Chapter 1

Introduction

Male subfertility is a very significant global problem. Epidemiological data show that approximately 1-in-7 couples are classed as subfertile [1]. Sperm dysfunction is the single most common cause of male subfertility. Indeed, studies using semen assessment criteria for male subfertility (sperm concentration $<20 \times 10^6$/ml) illustrate that 20% of 18-year-olds are classed as subfertile [2]. Although it is too simplistic to base a classification as subfertile solely on sperm concentration, the reported frequency of male subfertility points to a high proportion of the population being affected, compared with other prevalent diseases such as diabetes. Moreover, what is more worrying is the possibility that the prevalence of male subfertility is increasing [3]. Andrology is therefore a pivotal discipline in modern medicine, and it is against this background that we have assembled this handbook.

Semen analysis provides a comprehensive view of the reproductive functioning of the male partner of the subfertile couple. It includes assessments of sperm count (which examine sperm production, transport through the male genital tract and ejaculatory function), sperm motility (a basic functional marker of likely sperm competence), sperm vitality (to distinguish between dead spermatozoa and live, immotile spermatozoa), sperm form (aspects of sperm production and maturation), and the physical appearance of the ejaculate (semen production). In addition to this basic semen assessment there are further tests that can be performed – what we have termed extended semen analysis – permitting further analyses that assess more functional aspects of the semen sample. Such tests include biochemical examinations to evaluate the secretions from the auxiliary sex glands, the detection of antisperm antibodies, and the use of computer-aided sperm analysis (CASA) to examine sperm motility patterns (kinematics).

A high quality, comprehensive semen assessment is not just the cornerstone of the diagnosis of male subfertility; it is also the starting point for providing prognostic information. While the basic semen assessment has been performed for over 60 years, there have been a number of studies questioning the value of the traditional semen characteristics (sperm concentration, motility, and morphology) in the diagnosis and prognosis of male subfertility [4]. Partly, this is the result of an incomplete understanding of what clinical information a semen assessment can provide (see below), but primarily it is because the basic assessments are usually performed using inadequate methods with limited understanding of the technical requirements, and poor quality assurance. An example of this is the UK survey of laboratories performing andrology tests, which showed dramatic variation from World Health Organization (WHO) standardized procedures leading to uncritical reporting of results [5]. In this handbook we provide a detailed, step-by-step guide using robust methods for examining human semen. We have also included comprehensive documentation for training of staff and sections on quality control (QC), quality assurance (QA), and quality improvement (QI). Adoption of such methods and procedures will lead to a significant

improvement in the quality of the data produced by the andrology laboratory, and therefore more robust clinical information.

One matter that has been discussed in relation to semen analysis is the number of samples that must be analyzed from each individual. Quite often, at least two samples are said to be required to get a representative result for the individual [6,7]. However, when based on laboratory data, a considerable portion of the variability in results could be ascribed to technical variability due to substandard laboratory methods. Thus, with poor technical quality (including low numbers of spermatozoa assessed) investigations of multiple samples from the same man can, at least in part, compensate – but it is cost-efficient for neither the patient and his partner, nor the laboratory. The reason why, in epidemiological studies, men investigated for possible reproductive toxicological effects only need to produce one sample is most likely that the variability in individual samples will "disappear" when the average is used and differences in averages between groups can be analyzed [8]. Although there is a considerable biological variability in semen analysis results (see Chapter 2), especially concerning sperm concentration, the clinical evaluation of the man does not always require analyses of several repeated samples. For the primary investigation of the man in a subfertile couple, information from the very first semen sample can be enough to direct the continued investigation – either a very poor result indicating the need for direct clinical andrological investigations, or a very good result indicating that further basic semen analyses will not reveal any more information [9,10]. In the group of men with intermediate results, valuable information can be gained from repeated semen analysis. The methods described in this handbook have been developed to minimize the variability due to technical factors, to optimize the evaluation of the man as well as the laboratory work.

For the proper use of semen analysis results, appropriate interpretation is fundamental. With a few clear exceptions (e.g. azoospermia), the data cannot provide unambiguous information about the chances of future conception, either *in vivo* or *in vitro*. Currently, there is a clear tendency to overemphasize the value of a single parameter, e.g. sperm morphology with strict cut-offs as used for assisted reproductive technology (ART). However, as there is a considerable overlap between the semen characteristics of fertile and subfertile men, no single parameter can be used to provide prognostic information about the fertility potential of the couple. A combination of several variables (motility, morphology, and concentration) does give more accurate diagnostic and prognostic information, although there will always be overlaps between what is considered fertile and subfertile [11,12]. Irrespective of the low predictive value for the reproductive success of the couple, a comprehensive semen analysis provides information about the status of the male reproductive organs, and this is important in the well-being of the man. The results of a semen analysis are often used as a sentinel marker for the potential treatment pathway for patients. For example, a primary question in ART clinics remains: is the semen of this man suitable for intrauterine insemination (IUI), or is in-vitro fertilization (IVF), or even intracytoplasmic sperm injection (ICSI) needed? Primarily, what the clinic is trying to do is determine whether there are indications that the man will have a high likelihood of *failure* using a particular treatment modality, i.e. the man's spermatozoa are unsuitable for insemination by IUI, and IVF is indicated. However, despite the plethora of literature surrounding this area, there are still no simple answers. For example, a meta-analysis of the literature trying to ascertain the number of spermatozoa that have been (can be) used as a cut-off for IUI success concluded that there was no such cut-off, and that the data available were of insufficient

quality to provide a robust answer [13]. Of course, the quality of the sperm preparation methodology (and also the products used) will also impact on treatment outcome, confounding any simple relationship between pre-treatment semen characteristics and treatment outcome.

For the comprehensive investigation of a man's fertility potential it is essential not only to perform a semen analysis, but also that a physical examination be performed, a complete medical carried out, and a history taken [14]. Accurate interpretation of a semen analysis cannot be made without knowing the patient's history, and having information from a physical examination and other laboratory investigations, e.g. hormone analysis. Reduced fertility potential can be secondary to other diseases that should be properly investigated and treated. It is thus irresponsible and unethical to embark upon an infertility work-up without a complete physical examination and history.

A common source for misunderstandings and misinterpretations is the use of qualitative terms such as oligozoospermia and asthenozoospermia. Originally, these terms were used to characterize laboratory findings before the quantitative measures had become usable and reliable. Subsequently, these terms have been given precise limits on quantitative scales, creating the false impression of dichotomy (two clearly separated outcomes, such as subfertile and fertile) based on semen characteristics – as opposed to the true situation of a sliding scale between severely infertile (but not sterile) and fertile. In an effort to reduce such confusion in the future, we have abandoned the use of all such qualitative terms and urge everyone working in the field to do likewise: just describe what you see, as objectively and quantitatively as possible, and interpret the test results within the holistic medical context for the man, especially the particular circumstances that exist within the reproductive unit of which he is part, i.e. with his partner, since (sub)fertility is always a feature of the couple.

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Chapter 2

Basic physiology

What are gametes good for? Protection against microorganisms

One prerequisite for multi-cellular organisms to survive is to be able to repulse attacks by microorganisms; attacks by their deoxyribonucleic acid (DNA), ribonucleic acid (RNA), and proteins and prions. Every individual multi-cellular organism has developed an immunological defense system that is directed towards everything but itself. However, to discriminate *foreign* cells and microorganisms from cells belonging to itself it is essential to be unique. The problem of becoming unique was solved some 600 million years ago with the evolution of a new type of cell division, meiosis, which enabled the formation of genetically unique gametes. Coupled with this, the fusion (fertilization) of two genetically unique gametes (the spermatozoon and the oocyte), results in a genetically unique, new individual. Due to meiotic recombination, every gamete is supplied with one out of four unique DNA molecules for every chromosome pair. In human beings there are 23 pairs of chromosomes, so the number of possible DNA combinations in any gamete is 4^{23}, i.e. any gamete achieves 1 out of 70×10^{12} combinations (one out of seventy million million) of genetic material. At fertilization, gametes from two different individuals fuse and form one new individual; the genetic composition of the child is thus one combination out of 4900×10^{24} possible combinations. A man produces some 100 million unique genetic lots per day. A woman produces one mature oocyte a month; and again, each oocyte is genetically unique.

Thus, the evolution of meiosis and unique gametes was a prerequisite for an individual immune defense, which in turn was a prerequisite for the evolution and survival of multi-cellular organisms exposed to endless attacks by microorganisms [1].

Outside the body the laboratory staff must protect the gametes

Outside the body there is no immune system or reproductive tract to protect the gametes. The laboratory must therefore fulfill these functions to protect gametes and embryos. Assisted reproduction *in vitro* would be impossible if microorganisms were not actively combated. Laminar-air-flow benches, UV-light protected airlocks, sterile and controlled handling and culture media, rooms with controlled air purity and special clothing for the involved staff are some precautions regulatory bodies seek. Sometimes chemical weapons such as antibiotics are necessary, yet micro-organisms with foreign DNA and RNA may still manage to invade our culture media and become incorporated into embryos and thereby future generations [1].

Every man is a unique experiment of nature

Most multi-cellular organisms have both testes and ovaries, usually (but not always) in different individuals, of the male and female genders, respectively. Since oocytes are immobile these organisms exchange spermatozoa either while they are in direct contact or with the aid of wind, water or other vectors.

In mammals, some 300 million years ago the genes controlling sperm production were transferred from one of the two ancient X-chromosomes onto a "shortened X-chromosome", today called the Y-chromosome. The Y-bearing organism developed into a mobile, sperm-producing individual. In order for the "species" to survive, the sperm producer must succeed in finding signs of ovulation and be able to deliver spermatozoa for fertilization of the oocytes. In mammals, the default development (phenotype) is the female development. The development of a fertile man able to react to signs of ovulation requires the selection of a handful of specific male development routes during embryonic, fetal, and early childhood development [2,3]. Many of these traits are known to be dependent on testosterone. The traits result in the development of (a) the testes, (b) inner male genitalia, (c) outer male genitalia, (d) male sexual identity and (e) "oocyte-bearing" women as a sexual preference.

Production of the male gamete or spermatozoon

Spermatogenesis is the process by which spermatozoa are produced from spermatogonia in the testis. The light microscopic examination performed during semen analysis aims to give information about the success of spermatogenesis including the number of spermatozoa, their morphology and motility. A more thorough evaluation of the ejaculate can reveal a variety of disturbances originating in the different steps of spermatogenesis, and might shed light on disturbed testicular function, or even disclose the presence of early testis cancer [3,4].

Spermatogenesis preparations occur in the embryo

Already during the embryonic and fetal stage, preparations for spermatogenesis are being made. Immature germ cells from the epiblast migrate from the yolk sac and invade the seminiferous cords (which will become tubules at puberty) in both testes, and start to proliferate up to week 18 in the fetus. The other cells inside the seminiferous cords, the Sertoli cells, also multiply. The somatic Sertoli cell and its spermatogonia could be regarded as a unit for future sperm production. If the migration of germ cells is disturbed, or if the germ cells degenerate, few or no spermatogonia will be left for sperm production, and the only cells left would be the Sertoli cells (known clinically as the "Sertoli cell-only" syndrome).

Spermatogenesis is comprised of five different processes

Renewal of stem cells

There are two types of spermatogonia type A: dark and pale (Figure 2.1). Both belong to the stem cell population and are continuously renewed by mitotic divisions. It is estimated that spermatogonia undergo some 20 mitotic cell divisions a year, so at 35 years of age the spermatogonia have undergone some 400 mitotic cell divisions, whereas the female oocyte rests from embryonic week 10 until ovulation. Thus, hazards linked to cell division events (mutations in DNA, aneuploidy, mutations and deletions in mitochondrial DNA) are more likely to affect spermatogenesis than oogenesis.

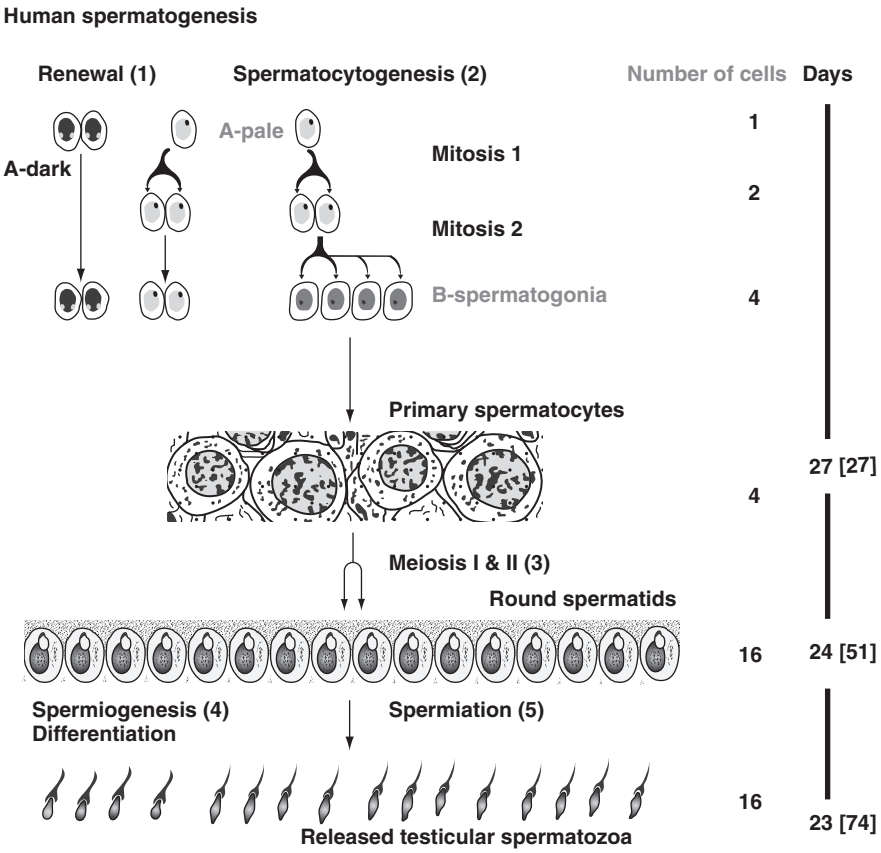

Figure 2.1 A schematic drawing of human spermatogenesis compiled from data given by references [4] and [5]: (1) shows that A-pale spermatogonia renew by mitosis and that A-dark spermatogonia mainly rest; (2) outlines that another A-pale spermatogonium is chosen to undergo two mitotic cleavages into four B-spermatogonia and that B-spermatogonia differentiate into primary spermatocytes (spermatocytogenesis); (3) shows the two meiotic divisions of each of the primary spermatocytes into four round spermatids, and finally (4) the differentiation of round spermatids into elongated spermatids (spermiogenesis) that through (5) spermiation are released as testicular spermatozoa in the lumen of the seminiferous tubule. Number of cells refers to the number of daughter cells (finally spermatozoa) resulting from one spermatogonium. Days mark the duration of each step and, in brackets, the accumulated duration.

Spermatocytogenesis

An important process is an exponential increase in number of spermatozoa; in the human testis two consecutive mitotic divisions prepare for the meiotic cell division. One spermatogonium A pale is recruited for sperm production and undergoes two mitotic cell divisions, resulting in a clone of four spermatogonia B. Each of these then differentiates into four primary spermatocytes. The latter two mitotic cell divisions increase the possible number of spermatozoa by a factor of four. If one mitotic division does not occur then only half the number of spermatozoa can be produced. It means that missing mitotic divisions could be one cause of low sperm numbers. In rodents there may be 11 mitotic divisions before the meiotic divisions begin, and in the rhesus monkey there may be five mitotic divisions. Thus,

in those species, one A-spermatogonium will theoretically result in 8192 and 128 spermatozoa, respectively, whereas the number of resulting spermatozoa in man is 16 [5].

Meiosis

The purpose of this process is to ensure that every spermatozoon achieves (a) a unique combination of DNA, and (b) a haploid genome in which the original 23 pairs of chromosomes are reduced to 23 single copies of the DNA. Each of the four primary spermatocytes in a clone undergoes the two meiotic divisions. The eight secondary spermatocytes finally result in the formation of 16 round spermatids.

Spermiogenesis

This is the process where the round spermatid transforms (differentiates) into a functional messenger cell called the testicular spermatid, which is still attached to the Sertoli cell (Figure 2.2).

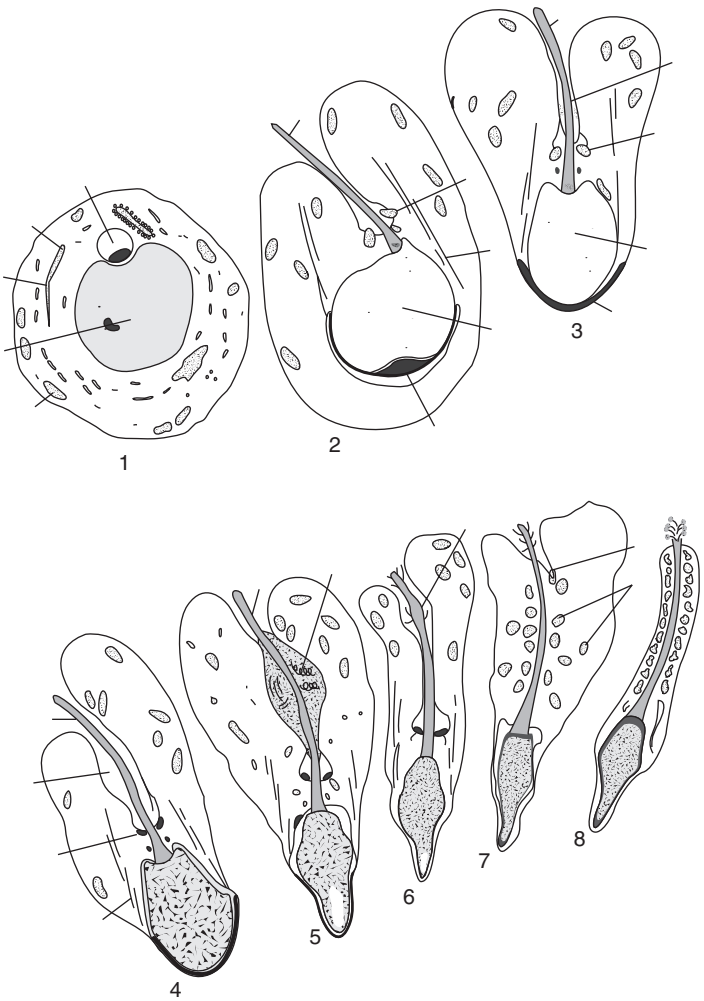

Figure 2.2 The steps of spermiogenesis. (1) Immature spermatid with round shaped nucleus. The acrosome vesicle is attached to the nucleus; the tail anlage still fails contact to the nucleus. (2) The acrosome vesicle is increased and flattened over the nucleus. The tail establishes contact with the nucleus. (3–8) Acrosome formation, nuclear condensation, and development of tail structures take place. The mature spermatid (8) is delivered from the germinal epithelium. Semi-schematic drawing on the basis of electron micrographs by AF Holstein [4].

Spermiation

Initiated by the Sertoli cell, testicular spermatozoa are released from the Sertoli cells, which take up the surplus cytoplasm and membrane from the sperm midpiece (the residual body).

Spermatogenesis takes place in the seminiferous tubules

A normal tubule has a diameter of about 180 μm, and the diameter is decreased when spermatogenesis is impaired (Figure 2.3). The tubule walls are composed of five layers of myofibroblasts in connective tissue, which cause peristaltic waves of contraction to transport the immotile testicular spermatozoa to the rete testis for further transportation, via the efferent ducts, to the caput of the epididymis. The thickness of the peritubular tissue is 8 μm, corresponding to the size of the neighboring spermatogonia. A thickened wall is associated with impaired spermatogenesis [4].

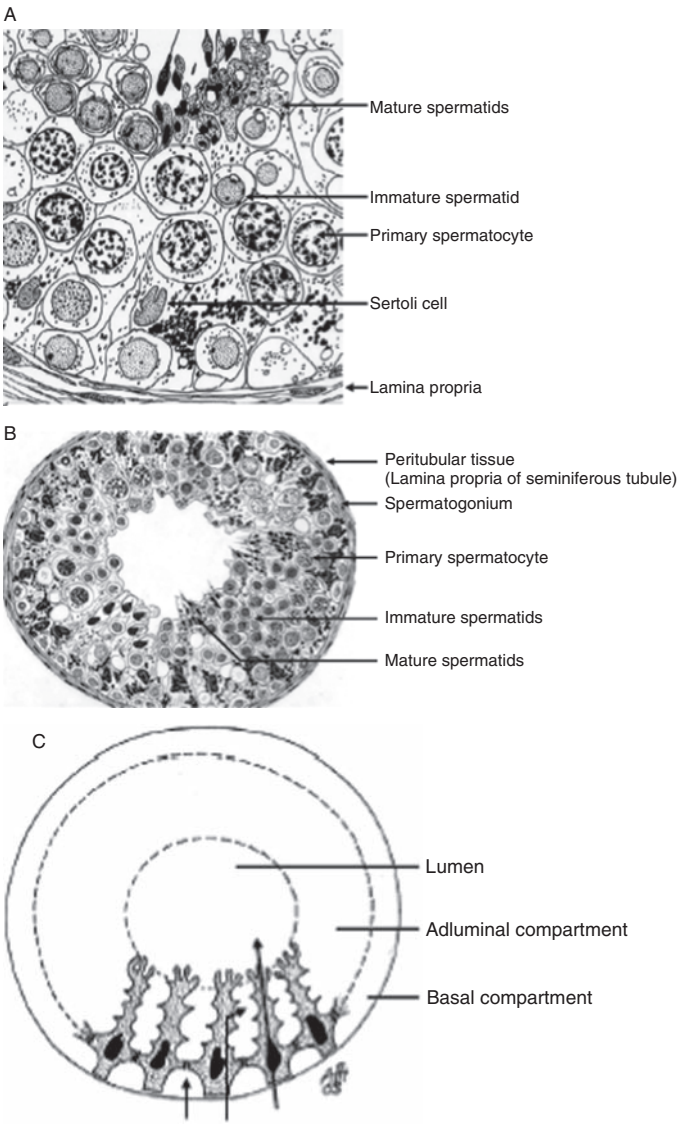

Figure 2.3 (A) Cross-section of a seminiferous tubule of a fertile man 32 years of age. Drawing of a semithin section ×300. (B) A sector of the germinal epithelium in the seminiferous tubule. Drawing on the basis of a semithin section ×900. (C) Sertoli cells divide the germinal epithelium in a basal and adluminal compartment. Arrows indicate that the passage of substances from the outside stops at the tight junctions in the basal compartment, and that the adluminal compartment and the lumen can only be reached by transport through the Sertoli cells. Drawings by AF Holstein [4].

The Sertoli cell

The Sertoli cell is the dominating cell in the seminiferous cords and tubuli. It is a supporting cell that provides nutrition and mediates paracrine signals for spermatogenesis, as well as protection of the developing germs cells from the immune system. The Sertoli cell communicates, via factors and testosterone, with the Leydig cells in the interstitium between the seminiferous tubules. It produces inhibin-B, which exerts a negative feedback on the follicle-stimulating hormone (FSH) secretion from the pituitary gland. In about half of men with azoospermia, the Sertoli cells produce low levels of inhibin-B, resulting in elevated FSH levels in blood. However, low levels of inhibin-B and high levels of FSH do not exclude that focal spermatogenesis can be found at testicular biopsy, making sperm retrieval and ICSI a possible treatment [6,7]. Protection from the immune system is mediated by neighboring Sertoli cells forming tight junctions between them, thereby dividing the seminiferous tubules into two compartments (Figure 2.3C): (a) the basal compartment facing the *inside* where the immune system can act against *foreign* objects, and (b) the one facing the *outside world* – the adluminal compartment, from which the spermatozoa are released and transported out of the man. At meiosis, the primary spermatocytes, which soon are going to give rise to spermatids with unique DNA (and therefore will appear as *foreign* to the immune system), are transferred from the basal to the adluminal (outside) compartment, thus escaping the risk of being attacked by the immune system. The so-called blood-testis barrier consists of a combination of these inter-Sertoli cell connections, the peritubular tissue in the walls of the tubules, and the endothelium of the testicular capillaries in the interstitium between the tubules [4].

The testicular interstitium and the Leydig cells

Besides spermatogenesis the testis has another vital physiological role: testosterone production by the Leydig cells which surround the capillaries in the interstitium.

The embryonic male

In response to human chorionic gonadotrophin (hCG) from the placenta anlagen (chorion cells) and thereafter the developing placenta of the embryo, Leydig cells produce testosterone necessary for the differentiation and development of a fertile man. Disturbed function of the placenta during critical time intervals of the embryonic development can jeopardize the specific male development and hence future male fertility.

The adult man

At puberty, gonadotrophin-releasing hormone (GnRH) is secreted from the hypothalamus in isolated peaks (once every 90 minutes), which stimulates the pituitary to secrete FSH and LH (named luteinizing hormone from its effect in females). LH stimulates the Leydig cells to produce testosterone. No less than 90% of the testosterone is taken up by the Sertoli cells in the tubules and is used for spermatogenesis and by luminal flow for the androgen-specific functions of the excurrent duct system up to the corpus of the epididymis. Some 10% is delivered to the capillary blood and exerts systemic androgen effects on the man, including male secondary sex characteristics such as body and face hair, deep voice, increased muscle mass, decreased body fat, increased male hemoglobin, and reinforced skeleton, as well as brain function resulting in “male temper” [3].