PRINCIPLES OF MEDICAL BIOLOGY

Edited by

E. EDWARD BITTAR

NEVILLE BITTAR

REPRODUCTIVE ENDOCRINOLOGY AND BIOLOGY

Reproductive Endocrinology and Biology

PRINCIPLES OF MEDICAL BIOLOGY
A Multi-Volume Work, Volume 12

Editors: **E. EDWARD BITTAR**, *Department of Physiology, University of Wisconsin, Madison*
NEVILLE BITTAR, *Department of Medicine, University of Wisconsin, Madison*

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Principles of Medical Biology
A Multi-Volume Work

Edited by **E. Edward Bittar**, *Department of Physiology, University of Wisconsin, Madison and*
Neville Bittar, *Department of Medicine University of Wisconsin, Madison*

This work provides:

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* Over 700 internationally acclaimed medical scientists, pathologists, clinical investigators, clinicians and bioethicists are participants in the undertaking.

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Reproductive Endocrinology and Biology

Edited by **E. EDWARD BITTAR**
Department of Physiology
University of Wisconsin
Madison, Wisconsin

NEVILLE BITTAR
Department of Medicine
University of Wisconsin
Madison, Wisconsin

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LIST OF CONTRIBUTORS

Robert A. Brace	Department of Reproductive Medicine University of California at San Diego La Jolla, California
Henry G. Burger	Prince Henry's Institute for Medical Research Clayton, Victoria, Australia
Ivan Damjanov	Department of Pathology and Laboratory Medicine University of Kansas Kansas City, Kansas
Douglas R. Danforth	Department of Obstetrics and Gynecology The Ohio State University Hospitals Columbus, Ohio
Jurrien Dean	Laboratory of Cellular and Developmental Biology National Institutes of Health Bethesda, Maryland
Timothy G. Dinan	Department of Psychological Medicine St. Bartholomew's Hospital London, England
Bruce A. Federson	Department of Pathology, Anatomy, and Cell Biology Thomas Jefferson University Philadelphia, Pennsylvania
Timothy J. Gelety	Department of Obstetrics and Gynecology The University of Arizona Health Sciences Center Tucson, Arizona

Ann M. Ginsberg
Laboratory of Cellular and Developmental Biology
National Institutes of Health
Bethesda, Maryland

Roger A. Gorski
Department of Anatomy and Cell Biology
UCLA Medical Center
Los Angeles, California

Mark P. Hedger
Institute of Reproduction and Development
Monash Medical Centre
Clayton, Victoria, Australia

Bozidar Horvat
Department of Pathology, Anatomy, and Cell Biology
Thomas Jefferson University
Philadelphia, Pennsylvania

Mohammed Kalimi
Department of Physiology
Medical College of Virginia
Richmond, Virginia

Mark McLean
Endocrine Unit
John Hunter Hospital
Newcastle, Australia

Bryan F. Mitchell
The Perinatal Research Centre
The University of Alberta
Edmonton, Alberta, Canada

Jamil Mroueh
Department of Obstetrics and Gynecology
The Ohio State University Hospitals
Columbus, Ohio

V. O'Keane
St. James's Hospital
Dublin, Ireland

Catherine Racowsky
Department of Obstetrics and Gynecology
Center for Reproductive Medicine
Brigham & Women's Hospital
Boston, Massachusetts

Russel J. Reiter
Department of Anatomy
The University of Texas Medical School
San Antonio, Texas

Michael G. Ross
Department of Reproductive Medicine
University of California at San Diego
La Jolla, California

B.V.Rama Sastry
Department of Anesthesiology
Vanderbilt University Medical Center
Nashville, Tennessee

Daniel S. Seidman
Department of Obstetrics and Gynecology
Chaim Sheba Medical Center
Tel Hashomer, Israel

Charles Siles
Department of Perinatal Medicine
The Royal Women's Hospital
Carlton, Victoria, Australia

Roger Smith
Endocrine Unit
John Hunter Hospital
Newcastle, Australia

William A.W. Walters
Division of Obstetrics and Gynaecology
John Hunter Hospital
Newcastle, NSW, Australia

Tamas Zakar
The Perinatal Research Centre
The University of Alberta
Edmonton, Alberta, Canada

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PREFACE

We have now reached the mid-point of our editorial task of putting together the compendium, Principles of Medical Biology, which is supposed to be composed of twenty-five modules. The present single-volume module on reproductive endocrinology and biology is in more than one respect a continuation of Module 10 (in two volumes) dealing with molecular and cellular endocrinology. In addition, it intersects, as it should, with various parts of obstetrics and gynecology, both of which are abetted by technology. One has only to recall that the practical benefits of ultrasound in perinatal medicine and in vitro fertilization are the outcome of the technological revolution in biomedicine. Whether we are approaching a new era in reproductive biology following the invention of animal cloning is still hard to tell. For some people, it remains an article of faith that cloning of the human being is highly probable. For others, asexual reproduction is anathema. It should surely be obvious to us all that somatic cell nuclear transfer technology (SCNTT) is going to be at its strongest in dealing with husbandry. Whether this and several social forces will alter our modern outlook, there can be little doubt.

As in diverse clinical and basic research, so in obstetrics, animals are used as a model. The data thus obtained is extrapolated, if valid, to the mother and fetus. The success of this approach is exemplified in studies carried out on sheep as a model. On the whole, it is also quite apparent that progress in the field of reproductive biology is to a large extent ascribable to the discovery in other disciplines of new hormones, as well as the introduction of new tools and recent improvements in laboratory methods including measurement of hormones.

However unorthodox it may appear, Chapter 1 is not out of place. In fact, the time seems ripe to regard the subject of sexual differentiation of the brain as falling within the confines of reproductive endocrinology and biology. Arguably, the problem might involve hard wiring between several parts of the brain or might entail a somewhat complex phenomenon at the interface of human endocrinology and developmental neurobiology. In either case, nothing is gained by omitting the topic.

We should like to express our gratitude to the various contributors for their worthy manuscripts, cooperation, and patience. We thank also Mr. Christian N. Costeines and the staff members of JAI Press for their assistance and courtesy.

E. EDWARD BITTAR
NEVILLE BITTAR

Chapter 1

Sexual Differentiation of the Brain

ROGER A. GORSKI

INTRODUCTION

Human beings grow and differentiate from embryo to fetus, are born and reach sexual maturity. At a hopefully appropriate age, they reproduce and the reproductive

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cycle of human life continues in another individual. The parent, however, continues his/her life sometimes for many decades during which additional offspring may or may not be produced. The subject of this chapter is not the process of reproduction per se, but the influence of gonadal hormones on the individual during development and adulthood. What are the possible consequences of reproductive hormone action on human existence?

Although one might be tempted to answer this question in a very restricted manner, e.g., the condition of reproductive infertility and what that means to many couples, there are much broader issues to consider. What are the potentially life-long psychological consequences of a genital malformation which leads to an incorrect sex assignment? Why are men generally erotically attracted to women and vice versa? What can be done for an individual who believes (rightly or wrongly) that he/she is a member of the sex opposite to his/her phenotypic sex (i.e., the external genitalia)? What can be done for a man or woman who is attracted to members of the same sex, but so frightened of possible social rejection that suicide is attempted? Beyond sexuality per se, are there effects of gonadal hormones on other brain functions such as cognitive abilities that might impact on the quality of life?

Given the dramatic development over the last 40 years or so of the field of neuroendocrinology, which has had tremendous significance clinically, most medical students, if not already so, will soon be aware of the important role the brain plays in the regulation of the reproductive and other endocrine systems. A corollary of neuroendocrine regulation is that hormones must modify brain function, either by hormonal feedback actions or the modification of behavior. What may be new to some is that even in the adult, hormones can change brain structure and, perhaps even more surprising, during development hormone action can *permanently* change brain structure and function, perhaps lending considerable complexity to many clinical situations.

The ability of gonadal hormones to permanently change brain function and structure has led to the concept of the sexual differentiation of the brain, the topic of this discussion. According to this concept, the brain is inherently female (or perhaps neuter) and for the establishment of functional and structural features characteristic of the male of a species, the brain must be exposed to testicular hormones during a critical period(s) of development. In research animals such as the laboratory rat, sexual differentiation of the brain has been firmly established from the results of experiments in which the hormonal environment was manipulated, e.g., androgen, or perhaps somewhat surprisingly, estrogen injection in perinatal females or castration of the newborn male. In the case of human beings, such procedures are clearly unethical. Thus, direct proof that the human brain undergoes the process of sexual differentiation is not available. Instead, one must make inferences from the animal literature as well as from data from the study of so-called "Experiments of Nature," e.g., individuals who have genetic mutations or other problems which alter hormonal production or responsiveness during development and present with specific clinical features.

It must be emphasized that the sexual differentiation of the human brain is a complex and controversial process, but one that has potential implications for the quality of life of an individual and, as well, significant implications for society in general. Because of this, a consideration of the sexual differentiation of the human brain must be delayed until fundamental concepts based on animal research are presented.

SEXUAL DIFFERENTIATION OF THE REPRODUCTIVE SYSTEM

The Gonads

Why begin a consideration of the sexual differentiation of the brain with a nonneural system? The answers to this are threefold: sexual differentiation of the reproductive system is well understood; it clearly applies to human beings, and, finally, in a very real sense, certain components of the brain are an integral part of the reproductive system.

The gonad of the male (testis) and female (ovary) develop from a common anlage—the indifferent gonad, although the testis is a further development of the medulla of the indifferent gonad and the ovary, of the cortex, i.e., the gonads actually arise from anatomically distinct anlagen. The term indifferent gonad indicates that for a time during prenatal development, one cannot determine sex by the structure of the gonad. On the short arm of the Y chromosome is a gene which induces, by still unknown mechanisms, the formation of the testes. The gene was called the testis determining factor when it was only a theoretical concept, and now that the gene has been isolated (Gubbay et al., 1990), it is called *sex-determining region, Y chromosome* (SRY). Without the activity of this gene, the individual, regardless of chromosomal "sex" as XY, will develop ovaries and become female. How might this occur? As with any gene, mutations of SRY can occur and disrupt its function. In addition, SRY resides within a region of the Y chromosome that has sufficient homology with the X chromosome such that crossing over of chromosomal matter can occur placing SRY on an X chromosome. The XX individual who has an X chromosome bearing SRY will develop testes and become male.

There obviously must be genetic messages that lead to the differentiation of the ovary from the indifferent gonad, but SRY appears to be the master switch. Is there a master switch in terms of the sexual differentiation of the internal and external genitalia? The answer is yes, but in this case the master switch is hormonal production by the testes.

The Internal Genitalia

For a time during development it is also not possible to sex an individual from his/her internal reproductive organs and, as in the case of the gonads, two distinct

anlagen are present in both sexes, although they are physically separate: the mesonephric or Wolffian duct and the paramesonephric or Müllerian duct. The Wolffian duct is the anlage for the epididymis, ductus (vas) deferens and seminal vesicles while the Müllerian duct is the anlage for the oviducts, uterus, cervix and deepest part of the vagina. Nature's "default program or blueprint" favors Müllerian duct differentiation in that no exogenous factors (like gonadal hormones) appear to be necessary. In males, however, the testes secrete two hormones which are critical for masculine differentiation of the internal reproductive organs. The testes produce Müllerian duct inhibiting hormone (MIH), a polypeptide which prevents the development of the Müllerian duct and leads to its death, and testosterone (T) which promotes the development and differentiation of the Wolffian duct.

Interestingly, both males and females use the Wolffian duct as part of a functional urinary system for a time prenatally, and in the male, the testes take advantage of this existing pathway to the outside world. However, in both sexes the ureteric bud is an outgrowth of the Wolffian duct and ultimately forms the ureter and collecting system of the adult kidney. It is not known why this urinary derivative of the Wolffian duct remains or becomes independent of T, while its reproductive derivatives remain or become dependent on T for survival.

The External Genitalia

With respect to the external genitalia the story is similar; for a time during prenatal life one cannot sex an individual by his/her external genitalia. However, there is one major difference. In gonadal development, anatomically distinct components become either the ovary (cortex) or testis (medulla) and in the case of the internal genitalia, two separate ducts exist; for the external genitalia, there is a single phallic tubercle which forms either the clitoris or penis, and scrotal/labial folds which form either the labia majora or scrotum. Once again, Nature's blueprint appears to be feminine and female genitalia develop in the apparent absence of any external hormonal stimuli. In the male testicular production of T is critical for the masculine differentiation of his genitalia, but T is a prohormone and is converted locally by the enzyme 5-α reductase into 5-α dihydrotestosterone (DHT) which is the hormone that actually masculinizes the external genitalia.

What would be the consequence to a male individual if this enzyme was defective, presumably because of a genetic mutation, and DHT was not formed during development? Does this actually occur? The answer is yes and is the first Experiment of Nature we will consider. Two others and a fourth condition, actually iatrogenic in nature, offer perhaps the best opportunity to evaluate the influence of hormones on the developing human brain. At this point, these clinical syndromes will be described only from the point of view of the sexual differentiation of the reproductive system. Later, these syndromes will be considered in terms of psychosexual differentiation. A major question is

whether an understanding of hormone-induced sexual differentiation of the brain in the laboratory rat will predict the psychosexuality of individuals with these syndromes.

5-α Reductase Deficiency

Without normal activity of this enzyme, DHT is not produced and the external genitalia of the XY individual fail to masculinize and appear female at birth. Such babies are usually assigned to the female sex. However, the external genitalia may not be completely normal since there may be no external urethral opening. Instead, the urethra opens into a vagina-like urogenital sinus. Since MIH is produced, the Müllerian duct derivatives fail to develop. The Wolffian derivatives are normal and the testes descend usually into the pelvic region. Quite interestingly, as puberty occurs, the testes descend into the "scrotum," the clitoris enlarges and the body habitus can become quite masculinized.

Androgen Insensitivity

This was formerly called testicular feminization. In this case, because of a genetic mutation(s), and in its complete form, androgen receptors are not functional (McPhaul et al., 1991). Thus, these XY individuals are born looking female in terms of their external genitalia. Since MIH is produced and acts independent of the androgen receptor, the Müllerian derivatives fail to develop and the vagina is underdeveloped. Wolffian derivatives are normal and the testes descend, commonly into the inguinal region. At puberty testicular hormonal activity increases, but there is no response to androgens. However, due to the aromatization of T to estrogen (E), distinctly feminine breasts develop. Axillary and pubic hair are scant if present.

Congenital Adrenal Hyperplasia

Although this syndrome, formerly called the congenital adrenogenital syndrome, occurs in both sexes, we will consider only the female. In this syndrome there are one or more defects in the enzyme system necessary for the synthesis of cortisol, the adrenal cortical hormone regulated by its negative feedback action on the hypothalamo-hypophyseal axis. Without normal negative feedback, there is excess secretion of adrenocorticotropic hormone and enhanced steroidogenesis in the adrenal cortex resulting in the release of androgenic adrenal hormones. Prenatal virilization of the external genitalia may be sufficient to cause a female baby to be sex-assigned as male. Müllerian duct derivatives are essentially normal and Wolffian derivatives do not develop. The syndrome cannot be cured but its symptoms caused by the excessive release of adrenal androgens can be ameliorated by the lifelong administration of cortisol.

In some individuals the synthesis of aldosterone is also affected and such individuals may suffer sufficient salt loss that the condition is recognized while the baby is still in the hospital after birth. By supplying the missing hormones from birth, these women will have been exposed to excess androgen, although of adrenal origin, only during pregnancy. As will be discussed below, these females may most closely form the human counterpart of female rats injected with T only during the critical period for the sexual differentiation of the brain.

Exposure to Diethylstilbestrol (DES) Prenatally

In the 1950s and 1960s, many pregnant women who threatened to abort were treated with DES, a potent synthetic E. Although most of the offspring of these pregnancies, of either sex, appeared normal in terms of their genitalia, later in life in women so exposed there was a significant increase in a relatively rare type of clear cell adenocarcinoma of the cervix and vagina (see Reinisch and Sanders, 1992). Males that were exposed to DES prenatally displayed a somewhat higher incidence of reproductive abnormalities. What will be important for this discussion, however, is the psychosexual and cognitive development of these individuals.

SEXUAL DIFFERENTIATION OF THE RAT BRAIN

Brain Function

In any consideration of sex differences in brain function, the nature of hormone action on the brain must be understood and this involves the concept of the organizational versus activational effects of gonadal hormones on the developing and mature brain, respectively. This concept was proposed more than 30 years ago and is still relevant today, although it is not known whether these two types of hormone action actually differ mechanistically. By activational is meant the transient modification of brain function (and possibly structure), by organizational, the permanent modification of brain function and structure.

Why is this concept important for a consideration of the sexual differentiation of the brain? Although it is common to refer to T as the male sex hormone and E and progesterone as female sex hormones, this is quite simplistic. What differs between the postpubertal male and female is the quantitative balance of these sex steroids, not an all or none qualitative difference. Nevertheless, the hormonal milieu to which the male and female brain are exposed is indeed different. Thus, if one observes a functional sex difference in intact adults, is that difference imposed on a similar brain by the differing hormonal environment in the activational sense, or is there a true sex difference in the functional capacity of the adult brain?

In laboratory animals such as the rat, it is fairly easy to distinguish between these two options. If adult male and female rats are gonadectomized and both are given intraocular grafts of ovarian tissue from newborn females, the grafts will take in both sexes, but ovulation, as determined by corpora lutea formation, will occur only in females. Since it is now known that ovulation is caused by estrogen positive feedback (a certain plasma level of E coupled with a photoperiodic signal triggers a surge of luteinizing hormone [LH] releasing hormone from the hypothalamus which, in turn, causes the ovulatory surge of LH), one can merely inject male and female rats gonadectomized as adults with E and measure plasma LH at appropriate time intervals afterwards. The female rat exhibits estrogen-induced positive feedback, but the male does not.

Similar approaches have been taken with other functional systems. Treating females ovariectomized as adults with T will enhance their masculine copulatory behavior but either not as much or not as rapidly as in males. Treating a male rat castrated as an adult with E and progesterone will increase their expression of female sexual behavior as measured by the lordosis reflex when mounted by a stud male, but again the level of lordosis responsiveness is lower than in females and/or develops more slowly.

With this approach of testing male and female animals under the same hormonal conditions, sex differences in the following functional parameters have been reported many times and in numerous species, although not all in the same species: aggressive, parenting, social and play behaviors, which may in general terms be related to reproduction. In addition, however, there are sex differences in brain function which are currently difficult to interpret in terms of reproduction, e.g., regulation of food intake and body weight, open field and learning behavior and learning strategies.

What may be surprising to some is that these functional sex differences can be eliminated or actually reversed by castrating newborn males or injecting females with T at an appropriate postnatal age. Thus, a male rat that is castrated within the first few days of postnatal life, when adult and treated with E and progesterone, will exhibit normal female levels of lordosis responding and a normal female estrogen-feedback response; neonatally castrated males also can ovulate if given ovarian grafts. Nature's default blueprint for brain function is female! As predicted, therefore, giving a female rat as few as one injection of T within the first week of postnatal life suppresses lordosis responsivenss and estrogen-induced positive feedback and enhances the masculine copulatory response to T injection during adulthood. Data of this nature have led to the concept stated above, namely, that the brain is inherently female and functional characteristics typical of the male are imposed on the developing brain (of either sex) by exposure to T during early postnatal development.

Brain Structure

How does hormone action during development permanently alter brain function? There are sex differences in the neurochemistry of the brain, but that topic re-

quires a chapter in itself (see De Vries, 1990). Since the focus of the present discussion is on the application of the concept of sexual differentiation to the human brain, its main emphasis will be on the possibility that structural sex differences underlie many of the known functional sex differences and that brain structure per se undergoes the process of sexual differentiation. Put quite simply, for many nuclei and regions of the rat brain, Nature's default blueprint is indeed female.

When structural sex differences were first identified, they were linked to the organizational action of steroids. In fact, the two became almost synonymous. In other words, structural modifications produced by gonadal hormones were thought to occur only during development. This clearly is not true and two examples from very different species illustrate this quite well. In the canary, there are marked structural sex differences in components of the song control/production system. Since this species is a seasonal breeder, during the non-breeding season the testes become inactive and involute and the volume of several brain areas decrease. As Spring arrives and the testes become hormonally active again, these brain regions enlarge, dendrites lengthen and new synapses form (De Voogd, 1991). Even in the adult mammalian brain (e.g., the stratum radiatum of the CA1 region of the hippocampus) the estimated density of synapses on dendritic spines changes significantly, apparently under the influence of E, over the four day estrous cycle of the rat (Woolley and McEwen, 1992).

At this point, suffice it to say that the caveat about the possible activational effects of the differing hormonal environments of the male and female brain also clearly applies to brain structure. Nevertheless, there is a growing list of structural sex differences in the rat central nervous system (CNS) which have been shown to be determined permanently by the hormonal environment perinatally (Table 1).

Three of these structural sex differences illustrate several important points. The sexually dimorphic nucleus of the preoptic area (SDN-POA) is perhaps the most marked structural sex difference in the mammalian brain being about fives times or more larger in the genetic male. The volume of the SDN-POA and, by inference, the number of neurons it contains, can be determined exclusively by the hormonal environment perinatally (Figure 1). Much is known about the development of the SDN-POA: a significant percentage of its neurons are born (i.e., become postmitotic) several days later than surrounding neurons; the origin and migratory pathway of SDN-POA neurons are known as is at least one probable mechanism of hormone action, i.e., hormone-induced prevention of programmed neuronal death by apoptosis (Davis et al., 1996a). What is not so clear is the function(s) of the SDN-POA in rats. The most recent and still unpulished data from the author's laboratory (Hori, unpublished observations) strongly suggest that the SDN-POA may be critically involved in masculine sexual behavior, particularly ejaculatory behavior. This conclusion is based on the marked enhancement of ejaculatory behavior in adult intact males in which the region of the SDN-POA was unilaterally electrically stimulated.

Table 1. Structural Sex Differences in the Rat Central Nervous System That Are Determined by Testicular Hormones During Perinatal Development

Volume or Size:	
Male greater than female	
Accessory olfactory bulb	Bed nucleus of olfactory tract
Bed nucleus of stria terminalis	Medial nucleus of amygdala
Medial preoptic nucleus	Sexually dimorphic nucleus of the preoptic area
Spinal nucleus of the bulbocavernosus	Ventromedial nucleus
Vomeronasal organ	
Female greater than male	
Anteroventral periventricular nucleus	
Locus coerulus	
Parastrial nucleus	
Synaptic Morphology	
Arcuate Nucleus	Lateral septum
Medial nucleus of amygdala	Medial preoptic area
Sexually dimorphic nucleus of the preoptic area	Ventromedial nucleus

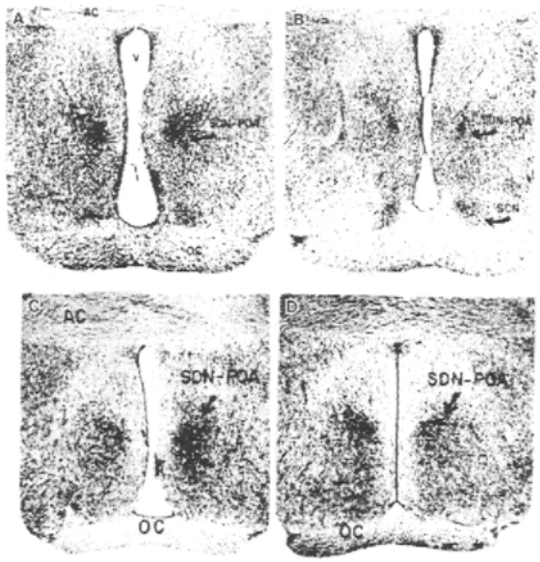

Figure 1. Representative photomicrographs through the sexually dimorphic nucleus of the preoptic area (SDN-POA) in adult male (**A**) and female (**B**) rats. Also illustrated are sections from adult female rats in which the SDN-POA was sex-reversed by treatment perinatally with testosterone (T) or the synthetic estrogen, diethylstilbestrol (D). Abbreviations: AC, anterior commissure; OC, optic chiasma; V, third ventricle.

The anteroventral periventricular nucleus (AVPv) is one of the few nuclei thus far found to be larger in volume in the female (see Table 1). In this case, T exposure appears to promote cell death. There most likely is not a single mode of action of steroid hormones on the brain. Interestingly, although gonadal steroids clearly act postnatally, the sex dimorphism in the AVPv does not develop until about the time of puberty (Davis et al., 1996b). This is markedly different from the SDN-POA in which the sex difference in volume develops over the course of the first week or so of postnatal life, the critical period for the sexual differentiation of brain function. The AVPv may be related to the cyclic release of LH or estrogen-induced positive feedback since lesions in this area block ovulation (Terasawa and Davis, 1983). Finally, the spinal nucleus of the bulbocavernosus is sexually dimorphic in the rat, but its larger volume and number of neurons appear to be due to a direct action of androgen on penile musculature rather than on the spinal cord itself (Goldstein and Sengelaub, 1994).

Thus, although testicular hormones appear to determine the structure of a number of regions of the CNS, no single mechanism appears to be responsible. In fact, no single hormonal species may be involved. Why raise this point? If testicular T is the masculinizing hormone for the rat CNS, then it is perhaps logical to assume that E either has no role or perhaps is a feminizing hormone, but neither appears to be correct.

Is the Rat Brain Inherently Female or Neuter?

Our consideration of the possible role of E in the development and/or differentiation of the rat brain has been delayed until both functional and structural sex differences were introduced. This is because for both processes, T appears to be basically a prohormone. The aromatization of T to E is, in fact, a prerequisite for much of the masculinization of the rat brain. As will be discussed below, this observation appears to have important implications for the possible sexual differentiation of the human brain. At least in the rat, the administration of E at doses lower than that of T masculinizes brain function and structure, and treatment with antiestrogen or an aromatase inhibitor blocks masculine differentiation in the intact male.

These observations pose not only a problem for our understanding of sexual differentiation of the human brain (see below), but also problems for the rat, too. The reason for this is that E levels are apparently very high in the plasma of neonatal rats of both sexes. This raises an important question: if E is the masculinizing hormone and plasma levels of E are high in both male and female rats during the process of sexual differentiation of the brain, how can normal females exist?

This question has not yet been answered satisfactorily. The first and simplest potential explanation came from the observation that for the first few weeks of postnatal life a liver protein, α-fetoprotein (AFP), is present in high amounts in the blood and AFP binds E. Thus, it was suggested that the role of AFP in the neonatal rat is to

bind plasma E so that it cannot enter neurons. This allows the inherently female nature of the rat brain to be expressed. Since AFP does not bind T, this testicular product is free to enter neurons where it can be aromatized to the functional masculinizing form, E. However, data also became available which suggested that this protection hypothesis was not true, or at least not the entire story. The results of *in vitro* studies indicated that E stimulates neurite outgrowth from estrogen-sensitive neurons and is actually required for such neurite outgrowth. (Note: the growth of neural process and the attainment of appropriate connections is one proposed mechanism for neuronal survival during the developmental process of "programmed" cell death.) In addition, treatment of neonatal female rats with anti-estrogen blocks ovulation and reduces female sexual behavior in adulthood, but without enhancing male copulatory behavior. That is to say the animals were defeminized but not masculinized. Treatment with anti-estrogen postnatally reduces SDN-POA volume in males as predicted, but it does so in females as well.

Taken together, these results suggest that the rat brain is not inherently female but rather is neuter or undifferentiated. Thus, in species like the rat in which sexual differentiation of the brain occurs postnatally, at least in part, some mechanism is needed to prolong high plasma levels of E beyond parturition, i.e., to provide enough E to ensure that the female brain develops normally; AFP may serve that role. Of course, the male would still be exposed to greater levels of E because of the local aromatization of T. Note that in a species such as the guinea pig, in which sexual differentiation is exclusively prenatal, exogenous E still masculinizes the brain (Hines et al., 1987). Although it may be somewhat uncertain what role E plays in the development of the normal female brain in laboratory animals, higher levels of E due to the aromatization of T produced by the testes are clearly necessary for masculine differentiation of the rat brain.

At this point, it may be useful to summarize what has been discussed thus far, before attention is focused on the human species. In human beings and other animals, chromosomal genetics determine whether a given individual will develop testes. The SRY gene appears to be the master switch in this regard. Once normal testes form, sexual differentiation of the internal and external genitalia are due to the production and action of two hormones, MIH and T, and on the ability of tissues to respond to these hormones. Nature's blueprint appears to be female and this clearly applies to the brain of animals such as the laboratory rat, both in terms of brain function and structure. In rats, the major brain masculinizing hormone is actually E derived from the aromatization of T produced by the testes.

STRUCTURAL SEX DIFFERENCES IN THE HUMAN BRAIN

Structural sex differences have been reported in the human brain (Table 2). However, by comparing Tables 1 and 2, one notes that such differences in the human brain are fewer in number than in the rat. Figure 2 illustrates the magnitude of sev-

Table 2. Reported Sex Differences in the Human Central Nervous System

Greater volume in the male than in the female
Sexually dimorphic nucleus of the preoptic area (also called the first interstitial nucleus of the anterior hypothalamus)
Second interstitial nucleus of the anterior hypothalamus
Third interstitial nucleus of the anterior hypothalamus
Darkly staining component of the bed nucleus of the stria terminalis
Central component of the bed nucleus of the stria terminalis
Onuf's nucleus in spinal cord
Greater in the female than in the male
Anterior commissure (midsagittal area)
Corpus callosum (midsagittal area)
Isthmus of the corpus callosum (midsagittal area; compared only to consistently right-handed men)
Massa intermedia (midsagittal area, and incidence)
Greater asymmetry in the male
Planum temporale
Shape
Splenium of the corpus callosum (more bulbous in females)
Suprachiasmatic nucleus (more elongated in females)

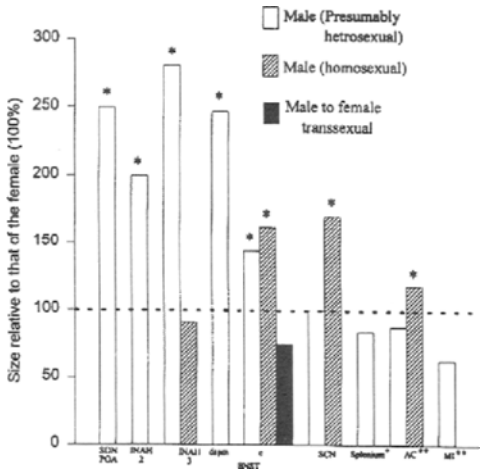

Figure 2. Relative magnitude of reported differences in the volume (unless indicated otherwise) of components of the human brain with respect to sex, homosexuality and transsexuality. Refer to text for multiple references. Abbreviations: BNST, bed nucleus of the stria terminalis (dspm, darkly staining posteromedial component; c, central subdivision); INAH-2 and INAH-3, second and third interstitial nucleus of the anterior hypothalamus; SCN, suprachiasmatic nucleus; SDN-POA, sexually dimorphic nucleus of the preoptic area. **Midsagittal area. +Bulbosity.

eral of the sex differences in the human brain which are smaller than many in the rat and much more controversial (see below). The smaller number of structural sex differences, however, may merely reflect the fact that the search for sex differences in the rat brain has at least a 10 year head start in terms of modern research. Moreover, the study of human postmortem brain tissue is fraught with potential problems that may have discouraged research and/or precluded meaningful results.

If one wants to study brain anatomy in the rat, the experimenter chooses the time of sacrifice, perhaps at a given time of day, at a certain stage of the estrous cycle in females, or after a comparable period following gonadectomy or some other intervention. The animal is anesthetized and, while still alive, the brain is flushed with physiological saline and immediately perfused with fixative. In the case of a human being, however, often heroic efforts are made to maintain life, thereby increasing the possibility of age-related neuropathology or drug-induced alterations in neural structure. When death does occur randomly, it takes time for the brain to be removed and possibly examined by a pathologist in an autopsy. Fragments of brain tissue are then immersed in fixative, but diffusion is a relatively slow process. The time interval between death and fixation adequate to stop degenerative processes can be quite variable and clearly not controlled as in the case of laboratory animals. In addition, the aging process can cause changes in brain structure but chronological age is not necessarily a good indicator of aging per se. Finally, the life style of each individual may impart its signature on brain structure. In rats, for example, the cortex of animals housed in an enriched environment differs anatomically from that of animals housed in a more sterile environment. The consequence of these caveats is that reports of structural sex differences in the human brain must be replicated a number of times before being fully accepted.

An example of the need for replication is an initial report that the corpus callosum (CC) of the human female was larger than that of the male in midline and that its posterior fifth (the splenium) was more bulbous in shape in women. After this report, a dozen or so studies failed to confirm this observation (see Allen et al., 1991). Note, however, that in the subsequent studies, subjects were not always age-matched. (Although age may not be the most reliable indicator of the aging process, age-matched subjects are likely to provide more reliable data than those from experiments in which age is not taken into account at all.) In addition, the CC was partitioned into various components for measurement, e.g., the splenium, by two different methods. A recent study—using magnetic resonance images in age-matched living subjects and analysis of CC size or that of its subcomponents after subdivision by both published techniques—failed to reveal any sex difference in size, although the splenium was confirmed to be more bulbous in females (Allen et al., 1991). On the other hand, Witelson (1991) has reported that the isthmus of the CC is larger in women (regardless of handedness) than in consistently right-handed men. Apparently, handedness may have to be added to the parameters which might influence brain structure.

Of greater relevance to the present discussion are reports of sex differences in several small hypothalamic nuclei. The first of these was found to be significantly larger in males than in females and was labeled the human SDN-POA (Swaab and Fliers, 1985). Its name should not be interpreted to mean that the human SDN-POA is homologous to the SDN-POA of the rat. Although that certainly could be true, a sex difference in nuclear volume is not an acceptable criterion for homology. Moreover, Allen and her colleagues (Allen et al., 1989) concurrently, but independently, analyzed four small nuclei which they labeled the interstitial nucleus of the anterior hypothalamus one to four (INAH–1-4). Although INAH-1 was not found to be sexually dimorphic in this study, there is general agreement that INAH-1 and the SDN-POA are the same structure.

Not only were these two studies performed on subjects from different countries, the methodology varied. In the first (Amsterdam) study, histological sections were cut at 6 μm, and every 25th section was analyzed. In the Los Angeles study, sections were cut at 60 μ and INAH-1 was evaluated for area on every section in which it appeared. The SDN-POA of the rat is not a homogeneous structure and if that applies to the human hypothalamic nucleus, the differences in methodology could perhaps explain the disagreement as to whether the SDN-POA or INAH-1 is or is not sexually dimorphic. There is yet another potential explanation for this disagreement. The human SDN-POA has been shown to vary with age and the age-distribution of the Los Angeles subjects may have been such as to bias against finding a sex difference even though the subjects were age-matched (see Swaab et al., 1992). In biology, a statistically significant difference usually carries more weight than a negative finding. Thus, awaiting further study, it can be assumed that INAH-1 (the SDN-POA) is significantly larger in volume in males.

Allen et al. (1989) reported that only INAH-2 and INAH-3 were significantly larger in men than in women (Figure 2). LeVay (1991), in an independent study but basically using the Allen technique, found that neither INAH-1, INAH-2, nor INAH-4 were sexually dimorphic. However, he confirmed that INAH-3 was larger in men than in women and went on to report that INAH-3 was larger in apparently heterosexual men than in men identified as homosexual from hospital records (see Figure 2 and below). It has also been reported that the midsagittal area of the anterior commissure is larger in women than in men (Allen and Gorski, 1991). None of these reports, except perhaps for the bulbosity of the splenium of the CC and the sex difference in the volume of INAH-3, have been replicated, although in contrast to the study of the CC, no reports of failed attempts to replicate the hypothalamic sex differences have been published thus far. The caveats with respect to the study of postmortem human brain tissue have already been touched upon. In the context of the concept of the sexual differentiationn of the brain, note that there is absolutely no information about the possible role of gonadal hormones—either organizationally or activationally—in establishing and/or maintaining these putative sex differences in the human brain.

Let us assume, then, for the purpose of discussion, that these sex differences in human brain structure are real. The question thus arises: what do they mean? Could they be related to sex differences in cognitive function or sexuality?

Sex Differences in Cognitive Function

On average, men and women exhibit the following differences in cognitive abilities: men perform better than women on some visuospatial tasks while women perform better than men on some verbal tasks. That these sex differences are task specific is not surprising because it is likely that two different tests of visuospatial or verbal ability actually differ in terms of how the brain processes the tasks. An experimenter may label two tasks as tests of verbal cognitive function but the brain may process the tasks in markedly different ways. Boys outnumber girls in performance in mathematical reasoning, but boys also outnumber girls at the low end of performance. Male brain function is generally considered to be more lateralized than in women, a conclusion recently supported by functional magnetic imaging studies (Shaywitz et al., 1995).

Could any of the putative structural sex differences in the human brain be related to these cognitive sex differences? Note, however, that the cognitive differences are by and large statistical differences with considerable overlap. Thus, a man and woman selected at random may exhibit a difference in cognitive ability actually opposite to the statistical prediction. In this regard, and confoundingly so, there is also significant overlap in the putative structural sex differences.

If brain function is less lateralized in women one might predict that the CC or some of its components would be larger in women in order to foster more "cross-talk" between the two hemispheres. However, a size or shape difference in the CC could reflect the number or size of axons and/or their degree of myelination. We are far from understanding the relationship between the putative sex differences in human brain structure and cognitive ability, but that does not mean that there is no relationship.

Sexuality

In comparison to the often subtle and overlapping differences in cognitive function, sex differences in human sexuality are typically profound. Is it possible, therefore, that structural sex differences in the human brain could be more easily related to sexuality? To date, however, there is no compelling evidence to link any structural sex difference in brain structure to differences in sexuality (see discussion of homosexuality below for further comments). Nevertheless, the so-called Experiments of Nature described above provide the best data available to challenge the applicability of the concept of the sexual differentiation of the brain to our species.

The origins of human sexuality have been debated for many years and two general theories have evolved in modern times. Either Nature (e.g., hormones and/or

genetics) or nurture (the complex interaction of experiential factors during one's developmental years) determine sexuality. To be more realistic, these concepts were applied to the etiology of homosexuality more so than to that of the more typical heterosexuality. In a way, one born with a penis is just expected to be psychosexually male and sexually attracted to women; one born without a penis is expected to be psychosexually female and sexually attracted to men. Although one cannot argue against the importance of experiential, cultural or societal influences, do they in fact determine sexuality?

One famous "experiment" (actually an accident) seemed to prove the critical role of rearing in human psychosexual differentiation. Identical twin infant boys were subjected to circumcision by cautery, but in one the penis was ablated. The decision was made to castrate this infant and raise him as a girl. The initial results were strongly supportive of the nurture theory. However, as the girl grew to maturity she wanted to be more like her brother and is now living as a man after reconstructive surgery. In fact, he reports that he never was happy as a girl, but felt pressured to state that he was (see Diamond, 1992).

Earlier in this chapter several experiments of Nature were introduced. Let us look at these again, but this time from the perspective of psychosexual differentiation and the possible hormone-dependent sexual differentiation of the brain.

5-α-Reductase Deficiency

Individuals with this condition are among the most interesting since although assigned as females, their brains were presumably exposed to T or its metabolites prenatally. In a group of such individuals from the Dominican Republic, a subset of 18 were studied by Imperato-McGinley et al. (1979). Although purportedly raised "unambiguously" as females, when their bodies masculinized at puberty, 17 of these "females" became psychosexual males. Although this result appears to support the paramount importance of hormonal activity during development (and perhaps at puberty) over developmental experiential factors, this has not been conclusively proven by any means. Forty to 60 year-old individuals who have been ridiculed by their social peers because they "changed" from female to male after puberty, are hardly the most reliable retrospective judges of the nature of their early rearing, nor are their parents. In addition, in the Dominican Republic it is socially advantageous to be male. Often it is the most postpubertally virilized individuals who opt to be male, perhaps as a psychological adjustment to the masculinization of their bodies. Less virilized individuals may recognize that a somewhat enlarged clitoris does not constitute a functional penis. Nevertheless, the standard procedure when this syndrome is discovered, i.e., castration and continued assignment as female, may not be appropriate for all individuals. To this author's knowledge, no test of cognitive abilities have been performed on individuals with this syndrome who, after puberty, chose to become males or remained as females.

Androgen Insensitivity

These XY individuals are psychosexually female, but again, this author knows of no detailed analyses of their cognitive abilities. One might argue that such individuals offer little to help us understand sexual differentiation of the human brain: the brains of these individuals cannot respond to androgen, they are born looking like females, are raised as females and they become women. However, remember that the major masculinizing hormone in the rat brain is E. Clearly, after puberty, these androgen insensitive XY women produce sufficient E to feminize the body. Do they, or do they not, produce sufficient estrogen during prenatal development to masculinize brain function if E is the masculinizing hormone? In this case, at least, is nurture more powerful than Nature? Is the brain masculinizing hormone in the human not E? Is there some protective mechanism, possibly akin to AFP, in the human fetus? Or finally, does the process of the hormone-dependent sexual differentiation of the brain actually apply to human beings? None of these questions can be answered definitively at this time.

Congenital Adrenal Hyperplasia (CAH)

In this case, women with this syndrome who have been treated with the missing hormone, cortisol, have been studied more extensively. In one study (Ehrhardt and Baker, 1974), these women were judged to be tomboys but by criteria that may not be as appropriate in 1997. Clearly, our social mores have changed and a woman who wears slacks rather than a dress or blouse and skirt is certainly not unusual, and certainly not necessarily a tomboy. Nevertheless, women with this syndrome appear to be shifted somewhat towards the masculine direction in a continuum of behavior. It must be noted that these women must be on cortisol therapy throughout life. Even if it were a simple matter of maintaining an adequate therapeutic regime of cortisol administration, which is apparently not the case, these women know that they are different and dependent upon permanent cortisol therapy, knowledge that may affect their self-image and/or behavior. However, as youngsters, girls with CAH play signficantly more with toys ordinarily preferred by boys and show a possible postpubertal spatial advantage. Interestingly, there is also an apparent increase in lesbian fantasies and lesbian behavior.

Exposure Prenatally to DES

It is difficult to analyze the effects of prenatal exposure to DES because this treatment was for therapeutic, not experimental, purposes. Thus, the dose of hormone varied from patient to patient as did the precise period during pregnancy when it was administered. Finally, there were basically no controls. Nevertheless, it appears that prenatal exposure to DES in females masculinizes brain asymmetry as determined by a dichotic listening test, but does not appear to influence cognitive

function. Although DES exposure is associated with an increase in bisexuality and homosexuality in women, it apparently does not affect sexual orientation in men. These results are quite consistent with DES being a masculinizing hormone for brain function. However, DES exposure in males has been reported to reduce hemispheric laterality and lower spatial ability, i.e., changes more in the female direction (Reinisch and Sanders, 1992).

Structural Differences in the Brains of Men Who Display Atypical Behavior

Since we do not understand the etiology of heterosexuality, perhaps it is premature to attempt to interpret these syndromes in this context. A consideration of atypical sexual behavior may be more productive. In this regard, two such behaviors will be considered: transsexuality and homosexuality.

The transsexual is the individual who believes (or if a repressed homosexual hoping to escape societal rejection, wants to believe) that he/she is a person of the sex (i.e., brain sex) opposite his/her phenotypic sex and opts for surgical sex conversion. Since brain sex can be experimentally manipulated in rats independent of phenotypic sex, the rat may well be a useful model for human transsexualism. Recently, Swaab and his colleagues (Zhou et al., 1995) have reported a potentially significant observation. They found a structure (the central subdivision of the bed nucleus of the stria terminalis [BNSTc]) which is sexually dimorphic (larger in volume in men than in women), but which does not vary with sexual orientation in men. (Note that the true sexual orientation of transsexuals can be difficult to ascertain.) However, in six postoperative male-to-female transsexuals, the BNSTc was female in volume (Figure 2). Although the BNSTc is one very small region of the brain, its feminine volume is consistent with the male-to-female transsexual having female components of brain structure.

Structural differences in the brains of homosexual and apparently heterosexual men have also been reported recently. (As far as this author knows, no studies comparing the brains of lesbian women have been performed.) At least initially, a search for structural differences in the brains of heterosexual and homosexual men seems ill-conceived. Homosexual men basically consider themselves male and there is not the discrepancy between "brain sex" and phenotypic sex that is characteristic of the transsexual. However, in laboratory animals, sexual differentiation of the brain has multiple components (e.g., masculinization, defeminization) which appear to be temporally and neuroanatomically distinct. Thus, it is possible that hormonal fluctuations at a given time in development might change a specific brain structure in a way that might lead, or predispose, one to become homosexual. To date there are three reports of structural differences between the brains of homosexual and heterosexual men (Figure 2). The first was the report that the suprachiasmatic nuclei (SCN) of homosexual men are larger in volume than that of a reference group (Swaab and Hofman, 1990). Although the SCN exhibit a sex difference in shape

(more elongated in females), they have not been found to differ between the two sexes in volume (Hofman et al., 1988; Figure 2). Moreover, since the SCN in the human brain are difficult to identify under regular Nissl staining, these studies utilized immunohistochemical identification of vasopressin cells to identify the SCN and quantitate its volume. The neuropeptide expression of neurons can be state dependent. Thus, it is still not known if the SCN differ between homosexual men and heterosexual men and women anatomically or just neurochemically.

As mentioned above, LeVay (1991), after confirming that INAH-3 is larger in volume in men than in women, also reported that INAH-3 was larger in heterosexual men than in homosexual men (Figure 2). Subsequently, Allen and Gorski (1992) reported that the midsagittal area of the anterior commissure, which is larger in women than in men, is even larger in homosexual men (Figure 2). In these studies as well as those which found significant structural sex differences, there is overlap between the groups, sometimes considerable overlap. Therefore, at present it is not possible to look at a specific brain structure and determine whether that individual was male or female, let alone predict sexual orientation.

Studies of the brains of homosexuals are subject to the caveats about postmortem brain tissue per se, including the need for replication but, in addition, these three studies were potentially confounded by AIDS. Moreover, one must ask which comes first: a structural difference in the brain which leads to homosexuality or homosexuality which leads to a structural brain difference. Since these were studies of postmortem brain tissue essentially nothing is known about the specific behaviors of the homosexual men.

GENETICS AND HOMOSEXUALITY

As an hopefully logical extension of a consideration of the sexual differentiation of the brain, this chapter has discussed the possible hormonally-based etiology of homosexuality versus that of a nurture-based etiology which by itself currently seems inadequate at best. However, it must be emphasized that genetic factors may also contribute to the origins of homosexuality. Briefly, the concordance rate for homosexuality among identical twins of either sex is about 50–75%, suggesting a very high incidence of homosexuality among identical twins (Bailley and Pillard, 1991; Bailey et al., 1993; Whitman et al., 1993). The concordance rate drops markedly for non-identical twins and even further for adopted siblings. Concordance is simply the chance that the second individual in a pair will also be homosexual if the first is. The high concordance rate in identical twins suggests a substantial role for genetic factors in the etiology of homosexuality; yet up to 50% of identical twins are not concordant for sexual orientation. Some other factor(s) must be involved.

Hamer et al. (1993) analyzed the family pedigrees of a selected group of homosexual men and observed a higher incidence of homosexuality in maternal uncles and cousins of homosexual men. This led them to a study of the X chromosome and

they found that in 33 of 40 pairs of homosexual brothers, the two brothers had inherited the same markers for a region of the X chromosome called Xq28. This laboratory has replicated this study with new subjects. It may be that within Xq28 there is a gene(s) which contributes in some significant way to the etiology of homosexuality. Even in this case, however, about 20% of the homosexual brothers had not inherited the same chromosomal markers.

Thus, there is some evidence for a genetic component to the etiology of homosexuality. However, steroid hormones are known to modify genomic activity. Thus, genetic influences may be independent factors in the etiology of homosexuality and perhaps in the sexual differentiation of brain structure and function which must involve alterations in gene function, or genomic influences may represent one way hormones act.

The author has not discussed the potential role of environmental or experiential factors in the etiology of human sexuality because this area is well beyond his expertise. However, in such a complex behavior as human sexuality, it is unlikely that a single factor, be it a hormone, a gene or some experience is the determinant. It is most likely that sexuality arises from a complex interaction of many factors.

SUMMARY AND CONCLUSIONS

This discussion has documented the sexual differentiation of the reproductive system in mammals including the human being. A gene (SRY) which is normally located on the Y chromosome is the master switch; its normal expression leads to the development of the testes. The testes in turn produce two hormones, MIH and T, which elicit masculine differentiation of both the internal and external genitalia. In contrast to masculine differentiation, no external stimulus such as hormones appears to be needed for female development. Nature's blueprint for the reproductive system is female.

The brain is an integral component of the reproductive system and perhaps because of this, it also undergoes the process of hormone-dependent sexual differentiation, at least in model species like the laboratory rat. However, sexual dimorphisms exist in areas of brain function which do not currently appear to be related to reproduction. Nevertheless, Nature's blueprint for brain development does appear to be female, although it is still possible that some hormone action is required for the normal development of the female brain. It is clear that in rats T serves as a prohormone and that it is actually E which induces most of the masculine differentiation of the brain. Although the concept of the sexual differentiation of the brain initially arose from studies of sex differences in brain function and the permanent or organizational effects of gonadal hormones on these functions, the results of more recent studies indicate that specific brain structures or regions are also subject to this process of hormone-dependent sexual differentiation.

A significant advantage of studies using laboratory animals is that the experimenter can modify the hormonal environment during development and then evaluate the consequences on brain structure and function. Such experiments cannot be performed with human beings. Nevertheless, structural sex differences have been identified in the human brain. Although there are no data to prove that these structural sex differences are hormone dependent, that remains a distinct possibility.

The functional significance of structural sex differences in the human brain remains unknown. Although sex differences in cognitive function do exist, it is not currently possible to link the two—structural and cognitive sex differences—together. There are also clinical syndromes which are due to disturbances in hormonal production or responsiveness but, to date, these give only minimal support for a role of gonadal hormones in the sexual differentiation of the human brain. Perhaps a more useful tool in trying to understand human brain development is the study of the etiology of atypical sexuality. One recent study of the brains of transsexual men provides some support for the sexual differentiation of the human brain, but only when interpreted in terms of concepts derived from the study of models such as the laboratory rat. Structural differences also appear to exist in the brains of heterosexual and homosexual men, but any causal relationship between their existence and sexual orientation remains obscure.

In a similarly incomplete way, the results of genetic studies suggest a possible involvement of genetic factors in the etiology of homosexuality and, by conceptual extension, in the etiology of heterosexuality. Perhaps hormonal, genetic and experiential factors interact to contribute to the origins of sexuality in human beings. Whether one accepts the model of sexual differentiation of the brain derived from the study of laboratory animals and applies it to the human brain remains a matter of subjective opinion. However, continued study, perhaps stimulated by the controversial data and conclusions generated by the results discussed here, will lead both to a greater understanding of the process of the sexual differentiation of the brain and its potential significance in human development and in the quality of life.

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Chapter 2

Hypophyseal-Gonadal Relationships in the Male

MARK P. HEDGER

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INTRODUCTION

The testis consists of two anatomically discrete compartments: the seminiferous tubules which constitute the majority of the adult testicular mass, and the intertubular, or interstitial, tissue. The seminiferous tubules are lined by a complex stratified epithelium comprising successive layers of developing germ cells embedded within the cytoplasmic processes of supporting Sertoli cells. In this epithelium, primitive germ cells, or spermatogonia, give rise to the highly differentiated male gametes, the spermatozoa, through a series of cell divisions and transformations that are collectively known as the process of spermatogenesis (Clermont, 1972). The interstitial tissue comprises a loose connective tissue network surrounding the seminiferous tubules, containing the steroidogenic Leydig cells, testicular vasculature, lymphatics and nerves.

In humans, the testes are located in a pendulous sac, or scrotum, outside the abdominal cavity. This organization is important for maintaining a low intratesticular temperature, known to be essential for spermatogenic development. The testis is enclosed within a connective tissue capsule, the tunica albuginea. Connective tissue septae arising from the internal surface of the tunica albuginea separate the testis into lobules. Within the lobules, the seminiferous tubules are highly convoluted and form close-ended loops connecting with the rete testis, an epithelial-lined reticular structure located along the posterior margin of the testis. Spermatozoa produced within the tubules drain directly into the rete testis and pass out through the testicular capsule via the efferent ducts into the adjacent epididymis. The efferent ducts fuse to form the long single epididymal duct which traverses the entire length of the epididymis. Spermatozoa are carried through the epididymal duct, maturing

and becoming fully motile as they do so, and are stored until ejaculation in the caudal region of the epididymis.

The development and adult function of the testis is regulated by the gonadotropic hormones, luteinizing hormone (LH) and follicle-stimulating hormone (FSH), which are secreted by the anterior pituitary (anterior hypophysis). Both hormones are dimeric glycoproteins consisting of a shared α-subunit and dissimilar β-subunits which determine their different biological functions (Pierce, 1971). These hormones are identical to the gonadotropic hormones of the female, and have retained the names originally ascribed to them for their actions upon the female reproductive system. Spermatogenesis has an absolute requirement for androgens, specifically testosterone, which are secreted by the testicular Leydig cells under LH stimulation (Christensen and Mason, 1965; Clermont and Harvey, 1965). Androgens also are required for maintenance of the accessory sexual organs and male secondary sexual characteristics. In addition to these hormone regulatory mechanisms, there is considerable evidence that intercellular communication occurs between the two compartments of the testis, and the somatic and germinal cells within those compartments. Locally produced cytokines direct and coordinate the cellular responses of the testicular cells to the regulatory influence exerted by the pituitary gonadotropic hormones and androgens.

Most of the information that we have about the function and regulation of the testis has been derived from animal studies, but these data have been supported by the results of clinical studies. In the following discussion, where data obtained from animal experiments is discussed, it can generally be assumed that the results as described are consistent with, and applicable to, the situation in the human testis.

THE HYPOTHALAMIC-PITUITARY-TESTICULAR AXIS

Gonadotropin-Releasing Hormone and the Hypothalamus

The role of the hypothalamus in the regulation of testicular function is to integrate input from the higher brain centers and the periphery, and modulate the release of the primary reproductive neuropeptide, luteinizing hormone-releasing hormone or gonadotropin-releasing hormone (GnRH). GnRH is a decapeptide which stimulates the release and synthesis of both LH and FSH, and attempts to isolate a separate FSH-releasing hormone have not been successful. Biosynthesis of GnRH occurs in the cell bodies of neurosecretory cells which possess axonal projections into the median eminence of the hypothalamus, although significant amounts of this peptide are also found throughout the central nervous system (CNS) (Sternberger and Hoffman, 1978). GnRH is synthesized as a larger molecular weight precursor and is processed into the native peptide during axonal transport (Seeburg and Adelman, 1984). The release of GnRH by the hypothalamic neurosecretory cells is itself under neural control, chiefly by noradrenergic neurones which

exert a stimulatory effect on GnRH release (Kordon et al., 1994). However, other neurotransmitters, including dopamine, serotonin and neuropeptides, also exert both stimulatory and inhibitory effects on the GnRH-secreting cells. The influence of light, emotions and olfaction on male reproductive function are presumably mediated by the activity of these neurones.

The Anterior Pituitary and Gonadotropin Secretion

The hypothalamo-hypophyseal portal blood system transports GnRH from the median eminence to the anterior pituitary (Figure 1). Direct measurement of the levels of GnRH in the portal blood of several species indicates that it is released in a pulsatile fashion. The gonadotropins, in turn, are released in a pulsatile pattern that corresponds with the pulsatile release of GnRH (Clarke and Cummins, 1982). The initial cellular events leading to the stimulation of gonadotropin release by the anterior pituitary are the binding of GnRH to specific receptors on the plasma membrane of the gonadotropin-secreting cells, or gonadotropes (Hopkins and Gregory, 1977).

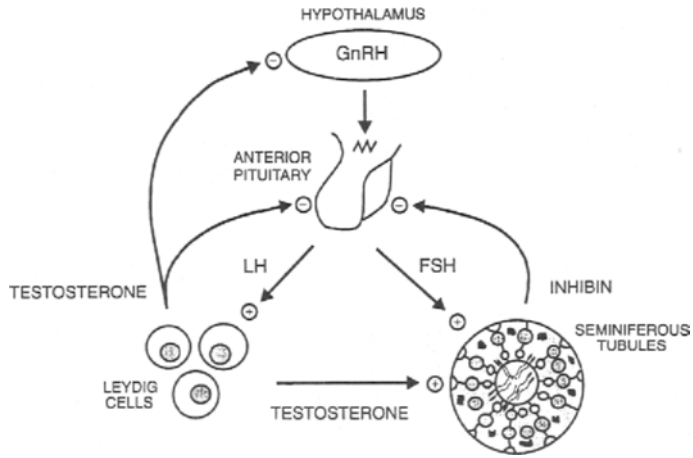

Figure 1. Schematic representation of the hypothalamic-pituitary-testis axis. Under influence of higher brain centers, the hypothalamus secretes gonadotropin-releasing hormone (GnRH) into the pituitary portal blood in a pulsatile manner to stimulate the release of luteinizing hormone (LH) and follicle-stimulating hormone (FSH). These hormones travel via the circulation to the testes where they stimulate androgen production by the Leydig cells, and germ cell development in the seminiferous tubules, respectively. Normal spermatogenesis also requires androgens (testosterone) for production of mature spermatozoa. As part of a negative feedback regulatory loop, testosterone acts at the pituitary and hypothalamic levels to inhibit LH and FSH synthesis and release, while inhibin produced by the seminiferous tubules acts predominantly on the anterior pituitary to modulate FSH.

Both LH and FSH are synthesized and released from the same cell type in the anterior pituitary, the gonadotrope, found throughout the glandular parenchyma. Immunocytochemical data has clearly demonstrated that both gonadotropins are present in the same cells (Nakane, 1970). GnRH acting upon the gonadotrope induces microaggregation of the surface GnRH-receptors, which is the key event required for its action. Microaggregation leads to secretion of both LH and FSH, as well as stimulating gonadotropin subunit mRNA synthesis and translation (Marshall et al., 1991). The action of GnRH on LH- and FSH-release is calcium-dependent and protein kinase C-independent, while the actions of GnRH on gonadotropin subunit synthesis appear to involve a separate pathway via protein kinase C activation (Conn, 1994). Differences in the amplitude and frequency of the GnRH pulses can modulate the differential synthesis of LH and FSH (Marshall et al., 1991). In general, slow frequencies favor FSH synthesis and higher frequencies are necessary to maintain LH, while the LHβ subunit is also sensitive to changes in GnRH pulse amplitude.

Once released by the pituitary, the gonadotropins enter the blood stream and travel to the testis via the peripheral circulation. At the testis level, LH acts directly on the Leydig cell to maintain its trophic characteristics and to stimulate steroidogenesis (Christensen and Mason, 1965). Both FSH and testosterone are required for maintenance of spermatogenesis, acting via specific receptors on the Sertoli cells of the seminiferous tubules (Clermont and Harvey, 1965; Cunningham and Huckins, 1979).

The Importance of Pulsatile GnRH Secretion in the Adult

The pulsatile pattern of GnRH secretion by the hypothalamus is critical to normal reproductive function. Intermittent exposure to endogenous GnRH *in vivo* and *in vitro* enhances both the responsiveness of the anterior pituitary to GnRH-stimulation, and induces an increase in GnRH receptor numbers. In contrast, continuous infusion of GnRH or the administration of large doses of GnRH or its agonists causes a reduction in GnRH receptor numbers, and a loss of responsiveness of the anterior pituitary to further stimulation by GnRH (Belchetz et al., 1978; Clayton, 1982). This "desensitization" of the anterior pituitary is primarily related to the internalization of the occupied GnRH receptors, rather than depletion of LH or FSH stores. The ultimate consequence of this desensitization is loss of fertility.

FETAL AND POSTNATAL DEVELOPMENT OF THE TESTIS

Events During Fetal Development of the Testis

In the male, the indifferent gonad begins to develop into a recognizable testis around the seventh week of gestation, with the formation of the testicular cords (Figure 2). By this time, the primordial germ cells have migrated into the genital

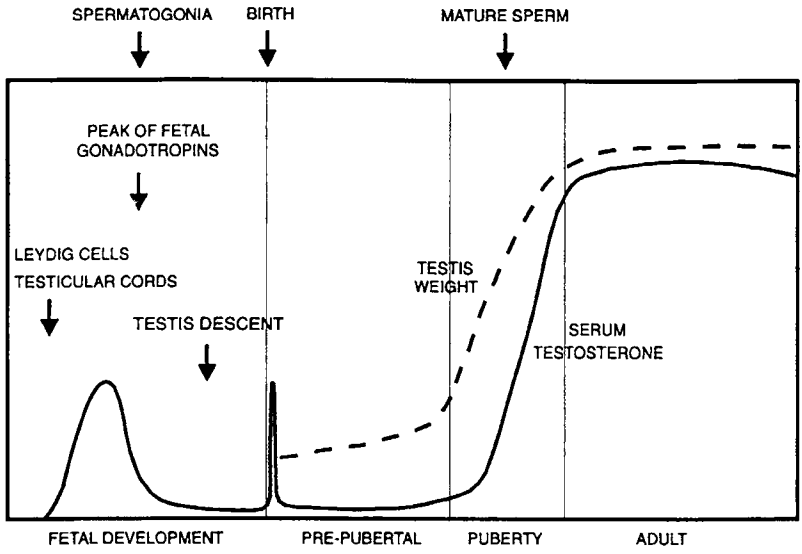

Figure 2. Summary of testicular events that occur during development and early adulthood in the human male. The relative changes in testicular weight and serum testosterone levels are shown to scale, but note that the time-scales are not consistent from different periods of life.

ridge from their original site in the yolk sac and are surrounded by supporting cells which will eventually develop into the Sertoli cells of the adult testis (Wartenberg, 1978). The germinal cells develop the characteristic appearance of spermatogonia and undergo proliferation. In contrast to the situation in the female, however, these cells do not enter into meiosis during fetal life.

The interstitial cells also proliferate, and identifiable Leydig cells fill the tissue between the cords (Pelliniemi and Niemi, 1969). These fetal Leydig cells begin to secrete androgens, reaching a peak of activity at 12–14 weeks of gestation. This peak of testosterone production is temporally related to the pattern of maternal chorionic gonadotropin (hCG) concentrations. Chorionic gonadotropin is able to bind to, and activate, the LH-receptor, suggesting that this externally derived hormone is the major stimulant of this early differentiation of the Leydig cells and androgen production in the fetus (Reyes et al., 1974; Clements et al., 1976). However, both LH and FSH can be detected in the fetal pituitary around this time, and fetal LH may be involved in stimulating Leydig cell steroidogenesis later in gestation (Hagen and McNeilly, 1975; Kaplan et al., 1976).

After this early period of development, the Leydig cells regress and become dedifferentiated (Mancini et al., 1963). Fetal pituitary activity also declines. The testicular cords remain essentially unchanged in structure throughout this period, although the germ cells and supporting cells undergo steady proliferation. At

between 27 weeks and 35 weeks of gestation the testis usually migrates down the inguinal canal to its adult position in the scrotal sac (Rajfer and Walsh, 1977; Hutson and Donahoe, 1986).

Regulation of Fetal Development of the Testis

Much of the early development of the fetal testis is genetically determined by sex-determining genes located on the Y-chromosome, in particular the sex-determining region, Y chromosome (1) SRY gene (Harley, 1993). Hormonal influences gradually take over this process as development proceeds. There is some evidence from anencephalic and hypophysectomized animals that fetal FSH may be responsible for directing the differentiation and proliferation of the supporting, pre-Sertoli cells, as well as the transformation and proliferation of primordial germ cells into spermatogonial-like cells (Beam, 1968; Gulyas et al., 1977); however, the development of the fetal testis is primarily directed by androgens and a locally produced glycoprotein hormone, Müllerian inhibitor substance or anti-Müllerian hormone (MIS or AMH) (Jost et al., 1973).

AMH is produced by the Sertoli cells during fetal life around the time of cord formation. It has been purified, cloned and sequenced, and is a glycosylated disulfide-linked protein dimer of molecular weight 140,000 (Cate et al., 1986). The main function of this hormone is to stimulate regression of the rudimentary female tract, but it may be involved in regulating other fetal testicular events, including testicular descent and the inhibition of meiosis in the fetal testis (Hutson and Donahue, 1986; Tran et al., 1986). During fetal development, androgens are primarily responsible for male genital tract development and masculinization, and are also believed to be important in testicular descent (Jost et al., 1973; Rajfer and Walsh, 1977).

GnRH immunoreactivity is detectable in the fetal hypothalamus, several weeks prior to the appearance of LH and FSH in the pituitary and development of the hypothalamo-pituitary portal system (Clements et al., 1976; Kaplan et al., 1976). The fetal pituitary is responsive to GnRH, and episodic LH secretion has been measured in the ovine fetus, indicating pulsatile release of GnRH (Clark et al., 1984). These data indicate that an intact hypothalamo-pituitary axis exists very early in development, even though gonadotropin secretion is relatively low.

Postnatal Development of the Testis

At the time of birth, there is a brief period of activation of the pituitary-testis axis, with an increase in LH, FSH and testosterone secretion, and germ cell proliferation (Faiman and Winter, 1971; Kaplan et al., 1976). After the first few months of life, however, gonadotropins and testosterone production return to very low levels. Between the neonatal period and puberty, there is little or no germ cell differentiation,

and while germ cell and Sertoli cell numbers steadily increase (Müller and Skakkebæk, 1983), Leydig cells are only occasionally observed in the testis at this time (Mancini et al., 1963).

Hypophyseal-Testicular Events at Puberty

Between the neonatal period and puberty there is a gradual continuous rise in both LH and FSH levels in serum, proliferation of germ cells and Sertoli cells, and an increase in testicular volume (Müller and Skakkebæk, 1983). The onset of puberty is indicated by an increase in nocturnal pulses of serum LH (Boyar et al., 1974), suggesting an increase in hypothalamic activity, and the pulses gradually become continual. These data suggest that puberty in the male is due to increased CNS activity leading to increased pulsatile activity of the hypothalamus, and priming of the anterior pituitary. In turn, the increased gonadotropin levels induce maturation of the androgen-secreting Leydig cells, a dramatic increase in testosterone release, and development of the accessory sexual organs and characteristics.

Under the influence of testosterone and FSH, Sertoli cell proliferation ceases before puberty and these cells develop their mature morphological characteristics. At the same time, the process of spermatogensis begins in focal locations throughout the testis, and the testicular cords develop a lumen, thereby becoming the seminiferous tubules (Müller and Skakkebæk, 1983).

THE ORGANIZATION OF THE ADULT TESTIS

The Seminiferous Epithelium

In the adult testis, the Sertoli cells rest upon the basement membrane of the seminiferous epithelium in a columnar array, their apical cytoplasm extending to the tubule lumen. The spermatogonia are arranged in a network around the base of the Sertoli cells (Fawcett, 1975). During their development, the spermatogenic cells are displaced upward through the seminiferous epithelium, between the Sertoli cells, maintaining at all times a close association with the surrounding Sertoli cell processes. The principal functions of the Sertoli cell are to act as a supportive cell for spermatogenic development, to secrete the fluid required to support the lumen of the tubule and drainage of spermatozoa towards the epididymis, and to phagocytose degenerating germ cells and the cast-off residual cytoplasm of developing spermatozoa (Jégou, 1992). However, it is generally accepted that they do much more than simply perform these housekeeping functions, possibly even directing and coordinating the process of spermatogenesis itself. The seminiferous tubules themselves are bounded by a thin layer of myoid-like peritubular cells, which have contractile properties.

Spermatogenesis and Spermiogenesis

Spermatogonia sit on the basement membrane of the seminiferous epithelium surrounding the Sertoli cells (Clermont, 1972; Fawcett, 1975). These primitive germ cells are the cellular reservoir from which mature spermatozoa develop, and must constantly divide by mitosis to maintain their numbers. Loss of spermatogonia from the epithelium will result in azoospermia and permanent sterility. Spermatogonia are often differentiated by their nuclear chromatin morphology into dark and light finely-grained type A spermatogonia, type B spermatogonia with coarse peripheral chromatin granules, and intermediate-type spermatogonia, which appear to be sequences in the development of the spermatogonia towards spermatogenesis (Clermont, 1972; Kerr, 1989). When the spermatogonia proliferate they undergo incomplete cytokinesis, remaining connected by cytoplasmic bridges.

In response to an as yet unidentified local signal, basally-situated spermatogonia cease mitotic division and enter into meiotic prophase, thereby beginning the process of spermatogenesis. The spermatogonia in any given region of the tubule enter this process at regular fixed intervals, so that the epithelium of the adult seminiferous tubules contains several different germ cell types arranged in a stratified pattern (Clermont, 1972). Each layer comprises germ cells at the same stage of differentation, and each layer represents a different successive level of development, or generation. The developing germ cells from each cluster of spermatogonia within each layer remain interconnected throughout their development by their cytoplasmic bridges.

Once the germ cells commence spermatogenic development, they are called primary spermatocytes (Figure 3). As in mitosis, the chromosomes double their chromatin content by DNA synthesis (preleptotene phase) to form chromatid pairs

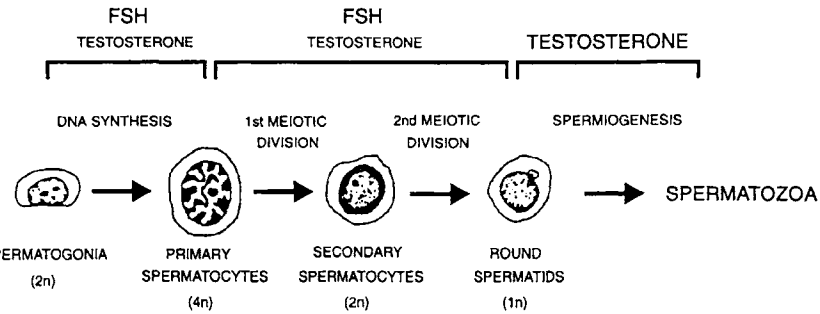

Figure 3. Representation of spermatogenic development in the human, and its hormonal correlates. Current data suggest that both FSH and testosterone are required for normal spermatogenesis, but FSH acts principally during the early events of spermatogenesis, while testosterone is less important at this time. Testosterone is required for the conversion of round spermatids into elongating spermatids (spermiogenesis).

joined together at the centromere (Kerr, 1989). Instead of dividing immediately, however, the chromosomes condense (leptotene), and each pair of homologous chromosomes comes together in the process of synapsis. During synapsis, "crossing-over" or transfer of genetic material from one chromosome to the other occurs (zygotene). This is followed by a long phase of nuclear and cytoplasmic growth (pachytene), ending with partial separation of the chromosomes (diplotene). The nuclear membrane dissolves, the homologous chromosomes separate and the cells divide, forming secondary spermatocytes which contain only one of each of the original chromosome pairs. These cells are haploid, because they contain only half the chromosome complement of a normal cell, i.e., only one copy of each maternally or paternally derived chromosome, although they still contain the same amount of DNA as a normal somatic cell. The secondary spermatocytes rapidly divide once more, and the duplicated chromatids separate to generate haploid offspring with only half the normal DNA content, the round spermatids.

The round spermatids enter into a process of extensive morphological differentiation involving elongation, nuclear condensation, development of the acrosome, removal of most of the cytoplasm, and formation of the sperm tail. This complex differentiation phase is called the process of spermiogenesis (Clermont and Leblond, 1955; Kerr, 1989). At the end of their differentiation, the spermatids have the appearance of mature spermatozoa, and are released from the apical cytoplasm of the Sertoli cell into the tubule lumen. The cast-off, or residual, cytoplasm of the spermatids is phagocytosed by the Sertoli cell, and the next generation of developing germ cells becomes the most mature germ cell type present in that region of the seminiferous epithelium.

The Cycle of the Seminiferous Epithelium

As a consequence of the fact that entry of spermatogonia into the process of spermatogenesis occurs at fixed regular intervals, and because the time taken for spermatogenic development is more or less constant, the succesive germ cell generations form specific cellular associations within the seminiferous epithelium (Clermont, 1972). This means that particular stages of germ cell development will always occur together in any given region of the seminiferous epithelium. Consequently, the seminiferous epithelium passes through a continuous series of these cellular associations, which constitutes the cycle of the seminiferous epithelium. This also means that the Sertoli cell in any given segment of tubule is in intimate contact with several different types of developing germ cells with potentially different functional characteristics and activities. There is evidence that the Sertoli cell also passes through a cycle of morphological and functional changes related to the germ cell cycle (Kerr and de Kretser, 1975; Parvinen, 1982).

Adjacent segments of the seminiferous epithelium contain successive stages of development, indicating a spatial relationship between developing clones of cells along the seminiferous tubule, sometimes called the "wave" of

spermatogenic development (Schulze, 1982). In the human testis, several waves of development appear to spiral around one another along the length of the tubule, so that a cross-section taken at any point along the tubule will reveal several different stages of the cycle. This relationship indicates that coordination of the spermatogenic process occurs both vertically through the successive layers of the seminiferous epithelium, and longtitudinally, along the length of the tubule.

The Interstitial Tissue

In between the tubules is a loose connective tissue network containing clusters of Leydig cells, as well as fibroblasts, mesenchymal-like cells and macrophages (de Kretser, 1967; Fawcett et al., 1973). This interstitial tissue also contains the testicular vasculature, its associated innervation, and the lymphatic vessels. Consequently, all hormonal communication from outside the testis, particularly blood-borne input, must pass through the interstitium before reaching the seminiferous tubules and the developing germ cells. While the capillary endothelium of the testis appears to display many characteristics of a highly selective blood-tissue barrier, many blood-borne molecules including proteins appear to have relatively free access into the interstitial space of the testis (Setchell et al., 1969). This suggests that there may be relatively little regulation of the entry of specific proteins into the testis, at least under normal conditions. However, there is considerable evidence that testicular blood flow characteristics and interstitial fluid volume are under the control of both pituitary LH and local factors produced by the seminiferous tubules (Widmark et al., 1986; Maddocks and Sharpe, 1989). Alterations of these fluid parameters may alter the concentration of active hormonal factors and nutrients within the interstitial tissue.

Compartmentalization of the Testis

With the exception of the basally situated spermatogonia and the early spermatocytes, the developing germ cells are effectively isolated from the testicular interstitium by junctional specializations between the adjacent Sertoli cell membranes in the basal region of the epithelium (Setchell et al., 1969). These junctions, together with the adventitial layer of myoid cells surrounding the seminiferous tubules, form the blood-testis barrier and effectively compartmentalize the testis into an intertubular compartment and a tubular compartment.

One important consequence of this physiological isolation of the later-stage developing germ cells is that the fluid surrounding these cells is markedly different in ionic and protein composition from the extracellular fluids of the interstitial tissue and serum (Setchell et al., 1969). This barrier also means that all nutrients and hormonal influences acting upon these germ cells have to pass through the Sertoli cell cytoplasm, implying an essential regulatory role for the Sertoli cell. Consequently,

it has long been suggested that the Sertoli cell, responding to hormonal and local stimulii, regulates and coordinates the process of spermatogenesis by creating and maintaining a specific microenvironment for the developing germ cells.

In addition to secreting the seminiferous tubule fluid, the Sertoli cell is actively engaged in modifying the content of this fluid. Potassium levels are much higher in the tubules than in either interstitial tissue fluid or blood, and are possibly maintained by the action of a Na^+-K^+ translocating ATPase pump located on the Sertoli basement membrane (Jégou, 1992). Protein levels in the tubule fluid are low, and it has been suggested that most, if not all, of the proteins in tubule fluid may be produced by the Sertoli cells. The proteins secreted by the Sertoli cell include an androgen-binding protein (ABP), which is distinct from the circulating sex hormone binding globulin (Hagenäs et al., 1975), as well as other transport proteins for essential cellular nutrients: transferrin (iron-transport), ceruloplasmin (copper-transport), retinol-binding protein, an albumin-like protein, and α2-macroglobulin (Griswold, 1988). In addition, Sertoli cells convert acetate to lactate and pyruvate, the preferred energy substrates for spermatocytes and spermatids (Jutte et al., 1983). The regulatory role of the Sertoli cell is supported by the observation that they also produce and secrete several growth factors, steroids and polyamines (Christensen and Mason, 1965; Jégou, 1992).

TESTICULAR STEROIDOGENESIS

The Leydig Cell

The Leydig cell is the most prominent cell type found in the adult intertubular tissue. These cells are epithelioid in shape and are generally found in clusters associated with the lymphatics and blood vessels. They possess the characteristic appearance of steroidogenically active cells, including prominent mitochondria with tubular cristae, abundant smooth endoplasmic reticulum and lipid droplets. The principal function of the Leydig cell is the synthesis of androgens, and this cell represents the chief source of testosterone production in the adult testis (Morris and Chaikoff, 1959; Christensen and Mason, 1965).

Production of Testicular Steroids

In the Leydig cell, cholesterol is synthesized *de novo* from acetate in the endoplasmic reticulum and mitochondria (Morris and Chaikoff, 1959). Some cholesterol may also be derived from lipoprotein sources, particularly under rate-limiting conditions of high steroid output *in vitro*, although the significance of this source *in vivo* remains unknown (Hedger and Risbridger, 1992). The pathway of androgen synthesis by the Leydig cell has been well-documented (Hall, 1994) (Figure 4). The cholesterol side-chain is removed by a cytochrome P450-containing enzyme com-

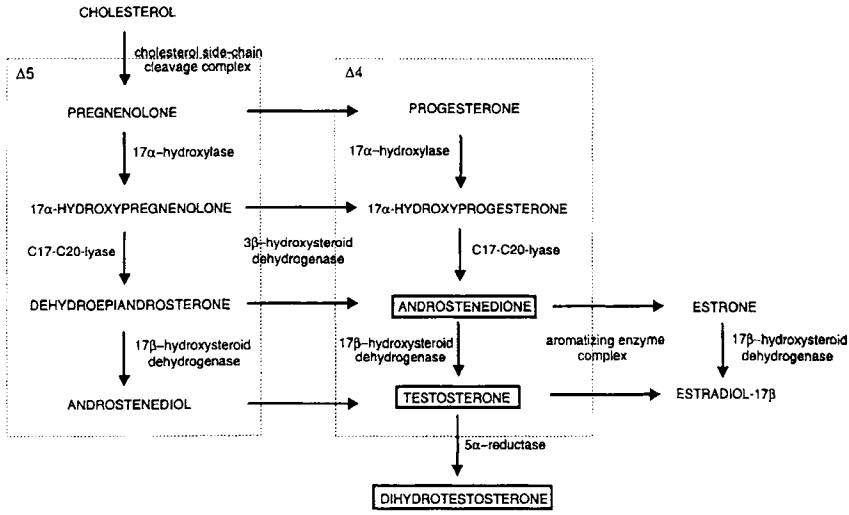

Figure 4. Synthesis of androgenic steroids in the human testis. In the human, the predominant pathway involves the Δ^5 intermediates, via dehydroepiandrosterone. The major androgens produced are androstenedione and testosterone. Testosterone can be further metabolized to the more potent androgen, dihydrotestosterone, by 5α-reductase in peripheral tissues, or to estradiol-17β, by the aromatizing enzyme complex.

plex within the inner mitochondrial membrane. The resulting pregnenolone passes back into the cytosol for conversion to testosterone via a series of enzymes bound to the endoplasmic reticulum. Biosynthesis of testosterone proceeds via Δ^5 (double bond in the B ring) intermediates (pregnenolone → 17α-hydroxy pregnenolone → dehydroepiandrosterone → 5-androstene-3β,17β-diol) or Δ^4 (double bond in the A ring) intermediates (progesterone → 17α-hydroxy progesterone → 4-androstene-3,17-dione), but in the human the Δ^5 pathway appears to be more important as progesterone levels are non-detectable.

The conversion of the less active Δ^5 precursors (pregnenolone, dehydroepiandrosterone and 5-androstenediol) to the more active Δ^4 steroids involves oxidation of the 3β-hydroxyl group to a ketone and rearrangement of the double bond by Δ^5-Δ^4 isomerase activity by a single enzyme, 3β-hydroxysteroid dehydrogenase (Lorence et al., 1990). Studies on the microsomal enzymes have indicated that a single cytochrome P-450-containing enzyme complex catalyzes the conversion of pregnenolone/progesterone to their 17α-hydroxy forms and then to dehydroepiandrosterone or androstenedione (C_{17}-C_{20} lyase), respectively (Nakajin et al., 1981). The enzyme 17β-hydroxysteroid dehydrogenase catalyzes the conversion to androstenediol (Δ^5) and testosterone (Δ^4). In the adult testis, the Leydig cell also produces estrogens, principally estradiol in the human, via the action of the aromatizing enzyme system (Payne et al., 1976).

Regulation of Steroid Production

The principal hormonal control of the Leydig cell is via LH, which acts through specific high-affinity receptors on the plasma membrane. This hormone is also essential for the development and maintenence of Leydig cell morphology (de Kretser, 1967) and for maintaining and regulating the enzymes involved in steroidogenesis (Hall, 1994). The LH receptor has been extensively studied in recent years. It belongs to the seven-transmembrane helix-G protein coupled receptor family, and posseses a very long (64 kDa) extracellular domain which is the site of LH binding (McFarland et al., 1989). The action of this hormone involves activation of a membrane G protein coupled to adenylyl cyclase and protein kinase A-mediated protein phosphorylation of proteins. However, other signal tranduction pathways also appear to be involved, particularly at lower LH concentrations, including Ca^{2+}, phospholipids and protein kinase C, and arachidonic acid metabolites (Nikula and Huhtaniemi, 1989; Cooke, 1990). The steps of the steroidogenic pathway which appear to be regulated by LH action are the transport of cholesterol into the mitochondria and the activity of the cholesterol side-chain cleavage complex (Hall and Young, 1968). However, the intracellular levels of the other steroidogenic enzymes are also LH dependent.

Leydig Cell Downregulation

Overstimulation of the Leydig cell by exogenous LH or hCG leads to a refractory period during which the Leydig cells are insensitive to further stimulation *in vitro* and *in vivo*. This desensitization is accompanied by a loss of LH-receptors from the cell surface, uncoupling of the G-protein-adenylyl cyclase transducing mechanism and reduced transcription of RNA species encoding the active receptor, mediated via a cAMP-mediated mechanism (Wang et al., 1991). Several other factors have been implicated in LH/hCG-induced Leydig cell downregulation, particularly increased oxygen-mediated damage to the P-450 enzymes, specific negative feedback on steroidogenesis and exhaustion of cholesterol stores (Dufau, 1988), although these mechanisms may actually be more important under *in vitro* conditions.

The Role of Other Pituitary Hormones

While replacement of LH alone can maintain the normal steroidogenic function of the Leydig cell in the hypophysectomized rat (El Safoury and Bartke, 1974; van Beurden et al., 1976; Zipf et al., 1978) there is evidence that other anterior pituitary hormones may also be involved in its control. If the testis is allowed to regress following hypophysectomy, FSH alone, or in synergism with LH, has the capacity to stimulate testicular steroidogenesis and LH-receptor numbers (El Safoury and Bartke, 1974; McNeilly et al., 1979). However, as the Leydig cell does not appear to possess FSH receptors, the effects of FSH on Leydig cell function are probably indirect, and mediated through the Sertoli cell (Orth and Christensen, 1977).

In contrast to FSH, the Leydig cells do possess functional receptors for another anterior pituitary hormone, prolactin (Charreau et al., 1977). Prolactin appears to be involved in the regulation of LH-receptor numbers and in augmentation of the steroidogenic responsiveness of the Leydig cell to LH (Zipf et al., 1978). In contrast, high levels of prolactin have an inhibitory effect on androgen production by the Leydig cell (Purvis and Hansson, 1978). However, while hyperprolactinemia is accompanied by testicular dysfunction, it is not clear whether these effects involve a direct action of prolactin on the testis, or effects on pituitary gonadotropin secretion.

Growth hormone is yet another anterior pituitary hormone which has a stimulatory effect on Leydig cells especially during pubertal maturation, most probably acting through intratesticular insulin-like growth factor-1 (IGF-1) production (Zipf et al., 1978; Chatelain et al., 1991). In addition, the posterior pituitary hormone, arginine vasopressin, has an inhibitory action on testosterone production by the Leydig cell (Kasson et al., 1986), although this hormone also appears to be synthesized within the testis and its role may be as a paracrine control mechanism.

In addition to external regulation, there is evidence for autoregulation of Leydig cell function (Hedger and Eddy, 1990). Androgens, themselves, act as an ultrashort feedback loop on Leydig cell steroidogenesis, and androgen receptors have been found in the Leydig cell (Wilson and Smith, 1975). Estrogens also have an inhibitory action on Leydig cell steroidogenesis, and aromatization of testosterone to estradiol-17β in the Leydig cell has been implicated in the mechanism of Leydig cell down-regulation after hyperstimulation with LH (van Beurden et al., 1976). These data suggest that autoregulation of steroidogenesis within the Leydig cell may be a physiological mechanism for controlling androgen production by the testis.

HORMONAL CONTROL OF SPERMATOGENESIS

The principal hormone involved in spermatogenic development is testosterone produced by the Leydig cells. In the hypophysectomized immature rat, testosterone alone is required for the initiation of spermatogenesis and subsequent germ cell development up to the early spermatid stage (Clermont and Harvey, 1965). Qualitatively normal spermatogenesis, once initiated in the presence of LH and FSH, can also be maintained in gonadotropin-deprived animals by high testicular concentrations of exogenous testosterone alone, providing the administration of testosterone begins immediately following gonadotropin withdrawal (Davies et al., 1974).

The Sertoli cell possesses receptors for androgen (Sanborn et al., 1977) and constitutes the primary target for testosterone and other androgens within the seminiferous epithelium. It has not been established if the germ cells are an additional target for androgen action, and it is believed that the effects of androgens on spermatogenesis are mediated almost exclusively via the Sertoli cells. High-affinity re-

ceptors for FSH are also found on the Sertoli cells, and these cells are responsive to stimulation by FSH (Hagenäs et al., 1975). The FSH receptor has been cloned, and belongs to the same G protein-coupled receptor family as the LH receptor (Sprengel et al., 1990). There is considerable evidence that fluid and protein production by the Sertoli cells *in vitro* is under hormonal regulation by FSH, but the vast majority of studies have used Sertoli cells from immature animals for these studies. Recent development of cultures of functional adult rat Sertoli cells may help to extend this information into the adult situation (Wright et al., 1989; Simpson et al., 1992).

The Relative Importance of Testosterone and FSH in Adult Spermatogenesis

While FSH is essential for the completion of the initial spermatogenic wave in the developing testis and for restoration of spermatogensis following complete regression of the seminiferous epithelium (Steinberger and Duckett, 1967; Lostroh, 1969), the importance of FSH in the maintenance of adult spermatogenic function has not been so well-defined (Figure 3). However, *in vitro* studies have shown that FSH receptor numbers vary during the spermatogenic cycle in the rat (Parvinen, 1982), and that cultures of adult seminiferous tubules and isolated Sertoli cells from mature testes are FSH-responsive (Gonzales et al., 1988; Wright et al., 1989; Simpson et al., 1992). In primates, passive immunization against FSH causes a significant decrease in spermatogenesis (Murty et al., 1979; Srinath et al., 1983), while Cunningham and Huckins (1979) showed that in hypophysectomized rats, FSH could stimulate spermatogenesis above the level provided by testosterone replacement alone. These studies have provided convincing evidence for the role of FSH in maintaining spermatogenic development in the adult testis.

It has long been known that the levels of testosterone inside the testis are several times higher than those found in the periphery and accessory organs. While it was originally believed that these high levels were essential, spermatogenesis can be maintained in the rat with levels of testosterone that are much lower than those found inside the testis, even when FSH levels are low (Sharpe et al., 1987). Moreover, it was shown that by giving large doses of testosterone by injection into rats deprived of endogenous testosterone by treatment with a Leydig cell-specific cytotoxin, ethane dimethane sulfonate (EDS), almost completely normal spermatogenesis could be maintained, providing a critical intratesticular concentration was achieved (Sharpe et al., 1987). More recent data support a role for both FSH and testosterone: FSH maintains the levels of the earlier stages of spermatogenesis, chiefly spermatogonial renewal, while testosterone acts principally on the process of spermiogenesis at the level of conversion of round spermatids to spermatozoa (Sun et al., 1990). It is generally accepted, however, that both hormones act principally through the Sertoli cell, and can also to some extent compensate for one another (de Kretser et al., 1992).

Posttesticular Events and the Reproductive Tract

In contrast to events within the testis, the hormonal regulation of later sperm development events have received relatively little attention. Maturation of the sperm and attainment of motility occurs principally in the epididymis, and this organ is dependent upon testosterone support (Dyson and Orgebin-Crist, 1973; Foldesy and Bedford, 1982). However, there is evidence that additional testicular factors may be important in later sperm development and epididymal function.

FEEDBACK CONTROL OF THE HYPOTHALAMO-HYPOPHYSEAL AXIS

The Role of Testicular Steroids

Castration leads to increased secretion of gonadotropins by the anterior pituitary, which can be reversed by the administration of testicular steroids (Swerdloff and Walsh, 1973). This indicates the existence of a steroid-mediated negative-feedback mechanism on the hypothalamus-anterior pituitary axis (Figure 1). This feedback is mediated, principally, by testicular androgens (Naftolin and Feder, 1973). Estradiol-17β, produced in the testis or by aromatization of androgens in the CNS and peripheral tissues, is also a potent inhibitor of gonadotropin secretion in the male (Swerdloff and Walsh, 1973; Santen, 1977). Divergent actions of testosterone and estradiol on the hypothalamo-pituitary axis have been indicated by the observation that testosterone decreased LH pulse frequency and amplitude when infused into normal men, while estradiol decreased pulse amplitude and had no effect on pulse frequency (Santen, 1977). Moreover, estradiol inhibited the response of the anterior pituitary to GnRH, while testosterone had no effect, in contrast to *in vitro* results. These observations suggest, in the human, that testosterone acts at the hypothalamic level to inhibit GnRH release, while estradiol acts principally on the anterior pituitary and LH/FSH. However, the relative importance of androgens and estradiol in the control of gonadotropin secretion in the male remains to be clarified.

During the pre-pubertal period, the pituitary appears to be particularly sensitive to androgen feedback, leading to very low LH and FSH levels in the face of low serum testosterone (Faiman and Winter, 1971). One of the key events at puberty appears to be a major change in the sensitivity of the hypothalamic-pituitary axis to testosterone. Post-puberty, the adult mechanism of testosterone modulation of LH and FSH develops.

Steroids have a dual effect on gonadotropin secretion. They inhibit GnRH release by the hypothalamic neurosecretory cells and act directly on the pituitary to regulate gonadotropin subunit synthesis. Castration in male rats leads to an increase in both α-subunit and LH and FSH β-subunit mRNA levels, which in the case of LH, at least, can be reversed by providing testosterone (Marshall et al., 1991). FSH β-subunit synthesis, however, does not appear to be as effectively controlled by tes-

tosterone alone (Marshall et al., 1991), and may actually be stimulated by testosterone at the pituitary level (Gharib et al., 1990).

Nonsteroidal Feedback

Evidence for a separate regulation of FSH by factors other than androgen has been accumulated over a period of many years. While the inhibitory action of testosterone on LH secretion has been clearly demonstrated, comparatively high levels of this steroid are required to suppress FSH secretion (Swerdloff and Walsh, 1973). In men with infertility, a negative relationship between degree of seminiferous epithelial damage and serum FSH levels has been observed (de Kretser et al., 1972). In adult rats, destruction of the seminiferous epithelium by a number of procedures which do not alter serum testosterone levels causes a selective elevation of plasma FSH (Rich and de Kretser, 1977). These studies indicate that the seminiferous tubules produce a factor which selectively inhibits FSH secretion by the anterior pituitary.

The active factor has been isolated and is a glycoprotein hormone called inhibin. Inhibin comprises a heterodimer of two disulphide-linked homologous subunits (α and β), and preferentially suppresses pituitary FSH secretion *in vivo* and *in vitro* (Robertson et al., 1985). Activin is a homodimer of the β-subunits which opposes the action of inhibin by stimulating FSH secretion (Ling et al., 1986; Vale et al., 1986). Two β-subunit genes have been identified (β_A and β_B), and their products can combine with the α-subunit or each other to form inhibin-A ($\alpha\beta_A$) and inhibin-B ($\alpha\beta_B$), or activin-A ($\beta_A\beta_A$), activin-B ($\beta_B\beta_B$) and activin-AB ($\beta_A\beta_B$), respectively. Both inhibin, activin and their receptors display considerable structural and sequence homology with the transforming growth factor-β (TGF-β) family of growth factors and the TGF-β receptor family (Mathews and Vale, 1991). Inhibin and activin are produced by Sertoli cells from both immature and adult testes (Risbridger et al., 1989; de Winter et al., 1993). In addition, inhibin and activin subunit proteins and mRNA have been confirmed in tumor and immature Leydig cells *in vitro*, although production by adult Leydig cells of the β-subunit, or β-subunit containing proteins, remains controversial (Lee et al., 1989; de Winter et al., 1992).

While a clear role for inhibin in the regulation of FSH in the female and in the prepubertal male testis has been established, the role of inhibin in the adult male remains to be clarified. Levels of inhibin rise during puberty in parallel with LH, FSH and testosterone (Burger et al., 1988). However, studies have shown no decrease in immunoreactive serum inhibin levels in men with varying degrees of testicular dysfunction or damage (de Kretser et al., 1989). Part of the problem lies with the nature of the molecules and the immunoassay methods employed to measure them. The antisera currently in use cross-react with the inhibin α-subunit, and nonbioactive inhibin α-subunit proteins appear to circulate in the blood, while the sensitivity of the current bioassays means that they cannot be applied to biological fluids.

Following passive immunization with inhibin antiserum, FSH levels are elevated in immature, but not adult, rats (Culler and Negro-Vilar, 1988), although in animals in

which EDS has been used to remove the Leydig cells and in male monkeys, inhibin antiserum does cause a specific increase in FSH (Culler and Negro-Vilar, 1990; Medhamurthy et al., 1990). This is consistent with the fact that, in EDS-treated adult rats, serum FSH levels rise but not into the castrate range, indicating the involvement of an additional non-Leydig cell-derived factor in the control of FSH. In the primate, both LH and FSH levels rise after castration, but only LH is regulated by testosterone administration (Dubey et al., 1987). However, an inhibin-containing preparation did prevent the rise in FSH after castration of rhesus monkeys receiving an intravenous GnRH-infusion (Abeywardene and Plant, 1989). Thus, a role for inhibin in the adult is clearly indicated, and it is anticipated that the development of assays that are specific for the bioactive inhibin molecule will resolve this controversy.

While the role of inhibin is indirectly indicated, the role of activin as a circulating hormone from the testis controlling FSH is more uncertain. In addition to their functions in FSH-regulation, however, both inhibin and activin have a broad range of actions on growth and differentiation of several different cell and tissue systems. Local regulatory roles in the gonads, on steroidogenesis and germ cell development, also have been demonstrated (Hsueh et al., 1987; Mather et al., 1990). Immunomodulatory actions of these proteins *in vitro* indicate that they may be involved in regulating immune responses within the testis (Hedger et al., 1989). Recent reports indicate that another gonadal protein with FSH-regulating activity, follistatin, is involved in regulating the bioactivity of activin, by acting as a specific activin-binding protein (Mather et al., 1993). The testicular source of this protein has yet to be established, although follistatin mRNA is present in the testis (Michel et al., 1990). It now appears that the most significant role of these proteins in the testis may be in regulating local communication within the testis.

The Role of Activin in the Anterior Pituitary

While the role of activin as a circulating regulator of gonadotropin secretion is uncertain, recent studies have indicated that activin is a locally produced endogenous regulator of FSH secretion by the pituitary. The mRNA for inhibin α-and β-subunits has been demonstrated in the pituitary (Meunier et al., 1988), and the subunits have been localized to the gonadotropes by immunocytochemistry (Roberts et al., 1988). Secondly, FSH secretion decreases following addition of activin-B antibody to anterior pituitary cell cultures (Corrigan et al., 1991). This may explain why, in the absence of GnRH, the pituitary continues to secrete FSH, while LH secretion declines (Farnworth et al., 1988).

COMMUNICATION BETWEEN CELLS IN THE TESTIS

The regulation of spermatogeneis by testosterone is an example of local control of one compartment of the testis by another. However, there is considerable local regu-

lation of the responsiveness of the testis, involving communication between the compartments and the cells within the compartments (Figure 5). These autocrine and paracrine interactions are likely to be just as important to normal testicular function as the classical central endocrine control mechanisms involving gonadotropins and testosterone.

Seminiferous Tubule–Leydig Cell Interactions

There is considerable evidence that the seminiferous tubules secrete factors which modulate steroidogenesis by the Leydig cells. Damage to the tubules causes localized hypertrophy of the Leydig cells, indicating a direct communication responsible for stimulating intratesticular androgen levels under these conditions (Aoki and Fawcett, 1978). As already described above, FSH also has an indirect stimulatory effect on Leydig cell steroidogenesis. Both Leydig cell stimulatory and inhibitory factors have been identified in Sertoli cell and tubule cultures and in the testicular interstitial fluid, and the overall stimulatory activity of these factors is increased in conditions of germ cell damage and by FSH-treatment *in vitro* (Benahmed et al., 1985; Hedger et al., 1994). The nature of these factors remains to be established, although numerous factors known to be produced by the Sertoli cells which modulate Leydig cell steroidogenesis *in vitro*, including interleukin-1 (IL-1), activin, and inhibin, IGF-1 and basic fibroblast growth factor (bFGF), may be involved (Verhoeven, 1992).

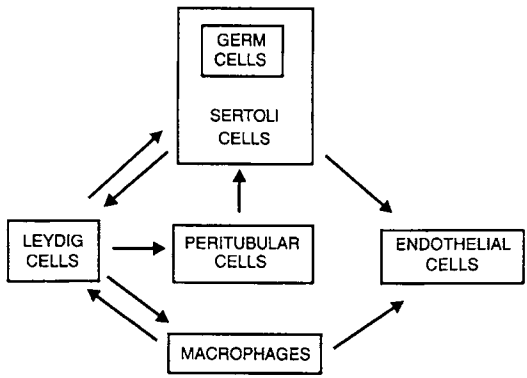

Figure 5. Intercellular interactions which have been identified within the testis. These are discussed in more detail in the text. Testosterone produced by the Leydig cells stimulates Sertoli cell function and production of a Sertoli cell-modulating protein by the peritubular cells (P-mod-S). The Sertoli cell, in turn, secretes factors which modulate testosterone production by the Leydig cells. Resident macrophages produce factors which have a direct effect on Leydig cell steroidogenesis, and which may be critical during intratesticular inflammation. Both macrophage and seminiferous tubule factors influence endothelial cell permability, controlling fluid volume and the concentration of regulatory factors and nutrients in the interstitial fluid.

Steroid-Germ Cell Interactions

The close association between the Sertoli cell and developing germ cells, and the observation that Sertoli cell functions are affected during the spermatogenic cycle and by germ cell depletion have led to studies trying to identify factors involved in communication between these cells. Communication by direct contact between these cells is implied by surface specializations between the Sertoli cells and spermatocytes/spermatids. Evidence for direct communication from the germ cells to the Sertoli cells, however, has been by inference from studies of the effects of germ cell depletion on Sertoli cell function, rather than detection of specific factors or molecules. Nonetheless, germ cells do secrete factors which influence Sertoli cell functions *in vitro*. Spermatogenic cell products which affect Sertoli cell functions, identified in recent years, are IL-1, bFGF and nerve growth factor (Ayer-Le Lievre et al., 1988; Mayerhofer et al., 1991; Haugen et al., 1994).

Although control is exerted by the Sertoli cells over germ cell development by transport proteins and nutrients, there is also evidence for paracrine control of germ cell development involving specific cytokines. These Sertoli cell products include IGF-1, which potenially acts on meiotic cells via specific receptors (Vannelli et al., 1988), inhibin and activin which have opposing actions on spermatogonial proliferation (Mather et al., 1990), and the stem cell factor (SCF) which interacts with its receptor (c-Kit) on type A spermatogonia (Rossi et al., 1993). In mutant mouse strains deficient in SCF, germ cells degenerate around the time of birth, and these animals are sterile (Brannan et al., 1992), while administration of an antibody to c-Kit prevents spermatogonial type A proliferation in mice (Yoshinaga et al., 1991).

Peritubular-Sertoli Cell Interactions

In addition, the peritubular cells which are androgen-responsive have an influence on Sertoli cell function. A specific protein secreted by the peritubular cells under androgen regulation stimulates protein secretion by the Sertoli cells, and is called P-Mod-S (Skinner and Fritz, 1985). This factor presumably mediates communication between these two cell types *in vivo*. Moreover, both cell types produce the extracellular matrix components of the basement membrane, which determines many of the vectorial functions of the Sertoli cell, and has a direct effect on the cAMP response of the Sertoli cell (Dym et al., 1991).

Importance of Paracrine and Autocrine Mechanisms in the Testis

In fact, there is evidence for direct communication between all the somatic and spermatogenic cell types within the testis, and an ever-increasing list of cytokines have been identified as playing a potential role in this communication. While all of these factors may play a role under certain conditions, there is evidence from mu-

tant models that at least one of these growth factors, c-Kit/SCF, appears to be essential for fertility (Brannan et al., 1992). Whether essential or not, however, there is no doubt that local communication is an important part of regulating moment to moment functioning of the testis.

INFLUENCE OF OTHER ORGANS ON TESTICULAR FUNCTION

In addition to the pituitary, other organ systems have a direct effect on testicular function. Of these, the most important, or at least the best studied, are the adrenal glands and the immune system. Glucocorticoids inhibit Leydig cell and pituitary function, and may mediate the effects of stress on testosterone production (Bambino and Hseuh, 1981). In systemic and testicular inflammation, Leydig cell function is also down-regulated (Adamopoulos et al., 1978), possibly due to the production of inflammatory cytokines by intratesticular macrophages, such as IL-1 and tumor necrosis factor-α which negatively regulate steroidogenesis (Calkins et al., 1990). In turn, there is increasing evidence that testicular secretions influence the functions of macrophages and lymphocytes within the intertubular tissue (Hedger et al., 1989; Wang et al., 1994).

The importance of the thyroid to testicular function, particularly during development, has also been indicated by the observation that testis size, and hence the spermatogenic potential of the testis, is enhanced by experimental hypothyroidism in developing rats (Cooke and Meisami, 1991). In addition, although it has had very little study, neural input can be expected to have an effect, particularly on vascular functions of the testis, and serotonergic as well as adrenergic innervation of the testis has been described (Davis, 1992). Thus, as would be expected, the testis does not function in isolation from the remainder of the body, and is affected by changes in other organ systems.

DISORDERS OF THE PITUITARY-TESTIS AXIS

Testicular dysfunction can occur due to both endocrine and non-endocrine factors, and manifests as reduced fertility or sterility. Common non-endocrine causes of testicular failure are physical trauma, vascular damage, immunological responses to sperm, obstruction or absence of the reproductive tract, and cytotoxic agents.

Cryptorchidism, or the failure of the testes to descend during fetal or postnatal life, is a common cause of male infertility. This descent is believed to be a hormone-dependent event requiring androgens and, possibly, anti-Müllerian hormone. If not corrected early in postnatal life, this condition leads to sterility, as spermatogenesis cannot proceed at normal body temperature. Another primary endocrine cause of infertility in men is Leydig cell failure. This is often associated

with the chromosomal aberration, Klinefelter's syndrome (XXY), or cytotoxic agents, and is usually untreatable (Baker, 1986). Similarly, androgen insensitivity due to spontaneous mutations in the androgen receptor gene will also result in permanent spermatogenic failure (Griffin and Wilson, 1984).

Hypogonadotropic hypogonadism, or secondary testicular failure, is due to low levels of gonadotropin secretion, and is usually detected early in life as delayed puberty. This condition can often be reversed by treatment with GnRH agonists or urinary gonadotropins (Burger and Baker, 1984). Other causes of pituitary gonadotropin deficiency are congenital adrenal hyperplasia and prolactin-secreting tumors, leading to reduced pituitary LH secretion and Leydig cell function (Wischusen et al., 1981; Davis, 1982).

At present, however, more than half of all cases of male infertility are due to unexplained germ cell failure (idiopathic azoospermia), critically reduced sperm number (oligospermia), or aberrent sperm movement (asthenozoospermia) or morphology (teratospermia). Most of these conditions do not have a primary or obvious endocrine cause. While the evidence is indirect, it remains likely that many of these conditions of spermatogenic arrest or dysfunction may be due to the failure of crucial intragondadal regulation mechanisms. Considerable further investigation of the local testicular control mechanisms is necessary, if treatment of these conditions is to become possible (See Chapter 17).

SUMMARY

The development and adult function of the testis is directed by gonadotropin secretion from the anterior pituitary, stimulated by pulsatile release of the hypothalamic decapeptide, GnRH. The gonadotropins, LH and FSH, act on the Leydig cells, and the Sertoli cell, respectively, to direct differentiation of the testis during fetal and prepubertal life, and to regulate the two functions of the adult testis, androgen production and spermatogenesis. Spermatogenesis in the adult is primarily directed by androgens produced by the Leydig cells under LH-stimulation, although FSH in the adult appears to regulate early spermatogenic events. Negative feedback control of GnRH and gonadotropin secretion occurs via testosterone, and a protein hormone called inhibin. In addition, there is considerable local control and integration of these functions involving other cytokines and local factors.

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Chapter 3

Hypophyseal-Ovarian Relationships

JAMIL MROUEH and DOUGLAS R. DANFORTH

INTRODUCTION

The endocrine regulation of reproductive function involves multiple feedback loops coordinating the hypothalamus, pituitary, and ovary. Each of these organs

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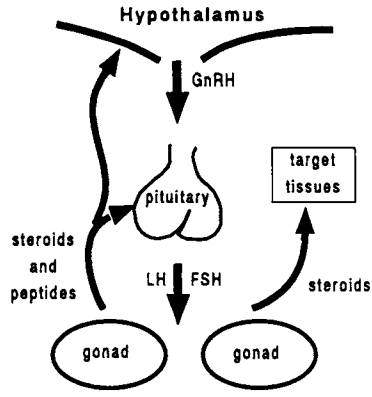

Figure 1. Hypothalamic-pituitary-ovarian axis. GnRH released from the hypothalamus stimulates LH/FSH release from the pituitary. In turn, pituitary gonadotropins stimulate the endocrine and gametogenic functions of the gonads. Steroid and protein hormones secreted by the gonads feed back to the hypothalamus and pituitary to regulate GnRH and LH/FSH secretion.

plays a vital role in the successful establishment and maintenance of normal reproductive function. The hypothalamus secretes releasing and inhibiting factors which modulate the release of pituitary hormones. These in turn directly regulate the gametogenic and secretory functions of the ovary. In response, the ovarian secretory products (steroid and non-steroidal hormones) act on the hypothalamus and pituitary, closing the feedback loop for maintenance of reproductive function (Figure 1).

During the past two decades our knowledge has greatly advanced regarding the complex interactions of the hypothalamus, pituitary, and ovaries which regulate the normal menstrual cycle. In addition, our understanding of the pathophysiology of the hypothalamic-pituitary-ovarian axis has resulted in a variety of new treatment modalities for reproductive disorders and problems of infertility. In this chapter we will discuss the basic tenets of hypophyseal-ovarian relationships from fetal life through menopause. In addition, at the end of the chapter we include clinical correlates which highlight fundamental aspects of hypothalamic-pituitary-ovarian function.

HYPOTHALAMIC-PITUITARY AXIS OVERVIEW

The hypothalamus is located at the base of the brain and serves as the major pathway between the brain and the pituitary gland. It is extensively connected with other brain areas and thus has a vital role as an endocrine, autonomic, and behavioral organ. The hypothalamus is arbitrarily divided into three longitudinal zones; a lateral zone, a medial zone, and a periventricular zone. These zones are further divided into

nuclei which are neuronal populations with relatively distinct functions. The nuclei of the lateral zone connect the limbic and brain stem areas with each other and with the medial zone. The medial and periventricular zones are more specialized for neuroendocrine and visceral functions. The hypothalamus regulates the pituitary gland by two distinct mechanisms, neural and endocrine (Figure 2).

The axons of the hypothalamic neurons regulate the posterior pituitary, or neurohypophysis via the neural pathway. Neurons in the paraventricular and supraoptic nuclei of the hypothalamus project through the median eminence and release vasopressin (also known as antidiuretic hormone, or ADH), oxytocin, and neurophysin from their axon terminals into the neurohypophysis. Neurophysins's only known function is the transport of oxytocin and ADH. Both oxytocin and vasopressin (and their respective neurophysins) are cleaved from larger precursor molecules during axonal transport to the neurohypophysis. Oxytocin causes smooth muscle contractions and plays an important role in breastfeeding and the conduct of labor. Nipple stimulation during nursing initiates the release of oxytocin from the neurohypophysis, which stimulates the myoepithelial cells in the mammary gland to contract and initiate milk let-down. Oxytocin stimulates myometrial contractions, and while im-

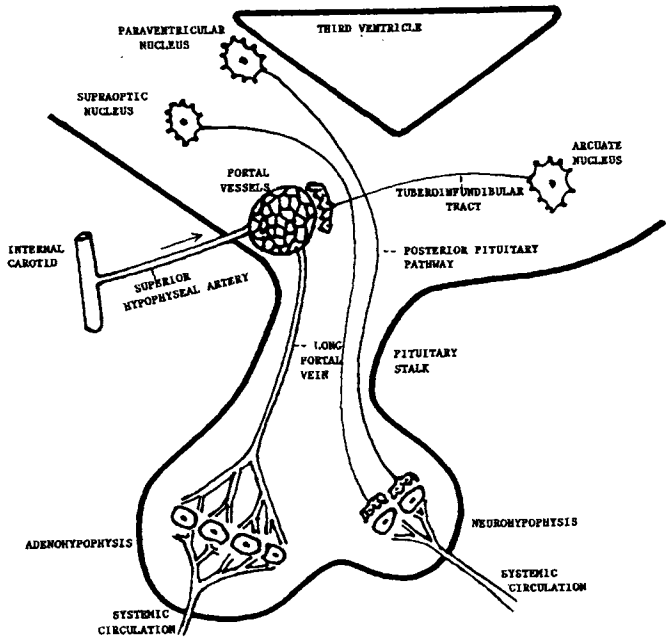

Figure 2. Hypothalamic control of pituitary function. The posterior pituitary hormones are synthesized in the hypothalamus and released at the axon terminals in the posterior pituitary. The synthesis and secretion of anterior pituitary hormones are regulated by releasing and inhibiting hypothalamic hormones secreted into the portal circulation.

portant for the maintenance of labor, it is probably not involved in the initiation of labor. Oxytocin is also produced by the ovary and high concentrations are present in follicular fluid and the corpus luteum, where it may modulate progesterone production. Oxytocin is also found in large concentrations in the oviductal tissue, and is thought to be involved in oviductal contractility. The primary physiologic function of vasopressin is the regulation of osmolality and blood volume. Vasopressin also acts on the pituitary gland and causes a decrease in prolactin secretion. Vasopressin is also found in the ovary, where it may play a role in the control of ovarian microcirculation.

The principal pathway of hypothalamic control of the pituitary involved in the regulation of ovarian function is via the portal vascular system providing the delivery of releasing hormones from the brain to the anterior pituitary, or adenohypophysis. Neurons in the hypothalamus secrete a variety of releasing hormones which regulate the secretion of anterior pituitary hormones (Table 1).

These regulating hormones flow downward along the nerve fibers and enter the microcirculation at the base of the hypothalamus. The capillaries converge and

Table 1. Hypothalamic Factors and Hormones

Hormone	*Structure*	*Pituitary Hormones Affected*
Corticotropin-releasing hormone (CRH)	SER-GLN-GLU-PRO-PRO-ILE-SER-LEU-ASP-LEU-THR-PHE-HIS-LEU-LEUARG-GLU-VAL-LEU-GLU-MET-THR-LYS-ALA-ASP-GLLN-LEU-ALA-GLN-GLN-ALA-HIS-SER-ASN-ARG-LYS-LEU-LEU-ASP-ILE-ALA-NH_2	Adrenocorticotropin β-Endorphins β Lipotropin α Lipotropin
Gonadotropin-releasing hormone (GnRH)	pGLU-HIS-TRP-SER-TYR-GLY-LEU-ARG-PRO-GLY-NH_2	Luteinizing hormone Follicle stimulating hormone
Growth hormone-releasing hormone (GHRH)	TYR-ALA-ASP-ALA-ILE-PHE-THR-ASN-SER-TYR-ARG-LYS-VAL-LEU-GLY-GLN-LEU-SER-ALA-ARG-LYS-LEU-LEU-GLN-ASP-ILE-MET-SER-ARG-GLN-GLN-GLY-GLU-SER-ASN-GLN-GLU-ARG-GLY-ALA-ARG-ALA-ARG-LEU-NH_2	Growth hormone
Growth hormone-inhibiting hormone (somatostatin)	ALA-GLY-CYS-LYS-ASN-PHE-PHE-TRP-LYS-THR-PHE-THR-SER-CYS	Growth hormone prolactin, Thyrotropin
Prolactin-inhibiting factor (PIF)	Dopamine	Prolactin
Thyrotropin-releasing hormone (TRH)	pGLU-HIS-PRO-NH_2	Thyrotropin Prolactin

form large parallel veins, or the portal vessels. Those in turn pass downward along the pituitary stalk and terminate in the capillaries of the anterior pituitary. Many studies have documented a retrograde blood flow, i.e., from the pituitary to the hypothalamus, which may indicate an ultrashort feedback effect of the pituitary hormones on hypothalamic function. At least four releasing hormones and two inhibiting hormones from the hypothalamus regulate pituitary function. Thyrotropin releasing hormone (TRH) and growth hormone releasing hormone (GHRH) act on the pituitary to release thyroid stimulating hormone (TSH) and growth hormone (GH), respectively. Corticotropin releasing hormone (CRH) stimulates the release of ACTH and β-endorphin by the anterior pituitary. The two inhibiting hormones from the hypothalamus, somatostatin and dopamine, inhibit GH and prolactin secretion, respectively. It is important to note that prolactin is the only anterior pituitary hormone under primarily inhibitory control from the hypothalamus. Thus, anything which inhibits hypothalamic-pituitary communication (e.g., tumor) usually results in elevated prolactin secretion. The principal hypothalamic hormone regulating reproductive function is gonadotropin releasing hormone (GnRH), also called luteinizing hormone releasing hormone, which is secreted by neurons originating primarily in the arcuate nucleus. GnRH stimulates the release of luteinizing hormone (LH) and follicle stimulating hormone (FSH) from the pituitary and will be discussed in detail later in this chapter.

Thus, directly through neural input and indirectly via releasing and inhibiting factors in the hypophyseal portal system, the hypothalamus maintains constant regulation of all aspects of normal pituitary function. Importantly, the hypothalamic-pituitary axis is outside of the blood-brain barrier and as such is subject to negative and positive feedback regulation by virtually all hormones produced by the ovary.

THE PITUITARY

The human anterior pituitary lies on the sella turcica near the hypothalamus and is under the direct control of the hypothalamus through the hypothalamic releasing hormones carried by the large portal vessels. At least five cell types exist in the human anterior pituitary. (Table 2). Light microscopic examination of stained sections reveals that both the somatotropes and lactotropes are eosinophilic cells and are localized predominantly in the lateral wings of the pituitary gland, whereas the basophilic cells—the corticotropes, thyrotropes, and gonadotropes—are predominantly in the middle.

The somatotropes, the most numerous cell type, secrete growth hormone under the inhibitory influence of somatostatin and the stimulatory influence of GHRH. GH stimulates the growth of almost all cells and tissues of the body; most of this growth effect is mediated through the production of growth factors. Growth factors are a group of peptides and polypeptides that promote cellular differentiation and

Table 2. Hypophyseal Cells and Hormones

Cell	*% Pituitary Population*	*Products Secreted/Molecular Weight*	*Target Organs*
Somatotrope	50	Growth hormone (GH), 22,000	All organs
Lactotrope	15	Prolactin, 22,000; 50,000	Breasts
Corticotrope	20	Adrenocorticotropin (ACTH), 4,500	Adrenal gland
Gonadotrope	10	Follicle-stimulating hormone (FSH)m 33,000 Luteinizing hormone (LH), 28,000	Ovaries
Thyrotrope	5	Thyrotropin (TSH), 28,000	Thyroid gland

mitosis in a variety of cell types. Two growth factors, insulin-like growth factor I and II (IGF-I, IGF-II), for example, are produced by the ovary and may play an important role in modulating steroid synthesis and follicular development. They are also present in the human endometrium and may be involved in mediating endometrial-trophoblast interactions.

The lactotropes synthesize and secrete prolactin. They are under the inhibitory influence of dopamine, also called prolactin inhibiting factor (PIF). Prolactin is a polypeptide hormone that circulates in two forms; the monomeric, or small form (molecular weight 22 kDa), which is biologically active, and the polymeric, or big form (molecular weight 50 kDa). Prolactin will stimulate the growth of mammary tissue as well as cause the production and secretion of milk into the alveoli of the breasts. In rodents, prolactin is an important luteotrophic hormone and is essential for maintenance of luteal function. In primates, however, prolactin is probably not a principal hormone regulating luteal function. Elevated prolactin levels can lead to reproductive disturbances, as will be discussed later in this chapter.

The corticotropes produce ACTH which acts on the adrenal cortex to stimulate the secretion of adrenocortical hormones including cortisol, which controls the metabolism of protein, carbohydrates, and fat. In addition, ACTH stimulates adrenal androgens, which can be utilized by the ovary as precursors for estrogen production. An elevated ACTH level is seen in cases of stress and is associated with menstrual abnormalities.

The thyrotropes secrete TSH which acts on the thyroid gland to cause thyroxin (T_4) and triiodothyronine (T_3) secretion. Although thyroid hormones probably do not directly participate in the regulation of reproductive function, both an excess of thyroid hormone or hypothyroidism can lead to reproductive dysfunctions.

The gonadotropes are gonadotropin producing cells, and both FSH and LH are secreted from the same cell type. These cells make up approximately 10% of the cell population of the anterior pituitary and are certainly the most important cell types in the pituitary regulating reproductive function. FSH and LH are glycosy-

lated proteins with molecular weights of approximately 28 kDa, and each hormone consists of two subunits, α and β. The α-subunit is identical in both FSH and LH (and also TSH); however, the β-subunits of these pituitary hormones have different amino acid sequences. As such, the specific biologic activities of FSH and LH are provided by the β-subunits; however, the intact αβ dimer structure is essential for biologic activity.

Due to the different β-subunit structures, LH has a short half-life of approximately 30 minutes, as compared to that for FSH, which is approximately four hours. Both FSH and LH are secreted in a pulsatile fashion by the anterior pituitary in response to pulsatile GnRH stimulation from the hypothalamus. The clearance of circulating FSH and LH occurs primarily through the liver and the kidney. Urinary excretion of intact gonadotropins is small; however, the measurement of the gonadotropin concentration in urine can give a good approximation of the rate of gonadotropin secretion, both in physiologic and pathologic conditions.

HYPOTHALAMIC REGULATION OF GONADOTROPIN SECRETION

Neurons in other areas of the brain terminate in the hypothalamic region and influence GnRH synthesis and release via catecholamine, dopamine, peptide, and endorphin-related mechanisms. GnRH is released into the portal vascular system; it reaches the anterior lobe of the pituitary gland and acts on target cells (gonadotropes) by binding to membrane receptors, where it stimulates the synthesis, storage, and secretion of FSH and LH. Primarily through the actions of these two hormones, GnRH regulates the function of the ovary. GnRH is a short peptide of 10 amino acid residues and has a very short half-life of (2–4 minutes) in the portal circulation. It is secreted by the hypothalamus in a pulsatile pattern and results in the pulsatile secretion of LH (Figure 3) and, to a lesser extent, FSH.

The arcuate nucleus of the hypothalamus is likely the site of the "pulse generator" which sets the timing and frequency of GnRH pulses. The pituitary requires the pulsatile presentation of GnRH, and changes in both amplitude and frequency of this pulsatile release have been observed in relation to the onset of puberty, pregnancy, and during different phases of the menstrual cycle. In experimental animals, as well as in humans, GnRH given continuously leads to inhibition of LH and FSH release (Figure 4). This process, called down-regulation or desensitization, is a result of internalization and loss of membrane-bound GnRH receptors which occurs if the pituitary is constantly exposed to GnRH. Thus, the pituitary absolutely requires that GnRH be presented in a pulsatile manner for normal gonadotropin production. The pulsatility of GnRH (and therefore LH) is modulated by ovarian steroids, the central nervous system (CNS), neurotransmitters, and endogenous opioids. During the early follicular phase of the menstrual cycle, GnRH pulses occur approximately once per hour and increase to two pulses per hour as the follicu-

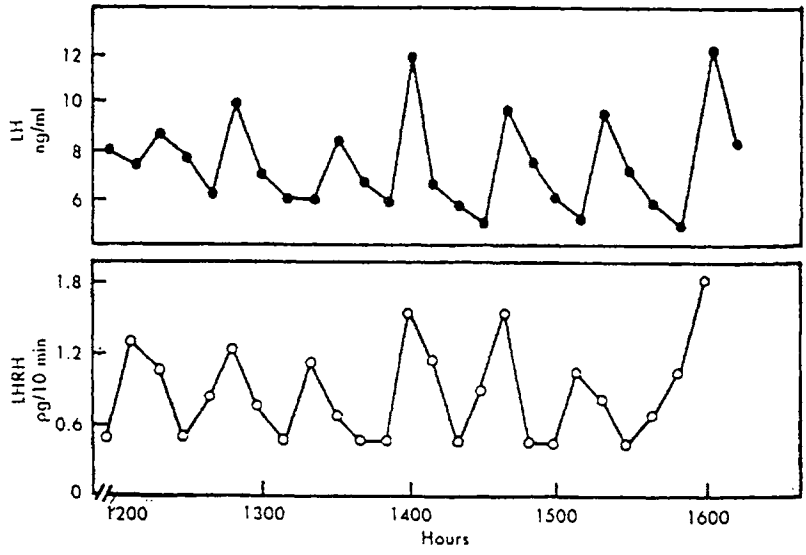

Figure 3. LH is released by the pituitary in a pulsatile fashion in response to pulsatile GnRH stimulation from the hypothalamus. From Levine et al (1982) Endocrinology 111, 1445-1455, with permission.

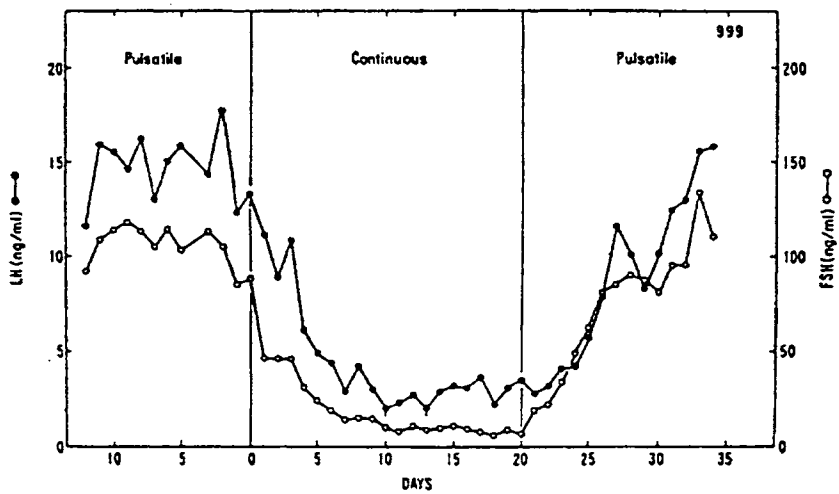

Figure 4. Comparison between pulsatile and continuous stimulation with GnRH in Rhesus monkeys. Note that GnRH given continuously leads to inhibition of FSH and LH release. From Belchetz et al. (1978) Science 202, 631-633, with permission.

lar phase progresses. As a result, there is an increase in the baseline LH levels as the follicular phase progresses. The increase in GnRH pulsatility is likely due to the action of increased estradiol on the hypothalamus. After ovulation and corpus luteum formation, GnRH pulsatility slows to approximately one pulse every four hours, presumably through the action of progesterone on the hypothalamus, with a corresponding decrease in basal LH secretion. The role of ovarian steroids in modulating GnRH secretion is indirect. GnRH-secreting neurons in the hypothalamus apparently do not contain classic nuclear receptors for estradiol or progesterone; therefore, the modulatory role of these ovarian steroids is likely mediated via other local factors, possibly catecholamines or opioids. The important neurotransmitter, dopamine, appears to inhibit the release of GnRH. Norepinephrine, on the other hand, can exert both an inhibitory and stimulatory effect on GnRH secretion. Earlier studies have shown that morphine-like substances inhibit GnRH release. The endogenous opioids endorphin, enkephalin, and dynorphin have a similar effect.

PITUITARY-OVARIAN REGULATION

The ovary (like the testis) has both an endocrine function (production of hormones) and a gametogenic function (maturation and ovulation of oocytes). Both the endocrine and gametogenic functions of the ovary are largely driven by the pituitary gonadotropins. LH from the pituitary acts on the theca cells of the ovaries to stimulate the production of androgens (testosterone and androstenedione). These androgens produced by the theca cells diffuse across the basement membrane to the granulosa cells. There, the androgens are aromatized to either estradiol or estrone under the action of FSH which activates the enzyme aromatase. This is the two-cell two-gonadotropin theory of ovarian steroid production. FSH also simulates the growth of the ovarian follicle by increasing the proliferation of granulosa cells and increases secretion of follicular fluid into the antrum of the follicle. Estrogen and progesterone feed back on the hypothalamic-pituitary axis to inhibit FSH and LH. Other ovarian hormones which may participate in the feedback regulation of pituitary gonadotropin secretion include the inhibins, activins, and follistatin. Inhibin is a heterodimeric protein composed of an $\alpha\beta$ subunit. There are at least two forms of biologically active inhibin, Inhibin A and Inhibin B. The α-subunits of the two inhibins are identical, while the β-subunits differ slightly in amino acid sequence. Inhibin A is primarily produced by the corpus luteum whereas Inhibin B is produced primarily by the developing follicle. *In vitro* experiments have clearly shown inhibin suppresses both synthesis and release of FSH. It has also been suggested that inhibin may act on the hypothalamus to inhibit GnRH secretion. Another FSH suppressing protein is follistatin; it has the same action as inhibin but is structurally dissimilar, and its *in vitro* potency is only 10–20% that of inhibin. Interestingly, follistatin is a binding protein for inhibin and activin and its primary physiological importance may be in regulating the availability of these two hormones. Activin is a

dimer composed of two β-subunits of inhibin. It is produced by the granulosa cells and stimulates FSH secretion by the pituitary and may also modulate follicle formation and oocyte maturation. It is noteworthy that these hormones may also be produced by the pituitary gland and may act in a paracrine fashion to modulate gonadotropin secretion.

THE PREPUBERTAL HYPOTHALAMIC-PITUITARY-OVARIAN AXIS

Data derived from human fetuses indicates that GnRH production begins in the neurons of the hypothalamus long before any vascular communication with the pituitary can be demonstrated. Despite this early presence of GnRH, it appears that the initiation of the synthesis of gonadotropin by cells of the developing pituitary is independent of the trophic influence of GnRH. At around 70 days of gestation, LH and FSH can be detected in the cells of the adenohypophysis, well before cellular differentiation within the pituitary has been accomplished. The precise time at which gonadotropin secretion comes under the control of GnRH is unknown, but there is a predictable increase in the gonadotropin content of fetal serum starting at around 100 days of gestation and reaching a plateau at 150 days. This is followed by a gradual decline in gonadotropin levels that will persist until birth. After birth, due to loss of the inhibitory action of the placental steroids, a rise in circulating gonadotropin concentration is noticed. This rise, however, will last only a few months, and usually by six months of life FSH and LH reach a nadir that will persist until puberty (Figure 5).

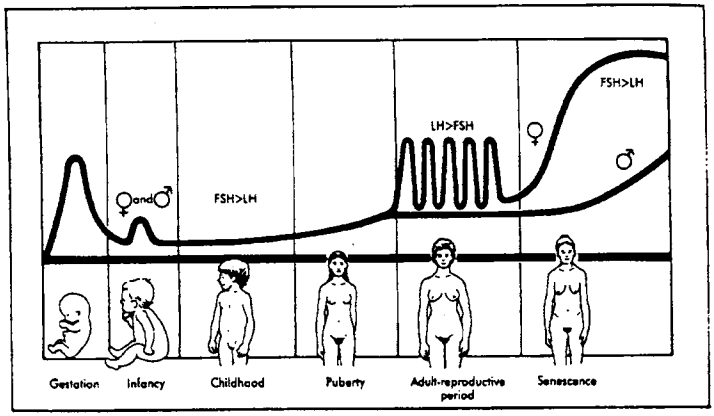

Figure 5. Patterns of LH and FSH secretion in the human female throughout life. Adapted from Genuth S.M. The reproductive glands. In: Physiology, (Berne, R.M. and Levy, M.N. eds.). 3rd edn., 1993, pp. 980-1024. Mosby Year Book, St Louis, with permission.

The prepubertal years are characterized by both a decreased responsiveness of the pituitary gland to the action of GnRH and a low level of hypothalamic GnRH output. GnRH remains at a very low level, despite low levels of ovarian estrogen and the apparent absence of negative feedback. Although the exact mechanism is unknown, the inhibition of GnRH secretion is likely due to CNS inhibition. At puberty, an increase in pulsatile GnRH release is evident. This is associated with an increase in pulsatile LH secretion during sleep and is a hallmark of the activation of the hypothalamic-pituitary-ovarian axis during puberty. During pubertal development the hypothalamic-pituitary-ovarian axis is not precisely synchronized, and the majority of these initial menstrual cycles may be anovulatory and/or infertile. Usually, most girls will begin ovulating within nine months of menarche (initiation of menses) and will establish regular ovulatory cycles within one year.

THE MENSTRUAL CYCLE

From menarche to menopause, the female reproductive function follows a cyclical pattern called the menstrual cycle. The duration of the menstrual cycle varies not only between different women, but also during the reproductive lifetime of an individual woman. The mean duration of the menstrual cycle is 28 ± 7 days and is divided into three phases; the follicular phase, ovulation, and the luteal phase. By convention, the first day of menstrual bleeding is considered the first day of the menstrual cycle.

The endocrine profile of a typical menstrual cycle is depicted in Figure 6. At the beginning of the follicular phase, a group or cohort of follicles which have been growing slowly for the previous several months will begin accelerated growth, possibly in response to the small elevations in FSH which occur at this time. Under the influence of FSH, these follicles will continue to develop until one of the cohort attains dominance. The dominant follicle, continues to grow in response to FSH stimulation and produces increasing amounts of estradiol. Increasing estradiol (and possibly inhibin) concentrations feed back to inhibit pituitary FSH production, which inhibits further development of the non-dominant follicles in the ovary. However, the dominant follicle is able to continue to grow in the face of decreasing FSH concentrations, possibly due to an enhanced ability to utilize FSH. As the dominant follicle grows, its granulosa cells gain responsiveness to LH via the acquisition of LH receptors, and at the end of the follicular phase the follicle begins to produce progesterone. The combination of rapidly rising estradiol production, together with increasing progesterone concentrations, feeds back at the level of the hypothalamus and pituitary to initiate the midcycle surges of LH and FSH. The LH and FSH surge is likely due to a dramatic release of GnRH from the hypothalamus (a GnRH surge) combined with an increased pituitary sensitivity to GnRH. Thus, the synergy of rapidly rising estradiol combined with progesterone switches a negative feedback signal to a positive feedback event. The cellular mechanisms respon-

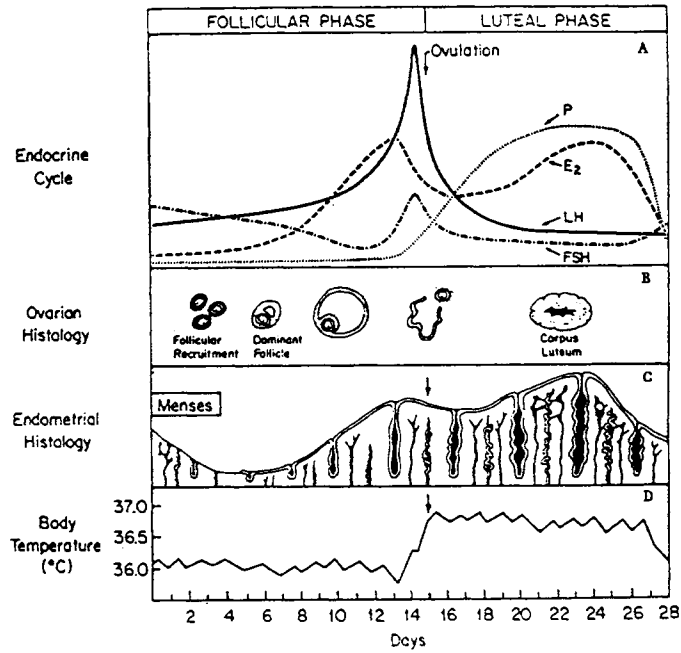

Figure 6. The ovulatory menstrual cycle: **A.** Pattern of hormone secretion in a normal ovulatory menstrual cycle. Note that if pregnancy occurs, progesterone will remain elevated. **B.** Selection of a dominant follicle, ovulation, and corpus luteum formation. **C.** Estrogen stimulates proliferation of the endometrium during the follicular phase. After ovulation, progesterone transforms the endometrium from a proliferative to a secretory type. **D.** The rise in basal body temperature after ovulation is due to the action of progesterone on the central thermoregulatory center. From Carr B.R. and Wilson, J.D. Disorders of the ovary and female reproductive tract. In: (Braunwald, E., Isselbacher, K.J., Petersdorf, R.G. et al., Eds.) pp. 1818-1837. Harrison's Principles of Internal Medicine, 11th edn., 1987: McGraw Hill, New York with permission.

sible for this switch are unknown. The net result is a 10- to 100-fold increase in circulating LH levels, which initiate the processes of ovulation of the oocyte and luteinization of the granulosa cells of the ovary. There is controversy as to whether humans and non-human primates have a GnRH surge, or simply manifest increased pituitary responsiveness to unchanging GnRH levels. Laboratory rats and domestic animals demonstrate a clear GnRH surge during midcycle. However, monkeys in which endogenous GnRH secretion is halted by ablation of the arcuate nucleus of the hypothalamus will demonstrate normal menstrual cycles with normal LH surges if given an unchanging pulsatile infusion of GnRH throughout the cycle. Recent evidence, however, indicates that elevations in GnRH concentrations do occur prior to the initiation of the LH surge in monkeys. These observations may suggest

that several redundant mechanisms (a GnRH surge, increasing pituitary sensitivity to GnRH, and possibly others), all act to ensure that the LH surge occurs at the appropriate time during the menstrual cycle, resulting in ovulation of a mature oocyte. In humans, the LH surge lasts for up to 48 hours. This prolonged surge (compared to that observed in rodents) may be important to ensure that the oocyte completes its resumption of meiosis, and that the granulosa cells become fully luteinized.

After ovulation, the majority of the granulosa and theca cells remain in the ovary and terminally differentiate to become the corpus luteum. The primary function of the corpus luteum is to produce progesterone, and the production of progesterone is dependent upon LH secretion from the pituitary. If pituitary LH secretion is stopped (for example, by hypohysectomy or administration of a GnRH antagonist), the corpus luteum will regress and cease progesterone production. However, the normal regression of the corpus luteum (luteolysis) at the end of the menstrual cycle is not due to the reduction of pituitary LH secretion. Indeed, the factors responsible for luteolysis in primates at the end of the menstrual cycle remain poorly understood. This is in contrast to domestic animals, where luteolysis is clearly initiated by an increase in uterine production of prostaglandins. As the corpus luteum regresses at the end of the menstrual cycle, progesterone secretion declines. Falling progesterone (and possibly inhibin) may signal the hypothalamus and pituitary to increase FSH secretion and, as such, initiate a new wave of follicle maturation for the next menstrual cycle.

If pregnancy occurs, the developing blastocyst will begin to secrete human chorionic gonadotropin (hCG) as early as six days after fertilization. hCG is a glycoprotein hormone with an α and β subunit structure similar to the pituitary glycoprotein hormones. In fact, the structure of hCG is virtually identical to that of LH, except that hCG contains a C terminal extension with three additional glycosylation sites, which inhibit degradation and increase its half-life in the circulation. hCG binds to the same ovarian receptors as LH and stimulates progesterone production by the corpus luteum. hCG secretion by the developing blastocyst rescues the corpus luteum from its natural regression and stimulates luteal progesterone production for several weeks. This continued production of progesterone is important for implantation and development of the early embryo in the uterus. Approximately 6–7 weeks after conception, the luteal-placental shift in progesterone production is essentially complete, and the placenta takes over steroid production for the remainder of the pregnancy.

MENOPAUSE

Menopause is the cessation of menstruation in the human female. It is a gradual and permanent process which occurs in American women around age 50. The events which lead to and initiate menopause are fairly well understood. Menopause is a result of the depletion of follicles (and oocytes) from the ovary. The human female is

born with approximately 700,000 oocytes, and the number declines gradually throughout her life. In addition, the follicles remaining in the ovary during the perimenopausal years demonstrate a reduced sensitivity to circulating FSH levels. Thus, through the decline in available follicles and the reduced sensitivity of those remaining, the ovary gradually loses its ability to respond to pituitary gonadotropins. This decline in ovarian follicle growth (and, more importantly, ovarian estrogen and inhibin production), results in reduced negative feedback at the hypothalamic-pituitary axis and a rise in circulating gonadotropins. Although the follicles no longer produce significant amounts of estradiol, postmenopausal ovarian stroma does produce testosterone and androstenedione. The peripheral conversion of androstenedione to estrone in adipose tissue accounts for the low level of circulating estrogen concentration present in postmenopausal women. Obese women generally have fewer menopausal symptoms due to adipose tissue conversion of androgens to estrone.

Menopause can be a very disturbing and stressful process because of the associated symptomatology. Symptoms occurring after surgical castration (i.e., removal of both ovaries) are usually more severe than those occurring after natural menopause due to a more gradual decline in the level of estrogen in the latter. Up to 75% of peri- and postmenopausal women will develop hot flushes. The hot flush is a sensation of burning involving the whole body followed by an outbreak of sweating; it frequently occurs at night, and may lead to insomnia. Hot flushes usually last 2–3 minutes and are associated with an increase in epinephrine, LH, and ACTH, but with no changes in FSH or estradiol levels. The factors responsible for hot flushes are not well understood, although it is thought to be due to a lowering of the thermoregulatory center in the hypothalamus by CNS catecholamines. The most effective treatment is estrogen and/or progesterone. Clonidine (an α-adrenergic agonist) has also been used successfully to reduce the incidence of hot flushes.

Bone resorption is accelerated after menopause. It is a serious public health problem, as 15,000 women in the U.S. die each year of osteoporosis and its complications. If left untreated, eventually 50% of trabecular bone and 30% of cortical bone mass can be lost. Estrogen replacement is an effective treatment to prevent postmenopausal bone loss, as it appears to prevent bone resorption. Estrogen receptors are present in bone, and estrogen appears to have a mild antagonistic action to that of parathyroid hormone. A sedentary lifestyle, cigarette smoking, belonging to the white or oriental race, or early menopause will all increase the risk of osteoporosis. Postmenopausal women should be encouraged to use long-term estrogen therapy as an acceptable, safe, and effective protection against osteoporosis. Other forms of treatment include fluoride, calcitonin, and biphosphonate. Adequate calcium and vitamin D intake, smoking cessation, and weight bearing exercise, will all help in the prevention of osteoporosis.

Estrogen increases the concentration of high-density lipoprotein cholesterol and decreases low-density lipoprotein cholesterol. For women in the U.S., the morbidity and mortality from coronary heart disease far exceeds that from breast cancer,

uterine cancer, and bone fractures combined. Observational data strongly suggest that estrogen replacement therapy reduces the incidence of ischemic heart disease in post-menopausal women by 40–50%.

Menopause has also been associated with painful sexual intercourse and vaginitis as well as incontinence, frequent urination, and an increased incidence of urinary tract infections. Estrogen replacement therapy started in the perimenopausal years will prevent these problems. Additional symptoms encountered in early menopause include fatigue, nervousness, headache, irritability, depression, and palpitations; these symptoms have been referred to as the "menopausal syndrome." Although estrogen replacement therapy appears to improve these symptoms, subjectivity of these complaints make it difficult to assess.

CLINICAL CORRELATES

Anovulation due to Malfunctioning of the Hypothalamic-Pituitary Axis

As previously mentioned, the normal menstrual cycle depends on the coordinated interaction of the hypothalamus, pituitary, and the ovaries. Compromise of any of these systems can lead to failure of ovulation, which is clinically manifested as amenorrhea, or menstrual irregularity. The hypothalamic-pituitary axis can be affected by anatomic lesions of the CNS as well as by functional causes like stress, weight loss or weight gain, physical activity, etc.

Anatomic Causes of Anovulation

Absence of the GnRH-producing cells in the hypothalamus, as in Kallman's syndrome, can cause amenorrhea and delay puberty. The most common CNS tumors, craniopharyngiomas, can lead to anovulation. These typically extrasellar masses interfere with GnRH synthesis, secretion, and stimulation of pituitary gonadotropins. Inflammatory lesions like tubercular or sarcoid granulomas of the CNS, hydrocephalus, or radiation therapy to the brain can also lead to hypothalamic-pituitary failure and anovulation.

Anatomic disorders can also affect pituitary function. Necrosis of the pituitary gland secondary to post-partum hemorrhage (Sheehan's syndrome), as well as the empty sella syndrome, can both lead to hypopituitarism. Tumors affecting the pituitary, the most common being prolactin secreting adenomas or prolactinomas, can also result in anovulation. Patients with this disorder may have not only an elevated prolactin level, but also an overproduction of GH, since the lactotropes and somatotropes are derived from a common progenitor cell. It is hypothesized that the elevated levels of prolactin will lead to an elevated level of dopamine (PIF) which will act on the hypothalamus to inhibit the release of GnRH thus leading to anovulation. Clinically, a patient presenting with galactorrhea and menstrual cycle irregularities

should have her prolactin level checked. If prolactin is elevated, the patient can be treated with bromocriptine, which is a dopamine receptor agonist.

Functional Causes of Anovulation

Stress. Many women get menstrual cycle disturbances when exposed to a stressful situation such as exams, divorce, etc. One hypothesis is that stress activates the hypothalamic release of corticotrophin releasing factor (CRF). CRF acts on the pituitary to cause the release of stress hormones including ACTH, GH, and prolactin. CRF will also activate the release of endogenous opioids, which can act on the hypothalamus to inhibit GnRH secretion, thus leading to anovulation and irregular menses.

Exercise. The increased participation of women in competitive sports has led to considerable data about exercise and menstrual disturbances. The incidence of menstrual abnormalities varies, depending upon the intensity of exercise; the duration of exercise (more abnormalities are seen at the end of the athletic season); the type of exercise (fewer irregularities in cyclists compared to joggers); the diet of the athlete (athletes on a high protein diet have less menstrual dysfunction); and the amount of weight loss. It has been hypothesized that a certain level of body weight, most importantly, lean-to-fat ratio, is needed to preserve regular menses. In patients with exercise-induced menstrual disorders, LH levels are reduced. This is thought to be due to the action of high levels of CRF on the hypothalamus. Athletes with menstrual irregularities are at higher risk for bone fractures because of osteoporosis resulting from decreased estrogen production by the ovaries due to the anovulatory state.

Anorexia Nervosa. This psychoneural endocrinologic disease develops in adolescents and young women and is the third most common chronic illness in teenage girls. In these patients weight loss is induced by severe dieting, self-induced vomiting, appetite suppressants, and purging with laxatives and diuretics. LH secretion levels are consistent with those seen in prepubertal girls and hypogonadotropism can be accompanied by a low thyroxin level and a high plasma cortisol level. This will help differentiate anorexia nervosa from pituitary insufficiency. Weight gain and pulsatile administration of GnRH will reverse gonadotropins to the normal, adult-like hormonal pattern.

Oral Contraceptives

In the early 1900s it was noted that giving ovarian extracts to animals would inhibit their fertility and thus the concept of oral contraceptives was born. Modern oral contraceptives are steroid preparations consisting of various combinations of estrogens and/or progestins. The progestin component acts on both the hypothala-

mus and the pituitary to suppress the release of LH, thus preventing ovulation. Progestins also cause decidualization of the endometrium, making it unreceptive to implantation and cause thickening of the cervical mucus, making it impervious to sperm penetration and transport.

The estrogen component affects both the pituitary and the hypothalamus to inhibit FSH secretion, thus limiting follicle growth. Estrogens provide stability to the endometrium to prevent unwanted breakthrough bleeding. The estrogen component may also increase the concentration of intracellular progesterone receptors, thus decreasing the need for higher doses of progesterone. Progestin-only mini pills do not contain the estrogen component and their efficacy is slightly less than the combination pill. The estrogen component of the pill has been associated with an increased incidence of thrombotic disease, and the progesterone component has been associated with increased androgenic effects (hair growth, weight gain, etc.). Decreasing the amount of these hormones may prevent or decrease those side-effects, and current oral contraceptives contain significantly smaller amounts of both estrogen and progestin than earlier formulations.

GnRH Analogues

GnRH antagonists bind to the GnRH receptor on the pituitary gonadotrope but do not stimulate the release of LH and FSH. As such, GnRH antagonists compete with endogenous GnRH for its receptor and block GnRH-stimulated gonadotropin secretion from the pituitary (Figure 7). The resulting decrease in circulating LH and, to a lesser extent, FSH, is rapid and essentially complete.

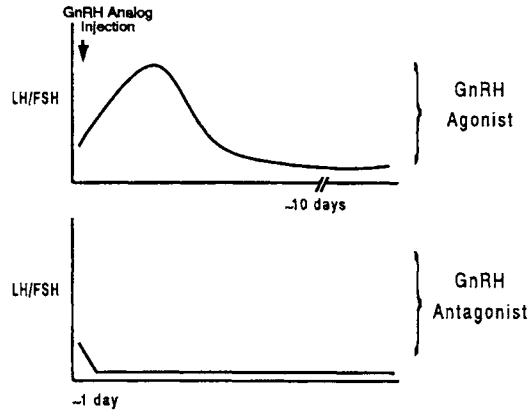

Figure 7. Effects of GnRH analogues on LH/FSH secretion. Note that with GnRH agonist treatment there is an initial stimulation of LH/FSH release followed by down-regulation and desensitization. In contrast, GnRH antagonists cause an immediate decrease in circulating LH/FSH.

In contrast, GnRH agonists bind to the GnRH receptor and initially stimulate LH and FSH secretion. However, because GnRH agonists (and antagonists) have been designed to have very long half-lives in the circulation, they provide constant occupancy of the GnRH receptor. Recall that the release of LH-FSH from the pituitary requires the pulsatile secretion of GnRH from the hypothalamus. GnRH agonists, when present in a continuous fashion, eventually lead to desensitization and down-regulation of the pituitary GnRH receptors. The result is that after a brief (7–10 days) stimulating phase, GnRH agonists eventually inhibit LH and FSH secretion to prepubertal levels. This medically induced hypogonadotropism is useful in the treatment of certain gynecological problems including precocious puberty, endometriosis, and uterine fibroids. Side effects of GnRH agonists due to the hypoestrogenic state similar to menopause may include hot flushes, osteoporosis, vaginal dryness, etc. GnRH agonists can be given in a pulsatile fashion to stimulate LH and FSH secretion and ovulation, and are sometimes used in cases of GnRH deficiency such as Kallmann's syndrome. Currently, only GnRH and GnRH agonists are clinically available.

Induction of Ovulation

Ovulatory dysfunction is present in up to 25% of patients presenting for infertility evaluation. Many protocols for inducing ovulation and superovulation in patients undergoing *in vitro* fertilization procedures are being used. They rely primarily on the use of four drugs.

GnRH given through a pump in a pulsatile fashion will often stimulate gonadotropin secretion and lead to ovulation, especially in patients with hypothalamic or CNS lesions. However, administration and compliance can be difficult, and thus GnRH is seldom used for ovulation induction. In contrast, the inhibitory action of prolonged GnRH agonist treatment is often exploited as adjunctive therapy with gonadotropins (see below) for ovulation induction. The down-regulation of GnRH receptors by GnRH agonists prevents premature LH surges and ovulation in patients receiving exogenous LH and FSH.

Human menopausal gonadotropin (hMG) is extracted from the urine of postmenopausal women which contains high amounts of both LH and FSH. hMG directly stimulates ovarian folliculogenesis. The LH component of hMG is probably unnecessary, and purified FSH preparations are available which are as effective as hMG for ovulation induction. Depending on the regimen, multiple follicle growth can be achieved which is especially useful for certain assisted reproduction techniques such as *in vitro* fertilization.

Clomiphene citrate is a nonsteroidal compound which binds to the estrogen receptor but has little estrogenic activity. It initiates ovulation by primarily acting on the hypothalamus, where it is thought to displace endogenous estrogen from hypothalamic receptor sites, thereby removing the negative feedback effect of estrogens.

The hypothalamic-pituitary-axis senses a low serum concentration of estrogen and stimulates the release of pulsatile GnRH. Clomiphene citrate is often the first drug of choice in ovulation induction protocols due to its relatively low cost as compared to hMG or FSH.

Human chorionic gonadotropin (hCG) is also used in ovulation induction protocols. Its action is identical to that of pituitary LH. Given intramuscularly, it will mimic the LH surge and cause ovulation within 36–48 hours after injection. hCG is used in ovulation induction so that precise timing of oocyte maturation can be achieved.

SUMMARY

Normal functioning of the hypothalamic-pituitary-ovarian axis requires the coordinated interaction of both neural and endocrine systems. Central inputs modify hypothalamic releasing and inhibiting factors which directly regulate pituitary function. In turn, hormones from the pituitary modulate ovarian function by direct action on the granulosa and theca cells and indirectly via other endocrine systems. In response, the ovary secretes a variety of steroidal and nonsteroidal hormones which act on the hypothalamus and pituitary to close the loop of feedback regulation of the hypothalamic-pituitary-ovarian axis. As such, the normal day-to-day regulation of reproductive function is precisely controlled.

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Chapter 4

The Biology of the Ovary

CATHERINE RACOWSKY and TIMOTHY J. GELETY

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PRENATAL PERIOD

The prenatal development of the human ovary progresses through discrete stages that ultimately result in germ cell formation, differentiation of the ovary, multiplication of the germ cells with their entry into oogenesis, and establishment of the early ovarian follicle.

Germ Cell Formation

As early as the fifth week of gestation, it is possible to distinguish among the three cell types from which the differentiated ovary will develop. These cell populations include the coelomic epithelial cells which later differentiate into the follicular granulosa cells; the mesenchymal cells of the gonadal ridge which will give rise to the ovarian stroma; and the primordial germ cells, from which will develop the mature female gametes, the definitive oocytes. Even at this early stage of intrauterine life, the gonadal ridge is visible as a region overlying the mesonephros, although it is not until 10–11 weeks of fetal life that it is morphologically distinguishable as a primordial ovary (Byskov and Hoyer, 1994).

The origin and movement of primordial germ cells can be traced by cytochemical localization of the enzyme, alkaline phosphatase (McKay et al., 1953; Hardisty, 1978). Human primordial germ cells originate in the endoderm of the yolk sac wall (Witschi, 1948) and can be distinguished from the surrounding region as a scattered population of ovoid, poorly differentiated cells (Franchi and Mandl, 1962) (Figure 1). Initially by passive movement, and subsequently by active amoeboid locomo-

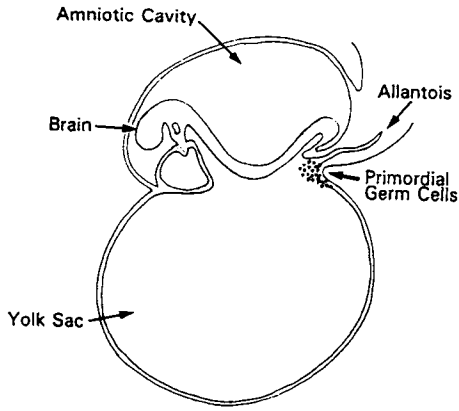

Figure 1. Primordial germ cells (black dots) differentiate in the endodermal layer of the yolk sac at 4–6 weeks of development.

tion, the primordial germ cells move from the yolk sac to the wall of the gut tube (Eddy et al., 1981). From here, they migrate via the mesentery to the dorsal body wall in the region of the gonadal ridge, the only site compatible with their survival. During their migration and for some time after they have reached the gonadal ridge, the germ cells complete their indifferent state and proliferate by many mitotic divisions, giving rise to a total of about 100,000 gonia (Baker, 1963).

When the germ cells arrive in the region of the presumptive gonadal ridge, they stimulate the surrounding coelomic epithelium and mesonephros to proliferate and to form strands of tissue, the primitive sex cords (Franchi et al., 1962; Gondos, 1978). As the sex cords continue to expand, they give rise to the genital ridges which represent the primordial gonads. In the female, the sex cords ultimately envelop the germ cells, giving rise to the ovarian follicles that will nourish and regulate the metabolism, and fate, of the developing sex cells (Eppig, 1985). The germ cells and cells of the sex cords have a symbiotic relationship. In the absence of sex cords, the germ cells fail to survive. However, if the germ cells fail to arrive in the region of the presumptive gonads, neither the gonads nor the sex cells will develop.

Differentiation of the Ovary

The mature ovary consists of two major portions, the outer cortex and the central medulla. However, it is the outer cortex that contains the structural units of the ovary, the follicles (Franchi et al., 1962; Zuckerman and Baker, 1977; Gondos, 1978). Therefore, in the absence of formation of medullary primary sex cords during testicular differentiation, the indifferent gonad will, by default, develop into an ovary. Thus, between 4–6 weeks of gestation, cortical dominance over the medulla is established (Baker and Scrimgeous, 1980), and estradiol synthesis begins which may play a role in subsequent differentiation of the ovary.

Oogonial Multiplication and Entry of Germs Cells into Meiosis

The first morphological evidence of ovarian differentiation occurs at 6–8 weeks, and is manifested by rapid mitotic multiplication of the germ cells so that their number increases from a mere 100,000 to a peak of as many as 7 million by the twentieth week of gestation (Baker, 1963) (Figure 2). The germ cells then differentiate into oogonia, are no longer mitotically active and immediately begin oogenesis by entering meiosis, only to become arrested in the diplotene stage of meiotic prophase I as primary oocytes (Ohno et al., 1962; Baker and Franchi, 1967; Albertini, 1993). However, the vast majority of oogonia succumb to the degenerative process of atresia so that only about 1 million primary oocytes are present by birth, 400,000 are present at menarche, and only a few remain at menopause (Figure 2). Thus, in contrast to the human male, the female is born with a finite number of germ cells.

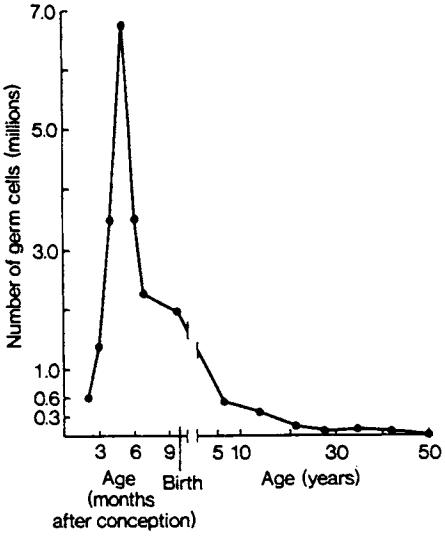

Figure 2. Changes in the total population of germ cells in the human ovary with increasing age. From T.G. Baker. [1972] Oogenesis and Ovulation. In: Germ Cells and Fertilization (Austin, C.R. & Short, R.V., eds.), pp. 14-45, Cambridge University Press, Cambridge.

Initiation of Folliculogenesis

As oogenesis gets underway, a single layer of perivascular cells of epithelial origin surrounds each oocyte that is then overlain with a more diffuse grouping of mesenchymal cells. The resulting unit is the primordial follicle—a structure delineated by a basal lamina and comprised of an oocyte and a single layer of presumptive granulosa cells (the epithelial cells) that are spindle-shaped (Peters, 1978). Surrounding these primordial follicles is an outer matrix of prethecal cells (the mesenchymal cells). Remaining mesenchyme not incorporated into the formation of primordial follicles forms the primitive ovarian stroma. The establishment of a layer of granulosa cells around the oocyte is a prerequiste not only for maintenance of oocyte viability, but also for mediating control of oocyte meiotic status. Oocytes not invested in granulosa cells either degenerate or else initiate premature meiotic resumption which subsequently results in degeneration (Baker, 1963; Peters et al., 1978). Completion of primordial follicle formation is accomplished at approximately six months after birth. The primordial follicles constitute the resting stockpile of nongrowing follicles that are progressively depleted during the reproductive lifespan.

With the establishment of primordial follicles, some leave this nongrowing pool to begin growth as their granulosa cells assume a cuboidal shape and undergo mito-

sis to form primary follicles (Zamboni, 1980). Concomitant with such follicular growth, the oocyte begins to grow, the nucleus migrates to an eccentric position, and the oocyte undergoes marked changes in ultrastructure, including the appearance of some novel organelles such, for example, as the zona pellucida and cortical granules (Szollosi, 1972; Zamboni, 1972; Wassarman and Josefowicz, 1978). Although granulosa cell proliferation may continue during the prenatal period, such follicles, however, are normally destined for atresia with the resultant demise of their enclosed oocytes.

The Principle and Fundamentals of Oogenesis

Oogenesis comprises the processes of meiosis and cytodifferentiation that together convert the oogonia into mature oocytes with full developmental competency. Meiosis is of fundamental importance to the success of sexual reproduction since it guarantees genetic diversity while controlling the chromosomal number characteristic of a species. Thus, the process involves a reduction, or halving, of the chromosomal complement of the diploid stem cells (the oogonia), to give rise to haploid oocytes. The term ploidy refers to the number of sets of chromosomes in a cell. A haploid cell (1n) contains only one set of chromosomes; a diploid cell (2n) contains two sets of chromosomes. Each chromosome may have only one copy of DNA (referred to as 1C), or it may have undergone replication but not segregation of the replicated copies, in which case it will be comprised of two copies of DNA (referred to as 2C).

Before entering meiosis, the oogonia undergo DNA replication to form primary oocytes containing two copies of the DNA of each chromosome. As with all human non-germ or somatic cells, each human oogonium contains 23 pairs of homologous chromosomes. Therefore, primary oocytes contain four copies of the DNA of each of the 23 chromosomes (i.e., are 4C), two copies from one homologue and two from the other. Each copy is referred to as a sister chromatid, and all four copies comprise a tetrad of chromatids, otherwise referred to as a bivalent of homologous chromosomes.

Meiosis comprises two divisions, the first of which is the reduction division in which the homologues of each of the 23 pairs of replicated chromosomes segregate to opposite ends of the meiotic spindle. Uneven cytokinesis then ensues resulting in the expulsion of one of the sets of chromosomes from the primary oocyte with a very small volume of cytoplasm to form the first polar body. The resulting secondary oocyte is haploid since it contains only one set of chromosomes, but it is 2C (Figure 3) since each chromosome still consists of two copies of each DNA strand. In contrast to the first meiotic division, the second division involves a halving of the DNA complement of each chromosome through separation and segregation of sister chromatids. Expulsion of one of the groups of sister chromatids as the second polar body then ensues to result in the formation of a haploid oocyte containing only one copy of DNA (1C). Accordingly, when such an oocyte (1n, 1C) undergoes syn-

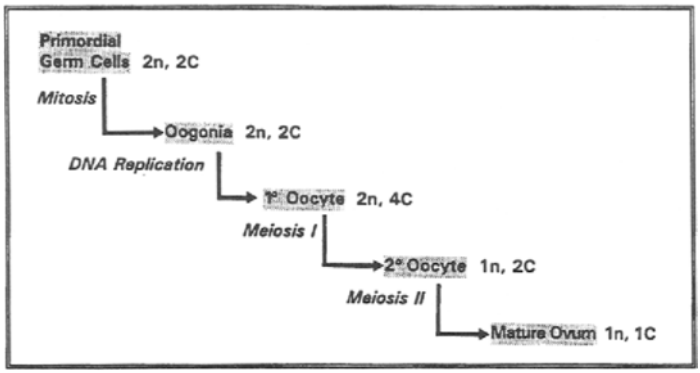

Figure 3. The ploidy and DNA content of female germ cells as they progress from the primordial germ cell stage of development through meiosis to become mature ova. n, the ploidy, or number of sets of chromosomes in the cell; C, the number of copies of DNA present.

gamy with a haploid sperm also containing only one copy of DNA (i.e., 1n, 1C), the normal diploid (2n) complement of chromosomes with 2C of DNA is restored.

Meiosis: A Discontinuous Process

Meiosis in the female is a discontinuous process characterized by three on-off set-points (Wassarman, 1994) (Figure 4). While initiated as early as the twelfth week of gestation, the process becomes arrested by birth in human females (Baker, 1963) when the oocyte is at diplotene of first meiotic prophase and exhibits an enlarged nucleus known as the germinal vesicle (GV) (Uebele-Kallhardt, 1978). Chiasma formation, and the occurrence of crossing over, exchange and genetic recombination between DNA segments of homologous chromosomes has been accomplished just prior to entry into this first meiotically arrested state. It should be noted that although the oocyte is meiotically arrested at this stage of development, metabolically, it is not in a state of quiescence. The decondensed chromatin of an arrested oocyte is active in RNA synthesis and, with resumption of folliculogenesis, the cell undergoes slow, but progressive, growth from a cell approximately 10 μm in diameter, finally reaching a diameter of 100-120 μm in preparation for the next phase of meiotic activity which occurs years later after menarche. Resumption of meiosis is triggered when a mature antral follicle is primed with the mid-cycle surge of preovulatory gonadotropin (Ayalon et al., 1972). However, the oocyte only progresses as far as metaphase II before it enters a second period of meiotic arrest (Figure 4). The so-called secondary oocyte is ovulated at this meiotic stage, and does not complete the meiotic process unless fertilization occurs which triggers the oocyte to traverse telophase II, thereby emitting the second polar body.

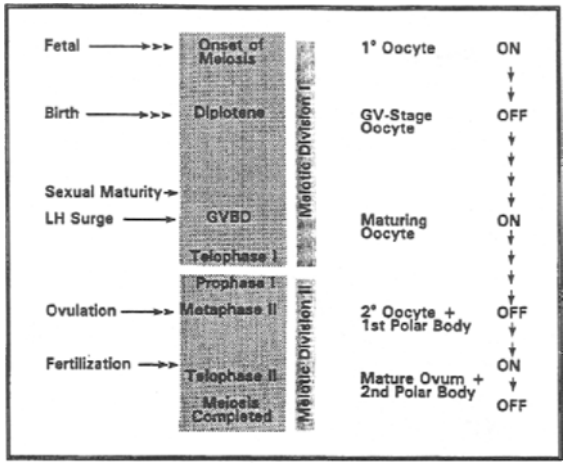

Figure 4. The meiotic progression of the female germ as it relates to development in the human. Note the three set-points through which the oocyte must traverse before it becomes a mature ovum.

NEONATAL PERIOD

Atresia: An Ongoing Process

Although as much as 85% of the total germ cell population has undergone degeneration by birth (Baker, 1963), developmental arrest and subsequent demise of developing folicles continues throughout childhood. This process, known as atresia, results in the ongoing loss of oocytes prior to their completion of cytoplasmic and nuclear maturation such that by the time of the first ovulation, shortly after menarche, only 400,000 follicles remain. During reproductive life, many more follicles are lost to atresia with each cycle, with normally only one achieving monthly ovulation. Shortly following the menopause, virtually no germ cells remain (Baker, 1963). An example of an apparent accelerated rate of germ cell atresia can be found in Turner's syndrome patients (45 XO), in whom normal ovarian formation and the presence of primordial follicles are seen *in utero*, but no oocytes remain at birth (Singh and Carr, 1966).

At birth, following delivery of the placenta, high levels of placental estrogen and progesterone decrease dramatically, releasing the neonatal hypothalamic-pituitary axis from the negative feedback characteristic of the late second and third trimester of pregnancy. This release of negative feedback inhibition results in a rise in the pituitary gonadotropins, luteinizing hormone (LH) and follicle-stimulating hormone (FSH), under the influence of pulsatile gonadotropin-releasing hormone (GnRH)

secretion from the hypothalamus. Pituitary gonadotropin stimulation of the newborn ovarian follicular apparatus results in follicular growth and significant gonadal steroid production (Winter et al., 1976). This abrupt increase in the stimulation to the newborn ovary may result in the formation of ovarian follicular cysts which may be apparent clinically on examination or by ultrasonography. Sufficient estrogen production by the neonatal ovary may be present to stimulate the reproductive tract, resulting in breast secretion ("witches milk") or rarely, vaginal bleeding.

Although gonadotropin secretion increases shortly after birth due to the loss of placental steroid negative feedback on the neonatal pituitary, pituitary gonadotropin secretion returns again to low levels within the first few years of life. This fall is due in part to an increased sensitivity of the hypothalamic-pituitary axis (estimated to be 6–15 times more sensitive than in the adult) to the negative feedback from very low levels of gonadal steroids (estrogens) characteristic of the prepubertal ovary (Winter and Faiman, 1973). However, such a decline in gonadotropin secretion is also observed in children without gonadal steroid production, as in the case of gonadal dysgenesis, or in children whose ovaries have been removed surgically during the neonatal period. Therefore, a steroid-independent mechanism may be operative at the level of the central nervous system to inhibit pulsatile secretion of GnRH from the hypothalamus which, in turn, results in inhibition of pituitary gonadotropin secretion prior to the onset of puberty. Infusion of GnRH to agonadal children has been shown to increase pituitary gonadotropin secretion (Roth et al., 1973), supporting a steroid independent mechanism.

Despite low levels of pituitary gonadotropin secretion during childhood, histologic evaluation of ovaries removed prior to the onset of puberty demonstrates active growth of follicles and ongoing atresia. However, due to the considerable proliferation of both follicles and stroma between birth and menarche, ovarian size increases with a resultant increase in weight from 250 mg to 4 gm (Peters et al., 1978). With the onset of puberty, under the influence of de-repressed pulsatile hypothalamic GnRH release, increases in cyclic pituitary secretion of both LH and FSH stimulate further ovarian follicle maturation. Further growth of ovarian follicles results in increasing gonadal steroid production which initiates the steroid-dependent changes characteristic of puberty (breast development, growth spurt, endometrial growth, and menstruation) and, ultimately, final oocyte maturation culminating in the ovulation of a fertilizable oocyte.

ADULT OVARY

Folliculogenesis

Initial follicular development occurs *in utero* during the second trimester of pregnancy associated with high levels of fetal gonadotropins. Subsequent limited follicular development and atresia continues independent of gonadotropin stimula-

tion during the third trimester and during childhood prior to the initiation of puberty. In the adult ovary, follicular development in various stages occurs continuously as in the early developing ovary. However, under the influence of coordinated pituitary gonadotropin stimulation, a follicle may develop fully to the point of ovulation of a mature ovum that has completed meiotic maturation and is capable of fertilization. Therefore, the process of follicle growth, although continuous, can be viewed in terms of gonadotropin-independent and gonadotropin-dependent events.

Gonadotropin-Independent Events

Although it is established that growth of primordial follicles during the preantral stage begins *in utero* and continues uninterrupted throughout life until exhaustion of the follicular pool, the mechanism controlling the initiation of this growth process is not clear. However, the growth is largely independent of gonadotropin regulation and the number of follicles initiating growth in a given cycle appears to be determined by the size of the pool of residual follicles (Peters et al., 1975). The first sign of initiation of follicular growth or follicular recruitment is the transformation of the primordial follicle to the primary follicle. The primary follicle is characterized by differentiation of the spindle-shaped granulosa cells to those with a cuboidal shape (Figure 5). The oocyte enlarges and secretes a glycoprotein forming the zona pellucida which separates the oocyte from the granulosa cells.

Proliferation of the granulosa cells by mitosis results in several distinct layers of cuboidal granulosa which characterize the secondary follicle (Figure 5). Due to an increase in granulosa cell mass and growth of the oocyte, the secondary follicle may reach 120 μm in diameter. Secondary follicles become associated with presumptive theca cells which can be identified as distinct from the surrounding stroma at this stage. With further development and differentiation, the layer of cells adjacent to the basal lamina become identifiable as the theca interna, and the outer layers of cells, which appear more spindle-shaped, form the theca externa. Secondary follicles become associated with their own blood supply through establishment of arteriolar branches that reach the basal lamina. The granulosa layers and oocyte remain avascular. However, a system of gap junctions forms which facilitates nutrient transfer and cellular communication.

Follicular development from primordial to secondary follicles is associated with a dramatic increase in oocyte size as well as proliferation of the granulosa and developing thecal compartments. Coincident with this growth is a migration of the growing follicle from the periphery of the outer ovarian cortex towards the inner medulla. Further growth of the follicle results from a proliferation of both the granulosa and theca cell layers. This stage is characterized by the appearance of pockets of fluid within the granulosa cell layer that ultimately coalesce to form a fluid-filled cavity, the antrum. The antral fluid is composed of serum transudate as well as the secretory products of the granulosa cells (Table 1). Granulosa secretory

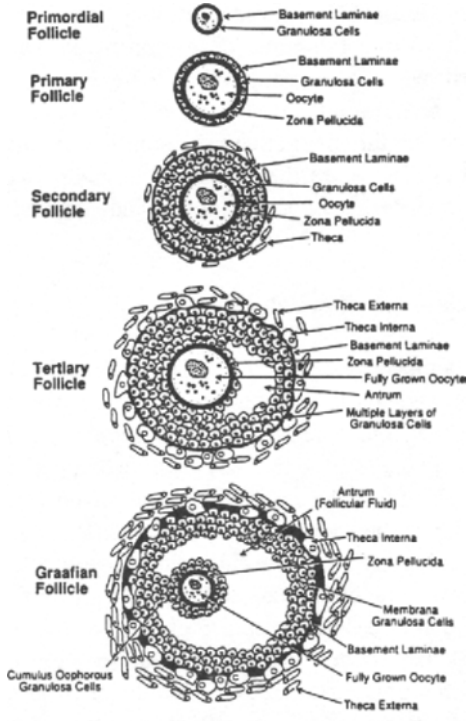

Figure 5. The structure and classification of the ovarian follicle during growth and development in the human. From Erickson et al., 1985.

products such as estrogens and proteinaceous growth factors are found in concentrations several times greater than in peripheral blood (Erickson et al., 1985).

Formation of the antrum is the hallmark of the antral stage of follicular development which is characterized by a rapid increase in follicle size under the influence of gonadotropin stimulation resulting in formation of the mature, or Graafian, follicle. Granulosa cell proliferation may result in a 600-fold increase in cell number. This increase is associated with a progressive enlargement of the antrum resulting in a more than 15-fold increase in follicle diameter. The oocyte becomes eccentrically located within the antrum and surrounded by two to three layers of granulosa cells known as the cumulus oophorus (Figure 5).

Gonadotropin-Dependent Follicular Development

Progression of follicle growth beyond the preantral stage is dependent on pituitary gonadotropin stimulation (LH and FSH). Such progression is associated with the proliferation of the thecal and granulosa cell layers and production of sex ster-

Table 1. Substances Found in Follicular Fluid[a]

Plasma proteins
Steroid-binding protein
Enzymes
Side-chain cleavage enzymes
3β-hydroxysteroid dehydrogenase
17α-hydroxylase
17,20 lyase
17β-hydroxysteroid dehydrogenase
20α-hydroxysteroid dehydrogenase
Aromatase
Plasminogen (proteases)
Micropolysaccharides (proteoglycans)
Hyaluronic acid
Chondroitin sulfate acid
Heparan sulfate
Steroids
Estrogens
Progestins
Androgens
Pituitary hormones
Follicle stimulating hormone
Luteinizing hormone
Prolactin
Oxytocin
Vasopressin
Nonsteroidal ovarian factors
Inhibin
Follicular protein (aromatase inhibitor)
Oocyte meiosis inhibitor
Luteinization inhibitor
Luteinization stimulator

Note: [a] From Yen (1986).

oids (androgens and estrogens). With differentiation of the outer thecal cell layer, the follicle acquires the enzymatic machinery necessary for conversion of the 27-carbon (C-27) cholesterol to the C-21 and C-19 steroids, principally androgens. Thecal cells demonstrate cell-surface receptors for LH, the binding of which activates the cAMP-protein kinase A (PKA) signal transduction pathway, increasing specific enzyme production via translation of RNA and protein synthesis, as well as increasing enzyme activity (Jia et al., 1991).

The granulosa cell compartment, under the influence of FSH, is responsible primarily for the conversion of C-19 androgen precursors to C-18 estrogens. FSH also acts via binding of specific cell-surface FSH-receptors utilizing the cAMP-PKA signal transduction pathway. Follicular estrogen, therefore, is derived principally from the action of the granulosa cell aromatase enzyme system under the influence of FSH, using androgen precursors derived from the thecal compartment under the

influence of LH. This concept, in which the individual gonadotropins stimulate separate cellular compartments within the follicle, constitutes the two-cell theory of follicular steroid production (Hsueh et al., 1984) (Figure 6).

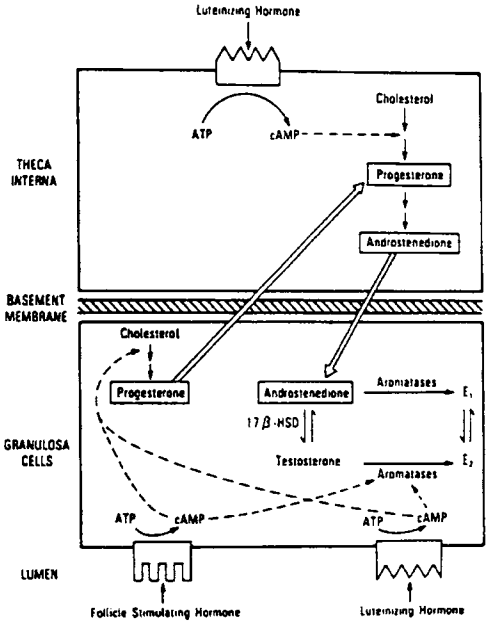

Figure 6. A two-cell, two-gonadotropin hypothesis of gonadotropin control of ovarian estrogen biosynthesis. From Hsueh et al., 1984.

Table 2. Intraovarian Modulators of Gonadotropin Action[a]

Modulator	Origin	Action	References
Activin	GC	Stimulation of FSH release	Ling et al., 1986
Angiotensin II	GC/TC/FF	Sensitization to LH	Do et al., 1988
Catecholamines	Nerves	Augmentation of androgen	Magoffin and Erickson, 1981
Inhibin	GC	Inhibition of FSH release	McLachlan et al., 1987
EGF	GC	Stimulation of GC growth	Hernandez et al., 1988
IGF-I	TC	Augmentation of FSH	Hernandez et al., 1988
TGF-α	TC	Stimulation of steroidogenesis Inhibition of androgen synthesis	Magoffin, 1989
TGF-β	TC/GC	Inhibition of androgen synthesis	Magoffin, 1989

Note: [a] GC, granulosa cells; TC, theca cells; FF, follicular fluid; EGF, epidermal growth factor; IGF-I, insulin-like growth factor; TGF-α, transforming growth factor-alpha; TGF-β, transforming growth factor-beta.

In addition to these direct pituitary gonadotropin effects, there exist a number of local intraovarian regulatory mechanisms, both autocrine and paracrine, by which the granulosa and thecal compartments can modulate the actions of gonadotropins. A variety of molecules have been demonstrated to influence ovarian action, including the locally produced polypeptide growth factors. Many of these peptides have been implicated in intraovarian regulatory mechanisms involving both the thecal and granulosa cell compartments (Table 2). Although these peptides exhibit activity via binding to specific receptors, they differ from classical endocrine effectors by acting locally in an autocrine (on the same cell) or a paracrine (on an adjacent cell) manner.

Follicular Dominance

With sexual maturity, follicular growth is regulated by cyclical pituitary release of LH and FSH, under the influence of pulsatile hypothalamic gonadotropin-releasing hormone (GnRH). This coordinated series of events comprises the menstrual cycle in which a single dominant follicle achieves full maturity culminating in the ovulation of a fertilizable oocyte. Prior to the start of each such cycle, under the influence of rising pituitary gonadotropin secretion, a cohort of follicles within both ovaries will initiate this phase of continued follicular development. Follicles that are capable of responding with each cycle have attained a sufficient stage of growth such that they exhibit cell surface receptors for FSH as well as the enzymatic machinery necessary for steroid (principally estrogen) production.

Both FSH and estrogens act to increase the activity of the granulosa cell aromatase system, as well as to increase cell-surface FSH receptor acquisition. With increased conversion of thecal androgen precursors, the follicle acquires a predominantly estrogenic microenvironment (Erickson, 1986). Estrogen concentrations within follicular fluid may reach several-fold those levels found in the peripheral blood. Rising follicular steroid production serves as negative feedback inhibition on pituitary FSH release. Thus, during rapid follicular growth under the influence of gonadotropins, ovarian steroid production effects negative feedback, resulting in a withdrawal of gonadotropin support.

Withdrawal of FSH results in a relative decrease in granulosa cell aromatization of androgens to estrogens in all but the single dominant follicle which has acquired sufficient levels of receptors and enzymatic machinery to maintain an estrogenic environment in the face of declining levels of serum FSH. The remainder of the follicles in the developing cohort, due to a lack of aromatization of thecal precursors, are characterized, principally, by androgen production. These follicles follow a limited life span and demonstrate morphologic evidence of atresia (Hodgen, 1982). Thus, through this process known as follicle selection, usually only a single dominant follicle will attain full maturity and eventual ovulation in any given cycle of follicular development; the remainder of the cohort will undergo atresia, further depleting the follicular pool (Figure 7). Using the technique of ovarian vein cannula-

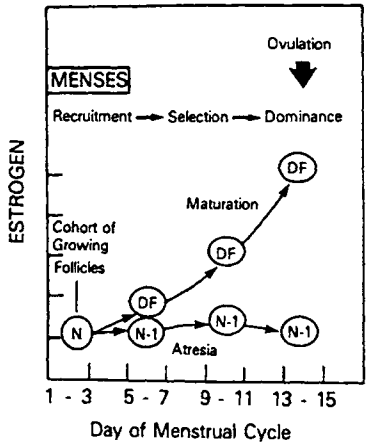

Figure 7. Time course for the recruitment, selection, and ovulation of the dominant follicle (DF) with the onset of atresia among the other follicles of the cohort (N-1). From Hodgen, 1982.

tion, a distinct unilateral rise in ovarian estrogen production has been demonstrated by the fifth day of the human menstrual cycle (Chikasawa et al., 1986), indicating that selection of the dominant follicle is determined early in the cycle.

Once selected, the dominant follicle undergoes rapid growth due both to proliferation of the thecal and granulosa cell compartments and to accumulation of follicular fluid. Follicles which average 8–10 mm in diameter at this stage (cycle day 5) will reach 16–26 mm in diameter prior to ovulation (cycle day 12). The theca layer becomes well vascularized, a feature which aids in gonadotropin delivery. The granulosa cells proliferate and estrogen production parallels follicular growth at this stage, reaching peak levels prior to ovulation. The volume of follicular fluid increases to a maximum of 6–7 ml prior to ovulation.

With further growth of the preovulatory follicle, the granulosa cells acquire cell-surface receptors for LH. Sustained elevated levels of estradiol in the peripheral blood (greater that 200 pg/ml for more than 50 hours) have been demonstrated to be responsible for the change from negative feedback on pituitary gonadotropin secretion to a positive feedback mechanism operating at the level of the hypothalamus. Furthermore, the elevated estradiol exerts a positive feedback on the pituitary to initiate the preovulatory LH surge (Liu and Yen, 1983). The LH surge is responsible for the process of luteinization of the follicle and resumption of meiosis in the oocyte. With the appearance of LH receptors, granulosa cells intitiate progesterone synthesis by the follicle, which begins prior to ovulation and may augment pituitary LH release (Veldhius et al., 1988). The LH surge reliably predicts ovulation, with ovum release occurring 34–36 hours following detection of a rise of LH in the peripheral blood. Although the precise mechanism responsible for ovum release in the human is unclear,

activation of proteolytic enzymes, as well as production of prostaglandins, progesterone, and cAMP all play a role in the process (Erickson, 1986).

Following ovulation, the collapsed follicle involutes and becomes highly vascularized, with capillaries penetrating into the central cavity which may fill with blood. The thecal cell layer continues to be active in steroid production, now comprised of theca-lutein cells. The granulosa cells enlarge and contain lipid-laden vacuoles characteristic of steroid-secreting cells. The accumulation of this material, which yields a yellow pigmentation, has given rise to the term corpus luteum (yellow body). The human corpus luteum, like the follicle, is capable of producing all three classes of steroids (C-21 progestogens, C-19 androgens, and C-18 estrogens). Estrogen production, which peaks prior to ovulation, falls rapidly following ovulation. However, the human corpus luteum is responsible for significant estrogen secretion, albeit at levels well below those observed during the follicular phase. Progesterone secretion, which begins prior to ovulation, rises dramatically and reaches a peak approximately eight days following the LH surge (Figure 8). High levels of progesterone produced by the corpus luteum maintain negative feedback

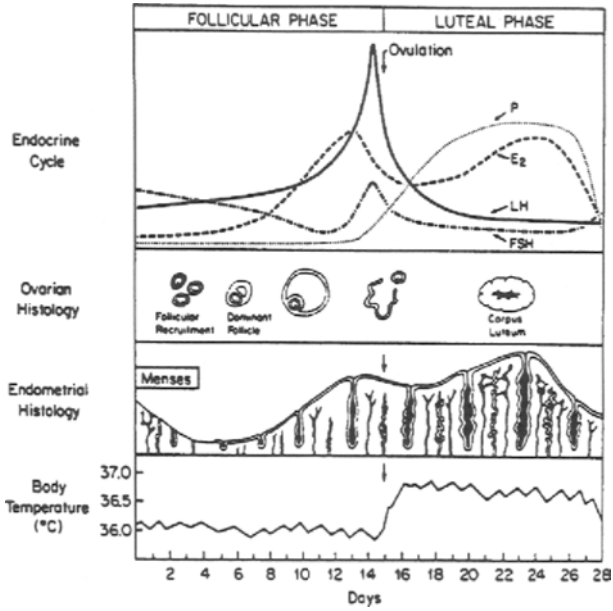

Figure 8. Hormonal, ovarian, endometrial, and basal body temperature changes and relationships throughout the normal menstrual cycle. From Carr, B.R. [1987]. Disorders of the ovary and female reproductive tract. In: Harrison's Principles of Internal Medicine, 11th edn. (Braunwald, E., Isselbacher, K.J., Petersdorf, R.G., eds.), pp. 1818-1837, McGraw-Hill, New York.

inhibition on pituitary gonadotropins, thereby inhibiting significant gonadotropin-dependent follicular growth.

If pregnancy occurs, rapidly differentiating trophoblastic tissue will produce human chorionic gonadotropin (hCG) which binds avidly to LH-receptors on the corpus luteum and maintains progesterone secretion, thereby inhibiting further follicular development and suppressing menstrual bleeding. In the absence of pregnancy, the corpus luteum undergoes a highly predictable demise 9–11 days following ovulation. The mechanism underlying luteolysis in the human ovary has not been fully elucidated, whereas in large ruminant species, luteolysis is under the influence of prostaglandin production (PGF2α), derived from local utero-ovarian circulation (Gelety and Chauduri, 1992). With regression of the corpus luteum, ovarian steroid production (principally progesterone) falls rapidly, releasing pituitary gonadotropin secretion from feedback inhibition. The resulting rise in FSH initiates the next wave of follicle recruitment.

The withdrawal of estrogen and progesterone support to the uterine endometrium is responsible for menstrual bleeding. Thus, the menstrual cycle can be divided into a follicular and a luteal phase, separated by ovulation (Figure 8). In a normal menstrual cycle, the interval from the midcycle surge of LH to the occurrence of menses is remarkably constant at 14 days. Therefore, differences in human menstrual cycle length (25–30 days, mean 28 days) are attributable to variations in the duration of the follicular phase.

Following menstrual bleeding and sloughing of the uterine endometrial lining, endometrial proliferation occurs under the mitotic influence of rising follicular phase estrogen levels, referred to as the proliferative phase of endometrial growth. Following ovulation, ovarian luteal progesterone secretion limits unchecked endometrial proliferation and induces a differentiation of endometrial glandular components resulting in copious glycogen-rich secretory activity, known as the secretory phase of endometrial growth. Withdrawal of estrogen and progesterone support following ovarian luteolysis results in an orderly breakdown of the endometrium and menstrual bleeding.

Oocyte Meiotic Maturation

Definition

Oocyte meiotic maturation is the process whereby the arrested, germinal vesicle (GV) stage oocyte enters a period of irreversible commitment to resume meiosis and subsequently undergoes germinal vesicle breakdown (GVBD), and progression through diakinesis, metaphase I, anaphase I, telophase I, and finally to traverse rapidly through prophase II to reach metaphase II (Figure 9). Interestingly, while the oocyte has acquired nuclear meiotic competence when it reaches metaphase II, the lapse of a few additional hours is required before it achieves full cytoplasmic competence (Racowsky, 1991). Thus, in order for an oocyte to realize full developmental competency it

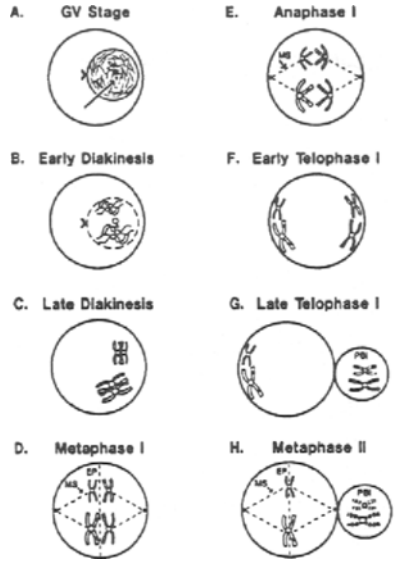

Figure 9. Schematic diagram showing changes in the configuration of chromatin as a human oocyte progresses through meiotic maturation. For clarity only two of the 23 pairs of homologous chromosomes are shown. **A**. The GV-stage showing a typical fibrous chromatin configuration, an intact nuclear membrane (arrowhead) and the densely staining nucleolus (arrow). **B**. Early diakinesis, which marks the onset of meiotic resumption in which chromatin condensation, homologue alignment, and chiasmata formation occur before nuclear membrane dissolution (arrowhead) and disappearance of the nucleolus. **C**. Late diakinesis, in which crossing-over occurs between homologous chromosomes. **D**. Metaphase I, characterized by formation of the meiotic spindle (MS) and alignment of bivalents on the equatorial plate (EP). **E**. Anaphase I, in which homologous chromosomes of each pair of bivalents migrate to opposite poles of the meiotic spindle. **F**. Early telophase I, in which homologous chromosomes have segregated to opposite poles. **G**. Late telophase I, in which one group of homologous chromosomes is eliminated from the oocyte within the first polar body (PBI). **H**. Metaphase II, characterized by formation of the second meiotic spindle and alignment of univalents on the second equatorial plate.

must complete nuclear maturation and acquire cytoplasmic competence. Accordingly, if a meiotically mature oocyte is inseminated too soon after completion of maturation *in vitro*, normal fertilization and development are compromised (Veeck, 1991).

Follicular Regulation of Meiotic Maturation

The follicular mechanisms that regulate oocyte meiotic resumption remain to be fully elucidated (Racowsky, 1991). It has been recognized for more than sixty years

that follicular cells are crucial for the maintenance of oocyte meiotic arrest *in vivo*, since Pincus and Enzmann (1935) reported that removal of an oocyte from the antral follicular environment resulted in meiotic resumption. Thus, it has become dogma that the oocyte *in vivo* is maintained in meiotic arrest by the action of a follicular "arrester" of granulosa origin, the effect of which is somehow overcome after exposure of the mature antral follicle to preovulatory gonadotropin.

The nature of the follicular meiotic arrester remains to be definitively proven, although numerous studies support a meiotic arresting role for cAMP (Racowsky, 1993), mediated by PKA. Furthermore, whether meiotic resumption is a consequence of removal from the influence of meiotic arrester, or results from a gonadotropic-mediated production of a meiotic stimulator is unclear. Evidence in favor of both possibilities exists. The former is supported by several observations that reveal a gonadotropin-induced disruption in the heterologous gap junctional network that exists between the oocyte and its surrounding granulosa cells (Larsen et al., 1986, 1987; Racowsky et al., 1989) which, in turn, would result in interruption of cAMP transfer from the somatic compartment to the oocyte. However, production of a meiotic stimulator is supported by other studies revealing a meiotic stimulating activity of, for example, EGF, prostaglandins, protein kinase C and, paradoxically, cAMP itself (Downs et al., 1988; Downs, 1989). Indeed, the role of cAMP-PKA may be dichotomous: a tonic level of intracellular cAMP appears to be required to maintain meiotic arrest, while a transient rise in cAMP such as that which occurs following LH-binding to its receptor protein may provide the signal for the onset of meiotic resumption.

At the molecular level, activation of maturation promoting factor (MPF) appears to be a ubiquitous requirement for entry either into meiotic resumption and/or through metaphase in oocytes of all mammalian species so far studied (Albertini, 1993). MPF is a heterodimer composed of P34cdc2 (P34) and cyclin B. The catalytic subunit, P34, displays a kinase activity, the regulation of which involves both phosphorylation/dephosphorylation on specific residues, as well as association with the regulatory subunit, cyclin B. In species such as the mouse, stores of inactive pre-MPF are activated rapidly at the onset of meiotic resumption through a mechanism independent of protein synthesis that appears to involve the c-mos protooncogene (Mutter et al., 1988; Paules et al., 1989). In contrast, in the larger domestic species such as cow, MPF activation appears linked to the synthesis of the cyclin B regulatory subunit of MPF. The mediating role of MPF in human oocyte maturation remains unknown. However, in view of similarities in the kinetics of meiotic progression between human oocytes and those of the larger domestic species, GVBD may also be dependent upon cyclin B synthesis in our species.

Oocyte Meiotic Errors and Their Consequences

Gross chromosomal abnormalities in the meiotic process can occur either before anaphase I, or when the oocyte has entered anaphase I/telophase I. In the former

case, several possibilities exist (Racowsky and Kaufman, 1992). One is that the oocyte may become arrested at metaphase I or that anaphase I may be initiated but may fail so that some of the homologous chromosomes will have segregated. A third possibility is that homologue segregation may be complete but movement of the chromosomes to opposite ends of the spindle may fail, thereby giving rise to a diploid metaphase II configuration. In addition, an aberration may occasionally occur where the pairs of homologous chromosomes segregate, rather than the homologues themselves (Racowsky and Kaufman, 1992). All these meiotic abnormalities are incompatible with normal fertilization.

In the case of errors occurring after the oocyte has entered anaphase I/telophase I, abnormalities include the condition of nondisjunction (Zenzes and Casper, 1992) in which there is failed segregation of homologous chromosomes and, more rarely, failed emission of the first polar body. The former results in aneuploidy in which the oocyte either has too few chromosomes (i.e. is hypohaploid) or too many (i.e., is hyperhaploid). If a normal haploid sperm fertilizes an oocyte with < 23 chromosomes, the absence of the specific chromosome or chromosomes will lead to a zygote that is monosomic for the chromosome(s) involved. Conversely, if an oocyte has two of the same chromosome (i.e., has > 23 chromosomes), the resulting zygote will be trisomic.

Clearly, the presence of the first polar body only indicates that an oocyte has completed meiotic maturation; it does not confirm that chromosomal segregation to the oocyte and polar body has been equal. Indeed, non-disjunctional errors alone result in an aneuploidy rate as high as 30% (Boue et al., 1985) and account for the great majority of spontaneous abortions attributable to chromosome imbalance (Hassold, 1986; Warburton, 1989).

While many aneuploidy errors are lethal, some are not (Schnizel, 1984). When monosomy involves one of the non-sex, or autosomal, chromosomes, the resulting condition is generally incompatible with embryonic survival. In contrast, autosomal trisomy is variably lethal depending upon which chromosome is involved, and X-chromosome trisomy is not lethal, although it is associated with reduced fertility. Generally speaking, the larger the chromosome, the more severe the clinical syndrome associated with autosomal trisomy. Thus, Patau's syndrome, which involves the relatively large chromosome 13, is associated with very severe mental retardation, gross somatic malformations, and a lifespan of only a few months. In contrast, Down's syndromy, which involves the smallest autosome 21, is associated with a longer life expectancy, and varying degrees of mental retardation and somatic anomaly.

In addition to gross chromosomal abnormalities, other types of meiotic error may occur during meiotic maturation (Schnizel, 1984). These include translocations in which all or part of a chromosome may become attached to another chromosome; inversions of lengths of chromosomes, or the formation of ring chromosomes; and partial monosomies or trisomies in which, respectively, deletions or duplications of specific chromosomes may occur.

PERIMENOPAUSAL/MENOPAUSAL OVARY

Demise of the Follicular Pool

It is clear that the number of germ cells is determined early in gestation reaching a peak by 20 weeks, and that continuous follicular development, arrest, and germ cell loss are ongoing processes that occur during a period spanning over 50 years. Shortly after the menopause (average age 51.5 years), the ovarian germ cell complement is exhausted and, due to the absence of follicular development, ovarian estrogen production ceases. At the time of menarche (average age 12.7 years), the ovaries contain roughly 400,000 germ cells, only one of which is released each month for potential fertilization over the next 40 years. The remainder, therefore, are inexorably lost through the process of atresia. The age of menopause when follicular exhaustion occurs appears to be unaffected by factors affecting the frequency of ovulation including the number of pregnancies attained, or duration of oral contraceptive use (Utian, 1980). Calculations of the expected rate of loss of germ cells during regular cyclic menses have suggested that the rate of loss is dramatically accelerated during the decade prior to the menopause (Hertig, 1944). It has been suggested that follicles remaining until the end of reproductive life may be less responsive to gonadotropin stimulation.

Hormonal Consequences

In the years prior to the menopause, known as the perimenopause, the remaining ovarian follicles and germ cells perform less well. Poor follicle development results in lower levels of estrogen attained during the follicular phase, due to diminished follicular granulosa cell functioning. Decreased estrogen production, as well as a decrease in the granulosa cell peptide, inhibin, results in inadequate negative feedback and a persistent elevation in pituitary FSH secretion, most evident during the early follicular phase (day 3). High levels of FSH occurring early during follicular development may paradoxically lead to accelerated follicle growth and a shortening of the follicular phase. Consequently, the overall menstrual interval is shortened, a characteristic of the perimenopausal period. Poor follicular growth may also result in poor functioning of the corpus luteum with an inadequate production of progesterone to maintain endometrial integrity. This may lead to menstrual cycle irregularities, as well as decreased fertility. With declining follicle function, sufficient estrogen production necessary for induction of an LH surge may not occur. Thus, anovulation ensues further contributing to abnormalities in menstrual cyclicity.

With exhaustion of the follicular pool following menopause, ovarian estrogen and progesterone production ceases, which results in cessation of cyclic uterine endometrial bleeding. Pituitary FSH secretion rises prior to the menopause due to deficient estrogen and inhibin secretion by the granulosa cells

(Sherman et al., 1976). The perimenopausal rise in FSH in the face of low levels of pituitary LH is the major piece of evidence leading to the elucidation of inhibin as a unique nonsex-steroid regulator of cyclic pituitary gonadotropin secretion. With follicular depletion, FSH levels continue to rise and reach a peak 1–3 years post-menopausally of an average of 130 mIU/ml. LH levels also rise, but not to the same degree, reaching an average serum concentration of 90 mIU/ml.

With loss of the follicular pool, the cortex of the postmenopausal ovary becomes markedly reduced in size. In contrast, the ovarian stroma becomes dominant, with prominent interstitial cells and ovarian hilar cells. In the absence of ovarian follicles, elevated gonadotropin levels maintain stimulation to the remaining stromal tissue. Following the menopause, serum testosterone levels do not decline and ovarian testosterone secretion may be increased relative to the premenopausal ovary. Androstenedione levels, however, which are derived equally from the ovary and the adrenal prior to the menopause, decrease by 50% following the menopause (Judd, 1976).

Reduction in Egg Quality

The well-recognized decline in fertility with advancing female age may be due, in part, to poor follicular functioning resulting in inadequate hormonal support for the fertilized ovum to achieve implantation and maintain early embryonic growth. However, several lines of evidence suggest that a dramatic decline in oocyte quality per se is also a major factor in declining fertility associated with advancing reproductive age. The dramatic increase in the incidence of chromosomal aneuploidy with increasing maternal age is well recognized and serves as the basis for recommending amniocentesis to all expectant mothers over the age of 35. Fetal aneuploidy is strongly correlated with maternal age with an incidence of 1:500 at age 22, increasing to 1:200 by age 35 and 1:40 at age 42. In addition to this age-related increased incidence in fetal aneuploidy detected during the second trimester of an ongoing pregnancy, early spontaneous pregnancy loss also increases dramatically with maternal age. The incidence of clinically apparent early pregnancy wastage increases from 15–20% during the third decade of life to as high as 50% after age 45. As many as 50–60% of these losses are associated with chromosomal abnormalities. Taken together, this evidence suggests an increase in meiotic aberrations and aneuploidy in the remaining ovarian germ cells with advancing reproductive age.

The most compelling evidence for an age-related decline in oocyte quality comes from experience using the technique of oocyte donation. Oocytes obtained from younger reproductive aged women, fertilized *in vitro*, and transferred to the uterus of postmenopausal recipients who have received appropriate exogenous estrogen and progesterone priming, will result in successful pregnancies at rates comparable to those of younger, fertile women. In addition, rates of early pregnancy

wastage are not increased (Sauer et al., 1993), suggesting a primary role for age-related oocyte "quality" in reproductive outcome.

SUMMARY

The mammalian ovary provides an ideal example of a dynamic organ that evolves through a characteristic life cycle which closely follows the life cycle of the female. This cycle is initiated during prenatal life and progresses through the neonatal interval to reach the climax of its activity during the period of sexual maturity prior to entry into a phase of gradual senescence before reaching menopause. The prenatal period is characterized by ovarian differentiation, germ cell formation, and the entry of a finite number of germ cells into oogenesis. While follicle formation begins prenatally, so also does the process of follicular atresia, whereby there is a gradual attrition of the predetermined pool of germ cells. During the neonatal interval, the cycle of folliculogenesis and atresia continues resulting in a progressive decrease in the number of germ cells, and thus the reproductive potential of the individual.

Superimposed on the period of sexual maturity is the menstrual cycle which, in contrast to the other phases of the ovarian cycle, is measured in days, rather than in months or years. It is during the menstrual cycle that the ovary fulfills its two primary physiological responsibilities: the release of a meiotically mature haploid egg with full developmental competency, and the production of the two steroid hormones, estradiol and progesterone. Both these activities are accomplished in an integrated, repetitive fashion involving a variety of heterogenous cell types organized into follicular units embedded within the ovarian stroma. The regulation of these activities is predominantly via the hormonal interplay between the ovary and the hypothalamic-pituitary axis, but is defined by a single, transitory subunit, the dominant follicle.

During the perimenopausal period, follicular function wanes, diminishing steroid production and overall reproductive potential. An accelerated attrition of the germ cell pool, as well as a decline in the quality of the remaining oocytes, are also major factors in the decline in fecundity. Such decline culminates, following the menopause, in exhaustion of the germ cell pool and cessation of ovarian steroid function.

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Chapter 5

Biology of Human Fertilization: Sperm–Egg Interactions and Early Development

ANN M. GINSBERG and JURRIEN DEAN

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INTRODUCTION

Mammalian fertilization initiates with sperm binding to an egg in the oviduct and terminates with the fusion of male and female pronuclei to create a one-celled zygote. This process accomplishes three vital tasks: (1) creation of unique haploid genomes via 40–150 genetic recombinations and independent assortment of chromatids during the two meiotic divisions of gametogenesis; (2) sex determination of the resulting fetus depending on the participation of a sperm bearing an X or a Y chromosome to create a female or a male embryo, respectively; and (3) formation of a diploid embryo by joining male and female haploid pronuclei. As will be discussed, normal development depends on a genetic contribution from each pronucleus.

Today we have the means to exercise unprecedented control over the reproductive process, due to advances in understanding the basic biology of fertilization and technical progress enabling us to manipulate this crucial function. While progress has been impressive, there is still much to be learned. In surveys completed in 1995, 10% of U.S. women (6.1 million of all marital status) in their fertile years (15–44 years old) have impaired fecundity with difficulty in becoming pregnant or carrying a fetus to term, 44% of these women have sought medical advice for reduced fertility and only half of couples who report a period of infertility eventually conceive (Jones and Toner, 1993; Schmidt and Munster, 1995; Mosher and Bachrach, 1996). While some are struggling to have children, others need improved methods to prevent unwanted pregnancies. The world population is growing at a staggering rate and is expected to exceed six billion by the year 2000. Half as many people will be born in the last 20 years of this century as were born between the origin of man and 1980 (Diczfalusy, 1993). These statistics add urgency to seeking a fuller understanding of fertilization in order to develop both better therapies for infertility and improved means of family planning.

Due to the difficulty of observing human fertilization *in vivo* and acquiring the necessary tissues for research, our current knowledge of reproductive biology is based largely on studies of non-human mammals. Therefore, much of what will be discussed in this chapter was derived from investigations in mice, hamsters, and rabbits.

GAMETE MATURATION

Primordial germ cells are allocated early in embryogenesis from a small pool of cells in the extraembryonic mesoderm (Lawson and Hage, 1994). A large, round shape and high alkaline phosphatase activity have facilitated investigations into their proliferation and migration from the allantois to the urogenital ridges. Upon arriving into the primitive gonad during the sixth week of human development, the primordial germ cells become incorporated into the primary sex cords of the indifferent gonad (Figure 1). At this point in development, the male and female germ

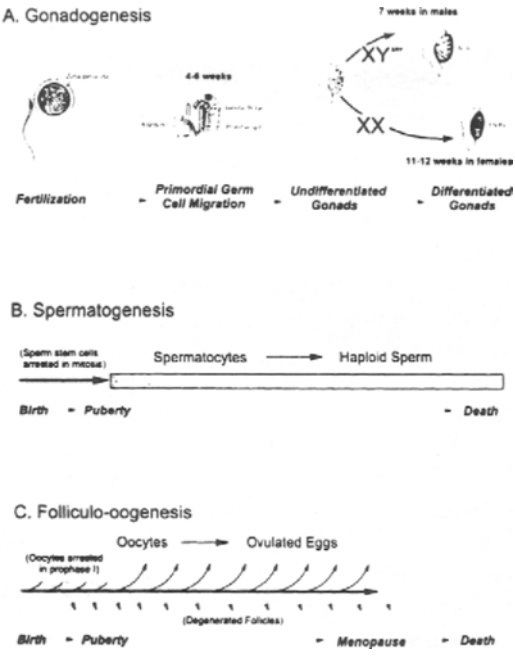

Figure 1. Timelines of human gonadogenesis and gametogenesis. (**A**). Gonadogenesis. The male and female gonads derive from the genital ridges of the developing embryo. Germ cells of each sex develop from primordial germ cells (PGCs), which migrate early in embryonic development from the allantois into the genital ridge. By six weeks of development, the migration is complete and the PGCs become incorporated into the primary sex cords. Male and female gonads appear morphologically identical (undifferentiated gonads) until approximately seven weeks of development. At this time the male sexual determination cascade is turned on, apparently under the influence of the SRY gene product (see text), and testicular cords can first be identified. The developing female gonad remains morphologically undifferentiated until 11–12 weeks of development. (**B**). Spermatogenesis. Spermatogonial stem cells in the sex cords of the developing testis become mitotically arrested before birth and remain in arrest until just before puberty. At puberty they divide mitotically to become primary spermatocytes which eventually undergo two meiotic divisions to form haploid spermatids. During subsequent spermiogenesis round spermatids differentiate into mature spermatozoa. In humans, development of mature sperm from a proliferating stem cell takes 74 days and occurs continually from puberty to death. (**C**). Folliculo-oogenesis. Most oocytes degenerate before birth (see text); the remainder become arrested pre-natally in Prophase of Meiosis I (Dictyate), where they remain until just prior to ovulation (any time from puberty to menopause). Although there is some follicular growth prior to puberty, all follicles degenerate (dotted arrows) if they are not ovulated. At puberty, groups of follicles resume growth, but only a dominant follicle (solid arrows) develops into the Graafian follicle; the rest degenerate (dotted arrows). The egg in the dominant follicle completes Meiosis I and is subsequently ovulated. Folliculo-oogenesis and ovulation end with menopause.

cells are indistinguishable. However during subsequent gonadogenesis, male germ cells enter into mitotic arrest where they remain until just before puberty, whereas female germ cells enter into meiotic arrest which persists until just prior to ovulation.

Male (XY) versus female (XX) gonadal differentiation is dependent on the presence or absence, respectively, of the *SRY* gene located on the short arm of the Y chromosome. Thus, XY fetuses that are missing *SRY* commonly develop into phenotypic females and XX fetuses containing *SRY* (for example, due to a chromosomal translocation) can develop into phenotypic males. The expression of *SRY* in the somatic cells of the developing male gonad appears to initiate events that result in formation of the testis and expression of the male phenotype. Except for a canonical HMG box implicated in bending DNA, the SRY protein is poorly conserved between man and mouse and is lacking in some mammalian species. Current investigations are directed at determining downstream targets of SRY that activate male-specific pathways, inhibit female-specific pathways or both. Other than the required absence of the Y chromosome, little is known about female sex differentiation and gonadogenesis. *DAXI* (a member of the nuclear hormone receptor family) located at the dosage-sensitive sex reversal *(DSS)* locus at Xp21is temporally expressed just after *SRY,* but in both male and female genital ridges. Its abundance falls off rapidly in males expressing *SRY* but remains high in female mice suggesting a role for *DAXI* in gonadal sex differentiation. Additional genes and factors that must play crucial roles in gonadogenesis are currently being sought and investigated (Harley et al., 1992; Lovell-Badge and Hacker, 1995; McElreavey et al., 1995; Swain et al., 1996).

Sperm Development

In humans, testicular differentiation begins in the seventh week of development (Figure 1A). Germ cells and presumptive Sertoli cells form testicular cords, the precursors of adult seminiferous tubules. Leydig cells develop within the testicular interstitium and secrete testosterone, the hormone responsible for the male phenotype. Spermatogenesis, the process by which spermatogonia develop into mature sperm (spermatozoa), begins just before puberty and continues throughout adult life (Figure 1B). At puberty, spermatogonia resume mitosis and begin to proliferate, regenerating stem cells and dividing to form primary spermatocytes. Four functionally equivalent haploid spermatids are produced from each primary spermatocyte via two meiotic divisions, which last 12–14 and 24 days, respectively. Each round spermatid then elongates, develops flagella, and forms an acrosome (an acidic organelle derived from the Golgi complex) overlying the nucleus in the sperm head. As spermatids mature, the nuclear chromatin undergoes reorganization, resulting in genome inactivation and nuclear condensation. Protamines, small basic proteins rich in arginine and cysteine residues, replace the histones that otherwise package nuclear DNA. Disulfide bonds form between protamines during

epididymal maturation, stabilizing the characteristic dense nuclei of mammalian sperm.

The fully developed sperm are released from their supporting Sertoli cells into the seminiferous tubule's lumen in a process called spermiation. Mature sperm then travel via the efferent ductules through the tubuli recti and rete testes into the epididymis. There they undergo "epididymal maturation," a poorly understood process during which the sperm membrane is modified and the sperm becomes motile. These two changes markedly enhance the ability of sperm to bind to the zona pellucida at the time of fertilization. Sperm maturation is completed in the distal cauda epididymis of most mammals and an average human adult produces approximately one hundred million sperm per hour per testis.

Spermatozoa, released by ejaculation (typically, 200 million sperm per human ejaculate), undergo capacitation in the female genital tract (Figure 2). Changes produced by this process have not been fully defined, although capacitation can be mimicked *in vitro* by incubation in artificial media. Capacitation appears to reveal sperm receptors important for binding to the egg zona pellucida and induces

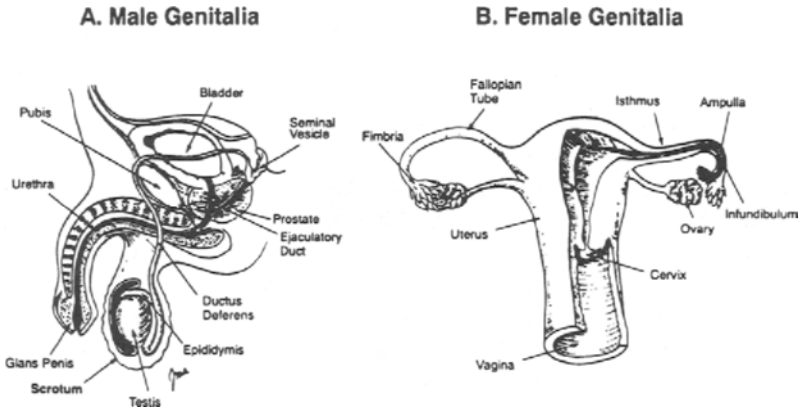

Figure 2. Male and female genital organs. (**A**). Male Genitalia. Spermatogenesis occurs in the seminiferous tubules of the mature testis The resultant spermatozoa undergo "maturation" in the epididymis where they are stored (see text). During coitus sperm are ejaculated via the ductus deferens to the seminal vesicle, where they mix with seminal fluid and pass down the ejaculatory duct through the prostate gland and the urethra. Each ejaculation releases approximately 200 million sperm. Unejaculated sperm are either resorbed or excreted in urine. (**B**). Female genitalia. Ovulation can occur from any part of the ovarian surface. The ovulated egg is picked up by the fimbria of the Fallopian tube (oviduct) and swept into the infundibulum of the tube, from which it enters the ampulla. Fertilization in humans usually occurs in the ampulla. The resultant zygote undergoes cleavage as it passes down the Fallopian tube. Implantation into the wall of the uterine cavity occurs at 7–12 days of development.

changes that enable the sperm to undergo the acrosome reaction. It is clear that capacitation is not species-specific as sperm from one species can be capacitated in the female genital tract of another. Sperm are capable of fertilizing eggs only after completing both epididymal maturation and capacitation.

Egg Development

Morphologic differentiation of the human ovary begins during weeks 11–12 of embryogenesis (Figure 1A), several weeks after initiation of testicular differentiation. Early in fetal development, oogonia undergo multiple mitotic divisions and proliferate rapidly. The initiation of meiosis marks their transition from oogonia into oocytes. They enter prophase of Meiosis I at approximately 15 weeks in response to as yet unidentified signal(s), and become arrested in the diplotene stage where they remain throughout their growth until just prior to ovulation (Figure 1C). By 20 weeks of development, primitive sex cords have developed into primordial follicles. Each primordial follicle consists of a 15–20 μm diameter oocyte surrounded by a single layer of flattened follicular cells: these latter cells are derived from the secondary sex cords.

The human ovary contains several million oocytes, but the vast majority degenerate prenatally, and at birth the human ovary contains less than two million oocytes. By menarche, only 200,000–400,000 remain of which fewer than 500 are ovulated (Erickson, 1986). During adult reproductive life, growing oocytes synthesize and secrete the zona pellucida that forms an extracellular matrix between the oocyte and its surrounding granulosa cells. As the oocyte grows, the single layer of granulosa cells becomes cuboidal and together these and the enlarging oocyte form the primary follicle, a structure approximately 50 μm in diameter. The granulosa cells and oocyte communicate with each other via specialized complexes in their plasma membranes, known as gap junctions. The follicle continues to enlarge as the oocyte grows and the granulosa cells proliferate into several layers. The cells surrounding the follicle simultaneously differentiate into steroidogenic thecal cells that form the theca interna.

The initial growth of human follicles is continuous and independent of follicle stimulating hormone (FSH) and luteinizing hormone (LH). After puberty cohorts of 5–20 primary follicles (the number decreases with age) are periodically recruited and grow to the pre-antral stage (200 μm) during each menstrual cycle. Rising FSH levels stimulate the follicular granulosa cells to produce estradiol 17β by aromatizing androgens produced by the theca interna cells responding to LH-induced signaling. As the follicles continue to grow and accumulate follicular fluid in the antrum, they are termed secondary follicles (500 μm). Only one of each cohort will be selected as the Graafian, or dominant, follicle; the others in the cohort, including those in the contralateral ovary, become atretic. The signals regulating this process remain incompletely understood (Erickson and Danforth, 1995). The Graafian follicle continues to grow and secrete estrogens, which eventually reach a critical cir-

culating threshold, stimulating the LH surge. Just before ovulation, the gonadotropin rise activates maturation promoting factor (MPF), comprised of two proteins, cdc2 and cyclin, which are highly conserved evolutionarily (yeast to humans). MPF triggers oocyte nuclear (germinal vesicle) breakdown and resumption of meiosis. The first meiotic division produces two asymmetric daughter cells, the secondary oocyte and the much smaller polar body. The oocyte proceeds through Meiosis II, arresting in metaphase. Ten to 12 hours after peak LH levels, the Graafian follicle (15–20 mm) ruptures, releasing the fully grown oocyte (120–140 μm) into the oviduct. The ovulated egg remains enclosed in the zona pellucida, which in turn is surrounded by a mass of cumulus cells in a hyaluronic acid matrix. This complex is known as the cumulus oophorus (Erickson, 1986).

FERTILIZATION

In humans, fertilization generally occurs in the ampulla of the Fallopian tube (oviduct) within 24 hours of ovulation, beginning when a capacitated sperm encounters a cumulus oophorus (Figure 3A). Despite the enormous numbers of sperm contained in the typical male ejaculate, 100 sperm or fewer are estimated to reach the ampulla, most of the ejaculated sperm being stored in the cervix (Williams et al., 1993). Invertebrate gametes appear to signal each other via secretion of chemoattractants, increasing the efficiency with which sperm and egg "find" each other. Some evidence suggests that human follicular fluid may contain a similar substance, and that the ability of follicular fluid to attract sperm correlates with the ability of the egg from that follicle to be fertilized (Cohen-Dayag et al., 1995).

After reaching the cumulus oophorus, the sperm passes through the cumulus cell mass. It then encounters the zona pellucida surrounding the egg and binds to it, inducing the sperm-acrosome reaction (Figure 3B). Acrosomal contents are released which are believed to aid the sperm in crossing the zona matrix either by modifying the surface of the sperm, the zona pellucida matrix or both. Once through the zona pellucida, the sperm enters the perivitelline space, where it binds to and fuses with the egg plasma membrane. Fusion stimulates the egg cortical reaction and the zona reaction, resulting in the establishment of the block to polyspermy. The egg then undergoes activation (including completion of Meiosis II), the sperm nucleus decondenses, and sperm and egg haploid genomes develop into male and female pronuclei, respectively. The pronuclei migrate toward the egg's center and fuse, completing fertilization.

Zona Pellucida—Sperm Binding

The zona pellucida plays a major role in species-specific fertilization. In humans and mice, this extracellular matrix surrounding the ovulated egg is formed by three major glycoproteins designated ZP1, ZP2, and ZP3 (Bleil and Wassarman, 1980a;

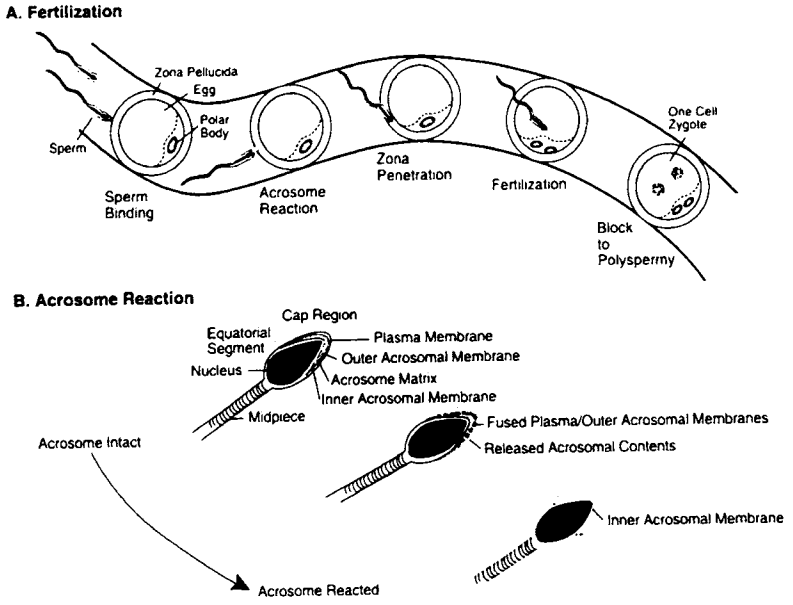

Figure 3. Mammalian fertilization. (**A**). Fertilization. The process of fertilization (depicted schematically in the Fallopian tube) begins with sperm binding to the zona pellucida via ZP3 and its receptor(s) on the sperm head. Binding to the zona induces the sperm acrosome reaction, causing release of lytic enzymes that in combination with sperm propulsion enable zona penetration by the sperm. Fertilization results when the sperm crosses the perivitelline space and fuses with the egg plasma membrane; sperm contents enter the egg cytoplasm, male and female pronuclei form, egg activation occurs and the pronuclei undergo syngamy. Subsequent to the cortical granule reaction (part of egg activation) a block to polyspermy is created by biochemical modification of the zona pellucida (and perhaps modification of the plasma membrane). (**B**). Acrosome reaction. Binding of sperm to the zona pellucida via receptor(s) in the cap region induces fusion of the outer acrosomal membrane and plasma membrane, causing release of the acrosomal contents. The contents include lytic enzymes important for zona penetration. Acrosome-reacted sperm bind the egg plasma membrane, initially in the region of their equatorial segment, leading to egg-sperm fusion.

Shabanowitz and O'Rand, 1988). The mouse zona pellucida is 7 μm thick (the human zona is 20–25 μm) and sufficiently porous to allow passage of macromolecules up to 170 kDa (Legge, 1995). A current model in mice suggests that the zona is comprised of long filaments of ZP2 and ZP3, interacting in repeating units via non-covalent interactions, and interconnected by ZP1 "bridges" (Greve and Wassarman, 1985).

The primary structure of human ZP1 (540 aa), ZP2 (745 aa), and ZP3 (424 aa) proteins have been deduced from their respective genes and transcripts (Chamberlin and Dean, 1990; Liang and Dean, 1 993; Harris et al., 1994) (Figure 4). All three

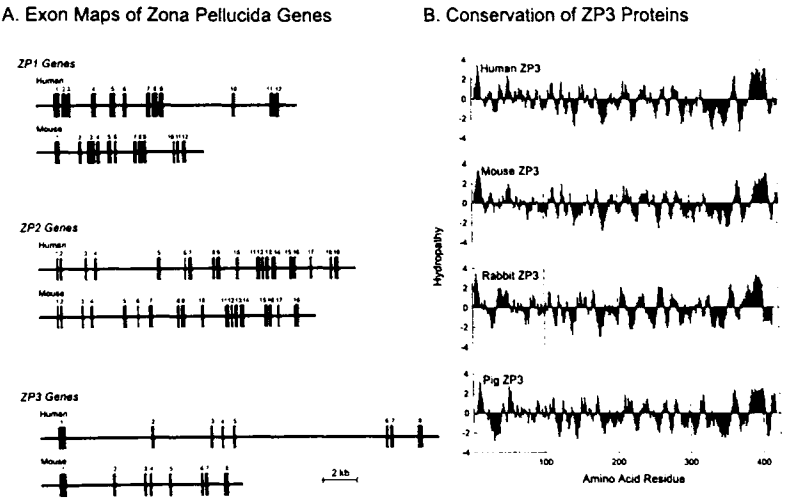

Figure 4. Structure and conservation of zona pellucida genes and proteins. (**A**). Exon maps of zona pellucida (ZP) genes. The human *ZP1* gene contains 12 exons encoding a 540 amino acid protein; mouse *Zp1* also has 12 exons but encodes a polypeptide chain of 623 amino acids. Human *ZP2* (19 exons) and mouse *Zp2* (18 exons) genes encode 745 and 713 amino acid proteins, respectively. Both human *ZP3* and mouse *Zp3* genes contain 8 exons and encode proteins of 424 amino acids (Kinloch et al., 1988; Chamberlin and Dean, 1989, 1990; Liang et al., 1990; Liang and Dean, 1993; Harris et al., 1994; Epifano et al., 1995). (**B**). Conservation of ZP3 protein. Hydropathicity plots comparing the secondary structure of four mammalian ZP3 proteins: human (424 amino acids), mouse (424 amino acids), rabbit (415 amino acids), and pig (421 amino acids). Note the overall conserved structure of these proteins, which are 66–74% conserved (at the amino acid level) among these four species. All have major hydropathic peaks in their signal peptides and near their carboxyl termini (Ringuette et al., 1988; Chamberlin and Dean, 1990; Harris et al., 1994).

proteins are secreted by the human oocyte and incorporated into an extracellular matrix, the zona pellucida. Although zona proteins are conserved in their amino acid sequences among mammals (Figure 4B), zona-sperm binding is relatively species-specific. It is not known what accounts for the specificity; however, the tools of molecular biology should enable investigators ultimately to elucidate precisely which peptide and carbohydrate moieties carry out these specific biological functions. Least conserved domains may be crucial for conferring species-specificity to sperm-zona binding, while highly conserved regions are more likely to play structural roles and participate in evolutionarily shared interactions.

Our knowledge of the role of individual zona protein(s) in fertilization continues to evolve. *In vitro* evidence from mouse experiments indicates that sperm-egg interactions are initially mediated by sperm binding to ZP3 via O-linked oligosaccha-

rides terminating in α1,3-linked galactose (Florman and Wassarman, 1985; Bleil and Wassarman, 1988). However, mice lacking α1,3-galactosyl transferase (the enzyme required to add α1,3-terminal galactose) are fertile, suggesting that additional molecules may mediate fertilization *in vivo* (Thall et al., 1995). In other species, rabbits and pigs, there are data to suggest that the ZP1 homologue participates in sperm binding (Sacco et al., 1989; Yonezawa et al., 1995; Prasad et al., 1996). It is not yet clear which of the human zona proteins is(are) critical for sperm binding to the zona pellucida. The observation that human sperm will not normally bind to mouse eggs and the ability to create mouse lines in which individual zona proteins are replaced with the corresponding human protein should provide greater insight into the molecular basis of human fertilization.

The identification of the receptor(s) that bind the zona pellucida ligand(s) to the sperm remains controversial. Based on *in vitro* experiments, 1,4 β-glactosyltransferase, sperm receptor kinase (ZRK), and sp56 have been proposed as the sperm receptor for ZP3 (Leyton and Saling, 1989; Bleil and Wassarman, 1990; Miller et al., 1992). While there is experimental support for the candidacy of each, the importance of substantiating their role *in vivo* has become increasingly apparent (Snell and White, 1996). Male mice genetically altered to lack 1,4 β-galactosyl--transferase remain fertile, albeit siring smaller litters one day later than control mice (Lu and Shur, 1997). The *in vivo* roles of the other two candidates will become more apparent when their functions are similarly assessed in genetically-altered mice. Although continued *in vivo* fertility in the absence of a candidate protein indicates it is not essential for fertilization, it may be that fertilization is sufficiently critical to the survival of a species that more than one mechanism, involving different sperm surface molecules, exists to ensure proper sperm-egg interactions.

Sperm Acrosome Reaction and Passage Through the Zona Pellucida

The acrosome is a membrane-bound secretory granule overlying the nucleus in the sperm head of eutherian mammals and has two distinct portions, a cap and an equatorial segment (Figure 3B). In humans, acrosome-reacted as well as acrosome-intact sperm can bind to the zona pellucida *in vitro* but in mice, only acrosome-intact sperm bind to the zona matrix. Using solubilized zonae from unfertilized mouse eggs, but not from two-cell embryos, the sperm acrosome reaction can be induced. According to one model, this causes acrosomal exocytosis via aggregation of receptors in the acrosomal membrane and transduction of a G-protein mediated signal, resulting in an influx of Ca^{2+} ions and an increase in intracellular pH. These reactions induce fusion of the outer acrosomal membrane with the sperm head plasma membrane to form fenestrations through which acrosomal contents, including lytic enzymes such as acrosin and glycosidases, escape (Morales et al., 1989; Saling, 1991; Ward and Kopf, 1993).

During mammalian fertilization, only acrosome-reacted sperm of the same species are observed in the perivitelline space. Although the mechanism by which

sperm traverse the zona remains unclear, only highly motile sperm with vigorous flagellar activity (requiring ATP as an energy source) pass through the zona. While acrosin has variously been described as modifying the sperm surface and/or cleaving zona pellucida proteins to facilitate zona penetration, genetically-altered mice lacking acrosin have normal *in vivo* fertility (Babe et al., 1994). Thus, it seems likely that a major determinant of sperm passage through the zona involves mechanical propulsive forces.

Sperm Fusion with the Egg Vitelline Membrane

After penetrating the zona pellucida, a sperm arrives in the perivitelline space between the zona and the egg plasma membrane. Binding to the vitelline membrane, presumably via receptor(s) on the sperm head, is followed by fusion of the sperm and egg plasma membranes. Fusion can occur anywhere on the egg surface, except in the region overlying the metaphase spindle, the only region of the vitelline membrane not covered with microvilli. Fusion begins with the area of the plasma membrane overlying or just posterior to the equatorial segment of the sperm, and results in the sperm contents being incorporated into the egg cytoplasm (Myles, 1993; Allen and Green, 1997).

Macromolecules on the posterior head of the sperm are candidates for mediating sperm-egg plasma membrane binding and fusion. Two, fertilin-β (Blobel et al., 1992) and cyritestin (Yuan et al., 1997) are transmembrane α/β -heterodimers of the ADAM (a disintegrin and metalloprotease) protein family. The β-chain of each contains a disintegrin domain capable of binding to integrins on the plasma surface of the egg (Almeida et al., 1995), and the α-chain of fertilin has a fusion peptide domain implicated in promoting the fusion of the egg and the sperm membranes. It is likely that additional macromolecules will be implicated in these sperm-egg interactions.

Egg Activation and the Postfertilization Block to Polyspermy

Fusion of the egg and sperm plasma membranes induces egg activation, a series of changes that includes the cortical granule reaction, resumption of meiosis and the establishment of the post-fertilization block to polyspermy. The mechanisms underlying egg activation have not been well delineated, but are thought to include a rapid, marked rise in intracellular Ca^{2+} concentration. In mammals, this rise is seen as multiple waves of increasing Ca^{2+} concentration traversing from the site of initial sperm-egg fusion across the egg cytoplasm, initiating seconds after fusion begins and lasting as long as 100 minutes. This dramatic but transient increase in Ca^{2+} concentration is believed to induce the cortical granule reaction and cell cycle progression (Home, 1995).

During the cortical granule reaction, the cortical granules, small-membrane-bound organelles underlying the egg plasma membrane, fuse with the plasma mem-

brane, undergo exocytosis and release their contents (a variety of hydrolytic enzymes and saccharides) into the perivitelline space. The cortical granule contents are thought to biochemically modify the zona proteins so that sperm no longer bind to the modified zona surrounding two-cell embryos and the modified zona is no longer capable of inducing the sperm acrosome reaction (Bleil and Wassarman, 1980b, 1983). These changes establish a potent block to polyspermy at the levels of sperm-egg binding and zona penetration. There is also evidence of a secondary block to polyspermy existing at the level of the plasma membrane that inhibits binding and/or fusion of any supernumerary sperm that manages to traverse the zona despite, or before establishment of, the primary block to polyspermy (Ducibella, 1996).

The mammalian egg's ability to undergo the cortical granule reaction develops just before ovulation and lasts only a short time. Delayed ovulation or delayed mating can result in cortical degeneration and an increased incidence of polyspermy, creating embryos incapable of normal development. Understanding the mechanisms of egg activation and aging are increasingly important as eggs are manipulated for infertility therapies.

Syngamy and the Establishment of the One-Cell Zygote

Sperm-egg fusion triggers not only the cortical granule reaction, but also resumption of meiosis. The second meiotic division produces a haploid egg nucleus, which becomes the female pronucleus, and the second polar body, which is eventually extruded from the egg cytoplasm. The sperm nucleus, induced by unknown mechanism(s), decondenses once it is within the egg cytoplasm, the paternally-derived chromatin is modified by factors within the egg cytoplasm and the male pronucleus forms. These modifications include eventual replacement of sperm chromatin-bound protamines with maternal histones.

Male and female pronuclei are not functionally equivalent, and normal embryonic development depends on the contributions of both. Paternally-derived DNA is required early in development for formation of extra-embryonic structures and maternally-derived DNA is crucial to normal development of the embryo itself, phenomena jointly known as genetic imprinting (Reik, 1996). The structural basis for imprinting appears to occur during gametogenesis and is a very active area of current research. Faulty imprinting has been demonstrated to play a role in the pathogenesis of some cancers, malformation syndromes, and other chromosomal disorders. Angelman Syndrome and Prader-Willi Syndrome, for example, are both due to a genetic abnormality at the 15q11-q13 chromosomal locus, but which of these two phenotypically very distinct syndromes arises depends on the parental origin of the abnormal locus.

DNA replication and chromosomal duplication are normal components of male and female pronuclear development. Once formed, the male and female pronuclei migrate toward the center of the egg, in a process that involves both microtubules

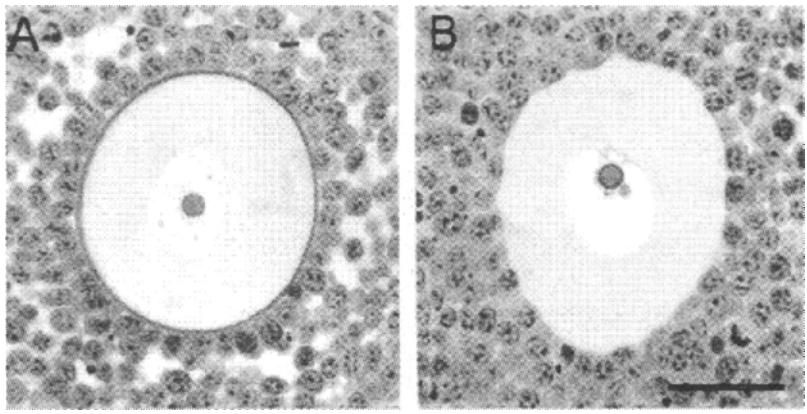

Figure 5 Follicular histology of mice lacking *ZP3*. (**A**) Normal mice. Mid-sized follicle from wildtype ($Zp3^{+/+}$) mice containing a 70–80 μm growing oocyte with an intact nucleus and a zona pellucida surrounded by multiple layers of granulosa cells. The first layer of granulosa cells is well-organized into the corona radiata and tightly adherent to the zona pellucida. (**B**) Mutant mice. Mid-sized follicle from mice lacking a functional *Zp3* gene ($Zp3^{-/-}$) containing a growing oocyte with no visible zona pellucida (arrow) and a disorganized corona radiata. These mice ovulate relatively few eggs and are uniformly infertile. Scale bar = 40 μm (modified from Rankin et al., 1996).

and microfilaments. In the center of the zygote, their nuclear membranes disintegrate and their chromosomes intermix, marking the end of fertilization and the initiation of embryonic development. In humans, the first embryonic mitotic cleavage begins 24–36 hours after sperm-egg fusion. The early embryo travels down the oviduct still surrounded by the zona pellucida which is critical for its survival. Mice that do not synthesize a zona, due to inactivation of the *Zp3* zona pellucida gene, develop mature oocytes that can be ovulated and fertilized *in vitro* (Figure 5). However, following mating, no two cell embryos are recovered from their oviducts and no births have been observed (Liu et al., 1996; Rankin et al., 1996). It is likely that an analogous genetic cause of infertility occurs in women, but has not yet been clinically described.

CLINICAL IMPLICATIONS

Increased understanding of the mechanisms underlying human gamete development and fertilization have derived largely from studies of other, more manipulable mammalian systems. New molecular genetic techniques hold out the promise of our being able to analyze the functional roles of individual proteins and their modifications ever more precisely. The new knowledge gained has already had profound implications for our ability to manipulate the reproductive process and provide aid

to infertile couples. In 1978 the first human birth resulting from *in vitro* fertilization (IVF) was reported (Steptoe and Edwards, 1978), and since then numerous related techniques have been developed and successfully applied to human reproduction—IVF and embryo transfer (IVF-ET), gamete intrafallopian transfer (GIFT), and zygote intrafallopian transfer (ZIFT)—resulting in thousands of healthy babies being born to previously infertile couples. While human couples regularly engaging in sexual intercourse without contraception have an estimated 25–30% chance of becoming pregnant each cycle (Chard, 1991), defects in the fertilization pathway and early fetal loss result in 20–35% of couples, at some point during their reproductive lives, being unable to conceive for at least a 12 month period (Azziz, 1993). Diagnosis and treatment of male causes of infertility lags behind, but the advent of intracytoplasmic sperm injection (Palermo et al., 1992) in which sperm from infertile males are injected into eggs *in vitro,* is contributing to physicians' ability to aid infertile couples.

Progress in understanding the molecular and cellular bases of fertilization and preimplantation development have led to, and will continue to lead to, the development and/or improvement of these and other therapies. These rapid advances in technology underscore the need to discuss and resolve ethical questions raised by our ability to freeze embryos, reduce embryo numbers postimplantation, twin or clone mammalian embryos, influence sex determination by preselection of sperm, and introduce life-saving techniques such as *in utero* gene therapy. Resolution of these issues will occur incrementally and will require an on-going dialogue between professionals in the field, ethicists, elected officials, and the lay public.

The ability to interrupt the reproductive cycle has far-reaching consequences for the human species and our world as a whole, and is an important option in many patients' lives. To date, contraceptive methods are available that inhibit ovulation and/or implantation (hormonal therapies), and that kill sperm and/or block their progress through the female genital tract (spermicides and barrier methods). However, possibilities for novel methods include inhibiting gamete maturation, blocking chemoattractant agents, disrupting normal follicular maturation in the ovary, blocking sperm-egg binding, and preventing implantation. A number of these possibilities are being approached via immunologic methods, in which antibodies directed at a reproductive hormone (Griffin, 1994), sperm or egg antigen (Primakoff et al., 1988; Millar et al., 1989), or other crucial reproductive tract macromolecule, would block fertilization and/or maintenance of pregnancy.

SUMMARY

Fertilization is a multi-step process that involves the interaction of a mature, capacitated spermatozoon and ovulated egg. The sperm and egg extracellular matrix bind in a relatively species-specific manner via glycoproteins in the zona pellucida and receptors on the sperm head. Sperm acrosomal contents are released and the sperm

progresses through the zona pellucida and perivitelline space to reach the egg plasma membrane. Sperm and egg plasma membranes bind and fuse, resulting in incorporation of sperm contents into the egg cytoplasm. Fusion triggers egg activation and establishment of a block to polyspermy. Finally, male and female pronuclei migrate to the center of the egg and undergo syngamy. This complex process involves numerous carefully regulated interactions between sperm and egg macromolecules and elements of the male and female genital tracts. Improving reproductive options depends on our elucidating the molecular mechanisms underlying these reactions.

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Chapter 6

Uterine Environment during the Implantation of The Embryo

IVAN DAMJANOV, BOZIDAR HORVAT, and BRUCE A. FENDERSON

INTRODUCTION

Implantation of the embryo into the uterus includes several consecutive events which culminate in formation of the placenta and establishment of the embryo

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maternal functional unit. This makes possible the intrauterine development of the eutherian conceptus, and is thus essential for the successful establishment of the postimplantation stages of pregnancy. This brief overview of uterine changes during implantation was written to summarize the current views and some of the salient facts essential for understanding early pregnancy. Additional details can be found in excellent reviews (Weitlauf, 1988; Armant and Diaz, 1990; Glasser et al., 1991; Fenderson, 1993).

At the outset, it is important to recognize that the mechanism of implantation and consequent placentation varies among different species (Wimsatt, 1975). This precludes generalizations concerning eutherian mammals. Therefore, we will limit our discussion to rodents with only occasional references to implantation in humans and domestic animals. Rodents and humans both form hemochorial placentae, which makes studies on mice and rats highly revelant for human reproductive biology. On the other hand, humans and rodents have distinctly different placentae which mitigates against such an opinion. However, on the positive side, it should be noted that the early stages of intrauterine pregnancy are not so different among these species, and that the knowledge gained in one system may be applied to the other as well.

Definitions

Implantation is based on the interaction of the embryo and the maternal organism. The term *embryo* is used here as a synonym for fetus, since there is no clear demarcation between the embryonic and fetal stages of development, and both terms are used imprecisely to describe the same entity (Biggers, 1990). The maternal organism includes the uterus, as well as the endocrine and immune systems of the pregnant female from which the ovum was derived, but also refers to surrogate mothers who were hormonally-primed prior to transfer of embryos from other donors. A pregnant uterus will sometimes be distinguished from pseudopregnant uteri which have been artificially-induced by hormones or mechanical stimuli to undergo changes similar to those occurring in pregnancy. Implantation will be used to denote the beginning of the uterine stages of pregnancy and the establishment of physical contact between the embryo and the maternal organism. Implantation can also be extrauterine, as it occurs sometimes under pathological conditions in the human fallopian tube or on the surface of the peritoneal cavity (reviewed in Kraus et al., 1991).

Certain aspects of implantation can be reproduced *in vitro*. These include: i) hatching from the zona pellucida that surrounds the preimplantation embryo (Perona and Wassarman, 1986); and ii) adhesion of the external trophectodermal epithelium to solid surfaces such as matrix-coated plastic and cultured uterine epithelial cells (Glasser and Mulholland, 1993). These processes have been studied by explanting preimplantation mouse embryos (Skreb et al., 1991) and human embryos produced by *in vitro* fertilization (Hardy et al., 1989).

MECHANISM OF IMPLANTATION

Implantation occurs only if there is complete synchrony between the development of the embryo and the response of the hormonally-primed uterus to maternal and embryonic signals. In practical terms, this means that the embryo has reached the stage of expanded blastocyst and that the uterus has been hormonally-primed to allow the embryo to establish contact with the endometrial surface epithelium. The period of uterine receptivity is relatively short, and therefore it is essential that the embryo reach the uterus during this receptive "window" (Finn and Martin, 1972, 1974). Mouse embryos enter the uterine cavity approximately 72 hours after ovulation (Johnson, 1981). The embryo hatches and begins implanting during the next 24 hours. By the end of the fourth day, mouse embryos have undergone nidation (*L.*, nest building) in the uterine crypts and cannot be removed easily. Human embryos also reach the uterine cavity 3–4 days after ovulation, but the implantation is typically delayed for another 2–3 days (Diaz et al., 1980; Edwards, 1988; Paulson et al., 1990).

Implantation includes several sequential events: i) hatching of the embryo from the zona pellucida; ii) apposition of the trophectoderm to the endometrial surface; iii) adhesion between trophectoderm/trophoblast and the endometrial luminal epithelium; iv) passage of the embryo through the luminal epithelium and basement membrane; and v) invasion of the endometrial stroma (reviewed in Schlafke and Enders, 1975; Schlafke et al., 1985; Aplin, 1991).

Uterine Receptivity

The nonpregnant uterus cannot establish contact with the embryo and is thus considered "non-receptive" (Mulholland and Glasser, 1991). The hormonal changes that follow ovulation change the endometrium and alter the endometrial response, but receptivity develops only if the uterus is exposed to a sequential influence of progesterone and estrogens. In mice, this includes priming with progesterone for at least 48 hours followed by a pulse of estrogen which provides the nidatory signal. Estrogen-induced receptivity develops 12–24 hours after the surge of estrogen and lasts several hours. If implantation does not occur within 30–36 hours of the surge of estrogen, the uterus reverts to its non-receptive state (Gidley-Baird et al., 1986; Hearn et al., 1988).

Uterine receptivity, as well as refractoriness, is hormonally-mediated (Psychoyos, 1973; DeHertogh et al., 1986). Blastocysts transferred to hormonally-primed uteri will implant, but only during the ovo-receptive period. Periimplantational administration of progesterone will inhibit implantation because hormone treatment disrupts the development of uterine receptivity in synchrony with embryonic development (Psychoyos, 1976). Antiprogesterone antibodies administered to rodents also change the period of uterine receptivity (Rider et al., 1987).

The critical effect of nidatory estrogen is best illustrated in experimentally-induced delayed implantation. This can be induced by ovariectomizing mice on the

day immediately after copulation and providing the necessary priming effect with exogenous progesterone (McLaren, 1971). Blastocysts that reach the uterus remain dormant and do not attach to the uterine surface epithelium unless a nidatory pulse of estrogen is given. Following estrogen administration, blastocysts attach to the endometrium and implant in a sequence similar to normal pregnancy.

The effects of sex hormones may be direct or indirect, e.g., mediated by secretory products of the uterine glands or other cells in the endometrium (Gupta et al., 1989; Haimovici et al., 1991). Among the many uterine secretory products (see below), recent interest has focused on a cytokine known as leukemia inhibitory factor (LIF). This 50 kDa glycoprotein is produced by uterine glands and its synthesis surges coincident with implantation of the mouse embryo on the fourth day of pregnancy (Bhatt et al., 1991). The uteri of transgenic mice deficient in LIF are completely resistant to blastocyst implantation (Stewart et al., 1992), suggesting that LIF has a crucial role in mediating receptivity of the uterus. Since LIF appears in glandular secretions, it could act to increase the adhesiveness of trophectodermal cells of the embryo or luminal cells of the uterus. However, the exact mechanism of LIF action remains unknown.

Uterine Proteins

Uterine receptivity and early pregnancy are accompanied by changes in protein synthesis and secretion (reviewed in Bell, 1988; Roberts and Bazer, 1988). One of the best characterized secretory proteins is uteroglobulin, the most prominent component of luminal fluid in the rabbit (Beier, 1968). Uteroglobulin (also known as blastokinin) has been shown to stimulate the growth and cavitation of rabbit blastocysts *in vitro* (Krishnan and Daniel, 1967). Another well-characterized secretory protein is uteroferrin, an acid phosphatase that is produced by the pig uterus under the influence of progesterone (Roberts and Bazer, 1984). Uteroferrin is involved in maternal-embryo iron transfer, a process that may be especially important in species such as the pig in which implantation is of the superficial, epitheliochorial type. Serine protease inhibitors have also been identified in uterine luminal secretions during pregnancy (Ing and Roberts, 1989). The function of these "uterine milk proteins" remains unknown; however, embryo transfer experiments in ovariectomized sheep have shown that preimplantation development and implantation are dependent on hormonally-regulated changes in uterine secretions, including the appearance of the uterine milk proteins.

The major secretory product of the decidualized human endometrium is an insulin-like growth factor binding protein (IGFBP-1), also known as placental protein 12 (Bell et al., 1989). This 25 kDa glycoprotein can be detected by radioimmunoassay in fetal serum, maternal serum, and amniotic fluid. Its function is to bind insulin-like growth factor (IGF) which, in turn, regulates endometrial cell proliferation and stromal cell decidualization. Thus, the human uterus has the ability to locally control placental growth and may influence fetal metabolism as well.

Membrane-bound and cytosolic proteins of the endometrium also change during the period of uterine receptivity. For example, we recently reported a two-dimensional map of mouse endometrial proteins during the estrous cycle (Horvat et al., 1992) and early pregnancy (Horvat and Damjanov, 1993). Several unique proteins appeared during the pre- and immediate post-implantation period. Some of these proteins were hormonally-regulated; others were deciduoma-associated; while other proteins showed a more complex pattern of regulation. The results suggest that a proper uteine biochemical milieu is necessary for development of the preimplantation embryo and initiation of the intrauterine phase of pregnancy.

Embryonic Signals

The embryo within the uterus appears to have an influence on uterine receptivity, although it is not known whether this influence is mediated primarily by physical or chemical stimuli (Kennedy, 1983; Weitlauf, 1989; Glasser, 1990). Mechanical stimulation of the hormonally-primed mouse uterus can induce decidualization, supporting the notion of a physical nature of embryonic signals. A decidual reaction can also be elicited by inserting plastic beads or injecting mineral oil into the cavity of hormone-pretreated uteri. However, these physical signals are not adequate, as evidenced by the lack of implantation in delayed implantation models. Thus, a dormant blastocyst may persist in the uterine lumen for several days and be fully incapable of implanting unless the uterine lining changes and makes apposition possible.

Although chemical signals released from the blastocyst remain poorly understood, several substances produced by trophoblastic cells have been implicated in implantation. These include, among others: histamine, embryo-derived estrogen, prostaglandins, and interferon-like substances such as ovine trophoblastic protein-1 (Roberts and Bazer, 1988). The specific roles of these mediators have not yet been elucidated. Indeed, the evidence favoring their role in embryonic signaling is counterweighted by arguments indicating the reverse. These arguments have been reviewed in detail by Glasser et al. (1991), who conclude that "no first order cause and effect relationship can currently be deduced." Nonetheless, some of these biologically-active substances may have important secondary roles and indirectly regulate the establishment of the embryo-maternal functional unit (Hunt and Roby 1994; Hunt et al., 1996).

Apposition and Attachment

The hormonal changes of pregnancy and putative embryonic signals make possible the first, close contact between the embryo and the endometrium known as apposition. After hatching from the zona pellucida, the embryo comes into direct contact with the luminal epithelium. Microvilli of trophectodermal cells intertwine with the corresponding surface of uterine luminal cells (Schlafke and Enders, 1975;

Morris et al., 1983). Apposition is associated with localized swelling of the endometrium which "clasps" the embryo and immobilizes it on the anti-mesometrial side of the narrowed lumen. Within hours, the lumen of the swollen uterus becomes almost completely obliterated and an implantation chamber is formed in which the embryo and the uterine luminal cells undergo an attachment reaction. Attachment is associated with increased permeability of the endometrial blood vessels at the site of implantation (McRae and Heap, 1988). This can be demonstrated by intravenous injection of dyes like Pontamine Blue, which extravasate at the site of implantation making it visible as a small blue dot. At the same time, the microvilli of the luminal uterine cells and trophoblastic cells are lost, and the smooth external surfaces of the adjacent embryonic and maternal cells become closely apposed.

Apposition and attachment of the blastocyst to the endometrium involves a series of complex cell-cell interactions. The mechanisms of these interactions are not fully understood; however, cell adhesion molecules such as cadherins and integrins, adhesive pericellular matrix proteins such as laminin, and plasma proteins such as fibronectin and vitronectin (all of which mediate cell contact in other systems) are not involved in implantation (reviewed in Carson et al., 1991). Rather, the cumulative evidence indicates that the oligosaccharide components of surface membranes (glycolipids and glycoproteins) might play the most important role. In support of this view are profound changes involving the biochemical composition of cell membranes of the implanting embryo and the uterine luminal epithelium, in the apical glycocalyx; and changes in the glycoproteins and proteoglycans in uterine secretions. These changes have been reviewed (Fenderson et al., 1990; Fenderson, 1993) and only the most salient features are listed here as follows.

The luminal epithelium of the nonpregnant uterus is nonreceptive to blastocysts and actually represents a barrier to blastocyst attachment and invasion. The surface of these uterine cells is covered with a thick, negatively-charged glycocalyx which can be demonstrated histochemically using cationic dyes (Anderson and Hoffman, 1984). Both N-linked and O-linked oligosaccharides have been detected in the mucinous glycoproteins of nonpregnant mouse uterine luminal cells (Carson et al., 1990; Valdizan et al., 1992). This mucin appears to form an effective barrier that prevents adhesion of trophoblastic cells to endometrial cells in nonpregnant mice. Heparan sulfate proteoglycans have also been detected on the apical surface of the uterine epithelium (Wilson et al., 1990).

Receptivity of the endometrium in pregnancy is marked by a decreased thickness of the apical glycocalyx and a dramatic loss of negative surface charge (Morris and Potter, 1984). This is accompanied in rodents by a loss of sialic acid residues and an increased expression of terminal galactose residues as demonstrated by lectin affinity histochemistry (Chavez and Anderson, 1985; Anderson et al., 1986). The equivalent stage of pregnancy in humans is also marked by a decline in sialylated glycolipids (gangliosides) and an increase in neutral glycolipids (Zhu et al., 1990). A decline in the negative surface charge of trophectodermal cells, which occurs simultaneously (Nilsson and Hjerten, 1982), may account for the increased adhesiveness between the

embryo and uterus that typically occurs at this stage of implantation. Inhibition of mucin assembly with benzyl-2-acetamido-2-deoxy-α-D-galactopyranoside facilitates the adhesion of blastocysts to polarized uterine epithelial cells *in vitro*, further supporting the notion that cell surface glycoconjugates play a critical role in regulating blastocyst attachment (Valdizan et al., 1992).

Monoclonal antibodies to oligosaccharides have been used to monitor changes in the composition of endometrial epithelial glycoconjugates during pregnancy (Table 1). Several fucosylated oligosaccharides such as Le^x, Le^y, and H type 1 chain have been found on uterine epithelial cells in the mouse and rabbit (reviewed by Fenderson, 1993). The expression of these glycans is under the control of estrogens and progesterone (Kimber and Lindenberg, 1990) and their distribution changes at the time of implantation. For example, H type 1 chain structure is present on all uterine luminal cells in nonpregnant mice but its distribution becomes patchy during the periimplantation period of pregnancy. Fucosylated glycoconjugates that are

Table 1. Carbohydrate Differentiation Antigens of the Mouse Blastocyst and Uterine Epithelium

		Expression[a]	
Antigen	*Carbohydrate Structure*	*Blastocyst*	*Uterus*
Lacto-series Oligosaccharides			
Le^x (SSEA-1)	Galβ1→4GlcNAcβ1→3Gal 3 ↑ Fucα1	—	+
Le^y	Galβ1→4G1cNAcβ1→3Gal 2 3 ↑ ↑ Fucα1 Fucα1	++	++
H (type 1 chain)	Galβ1→3G1cNAcβ1→4Gal 2 ↑ Fucα1	—	+
Globo-series Oligosaccharides			
SSEA-3	Galβ1→3GalNAcβ1→3Galα1→4Gal	+	—
SSEA-4	Galβ1→3GalNAcβ1→3Galβ1→4Gal 3 ↑ NeuAcα2	—	—
Forssman	GalNAcα1→3GalNAcβ1→3Galα1→4Gal	++	—

Note: [a] Results of indirect immunofluorescence assays on early embryos and uterine frozen sections using monoclonal anticarbohydrate antibodies. Data are summarized from published results (see Fenderson, 1993 for references).

present in nonpregnant uteri are replaced by galactose-rich oligosaccharides at the time of implantation (Lee et al., 1983). It is plausible that fucosylated glycans prevent cell-to-cell adhesion, and that the loss of fucosyl and sialic acid residues is associated with increased adhesiveness between trophoblastic and uterine luminal cells (Wu et al., 1993).

The galactose-rich glycoproteins of the mouse endometrium have been characterized by Horvat (1993). Numerous galactoproteins were identified by Western blotting using galactose-specific lectins (e.g., RCA). Two prominEnt species with molecular weights of 35 kDA and 64 kDA were specifically detected on days 3–4 of pregnancy (and pseudopregnancy). The 64 kDA galactoprotein, termed "metroglandin," is present in the endometrial glands. It is heavily N-glycosylated with a core-protein molecular weight of 50 kDA. Metroglandin appears to be a useful marker of uterine receptivity in the mouse.

Lactosaminoglycans (large N-linked polymers of galactose and N-acetylglucosamine) are copiously expressed on uterine luminal cells (Dutt et al., 1987; Dutt and Carson, 1990). Hypothetically, these glycans could serve as ligands for cell surface $\beta 1 \rightarrow 4$ galactosyltransferase, an enzyme that has been shown to mediate cell adhesion in several systems (Hathaway and Shur, 1988; Hathaway et al., 1989). We have proposed that simple sugar modifications of lactosaminoglycans such as fucosylation, sulfation, or sialylation could abrogate or strengthen intercellular adhesion and thereby render uterine epithelial cells either receptive or nonreceptive to blastocyst adhesion (Fenderson, 1993).

The attachment of the embryo to the receptive endometrium would not be possible without corresponding changes in the surface properties of the implanting embryo (Fenderson et al., 1990; Kimber, 1990). Changes in the composition of carbohydrate moieties on the external surface of the blastocyst have been studied using monoclonal antibodies and lectins. Several globo-series antigens, including stage-specific embryonic antigens three and four (SSEA-3 and SSEA-4), which are found on early cleavage-stage embryos, disappear from the embryonic surface at the time of implantation and are replaced by lacto-series antigens such as Le^x and Le^y (Figure 1). These antigens represent terminal oligosaccharides carried on large lactosaminoglycans which are constantly expressed on embryonic cell surfaces from the time of zygote formation until neurulation. The fact that these antigens become detectable by monoclonal antibodies or lectins at a specific stage of development indicates that these embryo-glycans have been modified enzymatically during synthesis in the Golgi or (perhaps) at the cell surface. This restructuring could have an important physiological function and could mediate implantation.

Among the various lacto-series antigens of the embryo, the Le^y antigen appears to correlate best with implantation (Fenderson et al., 1986; Zhu et al., 1995). This epitope is not present on embryos flushed from the oviduct, but is found on all intrauterine blastocysts. Furthermore, oviductal blastocysts explanted *in vitro* do not acquire this antigen, which indicates that its expression occurs only under the influence of uterine factors. A uterine ligand for embryonic Le^y has not been identified,

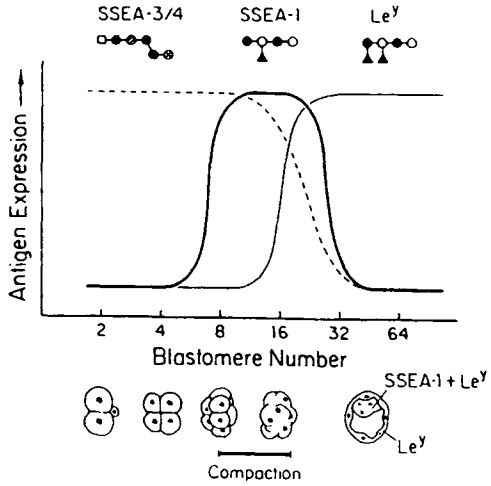

Figure 1. Changes in carbohydrate antigen expression during preimplantation mouse development. Stage-specific embryonic antigens three and four (SSEA-3 and -4) disappear, while SSEA-1 (Le^x) and Le^y appear at the 8- and 16-cell stages, respectively. Le^x antigen disappears from the outer tier of cells following compaction as these cells differentiate into trophectoderm. Symbols are defined as follows: ○, glucose; ○, N-acetylglucosamine; ●, galactose; ○, N-acetylgalactosamine; □, sialic acid; ▲, fucose. From Rosenman et al. (1988) with permission of Professional Postgraduate Services, Tokyo.

but maternal epigenetic modification of embryonic surface oligosaccharides may be important for implantation. These findings also point to the complexity of interaction between the implanting embryo and the uterus.

The possibility that carbohydrates mediate contact between the embryo and uterine cells has been tested. H type 1 chain oligosaccharides (i.e., lacto-*N*-fucopentaose I) and anti-H monoclonal antibodies have been reported to inhibit the outgrowth of mouse blastocysts on monolayers of uterine epithelial cells *in vitro* (Lindenberg et al., 1988). This has led to the hypothesis that recognition proteins on the surface of the blastocyst bind H oligosaccharides on the uterine epithelium to initiate implantation. We have proposed an alternative hypothesis in which H oligosaccharides on the uterine cells interact with complementary oligosaccharides (e.g., Le^y) on the blastocyst surface, forming a carbohydrate-to-carbohydrate link (Zhu et al., 1995). These possible mechanisms of carbohydrate recognition are illustrated in Figure 2. Changes in the composition of surface oligosaccharides of the embryo and the uterine lining cells may account for the development of uterine receptivity, attachment of trophoblast to uterine epithelial cells, and cell-to-cell adhesion, all of which are essential for the next stage of implantation: penetration of the epithelium and invasion of the uterus.

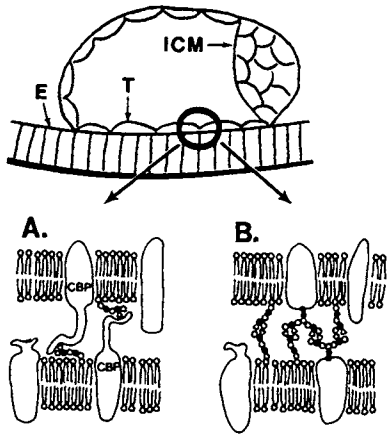

Figure 2. Two possible mechanisms for the involvement of surface carbohydrates in implantation. **A.** Carbohydrate-protein interaction. Oligosaccharide determinants on the surface of the blastocyst or the uterine lumine could provide ligands for complementary, carbohydrate-binding proteins. **B.** Carbohydrate-carbohydrate interaction. A decrease in the negative charge of the uterine epithelium at the receptive stage could permit multivalent, carbohydrate-carbohydrate interactions to anchor the blastocyst to the uterine wall. Subsequent events in implantation may include the activation of relatively nonspecific, but stronger cell adhesion molecules, including gap junction proteins and integrin-family receptors. Abbreviations: ICM, inner cell mass; T, trophectoderm; E, uterine epithelium.

Penetration of the Epithelium

Except for species in which placentation is of the superficial epitheliochorial type, e.g., lemurs, moles, and hoofed animals, implantation of all other mammals is associated with penetration of the uterine luminal epithelium (reviewed by Weitlauf, 1988; Armant and Diaz, 1990). Schlafke and Enders (1975) have described three basic types of embryo-maternal interaction (Figure 3):

1. Intrusive penetration, as seen in guinea pits, ferrets, and Rhesus monkeys, is marked by extension of trophoblastic cell cytoplasm between uterine epithelial cells to the epithelial basement membrane. The epithelium thus provides anchoring support to the embryo which follows the path produced by the invading trophoblastic wedge which passes through the basement membrane and ends below an intact surface epithelium within the endometrium.
2. Displacement penetration, as seen in rodents, is marked by apoptosis, i.e., programmed cell death of epithelial cells at the site of the apposition of the blastocyst (Parr et al., 1987). The basement membrane denuded of epithelial

cells is then breached, and trophoblastic cells lead the embryo by invading the underlying stroma which has undergone primary decidualization (Parr and Parr, 1989). The result is a compact implantation chamber. Apoptosis of the endothelium occurs only on the limited surface area at the antimesometrial side of the uterus that is in contact with the abembryonic portion of the blastocyst. The human embryo penetrates the uterine surface epithelium in a similar manner and, like rodents, humans also form a yolk sac and hemochorial placenta (King and Enders, 1993). However, implantation of the human embryo is less regulated and the orientation is less stringent than in rodents. The development of the placenta also follows different routes in the two species.

3. Fusion penetration, as seen in rabbits, is marked by fusion of apposed trophoblastic and uterine epithelial cells. This results in the formation of a multinucleated syncytium which forms knobs. These knobs then invade the uterine stroma and form symplastic structures in the future placental bed.

In each of these types of embryo-maternal interaction, invasion is accomplished through the direct action of the embryo on uterine epithelial cells and their underlying basement membrane (Blankenship and Given, 1992). It is presumed that this includes the action of enzymes released by trophoblastic cells. Cytokines and other

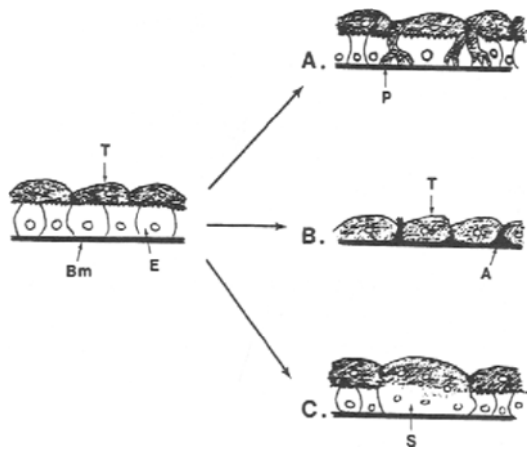

Figure 3. Mechanisms for embryo penetration of the uterine epithelium. **A.** Intrusion penetration (guinea pigs, ferrets, and Rhesus monkeys). **B.** Displacement penetration (rats and mice). **C.** Fusion penetration (rabbits). In each case, invasion of the uterus is accomplished through the direct action of the embryo on uterine epithelial cells and their underlying basement membrane. Abbreviations: Bm, basement membrane; T, trophoblast; E, uterine epithelium; P, cytoplasmic processes; A, apoptotic cell; S, syncytium. After Schlafke and Enders, (1975).

biologically-active substances may also serve as signaling molecules and, in the case of endometrial cell apoptosis, influence the behavior of uterine cells. The release of enzymes such as plasminogen activator must be controlled, and is thus associated with the subsequent release of specific inactivators (Kliman and Feinberg, 1990; Herz et al., 1992). A localized decidual reaction around the implantation chamber also controls embryonic outgrowth, however, the mechanisms are not completely understood (Parr and Parr, 1989).

Invasion

The embryo that has penetrated through the uterine epithelium must establish contact with the materal circulation by reaching blood vessels in the uterine stroma. This is achieved by the invasive action of trophoblastic cells; however, the extent of this invasion appears to be controlled by the maternal organism. Locally, in the endometrium, this is accomplished by: i) decidualization of the uterine stroma; ii) an influx of immunologically-competent cells; and iii) restructuring of the uterine microenvironment.

Invasion of the endometrium is mediated by cell surface receptors for various extracellular matrix (ECM) glycoproteins (Armant et al., 1986; Damjanov et al., 1986; Sutherland et al., 1988) and by proteolytic degradation of the uterine stroma (reviewed by Aplin, 1991; Strickland and Richards, 1992). Matrix-degrading enzymes have been demonstrated in both murine (Glass et al., 1983; Behrendtsen et al., 1992) and human trophoblasts (Kliman and Feinberg, 1990); they include nonspecific proteases, collagenases, stromolysin, and urokinase-type plasminogen activator. The action of these proteolytic enzymes is counteracted by inhibitors such as plasminogen activator inhibitors and tissue inhibitors of metalloproteinases (Herz et al., 1992), as well as by new ECM that forms in the decidua. For example, chondroitin sulfate proteoglycan, a major product of uterine stromal cells (Jacobs and Carson, 1991) has been found to inhibit the outgrowth of explanted mouse blastocytes (Carson et al., 1992). This and other secretory products of the pregnant uterus could serve to attenuate the invasiveness of the trophoblasts.

Our data indicate that hyaluronan could also control the invasive growth of trophoblastic cells in the pregnant uterus (Fenderson et al., 1993). Hyaluronan is an extracellular glycosaminoglycan that is bound by cell surface receptors and ECM proteins to form a stable, macromolecular lattice around cells. It consists of linear polysaccharide chains with repeating glucuronic acid ($\beta1\rightarrow3$) N-acetylglucosamine ($\beta1\rightarrow4$) structure. It is known to facilitate cell migration by creating a low-density, water-rich microenvironment. Hyaluronan-rich matrices may also regulate growth and differentiation by sequestering polypeptide growth factors (reviewed in Ruoslahti and Yamaguchi, 1991).

Carson and co-workers (1987) have shown that mouse blastocysts will use hyaluronan as a substrate for attachment and outgrowth *in vitro* suggesting that hyaluronan plays a key role in implantation. On the other hand, Brown and Papaioannou

(1992) and Fenderson et al. (1993) reported that decidualization is accompanied by a rapid loss of hyaluronan from the ECM of endometrium surrounding the mouse embryo. A similar, rapid redistribution of hyaluronan was also observed in oil-induced deciduomas. The results suggest that clearing of hyaluronan from the ECM of decidualized cells restricts trophoblast invasion by creating a non-permissive environment for trophoblastic cell migration. Redistribution of hyaluronan in the pregnant mouse uterus could also direct the growth of parts of the embryo towards the mesometrium and thus assure the establishment of contact between the embryo and the maternal circulation. In this connection, hyaluronan could facilitate the ingrowth of blood vessels into the future placental site by concentrating angiogenic growth factors such as transforming growth factor beta (TGFβ) and basic fibroblast growth factor (bFGF).

DECIDUALIZATION

Stromal cells of the endometrium when stimulated by the hormones of pregnancy undergo a sterotypic transformation which in humans forms the decidual membrane (Damjanov, 1985) and in mice a nodular outgrowth enclosing the implanted embryo called "deciduoma" (Welsh and Enders, 1985). Deciduomas can be induced in hormonally-primed rodent uteri by mechanical means: the embryo is not essential for its formation. In human uteri, decidual transformation of stromal cells occurs in the late secretory phase, but the completely formed decidual membrane is found only in pregnancy (Wewer et al., 1985). Deciduomas of rat and mouse can be induced mechanically during a limited time of pregnancy or during pseudopregnancy induced by mating females with infertile, vasectomized males (reviewed by Bell, 1983; Damjanov and Wewer, 1991). To this end, one may introduce into the uterine lumen plastic beads, thread, or oil. Non-traumatic stimulation, such as intraperitoneal injection of pryathiazine or chemical stimulation of the endometrium can achieve the same biological effect, but is less efficient and is limited to only several hours corresponding to the time of highest uterine receptivity (Finn and Martin, 1972). The formation of deciduomas depends critically on priming of the uterus with progesterone, whereas the estrogens have only a modifying role. Progesterone and estrogen receptors are found on both stromal endometrial cells and decidual cells. In pseudopregnancy, progesterone receptor levels remain constant while estrogen receptor levels decline (Chen et al., 1989).

Decidual transformation of endometrial stromal cells is marked by enlargement of cells, morphological changes seen by electron microscopy, and dramatic changes in protein biosynthetic activity (Wewer et al., 1985, 1986; Damjanov and Wewer, 1991). In the nonpregnant uterus, the endometrial stroma is composed of fibroblast-like cells that have elongated nuclei and very little cytoplasm (Karkavelas et al., 1988). The interstitial spaces contain loosely-arranged ECM fibrils and amorphous material rich in hyaluronan, plasma proteins, and chondroitin sulfate

proteoglycan (Carson et al., 1992). Decidual transformation leads to the enlargement of the cells, which become polygonal and acquire considerable amounts of cytoplasm (Welsh and Enders, 1985). Decidual cells are surrounded by an extracellular, basement membrane-like material (Figure 4). This ECM binds the cells together, accounting for the membrane-like appearance of the decidua in humans (Damjanov, 1985) and the compactness of the murine deciduoma.

Decidual cells are active secretors of ECM molecules such as laminin, fibronectin, collagen type IV, and osteonectin (Wewer et al., 1985 and 1986; Glasser et al., 1987a, 1987b). Decidual cells also secrete hormones such as prolactin (Soares et al., 1991) and growth factors such as IGF (Bell et al., 1989), TGFα (Bonvissuto et al., 1992), and TGFβ (Tamada et al., 1990), as well as many other proteins (Fay and Grudzinskas, 1991). In addition, decidual cells express receptors for polypeptide growth factors (Rutanen et al., 1986) and accumulate various plasma proteins such as corticosteroid-binding globulin (Selcer and Leavitt, 1988).

The decidual response to implantation probably has multiple functions, including: i) mechanical/structural support for the embryo-maternal unit; ii) barrier against the invasion of trophoblastic cells; iii) storage site for growth factors and nutrients; iv) source of hormones and growth factors necessary for embryonic development. However, the exact role of the decidua in maintaining pregnancy remains unknown.

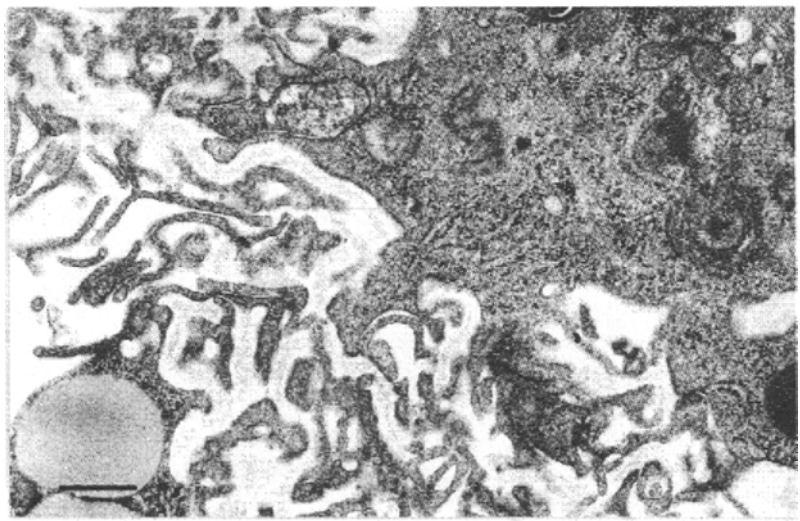

Figure 4. Electron photomicrograph of a decidual cell isolated from a 7-day pregnant uterus cultured *in vitro* for 7 days. Note the pericellular matrix surrounding the cytoplasmic processes. Bar, 0.5 μm; magnification, x 24,000. From Wewer et al. (1986) with permission of Springer-Verlag, Heidelberg.

SUMMARY

The implantation of the embryo into the uterus is a complex event that involves several coordinated sequences that occur simultaneously in the embryo and the uterus. Implantation is the essential prerequisite for the formation of the embryo-maternal unit and the intrauterine progression of pregnancy, culminating in the delivery of a newborn.

Implantation is accompanied by typical cellular and biochemical changes in the uterine epithelium and stroma, which evolve sequentially and in synchrony with development of the implanting embryo. These events include hormonal priming of the uterus, development of uterine receptivity, action of the embryo on the uterus (and *vice versa*), apposition of the embryo to the uterine epithelium, trophectoderm attachment, and embryonic invasion of the endometrium. These events are marked by typical morphological and biochemical changes which allow implantation, regulate embryonic invasion, and provide stimuli for the formation of the functional maternal-embryonic unit. Disturbances of implantation may be related to abnormal hormonal priming of the uterus, abnormal interactions between the embryo and the pregnant uterus, and asynchrony of events that are essential for implantation. Understanding of implantation is essential for the treatment of infertility, the regulation of fertility, and the successful application of reproductive technology to animal husbandry and human assisted reproduction.

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Chapter 7

The Pineal Gland, Melatonin, and Reproduction

RUSSEL J. REITER

INTRODUCTION

Shortly after the discovery of the pineal hormone melatonin (Lerner et al., 1959), it was shown that the biochemical activity of the pineal gland was regulated by the light:dark cycle and that the production of melatonin was likely confined to the daily dark period in experimental animals (Wurtman et al., 1964). About the same time it was observed that the photoperiodic environment to which animals were exposed was important in determining the functional status of their reproductive organs (Reiter and Fraschini, 1969). Thus, the exposure of long day breeding rodents such as the Syrian hamster to winter-type day lengths, i.e., short days, was followed

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by involution of the reproductive system in both males and females (Hoffman and Reiter, 1965, 1966). Furthermore, it was shown that the short day mediated suppression of reproductive physiology in these animals required an intact and functionally innervated pineal gland since, in the case of either surgical removal of the pineal gland (Hoffman and Reiter, 1965), or after the sympathetic innervation of the organ (Reiter and Hester, 1966), short days were totally incapable of exerting any control over the neuroendocrine-reproductive axis. These findings, besides proving the pineal gland was involved in mediating photoperiodic control of reproductive physiology, also implicated melatonin as the hormone responsible for these effects. Since there was no means at that time to directly quantify melatonin production, it was only surmised that it was the agent responsible for inducing gonadal regression in short day exposed animals. Furthermore, tests of melatonin's efficacy as an inhibitor of gonadal function following its chronic administration showed that it only had modest effects on the function of the sexual organs (Wurtman et al., 1963). Final proof that melatonin was the pineal hormone which accounted for the effects of the gland on the reproductive system was provided by observations that properly-timed, daily melatonin injections totally suppressed neuroendocrine-reproductive function in hamsters (Reiter et al., 1976; Tamarkin et al., 1976).

The melatonin-induced change in these studies were similar in terms of the period required to suppress reproduction and the magnitude of the response to those caused by the functional pineal gland in short day exposed animals (Reiter, 1980). At this point, the endocrine nature of the pineal gland was firmly established and the evidence linking the pineal and its hormone melatonin to reproductive physiology was unequivocal.

As of this writing, it is well accepted that melatonin has a major role in the neuroendocrine-reproductive physiology of animals. For example, it is known that in photoperiodic seasonal breeding mammals the pineal translates the changing photoperiods into a hormonal message, i.e., melatonin, with this hormone subsequently acting on the hypothalamo-pituitary unit to control the waxing and waning of the reproductive system (Reiter, 1980, 1993; Goldman, 1983; Bittman, 1985). The receptors that mediate some of the effects of melatonin on the hypothalamo-pituitary-gonadal axis are possibly located in the parts tuberalis of the anterior pituitary gland on the base of the median eminence (Morgan, 1991).

In humans, the pineal gland is widely accepted as being a highly active organ of internal secretion with melatonin exerting a number of important actions (Vaughan, 1984; Reiter, 1991, 1993). Whereas the pineal gland clearly has physiological effects that transcend reproductive physiology, only the potential relationships of the gland to the sexual organs will be considered in this chapter. For a readily accessible introduction to the human pineal gland and its hormone melatonin, the reader is referred to another chapter by the same author in volume 10.

MELATONIN LEVELS BEFORE PUBERTY

Whereas *in utero,* the human fetal pineal gland is generally considered to be nonfunctional and incapable of producing melatonin, the fetus itself, because of the transfer of maternal melatonin through the placenta, is presumably exposed to the same circadian melatonin rhythms as is the mother (Zemdegs et al., 1988). In nonhuman animals, the melatonin rhythm of the mother does exert physiological effects on the fetus. Whereas this possibility exists for the human as well, no actual studies of this have been performed.

There are rather few studies on the circadian melatonin rhythm in pregnant women and those that have appeared are not particularly consistent in terms of the results. In general, early pregnancy does not alter the melatonin rhythm of the mother although by the third trimester the amplitude of the nighttime melatonin peak may increase (Kivelä, 1991). During childbirth the melatonin cycle of the mother persists (Kivelä et al., 1990) with the levels of melatonin in the umbilical vessels exhibiting day:night differences (Munoz Hoyos et al., 1992).

In newborns, a day:night melatonin cycle is absent for the first two or three months after birth. This is based on the absence of measurable differences in blood melatonin levels during a given 24 hour period (Attanasio et al., 1985, 1986; Munoz Hoyos et al., 1993) as well as arrhythmic urinary excretion of 6-hydroxymelatonin sulfate (Kennaway et al., 1992), the chief metabolite of melatonin. After roughly three months of age, the melatonin cycle appears and continues to develop, such that by 12 months, the rhythm is well developed with highest levels being present at night. The actual onset of melatonin rhythmicity in the newborn seems to be more closely related to the date of conception as opposed to the date of birth (Kennaway et al., 1992) suggesting to the authors that it is a genetically determined event.

Throughout childhood, and perhaps during adulthood as well, daytime levels of circulating melatonin are uniformily low. Although some individuals have described a progressive reduction in already low daytime serum melatonin levels during childhood, in general, this has not been uniformly experienced and when a reduction was measured the magnitude was so minor as to probably be inconsequential. Daytime blood melatonin levels are usually near the detection limits of assays used to measure them.

Highest nighttime melatonin levels during life seem to occur in children 1–3 years of age (Waldhauser et al., 1988). Thereafter, as much as an 80% drop in measured nocturnal melatonin levels has been reported in children between the age of three years and adolescence. During this period of roughly a decade there are no sudden reductions in nocturnal melatonin levels. Although the childhood related decline in nocturnal melatonin has been observed in several studies (Waldhauser et al., 1988) there are also reports to the contrary (Cavallo, 1992, 1993).

MELATONIN LEVELS DURING PUBERTY

Because of studies using non-human mammals wherein it was shown that either melatonin administration or the exposure of animals to environmental photoperiods which maximize endogenous melatonin production delay pubertal onset, the levels of this indole in the blood of children passing through puberty was frequently examined. Some of the very earliest reports implicated the human pineal in the control of sexual maturation (Huebner, 1898; Marburg, 1909). In primates, generally, there is considerable evidence for an active steroid-independent mechanism that restrains gonadotropin and sex steroid secretion during childhood (Plant, 1988). That this steroid-independent mechanism of gonadotropin suppression may involve melatonin was initially proposed by Waldhauser and colleagues (1984). Although this is an attractive hypothesis and supported by some experimental data, not all clinical studies related to melatonin measurements during puberty have come to this same conclusion.

There are certainly reports of a negative correlation between nocturnal blood melatonin levels and gonadotrophin secretion in children and young adults (Waldhauser and Dietzel, 1985; Waldhauser et al., 1984, 1988; Waldhauser and Steger, 1986). The implication of these findings, and certainly one espoused by the authors of these reports, is that the onset and continuance of sexual maturation in the human may relate to the rather rapid and substantial drop in nocturnal circulating melatonin levels while individuals are progressing through puberty (Figure 1).

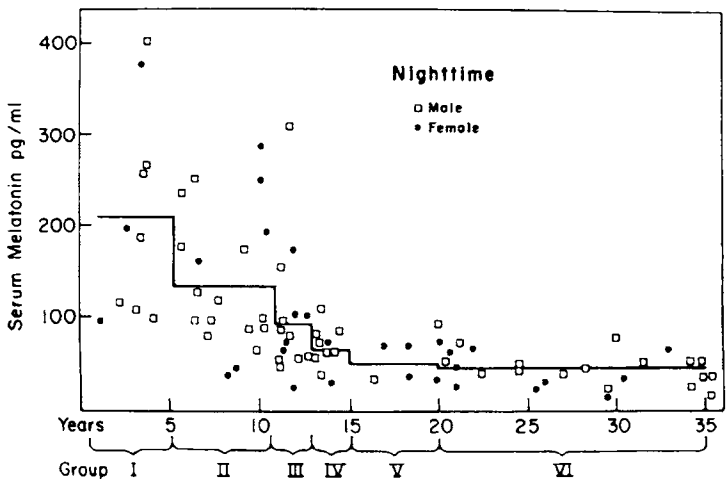

Figure 1. Individual nighttime serum melatonin levels in subjects during various pubertal stages (I–VI). The solid line represents the mean nighttime melatonin levels in the subjects at each pubertal stage. As seen from these data, nighttime melatonin levels drop as subjects go through puberty. From Waldhauser and Steger, 1986.

The rather pronounced fall in melatonin observed during late childhood and pubescence may be explained by at least two factors, namely, a reduction in the actual production of melatonin by the pineal gland or rapid increases in body size during the period of sexual maturation with no change in the actual quantity of melatonin being produced. While human body size typically increases 500–800% between early childhood and adolescence, the size of the pineal gland (Rodin and Overall, 1967) as well as its ability to produce and secrete melatonin change minimally during this period (Tetsuo et al., 1982; Young et al., 1988). Thus, the decline in blood melatonin levels during this critical interval is probably primarily accounted for by a rather uniform production of melatonin accompanied by a marked increase in the distribution volume for the hormone in the blood.

Anticipating a possible association of melatonin levels with pubertal development, several groups have examined circulating levels of the indole in individuals experiencing precocious sexual maturation. However, these studies failed to lead to any definitive conclusions: two of the reports (Ehrenkranz et al., 1982; Berga et al., 1989) claimed no marked change in the circadian melatonin rhythm as a consequence of precocious maturation of the neuroendocrine-reproductive axis in children, while the second study (Waldhauser et al., 1991) claimed a premature drop in nocturnal melatonin levels in children with central precocious puberty. On the other hand, a delayed drop in melatonin is a reasonably consistent finding when measurements are made in children who experience retarded sexual development (Cohen et al., 1982; Arendt et al., 1989; Puig-Domingo et al., 1992). The most remarkable case of delayed puberty associated with elevated melatonin levels is that reported by Puig-Domingo and colleagues (1992). They studied a male subject who, in his third decade of life, was still prepubertal. An examination of his circadian melatonin cycle revealed that both daytime and nighttime levels were far above normal (Figure 2). Over the next seven-year interval the high melatonin levels began to drift downward spontaneously. As the levels approached normal values for an individual of his age, the subject entered puberty and became sexually mature. Thus, the drop in melatonin was highly correlated with sexual maturation in an individual who, until that point, had exhibited markedly delayed puberty.

Blindness, which maximally stimulates melatonin production in the pineal gland, is accompanied by transient gonadal regression in a number of non-human species. In humans as well, the melatonin rhythm persists in blind subjects (Lewy and Newsome, 1983) and possibly the duration of elevated melatonin is prolonged (Wehr, 1991). Considering this, a number of individuals have examined menarchial onset in children who were blinded at birth or during childhood. The conclusion of some early studies was that blindness is associated with accelerated menarche (Zacharias and Wurtman, 1969), but more recent investigations indicate that the lack of photic input delays puberty in both maturing boys and girls (Bellastella et al., 1987, 1989). Specifically in blind prepubertal boys, Bellastella and co-workers (1987) measured lower circulating gonadotropin levels and reduced responsiveness of these individuals to gonadtropin-releasing hormone when compared to sighted boys.

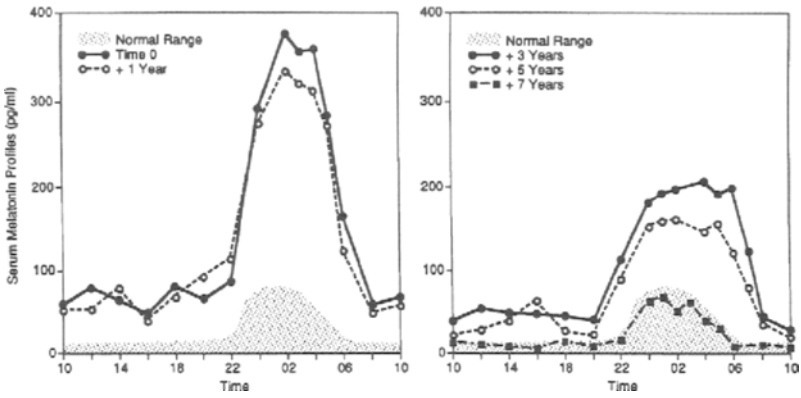

Figure 2. Daytime and nighttime melatonin levels in a young adult male subject who had hypermelatoninism and delayed puberty. When he initially entered the hospital (time 0), blood melatonin levels were very high (shaded area represents normal range of blood melatonin in 20–30 year old males). During the next seven years his blood melatonin levels dropped; he went through puberty and he fathered a child. Redrawn from Puig-Domingo et al., 1992.

In summary, although there certainly are some indications that pubertal onset in humans is influenced by the status of the pineal gland and its hormone melatonin, collectively the results are sufficiently inconsistent that a clear picture has not emerged. Obviously, certain key experiments have not been (and likely will not be) done. For example, whether the exogenous administration of melatonin to humans during the critical period of sexual maturation would influence the age or rate at which children become sexually mature has not been examined. Likewise, the consequence of premature pinealectomy on sexual maturation in the human has yet to be described. Strong justification would be required before either of these studies could be considered.

MELATONIN LEVELS IN RELATION TO REPRODUCTION DURING ADULTHOOD

After adulthood is achieved the melatonin rhythm that developed shortly after birth persists but with a lower amplitude than before puberty. The incremental rise in nocturnal melatonin has been reported to be highly stable within an individual but markedly different between individuals (Brzezinski and Wurtman, 1988; Reiter, 1990, 1993). Because of the great between-individual variation in the amplitude of the nocturnal melatonin rise in adults, the large error associated with grouped levels has made it difficult to statistically verify differences under certain conditions, such as during various states of the menstrual cycle. In general, the best studies are probably those that examined the melatonin rhythm in the same group of individuals over time.

Again, based on data accumulated from animal studies (Reiter, 1980; Goldman, 1983), it was assumed that melatonin in adult humans may have some effects on normal reproductive physiology. This idea possibly was strengthened by the observation that melatonin levels are higher in ovarian preovulatory follicular fluid than are concentrations in the serum of adult females (Figure 3) (Brzezinski et al., 1987). In the 15 subjects in which these measurements were made, the follicular fluid was obtained from infertile women who were undergoing *in vitro* fertilization and embryo transfer. Also, because of the observation that ovulation as well as the surge of ovulatory hormone was suppressed in experimental animals treated with melatonin (Reiter and Sorrentino, 1971), there was considerable interest in the possible variations of melatonin during the human menstrual cycle.

Besides possibly influencing ovulation and/or steroid hormone production in the ovary, recent data suggest another potential function for the elevated levels of melatonin in follicular fluid. Since melatonin has now been shown to be a free radical scavenger (Reiter et al., 1997) and anti-inflammatory agent (Cuzzocrea et al., 1997), the high melatonin levels in the follicular fluid could protect the ovum from excessive oxidative stress that may occur during oculation (Espey, 1980). Oxidative damage to the genome of the ovum could have disastrous effects since this genetic material is passed to the fetus.

Several of the early studies that examined possible variations in the amplitude of the melatonin rhythm during the menstrual cycle yielded indecisive findings for various reasons possibly including rather infrequent blood sampling times, techni-

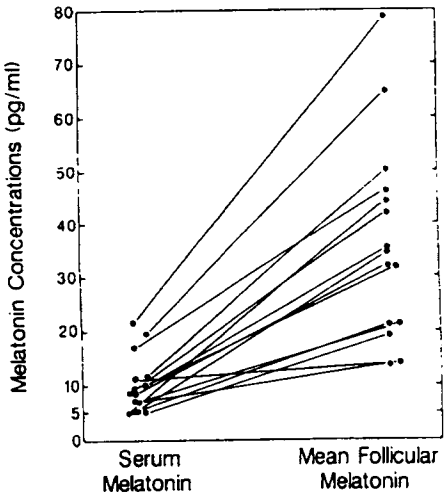

Figure 3. Near simultaneous serum and ovarian follicular melatonin levels in 15 women who were undergoing *in vitro* oocyte retrieval. In each case the level of melatonin in the follicular fluid exceeded that in the serum. From Brezezinski et al., 1987.

cal problems associated with the melatonin assay, wide between-individual variations in the rhythm and indirect measures of pineal melatonin production by assessing urinary 6-hydroxymelatonin (the chief urinary metabolite of melatonin) levels (Wetterberg et al., 1976; Fellenberg et al., 1982; Webley and Leidenberger, 1986). Of the most complete studies that have been accomplished, that of Hariharasubramanian et al. (1986) reported lower midcycle (near the time of expected ovulation) nocturnal melatonin levels than those measured during the luteal phase of the cycle. In a more recent study where four healthy women were sampled at two hour intervals over a 24 hour period at each of the early follicular (days 3–6), periovulatory (days 13–16) and midluteal (days 21–25) phases, no menstrual cycle dependent variations were reported (Brzezinski and Wurtman, 1988). Perhaps in the most complete study conducted to date, Berga and Yen (1990) estimated nocturnal melatonin levels (blood samples collected at 0.5 hour intervals) in eight women

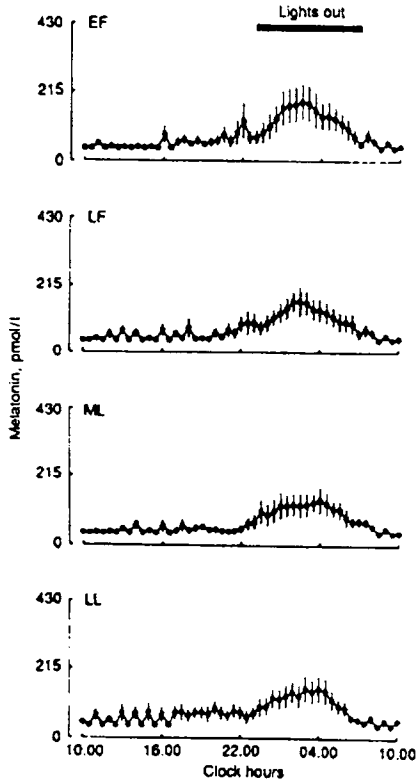

Figure 4. Mean (± SE) 24 hour melatonin levels in eight women at each of four stages of their menstrual cycle, i.e., early follicular (EL), late follicular (LF), midluteal (ML) phase, and late luteal (LL) phase. The "lights out" bar at the top signifies the period of darkness which extended from 23.00–07.00 hours. From Berga and Yen, 1990.

during four phases of the menstrual cycle: early follicular phase, late follicular phase, midluteal phase, and late luteal phase. In these subjects no consistent variation in plasma melatonin patterns related to the menstrual phase was found (Figure 4). Besides finding no menstrual cycle phase dependent amplitude variations in the melatonin rhythm, no differences in the duration of elevated melatonin levels were noted.

Whereas these studies indicate there may be no association between the circadian production and secretion of melatonin and normal menstrual cyclicity, other reports suggest that under some conditions excessive secretion of melatonin may alter menstrual cyclicity and possibly even ovulation. In adult females, strenuous exercise was reported to increase blood melatonin levels (Strassman et al., 1989). In this case the strenuous exercise was running a 28.5 mile mountain race; the sample size consisted of 12 women, age 25–55 years, who were unselected with regard to their menstrual cycle status. The post-race daytime blood levels were twice those measured before the race. Based on these findings, the authors surmised that augmented melatonin secretion in women who routinely engage in heavy exercise may relate to the menstrual cyclicity seen in some of these individuals. Finally, late luteal phase dysphoria, i.e., premenstrual syndrome, was claimed by one group to be related to changes in the melatonin cycle (Parry et al., 1990).

In individuals where menstrual cyclicity is typically altered because of a specific related endocrine condition, there is also not total agreement on the status of the melatonin cycle. Thus, in young females with anorexia nervosa both exaggerated (Brambilla et al., 1987; Tortosa et al., 1989) and unaltered (Mortola et al., 1993) melatonin rhythms have been described. In the one report of patients with bulimia nervosa, their melatonin cycles could not be distinguished from those of control subjects.

A possible relationship between melatonin levels and menstrual cyclicity seems to have been verified by Laughlin and co-workers (1991) who compared the 24 hour melatonin profiles in 10 cyclic athletic women with those of eight amenorrheic athletic women. The amenorrheic women in this study had nocturnal melatonin levels twice as high as those of the women who had regular menstrual cycles despite their frequent athletic activity (Figure 5). It is conceivable, although not proven by these observations, that the excessively high melatonin levels measured in the acyclic women could be causative of this condition.

Women who are amenorrheic for other reasons seem also to have abnormally elevated nocturnal melatonin levels. Thus, in two reports of the melatonin profiles in women suffering from functional hypothalmic amenorrhea, the melatonin secretion patterns were found to be markedly similar to those of the acyclic atheletes (Berga et al., 1988; Brzezinski and et al., 1988). As mentioned above, in animals high melatonin levels are not uncommonly found to be associated with excessively high on prolonged melatonin secretion. These similarities with the human findings suggest that melatonin may also suppress human menstrual cyclicity and ovulation, just as it inhibits estrous cyclicity and ovulation in non-human mammals.

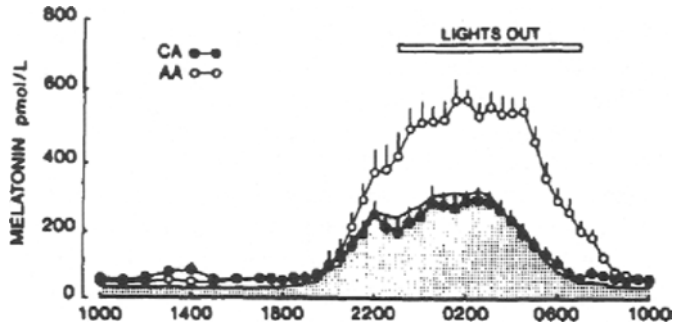

Figure 5. Mean (± SE) melatonin levels in 10 women who were athletes **but** had regular menstrual cycles (CA), eight women who were athletes and were acyclic (AA), and 10 women who served as normal controls (shaded area). The elevated levels of melatonin in the non-cyclic subjects suggest that higher than normal nocturnal melatonin levels may inhibit ovulation and menstrual cyclicity. From Laughlin et al., 1991.

The above mentioned findings have prompted the development of a melatonin-containing product as a contraceptive agent. Voordouw and colleagues (1992) have proposed and tested the use of a melatonin-progestin combination pill for its utility as a contraceptive agent in adult human females. It was in fact found to be effective in inhibiting ovulation and in suppressing the associated rise in ovulatory hormone levels in the blood. However, this concoction has not yet become available on the U.S. market.

Whether the cessation of ovulation and the interruption of menstrual cyclicity at menopause may be related to any aspect of the circadian melatonin rhythm is not known. This possibility has been rarely explored.

There is meager information about the circadian melatonin rhythm in males with reproductive aberrations. The one publication that reportedly defined the rhythm in adult males with either aspermia or zoospermia claimed that the sperm count in semen samples was inversely related to the melatonin levels. Thus, aspermic males had the highest mean levels of melatonin at night while normospermic subjects had the lowest levels (Karasek et al., 1990). Those of zoospermic subjects were intermediate between these extremes. Confirmation of this finding in additional subjects would be an important contribution.

CONCLUDING REMARKS

The reports summarized in this chapter are not totally uniform in terms of their outcomes. Also, the studies are limited by lack of experimental interventions which are commonly used to investigate such interactions in other mammals but not in man,

e.g., pinealectomy, melatonin replacement, etc. Thus far, studies have been primarily descriptive in terms of the circadian melatonin cycle. Despite this, the data collectively suggest there may be a general inhibitory effect of melatonin on human reproductive physiology. What is badly needed is a further definition of the interactions of melatonin with reproductive physiology in humans. As melatonin finds progressively wider interest among endocrinologists, these reports will surely appear and a clearer picture will emerge. Of particular interest are the rapid advances that are being made in terms of melatonin receptors, which may mediate the effects of the indole on reproductive physiology as well as on other processes (Reppert et al., 1997).

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Chapter 8

The Endocrinology of Pregnancy

ROGER SMITH and MARK McLEAN

INTRODUCTION

Mammalian pregnancy (except for the monotremes) requires an extraordinary level of communication between embryo and mother to create a successful symbiotic relationship. Normally communication within animals may occur by nervous or endocrine mechanisms and yet there are no nervous connections between mother and fetus. The entire weight of the communication burden between mother and fe-

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tus therefore rests on the endocrine system. To undertake this task, mammalian evolution has created an entirely novel organ: the placenta. The placenta lies at the interface between mother and fetus. The placenta is much more than a conduit for the transport of nutrients and metabolites between mother and fetus; it is a dynamic control system, the central computer of pregnancy, receiving and integrating information from both mother and fetus and co-ordinating the progressive changes of pregnancy in each participant.

An essential aspect of the maternal-fetal relationship is its dynamism and the need for the orderly attainment of the series of goals that lead from conception to delivery. The endometrial lining of the uterus must be prepared for successful implantation of the embryo and the maternal immune system persuaded to tolerate the foreign genome bearing embryo and placenta. Maternal metabolism must be altered to provide the flow of nutrients to permit fetal growth. To allow transport of an increasing volume of nutrients, the growth and development of the placenta must be balanced with the needs of the fetus and the ability of the mother to supply nutrients. Part of this process is the regulation of placental blood flow and the regulation of fetal growth itself. Finally the processes of parturition must ensure that both mother and fetus are co-ordinated in their preparation for delivery. Intriguingly, this series of achievements is orchestrated differently in different mammals. Thus, development and maintenance of the endometrium in some relies on the corpus luteum, while in others the trophoblast regulates this function; in some, parturition is dependent on progesterone withdrawal while other mechanisms are utilized in different species. From the medical perspective, this unfortunately means that only limited information can be obtained from animal studies and that extrapolation to human biology must be cautious.

DEVELOPMENT OF THE PLACENTA

The human placenta develops from the outer ectodermal layer of the blastocyst. The cytotrophoblasts are individual progenitor cells which fuse to form the multinucleate syncytiotrophoblast cells which are responsible for the majority of peptide and steroid hormone production by the placenta. As yet, little is known of the transcription factors which determine the expression of the placental peptide hormones and the enzymes responsible for placental steroid hormone synthesis. Recently, however, Pit-1, the transcription factor which determines the pituitary expression of prolactin, growth hormone, and thyroid stimulating hormone (TSH), has been identified in syncytiotrophoblast cells and several transcription factors which bind to placental cytochrome P450 steroid synthetic enzyme promoter sequences have been detected. There seems little doubt that the next few years will see a rapid growth in our knowledge of the transcription factors that determine which hormones and enzymes of the human genome are expressed at which stage of the development of the human placenta.

The structure of the multinucleate hormone producing cells of the syncytiotrophoblast is of relevance to the endocrine function of these cells. Classical peptide producing cells such as those of the pituitary or pancreatic islets are full of secretory vesicles which contain mature stored peptide hormone ready for signal induced release; in contrast, syncytiotrophoblast cells contain relatively few secretory vesicles suggesting that regulation of placental peptide hormone release occurs at the transcriptional level and that relatively little hormone is stored in a mature form ready for stimulated release in response to signals (see Figure 1A, B and Figure 2).

Emphasising the need for placenta-specific regulation, many placental hormones appear to represent gene duplication of pituitary hormones or alternatively spliced variants. The placental hormone genes with subsequent modifications of promoter and coding sequences allow for placental hormones to be regulated independently of their pituitary homologues and perhaps to exhibit altered bioactivity. An example is the growth hormone–placental lactogen (GH–PL) locus on chromosomal 17, Viz.

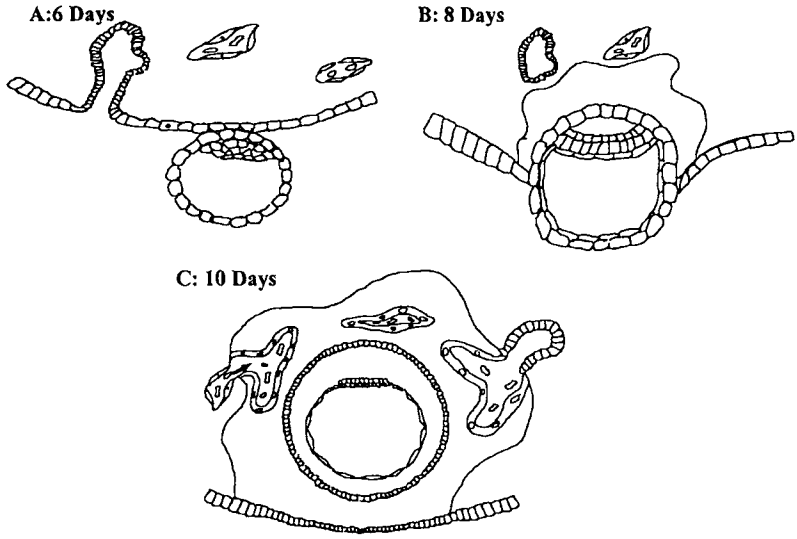

Figure 1. Diagrammatic representation of the invasive implantation of the human embryo. At day six (**A**) the embryo is surrounded by trophoblast cells and loosely attached to the decidua. Two days later (**B**) the embryo is partially buried in the decidua and invasive trophoblast has penetrated the maternal tissue towards the maternal endometrial glands and blood vessels. By 10 days (**C**) the maternal blood vessels and endometrial glands are eroded so that maternal blood is discharged directly into lacunae surrounded by fetal syncytiotrophoblast and the embryo is completely buried in the decidua. (Courtesy of Dr. J. Falconer, University of Newcastle).

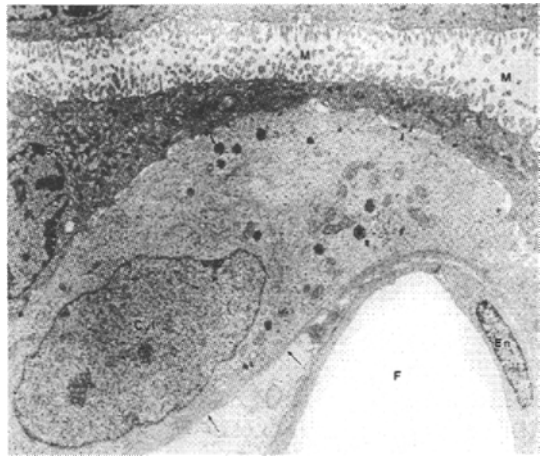

Figure 2. Electronmicrograph showing placental structure. Syt, Syncytiotrophoblast; Cyt, Cytotrophoblast; M, Maternal blood space; F, Fetal capillary lumen En, Fetal Endothelial cell; Arrow, Basal lamina of Cytotrophoblast, Arrowhead, Desmosome. (Courtesy of Dr. Gerald Little, University of Newcastle).

5'---GH-N (Pituitary)---PL (Pseudogene)--- PL---A-GH-V (Placenta)---PL-B---3'

The GH-N gene is expressed in the pituitary while the PL-A, PL-B, and GH-V genes are expressed in the placenta, although the nature of the transcription factors and the binding sites on the promoter sequences which determine this placenta specific expression is unknown.

The interplay between developmental expression factors and maternal and fetal signals results in a gestational pattern of secretion which varies with each placental peptide and steroid hormone. As the number of peptide hormones and factors identified is now very large only the pattern of secretion for a representative selection is portrayed (see Figure 3).

PREPARATION OF THE ENDOMETRIUM AND RECOGNITION OF PREGNANCY

The human menstrual cycle is associated with the orchestrated development of two processes: the maturation and release of an ovum and the concurrent alterations in the endometrium to potentially receive and nourish the ovum, if fertilized. Following the menstrual shedding of the endometrial lining of the previous cycle, estrogen production from the granulosa cells of the dominant follicle stimulates the generation of a proliferative endometrium. After ovulation, under the stimulus of maternal luteinizing hormone (LH) and follicle stimulating hormone (FSH), the corpus luteum pro-

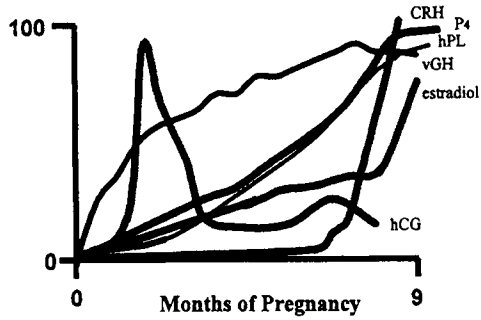

Figure 3. Comparison of the changes occurring during pregnancy in various hormones. vGH is placental variant growth hormone which shows a progressive rise during gestation. Placental lactogen (hPL) and progesterone (P4) also show a gradual rise from early pregnancy while hCG has an early peak with lower levels later in gestation. Estradiol also rises gradually but with a sharper rise later in gestation while CRH has a very rapid rise in late gestation. (Courtesy of Dr. J. Falconer, University of Newcastle).

duces progesterone, a crucial hormone for the creation and maintenance of pregnancy. The progesterone acts on the cells of the proliferating endometrium to create the secretory endometrium required for implantation. This secretory endometrium is characterized by the formation of glycogen filled vacuoles and an alteration in the microvilli of the epithelial cells. The endometrium can receive the blastocyst between days 16–19 of the menstrual cycle. The endometrium requires the continued presence of progesterone and this is provided by the corpus luteum which is maintained after fertilization of the ovum by human chorionic gonadotropin (hCG). hCG is a dimer formed by an α-chain identical to the α-chain of LH and a β-chain with high homology to but distinguishable from that of LH. hCG is one of the first endocrine products of the preimplantation blastocyst and can be detected in the maternal circulation 6–8 days after fertilisation. In mammals other than man, research has suggested that prostaglandins, histamine, estrogen, platelet-activating factor (PAF) and a variety of other factors and peptides play a role in preparing the endometrium for implantation and signaling to the mother the presence of a conceptus. However, interspecies variations are considerable and the role of these factors in human pregnancy is unclear. A second controversial area is the role of embryonic products in preventing maternal immune destruction of the genetically foreign embryo and placenta. While many embryonic products such as interleukins, prolactin, adreno-cortico-hormone (ACTH), and corticotropin releasing hormone (CRH) are reported to have immune system regulatory functions, it seems likely that the prime defence of the fetus is a "stealth" system which prevents maternal immune system recognition of the conceptus. The entire conceptus is contained within a trophoblastic shell which lacks the usual histo-compatibility locus-A (HLA) markers which facilitate immune attack, although an unusual non-polymorphic form of the HLA molecule (HLA-G) has been identified on the trophoblast.

TRANSFORMING MATERNAL METABOLISM

During human pregnancy the locus of control for much of maternal metabolism shifts from the maternal hypothalamus to the placenta. This is necessary for the maintenance and development of the fetus.

THE HYPOTHALAMIC-PITUITARY-GONADAL AXIS IN PREGNANCY

The hypothalamic-pituitary-gonadal axis is altered profoundly by the events of pregnancy. As early as six days post-conception the blastocyst usurps the pituitary by producing hCG to maintain the corpus luteum production of progesterone. FSH production from the pituitary is then suppressed by placental production of inhibin. These changes terminate the ovarian cycle for the duration of pregnancy and commence the suppression of pituitary gonadotroph function which is perpetuated post-partum by the hyperprolactinemia associated with breast-feeding. The high concentrations of hCG may also have effects on the thyrotrophs of the maternal pituitary by stimulating the maternal thyroid producing mild thyrotoxicosis and TSH suppression. This occurs because of the close similarity in structure between hCG and TSH.

In contrast to the suppression of the gonadotrophs, pituitary lactotrophs are stimulated to enlarge and multiply by the endocrine environment of pregnancy as both estrogen and progesterone are trophic to these cells. As a consequence maternal plasma prolactin concentrations rise several-fold during pregnancy. However, during pregnancy prolactin production is not limited to the pituitary and decidual prolactin production leads to high concentrations in amniotic fluid. The decidual prolactin is the product of translation of a longer form of mRNA produced by initiation of transcription at an alternative start site in the single prolactin gene. The physiological role of the decidual prolactin and the degree to which it contributes to circulating maternal concentrations remains obscure, although an effect on electrolyte and fluid transport into the amniotic fluid has been proposed.

While progesterone production from the corpus luteum of the maternal ovary is crucial to the successful implantation of the conceptus and the maintenance of the decidua the site of progesterone production is soon transferred to the placenta. Placental production of progesterone is dependent on the provision of cholesterol from maternal low-density lipoprotein (LDL). Cholesterol is converted by the cytochrome P450 side-chain cleavage enzyme into pregnenolone. Pregnenolone is subsequently converted by 3β-hydroxysteroid dehydrogenase into progesterone. The progesterone is required for the maintenance of pregnancy through its action on the endometrium and its effects in promoting uterine quiescence. In many species progesterone withdrawal precipitates labor but in humans antiprogestational agents are effective abortifacients only in the first and second trimester.

While the locus for estrogen synthesis is also transferred from the maternal ovary to the placenta, the synthetic process has some interesting complexities. The placenta lacks 17 α-hydroxylase enzymatic activity and is dependent on the provision of androstenedione and DHEA, sulphated in the fetal liver, as substrates for placental sulphatase and aromatase for the ultimate formation of estradiol. This requirement for both fetal and placental contributions to estrogen synthesis makes estrogen synthesis a marker for the well-being of the feto-placental unit which has been used clinically. The linkage also permits communication between fetus and mother, as increasing maturity of the fetal hypothalamic-pituitary-adrenal axis leads to increases in the size of the fetal zone of the adrenal and increased fetal production of DHEA and androstenedione. A consequence of the increased supply of fetal androgens at the end of pregnancy is a rising production of placental estrogen which appears to play a role in preparing the uterus for parturition by increasing myometrial expression of oxytocin receptors among other mechanisms.

THE MATERNAL HYPOTHALAMIC-PITUITARY-ADRENAL AXIS

In the non-pregnant human the hypothalamic-pituitary-adrenal axis forms a key part of the endocrine response to stress and exhibits a diurnal variation. CRH, a 41 amino acid polypeptide, and vasopressin (a nona-peptide), both produced in the paraventricular nucleus of the hypothalamus, combine to stimulate production and release of ACTH and other fragments of the pro-opiomelanocortin precursor from the corticotropes of the anterior pituitary. ACTH then stimulates production of cortisol from the adrenal cortex. During pregnancy the placenta also synthesizes CRH and production follows an exponential curve with maternal plasma levels undetectable in early pregnancy but peaking at the time of delivery. The placental CRH causes a gradual increase in maternal plasma ACTH, β-endorphin, and cortisol during pregnancy. The increase in ACTH, β-endorphin, and cortisol is relatively small in spite of the great increase in CRH concentrations. This is primarily due to desensitization of the corticotrophs of the pituitary to CRH occasioned by the persistent high circulating concentration. Late in pregnancy exogenous injections of CRH have negligible effects on pituitary ACTH release illustrating this desensitization. The effects of circulating CRH are also buffered by the CRH-binding protein found in human plasma. Late in pregnancy the high level of saturation of the CRH-binding protein leads to more rapid clearance of the binding protein and consequently even higher "free" concentrations of CRH. During these changes the maternal pituitary retains its ability to respond to stress and continues to maintain a diurnal rhythm, presumably through the action of vasopressin to which the maternal pituitary remains responsive.

The biological role of the placental CRH remains obscure although its production follows a clear exponential curve in each pregnancy such that its measurement

in maternal plasma can be a guide as to the likely time of delivery. CRH has also been shown to be a potent vasodilator in the placental vasculature. In the placenta CRH is also capable of stimulating production of proopiomelanocortin related peptides from placental cells although whether significant release of these peptides occurs into the maternal or fetal plasma remains unclear, but the majority of evidence favors the extensive cleavage of the peptides and their α-amidation in a similar fashion to that observed in the neurointermediate lobe of the pituitary in many animals with only low levels of secretion. Such α-amidation of the melanocyte stimulating hormones (MSH) renders them more potent and these peptides from the placenta or the increased ACTH production from the maternal pituitary may be responsible for the darkening of the maternal nipples and the midline, linea nigra ,often observed during pregnancy. Placental CRH also enters the fetal circulation. Again the physiological role of this CRH is obscure but it may stimulate fetal production of ACTH and related factors which promote the development of the fetal zone of the fetal adrenal and its synthesis of androgens which are converted in the placenta by aromatase enzyme into estrogens.

GROWTH HORMONE, PLACENTAL LACTOGEN, GROWTH FACTORS, AND FETAL GROWTH

Of all the endocrine changes of pregnancy one of the most profound occurs in the growth promoting axis. An essential aspect of mammalian pregnancy is the transfer of nutrients from the mother via the placenta to the fetus to permit fetal growth. For this to occur the endocrine environment of the mother must be altered to increase the availability of nutrients, especially amino acids, glucose, and fats. Placental growth must be sufficient to provide an adequate conduit to the fetus and yet not consume nutrients at the expense of the fetus. This metabolic balancing act is regulated by a complex series of endocrine interrelationships which is, as yet, poorly understood. The focus of control appears to be the placenta. As previously identified, gene duplication on chromosome 17 has led to a placenta specific GH variant and three placental lactogen genes, two of which lead to placenta specific expression of peptide hormones.

As pregnancy progresses pituitary secretion of GH declines and placental production of the growth hormone variant gene (GH-V) increases. Maternal concentrations of insulin-like growth factor-1 (IGF-1) increase during pregnancy and correlate with those of GH-V, strongly suggesting that maternal IGF-1 levels are regulated by placental secretion of GH-V. Through this action GH-V is likely to promote lipolysis, promote nitrogen retention (in association with protein) and antagonize the actions of maternal insulin. All these effects will tend to increase the availability of nutrients for transport to the fetus. This task seems sufficiently important that it cannot be left to one hormone, and PL (products of the PL-A and PL-B genes) has very similar effects promoting nitrogen retention, lipolysis, and

antagonizing the effects of insulin. Considerable redundancy appears to exist in the system as occasional PL deficient pregnancies give rise to normal neonates as do those deficient in GH-V. The crucial role of placental GH is, however, emphasized by the correlation which exists between maternal IGF-1 levels and birth weight which appears to be due to effects on maternal metabolism and placental growth as maternal IGF-1 does not cross the placenta. The metabolic role of PL is supported by the prominent effect of high-density lipoproteins in stimulating its release from cultured placental cells; perhaps *in vivo* this would lead to further lipolysis and consequently increased availability of LDL for placental and maternal metabolism. PL also has an important function in promoting glandular development of the breasts while lactation is inhibited by progesterone.

The placenta itself is metabolically active and follows a plan for growth and development, the success of which determines the welfare of the fetus. Intra-uterine growth retardation and toxemia of pregnancy are both frequent causes of low birth weight babies and frequently thought to be a consequence of inadequate placental development or inadequate placental function. The growth of the placenta is dependent on the mitotic activity of the cytotrophoblasts which both invade the maternal endometrium, eroding the spiral arteries to form the placental sinusoids and fuse to form the terminally differentiated multinucleate hormone producing and nutrient transferring syncytiotrophoblast. The factors which determine the invasiveness of cytotrophoblast are uncertain and, while several hormones and factors which influence trophoblast differentiation *in vitro* have been described (including hCG, colony stimulating factor 1 and granulocyte-macrophage-colony stimulating factor and hypoxia), their physiological significance is uncertain. The physical growth of the placenta itself is correlated with maternal plasma concentrations of PL and GH-V and the placenta possesses receptors for IGF-1 and IGF-2. The placenta synthesizes a number of growth factors including IGF-1, IGF-2, IL-2, IL-6, platelet derived growth factor B, transforming growth factor B and especially epidermal growth factor and receptors for many of these factors, though their roles are unclear.

The blood flow through the placenta is another important determinant of nutrient transfer from mother to fetus and is also regulated. As the placenta lacks innervation, which in other vascular beds exerts an important influence, the only control over placental vascular tone is exerted by hormonal and local autocoids. Generally the fetal placental vascular tree appears to be maintained in a vasodilated state by the hormonal and autocoid milieu. Several factors have been identified within the placenta with powerful vasodilator actions including nitric oxide (NO), prostacyclin, PTHrP and CRH. The endothelial form of NO synthase (calcium and calmodulin sensitive) is found in syncytiotrophoblast which also synthesizes CRH and PTHrP. Prostacyclin production also occurs in endothelial cells. These vasodilators are potentially antagonized by vasoconstrictors such as platelet derived prostaglandins and members of the endothelin family. The balance between these dilator and constrictor influences is clearly important but the determinants of the balance are presently not known.

Fetal growth itself is determined by nutrient supply and endocrine factors. Insulin, IGF-1, and IGF-2 are all stimuli to fetal mitosis and growth. However, how these growth factors are regulated is problematic; the fetal pituitary produces GH from 12 weeks of age but anencephaly is associated with only a modest reduction in fetal growth as is Laron dwarfism (with dysfunctional GH receptors) while placental GH has not been identified in fetal blood. In contrast, PL is found in fetal blood and PL receptors in fetal tissues, and PL is capable of stimulating IGF-1 release. Nevertheless, deficiency of PL does not appear to have profound effects on fetal growth. Pancreatic agenesis and consequent insulin deficiency is associated with profound intrauterine growth retardation. The best explanation for current data appears to be that redundancy exists in the factors regulating fetal growth.

MATERNAL POSTERIOR PITUITARY FUNCTION

Post-conception plasma osmolality falls by approximately 10 mOsm/kg. The precise mechanism is unknown but a new setting for central osmoreceptors is established and maintained throughout pregnancy. As pregnancy advances the placental release of amniopeptidase leads to a reduction in the half-life of vasopressin. Rarely, diabetes insipidus develops in late pregnancy perhaps due to the reduction in vasopressin half-life. Oxytocin production from the maternal pituitary is episodic and in monkeys nocturnal release in the last days of pregnancy appears crucial in co-ordinating uterine contractions. In humans the role of oxytocin produced from the posterior pituitary and also the endometrium is less clear as hypophysectomized subjects deliver normally but are unable to lactate.

CALCIUM METABOLISM

The fetus has a significant requirement for calcium (120–150 mg per day) especially in the second half of pregnancy. To meet this fetal need the placenta maintains a transplacental calcium gradient. Fetal parathyroid production of PTH is important in some species and perhaps in man. The human placenta is also capable of synthesizing PTHrP and contains the 1-hydroxylase enzyme required for the production of 1,25 dihydroxycholecalciferol. The alterations in calcium metabolism continues post-partum as breast tissue also expresses PTHrP.

A PLACENTAL HYPOTHALAMUS

In addition to the placental analogues of pituitary hormones (and the generation of placenta specific mRNA transcripts of genes also expressed in the pituitary), the placenta produces many hypothalamic peptides including CRH (whose function

has been discussed), gonadotropin releasing hormone (GnRH), growth hormone releasing hormone, and neurotransmitters such as acetyl choline and NO. Many of these hypothalamic releasing hormones have been suggested to control placental production of the placental analogues of pituitary hormones; for example, GnRH stimulates hCG release from cultured placental cells, while NO inhibits CRH. While these data are of interest, their physiological significance is presently uncertain.

SUMMARY

Successful completion of mammalian pregnancy requires a reorganization of the maternal physiology to create an environment optimal for the growth and development of the fetus. To a large extent this physiological transformation is effected by the specialized endocrine functions of the placenta. The ability of the simple structure of the placenta—primarily cytotrophoblast and syncytiotrophoblast to produce a complex array of peptide and steroid hormones each with a specific gestationally determined production pattern—remains a fascinating enigma.

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Chapter 9

The Endocrinology of Late Pregnancy and Parturition*

TAMAS ZAKAR and BRYAN F. MITCHELL

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INTRODUCTION

Endocrine systems in pregnancy can be considered to have two distinct functions: homeostatic regulation to ensure that the nutritional and oxygen supply to the developing conceptus is sufficient to support optimal growth and development, and to

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maintain the pregnant uterus in a state of relative quiescence throughout pregnancy to allow fetal development and maturation and then to cause birth at the time optimal for extrauterine existence of the newborn. The former of these functions has largely been dealt with in earlier chapters on implantation and other specific endocrine systems. This chapter will deal predominantly with the latter function—the maintenance of pregnancy and regulation of the timing of parturition. Disorders of these physiologic mechanisms constitute the major problem in modern obstetrics. Although there is increasing understanding of these mechanisms in some animal species, it is becoming clear that there are many differences between these species and humans. Where possible, we shall provide information from human experimentation. However, there remain many gaps in our understanding of the regulation of human parturition. What is becoming clearer is that many important processes are not occurring in a classic endocrine fashion but rather as paracrine events where products from one cell or tissue type are secreted to influence activity in adjacent cells or tissues. Such processes may not be reflected in circulating blood and are therefore very difficult to study.

Important components of intrauterine paracrine systems include the human fetal membranes: the amnion and the chorion. Human implantation occurs as an interstitial process. The fetus and amniotic fluid are contained within the amnionic membrane. This membrane is composed of a single layer of cuboidal epithelial cells with an underlying stroma of very tough collagenous tissue and a few fibroblasts. Human amnion produces large quantities of prostaglandins that generally are thought to play some role in parturition (Challis and Olson, 1988). As the conceptus grows, the amniotic cavity expands and takes with it the overlying layer of trophoblast tissue which becomes the chorionic membrane. This membrane is several cell layers thick and as it stretches around the expanding conceptus, it becomes avascular. The cytotrophoblast cells of this membrane are functionally distinct from the placental cytotrophoblasts. The membrane also contains cells of mesenchymal origin which have not been well characterized. Human chorion may be involved in production of several paracrine factors, particularly steroids, but also prostaglandins and peptides. However, it may be more important as a site of metabolism of hormones, particularly steroids and prostaglandins.

The endometrium of human pregnancy is termed decidua. This is a complex tissue situated at the interface of the fetal-maternal communication system. It is composed of modified glandular and stromal cells of the endometrium as well as abundant vascular tissue. At term, almost half of the decidua is composed of bone marrow-derived cells including macrophages, lymphocytes, and neutrophils. These cells, usually associated with the immune system, produce several cytokines that may be important in mechanisms regulating myometrial contractility. The decidua also produces a host of other hormones, including proteins, steroids and prostaglandins, that may influence the timing of labor onset.

Human parturition can be considered as a sequence of five distinct but likely interrelated events (Figure 1). a) The activation phase where the uterus is prepared to give maximal response to a stimulant. This phase includes such processes as syn-

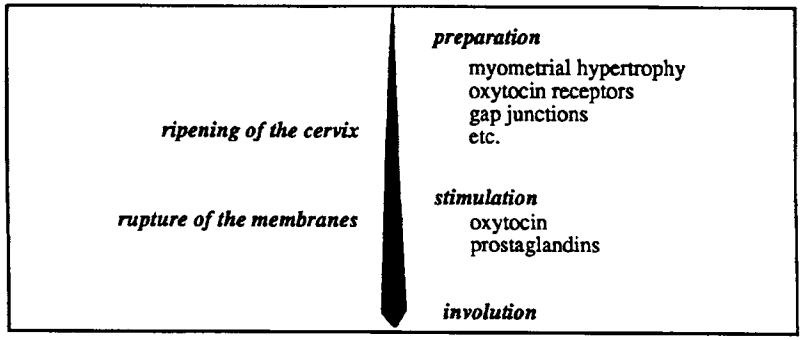

Figure 1. Parturition involves a number of discrete events that occur in an orderly sequence. Pregnancy complications occur when this sequence is delayed or prematurely activated.

thesis of oxytocin receptors and assembly of myometrial gap junctions. b) Ripening of the cervix where the collagen matrix of the cervix is broken down and the water content increased. This renders the cervix soft and pliable and therefore easily effaced and dilated, c) Rupture of the membranes usually occurs prior to delivery and often heralds the onset of labor. The mechanisms regulating degradation of the amnio-chorionic membrane are not well understood, d) The stimulation phase characterized by short, frequent high amplitude contractions causing delivery of the fetus. It is not clear whether this phase is caused by a discrete stimulant(s) or whether it represents escape from the mechanisms maintaining uterine quiescence prior to labor onset. e) The involution phase where the uterine changes regress and the uterus returns to its non-pregnant state. A large number of hormones are likely to play important roles in these processes. The major components of these hormonal regulatory systems will be discussed in this chapter.

STEROIDS

In almost all species, parturition is accompanied by a decline in the production and maternal plasma concentrations of progesterone. In most, there is a concomitant rise in estrogen. In sheep, the most extensively studied animal model of parturition, estrogen and progesterone are produced by the placenta. The key event triggering parturition is maturation of the fetal hypothalamo-pituitary-adrenal axis. The resultant increase in fetal cortisol secretion induces transcription of the 17-hydroxylase/17,20 desmolase gene in the placenta. The increasing activity of this enzyme causes metabolism of progesterone leading to a decrease in maternal plasma concentrations. The progesterone metabolites are estrogen precursors which are subsequently converted in the placenta to estrogen. The increasing estrogen/progesterone ratio leads to several events including synthesis of gap junctions which couple myometrial cells

electrically and metabolically enabling powerful, coordinated contractions. The changing steroid milieu also stimulates production of oxytocin receptors and stimulatory prostaglandins. Thus, the changes in estrogen and progesterone are likely responsible for both activation and stimulation of the uterus culminating in the onset of labor in the sheep (Figure 2). The presence of defects causing failure of the fetal hypothalamo-pituitary-adrenal axis, or administration of exogenous progesterone to the ewe, will postpone labor onset indefinitely.

Estrogen production in human pregnancy involves an interaction between the Fetus and placenta: the feto-placental unit (Figure 3). Pregnenolone is synthesized predominantly in the fetal adrenal from low-density lipoprotein cholesterol in the fetal circulation. The pregnenolone is converted in the fetal adrenal gland to the androgenic estrogen precursor dehydroepiandrosterone sulfate (DHAS). An hydroxyl moiety is attached by fetal hepatic 16-hydoxylase and the resultant 160H-DHAS is converted to the estrogen estriol in the placenta. Estriol is secreted into the maternal circulation where it is extensively conjugated to sulfates and glucuronides. This conjugation prolongs its half-life such that estriol accounts for approximately 90% of total circulating estrogens in human pregnancy. Its physiologic role is unknown. Estriol has less biological activity and the concentration of the unconjugated form is less than the major non-pregnancy estrogen, estradiol. The high estrogen levels may be important in maintaining uterine blood flow. Maternal se-

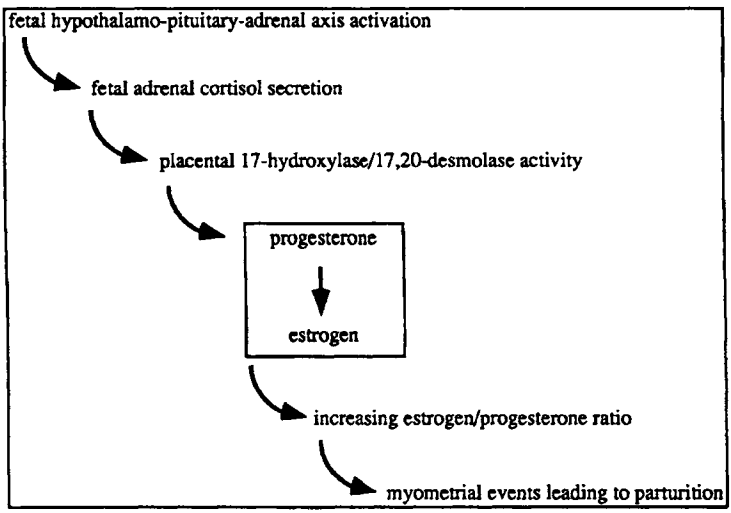

Figure 2. The sequence of endocrine events leading to parturition in the sheep. Maturation of the fetal hypothalamo-pituitary-adrenal axis causes an increase in fetal cortisol which induces synthesis of placental enzymes that result in metabolism of progesterone through to estrogens. The increasing estrogen/progesterone ratio leads to changes in the pregnant uterus that culminate in labor.

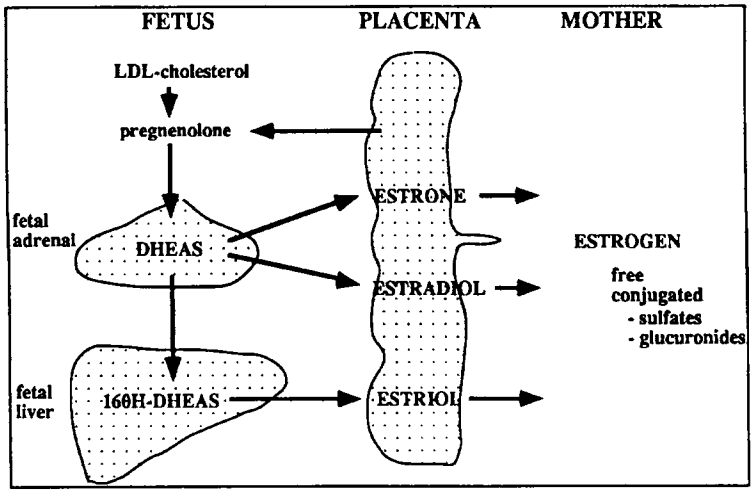

Figure 3. Estrogen synthesis in the human feto-placental unit. Pregnenolone derived largely from fetal low-density lipoprotein cholesterol is converted to dehydroepiandrosterone sulfate (DHEAS) in the fetal adrenal. DHEAS is converted in the placenta to estrone or estradiol or is 16-hydroxylated in the fetal liver for subsequent conversion in the placenta to estriol.

rum or urinary estrogen levels were formerly measured as an index of feto-placental function. This has largely been abandoned because of the high degree of variability, both within and between patients, and the resultant insensitivity in distinguishing normal from abnormal.

Estrone and estradiol also are synthesized in large quantities in human pregnancy. The androgenic precursors for these estrogens are derived in approximately equal amounts from both the fetal and maternal adrenal glands. They also do not require the 16-hydroxylation step which is predominantly found in the fetal liver. Thus, their production rates do not reflect function of the feto-placental unit to the extent that production of estriol does.

As in the sheep, progesterone production in late pregnancy is located in the placenta using maternal cholesterol as a substrate. Again, production rates are much higher than in animal models. In early pregnancy, progesterone is predominantly produced in the corpus luteum of the ovary and removal of the corpus luteum before six weeks menstrual age will result in abortion. However, exogenous progesterone will not delay parturition at term. Progesterone receptor blockers will increase human myometrial contractility. There is an uneasy consensus based on precious little human experimental evidence that, as in animal models, it is likely that progesterone is important in maintaining uterine quiescence during human pregnancy.

In contrast to the ewe, the role of estrogen and progesterone in human parturition is controversial. Maternal plasma levels of estrogen and progesterone do not

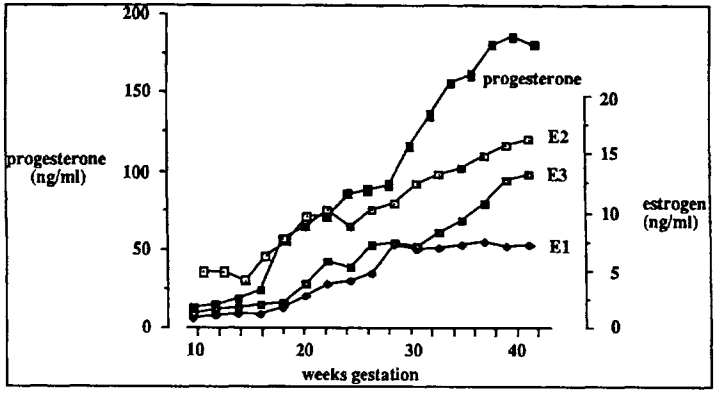

Figure 4. Maternal plasma concentrations of progesterone and unconjugated estrone (E1), estradiol (E2), and estriol (E3) through gestation showing no significant changes at the time of parturition. Modified from the data of Tulchinsky et al., 1972.

change significantly prior to parturition (Figure 4). However, it is well documented that there are significant diurnal variations in the concentrations of estrogen and progesterone in maternal plasma causing significant changes in the estrogen/progesterone ratio. This may correlate with the well described nocturnal peak in myometrial activity observed in the few days preceding human labor. Furthermore, the hypothesis has been proposed that the basic physiologic mechanisms regulating parturition in humans are similar to those in sheep. According to this hypothesis, the change in the estrogen/progesterone ratio in the human occurs within an intrauterine paracrine system involving the fetal membranes and decidua (Mitchell and Challis, 1988). Such an hypothesis is appealing because the critical changes would occur in the tissues that are in most intimate contact with the myometrium and ideally situated to transmit information between the mother and fetus. Human fetal membranes and decidua produce estrogen and progesterone and several changes in enzyme activity that could cause an increase in the local estrogen/progesterone ratio occur around the time of parturition. However, a great deal of work remains to prove this hypothesis. Because of the ethical considerations in human experimentation, it may prove a very difficult task to achieve a clear understanding of the role of the sex steroids in human parturition.

As in the sheep, glucocorticoids are important in human fetal maturation, particularly in the production of pulmonary surfactant. However, fetal cortisol does not appear to play an important role in the regulation of parturition. Fetal anomalies that result in fetal adrenal agenesis do not cause indefinite prolongation of pregnancy as in the sheep. Unlike the sheep, the human placenta freely transports cortisol in either direction. Indeed, the diurnal rhythms in maternal estriol concentrations probably reflect the influence of the maternal adrenal rhythm in cortisol transported across the placenta to cause a reverse rhythm in fetal adrenal estrogen precursor production.

Maternal total and free cortisol levels are increased in pregnancy perhaps because its metabolic effects help to ensure adequate nutritional supply to the fetus.

GONADOTROPHINS

The human placenta produces large quantities of human chorionic gonadotrophin (hCG). This glycoprotein hormone has biologic similarity to luteinizing hormone (LH) from which it differs biochemically by having a slightly longer b-subunit. It also is slightly more extensively glycosylated, giving it a longer metabolic half-life and greater biologic activity than LH. The major role for hCG appears to be the rescue of the corpus luteum. Under maternal pituitary gonadotrophin control, the corpus luteum spontaneously regresses after approximately 14 days. At this time, placental hCG takes over support of the corpus luteum to ensure continued progesterone production and pregnancy survival. Maternal plasma concentrations of hCG peak at approximately 10 weeks of gestation but significant quantities of hCG remain in the maternal circulation throughout gestation. Interestingly, a placental form of LH releasing hormone termed human chorionic gonadotrophin releasing hormone (hCGnRH) has been documented. It is unclear what role this hormone or other regulatory factors have in controlling hCG synthesis or release. There is no good evidence to support a significant role for hCG in steroid production in late pregnancy nor in the mechanism of parturition.

The placenta also produces large quantities of placental lactogen or human chorionic somatomammotrophin (hCS). Although in some species this hormone demonstrates gonadotrophic activity, there is no evidence that this is a significant function in human pregnancy.

OXYTOCIN

Oxytocin has been used pharmacologically since the early twentieth century to stimulate uterine contractions in pregnant women. This nonapeptide is synthesized in the paraventricular and supraoptic nuclei of the hypothalamus. It is transported down axons bound to its carrier protein neurophysin I and stored in the posterior pituitary. Prior to secretion, it is cleaved from neurophysin I and released into the maternal circulation as the active molecule. It has very specific and potent stimulatory activity on myometrial cells which contain specific receptors. These receptors increase in number in both the myometrium and decidua prior to human labor. Despite its obvious suitability as a physiologic regulator of human parturition, most investigators have discarded the notion that oxytocin has a key role in determining the time of human labor onset. These conclusions have been based on several pieces of scientific evidence. Several investigators have been unable to detect an increase in maternal plasma oxytocin concentrations until labor is well established. Even

then, maximal uterine activity occurs when plasma levels are two or three orders of magnitude lower than the K_d of the myometrial oxytocin receptor. Additionally, human pregnancy plasma contains a very active cysteine aminopeptidase, termed oxytocinase, that rapidly metabolizes circulating oxytocin. Furthermore, women with posterior pituitary dysfunction go into labor at the normal time. Although the fetus synthesizes oxytocin, there appears to be no way that it can escape placental metabolism to reach the myometrium intact. All of these findings suggest it is unlikely for pituitary oxytocin to be the key trigger to the stimulation phase of labor.

However, a recent finding could rationalize a role for oxytocin in human labor (see Hirst et al., 1993). Synthesis of mRNA for oxytocin was demonstrated in maternal decidua as well as in amnion and chorion in lesser amounts. The levels of oxytocin mRNA increased significantly around the time of labor. These findings were confirmed in the rat. This supports the hypothesis that oxytocin also could be involved in the paracrine network within fetal membranes and that this network could regulate the timing of the onset of human labor. In addition, sensitive radioimmunoassays have been used to measure small but significant increases in maternal plasma oxytocin levels associated with the nocturnal uterine activity that precedes labor. Oxytocin antagonists have been synthesized and these will block the nocturnal uterine contractions in subhuman primates. These new data suggest that oxytocin may indeed play a significant role in the initiation of human parturition by participation in an intrauterine paracrine network.

PROSTAGLANDINS

Prostaglandins are a group of locally acting regulatory factors derived from arachidonic acid, an esterified component of cellular phospholipids. Arachidonic acid is released by phospholipases and lipases, and is subsequently metabolized by various oxigenative enzymes. One such enzyme is cyclooxygenase, or COX, which attaches two oxygen molecules to the fatty acid at specific positions. The resulting compound, called prostaglandin G_2, is further modified by the peroxidase activity of COX itself and by other enzymes in sequential reactions, giving rise to a host of products termed prostaglandins and thromboxanes. Many of these have strong biological effects. Figure 5 shows the cascade leading to the formation of prostaglandins and thromboxanes. The reactions catalyzed by COX constitute the committing step of the biosynthesis, since they are upstream, pathway-specific, and irreversible. Such reactions are often subject to regulation. Two of the biologically active products, prostacyclin and thromboxane A_2 are chemically unstable, the others are rapidly inactivated by enzymes. Because of the ubiquitous presence of their precursors, arachidonic acid, and oxygen, and because of their fast decay rate, these compounds are ideally suited to their role, which is the short-term local regulation of various organ and tissue functions. Prostaglandins, prostacyclin, and thromboxane are involved in the control of vascular tone, haemostasis, cytoprotection, sleep/wake states, kidney function,

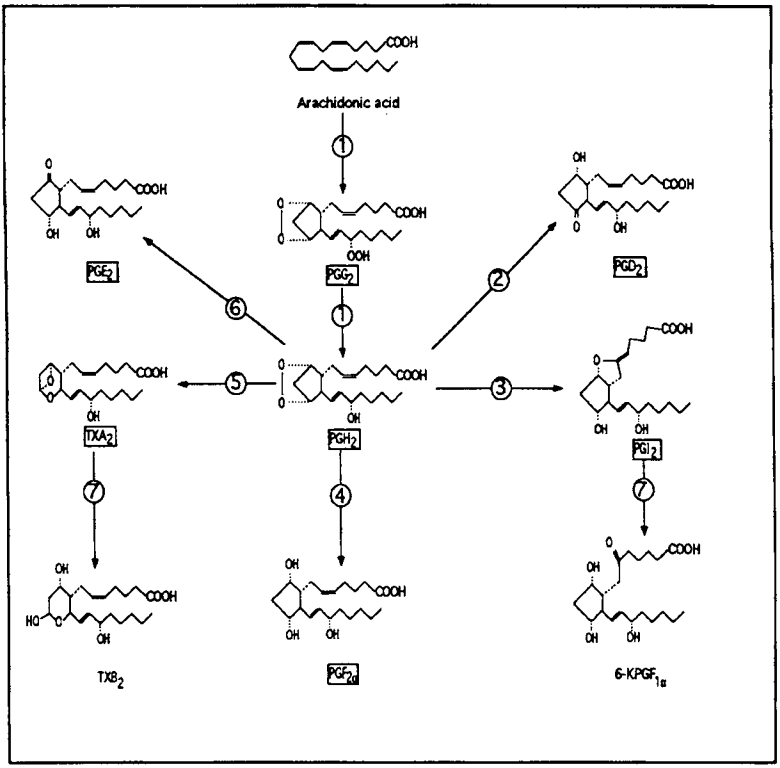

Figure 5. The conversion of arachidonic acid to prostaglandins and thromboxanes. Arachidonic acid is oxygenated to the prostaglandin endoperoxides prostaglandin G_2 (PGG_2) and prostaglandin H_2 (PGH_2) in sequential reactions by the enzyme cyclooxygenase (1). PGH_2 is metabolized further to prostaglandin D_2 (PGD_2) and prostaglandin E_2 (PGD_2) by enzymes with PGD-synthase (2) and PGE-synthase (6) activities, respectively. Prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$) is formed from PGH_2 by prostaglandin endoperoxide reductase (4). Prostacyclin (PGI_2) and thromboxane A_2 (TXA_2) are generated from PGH_2 by prostacyclin synthase (3) and thromboxane synthase (5), respectively. Non-enzymatic reactions (7) result in the formation of 6-ketoprostaglandin $F_{1\alpha}$ (6-$KPGF_{1\alpha}$) from PGI_2 and thromboxane B_2 (TXB_2) from TXA_2. Boxes mark the compounds with biologic activities.

thermoregulation, inflammation, cell proliferation, ovulation, and implantation. An excellent overview of arachidonate release and metabolism to prostaglandins and thromboxanes has been published by Smith and colleagues (1991).

In humans, three lines of evidence suggest that prostaglandins are key regulators of uterine function in late pregnancy and at parturition (Challis and Olson, 1988; Keirse, 1990). Firstly, the administration of prostaglandin E_2 or $F_{2\alpha}$ (PGE_2 and $PGF_{2\alpha}$) to pregnant women induces labor. The prostaglandins not only stimulate the

uterus to contract, but cause the uterine cervix to soften and efface, a process essential for natural birth to take place. Secondly, it was observed that the length of pregnancy as well as the duration of labor significantly increased in women who were treated with blockers of prostaglandin synthesis to alleviate the symptoms of chronic inflammatory diseases. Moreover, the administration of inhibitors of prostaglandin synthesis suppressed preterm labor and delayed preterm birth. Thirdly, the birth process is associated with increased levels of intrauterine prostaglandins. Specifically, the concentrations of prostaglandins E_2 and $F_{2\alpha}$ and of their precursor, arachidonic acid, rise progressively in the amniotic fluid as labor advances. The maternal plasma concentration and the urinary excretion of the metabolic product of prostaglandin $F_{2\alpha}$ increase at labor, also indicating the enhanced production of the endogenous prostaglandins. Notably, PGE_2 and $PGF_{2\alpha}$ levels begin to rise in the amniotic fluid shortly before the clinical onset of labor, phenomena suggestive of their involvement in the initiation process. Taken together, these findings firmly establish prostaglandins as principal factors controlling human birth.

Since strong evidence implicates prostaglandins as regulatory factors in parturition, important further questions arise. Which tissues are the sources of the intrauterine prostaglandins? How are the synthesis and the inactivation of prostaglandins controlled around the time of labor? Which tissues are the targets? What are the actions of the prostaglandins in the target tissues?

Research during recent years has made significant progress toward answering these questions (see Olson et al., 1993, for review). The amnion was shown to produce copious amounts of prostaglandin E_2, while the chorion laeve and decidua were identified as sources of both PGE_2 and $PGF_{2\alpha}$. The decidua releases prostacyclin and thromboxane too. It was demonstrated that the amnion synthesizes significantly more prostaglandin E_2 with labor than at any time during gestation. Metabolic inactivation does not occur in the amnion, but it is intensive in the chorion laeve.

More insight into the complicated tissue-specific regulation of intrauterine prostaglandin levels was obtained by the study of several key enzymes of prostaglandin synthesis and degradation. It was found that one of the isozymes of COX, called COX-2, is selectively induced in the amnion and the chorion laeve, but not in the decidua, at late gestation and during labor. This results in an increase of the capacity of these tissues to produce prostanoids. The cytosolic isoenzyme of phospholipase A_2, which specifically releases arachidonic acid from phospholipids, is also induced in the amnion at late gestation. On the other hand, 15-hydroxyprostaglandin dehydrogenase, the main enzyme of prostanoid degradation, exhibits lower activity in some cases of normal or pathological labor. Changes of enzyme expression thus play major roles in the control of fetal membrane prostaglandin production and inactivation.

The administration of prostaglandins to pregnant women induces uterine contractions suggesting that the myometrium is a target of prostaglandin action. Studies with synthetic prostaglandin and thromboxane analogs revealed that the human myometrium contains at least seven types of prostanoid receptors. These receptors bind prostaglandin E_2, $F_{2\alpha}$, D_2, thromboxane A_2, and prostacyclin with different af-

finities. The receptors are coupled to various signal transduction systems, and are capable of mediating the contraction as well as the relaxation of the tissue. Interestingly, isolated strips of pregnant human myometrium relax when exposed to prostaglandin E_2 or $F_{2\alpha}$. This indicates that, at least under in vitro conditions, the relaxing effects predominate. In vitro studies of prostaglandin transfer across the fetal membranes, and the decidua have demonstrated that decidual prostaglandins have unimpeded access to the myometrium because of the anatomical proximity of the two tissues. However, more than 90% of the amniotic prostaglandin E_2 is inactivated in the chorion before reaching the myometrium suggesting that the primary target of the prostaglandins produced by the amnion membrane is probably not the myometrium (Olson et al, 1993). Because of these observations, there is a growing consensus among investigators that the traditional view that labor is initiated by a rise in intrauterine prostaglandin levels that directly stimulate uterine contractions is too simplistic. It is more likely that prostaglandins act indirectly or in concert with other agonists to stimulate the uterus.

The uterine cervix and the amnion membrane are other likely targets of prostaglandins. Both of these structures undergo changes at parturition resulting in cervical softening and membrane rupture, respectively. The cervical changes include the remodeling of the collagen and glycosaminoglycan components of the extracellular matrix. This process is influenced by prostaglandins, especially PGE_2 (Challis and Olson, 1988; Huszar and Walsh, 1991). In fact, PGE_2 is used clinically to promote cervical pliability. The mechanism of prostaglandin action in these tissues is obscure, but it probably involves a local reaction resembling inflammation.

Various in vitro studies with perfused placentas, placental explants, cells, or homogenates have shown that the placenta produces measurable amounts of all major products of the prostanoid synthetic pathway (Figure 5) (Myatt, 1990). The majority of these compounds are most likely released from the cells of the placental vasculature, and participate in the regulation of fetal placental blood flow. The placental trophoblasts are very rich in prostaglandin-inactivating enzymes. Similar to the chorion, this high metabolic activity effectively separates the maternal and the fetal prostanoid pools.

The influence of hormonal and other factors on the prostanoid production of human gestational tissues has been extensively studied. Most of these experiments, reviewed recently by Olson and collealgues (1993), comprised the in vitro treatment of cells or tissues with hormonal or pharmacologic stimulants, followed by the determination of prostaglandin output or of other relevant parameters of prostanoid biosynthesis such as enzyme levels and arachidonate depletion. A variety of natural and synthetic factors have been found to modulate prostaglandin synthesis in these experiments. Among the steroids, cortisol was demonstrated to inhibit the prostaglandin output of amnion and placental explants, whereas progesterone or estrogens were not reported to affect prostaglandin production in term human gestational tissues. Oxytocin was shown to stimulate the prostaglandin production of decidual tissue and cultured amnion cells; however, these observa-

tions were not confirmed independently (Hirst et al., 1993). ACTH and CRH (corticotropin-releasing hormone) increased the prostaglandin output of amnion, chorion, decidua, and placenta at term pregnancy, whereas GnRH (gonadotropin-releasing hormone) stimulated the prostaglandin production of placental explants. Several cytokines, growth factors, and second messenger analogs also affect gestational tissue prostaglandin output; some of these will be discussed in more detail in later sections of this chapter.

Despite the wealth of information concerning the in vitro regulation of prostaglandin biosynthesis in the fetal membranes, placenta, and the decidua, little is known about the factors and mechanisms that control intrauterine prostaglandin production in vivo. Mechanical stimulation appears to play a role because artificial rupture of the membranes or distension of the cervix lead to increased prostaglandin levels in the maternal circulation and in the amniotic fluid. However, none of the hormones or paracrine factors that affect the prostanoid output of the gestational tissues in vitro have been demonstrated to be the physiological regulator(s) of the increasing intrauterine prostaglandin levels observed at labor. The criteria to be satisfied by such regulator(s) include (1) the changing endogenous production preceding or concomitant with the increase of intrauterine prostaglandin levels, (2) the ability to modulate prostaglandin levels upon exogenous administration, and (3) the blockade of the in vivo changes of prostaglandin synthesis by antagonists and/or synthesis inhibitors. Additionally, it is necessary to demonstrate that the agonist(s) gain access to the target tissues, the amnion, and the decidua, without metabolic inactivation.

These criteria are difficult to establish through experimentation because ethical considerations and technical difficulties often limit the scope of research involving human subjects. However, recent work involving mice lacking genes necessary for prostaglandin generation and action has provided strong in vivo evidence for a role of prostaglandins in parturition. Mice devoid of either the $PGF_{2\alpha}$ receptor or the arachidonic acid–specific cytosolic PLA_2 isozyme failed to deliver their otherwise healthy offspring at term. More detailed experiments have shown that the role of $PGF_{2\alpha}$ in murine parturition is to induce luteolysis, resulting in a drop of progesterone levels, an increase of oxytocin receptor abundance in the myometrium, and delivery (Sugimoto et al. 1997). The applicability of this information to humans still remains to be ascertained since the physiology of human parturition is considerably different from that of the mouse.

CYTOKINES

Cytokines are a group of polypeptide factors which orchestrate the response of an organism to injury, infection, or other effects threatening to upset its homeostasis. They are crucially important in the local as well as the systemic reactions of the acute phase response, and in both the cellular and humoral arms of the immune system. The inflammatory cytokines interleukin 1β (IL1β), tumor necrosis factor α

(TNFα), interleukin 6 (IL6), and the neutrophil chemoattractant cytokine IL8 accumulate in the amniotic fluid of pregnant women with intrauterine infection and chorioamnionitis. These conditions are often accompanied by preterm labor and elevated amniotic fluid levels of prostaglandins (Romero et al., 1988; Mitchell et al., 1991). All three inflammatory cytokines were shown to stimulate the prostaglandin output of amnion and decidua *in vitro*. The decidua, which contains bone marrow derived cells, produces TNFα and IL6 in response to bacterial endotoxin and other cytokines such as IL1β. Based on these observations it was proposed that cytokines are important mediators of preterm labor in the case of intrauterine infection and inflammation (Mitchell et al., 1991). It was suggested that the reaction evoked by the bacterial invasion of the uterine cavity leads to increased cytokine levels, which stimulate prostaglandin production. The prostaglandins induce labor and, as a result, the fetus is removed from the hostile intrauterine environment. Notably, normal term labor is accompanied by a rise in the number of cytokine producing proinflammatory cells in the decidua. However, the involvement of cytokines in normal labor has not been established.

Another phenomenon in which cytokines may be important is the adjustment of the immune system of the mother to allow tolerance of the fetal allograft (Colbern and Main, 1991). The suppression of the maternal TH1 lymphocyte dependent (cell-mediated) immunity and the relative prevalence of the TH2 lymphocyte dependent (antibody-mediated) immune response have been established in the pregnant mouse, and the existence of a similar adaptation in human pregnancy is postulated (Wegmann et al., 1993). A maternal immune system with decreased ability to mount a cell-mediated immune response could be advantageous for the maintenance of the pregnancy, because trophoblastic tissue may be susceptible to a cell-mediated immunoreaction. At the same time, enhanced atibody-mediated immunity may provide the newborn with increased protection against infection after birth. The TH1 cell-secreted cytokines IL2, interferon γ, and TNFβ, and the TH2 cell-secreted IL4, IL5, IL6, and IL10 are most likely involved in this function. The mediating role of prostaglandin E_2 is also conceivable, because this eicosanoid is a strong suppressor of natural killer cell activity.

CORTICOTROPIN RELEASING HORMONE (CRH)

The placenta and the other gestational tissues produce a variety of hypophysiotropic hormones such as thyrotropin-, gonadotropin-, and growth hormone–releasing hormones, as well as somatostatin, and CRH (Waddel, 1993). Due to increasing placental production, the concentration of CRH in the maternal and fetal circulation rises exponentially after 28 weeks of gestation. Furthermore, the level of the high affinity, CRH-binding protein falls in the maternal plasma at term, dramatically increasing the bioavailability of CRH prior to the onset of labor. Because the activation of the fetal hypothalamo-pituitary-adrenal axis has a well-established role in

the initiation of parturition in several nonprimate species, it was suggested that an analogous "hormonal axis" might be operating in the primate placenta and contributing to changes in hormone levels (Challis et al. 1995). Indeed, CRH has been shown to stimulate the production of ACTH by the trophoblastic tissue, and the placenta appears to provide trophic support for the maternal as well as the fetal adrenal. Contrary to the hypothalamus, placental CRH production is stimulated by glucocorticoids, implying that a feed-forward cascade may exist in the mother and the fetus, an event responsible for the increases of plasma CRH, ACTH, and cortisol levels as pregnancy advances. Preterm and postterm birth is often associated with a rise of plasma CRH levels that occurs earlier or later, respectively, than normal. This observation has led to the suggestion that a "placental clock" determines the length of gestation (McLean et al. 1995). The pace of this hypothetical clock is reflected by plasma CRH levels. CRH was also reported to have a direct stimulating action on the myometrium and was found to enhance the prostaglandin production of gestational tissues in vitro. Collectively, these actions have provided a basis to postulate a major role for placental CRH, ACTH, and cortisol in primate parturition. However, definitive proof for such a function in vivo has yet to be provided. Even more work is needed to establish the significance of the other hypophysiotropic factors produced by the placenta.

OTHER FACTORS

The vasoactive peptide *endothelin 1* is a potent stimulant of the myometrium. Endothelin 1 is synthesized by amnion cells and is present in term amniotic fluid. The chorion contains high levels of the enzyme enkephalinase, which degrades endothelin and thus prevents it from accessing the myometrium and causing contractions. The physiological function(s) of endothelin in pregnancy may include the regulation of placental blood flow and the closure of the ductus arteriosus (Mitchell, 1991).

Relaxin is a small polypeptide, produced by the myometrium, the gestational tissues, and the corpus luteum of pregnancy. Its role in preparing the uterus for labor in animals such as the pig or the rat is well documented (Challis and Olson, 1988). Relaxin has similar effects on the human uterus as it does in the animal models (Challis and Olson, 1988; Huszar and Walsh, 1991), but its importance in human parturition is not clearly established.

Prolactin concentration increases steadily in the maternal circulation during gestation and reaches levels approximately 10-fold higher than in nonpregnant women. The high prolactin level is one of the factors contributing to the decreased glucose tolerance and insulin sensitivity and to the increased lypolysis observed in women in late pregnancy. These metabolic changes are thought to facilitate the transport of nutrients to the fetus. The concentration of prolactin also increases in the fetal plasma during the last weeks of gestation.

Catecholamines are present in the amniotic fluid in increasing amounts with advancing gestation. The source of the amniotic fluid catecholamines is most likely the fetal urine. Adrenergic nerve terminals in the uterine cervix may also contribute to catecholamine levels in the uterus. α as well as β adrenergic receptors are present in the myometrium, the former mediating uterine contractions, the latter mediating relaxation (Huszar and Walsh, 1991). β adrenegic agonists are widely used in clinical practice to stop myometrial contractions in cases of preterm labor, although their efficacy diminishes after several hours of administration probably due to the desensitization of the myometrium.

SUMMARY

In late gestation, endocrine systems function to maintain uterine quiescence while preparing the myometrium and the cervix for impending labor. At the end of pregnancy, hormonal changes, which most likely involve paracrine interactions within intrauterine tissues, result in the stimulation of myometrial activity culminating in the expulsion of the fetus. Parturition is followed by involution of the uterus to its nonpregnant state.

Progesterone and estrogen levels are high in the maternal plasma in late pregnancy, and, contrary to well studied animal models such as the sheep, remain unchanged before labor. Progesterone is produced by the placenta; estrogen synthesis is the coordinated function of the fetal adrenal, fetal liver, and the placenta. Diurnal and/or local variations of the estrogen/progesterone ratio in the uterus possibly influence the contractility of the myometrium and the timing of labor. The human placenta produces large quantities of human chorionic gonadotrophin and placental lactogen, but the function of these hormones in late pregnancy is unknown.

Oxytocin is a powerful stimulant of the myometrium. Oxytocin secreted by the maternal and fetal hypophysis probably does not play a role in the onset of human labor, but locally produced oxytocin may function as a paracrine factor regulating myometrial contractility.

There is strong evidence suggesting that prostaglandins are important factors controlling human parturition. First, prostaglandins induce labor by promoting myometrial contractions as well as cervical ripening. Second, normal labor is associated with increased intrauterine prostaglandin synthesis. Third, inhibition of prostaglandin biosynthesis prolongs gestation and labor. Although a number of hormones were shown to affect the prostaglandin production of the gestational tissues, the *in vivo* regulation of intrauterine prostaglandin synthesis is unclear.

The inflammatory cytokines IL1, TNF, and IL6 accumulate in the amniotic fluid in pregnancies complicated with intrauterine infection and inflammation, and may have a primary role in the mechanism of infection-related preterm labor. Cytokines also are implicated in the modulation of the maternal immune system to tolerate the fetus and the placenta. Increasing placental production of corticotropin-releasing

hormone was postulated to signal and/or promote the onset of labor. Other factors such as endothelin, relaxin, catecholamines, and growth factors are also present in the uterus and influence certain aspects of uterine function in late pregnancy, but their physiological roles remain to be established.

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Chapter 10

Maternal Adaptation to Pregnancy

WILLIAM A. W. WALTERS

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INTRODUCTION

Maternal adaptation to pregnancy is apparent in all systems of the body. However, with the exception of the reproductive system itself, the circulatory system and its shared function with the respiratory system show the most striking changes, which will form the basis of this chapter. A knowledge of these physiological changes is essential for an understanding of both normal adaptation and maladaptation to pregnancy. Furthermore, such knowledge will be helpful in the clinical management of pregnant women with diseases of the circulatory and respiratory systems.

There is now good evidence, mentioned later, that normal adaptation to pregnancy occurs early in the embryonic period within the first trimester, long before the developing feto-placental unit is large enough to itself warrant the magnitude of the changes observed. Theoretically, this would suggest that the changes are preparatory to normal pregnancy and that if they do not occur or only occur partially, the future of the pregnancy may be in jeopardy. Indeed some studies (see below) have provided confirmation of this suggestion.

THE CIRCULATORY SYSTEM

Blood Volume

Both plasma volume and red cell mass (the total volume of red cells in contradistinction to red cell volume, which refers to the volume of an individual red cell) have usually been measured by methods based upon the indicator dilution principle. After injection of an indicator substance that is confined to the body compartment into which it is injected, the volume of fluid within the compartment can be calculated by measuring the dilution of the indicator. The volume of distribution of the indicator is equal to the amount injected, divided by the concentration of the indicator in a sample of fluid collected from the same compartment at a given time after injection to allow for adequate mixing of the indicator in the compartment.

Plasma volume has usually been measured by dyes that become bound to plasma protein, particularly Evans' blue (T-1824). Alternatively, radioactive iodinated serum albumin (RISA) has been used as the indicator. Neither of these indicators crosses the placental barrier. Once the plasma volume and the hematocrit (the percentage of the blood volume that is made up of cells and which can be determined from a blood sample) is known the total blood volume can be calculated by multiplying the plasma volume by 100 divided by 100 minus the hematocrit (Ganong, 1995). Similarly, the red cell mass has usually been measured by using the radioisotopes ^{51}Cr or ^{32}P to label a sample of the subject's red cells *in vitro* and then to inject these cells into the vascular compartment to act as the indicator. The red cell mass can also be determined indirectly by subtracting the plasma volume from the total blood volume. The total volume of white blood cells is ignored in studies of blood

volume (Ganong, 1995). For the most reliable results, subjects should only have blood volume measurements made when in a basal state, that is when fasting after a good night's sleep and in a temperature controlled environment.

Total Blood Volume

Ideally, both plasma volume and red cell mass should be measured simultaneously, but this has rarely been done. Commonly one has been measured and the other calculated indirectly from the hematocrit. Total blood volume increases progressively throughout pregnancy until the third trimester when it levels out until parturition, thereafter declining in the puerperium to pre-conceptional levels. The two major components of the blood volume, plasma volume and red cell mass, increase simultaneously but the increase in plasma volume is proportionately greater than that of the red cell mass (Chesley, 1972). This hemodilution is sometimes referred to as the "physiological anemia" of pregnancy. As it is not a true anemia, the term is best avoided.

Plasma Volume

During the first 30–34 weeks of a normal first pregnancy, plasma volume increases from a pre-conceptional level of about 2,600 ml by about 1,250 ml to 3,850 ml (Hytten and Leitch, 1971) and is then maintained at this level until term (Whittaker and Lind, 1993). The increase in plasma volume reaches significance between 7–12 weeks gestation (Whittaker and Lind, 1993). The mean maximum increase in plasma volume during pregnancy amounts to 42% (Chesley, 1972) (see Table 1 and Figure 1).

In a multiple pregnancy, plasma volume increases by a larger amount than in singleton pregnancies and in proportion to the number of fetuses present (Rovinsky and Jaffin, 1965; Fullerton et at., 1965; MacGillivray et al., 1971). However, the presence of a fetus is not essential for the increase in plasma volume which also occurs in women with a complete molar pregnancy (Pritchard, 1964).

Both the maximum increment in plasma volume (Hytten, and Paintin, 1963) and the maximum volume (Duffus et al., 1971) correlate closely with fetal weight at

Table 1. Changes in Plasma Volume During Gestation in Normal Women

Gestation Interval (weeks)	NP–7	7–12	12–20	20–28	28–36	26–PN
n	(47)	(55)	(67)	(67)	(64)	(64)
Changes in Plasma Volume (ml)	84	190*	454*	401*	139**	-1231*

Notes: * P<0.001 (paired t-test); ** P<0.05; NP = non-pregnant; PN = postnatal. From Whittaker and Lind (1993).

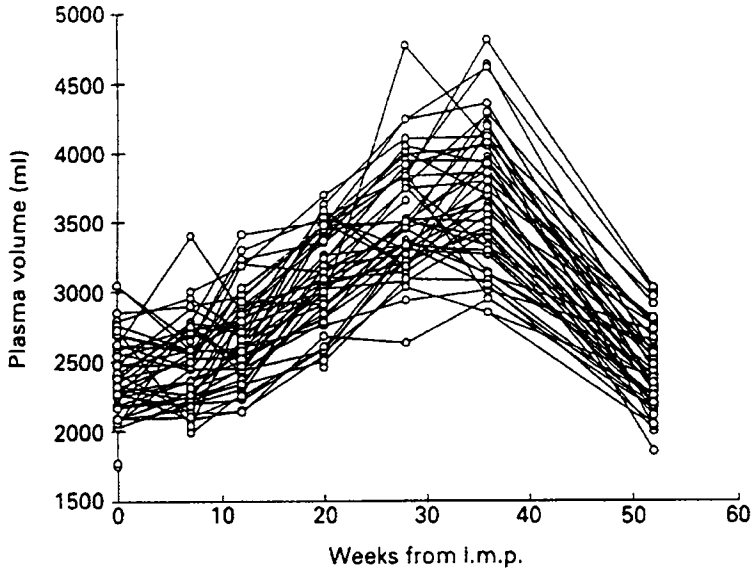

Figure 1. Serial measurements of plasma volume during normal pregnancy. From Whittaker and Lind (1993).

birth. This relationship also applies when fetal growth is impaired (Gibson, 1976). In fact women with a low pre-conceptional plasma volume often demonstrate a relatively small increase during pregnancy and deliver babies showing evidence of impaired growth (Croall et al., 1978).

Plasma volume before pregnancy and at 36 weeks gestation is also significantly correlated with maternal weight but this relationship does not hold for the increment in plasma volume during pregnancy. Furthermore, increasing parity has no significant effect on plasma volume (Whittaker and Lind, 1993).

Red Cell Mass

The total volume of the red cell mass in the healthy young woman before pregnancy is about 1,400 ml. During pregnancy this increases by about 240 ml (17% in the absence of any iron medication; when the latter is taken the increase in red cell mass is about 400 ml (28.5%) (Letsky, 1991). The pattern of the increase in red cell mass has not been adequately investigated but is likely to be similar to that of plasma volume. In keeping with plasma volume, red cell mass increases further in multiple pregnancy, the increase being directly proportional to the number of fetuses (Fullerton et al., 1965; Rovinsky and Jaffin, 1965). During pregnancy, the hematocrit falls gradually until about the thirtieth week and then rises gradually (Peeters and Buchan, 1989).

Blood Volume after Parturition

After normal labor and delivery (when blood loss does not exceed 500 ml) total blood volume decreases rapidly during the first 24 hours (Ueland, 1976) and then slowly decreases to normal pre-conceptional levels by the ninth week post-partum (Lund and Donovan, 1967).

The Cause of the Increase in Blood Volume

The explanation for the increase in blood volume during pregnancy is obscure. For plasma volume to increase there must be an increase in total fluid volume of the extracellular space and a normal distribution of fluid between the intravascular and extravascular compartments. One of the most important factors regulating the volume of extracellular fluid is the sodium balance, which is dependent upon factors promoting absorption and excretion of sodium in the kidney. During pregnancy, sodium excretion will be enhanced by the 50% increase in glomerular filtration rate and by the natriuretic effect of progesterone on the distal part of the convoluted tubule (Oparil et al., 1975). However, paradoxically, there is some evidence to suggest that progesterone may promote sodium retention by being converted to deoxycorticosterone (Winkel et al., 1980). Deoxycorticosterone production is increased as early as the eighth week of pregnancy and shows a greater percentage increase than other adrenal steroids (Wintour et al., 1978). Furthermore, the deoxycorticosterone is not suppressible by dexamethasone at any stage of pregnancy (Nolten et al., 1980), suggesting that it is of non-adrenal origin, perhaps from the feto-placental unit. In addition, the increased activity of the renin-angiotensin-aldosterone system during pregnancy favors sodium retention, in the same manner as does deoxycorticosterone, by its action on the distal renal tubule. Atrial natriuretic peptide is also significantly decreased during pregnancy, thereby favoring sodium and fluid retention (Thomsen et al., 1993).

The explanation for the increase in red cell mass during pregnancy is also obscure. Among other as yet unknown factors, the increased levels of erythropoietin circulating in pregnancy may be important in stimulating red cell production.

Benefits of Hypervolemia in Pregnancy

The increase in blood volume helps to maintain blood pressure in a vascular system of increased capacity, thereby protecting the mother and fetus from hypotension and reduced oxygen supply to the tissues. It also helps to compensate for blood loss at the time of delivery. The increase in red cell mass adequately provides for the increased oxygen requirement during pregnancy. The relatively greater increase in plasma volume compared with that of the red cell mass allows for a decrease in blood viscosity which, in turn, results in a decreased resistance to bloodflow and a decrease in cardiac work required to maintain the circulation. Further, because of

the 20% increase in basal metabolic rate during pregnancy, there is a need for dissipation of heat, which is achieved by the increased skin blood flow. In these circumstances, increased plasma volume is more important than any increase in cellular content of the blood. In addition, the increased renal bloodflow during pregnancy is required for increased excretion of waste products and here again the increase in blood volume requires that the plasma component, rather than the cellular component be increased.

Heart Rate

Heart rate at rest increases during pregnancy by an average of 15 beats per minute, typically from 70 to 85 beats per minute. It is significantly increased as early as 4–5 weeks after the first day of the last normal menstrual period (Clapp, 1985; Robson et al., 1989). The pattern of increase in heart rate is a gradual one from a median value of 79 beats per minute at 5–8 weeks, 83 beats per minute at 14 weeks, 87 beats per minute at 25 weeks, and 85 beats per minute at 35 weeks after the first day of the last normal menstrual period (Duvekot et al., 1993).

Cardiac Output

The cardiac output is the volume of blood expelled by either ventricle of the heart per unit of time, usually expressed as liters per minute. Normally the output of each ventricle is the same and averages 5.0 liters per minute in the non-pregnant subject at rest (Ganong, 1995). Earlier methods of measuring cardiac output were based upon the Fick principle which states that the amount of a substance taken up by an organ (or by the body) per unit time is equal to the arterial concentration of the substance minus its venous concentration (the arterio-venous difference) multiplied by the blood flow. This principle can be employed to determine cardiac output by measuring the amount of oxygen consumed by the body in a given time and dividing this value by the arterio-venous oxygen difference across the lungs. The arterial oxygen content can be obtained from a sample of blood collected from any artery and venous blood sample from the pulmonary artery is obtained by cardiac catheterization (Ganong, 1995).

A second commonly used method employed the Hamilton indicator dilution principle. A known amount of an indicator substance is injected intravenously and thereafter its concentration in serial samples of arterial blood is determined. The cardiac output is equal to the amount of indicator injected divided by its average concentration in arterial blood after its first circulation through the heart (Ganong, 1995).

Because of their invasiveness, these methods have been superseded by non-invasive methods employing ultrasound, namely echocardiography and Doppler blood flow measurements. Using these techniques, the velocity and volume of blood flow through the heart valves can be calculated. Doppler-determined flows

across the aortic, pulmonary, and mitral valves correlate closely with those obtained by the invasive measurements (Robson et al., 1989). Thus several independent estimates of cardiac output can be obtained from flow measurements made at different heart valves. Furthermore, cardiac chamber size and ventricular performance can be measured accurately by M-mode echocardiography (Murray et al., 1972).

Cardiac Output During Pregnancy

Cardiac output increases in early pregnancy (Walters et al., 1966), with this increase being significant five weeks after the last normal menstrual period (Capeless and Clapp, 1989; Robson et al., 1989; Duvekot et al., 1993). The increase continues until the end of the second trimester when the cardiac output is about 45% above pre-conceptional levels (Hunter and Robson, 1992). Thereafter, the cardiac output probably remains at much the same elevated level until term (Robson et al., 1989; Vered et al., 1991), although a recent study revealed a progressive increase in cardiac output until term (Mabie et al., 1994). The magnitude of the increase in cardiac output during pregnancy is of the order of 1.5–2.5 liters per minute (35–50%) (Robson et al., 1989; Easterling et al., 1990; Vered et al., 1991) (see Figure 2).

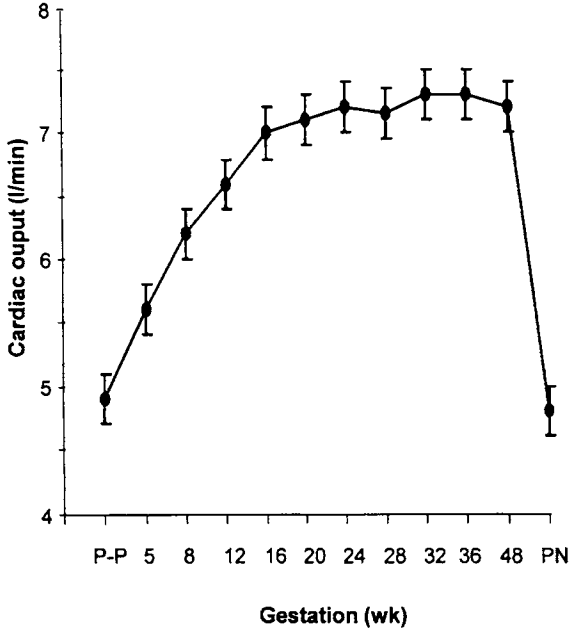

Figure 2. Cardiac output before, during, and after pregnancy. P-P = pre-pregnancy; PN = postnatal. From Hunter and Robson (1992).

Earlier studies of cardiac output in pregnancy found that it declined slightly after about 35 weeks gestation. Rather than being a true fall in cardiac output this was attributed to the supine position of the subjects, allowing the enlarged gravid uterus to fall back upon the inferior vena cava and reduce venous return to the heart (Lees et al., 1967). This matter is not settled, however, since some recent investigators have found a slight decline in cardiac output after 35 weeks gestation with subjects in a lateral position to avoid caval compression (McLennan et al., 1987; Easterling et al., 1990).

The increase in cardiac output during pregnancy appears to result from increases in heart rate and stroke volume together with a reduction in peripheral vascular resistance (see Figures 3 and 4). During the second trimester changes in chamber size suggest that stroke volume may be further augmented by increased venous return to the heart (Robson et al., 1989). In addition, there is some evidence to suggest that myocardial contractility is also increased during pregnancy. Systolic time intervals measured from simultaneous recordings of the electrocardiogram, the phonocardiogram, and the external carotid pulse have shown that the pre-injection period (the time during which the left ventricle is contracting before the aortic valve opens) is shortened during most of pregnancy indicating increased myocardial contractility. Along with this, the left ventricular ejection time (the time taken for the left ventricle to expel its contents) is somewhat increased due to the increased

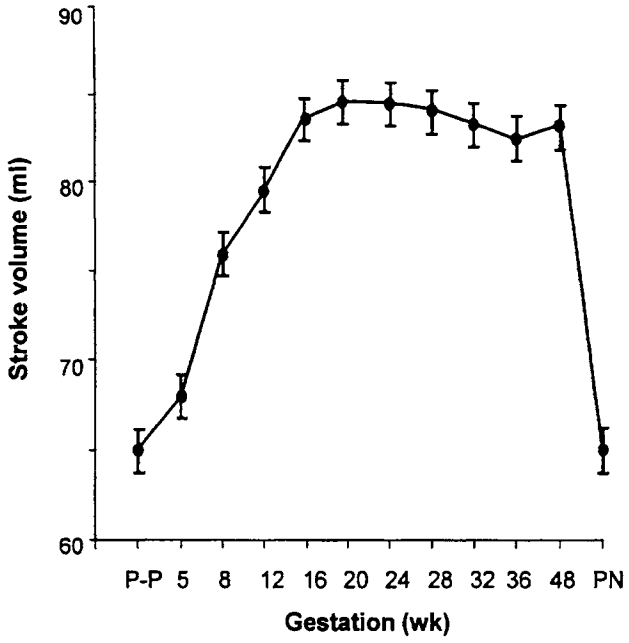

Figure 3. Stroke volume before, during, and after pregnancy. From Hunter and Robson (1992).

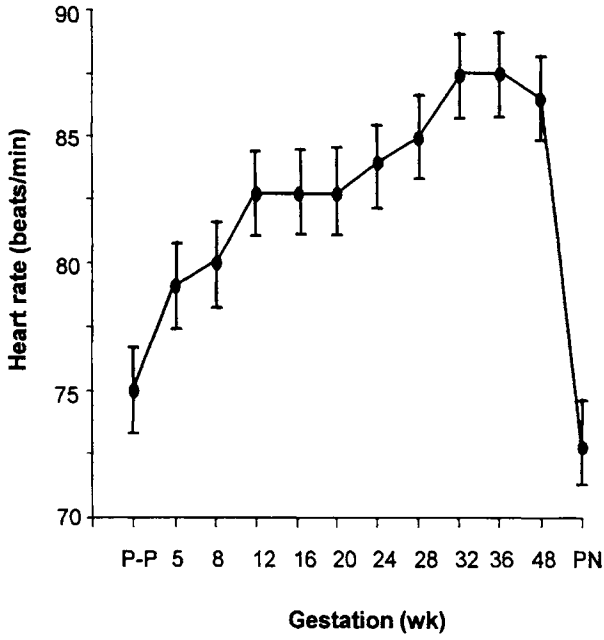

Figure 4. Heart rate before, during, and after pregnancy. From Hunter and Robson (1997).

stroke volume (Rubler et al., 1973; Burg et al., 1974; Walters and Lim, 1975). Furthermore, echocardiographic studies show an increase in the velocity of circumferential myocardial fiber shortening indicating increased myocardial contractility (Rubler et al., 1977).

Pregnancy is also accompanied by an increase in left ventricular wall thickness and mass during the second and third trimesters, indicative of myocardial hypertrophy (Robson et al., 1989). These observations are similar to those seen in athletes involved in isotonic exercise such as running (Lusiani et al., 1986). The analogy tempts one to suggest that pregnancy is comparable to a long period of exercise.

In summary, the increased cardiac output of pregnancy is accounted for by increases in heart rate, stroke volume, myocardial contractility, and venous return (pre-load) along with a decrease in peripheral vascular resistance (after-load). Overall, the hemodynamic changes accompanying pregnancy are consistent with the effects of chronic strain exerted on the heart and represent an obvious increase in its work output.

Cardiac Output in Labor

During the first stage of normal labor in women without epidural anesthesia, an increase in cardiac output of 12% between uterine contractions occurs. How-

ever, cardiac output during contractions increases progressively throughout labor: at the commencement of the second stage this increase is of the order of 34% (Hunter and Robson, 1992). Both an increase in stroke volume and heart rate are responsible for the increase in cardiac output during contractions (Hunter and Robson, 1992). In the first stage of labor a single uterine contraction can cause a transitory rise in cardiac output of as much as 2 liters per minute. This increase is mainly due to an increase in stroke volume consequent upon anxiety and pain, and perhaps partly due to an increased venous return caused by about 500 ml blood expelled from the uterus during a contraction (Hendricks and Quilligan, 1956; Ueland and Hansen, 1969a,b; Robson et al., 1987a). Both epidural and, to a greater extent, general anesthesia reduce the magnitude of the rise in cardiac output during labor (Robson et al., 1987a).

Maternal posture during labor also affects cardiac output, which is higher in the lateral than in the supine position between uterine contractions, with further increases during contractions (Ueland and Hansen, 1969a; Robson et al., 1987a). Heart rate increases after uterine contractions, as does blood pressure, which may show a mean rise of 35 mm Hg (systolic) and 25 mm Hg (diastolic) (Robson et al., 1987a).

Cardiac Output After Parturition

Immediately after delivery, cardiac output and stroke volume increase transiently to reach a maximum at 10–15 minutes post-partum and then decline to reach values seen before labor within one hour (Ueland and Hansen, 1969b; Robson et al., 1987b). Heart rate remains stable within the first few minutes after delivery and then gradually declines to approach normal pre-conceptional values by the tenth post-partum day (Robson et al., 1989). Left atrial diameter remains increased for 48 hours after delivery consistent with an increased venous return (Robson et al., 1987b). Subsequently stroke volume and cardiac output decrease rapidly in the first two weeks after delivery, thereafter continuing to decrease until 24 weeks post-partum before stabilizing (Robson et al., 1987b), indicating a normalization of circulating blood volume and pre-load.

At 12–24 weeks post-partum several hemodynamic variables have not reached a steady-state suggesting that not all cardiovascular parameters return to their pre-conceptional level, particularly stroke volume, cardiac output, and left ventricular dimensions; mild residual cardiac hypertrophy and lower cardiac contractility are seen when compared with a control group (Robson et al., 1987b).

Blood Pressure

During normal pregnancy systolic blood pressure does not change significantly whereas diastolic pressure decreases by about 10 mm Hg reaching a nadir in the middle trimester, and then rising to approach pre-pregnancy levels near term (Moutquin et al., 1985; Robson et al., 1989). Consequently mean arterial pressure is

lowest in the second trimester. Even by eight weeks gestation mean arterial pressure is decreased relative to pre-conceptional blood pressure (Clapp et al., 1988).

Indirect sphygmomanometric measurement of blood pressure in pregnancy is customarily taken with the subject in the sitting position with the arm at heart level. In the supine position blood pressure may fall because of compression of the inferior vena cava by the gravid uterus and a reduction in venous return to the heart. With the subject lying on the right or left side, the blood pressure is lowest (Wichman et al., 1984). The fall in arterial blood pressure (systolic 10%; diastolic 20–30%) when the patient lies on her side may be due to release of aortic compression by the enlarged uterus (Trower and Walters, 1968).

In pregnancy there is closer correlation with intra-arterial diastolic pressure when phase V rather than phase IV of the Korotkoff sounds is taken as the diastolic pressure (Johenning and Barron, 1992). Ginsburg and Duncan (1969) showed that in pregnancy, sphygmomanometry over-estimated systolic pressure by 7 mm Hg, and diastolic pressure by 12 mm Hg compared with direct intra-arterial measurement.

Peripheral Resistance

The peripheral vascular resistance is maintained by the arteriolar tone and calculated by dividing the mean arterial blood pressure by the cardiac output. In mid-pregnancy, total peripheral resistance (979–987 dynes/sec/cm^{-5}) is much below that in the non-pregnant state (1,700 dynes/sec/cm^{-5}). It rises gradually after 24 weeks to approach normal pre-conceptional levels but is still below these levels at 1,200–1,300 dynes/sec/cm^{-5} (Bader et al., 1955; Pyörälä, 1966).

The lowering of peripheral resistance is due to opening up of new vascular beds and a reduction in vascular smooth muscle tone. Even by five weeks gestation systemic vascular resistance has decreased significantly below pre-conceptional levels (Capeless et al., 1989; Robson et al., 1989; Duvekot et al., 1993). In fact systemic vascular resistance has already decreased during the late luteal phase of the menstrual cycle in women who conceive in that cycle (Veille et al., 1986). Thus it would appear that a lowering of systemic vascular resistance represents one of the earliest maternal adaptations to pregnancy. Its cause is obscure but one or more circulating vasodilator agents has been postulated (Schrier and Briner, 1991).

During pregnancy the pressor response to angiotensin II is reduced compared to the response in non-pregnant women (Gant et al., 1973) due to altered responsiveness of the arterioles (Gant et al., 1974). Autonomic ganglionic blockade in pregnant women compared with non-pregnant women results in a much greater fall in systolic and diastolic blood pressure in the former which increases as pregnancy advances (Assali et al., 1952), indicating that vascular tone is much more dependent on sympathetic nervous system control during pregnancy than in the non-pregnant state. This suggests that the autonomic nervous system may be attempting to compensate for the action of one or more circulating vasodilator agents in pregnancy,

whose unopposed influence could lead to hazardous maternal hypotension (de Swiet, 1991).

Venous Pressure and Distensibility

Venous pressure in the upper limbs is not altered during pregnancy but it is increased in veins of both lower limbs (McLennan, 1943). However, central venous pressure and right atrial pressure are not raised in pregnancy (Hamilton, 1949; Colditz and Josey, 1970). The rise in femoral venous pressure is due to venous obstruction caused by the enlarged gravid uterus compressing the abdominal part of the inferior vena cava, especially when the subject is lying supine. In addition, blood at relatively high pressure leaves the uterus particularly when a contraction occurs and this causes interference hemodynamically with femoral venous blood flow (Palmer and Walker, 1949). Furthermore, the rate of blood flow is reduced in the leg veins (Wright et al., 1950). Venous distensibility may also increase in veins of both upper and lower limbs during pregnancy (Goodrich and Wood, 1964) although this is disputed by other workers (Duncan, 1967; Sandström, 1974).

Regional Bloodflow

Uterine Bloodflow

Measurement of uterine bloodflow is technically difficult. All methods have their drawbacks and as far as the human is concerned involve hazards which are now regarded as ethically unacceptable. Therefore, the reliability of available data is questionable and, at best, provides only estimates of uterine bloodflow.

Assali et al. (1960) measured uterine bloodflow in 12 women between 10 and 28 weeks gestation at hysterotomy using electromagnetic flowmeter and nitrous oxide methods. The uterine bloodflow increased from about 70 ml per minute in the first trimester to about 200 ml per minute at 28 weeks gestation. In another study Romney et al. (1955) found that uterine bloodflow at term in human pregnancies was of the order of 500 ml per minute. However, Huckabee (1962) suggested that the figure was somewhat higher, around 700–800 ml per minute. Recent studies using the non-invasive method of Doppler ultrasound have shown that uterine artery bloodflow increases during pregnancy as demonstrated by an increasing diameter of the vessel and an increased flow velocity (Palmer et al., 1992). The latter workers found that uterine artery bloodflow increased as a consequence of increased common iliac artery flow and redistribution of pelvic flow from the external iliac to the uterine artery. Approximately half the increase was present by the twenty-first week of gestation and was due in equal proportions to increases in the uterine artery diameter and the mean flow velocity. A further increase in uterine artery bloodflow was found at 30–36 weeks gestation. This appeared to be mainly due to a faster uterine artery mean flow velocity. The increase in uterine artery and decrease in exter-

nal iliac artery volumetric flow implies that the redistribution of common iliac artery flow favors the uterine circulation at the expense of the external iliac (lower limb) vascular bed.

Renal Bloodflow

Effective renal plasma flow, which is approximately equal to total renal plasma flow, increases by 70–80% from about 1,200 ml per minute to 1,600 ml per minute between conception and 26 weeks gestation and then declines to a value which is still 50–60% above pre-conceptional levels near term (Dunlop, 1980). This decline in late pregnancy cannot be attributed solely to the supine position as it is still observed when subjects are in the lateral recumbent position (Ezimokhai et al., 1981).

The mechanism responsible for the rise in effective renal plasma flow and glomerular filtration rate is unknown. The trophoblast is not essential as similar changes have been observed in pseudo-pregnancy (Baylis, 1982). It is thought that the increase in glomerular filtration rate precedes that in blood volume and represents the renal expression of a primary generalized vasodilatation (Davison, 1987). The underlying mechanism of this vasodilatation remains obscure. After delivery, the glomerular filtration rate remains elevated for about a week and returns to pre-conceptional levels within one month (Krutzen et al., 1992).

Pulmonary Bloodflow

Robson et al. (1991) observed serial changes in pulmonary bloodflow in 13 women before conception, during pregnancy, and six months after delivery using the method of Doppler echocardiography. The mean pulmonary artery pressure in the pre-conceptional state was 13.8 mm Hg and no significant change was seen in pregnancy. Pulmonary bloodflow increased from 4.88 to 7.19 liters per minute during pregnancy. In addition, pulmonary vascular resistance decreased from 2.85 resistance units before pregnancy to 2.17 resistance units at eight weeks gestation. Thereafter there was no significant change with values returning to pre-conceptional levels by six months after delivery (Robson et al., 1991). Thus, the marked increase in pulmonary bloodflow does not lead to an increase in pressure. Indeed, this is in keeping with the known ability of the pulmonary vasculature to contain high bloodflow rates without any rise in pressure. Guyton et al. (1976) showed that the pulmonary vascular bed can sustain a fourfold increase in bloodflow above normal without any increase in pulmonary vascular pressure. The only way in which this can be achieved is for resistance to flow to decrease. As the pressure in the right ventricle, pulmonary artery, and pulmonary capillaries remains at the normal preconceptional level throughout pregnancy (Angelino et al., 1954; Bader et al., 1955), despite the markedly increased pulmonary bloodflow, there must be a reduction in pulmonary vascular resistance, probably achieved by vasodilatation and also possibly by the opening up of more capillaries.

Hepatic Bloodflow

It is not yet entirely clear whether hepatic bloodflow increases in pregnancy. Earlier workers found no change (Munnell and Taylor, 1947; Laakso et al., 1971) whereas Tindall (1975) found that hepatic bloodflow increased from 800 ml per minute in the non-pregnant to 1,400 ml per minute during normal pregnancy. On the other hand, a more recent study by Robson et al. (1990) using indocyanine green clearance and Doppler echocardiography at the aortic valve found no significant change in hepatic bloodflow during pregnancy.

Cerebral Bloodflow

Very few data on cerebral bloodflow in human pregnancy are available. Using the Fick principle and nitrous oxide in nine young gravidae between 34 and 40 weeks gestation, McCall (1949) found no difference in cerebral bloodflow between the pregnant women and normal men. Recently, Ikeda and Mori (1990) used Doppler velocimetry to assess internal carotid artery bloodflow in normal pregnant women and found that peak systolic and mean velocities in the first and second trimesters of pregnancy were almost the same as those in non-pregnant women, whereas they became slower in the third trimester.

In another study maternal middle cerebral artery bloodflow velocity was assessed transcranially in normal pregnant women using a 2 MHz pulsed probe (Williams and Wilson, 1994). Maximum systolic velocity fell significantly in the third trimester to its lowest value of 62 cm per second, whereas minimum diastolic velocity (28 cm per second) did not change with gestational age. Mean velocity fell significantly between 25 and 36 weeks gestation. It was concluded that systolic and mean maternal middle cerebral artery bloodflow velocity fell significantly with advancing gestational age even though diastolic velocity did not vary. These changes did not correlate with mean arterial pressure changes related to gestational age. The reasons for the changes in middle cerebral artery bloodflow velocity in late gestation are unknown. The decline in the third trimester may occur in response to a variety of chemical and neurological stimuli.

A recent study of maternal regional cerebral bloodflow found that it increased in early pregnancy between 7 and 19 weeks gestation compared with the non-pregnant state, the only exception being the bloodflow to the occipital lobes, which remained constant (Ikeda et al., 1993).

Skin Bloodflow

Bloodflow to the hand is increased six- or sevenfold during pregnancy (Burt, 1950; Ginsburg and Duncan, 1967) resulting in an increased skin temperature in the fingers (Burt, 1949; Herbert et al., 1958). Forearm bloodflow also increases slightly during pregnancy, probably due to an increase in forearm skin bloodflow, the mus-

cle bloodflow remaining constant, (Spetz and Jansson, 1969; MacGregor and Snodgrass, 1970). Bloodflow in the foot increases threefold (Ginsburg and Duncan, 1967) while bloodflow in the leg probably increases slightly (Herbert et al., 1958; Ginsburg and Duncan, 1967), similar to that in the forearm, again probably due largely to an increase in skin bloodflow. Bloodflow to the breast is also increased in pregnancy as can be seen by the distended veins which develop in the skin over the breast as pregnancy progresses. However, measurement of bloodflow to the human breast during pregnancy has not been reported.

Initiation of the Cardiovascular Changes in Pregnancy

Studies in early human and baboon pregnancy (Phippard et al., 1986) suggest that the cardiovascular changes are initiated by generalized vascular relaxation affecting both arterial and venous systems. The fall in arterial pressure and after-loads leads to a rise in cardiac output primarily through an increase in heart rate. The increased vascular capacitance activates volume retaining mechanisms such as the renin angiotensin axis and the osmoregulatory system. The induced volume retention enhances pre-load and stroke volume.

Vasorelaxation in pregnancy is now thought to be due to a circulating vasodilator substance, perhaps nitric oxide. The release of this autacoid from the endothelium is stimulated by calcitonin gene related peptide which is distributed in perivascular nerves, and plasma levels of this peptide have been found to increase throughout pregnancy (Stevenson et al., 1986). Presumably there are as yet unknown processes at the decidua-trophoblast interface which stimulate the release of the vasodilating agent (Duvekot and Peeters, 1994).

It is interesting to note that the cardiovascular system can be seen as a target tissue for estrogen. Estrogen receptors have been discovered in the cardiovascular system of humans (Karas et al., 1994; Losordo et al., 1994), as have progesterone receptors (Ingegno et al., 1988). Estrogen may play a significant role in regulating cardiovascular cell function (Lin and Shain, 1985). Furthermore, estrogen may exert a positive inotropic effect, possibly by improving cardiovascular biomechanics (Pines et al., 1992). It has long been recognized that estrogen administered to women (Walters and Lim, 1969, 1970; Lehtovirta, 1974) and to men (Slater et al., 1986) increases blood volume, cardiac output, and stroke volume, changes analogous to those seen in pregnancy when blood estrogen levels are increased. Furthermore, studies suggest that estradiol enhances vasodilatation by stimulating endothelial cell nitric oxide and prostacyclin production and by altering vascular smooth muscle cell responses to vasoconstrictor agents (White et al., 1995).

THE RESPIRATORY SYSTEM

Clearly the transport of oxygen to the tissues of the body is dependent on the adequacy of lung function, the efficiency of the circulation and the oxygen carrying capacity of

the circulating blood. Hence, functional changes that occur in the respiratory system should be considered along with the changes that occur in the circulatory system if the altered function of both systems during pregnancy is to be fully understood.

Lung Volumes

Vital capacity, which is the maximum volume of gas that can be expired after a maximum inspiration including all but the residual volume of gas remaining in the lungs (excluding the anatomical dead space of the large air passages), increases slightly by 100–200 ml during pregnancy (de Swiet, 1991). Between 80–85% of the vital capacity can be forcefully expired in one second (FEV_1) in non-pregnant women and this is not affected by pregnancy (Cameron et al., 1970).

The inspiratory capacity or maximum volume of gas which can be inspired from the resting end-expiratory position also increases progressively throughout pregnancy to reach about 300 ml in the last trimester (Knuttgen and Emerson, 1974). On the other hand, the expiratory reserve volume, which is the maximum amount of gas which can be expired from the resting end-expiratory position, is progressively reduced as pregnancy advances, the maximal reduction being approximately 200 ml (Knuttgen and Emerson, 1974). Likewise, the residual volume of gas remaining in the lungs at the end of maximal expiration is progressively reduced during pregnancy by about 300 ml (Knuttgen and Emerson, 1974). It follows that the functional residual capacity, comprising the residual volume plus the expiratory reserve volume, is reduced by about 500 ml during pregnancy.

The volume of gas inspired or expired in each respiration at rest, the tidal volume, increases throughout pregnancy from 560 ml before conception to 715 ml at term (Spätling et al., 1992). By 6–8 weeks postpartum it has resumed to preconceptional levels. As the respiratory rate of 14–15 respirations per minute does not change significantly during pregnancy, the minute ventilation increases with tidal volume from pre-conceptional levels of about 7.5 liters per minute to 10.5 liters per minute near term (Pernoll et al., 1975).

Alveolar ventilation represents ventilation at the point of gas exchange in the pulmonary alveoli. As early as 8–11 weeks gestation it has increased significantly by 80% to 6.2 liters per minute, thereafter remaining at much the same level throughout pregnancy (Spätling et al., 1992). It then decreases after delivery to 4.5 liters per minute by the sixth postpartum week and to 3.4 liters per minute 12 months after delivery (Spätling et al., 1992).

While mixing and distribution of gas in the lungs is more efficient in pregnant than in non-pregnant women (Cugell et al., 1953), gas transfer (pulmonary diffusing capacity) declines during pregnancy from pre-conceptional levels of 26.5 ml/min/mm Hg to 22.5 ml/min/mm Hg in late pregnancy (Lehman and Fabel, 1973). This reduction in gas transfer may be due partly to the lower hemoglobin concentration in circulating blood during pregnancy and partly to a hormonally induced alteration in the alveolar capillary wall (Gazioglu et al., 1970).

A flaring of the lower ribs begins early in pregnancy resulting in an increase in the subcostal angle from about 68° before conception to 103° in the third trimester (Thomson and Cohen, 1938). In addition the level of the diaphragm rises by a maximum of 4 cm and the transverse diameter of the chest increases by about 2 cm during pregnancy (Thomson and Cohen, 1938). At the same time, diaphragmatic excursion increases during respiration, indicating that breathing during pregnancy is more diaphragmatic than costal (Möbius, 1961)

Oxygen Consumption

Both basal oxygen consumption and oxygen consumption at rest increase during pregnancy, the magnitude of this increase being in some doubt but probably of the order of 40–120 ml per minute above that in the non-pregnant state (Pernoll et al., 1975; Spätling et al., 1992). Oxygen consumption increases significantly by as early as seven weeks gestation (Clapp et al., 1988).

Arterial Blood Gases

P_{CO_2}

Overbreathing is common in pregnancy and is thought to be due to a direct action of progesterone on the respiratory center. It leads to carbon dioxide being washed out of the lungs resulting in a lower concentration of carbon dioxide in the pulmonary alveoli and arterial blood than in the non-pregnant state. In late pregnancy the arterial P_{CO_2} is about 30 mm Hg, some 5–10 mm Hg lower than in non-pregnant women (Bouterline-Young and Bouterline-Young, 1956). The lowest arterial P_{CO_2} is found in the first trimester of pregnancy (Spätling et al., 1992). Indeed the decline in arterial P_{CO_2} is apparent in the luteal phase of the menstrual cycle before the fertilized ovum imbeds (Goodland and Pommerenke, 1952).

The reduction in arterial P_{CO_2} during pregnancy has been attributed to progesterone, which may stimulate the respiratory center leading to hyperventilation (Goodland et al., 1953). Exogenous progesterone administered to normal subjects stimulates ventilation (Skatrud et al., 1978). The mechanism whereby progesterone induces hyperventilation is not known but Bayliss and Millhorn (1992) suggest that it acts via progesterone receptors in the hypothalamus. The communicating link between the hypothalamus and the respiratory center in the medulla has yet to be discovered. In addition progesterone increases the level of carbonic anhydrase B in red cells, which facilitates transfer of carbon dioxide and tends to decrease arterial P_{CO_2} (Paciorek and Spencer, 1980).

P_{O_2}

Serial measurements of arterial P_{O_2} throughout pregnancy have revealed that mean values increase slightly, although variability between subjects is evident. The

arterial P_{O_2} is of the order of 106–108 mm Hg in the first trimester and 101–104 mm Hg in the third trimester (Andersen et al., 1969). The alveolar-arterial P_{O_2} gradient may also be slightly increased during pregnancy (Awe et al., 1979). This may tend to counteract the increase in arterial P_{O_2}. Both arterial P_{O_2} and the alveolar-arterial P_{O_2} gradient are influenced by the posture of the subject during pregnancy. In one study in late pregnancy a change in posture from the sitting to supine position was accompanied by a 13 mm Hg decrease in capillary P_{O_2} (Ang et al., 1969). Furthermore, with a similar change in posture, the alveolar-arterial P_{O_2} gradient has been found to increase from 14.3 to 20 mm Hg (Awe et al., 1979).

Acid–Base Status

Maternal arterial pH is not altered significantly during pregnancy, measuring about 7.40 units. The gestational decline in arterial P_{CO_2} is accompanied by an equivalent fall in plasma bicarbonate concentration, secondary to its renal excretion to compensate for the alkalosis resulting from hyperventilation.

Maternal Exercise

The physiological impact of exercise in pregnancy can be considered in terms of whether or not it costs more or is performed less efficiently than in the non-pregnant state. Any exercise which involves lifting the weight of the body will incur an inevitable extra cost during pregnancy due to increasing body weight. In most studies, only exercise involving lifting the body weight (e.g., treadmill walking) increases oxygen consumption, the increase being proportional to the increase in body weight (Knuttgen and Emerson, 1974; Hutchinson et al., 1981). By contrast, in most but not all studies, non-weight bearing exercise (e.g., cycling) has not been found to increase oxygen consumption.

In late pregnancy, the respiratory response to exercise reveals that pregnant women have a more rapid approach to the steady-state minute ventilation at the beginning of exercise; only 10 seconds is required to reach the steady-state ventilatory response during the third trimester (Edwards et al., 1981). Because the relative increase in cardiac output during exercise exceeds that of oxygen consumption, the arterio-venous oxygen difference at each work load is wider during pregnancy than in the non-pregnant state. This suggests that the supply of oxygen to the periphery is less efficient in pregnant compared with non-pregnant women (Veille et al., 1992).

During pregnancy, an increase in venous capacity induces an early increase in plasma volume along with a more gradual increase in red cell mass. When a regular exercise regimen is undertaken during pregnancy total blood volume increases by an additional 8–12% (Clapp et al., 1992). This may help to maintain uteroplacental blood flow during exercise when there is a redistribution of blood flow away from the visceral circulation to that of the skeletal musculature (Clapp et al., 1992).

Among pregnant women exercising regularly, the overall level of exercise performance decreases significantly with advancing gestation (Clapp and Capeless, 1991). In effect, this means that the overall physiological stress associated with exercise is reduced during pregnancy, thereby reducing the risk of any adverse effect on mother and fetus. Doppler ultrasound measurement of systolic-diastolic velocity ratios in umbilical and uterine arteries in 13 healthy women at around 29 weeks gestation undergoing exercise revealed no significant change compared with pre-exercise measurements (Allen et al., 1991).

While there is no good evidence to suggest that physical exercise in normal pregnancy has any adverse effects on pregnancy outcome (Erkkola, 1976), in abnormal pregnancies strenuous exercise may cause fetal heart rate accelerations or decelerations. Such fetal heart rate changes may indicate reduced blood or oxygen supply to the fetus and are associated with a higher risk of fetal distress in labor (Pomerance et al., 1974). Generally exercise has a beneficial effect during normal pregnancy and is to be encouraged in moderation. A healthy fetus is able to compensate for periods of transitory stress that may occur during maternal exercise (Schick-Boschetto and Rose, 1992).

EARLY PREGNANCY ADAPTATION

On the basis of a detailed study of 10 women in early pregnancy, Duvekot and colleagues (1993) hypothesize that an as yet unidentified endocrine stimulus initiates a widespread decrease in vascular tone leading to systemic vasodilatation and an increase in arterial compliance. Consequently, afterload falls stimulating an increase in stroke volume. The reduced vascular tone and accompanying vasodilatation may effect down-regulation of the baroreceptors and non-osmotic release of vasopressin. The resulting hemodilution renders the blood less viscous thereby potentiating the lowered vascular resistance. The overall result of these changes is the hyperkinetic circulation of early pregnancy characterized by a high rate flow and low resistance. The vasodilatation of afferent and efferent renal vessels and the increased renal blood flow secondary to an increase in cardiac output induce an increase in the glomerular filtration rate. The increase in left atrial diameter and the fall in plasma renin concentration between the fifth to eighth week of pregnancy and the leveling out of the glomerular filtration rate after the eighth week lend support to an increase in the vascular filling state, which might well be a compensatory development to the pre-existing hypovolemia.

The findings of Duvekot and colleagues (1993) strongly suggest that the changes in volume homeostasis and renal function in early pregnancy develop mostly secondary to the adaptative changes in maternal hemodynamics rather than independently in response to the same or a different stimulus. The same workers emphasized that the most important maternal adaptations to pregnancy occur in the first eight weeks.

There is good evidence to suggest that significant maternal physiological adaptations to pregnancy occur in several systems during the embryonic period (the first eight weeks of pregnancy) before they are functionally necessary. For example, in the serial study of 20 normal pregnant women by Clapp et al., (1988), significant changes were observed in body composition, cardiopulmonary function, and metabolism by the seventh week of gestation. Body fat increased 2%, plasma volume 11%, heart rate 16%, minute ventilation 24%, and oxygen consumption 10%, while mean arterial pressure fell 9%. Clapp et al., (1988) also found that in three of the pregnancies in their prospective serial study which later spontaneously aborted, no significant changes from the pre-conceptional values were observed in any parameter at the seventh week of gestation indicating failure of normal adaptation to pregnancy. The same workers hypothesized that quantitative aspects of one or more of these physiological parameters may be diagnostic of normal pregnancy and that any significant departure from these values could be diagnostic of abnormality.

In another serial study of eight normal pregnant women before and after conception by Capeless and Clapp (1989), cardiac output increased by about 1 liter minute at eight weeks gestation, representing greater than 50% of the total change seen during pregnancy (see Table 2). The increase in cardiac output was largely due to an increased stroke volume rather than to an increase in heart rate. By eight weeks gestation systemic vascular resistance had fallen to 70% of its pre-conceptional level. A statistically significant increase in heart rate did not occur until 16 weeks gestation.

Longo (1983) postulated that maternal blood volume and cardiac output are hormonally controlled by steroidogenesis in the fetal compartment which influences

Table 2. Cardiovascular Measurements before Pregnancy and during Early Pregnancy in Women [a,b]

	Before pregnancy	*8 Weeks Gestation*	*16 Weeks Gestation*	*24 Weeks Gestation*
Heart rate (beats per minute)	65 ± 3	68 ± 4	72 ± 3	73 ± 3
Cardiac output (liters per minute)	42 ± 0.4	5.2 ± 0.3	5.9 ± 0.4	5.7 ± 0.3
Stroke volume (millliliters)	65 ± 5	79 ± 5	83 ± 6	81 ± 5
Mean arterial pressure (mm Hg)	69 ± 3	62 ± 4	69 ± 2	67 ± 4
Systemic vascular (dynes. sec. cm^{-5})	1376 ± 143	969 ± 76	926 ± 68	930 ± 82

Notes: [a] Means ± standard error of the mean.
[b] From Capeless and Clapp (1989).

the maternal environment. The early onset of the changes, however, favor a hormonal mechanism which is more likely to be primarily maternal in origin. Spätling et al., (1992) pointed out that it is futile to attribute the changes to any or several of the hormones known to increase in pregnancy as there is no simple quantitative relationship between their concentrations and the cardio-pulmonary responses. Rather, they suggest that adaptation to pregnancy is caused by an intricate resetting of the hormonal balance which changes continuously as pregnancy progresses.

SUMMARY

The maternal circulatory and, to a lesser extent, the respiratory systems undergo significant changes during pregnancy, highlighted by a fall in total peripheral vascular resistance, an increase in blood volume and cardiac output, and increases in various regional bloodflows especially to the uterus, lungs, kidneys, and skin. The volume of air breathed per minute increases during pregnancy with an increase in alveolar ventilation. Oxygen consumption increases and the overall level of exercise performance decreases with advancing gestation. Many of these changes are apparent in the first few weeks of pregnancy during the embryonic period of development and would appear to be preparatory to a successful pregnancy. The earliest change is a reduction in systemic vascular tone. Its cause is unknown but is thought to be associated with one or more circulating vasodilator agents, possibly originating in the trophoblast. Whatever their cause, the various circulatory and respiratory changes occurring in the maternal organism during pregnancy are clearly designed to facilitate optimal conditions for fetal growth and development.

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Chapter 11

Amniotic Dynamics

ROBERT A. BRACE and MICHAEL G. ROSS

INTRODUCTION

Throughout the period of *in utero* development, the embryo and fetus are surrounded by amniotic fluid and this, in turn, is surrounded by the amniotic and chorionic membranes. In order for the fetus to develop normally, it is essential that

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amniotic fluid volume be within its normal range and perhaps of normal composition. The present review provides an overview of the amniotic fluid by addressing the following questions: What is the source of amniotic fluid? How does amniotic fluid volume change with fetal gestational age? What is the composition of amniotic fluid? What regulates both amniotic fluid volume and composition? What fetal abnormalities and pathologies are associated with abnormalities in the amniotic fluid?

Amniotic fluid is present during the embryonic period as well as during the fetal period. Hence, it is important to recall the meaning of these terms. The embryonic period is defined as the time after the long axis of the fetus appears until all major structures are represented. In humans, this corresponds to about two weeks after conception to the end of the seventh week. Thus, with the eighth week post-conception, the embryo is considered a fetus. However, fetal age as well as the length of gestation is timed not relative to conception but rather to the first day of the last menstrual cycle. Thus, at the end of the embryonic period, the fetus is in its tenth week of gestation.

As the amnion and chorion are forming during the embryonic period, two separate fluid sacs develop. First, amniotic fluid surrounds the embryo and is enclosed by the amniotic membrane. Second, coelomic fluid fills the space between the amnion and chorion, as illustrated in Figure 1. However, the coelomic fluid is only transitory as it completely disappears by 10–12 weeks of gestation. In contrast, the amniotic fluid volume expands throughout this period, a process which is critical for normal fetal development. This fluid which surrounds the fetus provides many important benefits to the fetus. First, it not only provides a space so that fetal movement can occur, but it also protects the fetus by providing a cushion to reduce external forces which may damage the fetus. Amniotic fluid also provides an important protection for the umbilical cord, preventing compression of the cord, and thus umbilical blood vessels, between the fetal body and uterine wall. By mechanisms which are not yet fully understood, the presence of normal amounts of amniotic fluid also promote the development and maturation of the fetal lungs. In contrast, with abnormally low volumes of amniotic fluid, the fetal lungs do not develop normally but instead remain hypoplastic and are unable to adequately ventilate the newborn at birth. In addition to the problems resulting from diminished amniotic fluid volume (oligohydramnios), excess amniotic fluid (polyhydramnios) also is associated with a variety of problems. For example, polyhydramnios not only makes it difficult for the pregnant woman to breath because the diaphragm is reflected upward, but also is associated with preterm delivery of the fetus. Finally, there are a large number of fetal diseases and genetic malformations associated with either too little or too much amniotic fluid (Liley, 1972; Brace et al., 1993). Thus, the maintenance of a normal progression of amniotic fluid volumes throughout gestation is critical for successful outcome for pregnancy.

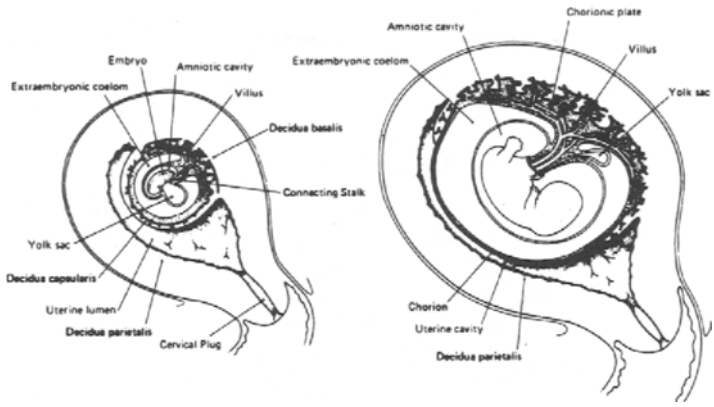

Figure 1. An illustration of the amniotic and coelomic fluid compartments in the embryonic and early fetal periods. From Faber and Thornburg (1984) Placental Physiology, Raven Press, New York.

AMNIOTIC FLUID SOURCES

There are multiple sources of amniotic fluid and the relative contribution of each source changes throughout pregnancy. During the embryonic period, the source of the amniotic fluid is thought to be transudation of fluid through the highly permeable embryonic skin and the fetal surface of the placenta, as well as transfer into the amniotic compartment across the amniotic membrane. During the embryonic period, the placenta is larger than the fetus and a large surface area of the placenta is in contact with the amniotic space. Thus, with fetal capillaries just below the fetal surface of the placenta, it is highly likely that the surface of the placenta is an important contributor to amniotic fluid during the embryonic period. At the end of the embryonic period and the beginning of the fetal period, there are major changes in the source of amniotic fluid. That is, at 9–11 weeks of gestation fetal urine first enters the amniotic compartment. At approximately the same time, the fetus begins to swallow amniotic fluid and the fetal lungs begin to secrete pulmonary liquid around mid-gestation (Abramovich, 1978, 1981; Brace. 1986; Brans, 1988; Brace et al., 1993). Although some of the secreted pulmonary fluid remains in the lung with expansion, a large portion of the fluid secreted by the lungs exits via the fetal trachea and is either swallowed or enters the amniotic compartment. Approximately half of the fluid that exits the lungs through the trachea is swallowed and half enters the amniotic compartment (Brace, 1995). Thus, by the early portion of the fetal period, all the major routes for fluid movement into and out of the amniotic compartment have begun to function. These routes are illustrated in Figure 2. At midgestation, there is an additional change which

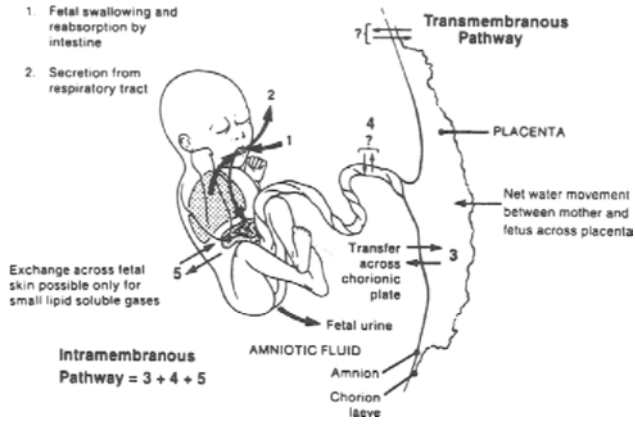

Figure 2. Pathways for fluid movement into and out of the amniotic space. From Brace (1995).

may have significant effects on amniotic fluid composition. That is, at approximately 23–24 weeks gestation, the fetal skin keratinizes and becomes impermeable to all substances except those with high lipid solubility such as CO_2.

Most studies of amniotic fluid volume and fluid fluxes have been conducted near-term and Figure 3 summarizes current estimates of amniotic flows in the near-term, healthy fetus (Gilbert et al., 1991; Brace, 1995). Presently, a similar graph for fetuses early in gestation cannot be made. There are several reasons for this. First, there is very little information available on fluxes which occur during the embryonic and early fetal period. Even in animal models, there is little data available from the first half of gestation. A second factor is that even in late gestation, it has been hard to quantify the fluxes which occur across either the fetal surface of the placenta or across the combined amnion and chorion between the maternal uterine wall and the amniotic fluid. The latter is difficult to quantify not only because of methodological limitations, but also because of the fact that fluxes across the placental surface and membranes may be small relative to the fluxes that occur due to fetal urinary excretion as well as swallowing and secretion from the fetal lungs. Thus, the relative magnitude of these fluxes make them difficult to study. Third, it has often been taught that, rather than secreting fluid, the fetal lungs absorb amniotic fluid. Over the past 25 years, the fluid secretion process of the lungs has been extensively studied. It is well established that the fetal lungs normally secrete a volume of fluid equal to 10% of fetal body weight per day over the last third of gestation and that the entry of amniotic fluid into the fetal lungs may occur during gasping in response to conditions of severe fetal distress such as asphyxia (Harding, 1994). Thus, during postmortem exams, it may not be unusual to find amniotic fluid within the fluid of the lungs although this would not normally occur in the healthy fetus.

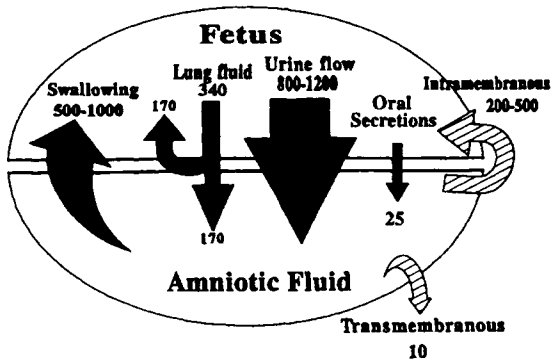

Figure 3. Current best estimates of flows into and out of the amniotic compartment in the near-term fetus. Arrows are proportional in size to flow rates. Solid arrows are measured flows and shaded arrows are estimated flows. From Brace (1995).

AMNIOTIC FLUID VOLUME

Because of the importance of amniotic fluid for the normal development of the fetus, amniotic fluid volume has been measured in a large number of pregnant women (Brace and Wolf, 1989). Figure 4 shows the average changes in amniotic volume as determined in over 700 measurements in women with normal fetuses. Note that, on average, amniotic fluid volume increases from approximately 20 ml at 10 weeks of gestation to 800 ml at 30 weeks of gestation. From 30 to 39 weeks, no statistically significant changes in amniotic fluid volume occur. However, amniotic fluid volume begins to decrease following 40 weeks and is often markedly reduced in post-term pregnancies (i.e., pregnancies that go beyond 42 weeks gestation). In individual cases the decrease can be severe, potentially resulting in cord compression and fetal death.

Although the above characterizes the average changes in amniotic fluid volume which occur, it is important to recognize two other facets of amniotic dynamics. First, in individual women, there is a wide range of amniotic fluid volumes. For example, in Figure 4, the 95% confidence interval at 40 weeks gestation covers the range from approximately 250 ml to 1,700 ml. The range of variance is even greater at 33 weeks of gestation, extending from approximately 300 ml to in excess of 2,000 ml. Patients with fluid volumes near or outside these ranges have an increased rate of perinatal complications. Second, in addition to the differences in amniotic volumes among women, within any individual pregnancy, there may be surprisingly large day-to-day variations in amniotic volume. This observation suggests that short-term variations in amniotic volume may be due to acute changes in the state of hydration of the mother. This most likely occurs via transfer of fetal and

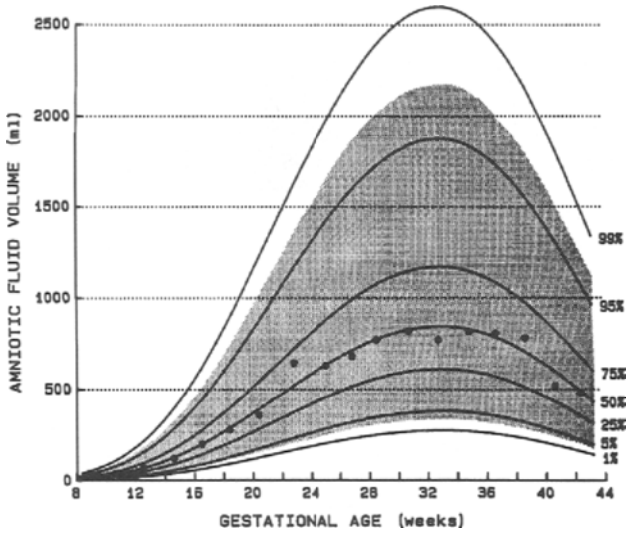

Figure 4. Average changes in amniotic fluid volume across gestation. Data from 705 measurements and each dot represents two-week mean values. The shaded area is the 95% confidence interval about the mean and individual confidence intervals as listed. From Brace and Wolf (1989).

maternal fluids across the placenta with secondary transfer between the fetus and the amniotic compartment as described below.

AMNIOTIC FLUID COMPOSITION

Essentially all of the constituents that are present in plasma are present in amniotic fluid. However, plasma and amniotic concentrations differ and there are major changes in amniotic fluid composition that occur throughout gestation (Liley, 1972; Seeds, 1980; Brace et al., 1989). These changes in composition most likely reflect the changes in the relative contribution of each of the individual sources to the amniotic compartment. For example, Figure 5 compares the changes in amniotic osmolality across gestation with maternal plasma osmolality. Note that osmolality is roughly equal to the total concentration (actually, the sum of individual chemical activities) of all solutes which are present in the amniotic fluid. When interpreting the curves in this Figure, note that plasma osmolality in women during pregnancy is decreased by about 10 mOsm/kg of water from the level in nonpregnant women of approximately 290 mOsm/kg. Recent research suggests that this decrease in maternal osmolality during pregnancy may be due to the physiological responses to increased concentrations of steroid hormones, chorionic gona-

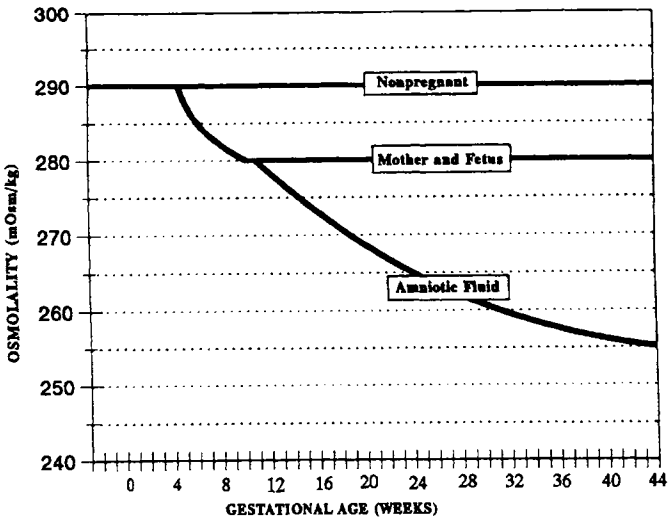

Figure 5. Osmolality of amniotic fluid and maternal and fetal plasma across gestation. From Gilbert et al., (1991).

dotropin and relaxin during pregnancy. Also note that, beginning at approximately 10–12 weeks of gestation, amniotic fluid osmolality begins to decrease below fetal and maternal plasma osmolality. This occurs at the time when fetal urine is beginning to enter the amniotic compartment. It should be recalled that fetal urine is unique in comparison with adult urine in that it is hypotonic relative to plasma, whereas adult urine is usually hypertonic relative to plasma. Thus the decrease in amniotic osmolality beginning at 10–12 weeks of gestation can be attributed to the low osmolality of the urine which enters the space. Further, as gestation proceeds, the fetus excretes progressively larger volumes of urine into the amniotic compartment, promoting a gradual reduction in amniotic osmolality. Further, with keratinization of the fetal skin at midgestation, there would be less water absorbed from the amniotic compartment across the fetal skin with the result that the fetal urine may be more effective at reducing amniotic osmolality. In comparison, fluid secreted by the fetal lungs is isotonic with fetal plasma and thus does not contribute to the changes in amniotic osmolality which are depicted in Figure 5. Even though amniotic osmolality falls from approximately 280 mOsm/kg at the beginning of the fetal period to approximately 260 mOsm/Kg at term, this is still far above the osmolality of fetal urine which averages approximately 140–150 mOsm/kg near term.

Sodium and chloride ions are the two major solutes which are present in the amniotic fluid. In early gestation, amniotic sodium and chloride concentrations are roughly the same as fetal plasma, i.e., 142 and 100 mEq/L, respectively (Wintour, 1986). Throughout gestation, amniotic sodium concentration decreases roughly in

parallel with the changes in osmolality shown in Figure 5. Thus, at term the amniotic sodium concentration averages approximately 125 mEq/L.

Many other solutes are present in the amniotic fluid including nutrients such as glucose and amino acids. However, the two major sources of the amniotic fluid in late gestation, i.e., fetal urine and lung fluid, have very low concentrations of both glucose and amino acids. Presumably, these solutes diffuse across the fetal surface of the placenta and enter the amniotic fluid. Although there is a great potential for movement of water and solutes between amniotic fluid and maternal blood within the wall of the uterus, essentially all physiologic studies to date indicate that there are only minor fluxes across the amnion and chorion (Brace, 1995). This has been difficult to interpret because permeabilities of the amnio-chorion measured *in vitro* suggest that moderate to large fluxes may occur. Presumably this discrepancy is due to a limited ability of water and solutes to move across the uterine wall rather than across the membranes.

REGULATION OF AMNIOTIC FLUID AND COMPOSITION

As described above, both amniotic fluid volume and amniotic composition undergo characteristic changes during the course of gestation (Gilbert et al., 1991). In addition, in individual pregnancies, amniotic volume can vary widely. However, amniotic concentrations of the major solutes remain surprisingly constant for any given gestational age. Thus, fetal diseases which are associated with abnormal amniotic volumes are not generally associated with abnormal amniotic compositions for the major solutes. An exception to these observations is that either just prior to or following fetal death, there may be changes in the amniotic composition. Further, in experimental animals, prolonged infusion of antidiuretic hormone into the fetal circulation results in increased urine osmolality and sodium concentration with smaller increases in amniotic osmolality and electrolytes (Ross et al., 1985). The mechanisms which maintain amniotic composition relatively constant in the face of widely varying inputs are unclear. Presumably this is due to a rapid movement of water between the amniotic compartment and fetal surface of the placenta, resulting in a relatively constant amniotic composition in view of the fact that fetal plasma composition remains relatively constant.

Even though amniotic composition remains relatively constant, both between and within individuals, amniotic volume varies considerably. There are two approaches which help generate an understanding of the mechanisms which determine amniotic volume. First, it is unlikely that amniotic volume is directly regulated. Although stretch of the membranes has been suggested as a sensor for amniotic volume, there currently is no support for the concept that a sensor mechanism exists. The second approach to understanding the regulation of amniotic volume is to examine the regulation of each of the individual flows into and out of the amniotic compartment. These will be discussed individually.

Urine Excretion

During the latter half of gestation, fetal urine is the major source of amniotic fluid (Brace et al., 1989). Thus it is not surprising to find that fetal renal function has been more extensively explored than any of the other pathways that contribute to amniotic volume. Studies to date have shown that there is one major difference and many similarities in renal function in the fetus versus the adult. The major difference is that fetal urine osmolality is far below plasma osmolality, averaging 150 mOsm/kg near-term compared to a plasma osmolality of 280 mOsm/kg, whereas adult urine osmolality is normally markedly hypertonic, averaging 600–800 mOsm/kg and sometime approaching 2,000 mOsm/kg. These differences in fetal and urine osmolalities occur in spite of the fact that plasma arginine vasopressin (AVP, or the antidiuretic hormone, ADH) is present in the same concentration in fetal and adult plasma. Thus, the fetal renal sensitivity to AVP may be different during the fetal period. Alternatively, the differences in fetal and adult urine osmolality may be due to immaturity of the concentrating mechanisms within the kidney since the fetal and adult kidneys are both sensitive to varying plasma concentrations of AVP. In other words, maximal osmolality in the renal interstitium is much lower in the fetus than in the adult and the maximal urine concentrating ability of the fetus is consequently much lower, averaging perhaps 350 mOsm/Kg near-term compared to 2,000 mOsm/kg in the adult.

This difference in urine osmolality is not reflected by different sensitivities to hormones by the fetal kidney. Thus, in addition to AVP, the fetal kidney also responds to aldosterone similar to adult responses. The fetal renal response to atrial natriuretic factor, the cardiac hormone which augments sodium excretion, is also similar to the adult responses. The only documented exception to similarities in fetal and adult sensitivities to circulating hormones is the fetal response to physiologic increases in cortisol. Cortisol induces both a natriuresis and a diuresis in the fetus, whereas these do not occur in the adult due to maturation of the tubular processes. Overall, there are many similarities between renal function in the fetus and the adult and a majority of the regulating processes are the same.

Lung Liquid Secretion

The lungs of the fetus are filled with liquid and this is in stark contrast with the air filled lungs of the adult. This liquid volume averages approximately 50 ml/kg of fetus during the last third of gestation and is characterized by a high turnover rate (Harding, 1994). This turnover occurs because the fetal lungs secrete fluid at a rate of approximately 100 ml/kg of fetal weight per day. Secretion is due to the active transport of chloride into the future airway of the fetus. Intravascular volume expansion of fetus does not increase lung fluid excretion, indicating that the lungs do not serve to regulate body fluid homeostasis. Rather pulmonary fluid is essential to expand the fetal lungs and permit normal growth and development. Pulmonary

fluid production must be inhibited and fluid resorption stimulated at the time of parturition. Beginning approximately 2–3 days before the onset of labor and delivery, the rate at which fluid is secreted by the fetal lung begins to gradually diminish. A number of fetal hormones, including cortisol, catecholamines, AVP, and atrial natriuretic factor suppresses lung fluid production. Notably, each of these factors increase in fetal plasma during labor, thus providing the impetus to clear the lungs for air breathing. In addition to the endocrine changes which occur during labor and delivery, fetal hypoxia is a powerful stimulus which augments the plasma concentrations of many of the vasoactive hormones. Fetal hypoxia is also associated with a reduced secretion rate of lung liquid or, with more severe hypoxia, reabsorption of lung liquid can be stimulated.

Of the large volumes of lung liquid which are secreted by the fetus, only approximately 1% is needed to expand the lungs with fetal growth. The other 99% flows out of the lungs by the trachea. Most of the outflow from the lungs occurs during episodes of fetal breathing movements and this is the same time period when most fetal swallowing occurs. Older studies in which hypertonic contrast medium was injected into the fetal trachea suggested that a large fraction of the tracheal outflow was swallowed. However, pulmonary surfactant is present in increasing amounts in the amniotic fluid, indicating that a substantial amount of the tracheal outflow must enter the amniotic space. Recent studies have shown that, over 24-hour periods, an average of 50% of the fetal lung outflow enters the amniotic space and the remaining 50% is swallowed directly (Brace, 1995).

Swallowing

The fetus swallows large volumes of fluid each day which is composed of approximately 85% amniotic fluid and 15% lung liquid (Brace, 1995). In humans, from studies in which radiolabeled red cells or labeled proteins were injected into the amniotic sac, the fetus was found to swallow a volume of amniotic fluid equivalent to 50–100% of amniotic fluid volume per day. Animal studies have suggested that the normal fetus may swallow a volume equivalent to 20–25% of body weight each day. Although this may be 10 times the volume of fluid intake in the normal adult relative to body weight, the fetus swallows less than the volume of urine which it produces each day. In addition to urine, a significant volume of lung fluid also enters the amniotic compartment. Thus, because swallowing is less than urinary output plus lung secretions, some mechanism in addition to swallowing must be responsible for the removal of large volumes of fluid each day from the amniotic compartment, and this most likely occurs across the fetal surface of the placenta. Nonetheless, fetal swallowing is the major route of removal of amniotic fluid and thus contributes greatly to the regulation of amniotic volume.

Much less is known about the regulation of swallowing (Ross et al., 1989) compared to the regulation of renal function of the fetus. Similar to outflow of lung fluid

from the trachea, the major episodes of fetal swallowing occur during periods of fetal breathing activity. Further, acute hypoxia suppresses not only secretion of fluid by lungs but also suppresses the volume of fluid swallowed by the fetus. Similar to the adult, central angiotensin II and hypertonicity act as fetal dipsogens (Ross et al., 1989). In addition, increases in plasma osmolality acutely stimulate swallowing by the fetus. Overall, the neurohumoral regulation of fetal swallowing is just beginning to be understood.

In addition to regulated swallowing activity, there appear to be other factors which determine the volume of fluid swallowed by the fetus. For example, one primary factor may be the volume of amniotic fluid which is present. That is, if there is little amniotic fluid present, there may be little fluid swallowed by the fetus even though swallowing activity is present.

Fetal Surface of the Placenta

Both water and solutes may move rapidly between fetal blood and the amniotic fluid across the fetal surface of the placenta (Gilbert and Brace, 1989; Brace, 1995). This is sometimes referred to as intramembranous exchange (see Figure 2). For water, the primary driving force would be the osmotic gradient between the amniotic fluid and fetal blood. Each mOsm/kg of the osmotic difference generates approximately 19.3 mm Hg in osmotic pressure at 37°C. Thus, there may be large volumes of water moving outward from the amniotic compartment into the fetal surface of the placenta. For electrolytes and small molecular weight solutes, concentration gradients in the opposite direction are fairly large and thus would tend to promote the movement of sodium, chloride, glucose, amino acids, etc. from fetal blood via diffusion into the amniotic sac.

Unfortunately, no studies have yet quantified the amount of solutes which are transferred into or out of the amniotic compartment via the intramembranous pathway. In comparison, several recent studies have suggested that approximately 200 ml/day of water may move outward via the intramembranous pathway so the potential for solute movement may also be large (Brace, 1995).

Membranes and Uterine Wall

Movement of water and solutes between the amniotic compartment and maternal blood within the uterine wall may occur as the surface area of the combined amnio-chorion is large (Wintour et al., 1986). This is often referred to as transmembranous exchange (see Figure 2). However, the studies conducted to date have largely found little or no transfer across the transmembranous pathway (Brace, 1995). For water, it has been estimated that approximately 10 ml/day might cross the transmembranous pathway in the near-term fetus. In contrast, studies of urea, carbon dioxide, and sodium have found transfer rates so low as to be undetectable.

PATHOPHYSIOLOGY OF THE AMNIOTIC COMPARTMENT

Abnormalities in amniotic fluid volume may have marked effects on the fetus and mother, while abnormalities of the fetus may have marked effects on the amniotic fluid (Liley, 1971; Abramovich, 1978, 1981; Seeds, 1980; Brace et al., 1993). Oligohydramnios, or reduced amniotic fluid volume, occurs in 1–2% of all pregnancies with an increasing incidence in post-term pregnancy. Though only a small portion of oligohydramnios is associated with fetal anomalies, reduced amniotic fluid in the first half of gestation may be secondary to an obstructed urinary tract or absent fetal kidneys, or a result of amniotic fluid leakage following rupture of the amnionic and chorionic sacs. Regardless of the etiology, complications include a syndrome of fetal structural malformations known as the oligohydramnios deformation syndrome. In the second trimester, reduced amniotic fluid may impair pulmonary development with resulting neonatal morbidity and mortality. Throughout the last third of gestation, oligohydramnios is associated with conditions of fetal stress, including intrauterine growth retardation and chronic hypoxia. Fetal endocrine responses to intrauterine stress, including increased AVP secretion, may account for reduced fetal urine production in growth retarded fetuses. Post-term pregnancies also exhibit a markedly increased incidence of oligohydramnios, with associated risks of umbilical cord occlusion, fetal distress in labor, meconium aspiration, operative deliveries, and stillbirth. Due in part to the adverse outcomes associated with oligohydramnios, current obstetrical management of intrauterine growth retarded or post-term pregnancies includes weekly or bi-weekly ultrasound assessments of amniotic fluid volume, and induction of labor if the amniotic volume is reduced. In laboring patients with oligohydramnios, saline infusions into the uterus, via a catheter inserted through the cervix, have been demonstrated to reduce umbilical cord compression, intrapartum fetal morbidity and the need for operative delivery. Although percutaneous (transabdominal) amniotic infusions have been utilized prior to rupture of membranes, there are no established therapeutic modalities to chronically increase amniotic fluid volume prior to labor or in patients with an intact amniotic sac. Percutaneous infusions also have been utilized in the first trimester to permit better ultrasound visualization of the fetus and diagnose congenital anomalies, and to differentiate if rupture of the amniotic sac has occurred. Despite the associations with intrauterine growth retardation and fetal stress, obstetricians are unable to accurately predict which women will develop oligohydramnios, either pre- or post-term. Thus, despite significant perinatal complications, therapeutic approaches for the prediction, prevention, and treatment of oligohydramnios remain limited.

Polyhydramnios, which occurs in 0.5 to 1% of pregnancies, is associated with a variety of fetal conditions (Brace et al., 1993). Fetal congenital anomalies that increase pulmonary fluid or urine production or decrease swallowing may result in excess amniotic fluid. Fetal swallowing may be reduced with fetal central nervous system abnormalities, neuromuscular disorders, chromosomal abnormalities, and

upper gastrointestinal tract obstructions or atresia. Fetal lung fluid may be increased with congenital cystic adematoid malformation of the lung, though polyhydramnios also may occur as a result of reduced swallowing with this condition. Kidney anomalies that result in impairment of renal tubular water and electrolyte resorption may increase urine flow rates. Fetal skeletal disorders, tumors, cardiac anomalies, and infectious diseases, as well as maternal diabetes, isoimmunization and multiple pregnancy also may result in polyhydramnios. In addition to the aforementioned malformations, approximately 30% of polyhydramnios is idiopathic, perhaps secondary to abnormalities of intramembranous flow.

There are a number of maternal complications of polyhydramnios including the previously mentioned difficulties in respiration due to the expanded uterine volume. Women with polyhydramnios often deliver preterm as a result of spontaneous preterm uterine contractions as well as preterm rupture of the amniotic sac. The fetus is often malpositioned resulting in an increased frequency of breech presentations. The free-floating fetus also permits the umbilical cord to prolapse, resulting in compromised fetal blood flow and oxygenation. Rapid decompression of the expanded uterus may result in the placenta shearing off the uterine wall (placental abruption) as well as an increased risk of postpartum hemorrhage.

Treatment for polyhydramnios has previously been limited to transcutaneous drainage of the uterine contents. As the fetus may produce 750 to 1,000 ml/day, drainage procedures are usually efficacious only for short durations. Repetitive needle insertions into the amniotic cavity are often associated with infection and spontaneous rupture of membranes. In recent years, maternal prostaglandin synthetase inhibitors have been demonstrated as effective treatments for polyhydramnios. After crossing the placenta to achieve pharmacologic fetal plasma concentrations, the prostaglandin inhibitors reduce fetal urine production. Unfortunately, complications of the prostaglandin inhibitors have included neonatal renal failure, oligohydramnios, intraventricular hemorrhage, and an increased incidence of necrotizing enterocolitis.

Thus, both polyhydramnios and oligohydramnios are associated with significant perinatal morbidity and mortality. Our increasing understanding of the mechanisms of amniotic fluid dynamics will potentially lead to the development of novel diagnostic and therapeutic approaches for these disorders.

SUMMARY

Amniotic fluid volume increases progressively through gestation until the middle of the third trimester when amniotic volume averages approximately 800 ml. There can be a wide range of volumes in individual pregnancies and within the same pregnancy over a period of time. Increases in amniotic fluid volume above normal as well as decreases below the normal range are associated with increased fetal and neonatal morbidity and mortality. In early gestation aberrations in amniotic volume

are often associated with fetal anomalies and a variety of genetic diseases. In late gestation, aberrations in amniotic volume are often associated with early delivery because of excess amniotic fluid or fetal stress because of umbilical cord compression when there is too little amniotic fluid. Amniotic fluid is derived from a combination of sources, including the fetal kidney, lung, and surface of the placenta. Clearance of the amniotic fluid occurs via fetal swallowing and reabsorption across the fetal surface of the placenta. There currently exists no consistently successful therapy for women with abnormal amniotic volumes including those with idiopathic abnormalities. Presently, bed rest and increased maternal fluid intake are sometimes tried as treatment for oligohydramnios whereas maternal indomethacin administration or drainage of excess amniotic fluid at the time of amniocentesis is used for treatment of polyhydramnios. Overall, there remains a great deal to be learned about the amniotic fluid compartment.

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Chapter 12

Ultrasound in Perinatal Medicine

CHARLES SILES

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INTRODUCTION

Ultrasound was first introduced into obstetrics in 1958 by Donald and co-workers in the investigation of abdominal masses. Since then its use has become commonplace in the field of obstetrics. Donald and co-workers suggested that the gravid uterus was an ideal target for ultrasound imaging with the "solid-fetus" being in a fluid-filled cavity. The main advantage at that time was the safety of using sound waves rather than ionizing radiation when assessing the fetus.

Diagnostic ultrasound uses sound energy with a wave frequency that exceeds 20,000 Hz, the audible limit in humans. When a sound beam crosses an interface between objects of different densities, some of the energy is reflected and some is transmitted. The amount of each is proportional to the difference in density. The reflected waves are detected by a transducer, which creates an image of the scanned object. More recent advances in technology and expertize have enhanced image quality. This has made ultrasound the modality of choice for fetal imaging. In the last decade, sonography has been used increasingly for prenatal diagnosis and treatment. The antenatal identification of an abnormal fetus allows the opportunity for prenatal counseling with a multidisciplinary team of experts, with a thorough discussion of pregnancy options. Furthermore, prenatal diagnosis can influence antepartum and intrapartum management, and permit planning of the mode and site of delivery, thus ensuring optimal care of the fetus and newborn. Its role in screening in a low-risk population is still being debated. The role of ultrasound in a high-risk population is less controversial.

The impact of high-resolution dynamic ultrasound imaging has given the perinatologist the ability to visualize the fetus and its activities. This allows us to perform a physical examination on the fetus, creating the concept of the fetus as a patient.

The use of Doppler ultrasound to study blood flow in obstetrics is of major importance because fetal inaccessibility precludes many other methods of study of the circulation. Doppler devices to detect motion of the fetal heart have been used for the past 25 years. Recent developments include the capacity to display the Doppler spectrum from a specific vessel imaged with the conventional B-mode ultrasound scan. Color Doppler has also been a significant advance.

CONFIRMATION OF FETAL LIFE

Ultrasound has the ability to establish rapidly and accurately whether a fetus is alive or dead. It is also able to predict the likely survival after a threatened miscarriage. The normal transvaginal ultrasound features in early pregnancy are included in Table 1.

Fetal life is simply confirmed by observation of fetal heart movements and fetal death by its absence. Early pregnancy failures include: i) blighted ovum (anembry-

Table 1. Transvaginal Ultrasound Features in Early Pregnancy

Gestation	*Ultrasound Appearance*
4 weeks	Thickened endometrium
5 weeks	3–5 mm gestational sac
6 weeks	Fetal heart movements seen

Table 2. Transvaginal Ultrasound Features of a Threatened Miscarriage in Early Pregnancy

Pregnancy Outcome	*Ultrasound Features*
Pregnancy failure	1) Yolk sac 5 mm or greater with no fetal heart movements or fetal pole 2) Gestational sac 15 mm or greater with no fetal heart movements or fetal pole
Poor pregnancy outcome	1) Fetal complex too big for the sac size (reduced fluid) 2) Fetal bradycardia (< 100 bpm) often erratic in rhythm 3) Irregular gestational sac 4) Disproportionately thick decidua for the gestational sac size 5) Small for dates (wrong dates must always be considered) 6) Gestational sac sliding up and down the uterine cavity 7) Gestational sac just above or in the cervical canal 8) Extra amniotic/retro placental fluid collection (hemorrhage) greater than 1/2 the sac size

onic pregnancy) which constitutes the largest group and are diagnosed by the absence of a fetal pole in the presence of a gestational sac larger than six weeks size (12–15 mm); and ii) missed abortion which constitutes the absence of fetal heart movements (FHM) in a fetus greater than six weeks size (5–6 mm), or a gestational sac greater than six weeks size (12–15 mm), or if a yolk sac greater than 6 weeks size (4 mm) were present. Ultrasound has rationalized the management of threatened miscarriage in early pregnancy. The ultrasound features of a threatened early pregnancy failure is described in Table 2.

If the pregnancy appears to be less than six weeks size (using the fetal pole, gestational sac and yolk sac measurements), then a repeat ultrasound examination would be required in 7–14 days to confirm a miscarriage or an ongoing pregnancy. One must always beware of the presence of an ectopic pregnancy in some of these cases.

ULTRASOUND DATING

One of the most important functions of ultrasound in pregnancy is the ability to date a pregnancy accurately. Epidemiological studies have shown that less than half the

Table 3. Accuracy of Ultrasound Relative to Gestation

Gestation	*Ultrasound Parameter (Biometry)*	*Ultrasound Accuracy*
First trimester	Crown-rump-length	± 5 days
Second trimester	Biparietal diameter, and/or femur length	± 10 days
Third trimester	Biparietal diameter, femur length	± 21 days

obstetric patients accurately recall the exact date of their last menstrual period. The importance of dating in perinatology is paramount, especially when delivery is to be timed. The accuracy of ultrasound dating is dependent on both the experience of the operator and on the quality of the ultrasound machine. Assuming that both these factors are optimal the quoted accuracy then depends on the gestation, as described in Table 3.

PLACENTAL LOCALIZATION

Localization of the placenta is of particular importance prior to chorion villous sampling (CVS), amniocentesis, antepartum hemorrhage, fetal malpresentation, and for patients with a history of uterine surgery so as to potentially exclude a placenta accreta. Factors that have been associated with placenta previa include advanced maternal age, multiparity, smoking, and prior caesarean section or uterine surgery.

Obstetric ultrasound has significantly improved the outcome of patients with placenta previa. Perinatal mortality for this condition is less than 5%, though in the 1950s it was as high as 40%. Other potential complications include intrauterine growth retardation (IUGR), premature delivery, placenta accreta, and vaginal bleeding. Vaginal bleeding complicates 3–5% of pregnancies in the third trimester. Placenta previa accounts for about 10% of these. Painless vaginal bleeding from placenta previa typically occurs during the third trimester due to uterine thinning leading to placental detachment and tears in the marginal and basilar veins. In 30% of cases vaginal bleeding initially occurs prior to the third trimester.

Placental localization is a topic that is often little understood. The problem lies in the fact that placenta previa is historically a clinical definition which predates ultrasound. The definition relies on the presence of a lower uterine segment which does not often form until after 28 weeks gestation. So to call a placenta, which appears low prior to the formation of a lower uterine segment, a placenta previa is incorrect. Because of this the terms "low-lying" placenta or "potential placenta previa" have been used. Three types of placenta previa need to be considered on ultrasound examination: they include complete, marginal, and partial. Table 4 describes the three types, their ultrasound features and their relative incidence in the third trimester.

Table 4. Ultrasound Features of Placenta Previa and their Relative Incidence in the Third Trimester

Type	*Ultrasound Features*	*Incidence*
Complete (total, major)	Placental tissue completely covering the internal cervical os	20%
Marginal (minor)	Placental tissue extending onto the cervix reaching the internal cervical os, but does not cover it	80%
Partial (minor)	Placental tissue partly extending onto the cervix but does not reach the edge of the internal cervical os	

We have found that the best way to describe the position of the placenta is to state to the clinician the distance of the lower edge of the placenta to the internal os (in centimeters). As a rough rule of thumb, if the lower edge is greater than 2.5 cm from the internal os, it is most unlikely to be of any clinical significance. This then avoids problems of nomenclature.

The phenomenon of placental migration is now well recognized: a placenta that appears low earlier in pregnancy may, with the formation of the lower uterine segment, appear to have risen on repeat ultrasound examination and thus no longer seems previa. At 18 weeks menstrual age, the incidence of a low-lying placenta is 5%. Of these only 10% will become placenta previa at 34 weeks. After 34 week placental migration is minimal so there is no need to re-ultrasound a patient after this time in the hope that further migration might occur. At 34 weeks the mode of delivery can therefore be arranged.

Two-thirds of false-positive diagnoses of placenta previa, on early scanning, could be attributed to technical artefacts resulting from an overly distended urinary bladder or focal uterine contractions of the lower uterine segment. The remaining cases can be attributed to placental migration. False positive diagnoses can lead to unnecessary additional sonograms, greater expense, needless parental anxiety, and even unnecessary caesarean delivery. To overcome the problem with a distended bladder we would recommend that either a translabial or transvaginal examination be performed, with an empty bladder. Using these two techniques one can also observe abnormal blood flow patterns often present in placenta previa. Also it is possible to diagnose vasa previa and placenta accreta using color or power Doppler.

ROLE OF ULTRASOUND IN THE ASSESSMENT OF CERVICAL INCOMPETENCE

Prevention of preterm labor is a major goal of perinatologists. One potentially preventable cause is cervical incompetence. Clinical cervical assessment (tracking)

with a digit or speculum examination has proven to be inaccurate. Ultrasound cervical tracking has the potential to improve this scenario.

Transabdominal cervical length assessment leads to apparent lengthening of the cervix with increasing volume of urine in the bladder. Since transabdominal ultrasound views of the cervix with an empty bladder are often suboptimal, transperineal or transvaginal cervical length assessments with an empty bladder should be performed. Until larger studies are completed, the management of patients at risk of cervical incompetence should be either transvaginal or transperineal ultrasound cervical length assessment (measuring from internal os to external os) every second week, and a cervical suture inserted if the cervical length is <2.5 cm, or significantly shortened since the previous examination. This is called ultrasound cervical tracking. Of course if cervical incompetence is diagnosed clinically, a cervical suture should still be inserted.

PRENATAL DIAGNOSIS OF MAJOR CONGENITAL MALFORMATIONS

Congenital malformations affect approximately 3% of all live births every year. It is important that as many of these are diagnosed *in utero*, and as early as possible, to allow adequate counseling of the mother and to permit preparation of the neonatal services to optimize the outcome.

There are a vast number of congenital anomalies that can be diagnosed on a thorough ultrasound examination, with properly qualified examiners using modern up-to-date equipment. These include abnormalities of the central nervous system, spine, neck, face, thorax, abdominal wall, gastro-intestinal tract, urinary tract, genitals, skeleton, hand, feet, and umbilical cord. Of all these congenital malformations, approximately 50% are aneuploidy (chromosomal abnormality) related. It is important to isolate them for genetic counseling, as they will have an impact on recurrent risks for future pregnancies. The most common chromosomal abnormalities are the trisomies. Trisomy 21 (Down syndrome) is the most common followed by trisomy 18 and 13. These are followed by Turner's syndrome and triploidy. It is important to remember that only the trisomies have an increase age-related risk. Table 5 1ists the typical features of each trisomy abnormality.

Often the ultrasound diagnosis of a fetus with chromosomal abnormality is made by a combination of abnormal ultrasound findings (aneuploidy markers), which give the examiner an idea on the likelihood of such an abnormality being present. Different chromosomal abnormalities often present with different combinations of structural anomalies (Table 5). It becomes more complicated as each chromosomal abnormality can present with different combinations of aneuploidy markers. Also, these markers are often present in nonaneuploic fetuses. So without proper karyotyping, caution must be taken before labeling a fetus. Despite this, over 90% of trisomy 13 and 18 have more than one detectable aneuploidy marker. So these should theoretically be difficult to miss on thorough ultrasound examination.

Table 5. Trisomy 13, 18, and 21 Pathological Features

Abn.[a]	*Trisomy 13*	*Trisomy 18*	*Trisomy 21*
CNS			
	Holoprocencephaly	Hydrocephalus	
	Agenesis of corpus	Myelomeningocele(10-20% callosum)	
CVS			
			AV septal defect
	VSD	VSD	VSD
	ASD	ASD	ASD
	PDA	PDA	PDA
		Double-outlet right Ventricle	
		Bicuspid aorta and pulmonary valves	
HEAD			
	Microcephaly	Microcephaly	
	Dolichocephaly	Brachycephaly	Brachycephaly
FACE			
	Cyclopsia	Micrognathia	Epicanthic folds
	Anophthalmia		Short nose
	Microphthalmia		Broad neck
	Cleft lip and palate	Cleft lip or palate	Protruding tongue
	Low set deformed ears	Low set (pixie) ears	Low set round ears
GIT			
	Omphalocele	Omphalocele	Omphalocele
	Umbilical hernia	Diaphragmatic hernia	Duodenal atresia
		Esophageal atresia	Esophageal atresia
		Anorectal atresia	Anorectal atresia
UG			
	Cystic renal dysplasia	Renal dysplasia	
	Hydronephrosis	Urethrovesical obstruction	
	Duplicated kidney	Horseshoe kidney	
	Ambiguous genitalia	Ambiguous genitalia	Undescended testes
Skeletal			
	Polydactyly (70%)	Short limbs	Short limbs
	Rockerbottom feet	Rockerbottom feet	Widening of iliac wings
	Scalp defects	Shortened first toe	Short, broad hands
		Overlapping fingers, flexed hands (>80%)	Clinodactyly
			Flattening of acetabular angles
			Increased space between first and second toes (sandle sign)

Continued

Table 5. Continued

Abn.[a]	*Trisomy 13*	*Trisomy 18*	*Trisomy 21*
Other			
	Single umbilical artery	Single umbilical artery (>80%)	
		Cystic hygroma	Cystic hygroma
		IUGR	Nuchal thickening
		Polyhydramnios	Hydrothorax
		Oligohydramnios	
		Nonimmune hydrops	Nonimmune hydrops

Notes: [a] Abbreviations: Abn., abnormal; PDA, patent ductus arteriosus; CNS, central nervous system; AV, atrioventricular; GIT, gastrointestinal tract; VSD, ventricular septal defect; UG, urogenital; ASD, atrial septal defect

The real problem lies with trisomy 21 where a large percentage present with either only soft signs of aneuploidy, often isolated, or with no aneuploidy markers at all. This is further complicated by the fact that the vast majority of fetuses with isolated soft markers are in fact normal. In general, the risk of aneuploidy increases with the number of markers detected on ultrasound examination:

2 markers = 10% risk
3 markers = 60% risk.

An aneuploidy marker should be seen as a beacon highlighting the need to look for other aneuploidy markers. As greater than 90% of trisomy 13 and 18 have more than one marker, one must be careful not to overemphasize the importance of isolated soft markers of aneuploidy. This is clinically important so as to avoid unnecessary parental anxiety, and unnecessary procedures arising from these anxieties. Procedures such as fetal karyotyping are not without risk, and repeated ultrasound examinations are not without cost to the patient or to the health care system.

ULTRASOUND ASSESSMENT OF FETAL WELL-BEING

The assessment of fetal well-being using ultrasound relies on the evaluation of i) fetal biophysical variables, ii) Doppler examination of the fetus, and iii) assessment of fetal cardiac arrhythmias, if present.

Fetal Biophysical Profile

The concept of performing a physical examination on the fetus was first proposed by Manning et al. (1980). Real time ultrasound permits the evaluation of multiple fetal activity (fetal movement, breathing, and tone) as well as the assessment of the in-

trauterine environment. The combination of these biophysical variables (fetal biophysical profile) was developed to decrease the false-positive results inherent in single variable biophysical assessment and to identify accurately the hypoxic fetus in the antepartum period. The predictive accuracy of each normal biophysical variable is high, and approximately equal among variables; however, the false-positive rate for each single abnormal variable is greater than 5%. The most important factor in the sensitivity of the testing method is the combination of acute (fetal heart rate reactivity, fetal movement, fetal breathing movements, and fetal tone) and chronic (amniotic fluid volume, fetal growth parameters, Doppler resistance indices, fetal echocardiography) markers of the fetal condition (Figure 1 and Table 6).

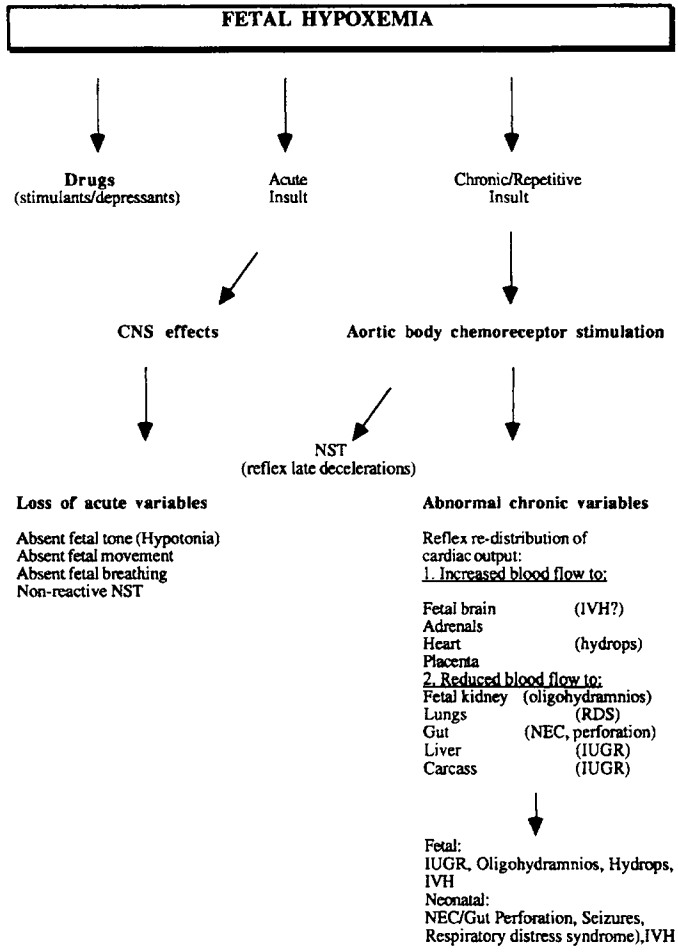


Figure 1. A schematic representation of the effects of fetal hypoxemia and drugs on the fetal acute and chronic markers of the fetal condition.

Table 6. Normal Fetal Movement Patterns Seen on Ultrasound

Gestation	*Fetal Movements Seen on Ultrasound*
7 weeks	Simple sporadic extremity motion, spastic
9 weeks	Twitching movements
10 weeks	Spasmodic jumps
12–16 weeks	Combined movements: trunk, head, and limbs
16–20 weeks	Combines movements: trunk, head, limbs, jaw, tongue movements, and swallowing
20 weeks	Fetal breathing movements
Late gestation	Trunk and extremity movements, high frequency movements

The acute markers of the fetal condition are dynamic activities that are initiated and regulated by complex integrated mechanisms of the fetal central nervous system (CNS). The presence or absence of these markers reflect fetal status at the time of testing. The presence of a normal biophysical activity is indirect evidence that the portion of the CNS that controls that activity is intact and functioning and therefore non-hypoxic. The absence of a biophysical activity, however, is more difficult to interpret as it may either reflect a pathological fetal status or else a normal cyclical physiological occurrence (normal periodicity). Cyclic variations are present for all acute biophysical markers of fetal well-being. Also CNS stimulants (catecholamines, hyperglycemia) may increase fetal biophysical activities, and CNS depressants (morphine, diazepam, barbiturates, halothane, heroin, methadone) may inhibit their activity (Tables 7 and 8, Figure 1). This reduction in fetal activity has also been observed with magnesium sulphate during the management of pre-eclampsia.

The effect of hypoxia on the fetal biophysical profile depends on the extent, duration, chronicity, and frequency of the insult. Fetal hypoxia may be transient without acidosis, or prolonged with associated metabolic or respiratory acidosis, consequently affecting multiple organ systems. Acute fetal hypoxia produces a dramatic decrease of fetal heart rate reactivity (non-reactive NST) and fetal breathing movements, and when severe will result in reduced or absent fetal movements and fetal tone (Table 7, Figure 1).

The biophysical activities that become active first in fetal development are the last to disappear when asphyxia arrests all activities. For example, the fetal tone (FT) center is the first to function at 7.5 to 8.5 weeks followed by the fetal movement (FM) center at 9 weeks gestation and they are the last to disappear during asphyxia. In contrast, fetal breathing movements (FBM), which start functioning after 20–21 weeks, and fetal heart rate reactivity (NST) starts operating late in the second trimester are the first to disappear during asphyxia (Table 8). The gestation of commencement of these activities in the fetus as with their sensitivity to hypoxia is directly related to the pO_2 level present and that which is required for each CNS center to function normally. This concept is of significant value in antepartum fetal well-being assessment and allows the clinician the ability to estimate the level of deterioration of the fetal condition and that of the fetal status at the time (Table 8).

Table 7. Factors Affecting Pattern of Acute Variables

Gestation	Amniotic fluid volume
Hypoxia	Maternal cigarette smoking
Drugs	Uterine contractions
Sleep/wake cycles	Fetal abnormality

Table 8. Acute Markers of Fetal Well-being

Acute Markers[a]	*CNS Region*	*Gestation First Function (Weeks)*	*$_pO^2$ Requirement in Order*	*Order Disappears With Hypoxia*
Fetal tone (FT)	Cortex (?subcortical)	7.5–8.5	4th	4th
Fetal movement (FM)	Cortex-nuclei	9	3rd	3rd
Fetal breathing movement (FBM)	Floor 4th ventricle	20–21	2nd	2nd
Fetal heart rate reactivity (FHRR)	Post hypothalamus, medulla	Late 2nd trimester	1st	1st

Notes: [a] FHRR and FBM cease: pH lower than 7.20. FM and FT begin to malfunction: pH 7.10–7.20. FM and FT cease: pH lower than 7.10.

Chronic fetal hypoxia or acidosis may produce protective redistribution (by aortic body chemoreceptor stimulation) of the cardiac output from non-vital organs (kidney, lung, gut, liver) to more vital organs (heart, brain, adrenals, placenta). With sustained hypoxia the redistribution may result in decreased urine production leading to oligohydramnios and to a reduction in glycogen deposition in the liver thus leading to a reduced fetal abdominal circumference (AC) (Figure 1). This can be easily measured and plotted on centile charts using ultrasound. With continued chronic hypoxia a more global reduction in fetal growth will eventually occur leading to IUGR. The fetus with oligohydramnios (reduced amniotic fluid volume) is not only chronically stressed but is also at risk of cord compression and pulmonary hypoplasia. The use of Doppler ultrasound in a potentially compromised fetus may be of help in deciding management, including timing of delivery. The presence of the chronic markers of hypoxemia imparts to the clinician a concept of reduced fetal reserve reflecting on the ability of the fetus to cope with any further stresses it may be exposed to *in utero*.

Ultrasound parameters such as abdominal circumference, fetal weight, fetal ponderal index, and femur length can all be measured and plotted on centile charts as guides to degree of fetal reserve. Similarly, the umbilical Doppler resistance index can be plotted and trends followed graphically. In this way, serial measurements will reflect a deterioration or improvement in the fetal condition. This becomes quite valuable in the difficult management of a fetus with potentially re-

duced fetal reserve, and of great use for the timing and mode of delivery, as well as for the frequency of further testing to be done.

In the scenario of a compromised fetus (reduced fetal reserve), absent or reverse diastolic flow on Doppler spectral ultrasound examination implies an absent (or at least extremely reduced) fetal reserve. The fetus is therefore severely hypoxemic. Immediate action must be undertaken by the clinician to avert fetal demise.

Fetal Biophysical Profile Scoring

Since the early 1980s an attempt was made to score each biophysical variable to determine a fetal biophysical profile score. As acute biophysical markers of fetal well-being can be equally explained by asphyxia or sleep (normal periodicity) it was necessary that the test would differentiate between these two conditions. This diagnostic dilemma was resolved by observing multiple biophysical variables and extending the observation period beyond that of a sleep-wake cycle: at least 30 minutes. Therefore two types of scoring systems were developed: one proposed by Manning et al. (1980) and the other by Vitzileos et al. Manning et al. allocated a score of 0 or 2 to each variable, while Vitzileos et al. allocated a score of 0, 1 or 2. In both, a total score of 8 or more was a reassurance of fetal well-being, while less than 8 suggested repeat testing or delivery.

Amniotic fluid volume is assessed using a semi-quantitative method: amniotic fluid volume is considered abnormal when the largest pocket of fluid measures less than 2 cm in two perpendicular axes (Table 9). This represents a change from the previously more stringent definition of oligohydramnios used originally by Manning et al. (1980) (vertical pocket < 1 cm).

These scoring systems are limited to patients at high risk of fetal compromise with recognized maternal or fetal high-risk factors. The minimum gestation for testing the biophysical profile by Manning et al. (1980) has been 25 weeks. In gen-

Table 9. Assessment of the Biophysical Profile[a]

Parameter	*Criteria*
Non-stress cardiotocography	At least 2 FHR accelerations of 150 bpm and at least 15 seconds duration in 40 mins
Amniotic Fluid Volume	A pocket of amniotic fluid of at least 2 cm in depth (excluding cord)
Fetal Tone	At least 1 episode of flexion-extension-flexion in 30 mins
Fetal movements	At least 3 gross body movements in 30 mins
Fetal breathing movements	At least 30 secs of sustained fetal breathing in 30 mins

Notes: [a] A score of 0 or 2 is given for each of the biophysical parameters. If the criteria are met score is 2; if they are not met the score is 0. Reference: Fleischer et al. (1991).

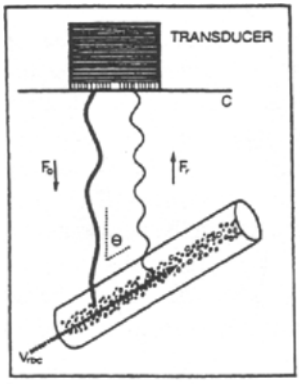
eral, Manning et al. schedules repeat testing on a weekly basis for all except post-dates, diabetics, and alloimmunized patients. The latter high-risk groups are recommenced to have at least a twice weekly examination and, in the case of severe rhesus disease the patients are seen daily. It is clear that a biophysical profile is conceptually very valuable, but its limitation lies in the need for a skilled time consuming examination.

Doppler Ultrasound

Physics

The Doppler effect is the change in frequency (transmitted verses received) of an acoustic or ultrasound wave that results when the total path length changes between transmitting and receiving sources (Figure 2). In clinical use, the transmitting and receiving sources are stationary and the change in path length results from movement of the target either towards or away from these transducers. In studies of blood flow, the moving column of blood within the interrogated vessels is the target. The red blood cells act as scatterers, reflecting the ultrasound beam. Fetal heart detectors record movement of the heart valves and chamber walls.

In obstetric applications, both continuous-wave and pulsed Doppler ultrasound systems are used, as well as color Doppler. Continuous-wave Doppler uses separate transmitting and receiving transducers, usually mounted side by side so that reflected signals from moving targets at any point along the ultrasound beam are recorded. Vessels can be located without prior imaging by adjusting the transducer



F_D = Doppler shift in frequency
$= F_s - F_r$
$= F_s \, 2\frac{V_{rbc}}{c} \cos \theta$
$F_D \propto V_{rbc}$

C = velocity of sound in medium
V_{rbc} = velocity of blood flow
θ = angle between ultrasound beam and direction of flow
F_s = transmitted ultrasound frequency
F_r = reflected ultrasound frequency

Figure 2. Diagram of the use of Doppler principle and Doppler velocity to study blood flow.

direction until the characteristic signal of the vessel to be studied is displayed (e.g., umbilical artery in the cord). This supplies the simplest Doppler display which is the audio signal. It is possible to provide an audio signal even if the ultrasound waves lie outside the audio frequency range, the Doppler shift frequency used in obstetric application lies within the audible range. But this system lacks spatial resolution in that the signals from all moving targets along the beam path are recorded. In obstetrics it is an important, inexpensive, easy-to-use tool often used to locate the fetal heart. Pulsed Doppler allows position and velocity information to be recorded. A sample volume or range gate can be located on a B-mode image so that only signals from that area are displayed. Pulsed Doppler systems have a limitation on the maximum Doppler frequency shift that can be detected before aliasing occurs. Real-time frequency spectrum analyzers are available to display the flow velocity waveform. Valuable clinical information can be obtained from the assessments of these Doppler frequency displays both during systole and diastole. Also the Doppler information can be stored for analysis. Measures such as mean frequency and various resistance indices can be calculated for immediate analysis and for future comparisons. Color Doppler uses detection of mean Doppler frequency shifts and assigns them a color according to the mean direction and magnitude. This flow information is then superimposed on the two-dimensional real-time images to form the complete color Doppler sonogram.

Clinical Information

The information about blood flow that Doppler studies give us consists of the flow velocity waveform and the volume of blood flow. Both will now be discussed.

Flow Velocity Waveform (FVW) The FVW contains information about the velocity of every blood cell within the blood vessel under interrogation by the ultrasound beam. Indices analyzing this waveform pattern have been developed as a measure of downstream resistance. Three indices, often referred to as resistance indices, are commonly used in perinatology: the systolic/diastolic (S/D) ratio, the pulsatility index (PI) and the pourcelot or resistance index (RI) (Figure 3). These indices are ratios, independent of the angle between the ultrasound beam and blood vessel. This is important when the angle is not known and so absolute velocity cannot be measured. This is the case with continuous-wave Doppler or when the vessel is small or tortuous and so unable to be adequately imaged (uterine artery and sometimes the umbilical artery).

There is good evidence to suggest that these indices do in fact reflect resistance downstream. In the umbilical artery waveform, a high resistance pattern may be recreated by embolization of the vascular bed. Theoretical proof is provided by mathematical analysis of a computer-based model of the umbilical vascular tree. The three indices are highly correlated with coefficients in excess of 0.9 having been reported. So it is unlikely that one index provides different or additional infor-

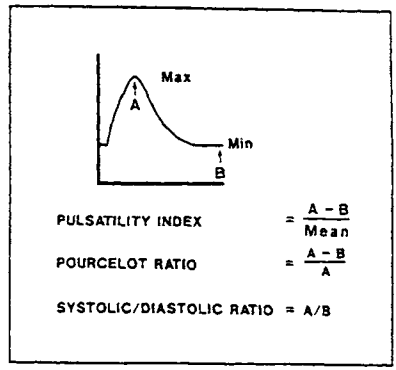


Figure 3. Diagram showing the method of calculating the three "resistance" indices.

mation than another. These indices are not normally distributed, so it is unlikely that the true mean velocity will be calculated accurately using the pulsatility index. Also error in the S/D ratio increases as the diastolic flow velocity becomes small, so commonly values above 6, for the umbilical artery, are grouped as extremely high. A further complication lies in the fact that there are two umbilical arteries known to supply different placental regions with variable overlap. Figure 4 shows how two umbilical arteries from the one umbilical cord provide different patterns. When following the progress of a fetus, interrogation of the same artery can not be guaranteed at each examination. Care must be taken with this phenomenon.

Volume Blood Flow (VBF). Volume blood flow (VBF) is measured as:

VBF(ml/min)= mean blood flow velocity x vessel area

There are several sources of error inherent in the methodology of pulsed-wave Doppler ultrasound when attempting to perform quantitative measurements of volume blood flow. These include:

1. The ability to measure vessel dimensions accurately.
2. The variation of blood velocity across the vessel cross-section.
3. Measurement of the angle between the ultrasound beam and blood vessel.
4. Position of the sample volume.
5. High-pass filtering of low-amplitude signals.
6. Attempt to normalize the VBF.

The physics involved with this new technique is beyond the scope of this chapter. Once perfected, though, it may well become an important adjunct to assessment of fetal well-being.

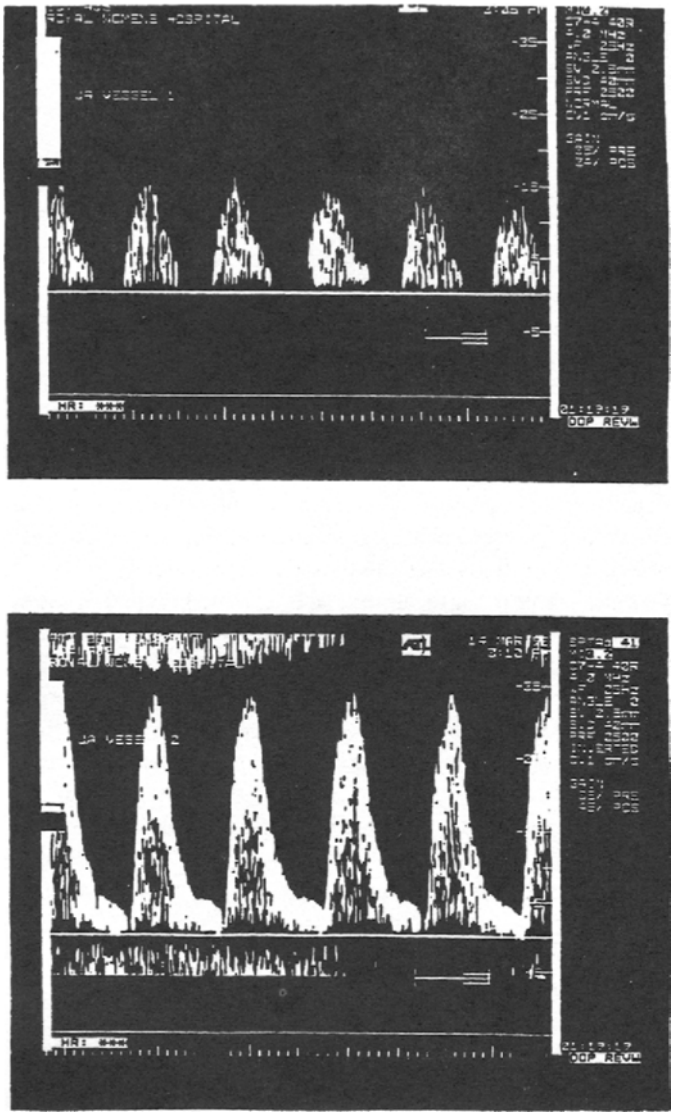


Figure 4. Two umbilical artery waveform patterns from the same umbilical cord but from the different arteries. The first shows absent diastolic flow.

Doppler Application in High Risk Pregnancy

Umbilical Artery Flow Velocity Pattern. The most important indicator of fetal compromise using Doppler waveform analysis is the presence of absent diastolic

flow (Figure 5) or reverse diastolic flow (Figure 6) as seen in the umbilical artery velocity waveform. Generally, however, S/D ratios above 6 are grouped as extremely high for which clinical intervention is often recommended.

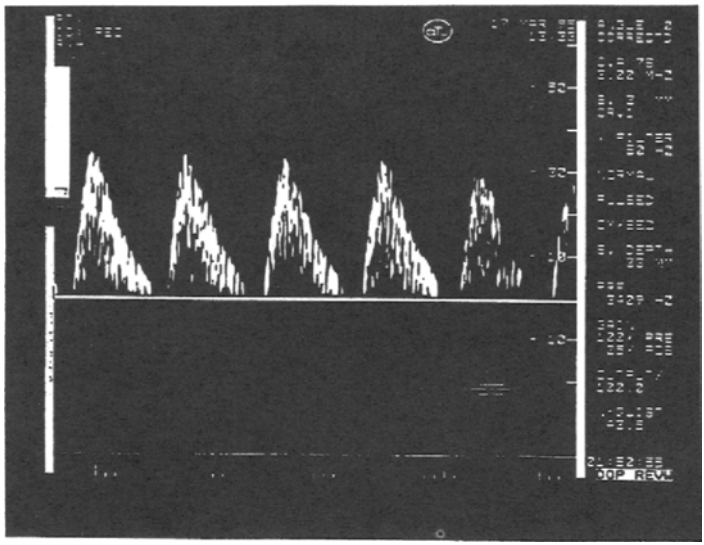


Figure 5. Doppler umbilical artery waveform pattern showing absent diastolic flow.

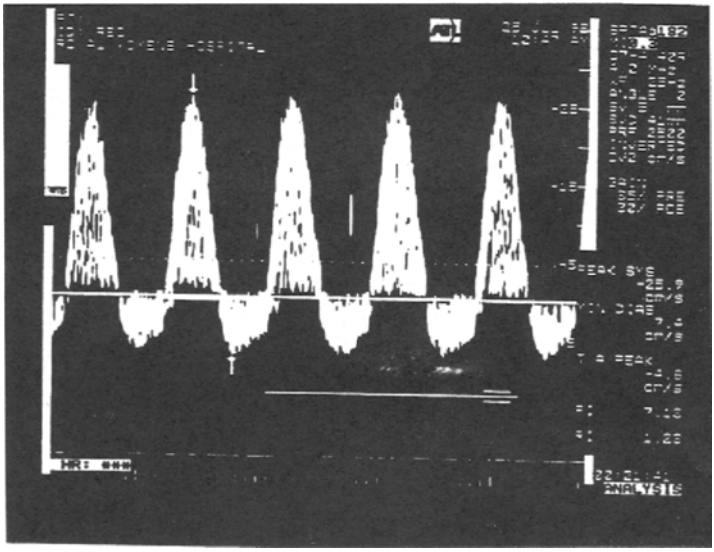


Figure 6. Doppler umbilical artery waveform pattern showing reverse diastolic flow.

Fetal Venous Flow. Recent studies suggest that assessment of the umbilical venous flow waveform may give indirect information on the presence of fetal compromise by the presence of reduced umbilical venous flow during arterial systole (seen in the umbilical artery). It is therefore important to insonate both the umbilical artery and vein simultaneously. The underlying mechanism is physiological tricuspid incompetence with increased venous pressure, which occurs as a consequence of increased cardiac intraventricular pressure as a response to the fetus being compromised. Ductus venosus venous flow pattern is also being assessed. Figure 7 shows abnormal ductus venosus flow pattern with reduced diastolic flow, supposedly representing a marker of fetal compromise.

Fetal Cardiac Assessment. Direct insonation of the fetal heart can give invaluable information on fetal well-being. The diagnosis of a structurally normal heart is paramount in perinatology especially with the high association between fetal cardiac structural anomaly and aneuploidy (about 25% association overall). This has major implications on pregnancy management. Also, fetal hypoxia results in increased resistance in non-vital organs with a subsequent increase in fetal cardiac output leading to increased intraventricular pressure causing physiological tricuspid incompetence. Tricuspid incompetence can be diagnosed using both spectral and color Doppler. An associated pericardial effusion can be seen using conventional B-mode imaging and the flow into the pericardial space has been visualized using color Doppler. This flow appears to be in a reverse direction to that in the ventricles during ventricular systole. The physiological assessment of

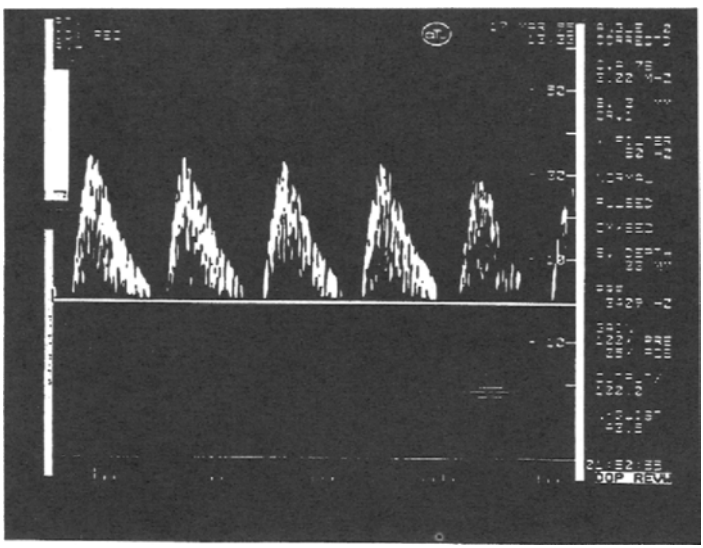


Figure 7. Doppler waveform pattern of abnormal ductus venosum venous blood flow suggestive of fetal cardiac compromise.

the fetal heart is therefore fast becoming an important biophysical marker of chronic fetal compromise, as seen in growth retarded fetuses and in cases of oligohydramnios.

Fetal Aorta Flow Velocity Waveform. There is, in fact, no evidence that the use of aortic flow velocity waveforms (FVWs) provides more information than the umbilical artery FVW.

Fetal Cerebral Circulation Flow Velocity Waveforms. Like other ultrasound variables of fetal well-being they should serve as a guide to management and always in conjunction with other ultrasound and clinical factors.

Maternal Uterine Circulation. Studies of the maternal uterine circulation have an uncertain clinical role. However, an important place could be its role as a potential early marker of pre-eclampsia. In the non-pregnant patient a diastolic notch normally is present in the uterine artery FVW. During the second trimester of pregnancy this notch normally disappears. The persistence of this diastolic notch in the second trimester has been used as a marker of pre-eclampsia.

Specific Clinical Application of Doppler Ultrasound

Diabetes Mellitus. Doppler FVWs are useful in cases of growth retardation. Cessation of fetal growth is associated with the development of a high-resistance pattern in the umbilical artery waveform. This is true whether the fetus is macrosomic or small (e.g., in mothers with diabetic vascular disease).

Maternal Lupus/Maternal Phospholipid Syndromes. Fetal deterioration is predicted by the development of an abnormal umbilical artery waveform; hence frequent examination of the fetus is recommended (at least weekly in the third trimester), as fetal demise may occur over a short period of time.

Major Fetal Structural Anomaly. Fetal growth retardation with high S/D resistance ratio is often seen, especially in the presence of oligohydramnios. Such a scenario in the later half of pregnancy should prompt the examiner to the risk of a major fetal anomaly.

Ultrasound Assessment of Fetal Cardiac Arrhythmias: M-mode, B-mode, Spectral, and Color Doppler

The indications for specific echocardiographic evaluation of heart rhythm are sustained fetal heart rates of less than 100 bpm, sustained heart rates of more than 180 bpm, repetitive irregular heart beats, and unexplained hydrops fetalis.

Systemic lupus erythematosus is a maternal condition that predisposes the fetus to heart block, so the fetus should be monitored for the development of bradycardia.

M-mode Echocardiography

M-mode echocardiography is performed using real time ultrasound by directing the recording line through the atrial free wall, atrial septum, AV valves, ventricular free walls, or semilunar (aortic, pulmonary) valves. The M-mode line inscribes the motion of these structures along a time base that can be varied according to the heart rate being examined. Once a recording is obtained appropriate measurements can be made.

B-mode Echocardiography

Fetus suspected of having an arrhythmia must be preceded by a detailed cardiac ultrasound B-mode examination. This is essential because certain arrhythmias (e.g., congenital complete heart block) are associated with serious congenital cardiac malformations. Hydrops fetalis may be manifest and associated with pericardial or pleural effusion and a dilated, poorly contractile heart, with or without evidence of physiological tricuspid incompetence. The atrial septum/foramen ovale must be examined for abnormal aneurysmal bulging into the left atrium, which may be the cause of the atrial arrhythmia. Tumors of the heart (rhabdomyomas, fibromas) may be associated with atrial or ventricular arrhythmias and can also be diagnosed.

Doppler Echocardiography

Spectral Doppler. This can be employed to interrogate blood flow through AV valves and used as an indicator of atrial contraction. Similarly, blood flow from ventricular outlets (great vessels) may be sampled as an indicator of ventricular contraction. By using a large sample range-gaited volume, Doppler flow patterns may be derived simultaneously from both inflow and outflow regions of the heart (e.g., mitral inflow and left ventricular outflow) allowing a comprehensive, quantitative definition of the atrial and ventricular contraction relationship. Abnormal regurgitant flow may also be noted through the AV valves (e.g., during diastole in patients with complete heart block). Additional pathological flow patterns such as reversal of flow in the inferior vena cave may indicate poor fetal hemodynamics resulting from the arrhythmia.

Color Doppler. Mapping can demonstrate valvular dysfunction and regurgitation. This assessment allows a qualitative appreciation of the severity of the arrhythmia and its hemodynamic compromise. Arrhythmia analysis may also be

aided by combining color flow Doppler with M-mode scanning. The resulting M/Q plot allows quantitative analysts of both the contractile events and the associated blood flow patterns.

PROCEDURAL ULTRASOUND

Karyotyping

The indications for fetal karyotyping are:

1. Advanced maternal age
2. Abnormal biochemical serum screening (Triple/Quadruple Test)
3. Family history
4. Past history
5. Anxiety
6. Ultrasound abnormalities

Advanced Maternal Age

It is well known that chromosomal abnormalities in the fetus increase with advancing maternal age. This is only true for the trisomies. Table 10 gives the maternal age-related risk of the trisomies at 9–14 weeks gestation, 14–20 weeks gestation, and at birth. This difference is explained by the high intrauterine spontaneous lethality for trisomies.

Traditionally, counseling parents as to the risk of fetal chromosomal abnormality depended on the provision of live birth incidences, mainly of trisomy 21. Table 10 allows us to properly counsel the patient prior to an invasive procedure by informing the patient of the trisomy risk to their fetus at the time of invasive testing. For CVS it would be that risk between 9–14 weeks (e.g., at 35 years it would be 1:122) and for amniocentesis it would be that risk between 15–20 weeks gestation (e.g., at 35 years it would be 1:200).

Table 11 demonstrates the fetal loss rate associated with diagnostic procedures. The risk of CVS between 9–14 weeks gestation would therefore approximate the risk of trisomy at a maternal age of 35 years. It would therefore be appropriate to offer CVS to a patient 35 or older. Similarly, the risk of amniocentesis between 14–20 weeks gestation would approximate the risk of trisomy at a maternal age of 35 years. It would therefore also be appropriate to offer amniocentesis to a patient 35 years or older. This is the rational used in genetic counseling when offering an invasive diagnostic procedure based on maternal age.

One of the problems with maternal age as a screening method for fetal aneuploidy is that there is less than 50% Maternal uptake of invasive testing and most pregnancies are to women less than 35 years of age. Overall only about 30% of tri-

Table 10. Maternal Age-Related Risk of Aneuploidy for Trisomies 13, 18, and 21 at 9–14 Weeks,15–20 Weeks Gestation and in Live Births

Age (Years)	*9–14 Weeks Gestation*	*15–20 Weeks Gestation*	*Live Births*
30	1:290	1:473	1:798
31	1:253	1:414	1:699
32	1:217	1:355	1:600
33	1:183	1:299	1:503
34	1:150	1:247	1:416
35	1:122	1:200	1:337
36	1:98	1:160	1:270
37	1:77	1:126	1:213
38	1:60	1:98	1:166
39	1:47	1:76	1:129
40	1:36	1:59	1:99
41	1:27	1:45	1:75
42	1:21	1:34	1:58
43	1:16	1:26	1:44
44	1:12	1:19	1:33
45	1:9	1:15	1:25

Table 11. Fetal Loss Rate Associated with Procedures

CVS	*Amniocentesis*	*Fetal Blood Sampling*
1:100–150	1:200	l:50

somies are diagnosed prior to birth. This is why methods are being investigated to increase the antenatal fetal abnormality pick-up rate.

Abnormal Biochemical Serum Screening Testing

The Triple Test includes (AFP) alpha fetal protein, free (b-HCG) b-chain human chorionic gonadotrophin, and estriol. With the maternal serum level of these three chemicals and accurate early pregnancy dating (menstrual dates or ultrasound), a risk score can be allocated to each pregnancy irrespective of maternal age. But the detection rate of fetal aneuploidy using the Triple Test in fact improves with increase in maternal age. The quoted results are shown in Table 12.

The Quadruple Test, which also includes maternal serum free a-HCG, has been used to improve detection rates and to reduce the false positive rate (Table 13). Both serum screening tests are also used to screen for neural tube defects using the AFP component.

Table 12. Detection Rate of Down Syndrome using Triple Serum Maternal Screening

	<37 Years	*>37 Years*	*Overall*
Detection rate	39%	71%	48%
False positive	3.3%	20%	
Odds of being affected	1:56	1:25	

Table 13. Screening Detection Rate using the Quadruple Test (1:300 Cut-off)

Maternal age group	<25	25–29	30–34	35–39	40–44	>44	All
Down syndrome detected	45%	50%	60%	80%	95%	>99%	65–70%

The acceptance rate of invasive testing with serum screening was 75% overall which is a significant improvement on the less than 50% rate using maternal age alone. The overall detection rate of 48% with the Triple Test and 65–70% with the Quadruple Test, compared to around 30% with maternal age alone, is also significant.

Family History

The following are the indications for karyotyping based on family history. a) Familial chromosome re-arrangement in any one of the parents. If any one member of a couple carries a chromosomal re-arrangement (balanced translocation) the risk that this re-arrangement might cause fetal aneuploidy is 1:10. b) Familial history of chromosomal abnormality which was not age-related, and in the absence of a chromosomal imbalance in either partner, then the risk is either 1:100 or the age-related risk for that patient (Table 10), whichever is greater. And, c) family history of chromosomal abnormality which was age-related, and in the absence of a chromosomal imbalance in either partner, then the risk is the age-related risk for that patient (Table 10).

Past History

Previous aneuploidy leads to a recurrent risk of 1:100 or the age-related risk (Table 10) whichever is greater, assuming there was no chromosomal imbalance in either partner.

Parental Anxiety

Despite explanation of the risks involved with the invasive procedures, and despite reassurance of low risk of a fetus with aneuploidy, some parents will still elect to have karyotyping performed. The most important point here is that the parents are aware of the implications of their decision and are comfortable with any possible outcome. Alleviation of anxiety is an important role of the perinatologist for any pregnant couple.

Ultrasound Abnormalities

The assessment of fetal nuchal translucency initially described by Nicolaides in 1992 has made a significant contribution to fetal chromosomal screening.

Nuchal translucency is the thickness of black space (fluid) in the back of the neck region of the fetus. Findings in more than 90,000 pregnancies worldwide have shown thus far that in most fetuses, some fluid can be seen and therefore measured. The test is simply an abdominal ultrasound measuring the thickness of this translucency. This test has the highest detection rate of any non-invasive test for chromosomal abnormalities. The detection rate is 80% compared to 65% for maternal serum screening and 30% for maternal age screening alone. The other advantage is that the test is performed between 11 and 13 weeks gestation. Other abnormalities such as spina bifida and anencephaly may also be excluded at this stage. It has also been shown that an increased nuchal translucency thickness is associated with fetal cardiac malformations.

Ultrasound features of chromosomal abnormalities have been described on pages 232-234.

Invasive Ultrasound-Guided Techniques for Karyotyping

Amniocentesis

Amniocentesis was first used in the 1880s for decompression of polyhydramnios. During the 1950s amniocentesis was used to measure the bilirubin concentration in rhesus disease. Its application to its most familiar role, fetal chromosome analysis, became important after 1956 when the number of chromosomes was first reported. Culturing and karyotyping amniotic cells was demonstrated by Steele and Berg in 1966. The first prenatal diagnosis of an abnormal karyotype (a balanced translocation) was reported by Jacobsen and Barter in 1967. Trisomy 21 was first detected prenatally by Valenti et al. in 1968. During the same year, the first diagnosis of a metabolic disorder was reported by Nadler.

During the 1960s amniocentesis was performed blindly. During the 1970s and early 1980s, ultrasound, initially static and subsequently real-time, was used to locate a placenta-free pocket of liquor as a needle-entry area. The position was marked on the maternal abdomen, and after a variable length of time, the operator would blindly insert the needle. However, in most centers today, amniocentesis is performed with continuous ultrasound guidance (Figure 8). The technique is achieved with either sector or linear-array transducers, and is done either freehand or with needle guides. See Table 11 for fetal loss rate. The major drawback with amniocentesis is that the procedure is performed in the second trimester. This delay increases emotional stress as well as there being an increased medical risk involved in performing dilatation and evacuation at this time if it were necessary.

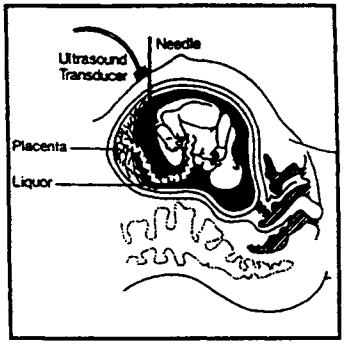


Figure 8. Technique of amniocentesis under ultrasound guidance.

Chorionic Villous Sampling

CVS overcomes the drawback associated with amniocentesis as the chorionic villi can be used as a source for fetal karyotyping during the tenth week of pregnancy. Hahnemann and Mohr first introduced the technique experimentally in 1968. The use of an endoscopic transvaginal approach was evaluated by the same group in 1974. In 1975 a Chinese group described the clinical use of chorionic villi for fetal sex determination by performing blind transvaginal aspiration without ultrasound examination. CVS became accepted for more widespread use when the ultrasound-guided technique for aspiration and the direct method for chromosome preparation were introduced.

CVS can be performed transvaginally and transabdominally, both under ultrasound control, with comparable safety and efficacy when performed by competent operators. CVS affords the choice and advantage of early prenatal diagnosis to women who have the appropriate indications for the procedure. See Table 11 for fetal loss rate. The list of conditions that can be DNA analyzed is expanding every year. Whether genetic testing is performed for any of these will be the decision of each individual following adequate counseling. As the list expands, inevitable social and personal ethical questions will need to be addressed. Such questions, though important, lie beyond the scope of this chapter. Examples of these conditions include: autosomal dominant (e.g., Huntington disease), autosomal recessive (e.g., cystic fibrosis), and X-linked recessive (e.g., Duchenne's muscular dystrophy) conditions.

Fetal Blood Sampling

In 1983, Daffos et al. first described a method of obtaining fetal blood under ultrasound guidance involving the transabdominal introduction of a 20-gauge spinal needle into the umbilical cord. The technique offered major advantages to the fetoscopic technique at the time. Subsequently, there have been variations to the tech-

nique, but they all still use this fundamental approach. Interchangeable terms to describe this technique include: percutaneous umblical blood sampling (PUBS), cordocentesis, fetal blood sampling (FBS), and funipuncture.

A major advantage of FBS is the ability to obtain rapid fetal karyotyping in less than seven days. The major disadvantages are the increase in fetal loss rate (Table 11), and the expertise required to perform the examination. General indications for FBS include: i) chromosome analysis for aneuploidy, ii) DNA analysis for other inheritable disorders, iii) fetal thrombocytopenia, iv) diagnosis of infectious disease, v) fetal well-being (of limited use at present), vi) fetal anemia, vii) fetal therapy (e.g., blood transfusion for rhesus affected fetuses and e.g., stem cell transplantation in the near future).

Fetal Biopsy

Skin. Fetal skin sampling is optimally performed between 17–20 weeks gestation, under ultrasound guidance. Examples of such skin conditions include: bullous congenital ichthyosiform erythroderma (autosomal dominant), and epidermolysis bullosa dystrophica (autosomal recessive).

Liver. Fetal liver biopsy is nowadays only performed for those families whose DNA analysis is not informative for the detected mutations in the ornithine transcarbamylase gene and the carbamyl phosphate synthetase gene. This technique is therefore rarely performed, but when it is, it is best performed between 17–20 weeks gestation using a 16.5-gauge thin-walled needle, under ultrasound guidance.

Muscle. The prenatal diagnosis of the X-linked recessive disease known as Duchenne's muscular dystrophy (DMD) is made using DNA analysis (amniocentesis, CVS, or FBS). When recombination occurs within the DMD gene, DNA analysis is uninformative, or carrier status cannot be ascertained. Fetal muscle biopsy with dystrophin analysis of the fetal muscle may provide the only method of prenatal diagnosis. Dystrophin deficiency is fully manifested by the late first trimester. The first successful prenatal diagnosis using dystrophin of fetal muscle biopsy was performed by Evans et al. in 1991. The technique is best performed between 17–22 weeks gestation using a 14-gauge Tru-cut biopsy needle under ultrasound guidance.

Ultrasound-Guided Fetal Surgery

Fetal Shunting Procedures

A new era in invasive fetal therapy began in the early 1980s, not only with the introduction of shunting procedures for hydrocephalus and hydronephrosis, but also with the introduction of fetal hydrothorax drainage as well as other miscellaneous fetal drainage procedures (e.g., fetal ovarian cyst aspiration). The literature experience, though limited, would suggest that with adequate patient selection, the potential benefit of ultrasound guided abnormal fetal fluid aspiration and shunt

placement far outweighs the risks of the procedure. Risks include: infection, bleeding, premature rupture of membranes, preterm labor, and fetal injury.

REASSURANCE/BONDING

An ultrasound examination has the potential to be an enjoyable experience for the parents, but it can also be a traumatic event. In the available studies, the majority of women valued the role of ultrasound in confirming the reality of the baby for them. The examination was often followed by a reduction in the anxiety and an increase in confidence. One study in the United States found that women perceived that nearly half the value of the examination pertained to issues outside the field of medical decision, such as knowing the sex of the child or having an early picture to show their children. This is a point that must be understood and acknowledged by the examiner. On the other hand, ultrasound can lead to psychological damage if a real or mistaken diagnosis of fetal abnormality is made. Another practical point is that the subjective experience of actually having a scan, even if the findings are normal, can be unpleasant because of uncommunicativeness on the part of the examiner. Proper allocation of examination time to each patient may help to alleviate this problem.

The only generalization that can be made based on available data is that there is a wide variation in women's views and expectations of an ultrasound examination. The obvious implication of this, to the examiner, is to take this variation into account when dealing with individuals. But health authorities, or care providers, must also be made aware of this, so that enough time is allocated to cater not only for medical requirements of the examination, but also to all the other needs of the parents.

NEONATAL TRANSCRANIAL ULTRASOUND (Intracranial Hemorrhage)

Intracranial hemorrhage (ICH) is the most common and serious cause of neurological morbidity and mortality in the newborn. With the advent of modern neonatal intensive care facilities younger infants are now surviving. As the immature brain is most susceptible to hemorrhage its incidence has therefore increased. It is particularly high in neonates born at less than 32 weeks gestation. The main cause is rupture of the fragile capillaries in the neonatal brain for various causes, with prematurity and birth asphyxia correlating most strongly. The germinal matrix becomes increasingly avascular with increasing gestation. Table 14 gives a summary of the ultrasound features of hemorrhage and Table 15 shows the gradings of ICH using these ultrasound features.

There are a few important points to remember: i) 30% of IVH is not associated with ventricular dilatation; ii) CPH can lead to IVH without underlying SEH; iii) hemorrhage, with any of the above gradings, can also occur (though rare) in the fetus; iv) the prognosis worsens as the grading increases; and v) the prognosis of IPH

Table 14. Ultrasound Features of Hemorrhage

Type		*Ultrasound Features*
Acute	(0–1 week)	Highly echogenic without shadowing
Subacute	(1–2 week)	Moderately echogenic with central sonolucency
Chronic	(>2 week)	Moderately echogenic with hematoma retraction
	(2–6 month)	Sonolucency (porencephaly)
Acute-on-chronic		Combination of above features

Table 15. Ultrasound Grading of Intracranial Hemorrhage

Grading	*Features*[a]
Grade 1	SEH
Grade 2	SEH +/or CPH + IVH
Grade 3	SEH +/or CPH + IVH + VD
Grade 4	SEH +/or CPH + IVH + IPH

Notes: SEH, Subependymal hemorrhage; CPH, Choroid plexus hemorrhage; IVH, Intraventricular hemorrhage; VD, Ventricular dilatation; IPH, Intraparenchymal hemorrhage.

depends on both the size and location of the hematoma. So it is important not only to quantify the IPH but also to note its location in the brain when performing a neonatal brain ultrasound examination.

As most ICH occur in the first three days of life, with 91% occurring by the fifth day, routine screening in the preterm neonate less than 32 weeks should be performed around day 5. If there is any evidence of respiratory distress, disseminated intravascular coagulation, or neonatal seizures then ultrasound should be performed earlier. A normal cranial ultrasound examination on day 1 does not exclude the development of intracranial hemorrhage later on. Most grades 3 or 4 intracranial hemorrhage occur in the first 24 hours of life. Table 16 shows the recommendations for neonatal head ultrasound in neonates <32 weeks old.

Sequelae of Intracranial Hemorrhage

ICH may lead to anatomical and neurological sequelae. The anatomical features that are detectable on ultrasound include subependymal cysts, porencephaly and hydrocephalus. In the presence of intraventricular hemorrhage weekly head ultrasounds are required to exclude hydrocephalus (Table 16), but the optimum time for diagnosis is two weeks. Often aqueduct stenosis occurs (secondary to either blood in the aqueduct or from ventriculitis). This can also be detected by ultrasound. The neurological sequelae depends on the ICH gradings. Grades 1 and 2 show good potential for normal development in early years. Grades 3 and 4 show increasing morbidity and mortality. Grade 4 has a 54% mortality rate, especially if there is either

Table 16. Recommendation for Neonatal Cranial Ultrasound in Neonates <32 Weeks

Day 1	To exclude grade 3, 4 ICH
Day 5–7	To exclude ICH
Any other time	Neonate respiratory distress, DIC, seizures, post-arrest. Some units also recommend an examination prior to discharge from the intensive care unit
Weekly	In the presence of IVH to exclude the development of hydrocephalus (ventricular dilatation). Usually this occurs 2 weeks after IVH

thalamic extension, posterior fossa extension, or a midline shift. Ultrasound is excellent at assessing these features. However, as there is a large variation in outcome in each grade and as there have not yet been adequate follow-up studies to determine long-term (adult) sequelae, caution should be exercised before predicting the outcome for an individual child purely on ultrasound examination.

Types of Intracranial Hemorrhage

Subependymal Hemorrhage (SEH)

This condition occurs predominantly in preterm neonates especially in the frontal and parietal lobes and is often seen on ultrasound adjacent to the frontal horns of the lateral ventricles. Extension of hemorrhage to the thalamic region, secondary to massive hemorrhage, often leads to severe hydrocephalus, and subsequent neonatal death. Hemorrhage in the occipital horn is rare as there is no germinal matrix, but extension from massive hemorrhage in the parietal lobe can involve the occipital lobe.

Periventricular Hemorrhagic Infarction (PHI)

This condition leads to periventricular leukomalacia (PVL). It is often located on ultrasound lateral to the frontal horns and to the bodies of the lateral ventricles. Ventricular rupture with IVH is uncommon and on ultrasound PHI appears as a region of high echogenicity often described as a flare.

Others

These include: (i) cortical hemorrhage, (ii) diffuse cerebral haemorrhage, (iii) cerebellar hemorrhage, and (iv) brain stem hemorrhage. Though they are all rare, they are able to be diagnosed using ultrasound imaging. Extracerebral hemorrhages are best diagnosed using CT scanning.

SUMMARY

Diagnostic ultrasound uses sound energy with a wave frequency that exceeds the audible limit in humans (20,000 Hz). Ultrasound is the modality of choice for fetal imaging. It is used in pregnancy to determine gestational age, detect multiple preg-

nancy, localize placentas, diagnose fetal anomalies, evaluate fetal well-being, and guide fetal diagnostic and treatment procedures. The antenatal identification of an abnormal fetus allows the opportunity for prenatal counseling with a multidisciplinary team of experts, with a thorough discussion of pregnancy options. The impact of high-resolution dynamic ultrasound imaging has given the perinatologist the ability to visualize the fetus and its activities. This allows us to perform a physical examination on the fetus, creating the concept of the fetus as a patient. The use of Doppler ultrasound to study blood flow in obstetrics is of major importance because fetal inaccessibility precludes many other methods of study of the circulation. This includes the use of spectral, color and power Doppler.

Ultrasound has the ability to rapidly and accurately establish the normality and well-being of a fetus or neonate. With properly qualified examiners using modern up-to-date equipment, there are a vast number of congenital anomalies that can be diagnosed on a thorough ultrasound examination. These include abnormalities of the central nervous system, spine, neck, face, thoracic, abdominal wall, gastro-intestinal tract, urinary tract, genitals, skeletal, hand, feet and umbilical cord. Real-time ultrasound permits the evaluation of multiple fetal activity (fetal movement, breathing, and tone) as well as the assessment of the intrauterine environment. The most important factor is the combination of acute (fetal heart rate reactivity, fetal movement, fetal breathing movements, and fetal tone) and chronic (amniotic fluid volume) markers of the fetal condition.

It is well known that chromosomal abnormalities in the fetus increases with advancing maternal age. Chorionic villous sampling, amniocentesis and fetal blood sampling allows us to confirm fetal normality in a select group of patients.

An ultrasound examination has the potential of being an enjoyable experience for the parents, leading to a reduction in the anxiety and an increase in confidence. However, ultrasound can also be unpleasant if a real or mistaken diagnosis of fetal abnormality is made. Communication is vital as well as is the proper allocation of examination time to each patient.

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Chapter 13

Placental Toxicology

B.V. RAMA SASTRY

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INTRODUCTION

Placenta Types and Functional Aspects

On a physiological basis, placentas are classified into four types: 1) hemochorial (human, rat, rabbit); 2) endotheliochorial (cat, dog); 3) syndesmochorial (sheep, ruminants); and 4) epitheliochorial (pig, horse). There are anatomical and functional differences among different types of placentas. Due to practical and technical advantages, animal placentas have been used to study placental function and toxicology. Although qualitative similarities among several types of placenta are present, identity of function and effects of toxicants should not be assumed.

The human placenta is of the hemochorial type, in which the fetal tissue is in direct contact with the maternal blood. The membrane separating the maternal and fetal compartments is thin and consists of only three layers: syncytiotrophoblast, connective tissue, and vascular fetal endothelium. The intensity of the passage of substances across the placenta is inversely proportional to the thickness of placental membranes. For example, the magnitude of Na^+ exchange across different types of placentas can be arranged in the following order: hemochorial > endotheliochorial > syndesmochorial > epitheliochorial. In all types of placentas, Na^+ exchange increases with gestation, reaching maximum shortly before term. In the hemochorial placenta, the exchange decreases at the end of gestation due to deposition of fibrinoid on the exchanging surface. Exchange involves not only physiological constituents, but also substances or elements which represent a pathologic risk for the fetus. Therefore, in assessing placental toxicology of chemicals, all physiological and functional variables should be taken into consideration.

Placenta and Dysmorphogenic Agents

The placenta is an important fetal accessory structure which brings maternal and fetal circulations and tissues into close relationship. Morphologically, it is partly of fetal origin (the trophoblast) and partly of maternal origin (from a transformation of the uterine mucosa). In humans, it is differentiated by the third month of pregnancy. During the embryonic period (the first two months of pregnancy), the embryo is morphologically defined, and its organs are outlined. During the fetal period (2–9 months), the organs undergo maturation at the histological (histogenesis) and functional levels. During this period, the fetus grows in size (crown-rump length), vol-

ume, and weight. During the embryonic period, there is no placental barrier. Drugs and environmental chemicals have easy access to the developing embryo and interfere with different stages of morphological differentiation. Chemical- or drug-induced dysmorphogenicity has been recognized in experimental animals for many years. However, the clinical implications of these experimental results have been widely recognized since 1961 due to the discovery of thalidomide-induced phocomelia and other embryopathies. The critical exposure of the embryo occurs *in utero* when the sedative drug thalidomide (100 mg) is ingested by a pregnant woman in the fourth and sixth weeks of pregnancy. About 8,000 victims of this teratogenic substance have been born (Rodin et al., 1962; Taussig, 1962).

Experiments in animals have revealed many chemical agents as well as physical and chemical factors which exhibit dysmorphogenic effects (Tuchmann-Duplessis, 1975). These include (a) physical factors, X-rays, and anoxia; (b) infection, rubella and toxoplasmosis; (c) endotoxins; and (d) chemicals, solvents, and dyes. Some of these chemical compounds have a low general toxicity. Others are highly cytotoxic. Although many dysmorphogens have been discovered in animals, proof of their noxious effects on the human embryo has been provided in only a few cases. For example, aminopterin, a cytotoxic drug, is an abortifacient in some humans (Thiersch, 1952, 1956). In some fetuses, it produces malformations in the central nervous system and considerable growth retardation. Thalidomide embryopathies are characterized by skeletal malformation, normal growth, and well-developed intelligence in humans. However, it induces nervous system anomalies in rabbits (Tuchmann-Duplessis, 1975). It is not possible to transfer information on the embryotoxic nature of drugs and chemicals from animal experiments to humans.

During the fetal period, the fully formed placenta plays an important role in the maintenance of nutrition to the fetus and in the secretory and regulatory functions that are essential for the maintenance of pregnancy. All supplies to the embryo and fetus—oxygen, water, electrolytes, nutrients, hormones, and antibodies—must pass through the placenta. Several enzymes involved in the four basic processes of drug biotransformation—oxidation, reduction, hydrolysis, and conjugation—are present in the placenta. The placenta has a significant role to play in the synthesis of steroid and protein hormones and of several regulatory factors including acetylcholine, opioid peptides, tachykinins, platelet activating factor (PAF), and prostaglandins. If the placenta is not functioning properly, it can be the limiting factor not only for fetal nutrition, but also for the maternal-fetal exchange of physiological constituents and fetal-maternal exchange of waste products that may represent a pathologic risk to the fetus.

The purpose of this chapter is to discuss the human placenta as a target organ for drug- and chemical-induced injury. The chapter also emphasizes those effects of abused pharmacologically active agents (morphine, cocaine, and components of tobacco smoke) on placental function that can ultimately lead to fetal growth retardation and other adverse reproductive effects in humans. Further, the adverse ef-

fects of several environmental chemicals like pesticides and herbicides on human placental function are examined.

ABUSED DRUGS AND PLACENTAL FUNCTION

There are several drugs which are abused by humans. These include nicotine (through tobacco smoking), morphine, cocaine, and alcohol. If pregnant women are exposed to abused drugs or alcohol, their placental functions will be compromised. The common effect of all these four drugs is intrauterine fetal growth retardation (IUGR). In IUGR, there is a decrease in all dimensions of fetal growth, indicating a common cause for the development of all organs of the fetus. One of the requirements for the growth and development of fetal organs is essential amino acids for protein synthesis. Therefore, effects of abused drugs on the placental transport of amino acids are emphasized.

Maternal Smoking, Placental Function, and Amino Acid Transport

The effects of maternal smoking and nicotine use on placental function have been reviewed by Sastry and Janson (1995). Maternal smoking alters many biochemical parameters in the maternal (e.g., blood carboxyhemoglobin), placental (e.g., lowered oxygen consumption), and fetal (e.g., decreased breathing movements) compartments. The final result of all these alterations is fetal growth retardation as indicated by low infant birth weight. Babies of smokers are, on average, 200 g lighter than babies born to comparable nonsmokers (Hasselmeyer et al., 1979). The whole weight distribution of babies of smokers is shifted downward compared with the weight distribution of babies of nonsmokers. Smoking-induced fetal growth retardation is not related to the characteristics of the smoker. The more the mother smokes, the greater the reduction in fetal growth. This fetal growth retardation may be related to disturbances in amino acid metabolism as indicated by depressed placental transport of amino acids in smokers.

The fetus is dependent on the placental transfer of amino acids from maternal to fetal circulation for its requirements of amino acids. The placental transfer of amino acids is a two-step process: (a) active uptake of amino acids by placental trophoblast cells from the mother's blood; and (b) passive diffusion of amino acids from placental trophoblast cells into umbilical venous blood. The first step is critical, and its efficiency could be compromised, resulting in depressed uptake into trophoblast under placental hypoxic conditions induced by maternal smoking. Nicotine and tobacco smoke components (carbon monoxide, cyanides, and nitrites) reduced active uptake of amino acids by isolated human placental villi (Rowell and Sastry, 1978; Barnwell and Sastry, 1983; Sastry et al., 1983; Sastry 1984). Exposure of human placental villi to nicotine inhibited the uptake of α5-aminoisobutyric acid (α-AIB) and decreased both maximum velocity (V_{max}, 71%) and Michaelis-Menten

constant (K_m, 67%) for uptake of α-AIB (Barnwell and Sastry, 1983). Part of the inhibition (16%) is not reversible, which may be of significance in chronic smoking (Sastry, 1991a). Concentrations of several essential amino acids (val, met, ileu, leu, tyr, phe, his) and nonessential amino acids (asp, glu, gly, ala, arg) in the placental villi of nonsmoking mothers were about 30–50% higher than those of smokers (Sastry et al., 1989b). Concentrations of thr and phe were about 14–15% higher in the placental villi of nonsmokers than in those of smokers. According to these observations, maternal smoking decreases the uptake of amino acids by the placenta and thus the net transfer of amino acids from maternal to fetal blood. Further, all the placental transfer of all essential amino acids is not affected equally, indicating that the nitrogen balance may be disturbed in the fetus. Therefore, fetal undernutrition for amino acids may provide partial explanation for fetal IUGR in tobacco smokers.

Maternal Smoking and the Regulatory Mechanisms for Amino Acid Uptake by the Placental Trophoblast

α-AIB, a nonmetabolizable amino acid, has been used as a model amino acid to study the mechanisms of amino acid uptake by human placental tissue. Four regulatory mechanisms have been proposed for the cellular uptake of amino acids in the placenta: (a) gammaglutamyl cycle for the regulation of amino acid transport (Meister and Anderson, 1983; Payne and Payne, 1984), (b) placental acetylcholine release and amino acid transport coupling (Harbison et al. 1975; Rowell and Sastry 1978, 1981; Sastry et al., 1983), (c) phospholipid N-methylation in the plasma membrane (Barnwell and Sastry, 1987), and (d) oxidative energy sources (Longo et al., 1973; Miller and Berndt 1974).

The gammaglutamyl (GC) cycle was postulated for amino acid transport by Meister and Anderson (1983). Gammaglutamyltranspeptidase (GGTP) plays a key role in the GC cycle. It is bound in the outside bilayer of the plasma membrane. Glutathione (gammaglutamylcysteinylglycine) is continuously secreted onto the cell surface, where GGTP transfers a glutamyl group to an incoming molecule of an amino acid. The products, gammaglutamyl-amino acid and cysteinylglycine, are reabsorbed into the cell. The bulk of the amino acid molecules is absorbed by independent transport systems. The number of these carrier molecules may be inversely related to the degree of absorption of gammaglutamyl-amino acid. During starvation of the cells for amino acids (or during low levels of extracellular amino acids), more transport carrier molecules are induced and incorporated into the plasma membrane. Thus, gammaglutamyl-amino acids may serve as transmembrane or environmental signals for the uptake of amino acids by cells (Payne and Payne, 1984). Cells can use the GC cycle for efficient recovery of cysteine. Human trophoblastic microvilli contain high levels of GGTP (Barnwell and Sastry, 1987). Maternal smoking during pregnancy decreases the activity and V_{max} of GGTP of the microvillus plasma membrane (Sastry and Horst, 1988; Horst and Sastry, 1988; Sastry et al., 1989a). This decreases the formation and absorption of gammaglutamyl-amino

acids, signaling the cell to induce the synthesis of more amino acid transport carriers and to insert them into the plasma membrane. There is an increase in the uptake of α-AIB, mainly due to an increase in V_{max}, in washed placental villi from smokers. The induction of placental α-AIB transport sites during maternal smoking can be partially explained by depression of the GC cycle. However, in the presence of tobacco-smoke components, the increased carrier systems do not fully compensate for the depression of the GC cycle. The effects of maternal smoking on components of the GC cycle other than GGTP have yet to be investigated.

Depression of the synthesis of acetylcholine (ACh) by inhibitors of choline acetyltransferase (ChA) (Rowell and Sastry, 1981; Sastry et al., 1983) and inhibition of the conversion of membrane phosphatidylethanolamine to phosphatidylcholine by phospholipid *N*-methyltransferases (PMTs) (Sastry et al., 1983; Barnwell and Sastry, 1987) decreases amino acid uptake by placental villi. Therefore, it has been postulated that the placental cholinergic system and phospholipid *N*-methylation regulate amino acid uptake by the placenta. Both ChA and the PMTs contain active -SH groups. The cadmium ions in tobacco smoke inhibit these enzymes (Sastry et al., 1984, 1985). The PMT activity of the placental plasma membrane is depressed in cigarette smokers (Horst and Sastry, 1989).

Oxygen consumption by placental slices from smoking mothers decreases in proportion to CoHb concentrations in maternal blood (Tanaka, 1965). The rate of oxygen consumption in placental slices from nonsmoking mothers (1.9 $\mu l\ mg^{-1}\ h^{-1}$) is about 30% higher than that in placental slices from smoking mothers (1.3 $\mu l\ mg^{-1}\ h^{-1}$, at an 8% maternal CoHb concentration). These observations indicate that the energy-dependent processes of placental cells may be depressed, and that the formation and concentrations of cellular adenosine 5'-triphosphate (ATP) may be affected by hypoxia. All of the above mechanisms for the regulation of amino acid transport are ATP-dependent. Three ATP molecules are required for operation of the GC cycle and the uptake of one molecule of gammaglutamyl-amino acid. Another ATP molecule is required to form one molecule of acetyl-coenzyme, a substrate for ChA to form one molecule of ACh. Still another ATP molecule is required to form one molecule of S-adenosyl-L-methionine (SAM). Three molecules of SAM are required to form one molecule of phosphatidylcholine by phospholipid methylation. Therefore, all postulated regulatory mechanisms of amino acid transport in the placenta may be indirectly affected by maternal smoking.

The Placental Opioid System and its Role in Amino Acid Transport

Different aspects of the human placental opioid system have been reviewed by Sastry (1995a) and Ahmed and Cemerikic (1995). When ingested by pregnant women, morphine and its analogues are known to retard intrauterine fetal growth, the extent of which is related to the degree of exposure. This raises questions about the role of the placenta in morphine-induced IUGR and about the occurrence and role of endogenous opioids. Generally, it is believed that enkephalins and endor-

phins may serve as neuromodulators and may regulate neurotransmitter or hormone release by positive or negative feedback systems (Goldstein, 1976). More specifically, enkephalins may regulate neuronal release of ACh and norepinephrine by negative feedback systems (Sastry, 1991b, 1995b). The occurrence of methionine enkephalin and β-endorphin in human placental villi has been demonstrated using sensitive and specific radioimmunoassays and bioassays. Immunoreactive corticotrophin, lipotrophin, and β-endorphin occur in whole placental extracts. Dynorphin 1-8 was also identified in placental extracts by mass spectroscopy. The opioid receptor of the placenta was also purified and identified as the kappa subtype (Ahmed et al., 1989a). When the floating villi, chorionic plate, and basal plate of the same human placenta were extracted and analyzed, the methionine enkephalin concentration was lower in the chorionic and basal plates. This suggests that the distribution of methionine enkephalin is similar to that of ACh in human placenta.

ACh occurs in high concentrations in the human placenta, which is not innervated. However, the release of ACh from human placental villi resembles that from the nerve in several respects (Olubadewo and Sastry, 1978). Isolated human placental villi contain 167 nmol ACh/g wet tissue. When such villi are incubated in a medium containing Krebs-Ringer bicarbonate buffer (pH 7.2–7.4, Ca^{2+} 2.54 mM), ACh is spontaneously released at a rate of 35 pmol/g/min. The rate of release of ACh was enhanced by: (a) depolarization with high concentrations of K^+ (17–63 mM); (b) increasing concentrations of Ca^{2+} (4.64–9.4 mM); and (c) nicotine. ACh was not released in the absence of extracellular Ca^{2+}. Depolarizing concentrations of K^+ and nicotine did not increase the rate of release of ACh in the absence of extracellular Ca^{2+}. Morphine and enkephalins inhibited ACh release in nervous tissue by inhibiting Ca^{2+} influx. The kappa opiate receptor agonist, ethylketocyclazocine (100 μM), depressed the rate of spontaneous release of ACh by about 50%. The kappa antagonist, (-)-2-(3-furylmethyl)-noretazocine (1 mM), enhanced the rate of ACh release by about 18-fold. These observations indicate that endogenous methionine enkephalin, β-endorphin, and other opiate agonists downregulate ACh release by a negative feedback mechanism. They may decrease Ca^{2+} influx into the trophoblast and decrease ACh release (Ahmed et al., 1989b).

ACh release, activation of a cholinergic receptor, Ca^{2+} influx, and amino acid transport are linked to one another in the uptake of amino acids by the human trophoblast. Atropine blocks ACh release and amino acid uptake by placental villi, indicating that ACh stimulates a muscarinic receptor and thereby regulates amino acid uptake by the trophoblast. Amino acid uptake by placental villi is also depressed in a Ca^{2+}-free medium and by morphine (Barnwell and Sastry, 1983). These observations indicate that, in morphine addicts, ACh-facilitated uptake of amino acids by the trophoblast is depressed. Therefore, fetal IUGR in morphine addicts can be partially explained by depressed amino acid transport in the placenta. These studies have yet to be supported by analysis of amino acids in the placentas of morphine addicts and nonaddicts. Some compensatory changes have been reported to occur in the placental opioid systems of mothers who used morphine-like com-

pounds during pregnancy. The number of kappa opiate receptors decreased in the placentas of mothers who used pentazocine or methadone during pregnancy. Morphine did not decrease ACh release from these placentas. These observations indicate that the negative feedback mechanism for the release of ACh is depressed or inactivated due to an inadequate number of kappa receptors in the placentaes of opiate addicts. This is a compensatory change to improve ACh-facilitated amino acid uptake by the placentas of opiate-addicted mothers. If there are any other compensatory changes (e.g., decrease in the synthesis of opioid peptides) in the opioid systems of opiate addicts, they have yet to be investigated.

Cocaine and Placental Amino Acid Transport

Cocaine abuse has become increasingly prevalent among certain urban populations. The subject of maternal cocaine use and fetal IUGR has been reviewed by Sastry (1995c). Investigators have focused on various complications of pregnancy and malformations in infants, but consistent findings are fetal growth retardation and prematurity. Cocaine seems to interfere with placental amino acid transport in a similar way as do nicotine and morphine. Cocaine acts as a Ca^{2+} antagonist in smooth muscle. It also decreases the release of ACh from placental villi (Sastry et al., 1977). This action may arise from the blockade of Ca^{2+} influx into the syncytiotrophoblast. Ca^{2+} is necessary for the release of ACh and the uptake of amino acids (Sastry et al., 1983). Cocaine depresses the uptake of neutral, acidic, and basic amino acids by the human placenta and thus the placental transport of these amino acids (Barnwell and Sastry, 1983). Therefore, cocaine-induced fetal growth retardation also can be partially explained by depression of placental amino acid transport.

Alcohol and Placental Function

The effect of alcohol on placental functions and fetal toxicity has been reviewed by Henderson and Schenker (1995). Maternal ethanol consumption, either continuously throughout pregnancy or in a "binge" pattern may produce adverse and lasting fetal outcome. In the human fetus, the most consistent impact is growth retardation, with specific detrimental impacts on the developing central nervous system (CNS). The overall effect of alcohol is described as fetal alcohol syndrome (FAS) which is characterized by: 1) growth retardation; 2) CNS abnormalities (which may include abnormal brain morphology, neurological abnormalities, developmental and intellectual impairment); and 3) the existing pattern of craniofacial abnormalities (Jones et al., 1973; Larrogue, 1992). These are associated with sustained heavy maternal ethanol consumption (Forrest et al., 1992). In addition, several extensive epidemiological studies over the past decade have provided evidence that, even in the absence of growth deficits and developmental delay, subtle but lasting behavioral and intellectual dysfunctions may persist (Committee on

substance Abuse and Committee on Children with Disabilities 1993). These fetal alcohol effects (FAE) include hyperactivity, attention and fine motor deficits, and a constellation of psychosocial disorders. A question arises about the contribution of the placenta to overall FAS. According to Henderson and Schenker (1995), there is an enormous body of evidence supporting direct impact of ethanol and acetaldehyde, a metabolite of alcohol, on a variety of fetal tissues and their components, and such direct effects may ultimately be proven to be the origin of toxicity of ethanol toward the fetus. In view of the central role of the placenta in maintenance of fetal development and the well-documented effects of ethanol on its three primary functions (transport, metabolism, and endocrine) ample evidence is provided that alterations of a variety of placental functions may contribute partly to the toxic effects of ethanol on the fetus.

Alcoholism is frequently associated with malnutrition and this, combined with the lack of normal growth and development of FAS children, suggests that impaired nutrition contributes to this syndrome. Thus, several studies have focused on the hypothesis that maternal ethanol consumption produces a suboptimal supply of nutrients needed for fetal growth. There is some evidence from animal and *in vitro* experiments using human placental tissues that ethanol affects nutrient transport of amino acids, glucose, some vitamins (vitamin B_6, folate, thiamine, and some trace elements e.g.., Zn). A small amount of ethanol is metabolized to acetaldehyde during placental transfer, which is toxic to the embryo and fetus.

Alcohol influences the effects of biogenic amines on human placental vasculature and therefore placental blood flow. It also influences membrane fluidity of placental membranes and thereby influences responsiveness of the placental vasculature to biogenic amines.

PLACENTAL VASCULATURE

Placental Blood Flow and Influence of Drugs

The placental vasculature and blood flow have been the subject of some reviews (Sastry and Sadavongvivad, 1979; Sastry and Owens, 1987). The transport of nutrients from trophoblast cells into the umbilical circulation occurs by diffusion. Placental blood flow facilitates this diffusion, but substances that cause vasoconstriction of placental blood vessels interfere with transplacental transport. An example of a substance that decreases placental blood flow and causes transplacental transport irregularities is 5-hydroxytryptamine (5-HT). 5-HT decreases perfusion flow through the umbilico-placental vasculature in the isolated human placenta. The fetal blood concentration of 5-HT increases before birth, which may prevent loss of blood through the placenta during birth. A single injection of 5-HT in pregnant mice increases death rate among the fetuses, with little effect on the mother. The increased rates of fetal deaths or congenital malformations caused by

5-HT could be attributed partially to a decrease in placental blood flow and transfer function. In view of the potent actions of 5-HT on uterine and placental vasculature, it has played an important role in several physiological and pathophysiological conditions related to pregnancy and uterine haemodynamics, including preeclampsia, abortion, and parturition. β-Adrenergic agonists are often employed to arrest premature labor. Among β-adrenergic agonists, salbutamol and ritodrine cause transitory decreases in human placental blood perfusion. Sodium triglycyl-[8-lysine] vasopressin, a vasopressin analogue, has been investigated clinically in the treatment of hemorrhage of the upper gastrointestinal tract. It caused a decrease in placental blood flow in the pregnant guinea pig. Because of this, its use contraindicated in pregnancy (Sjöquist et al., 1977).

Maternal tobacco smoking can also adversely affect placental blood flow. Upon smoking of a standard cigarette, an acute decrease in intervillous blood flow of approximately 15–20% is observed in healthy women. However, the blood flow returns to normal within 15 minutes after cigarette smoking. Nicotine is known to release 5-HT from nervous tissues (Hery et al., 1997). Nicotine enters the fetal circulation from the maternal blood of tobacco smokers. Released 5-HT may decrease fetal blood flow through the placenta and contribute to fetal hypoxia and growth retardation in babies of tobacco smokers. Nicotine may also exert a direct effect on placental blood vessels (Sastry and Owens, 1987).

Perfusion of isolated human placentae *in vitro* through the umbilical artery has shown that the placental vasculature is sensitive to a variety of compounds with vasoactive properties. This procedure provides useful information on the perfusion flow and overall resistance of the system. However, it does not distinguish the sites of vascular resistance or permit the quantifying of drug actions. Strips of umbilical arteries and veins have been examined for their sensitivity to drugs, but these studies reflect only the sensitivity of cord vessels per se and not that of the placental vasculature. Different regions of the placental vascular tree exhibit different sensitivities to vasoactive peptides (Tulenko, 1979). The small arteries or arterioles of the placental vasculature are the most probable sites of placental vascular resistance.

Three parts of the human placental vasculature—umbilical, chorionic, and villus-stem arteries—exhibit different sensitivities to 5-HT, nicotine, and ethyl alcohol (Sastry and Owens, 1987). Helically cut strips of vessels were used in these studies. Increased vascular resistance is indicated by increased tension or contraction of the vascular strips. According to these studies: 1) 5-HT is very potent for causing contractions of umbilical, chorionic and villus-stem arterial strips (EC_{50}: 84, 47, and 80 nM, respectively); 2) nicotine contracts umbilical, chorionic, and villus-stem arterial strips (EC_{50}: 2.4, 3.3, and 2.4 mM, respectively); 3) ethyl alcohol (ETOH; 0.5–2.0% v/v) exhibits differential effects on umbilical and chorionic arterial segments. The effect of ETOH on umbilical arterial strips consists of minimal contractions or flaccidity. 5-HT is less effective in umbilical arterial strips pretreated with ETOH; the contraction height induced by 5-HT is

reduced by about 50% in umbilical strips pretreated with ETOH. However, ETOH potentiates the contractile responses of umbilical strips to nicotine. ETOH by itself causes contractions of chorionic and villus-stem arterial strips and potentiates the effects of 5-HT and nicotine by about 115–160%, depending upon the concentration of ETOH and the nature of the strip. ETOH also enhances the effects of KCl on these strips.

Nicotine- and 5-HT-induced contractions may meet their Ca^{2+} requirement from different sources or storage sites of vascular tissue. The Ca^{2+} movements from its source required for nicotine-induced contractions may be facilitated in all vessels, whereas movements of Ca^{2+} from its sources for 5-HT-induced contractions are partially hindered in umbilical arteries. Because ETOH enhances the vasoconstrictive effects of both 5-HT and nicotine, maternal smoking combined with alcoholism may have hazardous effects on blood flow through the umbilico-placental vasculature and may contribute to 5-HT-induced complications of pregnancy (preeclampsia and abortion).

In perfusion studies with human full-term placentas, several opiates—morphine, meperidine, and codeine—exhibit a vasoconstrictive action on placental vessels (Gautieri and Ciuchta, 1962). This vasoconstrictive action may contribute to depressed transplacental transport to amino acids.

Placental Endothelial Cells and the Role of Acetylcholine

ACh is released from the trophoblast into both maternal blood (Olubadewo and Sastry, 1978) and fetal circulation (Raghavan and Sastry, 1970). Intravascular injection or infusion of ACh causes vasodilation in experimental animals. Therefore, it is possible that endogenously released ACh (82.4 ng/min/g at 37.5° C) causes vasodilation of the placental vasculature and decreases the resistance to fetal blood flow.

The effects of exogenously injected ACh on the perfused human placenta have been studied by several investigators (Euler, 1938; Eliasson and Aström, 1955; Ciuchta and Gautieri, 1964). In such experiments endogenous ACh is released in considerable amounts into the perfusion medium (Raghavan and Sastry, 1970) and the placental vasculature is already dilated, making it difficult to demonstrate the vasodilatory effects of exogenously injected ACh. The above investigators reported that there was no effect for exogenous ACh and that weak vasodilation or vasoconstriction was enhanced by physostigmine and abolished by atropine. Exogenously administered ACh (0.05 to 13 μM) caused vasodilation in 27% of perfused human term placentas, vasoconstriction in 11%, and had no effect in 56% (Euler, 1938; Sastry, 1991a). The vasoconstrictive response, normally observed with high concentrations of ACh, may be due to the release of vasoactive substances (e.g., catecholamines, 5-HT), or it may have causes that are as yet unexplained. *In vitro* preparations of the placental vasculature may be more suitable for these studies than perfused whole placentas.

Furchgott (1983) has reported that the presence of intact endothelial cells is necessary for the relaxation effect of ACh on isolated blood vessels. According to their investigations, (a) muscarinic receptors that are activated by ACh to release endothelium-derived relaxing factor (EDRF) are present on endothelial cells; and (b) EDRF mediates the ACh-induced relaxation of vascular smooth muscle. In the case of the human placenta, which is subjected to trauma during delivery, several questions need to be answered, including: (a) the status of endothelial cells in the human placental vasculature after delivery; (b) the effects of hypoxia due to tobacco smoke on endothelial cells; and (c) the effects of nicotine and other abused drugs and environmental chemicals on the cholinergic receptor and EDRF of endothelial cells.

Influence of Acute and Chronic Hypoxia on Endothelial Cells in Vascular Tissues

Intraluminal hypoxia relaxes smooth muscle cells and produces a vasodilatory response in dog (or rat) arterial segments (Busse et al., 1983). This dilatory response was abolished after the enzymatic or mechanical removal of endothelium. Theophylline and lipoxygenase inhibitors did not influence this dilation, but indomethacin reduced it significantly. These observations indicate that intact endothelium is necessary for hypoxia-induced vasodilation and that prostacyclin (PGI_2) may play a role in hypoxic endothelium-induced relaxation. The above reversible effects represent changes in vascular tissue due to acute hypoxia and they may be described as compensatory responses. During chronic hypoxia, endothelial cells are damaged (as described below) and the relaxation effect to hypoxia is lost. Changes in endothelial cells and their function in pulmonary arteries and capillaries of the rat due to acute or chronic hypoxia have been described (Kombe et al., 1980; Meyrick and Reid, 1980; Bisio et al., 1983). In the case of the human placenta, it is difficult to evaluate the hypoxic damage done to it after it is delivered and studied. Generally, the umbilical cord is squeezed to direct the blood into fetal circulation, and this manipulation may result in the loss of some endothelial cells and their muscarinic receptors in about 50% of placentas (Sastry, 1991a).

Effects of Smoking and Hypoxia on Human Placental Endothelial Cells

Chronic hypoxia or smoking induces marked changes in the vascular intima of human umbilical arteries (Asmussen and Kjeldsen, 1975; Asmussen, 1977). Scanning electron microscopy revealed swollen and irregular endothelial cells and cytoplasmic protrusions, or blebs, on their surfaces. Transmission electron microscopy demonstrated degenerative changes, including endothelial swelling and subendothelial edema. The vessels showed focal openings of intercellular junctions and collagen fibers. There were changes in the placental villi of smoking mothers, which

included broadening of the basement membrane of the placental villi, decreased vascularization, arterioles with intimal edema, a higher frequency of abnormal trophoblast cells, and clumping of nuclei in the syncytiotrophoblast. All of these changes demonstrate damaged endothelial cells and possible deficits in ACh-induced relaxation of the placental vasculature. ACh relaxes strips of chorionic vein in a concentration-dependent manner when the endothelial cells are intact. When the endothelial cells are damaged by rubbing, ACh enhances the tension developed in response to 5-HT (Sastry, 1991a).

Presence of endothelial cells is necessary for vasodilation of placental vasculature and facilitation of transport of amino acids from trophoblast cells into fetal circulation. As already discussed, chronic hypoxia due to maternal smoking damages endothelial cells in the placenta. Nicotine is known to increase the release of placental ACh (Sastry et al., 1977), which in turn increases the vasoconstrictive effects of endogenous 5-HT and other biogenic amines in vascular tissues denuded of endothelial cells. This vasoconstriction decreases fetal blood flow through the placenta. This means that the transfer of amino acids from the trophoblast cells to fetal blood decreases during maternal smoking. As described earlier, morphine and cocaine decrease ACh release due to their inhibitory effect on Ca^{2+} influx. Therefore, this vasodilatory effect is also prevented in morphine and cocaine addicts. Lack of ACh release (no vasodilation) with morphine and cocaine, or excess ACh release (vasoconstriction) with nicotine, has the same final effect on the placental vasculature: decreased blood flow and decreased placental transport of nutrients.

PLACENTA AS A METABOLIC ORGAN

Biotransformations in the Placenta

Most of the biotransformations which occur in the liver are also represented in the placenta. Induction compounds affecting liver enzymes also affect the inducible placental metabolic pathways (Sastry et al., 1981). This subject has been discussed in detail in several reviews and articles (Goodman et al., 1982; Juchau et al., 1987; Sastry, 1991a; Harbison et al. 1995; Juchau, 1995) and is beyond the scope of this text.

Glucuronidation, sulfation, and glutathione conjugation as well as reactions dependent upon cytochrome P-450, epoxide hydrase, catechol-O-methyltransferase, and monoamine oxidase occur in the placenta. However, the biotransformation capacity of the placenta is many times less than that of the liver. Comparisons in different experimental animals indicate that the placenta has about 3.3 to 2% or less of the activity that is present in liver tissue. The exception may be glucuronyltransferase activity, for which, in rats, placental activity is equivalent to liver activity. This suggests that, for the most part, xenobiotics may pass comparatively unchanged to the fetus. However, this cannot be generalized for all drugs and chemicals. There is

some evidence that the placenta is capable of metabolizing some toxic chemicals and/or forming toxic metabolites.

Benzo[a]pyrene, an environmental contaminant and also a component of cigarette smoke that is carcinogenic in animals, has been extensively studied in placental biotransformation. Benzo[a]pyrene metabolism, as measured by the formation of 3-hydroxybenzo[a]pyrene (a detoxifying pathway), is present at only a low level of activity in nonsmokers, but it may be increased 100-fold in smokers.

Human placental microsomes are capable of generating mutagenic metabolites of several polyaromatic hydrocarbons. Therefore, the placenta, like the liver, is capable of generating reactive metabolites of a number of compounds despite the lower activity of most placental metabolizing systems. Those factors affecting liver metabolism and hepatotoxic agents, such as multiple metabolic pathways and the induction of toxic versus nontoxic pathways, may be applied to explain chemically induced fetal toxicities. Placental-fetal biotransformation must be considered more seriously as a biochemical mechanism for chemical- and drug-induced fetotoxicities. In fact, the human placenta has been suggested as a model for the toxicity-screening of new drug molecules (Beaconsfield et al., 1987).

Phenoxyacetic Acid Herbicides and Formation of False Cholinergic Messengers

More recent investigations indicate that herbicides related structurally to phenoxyacetic acids enter the ACh synthetic pathway and interfere with the role of ACh in the placenta. The toxic symptoms of 2:4:5-trichlorophenoxyacetic acid (2:4:5-T; Agent Orange) include a reduction in metabolic rate and growth retardation. ACh serves as an essential growth factor to facilitate amino acid transport and to promote fetal growth. Hydatidiform mole lacks capacity for synthesis of ACh, and inhibition of ACh synthesis depresses placental amino acid transport. ACh is synthesized by two steps in the placenta and the nerve (Sastry, 1997): (1) formation of acetylcoenzyme A (ACoA) from acetate and coenzyme A (CoA) in the presence of acetylcoenzyme A synthetase (ACoAS); and (2) Formation of ACh from ACoA and choline in the presence of ChA. Sastry et al. (1995, 1996, 1997) studied the formation of 2:4:5-T-acetylcoenzyme A (2:4:5-T-CoA) by ACoAS and formation of 2:4:5-T-ACh by human placental ChA from 2:4:5-T-CoA and choline. In these studies, the widely used analogue of 2:4:5-T as a herbicide, 2:4-dichlorophenoxy acetic acid (2:4-D) was also included. These studies gave the following interesting results (M±S.D.; N,6):

1. The enzymatic rates of formation of acetyl-CoA, 2:4:5-T-CoA, and 2:4-D-CoA by ACoAS were 32±4, 23±3, and 26±8 nmol/mg protein/5 minutes, respectively.
2. There were no significant differences in the maximal amounts (nmol/mg protein) of acetyl-CoA (128±4), 2:4:5-T-CoA (125±8), and 2:4-D-CoA (96±6) formed during the reaction period of 50 minutes.

3. ^{14}C-2:4-ACh was formed from ^{14}C-2:4-D-CoA and choline by placental-ChA
4. Low concentrations (EC50 1–2 μM) of synthetic 2:4:5-T-ACh and 2:4-D-ACh decreased the contraction heights of the rat phrenic nerve-hemidiaphragm when the nerve or the muscle was electrically stimulated.
5. Similar results were obtained with other analogues of 2:4:5-T and 2:4-D.

Taken as a whole, these observations indicate that the chlorophenoxyherbicides form false coenzymes and cholinergic messengers in the nerve, muscle, and placenta. These false cholinergic messengers can be formed at both muscarinic and nicotinic synaptic sites and also in non-neuronal cells, where ACh plays an important regulatory role as a local hormone, and acts as a blocking agent. These observations may partially explain myotonia, ventricular fibrillation, and fetal growth retardation induced by these herbicides.

Covalent DNA Adducts and Binding Sites of Abused Drugs and Their Metabolites in the Human Placenta

In humans and animals, chemical carcinogenesis is a multistage process consisting of discrete events occurring over a considerable portion of the life span. The initial event (initiation) involves DNA damage, as indicated by the ability of a large number of chemical carcinogens to form covalent addition products (adducts) with DNA. Alternately, the presence of adducts in DNA samples from an individual indicates that the individual has been exposed to carcinogens. The concentrations of DNA adducts in the tissues of an individual probably represent a steady-state between the formation of new adducts and the loss of old ones through cell turnover and DNA repair. In studies involving DNA adducts, DNA is isolated from tissues and hydrolyzed to form mononucleotides. These nucleotides are then labeled with radioactive ATP and analyzed by thin-layer chromatography ([^{32}P]-postlabelling assay). DNA adducts have been studied in the placentas of smokers and nonsmokers by the [^{32}P]-postlabeling assay (Everson et al., 1986; Everson, 1987). Several modified nucleotides have been detected in the placentae of smokers, one of which (adduct 1) is strongly related to maternal smoking. The chemical identification of adduct 1 has yet to be accomplished.

All three drugs of abuse—nicotine (Olubadewo and Sastry, 1978; Rowell and Sastry, 1978; Sastry and Sadavongvivad, 1979), morphine (Ahmed et al., 1989a,b), and cocaine (Ahmed et al., 1990b)—have receptors or drug-binding proteins in the placenta. A muscarinic receptor has also been described to exist in the human placental plasma membrane (Rowell and Sastry, 1978; Fant and Harbison, 1981). Whether any of the metabolites of these abused drugs form DNA adducts has yet to be investigated.

PLACENTAL GROWTH FACTORS AND ENVIRONMENTAL AGENTS

Evaluation of placental growth factors and their importance for placental and/or fetal growth and agents interfering with growth factors is beyond the scope of this chapter. The reviews by Han (1993), Evain-Brion and Alsat (1994) and Fant (1996) may be consulted for details on this topic.

Development of the placenta involves proliferation and differentiation of several cell types, migration and invasion of fetal trophoblasts into maternal endometrium, modeling of the maternal endometrium and vasculature to prepare for interchange of nutrients and substrates between the maternal and fetal circulations, interaction between fetal and maternal cells to enable fetal cells to develop immunological privilege to survive within the maternal environment, and differentiation of trophoblasts into special endocrine cells for the maintenance of the pregnancy.

Growth factors are synthesized by the developing and mature placental tissues and regulate the proliferation of placental cells, which coordinate a wide variety of differentiative, endocrine, and immunological functions of the placenta. Growth factors play a role not only in the normal growth and development of the placenta, but also in the pathology of the placenta. The latter may lead not only to better understanding of pathological conditions like habitual abortions, premature labor, and IUGR, but also to the development of diagnostic tests and therapeutic regimens to treat these conditions.

Proper placental development is a prerequisite for normal fetal growth. Epidermal growth factor (EGF) plays a significant role in placental implantation, growth, and differentiation. It acts on placental target cells, the trophoblasts, via a specific receptor (EGFR). EGFR belong to the tyrosine kinase receptor family. The placental EGFR is located in the brush border at the fetomaternal interface. EGFR expression is modulated by trophoblast differentiation and by hormones or toxic substances such as smoke. In placental microvilli of infants with, (IUGR), a decrease or absence of tyrosine kinase activity is observed. This means that an alteration of EGFR biological activity will interfere with the fetoplacental unit development.

SUMMARY AND FUTURE DIRECTIONS

The functional toxicity of drugs and environmental chemicals on the placenta has received less attention than the corresponding toxicities to the fetus and the mother. This is justifiable in the sense that the placenta is discarded at the termination of pregnancy. However, proper function of the fully formed placenta during the fetal period (2–9 months) is critical to the fetus for nutrient support and as a protective barrier against environmental insults. Recent investigations indicate that the human placenta has the capacity to compensate for loss of function due to environmental

insults (Sastry et al. 1989a). For example, maternal smoking depresses amino acid uptake by the trophoblast and transfer of amino acids to the placental circulation, and it releases vasoactive biogenic amines that decrease placental blood flow. Compensation for this insult takes three forms: (a) an increase in the size of the placental to sustain a smaller fetus; (b) an increase in the number of amino acid carriers in the plasma membrane of the trophoblast to increase amino acid uptake; and (c) a decrease in the membrane fluidity of placental blood vessels so that they are less responsive to vasoconstriction by biogenic amines. All of these changes improve the placental transport of amino acids. This improvement is unmasked in the absence of tobacco-smoke compounds. Placentas from smoking women were washed free from components of tobacco smoke, and amino acid uptake by these placentas was measured. The above compensatory changes were observed in these placentas when they were compared to those of nonsmoking women. More detailed studies of such compensatory changes with other drugs of abuse, such as morphine, cocaine, and alcohol, and with environmental chemicals are needed.

Smoking increases the concentrations of several primary amino acids in the plasma of nonpregnant women, pregnant women, and umbilical arterial blood (Sastry et al., 1989a, c, d). This means that the utilization of amino acids by fetal as well as adult tissues decreases during smoking. The impact of nonutilization of amino acids may be greater for the fetus, which is developing and growing, than it is for adults.

Increased plasma concentrations of amino acids need not be harmful to the majority of people. However, individuals who are genetically predisposed to certain amino acid disorders have to be identified. This becomes more important for pregnant women. For example, women who are predisposed to metabolic disorders of phenylalanine resulting in phenylketonuria should be identified during prenatal care. Otherwise, damage may be done to the infant before it is born due to increase in the steady-state plasma concentrations of phenylalanine. This is important because more and more information is becoming available to indicate that there is a genetic component in certain tobacco-related diseases (e.g., lung and colon cancer). This means that certain population groups may be more susceptible to tobacco-related diseases than others.

Drug metabolism in the placenta has received little attention. Drugs and chemicals may undergo alterations not only in the maternal liver and tissues, but also in the placenta before they reach the fetus. The fetus may be more sensitive to certain drug metabolites than is the mother. Many of the drugs may also induce not only drug-metabolizing enzymes in the placenta but also enzyme systems involved in the function of the placenta. Drug metabolites deleterious to placental function are also hazardous to the fetus.

Although there are many studies on the deleterious effects of certain chemicals on embryonic development in the early stages of pregnancy before the placenta is fully formed, there are only a few studies on the molecular mechanisms of these deleterious effects. The question arises as to whether certain drugs interfere with the development of the placenta itself. The hydatidiform mole contains a negligible

amount of ChA, the enzyme that catalyzes the synthesis of ACh (Sastry et al., 1978). ACh seems to play a role in the maturation of the human placenta. Drugs that inhibit ChA may interfere with placental development. Pregnant women are exposed not only to drugs administered to them, but also many environmental chemicals including insecticides, herbicides, and utility chemicals used for cleaning and washing clothes in the home. Several gaps in the placental toxicology of all these chemicals have yet to be filled.

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Chapter 14

Preeclampsia and Eclampsia

DANIEL S. SEIDMAN

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INTRODUCTION

A 24-year-old woman, pregnant for the first time, is admitted to the delivery room at term, complaining of headaches and right upper quadrant pain. A physical examination reveals brisk reflexes and a blood pressure of 140/100 mm Hg. Before treatment is initiated, the patient suffers from violent generalized convulsions. Treatment is promptly provided and labor is induced. Following delivery the woman's complaints rapidly resolve, and the next day she and her baby leave the hospital in good health. This case, describing the characteristic and dramatic presentation of preeclampsia and eclampsia, serves to demonstrate one of the major complications of pregnancy. Preeclampsia, characterized by hypertension, proteinuria, edema and, at times, disturbances of coagulation and liver function, complicates 5 to 10% of pregnancies. Eclampsia, characterized by these abnormalities along with generalized convulsions is a rare event in pregnant women. The term eclampsia refers to the visual disturbance ("flashing lights") that often immediately precedes the generalized convulsions (Cooper et al., 1993).

Preeclampsia has traditionally been viewed as one of several forms of hypertension complicating pregnancy. Many terms have been used to classify the hypertensive disorders of pregnancy. For instance, the single entity designated here as "preeclampsia" has been previously called "pregnancy induced hypertension," "pregnancy associated hypertension," "gestosis," and "toxemia". Currently, the multisystem nature of this unique gestational disorder is being emphasized. This reflects an expanding knowledge of this disease, as well as current broadened understanding of the possible underlying pathophysiology. More recently, new treatments have been introduced following the prevailing concept of preeclampsia as an endothelial cell disease, with an associated deficiency in vasodilating prostaglandins.

THE CLINICAL SPECTRUM

Preeclampsia is unique to human pregnancy and complicates about 10% of gestations. It is a disease of first pregnancy (mainly, but not exclusively), and typically occurs after 20 weeks of gestation, most frequently near term. The two most important signs of preeclampsia are hypertension and proteinuria, and they serve as the basis for clinical diagnosis (Cunningham et al., 1997). The pregnant woman, however, is likely not to be aware of these two characteristic signs, and hence the utmost importance of prenatal care for the early detection and management of preeclampsia (Cunningham et al., 1997). The first sign of preeclampsia in some women is excessive weight gain. Normally, women gain about one pound per week, thus a weight gain exceeding two pounds in any given week or six pounds in a single month should raise suspicion that preeclampsia may be developing. The weight gain associated with preeclampsia is usually sudden and is almost entirely due to

abnormal fluid retention. The change in maternal body weight is usually noted before the visible signs of nondependent edema, such as swollen eyelids and puffy fingers. Edema was previously considered to be a diagnostic characteristic of preeclampsia, but is no longer considered to be a reliable sign, since most normal gravidas develop dependent edema, which becomes generalized in approximately 30% of patients. Furthermore, attempts to limit weight gain, in the mistaken belief that this would prevent preeclampsia, are currently viewed as detrimental, rather than beneficial, to both mother and fetus (Cunningham et al., 1997).

Additional characteristic symptoms include headache, upper abdominal pain, and visual disturbances. The headache is usually frontal and often not relieved by ordinary analgesics. The epigastric or right upper quadrant pain is usually attributed to stretching of the hepatic capsule. The visual dysfunction may include cortical blindness and retinal detachment (Seidman et al., 1991b). These symptoms tend to appear only late in the disease and are always ominous signs of severe preeclampsia. Other signs of the disease include hyperreflexia, hemoconcentration, low platelet count, cardiac decompensation, disturbed renal function, retinal hemorrhage, and fetal growth retardation (Cunningham and Lindheimer, 1992).

It is important to recognize the above signs and symptoms, as they may be indicative of imminent eclampsia, characterized by generalized convulsions. Preeclampsia can progress rapidly to eclampsia, one of the most dramatic and life-threatening complications of pregnancy. Although convulsions are usually preceded by premonitory signs, eclampsia may occasionally occur suddenly in asymptomatic women (Roybert et al., 1991). One form of preeclampsia that can appear deceptively benign is characterized by minimal changes in blood pressure, small decreases in platelet count, mild elevations in liver enzymes, and little renal dysfunction. This variant of the disease may progress rapidly to a life-threatening syndrome characterized by hemolysis, and both liver function and coagulation abnormalities: the so-called HELLP syndrome (hemolysis, elevated liver enzymes, and low platelet count).

DIAGNOSIS

The diagnosis of preeclampsia is based upon the identification of pregnancy-induced hypertension plus proteinuria and/or edema.

Pregnancy-Induced Hypertension

The classification of pregnancy induced hypertension is a controversial issue. This arises in part from the extraordinary difficulty in distinguishing, by clinical criteria alone, hypertension that is in some way induced by pregnancy from hypertension that merely coexists with it. One of the most commonly used classifications recommended by the American College of Obstetricians and Gynecologists identifies four categories of hypertension associated with pregnancy: 1) preeclampsia and eclamp-

sia; 2) chronic hypertension (of whatever cause); 3) preeclampsia superimposed on chronic hypertension; and 4) transient or late hypertension (Table 1).

The diagnosis of pregnancy-induced hypertension is made when blood pressure is 140/90 mm Hg or greater. Pregnancy induced hypertension has also been diagnosed with an increase of 30 mm Hg in systolic or 15 mm Hg in diastolic blood pressure over baseline levels, on at least two occasions, six or more hours apart. The diagnostic problem is related to the fact that an increase in systolic and diastolic blood pressure can be either a normal physiological change or a sign of developing pathology (Cunningham et al., 1997).

Systolic and diastolic blood pressure decreases in pregnancy by 5 to 15 mm Hg during the second trimester and returns to near-normal levels at term. Thus, diastolic blood pressure increases by approximately 10 mm Hg during the third trimester in normal gestation (Barron, 1992). It is therefore clear that the physician must closely observe any acute change in blood pressure, as the significance of such temporal changes may differ from one patient to the other. Awareness of the development of the signs and symptoms of preeclampsia is essential in order to allow prompt treatment of this life-threatening complication of pregnancy.

Proteinuria

Significant proteinuria is an important sign of preeclampsia and is usually considered as an indispensable part of the diagnosis. Proteinuria is defined as the excre-

Table 1. Classification of Hypertension Associated With Pregnancy

Preeclampsia and Eclampsia
Disorders unique to pregnancy. Occurs most often in first pregnancy, almost always after 20 weeks of gestation and typically near term. Characterized by hypertension plus proteinuria. Commonly associated with pathologic edema, decreased platelet count and disturbances of liver function. Eclampsia is characterized by these abnormalities along with generalized convulsions.
Chronic Hypertension
Disorders unrelated to pregnancy, in most cases the women have essential hypertension, and the pregnancy is uncomplicated. In unusual instances, there are specific causes that present serious risk for pregnancy outcome (e.g., Cushing's syndrome, pheochromocytoma, connective tissue disorders, and moderate to severe renal impairment).
Preeclampsia Superimposed on Chronic Hypertension
Women with chronic hypertension are more likely to have superimposed preeclampsia which is associated with a substantial risk to mother and fetus.
Transient or Late Hypertension
Increased blood pressure, usually mild, that occurs near term or immediately after delivery without other evidence of preeclampsia. The outcome of pregnancy is not affected appreciably, but hypertension often recurs during subsequent pregnancies. This may be harbinger of chronic on essential hypertension

tion of 300 mg or more of urinary protein per 24 hours or 100 mg/dL or more in at least two random urine specimens collected 6 or more hours apart. In the absence of proteinura, the clinical diagnosis of preeclampsia is questionable. However, as the degree of proteinuria may fluctuate dramatically over time, a random sample may fail to demonstrate significant proteinuria and, therefore, the diagnosis of preeclampsia should not be ruled out. Proteinuria may appear relatively late in the course of pregnancy-induced hypertension. Thus, from a pathophysiological and epidemiological perspective, it is evident that hypertension is the sine qua non of preeclampsia (Cunningham et al., 1997). In the case of proteinuria it is a sign of worsening hypertensive disease, specifically preeclampsia. Once proteinuria is overt and persistent, the risk for both mother and fetus is further increased beyond the dangers known to be related to the diagnosis of pregnancy-induced hypertension, and close observation and treatment is urgently needed.

Edema

The presence of generalized edema is, as stated earlier, such a common finding in pregnant women that its presence should not validate the diagnosis of preeclampsia any more than its absence should preclude the diagnosis (Cunningham et al., 1997). Edema of preeclampsia is pathological and not just dependent. Although both may involve the hands and face, this is more common in the former. A useful sign of non-dependent edema is a womans complaint that her rings have become too tight.

ETIOLOGY

Preeclampsia remains a disease of obscure etiology and, as such, many theories have been put forward since the recognition of this disease, with various treatment modalities assigned accordingly. Proposed etiologic factors have included everything from watermelon season to infestation with a worm (Chesley, 1974). Suggested treatment schemes have included a wide array of novel ideas such as renal decapsulation, mastectomy, oophorectomy, and alignment of the woman with earth's magnetic field.

Current understanding of the pathophysiology of preeclampsia, as outlined below, has greatly improved and scientifically supported preventive therapy is now under extensive investigation. However, the causative factor for the well-defined chain of events leading to preeclampsia remains poorly understood. Any sound etiologic theory must be compatible with the well-established epidemiologic observations associated with preeclampsia. The epidemiologic data mainly refers to the identification of the following groups at high risk for development of preeclampsia: (1) women pregnant for the first time; it seems that women pregnant for the second time have a lower incidence of preeclampsia regardless of whether their previous pregnancy was a full term gestation or ended in an abortion (Seidman et al., 1989; (2) women exposed to a super abundance of chorionic villi, a condition

known as hyperplacentosis; this situation refers to pregnancies associated with large placental tissue, for instance twins or hydatiform mole (a disease characterized by uncontrolled proliferation of placental chorionic villi; (3) familial aggregation of the disease pointing at a common genetic predisposition for developing hypertension during pregnancy; and (4) women with chronic vascular disease. In order to explain these well-founded observations, both immunological and genetic mechanisms have been proposed.

Immunologic Factors

The immunologic basis for the initiation of preeclampsia derives from the paradox of the fetal allograft. How does the mother manage to nourish within herself a fetus that is antigenically a foreign body? Exposure of the mother to the fetal trophoblast seems to play a fundamental role in the immunologic protection attained by the fetus. Therefore, the typical occurrence of preeclampsia in first pregnancies may be related to an aberrant immune reaction initiated at the first exposure to the foreign paternal and fetal antigens of the placenta (Zeeman and Dekker, 1992). This hypothesis is further supported by the increased incidence of preeclampsia following a change of partner and in a subsequent pregnancy after using birth control methods that prevent exposure to sperm.

Another immunologic mechanism assigns a central role to a disturbance in the formation of blocking antibodies by the mother to antigenic sites on the placenta. This would explain the enhanced risk of pregnancy-induced hypertension in circumstances where formation of the blocking antibodies might be impaired, for instance in women who receive immunosuppressive therapy to protect a renal transplant. Other conditions that may be associated with reduced formation of the blocking antibodies include first pregnancies in which effective immunization by a previous pregnancy is lacking or conditions where the placental tissue is unusually great, as in twins, thereby exceeding the amount of the blocking antibodies (Cunningham et al., 1997). It should be stressed that despite appealing theories attributing an immunological disorder to the etiology of preeclampsia, convincing proof of clinical significance is still absent (Cunningham et al., 1997).

Genetic Factors

Preeclampsia seems to be strongly heritable. The genetic defect may cause a dysfunction in the normal maternal immunological adaptation to the presence of a foreign antigenic load in her body during pregnancy, as outlined above. Markedly raised incidences of preeclampsia are seen in blood relatives (mothers, daughters, sisters, and granddaughters), but not in relatives by marriage (daughters-in-law, mothers-in-law) (Cooper et al., 1993). This suggests that the condition is caused by maternal genes (Seidman et al., 1991a). Other evidence, however, implicates the fetal genotype. The most decisive data supporting this shows a lack of concordance in

monozygous twins. Thus, the condition may be the result of a maternal-fetal genotype interaction, broadly analogous to Rhesus disease (Cooper et al., 1993).

Genetic analysis has showed primarily recessive inheritance of this disorder (Zeemar and Dekker, 1992). A lack of, or a defective, gene product has been postulated. The recessive model is currently seriously questioned in light of studies in monozygous twins demonstrating lack of concordance. Cooper et al. (1993) have interpretated this new data from twin pairs, in view of the confinement of the condition to pregnancy, as one of a maternal-fetal genotype by genotype interaction. That is, the mother must have a particular genotype and the fetus a particular genotype (usually different from the mother's) either at the same locus or at a separate one, for the phenotype to be manifested.

To date, most of the attempts to identify the genes involved focus on the human leukocyte antigen (HLA) system. It is clear that the maternal genes are not located in the HLA system, but the possibility that the postulated fetal genes are located there remains open (Cooper et al., 1993). Current work centers on linkage studies aimed at finding the responsible genes. The exact mode of inheritance and the interaction between maternal and fetal genotype remains to be determined.

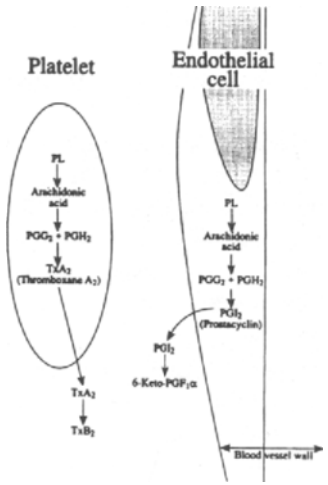

Figure 1. Production of the eicosaniods prostacyclin (PGI_2) and thromboxane A_2 (TxA_2) by platelets and by endothelial cells lining the vessel wall. When a blood vessel is injured, arachidonic acid is released from cellular phospholipids (PL) by enzymes called phospholipases. Arachidonic acid is then converted by the prostaglandin synthetase (also called cyclooxygenase) enzyme to two short-lived prostaglandin (PG) endoperoxides PGG_2 and PGH_2. In the platelet a thromboxane synthetase enzyme converts PGH_2 to TxA_2. Thromboxane A_2 is unstable and degrades rapidly to thromboxane B_2 (TxB_2). In endothelial cells, PGH_2 is converted by prostacyclin synthetase to PGI_2. Prostacyclin is unstable and is rapidly degraded to 6-keto- $PGF_{1\alpha}$. From Gerrard and colleagues, Pediatrics 90, 33, 1992.

PATHOPHYSIOLOGY

Placental Dysfunction

In normal pregnancy, implantation of the embryo is followed by migration of trophoblastic cells into the walls of the uterine spiral arteries. These vessels thereby lose their muscular media, which allows them to accommodate a 10-fold increase in uterine blood perfusion. A disturbance in this important process, which usually occurs between the 10th and 20th week of pregnancy, is believed to be one of the earliest pathophysiologic events in the development of preeclampsia (Barron, 1992). An additional obstructive vascular lesion, termed acute atherosis, characterized by fibriniod necorosis and accumulation of fat-laden macrophages, is observed in the placenta of some preeclamptic women. In combination, these vascular lesions may be responsible for the reduced uteroplacental blood flow, resulting in retarded fetal growth and adverse intrauterine development associated with preeclampsia. The cause of the failure of trophoblastic invasion of spiral arterial walls remains uncertain. It has been proposed, however, that this may be a consequence of altered immunologic interaction between fetal trophoblastic tissue and the maternal spiral arterial wall (Barron, 1992).

Increased Vascular Sensitivity

During the early stages of pregnancy there is a fall in blood pressure, a reduction in peripheral vascular resistance and a 40% increase in cardiac output. The vasodilatation is accompanied by the development of resistance to angiotesin II, so that plasma angiotensin II concentrations are two to three times higher in pregnant than nonpregnant women (Ferris, 1991). Since urinary excretion of 6-keto-prostaglandin $F_1\alpha$, the major metabolite of prostacyclin, increases in pregnancy, the resistance to angiotensin II is thought to be caused by increased endothelial cell synthesis of this vasodilating prostaglandin, which is also an inhibitor of platelet aggregation (Figure 2). Although the uterus and placenta are sites of extraordinarily high rates of prostaglandin synthesis during pregnancy, prostaglandins are thought to function primarily as autacoids, whose chief effect is at the site of synthesis. For instance, prostacyclin synthesis in smooth muscle cells may also account for the relaxation that occurs in the genitourinary tract, stomach, and gallbladder during pregnancy. How pregnancy increases prostacyclin synthesis is not known. Clinical findings implicate the uterus or placenta in the development of preeclampsia, since women with twin pregnancies, hydramnios, and hydatidiform moles have a higher incidence of the disease and preeclampsia resolves rapidly after delivery (Ferris, 1991).

The development of preeclampsia is accompanied by increased sensitivity to angiotensin II and decreased excretion of 6-keto- prostaglandin $F_1\alpha$. A decrease in prostacyclin synthesis could not only cause an increase in peripheral resistance, but

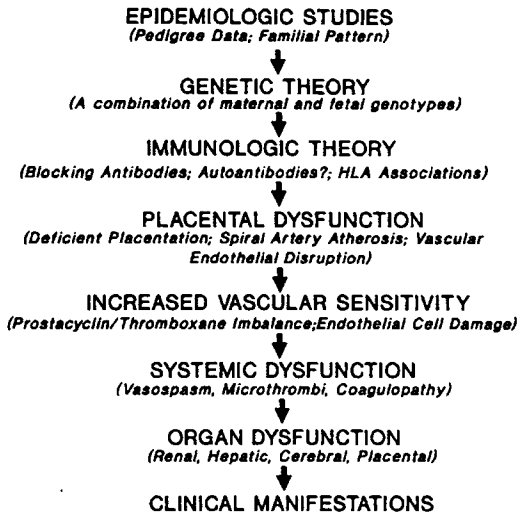

Figure 2. Proposed etiology and pathogenesis of pre-eclampsia. From Cooper et al., 1993.

also disturb the balance between the synthesis of thromboxane in platelets and that of prostacyclin in endothelial cells. This effect of pregnancy, in increasing the likelihood of platelet aggregation, was recognized years ago, when it was noted that the Shwartzman reaction occurred after one dose of endotoxin in pregnant animals, whereas repeated challenge was needed in nonpregnant animals (Ferris, 1991). Platelet aggregation in preeclampsia could account for the fibrin deposits found in the kidney and liver, as well as for the acute atherosclerosis that develops in the blood vessels of the placenta.

Endothelial Cell Dysfunction

The spectrum of endothelial disease could range from pregnancy induced hypertension to preeclampsia, with the hypertension associated with thrombotic thrombocytopenia. This hypothesis accounts for many of the clinical manifestations of preeclampsia. It has been suggested that preeclampsia may be characterized by the presence of a serum factor cytotoxic to endothelial cells, thus highlighting a difference in the mechanism leading to the increase in blood pressure from other hypertensive disorders of pregnancy (Tsukimori et al., 1992).

Preeclampsia results in an increase in systemic vascular resistance with a contracted plasma volume and reduced cardiac preload and cardiac output. It has been suggested that hypertension in pregnancy is the result of increased vessel tone due to systemic endothelial cell damage. This is in addition to the above de-

scribed imbalance in the relative production of prostacyclin and thromboxane A_2. In favor of the role of endothelial cell injury, other endothelial factors have been recognized that may contribute to the increase in vascular tone observed in preeclampsia. Increased concentrations of endothelin-1 have been demonstrated in women with preeclampsia (Cameron et al., 1993). This peptide is synthesized in endothelial cells and has potent vasoconstrictor and platelet-aggregating properties. Additionally, an increase in nitric oxide (NO) has been observed in hypertensive pregnant women (Cameron et al., 1993). NO, cleaved from L-arginine and activating soluble guanylate cyclase, has been identified as the endothelial derived vasodilating factor. It is, therefore, possible that a compensating increase in the synthesis of NO occurs in women with high blood pressure in pregnancy in an attempt to maintain homeostasis (Sladek et al., 1997). Others have suggested that a deficiency in NO may have a primary role in the pathophysiology of preeclampsia (Morris et al., 1996). It currently seems that preeclampsia is a complex endothelial cell dysfunction which results in a disturbance of the delicate balance between vasodilators such as prostacyclin and NO on one hand, and vasoconstrictors such as angiotensin II, thromboxane-A_2, and endothelin on the other (Morris et al., 1996).

PREVENTIVE TREATMENT

Calcium

Diminished dietary calcium intake has been implicated in pregnancy related hypertension and hypocalciuria has been demonstrated in patients with preeclampsia (Cunningham and Lindheimer, 1992). Since calcium supplementation has been shown to lower blood pressure in most studies of patients with essential hypertension, supplemental calcium is currently the focus of multicenter trials for the prevention of preeclampsia. One study found that calcium supplementation reduced the incidence of gestational hypertension and preeclampsia in a large group of pregnant women (Belizan et al., 1991). The mechanism by which calcium lowers blood pressure is unclear. Studies in pregnant women have shown that calcium supplementation decreases responsiveness to angiotensin II, suggesting an increase in endothelial cell synthesis of prostacyclin or NO. Urinary calcium excretion decreases with the development of preeclampsia, but this probably reflects an increase in proximal tubular absorption of calcium and sodium engendered by the reduction in plasma volume that accompanies preeclampsia. The same tubular response decreases urate clearance, causing the hyperuricemia characteristic of preeclampsia (Ferris, 1991). The definitive role of calcium in preventing hypertension during pregnancy is yet to be determined. However, one extensive randomized trial failed to demonstrate that calcium supplementation during pregnancy can prevent preeclampsia (Levine et al., 1997).

Aspirin

Aspirin affects platelet aggregation through interference with platelet synthesis of prostaglandin, presumably thromboxane A_2, a potent vasoconstrictor and promotor of platelet aggregation produced mainly by platelets. Aspirin irreversibly inhibits cyclooxygenase, and nonnucleated platelets cannot produce cyclooxygenase. Thomboxane synthesis by platelets can recover only as new platelets enter the circulation. The life span of platelets is approximately 10 days. The endothelial cells that produce prostacyclin can recover from aspirin suppression more rapidly and thus change the thromboxane/prostacyclin ratio in favor of prostacyclin. It seems reasonable therefore that aspirin may have a therapeutic role in the prevention of preeclampsia, a disease characterized by vasoconstriction and increased platelet aggregation (Imperiale and Pertulis, 1991). Low doses of aspirin (50–60 mg) were administered in a randomized fashion, early in pregnancy, in a number of studies, and a significant reduction in the occurrence of preeclampsia was observed (Benigini et al., 1989; Schiff et al., 1989; Hauth et al., 1993). However, not all of the studies found a significant beneficial effect (Italian Study of Aspirin in Pregnancy, 1993; CLASP, 1994). To date, the indications for treatment with aspirin during pregnancy have not been clearly defined. Further research is therefore needed in order to determine which high risk pregnant women may benefit from the use of low-dose aspirin in the prevention of preeclampsia, as well as other vascular disorders of pregnancy.

MANAGEMENT

Once the diagnosis of preeclampsia is suspected hospitalization is usually indicated. Delivery is the only definitive therapy for preeclampsia. Thus, if preeclampsia occurs beyond the 36th week of gestation, a point at which fetal lung maturity has generally occurred, labor should be induced. Delivery is indicated, regardless of the gestational week, if there is evidence that the fetus is in jeopardy or in cases of advanced maternal disease, such as severe hypertension persisting after 24 to 48 hours of treatment, thrombocytopenia, and progressive liver or renal dysfunction (Cunningham and Lindheimer, 1992). Another important indication for prompt delivery are signs of impending eclampsia, including headache, hyperreflexia, epigastric pain, and blurred vision. Theses premonitory signs of eclampsia also indicate that parenteral magnesium sulfate should be administered for prevention of eclamptic convulsions. Although magnesium is currently the drug of choice by most authorities in the U.S., there is disagreement regarding the management of eclamptic convulsions, stemming from the fact that their cause is poorly understood (Cunningham and Lindheimer, 1992). Furthermore, the mechanism responsible for the beneficial effects of magnesium has not

been fully elucidated. It must be stressed that any treatment provided for preeclamptic women, such as drug regulation of blood pressure, does not reverse the basic disease process and is only a temporary measure employed until the disease resolves following delivery.

SUMMARY

Preeclampsia and eclampsia are major complications of pregnancy and are associated with significant maternal and fetal morbidity. Much remains to be desired in regard to our limited knowledge of the etiology of this disease. Current research efforts have extended our understanding of the pathophysiologic mechanisms underlying this disease of unknown cause. More importantly treatment modalities, such as aspirin, based upon the concept of preeclampsia as being the result of endothelial cell dysfunction, seem to hold promise in prevention of the disease. The only definite therapy for preeclamptic mothers is delivery. The fetal outcome is thus determined to a large part by the gestational age. Adequate prenatal care is essential in order to ensure early recognition of preeclampsia, thereby preventing the development of eclampsia and achieving optimal maternal and fetal outcome.

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Chapter 15

The Premenstrual Syndrome

TIMOTHY G. DINAN and V. O'KEANE

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INTRODUCTION

The premenstrual syndrome (PMS) is best characterized as a psychoneuroendocrine disorder with significant emotional and behavioral changes which take place at a specific phase in the menstrual cycle. It was Robert Frank who, in 1931, in a paper to the New York Academy of Medicine provided the first formal description of the disorder, which was later coined the premenstrual syndrome by Greene and Dalton (1953). Frank himself had preferred the term premenstrual tension, but Greene and Dalton argued that the tension was only one psychological component of a syndrome which is characterized by emotional lability, weight gain, edema, lumbar pain, breast tenderness, abdominal pain, nausea, and headache.

DIAGNOSIS AND DEFINITION

There is still no universally accepted definition of the disorder, and although as a diagnosis it is gaining greater clinical acceptance, its validity is still questioned by some. The most widely held view is that it is a disorder, characterized by physical and psychological symptoms. Practitioners in psychiatry and psychology tend to place greater emphasis on the psychological nature of the symptoms, while those in gynecology frequently stress the physical symptoms.

The diagnosis of PMS should rest on the identification of a core symptom complex, which should include behavioral symptoms of either irritability, anxiety, depression, or fatigue. At least one core physical symptom such as bloating of the abdomen or extremities, breast tenderness, or headache are required to establish the diagnosis. Most women experience some breast tenderness premenstrually, but in severe PMS marked swelling may also be present.

Significant back ache is another common physical symptom. The psychological symptoms are varied and include tension, low mood, irritability, loneliness, anxiety, fatigue, insecurity, and general lability of mood. A proposed new category termed late luteal phase dysphoric disorder has been operationally defined in the diagnostic and statistical manual of the American Psychiatric Association (see Table 1).

Because the diagnostic criteria used from one study to the next frequently vary quite considerably, it is not surprising that the prevalence rates described are also extremely variable, ranging from as low as 20% to as high as 95%. Clearly when an extremely low threshold for defining a case is used, data which is of little clinical validity is obtained. In a study of factory workers 36% were found to have significant premenstrual symptoms, while in a study of university graduates, or the wives of male graduates, 52% had symptoms (see Table 2).

Results obtained are obviously dependent upon the population studied. Many researchers in this field have used populations to which they had easy access, for example university students. Comparing results from symptomatic women at a uni-

Table 1. Diagnostic Criteria For Late Luteal Phase Dysphoric Disorder

A In most menstrual cycles during the past year, symptoms in (B) occurred during the last week of the luteal phase and remitted within a few days after onset of the follicular phase.

B At least five of the following symptoms have been present for most of the time during each symptomatic late luteal phase. At least one of the symptoms being either (1), (2), (3), or (4):

(1) Marked affective lability
(2) Persistent and marked anger or irritability
(3) Marked anxiety or tension
(4) Marked depressed mood
(5) Decreased interest in usual activities
(6) Easy fatiguability or marked lack of energy
(7) Subjective sense of difficulty in concentrating
(8) Marked change in appetite, overeating, or specific food cravings
(9) Hypersomnia or insomnia
(10) Other physical symptoms, such as breast tenderness or swelling, headaches, joint or muscle pain

C The disturbance seriously interferes with work or with usual social activities or relationships with others.

D The disturbance is not merely an exacerbation of the symptoms of another disorder.

E Criteria are confirmed by prospective daily self-ratings during at least two symptomatic cycles.

Table 2. Prevalence Rates of Premenstrual Syndrome

Study	*Population*	*Prevalence (%)*
Andersch, 1980	Community survey	70 mild to moderate 2–3 severe
Appleby, 1960	GP attenders	29
Bickers and Woods, 1951	Factory workers	36
Clare, 1983	GP attenders	74
Halbrich and Endicott, 1985	Student nurses	51
Halbrich and, Endicott, 1985	Women executives	24
Lamb et al., 1953	Students nurses	73
Moos et al., 1969	University graduates	52
Pennington, 1957	Healthy women	96
Sutherland and Stewart, 1965	Nulliparous young women	97
van Keep and Lehert, 1981	Community survey	77

Note: GP: General Practitioner.

versity who have not sought treatment with PMS sufferers attending a specialist clinic is clearly inappropriate.

ASSESSMENT INSTRUMENTS

The most widely used scale for assessing symptom severity is the Menstrual Distress Questionnaire, developed by Moos. It consists of 47 items from which eight symptom groups emerge in factor analysis. As an instrument it tends to focus more heavily on somatic features, than on psychological. The Premenstrual Assessment Form was developed to give greater weighting to psychological symptoms. It has the advantage over the Moos questionnaire of including a greater assessment of mood and introducing bidirectionality in symptom assessment. Other commonly used instruments include the Menstrual Symptom Questionnaire, developed by Stephenson and the PMRS, a 36-item questionnaire developed by Steiner. For a general review of these instruments see Rubinow and Roy-Byrne (1984). For accurate diagnosis and assessment, it is imperative that a prospective procedure be followed. Such prospective ratings should take place over at least two full cycle lengths. Retrospective ratings are unreliable and should not be used for diagnostic purposes.

RELATIONSHIP BETWEEN PMS AND MOOD DISORDER

Most women with PMS experience some lowering or lability of mood. This alteration of mood is transient, and in general is relieved by the onset of menses. It is important in a clinical setting to distinguish this feature of PMS from the premenstrual exacerbation of an underlying mood disorder. Many women when clinically depressed show premenstrual exacerbation of their mood state. The onset of menses may result in a slight improvement in symptoms but the underlying depressive illness remains as before. Although there is clearly some overlap between these two clinical groups, they nonetheless remain two distinct clinical entities with significantly different management. Women who suffer from PMS are at a slightly greater risk of developing post-natal depression, but their overall risk of developing mood disorder is probably not significantly enhanced. The presence or absence of a history of depression (varying from 30% to 76%) in PMS samples suggests that whatever association may exist between depression and PMS pertains to a subgroup.

GENETICS OF PMS

Two recently published studies have thrown new light on genetic aspects of the condition. Condon (1993) examined PMS scores in two groups of twins, 157 pairs of

monozygotic twins and 143 pairs of dizygotic twins. The concordance rates in the monozygotic twins was considerably higher than that seen in the dizygotic twins. This suggests a significant genetic component to the disorder. Obviously, however, the fact that monozygotic twins may share greater environmental similarities than dizygotic twins needs to be considered.

In a similar study, Kendler and colleagues (1992) examined symptoms during the premenstrual and menstrual phases of the cycle in 827 pairs of female twins. A conventional factor analysis showed that the premenstrual and menstrual symptoms were relatively independent of one another and of baseline neurotic symptoms. This study also revealed distinct genetic and environmental factors for menstrual, premenstrual, and neurotic symptoms. Results from this study suggest that the genes which predispose to the development of premenstrual symptoms are largely distinct from those that predispose either to menstrual or to neurotic symptoms. Taken together, these results are consistent with the view that the expression of PMS is determined by several genetic factors, acting additively over a number of loci.

ENDOCRINE ALTERATIONS THROUGHOUT THE MENSTRUAL CYCLE AND PMS

Dramatic endocrine alterations occur during the menstrual cycle to initially induce ovulation and then to prepare the uterus for a possible pregnancy. Endocrine alterations related directly to these changes were logically the first factors to be implicated in the etiology of PMS. We will first consider these ovarian changes, then alterations in hypothalamic-pituitary function both of the gonadal and other axes, and, last, consider the effects of these alterations on central nervous system (CNS) neurotransmission. These physiological changes will be discussed in relation to the possible pathophysiology of PMS.

OVARIAN FACTORS

Relatively high plasma levels of estrogens in women with PMS compared to controls have historically been implicated in the etiology of this disorder. The cause of such high plasma levels of estrogens has variously been attributed to increased gonadotrophin secretion, impaired hepatic secretion, and decreased renal clearance. It has also been suggested that sex hormone binding globulin (SHBG) is decreased in PMS leading to greater circulating levels of unbound steroids. Other groups suggested that the syndrome was caused not so much by high estrogen levels as by a lack of progesterone to act as an antagonist. This progesterone deficiency hypothesis gave rise to the widely publicized, but poorly replicated, findings by Dalton's group of the efficacy of progesterone in the treatment of PMS. When one considers the hormonal surges during the menstrual cycle, with unopposed estro-

gen levels being highest mid-cycle following constant secretion of estrogen by the ovarian follicle, it makes an etiology of unopposed estrogen seem theoretically improbable. Following the postovulatory drop, estrogen levels rise in the luteal phase, but not to their mid-cycle levels and in the presence of rapidly increasing progesterone levels secreted by the corpus luteum. Symptoms should therefore theoretically occur at ovulation if unantagonized estrogen was responsible.

No consistent abnormalities in progesterone levels have been found in PMS. Failure to detect abnormality may be due in part to the inadequacy of the blood sampling procedures employed. In a recent study involving frequent blood sampling in the luteal phase, Facchinetti and colleagues (1993) found that the integrated progesterone levels in PMS patients were similar to health controls. However, the episodic secretion of the hormone is characterized by increased frequency and reduced amplitude. Carefully controlled studies such as this may reveal an abnormality in the secretion of the ovarian steroids. Certainly simple high estrogen or progesterone deficiency has not been supported by the vast majority of the studies and these hypotheses have largely been abandoned as theories of PMS.

More promising are the findings suggesting that it is the rate of decline in ovarian steroid levels, rather than the levels per se, that gives rise to premenstrual symptoms (Halbreicht et al., 1986). This invokes the concept of neuromodulators within the CNS being altered by the sex steroids in a cyclical manner leading to progressively more unstable neuromodulator systems: a paradigm similar to "kindling" models of epilepsy. This is a plausible theory given that both the intensity and incidence of PMS symptoms increases with age and there is a great deal of evidence to suggest that neurotransmitter systems altered by these steroids also alter behavior. It is further evident from the timing of this syndrome that PMS results from a decline or a withdrawal of some function mediated by the ovarian steroids rather than a simple excess or dearth of same.

CORPUS LUTEUM FACTORS

That PMS occurs in the luteal phase of the cycle has resulted in a search for factors which are specific to the luteal phase. Induced anovulation has been used as a treatment for PMS. When women with PMS are administered gonadotropin-releasing hormone agonist, it results in a downregulation of the pituitary gonadotropin cells and decreased secretion of follicle-stimulating hormone (FSH) and luteinizing hormone (LH), thus preventing ovulation. Such treatment leads to a marked reduction of premenstrual symptoms. Whether or not the improvements seen in such patients is due to anovulation is, however, a matter for debate. There are several reasons for questioning such a conclusion:

1. Combined oral contraceptives do not alter the frequency of premenstrual symptoms and may even exacerbate preexisting complaints.

2. There are reports of premenstrual symptoms occurring in well-documented anovulatory cycles
3. In the initial phase of treatment, when the analog is stimulatory, symptoms similar to PMS may occur. Overall it is clear that hormonal changes without ovulation and corpus luteum development can induce the symptoms of PMS.

There is no evidence to suggest that any other ovarian factors, such as inhibin, are altered in PMS sufferers.

ANDROGENS

Recent studies suggest a possible involvement of androgens in the pathophysiology of premenstrual irritability and dysphoria. Eriksson and colleagues (1992) reported higher levels of serum-free testosterone in patients with PMS than in health controls in the luteal phase of the cycle, a finding which was also present during the follicular phase and around the time of ovulation. Dehydroepiandrosterone (DHEA) was found to be elevated at the time of ovulation, while 17-OH-progesterone (17-OHP) levels were elevated in the luteal phase. These results are in marked contrast to those earlier studies which have reported negative findings and therefore need to be replicated.

HYPOTHALAMIC PITUITARY FUNCTION AND PMS

The ovarian steroids, and in particular the estrogens, exert profound effects within the CNS. Estradiol receptors are widely distributed throughout the brain, having a different distribution in males and females. Estradiol mediates its effects by binding to intracellular receptors and altering cellular function at a genomic level. Apart from direct feedback effects on the hypothalamic-pituitary-gonadal (HPG) axis, the sex steroid also alters the secretion of prolactin, adrenocorticotropic hormone (ACTH), growth hormone (GH) and possibly the posterior pituitary hormones. Each will be considered separately and in relation to PMS (see Table 3).

Hypothalamic-Pituitary-Gonadal Axis

The feedback mechanisms modulating the release of gonadotropins and the gonadal steroids within the HPG axis have been outlined elsewhere. Studies comparing the levels of FSH and LH throughout the menstrual cycle have found both decreased FSH and increased LH but these findings are preliminary and require replication. It is suggested that altered gonadotropin levels result from aberrant feedback of the ovarian steroids on the hypothalamo-pituitary axis.

Table 3. Neuroendocrine Studies in Premenstrual Syndrome

Axis Studied	*Authors*	*Findings (Compared to Controls)*
Thyroid	Schmidt et al., 1993	TSH response to TRH normal
Growth Hormone	Facchinetti et al., 1989	Response to (α2-adrenoceptor agonist) clonidine ↓
	Bancroft et al., 1991	Response to (serotonin precursor) L-tryptophan ↓
Prolactin	Steiner et al., 1984	Secretion normal
Cortisol	Rabin et al., 1990	Cortisol response to CRH ↑
	Redei and Freeman, 1990	ACTH levels ↓
Gonadotroph	Facchinetti et al., 1993	LH ↑

Notes: TSH, thyroid stimulating hormone; TRH, thyroid releasing hormone; CRH, corticotropin releasing hormone; ACTH, adrenocorticotropic hormone; LH, luteinizing hormone.

Thyroid Axis

There is a large amount of literature on the various associations between thyroidopathies and mood disturbance. Among the most striking of these associations are the findings that about 30% of patients suffering from clinical depression have blunted thyroid stimulating hormone (TSH) reponses to thyroid releasing hormone (TRH), referred to as sub-clinical hypothyroidism; and depression is a frequent finding in those with thyroid disease. The well-documented "myxoedematous madness" is a form of psychosis consequent upon severe hypothyroidism. This prompted the research into possible associations between thyroid disease and the mood disturbance of the premenstrum, with particular emphasis on possible sex steroid-mediated unmasking of sub-clinical hypothyroidism in the premenstrual period.

Thyroxine (T_4) and TSH levels remain constant throughout the menstrual cycle of healthy women. Neither are thyrotropin releasing hormone (TSH) responses to TRH altered by cycle phase. This suggests that the endocrine alterations of the HPG axis do not impinge on the thyroid axis and further suggests that the clinical manifestation of hypothyroid symptoms, if present, would be unlikely to be altered by cycle phase i.e., would be constant throughout the menstrual cycle. Many studies have now found that women with PMS have similar TRH/TSH responses in both the follicular and luteal phases of the cycle and these responses are normal, with no evidence for any abnormalities in either TSH or T_4 concentrations. There is thus no evidence that thyroid dysfunction underlies this disorder. This is supported by the inefficacy of T_4 replacement in the treatment of PMS (Schmidt et al., 1993).

Somatotroph Axis

It is well established that GH secretion is stimulated by estrogens. Pulsatile GH secretion is enhanced throughout the normal menstrual cycle by E_2 via an

amplitude-modulated effect on the endogenous GH pulse (Faria et al., 1992). The accelerated growth seen during puberty may be causally related to increased plasma GH concentrations consequent upon increased circulating E_2 levels. Administration of sex steroids results in increased GH secretion and suppression of gonadal steroids results in reduced GH secretion. Similarly, GH responses to a wide variety of challenge agents are primed by estrogens. GH responses to GH-releasing hormone (GHRH) are directly related to E_2 levels: peaking at mid-cycle when E_2 concentrations are at their highest. Estrogens also enhance the GH response to arginine and to physical exercise. GH response to the α2-adrenergic agonist clonidine are primed by rising levels of E_2 throughout the cycle of healthy females. The mechanism whereby estrogens modulate GH secretion has not been elucidated. E_2 increases insulin-like growth factor-1 via genomic pathways in certain selective target tissues in the rat and in osteoblastic cells from human long bones. It is possible that E_2 is exerting a similar effect on GH synthesis at the pituitary.

Some of these GH responses differ in women with PMS. The GH response to clonidine is reduced in women with certain premenstrual symptoms during the late luteal phase of the cycle compared to healthy controls (Facchinetti et al., 1989). The GH response to the serotonin (5-HT) precursor L-tryptophan is lower in women with PMS than controls at all phases of the menstrual cycle (Bancroft et al., 1991). This blunted response in the PMS group could result from either reduced GH secretion from the somatotroph or reduced sensitivity of the neurotransmitter systems stimulated, that is, either the 5-HT or the α2-adrenoceptors. This latter possibility is discussed below.

GH responses to the anticholinesterase pyridostigmine are not only primed by E_2 but are also independently augmented by rising circulating progesterone levels (O'Keane et al., 1992). This is interesting in view of the fact that progesterone frequently has an inhibitory effect on GH secretion (Faria et al., 1992). GH responses to the gamma-aminobutyric acid ($GABA_B$) receptor agonist baclofen follow a similar pattern: being stimulated by both E_2 and progesterone. It may be that progesterone is stimulatory to somatostatin-mediated pathways of GH secretion since both baclofen and pyridostigmine GH challenges utilize this pathway. There are no data on these responses in PMS sufferers.

It is unlikely that abnormalities in GH secretion are responsible for the differences in GH responses in PMS sufferers as outlined above since aberrations in GH secretion do not result in behavioral disturbances. Therefore, it is more likely that these differences reflect alterations in the neurotransmitter system challenged.

Prolactin and the Menstrual Cycle

E_2 increases prolactin pulse frequency throughout the menstrual cycle of healthy females (Marshall et al., 1988). This is effected by an E_2-induced increase in prolactin synthesis and secretion via estrogen receptors located on the lactotroph. Through a genomic mechanism E_2 increases prolactin mRNA and this is followed by cell proliferation.

E_2 thus predictably increases prolactin response to many stimuli: TRH, dopamine antagonists, gonadotropin, and the serotonin releasing agent d-fenfluramine (O'Keane et al., 1991). Prolactin responses to challenge agents have not been compared in these groups but there are no differences between prolactin levels throughout the menstrual cycle in healthy women and those with PMS (Steiner et al., 1984). In addition, women with hyperprolactinemia are not noted for having an increased incidence of mood disturbance. Prolactin is therefore unlikely to be causally implicated in PMS.

Hypothalamic-Pituitary-Adrenal Axis

The hypothalamic-pituitary-adrenal axis (HPA) is the core neuroendocrine axis activated during stress in man. In depressive illness major abnormality in the functioning of this axis is noted. In particular, depressed subjects show significant elevation in cortisol levels, frequently having values in the cushingoid range. Furthermore, a majority of severely depressed patients fail to suppress cortisol levels in response to challenge with the synthetic steroid dexamethasone. The release of ACTH from the anterior pituitary is blunted in response to corticotropin releasing hormone (CRH) challenge. The extent to which these abnormalities might exist in patients with PMS has been the subject of a number of recent investigations.

Both ACTH and cortisol plasma concentrations fluctuate throughout the menstrual cycle of healthy females suggesting that the endocrine changes of the menstrual cycle influence the activity of the HPA axis. Basal evening cortisol concentrations in plasma are reported as significantly decreased while the response of plasma cortisol to CRH is significantly increased in women with PMS. ACTH levels in a sub-group of women with PMS, compared to control women, are significantly lower. In healthy women luteal progesterone concentrations seem to exert an inhibitory effect on the HPA axis: this inhibition is not present in those with PMS (Rabin et al., 1990). High dexamethasone non-suppression rates, irrespective of menstrual cycle phase, have been reported in PMS sufferers. These findings suggest that PMS may be associated with subtle alteration in HPA activity in at least a subgroup of this population.

Whether these disturbances in HPA function seen in women with PMS are simply a biological indication of stress, not related to the core pathophysiology of the condition, or whether such endocrine disturbance is in fact of etiological significance remains to be determined.

Posterior Pituitary Hormones

The role of oxytocin and vasopressin in menstrual physiology has received little attention. It is known, however, that changes in the neurohypophyseal hormones do occur throughout the normal menstrual cycle and correlate with changes in estrogen. Vasopressin is a uterine stimulant and may contribute to the

symptom of dysmenorrhea. Females who experience severe dysmenorrhea have increased levels of both vasopressin and some prostaglandins compared to healthy controls (Stromberg et al., 1984). Women who suffer from severe pain premenstrually do not always have significant coexisting mood disturbance and it appears that while prostaglandins and vasopressin may be implicated in the symptom of dysmenorrhea, the syndrome of PMS cannot be explained by these alterations alone.

The other function of vasopressin in modulating water balance and thirst is frequently perceived as being altered by women who suffer from PMS. Fluid retention or bloating is one of the most commonly reported symptoms although this is not reflected objectively in increased weight gain in women who suffer from PMS compared to those who do not. Estrogens bring about an increase in aldosterone secretion by increasing plasma renin substrate, which in turn leads to enhanced angiotensin II and hence increased secretion of aldosterone. Progesterone also increases aldosterone secretion more directly. There is no difference, however, between aldosterone levels in women with and without premenstrual symptoms (Munday et al., 1981). There has thus been no alteration in water metabolism identified to date in women with PMS and no evidence that diuretics would consequently be of benefit.

NEUROTRANSMITTER FUNCTION AND PMS

Serotonin

Serotonin is an indolamine neurotransmitter present in the CNS and implicated in the modulation of many behaviors in humans including sleep, appetite, impulse control, and mood. 5-HT neurotransmission has been widely researched in mood disturbance and there is general consensus that 5-HT deficiency is associated with, and may be causally related to, depression. Since the mood and vegetative disturbances of PMS are similar in many ways to depression and there is an association between PMS and depressive illness, an abnormality of the 5-HT system has been hypothesized to play a role in the genesis and symptoms of PMS.

There is some evidence to support this hypothesis. Because of difficulties in accessing central 5-HT systems in humans, some studies have used the platelet as a peripheral marker since the 5-HT uptake system in platelets resembles that in brain presynaptic neurons. Many studies have now demonstrated that women with PMS have decreased platelet 5-HT uptake and content compared to controls (Steege et al., 1992). These 5-HT uptake abnormalities are similar to those of depression. One method of examining central 5-HT function involves administering 5-HT-mediated dynamic neuroendocrine challenges. One such challenge, mentioned above, has found reduced GH responses to L-tryptophan in the premenstrual period compared to control women (Bancroft et al., 1991). These findings suggest that

some aspects of 5-HT neurotransmission are different premenstrually in women suffering from PMS compared to control groups.

Plasma 5-HT alters throughout the normal menstrual cycle demonstrating an inverse relationship with E_2 levels (Blum et al., 1992). Additionally, many 5-HT-mediated neuroendocrine responses are dramatically altered by menstrual cycle-phase and are closely related to E_2 plasma concentrations (O'Keane et al., 1992). Animal studies suggest that E_2 may alter central 5-HT function by increasing the density of 5-HT receptors and their affinity for 5-MT. The suggestion that this steroid-induced alteration in 5-HT function may be abnormal in women with PMS is supported by the above findings and the fact that 5-HT levels are lower premenstrually in PMS sufferers compared to controls. Additionally, the selective serotonin reuptake inhibiting family of antidepressants is one of very few therapies found to be more effective than placebo in the treatment of this disorder.

Melatonin

Melatonin is a substance synthetized from 5-HT which regulates circadian and seasonal changes in many systems in animals, especially the reproductive system, and has been causally implicated in the etiology of seasonally determined affective disturbances in humans. There are no differences in the patterns of melatonin secretion between PMS and healthy women.

Catecholamines

Plasma noradrenaline (NA) levels also fluctuate throughout the normal menstrual cycle, correlating positively with E_2 levels (Blum et al., 1992). That the NA neurotransmitter system is affected by fluctuating levels of sex steroids is further suggested by the alterations in GH responses to the α2-noradrenergic challenges with desipramine and clonidine at differing menstrual cycle phases (Dinan and O'Keane, 1991). This latter response is reduced in women with PMS in the premenstrum compared to controls (Facchinetti et al., 1989). Estrogens may be mediating this effect on the NA system by altering the rate of transcription of the tyrosine hydroxylase gene: the rate-limiting step in the synthesis of NA (McEwan, 1988). Although this system has not been extensively explored in PMS, involvement of NA in the etiology of PMS appears to have some validity as this system has also been implicated in the pathogenesis of a wide variety of anxiety and depressive syndromes. Estrogens also have profound effects on central dopamine (DA) function but alterations in this system in PMS have not been looked at.

The sex steroids may additionally affect monoamine neurotransmission by altering levels of the monoamine oxidases (MAO): the enzymes responsible for the breakdown of the monoamines (McEwan, 1988). MAO activity differs premenstrually in PMS females compared to healthy controls.

Acetylcholine

Some have suggested that some of the behavioral changes of stress and depression may be mediated by this system and have extrapolated this theory to PMS. There is no scientific evidence to support this but the sex steroids do have a priming effect on cholinergically mediated GH responses (O'Keane and Dinan, 1992).

TREATMENT OF PMS

Effective management of PMS must involve a careful prospective evaluation of symptoms, together with a detailed psychosocial assessment of the circumstances in which they occur. A strictly biological approach to the management of symptoms is unlikely to be successful. A high placebo response rate is found in most studies and it is important to bear in mind that such placebo responses may be transitory. Many patients apparently treated successfully with placebo will relapse within a short period.

Of the numerous published pharmacological studies most are poorly designed with inappropriate diagnostic procedures and lack of placebo control. In fact few studies in this area stand up to a rigorous methodological assessment, resulting in the proliferation of a myriad of dubious treatment regimes. Recent evidence suggests that danazol may be effective and has been found superior to placebo. This inhibits pituitary gonadotrophins and combines androgenic activity with antiestrogenic and antiprogestogenic activity. Combination of gonadotropin-releasing hormone together with estrogen and progestin have also been reported as effective. A recent study comparing estradiol patches with placebo patches found the former to be effective.

There is an accumulating volume of evidence to indicate that the selective serotonin reuptake inhibitor fluoxetine is efficacious in a significant number of patients. The older tricyclic antidepressant, clomipramine, which also has serotonin reuptake inhibiting properties is also superior to placebo. Clomipramine is effective in very low doses and if administered only during the premenstrum.

Whether or not various nutritional supplements are of benefit is far from clear. Recent studies with primrose oil (efamorl) have found it to be no better than placebo. There is some evidence to support the view that magnesium supplementation may be helpful.

SUMMARY

The literature on the biology of PMS is resplendent with theory but rather short on fact. There is, however, an increasing volume of data to support the view that the condition is a psychoneuroendocrine disorder. The most consistent work impli-

cates abnormalities in serotonin neurotransmission as being associated with PMS and effective in its management. One major limitation of work in this area is the very complex alterations in the physiology of the normal menstrual cycle which are unfolding: particularly how endocrine fluctuations impinge on central neuromodulator function.

In a more general sense, it is imperative that future biological work in the area focus on the severe end of the spectrum, and as far as possible avoid studying women where social stress complicates the picture. After over 50 years since Frank originally described the core features of PMS, we are at last gaining some biological insights into the disorder. More rigorously conducted treatment studies are at least enabling us to treat sufferers on a more rational basis.

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Chapter 16

How RU 486 works

MOHAMMED KALIMI

INTRODUCTION

Steroid hormone antagonists are clinically important in the treatment of various steroid hormone related pathophysiological conditions. In addition, they serve as valuable tools to delineate the molecular mechanism of steroid hormone action. Therefore, the development and availability of various pure and potent steroid hormone antagonists are highly desirable both scientifically and clinically.

In 1980 researchers working at Roussel UCLAF, France, developed a synthetic compound designated RU 38486, also called RU 486 or mifepristone ([17 β-hydroxy-11α (4-dimethylaminophenyl)-17α-1-propynyl-estra-4,9-dien-3-one). Over the past decade, accumulated data has demonstrated that RU 486 possesses

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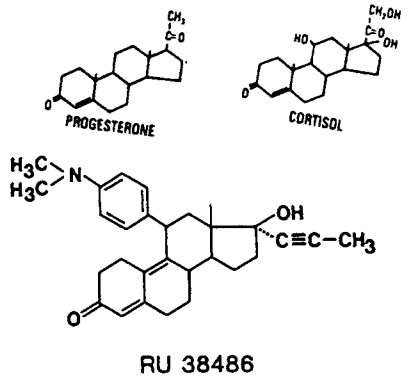

Figure 1. Chemical structures of RU 486, progesterone, and cortisol (a major glucocorticoid in human).

both antiprogestin and antiglucocorticoid activities. The chemical structures of RU 486, progesterone and cortisol are presented in Figure 1.

Animal trials followed by human studies have clearly shown the antiprogestin, abortifacient, and contraceptive properties of RU 486 (Ulmann et al., 1990). RU 486 also has a wide range of beneficial effects in the treatment of diseases such as breast cancer, endometriosis, meningioma, uterine fibroids, and hypercortisolemia or Cushing's syndrome (Nieman AND loriaux, 1987; Baulieu, 1989). In this chapter, the abortifacient properties and our current understanding of the molecular mechanism of RU 486 action are described.

THE ROLE OF PROGESTERONE IN THE FEMALE MENSTRUAL CYCLE AND DURING EARLY PREGNANCY

In order to understand the progesterone antagonistic effects of RU 486 in interrupting early pregnancy, the physiological role of progesterone in the female menstrual cycle and early pregnancy must be understood (see summary below and Figure 2).

During the first half of the menstrual cycle (days 1–14), that is, the follicular or proliferative phase, estrogen and other hormones promote the growth and development of a single ovarian follicle from a primordial follicle to a highly differentiated Graafian stage. The uterus also proliferates under the influence of estrogen, hence the designation proliferative phase. In addition, estrogen directs the synthesis and availability of progesterone receptors which is crucial for the development of progesterone responsiveness during the second half of the cycle.

On day 14, ovulation occurs with the release of the egg. The remaining Graafian follicle, minus the egg, develops into the corpus luteum during the second half of the menstrual cycle (days 15–28). This phase of the cycle is associated with a grad-

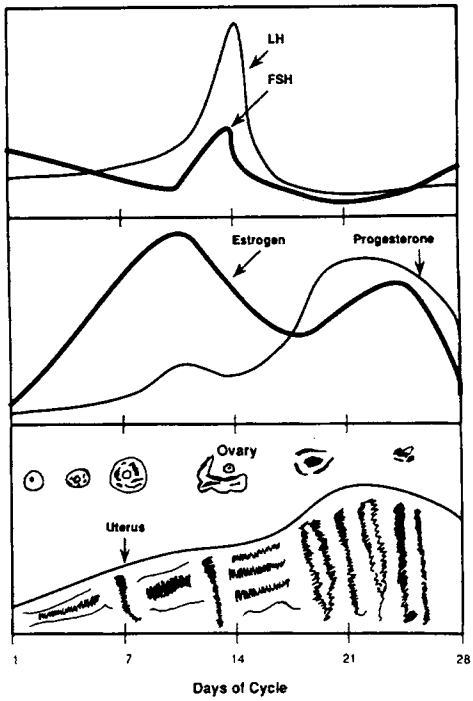

Figure 2. The female menstrual cycle.

ual increase in progesterone release and is known as the luteal or secretory phase. Peak levels of estrogen and progesterone occur between days 21–25 of the cycle. During the second half of the cycle, progesterone and estrogen further increase uterus vascularity, fluid accumulation, and endometrial proliferation as well as secretory activity including the accumulation of glycogen. The proliferative and secretory activity of the uterus is enhanced by progesterone during the second half of the cycle, which prepares it for implantation of the fertilized egg as well as the nourishment of the developing embryo, if fertilization should occur.

If, however, fertilization does not occur, the corpus luteum regresses (days 26–28) and the declining levels of progesterone and estrogen lead to menses on day 1 of the next cycle. Progesterone also decreases the threshold of sensitivity of the myometrial uterine musculature to contractions induced by prostaglandins and oxytocin. In addition, progesterone promotes the firming of the cervix by preventing its dilation. The relaxation of uterine muscle and the closing of the cervical os by progesterone prevents the expulsion of the embryo once it implants in the wall of the uterus.

Following fertilization, on day 14 or 15 of the cycle, the egg begins to divide and after about seven days post-ovulation, it implants in the dorsal wall of the uterus. At

this blastocyst stage, the fertilized egg is an embryonic structure containing an outer layer of trophoblast cells which gives rise to the placenta and an embryonic inner cell mass that will develop into the fetus.

Implantation involves the trophoblast-mediated invasion of the endometrium with eventual formation of a placenta. Progesterone is crucial for the survival and continued development of the fetus and its placenta for the next nine months. For this to happen early in pregnancy, progesterone is produced by the corpus luteum which is protected from regressing at the end of the cycle, if fertilization and implantation occur. This arrest of luteal regression is mediated by the trophoblast portion of the placenta which produces the hormone human chorionic gonadotrophin (hCG), providing for the continued production of progesterone through its interaction and maintenance of the corpus luteum. The increased levels of estrogen and progesterone under the influence of the corpus luteum suppress gonadotrophin (luteinizing hormone and follicle-stimulating hormone) levels which prevent the next cycle of folliculogenesis.

PROGESTERONE ANTAGONIST EFFECTS OF RU 486

It should be obvious from the above that (i) progesterone plays a crucial role in early pregnancy and (ii) RU 486 by its progesterone antagonistic actions, (a) inhibits progesterone-induced endometrial proliferation and secretory activity; (b) increases uterine myometrial contractions by potentiating oxytocin and prostaglandins effects and (c) facilitates dilation of the cervix if administered both before and during pregnancy. By these anti-progesteronic actions, RU 486 prevents implantation of the fertilized egg or detaches the embryo from the uterine wall. It seems that both RU 486 and progesterone must be present to antagonize progesterone action. Besides its direct action on the endometrium, RU 486 can also exert some indirect effect through a block to pituitary gonadotropin secretion which can inhibit ovulation as well.

In human subjects, administration of a single 600 mg dose of RU 486 results in successful termination of early pregnancy in almost 80% of volunteers. The procedure is considered successful if the embryo and a major portion of the endometrium is expelled; no follow up surgery, curettage or vacuum aspiration is needed. The 20% failure rate is attributed mainly to the fact that RU 486 alone is unable to induce the vigorous and frequent uterine myometrium contractions needed to completely expel the embryo and endometrial lining.

The more recent modified protocol consists of a single dose of 600 mg RU 486, followed by synthetic prostaglandin administration adopted for 36–48 hours later. This is now the clinical protocol inducing abortion. The combination of RU 486 plus prostaglandins facilitates complete ejection of both the embryo and endometrial lining within 24 hours of prostaglandin administration. This procedure's success rate is almost 95%.

Prostaglandin administration after RU 486 promotes cramps, nausea, and bleeding in some women. In extremely rare instances it can cause heart functional disturbances. Therefore the subject needs to be carefully monitored for several hours under proper clinical supervision after prostaglandin administration. RU 486, by itself, following its acute administration, does not show any anti-glucocorticoid effects such as changes in electrolyte balance or fatigue due to adrenal insufficiency, or any other noticeable adverse side effects.

The above procedure is only ideal for interruption of first trimester pregnancy since after three months of pregnancy the risks of infection, hemorrhage, and the ethical issues of late abortion become serious contraindications. However, in certain situations, such as serious malfunction of the fetus, *in vitro* death of the fetus, or when the health of the mother is in danger, RU 486 may be used beyond the first trimester of pregnancy.

In normal cyclic women, RU 486 administration promotes menstrual bleeding if given during the mid-luteal phase and delays the development of a secretory endometrium. This is particularly true when given during the early luteal phase. Thus, distinct from its role as an abortifacient, RU 486 may be very effective when given as a post-coital contraceptive or a once a month pill. At the other end of conception, it may be beneficial for the induction or facilitation of labor at term by enhancing myometrial contractions, and cervical dilation.

MOLECULAR MECHANISM OF RU 486 ACTION

Since RU 486 possesses so many useful and diverse clinical applications and also exhibits both antiprogestin and antiglucocorticoid properties in both a tissue and cell specific manner, intensive research has been devoted to understanding the precise mechanism of its action. RU 486 represents a multifunctional endocrine modulator of great versatility at the molecular level (Mao et al., 1992; Horwitz, 1992).

It is now generally recognized that progesterone, being hydrophobic, crosses the target cell's plasma membrane by simple diffusion and binds with high affinity and limited capacity to specific receptor proteins localized in the nuclear compartment (Carson-Jurica et al., 1990). The binding of progesterone to the receptor results in conformational changes called activation or transformation which enables the progesterone-receptor complex to dimerize. The subsequent binding of dimers to specific transcriptional enhancer DNA sequences called hormone-responsive elements (HRE) results in the activation of transcription of genes under the control of progesterone.

From the above outline of progesterone hormone action (see Figure 3), it is logical to assume that the difference between agonistic and antagonistic actions may occur at either one or more of the above mentioned steps. However, the precise molecular mechanism of RU 486 action is far from understood and no insight has yet been obtained to clearly delineate the step or steps where an antagonist precisely in-

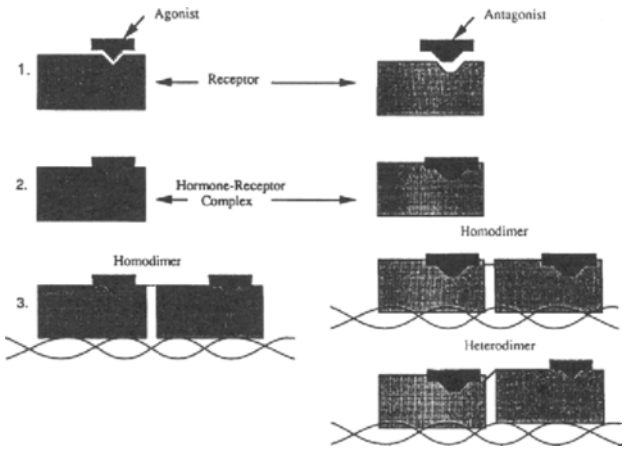

Figure 3. Schematic representation of antihormone action. At the receptor binding level (step 1), the antihormone (AH) may bind at the site distinct to that of agonist. At the activation level (step 2), distinct conformational changes may be induced by the agonist and antagonist following their binding to a progesterone receptor. At the HRE binding level (step 3), agonist-receptor complexes may display a distinct dimerization pattern as compared to the antagonist-receptor complex and/or differences in their binding to HRE. Beyond the HRE binding level, little is known about the mechanisms of antihormone actions.

hibits progesterone receptor actions (Figure 3). Briefly stated, the data obtained so far has demonstrated that RU 486 binds to the progesterone receptor with a higher affinity than that of progesterone itself. However, the receptor binding assay fails to reveal whether RU 486 binds to progesterone receptors at the same site as an agonist or at a different site. Answering this question will be one way to elucidate the mechanisms of the antagonistic functions of RU 486.

In an elegant study utilizing a library of a hormone binding domain-mutated human progesterone receptor B-subunit, Vegeto et al. (1992) concluded that there is a binding site difference between agonists and antagonists which may account for the observed biological differences between RU 486 and progesterone. Early studies concerning changes of RU 486-receptor complexes after initial binding suggest an almost normal activation of RU 486-PR complexes. These data seem to indicate that the activation process might not be the step by which RU 486 action is distinguished from the agonist actions.

Another possibility is that there are structural differences between the activated antihormone-receptor complexes and the activated hormone-receptor complexes which may result in changes in their ability to dimerize and /or in their binding to specific recognition sites involving expression of hormone inducible genes (DeMarzo et al., 1992), although they may recognize the same DNA binding site. Fi-

nally, it is possible that the antihormone action of RU 486 may exist beyond its binding to specific transcriptional enhancer DNA sequences or HREs (Truss et al, 1994).

SUMMARY

RU 486 is a potent synthetic antiprogesterone drug which provides an effective and almost safe clinical method of inducing abortion in early pregnancy. Alternatively, it can also be used as an effective postcoital pill blocking ovulation as well as nidation of the fertilized egg. However, despite its clinical value in interfering with gestation, the molecular mechanism of RU 486 is not clearly understood at the present time. Its antiglucocorticoid effects represent a separate but clinically important action distinct from its modulation of progesteronal physiology.

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Chapter 17

Infertility

HENRY G. BURGER

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INTRODUCTION: DEFINITIONS

Infertility is defined conventionally as the inability or failure of a couple to initiate a pregnancy after at least 12 months of unprotected sexual intercourse. The term "primary infertility" is used when no pregnancy has previously occurred in that union, whereas "secondary infertility" is the failure to initiate a pregnancy within 12 months after a previously successful pregnancy in that union. The definitions take no account of demonstrated fertility in a previous union.

Epidemiologic observations in a number of populations in developed Western societies indicate that 60 to 70% of couples in the general population will have initiated a pregnancy after 6 months of unprotected intercourse and 85 to 90% after 12 months. It is generally accepted that the average probability of initiating a clinical pregnancy in any given month is approximately 20 to 25%, although in couples of proven fertility that probability may be as high as 50 to 60% when intercourse occurs at the time of peak fertility during the menstrual cycle.

In some couples, pregnancies are successfully initiated, but recurrent abortion occurs. Such couples are clearly functionally infertile but the causes of recurrent miscarriage differ somewhat from those causing primary or secondary infertility and will not be considered further in this chapter.

Fertility is clearly a function of age, particularly of the female partner, with peak fertility rates being observed until approximately age 35 years, with a steady decline between 35 and 40 and a steeper decline thereafter. The most reliable data for the decline in fertility as a function of age has come from centers for donor insemination, where aging of the male partner can be corrected for. Various available data for population-related fertility rates indicate approximately 220 to 450 births per 1,000 married women per annum in the 30 to 34 year age group, 180 to 400 births in the 35 to 39 year age group, 100–220 births in the 40 to 44 year age group. Annual probabilities of pregnancy in the 45 to 49 year group are believed to be approximately 20–30 per 1,000. Modern tendencies in developed countries to delay the age of first planned pregnancy are thus associated with a somewhat higher probability of the occurrence of primary infertility.

It must be emphasized strongly that infertility is a problem of couples and that approaches to its assessment and management should be taken with the couple together, rather than with the individual members. It must also be emphasized that potential problems with fertility in one partner may become evident only following investigation of the other partner who appears primarily responsible for infertility, and that a relatively high level of fertility (difficult to define precisely scientifically) in one partner may compensate for impaired fertility in the other. Recent data from assisted reproductive technology (ART) programs (i.e., programs offering *in vitro* fertilization (IVF) and related procedures) suggest that when only few spermatozoa are available for IVF, only "high quality" oocytes are fertilized, whereas if many spermatozoa of good motility are available, lower quality oocytes are also fertilized. Infertility may result from an ob-

vious disturbance of reproductive capability in one of the partners, but may equally result in a couple where no obvious impairment of reproductive capacity is demonstrable.

REQUIREMENTS FOR FERTILITY

The fundamental requirement for normal fertility is the fertilization of an oocyte by a competent spermatozoon. The fertilized egg must then be able to be transported normally within the female reproductive tract to implant and to proceed with embryonic and fetal development. The requirements for normal fertilization clearly require the release of a mature oocyte from the ovary through the process of ovulation, the passage of that oocyte into the fimbrial end of the Fallopian tube, and along that tube in an environment free of adverse factors. Spermatozoa capable of fertilization must be deposited into the female tract close to the time of ovulation and must subsequently be capable of being transported through the female tract to the outer third of the Fallopian tube, and to undertake capacitation so as to successfully fertilize the egg.

Thus the requirements for normal fertility include a normal genital tract, normal ovarian follicular development and ovulation in the female, intact androgen production, and normal spermatogenesis and spermiogenesis in the male. Sexual intercourse must be appropriately timed and physically competent and there must be the appropriate passage of sperm through the female reproductive tract. Infertility will occur if there is a major obstacle to any of these functions.

APPROACHES TO THE ASSESSMENT OF THE INFERTILE COUPLE

Ideally, the couple are seen together at the initial consultation. The major objectives are assessment of each of the partners for the presence of factors likely to contribute to their infertility, assignment of a realistic prognosis where possible and provision of mutually significant information, which will allow the couple to make informed and responsible decisions about the extent of further investigation and treatment. For doctors specializing in the area of infertility, provision of the couple with a preliminary questionnaire in which information is sought about major likely contributory factors can be helpful and ensures that errors of omission will be unlikely to occur. Fundamental information sought from the couple at the outset includes the duration of their involuntary infertility and methods of fertility regulation previously used, the age of each of the members, any history of prior fertility in this or any other union, and the results of prior investigations including the circumstances in which they were conducted. The features specific to each member of the couple are considered below.

Male Partner

Information is sought first with regard to factors likely to affect testicular or reproductive tract function. Thus it is useful to obtain a history as to whether pubertal development was normal or required any therapy, and whether the testes have always been scrotal in position, or whether there is a history of cryptorchidism, likely to be an adverse factor in male fertility. Any history of testicular trauma, torsion or infection including epididymo-orchitis will be important. Urinary tract infections or urethral discharge may give a clue to possible etiologies of epididymal obstruction. Sexual function should be explored in terms of adequacy of erection, frequency of coitus and any difficulties encountered.

Questions should be asked regarding the man's general health, his use of any medications currently or in the past (e.g., antitumor therapy with agents such as cyclophosphamide can cause irreversible impairment of spermatogenesis, treatment of ulcerative colitis with salazopyrine or use of anabolic steroids can impair spermatogenesis) and the presence of diabetes, which can lead to problems such as impotence and retrograde ejaculation. Enquiry regarding the use of caffeine, alcohol and tobacco is of some importance as excessive alcohol intake can impair testicular function, and heavy cigarette smoking or caffeine intake may impair fertility, particularly in the female.

Physical examination of the male partner is directed particularly to the observation of any general signs of male hypogonadism such as poor beard development, diminished pubic hair, poorly developed musculature or eunuchoid proportions. A history regarding sense of smell can be important in raising the possibility of Kallmann's syndrome of hypogonadotropic hypogonadism with anosmia, and a story of chronic productive cough may suggest a cause of obstructive azoospermia. Gynecomastia may give a clue particularly to the possibility of Klinefelter's syndrome of seminiferous tubule dysgenesis, due to the presence of one or more extra X chromosomes.

Examination is directed particularly to the scrotal contents and the inguinal region. Testicular volume can be assessed by measurement of the length of the testis, or more satisfactorily by the use of a graded series of ellipsoids, an orchidometer, which allows a more accurate assessment of testicular volume. Reduced testicular volume is indicative of loss of seminiferous tubule tissue, a common association of impaired spermatogenesis. In contrast, normal testicular volume in the presence of azoospermia will suggest the diagnosis of duct obstruction. Palpation of the epididymis may reveal nodularity or dilatation of the head, suggesting epididymal obstruction. Palpation of the vas will assure its presence—congenital absence of the vas can be diagnosed at the bedside. Requests to the patient to perform Valsalva's maneuver can allow the identification of spermatic venous reflux or lead to distension of the spermatic veins when varicocele is present, usually on the left side. Examination of the penis will ensure normal positioning of the urethral meatus. The presence of scars in the inguinal region may give a clue to causes of surgical obstruction. Where suspected, more particular examination looking for physical signs of a pituitary tumor may be relevant in rare circumstances.

Female Partner

The history in the female partner is directed specifically to assessment of ovulatory function and of any circumstances which may predispose to tubal obstruction. Ovulatory function will be gauged from the history of menarche, the characteristics of the menstrual cycle and whether there are symptoms usually indicative of normal ovulatory function, e.g., premenstrual irritability, bloating, or mastalgia. It is useful to enquire whether the patient has learned about the symptoms of fertility, including the presence at mid-cycle of mucous discharge at the vulva, resembling raw egg white in being clear, slippery, and lubricative. A history of regular cycles with symptoms typical of the luteal phase will provide strong clinical evidence that an ovulatory disturbance is an unlikely contributor to the couple's infertility.

Enquiry regarding sexual function is again important in regard to frequency and particularly to the presence of dyspareunia, i.e., any discomfort, either superficial or deep, at intercourse. Dyspareunia may provide a clue to the possibility of endometriosis and may also occur with psychological disturbance. A history of marked pain on menstruation (dysmenorrhea) will provide additional evidence for the possibility of endometriosis. Particularly in the presence of menstrual irregularity or of secondary amenorrhea, a history of weight change or of hirsutism may help in providing clues about the possibility of hypothalamic amenorrhea or polycystic ovary syndrome, respectively. A lifelong history of oligomenorrhea is strongly suggestive of the latter.

A history of pelvic infection or of surgery for complicated appendicitis or other pelvic disorders will raise the possibility of tubal obstruction. A story of nonpuerperal galactorrhea will suggest the possibility of hyperprolactinemia.

Questioning should again be directed towards the female partner's general health and her use of alcohol and cigarettes. Excessive caffeine consumption has been associated with a lowered rate of fertility and should be enquired about.

Physical examination should pay particular attention to the patient's body mass (whether she is markedly underweight or overweight) and to the presence of hirsutism. Physical signs suggestive of hypothyroidism should be noted. Pelvic examination should be directed towards the size of the uterus, the palpability or otherwise of the ovaries, the presence of thickening in the fornices, or reduced uterine motility, suggesting possible pelvic inflammatory disease or hydrosalpinx, and particularly the presence of any pelvic pain or tenderness which would again suggest the diagnosis of endometriosis. Expressible nipple discharge can be sought as evidence of possible hyperprolactinemia.

Baseline Investigations

The minimal investigations of the infertile couple presenting for the first time include semen analysis in the male and hormonal assessment of ovulation, normally by determination of an appropriately timed serum progesterone concentration in

the female. In addition it is useful to check the serum prolactin concentration and in the presence of any clinical suspicion, to check baseline thyroid function. Assessment of tubal patency by laparoscopy or hysterosalpingography will be undertaken depending on the results of the other investigations and the overall formulation of the likely diagnosis.

Semen Analysis

It must be emphasized that the results of semen analysis must be interpreted with caution. It is generally recommended that at least two analyses are ordered, separated by an interval of at least two weeks. If the first analysis is entirely normal, it can be taken as indication that a significant male factor in the couple's infertility is unlikely, but the presence of any abnormal semen characteristics would lead to the obtaining of at least one further specimen, as semen parameters may show marked variability. It is noteworthy that a history of recent febrile illness is important as mild to moderate infections can produce a transient impairment in semen quality. The reported demonstration of azoospermia will of course indicate a clearcut male factor in the couple's infertility and requires further investigation to determine whether it is caused by disordered spermatogenesis or obstruction to the reproductive tract. Where azoospermia is due to impaired spermatogenesis, except in the rare case of hypogonadotropic hypogonadism, it is likely to be remediable only by assisted techniques (ART, see below). Obstructive azoospermia may be able to be corrected, or be managed also by ART.

Where spermatozoa are present in the ejaculate, the assignment of significance to the results of semen analysis is more problematic. A normal semen specimen usually ranges in volume from 2 to 6 ml, and contains a minimum of 20 million sperm/ml. At least 50% of the spermatozoa should be motile and at least 25% should show good forward progressive motility. Depending on the rigor of the criteria used, at least 20% of the spermatozoa should be of normal morphology. Where the sperm count is persistently below 1 million/ml a highly significant contribution to the couple's infertility may be assumed. Counts between 1 and 5 million/ml are also likely to represent a significant male contribution but in the range 5 to 20 million/ml, significance of oligospermia is more difficult to evaluate and requires a combined assessment of the couple's total situation. A routine screening investigation when sperm are present in the specimen, should be a search for the presence of antisperm antibodies, as immunological male infertility may be present in about 5% of infertile males and cannot be diagnosed without such assessment.

Endocrine Assessment of the Male Partner

Changing technologies for the management of male factor infertility are leading to a re-appraisal of the indications for endocrine assessment, particularly of the patient with normal secondary sexual characteristics and oligospermia. The most im-

portant single measurement is of serum follicle-stimulating hormone (FSH). For the male with azoospermia, serum FSH will assist in definition of the likely cause; a normal FSH will suggest the possibility of obstructive azoospermia, particularly if testicular volume is clearly normal. An elevated FSH level makes seminiferous tubule failure highly probable. In such cases, it is also important to measure luteinizing hormone (LH) and testosterone, as seminiferous tubule disease may be associated with some degree of Leydig cell failure, requiring androgen supplementation (e.g., in Klinefelter's syndrome). In the male with azoospermia and reduced testicular volume, serum FSH will provide a lead as to whether the disorder is primarily testicular (FSH raised) or whether it may be secondary to hypothalamic or pituitary disease (low or low normal FSH).

Whether serum prolactin should be measured routinely in the male partner who is found to have an abnormal semen analysis is controversial. In 1% to 2% of men with abnormal semen characteristics, serum prolactin is found to be mildly to moderately elevated and, in such instances, the possibility of a hypothalamo-pituitary lesion needs to be excluded. In the patient with varicocele and oligospermia, FSH measurement may also be of value in decision making about the indications to undertake varicocele ligation (or embolization); a normal FSH will favor intervention, an elevated FSH is sometimes regarded as a contraindication. In idiopathic oligospermia, or idiopathically reduced sperm motility, endocrine assessment is of little value.

Tests of Ovulation

Whereas charting of basal body temperature and demonstration of the presence of a hyperthermic phase provides useful data suggestive of normal ovulation, measurement of mid-luteal serum progesterone is the most useful diagnostic test to prove it. Occasional ovulation in the patient with oligomenorrhea may be more difficult to establish because of the problem of appropriate timing of the sample. Diagnostic progesterone values are obtainable only within 7 ± 3 days of the onset of the next menses and this may be impossible to predict in the patient with oligomenorrhea in whom weekly sampling may be helpful.

In the patient with amenorrhea the baseline estimation of serum FSH, LH, and prolactin is mandatory, particularly to exclude the possibility of primary (premature) ovarian failure, characterized by a clearly raised serum FSH level. An elevated ratio of LH to FSH with normal baseline follicular phase FSH concentration is compatible with the diagnosis of polycystic ovarian syndrome. An elevated prolactin concentration suggests the possibility of pituitary tumor, hypothalamic disease or use of dopamine-receptor blocking medications mainly for psychiatric disorders. Hyperprolactinemia is usually significant only if it causes oligomenorrhea or secondary amenorrhea.

More detailed aspects of the investigation of the female partner are beyond the scope of the present introductory chapter, but establishment of tubal patency is es-

sential in the couple with no other identified causes of infertility, and in those where the male partner has moderate oligospermia.

CAUSES AND APPROACHES TO MANAGEMENT OF MALE INFERTILITY

On the basis of history, physical examination and semen analysis, with or without serum FSH assays, and following the principles outlined above, it is usually possible to assign the man to a particular management category and to provide some assessment of prognosis. A decision can be made as to whether the male partner is likely to be a significant contributor to the couple's infertility. If he is, he may have a severe form of infertility which cannot be treated and for which the only appropriate management for the couple is the use of donor insemination. Alternatively, the male may have a potentially treatable disorder not involving recourse to ARTs and hence not involving direct treatment of the female partner. In many instances, the only treatment that may be suggested is the use of ARTs which then predominantly involve treatment of the female partner, as, usually, the only requirement from the male is the production of an appropriately timed ejaculate, or in some instances being subject to a surgical procedure to allow sperm collection from the testis.

Infertility Untreatable in the Male Partner

This situation is found in about one in eight men with male infertility. The major category is primary seminiferous tubule failure with azoospermia. This may result from chromosomal disorders, particularly micro deletions of the Y chromosome, not accompanied by any phenotypic abnormality. It may also occur in Klinefelter's syndrome, characterized by an extra X chromosome and various clinical abnormalities, such as eunuchoid proportions, gynecomastia, and testes < 6 ml in volume. It may follow severe orchitis, treatment with cytotoxic drugs or irradiation. Some patients with this disorder have associated impairment of interstitial cell function with borderline or low serum testosterone and may benefit from testosterone supplementation.

If acceptable to the couple and if the female partner is normal or can be rendered normal, insemination with donor sperm is the major option available to them, apart from the possibilities of adoption or acceptance of their childless state. However, ARTs with intra-cytoplasmic sperm injection (ICSI) may be an option if mature or even immature sperm are recoverable on open testicular biopsy.

Potentially or Possibly Treatable Infertility in the Male Partner

Potentially treatable disorders regrettably are again present in only about one in eight men with infertility. Causes include gonadotropin deficiency or suppression, occasional hyperprolactinemia, obstructive azoospermia, coital disorders due to

impotence, failure of ejaculation or retrograde ejaculation, sperm autoimmunity, and effects of some drugs, toxins, stress, or illness.

Gonadotropin Deficiency

Gonadotropin deficiency is rare but treatable either with gonadotropin replacement or with gonadotropin-releasing hormone administered in a pulsatile manner. At least 50% of affected individuals are likely to be rendered fertile. Recombinant gonadotropins are becoming available for this purpose.

Obstructive Azoospermia

The commonest cause of obstructive azoospermia is inflammatory blockage of the epididymis. Congenital absence of the vas is the commonest inherited cause of obstruction with a frequency of about 1 in 2,000 men. Such men are usually phenotypically normal and are heterozygous for a cystic fibrosis gene mutation. The semen analysis is characterized by the finding of a low semen volume, because of the associated absence of the seminal vesicles. Obstructive azoospermia may result from preceding vasectomy, where a change of mind about desired fertility has occurred or a new union entered.

Obstructive azoospermia may be approached by appropriate surgical techniques. Good results may be obtained if vasectomy reversal is undertaken within four to five years of the vasectomy, and results are generally poor if more than 10 years have elapsed. Even after early reversal, about 25% of men remain infertile, either because of surgical failure or because of diminished sperm function, resulting primarily from sperm autoimmunity. When surgery has failed or when it is likely to produce poor results, ARTs may be appropriate, with collection of sperm from the epididymal head, on the testicular side of the obstruction, followed by IVF or ICSI.

Coital Disorders

Serious coital disorders are a relatively uncommon cause of treatable male infertility. Management of impotence may now be achieved using direct intracavernosal injection of prostaglandin E or papaverine, while failure of ejaculation particularly in men with spinal cord injury is treatable with a vibrator or electrically induced ejaculation. Retrograde ejaculation, suggested by the presence of low seminal fluid volume, may be able to be treated by preparation of sperm from the urine, after suitable adjustment of urinary pH and osmolality.

Sperm Autoimmunity

Sperm autoimmunity may be idiopathic, postinflammatory, or a consequence of vasectomy. The presence of sperm antibodies alone is insufficient for the diagnosis

of this disorder which also requires demonstration of absence of sperm penetration of normal mid-cycle cervical mucus on at least two occasions. Approaches to therapy include the use of high dose corticosteroids to suppress the autoimmune process, but this has largely been replaced by the use of ART or ICSI.

Drugs, Toxins, and Transient Depression of Semen Quality

The possible influence of such agents has already been considered. It is of particular importance to ensure that semen collected for the purpose of fertility assessment must be collected in the absence of significant systemic illness. Even a prolonged sauna bath or an upper respiratory infection of moderate severity may produce transient changes in semen quality strongly suggestive of male factor infertility. Enquiry must therefore be made about the male partner's health and activities in the two months or so prior to collection of the ejaculate and repeated analyses made if necessary.

Male Infertility Potentially Treatable Using Assisted Reproductive Technologies Involving the Female Partner

When oligospermia, decreased motility, or non-obstructive azoospermia is present, a number of clinical associations may have been demonstrable in the history or the physical examination. These include a history of treated cryptorchidism, or the presence of varicocele. While it seems likely that the latter does contribute to causing male infertility (it is present in 5–10% of the normal fertile population of males in the community, but up to 40% of males seen in an infertility clinic), conflicting results have been reported as to whether correction of varicocele leads to a significant improvement in fertility. If a clear cut varicocele is present, accompanied by a testicular volume lower on the side of the varicocele than on the contralateral side, and if oligospermia is present with normal levels of serum gonadotropins, and normal or treatable female reproductive function, a trial of the results of varicocele ligation seems reasonable. If this does not lead ultimately to pregnancy, ART may again be recommended.

A large number of other treatments have been evaluated in the past for oligospermia or diminished motility of uncertain cause, including antibiotics, anti-inflammatory agents, various hormonal therapies and husband artificial insemination. None of these is of proven efficacy. There is however a growing body of evidence suggesting that ovulation stimulation in the female partner, with intrauterine insemination of washed male semen, may improve pregnancy rates in patients with this disorder.

For the vast majority of infertile men with depressed semen quality, initial attention should be paid to ensuring that all remediable factors in the female partner have been treated and that coitus is occurring with appropriate timing related to the fertile phase of the menstrual cycle. Once these factors have been taken into account a

realistic prognosis should be offered. On the basis of experience in the author's institution, average duration of infertility of more than 1,400 sub-fertile couples was 36 months, and the proportions of women conceiving within a year were as follows, based on the results of semen analysis:

15% in couples where the sperm count was 1 to 5 million/ml
22% with 5 to 20 million/ml
30% with sperm concentrations greater than 20 million/ml but impaired sperm motility (see Figure 1).

If no remediable factors are identified, the use of ART can then be carefully considered. The majority of men with oligospermia, significantly reduced sperm mo-

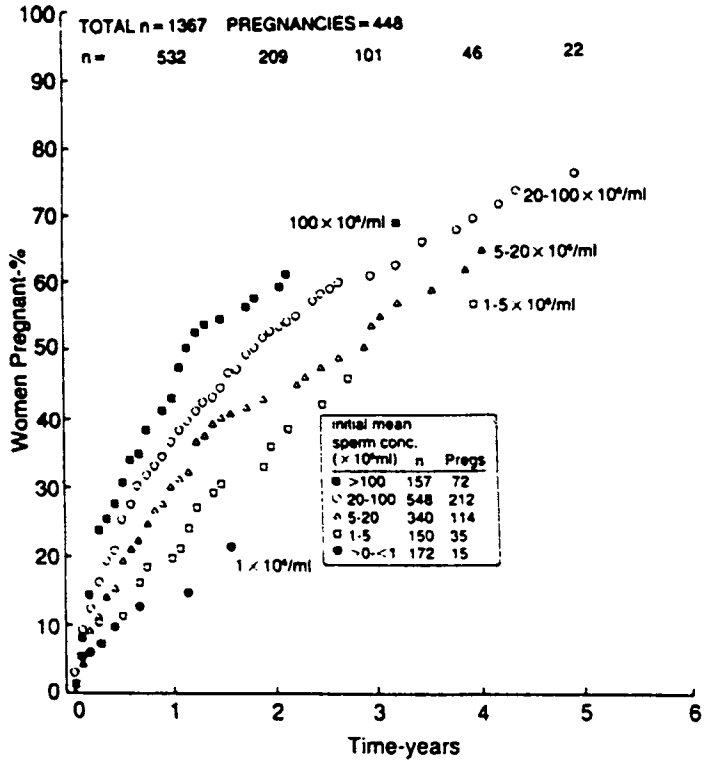

Figure 1. Pregnancy rate curves grouped according to average pretreatment sperm concentrations. The number of patients followed to the beginning of each year is shown above (n). The numbers of men and pregnancies in each sperm concentration group is shown in the inset table. Reproduced with permission from Baker, H.W.G. (1995). Male Infertility, In: DeGroot's Textbook of Endocrinology, W. B. Saunders Co., Philadelphia.

tility, or nonobstructive azoospermia have no clearly definable cause for the abnormality and in that situation in current practice, the use of ART can be recommended. Where at least 1 million motile sperm can be recovered from the ejaculate, the use of gamete intrafallopian transfer (GIFT) may be the preferred option, though many centers recommend an initial cycle in which IVF is attempted, in order to demonstrate the potential fertilizing capacity of the spermatozoa. If between 50,000 and 1 million sperm are available, IVF and embryo transfer (ET) may be appropriate. The results of ART depend on the severity of the defect in semen quality, with less than 20% of oocytes being fertilized in 20 to 40% of patients, particularly those with abnormal sperm morphology. When standard IVF fails, or when severe oligospermia is present, or if less than 5% of spermatozoa are morphologically normal, recently developed sperm microinjection techniques are indicated, the most promising being the direct ICSI, which provides fertilization rates of about 65%, with 90% of couples proceeding to ET, irrespective of sperm quality or site of collection. If sperm are collected directly from the testis or epididymis only ICSI is possible as such sperm are immotile.

Because of the expense and invasiveness of ARTs, the couple should be fully informed of these various aspects and be assured that the chances of obtaining a healthy child are significantly reduced, before a recommendation is made to proceed to IVF fertilization. This involves primarily significant hormonal therapy and investigation, often including laparoscopy, for the female partner for a disorder primarily resulting from problems in the male. The overall chance of success is 20 to 30% per attempt in most programs, i.e., similar to the natural fertility rate but relatively at great cost. Safety considerations regarding ARTs are important and include the possibility of an increased rate of breast or ovarian cancer resulting from ovarian hyperstimulation in the female, the possibility of transmitting of Y chromosomal defects to a male infant in men with severe oligospermia and the general lack of long-term data regarding the safety of ICSI.

In summary, specific therapies to correct the underlying cause of male factor infertility are applicable to only a minority of patients presenting with this disorder. The management of severe male factor infertility has been revolutionized by the application of ARTs.

CAUSES AND APPROACHES TO MANAGEMENT OF FEMALE INFERTILITY

The relative frequency of different causes of female infertility varies considerably according to the center reporting its data. Overall, it can be generalized that ovulatory disorders and tubal disease each contribute about 30% of cases of female factor infertility, endometriosis another 30%, and cervical factors the remainder. Approaches to the female will follow a classification similar to that used for the male.

Untreatable Female Infertility or Treatment Requiring the Use of Ovum Donation

The major untreatable (and fortunately rare) cause of female infertility is absence of the uterus. If the ovaries are absent or if premature ovarian failure has occurred, because of ovarian follicular depletion or refractoriness to gonadotropin stimulation, the possibility of ovum donation can be considered, using ART.

Female Infertility Treatable by Ovulation Induction

Primary amenorrhea is said to be present when no spontaneous menses have occurred after age 16, while secondary amenorrhea is the term used for the absence of menstruation for six months or more after at least one spontaneous menstrual period. Causes of amenorrhea may logically be assigned to the hypothalamus, the pituitary, the ovary, or to other endocrine disorders impacting on the hypothalamic-pituitary-ovarian axis. A detailed discussion of the causes of anovulation is beyond the scope of this chapter but a frequent cause of secondary amenorrhea is the occurrence of substantial alterations in body weight, usually weight loss. This is a particularly important consideration in patients who remain amenorrheic after discontinuation of the use of the oral contraceptive pill, when weight loss during pill taking may go unrecognized. In this case the amenorrhea is ascribed to a complication of the oral contraceptive, when in fact it is associated with weight loss and hence a functional hypothalamic disturbance.

A common cause of secondary amenorrhea or oligomenorrhea (menses occurring at intervals of six weeks to six months) is the polycystic ovary syndrome.This is characterized by the presence of one or more of the clinical features of oligomenorrhea, obesity and hirsutism; the biochemical features of elevation of serum LH and/or serum androgens; and the ultrasound features of peripherally situated multiple small ovarian cysts 2–8 mm in diameter, with increased stromal echogenicity and often increased ovarian volume.

Other more subtle causes of ovulatory disturbance include defective function of the corpus luteum, or failure of a follicle to rupture, the so-called luteinized unruptured follicle syndrome. Intensive studies of the mid-cycle period by hormonal and ultrasound assessment also suggest that subtle disturbances may sometimes be found in women who superficially appear to be ovulating normally. Hyperprolactinemia is an important cause of secondary amenorrhea and may result from the presence of pituitary tumor, either minute or large. Inappropriate galactorrhea is a frequent accompaniment of this disorder, which may also result from hypothalamic-pituitary disconnection or the use of dopamine blocking agents, e.g., psychotropic drugs.

Where an easily treatable cause of ovulatory failure is present, management is directed appropriately. Thus hyperprolactinema can be treated with the use of prolactin lowering agents, such as bromocriptine, and weight loss associated amenorrhea often responds to a program of weight gain. Where anovulation persists,

ovulation induction therapy can be offered, initially using ovulatory stimulant drugs such as clomiphene citrate. If this is not indicated or fails, gonadotropins can be used and in hypothalamic hypogonadotropic hypogonadism, pulsatile gonadotropin-releasing hormone presents an alternative possibility. The latter is associated with a low chance of the occurrence of multiple pregnancy.

Female Infertility Treatable by Surgical or Hormonal Methods

The major causes under this heading are tubal obstruction and/or endometriosis. The patency of the uterine tubes can be assessed by transcervical injection of a dye such as methylene blue and inspection of the passage of the dye through the uterine tubes at laparoscopy. Complete tubal obstruction may result from sexually transmitted infections, e.g., gonococcal and chlamydial, or may be secondary to pelvic peritonitis, or previous tubal ligation for the purpose of sterilization. The significance of other tubal disorders such as tortuosity, irregular dilatation, kinking or adhesions is less clear but in the absence of other causes of infertility may be regarded as having some substantial significance. Tubal obstruction may be treated either by tubal surgery aimed at restoring patency or by the use of ART especially IVF and ET, thus by-passing the tubal blockage. Surgical success depends on the nature of the obstruction and its site. For reversal of tubal ligation, appropriate surgery may restore patency in up to 90% of patients with about half of them becoming pregnant in two years. With other forms of obstruction results are less satisfactory and ART may be preferred. One of the major complications of tubal surgery is the subsequent occurrence of ectopic pregnancy.

For IVF, current "take-home baby" rates vary from 15 to 30% per transfer of two embryos, though this technology is associated with a spontaneous abortion rate of about 20% and ectopic pregnancy rate of about 6%. Pre-term labor and perinatal mortality were high in the past but have been reduced by reducing the number of embryos transferred, thus reducing the multiple pregnancy rate. The level of skill for neonatal intensive care has also improved the outlook.

Endometriosis, the presence of ectopic endometrial tissue in the pelvic peritoneum and in the ovaries, is associated with chronic pelvic pain, dyspareunia, dysmenorrhea, and infertility. The role of mild to moderate disease in causing female infertility is controversial and some authorities believe that it is insignificant. Severe endometriosis may clearly interfere with tubal or ovulatory function and its treatment can be surgical or with the use of drugs such as Danazol, the isoxazol derivative of 17α-ethinyltestosterone. Other ovulatory suppressing regimens can be used including high dose progestagens, or long acting gonadotropin-releasing hormone analogues or antagonists.

Other Treatable Causes of Female Infertility

The significance of factors involving cervical function is somewhat controversial. However occasional patients may have very thick and apparently impenetrable

cervical mucus or may have absence of mucus as a result of previous cervical surgery. Antisperm antibodies may also be present in cervical mucus. Management approaches include the use of intrauterine insemination or ARTs.

THE COUPLE WITH IDIOPATHIC INFERTILITY

In 10 to 20% of couples presenting with the complaint of infertility, detailed history and physical examination, followed by the major investigations outlined above, will fail to elicit any clear-cut explanation for the couple's problem. The diagnosis of infertility of unknown cause clearly depends on the degree and sophistication of investigations undertaken and on the strictness of the criteria of normality and the duration of infertility. As mentioned above, detailed assessment of the hormonal and ultrasonically detectable events surrounding ovulation may reveal subtle abnormalities in a woman otherwise characterized as having normal postovulatory serum progesterone levels in appropriately collected samples and in having no other apparent cause of infertility. Thus, the diagnosis of idiopathic infertility is one which will vary according to the depth of investigation undertaken. Because of the variability in diagnosis, the prognosis for such couples is also variably stated as being between 10 and 60% likelihood of conception within the 12 months after the diagnosis has been made.

For couples with idiopathic infertility of at least two years duration, it is customary to offer the possibility of the use of ARTs, particularly the GIFT technique, where pregnancy rates of the order of 30 to 35% per cycle of treatment have been reported. Superovulation using gonadotropin and combined with intrauterine insemination of prepared sperm, may be offered as initial therapy.

SUMMARY

Infertility is a common problem and is the problem of a couple. Its proper management involves the couple at all stages. In considering infertility management, it is most important to realize that pregnancies may occur independently of any therapy. One major study showed that pregnancy occurred in 41% of treated couples and 35% of untreated couples. Any new technologies introduced into the management of infertility must be rigorously assessed and subject to properly controlled trial. Management of male infertility in particular has been bedevilled by the use of untried and uncontrolled therapies. Couples in whom the prognosis for spontaneous fertility appears reasonable may elect to continue to attempt pregnancy while others may very quickly decide on donor insemination or the use of ARTs. This has made a major impact on infertile couples and has improved the chances of having children reasonably quickly. This chapter has not taken into account the need for the couple to adjust emotionally to their infertility and this may take time. The decision to opt

out of investigation or management should be handled with understanding, and supportive counselling. Even if one partner is believed to be the major reason for the couple's infertility, the other should be reviewed from time to time and detailed investigations may need to be repeated.

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