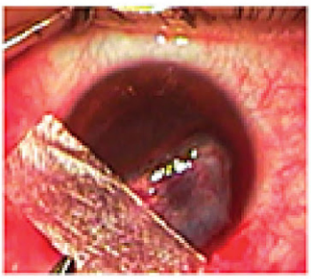
Human Amniotic Membrane

Basic Science and Clinical Application





Nazly Hilmy
Norimah Yusof
Aziz Nather
Editors

World Scientific

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Basic Science and Clinical Application

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Dedication

This book is dedicated to:

PIONEERS OF APASTB

- *Dr. Yongyudh Vajaradul,* Bangkok, Thailand
- *Dr. Aziz Nather,* Singapore
- *Dr. Moritoshi Itoman,* Tokyo, Japan
- *Dr. Norberto Agcaoili,* Manila, Philippines
- *Dr. Sun Shiquan,* Taiyuan, China



From left to right: Aziz Nather, Moritoshi Itoman, Sommart Kaerochana, Yongyudh Vajaradul, Norbeto Agcaoili, Sun Shiquan

20th Anniversary Lifetime Achievement Award

The Lifetime Achievement Award is presented by IAEA/NUS Regional Training Centre to give recognition to individuals who have made outstanding contributions to the Centre and in their own countries. The award is presented in celebration of the 20th Anniversary of the Training Centre, which trains tissue bank operators in the Asia Pacific Region (Nov 1997 to current).

20th Anniversary Lifetime Achievement Award

Dr. NAZLY HILMY
Consultant Radiation Biologist
Consultant, BATAN Research Tissue Bank
Centre for Research and Development
of Isotopes
Jakarta, Indonesia



- Pioneered efforts in tissue banking development in Indonesia.
- Head of BATAN Research Tissue Bank – first Tissue Bank set up in Indonesia (1990—2005).
- Introduced gamma irradiation for the sterilization of tissue grafts to promote safe tissue transplantation practice in Indonesia.
- Pioneered the procurement, processing and clinical transplantation of amniotic membrane in Indonesia.
- Conducted IAEA Expert Missions to several countries (1991–2009).
- Edited 3 textbooks on Tissue Banking:
  - — ***Radiation in Tissue Banking***. World Scientific Publishing Co Inc; 2007
  - — ***Allograft Procurement, Processing and Transplantation***. World Scientific Publishing Co Inc; 2010

— ***Human Amniotic Membrane — Basic Science & Clinical Applications***. World Scientific; 2017

- Lecturer and External Examiner to IAEA/NUS Diploma Course in Tissue Banking (2005—2017).

20th Anniversary Lifetime Achievement Award

Dr. NORIMAH YUSOF
Consultant Radiation Biologist
Adjunct Professor & Advisor, Bone Bank
National Orthopaedic Centre of Excellence in Research & Learning
Department of Orthopaedic Surgery, University of Malaya
Kuala Lumpur, Malaysia



- Pioneered efforts in tissue banking development in Malaysia
- Head of MINT Research Tissue Bank in Bangi, Malaysia (1990–2008).
- Introduced gamma irradiation for the sterilization of tissue grafts to promote safe tissue transplantation practice in Malaysia.
- Pioneered the procurement, processing and clinical transplantation of amniotic membrane in Malaysia.
- Conducted IAEA Expert Missions to several countries (1996–2009).
- Edited 3 textbooks on Tissue Banking:
  - — ***Radiation in Tissue Banking***. World Scientific Publishing Co Inc; 2007
  - — ***Allograft Procurement, Processing and Transplantation***. World Scientific Publishing Co Inc; 2010

— ***Human Amniotic Membrane — Basic Science & Clinical Applications***. World Scientific; 2017

- Won 2 awards:
  - — ***First Winner, Innovation Award of Selangor State 2001***: "Cost Effective Processing of Amnion for Wound Dressing"
  - — ***Gold Medal & Woman Inventor of the Year, Malaysian Technology Expo 2010***: "Development of Amnion and Chitosan Paste as Flexible Wound Dressings"
- Lecturer and External Examiner to IAEA/NUS Diploma Course in Tissue Banking (2005–2017).

20th Anniversary Lifetime Achievement Award

Professor HASIM MOHAMAD
Consultant Urologist
Lincoln University College
Department of Surgery
Hospital Raja Perempuan Zainab 11
Kelantan, Malaysia



- Pioneered efforts in tissue banking development in Malaysia
- Founded Tissue Bank in Kota Bahru, Kelantan in 1991; Inaugurated it as Malaysian National Tissue Bank (current USM Tissue Bank) in 1994.
- Pioneered the procurement, processing and clinical transplantation of amniotic membrane in Malaysia.
- First President Malaysian Cell and Tissue Society (2005).
- President of Asia Pacific Association of Surgical Tissue Banks (2000–2002)
- Chairman of 5th World Congress on Tissue Banks held in Kuala Lumpur, Malaysia in 2008

- Contributed 3 Book Chapters:
  - — "Anatomy & Embryology of Human Placenta, Amnion and Chorion"; *The Scientific Basis of Tissue Transplantation*; World Scientific Publishing Co Inc; 2001
  - — "Amnion for Treatment of Burns"; *Radiation in Tissue Banking*; World Scientific Publishing Co Inc; 2007
  - — "Amnion Processing"; *Allograft Procurement, Processing and Transplantation*; World Scientific Publishing Co Inc; 2010
- Lecturer to IAEA/NUS Diploma Course in Tissue Banking 4 times (2004–2014); Examiner to IAEA/NUS Diploma Course Examinations 2 times (2015, 2017)

20th Anniversary Lifetime Achievement Award

Dr. AZIZ NATHER
Senior Consultant Orthopaedic Surgeon
Director, NUH Tissue Bank
Department of Orthopaedic Surgery, National University Hospital
Singapore



- Pioneered efforts in tissue banking development in Singapore
- Set up first Singapore bone bank, NUS Research Bone Bank (1988). Inaugurated NUH Tissue Bank as a hospital cost centre (1998).
- Director IAEA/NUS Regional Training Centre for Tissue Bank operators in the Asia Pacific Region (1997).
- Director IAEA/NUS Diploma Course in Tissue Banking (1997–2017).
- President Asia Pacific Association of Surgical Tissue Banks (1990–1992).
- Conducted IAEA Expert Missions to several countries (1994–2009).
- Edited 5 textbooks on Tissue Banking:
  - — ***The Scientific Basis of Tissue Transplantation***; World Scientific Publishing Co Inc; 2001
  - — ***Bone Grafts and Bone Substitutes***; World Scientific; 2005

— ***Radiation in Tissue Banking***; World Scientific Publishing Co Inc; 2007
— ***Allograft Procurement, Processing and Transplantation***; World Scientific Publishing Co Inc; 2010
— ***Human Amniotic Membrane — Basic Science & Clinical Applications***; World Scientific; 2017

- ***Berita Harian Achiever of the Year Award 2000***, for developing the Spastic Children Association of Singapore, and Tissue Banking in Asia Pacific Region.

Preface

This book was launched during the opening ceremony of the 18th IAEA/NUS Diploma Course in Tissue Banking (12 September 2017) in commemoration of the 20th Anniversary of the IAEA/NUS Regional Training Centre for Tissue Bank Operators launched in November 1997.

This book is written as a comprehensive guide for all tissue bank operators to screen, procure and process amniotic membranes of the highest quality standards for safe amnion membrane transplantation practice.

It discusses the basic structure of amnion including the anatomy, histology and embryology of amnion as well as its electron microscopic structure. It also describes the biological properties and function of amnion with the cells and numerous growth factors present. The biomechanical properties are also described.

Clinical presentation includes donor selection criteria, exclusion criteria and laboratory investigations performed to evaluate donor suitability. Procurement methods include amnion from Caesarean Sections and from normal vaginal delivery. Processing techniques include freeze-dried and air-dried amniotic membranes. Sterilisation of grafts is performed using gamma irradiation. Quality control procedures and bioburden estimation method used are also discussed.

Amnion comes close to being the ideal biological membrane or dressing. It is readily available, inexpensive to use, easily processed and stored. It has low antigenicity, with anti-microbial potential and with the ability to enhance epithelialisation with marked relief of pain. Amnion is suita-

ble for temporary or long term coverage of wounds for various clinical conditions.

Its applications as dressings for wounds include burn wounds, Steven Johnson, flap surgery, traumatic soft tissue injury, leprous ulcers, diabetic ulcers, post-surgery wounds and radiation wounds in cancer patients.

Its ophthalmic applications include treatment of corneal ulcers and conjunctival tumour. Multi-layer amniotic membrane transplantation is also described.

Oral cavity applications include gingiva depigmentation and periodontal regeneration.

Amnion also play an important role as a biological scaffold for cell culture in tissue engineering. Its matrix component has been proven to be biocompatible in vitro, with no adverse effects on cellular proliferation or morphology. The amnion membrane has immense potential as a delivery system for epithelial cells.

Included in this book are the history and development of tissue banking in the Asia Pacific Region, the implementation of the IAEA Programme for the radiation sterilisation of tissue grafts and the Diploma Course Training for tissue bank operators by the IAEA / NUS Asia Pacific Regional training Centre for Tissue Bank Operators in Singapore.

It also documents Lifetime Achievement Awards presented by the Training Centre to give recognition to individuals for outstanding contributions to the Centre and to their own countries.

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- Head BATAN Research Tissue Bank (1990–2005)
- Consultant BATAN Research Tissue Bank (2006–current)
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- External Examiner to IAEA/NUS Examination for Diploma Course in Tissue Banking (2004–2017)
- Lecturer to IAEA/NUS Practical Tissue Bank Workshop (2004–2017)
- 20th Anniversary Lifetime Achievement Award presented by IAEA/NUS Regional Training Centre, Singapore (Sept 2017)

Edited

- ***Radiation in Tissue Banking***. World Scientific Publishing Co Inc; 2007
- ***Allograft Procurement, Processing and Transplantation***. World Scientific Publishing Co Inc; 2010
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- Head MINT (Malaysian Institute of Nuclear Technology) Research Tissue Bank, Bangi, Malaysia (1990–2008)
- Conducted IAEA Expert Missions to Sri Lanka (1996), Suzhou, China (1996), Myanmar (1998), Singapore (1998), Bangkok, Thailand (1999), Sao Paolo, IPEN, Brazil (2000), Cuba (2001), Buenos Aires, Argentina (2002) and Vienna, Austria (2009)
- External Examiner to IAEA/NUS Examination for Diploma Course in Tissue Banking (2004–2017)
- Lecturer to IAEA/NUS Practical Tissue Bank Workshop (2004–2017)
- 20th Anniversary Lifetime Achievement Award presented by IAEA/NUS Regional Training Centre, Singapore (Sept 2017)

Edited

- ***Radiation in Tissue Banking***. World Scientific Publishing Co Inc; 2007
- ***Allograft Procurement, Processing and Transplantation***. World Scientific Publishing Co Inc; 2010
- ***Human Amniotic Membrane — Basic Science & Clinical Applications***. World Scientific; 2017

Awards

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  "Cost Effective Processing of Amnion for Wound Dressing"
- Gold Medal & Woman Inventor of the Year, Malaysian Technology Expo 2010
  "Development of Amnion and Chitosan Paste as Flexible Wound Dressings."

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- Director, IAEA/NUS Regional Training Centre for Tissue Bank Operators in Asia Pacific Region (1997–2017)
- Director IAEA/NUS Diploma Course in Tissue Banking (1997–2017)
- Conducted IAEA Expert Missions to Malaysia (1994, 1996), Vietnam (1996), Zambia (1997), Myanmar (1997, 1998), Argentina (1997), Brazil (1997), Cuba (1997), Sri Lanka (1998), Sao Paolo, IPEN, Brazil (2000), Korea (2004, 2005) and Vienna, Austria (2009)
- Pioneer APASTB, Asia Pacific Association of Surgical Tissue Banking
- 20th Anniversary Lifetime Achievement Award presented by IAEA/NUS Regional Training Centre, Singapore (Sept 2017)

Edited

- ***The Scientific Basis of Tissue Transplantation***; World Scientific Publishing Co Inc; 2001
- ***Bone Grafts and Bone Substitutes***; World Scientific; 2005
- ***Radiation in Tissue Banking***; World Scientific Publishing Co Inc; 2007
- ***Allograft Procurement, Processing and Transplantation***; World Scientific Publishing Co Inc; 2010
- ***Human Amniotic Membrane — Basic Science & Clinical Applications***; World Scientific; 2017

Award

Berita Harian Achiever of the Year Award 2000
For developing the Spastic Children Association of Singapore, and Tissue Banking in Asia Pacific Region.

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Shaila Kothiwale

1

IAEA Programmes in Tissue Banking in Asia-Pacific Region

Nazly Hilmy[*,‡] *and Norimah Yusof*[†]

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Introduction

The ionising radiation technique had been used in several countries in the Asia-Pacific Region during the period of 1960s and 1970s mainly for the sterilisation of health care products and medical devices (IAEA 1998, 2007). Following this, International Atomic Energy Agency (IAEA) set up programmes on radiation and tissue banking in the region as previously described by Prof. Glynn Phillips and Dr Jorge Morales Pedraza (Phillips and Morales, 2003; Morales, 2006).

In the three decades of 1980s, 1990s and 2000s, the IAEA vigorously promoted the use of the ionising radiation technique for tissue sterilisation in more than 36 national, regional and inter-regional technical cooperation projects. This involved more than 70 tissue banks in 31 countries in 4 regions — the Asia Pacific Region, Latin America, Africa and Eastern Europe regions, involving more than 70 tissue banks. More than US$ 7 million were allocated to support the implementation of these projects.

IAEA Tissue Banking Projects

The first IAEA tissue banking programme in Asia-Pacific Region entitled *"Radiation Sterilisation of Tissue Grafts"* was implemented from 1985 to 2004 as national and regional projects. This was followed by regional and inter-regional projects.

The regional and inter-regional projects on tissue banking were as follows:

RAS/7/003: Radiation Sterilization of Tissue grafts (RCA),
RAS/7/008: Quality Assurance in Radiation Sterilization of Tissue Grafts (RCA)
INT/6/049: Interregional Centre of Excellence in Tissue Banking (inter-regional)
INT/6/052: Improving the Quality of Production and Uses of Radiation Sterilized Tissue Grafts (interregional).

The two projects under the Regional Cooperative Agreement (RCA) were successfully implemented for Asia-Pacific Regions:

- RAS/7/003 *"*Radiation Sterilisation of Tissue Grafts*"* (RCA) during the period of 1988–1998
- Followed by RAS/7/008 *"*Quality Assurance in Radiation Sterilisation of Tissue Graft*"* (RCA) during the period of 1997–2003.

The projects addressed main aspects of tissue banking including Public Awareness as well as Donor Promotion. For these purposes, two IAEA/RCA meetings and workshops were held:

- "Self –Assesment Review and Public Awareness in Tissue Banking", Kuala Lumpur in 1998
- "Present and Future Programmes of Public Awareness and Donor Promotion", Bangkok in 2001.

The projects were continued with another project on "Implementation and Improving Quality System in Tissue Banking" with the purpose of improving further and sustaining the tissue banking activities in the region.

National Coordinator Meetings attended by Member States representatives were held annually, and some of the meetings were jointly organised to coincide with Bi-annual Scientific Meetings/International Conference of Asia Pacific Association of Surgical Tissue Banks (APASTB).

In 1997, IAEA started to introduce tissue banking project at interregional level. The two interregional projects implemented were:

- INT/6/049 "Interregional Centre of Excellence in Tissue Banking" during the period of 1997–2003,
- INT/6/052 "Improving the Quality of Production and Uses of Radiation Sterilised Tissue Grafts" from 2002 to 2004.

The significant outcome of the projects was the formulation of an IAEA Code of Practice for Radiation Sterilization of Tissues Allografts. This offers guidelines and protocols for conducting radiation dose settings and dose validation experiments for tissue grafts. This protocol is suitable for far less number of tissue units per production/processing batch compared to manufactured medical devices.

The implementation of regional project of RAS/7/008 "Quality Assurance in Radiation Sterilization of Tissue Grafts" as the continuation of an earlier regional project of RAS/7/003 "Radiation Sterilization of Tissue Grafts" was in parallel with INT/6/052 "Improving the Quality of Production and Uses of Radiation Sterilised Tissue Grafts" at interregional level. Both projects were extended and eventually wrapped up in 2006 with great success.

Prior to the IAEA regional project, the first tissue banking project in Asia-Pacific Region supported by IAEA was BUR/7/004 a national project in Burma (now Myanmar) that was started in 1981. Under the project, the late Dr. U. Pe Khin, a Consultant Orthopaedic Surgeon founded the Burma Tissue Bank in Rangoon Orthopaedic Hospital in March 1983. This was the first tissue bank established in the region.

Among other pioneers in the region were Dr. Yongyudh Vajaradul who set up Bangkok Biomaterial Centre at the Siriraj Hospital, Mahidol University, Thailand in 1984. Another pioneer was the late Dr. Hudson Silva who previously set up the world famous Sri Lanka Eye Donation Society and

developed the Model Human Tissue Bank in 1993. This was achieved through the technical assistance of the IAEA. Undeniably, tissue banking in Thailand thereafter played a major role in stimulating the programme in Asia and the Pacific Regions (IAEA, 2007).

A number of IAEA technical staffs were involved in providing great technical supports to the projects. They were Dr. Ramen Mukherjee, Dr. John Easey. Dr. Vladimir Markovic, Dr. C.R. Aleta, Dr. J. Tatsuzaki, Dr. Jorge Morales and Prof Glynn O. Phillips, the IAEA Consultant for these Projects.

Chronology of IAEA Programmes on Radiation Sterilization of Biological Tissues in Asia Pacific Regions:

I. Early Period

In 1971, the IAEA Expert Group Meeting in Budapest resolved that there were advantages to be gained by using ionizing radiation to sterilize human and animal tissues.

In 1974, IAEA made the first declaration at "IAEA Symposium on Sterilization of Medical Products and Biological Tissues" in Bombay.

Experience in Tissue Banking and Surgical Transplantation throughout the world has shown that ionizing radiation, when applied correctly, can be a safe and reliable method for sterilizing human tissues for use in transplantation. Yet there remains uncertainty in the minds of many surgeons, mainly due to lack of readily available expert information on:

- Effect of radiation on tissues
- Changes in the physical, mechanical and biological properties of tissues irradiated

In some countries, experience was built up over the years. Since 1974 bone, fascia lata, duramater, peripheral nerves, skin, embryonic tissue, cartilage and tendons were already routinely obtained from suitable donors. These were sterilized by radiation and used safely for transplantation.

In 1976, IAEA Advisory Group Meeting in Athens (27 September to 10 October 1976), drew together clinicians, radiation chemists and biologists to review the available information about antigenic properties of irradiated biological tissues and their consequent utility in transplant surgeries.

The presentations were mostly on the effects of sterilizing radiation doses upon antigenic properties of protein and biological tissues. The Proceeding of the Advisory Group Meeting entitled "Radiation Sterilization: Irradiated Tissues and their Potential Clinical Use" was edited by Prof. G.O. Phllips, Prof. A. Tallentire and Prof. N. Triantafyllou (Phillips *et al.*, 1976). Among the pioneers attended the meeting were Prof. G.E. Friedlander, Dr. K.W. Sell, Dr. Michael Strong, Dr. K. Ostrowski, Dr. Sandor Pellet, Prof. Anna Goclawska, Prof. Pe Khin and Prof. N Triantafyllou (Phillips *et al.*, 1976). Thereafter, the programme gained momentum.

In 1978 and 1981, meetings under the auspices of the IAEA were held in the Republic of Korea and in Czechoslovakia respectively. It was concluded that the experience of the Eastern European countries was particularly helpful over this early period. In the 1980s, the IAEA started to consider the possibility to use the ionising radiation technique for the sterilisation of human and animal tissues. This was the terminal processing step undertaken following strict guidelines for donor selection and graft processing. All procedures were conducted within an overall quality system for which strict operational standards were specified (Easey, 1992).

In 1983, a conference on the subject of Tissue Banking held in Bombay. Sri Lanka was identified as a suitable country to develop a tissue bank. This is the country where the late Dr. Hudson Silva had already established Sri Lanka Eye Donation Society.

In the same year (1983), the first tissue bank in Asia was established in Burma by Dr. Pe Khin under the IAEA technical assistance (BUR/7/004), followed by Bangkok Biomaterial Center (BBC), Thailand which was established in December 1984 by Dr. Yongyudh Vajaradul.

II. Later Period

In 1984, IAEA Seminar on "Tissue Banking of Radiation Sterilized Grafts for Clinical Use for Asia and the Pacific" was held in Manila from 7 to 11 May, 1984. 10 countries were represented, namely: Bangladesh, India, Indonesia, Malaysia, Pakistan, the Phillippines, Sri Lanka, Thailand, United Kingdom and United States. As the outcome of the seminar, IAEA launched the regional project "Radiation Sterilization of Tissues Grafts" (RAS 7/003) from 1985 to 2004.

Starting 1985, "Tissue Banking" appeared as one of the IAEA official programme. Similarly "Tissue Banking" was used for the name of our association, APASTB, which is the acronym for Asia-Pacific Association of Surgical Tissue Banking. IAEA started the project RAS 7/003 promoting proper use of radiation sterilization on tissue grafts in initially nine member states. The project was implemented by the following individual countries independently yet with the convergent common goal to introduce tissue grafts sterilization by using ionizing radiation for health care use. Those projects were:

1. Bangladesh (BGD/7/005)
2. China (CPR/7/002)
3. Indonesia (INS/7/002)
4. Rep of Korea (ROK/7/RP 93–94)
5. Malaysia (MAL/7/002)
6. Pakistan (PAK/7/002)
7. Philippines (PHI/7/004)
8. Sri Lanka (SRL/6/017)
9. Thailand (THA/8/009)

In 1986, a National Coordinators Meeting of RAS 7/003 was held in Colombo, Sri Lanka.

In 1988, IAEA organised a Training Course on "Radiation Sterilization of Tissue Grafts" which was held in Taiyuan, Shanxi Province, China. Dr. Sun Shiquan was the course coordinator and organiser of this meeting.

In 1989, the first Workshop on "Radiation Sterilization of Tissue Grafts" was held in Bangkok under the auspices of the IAEA Regional Coordination Agreement (RCA). The first Meeting of the Asia-Pacific Association of Surgical Tissue Bangking (APASTB) was organised to coincide with the workshop. Dr. Yongyudh Vajaradul was the Chairman as well as the first President of APASTB.

In 1992, Project Formulation Meeting was held in Manila, 3–7 August 1992, compiling achievements of the project from 1985 to 1992, under the the coordination of Dr. John Easy from IAEA. This meeting was organised to coincide with the 4th APASTB Meeting. Dr. Norberto Agcaoili was the organiser of their meetup. Fourteen countries in the Asia–Pacific region that participated in this meeting were Bangladesh, China, India, Indonesia, Japan,

Korea, Malaysia, Myanmar, Pakistan, Phillippines, Singapore, Sri Lanka, Thailand and Vietnam where Myanmar, Singapore, Japan and Korea joined as new members.

Some remarkable outputs from the IAEA regional projects were:

- Radiation Sterilization of Tissue Allografts was successfully introduced and well adopted while the clinical applications of radiation sterilised tissue allografts were conducted in each of the member countries.
- Good Manufacturing Practice (GMP) in Tissue Banking was adopted and implemented.
- Quality Systems for Design/Development, Production, Installation and Servicing were implemented based on AS 3901:1987 or ISO 9001:1987.
- Reports from each country were presented and compiled (Easey, 1992).

Regional (RCA) Workshop on "Total Quality System for Tissue Banking of Radiation Sterilized Surgical Grafts" was held in Jakarta, Indonesia from 2 to 13 August 1993 (Figure 1). In the same year, Regional (RCA) Workshop on "Radiation Sterilization of Tissue Graft — An Open Learning Programme" was held in Ho Chi Minh City, Vietnam, from 15 to 26 November.



Figure 1. Tree planting by Dr R. Mukherjee and Prof G.O. Phillips from IAEA during the Regional (RCA) Workshop on Total Quality System for Tissue Banking of Radiation Sterilized Surgical Grafts in Jakarta, Indonesia, 2–13 August 1993

In 1994, Regional (RCA) Workshop on "Open Learning Technique Applied to Radiation Sterilization of Tissue Grafts" was held in Suzhou, China. The first curriculum on tissue banking was assembled in Suzhou, China with Prof. G.O. Phillips as the Editor. To deliver the training, the IAEA/RCA Regional Training Centre was established in National University Hospital (NUH), Singapore in the NUH Tissue Bank. The first IAEA/NUS Distance Learning Diploma Course in Tissue Bank for one-year duration was launched in November 1997.

In 1996, IAEA Regional (RCA) Workshop on "Total Quality System for Tissue Banking of Radiation Sterilized Surgical Grafts" was held in Gold Coast, Australia. It coincided with the First World Congress on Tissue Banking and the Sixth Scientific Meeting of APASTB. Dr. David Morgan was the Chairman and organiser of this meeting (Figure 2).

In 1997, Regional RCA Consultants Workshop on "Total Quality System in Producing Radiation Sterilised Tissue Grafts in Asia and Pacific Region" was held in Jakarta from 1 to 5 September. Dr. Nazly Hilmy was the Chairman and organiser of this workshop.

In 1998, Regional (RCA) Training Course for "Radiation Sterilization of Tissue Grafts" was held in Manila from 17 to 21 August. Dr. Norberto Agcaoili was the Chairman and organiser of this course.



Figure 2. IAEA/RCA Workshop in Gold Coast, Australia in 1996

In 1998, IAEA/RCA Workshop on "Self-Assesment Review and Public Awareness in Tissue Banking" was held in Kuala Lumpur from 23 to 27 November 1998 to coincide with the 7th APASTB Scientific Meeting (Figure 3 to 6). Dr. Hasim Mohamad was the Chairman and organiser of the workshop.



Figure 3. IAEA Meeting and 7th APASTB Conference, Kuala Lumpur, Malaysia, 1998



Figure 4. APASTB Board Members attending the 7th APASTB Conference and IAEA Workshop, Kuala Lumpur, Malaysia in 1998



Figure 5. Visiting Commercial Gamma Irradiator (Co-60) in Bangi, Selangor organised by Dr. Norimah Yusof during the 7th APASTB Conference and IAEA Workshop in Kuala Lumpur, 1998



Figure 6. IAEA experts Dr. M. Strong and Dr. Von Versen discussing with Dr. Chang Joon Yim from Korea during the visit to the Commercial Gamma Irradiator (Cobalt-60) in Bangi, Selangor during the 7th APASTB Conference and IAEA Workshop in Kuala Lumpur, Malaysia in 1998

In 1999, IAEA/RCA Regional Training Course on "Delivery of Curriculum to Tissue Bank Operator — First Phase" was held in Singapore from 19 to 30 April 1999. Dr. Aziz Nather was the Chairman and organiser of the course.

In 2000, IAEA/RCA Workshop on "Implementation of Quality System in Tissue Banking" was held in Bali, Indonesia from 22 to 24 October. Dr. Nazly Hilmy was the Chairperson and organiser of the Workshop (Figure 7 and 8). This workshop was coincided with the 8th APASTB International Conference on Tissue Banking where. Dr Abdurahman was the Chairman and organiser of the conference (Figure 9).

In 2001, IAEA/RCA Workshop on "Present and Future Programmes of Public Awareness and Donor Promotion" was held in Bangkok, Thailand from 5 to 9 November. Dr. Yongyudh Vajaradul was the Chairman and organiser of the workshop.

In 2002, Project Coordinators' Meeting for the IAEA/RCA Project RAS/7/008 was organised in Seoul, Korea to discuss "Improving Quality



Figure 7. Dr. Aleta from IAEA delivering the Opening Remarks (left photo) at IAEA/RCA Workshop on Implementation of Quality System in Tissue Banking, Bali, Indonesia. Delegates in the workshop (right photo)



(a) (b)

Figure 8. Dr R. Mukherjee and Dr R. Von Versen from IAEA attending the Workshop (a) with participants from the member countries (b) at the IAEA/RCA Workshop on Implementation of Quality System in Tissue Banking, Bali, Indonesia in 2000



(a) (b)

Figure 9. (a) Prof G.O. Phillips from IAEA (b) Prof Phillips with Conference VIPs and board members of APASTB

Assurance in Radiation Sterilization of Tissue Grafts". The meeting was chaired by Dr Jorge Morales from IAEA. This meeting coincided with the 9th APASTB International Conference on Tissue Banking. Dr. Chang Joon Yim was the Chairman and organiser of the conference.

In 2002, Third World Congress on Tissue Banking was held in Boston from 23 to 27 August. It was supported by American Association of Tissue Bank (AATB), Asia-Pacific Association of Surgical Tissue Banking (APASTB), European Association of Tissue Bank (EATB) and IAEA. Dr. Samuel Doppelt, President of AATB was the Chairman of the Congress. Prof. Phillips and Dr. Morales coordinated the IAEA meeting involving Asia-Pacific Region and Latin America.

In 2003, an International Conference on "Sterilization of Tissues Using Ionizing Radiation in conjunction with the 13th International Cellucon Conference was held in Wrexam, Wales. It was jointly organised by IAEA. Prof G.O. Phillips was the Organiser. During the conference, IAEA International Standards for Tissue Bank prepared by the Member States was launched by Dr B. Loty of IAEA (Loty, 2005).

Up to 2003, all participants who were National Coordinators from each Member State joined almost all APASTB Meeting as the meetings were jointly supported by IAEA and the local governments. Their continuous commitment and dedication were continued until the end of RAS/7/003 and RAS /7/008 programmes. Beginning 2004, when IAEA no longer supported the projects, all the participants who joined almost all the previous APASTB Meetings realized that they needed to support themselves to

attend the meetings. Due to shortage of funds and lack of support from the local govermnments, many National Coordinators could not join APASTB Meetings. With the closing of IAEA/RCA Regional Programme in 2004 and IAEA Interregional Programme in 2006, tissue banking activites in Asia-Pacific Region were severely affected. The activities decreased tremendously in several countries. Countries like Korea, Indonesia, India, Malaysia, Taiwan, HongKong, China, Thailand and Singapore remained active. Some countries progressed and advanced their tissue bank activities. Japan and Australia maintained their progress although they were previously not financially supported by IAEA.

During the last three decades, the IAEA undoubtedly managed to demonstrate that the sterilisation of tissues using the ionising radiation technique offered a clear advantage in terms of safety compared with other sterilisation techniques now in use in different countries (IAEA, 2007; Yusof and Hilmy, 2007). Fortunately, most tissues, including bone, skin and amnion, can be treated with ionising radiation to kill microorganisms, without affecting their functionality. The use of the ionising radiation technique, in the case of skin and amnion, requires the presence of a cryoprotectant for preserving cell morphology. At the same time, it is important to stress that the use of this sterilisation technique significantly reduces the risk of transferring communicable diseases to the recipient.

Other methods of tissue sterilisation namely heat and chemicals, which have also been used in some countries for a long period of time, have been demonstrated to cause damage to biological and physical properties of tissues.

Beneficial Results of IAEA Tissue Banking programmes to Asia-Pacific regions implemented during 1984 to 2004

The IAEA programme on tissue banking has benefitted the Asia-Pacific region generally in the medical field and specifically patient health care. Some of the benefits are as follow:

1. Each member country has set-up a National Tissue Bank with initial technical support from IAEA.
2. Each tissue bank has adopted GMP and Quality System in ensuring of high quality products. Quality documents such as Quality Assurance

Manual, Procedural Manual, Standard Operating Procedures and Work sheets have been established in accordance to international standards.

3. Several IAEA publications prepared by representatives of Member States working together with experts have been published as a guide to tissue bankers, regulators, decision makers and technologists in sustaining the implemented projects. The five publications are:
   - IAEA International Standards for Tissue Banks 2002,
   - IAEA Code of Practice for the Radiation Sterilisation of Tissues with latest edition in 2007,
   - Handbook for Public and Professional Awareness 2000,
   - IAEA Modules for Distance Learning Programme on Tissue Banking,
   - Advances in Tissue Banking Volume 1 to 7.
4. Trained human resource in Tissue Banking include both professionals/ surgeons (graft user) and tissue bank operators (graft producer). Most of them were trained in more advanced tissue banks in USA and Europe for training and to develop collaboration. In later period, tissue banks in Asia-Pacific region, like Singapore, Malaysia, Indonesia, Thailand and Australia were able to offer training place as well. IAEA/NUH Distance Learning Diploma Course on Tissue Banking had been held every year in Singapore, starting from 1998 until 2004. National University of Singapore has continued to conduct the Diploma Course after 2004 ever since IAEA closed its tissue banking programme. NUH Bone Bank has been the referral centre for tissue bank course in Latin America and Korea for regional and national level respectively.
5. Several APASTB staff members became the IAEA field experts to several countries in Asia-Pacific Region and Latin America, among others were Prof. Yongyudh Vajaradul, Prof. Aziz Nather, Dr. Norimah Yusof and Dr. Nazly Hilmy.

Highlights of IAEA Publications in Tissue Banking

IAEA publications has been used as references and guidelines in the field of tissue banking.

(a) IAEA International Standards for Tissue Bank:
There was a great deal of variations among tissue banks standards. IAEA Expert and regional groups took initiative to review the present standards

and decided to adopt USA and European standards and practices during the implementation of IAEA projects. IAEA International Standard that was eventually established could be easily adopted by the regional tissue banks without incompromising any importance aspects of tissue banking.

(b) IAEA Code of Practice for the Radiation Sterilisation of Biological Tissues (prepared under IAEA INT/6/052, 2004)
No international body could have undertaken this important function, which is definitely within the IAEA technical competence. This Code of Practice first released in 2002 was then introduced into the regions for practical evaluation before reviewed and published in 2007 (Morales *et al.*, 2011). It has been very useful to address problems faced by tissue bankers in validating radiation doses with small numbers of samples.

(c) The IAEA Handbook for Public and Professional Awareness
Public and professional awareness is the major challenge in tissue banking and is crucial to the further extension of the use of radiation sterilised grafts in individual countries. Education is needed at all levels. This handbook provides documentations to assist workshops and national awareness programmes and it has been adapted by the regions in several languages.

(d) Internet training
Internet training was introduced in IAEA training courses all over the world. This is a logical extension and formed the basis for developing the IAEA Distance Learning Programmes in tissue banking which is still being used. The rich sources of the multi-media curriculum and its recent update needed to be exploited in this way to give full benefits to the region.

(e) Technical books
Chapters in Advances in Tissue Banking (7 Volumes) were mostly prepared and written by experts and consultants under the IAEA programme. The books are excellent references and can be used to guide newcomers and member countries in Tissue Banking.

IAEA New Projects Related to the Application and the Improvement of Products of Tissue Banking

Having completed regional and interregional projects in tissue banking, IAEA created two Coordinated Research Project (CRP) and invited specialists

and researchers from Asia-Pacific, Latin America and Europe to conduct studies on identified issues (Wondergem, 2010):

1. "Improving Outcomes in Radiotherapy using Novel Biotechnologies: Modification of Tissue Reactions and the Use of Stem Cell Therapeutics" (*E3.30.32*)
   Asia Pacific countries joint the project were China, India, Indonesia, Malaysia and Korea.
2. "Safety and Optimisation Radiation Sterilization in Tissue Banking: Studies on Functional Properties of Irradiated Tissue Grafts" (2010–2014) (*E3.10.06*)
   Asia Pacific Countries joint the project were Australia, Indonesia, India, Malaysia and Singapore.

Conclusion

The IAEA Programmes on Radiation and Tissue Banking for the Asia-Pacific Region is beneficial and have supported the Health Care Programme in each of the member countries.

References

Easey J (1992) IAEA Report. Presented at the *Formulation Meeting: Regional RCA Project on Radiation Sterilization of Tissue Grafts*, Manila, Phillipines.

IAEA (2007) International Atomic Energy Agency: Thematic Planning Reports on Tissue Banking. IAEA, Vienna, Austria.

Loty B (2005) IAEA International Standards for Tissue Banks. In: Kennedy JF, Phillips GO and William PA (eds.), *Sterilisation of Tissues Using Ionising Radiation*, CRC Woodhead Publishing, England, pp. 3–38.

Morales PJ (2006) The IAEA Program on Tissue Banking. A Successful Program for Developing Countries. *Cell Tissue Bank*, **7**: 237–258.

Morales PJ, Yusof N and Hilmy N (2011) A Review of the International Atomic Energy Agency (IAEA) Code of Practice for the Radiation Sterilisation of Tissue Allografts. *J. Cell Sci. Ther.*, **2**: 105.

Phillips GO and Morales PJ (2003) The International Atomic Energy Agency (IAEA) Program in Radiation and Tissue Banking: Past, Present and Future. *Cell Tissue Bank*, **4**: 69–76.

Phillips GO, Tallentire A and Triantafyllou N (1976) Proceeding on Radiation Sterilization: Irradiated Tissues and Their Potential Clinical Use. IAEA, Vienna, Austria.

Wondergem J (2010) IAEA Programme in Stem Cell and Tissue Banking. Presented at the *13th APASTB International Conference*, Bukittinggi, Indonesia.

Yusof N and Hilmy N (2007) Need for Radiation Sterilisation of Tissue Grafts. In: Nather A, Yusof N and Hilmy N (eds.), *Radiation in Tissue Banking*, World Scientific, Singapore, pp. 11–21.

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Tissue Banking in Asia Pacific Region: Past, Present, Future

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Introduction

The Asia Pacific Region is grateful to Professor Glyn O Phillips (Fig. 1) for his vision and tireless efforts for developing tissue banking in thirteen countries.

He showed great leadership in promoting the use of gamma irradiation for the production of safe and high quality tissue grafts. Professor Phillips pioneered the development of a Curriculum for training Tissue Bank Operators — a first in the world. This is crucial to improving the quality of tissue grafts produced by tissue banks in our region.

Singapore is particularly grateful to Professor Phillips for choosing the NUH Tissue Bank to be the Regional Training Centre to run this education



Figure 1. Professor Phillips (left) Mrs. Rhiain Phillips (centre) and Mr Ramen Mukherjee during Conferment Ceremony of Honorary Membership of APASTB in Bali, Indonesia in 2000

programme. Singapore ran an IAEA/NUS Distance Learning Diploma Course on Tissue Banking from 1997 to current day. 17 annual diploma courses have been completed with a total of 221 tissue bank operators graduating from this centre — 194 from Asia Pacific Region itself, 6 from Latin America, 10 from Africa, 9 from Europe and 2 from Australia.

Historical Perspectives

The history of tissue banking in the Asia Pacific Region is shaped by 2 driving forces:

1) International Atomic Energy Agency (IAEA) via Regional Co-operative Agreement (RCA) Projects
2) Asia Pacific Association of Surgical Tissue Banking (APASTB)

It is best written in 3 separate sections:

I. History of Tissue Banking in Member Countries
II. History of APASTB
III. History of IAEA Programme in Asia Pacific Region

I. History of Tissue Banking in Member Countries

Myanmar

Tissue banking began in the Asia-Pacific region in Burma in 1981, when Dr. U. Pe Khin, a Consultant Orthopaedic Surgeon, founded the Burma Tissue Bank in Rangoon, Burma. Dr. U. Pe Khin had been the Medical Superintendent at the Rangoon Orthopaedic Hospital when he became the Chairman of the Burma Tissue Bank. It was built with IAEA Technical Assistance BUR/7/004.

After his sudden death in 1987, Dr. Myo Mint succeeded him in 1992 and reactivated the tissue bank project. However, his efforts were short-lived as he was transferred to Mandalay in 1995. Dr. Myo Mint was succeeded by Dr. Khin Maung Han.

There is no law for tissue donation in Myanmar, apart from the Eye Donation Law. Approximately 85% of the population are Buddhists, who favour tissue donation. Myanmar has a gamma irradiation facility, a gamma chamber belonging to the Department of Agriculture, available for use.

Thailand

Following the major setback suffered by Burma with the demise of its pioneer Dr. U. Pe Khin, Thailand became the forerunner of the region. In December 1984, Dr. Yongyudh Vajaradul, a Consultant Orthopaedic Surgeon, set up the Bangkok Biomaterial Centre at the Siriraj Hospital, Mahidol University, Thailand.

Like Myanmar, there is no tissue donation law in Thailand. Thailand is a predominantly Buddhist country favouring tissue donation. The radiation sterilisation of tissue grafts started in Thailand in 1986, when the bank acquired its own gamma chamber.

China

The China Institute for Radiation Protection (CIRP) Tissue Bank was set up in 1988 in Taiyuan, Shanxi Province, by Dr. Sun Shiquan and Dr. Li Youchen. This was quickly expanded to become the first provincial tissue bank in China, the Shanxi Provincial Tissue Bank, in July 1993. In 1994, it secured approval from the government to distribute its tissue grafts to Beijing and to

other cities in China. Although China does not have a human transplantation act, tissue banks in China follow the principle of obtaining written consent from donors before the procurement of tissues.

All tissue grafts are gamma-irradiated at the CIRP. The CIRP has been conducting national training courses for tissue bank operators in China for several years. In 2000, the Shanxi Provincial Tissue Bank was privatised by OsteoRad Biomaterial Co. Ltd. and fitted with modern up-to-date facilities with Dr. Li Bao Xing as its Executive Director.

India

The late Dr. N. M. Kavarana founded The Tata Memorial Hospital Tissue Bank in 1988 at the Tata Memorial Hospital, Mumbai, in collaboration with the IAEA. Tissue banking follows the Bombay Anatomy Act 1949, which covers the use of unclaimed or donated bodies for therapeutic purposes, medical education, or research; and the more recent Transplantation of Human Organs Act 1994, in which consent is required from the donor or next of kin. The irradiation of tissue grafts is performed in a Gamma Chamber 900 donated by the Atomic Energy Commission, Government of India. The current director of this tissue bank is Dr. Astrid Lobo Gajiwala.

In 2006, there was much renewed interest in the setting up tissue banks in many parts of India, such as Chennai (Dr. Mayil Natarajan), Coimbatore (Dr. S Rajasekaran), New Delhi (Dr. Surya Bahn), Kerala, Mumbai and Kolkata.

Singapore

The National University of Singapore (NUS) Bone Bank was set up in October 1988 as a research tissue bank for the Department of Orthopaedic Surgery by Dr. Nather using an NUS Research Grant, RP 880334 "Bridging of Large Bone Defects by Allografts" (Nather and Wang 2002). The bank was pushed into clinical activity fairly quickly with the rapid rise in clinical demand for tissue allografts.

In 1994, the bank was awarded a S$239 965 grant by the Ministry of Education (Totalisator Board) to upgrade its clinical facilities and functions, and to start the production of lyophilised gamma-irradiated morsellised bone

allografts. It acquired two sets of lyophiliser units (each lyophiliser unit included a band saw, a shaker bath, a lyophiliser, and a laminar flow cabinet) and two new freezers (in addition to its original two). It was inaugurated as the National University Hospital Tissue Bank in 1955 and became a hospital cost centre in 1998. That same year, the NUS Bone Bank became the national bone bank supplying bone and soft tissue allografts to all nine hospitals in the country.

Tissue donation follows the Medical (Therapy, Education and Research) Act of 1972, whereby any person of sound mind and 18 years of age or above may give all or any part of his or her body for education or transplantation. It is an "opting-in" law, requiring written consent from the donor or next of kin.

In September 1992, Singapore began irradiating deep-frozen long bones by sending the bones in Polylite containers packed with dry ice by air to the Malaysian Nuclear Agency (MINT) in Bangi, Selangor. They are irradiated in a cobalt-60 plant (Sinagama) by Dr. Norimah Yusof.

The National University Hospital (NUH) Tissue Bank was officially inaugurated in September 1995, and became a hospital cost facility in 1998. The bank has a wet processing laboratory, a dry processing laboratory, a documentation room, and a reception area with a total floor space of about 2000 square feet (Nather 2004).

The bank also became the Regional Training Centre for the Asia-Pacific region in 1997. The first graduates were convocated in 1998.

Indonesia

Indonesia's first surgical tissue bank, the BATAN Research Tissue Bank, at the Centre for Application of Isotopes and Radiation (CAIR), National Atomic Energy Agency (BATAN), was established by Dr. Nazly Hilmy in Jakarta in 1990. Initially, due to the shortage of deceased donors, the bank processed both human and bovine bone grafts for clinical application in addition to processing human amnion. The Indonesia 1992 Health Regulation is an "opting-in" law that allows retrieval of tissues from living donors only.

In 1986, Dr. Nazly Hilmy managed to enact a "Fatwa for Bone, Skin, and Amnion", thus permitting procurement from deceased donors as well.

However, this religious breakthrough did not cause any significant change in the attitude of donors due to cultural factors. Hence, the shortage of deceased donors in Indonesia persists.

A second bone bank, the Dr. M. Djamil Hospital Tissue Bank, has been set up in Padang, Sumatra, by Dr. Menkher Manjas, a Consultant Orthopaedic Surgeon. In addition, the late Dr. Abdurrahman, a Consultant Orthopaedic Surgeon and Musculoskeletal Oncologist, inaugurated the Dr. Soetomo Hospital Tissue Bank in the Department of Orthopaedic Surgery, Air Langga University, Surabaya, during the 8th Scientific Meeting of the APASTB in Bali in 2000.

Dr. Abdurrahman also set up the Indonesian Association of Tissue Banks and became its first president in 2001. He passed away on 18 February 2005 — a great loss to Indonesia and to the Asia Pacific Region. He was succeeded by Dr. Ferdianshah, another Consultant Orthopaedic Surgeon and Musculoskeletal Oncologist.

Hong Kong

Two tissue banks have been set up at the two medical universities in Hong Kong. In 1990, Dr. David Fang formalised a regional musculoskeletal tissue bank at the Queen Mary Hospital, University of Hong Kong. Dr. T. L. Poon took over as Director in 1994. In 1992, Dr. Shekhar Kumta formalised another regional musculoskeletal tissue bank at the Sir Y. K. Pao Centre for Cancer, Prince of Wales Hospital, Chinese University of Hong Kong.

Both banks are supported by the Hong Kong Government. Tissue banking in Hong Kong follows the Human Organ Transplantation Ordinance 1997, an "opting-in" law requiring consent from the donor or next of kin.

Korea

The Korea Biomaterial Research Institute was established in 1990 by Dr. Chang Joon Yim, a Dental Surgeon, at the College of Dentistry, Dankook University, in Cheonan, Korea. The tissue grafts are irradiated by the Korea Atomic Energy Research Institute (KAERI) in Taejon, Korea.

Brain death was legally recognised in Korea in February 2000. Since then, there has been tremendous activity in setting up new tissue banks in

Korea, notably at the St. Vincent's Hospital, Catholic Medical University, in Seoul, Korea, by Professor Yong Koo Kang. More than 20 new tissue banks were established.

With the support of IAEA, Dr. Chang translated the IAEA Curriculum into Korean. Dr. Kang became the Director of Korean National Training Centre (2003–2006) to run Korean Diploma Courses in Tissue Banking. 12 KNTC courses have been held till 2015.

Japan

The Kitasato University Hospital Bone Bank was set up in April 1991 by Dr. Moritoshi Itoman. Tissue procurement is illegal in Japan, except for cornea as stated in the Law for Transplantation of Kidneys and Corneas 1979. Radiation sterilisation was introduced by Dr. Itoman in 1994, with the cooperation of the Japan Atomic Energy Research Institute (JAERI).

Few organs have been procured despite the 1997 legislation recognising the concept of brain death. In a landmark event on March 1, 1999, a liver, heart, and both kidneys were procured from a brain-dead donor in Kochi and airflown for transplantation to four recipients, each in a different city (Reuters). While it was hoped that the event would spur tissue banking activity in Japan, this did not happen.

Urabe *et al.* reported that in the period between years 2000 and 2004, a total of 134 782 bone grafts have been performed in 2239 institution. Of this, 4% of the bone grafts utilised were banked bone grafts. Only 9% (≈200) of the 2239 institutions have their own bone banks. More importantly, only 3 regional bone banks procured, processed and preserved banked allografts from cadaveric donors for supply to other institutions.

Progress had been made with the legislation of Advanced Medical Treatment in December 2004, which allowed approved institutions to charge the expenses of authorised treatment directly to the patient. Prior to this legislation, bone banks had to bear all expenses, with one implantation costing 282 588 yen (1760 euro) (Urabe *et al.*). In March 2007, cryopreserved allogenic tissue from cadaveric donors was approved as advanced medical treatment.

However, the number of institutional and regional bone banks in Japan remained low. Urabe *et al.* attributed this to the high expenditure borne by

bone banks, and to the low number of regional banks, which was insufficient in supporting over 200 institutional banks.

Philippines

In Manila, the University of Philippines General Hospital (UPGH) Tissue Bank was set up by the late Dr. Norberto Agcaoili in May 1990 at the Department of Orthopaedic Surgery, University of Philippines College of Medicine. Tissue donation follows the Republic Act 7170, 1991, an "opting-in" law whereby the legacy or donation of all or part of a human body after death for specified purposes must be authorised by the donor or next of kin. While Filipinos are predominantly Catholic, their cultural attitudes do not favour donation. All tissues are gamma-irradiated at the Philippines Nuclear Research Institute (PNRI) in Manila.

Malaysia

In Malaysia, two tissue banks were set up in 1991:

1. The Malaysian National Tissue Bank (MNTB) by Dr. Hasim Mohamad, a General Surgeon, at the University of Science Malaysia, Kota Bahru, Kelantan
2. The Malaysian Institute for Nuclear Technology Research (MINT) Tissue Bank by Dr. Norimah Yusof at the Malaysian Institute for Nuclear Technology, Bangi, Selangor (later renamed the Malaysian Nuclear Agency or MNA in 2006)

As with other countries, tissue banking in Malaysia follows an "opting-in" law, the Laws of Malaysia, Act 130, 1974.

In September 1995, the Malaysia Islamic Centre passed a "Fatwa on Bone, Skin, and Amnion"—a religious ruling allowing Muslims to donate. Despite this, there has been little change in the attitude of Muslims towards tissue donation. Hence, the shortage of donors in Malaysia persists.

Dr. Hasim Mohamad helped inaugurate the Malaysian National Tissue Bank in Kota Bahru, Kelantan on November 5, 1994. A third tissue bank, the General Hospital Kuala Lumpur Bone Bank, was inaugurated by

Dr. Ruzlan in 1998 during the 7th Scientific Meeting of the APASTB held in Kuala Lumpur. Two more new tissue banks are being set up: one at the University of Malaya Medical Centre, Kuala Lumpur; and one at the International Islamic University of Malaysia, Kuantan. All tissue allografts are sent to Dr. Norimah Yusof at the MNA for gamma irradiation.

Under the leadership of Dr. Hasim Mohamad and Dr. Norimah Yusof, the development of tissue banking in Malaysia expanded tremendously. The Malaysian Association for Cell and Tissue Banking was established in 2005 with Dr. Hasim Mohamad as the Inaugural President, Dr. Zulmi Wan as first Vice President, Dr. Norimah Yusof as second Vice President and Dr. Kamarul Zaman as Secretary. Professor Glyn Phillips and Dr. Aziz Nather were appointed as international advisors.

Vietnam

Dr. Tran Bac Hai led the development of the Biomaterial Research Laboratory University Training Centre for Health Care Professionals in Ho Chi Minh City in January 1993. The bank procures and processes amnion and lyophilised morsellised chip grafts. All the grafts are sent to Dalat for irradiation.

During the same period, the Hanoi Tissue Bank — a skin bank — was set up at the Laboratory of Biomaterial Preparation (Vinatom) by Dr. Pham Quang Ngoc. The bank processes skin and amnion grafts. All tissues are sent to the Hanoi Irradiation Centre for irradiation.

Tissue banking in Vietnam follows 2 laws: Article 32 of the Civil Code, Chapter 2, on tissue donation; and The People's Health Protection Code, Chapter 4, on tissue transplantation.

Sri Lanka

The Sri Lanka Model Human Tissue Bank was set up by the late Dr. Hudson Silva and his wife. Dr. Silva is renowned for having procured 43 000 corneas for distribution to several countries in the world on humanitarian grounds. In 1993, an agreement was signed between the IAEA and the Ministry of Health in Sri Lanka, upon which the Government allotted a large piece of land for the development of the tissue bank. The bank was inaugurated on May 8, 1996, by the Prime Minister of Sri Lanka.

This project was conceptualised by as a "Model Project" because there were many donors in this predominantly Buddhist country. Sri Lanka provided the country with plenty of donors. It could supply the much-needed grafts to countries in the whole region where there generally is a great shortage of donors.

Sri Lanka follows an "opting-in" law, the Human Tissue Transplantation Act No. 48 of 1987, requiring consent from the donor or next of kin. The bank has its own gamma irradiation facility — a Gamma Cell 200 — in its own premises.

Currently, more professional education is needed in Sri Lanka to promote the utilisation of tissue grafts in the country.

Australia

Four tissue banks in Australia incorporate radiation processing for the terminal treatment of grafts. Irradiation is performed by the Australian Nuclear Science and Technology Organisation (ANSTO). These 4 banks include:

1. Queensland Bone Bank, led by Dr. David Morgan
2. Donor Tissue Bank of Victoria, led by Dr. Lyn Ireland, succeeded by Dr. Marissa Herson
3. Perth Bone and Tissue Bank, led by Dr. John Pearman
4. South Australia Tissue Bank, led by Dr. Steven Nailer

Tissue banks in Australia have to comply with the Australian Code of Good Manufacturing Practice for Therapeutic Goods — Human Tissues, September 1995. This Code adopts and applies basic quality system principles from the ISO 9000 series of standards to tissue banking.

The strength of tissue banking in Australia lies in the high standards these tissue banks comply with. They have to conform to standards spelt out by the Therapeutic Goods Administration (TGA) Act, in order to obtain yearly licensing. Annual audits of the banks are performed. Two new tissue banks have been added:

1. The New South Wales Bone Bank, a private tissue bank, with two clean rooms has recently been developed in New South Wales at the cost of about A$1 million. The bank is run by Ms Sharon Bryce.

2. The Queensland Bone Bank and Queensland Skin Bank — costing A$12 million — has also been set up in Brisbane by the Government of Queensland, with six clean rooms and state-of-the-art facilities. It is led by Dr. David Morgan.

II. History of APASTB

Pan-Asia Tissue Bank Organisation (PATBO) 1985

In the last meeting of the Advisory Group of IAEA in Colombo, Sri Lanka, 1–5 Sept 1985, it was agreed that an association among ASEAN countries should be established for tissue banking. At the 8th Congress of Western Pacific Orthopaedic Association held in Royal Orchid Sheraton Hotel, Bangkok on 29 Nov 1985, a meeting was held amongst members of the WPOA interested in tissue banking. 5 countries participated, namely Thailand, France, Philippines, Malaysia and Indonesia. Members present included Dr. Yongyudh Vajaradul (Thailand), Dr. Alain Patel (France) and Dr. Norberto Agcaoili (Philippines). A Pan-Asiatic Tissue Bank Organisation (PATBO) was set up by Dr. Yongyudh Vajaradul with its secretariat in Bangkok Biomaterial Centre, Siriraj Hospital, Thailand. A draft constitution was prepared by Dr. Noberto Agcaoili.

World Locomotor Tissue Bank Meetings (1987–1988)

In 1987, a World Locomotor Tissue Bank Meeting was held in Marseilles, France with Professor Dominic Poitout as the Organising Chairman. Dr. Vajaradul (Thailand) and Dr. Moritoshi Itoman (Japan) attended this meeting.

Beginning of Asia Pacific Association of Surgical Tissue Banks, 1988

Dr. Vajaradul organised the **3rd World Locomotor Tissue Bank Meeting in Ambassador Hotel, Bangkok, Thailand on 27–28 Oct 1988**. The meeting was attended by about 200 participants from Europe and the Asia Pacific. During this meeting it was decided to rename Pan Asiatic Tissue Bank Organisation to Asia Pacific Association of Surgical Tissue Banks (APASTB). Office bearers appointed for this association included Dr. Yongyudh

Vajaradul, Thailand (President), Dr. Aziz Nather, Singapore (First Vice President), Dr. Jiun Jer Wu, Taiwan (Second Vice President), Dr. Moritoshi Itoman, Japan (Third Vice President) and Dr. Sommart Keorochana, Thailand (Treasurer). Council Members included Dr. Sun Shiquan (China) and Dr. Roger Oakershott (Australia) Dr. Agcaoili was unable to attend this meeting. But his draft constitution for PATBO was amended by Dr. Aziz Nather and adopted as Constitution for APASTB.

Inaugural Meeting of APASTB (Fig. 2)

The Inaugural Meeting of APASTB was held in Central Plaza Hotel, Bangkok, 14–15 Nov 1989 in conjunction with the 8th Joint Congress of Asian and Pacific Federations of the International College of Surgeons with Dr. Yongyudh Vajaradul as Organising Chairman.

Dr. Vajaradul was inaugurated as **First President** of APASTB (1988–1990) with Dr. Nather as First Vice President.

The second annual meeting of APASTB was held in Pan Pacific Hotel, Singapore from 2–5 August 1990 with Dr Nather as Organizing Chairman under the auspices of the Singapore Orthopaedic Association annual scientific meeting (Fig. 3). Dr Nather was elected as second president of APASTB (1990–1992).



Figure 2. Inaugural meeting in Bangkok in 1989



Figure 3. Second meeting of APASTB in Singapore in 1990



Figure 4. Third meeting of APASTB in Tokyo in 1991

The third meeting was held in Keio Plaza Intercontinental Hotel in Tokyo, Japan on 1–3 August 1991 with Dr Moritoshi Itoman as Organizing Chairman (Fig. 4), supported by the Japanese Orthopaedic Association and the Japan Society of Transplantation.

The fourth meeting of APASTB was held in the Westin Philippine Plaza Hotel in Manila from 6–8 August 1992 with Dr Norberto Agcaoili as Organizing Chairman (Fig. 5). Dr Moritoshi Itoman was elected as third president (1992–1994).



Figure 5. Fourth meeting of APASTB in Manila in 1992

Tables 1, 2 and 3 summarise the Scientific Meetings of APASTB, the Presidents of APASTB and the Current Office-Bearers of APASTB for 2016–2018.

The 16th Annual Scientific Meeting on APASTB was held in Gifu, Japan with Dr Yudo Hachiya as the Organising Chairman (Table 1, Fig. 6).

Dr Ken Urabe was elected as the 15th and current President of APASTB (2016–2018) (Table 2).

Special lectures

Three special lectures were inaugurated during the First World Congress held in 2008 in Malaysia — the **Glyn Phillips Lecture**, **Abdurrahman Lecture** and **Hudson Silva Lecture**.

Table 1. Scientific meetings of APASTB

	Host country	Year	Organising chairman
Inaugural	Bangkok, Thailand	1989	*Yongyudh Vajaradul*
2nd	Singapore	1990	*Aziz Nather*
3rd	Tokyo, Japan	1991	*Moritoshi Itoman*
4th	Manila, Philippines	1992	*Norbeto Agcaoili*
5th	Suzhou, China	1994	*Tang Zhong Yi*
6th	Gold Coast, Australia	1996	*David Morgan*
7th	Kuala Lumpur, Malaysia	1998	*Hasim Mohamad*
8th	Bali, Indonesia	2000	*Abdurrahman*
9th	Seoul, Korea	2002	*Chang Joon Yim*
10th	Hong Kong	2004	*Shekhar Kumta*
11th	Mumbai, India	2006	*Krishna Sainis/Astrid Lobo Gajiwala*
12th	Malaysia	2008	*Hasim Mohamad*
13th	Bukit Tinggi, Indonesia	2010	*Menkher Manjas*
14th	Sydney, Australia	2012	*Sharon Bryce*
15th	Gifu, Japan	2014	*Yudo Hachiya*
16th	Seoul, Korea	2016	*Yong Koo Kang*
17th	Malaysia	2018	*Suzina Hamid*

Table 2. Presidents of APASTB

	Name	Country	Year
Founding President	Dr Yongyudh Vajadul	Thailand	1988–1990
2nd	Dr Aziz Nather	Singapore	1990–1992
3rd	Dr Moritoshi Itoman	Japan	1992–1994
4th	Dr Norberto Agcaoili	The Philippines	1994–1996
5th	Dr Sun Shiquan	China	1996–1998
6th	Dr David Morgan	Australia	1998–2000
7th	Dr Hasim Mohamad	Malaysia	2000–2002
8th	Dr Abdurrahman	Indonesia	2002–2004
9th	Dr Chang Joon Yim	Korea	2004–2006
10th	Dr Shekhar Kumta	Hong Kong	2006–2007
Acting 10th	Dr Astrid Lobo Gajiwala	India	2007–2008

(*Continued*)

Table 2. (*Continued*)

	Name	Country	Year
11th	Dr Astrid Lobo Gajiwala	India	2008–2010
12th	Dr Yong Koo Kang	Korea	2010–2012
13th	Dr Menkhar Manjas	Indonesia	2012–2014
14th	Dr Sharon Bryce	Australia	2014–2016
15th	Dr Ken Urabe	Japan	2016–2018

Table 3. Current office bearers of APASTB: 2016–2018

Immediate past president	Sharon bryce	Australia
President	Ken Urabe	Japan
First Vice President	Heri Suroto	Indonesia
Second Vice President	Suzina Sheikh A Hamid	Malaysia
Secretary-General	Jinyoung Jeong	Korea
Treasurer	Azura Mansor	Malaysia



Figure 6. 16th Meeting APASTB in Gifu in 2016

Table 4 shows the guest speakers invited to deliver these lectures.

APASTB newsletters

In the early years, APASTB Newsletters were produced with Dr. Hasim Mohamad and Dr. Norimah Yusof as editors from 1999–2003 and later Dr. Aziz Nather, Dr. Mohamed and Dr. Yusof as editors from 2004–2006.

Cell and tissue banking journal

Cell and Tissue Banking Journal was adopted as the official journal of APASTB, its editor being Dr Rudiger von Versen in Berlin, Germany. This journal has proven to be a very useful avenue to update our knowledge and share our research and best practices on tissue banking and tissue transplantation. A special issue in 2017 is dedicated to celebrating the 80th birthday of Professor Glyn Phillips, instrumental to the development of tissue banking in the Asia Pacific Region.

APASTB general standards

The APASTB Standards Subcommittee started work on the APASTB's own standards during the 9th APASTB Scientific Meeting in Korea, 2002. The Standards was drafted according to the template of the "IAEA International Standards in Tissue Banking", developed under the IAEA INT/6/052 Interregional Programme.

The first draft of APASTB Standards was completed during the 10th APASTB Scientific Meeting in Hong Kong, 2004. The first edition of the

Table 4. Special lectures of APASTB

	Glyn phillips lecture	Abdurrahman lecture	Hudson silva lecture
5th World Congress, 2008, 12th APASTB, Kuala Lumpur, Malaysia.	*Aziz Nather*	*Zulmy Wan*	*Glyn Phillips*
13th APASTB, 2010, Bukit Tinggi, Indonesia	*Ted Eastlund*	*Jan Wondergem*	*Scott Brubaker*
14th APASTB, 2012, Sydney, Australia	*Ted Eastlund*	*Nazly Hilmy*	*Greame Pollock*

APASTB General Standards was printed in January 2007 in the textbook "Radiation in Tissue Banking", edited by A Nather, N Yusof and N Hilmy. The Standards were revised in 2016 during the 16th APASTB meeting in Korea chaired by Dr. Yong Koo Kang and printed in January 2017.

Honorary Members of APASTB

Glyn Phillips	Cardiff, UK (IAEA) (Fig. 1)
Ramen Mukherjee	India, Vienna (IAEA) (Fig. 1)
Hudson Silva	Colombo, Sri Lanka

Friends of APASTB

Rudiger Von Versen	Berlin, Germany (IAEA)
Michael Strong	Seattle, USA (IAEA)
Anna Goclawska	Warsaw, Poland (IAEA)

Pioneers of APASTB (Fig. 7)

Yongyudh Vajaradul	Bangkok, Thailand
Aziz Nather	Singapore
Moritoshi Itoman	Tokyo, Japan
Norbeto Agcaoili	Manila, Philippines
Sun Shiquan	Taiyuan, China



Figure 7. Pioneers of APASTB
From left to right: Nather, Itoman, Sommart Kaerochana, Vajaradul, Agcaoili, Sun Shiquan

III. History of the IAEA Programme

IAEA (in particular the Regional Co-operative Agreement Projects) played a significant role in developing tissue banking in the Asia Pacific Region.

RCA Projects

IAEA successfully implemented 2 RCA Projects:

1. **RAS 7/003 "Radiation Sterilisation of Tissue Grafts".**
   This programme started in 1988 to promote the use of radiation sterilisation of tissue grafts in initially 9 member states.
2. **RAS 7/008 "Quality Assurance in Radiation Sterilisation of Tissue Grafts".**
   It produced the IAEA Draft Curriculum for training tissue banks in Suzhou, China in 1985. A "Train the Trainers Workshop" was conducted in Singapore in 1986 to pilot the delivery of this curriculum. NUH Tissue Bank was subsequently inaugurated as the IAEA/NUS Regional Training Centre to train tissue bank operators in the Asia Pacific Region in November 1997.

IAEA/NUS Internet Diploma Courses for Asia Pacific Region

From 1997 to 2016, 17 annual Diploma Courses were held from 1997 to 2016. 221 tissue bank operators have convocated including 194 from countries in Asia Pacific Region itself. In addition, there are 6 graduates from Latin America, 10 from Africa, 9 from Europe and 2 from Australia. The 18th Course started in October 2016 with its examination to be held in October 2017.

IAEA Interregional Projects

Two IAEA Interregional Projects also contributed to the development of tissue banking in the Asia Pacific Region.

INT/6/049 Interregional Centre of Excellence in Tissue Banking
INT/6/052 Improving the Quality of Production and Use of Radiation Sterilized Tissue Grafts

The project was responsible for developing the Diploma Training Course into an Internet course.

Recent Developments

Post IAEA Era (Post 2004)

2004 was a challenging year. IAEA withdrew financial support for tissue banking in member states. IAEA no longer sponsored tissue bankers to attend APASTB scientific meetings. It also stopped providing fellowships and expert missions to the member states. RCA member states struggled to survive. Bangladesh, China, Hong Kong, Pakistan, Thailand and Vietnam stopped participating in annual scientific meetings of APASTB. Fortunately, Indonesia, Malaysia, Australia, Singapore, Korea, Japan and India continued to actively participate in APASTB meetings and continued to develop the association.

Tissue Engineering Era (21st Century)

With the advent of "tissue engineering" in the 21st Century, bone banks took a back seat as stem cell centres bloom. The much needed research grant is lost to many banks as priorities were given to stem cell research. However, some bone banks survived on tissue engineering after nearly two decades did not deliver their much looked forward to results. They have still not succeeded to develop a good scaffold for bone. Autologous chondrocyte implantation went into decline. Today because of failure to modulate antigenicity of mesenchymal stem cells, only autologous MSCs can be used. BMPs were found to cause complications and PRP did not deliver the much required outcome.

Bone allografts remain the best scaffolds for bone — "God's scaffolds". There is promise of combining bone banking (bone allograft) with stem cell centre (adult MSCs) to fabricate "engineered bone". So long as the search for a bone scaffold continues, bone allografts remain the best option for developing bone substitutes in combination with autografts or adult mesenchymal stem cells.

Future Challenges

Succession

A major problem faced by tissue banks is the search for successors to continue the work achieved by tissue banks in the Asia Pacific region. Several

first generation leaders have retired including Dr. Yongyudh Vajaradul (Thailand), Dr. Moritoshi Itoman (Japan), Dr. Nazly Hilmy (Indonesia) and Dr. Norimah Yusof (Malaysia). We need young leaders who are committed and trained to continue to lead us. This is a key challenge for the future.

Other Issues

Other issues that need to be addressed are:

— Seek more support from institutions/hospital
— Seek Government Support
— Seek Legal Support
— Employ Tissue Transplant Coordinator
— Fight the threat of imported allografts in the country
— Focus on production of small bone grafts instead of massive long bone allografts
— Produce Demineralized Bones

Conclusions

The Asia Pacific Association of Surgical Tissue Banks has gone a long way since its beginning in Bangkok in October 1988. 26 years have since passed. APASTB has successfully organised 15 Scientific Meetings/Conferences and inaugurated 14 Presidents.

IAEA played a catalytic role in the early years (1994–2002) in promoting the growth of APASTB. Despite the withdrawal of support and funding from IAEA, the association continued to grow to its present form.

We are now witnessing the entrance of the younger generation into the arena. Meanwhile, the pioneers are slowly taking a back seat, allowing the new generation leaders to come forth and bring the association to greater heights.

The future of bone banks lies in forging strategic partnerships — combining stem cell centre with bone bank or combining bone bank with skin bank and heart valve bank to form a “tissue centre”. Combined centres will provide better administration, reduced manpower costs, improved facilities and better quality of tissues produced.

References

Anna D Goclowska, First World Congress of Tissue Banks. Annals Transplantation. 1997; **2**(1): 68.

APASTB Newsletter July 1999 (No. 1).

APASTB Newsletter Dec 2001 (No. 3).

APASTB Newsletter Oct 2003 (No. 5).

APASTB Newsletter Dec 2005 (No. 7).

APASTB Newsletter May 2006 (No. 8).

Bangkok Biomaterial Centre & Pan-Asiatic Tissue Bank Organisation 1984. Bangkok Biomaterial Centre Annual Report.

Nather A (1999) Tissue banking in the Asia Pacific region — the Asia Pacific Association of Surgical Tissue Banking. In: Phillips GO, Strong DM, von Versen R, and Nather A (eds.), *Advanced in Tissue Banking*, Vol. 3, World Scientific, Singapore, pp. 419–425.

Nather A, Phillips GO, Chang HF and Lim MYL (2001) Development of IAEA/NUS Internet Diploma Course in Tissue Banking. *ASEAN Orthop Assoc.*, **14**, 5–7.

Nather A, Phillips GO and Morales J (2003) IAEA/NUS distance learning diploma training course for tissue bank operators — past, present and future. *Cell Tissue Bank*, **4**, 77–84.

Nather A, Yusof N and Hilmy N (2007) Radiation in tissue banking. Basic sciences and clinical applications of irradiated tissue allografts. World Scientific, Singapore.

Nather A, Aziz Z and Zheng SS (2010) Asia Pacific Association of Surgical Tissue Banks — Past, Present and Future. In Nather A, Yusof N and Hilmy N. Allograft Procurement, *Processing and Transplantation. A Comprehensive Guide for Tissue Bank Operator*. World Scientific, New Jersey, London, Singapore.

Phillips GO and Pedraza JM (2003) The International Atomic Energy Agency (IAEA) Programme in radiation and tissue banking: Past, present and future. *Cell Tissue Bank*, **4**, 69–76.

Programme & Handbook of Abstracts: 13th Singapore Orthopaedic Association Meeting in conjunction with 2nd APASTB Meeting. 2–5 Aug 1990, Pan Pacific Singapore Hotel.

Proceedings of 3rd Meeting of APASTB (1991) Musculoskeletal Tissue Banking and Grafting edited by M. Itoman.

Programme & Abstract Book: 5th APASTB Meeting, 15–17 June 1994 Suzhou, China, Tong Zhong Yi.

Programme & Abstract Book: 9th International Conference of APASTB in co-ordination with IAEA/RCA Project Co-ordinaries Meeting 28–30 September 2002, Olympic Parktel, Seoul, Korea.

Programme & Abstract: 10th International Conference of APASTB, Prince of Wales Hospital, The Chinese University of Hong Kong, 6–8 Dec 2004.

3

Training Tissue Bank Operators — 20 Years of Experience by IAEA/NUS Regional Training Centre

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Introduction

This tissue banking community in Singapore and the Asia Pacific Region would like to dedicate this article to Professor Glyn Phillips for his vision and mission in pioneering the distance learning package for training tissue bank operators in the region.

It was a long journey from assembling the package in Suzhou, China in June 1994, the piloting of this curriculum in the Train the Trainers Workshop in Singapore in September 1995 to the launch of IAEA/NUS Diploma Course in Tissue Banking in November 1997. The Regional Training Centre for the Asia Pacific Region was inaugurated during this event.

The draft curriculum was succeeded by IAEA/NUS Multi-Media Curriculum printed by the training centre in Singapore. Over 20 years, this centre has trained a total of 254 participants. 221 tissue bank operators have



Figure 1. Professor Phillips (left), Mrs Rhiain Phillips (middle), and Mr Ramen Mukherjee (right) during the conferment ceremony for Honorary Membership in Bali

convocated including 194 from countries in Asia Pacific Region itself. A total of 17 training courses have been conducted. The 18th Course started in October 2016.

For his vision and tireless efforts to develop tissue banking in the region, Professor Phillips was conferred Honorary Membership of Asia Pacific Association of Surgical Tissue Banking during the 8th APASTB conference in Bali, Indonesia in 2000 (Fig. 1).

Background

In many regions of the world — Asia-Pacific, Latin America, Africa, and Eastern Europe — the directors of tissue banks are mostly part-time volunteers (Nather 2000). Only the technologists are employed as full-time staff. This is in contrast to large banks in the USA and Europe, many of which are run by large corporations as business ventures.

Furthermore, the responsibility of performing the day-to-day activities of a tissue bank lies with the **technologists**. Therefore, it is of utmost importance that they are well trained to perform all the duties required of them. These include the following:

- Screening of potential donors (both living and deceased)
- Performing serological investigations

- Procurement of tissues
- Processing of tissues
- Documentation
- Distribution of tissues
- Promotion of public awareness of tissue banking and transplantation
- Promotion of professional awareness of tissue banking and transplantation

In the past, the available **training programmes** were limited to a short 2-week course conducted by the American Association of Tissue Banks. There was therefore demand for a structured **year-long** training programme with a **comprehensive curriculum** leading to **diploma certification** by an **internationally recognised** university.

RAS 7/008: IAEA/RCA Programme on "Radiation Sterilisation of Tissue Grafts"

Since 1985, the International Atomic Energy Agency (IAEA)—under the Regional Cooperative Agreement (RCA) for member states in the Asia-Pacific region — began running a programme on the "Radiation Sterilisation of Tissue Grafts" (RAS 7/008) involving tissue banks in 13 countries, namely Bangladesh, China, India, Indonesia, Korea, Malaysia, Myanmar, Pakistan, the Philippines, Singapore, Sri Lanka, Thailand, and Vietnam (Nather 1999; Nather 2000; Nather *et al.* 2003).

RAS 7/008 was refined to a Thematic Model Project under the leadership of Professor Glyn Phillips, Technical Advisor to the Deputy Director-General, Department of Technical Cooperation, IAEA (Mr Qian Jihui). The objective was to raise the quality standards of tissue banking to an international level. Efforts were directed to harmonise the quality standards of tissue banks in the region so as to facilitate the exchange of grafts from one country to another in the long term.

National Coordinators from each member state spent several years developing and writing an IAEA/RCA Draft Curriculum on Tissue Banking, with Professor Phillips as the Coordinating Editor. The first draft curriculum was successfully assembled during the RCA Workshop in Suzhou, China, in 1994, and was the first of its kind in the world.

The curriculum was piloted in Singapore during the IAEA/RCA Regional Workshop on the "Dissemination of Information on Procedures for



Figure 2. The IAEA/RCA Regional Workshop on "Dissemination of Information on Procedures for Production and Radiation Sterilisation of Tissue Allografts" in 1995

Production and Radiation Sterilisation of Tissue Allografts" in September 1995 (Fig. 2). 21 trainers used the curriculum to teach 35 trainees. This was the largest workshop ever held for curriculum training and it was deemed to be successful, as the curriculum was found to be effective and very suitable for training **tissue bank operators**. The NUH Tissue Bank was inaugurated as a hospital tissue bank during the opening ceremony of this workshop (Nather 2000).

Development of Singapore as the Regional Training Centre (RTC) for the Asia-Pacific Region in Singapore

In September 1996, the NUH Tissue Bank was appointed by the IAEA to become the IAEA /NUS Regional Training Centre (RTC) for training tissue bank operators in the Asia-Pacific region (Nather 1999; Nather 2000). The Government of Singapore (represented by the Ministry of Environment), with the National Science and Technology Board (NSTB) as the funding agency, awarded a S$225 500 grant to build a new purpose-built tissue bank cum Regional Training Centre. The 2000 square feet centre is located on the 2nd level of the National University Hospital and has separate wet and dry processing laboratories, a documentation/distribution room, and a reception area.

The IAEA/NUS Regional Training Centre was launched during the IAEA/RCA Regional Training Course for the "Delivery of Curriculum to Tissue Bank Operators" on November 3, 1997. It was inaugurated by the Deputy Vice Chancellor of NUS, Professor Chong Chi Tat. The IAEA was represented by Mr Thomas Tisue, special advisor to the Deputy Director-General (Mr Qian Jihui). At the same time, the first ever IAEA/NUS Diploma Course on Tissue Banking was launched — another first in the world.

NUS Diploma Course in Tissue Banking

The NUS Tissue Banking Course is a 1-year distance learning diploma course. The minimum criteria for admission are at least five passes in the GCE O-Level Examination (or its equivalent), experience in working in a tissue bank or association with a tissue bank for at least 1 year, and proficiency in English. The course fee is now US$500.

The curriculum for the NUS Diploma course includes the following:

- The conversion of the IAEA Draft Curriculum on tissue banking into a Multi-Media Curriculum (Figure 3), which consists of 8 modules, accompanying sets of slides, 7 video demonstrations, and 1 audio cassette. The components of each module are contained in specially designed box containers (Nather 2000; Nather *et al.* 2003). The production costs



Figure 3. The IAEA/NUS Multi-Media Curriculum produced by Singapore

of this curriculum (about S$100 000) are borne by the NSTB. The eight modules comprise the following:

Module : Guide to Curriculum
Module 0: Historical Background
Module 1: Rules and Regulations
Module 2: Organisation
Module 3: Quality Assurance
Module 4: Procurement
Module 5: Processing
Module 6: Distribution and Utilisation
Module 7: Future Developments in Tissue Banking

- Lectures on Basic Sciences. The Basic Science subjects include Basic Anatomy, Basic Microbiology, Introduction to Transmissible Diseases, Basic Immunology, Principles of Sterile Technique, Basic Radiation Science, Biology of Healing of Tissue Transplantation, and Biomechanics of Tissue Transplantation.
- Recommended textbook: *The Scientific Basis of Tissue Transplantation, Advances in Tissue Banking*, Vol. 5 (A Nather, ed., World Scientific, Singapore, 2002).

The course structure consists of three components:

- 2-week **foundation course** at the RTC, Singapore, with lectures and practical demonstrations, ending with a **theory and practical** (OSPE) examination (phase I)
- Three assignments given at quarterly intervals, the last assignment being a practical assignment
- A **terminal examination** conducted by the NUS over 1 week at the RTC (phase II)

The marking allocation scheme for the diploma course is as follows:

- Foundation course exam (theory, practical) 20%
- Assignments 40%
- Terminal NUS Exam (theory, practical, viva) 40%

The NUS Diploma in Tissue Banking is awarded in three categories:

- Distinction >80 marks
- Credit 70–79 marks
- Pass 50–69 marks

Unsuccessful candidates are allowed to resit for the examination up to a maximum of three attempts during the main or supplementary examinations.

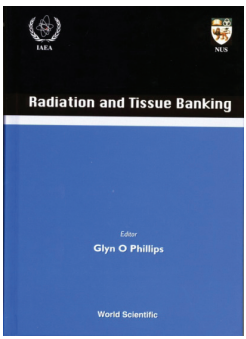
Curriculum Update

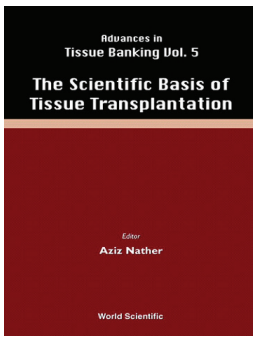
The curriculum has been updated in 3 phases (Nather *et al.* 2001):

- Phase 1
  - — Seven video tape demonstrations on the Procurement, Processing, and Transplantation of Tissues were converted into two compact discs in March 2000 (Fig. 3C).
- Phase 2
  - — A companion book, "*Radiation and Tissue Banking*" (GO Phillips, ed., World Scientific, 2000), (Fig. 4).
  - — A textbook on Basic Sciences "*The Scientific Basis of Tissue Transplantation*", in January 2002 (Fig. 4).
- Phase 3
  - — A textbook on "Radiation in Tissue Banking", (A Nather, N Yusof, N Hilmy eds., World Scientific, 2000) (Fig. 5)
  - — A new textbook on bone allografts "Allograft Procurement, Processing and Transplantation. A Comprehensive Guide for Tissue Bank Operators", (A Nather, N Yusof, N Hilmy eds. World Scientific 2010 (Fig. 5)
  - — A new textbook on Amnion Allografts "Amnion Allograft — Procurement, Processing and Clinical Applications. A Comprehensive Guide for Tissue Bank Operators", (N Hilmy, N Yusof, A Nather eds. World Scientific In Press 2017) (Fig. 5)



March 2000





July 2000 January 2002

Figure 4. Phase 2 of Curriculum Update

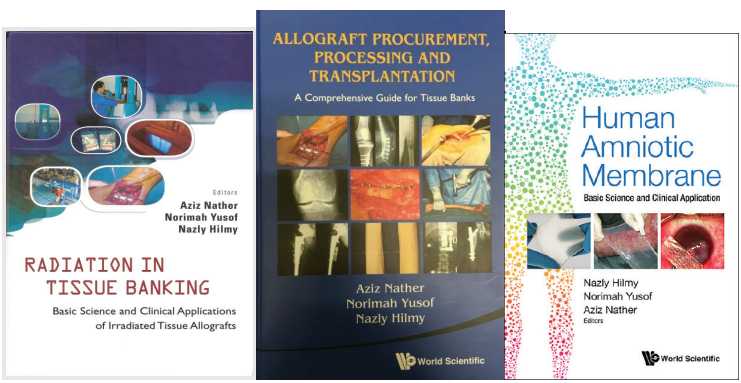


Figure 5. Phase 3 of Curriculum Update

IAEA/NUS Diploma Courses Conducted (1997–2003)

The first Diploma course was launched on November 3, 1997, with 17 candidates; and the first NUS Diploma examination was held in October 1998. Overall, 12 candidates graduated; of these, 4 passed with distinction, 5 with

credit, and 3 with pass only (Nather 2000, Nather *et al.* 2003). Diploma courses have since been held every consecutive year.

Figures 6 to 17 showed the convocations of the 1st to the 12th batches of students (October 1998 to September 2011).



Figure 6. 1st Convocation — October 1998: 4 Distinctions, 5 Credits, 3 Pass



Figure 7. 2nd Convocation — March 2000: 2 Distinctions, 5 Credits, 8 Pass



Figure 8. 3rd Convocation — March 2001: 1 Distinction, 2 Credits, 11 Pass



Figure 9. 4th Convocation — March 2002



Figure 10. 5th Convocation — April 2002



Figure 11. 6th Convocation in Feb 2004 — 1st Internet Course: 12 students convocated



Figure 12. 7th Convocation in March 2006 — 2nd Internet Course : 13 students convocated



Figure 13. 8th Convocation in April 2007 — 3rd Internet Course: 12 students convocated



Figure 14. 9th Convocation in Aug 2008 — 4th Internet Course: 6 students convocated



Figure 15. 10th Convocation in August 2009 — 5th Internet Course: 15 students convocated



Figure 16. 11th Convocation in October 2010 — 6th Internet Course: 6 students convocated



Figure 17. 12th Convocation in Sept 2011 — 6th Internet Course: 9 students convocated

The 6th to 12th batches were Internet Courses (1st to 7th Internet Courses) run by IAEA in conjunction with the IAEA/NUS Regional Training Centre in Singapore.

Transformation of Diploma Course into Internet Course

To save costs IAEA embarked on a programme to convert the Diploma Course into an on-line internet course. Large costs were saved by not needing to run a face-to-face two-week foundation course in Singapore. All that was needed was to run a face-to-face terminal Examination by the National University of Singapore.

The instruction materials that have been converted for internet delivery included the IAEA/NUS Multi-Media Curriculum — eight modules (text booklets), and two compact discs. In addition, textbooks were recommended to supplement the online curriculum. These include *Scientific Basis of Tissue Transplantation* and *Radiation in Tissue Banking.*

Internet Diploma Course in Tissue Banking (2004 onwards)

IAEA converted the distance learning programme into an internet course using funds from the IAEA/NT/6/052 Inter-Regional Programme directed by Dr J Morales. The internet course was piloted with the 4th batch in April 2001 (Fig. 18) and with the 5th batch in 2002. The first internet course was launched successfully with the 6th batch on February 9, 2004, with 16 students sponsored by IAEA. The terminal examination for this first internet course was held in February 2005.



Figure 18. Mr Jorge Morales, Director Inter-Regional Project on Tissue Banking (left), Internet Course piloted (middle and right photos)

Results of IAEA/NUS Diploma Training Course

Between 1997 and 2009, ten courses were conducted by the RTC with a total of 227 tissue bank operators (Table 1). Of these, 193 were from the Asia-Pacific Region (13 countries), 9 from Latin America (Brazil, Chile, Cuba, Peru, Uruguay), 11 from Europe (Greece, Slovakia, Poland, Ukraine), 11 from Africa (Zambia, Libya, Egypt, Algeria) and 3 from Australia. Table 2 showed the participation of students from various countries in the region including students from Latin America, Africa, Europe and Australia.

Table 1. Results of IAEA/NUS diploma courses conducted (1997–2009)

No. of training courses	No. students registered	No. students convocated	Results			
			Distinction	Credit	Pass	Fail
First Course (Nov 97–Oct 98)	18	12	4	5	3	6
Second Course (April 99–March 2000)	17	15	2	5	8	2
Third Course (April 2000–March 2001)	21	17	1	5	11	4
Fourth Course (April 2001–March 2002)	24	19	1	11	7	5
Fifth Course (April 2002–August 2003)	21	14	3	8	3	7
Sixth Course (Feb 2004–Feb 2005)	19	12	4	5	3	7
Seventh Course (Mar 2005–March 2006)	18	13	5	6	2	5
Eighth Course (April 2006–April 2007)	22	18	3	10	5	4
Ninth Course (April 2007–August 2008)	20	6	3	3	0	0
Tenth Course (Aug 2008–Aug 2009)	27	15	3	10	2	3
Eleventh Course (Aug 2009–Aug 2010)	18					
Total	**225**	**141**	29	68	44	43

Table 2. Regional distribution of tissue bank operators registered (1997–2009)

	No. students registered				
Batch No.	Asia-Pacific	Latin America	Africa	Europe	Australia
First Batch (Nov 97–Oct 98)	18	0	0	0	0
Second Batch (April 99–March 2000)	16	0	0	1 (Slovakia)	0
Third Batch (April 2000–March 2001)	19	2 (1 Brazil) (1 Chile)	0	0	0
Fourth Batch (April 2001–March 2002)	19	0	3 (2 Zambia) (1 Algeria)	2 (1 Greece) (1 Slovakia)	0
Fifth Batch (April 2002–August 2003)	14	3 (1 Cuba, 1 Peru, 1 Uruguay)	3 (1 Egypt, 1 Libya, 1 Zambia)	1 (Poland)	0
Sixth Batch (Feb 2004–Feb 2005)	12	0	2 (1 Libya) (1 Algeria)	5 (3 Slovakia, 1 Ukraine, 1 Poland)	0
Seventh Batch (Mar 2005–March 2006)	17	1 (1 USA)	0	0	0

(*Continued*)

Table 2. (*Continued*)

Batch No.	No. students registered				
	Asia-Pacific	Latin America	Africa	Europe	Australia
Eighth Batch (April 2006–April 2007)	18 (2 Iran)	0	2 (South Africa)	0	2
Ninth Batch (April 2007–August 2008)	20	0	0	0	0
Tenth Course (Aug 2008–Aug 2009)	24	1	1	0	1
Eleventh Course (Aug 2009–Aug 2010	16	2	0	0	0
Total	**193**	9	11	9	3

Withdrawal of Support from IAEA

IAEA stopped sponsoring students to attend the Diploma Course in Singapore from 2004 onwards, (last batch sponsored being the sixth batch of students — First Internet Course in Feb 2004).

Since 2005, students enrolling for the internet diploma course came on their own accord — paying all expenses on their own: airfare, hotel accommodation and registration fees (US $500/-). The Centre continued to run IAEA/NUS Internet Diploma Course for the next 11 years from 2006 (7th Batch) to 2017 (14th Batch)

Partnership of Singapore, Indonesia, Malaysia

Singapore training centre forged a vital partnership with 2 countries in the Asia Pacific Region — a key strategy to continue keeping the training centre self-sufficient. Revenue collected from registration fees of students were sufficient to pay for external examiners and other miscellaneous examination costs. This partnership worked because of 2 vital factors:-

1. **Examination Board** is formed involving 3 key personnel:

Dr Aziz Nather	(Chief Examiner, Orthopaedic Surgeon)
Dr Norimah Yusof	(External Examiner, Radiation Biologist, MINT (Malaysian Institute of Nuclear Technology) Champion for Malaysia
Dr Nazly Hilmy	(External Examiner, Radiation Biologist, BATAN (National Atomic Energy Agency Jakarta), Champion for Indonesia

Examiners from Indonesia and Malaysia did not incur high travel costs because of proximity of these countries to Singapore.

2. **Students from Indonesia and Malaysia**
   By appointing 2 champions to the Examination Board and to be part of the Faculty of the course, both Indonesia and Malaysia were encouraged to send more students to the Diploma Course. In addition, at least 3–5 students came from India to join the courses annually.

Results of IAEA/NUS Diploma Courses

Up to 2017, 17 Diploma Training Courses have been held for the Asia Pacific Region with 227 tissue bank personnel. 194 students have convocated.

In addition, students included 6 form Latin America, 10 from Africa, 9 from Europe and 2 from Australia. Up to 2017, a total of 254 participants have registered and 221 students have convocated.

Transfer of Technology to Latin America

In October 1998, an IAEA Inter-Regional Trainers Workshop on the "Distant Learning Use of the Curriculum Package on Tissue Banking" was conducted by Dr Phillips and Dr Nather in Singapore (Fig. 19) with participant trainers from Argentina, Brazil, Chile, Cuba, Mexico, and Peru (Nather 2000). One copy of the Multi-Media Curriculum (English version) was presented to each trainer from Latin America with the compliments of the Singapore Government. The curriculum was subsequently translated into Spanish for use by Latin American countries, with Argentina established as the RTC for Latin America in 2002.

Transfer of Technology to Africa

From 3–7 June 1999, Dr Phillips and Dr Nather conducted the "IAEA Regional Training Course on Tissue Banking" in Algiers, Algeria. The participants included representatives from Algeria, Egypt, Ghana, Libya, Zambia and Nigeria. However, no further development occurred following the workshop.



Figure 19. IAEA Inter-Regional Trainers Workshop, October 1998, Singapore

National Training Programmes

The Korean National Training Centre is set up in St Vincent's Hospital, Catholic Medical University, Seoul, Korea, with Professor Yong Koo Kang as the Director. It is jointly run by the Korean Association of Tissue Banks (KATB) and the Korean Musculoskeletal Transplantation Society (KMTS). It uses the IAEA Multi-Media Curriculum translated into the Korean language with funds from the IAEA, largely due to the efforts of Dr Chang Joon Yim (Korea), Dr Glyn Phillips (RCA), and Mr Jorge Morales (Inter-Regional Programme) (Fig. 20). The first Korean National Training Course (KNTC) was launched in 2003 with support from IAEA. Eleven students participated using the Multi-Media Curriculum printed in Korean.

The structure of the course (also a 1-year distance learning programme) is similar to the IAEA/NUS Diploma Course run in Singapore, and consists of the following:

- A 1-week foundation course (optional)
- Three assignments
- Three weekend courses (with lectures, assignments, and practical demonstrations) (Figs. 21, 22)
- A terminal face-to-face examination

The KNTC is run in collaboration with the IAEA/NUS Training Centre in Singapore. The latter provided all students with the internet curriculum in English.

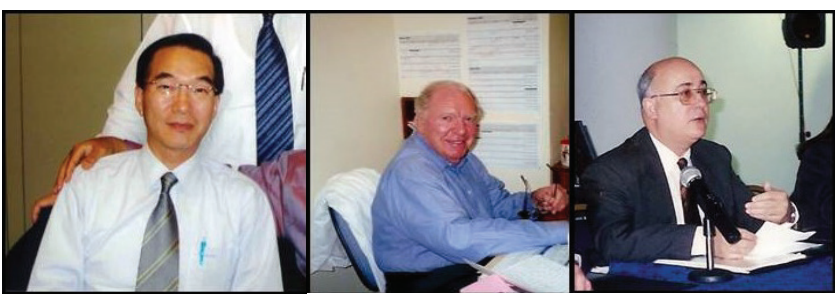


Figure 20. Korean Diploma Course — Dr Chang Joon Yim (left), Dr Glyn Phillips (middle), Mr Jorge Morales (right)

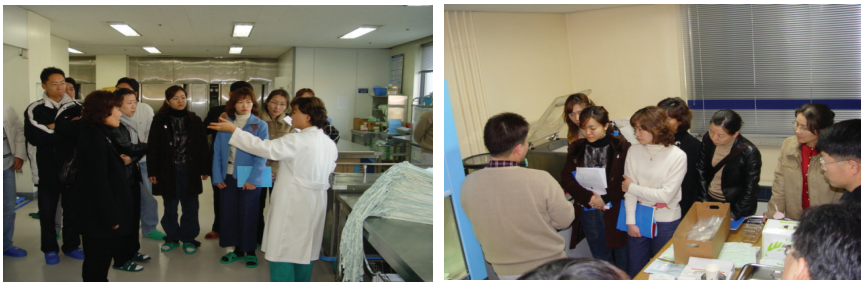


Figure 21. Practical Demonstrations for Processing (left). Viva for students in the course (right)



Figure 22. Practical Demonstration for Procurement using Cadavers.

The first Pilot Training Course was launched in December 2003 with 12 students at the Catholic University of Korea, in St Vincent's Hospital Tissue Bank (Fig. 23).

The first examination was held at St. Vincent's Hospital in November 2004, with Dr Nather as the IAEA consultant and external examiner. All 11 students passed, with 1 Top student and 2 Excellent student awards (Fig. 24).

Altogether, 12 courses have been completed with a total enrolment of 177 students. 153 have convocated and were certified as "Tissue Bank Specialists".

Indonesian Training Workshops

The first Indonesian Training Course was conducted in Surabaya, 1–3 October 2012 in the First National Workshop of Tissue Bank (Fig. 25).



Figure 23. First batch of students in the Pilot Training Course, December 2003 (left). Concert organized by students to commemorate course (right).



Figure 24. First Convocation in December 2004

Cell &Tissue Bank in Dr Soetomo Hospital

Surabaya ran several wokshops

- Stem Cell Workshop — 7 batches
- Biomaterial Workshop — 2 batches



Figure 25. First Indonesian Training Course, October 2012, Surabaya

Batan Research Tissue Bank Course was conducted in 2012 by Febrida (Amnion) and Basril (Bone) for continuing education of 12 participants (staff).

Malaysian Training Workshops

The ***First Bone Procurement workshop*** was conducted in August 2013 in MAHSA University by Forensic Pathologist Experts with 20 doctors and 22 paramedics attending (Fig. 26).

The ***First International Tissue Procurement Workshop (Edition: Bone & Cornea)*** was conducted on 15 Aug 2015 in Forensic Suite of Penang Hospital — attended by 27 doctors and 36 paramedics (Fig. 27).

Conclusion

Singapore played a vital role in the global training of tissue bank operators over the last 20 years, providing training not only to the students in the Asia-Pacific region, but also to Latin America, Africa, and Europe.



Figure 26. First Bone Procurement workshop in August 2013, MAHSA





Figure 27. Procurement of bone using cadavers (left). First International Tissue Procurement Workshop, Edition: Bone & Cornea (right).

Despite the ending of the IAEA programme in 2004, the centre continued the training courses on its own. It strategically forged key partnerships with Indonesia and Malaysia. This promoted participation of tissue bank personnel from both these countries in our diploma courses. Thus far, we have successfully completed 17 annual training courses for tissue bank operators. 194 tissue bank operators from Asia Pacific Region have convocated with Diploma Course Certificates from the National University of Singapore.

References

Nather A (1999) Tissue banking in the Asia Pacific region — the Asia Pacific Association of Surgical Tissue Banking. In: Phillips GO, Strong DM, von Versen R, and Nather A (eds.), *Advances in Tissue Banking*, **Vol. 3**, World Scientific, Singapore, pp. 419–425.

Nather A (2000) Diploma training for the technologists in tissue banking. *Cell Tissue Bank*, **1**: 41–44.

A Nather (ed.) (2002) *Advances in tissue banking*, **Vol. 5**, World Scientific, Singapore.

Nather A, Phillip GO, Cheong HF and Ling MY (2001) Development of IAEA/NUS Internet Diploma Course in Tissue Banking. *ASEAN Orthop Assoc.*, **14**: 5–7.

Nather A, Phillips GO and Morales J (2003) IAEA/NUS distance learning diploma training course for tissue bank operators — past, present and future. *Cell Tissue Bank*, **4**: 77–84.

4

Historical Development of Amnion

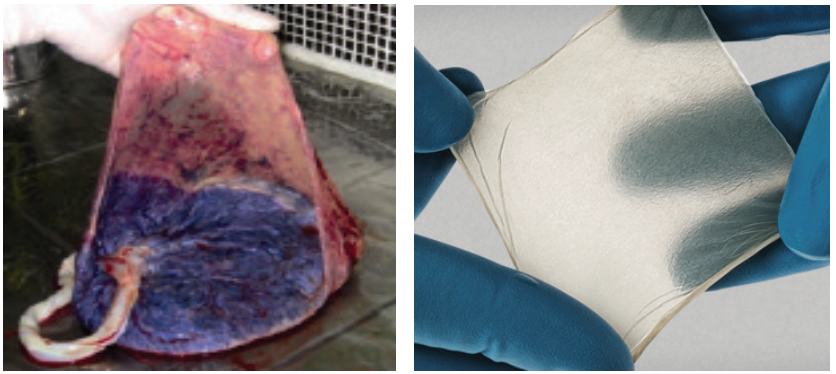
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Introduction

The earliest grafting of soft tissue was described in the *Sushruta Samhita* manuscript (ca. 600 BCE) when skin graft was used for nasal reconstruction in India (Nather & Zheng, 2010). Fresh amniotic membranes were first used in the transplantation of skin in 1910 as reported by J.S. Davis at Johns Hopkins Hospital, US (Davis, 1910). Three years later the fresh amniotic membrane was used as a wound dressing in treating burn patients (Sabella, 1913; Stern, 1913) and first used in ophthalmology in 1940 for the treatment of an ocular burn wound (de Rotth, 1940). However, there were few clinical reports on the use of amnion until the early 1990s when improved processing techniques and storage procedures were developed. The clinical uses of amniotic membranes and amnion grafts increased and since then, numerous clinical procedures have been published.

Amniotic membranes, which were previously discarded from hospital labour rooms, are now collected and processed into biological dressing. Under the Regional Programme for Radiation Sterilisation of Human Tissue Grafts by the International Atomic Energy Agency (IAEA), initiated in 1989



(a) (b)

Figure 1. Amniotic membrane: (a) Separation from placenta, (b) After processing

in 13 member countries of the Asia Pacific region, amnion grafts were produced by the newly established banks and radiation was the choice to sterilise them for safe clinical use. Amniotic membranes were taken from placenta of healthy screened mothers either through normal or caesarean delivery (Figure 1). The membranes were processed and subjected to several methods of preservation: deep freezing, air-drying, freeze-drying, oven-drying and glycerol preservation. Amnion grafts were then introduced to local hospitals and clinicians.

Clinical uses of amnion grafts in the Asia Pacific region started in the middle of 1990s. The clinical applications of amnions were initially as wound dressing in the treatment of burn wounds, scalds, chronic ulcers, dermal injuries and contaminated wounds. Amnions have also been used in post-wound surgery, diabetic and leprosy wounds and as a barrier to the prevention of surgical adhesions. Use of amnions in ophthalmology, plastic surgery and dental surgery encourages further production of amnion grafts. Recent literatures indicate the potential use of the amnion as a scaffold in tissue engineering for transplantation and regenerative medicine.

Clinical Outcomes of Using Amnion

Here are some reports on clinical outcomes and experience in using amnion grafts in a variety of clinical applications.

1. Wound Dressing

Dried and radiation sterilised amnions have been reported to be an effective dressing in the management of superficial and partial-thickness burns. Figure 2 illustrates three types of burn wound: superficial (first degree), partial thickness (second degree) and full thickness (third degree) based on level of injured skin.

The amnion flakes off from the healed burn wound surface, leaving no ugly scars (Mohamad, 2007; Bujang-Safawi *et al.*, 2010; Branski *et al.*, 2008). Amnion as a biological dressing is safe, simple to use, effective in promoting wound healing and able to prevent wound infection (Figure 3). Healing time is shortened from 7 to 5 days.

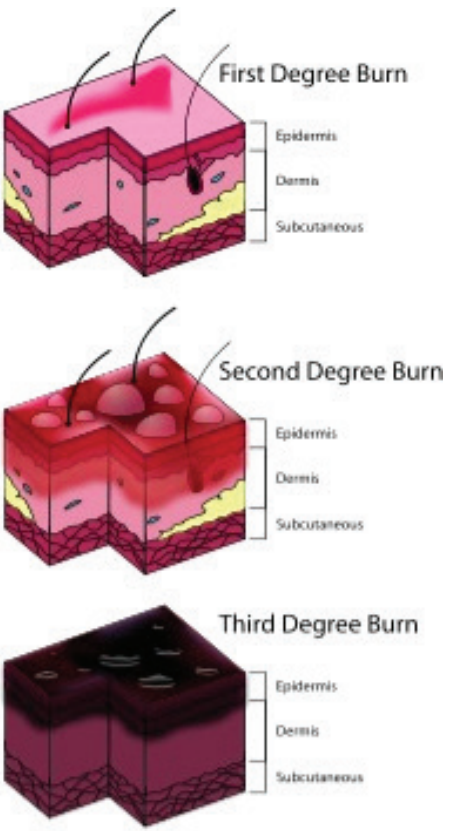


Figure 2. Three types of burn wound: superficial (first degree), partial thickness (second degree) and full thickness (third degree), based on levels of injured skin

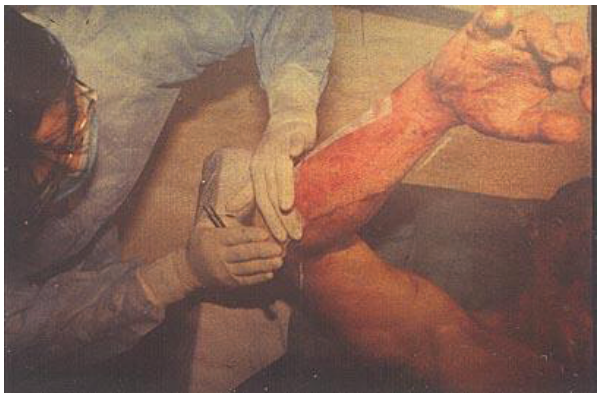


Figure 3. Amnion is used as a biological wound dressing in treating burn

Amnion can act as a temporary dressing for full-thickness skin defects (Maral *et al.*, 1999) and as a cover for skin grafts (Subrahmanyam, 1995). Its role is to prevent infection or as a barrier to contamination from the environment while epithelisation and wound granulation are taking place (Figure 4).

Some of the advantages in using amnions to treat wounds have been reported including the following (Mohamad, 2007; Halim *et al.*, 2007; Manjas *et al.*, 2010; Rodriguez-Ares *et al.*, 2009):

- good adherence — conform easily to wound bed and body contour
- effective barrier — reduce heat, prevent loss of fluid and electrolytes
- no immunological reaction
- bacteriostatic effect — reduce contamination and infection rate
- analgesic effect — reduce pain
- reduce the need for intensive nursing care
- promote healing by maintaining physiologically moist microenvironment
- able to absorb exudates
- semi permeable — allow oxygen and water transmission

Amnion can reduce formation of hypertrophic scar and contractures thus less formation of keloids (Halim *et al.*, 2007).



Figure 4. Amnion as a cover for skin graft

2. Leprosy Ulcer

The first report on the use of amnion as a dressing for chronic wound such as leprosy ulcer was in 1913 (Sabella, 1913). In the Asia Pacific region, amnion has been used to treat leprosy patients mainly in Indonesia and Bangladesh (Tarusaraya and Hilmy, 1998; Bari and Begum, 1999). Air-dried and freeze dried amnions can easily be stored at room temperature and are convenient for long distance despatch. Figure 5 shows the application of amnion on leprosy patients in Indonesia.

3. Diabetic Ulcer

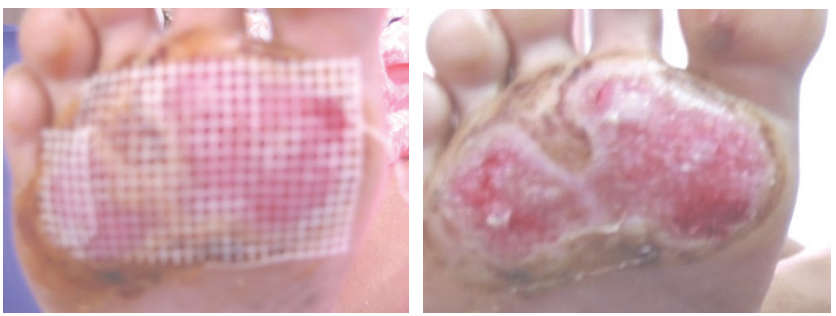
Many diabetic patients in Indonesia with foot ulcers have been treated with amnion (Dona *et al*. 1996). The diabetic ulcers treated with amnion showed a higher histopathology grade than those using conventional bandage. The average healing was 19 days, faster than the normal healing of all other wounds ranging from 21 to 26 days (Manjas *et al.*, 2010). The epithelialisation is higher in wounds treated by amnion as compared to those treated by conventional dressing. Clinical application on diabetic patients is shown in Figure 6.

Other than leprosy and diabetic ulcers, amnion has been used as a biological dressing to treat chronic ulcer and pressure sores. Amnion is also



(a) (b)

Figure 5. Amnion is used to treat leprosy ulcers in Indonesia: (a) Before apply amnion (b) Healed after 90 days (Tarusaraya and Hilmy, 1998)



(a) (b)

Figure 6. Amnion is used to treat diabetic ulcers: (a) Covered by amnion on 0 day (b) Healed without scars after 15 days (Manjas *et al.*, 2010)

effective in treating radiation-induced ulcers among patients receiving radiotherapy (Gajiwala dan Sharma, 2003).

4. Ophthalmology

Amniotic membrane remains useful in the treatment of several ophthalmic conditions, especially for ocular surface (Hilmy *et al.,* 2007). The membrane

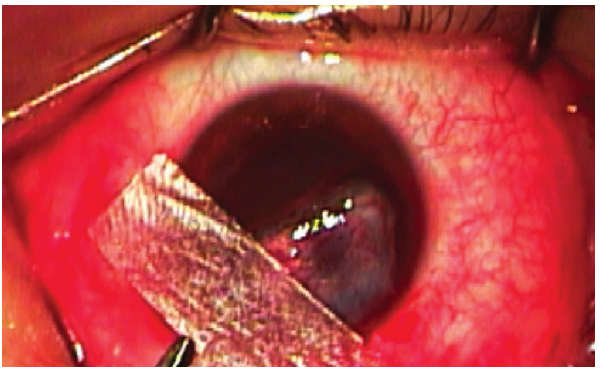


Figure 7. Amnion is used in ophthalmology

was first used in ophthalmology in 1940 when de Rotth reported partial success in the treatment of conjunctival epithelial defects using fresh membrane following his success in treating chemical burn on ocular surface (de Rotth, 1940). After many years of silence, Prasad *et al.* reintroduced the use of amnion for ocular surface reconstruction in 1986 (Prasad *et al.,* 1986). Subsequently, many reported the use of single- and multi-layered amnion for treating persistent corneal epithelial defects, severe ulceration of the cornea and sclera, and corneal perforations. In the beginning, fresh and frozen amnions were used but lately due to progress in preservation technique, glycerolised preserved amnion is the main choice. It is believed that the avascular stromal layer of amnion can reduce inflammation, promote neovascularisation and fibrosis hence stimulating re-epithelisation. Amnion is therefore useful in treating common ophthalmic problems especially those related to ocular surface reconstruction (Figure 7).

The variable clinical outcomes could be related to inter and intra donor variations in the membrane and the depletion or alterations in its constituents subsequent to processing and storage (Rahman *et al.*, 2009; Dua *et al.,* 2004).

5. Plastic Surgery

Skin substitutes and biological dressings play a major role in plastic and reconstructive surgeries (Halim *et al.*, 2007). Amnion has histological characteristics quite similar to those of skin although its thickness is only one

third of the thickness of the human skin. Amnion can be used in plastic surgery (Figure 8) for the following conditions:

- burns
- treatment of leg ulcers
- extensive skin loss in Stevens-Johnson syndrome
- dressing for split-thickness skin graft donor sites
- reconstruction of pelvic floor following pelvic exenteration
- oral cavity reconstruction
- flap dressing
- neurolysis and tenolysis
- vaginal epithelisation
- intra-abdominal and peritoneal defects
- intracranial implantations
- replacement of nasal mucosa in Rendu-Osler-Weber disease



Figure 8. Amnion was used in treating 4% partial thickness burn on facial area, wound healing evident on day 7 post-burn and complete healing without hypertrophic scar at 2-month follow up after injury (Bujang-Sapawi *et al.*, 2010).

- traumatic soft tissues
- tissue engineering (as a carrier for keratinocytes and fibroblasts)

6. Periodontal Surgery

Periodontal surgery is the surgical manipulation of periodontal tissue and bone by excising diseased gingival tissue and removing necrotic bone respectively. Although bone allografts have been used in maxillofacial surgery since 1950, amnions were introduced to periodontics only in the early 1970s (Tantin *et al.*, 2010). Amniotic membranes have been routinely used for periodontal cases including depigmentation of gingiva in the Asia Pacific region since 2000 (Figure 9). No rejection occurred in all of these applications.

7. Post-Surgery Wound

Amnion has been used as a wound dressing in several types of post-wound surgery, such as post-caesarean operation, post-skin grafting, post-circumcision, and clean wound in daily operations (Manjas *et al.*, 2010). No complications such as severe wound infection, secondary closing of wound, wound dehiscence, allergic reaction or bad appearance of wound were reported. In many cases, amnion dressing provides better results as compared to



(a) (b)

Figure 9. Amnion is used to treat gingiva for depigmentation: (a) Before treatment (b) After treatment (Courtesy: Dr Tantin, BATAN, Indonesia)

conventional dressing in terms of rate of infection, rate of wound healing and rate of complications.

8. Tissue Engineering

The Extra Cellular Matrix Composition (ECM) of the amnion and its components, such as growth factors, suggest that the amnion is an excellent candidate as a native scaffold for tissue engineering. In addition, the amnion is a biomaterial that can be easily obtained, processed and transported (Hilmy *et al.,* 1987; Niknejad *et al*., 2008).

In tissue engineering, amnion can be used as a carrier or scaffold for keratinocytes and fibroblast cells (Halim *et al*., 2007). Amnion cells have a variety of growth factors, all of which can attribute to cell growth and proliferation. Amniotic membrane has therefore the potential to become a scaffold for cell culture (Hodde, 2002). The matrix component of the amniotic membrane is biocompatible in vitro, and has no adverse effects in cellular proliferation or morphology (Wilshaw *et al*., 2006). The culture of human corneal epithelial cells (Kobayashi *et al*., 2006) and chondrocyte cells for cartilage transplantation (Krishnamurtithy *et al*., 2011) on amniotic membrane have been established. The epithelium and stroma are endowed with a number of cytokines and growth factors, among them are transforming growth factor ß and epidermal growth factor. These biological constituents allow the amniotic membrane to act as a substrate, which is very conducive to epithelial cell migration and attachment although the exact mechanism of action is still not clear (Rahman *et al.,* 2009).

Naturally occurring biopolymer scaffolds such as amnion are viable alternatives to synthetic polymers for tissue engineering cell growth substrates.

Advantages in Using Amnion for Clinical Applications

The processed amnion grafts have several advantages in clinical applications, as follow:

- Ethically non-controversial and has been widely used in treating patients
- Potential source for scaffolding tissues and adult stem cells

- Has biological properties such as anti-inflammatory, anti-microbial, anti-fibrosis; anti-scarring, low immunogenicity (not expressing HLA-A, B or DR antigens)
- Tough, porous, shiny membrane that makes it suitable for dressing
- Absence of blood vessels, lymphatic or nerve cells therefore no rejection

Halim *et al.* (2007) described the biological functions of amnion that are beneficial in clinical uses, as follows:

- Decrease of the bacterial count in the wound
- Reduction of fluid loss
- Promotion of wound healing
- Protection of growing epithelium
- Tight adherence to the wound surface
- Increased mobility and diminished pain
- Help in predicting the readiness for grafting
- Preparation of skin defects for closure
- Decrease in physiological stress for the patient
- Stimulation of neovascularisation

By having these properties, the membrane is useful in the management of burns, surgical dressings, reconstruction of oral cavity, bladder and vagina, tympanoplasty, arthroplasty and so forth (Fernandes *et al.*, 2005). Specifically, the amniotic membrane has gained importance because of its ability to reduce scarring and inflammation, enhance wound healing and serve as a scaffold for cell proliferation and differentiation as a result of its antimicrobial properties.

Conclusion

Amnion being histologically so close to skin has many advantages as wound dressing. However, use of amnion has been referred as grafting rather than transplantation. It is easily available, inexpensive to process, easily to preserve and store. It is low in antigenicity, has antimicrobial property and has been proven to enhance healing. Having a large variety of growth factors amnion has great potential to be a carrier or scaffolding matrix for cell growth and proliferation in tissue engineering.

References

Bari MM and Begum R (1999) Use of radiation-sterilised amniotic membrane grafts as temporary biological dressings for the treatment of leprotic ulcer. In: Phillips GO, Kearney JN, Strong DM, von Versen R and Nather A (eds.), *Advances in Tissue Banking*, Vol. 3, World Scientific, Singapore, pp. 477–483.

Branski LK, Herndon DN, Celis MM, Norbury WB, Masters OE and Jeschke MG (2008) Amnion in the treatment of pediatric partial-thickness facial burns. *Burns*, **34**: 393–399.

Bujang-Safawi E, Halim AS, Khoo TL and Dorai AA (2010) Dried irradiated human amniotic membrane as a biological dressing for facial burns — A 7-year case series. *Burns*, **36**(6): 876–882.

Davis JS (1910) Skin transplantation with a review of 550 cases at the Johns Hopkins Hospital. *Johns Hopkins Med. J*., **15**: 307.

de Rotth A (1940) Plastic repair of conjunctival defects with fetal membranes. *Arch. Ophthalmol*., **23**: 522–525.

Dona A (1996) Diabetic foot of NADDIM patient in the internal medicine department, Dr M Djamil Hospital 1990–1994. *Acta Med. Indones*., **27**: 1341–1346.

Dua HS, Gomes GAP, King AJ and Maharajan VS (2004) The amniotic membrane in ophthalmology. *Survey of Ophthalmology*, **49**(1): 51–77.

Fernandes M, Sridhar MS, Sangwan VS and Rao GN (2005) Amniotic membrane transplantation for ocular surface reconstruction. *Cornea*, **24**: 643–653.

Gajiwala AL and Sharma V (2003) Use of irradiated amnion as a biological dressing in the treatment of radiation induced ulcers. *Cell Tissue Bank*, **4**: 147–150.

Halim AS, Leow AM, Dorai AA and Wan Sulaiman WA (2007) Clinical applications of irradiated amnion grafts: Use of amnion in plastic surgery. In: Nather A, Yusof N and Hilmy N (eds.), *Radiation in Tissue Banking*, World Scientific, Singapore, pp. 365–379.

Hilmy N, Siddik S, Gentur S and Gulardi W (1987) Physical and Chemical Properties of Freeze Dried Amnion Membranes Sterilised by Irradiation. *J Atom Indonesia*, **13**(2): 1–14.

Hilmy N, Pandansari P, Ibrahim GS, Indira S, Bambang S, Sunarti R and Heryati S (2007) Use of freeze-dried irradiated amnion in ophthalmologic practices. In: Nather, A, Yusof, N and Hilmy, N (eds.), *Radiation in Tissue Banking*, World Scientific, Singapore, pp. 355–364.

Hodde J (2002) Naturally occurring scaffolds for soft tissue repair and regeneration. *Tissue Eng*., **8**: 295–308.

Kobayashi A, Yoshita T and Sugiyama K (2006) *In vivo* confocal microscopic analysis of human corneal epithelial sheet cultured on amniotic membrane. *Ophthalmic Surg. Lasers Imaging*, **37**: 304–309.

Krishnamurtithy G, Shilpa PN, Ahmad RE, Sulaiman S, Ng CLL and Kamarul T (2011) Human amniotic membrane as a chondrocyte carrier vehicle/substrate: In vitro study. *J. Biomat. Mater. Res.*, **99**(3): 500–506.

Manjas M, Tarusaraya P and Hilmy N (2010) Clinical applications of irradiated amnion grafts. In: Nather A, Yusof N and Hilmy N (eds.), *Allograft Procurement, Processing and Transplantation — A Comprehensive Guide for Tissue Banks*, World Scientific, Singapore, pp. 501–515.

Maral T, Borman H, Arslan H, Demirhan B, Akinbingol G and Haberal M (1999) Effectiveness of human amnion preserved long-term in glycerol as a temporary biological dressing. *Burns*, **25**(7): 625–635.

Mohamad H (2007) Amnion for treatment of bones. In: Nather A, Yusof N and Hilmy N (eds.), *Radiation in Tissue Banking*, World Scientific, Singapore, pp. 343–354.

Nather A and Zheng S (2010) Evolution of Alograft Transplantation. In: Nather A, Yusof N and Hilmy N (eds.), *Allograft Procurement, Processing and Transplantation — A Comprehensive Guide for Tissue Banks*, World Scientific, Singapore, pp. 3–42.

Niknejad H, Peirovi H, Jorjani M, Ahmadiani A, Ghanavi J and Seifalian MA (2008) Properties of the amniotic membrane for potential use in tissue engineering. *European Cells and Materials*, **15**: 88–89.

Prasad JK, Feller I and Thompson PD (1986) Use of amnion for the treatment of Stevens-Johnson syndrome. *J Trauma*, **26**: 945–946.

Rahman I, Said DG, Maharajan VS and Dua HS (2009) Amniotic membrane in ophthalmology: indications and limitations. *Eye*, **23**: 1954–1961.

Rodriguez-Ares MT, Lopez-Valladares MJ, Tourino R, Vieites B, Gude F, Silver MT and Couceiro J (2009) Effects of lyophilisation on human amniotic membrane. *Acta Ophtalmol.*, **87**: 396–403.

Sabella N (1913) Use of foetal membranes in skin grafting. *Med. Rec. NY*, **83**: 478–480.

Stern W (1913) The grafting of preserved amniotic membrane to burned an ulcerated skin surfaces substituting skin grafts. *JAMA*, **13**: 973–974.

Subrahmanyam M (1995) Amniotic membrane as a cover for microskin grafts. *British Journal of Plastic Surgery*, **48**: 477–478.

Tantin RD, Pandasari P, Abbas B and Hilmy N (2010). Clinical applications of radiation-sterilised grafts in periodontal surgery. In: Nather A, Yusof N and

Hilmy N (eds.), *Allograft Procurement, Processing and Transplantation — A Comprehensive Guide for Tissue Banks*, World Scientific, Singapore, pp. 517–525.

Tarusaraya P and Hilmy N (1998) Comparison study using freeze dried amnion membrane and ZnO ointment as wound covering for leprocy ulcer. Presented at the *7th International APASTB Conference on Tissue Banking*, Kuala Lumpur, Malaysia.

Wilshaw SP, Kearney JN, Fisher J and Ingham E (2006) Production of an acellular amniotic membrane matrix for use in tissue engineering. *Tissue Eng*., **12**: 2117–2129.

5

Anatomy and Histology of Amnion

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Introduction

Amniotic membrane is the innermost layer of the placenta that encloses the embryo and which forms a sac filled with amniotic fluid that surrounds the embryo and later the foetus as it enlarges. The membrane is collagen-rich, thin, transparent and tough, lining the chorion laeve. The placenta produces amniotic fluid at the earliest period of foetal development. The main function of amniotic membrane is to protect the foetus from unwanted materials during intrauterine development.

Terminologies that have been used other than amniotic membrane (AM) are foetal membrane, extraembryonic tissue (EET) and extraembryonic membrane (EEM). This foetal membrane consists of chorion and amnion layers. The term "extraembryonic tissue" (EET) refers to placenta, amnion and chorion. The term "extraembryonic membrane" (EEM) or amniotic membrane denotes a general name encompassing an inner membrane, the amnion and an outer membrane, the chorion, which is in contact with the maternal decidua during gestation (Figure 1). The term "amnion" is reserved for the inner layer of EEM as obtained when the amniotic epithelium and its

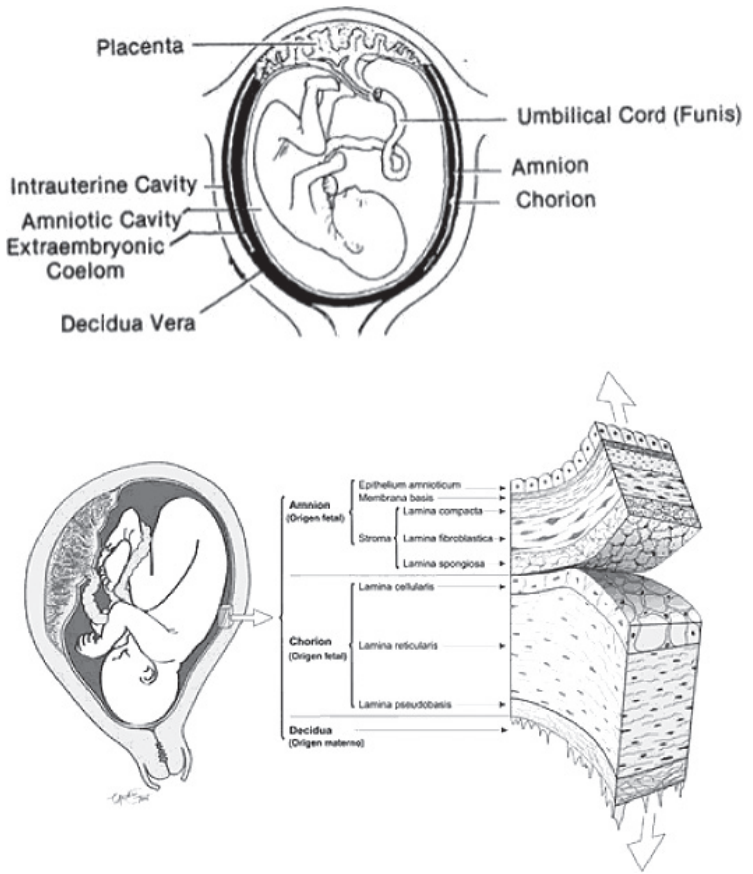


Figure 1. Extraembryonic membrane (EEM) or amniotic membrane encompasses an inner membrane, the amnion and an outer membrane, the chorion (Bourne, 1962)

underlying layers are stripped from the chorion without using any elaborate techniques of separation (Halim *et al.*, 2007; Hilmy *et al.,* 2007).

Amniotic Fluid

Amniotic fluid can be detected from the very beginning of the formation of the chorionic cavity or gestational sac. This water-like fluid originates from the maternal plasma, and passes through the foetal membrane by osmotic and hydrostatic forces. As the placental and foetal vessels develop, the fluid passes through the foetal tissue, as the exsudatum of the skin. After the

20–25th week of pregnancy when the keratinisation of skin occurs, amniotic fluid begins to produce depending on the factors that comprise the circulation of the amniotic fluid. At first, the amniotic fluid is mainly water with electrolytes, but by about the 12–14th week the liquid also contains proteins, carbohydrates, lipids and phospholipids and urea, all of which aid in the growth of the foetus. The volume of the amniotic fluid is positively correlated with the growth of the foetus. From the 10th to the 20th week it increases from 25ml to 400ml approximately. From the 8th week, when the foetal kidneys begin to function, foetal urine is also present in the amniotic fluid. In the 10th week the breathing and swallowing of the foetus causes a slight decrease in the amount of the fluid. Neither urination nor swallowing contributes significantly to the fluid quantity changes, until the 25th week, when keratinisation of skin is complete. Then the relationship between the amniotic fluid and foetal growth stops. It reaches the plateau of 800ml at the 28th week of gestational age (GA). The amount of fluid declines to roughly 400 ml at the 42nd week of GA. That is the reason why amniotic fluid as well as amniotic membrane have stem cells that come from the foetus through the umbilical cord. Amniotic stem cells are multipotent stem cells of mesenchymal origin extracted from amniotic fluid (Fauza, 2004). Two types of stem cells have been obtained from amnion:

- amnion epithelial stem cells (AESC) from the epithelial surface next to the amnion fluid and
- amnion mesenchymal stem cells (AMSC) in the basement membrane stroma.

They are able to differentiate into various tissue types, such as skin, cartilage, cardiac tissue, nerve, muscle, and bone and have potential medical applications, especially in organ regeneration (Bennet *et al.*, 2008; Eastlund, 2010; Wikipedia.org, 2015).

Embryology

Human foetus inside the amniotic membrane with the amnion lining the chorion laeve and placenta surrounded by amniotic fluid is shown in Figure 2. The umbilical cord, connecting the placenta and the foetus contains stem cells from the foetus.

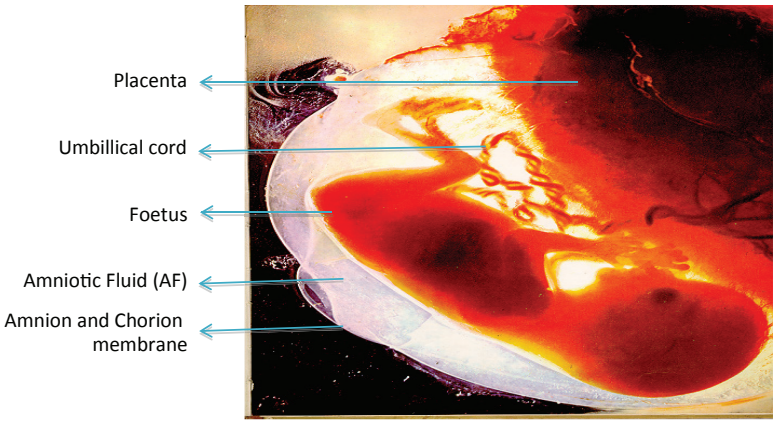


Figure 2. Human foetus inside the amniotic membrane (Hilmy *et al.*, 2007)

Anatomy and Development of Placenta

In the early phase of foetal development, the gestational sac is covered by villi formed all over the surface. Between the 12th and the 16th weeks of pregnancy, the villi on the capsular surface rapidly degenerate while those on the decidua basalis are compensated by hypertrophy and become a solid structure called placenta (Figure 3). The placenta is circular in shape at term and forms a spongy disc about 20 cm in diameter and 2.5 cm in thickness in the centre and slightly thinner at the edge. The placenta is actually a part of the chorion that serves the foetus (Mohamad, 2001).

After the fertilisation process, the ovum moves from the fallopian tube to the uterine cavity within 5 to 6 days. During the passage, the ovum starts to divide into 2, 4, and 8 cells and so on to form zygote. Through the process of segmentation, the zygote forms the morula, which then develops further into the blastocyst while still in the fallopian tube. The outermost cells of the blastocyst form the trophoblast, which is capable of eroding and digesting the outer epithelial surface of the uterine decidua and the fertilised ovum sinks into the inner layer of the decidua. The growing zygote enlarges and pushes aside the endometrial glands. The inner cell mass develops into the embryo with two cavities. These cavities develop further into the amniotic cavity and yolk sac. The amniotic cavity is lined by ectodermal cells and the yolk sac is lined by endodermal cells. Between these cavities lie the mesodermal cells.

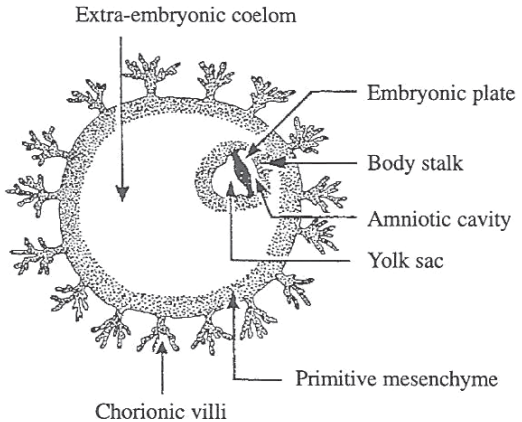


Figure 3. Development of the placenta, the early changes in the blastocyst, which later develops into the foetus (Mohamad, 2001)

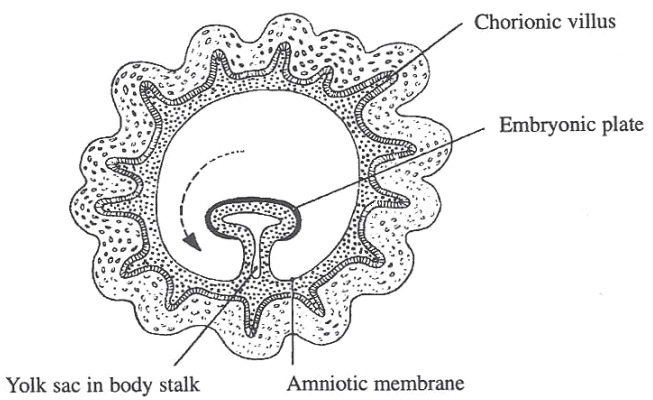


Figure 4. Early formation of amniotic membrane, chorionic villi and body stallk (Mohamad, 2001)

Therefore this region (see Figure 3) called the embryonic plate has three types of cells: ectoderm, endoderm and mesoderm cells. These three types of cells are necessary for the formation of a foetus. Ultimately, the amniotic cavity develops into foetal membrane surrounding the embryo and linked to the embryonic ectoderm at the umbilical cord. The junction between the trophoblast and the inner cell mass forms the body stalk, which ends as the umbilical cord (Figures 4).

Anatomy of Amniotic Membrane

Amniotic membrane developed from extraembryonic tissue consists of both foetal (embryonic plate) and maternal components (decidua). These two components are held together by the chorionic villi and connect the cyto-trophoblastic shell of the chorionic sac to the decidual basalis (Niknejad *et al.*, 2008).

Amniotic membrane with a total thickness of 0.02 to 0.5mm consists of amnion and chorion. The amnion is the inner part, which is smooth, glistering and tough while the chorion is external and weaker, easy to break during processing. The amnion has five layers and the chorion has three layers, as illustrated in Figure 5. Amnion consists of five layers as below:

- Inner layer of cuboidal and flattened polygonal **ephithelium cells.**
- **Basement membrane**: with two layers, lamina lucida and lamina densa, contains collagens type III, IV, V, VII, laminin-1, laminin-5, fibronectin allantoin, lisozim, transferin, progesteron and various growth factors.

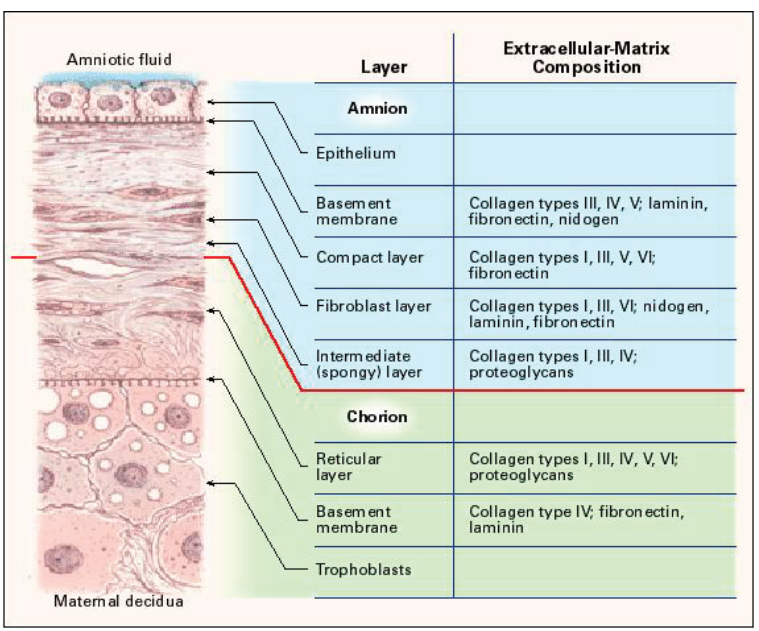


Figure 5. Schematic representation of the structure of the foetal membrane at term with the extracellular matrix components of each layer (Niknejad *et al.*, 2008)

- **Compact layer**: with a thin acellular and strong fibre, contains collagen type I, III, V and VI, fibronectin.
- **Fibroblast layer**: contains collagen I, III and VI, laminin, fibronectin as well as Hoffbauer cells.
- **Spongy layer**: compressed between amnion and chorion sac, consists of network of fibril and mucus and the fibril contains reticulin and collagen type I and II. Its viscoelastic function protects amnion against trauma and rupture. It also contains fibroblast and Hoffbauer cells (see Figure 4).

The size of the amniotic membrane can reach 1,500 cm sq. of which 1,200 cm sq. is the reflected amnion (Farazdaghi *et al.*, 2001). There is a variation of shape and size between cells of the placental as compared to the reflected portion of the membrane (Thomas, 1965). The epithelial cells are more cuboidal in shape and of more uniform size for membranes delivered by caesarian section than those of normal labor (Thomas, 1965). The membrane has no blood vessels, nerves or lymphatic channels. Therefore it has no direct supply of blood (Dua *et al.*, 2004).

Anatomy of Chorion Membrane

Chorion membrane is a thin membrane between the amnion and the decidua. It is an opaque fibrous membrane and lines the uterine decidua. Its thickness varies from 0.02–0.2mm with weak tensile strength. It easily breaks. It has no blood vessels, lymphatic or nerve cells. It consists of three layers:

- Reticular layer
- Pseudo-basement membrane
- Trophoblasts

Some authors consider the chorion to have four layers by adding Cellular layer as an additional layer (Hilmy *et al.*, 2007; Mohamad, 2007; Niknejad *et al.,* 2008).

Based on biological composition and mechanical properties, mainly the amnion is used for clinical applications. However the chorion has also been used for limited purposes (D'Lima *et al.*, 2014).

Histology of Amniotic Membrane

The amniotic membrane is histologically quite similar to skin. There are many similarities between human foetal skin and amnion of early pregnancy. The amnion layer is composed of a layer of epithelium while the chorion layer is composed of transitional epithelial cells (Mohamad, 2007).

Histological studies showed that the amnion has 5 layers (Bourne, 1960; von Versen-Hoynck *et al.,* 2004; Riau *et al.*, 2010). Amnion consists of the following layers: epithelium, basement membrane, compact layer, fibroblast layer and spongy layer (Figure 6).

By examining histological sections of amnions stained with Haematoxylin & Eosin, five layers can be seen as previously described (Figure 7). The amnion comprises of epithelial layer, which is attached to basement membrane and three layers of avascular stroma i.e. compact layer, fibroblast layer and spongy layer. The compact layer of stroma beneath the epithelium is likely to make an important contribution to the tensile strength of the amnion thus providing the mechanical strength to maintain the integrity of the foetal sac up to parturition (Aplin *et al.*, 1985; Bourne, 1960).

1. **Epithelial** layer has an epithelium with polygonal shaped cells and vary from columnar over the placenta to cuboidal or flat cells when away

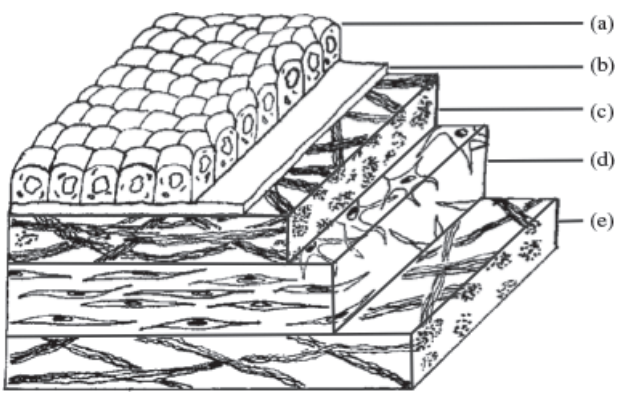


Figure 6. Schematic representation of the human amnion: (a) epithelial layer (b) basement membrane (c) compact layer (d) fibroblast layer (e) spongy layer (von Versen-Hoynck *et al.*, 2004)

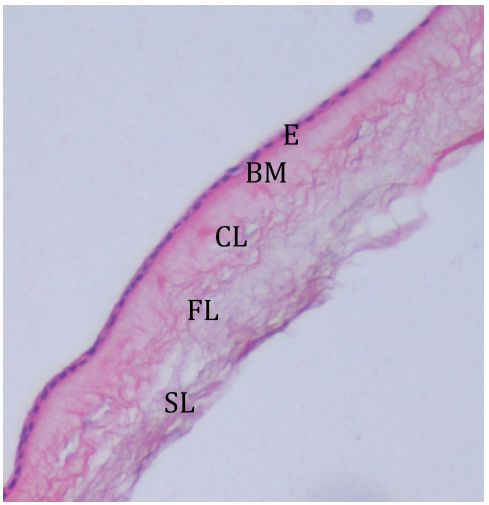


Figure 7. Air-dried amnion comprises of epithelial layer (E), which is attached to basement membrane (BM) and avascular stroma (compact layer (CL), fibroblast layer (FL) and spongy layer (SL)) under Light Microscope, Magnification 40x (Courtesy: Bone Bank, University Malaya, Malaysia)

from the placenta (Dua *et al.*, 2004). The amnion is a single layered **epithelium** with an occasional area of multiple cell layers away from placenta and umbilical cord (Figure 8).

2. **Basement membrane** consists of mesenchymal connective tissue and an a vascular stroma layer. Histologically, it is a thin a cellular structure of reticular tissue adhered to the base of the amniotic epithelium.
3. **Compact layer** is a thin acellular structure, uniform in depth and density. It is a network of reticular fibres and is interposed between the basement membrane and the underlying fibroblast layer.
4. **Fibroblast layer** is the thickest layer made of loose reticular network with sporadic fibroblasts. It has a thickness of 0.05 to 0.5 mm.
5. **Spongy layer** is the outmost layer connecting amnion to chorion and is made up of fine fibrils complex bathed in mucin. It also contains fibroblasts and Hoffbauer cells.

Electron microscopy of amnion (Figure 9) shows epithelium of cuboidal to polygonal cells covered by numerous microvilli (Farazdaghi *et al.*, 2001).

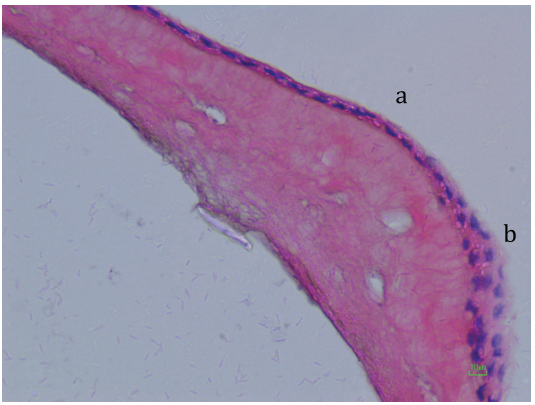


Figure 8. Air-dried amnion with single (a) and multilayered (b) epithelial cells. (Light Microscope, Magnification 40x) (Courtesy: Bone Bank, University Malaya, Malaysia)

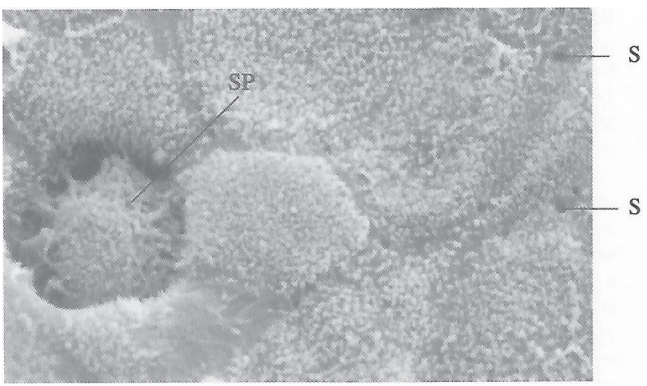
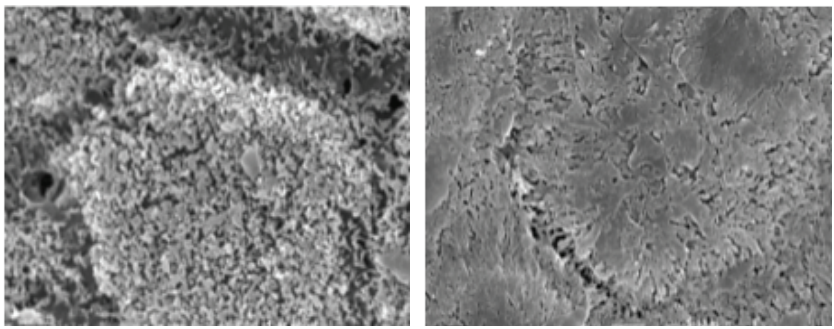


Figure 9. Polygonal epithelial cells with rounded corners and convex roof. Rough surface of cells demonstrates presence of microvilli. Stoma (S) at multicellular junctions. Spider cell (SP) as a precursor to epithelial cell degeneration (SEM, Magnification 3000X) (Farazdaghi *et al.*, 2001)

The height of these cells varies from 4 to 15 µm. Spider cells as a precursor to epithelial cell degeneration are also seen.

The morphological structure of amnion is influenced by the preservation method used and the sampling area chosen. Under scanning electron microscopy (SEM), glycerol preserved cells appeared to be homogenous in size and tended to round up, while the cell structure of the air dried amnion seemed



(R) (L)

Figure 10. Surface cell morphology of glycerol preserved (R) and air-dried amnion (L) (SEM, Magnification 10,000x) (Ab Hamid *et al*., 2014)

to be flattened and dehydrated (Ab Hamid *et al.,* 2014). As in Figure 10, the glycerol preserved amnion has a stucture almost undisturbed and similar to fresh unprocessed amnion.

The air-drying process clearly caused condensation of microvilli and intercellular channels while glycerol preserved the cell morphology. Therefore, glycerol preservation method is recommended for preserving the cell morphological structure of the amniotic membrane mainly for use in ophthalmology surgery (Ab Hamid *et al*., 2014). However the air-drying preservation technique is still acceptable for amnions to be used as a wound dressing because of cheaper processing cost and ease of storage.

Electron micrograph of processed amnion as in Figure 11 showed that surface texture of air-dried amnion is different from freeze-dried amnion (Tan *et al*., 2011). However cell attachment to air-dried and freeze-dried amnion was qualitatively comparable on Day 15. Thus, processed amniotic membranes are potentially valuable as a cell-carrier for mesenchymal stem cells (MSC) in tissue engineering work.

Ultrastructurally, the cells of the fresh human amnion under TEM are high cubical cells (Al-Yahya and Makhlouf, 2013). As in Figure 12, the apical cell membrane exhibits numerous long microvilli and the lateral cell membrane shows complex interdigitation. The basement membrane is thick and formed of collagen fibers. The connective tissue stroma contains collagen fibers and undifferentiated mesenchymal cells.

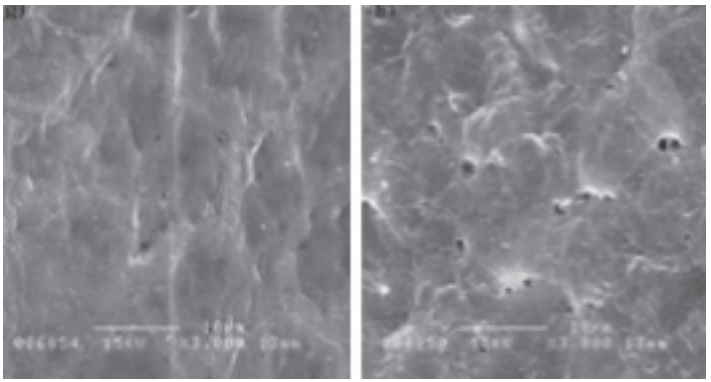


Figure 11. Surface texture of air-dried (R) and freeze-dried amnion (L) under SEM, Magnification 1,000x (Tan *et al.*, 2011)

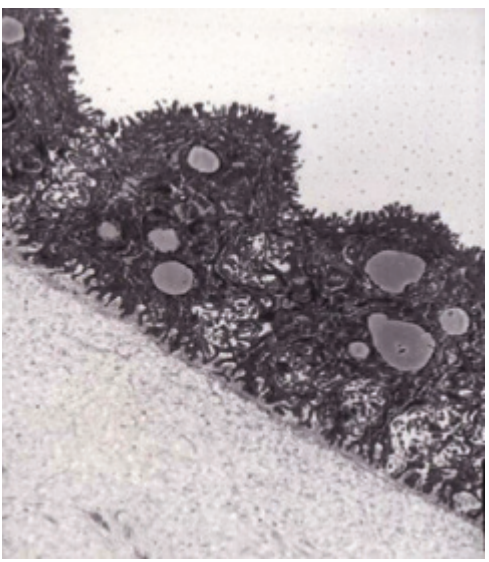


Figure 12. An electron micrograph of fresh amnion: the epithelial lining and underlying connective tissue under TEM, Magnification 3,000x (Al-Yahya and Makhlouf, 2013)

Atomic Force Microscope (AFM)

Atomic force microscopy produced topographic images of amnion and characterised amniotic membrane in terms of cell attachment and proliferation efficiencies for cells in tissue engineering such as limbal epithelium cell culture (Zhang *et al.*, 2013). Topography images of AFM have been used to

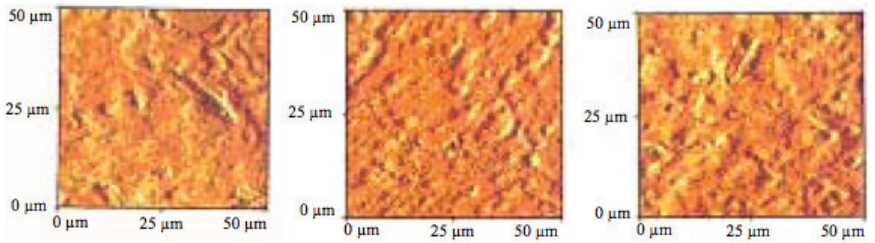


Figure 13. Molecular topography of non-irradiated (A) and irradiated (B: 25 kGy, C: 35 kGy) freeze-dried amnions viewed under an atomic force microscope in a 50x50 μm^2 window (Deocaris *et al.*, 2005)

study biophysical properties such as elasticity and molecular organisation of the amnion surface. In Figure 13, molecular topography of freeze-dried amnions viewed under an atomic force microscope showed ridges to be less pronounced, in terms of depth and density, in the non-irradiated (0 kGy) when compared to irradiated samples at 25 and 35 kGy. These changes are subtle indications of micro- damage to the material due to radiation (Deocaris *et al.*, 2005)

Conclusion

The amniotic membrane is the innermost layer of the fetal membrane. The membrane is derived from foetal ectoderm by cavitation and comprises of the amnion and the chorion. The amnion is a translucent membrane compost of an inner layer of epithelial cells, attached to a basement membrane and three layers of avascular stroma i.e. compact layer, fibroblast layer and spongy layer. Outside the amnion is the chorion laeve comprising of connective tissue containing the foetal vessels. The chorion is close to the decidua basalis, the outermost layer of the foetal membrane. The anatomy and histology of the amnion have been investigated under light microscope, scanning electron microscope and transmission electron microscope.

References

Ab Hamid SS, Zahari NK, Yusof N and Hassan A (2014) Scanning Electron Microscopic Assessment on Surface Morphology of Preserved Human Amniotic Membrane after Gamma Sterilisation. *Cell Tissue Banking*, **15**: 15–24.

Al-Yahya ARAI and Makhlouf MMM (2013) Characterisation of the human amniotic membrane: Histological, immunohistochemical and ultrastructural studies. *Life Sc. J*., **10**(4): 3701–3710.

Aplin JD, Campbell S and Allen TD (1985) The extracellular matrix of human amniotic epithelium: Ultrastucture, composition and deposition. *J. Cell Sci*., **79**: 119–136.

Bennet S, Cullen J, Sherer D and Wood J (2008) Amniotic Fluid. *American Journal of Perinatology*, **10**(2): 101–104

Bourne GL (1960) The microscopic anatomy of the human amnion and chorion. *Am J Obstet. Gynecol*., **79**: 1070–1073.

Bourne GL (1962) The foetal membranes. *Postgrad. Med. J*., **38**(438): 193–201.

D'Lima C, Gajiwala AL and Samant U (2014) Chorionic Membrane: An Efficient Barrier Membrane in Guided Tissue Regeneration. Presented at 15th APASTB/ 13th JSTT Combined Scientific Meeting, Gifu, Japan, 27–29 August 2014.

Deocaris (2005) Structural Damage in Irradiated Human Amnions. *Philippine Journal of Science*, **134**(1): 45–50.

Dua HS, Gomes GAP, King AJ and Maharajan VS (2004) The amniotic membrane in ophthalmology. *Survey of Ophthalmology*, **49**(1): 51–77.

Eastlund TD (2010) Stem cells in Amniotic Membrane. Presented at the 13th International Conference of Asia Pacific Association of Surgical Tissue Bank (APASTB), Bukittinggi, Indonesia.

Farazdaghi M, Adler J and Farazdaghi SM (2001) Electron microscopy of human amniotic membrane. In: Nather A (ed.) *The Scientific Basis of Tissue Transplantation*, World Scientific, Singapore, pp. 149–171.

Fauza D (2004) Amniotic Fluid and Placenta Stem Cells. *Best Practice & Research Clinical Obstetices and Gynaecology*, **18**: 877–891.

Halim SA, Leow AM, Dorai AD and Wan Azman WS (2007) Clinical Application of Irradiated Amnion Grafts: Use of Amnion in Plastic Surgery. In: Nather A, Yusof N and Hilmy N (eds.), *Radiation in Tissue Banking*, World Scientific, Singapore, pp. 365–379.

Hilmy N, Abbas B, and Anas F (2007) Validation for Processing and Irradiation of Amnion Grafts, In: Nather A, Yusof N and Hilmy N (eds.), *Radiation in Tissue Banking*, World Scientific, Singapore, pp. 237–248.

Mohamad H (2001) Anatomy and Embryology of Human Placenta, Amnion and Chorion. In: Nather A (ed.) *The Scientific Basis of Tissue Transplantation*, World Scientific, Singapore, pp. 237–248.

Mohamad H (2007) Amnion for treatment of bones. In: Nather A, Yusof N and Hilmy N (eds.), *Radiation in Tissue Banking*, World Scientific, Singapore, pp. 343–354.

Niknejad H, Peirovi H, Jorjani M, Ahmadiani A, Ghanavi J and Seifalian MA (2008) Properties of the Amniotic Membrane for Potential Use in Tissue Engineering. *European Cells and Materials*, **15**: 88–9.

Riau AK, Beuerman RW, Lim LS, Mehta JS (2010) Preservation, sterilisation and de-epithelialisation of human amniotic membrane for use in ocular surface reconstruction. *Biomaterials*, **31**: 216–225.

Tan SL, Sulaiman S, Pingguan-Murphy B, Selvaratnam L, Tai CC and Kamarul T (2011) Human amnion as a novel cell delivery vehicle for chondrogenic mesenchymal stem cells. *Cell Tissue Bank*, **12**: 59–70.

Thomas CE (1965) The ultrastructure of human amnion epithelium. *J. Ultrastruct. Res.*, **13**: 65–84.

von Versen-Hoynck F, Syring C, Bachmann S and Moller DE (2004) The influence of different preservation and sterilisation steps on the histological properties of amnion allografts — light and scanning electron microscopic studies. *Cell and Tissue Banking*, **5**: 45–56.

Wikipedia.org (2015) Amnion Fluid http://en.wikipedia.org/wiki/Amniotic_fluid

6

Biological Properties and Functions of Amnion

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Introduction

Recently cellular therapy has emerged as a new potential tool for treatment of a wide range of degenerative diseases. Regenerative medicine requires cell sources in addition to biomaterial scaffolds that contain growth factors/cytokines. Many potential cell sources for cell replacement therapy have to be safe and sufficient cell supply is needed for clinical application. One of the choices to overcome this problem is the utilisation of amniotic membranes.

Amniotic membrane (AM) or amnion is a thin membrane on the inner side of the fetal placenta; it completely surrounds the embryo and delimits the amniotic cavity, which is filled by amniotic liquid. It is easy to procure from the placenta and its use has no ethical issues since it is a discarded tissue after parturition. In recent years, the structure and function of the amnion have been investigated, which are an attractive source for tissue transplantation. AM has anti-inflammatory, anti-bacterial, anti-viral and low immunogenicity characteristics, as well as anti-angiogenic and proapoptotic features.

AM is a promoter of epithelialisation and non-tumorigenic tissue (Mamede *et al.*, 2012; Francisco *et al.*, 2013).

The amniotic membrane (AM) is a tissue of particular interest because its cells also possess characteristics of stem cells with multipotent differentiation ability. Amniotic membrane (AM) has been employed in the treatment of wounds for several decades. AM is rich in collagen and various cytokines and growth factor that improve the healing process and reduce scar formation (Carmen, 2010).

The application of this material nowdays has been extended to include wounds of other areas, including diabetic neurovascular ulcers, postoperative/ posttraumatic or burn wounds, and other potential uses as a reparative membrane in a number of other medical fields. (Donald, 2012).

Amniotic Membrane Basic Structure

The amnion membrane is the internal layer of the placenta and is in contact with the amnion liquid. Macroscopically the amnion membrane is thin, transparent, resistant, and adherent to the chorion. The normal amnion membrane is 0.02–0.05 mm thin, which is equivalent to 6–8 rows of cells, and has a surface of 1600 cm^2 (Andonovska D. *et al.*, 2008). The amnion is the fetal side of the membrane, and chorion is the maternal side. They are separated by a jelly like matrix.

Functionally AM not only provides a physical support for the fetus, but also serves as a metabolically active filter with a direct interaction with the amniotic fluid (Kerry Rennie *et al.*, 2012).

Human AM is composed of a layer of simple epithelium cells firmly adhered to the mesenchymal tissue, composed of three layers: compact, spongy and fibroblastic layer. These layers contain large amounts of collagen (type IV and V) laminin and a thick basal avascular layer. Externally, the amnion is located in the chorion, comprising a connective tissue that presents fetal vessels (chorioallantoic) (Francisco *et al.*, 2013).

The AM contains no blood vessels or nerves. Instead, the nutrients it requires are supplied directly by diffusion of the amniotic fluid and from the underlining decidua.

Histologically, the amnion membrane consists of a thin cuboidal epithelium which expresses cytokeratin 7, a thick basal membrane and an avascular stromal matrix (mesenchymal layer) which contains vimentin (Figure 1).

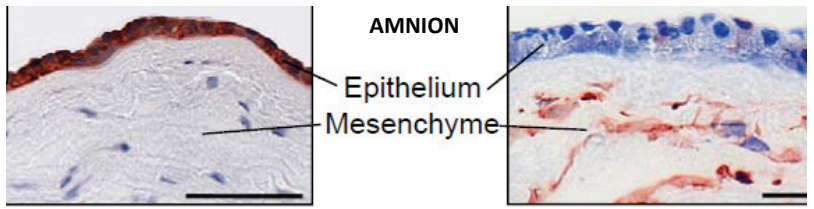


Figure 1. Overview of Amniotic membrane. (a) Epithelium layer expressing cytokeratin-7. (b) Mesenchymal layer which cells stained by anti-vimentin

Table 1. Structure and composition of amniotic membrane (Gupta, 2015)

Placental membrane	Layers of placental membrane	Extracellular matrix component
Amnion	1. Epithelium monolayer	Single layer of cells
	2. Basement membrane	Collagen types III,IV,V, Fibronectin, laminin, nidogen
	3. Compact layer	Collagen type I,III,V,VI, and fibronectin
	4. Fibroblast layer	Collagen types I,III,VI, laminin, nidogen
	5. Intermediate/spongy layer	Collagen type I,III,IV and proteoglycan
Chorion	1. Reticular layer	Collagen types I,III,IV,V,VI and proteogylcan
	2. Basement membrane	Collagen type IV, fibronectin and laminin
	3. Trophoblast	

Structure and composition of amniotic membrane showed in Table 1. Amniotic membrane is composed of 3 types of material:

- Structural collagen and extracellular matrix
- Biologically active cell
- Regenerative biomolecule layer (Donald, 2012).

Biological Properties of Amniotic Membrane and Functions (Table 2)

Amniotic Membrane has multiple metabolic functions — its role in water and soluble material transportation and the production of various growth

Table 2. Biological properties of amniotic membrane and function (Gupta, 2015)

Immune system	Epithelial tissue	Mesenchymal tissue	Biomechanical properties
Suppression of inflammation	Stimulation of growth epithelial cells	TGFβ: suppression of myofibroblastic differentiation	Avascular, devoid of cells, no immune response
Anti-bacterial and anti-viral factors	Epithelial growth factor (EGFR) and keratinocyte growth factor (KGF)	Reduction of scarring	Durable and puncture resistance
Induction of apoptosis of inflammatory cells		Axonal regeneration	Elastic and translucent
		Neural growth factor	Serve as a physical barrier

factors, cytokines and bioactive peptides (Gupta, 2015). The functions of biological properties in AM could be used as curative effect of AM. Literature revealed growth factors and cytokines to be present in amniotic membrane. They include epidermal growth factor (EGF), basic fibroblast growth factor (bFGF), keratinocyte growth factor (KGF), transforming growth factor (TGF) alpha and beta, hepatocyte growth factor (HGF), and nerve growth factor (NGF). They have been identified in fresh and preserved amniotic tissues (Koob TJ *et al.*, 2014).

The functions of utilisation of Amniotic Membrane: (Donald 2012).

1. Promotes epithelialisation

Amniotic membrane facilitates epithelial cell migration, strengthens basal cell adhesion, promotes epithelial cell differentiation and prevents of epithelial apoptosis. AM promotes epithelialisation of wound healing. Regenerative biomolecules important in the healing and growth process are concentrated in the amniotic membrane. These include epidermal growth factor (EGF), transforming growth factor (TGF) beta, fibroblast growth factor (FGF),

platelet derived growth factor (PFGF), metalloproteinases, and tissue inhibitor metalloproteinases (TIMPS) (Donald, 2012).

Amniotic membrane acts as a scaffold for proliferation and differentiation. This is due to the basement membrane containing collagen type IV, V and VII which facilitates epithelial cell migration. It also contains fibronectin, elastin, nidogen, hyaluronic acid. Nidogen is abundant in the stroma of AM (Rashedul, 2015).

2. Inhibits fibrosis

Fibroblasts are responsible for scar formation during wound healing, activated by TGFβ. Amniotic membrane reduces the risk of fibrosis by the effect of down-regulation of TGFβ. AM down-regulates TGF-beta and its receptor expression by fibroblasts causes a reduced fibrosis. This also modulates the healing of a wound by promoting tissue reconstruction.

AM secretes vascular endothelial growth factor (VEGF) and hepatocyte growth factor (HGF) which maintains a proper balance between TGF-1and TGF-3 to prevent scarring. Various immune cells like T cells, dendritic cells and B cells are actively suppressed. This prevents pathological remodeling and excessive fibrosis (Supriya Mishra, 2014).

3. Inhibits angiogenesis and inflammation

Anti-angiogenic chemicals identified in both epithelial and mesenchymal cells of AM are thrombospondin-1, endostatin and 4 types of tissue inhibitors of metalloproteinases (TIMP-1, 2, 3 and 4) (Rashedul, 2015).

Amniotic membrane contains cytokines that can suppress inflammatory process i.e Interleukin 10 (IL-10) and Interleukin-1 receptor antagonist (IR-1RA). IL-10 can suppress the effect of pro-inflammatory cytokine IL-6 and tumor necrosis factor α (THFα). It also suppresses the production of IL-8 a pro-inflammatory chemokine which attracts migration of neutrophil. ELISA assays identified the presence of ILs 4, 6, 8 and 10, and TIMPs 1, 2 and 4 (Koob, 2013). Human Amniotic Epithelial Cells (hAEC) have anti-inflammatory properties that inhibit the migration of neutrophils and macrophages to the site of injury, mediated by Migration Inhibitory

Factor (MIF) as well as the suppression of IL-1a, IL-1b and proteinase activity.

hAEC also releases several anti-inflammatory factors that have effects:

- reduces HLA Class II antigen presenting cells present at site of injury
- prevents apoptosis
- enhances wound healing and host cell proliferation
- suppresses pro-fibrotic TGFb isoforms and type II TGFb receptors in myofibroblast cells that deposit collagen which lead to fibrosis.

4. Lack of immunogenicity

Amniotic Epithelial Cells do not express HLA-A,-B,-D and DR antigen and beta2 macroglobulin. This combined with the presence of immunosuppressive cytokine IL-4, IL-10 and TGF results in "immunologically privileged" material. This may be the reason for lack of occurrence of immune rejection.

5. Anti-bacterial properties

AM contains natural anti-microbial molecules, which are part of an innate immune system and acts against Gram positive and Gram negative bacteria, viral and fungal infections. AM also have the ability to produce β defensin, a major group with anti-microbial and anti-viral properties: cystatin E, the analog cysteine proteinase inhibitor. AM plays a role as a barrier to prevent infiltration and adhesion of microorganism to the wound surface. The collagen fibers of AM basement membrane have haemostatic properties. This reduces bacterial load and risk of infection by preventing accumulation of microbes.

The adhesion of the wound surface also prevents formation of dead space and accumulation of serous discharge. This mechanism also prevents infection. Bacterial entrapment and stimulation of migration of phagocytes occur by fibrin filaments formed during wound healing. The bacterial count is reduced in wounds covered by AM (Gupta, 2015).

6. Reduces pain at the site of application

It diminishes inflammation and provides a better state of hydration that soothes the wound bed to promote faster healing. The soft mucoid lining of

amniotic membrane protects the exposed nerve endings from external irritants that help to decrease pain sensation (Supriya Mishra, 2014).

7. Contains essential growth factors and cytokines

The amniotic membrane is a metabolically active tissue that continually remodels and grows to accommodate the growing conceptus. Remodeling of the tissue is conducted by cytokines, growth factors, chemokines, and related regulatory factors produced by the endogenous cells in the amniotic membrane (Koob TJ *et al.*, 2014).

Amnion-produced growth factors participate in angiogenesis, re-epithelialisation, and immunomodulation as shown in *in vitro* studies. Some factors stimulate cell differentiation and proliferation, like macrophage colony-stimulating factor (M-CSF), which was detected in cultures of human amniocytes. Other factors, such as IGF-2, were found in mRNA of human amniotic epithelial cells, where they may promote proliferation. Cultured amniotic epithelial cells secrete various morphogens and growth factors such as epidermal growth factor (EGF), PDGF, Noggin, Activin, VEGF, TGFβ2, and TIMPS (Zofia Grzywocz *et al.*, 2008).

Amniotic Membrane Derived Stem Cells

Amniotic epithelium is a tissue of epiblastic origin. The stem cell characteristics of the amniotic epithelium demonstrated their ability to differentiate into the three germinal layers: ectoderm, mesoderm and endoderm (Miki *et al.*, 2005; Miki and Strom, 2006). Amniotic epithelial cells isolated from AM express stem cell markers associated with human embryonic stem cells such as : octamer-4 OCT-4, sex determining region Y-box2 Sox-2, FGF-4, Rex-1, CFC-1, ABCG2/BCRP (a member of ATP-binding superfamily that works as efflux pump), CD9, CD24,E-Cadherin, Integrin α 6 and β, c-met (receptor growth factor of hepatocyte), NANOG, Lefty-A, DPPA3, PROM1, PAX6, SSEA-3, SSEA-4, tumor rejection antigen (TRA1-6), TRA1-81, terato carcinoma-derived growth factor 1 (TDGF-1) and GCTM2 (confirmed by RT-PCR and immunocytochemistry technique).

The isolated human Amniotic Epithelial Cells (hAECs) show a heterogenous phenotype, suggesting that there is a different sub-population of cells.

The purity of isolates of AM epithelial cells can be assessed by flow analyses for markers such as HLA-G, cytokeratins 7, 8 and 18. The majority of the epithelial cells also express a typical stromal marker-vimentin (Ilancheran, 2008).

The ratio of stem cell positive marker AE cells is 5%–50%. It is considerably higher than other somatic/ tissue stem cells. Fetal amniotic epithelium contains more stem cells than amniotic epithelium (Ilancheran, 2008; Miki, 2011; Carmen *et al.*, 2010).

Biological Contents of Irradiated Freeze-Dried Amnion Membrane and Glycerol Cryopreserved Amnion Membrane

Ideally, for clinical use, AM should be sterile and free of contamination. It should also be easy to obtain, transport, and store for long periods without deterioration. Therefore, irradiated sterilised dehydrated AM has recently been used for several cases of wounds. The human AMs were obtained at the time of elective Cesarean Section in volunteers who were seronegative for Human Immunodeficiency Virus, Human Hepatitis B and C, and Syphilis. After several steps of processing, finally irradiation (25 kGy) was used to sterilise the resultant Freeze-Dried (FD) AM. Bacteriologic tests were performed on the cultures to confirm sterilisation.

The sterilised, Freeze-Dried AM retained most of the physical, biological, and morphologic characteristics of cryopreserved AM. Consequently, it is a useful biomaterial for ocular surface reconstruction.

The extracellular matrix macromolecules play an important role in the physical and biological properties of AM. Immunohistochemistry study demonstrates that collagen (types I, III, IV, and V) and fibronectin are expressed in the whole FD-AM. In contrast, collagen- VIII and laminin-5 were observed in the basement membrane side of FD-AM. These results are similar to those of cryopreserved AM. On the basis of these physical, immunohistochemical, and microscopic examinations, the process of drying and irradiation does not affect the physical or biological properties of AM (Nakamura, 2004). Determination of mRNA gene expression of growth factors in glycerol cryopreserved human amniotic membrane (HAM), gamma irradiated at 15 kGy has been done by Rusidah *et al.*, 2014. Cryopreserved fresh amnion was used for comparison. The results showed

up-regulation of HGF, KGF, KGFR, TGF-B2, TGF-B3, and bFGF and down-regulation of EGF, HGFR, TGF-a, and TGF-B1. The expressions were not significantly different compared to the fresh group. It can be concluded that the growth factors were not affected by irradiation at 15 kGy i.e. radiation sterilisation dose.

Amniotic membrane: Clinical Application

Biocompatibility of scaffold is required for tissue engineering. Biocompatibility refers to the property of being biologically compatible and not producing any toxic, injurious, carcinogenic, mutagenic or immunological response in living tissue (Weiss *et al.*, 2008). Mechanical properties of AM also added to the advantages of AM (Baguneid *et al.*, 2006; Yang *et al.*, 2001):

- Permeability
- Stability
- Elasticity
- Flexibility
- Resorbability

Amniotic membrane had been used for treatment of burns. The main principles in the treatment of minor burns are control of pain, control of infection, promotion of healing, and decreasing repeated trauma to the burn surface. All of these goals can be achieved by using amniotic membrane dressing. It had been investigated by comparing burns treated with silver sulfadiazine versus burns treated with amniotic membrane dressings. The wounds treated with AM showed better results (A.A.Mohammadi, 2009).

The components of ECM in AM, e.g collagen, laminin, fibronectin and vitronectin within the basement membrane promotes adhesion and growth of the overlying stem cells (Niknejad *et al.*, 2008). It has been shown that epithelial and mesenchymal cells on amnion scaffold are highly interconnected and capable of penetrating the porous structure of scaffold. Amnions are frequently used in reconstruction of ocular surface and skin (Yang *et al.*, 2001, 2006; Fatima *et al.*, 2006). Collagen type IV, V and VII are important components for structural integrity of the membrane.

Conclusion

The amniotic membrane has several advantages because its cells have characteristics of stem cells with multipotent differentiation ability. It has low immunogenicity and is easy to procure. There is little ethical barrier to their use. The biological properties of amniotic membrane have specific characteristics that promote wound healing. Amniotic membrane is a good material to use with wide clinical applications in cellular therapy and regenerative medicine.

References

Andonovska D, Dzokic G, Spasevska L, Trajkovska T, Popovska K, Todorov I, Petrovski P, Kondov G, Sapova B, Marcikic G, Atanasova E, Obocki E, J Ugrinovska J, Vasilevska V and Zogovska EM (2008) The advantages of the application of amnion membrane in the treatment of burns. *Sec. Biol. Med. Sci.*, **1**: 183–198.

Carmen MM, Ciuca DR, Soritau O, Susman S and Mihua D (2009) Isolation and characterization of mesenchymal stem cells from the amniotic membrane. *Romanian Journal of Morphology and Embryology*, **50**(1): 73–77.

Fetterolf DE and Snyder RJ (2012) Membrane in wound management. *Wounds*, **24**(10): 299–307.

Francisco JS, Cunha RC, Simeoni RB, Guarita-Souza LC, Ferreira RJ, Irioda CM, Maria CC, Souza O, Srikanth GVN, Nityanand, Chacques JC and de Carvalho AT (2013) Amniotic membrane as a potent source of stem cells and a matrix for engineering heart tissue. *J. Biomedical Science and Engineering*, **6**: 1178–1185.

Grzywoczb Z, Pius-Sadowska E, Klos P, Gryzik M, Wasilewska D, Aleksandrowicz B, Dworczynska M, Sabalinska S, Hoser G, Machalinski B and Kawiak J (2008) Growth factors and their receptors derived from human amniotic cells *in vitro*. *Folia Histochemica*, **52**(3): 183–198.

Gupta A, Kedige SD and Jain K (2015) Amnion and chorion membranes: Potential stem cell reservoir with wide applications in periodontics. *Hindawi Publishing Corporation International Journal of Biomaterials*, 1–9.

Ilancheran S, Moodley Y and Manuelpillai U (2009) Human fetal membranes: A source of stem cells for tissue regeneration and repair?. *Placenta*, **30**: 2–10.

Insausti CL, Blanquer M, Bleda P, Iniesta P, Majado MJ, Castellanos G and Moraleda JM (2010) The amniotic membrane as a source of stem cells. *Histol. Histopathol.*, **25**: 91–98.

Islam R, Rahman S, Asaduzzaman SM and Rahman MS (2015) Properties and therapeutic potential of human amniotic membrane. *Asian Journal of Dermatology*, **7**(1): 1–12.

Koob TJ, Rennert R, Zabek N, Massee M, Lim JJ, Temenoff JS, Li WW and Gurtner G (2013) Biological properties of dehydrated human amnion/chorion composite graft: Implications for chronic wound healing. *Int. Wound J*., 1–8.

Koob TJ, Lim JJ, Zabek N and Massee N (2014) Cytokines in single layer amnion allografts compared to multilayer amnion/chorion allografts for wound healing. *J. Biomed. Mater. Res. Part B*., **103B**: 1133–1140.

Koob TJ, Lim J, Massee M, Zabek N, Rennert R, Gurtner G and Li W (2014) Angiogenic properties of dehydrated human amnion/chorion allografts: Therapeutic potential for soft tissue repair and regeneration. *Vascular Cell*, **6**: 1–10.

Lindenmair A, Hatlapatka T, Kollwig G, Hennerbichler S , Gabriel C, Wolbank S, Redl H, Kasper C, Hatlapatka T and Kollwig G (2012) Mesenchymal stem or stromal cells from amnion and umbilical cord tissue and their potential for clinical applications. *Cells*, **1**: 1061–1088.

Mamede C, Carvalho MJ, Abrantes AM, Laranjo M, Maia CJ and Botelho MF (2012) Amniotic membrane: From structure and functions to clinical applications. *Cell Tissue Res*., **349**: 447–458.

Miki T (2011) Amnion-derived stem cells: in quest of clinical applications. *Miki Stem Cell Research & Therapy*, **2**: 25.

Mishra S and Singh S (2014) Human amniotic membrane: can it be a ray of hope in periodontal regeneration. *PARIPEX — Indian Journal of Research 3*.

Mohammadi AA, Sabet B, Riazi H, Tavakkolian AR, Mohammadi MK and Iranpak S (2009) Human amniotic membrane dressing: An excellent method for outpatient management of burn wounds. *Iran J. Med. Sci*., **34**(1): 61–64.

Nakamura T, Yoshitani M, Rigby H, Fullwood NJ, Ito W, Inatomi T, Sotozono C, Nakamura T, Shimizu Y and Kinoshita S (2004) Sterilized, Freeze-Dried Amniotic Membrane: A Useful Substrate for Ocular Surface Reconstruction. *IOVS*, **45**(1): 92–99.

Niknejad H, Peirovi H, Jorjani M, Ahmadiani A, Ghanavi J and Seifalian AM (2008) Properties of the amniotic membrane for potential use in tissue. *European Cells and Material*, **15**: 88–99.

Rennie K, Gruslin A, Hengstschlager M, Pei D, Cai J, Nikaido T and Yaghoub MB (2012) Applications of amniotic membrane and fluid in stem cell biology and regenerative medicine. *Hindawi Publishing Corporation Stem Cells Scientific and Clinical Support for the Use of Dehydrated Amniotic.*

Rusidah MY, Kannan TP, Suzina SAH and Hilda S (2014) Gene expression of Growth Factors in Irradiated Glycerol Cryopreserved Human Amniotic Membrane. Presented at the *15th APASTB /JSTT Combined Scientific Meeting*, Gifu, Japan.

Silini A, Parolini O, Huppertz B and Lang I (2013) Soluble factors of amnion-derived cells in treatment of inflammatory and fibrotic pathologies. *Current Stem Cell Research & Therapy*, **8**: 6–14.

7

Physical Properties of Amnion

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Introduction

Amnion is comparable to other wound dressings, easy to obtain and has ideal biological properties for promoting wound and ulcer healing. It adheres tightly to wound surfaces, conform to wound contours and is able to maintain moist environment which is required for healing process. Its ability to shield the exposed nerve endings from external elements reduces or diminishes the pain (Halim *et al.*, 2007). However all activities involving processing, preservation and sterilisation of amniotic membranes are equally responsible in determining final properties and morphological structure of amnion grafts as end products. Preservation techniques such as freezing, freeze-drying, air-drying, oven-drying and soaking in glycerol are vital for long term storage whilst sterilisation by radiation will prolong shelf life.

Properties of Amnion

Amnion has physical and biological properties of required criteria for an ideal biological dressing as shown in Table 1 (Mohamad, 2007).

Table 1. Physical and biological properties of amnion as an ideal biological dressing

Properties	Effects
1. Effective barrier	Reduces heat, fluid and protein loss
2. Good adherence and durability	Reduces contamination and provide moist environment which enhances healing process
3. Bacteriostatic effect	Reduces incidence of infection and septicemia
4. Analgesic effect	Reduces pain and analgesic usage
5. Non-antigenic effect	No immunologic effect on patients
6. Lightweight and elastic material	Conforms easily to the body surface and contours
7. Thin, flexible and easily cover joints	Allows early mobilisation of patients
8. Transparent	Easy to monitor the healing process

Amniotic membrane does not have blood vessels, lymphatic vessels and nerve tissue. Therefore the membrane has no immunological response and is non-antigenic hence suitable for transplantation (Farazdaghi *et al.*, 2001). The membrane is also angiogenic which stimulates neovascularisation and formation of new blood vessels as it can generate cell granulation in treating ulcer. On the other hand, the membrane can also act as anti-angiogenic as it releases anti-angiogenic chemicals from epithelium and mesenchymal cells, the property which is mainly demonstrated by chorion layer. Allantoin, lysozyme, transferrin and progesterone found in basement membrane are responsible in healing mechanism and giving bacteriostatic and bactericidal effects. Anti-inflammatory and anti-scarring properties are closely related to amnion effect on apoptosis or the death process of keratocyte cells (Dua *et al.*, 2004).

Physical Properties

The physical properties of preserved amnion that have been widely studied are:

- Thickness
- Biomechanical properties — tensile strength and elongation. Even though amnion is not stretched out when applied to wound, some strength is required during handling.

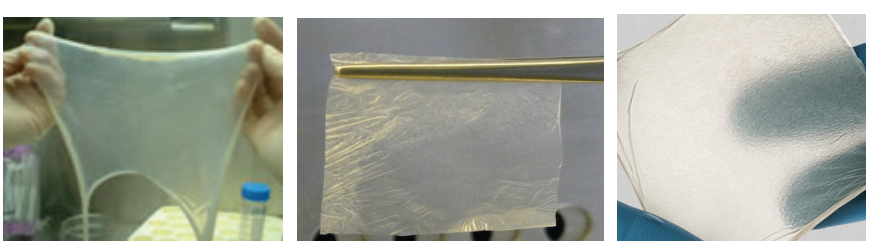
- Water Absorption (WA)
- Water Vapour Transmission Rate (WVTR)
- Oxygen Transmission Rate (OTR)

Among these, permeability of amnion to oxygen and water vapour are important properties to determine the effective clinical use of amnion as biological wound dressing. However its structure should be impermeable to microbes (Singh *et al.*, 2003). These properties enable amnion to function as a biological barrier to external contamination while providing moist environment to the wound surface for healing process to take place (Singh and Charcharkar, 2011).

1. Thickness

Despite being thin and transparent, amniotic membrane is rather tough and is able to protect growing foetus during pregnancy (Figure 1). The membrane is strong and yet elastic and soft (Arya *et al.*, 2010). The thickness is very much influenced by processing techniques (Figure 2). This collagen-rich membrane with thickness ranging from 0.02 to 0.5 mm (20 to 500 μm) after processing has been used widely as a biological dressing since early 20th century (Manjas *et al.*, 2007; Mohamad, 2007). Air-dried amnion has thickness ranging from 16.3 to 27.2 μm (Yusof, 1997).

Freeze-drying technique for preservation reduced the thickness of fresh amnion from 65 μm to 20 μm. However, the thickness increased to 463 μm with freezing (John and John, 2001). This thick frozen amnion is suitable in



(a) (b) (c)

Figure 1. Amnion: (a) Fresh after processing, (b) After air-drying, (c) After glycerol-preservation



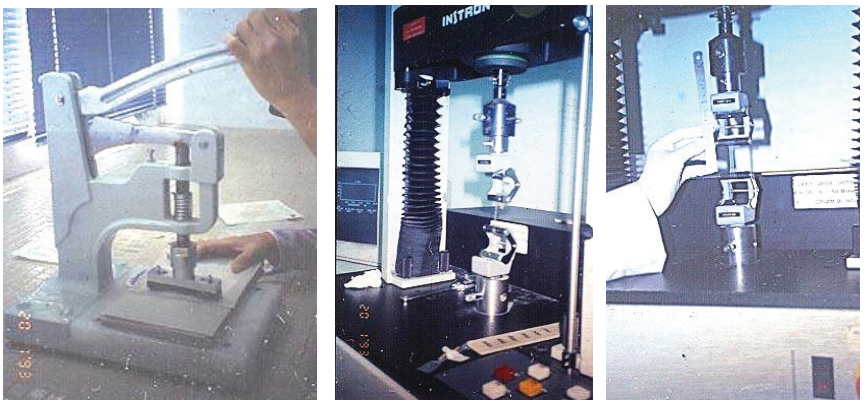
Figure 2. Measuring thickness of amnion using thickness gauge

ophthalmologic surgeries for reconstruction of ocular surface and pterygium surgery.

The glycerolised amnion is thicker (45–50 μm) than the freeze dried amnion (20–30 μm) (von Versen-Hoeynck *et al.,* 2004). Glycerolisation leads to an insertion of hydrophilic glycerol causing the cells to swell while drying leads to a withdrawal of liquid from the membrane (von Versen-Hoeynck *et al.*, 2008). Confirming this, under the scanning electron microscope (SEM) the morphological structure of epithelium cells of the glycerolised amnion is well preserved while the cells after air-drying are flattened (Ab Hamid *et al.*, 2014).

2. Biomechanical Properties

Biomechanical properties namely tensile strength and elongation are influenced by processing, preservation and sterilisation (Figure 3). The tests are useful in establishing and validating processing procedures and assisting tissue bankers in identifying suitable radiation doses that will not cause detrimental effects while sterilising the amnions (Yusof and Hilmy, 2007). The properties will also indicate stability of amnion after irradiation and storage thus determining shelf life of the sterile amnion grafts.



(a) (b) (c)

Figure 3. Universal testing machine for tensile strength determination: (a) Amnion is cut into dumbell shape, (b) Amnion is placed in the sample holders of universal testing machine and streched at the set speed and load, (c) The distance between the holders is measured after the amnion ruptured

Table 2. Effect of radiation on tensile strength of freeze-dried and air-dried amnions (kg/m^2)

	Non-irradiated	Irradiated	References
Freeze-dried amnion	0.023	0.035 (at 25 kGy) 0.009 (at 35 kGy)	Deocaris *et al.* (2005)
Air-dried amnion	0.033	0.038 (at 17 kGy)	Yusof and Hilmy (2007)

In Table 2, radiation at 25 and 17 kGy showed no effect on the tensile strength of freeze-dried and air-dried amnions, respectively. However, the tensile strength significantly decreased after 35 kGy (Deocaris *et al.* 2005; Yusof and Hilmy, 2007).

In Table 3, the air-dried amnion sterilised at 17 kGy and stored up to 20 months showed mechanical properties comparable to freshly processed amnions (Yusof, 1997). However, the tensile strength started to reduce after 34 months (Yusof and Ozkara, 2000). Air-dried sterile amnions maintain their colour, physical appearance and biomechanical properties even after 6 years of storage at room temperature as compared to fresh amnions which can only be stored for less than a week (Yusof and Hilmy, 2007). Therefore, there is no effect of storage up to 2 years on biomechanical properties of amnion.

Table 3. Biomechanical properties of air-dried amnion gamma irradiated (17 kGy) and stored at room temperature (Yusof, 1997; Yusof and Ozkara, 2000)

Storage (month)	Tensile strength (kg/cm^2)	Elongation (%)
0 (control)	209.00	13.45
4	304.97	12.25
6	405.51	12.73
8	239.00	9.81
12	488.60	12.46
16	279.89	15.82
18	327.27	14.05
20	267.50	14.38
34	64.39	11.30
36	78.90	31.30
49	147.76	20.97
52	158.78	20.07
54	154.63	18.97
70	102.87	5.90
72	205.43	16.90

In freeze-dried gamma irradiated (30 kGy) amnions, tensile strength and elongation decreased by 50% after a year of storage (Hilmy *et al.*, 1987; 1992).

3. Water Absorption (WA)

Water absorption (WA) is the capacity of amnion to absorb fluid. This property contributes to the ability of amnion to absorb wound exudate and also in creating moist microenvironment. The WA is not affected by:

- **Drying technique** — The WA capability of air-dried ($2.33\%/cm^2/h$) and the freeze-dried amnion ($2.725\%/cm^2/h$) are almost similar (Yusof and Hilmy, 2007).

- **Gamma radiation** — Doses of 25, 35 and 50 kGy did not change the WA of air-dried amnions (0.122–0.139 g/cm^2/h) (Singh *et al.*, 2007).
- **Storage** — The WA of amnions remained unchanged after 6-month storage at different temperatures and humidities (Singh *et al.*, 2004).

4. Water Vapour Transmission Rate (WVTR)

Amnion as a biological wound dressing is able to reduce fluid and electrolyte loss while allowing water vapour transmission in order to prevent dehydration and keep the wound surface moist conducive for healing process (Singh *et al.*, 2007). Water vapour transmission rate (WVTR) is performed according to ASTM E96, the standard for determining water vapour transmission property for thin film.

The freeze-dried amnion has the WVTR higher than the air-dried amnion. However, the WVTR for both were equalised after leaving for 24 hours at room temperature (Yusof and Hilmy, 2007), suggesting that the better preserved cell structure with freeze-drying allowed the membrane to transmit more water molecules initially.

The water vapour permeability of the glycerolised and the air-dried amnions are improved as the WVTRs are higher than the fresh or frozen amnions (Nor Kamalia *et al.*, 2014; von Verson-Hoeynck *et al.,* 2008). Therefore, the preservation techniques have influence on the physical properties of the amnion.

As in Table 4, the improvement in the WVTR by glycerolisation and air-drying preservation techniques does not appear to be in direct correlation with the thickness of the amnions. Even though the amnion is flattened after air-drying, the water vapour still can transmit through the intercellular gaps.

Table 4. Thickness and WVTR of amnions after different preservation techniques (Nor Kamalia *et al.*, 2014)

Preservation techniques	Median ± SD (μm)	WVTR at 24 h (g/cm2/h)
Fresh amnion	40 ± 10.98	0.0168 ± 0.0151
Air-dried amnion	10 ± 5.94	0.0252 ± 0.0258
Glycerolised amnion	70 ± 17.90	0.0296 ± 0.0283

The **WVTR** of air-dried amnions is not significantly affected by 25, 36 and 50 kGy (Singh *et al.*, 2007) and the property remains unchanged after 6-month storage at different temperatures and humidity (Singh *et al.*, 2004). However other studies reported that gamma radiation at 15 and 25 kGy reduced the WVTR of air-dried amnion while the WVTR of glycerolised amnion was not affected at 25 and 35 kGy (Zahari *et al.*, 2015). Glycerol having hydrophilic property seemed to attract water molecules to enter and pass through the cells and at the same time acts as radioprotectant.

5. Oxygen Transmission Rate (OTR)

Availability of oxygen is important for accelerating epithelialisation (von Verson-Hoeynck *et al.*, 2008) and collagen synthesis (Nangia *et al.*, 1991) during the healing of wound. High water content in amnions helps to restore the epithelial health by permitting high oxygen permeability (Yoshita *et al.*, 2004). Oxygen transmission rate (OTR) is the measurement of oxygen permeability. Analysis of oxygen transmission rate (OTR) can be performed according to international standards such as ASTM D 3985-95, the standard test method for oxygen gas transmission rate through plastic film and sheet using a coulometric sensor and changes in the OTR value are evaluated using Oxygen Permeation Analyser.

As in Table 5, the air-dried amnion is almost three times more permeable to oxygen than the glycerolised amnion (von Verson-Hoeynck *et al.*, 2008; Zahari *et al.*, 2015). The preservation techniques tend to reduce the ability of amnion to transmit oxygen.

The OTR of the amnions seemed to be influenced by the preservation methods due to changes in the cellular properties. Air-drying process at room

Table 5. Oxygen Transmission Rate in amnions after different preservation techniques

References	Fresh	Air-dried	Glycerolised
von Verson-Hoeynck *et al.* (2008) — DIN 53380-3		1.54L/(m^2.24h)	0.54L/(m^2.24h)
Zahari *et al.* (2015) — ASTM D 3985-95	16.67 cc/m^2/day	15.55 cc/m^2/day	4.28 cc/m^2/day

temperature reduces the water content thus the dehydration causes the cells to collapse. The air dried amnion appeared as a thin film with lesser intercellular gaps than the fresh, causing less oxygen transmission compared to the fresh amnion (Zahari *et al.,* 2015). Instead, the cell structure of the glycerolised amnion is rounded and close to each other that might narrow the gaps between cells as observed under the SEM thus limiting further oxygen transmission (Ab Hamid *et al.*, 2014).

6. Other Properties

The amniotic membranes were found to be impermeable to different strains of bacteria — *Bacillus*, *Eschericia coli, Pseudomonas, Citrobacter, Flavimonas* and *Staphylococcus* and no effect on the microbial impermeability of the membrane was observed after 6 months storage (Singh *et al.*, 2003).

Gamma radiation at 25, 36 and 50 kGy did not cause any degradation based on the unchanged characteristic of amide using FTIR therefore radiation sterilisation does not evoke undesirable changes in the functional properties of the amnion (Singh *et al.*, 2007).

The morphological structure of amnion is influenced by the preservation methods hence changing its physical properties. Cells of air-dried amnion with more intercellular gaps allow higher oxygen transmission compared to glycerolised amnion with lesser intercellular gaps between the rounded up cells. On the contrary, higher water vapour is transmitted through well-structured cells containing glycerol compared to flattened cells in air-dried amnion. Large intercellular gaps due to direct effects of radiation at 35 kGy seemed to allow higher WVTR and OTR in both air dried and glycerolised amnions (Zahari *et al.*, 2015).

Radiation at 25kGy may cause further changes in the structure of the epithelium of the glycerolised amnion (Rejzek *et al.*, 2001). However, it does not cause gross damage and only produces a rough surface with bigger gaps at 35 kGy (Ab Hamid *et al.*, 2014).

Conclusion

Preservation methods influence the morphological structures of amnion hence its physical properties. Drying techniques reduce the thickness of

amnion. However, the technique is cheap and can be used for processing amnions for use as biological dressings. Glycerol preservation is recommended for processing amniotic membranes for use in ophthalmic surgery where the amnion is thicker and the cell structure well preserved.

References

Ab Hamid SS, Zahari NK, Yusof N and Hassan A (2014) Scanning Electron Microscopic Assessment on Surface Morphology of Preserved Human Amniotic Membrane after Gamma Sterilisation. *Cell Tissue Banking*, **15**: 15–24.

Arya SK, Bhala S, Malik A and Sood S (2010) Role of amniotic membrane transplantation in ocular surface disorders. *Nep J. Oph.*, **2**(4): 145–153.

Deocaris CC, Abad LB, Enriquez EP, De Guzman ZM, Aliganga AA, Tangonan MB, Tolentino MM, Ignacio LV and Deocaris CC (2005) Radiolytic damage to freeze-dried human amniotic membrane. *Philippine Journal of Science*, **134**(1): 45–50.

Dua HS, Gomes GAP, King AJ and Maharajan VS (2004) The amniotic membrane in ophthalmology. *Survey of Ophthalmology*, **49**(1): 51–77.

Farazdaghi M, Adler J and Farazdaghi SM (2001) Electron microscopy of human amniotic membrane. In: Nather A (ed.), *The Scientific Basis of Tissue Transplantation*, World Scientific, Singapore, pp. 149–171.

Halim AS, Leow AM, Dorai AA and Sulaiman WA (2007) Clinical applications of irradiated amnion grafts: Use of amnion in plastic surgery. In: Nather A, Yusof N and Hilmy N (eds.), *Radiation in Tissue Banking*, World Scientific, Singapore, pp. 365–379.

Hilmy N, Basril A and Febrida A (1992) Effects of procurement, packaging, storage time and irradiation dose on physical properties of amnion membranes. *Proceedings of the IAEA/PFM on Radiation Sterilisation of Tissue Grafts*, Manila, Philippines.

Hilmy N, Siddik S, Gentur S and Gulardi W (1987) Physical and chemical properties of freeze-dried amnion chorion membranes sterilised by gamma irradiation. *Atom Indonesia*, **13**(2): 1–14.

John T and John OC (2001) Ultrastructural characteristics of four types of human amniotic membranes. *Invest. Ophthalmol. Vis. Sci.*, **42**: 271.

Manjas M, Tarusaraya P and Hilmy N (2007) The use of irradiated amnion in wound healing. In: Nather A, Yusof N and Hilmy N (eds.), *Radiation in Tissue Banking*, World Scientific, Singapore, pp. 329–342.

Mohamad H (2007) Amnion for treatment of bones. In: Nather A, Yusof N and Hilmy N (eds.), *Radiation in Tissue Banking*, World Scientific, Singapore, pp. 343–354.

Nangia A, Gambhir R and Maibach H (1991) Factors influencing the performance of temporary skin substitutes. *Clinical Materials*, **7**: 3–13.

Nor Kamalia Z, Suzina SABH and Norimah Y (2014) Changes in biophysical properties of human amniotic membranes after different preservation methods and radiation sterilisation. *Regenerative Research*, **3**(1): 64–70.

Rejzek A, Weyer F, Eichberger R and Gebhart W (2001) Physical changes of amniotic membranes through glycerolisation for the use as an epidermal substitute. Light and electron microscopic studies. *Cell and Tissue Banking*, **2**: 95–102.

Singh R, Gupta P, Kumar P, Kumar A and Chacharkar MP (2003) Properties of air dried radiation processed amniotic membranes under different storage conditions. *Cell and Tissue Banking*, **4**: 95–100.

Singh R, Chouhan US, Purohit S, Gupta P, Kumar P, Kumar A, Charcharkar MP, Kachhawa D and Ghiya BC (2004) Radiation processed amniotic membranes in the treatment of non-healing ulcers of different etiologies. *Cell and Tissue Banking*, **5**: 129–134.

Singh R, Purohit S and Chacharkar MP (2007) Effect of high doses of gamma radiation on the functional characteristics of amniotic membrane. *Radiation Physics and Chemistry*, **76**: 1026–1030.

Singh R and Charcharkar MP (2011) Dried gamma-radiated amniotic membrane as dressing in burn wound care. *Journal of Tissue Viability*, **20**: 49–54.

von Versen-Hoeynck F, Steinfeld AP, Becker J, Hermel M, Rath W and Hesselbarth U (2008) Sterilisation and preservation influence the biophysical properties of human amnion grafts. *Biologicals*, **36**: 248–255.

von Versen-Hoeynck F, Syring C, Bachmann S and Moller DE (2004) The influence of different preservation and sterilisation steps on the histological properties of amnion allografts-light and scanning electron microscopic studies. *Cell Tissue Banking*, **5**: 45–56.

Yoshita T, Kobayashi A, Sugiyama K and Tseng SC (2004) Oxygen permeability of amniotic membrane and actual tear oxygen tension beneath amniotic membrane patch. *Am. J. Ophthalmol.*, **138**(3): 486–487.

Yusof N (1997) Effect of gamma irradiation and storage on biomechanical properties of human amniotic membrane. *Proceedings of the 9th International Conference on Biomedical Engineering*, Singapore.

Yusof N and Hilmy N (2007) Physical and mechanical properties of radiation sterilised amnion. In: Nather A, Yusof N and Hilmy N (eds.), *Radiation in Tissue Banking*, World Scientific, Singapore, pp. 155–168.

Yusof N and Ozkara T (2000) Biomechanical properties of gamma irradiated amnion after storage. *Proceedings of the 8th International Conference on Tissue Banking*, Bali, Indonesia.

Zahari NK, Ab Hamid SS and Yusof N (2015) Effects of different doses of gamma irradiation on oxygen and water vapour transmission rate of preserved human amniotic membrane. *Cell and Tissue Banking*, **16**(1): 55–63.

8

Transmissible Diseases

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Introduction

A transmissible disease spreads from one person to another through several ways such as — contact with blood and bodily fluids, breathing in an airborne virus, or being bitten by an insect. With amniotic membrane transplantation, there is a small risk of transmitting diseases such as Human Immunodeficiency Virus (HIV), Hepatitis B and Hepatitis C, Syphilis and Tuberculosis. It is the fear of disease transmission that makes many surgeons shun amnion grafts and use other options instead including skin substitutes and cultured epithelial cells. This chapter describes the common diseases that could be transferred, their mode of transmission and clinical presentation and how we can minimise such risks.

Hepatitis B

What is Hepatitis B?

Hepatitis B is a potentially life-threatening liver infection caused by Hepatitis B Virus (HBV), a small, circular, partially double-stranded DNA virus in the Hepadnaviridae family (Figure 1). It can lead to chronic infection and results in a high risk of death from cirrhosis and liver cancer.

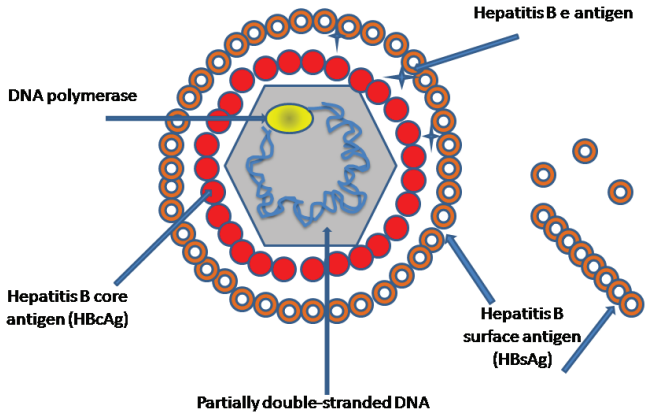


Figure 1. Hepatitis B Virus. (Source: https://www.herpes-coldsores.com/std/hepatitis_pictures.htm/hepatitis-b-diagram-virus/)

Geographical Distribution

Approximately 240 million people have chronic HBV infection globally. Figure 2 shows the estimated prevalence of chronic HBV infection by country. Hepatitis B is most prevalent in Sub-Saharan Africa and East Asia, where 5–10% of adults are chronically infected. High rates of chronic infections are also found in the Amazon and the southern parts of Eastern and Central Europe. In the Middle East and the Indian subcontinent, 2–5% of the general population is chronically infected. Less than 1% of the population of Western Europe and North America is chronically infected.

Transmission

HBV is found in the blood of people with HBV infection. Thus, it can be transmitted by contact with contaminated blood, blood products and other body fluids.

Some examples of transmission methods include:

- Birth — spread from an infected mother to her baby during birth.
- Unprotected sexual contact.
- Sharing needles, syringes or other drug-injection equipment.

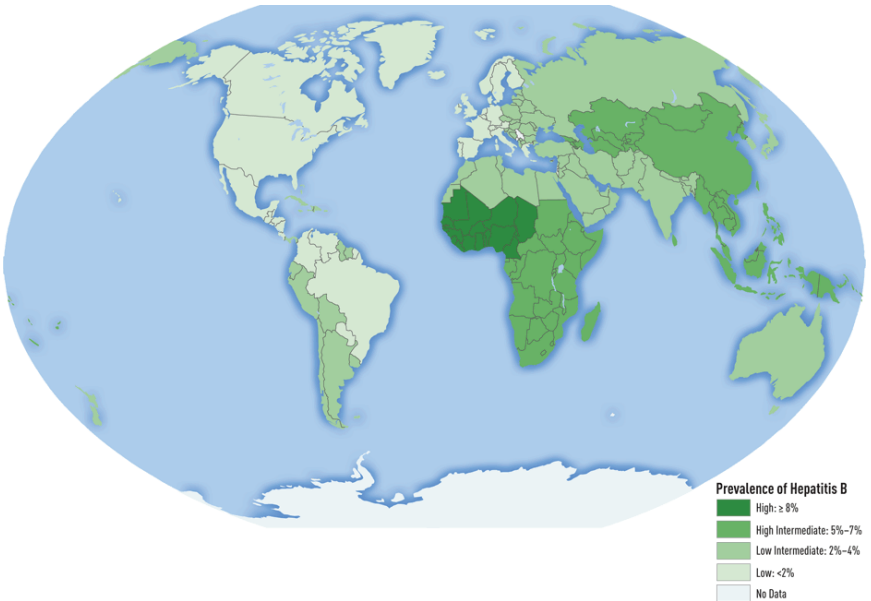


Figure 2. World prevalence of chronic hepatitis B virus infection among adults based on 2012 figures. (Source: https://www.cdc.gov/travel-static/yellowbook/2016/map_3-04.pdf)

- Body piercings and tattoos — the virus may be spread when needles used are not sterilised and infected blood enters another person.
- Toiletries — grooming items such as razors and toothbrushes can spread the virus if they carry blood from an infected person.

The HBV can survive outside the body for at least 7 days. During this time, the virus can still cause infection if it enters the body of a person who is not protected by the vaccine. The average incubation period of the HBV is 75 days, but can vary from 30 to 180 days. The virus may be detected within 30 to 60 days after infection and can persist and develop into chronic hepatitis B.

Acute HBV infection is diagnosed by detecting the presence of HBsAg and immunoglobulin M (IgM) antibody to the core antigen, HBcAg during the window period (Figure 3). During the initial phase of infection, patients are also seropositive for hepatitis B e antigen (HBeAg). HBeAg indicates that the blood and body fluids of the infected individual are highly contagious.

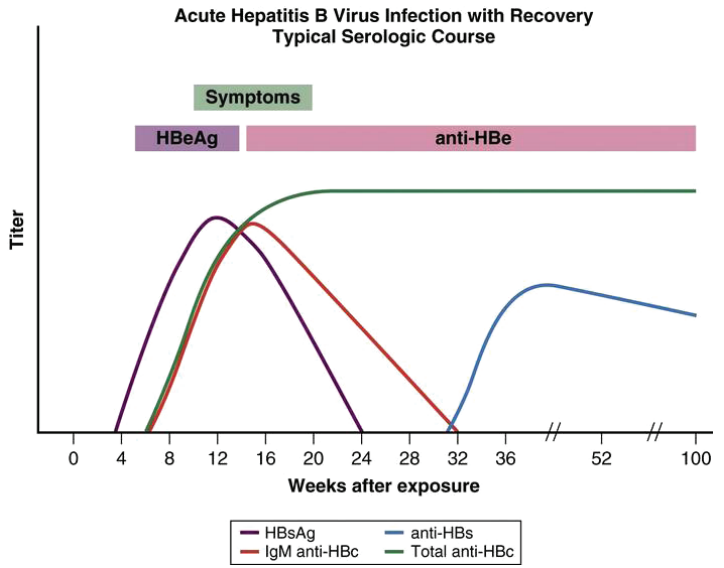


Figure 3. Serologic course of acute hepatitis B. (Source: http://www.clevelandclinicmeded.com/medicalpubs/diseasemanagement/hepatology/hepatitis-B/)

Chronic infection is characterised by the persistence of HBsAg in serum for more than 6 months (Figure 4). This is the principal marker of risk for developing chronic liver disease and liver cancer (hepatocellular carcinoma) later in life.

Laboratory Test for Hepatitis B Screening

HBsAg is a very sensitive assay that uses ELISA to detect Hepatitis B Surface Antigen in serum. HBsAg develops between 6 weeks and 6 months following infection, but prior to the onset of symptoms.

Symptoms

On average, symptoms appear 90 days (or 3 months) after exposure, but they can appear any time between 6 weeks and 6 months after exposure. Blood, semen and vaginal fluids, whether fresh or dried, are highly contagious during this period and for several weeks after the start of symptoms.

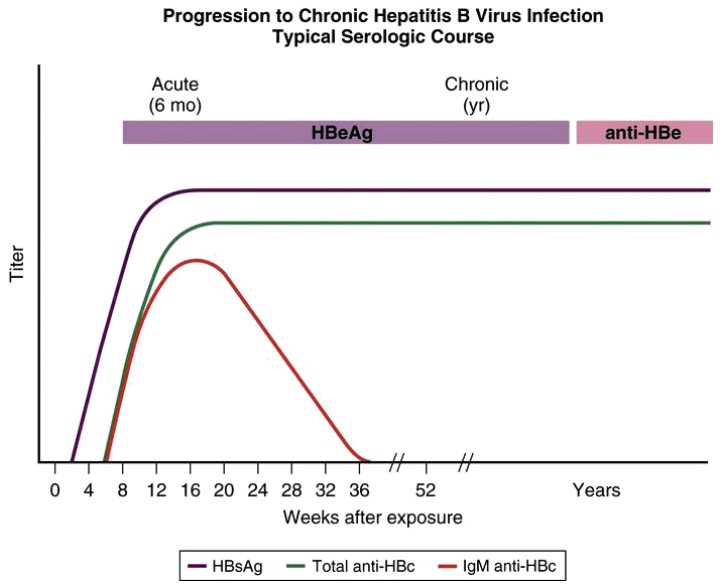


Figure 4. Serologic course of chronic hepatitis B. (Source: http://www.clevelandclinicmeded.com/medicalpubs/diseasemanagement/hepatology/hepatitis-B/)

During the acute phase (first 6 months after infection), most persons have no symptoms or might experience a mild illness. Symptoms of acute HBV infection may include:

- Jaundice — yellowing of the skin and eyes.
- Extreme fatigue
- Abdominal pain
- Nausea
- Diarrhoea
- Fever
- Dark urine

During the chronic phase (more than 6 months after infection), hepatitis B usually progresses silently, with no symptoms at all during the first 10 to 20 years. Chronic HBV infection can also lead to serious liver disease, liver scarring (cirrhosis) and hepatocellular (liver) cancer.

Treatment

There is no specific treatment for acute hepatitis B. Thus, care is aimed at maintaining comfort and adequate nutritional balance, including replacement of fluids lost from vomiting and diarrhea. On the other hand, chronic hepatitis B infection can be treated with drugs, including oral antiviral agents. Treatments can slow the progression of cirrhosis, reduce incidence of liver cancer and improve long term survival.

Hepatitis C

What is Hepatitis C?

Hepatitis C is a contagious liver infection caused by the hepatitis C virus (HCV), a spherical, enveloped, positive-strand RNA virus (Figure 5). HCV can cause both acute and chronic hepatitis infection, ranging in severity from a mild illness lasting a few weeks to a serious, lifelong illness.

Geographical Distribution

Hepatitis C is found worldwide. The most affected regions are Africa and Central and East Asia (Figure 6). Hepatitis C infection can be concentrated in certain populations (e.g. among people who inject drugs) or in general

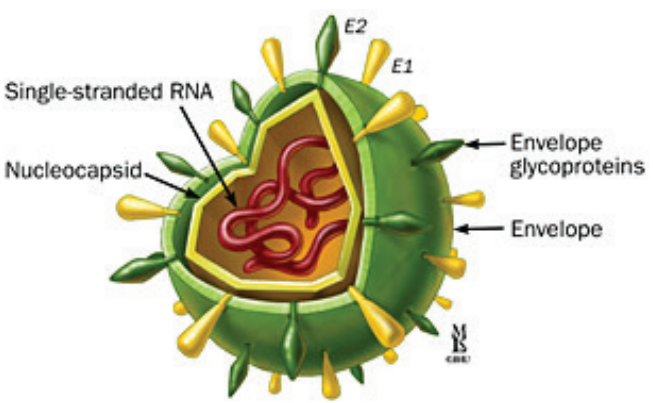


Figure 5. Hepatitis C Virus. (Source: https://www.jhmicall.org/GDL_Disease.aspx?CurrentUDV=31&GDL_Disease_ID=F90D3628-F21C-41B8-873E-FFFD82A8AF4C&GDL_DC_ID=9AA60584-3607-4D15-A459-BD3F67A3A4A7)

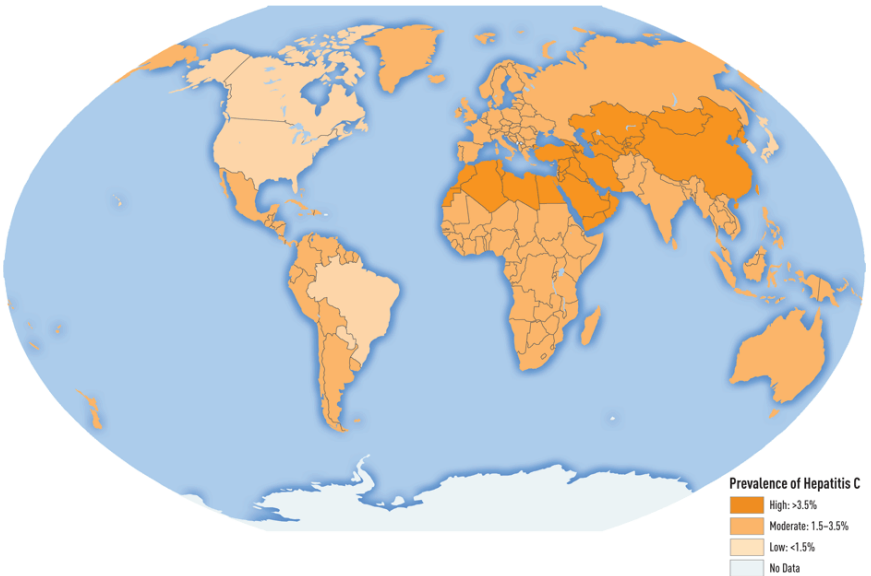


Figure 6. World prevalence of Hepatitis C based on 2013 figures. (Source: https://www.cdc.gov/travel-static/yellowbook/2016/map_3-05.pdf)

populations, depending on the country. There are multiple strains or genotypes of the HCV and their distribution varies by region.

Transmission

The HCV is a bloodborne virus. It is most commonly transmitted through:

- Injecting drug use through the sharing of injection equipment
- The re-use or inadequate sterilization of medical equipment, especially syringes and needles in healthcare settings
- The transfusion of unscreened blood and blood products.

HCV can also be transmitted sexually and it can be passed from an infected mother to her baby. However, these modes of transmission are much less common.

The window period for hepatitis C is generally in the range of 30–45 days. The vast majority of people develop detectable antibodies within 60 days (8 weeks) after infection with HCV (Figure 7).

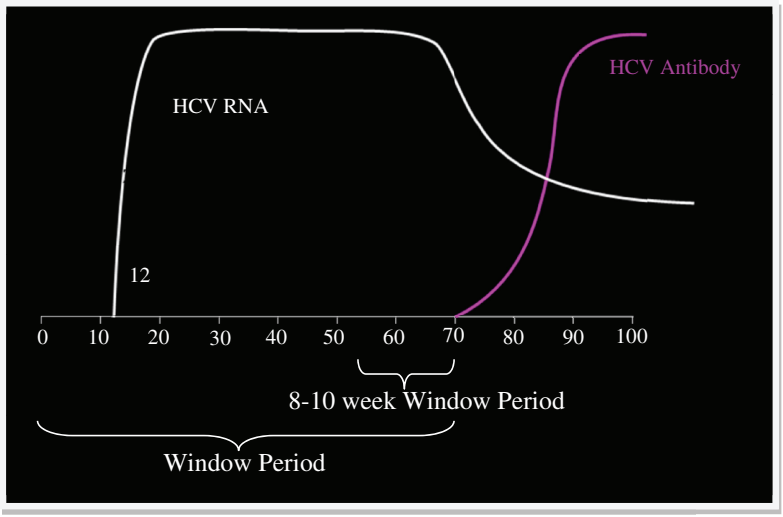


Figure 7. Graph demonstrating the window period of serological tests for Hepatitis C Virus (HCV). The window period is defined as the time between infection with HCV (Day 0) and the development of a detectable HCV-antibody response (Day 70). (Source: "The biology of window period infections: implications for donor screening and inventory hold" by Dr. Steven Kleinman during the FDA BPAC meeting, September 21, 2001)

Laboratory Tests for Hepatitis C Screening

- Anti-HCV
- Confirmatory Test- Recombinant Immunoblot Assay (RIBA)

Anti-HCV uses ELISA to test for antibodies against the Hepatitis C virus. Antibodies to Hepatitis C virus are produced by the immune system after more than 2 months of infection (MØller and Krarup, 2003).

Fourth generation ELISA tests for Anti-HCV are already available. The new assays include a greater number of HCV-encoded antigens to allow more specific antibody detection and to improve sensitivity.

If the results of Anti-HCV are positive, Recombinant Immunoblot Assay (RIBA) is used as a confirmatory test. RIBA is a qualitative ELISA test in which 4 recombinant HCV-encoded antigens immobilized as individual bands on test strips. The strips are incubated with the patient's serum. Any HCV antibodies present in the serum would then bind to the corresponding antigen bands on the strip, and show up as bands on the strip.

Symptoms

The natural course of hepatitis C disease varies from one person to another. During the acute phase (first 6 months after infection), most people (80%) have no symptoms or might experience a mild illness. Symptoms of acute HCV infection may include loss of appetite, abdominal pain, fatigue, nausea, dark urine and jaundice.

In 15-40% of persons with acute hepatitis C, the immune system successfully fights off the infection. The virus is cleared from the body within 6 months. The liver heals completely. In everyone else, hepatitis C infection persists past 6 months. This persistent state is known as chronic hepatitis C. Fatigue is the most common symptom among those who develop chronic HCV infection. HCV is also a major cause of cirrhosis and hepatocellular cancer.

Treatment

Anti-viral drugs can cure approximately 90% of persons with hepatitis C infection. When treatment is successful, liver scarring and damaged are reduced, thereby reducing the risk of death from liver cancer and cirrhosis. The success of the treatment depends on many factors, including the HCV genetic type (genotype). Also, access to diagnosis and treatment is low. There is currently no vaccine to prevent hepatitis C. However, research in this area is ongoing.

Human Immunodeficiency Virus (HIV)

What is HIV?

HIV is a single-stranded, positive-sense, enveloped RNA virus in the genus *Lentivirus* (Figure 8). HIV targets the immune system and weakens the people's defence systems. Immunodeficiency results in an increased susceptibility to a wide range of infections, cancers and other diseases that people with healthy immune systems can fight off.

The most advanced stage of HIV infection is Acquired Immunodeficiency Syndrome (AIDS) which can take 2 to 15 years to develop, depending on the individual. AIDS is defined by the development of certain cancers, infections, or other severe clinical manifestations.

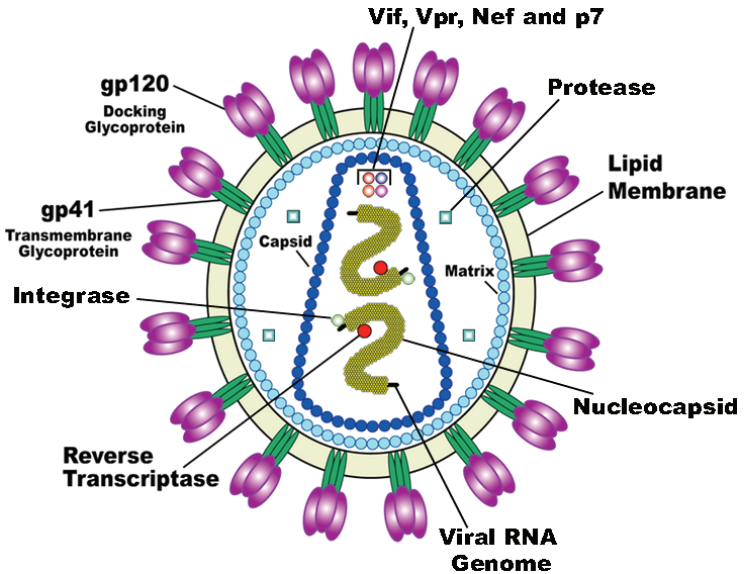


Figure 8. Human Immunodeficiency Virus. (Source: https://medicalxpress.com/news/2016-08-discovery-key-component-hiv-virus.html)

Geographical Distribution

HIV infection occurs worldwide and remains as a major global public health issue, having claimed more than 35 million lives so far. There were approximately 36.7 million people living with HIV at the end of 2015. Sub-Saharan Africa is the most affected region, with nearly 1 in every 25 adults (4.4%) living with HIV (Figure 9) and accounting for nearly 70% of the people living with HIV worldwide.

Transmission

HIV can be transmitted via the exchange of body fluids from infected individuals, such as sexual contact, needle- or syringe-sharing, medical use of blood or blood components, organ or tissue implantation, etc. It can also be transmitted from mother to child during pregnancy, at birth, and postpartum through breastfeeding.

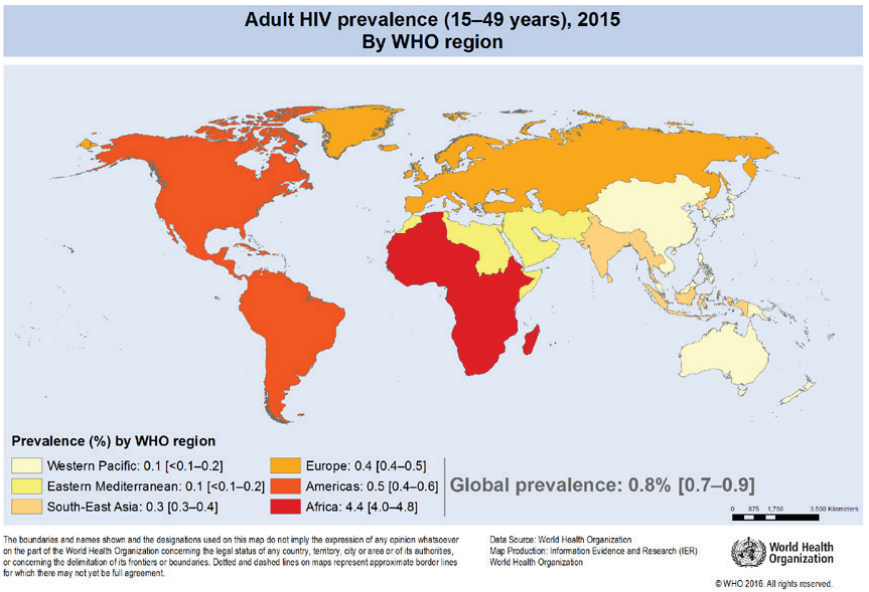


Figure 9. World prevalence of HIV based on 2015 figures. (Source: http://www.who.int/gho/hiv/en/)

Serological tests detect the presence or absence of antibodies to HIV-1/ HIV-2 or HIV p24 antigen. Antibody tests measure antibodies to HIV, which are proteins made by the immune system when the body is exposed to an infection. The antigen test measures a viral protein called p24 antigen, which shows up in the blood soon after a person gets HIV. Most individuals develop antibodies to HIV-1/ HIV-2 within 28 days. Therefore, antibodies may not be detectable early after infection. This is known as the window period. This early period of infection also represents the time of greatest infectivity. However, HIV transmission can occur during all stages of the infection.

Laboratory Tests for HIV-1 Screening

- Anti-HIV_1: ELISA
- Confirmatory Test — Western Blot

For the initial screening of HIV, Anti-HIV_1 is used. It uses Enzyme-Linked Immunosorbent Assay (ELISA) to test for antibodies to HIV-1 using plated HIV antigens. If ELISA is positive, then Western Blot is used as confirmatory test. Western Blot is a technique to test for HIV antibodies by using HIV proteins attached on a membrane.

However, Anti-HIV_1 is limited by its window period, which is defined as the time from infection to appearance of detectable antibodies (Figure 10). The figure for the window period of HIV using current Anti-HIV_1 test is 22 days, though it is expected to drop with the introduction of new generation tests which are more sensitive in their detection of HIV antibodies. HIV-infected donors who donate during the window period do not have sufficient HIV antibodies to test positive in the antibody test. This leads to false negatives.

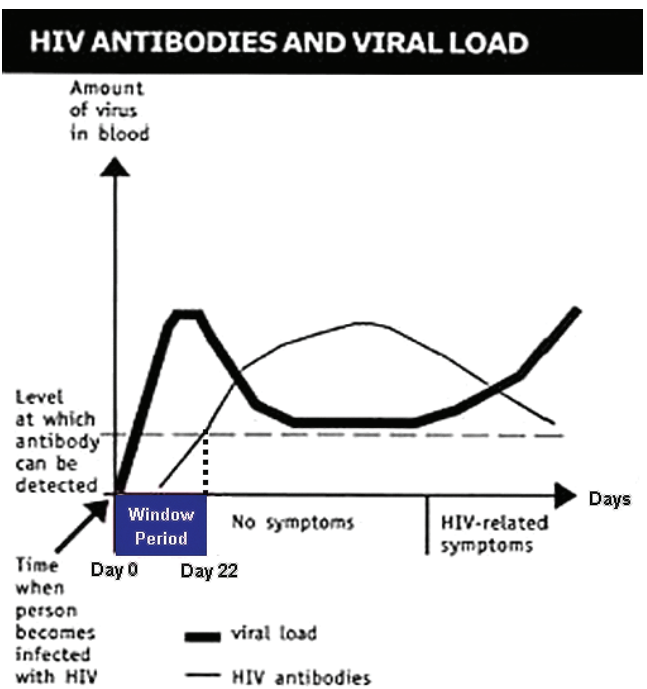


Figure 10. Graph demonstrating the window period of HIV. (Source: http://aidsaction.net/ht/section2.html)

Laboratory Test for HIV-2 Screening

- Anti-HIV_2

For the screening of HIV-2, HIV-1/HIV-2 ELISA is used to simultaneously test for antibodies against HIV-1 and HIV-2.

Symptoms

The symptoms of HIV vary depending on the stage of infection. The first few weeks after initial infection, individuals may experience no symptoms or an influenza-like illness including fever, headache, rash or sore throat. As the infection progressively weakens the immune system, other signs and symptoms may develop. These include swollen lymph nodes, weight loss, fever, diarrhoea and cough. Without treatment, severe illnesses such as tuberculosis, cryptococcal meningitis and cancers such as lymphomas and Kaposi's sarcoma may develop.

Treatment

HIV can be suppressed by combination Anti-Retroviral Therapy (ART) consisting of 3 or more Anti-Retroviral Drugs. ART does not cure HIV infection but controls viral replication within a person's body. This allows an individual's immune system to strengthen and regain the capacity to fight off infections. Effective treatment can also substantially reduce the risk of HIV transmission to others.

Syphilis

What is Syphilis?

Syphilis is a highly contagious sexually transmitted disease (STD) caused by the *Treponema pallidum* (Figure 11), a gram-negative spirochaete bacterium. Syphilis can cause long-term complications if it is not treated correctly.

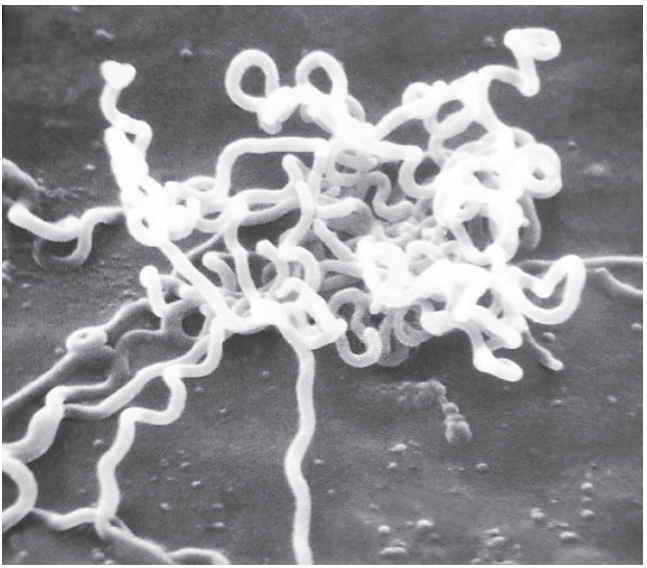


Figure 11. Treponema pallidum. (Source: http://www.ppdictionary.com/bacteria/bwum/pallidum.htm)

Transmission

Syphilis can be transmitted via direct contact with a syphilitic sore, known as a chancre, during vaginal, anal or oral sex. Chancres occur mainly on the external genitals, vagina, anus, or in the rectum, but can also occur on the lips and in the mouth. Syphilis can also be spread from an infected mother to her unborn baby.

Laboratory Tests for Syphilis Screening

- Rapid plasma reagin (RPR)

For the intial screening of syphilis, RPR is used to detect non-specific treponemal antibodies (reagin) by using cardiolipin antigens. When the test serum is added to a suspension containing carbon particles and cardiolipin antigen, any antibodies present would bind to the antigen and clump together, forming a lumpy mass (flocculate) that is made visible to the eye by

the carbon particles. In contrast, no flocculation is seen in a test serum without non-specific treponemal antibodies.

If RPR is positive, *Treponema pallidum* Haemagglutination (TPHA) Test is carried out as confirmatory test.

- Venereal Diseases Research Laboratory (VDRL)

VDRL is an alternative test for the initial screening of syphilis. It runs on the same principle as RPR, but it is an older test. Like RPR, it is a flocculation test that uses cardiolipin-antigen to detect non-specific treponemal antibodies.

If VDRL is positive, TPHA is carried out as confirmatory test.

- Confirmatory Test — *Treponema pallidum* Haemagglutination Assay (TPHA)

TPHA detects specific treponemal antibodies by using treponemal antigens attached to gelatin particles to test the patient's serum. When the test serum is added to the mixture of gelatin and antigens, any antibodies in the serum would cause agglutination and the formation of a mat across the bottom of the testing well. In contrast, no agglutination is seen in a test serum without specific treponemal antibodies.

Symptoms

Primary stage:

- Appearance of a single chancre that is usually firm, round and painless
- Chancre lasts 3 to 6 weeks and heals regardless of whether a person is treated or not

Secondary stage:

- Skin rashes and/ or mucous membrane lesions (sores in the mouth, vagina or anus)
- Rash may appear as rough, red or reddish brown spots both on the palms of the hands and the bottoms of the feet
- Fever, swollen lymph glands, sore throat, patchy hair loss, headaches, weight loss, muscle aches and fatigue

Latent and late stages:

The latent (hidden) stage of syphilis begins when primary and secondary symptoms disappear. Early latent syphilis is latent syphilis where infection occurred within the past 12 months. Late latent syphilis is latent syphilis where infection occurred more than 12 months ago. Latent syphilis can last for years.

In the late stages of syphilis, there may be difficulty in coordinating muscle movements, paralysis, numbness, gradual blindness, and dementia. This damage may be serious enough to cause death.

Treatment

Penicillin is usually used to treat syphilis. A single intra-muscular injection of long acting Benzathine Penicillin G will cure a person who has primary, secondary or early latent syphilis. Three doses at weekly intervals are recommended for individuals with late latent syphilis. Treatment will kill the bacteria and prevent further damage. However, it will not repair damage that has already occurred.

Tuberculosis

What is Tuberculosis?

Tuberculosis is caused by *Mycobacterium tuberculosis*, a rod-shaped bacterium that most often affects the lungs (Figure 12).



Figure 12. TB bacteria. (Source: http://www.histerius.com/hs0416/tb_bacteria.jpg)

Geographical Distribution

TB occurs in every part of the world, but the incidence varies. In the United States, the annual incidence is less than 4 per 100,000 population, but in some countries in Sub-Saharan Africa and Asia, the annual incidence is several hundred per 100,000 (Figure 13). In 2015, the largest number of new TB cases occurred in Asia, with 61% of new cases, followed by Africa, with 26% of new cases. Six countries accounted for 60% of the new TB cases: India, Indonesia, China, Nigeria, Pakistan and South Africa.

Transmission

TB is spread from person to person through the air. When people with lung TB cough, sneeze or spit, they propel the TB germs into the air. Bovine TB (caused by the closely related *M. bovis*) can be transmitted by consuming non-pasteurised dairy products from infected cattle.

People infected with TB bacteria have a 10% lifetime risk of falling ill with TB. However, persons with compromised immune systems, such as

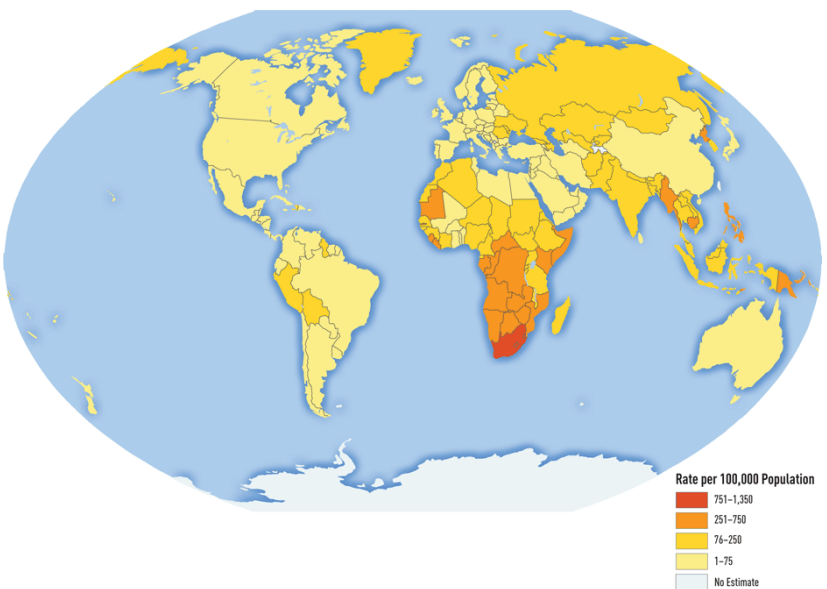


Figure 13. Estimated tuberculosis incidence rates, 2012. (Source: https://www.cdc.gov/travel-static/yellowbook/2016/map_3-13.pdf)

people living with HIV, malnutrition or diabetes, or people who use tobacco, have a much higher risk of falling ill.

Laboratory Tests for Tuberculosis

TB bacteria can live in the body without making a person sick. This is known as latent TB infection. People with latent TB infection do not have TB symptoms and cannot spread the bacteria to others. On the other hand, people with TB disease can spread the bacteria to others, feel sick, and suffer from TB symptoms.

The two tests that are used to determine if a person has been infected with TB bacteria are:

- Tuberculin Skin Test (TST)

The TB skin test is also known as the Mantoux tuberculin skin test. It is used most often. A small amount of tuberculin is injected into the skin on the lower part of the arm. After 48–72 hours, the result of the test depends on the size of the raised, hard area or swelling. A positive skin test means that the person's body was infected with TB bacteria. Additional tests are then required to determine if the person has latent TB infection of TB disease. A negative test means the person's body did not react to the test, and that latent TB infection or TB disease is unlikely.

- TB Blood Test

TB blood tests are also known as Interferon Gamma Release Assays (IGRAs). An IGRA measures how strong a person's immune system reacts to TB bacteria by testing the person's blood in a laboratory. The immune system produces cytokines and these TB tests work by detecting a cytokine called the interferon gamma cytokine.

To determine if someone has TB disease, other tests like chest x-ray and a sample of sputum (phlegm that is coughed up from deep in the lungs) may be needed. A PCR of the sputum could be done.

Symptoms

TB disease can affect any organ but most commonly occurs in the lungs (70–80%). Common symptoms of active lung TB are cough with sputum

and blood at times, chest pains, weakness, weight loss, fever and night sweats.

Treatment

TB is a treatable and curable disease. Active, drug-susceptible TB disease is treated with a standard 6 month course of 4 antimicrobial drugs: isoniazid, rifampin, ethambutol and pyrazinamide. These medications are provided with information, supervision and support to the patient by a health worker or trained volunteer.

References

MØller JM and Krarup HB (2003) Diagnosis of acute hepatitis C: Anti-HCV or HCV-RNA?. *Scand J Gastroentero*, **38**(5): 556–558.

https://wwwnc.cdc.gov/travel/yellowbook/2016/infectious-diseases-related-to-travel/hepatitis-b

http://www.who.int/mediacentre/factsheets/fs204/en/

http://www.sfcdcp.org/hepatitisb.html

http://www.sfcdcp.org/hepatitisc.html

https://wwwnc.cdc.gov/travel/yellowbook/2016/infectious-diseases-related-to-travel/hepatitis-c

http://www.who.int/mediacentre/factsheets/fs164/en/

http://www.openaidalliance.org/find-help/testing/

https://smartsexresource.com/topics/hiv-window-periods

https://wwwnc.cdc.gov/travel/yellowbook/2016/infectious-diseases-related-to-travel/hiv-infection

http://www.who.int/mediacentre/factsheets/fs360/en/

https://www.cdc.gov/std/syphilis/stdfact-syphilis.htm

https://www.cdc.gov/std/syphilis/stdfact-syphilis-detailed.htm

http://www.who.int/mediacentre/factsheets/fs104/en/

https://wwwnc.cdc.gov/travel/yellowbook/2016/infectious-diseases-related-to-travel/tuberculosis

https://www.cdc.gov/tb/topic/testing/tbtesttypes.htm

http://www.tbfacts.org/tb-tests/

https://www.cdc.gov/tb/publications/factsheets/testing/tb_factsheet.pdf

9

Donor Suitability

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DONOR SUITABILITY

Introduction

Autografts remain the best choice for clinical implantation/transplantation and does not pose any risk of transmitting diseases. It is only when autografts are not sufficient and available that allografts are used instead. Xenografts should never be considered as an option for fear of transmission of animal diseases to man.

The tissue bank must produce a tissue graft of the highest quality and with the smallest risk of disease transmission in order to achieve safe tissue transplantation practice.

Strict donor selection criteria is the first and most critical step to evaluate donor suitability. Detailed history taking and meticulous clinical examination is a must. Even when the clinical evaluation is positive, laboratory investigations must be performed to completely evaluate donor suitability.

Donor suitability criteria shall be established by medical director and follow APASTB Standards (APASTB Standards for Tissue Banking, 2017). Each donor shall be evaluated according to established criteria.

Indeed, the quality of a tissue bank can be gauged from the strictness with which it assesses donor selection criteria and the laboratory investigations it employs to investigate donor suitability.

Medical and Behavioural History

Donor History Review

Donor evaluation includes an interview of the potential living donor, performed by suitably trained personnel, using a questionnaire.

A qualified physician shall approve the donor evaluation process. At the time of the interview, the risk of transmission of these infections via tissue donation and the need to avoid transplantation of tissues from individuals who have engaged in high-risk behaviour should be explained to the living donor.

Data pertaining to the donor's age, medical history, medications taken, family medical history, sexual history and exposure to toxic materials are recorded. If any adverse information is found in the Donor Exclusion Criteria (see below), the procurement is not performed.

Amnion-Specific Donor Exclusion Criteria

The following exclusion criteria are specific for amnion (Nor Kamalia Z *et al.*, 2014; Suzina SAH *et al.*, 2012)

- Donors must have no history of drug or alcohol abuse
- Donors cannot be single mothers
- Donors cannot have multiple sexual partners
- Donors cannot have medical records of communicable diseases
- Donors should not have undergone chemotherapy treatment
- Donors should not be on prolonged steroid treatment
- Consent for donation must have been obtained from potential mothers before the serological test

- Donor cannot have tuberculosis or contact with a patient who has tuberculosis

The inclusion and exclusion criteria for amnion are as follows:-

Inclusion Criteria

- Caesarian Sections are ideal — amnion procured with strict sterile technique
- For vaginal deliveries, amniotic membrane should be ruptured less than 12 hours before delivery

Exclusion Criteria

— Meconium-stained foetus
— Dysmorphic foetus
— Chorio-amnionitis
— Endometriosis
— Prolonged labour
— Toxaemia of pregnancy
— Septicaemia
— Drug abuse
— History of venereal disease
— Seropositive for Hepatitis
— Seropositive for aids
— Seropositive for VDRL/TPHA

Physical Examination

Prior to procurement of tissue, the donor body shall be examined for general exclusion signs and for signs of infection, trauma or medical intervention. This examination confirms the patient's age, nutritional status and general well-being.

A qualified physician shall perform the physical examination. Special attention is given to the presence of infected wounds, tattoos, decubitus ulcers and abscesses.

If any of the following signs are observed or noted in any available record, and are deemed to be an indication of the risks, then the amnion cannot be procured.

- Physical evidence for risk of sexually transmitted diseases such as genital ulcerative disease, herpes simplex, chancroid.
- Physical evidence consistent with anal intercourse including perianal condyloma
- Physical evidence of non-medical percutaneous drug use such as needle tracks, including tattoos
- Disseminated lymphadenopathy
- Unexplained oral thrush
- Physical evidence of sepsis, such as generalized rash/petechiae
- Generalized vesicular rash
- Blue or purple spots on the skin or mucous membranes typical of Kaposi's sarcoma
- Unexplained fever >38.6 ^{o}C for > 10 days
- Unexplained jaundice, hepatomegaly, ascites or stigmata of chronic liver disease

Transmissible Diseases Blood Tests

To screen donors thoroughly, it is imperative to conduct serological as well as microbiological tests. A qualified physician shall obtain the blood sample from the patients.

Law and Practice

Tissues shall be tested for transmissible diseases in compliance with Law and practice in the country concerned. Applicable consent procedure for blood testing must be followed.

Tests

Tests shall be performed and found acceptable on properly identified blood samples from the donor using recognized, and if applicable, licensed tests

and according to manufacturer's instructions. Tests shall be performed by a qualified, and if applicable, licensed laboratory and according to Good Laboratory Practice (GLP).

Timing of Blood Sampling

Blood specimens for donor screening must be taken at the time of amnion procurement.

Notification of Confirmed Positive Test Results

The donor's physician shall be notified in accordance with National Laws of confirmed positive results having clinical significance.

Confirmed positive donor infectious disease tests shall be reported to Local/National Health Authorities, when required.

Blood Tests

Minimum Blood Tests

The extent and number of laboratory tests done varies among countries and among donors. Even so, a set of minimum requirements exist. Minimum Blood Tests shall include:

- Human Immunodeficiency Virus Antibodies (HIV-1/2-Ab)
- Hepatitis B Virus Surface Antigen (HBs-Ag)
- Hepatitis C Virus Antibodies (HCV-Ab)
- Syphilis: nonspecific (eg. VDRL) or preferably specific (eg. TPHA)

The Table 1 demonstrates the difference in minimum testing requirements between the Asia-Pacific Association of Surgical Tissue Banks (APASTB), American Association of Tissue Banks (AATB), Australasian Tissue Banking Forum (ATBF) and European Association of Tissue Banks (EATB). NAT testing is not performed in mothers (donors) in Asia Pacific countries.

Table 1. Comparison of minimum testing requirement between APASTB, AATB, ATBF and EATB

APASTB	AATB	ATBF	EATB
anti-HIV_1 & anti-HIV_2	anti-HIV_1 & anti-HIV_2	anti-HIV_1 & anti-HIV_2	anti-HIV_1 & anti-HIV_2
anti-HCV	anti-HCV	anti-HCV	anti-HCV
HBsAg	HBsAg	HBsAg	HBsAg
syphilis antibody	syphilis antibody	syphilis antibody	syphilis antibody
	anti-HBc	anti-HBc	anti-HBc
	anti-HTLV-I & anti-HTLV-II	anti-HTLV-I & anti-HTLV-II	anti-HTLV-I**
	NAT for HIV_1 and HCV*		

Living Donors Retesting

Retesting of living donors for HIV, HBV and HCV at 180 days is recommended. If another method of increasing safety, rather than retesting (antigen testing, molecular biology or viral inactivation method) is used (and allowed by applicable Regulation), it shall be documented and validated.

In Singapore, Malaysia and Indonesia, for mothers donating amnion, repeat testing is not performed.

Rejection of Amnion Grafts Procured

Positive results for HIV_1, HIV_2, Hepatitis B, Hepatitis C and Syphilis are absolute contra-indications for using the grafts. The amnion procured must not be processed. It must be discarded.

Bacteriological Studies of Donor and Tissues

Bacteriological Testing Methods

In accordance with the APASTB General Standards, under the section *B 1.610 Bacteriological Testing Methods:*

"Representative samples of each retrieved tissue have to be cultured, if the tissues are to be aseptically processed without terminal sterilisation. Samples shall be taken prior to exposure of the tissue to antibiotic containing solution."

Microbiological testing should be carried out on small donor tissue samples or swabs. The culture medium should allow the detection of aerobes, anaerobes and fungi. The results of the culture are to be documented in the donor record.

Bacteriological Bioburden Limits

If bacteriological testing of amnion tissue samples obtained at the time of donation reveals growth of low virulence microorganisms, which are commonly considered nonpathogenic, the tissue may not be distributed without being further processed in a way that effectively decontaminates the tissue.

Tissue from which high virulence microorganisms have been isolated are not acceptable for transplantation, unless the procedure has been validated to effectively inactivate the organisms without harmful potential effects, taking in account possible endotoxins.

Conclusion

Donor suitability criteria established by the tissue bank is a very important part of the tissue banking protocol to procure, process and transplant amnion of the highest quality and safety. It is important to minimize the risk of disease transmission by the amnion allograft used.

Donor suitability is dependent on a detailed medical and behavourial history, a meticulous review of the mother's medical records, a good physical examination of the mother and the performance and evaluation of minimum blood tests and microbiological cultures of amnion tissue procured.

References

APASTB (2017) Asia Pacific Association for Surgical Tissue Banks Standards for Tissue Banking. Second Edition.

AuBuchon JP, Birkmeyer JD and Busch MP (1997). Safety of the blood supply in the United States: Opportunities and controversies. *Ann. Intern. Med.*, **12**: 904–909.

Martin P, Fabrizi F, Quan S, Brezina M, Kaufman E, Sra K, DiNello R, Polito A, and Gitnick G (1997). Automated RIBA hepatitis C virus (HCV) strip immunoblot assay for reproducible HCV diagnosis. *J Clin Microbiol.*, **36**(2): 387–390.

Møller JM and Krarup HB (2003). Diagnosis of acute hepatitis C: Anti-HCV or HCV-RNA?. *Scand J Gastroentero.*, **38**(5): 556–558.

Nor Kamalia Z, Suzina SAH and Norimah Y (2014) Scanning electron microscopic assessment on surface morphology of preserved human amniotic membrane after gamma sterilisation. *Regenerative Research*, **3**: 64–70.

Scott JD and David RG (2007). Molecular diagnostics of hepatitis C virus infection: A systemic review. *J Am Med. Assn.*, **297**: 724–732.

Suzina SAH, Nor Kamalia Z, Yusof N and Asnah H (2012) Scanning electron microscopic assessment on surface morphology of preserved human amniotic membrane after gamma sterilisation. *Cell and Tissue Banking*, **15**: 15–24.

U.S. Food and Drug Administration (2002). FDA approves first nucleic acid test (NAT) system to screen whole blood donors for infections with human immunodeficiency virus (HIV) and hepatitis C virus (HCV). *U.S. Food and Drug Administration*. Retrieved from: http://www.fda.gov/default.htm.

Warren J (2004, Sept 30). Beating FDA to the punch, AATB requires accredited tissue banks to implement NAT testing for HIV-1 and HCV. *Transplant News*. Retrieved from: http://findarticles.com/p/articles/mi_m0YUG/is_18_14/ai_n17208495.

10

Procurement and Processing of Amniotic Membrane

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Introduction

The risk of infectious disease transmission through tissue allograft is a major concern in tissue banking. The possibility of bacterial, fungal, and viral infections of donor origin such as human immunodeficiency virus (HIV), hepatitis B and C (and HCV), cytomegalovirus (CMV), rabies, and prion diseases cannot be ignored. Contamination originating from the donor can be avoided through stringent screening of donors before procurement and processing. Detection of diseases using a highly sensitive method such as nucleic acid test (NAT) can help to exclude donors who carry any transmitted diseases. Contamination during procurement and processing is equally important. Procurement conducted in an open space, not in a properly controlled area such as an operating theatre, will lead to high contamination coming from the environment (Yusof and Hilmy, 2014).

Environment Control

Microbial contamination during procurement and processing, mainly bacteria, fungi, and mold, can come mostly from several main sources and some efforts must be taken, such as:

1. Environment in Procurement and Processing Area must be controlled by taking air sample regularly.
2. Processing Room surfaces i.e. tables, walls and floor must be cleaned and disinfected, and check microbial counts regularly.
3. Personnel or workers should wear protective clothing, and technical barriers must be checked, as well as routine medical checkups for the worker.
4. Gowning and hygienic practices must be well practiced through training and retraining of personnel.
5. Resources/raw materials such as:
   - Tissues — must check donor screening report.
   - Water — must check quality and conduct microbial monitoring.
   - Supplies and packaging materials — must check sterilisation records and certificates.
   - Equipment and instruments — must be calibrated and validated before use; must conduct maintenance schedule.
   - Tools — must be clean thoroughly before and after use.
6. Cross contamination of tissues from different donors must be avoided through non-pooling system i.e. tissues from different donors should not be mixed and must be individually packed (Hilmy and Yusof, 2010).

Proper gowning as in Figure 1 is important not only to avoid contamination to the procured amnion but also to protect tissue bank operators. Procurement of amnion must be conducted in a clean area.

Tissue banking has many similarities with the pharmaceutical and the medical device manufacturing industry, in particular the safety aspects. The biological tissues from which virulent micro-organisms have been



Figure 1. Proper gowning must be put on before processing tissues

isolated are not acceptable for transplantation, unless the procedure has been validated to effectively inactivate the microorganisms without leading to harmful potential effects (AATB, 2006; APASTB, 2016).

Quality Management

Before starting with routine procurement and processing, the Quality Management in procurement and processing should be set up as follows:

- Standard Operating Procedures (SOPs) for procurement and processing of donated tissues should be established,
- Each step of work flow should be documented,
- All work procedures should be carried out under aseptic condition according to GMP,
- Amniotic membranes from different donors should be treated and processed separately and each membrane from a single donor is processed as one batch,
- Tools and equipment should be calibrated,
- Donor consents form should be signed according to donor screening criteria,
- Workers should be protected from contaminated samples obtained from donors.

Important minimum tools, materials, chemicals needed in procurement and processing are:

TOOLS:	Forceps, low temperature container for amnion transfer, scissors, tray.
MATERIALS:	Sterile gloves, sterile cotton gauze, masks, sterile plastic bags, coats, log books for documentation.
CHEMICALS:	Sterile saline solution, sterile distilled water, 2% glutaraldehyde solution and 70% ethanol, media for microbes (aerobe/ anaerobe), 0.05% sodium hypochlorite.

Procurement

Each donor's serum must be subjected to serological donor screening tests that consist of obligatory blood tests such as HIV 1–2, VDRL, hepatitis B, hepatitis C, syphilis and optional tests such as CMC antibodies. Microbiology swab tests of the amniotic membrane should be negative (APASTB, 2016).

Those tests can be done at the hospitals or outsourced to any certified laboratory.

Fresh placenta is collected aseptically from labour room under aseptic procurement or from the operation theatre (OT) for caesarean section under sterile procurement. The amniotic membrane is placed in a sterile basin or wide-mouthed bottle filled with sterile saline (Nather and Chong, 2010). There is no difference between the biological and mechanical properties of both membranes. However, the vaginally delivered amniotic membranes have higher microbial content compared to those obtained from caesarean section (Hilmy *et al.*, 1987).

Procurement of amniotic membranes is summarised in Figure 2.

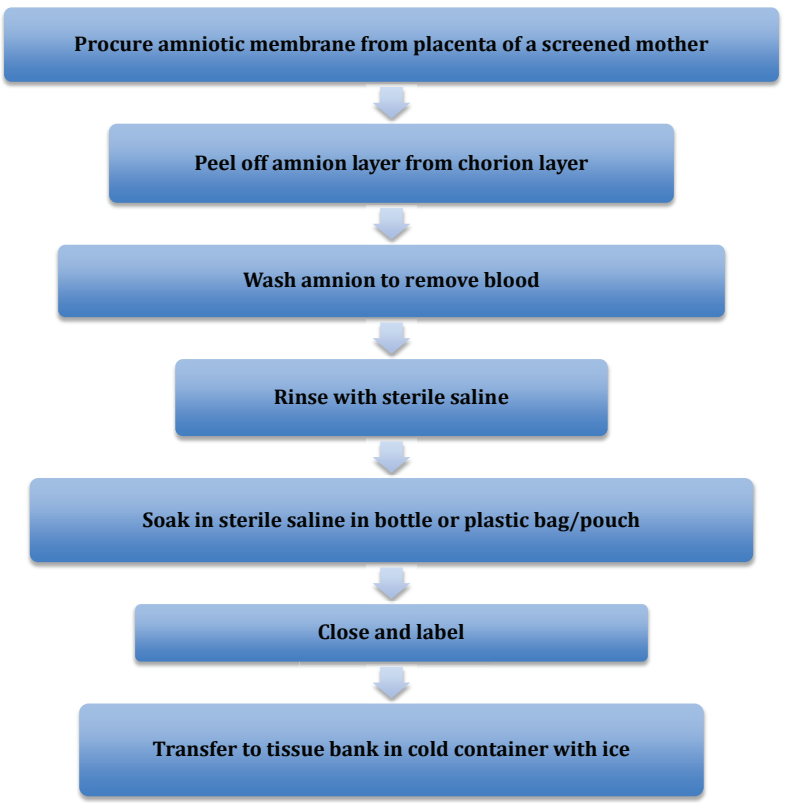


Figure 2. Steps in procurement of amniotic membranes

Sterile Procurement from Caesarean Deliveries

Fresh placentae are collected under sterile condition in the OT from pregnancies undergoing caesarean sections. In a clean area, the amniotic membrane is carefully released from the placenta. Amnion, the glistening layer, is separated from chorion, the dull layer of the amniotic membrane, by peeling it aseptically as shown in Figure 3. The amnion layer is then cleaned with sterile normal saline or sterile water to remove blood clot and mucus. The cleaned amnion is rinsed with sterile saline, placed in a sterile bottle or a plastic bag containing sterile saline and the container is closed. Amniotic membranes from different mothers are packed in separate bags as in Figure 4 to avoid cross-contamination.

The packed amnions are sent to tissue bank in a cold container with ice at temperature around 4°C. The bottle/plastic bag and the container transferring them should be labelled according to the SOPs.

Clean Procurement from Normal Delivery

Fresh placentae are procured in the labour room under clean condition after normal vaginal delivery. The amniotic membrane is immediately separated from the placenta mass in clean area close to the labour room (Figure 5). The membrane is made up of chorion and amnion. The amnion layer (the glistening layer of the amniotic membrane) is separated from the



(a) (b)

Figure 3. Procurement of amniotic membrane (a) Peeling off the membrane from fresh placenta (b) Separating amnion from chorion



Figure 4. Amniotic membranes individually packed in separate bags

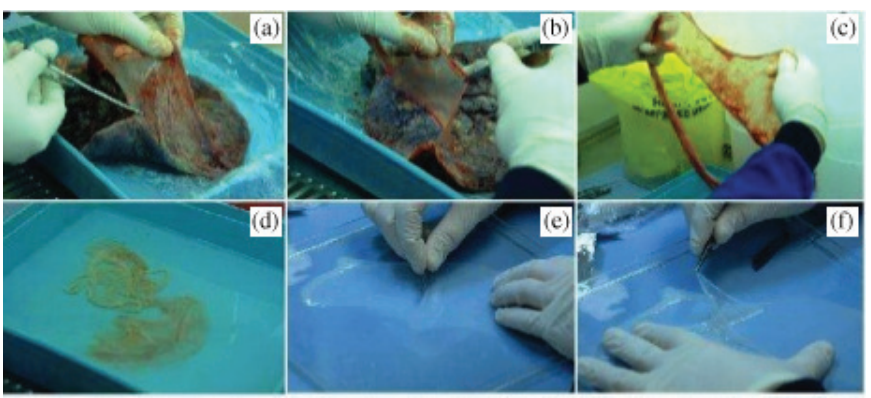


Figure 5. Procurement of amniotic membrane: a & b — Peeling off amniotic membrane from placenta and separate amnion from chorion, c — Amnion with blood and mucus, d — Amnion after washing and cleaning, e — Further removal of mucus from amnion, f — Cut cleaned amnion into specific size

chorion layer (the dull layer of the amniotic membrane) by peeling off as above. The amnion is then cleaned with normal saline to remove blood and other blood products. The amnion is rinsed further with saline solution to eliminate blood clots and mucus before being immersed in sterile normal saline in a sterile bottle or polyethylene bag. It is then sealed, labelled and transferred to the tissue bank in a cooler box containing ice at low temperature — 4°C according to validated Standard Operating Procedure (SOP). The procurement of amnion must be done as soon as possible, not more than 6 hours after labour to prevent microbial contamination and problems in separating the amnion from the chorion (Hilmy *et al.*, 2000 and 2003). The membranes are either stored at 4°C in the tissue bank if the processing is to be carried out within 2 to 3 days. It is kept frozen if the processing is delayed.

Labelling

The container should be labelled according to the SOP:

- Type of human tissue i.e. amniotic membrane,
- Donor documentation i.e. patient's Registration No. (R/N),
- Name and particulars of the donor,
- Tissue identification number,
- Date and time of procurement,
- Name & address of the hospital.

Only amniotic membranes of healthy patients who are free from infectious diseases with negative serology tests as well as negative swab test are processed to produce sterile amnion grafts.

Donor documentation should be completed i.e. sex of child, time and date of delivery, time of amnion collection, name of donor, type of delivery, name and address of the hospital and name and address of the tissue bank.

As soon as the amnions arrive at the tissue bank, they are stored in Quarantine compartment of a freezer or fridge with colour coding while waiting for results of serology and swab tests. Before processing, the tissues

in the sterile pouch or bag containing sterile saline solution are stored at low temperature to prevent from microbial proliferation and action of proteolysis enzymes. The pouch and the container should be labelled. The temperature of the container should be calibrated. Each step of work should be validated and documented according to quality management system (ISO, 2008).

Steps of quarantines for storing amnion at low temperature in procurement and processing procedures are as follows:

Quarantine 0: Rejected sample, marked with red colour and ready for disposal.

Quarantine 1: Received sample waiting for serology and swab test results, marked with yellow colour and store.

Quarantine 2: Serology and swab tests are negative i.e. tissues are free from viruses and pathogenic bacteria, marked with green colour and store while waiting for processing or during processing.

Quarantine 3: Washing and processing completed, marked with green colour while waiting for freezer drying (keep it at −80°C overnight) or other processing.

Quarantine 4: Freeze-drying or other processing completed, marked with green colour while waiting for cutting, packaging and labelling.

Quality controls of fresh amniotic membranes include the following (Hilmy *et al.*, 2007):

- Signed donor acceptance form,
- Donor screening — Results of serological test should be negative and results of swab test should be free from contaminated pathogenic bacteria. Exclusion criteria for swab test: tissues contaminated with *Streptococcus aureus*, *E. coli, Pseudomonas* sp (AATB, 2006).
- Appearance of the amniotic membranes: Should be colourless, clear and transparent. Meconium stained amnions should be discarded (keep it in Quarantine 0). Amniotic membranes with negative results of serological and swab test are allowed for processing.
- Steps of work should be documented.

Processing

Only amnions that have already passed donor screening test or **WELL SCREENED DONORS** are allowed for processing. No criteria on age of donors (mothers) and sex of babies.

During processing, amnions are subjected to a series of washing and cleaning steps. Pasteurisation and/or chemical treatment with sodium hypochlorite are necessary to inactivate viruses including HIV. The chemical treatment must be followed by several rinsings in sterile saline or water to ensure all chemical residues are removed. Main steps in processing amnions are as in Figure 6.

I. Washing

Washing process is carried out by using sterile water until all of blood particles and microbes are eliminated. Washing process should be validated. The amnion should be free from blood particles, free from contaminated microbes and free from residual of chemicals used in the washing process.

According to Hilmy's Method (Hilmy *et al*., 2000, Hilmy *et al*., 2003), washing is repeated 9 times, using 9 bottles whereby each bottle contains 400 ml of sterile water, except for bottle no. 5 which contains 0.05% sodium hypochlorite (NaOCl) solution in sterile water (Figure 7):

1. Amnion is washed and shaken well four times in 400 ml sterile water (bottles no. 1 to 4).
2. Amnion is immersed in 400 ml of 0.05% sodium hypochlorite solution (bottle no. 5) and gently shaken.
2. Amnion is washed again 4 times in 400 ml of sterile water in bottles no. 6 to 9. Each bottle is shaken for 10 minutes.
4. The used washing water in bottle no. 9 or the last rinse is taken for QC or validation: bioburden, blood and hypochlorite residues, as shown in Figure 8.

After early stage of washing, the real colour of amnion can still be seen. After last stage of washing the amnions are more transparent with no residual of blood particles (Figure 8). After washing, amnions are processed into viable or non-viable grafts.

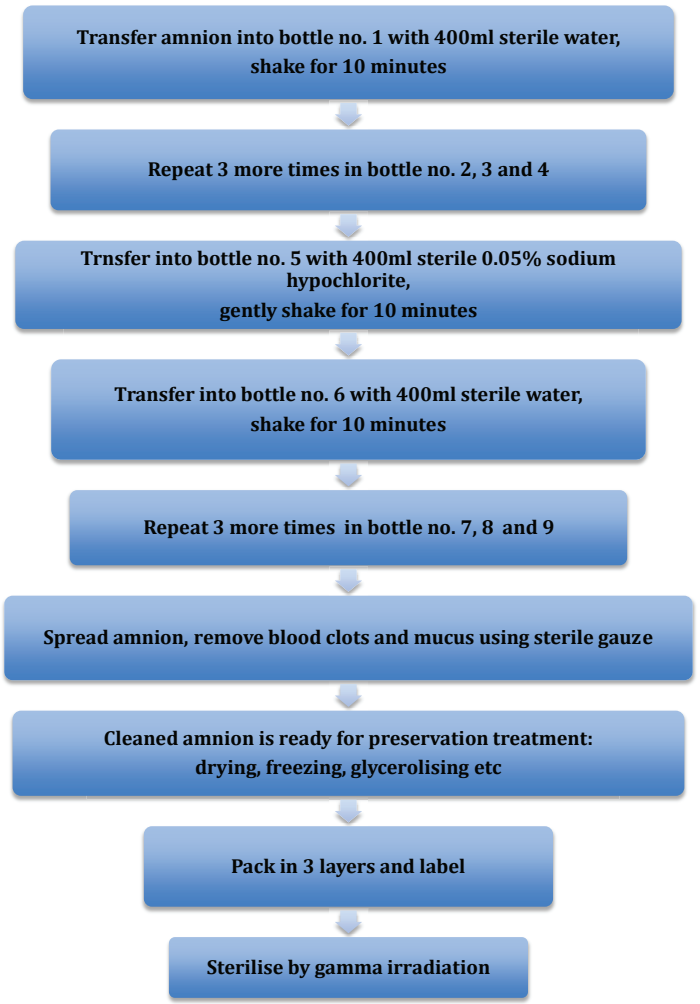


Figure 6. Main steps in processing amnion according to Hilmy's method

II. Preservation

After washing, several preservation methods can be used to produce amnion grafts in different forms for clinical applications:

1. Viable grafts *(Hypothermal preservation)*
   - Short-term storage in saline or combined with antibiotic, and stored at 4°C in a refrigerator, for 14 days, subject to validation.



Figure 7. Washing Process using 9 bottles of sterile water, except bottle number 5 contains 0.05% sodium hypochlorite solution (Hilmy *et al.*, 2007)

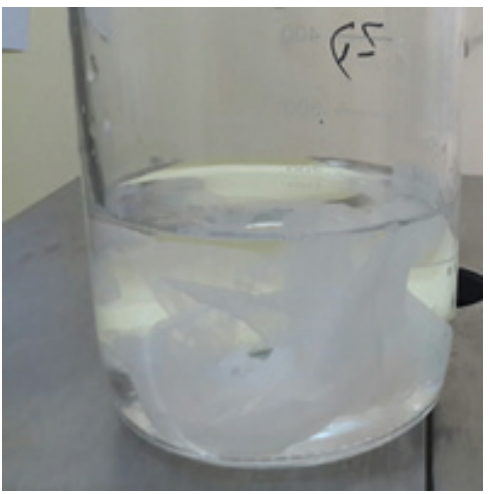
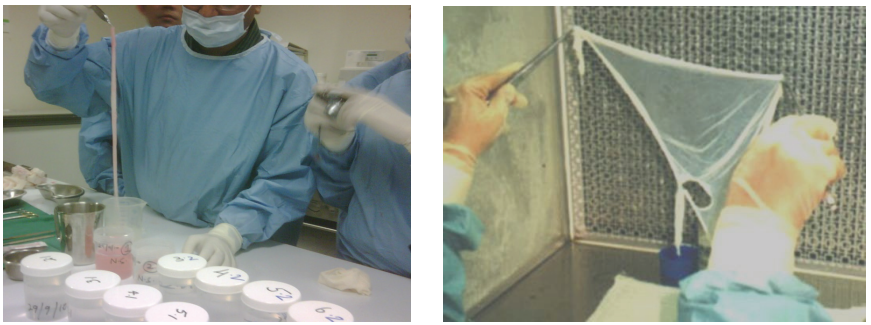


Figure 8. The used washing water in bottle no. 9 or the last rinse is taken for QC or validation



(a) (b)

Figure 9. After early stage of washing amnion is still with blood stain (a) and after final washing, amnion is white, clean, transparent and no residual of blood particles (b)

- Long-term storage using cryopreservation technique that has been subjected to validation.

2. Non-viable/preserved grafts
   - Freeze-, air- or oven-drying, or deep freezing then sterilised by radiation or other sterilisation methods.
   - Glycerolisation, and then sterilisation

III. Preparation of Non-viable Grafts:

A. Freeze Drying

Steps involved in freeze drying of amnions can be seen in Figures 10–13.

1. After washing, amnions are spread out and stretched in a clean room or in a laminar air flow cabinet (Figure 10).
2. The stretched amnion is spread over a sterile cotton gauze with chorion side facing the cotton gauze. The cotton gauze with amnion is mounted on a metal tray of the freeze-dryer (Figure 11). Cover the tray and freeze at −40°C overnight (Figure 12).
3. Amnions are freeze-dried at −30°C at a pressure of 0.370 mbar for 6 hours or until the moisture content is reduced to about 5–7%, subject to validation, to ensure the amnions are sufficiently dried for storage (Figure 13).

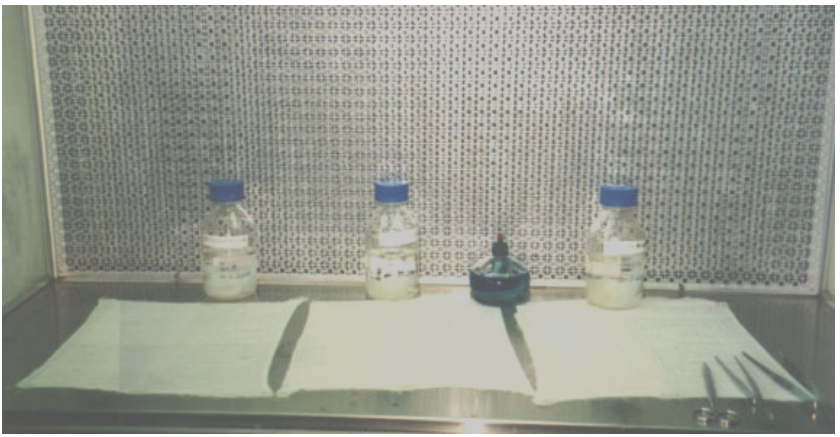


Figure 10. After washing amnions are stretched under aseptic condition under laminar air flow or in clean room (Courtesy: BATAN Tissue Bank, Indonesia)

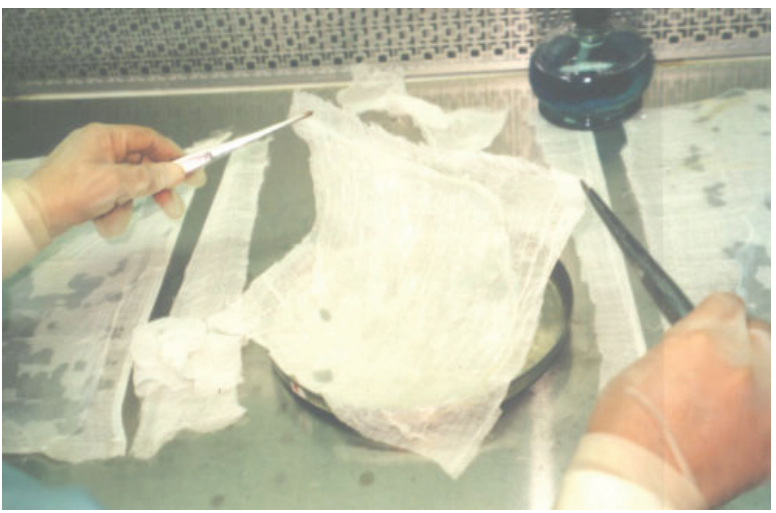


Figure 11. Mounting amnion with gauze on a metal tray of the freeze dryer

B. Air Drying

For air drying, the processed amnions directly after stretching are hung in the laminar air flow cabinet overnight as shown in Figure 14.



Figure 12. Freeze the covered tray containing amnion on gauze at −40°C overnight (24 hours)



Figure 13. Freeze dry amnion for about 6 hours until the water content of the dried amnion is about 5–7% (subject to validation)



Figure 14. Cleaned amnions are air-dried under laminar air flow bench to reduce water content between 5 to 7% (Hilmy *et al.*, 2007)

IV. Cutting and Packing

After drying, the amnions are cut according to required sizes and packed in 3 layers of plastic pouches and then vacuum sealed (plastic pouched and sealed are subject to validation).

In some countries, amnion is oven dried, for instance in Bangladesh the processed amnion is dried in oven at 40°C until the water content is less than 10%. Packed amnions are sent for sterilisation generally by gamma irradiation at 25 kGy, as in Figure 15.

C. Glycerolisation

After washing the amnion is preserved in 85% glycerol after a serial soaking in different concentrations of glycerol (Ab Hamid *et al.*, 2014):

1. Amnion is firstly submerged in 40% glycerol for 18 to 24 hours at room temperature.
2. Amnion is then submerged in 60% glycerol for 18 to 24 hours.
3. Amnion is then submerged in 80% glycerol for 18 to 24 hours.



(a)



(b)



(c)

Figure 15. Finished amnion products: (a) Label for amnion graft (b) Amnion is packed in three layers with radiation indicator which turn from yellow (before) to red (after sterilisation by gamma rays (c) A box of packed sterile amnions ready



Figure 16. Glycerolised amnions after sterilised by gamma irradiation at 25 kGy (with radiation indicator in red colour)

4. Amnion is eventually immersed and stored in 80% glycerol in a plastic bottle.
5. Each bottle is wrapped in double to triple sterile plastics. It is important to ensure the packaging is sterile, and properly sealed.

The procedures must be validated for proper preservation, packaging and labelling. Glycerol preserved amnions can be sterilised by gamma irradiation at 25 kGy (Figure 16).

Sterile amnion grafts (dried and glycerolised) are stored at room temperature in cool place until ready for despatch. Proper preservation of tissues is vital for long term storage whilst sterilisation will prolong shelf life of tissues.

Bioburden Estimation

Regardless of precautions taken, manufactured/processed tissues may still carry some microorganisms that are called bioburden. Therefore, any processed tissue still requires sterilisation treatment to ensure safety for clinical use (ISO, 2013). Bioburden estimation is carried out according to ISO 11737-1:1995 (ISO, 1995; Yusof *et al.*, 2010). According to ISO 11137:2013,

sterilisation dose of 15 or 25 kGy can be substantiated and validated based on bioburden:

- Dose 25 kGy is for products with bioburden less than 1,000 cfu/product unit such as air-dried, freeze-dried and frozen amnions.
- Dose 15 kGy for products with bioburden less than 1.5 cfu/product unit such as fresh amnions and other soft tissues.

Conclusion

Amniotic membranes are procured only from well-screened mothers, free from any transmitted diseases and not contaminated with pathogenic microbes. Amnion layer is immediately peeled from chorion layer by aseptic handling preferably in a clean area. The amnions are processed according to validated procedures — cleaning, removing of blood and mucus, chemical treatment to inactivate HIV, washing and many steps of rinsing. The processed amnion can be preserved in many ways depending on the end use of the grafts. For use as a biological dressing for treatment of wounds or as scaffolds in tissue engineering, they are best preserved by freeze-drying, air drying or oven drying. Preservation in glycerol is best reserved for ophthalmology or eye treatment. After being processed and preserved, all amnions are subjected to radiation sterilisation to ensure sterility and safety for clinical applications.

References

AATB (2006) Standards for Tissue Banking, American Association of Tissue Banks, McLean, VA, USA.

Ab Hamid SS, Zahari NK, Yusof N and Hassan A (2014) Scanning Electron Microscopic Assessment on Surface Morphology of Preserved Human Amniotic Membrane after Gamma Sterilisation. *Cell Tissue Banking*, **15**: 15–24.

APASTB (2016) Standards for Tissue Banking, Second Edition, Asia Pacific Association for Surgical Tissue Banking. December 2016.

Hilmy N, Abbas B and Anas F (2007) Validation for Processing and Irradiation of Amnion Grafts, In: Nather A, Yusof N and Hilmy N (eds.), *Radiation in Tissue Banking*, World Scientific, Singapore, pp. 237–248.

Hilmy N, Febrida A and Basril A (2000) Validation of Radiation Sterilisation Dose for Lyophilized Amnion and Bone Grafts. *Cell Tissue Bank*, **1**(2): 143–148.

Hilmy N, Febrida A and Basril A (2003) Indonesia: Statistical Sampling Technique in Validation of Radiation Sterilisation Dose of Biological Tissue, *Cell and Tissue Bank*, **4**(2): 185–191.

Hilmy N, Siddik S, Gentur S and Gulardi W (1987) Physical and Chemical Properties of Freeze Dried Amnion Membranes Sterilised by Irradiation. *J Atom Indonesia*, **13**(2): 1–14.

Hilmy N and Yusof N (2010) Environmental Management: Control and Safety. In: Nather A, Yusof N and Hilmy N (eds.) *Allograft Procurement, Processing and Transplantation*, World Scientific, Singapore, pp. 479–488.

ISO (1995) Sterilisation of Medical Devices — Microbial Methods — Part 1: Estimation of Population of Microorganism on Product, ISO 11737-1:1995, International Organization for Standardization, Geneva.

ISO (2008) International Organisation for Standardisation (ISO) 9001 — Quality Management Systems — Requirements, Switzerland.

ISO (2013) Sterilisation of Health Care Products — Radiation — Part 2: Establishing the Sterilisation Dose, ISO 11137:2013, International Organisation for Standardisation, Switzerland.

Nather A and Chong CL (2010) Amnion Procurement. In: Nather A, Yusof N and Hilmy N (eds.), *Allograft Procurement, Processing and Transplantation*, World Scientific, Singapore, pp. 321–323.

Yusof N, Hassan A and Hilmy N (2010) Bioburden Estimation in Tissue Banking. In: Nather A, Yusof N and Hilmy N (eds.), *Allograft Procurement, Processing and Transplantation*, World Scientific, Singapore, pp. 419–433.

Yusof N and Hilmy N (2014) Radiation Biology of Tissue Radiosterilisation. In: Brahme A (ed.). *Comprehensive Biomedical Physics*, Elsevier, Amsterdam, **7**: 263–287.

11

Sterilisation of Amnion

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Introduction

Sterilisation is a process to eliminate or kill all viable microorganisms in amnion grafts and to make the graft sterile. Sterilisation process renders the grafts sterile by inactivating all the microorganisms in term their ability to proliferate. A sterile product may be defined as "free from living microorganisms". Terminal sterilisation is a treatment given immediately after processing and preservation when amnions are in their final packaging. In tissue banking, sterilisation process is an important step to ensure tissue products are safe to be used in patients. It is important that tissue grafts that to be used by surgeons for transplantation are sterile at high sterility assurance level. Sterilisation process is not to replace but rather to complement other steps in tissue banking including donor screening and selection, procurement, processing, chemical or antibiotic treatment, preservation, packaging and storage.

Sterilisation is essential in order to avoid transferring disease. Sterilisation is required for cadaveric tissues retrieved outside of the operating theatre and also amnions, which are obtained in the labour room from normal birth. Moreover in order to distribute the safe banked tissues, sterilisation is required as an important safety measure against infection in all cases. Ultimately, banked tissues must be sterile and of high quality.

Different procedures are available to inactivate bacteria and fungi, including their spores, as well as viruses in the context of the production of human tissue transplants. The most efficient methods are considered to be gamma irradiation, thermal inactivation and chemical sterilisation such as ethylene oxide gas and peracetic acid–ethanol treatment (PES) under negative pressure conditions. Accordingly, special requirements have to be made on the biological safety of the tissues (Pruss *et al.*, 2001). Apart from a validated clinical effectiveness of the transplants, the quality assurance measures must focus on the validation of the sterilisation process. The selected spectrum of microorganisms covers the clinically relevant pathogens (*in-vivo* infection, aerosol contaminants) as well as spores, which are prescribed as mandatory for validating sterilisation procedures.

Importance of Sterilisation

There are many reasons why sterilisation is necessary:

Although tissues are only procured from a healthy donor who has been strictly screened and is free from transmissible diseases, sterilisation is still necessary to reduce further risks of transferring unforeseen and new diseases. The risk of diseases transmitted through allograft transplantation is a major concern in tissue banking (Yusof & Hilmy, 2010).

In addition, sterilisation can reduce the risk of transmitting new microbes and viruses that cannot be inactivated by current chemical and physical treatments during tissue processing despite the initial swab test showing the procured tissues to be free from any microbial contamination.

During processing, contamination may come from any of the followings:

- environment or surrounding,
- tools and equipment,
- personnel,
- materials such as chemicals,
- water,
- packaging materials and
- tissue itself.

Any processed products will have some microorganisms on it which is known as bioburden. Total bioburden expressed as colony forming unit (cfu) per product item will influence the efficiency of sterilisation techniques to achieve the desired sterility assurance level.

Sterility Assurance Level (SAL)

Inactivation of microorganisms by any sterilisation methods follows exponential law (Yusof and Hilmy, 2010). Therefore there is always a finite probability of a microorganism survives on a product after sterilisation process. Sterilisation will reduce the microbial count not only to zero, but even further down to reach certain probability levels which are identified as sterility assurance level or SAL. The SAL is defined as the probability or the chance to get a non-sterile amnion among a population of amnion grafts.

Clearly, the concept of sterility is a matter of probability, not of absolutes, which is represented by the desired SAL (sterility assurance level).

Two levels of sterility assurance level commonly used are:

1. SAL 10^{-3} is the probability of getting one non-sterile product in one thousand (10^3) products after the sterilisation process. This level is normally applied to products which are not intended to come into contact with compromised tissues that have lost the protection of the natural body barriers. Examples: disposable items for laboratory use such as petri dishes, urine bags, packaging materials, cotton buds etc.
2. SAL 10^{-6} is the probability of getting one non-sterile product in one million (10^6) products after the sterilisation process. This level is applied to medical products which are intended to come into contact with compromised tissues or open body parts. Examples: disposable items in contact with inside the body such as surgical gloves, medical products such as catheters and syringes, prostheses and tissue grafts.

Most medical devices are sterilised to SAL 10^{-6}, meaning that probability is one in a million that an item is non-sterile.

Industrial Sterilisation Technique

Manufacturers or the tissue bankers are responsible for selecting the appropriate sterilisation technique for their products so that the desired SAL is achievable. There are three types of validated sterilisation techniques that are commonly used on a commercial scale for medical products:

1. Thermal technique e.g. Moist heat (autoclave) and Dry heat (oven)
2. Chemical technique e.g. ethylene oxide (EO) gas
3. Physical or Non Thermal technique e.g. Radiation

Large scale facilities for sterilisation can be either in-house such as the autoclave machine and ethylene oxide (EO) chamber or outsourced to radiation service providers such as gamma irradiator.

Thermal Sterilisation

Steam sterilisation allows for high processing throughput at low costs with no known toxic effects. Steam sterilisation requires high temperatures and high moisture (Figure 1). Boiling or autoclaving reduces the osteoinductive activities of bone. So, this method cannot be recommended as sterilisation process for tissues.

Dry heat or thermal treatment is widely used in Germany, but is presently restricted exclusively to the disinfection of femoral heads obtained from total endoprosthesis operations. However, the validation methods described for this procedure do not completely meet the requirements of the national and international standards, particularly regarding the spectrum of the test organisms or "worst case" conditions (direct contact of the microorganisms with the bone tissue) (Pruss *et al*., 2003).

Thermal technique will denature tissue grafts because tissues are biological materials which mainly contain proteins and amino acids. High temperatures will also cause physical damage and weaken biomechanical properties.

Chemical Sterilisation

Ethylene oxide (EO) sterilisation has been in use for more than 60 years and approximately 50% of manufactured healthcare devices and instruments are



Figure 1. Industrial autoclave for steam sterilisation

sterilised in this way (Figure 2). EO sterilisation is efficient against every bacillus spore and virus due to a chemical reaction affecting the DNA of bacteria, spores and viruses. However EO sterilisation effect depends on time, temperature, product size, water and fat content.

EO is a low-temperature process that includes:

1. Preconditioning: Products are heated and humidified to aid the effectiveness of the sterilant gas to optimise the sterilisation process.
2. Sterilisation: The EO gas is introduced to the products under vacuum in a sealed chamber and held for a predefined dwell time validated to deliver a Sterility Assurance Level (SAL) of 10^{-6} and
3. De-Gassing: This final stage uses circulated heated air to allow gas dispersion and to remove residual EO from the product to ensure that it meets specified EO residual limits outlined in ISO10993-7.

The EO penetrates the packaging and sterilises all accessible surfaces of the products to render the products sterile by alkylation of proteins essential for cell reproduction. The aeration period is required to remove the gas from implants/products to allow for safe handling and to avoid chemical burns.



Figure 2. Ethylene oxide chamber for chemical sterilisation

The turnaround time for EO is normally longer than for steam or gamma radiation sterilisation. Possible problems with EO include toxic residuals and less penetration.

EO gas leaves toxic residuals that will interact with soft tissues. Hence, a quarantine period is needed before the products can be used. Due to its carcinogenic and mutagenic effects, EO sterilisation as well as other chemical treatments such as using beta-propiolactone and formaldehyde are no longer considered as suitable methods for sterilisation in many countries such as Germany (Pruss *et al.*, 2001). In recent years the technology has come under pressure as a sterilisation method from a number of sources including alternative sterilisation methods and tighter regulatory and environmental controls.

Moreover, the toxic residuals remain in water and fat components. It is very likely the EO residuals remain in tissues after sterilisation, if defatting and dehydration processing is incomplete.

The use of chemicals such as peracetic acid (PAA), hydrogen peroxide (H_2O_2) and alcohol during processing has been reported but these chemicals

are still considered as decontaminants and therefore need validation tests to prove their effectiveness as sterilants (Pruss *et al.*, 2001).

In the last 20 years, ethanol and peracetic acid have been increasingly used for tissue sterilisation. However, the diffusion of these chemicals into tissues is limited. Penetration-inhibiting fat barriers must be removed first by treating the bone with a chloroform–methanol mixture or by an equivalent validated procedure. Sterilisation of amnion in peracetic acid–ethanol mixture (PES) is performed for 4 hours at room temperature in 12.5 ml peracetic acid, 125 ml ethanol, 96% vol. and 362.5 ml distilled water (von Versen-Hoeynck, 2008). Subsequently the amniotic membrane samples are rinsed three times in sterile physiological saline solution to remove the peracetic acid or ethanol residuals. If required, another rinsing step is performed to attain a pH value of 7.0. Routine sterility tests are performed by an independent certified laboratory for each batch of finished tissues.

Radiation Sterilisation

Radiation technique is widely used to sterilise disposable products mainly made of polymers or plastic that are heat sensitive. Radiation is therefore the best method to sterilise the preserved allografts concerning safety and quality. This method of sterilisation came into wide use in the American and European Tissue Banks in the 1990s. Most of the tissue banks in the Asia Pacific region and in Latin America have been using radiation technique to sterilise their tissue allografts. The International Atomic Energy Agency (IAEA) under its regional and inter-regional programmes (1988–2008) assisted many developing member states in establishing tissue banks and introduced the proper use of ionising radiation as a terminal sterilisation process for tissue products (Phillips, 2013). Radiation allows sterilisation of the tissues in their final packaging and the sterile tissue grafts are ready to use immediately after sterilisation. This technique will be described further in this chapter.

Thus radiation offers the best sterilisation technique for tissues. However, the use of high doses must be avoided as they will cause physical damages especially to soft tissues. There are reports on detrimental effects of this method on biological and mechanical properties of tissues. Radiation seems to have effects on physical properties through direct and indirect effects, also

depending on the changes of cell structure and collagen arrangements caused by the processing and preservation methods. Amnion grafts can still be sterilised at 25 kGy for safe clinical use without any gross changes in the cell morphology.

Advantages and limitation of these sterilisation techniques are compared in Table 1.

Lately, tissue bankers are searching for an alternative sterilisation technique to sterilise soft tissues specifically tendon, ligament and cartilage. Limitations in validating radiation doses lower than 10 kGy has urged some tissue banks to consider new techniques such as supercritical carbon dioxide ($SCCO_2$) which has recently been installed and used routinely by a tissue

Table 1. Comparison of different sterilisation techniques

	Thermal — Autoclave	Chemical — EO	Radiation — Gamma
Tissue materials	Damaged and denatured	Residuals interact with soft tissue	Damages at high doses
Packaging	Heat resistant	Porous type for gas penetration	Radiation compatible
Parameters to control	Temperature Vacuum Pressure Humidity Time	Temperature Vacuum Pressure Humidity Time EO concentration	Exposure time
Residuals	None	Yes	None
Quarantine	7–14 days	14 to 30 days	None
Post sterility test	Desirable	Required	None
Reliability	Good	Good	Excellent
Continuous operation	No	No	Yes
Environment friendly	Yes	Poor — toxic residuals	Yes
Operational safety and risk to workers	Good	Poor	Good



Figure 3. Supercritical carbon dioxide ($SCCO_2$) chamber

bank in Australia (Figure 3). The technique has been proven to effectively remove cells and fats from tissues including bone and cartilage without changing the specific characteristics of the tissues (Fages *et al.*, 1994; Link *et al.*, 2014; Lovric *et al.*, 2014a). No adverse reactions in animal study have been reported (Lovric *et al.*, 2014b). Carbon dioxide exists in both liquid and gas states. It is able to diffuse into microporous structure of tissues especially in bone while dissolving lipids and removing unwanted antigenic materials and organic and cellular debris. It also increases permeability (Lovric *et al.*, 2014a).

Types of Radiation

Two main types of radiation that are available for commercial scale:

1. Gamma rays — are emitted either from radioactive either cobalt-60 (^{60}Co) or caesium-137 (^{137}Cs). Most of commercial gamma irradiators worldwide are using ^{60}Co as the radiation source (Figure 4). The radioactive source has half life of 5.27 years meaning that the source will deplete about 12% per year by which source replenishment is needed. Gamma rays have high penetration capability and can sterilise non-uniform and high density products even in their final boxes.
2. Electron beams — are generated from a man-made accelerator which can be switched on and off when needed (Figure 5). The penetration is

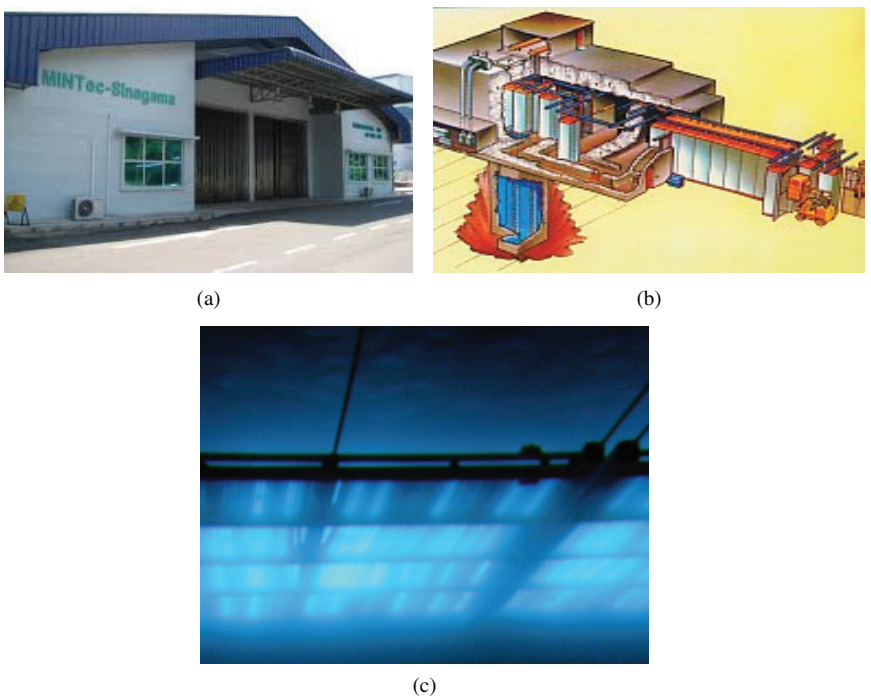
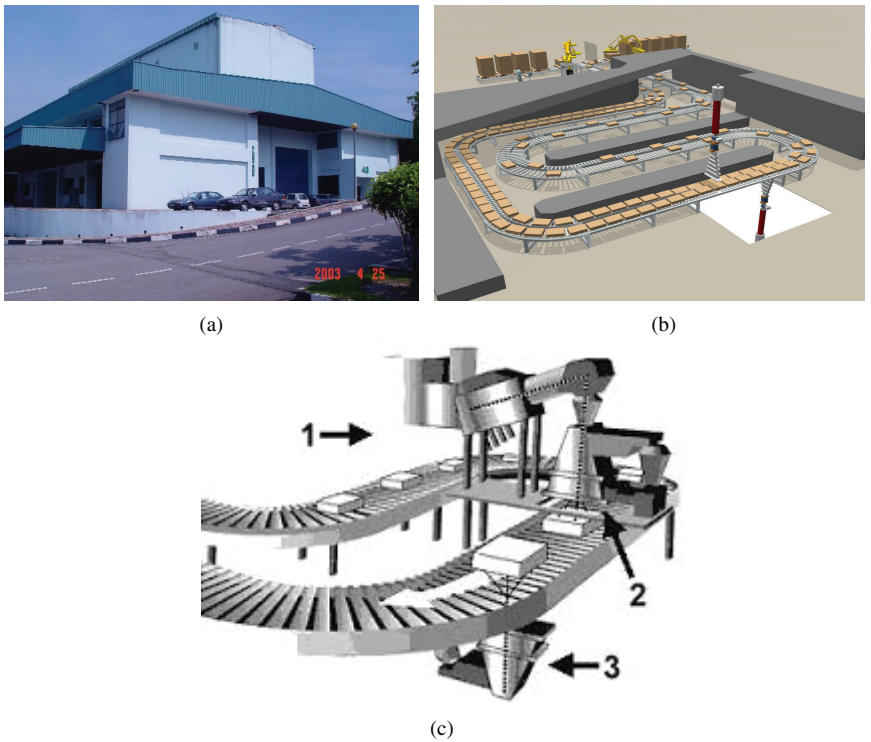


Figure 4. Gamma irradiation facility (a) MINTec-Sinagama Facility in Malaysian Nuclear Agency (b) Schematic diagram of a gamma irradiator room with conveyor system (c) Radioactive cobalt-60 is placed in water pool when not in use



(a) (b)

(c)

Figure 5. Electron beams facility: (a) Alurtron facility at Malaysian Nuclear Agency (b) Set up of conveyor system into irradiation chamber (c) products are exposed to electron beam while passing under two scanhorns from up (2) and bottom (3)

low and increases with the increasing energy level of the machine. Amnion can be irradiated by putting the packed amnions in a single layer on the conveyor. The amnions will be packed in boxes after sterilisation. Nevertheless, in terms of dose rate, electron beams have a high dose rate which will enable sterilisation process to be completed in minutes instead of hours of irradiation using gamma rays.

Gamma Radiation

The most common form of radiation sterilisation is gamma radiation. Gamma radiation has ionising energy to deeply penetrate the target and is

therefore a good fit for products with various densities and packaging types. It is economical for high- and low-volume operations, particularly for single-use medical supplies such as syringes, and catheters. Particular drawbacks are changes in coloration and mechanical properties. The material selected must be radiation compatible. Gamma radiation is also useful for biological materials such as tissues for transplantations, although it affects structural integrity at high doses. This sterilisation method decreases the risk of bacterial infection and may inactivate HIV at relatively high doses.

Electron Beam

Electron beam radiation does not penetrate as deeply as gamma rays. Its higher dose rate is useful for both high-volume, low-value products (example: syringes) and for low-volume, high-value products (example: cardiothoracic devices). E-beam radiation performs best on low density, uniformly packaged products and requires shorter exposure time than gamma radiation, thus having fewer oxidative effects on certain materials.

Radiation Process

Radiation is a cool process as there is no significant increase in temperature of a product to cause any changes in biological and physical properties of amnions. This reliable method allows for dosimetric monitoring. Dose dependent sterilisation means that the amount of radiation can be easily and quickly validated as proof of efficacy. Post-sterilisation quarantine or treatment is not required.

Prior to sending amnions for sterilisation by radiation, tissue bankers should be aware of the following aspects:

Dose Mapping

Different materials have different radiation absorption properties. Therefore, it is necessary to determine distribution of absorbed dose in a particular product or a package of products. Dose mapping is an exercise to predetermine dose distribution throughout a reference product package, e.g. a box of amnion grafts with a particular product arrangement, configuration

or loading. This product validation exercise must be conducted together with personnel of the radiation facility for any new products before starting routine sterilisation. It is an integral part of performance qualification according to ISO11137:2013 (ISO, 2013). Further advice on suitable packaging materials to be used can be obtained from the irradiation facility.

An example for conducting a dose mapping exercise with a commercial radiation facility is shared here:

(i) Fill up a product box with amnion products. Each amnion is triple packs: dried amnion is packed in 3 layers of polyethylene (PE) plastic bags, glycerol preserved amnion in plastic container with glycerol is packed with 2 layers of PE bags, and frozen amnion in 3 layers of PE bags. Amnions in different forms due to different methods of preservation cannot be mixed in one box except in dry form. Dried amnions either air-dried, freeze-dried or oven dried can be mixed in one box as the density is almost similar. Arrangement of the amnion products in the box must be noted and it remains for future routine irradiation. Amnions can be replaced with a dummy material of almost same size and weight if shortage of amnion samples occur.

(ii) Place routine dosimeters in between the product packages in the centre area of the box at specified points and also outside the box. For frozen amnions, the box is filled up by cooling materials with density (in term of volume and weight) is almost similar to dry ice to simulate the real situation. A reference point outside the box will be identified by the radiation facility personnel whereby dosimeters will be put only at that particular point during routine sterilisation process. Weight of the box is recorded after sealing the box properly. Figure 6 shows dose mapping is being conducted on boxes containing air-dried amnions to be irradiated in a tote box in a gamma irradiator facility.

(iii) Irradiation: Boxes of amnions are irradiated at room temperature to the minimum dose selected by the tissue bank as the sterilisation dose. Radiation cycle times and dose rate are noted by the facility operators.

(iv) After irradiation, all dosimeters are removed for analysis. Results of the absorbed doses for the dosimeters will provide data on: minimum dose inside the box (Dmax), maximum dose inside the box (Dmax) and



Figure 6. Conducting dose mapping for boxes containing air–dried amnions

absorbed dose at reference point outside the box (Dref). The following can be determined:

$$\text{Dose Uniformity Ratio (DUR)} = \text{Dmax/Dmin}$$

A DUR closer to 1 indicates that the dose distribution in the box is uniform as the different between the maximum and the minimum is very small. The irradiator personnel will be able to advise to narrowing the difference between minimum and maximum doses when the DUR is high.

$$\text{Correction Factor for Dmin at reference point} = \text{Dmin/Dref}$$

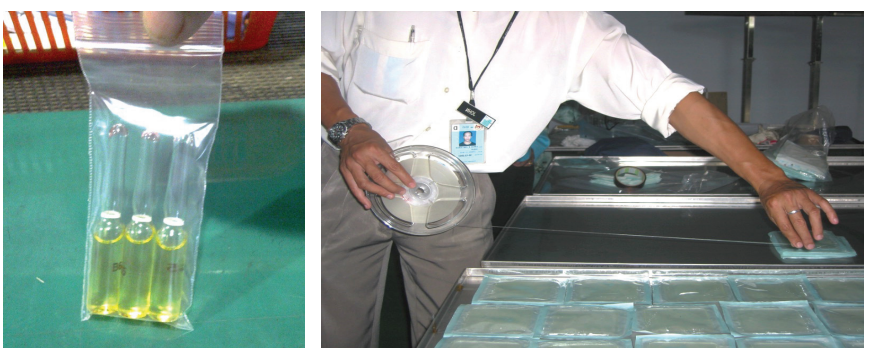
$$\text{Correction Factor for Dmax at reference point} = \text{Dmax/Dref}$$

During routine irradiation, Dmin and Dmax will be determined by applying these correction factors to the absorbed dose measured at the reference point.

(v) Repeat the exercise for reproducible results. Based on the dose mapping exercise, the tissue bank can prepare a standard operating procedure (SOP) on handling of the amnion products for radiation sterilisation. A copy of the SOP will be sent to the radiation facility for reference.

Dosimetry

Dosimetry is a system consisting of dosimeters, measurement instruments and their associated reference standards and procedures for using the system (Mod Ali, 2007). Dosimeters are devices that provide a quantitative and reproducible measurement of dose through a change in one or more of the physical properties of the dosimeters in response to the exposure to radiation energy (Figure 7). The selection of dosimeters will depend on the type of irradiator and range of dose to be measured. For measuring 25kGy by gamma rays, dosimeter ceric-cerous (ce/ce) can be used as the routine while ferrous sulphate (Fricke) as the reference standard dosimeter. Dosimeters for electron beams will be cellulose triacetate (CTA) film for the routine and graphite calorimeter as the primary or reference standard. Figure 4 shows dosimeters which are commonly used in commercial irradiators.



(a) (b)

Figure 7. Dosimeters for measuring absorbed dose: (a) ceric-cerous solutions for gamma rays and (b) CTA film for electron beams

Labelling of Amnion

Each package of amnion normally carries a label indicating graft no., type and size of amnion, expiry date and name of tissue bank. The label must also have go-no-go radiation indicator (sticker) which is usually yellow in colour. The indicator will turn red after radiation (Figure 8). The indicator does not inform the amount of absorbed dose but the changes in colour will assist the users to distinguish irradiated products from non irradiated ones.

Transportation to and from Irradiation Facility

A box of air-dried/freeze-dried and glycerolised amnions can be sent to a radiation facility by car, air or even by post (Figure 9). Some radiation facilities usually can arrange the return of the box by any modes of transportation as requested by the tissue bank. The situation is critical when handling frozen tissues as the amnions must be packed in dry ice and the frozen temperature must be maintained throughout irradiation time and transport back to tissue bank. Validation of temperatures for the whole duration must be conducted to show that the amnions are kept in frozen form below −40°C until arrive back and immediately return to deep freezer −80°C at the bank.



Figure 8. Go-no-go radiation indicator: before (yellow) and after (red) irradiation



(a) (b)

Figure 9. A box contains (a) air-dried amnion and (b) glycerolised amnions packed individually ready for gamma irradiation at room temperature

Documentation on Radiation

All the findings must be documented and become a part of quality records for validation. During an audit visit, validation data is equally as important for review as are all other quality records.

Irradiation Condition

Preservation technique used and temperature during irradiation have an influence on radiation effects. Therefore it is very important to specify the requirements during irradiation.

Irradiation for dried amnions is carried out at room temperature. Direct effects of radiation will cause lesion, scission and cross-linking of biopolymeric structure (Yusof and Junaidi, 2010). These damages will result in the weakening of biomechanical properties. Similar effects in viable microorganisms will lead to cell death due to damages occurring in critical components when deflected electrons of radiation directly attack mainly DNA molecules (Figure 10). Therefore the radiation dose selected for sterilisation must be effective enough to kill microorganisms but at the same time causing only minimal damages if any.

The processed amnions must have a moisture content of less than 10% to avoid any indirect effects of radiation. When radiation passes through

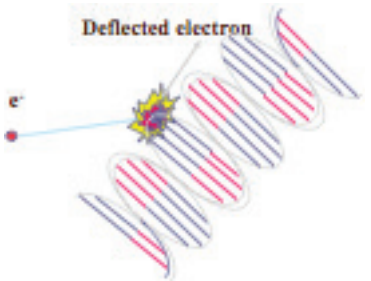


Figure 10. Direct effects of radiation: deflected electron of radiation directly attack critical components mainly DNA molecules

amnions, energy deposited in the tissue will initiate ionisation and excitation processes while the energy gets progressively weaker as radiation goes deeper inside the box. When the tissue moisture content exceeds 10%, there will be more interactions between radiation and water molecules producing free radicals and molecules which will interact with each other to form more free radicals. These free radicals will interact with critical components of the cells such as proteins, amino acids, enzymes, metabolites and nucleic acids that result in changes in biological components. Interaction of free radicals with oxygen molecules will produce hydroperoxy radicals (HO_2) and hydrogen peroxide (H_2O_2). Interaction with organic free radicals will produce organic peroxy radical (RO_2). These chain reactions will lead to greater damages and are held responsible for indirect effects of radiation (Figure 11). Therefore it is very important to keep the moisture content of air-dried and freeze-dried amnion in the range of 4–7% to lessen the indirect effects of radiation.

Glycerol preserved amnions have only direct effects of radiation because water molecules have been replaced by glycerol which is a radiation protectant. Fresh amnions must be kept frozen during irradiation so that water molecules are not free to interact with radiation to form free radicals. Amnions in frozen state will have radiation damages through direct effects which only occur at much higher doses exceeding 50 kGy. However effects on strength are not a big deal for amnions which are mainly used to cover wounds.

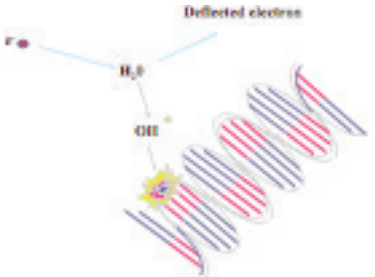


Figure 11. Indirect effects of radiation: interaction of deflected electron of radiation with water molecules results in chain reactions that produce free radicals causing greater damages

Radiation Certificate

Radiation facility will issue certificate or results of dosimetry analysis for each radiation batch. The certificate must indicate the following information for each batch:

Minimum dose — the minimum absorbed dose cannot be lower than the sterilisation dose as requested by the tissue bank.
Maximum dose — the maximum absorbed dose should not be too high such that it can cause detrimental effects on amnion.

Selection of Radiation Dose

Radiation dose of 25kGy is the most commonly used to sterilise medical products worldwide. However, sterilisation dose can actually be selected based on the cleanliness or microbiological quality of the products. Only clean products can be sterilised. Dirty products must be cleaned or decontaminated and require a very high dose for sterilisation. The cleanliness of a product is determined by measuring total microorganisms it contains. The number of microorganisms expressed as colony forming unit (cfu) per product item prior to sterilisation is called bioburden.

A tissue banker or product manufacturer can select sterilisation dose based on the information on bioburden. They are responsible for conducting validation experiments before the dosage is selected and used for routine

sterilisation. The International Organisation for Standardisation (ISO), a worldwide federation of national standard bodies provides ISO document no. 11137 as a guide to do validation experiments in establishing radiation doses for sterilising medical products (ISO, 2013). The document offers two methods based on a probability model for the inactivation of microbial population:

- Method 1 (products having microbial population of standard distribution of microbial resistance or SDR) is based on bioburden determination of the products;
- Method 2 (products with microbial population not having the SDR) requires products to be exposed to a series of incremental doses of radiation.

However both methods require large number of samples, 130 and 380 product items for Method 1 and Method 2, respectively. It is impossible for tissue banks to get sufficient samples. Due to this difficulty, the International Atomic Energy Agency (IAEA) issued a Code of Practice in 2007 (IAEA, 2007) to allow tissue bankers to conduct validation experiment using a small number of samples. The Code offers Method A (products having microbial population of standard distribution of microbial resistance or SDR) which has similar approach as Method 1 of ISO 11137:2013. It also offers Method A(i) (products with microbial population not having the SDR), which is similar to Method 2 of ISO 11137:2013. Method A and A(i) require only 20 samples for the validation experiments.

ISO 11137:2013 provides two other methods to achieve SAL 10^{-6} i.e. VD^{15}_{max} and VD^{25}_{max} to substantiate radiation doses of 15 and 25 kGy, respectively. Tissue bankers who wish to use 15 or 25 kGy to sterilise amnion grafts will substantiate the doses before the doses can be used for routine sterilisation of the grafts. However, prior to the dose validation, bioburden of the processed amnions must be consistently below 1.5 and 1,000 cfu per product for 15 and 25 kGy respectively (ISO, 2013).

The followings are work examples for methods to establish or select and validate sterilisation dose.

1. Establishment of Sterilisation Dose Based on Bioburden

Refer to Table 2 (Method A of IAEA Code of Practice IAEA, 2007):

Ten samples are required for bioburden determination. Another ten samples are exposed to a verification dose for SAL 10^{-1} as provided in Table III of the Code and then subjected to sterility test. When none or only one of the 10 samples has positive growth, the experiment is accepted. Refer to Table III again to identify the sterilisation dose at SAL 10^{-6}.

2. Selection and Substantiation of 25kGy as Radiation Dose

Refer to Table 3 (Method VD^{25}_{max} of ISO 11137:2013 to substantiate 25 kGy)

This method is applicable to products with an average bioburden of less than or equal to 1,000 cfu per item. Ten samples from 3 different batches are required for bioburden determination. Another ten samples from a single batch are exposed to a verification dose for SAL 10^{-1} as provided in Table 9 of the ISO and then subjected to sterility test. Sample item portion (SIP) = 1 or whole sample is required when bioburden is less than 0.9 cfu per item. When none or only one of the samples has positive growth, the experiment is accepted whereby 25kGy is now substantiated as the sterilisation dose to achieve SAL 10^{-6}.

Table 2. Work example using Method A of IAEA Code of Practice (Yusof and Hilmy, 2010)

Sample	Air-dried amnions with size 10×10 cm
Average bioburden of 10 samples from 3 batches	7.6 cfu/amnion
Verification dose (at SAL 10^{-1})	2.8 kGy
Sterility test	No positive
Therefore, Sterilisation Dose at SAL 10^{-6}	**17.2 kGy**

Table 3. Work example using Method VD_{max}^{25} (Yusof and Hilmy, 2010)

Sample	Air-dried amnions with size 10×10 cm
Overall Average bioburden of 3 batches (10 samples/batch)	7.63 cfu/amnion
Verification dose (at SAL 10^{-1})	6.9 kGy
Sterility test	No positive
Therefore,	
Substantiate Sterilisation Dose at SAL 10^{-6}	**25 kGy**

3. Selection and Substantiation of 15 kGy as Radiation Dose

Refer to Table 4 (Method VD_{max}^{15} of ISO 11137:2013 to substantiate 15 kGy).

This method is applicable to products with an average bioburden of less than 1.5 cfu per item. Ten samples from 3 different batches are required for bioburden determination. Another ten samples from a single batch are exposed to a verification dose for SAL 10^{-1} as provided in Table 10 of the ISO and then subjected to sterility test. Sample item portion (SIP) = 1 or whole sample is required. When none or only one of the samples has positive growth, the experiment is accepted whereby 15kGy is now substantiated as the sterilisation dose to achieve SAL 10^{-6}.

Tissue banker personnel must work closely with radiation facility in conducting these dose validation experiments because the exercises require delivery of verification doses within 10% variation. An independent and certified microbiology laboratory must be appointed to conduct sterility tests on the irradiated samples. Complete reports on validation experiments must be kept properly as they are part of the quality documentation of a bank. The reports act as evidence that the established and selected dosage has been validated before use for routine sterilisation.

A validation exercise must be conducted whenever bioburden cannot be maintained or when there are changes in processing procedures.

Tissue bankers are responsible for clearly indicating the type of sterilisation technique used on the label of the grafts or in the product insert. All records regarding name of the sterilisation facility, data on sterilisation process

Table 4. Work example using Method VD_{max} [15] Yusof and Hilmy, 2010

Sample	Air-dried amnions with size 10×10 cm
Overall Average bioburden of 3 batches (10 samples/batch)	0.73 cfu/amnion
Verification dose (at SAL 10^{-1})	2.3 kGy
Sterility test	No positive
Therefore,	
Substantiate Sterilisation Dose at SAL 10^{-6}	**15 kGy**

such as results of dosimetry analysis for radiation sterilisation (maximum and minimum doses) must be properly filed for audit and traceability.

Conclusion

Sterilisation is a validated process to inactivate or to kill microorganisms on and in a product and it is necessary to render a sterility assurance level of 10^{-6} for amnion grafts. Radiation technique using gamma rays or electron beams is a physical method which provides an effective terminal sterilisation for amnions in final packaging. The technique must be validated whereby the established/selected doses must be verified or substantiated prior to using the radiation dose for routine terminal sterilisation process. Amnions with low bioburden can easily be irradiated at 25kGy to achieve sterility assurance level (SAL) of 10^{-6} and for amnions with very low bioburden (less than 1.5 cfu), low dose of 15 kGy can be considered. The sterilisation of tissues is definitely very important, but we must not forget that it is by no means a substitute for proper screening, sterile retrieval and hygienic handling.

References

Fages J, Marty A, Delga C, Condoret JS, Combes D and Frayssinet P (1994) Use of supercritical CO2 for bone delipidation. *Biomaterials*, **15**: 650–656.

IAEA (2007) Radiation sterilisation of tissue allografts: Requirements for validation and routine control — A Code of Practice, International Atomic Energy Agency, Vienna.

ISO (2013) Sterilisation of health care products — Radiation — Part 2: Establishing the Sterilisation Dose, ISO 11137:2013, International Organisation for Standardisation, Switzerland.

Link D, Siddappa R, Thio M, Babychan B and Valster H (2014) Supercritical CO_2: The future for cleaner and safer tissues for regenerative medicine. Presented at the 7th *World Congress on Tissue Banking*, Melbourne, Australia, p. 22.

Lovric V, Irani M and Walsh WR (2014a) Effects of 25kGy gamma irradiation and supercritical CO2 sterilisation techniques on the permeability of bovine cortical bone. Presented at the 7th *World Congress on Tissue Banking*, Melbourne, Australia, pp. 27–28.

Lovric V, Yu Y, Anwar R and Walsh WR (2014b) Effects of preservation and sterilisation techniques on the *in vivo* response of allograft bone in the treatment of osseous defects. Presented at the 7th *World Congress on Tissue Banking*, Melbourne, Australia, pp. 23–26.

Mod Ali N (2007) Dosimetry and requirements for process qualification. In: Nather A, Yusof N and Hilmy N (eds.), *Radiation in Tissue Banking*, World Scientific, Singapore, pp. 171–186.

Phillips GO (2013) Jorge Morales Pedraza: The Use of the Ionization Technique for Tissue Sterilisation: the International Atomic Energy Agency (IAEA) experience. *Cell Tissue Bank*, **14**: 341–348.

Pruss A, Baumann B, Seibold M, Kao M, Tintelnot K, von Versen R *et al.* (2001) Validation of the sterilisation procedure of allogeneic avital bone transplants using peracetic acid–ethanol. *Biologicals*, **29**: 59–66.

Pruss A, Kao M, von Garrel T, Frommelt L, Gürtler L, Benedix F and Pauli G (2003) Virus inactivation in bone tissue transplants (femoral heads) by moist heat with the 'Marburg bone bank system'. *Biologicals*, **31**: 75–82.

von Versen-Hoeynck F, Steinfeld AP, Becker J, Hermel M, Rath W and Hesselbarth U (2008) Sterilisation and preservation influ- ence the biophysical properties of human amnion grafts. *Biologicals*, **36**: 248–255.

Yusof N and Hilmy N (2010) Principle Concepts of Radiation Sterilisation for Tissue Allografts. In: Nather A, Yusof N and Hilmy N (eds.), *Allograft Procurement, Processing and Transplantation — A Comprehensive Guide for Tissue Banks*, World Scientific, Singapore, pp. 371–383.

Yusof N and Junaidi AHA (2010) Interaction of radiation with tissues. In: Nather A, Yusof N and Hilmy N (eds.), *Allograft Procurement, Processing and Transplantation — A Comprehensive Guide for Tissue Banks*, World Scientific, Singapore, pp. 455–466.

12

Routine Quality Control of Processed Amniotic Membrane

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Introduction

Quality control (QC), a component under Quality Assurance (QA), is mainly conducted on in-coming materials (starting material QC), during processing (in-process QC) and at the end of a processing line (processed product QC). According to American Association of Tissue Banks (AATB) Standards, specific tests defined by the QA Programme are to be performed to monitor recovery, processing, preservation and storage, tissue quality, and test accuracy (AATB, 2008). These may include but are not limited to, performance evaluations, inspection, testing, and controls used to determine the accuracy and reliability of the tissue bank's equipment and operational procedures, as well as the monitoring of supplies, reagents, equipment, and facilities (AATB, 2008). The QC is a part of quality management defined as operational techniques and activities that are used to fulfill requirements for quality (APASTB, 2016). The QC is performed to assure that the processed

tissue has the identity, characteristics and quality intended and within established tolerance limits.

Measurable parameters to be carried out for the QC must be relevant to the end function of the product. For the processing of amniotic membrane, two parameters are recommended namely Water Content (Yusof and Hilmy, 2007) and Bioburden (Yusof and Hassan, 2007) as the routine laboratory QCs. The amnion samples are taken randomly immediately after the processing and before sterilization and will be subjected to the QC tests. Results of the tests shall be documented and reviewed.

Water Content

Water content or moisture content is the amount of water in a sample after processing. During processing, the amniotic membrane is washed followed by chemical treatment to inactivate HIV thus reducing any risk of HIV contamination. For preservation, processed amnions are subjected to dehydration to reduce residual water content, which is necessary for long-term storage at ambient temperature or colder. Dehydration can be achieved either by freeze drying using lyophilizer, air drying in a laminar flow cabinet or oven drying at 40°C. The main purpose of dehydration or drying is to reduce water content to less than 10%, preferably 6 to 7% (Yusof and Hilmy, 2007). Failing to reduce water content will lead to fungal and bacterial growth when there is delay in sterilization process. At this low residual water content, the indirect effect of radiation due to free radical formation during sterilization by gamma irradiation will be minimized (Yusof and Hilmy, 2010). A high amount of residual water will result in more free radical formation following more interactions of ionizing radiation with water molecules. Interactions of free radicals with tissue components will result in changes in tissue properties (Yusof and Hilmy, 2014).

Under QC programmes, performance of either a lyophilizer or a dehydrator shall be monitored and documented. A representative sample for the dried amnion shall be sent for routine QC test for residual moisture or water content. According to the AATB Standards, the residual moisture of lyophilized musculoskeletal tissue shall not exceed 6% of initial weight by gravimetric or Karl Fischer analysis or 8% by nuclear magnetic resonance spectrometry (NMR) (AATB, 2008). Since no specific value for residual

water content in amnion is given by any standards, each tissue bank must establish and validate the level for their processed amnion, which should not exceed 10%.

Water Content by Gravimetric Method

The most common method employed by tissue banks in Asia Pacific region to measure residual water content is gravimetric method (Yusof and Hilmy, 2007). Example of a procedure determining water content by oven drying to a constant weight is given below:

I. Equipment

Oven : Preheat oven at 60°C

Balance : Must be sensitive to 0.1 % of the minimum weight of the sample to be weighed.

II. Sampling

Select a representative quantity of the processed amnion in the final packaging.

III. Methodology

1. Dry container (e.g. crucible or petri dish) in a 105°C oven, preferably heated overnight until its weight is constant. Place in desiccator for about 20 minutes before weigh to stabilize the reading (W*c*).
2. Place amniotic sample (minimum weight 0.1 to 0.2 g) in the container and weigh (W*i*).
3. Dry the container with the sample in oven at 60°C for about 4 to 6 hours (or until the weight is constant), transfer to dessicator and weigh the cooled amnion (W*t*).
4. Calculate the percentage of water content.

IV. Calculation

The water content of the amnion is calculated using the following equation:

$$\% \text{ Water Content} = \frac{\text{Weight of water released after drying (g)} \times 100}{\text{Weight of amnion before drying (g)}}$$

$$\% \text{ Water Content} = \frac{(Wi - Wt)}{(Wi - Wc)} \times 100$$

Where

Wi is initial weight of container and sample

Wt is weight of container and sample after drying at 't' hour when the weight is constant

Wc is weight of container

Steps involved in determination of residual water content for pericardium samples are shown in Figure 1. Similar steps are carried out to determine residual water content in other membrane tissues including amnions.

Bioburden

Bioburden is defined as the number of contaminating organisms found on a given amount of tissue prior to undergoing a sterilizing procedure (AATB, 2008). Bioburden estimation is an important tool

- to determine the level of hygiene of tissue products, also
- to determine the effectiveness of radiation doses for sterilisation.

Bioburden must be kept low (microbial counts per graft) before the product is accepted for sterilisation in order to achieve a high sterility assurance



(a) (b) (c)

Figure 1. Determination of residual water content in freeze dried pericardium: (a) Pericardium samples are dried in oven at 60°C (b) Oven dried pericardiums are placed in dessicator before weigh (c) Pericardiums are weighed before and after drying

level (SAL) (Yusof and Hilmy, 2014). Bioburden reflects the cleanliness of a product and quantifies microbiological contamination level it.

Bioburden estimation of tissue shall be performed by a laboratory that is either certified or accredited by national laboratory accrediting organization. If microbiologic testing is to be performed by the tissue bank itself, the requirements as outlined by the local authority on QC laboratory shall apply. If the services of an outside laboratory are used, the procedures used and test results by that laboratory should be reviewed by the person responsible at the tissue bank (AATB, 2008). The bank must also have a standard operating procedure (SOP) for determining the acceptability of outside testing laboratories (AATB, 2008).

Microbiological standards available for estimating bioburden include the International Standard ISO 11737- Part 1 and for performing sterility tests, it is in accordance with ISO 11737- Part 2.

The ISO 11737 – Part 1 (2006) describes in detail the methodologies, recommended diluents and media as well as how a laboratory can conduct validation in choosing media and establish their own working procedures (ISO, 2006).

Methods for Bioburden Estimation

Three methods commonly used to estimate microbiological colonies of any samples where removal of microoganisms by elution/suspension is used, are:

1. Pour Plate method

An aliquot of microbial suspension is mixed with molten agar at temperature of approximately 45°C; the mixture is allowed to solidify in the plate, incubated and the microbial colonies grow within and on the surface of agar medium. The amount of eluent that can be pour-plated is limited therefore this method may not have the desired sensitivity for suspensions with low concentrations of microorganisms.

2. Spread Plate method

An aliquot of microbial suspension is spread on the agar surface using a sterile spreading device so colonies grow on the surface of agar medium. The aliquot of suspension that has been spread on the surface of the medium must be absorbed so that discrete colonies can develop; the need for absorption

governs the volume of the aliquot that can be spread using one plate. The amount of eluent that can be spread-plated is limited therefore this method might not have the desired sensitivity for suspensions with low concentrations of microorganisms.

3. Filtration method

An aliquot of suspension is filtered through a membrane and then the membrane is transferred onto agar medium. This method is more sensitive and particularly useful for products of low bioburden.

Petri dishes with microbial suspension or filter papers are incubated up to 5 to 7 days until a single microbe proliferates to form a colony. The colonies per plate can easily be counted with the naked eye and scored as a colony forming unit (cfu). The cfu per sample product can then be estimated.

For those samples suspected containing high microbiological count, serial dilution is required and the first two methods i.e. Pour Plate and Spread Plate can be considered. Only a microbial count between 30 to 300 colonies per plate is acceptable for these Pour Plate and Spread Plate method. Therefore for samples with very low bioburden, less than 30 counts, such as tissues and medical devices, filtration method is the most suitable.

For products with very low bioburden and methods where removal of microorganisms by elution/suspension is not used, these 3 methods are suggested (ISO, 2006):

1. Contact plating

Contact plates or slides are means by which solidified culture medium can be applied to a surface with the intention that viable microorganisms will adhere to the surface of the medium. The plate or slide is then incubated to produce colonies that are enumerated. This method should be used only when other methods are not applicable because the efficiency is generally low. Contact plates and slides are generally only useful on flat or at least regular surfaces.

2. Agar overlaying

Agar overlaying involves coating the surfaces of a product with a molten agar medium (at a temperature of approximately 45°C) and allowing it to solidify, followed by incubation to produce visible colonies. This method may be applicable when the bioburden is low and the product configuration

is suitable. The natural clumping of cells on surfaces, spreading of colonies at the agar interfaces, drying out of the agar, and possibility of anaerobic locations are potential disadvantages.

3. Most Probable Number (MPN)

The MPN method is a well-established and fully documented method of estimating the number of viable microorganisms in product in which the microorganisms are randomly distributed. Its principal uses are in the food and water industries where it is used with liquid, powdered and semi-solid products or raw materials. The method is particularly appropriate for a product with a low mean bioburden.

The method consists of taking replicate samples of product (by volume or weight) which contain, on average, the same number of viable microorganisms in each of them (hence the requirement for randomness of distribution) and scoring each sample individually for the presence of viable microorganisms by means of transferring to liquid growth media and incubation. When a sufficient volume of eluent is available, a range of dilutions can be inoculated into nutrient medium such that a fraction of the inoculated media does not produce visible growth on subsequent incubation. From the frequency of the occurrence of positive tests within a set of replicates, an estimate is made of the number of viable microorganisms present in the sample or the bulk product from which the sample has been taken; the 95% confidence limits about the estimate are relatively wide. The estimate and its confidence limits are derived from published MPN Tables, which have been developed on the assumption that numbers of viable microorganisms present in replicate samples are distributed around a mean number in accordance with Poisson distribution.

MPN methods are simple to perform, and the statistical basis for the method makes it more appropriate for general assessment rather than accurate determinations.

Stages in Bioburden Estimation

Figure 2 shows the four stages in bioburden estimation. It is encouraged to identify and characterise the most commonly found microorganisms of a particular tissue product.

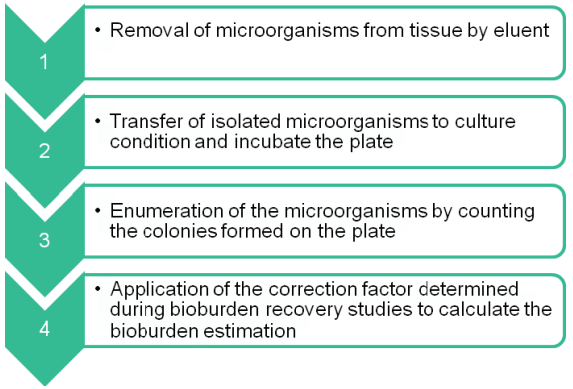


Figure 2. Four stages in bioburden estimation

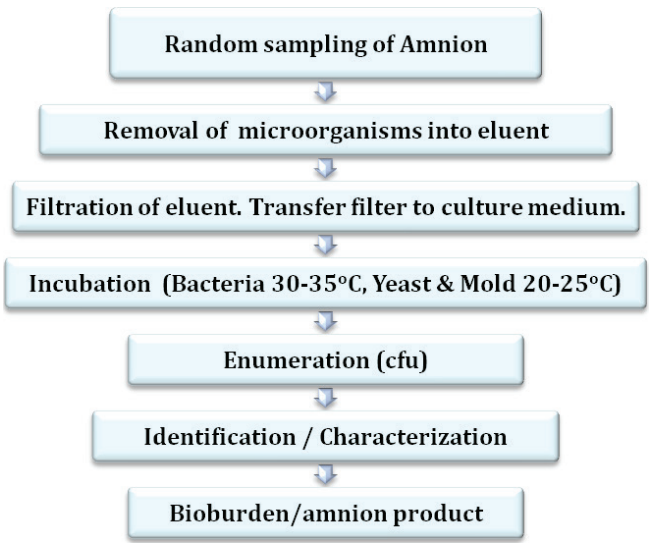


Figure 3. Work flow in conducting the bioburden estimation of an amnion sample using filtration method

As a tissue product, amnion is processed under clean environment. In principle, the bioburden should be low therefore filtration method is highly suggested. Figure 3 describes the workflow involved in conducting the microbiological test using filtration method.

Requirements for Bioburden Estimation

Details of requirements for bioburden estimation are described in the ISO 11737-Part (ISO, 2006). The following components are discussed as a guide for tissue bankers to prepare in establishing in-house QC laboratory for bioburden analysis (Yusof *et al.*, 2010): -

- Basic Equipment, Tools and Consumables for Bioburden Estimation
- Eluents and Diluents in Bioburden Estimation
- Media and Incubation Conditions for Bioburden Estimation

Basic Equipment, Tools and Consumables for Bioburden Estimation

Basic equipment, tools and consumables required in a microbiology laboratory are listed in Table 1. Scheduled maintenance for equipment must be

Table 1. Basic laboratory equipment and tools for bioburden estimation work

Equipment	Tools	Consumables
Laminar Airflow Cabinet	Membrane filtration set and vacuum pumps	Surgical gloves
Incubators	Anaerobic jars	Head covers
Autoclave	Forceps	Shoe covers
Water Distiller	Scissors	Laboratory coats
Water bath	Spreaders	Face masks
Microscopes	Inoculating loops	Disinfectants
Dry sterilizer (Oven)	Thermometers	Hand soaps and creams
Refrigerator & Freezer	Petri dishes	Staining kit
Vortex mixer	Class slides and covers	Eluent and Media
Hot plates & Magnetic stirrer	Glassware (bottles, beakers, flasks, test tubes, pipettes, glass etc.)	Chemicals
Ultrasonic cleaner	Pipettes and tips	Identification kits
Sealing machine	Bunsen burner and gas tank	Membrane filters 0.2 μm pore size
pH meter	Sprayer	Cotton gauze
Electronic balances		
Colony counter		

conducted in accordance with documented procedures and records of maintenance shall be retained. Calibration must be carried out on equipment with measurement or control functions.

Eluents and Diluents in Bioburden Estimation

All microbiological eluents and diluents needed to remove microorganisms from products and used for serial dilutions should be prepared sterile. The eluents or transport media should be such that it maintains the viability of aerobic and anaerobic, bacterial and fungal organisms. The compositions of selected eluents and diluents should not cause any proliferation or inactivation of microorganisms. The use of proper eluents and diluents can influence the overall efficiency of the bioburden method used. Commonly used eluents and diluents as listed in Table 2 can be considered for use.

When a liquid is used for removal of microorganisms from solid surfaces, the addition of a surfactant such as polysorbate (Tween) 80 into the selected eluent may be considered. A concentration of between 0.01% and 0.1% is generally used, depending upon the specific application.

Recovery of microorganisms from product surfaces may be enhanced by the presence of a surface-active agent in the eluent and can be improved

Table 2. Examples of commonly used eluents and diluents as listed by ISO 11737-1:2006

Solution	Concentration in water	Applications
Buffered peptone water	0.067 M phosphate 0.43% sodium chloride 0.1% peptone	General
Calgon Ringer	1/4 strength	Disolution of calcium alginate swabs
Peptone water	0.1% to 1.0%	General
Phosphate buffered Saline	0.02 M phosphate 0.9% sodium chloride	General
Ringer	1/4 strength	General
Sodium chloride	0.25% to 0.9%	General
Thiosulfate Ringer	1/4 strength	Neutralisation of residual chlorine.
Water	NA	Dilution of aqueous samples. Preparation of isotonic solutions of soluble materials prior to counting.

further by subjecting the sample to a physical treatment while in the eluent such as stomaching, ultrasonicating and shaking. Any eluents and diluents to be used in routine work must be validated to show their efficiency in giving highest bioburden count.

Media and Incubation Conditions for Bioburden Estimation

The selected microbiological growth media should be able to provide the optimal conditions to support growth of aerobic and anaerobic microorganisms specifically bacterial and fungal organisms commonly encountered by the amniotic membrane. A growth promotion test is done on each batch of medium using an inoculum of low numbers between 10 and 100 colony forming units (cfu) of a selected microorganism. Examples of media and incubation conditions as suggested in Table 3 can be used as a guide in choosing media according to ISO 11737-1:2006. All non-selective

Table 3. Examples of media and incubation conditions as recommended by ISO 11137-1: 2006

Types of microorganisms	Solid media	Liquid media	Incubation conditions[a]
Facultative, non-fastidious, aerobic bacteria	Soybean casein digest agar (Tryptone soya agar) Nutrient agar Blood agar Glucose tryptone agar	Soybean casein digest broth (Tryptone soya broth) Nutrient broth	30–35°C for 3–7 days
Yeast and molds	Sabouraud dextrose agar Malt extract agar Rose Bengal agar Chloramphenicol agar Soybean casein digest agar (Tryptone soya agar) Potato dextrose agar	Sabouraud dextrose broth Malt extract broth Soybean casein digest both (Tryptone soya broth)	20–25°C for 5–7 days
Anaerobic bacteria	Reinforced clostridial agar[b] Schaedler agar[b] Pre-reduced blood agar[b] Fastidious anaerobe agar[b] Wilken-Chalgren Agar[b]	Robertson's cooked meat broth Fluid thiglycollate broth	30–35°C for 3–7 days

[a] The incubation conditions listed are those which are commonly used for the types of microorganisms listed.
[b] Cultured under anaerobic conditions.

anaerobic culture methods may also permit the growth of facultative anaerobic organisms.

Tissue banks or contracted microbiology laboratories shall follow standards developed by the National Laboratory Standards, if any, specifying the requirements for *Quality Assurance* testing of culture media. Tissue banks that prepare their own growth medium must perform the following QC checks on each batch of media prepared:

1) Check each batch of media that is autoclaved or filtered during preparation for sterility; and
2) Check each batch of media for ability to support growth, using *Staphylococcus aureus* ATCC 25923 for aerobic growth and *Bacteroides fragilis* ATCC 23745 or 25285 for anaerobic growth.

If the batch or *Lot* is not used within 30 days, another check is recommended. Records must be kept for all QC procedures listed above. Batches of media that do not meet the above criteria shall not be used.

Sampling of Product

During sampling and handling the sample, any introduction of advertent contamination and significant alterations to the numbers and nature of microorganisms in the sample must be avoided. Sampling procedures should be consistent thus comparisons of data can be made over a period of time.

The bioburden estimation should utilise the whole product whenever practical. If not feasible such as when dealing with large amnion, a portion can be used. The selected portion must be able to reflect as closely as possible the bioburden of the whole packed product. Ideally, bioburden should be estimated for each production batch or on a regular basis. Sampling may be performed at a frequency based on time such as monthly or based on production batch such as every alternate batch or every 3 to 5 batches. Initially, in order to establish baseline levels, it is advisable to perform bioburden estimations for every production batch especially during the initial production of a new tissue product. The frequency can be reduced as the knowledge of the bioburden develops or the bioburden shows consistency. The frequency

of bioburden estimations should allow for the detection of changes in bioburden due to

- changes in processing/manufacturing,
- changes in materials,
- changes of personnel or
- even changes in environment.

Bioburden estimation is therefore a useful indicator for monitoring the production process. Bioburden can be used as a sensitive tool for detecting changes in processing, personnel and changes in the environment (Yusof *et al.*, 2010).

Bioburden Estimation using Membrane Filtration

Filtration of an eluent, followed by incubation of the filter on an appropriate growth medium to give visible colonies, is an effective means of enumerating viable microorganisms. A filter of appropriate nominal pore size not greater than 0,45µ is generally adequate to capture microorganisms. A vacuum source is usually required. Care should be exercised in order to avoid excessive backpressures, which can cause distortion of or damage to the membrane filter. Membrane filtration is particularly useful for suspensions of low concentrations of microorganisms.

Here is an example of a procedure to conduct a routine bioburden estimation using membrane filtration method:

I. Preparation of Solutions

Disinfectant: Alcohol 70%
Saline polysorbate solution (0.01%) as diluent: Mix 9.0 gm sodium chloride with 0.1 ml polysorbate solution (tween 80) in 1000 ml of distilled water. Stir on hot plate. Dispense 150 ml into 200ml bottles. Autoclave at 121°C for 15 minutes.

II. Preparation of Media

Plate Count Agar (PCA): Mixed 23.5 gm PCA agar powder with 1000 ml of distilled water. Heat the mixture on hot plate stirrer. Boil until the agar

dissolves completely. Dispense into a 1 litre bottle or conical flask. Cap the bottle or plug the cornical flask and autoclave for 15 minutes at 121°C. Pour approximately 15 to 20 ml of the media into sterile petri dishes aseptically (in the laminar flow cabinet). Allow the agar to solidify. Incubate the plates upside down in incubator at 32–35°C for 24 hours. Discard plates with growth. Keep clean plates in sealed plastic bag at room temperature (for short term storage) or in refrigerator (for long term storage).

III. Sampling of Amnion

Pick random samples of amnion for testing from each processing batch and measure the size of the amnion.

IV. Methodology

1. Switch on laminar flow cabinet at least 20 minutes before working. Disinfect the cabinet with 70% alcohol.
2. Assemble all the instruments needed in the laminar flow cabinet.
3. Disinfect the outside of the packaging with 70% alcohol.
4. Cut the packaging, transfer the sample with sterile forcep into saline polysorbate solution.
5. Mix for 5 minutes on vortex mixture. Repeat if necessary. Leave to stand for a short while.
6. Place the sterile membrane filter (pore size 0.2 or 0.45µm) on the filtration unit with sterile forceps. Filter certain volume of aliquot or cell suspension depending on the preset results. The membrane filter captures the microorganisms that are passing through. Pour sterile water to wash down the aliquot.
7. Remove the filter with sterile forceps. Roll the filter slowly onto surface of agar plate from the edge to eliminate the air bubbles. Label the plates.
8. Rinse the filteration unit with sterile water after each filteration and before filtering a new sample.
9. Incubate the plates invertedly for 5–7 days at 30 to 35°C for total aerobic bacteria (TB) and at 20 to 25°C for fungus, yeast and mould (TFYM). Place in anaerobic jar and incubate at 32–35°C for 5–7 days for anaerobic bacteria (TAB).
10. Count the colonies that appear on the filter. The count is expressed as colony forming unit (cfu). Colonies can be isolated for characterisation and identification. It is not necessary to isolate and identify each type

of the contaminants. Identification of the most commonly found contaminants is sufficient and it is preferable to identify the most resistant colonies to the selected sterilisation treatment. In the case of radiation as the sterilisation treatment, some *Bacillus* sp. are radiation resistant. For pathogenic bacteria such as *Streptococcus species*, *Pseudomonas species*, *Staphylococcus species*, coliform bacteria and *Escherichia coli*, isolate them using selective media. If coliform bacteria and *E.coli* are found in the sample, all grafts from a particular donor should be discarded.

V. Calculation

Calculate the Total Microbial Count (TMC) as follows:

TMC = TB + TFYM + TAB

If filtering only a portion of aliquot/eluent, calculate total microbial count according to the initial volume of the aliquot and the dilution factor must be taken into account if a serial dilution is done.

The final bioburden estimation of a product is obtained by applying a correction factor to the total microbial count, compensating for the recovery efficiency of the observed colonies. It is important that an accurate bioburden estimate is obtained when bioburden estimates are being used to establish the extent of the sterilisation treatment /process such as in determining radiation dose. In fact the bioburden estimate is required to confirm the adequacy of an established sterilisation process.

As the radiation dose is determined and validated based on bioburden performance, the record on bioburden is important to confirm whether the product is sterile. Sterility tests on random samples after radiation process are no longer necessary.

Steps involved in bioburden estimation of amnion samples are shown in Figure 4. The analysis was done in laminar air flow cabinet.

Recovery Efficiency of Bioburden Estimation

The validation of bioburden estimation techniques largely depends upon:

- The ability of the selected media to support growth
- The ability of the selected temperature and incubation time to support growth in the selected medium.



Figure 4. Bioburden estimation of the amnion using membrane filtration method: (a) Disinfect the package of the amnion with 70% alcohol before cutting and removing the amnion. (b) Immerse amnion into 0.01% sterile saline polysorbate. (c) Shake the amnion for 15 to 20 minutes to remove microorganisms. (d) Filter the aliquot of microorganism suspension through a membrane filter. (e) Place the membrane filter on agar plate. (f) Incubate the plates at 32 to 35 degrees Celsius upto 5 to 7 days. Colonies that appear on the filter are counted

The validation of the bioburden estimation techniques should ultimately lead to an insight into the microflora existing on a product. The ISO 11737-Part 1 requests for all the techniques used to be validated and the recovery efficiency to be determined. Two approaches are available for the validation of the efficiency of removal of microorganisms from the amnion:

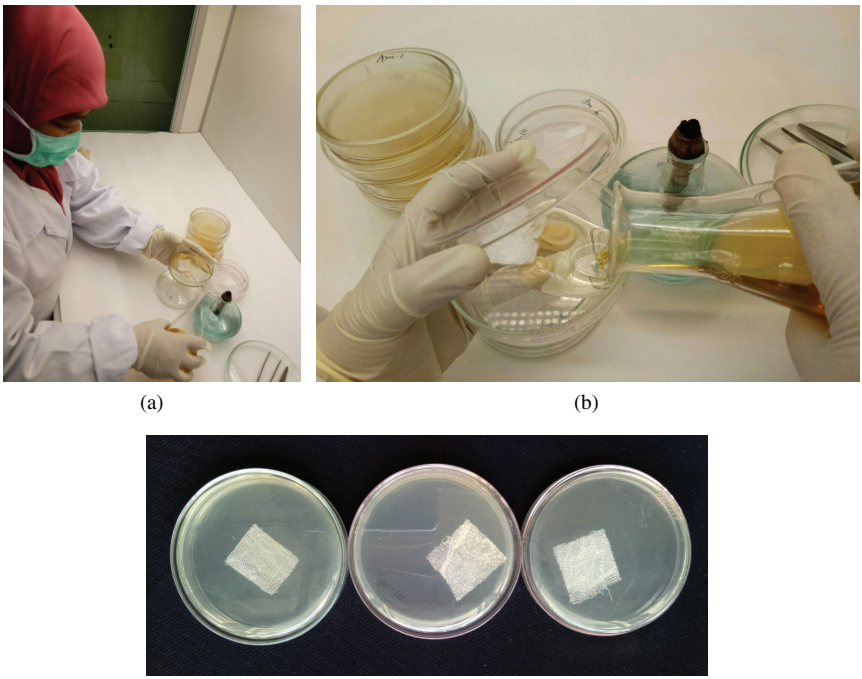
- Repetitive treatment of a sample product — This approach has the advantage of using the naturally occurring microbiological contamination but usually needs a relatively high initial bioburden.
- Product inoculation with known levels of microorganisms — This approach creates a model system for testing purposes suitable for low levels of natural contamination. However, this approach cannot be used for products with low levels of bioburden such as tissue grafts.

Repetitive Recovery Method

This approach uses the bioburden as it occurs naturally on the product, sometimes referred to as an "exhaustive recovery". The bioburden estimation method should be repeated until there is no significant increase in the accumulated number of microorganisms recovered. After each repetition, the eluent is totally recovered from the product. Results accumulated from the consecutive recoveries are compared.

An example as described in ISO 11737-1:2006 is presented here to illustrate how the correction factor is determined using repetitive recovery method. Bioburden estimates of 5 replicates of a tissue product as shown in Table 4. In each replicate, bioburden estimate is repeated 4 times with fresh eluent for each treatment. At the final stage, agar overlay is conducted by coating the surfaces of the product with a molten agar culture medium (at a maximum temperature of 45°C) and incubating it to produce visible colonies (Figure 5). Percent of recovery is calculated by dividing colony count in the first treatment with total colony count for each replicate. The average recovery from 5 replicates is then obtained. The correction factor is determined as follow:

$$\text{Correction factor (CF)} = 100/\text{Average recovery}$$



(a) (b)

(c)

Figure 5. Agar overlay is conducted by (a) placing amnion in a plate, (b) coating the surfaces of the amnion with a molten agar culture medium (at a maximum temperature of 45°C) and (c) incubating the plates to produce visible colonies

Table 4. Colony counts determined from repetitive treatment for 5 replicates of a product and calculation of correction factor (ISO 11737-1:2006)

	Replicate count				
Treatment	1	2	3	4	5
1	60	50	70	55	45
2	10	12	5	2	3
3	1	0	2	0	0
4	0	1	0	0	1
Agar overlay	2	1	2	1	0
Total colony count	73	64	79	58	49
% Removal*	82	78	89	95	92

* %Removal = [Count in Treatment 1/Total colony count] × 100.

In routine bioburden estimation, the colony counts (cfu) of the product are multiplied with the correction factor to get the bioburden estimation, as follow:

Bioburden estimate = [Colony count at presterilisation × CF] cfu

Average recovery by first treatment = 87.2% (Range = 78% to 95%)

Correction factor (CF) = 100/Average recovery = 100/87.2 = 1.15

In a routine bioburden estimation, the colony counts (cfu) of the product are multiplied with the correction factor to get the bioburden estimation, as follows:

Bioburden estimate = [Colony count at presterilisation × 1.15] cfu

Product Inoculation Method

An artificial bioburden is created by inoculating a known number of a selected microorganism on to a product in order to establish recovery efficiency. The microorganisms can be vegetative cells but the most common approach utilizes aerobic bacterial spores. The use of vegetative microorganisms is difficult in practice because loss of viability can occur on drying. Therefore aerobic bacterial spores are commonly used for inoculation, as they are capable of resisting drying.

A suspension of the microorganisms is prepared and its viable count determined. The number of microorganisms comprising the artificial bioburden should be of the same order of magnitude as the natural contamination on a product. For items with a low bioburden, a volume of suspension of suitable concentration that deposits less than 100 viable microorganisms on the product might be appropriate. A number of sterile products, or parts thereof, for which the recovery efficiency is to be determined, are selected. Each product is inoculated with a volume of the suspension of microorganisms and allowed to dry under laminar airflow cabinet. The suspension should be distributed on the product in such a way that the part from which it is most difficult to remove natural contamination is included.

The defined method of determination of bioburden is used to find the number of inoculated microorganisms that are removed from the product. The number of microorganisms removed is expressed as a fraction of the

number inoculated on to the product. This fraction is calculated for each product and is used to establish recovery efficiency. However, the results derived from the validation of bioburden recovery involving direct inoculation cannot mimic exactly the recovery of the true bioburden.

An example as described in ISO 11737-1:2006 is presented here to illustrate how the correction factor is determined using product inoculation method.

An aqueous suspension of *Bacillus subtilits* var. Niger is prepared and the viable count of the suspension is determined. A dilution of the suspension is prepared such that 0.1ml aliquots contain 100 spores. A selected portion of the sample or the whole product is inoculated with 0.1ml of this diluted suspension and allowed to dry under laminar airflow. The inoculated product is subjected to the chosen removal technique and the mean number of the *B subtilis* spores removed is obtained. Therefore the correction factor for recovery efficiency is calculated as shown below:

Number of *B subtilis* spore inoculated = 100 cfu

Number of *B subtilis* spore removed = 35 cfu

(with the range from 25 to 40)

Therefore,

Correction factor = 100/35 = 2.9

In routine bioburden estimation, the colony counts (cfu) of the product are multiplied with the correction factor to get the bioburden estimation, as follow:

Bioburden estimate = [Colony count at presterilisation × 2.9] cfu

Some bioburden estimates of amnions reported by several tissue banks are summarised in Table 5. Not all mentioned the correction factor.

The bioburden of processed amnions in the early days of tissue banking in Indonesia was high as reported by Hilmy *et al.* In 1987, however, due to proper handling using validated procedures and presence of trained personnel, the bioburden reported by the same group in 2007 was greatly reduced (Table 5). Amnion routinely processed under laminar air flow had low bioburden as reported by the two groups, Yusof (2005) and Marsit *et al.* (2014). Singh *et al.* (2007) reported a wide range of bioburden for a large

Table 5. Bioburden estimations of amnions and correction factor for recovery efficiency by several tissue banks

Authors	Tissues	Bioburden estimates cfu/ product unit or size	Correction factor
Hilmy *et al.* (1987)	Amnion (Freeze dried)	7×10^4/100 cm^2 aerobic 5×10^4/100 cm^2 anerobic	NA
Yusof (2005)	Amnion (air dried)	14.5 cfu/100 cm^2	NA
Hilmy *et al.* (2007)	Amnion (freeze dried)	32.5 cfu/packet	2.5
Singh *et al.* (2007)	Amnion (air dried)	10–10^3/100 cm^2	3.5
Marsit *et al.* (2014)	Amnion (air dried)	18.35 cfu/201 cm^2	NA

number of amnion they processed in their early work on amnion. Having strict screening criteria for donors, trained personnel, validated procedures, clean equipment & tools and a controlled environment are among many factors that should be considered for routine production of amnion grafts.

Conclusion

The Quality Control on processed amnion is a part of quality management to monitor the production process. The QC results can assure that the processed tissue has the identity, characteristics and quality intended and within established tolerance limits. For dried amnions, the residual water content reflects the preservation of the physical property while bioburden estimate reflects the sterility level that is achieved after sterilisation.

References

AATB (2008) Standards for Tissue Banking. American Association of Tissue Banks, McLean, VA.

APASTB (2016) Standards for Tissue Banking, Second Edition, Asia Pacific Association for Surgical Tissue Banking.

Hilmy N, Siddik S, Gentur S and Gulardi W (1987) Physical and chemical properties of freeze dried amnio-chorion membranes sterilized by gamma irradiation. *Atom Indonesia*, **13**(2): 1–14.

Hilmy N, Febrida A and Basril A (2007) Experiences in using IAEA code of practice for radiation sterilization of tissue allografts: Validation and routine control. *Radiation Physics and Chemistry*, **76**: 1751–1755.

ISO (2006) Microbiology, ISO 11737-1:2006. Sterilization of medical devices — Microbiological methods — Part 1: Determination of a population of microorganisms on products, Switzerland.

Marsit N, Dwejen S, Saad I, Abdalla S, Shaab A, Salem S, Khanfas E, Hasan A, Mansur M and Abdul Sammad M (2014) Substantiation of 25 kGy radiation sterilization dose for banked air dried amniotic membrane and evaluation of personnel skill in influencing finished product bioburden. *Cell Tissue Bank*, **15**: 603–611.

Singh R, Purohit S, Chacharkar MP, Bhandari PS and Bath AS (2007) Microbiological safety and clinical efficacy of radiation sterilised amniotic membranes for treatment of second-degree burns. *Burns*, **33**: 505–510.

Yusof N (2005) Is the irradiation dose of 25 kGy enough to sterilise tissue grafts? In: Nather A (ed.) *Bone Grafts and Bone Substitutes — Basic Science and Clinical Applications*. World Scientific, Singapore, pp. 189–212.

Yusof N and Hassan A (2007) Importance of microbiological analysis in tissue banking. In: Nather A, Yusof N and Hilmy N (eds), *Radiation in Tissue Banking: Basic science and clinical applications of irradiated tissue allografts*. World Scientific Publishing, Singapore, pp. 201–217.

Yusof N, Hassan A and Hilmy N (2010) Bioburden estimation in tissue banking. In: Nather A, Yusof N and Hilmy N (eds.), *Allograft Procurement, Processing and Transplantation*, World Scientific, Singapore, pp. 419–433.

Yusof N and Hilmy N (2007) Physical and mechanical properties of radiaiton-sterilized amnion. In: Nather A, Yusof N, Hilmy N (eds). Radiation in Tissue Banking: Basic science and clinical applications of irradiated tissue allografts. World Scientific Publishing, Singapore, pp 155–168.

Yusof N and Hilmy N (2010) Principle Concepts of Radiation Sterilisation for Tissue Allografts. In: Nather A, Yusof N and Hilmy N (eds.), *Allograft Procurement, Processing and Transplantation*, World Scientific, Singapore, pp. 371–383.

Yusof N and Hilmy N (2014) Radiation Biology of Tissue Radiosterilization. In: Brahme A (ed.), *Comprehensive Biomedical Physics*, Elsevier, Amsterdam, **7**: 263–287.

13

Use of Amnion in Plastic Surgery

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Introduction

A plethora of synthetic dressings are available, most of which are beneficial, accounts for a substantial portion of any health budget. One of the natural occurring products, the human afterbirth, is constantly available worldwide but is almost always discarded despite its membranes' established efficacy as a true biological dressing (Trelford and Trelford-Sauder, 1979; Matthews *et al.,* 1982).

In 1910, William Thornton who was a final year medical student at Johns Hopkins Hospital has suggested to Dr. Staige Davis that fetal membranes might be useful for wound healing. Although the membrane used at that time did not provide permanent cover on the only recorded case in

which Davis applied, he was enthusiastic about the concept and recommended further investigations (Davis, 1910).

The term "amniotic membrane" and "amnion" are used loosely in literature. Failure to recognize the appropriate terminology has bedeviled interpretation of clinical results and their outcomes as highlighted by Trelford. Thus, these terminologies become important for accurate definition of clinical practice and subsequent assessment of results. The term "extra-embryonic tissue" (EET) is referred to placenta, amnion and chorion. "Extra-embryonic membranes" (EEM) or amniotic membrane denotes a general name encompassing an inner membrane, the amnion and an outer membrane, the chorion, which is in contact with the maternal decidua during gestation. Hence, the term "amnion" is reserved for the inner layer of EEM as obtained when the amnion epithelium and its underlying layers are stripped from the chorion without using any elaborate techniques of separation, although this does not result in absolutely anatomical separation in histologic terms (Matthews *et al.,* 1982).

Procurement of Amnion for Clinical Usage

The real possibilities of transmitting diseases via human blood, sera and tissue products in the modern era cannot be ignored. Therefore, all the potential donors of placenta should be stringently screened for the possibility of blood borne diseases such as HIV, HbSAg, HCV, Syphilis and Gonorrhoea.

To obtain the membranes, EEM is cut from the edge of placenta and the reflected portion of the membrane is stripped from the placenta surface to the base of the umbilical cord. Strict aseptic technique should be exercised throughout. Ideally, only membranes obtained at Caesarean Sections are used, but vaginally delivered tissues have been widely applied without complications. For the vaginal delivered tissue, the amniotic membrane should be ruptured less than 12 hours before delivery. There are various conditions where the membranes are not suitable such as meconium-stained or dysmorphic, those from the mothers with seropositive blood screening or a history of venereal disease, endometriosis, prolonged labour, or toxemia. The membranes obtained are thoroughly rinsed in a normal saline to remove all blood traces, once in 0.025% sodium hypochlorite, and again several times in normal saline, before refrigeration at 4°C (Robson and Krizek, 1973) the addition of antibiotics being optional (Shun and Ramsey-Stewart, 1983).

Specific Features of Amnion Relevant to Plastic Surgical Practice

Skin substitutes and biological dressings play a major role in plastic and reconstructive surgery. The histological characteristic of amnion is quite similar to skin. The normal amnion is 0.02–0.05 mm thick and it has the thickness of only one third the thickness of the stratum corneum epidermis (Rao and Chandrasekharam, 1981). Among the various types of biological dressings, human amnion membrane play a vital role in facilitating wound healing. Amnion seems to satisfy all the criteria of an ideal biological dressing. It is thin, elastic and adherence to the wound surface. It provides an effective vapor barrier, a durable cover for raw surfaces, substantial pain relief with potential bacteriostatic properties and facilitates rapid re-epithelialisation (Rao and Chandrasekharam, 1981; Colocho *et al.,* 1974; Talmi *et al.,* 1990; Unger and Roberts, 1976). One of the most important principles in plastic surgery is to achieve rapid and complete wound healing in the shortest possible time. Any method that promotes and enhances rapid wound healing has an enormous potential for research and clinical use. Human amnion membrane has an inherent angiogenic property to increase the rate of healing and epithelialisation. Human amnion membrane has long been used for various clinical applications in the field of plastic surgery. Human amnion membrane has been widely used to treat superficial and partial thickness burns in many centers worldwide (Subrahmanyam, 1995). Amnion can also be used as a biological dressing in the treatment of chronic ulcers and pressure sores (Ward *et al.,* 1989). Skin graft donor sites can also be effectively treated by using human amnion membrane. Recently, radiation induced ulcers have been effectively treated using human amnion membrane (Gajiwala and Sharma, 2003). Human amnion thus has the potential to become an attractive biomaterial which is inexpensive and easily obtainable.

Type of Amnion Preparations Available to Clinical Applications

Amnion is mostly used as fresh, but dried (Rao and Chandrasekharam, 1981) frozen (Colocho *et al.*, 1974) irradiated (Rao and Chandrasekharam, 1981) or lyophilised (Burgos and Sergeant, 1983) preparations have also been used.

Factors that Contribute to the Success of Amnion in Plastic Surgical Practice

A. Anti-bacterial Properties

Theories of mechanism of the anti-bacterial effect observed in EEM and amnion are plentiful. Many authors have concurred that EEM and amnion are effective biological dressings since both membranes fall intimately into the contour of the wound to lie in close apposition to its surface (Trelford *et al*., 1973; Gruss and Jirsch, 1978; Bose, 1979). It has been noted that the degree of bacterial contamination was inversely proportional to the number of leucocytes beneath a graft (Gruss and Jirsch, 1978). Saymen *et al.* found that leucocytes migrate toward the surface of a covered wound and away from it if it is uncovered (Saymen *et al.*, 1973). Thus the precise elimination of dead space is considered important. Amnion alone, being thinner and even pliable than EEM, is probably the most potentially effective dressing available in this context. Simko *et al.* added the surface pH fell after covering with amnion and that the temperature in the underlying granulation tissue rose, increasing phagocytosis and reducing the exudation of interstitial fluid.

Robson and Krizek has found that EEM was superior to allograft (homograft) and to xenograft (heterograft) skin and as effective as autograft skin in lowering the bacterial count when applied to 20% rat scalds topically inoculated with *Pseudomonas aeruginosa* (Burgos and Sergeant, 1983). They concluded that no such anti-bacterial substance existed. However, a substance that enhanced leucotaxis or angiogenesis, with subsequent leucotaxis and promotion of host defense mechanisms, would be important (Robson and Krizek, 1973). Similarly, a substance depressing the normal host rejection to a "foreign" surface dressing and thus permitting continued close surface adherence with at least the aforementioned mechanical benefits, would achieve an additional anti-bacterial effect. The presence of a weak antibiotic-like substance in amniotic fluid has also been demonstrated, and the possibility of its elaboration in EEM epithelium remains strong (Galask and Synder, 1970; Sachs and Stern, 1970).

B. Effect of Amnion on Vessel Development

The vascular response of amnion on the wound bed has been extensively studied using standard histologic staining and immunohistologic techniques (Faulk *et al.*, 1980) It was found that not only was there a considerable

proliferation of capillary vessels towards the surface of leg ulcers following 5 days of tissue-culture-preserved EEM application, but also found that the blood vessel walls became thinner and more regular and their lamina were observed to be more consistently patent. Increased vascularity in burns to which EEM was applied has also been noted microscopically (Kirschbaum and Hernandez, 1963).

Proteins elaborated by amniotic epithelial cells have angiogenic effects, but the precise mode of action remains unknown (Burgos, 1983).

C. Alterations in Connective Tissues

Studies have shown that Reticulin Stain was able to reveal a marked loosening of the thick, tightly-woven tissue pattern often seen in the bed of chronic refractory skin ulcers (Faulk *et al.,* 1980). With this tight, fibrous matrix, blood vessels are formed in groups, their lumina often occluded, and their walls thickened and multi-layered with tissue of a density similar to that of the bands seen in the interstitium. Five days of EEM application results in loosening of the connective tissue matrix, thinning and shortening of the interstitial fibers, and a more widespread dispersal of smaller blood vessels with better defined walls and more consistency patency (Faulk *et al.*, 1980).

D. Immunosuppressive Role of Amnion

Elucidation of the fetomaternal relationship has led to the comparison of the fetus with existence of a parasite that successfully avoids host rejection (Faulk and Galbraith, 1979). A glycoprotein occurring in trophoblast and amniochorion is considered to be very important in suppressing detection of the fetus as "foreign" to the mother, by acting on maternal lymphocytes and preventing rejection throughout pregnancy (McIntyre and Faulk, 1979). The role of immunodiagnostic techniques continues to be important in the effort to ascertain the precise action of immunobiologically active glycoproteins of the EET as well as in the study of changes in the granulation tissue itself.

The Biological Functions and Benefits of Amnion as Wound Dressings

- Decrease the bacterial count in the wound
- Reduction of fluid loss
- Promotes wound healing

- Protects growing epithelium
- Tight adherence to the wound surface, increase in mobility and diminished pain
- Help predict the readiness for grafting
- Prepares skin defect for closure
- Reduce physiological stress for the patient
- Stimulates neovascularisation.

Specific Uses of Amnion in Plastic Surgery

- Burns
- Treatment of leg ulcers
- Extensive skin loss in Steven Johnson disease
- Dressing for split thickness skin graft donor sites
- Reconstruction of pelvic floor following pelvic exenterations
- Oral cavity reconstruction
- Flap dressing (temporary dressing following flap necrosis)
- Neurolysis and tenolysis
- Vaginal epithelialisation
- Intra-abdominal and peritoneal defect
- Intracranial implantation
- Replacement of nasal mucosal in Rendu-Osler-Weber disease
- Tissue engineering (as a carrier for keratinocytes, fibroblasts)

Specific Clinical Applications

Burn and Steven Johnson

Burn injury produces a significant loss of the protective layer of the skin's barrier to infection. The loss of this protective layer can lead to several detrimental effects. Superficial and partial thickness burns usually heal by 5–21 days. The ideal dressing material is that which provides a moist wound environment and does not interfere with the process of epithelialisation. The dressing material should also be conformable and well adherent to the wound bed.

Human Amnion membrane is a temporary biological dressing which is thin and pliable and easily adheres to the surface of the burn wound. It can be easily applied after the first 24 to 72 hours of burn injury after the oedema has reduced. It remains attached to the burn wound until the wound has completely healed. A secondary dressing is needed to absorb the moisture and exudate from the wound. Dressing changes are also not required frequently if the secondary layer is adequate. Normally the dressing changes are done every three to five days, depending on the amount of exudates. During dressing changes if the amnion remains well adherent to the wound surface, it denotes that the wound has not epithelialised completely. If the amnion membrane peels off on its own it clearly denotes that the wound has healed well. Being semi-permeable in nature, the amnion membrane allows exchange of gases and liquids across the membrane. The advantages of amnion membrane in burn injuries are that it forms a protective layer against micro-organisms that can enter the wound from the environment. It minimises the loss of body fluids and proteins and thereby reducing the incidence of electrolyte imbalance which can be a major problem in burns. It provides an ideal substrate for rapid wound healing and hence significantly reduces hypertrophic scar formation and contractures. Studies have showed that the formation of keloid and contractures were significantly reduced when the burn wounds were treated with fresh amniotic membrane (Sinha, 1990). One of the most significant advantages of using the amnion membrane in acute burns is it's pain less application and removal which does not require analgesia. Also the subsequent dressing changes are relatively pain less as the amnion membrane usually sticks well to the burn wound and only the outer layer needs to be changed. This eventually does not disturb the process of epithelialisation and leads to rapid wound healing. Hence as the wound surface is not exposed during each dressing change bleeding from the wound can be minimised.

As amnion membrane is readily available and processed, significant reduction in hospitalisation costs is possible (Subrahmanyam, 1995).

Adjunct Dressing in Flap Surgery

Flap is a process of transferring a tissue with its own vascular supply from one area (donor site) to a wound defect (recipient site). Flap is always at a risk of

failure from reversible congestion to irreversible flap necrosis. Continuous monitoring is vital to avoid necrosis. Flap color is one of the most important clinical parameters to monitor the flap well being.

Amnion dressing offers a translucent adjunct dressing when in contact with wound bed which facilitate continuous flap monitoring (Figure 1a). It contours well with a wound bed resulting in easy application and holistic flap monitoring comparable with opsite dressing. Amnion adhered well to the wound and acts as a barrier of protection from wound contamination while keeping the wound moist (Figure 1b). Amnion can also be used as a dressing material for flap donor site wound (Figure 2), with an additional advantage of pain reduction.

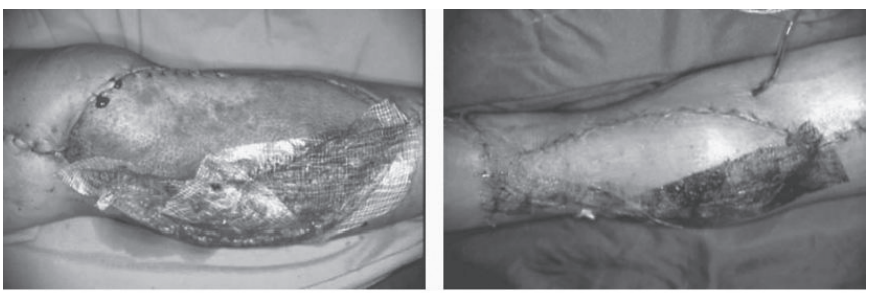


Figure 1. Application of amnion on residual wound of flap recipient site. (a) Immediately after application on a revised flap. (b) Three days after amnion application on the residual wound of another free fibula flap

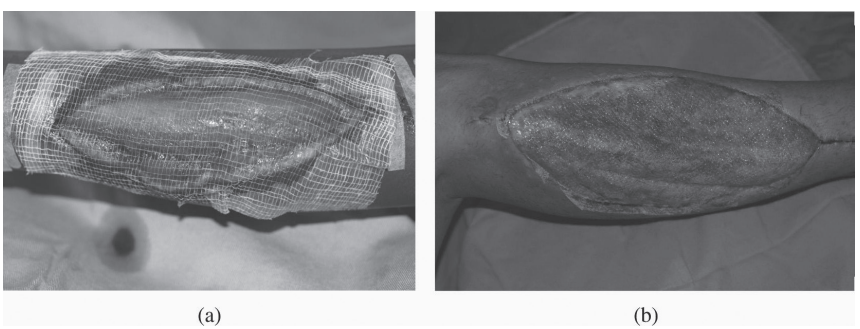


Figure 2. Amnion as a flap donor site dressing material. (a) Immediately after application. (b) Five days after application on another patient

Traumatic Soft Tissue Injury

Abrasion injury described as partial skin loss is similar to partial thickness burn (Figure 3a). It ranges from epidermal loss to deep dermal loss. It heals by re-epithelialisation with minimal to moderate scarring depending on its thickness.

Amnion is suitable as an abrasion wound dressing material because it contours well with the wound, resulting in maximum wound-amnion contact (Figure 3b). It keeps the wound moist for better re-epithelialisation and as a barrier of contamination. Its translucency gives an extra advantage of wound inspection without the need of dressing change. The end result of abrasion managed with amnion dressing is comparable with other commercial dressings (Figure 3c).

However, amnion is less favourable when used in deep or contaminated abrasion because amnion had minimal absorptive capacity and can be biodegraded leaving its outer layer impregnated into the granulating wound. Intense tissue reaction can give rise to unfavourable scarring.

Amnion as Scaffold in Tissue Engineering

Human amniotic membrane is the innermost layer of the placental membranes. It is a thin semi-transparent membrane. It is tough and is devoid of blood vessels, lymphatics and nerves. The amnion comprises a single layer of ectodermally derived columnar epithelial cells adhered to a basement membrane largely composed of collagen I, collagen III, collage IV, laminin

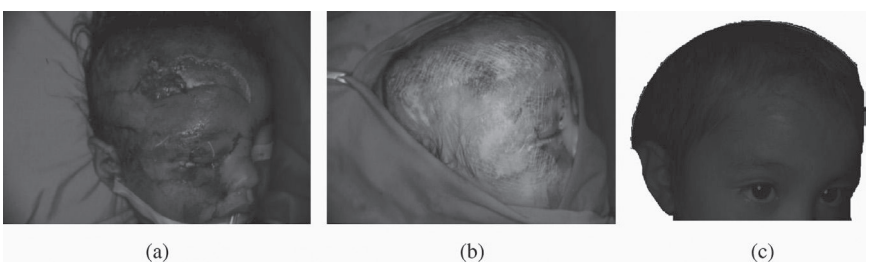


Figure 3. Facial trauma with laceration and abrasion wound. (a) Wound prior to repair. (b) Immediately after repair and application of amnion. (c) Results 3 months after injury with minimal scarring

and fibronectin, which in turn is attached to an underlying layer of connective tissue. The connective tissue has three components: an acellular compact layer composed of reticular fibres; a fibroblast layer, which is a loose reticular network containing sporadic fibroblasts; and a spongy layer, which is in contact with the underlying chorion and consists of a complex network of fine fibrils surrounded by mucus.

Amnion cells produce and release a large variety of growth factors, for example epidermal growth factor, transforming growth factor Beta, insulin like growth factor, hepatocyte growth factor, nerve growth factor and vascular endothelial growth factor, which have been attributed to the proliferative influence of the amnion epithelium. The epithelium of the amnion is capable of storing lipids and glycogen as well as hemosiderin (Rejzek *et al.*, 2001).

Tissue engineering methods designed to restore diseased and damaged tissues depend on the presence of a matrix structure that is amenable to cell growth and proliferation. Hence the amnion membrane has the potential to become a scaffold for cell culture (Hodde, 2002). The matrix component of the amnion membrane has been proven to be biocompatible in vitro and has no adverse effects on cellular proliferation or morphology (Wilshaw *et al.*, 2006). The amnion membrane thus has an immense potential to be used as a delivery system for epithelial cells. Thus autologous cells can be grown to confluence and later have the potential to be returned to the patient as a living tissue. The culture of human corneal epithelial cells on amnion membrane has also been well established (Kobayashi *et al.*, 2006). The goals of tissue engineering are to incite the formation of tissue structures that humans are incapable of regenerating on their own, to do as effectively and efficiently as possible, and to do so while ensuring safety, minimising morbidity, and maximising quality of life for the recipient. Naturally occurring biopolymer scaffold materials that interact with the host and fasten tissue regeneration are viable alternatives to synthetic polymers for tissue engineering cell growth substrates.

Conclusion

Amnion comes close to the description of the ideal biological dressing. Being readily available, inexpensive to use, easily processed and stored, of

low antigenicity and with anti-microbial potential, ability to enhance epithelialisation with marked relief of pain, amnion is suited for temporary or long term coverage of wounds in various clinical conditions. Therefore with health service finances increasingly stretched to provide adequate quality care, it is time for surgeons and others to overcome an almost primitive reluctance to make use of the amnion for wound healing, when so much evidence indicates its merits, and this truly biological dressing is needlessly going to waste.

References

Bose B (1979) Burn wound dressing with human amniotic membrane. *Ann. R. Coll. Surg. Engl.*, **61**: 444.

Burgos H and Sergeant RJ (1983) Lyophilised human amniotic membranes used in reconstruction of the ear (letter). *J. R. Soc. Med.*, **76**: 433.

Burgos H (1983) Angiogenic and growth factors in human amniochorion and placenta. *Eur. J. Clin. Invest.*, **13**: 289.

Colocho G, Graham WP III, Greene AE, *et al.* (1974) Human amniotic membrane as a physiological wound dressing. *Arch. Surg.*, **109**: 370.

Davis JS (1910) Skin transplantation with a review of 550 cases at the John Hopkins Hospital. *John Hopkins Hospital Report.*, **15**: 307.

Faulk WP and Galbraith GMP (1979) Trophoblast transferring and transferring receptors in the host-parasite relationship of human pregnancy. *Proc. R. Soc. Lond. (Biol.)*, **204**: 83.

Faulk WP, Matthews RN, Stevens PJ, *et al.* (1980) Human amnion as an adjunct in wound healing. *Lancet*, **1**: 1156.

Gajiwala AL and Sharma V (2003) Use of irradiated amnion as a biological dressing in the treatment of radiation induced ulcers. *Cell and Tissue Banking*, **4**: 147–150.

Gajiwala K and Gajiwala AL (2003) Use of banked tissue in plastic surgery. *Cell and Tissue Banking*, **4**: 141–146.

Galask RP and Synder IS (1970) Antimicrobials in amniotic fluid. *Am. J. Obstet. Gynecol.*, **106**: 59.

Gruss JS and Jirsch DW (1978) Human amniotic membranes: a versatile wound dressing. *Can. Med. Assoc. J.*, **118**: 1237.

Hodde J (2002) Naturally occurring scaffolds for soft tissue repair and regeneration. *Tissue Engineering*, **8**(2): 295–308.

Kirschbaum SM and Hernandez H (1963) Use of amnion in extensive burns. In: Broadbent TR (ed.), *Proceedings of the 3rd International Congress in Plastic Surgery*, Excerpta Medica, Amsterdam, pp. 152.

Kobayashi A, Yoshita T and Sugiyama K (2006) In Vivo Confocal Microscopic Analysis of Human Corneal Epithelial Sheet Cultured on Amniotic Membrane. *Ophthalmic Surg. Lasers Imaging*, **37**(4): 304–309.

Matthews RN, Faulk WP and Bennett JP (1982) A review of the role of amniotic membranes in surgical practice. In: Wynn RM (ed.), *Obstetrics and gynaecology annual*, Appleton-Century-Crofts, New York, pp. 31.

McIntyre JA and Faulk WP (1979) Antigens of human trophoblasts: effects of heterologous and anti-trophoblast sera on lymphocyte responses *in vitro*. *J. Exp. Med.*, **149**: 824.

Rao TV and Chandrasekharam V (1981) Use of dry human bovine amnion as a biological dressing, *Arch. Surg.*, **116**: 891.

Rejzek A, Weyer F, Eichberger R and Gebhart W (2001) Physical changes of amniotic membranes through glycerolisation for the uses as an epidermal substitute, Light and electron microscopic studies. *Cell and Tissue Banking*, **2**: 95–102.

Robson MC and Krizek TJ (1973) Amniotic membranes as a temporary wound dressing. *Surg. Gynecol. Obstet.*, **136**: 904.

Sachs BP and Stern CMM (1970) Activity and characterisation of a low molecular weight fraction in human amniotic fluid with broad spectrum antibiotic activity. *Brit. J. Obstet. Gynecol.*, **86**: 81.

Saymen DG, Nathan P, Holder IA, *et al.* (1973) Control of surface wound infection: skin versus synthetic grafts. *Appl. Environ. Microbiol.*, **25**: 921.

Shun A and Ramsey-Stewart G (1983). Human amnion in the treatment of chronic ulceration of the legs. *Med. J. Austr.*, **2**: 279.

Sinha R (1990) Amniotic membrane in the treatment of burn injury. *Indian J. Surg.*, January: 11–17.

Subrahmanyam M (1995) Amniotic membrane as a cover for microskin grafts. *British Journal of Plast. Surg.*, **48**: 477–478.

Talmi YP, Finkelstein Y and Zohar Y (1990) Use of human amniotic membrane as a biological dressing. *Eur. J. Plast. Surg.*, **13**: 160.

Trelford JD, Hanson FW and Anderson DG (1973) Amniotic membrane as a living surgical dressing in human patients. *Oncology*, **28**: 358.

Trelford JD and Trelford-Sauder M (1979) The amnion in surgery, past and present. *Am. J. Obstet. Gynecol.*, **134**: 833.

Unger MG and Roberts M (1976) Lyophilised amniotic membranes on graft donor sites. *Br. J. Plast. Surg*., **29**: 99.

Ward DJ, Bennett JP, Burgos H and Fabre J (1989) The healing of chronic venous leg ulcers with prepared human amnion. *Br. J. Plast. Surg*., **42**: 463–467.

Wilshaw SP, Kearney JN, Fisher J and Ingham E (2006) Production of an Acellular Amniotic Membrane Matrix for use in Tissue Engineering. *Tissue Engineering*, **12**(8): 2117–2129.

14

Role of Amnion for Treating Burns

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Introduction

For the treatment of partial thickness burns, amniotic membranes have been found to be effective as temporary dressings. As a dressing it benefits the patient as well as the nursing staff. A third advantage is its low treatment cost. The amniotic membrane flakes off from the healed burn wound surface with time.

Amniotic membranes are procured from the placentae of mothers who were antenatally screened from communicable or infectious diseases. Membranes from placentae with intrapartum complications are excluded. Processing of the membrane involve thorough washing with nomal saline, soaking in 0.05% sodium hypochorite solution for half to one hour, shaking several times with normal saline, drying, packing and gamma radiation at a degree of 25 KGray or lower according to the bioburden estimated in the tissue processed.

The ideal burn wound dressing is autologous skin. However, the supply of this material may be inadequate for treating an extensive burn. The search for alternative biological dressings which can mimic autologous skin has been going on since early this century.

Various substances, such as allograft and xenograft skins have been used in an attempt to treat burn wounds with minimal success. Problems of availability and immunological rejection of both allograft and xenograft skin had led to further research on artificial skin for burn wound coverage. Until permanent repair with autologous skin can be instituted, temporary biological burn wound dressings are life saving and essential especially for extensive burns. Amniotic membranes, which were previously discarded from the hospital labour room have now been found to be useful as an alternative to autologous skin for temporary wound dressings for burns, scalds, chronic ulcers and dermal injuries as well as for contaminated wounds. Amniotic membrane is a versatile and effective biological dressing for both superficial and deep burn wounds. Clinical trials have confirmed these findings.

Procurement of Amnion

Figure 1 shows the harvesting of an amniotic membrane from the placenta of a mother undergoing normal vaginal delivery.

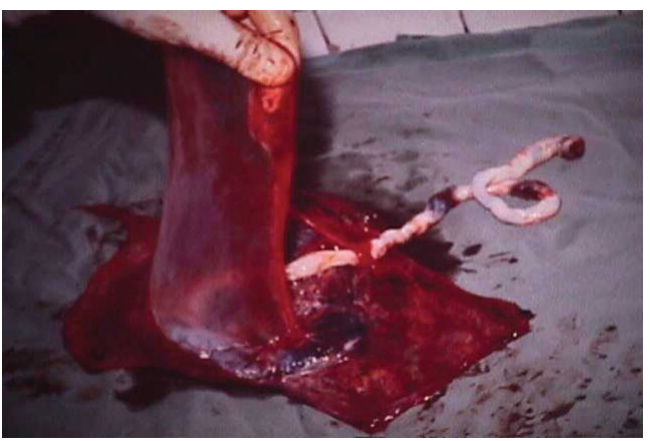


Figure 1. Harvesting the amniotic membrane from the placenta

Methods of Processing of Amnion

Amniotic membrane can be processed by air-drying or by freeze-drying method. Figure 2 outlines the steps in the processing of amniotic membrane allografts.

Results

For Partial Thickness Burn or Scalds

Under aseptic technique, the recipient wound is thoroughly and gently cleansed with normal saline, to remove all debris and dead skin. The radiated amniotic membranes are then applied with the glistening side against

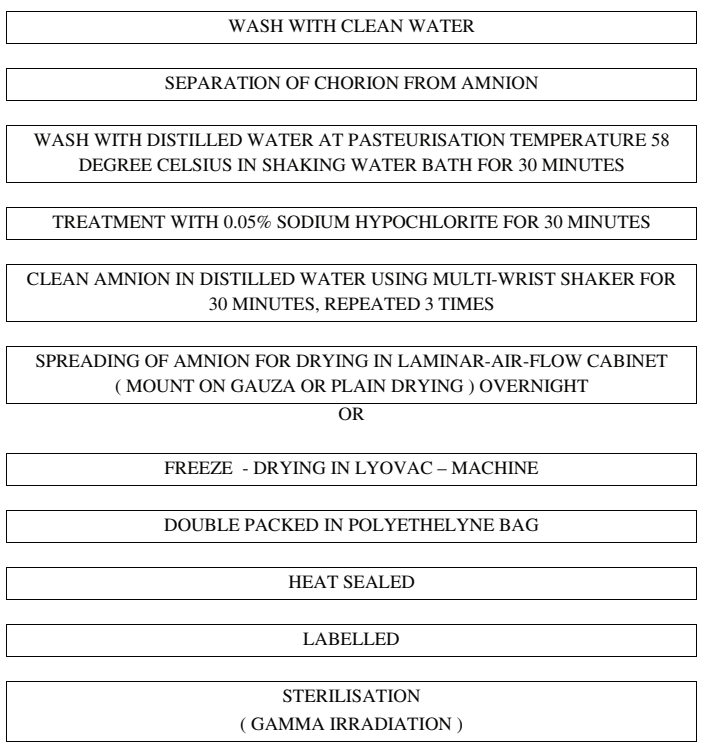


Figure 2. Steps in processing of amnion membrane grafts

the wound surface, and are allowed to remain in place until they separate spontaneously. All air bubbles and excess fluid should be smoothed out to ensure good contact.

However, whenever necessary, the membranes can be changed, especially if the wound does not "take". Usually the membranes immediately adhere to the wound upon application and they desiccate. This property of the membrane helps to prevent excessive loss of fluids and electrolytes from the wounds. If there are signs of infection, the patient is febrile or there is fluid or pus collection below the membrane, the burn wound needs to be washed thoroughly and gently and re-dressed. The use of antibiotic cream is not necessary and is not advisable. Systemic antibiotics are given only when indicated. Another dressing applied over the membrane is not necessary. In some situations, light gauze dressing can be applied to ensure the amniotic membrane is in place, e.g. on the patient's back. After application of the membrane, the recipient's wound would epithelise below the membrane and within a week or so, the desiccated membrane will "flake-off". It results in a shiny wound scar.

Figure 3 shows the application of amnion membrane dressing in a 5 year old sustaining a partial thickness burn wound as a result of an accident due to scalding with hot water.

Figure 4 shows the application of amnion dressings in a 13 year old boy sustaining burns. A major advantage is that this patient can have changes of dressing without pain. The patient is very comfortable with this pain-free dressing.



Figure 3. Burn wound before and after amniotic membrane application in a 5 year old boy



Figure 4. Clinical application of amniotic membrane, as a versatile dressing in a 13 year old boy for severe burns. Patient looks comfortable and is pain free

Steps for Clinical Application of Amnion Membrane Grafts

The steps for application of amnion grafts are:-

- The wound is cleansed with normal saline
- Amniotic membrane is applied over the burn wound
- The dressing then can be covered with thick gauze or bandage if necessary
- If there is no complications the burn wound is inspected on the fourth or fifth day
- Membrane that stuck to the burn wound surface should be left alone and the edge of the amnion that peeled–off should be trimmed using scissors.

For Full Thickness Burn Defects

Similarly, the radiated amniotic membranes may be applied over full-thickness skin defects.

However in this case, frequent changes of radiated membranes is necessary. Occasionally, the membrane might "dissolved". Its main role here is to prevent infection or contamination from the environment. Later, skin grafting is indicated when wound granulation had taken place.

Discussion

The radiated amniotic membranes, being thin film dressings, are easy to use clinically. They are also easy to apply onto the burn surface due to their good conformability. Radiated amnions adhered firmly to the wound. They become dry membranes covering the wound and protect the wound from contamination and infection. Amniotic membranes are not rejected by the host immune system. They not only provide excellent protection to the underlying fresh growing epithelium from infection and trauma but also stimulates the ideal condition for epithelial growth. No neovascularisation takes place on the overlying amniotic membranes. These dry membranes "peeled — off" spontaneously after wound healing had taken place. There is no need for re-grafting of the recipient wound due to a partial thickness burn or a scald. In cases of full — thickness burn, autologous skin grafting is necessary once granulation tissue has formed.

Clinically, immediate coverage of the open burn wound is necessary to ensure a satisfactory outcome. This is the most important factor determining the recovery of the patient. Superficial partial thickness burns may be contaminated or infected and progress to full — thickness lesions resulting in unwanted scars or disabilities.

To achieve acceptable clinical outcome, autograft skin is the best to use. Several biological dressings had been developed in the past decades to overcome the shortage of autografts. Immediate coverage of open wound has the following advantages.

1. prevents burn wound from contamination and infection
2. prevents loss of fluid and electrolytes and
3. prepares the wound for definite closure at a later date when autografts are available

Allograft (cadaveric) skin, satisfies the requirement but has a disadvantage of developing rejection. It needs frequent removal and replacement after about five days from the time of application. There is therefore a need for a constant supply of cadaveric skin grafts from a skin bank. Reports of successful use of allograft skin had been reported in the medical literature.

Likewise, xenograft skin (mainly porcine) had also been successfully used in the treatment of burn wounds. It has more favourable characteristics compared to cadaveric skin or xenographs from other animal tissues. However, porcine graft is not always socially or religiously acceptable. It is also expensive to procure and to process.

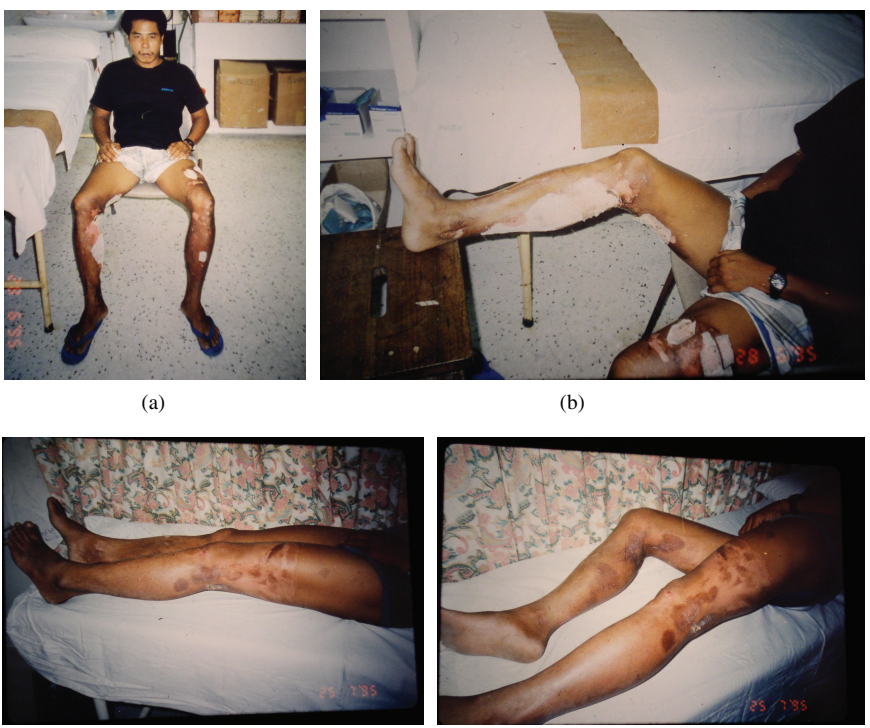
Artificial skin has recently been manufactured. The cost is exorbitant, hence limiting its clinical use. Amniotic membrane is a good alternative to other biological dressings. It is easily available and incurs little costs. Usage of both wet and dried amnion membranes has been sporadically reported in the medical literature with success (Robson, Samburg and Krizek, 1972) have reported in a comparative study that amniotic membranes were found to be equal to isografts and superior to both allografts and xenograft skin in decreasing bacterial levels in full thickness skin defects in rats. Upon application, it alleviates pain. This has tremendous beneficial effects, especially for application in children. Studies has shown that it inhibits infection and this antibacterial property is believed to be due to lysozymes and progesterone present in the amniotic fluid. Its adherence to the wound surface prevents the accumulation of fluid and pus on the granulation tissues. This is probably the most prominent anti-bacterial effect. Radiation of the amniotic membrane does not cause any physical damage or damage to its antibacterial property.

- Advantages of radiated amnion membranes are:-
- Easy to procure and process: Ease of storage and long shelf — life
- Cheap and cost effective: Reduces length of hospital stay
- Reduces contamination and infection rate
- Reduces need for intensive nursing care
- Potential for use in general surgical or burn ward
- Potential for use in peripheral or district hospitals
- Availability of good quality amnion from hospitals with a high delivery rate.

Figures 5, 6 and 7 show patients who have benefitted from dressings with amniotic membranes — burn over the back in one patient, burns in both legs in another patient and a facial burn (scald) in a child.



Figure 5. Amniotic membrane without a layer of gauze for a burn over the back



(a) (b)

(c) (d)

Figure 6. (a) Amniotic membrane applied to burns involving both legs. (b) Amniotic membrane applied over the right leg. (c) Healed wounds after a month in both legs. (d) Showing no hypertrophic scars in both legs after a month.



(a) (b)

Figure 7. (a) Membrane applied on a facial burn wound. (b) Showing face with healed wound

Conclusion

Carefully selected and procured radiated amniotic membranes with their long shelf — life, definitely play an important role in the treatment of burn wounds. Its clinical usage as a burn wound dressing has been proven beyond doubt especially for superficial and partial deep burn wounds. It is specially useful in the management of burns in children and teenagers especially for the alleviation of pain. Clinically its single application to the wound without the need for re-dressing the burn wound makes it popular among the nursing staff. Much time needed to nurse the wounds is saved. Silver sulphadiazine dressing used daily needed frequent changing of dressings.

Acknowledgement

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References

Bourne GL (1960) The Microscopic Anatomy of the Human Amnion and Chorion. *Annal Obstet Gynecol*, **79**: 1070.

Colocho G, Graham WP, Greene AE *et al*. (1974) Human amniotic membrane — as a physiologic wound dressing. *Archives of Surgery*, **109**: 370.

Hilmi N, Siddik S, Gentur S and Gulardi W (1987) Physical and Cheminal properties of Freeze Dried Amniochorion membrane sterilized by irradiation. *Atom Indonesia*, **13**(2) pp. 1–14.

Robson MC, Samburg JL & Krizek TJ (1972) Quantitative comparison of biologic dressings. *Surgical Forum*, **23**: 503–507.

Robson MC, Krizek TJ, Koss N *et al*. (1973) Amniotic membrane as a temporary dressing. *Surgery Gynecology and Obstetrics*, **136**: 478.

Sharma SC, Bagree MM, Bhat AL, Banga BB, Sing MP (1985) Amniotic membrane as an effective burn dressing material. *Japanese Journal of Surgery*, **15**: 40–43.

15

Role of Amnion for Healing of Wounds

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Introduction

The amnion is one of nature's finest biological membranes. It is a collagen rich, thin, transparent and tough membrane, lining the chorion laeve and placenta that produces the amniotic fluid in the earliest period of fetal development. It is the innermost layer of fetal membranes. The function is to protect the fetus from unwanted materials during intrauterine development.

The amniontic membrane consists of a thick basement membrane and an avascular stroma, with thickness of 0.02 to 0.5 mm. Basement membrane

contains: type IV and VII collagen, laminin 1, laminin 5, fibronectin, allantoin, lysozime, transferine, progesterone and several kinds of growth factors. Collagen IV and VII sub-chains are identical to that of conjunctiva. Laminin facilitates corneal epithelial cell adhesion, and play and importance role in ophthalmic surgery. Avascular stroma contains: growth factors and anti-inflammatory proteins as well as act as natural inhibitors to various proteases (Kamardi *et al.*, 1993; Panakove *et al.*, 1997; Kohler *et al.*, 1998). The angiogenetic capability of amnion membrane stimulates neovascularization and induces the development of new blood vessels. Allantoin, lysozime, transferine and progesterone play an important part in ulcer healing. They provide bacteriostatic and bactericidal functions (Rao V.T. and Chandra S.V, 1981; Nursal H., 1993). According to Gruss JS 1978, the allantoin serves as antibody generator. High concentration of lysozymes present provide bacteriostatic and bacteriolyctic functions. Meanwhile, progesterone is bacteriostatic in nature against gram-positive bacteria. In all the layers in the amnion, there is no identifiable structure of blood vessels, lymphatic vessels or nervous tissue. From the immunological point of view, amnion is a suitable transplant material since it does not express HLA-A, B or DR antigens and therefore rejection does not occur (Farazdaghi *et al.*, 2001).

The amniotic membrane promotes wound healing and tight adherence to the wound surface. It is soft, easily shapes the wound surface and has satisfactory adhesive properties. It shows good elasticity and sufficient transparency to allow wound healing to be seen without need for a secondary re-dressing. It increases mobility and diminishes pain. It prepares the skin defect for closure. It also stimulates neo-vascularization. The amniotic membrane can be used as a wound covering for ulcer healing (Hilmy *et al.*, 1987; Hilmy *et al.*, 1994; Menkher *et al.*, 2001).

Fresh amnion membranes have been used as biological dressings since 1913 (Stern W). Fresh amnion membranes have been used in ophthalmic surgery since 1940 (DeRoth).

Since 1989, preserved amnion graft i.e. lyophilised, freeze-dried irradiated amniotic membrane grafts and air-dried irradiated amniotic grafts were produced by several tissue banks in Indonesia such as Batan Research Tissue Bank, M. Djamil Hospital Tissue Bank, Soetomo Hospital Tissue

Bank and Sitanala Hospital Tissue Bank. These grafts have been applied as wound dressings for *burn wound, open wound, after surgical wound, diabetic ulcers and leprosy ulcers*. Such preserved amnion grafts can be kept at room temperature up to two years. Lyophilised grafts were used for ophthalmic and dental surgery. Air-dried grafts were mostly used as dressings for all kinds of wounds. In 2002, the tissue banks produced more than 8000 pieces of amnion grafts per year. The grafts were used as dressings for all kinds of wounds and ulcers, as well as for clinical applications in ophthalmology and maxillofacial surgery in over 50 hospitals in Indonesia. The healing time of the wound using amnion membranes is reduced up to about 50% compared to the healing time using conventional wound dressing (Hilmy *et al.*, 1987; Thalut K *et al.*, 1993; Tarusaraya *et al.*, 1998). Indira *et al.*, 2000, reported no difference in healing time between using fresh/frozen amnion and lyophilized amnion/air-dried amnion for ophthalmologic surgery.

This Chapter describes the applications of amnion grafts as wound dressings for leprosy ulcers, burn wounds, diabetic ulcers and dressing for post surgical wounds including post skin grafts.

Types of Amnion Grafts

The types of amnion grafts using for clinical applications include:-

1. Viable/fresh (hypothermal preservation)
   - Short-term storage in saline or combined with an antibiotic and then stored at 4°C in a refrigerator. It can be stored this way for up to 14 days.
   - Long-term storage by freezing at 85°C in DMSO (dimethylsulphoxide) or antibiotic solution.
   - Long-term storage by cryopreservation at 70°C.
   - Using glycerolisation method (amnion processed in 85% glycerol).
2. Processed and sterilization by gamma radiation
   - Freeze or air-dried/oven-dried grafts sterilised by irradiation.

Benefits of Amnion Membrane

Amnion promotes wound healing. It can be used as dressings for wounds and ulcers. This healing effect of amnion is due to the following chemical and biomechanical properties:-

- Anti-bacterial and angiogenetic effects
- Accelerates and protects the epithelisation and promotes granulation and tissue formation
- Stimulates neovascularization
- Decreases pain
- Heals without scar
- No rejection or immunological reaction
- Promotes tight adherence to the wound surface
- Increases mobility
- It is elastic, translucent, semi-permeable and biodegradable (Matthews RN, 1981; Panakova and Koller, 1997; Kohler J, 2001; Menkher *et al.*, 2001).

Clinical Applications

Applications of amniotic grafts as wound dressings include:-

- Post skin grafting and donor site (Hanfiah J, 1989)
- For Shallow clean second degree burn and post-burn deformities (Quinby WC 1982; Brown AS, 1986; Thalut *et al.*, 1993; Nursal H, 1993; Sjaifuddin N, 2001)
- For Chronic Ulcers (Troensagaard and Hansen E, 1950; Ward DJ, 1989; Henki J, 2004)
- Leprosy Wounds (Taruseraya *et al.,* 1994; Teruseraya *et al.*, 1998; Bari M *et al.*, 1999)
- Wound dressings after Caesarean Sections (Menkher *et al.*, 2001b)
- Post circumcision dressing (Menkher *et al.*, 2001a)
- For clean open wounds (Hanafiah, 1989)

Other applications of amnion grafts include:-

- Vaginal Reconstruction (Dhall K, 1984; Nisolle M *et al.*, 1986; Paraton H, 2001)

- As a mould for de-mineralized bone powder, it is biodegradable and compatible with oral tissues, because both are ectodermal in origin
- For eye surgery for conjunctiva surface reconstruction and corneal defects, excision of pterygium, excision of tumor and for lysis of symblepharon (Indira *et al.,* 2000; Djiwatmo, 2001; Getry Sukmawati IS, 2005)

Steps for Clinical Application

1. Immerse graft in sterile saline for about 5 minutes before use, except for opthalmology surgery.
2. Apply amnion-side onto the defect. This has proven to be more effective than putting the chorion-side onto the wound (Thalut *et al.,* 1993).
3. Apply chorion-side onto the defect. This has proven to be more effective than putting the amnion-side onto the wound for eye surgery (Indira *et al*., 2000, Getry Sukmawati IS, 2005).

Leprosy Ulcers

Amnion has been used as a dressing for leprosy ulcers since a long time ago. Fresh amnion membrane (Sabella, 1913) was used. The latest publication was repeated by Bari *et al.,* 1999. During 1997–1998, Sintanala Hospital in Tangerang, Indonesia, treated 85 patients with leprosy wounds. Their age ranged between 12 to 60 years. There were 65 males and 20 females. Air-dried amniotic membranes were used. Types of wound were reactive ulcers (38 cases) and simple ulcers (60 cases). Locations of wounds were in the legs (52 cases) and in the arms (33 cases). The average width of the wounds ranged between 0.5 to 36 cm^2. The area to be covered with air-dried irradiated graft was first debrided and cleaned. The grafts were regularly replaced every three to four days until complete healing was achieved (See Figure 1.) (Tarusaraya *et al*., 1998).

The types of dressings used were air-dried irradiated grafts and ZnO ointment. The parameters observed were:

- Healing Time correlated to Age (Table 1)
- Healing Time correlated to Type of Wound (Table 2)
- Effects of location of wound on length of healing using amnion dressing



Figure 1. Application of air-dried irradiated grafts onto leprosy wound at Sitanala Hospital in Tangerang, Indonesia.

Table 1. Relationship with age

Age (year)	Amnion membrane	ZnO ointment
<20	39.8 + 15.6	80.2 + 15.6
20–40	23.8 + 9.12	52.1 + 9.1
>40	27.5 + 11.6	60.1 + 11.6

Table 2. Relationship with types of wounds

Types of wound	Amnion membrane	ZnO ointment
Reaction	21.3 + 13.6	63.0 + 6.2
Simple Ulcer	39.4 + 4.6	82.6 + 4.6

It can be seen that the healing time of the wound in younger patients was longer compared to the time taken in old patients. This may be due to the fact that younger patients show more movement especially the wounds are in the legs. This occurred in both patients treated with amnion and with zinc oxide ointment. The healing time of the wounds treated with amniotic membranes was about 50% shorter compared to those treated with ZnO ointment.

Air-dried irradiated grafts can treat both kinds of wounds i.e. reactive wounds and simple ulcers. The healing time depends on the type of wound being treated.

Tarusaraya *et al.*, 1994, also described the speed of the healing time of leprosy wound using several kinds of dressings. This study involved simple plantar ulcers.

- ZnO ointment: 0.14 ± 0.10 cm^2/day
- MSGA (Magnesium Sulphat Glycerine Acriflavin mixture): 0.10 ± 0.06 cm^2/day
- Amnion sterile/AAS radiation: 0.29 ± 0.22 cm^2/day

Application of amniotic membranes to leprosy wounds showed several benefits: the healing time by using amnion is reduced by up to 50% compared to wounds treated with conventional dressings. The application of amniotic grafts gave more comfort to the patients. We found no difference in healing time between using air-dried irradiated amnion grafts compared to amnion preserved in 85% glycerol.

Burn Wounds

The use of freeze-dried and air-dried irradiated grafts began in Djamil Hospital in early 1990. It has several advantages when used for second degree burns:-

- Can cover whole surface of burn wound
- Reduces pain
- Prevents further infection
- Reduces evaporation of wound and can stimulate epithelization and granulation
- It is readily available at low cost (Koller J, 2001; Menkher M, 2001)

Thalut *et al.*, 1993, carried out a research study comparing air-dried irradiated amniotic membranes, antibiotic dressings and medicated dressings for grade 2 and 3 burn wounds caused by fire, electricity and hot water of 39 patients. Parameters studied included formation time of epithelization and granulation tissues, and angioneogenic effect on the wound. Results showed that the time taken for the formation of epithelial tissue was shorter when air-dried irradiated grafts were used instead of antibiotic dressings i.e average of 5 days and 7 days respectively. Granulation formation time was also

decreased from an average of 8 days to 6,5 days consecutively. A comparison study on angiogenetic effect (that induces epithelisation and granulation) using amniotic membrane and antibiotic dressing was done. The parameter studied was the total number of neovascularisation, using 30 samples for each kind of dressing. Total number of neovascularisation increased two times with air-dried irradiated grafts compared to those treated with antibiotic dressings as described by Nursal, 1993. The wound healing time by using air-dried irradiated dressings was reduced up to 45% compared to the healing time occurring with antibiotic wound dressing (Thalut *et al.*, 1993). The same results were also obtained by several authors as described by Koller J *et al.*, 1998.

During the period between 2000 to 2003, 63 patients were admitted with 2nd and 3rd degree burn wounds at Dr. M. Djamil Hospital in Padang, Indonesia. 76.3% were males. 70.9% of the patients were above 50 years of age. All wounds were treated with air-dried amniotic membranes. The average healing time of these wounds is 21–26 days. There was no complication. There was also no rejection.

Similar results were also described by Sjaifuddin Noer in 2001 in patients treated in Dr. Soetomo Hospital in Surabaya. Air-dried irradiated grafts were also successfully as biological skin substitutes for burn wounds since 2000.

Diabetic Ulcers

At least 15% of Diabetic patients from all over the world develop ulcers. 40%–70% of the cases underwent amputation (Amstrong *et al.,* 1998). The incidence was estimated to increase by 2 times by 2025. The cost of local management of each diabetic ulcer in Indonesia is relatively high. It is estimated to cost about US$15 per day.

During the period from 1997 to 2000, 37 cases of diabetic ulcers were treated in at Dr. M. Djamil Hospital in Padang, Indonesia. 62.3% were females. 50.9% were above 50 years of age. All wounds were treated using lyophilised irradiated grafts. 92% of the ulcers were on the leg. Average width of wounds is between 2 to 18 cm^2. The average healing time is between 21 to 26 days (Dona A, 1996).

In 2004, a comparison study was performed on Grade 2 diabetic ulcers (according to Wagner classification). 11 patients were treated with lyoph-

ilised irradiated grafts (Group 1). Another 11 patients were treated with conventional/medicated dressings (Group 2). The parameters recorded were histopathology examination and the length of wound healing. Results showed that histopathology examination grade of healing diabetic ulcers after using air-dried irradiated grafts gave higher grade than those using conventional dressings. Based on the research findings, it can be seen that air-dried irradiated grafts stimulate an increase in the number of cells from the adjacent tissues microscopically (Table 3). The average healing time was 25.36 ± 4.843 days (i.e. from 20 to 32 days). In comparison with the use of amnion grafts (Group 1) the average healing time was 19.56 + 2.035 days. With the use of conventional dressings (Group 11) as controls the healing time was 27.18 ± 2.897 days, see Figure 2 (Hengki J 2004).

Table 3. Comparison of histological examination and healing time in Group I and Group II

Histopathology description	Group I N = 11	Group II N = 11	T-test (p)
Granulation Tissue	16,73 ± 3,197	4,18 ± 2,786	0,000
Epithelial Cell	10,73 ± 2,328	3,82 ± 1,662	0,000
Neovascular	8,64 ± 2,420	2,73 ± 2,054	0,000
Lymphocyte Cell	11,73 ± 3,197	8,73 ± 1,954	0,015
PMN Cell	10,45 ± 2,423	7,73 ± 1,902	0,008



Figure 2. Diabetic Ulcer, application of lyophilised irradiated grafts as wound dressing for foot ulcer in Djamil hospital in Padang. Left: The wound covered with the graft. Right: wound healed without scar after about 15 days (M. Djamil Hospital doc.).

This study showed that the healing time of the ulcer by using lyophilised irradiated amnion grafts was faster compared to those treated with conventional sterile dressings.

Histological healing of skin ulcer can be detected by the increase in number of granulation tissue, epithelial cells, neovascularization, lymphocytes and polymorphonuclear cells (Bose B, 1979; Gruss J, 1978).

Post-Surgical Wounds

Amnion grafts can be applied to several types of post-surgical wounds — cesarean operation, skin grafting, circumcision and clean wounds in the course of routine surgery. During 1998 to 2002, 250 wounds were treated with air-dried irradiated amnion grafts — 58 cases with Caesarian Operation and 64 cases with circumcision.

Post Cesarean Operation

Wound is usually dressed with medicated gauze followed by application of abdominal strapping for three to four days. Sometimes the wounds heal by secondary healing. The latter leaves behind scars in the abdominal wall which is cosmetically not good for ladies. The operation was usually done with pfannenstiel incision. Abdominal strapping was used for flabby of abdominal walls. Radiation sterilized lyophilized amnion membranes produced by Dr. M. Djamil Hospital Tissue Bank were used in this study. The size of the amnion depends on the length of the wound. Most grafts were about 5 cm × 20 cm. All cases were followed up for about 8 days.

Results showed that, this technique is good in achieving the best results for wound healing. Technically, the application of amnion grafts is not difficult. Both emergency or elective surgery wounds, dirty or clean wounds gave good results of wound healing.

No complications were encountered, including severe wound infection, secondary closure of wound, wound dehiscence, allergic reaction, or unsightly appearance of wound. The authors recommended the amnion grafts as wound dressing for post Cesarean Section (Menkher *et al.,* 2001a).

Wound Dressing for Circumcision

The aim of using air-dried irradiated amnion graft is to achieve good wound healing free from infection, pain, as well as for earlier use of underpants after circumcision.

Dorsal circumcision technique was performed on 165 boys. Their ages ranged between 6–10 years. The technique was employed at Djamil Hospital for 58 boys (35.15%). In another 107 boys (64.85%) the technique was performed at Health Center or in other places where mass dorsal circumcision was performed.

After suturing the wound, amnion was used as wound dressing covering the glen penis to protect the glen from external irritation. No gauze or bandage was applied. The patient was allowed early mobilisation. Antibiotics were given prophylactically.

In all cases, the duration of wound healing occurred in less than 6 days. No difference in duration of wound healing was seen in cases done in hospitals and those in health centres ($\boldsymbol{P > 0.05}$), (Menkher *et al.*, 2001b).

Post Skin Grafting

Application of air-dried irradiated amniotic membrane dressing was used to cover skin defect at donor (Figure 3). The average wound healing without scar using air-dried grafts was 9 days compared to 11 days by conventional medicated dressing (Hanafiah D, 1989).

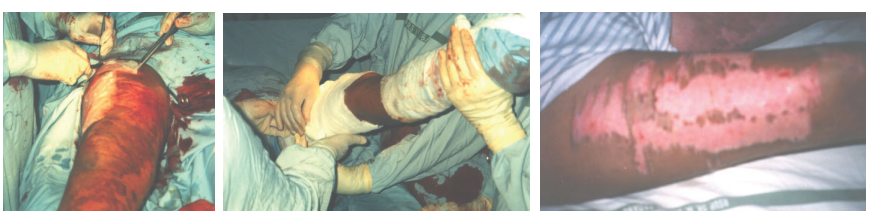


Figure 3. Post skin grafting. Application of air-dried irradiated amnion graft as dressing to donor site. Left: Wound covered by graft. Centre: Bandage applied. Right: Wound healing without scar (M. Djamil hospital doc.)

Conclusion

Lyophilised irradiated and air-dried irradiated amniotic membrane grafts could be applied on dressings for several types of wounds — leprosy ulcers, diabetic ulcers, burn wounds and for post-surgery wounds. It is a biological dressing and gives better results than non-biological conventional dressings. More research is needed to find out the exact bio-molecular mechanism which stimulates wound healing.

References

Armstrong DG and Lavery LA (1998) *Diabetic Foot Ulcers: Prevention, Diagnosis and Classification*. University of Texas Health Science Center at San Antonio and Diabetic Foot Research Group, San Antonio, Texas.

Bari MM and Begum R (1999) Use of Radiation — Sterilised Amniotic Membrane Grafts as Temporary Biological Dressings for the Treatment of Leprotic Ulcer, *Advances in Tissue Banking*. G.O. Phillips ed. Singapore (3): 477–483

Bose B (1979) *Burn Wound Dressing With Human Amniotic Membranes*, Annals of Royal College of Surgeon. 61st Ed. England. pp. 444–447.

Brown AS (1986) *Biological Dressing and Skin Substitute*. Clinic in Plastic Surgery. No 1., (13): 69–74.

Dhall K (1984) Amnion graft for treatment of congenital absence of the vagina. *Br. J. Obstet. Gynaecol.*, (91): 279–282.

Djiwatmo (2001) Transplantasi membran amnion. The 1st Indonesian Tissue Bank Scientific Meeting and Workshop on Biomaterial Application, Surabaya, 22–23 Sept. 2001. pp. 69–75.

Dona A (1996) Diabetic Foot of NIDDM Patient in the Internal Medicine. *Department Dr. M. Djamil Hospital 1990–1994*. Acta Medica Indonesiana. Indonesia (27): 1341–1346.

Farazdaghi M, Adler J and Farazdaghi SM (2001) Electron microscopy of human amniotic membrane, *Advance in Tissue Banking*. The Scientific Basis of Tissue Transplantation, Phillips GO, Nather A (eds.) *World Scientific*, Singapore (**5**): 149–171.

Getry SIS (2005) *Multilayered Amniotic Membrane Transplantation for the Treatment of Corneal Epithelial Defects and Ulcers*. Department of Ophthalmology Faculty of Medicine Andalas University/M Djamil Hospital Padang Indonesia, will be published.

Gruss JS *et al.* (1978) *Human Amniotic Membrane: A Versatile Wound Dressing.* CMA Journal. 118th Ed. pp. 1237–1254.

Hanafiah D (1989) *Clinical Studies on Application of Sterille Lyophilization Amnio-chorion Membrane on open Wound*. Presented at RCA Meeting on Radiation and Nuclear Teach for Sterillized and Clinical Quality Control of Tissue Graft. Bangkok.

Henky J (2004) *The Use of Freeze dried radiation sterilized amniotic membrane as wound covering for diabetic ulcer, Faculty of Medicine*, Andalas University, Padang, Indonesia.

Hilmy N, Siddik S, Gentur S and Gulardi W (1987) Physical and Chemical Properties of Freeze Dried Amnion Membranes Sterilized By Irradiation. *J. Atom Indonesia*, **13**(2): 1–14.

Hilmy N, Basril A and Febrida A (1994) *The Effects of Procurement, Packaging Materials, Storage And Irradiation Dose on Physical Properties of Lyophilised Amnion Membranes*, Proc. of IAEA Meeting of Radiation Sterilization of Tissue Grafts, Manila.

Indira S, Laksmi T and Bambang S (2000) *Freeze-dried and fresh amniotic membranes with limbal stem cell transplantation in severe conjunctival tumor and corneal defect*. The 8th International APASTB Conference on Tissue Banking, Bali 20–24 October 2000. p. 98.

Kamardi T, Nursal H and Nazly H (1993) *Clinical Studies on Application of Sterile Irradiated Freeze-Dryed Amnio-chorio Membranes on Burn Wound Treatment*. Presented at Seminar on Wound Treatment, Padang. Indonesia. pp. 5–24.

Koller J and Panakova E (1998) *Experiences in the use of foetal membranes for the treatment of burns and other skin defects.* Advance in Tissue Banking, Phillips GO (eds.), *World Scientific*, Singapore (2): 353–359.

Koller J (2001) *Healing of Skin and Amnion grafts,* Advance in Tissue Banking. The Scientific Basis of Tissue Transplantation, Phillips GO, Nather A (eds.), *World Scientific*, Singapore (5): 398–417.

Matthews RN (1981) *Wound Healing Using Amniotic Membrane*. British Journal of Plastic Surgery. 34th pp. 76–78.

Menkher M and Helfial Helmi (2001a) *Using amniotic membrane as wound covering after caesarian section operation*, Proc. Scientific Meeting Research and Development on Application of Isotopes and Radiation, Batan, Jakarta. pp. 169–173.

Menkher M, Ismal and Doddy E (2001b) *Experience of Using Amniotic Membrane After circumcision*, Proc. Scientific Meeting Research and Development on Application of Isotopes and Radiation, Batan, Jakarta. pp. 165–168.

Nisolle M and Donnez J (1992) Vaginoplasty using amniotic membranes in cases of vaginal agenesis or after vaginectomy. *J. Gynaecol. Surg*. (8): 25–30.

Nursal H (1993) *The Angioneogenic Effect and Decrease Population of Bacteria by Amniotic Membrane Conservation for Burn Wound in Dr. M. Djamil Hospital*. Seminar on Wound Treatment, Padang. Indonesia. pp. 25–32.

Panakova E and Koller J (1997) *Utilization of Foetal Membranes in Treatment of Burn and Other Skin Defect*. In: Advance in Tissue Banking. Phillips GO (eds.), *World Scientific*, Singapore. (1): 165–173.

Paraton H (2001) Management vaginal agenesis. The 1st Indonesian Tissue Bank Scientific Meeting and Workshop on Biomaterial Application, Surabaya, 22–23 Sept. pp. 77–83.

Quinby WC Jr., Hoover HC, Scheflan M, Philemon TW, Sumner AS and Conrado CB (1982) Clinical trials of amniotic membranes in burn wound care. *Plastic Reconstructive Surg*., (70): 711–716.

Rao VT and Chandra SV (1981) *Use of Dry Human Bovine Amnion as A Biological Dressing*. *Arch Surgeon*, 119th pp. 891–896.

Sabella N (1913) Use of foetal membranes in skin grafting. *Med. Records NY*, (83): 478–480.

Sjaifuddin N (2001) Clinical applications of Biomaterial for plastic surgery, in The 1st Indonesian Tissue Bank Scientific Meeting & Workshop on Biomaterial application, Surabaya 22–23 September, pp. 57–64.

Stern W (1913) The grafting of preserved amniotic membrane to burned and ulcerated skin surface substituting skin grafts. *JAMA*, (1): 973–974.

Tarusaraya P and Hilmy N (1998) Comparison study using Freeze dried amnion membrane and ZnO ointment as wound covering for Leprosy Ulcer, presented at the 7th International APASTB Conference on Tissue Banking, Kuala Lumpur.

Tarusaraya P and Halim PW (1994) Comparison study using Amnion membrane, ZnO ointment, Acriflavin Glyserine Magnesium Sulphat. On Ulcus Plantar of Leprosy patients. *Medical Journal of Indonesia*, Vol 44.

Troensagaard-Hansen E (1950) Amniotic Grafts in cronic ulceration., Lancet 1, pp. 859–860.

Ward DJ, Bennett JP, Burgos H and Fabre J (1989) The healing of chronic venous leg ulcers with prepared human amnion. *Br. J. Plastic Surg*., (42): 463–467.

16

Amnion Dressing in the Management of Radiation Skin Reaction Following Post Radiotherapy

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Introduction

Radiotherapy is an important component for treatment of cancer in addition to surgery or chemotherapy. However, even with the most modern radiotherapy techniques, like the use of linear accelerators, up to 90% of patients will suffer side effect with radiation induced skin reaction (RISR) at the site of radiation (Maddock *et al.*, 2005; Primavera *et al.*, 2006; MacBride *et al.*, 2008). The reactions usually manifest within 1–4 weeks after the start of radiation and persist for the duration of radiation therapy. It may then require 2–4 weeks to recover after radiotherapy is completed (Shimm and Canady, 1994). The reaction can be from a mild rash to severe ulceration. Acute radiation usually begins with erythema, itching and pain that can

cause delays in treatment, and diminished aesthetic appearance. Subsequently, it decreases the quality of life.

Generally, radiation skin reaction can be managed by preventive and curative strategies. Preventive strategies include minimising irritants to the irradiated skin such as those associated with particular hygiene regimens, minimising friction, reducing the frequency of washing, avoiding the use of soap, cream and deodorants, and avoiding sun exposure (Salvo *et al.*, 2010). Curative strategies of skin reactions focus on promoting healing, improving patient comfort, preventing infection and decreasing trauma to the area. Various dressings like topical preparations, hydrocolloid and gentian violet 1 % dressing have been used for its treatment (Dini *et al.*, 1993; Patwardhan *et al.*, 2002). Amnion membrane is known as the best biological dressing that can be used for local management of radiation skin reaction (Gajiwala and Sharma, 2003).

Radiation Skin Reaction

The radiobiological effects of radiotherapy treatment can be easily understood by knowing the normal anatomy and physiology of healthy skin regenerations. The skin is composed of two main layers, the epidermis (superficial layer) and the dermis (deep layer), as shown in Figure 1 below (Wough and Grant, 2001).

Normally, the new cells in the basal layer of the dermis replace the desquamation of skin superficial cells. The dermis, which contains blood vessels, glands, nerves and hair follicles, provides the supportive structure required for the epidermis to rebuild. Recovery of the entire epidermis takes approximately 3–4 weeks, although this process can be shorter during times of healing.

In general, the basal layer of the epidermis proliferates rapidly but it is particularly sensitive to radiotherapy. Ionising radiation essentially damages the mitotic ability. The DNA of clonogenic or stem cells within the basal layer were destroyed, thus preventing the process of repopulation. It can weaken the integrity of the skin. The degree to which a skin reaction develops is dependent on the survival of actively proliferating basal cells in the epidermis.

The basal layer of the skin began to be destroyed when it received radiation dose of 20–25 Gy. Maximum depletion of basal cells occurred when the patient had received a dose of 50 Gy (Archambeau *et al.*, 1995). Clinically,

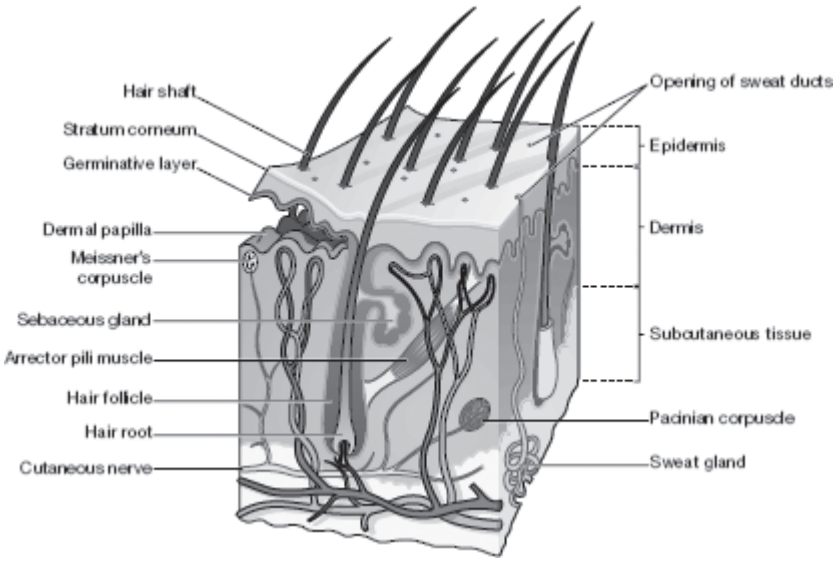


Figure 1. The skin and the main structure of dermis (Wough and Grant, 2001)

this means that skin reactions tend to become visible around the second to third week of radical radiotherapy, reaching a peak at the end or within 1 week of treatment completion (Arimoto *et al.*, 1989; Ratliff, 1990).

The majority of skin reactions will heal within 3-4 weeks after radiotherapy. Small areas of moist desquamation tend to heal from the basal layer, whereas large areas of broken epidermis require cells to migrate from the surrounding epidermis (Hopewell, 1990). Healing becomes visible as islands of epidermal cells expand and reform in central and peripheral regions of the desquamation. Initially, this reformed skin may be hyperpigmented, due to stimulation of melanocytes as a result of exposure to radiation (Cox *et al.*, 1986; Ratliff, 1990).

Skin reactions can range from mild erythema, dry desquamation (dry, flaky or scaly skin) to confluent moist desquamation, where blistering, peeling and sloughing of the skin occur. The most severe stage of necrosis is rarely seen nowadays. Usually it is possible to see a combination of erythema, dry and moist desquamation within a single treatment field. Although in a relatively short period, skin reactions are uncomfortable, itchy, and painful (Campbell *et al.*, 1992; Munro *et al.*, 1989).

There are two factors influencing the severity of skin reactions. First, intrinsic factors include age, general health, ethnic origin, coexisting diseases, ultra violet exposure, hormonal status, tumor site and genetic factors. Second, extrinsic factors include the dose, volume and fraction of radiation, radio-sensitisers, concurrent chemotherapy, and the site of radiotherapy.

Management of Radiation Skin Reaction

The management of moist skin desquamation (ulceration) in radiation therapy patients has been a concern since the inception of this mode of therapy (Margolin *et al.*, 1990). The problem is amplified when chemotherapy is used concurrently with radiation (Hansson, 1997; Bentzen *et al.*, 1989). With the advent of megavoltage machines with their skin sparing capabilities, the incidence of such ulcers has decreased but not disappeared completely. Management of radiation skin reaction can be preventive or therapeutic. The aims of treatment of skin reactions are to maintain the skin integrity, to stimulate recovery of skin function by maintaining skin hydration, providing comfort, reducing pain, preventing trauma, treating infection and creating a moist wound healing environment. The method of treatment is systemic or local treatment (Baskar *et al.*, 2012).

Systemic treatment could be done by oral or intravenous medications. Local treatment includes the use of topical drugs. To maintain skin hydration, a variety of moisturisers, barrier cream, aloe vera, lanolin and steroid cream are used.

Use of dressings in the treatment of skin reactions based on the understanding that a moist wound-healing environment will accelerate re-epithelisation. Keeping the wound moist will increase the wound healing time by 50%. Gajiwala *et al.,* 2003 used amnion dressing in moist desquamation. She found that such dressings can accelerate epithelisation. Amniotic dressing also provide an effective barrier against trauma and bacterial penetration. The treatment using amnion was also used by Menkher *et al.*, 2007 in Padang, West Sumatera, Indonesia.

Amniotic Membrane in Wound Management

Amnion is a collagen-rich, thin, transparent, and tough membrane lining the chorion laeve and placenta that produces amniotic fluid during the earliest fetal period. It is the innermost layer of fetal membranes. Its function is to

protect the fetus from unwanted material during intrauterine development. Amniotic membrane consists of two layers, the inner amniotic membrane and chorion as the outer layer. Amnion can be easily separated from the chorion laeve and placental amniotic separated along the umbilical cord. After separation, amnion will be thin and more elastic and easy to clean compared to thick chorion.

As a biological dressing amniotic membrane has several functions including barrier function, bacteriostatic function, reducing pain and accelerating wound healing. It can also be used for radiation induced skin reaction management. (Manjas *et al.*, 2007; Manjas and Henky, 2010) A very interesting effect noted by Faulk *et al.*, 1980, from the use of amniotic membrane in chronic leg ulcers, is the development of new blood vessels. This is caused by angiogenetic factors that play a role in the capillary endothelium. Burgos, 1983 also confirmed that there are angiogenic and mitogenic factors in the amniotic membrane. These factors contribute to acceleration of wound healing.

The amnion membrane consists of a thick basement membrane and an avascular stroma, with a thickness of 0,02-0,5 mm. Basement membrane contains type IV and type VII collagen, laminin-1, laminin-5, fibronectin, allantoin, lysozyme, transferine, progesterone, and several kinds of growth factors. The collagen IV and VII subchain are identical to that of conjunctiva and laminins, which facilitate corneal epithelial cell adhesion and play an important role in ophthalmic surgery. Avascular stroma contains growth factors and anti-inflammatory proteins, and acts as a natural inhibitor to various proteases. Amniotic membrane contains a number of critical cytokines such platelet-activating factor (PAF), basic fibroblast growth factor (b-FGF), hepatocyte growth factor (HGF), placental proliferin, transforming growth factor (TGF-b), and epidermal growth factor (EGF). Amniotic membrane also express pro-inflammatory cytokines (IL-1, IL-6, IL-8) and tumor necrosis factor (TNF), which activates local defense response in the event of invasion by bacteria and endotoxin.

Allantoin, lisozym, transferrin and progesterone play an important role in the mechanism of ulcer healing. They may have a bacteriostatic or bactericidal effect. According to Gruss and Jirsch, 1978, allantoin acts as an antibody generator. High concentrations of lisozym have bacteriostatic and bacteriolytic effect. Progesterone has a natural bacteriostatic against gram positive bacteria.

From the immunological perspective, amniotic membrane is a suitable transplantation material because it does not express HLA-A, B or DR antigen. There is therefore no rejection. Amniotic membrane adhere tightly to the wound surface. It is soft and easily molded to cover the surface of the wound. It has good elasticity. It reduces pain and stimulates neovascularization (Ganatra, 2003).

Amniotic Membrane in Skin Radiation Reaction

Amnion dressing for skin reaction after radiotherapy was first reported by Gajiwala *et al.*, 2003 from Bombay, India. It concluded that lyophilised, irradiated amnion was an effective temporary wound cover. In Jamil hospital in Padang, Indonesia doctors used amniotic dressings routinely for patients with radiation induced skin reactions. Interestingly they found that wound healing was faster using fresh amnion which may still contain stem cells (Manjas and Soeraya, 2013). The disadvantage is the odor of the fresh amnion. On the other hand, the lyophilised amniotic membrane influences faster healing compared to non-biological dressing such as Framycetin sulphate (Manjas *et al.*, 2014).

In our case series in 2015 (Table 1) (Figure 2), we compared the use of lyophilised amniotic membranes to patients treated with gentamycin ointment for radiation induced skin reactions. These reactions involved patients with breast cancer and head and neck cancer. We concluded that the use of lyophilised amniotic membrane significantly decreased the RISRAS scale faster compared to patients treated with gentamycin ointment.

Table 1. Comparison of RISRAS scale in patients treated with amnion versus those treated with gentamycin ointment

Time of Study	Lyophilised amnion	Gentamycin ointment	P
1st week			
Symptom	5,38 ± 1,41	2,75 ± 0,71	0,000
Professional assessment	1,88 ± 1,19	2,00 ± 1,10	0,830
RISRAS	7,38 ± 2,16	4,50 ± 1,44	0,007
2nd week			
Symptom	8,88 ± 1,36	5,63 ± 2,07	0,002
Professional assessment	4,38 ± 1,46	3,69 ± 1,87	0,426
RISRAS	12,06 ± 3,39	10,44 ± 2,82	0,315



Picture A. Before Applied Amniotic Membrane



Picture B. 7 Days After Treatment



Picture C. Before Applied Amniotic Membrane



Picture D. Amnion Membrane Applied on Affected Skin



Picture E. 14 Days After treatment

Figure 2. Pictures A, B, C, D and E: Treatment of radiation induced skin reactions with amniotic membrane

Conclusion

Radiation skin reactions remained a significant problem for patients undergoing radiotherapy treatment. Many techniques have been used to overcome this problem. It is best to prevent such reactions. However, once it

develops treatment must be given either systemically or topically. In the local treatment of such wounds, dressing with amniotic membrane is a useful method compared to treatment by conventional dressings.

References

Archambeau J O, Pezner R and Wasserman T (1995) Pathophysiology of irradiated skin and breast. *International Journal of Radiation Oncology, Biology, Physics*, **31**(5): 1171–1185.

Arimoto T, Maruhashi N and Takada Y *et al.* (1989) Acute skin reactions observed in fractionated proton irradiation. *Radiation Medicine*, **7**(1): 23–27.

Baskar R, Lee KA, Yeo R and Yeoh KW (2012) Cancer and Radiation Therapy. Current advances and future direction. *Int J Med Sci*., **9**(3): 193–199.

Bentzen SH, Overgaard M and Thames *et al.* (1989) Hodgkin's disease. Early and late normal tissue injury after post mastectomy radiotherapy alone or combined with chemotherapy. *Int J Radiat Oncol Biol Phys*., **56**: 711–715.

Burgos H (1983) Angiogenic and Growth factors in human amniochrorion and placenta. *Eur J Clin Invest*., **13**: 289–296.

Campbell J and Lane C (1996) Developing a skin-care protocol in radiotherapy. *Professional Nurse*, **12**(2): 105–108.

Cox J D, Byhardt R W and Wilson F *et al.* (1986) Complications of radiation therapy and factors in their prevention. *World Journal of Surgery*, **10**: 171–188.

D'Angio GJ, Faber S and Maddock CL (1959) Potentiation of x-ray effects by actinomycin D. *Radiology*, **73**(2): 175–180.

Dini D, Macchia R and Gozza A *et al.* (1993) Management of acute radiodermatitis. Pharmacological or nonpharmacological remedies? *Cancer Nurs*, **16**(5): 366–370.

Faulk WP, Mathews RN, Steven PJ, Bennet JP, Burgos H and His BL (1980) Human amnion as an adjuncy in wound healing. *Lancet*, **1**: 1156–1158.

Gajiwala AL and Sharma V (2003) Use of irradiated amnion as a biological dressing in the treatment of radiation induced ulcer. *Cell Tissue Bank*, **4**: 147–150.

Ganatra MA (2003) Amniotic Membrane in Surgery. Review Articles. *J Pak Med Assoc*., **53**(1): 29–32.

Gruss JS and Jirsch DW (1978) Human amniotic membranes: a versatile wound dressing. *Can Med Assoc J*, **118**: 237–240.

Hansson C (1997) Interactive wound dressings. A practical guide to their use in older patients. *Drugs Aging*, **11**(4): 271–284.

Hopewell J W (1990) The skin: its structure and response to ionizing radiation. *International Journal of Radiation Biology*, **57**(4): 751–773.

Khanna NR, Kumar DP, Laskar SG and Laskar S (2013) Radiation Dermatitis: An Overview. *Indian journal of Burns*, **21**: 24–31.

MacBride SK, Wells ME, Hornsby C, Sharp L, Finnila K and Downie L (2008) A case study to evaluate a new soft silicone dressing, Mepilex Lite, for patients with radiation skin reactions. *Cancer Nurs.*, **31**: E8–14.

Maddock–Jennings W, Wilkinson JM and Shillington D (2005) Novel approaches to radiation-induced skin reactions: a literature review. *Complement Ther Clin Pract.*, **11**: 224–31.

Manjas Menkher and Soeraya G. Clinical Use of Fresh Amnion Dressings in the Management of Local Skin Reaction Following Post Radiotherapy. Presented at Combined scientific meeting of the15th Asia-Pacific Association of Surgical Tissue Banking and the 13th Japanese Society of Tissue Transplantation.

Manjas M, Tarusaraya P and Hilmy N (2007) The Use of Irradiated Amnion Grafts in Wound Healing. Dalam (Nather A, Yusof N, Hilmy N, ed.) *Radiation in tissue banking*. World Scientific Publishing CO: 329–342.

Manjas M, Henky J and Agus S (2010) Use of Amnion Cream for Wound Healing in Wistar Mice. *Indonesia Medical Journal*, **60**(6): 268–272.

Margolin SG, Breneman JC and Denman DL *et al.* (1990) Management of radiation induced moist desquamation using hydrocolloid dressing. *Cancer Nurs.*, **13**(2): 71–80.

Mayer EG, Poulter CA and Aristizabal SA (1976) Complication of irradiation related to apparent drug potentiation by adriamycin. *Int J Radiat Oncol Biol Phys.*, **1**: 1179–1188.

Munro AJ, Biruls R and Griffin AV *et al.* (1989) Distress associated with radiotherapy for malignant disease: a quantitative analysis based on patient perceptions. *British Journal of Cancer*, **60**: 370–374.

Patwardhan A, Sharma V and Dinshaw KA (2002) Duoderm CGF vs Gentian Violet 1% dressing in treatment of radiation induced ulcers. *WCET J.*, **22**(2): 32–38.

Primavera G, Carrera M, Berardesca E, Pinnaró P, Messina M and Arcangeli G (2006) A double-blind, vehicle controlled clinical study to evaluate the efficacy of MAS065D (Xclair), a hyaluronic acid–based formulation, in the management of radiation-induced dermatitis. *Cutan Ocul Toxicol.*, **25**: 165–71.

Ratliff C (1990) Impaired skin integrity related to radiation therapy. *Journal of Enterostomal Therapy*, **17**(5): 193–198.

Salvo N, Barnes E, Dranen JV, Stacey E, Mitera G, Breen D, Giotis A, Czarnota G, Pang J and De angelis C (2010) Prophylaxis and management of acute

radiation-induced skin reaction: a systematic review of the literature. Radiation Oncology. *Current Oncology*, **17**(4): 94–112.

Sato Y, Morita M and Takahashi H *et al.* (1970) Combined surgery, radiotherapy, and regional chemotherapy in carcinoma of the paranasal sinuses. *Cancer*, **25**: 571–579.

Shimm DS and Canady JR (1994) The Skin. In: Fox JD (ed) Moss Radiation Oncology Rationale, Technique, Results. 7th edition, pp 99–100, chapter 4, The CV Mosby Company. St. Louis, Missouri, U.S.A.

17

Freeze-Dried Irradiated Amnion in Ophthalmic Surgery

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Introduction

Whilst fresh amnion membrane grafts have been used as biological dressings since 1913 (Stem, 1913), fresh amnion have also been applied to ophthalmology as early as 1940 (De Roth). De Roth reported partial success in the treatment of conjunctival epithelial defects after symblepharon using fresh amnion membrane. Lavery, 1946 and Sorsby and Symons, 1946 found that patients with caustic burns, lime burns of the conjunctiva with corneal involvement

could also be treated successfully using amniotic membranes. Fresh amnion membranes were used in all these cases.

In 1986, Prasad *et al.*, re-introduced amnion for ocular surface reconstruction i.e. for the treatment of Stevens-Johnsons syndrome. Subsequently Lee and Tseng, 1997, Prabhasawat *et al.*, 1997, Kruse *et al.*, 1999, Komolsuradej *et al.*, 2001 and Tseng 2005, used single and multi-layered amnion grafts for persistent corneal epithelial defect, severe ulceration of the cornea and sclera and for corneal perforations. Various authors have also reported the beneficial effects of human amnion membrane transplantation for ocular indications. These ocular applications involved using frozen or fresh amniotic membranes.

Since 1999, freeze-dried, irradiated amniotic membranes with a water content of less than 5% were used for ophthalmic surgery (e.g. for ocular surface reconstruction, severe conjunctival tumors, corneal ulcers, corneal perforations and pterygia) in several hospitals and eye centers in Indonesia with promising results.

Advantages of Amnion Grafts

The advantages of using freeze-dried amnion allografts include:

- Can be kept up to 2 years at room temperature.
- Easy to transport.
- Easy to store.
- Can be sterilised by irradiation at room temperature.

Advantages of Amnion Grafts for Ophthalmic Applications

Prabhasawat *et al.*, 1997, described the advantages of using amnion grafts for ophthalmologic practice to include:

- Heals all types of epithelial defects.
- Reduces inflammation and scarring.
- Promotes epithelial healing.
- Decreases irritation for painful bullae and band keratopathies.
- Prevents graft rejection.

- Covers defect in conjunctiva.
- Provides the perfect graft for conjuntival tumour.
- Serves as an adjunct to limbal transplantation.
- Enables anti-fibroblastic cell migration — promoting its activity

In addition, the amniotic membrane produces various growth factors such as basic fibroblast growth factor (FGF), hepatocyte growth factor (HGF) and transforming growth factor β (TGFB) which stimulate epithelialization (Tseng, 2005).

This chapter describes our experience in using freeze-dried, irradiated amnion membrane grafts in ophthalmic applications.

Preparation of Freeze-Dried, Radiation Sterilised Amniotic Membrane Grafts

The processing steps of amnion membranes after screening include:

1. Amniotic membrane procured from placenta of healthy mothers must be individually washed and processed. The Washing process is done nine times, using 9 bottles each containing 400 mL of sterile water, except for bottle no 5 which contains 0.05% sodium hypochlorite /NaOCl solution in sterile water. The amnion is washed and shaken well four times in 400 ml sterile water (bottles no 1–4) and then immerse in 0.05% sodium hypochlorite solution (bottles no 5) for 10 minutes and then wash again 4 times in bottles no 6–9. Each bottle is shaken for 10 minutes. The used washing water in bottle no 9 is subject to control for validation (IAEA/NUS, 1997; Hilmy *et al.*, 2000; Hilmy *et al.*, 2003).
2. The amnion is stretched and mounted on sterile cotton gauze or polyester net with the epithelial side of the amnion placed directly on the cotton gauze, and is then frozen (–70°C for 24 hours for lyophilised amnion grafts or keep it until sterilization for frozen amnion grafts).
3. Freeze-drying/lyophilising for 5 hours (validation by calculating the water content i.e. less than 5 %).
4. The amnion is cut into 4cm × 4 cm or another size.
5. Bio-burden estimation is performed on the amnion (validation is done according to ISO 11737-1, 1995)

6. The amnion is triple-packed in polyethylene pouch with the thickness of 0.1 mm.
7. The amnion is labeled.
8. The amnion is radiation sterilised at a dose of 25 kGy (validation is done according to IAEA Code of Practice 2004).
9. The amnion is stored at 5°C or at room temperature (24°C), protected from direct sunlight.

Freeze-Fried Amniotic Membrane Transplantation in Corneal Ulcer Management (Sunarti and Heryati, 2006)

The basement membrane, the migration of epithelial cells, reinforces the adhesion of basal epithelial cells, promotes epithelial differentiation and prevents epithelial apoptosis. The amnion membrane transplanted as basement membrane, acts as a new healthy substrate that is suitable for proper epithelization. Corneal ulcers are serious and urgent clinical problems that can threaten the patient's vision. Lee and Tseng, 1997, reported that amniotic membrane transplantation may be considered as an alternative substrate for treating persistent corneal epithelial defect. Amniotic membrane transplantations were performed when corneal ulcers persisted or become worse after medication given for a period of time, or when patients came with impending corneal perforation or after corneal perforation has developed.

Sunarti and Heryati, 2006, have carried out a study to determine the efficacy of radiation sterilised freeze- dried amniotic membrane transplantation in promoting wound healing in severe corneal ulcers. Freeze-dried radiation sterilised amniotic membranes produced by BATAN Research Tissue Bank were used in this case.

Method and Surgical Technique

A retrospective study of amnion membrane transplantation was performed on 18 eyes in 18 patients treated with corneal ulcer from January 2004 to December 2004 at Cicendo Eye Hospital, Indonesia. Of these, 16 were men and 2 were women. The age ranged from 8 to 68 years old. Transplantation was performed under general anesthesia. Eleven ulcers (61.12%) were located centrally in the cornea, 4 ulcers (22.22%) were located in the

periphery and 3 ulcers (16.66) were located mid-centrally. Pre-operative condition of 9 ulcers (50%) were perforated, 6 ulcers (33.33%) were with descemetocele, 2 (11.11%) have reached the profound stroma and 1 ulcer was found with corneal melting.

The base of ulcer was carefully debrided before amnion transplantation. Amnion membranes were trimmed to fit the ulcer's size, followed by placing the amnion with stromal side down layer by layer, to fill up the ulcer. Reaching the ulcer surface, amniotic membrane were cut into a bigger size than the ulcer and sutured with continuous 10-0 nylon suture. The top layer was sutured by 10-0 nylon suture. A bandage contact lens was applied on top of the membrane until the epithelial defect was healed. Topical medications after surgery were continued. Assessment of surgical outcome was determined by inflammation signs, healing time of area covered by membrane and recovered visual acuity. All patients were examined on postoperative day 1, and every week until 1 month. All operations had performed after obtaining informed consent.

Results and Discussion

Laboratory results indicated that KOH positive were found in 7 patients, Gram positive cocci were found in 4, Gram negative rods in 3 amd Gram negative diplococci in 2 patients. According to Jones's criteria, all of the ulcers were categorised as severe ulcers.

Pre-operative visual acuity in 14 patients (77.78%) was categorised as poor and the rest of the patients were categorised as good visual acuity. Post operatively, poor visual acuity was found in 11 patients (61.11%) and good visual acuity was found in 5 patients (27.78%). From these results, decrease of visual acuity was found in 5 patients, improvement in visual acuity in 5 patients and stable visual acuity was found in 6 patients.

Of the 18 eyes, 11 (61.11%) showed corneal re-epithelization and decreased inflammation. 3 (16.67%) showed worsening corneal defect, 1 (5.55%) developing endophthalmitis. 2 (11.12%) of them developed corneal staphyloma (one eye underwent evisceration, in another, corneal ulcer persisted after complete resolution of transplantation.

The goal of amniotic membrane transplantation was to seal the perforation or to act as a dressing to avoid perforation as well as to reduce inflammation

and to promote corneal tissue healing. Amniotic membrane transplantation gave an excellent result for two cases of peripheral corneal ulcers caused by Gram negative diplococci, both in terms of cornea re-epithelization and in terms of visual acuity recovery. Compared to purely bacterial ulcers, KOH positive ulcers received less benefit from amniotic membrane transplantation procedure. However, success was found in 3 cases. Therefore, this procedure could still be considered as an alternative therapy. Visual acuity was not an accurate parameter for this kind of surgical procedure. Since there were many factors affecting the visual outcome after resolution of corneal ulcers including position of corneal scar, the best visual acuity recovery was found in peripheral ulcers. Failure of epithelization may have been caused by imperfect surgical skill or a bad ulcer condition.

In conclusion, freeze-dried, radiation sterilised amniotic membrane can be considered as an alternative procedure in treating corneal ulcer to promote corneal wound healing.

Multi-layered Amniotic Membrane Transplantation and Tarsorrhaphy/MAMT for the Treatment of Corneal Epithelial Defects and Ulcers

Twenty two eyes (of 17 patients) with corneal epithelial defects and ulcers were studied from July 2005 to November 2005 (Getry SI, 2006). They were 13 males and 4 females: One patient (2 eyes) suffered from the corneal epithelial defects due to complication of Stevens-Johnson's Syndrome. 2 patients (4 eyes) suffered from limbal stem cell deficiency caused by alkali burns. 14 cases (16 eyes) suffering from severe corneal ulcers with hypopyon.

All of those 16 eyes were treated with Multi-layered Amniotic Membrane Transplantation and tarsorrhaphy. One patient (one eye) of Mooren's ulcer was treated by Multi-layered Amniotic Membrane Transplantation. Other indications included two patients (4 eyes) with corneal ulcers in Graves Hyperthyroidism, one unconscious patient (one eye) caused by head injury, another patient with one eye due to severe Neisseria Gonorrhoeceae's corneal ulcer, one eye with absolute glaucoma, two eyes with neurotrophic corneal ulcers caused by Herpes Simplex Virus and 3 patients of corneal ulcers caused by bacteria and fungal infections. All patients had been treated with eye

drops, eye ointment and systemic medication depending on the etiology of the disease.

Surgical Technique

The epithelial defect and/or the base of the stroma ulcer were debrided with a microsponge. The poorly adherent epithelium surrounding the defect or ulcer was removed. Freeze-dried, radiation sterilised amniotic membrane produced by Djamil Hospital Tissue Bank were used in this work. The amnion graft was peeled from its holding gauze, with epithelial side-up and was placed on the defect or stromal ulcer. One by one layer was applied to the defect. The entire surface of the cornea, and surrounding conjunctiva was covered by two or three layers of amniotic membrane. It was sutured with interrupted 10-0 nylon or 8-0 silk on the conjunctiva at 8 positions (Figure 1). The eye was then closed and patched. On the first and second day after operation, eye drops were instilled carefully through a small opened palpebral fissure (<1mm) to avoid damage or rupture of the amniotic membrane. On the third day, the eyes were inspected to see the process of healing of the ulcer and epithelialization using sterile flourescein staining. The sutures were removed after one to two weeks.

Clinical Results

The clinical results of the study showed that on the third days after multi-layered amniotic membrane transplantation, most of the cases showed corneal epithelializations. The mean time of the beginning of epithelialization was 3.9 ± 1.09 days (between 3 and 7 days). The mean time of complete epithelializations was 11.10 ± 4.95 days (between 4 and 21 days). The median time for complete epithelializations was 15 days. Improvement of visual acuity acquired in 64 % (14 eyes). In 27 % (6 eyes) of the cases, there was no worsening of the visual acuity. 9% (2 eyes) could not be evaluated i.e. one eye with absolute glaucoma and in one eye of unconscious patients suffering from severe head injury.

Most of the ulcerated eyes were of severe and deep type with hypopyon, 4 eyes suffered chemical and thermal burn. Two of the eyes had corneal erosion as a complication of Steven Johnson Syndrome. No post-operative

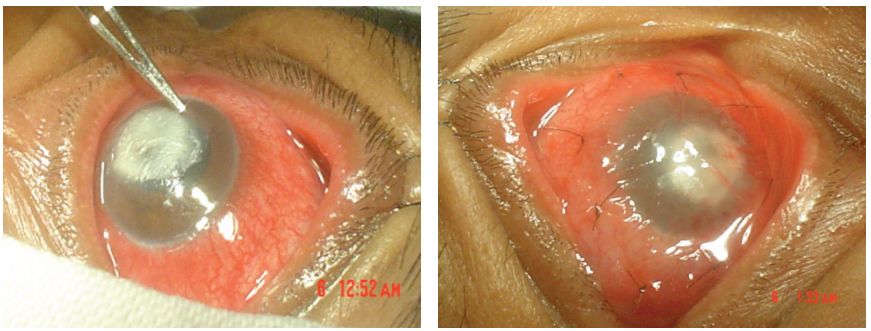


Figure 1. The surgical technique of freeze-dried, radiation sterilised multi-layer amniotic membrane transplantation was carried out at M. Djamil hospital. The freeze-dried amnion was peeled from the gauze, with epithelial side-up, and was placed on the defect or stromal ulcer one layer by one layer for filling up, grafting or patching of the ulcer. The entire surface of the cornea, and surrounding conjunctiva was then covered by two or three layer of amniotic membrane, and sutured with interrupted 10-0 or 8-0 silk on the conjunctiva at eight 8 positions

complications directly associated with the amnion were found such as super infection, new appearance of descemetocele, perforation, or graft rejection. However, in one case some hypopyon were still present. It was disappearing slowly within several days after MAMT.

It can be concluded that MAMT appeared to be a safe and effective method for restoring a stable corneal epithelium and for filling up of large corneal ulcers. Amnion was temporarily fixed/ stabilised on the corneal surface by way of suturing it to the conjunctiva. A pressure bandage was applied to the eyes for 3 days. No complications or failure was detected in this study.

Freeze-Dried and Fresh Amniotic Membranes with Limbal Stem Cell Transplantation for Severe Conjunctival Tumour and Corneal Defect

Limbus, is a junctional zone to separate the cornea from the conjunctiva, two distinctively different tissues. Limbal stem cell is the basal layer of limbal epithelium. It has two major functions i.e. as the ultimate source of cellular proliferation and differentiation of corneal epithelium and as the barrier to

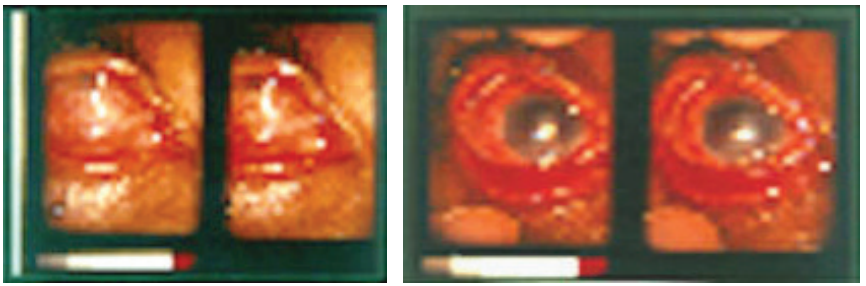


Figure 2. Patient with a large Symblepharon (left) before freeze-dried amnion transplant and (right) 6 weeks after amnion transplant (Cicendo Hospital, Bandung, Indonesia)

separate the corneal epithelium and conjunctive epithelium. It prevents conjunctiva from growing into a corneal epithelial defect. Limbal transplantation is to restore stem cells. Amniotic membrane transplantation is to restore basement membrane and stroma.

The aim of the work is to investigate the use of fresh amnion and freeze-dried, radiation sterilised amniotic membranes as transplants for ocular surface reconstruction. The amniotic membranes used in this work were produced by BATAN Research Tissue Bank.

Amniotic membrane with limbal stem cell transplantation has been performed in two groups of patients. Group A received fresh amnion. Group B received freeze-dried, radiation sterilised amniotic membranes. Each group consisted of 2 patients with large Symblepharon (Figure 2), 2 patients with large conjunctival tumour, 7 patients with corneal ulcer and 1 patient with Steven Johnson Syndrome. All the patients have corneal diseases with limbal deficiency.

The mean healing time on patients with large conjuntival tumour using fresh amniotic membranes transplantation was 4 weeks (SD, 1.41) and using freeze-dried amnion was 5 weeks (SD, 1.95). The mean healing time of patients with severe corneal defect using fresh amniotic membrane transplants was 3.43 weeks (SD, 2.44) and freeze dried amniotic membranes was 3.38 weeks (SD, 2.11). There was no significant difference on healing time of defects by transplanted fresh amnion or freeze-dried amnion. Both fresh and freeze-dried amniotic membranes can be used as transplants for ocular surface reconstruction.

Conclusions

Freeze-dried, radiation sterilised amnion membrane transplants provide a good alternative procedure for Ocular Surface Reconstruction for treating corneal defects. Multi-layer Amnion Membrane Transplantation/MAMT using freeze-dried amniotic membranes, provides a safe and effective method to restore a stable corneal epithelium. It also provides an adequate base for filling up large corneal ulcers. No significant difference in healing time was found in corneal defects treated with fresh and freeze-dried, radiation sterilised amnion grafts for ocular surgery transplantation.

References

De Rotth A (1940) Plastic repair of conjunctival defects with foetal membranes. Arch. *Ophthalmol.*, **23**: 522–525.

Getry Sukmawati Ibrahim S (2006) Multilayered Amniotic Membrane Transplantation for the Treatment of Corneal Epithelial Defects and Ulcers, Department of Ophthalmology Faculty of Medicine Andalas University/ M Djamil Hospital Padang Indonesia, (will be published).

Hilmy N, Febrida A and Basril A (2000) Validation of Radiation Sterilization Dose for Lyophilized Amnion and Bonegrafts. *J. Cell and Tissue Banking*, **1**(2): 143–148.

Hilmy N, Febrida A and Basril A (2003) Indonesia: Statistical Sampling Technique in Validation of Radiation Sterilization Dose of Biological Tissue. *Cell and Tissue Banking*, **4**(2): 185–191.

Indira S, Laksmi T and Bambang S (2000) Freeze dried and fresh amniotic membranes with limbal stem cell transplantation in severe conjunctival tumor and corneal defect. 8th International Conference on Tissue Banking, Bali 20–24 October 2000. p. 98.

International Atomic Energy Agency/ IAEA, Multi-Media Distance Learning Package on Tissue Banking (1997) Module 4, Procurement., IAEA/NUS, Interregional Training Centre, Singapore.

International Atomic Energy Agency/IAEA Code Of Practice for Radiation Sterilization of Tissue Allograft (2004) IAEA, Vienna.

Komolsuradej W, Tesavibul N and Prabhasawat P (2001) Single and Multilayer Amniotic Membrane Transplantation for Persistent Corneal Epithelial Defect. *Br. J. Ophthalmology*, **85**: 1455–1463.

Kruse FE, Rohrschneider K and Volcker HE (1999) Multi-layer amniotic membrane transplantation for reconstruction of deep corneal ulcers. *Ophthalmol.*, **106**: 1504–1511.

Lavery FS (1946) Lime burn of conjunctiva and cornea treated with amnioplastin graft. *Trans. Ophthalmol. Soc. UK*, **66**: 668.

Lee SH and Tseng SGD (1997) Amniotic Membrane Transplantation for Persistent Epithelial Defects with Ulceration. *American Journal of Ophthalmology*, **123:** 303–312.

Prabhasawat P, Barton K, Burkett G and Tseng SCG (1997) Comparison of conjunctival autografts, amniotic membrane grafts and primary closure for pterygium excision. *Ophthalmol.*, **104**: 974–985.

Prasad JK, Feller I and Thompson PD (1986) Use of amnion for the treatment of Stevens-Johnsons syndrome. *J. Trauma*, **26**: 945–946.

Radiah Sunarti and Susi Heryati (2006) Freeze-Dried Amniotic Transplantation In Corneal Ulcers Management, Cicendo Eye Hospital, Faculty of Medicine, Padjajaran University, Bandung, Indonesia (will be published).

Sorsby A and Symmons HM (1946) Amniotic membrane grafts in caustic burns of the eye (burns of second degree). *Br. J. Ophthalmol.*, **30**: 337–345.

Tseng SCG (2005) Literature Summary on Uses of Amniotic Membrane in Ocular Surface Reconstruction, Download from the URL http://www.ocularsurface.com/

18

Multi Layer Amniotic Membrane Transplantation (MLAMT) for Ocular Reconstruction

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Introduction

Amniotic Membrane (AM) is the innermost of the three layers forming the fetal membrane. It is a biological tissue that has been used as a graft for corneal and conjunctival reconstruction in a variety of ocular surface diseases. This membrane is avascular and it possesses anti-angiogenic, anti-scarring, anti-microbial, non-immunogenic and anti-inflammatory properties. It is not a substitute but rather a substrate upon which cells can migrate and regenerate, forming a new and healthy tissue. The AM is used as a biological patch, as a dressing, to treat inflammatory disorders.

The Amniotic Membrane Transplantation for Ocular Surface Reconstruction

In the period July 2005 to November 2014, Amniotic Membrane Transplantation was performed in 165 ocular cases (159 patients) at Dr M Djamil Hospital in Padang, West Sumatera Indonesia by the senior author (Getry Sukmawati). Freeze dried amniotic membranes sterilised by irradiation at 25 kGy produced by M Djamil Hospital in Padang (Figure 1) using the Multi-Layered Amniotic Membrane Transplantation (MLAMT) method were applied in all cases. The procedure was performed under topical or general anesthesia, to fill, graft and patch the corneal defect over the whole/part of the corneal surface and part of the conjunctiva.

The multi-layered AMT is useful in corneal ulceration, descemetocele, and corneal perforation. The success rate is 72% compared to 64% for mono-layered AMT (Thomas, 2003). Kruse *et al.* (2000) and Hanada *et al.* (2001) found the multi-layered AMT to be more successful compared to the mono-layered AMT. The latter disappears within a few weeks. Solomon *et al.* (2002) reported a success rate of 82.3% in using multi-layered AMT. Tseng (2001) found that the multi-layered corneal defect healed significantly faster than that of a single layer.

Several Applications of MLAMT

Corneal Surface Reconstruction

There were 87 cases of Corneal Ulcers with different depth and etiology, 14 cases of persistent epithelial defects and neurotrophic corneal ulcer,

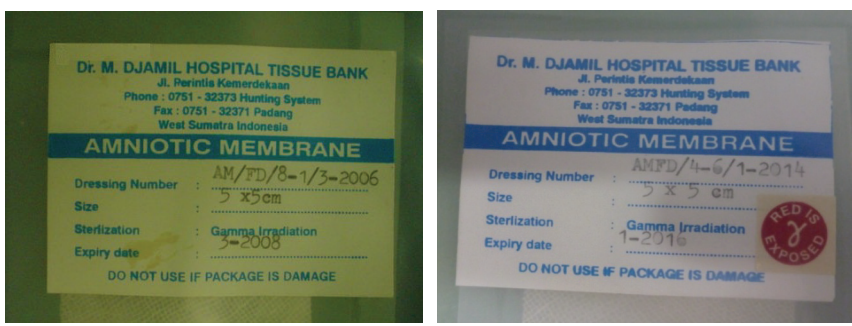


Figure 1. Freeze Dried Amniotic Membrane sterilised by irradiation at 25 kGy produced by M Djamil Hospital, Padang, West Sumatera, Indonesia in 2006 (left) and 2014 (right) used in our studies

4 cases of atheromatous corneal ulcer (include perforation), 8 cases of corneal defect with symblepharon (fornix plasty) caused by Steven Johnson Syndrome (SJS), 9 cases of Mooren's corneal ulcer and peripheral ulcerative keratitis, 3 cases of corneal perforation with iris prolapse, iris cyst or epithelial ingrowth, 1 case of corneal maceration with perforation after keratoplasty caused by long-term use of corticosteroid topical eye drops and 1 case of painful bullous keratopathy.

Conjunctival Surface Reconstruction

22 cases of pterygium surgery, 8 cases of conjunctival tumor excision, and 8 cases of Chemical burns were included in this study.

Methods of Reconstruction

Corneal ulcer is a defect of the corneal layer with epithelial loss, that can be caused by infection, or non-infection. Microorganisms that are often associated with infective corneal ulcers are bacteria, virus, fungi, or parasites. Non-infective or inflammatory causes induce hypersensitivity reaction, vitamin A deficiency, burn and chemical trauma.

MLAMT for corneal surface reconstruction was performed in 87 cases, with different etiologies and severity.

In general, the surgical technique employed depends on the depth of the corneal defect. After debridement and removal of poorly adherent and necrotic corneal epithelium surrounding the ulceration or defect, the amniotic membrane is applied on the defect of the cornea, layer by layer with the epithelial side up (up to 8 layers), for filling, grafting and patching the corneal surface. The last layer of amnion membrane for patching is applied to cover the conjunctiva surface to 2–3 mm of the posterior limbus. The amnion membrane was fixed by suturing to the conjunctiva to avoid corneal scar otherwise caused by the stitches. About 12–16 interrupted sutures with 8.0 silk/vicryl were stitched in 8–12 positions (Figure 2). A bandage is applied for three to four days after surgery to avoid breakage of the membrane and to maintain them stable on the cornea. The eye drops could be instilled through the eye lid fissures. After 3–4 days, epithelialisation appeared and is usually complete after 11.10 ± 4,95 days (Sukmawati, 2006). When there is no complication, the conjunctiva sutures are usually removed at 2 weeks.

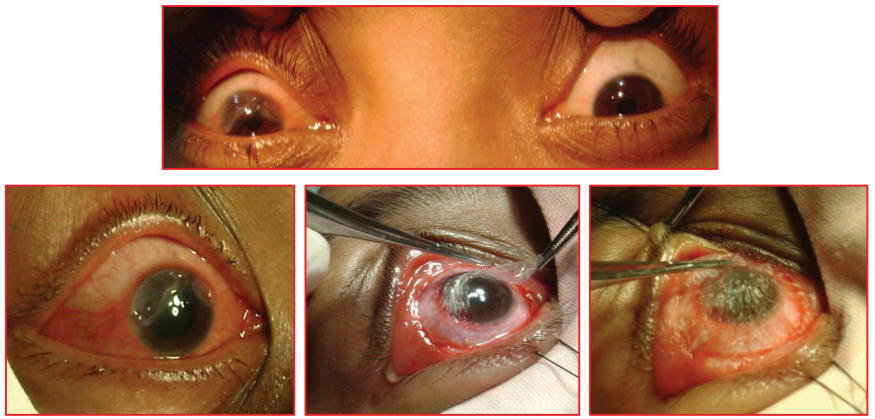


Figure 2. Showing surgical technique. A case of Mooren's Ulcer in right eye. The sclera was first bared and Multi Layer amnion Membrane Transplantation (MLAMT) was performed, for filling, patching and grafting of the corneal surface

For perforating corneal ulcer more than 3 mm, with or without iris prolapse, situational corneal suture is performed with 10.0 silk first, like a net, to help the amnion layer to be fixed and remain stable on the corneal surface (Figure 3) (Sukmawati, 2014).

Rodriguez-Ares *et al.* (2004) reported the use of AMT in treatment of corneal perforation of different sizes. They concluded that it is effective for treating corneal perforations with diameters of less than 1.5 mm. The method is unsuccessful when the perforation is 3 mm or more. Hick *et al.* (2004) proposed the use of AM and fibrin glue in the management of corneal ulcers for different diameters of perforation to up to 3 mm (Thomas, 2003).

In Case A, the corneal ulcer caused by Mooren's Ulcer, before MLAMT, total/partial conjunctival resection to bare sclera was performed to stop the immunological process between the cornea and antibodies from the vascular conjunctiva (Sukmawati and Havriza, 2008). The conjunctival flap can cause the ulcer to get worse as it can increase the auto-immune response — infiltration by lymphocytes, plasma cells, macrophages, and increased production of collagenase enzyme — and may result in lysis of the healthy cornea. Chen *et al.* (2003) reported the relapsing Mooren's ulcer after AMT combined with autografting of the conjunctiva (Sukmawati, 2008).

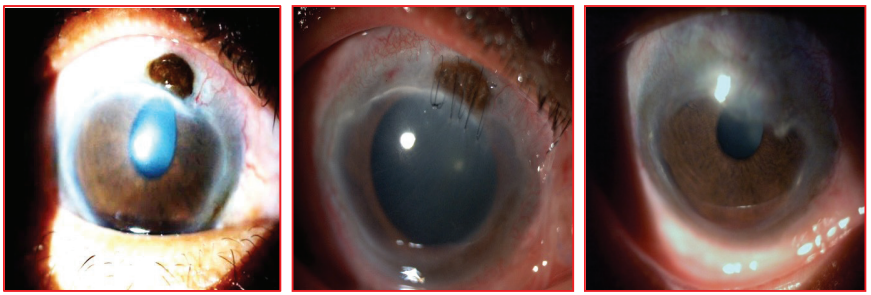


Figure 3. Case A. Perforated Mooren's ulcer. Left: before surgery. Middle and Right: after surgery

For cases with Grave's Ophthalmopathy, and for corneal exposure due to unconsciousness as a result of head injury, tarsorrhaphy was performed. Contact lens is not used.

In Case B due to chemical or thermal burn with corneal erosion and opacity with partial or total limbal ischemia, MLAMT was performed as soon as possible. The epithelialisation can occur and is complete by 21 days, depending on the destruction of the cornea and the extent of limbal ischemia.

Thomas (2003) used AMT for acute stage of chemical or thermal burn. AMT was applied between 7–10 days after the accident for maximal beneficial effect. The corneal epithelium consists of corneal stem cells at the limbus, transient amplifying cells (TAC), post mitotic cells (PMC), and terminally differentiated cells (TDC). Both stem cells and TAC are able to proliferate, whereas post-mitotic cells have lost this ability. In cases of limbal ischemia, amnion membrane transplantation can promote proliferation of TAC.

Joseph (2001) and Tamhane (2005) found different results. They found failure in more than 50% cases using AMT for ocular surface reconstruction in patients with severe acute chemical and thermal burns with total limbal ischemia. Tseng and Tsubota (2002) reported that AMT cannot be used to treat ocular surface disorders with total loss of limbal stem cells, stromal ischemia, severe aqueous tear deficiency, diffuse keratinisation, and absence of blinking in severe neurotrophic state.

Figure 5 showed our case with bullous keratopathy, unresponsive to medical treatment. Aspiration of the bullae was performed before amnion membrane was applied and sutured on the conjunctiva (Sukmawati, 2004).

Table 1. Some cases of MLAMT by authors. RE = right eye, LE = left eye

Cases	Age	Sex	Indication, ophthalmic finding	Vision pre op	Vision post op	Outcome
A	39 yo (Figure 3)	F	Perforated Mooren's Ulcer RE — Perforated cornea with iris prolapse			Significant Improvement. Corneal defect and iris prolapse were covered with amniotic membrane
B	6 yo	M	Alkali burn grade IV both eye — Total limbal ischemia — Corneal opacity	RE: 1 m counting finger LE: Light Perception	RE: 2/60, Two meter counting finger LE; HM (hand movement)	Improved (Corneal opacity decreased)
C	6 yo (Figure 4)	M	Alkali burn LE — Symblepharon — Trichiasis — Severe dry eye (schirmer test 1 mm, BUT and ferning test undetectable)	LE: hand movement	LE: 0,67	Significant Improvement (After 2nd MLAMT, significant improvement of corneal opacity and visual acuity) (Figure 4)
D	63 yo (Figure 5)	F	Bullous keratopathy with iris prolapse			Subjectively, patient feel better and no pain
E	64 yo (Figure 6)	M	Corneal maceration with perforation LE	RE: Light perception LE: hand movement	LE: 1 m counting finger	Improvement. Complete epithelialisation of the corneal surface and improved visual acuity

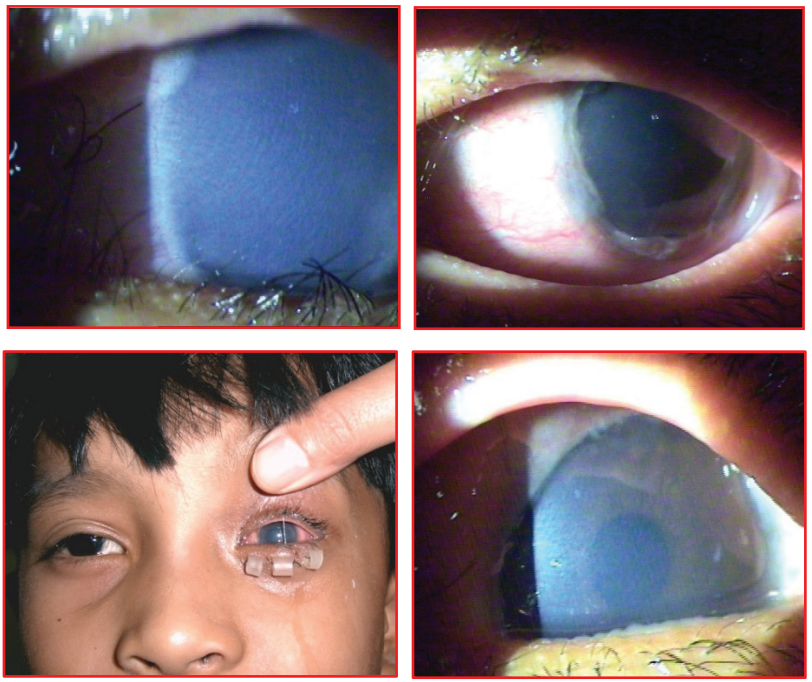


Figure 4. Case C. Boy, 6 years old, with alkali burn. MLAMT was performed twice. Two weeks after 2nd MLAMT (bottom), corneal opacity decreased and visual acuity improved

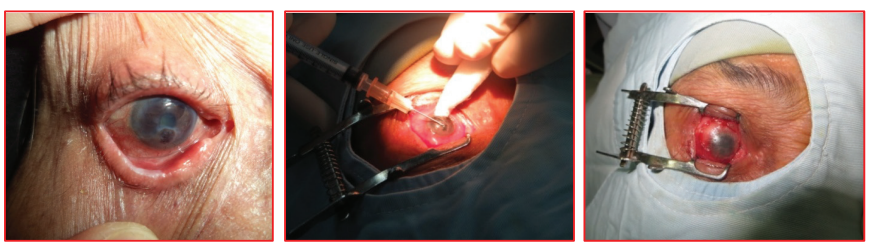


Figure 5. Case D. Female, 63 years old, bullous keratopathy with iris prolapse (One eye) July, 14-2014

Figure 6 showed our case with maceration on the paracentral cornea with perforation. This is caused by the use of topical corticosteroid eye drops on the left eye for 14 years after keratoplasty. MLAMT was performed after secondary infection was reduced (Figure 6).

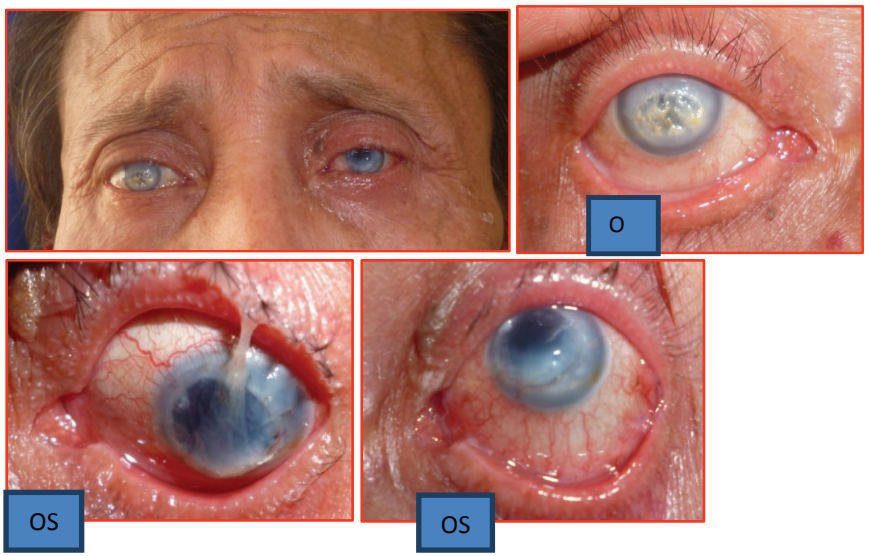


Figure 6. Case E. Male, 64 years old, corneal maceration with perforation. MLAMT performed resulting in epithelialisation of corneal surface and improvement of visual acuity

Conjunctiva Surface Reconstruction

Pterygium

Pterygium is a wing-shaped, degenerative, fibrovascular, hyperplastic proliferative tissue actively growing from the conjunctiva limbal area onto the cornea in the inter palpebra region. The etiology is still unknown. It is more frequent in areas nearer to the equator. It occurs with more exposure to ultraviolet (UV) radiation, and with hot, dry, windy, dusty and smoky environments. It occurs in people more exposed to the climatic conditions (pterygium belt/pterygium zone, 37° Latitude north and Latitude south) such as fishermen, farmers and construction workers, who are probably exposed to ultraviolet (UV) (Booth, 1985). UV-mediated limbal damage acts as a trigger for pterygium pathogenesis (Ooi *et al.*, 2007).

Amniotic Membrane Allograft Transplantation — MLAMT

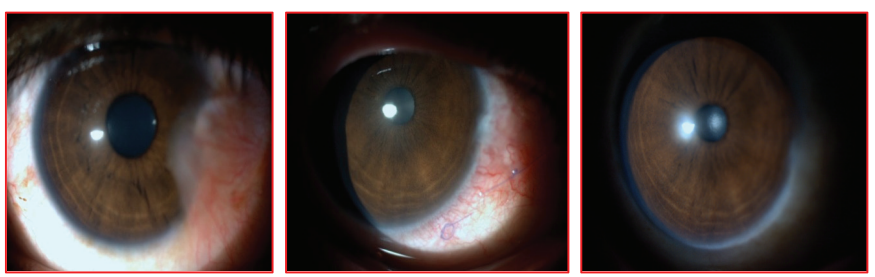
MLAMT can suppress transforming growth factor-ß signalling, proliferation, and myofibroblastic differentiation of normal human corneal and limbal

fibroblasts, conjunctival and pterygium body fibroblasts, to reduce scar formation after conjunctival reconstruction and prevent fibroblast activation and pterygium recurrence.

Bare scleral excision, with Amniotic Membrane Graft (AMT), or with Limbal Stem Cell Transplantation, had been used to prevent recurrence. Lamellar Keratoplasty is performed only for recurrent pterygium (Kaufman *et al.*, 2013). Intra or post operative Mitomycin C, Thiotepa, Daunorubicin and 5-FU were used to decrease recurrence rate (Dadeya and Kamlesh, 2002). Fibrin glue has been used as an alternative to sutures for securing conjunctiva grafts and also to reduce recurrence rate. Transplantation of Amniotic Membrane and Limbal Autograft was performed for patients with recurrent pterygium associated with symblepharon.

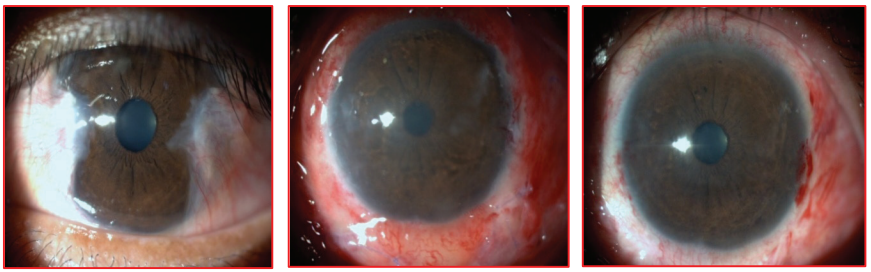
In our study, McReynolds Technique or Bare Scleral (conjunctiva resection) was performed in combination with MLAMT for pterygium surgery (Figures 7 and 8) (Sukmawati, 2014).

Pterygium excision with MLAMT was performed in 22 patients. Epithelialisation began on day 3. Complete epithelialisation occurred on day 4–7. Visual acuity improved in all patients after operation with minimal corneal scarring. Conjunctiva sutures were removed at one week. Recurrence occurred in 3 patients, between 1 week and 6 months after surgery. It is usually caused by outdoor activity. The recurrent pterygium did not progress. Second excision was not needed (Sukmawati, 2013). The complications included bleeding between amnion layers, sub conjunctiva hemorrhage and recurrent pterygium. Secondary operations were not needed.



(a) (b) (c)

Figure 7. Woman, 40 years old, Pterigium LE. (a) Pre-Operative, fibrovascular tissue on the cornea. (b) Three days after surgery, epithelialisation began and visual acuity improved. (c) Three years after surgery, cornea clear and visual acuity was normal



(a) (b) (c)

Figure 8. Male, 40 years old. Pterygium RE. (a) Pre-operation RE, fibrovascular tissue on both sides of the cornea RE. (b) Three days after surgery, epithelialisation begun. (c) Cornea is clearer with very minimal corneal scarring and with improvement of visual acuity

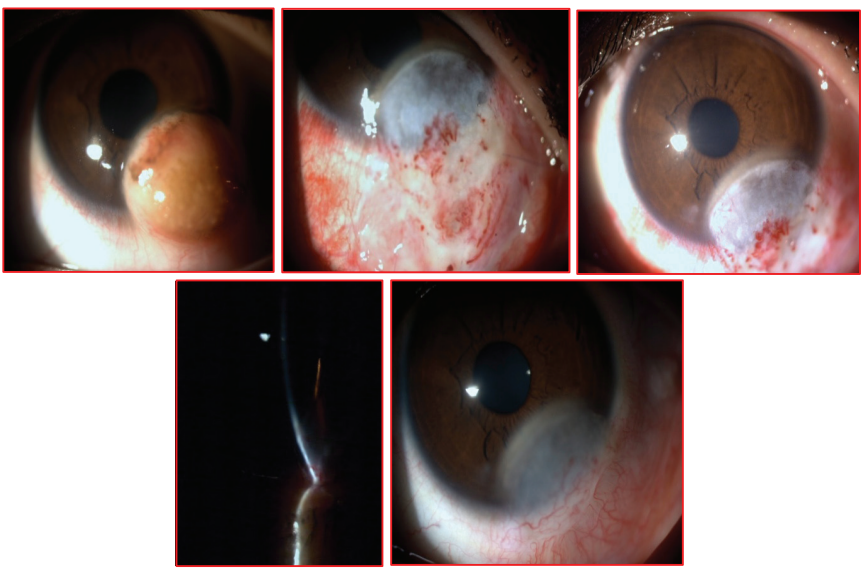


Figure 9. Jan, 12-2011, Male, 18 years old. Dermoid Cysta LE, complete epithelialisation 12 days after excision MLAMT performed. (Pathology: Fibro Epithelial Polyp)

Excision of tumor mass on conjunctiva or cornea was performed followed by application of amnion membrane layer by layer. The amnion was sutured onto the conjunctiva (Figure 9).

Complications and Failures

The use of AMT in ophthalmic surgery had been shown to provide an alternative for corneal and conjunctiva reconstruction, but it has its limitations and is not always successful for every condition. Some authors reported complications such as: recurrent hypopyon (BenEzra, 2003), graft failure, recurrent graft melts/corneal melts (Schechter *et al.*, 2003; Solomon, 2002), submembrane hemorrhage (Tseng, 2010), and corneal calcification (Anderson *et al.*, 2002).

Hanada *et al.* (2000), Kozak *et al.* (2000), Joseph *et al.* (2001), and Tamhane *et al.* (2005) reported failure with AMT in cases with total limbal deficiency. AMT restored the conjunctiva surface without symblepharon and reduced limbal stromal inflammation, but did not prevent limbal stem cell transplantation.

In our cases, we found few complications, such as: air bubble and hemorrhage between amnion layers, breakage of the amnion membranes, cornea calcification associated with antibiotic eye drops (Figure 10) and no graft rejection in 165 cases that had been performed (Sukmawati, 2006). We found failure in one patient with perforation corneal ulcer and iris prolapse, after corneal suture and MLAMT. Cornea epithelialisation did not occur and there was breakage in all of the layers of amnion membrane. We performed fascia lata autograft. The fascia lata graft failed 5 days after surgery. Second MLAMT was performed for reconstruction of the prolapsed iris and corneal defect. Keratoplasty cannot be performed. Availability of cornea donors is still a problem (Figure 11) (Sukmawati, 2014).

Results and Discussions

Multi-layered amniotic membrane transplantation can be used for ocular reconstruction successfully. It can improve visual acuity and promote corneal epithelialisation. In some conditions it improves activity of stem cells and activity of conjunctival goblet cells.

In this study, only one patient with pterygium treated with surgery using MLAMT developed recurrence one week after surgery. This is possibly caused by direct use of motorcycle. Corticosteroid and antibiotic eye drops

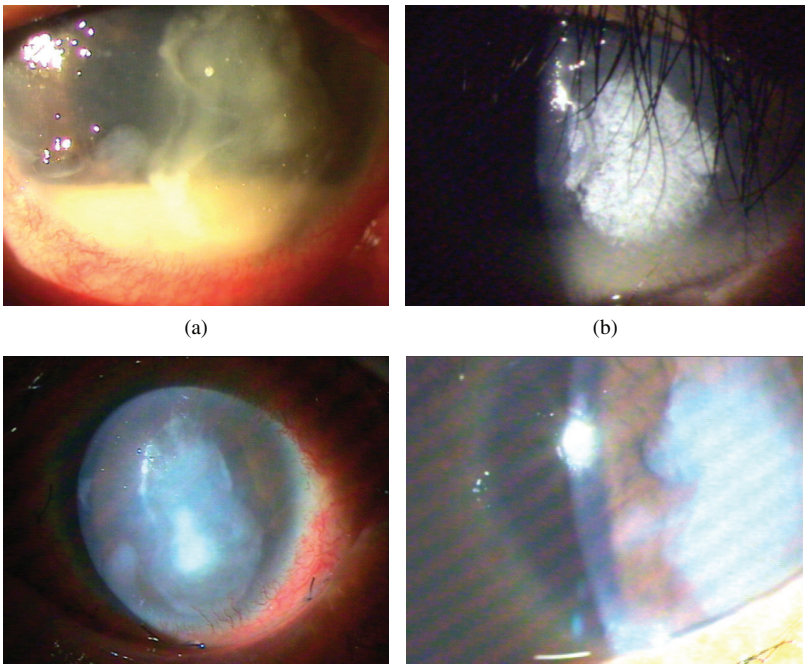


Figure 10. Female, 58 years old. (a) Corneal ulcer with Hypopyon (b) Corneal ulcer with corneal (drug) deposit, with calcification of cornea (c) Seven days after AMT. Plaque disappeared when eye drop was discontinued (d) Corneal scarring 10 months after AMT

were used but did not prevent recurrence. Our recommendation is after pterygium surgery with MLAMT the patient must avoid exposure to wind, UV light and outdoor activities.

Conclusion

Amniotic membrane transplantation can be used successfully for ophthalmic surgery. MLAMT is one of the surgical ocular reconstruction methods which can be applied, especially when keratoplasty cannot be performed for corneal defect. MLAMT can be done more than once and can be combined with another operation.

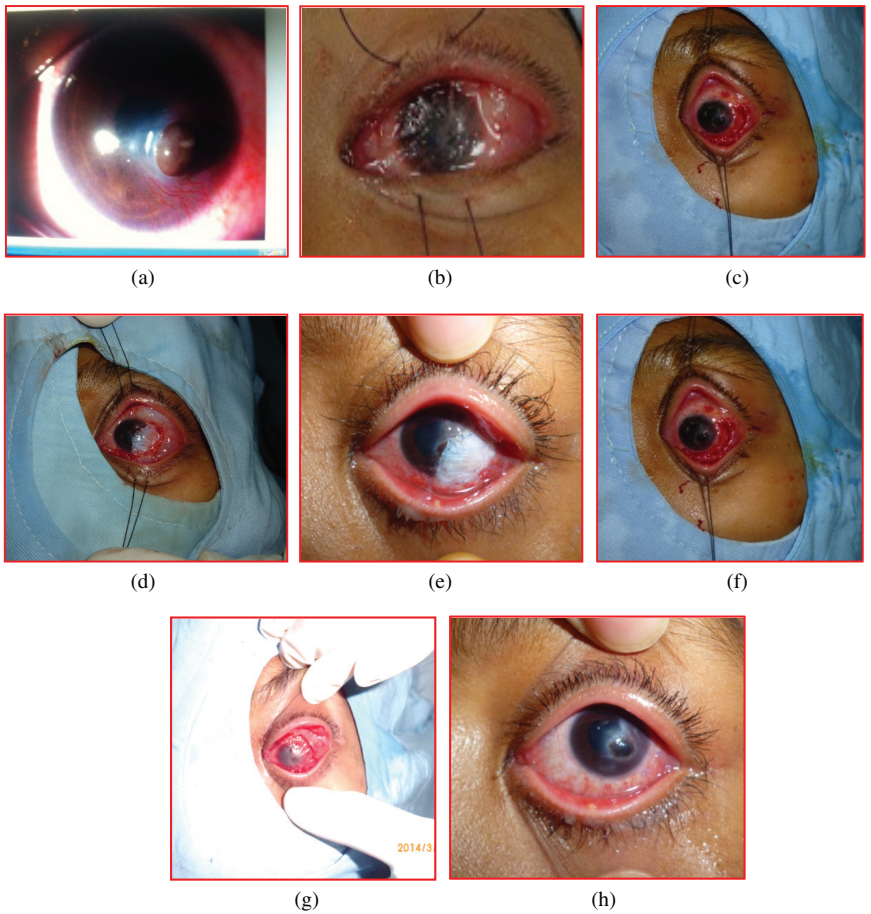


Figure 11. (a) Female, 11 years old. Perforation Corneal Ulcer with Iris Prolapse (3-4 mm). (b) Hechting of the cornea and MLAMT was performed. (c) Corneal suture was loose and Amnion Graft failed five days after surgery. (d) One month later, Autograft Fascia Lata was performed. (e) 4 days after Graft Fascia Lata: iris prolapse under the graft. Graft reposition was planned. (f) During operation, corneal melting under Fascia Lata Graft (rejection). (g) Second MLAMT performed. (h) 10 days after surgery, complete epithelialisation occurred. Visual acuity improved

Fresh oral mucosal auto graft can be combined with MLAMT to improve activity of conjunctival goblets cells. It is the other choice of surgical method for ocular surface reconstructions.

In our clinical experience there was no graft rejection or severe complication occurring after the use of Amnion Membrane for Ocular Reconstruction.

References

BenEzra D (2003) Amniotic Membrane Transplantation in Ocular Surface Inflammation, **4**: 246–252.

Booth F (1985) Heredity in One Hundred Patients Admitted for Excision of Pterygia. *Australian and New Zealand Journal of Opth.*, **13**: 59–61.

Dadeya S and Kamlesh (2002) Intraoperative daunorubicin versus conjunctival autograft in primary pterygium surgery. *Cornea*, **21**(8): 766–769.

Hanada K, Shimazaki J, Shimmura S and Tsubota K (2001) Multilayered Amniotic Membrane Transplantation for Severe Ulceration of the Cornea and Sclera. *Am. J. Ophthalmol.*, **131**: 324–331.

Hick S, Demers PE, Brunette I, La C and Mabon M (2005) Amniotic Membrane Transplantation and Fibrin Glue in the Management of Corneal Ulcers and Perforations. *Cornea*, **24**(4): 369–377.

Joseph A, Dua HS and King AJ (2001) Failure of Amniotic Membrane Transplantation in the Treatment of Acute Ocular Burns. *Br. J. Ophthalmol.*, **85**: 1065–1069.

Kaufman SC, Jacobs DS, Lee WB, Deng SX, Rosenblatt MI and Shtein RM (2013) Options and Adjuvants in Surgery for Pterygium. *Ophthalmology*, **120**: 201–208.

Kozak I, Trbolova A, Sevcikova Z, Juhas T and Ledecky V (2002) Superficial Keratectomy, Limbal Autotransplantation and Amniotic Membrane Transplantation in the Treatment of Severe Chemical Burns of the Eye. *Acta. Vet. BRNO*, **71**: 85–91.

Kruse FE, Joussen AM, Rohrschneider K, You L and Sinn B (2000) Cryopreserved Human Amniotic Membrane for Ocular Surface Reconstruction. *Graefe's Arch. Clin. Exp. Ophthalmol.*, **238**: 68–75.

Ooi JL, Sharma NS, Sharma S, Papalkar D, Oakey M, Dawes D and Coronea MT (2007) Ultraviolet Fluorescence Photography: Patterns in Establish Pterygia. *Am. J. Ophthalmol.*, **143**: 97–101.

Rodriguez-Ares TM, Tourino R, Lopez-Valladares JM and Gude F (2004) Multilayer Amniotic Membrane Transplantation in the Treatment of Corneal Perforations. *Cornea*, **23**(6): 577–583.

Schechter AB, Rand WJ, Nagler RS, Estrin I and Arnold SS (2005) Corneal Melt After Amniotic Membrane Transplant. *Cornea*, **24**(1): 106–107.

Solomon A, Meller D, Prabhasawat P, John T and Espana EM (2002) Amniotic Membrane Grafts for Non traumatic Corneal Perforations, Descemetoceles, and Deep Ulcers. *Ophthalmology*, **109**(4): 694–703.

Sukmawati G (2013) Use of Multi Layer Amniotic Membrane Transplantation (MLAMT) In Pterygium Excision. Presented at the Congress of Indonesian Ophthalmology Association (IOA), Surabaya, Indonesia.

Sukmawati G (2014) Non Infectious Corneal Ulcer. Presented at the Indonesia Ocular Infection Immunology Society (INNOIS), Solo, Indonesia.

Sukmawati G (2014) Update and Current Issue On Pterygium. Presented at the Sumatera Ophthalmology Meeting (SOM), Aceh, Indonesia.

Sukmawati G and Havriza V (2008) The Efficacy of Bare Sclera and Multi Layer Amniotic Membrane Transplantation (MLAMT) for Recurrent Mooren's Ulcer. Presented in Sumatera Ophthalmology Meeting (SOM), Medan, Indonesia.

Sukmawati G and Sastradiwirya I (2006) Multilayered Amniotic Membrane Transplantation for the Treatment of Corneal Epithelial Defects and Ulcers. Presented at the Congress of Indonesian Ophthalmology Association (IOA), Medan, Indonesia.

Tamhane A, Vajpayee RB, Biswas NR, Pandey RM and Sharma N (2005) Evaluation of Amniotic Membrane Transplantation as an Adjunct to Medical Therapy as Compared with Medical Therapy Alone in Acute Ocular Burns. *Ophthalmology*, **112**(11): 1963–1969.

Thomas J (2003) Human Amniotic Membrane Transplantation: Past, Present, and Future. *Ophthalmology Clinics of North America*, **16**: 43–65.

Tseng SCG (2001) Amniotic Membrane Transplantation for Persistent Corneal Epithelial Defect. *Br. J. Ophthalmol.*, **85**: 1400–1401.

Tseng SCG (2010) Literature Summary on Uses of Amniotic Membrane in Ocular Surface Reconstruction. www biotissue.com/AM % 20 literature % 20 summary % 2010-30-20.pdf

Tseng SCG and Tsubota K (2002) Amniotic Membrane Transplantation for Ocular Surface Reconstruction in Ocular Surface Disease. **IV**, **20**: 226–231.

19

Gingiva Depigmentation Using Amniotic Membrane

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Introduction

Gingiva depigmentation is a procedure used for cosmetic dentistry to eliminate dark spots or patches on the gingiva caused by excessive melanin. Basically, the normal colour of gingiva tissue is pink. Gingiva pigmentation causes discoloration of gingiva to brown or black. The color of gingiva depends on several factors: number and size of blood vessels, thickness of the epithelium, level of keratinisation and quantity of pigments (Balcheva and Balcheva, 2014). Factors that can lead to gingiva pigmentation include: heredity, smoking habits, and also the use of certain medications such as anti-malarial agent and tryciclic antidepressant (Almas and Sadig, 2002; Axell and Hedin, 1982; Araki *et al.*, 1983; Hedin and Axell, 1991; Hanioka *et al.*, 1993; Unsal *et al.*, 2001; Kaushik *et al.*, 2013; Hedin and Larsson, 1984; Halaban *et al.*, 2001).

Gingiva pigmentation is the result of melanin granules synthesized in melanosomes of melanocytes. Melanocytes are identified as dendritic cells in

the basal layer of the gingiva epithelium. Melanosomes are transferred to keratinocytes through dendritic processes by phagocytic activity, and are degraded due to rise to the surface. Melanin is synthesized from tyrosine and dihydroxyphenylalanine through dopaquinone as a result of oxidation activity of tyrosinase (Halaban *et al.*, 2001; Hedin *et al.*, 1993).

Melanin pigmentation of the gingiva is also correlated with active smoking. Some researchers found that the pigmentation of the gingiva smokers tend to be larger than non smokers (Araki *et al.*, 1983; Hedin and Axell, 1991; Hanioka *et al.*, 1993; Unsal *et al.*, 2001). After quitting smoking for several years, prevalence of gingiva pigmentation decreased (Mohammadi *et al.*, 2009). These findings suggest a relationship between cigarette smoking and melanin pigmentation in the gingiva. Gingiva pigmentation often occurred in the labial area of anterior teeth, and excessive pigmentation on the palatal musoca as a result of cigarette smoking. It is a rare phenomenon (Hanioka *et al.*, 1993; Unsal *et al.*, 2001).

To eliminate excessive pigmentation of the gingiva, depigmentation can be done. Gingiva depigmentation is a periodontal plastic surgery to eliminate and reduces gingiva hyperpigmentation. Some depigmentation techniques have been developed, such as using a scalpel technique, using high speed large round diamond burr and laser technique. The current method of pigmented gingiva cover areas that are not pigmented, such as gingiva graft and acellular dermal matrix allograft. Laser treatments are also reported to have given good results. However, not all hospitals and dental clinics have such expensive equipment (Prasad *et al.*, 2010; Gupta, 2011).

Repigmentation at the post-depigmentation of the gingiva may occur due to the penetration of chemicals that are difficult to control. To avoid fast repigmentation, a gingival graft can be used. However, the gingiva tissue sampling means increasing morbidity in extraction and in implantation .

The amniotic membrane (AM) is the innermost layer of the placenta and is composed of a single epithelial layer, a thick basement membrane and an avascular stroma. The special structure and biological viability of the AM allows it to be an ideal candidate for creating scaffolds for cell proliferation and differentiation. Amniotic membrane consists of three layers i.e epithelial layer, basement membrane and connective tissue matrix.

Epithelial cells derived from the AM have the advantages of stem cells and contain several components i.e collagen type III, IV, non collagenous

glycoproteins: fibronectin, vitronectin, laminin 5, TNF alpha, nidogen growth factor (NGF), and activin. Its functions include promoting cellular adhesion of gingiva cells, invasive growth of fibroblasts and angiogenesis in the early phases of wound healing.

The basement membrane layer of AM contain type IV collagen, type VII collagen, laminin 1, laminin 5, fibronectin, allantoin, lysozyme, transferrin, progesteron and several kinds of growth factors. These factors have a huge influence on the adhesion and growth of the overlying stem cells, as well as allowing the cells to attach and migrate.

The connective tissue layer contains mitogenic factors, anti-angiogenic and anti-inflammatory proteins and natural inhibitors to proteases, growth factors such as keratinocyte growth factor (KGF), basic fibroblast growth factor (b-FGF), transforming growth factor-beta (TGF-β), NGF, and epidermal derived growth factor (EDGF) and markers such as CD90+, CD105+, CD73+, CD44+, HLA I+, CD45, CD34, CD11b. These factors provide a natural healing environment, promoting anti-scarring properties and periodontal regeneration, and accelerating the inflammatory phase towards the proliferative phase allowing the wound to heal in a much faster and efficient way (Gupta, 2011; Niknejad *et al.*, 2008; Alireza *et al.*, 2007; Koizumi *et al.*, 2000).

AM has biological properties, including anti-inflammatory, anti-microbial, anti-fibrosis and anti-scarring properties. It also has reasonable mechanical properties and low immunogenicity. It also accelerates epithelialisation (Gupta, 2011; Niknejad *et al.*, 2008; Koizumi *et al.*, 2000).

AM has been used in various fields of clinical work, including management of burns, reconstruction of the oral cavity, bladder, vagina, tympanoplasty, arthroplasty and ophthalmology. Recently this graft has been used in the field of periodontics (Mishra and Singh, 2014; Agarwal *et al.*, 2014).

AM has been used as a skin substitute due to its effect in enhancing wound healing cascade. Amniotic derived cells have multipotent differentiation ability to regenerate oral and periodontal tissue. It provides good results in terms of root coverage, increased tissue thickness and increased attachment of gingiva tissue. It also produces excellent aesthetic results in terms of texture and matching of colour. It also acts as a barrier membrane for preventing the formation of intra bony defects. It also promotes

intraoral soft and hard tissue healing (Mishra and Singh, 2014; Agarwal *et al.*, 2014).

Since 1989, preserved AM, i.e lyophilised and radiation sterilised amnion (ALS radiation or AM sterile) and air dried radiation sterilised amnion (AAS radiation) were produced by several tissue banks in Indonesia including the BATAN Research Tissue Bank. AM is sterilised by gamma radiation at a dosage of 25 kGy. This product has been used as a dressing for many types of wounds and ulcers, as well as for ophthalmic and maxillofacial surgery in more than 50 hospitals in Indonesia (Hilmy *et al.*, 2000; Manjas, 2010).

Amniotic Membrane Transplantation for Gingiva Depigmentation (Tantin *et al.*, 2010)

Gingiva depigmentation has been used for more than 100 patients in several dental clinics in Jakarta. It has produced good results.

Ten patients with excessive gingival pigmentation (Figure 1a) were randomly evaluated from 100 patients. The area of gingiva to be removed was determined. After scraping, half of the gingiva was implanted with sterile AM and a periodontal pack was applied. The other half of the gingiva

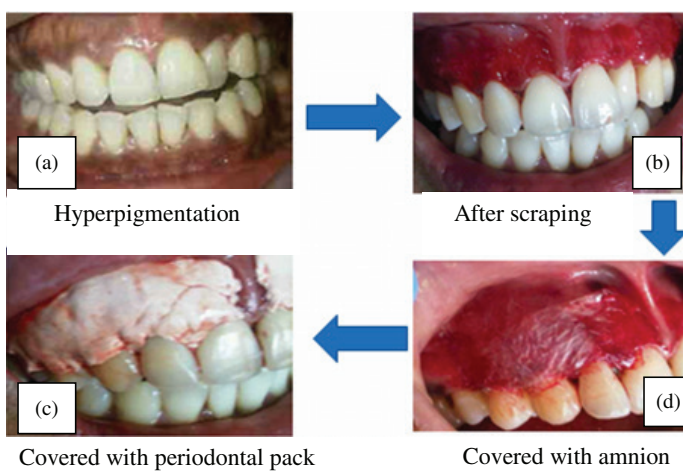


Figure 1. Process of depigmentation

was treated without AM. Only a periodontal pack was applied. The implantation of AM depends on the local condition. The operative procedure is as follows:

- Infiltration anesthesia on the gingiva
- Tissue scraping; using blunt burr and levelling with a blade
- Irrigation with colorless antiseptic and then drying. (Figure 1b)
- Implantation of amniotic membrane on the wound (Figure 1c)
- Application of a periodontal pack (Figure 1d).
- During the treatment, the patients were not allowed to smoke and eat coloured food and beverage. In addition, trauma occlusion and poor oral hygiene should be corrected.

The clinical parameters observed are:

- The time taken for wound healing after implantation (1, 2 and 4 weeks)
- The thickness of the gingiva were measured at 4 and 12 weeks post-implantation. For controls, the thickness of gingival after depigmentation was used (0 week)
- Colour of gingiva before and after implantation
- Recurrence of pigmentation

Depigmentation

Removing gingiva epithelium was done slowly until it reached the pigment without causing significant bleeding. Gauzes soaked in sterile saline solution were used to stop the bleeding. The work was done until all the pigment was removed. In the event of bleeding, it is stopped by pressing the source of the bleeding using a gauze tampon.

Figure 1 shows the process of depigmentation. It can be seen that after gingiva scraping, the wound looks fresh and there is no bleeding at all. In this study, the use of periodontal dressing gave better results.

The surface of the wound without staining can be seen in Figure 1b. The wound was covered with amniotic membrane. A periodontal dressing was applied (Figure 1c and 1d). After 2 weeks the wound healed.

Results

Post depigmentation, the gingiva was pink in color (Figure 2b), compared to before depigmentation (Figure 2a). It gave good results in aesthetic appearance.

The same appearance occurred in other cases shown in Figure 2c and 2e before depigmentation. Post-depigmentation, the color became pink. This was also seen in Figure 2d and 2e.



Figure 2. Appearance of gingiva pre-depigmentation (a), (c), (d) and post-depigmentation (b), (d), (e), (f)

Table 1. Comparison of time taken for wound healing with and without sterile Amniotic Membrane (+ = Healed; – = Not healed)

	With amnion			Without amnion		
Patients no.	1 week	2 weeks	4 weeks	1 week	2 weeks	4 weeks
1	+	+	+	–	+	+
2	+	+	+	–	+	+
3	+	+	+	–	+	+
4	+	+	+	–	+	+
5	+	+	+	–	+	+
6	–	+	+	–	–	+
7	+	+	+	–	+	+
8	+	+	+	–	–	+
9	+	+	+	–	+	+
10	+	+	+	–	+	+

Table 1 shows a comparison of time taken for wound healing between implantation of AM and without AM. The healing of wounds covered with AM occurred one week post-implantation compared to wounds without AM. The latter occured two weeks post-depigmentation. The whole wound healed within one month (Table 1).

Of the 10 cases, two of them are heavy smokers. Maxillary gingiva on the left was treated with AM and on the right without AM. After three months of post-depigmentation, repigmentation occurred in the right gingiva (without AM), while in those with AM, repigmentation did not occur (Figure 3).

Table 2 shows the thickness of the gingiva after 12 weeks of treatment. The maximum gingiva thickness occurred at four weeks. Depigmentation with AM increased the thickness of the gingiva up to 2mm, whereas without AM, the maximum thickness achieved was only 1mm.

The average thickness of gingiva with AM increased from 0.5 mm to 2 mm in 4 weeks. The thickness stopped increasing after 4 weeks (Figure 4). The thickness remained stable up to 48 weeks.

Using AM increased gingival thickness.



Figure 3. Repigmentation after 3 months post-depigmentation (shown by yellow arrow)

Table 2. Thickness of gingival pre- and post-depigmentation

	With amnion			Without amnion		
Patients No.	0 week	4 weeks	12 weeks	0 weeks	4 weeks	12 weeks
1	0,5 mm	2,0 mm	2,0 mm	0,5 mm	1,0 mm	1,0 mm
2	0,5 mm	2,0 mm	2,0 mm	0,5 mm	1,0 mm	1,0 mm
3	0,5 mm	2,0 mm	2,0 mm	0,5 mm	1,0 mm	1,0 mm
4	0,5 mm	2,0 mm	2,0 mm	0,5 mm	1,0 mm	1,0 mm
5	0,5 mm	2,0 mm	2,0 mm	0,5 mm	1,0 mm	1,0 mm
6	< 0,5 mm	2,0 mm	2,0 mm	0,5 mm	0,75 mm	0,75 mm
7	0,5 mm	1,5 mm	1,5 mm	0,5 mm	0,75 mm	0,75 mm
8	< 0,5 mm	1,5 mm	1,5 mm	0,5 mm	0,75 mm	0,75 mm
9	0,5 mm	1,5 mm	1,5 mm	0,5 mm	0,75 mm	0,75 mm
10	0,5 mm	1,5 mm	1,5 mm	0,5 mm	0,75 mm	0,75 mm

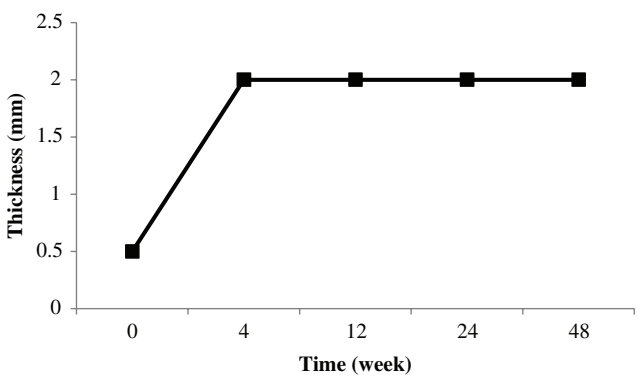


Figure 4. The thickness of the gingiva after using AM



(a) (b) (c)

Figure 5. Recurrence in heavy smokers, before depigmentation (a), 12 weeks post-depigmentation with AM implantation (b) and recurrence at 48 weeks post-depigmentation (with AM)

Recurrence occurred in heavy smokers earlier than in non smokers (Figure 5). After 12 weeks post-depigmentation the gingiva color remained pink (Figure 5b). After 48 weeks recurrence occurred. Hyperpigmentation for both treatments with and without AM can be seen in Figure 5c. In some patients with AM, recurrence did not occur after 2 years post-depigmentation.

Histopathological Examinations

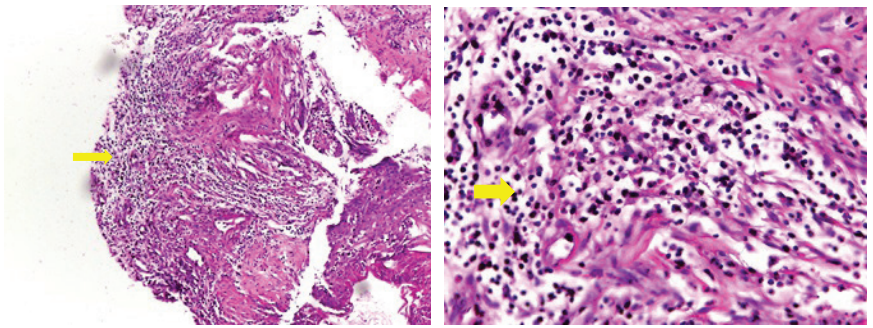
Histopathological evaluation was done by using Haematoxylin and Eosin (HE) staining. The work was carried out at 1 and 4 weeks after AM implantation.

At 1 week, moderate infiltration of polymorphonuclear (PMN) and lymphocyte inflammatory cells was seen in the connective tissue (Figure 6).

After 4 weeks, the comparison of histopathologic evaluation between unimplanted sites and implanted AM site was carried out. The AM grafted wound showed an increase in collagen bundles and fibroblasts in the connective tissue under the regenerated squamous epithelium. Capillary formation was also observed (Figure 7a). Microscopic appearance of ungrafted site showed hyperplasia of squamous epithelium with melanin pigment at the basement membrane. There was a scarcity of capillaries (Figure 7b). No inflammatory cells were found in both sites.

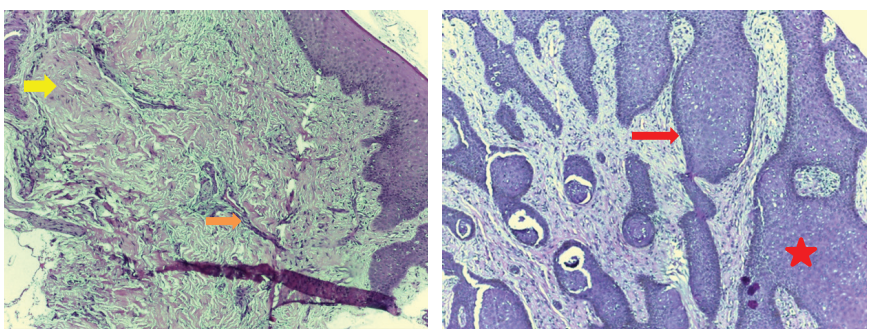
Discussion

Human amniotic membrane has been used successfully as biologic dressing in a variety of clinical situations. It serves specific functions such as



(a) (b)

Figure 6. HE stained histological section of AM treatment at 1 week. a: 40× magnification; b: 400× magnification



(a) (b)

Figure 7. a: Histological section of AM treatment at 4 weeks; b: Histological section without AM at 4 weeks. HE stained, 100× magnification. Collagen bundles (shown by yellow arrow) are more visible in grafted site. Capillary seen (shown by orange arrow). Ungrafted site was covered by thick squamous epithelium (shown by red star) with melanin pigment at basement membrane (shown by red arrow)

promotion of wound healing and the reduction of scarring and inflammation. In addition, the amnion also functions as a scaffold for proliferating and differentiating cells. Although clinical application of amniotic membrane in periodontal surgery had been used in recent years, few studies have been reported (Niknejad *et al.*, 2008; Koizumi *et al.*, 2000).

In this study, sterile, lyophilised amniotic membranes were used in cases of gingiva depigmentation. Results showed improvement of color appearance,

faster wound healing and increase in thickness of gingival after implantation of AM, compared to the ungrafted site.

The AM functions as a basal membrane that facilitates the migration of epithelial cells, strengthens basal epithelial cell adhesion and improves and prevents apoptosis of epithelial cells (Alireza *et al.*, 2007). In addition, the amnion also contains various growth factors such as Platelet Derived Growth Factor (PDGF), which encourages the proliferation of cells in tissue healing, Fibroblast Growth Factors (FGF), which increase cell proliferation and formation of collagen matrix, Transforming Growth Factor Beta (TGF-B) which supports wound healing, and Epidermal Growth Factors (EGF), which promotes proliferation of cell epithelium (Mishra and Singh, 2014).

In the case of re-pigmentation post-depigmentation, we found that so long as the smokers did not quit smoking, re-pigmentation may occur. The development of melanin pigmentation caused by smoking is highly dependent on the dose (Hanioka *et al.*, 1993).

Our results showed that amniotic membrane implantation induces rapid epithelialisation in gingiva.

Conclusion

The implantation of AM can accelerate wound healing, increase the thickness of gingiva and prevent repigmentation. More studies should be done regarding repigmentation.

References

Agarwal A, Shanker S, Singh G, Saxena P and Tahseen A (2014) Pleiotropic properties of amniotic membrane for modulation of periodontal healing. *Int. J. Dent. Med. Res.*, **3**: 110–117.

Alireza BR, Aghayan HR, Arjmand B and Javadi MA (2007) Amniotic Membrane Transplantation. *Iran J. Ophthalmic Res.*, **2**(1): 58–75.

Almas K and Sadig W (2002) Surgical treatment of melanin-pigmented gingiva: an aesthetic approach. *Int. J. Dent. Res.*, **13**(2): 70–73.

Araki S, Murata K, Ushio K and Sakai R (1983) Dose-response relationship between tobacco consumption and melanin pigmentation in the attached gingiva. *Arch Environ Health.*, **138**: 375–378.

Axell T and Hedin CA (1982) Epidemiologic study of excessive oral melanin pigmentation with special reference to the influence of tobacco habits. *Scand. J. Dent. Res.*, **90**: 434–442.

Balcheva G and Balcheva M (2014) Depigmentation of gingiva. *J of IMAB*, **20**: 487–489.

Gupta G (2011) Management of Gingival Hyperpigmentation by Semiconductor Diode Laser. *Journal of Cutaneous and Aesthetic Surgery*, **4**(3): 208–210.

Halaban R, Cheng E, Svedine S, Aron R and Hebert DN (2001) Proper folding and endoplasmic reticulum to Golgi transport of tyrosinase are induced by its substrates, DOPA and tyrosine. *J. Biol. Chem.*, **276**: 11933–11938.

Hanioka T, Tanaka M, Tamagawa H and Shizukuishi S (1993) Epidemiologic study of melanin pigmentation in the attached gingiva in relation to cigarette smoking [in Japanese]. *J. Dent. Health.*, **43**: 40–47.

Hanioka T, Tanaka M, Tamagawa H and Shizukuishi S (1993) Epidemiologic study of melanin pigmentation in the attached gingiva in relation to cigarette smoking. *J. Dent. Health*, **43**: 40.

Hedin CA, Axell T(1991). Oral melanin pigmentation in 467 Thai and Malaysian people with special emphasis on smoker's melanosis. *J Oral Pathol Med.*, **20**: 8–12.

Hedin CA and Larsson A(1984) The ultrastructure of the gingival epithelium in smoker's melanosis. *J. Periodont. Res.*, **19**: 177–190.

Hedin CA, Pindborg JJ and Axell T(1993) Disappearance of smoker's melanosis after reducing smoking. *J. Oral. Pathol. Med.*, **22**: 228–230.

Hilmy N, Febrida A and Basril A(2000) Validation of radiation sterilisation dose of lyophilised amnion and bonegrafts. *J. Cell and Tissue Banking*, **1**(2): 143–148.

Kaushik N, Srivastava N, Mayur K and Gaurav V (2013) Efficacy of different technique of gingival depigmentation: comparative evaluation with a case report. *Int. J. Laser Dent.*, **3**(2): 68–72.

Koizumi NJ, Inatomi TJ and Sotozono CJ (2000) Growth factor mRNA and protein in preserved human amniotic membrane. *Curr. Eye Res.*, **20**: 173–177.

Manjas M, Tarusaraya P and Hilmy N (2010) Clinical application of irradiated amnion grafts. In: Nather A, Yusof N and Hilmy N (eds.), *Allograft Procurement, Processing and Transplantation — A Comprehensive Guide for Tissue Banks*, World Scientific, Singapore, p. 501–504.

Mishra S and Singh S (2014) Human amniotic membrane : can it be a ray of hope in periodontal regeneration?. *PIJR.*, **3**(9): 118–121.

Mohammadi AA, Sabet B, Riazi H, Tavakkolian AR, Mohammadi MK and Iranpak S (2009) Human amniotic membrane dressing: an excellent method for outpatient management of burn wounds. *Iran J. Med. Sci.*, **34**(1): 61–64.

Niknejad H, Peirovi H, Jorjani M, Ahmadiani A, Ghanavi J and Seifalian AM (2008) Properties of amniotic membrane for potential use in tissue engineering. *European Cells and Materials*, **15**: 88–99.

Prasad SSV, Agrawal N and Reddy NR (2010). Gingival depigmentation: a case report. *PJSR.*, **3**(1): 27–29.

Tantin RD, Pandansari P, Abbas B and Hilmy N (2010) Clinical applications of radiation sterilised grafts in periodontal surgery. In: Nather A, Yusof N and Hilmy N (eds.), *Allograft Procurement, Processing and Transplantation — A Comprehensive Guide for Tissue Banks*, World Scientific, Singapore, pp. 517–524.

Unsal E, Paksoy C, Soykan E, Elhan AH and Sahin M (2001) Oral melanin pigmentation related to smoking in a Turkish population. *Community Dent. Oral Epidemiol.*, **29**: 272–277.

20

Amniotic Membrane in Periodontal Regeneration

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Introduction

Peridontium consists of the investing and supporting tissues of the tooth which include gingiva, periodontal ligament, cementum, and alveolar bone (Figure 1a, b).

Periodontal diseases are inflammatory disorders of tissues surrounding the teeth in adult oral cavity, According to periodontal disease classification system as proposed by the American Academy of Periodontology (AAP), periodontal diseases are generally grouped into two major types-gingivitis and periodontitis (Weibe *et al.*, 2000).

Gingivitis (Figure 2) is an inflammatory condition of soft tissues surrounding teeth without the loss of supporting tissues. Periodontitis (Figure 3) refers to inflammatory changes occurring in gingival tissues which ultimately lead to loss of periodontal ligament and the alveolar bone (Socransky *et al.*, 2008).



(a) (b)

Figure 1. (a) Parts of peridontium (b) Healthy peridontium



Figure 2. Gingivitis



Figure 3. Periodontitis

Periodontitis is clinically characterized by-

Periodontal Pocket (Figure 4)

Periodontal pockets (Figures 4, 5) are caused by microorganisms and their products present in plaque.

They produce pathological tissue changes in the soft tissue wall of the gingival sulcus leading to periodontal pocket. Thus, the periodontal pocket wall results from interplay of constructive and destructive changes.

The proliferative degenerative changes lead to the destruction of supporting periodontal structures resulting in tooth mobility.

Tooth mobility and radiographic evidence of bone destruction (Figure 6)

Bone Destruction

Bone destruction is caused by extension of gingival inflammation and is responsible for the reduction in the height of the alveolar bone. Trauma from occlusion causes bone destruction lateral to the root surface resulting in varied patterns of bone destruction.

The periodontal therapy can eliminate gingival inflammation, periodontal pockets, arrest the destruction of soft tissue and alveolar bone, reduce tooth mobility and they restore tissue destroyed by diseases.

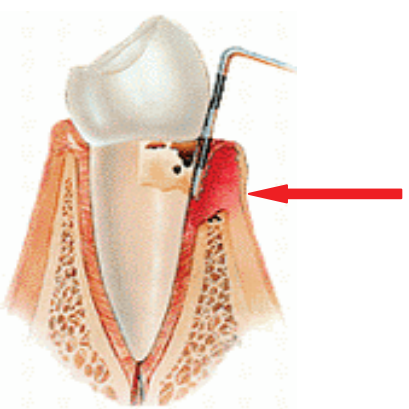
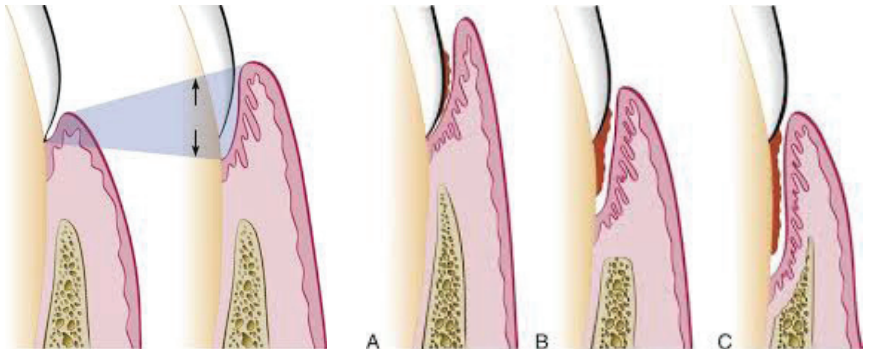


Figure 4. Periodontal pocket



(Carranza's Clinical Periodontlogy)

Figure 5. Diagrams indicating extension of inflammation from normal gingival sulcus to pocket formation

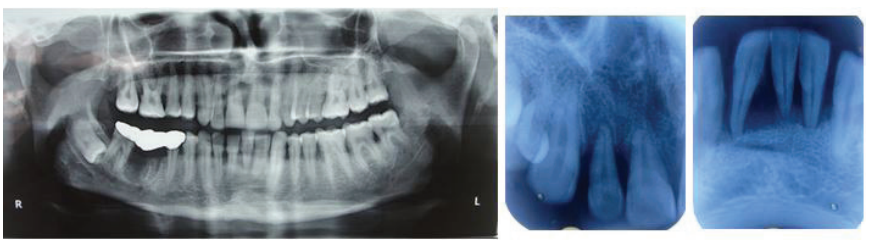


Figure 6. Radiographic evidence of bone destruction in an orthopantomograph and intra oral periapical radiograph

The periodontal therapy will also reestablish physiological the physiological gingival contour essential to preserve periodontal health, prevent reoccurrence of disease and tooth loss.

Periodontal therapy is divided into- non surgical and surgical therapy.

In surgical therapy, the purpose of surgical pocket therapy is to eliminate the pathological changes in the periodontal pocket wall, arrest the proliferative degenerative changes and promote periodontal regeneration.

The periodontal therapy with bone regeneration will result in obliteration of periodontal pockets and reconstruction of periodontium.

Reconstruction of periodontium is divided into-

1. Non graft associated regeneration
2. Graft associated regeneration

Non graft associated regeneration covers the rationale and techniques for removal of diseased periodontal pocket epithelium and prevention of their migration into the healing area.

The non-surgical non graft associated regeneration methods include curettage, chemical agents and ultrasonic methods.

The surgical methods include various flap technique. The flap techniques consists of elimination of the pocket epithelium, which may not be sufficient because the epithelium from the excised margin may rapidly proliferate apically to become interposed between the healing connective tissue and cementum.

To overcome this, Melcher(1976) introduced the concept of Guided Tissue Regeneration.

Guided tissue regeneration (Figure 7) — It is the prevention of epithelial migration along the cemental wall of the periodontal pocket consist of placing barriers of different types to cover the bone and the periodontal ligament. This technique is known as Guided Tissue Regeneration. This technique is based on the assumption that the periodontal ligament cells have potential for regeneration of the attachment apparatus of the tooth. Excluding the epithelium and the gingival connective tissue from the root surface during the post-surgical healing phase favours the repopulation of the area by periodontal ligament and bone cells.

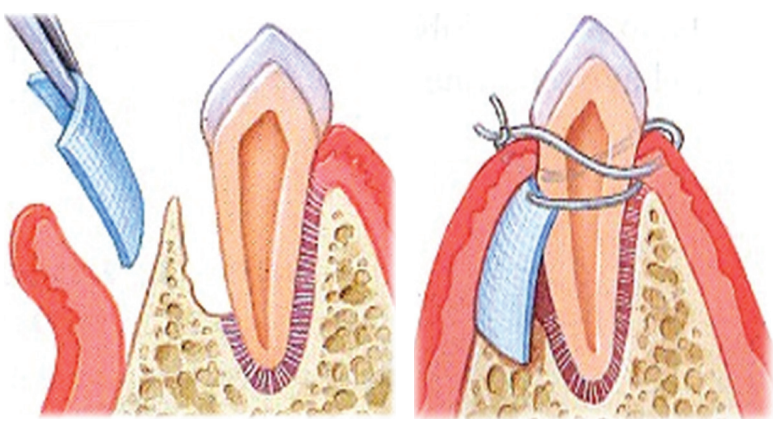


Figure 7. Guided tissue regeneration

Two types of barrier membranes are

1) Non-resorbable barrier membranes
2) Resorbable barrier membranes

Non-resorbable barrier membranes:

Nonresorbable materials are *cellulose* filters Millipore Filter, Millipore, expanded polytetrafluoroethylene (e-PTFE).The disadvantage of this membrane is that they require a second surgical intervention for their elimination at a specified interval of time after healing.

Resorbable Barriers

Resorbable membranes are resorbed during the healing phase and hence, do not require any secondary intervention. The bio-resorbable membranes are also called as second generation membranes. eg: collagen, polylactic acid, collagen membrane Clinical and histological studies have shown guided tissue regeneration results in gain in attachment level, histological studies have shown evidence of periodontal regeneration.

Hence, the clinical and radiological studies were carried out to investigate the effectiveness of amnion and chorion membranes as guided tissue regenerative membrane in periodontal regeneration and also enhance the gingival thickness to improve the periodontal health.

The following study has been undertaken to evaluate effects of amniotic membrane in periodontal regeneration

1) **The evaluation of chorionic membrane in guided tissue regeneration for periodontal pocket therapy: A clinical and radiographic study**

Introduction

Periodontitis is a globally prevalent inflammatory disease characterized by periodontal tissue destruction. The main purpose of periodontal treatment is to eliminate inflammatory processes in order to arrest the progression of the disease and keep the dentition in a state of the health (Cortellini *et al.*, 1993). Thus the ultimate goal of periodontal therapy is to prevent further

attachment loss and predictably restore the periodontal supporting structures that were lost because of disease or trauma in a way that the architecture and function of the lost structures can be re-established. Successful periodontal regeneration relies on the reformation of an epithelial seal, deposition of new acellular extrinsic fiber cementum and insertion of functionally oriented connective tissue fibers into the root surface, and restoration of alveolar bone height (Caton *et al.*, 1999)

The concept that the cells that repopulate the exposed root surface after periodontal surgery define the nature of the attachment that will form was extensively investigated (Melcher 1976, Gotlow *et al.*, 1982). Conventional periodontal therapies (including scaling, root planing and periodontal surgery) can arrest the disease process. However, healing occurs by a long junctional epithelium forming an attachment to the root surface, a process known as periodontal repair. The "repair" means the restoration of tissue continuity without its original architecture or function. This long epithelial attachment is less desirable because it eventually may lead to epithelial splitting and formation of a deep crevice (Ishikawa *et al.*, 2009). Gingival connective tissue cells can also populate the space adjacent to the denuded root surface after conventional periodontal treatment. Repopulation of the exposed root surface by gingival connective tissue cells is speculated to result in the formation of a connective tissue attachment followed by root resorption. Based on this speculation, Nyman *et al.*, (1982), suggested the placement of a physical barrier between the flap and the root surface to exclude gingival connective tissue and epithelium from the healing process, giving the periodontal ligament cells the opportunity to repopulate the coagulum on the root surface. This technique was named guided tissue regeneration (GTR) favouring the formation of a new periodontal attachment. The barrier membranes recommended for use in GTR, regardless of the material used, must be safe, biocompatible, non-toxic, not induce any inflammatory response, and be designed for clinical applicability based on the morphology of the osseous defects (Gotlow 1984). The barrier function must be maintained for a period sufficient to guide the tissue regeneration, preferably without creating gingival recession (Scantlebury 1992). One of the oldest biomaterials used for scaffolds is the foetal membrane. The foetal membrane was first used for the transplantation of skin in 1910. It is useful in the management of burns; creation of surgical dressings; as well as reconstruction of

oral cavity, bladder and vagina; tympanoplasty; and arthroplasty (Kothiwale *et al.*, 2009). It has gained importance because of its ability to reduce scarring and inflammation; enhance wound healing; and serve as a scaffold for cell proliferation and differentiation as a result of its antimicrobial properties. In addition, the chorionic membrane (CM) a foetal membrane is a biomaterial that can be easily obtained, processed and transported. The study was carried out with the concept of the GTR in periodontal pocket therapy. This was the foremost innovative study to use CM as a barrier membrane for the periodontal pocket therapy. Hence, the aim of the present clinical and radiographic study is to evaluate the effect of GTR using CM in the periodontal pocket therapy

Materials and Methods

Ten patients, 6 males and 4 females (25–55 years old; mean age 38.2 years) with moderate to severe periodontitis were selected in the single blind randomized controlled clinical trial. It was a split-mouth design study. The ethical clearance was obtained from the institutional ethical committee. The informed consent was taken from patients after explaining the procedure in their own language. No patients had any contraindications to periodontal surgery. The inclusion criteria were: (1) patients in good systemic health; (2) no use of drugs such as antibiotics, corticoids, chemotherapeutics or immunological modulators that might alter the expected response of the oral tissues; (3) no use of protective or orthodontic apparatus; (4) no smoking; (5) at least 2 mm of keratinized tissue; (6) probing depth C5 mm bilaterally; (7) radiographic evidence of bone loss; (8) compliance with maintenance programme.

Clinical Data

Following selection of patiets, clinical data included gingival index (GI), plaque index (PI), pocket probing depth (PPD) and relative attachment level (RAL) were collected at the baseline (before surgery) and 12 months post surgery. The PI and GI were recorded according the criteria described by Loe and Silness (1963) and Sillness and Loe (1964) respectively. The RAL and PPD were assessed using a pressure sensitive probe from the gingival margin

to the base of the pocket. Radiovisiograph (RVG) was taken at the baseline (before surgery), 6 and 12 months post surgery using paralleling technique. Initial therapy was performed, consisting of scaling and root planning.was performed in all the patients to eliminate local factors. The prematurities, caries and overhangs were treated followed by oral hygiene instructions. After 4–6 weeks following initial therapy, patient were reevaluated for periodontal pocket, RAL and oral hygiene maintenance following which periodontal pocket therapy was performed. Quadrants were divided into study and control sites in a split-mouth design. The study sites were treated by conventional therapy and a bioabsorbable membrane (CM) and the control sites were treated by conventional periodontal therapy alone. The surgeries were performed by the same operator and the measurements by a blinded examiner. The CM 3 × 3 cm (Figure 8) was obtained from, Tata Memorial Hospital Tissue Bank, Mumbai, India. The chorion membrane was dispatched for the clinical from the tissue bank. The processing procedures CM were used are based on the International Atomic Energy Agency (IAEA) recommendations and the Asia Pacific Association of Surgical Tissue Banks (APASTB) standards . (The placenta was procured under full informed consent from mothers who donate their placenta during delivery. The donors were screened for any history or presence of infection and transmissible diseases. Donor's blood was tested for HIV, hepatitis B, hepatitis C and VDRL. The chorion was cleaned of blood, pasteurized at 60 C in saline, treated with



Figure 8. Chorionic membrane

70 % alcohol, washed, lyophilized, packed and sterilized by gamma radiation at a dose of 25 kGy.)

Periodontal Pocket Therapy

The periodontal pocket therapy was performed with the administration of local anesthesia. Following the incisions a mucoperiosteal flap was reflected The complete debridement of inflamed granulation tissue was perfomed with scaling and root planing using curettes. Irrigation was done with normal saline. One quadrant in a patient was randomly selected to be treated with a CM for the study site (Figure 9). The CM was cut based on proper dimensions of surgical site and was then adapted over the alveolar bone extending from the base of the reflected flap to the alveolar crest (AC), flap was repositioned and sutured by interrupted suture technique. The periodontal

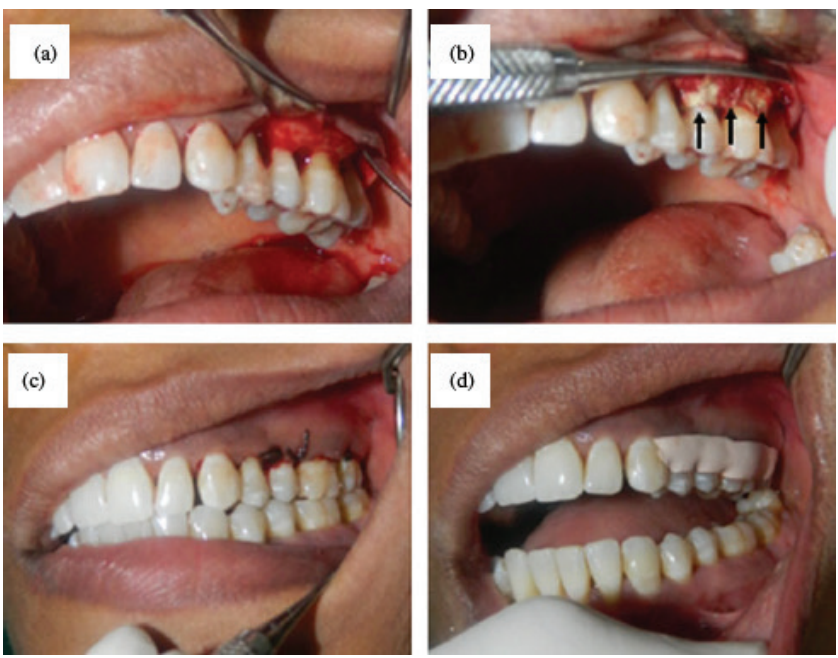


Figure 9. Periodontal pocket therapy (a) Flap reflection (b) Chorionic membrane placed (c) placement of Sutures (d) periodontal dressing placed

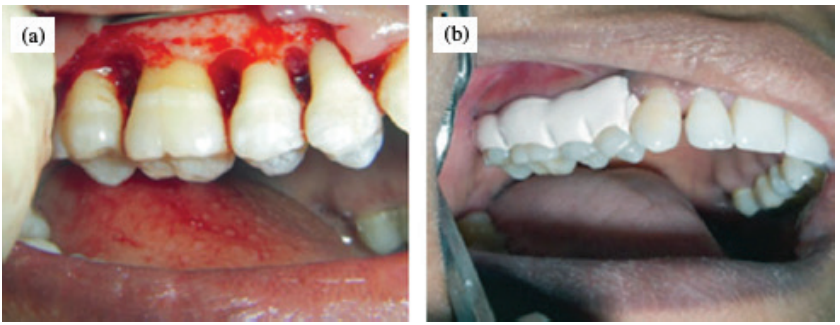


Figure 10. Periodontal pocket therapy of control site (a) Flap reflection (b) pack placed

dressing was placed. In the control site all surgical procedures were identical except no membrane placement (Figure 10). Patients received post-operative instructions and were prescribed antibiotic and analgesics. Patients were instructed to rinse twice daily with 0.2 % chlorhexidine gluconate for 6 weeks. The periodontal dressing and sutures were removed 7 days post-surgery. The patients were recalled at 6 and 12 months postoperatively to evaluate oral hygiene status. At 12 months all the parameters were reassessed.

Radiographic Assessment

The RVG, a digital radiography system, was used to obtain radiovisuographic images of each site before surgical procedure and at 12 months postoperatively using the parallel cone technique. For this purpose, RVG 5000 superb CMOS sensor was placed inside disposable polythene sleeve and positioned in the mouth. The radiovisiographic images stored in JPEG format were transferred to RVG software system which had in built densitometric analysis incorporated in greyscale units. The cementoenamel junction (CEJ), the base of the defect (BD) and the AC were located on the image. Using Kodak Software markings were made at CEJ to BD and from CEJ to AC respectively. The distance between the two points were displayed on screen. The depth of the osseous defect was obtained by subtracting the two measurements for pre and postsurgical radiographs. All clinical and radiographic measurements were performed by a single examiner who was blinded to the procedure (Figures 11, 12).

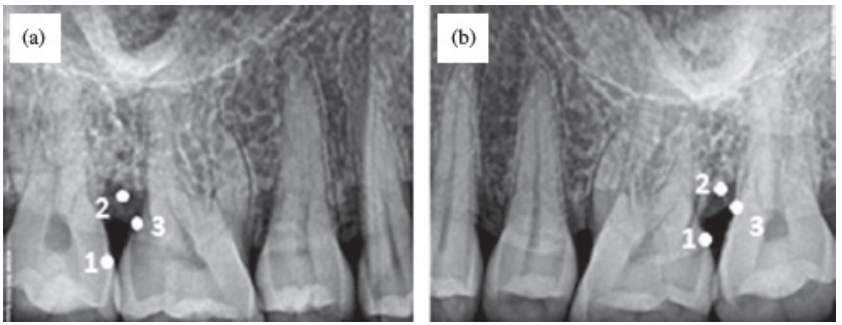


Figure 11. Presurgical linear measurement done using RVG software (a) control site, (b) study site. (1) cementoenamel junction (CEJ) (2) the base of the defect (BD), (3) alveolar crest (AC) were located on the image

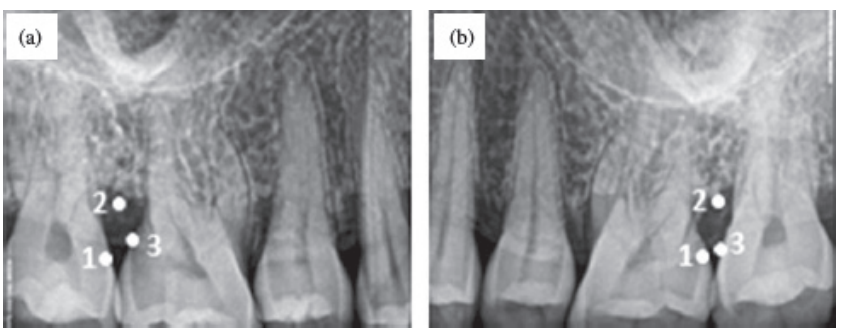


Figure 12. Postsurgical linear measurement done using RVG software (a) Control site, (b) Study site (1) cementoenaamel junction (CEJ), (2) the base of the defect (BD), (3) alveolar crest (AC) were located on the image

Statistical Analysis

The Student t test was used to analyze all parameters between study and control sites to determine the significance of changes over time. 95% of confidence interval (CI) was calculated. The significant level was fixed at 0.05 level. SPSS version 12 (trial version) was used to analyse the data.

Results

Table 1 show that the cases which had similar clinical readings were selected for the study to reduce bias in clinical outcomes. At the baseline both the

Table 1. Comparison between control and study sites at baseline

	Control		Study			
	Mean	SD	Mean	SD	*p* value	Inference
GI	2.43	0.33	2.32	0.43	0.5286	NS
PI	2.30	0.24	2.22	0.28	0.5035	NS
PPD	7.60	0.84	7.60	1.17	1.0000	NS
RAL	9.90	0.74	9.30	0.67	0.0739	NS

NS not significant.

Table 2. Comparison in study sites at baseline and 12 months

	Base		12th month			
	Mean	SD	Mean	SD	*p* value	Inference
GI	2.32	0.43	0.40	0.08	<0.0001	HS
PI	2.22	0.28	0.41	0.18	<0.0001	HS
PPD	7.60	1.17	2.50	0.53	<0.0001	HS
RAL	9.30	0.67	4.30	1.06	<0.0001	HS

HS highly significant.

groups exhibited no statistical difference in the mean ± SD of GI, PI, PPD and RAL suggesting the baseline values were similar. As shown in Table 2, in the study sites the mean ± SD at the baseline of the GI, PI, PPD and RAL reduced at 12 months with $p < 0.0001$. This reduction in clinical parameters was due to oral prophylaxis and periodontal pocket therapy. The mean ± SD values of above parameters shows that CM when used with periodontal pocket therapy can have better influence on clinical parameters. As shown in Table 3, the mean ± SD values of GI, PI, PPD and RAL at baseline and 12 months with a p value ($p < 0.0001$) indicates highly significant reduction in all clinical parameters. This reduction in scores was due to oral prophylaxis and periodontal pocket therapy in the control sites. Table 4 revealed, at 12 months the study sites showed the GI, PI, PPD were significantly reduced as compared to the control sites. The mean of the bone gain (BG) in the study sites 1.22 ± 0.35 mm (p\0.0001) was highly significant suggesting. Thus indicating good clinical outcome with CM used in periodontal pocket therapy. Table 5 shows that at 6 months in control and study sites the mean

Table 3. Comparison in control sites at baseline and 12 months

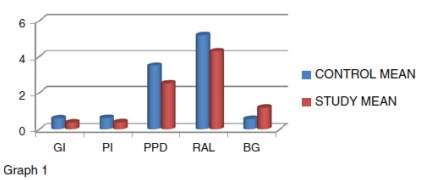
	Base		12th month			
	Mean	SD	Mean	SD	*p* value	Inference
Gl	2.43	0.33	0.61	0.10	<0.0001	HS
PI	2.30	0.24	0.63	0.19	<0.0001	HS
PPD	7.60	0.84	3.50	1.35	<0.0001	HS
RAL	9.90	0.74	5.20	1.40	<0.0001	HS

HS highly significant.

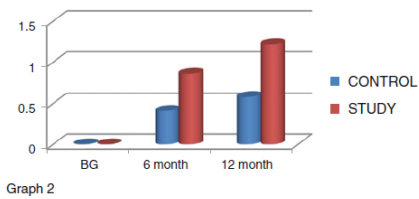
Table 4. Comparison between control and study sites at 12 months

	Control		Study			
	Mean	SD	Mean	SD	*p* value	Inference
Gl	0.61	0.10	0.40	0.08	0.0001	HS
PI	0.63	0.19	0.41	0.18	0.0155	S
PPD	3.50	1.35	2.50	0.53	0.0431	S
RAL	5.20	1.40	4.30	1.06	0.1221	NS
BG	0.58	0.23	1.22	0.35	<0.0001	HS

**HS* highly significant, ***S* significant, ****NS* not significant.



Graph 1



Graph 2

Table 5. Comparison BG in control and study site at 6 and 12 months

	Control		Study			
BG	Mean	SD	Mean	SD	*p* value	Inference
6 months	0.41	0.12	0.86	0.18	<0.0001	HS
12 months	0.58	0.23	1.22	0.35	0.0001	HS

BG was 0.41 ± 0.12 mm, 0.86 ± 0.18 mm respectively with $p < 0.0001$ indicating significant inference. At 12 months the mean BG of control sites 0.58 ± 0.23 mm, study sites 1.22 ± 0.35 mm ($p = 0.0001$) which was highly significant suggesting that the mean BG was increased in study sites as compared to control sites. Pre-surgical linear measurement were done using RVG software (a) control site, (b) study site. (1) cementoenamel junction (CEJ) (2) the base of the defect (BD), (3) alveolar crest (AC) were located on the image. Postsurgical linear measurement were done using RVG software (a) Control site, (b) Study site (1) cementoenamel junction (CEJ), (2) the base of the defect (BD), (3) alveolar crest (AC) were located on the image

Discussion

One of the goals in periodontal therapy is to restore periodontal tissues lost through periodontal disease. Regeneration is defined as the process by which architecture and function are completely renewed (American Academy of Periodontology 1992). By this definition, periodontal regeneration would include the formation of a new connective tissue attachment including new cementum and supporting bone. The regeneration of the periodontium is the result of electivecellular events that are facilitated by tissue exclusion using bioabsorbable or non-resorbable barriers. The results of our study showed that GTR using a bioabsorbable CM was more effective than conventional therapy for the periodontal pocket therapy particularly in regard to the amount of bone regeneration. The baseline clinical parameters on the sites treated with the CM were similar to conventional therapy (Table 1). The baseline mean values of both study and the control site when compared at 12 months, all the parameters showed significant improvement $p < 0.0001$ (Tables 2, 3). At the baseline GI, PI, probing pocket depth and RAL were similar suggesting that there is no difference between both the sites (Table 1). The GI (0.40 ± 0.08), PI (0.41 ± 0.18), PPD (2.5 ± 0.53), RAL (4.30 ± 1.06) showed significant improvement in both control and study site from baseline to 12 months (Table 4). The reduction in the above parameters can be attributed to elimination of local factors and eradication of periodontal pockets with periodontal pocket therapy. The advantage of GTR procedures using bioabsorbable CMs is that this technique does not require a second

surgical procedure, thus improving the clinical results without disrupting bone fill or attachment gain. PI and GI scores decreased from baseline to 12 months, indicating the maintenance of a good oral hygiene by the patient. In both sites, PPD reduction was found to be statistically significant after 12 months. The study sites showed a higher PPD reduction (2.5 ± 0.53 mm) as compared to the control sites (3.5 ± 1.35 mm). This may be credited for the presence of tissue inhibitor of metalloproteinases (TIMPs) in CM which suppresses matrix metalloproteinases (MMPs) and transforming growth factor beta (TGF-b) which stimulates the production of TIMPs from the surrounding tissue (Hao *et al*., 2000, Riau *et al*., 2010) Collectively, these proteins suppress inflammation and collagenous degradation, also the presence of intense concentrations of laminin and laminin-5 throughout the barrier is of particular importance due to its high affinity for binding gingival epithelial cells may contribute for better adaptation to the root surface (Pakkala *et al*., 2002). As epithelial cells quickly migrate across the CM barrier, they form a seal over the underlying bone graft and do not apically migrate into the defect. Histologic studies by Wallace and Cobb (2011), demonstrated this concept by the fact that when amnion CM was used as a barrier in site preservation, trephined bone core samples from healed surgical sites demonstrated new bone, residual bone graft, and connective tissue percentages comparable to those found in prior studies utilizing traditional GTR barriers. Additionally, this study found no events of adverse healing or epithelial invasion of the grafted sites. The radiovisiographic assessment in the control sites showed that the mean BG at 6 months was 0.41 ± 0.12 and at 12 months the mean of the BG was 0.58 ± 0.23 ($p = 0.028$) with a significant inference of the BG from baseline to 12 months. In study sites, at 6 months the mean of the BG was 0.86 ± 0.18 and at 12 months the mean of the BG was 1.22 ± 0.35 ($p = 0.0382$) with a significant inference of the BG from baseline to 12 months showing a highly significant BG in study sites in comparison with the control. At the study sites the bone density was increased as compared to the controls. The increase in bone density reflects a number of biomechanical properties, such as strength and modulus of elasticity of the bone in the study sites (Misch 20011). These findings demonstrated better results of the study sites compared to control sites (Table 5). This could be relevant for predicting the effectiveness of GTR. The intricacy of the periodontal attachment, as seen by the presence of a

variety of specialized cell types — the multifarious stromal-cellular interactions and the attachment complexes — is a major hindrance in attaining periodontal regeneration. Coupled with the avascular tooth-root surfaces and diverse oral microbial flora, the process of periodontal regeneration is further complicated. In order to overcome this inherent challenge, four fundamental components, namely cells, space creation, wound stability and blood supply, are required. The intimate interplay of these four basic elements in the regeneration of the periodontal attachment is facilitated by the use of GTR. The CM acted as a barrier membrane between the gingival epithelium and hard tissue to promote the periodontal ligament cells to form progenitor cells that can regenerate new tissues. Often the graft site must bear loads at or close to physiologic levels very soon after transplantation. CM when used as internal fixation provided early stability. CM is available in different length and width, sufficient to adapt at the site on both buccal and lingual aspect of the quadrant in a single strip with trimming at interdental sites. The barrier is placed dry and quickly hydrates with blood, becomes very pliable, and closely adapts to the contours of the underlying surface. It's thin, self-adherent nature do not compromise blood flow. CM will not easily displace from underneath the over laying flap and does not need to be fixed into place using sutures or tacks. Collectively, CM's unique biologic and physical attributes reduce the complexity of trimming, suturing and placement of barriers, minimizing the chances of post-operative complications (Holtzclaw *et al.*, 2012). Overall, the findings of this study suggested that placement of CM resulted in significant improvement in radiographic and clinical parameters when compared to periodontal pocket therapy alone. However, further long-term studies can evaluate the regenerative capabilities of this material by histological methods or surgical re-entry. These studies should determine the efficacy of CM as well as investigate to what extent CM may exert an influence on the healing dynamics following periodontal re-constructive procedures.

Conclusion

Conventional therapy is capable of controlling periodontal disease. Scaling and root planning allow reduction in probing depth with gain in clinical attachment level. The regeneration technique can promote bone fill, thus

improving the supporting structures. When GTR is applied to periodontal pocket therapy with high potential for regeneration these clinical objectives may be reached. From a clinical point of view, GTR using a bioabsorbable CM in periodontal pocket therapy demonstrated greater bone gain and good tissue integrity in clinical and radiographic evaluations than periodontal pocket therapy alone.

2) Enhancing gingival biotype through chorion membrane: innovative step in periodontal pocket therapy.

Introduction

The term periodontal *biotype* is used by Seibert and Lindhe, who classified the gingival as either thin or thick, where thin corresponds to scalloped type and thick to the flat (Seibert *et al.* 1989). A thick gingival biotype (Figure 13) is a requisite for good periodontal health. It has important role in resisting trauma and subsequent gingival recession, enables tissue manipulation and promotes creeping attachment (Barrington 1981, Harris 1997). The reason may be the presence of high volume of extracellular matrix and collagen, more layers of keratinisation which withstands collapse, contraction and inflammation of gingival tissue.

The thick gingival biotype is a significant predictor of clinical outcome of periodontal surgeries. The studies have shown better surgical outcome after periodontal therapy with thick gingival biotype. The explanation may be, it has increased vascularity, greater perfusion with enhanced oxygenation, clearance of toxic products, immune response and growth factor migration

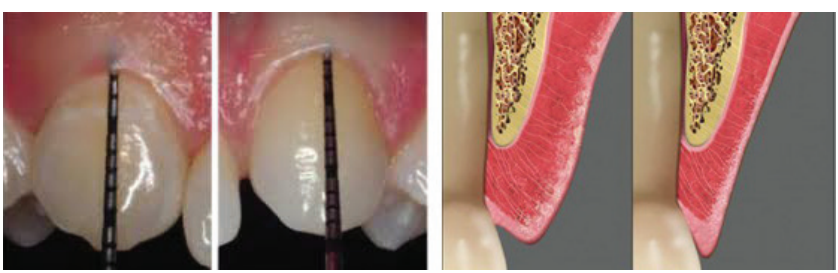


Figure 13. Thick and thin gingival biotypes

(Hwang *et al.* 2006). Hence, thick gingival biotype is essential for enhanced surgical outcomes.

There is a considerable intra-individual and inter-individual variation in both width and thickness of facial gingiva. The genetic and racial factors also influence gingival thickness. The facial gingiva is thicker in the maxilla than in the mandible. Gingiva is thinner in females than males.[20]

According to Weisgold, individuals with a thin, scalloped gingiva demonstrated a greater prevalence of recession (Weisgold 1997). Thin gingival biotype can affect the results of periodontal therapy, root coverage procedures, and implant placement (Hwang *et al.*, 2006, Hwang *et al.*, 2005, Clafeey *et al.*, 1983). It has been reported that patients with thin gingival biotype were more likely to experience gingival recession following nonsurgical periodontal therapy (Clafeey *et al.*, 1983). Mucogingival problems may result from orthodontic movement of teeth away from the alveolar process, particularly among patients with thin gingiva (Foushee *et al.*, 1985, Zachrisson *et al.*, 1997). Patients with thick gingiva appears less likely to experience gingival recession after surgical or restorative therapy (Anderegg *et al.*, 1995).

Various periodontal surgical techniques can enhance the gingival thickness and can result in constructive treatment outcome. Various surgical procedures used to increase the gingival thickness include the use of sub-epithelial connective tissue grafts, acellular dermal matrix and guided tissue regeneration (GTR).

In 2014, a study was performed with innovative step of placement of chorion membrane during the flap surgery for periodontal pocket therapy. The purpose of pocket therapy was to reduce or eliminate periodontal pocket and prevent the disease progression at disease site. The postoperative findings of the study reported, periodontal pocket reduction and an increase in degree of gingival thickness on visual examination. The study concluded the dual benefits of periodontal pocket reduction along with increase in thickness of gingival biotype (iwale 2014). Hence the present study incorporated similar innovative step of placement of chorion membrane to objectively evaluate the increase in thickness of gingival biotype during periodontal pocket therapy.

There are several methods, used to evaluate the gingival thickness. These methods include conventional histology on cadaver jaws, injection needles, transgingival probing, histologic sections, cephalometric radiographs, probe transparency, ultrasonic devices, and CBCT (Rezaei *et al.*, 2013).

The study was designed to objectively evaluate and compare the gingival thickness at surgical sites with and without placement of chorion membrane during periodontal pocket therapy in the mandibular anterior region using injection needle and digital Vernier caliper

Materials and Methods

Patient Selection

A total of five patients reporting to the Department of Periodontics, K.L.E's V. K Institute of Dental Science, between age group between 25 and 45 years with chronic periodontitis, (classification of American academy of periodontology, 1999) indicated for flap surgery were selected for the study. The inclusion criteria for the study were: (1) systemically healthy patients, (2) no use of drugs such as antibiotics, corticoids, chemotherapeutics, or immunological modulators that might alter the expected response of the oral tissues, (3) no use of protective or orthodontic apparatus, (4) non smoker, (5) minimum 2 mm of width of keratinized attached gingiva and (6) probing depth ≥5 mm. The ethical clearance was obtained from the institutional ethical committee. The informed consent was taken from patients after explaining the procedure in their own language.

Site Selection

A split-mouth randomized clinical trial was designed. Following selection of patient, the sites with pocket depth of 6–8 mm in the mandibular anterior teeth were allocated for the flap surgery. In a mandibular anterior region, two teeth were assigned as control site and two teeth as test sites using a flip coin method. The surgical procedure was performed by the single examiner. The chorion membranes (3 × 3 cm) were obtained from Tissue Bank of Tata Memorial Hospital, Mumbai, India.

AQ2

Pre-surgical Procedure

The initial therapy consisting of scaling and root planing was performed in patients who participated in the study. After 4–6 weeks following initial

therapy, patients were reevaluated for oral hygiene maintenance and probing pocket depth. The periodontal probing pocket depth in mandibular anterior region was measured using Williams graduated probe (Figure 14).

The gingival thickness was evaluated using injection needle and digital caliper.The markings of 0.1 mm were marked, at regular intervals, on injection needle of gauge 0.45 × 38 mm. Pre measurements of gingival thickness were taken with the help of these markings of the needle and rubber stopper (Figure 15) after anesthetizing the surgical site with 0.2 % lignocaine. The mid buccal measurements were assessed on the attached ginigva, half way between the mucoginigval junction and free gingival groove and mid papillary measurement were taken at the base of interdental papillae.

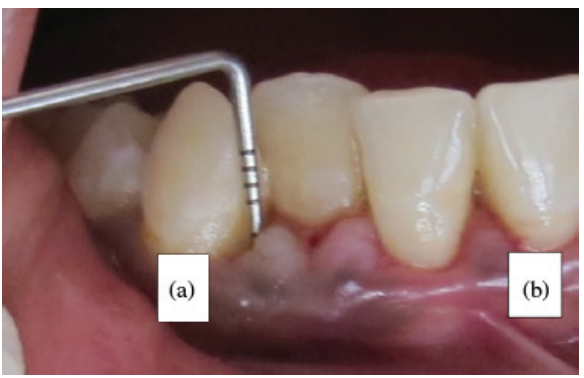


Figure 14. Preoperative radiograph a) control b) test

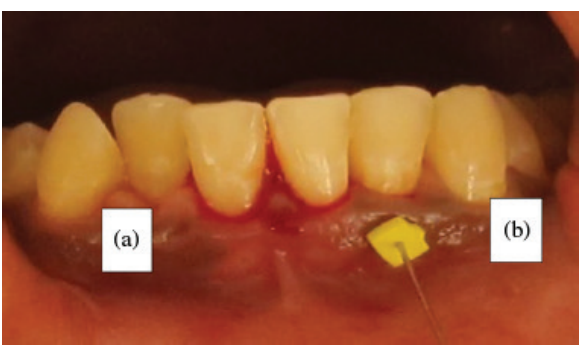


Figure 15. Measurement of gingival thickness.: a Control site and b test site

Thus the gingival thickness measurement was assessed, using a markings marked on injection needle and these markings were read using digital verniercaliper (Figure 16).

Surgical Procedure

After anaesthetizing the area, a full thickness flap was reflected in the selected area (Figure 17), with complete debridement of inflamed granulation tissue. A tin foil was trimmed according to the dimensions of buccal and interproximal areas till the base of reflected mucoperiosteal flap at the test site.

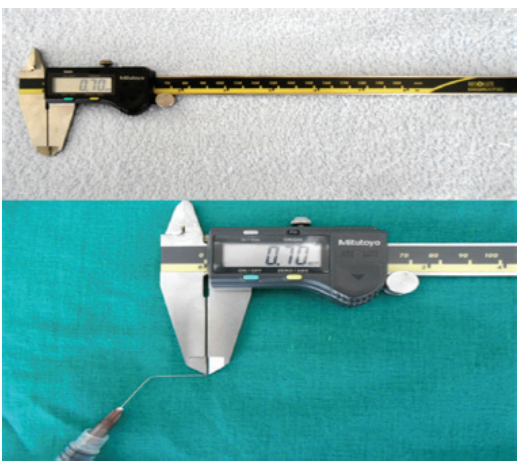


Figure 16. Measurement of gingival thickness. a Control site and b test site



Figure 17. Reflection of full thickness flap. a Control site and b test site

The chorion membrane was cut according to the dimensions of trimmed tin foil and adapted over the alveolar bone (Figures 18, 19). The flap was repositioned and sutured (Figure 20).

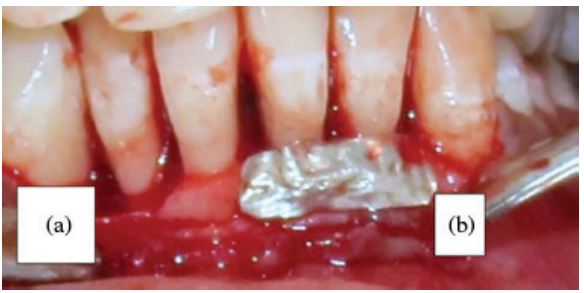


Figure 18. Placement of tin foil. a Control site and b test site



Figure 19. Placement of chorion membrane. a Control site and b test site



Figure 20. Flap re-positioned and sutured. a Control site and b test site

Post Operative Regime

Patients received post operative instructions and were prescribed antibiotic Amoxicillin 500 mg three times a day and analgesic consisting of combination of Ibuprofen (325 mg) and Paracetamol (400 mg) were prescribed for 5 days. Patients were instructed to rinse twice daily with 0.2% chlorhexidine gluconate for 6 weeks. The sutures were removed 7 days post surgery. The surgical sites were supervised for the oral hygiene maintenance at the intervals of 15 days for 6 weeks.

The post operative measurements were assessed at 6 weeks in the mid buccal, mid-papillary (Figure 21).

Statistical Analysis

Quantitative data was summarized as mean standard deviation. The intragroup comparison between baseline and 6 weeks values of both the group was done using Wilcoxson's sign rank test. Differences between the gingival thickness at test and control site was analyzed using Mann–Whitney *U* test.

AQ3

Results

The baseline values of gingival thickness at test site (1.04 ± 0.19) at mid buccal region, (1.24 ± 0.20) at mid papillary and control site (0.94 ± 0.11) at

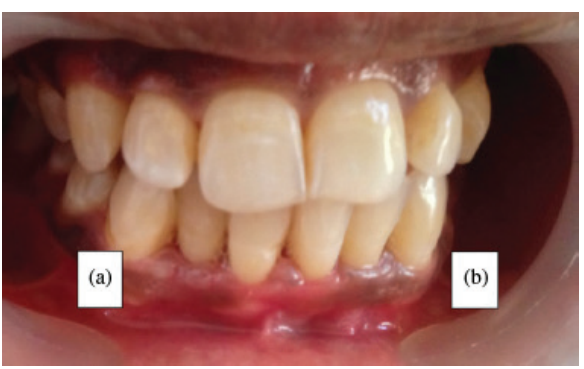


Figure 21. Post-operative view at follow up of 6 weeks after placement of chorion membrane. a Control site and b test site

mid buccal region, (1.14 ± 0.11) at mid papillary region showed no statistically significant difference. At control sites there was no statistically significant increase in gingival thickness at 6 weeks post treatment (1.06 ± 0.11 at mid buccal region, 1.24 ± 0.11 at mid papillary) as compared to test sites (1.36 ± 0.16 at mid buccal region and 1.48 ± 0.17 at mid papillary region), which showed statistically significant increase in gingival thickness ($p \leq 0.05$*). The 6 weeks post treatment values between test sites and control sites showed statistically significant difference ($p \leq 0.05$*) (Tables 6, 7).

Discussion

In periodontal flap surgeries, post operatively, along with the pocket reduction presence of minimal gingival recession is common due to the shrinkage of the gingival tissues (Barrington *et al.*, 1981). Gingival recession along with reduced gingival thickness is more prone for the recurrence of periodontal pocket as they are vulnerable for inflammation(Kassab *et al.*, 2003, Tugnait *et al.*, 2001). The use of chorion membrane in flap surgery was innovative step with added benefits of increase in the gingival thickness with average

Table 6. Mean gingival thickness at mid buccal region at control and test sites

Group	Baseline (mean ± SD)	Six weeks (mean ± SD)	Wilcoxon	*p* value
Control site	0.94 ± 0.11	1.06 ± 0.11	−1.190	0.234
Test site	1.04 ± 0.19	1.36 ± 0.16	−2.1	<0.05*
Mann–Whitney *U*	8.5	1		
p value	0.39	<0.05*		

Table 7. Mean gingival thickness at mid papillary region at control and test sites

Group	Baseline (mean ± SD)	Six weeks (mean ± SD)	Wilcoxon	*p* value
Control site	1.16 ± 0.11	1.24 ± 0.11	−2	0.56
Test site	1.24 ± 0.20	1.48 ± 0.17	−2.078	<0.05*
Mann–Whitney *U*	10	3		
p value	0.59	<0.05*		

reduction of 3–4 mm of pocket depth. The application of this technique especially in the mandibular region where gingival biotype is thin can increase the gingival thickness there by making the tissue more resistant to gingival recession.

The result of present study showed, the mean gingival thickness (1.06 ± 0.11 at mid buccal region, 1.24 ± 0.11 at mid papillary) in control site indicated no significant change in gingival thickness. There was increase (1.36 ± 0.16 at mid buccal region and 1.48 ± 0.17 at mid papillary region) in gingival thickness at test site significantly, which could be associated with chorion membrane.

The increase in gingival thickness using chorion membrane may be due to the presence of large number of pro-angiogenic growth factors, including angiogenein, angiopoietin-2, epidermal growth factor (EGF), fibroblast growth factor (bFGF), hepatocyte growth factor (HB-ECF, HGF), platlet derived growth factor (PDGF-BB), placental growth factor (PIGF) and vascular derived growth factor (VEGF). These growth factors promote endothelial recruitment and better vascularisation, promotes the potential blood supply to the flap connective tissue (Koob *et al.*, 2003).

Histologically, the reticular layer of chorion membrane contains collagen type 1, 2, 3, 4, 5, 6, 7 along with proteoglycans. In basement membrane collagen type 4, fibronectins and laminins are present. Immunohistochemical staining analysis of Chorion membranes showed intense concentrations of laminin and laminin-5 throughout the barrier (Bryant-Greenwood 1998). Laminin-5 is of particular importance due to its high affinity for binding gingival epithelial cells for better adaptation to root surface (Mullen *et al.*, 1999) (Figure 22).

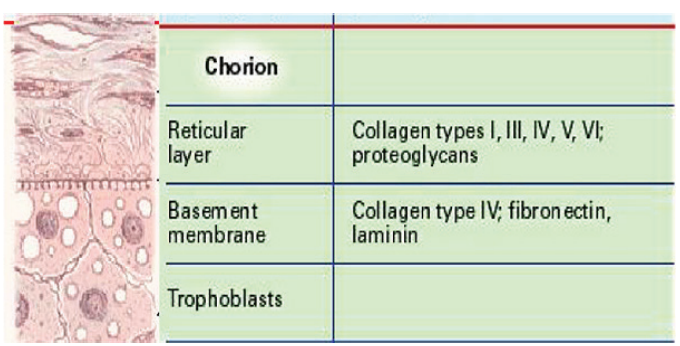


Figure 22. Layers of chorionic membrane

The chorion tissue contains tissue inhibitor of metalloproteinases (TIMPs) which suppress MMPs and transforming growth factor beta (TGF-β) which stimulates the production of TIMPs from the surrounding tissue. Hence, these proteins suppress inflammation and collagenous degradation (Koob *et al*., 2013)

The above properties of chronic membrane: anti-inflammatory, adhesion, vascularization, presence of collagen types with proteoglycans, adhesion with the help laminin might be responsible in increasing the thickness of gingiva thus making it as suitable for periodontal therapy.

Conclusion

Incorporation of chorion membrane as an innovative step during flap surgery results in reduction of periodontal pocket depth, increases gingiva thickness, and prenents post operative recession. Hence, adds to the prognostic value of periodontal therapy.

References

Anderegg CR, Metzler DG and Nicoll BK (1995) Gingiva thickness in guided tissue regeneration and associated recession at facial furcation defects. *J. Periodontol.*, **66**: 397–402.

Bryant-Greenwood GD (1998) The extracellular matrix of the human fetal membranes: structure and n. *Placenta.*, **19**: 1–11.

Barrington EP (1981) An overview of periodontal surgical procedures. *J. Periodontal.*, **52**: 518–528.

Claffey N and Shanley D (1986) Relationship of gingival thickness and bleeding to loss of probing attachment in shallow sites following nonsurgical periodontal therapy. *J. Clin. Periodontol.*, **13**: 654–657.

Cortellini P, Pini Prato GP and Tonetti MS (1993) Periodontal regeneration of human infrabony defects. I. Clinical measures. *J .Periodontol.*, **64**: 254–260.

Caton JG and Greenstein G (1998) Factors related to periodontal regeneration. *Periodontol*, *2000*, **1**: 9–15.

Foushee DG, Moriarty JD and Simpson DM (1985) Effects of mandibular orthognathic treatment on mucogingival tissues. *J. Periodontol.*, **56**: 727–733.

Gottlow J, Nyman S, Lindhe J *et al*., (1984) New attachment formation as the result of controlled tissue regeneration. *J. Clin. Periodontol.*, **19**(8): 494–503.

Gottlow J (1982) Guided tissue regeneration using bioresorbable and non-resorbable devices: Initial healing and long-term results. *J. Periodontol.*, **9**: 257–265.

Hao Y, Ma D, Hwang D, Kim W and Zhang F (2000) Identification of anti-angiogenic and anti-inflammatory proteins in human amniotic membrane. *Cornea*, **19**(3): 348–352.

Harris RJ (1997) Creeping attachment associated with the connective tissue with partial thickness double pedicle graft. *J. Periodontal.*, **68**: 890–899.

Holtzclaw D and Toscano N (2012) Amnion Chorion Allograft Barrier: Indications and techniques update. *J. Implant. Adv. Clin. Dent.*, **4**(2).

Huang LH, Neiva RE and Wang HL (2005) Factors affecting the outcomes of coronally advanced flap root coverage procedure. *J. Periodontol.*, **76**: 1729–1734.

Hwang D and Wang HL (2006) Flap thickness as a predictor of root coverage: A systematic review. *J. Periodontol.*, **77**: 1625–1634.

Ishikawa I, Iwata T, Washio K, Okano T, Nagasawa T, Iwasaki K and Ando T (2009) Cell sheet engineering and other novel cell-based approaches to periodontal regeneration. *Periodontology 2000*, **51**: 220–238.

Kassab MM and Cohen RE (2003) The etiology and prevalence of gingival recession. *J. Am. Dent. Assoc.*, **134**: 220–225.

Koob TJ, Rennert R, Zabek N, Massee M, Lim JJ, Temenoff JS and Gurtner G (2013) Biological properties of dehydrated human amnion/chorion composite grafts: Implications for chronic wound healing. *Int. Wound J.*, **10**: 493–500.

Koob TJ, Lim JJ, Massee M, Zabek N, Rennert R, Gurtner G and Li WW (2014) Angiogenic properties of dehydrated human amnion/chorion allografts: therapeutic potential for soft tissue repair and regeneration. *Vasc. Cell*, **6**: 10.

Kothiwale SV and Anuroopa P (2009) A clinical and radiographical evaluation of DFDBA with amniotic membrane versus bovine derived xenograft with amniotic membrane in human periodontal grade II furcation defects. *Cell Tissue Bank*, **10**(4): 317.

McIntyre J and Faulk W (1979) Antigens of human trophoblast. *J. Exp. Med.*, **149**(4): 824–836.

Kothiwale SV (2014) The evaluation of chorion membrane in guided tissue regeneration for periodontal pocket therapy: A clinical and radiographic study. *Cell Tissue Bank*, **15**: 145–152.

Melcher AH (1976) On the repair potential of periodontal tissues. *J. Periodontol.*, **47**(5): 256–260.

Misch C (2011) Contemporary implant dentistry, Elsevier, Canada.

Mullen IM, Richards DW and Quaranta V (1999) Evidence that laminin-5 is a component of the tooth surface internal basal lamina, supporting epithelial cell adhesion. *J. Periodontal Res.*, **34**(16): 24.

Nyman S, Lindhe J, Karring T and Rylander H (1982) New attachment following surgical treatment of human periodontal disease. *J. Clin. Periodontol.*, **9**: 290–296.

Pakkala T, Virtanen I, Oksanen J, Jones C and Hormia M (2002) Function of laminins and laminin-binding integrins in gingival epithelial cell adhesion. *J. Periodontal*, **73**(7): 709–719.

Rezaei Z, Kadkodazadeh M and Talbiardakani M (2013) Gingival biotype: a review. *Gen. Dent.*, **61**: 14–17.

Riau A, Beuerman R, Lim L and Mehta J (2010) Preservation, sterilization, and de-epithelialization of human amniotic membrane for use in ocular surface reconstruction. *Bio-materials*, **31**: 216–225.

Scantlebury TV (1993) 1982–1992. A decade of technology development for guided tissue regeneration. *J. Periodontol.*, **64**: 1129–1137.

Seibert JL and Lindhe J (1989) Esthetics and periodontal therapy. Lindhe textbook of clinical periodontology, Munksgaard, Copenhangen, pp. 477–514.

Socransky SS and Haffajee AD (2008) Periodontal infections. In: *Diagnosis and classification of periodontal diseases*. In: Lindhe J , Nika , Lang NP, Karring T (eds.), *Clinical Periodontology and Implant Dentistry*, Backwell Publishing Ltd., pp. 207–267.

Tugnait A and Clerehugh V (2001) Gingival recession — its significance and management. *J. Dent.*, **29**: 381–394.

Vandana KL and Savitha B (2005) Thickness of gingiva in association with age, gender and dental arch location. *J. Clin, Periodontal*, **32**: 828–830.

Wallace S and Cobb C (2011) Histologic analysis of trephine core samples from site preservations utilizing amnion chorion barriers. Presented at the *2011 American Academy of Periodontology Annual Meeting.*

Wiebe CB and Putnins EE (2000) The periodontal disease classification system of the American Academy of Periodontology-an update. *J. Canadian Dent. Assc.*, **66**(11): 594–599.

Weisgold A (1977) Contours of the full crown restoration. *Alpha Omegan*, **70**: 77–89.

Zachrisson BU (1997) Orthodontics and periodontics. In: Lindhe J, Karring T, Lang NP (eds), *Clinical periodontology and implant dentistry*, Munksgaard, Copenhagen, pp. 741–793.