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Ultrastructure of Spermatozoa from Infertility Patients

Elizaveta E. Bragina and Elena N. Bocharova

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Abstract

Standard examination of human semen currently remains a main test for examination of male fertility disorders. Although parameters of sperm quality in fertile men are generally higher than in sterile ones, there is a substantial overlap between the two populations, indicating that other important factors affect fertility, but are not assessed in conventional assay. Currently, tests determining the functional properties of sperm have been intensively developed. This review considers an electron microscopic examination of sperm, which assesses the structure and function of the sperm nuclear, penetration and motor apparatus. The detection of sperm chromatin structure can help to understand the causes of early embryonic malformation. Genetically caused and functional disorders of the structure and function of spermatozoa are discussed. Indications for electron microscopic examination of spermatozoa in fertility disorders are given.

Keywords: sperm ultrastructure, sperm chromatin, acrosome, asthenozoospermia

1. Introduction

Standard examination of human semen currently remains a main test for male fertility disorders. The concentration (total sperm count) and motility of spermatozoa and the content of morphologically normal (typical) spermatozoa are thought to reflect the fertilization potential of the semen [1]. Although their values in fertile men are generally higher than in sterile ones, there is a substantial overlap between the two populations, indicating that other important factors affect fertility, but are not assessed in conventional assay [2]. In this regard, methods to assess the functional properties of spermatozoa and thus to evaluate their reproductive (fertilizing) potential have intensely been developed in the past years.

Light microscopy, which is employed in a conventional sperm testing, reports the numbers of sperm heads and tails, their sizes and relative arrangement, the presence and sizes of the

acrosome and nuclear vacuole, and sperm movement. An ultrastructural examination makes it possible to look inside the spermatozoon and to study what is inaccessible by light microscopy, including the extent of chromatin condensation and the structures of the perinuclear theca (PT), its postacrosomal segment, the centriole, the axoneme, and periaxonemal elements of the tail.

Every function of the spermatozoon is now possible to attribute to a particular morphological structure owing to the achievements of modern molecular biology, cytology, and genetics. The morphology of spermatozoa reflects how competent they are to fertilize (enter) the oocyte and to provide for embryo development.

2. Sperm head

2.1. Chromatin structure in normal spermatozoa

The nucleus occupies a major part of the sperm head and contains condensed chromatin (**Figure 1a**), which is detected as an electron-dense homogeneous material, with small regions of lower electron density on ultrathin sections. Condensed chromatin is at least 10 times denser than ones in somatic cells [3].
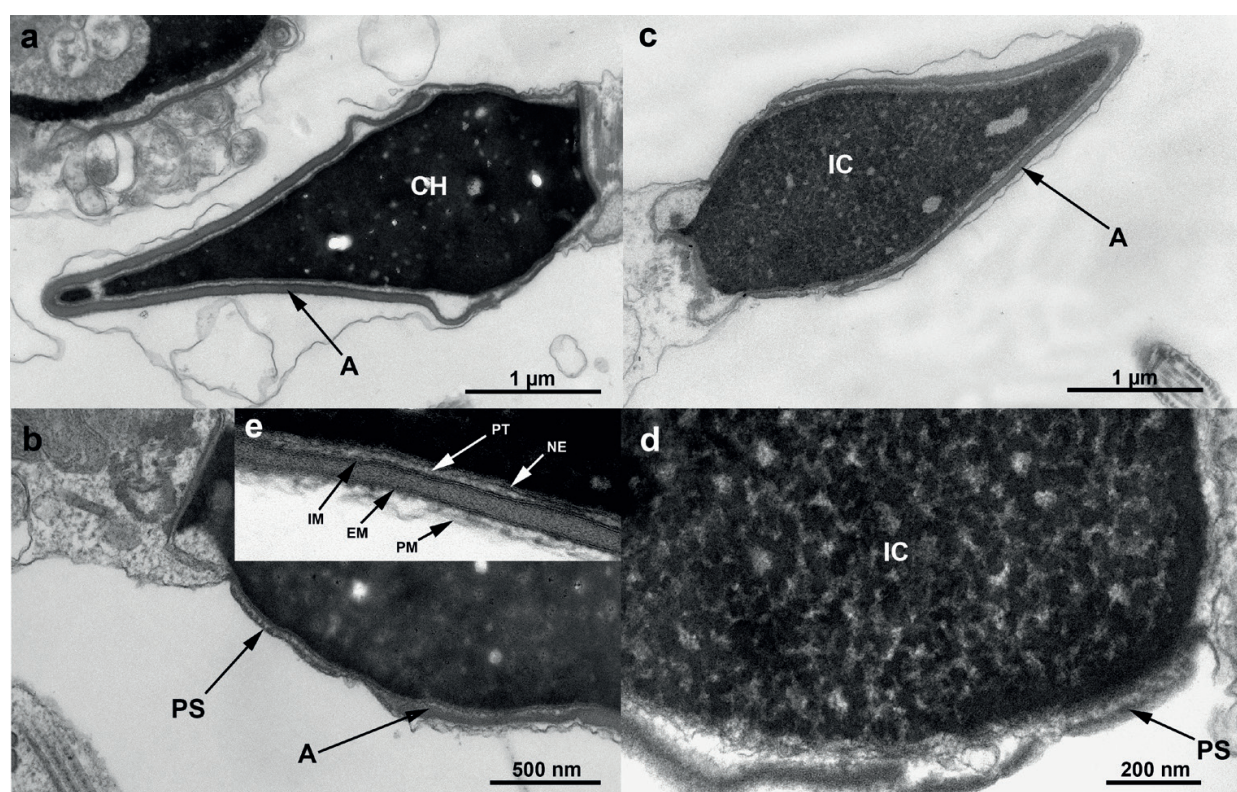

Figure 1. (a) Spermatozoon with condensed chromatin (CH) and normal acrosome (A). (b) Postacrosomal segment of perinuclear theca (PS) with characteristic intermittent striation. (c, d) Spermatozoon with immature chromatin (IC). (e) Fragment of sperm acrosome. NE, nuclear envelope; PT, perinuclear theca; IM, inner acrosome membrane; EM, extra acrosome membrane; and PM, plasma membrane.

To achieve this unique extent of compaction, sperm DNA is packaged in a specific manner, which substantially differs from chromatin packaging in somatic cells. In somatic cells, DNA is packaged to produce the so-called nucleosomes. The DNA double helix is wrapped around a specific complex of canonical histones (a histone octamer) [4].

During sperm maturation, canonical histones are replaced by testis-specific histones and then by protamines, basic proteins with lower molecular weight and high concentration of arginine and cysteine (for a review, see [5, 6]).

As spermatozoa progress through the epididymis, disulfide bridges form between cysteine residues of protamines to further stabilize the DNA-protamine complex and morphologically determine condensation of the dense nucleoprotamine complex in the sperm nucleus [7]. Sperm chromatin is decondensed and acquires a nucleosomal structure after fertilization. The organization of sperm chromatin facilitates the transfer of compacted DNA into the oocyte and ensures its reverse transformation so that genetic information becomes readily available in the developing embryo [8].

Approximately 5–10% of genomic DNA remains free of protamines and preserves a nucleosomal structure in mature human spermatozoa (for a review, see [9]). The role of the residual nucleosomes remained unclear until recently and was explained in three studies, which were published simultaneously in 2010 [10–12]. Residual nucleosomes were found to mark the genes for early embryo development factors and to perform an important function in the epigenetic regulation of embryo development. A gene distribution between protamine-associated and histone-containing (nucleosomal) regions of chromatin follows a certain pattern. Residual nucleosomes occur in the promoters of early developmental genes (e.g., *HOX* gene clusters), imprinted gene loci, and miRNA genes.

Condensation associated with histone-to-protamine replacement metabolically inactivates chromatin and, on the other hand, contributes to its mechanical and chemical stability, thus protecting the paternal genome from nucleases while spermatozoa travel through the male and female reproductive tracts and interact with the oocyte. Residual nucleosomes mark early developmental genes. Normal chromatin condensation is indicative of the sperm potential to produce a normally developing embryo.

2.2. Abnormal chromatin condensation in spermatozoa

Spermatozoa with incomplete chromatin condensation in the nucleus are almost always detectable in ejaculate samples from fertile donors. Granular and fibrillary structures of approximately 40 nm in diameter are seen in these cells. The chromatin structure observed in the spermatozoa is similar to that of elongated spermatids, and their chromatin is consequently known as immature chromatin (**Figure 1c**, **d**) [13].

What is a possible role of distorted chromatin compaction? The disturbance of chromatin condensation is a consequence of a reduced protamine content [14]. Hammoud et al. [15] have recently found that defects in histone-to-protamine exchange lead to a random distribution of nucleosomal (histone-associated and potentially active) chromatin in infertile patients, in contrast to a programmed nucleosomal chromatin distribution in fertile men. Distorted

chromatin compaction in spermatozoa seems to lead to substantial post-fertilization defects. Abnormal (insufficient) chromatin condensation was shown to delay the first cell division cycle and to subsequently cause damage to the embryo [16]. Such defects can be responsible for ART failures [17] and early pregnancy losses [18].

Higher percentage of spermatozoa with immature chromatin was observed in semen of the patients with arrest of embryonic development compared with fertile men, and the difference was statistically significant. Semen samples with increased percentage of spermatozoa with immature chromatin in the men with embryo development arrest in reproductive history were 2.2 times more frequent than in the control group (44 vs. 20%) [19].

The question arises as to whether defects in chromatin condensation are associated with DNA fragmentation in spermatozoa. An early hypothesis suggested that defects in histone-to-protamine exchange and, therefore, in chromatin condensation inevitably lead to higher sperm DNA fragmentation [20].

A higher count of spermatozoa with immature, insufficiently condensed chromatin in semen provides an independent diagnostic sign and shows no association with a higher count of spermatozoa with DNA fragmentation [19, 21]. Clinically, fertility disorders are associated with both higher percentage of spermatozoa with immature chromatin and higher percentage of spermatozoa with DNA fragmentation in the ejaculate, but the disorders differ in nature between the two cases. Diagnosing the nature of damage to sperm nuclear material makes it possible to choose a treatment adequate to the observed defect [22].

2.3. Vacuoles in the sperm nucleus

Hollows, which were initially described as vacuoles varying in size and location, can be detected in chromatin of sperm nuclei [23]. Vacuoles are actually indentations in the nucleus, as is seen on ultrathin sections. Chemes and Alvarez Sedò [24] have proposed the term lacunae or lacunar defects for nuclear vacuoles. Lacunae vary in location and texture. **Figure 2a**, **b** shows a lacuna surrounded by a membrane with membrane whorls (MWs), which consist of double membranes with septal complexes [25]; the lacuna is interconnected with nuclear pockets at the base of the head (**Figure 1a**).

Invaginations of another type can also be detected in sperm nuclei. The invaginations may occur in both basal and apical parts of the nucleus, are not surrounded by a membrane, and contain granular material (**Figure 2d**, **e**). A connection (contact) between a lacuna and the subacrosomal space cannot always be seen in ultrathin sections, and the lacuna consequently appears to be a vacuole in nuclear chromatin. DNA is absent from lacunae (**Figure 2c**) [26].

Moving sperm organelle morphology examination (MSOME) using high-resolution microscopy at magnifications exceeding 5000× makes it possible to select in vivo the vacuole-free spermatozoa and to perform intracytoplasmic morphologically selected sperm injection (IMSI) [27].

There are data that IMSI of spermatozoa without vacuoles or with one small vacuole substantially increases the yield of blastocysts as compared with spermatozoa containing large vacuoles or spermatozoa with more than two small vacuoles [28].
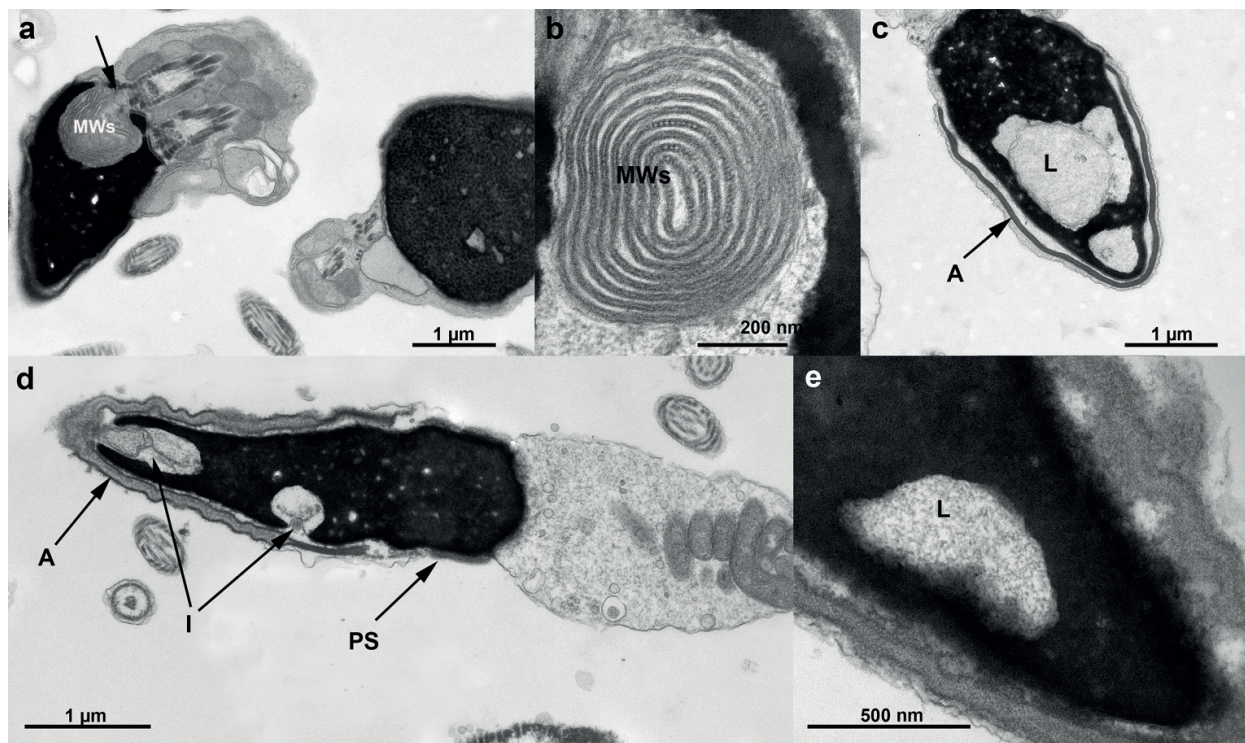
Figure 2. Vacuoles in the sperm nucleus. The lacuna surrounded by membrane with membrane whorls (MWs) (a, b). The lacuna is interconnected with nuclear pockets at the base of the head (a, arrow). Invaginations of the nucleus (I) not surrounded by membrane (d, e), and two large lacunae (L) without visible contact with the subacrosomal space (c). A, acrosome and PS, postacrosomal segment of perinuclear theca.

On the other hand, no correlation of the presence of large vacuoles in spermatozoa has been observed for spermiogram parameters, DNA damage, and live birth rate [29]. IMSI does not improve the outcome of ART after two successive IVF-ICSI failures [30].

Haraguchi et al. [31] have used immunochemistry with electron microscopy and detected proteasomes in nuclear vacuoles and clear spots of condensed chromatin. Nuclear vacuoles and nuclear pockets at the base of the nucleus were assumed to function as proteolytic centers to resorb the molecules (somatic and sperm-specific histones and transit proteins) that are released during chromatin reprogramming. A positive correlation between the presence of vacuoles and the acrosomal reaction [32], vacuoles and capacitation [33] similarly indicates that vacuoles are related to physiological properties of spermatozoa and has no effect on their fertilizing potential.

2.4. Acrosome and perinuclear theca

The acrosome is a secretory vesicle derived from the Golgi apparatus. The acrosome forms a cap on the anterior pole of the nucleus and consists of an outer membrane, inner membrane, and matrix. The outer acrosomal membrane is adjacent to the plasma membrane covering the head of the spermatozoon. A layer sandwiched between the inner acrosomal membrane and the nuclear envelope is known as the perinuclear theca (PT), which has a medium electron density and is approximately 200 nm thick (**Figure 1e**).

The acrosome covers the anterior two-thirds of the sperm head. Relative to the acrosome, the head can be divided into three regions: acrosomal, equatorial, and postacrosomal. Only the PT with its characteristic intermittent striation occurs between the nucleus and the plasma membrane in the postacrosomal region of the spermatozoon (**Figure 1b**).

Material contained in the lumen of the acrosome has a medium electron density and is known as the acrosomal matrix [34]. Zona pellucida (ZP)-binding proteins are found in the acrosomal matrix. Proacrosin is the most important of all ZP-binding proteins of the acrosome. Proacrosin was long believed to be a main lytic protein essential for sperm penetration through the ZP. However, proacrosin knockout mice were found to be fertile [35], although their spermatozoa penetrate through the ZP slower than spermatozoa of wild-type mice. Acrosin probably plays a role in maturation and packaging of other acrosomal matrix proteins. The acrosomal matrix contains several other ZP-binding proteins.

The acrosome of a capacitated spermatozoon interacts with ZP glycoprotein 1 (ZP1) of the oocyte to trigger fusion of the plasma and outer acrosomal membranes, the membrane ends fuse, and vesicles form. Then proteases are released from the acrosome and digest the ZP. The process is known as the acrosomal reaction, which consists in exocytosis and allows the spermatozoon to pass through the ZP. Acrosome-reacted spermatozoa subsequently bind with ZP2, another glycoprotein, which is responsible for sperm adhesion to the oocyte [36]. The inner acrosomal membrane remains intact.

The plasma membrane and the outer acrosomal membrane of the equatorial segment are not involved in forming vesicles during the acrosomal reaction. The equatorial segment is a region where fusion of the spermatozoon and oocyte plasma membrane is triggered. The sperm plasma membrane of the equatorial segment fuses with microvilli of the oolemma, the membranes fuse, and sperm components are thus delivered into the ooplasm. The equatorial segment protein (ESP) is found in the equatorial segment of the acrosome in human spermatozoa [37]. ESP is detectable throughout the acrosome biogenesis. It is thought that ESP plays a role in adhesion of the spermatozoon to the oocyte and their fusion at the oolemma level. Fujihara et al. [38] identified sperm equatorial segment protein 1 (SPESP1), which is specific to the equatorial segment. Spermatozoa of transgenic mice devoid of *SPESP1* (*Spesp1−/−*) fuse with eggs at a far lower rate. SPESP1 seems to be responsible for maintaining the integrity of the equatorial segment after the acrosomal reaction. Membranes of the equatorial segment are disrupted after the acrosomal reaction in Spesp1−/− mice, whereas the equatorial segment is preserved in wild-type mice.

An important role is ascribed to Izumo. The Izumo family includes four proteins, Izumo1-4. Izumo1 is a membrane immunoglobulin protein with an extracellular immunoglobulin domain of 145 residues and an N-terminal domain. The sperm protein Izumo1 on the equatorial segment of the acrosome-reacted spermatozoon recognizes its receptor, JUNO, on the oocyte surface. Human Izumo1 forms a high-affinity complex with the Juno receptor of the oocyte and changes its conformation [39].

2.5. Perinuclear theca

The PT is a cytoskeletal structure that harbors a specific oocyte-activating factor (for a review, see [40]). The PT and its postacrosomal segment remain associated with the sperm nucleus

and enter the oocyte upon fertilization. In contrast to the acrosome, which rapidly responds to exogenous factors, the PT is resistant to extraction with denaturing agents and high-salt buffers.

The putative oocyte-activating factor MN13 was found in the PT [40]. MN13 is located in periodic striations, which form the postacrosomal sheath of the PT.

Phospholipase C zeta (PLCζ) is another protein found in the postacrosomal segment of the PT and is thought to act as an oocyte-activating factor [41].

Thus, PLCζ and probably other proteins of the postacrosomal sheath of the PT act as oocyte-activating factors. The postacrosomal sheath is the first to contact the oocyte, and its dissolution (disassembly) is sufficient for triggering early events of oocyte activation. The oocyte-activating factors are transmitted from the sperm PT into the oocyte cytoplasm after the incorporation and rapid dissolution of the PT. In the normal fertilization cycle, the PT dissolves in the oocyte cytoplasm simultaneously with decondensation of the sperm nucleus and initiates division of the maternal pronucleus by hydrolyzing a membrane-bound phospholipid substrate, triggering cytoplasmic Ca^{2+} oscillations [42]. In the case of ICSI, activation occurs only in the oocytes that contain a partly or completely dissolved PT. When the PT dissolves only partly, the residual PT postacrosomal sheath may persist at the apical side of the paternal pronucleus and may delay or arrest zygote development [43]. Dissolution of the subacrosomal part of the PT is essential for complete DNA decondensation in the paternal pronucleus and the start of DNA synthesis in both pronuclei.

2.6. Acrosomal abnormalities

Electron microscopic examination of the acrosome provides an experimentally grounded alternative to sperm penetration assays. The method reliably reports the integrity of the acrosome and the status of its enzymatic system and the postacrosomal segment, which is involved in sperm attachment to the oocyte. A higher percentage of spermatozoa with abnormal acrosomes in an ejaculate sample can be responsible for idiopathic infertility when the spermiogram parameters are within the normal ranges.

2.6.1. Primary lack of an acrosome

Lack of an acrosome is identified as primary when resulting from spermiogenesis defects. Globozoospermia of a presumably genetic nature provides a classical example of the primary lack of an acrosome.

Globozoospermia is an uncommon male fertility disorder. Round-headed cells may account for up to 6% of the total sperm count in the ejaculate in fertile men [44], while 100% of spermatozoa have round heads in total globozoospermia. The sperm count and motility are not affected in globozoospermia. An ultrastructural examination shows that acrosomes are completely absent from round heads or that a rudimentary acrosome occurs at the nuclear pole opposite to the tail (**Figure 3a**).

Defects of chromatin condensation in the nucleus are additionally seen in the majority of ejaculate samples. Heterogeneity is also possible; i.e., spermatozoa with normal condensed chromatin and those with decondensed chromatin may be detected in one ejaculate sample. Both
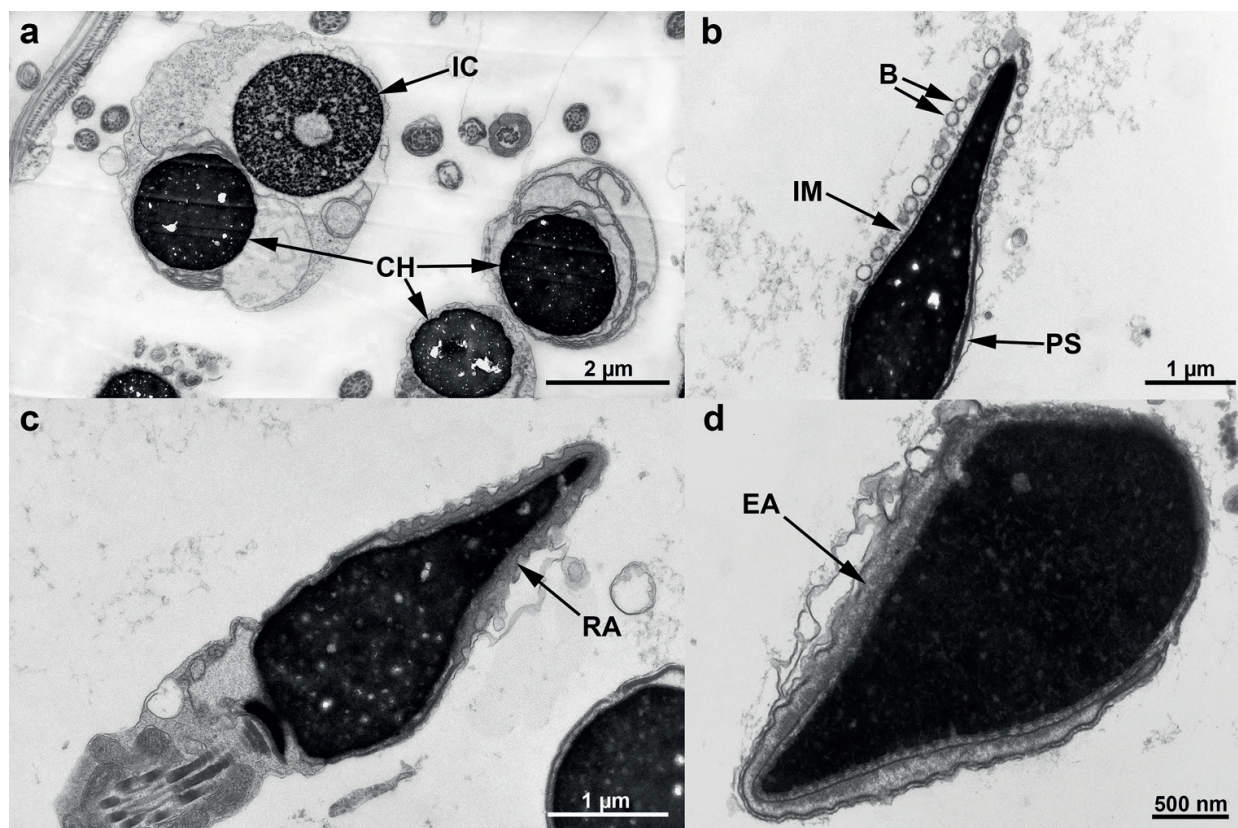
Figure 3. (a) Round acrosomeless sperm heads from globozoospermia. Some nuclei are with condensed chromatin (CH) and one nucleus with immature chromatin (IC). (b) Secondary lack of acrosome. The intact internal acrosomal membrane (IM) and postacrosomal segment (PS) are visible. The outer acrosomal membrane and the plasma membrane form bubbles (B). (c) Acrosome with irregular contours (RA); (d) "empty" acrosome (EA).

within- and between-sample heterogeneity are observed. Higher contents of spermatozoa with immature chromatin [45] were observed in globozoospermia in the majority of studies.

Kullander and Rausing [46] were the first to assume a genetic nature for globozoospermia. Cases with a family history of the disorder supported the assumption. Mutations or deletions of three genes—SPATA16, PICK1, and DPY19L2—were detected in globozoospermia in molecular genetic studies. A homozygous mutation of SPATA16 (3q26.32) was found in three brothers with globozoospermia [47]. A mutation of PICK1 (22q12.3-q13.2) was identified in a globozoospermia patient [48]. The protein products of the two genes occur in the Golgi apparatus and are involved in vesicular trafficking, which is necessary for acrosome biogenesis in spermatids during spermiogenesis [49]. A deletion of DPY19L2 was observed in the majority of total globozoospermia cases; Dpy19l3 protein is essential for a nuclear flattening and the formation of the acrosome [50].

The identification of the missense mutation L967Q of the gene VPS54 [51], gene GM130 inactivation [52], and some others lead to phenotypic globozoospermia in mouse model. These factors are related to the function of the Golgi apparatus vesicles, and these mutations are not identified in men.

The postacrosomal sheath of the PT is absent in patients with globozoospermia. PLCζ is found in extremely small, if any, amounts in spermatozoa of mice and human patients with a Dpy19l2 mutation and the globozoospermia phenotype [53]. Because these proteins possess

oocyte-activating activity, ICSI is insufficient in globozoospermia. The development of oocyte activation methods made it possible to achieve a better success rate [54].

2.6.2. Secondary lack of an acrosome

Secondary lack of an acrosome results from a premature acrosomal reaction, i.e., the acrosome is lost in acrosome-reacted spermatozoa (**Figure 3b**). Disruption of the plasma membrane is observed in this case, and the inner acrosomal membrane adjacent to the nuclear envelope is seen on the sperm surface in the acrosomal region. The outer acrosomal membrane and the plasma membrane form bubbles during the acrosomal reaction. In the case of a physiological acrosomal reaction, the postacrosomal segment and its plasma membrane are preserved in the live spermatozoon.

The percentage of spermatozoa with a secondary loss of the acrosome (i.e., acrosome-reacted spermatozoa) in ejaculate samples are $18.22 \pm 8.27\%$ in fertile men and $26.37 \pm 12.81\%$ in infertile patients with normal spermiogram parameters ($p < 0.05$) [52]. A higher percentage of acrosome-reacted spermatozoa (with acrosome degradation) in the ejaculate may impair its fertilization potential. Leukocytospermia with an enhanced production of reactive oxygen species by leukocytes is one of the possible causes of an early acrosomal reaction. Our findings indicate that bacterial microcolonies present in the ejaculate may also cause a premature acrosomal reaction, and their presence is not always accompanied by an inflammatory response. We analyzed the results of electron microscopic examinations of 746 semen samples from patients with fertility disorders. Bacterial microcolonies were detected in 186 of the 746 samples (25%), and a higher (more than 20%) content of spermatozoa with a secondary loss of the acrosome was observed in 112 of the 186 samples (60%). In the absence of bacterial infection, a higher content of acrosome-reacted spermatozoa was found in 117 of the 560 samples (20%) [55].

A higher leukocyte count in the ejaculate was detected in 36 of the 186 samples with bacterial microcolonies (19%).

Electron microscopy is a gold-standard test for acrosomal reaction, although a number of other tests are now available to assess the penetrating potential of spermatozoa.

2.6.3. Irregular acrosome

Irregular acrosome (**Figure 3c**) and lack of acrosomal contents (**Figure 3d**) (enzymatic insufficiency of the acrosome) are found in both pronounced teratozoospermia and normospermia. Proteolytic enzymes of the acrosome dissolve the zona pellucida to allow fusion of the spermatozoon and the oolemma. When the process is disturbed as a result of acrosome loss or dysfunction, spermatozoa lose their fertilizing potential. Irregularly T-shaped acrosomes can be detected in binuclear spermatozoa (**Figure 4a**).

2.6.4. Enlarged subacrosomal space and lack of a PT and postacrosomal segment

Spermatozoa that have an enlarged perinuclear space and lack the postacrosomal sheath of the PT account for 2–5% of the total sperm count in semen from fertile men (**Figure 4b–d**). The abnormality is often combined with the presence of excess residual cytoplasm on the
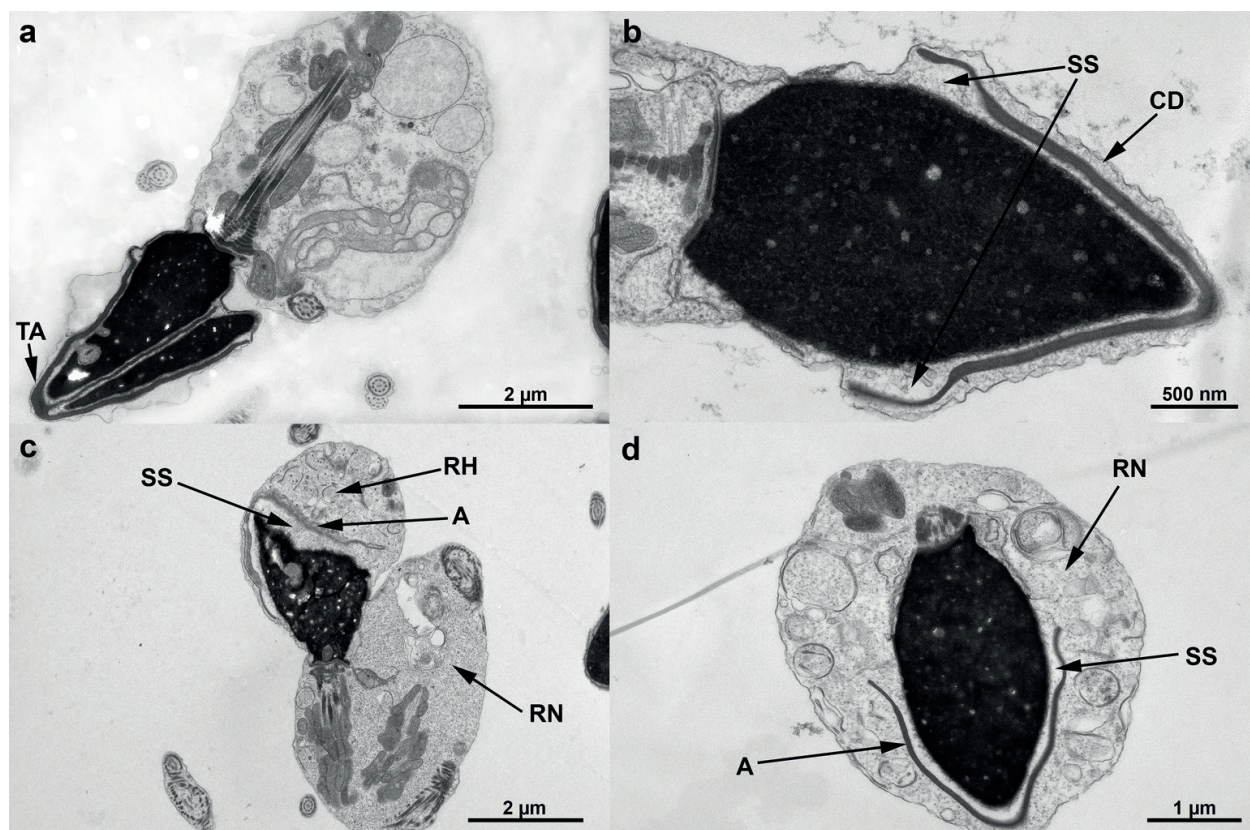
Figure 4. (a) Binuclear spermatozoa with T-shaped acrosome (TA). (b) Sperm with small cytoplasmic droplet on the head (CD) lacking the PT and its postacrosomal segment and with enlarged subacrosomal space (SS). A, irregular acrosome. (c, d) Spermatozoa with excess residual cytoplasm on the head (RH) and on the neck (RN), irregular acrosomes (A), and enlarged subacrosomal space (SS).

head (**Figure 4c**, **d**). The disorder is sometimes referred to as type II globozoospermia. Sperm heads appear to be spherical under a light microscope, but an ultrastructural study shows that spermatozoa have normal elongate nuclei, whereas their heads look round because of excess residual cytoplasm. This form of pathology also impairs fertility, but differs from globozoospermia [56] because lack of the PT and its postacrosomal segment suggests lack of the oocyte-activating factor.

In some cases, a small cytoplasmic droplet on the head is found in spermatozoa lacking the PT and its postacrosomal segment, so that the spermatozoa appear to be normal by light microscopy (**Figure 4b**). The pathology is detectable only by electron microscopy and may cause idiopathic infertility while the conventional spermiogram parameters are within the normal ranges. ICSI with the oocyte activation methods developed for patients with globozoospermia could solve the problem for these patients. A promising method was tested in a mouse model; i.e., recombinant PLCζ was injected to allow fertilization with spermatozoa of *PLCζ-/-I489F* mutant mice [57].

The acrosome is the most labile component of the spermatozoon. According to our data, the percentage of spermatozoa with abnormal acrosome shapes is 50.12 ± 8.70% in fertile men. Alterations of the acrosome shape or lack of the acrosomal contents are greater in men with

fertility disorders. Acrosomal hypoplasia is a common component of pronounced teratozoo-spermia, is well detectable by electron microscopy, and is essential to diagnose because acrosomal insufficiency is possible to correct using ICSI (for a review, see [58]).

3. Connecting piece (neck) of the spermatozoon

The connecting piece connects the head with the tail (**Figure 5a**). A thin basal plate occurs at the base of the head, it has a concave shape, forming an implantation fossa. The region beneath the basal plate harbors nine striated columns, which continue caudally as outer dense fibers. Striated columns are the part of the connecting structures of the neck. The basal plate is at the base of the head nucleus. A centriole is enclosed in an electron-dense capitulum. The centriole is a universal element of animal eukaryotic cells and plays a role in the formation of the mitotic spindle.

A typical centrosome (cell center) of immature germline cells consists of two cylindrical centrioles, each consists of nine symmetrically oriented microtubule triplets, of 0.5 μm in length and 0.2 μm in diameter. Two centrioles are positioned in an orthogonal orientation, the axis of the daughter centriole being perpendicular to that of the mother centriole. A typical centriole has a 9 + 0 organization of microtubule triplets. In a mature spermatozoon, the distal centriole gives
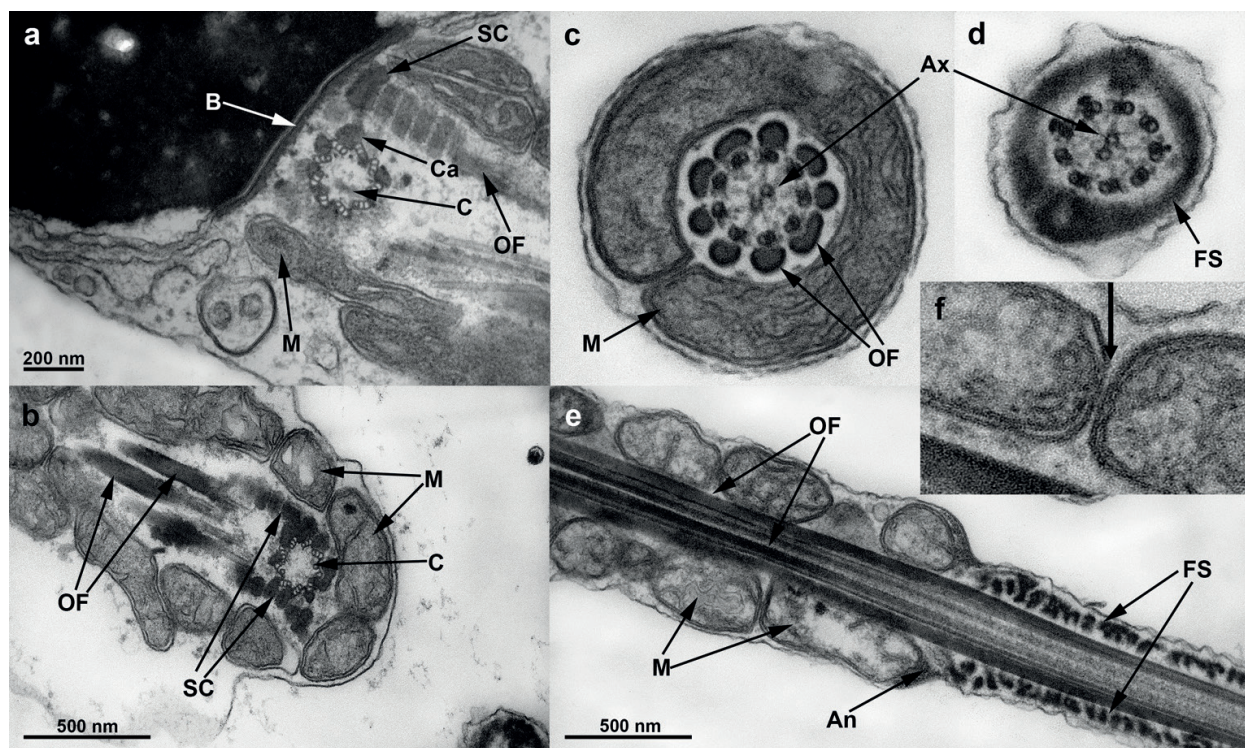

Figure 5. (a) The connecting piece of normal spermatozoon. (b) Decapitated spermatozoon. B, basal plate; C, centriole; Ca, capitulum; SC, striated column, OF, outer dense fibers; and M, mitochondria. (c) Transverse section through the midpiece of spermatozoon tail; (d) transverse section through the principal piece of spermatozoon; (e) longitudinal section through the middle and principal piece of the tail; (f) the site of contact between mitochondria (arrow). Ax, axoneme, dynein arms of peripheral microtubule doublets are visible. M, mitochondria; FS, fibrous sheath; and An, annulus.

origin to the tail axoneme and is reduced. The proximal centriole preserves its morphology, enters the oocyte upon fertilization, and plays a role in organizing the cleavage spindle [59].

The centriole is surrounded by striated columns, which are part of the connecting structures of the neck. The basal plate is at the base of the head nucleus. The centriole is capable of functioning as an organizing center during cell division only when having a normal morphology, as was demonstrated in many somatic cell studies [60].

The role of the sperm centriole has come into focus of research relatively recently, with the development of ART methods. A paternal inheritance of the centriole was then demonstrated for humans and large mammals as opposed to rodents [61]. The centriole organizes microtubule assembly to produce the sperm aster, which forms around the paternal pronucleus 6 h after fertilization [62] and gives origin to the first mitotic spindle. The main function of the centriole is to organize a network of microtubules, which originate from the oocyte.

3.1. Centrosome abnormalities

Centrosome abnormalities were described as a cause of unsuccessful fertilization and abnormal embryo development [61, 62]. Decaudated or decapitated sperm is a rare syndrome in humans and includes the absence of the implantation fossa and the basal plate. Morphological features of the human syndrome were described comprehensively, and ultrastructural defects of spermatozoa with an abnormal fragility of the head-tail junction were studied by electron microscopy (**Figure 5b**). The proximal centriole/centrosome, which induces the formation of the basal plate and the implantation fossa, was assumed to play an essential role in attaching the flagellum to the nucleus. Dysfunction of the proximal centriole/centrosome may alter the formation of tail attachment structures, leading to decapitated sperm. Spontaneous fertilization is impossible with such spermatozoa because the tail easily detaches from the head because of the neck fragility. ICSI is the only way of fertilization in this case, but rarely is successful. Chemes et al. [63] observed lack of cleavage after ICSI. Porcu et al. [64] reported successful ICSI in two infertile couples where the men were brothers and produced acephalic spermatozoa or spermatozoa with abnormal head-tail attachments, and one birth was published by Gambera et al. [65].

A genetic origin is now commonly accepted for the syndrome. Baccetti et al. [66] assumed that recessive autosomal mutations account for the majority of sperm genetic defects. However, the genes affected by the mutations are unknown. Light microscopic signs of the syndrome vary. Multiple motile tails with single, if any, tailless heads are observed in semen in the majority of cases. Kamal et al. [67] described 16 cases with a variant of the syndrome wherein the spermiogram parameters were normal, while minimal ICSI-related manipulations caused decapitation and immobilization of spermatozoa. The head and tail usually separate at the head-neck junction; the connecting piece is preserved; the basal plate and implantation fossa are absent from the caudal pole of the nucleus.

Several variants of decapitated sperm were described. Holstein et al. [68] reported a case where the basal plate and implantation fossa were normal in morphology, while separation occurred between the proximal and distal centrioles. Baccetti et al. [66] described a patient with sperm ruptures occurring between the nucleus and centriole region, between the anterior and caudal regions of the mid-piece, and between the mid-piece and principal piece. A number of variants are most likely possible for sperm decapitation.

Cases of familial incidence of teratozoospermia with acephalic sperm suggested a genetic nature for the disorder [69]. Homo- or heterozygous mutations of the spermatid-specific SUN5 gene were found in some patients with acephalic (decapitated) sperm syndrome [70]. The protein product of the gene occurs in the immediate vicinity of the head-tail junction, and proteins of its family are known as part of the contact system that connects the inner nuclear membrane with the cytoskeleton.

4. Tail

The intact tail of a human spermatozoon is approximately 50 μm in length and consists of four regions: a connecting piece, which is attached to the head; a mid-piece, which is 3–5 μm long; a principal piece, which accounts for approximately two-thirds of the tail length; and a short end piece. In contrast to cilia, which are covered by the plasma membrane, the sperm tail has not only the axoneme but also additional structures that surround the axoneme and are known as the periaxonemal structures. A mitochondrial helix and outer dense fibers surround the axoneme in the mid-piece and a fibrous sheath in the principal piece. The axoneme has no periaxonemal structures only in the short end piece.

4.1. Axoneme

The axoneme forms a core in cilia and flagella. The sperm axoneme consists of nine pairs of microtubules (doublets) that are arranged in a ring around two central singlet microtubules (9 + 2 arrangement). The doublets are numbered clockwise, starting from the site where two doublets overlay the central pair of microtubules; the right doublet is number one (**Figure 5c, d**). Each peripheral doublet consists of a complete microtubule (subunit A) and an adjacent incomplete microtubule (subunit B).

Two, outer and inner, arms consisting of the protein dynein (dynein arms) with ATPase activity extend from the A subunit of each doublet towards the B subunit of the next clockwise doublet. Each dynein arm is an intricate multiprotein complex and acts as a molecular motor [71]. The microtubule doublets are connected via thin bridges of the protein nexin (nexin bridges) and project radial spokes towards the two central microtubules. This sophisticated structure sustains sliding movements of the microtubules, thus providing for undulations of the tail. The axoneme is intricate molecular machinery wherein the inner and outer dynein arms generate forces to produce bending waves and the central apparatus and radial spokes play a regulatory role [72, 73].

4.2. Periaxonemal structures of the sperm tail

4.2.1. Mid-piece of the tail

The outer dense fibers are a morphological extension of the striated columns and capitulum, which are structural elements of the connecting piece of the sperm neck [74]. The outer dense fibers surround the axoneme in the mid-piece of the tail, one fiber overlaying one peripheral microtubule doublet. ODF1 is a major protein of the outer dense fibers (**Figure 5c, e**).

The number of mitochondria is reduced as a large portion of the cytoplasm is eliminated with residual bodies from spermatids in the course of spermiogenesis [75]. Up to 75 mitochondria are left in a mature spermatozoon with a minor cytoplasm amount and form a helix around the outer dense fibers and axoneme. The mitochondrial helix has 11–13 turns with two mito-chondria per turn. The mitochondrial helix length and the approximate number of turns are constant within a species [76].

The structure of the mitochondrial helix is stable owing to the so-called mitochondrial cap-sule, i.e., the outer mitochondrial membrane is coated with keratin-like molecules, which form disulfide bridges between cysteine- and proline-rich selenoprotein regions [77]. Contact zones form at the sites of contacts between mitochondria, indicating that the spermatozoon has a mitochondrial reticulum, similar to the mitochondrial network of the heart muscle rather than individual mitochondria (**Figure 5f**) [78, 79].

Active functional mitochondria were demonstrated to affect the sperm fertilizing potential in many studies. Ultrastructural defects in mitochondria are associated with lower sperm motil-ity. The available data on the role of mtDNA mutations are discrepant. Deletions from mtDNA were considered to be responsible for sperm dysfunction and infertility [80]. However, the difference was not confirmed for several mtRNAs by rtPCR.

Metabolism of sperm mitochondria is still a matter of discussion. It is commonly accepted that ATP produced by mitochondria provides a main source of energy for the dynein motor of the axoneme. In contrast, a compartmentalization hypothesis suggests that glycolysis is a main source of energy for tail movements [81]. Because discrepant experimental data were reported from different studies, the question is still open. It is possible that mitochondria are involved in basic redox processes, which determine the fertilizing potential and lifespan of the spermatozoon, rather than in energy metabolism as a main function.

The mid-piece and principal piece of the tail are separated by a ring structure known as the annulus (**Figure 5e**), which presumably performs a barrier function to prevent molecular dif-fusion between the two pieces [82].

4.2.2. Principal piece of the tail

The principal piece of the tail is distal of the mid-piece and is the longest tail segment. The mitochondrial sheath is not found in the principal piece, and a fibrous sheath as another cyto-skeletal element of the tail surrounds the axoneme. Two longitudinal columns of the fibrous sheath replace two opposite outer dense fibers and are connected together by numerous cir-cumferential ribs (**Figure 5d, e**).

A total of 18 polypeptides were identified in the fibrous sheath. The polypeptides form a scaffold for glycolytic enzymes and act as signaling molecules upon induction of sperm motil-ity (for a review, see [83]). A-kinase anchoring proteins 3 and 4 (AKAP3 and AKAP4) are major components of the fibrous sheath and probably form its integral cytoskeletal structure. AKAP3 and AKAP4 are associated with each other and bind to cAMP-dependent protein kinase A through its regulatory subunit. The AKAP3 and AKAP4 genes were sequenced, and the binding sites identified.

The principal piece of the tail harbors glycolytic enzymes, including sperm-specific hexokinase 1, lactate dehydrogenase, and sperm-specific glyceraldehyde 3-phosphate dehydrogenase (GAPDHs) [84].

4.3. Structural abnormalities of the tail

The complex system of tail elements with their concerted function provides the spermatozoon with the ability to move, that is, to reach and fertilize the oocyte. Any structural alteration of the system impairs sperm motility.

A functional variant of asthenozoospermia is the most common. Spermatozoa of patients display multiple heterogeneous ultrastructural changes in the axoneme and periaxonemal structures (**Figure 6a–e**), such as changes in the number and arrangement of the microtubule doublets, the shape of the outer dense fibers, or the architecture of the fibrous sheath. Quantitative changes in mitochondria and their altered localization were also associated with asthenozoospermia. The percentage of spermatozoa with ultrastructural tail defects is significantly higher in patients with asthenozoospermia. Ultrastructural defects of the tail axoneme were described in drug addicts [85]. Yet smoking and alcohol drinking were not found to affect the ultrastructural parameters of mature spermatozoa, lower sperm counts observed in alcoholics and smokers suggest testicular selection [86]. Functional asthenozoospermia can
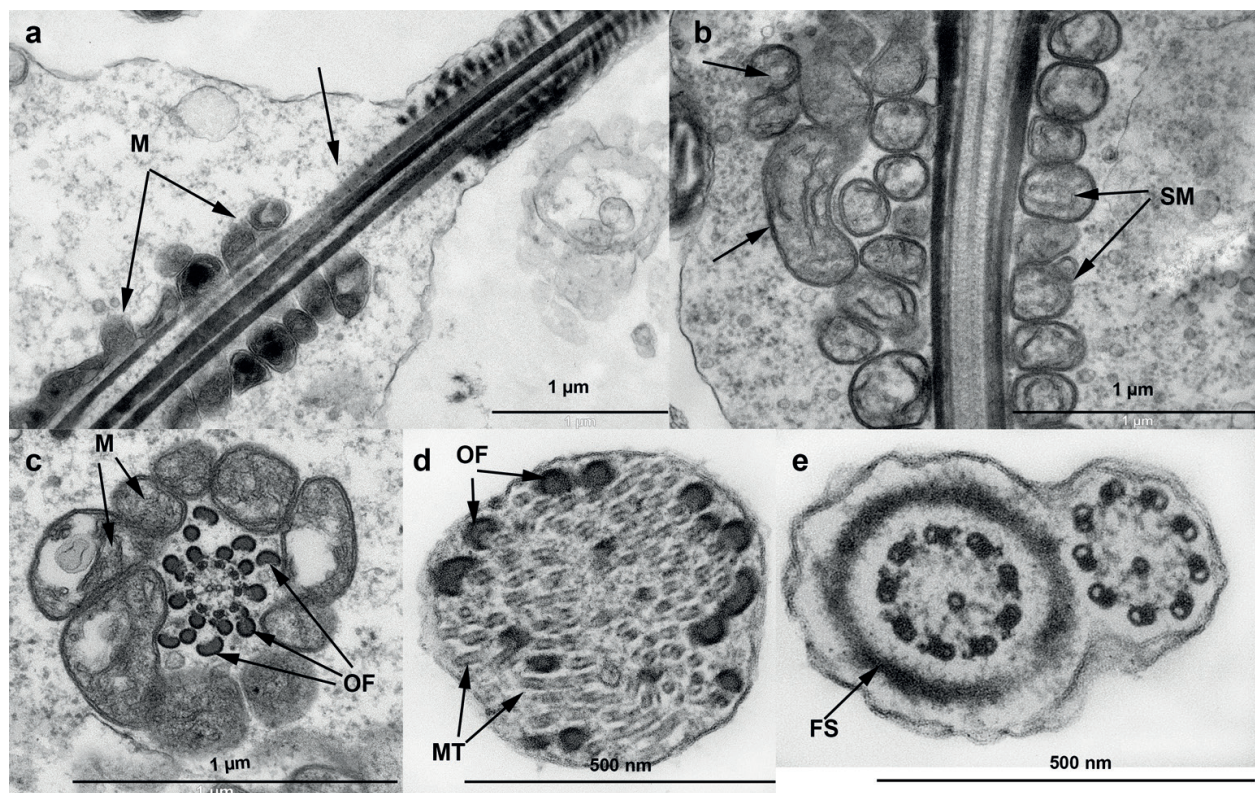

Figure 6. Longitudinal (a, b) and transverse (c–e) sections through abnormal sperm tails. (a) Lack of annulus (arrow) between the middle and principal piece of the tail; (b)swollen mitochondria (SM) and dislocation of mitochondria (arrow); (c) normal axonema structure and increased quantity of outer dense fibers (OF); (d) disorganization of axonemal microtubules (MT) and outer dense fibers (OF); (e) double tail with (9 + 1) microtubules. The absence of dynein arms in the right axoneme is revealed. FS, fibrous sheath.

be secondary to a varicocele, infections of reproductive organs, and exogenous exposures [58]. Spermiogram parameters are possible to correct with medications in men diagnosed with functional asthenozoospermia. When the treatment is ineffective, ICSI is likely to help.

4.4. Genetically determined forms of asthenozoospermia

4.4.1. Dysplasia of the fibrous sheath of the tail

Chemes et al. [87] proposed the term dysplasia of the fibrous sheath (DFS) for the disorder, which is also known as stump tail syndrome and short-tail spermatozoa. Spermatozoa have substantially reduced, if any, motility due to fibrous for DSF [88]. In spermatozoa, the location of longitudinal columns and transverse ribs of the fibrous layer has been disturbed. There are changes in the structure of the mitochondrial helix—a significant shortening and disruption of localization. Anomalies in the structure of the fibrous sheath often put together with the absence of a central pair of the axoneme microtubules (**Figure 7a–c**).

A mouse model of DFS was obtained by targeted disruption of the *Akap4* gene, and spermatozoa of mutant mice had short thick tails, which were morphologically identical to those in DFS patients [89]. However, consistent human data were reported from only one study. Baccetti et al. [90] used PCR and observed intragenic deletions of *AKAP4* and *AKAP3*, which code for major structural components of the fibrous sheath, in one DFS patient. No abnormality was detected in other samples.

We found a decrease in the activity of the glycolytic sperm-specific enzyme glyceraldehyde-3-phosphate dehydrogenase (sGAPD) and atypical localization of the enzyme. Mutations
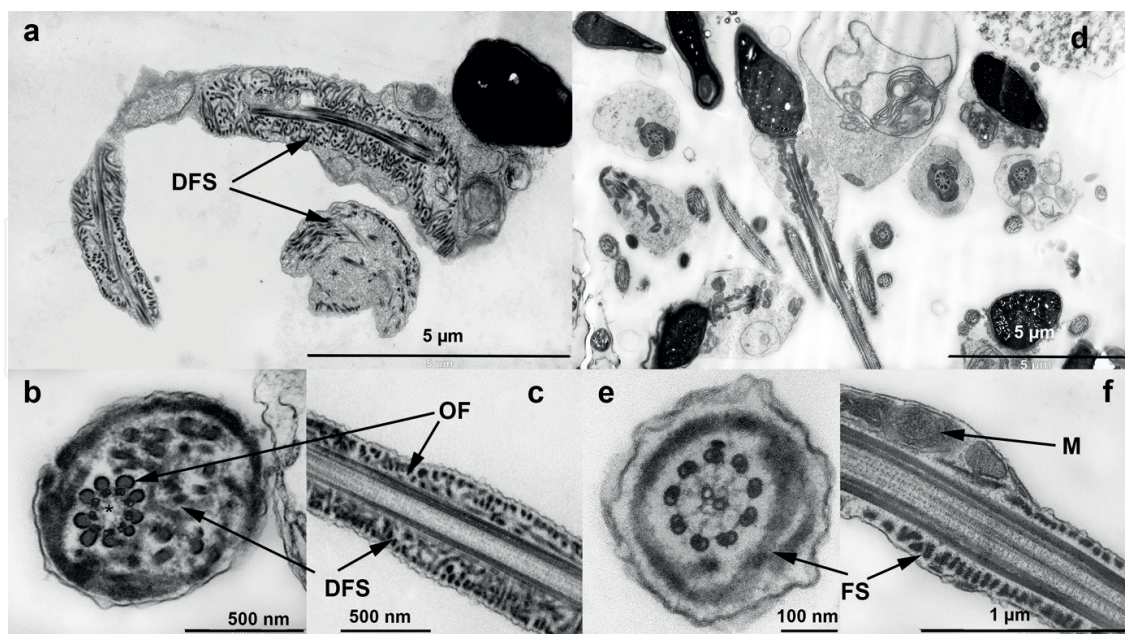

Figure 7. (a) Sperm with dysplasia of the fibrous sheath (DFS) of the tail. The lack of mitochondria is revealed (arrow). Transverse (b) and longitudinal (c) sections through the tail with DFS. The lack of the central pair of microtubules (asterisk). (d) Sperm with primary ciliary dyskinesia (PCD). Transverse (e) and longitudinal (f) sections through the tail with PCD. The absence of dynein arms is revealed on the transverse section. OF, outer dense fibers; FS, fibrous sheath; and M, mitochondria.

within human *GAPDS* gene were assayed. In all five studied semen DFS samples, a replacement of guanine by adenine was revealed in the intron region between the sixth and the seventh exons of *sGAPD* [91]. Pereira et al. [92] found heterozygous deletion in the *DNAH5* gene, but not mutations in *AKAP3* and *AKAP4* in four patients with DFS.

DSF has an autosomal recessive inheritance. The genetic risk is now impossible to estimate. A few cases of live births after ICSI with spermatozoa of DSF patients were reported in the medical literature [93].

4.4.2. Primary ciliary dyskinesia (PCD)

PCD is an autosomal recessive disorder and is highly heterogeneous genetically. PCD affects the axonemal structures (microtubules and dynein arms) of cilia and flagella (**Figure 7d–f**). Bronchial and pulmonary diseases are the main pathology in PCD because infections and bronchiectasis develop when respiratory cilia have motility defects or are immotile.

Headaches are common in PCD patients because lack of ciliary motility in the brain ventricles impairs circulation of the cerebrospinal fluid. Situs inversus is additionally observed in half of the PCD patients, possibly resulting from lack of ciliary motility in embryonic Hensen's node, which is responsible for the unidirectional fluid flow and thereby establishes left-right asymmetry [94]. The prevalence of PCD at birth is 1/10,000 to 1/20,000 [95].

Fertility is impaired in male patients because their spermatozoa are absolutely immotile or defects occur in efferent seminiferous ducts lined by ciliated epithelia. In a semen analysis, gross ejaculate parameters (volume, pH, viscosity, and color) and the concentration and count of morphologically normal spermatozoa are within the normal ranges.

Transmission electron microscopy (TEM) is commonly used to detect PCD. TEM reports lack of outer and/or inner dynein arms, the two central microtubules, or radial spokes and changes in microtubule arrangement.

Molecular methods to diagnose PCD have intensely been developed in the past years. Unicellular algae of the genus Chlamydomonas, which have two flagella, provide a convenient model to study the molecular composition of the axoneme. Axoneme protein genes identified in Chlamydomonas are candidate genes for PCD. A total of 16 mutations of PCD candidate genes were identified from 1999 to 2011 by genetic methods (analysis of linkage groups identified by homozygosity mapping), proteome analysis, and sequencing (mostly Sanger sequencing). Since 2011, mutations of 18 other genes have been described via whole-exome and whole-genome sequencing (for the review see [73, 96]).

PCD is genetically heterogeneous. Mutations of two genes, *DNAI1* and *DNAH5*, are the most common in PCD. *DNAI1* and *DNAH5* code for proteins of outer dynein arms of the axoneme. *DNAI1* mutations were found in 14% of PCD patients [97]. The *DNAH5* product is a major motor protein of outer dynein arms. Its mutations were observed in more than 25% of PCD patients [98].

Many proteins are involved in building the axoneme. Several proteins are common for epithelial cilia and sperm tails. Patients homozygous for mutations of their genes develop the total set of PCD signs, including bronchial and pulmonary diseases, changes in asymmetry of visceral organs, and immotile sperm. Other axonemal proteins are tissue specific, and mutations

of their genes cause mosaic ciliopathy, such as asthenozoospermia and anosmia, or astheno-zoospermia and swelling of the nasopharyngeal mucosa, which we identified in our patients.

The development of ICSI allowed men with pronounced asthenozoospermia, including forms with genetic causes, to have children. The consequences of using ICSI in PCD and DSF are poorly understood because the disorders are rare and only few live births after ICSI have been reported (20 cases according to PubMed). PCD patients with andrological symptoms naturally had no offspring before the advent of ICSI. PCD is an autosomal recessive disorder and is expressed only in homozygotes and compound heterozygotes, when both alleles of one gene are affected. This circumstance reduces PCD risk in ICSI offspring, but makes it more likely for the mutations to accumulate in the population and to occur in homozygote at a higher rate in the long term.

5. Virus infection of spermatozoa

Virus capsids morphologically identical to capsids of *Herpesviridae* family were detected in the nucleus and cytoplasmic droplet of infected spermatozoa by TEM (**Figure 8a**, **b**) (for the review see [99]). The structures shown in **Figure 8** (**8a**, **b**) are capsids of herpes simplex virus
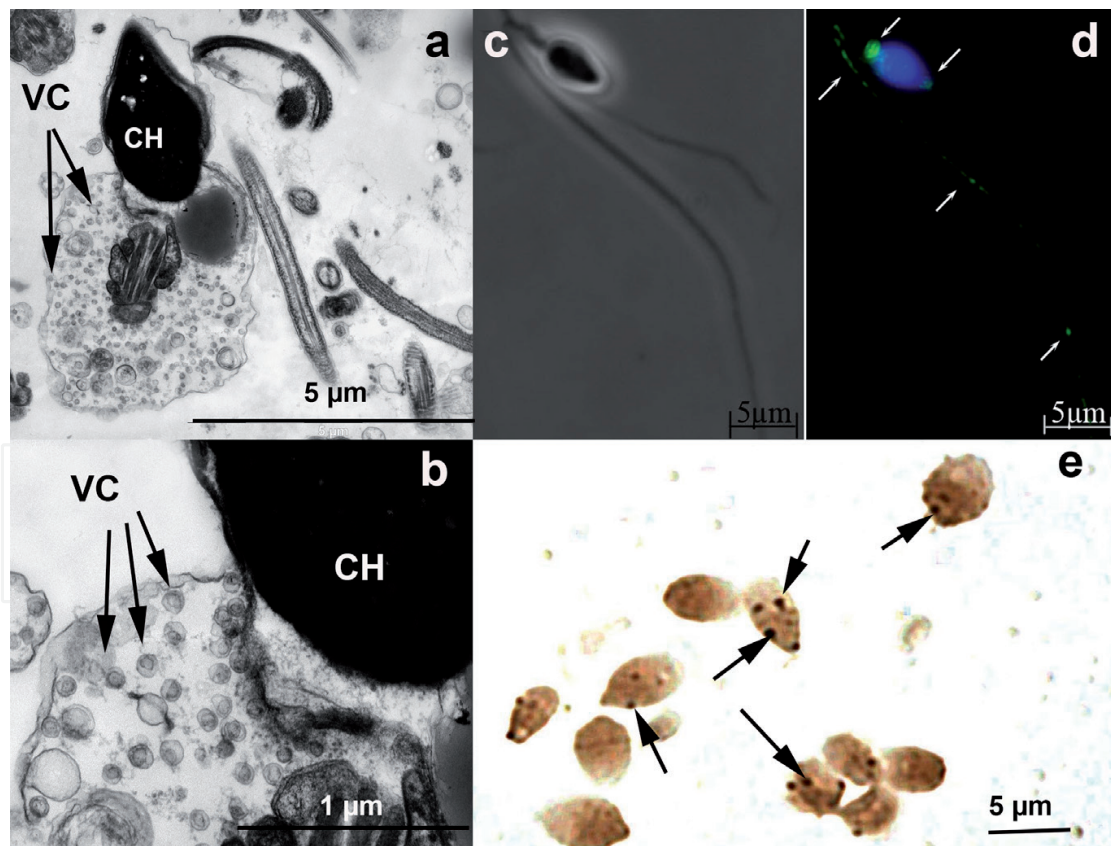

Figure 8. (a) Longitudinal section of normal sperm; (b) section through the middle piece of the tail of sperm. VC, HSV capsids; M, mitochondria; A, acrosome; CH, chromatin; and C, centriole. The hexagonal structure of some capsids is visible. Phase-contrast microscopy (c) and IF (d) of infected sperm. HSV antigen (arrows) and DAPI stain for DNA (blue); (e) FISH with probes to DNA of HSV. Localization of HSV DNA in sperm heads (arrows).

(HSV), we proved this by immunofluorescence (IF) using the monoclonal antibodies to HSV1 and HSV2 (**Figure 8c**, **d**) and *in situ* hybridization (ISH) with biotinylated probes for HSV (**Figure 8e**). Infection was observed in both the total sperm fraction and the isolated fraction of motile spermatozoa. The persistence of the HSV genome in motile, morphologically normal sperms indicates that the virus may be transmitted vertically to the offspring via natural fertilization or various ARTs, including IVF or ICSI.

Herpetic infection of spermatozoa was significantly more common in infertile men and men whose spouses had a history of spontaneous miscarriage or ART failure as compared with fertile men. Specific antiherpetic treatment of men diagnosed with HSV infection of spermatozoa results in a substantial, almost fivefold increase in the rates of blastocyst formation after ICSI and clinical pregnancy after ART [100].

6. Bacterial infection of the ejaculate

Bacterial colonies were detected in ejaculate samples from patients with fertility disorders. In the colonies, heteromorphic microorganisms were held together in a diffusive substance, probably of a polysaccharide nature, or covered with membranes as bacterial biofilms. The
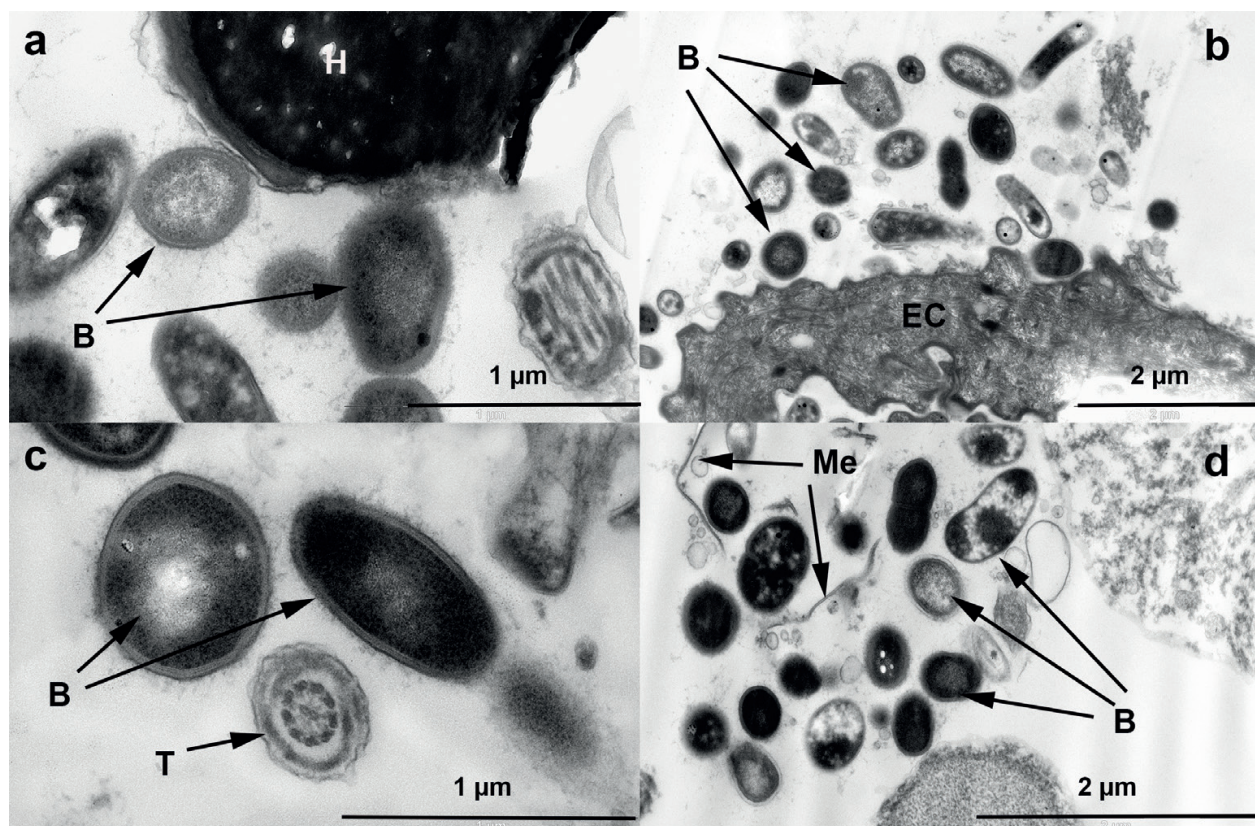

Figure 9. (a) Bacterial microcolony (B) attached to sperm head (H). (c) Bacterial microcolony (B) attached to sperm tail (T). (b, d) Bacterial microcolonies attached to the epithelial cells (EC). A diffuse substance (a–c) or membranes (Me) (d) are detected between bacterial cells.

majority of microcolonies were attached to squamous epithelial cells, whereas some were associated with sperm heads or tails (**Figure 9a–d**).

Moretti et al. [101] examined ejaculate samples from infertile patients and detected *Enteroccocus faecalis* and *Escherichia coli* most frequently and, at lower frequencies, *Streptococcus agalactiae*, *Ureaplasma urealyticum*, *Staphylococcus epidermidis*, *Streptococcus anginosus*, and *Morganella morganii*. With the exception of *U. urealyticum*, the microorganisms are classed with the non-specific saprophytic microflora and are not addressed in a conventional testing of infertility patients.

Bacteria may damage spermatozoa even in the absence of an overt inflammatory reaction. We observed higher contents of acrosome-reacted spermatozoa (i.e., those with a premature acrosomal reaction) in the ejaculate samples that contained bacterial microcolonies [55].

7. Conclusion

Despite the success of molecular biology, the morphological methods of research continue to play a considerable role in determining the reasons of male subfertility and infertility.

Indications for the studies using TEM are as follows:

1. Idiopathic infertility with normozoospermia or with small deviations in the semen parameters (revealing anomalies of spermatozoa that are not visible in the traditional spermiological study).

2. Examination of patients whose wives had a history of miscarriage due to abnormal embryo development, such as non-developing pregnancy or spontaneous abortion in the first trimester of natural pregnancy or ART failure.

3. Differential diagnosis of genetically determined and functional forms of asthenozoospermia and teratozoospermia.

Testing for ultrastructural sperm abnormalities makes it possible not only to identify the cause of infertility but also to choose proper clinical tactics, that is, to select treatments, to recommend ART using own spermatozoa, or to offer ART using donor sperm.

Acknowledgements

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