Trends in Andrology and Sexual Medicine
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Atlas of Human Semen Examination

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Società Italiana di Andrologia e Medicina della Sessualità

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Trends in Andrology and Sexual Medicine

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Preface

This new editorial venture by Springer is, necessarily, dedicated in its entirety to Loredana Gandini. Everything in this volume speaks of her: her initial idea, her hard work under the microscope taking the photos, the long hours she spent choosing the best images, the graphic design of the cover… Loredana did all this with the skill, scientific expertise, dedication and joy that characterized her entire scientific life.

We will never forget how her face lit up when the first edition of the *Atlante di Seminologia* was published in 1999, nor her immense satisfaction at its success—which prompted the publication of a second edition in 2010, for which the title was changed to *Diagnostica per immagini dello spermatozoo umano* ["Diagnostic imaging of human spermatozoa"]. This very success was the fruit of her tenacity in tracking down the clearest and most educational images for the readers. We will always remember the many hours we spent discussing the best shots and the right magnification for her beloved *goni*, *citi*, *tidi* and *zoi*, as she called them.

Sadly, Loredana's life was cut cruelly short before she could see the English edition of her labour of love, but we are still gratified by the great interest that this volume continues to arouse; this gives us the motivation and enthusiasm to continue building upon her remarkable achievement.

Rome, Italy

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Introduction

This volume is divided into two main parts. The first consists of a large collection of images of the main abnormal sperm forms and non-sperm cellular elements found in semen, including a short section dedicated to the basic concepts of spermatogenesis and structure of the male gamete: a reference to be kept by the microscope at all times. Seminal fluid examinations are all too often performed after only the most basic of training, as if they were a non-specialist analysis. There is a clear need to combine an objective cytomorphological evaluation with a functional interpretation, in order to assess the semen's fertilizing potential.

The interpretation above all of sperm morphology has varied so much over time that it is almost impossible to compare results from different retrospective studies and laboratories. This poor comparability also makes it difficult to properly diagnose and treat infertile patients. However, an adequate morphological evaluation, even under an optical microscope, can enable the identification of pathological monomorphisms that lead to congenital forms (e.g. Globozoospermia); these can then be subsequently confirmed using electron microscopy. No less important from both a diagnostic and speculative perspective is the identification of "round cells"—i.e. all cells present in the semen that are not mature gametes.

In relation to immature germ cells, our group has produced a number of articles (Gandini et al., Hum. Reprod., 14: 1022, 1999; Salanova M. et al., Lab. Invest. 79: 1127, 1999) for which the correct identification and selection of the germ cells present in the semen were essential. The identification of germ cells is particularly important in cases of azoospermia (subsequently confirmed with seminal biochemistry data), as a marker of the patency of the seminal tract, or as a response to gonadotropin therapy in patients with hypogonadotropic hypogonadism. The selection of germ cells is also a fundamental step for the most advanced genetic and molecular studies aiming to shed light on the still unsolved problems of human spermatogenesis.

The second part of this new volume concerns the evaluation of sperm chromatin integrity with TUNEL, one of the most commonly used methods in andrological research. It includes a description of the chromatin molecular structure and possible causes of nuclear fragmentation, as well as numerous images to aid the understanding and interpretation of the cellular signs induced by chromatin fragmentation. The study of sperm DNA damage is particularly relevant in an era when assisted reproductive techniques are widely used, leading to a need for a predictive test of success in terms of fertilization, embryo quality and implantation. Several studies in the literature have demonstrated high levels of sperm DNA damage in men with severe spermatogenetic disorders, while others have found that

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DNA fragmentation has a negative impact on the outcome of both natural and assisted fertilization. Numerous efforts have been made in recent years to identify a suitable test that evaluates sperm DNA integrity, although the clinical significance of such a test has not yet been established.

Human Semen Examination

1 Notes on Human Spermatogenesis

Spermatogenesis is a process involving the proliferation and differentiation of undifferentiated stem cells into spermatozoa. It takes place in the seminiferous tubules of the testis and is divided into three sequential phases: proliferation of spermatogonia, meiosis and spermiogenesis.

The proliferative phase takes place in the basal lamina of the seminiferous tubules. The clonal expansion of the spermatogonia in this phase allows both the production of germ cells destined to become primary spermatocytes (the first step of the spermatogenetic process) and the maintenance of a pool of stem cells. Spermatogonia are classified as type A, which may be dark (Ad, with dispersed, intensely coloured chromatin) or pale (Ap, with finely dispersed, weakly coloured chromatin), and type B. Following mitotic division, during differentiation type Ad spermatogonia produce both type Ap and further undifferentiated type Ad spermatogonia; the latter constitute the reserve of stem cells necessary for subsequent multiplication and differentiation cycles. Mitotic division of type Ap gives rise to type B spermatogonia; further mitosis produces primary spermatocytes (preleptotene) which, after duplicating their DNA, undergo the first meiotic division. There is no further development of germ cells until the beginning of puberty, when serum levels of gonadotropins and androgens rise and spermatogenetic activity resumes.

The prophase of the first meiotic division is rather long. It is characterized by a progressive increase in the size of the nuclei of the primary tetraploid spermatocytes and by the genetic recombination of the homologous chromosomes. It can be divided into five stages: leptotene, zygotene, pachytene, diplotene and diakinesis. In the leptotene stage, the chromosomes, consisting of two sister chromatids, appear as thin tangled filaments. During the zygotene stage, the homologous chromosomes appear to form the synaptonemal complex. In the pachytene stage the chromosomes shorten and thicken and DNA is exchanged between homologous chromosomes, the so-called crossing over; in this phase the cell size increases, and the pachytene spermatocyte is therefore the largest cell of the germ line. During the diplotene stage, the synaptonemal complex disappears and the chromosomes separate—except in the chiasm region, the area where the exchange of genetic material took place. In the final phase, diakinesis, the chromosomes condense.

The cells then proceed through the metaphase, in which the two centromeres of each bivalent align on the equatorial plate, and the anaphase, in which they separate and migrate to the opposite poles of the cell. In the telophase, the final stage of the first meiotic division, cytodieresis, takes place, leading to the formation of two daughter

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cells. It is interesting to note that cytodieresis of the germ cells, i.e. the separation of the cytoplasm at the end of cell division, is incomplete; the germ cells derived from a common spermatogonium thus remain in a syncytium (connected by cytoplasmic bridges) until the mature germ cells are released for spermiation. This syncytial structure allows the germ cells to "communicate" and coordinate their development synchronously. The two haploid daughter cells derived from the first meiotic division are called secondary spermatocytes.

Between the first and the second meiotic divisions there is a very short interphase in which no DNA is synthesized. The second meiotic division process begins almost immediately thereafter, and secondary spermatocytes progress from the prophase to telophase. The second meiotic division involves the separation of the sister chromatids along the centromere; at the end of this process, each secondary spermatocyte is divided and the daughter cells, called spermatids, contain a haploid genome. By the end of meiosis, each primary spermatocyte has thus split twice, giving rise to four spermatids.

Spermatids are initially round cells that are completely different from mature spermatozoa. They undergo a series of gradual modifications known as spermiogenesis, which transforms them into flagellated elongated cells with independent movement—spermatozoa. This transformation occurs through four successive phases, known as the Golgi, cap, acrosome and maturation phases.

In the Golgi phase, proacrosomal granules rich in carbohydrates (detectable by the periodic Schiff acid reaction, specific for polysaccharides) appear in the Golgi body and fuse into a single acrosomal granule. The cap phase involves the expansion of the membrane limiting the acrosomal vesicle, which reaches around the front two-thirds of the nucleus to form the so-called acrosome cap. At the same time, the centrioles migrate to the pole opposite the acrosome formation, where the distal centriole gives rise to the flagellum.

In the acrosome phase, considerable variations take place in the structures of the acrosome, nucleus and flagellum. The acrosomal granule widens until it fills the entire cap, the nucleus lengthens and migrates to the cell periphery, and the nuclear chromatin condenses into coarse grains. The cytoplasm moves towards the caudal pole of the nucleus and wraps around the proximal part of the flagellum, where the mitochondria are arranged like a sleeve.

Finally, in the maturation phase the spermatozoon assumes its final form. The nucleus becomes a compact, homogeneous structure, the tail undergoes its final differentiations and the excess cytoplasm is eliminated, and will subsequently constitute the so-called residual bodies that will be engulfed by the Sertoli cells.

As the cells progress through the spermiogenetic process, they are pushed by the tubular fluid towards the lumen of the seminiferous tubules, culminating in the release of mature spermatozoa into the lumen (spermiation). In humans, the entire process, from spermatogonium to mature sperm, takes about 72 days.

This process requires complex interactions between germ cells and the somatic cells in the seminiferous tubules, called Sertoli cells. These are large, coarsely cylindrical cells that do not divide; they go from the basal lamina to the lumen of the seminiferous tubule and wrap the cells of the spermatogenetic process (with the exception of spermatogonia, which rest directly on the basement membrane) through a complicated intertwining of cytoplasmic extensions. The cytoplasmic prolongations of the various Sertoli cells are interconnected by narrow junctions and constitute the so-called blood-testicular barrier, ensuring the specific microenvironment required for the correct development of germ cells inside the seminiferous tubules. The Sertoli cells offer a decisive contribution to this microenvironment by producing proteins and hormones and secreting paracrine factors that enable the exchange of information between germ and support cells and between peritubular and interstitial cells.

2 Morphological Structure of the Spermatozoon

Under an optical microscope the mature human spermatozoon appears to consist of a flattened oval apical portion, called the "head", a short neck and a long, thin flagellum, called the "tail". The head is formed by two domains, the nucleus and the acrosome. The compact nucleus contains condensed DNA with a haploid chromosomal structure and is two-third covered by the acrosomal complex. This cap-shaped structure consists of an internal acrosomal membrane in contact with the nuclear membrane and an external acrosome membrane positioned below the plasma membrane.

The acrosome, which derives from the spermatid's Golgi body, contains lytic enzymes—essential for oocyte penetration. The plasma membrane surrounding the acrosome and nucleus typically has a trilaminar appearance under the electron microscope, with a thick glycoprotein coating (sperm coating substances) on the external surface.

The short (just 1 micron) neck extends from the posterior part of the nuclear membrane to the point where the intermediate segment of the tail joins the head. The tail consists of the axial or axonemal filamentous complex, composed of a pair of microtubules located centrally and nine pairs arranged at the periphery (9 + 2 organization). This complex of filaments runs along the entire length of the tail, which is divided into an "intermediate segment" 5–6 μ long, a "main segment" about 45 μ long and a "final segment" about 5 μ long. In the intermediate segment the complex is surrounded by nine dense external fibres, which are thin where they are in contact with the microtubules and become thicker as they approach the external surface. These in turn are surrounded (in the intermediate segment only) by the mitochondrial sheath, essential for sperm respiration and for the production of energy. The intermediate segment is separated from the main segment by a structure called the annulus, a ring of dense material that adheres to the flagellum membrane.

In the main segment, the axoneme and external fibres are surrounded by a fibrous sheath composed of two columns of dense fibres. This fibrous sheath terminates abruptly at the junction between the main and terminal segments; the terminal segment itself is constituted by the axoneme covered by the plasma membrane alone.

Sperm morphology is evaluated fresh at 400× and on stained smears at 1000×. Numerous morphological classifications have been proposed. The WHO's current classification is as follows (2010):

Oval head: normal

Head defects
- large (macrocephalia)
- small (microcephalia)
- amorphous
- tapered
- pyriform
- round (no acrosome; small)
- vacuolated
- double head
- pinhead
- small or large acrosomal areas

Neck and midpiece defects
- bent neck
- asymmetrical insertion of the midpiece into the head
- thick insertion
- thin

Tail defects
- short
- looped
- coiled
- bent
- double, multiple
- broken

Excess residual cytoplasm