Animal Reproduction Science 194 (2018) 41–56

Contents lists available at ScienceDirect

Animal Reproduction Science

journal homepage: www.elsevier.com/locate/anireprosci

Phenotypic varieties of sperm pathology: Genetic abnormalities or environmental influences can result in different patterns of abnormal spermatozoa☆

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ARTICLE INFO

Keywords:
Ultrastructure of spermatozoa
Phenotypes of sperm pathology
Chromatin abnormalities
Globozoospermia
Acephalic spermatozoa
Dysplasia of the fibrous sheath (DFS)

ABSTRACT

The present paper reviews in detail ultrastructural and molecular studies addressed to characterize different phenotypes of sperm pathology in sterile men. In each case ultrastructural, immunocytochemical, molecular and genetic information is provided to differentiate two main kinds of sperm pathologies: systematic phenotypes with known or suspected genetic origin and non-systematic ones, usually secondary to different pathologies of the male reproductive system. Special attention is paid to detailed ultrastructural features profusely illustrated with electron micrographs. Diagnostic and fertility prognostic values of these phenotypes are also discussed and, when possible, comparison with similar pathologies in mammals and birds are discussed.

1. Introduction

The human desire to explore nature beyond the power of the naked eye has deep roots in human history. The Romans had invented glass during the 1st century and found that objects would look larger when observed through a rounded piece of glass that was made thicker in the center and thinner at the edges. These biconvex lenses were used to develop the first eyeglasses in the 13th century. During the late 16th century and throughout the 17th century great interest developed in designing instruments that would allow the observation of distant or minute objects. The Italian scientist Galileo Galilei built telescopes that revolutionized the knowledge of planets, moons and stars. Shortly thereafter the first microscopes were designed, but the magnifications were too low to show detailed microscopic structures. It was not until the late 17th century that Anton v. Leeuwenhoek, a Dutch silk tradesman and scientist, crafted minute and highly convex lenses that provided magnifications of over 200 times (Fig. 1). He was able to observe single unicellular organisms and produced the first detailed description of spermatozoa in human semen. In a letter to William Brounker dated November 1677 he wrote:

"What I investigate is only what, without sinfully defiling myself, remains as a residue after conjugal coitus ……." He described a multitude of "animalculi seminis (little animals in semen) less than a millionth the size of a coarse grain of sand and with thin, undulating transparent tails" (Leeuwenhoek, 1678).

His startling observations were subsequently submitted to the Royal Society of London, to which he shortly thereafter became a fellow. His microscope was only provided with a single lens, albeit a very small, exquisitely crafted and highly convex one. Leeuwenhoek documented his discovery with very detailed drawings that depicted with unusual detail the main characteristics of spermatozoa, including numerous examples of abnormally shaped cells (Fig.1). The remarkable morphological heterogeneity of his

☆ This research was supported by Grants from the National Research Council of Argentina (CONICET), PIP GI n° 11220120100279CO
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https://doi.org/10.1016/j.anireprosci.2018.04.074
Received 27 December 2017; Received in revised form 13 April 2018; Accepted 13 April 2018
Available online 20 April 2018
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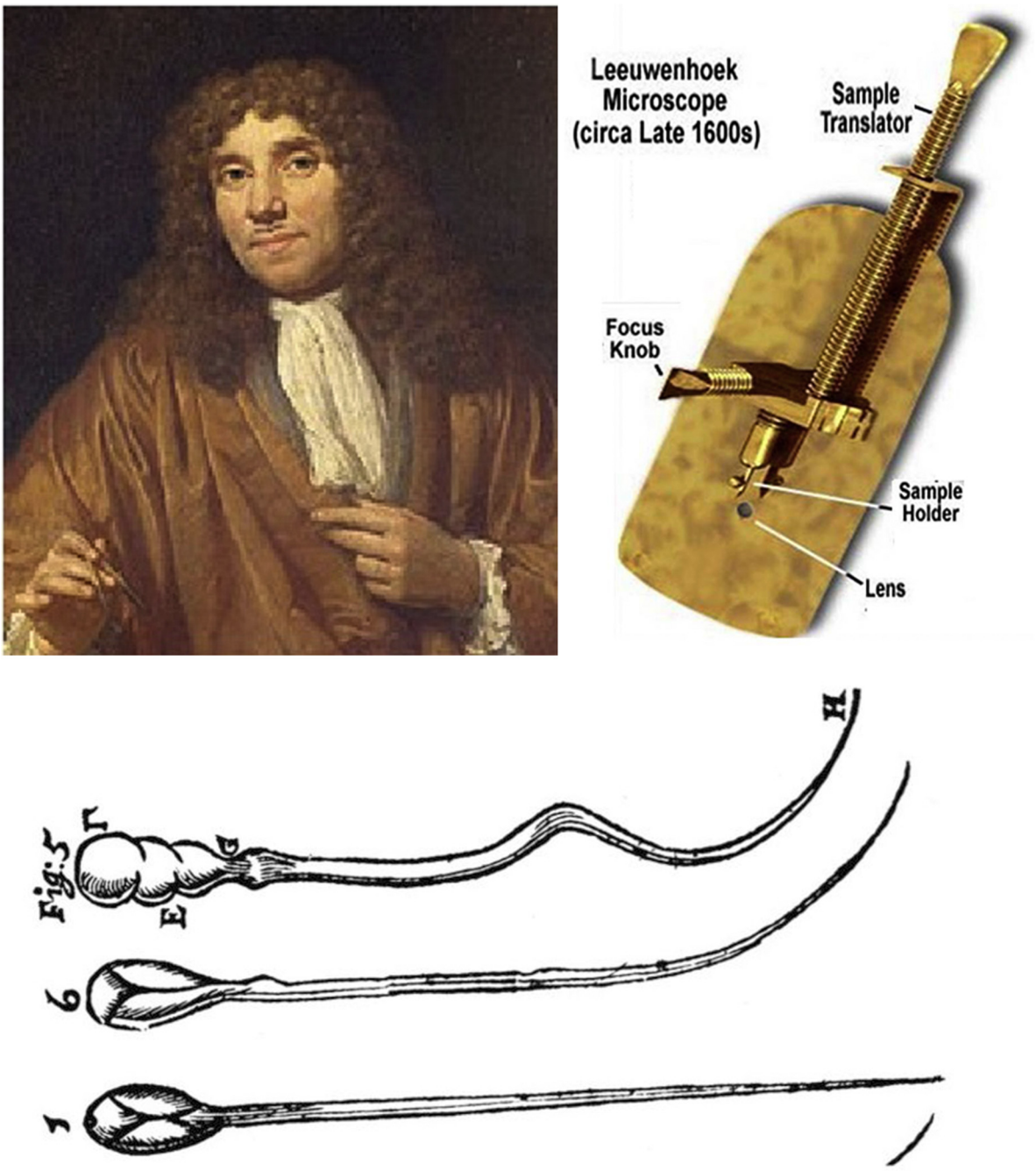

Fig. 1. The discovery of human spermatozoa. Portrait of Antonie van Leeuwenhoek painted by Jan Verkojie (circa 1684). To the right, the microscope he designed and used to first observe spermatozoa, documented below in his detailed original drawings depicting three different human spermatozoa. Notice the clear distinction of the head, midpiece and tail as well as their morphologic heterogeneity.

drawings is, aside from its partial inaccuracy, the first documentation of what is now called teratozoospermia (the increase of abnormal spermatozoa in semen. The very concept of teratozoospermia is something that needs to be revisited because it is based on the identification of atypical sperm shapes but does not recognize the cellular basis of their functional incompetence.

The term Sperm Pathology was proposed as the discipline of characterizing structural and functional deficiencies in abnormal spermatozoa (Chemes and Rawe, 2003). This concept complements that of sperm morphology which is mainly concerned with the external appearance of spermatozoa (Kruger et al., 1986). The following questions were posed: “what is wrong with wrong sperm shape? What hides behind a head-shape change in amorphous or tapering spermatozoa? In other words, what is it that impairs sperm function in morphologically abnormal sperm? Is it just abnormal shape or is there something wrong with specific sperm components?

…Ultrastructural evaluation of teratozoospermia coupled with immunocytochemistry and molecular techniques have allowed a precise definition of sperm abnormalities including their structural molecular and functional aspects". This approach goes beyond descriptive morphology of the appearance of spermatozoa. The main idea is to shed new light on the physiopathology of sperm abnormalities based on the study of the striking structural and chemical modifications that spermatid organelles and chromatin undergo during spermiogenesis. Different phenotypes involving the sperm head, the neck region/connecting piece, the midpiece and flagellum were re-evaluated considering their clinical manifestations, sperm morphology, ultrastructure, immunocytochemistry and genetic background (Chemes and Rawe, 2003; Chemes and Alvarez Sedo, 2012).

Following the concept of sperm pathology, two main variants of abnormal spermatozoa were distinguished. In the first and more frequent variety, a heterogeneous combination of different alterations is found randomly distributed in each individual and among different patients. These alterations can be referred to as nonspecific or non-systematic sperm defects and are usually secondary to various pathologies that affect the testis or the seminal pathway. The second variety presents with a characteristic phenotype that involves the vast majority of spermatozoa. These alterations may be called specific or systematic in the sense that there is a common sperm pattern that predominates in a given patient and repeats in other individuals suffering from the same condition. Systematic alterations tend to show family clustering and have proven or suspected genetic origin. This paper updates the available information concerning these phenotypes, and presents recent advances on their pathogenesis, genetic screening of infertile males suffering from well characterized sperm pathologies and genetic animal experimentation. This multidisciplinary approach affords a deeper understanding of the origin and development of different sperm abnormalities, highlighting normal and pathologic processes that underlay external appearances. These appearances, as seen in semen smears, may be deceiving since internal structures and molecular mechanisms are not readily apparent to light microscopy examination. As will be shown later, similar appearances may correspond to different pathologies.

2. The normal human spermatozoon

Human spermatozoa originate through spermatogenesis, a long process that takes place within testicular seminiferous tubules. Its duration has been estimated to be 64 days (Heller and Clermont, 1963), and comprises three sequential phases: 1) Multiplication and differentiation of spermatogonial stem cells to produce a large population of diploid B spermatogonia ready to enter the following phase. 2) Meiosis, two coupled reductional divisions leading to the formation of 4 haploid spermatids, and 3) Spermiogenesis, a division-free cell differentiation process during which round step 1 spermatids give rise to mature, elongated step 8 spermatids (Holstein, 1976; Fig. 2). Spermatid nuclei undergo progressive elongation, chromatin condensation and size reduction to change from a large round, euchromatic nucleus (step 1) to a completely elongated, heterochromatic, smaller one (step 8). Concomitant with these changes the Golgi derived acrosomic vesicle gradually flattens and spreads to cover the anterior 2/3 of the step 8 nuclear surface (Fig. 2). The centriole-flagellar complex migrates and attaches to the caudal pole of the spermatid nucleus to become the elongated sperm tail (steps 3, 5 and 8). Spermatid mitochondria, randomly distributed in the cytoplasm of steps 1, 3 and 5, migrate toward the initial part of the flagellum and, in step 8, organize helically around it to form the sperm midpiece.

After release from the germinal epithelium, normal human spermatozoa become free cells and migrate through the rete testis and the efferent ducts to the epididymis where they undergo maturational changes to acquire motility. The definition of a normal spermatozoon requires some considerations. The capacity to successfully fertilize an oocyte is certainly a most important one. However, since the advent of ICSI (Intra Cytoplasmic Sperm Injection) it became clear that sperm fertilizing capacity, although a necessary component, is not adequate as a definition of "sperm normality". Indeed, spermatozoa with serious deficiencies in PCD (Primary Ciliary Diskinesia), DFS (dysplasia of the fibrous sheath) or head-tail attachment defects cannot be considered "normal" although they are able to fertilize oocytes when microinjected. For the purpose of the present study "sperm normalcy" is referred to as that defined by structural considerations (including ultrastructure), without forgetting that normal motility, the capacity to undergo the acrosome reaction, to traverse the granulosa cell layer around the oocyte, interact with its plasma membrane and penetrate it are also necessary components.

According to Holstein and Roosen Runge (1981) and the WHO laboratory manual of human semen (2010), normal spermatozoa are composed of an oval head (approximately 4–5 μm long and 2.8 μm wide), a slender, cylindrical 4 μm long midpiece and a thin, undulating, 40–50 μm long tail. The shape of spermatozoa (the "sperm morphology") was carefully characterized and different denominations assigned to abnormal forms (amorphous, piriform, thick midpiece, short tails…). However, given the magnification limits of light microscopy and the fact that only the external appearance of sperm can be studied in semen smears, these denominations fail to convey a comprehensive account of the pathologies involved. As pointed out in the introduction, the higher resolution afforded by electron microscopy, coupled to immunocytochemistry, immunofluorescence and molecular studies, allows a more comprehensive view of normal spermatozoa and its pathologic variations.

A longitudinal view of the sperm head and beginning of the midpiece from a normal human spermatozoon is depicted in Fig. 2A. The chromatin has reached complete condensation with a uniformly dense heterochromatin. The very few small clear spots correspond to minute euchromatic areas where some important transcriptional activity may take place. The acrosome forms a flattened sac of dense content that covers the anterior 2/3 of the nuclear surface, including the distal equatorial segment. This is followed by the postacrosomal dense lamina (Holstein and Roosen Runge, 1981). Caudal to this point, the nuclear membrane separates from the chromatin forming the pore-rich redundant nuclear envelope, a possible site of nuclear exit for degradation products. The caudal aspect of the nucleus shows a shallow concavity, the implantation fossa, within which the connecting piece and proximal centriole are lodged. This head-tail articulation is followed caudally by the beginning of the flagellum and the mitochondrial sleeve of the midpiece, a spiral structure formed by 10–12 mitochondrial gyres that surrounds the axoneme and outer dense fibers. The midpiece

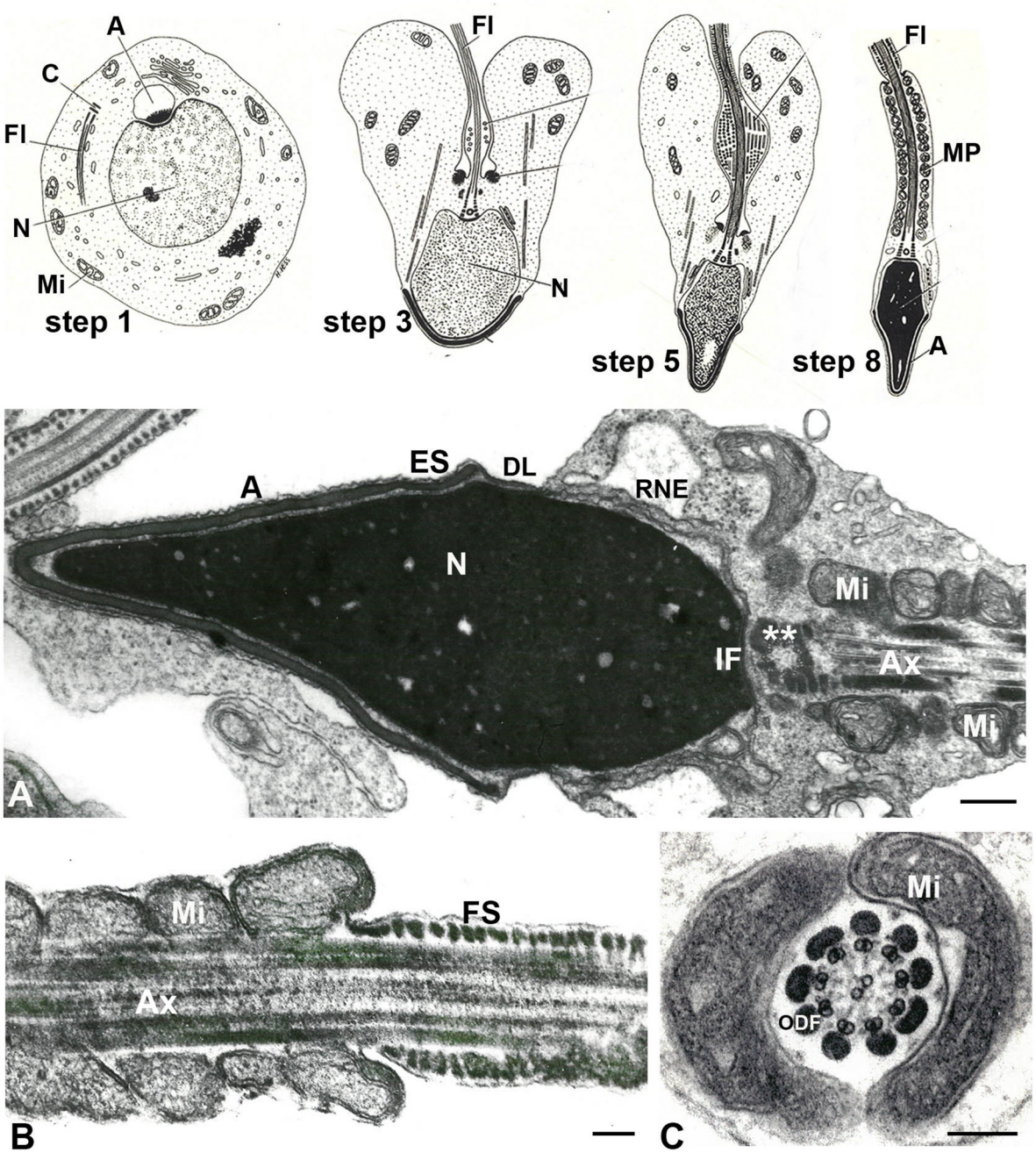

Fig. 2. Upper row: Schematic illustration of human spermiogenesis (steps 1, 3, 5 and 8, Holstein, 1976, see details in the text). Step 1 spermatids are round cells that evolve through a process of elongation and differentiation to become mature spermatids (step 8). A: acrosome, C: centrioles, Fl: flagellum, Mi: mitochondria, MP: midpiece, N: nucleus. Panel A: Longitudinal section of a normal human spermatozoon. N: nucleus (sperm head), A: acrosome, ES: equatorial segment, DL: dense lamina, RNE: redundant nuclear envelope, IF: implantation fossa, double white asterisks: proximal centriole, Mi: mitochondria. Ax: axoneme. Panel B: transition between the midpiece (left) and the tail principal piece (right). Note the axoneme (Ax) running longitudinally and surrounded by mitochondria (Mi) and the principal piece fibrous sheath (FS). C: Cross section through the midpiece. The central axoneme shows its 9 + 2 microtubular organization, the 9 associated outer dense fibers (ODF) and the mitochondrial sheath (Mi). A. Bar = 0.25 μm, B–C Bar = 0.1 μm.

spans for 3–5 μm, its distal end defined by the last turn of the mitochondrial sleeve and the location of the annulus, a ring-like cytoskeletal thickening of the cell membrane that marks the beginning of the tail principal piece. At this level, the axoneme and outer dense fibers are no longer surrounded by mitochondria but by the fibrous sheath of the tail principal piece (Fig. 2B). The axoneme has a 9 + 2 typical pattern constituted by 9 circumferentially arranged microtubular doublets surrounding a central pair of singlet

microtubules. Peripheral to each of the 9 microtubular doublets and running longitudinally in parallel to each of them there is one outer dense fiber (ODF, Fig. 2C). At the level of the principal piece ODFs 3 and 8 are replaced by the two lateral columns of the fibrous sheath (see below).

When assessing sperm normalcy some guidelines should be followed 1) the size of the acrosome (50–75 % of the nuclear surface) should only be determined in longitudinal sections of the head. Transversal sections of the caudal part of the head do not depict the acrosome because at this level the chromatin is only covered by the postacrosomal dense lamina. These images could be misinterpreted as round head acrosomeless spermatozoa. If this doubt arises the simultaneous presence in the same sample of elongated sperm heads with normal acrosomes clarifies the situation because the true lack of acrosomes affects most spermatozoa in a semen sample, 2) the chromatin should be mostly heterochromatic and homogeneously dense. In the next sections the question will be addressed on the differentiation of normal clear areas of the chromatin from pathologic “nuclear vacuoles”, 3) When a longitudinal section is analyzed the tip of the head and the midpiece should be on the same axis, otherwise, there may be a defect of head-tail attachment, but this should be a repetitive pattern to have pathologic significance. It is always important to remember that there may be isolated abnormal spermatozoa in normal semen from fertile individuals. 4) When determining the percentage of ultrastructurally-abnormal spermatozoa this will be valid within the context of the method used and should not be compared to the percentage of normal spermatozoa in routine semen analysis.

3. Pathologies of the sperm head: chromatin and acrosome anomalies

The main pathologies affecting sperm heads involve the chromatin and acrosome. The chromatin typical of mature spermatozoa results from important modifications that take place during spermiogenesis. After meiosis, the chromatin of testicular germ cells undergoes a series of complex changes in its chemical and macromolecular organization that lead to its characteristic compact state in mature spermatozoa. Chromatin is formed by the combination of DNA fibers and associated proteins. In most somatic cells and testicular germ cells up to early round spermatids, the DNA molecule associates with basic histones to form transcriptionally active euchromatin. A DNA filament formed of a stretch of 146 DNA base pairs wraps twice around a globular protein octamer composed by the association of two subunits of each of the four histones (H2A, H2B, H3 and H4) forming nucleosomes, the basic elements of euchromatin. The longitudinal arrangement of many nucleosomes results in a loose “beads in a string" configuration (Fig. 3) that exposes active DNA sequences facilitating transcription. During spermiogenesis nuclear histones are gradually replaced by intermediate proteins and finally by protamines, drastically changing protein-DNA interaction. Protamines are much smaller proteins than histones and associate tightly with the major groove of the DNA double helix inducing coiling into tightly packed toroids, donut shaped structures where DNA-protamine fibers are extremely compacted (Ward, 2017). While histone-associated DNA presents microscopically as micro-granular or filamentous in nature, protamine-DNA complexes yield a dense, homogeneous chromatin, transcriptionally quiescent and resilient to environmental destabilizing influences (Fig. 3A–D). This assures great stability of the genome during the long sperm transit through the male and female genital tracts before fertilization.

Alterations in chromatin condensation are among the most frequent pathologies in teratozoospermia. Although not necessarily associated with a specific nuclear shape they more frequently involve spermatozoa with irregular nuclear profiles (“amorphous”, Chemes, 2017). They are characterized by the presence of isolated or multiple nuclear rarefactions of variable size ranging from 1 to 2 μm and more frequently located at the tip of the sperm nucleus (Fig. 3E, F and I). Chromatin rarefactions display a clear granular or filamentous substructure, may be rounded or irregular in shape, isolated or multiple, and are frequently referred to as “vacuoles”, which is an inaccuracy because of the lack of a limiting membrane. They can appear in mature heterochromatic nuclei or in immature ones with insufficiently condensed chromatin (Fig. 3F, G and I) The origin of this abnormality resides in different pathologies that can interfere with normal spermiogenesis. This was confirmed by their presence in immature spermatids from testicular biopsies of infertile individuals (Francavilla et al., 2001). Haraguchi et al. (2007) described a normal process of protein degradation and removal from the nucleus during the histone to protamine transition. This process starts inside clear areas of the chromatin where proteasomes are preferentially located. It has been proposed that large chromatin rarefactions may be the pathologic counterpart of this physiologic protein turnover and reflect a proteasome hyper- activation that may affect chromatin stability (Chemes and Alvarez Sedo, 2012).

Grading the prevalence of nuclear vacuoles by real-time optical microscopy and comparing it with DNA fragmentation studied by neutral and alkaline Comet assays, Pastuszek et al. (2017) concluded that there is a direct association between medium sized and large nuclear “vacuoles” and high degrees of DNA fragmentation. Even though they are better visualized by electron microscopy, they can be identified by Nomarsky optics light microscopy of live spermatozoa (Bartoov et al., 2002). This technique has been used as a method to select the spermatozoon best suited for microinjection and increased fertilization rates and pregnancy outcomes have been reported, although these results have not gained universal acceptance.

The above considerations support the view that large nuclear rarefactions are indeed the pathologic expression of increased intranuclear proteolysis that disrupts chromosome integrity (Chemes and Alvarez Sedo, 2012). As a result of extensive ultrastructural investigations some authors consider that large nuclear vacuoles can occur both in fertile and infertile men (Holstein, 1975; Holstein and Roosen Runge, 1981). However, considering the high degree of chromatin compaction in the small volume of a human sperm head, it is difficult to accept that these “vacuoles” do not seriously compromise the integrity of sperm chromatin (and chromosomes).

Genetic manipulation or transgenic models of protamine and intermediate protein genes in experimental animals usually result in diminished fertility (Wu et al., 2000; Yu et al., 2000; Choi et al., 2001). Conversely, numerous studies in sterile men have not yielded conclusive results in spite of the occasional report of male infertility associated with genetic protamine abnormalities (Balhorn et al., 1988; deYebra et al., 1998; Bench et al., 1998; Ravel et al., 2007; Gázquez et al., 2008; Imken et al., 2009; Venkatesh et al., 2011). It

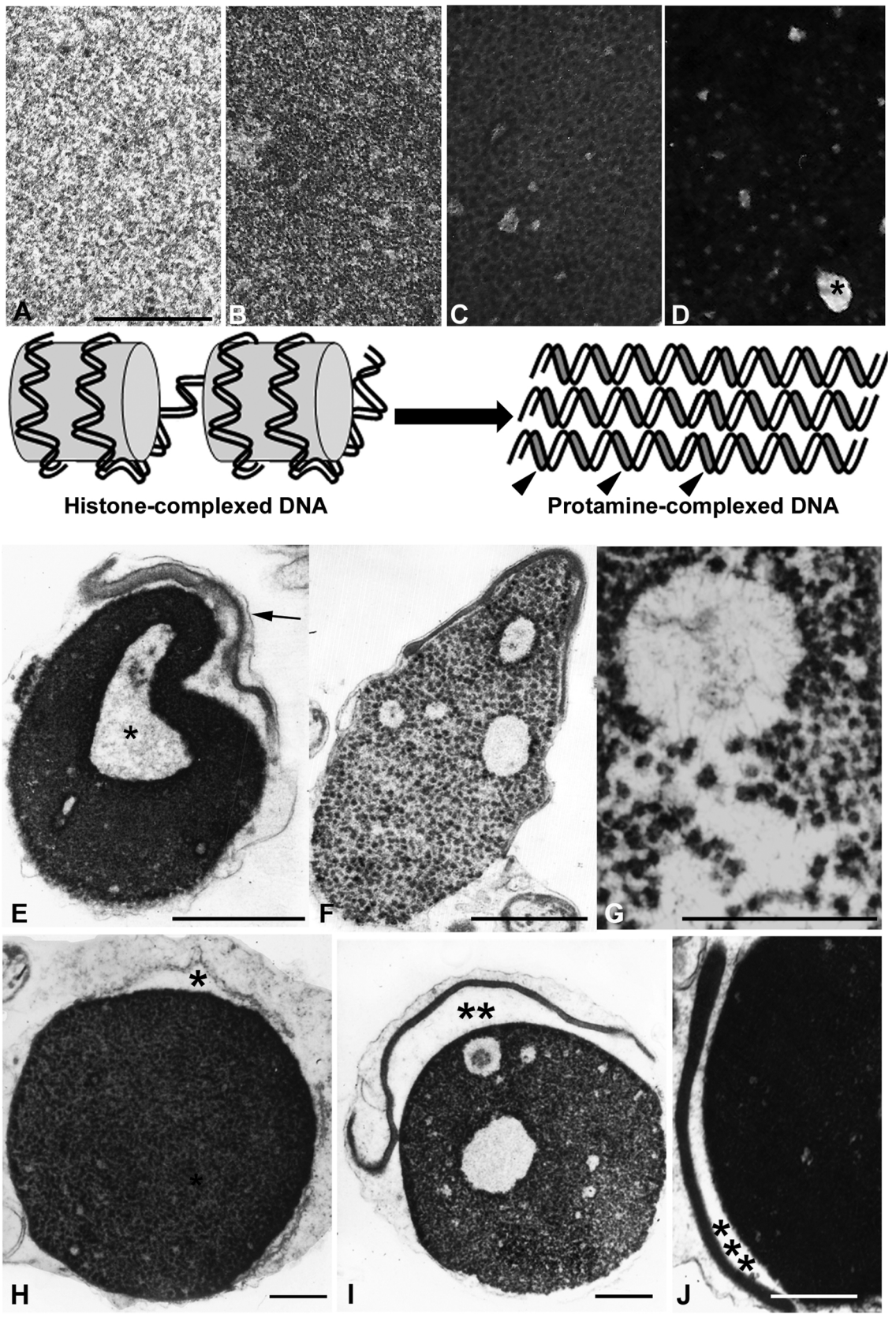

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Fig. 3. A–D. Successive steps of chromatin maturation and compaction from a euchromatic granular structure (A) to a dense, homogeneous heterochromatic appearance (D). The drawing depicts the association of histones (left) or protamines (right) with the DNA double helix. Histone-complexed DNA shows the loose "beads in a string" configuration typical of euchromatin (the nucleosome), while protamine-complexed DNA (heterochromatin) demonstrates a compact arrangement with small protamine molecules lodged into major DNA grooves (arrowheads). E–G: Three examples of defects in chromatin compaction. There is a large "vacuole" (E), multiple smaller ones (F), and a higher magnification detail (G) showing granulo-fibrillar substructure of nuclear rarefactions. The acrosomes in E and F are hypoplastic. Panels H–J show round heads with absent (H, one asterisk) or hypoplastic acrosomes (I and J, double and triple asterisks). The sperm head shown in I has chromatin rarefactions. A–D. Bar = 0,5 μm, E-G. Bar = 1,5 μm, H-I. Bar = 0,7 μm, J. Bar = 1 μm.

appears that chromatin rarefactions in sterile males are not due to abnormalities in the protamine genes. Unless newer investigations indicate otherwise it is reasonable to suggest that chromatin rarefactions are of a non-systematic nature, that is, not genetic but rather secondary to other reproductive pathologies.

In early spermatids the acrosome derives from dense-core vesicles emanating from the Golgi complex that grow in size and fuse to form a large pro-acrosomic vesicle. This structure migrates and attaches to the nuclear envelope, spreading over it to cover between half and 2/3 of its cranial surface. Acrosomal attachment to the nucleus takes place through the action of phospholipase C zeta (PLCζ), a perinuclear theca protein that activates the oocyte by triggering Ca^{2+} fluxes (Escalier, 1990; Oko et al., 2001; Nomikos et al., 2015). As the acrosome grows, a microtubular complex, the manchette, encircles the nucleus and migrates caudally exerting forces that remodel the nucleus from its spherical form to the oval - flattened shape of mature spermatozoa (Kierszenbaum and Tres, 2004; Kierszenbaum et al., 2011). This parallelism between acrosomal and manchette development has its pathological expression in the form of abnormal round-head acrosomeless spermatozoa.

The typical phenotype is that of sperm with spherical heads without acrosomes (globozoospermia, Fig. 3H–J). Small and/or abnormally shaped acrosomes are also found in isolated spermatozoa or immature spermatids in semen and in testicular biopsies from globozoospermic patients (Alvarez Sedó et al., 2012). A set of 6 subacrosomal Perinuclear Theca (PT) proteins, necessary for proper acrosome development were observed to be significantly diminished. The resulting abnormal acrosomes were mostly discarded in residual bodies after spermiation and therefore were not found in ejaculated spermatozoa (Alvarez Sedo et al., 2012). These studies indicated that lack of acrosomes in round headed-spermatozoa is due to abnormalities in perinuclear theca proteins like PLCζ. It is now accepted that this is the most common mechanism to generate acrosomeless spermatozoa, and not absent formation as had previously been proposed (discussed in Chemes, 2017).

In addition to absence of an acrosome in globozoospermia, detailed ultrastructural studies in teratozoospermia have disclosed spermatozoa with small, detached and/or thin acrosomes, that can be referred to as acrosomal hypoplasia (Fig. 3E, F, J). Small percentages of up to 5% of such cells are not uncommon, but should they increase to as much as 30–40% and associate with spheroidal head shapes their presence should be documented since they may fail to activate the oocyte after microinjection. The correct identification of globozoospermia and acrosomal hypoplasia is relevant, because the addition of Ca^{2+} ionophores or strontium to the incubation media can stimulate Ca^{2+} oscillations and overcome failed fertilization (Chemes and Alvarez Sedo, 2012).

Globozoospermia has a systematic phenotype with demonstrated family incidence. Mutations or deletions in genes DPY19L2, SPATA16 and PICK1 were reported in globozoospermic men (De Braekeleer et al., 2015), and 84% of the 31 patients analyzed by Coutton et al. (2015) carried a molecular alteration of DPY19L2. Moreover, acrosomal absence or hypoplasia associate with insufficient chromatin compaction (Fig. 3F and I). A recent report by Yassine et al. (2015) indicated that the histone-protamine transition is abnormal in DPY19L2-deficient mice and men.

In our experience acrosome hypoplasia occurs randomly in sterile patients with severe teratozoospermia which may indicate that this is not a genetically related sperm phenotype. A single publication by Baccetti et al. (1991) reported on a systematic variant of small acrosomes in a pair of brothers (referred to as "miniacrosome sperm defect"). With this exception there are no reports on family incidence or genetic abnormalities in acrosomal hypoplasia.

4. Pathologies of the sperm neck (head-tail connection)

The sperm neck is the site of articulation between heads and tails. The caudal pole of the sperm head depicts a concave depression, the implantation fossa, that is covered by the basal plate, a local dense reinforcement of the nuclear envelope. This shallow concavity accommodates the connecting piece, a compact proteinaceous structure organized around the sperm centrioles, a pair of perpendicular cylinders formed by 9 circularly arranged triplet microtubules. The proximal centriole, perpendicular to the sperm axis, is surrounded by 9 segmented columns, longitudinal pillars with periodic thick densities separated by thin lighter bands (Fig. 4). The distal centriole, parallel to the sperm axis disintegrates after giving rise to the sperm axoneme.

Pin-head spermatozoa were mentioned in the literature as an abnormality characterized by spermatozoa with minute heads (Zaneveld and Polakowsky, 1977). Shortly thereafter, LeLannou (1979) and Perotti et al. (1981) demonstrated that, regardless of the configuration of their cephalic end, there was no sperm head at all, and that these "minute heads" were actually small cytoplasmic droplets (Fig. 4B). They characterized the defect as "decapitated" spermatozoa. Further studies essentially confirmed the absence of heads and proposed alternate descriptive terminology such as "acephalic" and "abnormal development of the head-neck attachment" (Holstein et al., 1986; Baccetti et al., 1984, 1989; Chemes et al., 1987a, 1999).

During spermiogenesis, the axoneme starts developing from the distal centriole and both structures migrate to the plasma membrane where the sperm flagellum grows toward the extracellular space. The centriole-flagellar complex next travels back to the

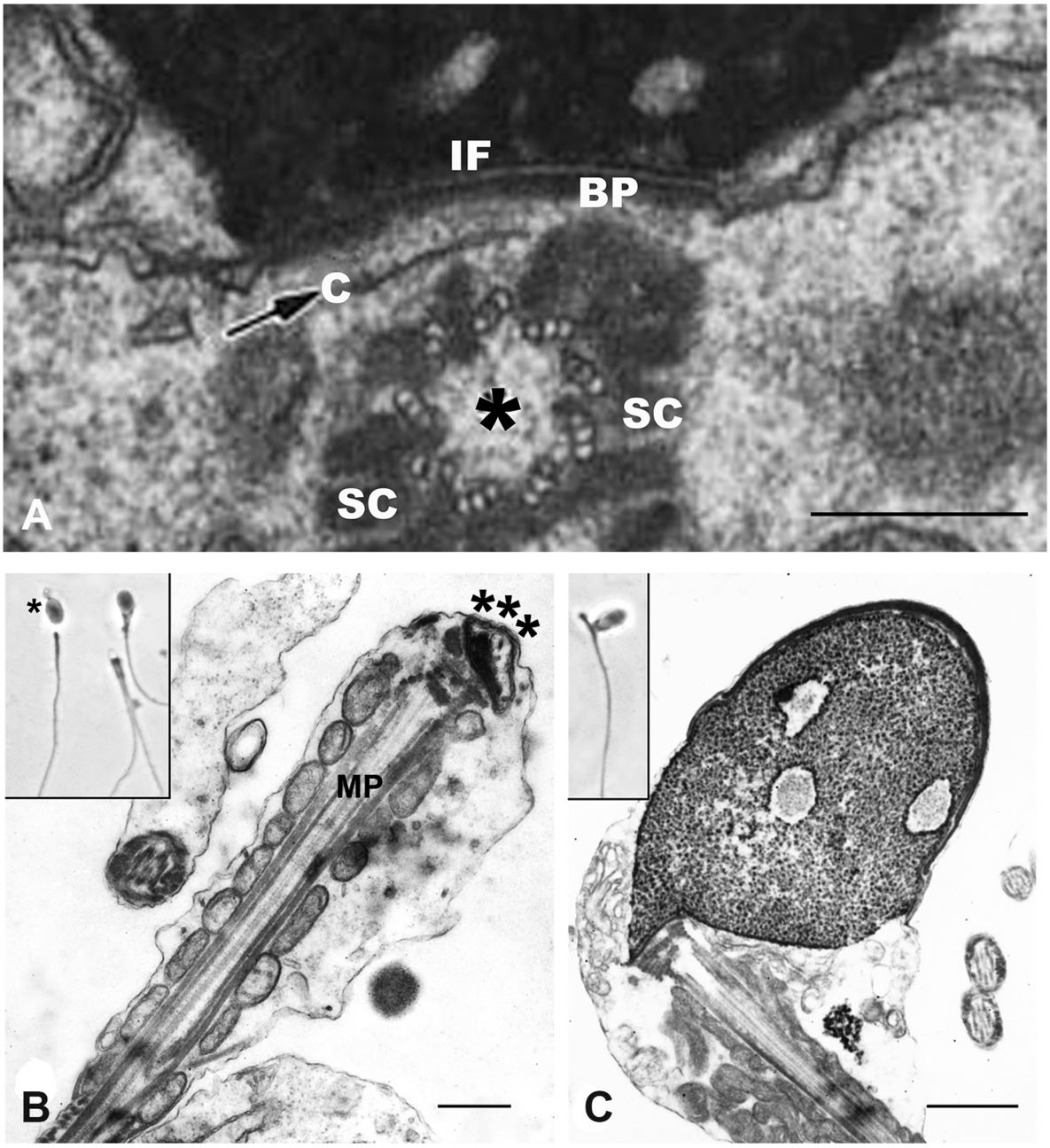


Fig. 4. A. High magnification of the neck region of a normal human spermatozoon. The shallow implantation fossa (IF) formed at the base of the nucleus is reinforced by the basal plate (BP), a thickening of the nuclear membrane. Within the fossa lies the capitulum (black arrow, C), a disk-like structure beneath which lie the transversally sectioned 9 triplet microtubules of the proximal centriole (asterisk). The segmented columns of the connecting piece (SC) surround the sperm centrioles. B and C. Two examples of pathologies of the neck region. B depicts an acephalic spermatozoon with normal midpiece (MP) but no head (triple asterisk). The inset shows two acephalic spermatozoa and one tail-less head (asterisk). C (and inset) show abnormal implantations of the head at right angles to the midpiece. Note the conspicuous nuclear vacuoles. A. Bar = 0.25 μm, B–C. Bar = 1 μm.

base of the spermatid nucleus where it inserts (Fig. 2). Acephalic spermatozoa and pathologies of the head tail attachment derive from alterations in this intricate journey. When the centriole-flagellar complex fails to reach the caudal pole, heads and tails develop independently and separate at spermiation. Headless tails become acephalic spermatozoa and heads are disposed of via residual bodies that are phagocytized by Sertoli cells. If the centrioles attach to the nucleus in a lateral position, heads and tails are not aligned along the same axis resulting in different degrees of angularity and increased fragility of the head-tail junction (Fig. 4C). In these cases, increased fragility of the head-tail connection becomes evident when spermatozoa are subjected to mechanical forces (Perotti

et al., 1981; Baccetti et al., 1984, 1989; Chemes et al., 1987a, 1999; Kamal et al., 1999). The site and nature of the adhesion between heads and tails is not yet clear, but it most probably resides between the basal plate and the neighboring densities of the connecting piece.

Spermatozoa showing abnormal head-midpiece attachment have been used for ICSI by various groups. After some failed attempts where fertilization and implantation took place but were not followed by evolving pregnancies (Saïas-Magnan et al., 1999; Rawe et al., 2002) some successful attempts with normal births were reported (Porcu et al., 2003; Emery et al., 2004; Gambera et al., 2010). This would indicate that in spite of serious sperm neck abnormalities, including centriole proteasome deficiencies (Rawe et al., 2008), ICSI success can be achieved in some patients.

Acephalic spermatozoa or abnormal sperm head positioning are conditions very stable in time, do not respond to therapeutic manipulations, have analogous characteristics in patients suffering from similar conditions and show family clustering, which suggests that it is a systematic sperm phenotype of genetic origin. Various groups reported similar phenotypes as a consequence of genetic manipulations in laboratory animals (Hook1: Mendoza-Lujambio et al., 2002, ODF1/HSPB10: Yang et al., 2014, Spata6: Yuan et al., 2015). Up to now, no alterations of these genes have been reported in the human syndrome, except for a recent publication by Hrdlička et al. (2016) on a Spata6 homozygote haplotype in two infertile males. However, in the last two years different gene anomalies have been reported that disrupt proteins localized to the sperm midpiece or to the head-tail junction in patients with acephalic spermatozoa or abnormal head-connecting piece relationships (SUN5: Zhu et al., 2016; Shang et al., 2017; Elkhatib et al., 2017; BRDT: Li et al., 2017, and TSGA10: Sha et al., 2017).

5. Pathologies of the sperm midpiece (mitochondrial sheath)

During late spermiogenesis the annulus, a cytoskeletal ring close to the sperm neck, migrates caudally allowing spermatid mitochondria to establish close contact and wrap helically around the proximal axoneme giving rise to the mitochondrial sheath (MS). This 4–5 μm long cylindro-conical rod formed as a spiral of 11–13 mitochondrial gyres extends from the sperm neck to the beginning of the principal piece of the tail (Fig. 5A). The mitochondrial sheath provides the energy needed for the regulation of flagellar motility via oxidative phosphorylation-generated ATP, the substrate of axonemal dynein-ATPases (Fawcett, 1975). However, more recent evidence suggests that the main source of ATP for sperm motility may come from glycolysis at the fibrous sheath of the sperm principal piece (Miki et al., 2004).

Mitochondrial anomalies of the principal piece are very infrequent in sterile males (Pedersen et al., 1971; Mc Clure et al., 1983; Bartoov et al., 1980; Wilton et al., 1992; Rawe et al., 2001). Two patients with primary sterility and severe asthenozoospermia due to sperm midpiece anomalies have been reported (Rawe et al., 2007). In normal controls, midpieces were visualized as 3–5 μm long, intensely fluorescent green rods formed by 11–13 regular mitochondrial gyres (Fig. 5A). In one of the patients, midpieces were 8–12 μm in length and were formed by 15–25 mitochondrial gyres with local aggregations that disrupted their regular cylindrical profiles. These midpieces were so extended that it was impossible to observe their full length on transmission electron microscopy (Fig. 5B). The other patient showed a completely opposite configuration, with absent or short midpieces due to the lack or extreme scarcity of mitochondria. This was evident by electron microscopy (Fig. 5C) and by the absence or punctate green fluorescence of the short midpieces. Lack of mitochondria resulted in thin and weak midpieces prone to acute bending. A different type of mitochondrial depletion is seen in sperm from patients with dysplasia of the fibrous sheath (DFS), a systematic sperm defect of genetic origin (see next section). Immotile DFS spermatozoa have a very short midpiece due to lack or insufficient caudal migration of the annulus. As a consequence, a limited number of mitochondria group at the connecting piece and are immediately followed by hyperplastic fibrous sheaths (Fig. 5D and E).

Men carrying sperm with severe midpiece anomalies are sterile due to immotility or extreme asthenozoospermia. However, the introduction of ICSI has shown that oocytes can be successfully fertilized by microinjection of such defective sperm, although in one patient, embryos so obtained implanted after being transferred, but pregnancy terminated in abortion at the end of the first trimester (Rawe et al., 2007). Sperm phenotypes with serious midpiece anomalies are very stable over time and probably result from genetic anomalies, although there are no reports on family incidence. The infrequency of this pathology has prevented further conclusions.

6. Pathologies of the sperm tail: axonemal and periaxonemal anomalies

The human sperm flagellum is a 40–50 μm long structure, with diameters decreasing from 0,3-0,5 μm at the beginning of the principal piece to 0,1 μm at the end piece. It is constituted by a central axoneme surrounded by various cytoskeletal components. The axoneme is a very thin cylinder formed by a set of 9 circumferentially-arranged microtubular doublets surrounding a central pair of singlet microtubules in the typical 9 + 2 configuration (Fig. 6A). Each peripheral doublet is formed by the fusion of two microtubular subunits, one complete (subunit A) and one incomplete (subunit B). In transverse sections of the axoneme viewed from the sperm neck, two small dynein arms protrude in a clockwise direction from one A subunit toward the B subunit of the next pair. The central pair is formed by two independent microtubules enclosed by a central sheath and connected to each peripheral doublet by a radial spoke. The integrity of this complex architecture is essential to produce the coordinated bending wave of tail movements.

The axoneme is continuous from the midpiece to the tail end piece, with each of the 9 peripheral pairs being associated with one outer dense fiber (ODF) at the level of the midpiece (Fig. 2C). At the transition between the mid and principal pieces, ODFs 3 and 8 are replaced by the lateral columns of the fibrous sheath that attach to axonemal pairs 3 and 8. The other 7 ODFs continue to the principal piece progressively diminishing in diameter. The fibrous sheath (FS) is an important cytoskeletal component of the sperm principal piece. It is formed by two longitudinal columns that continue in the position of ODFs 3 and 8 and associate to the

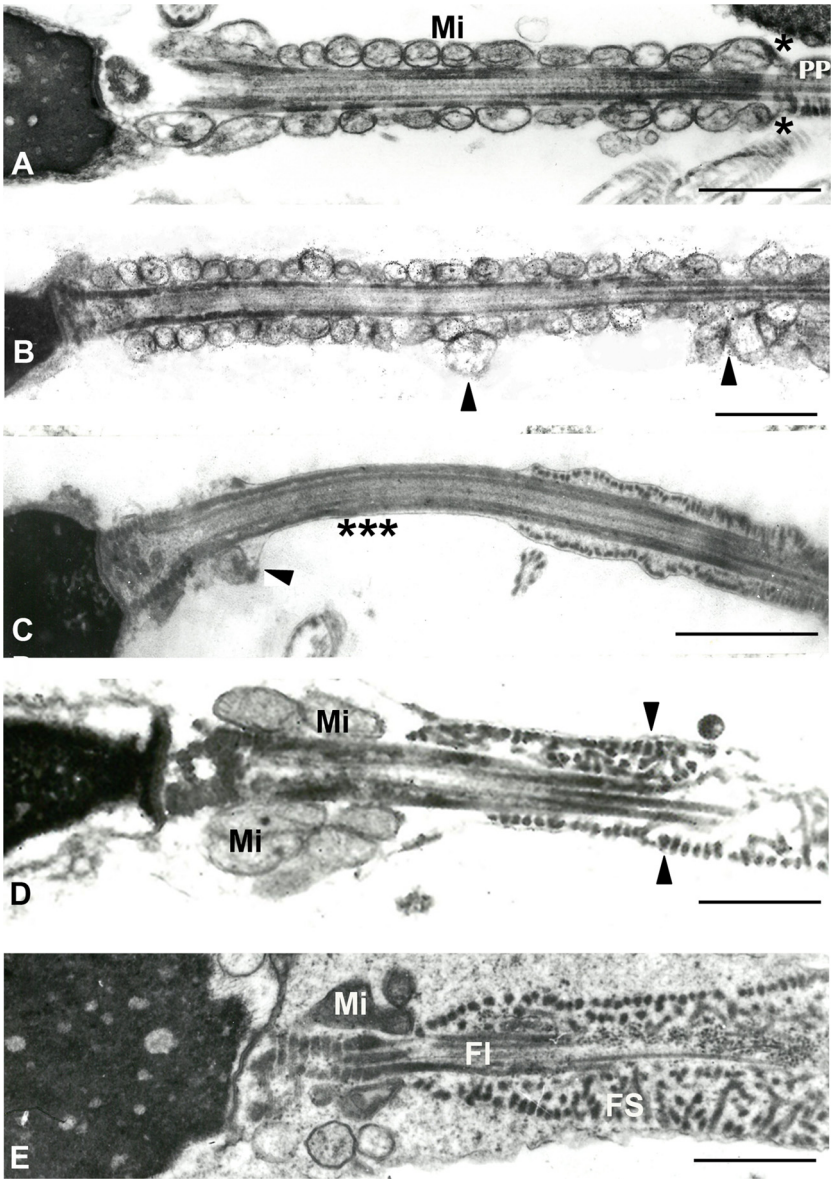
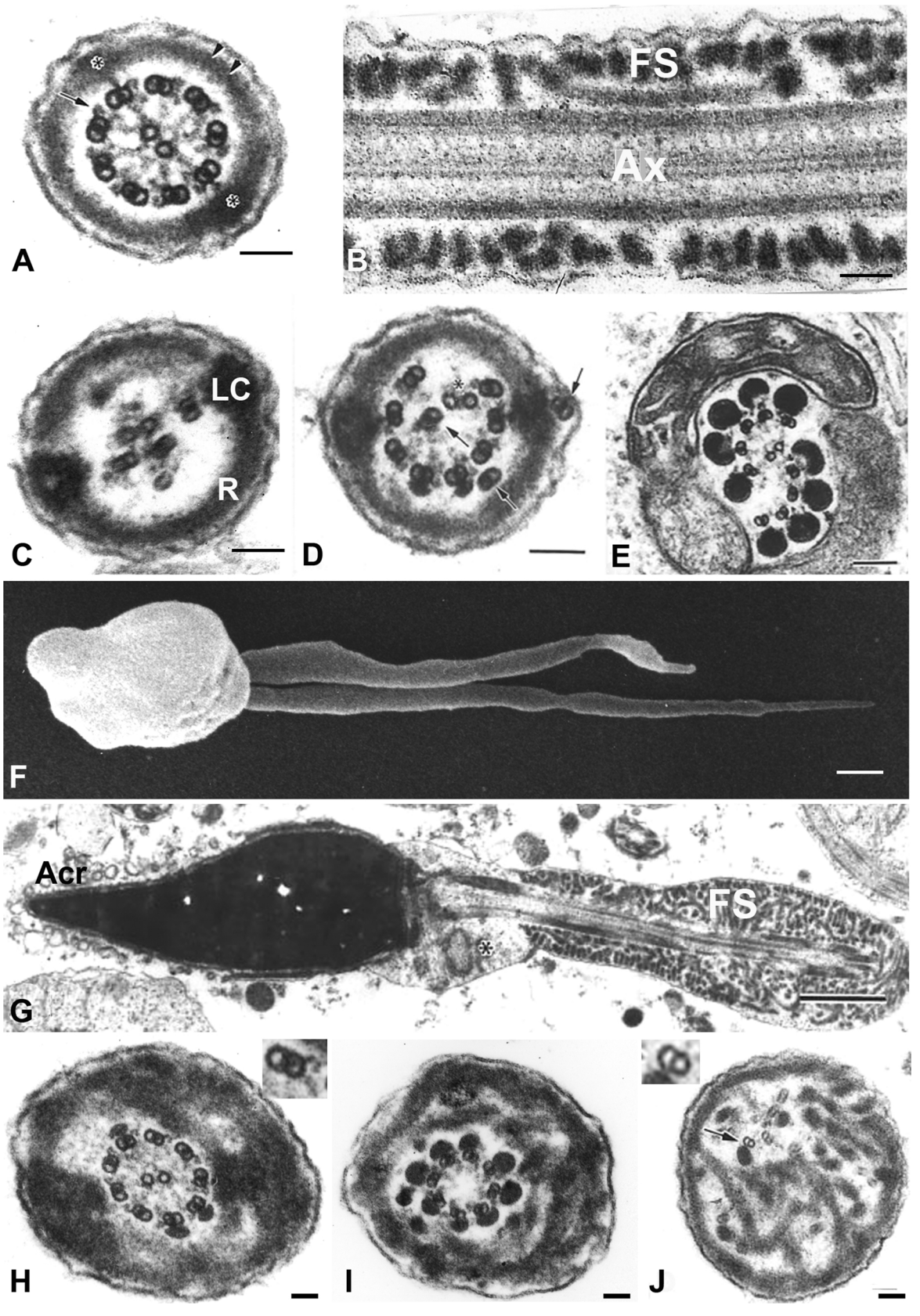


Fig. 5. Pathologies of the mitochondrial sheath (Mi). All micrographs show longitudinal sections through the midpiece with the sperm heads placed to the left. A. Normal spermatozoon. The mitochondrial sheath is formed by a single row of mitochondria forming a spiral of approximately 13 gyres. An electron-dense annulus (two asterisks) separates the midpiece from the principal piece (PP). B. An abnormally long midpiece is composed of at least 22 gyres around the axoneme. Mitochondrial clumping is occasionally present (arrowheads). C. Absence of the mitochondrial sheath. The axoneme is directly covered by the plasma membrane (three asterisks). A few mitochondria may be present near the neck region (arrowhead). D. an abnormally short and clumped mitochondrial sheath (Mi) followed by a duplicated, somewhat disorganized fibrous sheath (arrowheads). E. Another example of an extremely short mitochondrial sheath (Mi) followed by the flagellum (Fl) surrounded by a multilayered and disorganized fibrous sheath (FS). A. C and D. Bar = 1 μm, B. Bar = 1.2 μm, E. Bar = 0.5 μm.

corresponding microtubular pairs, and a succession of transversal semicircular fibers (the “ribs” of the FS) that run between both lateral columns (Fig. 6A). This is well appreciated in sections along the length of the axoneme where the individual “ribs” of the FS appear as a row of transversally sectioned dense units (Fig. 6B) (reviewed in Fawcett, 1975 and Holstein and Roosen Runge, 1981).

The formation and maintenance of the axoneme is assured by an assortment of molecular components organized to facilitate the dynamics of intra-flagellar transport. This is carried out by special types of dyneins and kinesins, molecular motors that transport specific protein cargos (axonemal precursors and signaling molecules) to the tip of the flagellum (kinesins) or in the opposite direction (dyneins). Specific details of this intricate mechanism are not yet fully understood.

Pathologies of the tail are responsible for asthenozoospermia (decreased or absent motility), which together with teratozoospermia are the major seminal causes of male infertility. There is extensive literature on studies relating asthenozoospermia



(caption on next page)

Fig. 6. Spermatozoa displaying pathologies of the sperm flagellum. A and B. Transverse and longitudinal sections through the principal piece. A. Typical arrangement of 9 doublet microtubules circularly arranged around a central pair of singlet microtubules (9 + 2 structure). Two dynein arms arise from each peripheral doublet toward the next doublet in a clockwise direction (arrow). Each peripheral pair is connected to the central pair by a radial spoke. This 9 + 2 complex is surrounded by the fibrous sheath formed by two longitudinal columns (asterisks) joined by interconnecting ribs (double arrowhead). In B the axoneme (Ax) is surrounded by a row of transversally sectioned ribs of the fibrous sheath (FS). C–E depict non-specific flagellar anomalies illustrated by a lack of microtubules (C and D), translocation (D) and "fractured" 9 + 2 axonemes (E). F and G show longitudinal views of abnormal spermatozoa from patients suffering from dysplasia of the fibrous sheath (DFS). F. spermatozoon with an amorphous head and two short, thickened flagella seen by scanning EM. This thickening is due (as shown in G) to a hyperplastic and disorganized fibrous sheath (FS). Disorganization and thickening of the fibrous sheath is accompanied by lack of a middle piece. H, I and J depict transverse sections of DFS flagella. The fibrous sheath is disorganized and thickened, and encircles a normal axoneme in H (the inset shows a doublet microtubule with normal dynein arms), an axoneme without the central microtubular pair and lack of peripheral pairs (I), and a disorganized axoneme with isolated doublet microtubules with lack of dynein arms (J and inset). A–E. Bar = 0,1 μm, F and G. Bar = 1 μm, H – J. Bar = 0.1 μm.

with changes in metabolic or molecular aspects, micro RNAs and phosphoprotein profiles or alterations in nitric oxide synthase or carbohydrate metabolism (Yunes et al., 2003; Buffone et al., 2009; Parte et al., 2012; Salas-Huetos et al., 2015; Zhang et al., 2015; Zhou et al., 2015; Bonanno et al., 2016). Conversely, since the mid 70´s considerable knowledge has accumulated on the ultra-structure of the sperm flagellum, including minute structures bordering the molecular level, like the dynein arms and their ATPase activity or the axonemal microtubules made of tubulin heterodimers.

In 1975 two independent reports from Scandinavian researchers disclosed the structure-functional nature of alterations in cilia and flagella in patients suffering from chronic respiratory disease, sperm immotility and abnormal visceral laterality (Afzelius et al., 1975; Pedersen and Rebbe, 1975). These studies introduced the concept of the Immotile Cilia Syndrome (ICS) later to be known as Primary Ciliary Dyskinesia (PCD) to acknowledge the "primary" (genetic) nature of the syndrome and the concept of dys-motility (Rossman et al., 1981). Dys-motility of cilia and flagella was due to the absence or scarcity of dynein arms / ATPases, an excellent example of structural – functional relationships.

Ultrastructure of sperm flagella in men with sperm motility disorders has been the subject of numerous reports. In particular, a very high incidence of flagellar alterations was found in a large series of 247 asthenozoospermic men. Two quite different sperm phenotypes were identified: non-specific flagellar anomalies (NSFA) and dysplasia of the fibrous sheath (DFS) (Chemes, 1991; Chemes et al., 1998).

NSFA were the predominant pathologies (fast forward progression 3.6%). In sperm smears NSFA spermatozoa displayed long slender tails of normal appearance. Ultrastructural examination disclosed a heterogeneous array of alterations in the number, to-pography and organization of axonemal microtubules (absence, dislocations or translocations). Modifications of the mitochondrial helix, outer dense fibers or the fibrous sheath could be present or not (Fig. 6C–E). These alterations combined randomly, and flagellar length and diameter remained unaffected. Patients in this group suffered from various andrological conditions usually affecting sperm motility such as varicocele, seminal infections or immune factors. The phenotype appeared as secondary and non-systematic, with no respiratory symptoms or family incidence.

Dysplasia of the fibrous sheath (DFS) is an entirely different phenotype usually associated with extremely low motility (fast forward progression 0.16%). Most DFS spermatozoa had short, thick, irregular tails and absent midpieces (Fig. 6F and G). The fibrous sheaths formed thick multilayered rings around axonemes (Fig. 6G–J). When present, lateral columns were misplaced and difficult to differentiate from the ribs and there was abnormal extension of ODF's 3 and 8 to the principal piece. Axonemes were rarely normal (Fig. 6H), frequently lacked central pairs or peripheral doublets (Fig. 6I) or were completely disorganized (Fig. 6J). Dynein arms were either present or missing (see insets in Fig. 6H and J). Respiratory involvement was documented in approximately 20% of the patients. These patients mostly belonged to mixed populations of European and Mid-Eastern origin (Chemes et al., 1987b, 1998, Chemes, 1991; Chemes and Rawe, 2003; Rawe et al., 2001). Similar characteristics have been reported by other groups (Escalier and David, 1984; Zamboni, 1987; Wilton et al., 1992).

The DFS phenotype is very stable over time, can be associated with lack of dynein arms in respiratory cilia and chronic respiratory disease (Chemes et al., 1990), does not respond to any therapeutic intervention, and shows family clustering (Chemes et al., 1998). Human Akap3 and Akap4 are the main structural proteins of the fibrous sheath. No alterations at the protein or gene levels were found in an early report of 9 DFS patients (Turner et al., 2001). Shortly after, Baccetti et al (2005), demonstrated intragenic deletions of the Akap3 and Akap4 genes and absence of the Akap4 protein in sperm flagella from a DFS patient. These factors indicate that it is a systematic sperm pathology of genetic origin as was further confirmed in the last few years by reports on deletions or mutations in various genes that code for axonemal or periaxonemal components (DNAH11: Bartoloni et al., 2002; DNAH1: Coutton et al., 2015; Amiri-Yekta et al., 2016; CFAP 43/44: Tang et al., 2017; AK7: Lorès et al., 2018; CCDC40: Yang et al., 2018).

Spontaneous fertility or successful ART can be achieved in patients with NSFA after treatment of asthenozoospermia according to their diverse etiologies (varicocele, seminal infections, immune factors, etc., Chemes et al., 1998). Provided that sperm heads are normal, flagellar pathologies do not compromise ICSI fertility outcome, even in patients with primary sperm phenotypes like DFS. This suggests that, after penetration into the oocyte, midpiece and tail components may not play a role in oocyte activation and early embryonic development (reviewed in Chemes and Alvarez Sedo, 2012).

7. Concluding remarks

What is the connection between genetic or environmental factors and sperm pathologies? Is it possible to conceive an interaction

between both pathogenic mechanisms? Comparative spermatology may provide some clues to this question.

The genetic origin of acephalic spermatozoa in humans may be compared with the sperm dimorphism that has been reported in Lepidoptera. Some butterflies and silk worms have heteromorphic spermatogenesis and produce nucleate (eupyrene) and acephalic (apyrene) spermatozoa. This divergence may be produced by a testicular factor that directs bi-potential spermatocytes to take one of two paths. While elongating eupyrene spermatids shift from lysine-rich to arginine-rich nucleoproteins, there is only lysine-rich chromatin in non-elongating apyrene spermatids that subsequently cast off their nuclei to become acephalic spermatozoa (Friedländer and Hauschteck-Jungen, 1982; Friedländer, 1997). High-throughput mass spectrometry analysis of these acephalic spermatozoa identified some proteins predominantly expressed in them. Coincidentally, apyrene spermatozoa greatly improve fecundity of females when inseminated together with cryopreserved normal (eupyrene) semen, which suggests that they possess apyrene-specific functions (Whittington et al., 2015).

Low percentages of acephalic sperm can also be observed in the Emu (du Plessis and Soley, 2011, 2012). A syndrome that strongly resembles the human one has been reported in bulls (Bloom and Birch Andersen, 1970) and boars (Toyama and Itoh, 1996). Therefore, in different species (insects, birds, non-human mammals, men), a somewhat similar phenotype may derive from genetic anomalies or from the interaction of genetic predisposition with the intra-testicular environment.

There is now abundant information on the genetic vs environmental influences in the pathogenesis of human sperm pathologies (see previous sections). Recognized genetic phenotypes in which gene mutations-deletions have now been confirmed include lack of acrosomes (globozoospermia), acephalic spermatozoa and DFS (dysplasia of the fibrous sheath). All these phenotypes are very stable in time and cannot be modified by any known therapeutic means, but the introduction of microinjection techniques (ICSI) have drastically changed their negative prognosis. In the case of globozoospermia, the identification of the importance of acrosome-derived factors in oocyte activation has open the way to their in vitro treatment in the course of intracytoplasmic sperm injection (reviewed in Chemes and Alvarez Sedo, 2012). There is an intermediate group (chromatin and midpiece anomalies) where positive identification of a genetic origin is suspected but has not been identified, either because genetic studies have been largely negative (defects of chromatin compaction) or because of its extreme rarity (midpiece anomalies). Finally, non-specific flagellar anomalies (NSFA) are definitively secondary to various andrological conditions and responsive to their treatment (Chemes et al., 1998). In summary, human sperm pathologies offer a good example of the benefits of intensive research in the identification of abnormal sperm phenotypes, their genetic or secondary etiology and diverse therapeutic possibilities.

One relevant question remains: how comparable are abnormal sperm phenotypes in humans and other animal species. The previous sections presented current knowledge on the clinical and experimental pathology of human spermatozoa, its impact on fertility and the subcellular basis of abnormal sperm function in sterile men. Confronted with their problem, infertile men seek medical advice and should be provided with a diagnosis that clarifies the reasons for sperm incompetence and hopefully provides clues that may help to overcome infertility.

Comparing human sperm phenotypes with published results of similar animal studies has proven more complex than anticipated. Sterility in a wild animal would probably end with the extinction of that individual genome and go unnoticed to scientists unless they receive a request to study a semen sample. This is certainly a problem with endangered wild animal species. Sterility in farm and domestic animals have received more attention that likely will expand in the future. Sperm phenotypes obtained by genetic manipulation are not considered here because, beyond their value as models to study gene function, they do not represent true sperm pathologies.

There is abundant information on abnormal animal sperm in the scientific literature that will be briefly summarized even if some important references may be omitted. The literature on animal sperm pathologies includes phenotypes with similarities with the human counterparts in different mammalian and bird species such as stallions (microtubular alterations, detached acrosomes: da Landim Alvarenga and Alvarenga 1997; Veeramachaneni et al., 2006; Pesch et al., 2006), dogs (coiling of tails, Immotile Cilia Syndrome and Primary Ciliary Diskinesia: Edwards et al., 1983, 1992, Rota et al., 2008), pigs and boars (Primary Ciliary Dyskinesia, decapitation, short tail sperm defect: Roperto et al., 1991, Toyama and Itoh, 1996, Sukura et al., 2002; Sironen et al., 2002; Sironen et al., 2006), bulls (midpiece and axonemal anomalies, defects of sperm heads and tails, “stump” tails and “dag defects”: Lorton et al., 1983; Barth and Oko, 1989; Vierula et al., 1983; Blom, 1966), goats (“dag like defects”: Molnár et al., 2001), and emus (bent heads, acephalic sperm, round heads: du Plessis and Soley, 2011, 2012). This is certainly a topic of great interest both in academia and in clinical practice in humans and other animals and will hopefully further develop in the future by more comparative studies exploring similarities and differences of abnormal sperm phenotypes in men and non-human species.

Conflicts of Interest

None.

Acknowledgments

In the Introduction and throughout the text there are quotations from previous publication of the author: Chemes and Rawe, 2003 (Human Reproduction Update, reproduced with permission from Oxford University Press), and Chemes & Alvarez Sedo, 2012 (Asian J. Androl., reproduced with permission from SIM, SJTU and Wolters Kluwer – Medknow). Portrait of A. van Leeuwenhoek and drawings of spermatozoa and microscope in Figure 1 are reproduced from https://es.wikipedia.org/wiki/Anton van Leeuwenhoek. Drawings in Figure 2 are reproduced from Holstein, 1976 (Andrologia, reproduced with permission from John Wiley and Sons). Figure 3, panels A to D and DNA drawings and Figure 3 (panels E-J) were previously published by Chemes and Alvarez Sedo, 2012 (Asian J. Androl, reproduced with permission from SIM, SJTU and Wolters Kluwer – Medknow). Figure 4 panel A was modified from a previous publication of Chemes et al., 1999 (Human Reproduction, reproduced with permission from Oxford University Press).

Figure 4 B and C were originally published by Chemes and Rawe, 2010 (Cell Tissue Res, published with permission from Springer Nature). Different panels in Figure 6 were previously published in Chemes et al., 1998 and Chemes and Rawe, 2003 (Human Reproduction and Human Reproduction Update, published with permission from Oxford University Press), or Linck et al., 2016 (J Assist Reprod Genet, published with permission from Springer).

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