Electron microscopy in the study of human sperm pathologies

E. Moretti1,2 and G. Collodel 1,2

1 Department of Biomedical Sciences, Applied Biology Section
2 Interdepartmental Centre for Research and Therapy of Male Infertility, University of Siena, viale Bracci, Ospedale Santa Maria alle Scotte, Siena

The aim of this study is to underline the role of transmission and scanning electron microscopy analysis in the diagnosis of human sperm pathologies. Among the possible causes of male infertility, defects of sperm morphology represent an important factor that may explain decreased fertilizing potential of sperm. Electron microscopy allowed us to identify systematic sperm defects that affect the vast majority of sperm in a semen sample and non-systematic sperm defects, a heterogeneous combination of randomly distributed alterations affecting the head and the tail organelles in a varied percentage of ejaculated sperm. Correct diagnosis of specific altered sperm phenotypes is important for the advancement of new therapies for treating male factor infertility and for the choice and outcome of assisted reproduction techniques.

Keywords electron microscopy; human sperm morphology, sperm pathology

1. Electron microscopy and human sperm morphology

Infertility can now be considered a topic of general health; male infertility is a significant problem in humans and it may be caused by different pathologies, such as anatomical problems, infections, hormonal imbalances, chromosomal alterations or gene anomalies, although the cause of infertility remains unknown for 30% of infertile men [1]. Sperm analysis is important to determine the fertilizing potential of sperm [2-4]. In current laboratory practice, semen evaluation is generally performed by light microscopy (LM), and sperm morphology is assessed in smeared and stained glass slides for evaluating morphometric parameters of head, midpiece and tail, according to World Health Organization (WHO) guidelines [5, 6]. The staining procedures recommended by the WHO are Papanicolaou, Shorr and Diff-Quick stain. However, although sperm analysis by LM allows for the examination of viable samples, it has obvious technical limitations in terms of resolution power and it cannot identify the entire variety of morphological defects that may occur in sperm organelles. Therefore, altered sperm heads and tails, highlighted at the level of LM, are the expression of anomalies of chromatin, acrosome, perinuclear theca, mitochondria, axonemal and periaxonemal structures that cannot be evaluated by this method.

The limits of LM can be overcome by the use of electron microscopy: transmission electron microscopy (TEM) permits the exploration of the “ultrastructure world” and the study of the different organelles rigorously characterising sperm abnormalities, and scanning electron microscopy (SEM) gives a three-dimensional image of the cell. Both techniques provide a more detailed evaluation of sperm characteristics. TEM and SEM are the best methods for studying teratozoospermia, a heterogeneous combination of defects in the shape of different sperm components that influence fertilizing potential. These approaches go beyond a descriptive morphology of the "look" of sperm and they play an essential role in the definition and the study of sperm pathology, the discipline that characterizes the structural and functional deficiencies of altered sperm [2, 7].

In sperm pathology, two main forms of ultrastructural sperm anomalies can be distinguished: non-systematic or non-specific sperm defects, and systematic sperm defects [2, 7]. The first and most frequent type includes a heterogeneous combination of alterations randomly affecting the head and the tail organelles in a varied percentage of sperm ejaculated. These alterations are not inherited in families, but might be related to andrological pathologies (i.e. infections, varicocele) or to other endogenous or exogenous factors [2, 8-10] and they may respond to different treatments. These alterations, that potentially affect every sperm organelle, are present in fertile individuals and, in higher percentages, in infertile individuals. They do not show a specific pattern and fluctuate in their occurrence among different patients and during the clinical evolution of the same individual. Among non-systematic sperm defects, necrozoospermia, a pathology involving the vast majority of a sperm population, can be identified. Sperm necrosis is thought to occur during epididymal transit or storage in which a hostile environment could be responsible for increased mortality and poor motility of ejaculated sperm [11]. Although a diagnosis of necrozoospermia can be performed by means of vital dyes, such as eosin, TEM analysis is the pivotal method for highlighting the status of sperm organelles in this pathology: the plasma membrane is broken, acrosome is reacted or absent, chromatin is disrupted, mitochondria is swollen and not assembled as a periaxonemal helix, and the axoneme, outer dense fibers (ODF) and the fibrous sheath (FS) are dislocated or disrupted.

TEM has proved to be the only tool able to specifically highlight the morphological features of the systematic sperm defects that are characterized by a predominant phenotype in all patients suffering from the same condition [2, 7]; these defects show family clustering and are significantly more frequent in individuals with a history of consanguinity. These alterations involve the sperm head, the head-neck attachment and the flagellum.
Concerning the head, the literature has reported: "globozoospermia" or round head acrosome-less spermatozoa [12, 13], "miniacroosome" [14], and a rare condition called "crater defect" [15]. Other systematic sperm defects of supposed genetic origin affect the head-neck attachment and the tail: "defects of head neck attachment" and "acephalic sperm" [2, 16-18], "Dysplasia of Fibrous Sheath" (DFS) [19-21], and "Primary Ciliary Dyskinesia" (PCD) [22-25]. In addition, electron microscopy played a central role in the characterization of other sperm defects, encompassing abnormally elongated midpiece concomitant with absence of the FS [26, 27] or with the absence of the axoneme and the ODF at the principal piece level [28] and in describing a new defect characterized by abnormal elongation of sperm tails which tend to break up or coil [29].

Before the advent of the IntraCytoplasmic Sperm Injection (ICSI) era, patients affected by these particular disorders were considered sterile; the introduction of ICSI had a huge impact on the field of assisted reproduction technologies (ART), removing all the barriers of natural selection between the oocyte and the sperm. In this way, the possibility that infertile people may reproduce has been one of the most important achievements in recent decades, although it has given rise to many questions and concerns from the ethical and evolutionary point of view in the possible enrichment of pathological genomes in future generations. In this scenario, the role of electron microscopy in the identification of specific altered sperm phenotypes is important for the advancement of new therapies targeted at treating severe male factor infertility and for the choice and outcome of assisted reproduction techniques.

In this chapter, an excursus on the systematic and non-systematic defects affecting the sperm organelles, such as head, neck, midpiece and tail, characterized by TEM and SEM, will be presented.

### 2. Sperm head anomalies

Non systematic sperm head anomalies, the most frequent finding in teratozoospermic patients, seriously affects fertility prognosis. LM examination essentially permits the identification of the external shape of sperm, and the variation in head form and size are generally the basis for classification of morphology [2, 5, 30]; however, the shape/size head defects usually labelled as "amorphous", "tapered" or "pyriform" do not show a direct association between the defined sperm anomalies and the characteristic sperm shape [31]. These limitations can be overcome by TEM analysis and a diagnosis, needed to improve the reproductive outcome, can be performed.

Ultrastructural analysis lends support in evaluating nuclear sperm shape and it is essential in the identification of multinucleated and binucleated sperm heads (Fig. 1a), characterised by the presence of the membranous septum between the nuclei, observed in patients with high prolactin levels [32] and in the presence of chromosomal translocations [33, 34].

![TEM micrograph of longitudinal sections of binucleated and multinucleated (arrow) sperm. The acrosome (A) is evident between the nuclei, a large vacuole (V) is present in the binucleated head; mitochondria (M), axoneme (AX).](image1a)

![TEM micrograph of longitudinal and cross sections of sperm showing different alterations: presence of cytoplasmic residues (CR) with coiled tails (AX), altered nuclei with uncondensed chromatin (uCh).](image1b)

![TEM micrograph of a necrotic sperm. The plasma membrane is broken (arrow), the chromatin is disrupted (dCh), the acrosome is absent, and the mitochondria (M) are swollen and disassembled. Bar 1 μm.](image1c)

The pathology of the sperm head essentially regards two components: the chromatin and the acrosome, structures easily identified by TEM. Alterations in maturation and compaction and the presence of "holes" in the chromatin are the most frequent nuclear defects. Chromatin holes were first identified as vacuoles [35, 36], and analysis of the nucleus at high magnification enables their characterization [37, 38]. However, TEM examination highlights the absence of plasma membrane surrounding these holes, thus they were more appropriately called "lacunar defects" or lacunae [31].
The origin and the consequences of sperm head lacunae are the subject of controversy. An acrosomal derivation of vacuoles localized at the front of sperm head has been recently proposed [39]. In addition, a particularly intriguing hypothesis from Chemes and Alvarez Sedò [31] has suggested that the lacunae may be the result of a deregulated ubiquitin proteasome system working during histone-protamine transition, a physiological process needed for chromatin condensation during spermiogenesis. Sperm with large lacunae were demonstrated to carry aneuploidies, chromatin condensation defects and acrosomal defects [40], and they are associated with a diminished fertilization rate, a negative influence on late embryo development or increased pregnancy loss [41-43]. Whether the presence of lacunae is associated with DNA fragmentation is still being debated [37, 40, 44, 45].

TEM analysis allows for the characterization of uncondensed chromatin with a granular texture (Fig. 1b) often associated with a roundish or elliptical nuclear shape, visible in a variable percentage in the ejaculates of fertile men, but particularly frequent in the presence of varicocele [46] and cryptorchidism [47], and in cases of chromosomal translocations [48, 49]. The chromatin of necrotic sperm (Fig. 1c) is also well recognizable as it shows a typical disrupted texture, often concomitant with the dissolution of plasma and acrosomal membranes, swelling of the mitochondria at midpiece level, and the disintegration of flagellar microtubules and periaxonemal structures [2, 50]. Even in this case, the percentage of necrotic sperm is low in normal ejaculates, but it increases considerably in the presence of bacterial infection detected in semen [51] or in epididymal dysfunction; this last condition may cause necrozoospermia [11, 52].

Nuclear alterations are often associated with acrosomal anomalies; in particular, the absence and the insufficient development of acrosomes are head defects that affect fertility. The most fitting example is the systematic sperm defect globozoospermia (Fig. 2 a, b) [revised by Chemes and Alvarez Sedò [31, 53], one of the first sperm pathologies that was identified, characterized by round-headed sperm without an acrosome which generally exhibit abnormalities in chromatin condensation, increased rates of DNA fragmentation and aneuploidies [54-56]. The only way a carrier of globozoospermia can reproduce is ICSI, but in many cases the oocytes remain unfertilized after injection. Some successful pregnancies have been reported after ICSI in cases of globozoospermia, in many cases after oocyte activation with calcium-ionophore [57-60]; the most recent successful childbirth was obtained by intracytoplasmic morphologically selected sperm injection (IMSI) that enables a more accurate analysis of the morphology of sperm to be injected [61].

![Fig. 2. a,b) TEM (a) and SEM (b) micrographs of a round headed sperm. In figure a, the absence of an acrosome and the presence of uncondensed chromatin (uCh) are evident. Bar 1μm.](image)

An interesting recent report demonstrated that the alterations observed during acrosome biogenesis in globozoospermia are derived from an anomalous development of Golgi-derived proacrosomic vesicles that fail to attach and/or extend around the spermatid nucleus [62]. The lack of attachment is probably due to defects of perinuclear techa, a cytosolic protein complex that drives acrosomal attachment and development and is involved in sperm-egg interaction [63]. These anomalies result in the absence of an acrosome in mature sperm and elimination of this organelle within the residual body before the sperm is released from the testis epithelium [31]. Recently, Dam et al. [64] defined partial globozoospermia as a distinctive seminal condition characterized by an increased percentage of round-headed acrosomeless sperm cells (>50%). TEM analysis is important for recognizing acrosome hypoplasia, an incomplete development of acrosome that appears small in size, generally combined with an elliptically shaped nucleus showing uncondensed chromatin and devoid of a postacrosomal sheath [65]. This anomaly has been reported in brothers and classified as systematic since the total sperm population showed the defect [7, 14], but it can be present in different percentages in ejaculates as an acquired and reversible condition [41]. Finally, rare acrosomal alterations have been described by TEM evaluation: the crater defect [15] that consists of a nuclear and acrosomal invagination present in 100% of the cells, and the presence of acrosomal inclusions [66]. Chemes and Alvarez Sedo [31] stressed the importance of a correct diagnosis of acrosomal alterations because the consequences of insufficient acrosomal function are sometimes treatable with methods that trigger Ca++ oscillations during ICSI, enabling the activation of oocyte.
3. Sperm neck anomalies

The neck is a joint between the sperm head and tail and it is composed of a centriole and a connecting piece. After fertilization, the proximal centriole duplicates and becomes the centrosome that assembles the first mitotic spindle of zygote. The conversion of the sperm head associated with the basal body into the male pronucleus associated centrosome is important for resetting egg polarity [67]. Abnormalities of head-neck attachment include varying degrees of alterations [2]. Decapitated or acetalic sperm end cranially with the midpiece, sometimes embedded in globular cytoplasmic droplets (Fig. 3) which, under the LM, may be confused with the sperm head.

![Fig. 3. SEM micrograph of acetalic sperm. They end cranially (arrows) with the midpiece. Bar 1μm.](image)

These spermatozoa can be found in the ejaculates of fertile individuals, but in a very small percentage, which can be up to 10-20% in subfertile men and can reach 90-100% of the sperm population in teratozoospermic men with a systematic defect [2]. The heads without a tail are rarely found in semen. Together with an acetalic form, it is possible to observe sperm with abnormal and fragile head-midpiece alignment; these forms are often called bent. Acaetalic spermatozoa originate in the testis when the centriole-tail attachment to the spermatid nucleus fails. In a few cases, head may be attached to the tip or sides of the midpiece and out of alignment with the sperm axis; this misalignment ranges from a complete lack of connection to lateral positioning of the nucleus at an angle of 90-180°. The lateral positioning of the nucleus could result from a dysfunction of the sperm proximal centriole, which does not migrate regularly to the caudal pole of the spermatid nucleus and fails to nucleate a functional sperm aster in the developing zygote, impairing normal syngamy and cleavage. The ICSI outcomes in patients with abnormal head-tail attachment have resulted in oocytes fertilization, but in embryo degenerations due to lack of syngamy of the two pronuclei [2, 68-70]. However, if the injected sperm head contains a normal centriolar region, pregnancies have been obtained [71-73].

Chemes and Alvarez Sedò [31] hypothesized an insufficient function of proteasomes, normally located near the centrosome in the neck area, probably involved in the post-fertilization release of the proximal centriole by proteolysis of the connecting piece. To support this hypothesis, a reduced activity of two proteasomal enzymes has been observed in sperm with abnormal head-tail attachments [74, 75]. TEM analysis is very important for understanding the real entity of this defect before undertaking ICSI procedure, due to the physiological role of paternal inheritance of the centriole for human fertilization and in early development [76, 77].

4. Sperm tail anomalies

4.1 The midpiece and mitochondria alterations

The midpiece contains mitochondria arranged in a spiral helix that completes 12 -13 turns around the axoneme and the ODF. The midpiece ends with an electron-dense ring-shaped structure containing septins, small GTPase proteins associated with membranes and cytoskeleton (reported in [78]). The lack of annulus and the disorganization of the midpiece-principal piece junction have been reported to be associated with asthenozoospermia [78] and this was the demonstration that, in humans, the integrity of the annulus is required for correct midpiece elongation and sperm motility. Even mitochondrial anomalies of the sperm midpiece are expected to play a role in motility disorders, but they have received relatively poor attention. Recently, a study from Pelliccione et al. [79] demonstrated that structural defects in mitochondrial membranes represent a main feature of severe, unexplained asthenozoospermia, although the pathogenetic mechanism has yet to be explored. Other conditions include the absence of mitochondria in two variants: in the first case the lack of mitochondria is part of the phenotype of a DFS sperm defect [2], in the second variety the mitochondria are absent [80] and the midpiece appears thin and frequently bent [66]. In addition, several other mitochondrial anomalies have been reported [81-83] including the abnormal elongation of midpiece concomitant with the absence of FS and the presence of supplementary axonemes [27], or with the absence of the axoneme and the ODF at the principal piece level [28, 84].
In conclusion, abnormalities of mitochondrial organization may play a role in impairing sperm motility. In particular, a lack of mitochondria in the midpiece and at the opposite side of the abnormal elongation of the mitochondrial sheath are both associated with asthenozoospermia [83].

4.2 Flagellar anomalies

A perfect assembly of sperm flagellum is the prelude for the normal and efficacious motility needed to reach and fertilize the oocyte. Most patients with severe asthenozoospermia have an increased number of sperm with non specific flagellar anomalies [41] and these defects have also been described, obviously in lower percentages, in fertile men. For this reason, a characterization of these anomalies in each patient is pivotal for making a reliable diagnosis [2]. Non specific defects of the sperm flagellum randomly affect axonemal or periaxonemal structures in a varying percentage of ejaculated spermatozoa without a definite pattern. Their incidence changes during the clinical evolution of the patient and it differs among patients. However, these altered flagella appear generally well shaped at the light microscopy level, and the anomaly is not evident on semen smears because microtubular alterations do not modify the flagellar diameter. For this reason, an ultrastructural analysis performed by TEM is required to detect structural defects of the sperm tail leading to more or less severe asthenozoospermia.

Axonemal anomalies mainly consist of alterations concerning the number of microtubules or their position in a 9+2 organization [2, 85]. Isolated abnormalities of the periaxonemal elements regarding the number, structure and organization of ODF and the spatial organization of FS are related to motility disturbances through flagellar diskinesia [86]. A frequent finding, in examining the ejaculates from infertile patients, is the presence of a variable percentage of coiled tails often embedded in large cytoplasmic residues (Fig. 1b); in these cases the axonemal and periaxonemal structures are generally severely compromised. This defect is frequent in immature spermatozoa detected in the presence of varicocele [46] or cryptorchidism [47] and when chromosomal alterations are present [48]. Extreme asthenozoospermia and total sperm immotility are conditions often caused by the presence of flagellar systematic sperm defects, when an identical and specific alteration affects the vast majority of a sperm population in infertile patients.

PCD is a condition of sperm immotility and recurrent respiratory tract infections, alterations caused by a compromised function of sperm flagella and respiratory cilia [22]. Systematic investigation in this field began in 1970s under the leadership of B. Afzelius, a Scandinavian researcher, who demonstrated that male infertility associated with chronic respiratory disease was caused by genetic-related dynein deficiency in the axonemes of immotile spermatozoa and respiratory cilia. Men with this pathological feature were originally referred to as having immotile cilia syndrome, more recently renamed as PCD [87] since residual motility is occasionally present in some affected infertile men (for review see [7]). At the LM level, sperm tails appear morphologically normal but stiff, and obviously the axonemal pattern cannot be highlighted. In this case, TEM plays a pivotal role in resolving the discrepancy between normal tail morphology and sperm immotility: missing outer or inner or both dynein arms, the absence of one or two central microtubules or radial spokes, transposed microtubules, a lack of axoneme and other fundamental components [2, 24, 25]. This defect is probably due to a recessive autosomal mutation with wide locus heterogeneity (revised by Chemes and Alvarez Sedò [31]).

Severe defects in the assembly of FS are characteristics of DFS systematic sperm defect (Fig. 4 a, b).

![Fig. 4. a,b) TEM (a) and SEM (b) micrographs of a DFS sperm. Figure a shows disassembled (arrows) axonemal components embedded in FS material; mitochondria (M) are disorganized. In figure b, a sperm with a normal head (h) but a short, thick tail (arrow) is shown. Bar 1μm.](image)

The denomination known as DFS was introduced by Chemes et al. [19] and it described sperm showing short, thick and irregular tails containing a disorganized and hyperplasic FS. Ultrastructural studies have highlighted that, despite general maturity of the head region, the axonemal components are generally disassembled, sometimes devoid of dynein arms, and embedded in FS material, invading the whole space of the short tail. Mitochondria are not assembled as a periaxonemal helix, but are sparse or sometimes concentrated close to the nucleus and the ODF are also altered. The familial incidence of DFS suggests a genetic origin of the defect; it is probably a multigenic disease with recessive autosomic inheritance (revised by Chemes and Alvarez Sedò [31]). The presence of similar alterations described in sperm, observed in immature spermatids found in semen and in testicular biopsies of DFS patients, have highlighted a testicular origin of the defect (revised by Chemes and Rawe [7]). In two unrelated consanguineous patients, a
morphogenetic anomaly of FS affecting the normal development of the medium region of the ribs was described by Escalier and Albert [88] in spermatozoa with truncated tails. TEM is a particularly important diagnostic tool for determining the origin of short tailed sperm and to distinguish the defects of FS and other alterations due to necrozooospermia, in which the flagellar structures are destroyed, the tails can be broken and appear short at the LM level, or with sperm ageing in men with partial obstruction of the seminal pathway.

Among systematic tail sperm defects, PCD and DFS are the most common. The use of ICSI has modified the fertility prognosis of patients affected by these alterations: successful attempts have been made (revised by Chemes and Alvarez Sedó [31], [89, 90]) and the sperm have been able to fertilize the oocyte. Since PCD and DFS have a genetic basis, the question arises as to their possible transmission to the next generation. Unfortunately, genetic counselling for these kinds of defects will not be possible until the genes involved and the inheritance pattern are completely understood.

In addition, electron microscopy has played a central role in the characterization of other combinations of the same sperm defects that have been proposed to share a genetic basis. In two patients, a very rare alteration characterized by three major defects, such as absence of FS, the presence of supplementary axonemes and an abnormally elongated midpiece was characterized by TEM and immunocytochemical studies [26, 27]. In one of the cases, Baccetti et al. [27] demonstrated, in analysing the testicular tissue, a normal early spermatogenesis, and the presence of the defect at the spermatid stage. FS cytoskeletal components, produced during spermiogenesis, were observed in cytoplasmic residues of spermatids but were not organized around the principal piece axoneme. Remnants of FS were detected in residual bodies released by spermatids into the tubular lumen and in ejaculate.

Some years later, our group reported a sperm tail defect, encompassing abnormal elongation of the midpiece with supernumerary microtubules, organized in more or less complete axonemes, concomitant with the absence of the axoneme and the ODF at the principal piece level [28]. The axoneme seemed to reach the annulus and to continue up to the base of the head, where disorganized microtubules were found. Similar ultrastructural observations were described by Sauvalle et al. [84] in two infertile patients, and Rawe et al. [83] reported a case of a patient whose sperm showed a midpiece four times longer than normal, continuing a short and stiff principal piece, without disorganized supernumerary microtubules. The absence of axonemes had already been reported by Baccetti [91] but, in the described case the axoneme was absent for the entire tail length, whereas ODF were regularly present, sometimes showing an abnormal profile.

In addition, in a few patients, an unusual, simultaneous presence of systematic sperm defects has been observed: DFS and alterations in the head-neck junction [70, 92], ciliary diskinesia and DFS [24], “9+0” axoneme and thickness of FS and defective mitochondria (typical of the DFS defect) [93], and round acrosomeless heads and acalpehalic tails [94]. A normal live birth after testicular sperm extraction and ICSI was obtained with a variant of PCD sperm also showing typical characteristics of DFS, the lack of the central tubules and fragility in the head-tail junction [90]. In this context a new defect, characterized by abnormal elongation of the sperm tail, which was prone to ruptures, concomitant with tails coiled at different levels with a strongly rolled axoneme or with a curl in the final flagellar segment and bent tail, has been described by TEM and SEM [29].

Finally, another kind of sperm defect combination of supposed genetic origin was reported by Escalier [80] in a consanguineous patient. The sperm phenotype was characterized by failure of the annulus relocation, absence of the mitochondrial helix and an FS at the intermediate step of assembly. The distal pole of some mitochondria exhibited an unusually dense substance. The detection of these anomalies raised the question of the mechanisms that lead to the impairment of both the annulus relocation and the deposit of proteins on the FS during spermiogenesis.

5. Conclusions and perspectives

In clinical practice, the traditional manual-visual light microscopic methods for evaluating semen quality have a central role in the assessment of male fertility potential. However, in order to supply other indications to shed a light on a diagnosis of male infertility, additional tests are generally needed. Ultrastructural analysis is not widely applied in andrology laboratories because it requires highly trained examiners with considerable experience in this field and expensive equipment. However, it is noteworthy that the powerful resolution of TEM overcomes the limitations of LM and allows for excellent observation of the internal structure and spatial organization of sperm organelles. This methodology is essential in order to provide a more comprehensive approach to sperm pathology, avoiding an empirical analysis of sperm anomalies and allowing for correct diagnosis and a deeper understanding of the mechanisms of abnormal reproduction in infertile males. The correct diagnosis of sperm pathology is of pivotal importance for therapeutic options, either pharmacological or based on ART, and, in a case of systematic sperm defects, it enables the clinician to properly inform the patient of the potential risks involved in using abnormal sperm for assisted fertilization free of natural sperm selection barriers.

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(Sources continued...)


