [61]. Evidence in skeletal muscle and cerebral arteries implicates 20-HETE synthesis as one of the O_2-sensing mechanisms in vascular smooth muscle [61, 62]. 20-HETE contributes to the constriction induced by increases in oxygenation. For example, the transition from the fetal circulation to air breathing produces over a three-fold increase in arterial pO_2. Brain arterioles constrict to prevent increases in O_2 transport [63], and this constriction is blocked by an inhibitor of 20-HETE synthesis [64]. With hypoxia, decreases in 20-HETE may permit other hypoxic mediators to produce vasodilation [65]. Vascular smooth muscle also possesses K^+ channels that are sensitive to ATP (K_{ATP} channels). Blocking sulphonylurea receptors linked to these channels attenuates vasodilation during hypoxia [66, 67]. Because ATP concentration is not grossly decreased during hypoxia unless the hypoxia is sufficiently severe to produce ischemia, changes in ATP affinity for K_{ATP} channels may be one possible mechanism whereby K_{ATP} channels are activated during hypoxia. Finally, there is some evidence in newborn brain that EETs are important in hypoxic vasodilation [68]. The source of EETS in the brain may be hypoxic astrocytes [69]. Therefore, multiple mechanisms appear to contribute to regulating O_2 transport when CaO_2 is altered.

The efficiency of the signaling mechanisms in maintaining tight control of O_2 transport varies with the vascular bed. In metabolically active tissue, such as heart and brain, O_2 transport during hypoxia remains tightly matched to O_2 consumption such that O_2-extraction fraction is nearly unchanged during hypoxic hypoxia. In brain, the increase in cerebral blood flow is reciprocally related to CaO_2 such that O_2 transport and O_2 consumption are well-maintained during acute decreases in CaO_2 as low as 50% of normoxic levels. In individual brain capillaries, the lineal density of RBCs increases during hypoxia, and the flux of RBCs increases proportionately more in capillaries with low normoxic RBC flux than in those with relatively high normoxic flux [70]. Hence, capillary RBC flux becomes more homogenous.

With very severe reductions in CaO_2, however, cerebrovascular autoregulation to changes in perfusion pressure becomes limited by the vasodilation in hypoxic tissue. Consequently, the blood flow response in the brain becomes more dependent on cardiac function during severe hypoxia. Heart rate and cardiac output normally increase during hypoxic hypoxia, and the heart requires additional O_2 to perform increased work during hypoxia [71]. Thus, the heart requires potent vasodilation to increase O_2 transport when CaO_2 decreases. When the vasodilatory reserve is exhausted and the increase in heart rate limits the duration of diastole necessary for perfusing the left ventricular subendocardium, cardiac function becomes impaired and arterial blood pressure falls. A fall in arterial pressure will limit the increase in blood flow to the brain. Thus, with severe hypoxia (typically CaO_2 less than 50% of normal), O_2 transport to the brain can fall.

The situation is somewhat different with breath-hold diving. In this case, bradycardia occurs instead of tachycardia. The bradycardia reduces cardiac O_2 consumption, increases the duration of diastolic coronary perfusion, and thereby permits cardiac function to be sustained at very low CaO_2 levels. The switch from tachycardia to bradycardia during diving is the result of the carotid body chemoreflex being unopposed by vagal afferents that sense lung inflation and by nasal afferents sensing cold water. Furthermore, the

balance of vasoconstrictor peripheral chemoreflexes and the vasodilatory lung-inflation reflex modulates sympathetic vasoconstriction, particularly in the renal and splanchnic circulation [71]. Hence, hyperventilation during hypoxemia, as occurs during the ascent to altitude, limits peripheral vasoconstriction, whereas hypoxemia during breath-holding augments peripheral vasoconstriction. This peripheral vasoconstrictor response is well developed in diving mammals and redirects the limited O_2 stores to brain and heart. Therefore, the integrated response to hypoxic hypoxia is complex, and the neurohumoral effects can override the local mediators of vasodilation in specific vascular beds.

Changes in Hb O_2 affinity influence O_2 delivery during hypoxic hypoxia. For an equivalent reduction in arterial pO_2, a decrease in p50 results in a greater CaO_2. Thus, a smaller degree of vasodilation is required to maintain O_2 transport. Consequently, greater altitude can be attained because of the leftward shift of the O_2-dissociation curve produced by hyperventilation-induced alkalosis during severe hypoxemia. The leftward shift of the O_2 dissociation curve of fetal Hb is also beneficial to the fetus in maintaining a higher CaO_2 and permitting a greater vasodilatory reserve when placental blood flow becomes impaired. After birth, the p50 increases as fetal Hb is replaced by adult Hb. This increase in p50 under normoxic conditions will increase O_2 flux into the tissue, produce counterregulatory constriction, and increase fractional O_2 extraction. This effect is most apparent in perinatal brain, where blood flow decreases when low-p50 fetal Hb is replaced by high-p50 adult Hb [72, 73]. This effect of p50 on cerebral blood flow persists during hypoxemia when adjusting for changes in CaO_2 (Figure 3.3). For a given reduction in CaO_2, cerebral blood flow is lower and fractional O_2 extraction is higher when p50 is increased [72]. Therefore, the cerebral blood flow response to hypoxemia can be described by independent effects of CaO_2 and p50. These independent effects on blood flow are presumably due to independent effects on tissue pO_2, which feeds back to regulate arteriolar tone.

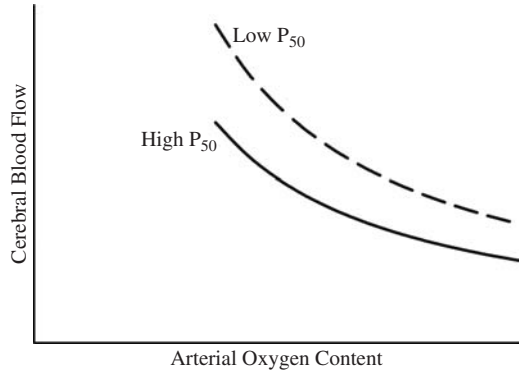

Figure 3.3 *Relationship of cerebral blood flow to arterial oxygen content during hypoxic hypoxia when the p50 of the RBC-based Hb is high (solid line) and low (dashed line). The relationship can be fit by a rectangular hyperbola, where the product of flow and oxygen content equals a constant and that constant is equivalent to oxygen transport. The low p50 increases the oxygen transport level at which the gain of the blood flow response is regulated during normoxia and hypoxia. (Based on data from [72]).*

3.9 Carbon Monoxide Hypoxia

Carbon monoxide hypoxia produces both a decrease in CaO_2 and a decrease in p50 and results in increased blood flow to brain, heart, skeletal muscle, and small intestine [74]. For a given reduction in CaO_2, the increase in cerebral blood flow produced by CO hypoxia (Figure 3.4) is greater than the increase in flow produced by hypoxic hypoxia [75, 76] or by methemoglobinemia [77]. This augmented response to CO hypoxia is partly attributed to the concurrent decrease in p50, because preventing the decrease in p50 lowers the increase in cerebral blood flow to levels seen with hypoxic hypoxia [78]. In addition, CO can have direct effects in dilating arteries by increasing cyclic GMP and opening K_{Ca} channels in vascular smooth muscle [79, 80].

Because of the enhanced vasodilation occurring with CO hypoxia, the vasodilatory reserve becomes exhausted more quickly with progressive reductions in CaO_2 compared to hypoxic hypoxia. In the heart, O_2 delivery becomes compromised when vasodilation can no longer compensate for the concurrent decrease in CaO_2 and p50. As cardiac function becomes impaired during severe CO hypoxia, arterial blood pressure falls, peripheral blood flow decreases, and O_2 delivery is no longer adequate to meet O_2 demand. In brain, the increase in cerebral blood flow becomes impaired when hypotension occurs. Then cerebral O_2 consumption eventually falls, and consciousness is lost. Prolonged periods of arterial hypotension accompanied by decreases in CaO_2 and p50 during severe CO hypoxia can produce permanent neuronal injury if CO is not rapidly cleared by the lungs and cardiac function is not restored.

3.10 Anemia

Anemia produces increases in blood flow, and polycythemia produces decreases in blood flow. In brain, the changes in blood flow nearly compensate for the changes in CaO_2,

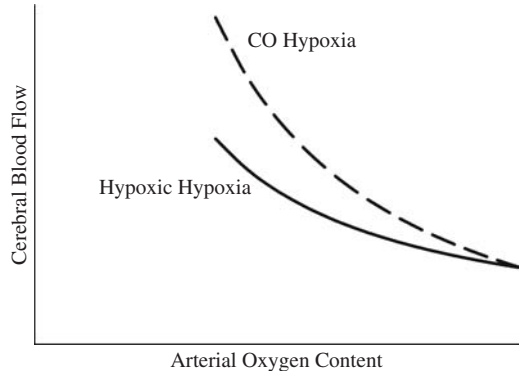

Figure 3.4 *Relationship of cerebral blood flow to arterial oxygen content during hypoxic hypoxia (solid line) when the p50 of the RBC-based Hb is constant and when cerebral oxygen transport is constant. With graded levels of CO hypoxia, arterial oxygen content and p50 decrease concurrently in a graded fashion and result in a progressively enhanced vasodilation and cerebral blood flow response (dashed line) that increases oxygen transport to compensate for the decrease in p50. (Based on data from [76, 78]).*

and cerebral O_2 transport and fractional O_2 extraction essentially are unchanged during anemia and polycythemia [81, 82]. However, the situation with anemia differs from hypoxic hypoxia in that the decrease in hematocrit during anemia decreases blood viscosity, which is an important component of vascular resistance. Hence, anemia does not require the same degree of vasodilation to maintain O_2 transport. In brain, the increase in blood flow during anemia is nearly the same as during hypoxic hypoxia at equivalent reductions in CaO_2 [81]. However, this increase in flow is achieved with either no change or only a small increase in arteriolar diameter [83–85]. When brain arterioles dilate during anemia, the dilation is blocked by inhibiting K_{ATP} channels [84]. Because K_{ATP} channels are important in vasodilation during hypoxic hypoxia, the K_{ATP} channel-dependent component of vasodilation during anemia may be attributable to tissue hypoxia.

The remaining nonvasodilatory component of the increase in cerebral blood flow is attributed to the decrease in viscosity. Viscosity is known to be important because transfusion of high-viscosity solutions under conditions of low hematocrit produces vasodilation to help maintain O_2 transport [86]. Viscosity influences shear stress along the endothelial wall, where mechanoreceptors regulate the production of endothelial-dependent vasodilators, such as NO. Therefore, the integrated response to anemia is the result of a balance between the physical effect of the decrease in hematocrit on viscosity, which acts to increase blood flow, and changes in vascular tone arising from tissue hypoxia (inducing decreased tone) and decreased endothelial-wall shear stress (inducing increased tone). The net effect is that O_2 transport to vital organs, such as brain and heart, is well maintained during anemia. Maintenance of bulk O_2 transport is also confirmed at the microcirculatory level. Hemodilution produces an increase in RBC velocity without a decrease in the lineal density of RBCs in individual brain capillaries, thereby increasing the capillary flux of RBCs [87]. In the mesenteric circulation, a more homogeneous distribution of RBCs has been described after hemodilution [88].

Cell-free Hb solutions can be administered as an exchange transfusion or as a topload transfusion. Exchange transfusion permits greater plasma Hb concentrations to be achieved without expanding blood volume, whereas topload transfusion is preferred for rescuing from extreme anemia. Exchange transfusion decreases hematocrit but does not produce the same increase in blood flow as that occurring with anemia [34]. In brain, O_2 transport remains essentially unchanged after exchange transfusion with acellular Hb [89, 90]. When the decrease in O_2 carried by RBCs is offset by the increase in O_2 carried by plasma-based Hb, cerebral blood flow remains unchanged. However, because blood viscosity can decrease when hematocrit decreases after exchange transfusion, maintenance of cerebral blood flow and O_2 transport requires vasoconstriction to offset the decrease in viscosity [40, 90]. This constriction is attributable to O_2-dependent synthesis of the vasoconstrictor 20-HETE rather than to scavenging of NO [84]. Thus, the 20-HETE-dependent constriction appears to represent a homeostatic regulatory mechanism to prevent over-oxygenation when erythrocyte-based Hb is replaced by cell-free Hb. Measurement of pO_2 in small cortical venules confirms that cell-free Hb better maintains oxygenation at reduced hematocrit [85].

Increasing plasma viscosity at reduced hematocrit augments vasodilation and, in the presence of acellular Hb, converts the vasoconstrictor response to a vasodilatory response to help maintain cerebral O_2 transport [86, 90]. This effect is somewhat unexpected

because mathematical modeling indicates that any increase in NO production stimulated by increased endothelial-wall shear stress will be scavenged by acellular Hb, and NO bioavailability will not increase in vascular smooth muscle [91]. However, increased shear stress may recruit vasodilators other than NO (Figure 3.2). Moreover, PEGylated-Hb solutions have been developed to provide greater viscosity than pure electrolyte solutions. In the peripheral circulation, PEGylated-Hb solutions appear to be superior in preserving a vasodilated state, possibly due to greater viscosity effects on endothelial-wall shear stress and to their low p50 [10, 92]. Maintaining wall shear stress may be important when using cell-free Hb to rescue tissue from severe anemia or from the inflammatory effects of sickle cell disease.

Hypoxic hypoxia can also be superimposed on to anemia. In this case, cerebral blood flow at low hematocrit remains higher than that at normal hematocrit for any given level of arterial pO_2 (Figure 3.5). However, at any given level of CaO_2, cerebral blood flow is equivalent at low and normal hematocrit during hypoxic hypoxia [81]. Moreover, when O_2 carrying capacity of whole blood at low hematocrit is restored by cell-free Hb, cerebral blood flow is no longer higher at each level of arterial pO_2, but rather remains appropriate for the level of CaO_2 [93]. Consequently, cerebral blood flow is a single function of CaO_2 at different levels of hematocrit, and cerebral O_2 transport is maintained constant during hypoxic hypoxia when hematocrit is reduced without a decrease in O_2 carrying capacity over a wide range of arterial pO_2. Importantly, plasma-based Hb does not interfere with cerebral or coronary vasodilation evoked by hypoxic hypoxia [93] or cerebral vasodilation evoked by direct activation of K_{ATP} channels [36], which participate in hypoxic vasodilation.

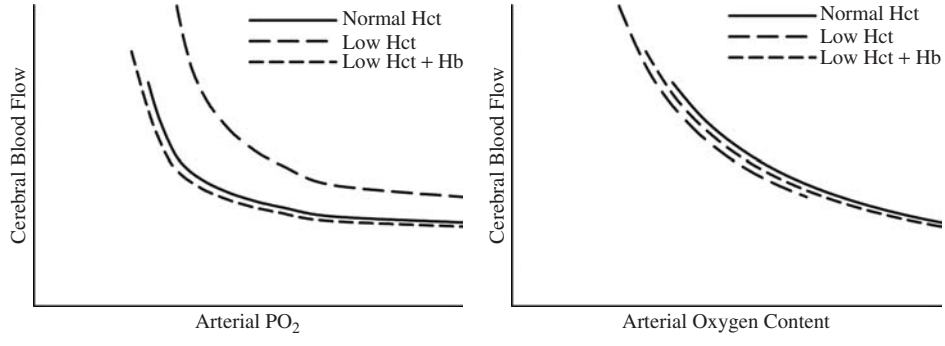

Figure 3.5 *Idealized relationship of cerebral blood flow to arterial pO_2 (a) and to arterial oxygen content (b) during hypoxic hypoxia under conditions of normal hematocrit, low hematocrit, and low hematocrit in which RBC-based Hb is replaced by cell-free Hb. Blood flow is higher with low hematocrit at every level of arterial pO_2 than with normal hematocrit or with low hematocrit at normal O_2 carrying capacity, but blood flow forms a single relationship when plotted against arterial O_2 content. (Based on data from [81, 93]).*

3.11 Conclusion

Vascular smooth muscle possesses intrinsic myogenic tone that varies directly with transmural pressure and contributes to the phenomenon of blood flow autoregulation in response to alterations in arterial pressure. Parenchymal cells release vasoactive signaling molecules in response to large reductions in arterial pressure, increases in energy metabolism, and decreases in CaO_2. However, the profile of signaling molecules can differ with each of these types of perturbation. Vasodilation in response to increased energy metabolism does not necessarily require tissue hypoxia. No evidence to date indicates that the presence of acellular Hb in the plasma space interferes with the myogenic response or parenchymal cell regulation of vasoactive control, although more work is needed in this area. Vascular smooth muscle also receives signals from the endothelium, which in turn are activated by signals from luminal mechanoreceptors, RBCs, platelets, and neurohumoral factors. Extravasation of a small fraction of transfused acellular Hb will scavenge tonically and dynamically released NO and result in tonic constriction and impaired dynamic vasodilation. In the absence of extravasation, Hb in the plasma space is thought to reduce NO bioavailability on the abluminal side, but this reduction does not appear to be sufficient to interfere with normal endothelial-dependent vasoactivity, possibly because of compensation by other signaling mechanisms. However, in situations with vascular disease where ROS are elevated and NO bioavailability is reduced, the addition of Hb to the plasma space may reduce NO bioavailability in vascular smooth muscle to critically low levels and impair normal vascular control.

References

[1] Gebremedhin, D., Lange, A.R., Lowry, T.F. *et al*. Production of 20-HETE and its role in autoregulation of cerebral blood flow, *Circ. Res*., **87**, 60–65 (2000).

[2] Roman, R.J. P-450 metabolites of arachidonic acid in the control of cardiovascular function, *Physiol. Rev*., **82**, 131–185 (2002).

[3] Harder, D.R. Pressure-dependent membrane depolarization in cat middle cerebral artery, *Circ. Res*., **55**, 197–202 (1984).

[4] Weir, E.K., Lopez-Barneo, J., Buckler, K.J. and Archer, S.L. Acute oxygen-sensing mechanisms, *N. Engl. J Med*., **353**, 2042–2055 (2005).

[5] Hughes, G.S., Yancey, E.P., Albrecht, R. *et al*. Hemoglobin-based oxygen carrier preserves submaximal exercise capacity in humans, *Clin. Pharmacol. Ther*., **58**, 434–443 (1995).

[6] Pittman, R.N. Oxygen transport and exchange in the microcirculation, *Microcirculation*, **12**, 59–70 (2005).

[7] Page, T.C., Light, W.R., McKay, C.B. and Hellums, J.D. Oxygen transport by erythrocyte/hemoglobin solution mixtures in an *in vitro* capillary as a model of hemoglobin-based oxygen carrier performance, *Microvasc. Res*., **55**, 54–64 (1998).

[8] Hellums, J.D. The resistance to oxygen transport in the capillaries relative to that in the surrounding tissue, *Microvasc. Res*, **13**, 131–136 (1977).

[9] Carvalho, H. and Pittman, R.N. Longitudinal and radial gradients of PO(2) in the hamster cheek pouch microcirculation, *Microcirculation*, **15**, 215–224 (2008).
[10] Tsai, A.G., Vandegriff, K.D., Intaglietta, M. and Winslow, R.M. Targeted O_2 delivery by low-P50 hemoglobin: a new basis for O_2 therapeutics, *Am. J. Physiol Heart Circ. Physiol*, **285**, H1411–H1419 (2003).
[11] McCarthy, M.R., Vandegriff, K.D. and Winslow, R.M. The role of facilitated diffusion in oxygen transport by cell-free hemoglobins: implications for the design of hemoglobin-based oxygen carriers, *Biophys. Chem*., **92**, 103–117 (2001).
[12] Page, T.C., Light, W.R. and Hellums, J.D. Prediction of microcirculatory oxygen transport by erythrocyte/hemoglobin solution mixtures, *Microvasc. Res*., **56**, 113–126 (1998).
[13] Vadapalli, A., Goldman, D. and Popel, A.S. Calculations of oxygen transport by red blood cells and hemoglobin solutions in capillaries, *Artif. Cells Blood Substit. Immobil. Biotechnol*., **30**, 157–188 (2002).
[14] Tsoukias, N.M., Goldman, D., Vadapalli, A. *et al*. A computational model of oxygen delivery by hemoglobin-based oxygen carriers in three-dimensional microvascular networks, *J. Theor. Biol*., **248**, 657–674 (2007).
[15] Federspiel, W.J. and Popel, A.S. A theoretical analysis of the effect of the particulate nature of blood on oxygen release in capillaries, *Microvasc. Res*., **32**, 164–189 (1986).
[16] Golub, A.S. and Pittman, R.N. Erythrocyte-associated transients in PO_2 revealed in capillaries of rat mesentery, *Am. J. Physiol. Heart Circ. Physiol*., **288**, H2735–H2743 (2005).
[17] Tsai, A.G., Cabrales, P., Johnson, P.C. *et al*. Effect of oxygen consumption by measuring method on PO_2 transients associated with the passage of erythrocytes in capillaries of rat mesentery, *Am. J. Physiol. Heart Circ. Physiol*., **289**, H1777–H1779 (2005).
[18] Hudetz, A.G. Blood flow in the cerebral capillary network: a review emphasizing observations with intravital microscopy, *Microcirculation*, **4**, 233–252 (1997).
[19] Villringer, A., Them, A., Lindauer, U. *et al*. Capillary perfusion of the rat brain cortex: an *in vivo* confocal microscopy study, *Circ. Res*., **75**, 55–62 (1994).
[20] Cabrales, P., Tsai, A.G. and Intaglietta, M. Microvascular pressure and functional capillary density in extreme hemodilution with low- and high-viscosity dextran and a low-viscosity Hb-based O_2 carrier, *Am. J. Physiol. Heart Circ. Physiol*., **287**, H363–H373 (2004).
[21] Ellsworth, M.L., Ellis, C.G., Goldman, D. *et al*. Erythrocytes: oxygen sensors and modulators of vascular tone, *Physiology*, **24**, 107–116 (2009).
[22] Sprague, R.S., Hanson, M.S., Achilleus, D. *et al*. Rabbit erythrocytes release ATP and dilate skeletal muscle arterioles in the presence of reduced oxygen tension, *Pharmacol. Rep*., **61**, 183–190 (2009).
[23] Liu, Y. and Gutterman, D.D. Vascular control in humans: focus on the coronary microcirculation, *Basic Res. Cardiol*., **104**, 211–227 (2009).
[24] Mendoza, S.A., Fang, J., Gutterman, D.D. *et al*. TRPV4-mediated endothelial Ca^{2+} influx and vasodilation in response to shear stress, *Am. J. Physiol. Heart Circ. Physiol*., **298**, H466–H476 (2010).

[25] Alonso-Galicia, M., Hudetz, A.G., Shen, H. *et al*. Contribution of 20-HETE to vasodilator actions of nitric oxide in the cerebral microcirculation, *Stroke*, **30**, 2727–2734 (1999).
[26] Sun, C.-W., Alonso-Galicia, M., Taheri, M.R. *et al*. Nitric oxide-20-hydroxyeicosatetraenoic acid interaction in the regulation of K^+ channel activity and vascular tone in renal arterioles, *Circ. Res*., **83**, 1069–1079 (1998).
[27] Fisslthaler, B., Popp, R., Kiss, L. *et al*. Cytochrome P450 2C is an EDHF synthase in coronary arteries, *Nature*, **401**, 493–497 (1999).
[28] Campbell, W.B., Gebremedhin, D., Pratt, P.F. and Harder, D.R. Identification of epoxyeicosatrienoic acids as endothelium-derived hyperpolarizing factors, *Circ. Res*., **78**, 415–423 (1996).
[29] Dietrich, H.H., Horiuchi, T., Xiang, C. *et al*. Mechanism of ATP-induced local and conducted vasomotor responses in isolated rat cerebral penetrating arterioles, *J. Vasc. Res*., **46**, 253–264 (2009).
[30] Larsen, B.T., Gutterman, D.D., Sato, A. *et al*. Hydrogen peroxide inhibits cytochrome p450 epoxygenases: interaction between two endothelium-derived hyperpolarizing factors, *Circ. Res*, **102**, 59–67 (2008).
[31] Larsen, B.T., Bubolz, A.H., Mendoza, S.A. *et al*. Bradykinin-induced dilation of human coronary arterioles requires NADPH oxidase-derived reactive oxygen species, *Arterioscler. Thromb. Vasc. Biol*., **29**, 739–745 (2009).
[32] Forstermann, U. and Munzel, T. Endothelial nitric oxide synthase in vascular disease: from marvel to menace, *Circulation*, **113**, 1708–1714 (2006).
[33] Ryoo, S., Lemmon, C.A., Soucy, K.G. *et al*. Oxidized low-density lipoprotein-dependent endothelial arginase II activation contributes to impaired nitric oxide signaling, *Circ. Res*., **99**, 951–960 (2006).
[34] Ulatowski, J.A., Nishikawa, T., Matheson-Urbaitis, B. *et al*. Regional blood flow alterations after bovine fumaryl ββ-crosslinked hemoglobin transfusion and nitric oxide synthase inhibition, *Crit. Care Med*., **24**, 558–565 (1996).
[35] Matheson, B., Kwansa, H.E., Bucci, E. *et al*. Vascular response to infusions of a nonextravasating hemoglobin polymer, *J. Appl. Physiol*., **93**, 1479–1486 (2002).
[36] Sampei, K., Ulatowski, J.A., Asano, Y. *et al*. Role of nitric oxide scavenging in vascular response to cell-free hemoglobin transfusion, *Am. J. Physiol. Heart Circ. Physiol*., **289**, H1191–H1201 (2005).
[37] Gulati, A., Sharma, A.C. and Singh, G. Role of endothelin in the cardiovascular effects of diaspirin crosslinked hemoglobin, *Crit. Care Med*., **24**, 137–147 (1996).
[38] Olofsson, C., Nygards, E.B., Ponzer, S. *et al*. A randomized, single-blind, increasing dose safety trial of an oxygen-carrying plasma expander (Hemospan) administered to orthopaedic surgery patients with spinal anaesthesia, *Transfus. Med*., **18**, 28–39 (2008).
[39] Kavdia, M., Tsoukias, N.M. and Popel, A.S. Model of nitric oxide diffusion in an arteriole: impact of hemoglobin-based blood substitutes, *Am. J. Physiol. Heart Circ. Physiol*., **282**, H2245–H2253 (2002).
[40] Asano, Y., Koehler, R.C., Ulatowski, J.A. *et al*. Effect of crosslinked hemoglobin transfusion on endothelial dependent dilation in feline pial arterioles, *Am. J. Physiol. Heart Circ. Physiol*., **275**, H1313–H1321 (1998).

[41] Rebel, A., Cao, S., Kwansa, H. *et al*. Dependence of acetylcholine and ADP dilation of pial arterioles on heme oxygenase after transfusion of cell-free polymeric hemoglobin, *Am. J. Physiol. Heart Circ. Physiol*., **290**, H1027–H1037 (2006).

[42] Qin, X., Kwansa, H., Bucci, E. *et al*. Role of heme oxygenase-2 in pial arteriolar response to acetylcholine in mice with and without transfusion of cell-free hemoglobin polymers, *Am. J. Physiol. Regul. Integr. Comp. Physiol*., **295**, R498–R504 (2008).

[43] Doherty, D.H., Doyle, M.P., Curry, S.R. *et al*. Rate of reaction with nitric oxide determines the hypertensive effect of cell-free hemoglobin, *Nat. Biotechnol*., **16**, 672–676 (1998).

[44] Resta, T.C., Walker, B.R., Eichinger, M.R. and Doyle, M.P. Rate of NO scavenging alters effects of recombinant hemoglobin solutions on pulmonary vasoreactivity, *J. Appl. Physiol*., **93**, 1327–1336 (2002).

[45] Kawaguchi, A.T., Nakai, K., Fukumoto, D. *et al*. S-nitrosylated pegylated hemoglobin reduces the size of cerebral infarction in rats, *Artif. Organs*, **33**, 183–188 (2009).

[46] Otani, H., Jesmin, S., Togashi, H. *et al*. An S-nitrosylated hemoglobin derivative protects the rat hippocampus from ischemia-induced long-term potentiation impairment with a time window, *J. Pharmacol. Sci*., **96**, 188–198 (2004).

[47] He, X., Azarov, I., Jeffers, A. *et al*. The potential of Angeli's salt to decrease nitric oxide scavenging by plasma hemoglobin, *Free Radic. Biol. Med*., **44**, 1420–1432 (2008).

[48] Minneci, P.C., Deans, K.J., Shiva, S. *et al*. Nitrite reductase activity of hemoglobin as a systemic nitric oxide generator mechanism to detoxify plasma hemoglobin produced during hemolysis, *Am. J. Physiol. Heart Circ. Physiol*., **295**, H743–H754 (2008).

[49] Baldwin, A.L. Modified hemoglobins produce venular interendothelial gaps and albumin leakage in the rat mesentery, *Am. J. Physiol*., **277**, H650–H659 (1999).

[50] Baldwin, A.L., Wiley, E.B. and Alayash, A.I. Comparison of effects of two hemoglobin-based O(2) carriers on intestinal integrity and microvascular leakage, *Am. J. Physiol. Heart Circ. Physiol*., **283**, H1292–H1301 (2002).

[51] Baldwin, A.L., Wiley, E.B., Summers, A.G. and Alayash, A.I. Sodium selenite reduces hemoglobin-induced venular leakage in the rat mesentery, *Am. J. Physiol. Heart Circ. Physiol*., **284**, H81–H91 (2003).

[52] Baldwin, A.L., Wiley, E.B. and Alayash, A.I. Differential effects of sodium selenite in reducing tissue damage caused by three hemoglobin-based oxygen carriers, *J. Appl. Physiol*., **96**, 893–903 (2004).

[53] Baldwin, A.L., Demaria, L. and Wiley, E.B. Effects of phosphorothioate oligodeoxynucleotide on hemoglobin-induced damage to intestinal mucosa, *Artif. Cells Blood Substit. Immobil. Biotechnol*., **33**, 163–186 (2005).

[54] Burke, T.K., Teng, X., Patel, R.P. and Baldwin, A.L. Effects of S-nitrosation on hemoglobin-induced microvascular damage, *Antioxid. Redox Signal*., **8**, 1093–1101 (2006).

[55] Przybelski, R.J., Daily, E.K., Kisicki, J.C. *et al*. Phase I study of the safety and pharmacologic effects of diaspirin cross-linked hemoglobin solution, *Crit. Care Med*., **24**, 1993–2000 (1996).

[56] Stamler, J.S., Jia, L., Eu, P. *et al*. Blood flow regulation by S-nitrosohemoglobin in the physiological oxygen gradient, *Science*, **276**, 2034–2037 (1997).

[57] McMahon, T.J., Moon, R.E., Luschinger, B.P. *et al*. Nitric oxide in the human respiratory cycle, *Nat. Med*., **8**, 711–717 (2002).

[58] Cosby, K., Partovi, K.S., Crawford, J.H. *et al*. Nitrite reduction to nitric oxide by deoxyhemoglobin vasodilates the human circulation, *Nat. Med*., **9**, 1498–1505 (2003).

[59] Basu, S., Grubina, R., Huang, J. *et al*. Catalytic generation of N_2O_3 by the concerted nitrite reductase and anhydrase activity of hemoglobin, *Nat. Chem. Biol*., **3**, 785–794 (2007).

[60] Pawloski, J.R., Hess, D.T. and Stamler, J.S. Export by red blood cells of nitric oxide bioactivity, *Nature*, **409**, 622–626 (2001).

[61] Harder, D.R., Narayanan, J., Birks, E.K. *et al*. Identification of a putative microvascular oxygen sensor, *Circ. Res*., **79**, 54–61 (1996).

[62] Lombard, J.H., Kunert, M.P., Roman, R.J. *et al*. Cytochrome P-450 omega-hydroxylase senses O_2 in hamster muscle, but not cheek pouch epithelium, microcirculation, *Am. J. Physiol. Heart Circ. Physiol*., **276**, H503–H508 (1999).

[63] Gleason, C.A., Jones, M.D. Jr, Traystman, R.J. and Notter, R.H. Fetal cerebral responses to ventilation and oxygenation *in utero*, *Am. J. Physiol. Regul. Integr. Comp. Physiol*., **255**, R1049–R1054 (1988).

[64] Ohata, H., Gebremedhin, D., Narayanan, J. *et al*. Onset of pulmonary ventilation in fetal sheep produces pial arteriolar constriction dependent on cytochrome p450 omega-hydroxylase activity, *J. Appl. Physiol*., **109**, 412–417 (2010).

[65] Gebremedhin, D., Yamaura, K. and Harder, D.R. Role of 20-HETE in the hypoxia-induced activation of Ca^{2+}-activated K^+ channel currents in rat cerebral arterial muscle cells, *Am. J. Physiol. Heart Circ. Physiol*., **294**, H107–H120 (2008).

[66] Taguchi, H., Heistad, D.D., Kitazono, T. and Faraci, F.M. ATP-sensitive K^+ channels mediate dilatation of cerebral arterioles during hypoxia, *Circ. Res*., **74**, 1005–1008 (1994).

[67] Tomiyama, Y., Brian, J.E. Jr and Todd, M.M. Cerebral blood flow during hemodilution and hypoxia in rats: role of ATP-sensitive potassium channels, *Stroke*, **30**, 1942–1947 (1999).

[68] Leffler, C.W., Smith, J.S., Edrington, J.L. *et al*. Mechanisms of hypoxia-induced cerebrovascular dilation in the newborn pig, *Am. J. Physiol. Heart Circ. Physiol*., **272**, H1323–H1332 (1997).

[69] Yamaura, K., Gebremedhin, D., Zhang, C. *et al*. Contribution of epoxyeicosatrienoic acids to the hypoxia-induced activation of Ca(2+)-activated K(+) channel current in cultured rat hippocampal astrocytes, *Neuroscience*, **143**, 703–716 (2006).

[70] Krolo, I. and Hudetz, A.G. Hypoxemia alters erythrocyte perfusion pattern in the cerebral capillary network, *Microvasc. Res*., **59**, 72–79 (2000).

[71] Koehler, R.C., McDonald, B.W. and Krasney, J.A. Influence of CO_2 on cardiovascular response to hypoxia in conscious dogs, *Am. J. Physiol*., **239**, H545–H558 (1980).

[72] Koehler, R.C., Traystman, R.J. and Jones, M.D. Jr. Influence of reduced oxygemoglobin affinity on cerebrovascular response to hypoxic hypoxia, *Am. J. Physiol. Heart Circ. Physiol*., **251**, H756–H763 (1986).
[73] Rosenberg, A.A., Harris, A.P., Koehler, R.C. *et al*. Role of O_2-hemoglobin affinity in the regulation of cerebral blood flow in fetal sheep, *Am. J. Physiol. Heart Circ. Physiol*., **251**, H56–H62 (1986).
[74] Koehler, R.C., Traystman, R.J. and Jones, M.D. Jr. Regional blood flow and O_2 transport during hypoxic and CO hypoxia in neonatal and adult sheep, *Am. J. Physiol. Heart Circ. Physiol*., **248**, H118–H124 (1985).
[75] Koehler, R.C., Jones, M.D. Jr and Traystman, R.J. Cerebral circulatory response to carbon monoxide and hypoxic hypoxia in the lamb, *Am. J. Physiol. Heart Circ. Physiol*., **243**, H27–H32 (1982).
[76] Koehler, R.C., Traystman, R.J., Zeger, S. *et al*. Comparison of cerebrovascular response to hypoxic and carbon monoxide hypoxia in newborn and adult sheep, *J. Cereb. Blood Flow. Metab*., **4**, 115–122 (1984).
[77] Hudak, M.L., Koehler, R.C., Rosenberg, A.A. *et al*. Effect of hematocrit on cerebral blood flow, *Am. J. Physiol. Heart Circ. Physiol*., **251**, H63–H70 (1986).
[78] Koehler, R.C., Traystman, R.J., Rosenberg, A.A. *et al*. Role of O_2-hemoglobin affinity on cerebrovascular response to carbon monoxide hypoxia, *Am. J. Physiol. Heart Circ. Physiol*., **245**, H1019–H1023 (1983).
[79] Jaggar, J.H., Leffler, C.W., Cheranov, S.Y. *et al*. Carbon monoxide dilates cerebral arterioles by enhancing the coupling of Ca^{2+} sparks to Ca^{2+}-activated K^+ channels, *Circ. Res*., **91**, 610–617 (2002).
[80] Jaggar, J.H., Li, A., Parfenova, H. *et al*. Heme is a carbon monoxide receptor for large-conductance Ca^{2+}-activated K^+ channels, *Circ. Res*., **97**, 805–812 (2005).
[81] Jones, M.D. Jr, Traystman, R.J., Simmons, M.A. and Molteni, R.A. Effects of changes in arterial O_2 content on cerebral blood flow in the lamb, *Am. J. Physiol. Heart Circ. Physiol*., **240**, H209–H215 (1981).
[82] Massik, J., Tang, Y.L., Hudak, M.L. *et al*. Effect of hematocrit on cerebral blood flow with induced polycythemia, *J. Appl. Physiol*., **62**, 1090–1096 (1987).
[83] Hurn, P.D., Traystman, R.J., Shoukas, A.A. and Jones, M.D. Jr. Pial microvascular hemodynamics in anemia, *Am. J. Physiol. Heart Circ. Physiol*., **264**, H2131–H2135 (1993).
[84] Qin, X., Kwansa, H., Bucci, E. *et al*. Role of 20-HETE in the pial arteriolar constrictor response to decreased hematocrit after exchange transfusion of cell-free polymeric hemoglobin, *J. Appl. Physiol*., **100**, 336–342 (2006).
[85] Vovenko, E., Golub, A. and Pittman, R. Microvascular PO_2 and blood velocity measurements in rat brain cortex during hemodilution with a plasma expander (Hespan) and a hemoglobin-based oxygen carrier (DCLHb), *Adv. Exp. Med. Biol*., **540**, 215–220 (2003).
[86] Rebel, A., Lenz, C., Krieter, H. *et al*. Oxygen delivery at high blood viscosity and decreased arterial oxygen content to brains of conscious rats, *Am. J. Physiol. Heart Circ. Physiol*., **280**, H2591–H2597 (2001).
[87] Hudetz, A.G., Wood, J.D., Biswal, B.B. *et al*. Effect of hemodilution on RBC velocity, supply rate, and hematocrit in the cerebral capillary network, *J. Appl. Physiol*., **87**, 505–509 (1999).

[88] Pries, A.R., Fritzsche, A., Ley, K. and Gaehtgens, P. Redistribution of red blood cell flow in microcirculatory networks by hemodilution, *Circ. Res*, **70**, 1113–1121 (1992).

[89] Ulatowski, J.A., Bucci, E., Nishikawa, T. *et al*. Cerebral O_2 transport with hematocrit reduced by cross-linked hemoglobin transfusion, *Am. J. Physiol. Heart Circ. Physiol*., **270**, H466–H475 (1996).

[90] Rebel, A., Ulatowski, J.A., Kwansa, H. *et al*. Cerebrovascular response to decreased hematocrit: effect of cell-free hemoglobin, plasma viscosity, and CO_2, *Am. J. Physiol. Heart Circ. Physiol*., **285**, H1600–H1608 (2003).

[91] Kavdia, M. and Popel, A.S. Wall shear stress differentially affects NO level in arterioles for volume expanders and Hb-based O_2 carriers, *Microvasc. Res*., **66**, 49–58 (2003).

[92] Tsai, A.G., Acero, C., Nance, P.R. *et al*. Elevated plasma viscosity in extreme hemodilution increases perivascular nitric oxide concentration and microvascular perfusion, *Am. J. Physiol. Heart Circ. Physiol*., **288**, H1730–H1739 (2005).

[93] Ulatowski, J.A., Bucci, E., Razynska, A. *et al*. Cerebral blood flow during hypoxic hypoxia with plasma-based hemoglobin at reduced hematocrit, *Am. J. Physiol. Heart Circ. Physiol*., **274**, H1933–H1942 (1998).

4

The Main Players: Hemoglobin and Myoglobin; Nitric Oxide and Oxygen

Tim J. McMahon[1] *and Joseph Bonaventura*[2]

[1]*Department of Medicine, Duke University and Durham VA Medical Centers, Durham, NC, USA*

[2]*The Nicholas School of the Environment Marine Laboratory, Beaufort, NC, USA and The Bonaventis Research Laboratory, Mayaguez, PR, USA*

4.1 Introduction

In this chapter we discuss the purpose and mechanistic functioning of hemoglobin (Hb) and myoglobin (Mb), emphasizing their interaction(s) with NO and O_2. An evolutionary view of the two hemoproteins' structures and functions sheds light on adaptive, blood flow-optimizing interactions with O_2, NO, and other gases and allosteric effectors, informing the rational development of therapeutics capable of delivering both O_2 and clinical benefit.

4.2 Role of Mammalian Mb in O_2 Homeostasis

In mammals, Mb is a hemoprotein found in muscle cells that serves a set of functions mirroring those of Hb [1]. Namely, Mb efficiently binds O_2 released from circulating red-blood-cell (RBC) Hb, and serves as a reservoir for O_2 in muscle so that the mitochondrial electron transport chain is supplied continuously and protected from fluctuations in O_2

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supply related to myocontractile or circulatory (pulsatile) changes. Mb differs from Hb in several respects. In humans, monomeric Mb assumes only one tertiary conformation; accordingly, the O_2-binding curve for Mb is in the shape of a rectangular hyperbola rather than being sigmoidal. Additional roles for Mb as an O_2 sensor or O_2 scavenger have also been proposed. Generally speaking, Mb is insensitive to allosteric effectors, with the important possible exception of the lactate anion, a byproduct of anaerobic metabolism. While an Mb "lactate effect" has been reported, some researchers (including one of us; J.B., personal communication) have been unable to show it for a variety of Mbs. The high O_2 affinity of Mb promotes the maintenance of a robust gradient in pO_2 from the extraerythrocytic plasma to the intracellular space of myocytes.

4.3 What's Missing in the Mb Knockout Mouse

New insights into the roles of Mb *in vivo* were facilitated by the production by two independent research groups of Mb-knockout mice [2, 3]. To the surprise of many, the mice appeared normal in nearly all respects under baseline conditions, according to initial studies. Additional studies revealed that the normal appearance masked a set of anatomical and physiological compensatory changes. Specifically, the "myo$^{-/-}$" mouse was characterized by increased capillary density in the Mb-rich skeletal muscle and mycocardium, decreased width of the cardiomyocyte, increased hematocrit, and increases in both basal coronary blood flow and coronary blood flow reserve. These changes had the common effect of steepening the pO_2 gradient from the blood to the mitochondrial electron transport chain, thereby compensating for the absence of Mb. Later work revealed that in spite of the various compensatory mechanisms identified, the myo$^{-/-}$ mouse was unable to match wild-type mice in cardiac contractility, cardiac output or exercise endurance.

Remarkably, humans are in the minority among vertebrates in that their Mb contains a reactive Cys (residue 110) capable of sustaining *S*-nitrosylation. Hence, human and a small number of other vertebrate Mb can bind NO at either its heme prosthetic group or at Cys110 [4–6]. Like that of Hb, Mb's unliganded heme can bind NO in either the reduced (ferrous) or oxidized (ferric) state, although the former is strongly favored. Reaction of NO with liganded MbO_2 yields NO_3^- and met-Mb, reminiscent of the "oxy-Hb" reaction of mammalian Hb. Formation of *S*-nitrosylated Mb requires conversion of NO into a nitrosating agent (that is, an NO derivative such as N_2O_3 that has nitrosonium (NO^+) character). Although kinetic modeling has suggested that when NO encounters oxy-Mb, *S*-nitrosothiol (SNO) formation can compete favorably with the nitrate-producing reaction, the relative yields of these species have not been carefully assayed under *in vivo* conditions. Whether SNO-Mb can, efficiently and under physiological conditions [7], release NO/SNO equivalents has also been examined. These findings suggest a functional purview beyond the NO scavenging and detoxification roles ascribed to SNO-Mb based on the known biochemistry [8]. Nevertheless, monomeric Mb has no quaternary structure and would probably not respond to the diverse set of biochemical cues to which Hb answers. Certainly, the proposed ability of Mb to divert NO from inhibiting cytochrome *c* oxidase (the terminal electron acceptor in mitochondria of red muscle) would be an important function in its own right. Interestingly, the inferior exercise endurance phenotype [9] documented in the myo$^{-/-}$ mice appears to be related not only to a weak link in the O_2 supply relay, but also to a reduced ability to scavenge excessive NO.

4.4 Evolutionary Origins of Mb and the Nitrogen Cycle

In an evolutionary sense, Mbs arose very early and their "original" role may well not have involved O_2 at all, but metabolism of diatomic gaseous molecules like NO, carbon monoxide, and other gaseous ligands [10]. In plants, precursors of Mb can be found in the high-affinity leghemoglobin (legHb) of the nodules of nitrogen-fixing plants. LegHb is consequently capable of maintaining the very low pO_2 necessary for nitrogen fixation, as well as supplying to bacteroids, via facilitated O_2 diffusion, the O_2 necessary for oxidative phosphorylation and thus the ATP needed for the nitrogenase. In contrast to the classical view that Mbs and Hbs uniformly originated from a common ancestor, the discovery of Mbs bearing structural and functional resemblance to the human indoleamine dioxygenase (IDO) further underscores the multiplicity of Mb reactivity toward the biological gases (CO, O_2, and NO) [11, 12].

The coelomic monomeric globin of annelid worms underscores further the role of Mb in facilitated diffusion: in those invertebrates, a vascular Hb relays O_2 to a higher-affinity coelomic Hb, storing the O_2 or transferring it later to tissue; this Hb's supreme O_2 affinity reinforces the O_2 gradient and thus its flow ultimately to mitochondria [13].

The complexity of invertebrate Hb structure is such that NO reactivity is easily altered and controlled [14]. This variety suggests that invertebrate Hbs may serve a multiplicity of roles, particularly within the realm of control of redox molecules. The first Hbs evolved before the presence of O_2 within our atmosphere. Furthermore, the principal function of the primordial prokaryotic Hbs appears to be consumption of NO equivalents to provide protection from nitrosative stress. Many of the invertebrate Hbs appear to contain structural elements designed to promote their redox activity. There are microorganism Hbs that additionally function to control nitrosative stress, plant Hbs that regulate NO levels, and the Hb from *Ascaris*, which uses NO to modulate O_2 levels [15–19]. These observations have led to the proposition that Hbs have evolved in part to handle NO and that their use as an O_2 transport protein is a relatively recent evolutionary development [15].

4.5 Human Hb: Evolved Sensor of pO_2 and Redox

By sensing ambient extra-RBC conditions via metabolically informative allosteric effectors such as CO_2 and proton concentration (pH), Hb's redox-sensing effectors serve to optimize delivery of the "nutrient" oxygen, thereby maintaining metabolic homeostasis. In the following sections we discuss how these metabolic signals influence the behavior of Hb, which in turn delivers O_2 directly and indirectly – via blood flow-augmenting (vasodilating) NO.

4.6 Broad Reactivity and Influence of NO: Lessons from the Microcosm Hb

Because of its rich redox chemistry, the small gaseous molecule NO interacts promiscuously with biological targets across a wide set of chemical and biochemical classes. The biological functions and organ systems subject to the influence of NO are accordingly

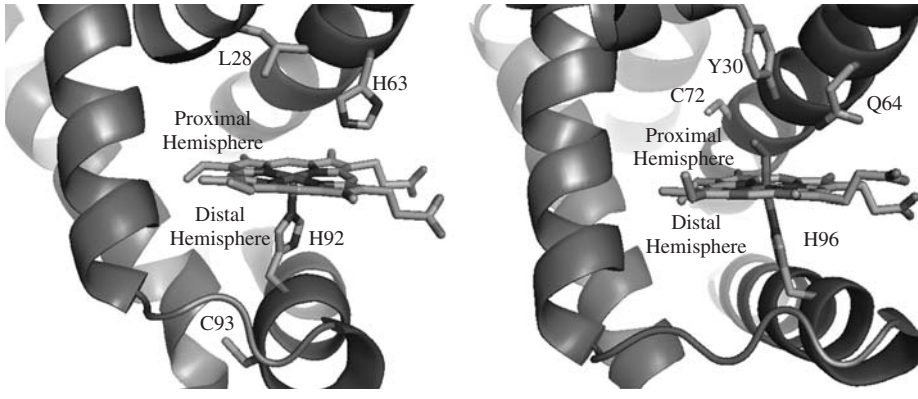

Figure 4.1 *The positioning of Cysβ93 in the proximal heme cavity of human Hb (PDB ID: 2DN1) (left) hinders reactions between Cysβ93 (NO) and heme-bound dioxygen, and thereby promotes the preservation and delivery of NO bioactivity. In contrast (at right), NO scavenging is favored by the close proximity of Cys(NO) and heme-bound dioxygen in* Ascaris *Hb (PDB ID: 1ASH). The stability of heme-bound O_2 is increased through electrophilic interaction with the hydroxyl side chain of Y30, and Q64 (position E10) enhances the rate of binding of NO to ferric hemes and thereby facilitates S-nitrosylation of C72. In human Hb versus* Ascaris *Hb, L28 is found at position B10 in place of Y30, and facilitates O_2 delivery by increasing k_{off}. Position E10 is occupied by H63 in human Hb and in all mammals and birds. C(β)93 and H92, which are located in the proximal hemisphere of the heme cavity, are similarly conserved.*

varied, including the cardiovascular, immunological, and nervous systems, to name just a few. A similarly broad purview of regulation by NO has increasingly been demonstrated throughout the phylogenetic tree, from microorganisms, plants, and an amazing variety of animal life, all the way to the human species. For decades, Hb has served as the "Type-Case" or even "Hydrogen Atom of Molecular Biology" (the term used by Max Perutz), demonstrating in its structure–function relationships fundamental life processes that are exemplified in proteins, nucleic acids, and other biological macromolecules. In the biology of NO too, the globins provide powerful examples. As a model O_2-transport protein and an enzymatic channel, Hb's rich biological life is evident.

An evolutionary examination of NO–Hb interactions demonstrates that the need to respond to and channel NO is such a key pressure in Hb's history that one can call Hb "**a protein that *also* binds oxygen**". Invertebrate Hbs in particular are remarkable for the ease with which their reactivity towards NO is altered and controlled (Figure 4.1). Specifically, coincident with the rise of the plant kingdom, when atmospheric O_2 became increasingly prevalent, primitive Hbs became O_2-detoxification enzymes, minimizing the nitrosative stress that would otherwise result from NO reacting with O_2 [14, 20].

4.7 Some Fish Demonstrate a Fundamental "Need" for Hb-dependent NO Cycling, as in Humans

The reactive cysteine residue conserved in the Hbs of all mammals and birds, Cys93 (in humans), appears to have earlier origins than were previously thought. Specifically, we

have identified in Hb of the Spot fish (*Leiostomus xanthuurus*), a Cys residue (alpha 133) with conformation-dependent reactivity toward NO species: when exposed to the (S)NO donor *S*-nitrosocysteine (SNO-Cys), this particular residue gained a 29 mass unit group according to electrospray mass spectroscopy, and the rate of protein *S*-nitrosylation was faster in the oxygenated conformation than in deoxy. Conversely (and in accordance with the principles of thermodynamic linkage predicting this reciprocal relationship), the rates of SNO group loss were faster in the deoxy state than in the oxy conformation. The existence of this SNO cycle *in vivo* was suggested by assays of Hb-bound SNO groups in the oxygenated and deoxygenated blood from freshly caught Spot fish, showing the abundance of SNO in the former and a paucity in the latter (J.B., unpublished observations). O_2 equilibrium curves constructed with NO-liganded and NO-free Spot Hb indicated a positive effect of *S*-nitrosylation on O_2 affinity in Spot Hb (Figure 4.2), and pointed to a T state-destabilizing effect as the basis for this, consistent with findings in mammalian Hb [21]. Taken together, these findings support the theory that the conformationally sensitive Cys residue in Hb does not need to be in the Cysβ93 position. Further studies are needed to define at what point the critical residue – regardless of position – was introduced. Interestingly, some fish, such as the Blue Fin tuna, have Hbs possessing the Cysβ93. In this fish, preliminary studies suggest that a "respiratory SNO cycle" like that of mammals and the Spot fish is operative. These phylogenetic findings promise to tell a very interesting story about the evolution of an SNO cycle in vertebrates that is so important that even if the "critical" β chain Cys93 is left out, another conformationally-sensitive Cys residue, even on a different subunit (the α chain), comes in to "rescue" it!

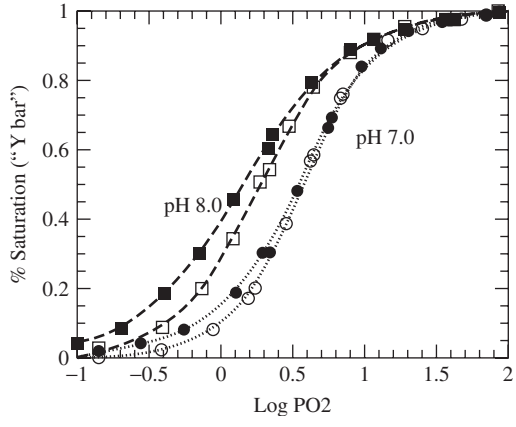

Figure 4.2 *Oxygen equilibrium curves (OECs) of unmodified Spot Hb (open symbols) and SNO-Spot Hb (closed symbols) at pH 7 (circles) and pH 8 (squares). S-nitrosylation shifts the lower part of the OECs toward higher O_2 affinity while having little or no effect on higher levels. This effect is just like that observed with human HbA, suggesting that S-nitrosylation stabilizes the oxygenated derivative of SNO-Hbs rather than destabilizing the deoxy form of the protein. (This figure originally appeared as Figure 9b in* Amer. Zool.*,* ***41****(2), (2001), pp. 346–359 [21] and was reproduced with explicit permission from Oxford University Press).*

4.8 Reactions of NO with Hb that Preserve NO Bioactivity

Earlier understanding held that the reaction between NO and hemoproteins, exemplified by Hb, led to irreversible or dead-end products. The classical example was the "oxy-hemoglobin" reaction of oxygenated Hb (oxy-Hb) with NO, leading to formation of oxidized Hb (met-Hb) and the relatively inert nitrate. A critical element in experiments illuminating the alternative processing by Hb of NO was the attention to *physiologically relevant* concentrations of Hb, NO, and the *ratio* of the two reactants [22]. Unlike O_2, whose concentration (tens of millimolar) and arterial-venous gradients (millimolar) reflect its role as a metabolic fuel, Hb-bound NO is a minority species, reflecting its role as the signaling arm in an O_2 delivery vehicle where Hb is the O_2 sensor. In humans, only ~1 in 1000 or fewer of the intra-RBC Hb tetramers bind an NO group under normal conditions [23]. When such conditions are accounted for experimentally, "NO addition" to Hb is demonstrated to compete favorably with the nitrate- and met-Hb-forming "oxy-Hb" reaction [24]. Specifically, even in arterial blood from healthy humans, oxygenated Hb makes up ~99.0% of the total Hb population, therefore the population of vacant hemes represented by the ~1% deoxygenated Hb still represents a substantial excess relative to the NO present in blood as a free radical.

Within the cardiorespiratory cycle, the binding and release of O_2 by Hb are linked to further channeling of NO in a manner that preserves, rather than quenches, its bioactivity (Figure 4.3). Namely, the allosteric transition from the T (tense, deoxygenated) to the R (relaxed, oxygenated) conformation in Hb triggers the conversion of Hb(FeII)NO (iron nitrosylhemoglobin, in which one or two of the remaining three hemes are typically vacant) into S-nitrosohemoglobin: $Hb([FeII]O_2)_4Cys\beta93NO$. Molecular O_2 can fulfill the requirement for an electron acceptor for this reaction, and erythrocytic superoxide dismutase (SOD) serves to eliminate superoxide formed from this reaction, as well as from autoxidation of Hb with respiratory cycling. Other electron sinks, such as the intra-RBC oxidized nicotinamide phosphates, might fulfill this important part of the chemical reaction.

4.9 Mammalian RBC/Hb–NO Interactions

The fate of all NO reacting with RBC Hb was once believed to be either (essentially irreversible) production of nitrate, through the "oxy-hemoglobin" reaction of NO with oxy-Hb (in which met-Hb is the other product), or the "addition" reaction in which NO binds to vacant hemes in Hb, forming iron nitrosyl-Hb (Hb[FeII]NO, also considered a "dead-end" product) [24, 25]. Stamler and colleagues reported in 1996 that, beyond these products, Hb in oxygenated R conformation binds NO equivalents at its highly conserved Cysβ93 residue, forming *S*-nitrosohemoglobin (SNO-Hb; see Figure 4.3) [26]. Conversely, upon deoxygenation-induced transition to T structure, Hb releases NO/SNO equivalents [26, 27]. In fact, in the deoxygenated T structure, SNO-Hb appears to be too unstable to be crystallized, whereas the crystal structure of an "SNO(oxy)-Hb" has been solved [28]. Parentheses are used here because the form that crystallized is not precisely the classical SNO derivative. The binding and release of NO equivalents by (SNO)-Hb is also sensitive to allosteric effectors, for example protons and CO_2. Thus, Hb/SNO-Hb is

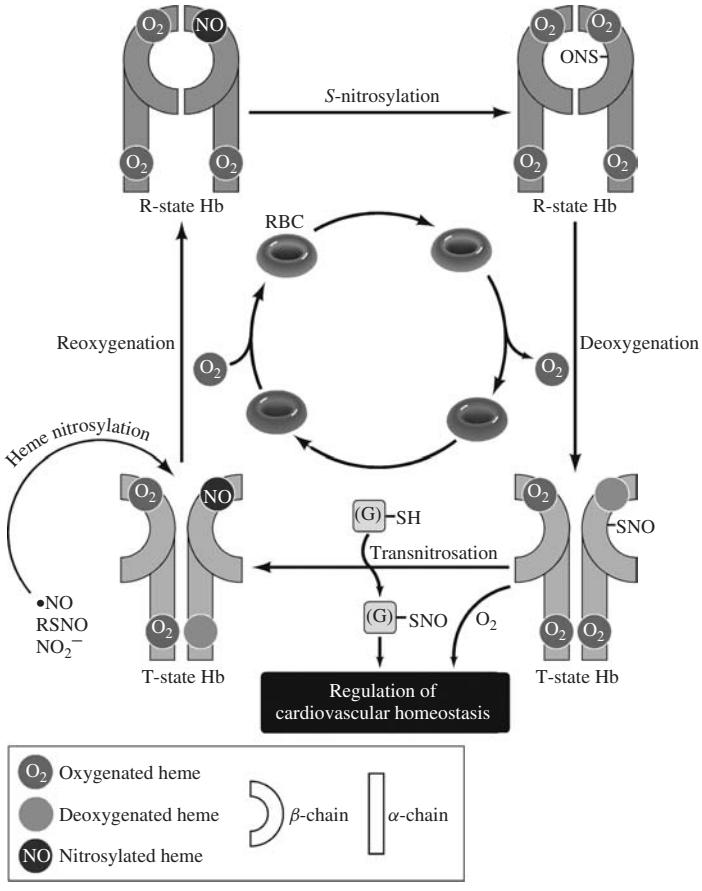

Figure 4.3 *Allosteric transitions of Hb during circulatory cycling regulate the delivery of (S)NO bioactivity and thus O_2 delivery. RBC Hb senses [O_2] and responds via allosterically governed (S)NO binding, formation, and release. At high pO_2 in the lungs, Hb is in the R state and the reactive Cys93 residue of the β chain is protected in a hydrophobic pocket in the vicinity of β hemes. On partial RBC deoxygenation in the peripheral circulation, Hb adopts the T structure and Cysβ93 is exposed to solvent. These allosteric transitions control the reactivity of Cysβ93. In the T state, a population of Hb reacts with •NO, NO_2^-, and/or low-mass SNOs to produce nitrosylated heme at the β chain (bottom left). Transition to R state, which brings Cysβ93 close to nitrosylated heme (top left), is followed by a nitrosylation reaction between NO heme and Cysβ93. This results in the formation of a covalently bound S-nitrosocysteine (top right). In the R state, SNO-Cysβ93 is protected from further reactions in a hydrophobic pocket. A potential direct nitros(yl)ation of Cysβ93 by higher oxides of nitrogen (NOx) or by metal catalysis, including by dinitrosyl iron complexes, is also possible. When Hb adopts the T conformation on partial deoxygenation (bottom right), SNO-Cysβ93 readily reacts with target nitrosothiols and initiates a cascade of transnitrosations (for example, with plasma), leading to the formation of SNOs outside RBCs. The precise identity of the SNOs formed remains unresolved, and the candidate GSNO is depicted here. SNO is at least as potent as •NO as a vasodilator and ensures •NO-independent functions aimed at preserving vascular homeostasis. RBCs thereby function as oxygen sensors to accurately regulate O_2 supply. (Reproduced with permission from [32]).*
For a better understanding of the figure, please refer to color plate 1.

poised to sense and respond to a number of biologically relevant metabolic stimuli [22, 23, 29]. Notably, RBC Hb may also utilize nitrite as a substrate for the formation of SNO equivalents destined for export in hypoxic vasodilation [30, 31]. This finding overcomes a fundamental problem with "nitrite reductase" activity alone: namely, that the NO so formed cannot escape the Hb-rich RBC [22, 32]. RBC membrane properties that govern RBC deformability are influenced at least in part by NO under basal and stimulated conditions [33, 34], but the role of Hb and SNO in this regulation remains unclear.

4.10 A Mutant Mouse Challenges the SNO-Hb Hypothesis, but does not Overthrow it

The SNO-Hb hypothesis has been challenged repeatedly (reviewed in [22, 32]). Most recently, findings were reported from a mutant mouse bearing human Hb in which the Cysβ93 residue was substituted by alanine [35]. Unfortunately, these experiments provide little insight into the function of mammalian SNO-Hb, for reasons including that: (i) the experiments lacked critical controls testing whether transgenic mouse RBCs expressing the nonmutated human Hb (labeled "control") behaved as true controls compared to either wild-type murine or human RBCs; (ii) compensatory changes such as S-nitrosylation of coexistent fetal Hb were not assessed with appropriate assay sensitivity; and (iii) whether human (SNO)Hb interacts with murine AE1 (anion exchanger type 1, the RBC membrane protein critical for SNO export from the RBC) was not assessed. When vasodilator responses to RBCs from the C93A mouse are compared to those from previously published experiments involving wild-type murine RBCs, the deficiency in the mutated RBCs supports an important role for SNO-Hb in hypoxic vasodilation [36, 37].

4.11 Signaling by Hb-derived SNO: A Metabolically Responsive, Regulated Pathway

Once formed, SNO-Hb is stable *in vitro*, but *in vivo* the deoxygenation-linked transition to the T state in Hb is accompanied by the release of the (S)NO equivalent. Therefore, release of Hb-derived SNO is coupled with the O_2-sensing function of Hb, and indeed, the release of SNO from free or erythrocytic Hb is a function of SO_2 (the O_2 saturation of Hb) rather than the pO_2 per se. Remarkably, hypoxia-induced increases in blood flow that match nutrient (O_2) supply with metabolic demand are similarly transduced by the Hb SO_2 rather than pO_2 in blood itself. The fate of SNO equivalents released from deoxygenating Hb is not random: the transition to T structure in Hb promotes the interaction of Hb with the membrane protein Band 3, or AE1 (anion exchanger 1), and reactive cysteine groups in AE1 accept the (S)NO group in transfer. Thus, NO/SNO groups leaving Hb have special protection from recapture by the heme groups in Hb via two mechanisms: first, the SNO/NO^+ chemical character of the group leaving the Cysβ93 residue has low affinity for hemes, and second, the specific protein–protein interaction between Hb and AE1, favored by deoxygenation, channels the SNO to a site where it is poised for export. SNO-Hb levels in the RBC are regulated in part by the enzyme GSNO reductase, which governs the levels of GSNO in most cells and, by extension,

governs protein-bound SNOs (including Hb-bound SNO) with which GSNO is typically in equilibrium [19, 38].

Beyond RBC membrane AE1, the details of the fate of Hb-derived SNOs are uncertain, but some clues have emerged. In blood plasma, reactive thiols in glutathione (GSH) or albumin may serve as SNO carrier molecules. For some RBC–SNO activities, direct cell–cell contact between RBCs and endothelial cells is possible, with SNO group transfer. Mechanisms of SNO entry into the target cell vary, and may require (i) cleavage from a larger to a smaller carrier molecule, exemplified in the requirement for cleavage from the tripeptide SNO GSNO to the dipeptide SNO Gly-Cys-NO in some cell types and events [39], and/or (ii) specific and active transport into cells, as by the L-type amino acid transporter (LAT) [40, 41].

4.12 Signaling by Hb-derived SNO: Pathway Complexity Revealed by Multiple Defects in Disease States

The sophisticated nature of metabolic sensing by RBC Hb coupled with extraerythrocytic delivery of vasodilator NO-equivalents is revealed by the varied lesions in the system associated with disease states. Lesions in the RBC–Hb–NO–SNO signaling pathway have been discovered either in ill humans or in mice modeling human disease, or both:

(i) Abnormally **low and high levels** of RBC SNO-Hb have been documented in the RBCs, respectively, of mice lacking eNOS, and in mice and humans with endotoxic sepsis (in which NOS is upregulated) [38, 42]. Elevated RBC SNO-Hb levels were also seen in mice lacking the enzyme GSNOR (GSNO reductase). GSNOR is responsible for dynamically regulating GSNO levels, which are in equilibrium with protein-SNOs, evidently including Hb [38].

(ii) Abnormally **low levels of SNO-Hb** in human RBCs stored in anticipation of transfusion were demonstrated in two independent studies [43, 44]. The precise mechanism is undetermined and under investigation. In addition, whether these changes may contribute to the disappointing clinical outcomes seen with RBC transfusion in the critically ill [45, 46] or in cardiac-surgery patients transfused with older stored RBCs is unknown [47]. Changes in stored RBCs are numerous and complex, but recent work illustrates for the first time functionally relevant changes in the ability of stored RBCs to respond to low-O_2 conditions with the appropriate (SNO-dependent) vasodilator activity [43, 44]. The reason this storage lesion was recognized only recently (in spite of the decades-old technique of RBC-banking and transfusion) is that the recognition of this activity was also very recent. Emerging work to improve the efficacy of both transfusion medicine and blood substitutes recognizes the duality of Hb's O_2-delivery activity via both O_2 dynamics and Hb–NO–SNO interactions that govern blood flow.

(iii) Deficient **formation** of SNO-Hb in the face of ample amounts of the canonical precursor Hb(FeII)NO ("iron nitrosylhemoglobin") – implying no limitation by eNOS-derived substrate – was demonstrated in **hypoxemic** patients with pulmonary arterial hypertension [48]. When RBC Hb is held at a static pO_2 similar to that in venous blood – rather than the normal cycling between arterial and venous pO_2s (and the 4–20 mm Hg encountered in capillaries) – subsequent SNO formation on

Hb is impaired [48]. Alternatively, deficient SNO-Hb **formation** may result in this case from unfavorable heme redox potential, as in the case of sickle hemoglobinopathy and disease [49].

(iv) Deficient **transfer** ("transnitrosylation") of (S)NO group equivalents from Hb to the membrane protein AE1 is a distinct lesion characterizing human sickle cell disease [49]. The impaired transnitrosylative transfer may be due to oxidative changes in AE1 that impact the Cys thiol target responsible for accepting the NO group.

(v) Deficient **transfer** of SNO equivalents from diabetic human RBCs has been linked with the known increase in O_2 affinity of glycosylated Hb. Despite higher than normal levels of SNO bound to Hb, the ability of diabetic RBCs to elicit relaxation of blood vessels was depressed [50].

4.13 Therapeutic Implications of the Hb–NO Signaling System

Hb can not only bind NO to its ferrous and ferric hemes with extremely high affinity, but also, simultaneously and in a coordinated fashion, orchestrate NO binding to a critical cysteinyl residue, β-chain 93. From Cysβ93, Hb delivers that bound NO equivalent to downstream targets as a function of tissue hypoxia, opening up opportunities to exploit this delivery system for therapeutic gain, correcting pathophysiological defects in a broad range of diseases. Interestingly, therapies already in clinical use may act, in part, via the RBC nitrosyl heme-thiol/nitrosothiol delivery system. For example, NO is inhaled (iNO) by neonates for the syndrome persistent pulmonary hypertension of the newborn (PPHN). The local action of NO in this setting (achieved not only topically, using inhalation as the delivery mode, but also purportedly reinforced by the heme-based scavenging capacity of circulating RBC Hb) has been considered a strength in that NO-related toxicity is limited spatially and temporally in the newborn. But remarkably, clinical studies investigating whether iNO can be applied in other neonatal diseases, for example in infants susceptible to bronchopulmonary dysplasia (BPD), have shown superior neurodevelopmental outcomes in infants inhaling NO compared to controls [51]. These findings could not be accounted for by an improvement in pulmonary outcomes.

4.14 HBOCs, NO, and SNO

The development and clinical application of Hb-based O_2 carriers (HBOCs) have been hampered by the very fact that these are, as it were, Hbs, and that they scavenge endothelium-derived NO in an unbridled manner. Wherever the extracellular Hb can go, intra- or extravascularly, it will scavenge NO. Efforts aimed at reengineering Hb so as to reduce the rate of Hb–NO reaction by substituting residues in the distal heme pocket – such as LeuB10 and ValE11 – that induce steric hindrance have proven effective [52–55]. The resulting Hb mutants induce correspondingly lower degrees of vasoconstriction, supporting the controversial mechanistic link between NO scavenging by HBOCs and the vasoconstriction they variably produce. Like native Hb, Hb of the

HBOC may also transfer the NO, typically as an NO^+ group equivalent, to the β93 thiol, which can, in turn, deliver vasoactive (S)NO. Such (S)NO delivery will ameliorate or antagonize the negative O_2-delivery effects of the HBOC products. HBOC studies, while typically very cognizant of the NO-related obstacles to efficacy, have generally not given this NO/SNO cycling due consideration, with a few recent exceptions [56, 57]. Ichinose and colleagues recently examined whether this challenge could be overcome by simultaneously administering inhaled NO gas to mice receiving an infusion of an HBOC, and indeed, under those conditions, the HBOC-induced vasoconstriction and hypertension were markedly attenuated [58]. Efforts are underway to test whether modifying HBOCs by *S*-nitrosylation directly will produce similar effects, and results from such studies are promising in this regard [56]. Downstream signaling by NO was the target of an analogous effort by Gotshall and coworkers [59] to offset the pulmonary and systemic hypertension accompanying administration of a glutaraldehyde-polymerized HBOC administered to rats: the type 5, cGMP-metabolizing PDE (phosphodiesterase) was inhibited by sildenafil (Viagra).

4.15 Other Gaseous Hb Ligands of Potential Therapeutic Significance

Hydrogen sulfide (H_2S) is increasingly appreciated as a potentially important modulator of protein function, in a manner reminiscent of the rich redox chemistry of NO's interactions with Hb at both metal and thiol sites. The vasoactivity of garlic extracts [60] is well established and has been suggested [61] to account for some of the health benefits of garlic ingestion, in part via its organic disulfides such as allicin, which in turn may be converted *in vivo* to H_2S. Oxygenated, but not deoxygenated, Hb oxidizes with RBC exposure to garlic extracts (J.B., unpublished observations), and the oxidation is prevented by preloading the RBCs with CO or NO, suggesting an O_2-dependence. In the deoxygenated state, exposure is accompanied by *S*-allylation of the Cysβ93 residue [62], which may explain the preferential conversion to H_2S of garlic derivatives by deoxygenated (vs oxy) RBCs [63]. Further work is needed to determine the biological and medical significance of these findings.

4.16 NO-related Enzymatic Activities of Hb: Reconciling Nitrite Reductase and SNO Synthase Functions

There is growing interest in the capacity of Hb to function as a "nitrite reductase", and some have touted this activity as a route to RBC-derived NO bioactivity. Specifically, nitrite originally bound to the heme groups can be converted to an NO equivalent through a deoxygenation–reoxygenation cycle. The resulting adduct is conventionally considered to be Hb(FeII)NO. Hb(FeII)NO produces no vasoactivity; however, from Hb(FeII)NO the formation of SNO-Hb offers a mechanism whereby the NO group can be salvaged for export [22, 64]. The relative importance of nitrite and NO itself as substrates for the ultimate formation of Hb-bound SNO is uncertain but could vary with diet and disease, redox, and oxygenation status.

4.17 Measuring Biologically Relevant Hb–NO Adducts

Progress in the field of interactions between NO and Hb depends crucially on the use and understanding of reliable, reproducible, sensitive, and specific detection and quantification methodologies. We suggest these criteria when adopting a particular assay or interpreting published work in the field:

(i) Relevant standards of biological relevance must be used to demonstrate the underlying sensitivity of an assay for a given analyte.
(ii) Sensitivity and specificity estimates should derive from experiments conducted with standards in a background of the biological matrix of interest. For example, an assay intended to measure SNO-Hb in an RBC lysate must be calibrated with RBC lysate in the standards. Implicit in this recommendation is that because Hb-bound NO adducts are an extreme minority (~1 NO group per 1000–10 000 Hb-tetramer molecules), standard curves and validation work must adopt these ratios. Whether NO detection follows photolytic liberation of the NO group or chemical liberation, NO tends strongly to rebind to the hemes (NO autocapture), and this must be taken into account when deciding whether signals from an unknown source can be reliably extrapolated against a standard curve [22, 32, 65].
(iii) Rigorous attention must be paid to the effects, intended and unintended, of preparative steps, reagents, exposure to inert and O_2-containing gases (including air), and dilution itself. Numerous examples of artifacts produced in Hb–NO analysis include the artifactual elimination of some SNOs in the presence of acidified sulfanilamide: this reagent is intended to remove nitrite only, but given the known equilibria between nitrite, NO, and SNOs (particularly at low pH), the assumed specificity for nitrite elimination is rarely justified [66]. Similarly, alkylating agents intended to block free thiols from intra-assay SNO formation can also displace existing SNO groups [66]. Exposure of RBC-derived samples to air – like exposure of Hb to air as RBCs traverse the lung capillaries – triggers the transition of Hb to the oxygenated R state, and thus the formation of SNO-Hb [23]. Indeed, exposure of samples to air (whether intentional or incidental) during an assay can eliminate the difference between venous and arterial blood samples (primarily a difference in O_2 content): venous blood becomes "arterialized" in this process.

4.18 Conclusion

Growth in our understanding of the rich, reversible chemistry between NO and Hb and Mb is actively reshaping approaches to O_2 delivery by native RBCs and blood substitutes. Just as Hb itself has evolved in response to selective environmental pressure, development of O_2 therapeutics informed by these interactions continues to hold great promise.

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References

[1] Wittenberg, B.A. and Wittenberg, J.B. Transport of oxygen in muscle, *Annu. Rev. Physiol*., **51**, 857–878 (1989).

[2] Garry, D.J., Ordway, G.A., Lorenz, L.N. *et al*. Mice without myoglobin, *Nature*, **395**, 905–908 (1998).

[3] Godecke, A., Flogel, U., Zanger, K. *et al*. Disruption of myoglobin in mice induces multiple compensatory mechanisms, *Proc. Natl. Acad. Sci. U S A*, **96**, 10495–10500 (1999).

[4] Witting, P.K., Douglas, D.J. and Mauk, A.G. Reaction of human myoglobin and nitric oxide: heme iron or protein sulfhydryl (s) nitrosation dependence on the absence or presence of oxygen, *J. Biol. Chem*., **276**, 3991–3998 (2001).

[5] Witting, P.K. and Mauk, A.G. Reaction of human myoglobin and H_2O_2: electron transfer between tyrosine 103 phenoxyl radical and cysteine 110 yields a protein-thiyl radical, *J. Biol. Chem*., **276**, 16540–16547 (2001).

[6] Witting, P.K., Mauk, A.G., Douglas, D.J. and Stocker, R. Reaction of human myoglobin and peroxynitrite: characterizing biomarkers for myoglobin-derived oxidative stress, *Biochem. Biophys. Res. Commun*., **286**, 352–356 (2001).

[7] Rayner, B.S., Wu, B.J., Raftery, M. *et al*. Human S-nitroso oxymyoglobin is a store of vasoactive nitric oxide, *J. Biol. Chem*., **280**, 9985–9993 (2005).

[8] Brunori, M. Nitric oxide, cytochrome-*c* oxidase and myoglobin, *Trends Biochem. Sci*., **26**, 21–23 (2001).

[9] Merx, M.W., Godecke, A., Flogel, U. and Schrader, J. Oxygen supply and nitric oxide scavenging by myoglobin contribute to exercise endurance and cardiac function, *FASEB J*., **19**, 1015–1017 (2005).

[10] Suzuki, T. and Imai, K. Evolution of myoglobin, *Cell. Mol. Life Sci*., **54**, 979–1004 (1998).

[11] Suzuki, T. and Takagi, T. A myoglobin evolved from indoleamine 2,3-dioxygenase, *J. Mol. Biol*., **228**, 698–700 (1992).

[12] Suzuki, T. Abalone myoglobins evolved from indoleamine dioxygenase: the cDNA-derived amino acid sequence of myoglobin from *Nordotis madaka*, *J. Protein Chem*., **13**, 9–13 (1994).

[13] Manwell, C. Histological specificity of pigments: I. Comparisons of the coelom and muscle haemoglobins of the polychaete worm *Travisia pupa* and the echinuroid worm *Arynchite pugettensis*, *Comp. Bioch. Physiol*., **1**, 267–276 (1960).

[14] Gow, A.J., Payson, A.P. and Bonaventura, J. Invertebrate hemoglobins and nitric oxide: how heme pocket structure controls reactivity, *J. Inorg. Biochem*., **99**, 903–911 (2005).

[15] Minning, D.M., Gow, A.J., Bonaventura, J. *et al*. Ascaris haemoglobin is a nitric oxide-activated "deoxygenase", *Nature*, **401**, 497–502 (1999).

[16] Angelo, M., Hausladen, A., Singel, D. and Stamler, J.S. Interactions of NO with hemoglobin: from microbes to man, *Methods Enzymol*., **436**, 131–168 (2008).

[17] Hausladen, A. and Stamler, J.S. Nitric oxide in plant immunity, *Proc. Natl. Acad. Sci. U S A*, **95**, 10345–10347 (1998).

[18] Liu, L., Zeng, M., Hausladen, A. *et al*. Protection from nitrosative stress by yeast flavohemoglobin, *Proc. Natl. Acad. Sci. U S A*, **97**, 4672–4676 (2000).

[19] Liu, L., Hausladen, A., Zeng, M. *et al*. A metabolic enzyme for *S*-nitrosothiol conserved from bacteria to humans, *Nature*, **410**, 490–494 (2001).
[20] Angelo, M., Hausladen, A., Singel, D.J. and Stamler, J.S. Interactions of NO with hemoglobin: from microbes to man, *Methods Enzymol*., **436**, 131–168 (2008).
[21] Bonaventura, J. and Lance, V.P. Nitric oxide, invertebrates,and hemoglobin, *Amer. Zool*., **41**, 346–359 (2001).
[22] Singel, D.J. and Stamler, J.S. Chemical physiology of blood flow regulation by red blood cells, *Annu. Rev. Physiol*., **67**, 99–145 (2005).
[23] McMahon, T.J., Moon, R.E., Luschinger, B.P. *et al*. Nitric oxide in the human respiratory cycle, *Nat. Med*., **8**, 711–717 (2002).
[24] Gow, A.J., Luchsinger, B.P., Pawloski, J.R. *et al*. The oxyhemoglobin reaction of nitric oxide, *Proc. Natl. Acad. Sci. U S A*, **96**, 9027–9032 (1999).
[25] Gow, A.J. and Stamler, J.S. Reactions between nitric oxide and haemoglobin under physiological conditions, *Nature*, **391**, 169–173 (1998).
[26] Jia, L., Bonaventura, C., Bonaventura, J. and Stamler, J.S. S-nitrosohaemoglobin: a dynamic activity of blood involved in vascular control, *Nature*, **380**, 221–226 (1996).
[27] Stamler, J.S., Jia, L., Eu, J.P. *et al*. Blood flow regulation by S-nitrosohemoglobin in the physiological oxygen gradient, *Science*, **276**, 2034–2037 (1997).
[28] Chan, N.L., Kavanaugh, J.S., Rogers, P.H. and Arnone, A. Crystallographic analysis of the interaction of nitric oxide with quaternary-T human hemoglobin, *Biochemistry*, **43**, 118–132 (2004).
[29] McMahon, T.J., Stone, A.E., Bonaventura, J. *et al*. Functional coupling of oxygen-binding and vasoactivity in S-nitrosohemoglobin, *J. Biol. Chem*., **275**, 16738–16745 (2000).
[30] Angelo, M., Singel, D.J. and Stamler, J.S. An *S*-nitrosothiol (SNO) synthase function of hemoglobin that utilizes nitrite as a substrate, *Proc. Natl. Acad. Sci. U S A*, **103**, 8366–8371 (2006).
[31] Huang, Z., Shiva, S., Kim-Shapiro, D.B. *et al*. Enzymatic function of hemoglobin as a nitrite reductase that produces NO under allosteric control, *J. Clin. Invest*., **115**, 2099–2107 (2005).
[32] Sonveaux, P., Lobysheva, I.I., Feron, O. and McMahon, T.J. Transport and peripheral bioactivities of nitrogen oxides carried by red blood cell hemoglobin: role in oxygen delivery, *Physiology*, **22**, 97–112 (2007).
[33] Kleinbongard, P., Schulz, R., Rassaf, T. *et al*. Red blood cells express a functional endothelial nitric oxide synthase, *Blood*, **107**, 2943–2951 (2006).
[34] Bor-Kucukatay, M., Wenby, R.B., Meiselman, H.J. and Baskurt, O.K. Effects of nitric oxide on red blood cell deformability, *Am. J. Physiol. Heart Circ. Physiol*., **284**, H1577–H1584 (2003).
[35] Isbell, T.S., Sun, C.W., Wu, L.C. *et al*. SNO-hemoglobin is not essential for red blood cell-dependent hypoxic vasodilation, *Nat. Med*., **14**, 773–777 (2008).
[36] Diesen, D.L., Hess, D.T. and Stamler, J.S. Hypoxic vasodilation by red blood cells: evidence for an *S*-nitrosothiol-based signal, *Circ. Res*., **103**, 545–553 (2008).
[37] Stamler, J.S., Singel, D.J. and Piantadosi, C.A. SNO-hemoglobin and hypoxic vasodilation, *Nat. Med*., **14**, 1008–1009; author reply 1009–1010 (2008).

[38] Liu, L., Yan, Y., Zeng, M. *et al*. Essential roles of *S*-nitrosothiols in vascular homeostasis and endotoxic shock, *Cell*, **116**, 617–628 (2004).
[39] Lipton, A.J., Johnson, M.A., Macdonald, T. *et al*. *S*-nitrosothiols signal the ventilatory response to hypoxia, *Nature*, **413**, 171–174 (2001).
[40] Li, S. and Whorton, A.R. Identification of stereoselective transporters for *S*-nitroso-L-cysteine: role of LAT1 and LAT2 in biological activity of *S*-nitrosothiols, *J. Biol. Chem*., **280**, 20102–20110 (2005).
[41] Brahmajothi, M.V., Mason, S.N., Whorton, A.R. *et al*. Transport rather than diffusion-dependent route for nitric oxide gas activity in alveolar epithelium, *Free Radic. Biol. Med*., **49**, 294–300 (2010).
[42] Palmer, L.A., Doctor, A., Chhabra, P. *et al*. *S*-nitrosothiols signal hypoxia-mimetic vascular pathology, *J. Clin. Invest*., **117**, 2592–2601 (2007).
[43] Bennett-Guerrero, E., Veldman, T.H., Doctor, A. *et al*. Evolution of adverse changes in stored RBCs, *Proc. Natl. Acad. Sci. U S A*, **104**, 17063–17068 (2007).
[44] Reynolds, J.D., Ahearn, G.S., Angelo, M. *et al*. *S*-nitrosohemoglobin deficiency: a mechanism for loss of physiological activity in banked blood, *Proc. Natl. Acad. Sci. U S A*, **104**, 17058–17062 (2007).
[45] Hebert, P.C., Wells, G., Blajchman, M.A. *et al*. A multicenter, randomized, controlled clinical trial of transfusion requirements in critical care: transfusion requirements in critical care investigators, Canadian Critical Care Trials Group, *N. Engl. J. Med*., **340**, 409–417; published erratum 1056 (1999).
[46] Lacroix, J., Hebert, P.C., Hutchison, J.S. *et al*. Transfusion strategies for patients in pediatric intensive care units, *N. Engl. J. Med*., **356**, 1609–1619 (2007).
[47] Koch, C.G., Li, L., Sessler, D.I. *et al*. Duration of red-cell storage and complications after cardiac surgery, *N. Engl. J. Med*., **358**, 1229–1239 (2008).
[48] McMahon, T.J., Ahearn, G.S., Moya, M.P. *et al*. A nitric oxide processing defect of red blood cells created by hypoxia: deficiency of S-nitrosohemoglobin in pulmonary hypertension, *Proc. Natl. Acad. Sci. U S A*, **102**, 14801–14806 (2005).
[49] Pawloski, J.R., Hess, D.T. and Stamler, J.S. Impaired vasodilation by red blood cells in sickle cell disease, *Proc. Natl. Acad. Sci. U S A*, **102**, 2531–2536 (2005).
[50] James, P.E., Lang, D., Tufnell-Barret, T. *et al*. Vasorelaxation by red blood cells and impairment in diabetes: reduced nitric oxide and oxygen delivery by glycated hemoglobin, *Circ. Res*., **94**, 976–983 (2004).
[51] Schreiber, M.D., Gin-Mestan, K., Marks, J.D. *et al*. Inhaled nitric oxide in premature infants with the respiratory distress syndrome, *N. Engl. J. Med*., **349**, 2099–2107 (2003).
[52] Doherty, D.H., Doyle, M.P., Curry, S.R. *et al*. Rate of reaction with nitric oxide determines the hypertensive effect of cell-free hemoglobin, *Nat. Biotechnol*., **16**, 672–676 (1998).
[53] Eich, R.F., Li, T.S., Lemon, D.D. *et al*. Mechanism of NO-induced oxidation of myoglobin and hemoglobin, *Biochemistry*, **35**, 6976–6983 (1996).
[54] Hartman, J.C., Argoudelis, G., Doherty, D. *et al*. Reduced nitric oxide reactivity of a new recombinant human hemoglobin attenuates gastric dysmotility, *Eur. J. Pharmacol*., **363**, 175–178 (1998).

[55] Olson, J.S., Foley, E.W., Rogge, C. *et al*. NO scavenging and the hypertensive effect of hemoglobin-based blood substitutes, *Free Radic. Biol. Med*., **36**, 685–697 (2004).
[56] Irwin, D., Buehler, P.W., Alayash, A.I. *et al*. Mixed S-nitrosylated polymerized bovine hemoglobin species moderate hemodynamic effects in acutely hypoxic rats, *Am. J. Respir. Cell. Mol. Biol*., **42**, 200–209 (2010).
[57] Sonveaux, P., Kaz, A.M., Snyder, S.A. *et al*. Oxygen regulation of tumor perfusion by S-nitrosohemoglobin reveals a pressor activity of nitric oxide, *Circ. Res*., **96**, 1119–1126 (2005).
[58] Yu, B., Raher, M.J., Volpato, G.P. *et al*. Inhaled nitric oxide enables artificial blood transfusion without hypertension, *Circulation*, **117**, 1982–1990 (2008).
[59] Gotshall, R.W., Hamilton, K.L., Foreman, B. *et al*. Glutaraldehyde-polymerized bovine hemoglobin and phosphodiesterase-5 inhibition, *Crit. Care Med*., **37**, 1988–1993 (2009).
[60] Kaye, A.D., Nossaman, B.D., Ibrahim, I.N. *et al*. Analysis of responses of allicin, a compound from garlic, in the pulmonary vascular bed of the cat and in the rat, *Eur. J. Pharmacol*., **276**, 21–26 (1995).
[61] McMahon, F.G. and Vargas, R. Can garlic lower blood pressure? A pilot study, *Pharmacotherapy*, **13**, 406–407 (1993).
[62] Bonaventura, J., Rodriguez, E.N., Beyley, V. and Vega, I.E. Allylation of intraerythrocytic hemoglobin by raw garlic extracts, *J. Med. Food*., **13**, 943–949 (2010).
[63] Benavides, G.A., Squadrito, G.L., Mills, R.W. *et al*. Hydrogen sulfide mediates the vasoactivity of garlic, *Proc. Natl. Acad. Sci. U S A*, **104**, 17977–17982 (2007).
[64] Luchsinger, B.P., Rich, E.N., Yan, Y. *et al*. Assessments of the chemistry and vasodilatory activity of nitrite with hemoglobin under physiologically relevant conditions, *J. Inorg. Biochem*., **99**, 912–921 (2005).
[65] Doctor, A., Platt, R., Sheram, M.L. *et al*. Hemoglobin conformation couples erythrocyte *S*-nitrosothiol content to O_2 gradients, *Proc. Natl. Acad. Sci. U S A*, **102**, 5709–5714 (2005).
[66] Hausladen, A., Rafikov, R., Angelo, M. *et al*. Assessment of nitric oxide signals by triiodide chemiluminescence, *Proc. Natl. Acad. Sci. U S A*, **104**, 2157–2162 (2007).

5

The Role of Reactive Oxygen and Nitrogen Species in Ischemia/Reperfusion Injury

Ester Spagnolli and Warren M. Zapol

Department of Anesthesia and Critical Care,
Massachusetts General Hospital, Harvard Medical School,
Boston, MA, USA

5.1 Introduction

Prolonged interruption of arterial blood flow to an organ or tissue causes ischemia and rapidly leads to a loss of function, followed by cell death. Reperfusion is necessary to save ischemic tissues from an otherwise certain death. However, reperfusion itself causes additional injury that jeopardizes viable cells. In several common clinical scenarios, such as reperfusion therapies for acute myocardial infarction and organ transplants, reperfusion injury plays an important role in worsening the outcome of patients [1–3].

Reperfusion of ischemic tissues is characterized by an acute burst of reactive oxygen species (ROS) and reactive nitrogen species (RNS) that can induce additional damage to the cellular lipids, proteins, and DNA of post-ischemic tissues [4]. Nonetheless, ROS and RNS can also play beneficial roles in preventing reperfusion injury [4, 5].

This chapter will focus on a brief description of the most important ROS and RNS in biological environments, the central mechanisms of reperfusion injury (with special emphasis on the role that ROS and RNS play in establishing this form of damage), some mechanisms of protection that have been discovered, and current and emerging treatment options related to ROS and RNS signaling.

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5.2 Redox System and Free Radicals in Biological Systems

The term *redox system* groups all the biological reactions that have pro-oxidant and antioxidant effects in a given biological environment; the term *redox homeostasis* indicates the existence of an equilibrium state between pro-oxidant and antioxidant species. Alterations of this equilibrium can be caused by excessive production of oxidant molecules or an insufficient antioxidant capacity within the system. The term *oxidative stress* indicates the harmful consequences that high levels of oxidant molecules induce when not neutralized by antioxidant species [6, 7]. Advantageous biological effects of both ROS and RNS have also been described and the term *redox signaling* refers to the important role that free radicals have, as molecular messengers, in biological reactions [8].

Antioxidant molecules are fundamental in maintaining redox homeostasis of biological organisms. Many endogenous molecules have antioxidant functions, including catalase, selenium-dependent glutathione peroxidase, copper and zinc-dependent superoxide dismutase (SOD), mitochondrial manganese-dependent SOD, vitamin E, vitamin C, vitamin A, and melatonin [9].

Free radicals, characterized by having one or more unpaired electrons, have a high degree of oxidant activity towards other molecules. Free radicals that derive from oxygen are called ROS and free radicals that derive from nitric oxide are called RNS [6]. Among ROS, the most biologically important are: superoxide anion ($O_2^{\bullet -}$), hydroxyl radical (HO^-), hydrogen peroxide (H_2O_2), and hypochlorous acid (HOCl); among RNS, the most important are nitric oxide (NO^-, hereafter denoted NO) and peroxynitrite ($ONOO^-$) [6].

Of particular interest in this regard is NO. This small free-radical molecule is characterized by a very short half-life in biological fluids (ranging from 3.8 to 6.2 seconds in aqueous solution at 150–700 mm Hg partial oxygen pressure) and a diffusion constant in aqueous solution (3300 $\mu M^2/s$ at 37 °C) that is 1.4-fold greater than that of oxygen. Given its high diffusion capacity, NO can rapidly reach its targets by passing through tissue layers, as it does for example in the cardiovascular system. Indeed, under physiological conditions and in the absence of avid NO scavengers (e.g. oxy-hemoglobin), NO diffuses rapidly from the endothelium, where it is produced, into arterial smooth-muscle cells, inducing vasorelaxation [10]. NO is synthesized from L-arginine and oxygen by a family of three NO synthases (NOSs): neuronal NOS (NOS1 or nNOS), inducible NOS (NOS2 or iNOS), and endothelial NOS (NOS3 or eNOS). In order to produce NO, NOS enzymes need oxygen and L-arginine as substrates, as well as cofactors such as tetrahydrobiopterin, flavin mononucleotide and flavin adenine dinucleotide. Moreover, the presence of zinc and reduced thiols is required. Insufficient concentrations of cofactors can cause NOS to transfer free electrons to oxygen, producing $O_2^{\bullet -}$ rather than NO [11]. While NOS1 and NOS3 are constitutively expressed in the cardiovascular system, produce low concentrations of NO (<100 nM), and are subject to calcium (Ca^{2+}) and calmodulin regulation, NOS2, in contrast, is an inducible isoenzyme that can produce very high concentrations of NO (>1 μM) in response to inflammatory mediators and is not under Ca^{2+} and calmodulin regulation [12, 13].

5.3 Pathophysiology of Ischemia/Reperfusion Injury

The continuous delivery of oxygenated blood to vital organs is fundamental for tissue survival. Significant and prolonged interruption of arterial blood flow can cause ischemia, leading to cell death and loss of vital functions within minutes. Rapid reperfusion is required to spare at least some of the ischemic tissue from irreversible damage. However, from initial experimental observations [14] and recent clinical studies [15], it has become clear that reperfusion itself can produce additional injury. The severity of the damage due to reperfusion is proportional to the length of ischemia; this is therefore commonly referred to as ischemia/reperfusion (I/R) injury [4, 16]. Acute myocardial infarction (AMI), ischemic stroke, and organ transplants are common clinical situations in which I/R injury can significantly limit the benefits of reperfusion therapies. Indeed, an abrupt reintroduction of oxygen and substrates through restoration of arterial blood flow (as occurs with percutaneous coronary intervention or pharmacological thrombolysis in the clinical setting of AMI) can induce a lethal insult to cells that were still viable prior to reperfusion [1]. AMI is the leading cause of morbidity and mortality in adults of Western societies [17] and thus an extensive body of literature on cardiac I/R injury exists. We will primarily refer to evidence collected in the cardiac field in describing the pathophysiology of I/R damage and some of the mechanisms of protection that have been discovered. Moreover, current and experimental therapies against I/R injury will be presented, with special emphasis on the roles of ROS and RNS, particularly NO.

Two types of damage can be recognized in tissues affected by I/R injury: an intracellular activation of apoptotic and necrotic molecular pathways, and an inflammatory response at the microcirculatory level, primarily involving circulating bone marrow-derived cells [1].

5.3.1 Cell Death

The electron transport chain (ETC), located on the inner membrane of mitochondria, transports electrons to pump protons into the mitochondrial intermembrane space. This activity produces a differential potential between the mitochondrial intermembrane space and the matrix. Following the electrical gradient, a flow of protons passes through F_1F_0-ATPase, releasing the energy required for ATP production. Under normal conditions, a small number of electrons escape the ETC to combine with oxygen and form $O_2^{\bullet-}$, the concentrations of which are reduced to lower levels by SOD [9, 16]. However, during an ischemic insult, the ETC undergoes damage that is proportional to the length of ischemia [16]. The abrupt reperfusion of an ischemic organ rapidly reactivates mitochondrial metabolism and enhances electron leakage, with a consequent burst of $O_2^{\bullet-}$ production [16, 18]. High concentrations of $O_2^{\bullet-}$ can exceed the capacity of SOD to metabolize it into oxygen or H_2O_2 [9]. The oxidative stress induced by high levels of $O_2^{\bullet-}$ intensifies ETC inefficiency, alters protein functions, and damages intracellular membranes. The consequent oxidation of ion-channel proteins, together with a major discrepancy between intra- and extracellular pH (extracellular pH normalizes quickly after reperfusion, while the intracellular environment remains severely acidotic for a

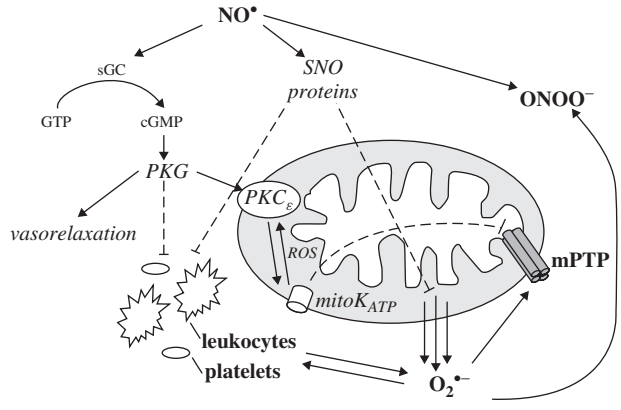

Figure 5.1 *The dual role of ROS and RNS in I/R injury. At reperfusion, a burst of ROS causes intracellular organelle damage and inflammatory cell recruitment. NO inhibits mPTP opening through activation of the sGC-cGMP–PKG–PKC–ROS–mitoK$_{ATP}$ cascade and inhibits platelet activation and recruitment of inflammatory cells through cGMP-dependent pathway and protein S-nitrosylation. Protein S-nitrosylation of mitochondrial ETC components reduces the electron leakage responsible for the superoxide ($O_2^{\bullet -}$) burst. Solid lines indicate activation or increased activity; dashed lines indicate inhibition or reduced activity. NO, nitric oxide; SNO-proteins, S-nitrosoproteins; sGC, soluble guanylate cyclase; GTP, guanosine triphosphate; cGMP, cyclic guanosine triphosphate; PKG, protein kinase G; PKC$_\epsilon$, protein kinase C epsilon; mitoK$_{ATP}$, mitochondrial potassium ATP-dependent channel; mPTP, mitochondrial permeability transition pore; $O_2^{\bullet -}$, superoxide anion; $ONOO^-$, peroxynitrite.*

longer time), and ATP depletion, induces severe intracellular ion disequilibrium, resulting in a characteristic state of intracellular Ca^{2+} overload. High intracellular levels of $O_2^{\bullet -}$ and Ca^{2+} induce the opening of a mitochondrial multiprotein megachannel, known as the mitochondrial permeability transition pore (mPTP) (Figure 5.1). The opening of the mPTP connects the mitochondrial matrix and the cytosol, causing a loss of the ion gradient. Consequently, the drop of the electric potential across the mitochondrial membrane causes further impairment of ATP production, mitochondrial swelling, membrane rupture, and subsequent release of cytochrome *c* into the cytosol. Once in the cytosol, cytochrome *c* activates apoptotic signaling pathways [4].

Wildhirt *et al*. have shown, in a rabbit model of cardiac I/R injury, an increased activity of NOS2 during the first 48 hours of reperfusion. Selective inhibition of NOS2 during this time period ameliorated myocardial function and coronary blood flow. Leukocyte infiltrate and infarct extent were also markedly reduced [19]. Indeed, when present at high concentration, as in the presence of induced NOS2, NO can exert cytotoxic effects. For example, NO binds to cytochrome *c* oxidase in the mitochondria – in competition with oxygen – and inhibits mitochondrial respiration [20]. Although NO inhibition of mitochondrial respiration represents a defense mechanism of cells to face hypoxic conditions, prolonged inhibition can turn harmful, leading the cell to metabolic failure [19]. In addition, NO induces increased production of cyclic guanosine monophosphate (cGMP) via activation of soluble guanylate cyclase (sGC). cGMP is recognized to be a negative inotrope on cardiomyocytes, and its high levels during reperfusion,

induced by excess NO production, can in part explain the myocardial dysfunction that characterizes cardiac I/R injury [19]. Moreover, when oxygen and NO are both present at high concentrations, they react together to produce $ONOO^-$, a potent cytotoxic agent. $ONOO^-$ can then react with deoxyribose, causing mutations in DNA, and with cellular lipids, inducing membrane disruption and cellular necrosis [4, 21]. In addition, $ONOO^-$ can cause protein nitration by irreversibly reacting with tyrosine residues of proteins. This reaction can induce a loss of function of target proteins; during I/R injury, nitration of mitochondrial SOD is thought to participate in mitochondrial dysfunction [16].

5.3.2 The Inflammatory Response

When cell necrosis occurs, the intracellular contents that are released provide an important stimulus for the activation of innate immunity and thus for initiation of an intense inflammatory response [22]. The high level of ROS that characterizes the first moments of reperfusion promotes the expression of platelet-activating factor by endothelial cells and enhances endothelial cell-dependent neutrophil adhesion [23]. Indeed, within the first few minutes of reperfusion, activated platelets are recruited into post-ischemic tissues [22, 24]. Thereafter, the expression of P-selectin on activated platelets promotes their binding to P-selectin glycoprotein ligand-1, located on T-lymphocytes, monocytes, and neutrophils. As a result, leukocyte activation, interaction with endothelial cells, and sequestration in post-ischemic tissues are all enhanced [24, 25]. Once recruited, activated neutrophils and macrophages produce and release inflammatory cytokines, lysosomal enzymes, and ROS and NO at high concentration, starting a vicious circle that amplifies the initial damage (Figure 5.1) [22]. Moreover, platelet activation and the accumulation of inflammatory cells can induce thrombosis of the microcirculation, causing microvascular obstruction, the "no-reflow" phenomenon. Despite timely and apparently successful medical interventions, the "no-reflow" phenomenon can severely reduce the efficacy of reperfusion strategies [1].

5.4 Protection Against I/R Injury

Oxidative stress plays a pivotal role in the establishment of I/R damage. However, some ROS and RNS also play a positive role as important second messengers in pathways that are activated during stress conditions and can protect the cell. Molecular pathways that preserve organs from I/R injury have been discovered in the last four decades. This section describes the protective roles that ROS and RNS can play as molecular messengers in these protective cascades.

5.4.1 Ischemic Pre- and Post-conditioning

In 1986, Murry *et al*. observed in an animal model of cardiac I/R injury that brief cycles of ischemia and reperfusion commencing before a sustained ischemic period can diminish lethal cell damage [26]. The investigators referred to this phenomenon of induced protection as ischemic preconditioning (IPC). About ten years ago, another protective phenomenon called ischemic postconditioning (IPost) was described by Vinten-Johansen's group. Consisting of brief periods of repetitive reperfusion and ischemia, produced just

prior to continued reperfusion, IPost also reduced the size of infarcted tissue in dogs exposed to cardiac I/R injury [14].

During the last two decades, many investigations have focused on the molecular mechanisms of IPC and IPost. It has become clear that IPC and IPost share many steps in their activated signaling pathways. Interestingly, and in line with the sharing of the molecular mechanisms, experimental protocols in which IPC and IPost were combined did not show any additional benefits against I/R injury as compared to protocols in which IPC or IPost alone were applied [27]. Moreover, it is well accepted that for both IPC and IPost the intracellular signaling that occurs in the first minutes of reperfusion are crucial to enabling protection against I/R injury [4]. Of particular interest regarding the molecular mechanisms of IPC and IPost is a group of kinases, known as phosphatidylinositol-3-OH kinase (IP3K)-Akt and p42/p44 extracellular signal-regulated kinases (Erk1/2), that have been observed to be activated when either IPC or IPost is applied; these kinases are therefore commonly referred to as reperfusion injury salvage kinases (RISK) [4, 28, 29]. The molecular cascades activated by RISK share a common final mechanism conferring protection: the inhibition of mPTP opening and, thus, inhibition of apoptosis. Interestingly, in the signaling cascade activated by IP3K-Akt, NO plays a pivotal role. Indeed, upon phosphorylation of NOS3 by Akt, the NO level increases, activating sGC, which in turn increases cGMP levels. The phosphorylation of protein kinase G (PKG) by cGMP leads to protein kinase C epsilon (PKC_ϵ) activation and translocation from the cytosol into the mitochondria. Once in the mitochondria, PKC_ϵ activates the mitochondrial ATP-dependent potassium ($mitoK_{ATP}$) channel. Activation of the $mitoK_{ATP}$ channel helps to preserve the mitochondrial membrane potential. As a consequence, Ca^{2+} and H^+ physiological concentrations in the mitochondria are maintained and mPTP pore opening is inhibited (Figure 5.1) [4, 5].

5.4.2 Pharmacological Conditioning

Discovery of the RISK pathways opened up possibilities for pharmacologic activation, producing *pharmacological conditioning*. Thus far, many molecules have been tested for protective effects in reducing I/R injury, and several have been demonstrated to be beneficial in animal studies [4]. Nevertheless, the translation of these molecules from bench to bedside has proven difficult because of the variability and complexity of clinical scenarios (etiology of disease, comorbidity, demographic profile, and timing of the intervention). These complexities are usually avoided in animal models of I/R injury [30]. We will focus our attention upon experimental and clinical studies that illuminate the protective roles that ROS, antioxidants, and NO play in the pleiotropic scenario of I/R injury.

5.4.2.1 The Protective Role of ROS and Antioxidants

It has been observed that complete ROS scavenging nulls the beneficial effects of IPC, showing the importance of redox signaling in cardioprotection [31]. Recently, Costa *et al*. demonstrated that in the IP3K–Akt cascade, activated by conditioning protocols, the activation of the $mitoK_{ATP}$ channel by PKC_ϵ occurs through redox signaling, which requires a low concentration of ROS [4, 5].

As previously noted, to maintain redox homeostasis there must be a balance between the production rate of oxidants and the concentration of antioxidant molecules that can

inhibit oxidative damage. However, antioxidant capacity can easily be saturated in the presence of high rates of ROS and RNS production [32]. It has been shown, in animal models of I/R injury, that endogenous antioxidants are depleted in proportion to the length of ischemia [33]. Thus, several investigators have focused on giving antioxidants to improve the fate of organs undergoing I/R injury. However, contradictory results, both in laboratory animal models of I/R injury and in clinical trials of patients undergoing cardiac reperfusion, have not allowed the scientific community to reach a common consensus on the efficacy of antioxidants in protecting against I/R injury [33, 34]. Melatonin has recently been studied in several laboratories for its remarkable antioxidant properties [35]. It has been shown that exogenous administration of melatonin ameliorates the fate of reperfused cells, tissues, and organs that were previously exposed to hypoxia. The antioxidant behavior of melatonin is pleiotropic: it efficiently neutralizes both HO^- and $ONOO^-$, induces the genetic expression of SOD, and increases tissue levels of glutathione [35]. Petrosillo *et al*. have recently reported that peroxidized cardiolipin, a reperfusion-induced oxidative-stress product, induces mPTP opening [36]. The same group subsequently demonstrated that melatonin administration preserves cardiolipin from oxidation and thereby diminishes mPTP opening [37]. In 2007, the first randomized clinical trial of melatonin as an adjunctive therapy for patients with acute myocardial infarction undergoing percutaneous coronary intervention was designed [38].

5.4.2.2 The Protective Role of NO

NO plays key roles in both inducing damage and protecting cells from I/R injury. This dual role is not surprising: many molecules that were once believed to be harmful have since been proven to have a normal physiological role and/or beneficial effects depending upon the concentration, location, and timing of their production and/or administration. Since 1986, NO has been recognized as a fundamental messenger and reactive molecule in normal and pathological states [39, 40]. Since early in the 20th century it has been known that organic nitrates are beneficial in treating angina pectoris by increasing coronary blood flow [41]. However, only in the 1980s was it demonstrated that the cardiovascular effects of nitrates were due to their release of NO [39]. Organic nitrates are still prescribed worldwide for the treatment of both angina and AMI. The vasorelaxant effect and the ability to reduce myocardial oxygen consumption, induced by the reduction of left-ventricular filling pressure, have been the rationale for the use of organic nitrate in angina and AMI treatment during the last century [42, 43]. However, in the last two decades new protective mechanisms have been attributed to NO-releasing drugs. Indeed, the discovery that NO takes part in one of the RISK cascades, the IP3K–Akt pathway, raised the idea that organic nitrites may be protective against I/R injury due to activation of a conditioning program. Studies on genetically engineered mice have provided additional evidence for an essential role of NO in cardioprotection: the absence of NOS3 expression worsens I/R injury [44, 45]; on the other hand, NOS3 overexpression has been shown to be protective against both cardiac and hepatic I/R damage [46, 47].

The biological actions induced by NO are typically classified by whether or not the effects are exerted via a cGMP-dependent cascade through the activation of the specific target enzyme sGC, or via cGMP-independent pathways.

In the classical cGMP-dependent pathway, NO activates sGC, a ferrous heme-containing enzyme [39, 48], increasing the level of cGMP, which in turn phosphorylates

PKG. PKG then mediates many of the well-known biological effects of NO. For example, NO-induced vasodilatation results from the reduction of Ca^{2+} concentration in smooth-muscle cells. Reduction of intracellular Ca^{2+} is due to several mechanisms that are all dependent on PKG: activation of Ca^{2+}-dependent K^+ channels (the consequent hyperpolarization of the cell membrane diminishes the effect of the depolarizing signal that induces intracellular Ca^{2+} release), inhibition of voltage-gated Ca^{2+} channels, and activation of the ATPase that sequesters Ca^{2+} in the sarcoplasmic reticulum [49]. Via the sGC–cGMP cascade, NO also inhibits platelet aggregation, preventing T-lymphocyte, monocyte, and neutrophil sequestration in damaged tissues [24, 25]. Moreover, as previously mentioned, the sGC–cGMP pathway plays a fundamental role in one of the RISK cascades that is activated by either IPC or IPost [4].

Although sGC was the first identified receptor for NO, increasing evidence suggests that NO also exerts its biological effects through cGMP-independent mechanisms. Several investigators report that NO can directly act upon mitochondria (which have many ferrous heme-containing enzymes), independently of the sGC pathway, thereby affording acute cardioprotection against I/R injury [50]. Moncada *et al*. have shown that NO modulates cellular respiration through reversible direct inhibition of mitochondrial cytochrome *c* oxidase [20]. This hypothesis is further supported by studies in which resistance to apoptosis induced by various kinds of stressors, such as hypoxia or intracellular Ca^{2+} overload, has been associated with the interaction between NO and cytochrome *c* oxidase [20]. In addition to the direct interaction of NO with mitochondrial components, S-nitrosylation of proteins (SNO-proteins) is considered another major mediator mechanism of the non-enzymatic activity of NO [51, 52]. The term *S-nitrosylation* indicates the reversible covalent modification of a cysteine-thiol group by NO. S-nitrosylation is recognized to be an important regulator of both adrenergic receptor signaling and calcium cycling in the heart. Moreover, in the peripheral vasculature, S-nitrosylation of signaling molecules involved in inflammation and apoptosis – e.g. SNO-caspase – mitigates these events (Figure 5.1). In addition, S-nitrosylation of hemoglobin (SNO-Hb) has also been linked to modulation of blood flow and oxygen delivery [53].

5.4.2.3 NO-based Therapies for I/R Injury

Several new and old drugs are used to enhance or replace the biological functions of endogenous NO production.

Nitroglycerin (NTG) is the most commonly used NO-releasing agent administered during unstable angina and AMI [43]. As mentioned before, NTG-induced vasorelaxation activates the sGC–cGMP pathway in vascular smooth-muscle cells. Evidence also indicates that short-term NTG infusion, at the time of cardiac reperfusion, reduces short-term mortality and left ventricle remodeling. Moreover, long-term infusions (24–48 hours) of NTG have protective effects in term of augmenting and preserving left-ventricular function and reducing infarct size [43]. However, sufficient clinical studies of long-term benefits of NTG are lacking [54]. It is well known that patients treated with a continuous infusion of organic nitrate will rapidly develop tolerance to the vasodilator effect of NTG [55, 56], and that frequent side effects like headaches and systemic hypotension, can be experienced [57].

Nitrite is a metabolite of NO that can be oxidized to nitrate by oxy-hemoglobin, or can be reduced back to NO by deoxy-hemoglobin or xanthine oxidoreductase [10]. Nitrite is

also produced by the reduction of dietary nitrate by commensal bacteria in the mouth and gastrointestinal tract. Nitrite is normally present at micromolar concentrations in plasma and tissues. Thought to be an inert metabolite of NO for many years, nitrite has been recently discovered to be an important form of NO storage in tissues. Indeed, in hypoxic conditions, nitrite is reduced to NO, which in turn exerts its biological effects [58]. The cytoprotective effect of nitrite administration in numerous animal models of diseases is well documented. For example, a protective effect of nitrite administration, when given just prior to reperfusion, has recently been shown in animal models of cardiac and liver I/R injury [59]. Clinical trials of nitrite-based therapies in settings characterized by the presence of I/R injury are planned [58].

Phosphodiesterase type-5A (PDE5) degrades cGMP produced by sGC in blood vessels upon NO stimulation. Sildenafil, marketed as Viagra, is a selective inhibitor of PDE5 and has been shown to induce cardioprotection in cardiac I/R injury models when given at reperfusion [60–62]. Further investigations are needed to clarify the clinical value of these experimental observations [63].

sGC contains a heme molecule that can exist in either a reduced (Fe^{2+}) or an oxidized (Fe^{3+}) state. When sGC is in its oxidized state, it no longer responds to NO activation. BAY 58-2667 is a molecule that can activate sGC both in redox states and in an NO-independent manner [64]. Krieg *et al*. have demonstrated, in a rabbit model of cardiac I/R injury that BAY 58-2667 administration during reperfusion protects the myocardium via the sGC–cGMP–PKG–mitoK_{ATP} pathway, closely mimicking IPost molecular mechanisms [65]. However, like NTG, systemic administration of BAY 58-2667 can easily produce systemic vasodilatation and hypotension. Thus, doubts have been raised about the safety of BAY 58-2667 for clinical use.

Interestingly, when NO is inhaled, even at a relatively high concentrations (e.g. 80 ppm), it does not produce systemic vasodilatation. When in 1990 inhaled NO started to be used in patients, it was believed that all of its effects were mediated via sGC/cGMP. It has subsequently become clear that inhaled NO also exerts its effects via cGMP-independent pathways [66]. Since NO has a short half-life in blood, it was also believed that inhaled NO actions would be limited to the lungs. Nevertheless, since the mid to late 1990s, evidence has accrued that NO inhalation can produce extra-pulmonary effects [67, 68]. In 2006, Hataishi *et al*. demonstrated that breathing NO conferred cardioprotection in a murine model of cardiac I/R injury when continuously administrated for 24 hours after reperfusion. The authors reported that the number of neutrophils in reperfused cardiac tissues of mice breathing air with 80 ppm NO was markedly reduced as compared with mice breathing only air. They concluded that neutrophils are required by inhaled NO to protect the heart from I/R injury [69]. One year later, Janssens's group confirmed the ability of NO inhalation begun immediately prior to coronary reperfusion to significantly reduce infarct size and improve microvascular perfusion in a swine model of cardiac I/R injury [70]. Nagasaka *et al*. subsequently demonstrated a rapidly – within 15 minutes – increased level of NO metabolites (nitrite, nitrate, nitrosyl-heme species, N-nitrosamines, and S-nitrosothiols) in the red blood cells (RBCs), plasma, and tissues of mice breathing 80 ppm NO. The same authors also demonstrated that brief periods of NO inhalation at the time of reperfusion still conferred protection against cardiac I/R injury [71]. Interestingly, Ng *et al*. have shown in a feline model of intestine I/R injury that NO inhalation significantly increases the arterial-venous gradient of the mesenteric

circulation for S-nitrosylated albumin (SNO-Alb) [72]. Moreover, a significant increase in mesenteric blood flow, together with increased SNO-Alb consumption and nitrite generation, was selectively observed in intestinal tissues undergoing I/R injury [72]. This study corroborated the hypothesis that inhaled NO increases the formation of nitrosothiols and that SNO-proteins provide an NO delivery system during acute I/R injury. Considering these complex reactions, when inhaled, NO is likely to be transported as an NO metabolite in the form of nitrite or nitrate, or bound to proteins (SNO-proteins), and delivered to peripheral tissues. In the presence of hypoxia, deoxy-hemoglobin can reconvert NO metabolites back to NO [58]. A recent clinical trial of inhaled NO, given as an extra-pulmonary therapeutic agent seeking to improve post-transplant liver function, showed encouraging results. Further clinical studies of inhaled NO in liver transplant [51] and cardiac I/R injury are ongoing (Janssens, S., personal communication).

5.5 Conclusion

I/R injury jeopardizes vital organ function in common clinical scenarios like AMI and organ transplantation. Greater understanding of the mechanisms responsible for I/R damage has pointed investigators to novel protective strategies. Many of these approaches have aimed at neutralizing oxidative stress or increasing the resistance of mitochondrial components against high and harmful intracellular concentrations of ROS and Ca^{2+}, which characterize the early reperfusion phase. However, translation of successful anti-I/R injury strategies from the bench to the bedside is challenging due to the extreme heterogeneity of the clinical disease and the occurrence of side effects of therapeutic agents. Whereas antioxidant-agent approaches have left many doubts about their clinical applications, NO-based therapies have provided more encouraging results. Further clinical and laboratory investigations are needed to fully elucidate the metabolic, cellular, and molecular targets of NO, and to optimize the route, dose, and timing of NO-based therapies.

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References

[1] Yellon, D.M. and Hausenloy, D.J. Myocardial reperfusion injury, *N. Engl. J. Med*., **357**, 1121–1135 (2007).
[2] Piot, C., Croisille, P., Staat, P. *et al*. Effect of cyclosporine on reperfusion injury in acute myocardial infarction, *N. Engl. J. Med*., **359**, 473–481 (2008).
[3] Lang, J.D. Jr, Teng, X., Chumley, P. *et al*. Inhaled NO accelerates restoration of liver function in adults following orthotopic liver transplantation, *J. Clin. Invest*., **117**, 2583–2591 (2007).
[4] Murphy, E. and Steenbergen, C. Mechanisms underlying acute protection from cardiac ischemia-reperfusion injury, *Physiol. Rev*., **88**, 581–609 (2008).

[5] Costa, A.D., Pierre, S.V., Cohen, M.V. *et al*. cGMP signalling in pre- and post-conditioning: the role of mitochondria, *Cardiovasc. Res*., **77**, 344–352 (2008).
[6] Valko, M., Leibfritz, D., Moncol, J. *et al*. Free radicals and antioxidants in normal physiological functions and human disease, *Int. J. Biochem. Cell Biol*., **39**, 44–84 (2007).
[7] Kovacic, P. and Jacintho, J.D. Reproductive toxins: pervasive theme of oxidative stress and electron transfer, *Curr. Med. Chem*., **8**, 863–892 (2001).
[8] Droge, W. Free radicals in the physiological control of cell function, *Physiol. Rev*., **82**, 47–95 (2002).
[9] Dhalla, N.S., Elmoselhi, A.B., Hata, T. and Makino, N. Status of myocardial antioxidants in ischemia-reperfusion injury, *Cardiovasc. Res*., **47**, 446–456 (2000).
[10] Kelm, M. Nitric oxide metabolism and breakdown, *Biochim. Biophys. Acta*, **1411**, 273–289 (1999).
[11] Munzel, T., Daiber, A., Ullrich, V. and Mulsch, A. Vascular consequences of endothelial nitric oxide synthase uncoupling for the activity and expression of the soluble guanylyl cyclase and the cGMP-dependent protein kinase, *Arterioscler. Thromb. Vasc. Biol*., **25**, 1551–1557 (2005).
[12] Ziolo, M.T., Kohr, M.J. and Wang, H. Nitric oxide signaling and the regulation of myocardial function, *J. Mol. Cell Cardiol*., **45**, 625–632 (2008).
[13] Schulz, R., Kelm, M. and Heusch, G. Nitric oxide in myocardial ischemia/reperfusion injury, *Cardiovasc. Res*., **61**, 402–413 (2004).
[14] Zhao, Z.Q., Corvera, J.S., Halkos, M.E. *et al*. Inhibition of myocardial injury by ischemic postconditioning during reperfusion: comparison with ischemic preconditioning, *Am. J. Physiol. Heart Circ. Physiol*., **285**, H579–H588 (2003).
[15] Ivanes, F., Mewton, N., Rioufol, G. *et al*. Cardioprotection in the clinical setting, *Cardiovasc. Drugs Ther*., **24**, 281–287 (2010).
[16] Burwell, L.S. and Brookes, P.S. Mitochondria as a target for the cardioprotective effects of nitric oxide in ischemia-reperfusion injury, *Antioxid. Redox Signal*., **10**, 579–599 (2008).
[17] National Heart, Lung, and Blood Institute. *Morbidity and Mortality: 2009 Chart Book on Cardiovascular, Lung, and Blood Diseases*. National Institutes of Health, Bethesda, MD (2009).
[18] Chen, Q., Camara, A.K., Stowe, D.F. *et al*. Modulation of electron transport protects cardiac mitochondria and decreases myocardial injury during ischemia and reperfusion, *Am. J. Physiol. Cell Physiol*., **292**, C137–C147 (2007).
[19] Wildhirt, S.M., Weismueller, S., Schulze, C. *et al*. Inducible nitric oxide synthase activation after ischemia/reperfusion contributes to myocardial dysfunction and extent of infarct size in rabbits: evidence for a late phase of nitric oxide-mediated reperfusion injury, *Cardiovasc. Res*., **43**, 698–711 (1999).
[20] Erusalimsky, J.D. and Moncada, S. Nitric oxide and mitochondrial signaling: from physiology to pathophysiology, *Arterioscler. Thromb. Vasc. Biol*., **27**, 2524–2531 (2007).
[21] Ma, X.L., Lopez, B.L., Liu, G.L. *et al*. Peroxynitrite aggravates myocardial reperfusion injury in the isolated perfused rat heart, *Cardiovasc. Res*., **36**, 195–204 (1997).

[22] Steffens, S., Montecucco, F. and Mach, F. The inflammatory response as a target to reduce myocardial ischaemia and reperfusion injury, *Thromb. Haemost*., **102**, 240–247 (2009).
[23] Lewis, M.S., Whatley, R.E., Cain, P. *et al*. Hydrogen peroxide stimulates the synthesis of platelet-activating factor by endothelium and induces endothelial cell-dependent neutrophil adhesion, *J. Clin. Invest*., **82**, 2045–2055 (1988).
[24] Xu, Y., Huo, Y., Toufektsian, M.C. *et al*. Activated platelets contribute importantly to myocardial reperfusion injury, *Am. J. Physiol. Heart Circ. Physiol*., **290**, H692–H699 (2006).
[25] Mellion, B.T., Ignarro, L.J., Ohlstein, E.H. *et al*. Evidence for the inhibitory role of guanosine 3′, 5′-monophosphate in ADP-induced human platelet aggregation in the presence of nitric oxide and related vasodilators, *Blood*, **57**, 946–955 (1981).
[26] Murry, C.E., Jennings, R.B. and Reimer, K.A. Preconditioning with ischemia: a delay of lethal cell injury in ischemic myocardium, *Circulation*, **74**, 1124–1136 (1986).
[27] Yang, X.M., Proctor, J.B., Cui, L. *et al*. Multiple, brief coronary occlusions during early reperfusion protect rabbit hearts by targeting cell signaling pathways, *J. Am. Coll. Cardiol*., **44**, 1103–1110 (2004).
[28] Hausenloy, D.J. and Yellon, D.M. New directions for protecting the heart against ischaemia-reperfusion injury: targeting the reperfusion injury salvage kinase (RISK)-pathway, *Cardiovasc. Res*., **61**, 448–460 (2004).
[29] Hausenloy, D.J. and Yellon, D.M. Preconditioning and postconditioning: underlying mechanisms and clinical application, *Atherosclerosis*, **204**, 334–341 (2009).
[30] Ludman, A.J., Yellon, D.M. and Hausenloy, D.J. Cardiac preconditioning for ischaemia: lost in translation, *Dis. Model Mech*., **3**, 35–38 (2010).
[31] Cohen, M.V., Yang, X.M., Liu, G.S. *et al*. Acetylcholine, bradykinin, opioids, and phenylephrine, but not adenosine, trigger preconditioning by generating free radicals and opening mitochondrial K(ATP) channels, *Circ. Res*., **89**, 273–278 (2001).
[32] Haramaki, N., Stewart, D.B., Aggarwal, S. *et al*. Networking antioxidants in the isolated rat heart are selectively depleted by ischemia-reperfusion, *Free Radic. Biol. Med*., **25**, 329–339 (1998).
[33] Dhalla, N.S., Temsah, R.M. and Netticadan, T. Role of oxidative stress in cardiovascular diseases, *J. Hypertens*., **18**, 655–673 (2000).
[34] Boaz, M., Smetana, S., Weinstein, T. *et al*. Secondary prevention with antioxidants of cardiovascular disease in endstage renal disease (SPACE): randomised placebo-controlled trial, *Lancet*, **356**, 1213–1218 (2000).
[35] Reiter, R.J. and Tan, D.X. Melatonin: a novel protective agent against oxidative injury of the ischemic/reperfused heart, *Cardiovasc. Res*., **58**, 10–19 (2003).
[36] Petrosillo, G., Di Venosa, N., Pistolese, M. *et al*. Protective effect of melatonin against mitochondrial dysfunction associated with cardiac ischemia-reperfusion: role of cardiolipin, *FASEB J*., **20**, 269–276 (2006).
[37] Petrosillo, G., Colantuono, G., Moro, N. *et al*. Melatonin protects against heart ischemia-reperfusion injury by inhibiting mitochondrial permeability transition pore opening, *Am. J. Physiol. Heart Circ. Physiol*., **297**, H1487–H1493 (2009).

[38] Dominguez-Rodriguez, A., Abreu-Gonzalez, P., Garcia-Gonzalez, M.J. *et al*. A unicenter, randomized, double-blind, parallel-group, placebo-controlled study of Melatonin as an Adjunct in patients with acute myocaRdial Infarction undergoing primary Angioplasty: the Melatonin Adjunct in the acute myocaRdial Infarction treated with Angioplasty (MARIA) trial: study design and rationale, *Contemp. Clin. Trials*, **28**, 532–539 (2007).

[39] Mittal, C.K. and Murad, F. Properties and oxidative regulation of guanylate cyclase, *J. Cyclic Nucleotide Res*., **3**, 381–391 (1977).

[40] Culotta, E. and Koshland, D.E. Jr. NO news is good news, *Science*, **258**, 1862–1865 (1992).

[41] Marsh, N. and Marsh, A. A short history of nitroglycerine and nitric oxide in pharmacology and physiology, *Clin. Exp. Pharmacol. Physiol*., **27**, 313–319 (2000).

[42] Laskey, W.K., Reichek, N., St John Sutton, M. *et al*. Matching of myocardial oxygen consumption to mechanical load in human left ventricular hypertrophy and dysfunction, *J. Am. Coll. Cardiol*., **3**, 291–300 (1984).

[43] Flaherty, J.T. Role of nitrates in acute myocardial infarction, *Am. J. Cardiol*., **70**, 73B–81B (1992).

[44] Sharp, B.R., Jones, S.P., Rimmer, D.M. and Lefer, D.J. Differential response to myocardial reperfusion injury in eNOS-deficient mice, *Am. J. Physiol. Heart Circ. Physiol*., **282**, H2422–H2426 (2002).

[45] Jones, S.P., Girod, W.G., Palazzo, A.J. *et al*. Myocardial ischemia-reperfusion injury is exacerbated in absence of endothelial cell nitric oxide synthase, *Am. J. Physiol*., **276**, H1567–H1573 (1999).

[46] Jones, S.P., Greer, J.J., Kakkar, A.K. *et al*. Endothelial nitric oxide synthase overexpression attenuates myocardial reperfusion injury, *Am. J. Physiol. Heart Circ. Physiol*., **286**, H276–H282 (2004).

[47] Duranski, M.R., Elrod, J.W., Calvert, J.W. *et al*. Genetic overexpression of eNOS attenuates hepatic ischemia-reperfusion injury, *Am. J. Physiol. Heart Circ. Physiol*., **291**, H2980–H2986 (2006).

[48] Ignarro, L.J. Signal transduction mechanisms involving nitric oxide, *Biochem. Pharmacol*., **41**, 485–490 (1991).

[49] Gewaltig, M.T. and Kojda, G. Vasoprotection by nitric oxide: mechanisms and therapeutic potential, *Cardiovasc. Res*., **55**, 250–260 (2002).

[50] Brown, G.C. and Cooper, C.E. Nanomolar concentrations of nitric oxide reversibly inhibit synaptosomal respiration by competing with oxygen at cytochrome oxidase, *FEBS Lett*., **356**, 295–298 (1994).

[51] Gaston, B.M., Carver, J., Doctor, A. and Palmer, L.A. S-nitrosylation signaling in cell biology, *Mol. Interv*., **3**, 253–263 (2003).

[52] Hess, D.T., Matsumoto, A., Kim, S.O. *et al*. Protein S-nitrosylation: purview and parameters, *Nat. Rev. Mol. Cell Biol*., **6**, 150–166 (2005).

[53] Lima, B., Forrester, M.T., Hess, D.T. and Stamler, J.S. S-nitrosylation in cardiovascular signaling, *Circ. Res*., **106**, 633–646 (2010).

[54] Nolan, J.P., Hazinski, M.F., Billi, J.E. *et al*. Part 1. Executive summary: 2010 International Consensus on Cardiopulmonary Resuscitation and Emergency

Cardiovascular Care Science With Treatment Recommendations, *Resuscitation*, **81**(Suppl 1), e1–e25 (2010).

[55] Hill, M., Takano, H., Tang, X.L. *et al*. Nitroglycerin induces late preconditioning against myocardial infarction in conscious rabbits despite development of nitrate tolerance, *Circulation*, **104**, 694–699 (2001).

[56] Chen, C.H., Budas, G.R., Churchill, E.N. *et al*. Activation of aldehyde dehydrogenase-2 reduces ischemic damage to the heart, *Science*, **321**, 1493–1495 (2008).

[57] Zimrin, D., Reichek, N., Bogin, K.T. *et al*. Antianginal effects of intravenous nitroglycerin over 24 hours, *Circulation*, **77**, 1376–1384 (1988).

[58] Calvert, J.W. and Lefer, D.J. Myocardial protection by nitrite, *Cardiovasc. Res*., **83**, 195–203 (2009).

[59] Duranski, M.R., Greer, J.J., Dejam, A. *et al*. Cytoprotective effects of nitrite during in vivo ischemia-reperfusion of the heart and liver, *J. Clin. Invest*., **115**, 1232–1240 (2005).

[60] Elrod, J.W., Greer, J.J. and Lefer, D.J. Sildenafil-mediated acute cardioprotection is independent of the NO/cGMP pathway, *Am. J. Physiol. Heart Circ. Physiol*., **292**, H 342–347 (2007).

[61] Das, S., Maulik, N., Das, D.K. *et al*. Cardioprotection with sildenafil, a selective inhibitor of cyclic 3′,5′-monophosphate-specific phosphodiesterase 5, *Drugs Exp. Clin. Res*., **28**, 213–219 (2002).

[62] du Toit, E.F., Rossouw, E., Salie, R. *et al*. Effect of sildenafil on reperfusion function, infarct size, and cyclic nucleotide levels in the isolated rat heart model, *Cardiovasc. Drugs Ther*., **19**, 23–31 (2005).

[63] Reffelmann, T. and Kloner, R.A. Phosphodiesterase 5 inhibitors: are they cardioprotective?, *Cardiovasc. Res*., **83**, 204–212 (2009).

[64] Stasch, J.P., Schmidt, P., Alonso-Alija, C. *et al*. NO- and haem-independent activation of soluble guanylyl cyclase: molecular basis and cardiovascular implications of a new pharmacological principle, *Br. J. Pharmacol*., **136**, 773–783 (2002).

[65] Krieg, T., Liu, Y., Rutz, T. *et al*. BAY 58-2667, a nitric oxide-independent guanylyl cyclase activator, pharmacologically post-conditions rabbit and rat hearts, *Eur. Heart J*., **30**, 1607–1613 (2009).

[66] Bloch, K.D., Ichinose, F., Roberts, J.D. Jr and Zapol, W.M. Inhaled NO as a therapeutic agent, *Cardiovasc. Res*., **75**, 339–348 (2007).

[67] Hogman, M., Frostell, C., Arnberg, H. *et al*. Prolonged bleeding time during nitric oxide inhalation in the rabbit, *Acta Physiol. Scand*., **151**, 125–129 (1994).

[68] Guery, B., Neviere, R., Viget, N. *et al*. Inhaled NO preadministration modulates local and remote ischemia-reperfusion organ injury in a rat model, *J. Appl. Physiol*., **87**, 47–53 (1999).

[69] Hataishi, R., Rodrigues, A.C., Neilan, T.G. *et al*. Inhaled nitric oxide decreases infarction size and improves left ventricular function in a murine model of myocardial ischemia-reperfusion injury, *Am. J. Physiol. Heart Circ. Physiol*., **291**, H379–384 (2006).

[70] Liu, X., Huang, Y., Pokreisz, P. *et al*. Nitric oxide inhalation improves microvascular flow and decreases infarction size after myocardial ischemia and reperfusion, *J. Am. Coll. Cardiol*., **50**, 808–817 (2007).

[71] Nagasaka, Y., Fernandez, B.O., Garcia-Saura, M.F. *et al*. Brief periods of nitric oxide inhalation protect against myocardial ischemia-reperfusion injury, *Anesthesiology*, **109**, 675–682 (2008).

[72] Ng, E.S., Jourd'heuil, D., McCord, J.M. *et al*. Enhanced S-nitroso-albumin formation from inhaled NO during ischemia/reperfusion, *Circ. Res*., **94**, 559–565 (2004).

Part II
Medical Needs for Oxygen Supply

6

Acute Traumatic Hemorrhage and Anemia

Lena M. Napolitano

Department of Surgery, University of Michigan, Ann Arbor, MI, USA

6.1 Introduction

Traumatic injuries are common and account for almost 15% of all allogeneic red blood cell (RBC) transfusion use in the United States. Control and treatment of hemorrhage is a critical aspect of trauma care, particularly in patients with hemorrhagic shock [1]. Despite major advances in the treatment of hemorrhagic shock, hemorrhage remains a leading cause of early death in both civilian and military trauma. Failure to control and treat hemorrhage promptly in such patients results in a "bloody vicious cycle" (Figure 6.1) of hemorrhage, resuscitation, hemodilution, and coagulopathy, leading to more hemorrhage [2–4].

At present, the only oxygen-carrying resuscitation fluid available for use in patients with traumatic injuries is allogeneic RBC transfusion. The aim of treatment of hemorrhagic shock with RBC transfusion is the rapid and effective restoration of an adequate blood volume to maximize tissue oxygen delivery. Furthermore, the goal of transfusion of blood and blood products is to maintain the patient's blood composition within safe limits with regard to hemostasis, oxygen-carrying capacity, oncotic pressure, and biochemistry. Therefore, the additional administration of other blood components (in addition to RBCs) is necessary for the prevention of dilutional coagulopathy and dilutional thrombocytopenia.

The Advanced Trauma Life Support (ATLS) course of the American College of Surgeons recommends starting two large-bore IVs in patients who are significantly injured and, if they are hypotensive, giving two liters of isotonic crystalloid solution [11].

Chemistry and Biochemistry of Oxygen Therapeutics: From Transfusion to Artificial Blood, First Edition.
Edited by Andrea Mozzarelli and Stefano Bettati.

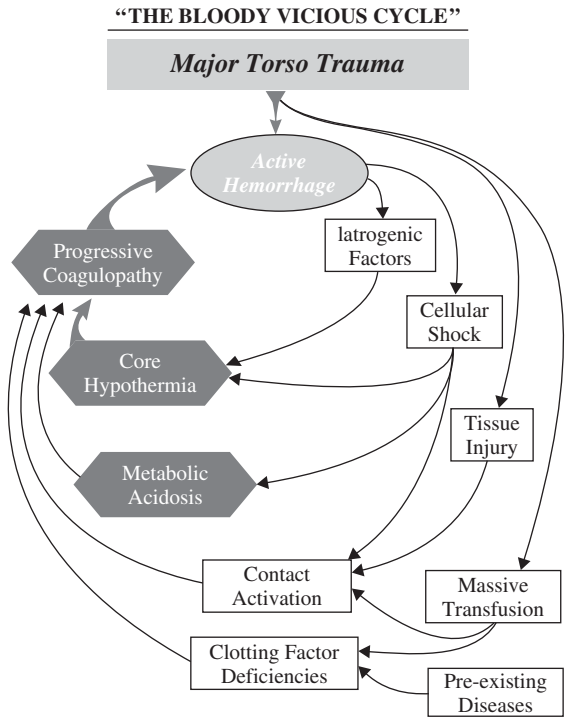

Figure 6.1 *The pathogenesis of the "bloody vicious cycle" following major torso trauma is multifactorial, but usually manifests as a triad of refractory coagulopathy, progressive hypothermia, and persistent metabolic acidosis. (Reprinted from [3], with permission from Elsevier).*

If the patient remains hypotensive in shock, then RBCs should be given. These guidelines were based on the recognition that prolonged shock frequently leads to organ failure that can be prevented by fluid resuscitation. Most frequently, uncrossmatched RBC units are administered to trauma patients requiring emergent RBC transfusion, and the increasing use of uncrossmatched RBC transfusion has been identified as an independent predictor of mortality and the need for massive transfusion (MT) [5]. The use of fresh whole blood has also been required in the combat casualty setting due to a lack of sufficient stored blood products, and therefore these combat casualty reports also document administration of fresh whole blood [6].

Recent evidence-based guidelines for RBC transfusion in adult trauma and critical care [7, 8] recommended the following:

(i) RBC transfusion is indicated for patients with evidence of hemorrhagic shock.
(ii) RBC transfusion may be indicated for patients with evidence of acute hemorrhage and hemodynamic instability or inadequate oxygen delivery.
(iii) A "restrictive" strategy of RBC transfusion (transfuse when Hb < 7 g/dL) is as effective as a "liberal" transfusion strategy (transfusion when Hb < 10 g/dL) in critically ill patients with hemodynamically stable anemia, except possibly in patients with acute myocardial ischemia.

(iv) The use of only Hb level as a "trigger" for transfusion should be avoided. Decision for RBC transfusion should be based on the individual patient's intravascular volume status, evidence of shock, duration and extent of anemia, and cardiopulmonary physiologic parameters.
(v) In the absence of acute hemorrhage, RBC transfusion should be given as single units.
(vi) RBC transfusion should not be considered as an absolute method to improve tissue oxygen consumption in critically ill patients.

6.2 Blood Transfusion in Trauma

6.2.1 Massive Transfusion

MT is required as a treatment for uncontrolled hemorrhage, and trauma is the most common etiology [9–11]. Massive blood transfusion is commonly defined as administration of $\geq$10 units of allogeneic RBCs to an individual patient or transfusion of more than one blood volume in 24 hours. Other "dynamic" definitions of MT have been used, particularly in order to initiate MT institutional protocols [12–18]. Risk factors for MT in trauma include tachycardia, hypotension, acidosis, penetrating mechanism, hemoperitoneum by sonography, and anemia [19]. Several predictive models for MT have been developed, with all variables necessary to calculate the score easily available upon emergency room arrival [20–22]. Trauma patients requiring MT have high mortality rates, ranging from 19 to 84%. Data from more recent studies document a significant reduction in mortality in trauma patients requiring MT, with most recent series reporting a mortality rate of approximately 30–58% [23]. Mortality is directly related to the severity of the hemorrhagic shock and the total number of packed RBC (PRBC) units transfused [24]. Interestingly, in a single-center retrospective study of trauma patients requiring MT of more than 50 units of blood products in the first 24 hours, the overall mortality was 57% but there was no significant difference in mortality rate between patients who received >75 units of blood products in the first day versus those who received 51–75 units [25].

6.2.2 Massive Transfusion and Coagulopathy

The standard goal of MT in past years was to supply isotonic crystalloids and plasma-poor RBC concentrates to maintain normovolemia and tissue oxygen supply. This, however, frequently led to dilutional coagulopathy, which was frequently aggravated and accelerated by hypothermia, acidosis, shock-induced impairment of hepatic function, disseminated intravascular coagulation due to tissue injury, and increased consumption of clotting factors and platelets at extensive wound sites in injured patients.

We now recognize that patients who have sustained severe hemorrhage and require MT commonly have an early and profound coagulopathy, which is present on admission and worsens with PRBC transfusion due to dilutional and consumptive coagulopathy [26–29]. Traditional resuscitation techniques using large amounts of crystalloid and PRBCs without other blood products can exacerbate this coagulopathy [30].

Therefore, another key aim of modern MT protocols is the timely administration of plasma and platelet concentrates as required to halt microvascular bleeding induced by impaired hemostasis [31, 32].

6.2.3 Hypotensive or Delayed Resuscitation

The concept of delaying resuscitation or only resuscitating to a low to low–normal blood pressure ("hypotensive resuscitation") in the actively hemorrhaging patient until definitive hemorrhage control is achieved has been advocated based on a number of preclinical studies which documente that vigorous fluid resuscitation in uncontrolled hemorrhagic shock is associated with increased hemorrhage and decreased survival [33]. Maintaining a low-blood-pressure goal with "hypotensive resuscitation" aims to reduce the amount of blood lost through the site of injury until definitive hemorrhage control is achieved.

In a randomized prospective clinical trial of immediate versus delayed fluid resuscitation in patients ($n = 598$) with penetrating torso trauma who presented with a prehospital systolic blood pressure of ≤ 90 mmHg with an overall mortality rate of 34%, there were significantly increased mortality (38% vs 30%, $p = 0.04$), length of stay, and postoperative complication rates in the immediate versus the delayed group [34].

In a single-center study that randomized patients ($n = 110$) presenting in hemorrhagic shock to one of two fluid resuscitation protocols (target systolic blood pressure >100 mmHg vs 70 mmHg) titrated to this endpoint until definitive hemostasis was achieved, no difference in overall survival (92.7% in both groups) was identified. Although no mortality benefit was identified with hypotensive resuscitation in this study, it was noted that a number of study limitations were present: failure to achieve target systolic blood pressure in the hypotensive group (mean systolic blood pressure 100 mmHg vs 114 mmHg in control group), small sample size, mix of blunt (49%) and penetrating (51%) trauma patients, and lengthy time for duration of active hemorrhage (2.97 ± 1.75 hours vs 2.57 ± 1.46 hours, $p = 0.20$). Despite the limitations of these clinical studies, "hypotensive" resuscitation has become increasingly accepted in the prehospital resuscitation phase of trauma, prior to definitive hemorrhage control, since aggressive fluid resuscitation may increase bleeding [35].

6.2.4 Hemostatic Resuscitation

Acidosis, hypothermia, and coagulopathy were identified more than 20 years ago as a deadly triad for patients presenting with exsanguinating hemorrhage. This led to fundamental changes in initial management of severely injured patients. Despite these major advances, hemorrhage remains a leading cause of early death in trauma patients. Recent studies report most severely injured patients to be coagulopathic at admission, before resuscitation interventions, and that traditional MT practices grossly underestimate what is needed to correct the coagulopathy.

Since hemorrhage is a major cause of trauma deaths and coagulopathy exacerbates hemorrhage and is commonly seen during major trauma resuscitations, prompt reversal of coagulopathy using "hemostatic resuscitation" has been advocated as the optimal practice for MT in trauma [36, 37]. Reversal of coagulopathy involves normalization of body temperature, elimination of the causes of disseminated intravascular coagulation, and transfusion with fresh frozen plasma (FFP), platelets, and cryoprecipitate as needed. Some have advocated that coagulopathy can best be avoided or reversed when severe trauma victims are transfused with at least the equivalent of whole blood [38, 39]. A study in combat casualty care identified that the ratio of blood products transfused affected

mortality in patients receiving MTs at a combat support hospital [26]. They performed a retrospective chart review of 246 patients at a US Army combat support hospital, each of who received an MT (≥10 units of PRBCs in 24 hours). They identified that a high 1 : 1.4 plasma to PRBC ratio was independently associated with improved survival to hospital discharge, primarily by decreasing death from hemorrhage. The authors concluded that MT protocols should utilize a 1 : 1 ratio of plasma to PRBCs for all patients who are hypocoagulable with traumatic injuries [40, 41].

The practice of hemostatic resuscitation was initiated in military combat casualty care [42], but has also been examined in civilian trauma, and the concept is now being applied to other patient populations requiring massive blood transfusion for severe hemorrhage. Multiple clinical studies in civilian trauma patient populations have addressed the topic of hemostatic resuscitation as well [43–48]. A recent systematic review of 37 studies, most of which were observational in nature, documented that in patients undergoing MT, plasma infusion at high plasma to RBC ratios was associated with a significant reduction in the risk of death (odds ratio (OR) 0.38, 95% CI 0.24–0.60) and multiple organ failure (OR 0.40, 95% CI 0.26–0.60). However, the quality of this evidence was very low due to significant unexplained heterogeneity and several other biases. In patients undergoing surgery without MT, plasma infusion was associated with a trend toward increased mortality (OR 1.22, 95% CI 0.73–2.03). Plasma transfusion was associated with increased risk of developing acute lung injury (OR 2.92, 95% CI 1.99–4.29) [49]. Evidence-based practice guidelines for plasma transfusion by the American Society of Hematology recommended that plasma be transfused to patients requiring MT, but could not recommend a specific plasma to RBC ratio [50]. Despite significant controversy, an emerging consensus for **hemostatic resuscitation** in patients requiring MT is as follows:

- Expedite control of hemorrhage to prevent consumptive coagulopathy and thrombocytopenia and reduce the need for blood products.
- Limit isotonic crystalloid infusion to prevent dilutional coagulopathy and thrombocytopenia.
- Carry out hypotensive resuscitation (systolic blood pressure 80–100 mm Hg) until definitive hemorrhage control is established.
- Transfuse blood products in a 1 : 1 : 1 ratio of PRBC : FFP : platelets.
- Carry out frequent laboratory monitoring (arterial lactate to assess adequacy of resuscitation, ionized calcium, electrolytes).

Although hemostatic resuscitation has been associated with reduced mortality in these retrospective studies, we must recognize that there are potential adverse effects associated with transfusion-of-blood-component therapy, including FFP and platelets. A number of studies have documented increased risk for acute lung injury and acute respiratory distress syndrome with both blood and plasma transfusions [51–53]. Transfusion-related acute lung injury (TRALI) is now the leading cause of transfusion-associated mortality, even though it is probably underdiagnosed and underreported [54–56].

6.2.5 Massive Transfusion Protocols

MT protocols have long been in place at major trauma centers for the treatment of patients with severe hemorrhagic shock [57]. In the past, MT protocols provided PRBCs, but still required the clinician to issue specific requests for other blood-component therapy, including FFP and platelets. Furthermore, it was recommended that transfusion of these additional blood components wait until laboratory evidence of dilutional and consumptive coagulopathy and thrombocytopenia were present. In the current era, MT protocols now focus on *prevention* of coagulopathy and thrombocytopenia.

As noted above, a 1 : 1 : 1 ratio (i.e. equal parts PRBCs, FFP, and platelets) for blood component therapy is now recommended for MT based on a more physiologic regimen similar to whole-blood transfusion. This approach has been named "hemostatic resuscitation" and focuses on the early correction of coagulopathy, which is thought to be associated with improved survival. MT protocols have now been revised to include other blood-component therapy in addition to RBC units, and have been associated with improved outcomes in trauma [58–61].

A multicenter prospective observational study of severely injured trauma patients who require blood transfusions is underway (PROMMTT, Prospective Observational Multicenter Massive Transfusion sTudy) [62], aiming to further investigate MT protocols associated with improved outcome. The results of this observational study will inform the development of a future randomized clinical trial which will test these protocols.

6.2.6 Transfusion after Hemorrhage Control

Once definitive hemorrhage control has been established, a restrictive approach to blood transfusion should be implemented. Guidelines for Transfusion in the Trauma Patient have been established as a standard operating procedure to guide RBC transfusion therapy for critically ill patients after the immediate resuscitation phase and to minimize the adverse consequences of potentially unnecessary transfusions. This protocol considers that the acute hemorrhage has been controlled, the initial resuscitation has been completed, and the patient is stable in the intensive care unit, with no evidence of ongoing bleeding (Figure 6.2). This guideline advocates a trigger for PRBC transfusion of hemoglobin (Hb) <7 g/dL (or a hematocrit $<21\%$), even in patients with a history of cardiovascular disease [63].

The recent report of the findings of the transfusion trigger trial for Functional Outcomes in Cardiovascular patients Undergoing Surgical hip-fracture repair (FOCUS, sponsored by the National Heart, Lung and Blood Institute) [64] confirms use of a lower transfusion trigger in patients with stable anemia. This trial randomized 2016 patients with asymptomatic anemia to a transfusion Hb trigger <8 vs <10 g/dL in 47 medical centers in the USA and Canada (8/2004-2/2009). No difference in cardiovascular outcomes (4.3 vs 5.2%) or in-hospital mortality (2 vs 1.4%) was identified [65].

6.2.7 Efficacy of RBC Transfusion in Trauma and Associated Risks

Adverse effects may occur with the transfusion of stored human RBCs. It has been documented that as human blood is stored, hemolysis occurs, and increased concentrations of free Hb are found in such stored blood [66] (see Chapters 17 and 18). Abnormal

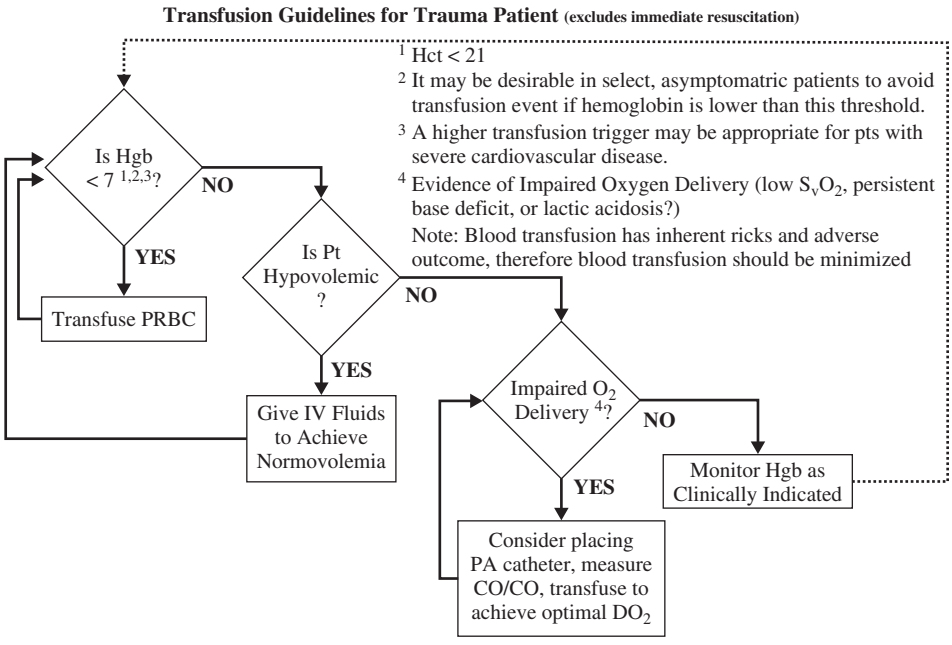

Figure 6.2 *Guidelines for Transfusion in the Trauma Patient have been established as a standard operating procedure to guide PRBC transfusion therapy for critically ill patients after the immediate resuscitation phase and to minimize the adverse consequences of potentially unnecessary transfusions. (Reprinted from [63], with permission from Wolters Kluwer Health).*

hemolysis in an individual RBC unit may be caused by several factors, including inappropriate handling during processing of blood, inappropriate or extended duration of storage, bacterial hemolysins, antibodies that cause complement lysis, defects in the RBC membrane, or an abnormality in the blood donor. The acceptable level of hemolysis has not been established in North America, but the value of 1% is currently used to assess biocompatibility of blood storage materials [67]. Increasing free plasma Hb in aged stored RBC units, in addition to generating reactive oxygen species such as the hydroxyl and superoxide radicals, is also a potent scavenger of nitric oxide. Nitric oxide, which is normally produced by the endothelium, regulates basal vasodilator tone, inhibits platelet and hemostatic activation, and reduces superoxide levels through radical–radical scavenging. The vasodilator activity of nitric oxide is possible only because most Hb is normally compartmentalized within erythrocytes. As free-Hb concentration increases in aged stored allogeneic RBCs, a vasoconstrictive effect is evident (Figure 6.3). One trial documented that RBC transfusion was associated with a significant increase in pulmonary vascular resistance index, which was not evident in the control cohort that received 5% albumin instead [68]. The clinical consequences of red-cell storage in trauma and the critically ill are particularly concerning [69]. Duration of red-cell storage has been associated with adverse outcomes including increased mortality [70]. In patients undergoing cardiac surgery, transfusion of red cells that had been stored for more than 2 weeks was associated with a significantly increased risk of postoperative complications

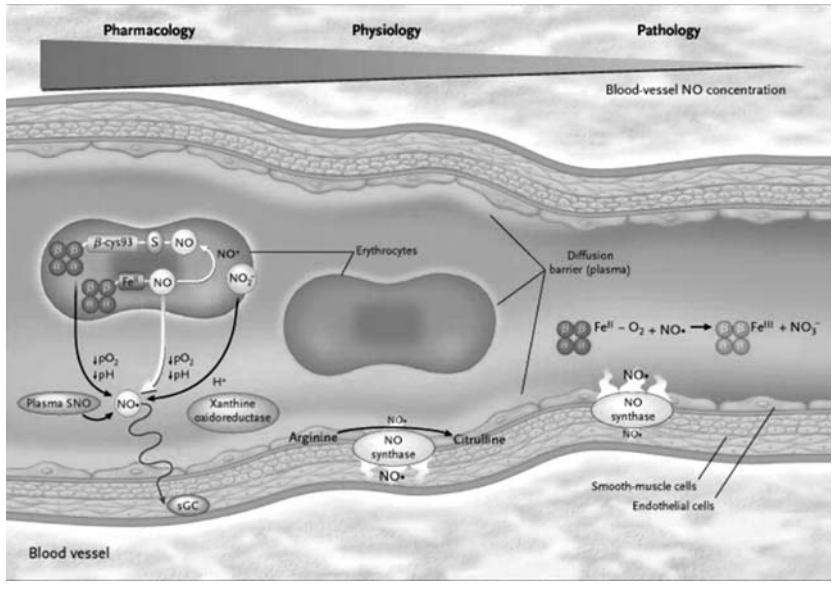

Figure 6.3 *A model of the interactions of nitric oxide (NO) with erythrocytes and cell-free Hb in an arterial blood vessel. The diagram illustrates the major processes regulating NO levels in blood vessels during pharmacologic NO delivery (left), under normal conditions (center), and under pathologic conditions, such as acute or chronic hemolysis (right). The overall blood-vessel NO concentration is depicted by the width of the blue band above the vessel. Within the vessel, smooth-muscle cells and a layer of endothelial cells are shown. Free Hb binds NO, resulting in vascular constriction on the right side of the figure. Aged stored RBC units with increased hemolysis and early-generation HBOCs may both have increased free-Hb concentrations and induce vasoconstriction in blood vessels. (Reproduced with permission from* The New England Journal of Medicine *[145]).*
For a better understanding of the figure, please refer to color plate 2.

as well as reduced short-term and long-term survival [71]. The multicenter Age of Blood Evaluation (ABLE) trial in the resuscitation of critically ill patients is ongoing, randomizing patients to the transfusion of fresh leuko-reduced RBCs (stored less than 8 days) versus transfusion of standard-issue RBCs stored 2–42 days [72]. Additional studies have documented significant risks associated with RBC transfusion [73–75] and lack of efficacy [76, 77]. Recent evidence suggests that in adult trauma patients, when used for treatment of anemia, RBC transfusions are associated with increased morbidity and mortality and therefore current RBC transfusion practices may require reevaluation, and alternative oxygen-carrying resuscitation fluids warrant investigation.

6.3 Oxygen Therapeutics in Trauma

The need for an alternative to allogeneic RBCs for transfusion has been recognized for over a century [78–80]. Since the inception of allogeneic RBC transfusion, the search for an alternative to the use of stored RBCs has been underway [81, 82]. Concerns

about the infectious and immunosuppressive risks of allogeneic blood products persist, and the increased disproportion of blood donation and consumption has reinforced the search for alternative erythrocyte transfusion strategies in recent years. The most serious motivation for the development of a blood substitute is the worldwide shortage of safe and viable allogeneic donor blood [83]. Donated allogeneic blood shortage is a significant issue, particularly in planning for disasters [84–86]. In addition, the stress on the donated blood supply is projected to increase in the coming years [87]. Interestingly, even though blood transfusions remain the standard of care, the efficacy and safety of allogeneic RBC therapy has never been rigorously tested via the clinical trial process [88–90]. Thus, comparing the safety and efficacy of a blood substitute to the standard of care may prove to be difficult.

Oxygen therapeutics that have been developed as RBC substitutes are the acellular oxygen carriers, which can be categorized into two types: perfluorocarbons (PFCs, completely synthetic) and hemoglobin-based oxygen carriers (HBOCs). HBOCs are oxygen carriers that use purified human, animal, or recombinant Hb in a cell-free Hb preparation. They are infusible oxygen-carrying fluids that have long shelf lives, have no need for refrigeration or crossmatching, could be in abundant supply, and are ideal for treating hemorrhagic shock in remote settings where blood is not available. Hb is a logical choice for a red-cell substitute because of its high capacity to carry oxygen and its oncotic properties [91]. It also lacks the numerous and complex antigens of the RBC membrane, hence it is universally compatible. It is a robust molecule that withstands rigorous purification and viral inactivation processes, and it is stable under ordinary storage conditions.

The ideal intravenous fluid for trauma resuscitation would have the following properties: provide volume expansion, carry oxygen, possess a long shelf-life at room temperature, not affect coagulation, and be universally compatible, non-antigenic, non-infectious, and inexpensive. HBOCs are, in theory, the ideal intravenous fluid for trauma resuscitation since they possess many of these ideal properties [92].

In spite of significant effort in the development of HBOCs, currently no such product is approved for use in North America or Europe. Only one product (Hemopure, HBOC-201, polymerized bovine Hb, Biopure Corporation) is approved for use in South Africa for adult surgery patients, to treat acute anemia and reduce the need for allogeneic RBCs [93, 94]. Biopure filed for bankruptcy in July 2009 and as of December 2009, Hemopure is unavailable. In patients where RBC transfusion is not an option (i.e. Jehovah's Witness patients) and others for whom blood is not available (difficult crossmatch), HBOC use may be life-saving [95].

In March 2006, a workshop sponsored by the National Heart, Lung and Blood Institute was convened to identify the role of basic science in clarifying the issues that are impeding progress in the development of HBOC solutions [96]. These discussions resulted in a consensus that, although HBOCs have shown clinical promise, various side effects have inhibited further development and regulatory approval, with cardiovascular events being of particular concern [97, 98]. Specific recommendations from this group included better understanding of the impact of HBOC infusion on human physiology, the need for rapid non-invasive methods for the measurement of tissue oxygenation in human patients to better inform transfusion decisions, further investigation of routes and consequences of Hb metabolism, optimization of clinical protocols for HBOC use, and assessment of the impact of HBOC formulation excipients.

A recent report of a 2008 conference sponsored by the Food and Drug Administration (FDA) and the National Institutes of Health (NIH) reviewed the current status and future directions in research of HBOCs [99]. The participants concluded that the effects observed in preclinical animal studies did not mimic those of the clinical trials, and they considered the feasibility of, obstacles to, and ethical issues related to future clinical trials of HBOCs. A brief review of the results of HBOC trials in trauma is presented below. PolyHeme and HemAssist are the only HBOCs for which there are published studies involving trauma, but a trauma trial was proposed for Hemopure as well, and a recent trauma trial was completed for MP4OX.

6.3.1 Diaspirin Crosslinked Hb

Diaspirin Crosslinked Hb (DCLHb, HemAssist) was a first-generation HBOC developed by Baxter Hemoglobin Therapeutics during the 1990s. This product circumvented the safety concerns surrounding dimerization of the Hb tetramer by crosslinking the alpha chains chemically. Animal studies were promising [100]. However, the clinical studies were stopped because of an increased mortality in patients who received DCLHb after stroke and traumatic shock [101–104]. Because of an observed increased mortality in the DCLHb-treated patients, the US study was terminated by the Data Safety Monitoring Board after the enrollment of 98 patients. At that time, the EU study was also halted and analysis of the EU data demonstrated no benefit with the use of DCLHb, resulting in the final termination of the study after the enrollment of 121 patients. Additional studies in cardiac and noncardiac surgery documented additional safety concerns, with early study termination related to serious adverse events (SAEs) [105–107].

Interestingly, a recent post hoc analysis of the data from the two multicenter trauma trials (US in-hospital study in emergency departments (2/1997–1/1998) and European prehospital study (7/1997–5/1998)) with 219 patients enrolled was performed. This analysis documented that, although patients who died had more greatly altered perfusion than those who survived, DCLHb treatment of traumatic hemorrhagic shock patients was not associated with base deficit (BD) or lactate abnormalities that would indicate poor perfusion [108]. Also, resuscitation blood pressures from these two studies did not demonstrate a consistent DCLHb pressor effect [109].

6.3.2 Hemopure

HBOC-201 is a polymerized bovine Hb product (Hemopure, polymerized bovine Hb, Biopure Corporation, Cambridge, MA). It has an intravascular half-life of 8–23 hours and a shelf life of 36 months at room temperature. One unit of Hemopure contains 30 g of ultrapurified, chemically crosslinked Hb in 250 ml of a balanced salt solution. When infused, these linked Hb molecules circulate in the plasma. They are smaller, have a lower viscosity, and release oxygen to tissues more readily than allogeneic RBCs [110]. The product is compatible with all blood types, and is purified through patented techniques that are validated to remove infectious agents, including bacteria, viruses, prions, and other potential contaminants. A similar bovine Hb substitute is used in veterinary medicine as Oxyglobin.

Phase II and III studies have documented that HBOC-201 can avoid or reduce allogeneic blood-transfusion needs in specific perioperative settings including infrarenal

aortic surgery [111], in surgical patients with intraoperative administration [112], in cardiac surgery patients requiring cardiopulmonary bypass [113], and in noncardiac surgical patients. Most recently, the report of the largest clinical trial (US phase III Orthopedic Surgery Trial, initiated in 1998) was published [114]. The ability of HBOC-201 to safely reduce and/or eliminate perioperative transfusion was studied in orthopedic surgery patients. This study was relevant to trauma, as many of the orthopedic surgical patients had traumatic orthopedic injuries. A randomized, single-blind, RBC-controlled, parallel-group multicenter study was conducted. A total of 266 patients were randomized to treatment with HBOC-201 (H, $n = 350$) or RBC (R, $n = 338$) at the first transfusion decision. Primary endpoints were transfusion avoidance and blinded assessment of safety non-inferiority. A total of 59.4% of patients in the H arm avoided PRBC transfusion. Adverse events (8.47 vs 5.88) and SAEs (0.35 vs 0.25) per patient were higher in the H versus R arms ($p < 0.001$ and $p < 0.01$). HBOC-201 eliminated transfusion in the majority of subjects. The between-arms (H vs R) safety analysis was unfavorable and likely related to patient age, volume overload, and undertreatment, and was isolated to patients who could not be managed by HBOC-201 alone. However, patients <80 years old with moderate clinical need may safely avoid transfusion when treated with up to 10 units of HBOC-201.

Hemopure has been approved (since 2001) in South Africa for the treatment of adult surgical patients who are acutely anemic, with the intention of eliminating or reducing the need for allogenic RBC transfusions. Hemopure has been administered to more than 800 human subjects in 22 completed clinical trials, including four advanced, RBC-controlled trials in cardiac, vascular, general noncardiac, and orthopedic surgery, respectively. These trials represented a logical progression in the study design that has expanded the dosing limits from 4 units (120 g Hb) of Hemopure administered after surgery over a 3-day maximum period to 10 units (300 g Hb) administered before, during, or after surgery over a 6-day period [115]. In March 2003, the US Naval Medical Research Center (NMRC) signed a collaborative research and development agreement with Biopure to help fund and conduct a trial on the effects of Hemopure in out-of-hospital resuscitation of patients with severe hemorrhagic shock. This trial was named "Restore Effective Survival in Shock" (RESUS) and over $14 million in Congressional, Navy, Army, and Air Force funding was given to support the trauma development program for Hemopure. In December 2006, the Blood Products Advisory Committee of the FDA voted against recommending that the US Navy proceed with late-phase clinical trials of Hemopure. The main reason for this decision was the adverse effect profile of the compound, since previous studies had shown that Hemopure could increase the risk of strokes and myocardial infarction. In the United States, phase III trials were put on hold due to safety issues. The manufacturer (Biopure) filed for bankruptcy in July 2009 and as of December 2009, Hemopure is unavailable for use in South Africa.

6.3.3 PolyHeme

PolyHeme (Northfield Laboratories Inc., Evanston, IL) is a first-generation pyridoxylated polymerized Hb made from outdated human blood. PolyHeme originally began as a military project following the Vietnam War and has since shown great potential for both military and civilian use. It has a half-life of 24 hours, a shelf life longer than

12 months when refrigerated, and a p50 of 28–30 mm Hg. The extraction and filtration of human Hb from RBCs is the first step in PolyHeme production. Then, using a multistep polymerization process, the purified Hb is associated into tetramers and, as the final step, is incorporated into an electrolyte solution.

PolyHeme was developed as a temporary solution to blood loss. As a military project, the focus was to develop a blood substitute to keep trauma patients alive in remote areas where allogeneic blood is not available. It is recognized that PolyHeme has a short circulation half-life of only 24 hours. Conditions requiring blood for longer than the circulation time of PolyHeme would require repeated transfusions of PolyHeme or later replacement with donor blood. Another factor that can limit the effectiveness of PolyHeme is the fact that it is manufactured using human Hb. While this Hb can be reclaimed from expired RBC products, it does not completely eliminate the need for donors because there must be a source of the outdated erythrocytes. The use of human Hb could limit the supply and manufacturing potential of PolyHeme.

In an initial phase II clinical trial ($n = 39$), PolyHeme was administered in up to 6 units in patients after acute trauma and surgery. No safety issues related to PolyHeme were reported. Plasma Hb mean was 4.8 ± 0.8 g/dL (reflecting PolyHeme Hb concentration), red-cell Hb fell to 2.9 ± 1.2 g/dL (reflecting patients' endogenous RBC Hb), but total Hb was maintained at 7.5 ± 1.2 g/dL with infusion of 6 units (300 g) of PolyHeme [116]. In a phase II randomized trial in 44 patients with acute trauma, PolyHeme reduced the required number of allogeneic RBC transfusions [117]. Forty-four trauma patients (33 male, 11 female) aged 19–75 years with an average Injury Severity Score (ISS) score of 21 ± 10 were randomized to receive red cells ($n = 23$) or up to 6 units (300 g) of PolyHeme ($n = 21$) as their initial blood replacement after trauma and during emergent operations. There were no serious or unexpected adverse events related to PolyHeme. The PolyHeme infusion of 4.4 ± 2.0 units (mean $\pm$ SD) resulted in a plasma Hb of 3.9 ± 1.3 g/dL, which accounted for 40% of the total circulating Hb. There was no difference in total Hb between the groups before infusion (10.4 ± 2.3 g/dL control vs 9.4 ± 1.9 g/dL experimental). At end-infusion, the experimental RBC Hb fell to 5.8 ± 2.8 g/dL versus 10.6 ± 1.8 g/dL ($p < 0.05$) in the control, although the total Hb was not different between the groups or from pre-infusion. The total number of allogeneic red-cell transfusions for the control and experimental groups was 10.4 ± 4.2 units versus 6.8 ± 3.9 units ($p < 0.05$) through day 1, and 11.3 ± 4.1 units vs 7.8 ± 4.2 units ($p = 0.06$) through day 3. This study documented that PolyHeme was safe in acute blood loss, maintained total Hb in lieu of red cells despite the marked fall in RBC Hb, and reduced the use of allogeneic blood. PolyHeme appeared to be a clinically useful HBOC.

A nonrandomized prospective trial enrolled 171 trauma or surgical patients who received rapid infusion of 1–20 units (1000 g, 10 L) of PolyHeme in lieu of RBCs as initial oxygen-carrying replacement in trauma and urgent surgery [118]. The protocol simulated the unavailability of RBCs, and the progressive fall in RBC Hb in bleeding patients was quantified. Thirty-day mortality was compared with a historical control group of 300 surgical patients who refused red cells on religious grounds. A total of 171 patients received rapid infusion of 1–2 units ($n = 45$), 3–4 units ($n = 45$), 5–9 units ($n = 47$), or 10–20 units ($n = 34$) of PolyHeme. Forty patients had a nadir RBC Hb ≤ 3 g/dL (mean 1.5 ± 0.7 g/dL), but total Hb was adequately maintained (mean 6.8 ± 1.2 g/dL) because of plasma Hb added by PolyHeme. The 30-day mortality was 25.0%

(10/40 patients) compared with 64.5% (20/31 patients) in historical control patients at these low RBC Hb levels. Additionally, 75% of patients with RBC Hb levels less than 1% survived traumatic injury after receiving PolyHeme as compared to 16% of historical controls at the same RBC Hb level. The authors concluded that PolyHeme increased survival at life-threatening RBC Hb by maintaining total Hb (plasma and RBC Hb) in the absence of red-cell transfusion. PolyHeme could be useful in the early treatment of urgent blood loss and resolve the dilemma of unavailability of red cells.

The US Multicenter PolyHeme Trauma Trial was the first trial in the United States of aN HBOC in the prehospital setting using waiver of informed consent [119, 120]. This was a phase III trial in trauma patients (n = 714 enrolled at 29 urban level 1 trauma centers) who were randomized to receive either PolyHeme or standard of care at the time of injury [121]. On reaching the hospital, patients in the control arm received allogeneic RBC transfusion as indicated, whereas patients in the PolyHeme arm continued to receive PolyHeme for 12 hours, and then receive allogeneic RBCs, as indicated. There was no significant difference between day 30 mortality in the as-randomized (13.4% PolyHeme vs 9.6% control) versus per-protocol (11.1% PolyHeme vs 9.3% control) cohorts. Allogeneic RBC use was lower in the PolyHeme group (68% vs 50% in the first 12 hours). The incidence of multiple organ failure was similar (7.4% PolyHeme vs 5.5% control). Adverse events (93% vs 88%; p = 0.04) and SAEs (40% vs 35%; p = 0.12), as anticipated, were frequent in the PolyHeme and control groups, respectively. Although myocardial infarction was reported by the investigators more frequently in the PolyHeme group (3% PolyHeme vs 1% control), a blinded committee of experts reviewed records of all enrolled patients and found no discernable difference between groups [122]. A number of criticisms of this clinical trial have emerged. Some have commented that it was unethical to continue the study protocol for 12 hours of the in-hospital phase of the study; that is, to not require administration of allogeneic RBCs to trauma victims upon hospital arrival [123–126]. In a published open letter to institutional review boards considering Northfield Laboratories' PolyHeme trial, some argued that the in-hospital stage of the study failed to meet ethical and regulatory standards governing the waiver of consent, which restrict its applicability to circumstances where proven, satisfactory treatments are unavailable. Blood – the standard treatment for hemorrhagic shock – is not available in ambulances but is available in hospitals [127].

6.3.4 MP4OX

MP4OX (Sangart Inc., San Diego, CA) is a PEG-conjugated human Hb currently undergoing clinical trials in the USA and Europe [128]. To further increase the circulation time, Hb can be linked to a macromolecule to increase its size. Human or bovine Hb that is conjugated with polyethylene glycol (PEG) is protected from renal excretion. The PEG-Hb has a larger molecular size and a higher viscosity. MP4OX was developed by introducing additional surface thiols with iminothiolane on to the Hb molecule. This process usually adds about five additional thiols, and Hb is linked to PEG-5000. MP4OX then requires no more purification steps. MP4OX has a lower Hb concentration, higher viscosity, higher oxygen affinity, and higher colloidal oncotic pressure than most other HBOCs in development. MP4OX did demonstrate an improvement in microcirculatory blood flow and tissue oxygenation in animal studies. In animal models, MP4OX

has been shown to be effective in cases of hemorrhagic shock [129]. Adverse effects associated with the vasoactive properties of first-generation blood substitutes are not seen with MP4OX. At relatively low concentrations, MP4OX is capable of transporting large amounts of oxygen. Pharmacokinetic analysis of plasma Hb yielded an estimated half-life of 43 hours in 100 mg/kg subjects in their phase I trial. To date, 1026 subjects have been enrolled in clinical trials with MP4OX. Sangart Inc. announced positive results and no safety concerns from phase I and II trials in orthopedic surgery patients, hip arthroplasty patients, and radical prostatectomy patients (ClinicalTrials.gov Identifier NCT00425334) [130–132]. A phase II study also compared MP4OX with colloid (Voluven) to evaluate vascular resistance and blood flow in the forearm, and to assess local skin blood flow and tissue oxygenation in an ischemic region of the foot in patients with chronic critical limb ischemia, and identified no evidence of increased vascular resistance following MP4OX administration (ClinicalTrials.gov Identifier NCT00633659).

A phase III orthopedic surgery *prevention trial* completed enrollment of 376 patients at 18 centers in 6 countries in May 2008, and evaluated the ability of MP4OX to prevent acute hypotension in orthopedic surgery patients undergoing first-time hip-replacement procedures under spinal anesthesia. This study demonstrated that MP4OX was superior to Voluven (a commercial starch-based plasma expander) for *preventing* hypotensive episodes during the operative and early postoperative period. The secondary objective, to show that MP4OX can also reduce the incidence of operative and postoperative morbidity, was not achieved. No safety concerns were identified. Additional clinical trial details can be found at http://clinicaltrials.gov/ct/show/NCT00421200?order=2.

Another phase III orthopedic surgery *treatment trial* completed enrollment of 474 patients (4/2007–4/2008) with the primary objective of demonstrating that MP4OX is superior to Voluven for *treating* hypotensive episodes during the operative period. MP4OX was superior to Voluven for the treatment of acute hypotensive episodes during anesthesia/surgery and throughout the postoperative period, and the total duration of hypotensive episodes was significantly shorter in the MP4OX group. The secondary objective was to show that MP4OX can also reduce the incidence of operative and postoperative morbidity, but this was not achieved. Additional details can be found at http://clinicaltrials.gov/ct/show/NCT00420277?order=1.

At present, MP4OX is undergoing testing to assess its efficacy to improve the perfusion and oxygenation of ischemic tissues by travelling where RBCs cannot reach and improving oxygen delivery. It is no longer being considered as a replacement for RBCs. To that end, a phase IIa trauma trial was completed in Europe and South Africa (enrollment 12/2009–6/2010) as a dose-finding study to evaluate the safety and efficacy of MP4OX plus standard of care in trauma patients with lactic acidosis due to hemorrhagic shock. Patients eligible for randomization were in hemorrhagic shock, with evidence of lactic acidosis (serum lactate level $\geq$5 mmol/L; equivalent to $\geq$45 mg/dL) within 2 hours after arrival at study hospital and within 4 hours of traumatic injury. Initial dose of either 250 mL of MP4OX plus 250 mL of Ringer's lactate, or 500 mL dose of MP4OX, or 500 mL of Ringer's lactate, was initiated within 30 minutes of randomization. The primary objective in this study is to evaluate the safety and efficacy of MP4OX treatment at two dose levels (250 and 500 mL) compared to isotonic Ringer's lactate solution, in addition to standard of care, in severely injured trauma patients exhibiting lactic acidosis within the first 2 hours after arrival at the hospital

and within 4 hours of trauma. The secondary objectives will be to evaluate early and late mortality, time to transfer from ICU and discharge from hospital, the duration of organ failure as measured by serial SOFA, modified Denver Score and days on mechanical ventilation, renal replacement therapy or vasopressors, and a morbidity outcome composite endpoint of time to complete organ-failure resolution, defined as the last day on which the patient no longer needs any vasoactive agents, mechanical ventilation, or renal-replacement support through study day 14 or day 21. SAEs and mortality outcome will be evaluated through day 28. Additional details about this protocol can be found at http://clinicaltrials.gov/ct/show/NCT01004198?order=3.

6.3.5 Recombinant Human Hb

Recombinant human Hb (rHb) is manufactured from *E. coli* with recombinant technology. This technology can be used to induce a variety of cell types to synthesize functional Hb. In addition, modifications of the Hb molecular structure can alter the properties of the molecule, allowing researchers to create Hbs with improved functionality or enhanced safety when used as Hb therapeutics. One very positive feature of rHb is that it can be manufactured, resulting in an unlimited supply.

Two first-generation HBOCs were under development by Baxter Hemoglobin Therapeutics and Somatogen during the 1990s, DCLHb (HemAssist) and a modified rHb (rHb1.1, Optro). Each of these products circumvented the safety concerns surrounding dimerization of the Hb tetramer by crosslinking the alpha chains (either chemically in the case of DCLHb, or through recombinant engineering with rHb1.1) [133].

rHb1.1 was a first-generation HBOC with a nitric oxide scavenging rate similar to that of native human Hb. rHb2.0 was a second-generation HBOC, created via genetic manipulation of the distal heme pocket of both the alpha and beta subunits of Hb, leading to steric hindrance for nitric oxide entry, with a nitric oxide scavenging rate 20- to 30-fold lower than rHb1.1 but maintenance of effective oxygen binding and release [134]. Preclinical animal studies were promising. rHb2.0 was associated with decreased pulmonary hypertension, diminished capacity to scavenge nitric oxide, and lack of modulation of pulmonary vascular permeability. These findings lend promise to the use of HBOCs with low nitric-oxide reactivity as oxygen therapeutics.

rHb2.0 has been investigated in a swine model of uncontrolled hemorrhage. rHb2.0 performed as well as heterologous blood for resuscitation in hemorrhage, did not cause sustained pulmonary hypertension, maintained adequate cardiac output and oxygen delivery, and was superior to lactated Ringer's solution and the first-generation HBOC DCLHb in survival [135]. Additional preclinical studies documented positive results [136–139]. Although rHb2.0 appeared promising, no clinical trials were performed and Baxter suspended funding of this initiative.

6.3.6 Adverse Effects of HBOCs

A number of adverse effects associated with HBOCs have been reported, including hypertension, abdominal pain, skin rash, diarrhea, jaundice, hemoglobinuria, oliguria, fever, stroke, and laboratory anomalies such as an elevation in lipase levels. Although most of these side effects were transient and clinically asymptomatic, many clinical trials involving these agents have been discontinued or held due to the associated adverse effects.

Although current formulations appear to cause fewer severe effects, concerns persist related to the side effects of HBOCs, including vasoactivity (vasoconstriction, hypertension, cardiac effects), increased hemostatic effect (platelet aggregation), gastrointestinal effects (nausea, vomiting, diarrhea, bloating), and interference with laboratory assays due to high concentrations of Hb in plasma.

It has been difficult to discern whether the adverse events that have been observed following the infusion of HBOCs in patients are related solely to the HBOCs or to other treatments administered to these patients during their routine care. Along with all three of the HBOCs studied in clinical trials, the patients received Ringer's d,l-lactate as the resuscitative fluid, Ringer's d,l-lactate in the excipient medium for the HBOC, and liquid-preserved RBCs that had been stored at 4 °C for longer than 2 weeks. The Ringer's d,l-lactate solution has been shown to be toxic in both animals and patients. The current formulation of Ringer's lactate contains only the l-isomer, which has been shown in animals to be less toxic than the d-isomer of lactate. In a recent publication, morbidity and mortality have been reported associated with the length of storage of RBCs at 4 °C in patients subjected to reoperative cardiac surgery. Current clinical studies to assess the safety and therapeutic effectiveness of an HBOC must consider the effects of the composition of the resuscitation solution (Ringer's l-lactate), the composition of the excipient medium (Ringer's l-lactate or 0.9% NaCl) for the HBOC, and the length of storage of the liquid-preserved RBCs infused with the HBOC [140].

A recent meta-analysis reviewed data on death and myocardial infarction as outcome variables in 16 trials in adult patients (n = 3711) involving five different HBOCs in varied patient populations [141]. They reported a statistically significant increase in the risk of death (164 vs 123 deaths; RR 1.30; 95% CI 1.05–1.61) and the risk of MI (59 vs 16 myocardial infarctions; RR 2.71; 95% CI 1.67–4.40). There are, however, many limitations to this analysis [142], such as: the inclusion of multiple products (HemAssist, PolyHeme, Hemolink, Hemopure, Hemospan) in the analysis; lack of consistent monitoring of cardiac events in the studies; lack of consistent treatment in the perioperative period to prevent cardiac events in the surgical studies; no identification of specific cardiac risk in patients enrolled in the studies; and lack of control for risk of myocardial events and mortality that might have been related to allogeneic transfusion [143, 144].

6.3.7 HBOCs in Trauma: A Way Forward?

Trauma surgeons will attest that delays in RBC availability for acute life-threatening hemorrhagic shock occur in both rural and urban centers today, and are particularly problematic in the combat casualty care environment. Furthermore, the transfusion of uncrossmatched RBCs in the prehospital environment for the acute care of trauma victims is not common. Perhaps a logical way forward is to consider the use of HBOCs in trauma victims with hemorrhagic shock who are at risk of need for MT, and therefore are predicted to have a high mortality rate. These patients can be identified by the use of a simplified score (ABC, Assessment of Blood Consumption) which uses four nonlaboratory, nonweighted parameters (penetrating mechanism, positive focused assessment with sonography for trauma confirming hemoperitoneum, arrival systolic blood pressure of 90 mm Hg or less, and arrival heart rate 120 bpm or greater). This score has not yet been validated in the prehospital environment, but three of these variables are easily available in the field.

6.4 Conclusion

Transfusion of the bleeding patient with RBCs and other blood products requires careful vigilance during the acute resuscitative and recovery phases. At present, RBC transfusion is the only oxygen-carrying resuscitation fluid for treatment of severe hemorrhagic shock. Newer protocols using "hemostatic resuscitation" advocate concomitant early use of plasma, and some studies report improved survival. This strategy has resulted in more liberal use of RBCs and blood products in acute resuscitation for massive hemorrhage. Additional multicenter studies are warranted to confirm this survival benefit with hemostatic resuscitation. However, following definitive cessation of hemorrhage, all efforts to minimize the use of RBC transfusion are warranted. There is a significant unmet medical need for HBOCs in trauma and hemorrhagic shock when RBCs are not available. Additional viable approaches to modifying the intrinsic biologic properties of Hb in order to produce improved HBOCs are now available. The ultimate goal is availability of an HBOC for use in the world in appropriate clinical situations. Despite the appeal, the scientific design and ethical conduct of clinical trials to establish the efficacy of HBOCs when RBCs are unavailable remains a significant challenge.

References

[1] Holcomb, J.B. Methods for improved hemorrhage control, *Crit. Care*, **8**, S57–S60 (2004).

[2] Cosgriff, N., Moore, E.E., Sauaia, A. *et al*. Predicting life-threatening coagulopathy in the massively transfused trauma patient: hypothermia and acidoses revisited, *J. Trauma*, **42**, 857–861 (1997).

[3] Moore, E.E. and Thomas G. Orr Memorial Lecture. Staged laparotomy for the hypothermia, acidosis, and coagulopathy syndrome, *Am. J. Surg.*, **172**, 405–410 (1996).

[4] McKinley, B.A., Gonzalez, E.A. and Balldin, B.C. Revisiting the "Bloody Vicious Cycle", *Shock*, **21**(Suppl 2), 47 (2004).

[5] Inaba, K., Teixeira, P.G., Shulman, I. *et al*. The impact of uncross-matched blood transfusion on the need for massive transfusion and mortality: analysis of 5,166 uncross-matched units, *J. Trauma*, **65**, 1222–1226 (2008).

[6] Repine, T.B., Perkins, J.G., Kauvar, D.S. and Blackborne, L. The use of fresh whole blood in MT, *J. Trauma*, **60**, S59–S69 (2006).

[7] Napolitano, L.M., Kurek, S. and Luchette, F.A. American College of Critical Care Medicine of the Society of Critical Care Medicine; Eastern Association for the Surgery of Trauma Practice Management Workgroup. Clinical practice guideline: red blood cell transfusion in adult trauma and critical care, *Crit. Care Med.*, **37**, 3124–3157 (2009); erratum, *Crit. Care Med.*, **38**, 1621 (2010).

[8] Napolitano, L.M., Kurek, S. and Luchette, F.A. EAST Practice Management Workgroup; American College of Critical Care Medicine (ACCM) Taskforce of the Society of Critical Care Medicine (SCCM). Clinical practice guideline: red blood cell transfusion in adult trauma and critical care, *J. Trauma*, **67**, 1439–1442 (2009).

[9] Sihler, K.C. and Napolitano, L.M. Massive transfusion: new insights, *Chest*, **136**, 1654–1667 (2009).
[10] Sihler, K.C. and Napolitano, L.M. Complications of massive transfusion, *Chest*, **137**, 209–220 (2010).
[11] Harvey, M.P., Greenfield, T.P., Sugrue, M.E. and Rosenfeld, D. Massive blood transfusion in a tertiary referral hospital: clinical outcomes and haemostatic complications, *Med. J. Aust.*, **163**, 356–359 (1995).
[12] Mollison, P.L., Engelfreit, C.P. and Contreras, M. Transfusion in oligaemia, in *Blood Transfusion in Clinical Medicine* (ed. H.G. Klein and D.J. Anstee), Blackwell Science, Oxford, UK, pp. 48–75 (1987).
[13] Levy, J.H. Massive transfusion coagulopathy, *Semin. Hematol.*, **43**, S59–S63 (2006).
[14] Lim, R.C., Olcott, C., Robinson, A.J. and Blaisdell, F.W. Platelet response and coagulation changes following massive blood replacement, *J. Trauma*, **13**, 577–582 (1973).
[15] *Advanced Trauma Life Support for Doctors: Student Course Manual*, 8 edn, American College of Surgeons, Chicago, USA (2008).
[16] Wudel, J.H., Morris, J.A., Yates, K. *et al*. Massive transfusion: outcome in blunt trauma patients, *J. Trauma*, **31**, 1–7 (1991).
[17] Moltzan, C.J., Anderson, D.A., Callum, J. *et al*. The evidence for use of recombinant factor VIIa in massive bleeding: development of a transfusion policy framework, *Transfus. Med.*, **18**, 112–120 (2008).
[18] Fakhry, S.M. and Sheldon, G.F. MT in the surgical patient, in *Massive Transfusion* (ed. L.C. Jeffries and M.E. Brecher), American Association of Blood Banks, Bethesda, MD, USA, pp. 135–156 (1994).
[19] Schreiber, M.A., Perkins, J., Kiraly, L. *et al*. Early predictors of massive transfusion in combat casualties, *J. Am. Coll. Surg.*, **205**, 541–545 (2007).
[20] Cotton, B.A., Dossett, L.A., Haut, E.R. *et al*. Multicenter validation of a simplified score to predict massive transfusion in trauma, *J. Trauma*, **69**(Suppl 1), S33–S39 (2010).
[21] Maegele, M., Lefering, R., Wafaisade, A. *et al*. The Trauma Registry of the Deutsche Gesellschaft fur Unfallchirurgie (TR-DGU). Revalidation and update of the TASH score: a scoring system to predict the probability for massive transfusion as a surrogate for life-threatening haemorrhage after severe injury, *Vox Sang.* (2010) (Epub ahead of print).
[22] Nunez, T.C., Voskresensky, I.V., Dossett, L.A. *et al*. Early prediction of massive transfusion in trauma: simple as ABC (assessment of blood consumption)?, *J. Trauma*, **66**, 346–352 (2009).
[23] Mahambrey, T.D., Fowler, R.A., Pinto, R. *et al*. Early massive transfusion in trauma patients: Canadian single-centre retrospective cohort study, *Can. J. Anaesth.*, **56**, 740–750 (2009).
[24] Como, J.J., Dutton, R.P., Scalea, T.M. *et al*. Blood transfusion rates in the care of acute trauma, *Transfusion*, **44**, 809–813 (2004).
[25] Vaslef, S.N., Knudsen, N.W., Neligan, P.J. and Sebastian, M.W. Massive transfusion exceeding 50 units of blood products in trauma patients, *J. Trauma*, **53**, 291–296 (2002).

[26] Brohi, K., Cohen, M.J., Ganter, M.T. *et al*. Acute coagulopathy of trauma: hypoperfusion induces systemic anticoagulation and hyperfibrinolysis, *J. Trauma*, **64**, 1211–1217 (2008).

[27] Cohen, M.J., Brohi, K., Ganter, M.T. *et al*. Early coagulopathy after traumatic brain injury: the role of hypoperfusion and the protein C pathway, *J. Trauma*, **63**, 1254–1261; disc. 1261–1262 (2007).

[28] Frith, D., Goslings, J.C., Gaarder, C. *et al*. Definition and drivers of acute traumatic coagulopathy: clinical and experimental investigations, *J. Thromb. Haemost.*, **8**, 1919–1925 (2010).

[29] MacLeod, J.B., Lynn, M., McKenney, M.G. *et al*. Early coagulopathy predicts mortality in trauma, *J. Trauma*, **55**, 39–44 (2003).

[30] Hardy, J.F., DeMoerloose, P. and Samama, C.M. The coagulopathy of massive transfusion, *Vox Sang.*, **89**, 123–127 (2005).

[31] Hellstern, P. and Haubelt, H. Indications for plasma in MT, *Thromb. Res.*, **107**(Suppl 1), S19–S22 (2002).

[32] Erbert, W.N. and Perry, D.J. Plasma and plasma products in the treatment of massive hemorrhage, *Best Pract. Res. Clin. Haematol.*, **19**, 97–112 (2006).

[33] Stern, S.A. Low-volume fluid resuscitation for presumed hemorrhagic shock: helpful or harmful?, *Curr. Opin. Crit. Care*, **7**, 422–430 (2001).

[34] Bickell, W.H., Wall, M.J., Pepe, P.E. *et al*. Immediate versus delayed fluid resuscitation for hypotensive patients with penetrating torso injuries, *N. Engl. J. Med.*, **331**, 1105–1109 (1994).

[35] Napolitano, L.M. Resuscitation endpoints in trauma, *Transfus. Alt. Transfus. Med.*, **6**, 6–14 (2005).

[36] Holcomb, J.B., Hoyt, D. and Hess, J.R. Damage control resuscitation: the need for specific blood products to treat the coagulopathy of trauma, *Transfusion*, **46**, 685–686 (2006).

[37] Johansson, P.I. and Stensballe, J. Hemostatic resuscitation for massive bleeding: the paradigm of plasma and platelets: a review of the current literature, *Transfusion*, **50**, 701–710 (2010).

[38] Ho, A.M., Karmakar, M.K. and Dion, P.W. Are we giving enough coagulation factors during major trauma resuscitation?, *Am. J. Surg.*, **190**, 479–484 (2005).

[39] Ho, A.M., Dion, P.W., Cheng, C.A. *et al*. A mathematical model for fresh frozen plasma transfusion strategies during major trauma resuscitation with ongoing hemorrhage, *Can. J. Surg.*, **48**, 470–478 (2005).

[40] Holcomb, J.B., Hess, J.R. (eds). Early MT: state of the art, *J. Trauma*, **60**, S1–S2 (2006).

[41] Fox, C.J., Gillespie, D.L., Cox, D. *et al*. Damage control resuscitation for vascular surgery in a combat support hospital, *J. Trauma*, **65**, 1–9 (2008).

[42] Simmons, J.W., White, C.E., Eastridge, B.J. *et al*. Impact of policy change on US Army combat transfusion practices, *J. Trauma*, **69**(Suppl 1), S75–S80 (2010).

[43] Gonzalez, E.A., Moore, F.A., Holcomb, J.B. *et al*. Fresh frozen plasma should be given earlier to patients requiring MT, *J. Trauma*, **62**, 112–119 (2007).

[44] Holcomb, J.B., Wade, C.E., Michalek, J.E. *et al*. The Trauma Outcomes Group. Increased plasma and platelet to RBC ratios improves outcome in 466 massively transfused civilian trauma patients, *Ann. Surg.*, **248**, 447–458 (2008).

[45] Scalea, T., Bochicchio, K.M., Lumpkins, K. *et al*. Early aggressive use of fresh frozen plasma (FFP) does not improve outcome in critically injured trauma patients, *Ann. Surg.*, **248**, 578–584 (2008).

[46] Duchesne, J.C., Hunt, J.P., Wahl, G. *et al*. Review of current blood transfusion strategies in a mature level 1 trauma center: were we wrong for the last 60 years?, *J. Trauma*, **65**, 272–276 (2008).

[47] Maegele, M., Lefering, R., Paffrath, T. *et al*. The Working Group on Polytrauma of the German Society of Trauma Surgery (DGU). Red blood cell to plasma ratios transfused during massive transfusion are associated with mortality in severe multiple injury: a retrospective analysis from the Trauma Registry of the Deutsche Gesellschaft fur Unfallchirurgie, *Vox Sang.*, **95**, 112–119 (2008).

[48] Sperry, J.L., Ochoa, J.B., Gunn, S.R. *et al*. Inflammation the Host Response to Injury Investigators. An FFP : PRBC transfusion ratio >/=1 : 1.5 is associated with lower risk of mortality after massive transfusion, *J. Trauma*, **65**, 986–993 (2008).

[49] Murad, M.H., Stubbs, J.R., Gandhi, M.J. *et al*. The effect of plasma transfusion on morbidity and mortality: a systematic review and meta-analysis, *Transfusion*, **50**, 1370–1383 (2010).

[50] Roback, J.D., Caldwell, S., Carson, J. *et al*. American Association for the Study of Liver; American Academy of Pediatrics; United States Army; American Society of Anesthesiology; American Society of Hematology. Evidence-based practice guidelines for plasma transfusion, *Transfusion*, **50**, 1227–1239 (2010).

[51] Khan, H., Belsher, J., Yilmaz, M. *et al*. Fresh-frozen plasma and platelet transfusions are associated with development of acute lung injury in critically ill medical patients, *Chest*, **131**, 1308–1314 (2007).

[52] Gajic, O., Yilmaz, M., Iscimen, R. *et al*. Transfusion from male-only versus female donors in critically ill recipients of high plasma volume components, *Crit. Care Med.*, **35**, 1645–1648 (2007).

[53] Netzer, G., Shas, C.V., Iwashyna, T.J. *et al*. Association of RBC transfusion with mortality in patients with acute lung injury, *Chest*, **132**, 1116–1123 (2007).

[54] Toy, P., Popovsky, M.A., Abraham, E. *et al*. National Heart, Lung and Blood Institute Working Group on TRALI. Transfusion-related acute lung injury: definition and review, *Crit. Care Med.*, **33**, 721–726 (2005).

[55] Gajic, O., Rana, R., Winters, J.L. *et al*. Transfusion-related acute lung injury in the critically ill: prospective nested case-control study, *Am. J. Respir. Crit. Care Med.*, **176**, 886–891 (2007).

[56] Rubenfeld, G.D., Caldwell, E., Peabody, E. *et al*. Incidence and outcomes of acute lung injury, *N. Engl. J. Med.*, **353**, 1685–1693 (2005).

[57] Malone, D.L., Hess, J.R. and Fingerhut, A. MT practices around the globe and a suggestion for a common massive transfusion protocol, *J. Trauma*, **60**, S91–S96 (2006).

[58] Cotton, B.A., Gunter, O.L., Isbell, J. *et al*. Damage control hematology: the impact of a trauma exsanguination protocol on survival and blood product utilization, *J. Trauma*, **64**, 1177–1182 (2008).

[59] Johansson, P.I., Stensballe, J., Rosenberg, I. *et al*. Proactive administration of platelets and plasma for patients with a ruptured abdominal aortic aneurysm: evaluating a change in transfusion practice, *Transfusion*, **47**, 593–598 (2007).

[60] Johansson, P.I. The blood bank: from provider to partner in treatment of massively bleeding patients, *Transfusion*, **47**, S176–S181 (2007).
[61] Johansson, P.I., Bochsen, L., Stensballe, J. and Secher, N.H. Transfusion packages for massively bleeding patients: the effect on clot formation and stability as evaluated by TEG, *Transfus. Apher. Sci.*, **39**, 3–8 (2008).
[62] http://www.uth.tmc.edu/cetir/PROMMTT/index.html
[63] West, M.A., Shapiro, M.B., Nathens, A.B. *et al*. Inflammation and the Host Response to Injury Collaborative Research Program. Guidelines for transfusion in the trauma patient: inflammation and host response to injury, a large-scale collaborative project: patient-oriented research core: standard operating procedures for clinical care, *J. Trauma*, **61**, 436–439 (2006).
[64] Carson, J.L., Terrin, M.L., Magaziner, J. *et al*. FOCUS Investigators. Transfusion trigger trial for functional outcomes in cardiovascular patients undergoing surgical hip fracture repair (FOCUS), *Transfusion*, **46**, 2192–2206 (2006).
[65] Carson, J.L. *et al*. Transfusion Trigger Trial for Functional Outcomes in Cardiovascular Patients Undergoing Surgical Hip Fracture Repair (FOCUS) trial results. Late-breaker presented at the American Heart Association Scientific Session 2009, Orlando, FL, USA.
[66] Gammon, R.R., Strayer, S.A., Avery, N.L. and Mintz, P.D. Hemolysis during leukocyte-reduction of stored red blood cells, *Ann. Clin. Lab. Sci.*, **30**, 195–199 (2000).
[67] Sowemimo-Coker, S.O. Red blood cell hemolysis during processing, *Transfus. Med. Rev.*, **16**, 46–60 (2002).
[68] Fernandes, C.J. Jr, Akamine, N., De Marco, F.V. *et al*. Red blood cell transfusion does not increase oxygen consumption in critically ill septic patients, *Crit. Care*, **5**, 362–367 (2001).
[69] Tinmouth, A., Fergusson, D., Yee, I.C. and Hebert, P.C. ABLE Investigators; Canadian Critical Care Trials Group. Clinical consequences of red cell storage in the critically ill, *Transfusion*, **46**, 2014–2027 (2006).
[70] Spinella, P.C., Carroll, C.L., Staff, I. *et al*. Duration of red blood cell storage is associated with increased incidence of deep vein thrombosis and in-hospital mortality in patients with traumatic injuries, *Crit. Care*, **13**, R151 (2009).
[71] Koch, C.G., Li, L., Sessler, D.I. *et al*. Duration of red cell storage and complications after cardiac surgery, *N. Engl. J. Med.*, **358**, 1229–1239 (2008).
[72] Age of Blood Evaluation (ABLE) trial. http://www.controlled-trials.com/ISRCTN44878718/
[73] Napolitano, L.M. Cumulative risks of early red blood cell transfusion, *J. Trauma*, **60**, S26–S34 (2006).
[74] Malone, D.L., Dunne, J., Tracy, J.K. *et al*. Blood transfusion, independent of shock severity, is associated with worse outcome in trauma, *J. Trauma*, **54**, 898–905 (2003).
[75] Dunne, J.R., Malone, D.L., Tracy, J.K. and Napolitano, L.M. Allogeneic blood transfusion in the first 24 hours after trauma is associated with increased SIRS and death, *Surg. Infect. (Larchmt.)*, **5**, 395–404 (2004).
[76] Napolitano, L.M. and Corwin, H.L. Efficacy of blood transfusion in the critically ill, *Crit. Care Clin.*, **20**, 255–568 (2004).

[77] Marik, P.E. and Corwin, H.L. Efficacy of red blood cell transfusion in the critically ill: a systematic review of the literature, *Crit. Care Med.*, **36**, 2667–2674 (2008).
[78] Winslow, R.M. Current status of oxygen carriers ("blood substitutes"): 2006, *Vox Sang.*, **91**, 102–110 (2006).
[79] Habler, O., Pape, A., Meier, J. and Zwissler, B. Artificial oxygen carriers as an alternative to red blood cell transfusion, *Anaesthesist*, **54**, 741–754 (2005).
[80] Ness, P.M. and Cushing, M.M. Oxygen therapeutics: pursuit of an alternative to the donor red blood cell, *Arch. Pathol. Lab. Med.*, **131**, 734–741 (2007).
[81] Eastman, A.L. and Minei, J.P. Comparison of hemoglobin-based oxygen carriers to stored human red blood cells, *Crit. Care Clin.*, **25**, 303–310 (2009).
[82] Napolitano, L.M. Hemoglobin-based oxygen carriers: first, second or third generation? Human or bovine? Where are we now?, *Crit. Care Clin.*, **25**, 279–301 (2009); erratum, *Crit. Care Clin.*, **25**, xiii (2009).
[83] Napolitano, L.M. The most serious motivation for the development of a blood substitute is the worldwide shortage of safe and viable allogeneic donor blood, Preface, *Crit. Care Clin.*, **25**, xi–xii (2009).
[84] Sullivan, M.T., Cotton, R., Read, E.J. and Wallace, E.L. Blood collection and transfusion in the United States in 2001, *Transfusion*, **47**, 385–394 (2007).
[85] Tabatabaie, M., Ardalan, A., Abolghasemi, H. *et al*. Estimating blood transfusion requirements in preparation for a major earthquake: the Tehran, Iran study, *Prehosp. Disaster Med.*, **25**, 246–252 (2010).
[86] Abolghasemi, H., Radfar, M.H., Tabatabaee, M. *et al*. Revisiting blood transfusion preparedness: experience from the Bam earthquake response, *Prehosp. Disaster Med.*, **23**, 391–394 (2008).
[87] Zou, S., Musavi F., Notari, E.P. 4th and Fant, C.T. ARCNET Research Group. Changing age distribution of the blood donor population in the United States, *Transfusion*, **48**, 251–257 (2008).
[88] Spiess, B. D. Risks of transfusions: outcome focus, *Transfusion*, **4**, 4S–14S (2004).
[89] Sazama, K. The ethics of blood management, *Vox Sang.*, **92**, 95–102 (2007).
[90] Cable, R., *Practice Guidelines for Blood Transfusion: A Compilation from Recent Peer Reviewed Literature*, American National Red Cross, Washington, DC, USA (2002).
[91] Tsai, A.G., Cabrales, P. and Intaglietta, M. Oxygen-carrying blood substitutes: a microvascular perspective, *Expert Opin. Biol. Ther.*, **4**, 1147–1157 (2004).
[92] Moore, E.E. Blood substitutes: The future is now, *J. Am. Coll. Surg.*, **196**, 1–17 (2003).
[93] http://www.hemopure.co.za/
[94] Stowell, C.P. What happened to blood substitutes?, *Transfus. Clin. Biol.*, **12**, 374–379 (2005).
[95] Mackenzie, C.F., Moon-Massat, P.F., Shander, A. *et al*. When blood is not an option: factors affecting survival after the use of a hemoglobin-based oxygen carrier in 54 patients with life-threatening anemia, *Anesth. Analg.*, **110**, 685–693 (2010).

[96] Estep, T., Bucci, E., Farmer, M. *et al*. Basic science focus on blood substitutes: a summary of the NHLBI Division of Blood Diseases and Resources Working Group Workshop, March 1, 2006, *Transfusion*, **48**, 776–782 (2008).
[97] Winslow, R.M. Vasoconstriction and the efficacy of hemoglobin-based blood substitutes, *Transfus. Clin. Biol.*, **1**, 9–14 (1994).
[98] Standl, T. Haemoglobin-based erythrocyte transfusion substitutes, *Expert Opin. Biol. Ther.*, **1**, 831–843 (2001).
[99] Silverman, T.A. and Weiskopf, R.B. Hemoglobin-based oxygen carriers current status and future directions, *Anesthesiology*, **111**, 946–963 (2009).
[100] Meisner, F.G., Kemming, G.I., Habler, O.P. *et al*. Diaspirin crosslinked hemoglobin enables extreme hemodilution beyond the critical hematocrit, *Crit. Care Med.*, **29**, 829–838 (2001).
[101] Sloan, E.P., Koenigsberg, M., Gens, D. *et al*. Diaspirin cross-linked hemoglobin (DCLHb) in the treatment of severe traumatic hemorrhagic shock: a randomized controlled efficacy trial, *JAMA*, **282**, 1857–1864 (1999).
[102] Kerner, T., Ahlers, O., Veit, S. *et al*. DCL-Hb for trauma patients with severe hemorrhagic shock: the European "On-Scene" multicenter study, *Intensive Care Med.*, **29**, 378–385 (2003).
[103] Sloan, E.P., Koenigsberg, M., Brunett, P.H. *et al*. DCLHb Traumatic Hemorrhagic Shock Study Group. Post hoc mortality analysis of the efficacy trial of diaspirin cross-linked hemoglobin in the treatment of severe traumatic hemorrhagic shock, *J. Trauma*, **52**, 887–895 (2002).
[104] Saxena, R., Wihnhoud, A.D., Carton, H. *et al*. Controlled safety study of a hemoglobin-based oxygen carrier, DCLHb, in acute ischemic stroke, *Stroke*, **30**, 993–996 (1999).
[105] Schubert, A., Przybelski, R.J., Eidt, J.F. *et al*. Perioperative Avoidance or Reduction of Transfusion Trial (PARTT) Study Group; Perioperative Avoidance or Reduction of Transfusion Trial (PARTT) Study Group. Diaspirin-crosslinked hemoglobin reduces blood transfusion in noncardiac surgery: a multicenter, randomized, controlled, double-blinded trial, *Anesth. Analg.*, **97**, 323–332 (2003).
[106] Lamy, M.L., Daily, E.K., Brichant, J.F. *et al*. Randomized trial of diaspirin cross-linked hemoglobin solution as an alternative to blood transfusion after cardiac surgery: the DCLHb Cardiac Surgery Trial Collaborative Group, *Anesthesiology*, **92**, 646–656 (2000).
[107] Schubert, A., O'Hara, J.F. Jr, Przybelski, R.J. *et al*. Effect of diaspirin crosslinked hemoglobin (DCLHb HemAssist) during high blood loss surgery on selected indices of organ function, *Artif. Cells Blood Substit. Immobil. Biotechnol.*, **30**, 259–283 (2002).
[108] Sloan, E.P., Koenigsberg, M.D., Philbin, N.B. and Gao, W. DCLHb Traumatic Hemorrhagic Shock Study Group; European HOST Investigators. Diaspirin cross-linked hemoglobin infusion did not influence base deficit and lactic acid levels in two clinical trials of traumatic hemorrhagic shock patient resuscitation, *J. Trauma*, **68**, 1158–1171 (2010).

[109] Sloan, E.P., Philbin, N.B., Koenigsberg, M.D. and Gao, W. The lack of consistent DCLHb infusion blood pressure effects in the US and EU Traumatic Hemorrhagic Shock Clinical Trials, *Shock*, **33**, 123–133 (2010).
[110] Cabrales, P., Tsai, A.G. and Intaglietta, M. Balance between vasoconstriction and enhanced oxygen delivery, *Transfusion*, **48**, 2087–2095 (2008).
[111] LaMuraglia, G.M., O'Hara, P.J., Baker, W.H. *et al*. The reduction of allogeneic transfusion requirements in aortic surgery with a hemoglobin-based solution, *J. Vasc. Surg.*, **31**, 299–308 (2000).
[112] Sprung, J., Kindscher, J.D., Wahr, J.A. *et al*. The use of bovine hemoglobin glutamer-250 (Hemopure) in surgical patients: results of a multicenter randomized single-blinded trial, *Anesth. Analg.*, **94**, 799–808 (2002).
[113] Levy, J.H., Goodnough, L.T., Greilich, P.E. *et al*. Polymerized bovine hemoglobin solution as a replacement for allogeneic red blood cell transfusion after cardiac surgery: results of a randomized, double-blind trial, *J. Thorac. Cardiovasc. Surg.*, **124**, 35–42 (2002).
[114] Jahr, J.S., MacKenzie, C., Pearce, L.B. *et al*. HBOC-201 as an alternative to blood transfusion: efficacy and safety evaluation in a multicenter phase III trial in elective orthopedic surgery, *J. Trauma*, **64**, 1484–1497 (2008).
[115] Jahr, J.S., Moallempour, M. and Lim, J.C. HBOC-201, hemoglobin glutamer-250 (bovine), Hemopure (Biopure Corporation), *Expert Opin. Biol. Ther.*, **8**, 1425–1433 (2008).
[116] Gould, S.A., Moore, E.E., Moore, F.A. *et al*. Clinical utility of human polymerized hemoglobin as a blood substitute after acute trauma and urgent surgery, *J. Trauma*, **43**, 325–331; disc. 331–332 (1997).
[117] Gould, S.A., Moore, E.E., Hoyt, D.B. *et al*. The first randomized trial of human polymerized hemoglobin as a blood substitute in acute trauma and emergent surgery, *J. Am. Coll. Surg.*, **187**, 113–120; disc. 120–122 (1998).
[118] Gould, S.A., Moore, E.E., Hoyt, D.B. *et al*. The life-sustaining capacity of human polymerized hemoglobin when red cells might be unavailable, *J. Am. Coll. Surg.*, **195**, 445–452; disc. 452–455 (2002).
[119] Moore, E.E., Cheng, A.M., Moore, H.B. *et al*. Hemoglobin-based oxygen carriers in trauma care: scientific rationale for the US multicenter prehosptial trial, *World J. Surg.*, **30**, 1247–1257 (2006).
[120] Moore, E.E., Johnson, J.L., Cheng, A.M. *et al*. Insights from studies of blood substitutes in trauma, *Shock*, **24**, 197–205 (2005).
[121] Moore, E.E., Moore, F.A., Fabian, T.C. *et al*. PolyHeme Study Group. Human polymerized hemoglobin for the treatment of hemorrhagic shock when blood is unavailable: the USA Multicenter Trial, *J. Am. Coll. Surg.*, **208**, 1–13 (2009).
[122] Moore, E.E., Johnson, J.L., Moore, F.A. and Moore, H.B. The USA multicenter prehospital hemoglobin-based oxygen carrier resuscitation trial: scientific rationale, study design, and results, *Crit. Care. Clinic.*, **25**, 325–356 (2009).
[123] Apte, S.S. Blood substitutes: The PolyHeme Trials. Crossroads: where medicine and the humanities meet, *McGill J. Med.*, **11**, 59–65 (2008).
[124] Chen, J.Y., Scerbo, M. and Kramer, G. A review of blood substitutes: examining the history, clinical trial results, and ethics of hemoglobin-based oxygen carriers, *Clinics (Sao Paulo)*, **64**, 803–813 (2009).

[125] Dickert, N.W. and Sugarman, J. Getting the ethics right regarding research in the emergency setting: lessons from the PolyHeme study, *Kennedy Inst. Ethics J.*, **17**, 153–169 (2007).
[126] McKenna, M. Polyheme and the ethics of informed consent, *Ann. Emerg. Med.*, **48**, 563–566 (2006).
[127] Kipnis, K., King, N.M. and Nelson, R.M. An open letter to institutional review boards considering Northfield Laboratories' PolyHeme trial, *Am. J. Bioeth.*, **6**, 18–21 (2006).
[128] Vandegriff, K.D., Malavalli, A., Wooldridge, J. *et al*. MP4, a new nonvasoactive PEG-Hb conjugate, *Transfusion*, **43**, 509–516 (2003).
[129] Young, M.A., Riddez, L., Kjellström, B.T. *et al*. MalPEG-hemoglobin (MP4) improves hemodynamics, acid-base status, and survival after uncontrolled hemorrhage in anesthetized swine, *Crit. Care Med.*, **33**, 1794–1804 (2005).
[130] Bjorkholm, M., Fagrell, B., Przybelski, R. *et al*. A phase I single blind clinical trial of a new oxygen transport agent (MP4), human hemoglobin modified with maleimide-activated polyethylene glycol, *Haematologica*, **90**, 505–515 (2005).
[131] Olofsson, C., Nygårds, E.B., Ponzer, S. *et al*. A randomized, single blind, increasing dose safety trial of an oxygen-carrying plasma expander (Hemospan®) administered to orthopedic surgery patients with spinal anesthesia, *Trans. Med.*, **18**, 28–39 (2008).
[132] Olofsson, N., Ahl, T., Johansson, T. *et al*. A multi-center clinical study of the safety and activity of maleimide-polyethylene glycol hemoglobin (Hemospan®) in patients undergoing major orthopedic surgery, *Anesthesiology*, **105**, 1153–1163 (2006).
[133] Burhop, K.E. The development of a second-generation, designer, recombinant hemoglobin, in *Artificial Oxygen Carrier*, Keio University International Symposia for Life Sciences and Medicine, Volume 12, Springer, Tokyo, Japan, pp. 127–134 (2005).
[134] Resta, T.C., Walker, B.R., Eichinger, M. and Doyle, M.P. Rate of NO scavenging alters effects of recombinant hemoglobin solutions on pulmonary vasoreactivity, *J. Appl. Physiol.*, **93**, 1327–1336 (2002).
[135] Malhotra, A.K., Kelly, M.E., Miller, P.R. *et al*. Resuscitation with a novel hemoglobin-based oxygen carrier in a swine model of uncontrolled perioperative hemorrhage, *J. Trauma*, **54**, 915–924 (2003).
[136] Fronticelli, C., Koehler, R.C. and Brinigar, W.S. Recombinant hemoglobins as artificial oxygen carriers, *Artif. Cells Blood Substit. Biotechnol.*, **35**, 45–52 (2007).
[137] Hermann, J., Corso, C. and Messmer, K.F. Resuscitation with recombinant hemoglobin rHb2.0 in a rodent model of hemorrhagic shock, *Anesthesiology*, **107**, 273–280 (2007).
[138] Raat, N.J., Liu, J., Doyle, M.P. *et al*. Effects of recombinant hemoglobin solutions rHb2.0 and rHb1.1 on blood pressure, intestinal blood flow and gut oxygenation in a rat model of hemorrhagic shock, *J. Lab. Clin. Med.*, **145**, 21–32 (2005).
[139] Von Dobschuetz, E., Hutter, J., Hoffmann, T. and Messmer, K. Recombinant human hemoglobin with reduced nitric oxide scavenging capacity restores effectively pancreatic microcirculatory disorders in hemorrhagic shock, *Anesthesiology*, **100**, 1484–1490 (2004).

[140] Valeri, C.R., Ragno, G. and Veech, R.L. Severe adverse events associated with hemoglobin-based oxygen carriers: role of resuscitative fluids and liquid preserved RBC, *Transfus. Apher. Sci.*, **39**, 205–211 (2008).
[141] Natanson, C., Kern, S.J., Lurie, P. *et al*. Cell-free hemoglobin-based blood substitutes and risk of myocardial infarction and death, *JAMA*, **299**, 2304–2312 (2008).
[142] Fergusson, D.A. and McIntyre, L. The future of clinical trials evaluating blood substitutes, *JAMA*, **299**, 2324–2326 (2008).
[143] Rao, S.V., Jollis, J.G., Harrington, R.A. *et al*. Relationship of blood transfusion and clinical outcomes in patients with acute coronary syndromes, *JAMA*, **292**, 1555–1562 (2004).
[144] Gerber, D.R. Transfusion of packed red blood cells in patients with ischemic heart disease, *Crit. Care Med.*, **36**, 1068–1074 (2008).
[145] Schechter, A.N. and Gladwin, M.T. Hemoglobin and the paracrine and endocrine functions of nitric oxide, *N. Engl. J. Med.*, **348**, 1483–1485 (2003).

7

Diagnosis and Treatment of Haemorrhages in 'Nonsurgical' Patients

Umberto Rossi[1] *and Rosa Chianese*[2]

[1]*European School of Transfusion Medicine (ESTM), Italy*
[2]*Transfusion Medicine and Haematology Service, Hospital of Ivrea (Torino), Italy*

7.1 Introduction

Haemorrhages in bleeding disorders neither requiring nor correlated with surgical intervention are numerous. Some of these 'nonsurgical' bleeding disorders are characterized by such a severe bleeding as to require transfusion treatment to compensate blood component loss (e.g. haemorrhages in vascular disorders). In these cases, transfusion therapy is a symptomatic therapy.

In some cases, treatment with blood components such as plasma or platelets, or coagulation factors, is indicated to correct the defect or abnormality of coagulation and/or haemostasis involved in the pathogenesis of the haemorrhagic disorder.

A correct transfusion approach requires a proper diagnosis and a nosologic classification of the haemorrhage.

7.1.1 Aetiopathogenetic Classification

From the aetiopathogenetic point of view, haemorrhages can be classified according to two criteria.

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Edited by Andrea Mozzarelli and Stefano Bettati.

The first criterion is pathogenetic:

(i) monofactorial pathogenesis: alterations of single physiological events that contribute to normal homeostasis of coagulation and haemostasis:
  (a) alterations of coagulation and of fibrinolysis (plasma factors);
  (b) haemostatic alterations (thrombocytopenias and thrombocytopathies);
  (c) vascular alterations;
(ii) multifactorial pathogenesis.

The other criterion is aetiological. Haemorrhages can accordingly be divided into:

(i) congenital;
(ii) acquired.

The observed deficiency may be quantitative or qualitative (see Tables 7.1 and 7.2).

7.1.2 Multifactorial Pathogenesis

The pathogenesis of a bleeding disorder cannot always be attributed to a single mechanism, especially in the *acquired forms secondary to systemic diseases*, where the pathogenesis of bleeding is *multifactorial*.

Acquired bleeding disorders are characterized by absence of family history of bleeding and absence of significant bleeding after surgery or trauma. They are frequently secondary to, or symptomatic of, a systemic disease, whose symptoms often dominate the clinical picture. They are more common in adulthood and are associated with liver disease, malabsorption, collagen and autoimmune diseases, neoplasias, myeloproliferative disorders and other oncohaematological diseases (acute promyelocytic leukaemia, myeloma, etc.), obstetric complications, sepsis, renal failure (uremia, dialysis patients), disseminated intravascular coagulation (DIC), thrombotic thrombocytopenic purpura (TTP), patients under antibiotic or cytostatic treatment.

These diseases may cause or facilitate the onset of one or more of the following conditions: (i) reduced synthesis of coagulation factors; (ii) reduced gamma-carboxylation of vitamin K-dependent coagulation factors; (iii) formation of acquired inhibitors of coagulation factors; (iv) hyperfibrinolysis; (v) reduced production of platelets; (vi) reduced platelet activity; (vii) increased destruction of platelets; (viii) consumption of coagulation factors and platelets; (ix) vasculopathy.

Haemorrhagic syndromes may also occur as a complication of treatments that impair the mucous membranes (steroids, antiblastic drugs such as methotrexate, etc.).

Finally, some haemorrhages occur in the context of haemorrhagic-thrombotic syndromes (e.g. DIC, TTP, Lupus anticoagulant-hypoprothrombinemia (LAHPS) syndrome, acquired specific inhibitors of coagulation factors).

7.1.3 Haemorrhagic Syndromes from Antithrombotic Treatment or Prophylaxis

One has finally to consider the haemorrhagic syndromes whose aetiopathogenesis depends on side-effects of antiaggregating (dipyridamole, sulfinpyrazone, ticlopidine, ASA, FANS), anticoagulant (heparin, oral anticoagulants) or fibrinolytic (urokinase) therapy.

Table 7.1 *Haemorrhages from congenital deficiency of coagulation plasma factors.*

Disease	Inheritance	Clinical features
Haemophilia A (FVIII:C deficiency) Incidence 1 : 10 000 people Haemophilia B (FIX:C deficiency) or Christmas disease Incidence 1 : 100 000 male babies	Sex-linked recessive transmission, diaginic transmission. The genes for factor VIII and factor IX are located at the end of the long arm of chromosome X. Mutations can be of various kinds: deletions, inversion, insertions, missense mutations, nonsense mutations.	Male subjects are affected. The few affected women are the result of a haemophilic father and a carrier mother, or of *de novo* gene mutations (so-called 'sporadic' cases). The activity of the deficient factor is <25%. Characteristic clinical features are: haemarthroses (evolving to joint ankylosis), muscle haematomas, internal bleedings, delayed, prolonged and recurrent bleeding after trauma, little response to local tamponade. Cerebral haemorrhage is uncommon. Depending on the severity of haemorrhagic manifestations or on plasma level of the specific procoagulant factor, haemophilias are classified as 'mild', 'moderate' or 'severe'. Mild haemophilias may remain undiagnosed until adult age. Haemophilia B is more rare and usually has clinical manifestations milder than those of haemophilia A
Haemophilia C (FXI:C deficiency)	Autosomal recessive transmission.	Affects both sexes. The haemorrhagic syndrome is not related to plasma levels of factor XI and is usually less important than haemophilia A, and only rarely is there haemarthroses or spontaneous bleeding.
FII/FV/FVII/FX/FXI deficiency	Autosomal recessive transmission. The parents of the affected person usually have 30–70% of normal levels of deficient factor and are asymptomatic.	Both men and women can be affected.

(*continued overleaf*)

Table 7.1 *(continued)*

Disease	Inheritance	Clinical features
von Willebrand disease (vWF quantitative or qualitative deficiency). In some forms, a defective release of vWF by the endothelium or an abnormal proteolysis has been supposed Prevalence 1 : 100	Autosomal transmission (gene located on chromosome 12), with variable clinical expression. Type 1 (variants with quantitative defect): autosomal dominant. Type 2 (variants with qualitative defect): variant 2A, 2B, 2M autosomal dominant; variant 2A rarely recessive; variant 2N autosomal recessive. Type 3 (severe quantitative defect/absence of vWF): autosomal recessive, from partial or total gene deletion.	Both men and women can be affected. Spontaneous bleedings (menometrorrhagias, epistaxis) or induced by small traumatic lesions, tooth extractions, tonsillectomy, etc. Type 3 (recessive) is the most serious, with very severe haemorrhagic symptoms. Types 1 and 2N are usually mild in many patients.
Dysfibrinogenemia, hypofibrinogenemia, afibrinogenemia, FXIII deficiency	Autosomal dominant transmission.	Both men and women can be affected. In dysfibrinogenemia, afibrinogenemia and congenital factor XIII deficiency, scar bleeding after the fall of the umbilical cord and abnormalities in wound cicatrisation are typical.
Hereditary haemorrhagic telangiectasia	Autosomal dominant transmission.	Both men and women can be affected. Epistaxis and recurrent gastrointestinal haemorrhages are common, in the absence of other haemorrhagic manifestations.

Table 7.2 *Haemorrhages from congenital deficiency of plasma factors of the fibrinolytic system.*

Deficiency of alpha2-antiplasmin (quantitative or qualitative)	Autosomal recessive transmission.	Both men and women can be affected. Often associated with severe haemorrhagic tendency.

7.2 Clinical Assessment

Clinical assessment of haemorrhages requires a careful medical history, a physical examination of haemorrhagic manifestations, and the investigation of a potential associated systemic disease.

Local spontaneous bleedings (menorrhagia, metrorrhagia, haematemesis, melena, bilateral epistaxis, gingival bleeding, prolonged isolated haematuria) may occur in any form of haemorrhagic diathesis due to coagulation or platelet or vascular defect.

Severe methrorragia may be the only symptom presented by a woman with von Willebrand disease, or else mild thrombocytopenia or an autosomal coagulation defect.

Cerebral haemorrhage may have different clinical presentations and may be classified as: (i) primitive intraparenchymal (represents about 80% of all cerebral haemorrhages and is due to alterations of small vessels, the most common causes being arterial hypertension and amyloid angiopathy); (ii) secondary intraparenchymal, usually symptomatic of arteriovenous malformations, aneurysms, tumours, coagulopathies (mainly FXIII, FVII and FX deficiencies); or (iii) subarachnoidal haemorrhage (SAH) (85% of spontaneous SAHs are due to rupture of aneurisma, 10% are idiopathic and 5% are associated with rare causes such as arteriovenous malformations, dural arteriovenous fistulae or arterial dissection).

Oral anticoagulant therapy is associated with a risk of intracranial bleeding (usually intraparenchymal) amounting to 0.3–2.5 per 1000 patients per year, with an increased relative risk of 15.3 times compared to untreated patients.

7.2.1 Medical History

Medical history helps to distinguish between acquired and congenital defects.

7.2.1.1 Congenital defects of plasma coagulation factors

- *Family history:* usually negative in case of autosomal recessive inheritance, negative in female ancestors or family members, present instead in men in the case of sex-linked recessive disease. Positive in the case of a disease with autosomal dominant transmission.
- *At birth:* possible cephalohaematoma and haemorrhage at the umbilical cord's fall.
- *In the first months of life:* gingival bleeding at teething, or in the case of circumcision; bleeding sores of the lips or of the fraenum of the tongue associated with oral introduction of sharp objects.
- *When walking:* haemarthroses.
- *During growth and adulthood:* bleeding and/or defective healing in the case of tooth avulsion or tonsillectomy, appendectomy or other surgical interventions; metrorrhagia or menorrhagia.

Moreover, medical history may reveal the use of *drugs* and/or the presence of *signs and symptoms that correlate with other diseases*, of which *haemorrhagic disease is symptomatic*.

7.2.2 Physical Examination

Physical examination helps to frame the pathogenesis of the defect (plasmatic-coagulative, platelet, vascular) and any possible concomitant diseases to which the bleeding disorder may be secondary.

One must search for cutaneous, mucosal, muscular and joint manifestations, and for signs and symptoms of concomitant diseases (e.g. liver diseases, haemopathies, collagen and immune diseases, neoplasias, nephropathies). Signs can be: bruises, haematomas, petechiae (purpura), bleeding.

- *Petechiae* should be searched for in the perimalleolar or anyhow lower regions, and in areas of skin subject to mechanical stress (e.g. compression by tight clothing). Small bruises with scattered petechiae suggest a low platelet count. Petechiae on an erythematous, maculopapular, pomphoid basis, sometimes associated with small areas of skin necrosis and stable hyperpigmentation by haemosiderin deposits, with symmetrical bilateral distribution, suggest a vasculopathy. Perifollicular petechiae are typical of scurvy.
- *Petechiae and haemorrhagic blisters in the oral mucosa* are typical of thrombocytopenias.
- *Skin-mucosal bleeding, spontaneous haematomas, haemarthrosis* suggest haemophilia, but may also be present in other conditions such as Ehlers–Danlos syndrome (vascular type), which is accompanied by abnormal laxity of cutaneous and mucosal connective tissue.
- *Pain or stiffness of limb movements*, which are often accompanied by *skin hypoaesthesia or anaesthesia*, may be a sign of deep-muscle haematomas compressing a neurovascular bundle, and suggest a haemophilia.
- *Large bruises* are typical of multiple coagulation defects, of the presence of acquired haemophilia and of hyperfibrinolysis.
- *Late bruises* (appearing with a colour witnessing their nonrecent formation, being signs of deep blood deposits or extravasations) are common in haemophilia and other bleeding disorders.
- *Acute abdomen* may be a sign of intramural intestinal bleeding, of retroperitoneal bleeding or of haematomas of the ileo-psoas muscle, suggesting a differential diagnosis: haemophilias, hereditary haemorrhagic telangectasias, Henoch–Schönlein purpura (which may be accompanied by intestinal intussusception) and acquired inhibitors.
- *Haemangiomas and telangiectasias* of the skin and mucous membranes, clearly visible on the hands, fingertips, toes, lips, tongue and nasal mucosa, are indicative of hereditary haemorrhagic telangiectasia (Rendu–Osler–Weber Disease or HHT). Patients may experience recurrent, profuse nosebleeds and recurrent gastrointestinal bleeding. Diagnosis is based (besides familiarity criteria) on the finding of characteristic *telangiectasias* on the face, mouth, nose and fingers and of arteriovenous malformations in the internal organs: gastrointestinal, liver, lung, brain and spine. Endoscopy or angiography is sometimes needed, however. Laboratory findings are usually normal, except for iron-deficiency anaemia in most patients. Wider recognition of the condition and awareness of its sequelae can help avoid the considerable risks associated with its mismanagement.
- *Neurological disorders* may be related to cerebromeningeal haemorrhage or to consumption coagulopathy or thrombocytopenia such as DIC and TTP.

Table 7.3 *Clinical differences between haemorrhagic disorders due to alterations of the platelet or vascular phase, or of the coagulation phase.*

	Platelet-vascular defect	Coagulation defect
Haemorrhagic family history	Rare	Frequent
Prevalence by sex	Women	Men
Type of bleeding	Skin and mucous membranes; presence of petechiae and ecchymoses, usually small and isolated	Deep tissues, muscle haematomas and haemarthroses, haematomas, large and confluent bruises
Onset of bleeding	Spontaneous appearance, immediately after traumas, persistent	Onset after traumas, delayed, prolonged and recurrent
Local tamponade	Usually effective	Usually not effective

- *Blood dripping in venipuncture site* or co-existence of thromboembolic phenomena and bleedings are present in consumption coagulopathies and/or thrombocytopenias.

Table 7.3 lists some clinical differences between different types of haemorrhagic disorder.

7.3 Laboratory Tests

7.3.1 Screening Tests

The contribution of screening tests of haemostasis and coagulation in the diagnostic orientation between bleeding disorders via alteration of the plasma coagulative or of the platelet phase is enumerated below:

(i) Blood count: platelet and/or red cell count.
(ii) Coagulation screening tests: PT, aPTT, fibrinogen.
(iii) Antithrombin assay (one of the physiological or 'natural' coagulation inhibitors) and D-dimer (fibrin degradation product stabilized by covalent crosslinks) are indicators of activation of the coagulation cascade.
(iv) The D-dimer is an indicator of activation of fibrinolysis, thus indirectly it also indicates the activation of coagulation.

It is very important to assess the selective or combined alterations of these parameters (Tables 7.4–7.6).

7.3.2 Second-level Laboratory Tests

The diagnosis of *factor deficiency* is carried out with the measurement of individual factors, by testing the correction of deficient plasma diluted with normal plasma. Failure to correct on mixing equal parts of patient plasma and normal plasma is indicative of the presence of inhibitors.

In the presence of *inhibitors*, it is necessary to distinguish between *acquired inhibitors specific to a single factor* and *inhibitors that interfere with the anionic phospholipids* (essential components in the transformation of prothrombin into thrombin); i.e., LACs

Table 7.4 *Conditions in which an altered platelet count can occur.*

Reduced platelet count	**Thrombocytopenia** (i) Reduced production: due to bone marrow replacement by haematologic malignancies, or to myelosuppressive/ myeloablative effect of chemotherapy, or to bone marrow aplasia or hypoplasia by antibiotics or other drugs. (ii) Increased destruction: by immunological cause (ITP), by hypersplenism, sepsis, antimycotics (e.g. amphotericin B), by consumption (DIC, TTP). (iii) Heparin therapy (mainly calcium-UFH heparin): immune-mediated (anti-'PF4-heparin' complex IgG), or with multifactorial pathogenesis, associated to a prothrombotic condition with abnormal generation of thrombin.
Increased platelet count	**Thrombocytosis** of chronic myeloproliferative diseases (TE, CML, PV, IM).

Table 7.5 *Presumptive diagnosis of some congenital disorders of haemostasis according to the results of screening tests.*

Disease	Platelet count	PT	aPTT	PFA-100[a] closure time	Bleeding time
Hereditary deficiency					
FVIII (haemophilia A)	normal	normal	prolonged	normal	normal
FIX (haemophilia B)	normal	normal	prolonged	normal	normal
FXI (haemophilia C)	normal	normal	prolonged	normal	normal
FX, FV, FII	normal	prolonged	prolonged	normal	normal
FVII	normal	prolonged	normal	normal	normal
Von Willebrand disease	normal	normal	normal or prolonged	prolonged	prolonged

[a]PFA-100 (Platelet Function Analyser PFA-100, Dade-Behring) makes use of the so-called 'closure time': it employs special cartridges coated with ADP collagen (CADP) or epinephrine collagen (CCE) membranes, which have a hole at their centre of a calibrated diameter of 150 μm, through which the blood flows with a standardised flow speed. The speed is controlled by the analyser, which measures the closing time by the formation of a platelet aggregate as an answer to the stimulation by collagen ADP or epinephrine collagen. The speed of formation of the platelet aggregate (of standard size) is therefore independent of the speed of blood flow.

(and ACAs) that are associated with thromboembolic and usually not with bleeding events. Characteristics of the tests for correction of deficient plasma with normal plasma are: sensitivity to incubation time (LAC-insensitive) and sensitivity to dilution and temperature of the phospholipid component of the *in vitro* reaction system (LAC-sensitive).

7.3.3 Other Tests

- *von Willebrand disease*: PFA-100, introduced in recent years, shows a diagnostic sensitivity substantially higher than the (elongated) bleeding time, and also allows monitoring of the therapeutic response to desmopessin. Diagnosis is reached by specific *second-level* laboratory tests, where as a measure of 'activity' of von Willebrand factor, its action as cofactor in the normal platelet aggregation induced by Ristocetin is taken (Ristocetin-cofactor 'vWF-A: Rcof').

Table 7.6 *Alterations of haematological laboratory tests in patients with acute haemorrhage.*

Laboratory parameter	Platelet count	PT ratio	aPTT ratio	Fibrinogen	Antithrombin	D-dimer
Consumption coagulopathy (DIC)	↓↓↓	↑↑↑	↑↑↑	↓↓↓	↓↓↓	↑↑↑
Consumption thrombocytopenia (TTP)	↓↓↓	N	N/↑	N	N/↓	↑↑↑
Hyperfibrinolysis (α_2-antiplasmin deficiency)	N	N	N	↓↓↓	N/↓	↑↑↑
Acquired inhibitors, or deficiency of factors of the coagulation common pathway (FX, FV, FII), with residual activity <30–40% of normal	N	↑ of variable entity	↑ of variable entity Not sensitive to deficiencies or inhibitors of FXIII	N	N	↑↑↑ in the presence of acquired inhibitors N in factor deficiencies
Presence of acquired specific inhibitors, or of deficiency of contact factors of the intrinsic pathway: prekallikrein, high-molecular-weight kininogen (HMWK), FXII, FXI, FIX, FVIII, with residual activity <30–40% of normal LAC	N	N	↑ of variable entity (in case of inhibitors, aPTT is not corrected with the mixture 1 : 1 after 2h at 37 °C)	N	N	↑↑↑ in the presence of acquired inhibitors N in factor deficiencies
Presence of specific acquired inhibitors, or of deficiency of factors of the extrinsic pathway: FVII. Residual activity <30–40% of normal values	N	↑ of variable entity (in case of PT inhibitors, do not correct with the mixture 1 : 1)	Not sensitive to deficiencies or inhibitors of FVII	N	N	↑↑↑ in the presence of acquired inhibitors N in factor deficiencies

(*continued overleaf*)

Table 7.6 *(continued)*

Laboratory parameter	Platelet count	PT ratio	aPTT ratio	Fibrinogen	Antithrombin	D-dimer
Congenital afibrinogenemia	N	↑↑↑	↑↑↑	↓↓↓	N	↓ of variable entity
Hypofibrinogenemia Dysfibrinogenemia	N	↑ if fibrinogen <100 mg/dL	N/↑	↓ of variable entity	N	↓ of variable entity
Defect of (hepatic) gamma-carboxylation of vitamin-K-dependent factors (which remain at the state of PIVKA: VII, IX, X, II), by: • low intake of vitamin K • malabsorption • vitamin K inhibitors	N	↑ of variable entity	N/↑	N	N	N
Chronic liver disease	N/↓	↑ of variable entity	↑ of variable entity	↓	↓	↑
Acute massive hepatic necrosis	↓	↑↑ of variable entity	↑↑ of variable entity	↓↓ of variable entity	↓↓ of variable entity	↑↑ of variable entity
Oral anticoagulant treatment	N	↑ (therapeutic range)	N ↑	N	N	N
Heparin treatment	N or decreased (heparin thrombocytopenia)	N	↑ (therapeutic range)	N	N	N
Urokinase treatment	N	↑ of variable entity	↑ of variable entity	↓ (monitoring: >100 mg/dL)	N/↓	↑↑↑

- *Morphological observation of peripheral blood smear* may be useful to provide guidance in:
  - differential diagnosis between real thrombocytopenia and pseudothrombocytopenia (platelet aggregates);
  - thrombocytopathies (giant platelets in Bernard–Soulier syndrome);
  - thrombocytopenias from increased destruction (platelet macrocytosis: young platelets);
  - presence of schistocytes in microangiopathic forms (DIC and above all TTP);
  - chronic myeloproliferative disorders, leukaemias (particularly important for bleeding complications is acute promyelocytic leukaemia), leukaemized lymphomas: presence of immature cells and morphological changes related to the haematological disease.

7.4 Haemorrhagic Syndromes Clinically Indicative of Systemic Defects with Normal Screening Tests

(i) Vasculopathies:
  (a) hereditary haemorrhagic telangiectasia: vascular anatomic malformation in which haemorrhagic manifestations may be the only clinically relevant manifestation of the disease. Diagnosis in these cases is based on physical examination (see Section 7.2.2);
  (b) vascular purpuras: syndromes associated with systemic disease in which haemorrhagic clinical manifestations usually consist of purpura, which can be a major manifestation of the main disease (Schönlein–Henoch or allergic purpura, purpura simplex, senile purpura, cryoglobulinemic purpura, etc.) or a minor and unstable event. In these cases, a fragility test may prove positive (e.g. armlet test), as may the bleeding time on occasion. Diagnosis is generally made on clinical criteria and on investigations aiming to diagnose the underlying disease.

(ii) Alterations of the clot-stabilisation phase, such as congenital FXIII deficiency. The association between bleeding at the umbilical cord's fall and defective wound healing is clinically indicative. Diagnosis is made through specific laboratory tests of fibrin clot solubility.

(iii) Haemorrhagic syndromes with patient's suggestive clinical history of excessive spontaneous mucosal bleeding and disproportionate haemorrhages at traumas or during surgical interventions, with repeatedly normal screening tests and no clinical evidence of systemic primary disease. In such cases, second-level laboratory tests should be performed to investigate the possible existence of:
  (a) congenital thrombocytopathies;
  (b) defects of coagulation factors, particularly factor VIII, IX, XI (if the residual activity is higher than 30–40% it cannot be detected by screening tests);
  (c) von Willebrand disease;
  (d) deficiency of alpha-2-antiplasmin.

(iv) Dysfibrinogenemia.

(v) Platelet hypoaggregability due to drugs (NSAIDs, ASA, dipyridamole, ticlopidine, sulfinpyrazone).

7.5 Blood and Blood Components in the Treatment of Haemorrhagic Syndromes

The use of blood and blood components in the treatment of haemorrhagic syndromes should be viewed in a broader therapeutic context. Treatment of haemorrhagic syndromes in its full complexity is not the subject of this chapter. Here we consider only a few therapeutic aspects, or better, only some fundamental criteria for an effective and safe transfusion.

The rational use of blood and blood components in the treatment of 'nonsurgical' haemorrhagic syndromes is based on the following points:

(i) Targeted correction of blood components lost as a result of bleeding, to maintain the oxyphoretic and haemostatic capacity of blood (not as a substitute for hypovolaemia). Indications of the type (red cells, plasma, platelets) and quantity of blood components depend on the amount of bleeding. In case of bleeding, transfusion of plasma should be timely proportionate to the use of red cells as well as of platelets. During haemorrhage, it should be noted that haemoglobin or haematocrit measurements may not be representative of the actual loss of red cells, and therefore of the real blood oxyphoretic capacity, which in turn is correlated with the onset of anaerobic metabolism and with a status of metabolic acidosis, better assessed with arterial pO_2 and venous pCO_2.

(ii) Targeted correction of the deficiencies of blood components or coagulation factors that determine the pathogenesis of the haemorrhage. Adequate therapy with blood components (plasma, platelets) or blood products (prothrombin complex or individual coagulation factors) depends on the correct diagnosis of the defect.

(iii) Modification of a possible anticoagulant treatment for antithrombotic prophylaxis or therapy. The criteria for therapy suspension depend on the intensity and site of bleeding (intracranial haemorrhage, major haemorrhages, minor haemorrhages), on the INR or aPTT of the patient and on the thrombotic risk. Any treatment with heparin or antiaggregant therapy should be suspended in case of major haemorrhage and recoagulation should be carried out by adequate antidote of the anticoagulant drug used (e.g. vit-k), prothrombin complex concentrate[1] or, if this is not available, by plasma.

Further Reading

Agencie Française de Sécurité sanitaire des Produits de Santé. Transfusion de plasma frais congelé: produits, indications, *Transfus. Clin. Biol*., **9**, 322–332 (2002).

Cattaneo, M., Federici, A.B., Lecchi, A. *et al*. Evaluation of PFA-100 system in the diagnosis and therapeutic monitoring of patients with von Willebrand disease, *Thromb. Haemost*., **82**, 35–39 (1999).

[1] Indications by the 'Federazione Centri per la diagnosi della trombosi e la sorveglianza delle terapie antitrombotiche' (FCSA).

Colman, R.W., Marder, V.J., Salzman, E.W. and Hirsh, J. Overview of the hemostasis, in *Hemostasis and Thrombosis: Basic Principles and Clinical Practice*, Lippincott (1994).

Federazione Centri per la diagnosi della trombosi e la Sorveglianza delle terapie Antitrombotiche (FCSA) Web site: http://www.fcsa.it/.

Federici, A.B. and Canciani, M.T. Clinical and laboratory versus molecular markers for a correct classification of von Willebrand disease, *Haematologica*, **94**, 610–615 (2009).

Gonzales, E.A., Moore, F.A., Holcomb, J.H. *et al*. Fresh frozen plasma should be given earlier to patients requiring massive transfusion, *J. Trauma*, **62**, 112–119 (2007).

Goodnight, S.H., Hathaway, W.E. Jr. *Disorders of Hemostasis and Thrombosis: A Clinical Guide* (2 edn), The McGraw-Hill Companies (2001).

Grottke, O., Henzler, D. and Rossaint, R. Activated recombinant factor VII (rFVIIa), *Best Pract. Res. Clin. Anaesthesiol*., **24**, 95–106 (2010).

Hardy, J.F., de Moerloose, P. and Samama, M. Massive transfusion and coagulopathy: pathophysiology and implications for clinical management, *Can. J. Anesth*., **51**, 293–310 (2004).

Kashuk, J.L., Moore, E.E., Johnson, J.L. *et al*. Postinjury life threatening coagulopathy: is 1 : 1 Fresh Frozen Plasma: Packed Red Blood Cells the answer?, *J. Trauma*, **65**, 261–271 (2008).

Kempton, C.L. Inhibitors in previously treated patients: a review of the literature, *Haemophilia*, **102**, 61–65 (2010).

Liumbruno, G., Bennardello, F., Lattanzio, A. *et al*. The Italian Society of Transfusion Medicine and Immunohaematology (SIMTI) Working Party. Recommendations for the transfusion of plasma and platelets, *Blood Transfus*., **7**, 132–150 (2009).

Martinowitz, U. and Michael M., on behalf of the Israeli Multidisciplinary rFVIIa Task Force. Guidelines for the use of recombinant activated factor VII (rFVIIa) in uncontrolled bleeding: a report by the Israeli Multidiscilinary rFVIIa Task Force.

Santagostino, E., Mancuso, M.E., Tripodi, A. *et al*. Severe hemophilia with mild bleeding phenotype: molecular characterization and global coagulation profile, *J. Thromb. Haemost*., **8**, 737–743 (2010).

Schols, S.E.M., van der Meijden, P.E.J., van Oerle, R. *et al*. Increased thrombin generation and fibrinogen level after therapeutic plasma transfusion: relation to bleeding, *Thromb. Haemost*., **99**, 64–70 (2008).

Stainsby, D., MacLennan S., Thomas, D. *et al*. The British Committee for Standards in Hematology Writing Group. Guidelines on the management of massive blood loss, *Br. J. Haematol*., **135**, 634–641 (2006).

Stanworth, S.J. The evidence-based use of FFP and cryoprecipitate for abnormalities of coagulation tests and clinical coagulopathy, *ASH Educational*, 179–186 (2007).

8

Management of Perioperative Bleeding

Sibylle A. Kozek-Langenecker

Department of Anesthesia and Intensive Care, Evangelical Hospital Vienna, Vienna, Austria

8.1 Introduction

The objectives of an early goal-directed coagulation therapy in massive perioperative bleeding are: (i) a restrictive use of allogeneic blood products; (ii) an individualized and prompt coagulation management based on point-of-care tests (POCTs) sensitive for the complex bleeding pathomechanisms; and (iii) a goal-directed procoagulant therapy based on efficacious factor concentrates. The main goal of the perioperative management of hemostasis is to increase the safety of patients undergoing surgical procedures. In this chapter, pathomechanisms of perioperative coagulopathy, means of coagulation monitoring, and procoagulant optimization will be reviewed [1–3].

8.2 Pathomechanisms of Coagulopathy in Massive Bleeding

Massive transfusion is commonly defined as the replacement of one blood volume over a period of 24 hours or transfusion of at least four red blood cell (PRBC) concentrates within 1 hour when ongoing need is foreseeable. In elective surgery, tissue trauma is more controlled, tissue anoxia is better avoided, blood losses are replaced in a timely manner, and coagulopathy is treated at earlier stages. Massively transfused patients will show evidence of coagulopathy in a high percentage of cases, with an interindividual variability [4, 5]. Uncontrolled bleeding initially leads to loss of coagulation factors and

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platelets [6, 7]. Exposure of the thromboplastin-rich subendothelial tissue to flowing blood induces the activation of coagulation [8], which may trigger consumptive coagulopathy [9–11]. The majority of blunt-trauma and brain-injury victims are hypercoagulable early after trauma, with tissue trauma being the key stimulus for coagulation [9, 12, 13]. In hypocoagulable patients, the remaining procoagulatory potential is reduced by dilution during the fluid resuscitation required to restore intravascular volume and to maintain hemodynamic stability. The degree of dilutional coagulopathy also depends on the type of fluid used: hydroxyethyl starch solutions, gelatins, and dextrans impair platelet function, inhibit fibrin polymerization, and induce an acquired von Willebrand syndrome at varying degrees, depending on the physicochemical characteristics of the colloidal solution [14, 15]. Tissue injury in trauma or surgery may lead to the exposure of tissue plasminogen activator, resulting in hyperfibrinolysis if the delicate balance between coagulation and fibrinolysis is lost [16]. Coagulopathy is confounded by hypothermia, acidosis, and preexisting disorders: trauma patients are prone to hypothermia, which slows down enzymatic reactions [17], modifies platelet function [18, 19], decreases platelet counts [20], and stimulates fibrinolysis [21]. Acidosis worsens fibrin polymerization strengthening the clot [11, 22]. Low ionized calcium (after massive PRBC transfusions containing citrate) and low hematocrit (<30%) further aggravate bleeding diathesis. Red cells contribute to the margination of platelets against the vessel wall and their availability to act at the site of a vascular lesion [23]. They have also been shown to modulate the biochemical and functional responsiveness of activated platelets. Activation and consecutive exhaustion of platelet function after extracorporeal circulation and anticoagulation and its reversal are additional etiologies for cardiopulmonary bypass-induced hemostatic defects. Since surgery and trauma are not restricted to previously healthy people, the increasing number of patients taking oral anticoagulants and platelet-inhibiting drugs poses a rapidly increasing problem [24]. Patients with inherited coagulation defects may exsanguinate with trauma or major surgery unless specific factor replacement is provided.

The vicious cycle of coagulopathy in massive transfusion results in: (i) a defect in clot firmness due to fibrinogen deficiency (an early phenomenon) and thrombocytopenia; (ii) prolonged clot generation due to various coagulation factor deficiencies; and (iii) impaired clot stability due to factor XIII deficiency (a late phenomenon) and hyperfibrinolysis (Figure 8.1).

8.3 Perioperative Coagulation Monitoring

Diagnosis of intra- and postoperative coagulopathy in massive transfusion needs to be verified by appropriate coagulation tests [25] and perioperative coagulation monitoring

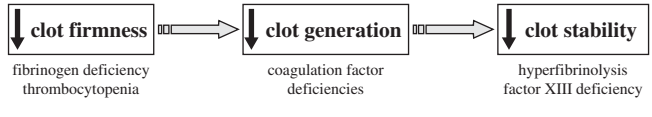

Figure 8.1 *Phenomena of acquired perioperative coagulopathy.*

is the rational basis for pro- and antithrombotic interventions. Currently, a change in paradigms is occurring, with (i) increasing implementation of an evidence-based approach to preoperative patient evaluation with laboratory coagulation testing secondary to the results of the standardized bleeding history, and (ii) awareness of the limitations of routine coagulation tests in guiding coagulation management in massive bleeding [26, 27]. Viscoelastic point-of-care monitoring is increasingly used worldwide. This innovative methodology triggers a trend towards an "early goal-directed coagulation management" focusing on potent coagulation factor concentrates. Practicability, cost-effectiveness, safety, and – above all – growing scientific evidence support this concept.

8.4 Limitations of Routine Coagulation Tests in the Perioperative Setting

In the perioperative setting, where events may proceed at a fast and dramatic pace, real-time monitoring of the patient's coagulation profile and repeated laboratory tests are vital in administrating proper replacement therapy. However, test results of routine coagulation monitoring performed at a hospital's central laboratory are generally only available with a delay of at least 30–60 minutes (sample preparation, including centrifugation and buffering, transportation of blood samples and test results) [28]. The most important limitation of routine coagulation tests is the fact that the predominant pathomechanism of bleeding in the complex scenario of perioperative coagulopathy cannot be differentiated: prolonged activated partial thromboplastin time (aPTT) may be due to "intrinsic coagulation factor" deficiency requiring specific substitution, fibrinogen deficiency requiring fibrinogen substitution, hypothermia requiring rewarming, heparinization requiring protamin reversal, or hyperfibrinolysis requiring antifibrinolytic drugs. Thus, a false differential diagnosis may lead to therapeutic misadventures. Routine coagulation tests are performed in plasma at a standardized temperature of 37 °C, without the presence of platelets and other blood cells. Accordingly, routine laboratory tests cannot assess the effect of hypothermia on hemostasis in hypothermic patients. Furthermore, fibrinolysis and platelet dysfunction pose diagnostic gaps. Since the hemostatic response to injury or surgery is a complex interaction of plasma proteins, platelets, and the vessel wall, according to the present well-accepted cell-based model of hemostasis, it cannot be depicted by tests performed in plasma. Fibrinogen-level determination by Clauss method may be false high in the presence of synthetic colloidal solutions, especially hydroxyethyl starches, which are clinically required for fluid resuscitation [29, 30]. Routine tests pick up abnormalities in hemostasis due to single or multiple deficiencies in coagulation factors, but do not identify them. Prothrombin time (PT) and aPTT assess only the speed of fibrin-strand formation, not the mechanical and functional properties of the clot over time (Figure 8.1). Functional fibrin polymerization may be impaired despite normal fibrinogen concentration. Routine coagulation tests are poor predictors of bleeding and mortality [31–33].

Methods of platelet function monitoring have been reviewed elsewhere [34], including the Platelet Function Analyzer PFA-100 (Dade). Major limitations of the PFA-100 as an intraoperative point-of-care system in massive transfusion include its strong dependence on platelet count (>100 G/l) and hematocrit (>30%).

8.5 Thromboelastography (TEG) and Rotational Thromboelastometry (ROTEM)

TEG and ROTEM measure the viscoelastic properties of non-anticoagulated or (citrate) anticoagulated blood after induction of clotting under low shear conditions, resembling the rheologic properties in venous vessels *in vivo*. The pattern of changes in viscoelasticity reflects the kinetics of all stages of thrombus formation (r and k time, clotting time (CT) and clot formation time (CFT)), the stability and firmness of the clot – a function of platelet–fibrin interaction and fibrin polymerization (maximum amplitude (MA), maximum clot firmness (MCF)), and dissolution (fibrinolysis) [35]. The increasing firmness of the clot gradually reduces the movement of a rotating pin, which is continuously detected. ROTEM (TEM International, Germany) permits differential diagnosis of the pathomechanism of coagulopathy by implementing test modifications [29, 36]. EXTEM is a baseline test that uses recombinant tissue factor to activate coagulation (comparable to the PT), which causes rapid generation of the clot. The clotting time (EXTEM CT) gives information about the initial activation and dynamics of clot formation, thus allowing analysis of factor deficiencies (and the detection of anticoagulants). The critical cut-off value for CT, indicating the necessity of administering prothrombin complex concentrates (PCC) or fresh frozen plasma, appears about 80 seconds after test initiation. Disposable wells containing cytochalasin D, a platelet inhibitor, are used in the fibrinogen-sensitive FIBTEM test. Critical FIBTEM MCF cut-off values, indicating the need for fibrinogen substitution, appear within 10–15 minutes after test initiation. Wells containing aprotinin facilitate the detection of hyperfibrinolysis (APTEM test) (Figure 8.2).

TEG/ROTEM measurements should be performed at the bedside at baseline of surgery, when clinically abnormal bleeding occurs, and after therapeutic interventions. Normal viscoelastic test results are unlikely to coincide with bleeding (high negative predictive value) [37]. As a consequence, another important implication of TEG/ROTEM monitoring is the immediate initiation of surgical reexploration if no hemostaseological cause of bleeding is observed.

8.6 Procoagulant Interventions

Indications for procoagulant interventions are (i) the correction of bleeding, and (ii) the prevention of bleeding before invasive procedures. Contraindications have to be considered before institution of procoagulant interventions [38]. Therapeutic options in perioperative coagulation management have been reviewed previously [4, 28, 39–42].

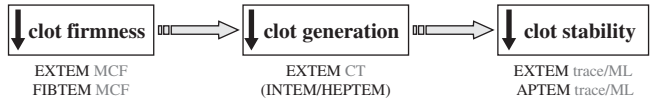

Figure 8.2 *Monitoring options using ROTEM to detect relevant phenomena of acquired perioperative coagulopathy. MCF, maximum clot firmness; CT, clotting time; ML, maximum lysis. Tests are explained in the text.*

The motto of a rational coagulation management in the bleeding patient is:

If you don't measure it, you can't manage it!
Replace what is missing!

A scheme of procoagulant interventions based on the pathomechanisms of bleeding is shown in Figure 8.3.

Fibrinogen, the final effector of the clotting system, is vulnerable in trauma-associated coagulability because it reaches critical values before several other coagulation factors [43, 44]. Replacement of only one blood volume may lead to a clinically relevant fibrinogen deficiency [31, 43]. The administration of virus-inactivated fibrinogen concentrates is faster and more effective in reversing a fibrinogen deficiency than the administration of fresh frozen plasma (FFP) [45]. Alternatively, cryoprecipitate containing factor VIII, von Willebrand factor and (lower amounts of) fibrinogen is used in the Anglo-American literature in acquired fibrinogen deficiency [46]. Empiric transfusion triggers in bleeding trauma patients are fibrinogen <100 mg/dl or alternatively in a ROTEM-based transfusion algorithm, EXTEM MCF <50 mm plus FIBTEM MCF <12 mm [47, 48]. Fibrinogen substitution partially reverses the dilutional coagulopathy induced by crystalloids and colloids *in vitro* and *in vivo* [49].

Replacement of 2.5 blood volumes may lead to clinically relevant thrombocytopenia [43]. Empiric indicators for transfusion of platelet concentrates are platelet counts <50 (−100) G/l [50] or EXTEM MCF <45 mm combined with FIBTEM MCF >12 mm. Bearing in mind the individual response (release and sequestration of platelets), recommendations for fixed transfusion ratios for platelet concentrates versus PRBC (ratio of 0.5 : 0.8) are useless [28, 51]. Prophylactic platelet administration fails to prevent massive transfusion [52].

In contrast to fresh frozen plasma, prothrombin complex concentrates (PCC) are rapidly available in the trauma unit or OR. Empiric transfusion triggers are aPTT or PT >1.5 (−1.8) times normal or EXTEM CT >80 seconds, which indicates clinically relevant loss of coagulation factors II, VII, IX, X. Co-administration of PCC and antithrombin is not required in massively bleeding patients. Adequate doses to increase coagulation factor levels by FFP are >20–30 ml/kg [45]. Prophylactic administration of FFP has been shown to be ineffective in massively transfused patients [32, 51, 52]. FFP transfusion is indicated for correction of known coagulation deficiencies (e.g. factor V) for which specific concentrates are unavailable.

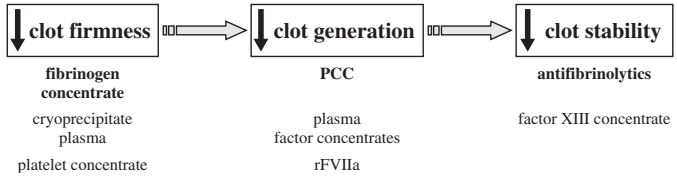

Figure 8.3 *Procoagulant interventions based on detected phenomena of acquired perioperative coagulopathy. PCC, prothrombin complex concentrate; rFVIIa, recombinant activated factor VII.*

The efficacy of recombinant activated factor VIIa (rFVIIa) is used off-label in nonhemophilic patients with severe trauma. rFVIIa at supraphysiological doses (up to 90 μg/kg BW per bolus) binds to activated platelets and induces the generation of a stable fibrin clot via a thrombin burst. Severe coagulopathy may render rFVIIa ineffective. Before rFVIIa, transfusion replacement should aim at fibrinogen levels >100 mg/dl, platelet counts >50 (−100) G/l, and PT and aPTT approximating normal [24]. It must be kept in mind that patients, if oversupported in the pre- and intraoperative period, may rapidly swing back to a thrombotic state postoperatively, with the risk of myocardial infarction, pulmonary embolism, or deep vein thrombosis.

During massive transfusion and dilutional coagulopathy, factor XIII deficiencies may occur, which can be treated by a specific factor concentrate [53]. Monitoring of factor XIII levels (or EXTEM assay with *ex vivo* spiking with factor XIII) may guide factor XIII concentrate supplementation. Patients with inherited coagulation defects may exsanguinate with trauma or major surgery unless specific factor replacement is provided (such as factor VIII, IX, von Willebrand factor concentrate).

Lysine analogues such as tranexamic acid and aminocaproic acid inhibit plasminogen activation. A meta-analysis of all randomized, controlled trials of the three most frequently used pharmacological strategies to decrease postoperative blood loss – aprotinin, lysine analogues, and desmopressin – showed that aprotinin decreased mortality in cardiac surgery almost twofold compared with placebo [40]. If detected in the ROTEM or TEG, first-line therapy has to correct hyperfibrinolysis, followed by replacement of consumed coagulation factors.

Desmopressin (DDAVP) is a vasopressin analogue that induces the release of von Willebrand factor and factor VIII from endothelial cells. Desmopressin results in a small decrease in blood loss in cardiac surgery, but is not associated with a beneficial effect on other clinical outcomes [40]. Indications in massively bleeding patients are limited to inherited and acquired platelet dysfunctions, von Willebrand syndrome, and preexisting antiplatelet medication. In patients with preoperatively identified platelet dysfunctions and without contraindications against desmopressin, shortening of the PFA-closure time after desmopressin infusion should be assessed ("desmopressin response test") [54].

Adequate hematocrit, adequate tissue perfusion, and prevention of acidosis are crucial in the correction of bleeding. PRBC should be transfused to correct a physiological deficit likely to be detrimental to the patient [55]. The optimal hematocrit to sustain hemostasis in the context of massive transfusion remains unknown.

Maintaining a normal body temperature is a first-line and effective strategy to improve hemostasis during massive transfusion. There is a limit on the level of hemostasis that can be restored by replacement therapy in the presence of hypothermia and acidosis.

Novel rapidly degradable hydroxyethyl starch solutions or gelatins with negligible effects on plasmatic coagulation and platelet function are preferred for the resuscitation of patients requiring massive transfusion.

8.7 Algorithm for Coagulation Management

Viscoelastic point-of-care coagulation monitoring embedded into a management algorithm (evidence-based medicine level Ib) is highly recommended [1]. The institution of

transfusion algorithms based on thromboelastographic parameters reduced transfusion requirements (and in some study designs also blood loss) in both routine and high-risk cardiac surgery in adults and children, and liver transplantation [56–63]. Transfusion requirements before and after the implementation of the ROTEM were significantly lower [64]. Since the point-of-care based coagulation management helps to minimize direct costs of blood products, avoids costly adverse effects of transfusion, and shortens surgical procedures, frequency of reopenings, and intensive care stay, it has a massive potential for significant cost savings.

References

[1] Fries, D., Innerhofer, P., Perger, P. *et al*. Coagulation management in trauma-related massive bleeding: recommendations of the Task Force for Coagulation of the Austrian Society of Anaesthesiology, Resuscitation and Intensive Care Medicine (ÖGARI), *AINS*, **45**, 552–561 (2010).
[2] OEGARI. http://www.oegari.at/arbeitsgruppe.asp?id=116.
[3] http://www.perioperativebleeding.org (2009).
[4] Hardy, J.F., de Moerloose, P. and Samama, C.M. Massive transfusion and coagulopathy: pathophysiology and implications for clinical management, *Can. J. Anaesth*., **53**, S40–S58 (2006).
[5] Levy, J. Massive transfusion coagulopathy, *Semin. Hematol*., **43**, S59–S63 (2006).
[6] Lynn, M., Jeroukhimov, I., Klein, Y. and Martinowitz, U. Updates in the management of severe coagulopathy in trauma patients, *Intensive Care Med*., **28**(Suppl 2), S241–S247 (2002).
[7] Aucar, J.A., Norman, P., Whitten, E. *et al*. Intraoperative detection of traumatic coagulopathy using the activated coagulation time, *Shock*, **19**, 404–407 (2003).
[8] Gando, S., Tedo, I. and Kubota, M. Posttrauma coagulation and fibrinolysis, *Crit. Care Med*., **20**, 594–600 (1992).
[9] Ungerstedt, J.S., Grenander, A., Bredbacka, S. and Blomback, M. Clotting onset time may be a predictor of outcome in human brain injury: a pilot study, *J. Neurosurg. Anesthesiol*., **15**, 13–18 (2003).
[10] Hulka, F., Mullins, R.J. and Frank, E.H. Blunt brain injury activates the coagulation process, *Arch. Surg*., **131**, 923–927; disc. 927–928 (1996).
[11] Cosgriff, N., Moore, E.E., Sauaia, A. *et al*. Predicting life-threatening coagulopathy in the massively transfused trauma patient: hypothermia and acidosis revisited, *J. Trauma*, **42**, 857–861; disc. 861–862 (1997).
[12] Schreiber, M.A., Differding, J., Thorborg, P. *et al*. Hypercoagulability is most prevalent early after injury and in female patients, *J. Trauma*, **58**, 475–480; disc. 480–481 (2005).
[13] Kaufmann, C.R., Dwyer, K.M., Crews, J.D. *et al*. Usefulness of thrombelastography in assessment of trauma patient coagulation, *J. Trauma*, **42**, 716–720; disc. 720–722 (1997).
[14] Kozek-Langenecker, S.A. Effects of hydroxyethyl starch solutions on hemostasis, *Anesthesiology*, **103**, 654–660 (2005).

[15] deJonge, E., Levi, M. and Berends, F. Impaired haemostasis by intravenous administration of a gelatin-based plasma expander in human subjects, *Thromb. Haemost*., **79**, 286–290 (1998).
[16] Enderson, B.L., Chen, J.P., Robinson, R. and Maull, K.I. Fibrinolysis in multisystem trauma patients, *J. Trauma*, **31**, 1240–1246 (1991).
[17] Rohrer, M. and Natale, A. Effect of hypothermia on the coagulation cascade, *Crit. Care Med*., **20**, 1402–1405 (1992).
[18] Scharbert, G., Kalb, M., Marschalek, C. and Kozek-Langenecker, S.A. The effects of test temperature and storage temperature on platelet aggregation: a whole blood in vitro study, *Anesth. Analg*., **102**, 1280–1284 (2006).
[19] Valeri, C., Feingold, H., Cassidy, G. *et al*. Hypothermia-induced reversible platelet dysfunction, *Ann. Surg*., **205**, 175–181 (1987).
[20] Pina-Cabral, J.M., Ribeiro-da-Silva, A. and Almeida-Dias, A. Platelet sequestration during hypothermia in dogs treated with sulphinpyrazone and ticlopidine: reversibility accelerated after intra-abdominal rewarming, *Thromb. Haemost*., **54**, 838–841 (1985).
[21] Yoshihara, H., Yamamoto, T. and Mihara, H. Changes in coagulation and fibrinolysis occurring in dogs during hypothermia, *Thromb. Res*., **37**, 503–512 (1985).
[22] Engstrom, M., Schott, U., Romner, B. and Reinstrup, P. Acidosis impairs the coagulation: a thromboelastographic study, *J. Trauma*, **61**, 624–628 (2006).
[23] Quaknine-Orlando, B., Samama, C.M., Riou, B. *et al*. Role of the hematocrit in a rabbit model of arterial thrombosis and bleeding, *Anesthesiology*, **90**, 1454–1461 (1999).
[24] DeLoughery, T.G. Coagulation defects in trauma patients: etiology, recognition, and therapy, *Crit. Care Clin*., **20**, 13–24 (2004).
[25] Practice guidelines for perioperative blood transfusion and adjuvant therapies: an updated report by the American Society of Anesthesiologists Task Force on perioperative blood transfusion and adjuvant therapies, *Anesthesiology*, **105**, 198–208 (2006).
[26] Kozek-Langenecker, S. Management of massive operative blood loss, *Minerva Anestesiol*., **73**, 401–415 (2007).
[27] Kozek-Langenecker, S. Monitoring of hemostasis in emergency medicine, in *Yearbook of Intensive Care and Emergency Medicine* (ed. J.L. Vincent), Springer-Verlag, Berlin-Heidelberg, Germany, pp. 847–860 (2007).
[28] Ketchum, L., Hess, J.R. and Hiippala, S. Indications for early fresh frozen plasma, cryoprecipitate, and platelet transfusion in trauma, *J. Trauma*, **60**, S51–S58 (2006).
[29] Fries, D. Diagnostik beim Blutungsnotfall, in *Gerinnungsmanagement beim perioperativen Blutungsnotfal*, 1 edn (ed. B. Heindl), Uni-Med Verlag AG, Bremen, Germany, pp. 60–65 (2008).
[30] Hiippala, S. Dextran and hydroxyethyl starch interfere with fibrinogen assays, *Blood Coagul. Fibrinolysis*, **6**, 743–746 (1995).
[31] Ciavarella, D., Reed, R., Counts, R. *et al*. Clotting factor levels and the risk of diffuse microvascular bleeding in the massively transfused patient, *Br. J. Haematol*., **67**, 365–368 (1987).
[32] Counts, R.B., Haisch, C., Simon, T.L. *et al*. Hemostasis in massively transfused trauma patients, *Ann. Surg*., **190**, 91–99 (1979).

[33] Yuan, S., Ferrell, C. and Chandler, W.L. Comparing the prothrombin time INR versus the APTT to evaluate the coagulopathy of acute trauma, *Thromb. Res*., **120**, 29–37 (2007).

[34] Kozek-Langenecker, S. Monitoring of hemostasis in the perioperative setting, in *Alternatives to Blood Transfusion in Transfusion Medicine* (ed. A. Maniatis, J. Hardy and P. Van der Linden), Blackwell Publishing, UK (2010).

[35] Di Benedetto, P., Baciarello, M., Cabetti, L. *et al*. Thrombelastography: present and future perspectives in clinical practice, *Minerva Anestesiol*., **69**, 501–509, 509–515 (2003).

[36] Luddington, R.J. Thrombelastography/thromboelastometry, *Clin. Lab. Haematol*,. **27**, 81–90 (2005).

[37] Cammerer, U., Dietrich, W., Rampf, T. *et al*. The predictive value of modified computerized thromboelastography and platelet function analysis for postoperative blood loss in routine cardiac surgery, *Anesth. Analg*., **96**, 51–57 (2003).

[38] Mangano, D.T., Tudor, I.C. and Dietzel, C. The risk associated with aprotinin in cardiac surgery, *N. Engl. J. Med*., **354**, 353–365 (2006).

[39] Fries, D., Haas, T., Salchner, V. *et al*. Management of coagulation after multiple trauma, *Anaesthesist*, **54**, 137–144 (2005).

[40] Levi, M., Cromheecke, M.E., de Jonge, E. *et al*. Pharmacological strategies to decrease excessive blood loss in cardiac surgery: a meta-analysis of clinically relevant endpoints, *Lancet*, **354**, 1940–1947 (1999).

[41] Spivey, M. and Parr, M.J. Therapeutic approaches in trauma-induced coagulopathy, *Minerva Anestesiol*., **71**, 281–289 (2005).

[42] Erber, W.N. and Perry, D.J. Plasma and plasma products in the treatment of massive haemorrhage, *Best Pract. Res. Clin. Haematol*., **19**, 97–112 (2006).

[43] Hiippala, S.T., Myllyla, G.J. and Vahtera, E.M. Hemostatic factors and replacement of major blood loss with plasma-poor red cell concentrates, *Anesth. Analg*., **81**, 360–365 (1995).

[44] Murray, D.J., Pennell, B.J., Weinstein, S.L. and Olson, J.D. Packed red cells in acute blood loss: dilutional coagulopathy as a cause of surgical bleeding, *Anesth. Analg*., **80**, 336–342 (1995).

[45] Chowdhury, P., Saayman, A.G., Paulus, U. *et al*. Efficacy of standard dose and 30ml/kg fresh frozen plasma in correcting laboratory parameters of haemostasis in critically ill patients, *Br. J. Haematol*., **125**, 69–73 (2004).

[46] Pantanowitz, L., Kruskall, M.S. and Uhl, L. Cryoprecipitate: patterns of use, *Am. J. Clin. Pathol*., **119**, 874–881 (2003).

[47] Goerlinger, K., Dirkmann, D., Kiss, G. *et al*. ROTEM-based management for diagnosis and treatment of acute haemorrhage during liver transplantation. A322. *Eur. J. Anaesthesiol*., **23**, 85 (2006).

[48] Coakley, M., Reddy, K., Mackie, I. and Mallett, S. Transfusion triggers in orthotopic liver transplantation: a comparison of the thromboelastometry analyzer, the thromboelastogram, and conventional coagulation tests, *J. Cardiothorac. Vasc. Anesth*., **20**, 548–553 (2006).

[49] Fries, D., Krismer, A., Klingler, A. *et al*. Effect of fibrinogen on reversal of dilutional coagulopathy: a porcine model, *Br. J. Anaesth*., **95**, 172–177 (2005).

[50] Dzik, W., Arkin, C., Jenkins, R. and Stump, D. Fibrinolysis during liver transplantation in humans: role of tissue-type plasminogen activator, *Blood*, **71**, 1090–1095 (1988).
[51] Mannucci, P.M., Federici, A.B. and Sirchia, G. Hemostasis testing during massive blood replacement: a study of 172 cases, *Vox Sang*., **42**, 113–123 (1982).
[52] Reed, R.L. 2nd, Ciavarella, D., Heimbach, D.M. *et al*. Prophylactic platelet administration during massive transfusion: a prospective, randomized, double-blind clinical study, *Ann. Surg*., **203**, 40–48 (1986).
[53] Gerlach, R., Raabe, A., Zimmermann, M. *et al*. Factor XIII deficiency and postoperative hemorrhage after neurosurgical procedures, *Surg. Neurol*., **54**, 260–264; disc. 264–265 (2000).
[54] Koscielny, J., von Tempelhoff, G.F., Ziemer, S. *et al*. A practical concept for preoperative management of patients with impaired primary hemostasis, *Clin. Appl. Thromb. Hemost*., **10**, 155–166 (2004).
[55] Escolar, G., Garrido, M., Mazzara, R. *et al*. Experimental basis for the use of red cell transfusion in the management of anemic-thrombocytopenic patients, *Transfusion*, **28**, 406–411 (1988).
[56] Shore-Lesserson, L. Evidence based coagulation monitors: heparin monitoring, thromboelastography, and platelet function, *Semin. Cardiothorac. Vasc. Anesth*., **9**, 41–52 (2005).
[57] Shore-Lesserson, L., Manspeizer, H.E., DePerio, M. *et al*. Thromboelastography-guided transfusion algorithm reduces transfusions in complex cardiac surgery, *Anesth. Analg*., **88**, 312–319 (1999).
[58] Avidan, M.S., Alcock, E.L., Da Fonseca, J. *et al*. Comparison of structured use of routine laboratory tests or near-patient assessment with clinical judgement in the management of bleeding after cardiac surgery, *Br. J. Anaesth*., **92**, 178–186 (2004).
[59] Royston, D. and von Kier, S. Reduced haemostatic factor transfusion using heparinase-modified thrombelastography during cardiopulmonary bypass, *Br. J. Anaesth*., **86**, 575–578 (2001).
[60] Nuttall, G.A., Oliver, W.C., Santrach, P.J. *et al*. Efficacy of a simple intraoperative transfusion algorithm for nonerythrocyte component utilization after cardiopulmonary bypass, *Anesthesiology*, **94**, 773–781; disc. 775A–776A (2001).
[61] Spiess, B.D., Gillies, B.S., Chandler, W. and Verrier, E. Changes in transfusion therapy and reexploration rate after institution of a blood management program in cardiac surgical patients, *J. Cardiothorac. Vasc. Anesth*., **9**, 168–173 (1995).
[62] Miller, B.E., Guzzetta, N.A., Tosone, S.R. and Levy, J.H. Rapid evaluation of coagulopathies after cardiopulmonary bypass in children using modified thromboelastography, *Anesth. Analg*., **90**, 1324–1330 (2000).
[63] Kang, Y. Coagulation and liver transplantation: current concepts, *Liver Transplant. Surg*., **3**, 465–467 (1997).
[64] Anderson, L., Quasim, I., Soutar, R. *et al*. An audit of red cell and blood product use after the institution of thromboelastometry in a cardiac intensive care unit, *Transfus. Med*., **16**, 31–39 (2006).

9

Oxygenation in the Preterm Neonate

Vidheya Venkatesh[1], *Priya Muthukumar*[1], *Anna Curley*[1] *and Simon Stanworth*[2]

[1]*Neonatal Unit, Cambridge University Hospitals NHS Trust, Cambridge, UK*
[2]*NHSBT Oxford Centre, John Radcliffe Hospital, Oxford, UK*

9.1 Introduction

Neonatal intensive care has witnessed many advances over the last few decades but the cardinal importance of adequate respiratory support and oxygenation has been recognized for a long time. Indeed, as early as 400 BC Hippocrates described cannulation of the trachea to support ventilation [1]. Advances in neonatal care since then have paralleled the advances made in the areas of physics, chemistry, physiology and most importantly obstetric care.

In 1772 a Scottish obstetrician, William Smellie, developed the first straight endotracheal tube for the resuscitation of neonates [2]. Later, in 1774, Joseph Priestley discovered the gas oxygen [3]. Lavoisier gave the gas its name and was the first person to propose oxygen played a crucial role in human metabolic processes [4]. In 1891, Bonnair published the first clinical report of the use of oxygen in preterm neonates [5]. It was only after the 1930s, however, that routine use of oxygen in the care of preterm neonates became widespread.

By the 1950s, the benefits of supplemental oxygen therapy were increasingly recognized, such as normalizing the irregular pattern of breathing in the preterm neonate. Observational studies reported improved sleep patterns, growth and neurodevelopmental outcome in neonates who were administered supplemental oxygen [6, 7], and from the 1960 to the 1980s studies demonstrated that supplemental oxygen could decrease neonatal mortality and incidence of cerebral palsy [8–11]. However, with increasing experience,

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clinicians became more aware of the potential for side effects. Even today there are many unanswered questions about what is safe and optimal postnatal oxygen therapy.

9.2 Physiology of Oxygen Transport in Fetal and Postnatal Life

9.2.1 Oxygenation of the Fetus

A clear understanding of the physiology of oxygen transfer, oxygen carrying capacity, diffusion across the placenta and the factors that influence oxygen transfer in the fetus is important. Maternal, placental and fetal factors combine to play an important role in maintaining fetal tissue oxygenation. Evolution has in fact made some adaptations in the fetus that facilitate adequate tissue oxygenation. The fetal circulation ensures that oxygenated blood from the placenta flows from the inferior vena cava through the foramen ovale to the left atrium and ventricle to preferentially supply oxygen to the brain and heart. Less-oxygenated blood flows from the superior vena cava to the right atrium and ventricle, and the pulmonary artery and ductus arteriosus to the aorta.

In adults, each hemoglobin molecule is a tetramer composed of two α and two β chains attached to a heme moiety. In contrast, fetal hemoglobin (HbF) is a tetramer of two α and two γ chains, and newborns have between 65 and 95% of fetal haemoglobin at birth [12], which falls progressively during the first year of life [13, 14]. Due to the presence of HbF, the fetal oxyhemoglobin dissociation is left-shifted and facilitates the transfer of oxygen from the mother to the fetoplacental circulation. Moreover, as the hemoglobin binds the greater levels of carbon dioxide present in the fetus, oxygen affinity is decreased, favouring oxygen diffusion to the fetal tissues. In the intervillous circulation, the opposite effect – offloading of carbon dioxide from the fetal to the maternal circulation – promotes oxygen binding (the double Bohr effect), which can actually double the oxygen tension gradient [15].

The globin genes were among the first genes to be sequenced and analysed and have provided a paradigm for understanding gene expression, such as the structure of introns and the functions of critical regulatory elements. Fundamental questions still remain about how the characteristic patterns of tissue and developmental-stage specificity for expression of the different embryonic and fetal and adult globin genes are achieved [16]. Switching from the fetal to adult globin genes may not appear to be related to changes at birth itself, but rather to be directed by intrinsic mechanisms, or even developmental clocks. The clinical importance of understanding these observations is that slowing or reactivating fetal hemoglobin production may benefit certain groups of infants and patients [17].

Many other factors influence placental oxygen transfer and oxygen transfer to the fetus [18]. It is well known that intrauterine oxygen saturation in the human fetus is between 60 and 80% and that growth is maintained with a fetal pO_2 of 3.3 kPa [19, 20]. The fetus can adapt to a reduction of fetal and maternal blood flow by up to 50% by increasing the fractional extraction of oxygen, resulting in minimal changes in fetal oxygen uptake. Fetal hypoxia causes a redistribution of cardiac output to the brain, heart and other important organs [21–23]. Several studies suggest a critical level for fetal oxygen delivery of 6 mmol/min/kg. When oxygen delivery is reduced below this level,

fetal oxygen uptake falls, resulting in tissue hypoxia and causing acidosis [24–28]. This can be acute or chronic and many studies have shown that a fetal oxygen saturation of less than 30% for more than 10 minutes during labor indicates fetal hypoxia and results in adverse neonatal outcome and acidosis [29–31].

9.2.2 Measuring Oxygenation in the Neonate

Measuring oxygen saturations (SpO_2) (the fraction of the hemoglobin molecules in a blood sample that are saturated with oxygen at a given partial pressure of oxygen) through the use of a saturation monitor has become the standard noninvasive method of measuring oxygen levels in the neonate, in view of its ease of use and relative lack of heat-related side effects. Oxy- and deoxyhemoglobin have different absorption of infrared and red wavelengths. Oxy- and deoxyhemoglobin ratios can be measured by the pulse oximeter, using a probe positioned on either side of a pulsating arterial bed, with light-emitting-diode infrared and red light transmitted through the pulsating vascular bed to a photodetector positioned on the opposite site [32]. The major limitation of older pulse oximeters was the inability to correct any motion errors. The venous blood saturation, the magnitude of the motion and the arterial signal amplitude all contribute to the error in SpO_2 measurement. When motion is marked relative to peripheral arterial blood flow, the motion-added signal can predominate over the arterial pulse, causing a falsely low SpO_2 value to be displayed [32]. However, the advances made in this field mean that some of the new pulse oximeters are able to measure carboxyhemoglobin, methemoglobin and hemoglobin, and in addition to correct any signal-motion abnormalities using signal-extraction technology (SET) employing unique signal-processing algorithms (Masimo SET) that detect and ignore sources of SpO_2 and pulse rate (PR) interference. While the ability of such meters to detect hypoxia is no longer in doubt, there still remains a question concerning whether these monitors are able to detect hyperoxia (defined as an arterial tension of more than 80 mmHg), as it has been shown that fractional oxygen saturations of 93% are often associated with hyperoxia [33]. Oxygen can also be measured non-invasively through the transcutaneous route, although this is seldom relied upon in clinical practice [34]. In addition, the development of arterial indwelling catheters that can record oxygenation is of value in babies who have umbilical catheters.

Ultimately, what is important to neonatal care is a better and more direct understanding of tissue oxygen delivery. Many factors contribute to this, in addition to arterial oxygen saturation, including amount and type of hemoglobin, function of red cells (e.g. levels of 2,3-diphosphoglycerate) and cardiac output. Attempts to identify and target red-cell transfusions to neonates on the basis of indicators of peripheral oxygen delivery have been reported, with variable results, including the use of better measures of circulating blood volume or near-infrared spectroscopy to measure fractional oxygen extraction [35].

9.3 Oxygen Therapy in the Postnatal Period

The primary aim of oxygen therapy in preterm neonates is to maintain adequate tissue oxygenation without causing oxygen toxicity. The toxic effects of hyperoxia were first clearly established in the 1950s, when a clear link between oxygen and retinopathy of prematurity (ROP) was established. Further studies [36, 37] have demonstrated clearly

that chronic lung disease (CLD) is more prevalent in neonates where higher oxygen saturations are targeted. This section discusses the mechanisms of possible damage from oxygen.

9.3.1 Oxidative Stresses in the Newborn Period

Oxidative stress plays an important role in the pathogenesis of several neonatal disorders. Oxygen free radicals have been implicated in causing cellular damage through alteration of cell morphology and components by lipid and protein peroxidation. Oxygen free radicals include the superoxide, the hydroxyl radical and singlet oxygen, which are all produced by the mitochondria via hypoxanthine-xanthine oxidase, the p450 system, oxidation of arachidonic acid and activated phagocytes.

Oxygen free radicals cause mitochondrial injury by increasing calcium influx into the mitochondria, leading to uncoupling of respiration from ATP synthesis. The mitochondrial release of free radicals impairs the antioxidant systems and promotes mitochondrial membrane permeability, leading to cell death [38–41].

Hypoxanthine accumulates during hypoxia, and during reoxygenation superoxide radicals are produced [42, 43]. Reoxygenation causes the increased production of superoxide radicals, which lead to cell damage and death [42]. The hydroxyl radical is produced through Fenton chemistry, which might induce further oxidation, protein oxidation and DNA damage, and may also affect signal transduction [44].

Antioxidants like gluthathione and ascorbic acid protect against such stresses. Animal studies have shown that glutathione, with its antiperoxidation action, is one of the most important and abundant antioxidants. In addition to aiding in the regeneration of other antioxidants, glutathione helps in detoxification. Decreased antioxidant levels and function contribute to the reduced ability of preterm neonates to combat oxidative stress as it is only in the third trimester that the development of the antioxidant system takes place [45]. The fetal liver is not able to produce adequate amounts of glutathione in response to hyperoxia and this persists in the preterm neonate.

Saugstad [46] has hypothesized that many neonatal pathologies, such as ROP, CLD, periventricular leukomalacia (PVL), patent ductus arteriosus (PDA) and necrotizing enterocolitis (NEC), may be caused by oxidative stress in the neonate, especially the preterm neonate.

9.3.2 Clinical Sequelae of Hyperoxia

9.3.2.1 Retinopathy of Prematurity

ROP is a vasoproliferative disorder affecting preterm neonates. Prior to the introduction of cryotherapy and laser therapy it was the leading cause of childhood blindness. The relationship between oxygen and development of ROP is complex but well established [47, 48]. It is known that vasoactive endothelial growth factor (VEGF) and oxygen play an important role in the pathogenesis of this condition [34, 49]. In the premature neonate the retina is poorly vascularized and VEGF plays a crucial role in angiogenesis. Supplemental oxygen therapy after birth leads to a state of hyperoxia, which inhibits VEGF production and leads to arrest in the development of blood vessels in the retina. This leads to a relative state of hypoxia in the retina and increased production of VEGF,

which leads in turn to the unregulated production of new retinal vessels and ROP and, in the presence of persistently high levels of VEGF, fibrosis and retinal detachment. Another factor that can promote development of ROP is insulin-like growth factor (IGF). The interaction between IGF receptor 1 and VEGF helps in angiogenesis. It is known that the level of IGF1 falls in preterm birth, which leads to increased interaction between the VEGF and IGF receptor, leading on, in the presence of hyperoxia, to the development of severe ROP.

Phelps *et al*. demonstrated that targeting higher blood oxygen levels led to reduced ROP in an animal model [50, 51]. This led to the STOP ROP trial [36], a randomized controlled trial looking at the effect of higher saturation targeting and ROP. This study showed that while there was no significant difference in the incidence of ROP between the two saturation arms, there was a statistically significant difference in rates of CLD. Further studies have shown that the incidence of ROP and need for laser treatment can be significantly decreased by the use of a lower rather than higher oxygen saturation threshold [52, 53]. Tin *et al*. quantified this, showing that a higher oxygen saturation threshold of 88–98% versus 70–90% resulted in a fourfold increase in the risk of needing treatment for ROP [54].

9.3.2.2 Oxygen and Chronic Lung Disease

Bronchopulmonary dysplasia was first described in a report documenting the clinical, radiological and pathological features seen in preterm neonates with respiratory distress who were treated with high concentrations of oxygen. CLD or bronchopulmonary dysplasia is defined as the continued need for oxygen at 36 weeks post menstrual age [55]. It can also be defined as the need for oxygen at 28 days of life plus radiographic abnormalities and a history of mechanical ventilation. The overall incidence of CLD has increased since 1962–1965. It is now primarily seen in preterm babies who weigh less than 1 kg at birth and are at less than 28 weeks gestation, with a wide variation of reported incidences [56, 57]. In addition, 'new CLD' or atypical CLD is now being increasingly described. Typically these neonates will have had minimal ventilator requirements or oxygen therapies but have a progressive deterioration of their pulmonary function over time. Numerous studies have shown that CLD is associated with increased morbidity, with repeated respiratory tract infections, increased desaturations especially during feeds, poor weight gain and adverse neurodevelopment outcome [58]. CLD is therefore responsible for considerable morbidity and resource utilization in the care of such infants.

The pathogenesis of CLD is multifactorial. The primary factors that lead to CLD are prematurity, oxygen therapy (oxygen free radicals), mechanical ventilation, infection/inflammation and PDA. The relative contribution of these factors remains unknown but animal models have greatly contributed to our knowledge of the development and pathogenesis of CLD [59, 60]. Hyperoxia alone results in the pathological finding of CLD in various animal models. In term piglets it has been shown that oxygen can cause evidence of BPD, but this is greatly enhanced if oxygen is combined with hyperventilation leading to hypocarbia rather than hyperventilation alone. Studies on mice have also shown that administration of oxygen leads to the development of irreparable pulmonary injury and inhibited DNA synthesis [61]. DeLemos, in his experiment on newborn lamb lungs, showed that oxygen on its own can cause pathological damage [62].

Studies conducted in premature baboons at varying gestations have consistently shown that baboons ventilated with a higher concentration of oxygen show significantly more damage than those ventilated with lower oxygen concentrations. They also show that oxygen worsens the severity of CLD inversely proportional to the gestational age [58, 63]. Animal studies have also shown that oxygen free radicals can inhibit pulmonary surfactant function, which in turn leads to the development of CLD [64–66].

In preterm neonates, oxidative stress can cause lung damage. Increased levels of oxidative stress markers have been identified in the bronchopulmonary lavage of babies who go on to develop CLD [67]. Pitkanen *et al*. showed that the degree of lipoid peroxidation was higher in the babies who later went to have a poor outcome [68]. This was later confirmed by Varsila *et al*., who demonstrated high levels of ethane and pentane in the expired air of preterm infants in the first week of life compared to term neonates [69]. Similarly, studies conducted by Levine and Varsila have shown significantly higher levels of protein carbonylation in babies who progressed to develop CLD [70–72]. Other studies have shown elevated levels of other biochemical markers, such as allantoin and F2 isoprostanes (a product of arachidonic acid), in babies who developed CLD in the early weeks of life.

Despite such evidence, a recent Cochrane review to determine the efficacy of exogenous administration of superoxide dismutase in preterm babies at risk of developing CLD did not show sufficient evidence of reduction in CLD [73].

9.3.2.3 Oxygen and Periventricular Leukomalacia

PVL is a loss of cerebral white matter, characteristically associated with preterm infants. The development of PVL is complex and multifactorial. Cerebrovascular perfusion (impaired autoregulation in preterm infants), reperfusion injury mediated by oxygen free radicals (such as superoxide anion and hydrogen peroxide radicals), as well as fetomaternal infection and inflammation, all play important roles in its pathogenesis [74–79].

9.4 Oxygen and Resuscitation of the Newborn Infant

Physiological adaptation from a protected *in utero* environment to an *ex utero* environment at birth is rapid and complex. Usually, this adaptation is smooth and does not require any intervention. However, about 5–10% of newborns require some degree of resuscitation, ranging from simple stimulation to assisted ventilation. In 1998 the advisory statement from the International Liaison Committee on Resuscitation (ILCOR) recommended that 100% oxygen should be used in neonatal resuscitation [80]. Recent evidence however has shown that resuscitation with 100% oxygen in asphyxiated neonates causes hyperoxia and increased oxidative stress, which in turn are associated with delayed initiation of spontaneous respiration and irregularities in the cerebral circulation [81]. In light of this evidence, the ILCOR revised their guidelines in 2005 to state that excessive oxygen administration can cause tissue damage, especially in preterm infants [82]. To date, three meta-analyses have been conducted in this regard [83–85]. Although the 2005 Cochrane review did not make any recommendations for the use of room air over 100% oxygen, it did conclude, like other reviews, that a reduction in mortality was achieved with room air without evidence of harmful consequences in the short term [85].

9.5 Transfusion in the Newborn

During their stay on the neonatal unit, preterm infants receive numerous red-cell transfusions with adult donor-derived packed cells. The long-term effects on fetal oxygenation and development of replacing fetal blood with adult blood are not completely known, and it must also be appreciated that packed red cells are stored for variable periods of time in (artificial) additive solutions prior to transfusion. Human fetuses given intraperitoneal transfusions of adult red cells for erythroblastosis have survived with normal growth indices and normal acid–base values at birth [86]. Indeed, the effect of exchange transfusion may be less pronounced as the difference between fetal and maternal p50 is smaller [87]. Transfusion with adult packed cells may decrease the fetal hemoglobin content and increase the adult hemoglobin content, thereby increasing tissue oxygenation [87], and can also cause hemodynamic compromise by impairing cardiac function [88, 89]. Indeed, routine transfusion volumes on the basis of ml/kg body weight are high in neonates and newborns – it has been suggested that the 'standard' 15 ml/kg neonatal red-cell transfusion is equivalent to around three units for an adult. To prevent oxygen poisoning following blood transfusions in neonates, oxygenation status should be monitored closely, as right-shifting hemoglobin oxygen-binding curves results in more oxygen being released to the tissues [89, 90]. In view of the problems with assessing optimal oxygen delivery in neonates, and the risks related to transfusion, there has been a reevaluation of the benefit of red-cell transfusions through larger pragmatic trials.

9.6 ROP and Transfusions

Iron and blood transfusions have been implicated in the pathogenesis of ROP in numerous studies [91–94]. Blood transfusions could contribute to the development of ROP via the increase of oxygen delivery to the retina [93], secondary to the increased haematocrit and the lower oxygen affinity of adult haemoglobin. In addition, transfused cells have a shortened half-life and the iron they contain can cause iron overload. Brooks *et al*. reported that limiting packed-cell transfusions did not cause a decrease in ROP [95] but in contrast Dani *et al*. showed that blood transfusion volume and iron load by transfusion are associated with the risk of occurrence of ROP in neonates with a birth weight of less than 1250 g [96]. Two randomized controlled trials of packed-cell transfusions in neonates did not show any difference in the incidence of ROP in neonates in the liberal or restricted transfusion arms [97, 98].

9.7 Conclusion

The first reported use of oxygen in preterm neonates was in 1891. Since then, much has been learned about its beneficial and also its toxic side effects. In the fetus, maternal, placental and fetal factors combine to play an important role in maintaining tissue oxygenation. Fetal hemoglobin causes the fetal oxyhemoglobin dissociation to be left-shifted, facilitating the transfer of oxygen from the mother to the fetoplacental circulation.

The primary aim of oxygen therapy in preterm neonates is to maintain adequate tissue oxygenation without causing oxygen toxicity. Pulse oximetry has become the

standard noninvasive method of measuring oxygen levels in the neonate. In addition, the development of arterial indwelling catheters that can record oxygenation is of value in babies who have umbilical catheters.

Oxidative stress plays an important role in the pathogenesis of several neonatal disorders, such as ROP, CLD, PVL and NEC. Recent evidence has also focused on the role of oxygen in newborn resuscitation, concluding that a reduction in mortality is achieved with room air without evidence of harmful consequences in the short term. Although iron and blood transfusions have been implicated in the pathogenesis of some neonatal pathologies, such as ROP, the long-term effects of replacing fetal blood with adult blood are not completely known.

References

[1] Daily, W.J. and Smith, P.C. Mechanical ventilation of the newborn infant. I., *Curr. Probl. Pediatr*., **1**, 1–29 (1970).
[2] Smellie, W. *A Treatise on the Theory and Practice of Midwifery: Two Addenda with a Collection of Cases and Observations in Midwifery*, D. Wilson, London (1754).
[3] Priestley, J. *Experiments and Observations on Different Kinds of Air*, Johnson, London (1775).
[4] Lavoisier, A. *Traité Élémentaire de Chimie*, Paris (1789).
[5] Bonnaire, E. Des inhalations d'oxygene chez les nouveau-nes, *J. Med. (Paris)*, **3**, 312–314 (1891).
[6] Harris, M.A. and Sullivan, C.E. Sleep pattern and supplementary oxygen requirements in infants with chronic neonatal lung disease, *Lancet*, **345**, 841–842 (1995).
[7] Simakajornboon, N., Beckerman, R.C., Mack, C. *et al*. Effect of supplemental oxygen on sleep architecture and cardiorespiratory events in preterm infants, *Pediatrics*, **110**, 884–888 (2002).
[8] Avery, M. and Oppenheimer, E. Recent increase in the mortality from hyaline membrane disease, *J. Pediatr*., **57**, 553–559 (1960).
[9] McDonald, A.D. Cerebral palsy in children of very low birth weight, *Arch. Dis. Child*., **38**, 579–588 (1963).
[10] Groothuis, J.R. and Rosenberg, A.A. Home oxygen promotes weight gain in infants with bronchopulmonary dysplasia, *Am. J. Dis. Child*., **141**, 992–995 (1987).
[11] Hudak, B.B., Allen, M.C., Hudak, M.L. and Loughlin, G.M. Home oxygen therapy for chronic lung disease in extremely low-birth-weight infants, *Am. J. Dis. Child*., **143**, 357–360 (1989).
[12] Richardson, D.B., Wing, S., Lorey, F. and Hertz-Picciotto, I. Adult hemoglobin levels at birth and risk of sudden infant death syndrome, *Arch. Pediatr. Adolesc. Med*., **158**, 366–371 (2004).
[13] Stamatoyannopoulos, G. and Nienhuis, A.W. *Developmental Control of Globin Gene Expression*, Johns Hopkins University Press (1987).
[14] Stamatoyannopoulos, G., Nienhuis, A., Stamatoyannoupoulos, G. *et al*. *The Regulation of Hemoglobin Switching*, Johns Hopkins University Press, New York (1991).
[15] Metcalfe, J. Placental gas transfer, *Anesthesiology*, **26**, 460–464 (1965).
[16] Weatherall, D.J. and Clegg, J.B. *The Thalassaemia Syndromes*, Wiley-Blackwell (2001).

[17] Olivieri, N.F. and Weatherall, D.J. The therapeutic reactivation of fetal haemoglobin, *Hum. Mol. Genet*., **7**, 1655–1658 (1998).
[18] Carter, A. Placental oxygen transfer and the oxygen supply to the fetus, *Fetal Matern. Med. Rev*., **11**, 151–161 (1999).
[19] Askie, L.M, Henderson-Smart, D.J. and Ko, H. Restricted versus liberal oxygen exposure for preventing morbidity and mortality in preterm or low birth weight infants, *Cochrane Database Syst. Rev*. (1), CD001077 (2009).
[20] Low, J.A. Fetal oxygenation in normal obstetric patients, *Obstet. Gynecol*., **13**, 695–700 (1959).
[21] Itskovitz, J., LaGamma, E.F. and Rudolph, A.M. Effects of cord compression on fetal blood flow distribution and O_2 delivery, *Am. J. Physiol*., **252**, H100–H109 (1987).
[22] Jensen, A., Garnier, Y. and Berger, R. Dynamics of fetal circulatory responses to hypoxia and asphyxia, *Eur. J. Obstet. Gynecol. Reprod. Biol*., **84**, 155–172 (1999).
[23] Rurak, D.W., Richardson, B.S., Patrick, J.E. *et al*. Blood flow and oxygen delivery to fetal organs and tissues during sustained hypoxemia, *Am. J. Physiol*., **258**, R1116–R1122 (1990).
[24] Edelstone, D.I., Caine, M.E. and Fumia, F.D. Relationship of fetal oxygen consumption and acid–base balance to fetal hematocrit, *Am. J. Obstet. Gynecol*., **151**, 844–851 (1985).
[25] Edelstone, D.I., Darby, M.J., Bass, K. and Miller, K. Effects of reductions in hemoglobin-oxygen affinity and hematocrit level on oxygen consumption and acid–base state in fetal lambs, *Am. J. Obstet. Gynecol*., **160**, 820–826 (1989).
[26] Gu, W., Jones, C.T. and Parer, J.T. Metabolic and cardiovascular effects on fetal sheep of sustained reduction of uterine blood flow, *J. Physiol*., **368**, 109–129 (1985).
[27] Paulone, M.E., Edelstone, D.I. and Shedd, A. Effects of maternal anemia on uteroplacental and fetal oxidative metabolism in sheep, *Am. J. Obstet. Gynecol*., **156**, 230–236 (1987).
[28] Wilkening, R.B. and Meschia, G. Current topic: comparative physiology of placental oxygen transport, *Placenta*, **13**, 1–5 (1992).
[29] Kühnert, M., Seelbach-Göebel, B. and Butterwegge, M. Predictive agreement between the fetal arterial oxygen saturation and fetal scalp pH: results of the German multicenter study, *Am. J. Obstet.Gynecol*., **178**, 330–335 (1998).
[30] Seelbach-Göbel, B., Heupel, M., Kühnert, M. and Butterwegge, M. The prediction of fetal acidosis by means of intrapartum fetal pulse oximetry, *Am. J. Obstet. Gynecol*., **180**, 73–81 (1999).
[31] Leszczynska-Gorzelak, B., Poniedzialek-Czajkowska, E. and Oleszczuk, J. Intrapartum cardiotocography and fetal pulse oximetry in assessing fetal hypoxia, *Int. J. Gynaecol. Obstet*., **76**, 9–14 (2002).
[32] Hay, W.W., Rodden, D.J., Collins, S.M. *et al*. Reliability of conventional and new pulse oximetry in neonatal patients, *J. Perinatol*., **22**, 360–366 (2002).
[33] Castillo, A., Sola, A., Baquero. H. *et al*. Pulse oxygen saturation levels and arterial oxygen tension values in newborns receiving oxygen therapy in the neonatal intensive care unit: is 85% to 93% an acceptable range?, *Pediatrics*, **121**, 882–889 (2008).

[34] Flynn, J.T., Bancalari, E., Snyder, E.S. *et al*. A cohort study of transcutaneous oxygen tension and the incidence and severity of retinopathy of prematurity, *N. Engl. J. Med*., **326**, 1050–1054 (1992).
[35] Wardle, S.P., Garr, R., Yoxall, C.W. and Weindling, A.M. A pilot randomised controlled trial of peripheral fractional oxygen extraction to guide blood transfusions in preterm infants, *Arch. Dis. Child. Fetal Neonatal Ed*., **86**, F22–F27 (2002).
[36] The STOP-ROP Multicentre Group. Supplemental Therapeutic Oxygen for Prethreshold Retinopathy Of Prematurity (STOP-ROP), a randomized, controlled trial. I. Primary outcomes, *Pediatrics*, **105**, 295–310 (2000).
[37] Askie, L.M., Henderson-Smart, D.J., Irwig, L. and Simpson, J.M. Oxygen-saturation targets and outcomes in extremely preterm infants, *N. Engl. J. Med*., **349**, 959–967 (2003).
[38] Saugstad, O.D. Bronchopulmonary dysplasia-oxidative stress and antioxidants, *Semin. Neonatol*., **8**, 39–49 (2003).
[39] Kowaltowski, A.J., Castilho, R.F. and Vercesi, A.E. Mitochondrial permeability transition and oxidative stress, *FEBS Lett*., **495**, 112–115 (2001).
[40] Ermak, G. and Davies, K.J.A. Calcium and oxidative stress: from cell signaling to cell death, *Mol. Immunol*., **38**, 713–721 (2002).
[41] Maciel, E.N., Vercesi, A.E. and Castilho, R.F. Oxidative stress in Ca(2+)-induced membrane permeability transition in brain mitochondria, *J. Neurochem*., **79**, 1237–1245 (2001).
[42] Saugstad, O.D. and Aasen, A.O. Plasma hypoxanthine concentrations in pigs: a prognostic aid in hypoxia, *Eur. Surg. Res*., **12**, 123–129 (1980).
[43] Saugstad, O.D. and Sanderud, J. Circulatory effects of oxygen radicals. *Biomed. Biochim. Acta*, **48**, S20–S24 (1989).
[44] Wardman, P. and Candeias, L.P. Fenton chemistry: an introduction, *Radiat. Res*., **145**, 523–531 (1996).
[45] Chen, Y. and Frank, L. Differential gene expression of antioxidant enzymes in the perinatal rat lung, *Bioorg. Med. Chem*., **34**, 27–31 (1993).
[46] Saugstad, O.D. Oxidative stress in the newborn: a 30-year perspective, *Biol. Neonate*, **88**, 228–236 (2005).
[47] Smith, L.E.H. Pathogenesis of retinopathy of prematurity, *Semin. Neonatal*., **8**, 469–473 (2003).
[48] Smith, L.E., Shen, W., Perruzzi, C. *et al*. Regulation of vascular endothelial growth factor-dependent retinal neovascularization by insulin-like growth factor-1 receptor, *Nat. Med*., **5**, 1390–1395 (1999).
[49] Avery, M.E. Recent increase in mortality from hyaline membrane disease, *J. Pediatr*., **57**, 553–559 (1960).
[50] Phelps, D.L. and Rosenbaum, A.L. Effects of marginal hypoxemia on recovery from oxygen-induced retinopathy in the kitten model, *Pediatrics*, **73**, 1–6 (1984).
[51] Phelps, D.L. Reduced severity of oxygen-induced retinopathy in kittens recovered in 28% oxygen, *Pediatr. Res*., **24**, 106–109 (1988).
[52] Vanderveen, D.K., Mansfield, T.A. and Eichenwald, E.C. Lower oxygen saturation alarm limits decrease the severity of retinopathy of prematurity, *J. AAPOS*, **10**, 445–448 (2006).

[53] Chow, L.C., Wright, K.W. and Sola, A. CSMC Oxygen Administration Study Group. Can changes in clinical practice decrease the incidence of severe retinopathy of prematurity in very low birth weight infants?, *Pediatrics*, **111**, 339–345 (2003).
[54] Tin, W., Milligan, D.W., Pennefather, P. and Hey, E. Pulse oximetry, severe retinopathy, and outcome at one year in babies of less than 28 weeks gestation, *Arch. Dis. Child. Fetal Neonatal Ed*., **84**, F106–F110 (2001).
[55] Shennan, A.T., Dunn, M.S., Ohlsson, A. *et al*. Abnormal pulmonary outcomes in premature infants: prediction from oxygen requirement in the neonatal period, *Pediatrics*, **82**, 527–532 (1988).
[56] Corcoran, J.D., Patterson, C.C., Thomas, P.S. and Halliday, H.L. Reduction in the risk of bronchopulmonary dysplasia from 1980–1990: results of a multivariate logistic regression analysis, *Eur. J. Pediatr*., **152**, 677–681 (1993).
[57] Parker, R.A., Lindstrom, D.P. and Cotton, R.B. Improved survival accounts for most, but not all, of the increase in bronchopulmonary dysplasia, *Pediatrics*, **90**, 663–668 (1992).
[58] Coalson, J.J., Winter, V.T., Siler-Khodr, T. and Yoder, B.A. Neonatal chronic lung disease in extremely immature baboons, *Am. J. Respir. Crit. Care Med*., **160**, 1333–1346 (1999).
[59] Smith, J.L. The pathological effects due to increase of oxygen tension in the air breathed, *J. Physiol*., **24**, 19–35 (1899).
[60] Northway, W.H., Rosan, R.C., Shahinian, L. *et al*. Radiologic and histologic investigation of pulmonary oxygen toxicity in newborn guinea pigs, *Invest. Radiol*., **4**, 148–155 (1969).
[61] Bonikos, D.S., Bensch, K.G. and Northway, W.H. Oxygen toxicity in the newborn: reversal of inhibition of DNA synthesis in the mouse, *Pediatrics*, **57**, 41–46 (1976).
[62] DeLemos, R., Wolfsdorf, J., Nachman, R. *et al*. Lung injury from oxygen in lambs: the role of artificial ventilation, *Anesthesiology*, **30**, 609–618 (1969).
[63] Delemos, R.A., Coalson, J.J., Gerstmann, D.R. *et al*. Oxygen toxicity in the premature baboon with hyaline membrane disease, *Am. Rev. Respir. Dis*., **136**, 677–682 (1987).
[64] Holm, B.A., Matalon, S., Finkelstein, J.N. and Notter, R.H. Type II pneumocyte changes during hyperoxic lung injury and recovery, *J. Appl. Physiol*., **65**, 2672–2678 (1988).
[65] Beckman, J.S., Beckman, T.W., Chen, J. *et al*. Apparent hydroxyl radical production by peroxynitrite: implications for endothelial injury from nitric oxide and superoxide, *Proc. Natl Acad. Sci. U S A*, **87**, 1620–1624 (1990).
[66] Haddad, I.Y., Ischiropoulos, H., Holm, B.A. *et al*. Mechanisms of peroxynitrite-induced injury to pulmonary surfactants, *Am. J. Physiol*., **265**, L555–L564 (1993).
[67] Ogihara, T., Kim, H.S., Hirano, K. *et al*. Oxidation products of uric acid and ascorbic acid in preterm infants with chronic lung disease, *Biol. Neonate*, **73**, 24–33 (1998).
[68] Pitkänen, O.M., Hallman, M. and Andersson, S.M. Correlation of free oxygen radical-induced lipid peroxidation with outcome in very low birth weight infants, *J. Pediatr*., **116**, 760–764 (1990).
[69] Varsila, E., Pitkänen, O., Hallman, M. and Andersson, S. Immaturity-dependent free radical activity in premature infants, *Pediatr. Res*., **36**, 55–59 (1994).

[70] Varsila, E., Hallman, M. and Andersson, S. Free-radical-induced lipid peroxidation during the early neonatal period, *Acta Paediatr*., **83**, 692–695 (1994).
[71] Gladstone, I.M. and Levine, R.L. Oxidation of proteins in neonatal lungs, *Pediatrics*, **93**, 764–768 (1994).
[72] Varsila, E., Pesonen, E. and Andersson, S. Early protein oxidation in the neonatal lung is related to development of chronic lung disease, *Acta Paediatr*., **84**, 1296–1299 (1995).
[73] Suresh, G.K., Davis, J.M. and Soll, R.F. Superoxide dismutase for preventing chronic lung disease in mechanically ventilated preterm infants, *Cochrane Database Syst. Rev*. (1), CD001968 (2001).
[74] Armstead, W.M., Mirro, R., Busija, D.W. and Leffler, C.W. Postischemic generation of superoxide anion by newborn pig brain, *Am. J. Physiol*., **255**, H401–H403 (1988).
[75] Palmer, C., Towfighi, J., Roberts, R.L. and Heitjan, D.F. Allopurinol administered after inducing hypoxia-ischemia reduces brain injury in 7-day-old rats, *Pediatr. Res*., **33**, 405–411 (1993).
[76] Pourcyrous, M., Leffler, C.W., Bada, H.S. *et al*. Brain superoxide anion generation in asphyxiated piglets and the effect of indomethacin at therapeutic dose, *Pediatr. Res*., **34**, 366–369 (1993).
[77] Dammann, O., Kuban, K.C.K. and Leviton, A. Perinatal infection, fetal inflammatory response, white matter damage, and cognitive limitations in children born preterm, *Ment. Retard. Dev. Disabil. Res. Rev*., **8**, 46–50 (2002).
[78] Kadhim, H., Tabarki, B., Verellen, G. *et al*. Inflammatory cytokines in the pathogenesis of periventricular leukomalacia, *Neurology*, **56**, 1278–1284 (2001).
[79] Volpe, J.J. Neurobiology of periventricular leukomalacia in the premature infant, *Pediatr. Res*., **50**, 553–562 (2001).
[80] Kattwinkel, J., Niermeyer, S., Nadkarni, V. *et al*. Resuscitation of the newly born infant: an advisory statement from the Pediatric Working Group of the International Liaison Committee on Resuscitation, *Resuscitation*, **40**, 71–88 (1999).
[81] Vento, M., Asensi, M., Sastre, J. *et al*. Resuscitation with room air instead of 100% oxygen prevents oxidative stress in moderately asphyxiated term neonates, *Pediatrics*, **107**, 642–647 (2001).
[82] International Liaison Committee on Resuscitation. Part 7. Neonatal resuscitation, *Resuscitation*, **67**, 293–303 (2005).
[83] Saugstad, O.D., Ramji, S. and Vento, M. Resuscitation of depressed newborn infants with ambient air or pure oxygen: a meta-analysis, *Biol. Neonate*, **87**, 27–34 (2005).
[84] Rabi, Y., Rabi, D. and Yee, W. Room air resuscitation of the depressed newborn: a systematic review and meta-analysis, *Resuscitation*, **72**, 353–363 (2007).
[85] Tan, A., Schulze, A., O'Donnell, C.P.F. and Davis, P.G. Air versus oxygen for resuscitation of infants at birth, *Cochrane Database Syst. Rev*. (2), CD002273 (2005).
[86] Novy, M.J., Frigoletto, F.D., Easterday, C.L. *et al*. Changes in umbilical-cord blood oxygen affinity after intrauterine transfusions for erythroblastosis, *N. Engl. J. Med*., **285**, 589–595 (1971).

[87] Itskovitz, J., Goetzman, B.W., Roman, C. and Rudolph, A.M. Effects of fetal-maternal exchange transfusion on fetal oxygenation and blood flow distribution, *Am. J. Physiol*., **247**, H655–H660 (1984).
[88] Hay, W.W. The uses, benefits, and limitations of pulse oximetry in neonatal medicine: consensus on key issues, *J. Perinatol*., **7**, 347–349 (1987).
[89] James, L., Greenough, A. and Naik, S. The effect of blood transfusion on oxygenation in premature ventilated neonates, *Eur. J. Pediatr*., **156**, 139–141 (1997).
[90] DeHalleux, V., Truttmann, A., Gagnon, C. and Bard, H. The effect of blood transfusion on the haemoglobin oxygen dissociation curve of very early preterm infants during the first week of life, *Semin. Perinatol*., **26**, 411–415 (2002).
[91] Sullivan, J.L. Iron, plasma antioxidants, and the 'oxygen radical disease of prematurity, *Am. J. Dis. Child*., **142**, 1341–1344 (1988).
[92] Levine, R. L. Ischemia: from acidosis to oxidation, *FASEB J*., **7**, 1242–1246 (1993).
[93] Cooke, R.W., Clark, D., Hickey-Dwyer, M. and Weindling, A.M. The apparent role of blood transfusions in the development of retinopathy of prematurity, *Eur. J. Pediatr*., **152**, 833–836 (1993).
[94] Sacks, L.M., Schaffer, D.B., Anday, E.K. *et al*. Retrolental fibroplasia and blood transfusion in very low-birth-weight infants, *Pediatrics*, **68**, 770–774 (1981).
[95] Brooks, S.E., Marcus, D.M., Gillis, D. *et al*. The effect of blood transfusion protocol on retinopathy of prematurity: a prospective, randomized study, *Pediatrics*, **104**, 514–518 (1999).
[96] Dani, C., Reali, M.F., Bertini, G. *et al*. The role of blood transfusions and iron intake on retinopathy of prematurity, *Early Hum. Dev*., **62**, 57–63 (2001).
[97] Kirpalani, H., Whyte, R.K., Andersen, C. *et al*. The Premature Infants in Need of Transfusion (PINT) study: a randomized, controlled trial of a restrictive (low) versus liberal (high) transfusion threshold for extremely low birth weight infants, *J. Pediatr*., **149**, 301–307 (2006).
[98] Bell, E.F., Strauss, R.G., Widness, J.A. *et al*. Randomized trial of liberal versus restrictive guidelines for red blood cell transfusion in preterm infants, *Pediatrics*, **115**, 1685–1691 (2005).

10
Ischemia

Hooman Mirzakhani and Ala Nozari

Department of Anesthesia, Critical Care and Pain Medicine, Massachusetts General Hospital, Harvard Medical School, Boston, MA, USA

10.1 Introduction

Ischemia is a perturbation of the blood flow to the tissues, resulting in an insufficient supply of oxygen and energy substrates. The depletion of energy-rich substrates during ischemia can rapidly cause parenchymal injury through a necrotic pathway [1], or under certain circumstances, through apoptosis [2] or necrapoptotic cell death [3]. The severity of injury is primarily determined by the duration and intensity of ischemia [4, 5]. Therefore, it is prudent to restore tissue perfusion and oxygen supply to the ischemic tissue to prevent or mitigate the progression of the injury. Restoration of circulation may, nevertheless, provoke a series of postischemic circulatory and metabolic disturbances, referred to as the reperfusion injury (see Chapter 5). The outcome after an ischemic insult is therefore determined not only by the severity and duration of the ischemia, but also by the extent of reperfusion injury. Clinically, ischemic and reperfusion injuries are commonly encountered during myocardial infarction or stroke, or after organ transplantation, thrombolysis and revascularization procedures, aortic surgery, heart surgery, cardiac arrest and cardiopulmonary resuscitation, and hemodynamic instability and shock.

10.2 Pathophysiology

10.2.1 Energy Failure

Disruption of the oxidative phosphorylation and energy production that follows ischemia results in a rapid depletion of the cellular energy-rich substances, including

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phosphocreatine, ATP, and other nucleoside triphosphates (CTP, UTP, and GTP) [6, 7]. Anaerobic pathways, in particular the glycolytic pathway, then come into play (Figure 10.1). The glycolitic pathway produces hydrogen and lactate ions, which, in contrast to the end-products of aerobic metabolism – water and carbon dioxide – are not easily removed from the cells and body. As a consequence, the cytosolic pH decreases, which further limits ATP production by inhibiting the rate-limiting enzyme 6-phosphofructokinase. The fall in cellular energy leads to an impaired function of active ion pumps, including sodium–potassium and sodium–calcium exchange pumps, and an upsurge in the cytosolic sodium and calcium concentrations. An influx of sodium and calcium ions is accompanied by a passive flow of water, cytotoxic edema, and cell rupture, with the release of cytosol into the interstitial space [8, 9]. Dilation of the endoplasmic reticulum (one of the first ultrastructuaral changes evident in the reversible ischemic injury) causes detachment of the ribosomes and reduction of protein synthesis [10]. The disaggregation of ribosomes is also promoted by membrane damage. Vacuolization within the cytoplasm (which characterizes the hydropic change) and mitochondrial swelling occur if oxygen is not restored.

10.2.2 Cell Membrane Damage

Separating the interior of a cell from the outside environment, the cell membrane is selectively permeable to ions and organic molecules, and controls the movement of these important substances. Hypoxic injury damages the plasma, lysosomal, and other organelle membranes, with loss of membrane phospholipids. Morphologically, myelin figures (whorl-like structures originating from damaged membranes) and cell blebs (cell-surface deformities caused by disorderly function of the cellular cytoskeleton) may be seen, but the appearance of these structures does not indicate irreversible cellular injury.

10.2.3 Increased Cytosolic Calcium

Calcium is an important intracellular ion that mediates multiple cellular functions. In normal cells, it is bound to buffering proteins such as calbindin or paralbumin, and is contained in endoplasmic reticulum and the mitochondria. The cytosolic concentration of calcium is significantly lower than the extracellular environment. This concentration gradient is maintained by several mechanisms, including active extrusion from the cell by an ATP-driven membrane pump [11], exchange of calcium for sodium by ATP-dependent calcium pumps (Na^+/Ca^{2+} antiporter), sequestration in the endoplasmic reticulum by an ATP-driven process [12], and accumulation inside the mitochondria by an oxidation-dependent calcium-sequestration process [13]. Consequently, ATP depletion in ischemic cells leads to the disruption of the intracellular calcium homeostasis and intracellular shift of calcium into the cytosol, as well as its release from the endoplasmic reticulum (Figure 10.2). This change in the intracellular calcium concentration activates several enzyme systems, such as phospholipases, protein kinases, proteases, and nucleases [9, 14, 15]. Phospholipases (e.g. phospholipase-A2) break down membrane lipids (membrane damage by phospholipid degredation and loss) and release arachidonic acid metabolites. Protein kinases activate other enzymes such as NO synthase and xanthine oxidase, ultimately leading to the phosphorylation of proteins and chromatin fragmentation. Proteases break down the cell skeleton (cytoskeletal disassembly)

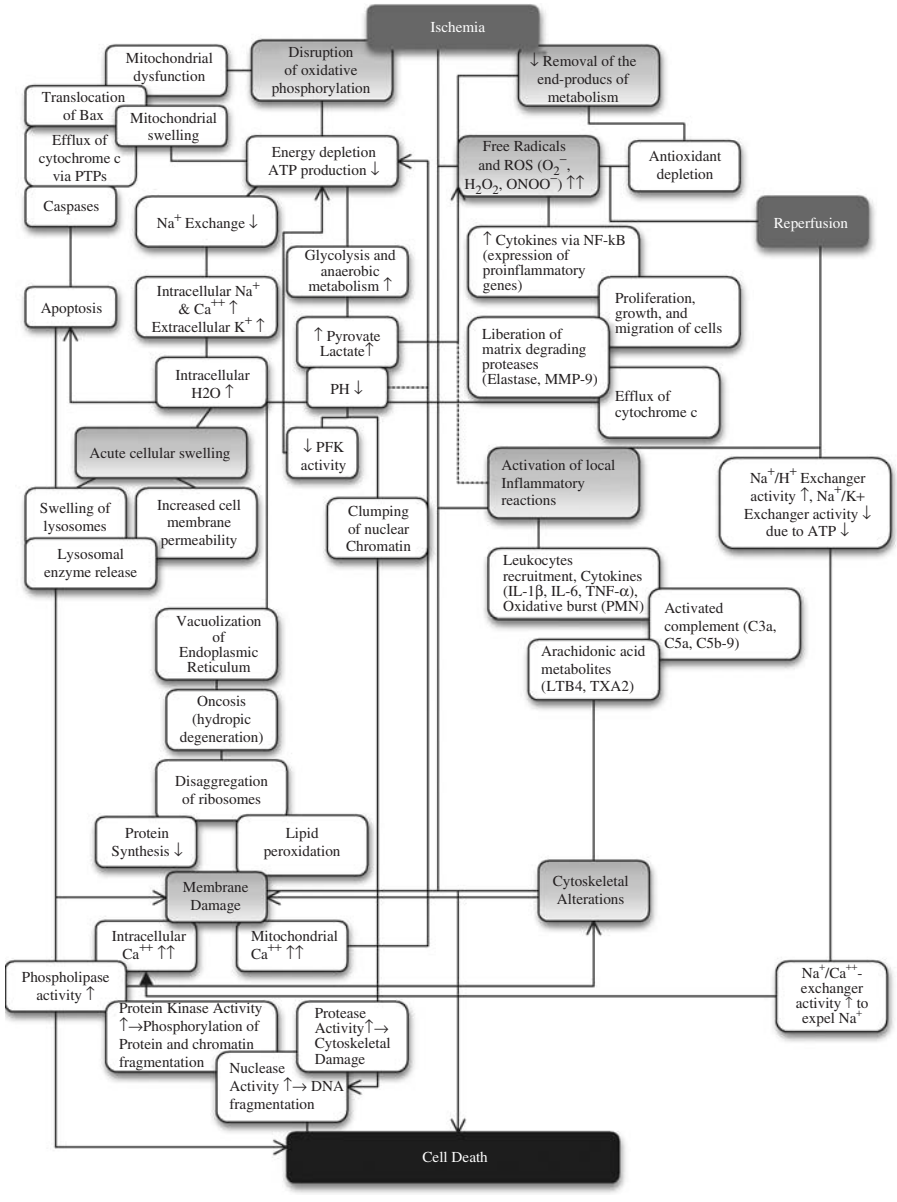

Figure 10.1 *Pathophysiology of ischemic injury. Ischemia-induced depletion of cellular energy provokes a cascade of pathophysiological reactions, leading to the failure of cellular ion pumps and an influx of sodium and calcium ions into the cell, an increase in cytosolic pH, cellular swelling, and cytoskeletal and membrane damage. ATP, adenosine triphosphate; PFK, phosphofructokinase; ROS, reactive oxygen species; H_2O, hydrogen oxide; H_2O_2, hydrogen peroxide; bax, Bcl-2-associated X protein; TXA2, thromboxane A2; C3a, C5a, complement proteins 3a and 5a; C5b-9, terminal C5b-9 complement complexes; LBT4, leukotrien B4; PMN, polymorphonuclear granulocytes; TNF-α, tumor necrosis factor-α; IL, interleukin; MMP-9, matrix metalloproteinase-9; NFkB, nuclear factor kB; O_2^-, superoxide anion; $ONOO^-$, peroxynitrite; PTP, permeability transition pore.*

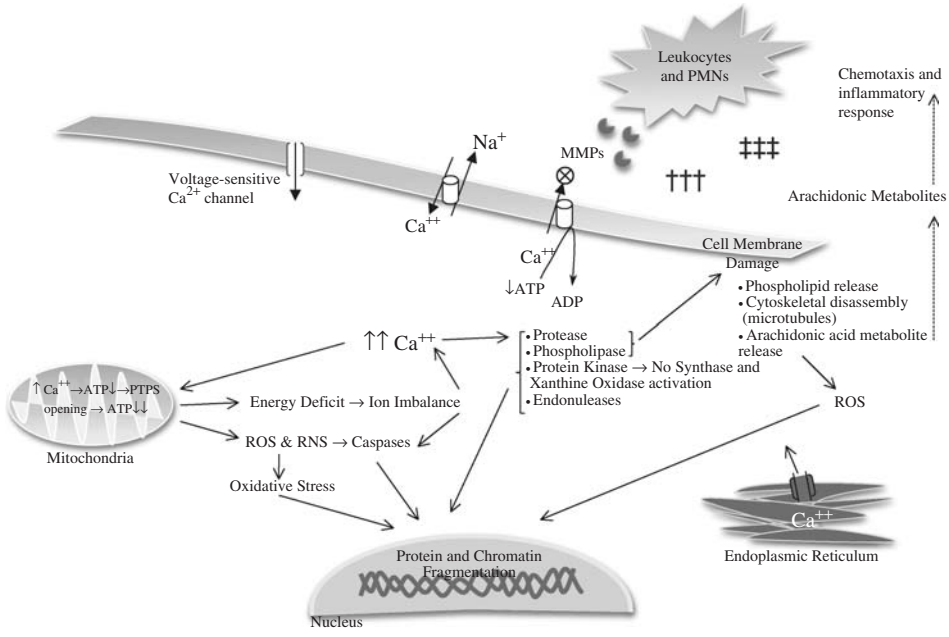

Figure 10.2 *The central role of cytosolic calcium in the pathophysiology of ischemic injury. The influx of Ca^{2+} across the cellular membrane, as well as its release from the sarcoplasmic reticulum, leads to a rapid buildup within the cytoplasm. It then activates several intracellular enzymes and can lead to the opening of the mitochondrial PTP pores. An increased oxidative stress promotes DNA damage and augments membrane injury. Local expression of pro-inflammatory stimuli and release of chemotactic factors provoke neutrophil and leukocyte appearance and release of cytotoxic factors.*

and endonucleases initiate the destruction of the cellular DNA (nucleus chromatin damage). Moreover, calcium buildup in the mitochondria induces the opening of the mitochondrial permeability-transition pores (PTPs), leading to voltage drop across the mitochondrial membrane and repealed ATP production. PTPs are multiprotein complexes that form nonselective pores in the inner mitochondrial membrane. PTP opening causes immediate depolarization of mitochondrial membrane potential and interruption of ATP production, water entry into the matrix, and outer mitochondrial membrane rupture, with the release of pro-apoptotic molecules, such as cytochrome *c*, apoptosis inducing factor, Smac/DIABLO, and APAF-1 [16].

10.2.4 Inflammation

Polymorphonuclear leukocytes, monocytes, and macrophages have been implicated as significant contributors to the injury after ischemia. Local expression of pro-inflammatory stimuli, such as cytokines, chemokines, and growth factors, and an upregulation of cell-adhesion molecules on endothelial cells, platelets, or leukocytes, induces leukocyte rolling and adherence to the endothelial cells. This is followed by neutrophil activation, occlusion of microvasculature, release of cytotoxic enzymes, and alteration of vasomotor

reactivity [17, 18]. Activated leukocytes generate large amounts of hydrogen peroxide. A large fraction of the hydrogen peroxide reacts with the halides Cl^-, Br^-, or I^- to produce their corresponding hypohalous acids [19]. Hypochlorous acid and other hypohalous acids are capable of damaging a wide range of organic molecules, including the cytoskeleton and the proteinaceous extracellular matrix [20]. Moreover, activated leukocytes can adhere to the microvascular endothelium of postcapillary venules [21, 22], and contribute to the manifestation of postischemic injury by plugging the microvessels, leading to the no-reflow phenomenon.

10.2.5 The No-reflow Phenomenon

Tissue reperfusion after ischemia is often characterized by localized or confluent areas of impaired vascular filling, referred to as the no-reflow phenomenon [23–25]. The production of chemical mediators, as described above, leads to the expression of adhesion molecule on the endothelial cells or polymorphonuclear leukocytes. Leukocytes adhere then to the microvascular endothelium and emigrate to the vascular tissue, releasing inflammatory mediators and cytotoxic enzymes [26–28]. The severity of no-reflow impairment depends on the duration of ischemia, the extent of hemorheological abnormalities, and reactivation of the coagulation system [24, 29–31]. High perfusion pressures, in combination with hemodilution through the administration of hypertonic solutions and the correction of systemic acidosis, have been suggested as potential treatments for this circulatory disorder [32].

10.2.6 Free Radicals and Reactive Oxygen Species

The important role of reactive oxygen species (ROS) and reactive nitrogen species (RNS) has been described in detail in Chapter 5. Under normal physiological conditions of respiration, aerobic cells produce the superoxide anion (O_2^-) by enzymes such as NADPH oxidases and xanthine oxidase, or non-enzymatically by redox reactive compounds such as the semiubiquinone compound of the mitochondrial electron transport chain. During ischemia, increased intracellular calcium enhances the conversion of xanthine dehydrogenase to xanthine oxidase. Upon reperfusion and reintroduction of oxygen, xanthine oxidase may produce superoxide and xanthine from hypoxanthine and oxygen [26, 33]. Production of the radicals may also occur via the ubiquinone cycle in the mitochondrial inner membrane and by excitatory amino-acid stimulation of arginin to nitric oxide formation [34, 35]. Superoxide dismutase (SOD) converts superoxide into hydrogen peroxide, which can react with cellular metals, particularly Fe^{2+} or Cu^+ ions, to produce the highly reactive hydroxyl radical (•OH) via the Fenton reaction [36]. The transition metals needed to drive this reaction are present in abundant quantities in bound form in living systems in the form of cytochromes, transferrin, and hemoglobin (Hb), and are released during anaerobic conditions [37–39]. Moreover, O_2^- may be formed by the actions of xantine oxidase and released from activated neutrophils, as described above. Oxygen radicals are destructive to cellular components, can impair capillary endothelial cell mechanisms that help maintain homeostasis of electrolytes and water [40], and appear to contribute to synaptic damage [41]. Furthermore, restoration of ATP levels during reperfusion allows active uptake of calcium by the mitochondria, with the consequence of massive calcium overload and destruction of the mitochondria [42].

10.2.7 Excitotoxicity

Excitotoxicity is recognized as a fundamental pathway of irreversible cellular injury, and involves the activation of glutamate receptors in the central nervous system [43]. Glutamate is an important neurotransmitter and the most abundant excitatory neurotransmitter in the vertebrate nervous system. It acts on several types of receptor on postsynaptic neurons, including N-methyl-D-aspartate (NMDA), ∝-amino-3-hydroxy-5-methyl-4-isoxazole propionic acid (AMPA), and kainate receptor, which directly control sodium and calcium ion channels, as well as the metabotropic receptors linked to the G-proteins that regulate such intracellular second messengers as guanosine-3,5-cyclic monophosphate (cGMP) [44]. The areas of the brain that show the most "selective vulnerability" to ischemia – the neocortex and hippocampus – are richly endowed with excitatory AMPA and NMDA receptors [45]. It has been suggested that blockade of the NMDA receptors can provide protection against delayed neuronal death following cerebral ischemia [46, 47]. Indeed, NMDA receptor-blocking drugs such as dextrorphan and MK-801 have been shown to ameliorate focal cerebral ischemic injury in experimental studies [48, 49]. However, restoration of blood flow after ischemia leads to a rapid inactivation of the NMDA receptors [50], limiting the potential role of the NMDA receptors in modulating the injury. Despite positive outcome data in rodents, more recent dog and primate studies have failed to show significant outcome benefit when NMDA-receptor inhibitors were used in global cerebral ischemia [51, 52].

10.3 Therapeutic Potentials

10.3.1 Preconditioning

Recent efforts to identify techniques for mitigation of the ischemic injury have led to the interesting treatment approaches of ischemic conditioning, or hypoxic conditioning. Exposure of tissues to short, intermittent ischemic stimuli has been shown to protect against subsequent damage from a similar, but more severe insult. Animal studies suggest that a preconditioning insult of 1 minute or longer induces an early and transient protective effect, followed by a second window of protection that can last up to 3–4 days. The protective mechanism appears to involve an upregulation of the transcription factor hypoxia-inducible factor (HIF). HIF stimulates the transcription of distinct subsets of genes, whose promoters include hypoxia-response elements such as erythropoietin and vascular endothelial growth factor [53]. Products of these genes mediate the restoration of tissue oxygenation and limit tissue damage.

Despite promising results from experimental studies, clinical application of preconditioning has been limited by several factors, including the timing of these interventions. Preconditioning needs to be applied before the prolonged "index" ischemia, which is often not an option in the clinical setting. Nevertheless, recent studies have shown that tissue salvage can similarly be achieved when the ischemic tissue is "postconditioned" by cycles of briefly interrupted perfusion during the early moments of reflow [54]. Clinically, postconditioning is of greater value, since it can be applied *after* a patient presents with organ ischemia, for example after acute myocardial infarction or stroke. Clinical use of ischemic postconditioning is nevertheless controversial, because it can potentially

aggravate the ischemic insult. Instead, it has been suggested that a non-ischemic organ or tissue be subjected to postconditioning in these patients. As an example, one extremity can be subjected to ischemic cycles in a patient who presents with symptoms of myocardial infarction. This approach is known as "remote ischemic postconditioning" and has the advantage that the ischemic organ is not subjected to additional insults. However, the effectiveness of this approach has as yet not been substantiated.

10.3.2 Antioxidants

As described above, ROS play an important role in the pathophysiology of ischemic injury. ROS can initiate several detrimental pathways, including apoptosis, mitochondrial permeability transition, and poly(ADP-ribose) polymerase activation for oxidative damage, and therefore provide new targets for postischemic therapeutic intervention. The major enzymatic defences against oxidative stress are provided by SOD, glutathione peroxidase, and catalase. SOD catalyzes the dismutation of O_2^- to H_2O_2 and O_2. Glutathione peroxidase and catalase, in turn, convert the potentially toxic H_2O_2 to H_2O and O_2. In animal models of myocardial ischemia, SOD and catalase have been shown to reduce the contractile dysfunction [55] but not the myocardial infarct size [56]. Despite abundant experimental evidence that these substances can mitigate the ischemic injury, no effect on outcome from human studies has been reported [57–59].

10.3.3 Anti-inflammation Therapy

Interventions aimed at decreasing the postischemic inflammation provide an attractive therapeutic strategy in humans. While animal studies provide promising results in leukocyte anti-adhesive strategy, inhibiting leukocyte–endothelial interactions has not been successful in clinical trials [60–62]. Anti-adhesive therapies may indeed improve the postischemic reperfusion, but these agents can potentially also increase the influx of ROS, resulting in the activation of deleterious oxidative stress-sensitive inflammatory pathways [63]. Therefore, treatments aimed at mitigating the oxidative stress may be considered in addition to anti-inflammatory treatments in these patients. Similarly, other pluripotent treatments with effects on both inflammation and oxidative injury (e.g. therapeutic hypothermia) may prove helpful in the search for effective techniques to mitigate the reperfusion injury.

10.3.4 Therapeutic Hypothermia

Pioneering works by Bigelow, Dripper, Safar, and Negovski in the 1950s and 1960s documented the neuroprotective effects of hypothermia, if it was induced *before* an ischemic insult. Resuscitative hypothermia – hypothermia induced *after* ischemia – was nevertheless not generally accepted until its safety and outcome benefits were established in survivors from cardiac arrest [64, 65]. Despite clear evidence of improved neurological outcome, clinicians are often cautioned against routine application of therapeutic hypothermia because of the potential for deleterious effects on various organ systems and physiological functions. As an example, a reduction in platelet count and platelet dysfunction is noted when the body temperature is lowered [66]. When the temperature is decreased below 33 °C, the synthesis and kinetics of clotting enzymes and

plasminogen activator inhibitors are also affected [67]. The pathophysiological response to hypothermia is modulated by numerous variables, including the presence of defensive mechanisms (shivering and increased metabolism), the extent of cooling (mild, moderate or profound hypothermia), the timing of its induction (before or after the ischemic insult), the rate of its induction and reversal, and the presence of associated injuries.

The protective mechanism of therapeutic hypothermia is complex and involves several different pathways. Decreased metabolism is probably an important effect of hypothermia. It has also been shown that hypothermia preserves cellular ATP levels, reduces acidosis and excitatory neurotransmitters, and modulates the activity of numerous enzymes (the Arrhenius effect). The postischemic inflammatory response is also mitigated, as is the extent of cellular edema and the blood–brain barrier disruption. An increasing body of evidence suggests that therapeutic hypothermia can improve the outcome after hemorrhagic shock, traumatic brain injury, stroke, spinal cord injuries, myocardial infarction, and status epilepticus.

10.3.5 Hydrogen Sulfide

A colorless, toxic, and flammable gas, hydrogen sulfide has been shown to possess anti-inflammatory and organ-protective effects when administered in low concentrations. It has been reported to induce a suspended animation-like state in mice, characterized by hypothermia, a reduction in respiratory and heart rates, and decreased metabolism [68, 69]. Animal experiments have also documented survival benefit as well as an improved myocardial and neurological outcome when hydrogen sulfide is administered after cardiac arrest [70]. The mechanisms through which hydrogen sulfide exerts its protective effects are not fully understood, but recent data indicate that it can attenuate the oxidative stress and may inhibit the mitochondrial permeability transition through a nitric oxide synthase 3-dependent mechanism [70].

10.3.6 Hyperoxia and Hyperbaric Oxygen

The application of normobaric and hyperbaric oxygen for ischemia is discussed in detail in Chapter 11. Many animal and human studies suggest that these treatments can salvage ischemic tissue and extend the time window during which therapeutic measures can be applied. Using a dog model of cardiac arrest, Rosenthal and associates documented an improved neurological outcome when animals were subjected to 2.7 atmospheres absolute for 1 hour [71]. Similarly, neurological and electrophysiological outcomes were better when dogs were treated with 3 atmospheres absolute after 15 minutes of global cerebral ischemia [72]. In a pilot study of 16 patients, Singhal and associates documented improved clinical features and MRI parameters with normobaric oxygen after hemispheric ischemic stroke [73]. Despite theoretical concerns regarding oxygen toxicity, no clinical or radiological evidence of side effects was identified in this study. The authors therefore suggest that stroke patients may benefit from receiving normobaric oxygen in the field, followed by hyperbaric oxygen upon arrival to the hospital [74].

10.3.7 Hemoglobin-based Oxygen Carriers

Recent studies have demonstrated that plasma can perfuse to an ischemic tissue despite impairment of the microcirculation [75, 76]. Delivery of soluble oxygen is

nevertheless very limited unless hyperbaric oxygen is used. Hemoglobin-based oxygen carriers (HBOCs) have been suggested as alternative carriers of oxygen to the ischemic tissue, because of their beneficial rheological properties and their small size (diameter in the nanometers) relative to the red blood cells (8 μm). Moreover, HBOCs with a lower p50 have a left-shifted oxygen dissociation curve, which is favorable for delivering oxygen to ischemic tissue [77, 78]. A lower viscosity is also expected to contribute to improvement of microcirculation. Indeed, in a rat model of middle cerebral artery occlusion, Kawaguchi and associates showed that liposome-encapsulated Hb reduces the size of cerebral cortical infarction. Similarly, a recent publication reported improved myocardial oxygenation and left-ventricular function after administration of the hemoglobin-based oxygen carrier HBOC-201 in a pig model of coronary artery occlusion [79]. Clinical application of HBOCs has nevertheless been hampered by their noxious side effect of induced vasoconstriction [80]. This is likely caused by scavenging of the endothelium-derived nitric oxide by cell-free Hb [81, 82]. Strategies to minimize the scavenging of nitric oxide have been developed and include crosslinking or encapsulating of the Hb (see Chapter 22). Administration of inhaled nitric oxide can also prevent systemic vasoconstriction [83]. Despite positive animal data, a safety trial in 85 patients with acute ischemic stroke showed an increased rate of adverse events and death, as well as a worse neurologic outcome when the HBOC DCLHb was administered [84]. Similarly, a study of 209 patients after cardiac surgery showed decreased cardiac output and an increased incidence of hepatic and renal complications when DCLHb was administered [85]. These studies highlight the importance of identifying underlying mechanisms for neuronal and cardiac toxicity by these compounds in the assessment of newer-generation HBOCs.

10.4 Conclusion

Ischemia leads to a rapid depletion of cellular energy, and provokes injury through a complex pathophysiological network. Pleiotropic approaches aimed at restoring tissue oxygenation, as well as mitigating the postischemic cascade of cellular injury, should be considered to restore the disturbed cellular homeostasis. Preconditioning, therapeutic hypothermia, anti-inflammation, and antioxidant therapies bear promise for improving the outcome after ischemia. A pleiotropic approach to treating ischemia may also include hydrogen sulfide, hyperoxia, and HBOCs. Studies aimed at identifying the pathophysiology of ischemia are warranted.

References

[1] Jaeschke, H. and Lemasters, J.J. Apoptosis versus oncotic necrosis in hepatic ischemia/reperfusion injury, *Gastroenterology*, **125**, 1246–1257 (2003).

[2] Kerkweg, U., Li, T., de Groot, H. and Rauen, U. Cold-induced apoptosis of rat liver cells in University of Wisconsin solution: the central role of chelatable iron, *Hepatology*, **35**, 560–567 (2002).

[3] El-Gibaly, A.M., Scheuer, C., Menger, M.D. and Vollmar, B. Improvement of rat liver graft quality by pifithrin-alpha-mediated inhibition of hepatocyte necrapoptosis, *Hepatology*, **39**, 1553–1562 (2004).

[4] Abramson, N.S., Safar, P., Detre, K.M. *et al*. Brain Resuscitation Clinical Trial I Study Group. Neurologic recovery after cardiac arrest: effect of duration of ischemia, *Crit. Care Med*., **13**, 930–931 (1985).
[5] Prough, D.S. and Zornow, M.H. Why is cardiac arrest lasting more than five minutes associated with poor neurologic outcome?, *Crit. Care Med*., **27**, 1398–1400 (1999).
[6] Norberg, K., Quistorff, B. and Siesjo, B.K. Effects of hypoxia of 10–45 seconds duration on energy metabolism in the cerebral cortex of unanesthetized and anesthetized rats, *Acta Physiol. Scand*., **95**, 301–310 (1975).
[7] Reichelt, K.L. The chemical basis for the intolerance of the brain to anoxia, *Acta Anaesthesiol. Scand. Suppl*., **29**, 35–46 (1968).
[8] Hossmann, K. Development and resolution of ischemic brain swelling, in *Dynamics of Brain Edema* (ed. H.M. Papius), Springer-Verlag, Berlin, pp. 219–227 (1976).
[9] Siesjo, B.K., Katsura, K., Zhao, Q. *et al*. Mechanisms of secondary brain damage in global and focal ischemia: a speculative synthesis, *J. Neurotrauma*, **12**, 943–956 (1995).
[10] Swann, J.D., Smith, M.W., Phelps, P.C. *et al*. Oxidative injury induces influx-dependent changes in intracellular calcium homeostasis, *Toxicol. Pathol*., **19**, 128–137 (1991).
[11] Carafoli, E. and Crompton, M. The regulation of intracellular calcium by mitochondria, *Ann. N Y Acad. Sci*., **307**, 269–284 (1978).
[12] Blaustein, M.P., Ratzlaff, R.W. and Kendrick, N.K. The regulation of intracellular calcium in presynaptic nerve terminals, *Ann. N Y Acad. Sci*., **307**, 195–212 (1978).
[13] Mitchell, P. and Moyle, J. Chemiosmotic hypothesis of oxidative phosphorylation, *Nature*, **213**, 137–139 (1967).
[14] Siesjo, B.K. Historical overview: calcium, ischemia, and death of brain cells, *Ann. N Y Acad. Sci*., **522**, 638–661 (1988).
[15] Choi, D.W. Calcium-mediated neurotoxicity: relationship to specific channel types and role in ischemic damage, *Trends Neurosci*., **11**, 465–469 (1988).
[16] Patterson, S.D., Spahr, C.S., Daugas, E. *et al*. Mass spectrometric identification of proteins released from mitochondria undergoing permeability transition, *Cell Death Differ*., **7**, 137–144 (2000).
[17] Kochanek, P.M. and Hallenbeck, J.M. Polymorphonuclear leukocytes and monocytes/macrophages in the pathogenesis of cerebral ischemia and stroke, *Stroke*, **23**, 1367–1379 (1992).
[18] Engler, R. Consequences of activation and adenosine-mediated inhibition of granulocytes during myocardial ischemia, *Fed. Proc*., **46**, 2407–2412 (1987).
[19] Klebanoff, S.J. Phagocytic cells: products of oxygen metabolism, in *Inflammation: Basic Principles and Clinical Correlates* (ed. J.I. Gallin, I.M. Goldstein and R. Snyderman), Raven Press, New York, NY, pp. 391–444 (1988).
[20] Test, S.T. and Weiss, S.J. Assay of the extracellular hydrogen peroxide pool generated by phagocytes, *Methods Enzymol*., **132**, 401–406 (1986).
[21] Granger, D.N., Benoit, J.N., Suzuki, M. and Grisham, M.B. Leukocyte adherence to venular endothelium during ischemia-reperfusion, *Am. J. Physiol*., **257**, G683–G688 (1989).

[22] del Zoppo, G.J., Schmid-Schonbein, G.W., Mori, E. *et al*. Polymorphonuclear leukocytes occlude capillaries following middle cerebral artery occlusion and reperfusion in baboons, *Stroke*, **22**, 1276–1283 (1991).
[23] Ames, A. 3rd, Wright, R.L., Kowada, M. *et al*. Cerebral ischemia. II. The no-reflow phenomenon, *Am. J. Pathol*., **52**, 437–453 (1968).
[24] Fischer, M. and Hossmann, K.A. No-reflow after cardiac arrest, *Intensive Care Med*., **21**, 132–141 (1995).
[25] Kagstrom, E., Smith, M.L. and Siesjo, B.K. Local cerebral blood flow in the recovery period following complete cerebral ischemia in the rat, *J. Cereb. Blood Flow Metab*., **3**, 170–182 (1983).
[26] McCord, J.M. Oxygen-derived free radicals in postischemic tissue injury, *N. Engl. J. Med*., **312**, 159–163 (1985).
[27] Suzuki, M., Inauen, W., Kvietys, P.R. *et al*. Superoxide mediates reperfusion-induced leukocyte-endothelial cell interactions, *Am. J. Physiol*., **257**, H1740–H1745 (1989).
[28] Mullane, K.M., Salmon, J.A. and Kraemer, R. Leukocyte-derived metabolites of arachidonic acid in ischemia-induced myocardial injury, *Fed. Proc*., **46**, 2422–2433 (1987).
[29] Fischer, E.G., Ames, A. 3rd and Lorenzo, A.V. Cerebral blood flow immediately following brief circulatory stasis, *Stroke*, **10**, 423–427 (1979).
[30] Gaszynski, W. Research work on blood clotting system during cardiorespiratory resuscitation, *Anaesth Resusc Intensive Ther*, **2**, 303–316 (1974).
[31] Lin, S.R. Changes of plasma volume and hematocrit after circulatory arrest: an experimental study in dogs, *Invest. Radiol*., **14**, 202–206 (1979).
[32] Hossmann, K.A. Ischemia-mediated neuronal injury, *Resuscitation*, **26**, 225–235 (1993).
[33] Kleihues, P., Kobayashi, K. and Hossmann, K.A. Purine nucleotide metabolism in the cat brain after one hour of complete ischemia, *J. Neurochem*., **23**, 417–425 (1974).
[34] Cino, M. and Del Maestro, R.F. Generation of hydrogen peroxide by brain mitochondria: the effect of reoxygenation following postdecapitative ischemia, *Arch. Biochem. Biophys*., **269**, 623–638 (1989).
[35] Schmidt, H.H., Nau, H., Wittfoht, W. *et al*. Arginine is a physiological precursor of endothelium-derived nitric oxide, *Eur. J. Pharmacol*., **154**, 213–216 (1988).
[36] Halliwell, B. Reactive oxygen species and the central nervous system, *J. Neurochem*., **59**, 1609–1623 (1992).
[37] Komara, J.S., Nayini, N.R., Bialick, H.A. *et al*. Brain iron delocalization and lipid peroxidation following cardiac arrest, *Ann. Emerg. Med*., **15**, 384–389 (1986).
[38] Babbs, C.F. Role of iron ions in the genesis of reperfusion injury following successful cardiopulmonary resuscitation: preliminary data and a biochemical hypothesis, *Ann. Emerg. Med*., **14**, 777–783 (1985).
[39] White, B.C., Krause, G.S., Aust, S.D. and Eyster, G.E. Postischemic tissue injury by iron-mediated free radical lipid peroxidation, *Ann. Emerg. Med*., **14**, 804–809 (1985).
[40] Lo, W.D. and Betz, A.L. Oxygen free-radical reduction of brain capillary rubidium uptake, *J. Neurochem*., **46**, 394–398 (1986).

[41] Pellmar, T.C. and Neel, K.L. Oxidative damage in the guinea pig hippocampal slice, *Free Radic. Biol. Med*., **6**, 467–472 (1989).
[42] Safar, P. Cerebral resuscitation after cardiac arrest: a review, *Circulation*, **74**, IV138–153 (1986).
[43] Lo, E.H., Dalkara, T. and Moskowitz, M.A. Mechanisms, challenges and opportunities in stroke, *Nat. Rev. Neurosci*., **4**, 399–415 (2003).
[44] Mitani, A., Yanase, H., Sakai, K. *et al*. Origin of intracellular Ca^{2+} elevation induced by in vitro ischemia-like condition in hippocampal slices, *Brain Res*., **601**, 103–110 (1993).
[45] Monaghan, D.T., Holets, V.R., Toy, D.W. and Cotman, C.W. Anatomical distributions of four pharmacologically distinct 3H-L-glutamate binding sites, *Nature*, **306**, 176–179 (1983).
[46] Barnes, D.M. NMDA receptors trigger excitement, *Science*, **239**, 254–256 (1988).
[47] Benveniste, H., Jorgensen, M.B., Diemer, N.H. and Hansen, A.J. Calcium accumulation by glutamate receptor activation is involved in hippocampal cell damage after ischemia, *Acta Neurol. Scand*., **78**, 529–536 (1988).
[48] Steinberg, G.K., Saleh, J., DeLaPaz, R. *et al*. Pretreatment with the NMDA antagonist dextrorphan reduces cerebral injury following transient focal ischemia in rabbits, *Brain Res*., **497**, 382–386 (1989).
[49] Ozyurt, E., Graham, D.I., Woodruff, G.N., and McCulloch, J. Protective effect of the glutamate antagonist, MK-801 in focal cerebral ischemia in the cat, *J. Cereb. Blood Flow Metab*., **8**, 138–143 (1988).
[50] Diemer, N.H., Johansen, F.F. and Jorgensen, M.B. N-methyl-D-aspartate and non-N-methyl-D-aspartate antagonists in global cerebral ischemia, *Stroke*, **21**, III39–42 (1990).
[51] Sterz, F., Leonov, Y., Safar, P. *et al*. Effect of excitatory amino acid receptor blocker MK-801 on overall, neurologic, and morphologic outcome after prolonged cardiac arrest in dogs, *Anesthesiology*, **71**, 907–918 (1989).
[52] Lanier, W.L., Perkins, W.J., Karlsson, B.R. *et al*. The effects of dizocilpine maleate (MK-801), an antagonist of the N-methyl-D-aspartate receptor, on neurologic recovery and histopathology following complete cerebral ischemia in primates, *J. Cereb. Blood Flow Metab*., **10**, 252–261 (1990).
[53] Althaus, J., Bernaudin, M., Petit, E. *et al*. Expression of the gene encoding the pro-apoptotic BNIP3 protein and stimulation of hypoxia-inducible factor-1alpha (HIF-1alpha) protein following focal cerebral ischemia in rats, *Neurochem. Int*., **48**, 687–695 (2006).
[54] Zhao, Z.Q., Corvera, J.S., Halkos, M.E. *et al*. Inhibition of myocardial injury by ischemic postconditioning during reperfusion: comparison with ischemic preconditioning, *Am. J. Physiol. Heart Circ. Physiol*., **285**, H579–H588 (2003).
[55] Gross, G.J., Farber, N.E., Hardman, H.F. and Warltier, D.C. Beneficial actions of superoxide dismutase and catalase in stunned myocardium of dogs, *Am. J. Physiol*., **250**, H372–H377 (1986).
[56] Watanabe, B.I., Premaratne, S., Limm, W. *et al*. High- and low-dose superoxide dismutase plus catalase does not reduce myocardial infarct size in a subhuman primate model, *Am. Heart J*., **126**, 840–846 (1993).

[57] Kavanagh, R.J. and Kam, P.C. Lazaroids: efficacy and mechanism of action of the 21-aminosteroids in neuroprotection, *Br. J. Anaesth*., **86**, 110–119 (2001).
[58] Haley, E.C. Jr. High-dose tirilazad for acute stroke (RANTTAS II). RANTTAS II Investigators, *Stroke*, **29**, 1256–1257 (1998).
[59] Warner, D.S., Sheng, H. and Batinic-Haberle, I. Oxidants, antioxidants and the ischemic brain, *J. Exp. Biol*., **207**, 3221–3231 (2004).
[60] Connolly, E.S. Jr, Winfree, C.J., Prestigiacomo, C.J. *et al*. Exacerbation of cerebral injury in mice that express the P-selectin gene: identification of P-selectin blockade as a new target for the treatment of stroke, *Circ. Res*., **81**, 304–310 (1997).
[61] Huang, J., Choudhri, T.F., Winfree, C.J. *et al*. Postischemic cerebrovascular E-selectin expression mediates tissue injury in murine stroke, *Stroke*, **31**, 3047–3053 (2000).
[62] Harlan, J.M. and Winn, R.K. Leukocyte-endothelial interactions: clinical trials of anti-adhesion therapy, *Crit. Care Med*., **30**, S214–S219 (2002).
[63] Wong, C.H. and Crack, P.J. Modulation of neuro-inflammation and vascular response by oxidative stress following cerebral ischemia-reperfusion injury, *Curr. Med. Chem*., **15**, 1–14 (2008).
[64] Bernard, S.A., Gray, T.W., Buist, M.D. *et al*. Treatment of comatose survivors of out-of-hospital cardiac arrest with induced hypothermia, *N. Engl. J. Med*., **346**, 557–563 (2002).
[65] Holzer, M., Cerchiari, E., Martens, P. *et al*. Mild therapeutic hypothermia to improve the neurologic outcome after cardiac arrest, *N. Engl. J. Med*., **346**, 549–556 (2002).
[66] Pina-Cabral, J.M., Ribeiro-da-Silva, A. and Almeida-Dias, A. Platelet sequestration during hypothermia in dogs treated with sulphinpyrazone and ticlopidine: reversibility accelerated after intra-abdominal rewarming, *Thromb. Haemost*., **54**, 838–841 (1985).
[67] Polderman, K.H. Mechanisms of action, physiological effects, and complications of hypothermia, *Crit. Care Med*., **37**, S186–S202 (2009).
[68] Blackstone, E., Morrison, M. and Roth, M.B. H_2S induces a suspended animation-like state in mice, *Science*, **308**, 518 (2005).
[69] Volpato, G.P., Searles, R., Yu, B. *et al*. Inhaled hydrogen sulfide: a rapidly reversible inhibitor of cardiac and metabolic function in the mouse, *Anesthesiology*, **108**, 659–668 (2008).
[70] Minamishima, S., Bougaki, M., Sips, P.Y. *et al*. Hydrogen sulfide improves survival after cardiac arrest and cardiopulmonary resuscitation via a nitric oxide synthase 3-dependent mechanism in mice, *Circulation*, **120**, 888–896 (2009).
[71] Rosenthal, R.E., Silbergleit, R., Hof, P.R. *et al*. Hyperbaric oxygen reduces neuronal death and improves neurological outcome after canine cardiac arrest, *Stroke*, **34**, 1311–1316 (2003).
[72] Takahashi, M., Iwatsuki, N., Ono, K. *et al*. Hyperbaric oxygen therapy accelerates neurologic recovery after 15-minute complete global cerebral ischemia in dogs, *Crit. Care Med*., **20**, 1588–1594 (1992).
[73] Singhal, A.B., Benner, T., Roccatagliata, L. *et al*. A pilot study of normobaric oxygen therapy in acute ischemic stroke, *Stroke*, **36**, 797–802 (2005).

[74] Singhal, A.B. A review of oxygen therapy in ischemic stroke, *Neurol. Res*., **29**, 173–183 (2007).
[75] Theilen, H., Schrock, H. and Kuschinsky, W. Gross persistence of capillary plasma perfusion after middle cerebral artery occlusion in the rat brain, *J. Cereb. Blood Flow Metab*., **14**, 1055–1061 (1994).
[76] Villringer, A., Them, A., Lindauer, U. *et al*. Capillary perfusion of the rat brain cortex: an in vivo confocal microscopy study, *Circ. Res*., **75**, 55–62 (1994).
[77] Linberg, R., Conover, C.D., Shum, K.L. and Shorr, R.G. Increased tissue oxygenation and enhanced radiation sensitivity of solid tumors in rodents following polyethylene glycol conjugated bovine hemoglobin administration, in vivo, **12**, 167–173 (1998).
[78] Contaldo, C., Plock, J., Sakai, H. *et al*. New generation of hemoglobin-based oxygen carriers evaluated for oxygenation of critically ischemic hamster flap tissue, *Crit. Care Med*., **33**, 806–812 (2005).
[79] Te Lintel Hekkert, M., Dube, G.P., Regar, E. *et al*. Preoxygenated hemoglobin-based oxygen carrier HBOC-201 annihilates myocardial ischemia during brief coronary artery occlusion in pigs, *Am. J. Physiol. Heart Circ. Physiol*., **298**, H1103–H1113 (2010).
[80] Yu, B., Bloch, K.D. and Zapol, W.M. Hemoglobin-based red blood cell substitutes and nitric oxide, *Trends Cardiovasc. Med*., **19**, 103–107 (2009).
[81] Reiter, C.D., Wang, X., Tanus-Santos, J.E. *et al*. Cell-free hemoglobin limits nitric oxide bioavailability in sickle-cell disease, *Nat. Med*., **8**, 1383–1389 (2002).
[82] Gladwin, M.T., Sachdev, V., Jison, M.L., Shizukuda, Y. *et al*. Pulmonary hypertension as a risk factor for death in patients with sickle cell disease, *N. Engl. J. Med*., **350**, 886–895 (2004).
[83] Yu, B., Raher, M.J., Volpato, G.P., Bloch, K.D. *et al*. Inhaled nitric oxide enables artificial blood transfusion without hypertension, *Circulation*, **117**, 1982–1990 (2008).
[84] Saxena, R., Wijnhoud, A.D., Carton, H. *et al*. Controlled safety study of a hemoglobin-based oxygen carrier, DCLHb, in acute ischemic stroke, *Stroke*, **30**, 993–996 (1999).
[85] Lamy, M.L., Daily, E.K., Brichant, J.F. *et al*. Randomized trial of diaspirin cross-linked hemoglobin solution as an alternative to blood transfusion after cardiac surgery. The DCLHb Cardiac Surgery Trial Collaborative Group, *Anesthesiology*, **92**, 646–656 (2000).

11

Normobaric and Hyperbaric Oxygen Therapy for Ischemic Stroke and Other Neurological Conditions

Ari Moskowitz[1], *Yu-Feng Yvonne Chan*[1] *and Aneesh B. Singhal*[2]

[1]*Department of Emergency Medicine, The Mount Sinai Medical Center, New York, NY, USA*

[2]*Department of Neurology, Massachusetts General Hospital, Boston, MA, USA*

"[we need to be] more responsive to the challenge of vascular disease. . .the debate on healthcare issues is gradually turning into questions directed at our attitudes and approaches as experts in the field. Congress a year ago was tempted to ask the National Institutes of Health: We doubled your budget in the last 10 years, have you doubled the cure?"

– Professor Antoine Hakim (2007 Willis Lecture, American Heart Association)

11.1 Introduction

According to the most recent Centers for Disease Control (CDC) estimates, cerebrovascular diseases are the third leading cause of death in the United States. In 2006, these diseases accounted for 137 119 fatalities in the USA alone [1]. Unfortunately, despite the great mortality and disability burden of stroke, there is a lack of effective treatment options. Recombinant tissue plasminogen activator (rt-PA), a thrombolytic agent introduced in 1996, remains the only FDA-approved treatment for acute ischemic stroke

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(AIS) [2]. This medication, while clearly beneficial when administered appropriately, is limited by a short therapeutic time window (up to 4.5 hours after symptom onset [3]) and a 10-fold higher risk of brain hemorrhage. Given the unmet need for AIS interventions, stroke researchers have developed and investigated a variety of so-called "neuroprotective" agents. These agents are designed to interrupt molecular pathways of ischemic cell death following AIS and, thereby, salvage ischemic neurons. Over the past two decades, nearly 100 neuroprotective agents have been tested but have failed to show efficacy in phase II and phase III trials despite success in preclinical studies [4, 5]. Contributing to the failure of these agents are a delayed time to treatment, failure to reach ischemic brain target tissue, and drug toxicity or side effects.

The results of several animal studies and pilot clinical trials conducted over the past three decades suggest that oxygen therapy may be an overlooked strategy for neuroprotection, with many "ideal" properties for a neuroprotective drug. Oxygen acts on virtually every pathway of ischemic cell death, has few known side effects, and can readily diffuse through the blood–brain barrier to reach the target ischemic tissue. To date, several routes of oxygen delivery have been explored in the literature, including hyperbaric oxygen (HBO), normobaric oxygen (NBO), perfluorocarbons, and aqueous oxygen solutions. The role of HBO and NBO has been investigated in animals and humans with ischemic stroke, while the other strategies are still in development. As opposed to these "therapeutic" oxygen delivery strategies, "supplemental oxygen" (SO) refers to the delivery of smaller quantities of oxygen in order to prevent or treat hypoxia. In this chapter we will explore the pathophysiological basis of oxygen therapy in ischemic stroke, describe the progress to date in both animal and human trials, and briefly explore the utilization of oxygen therapy in other neurological diseases.

11.2 Rationale of Oxygen Therapy in AIS

The first studies of induced hyperoxia in cerebral ischemia were performed using pressurized oxygen delivery chambers (HBO). HBO has the advantage of being the modality best suited to increasing plasma concentrations of dissolved oxygen in the ischemic brain as per Henry's Law (amount dissolved is proportional to partial pressure). In the infancy of oxygen therapy, the belief was that oxygen's efficacy in ischemic stroke depended directly on the restoration of oxygen levels in the ischemic "penumbra".[1] Given the 0.003 coefficient attached to pO_2 in the equation below, it was apparent that only large changes in oxygen pressure would affect overall oxygenation, and as a result HBO was used and other strategies such as NBO were overlooked. Nevertheless, a deeper understanding of oxygen physiology suggests that NBO may also have a role in treating brain ischemic. Room air has an FiO_2 of ~21% and will provide a pO_2 of approximately 80 mm Hg.

[1] Ischemic stroke in the acute setting leads to the formation of regions with variably compromised blood flow and oxygen supply distal to the site of arterial occlusion. The *core* of the ischemic territory refers to regions of severely compromised blood flow where cellular injury is irreversible and tissue is nonsalvageable. In such regions, cell death usually occurs within minutes. Surrounding the "core" regions are areas of reduced blood flow supported by collateral circulation, where tissue is at risk for infarction but still salvageable. This tissue is referred to as the *ischemic penumbra*. Results of PET and functional MRI studies suggest that the ischemic penumbra exists for several hours or more after symptom onset. With passage of time, there is a reduction in the volume of ischemic penumbra and growth of the infarct core.

Table 11.1 *Oxygen physiology.*

Key oxygen equations

Hemoglobin bound oxygen = 1.36 mL oxygen per 1 g hemoglobin
Dissolved oxygen = 0.003 mL/mm HgO_2/dL blood
Blood oxygen content (per dL) = 1.36 × [Hb × (saturation/100)] + 0.003 × pO_2 = 21 mL
Mixed venous blood pO_2 = 45 mm Hg
Oxygen utilization (%) = (arterial oxygen content − venous oxygen content)/(arterial oxygen content)
Oxygen consumption (mL/min/100 g brain tissue) = cerebral blood flow × (arterial oxygen content − venous oxygen content)

Oxygen transport

pO_2 (mmHg)	Hb sat	Hb-bound oxygen (mL/dL)	Dissolved oxygen (mL/dL)	Total oxygen (mL/dL)	Dissolved/ Hb-bound
760	99.9	~21	~2.5	23.5	11%
300	99.2	~21	~1.0	22	5%
100	97.5	20.67	0.33	21	1.5%
50	80	17.83	0.167	18	1%
26	50		(at pCO_2 = 40 mm Hg, pH = 7.4, 37 °C		

HBO (the inspiration of pure oxygen ($FiO_2$100%) at hyperbaric pressures), will yield a pO_2 of >1000 mm Hg, and NBO (the inspiration of pure oxygen ($FiO_2$100%) at atmospheric pressure) will yield a pO_2 of 300 mm Hg. However, as per the oxygen equations and Table 11.1, both HBO and NBO will have a significant effect on the total oxygen content, and on the ratio of dissolved to hemoglobin-bound oxygen. More importantly however, these equations are applicable to the physiological non-ischemic state; oxygen dissociation and offloading of oxygen molecules is enhanced in the ischemic tissues. Moreover, the critical oxygen tension required for mitochondrial function is extremely low [6]. These data suggest that NBO, like HBO, may also be effective in improving oxygenation in the ischemic brain.

The direct effects of increased dissolved oxygen on ischemic tissue have been explored in a number of studies. In trials of patients with traumatic brain injury, increased pO_2 via HBO improved the cerebral rate of oxygen consumption, decreased brain lactate and pyruvate levels, and improved mitochondrial function [7, 8]. Additionally, HBO has been shown in animals to reduce diffusion-weighted MRI (DWI) abnormalities following ischemic stroke, likely secondary to restoration of sodium-potassium ATPase function in ischemic regions [9–11]. PET imaging has further demonstrated restoration of glucose uptake in ischemic regions following oxygen therapy in rodent stroke models [12]. Recent studies exploring the use of NBO in cerebral ischemia have further demonstrated that even small increases in dissolved oxygen (approximately fivefold in NBO) may be sufficient to delay cell death in the ischemic setting.

In addition to these direct actions, however, there has been a growing emphasis in the literature on the multiple *indirect* neuroprotective effects of induced hyperoxia in the form of NBO and HBO. It is now widely understood that oxygen therapy plays a critical role in all the major pathways of cell death: excitotoxicity, oxidative/nitrative stress,

inflammation, and apoptosis [13]. Several studies have shown that oxygen therapy has potent antiapoptotic effects. In models of AIS [14], global cerebral ischemia/reperfusion, brain trauma [15], and neonatal hypoxic-ischemic brain injury [16], HBO was found to reduce the expression of a number of proapoptotic genes, including hypoxia-inducible factor-1 alpha, p53, caspase-9, and caspase-3. Recent evidence further indicates that oxygen therapy exhibits anti-inflammatory properties. Hyperoxia reduces cyclooxygenase-2 (COX-2) mRNA and MMP-9, reduces intercellular adhesion molecule-1 (ICAM-1) levels, and reduces leukocyte adhesion and infiltration via the induction of endothelial nitric oxide synthase (eNOS) pathways [17–19]. Further, oxygen therapy induces hypoxia-inducible factor-1α and Bcl-2, decreases blood viscosity, reduces platelet aggregation, and improves microcirculation. It has been shown to preserve blood–brain barrier integrity, reduce vascular permeability, decrease vasogenic brain edema, and increase the expression of the free-radical scavenger Mn-SOD [20–22]. Finally, a recent study in 2010 demonstrated that oxygen therapy in cerebral ischemia leads to the differential expression of thousands of genes in the rat transcriptome [23]. Collectively, these findings suggest that induced hyperoxia likely provides lasting neuroprotective effects and may influence neuronal recovery.

11.3 Hyperbaric Oxygen Therapy

The use of HBO in organ ischemia was first described in the 1960s by a cardiac surgeon, Professor L. Boerema. He hypothesized that placing infants with congenital heart defects in hyperbaric chambers could sufficiently "drench" tissues with oxygen to allow a surgeon the window to stop the heart and repair the defect. Professor Boerema published *Life without Blood*, which detailed how HBO chambers salvaged ischemic myocardial tissue in exanguinated pigs [24–26]. In 1961, Smith, Lawson and colleagues described the preservation of cortical electrical activity during cerebral ischemia using compressed oxygen [27, 28]. Following these promising results, a number of case studies appeared in the literature detailing improvement in neurological deficits and EEG findings when AIS patients were exposed to HBO [27, 28]. The largest case series to date was published in 1980 by Neubauer and End. In this study, 122 clinically diagnosed AIS patients were exposed to 10–20 rounds of HBO treatments at 1.5–2.0 atmospheres (ATA) [29]. The results of these early reports suggested a benefit of HBO in both acute and completed stroke and eventually led to randomized control trials (RCTs).

To date there have been four RCTs exploring the use of HBO in AIS [30–33]. The first was performed by Anderson *et al*. in 1991 [30]. In this pilot study, the researchers randomized 39 patients with AIS to receive either 100% HBO or compressed air. The average time from onset of symptoms to first treatment was 51.8 hours (study protocol called for a <6 hour delay) and many patients in both study arms did not reach the goal of 15 treatments. The study was stopped early due to an interim analysis showing a trend towards superior neurological outcomes in the air-treated patients. While this trend was eventually attributed to randomization, the study was not resumed due to patients' poor tolerance of hyperbaric treatments and logistical difficulty. In a second trial, performed in 1995 by Nighoghossian *et al*., 34 patients were randomized to HBO

versus compressed air for a total of 10 treatments over 10 days [31]. This trial also failed to show any benefit of HBO in AIS. A third trial, by Rusyniak *et al*., randomized 33 patients to receive either HBO at 2.5 ATA or HBO at 1.14 ATA [32]. Patients received just one round of therapy. At three months, the authors reported that patients receiving higher pressures had worse National Institutes of Health Stroke Scale (NIHSS) scores. Based on these results, the authors suggested that HBO offers no benefit in AIS and may in fact be harmful. Most recently, Imai *et al*. explored the use of HBO in combination with the free-radical scavenger Edaravone in AIS involving the anterior cerebral circulation [33]. The study involved 38 patients. Those in the treatment group received seven 1-hour sessions of HBO at 2.0 ATA with 30 mg of Edaravone administered before and after each treatment. The results showed no difference in NIHSS between the groups, although more patients in the HBO group (31%) had favorable functional outcomes, based on a 90-day modified Rankin scale, than in the control group (5%).

The failure of the above trials does not necessarily indicate that HBO is ineffective or unsafe. A number of alternative factors may have contributed to the failure. Specifically, these RCTs suffered from small sample sizes, relatively delayed stroke onset-to-treatment times, deviations from study protocols, inadequate patient selection criteria such as differentiation of stroke subtypes, and a failure to rule out hemorrhagic stroke via CT prior to study enrollment [34]. The chamber pressure used, and the number or duration of exposures, was largely arbitrary. In retrospect, these deficiencies are not surprising considering that animal studies of HBO in AIS have paradoxically lagged behind human studies. Preclinical testing and elucidation of the appropriate therapeutic time window, dosage, and recommended stroke subtypes may have provided for more strongly designed RCTs. Prior to 1990, only five animal trials of HBO in focal cerebral ischemia had been published [35–39]. Since 2000, however, more than 40 *in vivo* animal studies have been performed [9–12, 17–19, 40–63]. These studies have utilized a wide range of focal ischemic stroke models. Further, a number of different HBO durations (15 minutes to 36 hours), chamber pressures (1.5–3.0 ATA), and durations of ischemia (15 minutes to 6 hours) have been explored. Despite the wide variation in study methodology, the vast majority of studies have demonstrated a significant benefit of HBO in animal models of focal ischemic stroke. Recent reviews have suggested that HBO's efficacy can be maximized by minimizing the therapeutic time window (ischemic duration), the use of lower chamber pressures (1.5–2.5 ATA), and incorporating multiple treatment sessions [22, 64–67]. Notably, some rodent studies have indicated that the "neuroprotectant" time window for use of HBO is 6 hours and initiation of HBO beyond 12 hours may be harmful [16, 50].

One interesting direction of some recent animal studies is the use of HBO in ischemic preconditioning. Specifically, the "prophylactic" exposure of experimental animals to repeated HBO sessions appears to protect them from neurological deterioration during subsequent ischemic insults [42, 45, 68]. In one study of gerbils, multiple pretreatment courses of HBO led to an increased expression of manganese superoxide dismutase, a free-radical scavenger [69]. HBO has been shown to facilitate angiogenesis, inhibit pathways that block brain plasticity, and upregulate hypoxia-inducible factor, leading to increased erythropoietin production [70]. Human RCTs will be required to determine the usefulness of HBO as a prophylactic neuroprotectant in patients at high risk for stroke.

11.4 Normobaric Oxygen Therapy

Given the logistical difficulty and very limited availability of HBO, investigative focus in the field of oxygen therapy for AIS has turned to NBO. NBO therapy refers to 100% oxygen delivered at atmospheric pressure, usually via face mask. The advantages of NBO therapy include ease of administration, wide availability, low cost, and patient comfort [64–66, 71, 72]. The first focused study of NBO in cerebral ischemia was reported by Singhal *et al*. in 2002. Using pathological, advanced brain imaging, and functional outcome measures, the authors were able to demonstrate neuroprotective effects using NBO in rodent models of transient AIS [73, 74]. These findings have been replicated and extended in subsequent animal studies performed in different laboratories in the USA and Germany. The results of these studies have been nearly universally positive, but the benefits are time sensitive, most pronounced in the acute phase, and appear to be lost without prompt reperfusion [75–85].

The neuroprotective effects of NBO in ischemic stroke appear to be similar to those seen in studies of HBO therapy. While NBO does not increase dissolved oxygen to the levels obtained during HBO therapy, recent NBO trials in animals and humans have demonstrated prompt reversal of DWI lesions and reduced chronic infarct volumes, as well as acute improvement in stroke-related functional deficits. One possible explanation for this preservation of benefit is that the critical oxygen tension required for mitochondrial function is extremely low [13], and therefore even small increases in brain tissue oxygenation – achievable with NBO – might be adequate to overcome thresholds for ischemic cell death. NBO may also preserve cerebral blood volume in the ischemic brain, either by preventing neuronal death and promoting neurovascular coupling, or by preserving endothelial function and maintaining collateral vessels. Since hyperoxia is known to induce vasoconstriction in the normal brain, it is possible that NBO acts by diverting blood from non-ischemic brain into ischemic brain (a “reverse steal” or “Robin Hood” effect) [73].

Notably, Kim *et al*. explored the use of NBO in combination with reperfusion. Rats were randomized to receive a variable duration of cerebral ischemia (1, 2, 3 or 4 hours) and either NBO or room air during the ischemic period before reperfusion. Infarct volumes were similar in rats in the 1 hour of ischemia group. However, at 2 and 3 hours, those rats who received hyperoxia had smaller infarct volumes, indicating that NBO may extend the reperfusion time window (by a factor of three) following AIS [80]. Given that rt-PA is the only approved therapy for AIS, extension of its therapeutic window has potential to vastly increase its utilization, and perhaps the safety of rt-PA, by preventing acute ischemic cell death. In a recent animal study in 2009, Fujiwara *et al*. reported that the administration of NBO in combination with rt-PA was safe and did not change the efficacy of the rt-PA following simulated middle cerebral artery (MCA) stroke [78]. Further, in 2007, Henninger *et al*. explored NBO following permanent and transient MCA occlusion in rats. Using diffusion and perfusion weighted MRI, the authors were able to establish that NBO: (i) acutely preserved perfusion/diffusion mismatch ratios without changing cerebral blood flow; (ii) extended the time window for reperfusion following arterial occlusion; (iii) induced lasting protection against ischemic cell death following permanent MCA occlusion; and (iv) despite being capable of reducing cell death in hypoperfused tissue, induced a small amount of cell death in otherwise unaffected areas [79].

As with HBO therapy, the combination of NBO with a free-radical scavenger has shown benefit in animal studies. Furthermore, Bigdelli *et al*. suggest the possible utility of NBO in ischemic preconditioning. Repeated exposure to NBO triggers an upregulation in antioxidant enzymes, excitatory amino-acid transporters (EAATs), and increased serum TNF-α levels [86–88].

Human studies of NBO have been limited thus far. The first clinical trial of NBO involved 16 patients with hemispheric ischemic stroke of onset <12 hours [13]. All subjects had a perfusion/diffusion mismatch on initial MRI, indicating salvageable brain tissue. These patients were randomized to receive either NBO (45 L by face mask for 8 hours) or room air. Brain MRI was performed before starting treatment, halfway through therapy, and again at 24 hours, 1 week, and 3 months. Despite the small sample size, patients in the NBO group showed a greater improvement in NIHSS scores and reduced growth of DWI lesions both during therapy and in follow-up evaluations (Figure 11.1). Further, the results of serial MR-spectroscopy (performed in a subset) showed that NBO-treated patients had lower lactate levels, and higher n-acetyl aspartate levels, providing additional evidence for the acute neuroprotective effects of NBO in AIS. More comprehensive clinical trials of NBO are presently ongoing.

11.5 The Status of Supplemental Oxygen Delivery

At present, there exists tremendous variability in the use of SO therapy in presumptive and confirmed AIS. While the American Heart Association currently does not recommend the use of prehospital SO in nonhypoxic AIS patients (room air arterial oxygen saturation >92%), some regional prehospital protocols do not follow the guidelines. For example, the New York City prehospital protocol mandates 10–15 L/min 100% oxygen delivery via nonrebreather mask (NRM) to all presumptive stroke patients [89]. SO use within emergency departments appears variable as well. Few clinical studies address the frequency, effectiveness, and safety of the routine administration of SO in nonhypoxic AIS patients. In one frequently cited observational study [90], 550 patients were randomized to receive either 3 L/min of oxygen by nasal cannula or room air. Based on the finding of better outcome in the room-air group at 1 year post-intervention, the authors recommended against routine SO use in AIS. However, 13% of patients had hemorrhagic strokes, 18% did not receive the assigned therapy, oxygen was delivered for >24 hours at times, and 40% of participants had an unknown time of stroke onset or were treated after 12–24 hours. Therefore, one cannot ascertain the true safety and efficacy of SO in AIS on the basis of this study. Unfortunately, these results are often cited as evidence against SO administration in AIS. Another small feasibility study found lower mortality and complication rates in patients with severe MCA stroke treated with venturi-mask oxygenation [91].

11.6 Comparison of HBO and NBO in AIS

Researchers have increasingly begun to compare HBO and NBO in terms of safety and efficacy (Figures 11.2 and 11.3). In one study, Veltkamp *et al*. found that delivering HBO

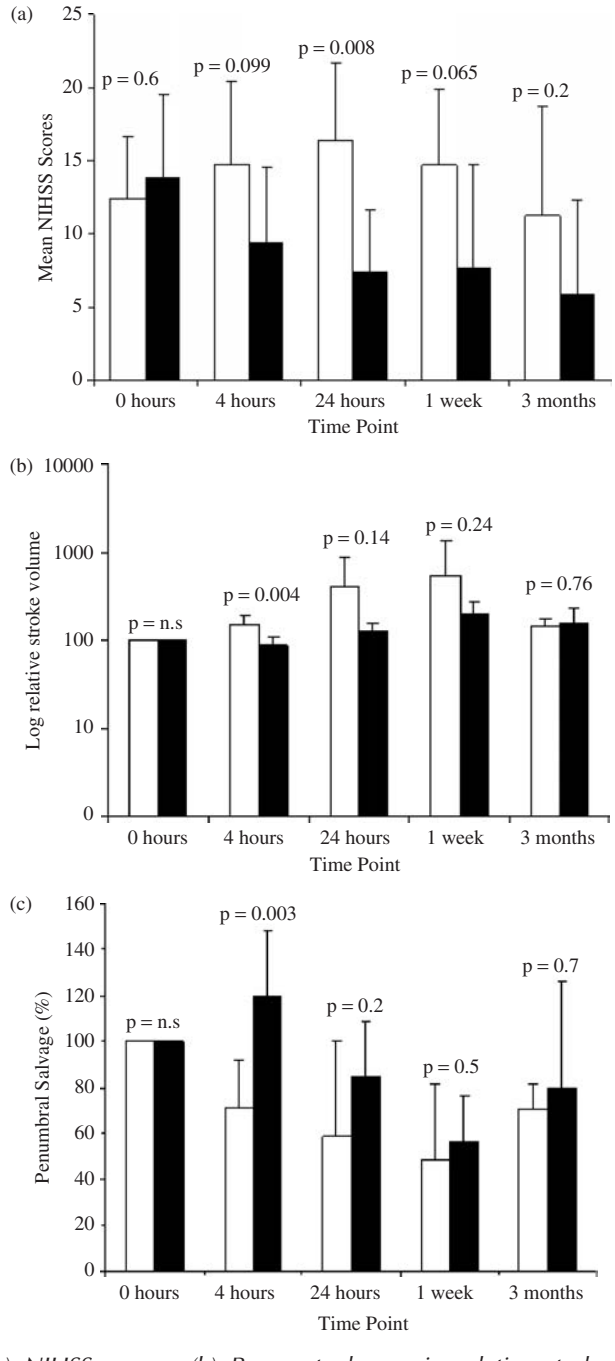

Figure 11.1 *(a) NIHSS scores. (b) Per cent change in relative stroke lesion volumes. (c) Penumbral salvage or the ratio of acutely hypoperfused tissue salvaged from infarction ((baseline MTT volume) – (infarct volume at later time point)) to the acute tissue at risk for infarction ((baseline MTT volume) – (DWI volume at baseline)). Controls, white bars; NBO, black bars; mean ± SD. (Reprinted from [13], with permission from Elsevier).*

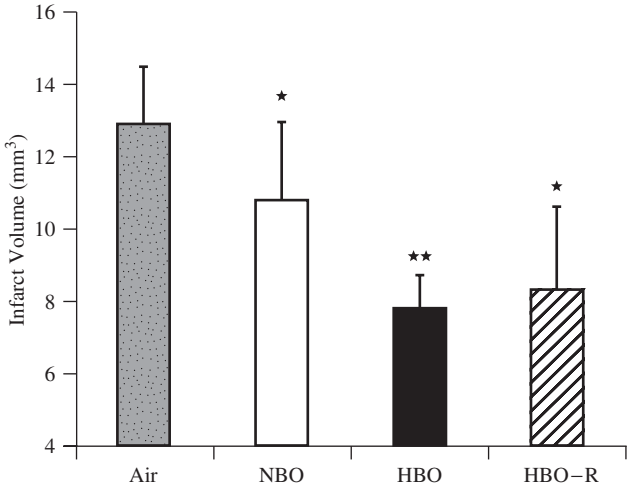

Figure 11.2 *Infarct volume on silver-stained sections 7 days after MCA coagulation. A single 60-minute treatment with either NBO or HBO (3 ATA) reduces infarct volume compared to air. Reduction of infarct size by HBO is larger than by NBO. Repeated HBO (HBO-R) administration on subsequent days fails to deliver an additional benefit (*p < 0.05 compared to air; **p < 0.05 compared to NBO; ANOVA). (Reprinted from [53]).*

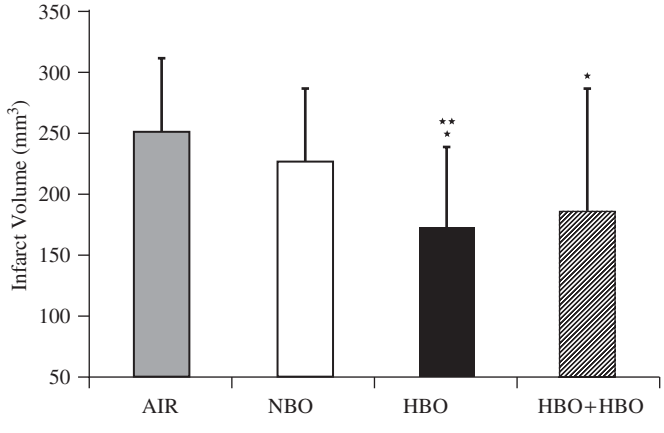

Figure 11.3 *Infarct volumes on silver stained-sections 7 days after MCA occlusion. HBO decreases infarct volume compared to air and NBO. Combination of HBO and NBO decreases infarct volume significantly compared to air, but the difference is not significant compared to NBO-treated animals (*p < 0.05 compared to air; **p < 0.05 compared to NBO; ANOVA and post hoc Fisher's protected least significant difference test). (Reprinted from [55], with permission from Elsevier).*

at 2.5 ATA following 75 minutes of focal cerebral ischemia was superior to either HBO at 1.5 ATA or 100% oxygen at 1.0 ATA (essentially NBO) [44]. Similarly, Eschenfelder *et al*. found that higher oxygen pressures (3.0 ATA) created long-lasting reductions in infarct volumes and improved clinical outcomes when compared with lower pressures (1.0 ATA) in rats exposed to 90 minutes of transient focal cerebral ischemia [59]. Beynon *et al*. hypothesized that the above studies favored HBO over NBO due to a failure of the authors to account for the increased efficacy of NBO when initiated early [55]. In their study, Beynon *et al*. compared NBO initiated at 30 minutes after cerebral ischemia to HBO started at 90 minutes. They too, however, demonstrated superior efficacy of HBO but no benefit of combining NBO and HBO. In separate experiments, the combination of NBO administered soon after occlusion followed by HBO therapy has been shown to reduce infarct size [53], leading to the suggestion that NBO initiated in the prehospital setting followed by HBO on hospital arrival may be beneficial. Significantly, a 2008 study in mice subjected to transient MCA occlusion found that HBO was significantly more effective in reducing markers of hypoxia (EF-5 and HIF-1α) in the ischemic penumbra [63]. Given the lack of human trials and somewhat contradictory nature of the above studies, additional research is needed to determine the ideal duration and modality of induced hyperoxia in AIS. Overall, however, it appears that while HBO may be a more effective modality for "neuroprotection", NBO is more practical and better tolerated by patients. Ultimately, a combination of NBO in the field followed by HBO upon hospital arrival may be the most effective approach to oxygen therapy in ischemic stroke.

11.7 Safety Concerns

In discussing the safety of oxygen therapy it is critical to differentiate between the safety of the *delivery method* and the safety of *hyperoxia*. With regards to the former, HBO requires the use of high-pressured chambers and places patients at risk for barotrauma (aural and pulmonary), progressive reversible myopia secondary to conformational changes in the lens, lower seizure thresholds, and claustrophobia. Barotrauma has been shown to be exceedingly rare in patients treated with HBO and is generally only seen in patients with underlying lung disease. The safety profile of invasive methods for oxygen delivery (e.g. aqueous oxygen solutions and perfluorocarbons) remains to be determined.

Hyperoxia can cause acute respiratory failure in patients suffering advanced chronic obstructive pulmonary disease. Additionally, in a normal brain, increased oxygen tension in the cerebral circulation can lead to vasoconstriction [92]. In the ischemic brain, however, as discussed above, hyperoxia may paradoxically act to shunt blood towards ischemic tissues [73, 93].

Prolonged oxygen administration can lead to increased generation of reactive oxygen radicals that can permanently damage the lung, eye, and brain [94]. Specifically, HBO given over prolonged periods has been shown to precipitate brain and spinal cord necrosis, neuronal loss, and limb paralysis [95]. This concern was demonstrated by a study by Mickel *et al*., in which gerbils exposed to 100% oxygen following temporary carotid occlusion increased lipid peroxidation and associated white-matter damage [96]. Studies in cancer patients have revealed that HBO can increase the toxicity of chemotherapeutic agents [95].

Critically, the above findings were most pronounced in patients receiving oxygen therapy for extended periods. Further, many of the studies cited used global (instead of focal) models of cerebral ischemia. In the setting of focal ischemia, the neuroprotectant effects of hyperoxia likely outweigh the free-radical toxicity, especially when shorter durations of therapy and lower pressures are used. In one study we demonstrated that NBO did not increase hydroethidine (a cellular marker of superoxide generation), did not increase indirect markers of oxidative stress (matrix metalloproteinase-2, MMP-9, heat-shock protein-32, protein carbonyl formation), and did not worsen blood–brain barrier damage [74]. These findings were replicated by Liu *et al*., who also found no increase in MMP-9 or caspase-8 levels [83, 84]. Importantly, there was no increase in cerebral hemorrhage when NBO was followed by rt-PA administration [82].

11.8 HBO and NBO in Other Conditions

The first major use of HBO was in deep-sea divers suffering from decompression sickness ("the bends") and arterial gas embolism [97–99]. Since that time, oxygen therapy has been successfully explored in a wide range of conditions, including wound healing, severe infections, myocardial infarction, and carbon-monoxide inhalation. Interestingly, both HBO and NBO have been studied for use following traumatic brain injury (TBI) in both acute and chronic settings [7, 100–105]. Importantly, hyperoxia following TBI has been shown to decrease intracranial pressure, improve brain lactate levels, reduce cerebral edema, and preserve the blood–brain barrier [104]. A recent prospective RCT performed by Rockswold *et al*. examined HBO versus NBO versus control in 69 patients with severe traumatic brain injury. Their results showed that both HBO and NBO acted favorably on markers of cerebral inflammation when compared with controls (Figure 11.4); HBO had a larger effect than NBO. The authors found no difference in CSF F2-isoprostane levels, microdialysate glycerol, or BAL inflammatory markers between the groups: all markers of oxygen toxicity [106]. A review of www.clinicaltrials.gov reveals at least nine ongoing clinical trials exploring the use of hyperoxia following TBI. The results of these studies may revolutionize the treatment of TBI, which accounts for up to 1 000 000 emergency department visits and costs >$56 billion in the USA annually. Lastly, hyperoxia has classically been used as a therapy in cluster headache. This use was supported by a recent trial published in 2009, in which 100% oxygen at 12 L/min was shown to be associated with more rapid pain relief in patients with cluster headache when compared to controls [107]. Additional trials of both HBO and NBO are being undertaken for use in cluster and migraine headaches.

11.9 Conclusion

The safety and clinical effectiveness of HBO in AIS patients remains uncertain at this time. The results of multiple RCTs have failed to show a clear benefit of HBO in cerebral ischemia, but these studies are flawed in trial design and execution. Animal studies have overwhelmingly shown promising results. Recent literature has suggested pretreatment with HBO in those at risk for stroke as a potential prophylactic "neuroprotectant".

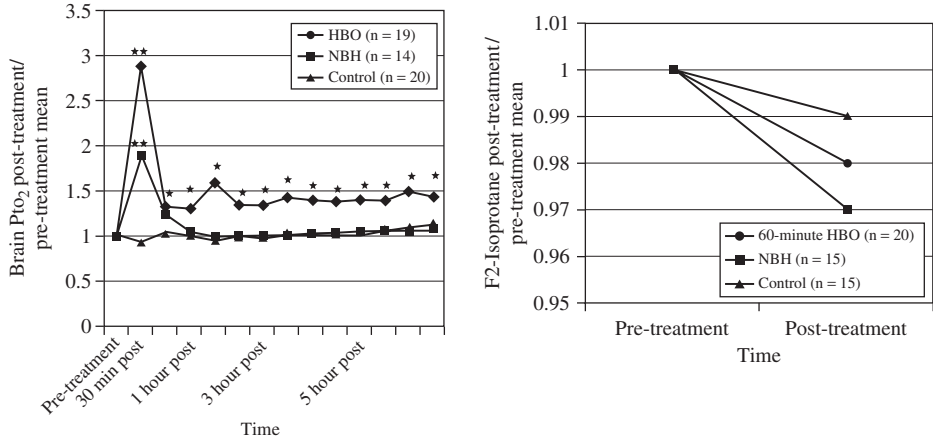

Figure 11.4 *Effects of HBO and normobaric hyperoxia (NBH) in patients with severe traumatic brain injury. Graphs show brain-tissue pO_2 and ventricular CSF F2-isoprostane levels with post-/pretreatment ratio means. A value >1 indicates the post-treatment value is > the pretreatment value, and a value <1 indicates the post-treatment value is < the pretreatment value. In the HBO group, compared with the control group, there was an increase of ~280% in brain-tissue pO_2 from pretreatment to 30 minutes post-treatment. The HBO group's brain-tissue pO_2 then remained higher than that in the control group by ~50% throughout the post-treatment period. In comparison with the control group, the NBH group had a 180% increase in brain-tissue pO_2 at 30 minutes post-treatment, but the brain-tissue pO_2 decreased to baseline levels within 1 hour after each treatment was completed. The levels of CSF F2-isoprostane did not change over time for either the HBO or the NBH group in comparison with the control group (*$p<0.05$ and **$p<0.0001$, compared with the control group; post, post-treatment). (Reproduced with permission from AANS.JNS. Rockswold, [106]).*

NBO therapy is more accessible, lower-cost, easier to administer, and more pleasant for patients than HBO. Although NBO does not result in the same level of pO_2 elevation as HBO, it appears to exert many of the same neuroprotective effects. Experimental studies have shown almost universally positive results (including potential for extending the reperfusion window) but human trials are ongoing.

SO therapy is widely used in the prehospital and emergency-department settings. Doses and durations of SO administration are variable. Few published studies have assessed the safety and efficacy of SO.

References

[1] Lloyd-Jones, D., Adams, R.J., Brown, T.M. *et al*. Executive summary: heart disease and stroke statistics. 2010 update: a report from the American Heart Association, *Circulation*, **121**, 948–954 (2010).

[2] The National Institute of Neurological Disorders and Stroke rt-PA Stroke Study Group. Tissue plasminogen activator for acute ischemic stroke, *N. Engl. J. Med*., **333**, 1581–1587 (1995).

[3] Lees, K.R., Bluhmki, E., von Kummer, R. *et al*. Time to treatment with intravenous alteplase and outcome in stroke: an updated pooled analysis of ECASS, ATLANTIS, NINDS, and EPITHET trials, *Lancet*, **375**, 1695–1703 (2010).

[4] Cheng, Y.D., Al-Khoury, L. and Zivin, J.A. Neuroprotection for ischemic stroke: two decades of success and failure, *NeuroRx*, **1**, 36–45 (2004).

[5] O'Collins, V.E., Macleod, M.R., Donnan, G.A. *et al*. 1026 experimental treatments in acute stroke, *Ann. Neurol*., **59**, 467–477 (2006).

[6] Hempel, F.G., Jobsis, F.F., LaManna, J.L. *et al*. Oxidation of cerebral cytochrome aa3 by oxygen plus carbon dioxide at hyperbaric pressures, *J. Appl. Physiol*., **43**, 873–879 (1977).

[7] Daugherty, W.P., Levasseur, J.E., Sun, D. *et al*. Effects of hyperbaric oxygen therapy on cerebral oxygenation and mitochondrial function following moderate lateral fluid-percussion injury in rats, *J. Neurosurg*., **101**, 499–504 (2004).

[8] Rockswold, S.B., Rockswold, G.L., Vargo, J.M. *et al*. Effects of hyperbaric oxygenation therapy on cerebral metabolism and intracranial pressure in severely brain injured patients, *J. Neurosurg*., **94**, 403–411 (2001).

[9] Schabitz, W.R., Schade, H., Heiland, S. *et al*. Neuroprotection by hyperbaric oxygenation after experimental focal cerebral ischemia monitored by MRI, *Stroke*, **35**, 1175–1179 (2004).

[10] Veltkamp, R., Siebing, D.A., Heiland, S. *et al*. Hyperbaric oxygen induces rapid protection against focal cerebral ischemia, *Brain Res*., **1037**, 134–138 (2005).

[11] Henninger, N., Kuppers-Tiedt, L., Sicard, K.M. *et al*. Neuroprotective effect of hyperbaric oxygen therapy monitored by MR-imaging after embolic stroke in rats, *Exp. Neurol*., **201**, 316–323 (2006).

[12] Lou, M., Zhang, H., Wang, J. *et al*. Hyperbaric oxygen treatment attenuated the decrease in regional glucose metabolism of rats subjected to focal cerebral ischemia: a high resolution positron emission tomography study, *Neuroscience*, **146**, 555–561 (2007).

[13] Singhal, A.B., Benner T., Roccatagliata, L. *et al*. A pilot study of normobaric oxygen therapy in acute ischemic stroke, *Stroke*, **36**, 797–802 (2005).

[14] Yin, D., Zhou, C., Kusaka, I. *et al*. Inhibition of apoptosis by hyperbaric oxygen in a rat focal cerebral ischemic model, *J. Cereb. Blood Flow Metab*., **23**, 855–864 (2003).

[15] Vlodavsky, E., Palzur, E., Feinsod, M. and Soustiel, J.F. Evaluation of the apoptosis-related proteins of the BCL-2 family in the traumatic penumbra area of the rat model of cerebral contusion, treated by hyperbaric oxygen therapy: a quantitative immunohistochemical study, *Acta Neuropathol*., **110**, 120–126 (2005).

[16] Calvert, J.W., Yin, W., Patel, M. *et al*. Hyperbaric oxygenation prevented brain injury induced by hypoxia-ischemia in a neonatal rat model, *Brain Res*., **951**, 1–8 (2002).

[17] Buras, J.A., Stahl, G.L., Svoboda, K.K. and Reenstra, W.R. Hyperbaric oxygen downregulates ICAM-1 expression induced by hypoxia and hypoglycemia: the role of NOS, *Am. J. Physiol. Cell. Physiol*., **278**, C292–C302 (2000).

[18] Yin, W.B.A., Mychaskiw, G. and Zhang, J.H. Down regulation of cox-2 is involved in hyperbaric oxygen treatment in a rat transient focal cerebral ischemia model, *Brain Res*., **926**, 165–171 (2002).

[19] Miljkovic-Lolic, M., Silbergleit, R., Fiskum, G. and Rosenthal, R.E. Neuroprotective effects of hyperbaric oxygen treatment in experimental focal cerebral ischemia are associated with reduced brain leukocyte myeloperoxidase activity, *Brain Res*., **971**, 90–94 (2003).
[20] Matchett, G.A., Martin, R.D. and Zhang, J.H. Hyperbaric oxygen therapy and cerebral ischemia: neuroprotective mechanisms, *Neurol. Res*., **31**, 114–121 (2009).
[21] Veltkamp, R., Siebing, D.A., Sun, L. *et al*. Hyperbaric oxygen reduces blood–brain barrier damage and edema after transient focal cerebral ischemia, *Stroke*, **36**, 1679–1683 (2005).
[22] Helms, A.K., Whelan, H.T. and Torbey, M.T. Hyperbaric oxygen therapy of cerebral ischemia, *Cerebrovasc. Dis*., **20**, 417–426 (2005).
[23] Rink, C., Roy, S., Khan, M. *et al*. Oxygen-sensitive outcomes and gene expression in acute ischemic stroke, *J. Cereb. Blood Flow Metab*., **30**, 1275–1287 (2010).
[24] Boerema, I., Meyne, N.G., Brummelkamp, W.H. *et al*. Life without blood, *Ned. Tijdschr. Geneeskd*., **104**, 949–954 (1960).
[25] Boerema, I. Pressurized oxygen for total body perfusion, *Lancet*, **ii**, 1459 (1961).
[26] Boerema, I. The use of hyperbaric oxygen, *Am. Heart J*., **69**, 289–292 (1965).
[27] Smith, G., Lawson, D.D., Renfrew, S. *et al*. Preservation of cerebral cortical activity by breathing oxygen at two atmospheres of pressure during cerebral ischemia, *Surg. Gynec. Obstetr*., **113**, 13–16 (1961).
[28] Illingworth, C. Treatment of arterial occlusion under oxygen at two atmospheres pressure, *Brit. Med. J*., **2**, 1271–1275 (1962).
[29] Neubauer, R.A. and End, E. Hyperbaric oxygenation as an adjunct therapy in strokes due to thrombosis: a review of 122 patients, *Stroke*, **11**, 297–300 (1980).
[30] Anderson, D.C., Bottini, A.G., Jagiella, W.M. *et al*. A pilot study of hyperbaric oxygen in the treatment of human stroke, *Stroke*, **22**, 1137–1142 (1991).
[31] Nighoghossian, N., Trouillas, P., Adeleine, P. and Salord, F. Hyperbaric oxygen in the treatment of acute ischemic stroke: a double-blind pilot study, *Stroke*, **26**, 1369–1372 (1995).
[32] Rusyniak, D.E., Kirk, M.A., May, J.D. *et al*. Hyperbaric oxygen therapy in acute ischemic stroke: results of the Hyperbaric Oxygen in Acute Ischemic Stroke Trial Pilot Study, *Stroke*, **34**, 571–574 (2003).
[33] Imai, K., Mori, T., Izumoto, H. *et al*. Hyperbaric oxygen combined with intravenous edaravone for treatment of acute embolic stroke: a pilot clinical trial, *Neurol. Med. Chir*., **46**, 373–378; disc. 378 (2006).
[34] Jain, K.K. and Toole, J.F. (eds). *Hyperacute Hyperbaric Oxygen Therapy for Stroke: Proceedings of a Conference Held at Bowman Gray School of Medicine, Winston-Salem, North Carolina, 2–4 Nov 1997*, Undersea and Hyperbaric Medical Society of the USA, Bethesda, MD, USA (1998).
[35] Jacobson, I. and Lawson, D.D. Effect of hyperbaric oxygen on experimental cerebral infarction in dog – with preliminary correlations of cerebral blood flow at 2 atmospheres of oxygen, *J. Neurosurg*., **20**, 849–859 (1963).
[36] Burt, J.T., Kapp, J.P. and Smith, R.R. Hyperbaric oxygen and cerebral infarction in the gerbil, *Surg. Neurol*., **28**, 265–268 (1987).

[37] Weinstein, P.R., Anderson, G.G. and Telles, D.A. Results of hyperbaric oxygen therapy during temporary middle cerebral artery occlusion in unanesthetized cats, *Neurosurgery*, **20**, 518–524 (1987).

[38] Kawamura, S., Yasui, N., Shirasawa, M. and Fukasawa, H. Therapeutic effects of hyperbaric oxygenation on acute focal cerebral ischemia in rats, *Surg. Neurol*., **34**, 101–106 (1990).

[39] Reitan, J.A., Kien, N.D., Thorup, S. and Corkill, G. Hyperbaric oxygen increases survival following carotid ligation in gerbils, *Stroke*, **21**, 119–123 (1990).

[40] Atochin, D.N., Fisher, D., Demchenko, I.T. and Thom, S.R. Neutrophil sequestration and the effect of hyperbaric oxygen in a rat model of temporary middle cerebral artery occlusion, *Undersea Hyperb. Med*., **27**, 185–190 (2000).

[41] Chang, C.F., Niu, K.C., Hoffer, B.J. *et al*. Hyperbaric oxygen therapy for treatment of postischemic stroke in adult rats, *Exp. Neurol*., **166**, 298–306 (2000).

[42] Prass, K., Wiegand, F., Schumann, P. *et al*. Hyperbaric oxygenation induced tolerance against focal cerebral ischemia in mice is strain dependent, *Brain Res*., **871**, 146–150 (2000).

[43] Sunami, K., Takeda, Y., Hashimoto, M. and Hirakawa, M. Hyperbaric oxygen reduces infarct volume in rats by increasing oxygen supply to the ischemic periphery, *Crit. Care Med*., **28**, 2831–2836 (2000).

[44] Veltkamp, R., Warner, D.S., Domoki, F. *et al*. Hyperbaric oxygen decreases infarct size and behavioral deficit after transient focal cerebral ischemia in rats, *Brain Res*., **853**, 68–73 (2000).

[45] Xiong, L., Zhu, Z., Dong, H. *et al*. Hyperbaric oxygen preconditioning induces neuroprotection against ischemia in transient not permanent middle cerebral artery occlusion rat model, *Chin. Med. J. (Engl)*., **113**, 836–839 (2000)

[46] Badr, A.E., Yin, W., Mychaskiw, G. and Zhang, J.H. Dual effect of HBO on cerebral infarction in MCAO rats, *Am. J. Physiol. Regul. Integr. Comp. Physiol*., **280**, R766–R770 (2001).

[47] Badr, A.E., Yin, W., Mychaskiw, G. and Zhang, J.H. Effect of hyperbaric oxygen on striatal metabolites: a microdialysis study in awake freely moving rats after MCA occlusion, *Brain Res*., **916**, 85–90 (2001)

[48] Hjelde, A., Hjelstuen, M., Haraldseth, O. *et al*. Hyperbaric oxygen and neutrophil accumulation/tissue damage during permanent focal cerebral ischaemia in rats, *Eur. J. Appl. Physiol*., **86**, 401–405 (2002).

[49] Yang, Z.J., Camporesi, C., Yang, X. *et al*. Hyperbaric oxygenation mitigates focal cerebral injury and reduces striatal dopamine release in a rat model of transient middle cerebral artery occlusion, *Eur. J. Appl. Physiol*., **87**, 101–107 (2002).

[50] Lou, M., Eschenfelder, C.C., Herdegen, T. *et al*. Therapeutic window for use of hyperbaric oxygenation in focal transient ischemia in rats, *Stroke*, **35**, 578–583 (2004).

[51] Gunther, A., Kuppers-Tiedt, L., Schneider, P.M. *et al*. Reduced infarct volume and differential effects on glial cell activation after hyperbaric oxygen treatment in rat permanent focal cerebral ischaemia, *Eur. J. Neurosci*., **21**, 3189–3194 (2005).

[52] Yin, D. and Zhang, J.H. Delayed and multiple hyperbaric oxygen treatments expand therapeutic window in rat focal cerebral ischemic model, *Neurocrit. Care*., **2**, 206–211 (2005).

[53] Veltkamp, R., Sun, L., Herrmann, O. *et al*. Oxygen therapy in permanent brain ischemia: potential and limitations, *Brain Res*., **1107**, 185–191 (2006).
[54] Acka, G., Sen, A., Canakci, Z. *et al*. Effect of combined therapy with hyperbaric oxygen and antioxidant on infarct volume after permanent focal cerebral ischemia, *Physiol. Res*., **56**, 369–373 (2007).
[55] Beynon, C., Sun, L., Marti, H.H. *et al*. Delayed hyperbaric oxygenation is more effective than early prolonged normobaric hyperoxia in experimental focal cerebral ischemia, *Neurosci. Lett*., **425**, 141–145 (2007).
[56] Hou, H., Grinberg, O., Williams, B. *et al*. The effect of oxygen therapy on brain damage and cerebral pO(2) in transient focal cerebral ischemia in the rat, *Physiol. Meas*., **28**, 963–976 (2007).
[57] Huang, Z.X., Kang, Z.M., Gu, G.J. *et al*. Therapeutic effects of hyperbaric oxygen in a rat model of endothelin-1-induced focal cerebral ischemia, *Brain Res*., **1153**, 204–213 (2007).
[58] Qin, Z., Karabiyikoglu, M., Hua, Y. *et al*. Hyperbaric oxygen-induced attenuation of hemorrhagic transformation after experimental focal transient cerebral ischemia, *Stroke*, **38**, 1362–1367 (2007).
[59] Eschenfelder, C.C., Krug, R., Yusofi, A.F. *et al*. Neuroprotection by oxygen in acute transient focal cerebral ischemia is dose dependent and shows superiority of hyperbaric oxygenation, *Cerebrovasc. Dis*., **25**, 193–201 (2008).
[60] Gu, G.J., Li, Y.P., Peng, Z.Y. *et al*. Mechanism of ischemic tolerance induced by hyperbaric oxygen preconditioning involves upregulation of hypoxia-inducible factor-1alpha and erythropoietin in rats, *J. Appl. Physiol*., **104**, 1185–1191 (2008).
[61] Li, J., Liu, W., Ding, S. *et al*. Hyperbaric oxygen preconditioning induces tolerance against brain ischemia-reperfusion injury by upregulation of antioxidant enzymes in rats, *Brain Res*., **1210**, 223–229 (2008).
[62] Li, Z., Liu, W., Kang, Z. *et al*. Mechanism of hyperbaric oxygen preconditioning in neonatal hypoxia-ischemia rat model, *Brain Res*., **1196**, 151–156 (2008).
[63] Sun, L., Marti, H.H. and Veltkamp, R. Hyperbaric oxygen reduces tissue hypoxia and hypoxia-inducible factor-1 alpha expression in focal cerebral ischemia, *Stroke*, **39**, 1000–1006 (2008).
[64] Singhal, A.B., Lo, E.H., Dalkara, T. and Moskowitz, M.A. Advances in stroke neuroprotection: hyperoxia and beyond, *Neuroimaging Clin. N. Am*., **15**, 697–720, xii–xiii (2005).
[65] Singhal, A.B. A review of oxygen therapy in ischemic stroke, *Neurol. Res*., **29**, 173–183 (2007).
[66] Singhal, A.B. Oxygen therapy in stroke: past, present, and future, *Int. J. Stroke*, **1**, 191–200 (2006).
[67] Zhang, J.H., Lo, T., Mychaskiw, G. and Colohan, A. Mechanisms of hyperbaric oxygen and neuroprotection in stroke, *Pathophysiology*, **12**, 63–77 (2005).
[68] Dong, H., Xiong, L., Zhu, Z. *et al*. Preconditioning with hyperbaric oxygen and hyperoxia induces tolerance against spinal cord ischemia in rabbits, *Anesthesiology*, **96**, 907–912 (2002).
[69] Wada, K., Miyazawa, T., Nomura, N. *et al*. Preferential conditions for and possible mechanisms of induction of ischemic tolerance by repeated hyperbaric oxygenation in gerbil hippocampus, *Neurosurgery*, **49**, 160–166; disc. 166–177 (2001).

[70] Zhou, C., Li, Y., Nanda, A. and Zhang, J.H. HBO suppresses Nogo-A, Ng-R, or RhoA expression in the cerebral cortex after global ischemia, *Biochem. Biophys. Res. Commun*., **309**, 368–376 (2003).

[71] Henninger, N. and Fisher, M. Normobaric hyperoxia: a promising approach to expand the time window for acute stroke treatment, *Cerebrovasc. Dis*., **21**, 134–136 (2006).

[72] Singhal, A.B. and Lo, E.H. Advances in emerging nondrug therapies for acute stroke 2007, *Stroke*, **39**, 289–291 (2008).

[73] Singhal, A.B., Dijkhuizen, R.M., Rosen, B,R. and Lo, E.H. Normobaric hyperoxia reduces MRI diffusion abnormalities and infarct size in experimental stroke, *Neurology*, **58**, 945–952 (2002).

[74] Singhal, A.B., Wang, X., Sumii, T. *et al*. Effects of normobaric hyperoxia in a rat model of focal cerebral ischemia-reperfusion, *J. Cereb. Blood Flow Metab*., **22**, 861–868 (2002).

[75] Flynn, E.P. and Auer, R.N. Eubaric hyperoxemia and experimental cerebral infarction, *Ann. Neurol*., **52**, 566–572 (2002).

[76] Liu, S., Shi, H., Liu, W. *et al*. Interstitial pO_2 in ischemic penumbra and core are differentially affected following transient focal cerebral ischemia in rats, *J. Cereb. Blood Flow Metab*., **24**, 343–349 (2004).

[77] Liu, S., Liu, W., Ding, W. *et al*. Electron paramagnetic resonance-guided normobaric hyperoxia treatment protects the brain by maintaining penumbral oxygenation in a rat model of transient focal cerebral ischemia, *J. Cereb. Blood Flow Metab*., **26**, 1274–1284 (2006).

[78] Fujiwara, N., Murata, Y., Arai, K. *et al*. Combination therapy with normobaric oxygen (NBO) plus thrombolysis in experimental ischemic stroke, *BMC Neurosci*., **10**, 79 (2009).

[79] Henninger, N., Bouley, J., Nelligan, J.M. *et al*. Normobaric hyperoxia delays perfusion/diffusion mismatch evolution, reduces infarct volume, and differentially affects neuronal cell death pathways after suture middle cerebral artery occlusion in rats, *J. Cereb. Blood Flow Metab*., **27**, 1632–1642 (2007).

[80] Kim, H.Y., Singhal, A.B. and Lo, E.H. Normobaric hyperoxia extends the reperfusion window in focal cerebral ischemia, *Ann. Neurol*., **57**, 571–575 (2005).

[81] Shin, H.K., Dunn, A.K., Jones, P.B. *et al*. Normobaric hyperoxia improves cerebral blood flow and oxygenation, and inhibits peri-infarct depolarizations in experimental focal ischaemia, *Brain*, **130**, 1631–1642 (2007).

[82] Henninger, N., Bratane, B.T., Bastan, B. *et al*. Normobaric hyperoxia and delayed tPA treatment in a rat embolic stroke model, *J. Cereb. Blood Flow Metab*., **29**, 119–129 (2009).

[83] Liu, W., Sood, R., Chen, Q. *et al*. Normobaric hyperoxia inhibits NADPH oxidase-mediated matrix metalloproteinase-9 induction in cerebral microvessels in experimental stroke, *J. Neurochem*., **107**, 1196–1205 (2008).

[84] Liu, W., Hendren, J., Qin, X. J. *et al*. Normobaric hyperoxia attenuates early blood-brain barrier disruption by inhibiting MMP-9-mediated occludin degradation in focal cerebral ischemia, *J. Neurochem*., **108**, 811–820 (2009).

[85] Nonaka, Y., Koumura, A., Hyakkoku, K. *et al*. Combination treatment with normobaric hyperoxia and cilostazol protects mice against focal cerebral

ischemia-induced neuronal damage better than each treatment alone, *J. Pharmacol. Exp. Ther*., **330**, 13–22 (2009).
[86] Bigdeli, M.R. Preconditioning with prolonged normobaric hyperoxia induces ischemic tolerance partly by upregulation of antioxidant enzymes in rat brain tissue, *Brain Res*., **1260**, 47–54 (2009).
[87] Bigdeli, M.R., Hajizadeh, S., Froozandeh, M. *et al*. Normobaric hyperoxia induces ischemic tolerance and upregulation of glutamate transporters in the rat brain and serum TNF-alpha level, *Exp. Neurol*., **212**, 298–306 (2008).
[88] Bigdeli, M.R., Hajizadeh, S., Froozandeh, M. *et al*. Prolonged and intermittent normobaric hyperoxia induce different degrees of ischemic tolerance in rat brain tissue, *Brain Res*., **1152**, 228–233 (2007).
[89] Adams, H.P. Jr, del Zoppo, G., Alberts, M.J. *et al*. Guidelines for the early management of adults with ischemic stroke: a guideline from the American Heart Association/American Stroke Association Stroke Council, Clinical Cardiology Council, Cardiovascular Radiology and Intervention Council, and the Atherosclerotic Peripheral Vascular Disease and Quality of Care Outcomes in Research Interdisciplinary Working Groups: the American Academy of Neurology affirms the value of this guideline as an educational tool for neurologists, *Stroke*, **38**, 1655–1711 (2007).
[90] Ronning, O.M. and Guldvog, B. Should stroke victims routinely receive supplemental oxygen? A quasi-randomized controlled trial, *Stroke*, **30**, 2033–2037 (1999).
[91] Chiu, E.H., Liu, C.S., Tan, T.Y. and Chang, K.C. Venturi mask adjuvant oxygen therapy in severe acute ischemic stroke, *Arch. Neurol*., **63**, 741–744 (2006).
[92] Watson, N.A., Beards, S.C., Altaf, N. *et al*. The effect of hyperoxia on cerebral blood flow: a study in healthy volunteers using magnetic resonance phase-contrast angiography, *Eur. J. Anaesthesiol*., **17**, 152–159 (2000).
[93] Nakajima, S., Meyer, J.S., Amano, T. *et al*. Cerebral vasomotor responsiveness during 100% oxygen inhalation in cerebral ischemia, *Arch. Neurol*., **40**, 271–276 (1983)
[94] Jenkinson, S.G. Oxygen toxicity, *New Horiz*., **1**, 504–511 (1993).
[95] Balentine, J.D. Pathologic effects of exposure to high oxygen tensions: a review, *N. Engl. J. Med*., **275**, 1038–1040 (1966).
[96] Mickel, H.S., Vaishnav, Y.N., Kempski, O. *et al*. Breathing 100% oxygen after global brain ischemia in Mongolian gerbils results in increased lipid peroxidation and increased mortality, *Stroke*, **18**, 426–430 (1987).
[97] Gill, A.L. and Bell, C.N. Hyperbaric oxygen: its uses, mechanisms of action and outcomes, *QJM*, **97**, 385–395 (2004).
[98] Benson, J., Adkinson, C. and Collier, R. Hyperbaric oxygen therapy of iatrogenic cerebral arterial gas embolism, *Undersea Hyperb. Med*., **30**, 117–126 (2003).
[99] Yarbrough, O.D. and Behnke, A.R. Treatment of compressed air illness utilizing oxygen, *J. Indust. Hyg. Toxicol*., **21**, 213–218 (1939).
[100] Nortje, J., Coles, J.P., Timofeev, I. *et al*. Effect of hyperoxia on regional oxygenation and metabolism after severe traumatic brain injury: preliminary findings, *Crit. Care Med*., **36**, 273–281 (2008).

[101] Palzur, E., Vlodavsky, E., Mulla, H. *et al*. Hyperbaric oxygen therapy for reduction of secondary brain damage in head injury: an animal model of brain contusion, *J. Neurotrauma*, **21**, 41–48 (2004).

[102] Rockswold, G.L., Ford, S.E., Anderson, D.C. *et al*. Results of a prospective randomized trial for treatment of severely brain-injured patients with hyperbaric oxygen, *J. Neurosurg*., **76**, 929–934 (1992).

[103] Tisdall, M.M., Tachtsidis, I., Leung, T.S. *et al*. Increase in cerebral aerobic metabolism by normobaric hyperoxia after traumatic brain injury, *J. Neurosurg*., **109**, 424–432 (2008).

[104] Tolias, C.M., Reinert, M., Seiler, R. *et al*. Normobaric hyperoxia: induced improvement in cerebral metabolism and reduction in intracranial pressure in patients with severe head injury: a prospective historical cohort-matched study, *J. Neurosurg*., **101**, 435–444 (2004).

[105] Zhou, Z., Daugherty, W.P., Sun, D. *et al*. Protection of mitochondrial function and improvement in cognitive recovery in rats treated with hyperbaric oxygen following lateral fluid-percussion injury, *J. Neurosurg*., **106**, 687–694 (2007).

[106] Rockswold, S.B., Rockswold, G.L., Zaun, D.A. *et al*. A prospective, randomized clinical trial to compare the effect of hyperbaric to normobaric hyperoxia on cerebral metabolism, intracranial pressure, and oxygen toxicity in severe traumatic brain injury, *J. Neurosurg*., **112**, 1080–1094 (2010).

[107] Cohen, A.S., Burns, B. and Goadsby, P.J. High-flow oxygen for treatment of cluster headache: a randomized trial, *JAMA*, **302**, 2451–2457 (2009).

12

Transfusion Therapy in β Thalassemia and Sickle Cell Disease

Carlo Brugnara[1] *and Lucia De Franceschi*[2]

[1]*Departments of Pathology and Laboratory Medicine, Children's Hospital, Harvard Medical School, Boston, MA, USA*

[2]*Department of Medicine, University of Verona, Policlinico GB Rossi, Verona, Italy*

12.1 Introduction

Hereditary disorders of production and assembly of hemoglobin (Hb) chains represent the most common cause of monogenic inherited anemia. In fact, 4.83% of the world population has a globin variant: 1.67% is heterozygous for α or β thalassemia and 1.92% for sickle hemoglobin (HbS) [1–5]. Different gene mutations and pathophysiological events underlie sickle cell disease (SCD) and β thalassemia: both diseases are characterized by an abnormal erythrocyte cellular environment, which is responsible for premature red cell senescence and reduced erythrocyte survival. Thus, specific transfusion strategies have been designed for both hemoglobinopathies.

12.2 β Thalassemia and Transfusion

In β thalassemia, a variety of genetic mutations or deletions in either the β globin gene or in regulatory elements of its activity result in reduced or absent production of β globin chains, one of the two subunits, which come together in a tetramer to form adult hemoglobin A (HbA). While the disease is for all practical purposes clinically asymptomatic in the heterozygous state, it leads to severe anemia when the

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production of β chains is affected in both genetic loci. The anemia of homozygous β thalassemia is due to the combination of two factors: reduced survival of circulating erythrocytes, and ineffective erythropoiesis. In circulating red cells, the membrane damage induced by the excess of free α chains plays a crucial role in the shortening of erythrocyte life-span, but the relative contribution of the multiple membrane alterations to the pathogenesis of this disease still remains undetermined. Red cells from β-thalassemia intermedia patients show membrane clusters of hemichrome and band 3, presumably as a result of oxidative injury. Immunoglobulin and complement components localize at the membrane surface over these clusters, mediating the removal of the damaged β thalassemic erythrocytes by macrophages (Figure 12.1) [6–11]. In addition, membrane oxidative damage profoundly affects the regulation of ion transport across the red cell membrane, resulting in abnormal activation of K-Cl cotransport (KCC), a gradient-driven cation transport system that promotes K^+ and water loss from red cells. KCC activation is responsible for the reduced red cell K^+ content observed in β thalassemia (Figure 12.1) [12, 13]. Membrane lipid peroxidation, loss of phospholipid asymmetry, and externalization of phosphatidylserine (PS) have been demonstrated in β thalassemic erythrocytes. PS externalization in β thalassemic erythrocytes is believed to play a significant role in their premature removal and reduced survival. PS externalization may promote macrophage recognition with removal

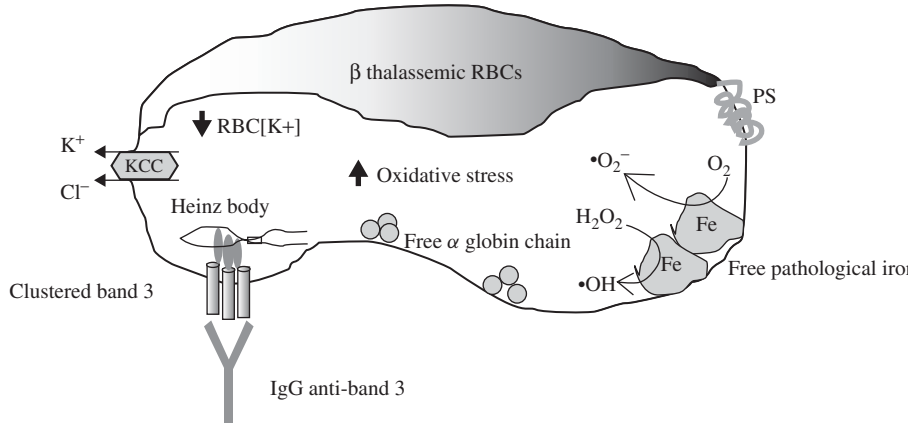

Figure 12.1 *Schematic diagram of abnormalities observed in β thalassemic red blood cells (RBCs). The presence of pathological free iron (Fe) adjacent to the membrane is involved in the Fenton reaction, producing reactive oxygen species (ROS, $\bullet O_2^-$), which contribute to the pro-oxidant environment of β thalassemic RBCs. The unbalance in α/β chain synthesis results in aggregation of highly oxidative α chains. The pro-oxidant environment is responsible for protein and lipid oxidative damage, favoring abnormal clustering of red cell-membrane proteins such as band 3 and exposure of PS. The abnormally clustered band 3 is recognized by anti-band 3 antibodies (IgG type). The severely damaged β thalassemic red cells have short life-spans and are removed by macrophages of the reticulo-endothelial systems through PS exposure and IgG anti-band 3-mediated mechanisms. The oxidative stress abnormally activates the KCC, which promotes K^+, Cl^-, and water loss, contributing to the reduced red cell K^+ content that characterizes β thalassemic red cells.*

of erythrocytes, cell apoptosis, and activation of coagulation. In human β thalassemia, the proportion of PS-positive erythrocytes varies from patient to patient and appears to be either distributed over the entire cell membrane or localized in areas with abundant deposits of α globin chains (Figure 12.1) [6, 7, 9, 10]. Different factors may modulate the extent of membrane oxidative damage and loss of phospholipid asymmetry, such as the co-inheritance of other mutations affecting the production of β or α chains, or the presence/absence of the spleen [11, 14]. The ineffective erythropoiesis can be massive in homozygous thalassemias, with most of the maturing erythroblasts being unable to complete their full differentiation into reticulocytes and undergoing cell death and destruction within the bone marrow. The resulting marrow hyperplasia plays a major role in the clinical manifestation of untreated severe thalassemias.

The clinical management of β thalassemia major is mainly based on chronic transfusion, unless conditions exist for a curative bone marrow transplantation. Patients are transfused generally at a pre-transfusion Hb level of 9–10 g/dL, aiming for a post-transfusional level of 13–14 g/dL. This approach prevents growth failure, organ damage, and bone deformities, with an improvement of patient quality of life (http://www.thalassemia.org.cy) [15]. The evaluation of transfusion therapy should be routinely carried out, considering pre- and post-transfusion patient Hb, hematocrit of the transfused units, and the interval between transfusions. These data allow the physician to establish the red cell requirement and monitor the accompanying iron load. With chronic transfusion, the amount of iron contained in the transfused red cells vastly exceeds the body requirements, resulting in iron overload and secondary hemochromatosis with related organ damage. If iron overload is left untreated, the accompanying damage of organs such as liver and heart is a major cause of death [16]. In β thalassemia, the spleen plays a crucial role in removal of damaged and precociously senescent red cells: thus, an increased consumption of transfused red cells should always be carefully considered. Once other additional hemolytic causes, such as immuno-mediated hemolysis, have been excluded, the increased transfusion requirement (more than 200 mL RBC/kg/year) is an indication for splenectomy. Other indications for splenectomy in β thalassemia major patients are symptoms of splenic enlargement, leucopenia, and/or thrombocytopenia.

Since β thalassemia patients are chronically transfused for life, the red cell units transfused need an extensive red cell antigen typing including at least Rh antigens, Duffy, Kidd, and Kell, to reduce alloimmunization against the transfused red cells. In addition, the general practice is to transfuse red cell units that are less than 2 weeks old, to avoid possible red cell changes due to longer storage, which can reduce their survival in peripheral circulation and may favor alloimmunization [17, 18]. A small subgroup of β thalassemia patients may develop sensitization to plasma proteins; in these cases, the use of washed red cells may facilitate the transfusional management.

In β thalassemia intermedia patients, the start of transfusional therapy is mainly determined by the severity of anemia, the appearance of growth failure and development, and extramedullary erythropoiesis [19–21].

In severe β thalassemia the high morbidity and mortality are mainly related to multiple organ damage, in part due to iron overload induced by the chronic transfusion therapy. Thus, iron chelation therapy represents an important approach to preventing these life-threatening complications for β thalassemia patients.

12.3 Sickle Cell Disease and Transfusion

A mutation in the gene for β globin resulting in the substitution of the native glutamic acid in the sixth amino acid position with valine is the proximate cause of SCD. HbS shows peculiar biochemical properties, and polymerizes when deoxygenated. Studies of the kinetics of HbS polymerization following deoxygenation have shown that the kinetics of polymer formation is a high-order exponential function of Hb concentration, thus demonstrating the crucial role of cellular HbS concentration in sickling. HbS polymerization is associated with a reduction in cell ion and water content (cell dehydration), increased red cell density, and further acceleration of HbS polymerization [22–24]. Dense, dehydrated erythrocytes are likely to undergo instant polymerization in conditions of mild hypoxia due to their high HbS concentration, and HbS polymers may be formed under normal oxygen pressure.

Pathophysiological studies have shown that the dense, dehydrated red cells play a central role in acute and chronic clinical manifestations of SCD, in which intravascular sickling in capillaries and small vessels leads to vaso-occlusion and impaired blood flow [22, 25]. However, the persistent membrane damage associated with HbS polymerization also favors the generation of distorted rigid cells and further contributes to vaso-occlusive events (VOCs) and cell destruction in the peripheral circulation. Thus, the two main clinical manifestation of SCD are the chronic hemolytic anemia and the acute VOCs (Figure 12.2).

SCD patients with complicated clinical manifestation are placed under hydroxyurea (HU) treatment, but the transfusion approach is used in management of both acute and chronic sickle cell-related clinical manifestations. Transfusional therapy has two major goals in SCD: (i) to restore the Hb levels in patients with acute exacerbation of the anemia, such as aplastic anemia or acute splenic sequestration in children with SCD; and (ii) to reduce the HbS in order to prevent sickling and decrease VOC complications such as ACS or stroke [26–28]. The management of transfusion therapy in SCD patients during acute events requires monitoring of both total Hb levels and HbS percentage. The goal is to restore the patient's Hb levels (not more than 10–11 g/dL) before the acute event in order to prevent possible complications related to blood hyperviscosity, while the percentage of HbS reflects the success of transfusion therapy, with HbS levels being maintained below 40% to avoid sickle-cell related complications [29–31]. There is still a large debate in the hematological community on clinical indication for transfusion therapy in SCD. However, three different transfusional strategies are available, with the following indications: (i) simple transfusion on demand, such as for symptomatic anemia, acute neurological events, acute chest syndrome (ACS), acute splenic or hepatic sequestration, or preparation for major surgery; (ii) chronic simple transfusion for prevention of recurrent stroke or ACS, symptomatic anemia with renal failure unresponsive to erythropoietin, or pulmonary hypertension; and (iii) exchange transfusion for treatment of acute neurological events, severe ACS, preparation for major surgery, or chronically to avoid iron overload in place of simple transfusion treatment [32, 33]. In addition to these conditions, there is a group of clinical issues related to SCD for which the use of transfusion therapy is still controversial, such as ophthalmological complication, recurrent pain events not responsive to HU treatment, recurrent priapism, and acute priapism.

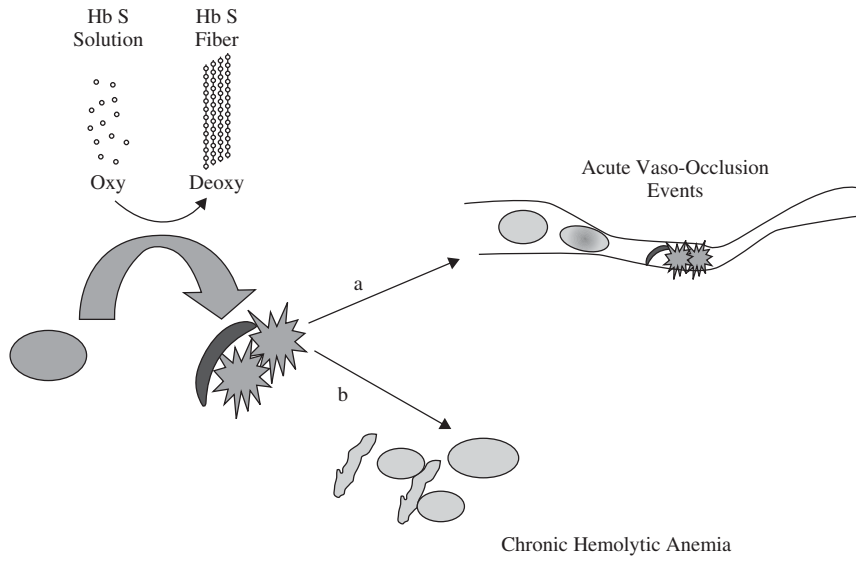

Figure 12.2 *Schematic diagram of the two major clinical manifestations of sickle cell disease. Hemoglobin S (HbS) under oxygenation (Oxy) condition is soluble in the cytoplasm, but when deoxygenated (Deoxy), HbS polymerizes, generating rigid fibers responsible for generation of abnormal red cell morphology (sickle red cells). (a) The microvascular system is the critical area where these events occur. The deoxygenated, dense, and abnormal red cells can easily adhere to the activated vascular endothelial surface, promoting a decreased flow rate, which then allows the entrapment of other sickled red cells and the development of acute VOCs. (b) The cyclic polymerization–depolymerization events result in chronic red cell membrane and lipid damage, with generation of distorted, dense red cells that show reduced red cell survival in the peripheral circulation and are responsible for the chronic hemolytic anemia characterizing SCD.*

The limited number of studies and the absence of clinical randomized trials mean these problems remain unsolved.

One of the major clinical complications of SCD is ACS, defined by the appearance of new pulmonary infiltrate and acute respiratory symptoms. ACS is associated with high mortality rate in young adult SCD patients [34, 35]. The transfusion approach in ACS might be either simple or exchange transfusion whenever there is respiratory compromise or general clinical deterioration. Vichinsky *et al*. have shown that the patient outcome following ACS is mostly related to age: more complications appeared in patients older than 20 years than in the younger SCD population [36]. An ancillary analysis of data from the STOP study (Stroke Prevention Trial in Sickle Cell Anemia) has shown that SCD children on transfusion therapy have a significant reduction in the frequency of ACS compared to the control group [37]. In another study, Hankins *et al*. reported a reduction of the incidence of ACS in SCD children under a chronic transfusion program, without significant changes in ACS clinical severity or in the period of hospitalization [38].

Another dramatic clinical complication of SCD is the ischemic stroke, especially when SCD children are involved. To prevent acute ischemic stroke in children with high risk based on transcranial Doppler (TCD) blood-flow velocity determination ($\geq$200 cm/s),

chronic transfusion treatment is warranted, with the objective of maintaining HbS <30% (multicentric STOP study, 1995–2000) [32, 39–43]. The STOP II study (2000–2005) showed that when chronic transfusion treatment was stopped in these patients, there was a reversion of the abnormalities in TCD to the high range of stroke, indicating the need for chronic transfusion treatment [39–43]. At present, the main problem is the long-term management of these patients, related to iron overload due to chronic transfusion therapy and alloimmunization.

Transfusion therapy in SCD is an important component in the management of SCD patients who need major surgery. In fact, studies have shown that administration of transfusions before surgery reduces the postoperative complications such as painful crisis or ACS [32, 44–46]. At present there are no data available to define the respective advantages or disadvantages of the two main transfusional approaches, which are either an intensive transfusion regimen or a more limited transfusion approach to maintain Hb levels of 10 g/dL [32, 44–46]. Vichinsky *et al*. have analyzed the surgery outcomes of young SCD patients (age 20) placed under either one of these two transfusional approaches. They showed that complications such as alloimmunization and hemolytic transfusion reactions were more prevalent in patients undergoing the more intensive transfusion program [44]. However, no data are available on other SCD populations. In support of the use of transfusion before surgery is the analysis of a series of intra-abdominal surgery procedures on SCD patients, showing increased complications such as painful crisis or ACS in not-transfused SCD subjects compared to transfused ones [32, 44–46]. To date, there is no solid evidence that the automated exchange transfusion approach has any major advantages compared to simple transfusion for SCD patients, except for the possible reduction in long-term iron overload. Recently, two reports have described intraoperative exchange transfusion before cardiopulmonary bypass in SCD patients undergoing open-heart surgery, with reduced red cell and clotting factor transfusion and prevention of cardiovascular stress associated with transfusion therapy induced before the operation [47, 48].

The management of pregnancy is still the subject of intense discussions in the scientific community, mainly due to the limited number of well-designed clinical trials. SCD women show increased tendency to preterm labor and pre-eclampsia and low-birth-weight babies, with neonatal mortality rate <5% [32, 49–53]. Villers *et al*. have recently analyzed the morbidity associated with SCD in pregnancy, showing increased complication rates related to either SCD or pregnancy complications such as abruption or ante-partum bleeding, but also cardiomiopathy and pulmonary hypertension at the delivery [54]. Studies in pregnant SCD women placed under either prophylactic chronic erythrocytopheresis or simple transfusion show a reduction in acute painful events, but none of these studies have clearly addressed whether these strategies yield any amelioration of babies' weight or perinatal mortality [32, 49–53]. Recently, Grossetti *et al*. analyzed a series of 55 pregnancies (1992–2004) treated with prophylactic simple transfusion, showing a reduction of acute VOCs and preterm delivers [55]. Since none of the studies were randomized, the use of prophylactic transfusion therapy in SCD patients during pregnancy still depends on physician judgment. In addition to prophylactic transfusion therapy, there are other indications for transfusion in pregnant SCD women: (i) the presence of anemia with cardiac or respiratory compromise; (ii) the severity of sickle cell-related clinical manifestations (ACS); (iii) refractory eclampsia; and (iv) preparation for Cesarean section [32, 49–53].

SCD patients undergoing a chronic transfusion program should be transfused with red cell units only after an extended red cell antigen typing is performed, including at least Rh antigens, Duffy, Kidd, and Kell, to reduce alloimmunization against transfused red cells. In addition, a growing problem for multiply transfused patients with SCD is immunization against Fy(a−, b−) blood antigen, which differs in people of African descent [56–58]. In fact, Africans show a complete absence of the molecule carrying the Duffy blood-group antigens due to a point mutation on GATA-1 on the binding site for Duffy gene promoter. The Fy(b−) phenotype results from the positive pressure of Malaria in the endemic areas [56–58]. Patients with SCD disease are mainly of African descent (Fy(a−, b−)), with high risk of developing multiple alloantibodies when placed in a chronic transfusion program due to the fact that the large majority of donors are Caucasian (Fy(a+, b+)) [59]. Genotyping of donors with a DNA array has been suggested to determine polymorphisms of the more frequent blood-group systems in order to identify a pool of blood donors to be used for SCD patients in order to reduce alloimmunization and transfusion reactions [60].

A clinically relevant complication of transfusion treatment in SCD is the delayed hemolytic transfusion reaction (DHTR). DHTR is a life-threatening complication characterized by acute hemolytic crisis due to the destruction of both transfused and endogenous red cells, associated with worsening of SCD symptoms [61–64]. The mechanism underling DHTR in SCD has not been completely defined, even if the presence of alloantibodies against red cells has been recognized as an important component of DHTR. Since SCD red cells show reduced red cell survival, mediated by reticuloendothelial removal via PS exposure and anti-band 3 antibody, and by intravascular hemolysis via activation of the alternative complement pathway, transfusion might favor auto-immunization against red cells in the context of alloantibodies production in SCD [61–64]. Recently, Chadebech *et al*. have suggested that in DHTR the transfused red cells might be severely damaged in the peripheral circulation of SCD patients, based on the increased percentage of PS-positive red cells measured after transfusion in the absence of detectable antibody [65]. Treatment of DHTR depends on the rate of hemolysis and the degree of anemia; subsequent transfusion may be required, with possible associated short-term immunoglobulin and methylprednisolone treatment or the anti-CD20 monoclonal antibody, rituximab, as second-line therapy [64, 66].

In SCD patients, a long-term complication of simple transfusion is chronic iron overload, when the amount of iron introduced by transfusion exceeds the capacity to transport and store iron in the body. Thus, iron chelation therapy should be considered in chronically SCD-transfused patients. Recently, Vichinsky *et al*. have reported a high proportion of SCD patients with hemosiderosis in a chronically transfused population of SCD patients, suggesting the need for better monitoring and treatment of iron overload in SCD patients [67].

12.4 Iron Chelation Tools

Three iron chelators are clinically available for use in chronically transfused patients: deferoxamine mesylate (desferoxamine, DFO, Desferal), deferipone (Ferriprox), and deferasirox (Exjade), but only deferoxamine and deferasirox have been approved by the

Food and Drug Administration in the USA [16, 68–73]. The current reference iron chelation therapy is that based on deferoxamine, which has been extensively used in patients with iron overload, showing significant morbidity and mortality benefits [74–77]. However, the frequent and prolonged subcutaneous infusion impacts on patient quality of life and compliance [74–77]. Thus the development of two new oral iron chelators has increased the possibility of effectively treating iron overload in these patients.

Deferoxamine is characterized by a very short plasma half-life (5–10 minutes) and needs a prolonged subcutaneous administration over a period of 8–12 hours in order to obtain an effective iron chelation [16, 78]. The routes of excretion are via urine and stools; major side effects are related to potential ear, eye, and neurological toxicity, and arthropathy [71]. The cardiac disease frequently observed in β thalassemic patients can be reversed by intensive infusion programs [71].

Deferipone is characterized by a plasma life of 47–134 minutes; thus it needs three times daily administration. The excretion is primarily by urine [71, 79, 80]. The major side effects are agranulocytosis, muscle, skeletal, and joint pain, and zinc deficiency [71, 79, 80]. A major concern in the chronic use of this compound is the lack of controlled long-term observations on its safety. Studies have shown that deferipone is effective in removing heart iron [72, 73]. Combined chelation with deferroxamine and deferipone has been evaluated in β thalassemic major patients with severe cardiac or endocrine complications, indicating amelioration of left-ventricular diastolic function and some beneficial effects on glucose intolerance [19, 73, 81].

Deferasirox is the most recently approved oral iron chelator. It has a plasma half-life of 8–16 hours, allowing a mono-dose administration for 24 hours' iron chelation [16, 68–70]. The primary route of excretion is the stool and the most common side effects are gastrointestinal symptoms and rash. The efficacy of deferasirox in iron chelation is similar to that of deferoxamine in chronically transfused patients, and it has been shown to effectively chelate myocardial iron deposition [16, 68–70, 79]. Renal and liver functions should be monitored monthly.

12.5 Conclusion

Transfusion therapy represents an essential therapeutic modality for both homozygous β thalassemia and SCD. The growing number of patients treated with chronic transfusion therapy is an implicit recognition of the success of this approach, which has extended life expectancy dramatically for patients with severe β thalassemias and has reduced mortality and morbidity in patients with sickle cell disease. Challenges remain in the selection of proper transfusion strategies, prevention of transfusion-related complications, and management and prevention of iron overload.

References

[1] Rund, D. and Rachmilewitz, E. Beta-thalassemia, *N. Engl. J. Med*., **353**, 1135–1146 (2005).

[2] Weatherall, D.J. The global problem of genetic disease, *Ann. Hum. Biol*., **32**, 117–122 (2005).

[3] Weatherall, D.J. and Clegg, J.B. Inherited haemoglobin disorders: an increasing global health problem, *Bull. World Health Organ*., **79**, 704–712 (2001).
[4] Modell, B. and Darlison, M. Global epidemiology of haemoglobin disorders and derived service indicators, *Bull. World. Health. Organ*., **86**, 480–487 (2008).
[5] Brousseau, D.C., Panepinto, J.A., Nimmer, M. and Hoffmann, R.G. The number of people with sickle-cell disease in the United States: national and state estimates, *Am. J. Hematol*., **85**, 77–78 (2010).
[6] Kuypers, F.A., Yuan, J., Lewis, R.A. *et al*. Membrane phospholipid asymmetry in human thalassemia, *Blood*, **91**, 3044–3051 (1998).
[7] Advani, R., Rubin, E., Mohandas, N. and Schrier, S.L. Oxidative red blood cell membrane injury in the pathophysiology of severe mouse beta-thalassemia, *Blood*, **79**, 1064–1067 (1992).
[8] Schrier, S.L., Bunyaratvej, A., Khuhapinant, A. *et al*. The unusual pathobiology of hemoglobin constant spring red blood cells, *Blood*, **89**, 1762–1769 (1997).
[9] Browne, P.V., Shalev, O., Kuypers, F.A. *et al*. Removal of erythrocyte membrane iron in vivo ameliorates the pathobiology of murine thalassemia, *J. Clin. Invest*., **100**, 1459–1464 (1997).
[10] Schrier, S.L. and Mohandas, N. Globin-chain specificity of oxidation-induced changes in red blood cell membrane properties, *Blood*, **79**, 1586–1592 (1992).
[11] Mannu, F., Arese, P., Cappellini, M.D. *et al*. Role of hemichrome binding to erythrocyte membrane in the generation of band-3 alterations in beta-thalassemia intermedia erythrocytes, *Blood*, **86**, 2014–2020 (1995).
[12] Olivieri, O., De Franceschi, L., Capellini, M.D. *et al*. Oxidative damage and erythrocyte membrane transport abnormalities in thalassemias, *Blood*, **84**, 315–320 (1994).
[13] De Franceschi, L., Turrini, F., Honczarenko, M. *et al*. In vivo reduction of erythrocyte oxidant stress in a murine model of beta-thalassemia, *Haematologica*, **89**, 1287–1298 (2004).
[14] Cappellini, M.D., Robbiolo, L., Bottasso, B.M. *et al*. Venous thromboembolism and hypercoagulability in splenectomized patients with thalassaemia intermedia, *Br. J. Haematol*., **111**, 467–473 (2000).
[15] Cazzola, M., Borgna-Pignatti, C., Locatelli, F. *et al*. A moderate transfusion regimen may reduce iron loading in beta-thalassemia major without producing excessive expansion of erythropoiesis, *Transfusion*, **37**, 135–140 (1997).
[16] Shander, A., Cappellini, M.D. and Goodnough, L.T. Iron overload and toxicity: the hidden risk of multiple blood transfusions, *Vox. Sang*., **97**, 185–197 (2009).
[17] Dzik, W. Fresh blood for everyone? Balancing availability and quality of stored RBCs, *Transfus. Med*., **18**, 260–265 (2008).
[18] Koch, C.G., Li, L., Sessler, D.I. *et al*. Duration of red-cell storage and complications after cardiac surgery, *N. Engl. J. Med*., **358**, 1229–1239 (2008).
[19] Farmaki, K., Angelopoulos, N., Anagnostopoulos, G. *et al*. Effect of enhanced iron chelation therapy on glucose metabolism in patients with beta-thalassaemia major, *Br. J. Haematol*., **134**, 438–444 (2006).
[20] Velati, C., Romano, L., Fomiatti, L. *et al*. Impact of nucleic acid testing for hepatitis B virus, hepatitis C virus, and human immunodeficiency virus on the safety of blood supply in Italy, a 6-year survey, *Transfusion*, **48**, 2205–2213 (2008).

[21] Angelucci, E., Muretto, P., Nicolucci, A. *et al*. Effects of iron overload and hepatitis C virus positivity in determining progression of liver fibrosis in thalassemia following bone marrow transplantation, *Blood*, **100**, 17–21 (2002).
[22] Steinberg, M.H. Management of sickle cell disease, *N. Engl. J. Med*., **340**, 1021–1030 (1999).
[23] Eaton, W.A. and Hofrichter, J. Sickle cell hemoglobin polymerization, *Adv. Protein Chem*., **40**, 63–279 (1990).
[24] Ballas, S.K. and Smith, E.D. Red blood cell changes during the evolution of the sickle cell painful crisis, *Blood*, **79**, 2154–2163 (1992).
[25] Solovey, A.A., Solovey, A.N., Harkness, J. and Hebbel, R.P. Modulation of endothelial cell activation in sickle cell disease: a pilot study, *Blood*, **97**, 1937–1941 (2001).
[26] Charache, S., Terrin, M.L., Moore, R.D. *et al*. Design of the multicenter study of hydroxyurea in sickle cell anemia. Investigators of the Multicenter Study of Hydroxyurea, *Control. Clin. Trials*, **16**, 432–446 (1995).
[27] Charache, S., Terrin, M.L., Moore, R.D. *et al*. Effect of hydroxyurea on the frequency of painful crises in sickle cell anemia. Investigators of the Multicenter Study of Hydroxyurea in Sickle Cell Anemia, *N. Engl. J. Med*., **332**, 1317–1322 (1995).
[28] Platt, O.S. Hydroxyurea for the treatment of sickle cell anemia, *N. Engl. J. Med*., **358**, 1362–1369 (2008).
[29] Jan, K., Usami, S. and Smith, J.A. Effects of transfusion on rheological properties of blood in sickle cell anemia, *Transfusion*, **22**, 17–20 (1982).
[30] Schmalzer, E.A., Lee, J.O., Brown, A.K. *et al*. Viscosity of mixtures of sickle and normal red cells at varying hematocrit levels: implications for transfusion, *Transfusion*, **27**, 228–233 (1987).
[31] Alexy, T., Pais, E., Armstrong, J.K. *et al*. Rheologic behavior of sickle and normal red blood cell mixtures in sickle plasma: implications for transfusion therapy, *Transfusion*, **46**, 912–918 (2006).
[32] Lottenberg, R. and Hassell, K.L. An evidence-based approach to the treatment of adults with sickle cell disease, *Hematology Am. Soc. Hematol. Educ. Program*., 58–65 (2005).
[33] Vichinsky, E.P., Luban, N.L., Wright, E. *et al*. Prospective RBC phenotype matching in a stroke-prevention trial in sickle cell anemia: a multicenter transfusion trial, *Transfusion*, **41**, 1086–1092 (2001).
[34] Platt, O.S., Brambilla, D.J., Rosse, W.F. *et al*. Mortality in sickle cell disease: life expectancy and risk factors for early death, *N. Engl. J. Med*., **330**, 1639–1644 (1994).
[35] Platt, O.S., Thorington, B.D., Brambilla, D.J. *et al*. Pain in sickle cell disease: rates and risk factors, *N. Engl. J. Med*., **325**, 11–16 (1991).
[36] Vichinsky, E.P., Neumayr, L.D., Earles, A.N. *et al*. Causes and outcomes of the acute chest syndrome in sickle cell disease. National Acute Chest Syndrome Study Group, *N. Engl. J. Med*., **342**, 1855–1865 (2000).
[37] Miller, S.T., Wright, E., Abboud, M. *et al*. Impact of chronic transfusion on incidence of pain and acute chest syndrome during the Stroke Prevention Trial (STOP) in sickle-cell anemia, *J. Pediatr*., **139**, 785–789 (2001).

[38] Hankins, J., Jeng, M., Harris. S. *et al*. Chronic transfusion therapy for children with sickle cell disease and recurrent acute chest syndrome, *J. Pediatr. Hematol. Oncol*., **27**, 158–161 (2005).

[39] Adams, J.R., Elting, L.S., Lyman, G.H. *et al*. Use of erythropoietin in cancer patients, assessment of oncologists' practice patterns in the United States and other countries, *Am. J. Med*., **116**, 28–34 (2004).

[40] Adams, R.J. Big strokes in small persons, *Arch. Neurol*., **64**, 1567–1574 (2007).

[41] Adams, R.J., McKie, V.C., Hsu, L. *et al*. Prevention of a first stroke by transfusions in children with sickle cell anemia and abnormal results on transcranial Doppler ultrasonography, *N. Engl. J. Med*., **339**, 5–11 (1998).

[42] Lee, M.T., Piomelli, S., Granger, S. *et al*. Stroke Prevention Trial in Sickle Cell Anemia (STOP): extended follow-up and final results, *Blood*, **108**, 847–852 (2006).

[43] Scothorn, D.J., Price, C., Schwartz, D. *et al*. Risk of recurrent stroke in children with sickle cell disease receiving blood transfusion therapy for at least five years after initial stroke, *J. Pediatr*., **140**, 348–354 (2002).

[44] Vichinsky, E.P., Haberkern, C.M., Neumayr, L. *et al*. A comparison of conservative and aggressive transfusion regimens in the perioperative management of sickle cell disease. The Preoperative Transfusion in Sickle Cell Disease Study Group, *N. Engl. J. Med*., **333**, 206–213 (1995).

[45] Neumayr, L., Koshy, M., Haberkern, C. *et al*. Surgery in patients with hemoglobin SC disease. Preoperative Transfusion in Sickle Cell Disease Study Group, *Am. J. Hematol*., **57**, 101–108 (1998).

[46] Haberkern, C.M., Neumayr, L.D., Orringer, E.P. *et al*. Cholecystectomy in sickle cell anemia patients: perioperative outcome of 364 cases from the National Preoperative Transfusion Study. Preoperative Transfusion in Sickle Cell Disease Study Group, *Blood*, **89**, 1533–1542 (1997).

[47] Bocchieri, K.A., Scheinerman, S.J. and Graver, L.M. Exchange transfusion before cardiopulmonary bypass in sickle cell disease, *Ann. Thorac. Surg*., **90**, 323–324 (2010).

[48] Adhikari, R.K., Maddali, M.M. and Zacharias, S. Preoperative exchange transfusion for Sickle Cell Disease patients undergoing open-heart surgery: an exception to the rule, *J. Card. Surg*., **25**, 691–693 (2010).

[49] Morrison, J.C., Morrison, F.S., Floyd, R.C. *et al*. Use of continuous flow erythrocytapheresis in pregnant patients with sickle cell disease, *J. Clin. Apher*., **6**, 224–229 (1991).

[50] Sun, P.M., Wilburn, W., Raynor, B.D. and Jamieson, D. Sickle cell disease in pregnancy: twenty years of experience at Grady Memorial Hospital, Atlanta, Georgia, *Am. J. Obstet. Gynecol*., **184**, 1127–1130 (2001).

[51] Serjeant, G.R., Loy, L.L., Crowther, M. *et al*. Outcome of pregnancy in homozygous sickle cell disease, *Obstet. Gynecol*., **103**, 1278–1285 (2004).

[52] Smith, J.A., Espeland, M., Bellevue, R. *et al*. Pregnancy in sickle cell disease: experience of the Cooperative Study of Sickle Cell Disease, *Obstet. Gynecol*., **87**, 199–204 (1996).

[53] Koshy, M., Burd, L., Wallace, D. *et al*. Prophylactic red-cell transfusions in pregnant patients with sickle cell disease: a randomized cooperative study. *N. Engl. J. Med*., **319**, 1447–1452 (1988).
[54] Villers, M.S., Jamison, M.G., De Castro, L.M. and James, A.H. Morbidity associated with sickle cell disease in pregnancy, *Am. J. Obstet. Gynecol*., **199**, 125, e1–5 (2008).
[55] Grossetti, E., Carles, G., El Guindi, W. *et al*. Selective prophylactic transfusion in sickle cell disease, *Acta Obstet. Gynecol. Scand*., **88**, 1090–1094 (2009).
[56] Anstee, D.J. The relationship between blood groups and disease, *Blood*, **115**, 4635–4643 (2010).
[57] Miller, L.H., Mason, S.J., Clyde, D.F. and McGinniss, M.H. The resistance factor to Plasmodium vivax in blacks: the Duffy-blood-group genotype, FyFy, *N. Engl. J. Med*., **295**, 302–304 (1976).
[58] Tournamille, C., Colin, Y., Cartron, J.P. and Le Van Kim, C. Disruption of a GATA motif in the Duffy gene promoter abolishes erythroid gene expression in Duffy-negative individuals, *Nat. Genet*., **10**, 224–228 (1995).
[59] Anstee, D.J. Red cell genotyping and the future of pretransfusion testing, *Blood*, **114**, 248–256 (2009).
[60] Ribeiro, K.R., Guarnieri, M.H., da Costa, D.C. *et al*. DNA array analysis for red blood cell antigens facilitates the transfusion support with antigen-matched blood in patients with sickle cell disease, *Vox Sang*., **97**, 147–152 (2009).
[61] Petz, L.D. Bystander immune cytolysis, *Transfus. Med. Rev*., **20**, 110–140 (2006).
[62] Wang, R.H., Phillips, G. Jr, Medof, M.E. and Mold, C. Activation of the alternative complement pathway by exposure of phosphatidylethanolamine and phosphatidylserine on erythrocytes from sickle cell disease patients, *J. Clin. Invest*., **92**, 1326–1335 (1993).
[63] Aygun, B., Padmanabhan, S., Paley, C. and Chandrasekaran, V. Clinical significance of RBC alloantibodies and autoantibodies in sickle cell patients who received transfusions, *Transfusion*, **42**, 37–43 (2002).
[64] Noizat-Pirenne, F., Bachir, D., Chadebech, P. *et al*. Rituximab for prevention of delayed hemolytic transfusion reaction in sickle cell disease, *Haematologica*, **92**, e132–e135 (2007).
[65] Chadebech, P., Habibi, A., Nzouakou, R. *et al*. Delayed hemolytic transfusion reaction in sickle cell disease patients: evidence of an emerging syndrome with suicidal red blood cell death, *Transfusion*, **49**, 1785–1792 (2009).
[66] Win, N., Sinha, S., Lee, E. and Mills, W. Treatment with intravenous immunoglobulin and steroids may correct severe anemia in hyperhemolytic transfusion reactions: case report and literature review, *Transfus. Med. Rev*., **24**, 64–67 (2010).
[67] Vichinsky, E.P. and Ohene-Frempong, K. Approaches to transfusion therapy and iron overload in patients with Sickle Cell Disease: results of an international survey, *Pediatr. Hematol. Oncol*., epub 30 Jul 2010 (doi: 10.3109/08880018.2010.505497).
[68] Cappellini, M.D. and Piga, A. Current status in iron chelation in hemoglobinopathies, *Curr. Mol. Med*., **8**, 663–674 (2008).
[69] Cappellini, M.D. Management of iron overload. Introduction, *Blood. Rev*., **22**(Suppl. 2), S13 (2008).

[70] Cappellini, M.D. Long-term efficacy and safety of deferasirox, *Blood Rev*., **22**(Suppl. 2), S35–S41 (2008).
[71] Cohen, A.R. New advances in iron chelation therapy, *Hematology Am. Soc. Hematol. Educ. Program*., 42–47 (2006).
[72] Galanello, R. Deferiprone in the treatment of transfusion-dependent thalassemia: a review and perspective, *Ther. Clin. Risk. Manag*., **3**, 795–805 (2007).
[73] Galanello, R., Agus, A., Campus, S. *et al*. Combined iron chelation therapy, *Ann. N Y Acad. Sci*., **1202**, 79–86 (2010).
[74] Cappellini, M.D. Overcoming the challenge of patient compliance with iron chelation therapy, *Semin. Hematol*., **42**, S19–S21 (2005).
[75] Mourad, F.H., Hoffbrand, A.V., Sheikh-Taha, M. *et al*. Comparison between desferrioxamine and combined therapy with desferrioxamine and deferiprone in iron overloaded thalassaemia patients, *Br. J. Haematol*., **121**, 187–189 (2003).
[76] Treadwell, M.J. and Weissman, L. Improving adherence with deferoxamine regimens for patients receiving chronic transfusion therapy, *Semin. Hematol*., **38**, 77–84 (2001).
[77] Brittenham, G.M., Griffith, P.M., Nienhuis, A.W. *et al*. Efficacy of deferoxamine in preventing complications of iron overload in patients with thalassemia major, *N. Engl. J. Med*., **331**, 567–573 (1994).
[78] Porter, J.B. Practical management of iron overload, *Br. J. Haematol*., **115**, 239–252 (2001).
[79] Vermylen, C. What is new in iron overload?, *Eur. J. Pediatr*., **167**, 377–381 (2008).
[80] Nielsen, P., Engelhardt, R., Fischer, R. *et al*. Noninvasive liver-iron quantification by computed tomography in iron-overloaded rats, *Invest. Radiol*., **27**, 312–317 (1992).
[81] Farmaki, K., Tzoumari, I., Pappa, C. *et al*. Normalisation of total body iron load with very intensive combined chelation reverses cardiac and endocrine complications of thalassaemia major, *Br. J. Haematol*., **148**, 466–475 (2010).

Part III

"Old" and New Strategies for Oxygen Supply

13

Transfusion: Political, Administrative and Logistic Issues

John R. Hess[1] *and Giuliano Grazzini*[2]

[1] *University of Maryland School of Medicine, Baltimore, MD, USA*

[2] *Italian National Blood Centre, Istituto Superiore di Sanità, Rome, Italy*

Disclaimer

Dr Hess notes the following financial conflicts. He is a US Government inventor who receives patent license royalties in accordance with US law for patents describing ways to store red cells longer. He also advises Hemerus, Inc., licensee of those US Government patents, and receives compensation.

Dr Grazzini does not note any financial conflict.

13.1 Introduction

Blood transfusion is a medical procedure with wide applicability and excellent efficacy but significant safety risks. Blood use enables fetal medicine, safe obstetrics, the correction of congenital defects, the treatment of severe injuries and acquired diseases such as cancer and heart disease, the expanding use of transplantation and stem cell-based reconstruction, and the palliation of diseases that cannot be cured. As a result of these many benefits, thoughtful patients and their families, medical systems, and governments want blood for transfusion to be available, safe, effective, and cheap [1].

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Blood is also a symbol of social cohesion. "Countries with safe blood" defines that part of the world where economic development and the rule of law allow the benefits of modern medical science to be delivered to ordinary citizens. Blood-safety crises have occurred in a number of developed countries, related to hepatitis in the 1970s, human immunodeficiency virus (HIV) in the 1980s, mad cow disease in the 1990s, and arboviruses in the 2000s [2]. The responsiveness of representative governments to these blood crises has been in proportion to the great importance that ordinary citizens place on the safety of blood when they seek care for themselves and their families.

Administratively, managing a blood system is an enormous task. Every donor, every component, and every recipient is unique. So are the specificities of the antibodies used in infectious disease testing and phenotyping, and the reagent cells and panels used in crossmatching. Donors are unique in their combinations of availability, health, social exposures, and travel histories. Individuals are occasionally highly desirable as donors because of genetic or phenotypic traits such as the blood group O RhD negative "universal donor" status or possession of the rare Bombay phenotype, whose carriers can only give blood to one another, with only 131 individuals and families identified worldwide. Components made from individual blood donations need to remain linked because the risks associated with mislabeling and contamination apply to all parts of a donation. Recipients too can have highly defined blood requirements, such as a need for phenotypically matched platelets or red cells. Recipients also need to be notified if risks are identified after the fact. Therefore, records need to be maintained for years, creating veritable mountains of paperwork and requiring powerful information technology systems [3].

Logistically, blood systems hope to benefit from economies of scale while still maintaining their highly personal interactions with donors, clinicians, and patients, as well as couriers, suppliers, maintenance technicians, and laboratory coworkers. The equipment necessary for modern blood banking – refrigerators and freezers, centrifuges, gamma-irradiators, robotic systems for blood testing, and information management systems – is most efficient if run at or near capacity, and yet all these systems need backup. The tradeoffs between efficiency and redundancy push for centralization, while the need for direct individual patient-care services can only be met by local floor-level management of highly technical services [4].

The interactions between the clinical details of blood transfusion, their administration and logistics, and the politics of blood safety and availability are the topics of this short paper. The authors are senior workers in the field of transfusion. They offer this work as an example of why a safe and effective blood substitute would have such value.

13.2 Blood Safety

Since 1943, we have known that the transfusion of blood can lead to an infectious hepatitis [5]. Experience with pooled plasma showed that the larger the number of donors in a pool, the greater the risk. However, it was the work of Alter at the US National Institutes of Health in the 1960s, showing that 23% of all patients who received transfusions in the cardiac surgery program developed elevations of liver enzymes, that led to the US national ban on paid blood donors for clinical transfusions [6]. With the development of screening tests for hepatitis B in the late 1960s and hepatitis C in the early

1990s, and widespread immunization for hepatitis B, the transmission of hepatitis in blood transfusions has largely disappeared [7].

In June of 1981, the first cases of the acquired immunodeficiency syndrome (AIDS) were described. A year later, in July of 1982, transmission of AIDS in blood transfusion was strongly suggested by its appearance in children with hemophilia. By August of 1983, the LAV/HIV virus was identified, and screening tests for the virus became available in May of 1985. In those years, more than 8000 recipients of blood transfusions in the USA became infected and an additional 13 000 hemophilia patients were infected from commercial pooled coagulation-factor concentrates. Since May of 1985, only 49 individuals in the US have become infected with HIV through transfusion, and only two in the last 6 years. The exclusion of donors with high-risk lifestyles and successively better viral testing have both played a role. Individual donor centers and organizations maintain lists of excluded donors, but the lists are not shared [8].

In 1986, the UK recognized that it was having an epidemic of bovine spongiform encephalopathy (BSE), mad cow disease. In 1995, cases of mad cow disease began to occur in humans. Overall, 179 000 infected cattle were identified and it is estimated that more than 400 000 such animals entered the food chain. Herds were culled of more than 4 million animals to stem the epidemic. Up to October 2009, 170 people in the UK are known to have been infected and 166 have died. The epidemic peaked in 2000, with 28 human deaths that year, and has now slowed to the rate of 2–3 deaths each year. However, four pairs of these infected people were epidemiologically linked as blood donor–recipient pairs, and transmission by transfusion of scrapie, a similar disease in sheep, has been demonstrated. The USA, most European countries, and several other countries have excluded potential blood donors from donating if they have a significant risk of exposure to British beef during the epidemic period. This has led to a requirement for an extensive travel history as part of the donation process [9]. In the UK, North American plasma and prion-reduction filters have been introduced to further reduce the risk of transmission.

In 1999, West Nile Virus (WNV) appeared in Long Island, and over the next two years it spread down the US east coast. In 2001, there were more than 4000 cases and 243 deaths, including deaths related to transfusion and transplantation. Additional questions were added to donor questionnaires and in 2003 nucleic acid testing (NAT) was added to routine virus screening [10]. In Europe, a few sporadic cases of WNV infection in humans have been identified in Portugal, Spain, France, Italy, Czech Republic, Romania, and Hungary in recent years, mostly during the latter half of the mosquito season, between the end of July and the end of September [11]. Since 2008, the observed epidemiological picture of WNV infection in Europe has appeared to be changing. In that year, clusters of human cases of WNV disease were reported in Italy [12], Hungary, and Romania. This was the first time that outbreaks with human cases were reported simultaneously from several European countries. In 2005–2006, 38% of the population of Reunion Island, a part of overseas France, was infected with Chikungunya virus, and cases in tourists have developed after return to Europe. As a result, France has instituted surveillance, a limited donor ban, and pathogen reduction for platelet transfusions. In summer 2007, a limited autochthonous epidemic outbreak of Chikungunya virus affected an Italian region as well, causing a significant impact on the local blood supply [13].

The cumulative result of more than four decades of blood crises has been to make politicians very wary of blood-safety issues. They have insulated themselves through the implementation of regulatory structures and oversight boards that have consistently advised greater restrictions on donation and more rigorous testing. All of this has added to the administrative complexity and logistic complications of giving blood and running blood banks. Completing a donor history now requires a considerable amount of time, and donor infectious disease testing tends to be highly centralized. All of this information must be assembled and checked before a blood component can be labeled in a donor center.

Non-infectious complications of transfusion have also gathered regulatory attention. These include acute hemolytic transfusion reactions from administering out-of-type units (classically group A units to group O patients), anaphylactic shock in patients with congenital IgA deficiency and anti-IgA antibodies who are given a unit of blood containing IgA, and transfusion-related acute lung injury (TRALI). TRALI was named by Propovsky and Moore in 1985 and has since come to be recognized as the leading cause of transfusion-related death [14]. It occurs most commonly when blood products from women donors who have previously been pregnant and developed high-titer antibodies to white-blood-cell antigens from the child's father are transfused to a patient who shares those white-cell antigens [15]. The antibodies activate recipient white cells, most noticeably those previously marginated on lung endothelium by the patient's underlying illness, and the white cells secrete peroxides which damage the underlying endothelium and make it leak plasma into the lung. A second mechanism for lung injury can occur when oxidative breakdown products of the cell membranes of stored red cells or platelets similarly activate the white cells on the lung endothelium. In the HLA-antibody-mediated form of TRALI, the donor may be identified as a dangerous donor and deferred from future donation, while the lysophospholipid-related cases suggest that we may be storing cellular blood products too long between collection and transfusion. Strategies have been proposed and actually implemented in several countries in order to prevent TRALI, such as transfusing male-only plasma [16], screening blood for antibodies to white cells [17], and using pharmaceutical preparations of solvent-detergent inactivated plasma in which antibodies are diluted to inefficient titers [18]. All of these preventive measures further add to the complexity and costs of blood management.

13.3 Blood Availability

The first blood transfusions were performed directly from the arm of the donor to the arm of the recipient. The discoveries of citrate as an anticoagulant and glucose as a red-cell nutrient in the period 1913–1915 allowed the collection and storage of blood in bottles and the separation of the donor and recipient in space and time. This separation allowed blood to be collected, typed, tested for syphilis, and assembled into blood banks. Blood banks transformed transfusion from a desperate clinical act on a sick patient to a planned therapy based on a supporting medical logistic activity [19].

The original form of blood stored in blood banks, whole blood in glass bottles, turned out to have many disadvantages. First, the bottles were heavy and they broke. Second, other than the injured and massively transfused, most patients who needed blood did not

need whole blood, but just one of its components. However, each of the component parts of whole blood has its own different storage requirement. Red blood cells (RBCs) are best stored at refrigerator temperatures, where the cold slows their metabolism, prolonging the time until the acid products of glucose metabolism inhibit that metabolism entirely. Platelets are best stored at room temperature, above 18 °C, where a phase change in their lipid membrane leads to the aggregation of platelet surface glycoproteins, which are recognized and removed by the recipient's immune system. Plasma is best stored frozen, where interactions between the proteins and the glass or plastic surfaces do not lead to activation or denaturation. Interconnecting plastic bags allow the sterile separation of the components, reduce weight and breakage in storage, allow multiple patients to benefit from a single donation, and actually improve the quality of the separate components.

Making blood components is now a batch or production-line process. Red cells are centrifugally separated – the iron makes them heavy – and the white cells are removed by passing them through a nylon filter. Removal of the white cells – leukoreduction – was originally done to prevent inflammatory reactions and immunization to the white-cell HLA antigens which make leukemia patients refractory to platelets, but it also removes a competing user of the bags' limited energy reserves and the source of oxidative materials and enzymes that damage the red-cell surface as the white cells break down. Leukoreduced RBCs have about 2% better recovery and half the hemolysis of RBCs stored with white cells [20]. RBCs can also be stored frozen, but the process is time consuming, expensive, and associated with 20% losses of the cells in the freeze–thaw–wash procedure, so it is normally only used for rare units of blood [21]. Platelets are separated and generally leukoreduced as well. They are stored in thin polyolefin or polyethylene bags with high surface area on rockers to facilitate the diffusion of oxygen and thus support their oxidative mitochondrial metabolism. Acetate, phosphate, and oxygen turn out to be the critical requirements for platelet storage. Plasma does best deeply frozen. In the USA it can be stored for a year at −18 °C or for 7 years at −65 °C, and in Europe for up to 3 years according to processes used for collection, processing, and storage [22].

Even with good storage systems, blood banks have to maintain a complex inventory of rapidly outdating products. RBCs can be kept liquid for 5 or 6 weeks or frozen for 10–30 years. Blood types complicate the RBC inventory situation, and although the uncommon group AB can accept blood from donors of the other types, and generally everyone can receive group O "universal donor" blood, to balance donation and use, and avoid waste of inventory, it is generally better if everyone gets their own type except in emergencies. Platelets are much more difficult to manage because they only have a functional 3 day shelf-life, and because total platelet usage is much lower than with RBCs [23].

Minor antigens can also cause problems in providing blood for specific patients and in blood-bank logistics. While we now realize that there are more than 1900 gene products associated with red cells, only 30 on the surface of red cells are associated with allelic phenotypes that give rise to the 270 different antigens that can raise alloantibodies which complicate transfusion [24]. Only 18 of these antigens are commonly clinically significant, but alloantibodies create demand for RBCs lacking the corresponding antigens in patterns which can be quite rare. Nationally, blood systems maintain rare donor files of units of blood recently donated by donors of known phenotype, frozen units, and donors willing to donate. Exchange of these rare units by air shipment, sometimes halfway around the world, is a monument to international cooperation. Platelets also have allelic

forms of their glycoproteins in the population, of which at least 16 different pairs form the HPA (human platelet antigen) system. These can be important in neonatal alloimmune thrombocytopenia purpura and post-transfusion purpura, uncommon diseases of the fetus and newborn, and of adults, respectively. Only a few locations worldwide have type-specific platelets for these conditions. More common are HLA alleles, which create the antibodies that cause platelet refractoriness in cancer and other multiply-transfused patients. Finding HLA-compatible platelets in a small blood center's platelet inventory is unlikely, so frequently a large HLA-typed platelet donor pool will have to be searched to find a compatible donor and that individual will have to be specially recruited to donate for a specific (but unknown to them) patient.

Overall, about 95% of RBCs and 85% of platelets collected generally find a recipient. For RBCs that rate can be as high as 99+% in a large tertiary care hospital or as low as 2% when supplying remote military outposts in a UN peacekeeping mission. For platelets, their short shelf life and the day-to-day changes in the conditions and numbers of cancer and transplant patients make their supply a constantly moving target. Plasma, because of its long shelf life, is generally easiest to manage.

13.4 Cost and Fairness

The safest blood, according to the World Health Organization's Global Blood Safety Program, comes from volunteer nonremunerated donors. The blood is freely given, and yet it costs money. The plastic bag costs about $20 US and the RBC leukocyte reduction filter about the same. The cost of the full battery of infectious disease tests is perhaps another $40. Nurses, donor techs, couriers, and drivers, as well as donor chairs, laptop computers, hemoglobin spectrophotometers, vehicles, and juice and cookies all cost more. Seventy per cent of the blood collected in the US is collected at blood-collection events at sites remote from the blood center. Despite the use of donated space and volunteers, the paid staff travel. The net result is that the cost of collecting a unit of blood is about $100, and the cost of processing it into components and of delivering it to the hospitals that use it is about $100 more. As a result, the net cost of a unit of RBCs is about $200; a unit of apheresis platelets, about $500; and a unit of plasma, about $40. In Europe the average costs are quite similar, ranging from €140 to €180 for a unit of RBCs (unpublished data). Whether the hospital pays the blood-donor center this cost and in turn bills it to the patient's health insurance or the national health care system pays the bill as cost of business, the fact is that the cost of blood is about 2% of the cost of a tertiary medical center and about 1% of the overall cost of all health care in the developed world.

In the hospital, it costs money to staff a transfusion service around the clock and to buy reagents and refrigerators. It takes about 40 people to run a hospital transfusion service that delivers 60 000 components a year, even with appropriate automation. So the cost of personnel is $2 million and reagents another $1 million annually. Amortized building costs, heat, air conditioning, electricity and water, and removal of medical waste and paper containing medical information all add to the costs. The shared cost of hospital computer systems and appropriate information technology is also large. On the wards far from the blood bank, nurses hang the blood and oversee the transfusions in ways described in transfusion protocols. It all costs money: about $80 per capita every year is

the blood piece of the $2.4 trillion total US health care expenditure. In a very recent study [25] aimed at accurately determining the overall cost of blood in a surgical population from a health-system perspective, an activity-based costing model was applied in two US and two European hospitals, yielding per-RBC-unit costs between $522 and $1183 (mean, $761 ± $294). This shows that blood utilization consumes more health care resources than previously estimated [25].

If, as part of their responsibility to promote the general welfare, governments have become the ultimate guarantors of blood safety, are they also liable for its failures? This was certainly the case with HIV and hemophilia in many Western countries and mad cow disease in Britain. The potential liability is limited only by the social contract.

All around the world, the populations of developed countries are aging. About half of all the blood transfused in the USA and Europe is given to patients older than 65 years. Their number is projected to increase by 50% between the years 2000 and 2025. Meanwhile, the fraction of the population between 17 and 60, the typical ages of blood donors, is going down. We will need to recruit more donors, both absolutely and relatively. The cost of providing the present level of care increases daily.

In middle-income countries, the choices are more focused. Enzyme-linked immunosorbant assays provide almost as much protection against AIDS and hepatitis as NAT testing at a fraction of the cost, and the extra money is far better spent on hepatitis B immunization as a national strategy. Leukoreduction can be focused to mothers, infants, and leukemia patients. However, the brain drain is a constant problem, and the hospital transfusion services of wealthy countries are filled with immigrant technologists.

In poor countries, the situation is even direr. Most spend less on health per capita than the wealth countries spend on blood. The lack of blood for even basic emergencies such as severe malarial anemia in infants, obstetric catastrophies, and work- and transport-related accidents contributes to their high rates of infant and perinatal mortality, and the vast toll of disability summed in the loss of years of productive life in such countries. However, even more basic needs for education, clean water, and food compete with health for limited resources. The effect of wealth on the rate of blood collection across 170 countries reporting to the WHO's Global Blood Resource Database is shown in Figure 13.1.

And in the kleptocracies, the situation is completely mad. In Pyongyang, blood collected from "volunteers" for the Great Leader sits in refrigerators in the Kim Jong Il Institute, awaiting the dictator's need. However, the electricity goes out too frequently to guarantee its quality, and the test kits probably were not in date anyway. In the absence of the rule of law, quality programs are meaningless.

13.5 Transfusion Medicine

Transfusion medicine is the medical specialty that relates to blood collection and transfusion. It concerns donor psychology and health, immunohaematology, and blood physiology, but also blood-program administration, logistics, and politics, as noted in this paper. There is a paucity of well-trained people who understand the full range of this specialty and academic institutions and research centers tend not to be very keen on it.

Transfusion-medicine specialists were among those who watched the development of hemoglobin-based blood substitutes most closely. They elucidated the multiple toxicities

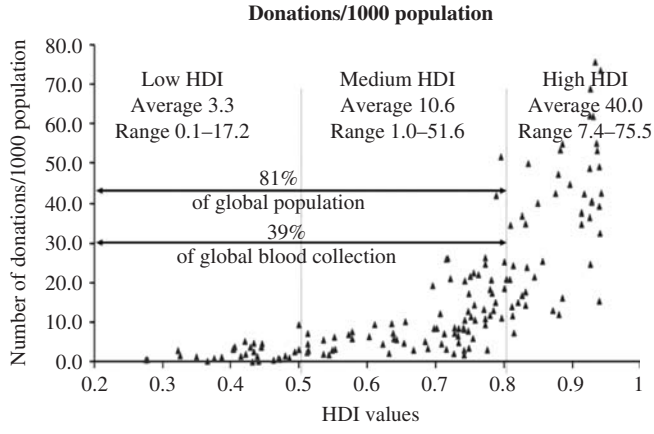

Figure 13.1 *This graph from the World Health Organization's Global Program for Blood Safety shows that 19% of the world's population lives in countries that provide 61% of all blood transfusions. Much of the world's population lives in countries that collect less than 1 unit of blood for every 100 members of their population. (Graph courtesy of Dr Neelam Dhingra, Global Program for Blood Safety).*

of hemoglobin A_0, the base material for all subsequent developments, and its recent modifications, which came to clinical trials in the 1980s through the 2000s. These toxicities including vasoconstriction, vasculitis, neurotoxicity, heightened reactivity to bacterial endotoxin, potentiation of bacterial infections, enhanced free-radical injury, and interference with blood coagulation [26]. Systematic exclusion of knowledgeable individuals who understood these issues from the meetings that supported recent developments in blood-substitute materials allowed 11 human trials to be conducted with excess mortality in every one [27].

Transfusion medicine is therefore with us for the foreseeable future. Donors will need to be recruited, questioned, bled, thanked, and followed. Human blood will need to be made into components for transfusion and deployed around the world. Patients with special needs will need to be evaluated and their complications followed. The blood system in all of its complexity will need to be administered, its logistics improved, and its political fights fought to livable conclusions.

References

[1] Collins, J.A., The surgeon's point of view, in *Preservation of Red Blood Cells* (ed. H. Chaplin Jr, E.R. Jaffe, C. Lanfant and C.R. Valeri), National Academy of Sciences, Washington, DC, USA, pp. 339–344 (1973).

[2] Dodd, R., Kurt Roth, W., Ashford, P. *et al*. Transfusion medicine and safety, *Biologicals*, **37**, 62–70 (2009).

[3] Sazama, K. Current good manufacturing practices for transfusion medicine, *Transfus. Med. Rev*., **10**, 286–295 (1996).

[4] MacIvor, D., Triulzi, D.J. and Yazer, M.H. Enhanced detection of blood bank sample collection errors with a centralized patient database, *Transfusion*, **49**, 40–43 (2009).
[5] Beeson, P.B. Jaundice occurring one to four months after transfusion of blood or plasma: a report of 7 cases, *JAMA*, **121**, 1332–1334 (1943).
[6] Alter, H.J., Holland, P.V., Purcell, R.H. *et al*. Posttransfusion hepatitis after exclusion of commercial and hepatitis-B antigen-positive donors, *Ann. Intern. Med*., **77**, 691–699 (1972).
[7] Alter, H.J. and Klein, H.G. The hazards of blood transfusion in historical perspective, *Blood*, **112**, 2617–2626 (2008).
[8] Busch, M.P. Transfusion and HIV, *Curr. Opin. Hematol*., **1**, 438–442 (1994).
[9] Lefrère, J.J. and Hewitt, P. From mad cows to sensible blood transfusion: the risk of prion transmission by labile blood components in the United Kingdom and in France, *Transfusion*, **49**, 797–812 (2009).
[10] Alter, H.J., Stramer, S.L. and Dodd, R.Y. Emerging infectious diseases that threaten the blood supply, *Semin. Hematol*., **44**, 32–41 (2007).
[11] European Centre for Disease Prevention and Control (ECDC). Expert consultation on West Nile virus infection. 8 Sept 2009. Available at http://www.ecdc.europa.eu/en/healthtopics/Pages/West_Nile_Fever.aspx (last consultation 31 May 2010).
[12] Grazzini, G., Liumbruno, G.M., Pupella, S. *et al*. West Nile virus in Italy: a further threat to blood safety, a further threat to the blood system, *Blood Transfus*., **6**, 235–237 (2008).
[13] Liumbruno, G.M., Calteri, D., Petropulacos, K. *et al*. The Chikungunya epidemic in Italy and its repercussion on the blood system, *Blood Transfus*., **6**, 199–210 (2008).
[14] Popovsky, M.A. and Moore, S.B. Diagnostic and pathogenetic considerations in transfusion-related acute lung injury, *Transfusion*, **25**, 573–577 (1985).
[15] Keller-Stanislawski, B., Reil, A., Günay, S. and Funk, M.B. Frequency and severity of transfusion-related acute lung injury: German haemovigilance data (2006–2007), *Vox Sang*., **98**, 70–77 (2010).
[16] Nakazawa, H., Ohnishi, H., Okazaki, H. *et al*. Impact of fresh-frozen plasma from male-only donors versus mixed-sex donors on postoperative respiratory function in surgical patients: a prospective case-controlled study, *Transfusion*, **49**, 2434–2441 (2009).
[17] Powers, A., Stowell, C.P., Dzik, W.H. *et al*. Testing only donors with a prior history of pregnancy or transfusion is a logical and cost-effective transfusion-related acute lung injury prevention strategy, *Transfusion*, **48**, 2549–2558 (2008).
[18] Sachs, U.J., Kauschat, D. and Bein, G. White blood cell-reactive antibodies are undetectable in solvent/detergent plasma, *Transfusion*, **45**, 1628–1631 (2005).
[19] Stansbury, L.G. and Hess, J.R. Blood transfusion in world war I: the roles of Lawrence Bruce Robertson and Oswald Hope Robertson in the most important medical advance of the war, *Transfus. Med. Rev*., **23**, 232–236 (2009).
[20] Hess, J.R. Red cell storage, *J. Proteomics*, **73**, 368–373 (2010).
[21] Hess, J.R. RBC freezing and it impact on the supply chain, *Transfus. Med*., **14**, 1–8 (2004).

[22] Commission Directive 2004/33/EC of 22 March 2004 implementing Directive 2002/98/EC of the European Parliament and of the Council as regards certain technical requirements for blood and blood components. *Official Journal of the European Union* L 91, 30 March 2004.
[23] Blake, J.T. On the use of Operational Research for managing platelet inventory and ordering, *Transfusion*, **49**, 396–401 (2009).
[24] Daniels, G. and Reid, M.E. Blood groups: the past 50 years, *Transfusion*, **50**, 281–289 (2010).
[25] Shander, A., Hofmann, A., Ozawa, S. *et al*. Activity-based costs of blood transfusions in surgical patients at four hospitals, *Transfusion*, **50**, 753–765 (2010).
[26] Hess, J.R. Blood Substitutes, *Sem. Hem*., **33**, 369–378 (1996).
[27] Natanson, C., Kern, S.J., Lurie, P. *et al*. Cell-free hemoglobin-based blood substitutes and risk of myocardial infarction and death: a meta-analysis, *JAMA*, **299**, 2304–2312 (2008).

14

Conscientious Objection in Patient Blood Management

Kenneth E. Nollet and Hitoshi Ohto

Department of Blood Transfusion and Transplantation Immunology, Fukushima Medical University, Fukushima, Japan

14.1 Introduction

Conscientious objection in patient blood management is an overarching description of thought and behavior that is commonly framed as religious refusal of blood transfusion. These ideas are related, but different. Religious refusal of blood transfusion should, ideally, be a conscientious decision rather than a blind act of obedience. Nonreligious objections to transfusion also exist, usually based on perceptions of risk and benefit that may or may not be accurate. Dealing constructively with either can consume time, which is arguably a health care provider's most precious resource. Reluctance, or refusal, to treat patients who object to certain interventions should also be conscientious, rather than self-serving or expedient. This chapter addresses both aspects of conscientious objection in patient blood management: patients for whom alternatives to blood transfusion are sought, and health care providers who may object to demands made by patients, parents, or guardians.

14.2 Conscientious Objection

A PubMed search of "conscientious objection" yielded 128 citations. The earliest two, from 1953, pertain to compulsory vaccination [1, 2]. As with transfusion, vaccination of

Chemistry and Biochemistry of Oxygen Therapeutics: From Transfusion to Artificial Blood, First Edition.
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minors is a matter not only between patient and provider, but also involving parents or guardians. Anxiety about vaccination safety persists today, seemingly informed by some science and a great deal of rhetoric [3–6]. Next, from 1972, is a doctor's letter about conscientious objection to abortion [7]. As with transfusion, religious convictions may dominate matters of reproductive health. Not only might patient or guardian convictions be at odds with provider recommendations, but also, provider convictions may be at odds with patient expectations [8–11]. This latter scenario may seem irrelevant to patient blood management, but a provider might legitimately object to demands that are perceived to be against the best interest or survival of the patient [12].

A 2009 guidance document by Ohto *et al*. [13] appears to be the first peer-reviewed publication in which religious refusal of blood transfusion is explicitly considered in the broader framework of conscientious objection. The guidance was co-developed and sanctioned by the Japan Society of Transfusion Medicine and Cell Therapy, the Japanese Society of Anesthesiologists, the Japan Pediatric Society, the Japan Society of Obstetrics and Gynecology, and the Japan Surgical Society. Thus, it is multidisciplinary in scope, but specific to the laws, culture, and health care delivery system of a single country [13]. In Japan and elsewhere, it still behooves institutional ethics committees to implement specific policies and procedures to address conscientious objection. Such policies and procedures should anticipate as many scenarios as possible in which conscientious objections may arise. Of equal importance, ethics committee guidance should be well enough written and well enough understood to ensure that unanticipated scenarios can be deliberated in a timely and effective manner. Although people who object to transfusion generally interact with specific individuals or departments during an episode of care, ethics committee oversight can ensure that policies and procedures are consistent with conscientious objection in other areas of health care. Thus, rather than depending solely on publications specifically related to transfusion therapy, the broader literature on conscientious objection should be consulted. From 128 PubMed citations, conscientious objection is considered in palliative care (5 citations, 4%), vaccination (8 citations, 6%), contraception (24 citations, 19%), and abortion (58 citations, 45%). These articles pertain not only to objections raised by patients, parents, and guardians, but also to objections raised by health care providers themselves. The original Oath of Hippocrates proscribed abortion, but modern health care providers more likely object to reproductive interventions on religious grounds. This may seem unrelated to providers who do not want to treat patients who refuse transfusion, but in either case an institution, guided by its ethics committee, must anticipate and address the situation.

14.3 Patient Blood Management

Patient blood management has gained favor over *transfusion medicine* or *transfusion practice* as a descriptor including all interventions for acute or chronic blood loss, anemia, thrombocytopenia, neutropenia, coagulopathy, and so on. In a surgical context, three elements of patient blood management are:

(i) Increasing preoperative red-cell mass.
(ii) Minimizing perioperative blood loss.
(iii) Accepting low-hemoglobin transfusion triggers.

Ideally, patient blood management is a cooperative *inter*disciplinary approach that seeks the best possible outcome for patients. In contrast, *multi*disciplinary approaches that are not cooperative may attempt to shift risk, effort, or expense from one specialty to another, resulting in suboptimal care. For example, in a patient care scenario where blood products and pharmaceuticals are charged to different cost centers, a clinical question of red-cell transfusion versus erythropoietin therapy may become an economic debate between a hospital's blood bank and pharmacy. Even if clinical and economic consensus can be achieved among providers, it is an incomplete consensus if it fails to take account of a patient's emotional needs and decision-making autonomy [14–16].

14.4 Jehovah's Witnesses

Jehovah's Witnesses are well known among health care providers for refusing whole-blood, red-cell, white-cell, platelet, and plasma transfusions. Minor fractions of blood, and organ transplants, are a matter of individual conscience. Definitive church teaching can be found at www.watchtower.org, or through local Hospital Liaison Committees [17, 18].

It can be argued that inconsistencies exist in the faith. One criticism is that the "blood ban" did not exist prior to 1945; another is that transplants, whether of solid organs, bone marrow, or peripheral blood stem cells, are not devoid of blood. In a survey of 30 Jehovah's Witnesses published in 1976, *all* Witnesses agreed that "[t]ransplanting human organs is against God's will" [19]. Now, transplants are regarded as a matter of individual conscience. A health care professional outside the faith might regard shifting doctrinal stances or other perceived inconsistencies as evidence against the validity of Witness beliefs. We propose a more conciliatory view. Founded in 1872, the faith predates modern blood transfusion. The Bible has a much longer history. Literal interpretation, a hallmark of fundamentalism in any religion, holds scripture to be inerrant and unchanging. But the circumstances of human existence change, so what is *literal* must still be *interpreted*. This prerogative belongs to faith, not science. (Medicine, it might be argued, falls somewhere in between.)

The "No Blood" card carried by faithful Jehovah's Witnesses, and available to others, is an advanced medical directive, but as such may be incomplete. Variations exist, including a double-printed page which folds into card size [20]. A Hospital Liaison Committee would likely provide sample cards to local health care providers. A crisis is not the ideal time to first see such a card. For this and other reasons, we support lines of communication – "diplomatic relations" – between transfusion services and Hospital Liaison Committees.

14.5 Will the Real Objection Please Stand Up?

Patients in general may object to blood transfusion for various reasons. Objections arising from fear or misinformation should be addressed as for other interventions. Jehovah's Witnesses have a well-defined and well-articulated doctrine on blood transfusion, which nevertheless accommodates a believer's own conscience in regard to the minor components of blood, such as immunoglobulin and coagulation factor concentrates.

A holistic approach for all patients is to consider not only specific objections, but also underlying motivations. In matters of religious faith, there may be differences between first- and second-generation believers [13]. Ultimately, matters of conscience are, by definition, unique to each individual. Attending to the emotional wellbeing of a patient, especially when family and co-religionists are involved, warrants attention to the motivations that underlie words and deeds. Examples outside of transfusion medicine may be instructive.

A couple known to one author (K.N.) illustrates this point. The husband had been a novice seminarian in a priestly order of the Roman Catholic Church. The wife was a nurse. Both were committed Catholics who embraced the principles and practice of natural family planning, but for distinctly different reasons. One might imagine that the seminary-trained husband would be motivated by religious doctrine, as articulated in documents such as the papal encyclical "Humanae Vitae" [21]. Likewise, the medically informed wife might presumably be motivated by her knowledge of side effects associated with artificial methods of birth control. In fact, the husband's overriding concern was for the health of his wife, and the wife's concern was not for her physical health per se, but for the spiritual health of their marital union.

This couple, fortunate in achieving healthy pregnancies at will, encountered no conflicts of conscience in dealing with their health care providers. Nevertheless, the example should remind health care providers that objection to transfusion therapy, even if articulated as a matter of faith, may arise from a variety of underlying motivations. Especially when an objection is to be overridden by court order, the emotional wellbeing of a patient may depend on how well providers can understand, and address, the underlying motivations of all participants.

14.6 Conscientious Objection in Relation to Oxygen Therapeutics and Other Innovations

Just as refusal of blood transfusion should be a conscientious decision rather than a blind act of obedience, acceptance of a transfusion alternative should be a conscientious decision rather than a blind act of enthusiasm. Perfluorocarbon solutions are not likely to arouse religious opposition, but environmental questions may arise about their use. Hemoglobin-based oxygen carriers (HBOCs), derived from human or animal blood, are more likely to attract religious scrutiny. Religious approval may in some respects be more problematic than religious prohibition. Tentative enthusiasm for HBOCs was expressed in an assembly of Jehovah's Witnesses to which one of us (K.N.) was an invited speaker. Then a member of the assembly asked about recent reports blaming "old" transfused red

cells for their effect on nitric oxide metabolism. This afforded a teaching moment, in which the potential hazards of cell-free hemoglobin could be discussed. At the same assembly, one of the church elders, not himself a health care professional, made a presentation on recombinant factor VII, using information provided by its manufacturer. In this particular instance, the presenter made a sincere effort to be objective, but one can also imagine that a nonmedical, religiously motivated person armed with commercial literature might, even unintentionally, convey a biased perspective. This is not so much a religious phenomenon as it is a human one.

Should developers and manufacturers of products for patient blood management directly engage patients? Many countries allow direct-to-consumer pharmaceutical advertising, and some readers may recall direct-to-consumer promotion of pathogen-inactivated plasma. One of us (K.N.) recalls the wrath of transfusion-service medical directors when a manufacturer (now defunct) of solvent-detergent plasma instructed marketing representatives to call directly on nurses, anesthesiologists, and others.

Guidelines for disseminating product information related to patient blood management should distinguish emotional appeal from informed consent (or informed *dissent*). New alternatives may appeal to a conscientious objector if the products are sufficiently different from those that are prohibited. The very fact that new products differ from old ones should be heeded as a warning: differences in efficacy and side-effect profiles must be considered. Faithful Jehovah's Witnesses cite the hazards of blood transfusion in support of their religious stance. Providers, especially those who maintain open lines of communication with Witnesses, can credibly educate them about the hazards of religiously acceptable alternatives. This is not unique treatment; this is informed consent. What *is* unique is that Jehovah's Witnesses perceive themselves as stakeholders in patient blood management, not only as individual patients when the need arises, but also as a community whose members anticipate their health care encounters. Providers should view this as an opportunity.

The Jehovah's Witness "blood ban" of 1945 can be criticized for costing lives, or commended for helping transfusion medicine evolve into patient blood management. To the extent that it has done the latter, the blood ban has saved lives in and out of the faith. Even outside the faith, we would find an abrupt end to the blood ban unsettling. But history suggests interesting possibilities. The blood ban predates modern developments such as pathogen inactivation and oxygen therapeutics. Thus, it is conceivable that new products could be deemed acceptable either as a matter of religious doctrine or as a matter of individual conscience. With or without religious motivation, the exercise of individual conscience should be respected as a patient's prerogative.

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References

[1] Knack, A.V. Compulsory vaccination with or without conscientious objection clause, *Hippokrates*, **24**, 565–567 (1953).

[2] Huber, H.G. Compulsory vaccination or conscientious objection clause, *Hippokrates*, **24**, 756–758 (1953).

[3] Hanna, J.N., Symons, D.J. and Lyon, M.J. A measles outbreak in the Whitsundays, Queensland: the shape of things to come?, *Commun. Dis. Int*., **26**, 589–592 (2002).

[4] Jacobson, R.M., Zabel, K.S. and Poland, G.A. The challenge of vaccine safety. *Semin. Pediatr. Infect. Dis*., **13**, 215–220 (2002).

[5] Salmon, D.A. and Siegel, A.W. Religious and philosophical exemptions from vaccination requirements and lessons learned from conscientious objectors from conscription, *Public Health Rep*., **116**, 289–295 (2001).

[6] Greenberg, S.B. "Bacilli and bullets": William Osler and the antivaccination movement, *South. Med. J*., **93**, 763–767 (2000).

[7] Walley, R.L. Conscientious objection to abortion, *Brit. Med.l J*., **4**, 234 (1972).

[8] Davidson, L.A., Pettis, C.T., Joiner, A.J. *et al*. Religion and conscientious objection: a survey of pharmacists' willingness to dispense medications, *Soc. Sci. Med*., **71**, 161–165 (2010).

[9] McHale, J.V. Conscientious objection and the nurse: a right or a privilege?, *Brit. J. Nurs*., **18**, 1262–1263 (2009).

[10] American Academy of Pediatrics Committee on Bioethics. Physician refusal to provide information or treatment on the basis of claims of conscience, *Pediatrics*, **124**, 1689–1693 (2009).

[11] Frader, J. and Bosk, C.L. The personal is political, the professional is not: conscientious objection to obtaining/providing/acting on genetic information, *Am. J. Med. Genet. C*, **151C**, 62–67 (2009).

[12] Mullan, K., Allen, W.L. and Brushwood, D.B. Conscientious objection to assisted death: can pharmacy address this in a systematic fashion?, *Ann. Pharmacother*., **30**, 1185–1191 (1996).

[13] Ohto, H., Yonemura, Y., Takeda, J. *et al*. Guidelines for managing conscientious objection to blood transfusion, *Transfus. Med. Rev*., **23**, 221–228 (2009).

[14] Isbister, J.P. Clinicians as gatekeepers: what is the best route to optimal blood use?, *Dev. Biologicals*, **127**, 9–14 (2007).

[15] Spahn, D.R., Moch, H., Hofmann, A. and Isbister, J.P. Patient blood management: the pragmatic solution for the problems with blood transfusions, *Anesthesiology*, **109**, 951–953 (2008).

[16] de Saint Maurice, G., Pequignot, F., Auroy, Y. *et al*. Patient blood management and transfusion, *Anesthesiology*, **111**, 444–445; author reply 445–446 (2009).

[17] Ariga, T. Refusal of blood by Jehovah's Witnesses and the patient's right to self-determination, *Legal Med*., **11**, S138–S140 (2009).

[18] Ariga, T. and Hayasaki, S. Medical, legal and ethical considerations concerning the choice of bloodless medicine by Jehovah's Witnesses, *Legal Med*., **5**, S72–S75 (2003).

[19] Cleveland, S.E. Jehovah's Witnesses and human tissue donation, J. *Clin. Psychol*., **32**, 453–458 (1976).

[20] MyBlood Advanced Medical Directive. www.mybloodsite.com/content/blood-cards. Accessed June 28, 2010.
[21] Humanae Vitae. www.vatican.va/holy_father/paul_vi/encyclicals/documents/hf_p-vi_enc_25071968_humanae-vitae_en.html. Accessed June 1, 2010.
[22] Nollet, J.A. White Plume Communications. www.whiteplumecommunications.com. Accessed August 12, 2010.

15

Red-cell Transfusion in Clinical Practice

Harvey G. Klein

Department of Transfusion Medicine, Clinical Center, National Institutes of Health, Bethesda, MD, USA

15.1 Introduction

The first transfusions of blood from sheep to man in the 17th century were marked by high expectations and unexpected, often lethal complications. Most were direct, vein-to-vein or artery-to-vein transfusions. The first indirect human transfusion, using a syringe to transfer blood from donor to recipient, is attributed to the English obstetrician James Blundell in 1818, although the first *successful* transfusion was not reported until 7 years later [1]. Blundell cautioned that transfusion should be reserved for patients *in extremis*. Half of Blundell's series of 10 patients died, although the mortality related to volume overload or transfusion of incompatible blood in these nearly moribund patients remains unknown. Those who expired could not have lived long enough to die from infectious causes related to the transfused blood [2].

The era of modern transfusion dates from the early 20th century with the identification of the ABO blood groups [3]. Subsequent use of Landsteiner's agglutination technique for compatibility testing improved blood safety dramatically, although recognition of the immunologic diversity of red blood cells and sophisticated crossmatching awaited the discovery of the direct antiglobulin (Coombs) test almost 50 years later [4]. The development of anticoagulant (1914) and preservative solutions (1916) led to the establishment of the modern blood bank, where blood could be stored after collection for several days prior to use [5]. The quality of these red cells was not documented, but from all reports,

Chemistry and Biochemistry of Oxygen Therapeutics: From Transfusion to Artificial Blood, First Edition. Edited by Andrea Mozzarelli and Stefano Bettati.

these early transfusions saved lives [5]. The therapeutic benefit of blood transfusion has seemed obvious from its earliest beginnings. No controlled studies of the effectiveness of whole-blood or red-cell transfusions have ever been conducted and no randomized trials are ever likely to be performed.

With the exception of a few thousand transfusions a year for far-front military casualties, transfusion of whole blood is largely confined to the developing world, where as much as 90% of blood collected is transfused as whole blood [6, 7]. In the developed world, systems of interconnected plastic blood bags developed in the 1960s ushered in the era of component therapy, which not only permitted efficient use of collections from a single blood donor, but also decreased the risks of bacterial contamination and volume overload from transfusion [8]. At about the same time, transfusion-transmitted hepatitis, recognized since World War II, was reported at inordinately high risk in recipients of blood from commercial sources [9]. As a result, developed countries have almost universally instituted all-volunteer blood-donation systems. However, the challenges of avoiding pathogen transmission through blood have moved to the forefront of blood safety, virtually dwarfing concerns about other risks and about a dwindling supply of blood and components.

15.2 Red-cell Use

More than 15 million units of blood were collected from 9.5 million volunteer donors in the US at the last national survey in 2007, about 8% more than was transfused [10]. Approximately 84 units were collected for every thousand Americans in the donating age group. The US collected slightly more blood per 1000 inhabitants (51 units) than did European countries (34–45 units), but both are relatively awash in blood compared to worldwide statistics, which indicate that of the 90 million units collected, there are only 12 units per thousand population [11]. The difference reflects the poverty of safe blood in the developing world, where far less is collected than the projected need.

Statistics about how blood is used are more difficult to come by, although the contrasts between the developed and developing world are equally compelling. In developed countries, about a third of red cells are used for surgery a third for medical indications, and about 6% for obstetrical needs. In the developing world, more than a third is used for pregnancy-related indications and 18% for trauma; close to 20% is used for children and only 14% for medical indications [7]. One quarter of all obstetrical deaths are due to bleeding. Differences between developed countries can be striking as well. On a per capita basis, 29% more blood units are transfused in Denmark than in Sweden [12]. Even within a single country, such as the USA, large differences exist in how red cells are used from institution to institution and in different geographical areas. Part of the disparity may be explained by patient mix and discrepant definitions and data analysis, but it is clear that much of the variation is not well understood.

In the developed world, the elderly use more blood. This is likely to present increasing challenges as populations continue to age. In a survey in France between the years 1994 and 2006, more than 50% of the blood transfused was to patients 70 years of age or older [13]. In Sweden and Denmark, at age 80, one in five inhabitants had received a blood transfusion [12]. Nevertheless, blood collections and use appear to be declining

in the United Kingdom, the Netherlands, France, and several other European countries that have national blood systems and detailed registries. This observation suggests that practices must be changing or perhaps that alternatives to blood are being used.

15.3 The Red-cell-transfusion Trigger

The term "transfusion trigger" is often misused (and abused). In 1980, Friedman *et al*. coined it in a review of the "factors that motivate physicians to order blood" in a study of 535 031 male and female surgical patients, noting that hematocrit or packed cell volume (PCV) was an important and arbitrary component of the physician's decision to transfuse [14]. Decades earlier, Lundy and Adams had advised, based on their totally empiric observation, that it was prudent to maintain a hemoglobin (Hb) concentration of 10 g/dL or a hematocrit of 30% in poor-risk surgical patients undergoing general anesthesia [15]. The so-called "10/30 rule" has been misinterpreted as the trigger to transfuse all patients – surgical or medical, young or old, stable or critically ill – regardless of clinical status. It was never intended to do so. The 1988 NIH Consensus Conference on Perioperative Red Blood Cell Transfusions suggested that no single criterion should be used as an indication for red-cell component therapy and that multiple factors related to a patient's clinical status and oxygen delivery needs should be considered [16].

What is known about the need for red cells, based on physiology, studies in a variety of animal species, and clinical experience, is that a "critical hemoglobin concentration" exists: the Hb level below which, after all compensatory mechanisms are exhausted, oxygen consumption becomes supply-dependent. This is unlikely to be a fixed value, since O_2 consumption varies among organs and depends on the metabolic activity of the tissue and oxygen extraction capabilities. Studies in dogs, pigs, and baboons find the critical Hb concentration in animal models to be surprisingly consistent, approximately 20–25% of normal, or about 3–4 g/dL in human patients [17]. In one of the most carefully studied clinical cases, an 82-year-old man who refused transfusion for religious reasons sustained a massive hemorrhage with a loss of 4.5 L of blood during surgery for a gastric tumor. During meticulous monitoring under anesthesia, and with stable intravascular volume, critical O_2 delivery was calculated at 184 mL/m^2/ min, and the critical Hb at 4.0 g/dL [18]. He expired as his Hb fell to 1.86 g/dL. Experience with other patients declining blood transfusion has further helped define the limits of human tolerance of severe anemia. A retrospective chart review of Jehovah's Witness medical and surgical patients identified 50 deaths, of which 23 were attributable to anemia. With the exception of three who died after cardiac surgery, all of the patients whose deaths were attributable to anemia had Hb levels of 5 g/dL or less. However, 25 patients survived, even with Hb of 5 g/dL or less [19]. Less rigorous clinical observations document that change in mental status and hypoxic muscle spasms occur at an Hb concentration of about 3 g/dL.

Anemia remains a poor prognostic factor in most clinical situations. A retrospective analysis of Medicare records found that anemic patients have a twofold increased two-year mortality compared to non-anemic controls and that anemia increases mortality in patients with chronic renal disease and congestive heart failure up to sixfold [20]. That is not to say that transfusion, or transfusion to normal levels, would have improved survival. The role of transfusion has not been studied rigorously. Nevertheless a large observational

study using Medicare billing data of 78 974 patients hospitalized for confirmed acute myocardial infarction did find that elderly patients with acute myocardial infarction and lower hematocrit on admission had higher 30-day mortality rates; red-cell transfusion was beneficial for short-term survival among those with hematocrit below 30–33% [21]. However, timing of transfusions and their impact on hematocrit was not assessed and associations were based solely on admission hematocrit. A retrospective observational cohort study of 1958 surgical patients refusing RBC transfusion concluded that overall mortality increased with decreasing preoperative Hb and that mortality increased at higher Hb levels in patients with ischemic heart disease [22]. A study of 4470 critically ill ICU patients found that patients with cardiac disease trended towards increased mortality when Hb levels fell below 9.5 g/dL, and that anemic patients with Acute Physiology and Chronic Health Evaluation (APACHE) II scores of >20 and a cardiac diagnosis had a significantly lower mortality rate when given 1–3 or 4–6 units of allogeneic red cells [23]. These and conflicting observational studies of anemia and transfusion have been reviewed elsewhere [24].

The most influential and largest controlled trial to date investigating the relationship between anemia and transfusion (Transfusion Requirements in Critical Care, TRICC) reported no significant difference in 30-day mortality in 838 ICU patients randomized between a liberal and a restrictive transfusion strategy [25]. In fact, the in-hospital mortality rate was lower in the restrictive group. A pediatric study with a design paralleling that of the TRICC trial came to a similar conclusion, that a "trigger" Hb of 7.0 g/dL is as safe and effective as one of 9.5 g/dL, and that substantially fewer red-cell units were administered to the restrictive group [26]. The findings of the TRICC trial have been questioned on the basis of flaws in trial design, in which noncomparable practice-misaligned treatment subgroups were inadvertently created [27]. However, these trials do suggest that lower levels of Hb than have previously been accepted are readily tolerated by critically ill patients.

The current guidelines for transfusion rest on a few basic principles: (i) mortality rises as preoperative Hb falls; (ii) a postoperative Hb below 5–6 g/dL is associated with high mortality; (iii) animal and human data suggest that patients with cardiovascular disease may be less tolerant of anemia than patients without CVD; and (iv) clinicians should consider transfusion when the Hb concentration falls below 7 g/dL and reconsider if the Hb is above 10 g/dL. However, there remains no single or set of objective laboratory or physiologic criteria on which to base a "transfusion trigger", and the decision to transfuse red cells continues to rely on evaluation of the individual patient by skilled clinicians at the bedside, who use Hb concentration and other laboratory measurements as no more than a helpful guide.

15.4 Risks of Red-cell Transfusion

The estimated current risks of red-cell transfusion are listed in Table 15.1. The history of these risks and the basis of the current estimates have recently been reviewed [28]. The threat of transfusion-transmitted pathogens has declined dramatically during the past half-century due to a combination of volunteer donors, meticulous screening, sensitive testing, and careful follow-up of recipients. Concerns for additional infectious risks,

Table 15.1 *Estimated risks of red-cell transfusion per unit.*

Fever/allergic reactions 1 : 200
Hemolytic transfusion reactions 1 : 6000
Fatal hemolytic reactions 1 : 1 000 000
HIV infection 1 : 1 900 000
HBV infection 1 : 180 000
HCV infection 1 : 1 600 000
Bacterial contamination 1 : 40 000
Acute Lung Injury (TRALI) 1 : 50 000
Circulatory overload 1 : 5000
Anaphylaxis 1 : 50 000
Graft-versus-host disease Rare
Immune suppression Unknown

for example from "imported" agents such as dengue and malaria, or newly recognized risks such as chikungunya virus, are well-founded, and the emergence of some totally unanticipated, highly lethal agent as happened with HIV in the 1980s remains a valid worry. As the number of screening tests multiplies, and as geographic exclusions of donors for such diseases as malaria add up, the number of eligible donors is increasingly restricted. Progressive elimination of groups of prospective donors raises concerns about blood availability, and at least in the USA, the difference between blood collected and blood needed is slim. An alternative to the system of surveillance, testing, and exclusion is to apply technologies (chemicals, light activation, irradiation, filters) to remove or inactivate infectious agents in the donated blood. Strategies to inactivate pathogens in blood have become a reality for plasma fractions, an evolving success story for plasma and platelet components, but still a pipe dream for red cells [29].

Non-infectious complications, such as hemolysis, alloimmunization, and transfusion-associated acute lung injury have recently been reviewed [28]. These risks are generally well-recognized, relatively uncommon, and amenable to technologic solutions. One of the more controversial and certainly hotly debated issues is whether allogeneic red-cell transfusion, or further the age of red cells stored in plastic containers, affects the morbidity and mortality of the recipient. Currently red cells may be stored for up to 42 days at refrigerated temperatures. Preservative solutions and container composition vary around the world. However, there is no question that red cells undergo progressive morphological, biochemical, structural, and functional deterioration during this six-week interval. Changes in the red-cell membrane, alterations in cell metabolism, Hb leakage, progressive component acidosis, loss of intracellular potassium, and lipid peroxidation, which all occur during storage of red cells, have been termed the "storage lesion" [30]. Analyses of numerous retrospective and prospective cohorts have arrived at differing conclusions regarding the clinical importance of these changes, although the majority of the reports suggest that morbidity and mortality among the critically ill increase with increasing red-cell transfusion and with the storage age of the transfused cells [31]. A recent analysis compared 2872 cardiac surgery patients who received red-cell transfusion stored for

less than 2 weeks with 3130 matched patients given blood stored for 14 days or longer [32]. At 1 year, mortality was significantly lower in patients given "fresher" blood, and transfusion of red cells that had been stored for more than 14 days was associated with a significantly increased risk of postoperative complications. All of these studies suffer from methodologic and statistical weaknesses. Such controversies can rarely be settled with more observational studies or without reference to prospective, randomized, blinded studies, and the latter are both difficult and expensive. Fortunately, in the area of the storage lesion, two such trials have recently been proposed, the Age of Blood Evaluation (ABLE) study in Canada and the Red Cell Storage Age Study (RECESS) in the USA.

15.5 Conclusion

Red-cell collection, storage, indications, and usage vary internationally and even within countries. Surprisingly few data-driven studies guide red-cell use. Although blood is safer than ever, particularly in developed countries, it is not zero-risk and cannot be rendered risk-free. In developed countries, the current risk of infection from blood is small, but the potential dangers from imported agents and emerging infections are significant. Non-infectious risks from administration of the wrong unit, transfusion-related acute lung injury, and graft-versus-host disease persist, but these can be improved and technology is promising in several areas. The importance of red cells as a toxin and of the "storage lesion" needs to be explored. The greatest potential for transfusion benefit is in the developing world, where blood is both scarce and hazardous. What blood is available is often untested and inadequately processed, stored, and transported. This is where a safe, effective, and affordable red-cell substitute might have a major impact.

Disclaimer

This article was written in a personal capacity and does not necessarily represent the opinions or endorsement of the NIH, DHHS, or the Federal Government.

References

[1] Waller, C. Case of uterine hemorrhage, in which the operation of transfusion was successfully performed, *Med. Phys. J*., **54**, 273–277 (1825).
[2] Blundell, J. Observations on the transfusion of blood, *Lancet*, **ii**, 321–324 (1828).
[3] Landsteiner, K. Uber Agglutinationserscheinungen normalen menschlichen Blutes, *Wien Klin. Wschr*., **14**, 1132–1134 (1901).
[4] Coombs, R.R.A., Mourant, A.E. and Race, R.R. A new test for the detection of weak and "incomplete" Rh agglutinins, *Brit. J. Exp. Path*., **26**, 255–266 (1945).
[5] Robertson, O.H. Transfusion with preserved red blood cells, *Brit. Med. J*., **1**, 691–695 (1918).
[6] Spinella, P.C., Perkins, J.G., Grathwohl, K.W. *et al*. Warm fresh whole blood is independently associated with improved survival for patients with combat-related traumatic injuries, *J. Trauma*, **66**, S69–S76 (2009).

[7] Marwaha, N. Whole blood and component use in resource poor settings, *Biologicals*, **38**, 68–71 (2010).
[8] Walter, C.W. Invention and development of the blood bag, *Vox Sang*., **47**, 318–324 (1984).
[9] Allen, J.G., Dawson, D., Sayman, W.A. *et al*. Blood transfusions and serum hepatitis: use of monochloroacetate as an antibacterial agent in plasma, *Ann. Surg*., **150**, 455–458 (1959).
[10] Whitaker, B.I., Green, J., King, M.R. *et al*. The 2007 National Blood Collection and Utilization Survey, http://www.hhs.gov/ash/bloodsafety/2007nbcus_survey.pdf.
[11] Sweeney, J.D. Control of blood utilization, *Transfus. Apher. Sci*., **39**, 139–144 (2008).
[12] Kamper-Jorgensen, M., Edgren, G., Rostgaard, K. *et al*. Blood transfusion exposure in Denmark and Sweden, *Transfusion*, **49**, 888–894 (2009).
[13] Quaranta, J.F., Berthier, F., Courbil, R. *et al*. Who uses blood? Results of a cross-sectional and nation wide survey, *Transfus. Clin. Biol*., **16**, 21–29 (2009).
[14] Friedman, B.A., Burns, T.L. and Schork, M.A. An analysis of blood transfusion of surgical patients by sex: a question for the transfusion trigger, *Transfusion*, **20**, 179–188 (1980).
[15] Adams R.C. and Lundy, J.S. Anesthesia in cases of poor risk: some suggestions for decreasing the risk, *Surg. Gynecol. Obstet*., **74**, 1011–1019 (1942).
[16] The 1988 NIH Consensus Conference on Perioperative Red Blood Cell Transfusions, *JAMA*, **260**, 2700–2703 (1988).
[17] Klein, H.G., Spahn, D.R. and Carson, J.L. Red blood cell transfusion in clinical practice, *Lancet*, **370**, 415–426 (2007).
[18] Van Woerkens, E.C.S., Trouwborst, A. and van Lanschot, J.J. Profound hemodilution: what is the critical level of hemodilution at which oxygen delivery-dependent oxygen consumption starts in an anesthetized human?, *Anesth. Analg*., 75, 818–821 (1992).
[19] Viele, M.K. and Weiskopf, R.B. What can we learn about the need for transfusion from patients who refuse blood? The experience with Jehovah's Witnesses, *Transfusion*, **34**, 396–401 (1994).
[20] Herzog, C.A., Muste, H.A., Li, S. and Collins, A.J. Impact of congestive heart failure, chronic kidney disease, and anemia on survival in the Medicare population, *J. Card. Fail*., **10**, 467–472 (2004).
[21] Wu, W.C., Rathore, S.S., Wang, Y. *et al*. Blood transfusion in elderly patients with acute myocardial infarction, *N. Engl. J. Med*., **345**, 1230–1236 (2001).
[22] Carson, J.L., Duff, A., Poses, R.M. *et al*. Effect of anaemia and cardiovascular disease on surgical mortality and morbidity, *Lancet*, **348**, 1055–1060 (1996).
[23] Hébert, P.C., Wells, G., Tweeddale, M. *et al*. Does transfusion practice affect mortality in critically ill patients? Transfusion Requirements in Critical Care (TRICC) Investigators and the Canadian Critical Care Trials Group, *Am. J. Respir. Crit. Care Med*., **155**, 1618–1623 (1997).
[24] Wang, J.K. and Klein, H.G. Red blood cell transfusion in the treatment and management of anaemia: the search for the elusive transfusion trigger, *Vox Sang*., **98**, 2–11 (2010).

[25] Hébert, P.C., Wells, G., Blajchman, M.A. *et al*. A multicenter, randomized, controlled clinical trial of transfusion requirements in critical care. Transfusion Requirements in Critical Care Investigators, Canadian Critical Care Trials Group, *N. Engl J. Med*., **340**, 409–417 (1999).

[26] Lacroix, J., Hébert, P.C., Hutchison, J.S. *et al*. TRIPICU Investigators, Canadian Critical Care Trials Group, Pediatric Acute Lung Injury and Sepsis Investigators Network. Transfusion strategies for patients in pediatric intensive care units, *N. Engl. J. Med*., **356**, 1609–1619 (2007).

[27] Deans, K.J., Minneci, P.C., Klein, H.G. and Natanson, C. The relevance of practice misalignments to trials in transfusion medicine, *Vox Sang*., **99**, 16–23 (2010).

[28] Alter H.J. and Klein, H.G. The hazards of blood transfusion in historical perspective, *Blood*, **112**, 2617–2626 (2008).

[29] Bryant, B.J. and Klein, H.G. Pathogen inactivation: the definitive safeguard for the blood supply, *Arch. Pathol. Lab. Med*., **131**, 719–733 (2007).

[30] Holme, S. Current issues related to the quality of stored RBCs, *Transfus. Apheresis Sci*., **33**, 55–61 (2005).

[31] Tinmouth, A., Fergusson, D., Yee I.C. and Hébert, P.C. ABLE Investigators; Canadian Critical Care Trials Group. Clinical consequences of red cell storage in the critically ill, *Transfusion*, **46**, 2014–2027 (2006).

[32] Koch, C.G., Li, L., Sessler, D.I. *et al*. Duration of red-cell storage and complications after cardiac surgery, *N. Engl. J. Med*., **358**, 1229–1239 (2008).

16

Causes and Consequences of Red Cell Incompatibility

Chisa Yamada and Robertson Davenport

University of Michigan Medical School, Ann Arbor, MI, USA

16.1 Introduction

For most of the 20th century, from the first description of blood groups by Karl Landsteiner, blood transfusion was the main treatment of anemia. Today, despite the development of pharmaceutical erythropoietin, transfusion remains the major therapeutic option for providing oxygen carrying capacity, particularly in acute anemia. Transfusion would be a relatively simple matter were it not for immunologic incompatibility. In this chapter we will summarize the blood groups and compatibility testing and the hemolytic transfusion reactions, the clinical consequence of red cell incompatibility.

16.2 Red Cell Antigens

A total of 29 blood group systems are recognized by the International Society of Blood Transfusion [1]. More than 200 red cell antigens have been described within these systems, in addition to antigens that have not been assigned to a known system. Blood group antigens vary considerably in frequency and in clinical importance.

16.2.1 ABO and the H System

A and B antigens are carbohydrates and controlled by genes at three loci, H, Se, and ABO, which encode for glycosyl transferases. H locus produces a transferase to create H

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antigen on red cells, and Se locus produces a transferase to create H antigen in secretions such as saliva. The A antigen is produced by adding N-acetylgalactosamine to the H antigen, while the B antigen results from adding galactose to the H antigen. The O blood type represents the absence of either A or B. O alleles do not produce a functional transferase and do not produce any additional antigens, so that only H antigen is present in an individual with only O alleles. More than 215 ABO alleles have been described [2]. The carbohydrate chains that carry ABH antigens are bound to either glycoproteins, glycosphingolipids, or glycolipids, and the former two with A, B, and H antigens are parts of the membranes of red, epithelial, and endothelial cells.

16.2.2 The Lewis System and Structurally Related Antigens

The Lewis (Le), I and i, and P antigens have the same carbohydrate precursor structure as ABO antigens. Le antigens are found as glycolipids in plasma and adsorbed on to red cells. The Le^a antigen is the product of the Lewis gene, and Le^b antigen is created by the Se gene by adding one more fucose on to Le^a antigen. Therefore, an individual who has a Lewis gene and an Se gene will have both Le^a and Le^b antigens in secretions; however, only Le^b antigen is detected on red cells in these individuals, since Le^b is favored for red cell adhesion sites over Le^a. I and i antigens have the same core precursor as ABH antigens. The fetal and cord red cells predominantly carry nonbranched structures expressed as i antigens, and the majority of adult red cells carry branched structures expressed as I antigens. The P blood group antigens are also synthesized by adding sugars to the same precursor substances.

16.2.3 The Rh System

Rh (Rhesus) antigens are proteins and are the second most important blood group system next to ABO due to the clinical significance of incompatibility. The antigens in this system are erythroid specific and are not found in other tissues. Currently more than 50 different Rh antigens have been identified [1, 3]. Of these, five (D, C, c, E, and e) are the most immunogenic, causing clinical problems when antibodies are present. These antigens are composed of numerous epitopes. Rh antigens are encoded for by two closely related genes, RhD and RhCE. C and c are antithetical antigens carried on the RhCE protein, as are E and e. The D antigen is carried on the RhD protein and has no antithetical antigen. “Rh-positive” and “Rh-negative” represent the presence or absence of the D antigen, respectively.

16.2.4 Other Blood Group Systems

The other most clinically significant red blood cell (RBC) blood groups are the Kell system, the Duffy (Fy) system, the Kidd (Jk) stytem, the MNS system, the Lutheran system, and the Chido/Rogers system, which are all located on glycoproteins. K in the Kell system has high immunogenicity and anti-K is developed by as much as 10% of chance when K-negative individuals receive K-positive RBCs. The Fy glycoprotein belongs to the superfamily of chemokine receptors and is known as the “Duffy antigen receptor for chemokines” [4]. The Fy(a−, b−) phenotype, resulting from non-expression of the Duffy glycoprotein on RBCs, found almost only in persons of central African ancestry,

confers resistance to malarial infection. The Jk antigens are urea transporters, therefore lack of these antigens on RBCs induces resistance to lysis in 2 M urea. However, Jk(a−, b−) phenotype usually does not cause any clinically significant dysfunctions. M and N are carried on glyophorin A. M and N are antithetical antigens and highly polymorphic. S and s are carried on glyophorin B and are also antithetical. The S−, s− phenotype, occasionally found in blacks, is extremely rare and lacks the high prevalence antigen U. The Luthran glycoproteins are adhesion molecules that bind to the extracellular matrix glycoprotein laminin. This complex is thought to play a role in organogenesis, vascular development, erythropoiesis, and smooth muscle development and organization, though the role of Luthran on RBCs is unclear. The nine antigens of the Chido/Rogers system are located on the component of C4d complement in plasma and adsorbed on to RBCs in a similar way to Lewis antigens, most of which are high incidence antigens.

16.3 Red Cell Antibodies

Exposure to antigen positive RBCs, through either transfusion or pregnancy, can cause antigen negative individuals to make red cell antibodies. In addition, some individuals produce antibodies to their own RBCs (autoantibodies) or to neoantigens induced by some drugs (drug dependent antibodies).

16.3.1 Naturally Occurring Antibodies and Immune Antibodies

Naturally occurring antibodies are produced even if the individual has not been exposed to other red cells by transfusions or pregnancies. These antibodies are produced in response to environmental antigens that are very similar to red cell antigens carried on bacteria or other environmental sources. This is the origin of the expected ABO antibodies. Consequentially, neonates have none or a very little amount of these antibodies. Naturally occurring antibodies are generally IgM cold agglutinins, which react at room temperature or lower, activate complement, and can cause intravascular hemolysis when reactive at body temperature. Antibodies to ABH, Lewis, I and i, P, M, and N are examples of this type. Among these, anti-A, anti-B, and anti-H are particularly important because they react well at body temperature and can cause immediate hemolysis. In contrast, immune antibodies are produced only when individuals are exposed to non-self red cell antigens. Most of these antibodies are IgG and react best at body temperature. Examples of clinically significant IgG red cell antibodies are antibodies to Rh, Kell, Fy, Jk, and MNS (other than M and N) systems. IgG antibodies usually do not activate complement, with rare exceptions, and they cause primarily extravascular hemolysis. Most of these antibodies are detectable for many years after the initial immune response, even in the absence of further immunologic stimulation, but antibodies to Jk system antigens tend to decrease to undetectable levels with time [5]. Since the immune responses after the second exposure are rapid and strong and cause hemolysis, a history of previous antibodies is important in compatibility testing. There are a number of IgG antibodies to other blood system antigens which do not activate complement and are not clinically significant. Antibodies in the Chido/Rogers blood group system and the Knopps/McCoy system are historically called high-titer, low avidity antibodies. Because these antibodies bind to antigen weakly, agglutination strength often shows 1+, but reacts with diluted

serum of 1 : 64 or higher. They are clinically insignificant. However, identifying these antibodies is important because they often create confusion in the detection of clinically significant antibodies.

16.3.2 Autoantibodies

Autoantibodies are produced in response to self antigens. These can result in hemolysis of autologous RBCs as well as transfused cells. Most auto antibodies do not have identifiable specificities and agglutinate all cells tested, which makes the detection of underlying alloantibodies difficult. Although RBC autoantibodies in many patients who have autoimmune diseases such as systemic lupus erythematosis or rheumatoid arthritis do not cause hemolysis, the presence of these antibodies is often detected in antibody identification testing in blood bank.

16.3.3 Drug Induced Antibodies

Drugs that bind to RBCs can cause formation of antibodies against the drug itself or against red cell membrane, which can result in hemolysis of red cells. Alternatively, anti-drug antibody–drug complexes formed in plasma which activated complement can bind to red cells. Many drugs have been reported to cause immune hemolysis, but the most common offenders are β-lactam antibiotics. Rarely, drugs can also induce the production of autoantibodies to red cell antigens.

16.4 Compatibility Testing

Pretransfusion compatibility testing is essential for successful transfusion for the prevention of hemolytic transfusion reactions, which can be life threatening.

16.4.1 ABO and Rh D Typing

ABO and Rh D typing are the first steps for compatibility testing. ABO typing includes testing for A and B antigens on RBCs, called forward typing, and testing for anti-A and anti-B in plasma, called reverse typing. Forward and reverse typing must be matched before the ABO typing can be concluded. ABO typing is usually straightforward, but occasional discrepancies can be seen, for multiple reasons. The determination of Rh status is done using anti-D blood typing reagents. Rh-positive indicates the presence of the D antigen. Typing for other RBC antigens is not routinely performed prior to transfusion, but may be indicated in special circumstances.

16.4.2 Antibody Screening and Identification

Antibody screening is the second step of pretransfusion testing. This test is to detect clinically significant unexpected antibodies in patients' sera. Cells from two or three donors with known different antigen sets are mixed and tested with the patient's serum. If the test is positive, showing agglutination with any of the screening cells, antibody identification testing is then performed. Several methods are available for this test, including low ionic strength solution anti-human globulin (AHG) methods, gel AHG methods, and other tube methods with albumin or polyethylene glycol enhancement. All of these tests

are performed to identify specific clinically significant antibodies. Once the specificity of the antibody is identified, red cells lacking the corresponding antigen are selected for transfusion. Finding compatible RBCs can be difficult if there are multiple antibody specificities, or if there is an antibody to a high incidence antigen.

16.4.3 Selection of Appropriate Blood

When the ABO group and Rh type are confirmed, group and type identical red cells are usually provided for transfusion. In emergency cases when there is not time for completion of compatibility tests, group O red cells may be given. If antibody screening is positive and a clinically significant specificity is identified, red cells without corresponding antigens are selected. However, a negative screening test does not guarantee the compatibility, due to antibodies to low incidence antigens, which can be clinically significant but are not identified in routine tests, or to variable reactivity with the method of antibody identification used. Therefore, a crossmatch will be performed as a final step.

16.4.4 Crossmatch Testing

The purpose of the crossmatch is final verification of compatibility. Two kinds of crossmatch testing are performed in regular blood bank practice: computer/electronic crossmatch and immediate spin/antiglobulin crossmatch. When no clinically significant unexpected antibodies are detected currently and historically, a computer/electronic crossmatch can be performed to match the ABO group and Rh type of the patient and those of red cell units using the data stored in a computer system. For other cases, immediate spin or antiglobulin crossmatches are performed. The immediate spin crossmatch is designed to detect mainly ABO incompatibilities between patient serum and donor red cells at room temperature, which is optimal for IgM activation. The antiglobulin crossmatch detects mainly IgG coated red cells by adding anti-human globulin and incubation at body temperature.

16.5 Hemolytic Transfusion Reactions

A hemolytic transfusion reaction (HTR) is the accelerated clearance or lysis of transfused RBCs due to immunologic incompatibility. While most HTRs are caused by RBC transfusion, occasional reactions result from transfusion of plasma containing blood components such as platelets. HTR may be an acute or delayed reaction, and may result in intravascular or extravascular hemolysis. Intravascular hemolysis is characterized by hemoglobinemia and hemoglobinuria, while extravascular hemolysis is characterized by shortened survival of transfused RBCs, along with the accumulation of hemoglobin breakdown products.

There is a broad range of initial clinical presentations of HTR. Most patients with intravascular hemolysis present with fever and/or chills. Nausea or vomiting, pain, dyspnea, and hypotension or tachycardia may also be seen. In extravascular hemolysis, fever and/or chills are the most commonly reported initial symptoms, although many patients are asymptomatic. An acute HTR usually presents during or shortly after transfusion. The time from transfusion to clinical presentation of delayed HTR is quite variable, though it mostly presents within two weeks after transfusion.

HTR may be particularly severe in patients with sickle cell disease [6]. In such reactions the degree of anemia may actually be greater than before transfusion, due to bystander hemolysis of autologous red cells. In some cases there is suppression of erythropoiesis, while in others the reticulocyte count and the absolute number of hemoglobin S-containing red cells increases during HTR [1, 7]. Pain crisis in a sickle cell patient following transfusion should suggest the occurrence of sickle cell hemolytic transfusion-reaction syndrome. Further transfusion in this setting may exacerbate the anemia and even prove fatal. Serologic studies may not provide an explanation for HTR in these patients. In addition, the presence of multiple alloantibodies may make the serologic diagnosis difficult.

16.5.1 Pathophysiology

HTRs proceed through three phases: antibody–antigen interaction, phagocytosis and inflammatory cell activation, and systemic response. Initially, there is a binding of antibody to red cell antigens, which can result in complement activation. Next, immunoglobulin and complement coated cells interact with phagocytes, resulting in clearance and activation. Finally, the inflammatory mediators produced in the first two phases act on a variety of cell types, causing a clinical manifestation of HTR.

The course of immune hemolysis is determined by antigen site density, the immunoglobulin class of the alloantibody, and activation of complement. ABO antigens are present in high numbers on red cell surfaces, while Rh, Kell, Kidd, and Duffy antigens are 10–100 fold less abundant. Complement fixation is facilitated by close proximity of antigens, which allows bridging of IgG molecules by C1q, although IgM antibodies can fix complement without requiring bridging between molecules. IgM antibodies are common in the ABO system, but relatively unusual as alloantibodies to other antigens. Activation of the classical pathway of complement proceeds from C1q binding through C3 activation. Cleavage of C3 results in C3a liberation into circulation and C3b deposition on the red cell membrane. Activated C3 may then cleave C5 with release of C5a. Assembly of the membrane attack complex may then proceed with resultant intravascular hemolysis. Factor I is the major regulator of C3b activity. Cleavage of membrane bound C3b by factor I results in the generation of iC3b and release of the small peptide fragment C3c. This terminates the complement cascade, since iC3b is enzymatically inactive. iC3b is further degraded into C3dg and C3d by factor I and trypsin-like proteinases. Red cell bound C3d is responsible for complement reactivity in the indirect antiglobulin test (Coombs' test).

Erythrophagocytosis results from interaction of immunoglobulin and/or complement coated red cells with phagocyte receptors. Cell bound antibodies promote red cell clearance primarily through interaction of the Fc portion of IgG with specific receptors. FcγRIII appears to be the most important IgG receptor on splenic macrophages in alloimmune and autoimmune red cell clearance, as well as in autoimmune thrombocytopenia [8–10]. The principal complement receptor expressed by macrophages and monocytes, CR3, primarily recognizes iC3b. Receptors for C3a and C5a are present on a wide variety of cells, including monocytes, macrophages, neutrophils, platelets, endothelium, and smooth muscle. The physiologic effects of C3a and C5a include oxygen radical

production, granule enzyme release, leukotriene production, nitric oxide production, and cytokine production.

Immune hemolysis stimulates the production of a variety of cytokines that are crucial to the initiation, maintenance, and ultimate resolution of HTR. ABO incompatibility strongly stimulates production of tumor necrosis factor-α (TNF-α) and the chemokines CXCL-8 and CCL-4 [11–14]. TNF-α is an early response, appearing in plasma within 2 hours, and has potent proinflammatory effects, including pyrogenic activity, leukocyte activation, stimulation of procoagulant activity, and expression of a large number of gene products related to the inflammatory response. TNF-α produced in blood during ABO incompatibility can stimulate endothelial cells to express leukocyte adhesion molecules, chemotactic cytokines, and procoagulant activity [15]. CXCL-8 and CCL-4 produced during ABO incompatibility appear later than TNF-α and can reach very high levels. CXCL-8 primarily activates neutrophils to undergo the respiratory burst, release granule contents, and alter surface adhesion molecules [16]. CCL-4 is primarily a chemotactic and activating factor for monocytes [17].

In IgG mediated extravascular hemolysis there are two categories of cytokine responses: those produced at high levels, greater than 1 ng/ml by 24 hours, and those produced at lower levels, in the range of 100 pg/ml [13, 18, 19]. Low level cytokine responses include IL-1β, IL-6, and TNF-α. CXCL-8 is a high level response with a time course similar to that of ABO incompatibility. In contrast to the setting of ABO incompatibility, TNF-α is produced in a delayed fashion in response to IgG coated red cells, achieving a level of less than 100 pg/ml. However, cell associated TNF-α can be demonstrated by immunocytochemical staining in monocytes engaged in erythrophagocytosis.

While the *in vitro* models employed in these studies are not directly comparable, these findings do suggest a possible reason for the clinical differences between intravascular and extravascular hemolytic transfusion reactions. In the former case, TNF-α is released into systemic circulation, where it can have diverse effects on many cell types, whereas in the latter case, TNF-α effects may be confined to local effects at the site of erythrophagocytosis, primarily the spleen. Both IL-1β and IL-6 produced by monocytes in response to IgG coated red cells increase progressively over 24 hours to levels approximating 100 pg/ml. Since IL-1α and IL-6 are B-cell growth and differentiation factors, the production of these two cytokines may promote the production of red cell allo- and autoantibodies, which are often associated with delayed HTRs. IgG-mediated hemolysis also results in the production of the IL-1β inhibitor IL-1ra, in parallel with IL-1β [20]. Neutralization of IL-1β does not suppress either IL-1ra or IL-1β gene expression in this setting. Therefore, it appears that IL-1ra production is a primary response to the IgG coated red cell stimulus, rather than an autocrine phenomenon induced by initial IL-1β production. Thus, the clinical variability of delayed HTR, and some of the clinical differences from intravascular HTR, may be accounted for, in part, by the relative balance of IL1-β and IL-1ra production.

Labile blood pressure is a feature of severe HTR, particularly with intravascular hemolysis. Both complement activation products such as C5a, and cytokines such as IL-1β and TNF-α can contribute to hypotension. The common pathway of these mediators is the production of nitric oxide by endothelial cells. Nitric oxide, in turn, causes relaxation of vascular smooth muscle. Hypotension and deposition of thrombi in arterioles, which

impair cortical blood flow, are the major factors that contribute to renal failure. In addition, there may be direct effects of inflammatory mediators on the kidneys.

16.5.2 Prevention

Hemolytic transfusion reactions could be avoided entirely if the responsible antigens on the red cell surface could be removed or camouflaged. One possible strategy is treating red cells with polyethylene glycol (PEG) as a means of camouflaging antigens [21]. PEG modification appears to work by creating a sphere of hydration around the red cell that effectively excludes IgG or IgM from coming into contact with antigenic structures on the membrane surface. PEG treated red cells have size, shape, intracellular ion content, and oxygen binding properties that are identical to those of untreated red cells. However, PEG treated red cells have a low shear viscosity compared to normal red cells. This may be advantageous in sickle cell disease, in which increased blood viscosity within capillaries can result in occlusive crises.

The effectiveness of PEG modification is dependent on the molecular weight and branching characteristics of PEG molecules and the chemistry of covalent attachment [22]. Use of a dichlorotriazine derivative of 5 kDa PEG results in complete inhibition of direct agglutination by anti-D. However, such cells are still agglutinated by anti-D in the indirect antiglobulin test. A and B epitopes are partially, but not completely, masked. In contrast, RBCs coated with branched chain 10 kDa PEG after treatment with succinimidyl propionate modified 20 kDa PEG are not agglutinated by anti-A, anti-B, or anti-D.

Group A and group B RBCs can be converted to group O by enzymatic cleavage of terminal determinant saccharides with α-N-acetylgalactosaminidase or α-D-galactosidase [23–25]. The use of such technology for large scale conversion of RBCs raises the possibility that acute HTR due to ABO incompatibility may be completely avoidable in the future. However, there are issues with regard to the completeness of antigen removal and the possibility of exposure to neoantigens by enzymatic treatment. Treatment of red cells with α-N-acetylgalactosaminidase results in rapid loss of A epitopes binding Dolicos biflorus lectin [18]. Inhibition of complement mediated hemolysis is somewhat slower. However, the epitopes of A antigen that react with human source anti-A are relatively resistant to enzymatic degradation. Additionally, there are differences in the enzymatic sensitivity of A epitopes on the red cell membrane. Glycosphingolipids with short oligosaccharide chains display the greatest resistance to enzymatic treatment.

Clinical trials with enzyme converted group O (ECO) RBCs have shown promise [26, 27]. An initial trial of a two unit transfusion of ECO RBC to group O subjects demonstrated good 24 hour post-transfusion survival, 95%, with a half-life of 29.5 days [21]. There was no clinical or laboratory evidence of hemolysis. A subsequent study with larger volume transfusions had similar results. Subjects who received a second transfusion did not show evidence of alloimmunization or increase in anti-B titer [22].

References

[1] Daniels, G.L., Fletcher, A., Garratty, G. *et al*. Blood group terminology 2004: from the International Society of Blood Transfusion committee on terminology for red cell surface antigens, *Vox. Sang*., **87**, 304–316 (2004).

[2] Storry, J.R. and Olsson, M.L. The ABO blood group system revisited: a review and update, *Immunohematology*, **25**, 48–59 (2009).
[3] Daniels, G.L., Castilho, L., Flegel, W.A. *et al*. International Society of Blood Transfusion Committee on terminology for red blood cell surface antigens: Macao report, *Vox. Sang*., **96**, 153–156 (2009).
[4] Hadley, T.J. and Peiper, S.C. From malaria to chemokine receptor: the emerging physiologic role of the Duffy blood group antigen, *Blood*, **89**, 3077–3091 (1997).
[5] Mollison, P.L. and Engelfriet, C.P. and Contreras, M. *Blood Transfusion in Clinical Medicine*, 11 edn, Blackwell Science, Oxford, UK, p. 217 (2005).
[6] Petz, L.D., Calhoun, L., Shulman, I.A. *et al*. The sickle cell hemolytic transfusion reaction syndrome, *Transfusion*, **37**, 382–392 (1997).
[7] King, K.E., Shirey, R.S., Lankiewicz, M.W. *et al*. Delayed hemolytic transfusion reactions in sickle cell disease: simultaneous destruction of recipients' red cells, *Transfusion*, **37**, 376–381 (1997).
[8] Davenport, R.D. and Kunkel, S.L. IgG receptor roles in red cell binding to monocytes and macrophages, *Transfusion*, **34**, 79S (1994).
[9] Unkeless, J.C. Function and heterogeneity of human Fc receptors for immunoglobulin G, *J. Clin. Invest*., **83**, 355–361 (1989).
[10] Clarkson, S.B., Kimberly, R.P., Valinsky, J.E. *et al*. Blockade of clearance of immune complexes by anti-Fc gamma receptor monoclonal antibody, *J. Exp. Med*., **164**, 474–489 (1986).
[11] Butler, J., Parker, D., Pillai, R. *et al*. Systemic release of neutrophil elastase and tumour necrosis factor alpha following ABO incompatible blood transfusion, *Br. J. Haematol*., **79**, 525–526 (1991).
[12] Davenport, R.D., Strieter, R.M. and Kunkel, S.L. Red cell ABO incompatibility and production of tumour necrosis factor-alpha, *Br. J. Haematol*., **78**, 540–544 (1991).
[13] Davenport, R.D., Strieter, R.M., Standiford, T.J. and Kunkel, S.L. Interleukin-8 production in red blood cell incompatibility, *Blood*, **76**, 2439–2442 (1990).
[14] Davenport, R.D., Burdick, M.D., Strieter, R.M. and Kunkel, S.L. Monocyte chemoattractant protein production in red cell incompatibility, *Transfusion*, **34**, 16–19 (1994).
[15] Davenport, R.D., Burdick, M. and Kunkel, S.L. Endothelial cell activation in hemolytic transfusion reactions, *Transfusion*, **32**, 53S (1992).
[16] Matsushima, K. and Oppenheim, J.J. Interleukin 8 and MCAF: novel inflammatory cytokines inducible by IL 1 and TNF, *Cytokine*, **1**, 2–13 (1989).
[17] Jiang, Y., Beller, D.I., Frendl, G. and Graves, D.T. Monocyte chemoattractant protein-1 regulates adhesion molecule expression and cytokine production in human monocytes, *J. Immunol*., **148**, 2423–2428 (1992).
[18] Davenport, R.D., Burdick, M., Moore, S.A. and Kunkel, S.L. Cytokine production in IgG mediated red cell incompatibility, *Transfusion*, **33**, 19–24 (1993).
[19] Hoffman, M. Antibody-coated erythrocytes induce secretion of tumor necrosis factor by human monocytes: a mechanism for the production of fever by incompatible transfusions, *Vox Sang*., **60**, 184–187 (1991).

[20] Davenport, R.D., Burdick, M.D., Strieter, R.M. and Kunkel, S.L. In vitro production of Interleukin-1 receptor antagonist in IgG mediated red cell incompatibility, *Transfusion*, **34**, 297–303 (1994).
[21] Scott, M.D., Bradley, A.J. and Murad, K.L. Camouflaged blood cells: low-technology bioengineering for transfusion medicine?, *Transfus. Med. Rev*., **14**, 53–63 (2000).
[22] Fisher, T.C., Armstrong, J.K., Meiselman, H.J. *et al*. Second generation poly(ethylene glycol) surface coatings for red blood cells, *Transfusion*, **40**, 119S (2000).
[23] Hoskins, L.C., Larson, G. and Naff, G.B. Blood group A immunodeterminants on human red cells differ in biologic activity and sensitivity to alpha-N-acetylgalactosaminidase, *Transfusion*, **35**, 813–821 (1995).
[24] Hobbs, L., Mitra, M., Phillips, R. *et al*. Deantigenation of human type B erythrocytes with Glycine max alpha-D-galactosidase, *Biomed. Pharmacother*., **49**, 244–250 (1995).
[25] Zhu, A., Leng, L., Monahan, C. *et al*. Characterization of recombinant alpha-galactosidase for use in seroconversion from blood group B to O of human erythrocytes, *Arch. Biochem. Biophys*., **327**, 324–329 (1996).
[26] Lenny, L.L., Hurst, R., Goldstein, J. and Galbraith, R.A. Transfusions to group O subjects of 2 units of red cells enzymatically converted from group B to group O, *Transfusion*, **34**, 209–214 (1994).
[27] Lenny, L.L., Hurst, R., Zhu, A. *et al*. Multiple-unit and second transfusions of red cells enzymatically converted from group B to group O: report on the end of phase 1 trials, *Transfusion*, **35**, 899–902 (1995).

17

Biochemistry of Storage of Red Blood Cells

Ryan Stapley, Dario A. Vitturi and Rakesh P. Patel

Department of Pathology and Center for Free Radical Biology, University of Alabama at Birmingham, AL, USA

17.1 Introduction

Contrary to the general notion that the red blood cell (RBC) is simply a 'dead' bag that carries hemoglobin, it is now clear that these cells play critical roles in many vascular processes. These encompass the primary function of reversible oxygen binding by hemoglobin via allosterically regulated processes which ensure the matching of oxygen delivery to metabolic need in all tissues. In addition to transport of other respiratory gases (e.g. carbon dioxide), other well-known functions include blood buffering, regulation of ion transport and encapsulating hemoglobin, thereby preventing direct heme-based toxicity to other vascular cells, as discussed in detail elsewhere in this book. It has emerged that RBCs and hemoglobin also regulate vascular nitric oxide (NO) metabolism and thereby can impact the multiple homeostatic roles this free radical plays, including vasodilation, platelet aggregation and inflammation, to name but a few [1]. In the latter context, RBCs may also impact vascular inflammation by modulating circulating cytokine levels and by regulating oxidative/nitrosative stress [2, 3]. Given the multiple physiological roles in the vasculature for RBCs, it has been postulated that dysfunction in these roles during RBC storage underlies the etiology of the so-called 'RBC storage lesion', which defines toxicities associated with transfusion of older units of stored RBCs. In this chapter, we will first briefly discuss the storage lesion and then focus on the biochemical changes that occur in RBCs during storage and how these impact their multiple

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physiological functions. Our goal is to highlight the current hypotheses and proposed mechanisms by which RBC storage may contribute to transfusion-related toxicities.

17.2 Pathologic Consequences of Transfusion with Aged RBCs

Resuscitation with banked RBCs is a frontline therapy for improving tissue oxygenation after blood loss. However, recent studies have demonstrated an increase in transfusion-related toxicities (e.g. vascular dysfunction, lung injury) after resuscitation with older RBC units [4], a clinical phenomenon referred to as the 'RBC storage lesion'. Current blood-storage techniques have extended the shelf life of packed red-cell units to 42 days but significant deleterious effects are observed with 14–21-day-old RBCs [4, 5]. This, coupled with limitations of donor supply and the time taken for RBC processing post-collection (~7–14 days), indicates that a large majority of transfusions are performed with RBCs that may have deleterious consequences. Initial studies pointed towards white blood cell-derived products generated during storage (including reactive lipids, cytokines) in mediating the storage lesion by stimulating inflammatory processes, a concept that has led to the clinical practice of leukodepletion [5]. Despite extensive leukoreduction, significant transfusion-related toxicities still remain with the use of older RBC units, suggesting factors intrinsic to the RBC itself are also altered during storage and play key roles in storage lesion. Current concepts suggest that during storage, biochemical changes in the RBC lead to dysfunction in the multiple biological roles that RBCs play in the vasculature, which include but also go beyond oxygen binding. We will discuss these changes next, and how they may alter the physiological functions of RBCs and thereby promote toxicity.

17.3 Changes in Oxygen Affinity During RBC Storage

A primary concern in the storage of RBCs is loss of oxygen-binding capacity and increase in hemoglobin oxygen affinity, leading to an RBC that is unable to adequately deliver oxygen to respiring tissues. Reversible oxygen binding occurs with ferrous (Fe^{2+}) hemoglobin, however hemoglobin also continually becomes oxidized by oxygen to form met- or ferric hemoglobin (Fe^{3+}), which is unable to bind oxygen. In the RBC, the enzyme metHb reductase reduces ferric hemoglobin back to ferrous hemoglobin, thereby ensuring that oxygen-binding capacity is not compromised. However, this system is lost during storage, leading to the accumulation of ferric hemoglobin. Interestingly, anaerobic storage preserves oxygen-binding affinity by limiting hemoglobin autoxidation and oxidative stress [6, 7]. In addition to loss of oxygen carrying capacity, dysfunction in the allosteric mechanisms that regulate the oxygen affinity of hemoglobin is also observed, leading to a mismatch between oxygen delivery and tissue requirements. These include decreased concentrations of 2,3-bisphosphoglycerate (2,3-BPG) and ATP, which decrease oxygen affinity. The loss of 2,3-BPG in RBC during storage is rapid, decreasing to undetectable within 1 week of storage [8]. However, upon transfusion, *de novo* synthesis of 2,3-BPG will restore levels to normal within 48 hours, but importantly, over this initial period after delivery and depending on the amount of blood transfused, the overall

oxygen affinity will remain elevated and can cause tissue hypoxia [9]. Similar changes occur in ATP, which in addition to affecting oxygen affinity will also compromise other RBC functions such as active transport, oxidative stress defense mechanisms, membrane stability and phospholipid distribution, among other energy-requiring reactions. This in turn increases the vulnerability to cell stresses encountered in the circulation, resulting in increased lysis [10]. As blood is stored, the cell's ATP is used in metabolic processes to maintain the conditions aforementioned. To help compensate for the loss of ATP, erythrocytes are stored in a media that contain sugars (e.g. glucose or dextrose) and adenine to enable the synthesis of ATP. These two components will ameliorate the loss of ATP levels but will not completely restore them.

Currently employed storage solutions have been developed with the primary goal of preventing hemoglobin oxidation and slowing the loss of allosteric effectors. Despite these efforts, emerging evidence indicates that other changes in the RBC still occur that could lead to dysregulated oxygen delivery. For example, expression of the RBC membrane protein anion exchanger 1 (AE1, or band 3), which is involved in mediating the exchange of chloride and bicarbonate ions across the RBC membrane, decreases by ~50% over a 6-week storage time [11]. Loss of AE-1 appears to be a result of proteosomal activity. The precise functional consequences of such a dramatic loss of AE-1 on RBC function after resuscitation are not clear, but underscore the concept that multiple biochemical changes during storage may compromise RBC functions in the vasculature.

17.4 Role of Oxidative Damage During RBC Storage

Many studies have documented increased oxidative stress in various RBC components during storage time and with different storage solutions [12–15]. This is typically characterized by an increase in markers of oxidative stress (e.g. increase in protein carbonyls, thiol and lipid oxidation products) and by a loss of reductants, antioxidants and antioxidant enzyme activities (e.g. glutathione, glutathione peroxidase, peroxiredoxin). Redox-cycling reactions between hemoglobin and lipid hydroperoxides and/or hydrogen peroxide are central in mediating oxidative damage to red-cell components [16, 17], with the consequence being manifold depending on the target, extent and nature of the oxidative event. One example pertinent to oxygen carrying capacity is loss of NAD(P)H reducing equivalents, which compromises the methemoglobin reductase system and thereby leads to an accumulation of methemoglobin. Similarly, a RBC with compromised antioxidant defenses is also more susceptible to damage by exogenous reactive species, which may be encountered in the circulation and especially so in patients who have vascular inflammation, which likely encompasses many groups who would require transfusions. Moreover, a common property of stored RBCs is increased fragility, which can arise from lipid peroxidation and/or oxidative modification of cytoskeletal proteins. Therefore, preventing oxidative stress during storage is an important therapeutic goal that could limit storage-related changes in the RBC. Strategies to inhibit hemoglobin redox cycling are also likely to affect oxygen binding and therefore are not clinically useful. However, storage of RBCs at low-oxygen tensions as a strategy to limit reactive oxygen species formation has been recently shown to significantly attenuate oxidative damage [18, 19]. Other studies on the other hand have suggested that chronic hypoxia

in fact compromises RBC-reducing equivalents required for antioxidant protection, by inhibiting flux through the hexose monophosphate pathway [20]. The potential for low-oxygen storage therefore requires further evaluation. Finally, several studies have tested antioxidant repletion strategies during storage. For example, supplementing storage solution with amino-acid precursors for glutathione synthesis prevented loss of several oxidant sensitive proteins in the RBC [21, 22]. Together, these studies provide proof of concept that modulating oxidative stress in the RBC during storage is possible, but whether this translates to improved function and prevention of toxicity post-transfusion remains to be determined.

17.5 Changes in the Physical Properties of RBCs During Storage

The biconcave disc shape of RBCs is essential for function as it increases the surface area-to-volume ratio, thereby facilitating RBC deformability and allowing RBC transit through smaller capillaries. As a RBC ages *in vivo* or during storage, its shape changes to an echinocyte and then spheroechinocyte and becomes less deformable and more fragile. These changes are important for the expression of epitopes that target the RBC for removal by the immune system but also result in a higher probability for lysis, both during storage and after transfusion. In fact, hemolysis during storage varies between 1 and 5.5% depending on the storage medium and length. This is important because cell-free hemoglobin is a potent oxidizing agent and scavenger of NO in the vascular compartment which may play a key role in the pathologic mechanisms of the storage lesion. How hemolysis can be modulated specifically is not clear, since unlike changes in oxygen affinity, the physical changes that occur in RBCs are not readily reversible, suggesting prevention as the only viable therapeutic strategy.

17.6 RBCs as Modulators of Vascular Flow

Blood flow across individual vascular beds must accommodate for changes in metabolic rates of the different tissues, matching oxygen and nutrient supply with demand. Interestingly, studies in which the relative role of dissolved oxygen tension and hemoglobin-bound oxygen (oxygen content) as mediators of hypoxic vasodilatation is assessed show that oxygen fractional saturation is the main determinant of changes in exercise induced vascular blood flow in humans [23, 24]. These observations suggest that RBCs are crucial mediators in the modulation of hypoxic blood flow. In this regard, three hypotheses have been put forward proposing mechanisms by which RBCs might affect vascular tone.

17.6.1 ATP Release Hypothesis

According to the model proposed by Ellsworth and Sprague, RBC deoxygenation [25–27], and more precisely hemoglobin desaturation [28], activates a cAMP-dependent signaling pathway that leads to the release of micromolar concentrations of ATP into the vasculature, stimulating vasodilatation [29]. Once in the circulation, ATP binds P_{2Y} purinergic receptors in the endothelium [30], leading to the activation of eNOS and

production of NO. Since the signal that stimulates ATP release is dependent on the fractional saturation of hemoglobin, an altered P50 could potentially uncouple regulated ATP release. Moreover, storage of RBCs leads to a progressive decline in glucose uptake [6] and ATP levels [7, 31]. This observation suggests that in addition to altered oxygen sensing, stored RBCs might even have decreased availability of ATP to release. In fact, it has been shown that inhibition of glycolysis leads to a decreased ability of RBCs to release ATP upon exposure to hypoxia despite the presence of millimolar levels of ATP still available in the cytosol [28]. Also, RBC storage is associated with increased lactate levels (from 1 to 12 mM in the first 10 days) [7, 8, 31], which has been shown to inhibit ATP release at similar concentrations [32]. As a result, it is possible that transfused RBC might not be able to release ATP until the excess lactate is metabolized by the donor.

Finally, whereas DPG and ATP levels can be restored within 48 hours post-transfusion [19, 33], there are other storage-associated alterations in the RBC that are thought to be irreversible. There is ample evidence that storage of RBCs leads to aggregation [12, 34, 35] and degradation of the AE1 [12, 35]. Moreover, these results correlate with the observation of a progressive loss of oxygen-sensitive regulation of glycolysis and the pentose phosphate pathways [6], which in circulating RBCs is thought to be mediated by the displacement of glycolytic enzymes from their binding site at the cytoplasmic domain of AE1 by deoxyhemoglobin [36, 37]. Notably, this competition between deoxyhemoglobin and glycolytic enzymes has been proposed to be involved in the synthesis of the releasable ATP pool [28]; it is possible therefore that the ability of RBCs to stimulate hypoxic blood flow by ATP release could result in irreversible impairment during storage despite regeneration of cytosolic ATP levels after transfusion.

17.6.2 SNO-hemoglobin Hypothesis

Another mechanism implicating RBCs as physiologically relevant modulators of blood flow has been postulated by Stamler and colleagues. According to this hypothesis, the transition between R-state and T-state hemoglobin is associated with the release of NO equivalents derived from the reactions of an S-nitrosothiol located on a conserved Cys residue at the 93rd position of the beta chains of hemoglobin (SNOHb) [38–44].

With regards to a possible alteration of this pathway as a result of RBC storage, two recent studies [8, 45] suggested that the level of S-nitrosated hemoglobin decreased within hours of storage and remained below basal for the remainder of the storage time. Moreover, these stored RBCs had diminished capacities to elicit hypoxic vasorelaxation [8, 45], and repleting S-nitrosohemoglobin (and nitrosylhemoglobin) to levels similar to those observed in fresh RBCs restored their ability to elicit vasorelaxation under hypoxia [45]. Unfortunately, the levels of nitrite, a common contaminant found in NO solutions, introduced during treatment of RBCs with NO were not assayed, thus preventing a potential contribution of nitrite-mediated vasodilatation from being confidently disregarded. Moreover, potential contributions from ATP release were not tested either. Other biochemical changes that normally take place during RBC storage may contribute to decreased NO delivery via the SNO-hemoglobin mechanism. For instance, AE1 has been reported to be involved in the export of NO activity by RBCs [43] and glutathione nitrosylation/denitrosylation [38, 46] might also play a role in this mechanism. Both the

total concentration and the aggregation state of AE1 changes as a function of RBC storage [34, 35, 47] and glutathione levels are also decreased under this same conditions [15].

Finally, whereas these reports suggest that deficiencies or alterations in SNOHb homeostasis are associated with the loss of physiological activity of banked blood, a recent publication from our group shows that mice in which the β93Cys has been replaced with alanine, precluding S-nitrosohemoglobin formation, develop normally and are physiologically indistinguishable from mice that do express β93Cys [48]. Moreover, we observed that RBCs isolated from animals lacking the β93Cys are able to induce hypoxic vasorelaxation *ex vivo* in a manner indistinguishable from wild type; we ascribe this observation to oxygen-dependent ATP release [48, 49]. The importance of the S-nitrosohemoglobin pathway, and subsequent defects in it, in affecting blood flow remains to be elucidated.

17.6.3 Nitrite Reductase/Anhydrase Hypothesis

The third hypothesis involves the reduction of the anion nitrite by deoxyhemoglobin to produce NO according to the following mechanism [50]:

$$NO_2^- + H^+ + Fe^{II}Hb \rightarrow \bullet NO + Fe^{III}Hb + OH^-$$
$$\bullet NO + Fe^{II}Hb \rightarrow Fe^{II}NOHb$$

The concept here is similar to that above in that hemoglobin deoxygenation triggers a mechanism leading to NO formation. Notably, the rate of this reaction depends on the quaternary conformation of hemoglobin, being faster when the heme centers are under the R-state conformation [49, 51, 52]. Coupled with the requirement for deoxygenation to provide substrate heme for nitrite reduction, the effective initial rate of nitrite reduction (determined by the product of deoxyhemoglobin concentration and rate constant) versus hemoglobin oxygen fractional saturation exhibits a bell-shaped profile with maximal rates between 40 and 60% saturation [49, 52]. This relationship is compliant with the physiological range of hemoglobin fractional saturations (~50–100%) and suggests that increased rates of nitrite reduction to NO parallel hemoglobin deoxygenation in the microcirculation. Evaluating this hypothesis remains an active area of investigation, with recent studies suggesting that following nitrite reduction by deoxyhemoglobin, subsequent reactions lead to the formation of the nitrosating agent dinitrogen trioxide (N_2O_3), which subsequently can decompose to yield NO either by homolysis or via the formation of a nitrosothiol intermediate [53].

There are no reports in the literature assessing the effects of RBC storage on its ability to induce hypoxic vasorelaxation via deoxyhemoglobin-mediated nitrite reduction, and similar to ATP, alterations in oxygen affinity will affect the coupling of hypoxia to nitrite reduction. Interestingly, we have recently reported that the interaction between deoxyhemoglobin and the cytoplasmic domain of AE1 inhibits nitrite export by RBCs under hypoxemic conditions (half-fractional saturations, 30 mm Hg), thereby allowing for more nitrite to be utilized for nitrite reduction and NO production [54]. In line with this observation, deoxyhemoglobin bound to AE1 has been shown to be a more efficient nitrite reductase than its cytosolic counterpart [55]. Taken together, these reports suggest that the alterations in AE1 levels and structures might influence the metabolism of nitrite by stored RBCs. Finally, the progressive crosslinking of oxidized/denatured hemoglobin to membrane proteins during storage [12] might also affect nitrite homeostasis in RBCs

by either decreasing the ability of red cells to transport nitrite across the membrane or by altering the global reactivity of the cells towards this anion.

Based on current understanding, it is clear that RBC storage has the potential to significantly alter all the pathways discussed above and thereby compromise the coupling of hemoglobin deoxygenation with increased NO-dependent blood flow. Since NO is critical in numerous other vascular processes, including limiting inflammation, it is tempting to speculate that dysfunction in the hemoglobin oxygen sensing–NO formation relationship will also promote inflammation that characterizes many of the toxic effects associated with the storage lesion.

17.7 RBC-dependent Modulation of Inflammation

Emerging evidence suggests that RBCs may be critical players in regulating vascular inflammation via two principal mechanisms. First is the ability of RBCs to affect NO concentrations. NO is an anti-inflammatory effector with multiple mechanisms described, ranging from antioxidant effects to downregulating inflammatory adhesion-molecule expression [56]. The ability of RBC to stimulate NO production is discussed above, and whether these pathways contribute to the anti-inflammatory effects of NO is currently unknown. Stronger data pointing towards a pro-inflammatory role for stored RBCs is associated with increased hemolysis. Specifically, cell-free hemoglobin is a more potent (~1000-fold) scavenger of vascular NO than erythrocytic hemoglobin [57]. Therefore, even low levels of hemolysis that increase circulating free hemoglobin to modest levels (5–10 μM) are sufficient to significantly scavenge and inhibit endogenous NO signaling. In this way, cell-free hemoglobin tilts the 'normal' balance of NO formation versus NO consumption to the latter and results in hypertension and pro-inflammatory effects. It is important to note that in addition to NO scavenging, cell-free hemoglobin can also promote oxidative damage to biomolecules, which itself can lead to inflammatory stress [16]. In summary, hemolysis and increased fragility associated with RBC storage are likely to play a central role in promoting inflammatory injury.

More recently, pro-inflammatory mechanisms independent of hemolysis have also been demonstrated. RBC possesses Duffy-antigen receptors for chemokines (DARC) located on the cell membranes. DARCs bind several chemokines and are viewed as nonspecific scavengers of these proteins, thereby limiting the magnitude of the inflammatory response [3, 58, 59]. Recent studies have shown that during RBC storage, the activity of DARCs is decreased [3]. In turn, this leads to higher concentrations of circulating chemokines, leading to more distal organ (e.g. lung) injury.

17.8 Conclusion

In the past few years there has been renewed interest in blood-storage techniques and investigation of the blood-storage lesion. As stated in this chapter, there are many aspects of the RBC that change during storage. In the past, little was known about these changes and even less about how they affect the multitude of functions that RBCs play. With emerging data highlighting new functions of RBCs (e.g. regulating blood flow and

inflammation), the need to understand these change during storage is clear. This will not only offer directions for developing strategies that maximize the effectiveness and safety of RBC transfusions but will also likely provide insights into mechanisms of other hematological diseases and complications.

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References

[1] Crawford, J.H., Chacko, B.K., Kevil, C.G. and Patel R.P. The red blood cell and vascular function in health and disease, *Antioxid. Redox Signal*., **6**, 992–999 (2004).
[2] Kiefmann, R., Rifkind, J.M., Nagababu, E. and Bhattacharya, J. Red blood cells induce hypoxic lung inflammation, *Blood*, **111**, 5205–5214 (2008).
[3] Mangalmurti, N.S., Xiong, Z., Hulver, M. *et al*. Loss of red cell chemokine scavenging promotes transfusion-related lung inflammation, *Blood*, **113**, 1158–1166 (2009).
[4] Weinberg, J.A., McGwin, G. Jr, Griffin, R.L. *et al*. Age of transfused blood: an independent predictor of mortality despite universal leukoreduction, *J. Trauma*., **65**, 279–282; disc. 282–284 (2008).
[5] Vamvakas, E.C. and Carven J.H. RBC transfusion and postoperative length of stay in the hospital or the intensive care unit among patients undergoing coronary artery bypass graft surgery: the effects of confounding factors, *Transfusion*, **40**, 832–839 (2000).
[6] Messana, I., Ferroni, L., Misiti, F. *et al*. Blood bank conditions and RBCs: the progressive loss of metabolic modulation, *Transfusion*, **40**, 353–360 (2000).
[7] Yoshida, T., AuBuchon, J.P., Dumont, L.J. *et al*. The effects of additive solution pH and metabolic rejuvenation on anaerobic storage of red cells, *Transfusion*, **48**, 2096–2105 (2008).
[8] Bennett-Guerrero, E., Veldman, T.H., Doctor, A. *et al*. Evolution of adverse changes in stored RBCs, *Proc. Natl. Acad. Sci. U S A*, **104**, 17063–17068 (2007).
[9] Stan, A. and Zsigmond, E. The restoration in vivo of 2,3-diphosphoglycerate (2,3-DPG) in stored red cells, after transfusion: the levels of red cells 2,3-DPG, *Rom. J. Intern. Med*., **47**, 173–177 (2009).
[10] Raat, N.J., Verhoeven, A.J., Mik, E.G. *et al*. The effect of storage time of human red cells on intestinal microcirculatory oxygenation in a rat isovolemic exchange model, *Crit. Care Med*., **33**, 39–45; disc. 238–239 (2005).
[11] Antonelou, M.H., Kriebardis, A.G., Stamoulis, K.E. *et al*. Red blood cell aging markers during storage in citrate-phosphate-dextrose-saline-adenine-glucose-mannitol, *Transfusion*, **50**, 376–389 (2010).

[12] Kriebardis, A.G., Antonelou, M.H., Stamoulis, K.E. *et al*. Progressive oxidation of cytoskeletal proteins and accumulation of denatured hemoglobin in stored red cells, *J. Cell. Mol. Med*., **11**, 148–155 (2007).
[13] Kriebardis, A.G., Antonelou, M.H., Stamoulis, K.E. *et al*. RBC-derived vesicles during storage: ultrastructure, protein composition, oxidation, and signaling components, *Transfusion*, **48**, 1943–1953 (2008).
[14] Korgun, D.K., Bilmen, S. and Yesilkaya A. Alterations in the erythrocyte antioxidant system of blood stored in blood bags, *Res. Commun. Mol. Pathol. Pharmacol*., **109**, 357–363 (2001).
[15] Dumaswala, U.J., Zhuo, L., Jacobsen, D.W. *et al*. Protein and lipid oxidation of banked human erythrocytes: role of glutathione, *Free Radic. Biol. Med*., **27**, 1041–1049 (1999).
[16] Alayash, A.I., Patel, R.P. and Cashon R.E. Redox reactions of hemoglobin and myoglobin: biological and toxicological implications, *Antioxid. Redox Signal*., **3**, 313–327 (2001).
[17] Rogers, M.S., Patel, R.P., Reeder, B.J. *et al*. Pro-oxidant effects of cross-linked haemoglobins explored using liposome and cytochrome c oxidase vesicle model membranes, *Biochem. J*., **310**(Pt 3), 827–833 (1995).
[18] D'Amici, G.M., Rinalducci, S. and Zolla L. Proteomic analysis of RBC membrane protein degradation during blood storage, *J. Proteome Res*., **6**, 3242–3255 (2007).
[19] Dumont, L.J., Yoshida, T. and AuBuchon, J.P. Anaerobic storage of red blood cells in a novel additive solution improves in vivo recovery, *Transfusion*, **49**, 458–464 (2009).
[20] Rogers, S.C., Said, A., Corcuera, D. *et al*. Hypoxia limits antioxidant capacity in red blood cells by altering glycolytic pathway dominance, *FASEB J*., **23**, 3159–3170 (2009).
[21] Dumaswala, U.J., Wilson, M.J., Wu, Y.L. *et al*. Glutathione loading prevents free radical injury in red blood cells after storage, *Free Radic. Res*., **33**, 517–529 (2000).
[22] Dumaswala, U.J., Zhuo, L., Mahajan, S. *et al*. Glutathione protects chemokine-scavenging and antioxidative defense functions in human RBCs, *Am. J. Physiol. Cell. Physiol*., **280**, C867–873 (2001).
[23] Gonzalez-Alonso, J., Mortensen, S.P., Dawson, E.A. *et al*. Erythrocytes and the regulation of human skeletal muscle blood flow and oxygen delivery: role of erythrocyte count and oxygenation state of haemoglobin, *J. Physiol*., **572**(Pt 1), 295–305 (2006).
[24] Roach, R.C., Koskolou, M.D., Calbet, J.A. and Saltin, B. Arterial O_2 content and tension in regulation of cardiac output and leg blood flow during exercise in humans, *Am. J. Physiol*., **276**(2 Pt 2), H438–H445 (1999).
[25] Ellsworth, M.L., Forrester, T., Ellis, C.G. and Dietrich H.H. The erythrocyte as a regulator of vascular tone, *Am. J. Physiol*., **269**(6 Pt 2), H2155–H2161 (1995).
[26] Sprague, R.S., Ellsworth, M.L., Stephenson, A.H. and Lonigro, A.J. Participation of cAMP in a signal-transduction pathway relating erythrocyte deformation to ATP release, *Am. J. Physiol. Cell. Physiol*., **281**, C1158–C1164 (2001).
[27] Sprague, R.S., Olearczyk, J.J., Spence, D.M. *et al*. Extracellular ATP signaling in the rabbit lung: erythrocytes as determinants of vascular resistance, *Am. J. Physiol. Heart Circ. Physiol*., **285**, H693–H700 (2003).

[28] Jagger, J.E., Bateman, R.M., Ellsworth, M.L. and Ellis, C.G. Role of erythrocyte in regulating local O_2 delivery mediated by hemoglobin oxygenation, *Am. J. Physiol. Heart Circ. Physiol*., **280**, H2833–H2839 (2001).
[29] McCullough, W.T., Collins, D.M. and Ellsworth, M.L. Arteriolar responses to extracellular ATP in striated muscle, *Am. J. Physiol*., **272**(4 Pt 2), H1886–H1891 (1997).
[30] Horiuchi, T., Dietrich, H.H., Tsugane, S. and Dacey, R.G. Jr. Analysis of purine- and pyrimidine-induced vascular responses in the isolated rat cerebral arteriole, *Am. J. Physiol. Heart Circ. Physiol*., **280**, H767–H776 (2001).
[31] Yoshida, T., AuBuchon, J.P., Tryzelaar, L. *et al*. Extended storage of red blood cells under anaerobic conditions, *Vox Sang*., **92**, 22–31 (2007).
[32] Rozier, M.D., Zata, V.J. and Ellsworth, M.L. Lactate interferes with ATP release from red blood cells, *Am. J. Physiol. Heart Circ. Physiol*., **292**, H3038–H3042 (2007).
[33] Beutler, E. and Wood, L. The in vivo regeneration of red cell 2,3 diphosphoglyceric acid (DPG) after transfusion of stored blood, *J. Lab. Clin. Med*., **74**, 300–304 (1969).
[34] Karon, B.S., Hoyer, J.D., Stubbs, J.R. and Thomas, D.D. Changes in Band 3 oligomeric state precede cell membrane phospholipid loss during blood bank storage of red blood cells, *Transfusion*, **49**, 1435–1442 (2009).
[35] Bosman G.J., Lasonder E., Luten M. *et al*. The proteome of red cell membranes and vesicles during storage in blood bank conditions, *Transfusion*, **48**, 827–835 (2008).
[36] Campanella, M.E., Chu, H., Wandersee, N.J. *et al*. Characterization of glycolytic enzyme interactions with murine erythrocyte membranes in wild-type and membrane protein knockout mice, *Blood*, **112**, 3900–3906 (2008).
[37] Low, P.S., Rathinavelu, P. and Harrison, M.L. Regulation of glycolysis via reversible enzyme binding to the membrane protein, band 3, *J. Biol. Chem*., **268**, 14627–14631 (1993).
[38] Jia, L., Bonaventura, C., Bonaventura, J. and Stamler, J.S. S-nitrosohaemoglobin: a dynamic activity of blood involved in vascular control, *Nature*, **380**, 221–226 (1996).
[39] Gow, A.J., Luchsinger, B.P., Pawloski, J.R. *et al*. The oxyhemoglobin reaction of nitric oxide, *Proc. Natl. Acad. Sci. U S A*, **96**, 9027–9032 (1999).
[40] Gow, A.J. and Stamler, J.S. Reactions between nitric oxide and haemoglobin under physiological conditions, *Nature*, **391**, 169–173 (1998).
[41] McMahon, T.J., Stone, A.E., Bonaventura, J. *et al*. Functional coupling of oxygen binding and vasoactivity in S-nitrosohemoglobin, *J. Biol. Chem*., **275**, 16738–16745 (2000).
[42] McMahon, T.J., Moon, R.E., Luschinger, B.P. *et al*. Nitric oxide in the human respiratory cycle, *Nat. Med*., **8**, 711–717 (2002).
[43] Pawloski, J.R., Hess, D.T. and Stamler, J.S. Export by red blood cells of nitric oxide bioactivity, *Nature*, **409**, 622–626 (2001).
[44] Singel, D.J. and Stamler, J.S. Chemical physiology of blood flow regulation by red blood cells: the role of nitric oxide and S-nitrosohemoglobin, *Annu. Rev. Physiol*., **67**, 99–145 (2005).

[45] Reynolds, J.D., Ahearn, G.S., Angelo, M. *et al*. S-nitrosohemoglobin deficiency: a mechanism for loss of physiological activity in banked blood, *Proc. Natl. Acad. Sci. U S A*, **104**, 17058–17062 (2007).
[46] Liu, L., Yan, Y., Zeng, M. *et al*. Essential roles of S-nitrosothiols in vascular homeostasis and endotoxic shock, *Cell*, **116**, 617–628 (2004).
[47] Kriebardis, A.G., Antonelou, M.H., Stamoulis, K.E. *et al*. Storage-dependent remodeling of the red blood cell membrane is associated with increased immunoglobulin G binding, lipid raft rearrangement, and caspase activation, *Transfusion*, **47**, 1212–1220 (2007).
[48] Isbell, T.S., Sun, C.W., Wu, L.C. *et al*. SNO-hemoglobin is not essential for red blood cell-dependent hypoxic vasodilation, *Nat. Med*., **14**, 773–777 (2008).
[49] Crawford, J.H., Isbell, T.S., Huang, Z. *et al*. Hypoxia, red blood cells, and nitrite regulate NO-dependent hypoxic vasodilation, *Blood*, **107**, 566–574 (2006).
[50] Gladwin, M.T., Grubina, R. and Doyle, M.P. The new chemical biology of nitrite reactions with hemoglobin: R-state catalysis, oxidative denitrosylation, and nitrite reductase/anhydrase, *Acc. Chem. Res*., **42**, 157–167 (2009).
[51] Huang, K.T., Keszler, A., Patel, N. *et al*. The reaction between nitrite and deoxyhemoglobin. Reassessment of reaction kinetics and stoichiometry, *J. Biol. Chem*., **280**, 31126–31131 (2005).
[52] Huang, Z., Shiva, S., Kim-Shapiro, D.B. *et al*. Enzymatic function of hemoglobin as a nitrite reductase that produces NO under allosteric control, *J. Clin. Invest*., **115**, 2099–2107 (2005).
[53] Basu, S., Grubina, R., Huang, J. *et al*. Catalytic generation of N_2O_3 by the concerted nitrite reductase and anhydrase activity of hemoglobin, *Nat. Chem. Biol*., **3**, 785–794 (2007).
[54] Vitturi, D.A., Teng, X., Toledo, J.C. *et al*. Regulation of nitrite transport in red blood cells by hemoglobin oxygen fractional saturation, *Am. J. Physiol. Heart Circ. Physiol*., **296**, H1398–H1407 (2009).
[55] Salhany, J.M. Kinetics of reaction of nitrite with deoxy hemoglobin after rapid deoxygenation or predeoxygenation by dithionite measured in solution and bound to the cytoplasmic domain of band 3 (SLC4A1), *Biochemistry*, **47**, 6059–6072 (2008).
[56] Patel, R.P., McAndrew, J., Sellak, H. *et al*. Biological aspects of reactive nitrogen species, *Biochim. Biophys. Acta*, **1411**, 385–400 (1999).
[57] Rother, R.P., Bell, L., Hillmen, P. and Gladwin, M.T. The clinical sequelae of intravascular hemolysis and extracellular plasma hemoglobin: a novel mechanism of human disease, *J. Am. Med. Assoc*., **293**, 1653–1662 (2005).
[58] Reutershan, J., Harry, B., Chang, D. *et al*. DARC on RBC limits lung injury by balancing compartmental distribution of CXC chemokines, *Eur. J. Immunol*., **39**, 1597–1607 (2009).
[59] Schnabel, R.B., Baumert, J., Barbalic, M. *et al*. Duffy antigen receptor for chemokines (Darc) polymorphism regulates circulating concentrations of monocyte chemoattractant protein-1 and other inflammatory mediators, *Blood*, **115**, 5289–5299 (2009).

18

Proteomic Investigations of Stored Red Blood Cells

Lello Zolla and Angelo D'Alessandro

Department of Environmental Sciences, Tuscia University, Viterbo, Italy

18.1 Introduction

Red blood cells (RBCs) are still the most transfused blood component worldwide [1]. Preservation and long-term storage of RBCs are impelling issues in transfusion medicine, in order to ensure a readily available, safe blood supply for clinical [2] or military purposes [3]. Indeed, although 92% of all RBC units that meet release criteria actually find a recipient [4], the demand from organizations for longer and better storage has recently increased.

On one hand, although the modern blood-banking establishment keeps pace with the current ordinary demand, it is not nevertheless tailored to meet the need for massive RBC supplies and for rare blood-group units under extraordinary circumstances, such as in a calamity or a disaster [5]. Nonetheless, seasonal shortages seldom occur, although local supplies occasionally run out.

On the other hand, a growing body of evidence has contributed to outlining a concrete relationship between the RBC lesions that accumulate over storage time and the undesired effects experienced by recipients after reinfusion of long-stored RBCs [6, 7]. Questions about the safety and viability of stored RBCs arise and persist, especially since RBC storage lesions have been linked to major complications in transfused recipients, although randomized prospective double-blind studies are currently *in fieri* [8, 9].

The standard protocol approved by the Council of Europe suggests a 42-day storage of RBCs at 4 °C, with an accepted *in vivo* recovery after 24 hours from reinfusion

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above the 75% threshold, with hemolysis under 0.8% upon the introduction of the 95/95 rule [10, 11].

18.2 RBC Ageing and Metabolism *in vivo*

RBCs play a pivotal role in gas transport (i.e. oxygen and carbon dioxide) and a minor, but no less important, role in a range of other functions, such as transfer of GPI-linked proteins and transport of iC3b/C3b-carrying immune complexes [12].

In humans, the circulating mature RBC is the end stage of a developmental process which starts in the bone marrow, as hematopoietic stem cells differentiate to enucleated reticulocytes [13]. After extrusion of nuclei and degradation of internal organelles and endoplasmic reticulum, reticulocytes emerge in the circulation, where they rapidly develop into mature RBCs. Through the duration of its lifespan of 120 ± 4 days, with 120 miles of travel and 1.7×10^5 circulatory cycles, the human RBC successfully copes with a number of dangers, such as passages across narrow capillaries and splenic slits, periodic high turbulences and high shear stresses, and extremely hypertonic conditions. Owing to their constant cytoskeleton rearrangement, RBCs are able to traverse passageways as narrow as 1 μm in diameter, by changing their shape from a biconcave disc of 8 μm diameter to a cigar shape [14]. The lack of internal organelles and nuclei intuitively hampers mRNA-targeting approaches, while making them an eligible target for early biochemical studies and for proteomic investigations, the latter of which have recently gained momentum [15–26].

Circulating RBCs *in vivo* undergo metabolic and physical changes associated with the process of senescence, viz membrane vesiculation, decrease in cell size, increase of cell density, alteration of cytoskeleton, enzymatic desilylation, and phosphatidylserine (PS) exposure, to mention just a few [27–29]. At the end of their lifespan, senescent RBCs are recognized and removed by the resident macrophages in the reticuloendothelial system (RES), mainly by Kupffer cells in the liver. It has been estimated that 5 million RBCs per second are endocytosed by RES macrophages [30].

More than one cause participates to the senescent/ageing process *in vivo*. Membrane and cytosolic proteins of RBCs are continuously stressed by oxygen radical attacks, which cause aminoacid modifications. The morphology, function, and metabolism of RBCs suffer from continuous alterations as the cell winds its way through the circulatory system. Basically, due to the lack of protein synthesis and inability to regenerate effective protein molecules, most notably enzymes, a multitude of alterations accumulate as the end of the RBC lifespan approaches. Neoantigens form from membrane proteins, especially through clustering of anion exchanger band 3 [31] and hemoglobin (Hb) denaturation [32]. Both proteins are closely related to gas transport [33, 34], cell homeostasis and shape [35], and glycolitic metabolism [36]. Thus, physiologically fundamental proteins fail to fulfill their biological goal. Furthermore, these senescent antigens which appear on oxidatively damaged old cells, radically accelerate RBC removal from blood flux through the activation of lifespan immunoregulation mechanisms, via triggering macrophage erythrophagocytosis, complement deposition, and immunoglobulin G (IgG) binding [37–39]. Antibody binding induces major alterations in membrane

organization as well as vesicle formation [40, 41]. Kupffer cells also remove RBC vesicles, with a major role for exposure of PS [42]. Taken together, all these data suggest more than a superficial resemblance between RBC ageing and apoptosis [43]. Particularly telling is the term "eryptosis", coined by Lang's group to identify that special form of apoptosis typical for the anucleated RBCs. [43]. Eryptosis is characterized by PS exposure, cellular shrinkage, membrane blebbing, ceramide formation, opening of cation channels, increase of intracellular Ca^{2+} activity, and activation of intracellular proteases such as μ-calpain, in the absence of hemolysis but ensuing into phagocytic recognition of exposed PS by a scavenger receptor on the macrophage [43]. This mechanism may be a parallel pathway leading to RBC removal without passing through IgG mediation [37–39].

To counteract oxidative damage, the anucleated RBC, which is unable to synthesize new proteins (except for the translation of the mRNA inherited from reticulocytes), is equipped with protective enzymes fully adequate to sustaining even excessive oxidative stress for limited time periods [44]. Indeed, it is not a coincidence that bioinformatic analyses of whole-RBC protein–protein interactions and functions have revealed a central core of proteins devoted to nascent protein folding or to refolding of denatured proteins (e.g. chaperonines, heat shock proteins, antioxidant proteins such as peroxiredoxins, catalases, glutathione peroxidases, superoxide dismutases) [14, 45].

Together with membrane and cytoskeleton alterations, senescence also provokes metabolic anomalies in RBCs. Similarly, the energyless RBC is inevitably lost [46]. Because of the lack of nuclei and mitochondria, mature RBCs are incapable of generating energy via the (oxidative) Krebs cycle. Nonetheless, there are four RBC metabolic pathways (Figure 18.1): the Embden–Meyerhof pathway (glycolysis), in which most of the RBC adenosine triphosphate (ATP) is generated through the anaerobic breakdown of glucose; the hexose monophosphate shunt (HMS), which produces NADPH to protect RBCs from oxidative injury; the Rapoport–Luebering shunt, responsible for the production of 2,3-diphosphoglycerate (DPG) for the control of Hb oxygen affinity; and finally, the methemoglobin (met-Hb) reduction pathway, which reduces ferric heme iron to the ferrous form to prevent Hb denaturation [47]. Glucose, the only fuel utilized by mature RBCs, is primarily metabolized via anaerobic glycolysis. Following facilitated diffusion, glucose is immediately converted to glucose-6 phosphate. Glucose can be transformed to lactate via glycolysis, or to ribulose-5-phosphate via the oxidative section of HMS. Ribulose-5-phosphate can reenter glycolysis via the nonoxidative section of HMS. Under normal steady-state conditions, 92% of glucose is metabolized along glycolysis and 8% along HMS. Under oxidant conditions, up to 90% of glucose can be metabolized along HMS. Net output of glycolysis is 2 M of ATP per mole of glucose metabolized. The main glycolytic pathway has two branching points: in the first, the product of hexokinase, glucose-6-phosphate, can be diverted to the HMS by glucose-6-phosphate dehydrogenase (G6PD); in the second, 1,3-DPG can be diverted by DPG-mutase to produce 2,3-DPG.

Maintenance of the RBC membrane system and Hb function is dependent on energy generation through RBC metabolic pathways.

Five metabolic intermediates are particularly important in RBCs: ATP, DPG, NADH, NADPH, and glutathione (GSH).

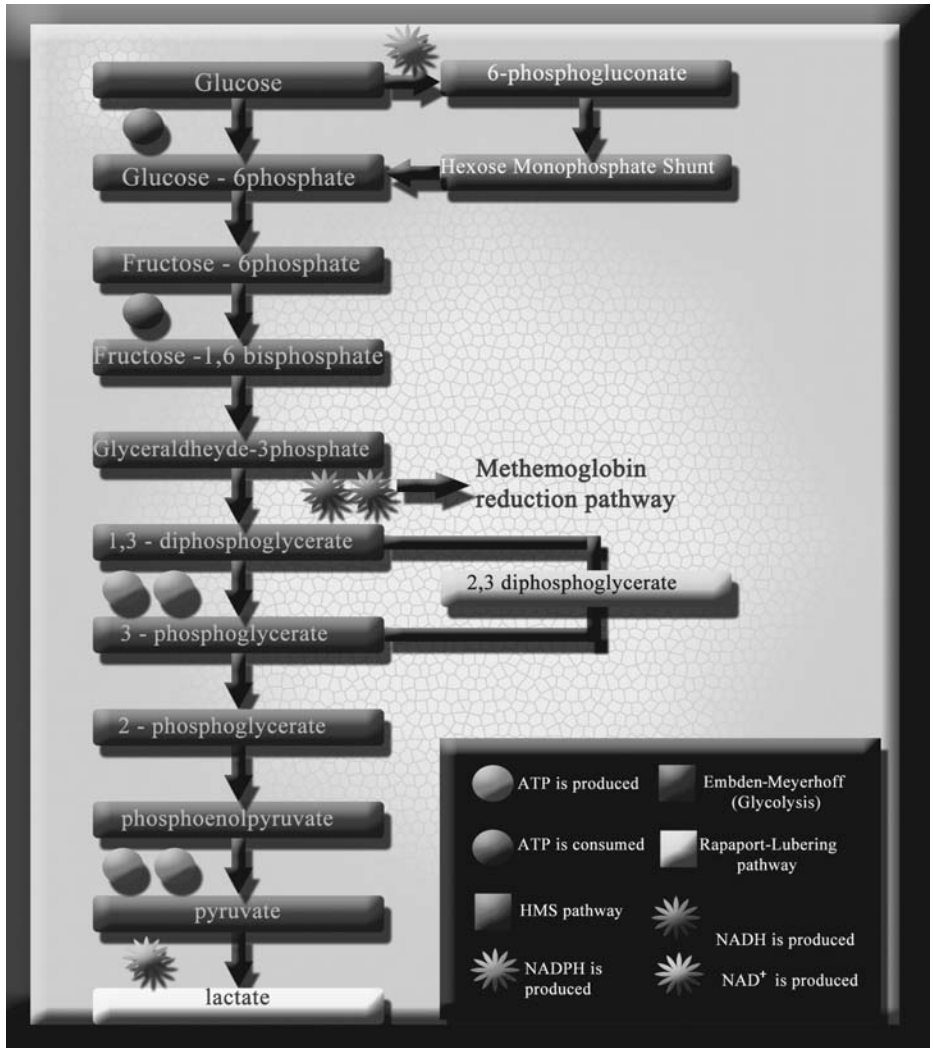

Figure 18.1 *RBC metabolism mainly gravitates towards four main pathways: the Embden–Meyerhof glycolytic pathway (for ATP production), the hexose monophosphate shunt (HMS) pathway (for NADPH generation), the Rapoport–Luebering pathway for 1,3-DPG conversion to 2,3-DPG, and the methemoglobin reduction pathway. When NADH is not fully oxidized back to NAD^+ through the methemoglobin reduction pathway, lactate is produced from pyruvate as a byproduct of anaerobic glycolysis.*
For a better understanding of the figure, please refer to color plate 3

(i) ATP, the primary energy intermediate, is essential to maintaining electrolyte balance by powering sodium–potassium cationic pumps, which are necessary to preserve the cytoplasmic ionic milieu, thus preventing colloidal osmotic lysis and, ultimately, conserving RBC shape and flexibility [48]; RBCs have an intrinsic program of cell death that is held in check by normal concentrations of RBC ATP. Normal

ATP concentrations are necessary to prevent calcium-induced membrane loss by microvesiculation and for active transport of negatively charged phospholipids, specifically PS, from the outer to the inner leaflet of the RBC membrane to prevent RBC clearance from the circulation by macrophages [49]. ATP is an essential resource for other focal physiological activities: the synthesis of GSH and other metabolites; purine and pyrimidine metabolism; the maintenance of Hb iron in its functional, reduced, ferrous state; the protection of metabolic enzymes, such as Hb and membrane proteins, from oxidative denaturation; and the preservation of membrane phospholipid asymmetry [50].

(ii) DPG, in association with pH and HCO_3^-/CO_2, modulates the position and shape of the oxygen dissociation curve [51]. When arterial blood arrives in peripheral capillaries, RBCs pass through the narrow capillaries one by one, CO_2 is rapidly hydrated to H_2CO_3 inside RBCs by carbonic anhydrase, and the H_2CO_3 promptly dissociates into H^+ and HCO_3^-. Band 3 protein, the major integral membrane protein of RBCs, exchanges the cellular HCO_3^- with Cl^- in plasma, a process that is conventionally known as the "chloride shift". As the result of the anion exchange, the weak acid H_2CO_3 is converted to the strong acid HCl, and consequently the intracellular pH of RBCs is rendered acidic. This acidification is the trigger for the dissociation of O_2 from oxyhemoglobin (HbO_2), and the dissociated O_2 is supplied to tissues that metabolically produce CO_2. Protons formed in RBCs are accepted by the groups of deoxyhemoglobin (HbH^+) participating in the "Bohr Effect", and the pH within the RBCs is restored in order to prevent further dissociation of oxygen from HbO_2. By means of the transient acidification triggered by the anion-exchange activity, tissues producing more CO_2 are supplied with more O_2 from HbO_2. The rapid disappearance of 2,3-DPG from preserved blood has not been of high concern because RBCs regain the ability to synthesize 2,3-DPG after transfusion. However, the restoration of 2,3-DPG *in vivo* requires up to 48 hours, and this period of altered oxygen affinity may be significant in certain clinical conditions [52]. Preserving RBC 2,3-DPG levels is therefore an essential element in maintaining the *ex vivo* quality of hypothermically stored RBCs. Inorganic phosphate is also added to the storage medium to act both as a buffer to the continuously decreasing pH and as a substrate for the synthesis of 2,3-DPG.

(iii) The ferrous iron of Hb is exposed continuously to high concentrations of oxygen and thereby oxidized slowly to met-Hb, a protein unable to carry oxygen. To restore Hb function, met-Hb (also known as ferrihemoglobin) must be reduced to Hb (ferrohemoglobin) [51]. Under physiological conditions, met-Hb reduction is accomplished mainly by red-cell NADH-dependent cytochrome b5 reductase (NADH-methemoglobin reductase) so efficiently that there are insignificant amounts of met-Hb in the circulating blood [53].

(iv) Under oxidative stress, Hb can be oxidized to met-Hb (as it regards its heme iron) and to hemichromes, variants of Hb in which cysteine thiol groups have been dangerously oxidized to form denatured Hb aggregates precipitating in inclusion bodies within RBCs, also known as "Heinz bodies". GSH is the main protector of thiol groups, a scavenger of oxides, peroxides, and oxidant radicals, and a detoxicant of foreign compounds. Glutathione cycling from oxidized glutathione (GSSG) to the reduced form (GSH) is dependent upon NADPH generation during the first

two reactions of HMS, by G6PD and 6-phosphogluconate dehydrogenase (6-PGD). NADPH is the substrate for GSH-reductase to regenerate glutathione after oxidant insults and protects catalase from inactivation [54].

$$\text{oxidized glutathione(GSSG)} + 2\text{NADPH} + \text{H}^+ \rightarrow 2\text{GSH} + 2\text{NADP}^+$$

(v) In concomitance with GSH-reductase, glutathione-S-transferase, peroxidases (namely peroxiredoxins and glutathione peroxidises), and superoxide dismutase, catalase is believed to be very important in cellular antioxidant defense and therefore prolongs RBC lifespan in mammals [55]. NADPH is also a protector of peroxiredoxin and catalase efficiency, and an insufficient concentration of NADPH causes an impairment of the catalase-dependent detoxication route [56].

Erythrocyte biopreservation is the ability to preserve the integrity of RBCs outside the native environment for extended periods. Its main end is to provide viable and functional RBCs for patients requiring a blood transfusion. The data provided in this section should help the reader glean an insight into RBC physiology, which is essential to assessing the effectiveness of a biopreservation approach, as well as the *in vitro* and *in vivo* quality of transfused RBCs [57].

18.3 RBC Storage Lesions Through Proteomics

At the macroscopic level, RBC cold-liquid storage induces a series of evident changes in the RBC shape [58] (Figure 18.2). During storage the erythrocyte shape visibly changes from a deformable discoid to a reversibly deformed echinocyte to an irreversibly deformed spheroechinocyte. These changes are triggered by the irreversible loss of membrane through the formation of vesicles, which is the likely cause of an increased osmotic fragility, a reduced deformability, and poor function after transfusion [59]. Vesicles are commonly grouped under two distinct categories: microvesicles and nanovesicles. The former have a mean diameter of approximately 180 nm, while the latter have an approximate diameter of 80 nm [25]. Though deriving from the shedding membranes, microvesicles display a particular protein composition in that they contain a low number of membrane proteins, a high number of metabolic enzymes, and an elevated content of Hb [25]. These vesicles contain almost no integral membrane proteins or cytoskeletal components, with the exception of band 3 and actin. The protein composition of the nanovesicles is quite different from that of the microvesicles and of the RBC membrane, with a conspicuously large number of complement and immunoglobulin proteins [25]. Raft formation may be involved in vesicle formation [60] and RBC storage has recently been shown to be associated with changes in the concentration of raft-associated proteins of the membrane fraction [25]. When examining the presence of the raft-associated proteins flotillin-1, flotillin-2, and stomatin, Bosman *et al*. found that, during storage, the RBC membrane content of flotillins and stomatin decreases, whereas the microvesicles become strongly (10-fold) enriched in stomatin, and that upon prolonged storage, the nanovesicles become 20-fold depleted of flotillins [25]. These main changes are accompanied by a wide range of other biochemical and molecular alterations.

Under a mere biochemical point of view, cooling below normal physiological temperatures inhibits metabolic processes and partially contrasts both the depletion of critical

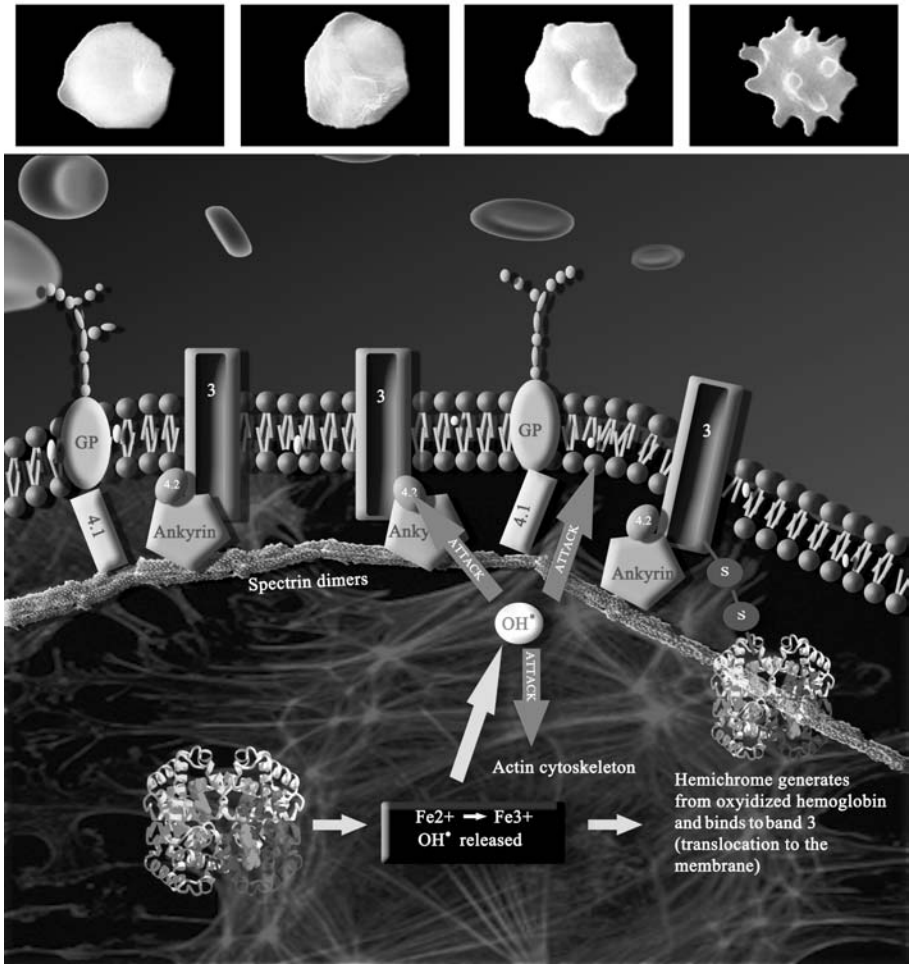

Figure 18.2 *A domino effect appears to be responsible for RBC storage lesions (upper panel shows shape of morphology-related lesions in time course, from left to right). Being stored in an oxidative environment, hemoglobin is slowly oxidized to met-Hb, and a low percentage of hemichrome aggregates form through disulfide bonds. In the meantime, heme iron is reduced from a ferrous to a ferric state via a Fenton's reaction, which produces $OH^{\bullet}$ radicals. A cascade of oxidative events takes place due to the spreading of reactive oxygen species (ROS). These events involve ROS attacks to the cytoskeleton and membrane (either lipid or protein fraction). A particularly eligible target appears to be the anion-exchanger band 3 and the proteins near to its cytosolic portion (band 4.2, ankyrin, and several enzymes such as glyceraldehyde-3-phosphate-dehydrogenase). Although RBCs are well equipped to cope with high oxidative stresses, prolonged storage periods end up exacerbating the oxidative phenomena, thus the outcome is a no-longer-functional or -vital RBC. Furthermore, the oxidative environment rapidly switches the metabolic trigger from the classic glycolytic pathway to the HMS pathway, with the result that the RBC experiences a total depletion of ATP. When transfused, the long-stored effete RBC is rapidly removed from the bloodstream or else it contributes to the promotion of untoward responses in the recipient.*
For a better understanding of the figure, please refer to color plate 4.

cellular metabolites and the accumulation of oxidative injuries. However, these benefits are counteracted by three effects: (i) the rate of met-Hb reduction by cytochrome b5 reductase is slowed; (ii) met-Hb may be more prone to denaturation, as suggested by lower thermodynamic stability of met-Hb at 4 °C; and (iii) the solubility of oxygen is doubled at 4 °C. As a result, oxidative damage can accumulate with refrigerated red-cell liquid storage [23].

Contrary to the senescence process, upon *in vitro* storage, RBCs lose potassium, DPG, ATP, and calcium stores. Moreover, RBCs undergo several changes, including alterations in cellular membrane, shape, phospholipid content, phospholipid asymmetry, and antigenic markers, while becoming more rigid and demonstrating reduced oxygen offloading [61]. RBCs also become more acidotic and the suspending fluid has a higher concentration of free Hb and biologically active lipids, and contains greater quantities of negatively charged microvesicles with pro-inflammatory and prothrombotic activity [62]. The potassium loss is a consequence of the altered metabolic activity upon cooling, while the loss of DPG and reduced glycolytic activity provoke a decrease in pH levels. DPG is typically gone by the 10th day of RBC storage, whereas ATP concentrations initially increase, due to precox DPG breakdown, or are stable during the first 2 to 4 weeks of storage, with generally declining concentrations thereafter. New experimental alkaline solutions, such as CPD and other phosphate-containing ASs, are aimed at further delaying the total ATP consumption [4]. It is noteworthy that, at the end of the RBC lifespan, its enzyme activities, ATP, and other crucial metabolites are still present in sufficient amounts and do not justify RBC death. On the contrary, upon storage, ATP and DPG are almost fully depleted and most of the membrane proteins are oxidized, as well as the lipids, while the cytosolic enzymes are only partly damaged.

Stored erythrocytes also undergo other irreversible damage, as exemplified by the hemolysis in the second half of the actual maximal blood-bank storage period. Determination of the degree of hemolysis is currently based on the amount of extracellular Hb. Nonetheless, the detection of extracellular Hb is not the most reliable criterion, since up to 50% of the extracellular Hb is contained within vesicles and cannot be detected by routine approaches [62].

HbO_2 is potentially harmful as it promotes the generation of reactive oxygen species (ROS), putatively $OH^{\bullet}$, after Fenton's reaction involving its heme iron. During RBC storage, Hb becomes associated with the membrane fraction, mainly with the cytoplasmic domain of band 3 [63], partially in a nonreducible, crosslinked form [64]. This association has been speculated to induce the generation of neoantigens that trigger immune recognition and removal of aged and/or damaged RBCs.

Regarding membrane-protein damage, it is well known that the etiology of lesions in RBC membranes is multifactorial, involving both ROS and proteolytic enzyme activity. Recently, in order to gather information on the time course of storage lesions, investigations on the relative contributions of oxidation and enzyme cleavage to this process and the fragmentation of RBC membranes have been documented by mapping the proteome changes over the storage period.

A comparative study of stored (at 0, 7, 14, and 42 days of storage) and fresh RBCs [23] was performed with two-dimensional gel electrophoresis, followed by in-gel digestion and electrospray ionization-tandem mass spectrometry. The former analysis showed

a diminished staining intensity of some spots over storage time, whereas other spots had anomalous electrophoretic migration patterns, as if their apparent molecular weights had changed upon storage, mainly due to fragmentation and aggregation phenomena. These phenomena are usually observed when proteins are exposed to ROS [65]. In fact, the presence of smearing, mobility shifts of intrinsic protein bands, aggregate formation, and also protein fragmentation, could be due to ROS generated during storage, which attack proteins in the cytoskeleton. ROS produced prevalently from HbO_2 and hemichromes – the final oxidized variant of denatured Hb – are likely to constitute the leading cause of the well-known morphological, biochemical, and metabolic changes in RBCs during storage, through an initial oxidation of amino-acid residues, with consequent protein fragmentation and/or aggregation phenomena. Consistent with this, most of the affected proteins investigated in the report from Zolla's group were located in the cytoskeleton, and oxidation occurred systematically after 10 days of storage [24]. During the first 7 days of storage, oxidative degradation was observed prevalently in band 4.2, and to a minor extent in bands 4.1 and 3 and in spectrin. Indeed, the most important RBC membrane protein, band 3, was found to decrease in the 75 kDa fraction, while it increased its presence in the 150 kDa one, as aggregation occurred during storage [24]. A band 3-centric process was therefore suggested to be the most relevant in complement activation and, ultimately, in RBC removal of senescent cells or transfused RBCs in the recipient. Indeed, antibodies which have too-weak affinities to bind to band 3 monovalently will avidly react with band 3 aggregates (bivalent interaction). These clusters show an enhanced affinity (more than three orders of magnitude) for normally circulating anti-band-3 antibodies, which in turn activate the complement system. It has been shown that $<1\%$ oligomerized band 3 was sufficient to elicit deposition of autologous anti-band-3 IgG. These few molecules were able to induce generation of large amounts of complement fragments via activation of the alternative pathway [66].

After 14 days, new fragments appeared from β actin, G3PD, band 4.9, and ankyrin, among others, as a clear symptom of fragmentation. Preliminary protein–protein crosslinked products, involving α and β spectrin, were also detected. The crosslinked products continued to increase over time [24].

ROS attack the protein fraction at the membrane levels, but also initiate lipid peroxidation reactions that lead to loss of membrane integrity and cell death. For example, malondialdehyde (MDA), a highly reactive bifunctional molecule, is an end product of membrane lipid peroxidation. Lipid derivatives of oxidant attack, most notably MDA, exert a number of detrimental effects on RBCs. MDA has been shown to crosslink erythrocyte phospholipids and proteins. MDA accumulation can affect the anion transport and function of the band 3-associated enzymes (G3PD and phosphofructokinase). MDA can damage the membrane structure via a series of cascade events: the formation of membrane pores, which increase potassium leakage and alter water permeability; the polymerization of membrane components and a decreased cell deformability; crosslinkage of membrane proteins; enhanced IgG binding and complement activation; and an enhanced exposure of PS on the outer cell surface [67]. We have already emphasized PS exposure as an alternative mechanism to explain RBC removal and, in general, as an apoptotic marker. CD47 has been shown to decrease in membranes over storage [68] and the likely mechanism seems to be vesiculation [25].

18.4 Conclusion

At the dawn of the RBC biopreservation era, major concerns regarded the possibility of separating the donor and the recipient in space and time. Hypothermic storage of RBCs has so far managed to meet this goal. The next concern in RBC biopreservation research has been and will be to focus on the "quality issue". Indeed, several contraindications have been listed for the use of long-term stored RBCs and the scientific milieu has begun to wonder whether to pursue a longer or a better storage. While some biochemical alterations accumulating over the storage period are reversible through the addition of peculiar rejuvenation solutions, ROS-induced fragmentation/aggregation events at the protein level are instead irreversible and ask for improved strategies aimed at their prevention.

Alternatives to current protocols for hypothermic storage of RBCs are currently under investigation, such as the anaerobic storage models independently proposed by Yoshida *et al*. [69] and Zolla *et al*. [24].

References

[1] Grazzini, G. The Italian Blood System, *Blood Transfus*., **1**, 4–5 (2009).
[2] Liumbruno, G.M. Proteomics: applications in transfusion medicine, *Blood Transfus*., **6**, 70–85 (2008).
[3] Fitzpatrick, G.M. Strategic reserves (liquid and frozen), in *Blood Safety Transcripts. Department of Health and Human Services*,*Advisory Committee on Blood Safety and Availability. Seventeenth Meeting*, Department of Health and Human Services, Washington, DC (2002). Available at: http://www.hhs.gov/ophs/bloodsafety/pastmeetings/transcripts/20020905.html. Last accessed March 16, 2010.
[4] Hess, J.R. An update on solutions for red cell storage, *Vox Sang*., **91**, 13–19 (2006).
[5] Ramsey, G. Frozen red blood cells: cold comfort in a disaster?, *Transfusion*, **48**, 2053–2055 (2008).
[6] Koch, C.G., Li, L., Sessler, D.I. *et al*. Duration of red-cell storage and complications after cardiac surgery, *N. Engl. J. Med*., **358**, 1229–1239 (2008).
[7] Lelubre, C., Piagnarelli, M. and Vincent, J.L. Association between duration of storage of transfused red blood cells and morbidity and mortality in adult patients: myth or reality?, *Transfusion*, **49**, 1384–1394 (2009).
[8] US Public Health Service. Department of Health & Human Services (2008). Available at: http://www.hhs.gov/ophs/bloodsafety/recommendations/resmay08.pdf. Last accessed March 16, 2010.
[9] Bennet-Guerrero, E., Stafford-Smith, M., Waweru, P.M. *et al*. A prospective double-blind, randomized clinical feasibility trial of controlling the storage age of red blood cells for transfusion in cardiac surgical patients, *Transfusion*, **49**, 1375–1383 (2009).
[10] Council of Europe. Guide to the preparation, use and quality assurance of blood components. Recommendation no R (95) 15 on the preparation, use and quality assurance of blood components, 14 edn, Council of Europe Press, Strasbourg (2008).

[11] Hess, J.R., Sparrow, R.L., van der Meer, P.F. *et al*. Red blood cell hemolysis during blood bank storage: using national quality management data to answer basic scientific questions, *Transfusion*, **49**, 2599–2603 (2009).
[12] Schifferli, J.A. and Taylor, R.P. Physiological and pathological aspects of circulating immune complexes, *Kidney Int*., **35**, 993–1003 (1989).
[13] Palis, J. Ontogeny of erythropoiesis, *Curr. Opin. Hematol*., **15**, 155–161 (2008).
[14] Goodman, S. R., Kurdia, A., Ammann, L. *et al*. The human red blood cell proteome and interactome, *Exp. Biol. Med*., **232**, 1391–1408 (2007).
[15] Rosenblum, B. B. Two-dimensional gel electrophoresis of erythrocyte membrane proteins, *Prog. Clin. Biol. Res*., **56**, 251–268 (1981).
[16] Aminoff, D., Rolfes-Curl, A. and Supina, E. Molecular biomarkers of aging: the red cell as a model, *Arch. Gerontol. Geriatr*., **15**, 7–15 (1992).
[17] Low, T.Y., Seow, T.K. and Chung, M.C. Separation of human erythrocyte membrane associated proteins with one-dimensional and two-dimensional gel electrophoresis followed by identification with matrix-assisted laser desorption/ionization-time of flight mass spectrometry, *Proteomics*, **2**, 1229–1239 (2002).
[18] Kakhniashvili, D.G., Bulla L.A. Jr, and Goodman, S.R. The human erythrocyte proteome: analysis by ion trap mass spectrometry, *Mol. Cell. Proteomics*, **3**, 501–509 (2004).
[19] Körbel, S., Büchse, T., Prietzsch, H. *et al*. Phosphoprotein profiling of erythropoietin receptor- dependent pathways using different proteomic strategies, *Proteomics*, **5**, 91–100 (2005).
[20] Anniss, A.M., Glenister, K.M., Killian, J.J. and Sparrow, R.L. Proteomic analysis of supernatants of stored red blood cell products, *Transfusion*, **45**, 1426–1433 (2005).
[21] Pasini, E.M., Kirkegaard, M., Mortensen, P. *et al*. In-depth analysis of the membrane and cytosolic proteome of red blood cells, *Blood*, **108**, 791–801 (2006).
[22] Kriebardis, A.G., Antonelou, M.H., Stamoulis, K.E. *et al*. Storage dependent remodeling of the red blood cell membrane is associated with increased immunoglobulin G binding, lipid raft rearrangement, and caspase activation, *Transfusion*, **47**, 1212–1220 (2007).
[23] Kriebardis, A.G., Antonelou, M.H., Stamoulis, K.E. *et al*. Progressive oxidation of cytoskeletal proteins and accumulation of denatured hemoglobin in stored red cells, *J. Cell. Mol. Med*., **11**, 148–155 (2007).
[24] D'Amici, G.M., Rinalducci, S. and Zolla, L. Proteomic analysis of RBC membrane protein degradation during blood storage, *J. Proteome Res*., **6**, 3242–3225 (2007).
[25] Bosman, G.J., Lasonder, E., Luten, M. *et al*. The proteome of red cell membranes and vesicles during storage in blood bank conditions, *Transfusion*, **48**, 827–835 (2008).
[26] Roux-Dalvai, F., Gonzalez de Peredo, A., Simó, C. *et al*. Extensive analysis of the cytoplasmic proteome of human erythrocytes using the peptide ligand library technology and advanced mass spectrometry, *Mol. Cell. Proteomics*, **7**, 2254–2269 (2008).
[27] Tannert, C., Schmidt, G., Klatt, D. and Rapoport, S.M. Mechanism of senescence of red blood cells, *Acta Biol. Med. Ger*., **36**, 831–836 (1977).
[28] Clark, M.R. and Shohet, S.B. Red cell senescence, *Clin. Haematol*., **14**, 223–257 (1985).

[29] Shinozuka, T. Changes in human red blood cells during aging in vivo, *Keio J. Med*., **43**, 155–163 (1994).
[30] Bratosin, D., Mazurier, J., Tissier, J.P. *et al*. Cellular and molecular mechanisms of senescent erythrocyte phagocytosis by macrophages: a review, *Biochimie*, **80**, 173–195 (1998).
[31] Kay, M.M. Generation of senescent cell antigen on old cells initiates IgG binding to a neoantigen, *Cell. Mol. Biol*., **39**, 131–153 (1993).
[32] Low, P.S. and Kannan, R. Effect of hemoglobin denaturation on membrane structure and IgG binding: role in red cell aging, *Prog. Clin. Biol. Res*., **319**, 525–552 (1989).
[33] De Rosa, M.C., Carelli Alinovi, C., Galtieri, A. *et al*. The plasma membrane of erythrocytes plays a fundamental role in the transport of oxygen, carbon dioxide and nitric oxide and in the maintenance of the reduced state of the heme iron, *Gene*, **398**, 162–171 (2007).
[34] Hamasaki, N. and Okubo, K. Band 3 protein: physiology, function and structure, *Cell. Mol. Biol*., **42**, 1025–1039 (1996).
[35] Jay, D.G. Role of band 3 in homeostasis and cell shape, *Cell*, **86**, 853–854 (1996).
[36] Low, P.S., Rathinavelu, P. and Harrison, M.L. Regulation of glycolysis via reversible enzyme binding to the membrane protein, band-3, *J. Biol. Chem*., **268**, 14627–14631 (1993).
[37] Kay, M. Immunoregulation of cellular life span, *Ann. N. Y. Acad. Sci*., **1057**, 85–111 (2005).
[38] Bosman, G.J. and Kay, M.M. Erythrocyte aging: a comparison of model systems for simulating cellular aging in vitro, *Blood Cells*, **14**, 19–46 (1998).
[39] Turrini, F., Arese, P., Yuan, J. and Low, P.S. Clustering of integral membrane proteins of the human erythrocyte membrane stimulates autologous IgG binding, complement deposition and phagocytosis, *J. Biol. Chem*., **236**, 23611–23617 (1991).
[40] Head, D.J., Lee, Z.E., Poole, J. and Avent, N.D. Expression of phosphatidylserine (PS) on wild-type and Gerbich variant erythrocytes following glycophorin-C (GPC) ligation, *Br. J. Haematol*., **129**, 130–137 (2004).
[41] Head, D.J., Lee, Z.E., Swallah, M.M. and Avent, N.D. Ligation of CD47 mediates phosphatidylserine expression on erythrocytes and a concomitant loss of viability in vitro, *Br. J. Haematol*., **130**, 788–790 (2005).
[42] Bosman, G.J., Willekens, F.L. and Werre, J.M. Erythrocyte aging: a more than superficial resemblance to apoptosis?, *Cell. Physiol. Biochem*., **16**, 1–8 (2005).
[43] Lang, F., Gulbins, E., Lerche, H. *et al*. Eryptosis, a window to systemic disease, *Cell. Physiol. Biochem*., **22**, 373–380 (2008).
[44] Siems, W.G., Sommerburg, O. and Grune, T. Erythrocyte free radical and energy metabolism, *Clin. Nephrol*., **53**, 9–17 (2000).
[45] D'Alessandro, A., Righetti, P.G. and Zolla, L. The red blood cell proteome and interactome: an update, *J. Proteome Res*., **9**, 144–163 (2010).
[46] van Wijk, R. and van Solinge, W.W. The energy-less red blood cell is lost: erythrocyte enzyme abnormalities of glycolysis, *Blood*, **106**, 4034–4042 (2005).
[47] Wiback, S.J. and Palsson, B.O. Extreme pathway analysis of human red blood cell metabolism, *Biophys. J*., **83**, 808–818 (2002).

[48] Cart, R. T. Red cell membrane changes during storage, *Transfus. Med. Rev*., **2**, 40–47 (1998).
[49] Kamp, D., Sieberg, T. and Haest, C.W. Inhibition and stimulation of phospholipid scrambling activity: consequences for lipid asymmetry, echinocytosis, and microvesiculation of erythrocytes, *Biochemistry*, **40**, 9438–9446 (2001).
[50] Rouault, C. Red cell oxygen delivery: effect of 2,3-diphosphoglycerate, *Postgrad. Med*., **53**, 201–203 (1973).
[51] Salhany, J.M., Mathers, D.H. and Eliot, R.S. Molecular basis for oxygen transport: hemoglobin function and controlling factors, *Adv. Cardiol*., **9**, 53–67 (1973).
[52] Valeri, C.R. and Collins, F.B. Physiologic effects of 2,3-DPG–depleted red cells with high affinity for oxygen, *J. Appl. Physiol*., **31**, 823–827 (1971).
[53] Mansouri, A. and Lurie, A.A. Concise review: methemoglobinemia, *Am. J. Hematol*., **42**, 7–12 (1993).
[54] Untucht-Grau, R., Schirmer, R.H., Schirmer, I. and Krauth-Siegel, R.L. Glutathione reductase from human erythrocytes: amino-acid sequence of the structurally known FAD-binding domain, *Eur. J. Biochem*., **120**, 407–419 (1981).
[55] Kurata, M., Suzuki, M. and Agar, N.S. Antioxidant systems and erythrocyte lifespan in mammals, *Comp. Biochem. Physiol. B*., **106**, 477–487 (1993).
[56] Winterbourn, C.C. Oxidative denaturation in congenital hemolytic anemias: the unstable anemias, *Semin. Hematol*., **27**, 41–50 (1990).
[57] Rous, P. and Turner, J.W. The preservation of living red blood cells in vitro, *J. Exp. Med*., **23**, 219–237 (1916).
[58] Scott, K.L., Lecak, J. and Acker, J.P. Biopreservation of red blood cells: past, present, and future, *Transfus. Med. Rev*., **19**, 127–142 (2005).
[59] Greenwalt, T.J. The how and why of exocytic vesicles, *Transfusion*, **46**, 143–152 (2006).
[60] Salzer, U., Hinterdorfer, P., Hunger, U. *et al*. Ca^{++}-dependent vesicle release from erythrocytes involves stomatin-specific lipid rafts, synexin (annexin VII), and sorcin, *Blood*, **99**, 2569–2577 (2002).
[61] Rensing, H. Storage dependent effects on the oxygen transport capacity of erythrocyte concentrates, *Anaesthesist*, **50**, 9–15 (2001).
[62] Greenwalt, T.J., McGuinness, C.G. and Dumaswala, U.J. Studies in red cell preservation. 4. Plasma vesicle hemoglobin exceeds free hemoglobin, *Vox Sang*., **61**, 14–17 (1991).
[63] Zhang, D., Kiyatkin, A., Bolin, J.T., and Low P.S. Crystallographic structure and functional interpretation of the cytoplasmic domain of erythrocyte membrane band 3, *Blood*, **96**, 2925–2933 (2000).
[64] Wolfe, L.C., Byrne, A.M. and Lux, S.E. Molecular defect in the membrane cytoskeleton of blood bank-stored red cells: abnormal spectrin-protein 4.1-actin complex formation, *J. Clin Invest*., **78**, 1681–1686 (1986).
[65] Sheehan, D. Detection of redox-based modification in twodimensional electrophoresis proteomic separations, *Biochem. Biophys. Res. Commun*., **349**, 455–462 (2006).
[66] Lutz, H.U. Innate immune and non-immune mediators of erythrocyte clearance, *Cell. Mol. Biol*., **50**, 107–116 (2004).

[67] Pantaleo, A., Giribaldi, G., Mannu, F. *et al*. Naturally occurring anti-band 3 antibodies and red blood cell removal under physiological and pathological conditions, *Autoimmun. Rev*., **7**, 457–462 (2008).
[68] Stewart, A., Urbaniak, S., Turner, M. and Bessos, H. The application of a new quantitative assay for the monitoring of integrin-associated protein CD47 on red blood cells during storage and comparison with the expression of CD47 and phosphatidylserine with flow cytometry, *Transfusion*, **45**, 1496–1503 (2005).
[69] Yoshida, T., AuBuchon, J.P., Tryzelaar, L. *et al*. Extended storage of red blood cells under anaerobic conditions, *Vox Sang*., **92**, 22–31 (2007).

19

Red Blood Cells from Stem Cells

Anna Rita Migliaccio[1], *Carolyn Whitsett*[1] *and Giovanni Migliaccio*[2]

[1]*Tisch Cancer Institute, Mount Sinai School of Medicine, New York, NY, USA*

[2]*Department of Cell Biology and Neurosciences, Istituto Superiore Sanità, Rome, Italy*

19.1 Introduction

Red cells are the cellular elements of the blood that perform the unique function of ensuring proper oxygen delivery to the tissues of the body [1]. An average transfusion provides $\sim 2.5 \times 10^{12}$ red cells, $\sim 1/10$ of the adult blood volume. Progress in understanding *ex vivo* cell production and establishment of good manufacturing practice (GMP) conditions have made possible the rapid development *ex vivo* of several cell-therapy products (cellular vaccines, hematopoietic stem cells and skin grafts, etc.) at a reasonable cost. With respect to these therapies, transfusion requires great numbers of red cells and, due to the large volume of media currently required for production, poses unique technical and financial challenges to *ex vivo* development of suitable products.

The concept of *ex vivo*-generated erythroid cells as a transfusion product was first hypothesized when culture conditions to produce erythroid cells improved to an extent that made generation of 2×10^{12} red cells *ex vivo* theoretically possible [2]. Studies demonstrating that mice with experimentally induced lethal anemia were protected by *ex vivo*-generated erythroid cells established a proof of concept for this therapy [3]. Several investigators are presently pursuing the development of production processes for the generation of transfusion products *ex vivo* (reviewed in [4, 5]). This chapter will review alternative sources of stem cells, improvements in culture conditions, properties of *ex vivo*-generated erythroid cells, and clinical applications for this product.

Chemistry and Biochemistry of Oxygen Therapeutics: From Transfusion to Artificial Blood, First Edition.
Edited by Andrea Mozzarelli and Stefano Bettati.

19.2 Stem-cell Sources for *ex vivo* Generation of Erythroid Cells as a Transfusion Product

Specific growth factors (GFs) instruct pluripotent hemopoietic stem cells (HSCs) to generate a hierarchy of progressively lineage-restricted erythroid progenitor cells (EPCs), a process termed "commitment" [1]. Although EPCs are morphologically indistinguishable from HSCs, they have specific antigen and mRNA expression profiles. After several divisions, EPCs give rise to the first morphologically recognizable erythroid cell, the pro-erythroblast (pro-EB). Pro-EBs are still capable of proliferation and of initiating a chain of events that generates progressively more mature EBs (basophilic, polychromatic, and orthochromatic EBs), which eventually lose proliferative potential and undergo enucleation. HSCs are present at low frequency in adult blood (AB) (approximately 2.5×10^3 cells/mL) but may be found in larger numbers in the blood of neonates (approximately 2.5×10^4 cells/mL) [6, 7]. The antigen recognized by the CD34 antibody represents the earliest marker for HSCs [1]. In addition to $CD34^{pos}$ EPCs, blood mononuclear cells (MNCs) contain unknown numbers of more mature CD34-negative EPCs that contribute to the generation of EBs *ex vivo* [4]. Both $CD34^{pos}$ cells and MNCs from AB and cord blood (CB) have been used for EB expansion *ex vivo* [4, 6]. For normalization purposes, generation of EBs in culture is expressed as fold increase: that is, the ratio between the number of cells observed at a given day in culture and that used to seed the culture. In general, greater fold increases (in the order of thousands) are observed in cultures initiated with $CD34^{pos}$ cells than in those seeded with mononuclear cells (in the order of tens). However, greater absolute numbers of EBs are generated by cultures seeded with MNCs (10^{10}–10^{11} EBs) than in those seeded with $CD34^{pos}$ cells (5×10^8–5×10^{10} EBs) (Figure 19.1) [4].

CB cells (both $CD34^{pos}$ cells and MNCs) generate on average 10 times more EBs than the corresponding cells from AB (1.7×10^{11} vs 2.5×10^{12} EBs per unit) (Figure 19.1) [4]. This greater proliferative potential of CB is usually explained by shorter doubling times, less stringent cytokine requirements, greater telomere length, and expression of the more active gamma isoform of the glucocorticoid receptor (GR) of neonatal EPCs compared to adult EPCs.

$CD34^{pos}$ cells or MNCs from the same donor generate similar numbers of EBs in cultures repeated over time. By contrast, great variability exists in the number of EBs generated in cultures from different donors [4]. These observations highlight the need to identify biomarkers to predict the potency of a donor to generate EBs *ex vivo*. Possible biomarkers may be represented by the frequency of EPCs present in the unit and/or genetic polymorphisms of the donors (length of the telomeres [8], and GR [9] and estrogen (ESR) [10] receptor loci). Telomere length of $CD34^{pos}$ cells has been reported to predict the numbers of EBs that are generated by different CB units in cultures [8], and telomerase expression, low in $CD34^{pos}$ cells, increases several fold with erythroid maturation [11]. *Ex vivo* EB expansion depends on activation of two highly polymorphic nuclear receptors, GR and ESR, both targeting GATA1 activity in EBs [12, 13] (Figure 19.2). These polymorphisms are emerging as the leading cause of variability in body mass, adipose tissue distribution, and response to glucocorticoids in depression and immunotherapies within the human population [9, 10]. Although the effect of these

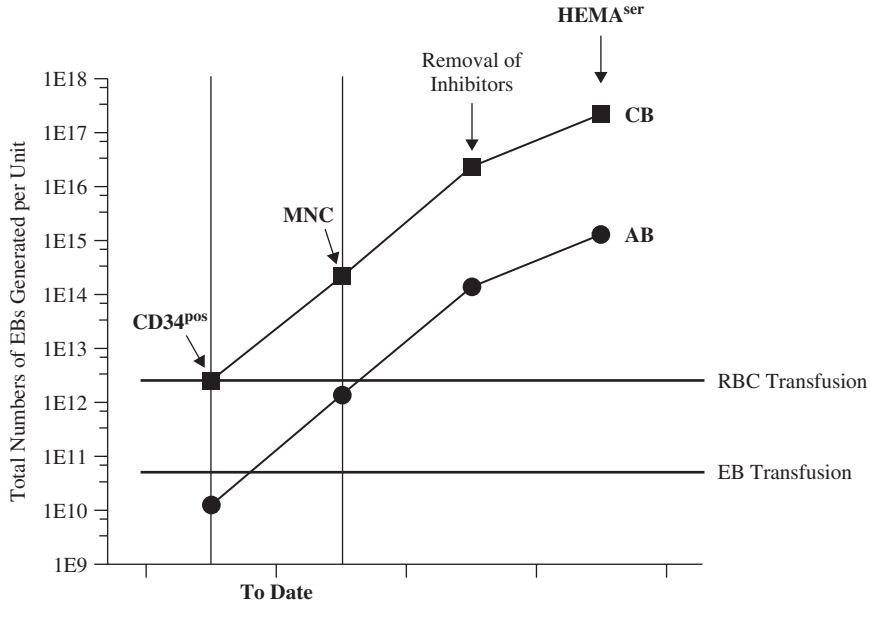

Figure 19.1 *Calculation of the numbers of red cells generated from EPCs contained in CD34^{pos} cells and MNCs from CB (squares) and AB (circles) with current techniques or by including in the production process procedures to remove growth inhibitors released during culture and humanized culture media (HEMA^{def}). The calculations assume that each red blood cell (RBC) transfusion requires 2.5 × 10^{12} cells. Since EBs are capable of additional divisions* in vivo *(6–64), therapeutic effects may be achieved by transfusion of approximately 5 × 10^{10} EBs. Current culture techniques are sufficient to produce at least one RBC transfusion from an average CB unit and one EB transfusion from the MNCs in an average AB donation [4, 5]. Production of RBC or EB transfusion product requires approximately 2500 or 50 L, respectively, of culture media, at an average cost, including GFs and other components, of $1000 per liter, and with an average production time of 30 and 10 days, respectively, indicating that production of* ex vivo*-generated transfusion products is costly.*

polymorphisms on the red-cell mass under steady-state conditions has not been investigated as yet, we hypothesize that different GR and ESR expression patterns in EPCs from donors may establish a range of alternative synergistic/antagonistic interactions that affect the efficiency of the EB production process.

In addition to hematopoietic stem cells, human embryonic stem cells (hESCs) [16–18] and induced pluripotency stem cells (iPSs) [19] are being actively investigated as a source of EPCs for EB expansion *ex vivo*. Since red cells do not have a nucleus, safety considerations suggest that red cells may represent the first cell-therapy product to be generated from hESCs and iPSs. Although these studies address important developmental biology issues, we believe that they are unlikely to advance our ability to generate EBs *ex vivo* for transfusion. On one hand, hESC cell lines are available in limited numbers and may generate type-O Rh-negative red cells (universal donor blood) but are unlikely to generate cells with the complex antigen phenotype necessary for matching of

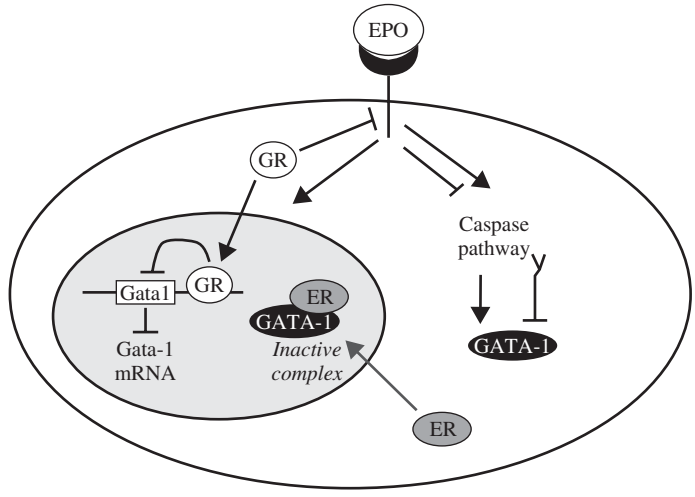

Figure 19.2 *A model for the mechanism of action of GR and ESR on erythroid maturation based on their activity on the transcription factor GATA1. GATA1 plays a major role in determining erythroid maturation. In mice, both hypomorphic (reduced-expression) and gain-of-function GATA1 mutations induce a lethal anemia phenotype (reviewed in [1]). In the case of the hypomorphic mutations, the anemia is induced by increased apoptosis rates at the basophilic EB stage due to insufficient expression of the anti-apoptotic gene* BCL_{XL}*. In the case of GATA1 overexpression, the anemia is due to insufficient red-cell production because of accelerated maturation. These observations in mice suggest that factors which control the biological activity of GATA1 regulate the numbers of EBs generated* in vitro *by tuning the equilibrium between promoting apoptosis and accelerating maturation. Although the major positive control on GATA1 activity is exerted by erythropoietin (EPO) [14, 15], both GR and ESR, when stimulated with their respective ligands, exert a negative regulation on GATA1 activity in EBs: GR destabilizes the protein [12], while ESR binds GATA1 into transcriptionally inactive complexes [13]. Generation of optimal numbers of EBs in cultures depends on the establishment of a delicate equilibrium between the effect of EPO (positive) and that of GR and ESR (both negative), which ensures that the levels of GATA1 activity remain within appropriate ranges.*

alloimmunized patients. On the other, iPSs may be generated from the mature somatic cells of any individual (fibroblasts or even blood cells) by inducing ectopic expression of the four genes Oct4, Sox2, Klf4, and cMyc [20]. Therefore, iPSs have the potential to generate matched red cells. However, variability is emerging in the ability of iPS lines derived from individual donors to generate EBs *ex vivo*. In addition, EBs generated from iPSs are similar to embryonic EBs [19] and may, therefore, express antigens not expressed by adult EBs and induce allo- and/or auto-immune reactions.

19.3 Conditions that Favor *ex vivo* Erythroid Cell Expansion

The minimal combination of GFs required to induce EPCs to generate EBs *ex vivo* is represented by stem cell factor (SCF), interleukin 3 (IL-3), and erythropoietin (EPO)

[1, 21]. $CD34^{pos}$ cells (or MNCs) from CB or AB cultured *ex vivo* with these cytokines generate within 12–15 days hematopoietic cells of multiple lineages, approximately half of which are represented by EBs at all stages of maturation. Within 18 days of culture, these EBs have matured to the stage where they cease to proliferate. Erythropoiesis is also regulated by the nuclear receptors GR and ESR [4]. The addition of DXM and ES to the combination of GFs used to stimulate EPCs results in the generation of larger numbers of EBs in culture [4, 5]. DXM and ES do not affect the number of EPCs recruited in the maturation process, but rather the cellular output from each EPC [4]. The two hormones act by delaying the transition of proEBs to the next maturation stage, favoring greater numbers of proliferative events (see Figure 19.2).

19.4 A Clinical-grade Production Process for *ex vivo* Generation of Red-cell Transfusion Products

The recognition that EPCs stimulated with DXM and ES in addition to GFs generate great numbers of EBs *ex vivo* represents the basis for a production process for *ex vivo* generation of EBs as a transfusion product [4, 5]. In the USA and Europe, *ex vivo*-generated red-cell transfusion products fall into the category of biological medicinal products. The identity of these products must be precisely documented by defining the nature of the production process, the cell composition of the product, and its potency in xenogeneic models.

19.4.1 The Nature of the Production Process

It is envisioned that the production process to be developed first will include only the EB expansion process, defined also as Phase I, **expansion**, or human erythroid massive amplification (HEMA) cultures (see Figure 19.3). The EPC source used to start the production process is discussed above. Inclusion of bovine components in the culture medium currently used for *ex vivo* EB expansion ($HEMA^{ser}$) precluded its use for production of a clinical product. The optimal GF and hormone combinations identified in $HEMA^{ser}$ were recently used by us to formulate a medium composed of pharmaceutical-grade human albumin, human albumin-based lipid liposomes, and iron-saturated recombinant human tranferrin ($HEMA^{def}$) [25]. In addition to being clinical grade, $HEMA^{def}$ sustains EB amplification from CB MNCs as efficiently as $HEMA^{ser}$, and from AB MNCs 10-fold higher than $HEMA^{ser}$. Moreover, the numbers of EBs generated in $HEMA^{def}$ by adult MNCs were similar to those generated by CB MNCs (750×10^6 vs 500×10^6 per 10^6 MNCs from AB and CB, respectively). Therefore, a production process under $HEMA^{def}$ conditions will produce similar numbers of EBs from AB and from CB.

Assuming that MNCs contain 10^2–10^3 EB progenitors ($CD34^{pos}$ cells represent 0.1% of MNCs and erythroid progenitors represent 10% of $CD34^{pos}$ cells) [6, 7], it was calculated that the generation of 750×10^6 EBs from the progenitors present in 10^6 adult MNCs requires 19–23 divisions, a number below the theoretical Hayflick's limit for somatic cell divisions (35) [26]. Therefore, it should be possible, by optimizing the production process, to further increase the number of EBs that can be generated *ex vivo* from AB, and possibly from CB (Figure 19.1). We believe that this improvement

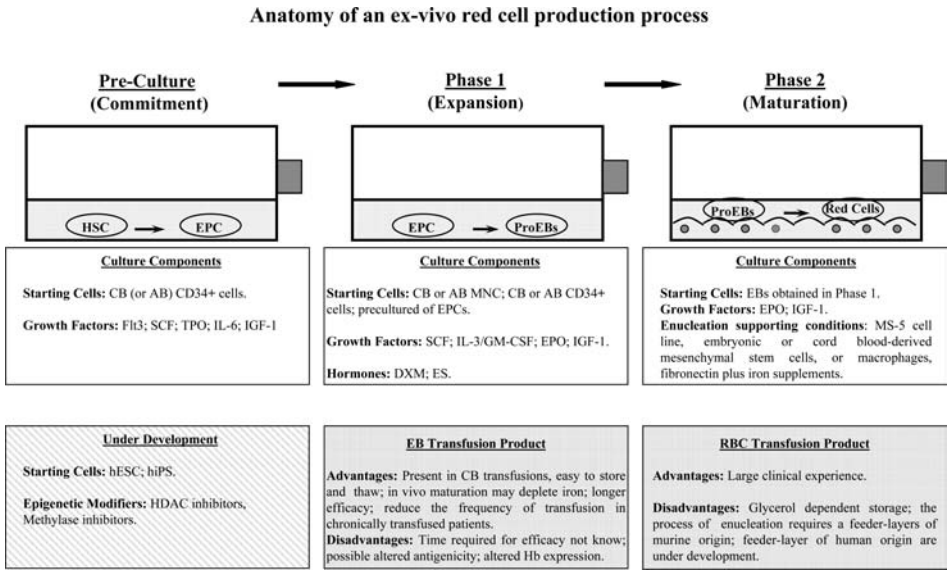

Figure 19.3 *Anatomy of a hypothetical* ex vivo *red cell production process. Based on the results obtained by our group and others (reviewed in [4, 5]), a process to produce* ex vivo *red cells will be composed of three sequential cultures: (i) preculture (from HSCs to EPCs); (ii) expansion (from EPCs to EBs); and (iii) maturation (from EBs to enucleated red cells) cultures. The boxes specify the components (starting populations, GFs, and additives) of each phase of the production, the promising areas of research in the development of preculture systems, and the advantages and disadvantages of using EBs and enucleated red cells as transfusion products. The very first process to generate* ex vivo *red-cell transfusion products may include only expansion cultures. In fact, preculture conditions are not completely developed and the present conditions that allow maturation of EBs into red cells are not clinical grade because they require either the murine MS-5 cell line or human mesenchymal stem cells generated in the presence of fetal bovine serum [22–24].*

will be achieved by interfering with the unique paracrine loop established among EBs as they mature [27]. The major obstacles in defining an *ex vivo* red cell production process, which cannot be surmounted with current knowledge, derive from the fact that cultured EBs release negative regulatory factors (TGF-β and TRAIL) that inhibit growth by accelerating maturation and/or inducing apoptosis [28, 29]. To reduce the effects of this paracrine loop, EBs must be maintained in culture at densities lower than 10^6 cells/mL. Therefore, *ex vivo* generation of a unit of blood (2.5×10^{12} EBs) would require one cubic meter of media (Figure 19.1). The identification of manipulations that increase the density at which EBs can be cultured (i.e. inhibition of the paracrine loop and/or removal/storage of EBs as they mature) will play a major role in developing an *ex vivo* red cell production process. Evidence for this hypothesis has recently been obtained through the observation that immature EBs sequentially sorted every 2 days generated fivefold more EBs than the corresponding unfractionated EBs (FI = 25 vs 5, respectively) [30]. Proof that greater generation potential is prevented due to direct inhibition of proliferation by mature EBs was provided by co-culture

experiments in which immature EBs were co-cultured with fluorochrome-labeled mature EBs for 4 days. Immature EBs cultured alone increased in number by twofold over this culture period. However, when co-cultured with mature EBs, they did not increase in number and became undetectable after 4 days (unpublished observation). The failure to detect cells after 4 days in co-culture experiments suggests that mature EBs induce death of the immature cells and that the number of cells generated may be increased by introducing into the process a device to maintain physical separation of immature and mature EBs.

EPCs represent a minority of $CD34^{pos}$ cells and culture conditions have been designed to induce $CD34^{pos}$ cells to generate EPCs for use in expansion cultures (Figure 19.3). The purpose of these **precultures/commitment cultures** is to increase the number of EPCs, and therefore of EBs, that can be generated *ex vivo* from a single blood donation. Preculture conditions have been developed for CB $CD34^{pos}$ cells [4, 5] but in principle the same conditions could be used to generate EPCs from AB $CD34^{pos}$ cells.

A different set of conditions have been designed to induce EB maturation (Figure 19.3). When *ex vivo*-expanded EBs are transferred into **maturation cultures** (Phase 2) stimulated with EPO and IGF1, they progress to the orthochromatic stage (Figure 19.3) [4, 5]. Enucleation remains inefficient (5–10%) and requires the addition of stromal cells, which probably exert *in vitro* the functions performed by macrophages *in vivo* (providing a surface to facilitate rolling-over during enucleation and/or to facilitate iron uptake) [22–24].

19.4.2 Cellular Composition of the Product

A conservative approach would be to define the cellular composition of *ex vivo*-expanded red-cell products as "enucleated red cells". However, in principle, *ex vivo*-generated EBs could also serve as the transfusion product. These cells are present in the circulation during fetal development, in neonates and adults with pyruvate kinase deficiency, hemolytic anemias, and other conditions of stress [1]. EBs are also present in large numbers ($\sim 4 \times 10^7$ cells) in CB, which is occasionally used for transfusions in developing countries [31]. The consideration that EBs may undergo 4–64 further divisions suggests that *ex vivo*-generated EBs may be more effective as transfusion products than enucleated red cells (Figure 19.1). The use of EBs rather than red cells may be particularly beneficial for those conditions that require chronic transfusion, because they would reduce therapy-related iron overload.

An additional advantage to the use of *ex vivo*-expanded EBs over red cells as a transfusion product is represented by the fact that EBs remain viable, retaining proliferative and maturation potential, after short- and long-term storage [32]. It has been shown that freshly expanded and short- and long-term-stored EBs equally doubled in number (fold increase = 2.4), retaining an immature phenotype, when cultured for 4 days under HEMA conditions, and matured when exposed for 4 days to EPO [32]. These characteristics will allow the establishment of banks of cryopreserved EB products for emergency use.

The definition of a production process includes the identification of biomarkers which characterize each stage of production during the manufacturing process. Biomarkers that can be used to monitor EB expansion during production are represented by antigenic and/or expression profiling. Antigenic profiling can be performed using two-color

flow cytometry based on the levels of CD235a (Glycophorin A) and either CD71 (transferrin receptor type I) or CD36 (thrombospondin receptor, also a receptor for malarial parasites) expression [1]. Both CD71 and CD36 expression are upregulated when progenitor cells become EPCs in response to EPO and represent early markers for erythroid commitment. These markers divide EBs into four populations: EPC ($CD36^{high}$ or $CD71^{high}/CD235a^{neg}$), proEBs ($CD36^{high}$ or $CD71^{high}/CD235a^{low}$), basophilic-polychromatophilic ($CD36^{high}$ or $CD71^{high}/CD235a^{high}$), and orthochromatic ($CD36^{neg}$ or $CD71^{low}/CD235a^{high}$) EBs. Reticulocytes may be identified as small $CD36^{neg}/CD235a^{high}$ cells that stain negative for DNA.

19.4.3 Functional Status of Product

Proof that *ex vivo*-expanded murine red cells are functional is provided by the observation that these cells protect mice from lethal bleeding [3]. Such proof is not available as yet for *ex vivo*-expanded human EBs. Whether *ex vivo*-expanded EBs will be functional *in vivo* is therefore inferred by comparative studies of their biological properties with those of their counterpart produced *in vivo*.

Preliminary data indicate that *ex vivo*-expanded human erythrocytes have membrane deformability, glucose-6-phosphate dehydrogenase, and pyruvate kinase levels similar to those of young RBCs, indicating that their metabolic activity is normal [5].

Size represents the most obvious difference between *ex vivo*- and *in vivo*-generated EBs (Table 19.1). EBs generated *ex vivo* from both CD and AB have a diameter threefold larger than that of the corresponding cells generated *in vivo*. However, the size of the cells becomes normal with maturation induced either *in vitro* or *in vivo* in xenogeneic transfusion models [4].

Ontogenetic differences exist among the biological properties of EBs generated *in vivo* [1]. These differences include levels of activity of the glycolytic enzymes (greater in CB cells) and of carbonic anhydrase (greater in AB cells), expression of different isozymes (phosphoglycerate kinase, acetylcholinesterase, etc.), hemoglobin (Hb; CB EBs express mainly fetal Hb – HbF – while adult EBs express adult Hb – HbA), and antigenic profiles (HLA class II antigens may be expressed by CB- but not AB-derived EBs; CB or AB EBs may express the i or I antigen). These differences are conserved in EBs generated *ex vivo* from CB and AB.

In addition, variability is observed in gene expression among EBs expanded *ex vivo* from different individuals. Hb is by far the protein most expressed by mature EBs (Table 19.1). Such high levels of expression are achieved through robust levels of globin gene activation. EBs expanded *ex vivo* from 19 different AB donors were found to express levels of globin mRNA spanning over 2 logs [33]. However, in all the cases, expression was robust and balanced. In particular, the levels of α-globin mRNA were correlated not only with those of non-α globins ($R2 = 0.93$, $p < 0.001$) but also with those of AHSP ($R2 = 0.86$, $p < 0.0001$), a protein essential to retaining α globin in solution and for proper assembly with the heme group and non-α globin chains into functional hemoglobin. Therefore, expression variability in globin-related genes observed among EBs derived from individual donors does not pose a limitation to their use as transfusion products (Table 19.1).

Table 19.1 *Properties of EBs generated* ex vivo *by EPCs from AB.* In vivo*-generated immature and mature EBs have a protein content of approximately 18 and 24 pg/cell, respectively [1]. The Hb content in these immature EBs is barely detectable, while that of mature EBs corresponds approximately to the total protein content. Although the total Hb content of* ex vivo*-generated EBs was not measured, it is also likely that in these cells the majority of the protein is represented by Hb. Therefore, it can be assumed that* ex vivo- *and* in vivo*-generated EBs have a similar Hb content. In fact, oxygen dissociation curves of* ex vivo*-expanded erythrocytes are similar to those of young RBCs [5]. The concentration of hemoglobin F (HbF) of* ex vivo*-generated adult EBs (1% of total Hb) is at least 10-fold greater than that of* in vivo*-generated cells.* Ex vivo*-generated immature EBs are three times bigger than the corresponding cells generated* in vivo *(40.1 ± 1.4 μm). These cells significantly reduce their diameter upon exposure to EPO, to 28.5 ± 2.0, 21.2 ± 0.7, and 11.6 ± 0.3 μm, by 48 and 96 hours ($p < 0.01$ in all cases) [4]. Although macrocytic (11.6 μm),* ex vivo*-generated orthochromatic EBs are smaller than embryonic (20 μm) and fetal (12.5 μm) EBs and slightly larger than adult normocytic red cells (8 μm) [1].*

	Ex vivo-generated EBs	
	Immature	Mature
Size (μm)	40.1 ± 1.4	11.6 ± 0.3
Total mRNA (ng/2 × 10^6 cells)	22 ± 4.2	9.5 ± 0.8
β-globin mRNA (arbitrary units)	3.4 ± 0.31	b.d.
γ-globin mRNA (arbitrary units)	0.69 ± 0.01	b.d.
Total protein (pg/cell)	18.7 ± 9	23.7 ± 9.5
Total HbF (pg/cell)	0.12 ± 0.014	0.20 ± 0.27
α/(γ + β) synthetic ratio	4.2 ± 2.5	4.0 ± 1.0
γ/(γ + β) synthetic ratio	0.1 ± 0.3	0.08 ± 0.08

19.4.4 Safety Considerations

Safety concerns for any cell therapy include possible exposure of the products during culture to xenogeneic substances and/or infectious agents, and neoplastic transformation [34–37]. Xenogeneic substances also expose the recipient to immunological risks [38, 39], which have been addressed by the development of complete humanized media for *ex vivo* expansion of EBs [25]. Transmission of known and unknown adventitious agents may also occur during manufacturing and is prevented by appropriate GMP procedures. Although it has been proposed that neoplastic transformation does not represent a concern for red cells as a product because they do not contain a nucleus, the process of transformation itself may alter the biological properties of the cells and therefore interfere with the number and functionality of the final product. Although EBs generated *ex vivo* remain chromosomally normal after 8-year storage [32], the safety of these cells must be further evaluated by cytogenetic/genotoxicity assays, in compliance with FDA guidelines [40].

Ex vivo-expanded EBs pose the same risk for infection, incompatibility, and antigenicity as any transfusion product. The risk for infection may be addressed following the EPC donor guidelines established for blood and hematopoietic stem-cell donors. However, current processes and assays to ensure compatibility of blood transfusion may be inadequate to address the immunogenicity risk of *ex vivo*-expanded EBs. In spite of precise matching of the EPC donor for blood-group antigens, antigenic variability may be induced by the *ex vivo* expansion process itself. Blood-group antigens encoded by specific genes may be altered by post-transcriptional modifications induced by the culture conditions. In addition, culture components may alter the levels of activity of transcription factors, such as EKLF – which when poorly expressed determines the rare blood group In(Lu) phenotype [41] – and C/EBPα – the level of phosphorylation of which determines the expression of the i or I phenotype [42]. In addition, matched EBs may induce auto-immunity and/or appear incompatible if expressing antigen levels greater than those normally present on *in vivo*-generated red cells. These concerns mandate a thorough analysis of the effect of culture conditions on antigen expression.

19.5 Time Line of the Clinical Application of *ex vivo*-generated Erythroid Cells

Cost and logistic considerations (see Figure 19.1) suggest that the first clinical application of *ex vivo*-expanded EBs will be drug discovery, followed by systemic drug delivery and then genotypically matched transfusions for alloimmunized patients.

19.5.1 Drug Discovery

In the future, *in vitro* assessment of toxicity and efficacy of new drugs will likely employ assays using panels of primary cells reflecting age, sex, and genetic polymorphism of human populations [43]. This approach could reduce animal experimentation and increase the sensitivity and robustness of preclinical toxicology and efficacy studies. *Ex vivo*-generated EBs were recently used to identify the mechanism of toxicity of the B19 parvovirus [44]. This infection induces a mild transient and usually benign anemia but may cause severe and possibly life-threatening anemia in immunosuppressed patients and in patients with decreased red-cell survival [45]. B19 parvovirus infection may also trigger/aggravate autoimmune diseases and cause severe anemia and nonimmune hydrops with fetal loss in pregnancy [45]. The lack of specific therapies for this infection is related to limited knowledge of the pathogenesis of the virus. B19 parvovirus shows a high tropism for the erythroid lineage, entering cells by adherence to the P blood-group antigen on the erythroid-specific receptor globoside [45]. The virus kills *ex vivo*-expanded human erythroid cells (human erythroid cell lines are resistant to B19 infection) by inducing caspase-dependent apoptosis through the 11 kDa viral-protein-mediated activation of caspase 10 [44]. The identification of this mechanism paves the way to structural modeling studies which may identify therapeutic drugs that by competing/inhibiting 11 KDa mediated caspase 10 activation will specifically prevent anemia induced by B19 infection. The use of *ex vivo*-expanded human erythroblasts as read-out assay will facilitate the identification of these drugs and also allow investigation

of individual susceptibility to the consequences of viral infection, opening the way for patient-specific treatment of B19 infection [43].

Another example of the use of *ex vivo*-expanded EBs for drug discovery is represented by the development of cellular-based malaria vaccines. Recently it was demonstrated that the malaria parasite is capable of infecting *ex vivo*-expanded EBs [46, 47], opening the way to the use of these cells to identify lentiviral-induced microRNA overexpression strategies that will interfere with the intracellular stage of the parasite.

19.5.2 Drug Delivery

The rationale to utilize red cells for systemic protein delivery, especially for proteins with short half lives that require continuous or frequent intravenous administration, has formidable potential. Since EBs may be generated from either EPCs or iPSs, they can be used in an autologous setting. Coopting the formidable protein synthetic capacity of EBs would eliminate the need for *in vitro* cGMP protein production and the need for regular intravenous injections.

The challenge is to establish robust, efficient, safe, and cost-effective methodologies for generating therapeutic EBs. The EBs can be generated *in vivo* or *ex vivo*. The former requires transplantation of molecularly engineered $CD34^{pos}$ hematopoietic progenitors from G-CSF- or Plerixafor (AMD3100, an inhibitor of the CXCR12L chemokine)-mobilized peripheral blood, bone marrow or CB. Since this approach is effective even in the absence of complete chimerism, it may be performed with reduced immunosuppressive regimens.

Hemophilia is an X-linked recessive congenital disorder of coagulation affecting 11–13 per 100 000 male births in the USA, depending on ethnicity [48]. The disease is due to factor VIII or IX deficiency and is manifested by frequent spontaneous intra-articular joint and soft-tissue bleeding episodes. Patients with less than 1% of normal factor VIII/IX activity have frequent severe bleeding episodes, requiring treatment with either plasma-derived or recombinant factor products. Patients with mild disease (5–30% of normal factor levels) typically have few spontaneous bleeding episodes but are still at risk for trauma-induced bleeding. Prophylactic factor infusion reduces the rate and severity of joint bleeding but is expensive and requires patient compliance. The goal for hemophilia treatment is to sustain long-term factor levels greater than 5%, converting severely affected patients to a milder phenotype.

Recently, Sadelain *et al*. demonstrated that transplantation of HSCs molecularly engineered to produce factor IX in red cells cured hemophilia B in mice [49]. *In vivo* therapeutic levels of factor IX were achieved when the engrafted murine EBs produced 600–800 ng/mL of human factor IX. Although the normal concentration of factor IX is 5000 ng/mL, the critical therapeutic threshold to reach is 5%; that is, 250 ng/mL. Assuming that similar levels of factor IX are produced by human EBs, approximately 30% of the EB pool would have to secrete factor IX to achieve therapeutic levels. Considering that humans make 2×10^{11} reticulocytes per day, and since *ex vivo*-generated EBs may be expected to generate *in vivo* 4–64 reticulocytes each, it can be anticipated that the injection of 30% of 2×10^{11} – that is, 9×10^{10} – factor IX-producing EBs will provide therapeutic amounts of factor IX for at least 4–64 days. *Ex vivo* generation of 9×10^{10} EBs is clearly obtainable with current expansion techniques.

19.5.3 *Ex vivo*-expanded EBs for Alloimmunized Patients

Patients who require chronic transfusion therapy to treat or prevent anemia often develop alloantibodies to minor group antigens (antigens other than ABO and RHD) [50–55]. These alloantibodies, which fluctuate in titer, may be undetectable with current techniques and may cause immediate or delayed hemolytic transfusion reactions and possibly trigger fulminant autoimmune hemolytic anemia. Alloimmunization occurs in many multitransfused patients but the prevalence of alloimmunization is especially high in patients with hemoglobinopathies. When prospective antigen matching is not performed, 30–40% of sickle cell anemia and approximately 20% of thalassemia patients develop antibodies. Identification of phenotypically identical or compatible blood for such highly immunized patients may require a search of national or international rare-donor registries. As an example, sickle cell anemia patients of African-American descent may lack the glycophorin B antigens S and s, and may also be negative for the high-incidence antigen U (usually expressed by most blood donors) and could become alloimmunized to these antigens. The rarity of the U-negative antigen phenotype is demonstrated by data provided by the Life-Share Blood Centers Web site (Shreveport, LA, USA); of 17 603 donors, only 101, all of African-American descent, were found negative for the U antigen, with a frequency of 1/250 in the Louisiana donor pool, which is relatively enriched for African-American donors [56]. Assuming that the frequency of the U-negative antigen phenotype in patients with sickle cell anemia is the same as in the general African-American donor population, there is potentially a relatively large demand for U-negative blood for patients who are served by a limited number of precisely matched blood donors. This strongly suggests that EBs expanded *ex vivo* from these rare donors will represent the first EB products to be used as alternatives to blood.

References

[1] Papayannopoulou, T., Abkowitz, J., D'Andrea, A. and Migliaccio, A.R. Biology of erythtropoiesis, erythroid differentiation and maturation, in *Hematology: Basic Principles and Practice* (ed. R. Hoffman, E.J. Benz, S.J. Shattil *et al*.), Elsevier, Philadelphia, PA, 5 edn, pp. 276–294 (2009).

[2] Mohandas, N. Banking on red blood cells, *Nat. Biotech*., **23**, 35–36 (2005).

[3] Hiroyama, T., Miharada, K., Sudo, K. *et al*. Establishment of mouse embryonic stem cell-derived erythroid progenitor cell lines able to produce functional red blood cells, *PLoS ONE*, **3**, e1544 (2008).

[4] Migliaccio, A.R., Whitsett, C. and Migliaccio, G. Erythroid cells in-vitro: from developmental biology to blood transfusion products, *Curr. Opin. Hematol*., **16**, 259–268 (2009).

[5] Douay, L., Lapillonne, H. and Turhan, A.G. Stem cells – a source of adult red blood cells for transfusion purposes: present and future, *Crit. Care Clin*., **25**, 383–398 (2009).

[6] Bender, J.G., Unverzagt, K.L., Walker, D.E. *et al*. Identification and comparison of CD34-positive cells and their subpopulations from normal peripheral blood and bone marrow using multicolor flow cytometry, *Blood*, **77**, 2591–2596 (1991).

[7] Wong, A., Yuen, P.M., Li, K. *et al*. Cord blood collection before and after placental delivery: levels of nucleated cells, haematopoietic progenitor cells, leukocyte subpopulations and macroscopic clots, *Bone Marrow Transplant*., **27**, 133–138 (2001).
[8] Bartolovic, K., Balabanov, S., Berner, B. *et al*. Clonal heterogeneity in growth kinetics of CD34+CD38 – human cord blood cells in-vitro is correlated with gene expression pattern and telomere length, *Stem Cells*, **23**, 946–957 (2005).
[9] van Rossum, E.F., Binder, E.B., Majer, M. *et al*. Polymorphisms of the glucocorticoid receptor gene and major depression, *Biol. Psychiatry*, **59**, 681–688 (2006).
[10] Almeida, S. and Hutz, M.H. Genetic variation of estrogen metabolism and the risks of cardiovascular disease, *Curr. Opin. Investig. Drugs*, **8**, 814–820 (2007).
[11] Prade-Houdellier, N., Frebet, E., Demur, C. *et al*. Human telomerase is regulated by erythropoietin and transforming growth factor-beta in human erythroid progenitor cells, *Leukemia*, **21**, 2304–2310 (2007).
[12] Chang, T.J., Scher, B.M., Waxman, S. and Scher, W. Inhibition of mouse GATA-1 function by the glucocorticoid receptor: possible mechanism of steroid inhibition of erythroleukemia cell differentiation, *Mol. Endocrinol*., **7**, 528–542 (1993).
[13] Blobel, G.A. and Orkin, S.H. Estrogen-induced apoptosis by inhibition of the erythroid transcription factor GATA-1, *Mol. Cell. Biol*., **16**, 1687–1694 (1996).
[14] Ribeil, J.A., Zermati, Y., Vandekerckhove, J. *et al*. Hsp70 regulates erythropoiesis by preventing caspase-3-mediated cleavage of GATA-1, *Nature*, **445**, 102–105 (2007).
[15] Zhao, W., Kitidis, C., Fleming, M.D. *et al*. Erythropoietin stimulates phosphorylation and activation of GATA-1 via the PI3-kinase/AKT signaling pathway, *Blood*, **107**, 907–915 (2006).
[16] Lu, S.J., Feng, Q., Park, J.S. *et al*. Biologic properties and enucleation of red blood cells from human embryonic stem cells, *Blood*, **112**, 4475–4484 (2008).
[17] Ma, F., Ebihara, Y., Umeda, K. *et al*. Generation of functional erythrocytes from human embryonic stem cell-derived definitive hematopoiesis, *Proc. Natl. Acad. Sci. U S A*, **105**, 13087–13092 (2008).
[18] Bhatia, M. Hematopoietic development from human embryonic stem cells. *Hematology Am. Soc. Hematol. Educ. Program*., 11–16 (2007).
[19] Chang, K.H., Huang, A., Hirata, R.K. *et al*. Globin phenotype of erythroid cells derived from human induced pluripotent stem cells, *Blood*, **115**, 2553–2554 (2010).
[20] Hochedlinger, K. and Plath, K. Epigenetic reprogramming and induced pluripotency, *Development*, **136**, 509–523 (2009).
[21] Migliaccio, G., Migliaccio, A.R. and Adamson, J.W. The biology of hematopoietic growth factors: studies in-vitro under serum-deprived conditions, *Exp. Hematol*., **9**, 1049–1055 (1990).
[22] Chasis, J.A. and Mohandas, N. Erythroblastic islands: niches for erythropoiesis, *Blood*, **112**, 470–478 (2008).
[23] Ledran, M.H., Krassowska, A., Armstrong, L. *et al*. Efficient hematopoietic differentiation of human embryonic stem cells on stromal cells derived from hematopoietic niches, *Cell Stem Cell*, **3**, 85–98 (2008).

[24] Miharada, K., Hiroyama, T., Sudo, K. *et al*. Efficient enucleation of erythroblasts differentiated in-vitro from hematopoietic stem and progenitor cells, *Nat. Biotechnol*., **24**, 1255–1256 (2006).
[25] Migliaccio, G., Sanchez, M., Masiello, F. *et al*. Humanized culture medium for clinical expansion of human erythroblasts, *Cell Transplant*., **19**, 453–469 (2010).
[26] Hayflick, L. and Moorhead, P.S. The serial cultivation of human diploid cell strains, *Exp. Cell. Res*., **25**, 585–621 (1961).
[27] Majka, M., Janowska-Wieczorek, A., Ratajczak, J. *et al*. Numerous growth factors, cytokines, and chemokines are secreted by human CD34(+) cells, myeloblasts, erythroblasts, and megakaryoblasts and regulate normal hematopoiesis in an autocrine/paracrine manner, *Blood*, **97**, 3075–3085 (2001).
[28] Bohmer, R.M. IL-3-dependent early erythropoiesis is stimulated by autocrine transforming growth factor beta, *Stem Cells*, **22**, 216–224 (2004).
[29] Zamai, L., Secchiero, P., Pierpaoli, S. *et al*. TNF-related apoptosis-inducing ligand (TRAIL) as a negative regulator of normal human erythropoiesis, *Blood*, **95**, 3716–3724 (2000).
[30] Sanchez, M., Masiello, F., Tirelli, V. *et al*. The final cellular output in human erythroid massive amplification culture (HEMA) is determined by dynamic interactions between immature and mature cell populations, *Blood*, **114**, 18318a (2009).
[31] Bhattacharya, N. A preliminary report of 123 units of placental umbilical cord whole blood transfusion in HIV-positive patients with anemia and emaciation, *Clin. Exp. Obstet. Gynecol*., **33**, 117–121 (2006).
[32] Migliaccio, G., Sanchez, M., LeBlanc, A. *et al*. Long term storage does not alter functionality of in vitro generated human erythroblasts: implications for ex vivo generated erythroid transfusion products, *Transfusion*, **49**, 2668–2679 (2009).
[33] Varricchio, L., Fabucci, M.E., Alfani, E. *et al*. Compensated variability in the expression of globin-related genes in erythroblasts generated ex vivo from different donors, *Transfusion*, **50**, 672–684 (2009).
[34] Turner, M. The impact of new-variant Creutzfeldt–Jakob disease on blood transfusion practice, *Br. J. Haematol*., **106**, 842–850 (1999).
[35] Tolar, J., Nauta, A.J., Osborn, M.J. *et al*. Sarcoma derived from cultured mesenchymal stem cells, *Stem Cells*, **25**, 371–379 (2007).
[36] Rubio, D., Garcia-Castro, J., Martin, M.C. *et al*. Spontaneous human adult stem cell transformation, *Cancer Res*., **65**, 3035–3039 (2005).
[37] Bernardo, M.E., Zaffaroni, N., Novara, F. *et al*. Human bone marrow derived mesenchymal stem cells do not undergo transformation after long-term in-vitro culture and do not exhibit telomere maintenance mechanisms, *Cancer Res*., **67**, 9142–9149 (2007).
[38] Selvaggi, T.A., Walker, R.E. and Fleisher, T.A. Development of antibodies to fetal calf serum with arthus-like reactions in Human Immunodeficiency Virus-infected patients given syngeneic lymphocyte infusions, *Blood*, **89**, 776–779 (1997).
[39] Sakamoto, N., Tsuji, K., Muul, L.M. *et al*. Bovine apolipoprotein B-100 is a dominant immunogen in therapeutic cell populations cultured in fetal calf serum in mice and humans, *Blood*, **110**, 501–508 (2007).
[40] FDA guidelines for cell therapy. Available online at: http://www.fda.gov/cber/ind/ind.htm.

[41] Singleton, B.K., Burton, N.M., Green, C. *et al*. Mutations in EKLF/KLF1 form the molecular basis of the rare blood group In(Lu) phenotype, *Blood*, **112**, 2081–2088 (2008).
[42] Twu, Y.C., Hsieh, C.Y., Lin, M. *et al*. Phosphorylation status of transcription factor C/EBPalpha determines cell-surface poly-LacNAc branching (I antigen) formation in erythropoiesis and granulopoiesis, *Blood*, **115**, 2491–2499 (2010).
[43] Migliaccio, G. and Migliaccio, A.R. Getting personal with B19 parvovirus, *Blood*, **115**, 922–923 (2010).
[44] Chen, A.Y., Zhang, E.Y., Guan, W. *et al*. The small 11 kDa non-structural protein of human parvovirus B19 plays a key role in inducing apoptosis during B19 virus infection of primary erythroid progenitor cells, *Blood*, **115**, 1070–1080 (2010).
[45] Young, N.S. and Brown, K.E. Parvovirus B19, *N. Engl. J. Med*., **350**, 586–597 (2004).
[46] Tamez, P.A., Liu, H., Fernandez-Pol, S. *et al*. Stage-specific susceptibility of human erythroblasts to Plasmodium falciparum malaria infection, *Blood*, **114**, 3652–3655 (2009).
[47] Jiang, L., López-Barragán, M.J., Jiang, H. *et al*. Epigenetic control of the variable expression of a Plasmodium falciparum receptor protein for erythrocyte invasion, *Proc. Natl. Acad. Sci. U S A*, **107**, 2224–2229 (2010).
[48] Ragni, M.V., Kessler, C.M. and Lozier, J.M. Clinical aspects and therapy for hemophilia, in *Hematology: Basic Principles and Practice* (ed. R. Hoffman, E.J. Benz and S. Shattil), 5 edn, Churchill Livingstone, New York, p. 1912 (2009).
[49] Chang, A.H., Stephan, M.T., Lisowski, L. and Sadelain, M. Erythroid-specific human factor IX delivery from in vivo selected hematopoietic stem cells following nonmyeloablative conditioning in hemophilia B mice, *Mol. Ther*., **16**, 1745–1752 (2008).
[50] Luzo, A.C., Pereira, F.B., de Oliveira, R.C. *et al*. Red blood cell antigen alloimmunization in liver transplant recipients, *Transplant. Proc*., **42**, 494–495 (2010).
[51] Schonewille, H., de Vries, R.R. and Brand, A. Alloimmune response after additional red blood cell antigen challenge in immunized hematooncology patients, *Transfusion*, **49**, 453–457 (2009).
[52] Vichinsky, E.P., Earles, A., Johnson, R.A. *et al*. Alloimmunization in sickle cell anemia and transfusion of racially unmatched blood, *N. Engl. J. Med*., **322**, 1617–1621 (1990).
[53] Rosse, W.F., Gallagher, D., Kinney, T.R. *et al*. Transfusion and alloimmunization in sickle cell disease. The Cooperative Study of Sickle Cell Disease, *Blood*, **76**, 1431–1437 (1990).
[54] Castro, O., Sandler, S.G., Houston-Yu, P. and Rana, S. Predicting the effect of transfusing only phenotype-matched RBCs to patients with sickle cell disease: theoretical and practical implications, *Transfusion*, **42**, 684–690 (2002).
[55] Singer, S.T., Wu, V., Mignacca, R. *et al*. Alloimmunization and erythrocyte autoimmunization in transfusion-dependent thalassemia patients of predominantly asian descent, *Blood*, **96**, 3369–3373 (2000).
[56] http://www.lifeshare.org/facts/raretraits.htm.

20

The Universal Red Blood Cell

Luca Ronda[1] *and Serena Faggiano*[2]

[1]*Department of Biochemistry and Molecular Biology, University of Parma, Parma, Italy*

[2]*MRC National Institute for Medical Research, London, UK*

20.1 Introduction

In blood transfusion, matching the blood between donor and transfused patient is a critical issue. The transfusion of ABO-incompatible blood is the leading cause of fatal transfusion reactions reported by the national hemovigilance systems and governmental regulatory bodies [1]. Donor and acceptor are considered compatible when matching occurs towards major blood group-specific antigens, ABH and D.

The effects of the infusion of ABO-incompatible red blood cells (RBCs) are caused by the reaction of the recipient's immune system: the recipient's ABO antibodies coat RBCs and destroy them due to complement activation and induction of membrane attack complexes (MAC) with the capacity to lyse cells. The resulting clinical syndrome is often an acute intravasal hemolytic transfusion reaction with symptoms including hemoglobinuria, back pain, chest pressure, and hypotonia [2] (see Chapter 16). Moreover, patients subjected to repeated blood transfusions develop alloimmunization against non-ABH/D antigens [3–5]. This issue is particularly evident in sickle cell patients, who are usually originated from Africa, when they receive blood from Caucasian donors. A different Rh-antigen distribution between donor and acceptor ethnic group is responsible for this alloimmunization [6, 7].

Group O Rh(D) RBCs, lacking the terminal immunodominant sugars, are referred to as "universal RBCs" since they can be safely transfused in individuals of any blood

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group. Moreover, group O RBCs avoid the issue of blood-group testing in case of trauma and/or shocks, saving time.

Many efforts have been made in recent years to set up a strategy for the large-scale production of "universal red blood cells", which limit the use of group O Rh(D^-) RBCs. This achievement would: (i) overcome the risk of hemolytic transfusions caused by ABO incompatibility, and the shortage of units with specific ABO blood groups, and (ii) reduce the wastage of excess blood donations of the less needed types, as well as the costs of the logistics associated with blood-group inventory management [1, 8]. Two different approaches have been proposed in order to develop a "universal blood": the elimination of the immunodominant sugars from group A and group B RBCs by means of specific glycosidases (named A-zyme and B-zyme, respectively), and the decoration of the RBC surfaces through addition of polyethylene glycol (PEG) molecules [9, 10].

20.1.1 ABO Antigens

The blood-type antigens A and B were originally described in 1900 by Karl Landsteiner, who was awarded the Nobel Prize in 1932 for this discovery [11]. These antigens are associated to glycoproteins and glycolipids attached to the cell membrane of erythrocytes [12]. The blood-group specificity is determined by the nature and linkage of monosaccharides at the ends of the carbohydrate chains (Figure 20.1). The monosaccharide determining type-A specificity is a terminal α-1-3-linked N-acetylgalactosamine (GalNAc), while the corresponding monosaccharide of B-type specificity is an α-1-3-linked galactose (Gal). Group O cells lack either of these monosaccharides and the oligosaccharide chains terminate with an α-1-2-linked fucose (Fuc) residue, called the H antigen [2, 13]. The immunodominant sugars GalNAc and Gal are located in terminal positions on the H oligosaccharide chain. The genes involved in the synthesis of the ABO epitopes encode for transferases responsible for the addition of the terminal immunodominant sugar. Antibodies against lacking antigens are developed by human newborns within 18 months after birth, probably due to the continuous exposure to microbial organisms carrying related carbohydrate structures. Group-A individuals possess in their plasma antibodies to B antigen. Conversely, individuals of blood group B carry in their plasma antibodies to A antigen. The blood of group AB has neither antibody, and the blood of group O has both. This is generally referred to as Landsteiner's rule [2].

There are several inherited variants of blood groups A and B. Group-B variants are very rare, while group A splits in three main subtypes, known as A_1, A intermediate (A_{int}), and A_2. These subtypes differ in the number of antigenic A sites, $A_1 > A_{int} > A_2$ [14]. Moreover, A_1 RBCs have a dual repeated-A structure attached on a subset of glycosphingolipids, while A_2 cells exhibit an H structure on an internal A structure on a similar subset of glycolipids [15–17].

20.1.2 The Rh System

The Rh blood-group system is a highly polymorphic human blood-group system, first discovered in 1940 by Landsteiner and Wiener, and consisting of at least 45 independent antigens. It is considered the most polymorphic human blood-group system. From a molecular point of view, Rh antigens are transmembrane proteins (Figure 20.1), with 12 membrane-spanning domains and 6 extracellular loops. The D antigen is the most

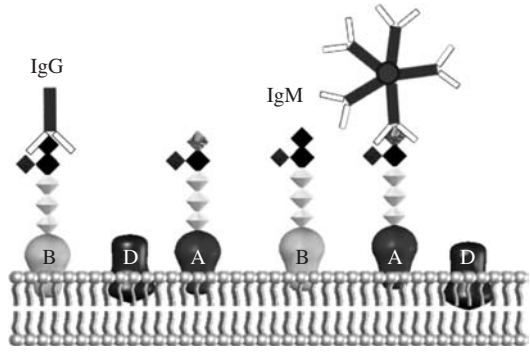

Figure 20.1 *RBC surface antigens exposed to mismatched blood produce antibody reactions with this complement activation.*

significant, and is routinely tested for blood compatibility. Other antigens, among them C and E, are also present.

Rh factors are involved in hemolytic transfusion reactions, and the incompatibility between mother and fetus is the major cause of hemolytic disease of the newborn (HDN). The relevance of Rh antigens to RBC membrane integrity is demonstrated by the fact that subjects deficient in all Rh antigens (Rh_{null} and Rh_{mod} rare phenotypes) show abnormalities in erythrocytes shape and function [18].

20.2 Enzymatic Removal of A and B Antigens

20.2.1 Conversion of B RBCs to Group O

In the first studies into the biochemistry of ABO blood-group antigens, Morgan [19] and Watkins [20] in the UK, and Iseki [21] in Japan, discovered that some bacterial enzymes from *Clostridium tertium, Clostridium welchii, Bacillus cereus*, and *Trichomonas foetus* specifically destroyed A, B, or H antigens. In the early 1980s, the enzymatic conversion of B RBCs to group O (production of so-called "B ECO RBCs", Figure 20.2) was carried out for practical purposes by Lenny and Goldstein, at the New York Blood Center, using either purified or recombinant coffee-bean (*Coffea canephora*) α-galactosidase [22, 23]. The first experiments were carried out with RBCs from gibbons [22]. Small infusions of enzyme-converted blood in the primates did not cause any significant adverse effect. Subsequently, as gibbons were declared on the border of extinction and thus not usable for scientific experiments, similar trials were performed on human volunteers [23]. Small amounts of ^{51}Cr-labeled ECO RBCs survived in the circulation and did not evoke adverse clinical reactions. The clinical trials scaled from small infusions to a single RBC unit and later to multiple and repeated transfusions. In 1991, group-A and -O healthy volunteers were transfused with a whole unit of group-B RBCs enzymatically converted to group O, and one recipient had two separate single-unit transfusions. RBCs survived normally and did not increase preexisting anti-B levels in all recipients [24]. Later the transfusion of two units was reported [25]. No clinical adverse reactions were observed, but one patient showed a rise of anti-B titers. In 1995, a report of a phase I

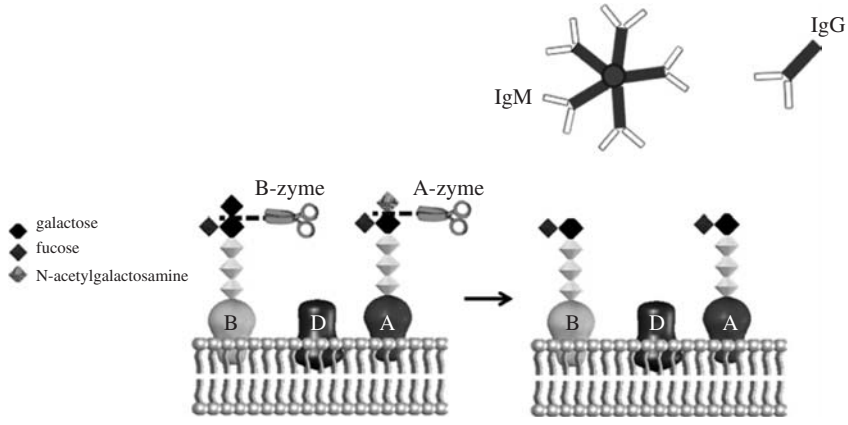

Figure 20.2 *RBCs reacted with α-galactosidase, which removes A and B antigenic determinants, do not lead to immune-system activation.*

trial was published [26]. Normal group-A and -O subjects received 3 units of B ECO cells, and group-O subjects underwent transfusion several months later. Clinical evaluation and serologic analyses did not reveal any evidence of subtle or acute transfusion reaction or significant increase in preexisting anti-B titer. In 1998, ZymeQuest, a US company, completely took over the process developed by Goldstein [10]. Two years later, a phase II trial utilizing recombinant enzyme was reported [27], in which enzyme-converted RBCs were given to 21 hospitalized group-A and -O patients. Five of the patients showed an increase in anti-B titers, and one of them developed a positive direct antiglobulin test (DAT). One patient was found to be serologically incompatible with the B ECO RBCs, even though routine compatibility tests were negative. Unexpectedly, 20% of group-A and 40% of group-O sera from random patients reacted *in vitro* with the enzyme-converted RBCs. Moreover, the large-scale production of universal RBCs from group-B blood using coffee-bean α-galactosidase showed some limitations, as it required large amounts of enzyme (6 mg/ml enzyme at 80–90% hematocrit) and showed a pH optimum at 5.5, not ideal for erythrocytes. Soy-bean (*Glycine max*), and more recently *Pseudoalteromonas* [28], α-galactosidases have been proposed in order to convert B cells in a more efficient way, but these protocols have not been completely evaluated by routine blood-typing tests or clinical trials. More recently, ZymeQuest screened 2500 fungal and bacterical isolates for A- and B-zymes [29]. The company searched for enzymes with selective substrate specificities for tetrasaccharide A and B structures, more complex with respect to the previously used monosaccharide *p*-nitrophenyl substrate. Moreover, it selected enzymes reacting with a high efficiency at neutral pH. Two enzymes with α-N-acetylgalactosaminidase activity were isolated. One B-zyme from *Bacteroides fragilis*, NCTC 9343 (designated "Frag A"), was successfully used to prepare B ECO RBCs that did not react with commercial B antibodies. The enzyme exhibits a pH optimum between 5 and 7.5, and its activity with the B tetrasaccharide substrate is 300-fold higher than that of coffee-bean galactosidase [29]. No clinical trials using B ECO cells obtained by *Bacteroides fragilis* glycosidase have been reported to date.

20.2.2 Conversion of A RBCs to Group O

Enzymatic conversion of group-A RBCs (Figure 20.1) was found to be more difficult due to the lack of appropriate glycosidases and to the more complex nature of the A antigens. In 1980, an α-N-acetylgalactosaminidase from *Clostridium perfringens* was described to remove A antigens from A cells, but the resulting RBCs showed a persistent group-A activity after the conversion [30]. The purified enzyme removed A antigens from A_2 cells, but not from A_1 cells, as proved later by ELISA assays [31]. No protocols for the conversion of intact cells were reported [32]. Goldstein screened many microbial and animal sources in order to find a suitable glycosidase, and finally discovered a chicken-liver α-N-acetylgalactosaminidase which catalyzed the conversion of weak A subgroups A_{int} and A_2, but only partially reduced the activity of the A_1 antigen [33, 34]. The enzyme has a pH optimum of 3.65 and requires more than 3 mg/ml enzyme protein to convert A_2 cells [35]; thus its use would be impractical for routine A ECO RBCs production. In 1991, an α-N-acetylgalactosaminidase from *Ruminococcus torques* was described, which reduces the A antigenic strength of A_1 cells to the level of A_2 cells [36, 37]. *Acremonium sp*. α-N-acetylgalactosaminidase was also reported to work on type A_1 cells, but the observed conversion was not complete [38]. Later, a novel α-N-acetylgalactosaminidase from the marine bacterium *Pseudoalteromonas sp*. was identified and purified [39]. This enzyme could perform the conversion to group O of both A_1 and A_2 RBCs at neutral pH, but the resulting ECO RBCs were not evaluated by routine typing procedures. The developed conversion protocol involved 24-hour incubation, and the functionality of the converted cells was not checked. The first recombinant α-N-acetylgalactosaminidase used to prepare A ECO cells infused to humans was isolated by ZymeQuest researchers from *Chryseobacterium spp*. This enzyme conveniently converts both A_1 and A_2 RBCs [2, 40]. The results of phase I and II clinical trials have not been published as yet (information available at: http://clinicaltrial.gov/ct2/show/study/NCT0261274). ZymeQuest's extensive search of new enzymes has recently led to the discovery of five other enzymes with α-galactosidase activity [29]. One suitable A-zyme was isolated from *Elizabethkingia meningosepticum* [41]; it is similar to an A-zyme from *E. meningosepticum* that had already been cloned. This enzyme proved to be very efficient at a neutral pH on both A_1 and A_2 RBCs (about 60 mg of enzyme required to convert 200 ml of A_1 RBCs; 15 mg for the conversion of A_2 RBCs). *E. meningosepticum* A-zyme, together with *B. fragilis* B-zyme (see above), was used to prepare groups A, B, and AB ECO RBCs using more than 200 blood samples from groups A_1 and A_2. The converted cells did not react with the most powerful commercial monoclonal anti-A, anti-B, or anti-AB antibodies. To date, no data have been published on the clinical trials using these new ECO RBCs.

20.3 RBC Camouflage Through PEGylation

One of the main drawbacks of the enzymatic conversion of A- and B-group RBCs into O-group is that this approach cannot be developed for protein antigens such as the Rh system. As a matter of fact, these proteins are very close to the cell membrane (Figure 20.1), and any attempt to remove these antigenic determinants could alter the integrity and/or the functional properties of erythrocytes.

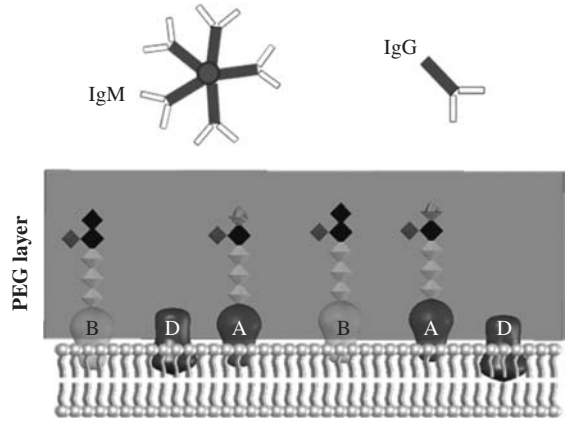

Figure 20.3 *Coating of RBC surfaces with PEG molecules masks antigens from antibody recognition.*

Immunocamouflaged RBCs – that is, RBCs whose surfaces are derivatized to mask their antigenic determinants with reactive polyethylene glycol (PEG) – are an alternative approach. PEG is a nontoxic polymer that has been conjugated to different molecular entities, from proteins [42, 43] and nucleic acids [44] to blood cells and tissues [45], in order to enhance their bioavailability, since its hydrophilic nature improves biocompatibility and reduces immunological recognition (Figure 20.3).

This immunocamouflage can also attenuate the risk of alloimmunization in patients requiring chronic RBC transfusions, such as patients with sickle cell anemia and thalassemias, as a significant number of these individuals, up to 35%, show clinically significant alloimmunization to non-ABO antigens [5, 46].

20.3.1 Functionalized Methoxy PEG

The effect of linker chemistry and polymer length on the immunoprotective properties of RBC PEGylation was studied, comparing erythrocytes modified with cyanuric chloride-activated methoxypolyethylene glycol (mPEG, 5 kDa), benzotriazole carbonate methoxy PEG (5 and 20 kDa), and N-hydrosuccinimidyl ester of mPEG propionic acid (2, 5 and 20 kDa). These different derivatives of mPEG, even though all react on amino groups (ε-amino groups of lysines residues and proteins amino-terminal groups), introduce different linkers with different efficiencies of linkage reaction and efficacies of immunocamouflage [47]. The modification of cells or tissues with mPEG limits immunological recognition and can facilitate tolerance induction through camouflaging of antigenic sites, membrane-surface charges, and attenuation of receptor–ligand and cell–cell interactions. PEGylation is effective in masking group antigens D, C, c, E, e, Fya, JKa, and Jkb [48–51]. This protection relates well with the experimental observation that fluorescein-PEG attachment on RCB surfaces is homogeneous and stable for at least 30 days [52].

The modification with mPEG was initially demonstrated to be effective in reducing the immunogenicity of allogeneic and xenogeneic RBCs in mouse models [49, 53]. The

effect of RBC PEGylation on microcirculation has been tested on hamsters, observing blood-flow velocities and the diameters of individual blood vessels after infusion of 0.05–5 mM RBCs conjugated with 5 or 20 kDa mPEG, demonstrating that they are well tolerated and do not cause any vasoconstrictive effect [54].

20.3.2 Cyanuric Chloride PEG

Cyanuric chloride PEG (CnCl-PEG) is widely used as a PEGylation reagent for RBCs [47–49]. This functionalized PEG predominantly reacts with protein amino groups [55]. The conditions of PEGylation reaction using the cyanuric chloride derivative of PEG 5 kDa were optimized for the masking effect on D antigens in terms of pH, temperature, and PEG concentration [56].

The conjugation of RBCs with CnCl-PEG 5 kDa was demonstrated to be able to mask Rh antigens to a reasonable extent, but to only partially mask A and B antigens, due to the absence or low accessibility of amino groups. Another concern is the size of the antigens: short D antigens are well camouflaged, but large antigens, such as ABH epitopes attached to oligosaccharide chains on glycoproteins or glycosphingolipids, are more extended and cannot be sufficiently camouflaged by cell-conjugated PEG chains [51].

Moreover, CnCl-PEG enhances cell lysis in mismatched ABO serum, which correlates with increased IgM binding and complement activation [51]. It was observed that upon increasing PEG 5 kDa concentration, an increased echinocytosis appeared [56].

20.3.3 Extension Arm-facilitated RBC PEGylation

An alternative PEGylation chemistry, also applied to hemoglobin in order to make it suitable as a blood substitute, is based on the use of maleimido-functionalized PEG (Mal-Phe PEG) on RBCs previously reacted with iminothiolane (IT). IT reacts on protein amino groups, adding a spacer arm ending with a thiol group. Mal-Phe PEG can then react with sulfydryl groups of cysteine side chains as well as with thiol groups from IT, increasing the number of conjugating sites [50]. However, the simple use of Mal-Phe PEG does not result in significant masking of the A or the D antigens, due to poor presence of thiol groups on RBC membranes. The reaction with Mal-Phe PEG 5 kDa and IT gave PEGylated cells exhibiting protection of the Rh antigens (D antigen and CE antigens), but ABH antigens remained unmasked.

The influence of PEG-chain length on ABH-antigen masking was also investigated. Rh antigens are multipass-transmembrane proteins, while ABH antigens are carbohydrates bound to proteins or lipids on the cell surface, thus the latter extend more towards the solvent than the former. Therefore, longer PEG chains were thought to be more efficient in epitope masking [50]. PEG 10 and 20 kDa were used in combination with IT, producing a strong reduction of anti-A antigen-induced agglutination, although not a complete inhibition. However, PEG 20 kDa, which was the most effective in protecting A antigens, exhibited a lower masking effect on D antigens than shorter PEG chains (5 and 10 kDa) and caused some autoaggregation of RBCs even in the absence of any antibody. In view of this observation, a mixture of reactive PEG chains was tested. It was found that a two-step PEGylation, where RBCs were reacted with Mal-Phe PEG 5 kDa and then with Mal-Phe PEG 20 kDa, gave a protection against anti-A, anti-B, and anti-D antibodies [50].

The effect of positively charged ε-amino group neutralization through PEGylation was also studied, comparing RBCs PEGylated with different reactants. Maleimido-PEG RBCs, prepared using IT as a thiolation reagent, which converts the ε-amino groups of proteins into substituted amidines with a charge conservation, were compared with RBCs PEGylated with 3,3′-dithiobis(sulfosuccinimidyl propionate) (DTSSP), which links through an isopeptide linkage, neutralizing the positive charge of lysine residues. Charge neutralization was shown to only affect the oxygen affinity of RBCs. This comparison demonstrated that extension-arm engineering per se, regardless of its chemistry, improves the capability of Rh-system antigen masking [57].

20.3.4 Increasing the Degree of RBC PEGylation

Recently, the problem of inefficient conjugation of hydrophilic molecules such as PEG to cell surface, due to repulsion, has been addressed by the use of "additive" polymers such as dextran and hyperbranched polyglycerol (HPG) in combination with reactive polymers. A primary amine reactive succinimidyl succinate-functionalized PEG (SS-PEG, 5 kDa) and HPG (SS-HPG, 25 kDa) were reacted with lysine residues. The enhanced modification efficiency (up to 10-fold with dextran) was explained by the synergistic effects of enhanced polymer transport and increased concentration of reactive polymer near the cell surface, due to an increased penetration into the glycocalix of cell membrane. The efficacy of this approach has been demonstrated with different cell types, such as RBCs, leukocites, platelets, and Jurkat cells, allowing a higher cell derivatization with a lower reactive polymer concentration, the latter minimizing toxicity and cost [58].

20.4 Conclusion

Although significant progress has been made towards developing a strategy to convert RBCs with different antigenicities to universally transfusable cells, at the moment no large-scale applicable protocol is available. The enzymatic removal of A and B antigens is the only approach for which human clinical trials have been performed. RBC camouflaging with PEG is a valuable alternative, offering the possibility of masking non-ABO antigenic determinants as well. The *in vivo* efficacy of this approach and the effect of PEG on RBCs' physical properties have still to be evaluated.

References

[1] Olsson, M.L. and Clausen, H. Modifying the red cell surface: towards an ABO-universal blood supply, *Br. J. Haematol*., **140**, 3–12 (2008).

[2] Olsson, M.L., Hill, C.A., de la Vega, H. *et al*. Universal red blood cells-enzymatic conversion of blood group A and B antigens, *Transfus. Clin. Biol*., **11**, 33–39 (2004).

[3] Blumberg, N., Ross, K., Avila, E. and Peck, K. Should chronic transfusions be matched for antigens other than ABO and Rho(D)?, *Vox Sang*., **47**, 205–208 (1984).

[4] Castro, O., Sandler, S.G., Houston-Yu, P. and Rana, S. Predicting the effect of transfusing only phenotype-matched RBCs to patients with sickle cell disease: theoretical and practical implications, *Transfusion*, **42**, 684–690 (2002).
[5] Vichinsky, E.P., Earles, A., Johnson, R.A. *et al*. Alloimmunization in sickle cell anemia and transfusion of racially unmatched blood, *N. Engl. J. Med*., **322**, 1617–1621 (1990).
[6] Issitt, P.D. Race-related red-cell alloantibody problems, *Br. J. Biomed. Sci*., **51**, 158–167 (1994).
[7] Sosler, S.D., Jilly, B.J., Saporito, C. and Koshy, M. A simple, practical model for reducing alloimmunization in patients with Sickle-Cell Disease, *Am. J. Hematol*., **43**, 103–106 (1993).
[8] Kruskall, M.S. and AuBuchon, J.P. Making Landsteiner's discovery superfluous: safety and economic implications of a universal group O red blood cell supply, *Transfus Sci*., **18**, 613–620 (1997).
[9] Garratty, G. Progress in modulating the RBC membrane to produce transfusable universal/stealth donor RBCs, *Transfus. Med. Rev*., **18**, 245–256 (2004).
[10] Garratty, G. Modulating the red cell membrane to produce universal/stealth donor red cells suitable for transfusion, *Vox Sang*., **94**, 87–95 (2008).
[11] Landsteiner, K. Zur Kenntnis der antifermentativen, lytischen und agglutinierenden Wirkungen des Blutserums und der Lymphe, *Zbl. Bakt*., **27**, 357–362 (1900).
[12] Ravn, V. and Dabelsteen, E. Tissue distribution of histo-blood group antigens, *Acta Pathol. Microbiol. Scand*., **108**, 1–28 (2000).
[13] Watkins, W.M. Biochemistry and genetics of the ABO, Lewis, and P blood group systems, *Adv. Hum. Genet*., **10**, 1–136, 379–385 (1980).
[14] Schachter, H., Michaels, M.A., Tilley, C.A. *et al*. Qualitative differences in the N-acetyl-D-galactosaminyltransferases produced by human A1 and A2 genes, *Proc. Natl. Acad. Sci. U S A*, **70**, 220–224 (1973).
[15] Clausen, H., Levery, S.B., Nudelman, E. *et al*. Repetitive A epitope (type 3 chain A) defined by blood group A1-specific monoclonal antibody TH-1: chemical basis of qualitative A1 and A2 distinction, *Proc. Natl. Acad. Sci. U S A*, **82**, 1199–1203 (1985).
[16] Clausen, H., Holmes, E. and Hakomori, S. Novel blood group H glycolipid antigens exclusively expressed in blood group A and AB erythrocytes (type 3 chain H). II. Differential conversion of different H substrates by A1 and A2 enzymes, and type 3 chain H expression in relation to secretor status, *J. Biol. Chem*., **261**, 1388–1392 (1986).
[17] Clausen, H., Levery, S.B., Kannagi, R. and Hakomori, S. Novel blood group H glycolipid antigens exclusively expressed in blood group A and AB erythrocytes (type 3 chain H). I. Isolation and chemical characterization, *J. Biol. Chem*., **261**, 1380–1387 (1986).
[18] Lomas-Francis, C. and Reid, M.E. The Rh blood group system: the first 60 years of discovery, *Immunohematology*, **16**, 7–17 (2000).
[19] Morgan, W.T. The human ABO blood group substances, *Experientia*, **3**, 257–267 (1947).
[20] Watkins, W.M. Changes in the specificity of blood-group mucopolysaccharides induced by enzymes from *Trichomonas foetus*, *Immunology*, **5**, 245–266 (1962).

[21] Aminoff, D. and Furukawa, K. Enzymes that destroy blood group specificity. I. Purification and properties of alpha-L-fucosidase from *Clostridium perfringens*, *J. Biol. Chem*., **245**, 1659–1669 (1970).
[22] Lenny, L.G.J. Enzymatic removal of blood group B antigen from Gibbon erythrocytes, *Transfusion*, **20**, 618 (1980).
[23] Goldstein, J., Siviglia, G., Hurst, R. *et al*. Group B erythrocytes enzymatically converted to group O survive normally in A, B, and O individuals, *Science*, **215**, 168–170 (1982).
[24] Lenny, L.L., Hurst, R., Goldstein, J. *et al*. Single-unit transfusions of RBC enzymatically converted from group B to group O to A and O normal volunteers, *Blood*, **77**, 1383–1388 (1991).
[25] Lenny, L.L., Hurst, R., Goldstein, J. and Galbraith, R.A. Transfusions to group O subjects of 2 units of red cells enzymatically converted from group B to group O, *Transfusion*, **34**, 209–214 (1994).
[26] Lenny, L.L., Hurst, R., Zhu, A. *et al*. Multiple-unit and second transfusions of red cells enzymatically converted from group B to group O: report on the end of phase 1 trials, *Transfusion*, **35**, 899–902 (1995).
[27] Kruskall, M.S., AuBuchon, J.P., Anthony, K.Y. *et al*. Transfusion to blood group A and O patients of group B RBCs that have been enzymatically converted to group O, *Transfusion*, **40**, 1290–1298 (2000).
[28] Bakunina, I.Y., Sova, V.V., Nedashkovskaya, O.I. *et al*. Alpha-galactosidase of the marine bacterium *Pseudoalteromonas sp*. KMM 701, *Biochemistry (Mosc)*, **63**, 1209–1215 (1998).
[29] Liu, Q.Y.P., Sulzenbacher, G., Yuan, H.P. *et al*. Bacterial glycosidases for the production of universal red blood cells, *Nat. Biotechnol*., **25**, 454–464 (2007).
[30] Levy, G.N. and Aminoff, D. Purification and properties of alpha-N-acetylgalactosaminidase from *Clostridium perfringens*, *J. Biol. Chem*., **255**, 11737–11742 (1980).
[31] Hsieh, H.Y., Calcutt, M.J., Chapman, L.F. *et al*. Purification and characterization of a recombinant alpha-N-acetylgalactosaminidase from *Clostridium perfringens*, *Protein Expr. Purif*., **32**, 309–316 (2003).
[32] Hsieh, H.Y. and Smith, D. *Clostridium perfringens* alpha-N-acetylgalactosaminidase blood group A2-degrading activity, *Biotechnol. Appl. Biochem*., **37**, 157–163 (2003).
[33] Goldstein, J. Preparation of transfusable red cells by enzymatic conversion, *Prog. Clin. Biol. Res*., **165**, 139–157 (1984).
[34] Goldstein, J. Conversion of ABO blood groups, *Transfus. Med. Rev*., **3**, 206–212 (1989).
[35] Zhu, A., Monahan, C., Wang, Z.K. and Goldstein, J. Expression, purification, and characterization of recombinant alpha-N-acetylgalactosaminidase produced in the yeast *Pichia pastoris*, *Protein Expr. Purif*., **8**, 456–462 (1996).
[36] Falk, P., Hoskins, L.C., Lindstedt, R. *et al*. Deantigenation of human erythrocytes by bacterial glycosidases-evidence for the noninvolvement of medium-sized glycosphingolipids in the *Dolichos biflorus* lectin hemagglutination, *Arch. Biochem. Biophys*., **290**, 312–319 (1991).

[37] Hoskins, L.C., Boulding, E.T. and Larson, G. Purification and characterization of blood group A-degrading isoforms of alpha-N-acetylgalactosaminidase from *Ruminococcus torques* strain IX-70, *J. Biol. Chem*., **272**, 7932–7939 (1997).

[38] Izumi, K., Yamamoto, K., Tochikura, T. and Hirabayashi, Y. Serological study using alpha-N-acetylgalactosaminidase from *Acremonium sp*., *Biochim. Biophys. Acta*, **1116**, 72–74 (1992).

[39] Bakunina, I.Y., Kuhlmann, R.A., Likhosherstov, L.M. *et al*. Alpha-N-acetylgalactosaminidase from marine bacterium *Arenibacter latericius* KMM 426T removing blood type specificity of A-erythrocytes, *Biochemistry (Mosc)*, **67**, 689–695 (2002).

[40] Olsson, M.L. New developments in immunohaematology, *Vox Sang*., **87**(Suppl 2), 66–71 (2004).

[41] Sulzenbacher, G., Roig-Zamboni, V., Peumans, W.J. *et al*. Crystal structure of the GalNAc/Gal-specific agglutinin from the phytopathogenic ascomycete *Sclerotinia sclerotiorum* reveals novel adaptation of a beta-trefoil domain, *J. Mol. Biol*., **400**, 715–723 (2010).

[42] Payne, R.W., Murphy, B.M. and Manning, M.C. Product development issues for PEGylated proteins, *Pharm. Dev. Technol*., published online (DOI: 10.3109/10837450.2010.513990) (2010).

[43] Jevsevar, S., Kunstelj, M. and Porekar, V.G. PEGylation of therapeutic proteins, *Biotechnol. J*., **5**, 113–128 (2010).

[44] Xu, L. and Anchordoquy, T. Drug delivery trends in clinical trials and translational medicine: Challenges and opportunities in the delivery of nucleic acid-based therapeutics, *J. Pharm. Sci*., **100**, 38–52 (2011).

[45] Scott, M.D. and Chen, A.M. Beyond the red cell: pegylation of other blood cells and tissues, *Transfus. Clin. Biol*., **11**, 40–46 (2004).

[46] Sirchia, G., Zanella, A., Parravicini, A. *et al*. Red cell alloantibodies in thalassemia major. Results of an Italian cooperative study, *Transfusion*, **25**, 110–112 (1985).

[47] Bradley, A.J., Murad, K.L., Regan, K.L. and Scott, M.D. Biophysical consequences of linker chemistry and polymer size on stealth erythrocytes: size does matter, *Biochim. Biophys. Acta*, **1561**, 147–158 (2002).

[48] Jeong S.T. and Byun, S.M. Decreased agglutinability of methoxy-polyethylene glycol attached red blood cells: significance as a blood substitute, *Artif. Cells Blood Substit. Immobil. Biotechnol*., **24**, 503–511 (1996).

[49] Scott, M.D., Murad, K.L., Koumpouras, F. *et al*. Chemical camouflage of antigenic determinants: stealth erythrocytes, *Proc. Natl. Acad. Sci. U S A*, **94**, 7566–7571 (1997).

[50] Nacharaju, P., Boctor, F.N., Manjula, B.N. and Acharya, S.A. Surface decoration of red blood cells with maleimidophenyl-polyethylene glycol facilitated by thiolation with iminothiolane: an approach to mask A, B, and D antigens to generate universal red blood cells, *Transfusion*, **45**, 374–383 (2005).

[51] Bradley, A.J., Test, S.T., Murad, K.L. *et al*. Interactions of IgM ABO antibodies and complement with methoxy-PEG-modified human RBCs, *Transfusion*, **41**, 1225–1233 (2001).

[52] Tan, Y., Qiu, Y., Xu, H. *et al*. Decreased immunorejection in unmatched blood transfusions by attachment of methoxypolyethylene glycol on human red blood cells and the effect on D antigen, *Transfusion*, **46**, 2122–2127 (2006).
[53] Murad, K.L., Mahany, K.L., Brugnara, C. *et al*. Structural and functional consequences of antigenic modulation of red blood cells with methoxypoly(ethylene glycol), *Blood*, **93**, 2121–2127 (1999).
[54] Chen, P.C.Y., Huang, W., Stassinopoulos, A. and Cheung, A.T.W. Effects of pegylated hamster red blood cells on microcirculation, *Artif. Cells, Blood Substit. Biotechnol*., **36**, 295–309 (2008).
[55] Roberts, M.J., Bentley, M.D. and Harris, J.M. Chemistry for peptide and protein PEGylation, *Adv. Drug Deliv. Rev*., **54**, 459–476 (2002).
[56] Hashemi-Najafabadi, S., Vasheghani-Farahani, E., Shojaosadati, S.A. *et al*. A method to optimize PEG-coating of red blood cells, *Bioconjug. Chem*., **17**, 1288–1293 (2006).
[57] Nacharaju, P., Manjula, B.N. and Acharya, S.A. Thiolation mediated pegylation platform to generate functional universal red blood cells, *Artif. Cells Blood Substit. Immobil. Biotechnol*., **35**, 107–118 (2007).
[58] Rossi, N.A.A., Constantinescu, I., Brooks, D.E. *et al*. Enhanced cell surface polymer grafting in concentrated and nonreactive aqueous polymer solutions, *J. Am. Chem. Soc*., **132**, 3423–3430 (2010).

21

Allosteric Effectors of Hemoglobin: Past, Present and Future

Martin K. Safo[1] *and Stefano Bruno*[2]

[1]*Department of Medicinal Chemistry, School of Pharmacy and Institute for Structural Biology and Drug Discovery, Virginia Commonwealth University, Richmond, VA, USA*

[2]*Department of Biochemistry and Molecular Biology, University of Parma, Parma, Italy*

21.1 Introduction

Hemoglobin (Hb) remains the most studied and understood example of an allosteric protein. It exists in equilibrium between a tense or T state (unliganded or deoxygenated Hb) and a relaxed or R state (liganded or oxygenated Hb). The atomic structural determination of Hb by Perutz and colleagues was not only one of the first three-dimensional atomic-level descriptions of a protein [1, 2] but, just as importantly, provided a firm foundation to unravel the function of all allosteric proteins. It has been said that the determination of the structure of Hb has the same special significance in protein chemistry as the description of the hydrogen atom in quantum mechanics. The crystal structures of the classical T and R states are composed of two αβ dimers arranged around a twofold axis of symmetry, resulting in a central water cavity. The α- and β-clefts define the two entry points into the central water cavity. The allosteric transition between the two states results in the rotation and translation of the $\alpha_1\beta_1$ dimer by $\sim$14° and $\sim$1 Å relative to the $\alpha_2\beta_2$ dimer [2]. This movement results in several notable differences between the two structures, including, but not limited to, the formation of a larger central water cavity

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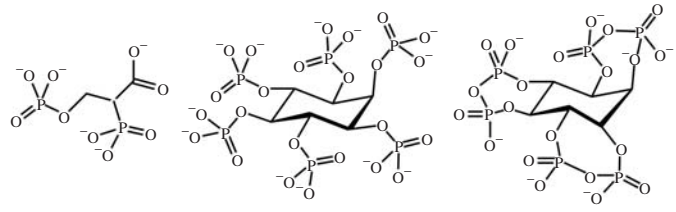

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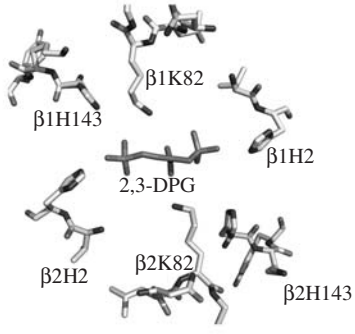

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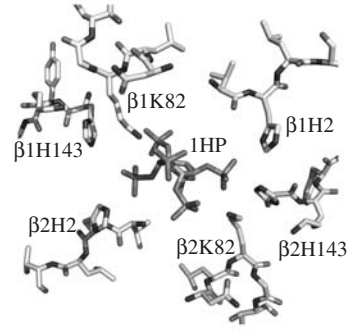

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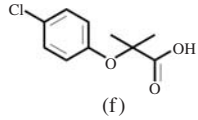

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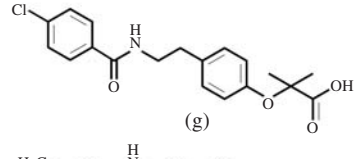

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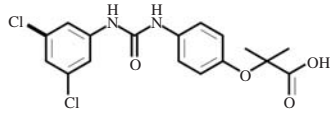

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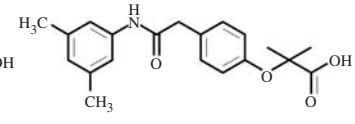

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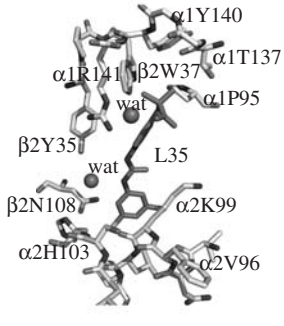

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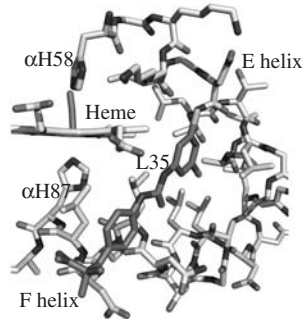

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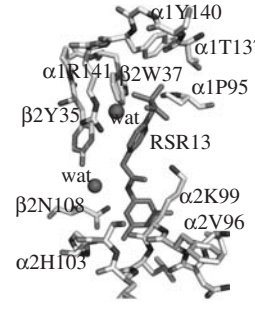


(l)

in the T state with respect to the R state; the breakage of several T-state interdimer hydrogen-bond and/or salt-bridge interactions; and the change in position of several αFG corner and αG helix residues, as well as of the C-terminal residue αArg141.

The classical T- and R-state Hb structures were used to formulate the two-state MWC model for the mechanism of allosteric proteins [3]. An alternative mechanism, known as the KNF model and refined by Max Perutz, was later proposed, also assuming the two-state classical T and R states [4–7]. Nevertheless, several functional and structural studies suggest that the R state of Hb is an ensemble of relaxed states with distinct quaternary structures, including but not limited to the classical R state, the R2 state, the RR2 state, and the R3 state [8–16]. The quaternary differences between some of these relaxed-state structures are as large, and in some cases even larger, than those between the T state and the classical R state [14, 16]. The relaxed structures also show significant differences in the central water cavities, interdimer interfaces, and the α- and β-clefts [14, 16]. A recent study has suggested that the different relaxed states may be uniquely involved in oxygen uptake, transport, and release [16].

The allosteric equilibrium of Hb can be modulated by effectors. The stabilization of the relaxed state shifts the oxygen equilibrium curve (OEC) to the left, producing a high-affinity Hb that more readily binds and holds oxygen. A shift toward the T state (right-shift) produces a low-affinity Hb that readily releases oxygen. The degree of shift in the OEC is reported as an increase or decrease in p50 (oxygen tension at 50% Hb O_2 saturation). The first allosteric effector of human hemoglobin (HbA) discovered was the natural effector 2,3-diphosphoglyceric acid (2,3-DPG; Figure 21.1a) [17]. Preferential binding of 2,3-DPG at the entrance of the larger β-cleft of the central water cavity in the T state leads to additional stabilization of this allosteric form relative to the R state, further decreasing the affinity of T-state Hb for oxygen and increasing tissue oxygenation [18]. A number of synthetic effectors have also been found to bind at the α- or β-cleft or the middle of the central water cavity and to shift the OEC either to the left or to the right. These compounds have long been recognized as potential therapeutic agents for the treatment of a variety of conditions for which a transient increase in oxygen delivery to tissues or an increase in the oxygen affinity of Hb is beneficial.

Figure 21.1 *(a–c) Structures of organic phosphates. (d) Binding of 2,3-DPG at the β-cleft of unliganded (T-state) Hb, making hydrogen-bond interactions with residues from both the β1- and β2-subunits (unpublished coordinate data, M.K. Safo). (e) Binding of IHP at the β-cleft of liganded (R-state) Hb, making interactions with both the β1- and β2-subunits (pdb code 3HXN). (f–i) Structures of right-shifting aromatic propionates. (j) Binding of L35 at the central water cavity of unliganded (T-state) Hb, making hydrogen-bond and hydrophobic interactions with residues from the α1-, α2-, and β2-subunits (pdb code 2D5Z). Water molecules are shown as spheres. The other L35 molecule (not shown) makes symmetry-related interactions with the protein. (k) Binding of L35 close to the α-heme pocket of liganded (R-state) Hb (pdb code 2D5X). (l) Binding of Efaproxiral at the central water cavity of unliganded (T-state) Hb, making hydrogen-bond and hydrophobic interactions with residues from the α1-, α2-, and β2-subunits (pdb code 1G9V). Water molecules are shown as spheres. The other Efaproxiral molecule (not shown) makes symmetry-related interactions with the protein. Note that Efaproxiral makes a unique hydrogen-bond interaction with αLys99, which is absent in the L35 complex.*

This chapter focuses on the chemistry and mechanism of the naturally occurring and synthetic small molecules that alter Hb allosteric properties, including organic phosphates, aspirin derivatives, bezafibrate derivatives, and aromatic aldehydes. These allosteric effectors serve both as tools to unravel the details of the allosteric transitions at the molecular level and as potential therapeutic agents for the treatment of hypoxic diseases, trauma, stroke, sickle cell anemia, and for the prevention and treatment of various cancers. Our purpose is to review the current status of allosteric inhibitors with the hope that further studies will add to the existing understanding, which may be useful in medicine and extrapolation to other allosteric systems.

21.2 Natural and Synthetic Allosteric Effectors

21.2.1 Organic Phosphates

Several organic phosphates, including natural and synthetic compounds (Figure 21.1a–c), are known to affect the oxygen affinity of Hb. In humans, as in most mammals, 2,3-DPG (Figure 21.1a) is the endogenous allosteric effector, forming a stoichiometric complex with Hb. The compound was first reported in 1967 by Reinhold and Ruth Benesch [17], and its mechanism of action and binding to HbA was subsequently determined by Arnone [18]. The structure of deoxygenated Hb in complex with 2,3-DPG (Figure 21.1d) shows the effector binding snuggly at the β-cleft (on the twofold axis) via salt bridges that tie the two β-subunits together, making it difficult for the T–R transition to take place. Even though 2,3-DPG is known to bind to liganded Hb, the significantly smaller β-cleft precludes an equally strong interaction. The preferential binding of 2,3-DPG to deoxygenated Hb stabilizes the T state relative to the R state and decreases the affinity of Hb for oxygen, inducing a right-shift of the Hb oxygen-binding curve, with the p50 increasing from 12 to 26 Torr at physiological temperature and pH. 2,3-DPG modulates the intrinsic affinity of the T and R quaternary states [10, 19] and affects the tetramer stability in both conformations [19]. It also binds free αβ dimers [20]. The highly charged groups on 2,3-DPG preclude this compound from being used therapeutically, as it does not penetrate into red blood cells (RBCs).

Among the structural analogs of 2,3-DPG, inositol hexaphosphate (IHP, Figure 21.1b) has been extensively employed in the investigation of the allosteric properties of Hb because of the strength of its complex, 1000 times more stable than that with 2,3-DPG [21, 22]. However, its therapeutic applications are prevented by its poor pharmacokinetic properties and, particularly, by its inability to cross the cell membranes of erythrocytes. For experimental purposes, IHP was incorporated into erythrocytes by electroporation or other *ex vivo* physical methods, allowing for a lasting modulation of the p50 of intracellular Hb [23, 24] and resulting in a higher oxygen release to tissues when IHP-containing erythrocytes are reinfused. *In vitro* sickling of erythrocytes from patients suffering from sickle cell anemia is reduced at the low oxygen pressures typical of microcirculation by addition of normal erythrocytes containing IHP [25]. This effect was associated to a transfer of oxygen from the IHP-containing, low-affinity erythrocytes to sickle cell erythrocytes, which therefore are never exposed to the low oxygen pressures at which sickle Hb undergoes polymerization. The crystal structure of IHP in complex with the classical R-state Hb has recently been deposited at the RCSB protein databank

(PDB code 3HXN) and shows IHP bound at the β-cleft (Figure 21.1e), as described above for 2,3-DPG binding to T-state Hb.

Unlike IHP, the recently discovered allosteric effector myo-inositol trispyrophosphate (ITPP, Figure 21.1c) is capable of crossing the membrane of erythrocytes, inducing a rightward shift of the blood oxygenation curve upon *in vivo* administration [26]. By increasing the oxygenation of the tissues, ITPP was suggested to be effective in preventing angiogenesis in solid tumors, a process triggered by hypoxia and associated to tumor growth [27]. ITPP was shown to enhance the exercise capacity in a murine model of heart failure [28].

21.2.2 Synthetic Aromatic Propionate Right-shifters

The discovery of 2,3-DPG prompted the search for synthetic Hb effectors that could be used to gain insight into Hb function, as well as potentially for therapeutic purposes, culminating in the discovery of several aromatic propionates (Figure 21.1f–i), among other classes of compounds. In the early 1980s, the antilipidemic drug clofibrate (CFA; Figure 21.1f) was tested and found to be an excellent antigelling agent for possible treatment of sickle cell anemia. Surprisingly, CFA right-shifted the oxygen curve toward a low-affinity Hb [29]. A low-resolution crystal structure of CFA with T-state Hb shows four molecules of CFA bound at two symmetry-related binding sites located in the central water cavity 20 Å away from the β-cleft binding site of 2,3-DPG and other organic phosphates [29]. The binding seems to tie the two dimers together, stabilizing the T state relative to the R state. CFA was tested by radiation oncologists after its discovery.

This discovery led to the testing of bezafibrate (Figure 21.1g), another aromatic propionate antilipidemic agent that exhibited a greater effect in right-shifting the oxygen-binding curve toward the T state [30]. A low-resolution structure of bezafibrate in complex with deoxygenated Hb shows the ligand bound to Hb in a 2 : 1 ratio, each molecule spanning two CFA binding sites to make interactions with several residues from two α-subunits and one β-subunit of the protein [31]. Bezafibrate analogs with much greater potency were next synthesized, specifically 2-[4-(3,5-dichlorophenylureido) phenoxy]-2-methylpropionic acid (L35; Figure 21.1h) [32]. L35 also binds Hb dimers and modulates the oxygen-binding properties of both the T and R states [32–34]. A high-resolution crystal structure of L35 in complex with T-state Hb (Figure 21.1j) shows similar binding interactions to bezafibrate [33]. Specifically, the methylpropionic acid moiety fits into a cavity surrounded by the residues αThr134, αPro95, αThr137, αTyr140, αArg141, and βTrp37 to make both hydrophobic and water-mediated interactions between the carboxylate moiety and the guanidinium group of αArg141. The phenoxy ring is engaged in hydrophobic contacts with αLys99, αArg141, βTyr35, and βTrp37. The chlorobenzene ring binds in a narrow hydrophobic pocket composed of the following FG-corner and G-helix residues: αVal96, αLys99, αLeu100, αHis103, and βAsn108. These interactions, which are also observed in the bezafibrate-deoxy-Hb structure, stabilize the T state by constraining movements of several of the above residues, which are known to shift significantly during the T–R transition.

Interestingly, the crystal structures of both L35 and BZF in complex with the classical R-state Hb show two symmetrically bound L35s or BZFs at the surface of the α-subunits, distinct from their T-state central water cavity binding site (Figure 21.1k) [33–35]. The bound molecules are located near the α-subunit E helix, with close contacts with the

heme. The mode of binding has resulted in the distal residue His58 (E7) moving further toward the Fe position, increasing steric hindrance between the bound ligand and the Fe and causing the observed decrease in liganded Hb affinity for ligand [35]. Unlike the organic phosphates, bezafibrate and L35 can cross cell membranes, thus potentially allowing their employment in therapy. Moreover, as these compounds bind to a different site from 2,3-DPG, they act synergistically [36]. Unfortunately, neither bezafibrate nor L35 is useful as a potential therapeutic agent due to their strong affinity for serum albumin, preventing their transport into RBCs.

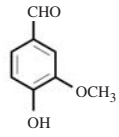
Efaproxiral, or RSR13 (licensed to Allos Therapeutics Inc., Westminster, CO, USA), is another bezafibrate derivative with strong allosteric properties *in vitro* and *in vivo* (Figure 21.1i). Structurally, it differs from bezafibrate in a reduction in the linking atoms of the phenyl ring from four to three. It also differs from both L35 and bezafibrate in the positions of the amide linking atoms, as well as in having one less amide nitrogen of the urea link exhibited in L35, which decreases its binding property to serum albumin. Efaproxiral binds to the same site as bezafibrate or L35, making similar protein interactions (Figure 21.1l) [37]. In addition, Efaproxiral makes a unique hydrogen-bond interaction between its carbonyl oxygen and the side-chain amino group of αLys99 (Figure 21.1l) [37]. In effect, it fits the binding cavity better than bezafibrate and L35. Efaproxiral was shown to have promise for several hypoxic diseases, including stroke, wound healing, and oxygen deprivation during exercise, and emerged as a candidate for several clinical applications, particularly as a radiation enhancer in the radiotherapy of hypoxic tumors [38–46]. The radiation oncologists have for decades believed that oxygenating tumors during radiation will enhance tumor death. In early *in vivo* experiments, Efaproxiral was shown to increase the release of oxygen to tissues [38, 39] and to induce hemodynamic changes associated with higher concentrations of circulating oxygen [40]. Following several promising phase II studies for various medical implications, it was tested in clinical trials as a radiation-sensitizing agent associated with radiotherapy of solid tumors. The Radiation Enhancing Allosteric Compound for Hypoxic Brain Metastases (REACH) phase III study tested the association of Efaproxiral with oxygen in whole-brain radiation therapy (WBRT) [41]. A concentration-dependent survival improvement was observed and the dosing requirements for efficacy were established [42]. Efaproxiral was also tested in a multicentre, randomized phase III clinical trial as an adjunct to WBRT in the treatment of brain metastases originating from breast carcinoma [43, 44]. An extended survival and an improved quality of life were observed [45]. However, a subsequent meta-analysis seemed to refute the efficacy of Efaproxiral and other radiation-sensitizing agents in the treatment of brain metastases [46]. Despite the focus on its radio-sensitizing activity, a possible application in the treatment of chronic heart failure has also been proposed [47]. Several structural modifications have been made to Efaproxiral, culminating in the discovery of dozens of other right-shifters [48–51]. However, none of these compounds have generated the same attention as Efaproxiral.

21.2.3 Aromatic Aldehyde Left-shifters

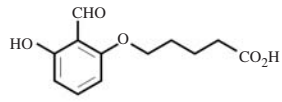
Several aromatic aldehydes are known to form Schiff-base interactions with the terminal amino groups of the two symmetry-related αVal1s of Hb and increase the protein affinity

for oxygen [15, 52–57]. A series of aspirin and furan aldehydes that left-shift the OEC have been investigated as potential treatments of sickle cell anemia (Figure 21.2a–c). These effectors include vanillin (Figure 21.2a) and tucaresol or 12C79 (Figure 21.2b), which have been studied for their antisickling activities [52, 53, 56, 57]. Binding of these compounds to Hb shifts the allosteric equilibrium to the relaxed state, increasing the high-O_2-affinity Hb fraction. The structural basis for the left-shifting effect of these aldehydes was first elucidated by Abraham *et al*. with the determination of deoxygenated Hb structure in complex with 12C79 [53]. A pair of symmetry-related 12C79s was observed at the α-cleft, forming a Schiff-based interaction with the two αVal1 N-terminal amino groups (Figure 21.2d). The authors noted that 12C79 binding did not lead to any additional intersubunit interaction, but rather to the disruption of a native water-mediated bridge between the αVal1 and αArg141 of the opposite α-subunit. The result is destabilization of the T state relative to the R state, and a left-shift of the OEC. A previous modeling study has suggested that the observed left-shifting property of 12C79 was due to a single 12C79 crosslinking the two symmetry-related αVal1s via a Schiff-based interaction between the aldehyde and the nitrogen of one of the αVal1s, and a hydrogen-bond interaction between the carboxylate and the other Val1 nitrogen, with a concomitant stabilization of the R state [56]. It was not until several years later that it was discovered that these left-shifting aldehydic agents bind to both liganded and unliganded Hb, stabilizing the former and destabilizing the latter [15]. Both vanillin and 12C79 have been tested clinically. However, the large doses of compounds needed to elicit therapeutic *in vivo* antisickling effects were not clinically acceptable, resulting in 12C79's case in side effects. Moreover, vanillin was poorly orally bioavailable.

In a recent discovery, 5-hydroxymethyl-2-furfural (5HMF; Figure 21.2c), a decomposition product of sugars and other furfural analogs, was remarkably found to be a stronger left-shifter than other known aldehydes [15, 54]. A single oral dose of 100 mg/kg of 5HMF was shown to be sufficient to protect transgenic sickle mice from death by acute pulmonary sequestration of sickle cells under hypoxic conditions [54], while chronic administration of up to 375 mg/kg/day of 5HMF for 2 years was nontoxic to rats and mice (NTP report, 2008). Unlike vanillin, 5HMF is orally bioavailable and highly specific for Hb, and plasma proteins do not inhibit its binding to intracellular Hb [54]. Crystallographic analysis of Hb in complex with 5HMF, as well as other furfurals, shows the compounds to bind at the α-cleft in a symmetry-related fashion, making a Schiff-base interaction with the N-terminal αVal1 amino group, as observed for 12C79 [15]. Binding to unliganded Hb did not add to the constraint of the T state, but rather destabilized it, as described above for 12C79. On the other hand, binding to liganded Hb led to stabilization of the relaxed state (in the form of R2 state), with intricate direct and water-mediated compound–Hb interactions that tie the two α-subunits together (Figure 21.2e) [15]. A follow-up study with vanillin and several of its pyridyl derivatives showed similar crystallographic results, suggesting that the left-shifting effect of these aldehydes is due to stabilization and destabilization of liganded and unliganded Hb, respectively (Safo, unreported data). Preclinical and phase I clinical antisickling studies of 5HMF by AesRx Inc. in conjunction with NIH are planned for the treatment of sickle cell anemia.



(a)



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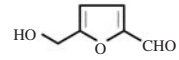

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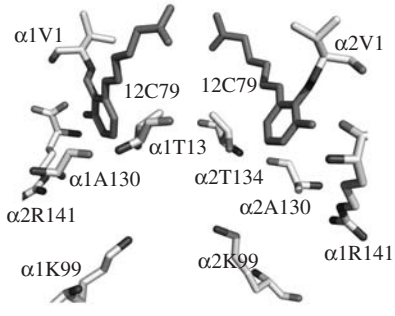
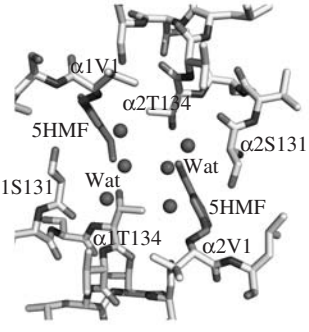
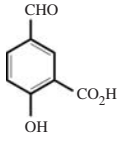


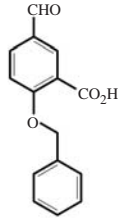
(d)



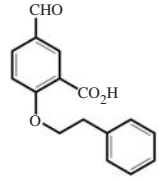
(e)



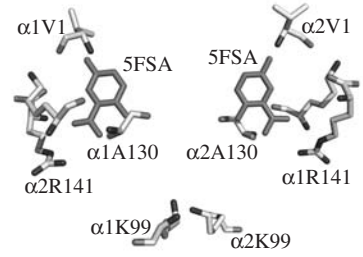
(f)



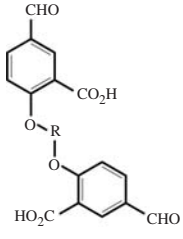
(g)



(h)

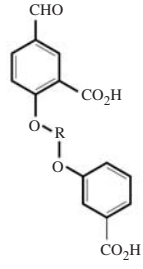


(i)



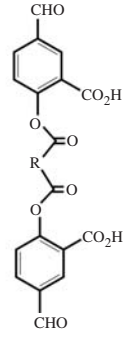
R = $(CH_2)n$; n = 1–10
or
R = meta $CH_2(C_6H_4)CH_2$
R = ortho $CH_2(C_6H_4)CH_2$
R = para $CH_2(C_6H_4)CH_2$
R = $CH_2CH{=}CHCH_2$

(j)



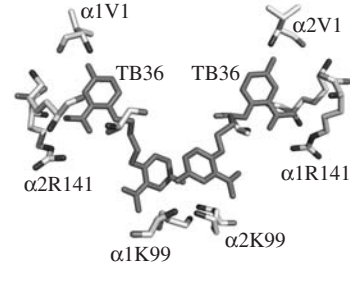
R = $(CH_2)n$; n = 1–5

(k)



R = $(CH_2)n$; n = 1–2
or
R = CH=CH
R = para C_6H_4

(l)



(m)

21.3 Molecular Mechanism of Action of Allosteric Effectors

21.3.1 Oxygen Binding Curve and Hb Structural Changes

As shown by Perutz, the low oxygen affinity of T-state Hb is due to a strain at the F helix that causes the proximal histidine to move the iron atom from the plane of the porphyrin ring, as well as to steric hindrance to ligand binding by the distal histidine, especially at the β-hemes [1, 5–7]. Binding of oxygen, first to the less sterically hindered α-heme, facilitates movement of the iron closer to the porphyrin plane in the α-heme and increase uptake of oxygen at the α-heme. This causes the αF helix and αFG corner to move toward and communicate with the opposite subunit, leading to a sequential communication between the four subunits. The effect is a sequential decrease in steric strain and/or steric hindrance and increase in oxygen uptake at the four hemes. Thus, the binding of oxygen is a cooperative process, with the binding of the first oxygen influencing the oxygen-binding properties of the other subunits. The result is a sigmoidal oxygen-binding curve.

Allosteric effectors have the ability to freeze the movements of the αF helix and αFG corner (as well as other parts of the Hb) in either direction, thus stabilizing one state relative to the other. These effectors are capable of binding to either liganded or unliganded Hb, or to both Hb forms, and the direction of the allosteric shift is dependent on preferential stabilization of one state over the other. The effectors may "freeze" one state relative to the other with several intricate hydrogen-bond and hydrophobic interactions that prevent the rotation and translation associated with the allosteric transition and/or "unlock" the other state by removing intersubunit interactions, which facilitates the allosteric movement. So far the left-shifting agents discovered are those compounds that are capable of forming a covalent adduct with the N-terminal αVal1 nitrogen to add further constraint to the relaxed state, while right-shifting agents are noncovalent compounds that bind either at the middle of the central water cavity or at the β-cleft and add constraint to the T state. As will be shown in Sections 21.3.2 and 21.3.3, covalent binders at the α-cleft and the central water cavity are also capable of right-shifting the OEC.

Figure 21.2 *(a–c) Structures of left-shifting aromatic aldehydes. (d) Binding of a pair of 12C79 molecules at the α-cleft of unliganded (T-state) Hb, making interactions with the α1- and α2-subunits (unpublished coordinate data, D.J. Abraham; see [53]). [59]) (e) Binding of a pair of 5HMF molecules at the α-cleft of liganded (R2-state) Hb, making interactions with the α1- and α2-subunits (pdb code 1QXE). Water molecules are shown as spheres and mediate intricate hydrogen-bond interactions between the two 5HMF and the two α-subunits. (f–h) Structures of mono-acid aldehyde right-shifters. (i) Binding of a pair of 5FSA molecules at the α-cleft of unliganded (T-state) Hb, making interactions with the α1- and α2-subunits (unpublished coordinate data, M.K. Safo and D.J. Abraham; see [58]). (j–l) Structures of bis-acid aldehyde right-shifters. (m) Binding of a pair of TB36 ((j), n = 3) molecules at the α-cleft of unliganded (T-state) Hb, crosslinking the α1- and α2-subunit via covalent linkage with α Val1 and the opposite α Lys99 amines (unpublished coordinate data, M.K. Safo and D.J. Abraham; see [59]).*

21.3.2 How Allosteric Effectors can Bind to the Same Site and Have Opposite Allosteric Properties

A group of mono-acid aldehyde right-shifters (Figure 21.2f–h) was discovered, interestingly, in the attempt to design more potent left-shifting aldehyde antisickling agents [58]. For example, some aldehydes originally designed to be used as left-shifters, such as 5-formylsalicylic acid (5FSA; Figure 21.2f), 2-(benzyloxy)-5-formylbenzoic acid (2BF; Figure 21.2g), and 2-(phenylethyloxy)-5-formylbenzoic acid (2PEF; Figure 21.2h), were shown to behave instead as right shifters [58], similarly to 2,3-DPG and bezafibrate. These aldehyde-based right-shifters, like the left-shifters, such as 5HMF and 12C79, also bind at the α-cleft of deoxygenated Hb to form a Schiff base with the terminal amino groups of the two symmetry-related αVal1s (Figure 21.2i). The surprising opposite effects produced by structurally related compounds binding to the same residue were explained as a different interaction with αArg141, which as described above linked to the αVal1 of the opposite α-subunit via a water molecule in native T-state Hb [58]. The carboxylate moiety that characterizes the right-shifting aromatic aldehydes replaces this water-mediated bond by forming an ionic bridge with the positively charged guanidinium group of αArg141 (Figure 21.2i). As this bond is stronger than the water-mediated one, it results in an increase in the stability of the T state. The left-shifting aldehydes do not possess a carboxylate group, or else the group is present but not correctly oriented to form an interaction with αArg141, and thus these compounds do not contribute to stabilization of the T state but rather to its destabilization.

21.3.3 Decreasing Subunit Mobility and Changes in Allosteric Properties: Molecular Ratchets

The structural studies of the mono-acid aldehydes described above with deoxygenated Hb showed the side chains of these compounds pointing toward the αLys99 of the opposite α-subunit (Figure 21.2i), and it was foreseen that the addition of a terminal group to these compounds, capable of forming an ionic or covalent bond with the αLys99, might result in a further stabilization of the T state. These compounds were shown, as predicted, to strongly bind Hb. Several series of mono-aldehyde bis-acids and bis-aldehyde bis-acids with varying chain lengths were synthesized (Figure 21.2j–l) and evaluated as allosteric effectors of Hb. Among the compounds capable of binding αLys99, the bis-aldehyde bis-acid allosteric effectors are stronger than the mono-aldehyde bis-acids [59]. It was shown by both functional and crystallographic studies that the bis-aldehydes formed Schiff-base interactions exclusively between the αVal1 and αLys99 of the opposite α chain, even though the two terminal αVal nitrogens are ideally spaced to also form crosslinks (Figure 21.2m). The mono-aldehyde bis-acids also formed Schiff-base interactions with αVall, with the acid group making ionic interaction with the αLys99.

The influence of chain direction was set by key substitutions on the bis-aldehyde molecule. These studies support the general conclusion that long, flexible molecules prefer to bind along cavity walls, like double-sided molecular sticky tape, rather than span large open spaces with few chances for interaction. A significant decrease in oxygen affinity was observed as the dimer subunits were linked with shorter chain lengths. The chain length acts as a molecular ratchet and dictates the degree of allosteric effect observed. The shorter the crosslink, the greater the constraint on the T state and the

stronger the allosteric effect that is produced. Nonetheless, several of these compounds, including the potent ones, suffer from their inability to cross the cell membrane. As noted above for Efaproxiral binding to deoxy-Hb, the importance of αLys99 on the allosteric equilibrium was confirmed.

21.4 The First Visualization of an Important Pharmacological Theory via Hb Allosteric Effector Binding

Hb allosteric effector studies have also helped form our understanding of the concept of intrinsic activity, which has had a long history in our understanding of pharmacological activity for various receptors. In the simplest case, the biological response to a drug is proportional to the amount of drug bound. However, the biological response is also mediated by the ability of a drug when bound to exert its maximum effectiveness. This effectiveness is termed the "intrinsic activity". Detailed oxygen-binding experiments combined with X-ray crystallographic studies on allosteric effectors of Hb demonstrated that certain Hb allosteric effectors bind at the same site in Hb with similar binding constants, yet shift the allosteric equilibrium and the oxygen affinity of Hb by different degrees or in opposite directions. Thus, some of the effectors with similar binding affinities for the same site exhibited varying degrees of effectiveness; that is, they possessed different intrinsic activities. These results afforded the first understanding at the molecular level of the concept of intrinsic activity. For example, the 3,5-disubstitution of methyl- or chloro- groups to the terminal phenyl ring in Efaproxiral and its analogs always caused one of the groups to be inserted into the G helix, preventing the rotation that occurs when Hb attempts the transition from the T to the R state (Figure 21.1j,i). It has been observed that different atom substitutions at the 3,5-position result in subtle but significant effects on the locking of the G helix in the T-state conformation, expressed in differences in right-shifting potencies.

21.5 The Clinical Importance of Hemoglobin Allosteric Effectors

Since the discovery of the natural Hb allosteric effector 2,3-DPG, there have been ongoing efforts to develop Hb-based drugs to treat several diseases using the allosteric properties of Hb. A number of synthetic effectors have been found which bind at the α- or β-cleft or the middle of the central water cavity to shift the OEC to the right. These compounds are potential therapeutic agents for the treatment of a variety of conditions for which a transient increase in oxygen delivery to tissues is beneficial. Since only 25% of Hb-bound oxygen is released to tissues in each circulation cycle, a pharmacologically induced increase in p50 translates into a significantly higher oxygen delivery, provided that the affinity remains high enough to allow oxygen saturation in the lungs. Such an approach has been particularly envisaged for the treatment of ischemic conditions or to sensitize solid tumors to oxygen-sensitive radiotherapy and chemotherapy, as exemplified by Efaproxiral or RSR13 [38–47, 60, 61]. As a matter of fact, local hypoxia in solid tumors is a widely accepted cause for a reduced sensitivity to radiotherapy, as oxygen is a key intermediate in the free radical-mediated DNA damage promoted by ionizing radiations.

At the other end of the spectrum, other synthetic and natural compounds have also been shown to bind in a covalent fashion to Hb, shifting the allosteric equilibrium to the left. Such compounds produce a high-O_2-affinity Hb to increase the fraction of the relaxed states. These compounds have been clinically evaluated as antisickling agents for treatment of sickle cell anemia, as high-O_2-affinity sickle Hb does not form polymers [52–57]. Fully-oxygenated RBCs typically release some oxygen in larger vessels, less at the arterioles, and most at the capillaries. This process is influenced by Hb oxygen affinity and oxygen sensors at the arterioles. In sickle-cell-anemia patients, because of the (possibly compensatory) low oxygen affinity, more oxygen is released at the arteries and arterioles. Regulatory vasoconstriction occurs at the arterioles to counter this process, which leads to cell sickling. However, in the presence of left-shifting compounds that increase Hb oxygen affinity, less oxygen should be released at the larger vessels, leading to increased availability of oxygen at the capillaries. Further release of oxygen at this level should not deoxygenate the cells sufficiently for sickling to occur, making the use of these compounds viable for sickle-cell-anemia treatment. A promising left-shifting and antisickling compound, 5HMF, is currently being studied for its toxicity profile, and phase I/II clinical trials are planned.

References

[1] Perutz, M. F. Stereochemistry of cooperative effects in hemoglobin, *Nature*, **228**, 726–734 (1970).
[2] Baldwin, J. and Chothia, C. Haemoglobin, the structural changes related to ligand binding and its allosteric mechanism, *J. Mol. Biol*., **129**, 175–220 (1979).
[3] Monod, J., Wyman, J. and Changeux J.P. On the nature of allosteric transitions: a plausible model, *J. Mol. Biol*., **12**, 88–118 (1965).
[4] Koshland, D. E. Jr, Némethy, G. and Filmer, D. Comparison of experimental binding data and theoretical 30 models in proteins containing subunits, *Biochemistry*, **5**, 365–385 (1966).
[5] Perutz, M. F. Nature of haem–haem interaction, *Nature*, **237**, 459–499 (1972).
[6] Perutz, M. F. Mechanisms of cooperativity and allosteric regulation in proteins, *Q. Rev. Biophys*., **22**, 139–237 (1989).
[7] Perutz, M. F., Wilkinson A. J., Paoli, M. and Dodson, G. G. The stereochemical mechanism of the cooperative effects in hemoglobin revisited, *Annu. Rev. Biophys. Biomol. Struct*., **27**, 1–34 (1998).
[8] Lukin, J. A., Kontaxis, G., Simplaceanu, V. *et al*. Quaternary structure of hemoglobin in solution, *Proc. Natl. Acad. Sci. U S A*, **100**, 517–520 (2003).
[9] Sawicki, C. A. and Gibson, Q. H. Quaternary conformational changes in human hemoglobin studied by laser photolysis of carboxyhemoglobin, *J. Biol. Chem*., **251**, 1533–1542 (1976).
[10] Jayaraman, V. and Spiro, T. G. Structure of a third cooperativity state of hemoglobin, ultraviolet resonance Raman Spectroscopy of cyanomethemoglobin ligation microstates, *Biochemistry*, **34**, 4511–4515 (1995).
[11] Perrella, M. and Di Cera, E. CO ligation intermediates and the mechanism of hemoglobin cooperativity, *J. Biol. Chem*., **274**, 2605–2608 (1999).

[12] Silva, M. M., Rogers, P. H. and Arnone, A. A third quaternary structure of human hemoglobin A at 1.7-Å resolution, *J. Biol. Chem*., **267**, 17248–17256 (1992).
[13] Mueser, T. M., Rogers, P. H. and Arnone, A. Interface sliding as illustrated by the multiple quaternary structures of liganded hemoglobin, *Biochemistry*, **39**, 15353–15364 (2000).
[14] Safo, M. K. and Abraham, D. J. The enigma of the liganded hemoglobin end-state, a novel quaternary structure of human carbonmonoxy hemoglobin, *Biochemistry*, **44**, 8347–8359 (2005).
[15] Safo, M. K., Abdulmalik, O., Danso-Danquah, R. *et al*. Structural basis for the potent antisickling effect of a novel class of 5-membered heterocyclic aldehydic compounds, *J. Med. Chem*., **47**, 4665–4676 (2004).
[16] Jenkins, J. D., Musayev, F. N., Danso-Danquah, R. *et al*. Structure of relaxed-state human hemoglobin, insight into ligand uptake, transport and release, *Acta Crystallogr. D*, **65**, 41–48 (2009).
[17] Benesch, R. and Benesch, R. E. The effect of organic phosphates from the human erythrocyte on the allosteric properties of hemoglobin, *Biochem. Biophys. Res. Commun*., **26**, 162–167 (1967).
[18] Arnone, A. X-ray diffraction study of binding of 2,3-diphosphoglycerate to human deoxyhaemoglobin, *Nature*, **237**, 146–149 (1972).
[19] Schay, G., Smeller, L., Tsuneshige, A. *et al*. Allosteric effectors influence the tetramer stability of both R- and T-states of hemoglobin A, *J. Biol. Chem*., **281**, 25972–25983 (2006).
[20] Tsuneshige, A., Kanaori, K., Samuni, U. *et al*. Semihemoglobins, high oxygen affinity dimeric forms of human hemoglobin respond efficiently to allosteric effectors without forming tetramers, *J. Biol. Chem*., **279**, 48959–48967 (2004).
[21] Yonetani, T. and Tsuneshige, A. The global allostery model of hemoglobin, an allosteric mechanism involving homotropic and heterotropic interactions, *C. R. Biologies*, **326**, 523–532 (2003).
[22] Gong, Q., Simplaceanu, V., Lukin, J. A. *et al*. Quaternary structure of carbonmonoxyhemoglobins in solution, structural changes induced by the allosteric effector inositol hexaphosphate, *Biochemistry*, **45**, 5140–5148 (2006).
[23] Teisseire, B., Ropars, C., Villereal, M. C. and Nicolau, C. Long-term physiological effects of enhanced O_2 release by inositol hexaphosphate-loaded erythrocytes, *Proc. Natl. Acad. Sci. U S A*, **84**, 6894–6898 (1987).
[24] Cabrales, P., Tsai, A. G. and Intaglietta, M. Modulation of perfusion and oxygenation by red blood cell oxygen affinity during acute anemia, *Am. J. Resp. Cell Mol*., **38**, 354–361 (2008).
[25] Bourgeaux, V., Hequet, O., Campion, Y. *et al*. Inositol hexaphosphate-loaded red blood cells prevent *in vitro* sickling, *Transfusion*, **50**, 2176–2184 (2010).
[26] Fylaktakidou, K. C., Lehn, J. M., Greferath, R. and Nicolau, C. Inositol tripyrophosphate, a new membrane permeant allosteric effector of haemoglobin, *Bioorg. Med. Chem. Lett*., **15**, 1605–1608 (2005).
[27] Kieda, C., Greferath, R., Crola da Silva, C. *et al*. Suppression of hypoxia-induced HIF-1alpha and of angiogenesis in endothelial cells by myo-inositol trispyrophosphate-treated erythrocytes, *Proc. Natl. Acad. Sci. U S A*, **103**, 15576–15581 (2006).

[28] Biolo, A., Greferath, R., Siwik, D. A. *et al*. Enhanced exercise capacity in mice with severe heart failure treated with an allosteric effector of hemoglobin, myo-inositol trispyrophosphate, *Proc. Natl. Acad. Sci. U S A*, **106**, 1926–1929 (2009).

[29] Abraham, D. J., Perutz, M. F. and Phillips, S. E. Physiological and x-ray studies of potential antisickling agents, *Proc. Natl. Acad. Sci. U S A*, **80**, 324–328 (1983).

[30] Perutz, M. F. and Poyart, C. Bezafibrate lowers oxygen affinity of haemoglobin, *Lancet*, **ii**, 881–882 (1983).

[31] Perutz, M. F. and Fermi, G. Hemoglobin as a receptor of drugs and peptides, X-ray studies of the stereochemistry of binding, *J. Am. Chem. Soc*., **108**, 1064–1078 (1986).

[32] Lalezari, I., Lalezari, P., Poyart, C. *et al*. New effectors of human hemoglobin, structure and function, *Biochemistry*, **29**, 1515–1523 (1990).

[33] Yokoyama, T., Neya, S., Tsuneshige, A. *et al*. R state haemoglobin with low oxygen affinity, crystal structures of deoxy human and carbonmonoxy horse haemoglobin bound to the effector molecule L35, *J. Mol. Biol*., **356**, 790–801 (2006).

[34] Chen, Q., Lalezari, I., Nagel, R. L. and Hirsch, R. E. Liganded hemoglobin structural perturbations by the allosteric effector L35, *Biophys. J*., **88**, 2057–2067 (2005).

[35] Shibayama, N., Miura, S., Tame, J. R. *et al*. Crystal structure of horse carbonmonoxyhemoglobin-bezafibrate complex at 1.55-Å resolution: a novel allosteric binding site in R state hemoglobin, *J. Biol. Chem*., **277**, 38791–38796 (2002).

[36] Ascenzi, P., Coletta, M., Desideri, A. *et al*. Effect of bezafibrate and clofibric acid on the spectroscopic properties of the nitric oxide derivative of ferrous human hemoglobin, *J. Inorg. Biochem*., **48**, 47–53 (1992).

[37] Safo, M. K., Moure, C. M., Burnett, J. C. *et al*. High resolution crystal structure of deoxy hemoglobin complexed with a potent allosteric effector, *Protein Sci*., **10**, 951–957 (2001).

[38] Grinberg, O. Y., Miyake, M., Hou, H. *et al*. The dose-dependent effect of RSR13, a synthetic allosteric modifier of hemoglobin, on physiological parameters and brain tissue oxygenation in rats, *Adv. Exp. Med. Biol*., **530**, 287–296 (2003).

[39] Khandelwal, S. R., Randad, R. S., Lin, P. S. *et al*. Enhanced oxygenation in vivo by allosteric inhibitors of hemoglobin saturation, *Am. J. Physiol*., **265**, H1450–H1453 (1993).

[40] Kunert, M. P., Liard, J. F. and Abraham, D. J. RSR13, an allosteric effector of hemoglobin, increases systemic and iliac vascular resistance in rats, *Am. J. Physiol*., **271**, H602–H613 (1996).

[41] Suh, J. H., Stea, B., Nabid, A. *et al*. Phase III study of efaproxiral as an adjunct to whole-brain radiation therapy for brain metastases, *J. Clin. Onc*., **24**, 106–114 (2006).

[42] Stea, B., Suh, J. H., Boyd, A. P. *et al*. Whole-brain radiotherapy with or without efaproxiral for the treatment of brain metastases, determinants of response and its prognostic value for subsequent survival, *Int. J. Radiat. Oncol. Biol. Phys*., **64**, 1023–1030 (2006).

[43] Kaal, E. C. and Vecht, C. J. CNS complications of breast cancer, current and emerging treatment options, *CNS drugs*, **21**, 559–579 (2007).
[44] Peacock, K. H. and Lesser, G. J. Current therapeutic approaches in patients with brain metastases, *Curr. Treat. Options Oncol*., **7**, 479–489 (2006).
[45] Scott, C., Suh, J., Stea, B. *et al*. Improved survival, quality of life, and quality-adjusted survival in breast cancer patients treated with efaproxiral (Efaproxyn) plus whole-brain radiation therapy for brain metastases, *Am. J. Clin. Oncol*., **30**, 580–587 (2007).
[46] Viani, G. A., Borges, G. M., Fonseca, E. C. *et al*. Whole brain radiotherapy with radiosensitizer for brain metastases, a meta-analysis of randomized controlled trials, *Exp. Clin. Canc. Res*., **28**, 1 (2009).
[47] Watanabe, T., Takeda, T., Omiya, S. *et al*. Reduction in hemoglobin-oxygen affinity results in the improvement of exercise capacity in mice with chronic heart failure, *J. Am. Coll. Cardiol*., **52**, 779–786 (2008).
[48] Safo, M. K., Boyiri, T., Burnett, J. C. *et al*. X-ray crystallographic analyses of symmetrical allosteric effectors of hemoglobin, compounds designed to link primary and secondary binding sites, *Acta Crystallogr*., **58**, 634–644 (2002).
[49] Abraham, D. J., Wireko, F. C., Randad, R. S. *et al*. Allosteric modifiers of hemoglobin, 2-[4-[[3,5-dimethylanilino[(carbonyl)methyl]-phenoxy]-2-methylpropionic acid derivatives that lower the oxygen affinity of hemoglobin in red cell suspensions, in whole blood, and in vivo in rats, *Biochemistry*, **31**, 9141–9149 (1992).
[50] Youssef, A. M., Safo, M. K., Danso-Danquah, R. *et al*. Synthesis and X-ray studies of chiral allosteric modifiers of hemoglobin, *J. Med. Chem*., **45**, 1184–1195 (2002).
[51] Phelps Grella, M., Danso-Danquah, R., Safo M. K. *et al*. Synthesis and structure–activity relationships of chiral allosteric modifiers of hemoglobin, *J. Med. Chem*., **43**, 4726–4737 (2000).
[52] Abraham, D. J., Mehanna, A. S., Wireko, F. C. *et al*. Vanillin a potential agent for the treatment of sickle cell anemia, *Blood*, **77**, 1334–1341 (1991).
[53] Wireko, F. C. and Abraham, D. J. X-ray diffraction study of the binding of the antisickling agent 12C79 to human hemoglobin, *Proc. Natl. Acad. Sci. U S A*, **88**, 2209–2211 (1991).
[54] Abdulmalik, O., Safo, M. K., Chen, Q. *et al*. 5-Hydroxymethyl-2-furfural modifies intracellular sickle haemoglobin and inhibits sickling of red blood cells, *Br. J. Haematol*., **128**, 552–561 (2005).
[55] Nnamani, I. N., Joshi, G. S., Danso-Danquah, R. *et al*. Pyridyl derivatives of benzaldehyde as potential antisickling agents, *Chem. Biodivers*., **5**, 1762–1769 (2008).
[56] Beddell, C. R., Goodford, P. J., Kneen, G. *et al*. Substituted benzaldehydes designed to increase the oxygen affinity of human haemoglobin and inhibit the sickling of sickled erythrocytes, *Br. J. Pharmacol*., **82**, 397–407 (1984).
[57] Zaugg, R. H., Walder, J. A. and Klotz, I. M. Schiff-base adducts of hemoglobin: modifications that inhibit erythrocyte sickling, *J. Biol. Chem*., **252**, 8542–8548 (1977).
[58] Abraham, D. J., Safo, M. K., Boyiri, T. *et al*. How allosteric effectors can bind to the same protein residue and produce opposite shifts in the allosteric equilibrium, *Biochemistry*, **34**, 15006–15020 (1995).

[59] Boyiri, T., Safo, M. K., Danso-Danquah, R. E. *et al*. Bisaldehyde allosteric effectors as molecular ratchets and probes, *Biochemistry*, **34**, 15021–15036 (1995).
[60] Rosenberg, A. and Knox, S. Radiation sensitization with redox modulators, a promising approach, *Int. J. Radiat. Oncol. Biol. Phys*., **64**, 343–354 (2006).
[61] Mehta, M. P. and Khuntia, D. Current strategies in whole-brain radiation therapy for brain metastases, *Neurosurgery*, **57**, S33–S44 disc. S1–S4 (2005).

22

Hemoglobin-based Oxygen Carriers: History, Limits, Brief Summary of the State of the Art, Including Clinical Trials

Jonathan S. Jahr[1], *Arezou Sadighi*[1], *Linzy Doherty*[1], *Alvin Li*[2] *and Hae Won Kim*[3]

[1]*David Geffen School of Medicine at UCLA, Ronald Reagan UCLA Medical Center, Los Angeles, CA, USA*

[2]*College of Letters and Science, UCLA, Los Angeles, CA, USA*

[3]*Department of Molecular Pharmacology, Physiology and Biotechnology, Brown University, Providence, RI, USA*

22.1 Introduction

For as long blood transfusion has been in medical practice, creating an infusible product that minimizes the complications associated with human blood products has been a laudable goal [1]. Over the past century, scientists have developed a number of alternate oxygen-carrying solutions; however, few of these have made it into clinical practice, largely because of their adverse effect profiles. Although transfusion with red blood cells (RBCs) may be life-saving, there are a number of limitations as well as multiple risks associated with transfusing blood [2, 3]. The issues surrounding blood transfusion have spurred much of the incentive to find a better, more efficient means of providing oxygen-carrying hemoglobin (Hb) to the patient in need. Hb-based oxygen carriers (HBOCs), though not perfected, are being evaluated as a life-saving intervention, and with continued research, HBOCs may soon be an integral part of transfusion medicine [2].

Chemistry and Biochemistry of Oxygen Therapeutics: From Transfusion to Artificial Blood, First Edition. Edited by Andrea Mozzarelli and Stefano Bettati.

22.2 American Society of Anesthesiologists Guidelines and Risks of Blood Transfusion

The American Society of Anesthesiologists has published guidelines and revised them regarding transfusion of RBCs [3]. While these guidelines recommend individualizing transfusion of red cells based on patient status, they indicate use is almost always necessary below an Hb of 6 g/dL, and rarely needed above an Hb of 10 g/dL. In fact, elegant work done by Weiskopf and colleagues has clearly defined the lower limits of survivability based on volunteer studies and the Jehovah's Witness population [4]. At Hbs of 3 g/dL or less, survival is unlikely, and between 3 and 7 g/dL, there may be a beneficial effect of viscous colloid solutions; however, these preclinical studies have not yet been validated in clinical models [5].

The risks of allogeneic RBC transfusion include: infectious transmission, crosstyping issues, immunomodulation, transfusion-related acute lung injury (TRALI), transfusion-associated circulatory overload (TACO), and stored RBC lesion [6]. While the risk of transmissible disease is extremely low, every single unit of allogeneic RBCs transfused increases the risk of postoperative wound infection, and decreases survival in multiple types of surgical cancer resections. Additionally, the incidence of TRALI at around 1 : 5000–1 : 70 000 units of transfused blood, with a mortality range of 5–14%, creates significant risk [6].

22.3 Limitations of Blood Transfusion

Many of the early limitations associated with blood-product transfusion, such as supply, storage, and administration, have been reduced with increasing accessibility and efficiency in modern medicine [7]. Other major limitations such as the longevity of blood and the rigorous crosstype matching that must be undertaken in order to administer a product with minimal crossreactivity risk to the patient [8] remain constant problems in transfusion medicine. Other underlying risks of blood-product transfusion are disease transmission and noninfectious transfusion reactions. For all of these reasons, developing an oxygen carrier with limited risks and limitations is a cornerstone of transfusion medicine. The benefits foreseen with a successful HBOC are multiple; however, above all it needs to deliver adequate oxygen supply to the body in an efficient manner and have an acceptable risk : benefit ratio as compared to Hb transported via RBCs.

22.4 History

Acellular Hb in solution has been studied as a potential blood substitute for close to a century. Notably, in 1934, Amberson purified bovine Hb and administered it to a population of canines [9]. Unfortunately there was significant nephrotoxicity and loss of Hb from the circulation that limited the original formulation [9]. He later studied purified human Hb and transfused this into anemic patients. Although the infusion restored blood volume and improved oxygen carrying capacity, these studies were again limited by poor outcomes in the study population. For the next few decades a number

Table 22.1 *Ideal characteristics of a blood substitute.*

Nonantigenic (universal compatibility)
Oxygen and carbon dioxide transport and delivery comparable to natural blood[a]
Does not cause abnormally high blood pressure
Therapeutically sufficient circulation half-life
Absence of toxicity (organ toxicity)
Immediate availability
Easy to administer
Does not impair host defense and hemostatic mechanisms
Long-term storage without refrigeration (stable at room temperature)
Does not form oxidized Hb derivatives, promote free-radical formation, or cause drug interactions

[a]This concept has been challenged in newer-generation HBOCs [23–26, 35].
Adapted from [2, 11].

of formulations came into development and clinical testing; however, none of these were successful at reducing morbidity associated with their use. Currently, the majority of research resides within two groups: cell-free Hb solutions and Hb encapsulated in lipid vesicles or polymers [2]. Ideal characteristics of a blood substitute are shown in Table 22.1.

22.5 Development

There have been a number of preparations of HBOCs, unmodified cell-free Hb, crosslinked Hb, polymerized Hb, recombinant Hb, and encapsulated Hb [2]. Hb is modified from either human or animal blood, or even recombinant Hb sources. These preparations have been modified in multiple ways to obtain a variety of properties and to minimize side effects [10]. Unfortunately, early preparations were limited by nephrotoxicity, thought to be secondary to red-cell stromal fragments in Hb solution; therefore, later preparations were made stroma-free. Early encapsulated Hb preparations had complications from the host-defense (reticuloendothelial) system that understandably limited their clinical usefulness; however, current Hb vescicles (HbVs) may have resolved this issue. Most recently, preparations have been faulted for their plausible vasoconstrictive properties. Hypoxic vasodilation is reported to be inhibited with the use of HBOCs secondary to the scavenging of nitric oxide, thereby causing a decrease in blood flow during states of hypoxia [11]. Although there have been a few successful solutions of HBOCs, as it stands, there is no current preparation that can replace our current standard of care. Table 22.2 shows methods for modifying Hbs.

Despite the above-mentioned issues, there is ongoing development to help eliminate these complications and develop a favorable risk : benefit ratio for the use of HBOCs. The previous trials have each left their mark on the current state of HBOCs and will eventually lead to a product that will replace the use of human blood-product transfusion as a gold standard. The most recent trials have provided us not only with information on what aspects of HBOCs are beneficial, but also a guideline for future practice protocols with regards to HBOCs.

Table 22.2 *Modified Hb solutions.*

From stroma-free hemoglobin (human, bovine)
Tetrameric stabilization (intramolecular crosslinking):
α−α crosslinked Hb: diaspirin crosslinked Hb (DCL-Hb)
β−β crosslinked Hb: 2-nor-2-formyl PLP-Hb (NFPLP-Hb)
Polymerization (intermolecular crosslinking):
Glutaraldehyde polymerized Hbs (HBOC-201, PolyHeme)
Conjugation to macromolecules: PEG-Hbs
Encapsulation of Hb (liposomes, polymersomes, nanocapsules)
Genetic engineering (recombinant human Hbs using bacteria, yeasts, plants, or animals)

Adapted from [2, 11].

Table 22.3 *Current and past HBOC products.*

Company	Product name	Modified Hb
Baxter	HemAssist® (DCL-Hb)[a]	Diaspirin crosslinked-hHb
Biopure (now OPKbiotech)	Hemopure® (HBOC-201)	Polymerized bHb
Curacyte/Apex	PHP/Hemoximer	PEG- hHb
Enzon	PEG-Hb[a]	PEG-bHb
Hemarina	M101	Arenicola marina Hb
HemoBiotech	Hemotech®	Polymerized/conj. bHb
Hemosol	Hemolink®[a]	hHb oligomer
Northfield	PolyHeme®[a]	Polymerized hHb
Oxyvita	OxyVita®	Zerolink bHb polymer
Prolong	Sanguinate®	PEG-bHb
Sangart	Hemospan® (MP4)	mPEG-hHb
Somatogen	Optro®[a]	Recombinant hHb
Synzyme	PNH	Polynitroxylated hHb

[a]Terminated development. h = human; b = bovine.
Adapted and updated from [34].

22.6 Definitive Clinical Trials

Table 22.3 lists HBOC products developed by industry to date.

22.6.1 Diaspirin Crosslinked Hemoglobin (DCLHb, HemeAssist, Baxter Laboratories, Deerfield, IL)

Among HBOCs, DCLHb, a purified, cell-free human Hb solution, has been shown to be effective in enhancing cellular perfusion in small volumes in preclinical models. Its use in animal stroke models led to a significant reduction in the extent of brain injury [12].

A clinical trial was conducted to assess the safety and tolerability of the repeated low-dose infusions of DCLHb in patients with acute ischemic stroke [12]. In this multicenter, randomized, single-blind, dose-finding, controlled safety trial, DCLHb or saline was administered to 85 patients with acute ischemic stroke in the anterior circulation within 18 hours of the onset of symptoms. The results showed that DCLHb produced a rapid increase in blood pressure and the duration of the effect was dose-dependent.

Although the hypertensive effect was not accompanied by complications such as hypertensive encephalopathy or hemorrhagic transformation of the infarction, some side effects observed (jaundice, hemoglubinuria, and lab abnormalities) were independently related to the use of DCLHb. In addition, the outcome at 3 months was significantly worse in the DCLHb treatment group and the cause of this worse outcome was unclear. Furthermore, based on the results, analogous to other stroke treatment, it was shown that it may be beneficial to administer DCLHb immediately after the onset of ischemia but harmful to give it during a later phase. In conclusion, infusion of low doses of DCLHb over 3 days adversely affected outcome in acute ischemic stroke patients and caused more serious adverse events, including increased mortality [12].

A major randomized controlled single-blinded efficacy trial was conducted between February 1997 and January 1998 at 18 US trauma centers to determine if the infusion of up to 1000 ml of DCLHb during the initial hospital resuscitation could reduce 28-day mortality in traumatic hemorrhagic shock patients [13]. The other end points included 28-day morbidity as measured by multiple-organ dysfunction score, 48-hour mortality, and the 24-hour lactate level. A total of 112 patients with traumatic hemorrhagic shock and unstable vital signs were randomized and 98 were infused with 500 ml of DCLHb or saline solution. Critically ill patients could have received up to an additional 500 ml during the 1-hour infusion period. The results showed that at 28 days, 46% of the patients infused with DCLHb died versus 17% of the patients infused with the saline solution. Also, the 28-day morbidity score was 72% higher in the DCLHb group, but there was no difference in adverse event rates or the 24-hour lactate level between the groups. Although, based on the results of this clinical trial, DCLHb did not appear to be an effective resuscitation fluid, the investigators believed that it was not possible to conclude definitely that the mortality imbalance was due solely to DCLHb, despite the results strongly suggesting an adverse treatment effect for this product [13].

In addition to these clinical trials, over 100 preclinical studies in several small and large animal species have been performed to evaluate the safety and efficacy of DCLHb as an oxygen therapeutic [14]. During these preclinical evaluations, the appearance of some myocardial lesions 24–48 hours following the administration of DCLHb to species such as rhesus monkeys or pigs attracted the investigators' attention. The left-ventricular myocardium was the most frequently affected region, followed by the interventricular septum and the right ventricle, while the left and right atria were usually not affected. Furthermore, even if increases in serum enzymes (AST, CK, LDH) were observed after infusion of DCLHb, the myocardial-related isoenzymes were not altered by these lesions, no EKG changes were noticed, and no observable adverse effect on myocardial function appeared.

Additionally, the investigators discovered that the polymerization of Hb diminished the extravasation of Hb from the vascular space and that it may also reduce both the severity and incidence of the myocardial lesions [14]. It is important to note that to date no evidence of Hb-induced myocardial lesions has been observed in man, and no increases have been seen in enzymatic markers of myocardial injury such as CK-MB or Troponin-I in any of the human clinical trials conducted with DCLHb. However, the presence of myocardial lesions in the preclinical studies represents a histopathological finding that must be considered during the preclinical testing and development of new HBOCs in the future [14]. The increased morbidity and mortality led Baxter to discontinue production

and development of DCLHb, and to abandon further work on its recombinant HBOCs developed through Somatogen as well [2].

22.6.2 Hemoglobin Raffimer (HR, Hemolink, Hemosol Inc., Ontario, Canada)

Continued concern over the safety of RBC transfusions has fostered alternative strategies for treating bleeding in massive hemorrhage (such as the bleeding and coagulopathy after coronary artery bypass grafting (CABG) that may be a major surgical complication), including blood conservation by intraoperative autologous donation (IAD) to reduce the patient's need for banked blood. This technique involves the removal of several units of a patient's blood immediately before operation. This collected blood is held in reserve in the operating room for retransfusion when the patient reaches a specified transfusion trigger. In the IAD procedure, the harvested blood volume is replaced with a plasma expander or an oxygen carrier [15].

A multicenter, randomized, double-blind, phase III clinical trial was performed to determine the efficacy and safety of Hb raffimer versus pentastarch 10% when used to facilitate IAD [16]. A total of 299 patients undergoing CABG were randomized to receive either 750 mL Hb raffimer or 750 mL pentastarch 10% after the harvest of 500–1500 mL of autologus blood at the start of cardiopulmonary bypass (CBP). Results were compared with transfusion requirements for 150 matched patients in the reference group, who were consecutive patients with similar demographics undergoing CABG surgery with CBP from a major university hospital database. Patients were also evaluated for the occurrence or avoidance of RBC and non-RBC blood-product transfusions during the course of hospitalization, the number of RBC and non-RBC blood products transfused, adverse events, vital signs, clinical chemistry, hematology, and urinanalysis [16]. Based on the results, the frequency of allogenic RBC transfusion in the Hb raffimer, pentastarch, and reference groups was 56%, 76%, and 95%. The number of allogenic red-cell units used was 49 in the Hb raffimer group, 104 in the pentastarch group, and 480 in the reference group. The total number of non-RBC units administered was 150 in the Hb raffimer group, 238 in the pentastarch group, and 270 in the reference group. Every patient in the study experienced at least one adverse event. The total number of adverse events, specifically hypertension, hyperbilirubinemia, elevated amylase, and urogenital events, was about 10% higher in the Hb raffimer group. Elevation in creatinine kinase-MB and troponin I was similar between the Hb raffimer and pentastarch groups. Also, ischemic changes in EKG were reported with similar frequency in both groups. One interesting finding in this clinical trial was the higher incidence of rehospitalization in the control group compared with the Hb raffimer-treated arm: 19 versus 11. One may speculate a potential improvement, decreased incidence, or less-severe sequelae to organ damage by ischemic events with the use of an oxygen therapeutic in this setting.

The findings in this study support the hypothesis that Hb raffimer may enhance the benefits of an IAD program in surgery with moderate blood loss. An individual's exposure to allogeneic transfusion-associated risks is reduced, and there is a reduced demand on the overall blood supply. These facts support a favorable balance of risks and benefits for Hb raffimer. They also suggest the probability that overall costs may be reduced by

an avoidance or reduction in the use of allogeneic blood and blood products [16]. However, concern over imbalance of cardiac events led to the discontinuation of the research program and failure of the company.

22.6.3 Human Polymerized Hemoglobin (PolyHeme, Northfield Laboratories, Evanston, IL)

Among the HBOCs, human polymerized Hb is a universally compatible, disease-free, immediately available, oxygen-carrying resuscitative fluid, intended for use as a red-cell substitute in the treatment of urgent hemorrhage, attendant to trauma and surgery, when red cells are unavailable [17]. The preparation of this "tetramer-free" Hb, which has been demonstrated to have life-sustaining capability and to attenuate the post-injury immunoinflammation, was designed to avoid the vasoconstriction issues seen with earlier tetrameric Hb preparations [18].

A critical unmet need for a universally compatible oxygen-carrying fluid encouraged the investigators to conduct a multicenter, randomized, phase III, open-label trial as a first US study to assess survival of patients resuscitated with human polymerized Hb starting at the scene of injury, comparing this product with standard care (crystalloid in the field followed by stored RBCs at hospital arrival) [18]. In total, 720 injured patients (48% blunt trauma versus 52% penetrating) with a systolic blood pressure $\leq$90 mm Hg were randomized in the study from January 2004 to July 2006 at 29 level I trauma centers to receive field resuscitation with human polymerized Hb or crystalloid. Excluding the 6 who received no treatment, the remaining 714 patients were analyzed according to the treatment to which they were randomized. Patients received up to 6 units (50 g Hb/unit) of human polymerized Hb beginning at the scene of the injury and during the first 12 hours post-injury. If needed, stored RBCs were given thereafter. Controlled patients received crystalloid in the field and stored RBCs as needed in the hospital. The primary efficacy end point in this trial was 30-day mortality, and the secondary efficacy end points were 30-day mortality for injury-type subgroups (blunt trauma versus penetrating), day-1 mortality, allogenic blood use through day 1, and the incidence of multiple organ failure (lung, kidney, liver, heart) through day 30.

In general, the results were not favorable for the use of human polymerized Hb as a blood substitute (30-day mortality: 13.4% human polymerized Hb versus 9.6% control; 30-day mortality for injury-type subgroups statistically higher in the human polymerized Hb group for both types of trauma; day-1 mortality: 10.0% human polymerized Hb versus 7.4% control; allogenic blood use through day 1 : 32.0% human polymerized Hb versus 50.0% control; incidence of multiple organ failure: 7.4% human polymerized Hb versus 5.5% control; adverse events: 93% human polymerized Hb versus 88% control; serious adverse events: 40.0% human polymerized Hb versus 35.0% control). In addition, myocardial infarction was reported by the investigators as a serious adverse event, occurring more frequently in the human polymerized Hb group (3.0% human polymerized Hb versus 1.0% control), but a blinded committee of experts reviewed records of all enrolled patients and found no discernable difference between groups [18].

There is an undisputed need for a universally compatible, oxygen-carrying product with reduced risk of disease transmission and long-term storage capability for use when RBCs are not available or not an option. It is theoretically possible for an HBOC such

as human polymerized Hb to provide a clinically meaningful benefit, despite outcomes that may be less favorable than those observed with stored blood. However, the studies performed to date do not document this. It is still unclear whether or not this product will perform as a useful blood substitute [19]. Concern over disproportionate mortality led to FDA disapproval and the company halted production of the product and is no longer in business.

22.6.4 Hemoglobin Glutamer-250 (Bovine) (HBOC-201, Hemopure, Biopure Corp., Cambridge, MA)

Another product from the HBOC family, HBOC-201 is a polymerized, iso-oncotic, high-molecular-weight, bovine Hb-based oxygen carrier for intravenous infusion and has been approved for the treatment of acute surgical anemia in South Africa since 2001. Of late, needs beyond surgical anemia, trauma, and hemorrhagic shock have evolved to include ischemic rescue and applications to cardiology and vascular surgery [20]. HBOC-201 has additional advantage over other oxygen therapeutics. Unlike HBOC products derived from human blood, HBOC-201 takes advantage of a plentiful supply of raw material (bovine Hb). In addition, it has a higher Hb concentration, which helps to maintain oxygen delivery to ischemic tissues with limited perfusion flow or in anemia, when early hemodilution with crystalloid or colloid solutions may complicate volume management. Also, unlike stored RBCs, which often have the potential to elicit inflammatory responses and induce diminished host-defense mechanisms, HBOC-201 is reported to be free of pro-inflammatory stimuli and possible infectious agents. It is compatible with all blood types and is the only HBOC demonstrated to be stable at 40 °C. It has the longest room temperature shelf life (3 years) of all HBOCs currently in development [20].

HBOC-201 has been administered to more than 800 human subjects in 22 completed clinical FDA phase I, II, and III trials. The most important of these trials are four advanced RBC controlled trials, in cardiac, vascular, general noncardiac, and orthopedic surgeries [21], in which the median or mean number of allogenic RBC units transfused was significantly lower for subjects randomized to HBOC-201 than for subjects randomized to RBCs [22].

The largest clinical trial of HBOC-201 was a randomized, single-blinded, multicenter, RBC controlled study in orthopedic surgical patients to evaluate the ability of HBOC-201 to reduce safely and/or eliminate perioperative transfusion in orthopedic surgery patients [21, 22]. In this study, 688 patients were randomized into two treatment groups (HBOC-201, $n = 350$, and packed red blood cells (PRBCs), $n = 338$) based on the investigators' assessment of the transfusion need and a total blood Hb < 10.5 g/dL. Patients were expected to require at least two units of PRBC transfusion before midnight of postoperative day 3. In case of clinical need, the patients in the HBOC-201 group had the possibility to be crossed over to treatment with PRBC, and there were no upper limits for the number of units of PRBC a patient could receive. The primary efficacy end point was transfusion avoidance and patients were evaluated before and after infusions on days 2 through 6, 24 hours and 48 hours after the final administration of HBOC-201 or PRBC, and 6 weeks postoperatively [21, 22]. The results demonstrated that the proportion of HBOC-201 patients not receiving allogeneic RBCs by day 1 was 96.3%, by day 7 was 67%, and by 6 weeks was 59%. Although there were no differences

in electrolytes, acid–base parameters, albumin, total bilirubin, alkaline phosphatase, lactate dehydrogenase, gamma glutamyl transferase, or glucose results between the two treatment groups, there were transient elevations in alanine aminotransferase, aspartate aminotransferase, lipase, creatinine kinase (CK-MB), and troponin in the HBOC-201 group, which were not related to liver failure, pancreatitis, or myocardial infarction. Some of these adverse events in the HBOC-201 group, including skin and scleral discoloration, GI side effects, and elevated blood pressure, are considered to be physiological effects of HBOC-201. The mortality in the HBOC-201 group in this clinical trial was higher than in the PRBC group (10 versus 6) but an independent safety end-point evaluation committee categorized no deaths as being associated with either treatment. However, in the patients over 80 years of age, there was a marked imbalance in mortality rate between the two groups (16.1% in HBOC-201 group versus 3.9% in PRBC group) [21].

This study, as the largest randomized controlled clinical study performed with an HBOC as an alternative to PRBC transfusion for elective surgery, demonstrates that patients less than 80 years old with moderate clinical need may safely avoid transfusion when treated with up to 10 units of HBOC-201. These results are encouraging for the use of HBOC-201 as a resuscitative fluid when compatible blood is not available or is not an option [21, 22], and also in the realm of the cardiac, peripheral ischemia, and orthopedic patients, for whom this oxygen therapeutic may prove to be an important tool in the armamentarium of the anesthesiologist, cardiologist, and surgeon [20]. Challenges with regulatory approval and concern over cardiac and other adverse events led to the product's current unavailability in South Africa, and sale of the company to OPK Biotech. However, HBOC-201 was reapproved by the Medicines Control Council of South Africa for human use in 2009, after initial approval in 2001, and the related veterinary product, HBOC-200 (hemoglobin gluatamer-200 (bovine), oxyglobin) was approved for canine anemia by the FDA in the United States in 1997 and the European Union in 1998.

22.6.5 Maleimide-polyethylene Glycol-modified Hemoglobin (MP4, Hemospan, Sangart Inc., San Diego, CA)

Maleimide-polyethylene glycol-modified Hb (MP4) is an oxygen-carrying plasma expander, prepared from human Hb, with unique oxygen-transport properties. In contrast to earlier-generation modified Hbs, MP4 is claimed to be not vasoconstrictive but slightly vasodilatatory [23]. Different clinical and preclinical studies have shown that this product produces neither systemic hypertension in human volunteers [24] nor systemic or pulmonary hypertension in pigs [25]. This product has successfully completed a phase I trial in healthy volunteers and the investigators performed a number of clinical trials in order to explore its safety further.

In a multicenter, phase II clinical study of the safety and activity of MP4, 90 male and female patients undergoing spinal anesthesia for major orthopedic surgery, aged 50–89 years, were randomly assigned into three groups to receive either 250 (Group A) or 500 ml (Group B) MP4 or ringer acetate (Group C) [26]. Patients were excluded from the study if they had clinical manifestations of uncontrolled metabolic, cardiovascular or psychiatric disorders, high blood pressure (>180/105), or a history of myocardial infarction or stroke within the preceding 6 months. The main goal of the study was to evaluate the safety of two dosages (250 and 500 ml) of MP4. The secondary goal

was to evaluate the potential for this product to improve hemodynamic stability during the surgical procedure, as measured by the incidence of hypotension (systolic blood pressure less than 90 mm Hg or 75% of baseline value) and the use of vasopressors. Safety assessment included vital signs and Holter monitoring from infusion to 24 hours, evaluation of laboratory values before anesthesia induction and 6 hours after the start of infusion, and fluid balance. All patients received midazolam or propofol intravenously for sedation before induction of the spinal block and during surgery. Control or study solution was infused before the initiation of the spinal block.

Based on the results of this study, three serious adverse events (death due to massive aspiration, death due to an extended posterior-lateral myocardial infarction, and a non-ST elevation myocardial infarction) were noted, none of which were deemed related to study treatment. Liver enzymes, amylase, and lipase increased transiently in patients in all three groups. No significant differences in electrocardiogram or Holter parameters were noted, but there was a suggestion of more bradycardic events in the treated groups, although hypotension was less frequent in the treated patients compared with controls. In summary, the study data provided an indication that MP4 was well tolerated in enrolled patients, but the fact that the study groups were not strictly comparable (for example, Group A was predominantly female whereas group B was predominantly male), along with the small number of the participants, decreased the statistical power of the results. Absence of a high frequency of serious adverse events brought to the scientists' attention that further clinical trials should be undertaken to confirm the above results [26].

Another phase Ib/II, randomized, controlled, single-blind, increasing-dose safety and tolerability trial was designed to compare five doses of MP4 with Ringer's lactate (RL) in 30 adult patients (older than 18) undergoing elective orthopedic surgery under spinal anesthesia in which anticipated blood loss was 250 mL or more [23]. In addition, adverse events, laboratory parameters, and plasma disappearance of MP4 were assessed. Patients were excluded from this clinical trial if they demonstrated any acute or chronic condition that would limit their completion of the study. Patients were randomized in five groups (A–E), each consisting of six patients, of whom four received MP4 in doses of 200, 400, 750, or 1000 ml, and two received RL immediately prior to induction of spinal anesthesia. All the patients received midazolam or propofol intravenously for sedation before induction of the spinal block.

There were no serious adverse events reported in the study. The observed adverse events (1.5 per patient in the MP4 group versus 1.4 per patient in the control group) were mild to moderate in intensity, and the most commonly reported adverse events were gastrointestinal, mainly nausea. One patient receiving 200 ml MP4 had elevated mean arterial pressure after dosing, but there were no elevations in any of the other patients. Apart from a transient elevation in the liver enzymes ALT, AST, and GGT in patients receiving 200 and 400 ml of MP4, a simultaneous decrease in ALP associated with all dose levels of MP4 treatment and a transient elevation in Troponin T in two patients receiving MP4 without any cardiac symptoms or electrocardiogram abnormalities, no clinically relevant abnormalities were seen in the study. The disappearance of MP4 from the plasma was dose-dependent, ranging from 14.1 to 23.0 hours at the lowest and highest doses studied. Also, it was noted that the plasma oxygen content was significantly enhanced, even with the modest doses of MP4; thus administration of MP4 might offer the possibility of increasing oxygen delivery to tissue, which could be of benefit for

patients who are unable to achieve elevated arterial pO_2. MP4 was well tolerated in the patients enrolled in this study [23].

In conclusion, a substitute for erythrocytes in transfusion is a long-sought-after goal. Nontheless, no product is currently approved for clinical use (except in South Africa), despite a considerable amount of research and development by both industry and academia.

22.7 Current Status and Future Directions of HBOCs

In 2008, immediately prior to an FDA meeting on the status of blood-substitute clinical trials, Natanson and colleagues published a meta-analysis of the morbidity and mortality of three generations of HBOCs [27a]. This article combined results then available to the authors, but not all subsequently published results. Combined results from three generations of HBOCs, including a third-generation drug currently undergoing phase III testing in Europe (Hemospan, Sangart, San Diego, CA, USA), were analyzed, and the report claimed that morbidity and mortality were higher with all three generations of compounds. Two second-generation products that failed clinical testing (Hb raffimer and human polymerized Hb) and the failed first-generation DCLHb were included in this meta-analysis, which looked at subset data analyses (myocardial infarction and mortality), not primary or secondary end points (which for most studies included the number of allogeneic units of RBCs avoided and so on). However, the study did highlight concern for cardiac safety with these products, and the need for basic science models specifically to look at the issue of cardiac ischemia and infarct [28].

In 2009, Silverman and Weiskopf published simultaneously in *Anesthesiology* and *Transfusion* the proceedings of the FDA meeting that occurred just after the publication of Natanson *et al*.'s controversial article [29] (there were multiple letters to the editor of *JAMA*, and a apology by one of the authors over lack of disclosure and subsequent amendment of the online version [27b–k]). The conclusions of the Silverman and Weiskopf article included the statement that all HBOCs tested to date, including the Sangart product – MP4 – caused vasoconstriction and elevations in blood pressure (see Table 22.4 for the FDA summary).

A number of new-generation products were being tested prior to the discontinued study of the two second-generation HBOCs, Hemosol [30] and Biopure [31], and even a modification of the MP4, which with proper chemical modification may eliminate the vasoconstriction [32].

The future of HBOC research must center on a concerted effort, with industry collaborating closely with academic centers, and an oversight committee to ensure that appropriate independent testing is undertaken and that the focus of regulatory approval is understood [33]. This also requires the cooperation of regulatory bodies to set clear guidelines for the process and adhere to them, assuming there is no growth in knowledge that would mandate a modification. Finally, funding sources from nonprivate mechanisms (public) must be in place to ensure that the research follows the highest possible principles. This paradigm has not been in place up to now, and the huge private investments from at least five companies have not led to a major regulatory approval, although significant new knowledge has been created in the process that should guide future development.

Table 22.4 *FDA summary of adverse events in HBOC clinical trials.*

	Apex		Baxter		Biopure		Enzon		Hemosol		Northfield		Sangart		Somatogen	
Cohort	T	C	T	C	T	C	T	C	T	C	T	C	T	C	T	C
Number of subjects	Not reported		504	505	708	618	Not reported		209	192	623	457	85	45	64	26
1. Death	*	*	78	61	25	14	*	*	1	4	73	39	2	0	*	*
2. Hypertension	*	*	76	38	166	59	*	*	113	75	*	*	7	1	8	0
3. Pulmonary hypertension	*	*	1	0	3	0	*	*	*	*	*	*	*	*	*	*
4. Chest pain/chest tightness	*	*	*	*	21	16	*	*	*	*	*	*	*	*	6	0
5. Congestive heart failure	*	*	0	1	54	22	*	*	0	2	17	20	*	*	*	*
6. Cardiac arrest	*	*	*	*	17	6	*	*	1	1	14	9	*	*	*	*
7. Myocardial infarction	*	*	6	1	14	4	*	*	14	7	29	4	2	0	*	*
8. Cardiac arrhythmias/ conduction abnormalities	*	*	23	17	153	100	*	*	1	1	*	*	15	5	1	1
9. Cerebrovascular accident, cerebrovascular ischemia, TIA	*	*	*	*	16	3	*	*	2	1	3	1	*	*	*	*
10. Pneumonia	*	*	*	*	35	22	*	*	*	*	27	21	*	*	*	*
11. Respitory distress/failure	*	*	*	*	22	12	*	*	*	*	21	17	*	*	*	*
12. Acute renal failure	*	*	1	3	10	4	*	*	2	2	*	*	*	*	*	*
13. Hypoxia, cyanosis, decreased oxygen saturation	*	*	*	*	76	35	*	*	1	1	*	*	*	*	3	1
14. Hypovolemia	*	*	*	*	19	4	*	*			*	*	*	*	*	*
15. Gastrointestinal	*	*	51	31	645	195	*	*	23	1	*	*			36	6
16. Liver, LFTs abnormal	*	*	27	8	20	5	*	*	8	0	*	*	57	20	6	3
17. Pancreatitis	*	*	11	0	5	3	*	*	1	0	*	*	*	*	*	*
18. Coagulation defect, thrombocytopenia, thrombosis	*	*	*	*	45	17	*	*	1	0	13	4	*	*	*	*
19. Hemorrage/bleeding/ anemia	*	*	33	22	108	55	*	*	1	1	20	17	*	*	*	*

20. Sepsis, septic shock, MOF	*	*	2	2	15	6	*	*	0	1	26	20	*	*	*	*
21. Pancreatic enzyme increase	*	*	13	4	3	0	*	*	*	*	*	*	*	*	*	*
22. Lipase increase	*	*	29	9	48	12	*	*	19	2	*	*	8	4	7	1
23. Amylase increase	*	*	48	45	*	*	*	*	35	20	*	*	7	2	4	1

Adverse events categories – terms included in each category:

1. Death.
2. Hypertension, blood pressure increased, hypertensive crisis, systolic hypertension, systemic vascular resistance (SVR) increased, malignant hypertension, postoperative hypertension, systolic blood pressure increased.
3. Pulmonary hypertension.
4. Chest pain, chest tightness.
5. Congestive failure – cardiac, cardiac failure, cardiorespiratory failure, left-ventricular failure, pulmonary edema, acute circulatory failure, rales, cardiac index decreased, cardiac output decreased, central venous pressure (CVP) increased, fluid overload, dyspnea.
6. Cardiac arrest, cardiopulmonary arrest, ventricular fibrillation.
7. Myocardial infarction.
8. Arrhythmias and conduction abnormalities.
9. Cerebrovascular accident, cerebral infarction, hemiparesis, hemiplegia, monoparesis, transient ischemic attack (TIA), reversible ischemic neurological deficit (RIND), transient cerebrovascular event.
10. Pneumonia, pneumonia-Klebsiella, pneumonia-Pseudomonas, pneumonia-Staphylococcus, aspiration pneumonia, pneumonitis.
11. Acute respiratory distress syndrome (ARDS), respiratory distress, respiratory failure.
12. Acute renal failure.
13. Hypoxia, decreased oxygen saturation, cyanosis.
14. Hypovolemia.
15. Gastrointestinal (GI) pain, GI pain – upper, esophageal spasm, vomiting, abdominal distension, dyspepsia, dysphagia, eructation, vomiting – aggravated, postoperative nausea, retching, abdominal pain – lower, nausea – aggravated, nausea, ileus.
16. Liver function tests (LFTs) abnormal.
17. Pancreatitis.
18. Coagulation disorder, disseminated intravascular coagulation (DIC), thrombocytopenia, thrombocythemia, activated partial thromboplastin time (aPTT) prolonged, bleeding time prolonged, fibrinogen decreased, D-dimer increased, prothrombin level decreased, petecchiae, purpura, thrombosis, arterial thrombosis-limb, deep venous thrombosis, pulmonary embolus, thromboembolism, thrombophlebitis – deep, PT change.
19. Anemia, duodenal ulcer hemorrhage, gastric ulcer hemorrhage, rectal hemorrhage, exsanguination, ulcer hemorrhage, intraoperative hemorrhage, postoperative hemorrhage, secondary anemia, hemoglobin decreased, vaginal hemorrhage, anemia aggravated.
20. Sepsis, septic shock, multiple organ failure (MOF).

Adapted from [29].

References

[1] Greenburg, A.G. and Kim, H.W. Hemoglobin-based oxygen carriers, *Crit. Care*, **8**(Suppl 2), S61–S64 (2004).

[2] Jahr, J.S., Walker, V. and Manoochehri, K. Blood substitutes as pharmacotherapies in clinical practice, *Curr. Opin. Anaesthesiol*., **20**, 325–330 (2007).

[3] American Society of Anesthesiologists Task Force on Perioperative Blood Transfusion and Adjuvant Therapies. Practice guidelines for perioperative blood transfusion and adjuvant therapies, an updated report by the american society of anesthesiologists task force on perioperative blood transfusion and adjuvant therapies, *Anesthesiology*, **105**, 198–208 (2006).

[4] Weiskopf, R.B., Viele, M.K., Feiner, J. *et al*. Human cardiovascular and metabolic response to acute, severe isovolemic anemia, *JAMA*, **279**, 217–219 (1998).

[5] Wettstein, R., Erni, D., Intaglietta, M. and Tsai, A.G. Rapid restoration of microcirculatory blood flow with hyperviscous and hyperoncotic solutions lowers the transfusion trigger in resuscitation from hemorrhagic shock, *Shock*, **6**, 641–646 (2006).

[6] Jahr, J.S., Moallempour, M. and Ellis, J. Transfusion update: triggers, targets and will we ever have a blood substitute?, *Curr. Rev. Clin. Anest*., **28**, 251–256 (2008).

[7] Cohn, S.M. Blood substitutes in surgery, *Surgery*, **127**, 599–602 (2000).

[8] Miller, R.D., Eriksson, L.I., Fleisher, L.A. *et al*. *Miller's Anesthesia*, 7 edn, Churchill Livingstone, Philadelphia, PA (2010).

[9] Amberson, W. Blood substitutes, *Biol. Rev*., **12**, 48–86 (1937).

[10] Spahn, D.R. and Kocian, R. Artificial O_2 carriers: status in 2005, *Curr. Pharm. Design*, **11**, 4099–4114 (2005).

[11] Jahr, J.S., Nesargi, S.B., Lewis, K. and Johnson, C. Blood substitutes and oxygen therapeutics: an overview and current status, *Am. J. Ther*., **9**, 438–439 (2002).

[12] Saxena, R., Wijnhoud, A.D., Carton, H. *et al*. Controlled safety study of a Hemoglobin-Based Oxygen Carrier, DCLHb, in acute ischemic stroke, *Stroke*, **30**, 993–996 (1999).

[13] Sloan, E.P., Koenigsberg, M., Gens, D. *et al*. Diaspirin Cross-Linked Hemoglobin (DCLHb) in the treatment of severe traumatic hemorrhagic shock: a randomized controlled efficacy trial, *JAMA*, **282**, 1857–1864 (1999)

[14] Burhope, K., Gordon, D. and Estep, T. Review of hemoglobin-induced myocardial lesions, *Artif. Cell. Blood Sub. Biotechnol*., **32**, 353–374 (2004).

[15] Cheng, D.C., Mazer, C.D., Martineau, R. *et al*. A phase II dose–response study of Hemoglobin Raffimer (Hemolink) in elective coronary artery bypass surgery, *J. Thorac. Cardiov. Sur*., **127**, 79–86 (2004).

[16] Greenburg, A.G. and Kim, H.W. Use of an oxygen therapeutic as an adjunct to intraoperative autologous donation to reduce transfusion requirements in patients undergoing coronary artery bypass graft surgery. The Hemolink Study Group, *J. Am. Coll. Surgeons*, **198**, 373–383 (2004).

[17] Gould, S.A, Moore, E.E., Hoyt, D.B. *et al*. The life-sustaining capacity of Human Polymerized Hemoglobin when red cells might be unavailable, *J. Am. Coll. Surgeons*, **195**, 445–455 (2002).

[18] Moore, E.E., Moore, F.A., Fabian, T.C. *et al*. Human Polymerized Hemoglobin for the treatment of hemorrhagic shock when blood is unavailable: the USA multicenter trial. The PolyHeme Study Group, *J. Am. Coll. Surgeons*, **208**, 1–13 (2009).

[19] Jahr, J.S. and Varma, N., PolyHeme Northfield Laboratories, *IDrugs*, **7**, 478–482 (2004).

[20] Jahr, J.S., Moallempour, M. and Lim, J.C. HBOC-201, hemoglobin glutamer-250, Hemopure, *Expert Opin. Biol. Th*., **8**, 1425–1433 (2008).

[21] Jahr, J.S., Mackenzie, C., Pearce, L.B. *et al*. HBOC-201 as an alternative to blood transfusion: efficacy and safety evaluation in a multicenter phase III trial in elective orthopedic surgery, *J. Trauma*, **64**, 1484–1497 (2008).

[22] Dube, G.P., Vranckx, P. and Greenburg, A.G. HBOC-201: the multi-purpose oxygen therapeutic, *Eurointervention*, **4**, 161–165 (2008).

[23] Olofsson, C., Nygards, E.B., Ponzer, S. *et al*. A randomized, single-blind, increasing dose safety trial of an oxygen-carrying plasma expander (Hemospan) administered to orthopedic surgery patients with spinal anesthesia, *Transfusion Med*., **18**, 28–39 (2008).

[24] Bjorkholm, M., Fargel, B., Przybelski, R. *et al*. A phase I single blind clinical trial of a new oxygen transport agent (MP4), human hemoglobin modified with maleimide-activated polyethylene glycol, *Haematologica*, **90**, 505–515 (2005).

[25] Drobin, D., Kjellstrom, B., Malm, E. *et al*. Hemodynamic response and oxygen transport in pigs resuscitated with maleimide-polyethylene glycol-modified hemoglobin (MP4), *J. Appl. Physiol*., **96**, 1843–1853 (2004).

[26] Olofsson, C., Ahl, T., Johansson, T. *et al*. A multicenter clinical study of the safety and activity of Maleimide-Polyethylene Glycol-Modified Hemoglobin (Hemospan) in patients undergoing major orthopedic surgery, *Anesthesiology*, **105**, 1153–1163 (2006).

[27] (a) Natanson, C., Kern, S.J., Lurie, P. *et al*. Cell-free hemoglobin-based blood substitutes and risk of myocardial infarction and death, *JAMA*, **299**, 2304–2312 (2008). (b) Fergusson, D. A. and McIntyre, L. The future of clinical trials evaluating blood substitutes, *JAMA*, **299**, 2324–2326 (2008). (c) Levien, L.J., Hodgson, R.E. and James, M.F.M. Letters: Hemoglobin-based blood substitutes and risk of myocardial infarction and death, *JAMA*, **300**, 1295 (2008). (d) Keipert, P.E., Olofsson, C. and Winslow, R.M. Letters: Hemoglobin-based blood substitutes and risk of myocardial infarction and death, *JAMA*, **300**, 1295–1296 (2008). (e) Lewis, R.J. and Fost, N. Letters: Hemoglobin-based blood substitutes and risk of myocardial infarction and death, *JAMA*, **300**, 1296 (2008). (f) Shander, A., Javidroozi, M. and Thompson, G. Letters: Hemoglobin-based blood substitutes and risk of myocardial infarction and death, *JAMA*, **300**, 1296–1297 (2008). (g) Sauaia, A., Moore, E.E. and Banerjee, A. Letters: Hemoglobin-based blood substitutes and risk of myocardial infarction and death, *JAMA*, **300**, 1297 (2008). (h) Sarani, B. and Gracias, V. Letters: Hemoglobin-based blood substitutes and risk of myocardial infarction and death, *JAMA*, **300**, 1297–1298 (2008). (i) Natanson, C., Lurie, P. and Wolfe, S.M. Letters: Hemoglobin-based blood substitutes and risk of myocardial infarction and death-reply, *JAMA*, **300**, 1298–1299 (2008). (j) Fergusson, D. and McIntyre, L. Letters: Hemoglobin-based blood substitutes and risk of myocardial infarction and death-reply, *JAMA*, **300**, 1299 (2008). (k) Natanson, C. Incomplete

financial disclosure in a study of cell-free hemoglobin-based blood substitutes and risks of myocardial infarction and death, *JAMA*, **300**, 1300 (2008).
[28] Jahr, J.S. Do approved blood substitutes reduce myocardial infarction size: is this the critical question?, *Brit. J. Anaesth*., **103**, 470–471 (2009).
[29] Silverman, T.A. and Weiskopf, R.B. Hemoglobin-based oxygen carriers: current status and future directions, *Anesthesiology*, **111**, 946–963 (2009).
[30] Crawford, M.W., Shichor, T., Engelhardt, T. *et al*. The novel hemoglobin-based oxygen carrier HRC 101 improves survival in murine sickle cell disease, *Anesthesiology*, **107**, 281–287 (2007).
[31] Fonseca, V., Avizinis, J., Moon-Massat, P. *et al*. Differential sensitivities of pulmonary and coronary arteries to hemoglobin-based oxygen carriers and nitrovasodilators: study in a bovine ex vivo model of vascular strips, *Vasc. Pharmacol*., **52**, 215–223 (2010).
[32] Dennis, A.M., Haselkorn, M.L., Vagni, V.A. *et al*. Hemorrhagic shock after experimental traumatic brain injury in mice: effect on neuronal death, *J. Neurotrauma*, **6**, 889–899 (2009).
[33] Jahr, J.S. Are newer generation hemoglobin-based oxygen carriers safe: do they offer an answer?, *Vasc. Pharmacol*., **52**, 214 (2010).
[34] Kim, H.W. and Greenburg, A.G. Artificial oxygen carriers as red blood cell substitutes: a selected review and current status, *Artif. Organs*, **28**, 613–828 (2004).
[35] Jahr, J.S., Weeks, D.L., Desai, P. *et al*. Does OxyVita, a new generation hemoglobin-based oxygen carrier, or oxyglobin acutely interfere with coagulation compared with normal saline or 6% hetastarch? An ex vivo thrombelastography study, *J. Cardiothorac. Vasc. Anesth*., **22**, 34–39 (2008).

23

Oxygen Delivery by Natural and Artificial Oxygen Carriers

Enrico Bucci

University of Maryland Medical School, Department of Biochemistry and Molecular Biology, Baltimore, MD, USA

23.1 Introduction

Oxygen is a toxic gas, indispensable for aerobic living. Its toxicity is inversely proportional to its partial pressure in the environment. In air it has a partial pressure near 20 kPa (150 Torr). For protection, besides retaining water, the relative impermeability of skin to oxygen lowers the partial pressure of oxygen to less than 1 kPa (10 Torr), just a few mm below its surface [1]. Metabolism needs and consumes a large amount of pure oxygen, about 1.0 L/min at 37 °C and atmospheric pressure. Therefore, the daunting task of physiology is to extract from air a large amount of oxygen, accumulating and storing it in a nonactive form (to prevent toxicity) and releasing it as a free active form as needed by metabolism, at the lowest possible partial pressure. Most importantly, the rate of delivery must match the speed of consumption. Irrespective of the quantity (mass) of available oxygen, a faster rate of delivery over consumption rate would produce hyperoxygenation, a slower rate would produce anoxia.

23.2 The Role of Oxygen Carriers

Oxygen is inactive (as if nonexistent) when bound to an oxygen carrier. When released, O_2 goes back into its free form, with all of its metabolic and toxic properties. The

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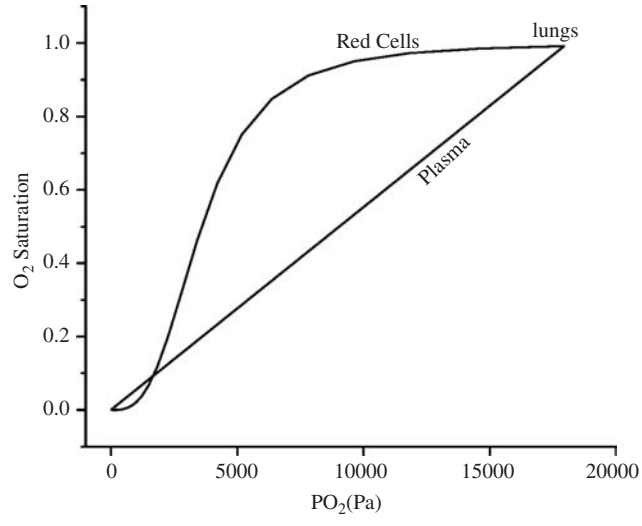

Figure 23.1 *Schematics of the longitudinal gradient of free oxygen through plasma compared to the oxygen binding and release by red cells in blood.*

relationship between bound oxygen and free oxygen *in vivo* is shown in Figure 23.1. In a schematic way, the straight line shows the gradient of free oxygen going from near 16 kPa (120 Torr) at the lungs to 300 Pa (2 Torr), or less, through plasma, to arrive in the parenchyma of muscle tissue. The gradient is linear and the oxygen there dissolved goes from the high to the low 10^{-5} M range. This amount of free-oxygen concentration would not be able to support metabolism. The top line represents the behavior of an oxygen carrier – the red cells in plasma – which binds oxygen at the lungs and keeps it sequestered and inactive until the partial pressure of the gradient becomes compatible with the oxygen affinity of the carrier. Once compatible, the carrier passively (it is not an oxygen pump) releases the bound oxygen, replacing molecule by molecule the oxygen consumed by metabolism. In so doing, a necessary large amount of oxygen is provided to the metabolism, released at low nontoxic pO_2. Oxygen replacement implies that during the process the partial pressure of oxygen does not decrease and remains buffered at a value within the range of the oxygen-binding equilibrium of the red cells: their buffer capacity.

23.3 The Role of Natural Cell-bound Oxygen Carriers

The buffer capacity of a carrier is its sensitivity to the partial pressure of oxygen and can be evaluated from the first derivative of its binding isotherm, as shown in its semilogarithmic form by:

$$BC = \partial S / \partial Log(PO_2) \tag{23.1}$$

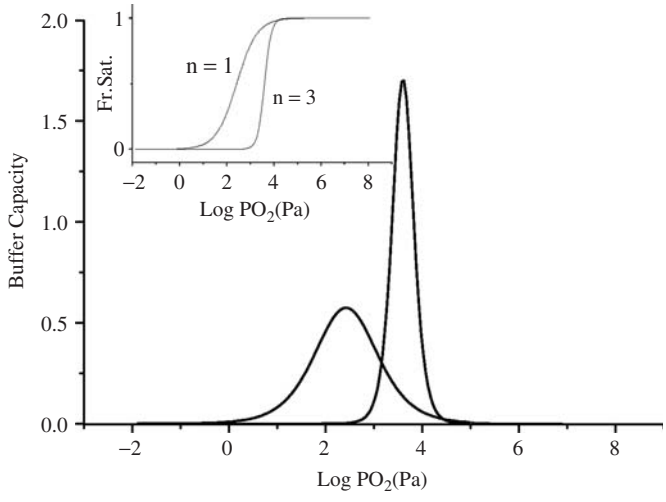

Figure 23.2 *Semilogarithmic profile of the buffer capacity of myoglobin (lower curve, p50 = 266 Pa, 2.0 Torr, n = 1.0) and red cells (hemoglobin) (upper curve, p50 = 3.9 kPa, 30.0 Torr, n = 3.0). The maximum strength is at the respective p50 values. The inset shows the isotherms used for computing the buffer capacities.*

where S is its fractional saturation with oxygen and BC is the buffer capacity. Following Equation 23.1, Figure 23.2 shows the plots against Log(pO_2) of the buffer capacities of red-cell hemoglobin and myocytes myoglobin, the cell-bound physiologic carriers present in blood and muscle tissue, respectively. Oxygen binding cooperativity makes the red-cell buffer capacity sharp and very strong, in contrast to the broad and lower strength of the buffer capacity of myoglobin, voided of binding cooperativity. The maximum capacity is at the respective p50 values, namely 4 kPa (30 Torr) for red cells and 270 Pa (2 Torr) for myoglobin.

On these premises, it would be expected that when red cells release their bound oxygen at the capillaries, the pO_2 of the oxygen gradient would be buffered near their maximum capacity, 4 kPa (30 Torr), and that when the released free O_2 diffused from capillaries into muscle parenchyma the gradient would be stabilized near 270 Pa (2 Torr) by myoglobin.

It should be stressed that in both cases, buffering near their p50 values leaves the carriers 50% saturated with oxygen. Thus only 50% of the respective bound oxygen is released. The excess bound oxygen remains available for increasing metabolic demands.

In fact, measurements from several laboratories reported by Tsay *et al*. [1] show that the pO_2 gradient forms a plateau near 4 kPa (30 Torr) at the capillaries, as in Figure 23.3. Furthermore, as reported by Wittenberg and Wittenberg [2], pO_2 in muscle tissues is near 270 Pa (2 Torr), the maximum buffer capacity of myoglobin.

Thus a large amount of oxygen replaces and copes with oxygen consumption, delivered first at a pO_2 of only 4 kPa (30 Torr) at the capillaries, then at a pO_2 near 270 Pa (2 Torr) in the muscle.

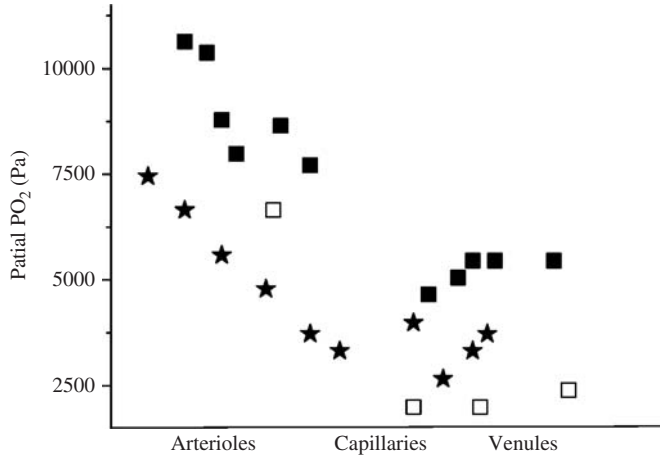

Figure 23.3 *Distribution of oxygen tension in the microcirculation of different animal models. □, dog gracilis ms; ★, hamster skinfold; ■, rat cerebral brain cortex. (Adapted from [1]).*

23.4 Matching the Rate of Oxygen Delivery with the Rate of Oxygen Consumption

23.4.1 The Imbalance

There is an imbalance between the rates of delivery and consumption of oxygen. The delivered oxygen from hemoglobin (inside the red cells) has to emerge from the internal viscosity of the cells, cross their membranes, navigate through plasma, and cross the capillary wall before reaching the muscle parenchyma. In the parenchyma, oxygen must still be bound and released by myoglobin and traverse the interstitial fluid, the cellular membranes, and their cytosol before it reaches a mitochondrion. Besides the long itinerary, oxygen diffusion is further delayed by its low solubility in watery environments. This retards considerably the arrival of oxygen to the mitochondria, and the related rate of oxygen replacement. No delay affects the rate of immediate oxygen consumption by cytochrome oxydase at the mitochondrion.

23.4.2 The Rate of Oxygen Release from the Red Cells

The rate of oxygen release $(O_2)_{del}$ by the red cells at the capillaries is a balance between the rate of their saturation at the lungs and that of their desaturation at the capillaries, as in:

$$(O_2)_{del,t} = \{Y_t BO_{tot}(\exp(-k_{off}t) - (1-Y)_t \underset{desaturation}{(BO_{tot})_{tiss}} \underset{resaturation}{\exp(k_{on}^{cap}t)} + (BO_{tot})_{alv} \exp(kt_{on}^{alv}t)\}. \quad (23.2)$$

where BO_{tot} is the potential total amount of oxygen carried by the red cells, Y their fractional saturation with oxygen, and (1−Y) their fractional desaturation.

On the right side of the equation, the first term on the left is the kinetics of oxygen release from the carrier (desaturation). It is a first-order kinetics, and is therefore dependent solely on the amount of oxygen-saturated carrier (YBO_{tot}) and on the standard k_{off} constant of hemoglobin.

The second (middle) term is the rebinding of oxygen by the red cells at the capillaries as part of the reversibility of the reaction. It is a pseudo first-order kinetics, where $k_{on}{}^{cap}$ is the standard k_{on} of hemoglobin modulated by the oxygen pressure prevailing at the capillaries. The rebinding contributes to the buffering action of oxygen release.

The third term is the oxygen uptake (resaturation) kinetics at the alveoli. It is also a pseudo first-order kinetics, where $k_{on}{}^{adv}$ is the standard k_{on} constant, this time modulated by the pO_2 present inside the alveoli.

23.4.3 Matching the Delivery/Consumption Rates

It appears that the rate of oxygen release by the red cells is regulated by the oxygen pressure prevailing either at the capillaries or inside the alveoli, and by the total amount of oxygen carried by hemoglobin inside the red cells, BO_{tot}.

It should be stressed that BO_{tot} corresponds to the total amount of hemoglobin carried by the red cells in blood; that is, by the hematocrit. This also means that, as shown in Equation 23.2, hematocrit is a very important parameter for regulating the rate of oxygen release at the capillaries.

Thus, the problem of the imbalance between the rates of delivery and consumption of oxygen, mentioned above, is solved by a large 40% hematocrit. It is a sheer mass action, necessary for increasing the amount of oxygen which diffuses through the distance between the red cells at the capillaries and the cell mitochondria, so as to match the amount and rate of oxygen consumption.

23.4.4 The Hematocrit is a Critical Parameter

When a hemoglobin mutation increases the oxygen affinity of hemoglobin, lowering its p50, the decreased value of the off-kinetics, k_{off}, of hemoglobion results in a decreased rate of desaturation, and consequently of oxygen release. There is a further delay to oxygen diffusion, compensated by the "mass" action of the resulting polycytemia.

Also, as mentioned, oxygen uptake by the red cells at the lungs (resaturation) depends on the pO_2 in the alveoli. When this decreases, downgrading the fractional resaturation of the carrier, the lower oxygen saturation of the red cells is compensated by an increased hematocrit. In fact, individuals living at high altitudes (3000 m), where there is a lower pO_2 in the air and in the lungs, have higher hematocrit values, above 50% [3, 4].

23.5 The Role of Artificial Cell-free Oxygen Carriers

23.5.1 Facilitated Diffusion

To the scenario of oxygen replacement by cell-bound carriers, cell-free oxygen carriers (HBOCs) add their Brownian motions in plasma. Using Brownian translational motion, oxygenated HBOCs carry bound oxygen through plasma, in practice increasing the rate

of diffusion of oxygen into watery environment. This phenomenon, called facilitated diffusion, was first described by Wittemberg and Wittemberg using hemoglobin solutions embedded in porous support [2]. Bucci *et al*. confirmed that, because of facilitated diffusion, HBOCs are much more efficient than suspensions of red cells in prolonging the survival and functionality of isolated organs (intestinal membranes) superfused by fluids equilibrated with low oxygen tensions [5].

By increasing the rate of diffusion of oxygen through plasma, facilitated diffusion, in practice, shortens the length of the itinerary from red cells to mithocondria, and also increases the amount of transported oxygen. This may modify the equilibrium of delivery/consumption rates in favor of delivery. While this assures the peripheric release of oxygen by HBOCs, it may also produce hyperoxygenation.

Hyperoxygenation is known to produce compensatory vasoconstriction. It can be monitored by observing changes in the diameter of the pial arteries. It was noted by Matheson *et al*., Asano *et al*., Sampei *et al*., Rebel *et al*., and Quin *et al*. [6–10] that, in animals exchange-transfused with a variety of HBOCs, compensatory vasocostriction was observed, indicating hyperoxygenation.

As a consequence of facilitated diffusion, oxygen delivery *in vivo* by HBOCs is relatively independent of their oxygen affinity. As shown by Koehler *et al*. [11], it appears that both high- and low-oxygen-affinity HBOCs are efficient in reducing the volume of cerebral infarcts in mice (Figure 23.4). It is interesting to observe that the higher-affinity HBOCs are more efficient.

23.5.2 Toxicity

The main toxicity of HBOCs is produced when they extravasate into the muscle layers of the vascular system, scavenging the relaxing factor NO. A systemic vasoconstriction is produced, which increases mean arterial pressure, and deprives of oxygen critical organs like intestine, kidneys, and the pituitary [12]. Extravasation occurs through large pores present in the endothelial layer of the vascular walls. Pores with diameters as large as 500 nm are present in several capillary beds [13]. This toxicity can be avoided by increasing the size of HBOCs above 500 kDa, preventing extravasation [6, 14]. Other factors, not well identified, may contribute to systemic vasoconstriction after HBOC infusions. NO scavenging remaining a principal event. Hyperoxygenation is also toxic, because it increases the formation of oxygen radicals. In a way, facilitated diffusion mimics reperfusion injuries. There are indications that, probably because of the formation of oxygen radicals, HBOCs per se damage endothelial tissues, producing extravasation [15, 16].

23.6 Other Parameters

Oxygen transport is a very complex physiologic event. Equation 23.2 does not include parameters which would refer to either hemoglobin effectors, like polyphosphates and anions, or vasoactivity and heart rate. These other parameters involve modification either of the on/off kinetic constants in Equation 23.2 (the effectors) or of blood flow, which mimics changes of the hematocrit (vasoactivity, heart rate). Therefore, either the effectors – by modulating the kinetics constants – or the blood flow – by modulating the hematocrit – are, indirectly, still included in Equation 23.2.

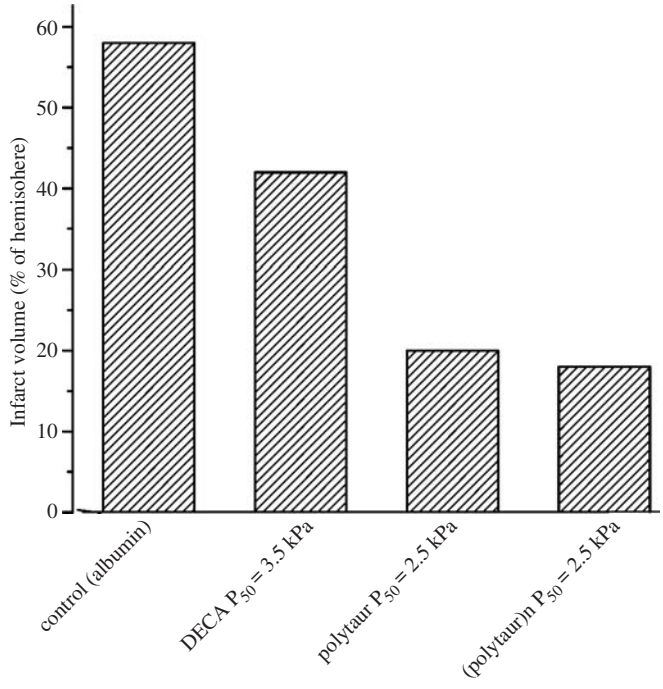

Figure 23.4 *Infarct volume after 2 hours of middle cerebral artery occlusion and 22 hours of reperfusion in mice exchange transfused during ischemia with: 5% albumin solution (controls, n = 13), 6% DECA (p50 3.5 kPa, 30 Torr, n = 5), 6% Hb Polytaur solution (p50 = 2.5 kPa, 18 Torr; n = 5), or 6% Hb (Polytaur)n (p50 = 2 kPa, 3 Torr; n = 5). DECA is a tretrameric human Hb intramolecularly crosslinked with sebacic acid [18]. Polytaur is a polymeric recombinant Hb including seven tetramers [19]. (Polytaur)n is a recombinant Hb with molecular size >500 kDa [19]. All HBOCs decreased the infarct volume with both high and low oxygen affinity. The high-affinity HBOCs were more efficient. (n = number of experiments). (Adapted from [11]).*

The trigger of all compensatory events appears to be the imbalance between the rates of delivery and consumption of oxygen. Hematocrit is a buffer which compensates all imbalances as in Equation 23.2. Hematocrit and its fractional saturation with oxygen are easily measured by the experimenter, offering a window on the status and variation of the overall oxygen delivery.

23.7 Clinical Use?

Whether HBOCs can be used in clinical settings is problematic. Even when extravasation is prevented, their increased or even excessive oxygen delivery bypasses physiologic regulations, and makes them a device of unknown efficiency and toxicity. Animal experimentation is of moderate help, because it is performed on young healthy subjects, limiting the scope of the results. In fact, in resuscitation attempts in the field, where "*previous*

conditions" and age are a problem, the use of HBOCs did not offer a statistical improvement over classic procedures, even resulting in increased risk of myocardial infarction [17]. In the opinion of this author, nonproper use, which did not consider possible hyperoxygenation and extravasation events, may have had a significance. Still, HBOCs offer the tantalizing possibility of administering therapeutic calibrated amounts of oxygen. It is necessary to learn how to use them in clinical settings.

Acknowledgments

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References

[1] Tsai, A.G., Vandegriff, K.D., Intaglietta, M. and Winslow, R.M. Targeted O_2 delivery by low-P_{50} hemoglobin: a new basis for O_2 therapeutics, *Am. J. Physiol. Heart Circ. Physiol*., **285**, H1411–H1419 (2003).

[2] Wittemberg, J.W. and Wittemberg, B.A. Myoglobin function reassessed, *J. Exp. Biol*., **206**, 2011–2020 (2003).

[3] Winslow, R.M., Chapman, K.V., Gibson, C.C. *et al*. Different hematologic response to hypoxia in Sherpas and Quechua Indians, *J. Appl. Physiol*., **66**, 1561–1569 (1989).

[4] Leon-Velarde, F., Gamboa, A., Chuquiza, J.A. *et al*. Hematological parameter in high altitude resident living at 4355, 4660, and 5500 meters above sea level, *High Alt. Med. Biol*., **1**, 97–104 (2000).

[5] Bucci, E., Watts, T.L., Kwansa, H. and Fasano, A. Different efficacy in vitro of hemoglobin based oxygen carriers and red cells, *Adv. Exp. Med. Biol*., **510**, 89–92 (2003).

[6] Matheson, B., Kwansa, H.E., Bucci, E. *et al*. Vascular response to infusions of a nonextravasating hemoglobin polymer, *J. Appl. Physiol*., **93**, 1479–1486 (2002).

[7] Asano, Y., Koehler, R.C., Ulatowski, J.A. *et al*. Effect of crosslinked hemoglobin transfusion on endothelial dependent dilation in feline pial arterioles, *Am. J. Physiol. Heart Circ. Physiol*., **275**, H1313–H1321 (1998).

[8] Sampei, K., Ulatowski, J.A., Asano, Y. *et al*. Role of nitric oxide scavenging in vascular response to cell-free hemoglobin transfusion, *Am. J. Physiol. Heart Circ. Physiol*., **289**, H1191–H1201 (2005).

[9] Rebel, A., Ulatowski, J.A., Kwansa, H. *et al*. Cerebrovascular response to decreased hematocrit: effect of cell-free hemoglobin, plasma viscosity, and CO_2, *Am. J. Physiol. Heart Circ. Physiol*., **285**, H1600–H1608 (2003).

[10] Qin, X., Kwansa, H., Bucci, E. *et al*. Role of 20-HETE in the pial arteriolar constrictor response to decreased hematocrit after exchange transfusion of cell-free polymeric hemoglobin, *J. Appl. Physiol*., **100**, 336–342 (2006).

[11] Koehler, R.C., Fronticelli, C. and Bucci, E. Independence of oxygen transport on oxygen affinity of Hemoglobin-Based Oxygen Carriers, *Biochim. Biophys. Acta*, **1784**, 1387–1394 (2008).
[12] Ulatowski, J.A, Nishikawa, T., Matheson-Urbaitis, B. *et al*. Regional blood flow alterations after bovine fumaryl beta beta-crosslinked hemoglobin transfusion and nitric oxide synthase inhibition, *Crit. Care Med*., **24**, 558–565 (1996).
[13] Rippe, B. and Haraldsson, B., Transport of macromolecules across microvascular walls the two-pore theory, *Physiol. Rev*., **74**, 163–219 (1994).
[14] Cabrales, P., Sun, G., Zhou, Y. *et al*. Effect of the molecular mass of tense-state polymerized bovine hemoglobinon on blood pressure and vasoconstriction, *J. Appl. Physiol*., **107**, 1548–1558 (2009).
[15] Dull, R.O., DeWitt, B.J., Dinavahi, R. *et al*. Quantitative assessment of hemoglobin-induced endothelial barrier dysfunction, *J. Appl. Physiol*., **97**, 1930–1937 (2004).
[16] Baldwin, A.L., Wiley, E.B., Summers, A.G. and Alayash, A.L. Sodium selinite reduces hemoglobin-induced venular leakage in the rat mesentery, *Am. J. Physiol*., **284**, H81–H91 (2003).
[17] Natanson, C., Kern, S.J., Lurie, P. *et al*. Cell free hemoglobin-based blood substitutes and risk of myocardial infarctions and death, *J. Am. Med. Assoc*., **299**, 2304–2312 (2008).
[18] Bucci, E., Razynka, A., Kwansa, H. *et al*. Production and characteristics of an infusible oxygen-carrying fluid based on hemoglobin intramolecularly cross-linked with sebacic acid, *J. Lab. Clin. Med*., **128**, 146–153 (1996).
[19] Fronticelli, C. and Koehler, R.C. Design of recombinant hemoglobins for use in transfusion fluids, *Crit. Care Clin*., **25**, 357–371 (2009).

24

Crosslinked and Polymerized Hemoglobins as Potential Blood Substitutes

Kenneth W. Olsen and Eugene Tarasov

Department of Chemistry, Loyola University, Chicago, IL, USA

24.1 Introduction

The development of hemoglobin-based oxygen carriers (HBOCs) has been a very active area of research since the early 1980s. The production of an HBOC requires modification of the hemoglobin (Hb) tetramer to give it the desirable properties required for a potential blood substitute. These modifications must alter the Hb to avoid tetramer dissociation, increase oxygen affinity, maintain colloid osmotic pressure, minimize vasoconstriction and redox chemistry, and be nonimmunogenic, sterile, and free of endotoxins. While making such an Hb may seem straightforward, the production of a clinically useful blood substitute has proven to be elusive.

Chemical modification of an Hb to produce an HBOC has been reviewed by Riess [1], Lowe [2] and Mozzarelli and coworkers [3]. This chapter will review the chemistry that has been used to produce HBOCs and assess the current status of the field with respect to what modifications are most likely to produce HBOCs with desirable properties. The reactions will include site-specific modifications, polymerization, and the addition of antioxidant enzymes to Hb polymers. Other approaches, such as recombinant Hbs, PEGylated Hbs, natural Hb polymers, and artificial red blood cells (RBCs) will be treated elsewhere in this volume.

Chemistry and Biochemistry of Oxygen Therapeutics: From Transfusion to Artificial Blood, First Edition.
Edited by Andrea Mozzarelli and Stefano Bettati.

24.2 Crosslinking the Hb Tetramer

Crosslinking Hb was a logical step in avoiding the extravasation of Hb into the tissue observed with stroma-free Hb. Since the tetramer can dissociate into αβ-dimers, which are small enough to be excreted by the kidney [4, 5], crosslinking the tetramer prevents both short serum lifetimes and nephrotoxicity. Many crosslinkers were developed, but the best studied was bis(3,5 dibromosalicyl) fumarate (DBSF). In the DBSF reaction the dibromosalicylate is displaced by the ε-amino group of a specific lysine (Figure 24.1). The reaction between DBSF and oxy-Hb produced crosslinking between Lys82β_1 and Lys82β_2 with high oxygen affinity and no cooperativity [6–8]. The X-ray crystal structure of the β82 crosslinked protein displayed both T- and R-state characteristics because it was crosslinked under oxy conditions but crystallized under deoxy ones [9]. Reaction between DBSF and deoxy-Hb produced crosslinking between Lys99α_1 and Lys99α_2, with a low oxygen affinity and lower cooperativity [10–13]. This crosslink did not perturb the T-state structure [9]. DBSF crosslinking eliminates tetramer dissociation and allows oxygen delivery to the tissues, but does not prevent vasoconstriction or extravasation [14–16]. The vasoconstriction has been attributed to NO binding [17, 18].

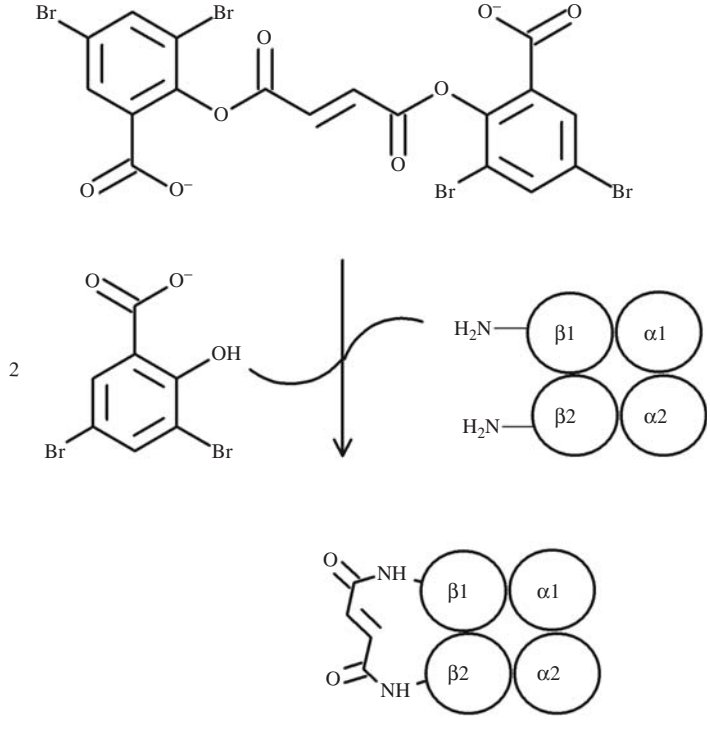

Figure 24.1 *DBSF crosslinking reaction for oxy-Hb links the two β82 lysine residues [6]. If the reaction is done under deoxy conditions, the predominant product is linked between the α99 lysine residues [10].*

Since a blood substitute was thought to require oxygen binding similar to erythrocytes, Baxter Healthcare tried to develop the α99 crosslinked Hb (DCLHb) as a potential blood substitute. The stability of DCLHb was significantly increased [19], allowing Baxter Healthcare to purify it from unreacted Hb while pasteurizing the product [20]. Unfortunately, myocardial lesions were observed in certain animal species [21], which may result from cytotoxicity to endothelial cells [22] and activation of platelet coagulation cascade due to scavenging of NO by the crosslinked Hb [23]. Nevertheless, early clinical trials on humans were encouraging, but phase III clinical trials for severe hemorrhagic-shock patients were terminated in the USA in 1998, due to cerebral and pulmonary edema, pancreatic insufficiency, myocardial ischemia, and death due to stroke [24]. Use of DCLHb resulted in a significantly higher percentage of mortality versus the control group [25]. European clinical trials continued slightly longer but ultimately were terminated due to the lack of efficiency in prevention of organ failure and mortality. Although adverse effects where observed, they were not statistically significant in comparison to the control (90% vs 76%) [26]. The disparity in the results between US and European studies was attributed to the differences in the study protocols. In the European model, DCLHb was administered directly at the scene of the traumatic incident, while in the USA it was only infused when the patient arrived at hospital. At the conclusion of both trials it was suggested that the main mechanism behind adverse reactions was NO scavenging.

An interesting variation of these crosslinking studies was the double crosslinking of the protein. Olsen and coworkers [27] first demonstrated double crosslinking of oxy-Hb both with DBSF and dimethylpimelimidate and with DBSF followed by deoxygenation and recrosslinking with DBSF. Both products had increased thermal stability, decreased cooperativity, and high oxygen affinity compared with singly-crosslinked Hbs. Jones and coworkers [28] doubly crosslinked Hb using trimesyl tris(3,5-dibromosalicylate) to bridge the two β82 Lys residues and DBSF to bridge the α99 Lys residues. Unlike the earlier doubly-crosslinked Hb, this species demonstrated low oxygen affinity and significant cooperativity. The concept of doubly crosslinking the Hb can be used in other contexts, such as crosslinking followed by PEGylation [29] to control oxygen affinity and add stability.

Acyl phosphates [30, 31] have been extensively used to crosslink Hbs (Figure 24.2). The resulting reagents often react at the same sites as DBSF, bridging either the β82 or the α99 Lys residues. By introducing a disulfide bond in the middle of the crosslink, Kluger and Li [32] found that β-mercaptoethanol would reduce the disulfide bond when the protein was not crosslinked, but not when it was. The stronger reducing agent dithiothreitol would reduce the disulfide in both cases.

Several other chemistries have been used to produce crosslinked Hbs. Hosmane and associates have used phosphinic acids to produce crosslinks involving Val1 or Lys82 on one β-subunit and Lys82 or Lys144 on the other [33, 34]. These products exhibited oxygen affinity similar to that of cell-free Hb but lacked cooperativity. Dialdehydes have also been used to crosslink Hb, allowing control of its allosteric properties [35]. The earliest specific crosslinking of Hb was done using 2-nor-2-formylpyridoxal 5'-phosphate to crosslink between the Lys82β_1 and Val1β_2 [36, 37]. Similar modifications using pyridoxal 5'-phosphate have been employed prior to polymerization with other reagents (see below).

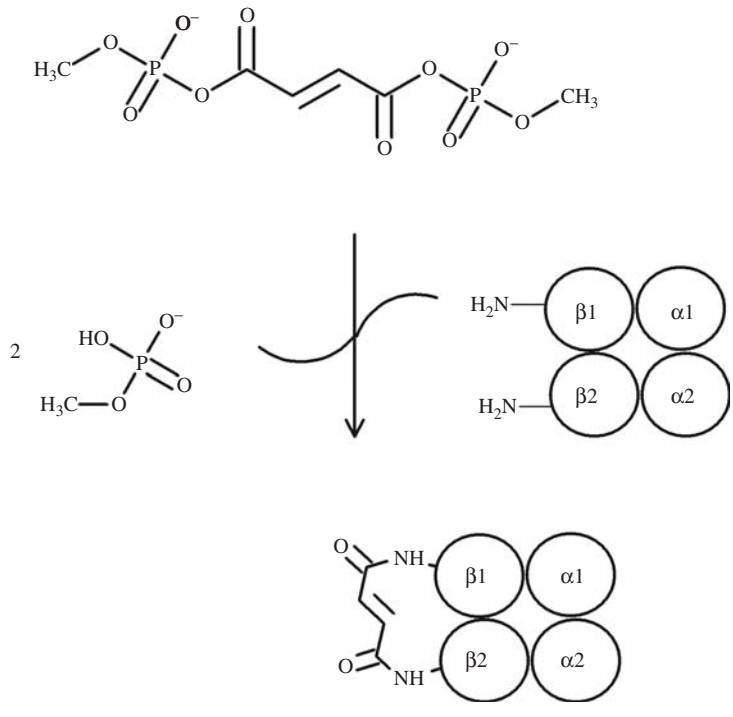

Figure 24.2 *Reaction of fumaroyl bis(methylphosphate) with oxy-Hb. Like DBSF, this reagent primarily links the two β82 lysine residues in oxy-Hb, but it links several places in deoxy-Hb [38].*

Several studies have been made of the effects of crosslinker length on the properties of the modified proteins. Jones and coworkers [28, 38] examined five different lengths of bis(methyl phosphate) reagents reacted with both carbon monoxy-Hb and deoxy-Hb and established linear relationships between log p50 and bridge length for three different types of Hb modification, enabling prediction of p50 from crosslinker length. Olsen and coworkers have investigated the variation of reaction yield, thermal stability, and autoxidation with crosslinker length [39, 40]. Yields of the modified proteins decreased with crosslinker length, which correlated with the finding predicted by molecular dynamics studies [39]. Autoxidation rates gradually increased with longer reagents, while thermal stability decreased sharply when the crosslinking length exceeded 10.5 Å [39]. Zhang and Olsen [41] observed that oxy-Hb crosslinked with bis(3,5-dibromosalicyl) sebacate had low oxygen affinity with reasonable cooperativity and high thermal stability. Bucci and coworkers showed that a linker this long could easily be incorporated into the T-state structure but not into the R- or R2-state structures [42].

Multilinking involves linking three or more groups on the surface of the Hb protein, a process developed mainly in the Kluger and Olsen laboratories. An advantage of multilinkers is that they provide a greater control over the oxygen affinity. The reaction chemistries used are the ones for bis(3,5-dibromosalicyl) esters and acyl phosphates (Figures 24.1 and 24.2), but the core of the multilinker allows a greater number of

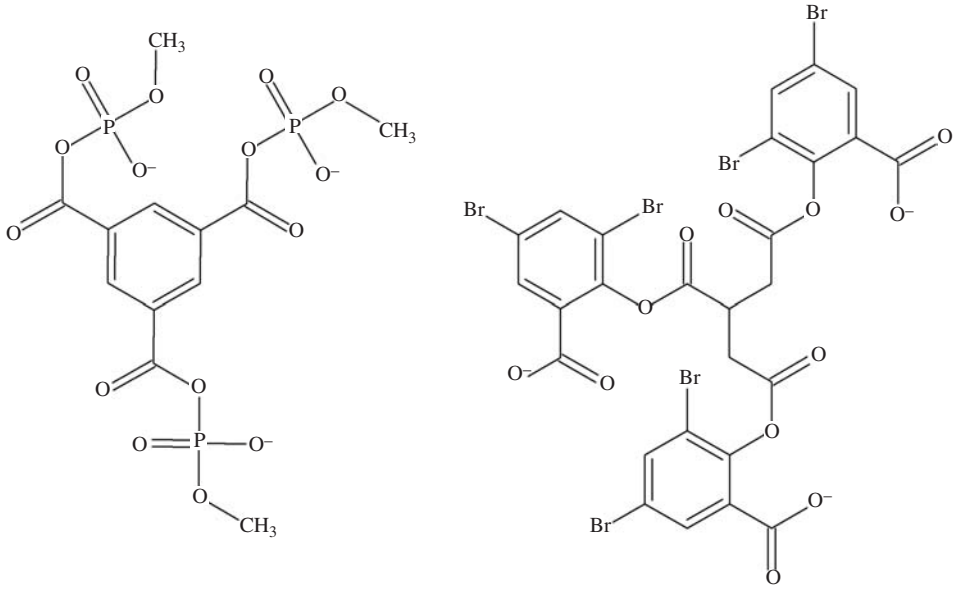

Figure 24.3 *Examples of multilinker structures. (a) Trimesoyl tris(methyl phosphate) [43]. (b) Tris(3,5-dibromosalicyl) tricarballylate [44].*

reactive groups (Figure 24.3). Kluger, Jones and coworkers [43] developed trimesoyl tris(methyl phosphate) to react selectively with the Val1 and Lys82 of one β-chain and the Lys82 of the other. This trilinked Hb has lower oxygen affinity (p50 = 18.1 mm Hg) than Hb while maintaining reasonable cooperativity (Hill n = 2.4). Zheng and Olsen [44], designed tris(3,5-dibromosalicyl) trimesate to stabilize R-state Hb and generate an Hb trilinked between those β-chains that exhibited low oxygen affinity and decreased cooperativity when reacted under either oxy or deoxy conditions. Paal, Jones and Kluger made 3,5,3',5'-biphenyltetracarbonyl tetrakis(3,5-dibromosalicylate), which only crosslinked between the two Lys82β residues, rather than tetralinking the tetramer [45]. The product had low affinity (p50 = 13.9 mm Hg) with reasonable cooperativity (n = 2.5). Huang, Zhang and Olsen [46] made tetrakis(3,5-dibromosalicyl) 3,3',4,4'-benzophenone tetracarboxylate, which was designed to react with both Lys82β residues and both Val1β residues in T-state Hb. This product had very low oxygen affinity (p50 = 27 mm Hg) but no cooperativity (n = 1.2).

The success of these multilinkers generated attempts to produce tetralinked Hb octamers. Kluger and coworkers [47] used the tetrafunctional reagent *N*,*N*-5,5-bis[bis (3,5-dibromosalicyl)isophthalyl] terephthalamide to form the octamer. Gel-filtration studies run in 0.5 M $MgCl_2$ to dissociate noncrosslinked αβ-dimers showed that a significant amount of octamer had been produced, although the majority of the Hb was not in that form. Formation of the octamer from bis(isophthalamide) crosslinked tetramers using a short linker caused a loss of cooperativity [48], while using longer, more flexible linkers did not [49]. More recently, Kluger and coworkers have

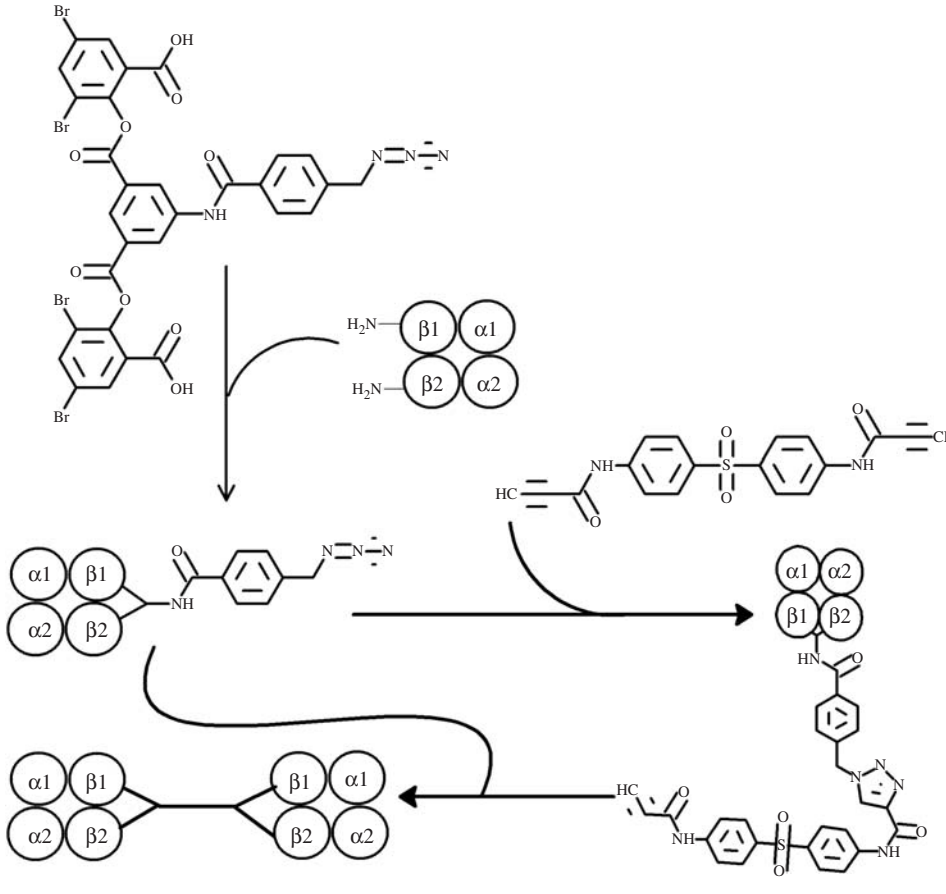

Figure 24.4 *Hb octamer formation using azide–alkyne click chemistry [50].*

increased the yield of octamer significantly by employing azide–alkyne click chemistry (Figure 24.4). In this reaction, the second step is promoted by the first one, giving a high yield of the desired product. Kluger and his associates have recently reviewed the research coming from his laboratory [50].

24.3 Hb Polymers

Large Hb oligomers have been developed using several methods of polymerization. The chemistries that have been employed include Schiff-base formation, "zero-length" amide bonds, and sulfhydryl–maleimide click chemistry.

O-raffinose polymerization (Hemosol Inc., Toronto, Canada) forms Schiff bases between the protein and the crosslinker, which are reduced by dimethylamine borane to avoid dissociation. The general Schiff-base chemistry, which is also used in glutaraldehyde polymerizations, is shown in Figure 24.5. The process results in a

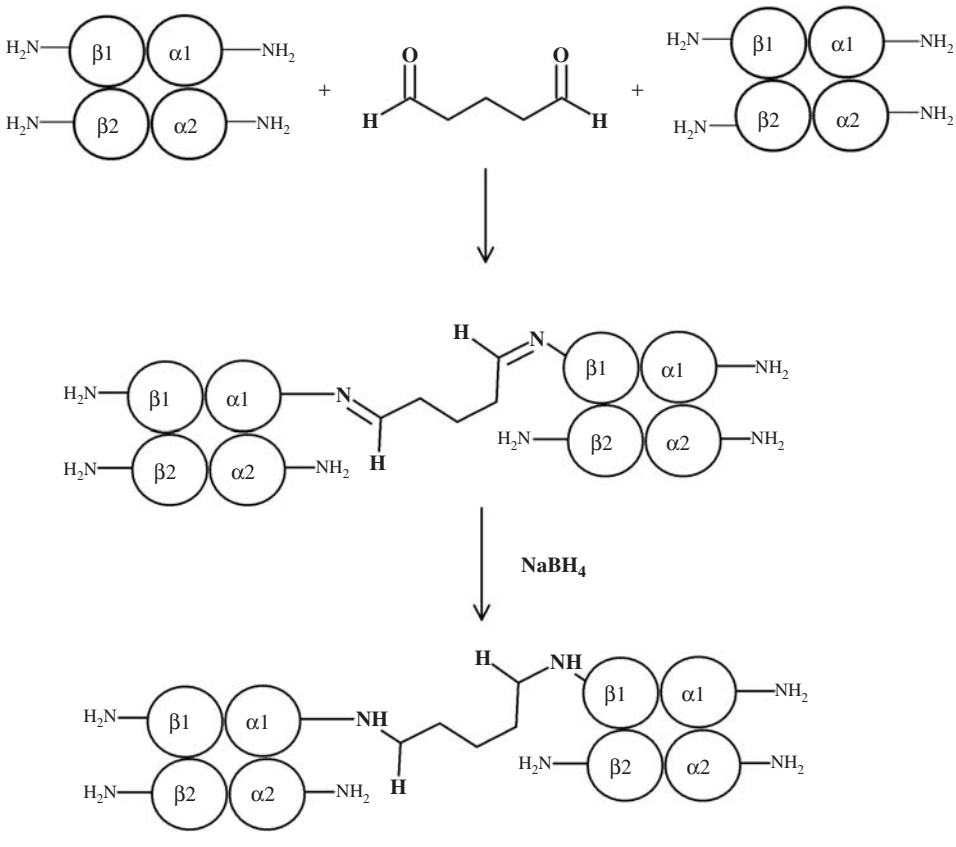

Figure 24.5 *Polymerization chemistry for glutaraldehyde. A Schiff base is first formed between amino groups on the protein and the aldehydes of the reagent. In a second step, the Schiff base is reduced, usually using $NaBH_4$. The chemistry for raffinose polymerization is similar.*

heterogeneous product with both intra- and intermolecular crosslinking, 33 and 66%, respectively. Size-exclusion chromatography showed a mass distribution between 128 kDa and 600 kDA [51, 52]. The half-life of o-raffinose-modified Hb was 14–20 hours in humans, with ~95% of the solution having a molecular weight greater than 64 kDa [53–55]. In dogs, the half-life of o-raffinose-modified Hb was directly proportional to the molecular weight of the fraction used [56]. Clinical trials with o-raffinose-modified Hb in coronary artery bypass surgery continued into phase III but were terminated when an increased number of patients suffered myocardial infarctions. The cardiac marker troponin was found in some patients, indicating cardiomyocyte damage [57]. Hemosol's facilities were purchaced by Therapure Biopharma in 2008, and the o-raffinose product is no longer in commercial production.

Hb has most often been polymerized using glutaraldehyde, a five-carbon dialdehyde that reacts with various residues, including lysines, cysteines, histidines, and tyrosines [58], producing a heterogeneous product. The synthesis (Figure 24.5) involves the

formation of a Schiff base between glutaraldehyde and lysine side chains of Hb, followed by reduction using sodium borohydride [59]. Glutaraldehyde undergoes an aldol condensation in alkaline solution, resulting in a polymer with multiple reactive aldehyde groups that provide longer crosslinks in proteins [60]. Unless reduced to amines, this bond can dissociate, resulting in release of glutaraldehyde and Hb [61]. Full reduction of the Schiff base is crucial for the stability of the product and is very difficult to achieve [62]. Thus, the safety of glutaraldehyde products has been questioned [52, 61]. The glutaraldehyde-Hb polymer has diverse properties, reflecting its heterogeneity. The p50 value is significantly left-shifted, depending on the amount of glutaraldehyde used. The cooperativity is almost non-existent. Two commercial products utilizing bovine or human Hb have been tested in clinical trials.

The bovine product was sold under the brand names Hemopure (HBOC-201) and Oxyglobin (HBOC-301) (BioPure, Cambridge, MA). The former was in development for use in humans, while the latter had been approved for veterinary use. The bovine product has some inherent advantages over the human Hb counterpart. Since the bovine Hb oxygen affinity is dependent on the presence of chloride ions, which are present at high concentrations in humans, there is no need to include affinity modifiers into the synthesis of HBOC-201 [63, 64]. In addition, since bovine RBCs are more available than human, there are no raw-product shortages. One major problem with bovine Hb, the possible presence of bovine spongiform encephalophathy (BSE), can be avoided by use of cattle from regions unaffected by this disease.

Hemopure was a mixture of variously sized oligomers, ranging from 130 to 500 kDa. It allowed oxygen delivery in multiple animal models but also produced vasoconstriction [65–68]. Vasoconstriction has been observed in a large number of studies in both humans and animals [17, 69–75], probably due to NO concentrations decreasing after Hemopure use [76]. Other studies showed conflicting results, which failed to see vasoconstriction or a decrease in NO concentration [65, 68, 77–79]. The rapid formation of met-Hb was also observed in various animal and human settings [73, 74, 80]. After a series of regulatory problems, Biopure filed for bankruptcy in July 2009, and its assets were purchased by OPK Biotech. Neither Hemopure nor Oxyglobin are currently available.

Northfield Laboratories Inc. (Evanston, IL) produced a glutaraldehyde-polymerized human Hb. This product was manufactured by initially reacting the Hb with pyridoxal 5'-phosphate, a natural coenzyme related to vitamin B6 and a functional analogue of 2,3-BPG, followed by glutaraldehyde polymerization. This extra step increases the p50 value from 18–22 to 28–30 mm Hg [81–83]. After undertaking some preliminary product testing, the company was granted an approval to conduct highly controversial clinical trials in emergency settings on people. In 2009, FDA concluded that the risks associated with the use of PolyHeme were greater than its benefits, since more patients in the phase III clinical trial died after having received this product than after getting a standard blood transfusion. As a result of these findings, the company had to close its doors.

Similar polymerization strategies are still under investigation in China. By doing the polymerization on a cation-exchange column, the size of the polymers can be mainly restricted to the range of 128–256 kDa [84]. Wang and coworkers argue that this relatively low molecular weight will be an advantage, due to low viscosity and vascular resistance, but it is not clear if this smaller species will provide protection from vasoconstriction as the larger polymers do. Glutaraldehyde-polymerized Hb may have enhanced

immunogenicity [85], but this may also be true for some of the other chemical polymerization methods since not all systems have been studied for their immunogenicity.

Several other polymerization methods are currently being investigated to provide a large polymer that will not extravasate or cause vasoconstriction and that will prevent oxidative damage due to redox chemistry at the heme sites. Sulfhydryl–maleimide click chemistry (Figure 24.6) has been used to generate large polymers (>700 kDa) [86]. Since more than one round of polymerization can be done, the structure can be made any size by adding additional layers of protein. The chemistry can be used to incorporate the antioxidant enzymes catalase and superoxide dismutase (SOD), which may help reduce the effects of heme redox reactions on tissues.

Another novel approach has been to attach Hb to nanoparticles. Marden and coworkers [87] have made poly(alkyl cyanoacrylate) nanoparticles decorated with polysaccharides. The synthesis is carried out in water, avoiding cosolvent contamination, using a redox-radical polymerization. The resulting core-shell nanoparticles have a hydrophobic poly(cyanoacrylate) core with a polysaccharide surface layer. This surface layer can adsorb Hb, giving an oxygen-carrying nanoparticle. While the chemistry of the adsorption has not been established, it may involve a Schiff base between the polysaccharides and the protein.

Bucci and coworkers [88] produced a zero-length crosslinked Hb polymer using chemistry that had been used to demonstrate interactions between proteins [89]. The method forms amide bonds between protein carboxylate and amino groups after activating the carboxylates with a water-soluble carbodiimide (Figure 24.7). After eliminating smaller components with a 300 kDa membrane, the polymer had an average hydrodynamic radius of 250 Å and did not extravasate [88]. The size of this polymer has been estimated between 17 and 25 MDa [90, 91], which accounts for the lack of increase in the mean arterial pressure when it is used [90]. The product has high oxygen affinity and low cooperativity [92]. The autoxidation rate is increased threefold compared to unmodified Hb but is not slowed greatly by the presence of catalase and SOD [92]. The product is being commercialized by OxyVita (New Winsor, NY).

Clearly, one of the current concerns in the development of HBOCs as potential blood substitutes is the redox chemistry of the heme. Yang and Olsen [93] showed that the autoxidation rate for α99 DBSF-crosslinked Hb was significantly faster than that of HbA, but that the rate for the β82 DBSF-crosslinked Hb was only slightly increased. Later spectroelectrochemical studies on normal and crosslinked Hbs showed that the chemical mechanism of oxygen dissociation and the accessibility of water and oxygen radicals to the heme site control autoxidation [94]. The reactions involved in autoxidation can generate several reactive oxygen species (ROS), such as superoxide ($O_2^{\bullet -}$) and hydrogen peroxide (H_2O_2), through Fenton-type chemistry, and lead to the generation of met-Hb [95, 96]. The ROS produced by extracellular HBOCs cause significant damage to the tissues, possibly via lipid peroxidation, hemin release, or bilirubin formation [97]. The consequences of this damage may be greater in situations like trauma or ischemia/reperfusion, when ROS are already overproduced [96]. The addition of catalase and SOD to the Hb solution greatly decreased the rates of autoxidation [98]. Several groups have produced HBOCs containing these antioxidant enzymes as a possible way of controlling the potential damage due to ROS. Chang and his coworkers made a glutaraldehyde-polymerized Hb–catalase–SOD complex that greatly decreased oxidative

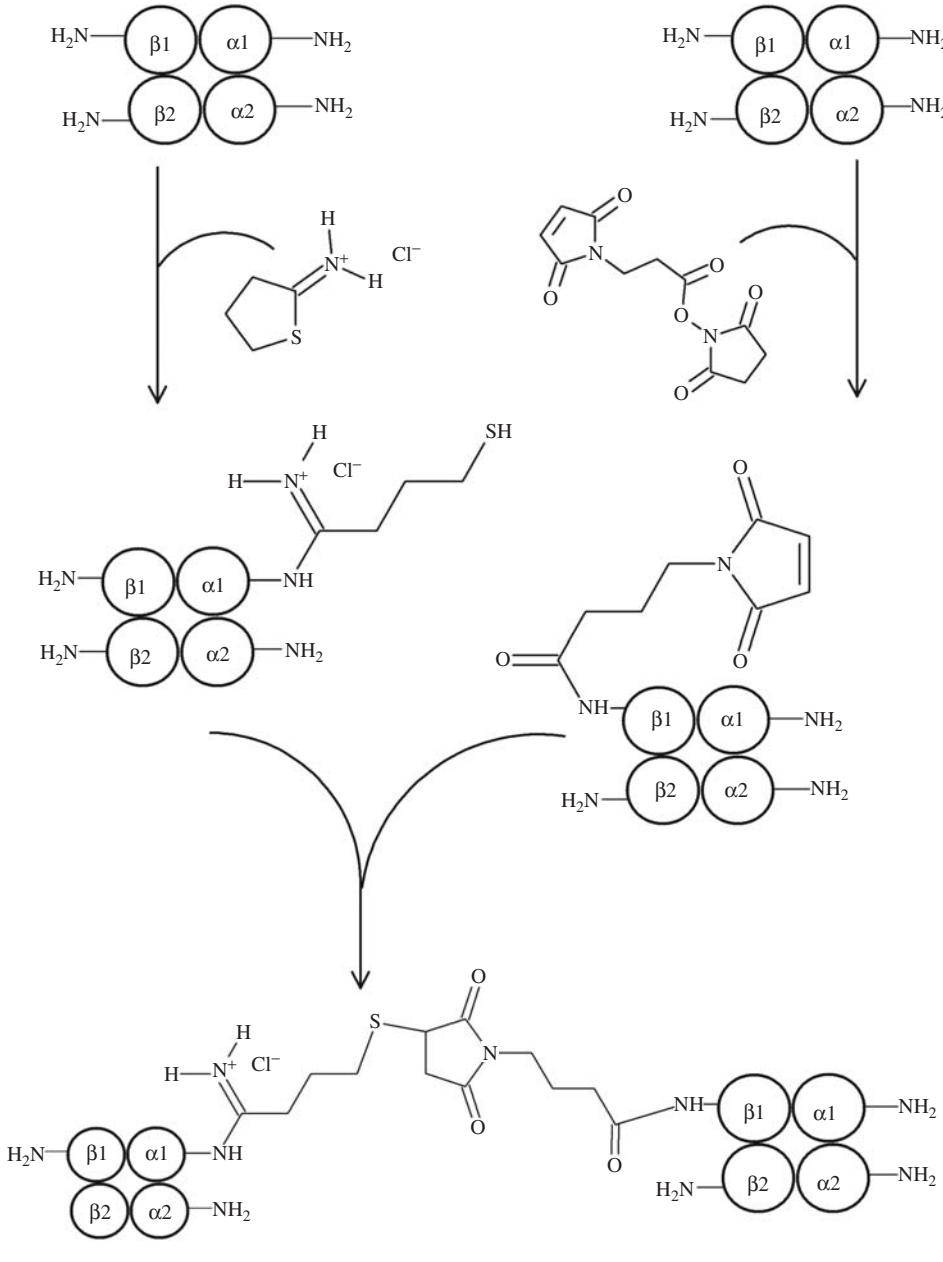

Figure 24.6 *Polymerization by sulfhydryl–maleimide click chemistry [86].*

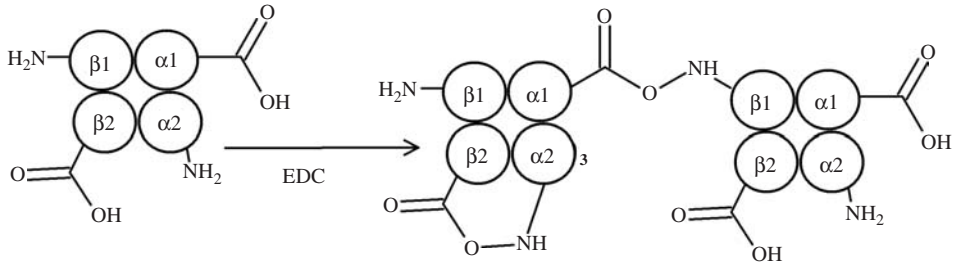

Figure 24.7 *Zero-length crosslinking of Hb [88].*

reactions involving the Hb component of the complex [99] and alleviated oxidative stress in an ischemia/reperfusion model [100]. Kluger and coworkers produced a 1 : 1 Hb–SOD complex using site-specific reagents to introduce a reactive thiol group on to the Hb and a maleimide group on the SOD [101]. An Hb–SOD fusion protein with enhanced antioxidative properties has also been genetically engineered [102]. Olsen and coworkers used thiol-maleimide chemistry to generate an Hb polymer containing both catalase and SOD [86]. While it is clear that these Hb–catalase–SOD conjugates must undergo significantly more biological testing to prove their worth, the fact that they decrease the autoxidation rates of the Hb component is encouraging.

24.4 Conclusion

The field of blood substitutes today is very diverse, with multiple strategies being utilized to address the complex physiology of blood. To say that one strategy is superior to another is difficult, with almost every attempt experiencing problems. A review of the literature suggests that there is a tendency to push newly developed compounds into human clinical trials too quickly. While this strategy has greatly increased our understanding of the effectiveness and safety profiles of these products, it has led to a series of failures in clinical trials. One reason for this rapidity of starting clinical trials might be the escalating costs of research. With low amounts of government funding for blood-substitute projects at present, most researchers take their products from academic research settings to the entrepreneurial realm (e.g. PolyHeme, HBOC-201, o-raffinose-modified Hb, MP4). The current state of blood substitutes is very troubling, considering the need for blood products is rising, but the amount of knowledge that has been generated as a result of all of these past strategies is significant.

The following considerations appear to be important in making a better potential blood substitute. First, it is important to have a large polymer to reduce vasoconstriction. Second, crosslinking the tetramer before polymerization is beneficial, because it stabilizes the protein and controls oxygen affinity. Third, a species with high oxygen affinity is probably preferable [103]. Fourth, adding catalase and SOD to the polymer will help prevent some of the oxidative problems associated with HBOCs and may decrease tissue damage. Fifth, the complex needs to be nonimmunogenic, which may require masking the chemical modification by PEGylating the complex. Even with all of these factors being met, the real proof will always be clinical testing.

References

[1] Riess, J.G. Oxygen carriers ("blood substitutes"): raison d'etre, chemistry, and some physiology, *Chem. Rev*., **101**, 2797–2920 (2001).

[2] Lowe, K.C. Blood substitutes: from chemistry to clinic, *J. Mater. Chem*., **16**, 4189–4196 (2006).

[3] Bruno, S., Ronda, L., Faggiano, S. *et al*. Oxygen delivery via allosteric effectors of hemoglobin and blood substitutes, in *Burgers Medicinal Chemistry, Drug Discovery and Development*, Vol. 4 (ed. D.J. Abraham), John Wiley & Sons Ltd., New York, pp. 1–51 (2010).

[4] Bunn, H.F., Esham, W.T. and Bull, R.W. The renal handling of hemoglobin. I. Glomerular filtration, *J. Exp. Med*., **129**, 909–923 (1969).

[5] Chan, W.L., Tang, N.L., Yim, C.C. *et al*. New features of renal lesion induced by stroma free hemoglobin, *Toxicol. Pathol*., **28**, 635–642 (2000).

[6] Walder, J.A., Zaugg, R.H., Walder, R.Y. *et al*. Diaspirins that cross-link beta chains of hemoglobin: bis(3,5-dibromosalicyl) succinate and bis(3,5-dibromosalicyl) fumarate, *Biochemistry*, **18**, 4265–4270 (1979).

[7] Walder, J.A., Walder, R.Y. and Arnone, A. Development of antisickling compounds that chemically modify hemoglobin S specifically within the 2,3-diphosphoglycerate binding site, *J. Mol. Biol*., **141**, 195–216 (1980).

[8] Zaugg, R.H., Walder, J.A., Walder, R.Y. *et al*. Modification of hemoglobin with analogs of aspirin, *J. Biol. Chem*., **255**, 2816–2821 (1980).

[9] Fernandez, E.J., Abad-Zapatero, C. and Olsen, K.W. Crystal structure of Lysβ_1 82-Lysβ_2 82 crosslinked hemoglobin: a possible allosteric intermediate, *J. Mol. Biol*., **296**, 1245–1256 (2000).

[10] Chatterjee, R., Welty, E.V., Walder, R.Y. *et al*. Isolation and characterization of a new hemoglobin derivative cross-linked between the alpha chains (lysine 99 α_1–lysine 99 α_2), *J. Biol. Chem*., **261**, 9929–9937 (1986).

[11] Highsmith, F.A., Driscoll, C.M., Chung, B.C. *et al*. An improved process for the production of sterile modified haemoglobin solutions, *Biologicals*, **25**, 257–268 (1997).

[12] Snyder, S.R., Welty, E.V., Walder, R.Y. *et al*. HbXL99 alpha: a hemoglobin derivative that is cross-linked between the alpha subunits is useful as a blood substitute, *Proc. Natl. Acad. Sci. U S A*, **84**, 7280–7284 (1987).

[13] Tye, R.W., Medina, F., Bolin, R.B. *et al*. Modification of hemoglobin: tetrameric stabilization, *Prog. Clin. Biol. Res*., **122**, 41–49 (1983).

[14] Keipert, P.E., Gomez, C.L., Gonzales, A. *et al*. Diaspirin cross-linked hemoglobin: tissue distribution and long-term excretion after exchange transfusion, *J. Lab. Clin. Med*., **123**, 701–711 (1994).

[15] Powell, C.C., Schultz, S.C., Burris, D.G. *et al*. Subcutaneous oxygen tension: a useful adjunct in assessment of perfusion status, *Crit. Care Med*., **23**, 867–873 (1995).

[16] Reah, G., Bodenham, A.R., Mallick, A. *et al*. Initial evaluation of diaspirin cross-linked hemoglobin (DCLHb) as a vasopressor in critically ill patients, *Crit. Care Med*., **25**, 1480–1488 (1997).

[17] Cole, D.J., Nary, J.C., Drummond, J.C. *et al*. Alpha-alpha diaspirin crosslinked hemoglobin, nitric oxide, and cerebral ischemic injury in rats, *Artif. Cells Blood Substit. Immobil. Biotechnol*., **25**, 141–152 (1997).

[18] Doherty, D.H., Doyle, M.P., Curry, S.R. *et al*. Rate of reaction with nitric oxide determines the hypertensive effect of cell-free hemoglobin, *Nat. Biotechnol*., **16**, 672–676 (1998).

[19] White, F.L. and Olsen, K.W. Effects of crosslinking on the thermal stability of hemoglobin. I. The use of bis(3,5-dibromosalicyl) fumarate, *Arch. Biochem. Biophys*., **258**, 51–57 (1987).

[20] Azari, M., Catarello, J., Burhop, K. *et al*. Validation of the heat treatment step used in the production of diaspirin crosslinked hemoglobin (DCLHb) for viral inactivation: effect of crosslinking, *Artif. Cells Blood Substit. Immobil. Biotechnol*., **25**, 521–526 (1997).

[21] Burhop, K., Gordon, D. and Estep, T. Review of hemoglobin-induced myocardial lesions, *Artif. Cells Blood Substit. Immobil. Biotechnol*., **32**, 353–374 (2004).

[22] Goldman, D.W., Breyer, R.J. III, Yeh, D. *et al*. Acellular hemoglobin-mediated oxidative stress toward endothelium: a role for ferryl iron, *Am. J. Physiol*, **275**, H1046–H1053 (1998).

[23] Olsen, S.B., Tang, D.B., Jackson, M.R. *et al*. Enhancement of platelet deposition by cross-linked hemoglobin in a rat carotid endarterectomy model, *Circulation*, **93**, 327–332 (1996).

[24] Saxena, R., Wijnhoud, A.D., Carton, H. *et al*. Controlled safety study of a hemoglobin-based oxygen carrier, DCLHb, in acute ischemic stroke, *Stroke*, **30**, 993–996 (1999).

[25] Sloan, E.P., Koenigsberg, M., Gens, D. *et al*. Diaspirin cross-linked hemoglobin (DCLHb) in the treatment of severe traumatic hemorrhagic shock: a randomized controlled efficacy trial, *JAMA*, **282**, 1857–1864 (1999).

[26] Kerner, T., Ahlers, O., Veit, S. *et al*. DCL-Hb for trauma patients with severe hemorrhagic shock: the European "On-Scene" multicenter study, *Intensive Care Med*., **29**, 378–385 (2003).

[27] Olsen, K.W., Zhang, Q.Y., Huang, H. *et al*. Stabilities and properties of multilinked hemoglobins, *Biomater. Artif. Cells Immobil. Biotechnol*., **20**, 283–285 (1992).

[28] Jones, R.T., Shih, D.T., Fujita, T.S. *et al*. A doubly cross-linked human hemoglobin. Effects of cross-links between different subunits, *J. Biol. Chem*., **271**, 675–680 (1996).

[29] Nacharaju, P., Friedman, J.M., Prabhakaran, M. *et al*. Combining the influence of two low O_2 affinity-inducing chemical modifications of the central cavity of hemoglobin, *Biochemistry*, **46**, 4554–4564 (2007).

[30] Kluger, R. 1994 Syntex award lecture: anionic electrophiles, protein modification, and artificial blood, *Can. J. Chem*., **72**, 2193–2197 (1994).

[31] Kluger, R. 2001 Lemieux award lecture: organic chemistry and hemoglobin: benefits from controlled alteration, *Can. J. Chem*., **80**, 217–221 (2002).

[32] Kluger, R. and Li, X. Efficient chemical introduction of a disulfide cross-link and conjugation site into human hemoglobin at beta-lysine-82 utilizing a bifunctional aminoacyl phosphate, *Bioconjug. Chem*., **8**, 921–926 (1997).

[33] Hosmane, R.S., Peri, S.P., Bhadti, V.S. and Macdonald, V.W. Bis[2-(4-carboxy phenoxy)carbonylethyl]phosphinic acid (BCCEP): a novel affinity reagent for the beta-cleft modification of human hemoglobin, *Bioorg. Med. Chem*., **6**, 767–783 (1998).

[34] Roach, T.A., Macdonald, V.W. and Hosmane, R.S. A novel site-directed affinity reagent for cross-linking human hemoglobin: bis[2-(4-phosphono oxyphenoxy)carbonylethyl]phosphinic acid, *J. Med. Chem*., **47**, 5847–5859 (2004).

[35] Marden, M.C., Cabanes-Macheteau, M., Babes, A. *et al*. Control of the allosteric equilibrium of hemoglobin by cross-linking agents, *Protein Sci*., **11**, 1376–1383 (2002).

[36] Arnone, A., Benesch, R.E. and Benesch, R. Structure of human deoxyhemoglobin specifically modified with pyridoxal compounds, *J. Mol. Biol*., **115**, 627–642 (1977).

[37] Benesch, R.E., Ikeda, S. and Benesch, R. Reaction of haptoglobin with hemoglobin covalently cross-linked between the alpha beta dimers, *J. Biol. Chem*., **251**, 465–470 (1976).

[38] Jones, R.T., Head, C.G., Fujita, T.S. *et al*. Modification of human hemoglobin with methyl acyl phosphates derived from dicarboxylic acids: systematic relationships between cross-linked structure and oxygen-binding properties, *Biochemistry*, **32**, 215–223 (1993).

[39] Bobofchak, K.M., Tarasov, E. and Olsen, K.W. Effect of cross-linker length on the stability of hemoglobin, *Biochim. Biophys. Acta*, **1784**, 1410–1414 (2008).

[40] Huang, H. and Olsen, K.W. Thermal stabilities of hemoglobins crosslinked with different length reagents, *Artif. Cells Blood Substit. Immobil. Biotechnol*., **22**, 719–724 (1994).

[41] Zhang, Q. and Olsen, K.W. The modification of hemoglobin by a long crosslinking reagent: bis(3,5-dibromosalicyl) sebacate, *Biochem. Biophys. Res. Commun*., **203**, 1463–1470 (1994).

[42] Bucci, E., Razynska, A., Kwansa, H. *et al*. Production and characteristics of an infusible oxygen-carrying fluid based on hemoglobin intramolecularly cross-linked with sebacic acid, *J. Lab. Clin. Med*., **128**, 146–153 (1996).

[43] Kluger, R., Wodzinska, J., Jones, R.T. *et al*. Three-point cross-linking: potential red cell substitutes from the reaction of trimesoyl tris(methyl phosphate) with hemoglobin, *Biochemistry*, **31**, 7551–7559 (1992).

[44] Zheng, Y. and Olsen, K.W. Tris(3,5-dibromosalicyl) tricarballylate crosslinked hemoglobin: functional evaluation, *Artif. Cells Blood Substit. Immobil. Biotechnol*., **24**, 587–598 (1996).

[45] Paal, K., Jones, R.T. and Kluger, R.A Site-specific tetrafunctional reagent for protein modification: cross-linked hemoglobin with two sites for further reaction, *J. Am. Chem. Soc*., **118**, 10380–10383 (1996).

[46] Huang, H., Zhang, Q.Y. and Olsen, K.W. Design and synthesis of hemoglobin multilinkers, in *Book of Abstracts*, *210th ACS National Meeting*, **210**, ORGN–377 (1995).

[47] Kluger, R., Lock-O'Brien, J. and Teytelboym, A. Connecting proteins by design: cross-linked bis-hemoglobin, *J. Am. Chem. Soc*., **121**, 6780–6785 (1999).

[48] Gourianov, N. and Kluger, R. Conjoined hemoglobins: loss of cooperativity and protein-protein interactions, *Biochemistry*, **44**, 14989–14999 (2005).
[49] Hu, D. and Kluger, R. Functional cross-linked hemoglobin bis-tetramers: geometry and cooperativity, *Biochemistry*, **47**, 12551–12561 (2008).
[50] Kluger, R., Foot, J.S. and Vandersteen, A.A. Protein-protein coupling and its application to functional red cell substitutes, *Chem. Commun*., **46**, 1194–1202 (2010).
[51] Lieberthal, W., Fuhro, R., Freedman, J.E. *et al*. O-raffinose cross-linking markedly reduces systemic and renal vasoconstrictor effects of unmodified human hemoglobin, *J. Pharmacol. Exp. Ther*., **288**, 1278–1287 (1999).
[52] Nelson, D., Hai, T., Srnak, A. *et al*. Synthesis and properties of polymerized, diaspirin cross-linked hemoglobins, *Biomater. Artif. Cells Immobil. Biotechnol*., **20**, 253–258 (1992).
[53] Carmichael, F.J., Ali, A.C., Campbell, J.A. *et al*. A phase I study of oxidized raffinose cross-linked human hemoglobin, *Crit. Care Med*., **28**, 2283–2292 (2000).
[54] Carmichael, F.J. Recent developments in hemoglobin-based oxygen carriers: an update on clinical trials, *Transfus. Apher. Sci*., **24**, 17–21 (2001).
[55] Cheng, D.C. Safety and efficacy of o-raffinose cross-linked human hemoglobin (Hemolink) in cardiac surgery, *Can. J. Anaesth*., **48**, S41–S48 (2001).
[56] Wicks, D., Wong, L.T., Sandhu, R. *et al*. The intravascular persistence and methemoglobin formation of Hemolink (hemoglobin raffimer) in dogs, *Artif. Cells Blood Substit. Immobil. Biotechnol*., **31**, 1–17 (2003).
[57] Hill, S.E., Gottschalk, L.I. and Grichnik, K. Safety and preliminary efficacy of hemoglobin raffimer for patients undergoing coronary artery bypass surgery, *J. Cardiothorac. Vasc. Anesth*., **16**, 695–702 (2002).
[58] Habeeb, A.J. and Hiramoto, R. Reaction of proteins with glutaraldehyde, *Arch. Biochem. Biophys*., **126**, 16–26 (1968).
[59] Eike, J.H. and Palmer, A.F. Effect of Cl^- and H^+ on the oxygen binding properties of glutaraldehyde-polymerized bovine hemoglobin-based blood substitutes, *Biotechnol. Prog*., **20**, 1543–1549 (2004).
[60] Wine, Y., Cohen-Hadar, N., Freeman, A. and Frolow, F. Elucidation of the mechanism and end products of glutaraldehyde crosslinking reaction by X-ray structure analysis, *Biotechnol. Bioeng*., **98**, 711–718 (2007).
[61] Beauchamp, R.O. Jr, St Clair, M.B., Fennell, T.R. *et al*. A critical review of the toxicology of glutaraldehyde, *Crit. Rev. Toxicol*., **22**, 143–174 (1992).
[62] Marini, M.A., Moore, G.L., Christensen, S.M. *et al*. Reexamination of the polymerization of pyridoxylated hemoglobin with glutaraldehyde, *Biopolymers*, **29**, 871–882 (1990).
[63] Fronticelli, C., Bucci, E. and Orth, C. Solvent regulation of oxygen affinity in hemoglobin: sensitivity of bovine hemoglobin to chloride ions, *J. Biol. Chem*., **259**, 10841–10844 (1984).
[64] Fronticelli, C. and Bucci, E. Conformational and functional characteristics of bovine hemoglobin, *Methods Enzymol*., **231**, 150–163 (1994).
[65] Harringer, W., Hodakowski, G.T., Svizzero, T. *et al*. Acute effects of massive transfusion of a bovine hemoglobin blood substitute in a canine model of hemorrhagic shock, *Eur. J. Cardiothorac. Surg*., **6**, 649–654 (1992).

[66] Hayward, R. and Lefer, A.M. Administration of polymerized bovine hemoglobin improves survival in a rat model of traumatic shock, *Methods Find. Exp. Clin. Pharmacol*., **21**, 427–433 (1999).

[67] Slanetz, P.J., Lee, R., Page, R. *et al*. Hemoglobin blood substitutes in extended preoperative autologous blood donation: an experimental study, *Surgery*, **115**, 246–254 (1994).

[68] Standl, T., Horn, P., Wilhelm, S. *et al*. Bovine haemoglobin is more potent than autologous red blood cells in restoring muscular tissue oxygenation after profound isovolaemic haemodilution in dogs, *Can. J. Anaesth*., **43**, 714–723 (1996).

[69] Botzlar, A., Steinhauser, P. and Nolte, D. Effects of ultra-purified polymerized bovine hemoglobin on local tissue oxygen tension in striated skin muscle: an efficacy study in the hamster, *Eur. Surg. Res*., **34**, 106–113 (2002).

[70] Caswell, J.E., Strange, M.B., Rimmer, D.M. III *et al*. A novel hemoglobin-based blood substitute protects against myocardial reperfusion injury, *Am. J. Physiol Heart Circ. Physiol*, **288**, H1796–H1801 (2005).

[71] Freilich, D., Branda, R., Hacker, M. *et al*. Decreased lactic acidosis and anemia after transfusion of o-raffinose cross-linked and polymerized hemoglobin in severe murine malaria, *Am. J. Trop. Med. Hyg*., **60**, 322–328 (1999).

[72] Gonzalez, P., Hackney, A.C., Jones, S. *et al*. A phase I/II study of polymerized bovine hemoglobin in adult patients with sickle cell disease not in crisis at the time of study, *J. Investig. Med*., **45**, 258–264 (1997).

[73] Lee, R., Neya, K., Svizzero, T.A. and Vlahakes, G.J. Limitations of the efficacy of hemoglobin-based oxygen-carrying solutions, *J. Appl. Physiol*, **79**, 236–242 (1995).

[74] Mullon, J., Giacoppe, G., Clagett, C. *et al*. Transfusions of polymerized bovine hemoglobin in a patient with severe autoimmune hemolytic anemia, *N. Engl. J. Med*., **342**, 1638–1643 (2000).

[75] Sprung, J., Kindscher, J.D., Wahr, J.A. *et al*. The use of bovine hemoglobin glutamer-250 (Hemopure) in surgical patients: results of a multicenter, randomized, single-blinded trial, *Anesth. Analg*., **94**, 799–808, table (2002).

[76] Fitzpatrick, C.M., Savage, S.A., Kerby, J.D. *et al*. Resuscitation with a blood substitute causes vasoconstriction without nitric oxide scavenging in a model of arterial hemorrhage, *J. Am. Coll. Surg*., **199**, 693–701 (2004).

[77] Hodakowski, G.T., Page, R.D., Harringer, W. *et al*. Ultra-pure polymerized bovine hemoglobin blood substitute: effects on the coronary circulation, *Biomater. Artif. Cells Immobil. Biotechnol*., **20**, 669–672 (1992).

[78] Knudson, M.M., Lee, S., Erickson, V. *et al*. Tissue oxygen monitoring during hemorrhagic shock and resuscitation: a comparison of lactated Ringer's solution, hypertonic saline dextran, and HBOC-201, *J. Trauma*, **54**, 242–252 (2003).

[79] Vlahakes, G.J., Lee, R., Jacobs, E.E. Jr *et al*. Hemodynamic effects and oxygen transport properties of a new blood substitute in a model of massive blood replacement, *J. Thorac. Cardiovasc. Surg*., **100**, 379–388 (1990).

[80] Henderson, C.L., Anderson, C.M., Sorrells, D.L. *et al*. The use of a hemoglobin-based oxygen-carrying solution (HBOC-201) for extracorporeal membrane oxygenation in a porcine model with acute respiratory distress syndrome, *Pediatr. Crit. Care Med*., **5**, 384–390 (2004).

[81] Gould, S.A. and Moss, G.S. Clinical development of human polymerized hemoglobin as a blood substitute, *World J. Surg*., **20**, 1200–1207 (1996).
[82] Gould, S.A., Sehgal, L.R., Sehgal, H.L. and Moss, G.S. Artificial blood: current status of hemoglobin solutions, *Crit. Care Clin*., **8**, 293–309 (1992).
[83] Sehgal, L.R., Sehgal, H.L., Rosen, A.L. *et al*. Characteristics of polymerized pyridoxylated hemoglobin, *Biomater. Artif. Cells Artif. Organs*, **16**, 173–183 (1988).
[84] Wang, X., Huang, L., Wang, J.F. and Yang, C.M. A novel dynamic heterogeneous phase polymerization reaction for poly-hemoglobin with narrow molecular weight distribution, *Artif. Cells Blood Substit. Immobil. Biotechnol*., **36**, 439–444 (2008).
[85] Zhu, X., Chu, W., Wang, T. *et al*. Variations in dominant antigen determinants of glutaraldehyde polymerized human, bovine and porcine hemoglobin, *Artif. Cells Blood Substit. Immobil. Biotechnol*., **35**, 518–532 (2007).
[86] Tarasov, E., Blaszak, M.M., LaMarre, J.M. and Olsen, K.W. Synthesis of a hemoglobin polymer containing antioxidant enzymes using complementary chemistry of maleimides and sulfhydryls, *Artif. Cells Blood Substit. Immobil. Biotechnol*., **35**, 31–43 (2007).
[87] Baudin-Creuza, V., Chauvierre, C., Domingues, E. *et al*. Octamers and nanoparticles as hemoglobin based blood substitutes, *Biochim. Biophys. Acta*, **1784**, 1448–1453 (2008).
[88] Matheson, B., Kwansa, H.E., Bucci, E. *et al*. Vascular response to infusions of a nonextravasating hemoglobin polymer, *J. Appl. Physiol*, **93**, 1479–1486 (2002).
[89] Heaphy, S. and Tregear, R. Stoichiometry of covalent actin-subfragment 1 complexes formed on reaction with a zero-length cross-linking compound, *Biochemistry*, **23**, 2211–2214 (1984).
[90] Bucci, E., Kwansa, H., Koehler, R.C. and Matheson, B. Development of zero-link polymers of hemoglobin, which do not extravasate and do not induce pressure increases upon infusion, *Artif. Cells Blood Substit. Immobil. Biotechnol*., **35**, 11–18 (2007).
[91] Harrington, J.P., Orlig, K., Zito, S.L. *et al*. Structural and redox behavior of OxyVita, a zero-linked polymeric hemoglobin: comparison with natural acellular polymeric hemoglobins, *Artif. Cells Blood Substit. Immobil. Biotechnol*., **38**, 64–68 (2010).
[92] Jia, Y. and Alayash, A.I. Effects of cross-linking and zero-link polymerization on oxygen transport and redox chemistry of bovine hemoglobin, *Biochim. Biophys. Acta*, **1794**, 1234–1242 (2009).
[93] Yang, T. and Olsen, K.W. The effect of crosslinking by bis(3,5-dibromosalicyl) fumarate on the autoxidation of hemoglobin, *Biochem. Biophys. Res. Commun*., **163**, 733–738 (1989).
[94] Dragan, S.A., Olsen, K.W., Moore, E.G. and Fitch, A. Spectroelectrochemical study of hemoglobin A, alpha- and beta-fumarate crosslinked hemoglobins; implications to autoxidation reaction, *Bioelectrochemistry*, **73**, 55–63 (2008).
[95] Alayash, A.I. Oxygen therapeutics: can we tame haemoglobin?, *Nat. Rev. Drug Discov*., **3**, 152–159 (2004).
[96] Buehler, P.W. and Alayash, A.I. Toxicities of hemoglobin solutions: in search of in-vitro and in-vivo model systems, *Transfusion*, **44**, 1516–1530 (2004).

[97] Everse, J. and Hsia, N. The toxicities of native and modified hemoglobins, *Free Radic. Biol. Med*., **22**, 1075–1099 (1997).
[98] Yang, T. and Olsen, K.W. Enzymatic protection from autoxidation for crosslinked hemoglobins, *Artif. Cells Blood Substit. Immobil. Biotechnol*., **22**, 709–717 (1994).
[99] D'Agnillo, F. and Chang, T.M. Cross-linked hemoglobin-superoxide dismutase-catalase scavenges oxygen-derived free radicals and prevents methemoglobin formation and iron release, *Biomater. Artif. Cells Immobil. Biotechnol*., **21**, 609–621 (1993).
[100] Razack, S., D'Agnillo, F. and Chang, T.M. Crosslinked hemoglobin-superoxide dismutase-catalase scavenges free radicals in a rat model of intestinal ischemia-reperfusion injury, *Artif. Cells Blood Substit. Immobil. Biotechnol*., **25**, 181–192 (1997).
[101] Alagic, A., Koprianiuk, A. and Kluger, R. Hemoglobin-superoxide dismutase chemical linkages that create a dual-function protein, *J. Am. Chem. Soc*., **127**, 8036–8043 (2005).
[102] Grey, M., Yainoy, S., Prachayasittikul, V. and Bulow, L. A superoxide dismutase-human hemoglobin fusion protein showing enhanced antioxidative properties, *FEBS J*., **276**, 6195–6203 (2009).
[103] Vandegriff, K.D., Malavalli, A., Wooldridge, J. *et al*. MP4, a new nonvasoactive PEG-Hb conjugate, *Transfusion*, **43**, 509–516 (2003).

25

Engineering the Molecular Shape of PEG-Hemoglobin Adducts for Supraperfusion

Seetharama A. Acharya[1], *Marcos Intaglietta*[2], *Amy G. Tsai*[2], *Kulal Ananda*[1] *and Fantao Meng*[1]

[1]*Departments of Medicine and of Physiology and Biophysics, Albert Einstein College of Medicine, Bronx, NY, USA*

[2]*Department of Bioengineering, University of California, San Diego, La Jolla, CA, USA*

25.1 Introduction

Initial blood losses can be corrected using volume-restitution fluids (plasma expanders, PEs), since the related anemia is usually inconsequential in challenging the capacity of the circulation to deliver O_2 and extract tissue metabolic byproducts [1]. As blood losses or anemia progress, the "transfusion trigger" is reached and the decrease in the O_2 carrying capacity increases the risk of hypoxia, necessitating blood transfusion. Restitution of circulatory volume and of O_2 carrying capacity in treating blood losses are intertwined and usually proceed sequentially.

In designing the blood substitute, both of these aspects of blood loss should be addressed simultaneously. However, derivatives of hemoglobin (Hb) designed early on as potential blood substitutes – intramolecularly crosslinked, oligomerized, and even recombinant Hbs – were not designed with the above concept in mind. The vasoconstrictive

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activity of most of the intramolecularly crosslinked and oligomerized Hbs has been the major impediment to the development of Hb-based blood substitutes, as these products induce hypertension when infused.

25.2 Enzon DecaPEGylated Bovine Hb is Nonhypertensive

The generation of polyethylene glycol (PEG)-conjugated Hb has profoundly influenced our thinking on the design of blood substitutes [2]. PEG bovine Hb is a conjugate of bovine Hb with the synthetic biopolymer PEG-5K (5 kDa PEG). Bovine Hb, a low-O_2-affinity Hb, has been conjugated with 10 copies of PEG-5K through urethane linkage in designing this product. The decaPEGylation of Hb induces PE-like solution properties to Hb. It induces an increase in colloidal osmotic pressure (COP), viscosity, and an unusually high molecular volume (for the mass of PEG conjugated) relative to the unPEGylated control, and is coupled with a much-reduced mean arterial pressure (MAP) as well as a high functional capillary density (FCD) in experimental animal models relative to nonPEGylated Hbs, just like high-viscosity PEs. Thus this PEGylated Hb is an unusual PE in that its viscosity is lower than that of the low-viscosity PEs such as dextran 70 kDa, but it exhibits increased FCD on infusion just like high-viscosity PEs such as dextran 500 kDa. PEGylation of Hb has essentially no influence on the NO scavenging activity of Hb *in vitro*. The unusually high hydrodynamic volume of the PEGylated sample attenuates its extravasation, and thus can reduce extravasation-mediated *in vivo* NO scavenging. This, together with the effect on the viscosity and COP, increases the FCD, making it a nonhypertensive derivative of Hb.

However, PEGylation of bovine Hb also significantly increases its O_2 affinity. The high oxygen affinity is another distinguishing feature of PEG-Hb derivatives as compared to other nonPEGylated Hb derivatives. Extensive microcirculatory studies with PEGylated Hb have established that PEGylation of bovine Hb increases FCD. Thus, in contrast to molecular approaches based on site-directed mutagenesis that tinker with the structure of the Hb to reduce NO binding activity, PEGylation appears to attenuate the vasoaconstrictive activity of acellular Hb by the autotransfusion facilitated increase in FCD and by reducing the autoregulatory activity associated with the low O_2 affinity of Hb derivatives. Accordingly, engineering of PE-like properties to Hb through PEGylation, along with an increase in the oxygen affinity of Hb, has been advanced as a new paradigm for the design of Hb-based nonhypertensive O_2-carrying PEs.

The Enzon PEGylated bovine Hb does not carry any intramolecular crosslinks, unlike other Hb derivatives developed previously as blood substitutes, but it appears to have very little nephrotoxicity. This is apparently due to its large hydrodynamic volume, which limits its kidney filtration. Ajinomoto PEG-Hb, which is a pyridoxylated, oligomerized, and PEG surface-decorated Hb generated using bifunctional bis succinimidyl PEG-3K [3, 4] shows only a transient period of nonhypertensive activity [5]. This product exhibits an O_2 affinity (measured as p50) around 20 mm Hg, intermediate to that of red blood cells (RBCs) and Enzon PEG bovine Hb. Given the lower affinity of the Ajinomoto product as compared to Enzon PEG-Hb, a total PEG mass of 30 kDa is probably not adequate, and a higher level of 50 kDa or more may be necessary to attenuate its vasoactivity [4].

25.3 EAF HexaPEGylated Hb (EAF P5K6-Hb) is Nonhypertensive

A new PEGylation platform referred to as Extension Arm Facilitated (EAF) PEGylation which simplifies the protein PEGylation protocol and simultaneously reduces the cost of large-scale production of PEGylated proteins was developed at Albert Einstein College of Medicine, Bronx, NY [5–9]. The new PEGylation platform involves the thiolation of the protein using a heterobifunctional reagent to obtain a desired level of a new functional group (thiol) to be used as the targeted site for conjugating the PEG chains via a complementary functionalized PEG. 2-iminothiolane (IT) has been chosen as the thiolation reagent in the initial studies. IT does not carry free thiols, and free thiol groups are generated *in situ* only after the reaction of the reagent with the protein amino groups. Accordingly, the protein can be incubated with IT in the presence of maleimide PEG without the danger of the free thiolating reagent consuming the maleimide PEG. In the thiolation reaction, the 2-IT reacts with the protein amino groups and converts them into positively charged amidine linkages. Thus, in the PEGylated protein, the original positive charges of the amino groups are conserved even though they are derivatized; accordingly, this approach is referred to as "Conservative EAF PEGylation" (Figure 25.1).

The thiolation-mediated EAF PEGylation of Hb has been optimized for hexaPEGylation of oxy-Hb [6, 7]. Hb is completely PEGylated in this reaction and no free Hb species are left behind in the product. The reaction generates a very narrow distribution of the PEGylated species, the hexaPEGylated being the most abundant molecule, with equal distribution of penta- and heptaPEGylated species. The PEGylated product is isolated from excess PEG reagent by tangential flow filtration. One-step EAF PEGylation protocol is the preferred mode as it is simpler for scaling up. A two-step process has also been developed, as the control experiments have established that the thiolated Hb is stable and does not generate disulfide bonds even under oxy conditions. In a two-step EAF PEGylation process the thiolation is carried out in the absence of maleimide, and once the thiolation is carried out, a concentrated stock solution of PEG maleimide is added to get the desired concentration of Hb. In the two-step process, thiolation is carried out at a higher protein concentration and the PEGylation at a lower protein concentration, thus a possibility exists that the distribution of the PEGylated Hbs in the one-step and two-step processes may be very distinct.

25.4 Molecular and Solution Properties of EAF HexaPEGylated Human Hb (EAF-P5K6-Hb)

The estimation of the number of thiols on Hb in the control thiolation experiment using 10-fold molar excess of 2-IT over Hb suggests the presence of six to seven titratable thiol groups per molecule, and accordingly a minimum of six to seven PEG-5K chains should be conjugated in the PEGylated Hb. Control experiments without thiolation have established the complete PEGylation of Cys-93(β) under the EAF PEGylation conditions. NMR estimation of the number of the PEG-5K chains confirmed that six to seven PEG 5K chains are in the product [6, 10].

Since the PEGylation of Cys-93(β) is essentially complete, the rest of the PEG chains are distributed on the thiols on the extension arms. The thiol-carrying extension arms

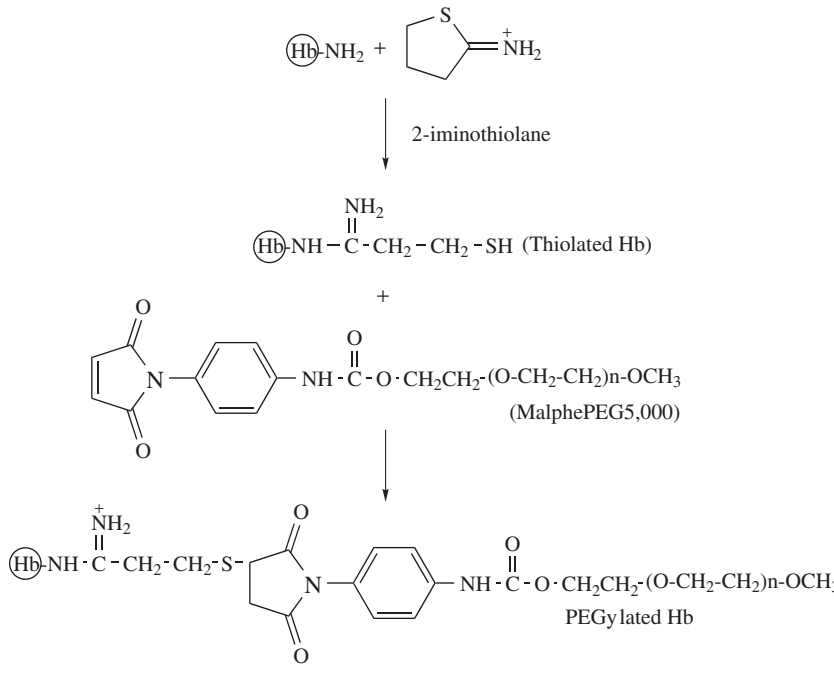


Figure 25.1 *Schematic representation of thiolation-mediated conservative EAF PEGylation. Hb is thiolated using 2-IT in the presence of maleimide PEG. The thiolating reagent, 2-IT, is a cyclic form of δ-mercapto-butyrimidate and carries no free thiol groups. But on reaction with protein amino groups, the thiol group is generated at the distal end of the extension arm. The nascent thiol generated* in situ *is the targeted functional group for reaction with maleimide PEG of desired molecular size. Since the thiol group is generated* in situ *on reaction of the reagent with protein, the thiolating reagent and PEG maleimide can be incubated together with the protein, without worry of the thiolating reagent consuming the PEG maleimide before it reacts with the protein. 2-IT derivatizes the amino group as substituted amine, and the positive charge of the ε-amino groups is conserved under the physiological conditions, and accordingly this approach has been referred to as "Conservative EAF PEGylation".*

are distributed over six to seven Lys residues; that is, the thiolation is restricted to a small number of reactive surface amino groups of Hb. Since the sites of thiolation are not quantitatively modified, and the thiolation of individual sites is partial, the EAF hexaPEGylated Hb is not a chemically homogeneous entity.

Nonetheless, EAF P5K6-Hb elutes as a nearly symmetrically homogeneous peak on Superose 12 columns. The elution position of the EAF hexaPEGylated Hb from a Superose 12 column corresponds to the position of four intramolecularly crosslinked Hb tetramers; that is, the hydrodynamic volume of Hb nearly quadrupled, assuming the hydrodynamic volume of a globular protein of a molecular mass of 256 kDa. Since the hexaPEGylation of Hb increases the molecular mass by only 30 kDa, it follows that PEG-5K chains are more than six times more efficient than the conjugation of a globular protein of molecular mass of 30 kDa in enhancing the hydrodynamic volume of the

Table 25.1 *Comparison of the EAF PEGylation-induced PE-like properties with those of SC PEGylated Bovine Hb.*

	Viscosity (cP)[a]	COP (mm Hg)[a]	Radius (nm)
HbA	0.84	8.6	3.2
EAF-P5K6-Hb	1.6	36	6.5
Prolong P5K10-BvHb[b]	2.1	51	5.5
Enzon P5K10-BvHb[c]	–	40	14.1[c]

[a]The protein concentration was 30 mg/mL. Viscosity was measured at 37 °C, COP was measured at room temperature.
[b]We thank Dr Abuchowski (Prolong Pharmaceuticals) for the samples of P5K10-BvHb.
[c]Data calculated from the COP data, assuming that no changes in the dissociation pattern of Hb have resulted from its PEGylation, and taken from [11].

protein when they are conjugated. PEGylation represents the engineering of a molecular shell of PEG chains of loose packing density around the central protein core of high packing density. The EAF PEG-Hb with multiple copies of PEG chains represents a unique class of semisynthetic hybrid biopolymers of an unusual structure, with a high packing density, rigid, central protein core, and an outer low-packing-density flexible PEG shell, distinct from that of conventional colloidal PEs.

The molecular radius of EAF hexaPEGylated Hb (EAF P5K6-Hb) is around 6 nm, as determined by dynamic light scattering – nearly double that of uncrosslinked Hb. The molecular radius of the Enzon PEG-Hb, which carries 10 copies of PEG-5K chains conjugated to the protein through urethane linkage, has been calculated to be around 15 nm from COP data (Table 25.1) and is significantly larger than the hexaPEGylated molecule designed by EAF PEGylation [11]. Thus the packing density of PEG chains in the PEG shell of Enzon PEG-Hb is considerably lower than that in the EAF P5K6-Hb. The lower efficiency of EAF PEGylation in increasing the hydrodynamic volume of Hb as compared to the direct PEGylation of bovine Hb apparently reflects the influence of the extension arm on the packing density of the PEG shell engineered around the molecule.

The viscosity of a 4% solution of EAF P5K6 Hb is around 2.2 cP and COP is 50 mm Hg. As judged by extreme hemodilution experiments in hamsters, this molecule is essentially vasoinactive and nonhypertensive. Attenuation of the vasoconstrictive activity of Hb derivative is the first critical requirement for its development as an O_2-carrying PE for transfusion.

25.5 High O_2 Affinity of EAF HexaPEGylated Hb and Tissue Oxygenation in Extreme Hemodilution

EAF hexaPEGylation of Hb increases its O_2 affinity; the p50 of Hb is lowered from about 14 mm Hg to about 8 mm Hg. This increase in the O_2 affinity is either a direct consequence of the chemical modification of the Cys-93(β) by PEG or an influence of the PEG shell on oxy–deoxy conformational transitions of Hb. The succinimidyl carbonate chemistry used for the preparation of Enzon PEG-Hb does not modify the crucial Cys-92(β) of bovine Hb; nonetheless the O_2 affinity is lowered to around 10 mm Hg, even though bovine Hb is a low-O_2-affinity Hb with an affinity close to that of αα-fumaryl Hb.

Can the high-O_2-affinity hexaPEGylated Hbs deliver O_2 to the target tissues? A comparison of the tissue oxygenation by EAF-P5K6-Hb in extreme hemodilution in hamster with a control run with 6% Dextran 70 shows that Hb content in plasma increases from 4% in control runs to about 5% using PEG-Hb samples, with the tissue pO_2 increasing from 1.8 to about 4 mm Hg. In these extreme hemodilution models, tissue pO_2 is considerably below the p50 of PEGylated Hb. Hence the low level of tissue oxygenation seen in these studies is not surprising. The important question as we design new PEG-Hbs is whether an O_2 affinity higher than the desired normal provides adequate levels of O_2 to the tissues when restoring the lost blood volume. It is our working hypothesis that the p50 of the PEG-Hb solutions should be slightly higher than the desired tissue pO_2, and be intermediate to that of RBCs and the desired tissue pO_2. p50 should not be too high; in this case, most of the carried O_2 is delivered in the arterial side of the circulation, triggering autoregulatory vasoconstriction.

25.6 Influence of Total PEG Mass Conjugated to Hb on O_2 Affinity and Tissue Oxygenation by PEG-Hbs

In designing new PEG-Hbs, it is also important to understand the role of the pattern of PEGylation and the total PEG mass conjugated to Hb on the delivery of O_2 to the tissues. The propensity of the PEG-Hbs to achieve a given level of tissue oxygenation could come from the pattern of PEGylation on O_2 affinity, the O_2 carrying capacity of the system (RBC in the system), the *in vivo* perfusion properties, or the tissue oxygenation that can be achieved by the RBC. If tissue pO_2 is higher than the p50 of PEG-Hb, it is unlikely that the PEG-Hb will contribute to tissue oxygenation. There appears to be a distinct influence of the pattern of PEGylation on tissue oxygenation: when the O_2 affinity is nearly the same, the PEG-Hbs with lower amounts of PEG mass achieve better tissue oxygenation. The tissue oxygenation by diPEGylated Hb (P5K2-Hb and P10K2-Hb) and by tetraPEGylated canine Hb (P5K4-CanHb) has also been compared in the extreme hemodilution model [12]. In all three PEGylated Hbs, Cys-93(β) is PEGylated, and all have nearly the same high O_2 affinity. Thus the diPEGylation pattern of P5K2-Hb gives the best tissue oxygenation; this correlates well with the blood flow [12].

In an attempt to increase the tissue oxygenation by PEG-Hbs, P5K2αα-fumaryl Hb with PEGylation on Cys-93(β) has been designed. The presence of the αα-fumaryl crosslink lowers the O_2 affinity of P5K2-Hb, the O_2 affinity of P5K2αα-fumaryl Hb being around 14 mm Hg. In spite of this reduction in the O_2 affinity of the P5K2-Hb molecule in αα-fumaryl intramolecular crosslinking, the efficacy of O_2 delivery by the diPEGylated Hb in the extreme perfusion model is not influenced. Therefore, the tissue oxygenation by PEG-Hb seen in the extreme hemodilution model is not a direct correlate of O_2 affinity of the PEG-Hb. A tetraPEGylated αα-fumaryl Hb generated by reaction of isothiocyanato phenyl PEG-5K with αα-fumaryl Hb has an O_2 affinity around 30 mm Hg. Also, (TCP PEG5K)$_4$-αα-fumaryl Hb with very low oxygen affinity did not improve the tissue oxygenation as compared to P5K2-Hb. These results suggest that tissue oxygenation to a level of 7 mm Hg represents the limit that can be achieved in the extreme hemodilution model, given the amount of RBCs remaining in this model. Recent studies have suggested that the RBC level in extreme hemodilution models is

indeed the limiting factor for tissue oxygenation [13]. Further studies with 50% exchange hemodilution model can help to map the correlation between the O_2 affinity of PEG-Hb and the tissue oxygenation.

25.7 Influence of PEGylation Chemistry on Structural, Functional, and Solution Properties of HexaPEGylated Hb

Five different conjugation chemistries have been used in our laboratory to prepare hexaPEGylated Hb using PEG-5K. In all reactions, PEGylation is targeted primarily to the surface amino groups. The approaches used to PEGylate Hb are: (i) 2-IT-mediated EAF-conservative PEGylation [6, 10, 14]; (ii) reductive alkylation chemistry [15, 16]; (iii) isothiocyanate chemistry [17]; (iv) acylation chemistry [18]; and (v) succinidimidyl carbonate chemistry. The first two PEGylation platforms do not neutralize the positive charge of the amino groups upon surface decoration of Hb with PEG chains; therefore they are c*onservative PEGylation platforms*. The PEG-Hb conjugates generated by the remaining three platforms neutralize the charge at the site of PEGylation, and are hence *nonconservative PEGylation platforms*. In the case of the first platform alone, extension arms are engineered between the protein amino group and the PEG chain.

Colligative, molecular, structural, and functional properties of hexaPEGylated Hb generated by all five PEGylation platforms have been extensively investigated and compared. Each hexaPEGylated Hb generated by different chemistries is very distinct in the perturbations induced at the heme center, as shown by CD spectroscopy at the Soret band and changes in the conformation at the $\alpha_1\beta_2$ interface and front-face fluorescence spectra of Trp-37(β) [15, 17].

One commonality in the PEGylation-induced changes of the functional properties of hexaPEGylated Hb (using different functionalized PEG-5K) is the increase in the O_2 affinity, irrespective of the PEGylation platform used. Apparently, the PEG shell generated on PEGylation of Hb with six copies of PEG-5K chains has a dominant influence on stabilization of the oxy conformation versus the deoxy conformation. Though the oxygenation-induced deoxy–oxy conformational transition of uncrosslinked Hb is strongly influenced by all PEGylation chemistries, the reductive alkylation and the thiocarbomoylation chemistry-based PEGylations induce the maximum influence, while the EAF PEGylation and acylation chemistry-based hexaPEGylation-induced increase in O_2 affinity is relatively lower [6, 15, 17, 18]. In the case of EAF PEGylation, the PEGylation-induced increase in the oxygen affinity is reduced only slightly, if Cys-93(β) is reversibly protected during PEGylation so that the thiol of Cys-93(β) is free in the final product. The increase in O_2 affinity of Hb on PEGylation is apparently a correlate of the PEG shell-induced overall conformational changes, coupled with the site-specific PEGylation. All hexaPEGylated Hbs generated by direct PEGylation platforms have free Cys-93(β), but still their O_2 affinity is slightly higher than that of the product generated by EAF PEGylation.

A stabilization of the oxy structure of Hb relative to the deoxy structure by a chemical modification generally increases the chemical reactivity of the thiol of Cys-93(β). The influence of direct hexaPEGylation on the O_2 affinity of Hb is higher than that of the product generated by EAF hexaPEGylation [18]. This suggests that all direct

hexaPEGylation protocols induce a significantly stronger oxy-conformation-stabilizing effect. This is surprising given that all direct PEGylations are directed to the amino groups, and Cys-93(β) is free in these hexaPEGylated Hbs; thus the change in the reactivity of Cys-93(β) appears to reflect the influence of the PEG shell on the quaternary structure of Hb.

In the uncrosslinked Hb molecule, the O_2 affinity of N-ethylmaleimide (NEM)-modified Hb is very close to that of EAF PEGylated Hb, implying that the influence of EAF hexaPEGylation at the ε-amino groups of Hb on O_2 affinity is not additive to that induced by PEGylation of Cys-93(β) using maleimide PEG.

The hexaPEGylation of αα-fumaryl Hb increases the reactivity of the thiol of Cys-93(β) to form mixed disulfide with 4,4'-dithiopyridine; that is, the oxy conformation of Hb is stabilized by PEGylation. This increase in the reactivity is also a function of PEGylation chemistry, and presumably reflects the overall impact of the PEG shell on the quaternary structure of crosslinked Hb. The reductive alkylation chemistry-based hexaPEGylation results in the highest increase. The high selectivity of reductive PEGylation for the α-amino groups, and the fact that reductive alkylation of the amino terminus of Hb with neutral aliphatic amino acids has an O_2-affinity-increasing effect, may be contributory for the PEGylation-induced high increase in O_2 affinity [19]. On the other hand, EAF PEGylation exclusively targeted to the ε-amino groups induces almost no change in the reactivity of Cys-93(β) to form mixed disulfide with dithiopyridine. The extension arms engineered between the PEG chains and the protein core attenuate the influence of the PEG shell on the oxygen affinity of Hb.

The molecular radius of the hexaPEGylated Hb (generated using direct PEGylation chemistry) is smaller than that of EAF hexaPEGylated Hb. The reductively hexaPEGylated Hb also exhibits the highest COP compared at 4% with other hexa-PEGylated Hbs we have generated. The COP of direct PEGylation products is higher than that of EAF hexaPEGylated Hb. This suggests that the COP of the PEGylated protein may be a function of the chemistry of PEGylation.

The COP of a solution is a colligative property – a function of the number of particles in the solution – therefore the higher COP of direct hexaPEGylated Hbs related to EAF hexaPEGylated Hb may be suggestive of a higher number of particles (molecules) in solution. Since Hb is a tetrameric protein, this interpretation implies that PEGylation influences the tetramer–dimer equilibrium in favor of dimer formation, which results in an increase in the number of molecules in solution at comparable levels of Hb concentration. The higher COP of hexaPEGylated Hb generated by direct PEGylation therefore suggests that PEGylation facilitates dissociation of tetramers. In this scenario, reductive hexaPEGylation is most efficient in favoring the dissociation of Hb into αβ-dimers.

25.8 Reductive PEGylation-induced Weakening of Interdimeric Interactions of Tetrameric Hbs

The influence of the stabilization of the quaternary structure of Hb by αα-fumaryl intramolecular crosslinks on reductive hexaPEGylation-induced promotion of the dissociation of the tetrameric structure (as reflected by the decrease in the hydrodynamic volume

and COP) has been investigated. Reductive hexaPEGylation exhibits high site selectivity for surface decoration of Hb with PEG; four of the six PEG chains conjugated to Hb are on the four α-amino-terminal residues of the molecule. This site selectivity of PEGylation is not influenced by the presence of an αα-fumaryl crossbridge in the molecule.

The reductively PEGylated αα-fumaryl Hb (Propyl-PEG5K)$_6$-αα-Hb elutes significantly earlier on Superose 12 column than does (Propyl-PEG5K)$_6$-Hb, reflecting the higher hydrodynamic volume of the crosslinked material. This suggests that the reductive hexaPEGylation promotes dissociation of tetramer. Analytical ultracentrifugal analysis of (Propyl-PEG5K)$_6$-αα-Hb shows that it sediments as stable tetrameric molecular species, while (Propyl-PEG5K)$_6$-Hb sediments as dimeric molecular species. This confirms that (Propyl-PEG5K)$_6$-Hb is essentially a PEGylated Hb dimer, not a tetrameric species [16].

The reductively hexaPEGylated αα-fumaryl Hb also exhibits larger molecular radius and lower COP than the hexaPEGylated uncrosslinked Hb (Propyl-PEG5K)$_6$-Hb. Reductive hexaPEGylation-induced perturbation of the heme environment, as well as the microenvironment of Trp-37(β) of the $\alpha_1\beta_2$ interface of Hb, is also reduced by αα-fumaryl intramolecular crosslinking [16]. The COP of the reductively hexaPEGylated Hb is also lowered by the αα-fumaryl crossbridge. These results are consistent with the attenuation of the PEGylation-induced promotion of the dissociation.

ββ-succinimidophenyl PEG-2000 Hb carries an intramolecular crosslink outside the central cavity between the two Cys-93(β) residues that increases its O_2 affinity. This crosslink also stabilizes the quaternary structure of (Propyl-PEG5K)$_6$-Hb, just like a αα-fumaryl crosslink [20]. These results establish the necessity of interdimeric intramolecular crosslinking if reductive PEGylation is the PEGylation platform of choice, for the attenuation of reductive PEGylation-induced promotion of the dissociation of Hb in generating hexaPEGylated Hbs.

25.9 PEGylation-promoted Dissociation of Hb Tetramer is Attenuated by the Extension Arms of EAF PEGylated Hbs

The weakening of the interdimeric interactions of Hb discussed above for reductive hexaPEGylation, resulting in the generation of PEGylated dimers, is true for all four direct platforms [16–18]. However, the extent to which hexaPEGylation of Hb promotes dissociation is a function of PEG conjugation chemistry. The weakening of the interdimeric interaction is maximum for reductive PEGylation, which may be a consequence of the site selectivity of reductive PEGylation [15, 16]. Even though the site selectivity of thiocarbamoylation-mediated hexaPEGylation [17] is essentially identical to that of reductive PEGylation, and all four α-amino groups of Hb are PEGylated quantitatively in both cases, the extent of weakening of interdimeric interactions of Hb appears to be lower in thiocarbmoylation chemistry-based PEGylation. The orientation of PEG chains dictated by conjugation chemistry dictates the extent of promotion of the dissociation seen on hexaPEGylation.

The elution patterns of EAF hexaPEGylated Hb and EAF hexaPEGylated αα-fumaryl Hb in FPLC are essentially identical. The results suggest extension-arm chemistry has essentially attenuated the promotion of the dissociation of Hb tetramer resulting from PEGylation.

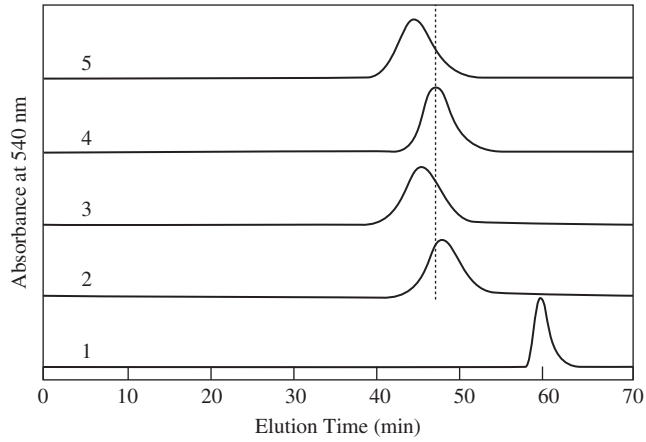


Figure 25.2 *Influence of αα-fumaryl intramolecular crosslinks of Hb on the hydrodynamic volume of hexaPEGylated Hb generated by a direct PEGylation platform using succinimidyl propyl (SP) and succinimidyl carbonate (SC) PEG-5K. A size-exclusion chromatographic analysis of Hb-PEG adducts in FPLC is shown. Curve 1, HbA control; curve 2, Hb-SCPEG5K6; curve 3, ααHb-SCPEG5K6; curve 4, Hb-SPAPEG5K6; curve 5, ααHb-SPAPEG5K6. The SEC analysis was carried out at room temperature on two HR10/30 Superose 12 columns connected in series. The columns were eluted with PBS, pH 7.4, at a flow rate of 0.5 mL/minute. Note that hexaPEGylated αα-fumaryl Hb exhibits a higher hydrodynamic volume than the hexaPEGylated uncrosslinked Hb. We thank Dr Abuchowski of Prolong Pharmaceuticals, NJ for the gift of SC PEG-5K.*

25.10 Does Urethane-linkage-mediated PEGylation of Hb Promote its Dissociation?

Enzon PEG bovine Hb is generated using succinimidyl carbonate chemistry, which results in a urethane linkage between PEG chains and the protein amino groups. The SEC patterns of SP (succinimido propyl) PEG- and SC (succinimido carbonate) PEG-based direct hexaPEGylated αα-fumaryl Hb have been compared with the corresponding patterns of hexaPEGylated Hb. HexaPEGylation of Hb by urethane chemistry also promotes dissociation of the Hb tetramer (Figure 25.2 and Table 25.2), just like other direct PEGylation platforms.

25.11 Hemospan: Prototype of EAF HexaPEGylated Hb Designed at Einstein

The basic PEGylation platform applied for the generation of Hemospan, a hexaPEGylated (using maleimide PEG-5K) Hb product of Sangart Inc., San Diego, CA, is EAF PEGylation. Instead of the maleimide phenyl PEG-5K used at Albert Einstein College of Medicine in the EAF PEGylation, Sangart has used PEG propyl maleimide for the preparation of this PEGylated Hb and has adopted a two-stage protocol for its larger-scale production [21–24]. This Hb represents the most extensively studied PEGylated

Table 25.2 *Comparison of the influences of isopeptide and urethane-linked PEG shell on the tetramer stability of the PEGylated tetramer.*

	Viscosity (cP)	COP (mm Hg)	Radius (nm)
HbA	0.86	9.6	3.2
EAF-P5K6 Hb	2.5	68.0	6.5
Propionyl-P5K6 Hb	2.1	55.4	5.5
Propionyl-P5K6 ααHb	2.0	41.4	6.0
Carbamoyl-P5K6 Hb	2.6	63.8	5.8
Carbamoyl-P5K6 ααHb	2.3	49.5	6.2

All measurements were made at a protein concentration of 40 mg/mL. All viscosity measurements were made at 37 °C, the COPs were measured at room temperature.

Hb and should be the best platform for the design and development of second-generation PEGylated Hbs with different O_2 affinities and/or with antioxidant adducts to overcome ischemia reperfusion injuries.

25.12 EAF HexaPEGylated Hb Compared to other Blood Substitutes of Earlier Designs

EAF hexaPEGylated Hb presents many advantages. Most formulations of EAF PEG-Hb are based on 4% solutions of the Hb derivative rather than a 13% solution of Hb derivatives (earlier designs of intramolecularly crosslinked and oligomerized Hbs). The use of a lower concentration of EAF PEG-Hb allows the use of the available blood to be expanded to meet increasing transfusion demands, while minimizing potential *in vivo* toxicities, in particular the autoxidation-mediated toxicities.

However, the consequences of the use of uncrosslinked EAF PEG-Hb in terms of autoxidation and the resultant toxicity are yet to be understood and require a comparison between the hexaPEGylated Hbs generated by direct PEGylation platforms and those by EAF PEGylation platforms, and also with crosslinked PEGylated Hbs. Given the higher hydrodynamic volume conferred by PEGylation to Hb, even a PEGylated dimer of Hb is expected to have a large enough molecular dimension to make it unlikely to filter through kidney and cause nephrotoxicity. However, the influence of the lack of crosslink on the autoxidation-mediated toxicity of PEGylated Hb *in vivo* is yet to be delineated.

25.13 Reversible Protection of Cys-93(β) during EAF PEGylation of Hb and Crosslinked Hbs: A Structural Requirement to Generate Medium- and Low-O_2-affinity PEG-Hbs

The PEGylated Hbs generated by the EAF PEGylation platform exhibit a high O_2 affinity, a consequence of PEGylation of Cys-93(β). PEGylation of a recombinant Hb (rHb) in which Cys-93(β) has been mutated to Ala (thereby avoiding the PEGylation of Cys-93(β) of Hb) also increased the O_2 affinity [14]. EAF PEGylation-induced increase in the O_2 affinity of Hb is a result of at least two structural aspects: PEGylation of Cys-93(β) and the PEGylation-induced structural/conformational perturbations of Hb.

The EAF PEGylation protocol has been modified to keep Cys-93(β) free in the final product by reversibly protecting the thiol groups of Cys-93(β) as mixed disulfide with 4-thiopyridine during PEGylation, so that EAF PEGylation is targeted exclusively to the ε-amino groups of Hb [25] (Figure 25.3). Even when Cys-93(β) is protected during PEGylation, and then regenerated after PEGylation, the O_2 affinity of Hb still increases from 14 to ~10 mm Hg (instead of 8 mm Hg when Cys-93(β) is not protected).

In EAF PEGylated Hb in which the PEGylation is targeted exclusively to the ε-amino groups of Hb, the perturbation of the circular dichroism of the Soret band (sensitive to the heme microenvironment) and of the Trp fluorescence (which probes the $\alpha_1\beta_2$ interface) is significantly reduced. The reactivity of the Cys-93(β) to form mixed disulfide with bis(dithio)pyridine is unperturbed by EAF PEGylation. This approach has been very useful in preparing the EAF PEGylated αα-Hb, which has medium O_2 affinity, lower than that of the species with PEG on Cys-93(β).

25.14 Engineering Extension Arms between the Protein Core and PEG Shell Attenuates PEGylation-promoted Tetramer Dissociation

Active ester chemistry-mediated PEGylation (using SPA-PEG) is the most widely used PEGylation platform for PEGylation of protein and peptide therapeutics. An EAF version of active ester chemistry-based PEGylation of Hb has been designed to establish that extension arms engineered between the PEG shell and protein core do indeed attenuate the PEGylation-induced weakening of interdimeric interactions in Hb. The strategy for this new EAF PEGylation platform involves the use of a small-molecular-weight heterobifunctional reagent, ε-maleimido caproic acid succinimidyl ester (EMCS), to introduce the extension arm. One end of EMCS carries an active ester, and this forms an isopeptide linkage with the amino groups of Hb, and the other end carries a maleimide moiety as the targeted functional group for PEGylation using thiol-PEG.

In this new EAF PEGylation platform, the thiol groups of the Cys-93(β) of Hb are first derivatized as mixed disulfide with thiopyridine, and then modified with EMCS to introduce the desired number of maleimide groups on the protein [26]. These maleimide functional groups are now the target sites for PEGylation using thiol-PEG (Figure 25.3). The new EAF PEGylation is a nonconservative PEGylation, just like PEGylation using SPA-PEG, and the chemical linkage between the EA arms and protein amino groups is identical to that in the direct PEGylation of Hb with SPA-PEG. A thiosuccinimide linkage is generated between PEG and the extension arm, but this linkage is in a reversed polarity as compared to that in conservative EAF PEGylation. The SEC and the COP of hexaPEGylated HbA have established that the nonconservative EAF PEGylation also attenuates the PEGylation-promoted tetramer dissociation of Hb. This establishes that engineering the EA between Hb and PEG chains attenuates the impact of the PEG shell on the interdimeric interactions in Hb, without significant influence on the PEGylation-induced PE-like properties of PEG-Hb adducts [26]. New heterobifunctional reagents with either an aldehyde or an isothiocyanate function on one end and either thiol (protected as mixed disulfide with thiopyridine) or maleimide have been developed to design EAF versions of reductive PEGylation and thiourethane-based EAF PEGylation platforms, respectively.

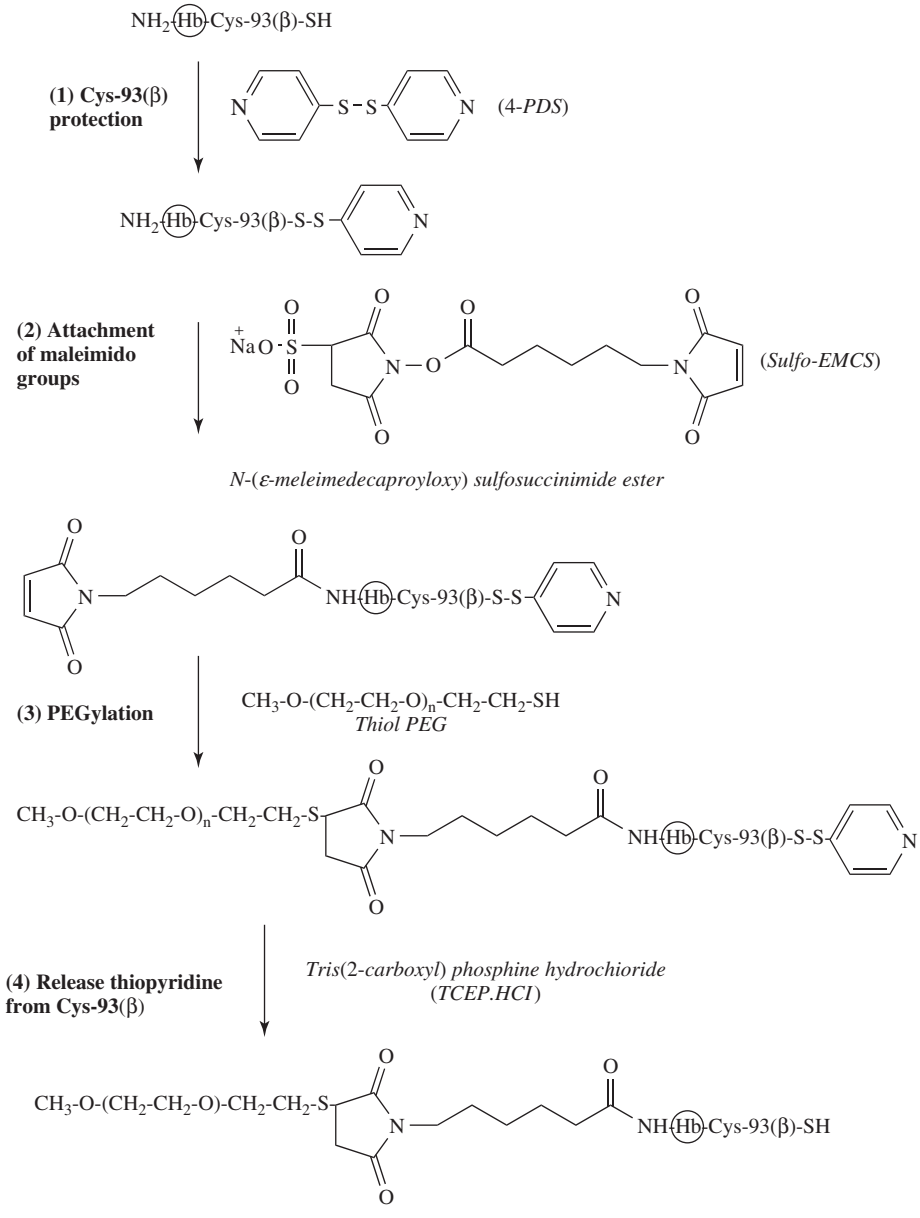
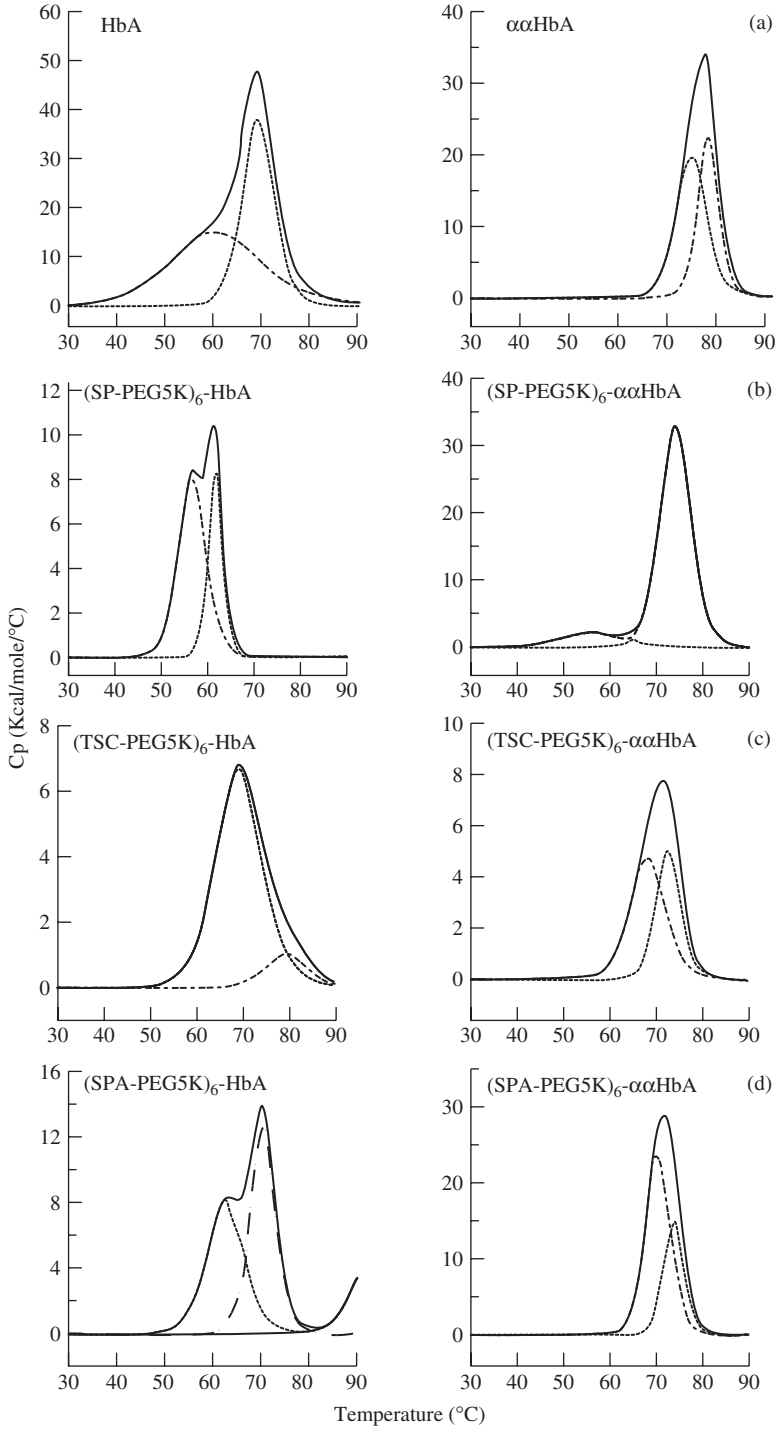


Figure 25.3 *Schematic representation of nonconservative EAF PEGylation of Hb. The extension arms are attached to the protein through active ester chemistry (succinimidyl ester). The new functional group introduced is not thiol, but a maleimide, the targeted site for PEGylation using thiol PEG. Accordingly, the polarity of the linkage in this case is reversed as compared to the 2-IT-based conservative PEGylation using maleimide PEG.*


HbA
αα HbA
(a)
(SP-PEG5K)6-HbA
(SP-PEG5K)6-αα HbA
(b)
(TSC-PEG5K)6-HbA
(TSC-PEG5K)6-αα HbA
(c)
(SPA-PEG5K)6-HbA
(SPA-PEG5K)6-αα HbA
(d)
Cp (Kcal/mole/°C)
Temperature (°C)

25.15 Attenuation of Direct HexaPEGylation-promoted Dissociation of Hb Tetramers by Increasing the Tetramer Stability Through Chemical Modification

Attachment of propyl PEG-5K to the α-amino group of Val-1(α) destabilizes the tetrameric structure of Hb extensively, and the diPEGylated Hb exists essentially as PEGylated dimers. In contrast, the propylation of Val-1(α) stabilizes the tetrameric structure of Hb by nearly an order of magnitude. The weakening of interdimeric interaction of Hb by PEG chains at the αα-end of the central cavity of Hb is so strong that the propyl chain-induced stabilization of tetramer is also neutralized. This site-specific effect of direct PEGylation of Val-1(α) on the quaternary structure is apparently the primary molecular aspect of the strongest effect of hexaPEGylation on the quaternary structure of Hb and the highest COP of solutions of reductively hexaPEGylated Hb [19]. The PEG-5K chains on the distal end of the propyl moiety, besides completely neutralizing the increased tetramer stability induced to the Hb tetramer in the oxy conformation by propylation of Val-1(α), also contribute to the weakening of the intrinsic interdimeric interactions of the tetramer. Stabilization of the quaternary structure by chemical modification or site-directed mutagenesis will be an alternative strategy for EAF PEGylation to attenuate the direct PEGylation-induced weakening of the tetramer stability.

25.16 Influence of the Extension Arm on the HexaPEGylation-enhanced Thermal Stability of Hb

The influence of conservative and nonconservative EAF PEGylation on the thermal stability of hexaPEGylated Hb has been investigated by differential scanning microcalorimetry. The thermal-transition patterns are deconvoluted using a non-two-state curve-fitting (Figure 25.4). The T_{m1} and T_{m2} for Hb are at 60 and 70 °C, respectively. Presumably, the first and second transitions correspond to the perturbations of inter-($\alpha_1\beta_2$) and intra-($\alpha_1\beta_1$) dimeric interactions. The T_{m1} and T_{m2} for conservatively EAF PEGylated Hb are at 57 and 62 °C, so that this PEGylation lowers the thermal transition temperature, as compared to the parent protein. T_{m1} and T_{m2} for nonconservatively EAF hexaPEGylated Hb are at 70 and 79 °C, so that this EAF PEGylation platform stabilizes the

Figure 25.4 *Influence of extension-arm chemistry on the thermal-transition curves of PEGylated Hbs. All samples (2 mg/mL) were dialyzed overnight with PBS and thermal-transition curves were obtained at a temperature range of 20 − 100 °C with a scanning rate of 90 °C/hour. Scans of dialyzed PBS were used for the baseline subtraction. Origin 7 software is used for the fitting of the experimental curves. The experimental curves are shown as continuous lines and the deconvoluted curves are shown as dotted lines. The left column presents hexaPEGylated uncrosslinked Hbs and the right column presents hexaPEGylated αα-fumaryl Hb. Panels (a), (b), (c), and (d) represent different PEGylation platforms used for surface decoration of Hb and αα-fumaryl Hb. (a) Control unPEGylated samples. (b) HexaPEGylation carried out using a conservative EAF PEGylation platform. (c) HexaPEGylation carried out using a nonconservative EAF PEGylation platform. (d) Direct PEgylation using SPA PEG.*

protein slightly. The first thermal transition of this nonconservative EAF PEG-Hb nearly corresponds to the second T_m of the uncrosslinked Hb. Thus, the nonconservative EAF PEGylation, but not the conservative one, slightly increases the thermal stability of uncrosslinked Hb. This may be a consequence of the differences in the site selectivity of PEGylation between the two platforms.

25.17 PEGylation of Hb Induces a Hydrostatic Molecular Drag to the PEG-Hb Conjugate

Sedimentation velocity of intramolecularly crosslinked Hb is reduced upon conjugation of PEG chains, even though the molecular mass of the PEG-Hb conjugate is nearly 50% higher than that of the unmodified sample [16]. This reflects the fact that PEGylation induces a resistance for the sedimentation of Hb in the centrifugal field. The major structural consequence of hexaPEGylation of Hb with PEG-5K chains is an unusual enhancement in the hydrodynamic volume of Hb as compared to the mass of conjugated PEG chains. Assuming that the overall packing of the protein core is not significantly perturbed, this unusual increase in the hydrodynamic volume implies very loose packing of the PEG chains in the PEG shell. This will impart the PEG shell more flexibility than the protein core. A consequence is that during sedimentation analysis, reorientation of the low-density PEG shell induces a "molecular drag" on the Hb. This molecular drag will make the covalently attached PEG chains behave as a parachute, increasing the hydrodynamic drag on the molecule and lowering the rate of sedimentation. We extrapolate that a similar influence is exerted by the PEG chains when the PEG-Hb is introduced into the circulatory system, and this can influence the flow properties, inducing enhanced shear stress and consequent NO production by the endothelium. This molecular effect may be expected to induce vasodilatory activity to PEGylated protein.

The influence of the "hydrodynamic molecular drag", if indeed it contributes to the vasodilatory effect of PEGylation of Hb (see discussion below), is expected to be high with EAF PEGylated Hbs compared to direct PEGylated products, as in the latter case PEGylation promotes the dissociation of the PEGylated Hb into the corresponding dimers. The influence of the hydrostatic pressure on the stability of EAF hexaPEGylated Hb tetramers has not yet been investigated. If it turns out that some dimeric species may be formed, intramolecularly crosslinked Hbs should be used for EAF PEGylation, instead of uncrosslinked Hbs.

25.18 EAF HexaPEGylated Hb is a Superperfusion Agent

Recent studies have established that EAF hexaPEGylated Hb and EAF hexaPEGylated albumin are a unique class of PEs as compared to the conventional ones, and distinguish themselves as novel perfusion agents. Although a 4% solution of these PEGylated proteins is a low-viscosity colloidal solution, they act as high-viscosity PEs and induce vasodilation as they restitute plasma expansion. How does a low-viscosity solution

of PEGylated Hb and PEGylated albumin mimic the physiological consequences of high-viscosity PEs?

The novel enhanced or superperfusion properties of PEGylated Hb and PEGylated albumin, as compared to the conventional low-viscosity colloidal PEs, are presumably a correlate of the unusual biophysical structure of these semisynthetic hybrid biopolymers. The unusual structural feature of EAF PEG-Hb and EAF PEG-albumin is that they are organized with a distinct central, high-packing-density, rigid protein core and a surrounding outer low-packing-density, deformable (flexible) PEG shell. In between these two distinct regions of the molecule is an intervening layer of extension arms that is mostly in the protein hydration layer. The presence of two regions with distinct packing densities makes this class of molecule unique compared to dextran molecules, which have a molecular domain of uniform packing density. Accordingly, the way the PEGylated proteins and the dextran respond to hydrostatic pressure should be very distinct.

The "superperfusion" achieved by PEGylated Hb represents a synergy of multiple physiological consequences, such as the increased FCD, primarily resulting from the autotransfusion (dictated by COP of the PEGylated proteins) *in vivo*, and the vasodilation of arteries and arterioles induced by shear stress generated by these molecules, dictated by the viscosity coupled with the "molecular drag" effect of the PEG shell under flow conditions. Each of these effects can be modulated by careful design strategies to optimize perfusion by PEGylated proteins.

25.19 EAF PEG-Hb-induced Vasodilation

The control of vasodilation *in vivo* involves the action of molecules like prostaglandin, adenosine, and NO, the intrinsic vasodilator. NO generated by the reduction of nitrite in the RBC has been proposed recently to contribute to this mechanism [27–29]. The reduction of nitrite to NO in the plasma by EAF PEG-Hb and the formation of nitrosothiols is one potential molecular mechanism for vasodilation or lowered vasoconstriction relative to unPEGylated Hb derivatives. It has been demonstrated that ββ-succinimidophenyl PEG-2000 Hb exhibits higher levels of *in vivo* nitrite reductase activity than EAF hexaPEGylated Hb [28].

25.20 *In vivo* Vasodilation by EAF PEG-Hb through its Enhanced Nitrite Reductase Activity

The nitrite reductase activity of EAF PEGylated Hb *in vivo* is a reflection of some common aspects of the conformation/structure induced to Hb by EAF PEGylation and/or ββ-succinimido phenyl PEG-2K crosslinking. The common structural features of these Hb derivatives are maleimide modification on Cys-93(β), high O_2 affinity, and increased nitrite reductase activity. However, the amount of PEG mass in the crosslinked molecule (bis maleimide PEG-2K crosslinked) is nearly 15 times higher than that in the EAF

Table 25.3 *Influence of maleimide modification of Cys-93(β) on the nitrite reductase activity of PEGylated Hbs.*

Sample	Rate (s^{-1})
HbA	4.5×10^{-4}
Bis thiopridyl HbA	1.3×10^{-4}
(Cys-93, ββ-succinimido phenyl P2K) Hb	2.0×10^{-3}
[Lys-82(β,β′)fumaryl] Hb	6.6×10^{-4}
(S-Ethyl)6-HbA	1.4×10^{-3}
Bis thiopyridyl (S-Ethyl)6 HbA	6.2×10^{-4}
EAF $P5K_6$ HbA	1.64×10^{-3}
EAF $P5K_4$ HbA Cys-93(free)	6.4×10^{-4}

Data kindly provided by Professor Joel Friedman, Albert Einstein College of Medicine, Bronx, NY, USA prior to publication.

hexaPEGylated Hb. This establishes that the enhancement in the nitrite reductase activity of Hbs seen on conjugation of PEG chains is not a direct correlate of the PEGylation reaction. This enhancement in the intrinsic nitrite reductase activity of Hb apparently balances *in vivo* NO scavenging effects of Hb due to its presence in plasma (autoxidation chemistry). The studies establish that PEGylation induces nitrite reductase activity of Hb in a PEGylation- and conjugation-chemistry-dependent fashion and introduces a new dimension in design-based *in vivo* manipulation of the influence of Hb on NO biochemistry of the vascular system (Table 25.3).

Kluger and colleagues [22] have suggested PEGylation of Hb, in particular PEGylation of Cys-93(β) of Hb by maleimide PEG, as the molecular basis of the enhanced nitrite reductase activity [27, 29]. However, our own *in vitro* studies have established that the enhanced nitrite reductase activity of the uncrosslinked EAF hexaPEGylated Hb is not a correlate of conjugation of PEG chains, but is a direct correlate of the maleimide modification of Hb at Cys-93(β). The modification of Cys-93(β) as a mixed disulfide with thiopyridine does not induce an enhancement in the intrinsic nitrite reductase activity of Hb (Table 25.3). Since the enhancement in the reductase activity is unique to the maleimide modification of Cys-93(β), this aspect of modulation of the NO biochemistry by PEGylated Hb will be unique to EAF PEGylated Hb generated only under oxy conditions, and is not an intrinsic property of other direct PEGylated Hbs, nor of EAF PEGylation of Hb when Cys-93(β) is reversibly protected during EAF PEGylation, nor of EAF hexaPEGylated Hb generated under deoxy conditions [30]. In all these cases the thiol of Cys-93(β) is free. Consistent with this, EAF PEGylation of ββ-succinimidophenyl Hb does not enhance the nitrite reductase activity beyond that of crosslinked Hb.

The presence of αα-fumaryl Hb also increases the nitrite reductase activity in spite of its low oxygen affinity (Table 25.4). However, the nitrite reductase activities of αα-fumaryl Hb and of its EAF hexaPEGylated form are not significantly different (Table 25.4), since the nitrite-reductase-enhancing propensity of maleimide modification of Hb at Cys-93(β) is essentially neutralized by the αα-fumaryl intramolecular crosslinking. On the other hand, reductive hexaPEGylation of αα-fumaryl Hb enhances

Table 25.4 *The influence of the chemistry of intramolecular crosslinking and of PEGylation on the nitrite reductase activity of Hb.*

Sample	Rate (s^{-1})
HbA	4.5×10^{-4}
Bis thiopridyl Hb	1.3×10^{-4}
ββ-succinimido phenyl P2K Hb	2.0×10^{-3}
Lys 82 (ββ)-fumaryl Hb	6.6×10^{-4}
αα-fumaryl HbA	1.93×10^{-4}
Bis thiopridyl αα-fumaryl Hb	2.6×10^{-4}
(Propyl PEG-5K)6 αα-fumaryl Hb	1.09×10^{-3}
(SP-PEG)P5K6 αα-fumaryl Hb	7.4×10^{-4}
(TCP-PEG5K)4 αα-fumaryl Hb	1.7×10^{-4}
(SP-PEG5K)4 αα-fumaryl Hb (Cys-93, free)	3.4×10^{-4}
(SP-PEG 5K)6 αα-fumaryl Hb	4.8×10^{-4}
(SE)6 αα-fumaryl Hb	3.3×10^{-4}

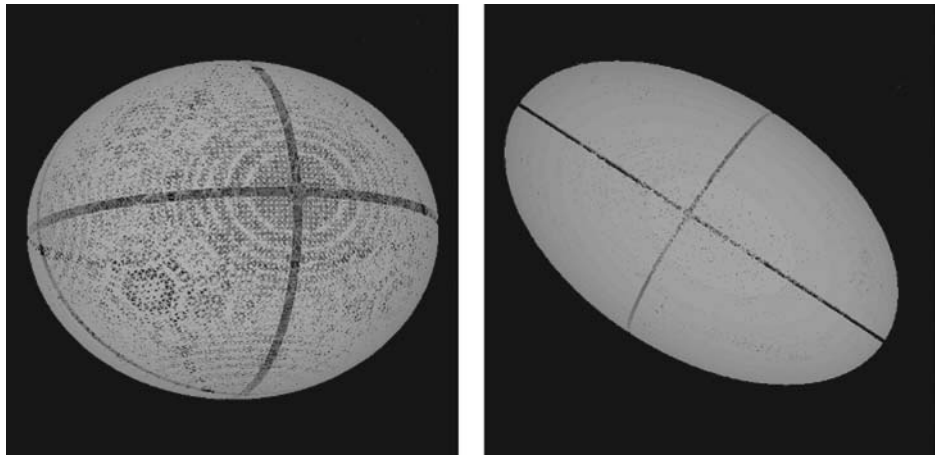
Data kindly provided by Professor Joel Friedman, Albert Einstein College of Medicine, Bronx, NY, USA prior to publication.

the nitrite reductase activity almost to the level of ββ-succinimidophenyl PEG-2000 Hb. Even though the site selectivity of thiocarbamoylation chemistry-based PEGylation of αα-fumaryl Hb is the same as the reductive alkylation, this PEGylation does not enhance the nitrite reductase activity. It is not the presence of PEG at the unique sites in these PEGylated proteins that dictates the nitrite reductase activity.

25.21 EAF PEG-Hbs as Mechanotransducers of e-NOS Activity

Upon infusion of animals with PEG-albumin, perivascular NO is increased, suggesting the *de novo* increased synthesis or decreased consumption of NO in animals getting PEGylated protein [31]. This could be a consequence of *in vivo* activation of endothelial nitric oxide synthase (e-NOS) activity. Mechanotransduction-mediated e-NOS-activated synthesis of NO is the obvious pathway for this physiological influence.

The shear stress (mechanotransduction)-induced production of NO will be dependent on the size, shape, and flexibility of the macromolecules in circulation. A comparison of molecular models of EAF PEGylated Hb with eight copies of PEG-5K chains and Hb PEGylated with two copies of PEG-20K chains on their Cys-93(β) [32] has reflected the distinct influence of the PEGylation pattern on the molecular shape of PEGylated proteins. The molecular envelopes of these two PEGylated Hbs are depicted in Figure 25.5. EAF hexaPEGylated Hb with eight copies of PEG-5K chains is more globular, while PEGylated Hb with two copies of PEG-20K on the two Cys-93(β) is ellipsoidal. Moreover, the shape of the PEG shell is deformable, as discussed above, to induce an additional "molecular drag" that may amplify the shear stress experienced by the endothelium under flow conditions.



(a) (b)

Figure 25.5 *Molecular envelopes of models of HbA (a) (a, b, and c axes of the molecular envelope are 37, 30, 32 Å, respectively) and EAF hexaPEGylated Hb (b) (a, b, and c axes of the molecular envelope are 93, 56, 58 Å, respectively), depicting their shapes. The molecular envelopes were kindly generated by Dr M. Prabhakaran of International University of Miami, FL, USA.*

25.22 The Pattern of PEGylation of Intramolecularly Crosslinked Hbs Influences the Viscosity of the PEG-Hb Solution

PEGylation of one of the Cys-93(β)s of αα-fumaryl Hb (P30K1-αα-Hb) using maleimide PEG-30K has been carried out recently. The asymmetrically PEGylated Hb exhibits a considerably lower p50 compared to that of EAF P5K6-αα-Hb. Furthermore, the viscosity of this asymmetrical molecule is higher than that of the EAF P5K6-αα-fumaryl Hb. The difference in molecular radius of the molecules, one with six copies of PEG-5K and one with one copy of PEG-30K, is not as significant as the O_2 affinity. The elution position of the molecule with PEG-30K on Superose 12 is earlier than that of the molecule with six copies of PEG-5K. The difference in COP is also marginal, but the viscosity difference is significant (Table 25.5). Reversible protection of thiols of Cys-93(β) during EAF monoPEGylation will facilitate the targeting of PEGylation to Lys residues. Such molecules will enable us to establish how the changes in viscosity of PEG-Hb, engineered through design strategies, could influence "*in vivo* molecular mimicry" of the high-viscosity PEs by EAF PEG-Hb to induce NO production by endothelium.

25.23 Conclusion

The paradigm for the development of new EAF PEG-Hbs as O_2-carrying PEs is depicted in Figure 25.6. Studies of EAF hexaPEGylated Hb and parallel studies with EAF hexaPEGylated albumin have added new insights into the structural and functional role of

Table 25.5 *Influence of the pattern of PEGylation on the molecular and solution properties of PEGylated Hb.*

Molecular Properties	αα-fumaryl Hb	(PEG5K)6-αα-fumaryl Hb[a]	(PEG30K)1-αα-fumaryl Hb[a]
Total PEG mass per ααHbA (Dalton)	0	30 000	30 000
Total number of oxyethylene units per ααHbA	N/A	682	682
Molecular radius (nm)	3.1	6.0	6.5
Molecular volume (nm^3)	125	903	1148
PEGylation-induced increase in molecular volume (nm^3)	N/A	778	1023
Molecular density of PEG shell (Dalton/nm^3)	N/A	39	29
Viscosity at 4% protein (cP)	0.97	2.2	3.55
COP at 4% protein (mm Hg)	12	65	60.3

[a]The total mass of PEG conjugated to αα-fumaryl Hb in the two PEGylated products is the same.

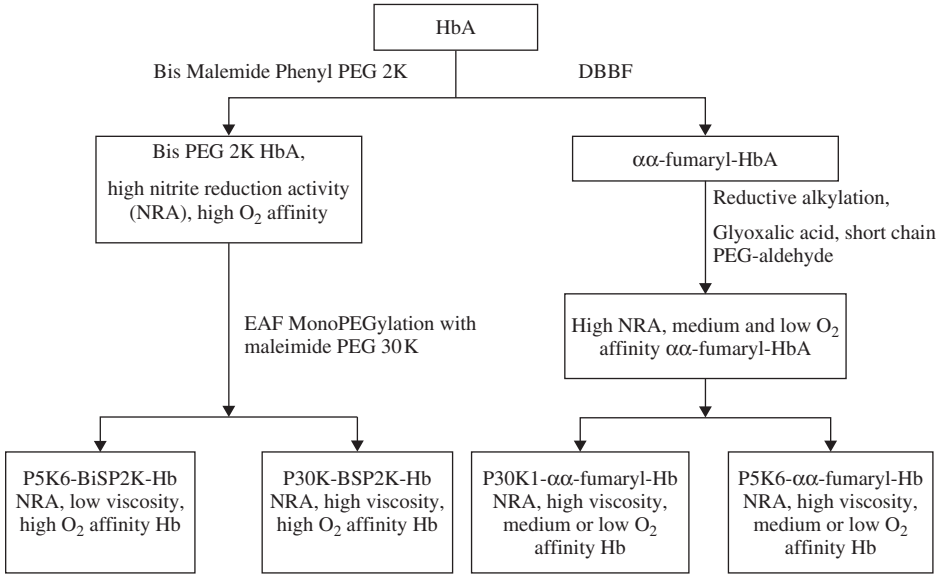


Figure 25.6 *Schematic representation of the future design of O_2-carrying PEs with superperfusion activity.*

extension arms in the EAF PEG proteins. The propensity of extension arms to attenuate the impact of the PEG shell on the structure/conformation of the protein core distinguishes the EAF PEG proteins from the other PEG proteins, which do not carry extension arms. EAF hexaPEGylated Hb is essentially a tetramer just like Hb, while other hexaPEgylated Hbs generated by direct PEGylation are essentially PEGylated dimers. This makes EAF PEG-Hb unique, and EAF PEG-Hb with Cys-93(β) free exhibits a tetramer–dimer dissociation behavior indistinguishable from the parent molecule.

The fascinating new development with EAF hexaPEGylated Hb and EAF hexaPEGylated albumin is that the low-viscosity solutions of these molecules have excellent perfusion properties, increasing the efficacy of O_2 delivery by RBCs. Current investigations are focused on understanding the "molecular mimicry" of the physiological consequences of the high-viscosity PEs by the low-viscosity solutions of PEG-Hbs. The ability to modulate the nitrite reductase activity of PEG-Hbs, the O_2-carrying PEs, by simple maleimide modification of Hb or reductive alkylation of the amino terminus of αα-crosslinked Hb, gives us another dimension in which to reengineer the structure of EAF PEG-Hbs as superperfusion agents.

The optimum O_2 affinity for the O_2-carrying PEs is still a matter of debate and has to be established by future investigations, but is expected to be a function of blood-volume loss and the replacement involved; that is, whether the transfusion trigger needs to be activated or not when the material has to be used. The current high-O_2-affinity O_2-carrying PEs are designed to target the O_2 delivery to the hypoxic regions, and are probably ideal in situations of low-blood-volume replacement. In this case, EAF PEG-Hb can perform two critical functions: enhancing perfusion and targeting O_2 delivery to the hypoxic regions of the body.

As the blood volume to be replaced increases and reaches the range of activating the transfusion trigger, it will become necessary to lower the O_2 affinity of more than the current versions of EAF PEG-Hbs, and in high-blood-volume-loss situations we envisage the use of mixtures of Hbs with high (p50 5–10 mm Hg), medium (p50 10–15 mm Hg), and low (p50 15–25 mm Hg) O_2 affinity. The design of these classes of molecule will consider the nitrite reductase activity, mechanotransduction-based NO production, and O_2 affinity. EAF PEGylation exclusively targeted to the amino groups (possibly single-site PEGylation to enhance mechanotransduction-induced NO production by the endothelium) of Cys-(ββ) succinimido phenyl PEG 2000 Hb, αα-fumaryl Hb with short (10–12 oxyethylene units) propyl-PEG chains on the α-amino groups (to induce high nitrite reductase activity), and carboxymethylated (on Val-1(β)) αα-fumaryl Hb is being contemplated as the starting material for the generation of low-, medium-, and high-O_2-affinity OCPEs, respectively.

Acknowledgments

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References

[1] Intaglietta, M., Cabrales, P. and Tsai, A.G. Microvascular perspective of oxygen-carrying and noncarrying blood substitutes, *Annu. Rev. Biomed. Eng*., **8**, 289–321 (2006).

[2] Conover, C.D., Linberg, R., Shum, K.L. and Shorr, R.G. The ability of polyethylene glycol conjugated bovine hemoglobin (PEG-Hb) to adequately deliver oxygen in both exchange transfusion and top-loaded rat models, *Artif. Cells Blood Substitut. Immobil. Biotechnol*., **27**, 93–107 (1999).

[3] Yabuki, A., Yamaji, K., Ohki, H. and Iwashita, Y. Characterization of a pyridoxalated hemoglobin-polyoxyethylene conjugate as a physiologic oxygen carrier, *Transfusion*, **30**, 516–520 (1990).

[4] Rohlfs, R.J., Brunner, E., Chiu, A. *et al*. Arterial blood pressure responses to cell-free hemoglobin solutions and the reaction with nitric oxide, *J. Biol. Chem*., **273**, 12128–12134 (1998).

[5] Acharya, S.A. and Manjula, B.N. Surface decoration of hemoglobin with polyethylene glycol, in *Blood Substitutes* (ed. R.M. Winslow), Elsevier, London, pp. 460–469 (2006).

[6] Manjula, B.N., Tsai, A.G., Intaglietta, M. *et al*. Conjugation of multiple copies of polyethylene glycol to hemoglobin facilitated through thiolation: influence on hemoglobin structure and function, *Protein J*., **24**, 133–146 (2005).

[7] Acharya, A.S., Manjula, B.N. and Smith, P. Hemoglobin crosslinkers, US patent 6017943, January 25, 2000.

[8] Acharya, A.S., Intaglietta, M., Tsai, A.G. *et al*. Enhanced molecular volume of conservatively PEGylated Hb: $(SP\text{-}PEG5K)_6$-HbA is non-hypertensive, *Artif. Cells Blood Substit. Immobil. Biotechnol*., **33**, 239–255 (2005).

[9] Li, D., Hu, T., Manjula, B.N. and Acharya, S.A. Extension arm facilitated PEGylation of hemoglobin: correlation of the properties with the extent of PEGylation, *Protein J*., **25**, 263–274 (2006).

[10] Li, D., Hu, T., Manjula, B.N. and Acharya, S.A. Extension arm facilitated pegylation of alphaalpha-hemoglobin with modifications targeted exclusively to amino groups: functional and structural advantages of free Cys-93(beta) in the PEG-Hb adduct, *Bioconjug. Chem*., **20**, 2062–2070 (2009).

[11] Vandegriff, K.D., McCarthy, M., Rohlfs, R.J. and Winslow, R.M. Colloid osmotic properties of modified hemoglobins: chemically cross-linked verses polyethylene glycol surface-conjugated, *Biophys. Chem*., **69**, 23–30 (1997).

[12] Acharya, S.A., Acharya, V.N., Kanika, N.D. *et al*. Non-hypertensive tetraPEGylated canine haemoglobin: correlation between PEGylation, O_2 affinity and tissue oxygenation, *Biochem. J*., **405**, 503–511 (2007).

[13] Cabrales, P., Meng, F. and Acharya, S.A. Tissue oxidative metabolism after extreme hemodilution with peg conjugated hemoglobin, *J. Appl. Physiol*., in press.

[14] Li, D., Manjula, B.N., Ho, N.T. *et al*. Molecular aspects of the high oxygen affinity of non-hypertensive hexa pegylated hemoglobin, (SP-PEG5K)(6)-[Hb], *Artif. Cells Blood Substit. Immobil. Biotechnol*., **35**, 19–29 (2007).

[15] Hu, T., Prabhakaran, M., Acharya, S.A. and Manjula, B.N. Influence of the chemistry of conjugation of poly(ethylene glycol) to Hb on the oxygen-binding and solution properties of the PEG-Hb conjugate, *Biochem. J*., **392**, 555–564 (2005).
[16] Hu, T., Manjula, B.N., Li, D. *et al*. Influence of intramolecular cross-links on the molecular, structural and functional properties of PEGylated haemoglobin, *Biochem. J*., **402**, 143–151 (2007).
[17] Meng, F., Manjula, B.N., Tsai, A.G. *et al*. Hexa-thiocarbamoyl phenyl PEG5K Hb: vasoactivity and structure: influence of rigidity of the conjugation linkage on the PEGylation induced plasma expander-like solution properties of PEG-Hb adducts, *Protein J*., **28**, 199–212 (2009).
[18] Li, D., Hu, T., Manjula, B.N. and Acharya, S.A. Non-conservative surface decoration of hemoglobin: influence of neutralization of positive charges at PEGylation sites, *Biochim. Biophys. Acta*, **1784**, 1395–1401 (2008).
[19] Hu, T., Li, D., Manjula, B.N. *et al*. PEGylation of Val-1(alpha) destabilizes the tetrameric structure of hemoglobin, *Biochemistry*, **48**, 608–616 (2009).
[20] Manjula, B.N., Malavalli, A., Smith, P.K. *et al*. Cys-93-betabeta-succinimidophenyl polyethylene glycol 2000 hemoglobin A: intramolecular cross-bridging of hemoglobin outside the central cavity, *J. Biol. Chem*., **275**, 5527–5534 (2000).
[21] Vandegriff, K.D., Malavalli, A., Wooldridge, J. *et al*. MP4, a new nonvasoactive PEG-Hb conjugate, *Transfusion*, **43**, 509–516 (2003).
[22] Winslow, R. and Kramer, G. Maleimide-polyethylene glycol-modified human hemoglobin, *Crit. Care Med*., **31**, 2815–2816 (2003).
[23] Winslow, R.M. MP4, a new nonvasoactive polyethylene glycol-hemoglobin conjugate, *Artif. Organs*, **28**, 800–806 (2004).
[24] Vandegriff, K.D., Malavalli, A., Minn, C. *et al*. Oxidation and haem loss kinetics of poly(ethylene glycol)-conjugated haemoglobin (MP4): dissociation between *in vitro* and *in vivo* oxidation rates, *Biochem. J*., **399**, 463–471 (2006).
[25] Li, D., Hu, T., Manjula, B.N. and Acharya, S.A. Extension arm facilitated pegylation of alphaalpha-hemoglobin with modifications targeted exclusively to amino groups: functional and structural advantages of free Cys-93(beta) in the PEG-Hb adduct, *Bioconjug. Chem*., **20**, 2062–2070 (2009).
[26] Ananda, K. and Acharya, S.A., Role of extension arm in PEG-Hb conjugates on the stability of the tetramer: non-conservative EAF maleimide thio-PEG mediated PEGylation, *Artif. Cells Blood Substit. Immobil. Biotechnol*., **36**, 499–512 (2008).
[27] Lui, F.E. and Kluger, R. Enhancing nitrite reductase activity of modified hemoglobin: bis-tetramers and their PEGylated derivatives, *Biochemistry*, **48**, 11912–11919 (2009).
[28] Cabrales, P. and Friedman, J.M. PEGylated hemoglobins mechanisms to avoid vasoconstriction and maintain perfusion, *Transfus. Altern. Transfus. Med*., **9**, 281–293 (2007).
[29] Lui, F.E., Dong, P. and Kluger, R. Polyethylene glycol conjugation enhances the nitrite reductase activity of native and cross-linked hemoglobin, *Biochemistry*, **47**, 10773–10780 (2008).
[30] Caccia, D., Ronda, L., Frassi, R. *et al*. PEGylation promotes hemoglobin tetramer dissociation, *Bioconjug. Chem*., **20**, 1356–1366 (2009).

[31] Tsai, A.G., Acero, C., Nance, P.R. *et al*. Elevated plasma viscosity in extreme hemodilution increases perivascular nitric oxide concentration and microvascular perfusion, *Am. J. Physiol. Heart Circ. Physiol*., **288**, H1730–H1739 (2005).

[32] Manjula, B.N., Tsai, A., Upadhya, R. *et al*. Site-specific PEGylation of hemoglobin at Cys-93(beta): correlation between the colligative properties of the PEGylated protein and the length of the conjugated PEG chain, *Bioconjug. Chem*., **14**, 464–472 (2003).

26

Hb Octamers by Introduction of Surface Cysteines

Véronique Baudin-Creuza[1], *Chien Ho*[2] *and Michael C. Marden*[1]

[1]*Inserm U779, CHU Bicêtre, Paris, France*
[2]*Department of Biological Sciences, Carnegie Mellon University, Pittsburgh, PA, USA*

26.1 Introduction

From the point of view of a hemoglobin (Hb) molecule in its natural environment, the most likely encounter is with another Hb molecule. Protected within the red blood cell (RBC), the high concentration leads to frequent encounters on a microsecond and millisecond timescale; there are many collisions, most likely with a tetramer, but also with Hb dimers, the natural effector 2,3-diphosphogycerate (DPG), and of course oxygen molecules. The Hb molecule should certainly not be considered in a static state; if oxygen is bound to a certain subunit, another look a few milliseconds later, and it may now be in the deoxy state. As the number of ligands per tetramer changes, the allosteric switch requiring about 100 μs will come into play. On a slower timescale, oxyHb separates into dimers within a few seconds, and an oxidized subunit requires the service of metHb-reductase an average of every few days; here again the cell holds all these elements together to maintain the Hb tetramer as the dominant species.

Consider the end of the cell's life, after typically 4 months, where lysis will liberate these components to the open blood vessel. Dissociation of the effector DPG may be irreversible. The transition to dimers will also be only slowly reversible; alternatively, the dimers may bind to haptoglobin irreversibly. The dimers oxidize more rapidly than tetramers, and the oxidized subunits tend to lose the heme, which may end up binding to

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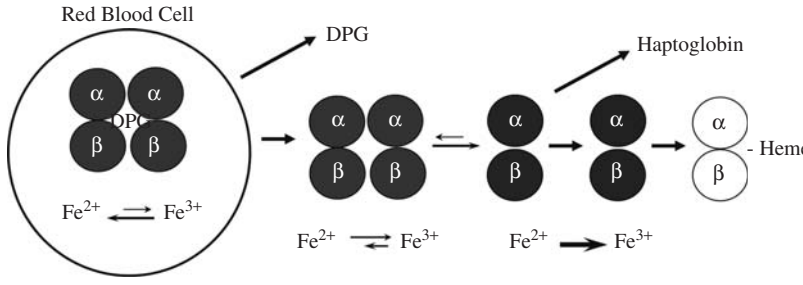

Figure 26.1 *Autocatalytic degradation of an RBC. Once exposed to the exterior environment, the enhanced dimerization will lead to a higher oxidation rate, which in turn leads to loss of the heme group. The weakly binding allosteric effector DPG will be easily lost, and free dimers will be captured by haptoglobin for elimination. Stabilization is thus critical for a cell-free oxygen carrier.*

serum albumin. This autocatalytic process of elimination of Hb (Figure 26.1) summarizes the difficulty of the survival of Hb-based blood substitutes (HBOCs).

It was clear from early attempts that unmodified Hb solutions were not stable enough. The circulation time was only about 30 minutes, with nephrotoxic side effects, most likely from the filtering of dimers. The crosslinked Hb was probably the first serious candidate as a blood substitute. With a simple chemical bridge holding the tetramer together [1], it behaved correctly in terms of oxygen binding and release [2]. Why did it not pass the final tests? Two explanations can be offered. Being smaller than a cell, the Hb tetramer may circulate much closer to the vessel wall and may cross the barrier; capture of NO, the vasodilation factor in the endothelial cells, would perturb the control of blood circulation, as shown by the jump in mean arterial pressure [3]. While the NO-binding explanation offers a detailed series of events, it is not clear that it is the only or even the main cause of the negative clinical results.

The other important factor is heme oxidation. The ferric iron no longer binds oxygen, so it is effectively in an inactive state. Furthermore, the Hb tetramer will no longer be in a fully deoxy state. The cooperativity which leads to efficient oxygen unloading is now hindered by the inactive partner within the tetramer. A critical question is the state of the Hb versus time after injection into the bloodstream, and the potential oxidative stress during the elimination of the degrading Hb.

For these reasons, much current research is based on larger transporters. Various methods of holding several Hb molecules together have been employed, such as small Hb oligomers and Hb within nanoparticles [4–7]. There are also many examples of recombinant Hb (rHb) oligomers [8] and natural giant Hbs such as that from the earthworm, which has Cys-based linker subunits [9]. Generally, cysteines are involved in bonds to stabilize a subunit or a superstructure; however, one can note the presence of an internal disulfide bond in neuroglobin and cytoglobin [10], which may modulate the oxygen affinity. In this chapter we will focus on the example of Hb Ta-Li [11], a mutant of human Hb with an exposed cysteine that may lead to the assembly of an octameric form [12].

26.2 Genetic Engineering of Proteins with Cysteines

In this chapter we will essentially focus on the properties of Hb octamers, based on point mutations of Hb. A first step is to consider the known globin sequences involving Cys residues. For this there are two databases to consider: the globins of different species, and the human Hb variants [13]; both currently have about 1000 entries [14]. In adult human Hb (HbA), there is one cysteine in the alpha chain at position α104 (G11), and two cysteines in the beta chain at β93 (F9) and β112 (G14), but none of these is involved in disulfide bridges. Overall there are a surprising number of examples: Cys has been observed in nearly half of the sequence positions of chordate globins. There are some 50 different positions in 280 different species of alpha or beta chains. While cysteines are rather rare in globins, the number is still quite variable. In myoglobin, the general rule is zero cysteines, and a Cys has been observed at only five different positions for 100 species. For the database of some 1000 species, the number of Cys per chain varies typically from zero to four, with cases of five in the alpha chain of some fish, six in a mouse theta-like chain, eight in Sabellida (*Sabellastarte indica*, chlorocruorin chain E precursor), and nine in *Toxocara canis* (canine roundworm).

Of particular interest for the genetic engineering of human Hb are the known variants; there are about 24 mutants of human Hb (Table 26.1) that involve an additional Cys residue (Figure 26.2). In particular, Hb Porto-Alegre, β9(A6)Ser → Cys HbA, which carries an extra thiol group oriented towards the exterior, spontaneously forms a polymer [15]. Indeed, the cytoplasm of the RBC is a reducing environment, and the polymerized Hb was observed only in the hemolysate, and mainly in the aged hemolysate. Others substitutions with Cys lead to the polymerization, such as Hb Mississippi β44(CD3)Ser → Cys [16] and Hb Ta-Li β83(EF7)Gly → Cys [11]. rHbs with additional cysteines have since been produced (Table 26.2). In particular, the group of Fronticelli has developed different rHbs, including an oligomeric rHb composed of seven to eight tetramers [7]. This molecule contains a supplementary cysteine at position β9, the site of the mutation of Hb Porto-Alegre, and the two natural cysteines in the human β chains were substituted. On the basis of bovine Hb, the same group achieved a hybrid rHb (human α, bovine β) composed of eight tetramers [17] (Table 26.2). The difficulty is to obtain a stable and homogenous molecule. Of particular interest was Hb Tali βG83C, reported to form a disulfide bond between Hb tetramers, which we will refer to as an Hb octamer. This variant has been expressed as a recombinant protein [12] associated with the βF41Y mutation [18] or not (Table 26.2), and its properties will be discussed in detail.

26.2.1 Protein Expression

The usual methods for expressing Hb in *E. coli* could be applied [19, 20]. A co-expression of the alpha and beta chains provides a better yield [20], as the isolated alpha chains are unstable. More recently, the chaperon protein for the alpha chain, AHSP [21], could also be used to obtain better yields for α-Hb [22]. However, despite all attempts, including transgenic plants [23], the overall yield is still low compared to the needs of a supply for a blood substitute.

Table 26.1 *Naturally occurring cysteines in human Hb.*

Native: α104 (G11), β93 (F9), and β112 (G14)			
Human Hb mutants:			
Alpha			
Bladensburg	14	A12	Trp
Lima	18	A16	Gly
Ramona	24	B5	Tyr
Nigeria	81	F2	Ser
Pak-Num-Po	132	H15	Val
Senlis	135	H18	Val
Ecuador	138	H21	Ser
Nunobiki	141	HC3	Arg
Beta			
Porto-Alegre	9	A6	Ser
Kent	37	C3	Trp
Ilmenau	41	C7	Phe
codon 42	42	CD1	Phe
Mississippi	44	CD3	Ser
Arta	45	DC4	Phe
Colima	49	CD8	Ser
Leeds	56	D7	Gly
Manhattan	109	G11	Val
Harrow	118	CH1	Phe
Montfermeil	130	H8	Tyr
Visayan	136	H14	Gly
Rainien	145	HC2	Tyr
Gamma			
F-Malaysia	1	NA1	Gly
Delta			
A2-Troodos	116	G18	Arg

The Hbs expressed in *E. coli* are recovered in a reducing environment. The iron atom is thus reduced and the Hb is in the oxy form; exposure to CO leads to the HbCO species, which in many cases is more stable and better suited for long-term storage. The cysteines are also apparently in a reduced state, a form which does not participate in disulfide bond formation. This is fortunate for the protein expression, avoiding unwanted bond formation, but a subsequent oxidation is required to produce the octamers via intermolecular S−S bond formation. Note that a final use of such octamers requires the opposing conditions of a reduced iron atom but oxidized cysteines. Thus much care must be taken concerning the sample environment.

26.2.2 Oligomer Size

The size of the Hb oligomer can be estimated by size-exclusion chromatography (SEC), as shown in Figure 26.3, or by dynamic light scattering. As shown in the figure, the HbA tetramers and octamers are clearly resolved. Several features can be studied by SEC analysis. A first test is a simple sample dilution; while the peak for HbA shifts to high elution volumes due to dimer formation, the peak position for the β83C-octamer

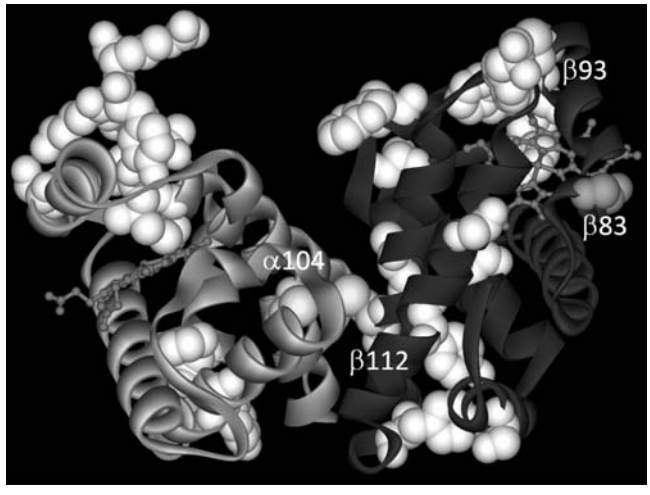

Figure 26.2 *Known positions of Cys residues (yellow, except for β83 of Hb Tali, in orange) in Hb alpha (green) and beta (dark blue) chains, based on the database of some 1000 mutants (see Table 26.1). Also shown are sites for wild-type human Cys (cyan, α104 G11, β93 F9, and β112 G14).*
For a better understanding of the figure, please refer to color plate 5.

Table 26.2 *Recombinant Hbs with additional cysteines.*

rHb	Species Hb	Oligomerization	Ref.
Prisca βS9C + C93A + C112G	α and β human	7–8 tetramers	[7]
βG83C	α and β human	2 tetramers	[12]
Polytaur αV1M+C104S; βA9C+C93A	α human, β bovine	8 tetramers 500 kDa	[17]
βG83C-F41Y	α and β human	2 tetramers	[18]
αN78C	α and β human	2 tetramers	

remains fixed. Similar results were obtained with an analogous mutation of the alpha chain, N78C.

A stronger test for dimer formation is to mix the Hb solution with haptoglobin, which has a high affinity for dimers [24]; in the case of HbA, the initial peak disappears and a peak for the complex (Figure 26.3) appears at low elution volume. The same experiment with the octamer shows little change after a 15 minute incubation with haptoglobin, but some complex formation can be observed after 24 hours.

26.2.3 Disulfide Bond Formation

Another important test for a perspective blood substitute is the stability of the octamer in the presence of fresh plasma, since a reducing component might lead to loss of the disulfide bonds. These tests did not indicate a transition from octamer to tetramers or smaller species. Note that the addition of reducing agents such as DTT leads to a

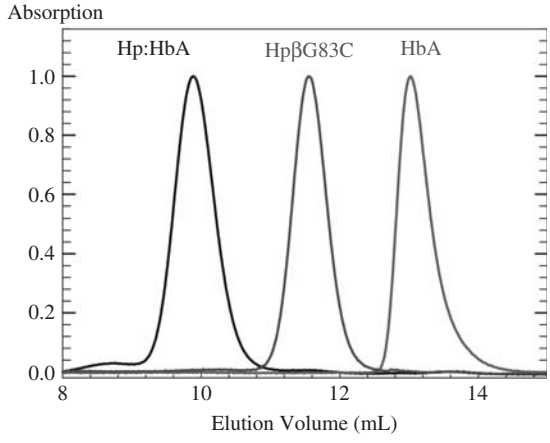

Figure 26.3 *Hb solutions can be studied by SEC to estimate the size or MW of various components. Tetramers are easily distinguished from the octamers or the complex of haptoglobin and Hb. The detection was at 280 nm for 10 μL samples on a Superose 12 HR 10/300 GL column at 25°C in 150 mM Tris-Acetate at pH 7.5, with a flow rate of 0.4 mL/minute.*

transition to a significant tetramer population within 1 hour. The reversible kinetics of the formation and loss of the disulfide bonds can thus be observed. The bond formation depends on the protein concentration, as expected for a bimolecular reaction, and requires about 24 hours at 10 μM protein on a heme basis.

26.2.4 Functional Properties of the Octamers

The oxygen equilibrium curves provide one of the best indications for the correct function of the cooperative Hb molecule. Indeed, it could not be predicted whether the allosteric transition would be hindered in the joined tetramers. The data (see Figure 26.4) indicate that both the alpha and beta octamers function correctly, with parameters similar to those of HbA tetramers. Relative to HbA, the beta octamer had a higher oxygen affinity, the alpha octamer slightly lower (higher p50).

Introduction of an additional mutation, such as βF41Y, could reduce the oxygen affinity [25], but it is not clear if matching the physiological parameters of an HBOC to those of Hb in physiological conditions is the best strategy. There is the argument that a slightly higher affinity would be better for oxygen delivery; if the oxygen is released too soon (in the arteries) then it might provide a misleading signal that oxygen is plentiful and induce a vasoconstriction [26]. There is also a general correlation between oxygen affinity and Hb auto-oxidation rate in that Hbs with a higher oxygen affinity will oxidize more slowly; this would be an important compromise since the ferric heme iron does not bind oxygen.

The CO rebinding kinetics after flash photolysis (Figure 26.5) also indicate a normal function for the octameric Hbs. The CO bound form is often used in place of oxy-Hb, since it provides a better signal for photodissociation, and there is a larger difference of rates (over an order of magnitude) in the R and T association rates. As seen for CO

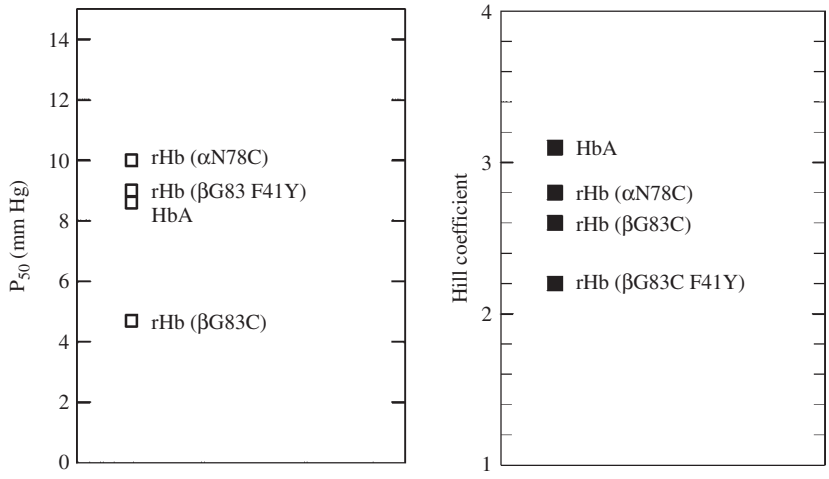

Figure 26.4 *Oxygen-binding properties of the various Hbs at pH 7.4. The oxygen affinity of the alpha octamer is similar to that of HbA, while that of the beta octamer is higher (lower p50 value). An additional mutation can be incorporated (βG83C and βF41Y) in order to approach the normal affinity. The cooperativity, as indicated by the Hill coefficient, is nearly always lower for recombinant Hbs.*

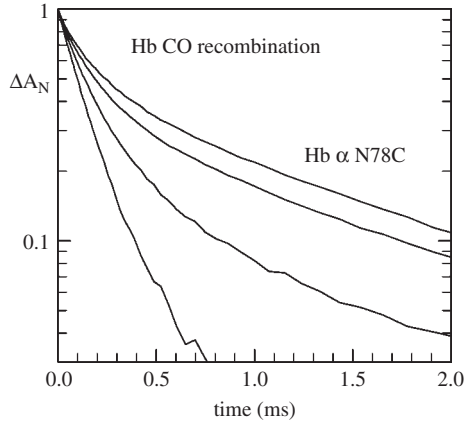

Figure 26.5 *The ligand rebinding kinetics after photodissociation provides direct information on the protein function. The normalized kinetics, shown here at 65, 57, 38, and 18% dissociation of the alpha octamer under pure CO at 37°C (from top to bottom), also probe the allosteric transition. At high dissociation levels (top curves), a significant population of deoxy and singly liganded tetramers is produced, which rapidly switches to the slowly reacting T-state conformation.*

rebinding to the mutant αN78C (Figure 26.5), there is the typical biphasic shape of the rebinding kinetics, characteristic of the two allosteric states.

If one considers the oxy and deoxy crystallographic structures for the Hb tetramer, there is a difference in distance between the critical residues forming the disulfide bond. This would imply that the two tetramers would be required to make the allosteric transition together, or possibly they could hinder the transition. As the sample is fully liganded before the actinic light pulse, the presence of the deoxy conformation indicates a rapid (μs) transition. Thus there does not appear to be a major steric hindrance to the allosteric transition of both tetramers within the octamer.

26.2.5 Octamer Properties

Protein-unfolding studies at high temperature indicate a higher stability for the octameric forms. As observed with DCL-Hb, a diaspirin crosslinked Hb, the additional bonds help maintain the overall structure, with a gain of about 6 °C in the thermal-transition temperature for Hb Ta-Li relative to HbA tetramers. This would be a benefit in the production and sterilization of the Hb solutions.

If the tetramers were joined by a single bond, one would expect that each tetramer could still easily lose a dimer. However, since there was no indication of dimers at low protein concentrations, and no interaction with haptoglobin, it seems that the octamer formation involves the symmetrical case of two bonds. The two disulfide bonds could explain the enhanced stability and octameric constraints that are observed. As this form is 100% protein (no linker), and the tetramers must make the allosteric transition together, it thus behaves as an octamer, rather than as tethered tetramers.

26.2.6 Octamer Constraint

Small changes were observed in ligand association rates in the various octamers. Specifically, the binding of the last ligand to an octamer or tetramer occurs with the rapid R-state rate. Addition of DTT, to form tetramers, led to a similar rate for all Hbs. The difference in rates is thus specific to the octameric form, and indicates some constraint due to the disulfide bonds.

26.3 Conclusion

There are still several factors hindering production of an HBOC. The fundamental properties, such as oxygen affinity, can be controlled, but the oxidation rate remains a problem. Larger molecules can be made, but recombinant technology for the Hb supply falls far short of the demand. On the other hand, novel molecules can be prepared, and perhaps a combination of methods may still lead to a real blood substitute.

References

[1] Chatterjee R., Welty, E.V., Walder, R.Y. *et al*. Isolation and characterization of a new hemoglobin derivative cross-linked between the alpha chains (lysine 99 alpha 1 → lysine 99 alpha 2), *J. Biol. Chem*., **261**, 9929–9937 (1986).

[2] Moore, E.E., Johnson, J.L., Cheng, A.M. *et al*. Insights from studies of blood substitutes in trauma, *Shock*, **24**, 197–205 (2005).
[3] Tsai, A.G., Cabrales, P., Manjula, B.N. *et al*. Dissociation of local nitric oxide concentration and vasoconstriction in the presence of cell-free haemoglobin oxygen carriers, *Blood*, **108**, 3603–3610 (2006).
[4] Chauvierre, C., Marden, M.C., Vauthier, C. *et al*. Heparin coated poly(alkylcyano acrylate) nanoparticles coupled to hemoglobin: a new oxygen carrier, *Biomaterials*, **25**, 3081–3086 (2004).
[5] Chauvierre, C., Leclerc, L., Labarre, D. *et al*. Enhancing the tolerance of poly(isobutylcyanoacrylate) nanoparticles with a modular surface design, *Int. J. Pharm*., **338**, 327–332 (2007).
[6] Zhao, J., Liu, C.S., Yuan, Y. *et al*. Preparation of hemoglobin-loaded nano-sized particles with porous structure as oxygen carriers, *Biomaterials*, **28**, 1414–1422 (2007).
[7] Chang, T.M.S. and Yu, W.P. Nanoencapsulation of hemoglobin and red blood cell enzymes based on nanotechnology and biodegradable polymer, in *Blood Substitutes: Principles, Methods, Products and Clinical Trials* (ed. T.M.S. Chang), Vol. 2, Chapter 11, Karger Landes Systems, Basel, Switzerland, pp. 216–231 (1998).
[8] Fronticelli, C., Arosio, D., Bobofchak, K.M. and Vasquez, G.B. Molecular engineering of a polymer of tetrameric hemoglobins, *Proteins*, **44**, 212–222 (2001).
[9] Hirsch, R.E., Jelicks, L.A., Wittenberg, B.A. *et al*. A first evaluation of the natural high molecular weight polymeric Lumbricus terrestris hemoglobin as an oxygen carrier, *Artif. Cells Blood Substit. Immob. Biotechnol*., **25**, 429–444 (1997).
[10] Hamdane, D., Kiger, L., Dewilde, S. *et al*. The redox state of the cell regulates the ligand binding affinity of human neuroglobin and cytoglobin, *J. Biol. Chem*., **278**, 51713–51721 (2003).
[11] Blackwell, R.Q., Liu, C.S. and Wang, C.L. Hemoglobin Ta-Li: 83 Gly leads to Cys, *Biochim. Biophys. Acta*., **243**, 467–474 (1971).
[12] Fablet, C., Marden, M.C., Green, B.N. *et al*. Stable octameric structure of recombinant hemoglobin alpha(2)beta(2)83 Gly → Cys, *Protein Sci*., **12**, 690–695 (2003).
[13] Patrinos, G.P., Giardine, B., Riemer, C. *et al*. Improvements in the HbVar database of human hemoglobin variants and thalassemia mutations for population and sequence variation studies, *Nucl. Acids Res*., **32**(Special Issue), D537–D541 (2004).
[14] Marden, M.C., Dewilde, S., Kiger, L. *et al*. Exploiting a list of protein sequences, *Gene*, **398**, 35–41 (2007).
[15] Tondo, C.V., Salzano, F.M. and Rucknagel, D.L. Hemoglobin Porto Alegre, a possible polymer of normal hemoglobin in a Caucasian Brazilian family, *Am. J. Hum. Genet*., **15**, 265–279 (1963).
[16] Adams, J.G. 3rd, Morrison, W.T., Barlow, R.L. and Steinberg, M.H. Hb Mississippi [beta 44(CD3)Ser → Arg]: a new variant with anomalous properties, *Hemoglobin*, **11**, 435–452 (1987).
[17] Bobofchak, K.M., Mito, T., Texel, S.J. *et al*. A recombinant polymeric hemoglobin with conformational, functional, and physiological characteristics of an in vivo O_2 transporter, *Am. J. Physiol. Heart Circ. Physiol*., **285**, 549–561 (2003).

[18] Vasseur-Godbillon, C., Sahu, S.C., Domingues, E. *et al*. Recombinant hemoglobin βG83CF41Y: an octameric protein, *FEBS J*., **273**, 230–241 (2006).
[19] Looker, D., Abbott-Brown, D., Cozart, P. *et al*. A human recombinant haemoglobin designed for use as a blood substitute, *Nature*, **35**, 258–260 (1992).
[20] Shen, T.J., Ho, N.T., Zou, M. *et al*. Production of human normal adult and fetal hemoglobins in Escherichia coli, *Protein Eng*., **10**, 1085–1097 (1997).
[21] Kihm, A.J., Kong, Y., Hong, W. *et al*. An abundant erythroid protein that stabilizes free α-haemoglobin, *Nature*, **417**, 758–763 (2002).
[22] Vasseur-Godbillon, C., Hamdane, D., Marden, M.C. and Baudin-Creuza, V. High-yield expression in Escherichia coli of soluble human α-hemoglobin complexed with its molecular chaperone, *Protein Eng. Des. Sel*., **19**, 91–97 (2006).
[23] Dieryck, W., Pagnier, J., Poyart, C. *et al*. Human haemoglobin from transgenic tobacco, *Nature*, **386**, 29–30 (1997).
[24] Nagel, R.L. and Gibson, Q.H. The binding of hemoglobin to haptoglobin and its relation to subunit dissociation of haemoglobin, *J. Biol. Chem*., **246**, 69–73 (1971).
[25] Baudin, V., Pagnier, J., Lacaze, N. *et al*. Allosteric properties of haemoglobin β41(C7) Phe → Tyr: a stable, low oxygen affinity variant synthesized in Escherichia coli, *Biochim. Biophys. Acta*, **1159**, 223–226 (1992).
[26] Winslow, R.M. Red cell substitutes, *Semin. Hematol*., **44**, 51–59 (2007).

27

Hemoglobin Vesicles as a Cellular-type Hemoglobin-based Oxygen Carrier

Hiromi Sakai[1,2], *Hirohisa Horinouchi*[3], *Eishun Tsuchida*[2,*] *and Koichi Kobayashi*[3]

[1] *Waseda Bioscience Research Institute in Singapore, Singapore, Republic of Singapore*

[2] *Research Institute for Science and Engineering, Waseda University, Shinjuku, Tokyo, Japan*

[3] *Department of Surgery, School of Medicine, Keio University, Shinjuku, Tokyo, Japan*

27.1 Introduction

Hemoglobin (Hb) is the most abundant protein in blood (12–15 g/dL), and should be the most essential protein. However, Hb becomes toxic once it is released from red blood cells (RBCs), which is evident in some pathological hemolytic diseases. Chemically modified cell-free Hb-based oxygen carriers (HBOCs), such as intramolecularly crosslinked, polymerized, and polymer-conjugated Hbs, have been synthesized to prevent the toxic effect of cell-free Hbs. However, no product is commercially available yet. Some safety issues arose during the final stage of clinical trials. It seems difficult to completely eliminate the side effect of cell-free Hbs by chemical modification. Now is the time to reconsider the physiological importance of the cellular structure of RBCs. Why is Hb compartmentalized in RBCs with such a complicated corpuscular structure? Hb vesicles

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(HbVs) are artificial oxygen carriers encapsulating concentrated Hb solution (35 g/dL) with a phospholipid bilayer membrane. HbVs are designed to mimic or overcome the function of RBCs. In this chapter, we focus on the concept of Hb encapsulation and recent topics concerning HbVs, especially reactions with gaseous molecules (O_2, NO, CO), which greatly relate to its safety and a new application.

27.2 The Concept of Hb Encapsulation in Liposomes

Hb encapsulation was first performed by Chang in the 1950s [1], using a polymer membrane. Some Japanese groups also tested Hb encapsulation with gelatin, gum Arabic, silicone, and so on. Nevertheless, it was extremely difficult to regulate the particle size to be appropriate for blood flow in the capillaries and to obtain sufficient biocompatibility. After Bangham and Horne reported in 1964 [2] that phospholipids assemble to form vesicles in aqueous media, and that they encapsulate water-soluble materials in their inner aqueous interior, it seemed reasonable to use such vesicles for Hb encapsulation. Djordjevici and Miller [3] prepared a liposome-encapsulated Hb (LEH) composed of phospholipids, cholesterol, fatty acid, and so on. Since then, many groups have tested encapsulated Hbs using liposomes [4–7]. Some failed initially, and some are progressing with the aim of clinical usage. The Naval Research Laboratory presented remarkable progress on LEH [8], but it suspended development about 10 years ago. What we call HbVs with high-efficiency production processes and improved properties have been established by our group, based on nanotechnologies of molecular assembly and pharmacological and physiological aspects [9]. In spite of such a large number of studies of HBOCs in general, no product has so far been tested clinically because of the difficulty of the production method. Chemically modified cell-free HBOCs are much easier to produce, therefore more researchers have tested the cell-free types, and they have been more advanced than the cellular type in entering clinical trials. However, during the long history of R&D, some unexpected problems arose for cell-free HBOCs, presumably due to the direct exposure of Hb to vasculature.

It has been well understood that the compartmentalization of Hb in RBCs is important for: (i) prevention of extravasation or excretion through renal glomeruli; (ii) preservation of the chemical environment in cells, such as the concentrations of electrolytes and enzymes; and (iii) rheology control of blood, an RBC dispersion, to a non-Newtonian viscous fluid. Moreover, for us it seems that RBCs are evolutionally designed for: (iv) retardation and targeting of O_2 unloading at microcirculation to avoid autoregulatory vasoconstriction; (v) reduction of a high colloidal osmotic pressure of Hb solution to zero, to increase blood Hb concentration; and (vi) modulation of reactions with NO as an endothelium-derived relaxation factor (EDRF). Now we have to consider the physiological importance of RBC structure, and mimic the structure to design the optimal HBOCs.

Our HbVs are artificial oxygen carriers encapsulating concentrated Hb solution (35 g/dL) with a phospholipid bilayer membrane [7]. Concentration of the HbV suspension is extremely high ([Hb] = 10 g/dL, [lipids] = 6 g/dL, volume fraction ~40%). HbV has an oxygen-carrying capacity that is comparable to that of blood. HbV is much smaller than RBCs (250 vs 8000 nm), but it recreates the functions of RBCs, as has been confirmed by many animal experiments testing its effectiveness as a resuscitative

Table 27.1 *Preclinical studies of HbV as a transfusion alternative and for other therapeutics.*

	Indication	Ref.
1.	Resuscitative fluid for hemorrhagic shock	[11–13]
2.	Hemodilution	[14]
3.	Priming fluid for extracorporeal membrane oxygenator (ECMO) for cardiopulmonary bypass	[10]
4.	Perfusate for resected organs	[15, 16]
5.	Oxygenation of ischemic brain (stroke)	[17]
6.	Oxygenation of ischemic skin flap (plastic surgery)	[18, 19]
7.	Tumor oxygenation for irradiation sensitization	[20]
8.	CO carrier for cytoprotection at reperfusion	[21]
9.	Measurement of brain oxygen consumption by positron emission tomography (PET)	[22]

fluid for hemorrhagic shock, hemodilution, and a prime for cardiopulmonary bypass [10–12] (Table 27.1). Other characteristics similar to those of RBCs include: (i) the rate of O_2 unloading is slower than Hb solution [23]; (ii) colloid osmotic pressure is zero at [Hb] = 10 g/dL, and it has to be co-injected with or suspended in a plasma substitute such as albumin or HES [24]; (iii) the resulting viscosity of an HbV suspension is adjustable to that of blood [25]; (iv) HbV is finally captured by RES and the components are degraded and excreted [13, 26, 27]; (v) the HbV particle of itself is not eliminated through glomeruli [28]; (vi) PLP is co-encapsulated as an allosteric effector, instead of 2,3-diphosphoglyceric acid, to regulate oxygen affinity [18, 29]; (vii) no hemolysis occurs during circulation and the lipid-bilayer membrane prevents direct contact of Hb and vasculature; and (viii) reaction of NO is retarded to some extent by an intracellular diffusion barrier, and HbV does not induce vasoconstriction [30–32].

In the next section we focus on the gas reactions of cell-free Hb and cellular HbV.

27.3 Hb Encapsulation Retards Gas Reactions

The major remaining hurdle before clinical approval of the earliest generation of HBOCs is vasoconstriction and resulting hypertension, which is presumably attributable to the high reactivity of Hb with NO [33]. It has been suggested that Hbs permeate across the endothelial cell layer to the space near the smooth muscle and inactivate NO. However, cellular HbVs induce neither vasoconstriction nor hypertension [30]. A physicochemical analysis using stopped-flow rapid-scan spectrophotometry clarified that Hb encapsulation in vesicles retards NO binding in comparison to Hb because an intracellular diffusion barrier of NO is formed. The requisites for this diffusion barrier are (i) a more concentrated intracellular Hb solution and (ii) a larger particle size [31, 32] (Figure 27.1a). Even though various kinds of liposome-encapsulated Hb have been studied by many groups [7], our HbV encapsulates a highly concentrated Hb solution (>35 g/dL) with a regulated large particle diameter (250–280 nm) and attains 10 g/dL Hb concentration in the suspension. The absence of vasoconstriction in the case of intravenous HbV injection might

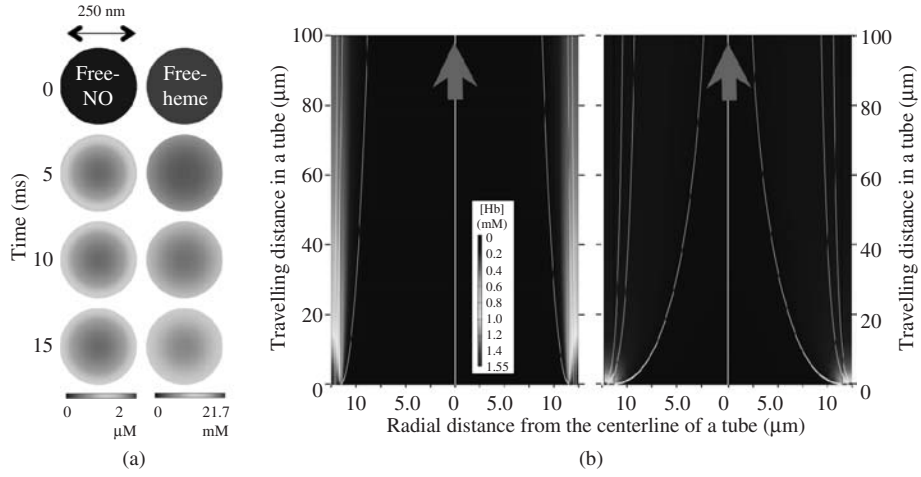

Figure 27.1 *Encapsulation of Hb in vesicles retards NO binding. (a) Schematic two-dimensional representation of the simulated time courses of distributions of unbound free NO and unbound free heme (deoxy form) in one HbV (250 nm) after immediate mixing of NO and HbV by stopped-flow method. Computer simulation shows that both free NO and unbound hemes are distributed heterogeneously. The concentration changes gradually from the surface to the core. The determinant factor of retardation of NO binding should be the intracellular diffusion barrier, which was induced by: (i) intrinsically larger binding rate constant of NO to a heme in an Hb molecule; (ii) numerous hemes as sites of gas entrapment at a higher Hb concentration; (iii) a slowed gas diffusion in the intracellular viscous Hb solution; and (iv) a longer gas diffusion distance in a larger capsule [31]. (b) Schematic representation of the simulated density distribution and track of HbV (left) and Hb (right) in a narrow tube (<100 μm traveling distance). We assumed that two different solutions with the same physicochemical properties enter and flow through the same tube. The radius of the tube was 12.5 μm: component 1 (blue color) enters the core of the tube (radial distance from the centerline, 0–11 μm) and component 2 (red color) enters near the wall (radial distance from the centerline, 11–12.5 μm). Finally, both components are mixed completely. The diffusivity of HbV is much slower than that of Hb, resulting in the retarded gas reactions in microvessels. The concentration of Hb is expressed as heme concentration ([Hb] = 1.55 mM at 10 g/dL) [39]. (Reproduced with permission from* Am J Physiol. Heart Circ. Physiol.*,* ***298****, H956–H965 (2010)).*

For a better understanding of the figure, please refer to color plate 6.

be related to the lowered NO binding rate constant, though it is much larger than that of RBCs, and the lowered permeability across the endothelial cell layer in the vascular wall.

The proposed mechanism of vasoconstriction induced by HBOCs in relation to gaseous molecules is not limited to NO scavenging. For example, endogenous carbon monoxide (CO) is produced by constitutive hemeoxygenase-2 in hepatocytes, serving as a vasorelaxation factor in hepatic microcirculation. Hb permeates across the fenestrated endothelium, scavenges CO, and induces constriction of sinusoids and augments peripheral resistance [15]. Oversupply of O_2 induces autoregulatory vasoconstriction to regulate the O_2 supply [34]. Injection of small HBOCs induces vasoconstriction, probably because of the facilitated O_2 transport [35].

These reports imply the importance of studying the reaction profiles of HBOCs with NO, CO, and O_2. Stopped-flow rapid-scan spectrophotometry and flash photolysis are common methods of defining the binding and dissociation rate constants of Hb [31, 32, 36, 37]. However, Hb concentration in a cuvette must be diluted extremely, for example to 2 μM heme concentration ([Hb] = 0.003 g/dL). On the other hand, gas-permeable narrow tubes enable the measurement of the O_2-releasing rates of HBOCs and RBCs during their flow through the tubes at a practical Hb concentration (6–13 g/dL) [23, 35, 38]. We used gas-permeable narrow tubes made of perfluorinated polymer to study not only O_2-release but also NO-binding and CO-binding profiles. We examined these gas reactions when Hb-containing solutions of four kinds were perfused through artificial narrow tubes at a practical Hb concentration (10 g/dL). Purified Hb solution, polymerized bovine Hb ($Poly_B$Hb), encapsulated Hb (HbV, 279 nm), and RBCs were perfused through a gas-permeable narrow tube (25 μm inner diameter) at 1 mm/second centerline velocity. The level of reactions was determined microscopically based on the visible-light absorption spectrum of Hb. When the tube was immersed in NO and CO atmospheres, both NO binding and CO binding of deoxygenated Hb and $Poly_B$Hb in the tube were faster than those of HbV and RBCs, and HbV and RBCs showed almost identical binding rates. When the tube was immersed in an N_2 atmosphere, oxygenated Hb and $Poly_B$Hb showed much faster O_2 release than did HbV and RBCs. $Poly_B$Hb showed a faster reaction than Hb because of the lower O_2 affinity of $Poly_B$Hb than of Hb [39].

The diffusion process of the particles was simulated using Navier–Stokes and Maxwell–Stefan equations (Figure 27.1b). Results clarified that small Hb (6 nm) diffuses laterally and mixes rapidly. However, the large-dimension HbV shows no such rapid diffusion. The NO and CO molecules, which diffuse through the tube wall and enter the lumen, would immediately react with Hb-containing solutions at the interface. Therefore, the fast mixing would be effective in creating more binding sites of these gas molecules. In the case of O_2 release, O_2 can be removed more easily at the tube wall, where the O_2 concentration gradient is the greatest. The fast mixing would create a higher concentration gradient and fast O_2 transfer. The purely physicochemical differences in diffusivity of the particles and the resulting reactivity with gas molecules are one factor inducing biological vasoconstriction of HBOCs.

27.4 HBOCs as a Carrier of not only O_2 but also CO

CO, biliverdin, and bililubin are produced during oxidative heme degradation that is catalyzed by a stress protein: heme oxygenase (HO). They mediate antioxidative, antiproliferative, and anti-inflammatory effects [40]. Endogenous CO shows a vasorelaxation effect, as does NO [41]. Motterlini *et al*. [42] synthesized a series of CO-releasing metal complexes; subsequent *in vivo* studies clarified some pharmacological effects. Despite the poisonous effect of CO gas, low-concentration CO inhalation (250 ppm) was tested in animal models of hemorrhagic shock, septic shock, and ischemia-reperfusion [43]. Some cytoprotective effects were obtained and the mechanism has been studied extensively. Cabrales *et al*. [44] recently reported CO-bound RBC injection to hemorrhaged hamsters and clarified its cytoprotective effect in subcutaneous microcirculation. These studies have led us to test intravenous injection of CO as a ligand of heme in HBOCs that have been extensively studied as transfusion alternatives.

A traumatic hemorrhage might cause a shock state, which subsequently causes a systemic inflammatory response, in some cases leading to multiple organ failure (MOF). Resuscitation with transfusion or HBOCs with an O_2-carrying capacity induces reperfusion injury, as evidenced by elevations of plasma enzyme levels and tissue cytokine levels [12, 45, 46]. Actually, we observed elevation of plasma enzyme levels 6 hours after resuscitation from hemorrhagic shock by administration of O_2-bound RBCs and HbVs in a rat model [11]. It is expected that co-injection of cytoprotective CO would improve resuscitative effects. For this study, using the same experimental model, we tested injection of CO-bound HbVs for the first time as an exogenous CO supplier for fluid resuscitation. In comparative experiments, we also tested empty vesicles (EVs) which carry neither O_2 nor CO, and CO-bound RBCs. All fluids showed restoration of blood-pressure and blood-gas parameters, and the rats survived for 6 hours of observation period. No remarkable difference was found among the groups, except that the EV group showed significant hypotension. Plasma enzyme levels (AST and ALT) were elevated, especially in the O_2-HbV, O_2-RBC, and EV groups. They were significantly lower in the CO-HbV and CO-RBC groups than in the O_2-bound fluids. Blood HbCO levels (26–39% immediately after infusion) decreased to less than 3% at 6 hours, while CO was exhaled through the lung, as detected by gas chromatography. Both HbV and RBC gradually gained the O_2 transport function. Accordingly, both CO-HbV and CO-RBC showed a resuscitative effect for hemorrhagic-shocked rats. They reduced oxidative damage to organs in comparison to O_2-HbV and O_2-RBC. Adverse and poisonous effects of CO gas were not evident in this experimental model [21].

Hemorrhagic shock and resuscitation typically entail systemic ischemia-reperfusion injury. Activated neutrophils and macrophages produce reactive oxygen species (ROS) [47], with NADPH-oxidase involved as a major source. This enzyme contains two hemes that catalyze the NADPH-dependent reduction of oxygen to form O_2^- [48]. However, CO can bind to the hemes and modulate the enzymatic activity [49]. During hemorrhagic shock, there should be an initiation of inflammatory cytokine production and NO release from the inducible form of NO synthase (NOS) in organs such as the liver and lung. In fact, CO gas potently inhibits the conversion of L-arginine to NO and citrulline by neuronal and macrophage NOS because two heme moieties are contained in the active enzymes. CO would modulate overproduction of NOS-derived NO [50]. Together, O_2^- and NO react to form peroxynitrate, $ONOO^-$, a potent cytotoxic molecule that promotes nitration of tyrosyl residues in proteins [51]. The possibility exists that the injected CO reduces production of both NO and O_2^-, and the resultant $ONOO^-$. Actually, our immunohistochemical observations of the liver and lung clarified that injection of CO-HbV and CO-RBC reduced the formation of nitrosotyrosine on the proteins.

To our knowledge, the present study is the first to use an HBOC to administer CO in a shock state for a pharmacological effect. Although further research is definitely necessary to clarify the mechanism and clinical relevance of our experimental results using small animals, the data would suggest that both RBCs and HBOCs can be effective CO-carriers. Vandegriff *et al*. also reported that CO-bound PEG-Hb reduces myocardial infarction [52]. The advantages of CO-bound HBOC injection are: (i) carbonylhemoglobin is stable for a longer-term storage; (ii) special equipment to inhale CO gas is not necessary in an emergency situation; (iii) the CO dosage is strictly definable; and (iv) the fluid functions initially as a CO carrier to prevent pro-oxidative damage and then as an O_2 carrier.

27.5 Conclusion

Historically, the starting point of the development of HBOCs simply aimed at transporting oxygen to peripheral tissues as blood does. However, the development became complicated after the discovery of endogenous NO and CO, which have strong affinity to HBOCs and influence on their safety. Actually, RBCs are designed to retard the gas reactions, and we have to reconsider the physiological significance of the RBC structure when designing HBOCs. In this chapter, we also demonstrated the potential of HBOCs as CO carriers. Of course, CO is a toxic gaseous molecule, but it shows cytoprotective effect depending on the dose.

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References

[1] Chang, T.M.S. Blood substitutes based on modified hemoglobin prepared by encapsulation or crosslinking: An overview, *Biomater. Artif. Cells Immobilization Biotechnol*., **20**, 159–182 (1991).

[2] Bangham, A.D. and Horne, R.W. Negative staining of phospholipids and their structure modification by surface-active agents as observed in the electron microscope, *J. Mol. Biol*., **8**, 660–668 (1964).

[3] Djordjevich L. and Miller, I.F. Lipid encapsulated hemoglobin as a synthetic erythrocyte, *Fed. Proc*., **36**, 567 (1977).

[4] Gaber, B.P., Yager, P., Sheridan, J.P. and Chang, E.L. Encapsulation of hemoglobin in phospholipid vesicles, *FEBS Lett*., **153**, 285–288 (1983).

[5] Djordjevich, L. and Ivankovich, A.D., Liposomes as carriers of hemoglobin, in *Liposomes as Drug Carriers* (ed. G. Gregoriadis), John Wiley & Sons Ltd, Chichester, pp. 551–567 (1988).

[6] Hunt, C.A., Burnette, R.R., MacGregor, R.D. *et al*. Synthesis and evaluation of prototypal artificial red cells, *Science*, **230**, 1165–1168 (1985).

[7] Sakai, H., Sou, K. and Tsuchida, E. Hemoglobin-vesicles as an artificial oxygen carrier, *Methods Enzymol*., **465**, 363–383 (2009).

[8] Rudolph, A.S., Klipper, R.W., Goins, B. and Phillips, W.T. In vivo biodistribution of a radiolabeled blood substitute: ^{99m}Tc-labeled liposome-encapsulated hemoglobin in an anesthetized rabbit, *Proc. Natl. Acad. Sci. U S A*, **88**, 10976–10980 (1991).

[9] Tsuchida, E. *Blood substitutes: Present and Future Perspectives*, Elsevier, Chichester (1998).

[10] Yamazaki, M., Aeba, R., Yozu, R. and Kobayashi, K. Use of hemoglobin vesicles during cardiopulmonary bypass priming prevents neurocognitive decline in rats, *Circulation*, **114**(Suppl. 1), I220–I225 (2006).
[11] Sakai, H., Masada, Y., Horinouchi, H. *et al*. Hemoglobin-vesicles suspended in recombinant human serum albumin for resuscitation from hemorrhagic shock in anesthetized rats, *Crit. Care Med*., **32**, 539–545 (2004).
[12] Sakai, H., Seishi, Y., Obata, Y. *et al*. Fluid resuscitation with artificial oxygen carriers in hemorrhaged rats: profiles of hemoglobin-vesicle degradation and hematopoiesis for 14 days, *Shock*, **31**, 192–200 (2009).
[13] Taguchi, K., Maruyama, T., Iwao, Y. *et al*. Pharmacokinetics of single and repeated injection of hemoglobin-vesicles in hemorrhagic shock rat model, *J. Control. Release*, **136**, 232–239 (2009).
[14] Cabrales, P., Sakai, H., Tsai, A.G. *et al*. Oxygen transport by low and normal oxygen affinity hemoglobin vesicles in extreme hemodilution, *Am. J. Physiol. Heart Circ. Physiol*., **288**, H1885–H1892 (2005).
[15] Goda, N., Suzuki, K., Naito, M. *et al*. Distribution of heme oxygenase isoforms in rat liver. Topographic basis for carbon monoxide-mediated microvascular relaxation, *J. Clin. Invest*., **101**, 604–612 (1998).
[16] Verdu, E.F., Bercik, P., Huang, X.X. *et al*. The role of luminal factors in the recovery of gastric function and behavioral changes after chronic Helicobacter pylori infection, *Am. J. Physiol. Gastrointest. Liver Physiol*., **295**, G664–G670 (2008).
[17] Komatsu, H., Furuya, T., Sato, N. *et al*. Effect of hemoglobin vesicle, a cellular-type artificial oxygen carrier, on middle cerebral artery occlusion- and arachidonic acid-induced stroke models in rats, *Neurosci. Lett*., **421**, 121–125 (2007).
[18] Plock, J.A., Tromp, A.E., Contaldo, C. *et al*. Hemoglobin vesicles reduce hypoxia-related inflammation in critically ischemic hamster flap tissue, *Crit. Care Med*., **35**, 899–905 (2007).
[19] Plock, J.A., Rafatmehr, N., Sinovcic, D. *et al*. Hemoglobin vesicles improve wound healing and tissue survival in critically ischemic skin in mice, *Am. J. Physiol. Heart Circ. Physiol*., **297**, H905–H910 (2009).
[20] Yamamoto, M., Izumi, Y., Horinouchi, H. *et al*. Systemic administration of hemoglobin vesicle elevates tumor tissue oxygen tension and modifies tumor response to irradiation, *J. Surg. Res*., **151**, 48–54 (2009).
[21] Sakai, H., Horinouchi, H., Tsuchida, E. and Kobayashi, K. Hemoglobin vesicles and red blood cells as carriers of carbon monoxide prior to oxygen for resuscitation after hemorrhagic shock in a rat model, *Shock*, **31**, 507–514 (2009).
[22] Tiwari, V.N., Kiyono, Y., Kobayashi, M. *et al*. Automatic labeling method for injectable ^{15}O-oxygen using hemoglobin-containing liposome vesicles and its application for measurement of brain oxygen consumption by PET, *Nucl. Med. Biol*., **37**, 77–83 (2010).
[23] Sakai, H., Suzuki, Y., Kinoshita, M. *et al*. O_2 release from Hb vesicles evaluated using an artificial, narrow O_2-permeable tube: comparison with RBCs and acellular Hbs, *Am. J. Physiol. Heart Circ. Physiol*., **285**, H2543–H2551 (2003).
[24] Sakai, H., Sato, A., Takeoka, S. and Tsuchida, E. Mechanism of flocculate formation of highly concentrated phospholipid vesicles suspended in a series of water-soluble biopolymers, *Biomacromolecules*, **10**, 2344–2350 (2009).

[25] Sakai, H., Sato, A., Takeoka, S. and Tsuchida, E. Rheological properties of hemoglobin vesicles (artificial oxygen carriers) suspended in a series of plasma-substitute solutions, *Langmuir*, **23**, 8121–8128 (2007).
[26] Sou, K., Klipper, R., Goins, B. *et al*. Circulation kinetics and organ distribution of Hb vesicles developed as a red blood cell substitute, *J. Pharmacol. Exp. Ther*., **312**, 702–709 (2005).
[27] Sakai, H., Horinouchi, H., Tomiyama, K. *et al*. Hemoglobin-vesicles as oxygen carriers: influence on phagocytic activity and histopathological changes in reticuloendothelial system, *Am. J. Pathol*., **159**, 1079–1088 (2001).
[28] Taguchi, K., Urata, Y., Anraku, M. *et al*. Pharmacokinetic study of enclosed hemoglobin and outer lipid component after the administration of hemoglobin-vesicles as an artificial oxygen carrier, *Drug Metab. Disposit*., **37**, 1456–1463 (2009).
[29] Wang, L., Morizawa, K., Tokuyama, S. *et al*. Modulation of oxygen-carrying capacity of artificial red cells (ARC), *Polymer Adv. Technol*., **4**, 8–11 (1992).
[30] Sakai, H., Hara, H., Yuasa, M. *et al*. Molecular dimensions of Hb-based O_2 carriers determine constriction of resistance arteries and hypertension, *Am. J. Physiol. Heart Circ. Physiol*., **279**, H908–H915 (2000).
[31] Sakai, H., Sato, A., Masuda, K. *et al*. Encapsulation of concentrated hemoglobin solutionin phospholipid vesicles retards the reaction with NO, but not CO, by intracellular diffusion barrier, *J. Biol. Chem*., **283**, 1508–1517 (2008).
[32] Sakai, H., Sato, A., Sobolewski, P. *et al*. NO and CO binding profiles of hemoglobin vesicles as artificial oxygen carriers, *Biochim. Biophys. Acta*, **1784**, 1441–1447 (2008).
[33] Natanson, C., Kern, S.J., Lurie, P. *et al*. Cell-free hemoglobin-based blood substitutes and risk of myocardial infarction and death: a meta-analysis, *JAMA*, **299**, 2304–2312 (2008).
[34] Harder, D.R., Narayaran, J., Birks, E.K. *et al*. Identification of a putative microvascular oxygen sensor, *Circ. Res*., **79**, 54–61 (1996).
[35] McCarthy, M.R., Vandegriff, K.D. and Winslow, R.M. The role of facilitated diffusion in oxygen transport by cell-free hemoglobins: implications for the design of hemoglobin-based oxygen carriers, *Biophys. Chem*., **92**, 103–117 (2001).
[36] Olson, J.S., Foley, E.W., Rogge, C. *et al*. NO scavenging and the hypertensive effect of hemoglobin-based blood substitutes, *Free Radic. Biol. Med*., **36**, 685–697 (2004).
[37] Rohlfs, R.J., Bruner, E., Chiu, A. *et al*. Arterial blood pressure responses to cell-free hemoglobin solutions and the reaction with nitric oxide, *J. Biol. Chem*., **273**, 12128–12134, (1998).
[38] Page, T.C., Light, W.R., McKay, C.B. and Hellums, J.D. Oxygen transport by erythrocyte/hemoglobin solution mixtures in an in vitro capillary as a model of hemoglobin-based oxygen carrier performance, *Microvasc. Res*., **55**, 54–66 (1998).
[39] Sakai, H., Okuda, N. Sato, A. *et al*. Hemoglobin encapsulation in vesicles retards NO- and CO-binding and O_2-release when perfused through narrow gas-permeable tubes, *Am. J. Physiol. Heart Circ. Physiol*., **298**, H956–H965 (2010).
[40] Ryter, S.W., Alam, J. and Choi, A.M. Heme oxygenase-1/carbon monoxide: from basic science to therapeutic applications, *Physiol. Rev*., **86**, 583–650 (2006).

[41] Morita, T., Perrella, M.A., Lee M.E. and Kourembanas, S. Smooth muscle cell-derived carbon monoxide is a regulator of vascular cGMP, *Proc. Natl. Acad. Sci. U S A*, **92**, 1475–1479 (1995).
[42] Motterlini, R., Clark, J.E., Foresti, R. *et al*. Carbon monoxide-releasing molecules: characterization of biochemical and vascular activities, *Circ. Res*., **90**, e17–e24 (2002).
[43] Morse, D., Pischke, S.E., Zhou, Z. *et al*. Suppression of inflammatory cytokine production by carbon monoxide involves the JNK pathway and AP-1, *J. Biol. Chem*., **278**, 36993–36998 (2003).
[44] Cabrales, P., Tsai, A.G. and Intaglietta, M. Hemorrhagic shock resuscitation with carbon monoxide saturated blood, *Resuscitation*, **72**, 306–318 (2007).
[45] Lehnert, M., Arteel, G.E., Smutney, O.M. *et al*. Dependence of liver injury after hemorrhage/resuscitation in mice on NADPH oxidase-derived superoxide, *Shock*, **19**, 345–351 (2003).
[46] Hierholzer, C. and Billiar, T.R. Molecular mechanisms in the early phase of hemorrhagic shock, *Langenbecks Arch. Surg*., **386**, 302–308 (2001).
[47] Jaeschke, H. and Farhood, A. Neutrophil and Kupffer cell-induced oxidant stress and ischemia-reperfusion injury in rat liver, *Am. J. Physiol. Gastroenterol. Liver Physiol*., **260**, G355–G362 (1991).
[48] Babior, B.M., Lambeth J.D. and Nauseef, W. The neutrophil NADPH oxidase, *Arch. Biochem. Biophys*., **397**, 342–344 (2002).
[49] Taillé, C., El-Benna, J., Lanone, S. *et al*. Mitochondrial respiratory chain and NAD(P)H oxidase are targets for the antiproliferative effect of carbon monoxide in human airway smooth muscle, *J. Biol. Chem*., **280**, 25350–25360 (2005).
[50] Ishikawa, M., Kajimura, M., Adachi, T. *et al*. Carbon monoxide from heme oxygenase-2 is a tonic regulator against NO-dependent vasodilatation in the adult rat cerebral microcirculation, *Circ. Res*., **97**, e104–e114 (2005).
[51] Kooy, N.W., Royall, J.A., Ye, Y.Z. *et al*. Evidence for in vivo peroxynitrite production in human acute lung injury, *Am. J. Respir. Crit. Care Med*., **151**, 1250–1254 (1995).
[52] Vandegriff, K.D., Young, M.A., Lohman, J. *et al*. CO-MP4, a polyethylene glycol-conjugated haemoglobin derivative and carbon monoxide carrier that reduces myocardial infarct size in rats, *Br. J. Pharmacol*., **154**, 1649–1661 (2008).

28

Animal Models and Oxidative Biomarkers to Evaluate Preclinical Safety of Extracellular Hemoglobins

Paul W. Buehler and Felice D'Agnillo

Laboratory of Biochemistry and Vascular Biology, Division of Hematology, Center for Biologics Evaluation and Research (CBER), Food and Drug Administration (FDA), Maryland, USA

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The findings and conclusions in this chapter have not been formally disseminated by the Food and Drug Administration and should not be construed to represent any agency determination or policy.

28.1 Introduction

The basis for all preclinical safety testing is to demonstrate reasonable safety risks to human subjects in various phases of clinical evaluation and post regulatory approval. Unique to the study of hemoglobin-based oxygen carriers (HBOCs) have been challenges in linking predictive safety from GLP (good laboratory practice) studies involving normal healthy animals to a heterogeneous population of subjects with disease-state complications and underlying comorbidities, which may limit the safety of HBOC product candidates. Decades of knowledge from human clinical trials regarding the nature of adverse events and a lack of corroborating overt toxicological response in normal animals dosed with HBOCs appear to underscore a clear disconnect between preclinical

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safety results and clinical outcomes with certain HBOCs. This suggests that refined and innovative approaches to assessing safety in human predictive species, validated animal models of disease, and validation of new markers of end-organ toxicity may be needed. Animal species/models that are relevant with regard to HBOC pharmacokinetics, pharmacodynamics, and physiologic response to HBOCs in humans would help to advance our understanding of HBOC safety. The publically recorded data on adverse-event profiles in clinical studies suggest that hemoglobin (Hb)-induced vascular injury is a major contributor to human toxicology [1, 2]. The origin of these events has focused primarily on nitric oxide (NO) scavenging and vasoconstriction as an important factor in the processes of early- and late-stage toxicity, with minimal emphasis on heme-induced oxidative stress.

The present chapter focuses on primary toxicities of Hbs/HBOCs, standard toxicology/safety pharmacology studies, and disease-state models in preclinical testing of HBOCs. Additionally, experimental approaches to toxicity testing using novel species selection, animal-model design, and markers of oxidative toxicity to evaluate responses to HBOC exposure will be discussed. Emphasis is placed on assessment of Hb-related toxicity from the perspective of oxidative stress.

28.2 HBOC Safety and Efficacy

28.2.1 Proposed Mechanisms of Toxicity

28.2.1.1 Hypertension

HBOCs are often described as problematic due to systemic and pulmonary hypertension (defined by increased vascular resistance), which occurs immediately upon administration. While numerous physiologic components can influence systemic and pulmonary blood pressure, the most accepted hypothesis suggests that Hb-induced hypertension is primarily caused by the interactions of NO with oxy- and deoxy-Hb [3, 4]. HBOC depletion of NO increases blood pressure by decreasing available NO for binding with the heme of vascular smooth-muscle (VSM) guanylate cyclase. The end result is loss of VSM relaxation mediated by cGMP and an increase in systemic vascular resistance [5, 6]. This physiologic response leads to a reduction in heart rate (HR), stroke volume (SV), and ultimately cardiac output distribution to vital organ systems. Hb effects on VSM have been suggested as a cause for myocardial lesions in certain animal species [7] and myocardial adverse events in some clinical trials [8]. Attempts at attenuation of HBOC-induced hypertension have focused on methods to increase NO bioavailabity and normalize vasoconstriction by either limiting NO/heme pocket interactions, thus employing an NO-sparing approach [3, 4, 9], or by pharmacologic methods of NO supplementation [10]. The NO interactions (i.e. NO dioxygenation) with oxy-Hb may in part lead to Hb oxidation ($HbFe^{2+}\text{-}O_2 + NO \rightarrow HbFe^{3+} + NO_3^-$) and could contribute to oxidative toxicity *in vivo*.

28.2.1.2 Oxidative Stress

Intracellular hydrogen peroxide (H_2O_2) is generated by dismutation of superoxide ($O_2^{\bullet -}$) either spontaneously or by superoxide dismutase. Its primary fate is intracellular

conversion to H_2O and O_2 by catalase and peroxidases and it is involved in cell signaling. Remaining H_2O_2 can be converted to reactive oxygen species (ROS) or secreted into the extracellular environment, typically in low (1–15 μM) quantities [11]. Additionally, lipid hydroperoxides in tissue compartments are important components influencing oxidative toxicity of Hbs [12]. During inflammatory states, certain cell types increase production and release of H_2O_2, as well as intracellular production and secretion of hypochlorus acid (HOCl) from H_2O_2 and chloride (Cl^-) [13, 14]. It is likely within localized tissue compartments and certain vascular sites that increased levels of prooxidants encounter extracellular Hb and HBOCs.

The mechanism of toxicity associated with Hb and potentially HBOC-dissociated tetramers [15] excreted by the kidneys may be caused by redox cycling and ferryl-Hb formation [12]. During rhabdomyolysis, redox cycling of ferric and ferryl myoglobin driven by lipid hydroperoxides enhances tissue oxidation and F_2-isoprostane release [12, 16]. Recent studies demonstrate extensive tissue protein adducts in renal proximal tubules following extracellular Hb exposures [17]. Observations of renal excretion of dimeric, tetrameric, and polymeric Hb as well as iron deposition and oxidative tissue modifications have been made in animals [18]. Within the gastrointestinal circulation of rats, an increase in microvascular permeability to fluorescently labeled albumin was reported to occur with several HBOCs [19, 20]. The effects appeared to be causally linked to rates of oxidation for individual HBOCs tested [21]. Co-administration of sodium selenite reduced mesenteric vascular leakage and mucosal epithelial damage [22, 23]. More recent data suggest increased heme exposure, as determined by heme oxygenase (HO-1) immunohistochemistry within the aortic wall of rodents exposed to a polymerized bovine Hb [24] (see Figure 28.1). *In vivo* studies do suggest a relationship between Hb and HBOCs in oxidative tissue toxicity. However, the effects of heme-related oxidative stress in standard GLP toxicology studies are typically not evaluated, therefore potential contribution to early- or late-stage safety signals may not be fully appreciated.

28.2.2 Safety Pharmacology and Toxicology Studies

The regulations of biologic products within the United States are governed by the Public Health Service Act as well as the Food Drug and Cosmetic Act. The Code of Federal Regulations (CFR) for biologics (21 CFR 601.2 (a)) states that manufacturers, in order to obtain a license for marketing, must submit preclinical data to support safety, purity, and potency. With regard to pharmacology and toxicology studies, the Investigational New Drug regulations under 21 CFR 312.23 (a) [8] state "the kind, duration and scope of animal and other tests required varies with the duration and nature of the proposed clinical investigation". Guidance on the type and scope of nonclinical safety studies is recommended by the International Conference on Harmonization (ICH, ICH S6, "Pre-clinical Safety Evaluation of Biotechnology-derived Pharmaceuticals") based on the recommendations of representatives from government, academia, and industry. Additionally, guidance documents are drafted within the US Food and Drug Administration (FDA) to reflect the current FDA thinking on a particular topic (http://www.fda.gov/RegulatoryInformation/Guidances).

The basis for safety pharmacology studies is to evaluate functional indices of toxicity in major organ systems. Historically, these studies have included physiologic monitoring

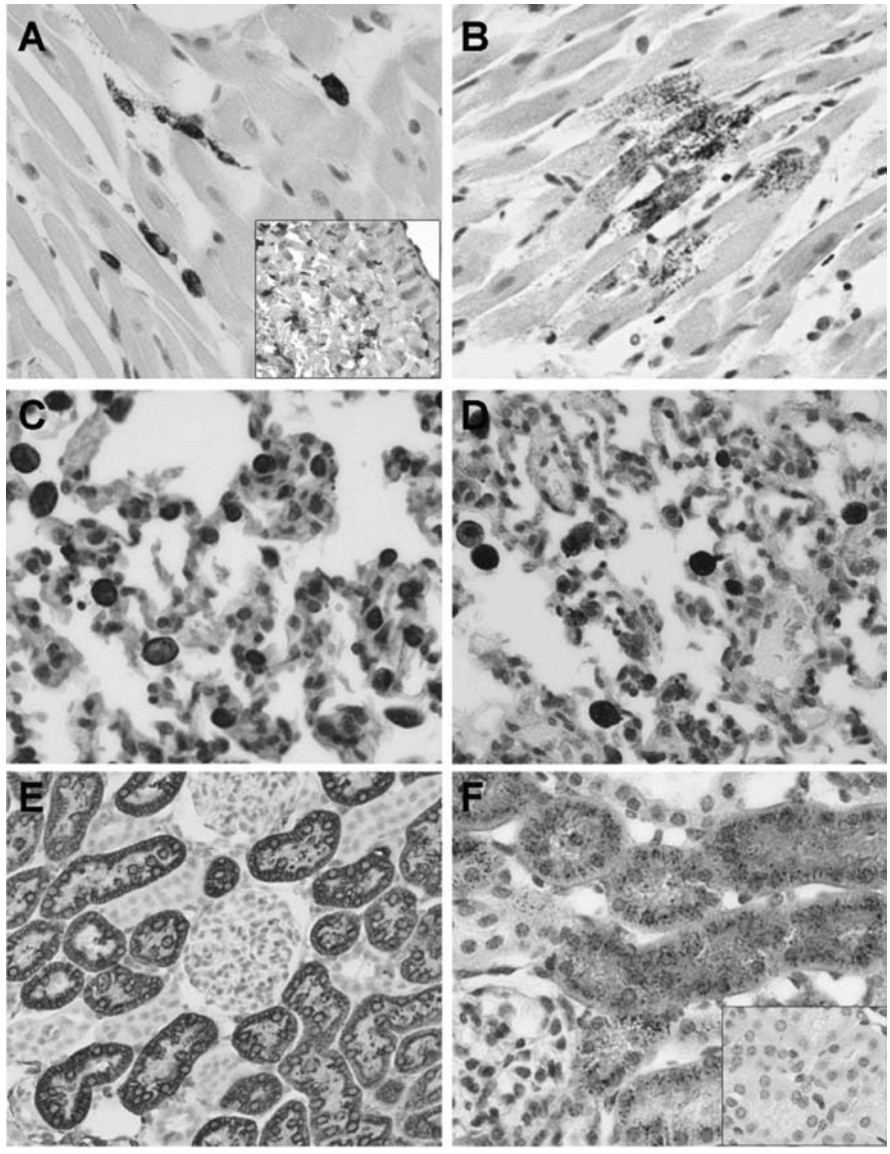

Figure 28.1 *HO-1 induction and non-heme iron deposition following HBOC infusion in rodents. Tissues were collected following 50% exchange transfusion of polymerized bovine Hb and analyzed for HO-1 ((a), (c), (d)) or Perls non-heme iron histochemistry with DAB intensification ((b), (e), (f)). (a), (b) Heart. HO-1 induced primarily in interstitial cells, possibly infiltrating phagocytes in myocardium and in perivascular sites in the adventitial layers of the aorta (inset). Positive Perls iron was observed in myocytes (brown). (c), (d) Lung. Large alveolar macrophages displaying HO-1 expression and non-heme iron deposits 12 hours after HBOC administration. (e), (f) Kidney. Striking HO-1 reactivity and iron deposits in renal proximal tubular epithelium but absent in glomeruli compared to nontreated kidney (inset). Magnification 400 ×.*
For a better understanding of the figure, please refer to color plate 7.

of the cardiovascular, renal, pulmonary, and central nervous systems, as well as clinical chemistries to assess potential safety concerns in specific organ systems. The findings from safety pharmacology studies have helped focus on organ systems and toxicity end points. Toxicology studies are typically performed under GLP in normal animals of both genders to understand the relationship between dosing levels and systemic as well as local toxicity when the intended clinical route, dosing regimen, and duration of exposure are evaluated. Historically, studies have employed toxicokinetic approaches to help understand the potential relationships between toxicity and pharmacokinetic parameters. It has been shown that under some circumstances, animal models of disease may help to understand the impact of a given product candidate on toxicity. One consideration to maximize the relevance and prevent repetition in nonclinical studies is to employ test material that is comparable to the final product used in clinical studies and proposed for licensure.

HBOCs have presented a challenge to the paradigm of standard safety pharmacology and toxicology testing. Parameters evaluated in animal studies have in several circumstances not adequately predicted adverse outcomes in later phases of clinical testing [1, 2, 25]. There are several factors that may account for this within the scope of HBOCs: (i) *Timing of toxicological assessment*: early time points (within 24 hours after dosing) of toxicological evaluation are not routinely performed and therefore indicators of acute responses in tissue may be undetected. (ii) *Sensitivity of standard toxicology*: standard testing generally does not employ specialized tissue-staining techniques to identify indicators of oxidative stress and inflammation in the vascular tissue of major blood vessels or within the vasculature of critical organ systems (heart, brain, and kidneys). (iii) *Specialized models*: animal models relevant to exacerbation of HBOC toxicity such as antioxidant depletion, early endothelial dysfunction, advanced endothelial dysfunction (atherosclerosis), and renal impairment have not been routinely used to evaluate safety. (iv) *Dosing*: HBOC dosing was, in some circumstances, estimated from animals rather than carefully designed dose-finding studies in humans; as a result, clinical response to certain HBOC therapies may have been compromised [2, 26, 27].

28.2.3 *In vivo* Models of Efficacy "Proof of Concept"

Models of efficacy in animals have been used to establish a proof of concept, demonstrate physiologic/pharmacologic effect, and suggest feasibility in support for use in human-specific indications. In combination with safety pharmacology and toxicology studies, proof-of-concept studies have provided a basis for progression into clinical trials. However, the main purpose of preclinical animal testing remains the assurance of safety in human subjects in all phases of clinical trials and this is defined primarily by preclinical toxicology data. Numerous proof-of-concept models have been studied in the nonclinical evaluation of HBOCs to establish physiologic effects on tissue blood flow and oxygenation, as well as efficacy in primary disease states for which HBOCs could be indicated.

28.2.3.1 Tissue Blood Flow and Oxygenation

Measurements of tissue blood flow and oxygenation (tPO_2) studies have helped define effective HBOC tissue perfusion. Electromagnetic flowmetry in large blood vessels [27]

as well as radioactive and fluorescent microsphere distribution techniques [28–33] have been utilized to determine macrocirculatory blood flow and cardiac output distribution following administration of HBOCs. Intravital microscopy as well as laser doppler flowmetry have been primary approaches to determining microcirculatory blood flow following HBOC administration [34–37]. In general, these techniques provide reliable estimates of blood flow to specific organs as well as cardiac output distribution to organs.

Measurements of tPO_2 in skeletal muscle [27], gastrointestinal tract [38, 39], heart [40], skin [34–37], liver [41], and brain [42, 43] following HBOC administration have been performed using embedded O_2 electrodes, optical O_2 electrodes, or fiber-optic palladium-porphyrin phosphorescence quenching. However, these techniques may raise significant reliability questions since they are limited to regions within a specific organ (often to the tip of a tissue-embedded electrode), they require invasive approaches, and data cannot be extrapolated to other tissues. Alternatively, protein- and gene-based biomarker approaches to tissue O_2 delivery have been attempted using hypoxia-inducible factor (HIF) and related downstream gene targets of oxygen regulation [44, 45]. This approach has been useful as a screening method in normal animals and has allowed for evaluation of response in multiple tissues. Molecular imaging with magnetic resonance (MRI) and positron-emission tomography (micro-PET and PET) have advanced to the point of utility in preclinical investigation [46] and have been shown to be particularly useful in HBOC proof of concept regarding oxygenation and tissue perfusion in normal and disease-state animal models [47].

28.2.3.2 Traumatic Hemorrhage

Animal models of either targeted blood-volume removal (40–50%) or pressure-controlled hemorrhage (30–40 mm Hg, MAP), followed by set time periods (30–90 minutes) prior to resuscitation, have been used in combination with tissue injury (surgical tissue damage, bone fracture, brain injury, and chest injury) to study the early and late physiologic effects of trauma in combination with blood loss. Several animal species, including rodents (mice and rats) [48–50], guinea pigs [51], dogs [52, 53], swine [54–57], sheep [58], and nonhuman primates [59], have been evaluated. Each species provides advantages and disadvantages in the study of hemorrhage and trauma. For example, mice and rats are advantageous due to being widely available and low-cost. Additionally, advances in transgenic manipulation and increasing commercial availability of specialized mice provide the advantage of being able to study the role of certain proteins in inflammatory [60] and the coagulation cascades [61]. Disadvantages of these species tend to be related to technical challenges regarding specialized surgical techniques; however, with the advent of long-term implantable pumps, telemetry probes, and specialized catheters, this issue is less relevant. The nonrodent species most popular and amenable to the study of HBOC safety in traumatic hemorrhage appears to be swine. Advantages include ease of monitoring, long-term monitoring, and similar cardiovascular and hemodynamic responses to human. Disadvantages suggested by unpublished observational data gathered from multiple studies indicate that the species can tolerate long durations with low hematocrits ($\sim$ 3 g/dL of Hb) and may therefore not extrapolate well to human transfusion and predictive survival. From a physiology and pharmacology

perspective, these models can provide valuable insight into the impact of HBOCs on short- and long-term physiologic consequences of traumatic hemorrhage. Additionally, animal models intended to mimic clinical indications for HBOCs may be useful in identifying preclinical safety signals for increased monitoring in selected clinical settings, or in some circumstance may suggest avoiding the use of a product candidate in a specific setting.

28.2.3.3 Local Ischemia

Several HBOCs have been proposed for use in ischemic stroke, myocardial infarction, and coronary-artery-bypass-graft (CABG) surgery. In ischemic stroke animal models, a proof-of-concept study that employed middle-cerebral-artery occlusion demonstrated exceptional treatment efficacy of DCLHb [62, 63]. However, evaluation of DCLHb in human subjects demonstrated worsening of ischemic stroke following treatment [64, 65]. An inability to predict from animal models the increased risk of ischemic insult caused by DCLHb may result from differences between humans with multiple vascular-disease contributing factors and animals subjected to periods of ischemia but otherwise in good health. The Stroke Therapy Academic Industry Roundtable (STAIR) issued guidelines in 1999 geared towards preclinical efficacy of therapeutics for use in stroke [66]. While these guidelines have not specifically been applied to HBOC products, a clear understanding of the potential for HBOCs to enhance local toxicity in stroke and other ischemic conditions remains unclear.

28.2.3.4 Sickle Cell Disease

Conceptually, HBOC delivery of O_2 during vaso-occlusion suggests promise in a disease state with limited therapeutic options. However, there are toxicity-based questions regarding the safety of extracellular Hb exposure in sickle cell disease, particularly since disease-state progression in sickle cell patients is in part due to extracellular Hb exposure. From a pharmacologic perspective, the conceptual advantages of HBOC may be in acute sickle cell crisis. An understanding of the potential toxicity of HBOC preparations in sickle cell models may help guide product development and clinical use to optimize benefit and limit risk. Options for preclinical study exist in transgenic mice, although these models are not widely available. Two examples of models used to evaluate toxicity and proof of concept include the S + S Antilles and BERK transgenic sickle cell mouse strains. The S + S Antilles strain is based on C57BL/6 mice but expresses human α, β^s, and $\beta^{s\text{-Antilles}}$ globin transgenes, with approximately 42% of β globin chains expressed as β^s and approximately 36% as $\beta^{s\text{-Antilles}}$ [67–69]. BERK mice express human α, β^s globin transgenes and represent the most severe model of disease [67, 70]. Both transgenic strains experience red blood cell (RBC) congestion, inflammation, ischemia, and tissue infarction similar to sickle cell patients. The S+S Antilles mouse provides an opportunity to understand oxidative and inflammatory mechanisms underlying the pathology of sickle cell disease and any benefit or risk imparted by HBOC formulations. Certain investigators have recommended that study designs should rely on two independent transgenic mouse strains, as each may demonstrate its own strengths and weaknesses [67].

28.2.4 Experimental Approaches to Assessing Preclinical Safety of HBOCs

28.2.4.1 Species Antioxidant Status (Natural Evolution)

Typically animal species for prediction of human toxicity response are specifically chosen based on pharmacokinetics, pharmacology, and physiology which may be relevant to humans. One lesser studied area with HBOCs is oxidative stress caused by heme exposure to vasculature as well as tissue-compartment parenchyma. The initiation of oxidative toxicity may be acute or long-term but is likely dependent on circulating as well as tissue antioxidant status. Evaluation of HBOCs in species or transgenic animals relevant to human antioxidant states may increase understanding of oxidative-stress safety signals when HBOCs are administered. Species such as nonhuman primates, Indian fruit bats, guinea pigs, and humans are unable to produce hepatic ascorbic acid (AA) due to an evolutionary loss of L-gulonolactone-γ- oxidase (LGO), which is the rate-limiting enzyme in AA synthesis, and therefore these species rely on dietary intake of this small-molecule antioxidant [71]. In the case of small animals, the guinea pig additionally has upregulated antioxidant systems (SOD and catalase), in agreement with human systems [72]. Guinea pig and human RBC reductive capacity similarities have also been suggested [73]. The rat is the prototype rodent species used in preclinical toxicology; however, rats possess very different antioxidant status in tissue and circulation compared to humans, generating AA at a rate of 38 μg AA/mg microsomal protein/hour in the liver and much higher amounts when subjected to physical stressors [72]. As a critical plasmatic small molecule responsible for limiting ferric Hb formation, AA, along with circulating RBC reductive capacity, may be important to predicting HBOC oxidation and tissue exposures to ferric Hb (Figure 28.2).

Other intriguing species include the osteogenic-disorder Shionogi rats, which were originally described in 1984 as a naturally occurring hereditary LGO-deficient colony of Wistar rats genetically susceptible to scurvy [74]. The loss of functional LGO was found to be caused by a single nucleotide alteration in the LGO mRNA [75]. These rats have been evaluated on a limited basis and primarily used as models of osteogenic disorders based on reduced collagen production. It remains unknown how these animals have compensated for loss of or reduced AA production. Nonetheless, they may offer benefit in better understanding certain pro-oxidative effects of HBOCs preclinically. Finally, with increasing ability to obtain knockout mouse phenotypes, the ability to combine single-LGO knockout or multiple-antioxidant knockout phenotypes is possible and may serve as a useful component to understanding the role of antioxidant status and HBOC toxicity.

28.2.4.2 Chemically Induced Antioxidant Depletion

A number of chemical approaches have been employed to deplete endogenous antioxidant status in cell culture and in animals, particularly for glutathione (GSH). Reduced GSH, the most abundant cellular thiol (0.5–10 mM), plays an important role in regulating the intracellular redox environment. GSH depletion occurs in ischemia, reperfusion, sepsis, organ transplantation, and myocardial conditions [76, 77]. A well-studied and reliable chemical approach used to deplete intracellular glutathione (GSH) stores involves using buthionine-[S,R]-sulfoximine (BSO), an inhibitor of gamma-glutamylcysteine synthetase, the enzyme catalyzing the rate-limiting step in GSH synthesis. *In vitro* studies using BSO

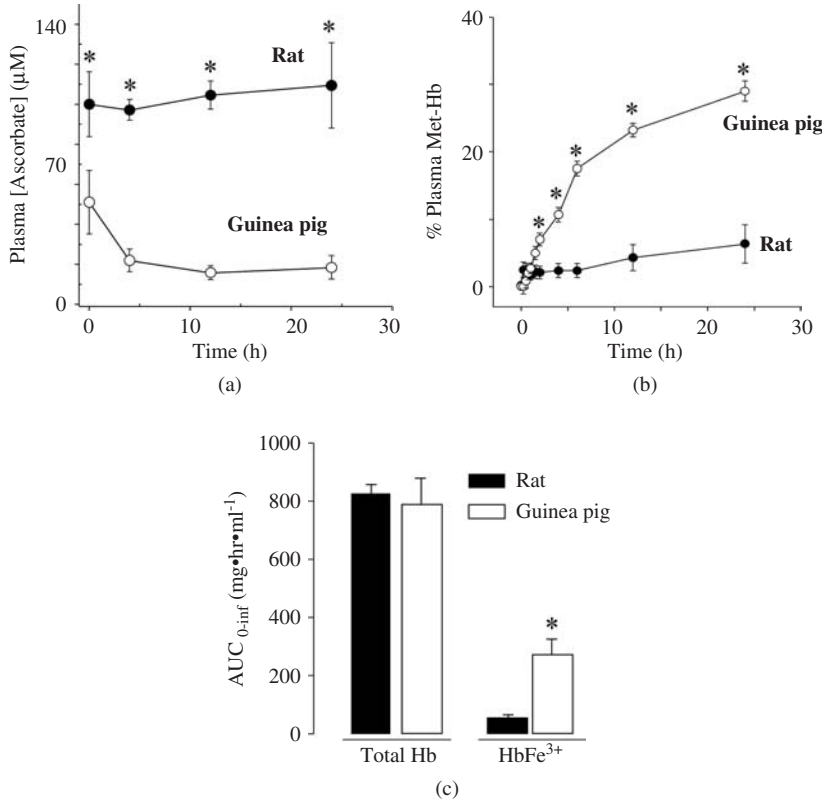

Figure 28.2 *(a) The potential influence of plasma AA levels on iron oxidation of circulating polymerized bovine Hb in AA-producing rats and non-AA-producing guinea pigs. Rats maintain constant AA levels despite a 50% dilution with polymerized bovine Hb, demonstrating their ability to synthesize AA, unlike the guinea pig. (b) The effect on plasma polymerized bovine Hb is seen with nearly 40% of the circulating polymerized bovine Hb in the ferric (met) form within 24 hours compared to less than 10% in the rat. (c) The pharmacokinetic estimates obtained from single-compartment modeling using Winonlin software indicate equal total polymerized bovine Hb AUCs in rat and guinea pig and fourfold greater ferric polymerized bovine Hb in guinea pig, indicating a four-times greater total dosing exposure.*

pretreatment rendered endothelial cells markedly more susceptible to the cytotoxicity induced by the redox cycling of Hb and HBOCs [78, 79]. Treatment with L-BSO i.p. produces reproducible GSH depletion in liver, heart, and kidney in rodent species [80, 81]. Preclinical models of antioxidant depletion may provide helpful tools to better evaluate and compare the oxidative toxicity of various HBOCs, and may at the same time offer insight into potential protective antioxidant strategies.

28.2.4.3 Endothelial Dysfunction

The vascular endothelium plays a key role in determining the overall response to HBOCs. *In vitro* studies largely describe underlying mechanisms of cytotoxicity caused

by interaction of ferryl heme and potentially ROS [79, 82]. The balance between NO and $O_2^{\bullet -}$ in the vasculature can be disrupted in the presence of cell-free Hb in favor of more powerful oxidants such as peroxynitrite and H_2O_2. It is likely that both decrease NO vascular function, and heme interactions with the vasculature, particularly in locations of plaque formation, exacerbate endothelial/vascular disease progression. In normal animals, HO-1 induction has been demonstrated within the tunica adventitia and media of large vessels [24] (Figure 28.1). Recent findings from our laboratory showed increased HO-1 expression in areas of necrosis and is suggestive of heme exposure. *In vivo* endothelial disease-progression models using atherogenic diet supplementation, long-term hypertension, and diabetes in animals have been established in the study of atherosclerosis. A mouse model of high-fat dietary supplementation and NO function was recently studied with regards to Hb and HBOCs [83]. This study demonstrated that the adverse hemodynamic effects of a dysfunctional vascular system were more pronounced than in normal dieted animals.

Models of cholesterol-induced atherosclerosis in animals can be designed to mimic human disease progression. For example, hypercholesterolemia models using rats, guinea pigs, and rabbits have been developed and could be employed to evaluate the potential safety risks of HBOCs in models consistent with progression of cholesterol-induced endothelial dysfunction in humans [84].

28.2.4.4 Sepsis and Endotoxemia

Sepsis and/or endotoxemia are generally accompanied by overproduction of ROS/reactive nitrogen species (RNS), antioxidant depletion, and the release of inflammatory mediators. Increased lipid peroxidation and marked depletion of antioxidants such as ascorbate, vitamin E, and GSH are observed in clinical and experimental sepsis [85]. Cell-free Hb or HBOCs can exacerbate the pathophysiology of sepsis or endotoxemia [86, 87]. A synergistic effect between Hb and lipopolysaccharide (LPS), a cell-wall component of gram-negative bacteria and the primary mediator of gram-negative sepsis, has been proposed [88, 89]. The redox cycling of various Hbs by a low level of oxidative stress, and not their direct interaction with LPS, has been shown to dramatically enhance LPS-induced apoptosis [78]. The ferryl heme species was indirectly detected in the peritoneal fluid of animals subjected to experimental *E. coli* peritonitis [90]. Clearly, the oxidative environment created by sepsis or endotoxemia could facilitate the pro-oxidant reactions of Hb. Studying the safety and efficacy of HBOCs in models of sepsis or endotoxemia could provide much-needed information on a potentially key and under-researched area associated with the administration of HBOCs.

28.3 Experimental Oxidative Biomarkers and Extracellular Hb Exposure

28.3.1 Heme Iron Oxidation

HBOCs can oxidize to their ferric iron oxidation state in circulation and in renal filtrate and may lead to dimerization or destabilization of chemical modification. Figure 28.2 demonstrates that polymerized bovine Hb oxidizes significantly to ferric polymerized bovine Hb in transfused guinea pigs [18, 91]. Over the time course of circulation,

the stabilized crosslink in the low-molecular-weight fraction of polymerized bovine Hb becomes dissociated to show both α and β globin chains in circulation (Figure 28.3a). This was not observed in rats and appeared to be associated with the extent of ferric polymerized bovine Hb formation in the guinea pig. This observation was extended to the renal filtrate, where ferric bovine Hb was observed in urine collected from guinea pigs. Guinea pig-excreted bovine Hb was detected as a heterodimer by size-exclusion chromatography and mass spectrometry with distinct α and β globin chain ion masses (Figure 28.3b).

28.3.2 Amino-acid Oxidation

When extracellular ferric Hb reacts with H_2O_2 the formation of a transiently stabilized ferryl heme protein radical ($\bullet$P-Fe^{4+}) occurs at tyrosine 42 in the α globin chains [92, 93]. Stabilization of a radical on the α globin tyrosine 42 residues, in combination with a transiently stabilized heme radical, forms a protein–heme crosslink that could be used as a biomarker for ferryl heme formation [12, 94–96]. Studies also suggest that ferrous Hb reacted with H_2O_2 produces a reproducible pattern of β globin-chain amino-acid oxidations [97] and relative quantification mass spectrometry studies suggest that 75% of βCys93 residues are irreversibly oxidized following exposure to glucose/glucose oxidase, generating physiologically relevant H_2O_2 concentrations [98]. Both Hb crosslinking and βCys93 oxidations have been observed in tissue from animal models of Hb exposure [17, 94]. Interestingly, while no α globin-chain amino-acid oxidations result from reactions with H_2O_2, extensive α globin–α globin crosslinks are observed [94, 97]. The detection of amino-acid oxidations, particularly at cysteine residues, and of heme-induced crosslink formation in tissue compartments, may serve as a biomarker for the severity of oxidative insult encountered by extracellular Hb and HBOCs post-infusion [91, 99].

28.3.3 Heme Catabolism and Iron Sequestration

Heme-catabolic and iron-sequestration systems likely play an important role in mediating the physiological and pathophysiological responses to HBOCs. Heme oxygenase (HO) is the primary enzyme of heme catabolism. The two main isoforms, HO-1 and HO-2, catalyze the degradation of heme into biliverdin, an intermediate in the production of bilirubin, ferrous iron, and carbon monoxide [100]. HO-2 is constitutively expressed, while HO-1 is induced by various stimuli, including heme, LPS, hypoxia, and heavy metals [100]. HO-1 induction is primarily considered a protective response, although, under some settings, HO-1 overexpression may have deleterious consequences. Significant research has been dedicated to understanding the regulation of HO and other heat-shock genes and their role in modifying tissue response to Hb- or heme-induced injury. The differential tissue expression of HO isoforms is also an important consideration for assessing the outcome of Hb exposure and whether HO induction is protective or harmful. For example, in kidney, remarkable HO-1 induction is associated with hemolysis or with the infusion of cell-free Hb or chemically modified Hbs in renal proximal tubular epithelium [18] (Figure 28.1). The extent of renal HO induction appears to correlate with the extent of HBOC oxidation and *in vivo* antioxidant status [18]. Besides the kidney, exchange transfusion with polymerized bovine Hb induces HO-1 in a number

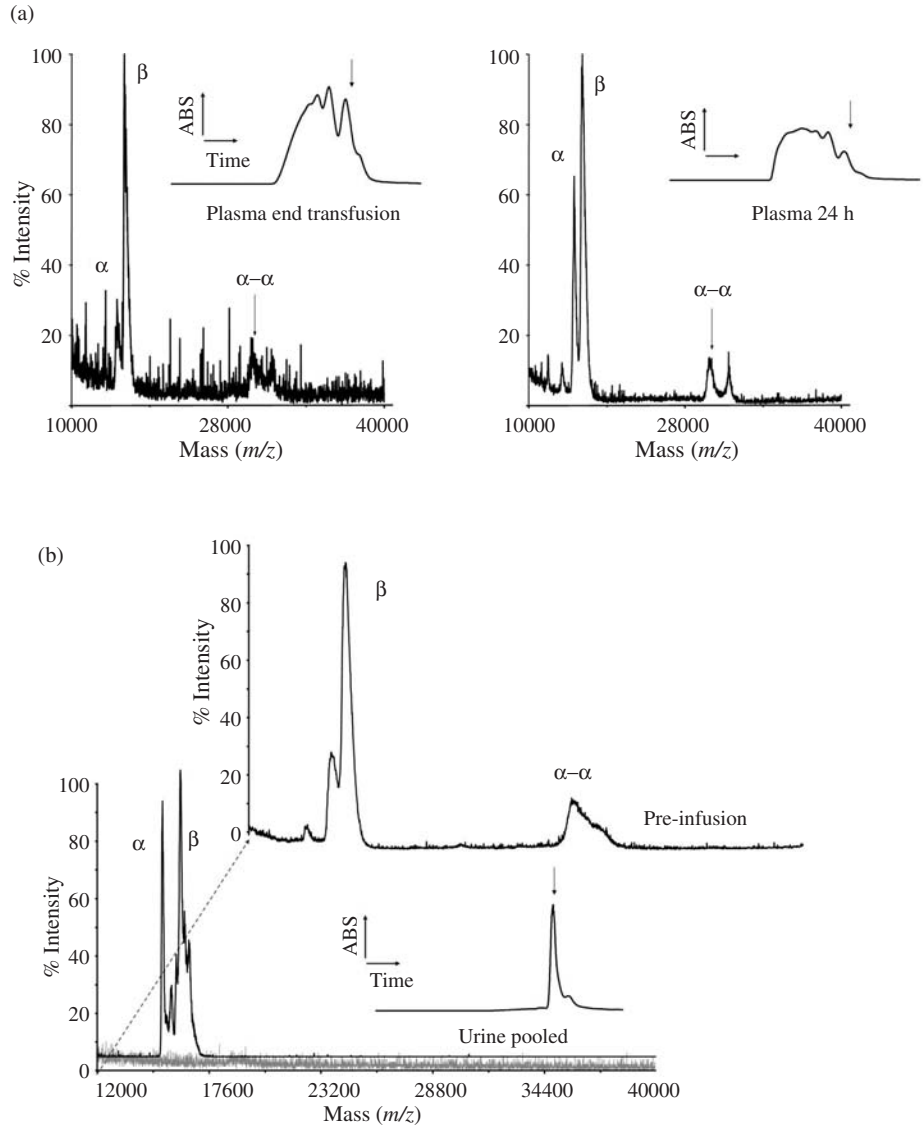

Figure 28.3 *(a) Monitoring of polymerized bovine Hb in plasma and urine of 50% blood--exchange-transfused guinea pigs demonstrates increased chemical destabilization of the tetrameric fraction in plasma over time. (b) At the end of transfusion (left) no destabilization is detected, however within 24 hours post-exchange (right) approximately all of the tetramer is destabilized to heterodimer. Pooled 24-hour urine collections analyzed by MALDI-MS are identified as α and β globin chains by mass spectrometry (bottom panel).*

of different organs and tissues (Figure 28.1). Clearly, the assessment of HO activity and HO-1 expression as a biomarker of Hb exposure and oxidative stress may prove useful in evaluating the responses to HBOCs.

Ferritin, the main intracellular iron-storage protein, is composed of 24 subunits of ferritin heavy (H-ferritin) and light (L-ferritin) chains. An acute response to short-term iron stress is usually accompanied by H-ferritin induction, while L-ferritin expression is typically associated with long-term iron storage [101]. Breakdown of ferritin to hemosiderin occurs in lysosomes. Together, ferritin and hemosiderin constitute important sources of intracellular non-heme iron. Tissue non-heme iron visualization using the conventional Perls histochemical method has been vastly improved in recent years with the advent of various intensification methods [102]. Surprisingly, studies evaluating non-heme iron deposition have been somewhat limited in the HBOC field despite the potential long-term consequence that excess iron deposition may have on tissue function or toxicity. Recent findings from our laboratory identified significant Perls iron deposition in many organs of both rats and guinea pigs following exchange transfusion with polymerized Hbs (Figure 28.1). In kidney, differential patterns of non-heme iron deposition were observed in rats and guinea pigs and appeared to correlate with the extent of ferritin induction in both species [18].

28.4 Markers of *in vivo* Oxidative Stress and Tissue Damage

28.4.1 4-hydroxy-2-nonenal (4-HNE) Protein Adducts

4-HNE, a major lipid peroxidation product of n-6 polyunsaturated fatty acids, contains a hydroxy group, a conjugated C=C double bond, and a carbonyl group, which renders this molecule highly reactive [103]. Significant attention has focused on the reaction of 4-HNE with proteins, and in particular with the side chains of three main amino acids: cysteine, lysine, and histidine (Figure 28.4). Measurement of 4-HNE-modified protein adducts has become one of the most reliable indices of *in vivo* oxidative stress. Recent data from our laboratory, using specific commercialized antibodies for 4-HNE adducts, have shown increased levels of 4-HNE adducts by immunohistochemistry and western blot in various tissues, including kidney, brain, and heart, following exchange transfusion with stroma-free Hb and polymerized Hbs (Figure 28.4) [17]. Several analytical approaches using mass spectrometry, HPLC, and certain colorometric assays have also been developed to measure 4-HNE adducts, with advantages and disadvantages associated with each method [104].

28.4.2 8-hydroxy-2′-deoxyguanosine (8-OHdG)

Oxidative DNA damage characterized by the formation of 8-OHdG is a prominent form of free-radical-induced oxidative damage that is widely used as a biomarker for oxidative stress. A widely used method of quantitative 8-OHdG analysis is high-performance liquid chromatography (HPLC) with electrochemical detection (EC), gas chromatography–mass spectrometry (GC-MS), and HPLC tandem mass spectrometry [105]. Monoclonal antibodies for 8-OHdG have been developed and are commercially available. Other oxidative reaction products such as oxidized low-density

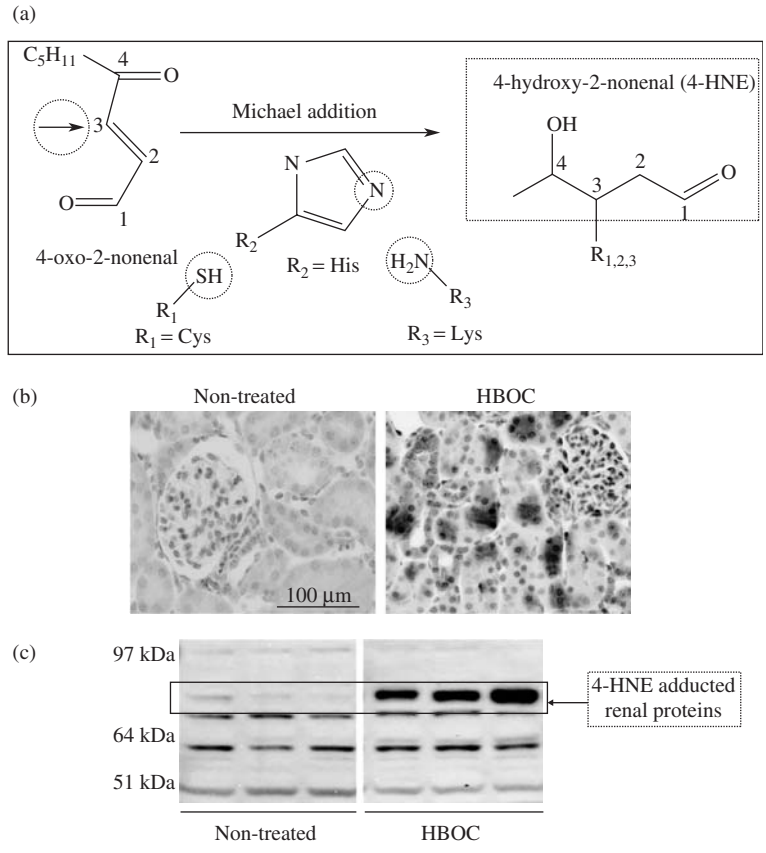

Figure 28.4 *4-HNE-modified adducts as reliable markers of lipid peroxidation. (a) Schematic representation of typical 4-HNE-adduct formation with amino-acid residues, histidine, lysine, and cysteine. (b) Immunohistochemical detection of 4-HNE-modified adducts in guinea pig kidney following transfusion with polymerized bovine Hb. (c) Western blot analysis of 4-HNE adducts in guinea pig kidney.*
For a better understanding of the figure, please refer to color plate 8.

lipoprotein (OxLDL) and isoprostanes may also be useful as predictive plasma markers for cardiovascular risk assessment [106].

28.5 Conclusion

Administration of HBOCs may lead to increased vascular toxicity, including hypertension, inflammation, and oxidative stress. Controlled clinical trials on HBOCs have provided the most in-depth information on the potential harmful effects of extracellular Hb, highlighted by severe adverse events (SAEs) such as myocardial infarction and stroke [2]. These SAEs were not predicted by preclinical studies performed in normal animals. Preexisting vascular diseases producing varying degrees of endothelial dysfunction are

key risk factors that can impact the safety profiles of HBOCs. The use of animal models, particularly of vascular disease, inflammation, and depletion of endogenous antioxidant defenses, as well as more specific markers for extracellular Hb toxicity or exposure, may improve the predictive value of preclinical studies for current or future HBOC candidates.

References

[1] Silverman, T.A. and Weiskopf, R.B. Hemoglobin-based oxygen carriers: current status and future directions, *Transfusion*, **49**, 2495–2515 (2009).

[2] Silverman, T.A. and Weiskopf, R.B. Hemoglobin-based oxygen carriers: current status and future directions, *Anesthesiology*, **111**, 946–963 (2009).

[3] Doherty, D.H., Doyle, M.P., Curry, S.R. *et al*. Rate of reaction with nitric oxide determines the hypertensive effect of cell-free hemoglobin, *Nat. Biotechnol*., **16**, 672–676 (1998).

[4] Olson, J.S., Foley, E.W., Rogge, C. *et al*. NO scavenging and the hypertensive effect of hemoglobin-based blood substitutes, *Free Radic. Biol. Med*., **36**, 685–697 (2004).

[5] Furchgott, R.F., Cherry, P.D., Zawadzki, J.V. and Jothianandan, D. Endothelial cells as mediators of vasodilation of arteries, *J. Cardiovasc. Pharmacol*., **6**(Suppl. 2), S336–S343 (1984).

[6] Furchgott, R.F. and Zawadzki, J.V. The obligatory role of endothelial cells in the relaxation of arterial smooth muscle by acetylcholine, *Nature*, **288**, 373–376 (1980).

[7] Burhop, K., Gordon, D. and Estep, T. Review of hemoglobin-induced myocardial lesions, *Artif. Cells Blood Substit. Biotechnol*., **32**, 353–374 (2004).

[8] Natanson, C., Kern, S.J., Lurie, P. *et al*. Cell-free hemoglobin-based blood substitutes and risk of myocardial infarction and death: a meta-analysis, *JAMA*, **299**, 2304–2312 (2008).

[9] Dou, Y., Maillett, D.H., Eich, R.F. and Olson, J.S. Myoglobin as a model system for designing heme protein based blood substitutes, *Biophys. Chem*., **98**, 127–148 (2002).

[10] Yu, B., Raher, M.J., Volpato, G.P. *et al*. Inhaled nitric oxide enables artificial blood transfusion without hypertension, *Circulation*, **117**, 1982–1990 (2008).

[11] Schroder, E. and Eaton, P. Hydrogen peroxide as an endogenous mediator and exogenous tool in cardiovascular research: issues and considerations, *Curr. Opin Pharmacol*., **8**, 153–159 (2008).

[12] Moore, K.P., Holt, S.G., Patel, R.P. *et al*. A causative role for redox cycling of myoglobin and its inhibition by alkalinization in the pathogenesis and treatment of rhabdomyolysis-induced renal failure, *J. Biol. Chem*., **273**, 31731–31737 (1998).

[13] Winterbourn, C.C. Biological reactivity and biomarkers of the neutrophil oxidant, hypochlorous acid, *Toxicology*, **181–182**, 223–227 (2002).

[14] Winterbourn, C.C. and Kettle, A.J. Biomarkers of myeloperoxidase-derived hypochlorous acid, *Free Radic. Biol. Med*., **29**, 403–409 (2000).

[15] Caccia, D., Ronda, L., Frassi, R. *et al*. PEGylation promotes hemoglobin tetramer dissociation, *Bioconjug. Chem*., **20**, 1356–1366 (2009).

[16] Boutaud, O., Moore, K.P., Reeder, B.J. *et al*. Acetaminophen inhibits hemoprotein-catalyzed lipid peroxidation and attenuates rhabdomyolysis-induced renal failure, *Proc. Natl. Acad. Sci. U S A*, **107**, 2699–2704 (2010).

[17] Boretti, F.S., Buehler, P.W., D'Agnillo, F. *et al*. Sequestration of extracellular hemoglobin within a haptoglobin complex decreases its hypertensive and oxidative effects in dogs and guinea pigs, *J. Clin. Invest*., **119**, 2271–2280 (2009).

[18] Butt, O.I., Buehler, P.W. and D'Agnillo, F. Differential induction of renal heme oxygenase and ferritin in ascorbate and nonascorbate producing species transfused with modified cell-free hemoglobin, *Antioxid. Redox Signal*., **12**, 199–208 (2010).

[19] Baldwin, A.L. Modified hemoglobins produce venular interendothelial gaps and albumin leakage in the rat mesentery, *Am. J. Physiol*., **277**(2Pt 2), H650–H659 (1999).

[20] Baldwin, A.L., Wilson, L.M. and Valeski, J.E. Ultrastructural effects of intravascularly injected polyethylene glycol-hemoglobin in intestinal mucosa, *Am. J. Physiol*., **275**(2Pt 2), H615–H625 (1998).

[21] Baldwin, A.L., Wiley, E.B. and Alayash, A.I. Comparison of effects of two hemoglobin-based O_2 carriers on intestinal integrity and microvascular leakage, *Am. J. Physiol. Heart Circ. Physiol*., **283**, H1292–H1301 (2002).

[22] Baldwin, A.L., Wiley, E.B. and Alayash, A.I. Differential effects of sodium selenite in reducing tissue damage caused by three hemoglobin-based oxygen carriers, *J. Appl. Physiol*., **96**, 893–903 (2004).

[23] Baldwin, A.L., Wiley, E.B., Summers, A.G. and Alayash, A.I. Sodium selenite reduces hemoglobin-induced venular leakage in the rat mesentery, *Am. J. Physiol. Heart Circ. Physiol*., **284**, H81–H91 (2003).

[24] Buehler, P.W. and D'Agnillo, F. Toxicological consequences of extracellular hemoglobin: biochemical and physiological perspectives, *Antioxid. Redox Signal*., **12**, 275–291 (2003).

[25] Buehler, P.W. and Alayash, A.I. Toxicities of hemoglobin solutions: in search of in-vitro and in-vivo model systems, *Transfusion*, **44**, 1516–1530 (2004).

[26] Sibbald, W.J., Messmer, K. and Fink, M.P. Roundtable conference on tissue oxygenation in acute medicine, Brussels, Belgium, 14–16 March 1998, *Intensive Care Med*., **26**, 780–791 (2000).

[27] Standl, T., Freitag, M., Burmeister, M.A. *et al*. Hemoglobin-based oxygen carrier HBOC-201 provides higher and faster increase in oxygen tension in skeletal muscle of anemic dogs than do stored red blood cells, *J. Vasc. Surg*., **37**, 859–865 (2003).

[28] Mongan, P.D., Moon-Massat, P.F., Rentko, V. *et al*. Regional blood flow after serial normovolemic exchange transfusion with HBOC-201 (Hemopure) in anesthetized swine, *J. Trauma*, **67**, 51–60 (2009).

[29] Barve, A., Sen, A.P., Saxena, P.R. and Gulati, A. Dose response effect of diaspirin crosslinked hemoglobin (DCLHb) on systemic hemodynamics and regional blood circulation in rats, *Artif. Cells Blood Substit. Immobil. Biotechnol*., **25**, 75–84 (2000).

[30] Sen, A.P., Dong, Y., Saxena, P.R. and Gulati, A. Modulation of resuscitative effect of diaspirin cross-linked hemoglobin by L-NAME in rats, *Shock*, **9**, 223–230 (1998).

[31] Meisner, F.G., Kemming, G.I., Habler, O.P. *et al*. Diaspirin crosslinked hemoglobin enables extreme hemodilution beyond the critical hematocrit, *Crit. Care Med*., **29**, 829–838 (2001).

[32] Creteur, J., Zhang, H., De Backer, D. *et al*. Diaspirin cross-linked hemoglobin improves oxygen extraction capabilities in endotoxic shock, *J. Appl. Physiol*., **89**, 1437–1444 (2000).

[33] Kingma, J.G. Jr, Sandhu, R., Hamelin, N.D. *et al*. The effects of hemodilution with Hemolink upon hemodynamics and blood flow distribution in anesthetized dogs, *Artif. Cells Blood Substit. Immobil. Biotechnol*., **30**, 137–154 (2002).

[34] Kerger, H., Tsai, A.G., Saltzman, D.J. *et al*. Fluid resuscitation with O_2 vs. non-O_2 carriers after 2h of hemorrhagic shock in conscious hamsters, *Am. J. Physiol*., **272**(1Pt 2), H525–H537 (1997).

[35] Tsai, A.G., Cabrales, P., Manjula, B.N. *et al*. Dissociation of local nitric oxide concentration and vasoconstriction in the presence of cell-free hemoglobin oxygen carriers, *Blood*, **108**, 3603–3610 (2006).

[36] Tsai, A.G., Vandegriff, K.D., Intaglietta, M. and Winslow, R.M. Targeted O_2 delivery by low-P50 hemoglobin: a new basis for O_2 therapeutics, *Am. J. Physiol. Heart Circ. Physiol*., **285**, H1411–H1419 (2003).

[37] Wettstein, R., Tsai, A.G., Erni, D. *et al*. Resuscitation with polyethylene glycol-modified human hemoglobin improves microcirculatory blood flow and tissue oxygenation after hemorrhagic shock in awake hamsters, *Crit. Care Med*., **31**, 1824–1830 (2003).

[38] Frankel, H.L., Nguyen, H.B., Shea-Donohue, T. *et al*. Diaspirin cross-linked hemoglobin is efficacious in gut resuscitation as measured by a GI tract optode, *J. Trauma*, **40**, 231–240, disc. 41 (1996).

[39] Raat, N.J., Liu, J.F., Doyle, M.P. *et al*. Effects of recombinant-hemoglobin solutions rHb2.0 and rHb1.1 on blood pressure, intestinal blood flow, and gut oxygenation in a rat model of hemorrhagic shock, *J. Lab. Clin. Med*., **145**, 21–32 (2005).

[40] van Iterson, M., Siegemund, M., Burhop, K. and Ince, C. Hemoglobin-based oxygen carrier provides heterogeneous microvascular oxygenation in heart and gut after hemorrhage in pigs, *J. Trauma*, **55**, 1111–1124 (2003).

[41] Knudson, M.M., Lee, S., Erickson, V. *et al*. Tissue oxygen monitoring during hemorrhagic shock and resuscitation: a comparison of lactated Ringer's solution, hypertonic saline dextran, and HBOC-201, *J. Trauma*, **54**, 242–252 (2003).

[42] Lee, S.K., Morabito, D., Hemphill, J.C. *et al*. Small-volume resuscitation with HBOC-201: effects on cardiovascular parameters and brain tissue oxygen tension in an out-of-hospital model of hemorrhage in swine, *Acad. Emerg. Med*., **9**, 969–976 (2002).

[43] Manley, G.T., Hemphill, J.C., Morabito, D. *et al*. Small-volume resuscitation with the hemoglobin substitute HBOC-201: effect on brain tissue oxygenation, *Adv. Exp. Med. Biol*., **530**, 311–317 (2003).

[44] Caretti, A., Bianciardi, P., Ronchi, R. *et al*. Phosphodiesterase-5 inhibition abolishes neuron apoptosis induced by chronic hypoxia independently of hypoxia-inducible factor-1alpha signaling, *Exp. Biol. Med*., **233**, 1222–1230 (2008).

[45] Manalo, D.J., Buehler, P.W., Baek, J.H. *et al*. Acellular haemoglobin attenuates hypoxia-inducible factor-1alpha (HIF-1alpha) and its target genes in haemodiluted rats, *Biochem. J*., **414**, 461–469 (2008).

[46] Krohn, K.A., Link, J.M. and Mason, R.P. Molecular imaging of hypoxia, *J. Nucl. Med*., **49**(Suppl. 2), 129S–148S (2008).

[47] Awasthi, V., Yee, S.H., Jerabek, P. *et al*. Cerebral oxygen delivery by liposome-encapsulated hemoglobin: a positron-emission tomographic evaluation in a rat model of hemorrhagic shock, *J. Appl. Physiol*., **103**, 28–38 (2007).

[48] Makley, A.T., Goodman, M.D., Friend, L.A. *et al*. Resuscitation with fresh whole blood ameliorates the inflammatory response after hemorrhagic shock, *J. Trauma*, **68**, 305–311 (2010).

[49] Schmand, J.F., Ayala, A. and Chaudry, I.H. Effects of trauma, duration of hypotension, and resuscitation regimen on cellular immunity after hemorrhagic shock, *Crit. Care Med*., **22**, 1076–1083 (1994).

[50] Capone, A., Safar, P., Stezoski, S.W. *et al*. Uncontrolled hemorrhagic shock outcome model in rats, *Resuscitation*, **29**, 143–152 (1995).

[51] Swafford, A.N. Jr, Bidros, D., Truxillo, T.M. *et al*. Ethanol intoxication and lactated Ringer's resuscitation prolong hemorrhage-induced lactic acidosis, *Shock*, **20**, 237–244 (2003).

[52] Woodman, O.L., Amano, J., Hintze, T.H. and Vatner, S.F. Augmented catecholamine uptake by the heart during hemorrhage in the conscious dog, *Am. J. Physiol*., **250**(1Pt 2), H76–H81 (1986).

[53] Capone, A., Safar, P., Radovsky, A. *et al*. Complete recovery after normothermic hemorrhagic shock and profound hypothermic circulatory arrest of 60 minutes in dogs, *J. Trauma*, **40**, 388–395 (1996).

[54] Manning, J.E., Katz, L.M., Brownstein, M.R. *et al*. Bovine hemoglobin-based oxygen carrier (HBOC-201) for resuscitation of uncontrolled, exsanguinating liver injury in swine. Carolina Resuscitation Research Group, *Shock*, **13**, 152–159 (2000).

[55] Hess, J.R., MacDonald, V.W. and Brinkley, W.W. Systemic and pulmonary hypertension after resuscitation with cell-free hemoglobin, *J. Appl. Physiol*., **74**, 1769–1778 (1993).

[56] Stern, S., Rice, J., Philbin, N. *et al*. Resuscitation with the hemoglobin-based oxygen carrier, HBOC-201, in a swine model of severe uncontrolled hemorrhage and traumatic brain injury, *Shock*, **31**, 64–79 (2009).

[57] Stern, S.A., Jwayyed, S., Dronen, S.C. and Wang, X. Resuscitation of severe uncontrolled hemorrhage: 7.5% sodium chloride/6% dextran 70 vs 0.9% sodium chloride, *Acad. Emerg. Med*., **7**, 847–856 (2000).

[58] Frithiof, R., Mats, R., Johan, U. *et al*. Comparison between the effects on hemodynamic responses of central and peripheral infusions of hypertonic NaCl during hemorrhage in conscious and isoflurane-anesthetized sheep, *Shock*, **26**, 77–86 (2006).

[59] Pretorius, J.P., Schlag, G., Redl, H. *et al*. The "lung in shock" as a result of hypovolemic-traumatic shock in baboons, *J. Trauma*, **7**, 1344–1353 (1987).

[60] Hsieh, C.H., Frink, M., Hsieh, Y.C. *et al*. The role of MIP-1 alpha in the development of systemic inflammatory response and organ injury following trauma hemorrhage, *J. Immunol*., **181**, 2806–2812 (2008).

[61] Pergolizzi, R.G., Jin, G., Chan, D. *et al*. Correction of a murine model of von Willebrand disease by gene transfer, *Blood*, **108**, 862–869 (2006).

[62] Cole, D.J., Schell, R.M., Drummond, J.C. and Reynolds, L. Focal cerebral ischemia in rats: effect of hypervolemic hemodilution with diaspirin cross-linked hemoglobin versus albumin on brain injury and edema, *Anesthesiology*, **78**, 335–342 (1993).

[63] Cole, D.J., Schell, R.M., Przybelski, R.J. *et al*. Focal cerebral ischemia in rats: effect of hemodilution with alpha-alpha cross-linked hemoglobin on CBF, *J. Cereb. Blood. Flow Metab*., **12**, 971–976 (1992).

[64] Saxena, R., Wijnhoud, A.D., Carton, H. *et al*. Controlled safety study of a hemoglobin-based oxygen carrier, DCLHb, in acute ischemic stroke, *Stroke*, **30**, 993–996 (1999).

[65] Saxena, R., Wijnhoud, A.D., Man in 't Veld, A.J. *et al*. Effect of diaspirin cross-linked hemoglobin on endothelin-1 and blood pressure in acute ischemic stroke in man, *J. Hypertens*., **16**, 1459–1465 (1998).

[66] Recommendations for standards regarding preclinical neuroprotective and restorative drug development, *Stroke*, **30**, 2752–2758 (1998).

[67] Belcher, J.D., Mahaseth, H., Welch, T.E. *et al*. Heme oxygenase-1 is a modulator of inflammation and vaso-occlusion in transgenic sickle mice, *J. Clin. Invest*., **116**, 808–816 (2006).

[68] Belcher, J.D., Mahaseth, H., Welch, T.E. *et al*. Critical role of endothelial cell activation in hypoxia-induced vasoocclusion in transgenic sickle mice, *Am. J.Physiol. Heart Circ. Physiol*., **288**, H2715–H2725 (2005).

[69] Fabry, M.E., Sengupta, A., Suzuka, S.M. *et al*. A second generation transgenic mouse model expressing both hemoglobin S (HbS) and HbS-Antilles results in increased phenotypic severity, *Blood*, **86**, 2419–2428 (1995).

[70] Paszty, C., Brion, C.M., Manci, E. *et al*. Transgenic knockout mice with exclusively human sickle hemoglobin and sickle cell disease, *Science*, **278**, 876–878 (1997).

[71] Chatterjee, I.B. Evolution and the biosynthesis of ascorbic acid, *Science*, **182**, 1271–1272 (1973).

[72] Nandi, A., Mukhopadhyay, C.K., Ghosh, M.K. *et al*. Evolutionary significance of vitamin C biosynthesis in terrestrial vertebrates, *Free Radic. Biol. Med*., **2**, 1047–1054 (1997).

[73] Su, D., May, J.M., Koury, M.J. and Asard, H. Human erythrocyte membranes contain a cytochrome b561 that may be involved in extracellular ascorbate recycling, *J. Biol. Chem*., **281**, 39852–39859 (2006).

[74] Mizushima, Y., Harauchi, T., Yoshizaki, T. and Makino, S. A rat mutant unable to synthesize vitamin C, *Experientia*, **40**, 359–361 (1984).

[75] Kawai, T., Nishikimi, M., Ozawa, T. and Yagi, K. A missense mutation of L-gulono-gamma-lactone oxidase causes the inability of scurvy-prone osteogenic disorder rats to synthesize L-ascorbic acid, *J. Biol. Chem*., **267**, 21973–21976 (1992).

[76] Franco, R., Schoneveld, O.J., Pappa, A. and Panayiotidis, M.I. The central role of glutathione in the pathophysiology of human diseases, *Arch. Physiol. Biochem*., **113**, 234–258 (2007).

[77] Circu, M.L. and Aw, T.Y. Glutathione and apoptosis, *Free Radic. Res*., **42**, 689–706 (2008).

[78] D'Agnillo, F. Redox active hemoglobin enhances lipopolysaccharide-induced injury to cultured bovine endothelial cells, *Am. J. Physiol. Heart Circ. Physiol*., **287**, H1875–H1882 (2004).

[79] D'Agnillo, F. and Alayash, A.I. Redox cycling of diaspirin cross-linked hemoglobin induces G2/M arrest and apoptosis in cultured endothelial cells, *Blood*, **98**, 3315–3323 (2001).

[80] Seckin, S., Dogru-Abbasoglu, S., Basaran-Kucukgergin, C. *et al*. Increased lipid peroxidation in the liver and kidney and their mitochondrial fractions following buthionine sulfoximine induced glutathione depletion in guinea pigs, *Res. Commun. Mol. Pathol. Pharmacol*., **109**, 299–308 (2001).

[81] Watanabe, T., Sagisaka, H., Arakawa, S. *et al*. A novel model of continuous depletion of glutathione in mice treated with L-buthionine (S,R)-sulfoximine, *J. Toxicol. Sci*., **28**, 455–469 (2003).

[82] D'Agnillo, F. and Alayash, A.I. A role for the myoglobin redox cycle in the induction of endothelial cell apoptosis, *Free Radic. Biol. Med*., **33**, 1153–1164 (2002).

[83] Yu, B., Shahid, M., Egorina, E.M. *et al*. Endothelial dysfunction enhances vasoconstriction due to scavenging of nitric oxide by a hemoglobin-based oxygen carrier, *Anesthesiology*, **112**, 586–594 (2010).

[84] Fernandez, M.L. and Volek, J.S. Guinea pigs: a suitable animal model to study lipoprotein metabolism, atherosclerosis and inflammation, *Nutr. Metab*., **3**, 17 (2006).

[85] Rinaldi, S., Landucci, F. and De Gaudio A.R. Antioxidant therapy in critically septic patients, *Curr. Drug Targets*, **10**, 872–880 (2009).

[86] Su, D., Roth, R.I., Yoshida, M. and Levin, J. Hemoglobin increases mortality from bacterial endotoxin, *Infect. Immun*., **65**, 1258–1266 (1997).

[87] Krishnamurti, C., Carter, A.J., Maglasang, P. *et al*. Cardiovascular toxicity of human cross-linked hemoglobin in a rabbit endotoxemia model, *Crit. Care Med*., **25**, 1874–1880 (1997).

[88] Kaca, W., Roth, R.I. and Levin, J. Hemoglobin, a newly recognized lipopolysaccharide (LPS)-binding protein that enhances LPS biological activity, *J. Biol. Chem*., **269**, 25078–25084 (1994).

[89] Kaca, W., Roth, R.I., Vandegriff, K.D. *et al*. Effects of bacterial endotoxin on human cross-linked and native hemoglobins, *Biochemistry*, **34**, 11176–11185 (1995).

[90] Yoo, Y.M., Kim, K.M., Kim, S.S. *et al*. Hemoglobin toxicity in experimental bacterial peritonitis is due to production of reactive oxygen species, *Clin. Diagn. Lab. Immunol*., **6**, 938–945 (1999).

[91] Buehler, P.W., D'Agnillo, F., Hoffman, V. and Alayash, A.I. Effects of endogenous ascorbate on oxidation, oxygenation and toxicokinetics of cell-free modified

hemoglobin after exchange transfusion in rat and guinea pig, *J. Pharmacol. Exp. Ther*., **323**, 49–60 (2007).

[92] Reeder, B.J., Cutruzzola, F., Bigotti, M.G. *et al*. Tyrosine as a redox-active center in electron transfer to ferryl heme in globins, *Free Radic. Biol. Med*., **44**, 274–283 (2008).

[93] Svistunenko, D.A., Reeder, B.J., Wankasi, M.M. *et al*. Reaction of *Aplysia limacina* metmyoglobin with hydrogen peroxide, *Dalton Trans*., **28**, 840–850 (2007).

[94] Buehler, P.W., Abraham, B., Vallelian, F. *et al*. Haptoglobin preserves the CD163 hemoglobin scavenger pathway by shielding hemoglobin from peroxidative modification, *Blood*, **113**, 2578–2586 (2009).

[95] Holt, S., Reeder, B., Wilson, M. *et al*. Increased lipid peroxidation in patients with rhabdomyolysis, *Lancet*, **353**, 1241 (1999).

[96] Reeder, B.J., Sharpe, M.A., Kay, A.D. *et al*. Toxicity of myoglobin and haemoglobin: oxidative stress in patients with rhabdomyolysis and subarachnoid haemorrhage, *Biochem. Soc. Trans*., **30**, 745–748 (2002).

[97] Jia, Y., Buehler, P.W., Boykins, R.A. *et al*. Structural basis of peroxide-mediated changes in human hemoglobin: a novel oxidative pathway, *J. Biol. Chem*., **282**, 4894–4907 (2007).

[98] Widmer, C.C., Pereira, C.P., Gehrig, P. *et al*. Hemoglobin can attenuate hydrogen peroxide-induced oxidative stress by acting as an antioxidative peroxidase, *Antioxid. Redox Signal*., **12**, 185–198 (2010).

[99] Buehler, P.W., Boykins, R.A., Jia, Y. *et al*. Structural and functional characterization of glutaraldehyde-polymerized bovine hemoglobin and its isolated fractions, *Anal. Chem*., **77**, 3466–3478 (2005).

[100] Abraham, N.G. and Kappas, A. Pharmacological and clinical aspects of heme oxygenase, *Pharmacol. Rev*., **60**, 79–127 (2008).

[101] Hintze, K.J. and Theil, E.C. Cellular regulation and molecular interactions of the ferritins, *Cell. Mol. Life Sci*., **63**, 591–600 (2006).

[102] Meguro, R., Asano, Y., Odagiri, S. *et al*. Nonheme-iron histochemistry for light and electron microscopy: a historical, theoretical and technical review, *Arch. Hystol. Cytol*., **70**, 1–19 (2007).

[103] Schaur, R.J. Basic aspects of the biochemical reactivity of 4-hydroxynonenal, *Mol. Asp. Med*., **24**, 149–159 (2003).

[104] Poli, G., Schaur, R.J., Siems, W.G. and Leonarduzzi, G. 4-hydroxynonenal: a membrane lipid oxidation product of medicinal interest, *Med. Res. Rev*., **28**, 569–631 (2008).

[105] Mateos, R. and Bravo, L. Chromatographic and electrophoretic methods for the analysis of biomarkers of oxidative damage to macromolecules (DNA, lipids, and proteins), *J. Sep. Sci*., **30**, 175–191 (2007).

[106] Tsimikas, S. In vivo markers of oxidative stress and therapeutic interventions, *Am. J. Cardiol*., **101**, 34D–42D (2008).

29

Academia–Industry Collaboration in Blood Substitute Development: Issues, Case Histories and a Proposal

Hae Won Kim[1], *Andrea Mozzarelli*[2], *Hiromi Sakai*[3] *and Jonathan S. Jahr*[4]

[1]*Department of Molecular Pharmacology, Physiology and Biotechnology, Brown University, Providence, RI, USA*

[2]*Department of Biochemistry and Molecular Biology, University of Parma, Parma, Italy, and National Institute of Biostructures and Biosystems, Rome, Italy*

[3]*Waseda Bioscience Research Institute in Singapore, Singapore, Republic of Singapore and Research Institute for Science and Engineering, Waseda University, Shinjuku, Tokyo, Japan*

[4]*David Geffen School of Medicine at UCLA, Ronald Reagan UCLA Medical Center, Los Angeles, CA, USA*

29.1 Introduction

Over the last 30 years, researchers in academia and industry have tirelessly worked together to achieve the goal of developing successful hemoglobin-based oxygen carriers (HBOCs) as safe and clinically effective therapeutics for the treatment of hemorrhagic shock, acute anemia, ischemia, and other conditions. Some leading products have reached the final stages of the development process and are closer than ever before to regulatory approval. However, higher incidences of adverse events (AEs) in the HBOC-treated group than in controls in recent clinical trials have hampered further progress of these candidate HBOCs toward regulatory approval [1–6]. In an effort to help identify the causes of HBOC-mediated AEs and to find ways to alleviate them, a FDA–NIH-sponsored workshop was recently held [7]. Publicly available information on

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the characteristics and clinical profiles of leading HBOC products were reviewed and potential mechanisms of toxicity discussed. The workshop concluded with a recommendation that further basic research is needed to identify the cause(s) of the AEs observed with current candidate HBOC products and to develop a new generation of safer and more effective products [7].

To elucidate the pathophysiologic mechanisms of AEs observed with HBOCs, it is essential to understand how HBOCs affect key organ systems and their physiologic function, not simply in normal subjects but in patients; studies conducted in models of healthy animals have failed to predict the pathophysiologic responses observed in actual patients, who often present with multiple comorbidities. It is essential that preclinical safety studies are conducted in animal models that closely simulate target patient conditions. Further investigations are required to establish the causality of AEs to an individual HBOC tested in clinical trials and to determine the mechanism involved. Only when armed with accurate knowledge of pathophysiologic mechanisms can we make appropriate modifications to the current HBOC products or develop new, safer products. However, there are barriers to investigations. The goal of this chapter is to initiate a constructive discourse on new ideas in academia–industry collaboration, in order to break down the barriers that impede development of viable HBOC products. Toward this goal, this chapter will: (i) briefly review and discuss generic issues in current academia–industry collaboration practices in a broader field; (ii) discuss issues relevant to academia–industry collaboration in HBOC development; and (iii) propose a new conceptual model for academia–industry collaboration.

29.2 Generic Issues in Academia–Industry Collaboration

To facilitate technology transfer, in 1980 the US government enacted an amendment to the Patent and Trademark Law (commonly known as the Bayh–Dole Act) which permits universities, small businesses, and nonprofit institutions to patent inventions arising from government-funded research [8]. The Bayh–Dole Act has made a significant impact on the academia–industry relationship as industry can no longer monopolize the benefits from research inventions. The benefits of academia–industry collaboration include pooling of expertise and resources (allowing increased chance of new and improved product development) and enhanced productivity and problem-solving capability. For an academic collaborator, industrial funding also allows support for additional students and staff to advance the research and increase productivity. On the other hand, such an industrial sponsorship may have a potentially detrimental effect on free scientific communication and the objectivity of research results. Of particular concern is that industrial priority on the protection of proprietary rights (‘proprietary science’) may impose constraints on free communication of new knowledge to peers, the scientific community, and the general public (‘public science’). Such constraints make it more difficult, if not impossible, for independent investigators to validate research results and expand and develop new applications [9]. Dissemination of study results (presentations and/or publications), even if allowed, is often delayed until well after project completion. If study results are negative or perceived as counterproductive, the sponsor may be less inclined to publish them, unless a priori agreement was explicitly made in the research contract to

allow publication regardless of study outcome. In general, industry-sponsored research contracts are short-term, often narrowly defined, and allow only limited flexibility in the conduct of research.

The primary rationale for the Bayh–Dole Act is to facilitate technology transfer and the commercialization of research results for the public good. Technology transfer is typically achieved through creation of start-up/spin-off companies or by partnering with an established industry that has the expertise and resources to quickly bring the research results to marketable products. Indeed, the enactment of the Bayh–Dole Act considerably enhanced academia–industry collaborations, resulting in a remarkable increase in the patent and licensing activities of universities and nonprofit research institutions. Recently, however, the heightened interest of academic institutions in intellectual property (IP) rights has caused an increasing number of disputes. Emphasis on IP has raised concerns regarding the core values of academic research, as securing IP rights often restricts free and objective communication of research results [10].

A variety of collaboration models (sponsored research, research partnerships/consortia, consulting, etc.) with various IP arrangements (joint ownership, licensing, first-refusal rights, etc.) are being evaluated to mitigate such conflicts and facilitate research collaboration and technology transfer. A key factor in a successful collaboration is building mutual trust based on understanding of the needs and expectations of the other party/ies) involved.

Another disadvantage of academia–industry collaboration is potential conflict of interest (COI). In a typical academia–industry research collaboration, academic investigators receive research funds from their industrial sponsor; they may thus be biased to produce and interpret research results that are more agreeable to that sponsor. In addition, academic collaborators often serve as a consultant or member of the board at a sponsoring company. In return, they may receive direct and indirect benefits (monetary and nonmonetary), raising further COI concerns. Growing emphasis on academia–industry collaboration, IP rights, and the commercialization of research findings have increased public awareness and concern about COI issues. In medical research, any serious compromise in research integrity could have grave consequences for patient treatment and public health in general. Recently, serious concerns about COI have been raised regarding pharmaceutical company-sponsored medical research/education and faculty/officers of medical institutions serving as paid consultants or as members of companies' boards [11, 12]. To protect study subjects/patients and preserve the integrity and objectivity of scientific research, the Association of American Medical Colleges (AAMC) and the Federation of American Societies for Experimental Biology (FASEB) recently issued a strong call for implementation of rigorous COI policies and guidelines in academic institutions [13, 14].

29.3 Academia–Industry Collaboration in HBOC Development

As in other fields, there are limitations and barriers to optimal academia–industry collaboration in HBOC development. First, thorough investigation of HBOC-mediated pathophysiologic responses requires full and detailed information concerning the observed AEs, including patient's clinical history, HBOC dose and rate, circumstances

around the incident, time course of events, concomitant medications, and other relevant information. Unfortunately, much of this information is often not made fully public. Of note, there is a strong ongoing ethical argument to disclose all adverse effects in clinical trials [15]. In addition, because test HBOCs are not yet commercially available, they are not generally available to independent investigators who might help raise and resolve the issues. Understandably, to protect proprietary information from competitors, HBOC producers rarely share their products with 'outsiders' beyond the circle of a small number of close collaborators who are bound by confidentiality agreements. A working group workshop organized by NIH–NHLBI in 2006 [16] explicitly discussed these issues and recommended, among other things, (i) funding of the production and distribution of highly purified 'generic' HBOC solutions in sufficient quantities to support research by independent investigators and (ii) that the FDA investigate ways of making information publicly available regarding the nature and incidence of AEs observed in clinical trials. Advancing these objectives would require breaking down the barriers between and improving the cooperation of both the HBOC producers and the academic investigators. However, because of the unique challenges involved in developing HBOCs as therapeutics, the current industry-driven collaboration model has so far not been able to produce a viable HBOC product even after decades of efforts and substantial private-sector investments.

To date, there have not been many notable IP right disputes in the HBOC field, perhaps in part due to a lack of successfully marketed products. However, COI is of concern since many academic HBOC experts often receive research funds and/or provide compensated services on behalf of one or more companies. Further, lack of transparency and clear open communications regarding some recent negative clinical results has fostered skepticism that HBOC products may have serious safety issues. Indeed, a recent study based on a meta-analysis of pooled AE data from 16 different clinical trials of five past and current HBOC products reported that use of HBOCs was associated with increased risk of serious cardiovascular events and death [17]. Pooling of data conducted with different HBOC formulations of distinct chemical compositions and physico-chemical characteristics is highly controversial. In addition, negative publicity in the media led to diminishing public trust and confidence in the whole class of HBOCs as potential oxygen therapeutics. This negative climate, coupled with recent economic downturn, seriously hampers further development of a safer new generation of HBOC products.

Notable academia–industry relationships began to develop in the early 1980s, when the first HBOC companies emerged (Baxter, Hemosol, Northfield, Biopure). Typically, researchers at academic institutions developed a noble idea of a new HBOC, did initial proof-of-concept work, and obtained IP rights. Later, the inventor(s) either started a new company or worked with an established company to develop further the work into a candidate therapeutic product, which had to meet the rigorous regulatory standards for biologic products. HBOC development efforts followed a typical academia to industry technology transfer pattern and stages. The leading role shifted from academia to industry as product development progressed from basic proof-of-concept work to developmental work for a marketable product that met GMP and other regulatory criteria (new idea/invention → proof-of-concept → further development → scale-up → product safety and effectiveness testing → regulatory approval). Both academia and industry play integral components in the development process. There can be no separate 'academic approach' and 'industrial approach' in HBOC development.

Despite the limitations, academia–industry collaborations have contributed greatly to HBOC development. Examples include: University of Chicago/Michael Reese Hospital–Northfield Inc., Letterman Army Institute of Research–Baxter International, Inc., University of California, San Diego–Sangart, Inc., University of Maryland–OxyVita, Inc., Texas Tech University–HemoBiotech, Inc., Naval Medical Research Center–Biopure Corp., Waseda and Keio Universities–industries. The achievements of the Waseda University–Keio University-initiated research collaborations have been remarkable and particularly productive. With continued government support and strong complementary expertise in sophisticated biosynthetic organic chemistry and animal experimentation, they have produced a host of promising candidate blood substitutes, including Hb-vesicles (HbVs), albumin-heme, and platelet substitutes, over the last 25 years (see Appendix). The EuroBloodSubstitutes consortium is a more recent effort, begun in 2004, consisting of 13 European academic and industrial teams aiming to develop improved blood substitutes. In just a few years, this effort produced new recombinant Hbs and Euro-PEG-Hb (see Appendix). Although these two efforts have not yet conducted any clinical trials, the entire HBOC community may benefit from their successful research collaboration experiences. Therefore, these two historic cases of HBOC research collaboration are described in more detail in the Appendix.

29.4 Proposal for a New Academia–Industry Collaboration Model in HBOC Development: an HBOC Research Consortium (a Conceptual Model)

29.4.1 Mission

- To facilitate development of candidate HBOCs to approved and clinically accepted therapeutic products.
- To resolve issues with current HBOC products collectively, and to identify clinical indications for which these products may be of therapeutic value.
- To develop a new generation of products with acceptable safety and efficacy profiles for one or more clinical indications.

29.4.2 Guiding Principles

- Sound scientific and ethical principles.
- Integrity and objectivity (avoid COI).
- Free and timely communication of research results.

29.4.3 Key Objectives

- Secure research funding (e.g. government and nonprofit foundation grants) for HBOC research.
- Develop standardized research designs/study protocols for key studies common to all HBOC products.
- Build a universally accessible repository for all consortium-generated research data and results for members of the consortium and for timely dissemination to the public.

- Eliminate COI by separation of industrial sponsor from investigators.
- Share HBOC products developed by consortium members with other member investigators.

29.4.4 Structure

In the early stage of HBOC development, a company's research collaboration is usually limited to one or two close collaborators. But as the company grows with substantial research revenue, the collaboration expands to multiple academic investigators (a traditional one industry–multiple investigator model; see Figure 29.1). In this industry-centered model, an academia–industry relationship is generally driven by the sponsoring industry, as the company selects projects/investigators based on its needs. The nature of developmental work and research results are generally shared only within the circle of collaborators, especially when problems are encountered or study outcomes are negative. Thus, unless specifically requested, there is virtually no possibility that outside investigators can contribute to the resolution of any issues associated with a particular product.

To help resolve the issues associated with the traditional collaboration model in HBOC development, we propose a new academia–industry collaboration model that is, at least in concept, designed to be more objective and transparent. We envision an HBOC research consortium consisting of multiple HBOC producers and multiple academic investigators

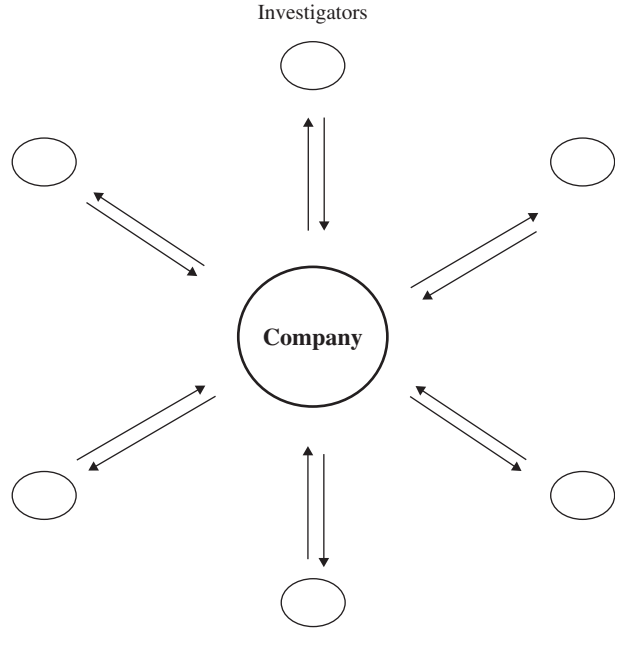

Figure 29.1 *A traditional model of academia–industry collaboration.*

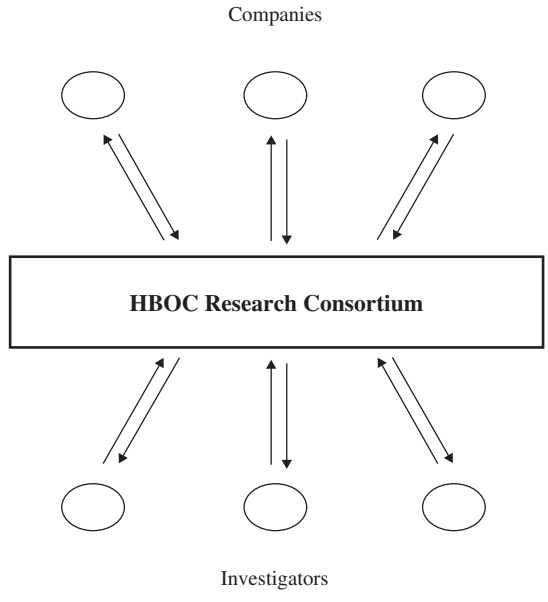

Figure 29.2 *A new model of academia–industry collaboration in HBOC development.*

in the field, each with distinct expertise (Figure 29.2). This approach will allow pooling of the expertise and resources needed to resolve issues associated with current HBOCs and to develop a new generation of improved products for eventual regulatory approval. In addition, the joint effort will enhance the chances of securing the research funds necessary for such work. The involvement of multiple HBOC companies which produce distinct HBOC products will enhance the chances of developing a successful product(s). As presented in the guiding principles, while honoring HBOC producers' IPs and other agreed-upon rights, the consortium will adopt an unrestricted publication policy for all study results, whether positive or negative in outcome. This will facilitate open discussion of any problems/issues and thereby ensure quicker resolution. In this model, HBOC producers do not have direct sponsor–investigator relationship with researchers. Rather, they are separated by a 'buffer', the consortium. This separation of sponsors and investigators will minimize potential COI and bias issues, thus increasing objectivity in the conduct of research studies and interpretation of results. If practical, the industrial developers and research investigators could be blinded to each other by the consortium by removing or codifying any words/phrases that would reveal their identity in the study protocol, product description, and communications.

29.4.5 Operation

The consortium may be based on an academic institution or it could be operated as a standalone nonprofit entity established for the purpose in a location convenient to its members and staff. The consortium will be run by a director under an operational policy/set of directives generated by a steering/executive committee. The consortium director must not have any COI issues with any of the industrial and academic participants. The executive

committee will be composed of participating academic and industrial members, as well as some independent members as appropriate (e.g. financial sponsors, selected independent investigators, concerned public). The executive committee may authorize establishment of specific task groups (e.g. a study review committee) as necessary to achieve the stated goals. A small full- or part-time support staff may assist in day-to-day operation. Ideally, the operating funds should come primarily from grants from government agencies and nonprofit foundations, but genuinely 'no-strings-attached' nonrestricted gifts/grants from industries may also be considered.

29.5 Discussion

Today, blood transfusion is generally safe and is a routine clinical therapy in developed countries. However, in many third-world countries, safe blood is scarce for transfusion because of the prevalence of AIDS, hepatitis, and other transfusion-transmittable infectious diseases in the donor pool and inadequate donor blood-screening due to limited resources [18]. Therefore, there is a need to develop alternatives to standard allogeneic donor blood, including safe and effective HBOCs [19]. The purpose of this chapter is to initiate discussion on how academia and industry may collaborate to facilitate development of viable HBOC products in the foreseeable future.

There have been many successful new product-development cases in engineering, biotechnology, pharmaceutical, and other fields as a result of successful academia–industry collaboration. However, although there are many similarities, there seems to be no standard pathway/formula that guaranties success; every case is different, depending on such factors as technical field, the nature of the product, and the goals of the parties involved. As in most other fields, there are some generic barriers to effective academia–industry collaboration in HBOC development, including disparities in cultures, time lines, missions/goals, management, and issues related to proprietary rights and ethics. The success of academia–industry collaboration will depend on surpassing these barriers [20].

Resolving issues related to COI and IP rights may be among the most complicated and difficult tasks. Recently, COI issues in biomedical research have been brought to public scrutiny as many prominent scientists and administrators have been reported to receive research/training funds from industry or to serve as paid consultants for companies. Results published by the investigators of industry-sponsored studies may be perceived as biased even when their COI statuses are clearly disclosed. In the proposed consortium model, such perceived or real bias/COI would be substantially reduced or eliminated, since the investigators and industrial sponsors are 'buffered' through a consortium. When an HBOC producer contracts the consortium for a specific study, the consortium will select an appropriate investigator to conduct the study. In addition, an investigator will not receive funds directly from the industrial sponsor, eliminating a key element of potential COI. Consequently, studies conducted in this way should be perceived as more objective. This is beneficial for the public good, as both positive and negative results will be reported without bias.

However, there are challenges with this model. It may be too idealistic and there may be difficulty in recruiting industrial members, for a couple of reasons. First, HBOC

producers will have virtually no influence or control over the conduct of the study or the interpretation of results, even though they may still be paying for the study. This could be perceived as too high a risk for a company run with funds from private and public investments. To alleviate this situation, it would be best that the consortium be operated with funds from government or nonprofit foundation grants, rather than with fees from industrial sponsors.

Another area of difficulty is IP rights resulting from this approach. Every institution has different IP policies and may require individual negotiations for each of the consortium members. This could lead to long and difficult negotiations for some consortium participants. Therefore, implementation of a standardized IP agreement for all members would be essential for success. The HBOC research consortium can be implemented with members across national borders, as successfully demonstrated by the Waseda–Keio and EuroBloodSubstitutes consortia, although their approaches are quite different. Relative to a domestic consortium, an international consortium consisting of members from multiple nations will be much more complicated as there are intercountry disparities in laws covering product approval/regulation, IP, material-transfer agreements, and other issues. However, with proper arrangements, it should be feasible to overcome these difficulties, as numerous preclinical and clinical trials of pharmaceuticals are currently being conducted across national borders, often involving sites in multiple countries.

Clearly, there are many issues that must be overcome before a workable model of an HBOC research consortium could be constructed. Of note, the proposed HBOC research consortium is designed primarily for academia–industry collaborations in early to mid stages of HBOC development, involving mostly product characterization and preclinical safety and efficacy studies. The intent of this chapter is not to present a blueprint of a perfected model but to present a concept from which debate and discussion will generate a new framework that can be transformed into a productive structure.

29.6 Conclusions

Observations of some serious AEs in recent HBOC clinical trials and failure to gain regulatory approval are hampering further development of HBOCs as viable oxygen therapeutics. HBOC developmental efforts to date are largely based on a traditional industry-centered research model. However, this traditional approach so far has not been successful in producing accepted HBOC products that are considered safe and effective by regulatory agencies in the USA and Europe. To facilitate progress of HBOC development, we propose an HBOC research consortium consisting of key academic laboratories and HBOC producers. In this model, ‘sponsors’ and ‘investigators’ are buffered by the consortium, thus minimizing potential COI while encouraging open communications and increased transparency and objectivity in the conduct of research. If properly structured and operationalized, an HBOC research consortium would greatly facilitate development of viable HBOC products through pooled expertise and resources. In addition, a research consortium approach would enhance the chances of securing research funds in this time of limited resources and increased competition. Finally, this concerted research effort may also reveal clinical niches for currently available HBOC products.

Appendix: Successful Academia–Industry Collaboration Cases in HBOC Development

Case A: Waseda–Keio–Industry Research Collaboration

Waseda University and Keio University have worked in close cooperation on artificial O_2-carrier research for over 25 years. Here is a brief summary of how this began and how the academic research group has been established with domestic and overseas research institutes.

Professor Tsuchida first started the study of synthetic hemes embedded in a hydrophobic cluster, and clarified that the electronic processes of the active sites are controlled by the surrounding molecular environment. He tried to reproduce the O_2-binding ability of red blood cells (RBCs); that is, the development of a synthetic O_2 carrier without using Hb. In general, the central ferrous iron of a heme is immediately oxidized by O_2 in water, preventing the O_2 coordination process from being observed. In 1983, Professor Tsuchida succeeded in producing reversible and stable O_2 coordination and preparing phospholipid vesicles embedded with amphiphilic-heme, known as lipidheme/phospholipids vesicles [21].

Soon after this invention, Professor Kobayashi of Keio University asked Professor Tsuchida for a chance to evaluate the lipidheme solution with *in vivo* experiments. Since that time, the joint research and collaboration on lipidheme/phospholipids vesicles has continued. In 1985, Dr Sekiguchi, the former director of Hokkaido Red Cross Blood Center (Sapporo, Japan), proposed that Professor Tsuchida consider the utilization of outdated RBCs and Hbs, because the totally synthetic system was definitely promising, but it appeared that it would take considerable time to arrive at a social consensus. Production of HbVs was started using purified Hbs and molecular-assembly technologies [22]. In the late 1990s, a mass-production system for recombinant human serum albumin was established by a Japanese pharmaceutical company. Albumin–heme hybrids using the nonspecific binding ability of hemes have been studied as an O_2-carrying plasma expander [23].

Based on the variety of new functional materials developed by Waseda University, and the evaluation system by Keio University using animal experiments, strong progress has been made in the scientific research of artificial oxygen carriers. During this period, the project has received grants from the Japanese government (Ministry of Health, Labor and Welfare, MHLW; Ministry of Education, Culture, Sports, Science and Technology, MEXT). In particular, since 1997 this research has continuously received a Health Sciences Grant from the MHLW (Principal Investigators: Professor Tsuchida, 1997–2002; Professor Kobayashi, 2003–2007; Associate Professor Horinouchi, 2008–present). This was a driving force in establishing the present interdisciplinary academic consortium, comprising researchers not only from Waseda University, Keio University, and Hokkaido Red Cross, but also from Kumamoto University, Higashitakarazuka-Sato Hospital, and National Defense Medical College. With the governmental support, some overseas experts were invited to Japan, and some young Japanese researchers were given opportunities to study in universities in the USA. Since then, collaborations have expanded to multiple international partners (University of California, San Diego; University of Texas, San Antonio; University of Berne; McMaster University; Cornell

University College of Medicine; and Massachusetts General Hospital) to undertake safety and efficacy evaluations of artificial oxygen carriers using various animal models.

Aiming at the industrialization of HbV, in the early 1990s Waseda collaborated with NOF Corp. (Tokyo, Japan). At that time it was believed that liposomes were unstable corpuscles. A polymerizable phospholipid bearing two dienoyl groups was utilized as a main membrane component, and the HbV obtained was irradiated with gamma rays to polymerize the membrane. The resulting particles were so stable that they were resistant to freeze-thawing and freeze-drying. However, the particles were not degraded easily and remained in the reticuloendothelial system for several months. Due to this problem, the collaboration was terminated. After a period of trial and error, the present optimal lipid composition (without polymerization) was found to enable long-term storage and prompt degradation in the reticuloendothelial system. A bioventure company, Oxygenix Corp. (Tokyo, Japan), was established in 2003, and production was transferred from Waseda University to Oxygenix. However, due to financial problems and its eventual bankruptcy, Oxygenix terminated development in 2008 before the product could enter clinical trials. Currently, Nipro Corp. (Osaka, Japan) is continuously developing HbV in order to establish an efficient production method.

The HbV is categorized as a molecular assembly and its production method is complicated in comparison to cell-free HBOCs. Moreover, its dosage as a blood substitute is estimated to be considerably larger than that of conventional liposomal drugs and it is difficult to design animal experiments for safety and efficacy evaluation. Therefore, the academic professionals in this field, including the material inventors and clinicians, have supported the initial industrial development of HbVs of pre-clinical stage. Waseda and Keio Universities individually contracted with Oxygenix and Nipro for technology transfer, license, or consultancy. The companies have participated in our consortium, supported by the MHLW. Recently, as concerns about COI have been heightened in Japan, all these relationships have been disclosed. All members signed collaborative research agreements, specifying confidentiality, material transfer, new IP filing, and submission of academic papers and presentations. Because the research is supported by grants from the MHLW, all results from the consortium are submitted to the Ministry. In the future, when this research enters the clinical-trial stage, it will be even more important to consider COI. Clinical trials will be conducted by appropriate investigators who are in a neutral position and are free of COI.

Apart from Waseda–Keio activity, there is a long history of R&D of artificial oxygen carriers in Japan, beginning in the 1950s. The perfluorocarbon emulsion of Green Cross Corp. was the first US FDA-approved oxygen carrier (though the production was terminated due to side effects and limited clinical usage). The pyridoxalated-Hb-poly(oxyethylene) conjugate (PHP) of Ajinomoto Corp. was the first HBOC approved for clinical trials in the USA, by the FDA. The Japanese researchers who are studying or interested in artificial blood (substitutes for all the components of blood) established the Society of Blood Substitutes Japan (SBSJ) in 1993 (Former President, Professor Tsuchida; Current President, Professor Kobayashi) [24]. Since then, annual meetings have been held, inviting researchers from both engineering and medical fields, industries, and governmental agencies. Joint symposia with the International Society of Blood Substitutes were held in 1997 and 2003.

After the experiences of an HIV epidemic due to unpasteurized blood products, the disaster of the Great Hanshin–Awaji Earthquake, and the global trend for artificial blood, the government decided to promote R&D of artificial blood, aiming at industrialization, as clearly stated in the Revised Pharmaceutical Affairs Act of 2002.

The development of HbV as a cellular-type HBOC goes far behind that of the cell-free HBOCs; however, it is quite interesting to note that the side effects of molecular Hb and the physiological importance of the cellular structure of RBCs have been recognized through R&D of artificial oxygen carriers. Due to the current economic depression and concerns over development of new drugs, especially those categorized as a biologic drugs, Japanese pharmaceutical companies seem reluctant to develop artificial red cells. Despite some failures for industrialization, the academic consortium in Japan continues the research with a strong will and the support of the government, aiming at the realization of artificial oxygen carriers, which will eventually benefit human health and welfare.

Case B: EuroBloodSubstitutes Consortium

In 2004, Dr Kenneth C. Lowe, University of Nottingham, led the organization of the EuroBloodSubstitutes consortium, a multicenter consortium of 13 European academic and industrial teams [25], most with a consolidated experience in hemoglobin research and very limited previous activities in blood substitutes. This was the first time that a European coordinated action towards the development of blood substitutes took place. The EuroBloodSubstitutes project was funded by a grant from the European Union's 6th Framework Programme. As stated in the final publishable activity report of the project, there were six key scientific and technological objectives (end results) of the EuroBloodSubstitutes project, which were to:

(i) Identify, using quantitative and qualitative approaches to data collection, the perceived benefits and risks among stakeholders (e.g. public, patient groups, health care professionals, regulatory agencies) of blood and blood-substitute use in European society.
(ii) Develop a technological platform and genomic basis for generating prototype native, modified, and variant heme proteins for improved formulations in blood substitutes to replace some blood uses.
(iii) Identify and overcome bottlenecks in the efficient production of prototype heme proteins using fungi and plants as expression systems.
(iv) Characterize, using appropriate biochemical and biophysical techniques, the prototype heme proteins.
(v) Develop and optimize standard assays for the biological characterization of the prototype heme proteins.
(vi) Disseminate, through reports, publications, and patents, the new knowledge and understanding on issues associated with the generation and stakeholder perception of blood-substitute materials.

The implementation of the activities within the EuroBloodSubstitutes consortium was carried out via eight Workpackages, which led to the following main results:

(i) Workpackage 1 *(Blood and blood substitutes in European Society)*: Definitive survey materials identifying prevailing attitudes and opinions of the UK public to the societal use of blood (e.g. in transfusion) and potential clinical use of different types of blood substitute were piloted, refined, and fully developed. The materials were distributed to 12 000 members of the UK public and in the Netherlands [26].

(ii) Workpackages 2, 3, and 7 *(Protein design, protein expression, fermentation and scale-up)*: Genes for native hemoglobin were obtained, expression of α- and β-chains of hemoglobin in the bacterium *Escherichia coli* was achieved, and new mutant hemoglobin subunits were synthesized and expressed, carrying modifications at α42, β37, α C-terminus and β C-terminus. The synthesis and characterization of a two-subunit, four-domain hemoglobin derivative was completed [27]. Recombinant hemoglobin was expressed in yeasts, optimizing the yields, by exploiting microarray technology.

(iii) Workpackages 4, 5, and 6 *(Protein modification and purification, biochemical, biophysical, and physiological characterization)*: Chemical procedures for the production under anaerobic conditions of PEGylated-hemoglobin were developed. Robust procedures for the biochemical, biophysical, and *in vitro* and *in vivo* physiological characterization of PEG hemoglobin derivatives were carried out [28–35].

(iv) Workpackage 8 *(Management and dissemination of information)*: Achievements throughout the project include several internal meetings and the organization of the International Visions on Blood Substitutes Conference – Hemoglobin-Based Oxygen Carriers: From Chemistry to Clinic in Parma, Italy, September 17–20, 2006. Based on the success of this conference, the XII International Symposium on Blood Substitutes was organized for the first time in Europe, in Parma, Italy, August 25–28, 2009.

The EuroBloodSubstitutes consortium was managed by a committee composed of workpackage leaders and by a committee composed of selected academic and industrial partners dealing with IP-related issues, such as data publications. Partners were bound to work toward achieving objectives, milestones, and deliverables defined at the time of proposal submission at the European FP6 commission. Partner meetings, held every six months, allowed monitoring of achievements, discussion of emerging issues, and definition of strategies to overcome difficulties. In parallel, scientific and financial reports were collected and reviewed by the EU commission. This thorough and time-consuming documentation made the consortium activities very transparent and verifiable at any stage. Consortium partners were also bound by the Consortium Agreement, which dictated rules on all issues associated with the project, including IP protection and data dissemination. Patents generated from activities carried out within the consortium belong to the universities involved, not to all partners. Before submission of a publication, authors were asked to receive an approval by the consortium committee. Products generated from consortium activities were freely shared among partners. For example, a novel PEGylated HBOC, called EuroPEG-Hb [29], developed by one partner was characterized at the biochemical and physiological levels by others partners.

The key feature of the consortium was the coordination of different laboratories with distinct expertises towards a common goal. This scheme has recently been proposed at the international level as a possible strategy to overcome the present fragmentation in blood-substitute research activities, shortage of funding, and unnecessary competition. A key lesson from the EuroBloodSubstitutes consortium is that a curiosity-driven research is much more rewarding and, in the long run, more likely to be successful than a market-driven research in solving basic research issues.

References

[1] Sloan, E.P., Koenigsberg, M., Gens, D. *et al*. Diaspirin cross-linked hemoglobin (DCLHb) in the treatment of severe traumatic hemorrhagic shock: a randomized controlled efficacy trial, *JAMA*, **282**, 1857–1864 (1999).

[2] Saxena, R., Wijnhoud, A.D., Carton, H. *et al*. Controlled safety study of a hemoglobin-based oxygen carrier, DCLHb, in acute ischemic stroke, *Stroke*, **30**, 993–996 (1999).

[3] Greenburg, A.G. and Kim, H.W. Use of an oxygen therapeutic as an adjunct to intraoperative autologous donation to reduce transfusion requirements in patients undergoing coronary artery bypass graft surgery. Hemolink Study Group, *J. Am. Coll. Surg*., **198**, 373–383 (2004).

[4] Jahr, J.S., Mackenzie, C., Pearce, L.B. *et al*. HBOC-201 as an alternative to blood transfusion: Efficacy and safety evaluation in a multicenter phase III trial in elective orthopedic surgery, *J. Trauma*, **64**, 1484–1497 (2008).

[5] Olofsson, C., Nygards, E.B., Ponzer, S. *et al*. A randomized, single blind, increasing dose safety trial of an oxygen-carrying plasma expander (Hemospan®) administered to orthopedic surgery patients with spinal anesthesia, *Trans. Med*., **18**, 28–39 (2008).

[6] Moore, E.E., Moore, F.A., Fabian, T.C. *et al*. Human polymerized hemoglobin for the treatment of hemorrhagic shock when blood is unavailable: the USA multicenter trial. PolyHeme Study Group, *J. Am. Coll. Surg*., **208**, 1–13 (2009).

[7] Silverman, T.A. and Weiskopf, R.B. Hemoglobin-based oxygen carriers- current status and future directions, *Anesthesiology*, **111**, 946–963 (2009).

[8] Thursby, J.G. and Thursby, M.C. University licensing and the Bayh–Dole Act, *Science*, **301**, 1052 (2003).

[9] Perkman, M. and Walsh, K. The two faces of collaboration: impacts of university-industry relations on public research, *Ind. Corp. Change*, **18**, 1033–1065 (2009).

[10] Kesselheim, A.S. and Avorn, J. University-based science and biotechnology products: defining the boundaries of intellectual property, *JAMA*, **293**, 850–854 (2005).

[11] Wilson, D. Harvard teaching hospitals cap outside pay, *The New York Times*, January 3, 2010.

[12] AAMC. Industry funding of medical education. Report of an AAMC Task Force, June 2008.

[13] AAMC. Protecting patients, preserving integrity, advancing health: accelerating the implementation of COI policies in human subjects research. A report of the AAMC-AAU Advisory Committee on Financial Conflict of Interest in Human Subject Research, February 2008.

[14] Brockway, L.M. and Furcht, L.T. Conflicts of interest in biomedical research: the FASEB guidelines, *FASEB J*., **20**, 2435–2438 (2006).
[15] Liao, S.M., Sheehan, M. and Clarke, S. The duty to disclose adverse clinical trial results, *Am. J. Bioethic*., **9**, 24–32 (2009).
[16] Estep, T., Bucci, E., Farmer, M. *et al*. Basic science focus on blood substitutes: a summary of the NHLBI Division of Blood Diseases and Resources Working Group Workshop, March 1, 2006, *Transfusion*, **48**, 776–782 (2008).
[17] Natanson, C., Kern, S.J., Lurie, P. *et al*. Cell-free hemoglobin-based blood substitutes and risk of myocardial infarction and death: a meta-analysis, *JAMA*, **299**, 2304–2312 (2008).
[18] WHO, Global database on blood safety 2004–2005, WHO Press, Geneva, Switzerland, 2008.
[19] CBER, FDA and NHLBI, NIH, and Office of Public Health and Science, DHHS. Safety of Hemoglobin-Based Oxygen Carriers (HBOCs), NIH, Bethesda, Maryland. http://www.fda.gov/BiologicsBloodVaccines/NewsEvents/WorkshopsMeetingsConferences/ucm092010.htm (accessed March 9, 2010).
[20] National Academy of Sciences. Overcoming barriers to collaborative research: report of a workshop, National Academy Press, Washington, DC (1999).
[21] Matsushita, Y., Hasegawa, E., Eshima, K. and Tsuchida, E. Synthesis of amphiphilic porphinatoiron complexes having phosphocholine groups, *Chem. Lett*., **12**, 1387–1389 (1983).
[22] Sakai, H., Sou, K., Horinouchi, H. *et al*. Hemoglobin-vesicles as artificial oxygen carriers: Present situation and future vision, *J. Intern. Med*., **263**, 4–15 (2008).
[23] Tsuchida, E., Sou, K., Nakagawa, A. *et al*. Artificial oxygen carriers, hemoglobin vesicles and albumin-hemes, based on bioconjugate chemistry, *Bioconjugate Chem*., **20**, 1419–1440 (2009).
[24] Tsuchida, E. Establishment of Society of Blood Substitutes Japan, *Artif. Blood*, **1**, 2 (1993).
[25] Lowe, K.C. Genomics and blood substitutes for 21st century Europe ('EuroBlood-Substitutes'), *Art. Cells Blood Subs. Biotech*., **34**, 589–599 (2006).
[26] Ferguson, E., Spence, A., Townsend, E. *et al*. What type of information is trusted by whom? A multilevel analysis of the stability of the information source-trust association for blood transfusion, *Transfusion*, **49**, 1637–1648 (2009).
[27] Panetta, G., Arcovito, A., Morea, V. *et al*. Hb(αα,ββ): a novel fusion construct for a dimeric, four-domain hemoglobin, *Biochim. Biophys. Acta*, **1784**, 1462–1470 (2008).
[28] Iafelice, R., Cristoni, S., Caccia, D. *et al*. Identification of the sites of deoxyhaemoglobin PEGylation, *Biochem. J*., **403**, 189–196 (2007).
[29] Portoro, I., Kocsis, L., Herman, P. *et al*. Towards a novel haemoglobin-based oxygen carrier: Euro-PEG-Hb, physico-chemical properties, vasoactivity and renal filtration, *Biochim. Biophys. Acta*, **1784**, 1402–1409 (2008).
[30] Caccia, D., Ronda, D., Frassi, R. *et al*. PEGylation promotes hemoglobin tetramer dissociation, *Bioconjug. Chem*., **20**, 1356–1366 (2009).
[31] Dunne, J., Caron, A., Menu, P. *et al*. Ascorbate removes key precursors to oxidative damage by cell-free haemoglobin *in vitro* and *in vivo*, *Biochem. J*., **399**, 513–524 (2006).

[32] Silaghi-Dumitrescu, R., Reeder, B.J., Nicholls, P. *et al*. Ferryl haem protonation gates peroxidatic reactivity in globins, *Biochem. J*., **403**, 391–395 (2007).
[33] Reeder, B.J., Grey, M., Silaghi-Dumitrescu, R.L. *et al*. Tyrosine residues as redox cofactors in human hemoglobin: implications for engineering nontoxic blood substitutes, *J. Biol. Chem*., **283**, 30780–30787 (2008).
[34] Cooper, C.E., Silaghi-Dumitrescu, R., Rukengwa, M. *et al*. Peroxidase activity of hemoglobin towards ascorbate and urate: a synergistic protective strategy against toxicity of Hemoglobin-Based Oxygen Carriers (HBOC), *Biochim. Biophys. Acta*, **1784**, 1415–1420 (2008).
[35] Caretti, A., Fantacci, M., Caccia, D. *et al*. Modulation of the NO/cGMP pathway reduces the vasoconstriction induced by acellular and PEGylated haemoglobin, *Biochim. Biophys. Acta*, **1784**, 1428–1434 (2008).

Index

Chemistry and Biochemistry of Oxygen Therapeutics: From Transfusion to Artificial Blood, First Edition.
Edited by Andrea Mozzarelli and Stefano Bettati.

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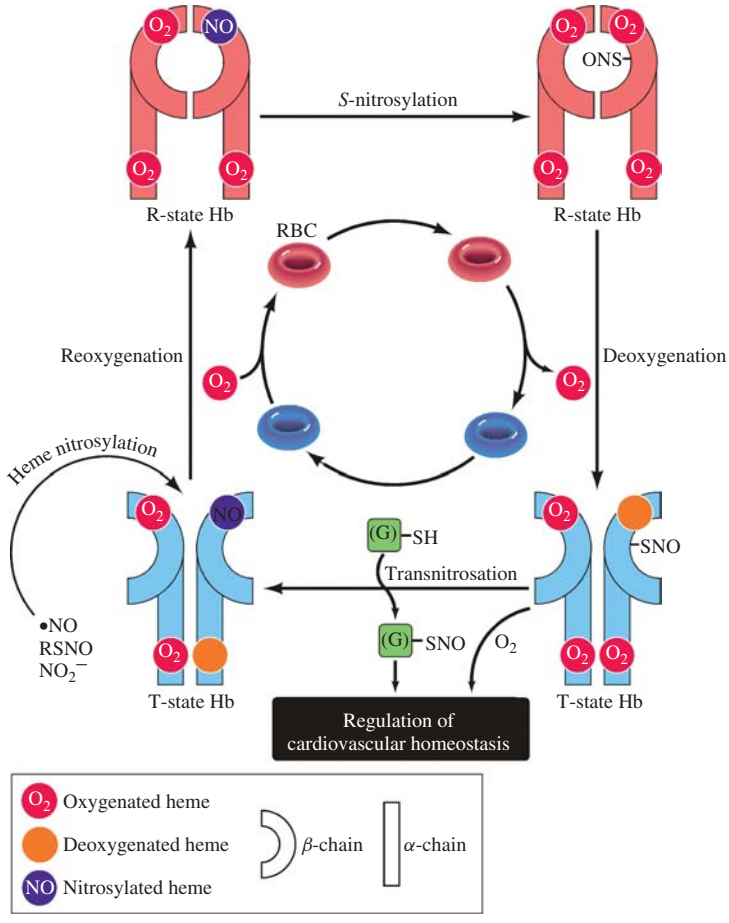
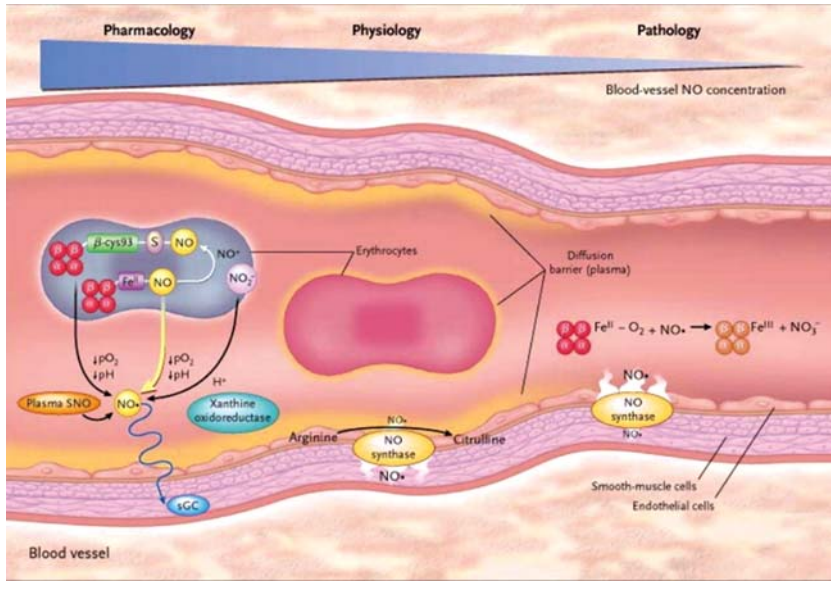
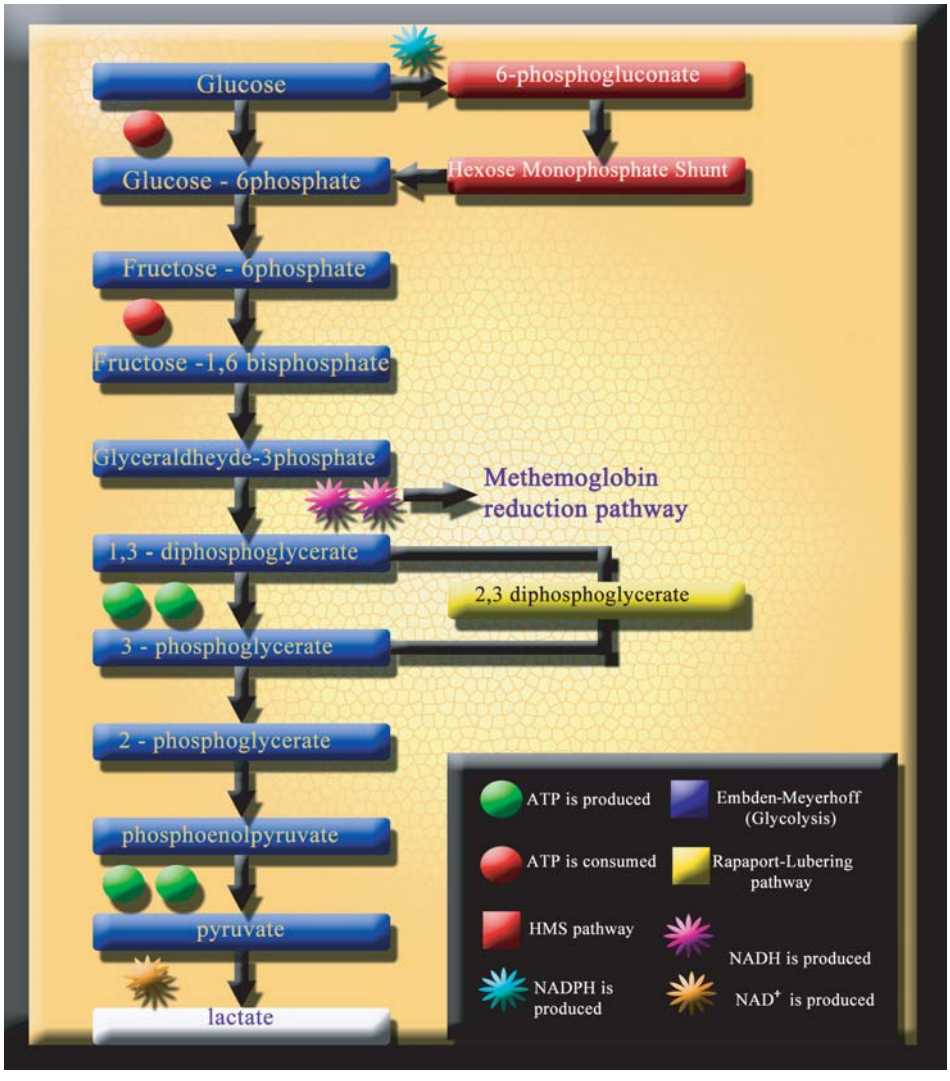


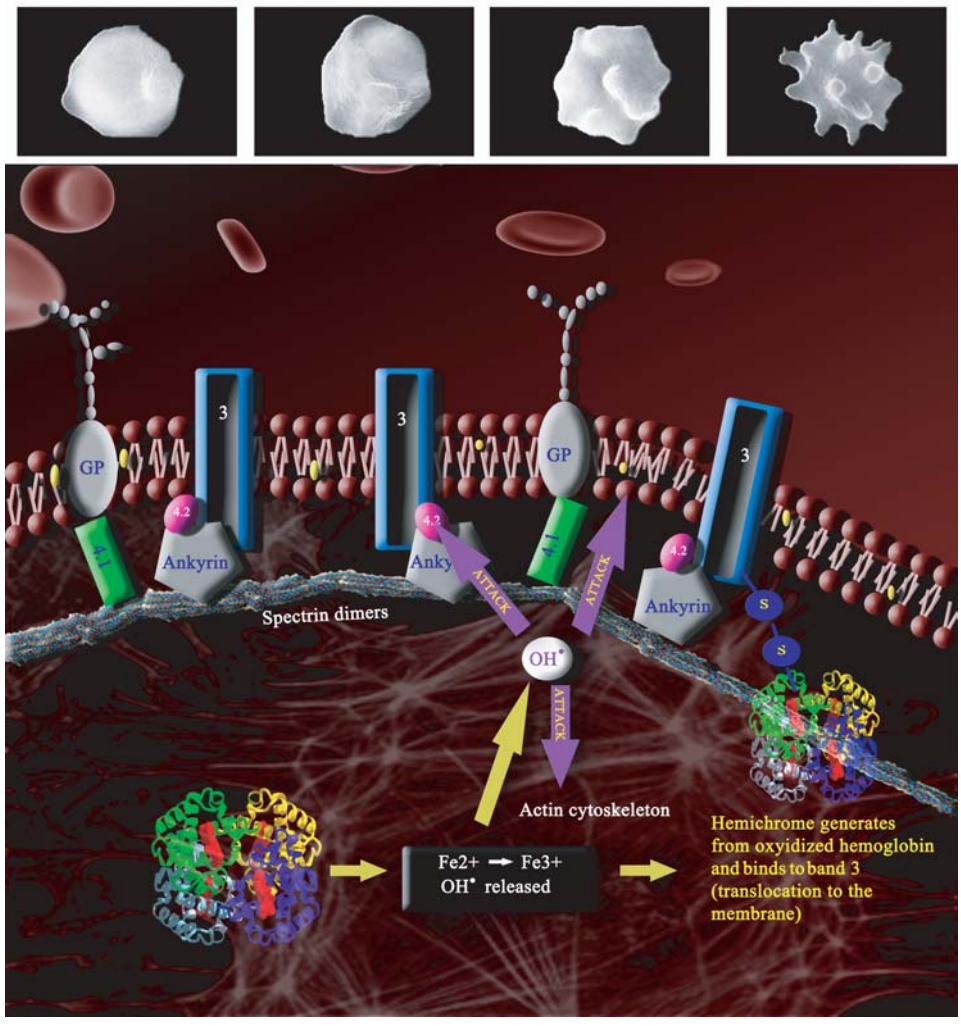
Color Plate 1: **Figure 4.3** *Allosteric transitions of Hb during circulatory cycling regulate the delivery of (S)NO bioactivity and thus O_2 delivery. RBC Hb senses [O_2] and responds via allosterically governed (S)NO binding, formation, and release. At high pO_2 in the lungs, Hb is in the R state and the reactive Cys93 residue of the β chain is protected in a hydrophobic pocket in the vicinity of β hemes. On partial RBC deoxygenation in the peripheral circulation, Hb adopts the T structure and Cysβ93 is exposed to solvent. These allosteric transitions control the reactivity of Cysβ93. In the T state, a population of Hb reacts with •NO, NO_2^-, and/or low-mass SNOs to produce nitrosylated heme at the β chain (bottom left). Transition to R state, which brings Cysβ93 close to nitrosylated heme (top left), is followed by a nitrosylation reaction between NO heme and Cysβ93. This results in the formation of a covalently bound S-nitrosocysteine (top right). In the R state, SNO-Cysβ93 is protected from further reactions in a hydrophobic pocket. A potential direct nitros(yl)ation of Cysβ93 by higher oxides of nitrogen (NOx) or by metal catalysis, including by dinitrosyl iron complexes, is also possible. When Hb adopts the T conformation on partial deoxygenation (bottom right), SNO-Cysβ93 readily reacts with target nitrosothiols and initiates a cascade of transnitrosations (for example, with plasma), leading to the formation of SNOs outside RBCs. The precise identity of the SNOs formed remains unresolved, and the candidate GSNO is depicted here. SNO is at least as potent as •NO as a vasodilator and ensures •NO-independent functions aimed at preserving vascular homeostasis. RBCs thereby function as oxygen sensors to accurately regulate O_2 supply. (Reproduced with permission from [32]).*



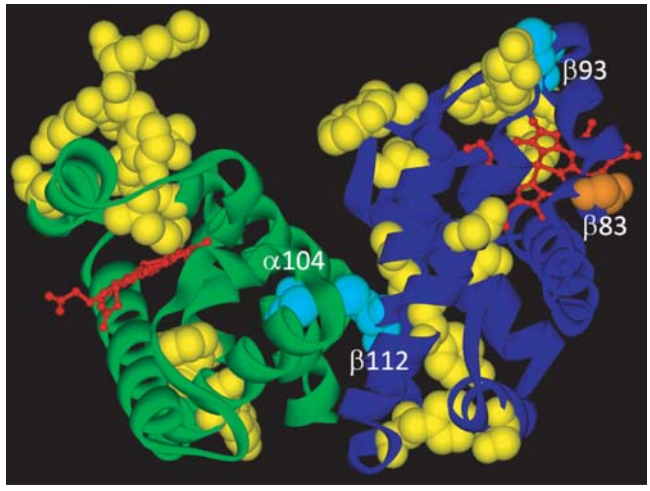
Color Plate 2: **Figure 6.3** *A model of the interactions of nitric oxide (NO) with erythrocytes and cell-free Hb in an arterial blood vessel. The diagram illustrates the major processes regulating NO levels in blood vessels during pharmacologic NO delivery (left), under normal conditions (center), and under pathologic conditions, such as acute or chronic hemolysis (right). The overall blood-vessel NO concentration is depicted by the width of the blue band above the vessel. Within the vessel, smooth-muscle cells and a layer of endothelial cells are shown. Free Hb binds NO, resulting in vascular constriction on the right side of the figure. Aged stored RBC units with increased hemolysis and early-generation HBOCs may both have increased free-Hb concentrations and induce vasoconstriction in blood vessels. (Reproduced with permission from The New England Journal of Medicine [145]).*



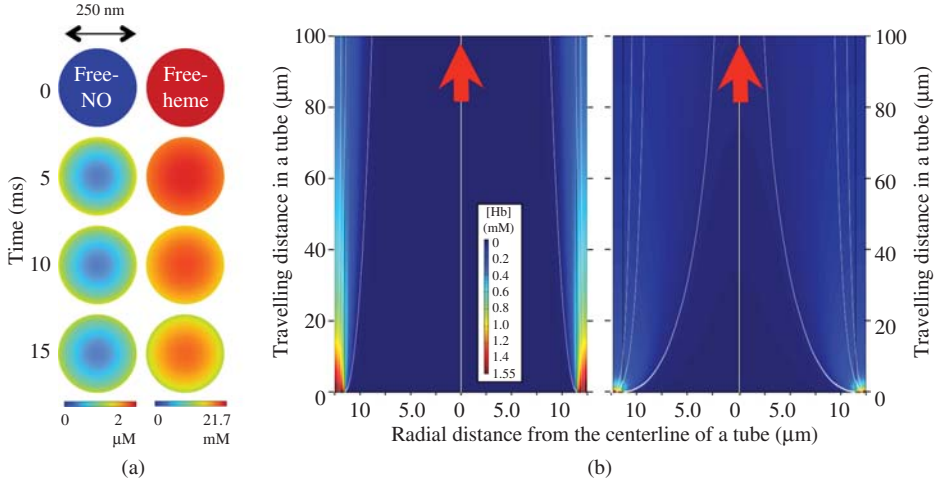
Color Plate 3: **Figure 18.1** *RBC metabolism mainly gravitates towards four main pathways: the Embden–Meyerhof glycolytic pathway (for ATP production), the hexose monophosphate shunt (HMS) pathway (for NADPH generation), the Rapoport–Luebering pathway for 1,3-DPG conversion to 2,3-DPG, and the methemoglobin reduction pathway. When NADH is not fully oxidized back to NAD^+ through the methemoglobin reduction pathway, lactate is produced from pyruvate as a byproduct of anaerobic glycolysis.*



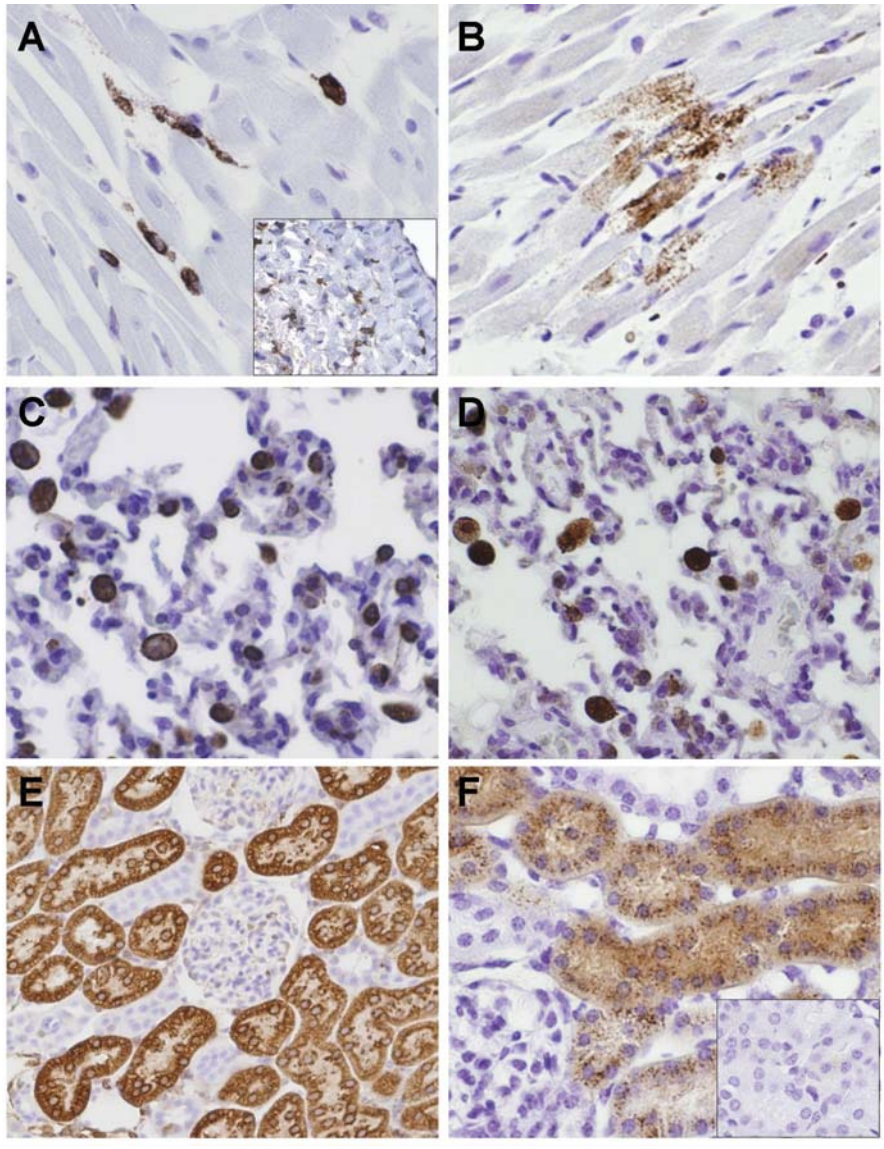
Color Plate 4: **Figure 18.2** *A domino effect appears to be responsible for RBC storage lesions (upper panel shows shape of morphology-related lesions in time course, from left to right). Being stored in an oxidative environment, hemoglobin is slowly oxidized to met-Hb, and a low percentage of hemichrome aggregates form through disulfide bonds. In the meantime, heme iron is reduced from a ferrous to a ferric state via a Fenton's reaction, which produces OH• radicals. A cascade of oxidative events takes place due to the spreading of reactive oxygen species (ROS). These events involve ROS attacks to the cytoskeleton and membrane (either lipid or protein fraction). A particularly eligible target appears to be the anion-exchanger band 3 and the proteins near to its cytosolic portion (band 4.2, ankyrin, and several enzymes such as glyceraldehyde-3-phosphate-dehydrogenase). Although RBCs are well equipped to cope with high oxidative stresses, prolonged storage periods end up exacerbating the oxidative phenomena, thus the outcome is a no-longer-functional or -vital RBC. Furthermore, the oxidative environment rapidly switches the metabolic trigger from the classic glycolytic pathway to the HMS pathway, with the result that the RBC experiences a total depletion of ATP. When transfused, the long-stored effete RBC is rapidly removed from the bloodstream or else it contributes to the promotion of untoward responses in the recipient.*



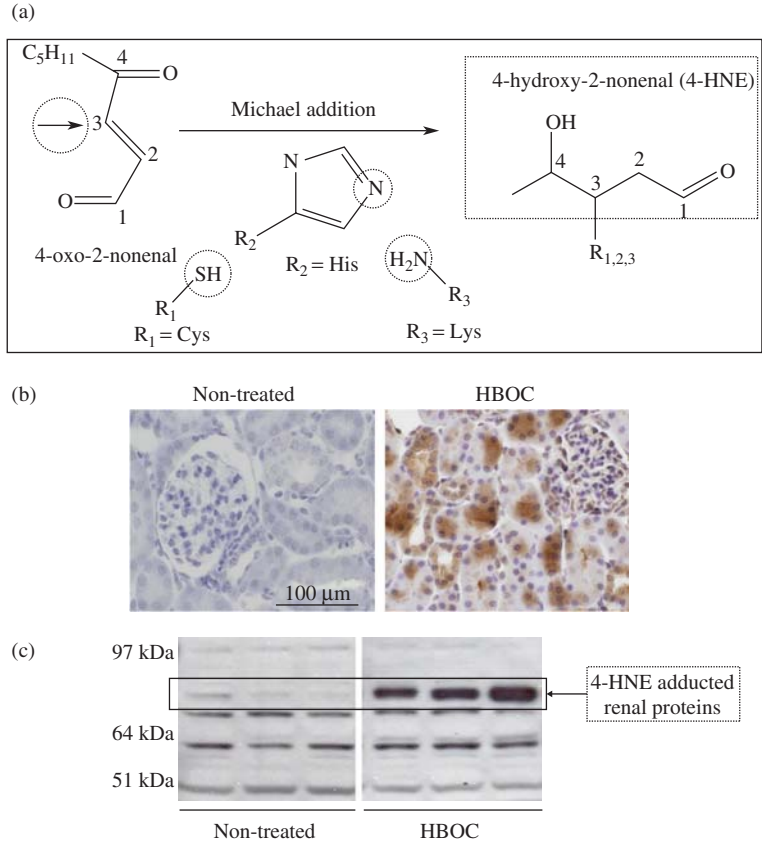
Color Plate 5: **Figure 26.2** *Known positions of Cys residues (yellow, except for β83 of Hb Tali, in orange) in Hb alpha (green) and beta (dark blue) chains, based on the database of some 1000 mutants (see Table 26.1). Also shown are sites for wild-type human Cys (cyan, α104 G11, β93 F9, and β112 G14).*



Color Plate 6: **Figure 27.1** *Encapsulation of Hb in vesicles retards NO binding. (a) Schematic two-dimensional representation of the simulated time courses of distributions of unbound free NO and unbound free heme (deoxy form) in one HbV (250 nm) after immediate mixing of NO and HbV by stopped-flow method. Computer simulation shows that both free NO and unbound hemes are distributed heterogeneously. The concentration changes gradually from the surface to the core. The determinant factor of retardation of NO binding should be the intracellular diffusion barrier, which was induced by: (i) intrinsically larger binding rate constant of NO to a heme in an Hb molecule; (ii) numerous hemes as sites of gas entrapment at a higher Hb concentration; (iii) a slowed gas diffusion in the intracellular viscous Hb solution; and (iv) a longer gas diffusion distance in a larger capsule [31]. (b) Schematic representation of the simulated density distribution and track of HbV (left) and Hb (right) in a narrow tube (<100 μm traveling distance). We assumed that two different solutions with the same physicochemical properties enter and flow through the same tube. The radius of the tube was 12.5 μm: component 1 (blue color) enters the core of the tube (radial distance from the centerline, 0–11 μm) and component 2 (red color) enters near the wall (radial distance from the centerline, 11–12.5 μm). Finally, both components are mixed completely. The diffusivity of HbV is much slower than that of Hb, resulting in the retarded gas reactions in microvessels. The concentration of Hb is expressed as heme concentration ([Hb] = 1.55 mM at 10 g/dL) [39]. (Reproduced with permission from Am. J. Physiol. Heart Circ. Physiol.,* **298***, H956–H965 (2010)).*



Color Plate 7: **Figure 28.1** *HO-1 induction and non-heme iron deposition following HBOC infusion in rodents. Tissues were collected following 50% exchange transfusion of polymerized bovine Hb and analyzed for HO-1 ((a), (c), (d)) or Perls non-heme iron histochemistry with DAB intensification ((b), (e), (f)). (a), (b) Heart. HO-1 induced primarily in interstitial cells, possibly infiltrating phagocytes in myocardium and in perivascular sites in the adventitial layers of the aorta (inset). Positive Perls iron was observed in myocytes (brown). (c), (d) Lung. Large alveolar macrophages displaying HO-1 expression and non-heme iron deposits 12 hours after HBOC administration. (e), (f) Kidney. Striking HO-1 reactivity and iron deposits in renal proximal tubular epithelium but absent in glomeruli compared to nontreated kidney (inset). Magnification 400×.*



Color Plate 8: **Figure 28.4** *4-HNE-modified adducts as reliable markers of lipid peroxidation. (a) Schematic representation of typical 4-HNE-adduct formation with amino-acid residues, histidine, lysine, and cysteine. (b) Immunohistochemical detection of 4-HNE-modified adducts in guinea pig kidney following transfusion with polymerized bovine Hb. (c) Western blot analysis of 4-HNE adducts in guinea pig kidney.*