Cell Tissue Res (2010) 341:349–357
DOI 10.1007/s00441-010-1007-3

REVIEW

The making of abnormal spermatozoa: cellular and molecular mechanisms underlying pathological spermiogenesis

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Received: 16 March 2010 / Accepted: 9 June 2010 / Published online: 2 July 2010

Abstract Fertilization in mammals occurs via a series of well-defined events in the secluded environment of the female reproductive tract. The mode of selection of the fertilizing spermatozoon nevertheless remains unknown. As has become evident during in vitro fertilization by sperm microinjection into the oocyte, abnormal spermatozoa can successfully fertilize oocytes. Under these extreme conditions, post-fertilization events, early embryonic development and implantation are significantly compromised indicating that the contribution of spermatozoa extends beyond sperm penetration. Microscopic identification of normal spermatozoa is a well-standardized procedure but insights into the mechanisms that lead to aberrant sperm differentiation and into the subcellular nature of sperm abnormalities have only recently begun to be obtained. The spermatozoon is the result of a complex development in which spermatid organelles give rise to various structural components with characteristic functions. Similar to other differentiated cells, the spermatozoon has a specific pathology that is most clearly identified by ultrastructural evaluation coupled with immunocytochemistry and molecular techniques. This multidisciplinary approach allows the precise characterization of sperm abnormalities, including structural, molecular and functional aspects. We summarize here studies of the physiopathology of spermiogenesis in two abnormal sperm phenotypes of infertile men: dysplasia of the fibrous sheath and acephalic spermatozoa/abnormal head-tail attachment. The characterization of the abnormalities of the tail cytoskeleton and centrioles has uncovered aspects of the subcellular basis of pathological spermiogenesis, has suggested experimental approaches to explore the nature of these anomalies and has opened the way for genetic studies that will ultimately lead to the design of the therapeutic tools of the future.

Keywords Abnormal spermiogenesis · Sperm pathology · Dysplasia of the fibrous sheath · Acephalic sperm · Sperm proteasomes · Human

This work was supported by grants from CONICET (PIP 2565), ANPCyT (PICT 9591 and 05-38229) and the CEGyR Foundation.

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Introduction

Fertilization in mammals is the result of a series of well-defined events, such as sperm capacitation and the acrosome reaction, sperm-zona binding, the fusion of gamete membranes and oocyte penetration and activation (Yanagimachi 1994). This intricate process has occurred across the millennia in the secluded environment of the female reproductive tract and the mode of selection of the fertilizing spermatozoon has evaded the inquiring eye of many generations of scientists. Since the introduction of in vitro fertilization by sperm microinjection into the oocyte (ICSI), reproductive biologists have been in a position to choose what they consider is the best-suited spermatozoon for microinjection. However, this selection has proven to be

extremely difficult and, at the same time, it has become evident that abnormal spermatozoa can successfully fertilize oocytes. Under these extreme conditions, post-fertilization events, early embryonic development and implantation are significantly compromised indicating that the contribution of spermatozoa extends beyond sperm penetration (Chemes and Rawe 2003). Microscopic identification of normal spermatozoa is a well-standardized procedure (Kruger et al. 1986; WHO 1999) but insights into the mechanisms that lead to aberrant sperm differentiation and into the subcellular nature of sperm abnormalities have only recently began to emerge. The spermatozoon is the result of a complex development in which spermatid organelles give rise to various structural components with characteristic functions. Similar to other differentiated cells, the spermatozoon has a specific pathology that is most clearly identified by the use of ultrastructural evaluation coupled with immunocytochemistry and molecular techniques. This multidisciplinary approach allows the precise characterization of sperm abnormalities including structural, molecular and functional aspects. This review summarizes our studies of the physiopathology of spermiogenesis in two abnormal sperm phenotypes of infertile men: dysplasia of the fibrous sheath (DFS) and acephalic spermatozoa/abnormal head-tail attachment.

Dysplasia of the fibrous sheath

Severe sperm tail abnormalities of infertile men are responsible for deficient or absent sperm motility. They comprise primary flagellar anomalies that are most likely of genetic origin and secondary anomalies caused by various andrological conditions. Primary anomalies arise as a consequence of systematic disorders of spermatid differentiation. Among them, primary ciliary diskinesia (formerly known as the immotile cilia syndrome) has been characterized as being the result of a lack of dynein arms in the axonemal microtubules of respiratory cilia and sperm flagella (Afzelius et al. 1975; Pedersen and Rebbe 1975). In 1987, we reported a group of infertile men with sperm immotility and introduced the term DFS to describe a primary flagellar anomaly affecting the sperm tail cytoskeleton, most prominently the fibrous sheath of the tail principal piece (Chemes et al. 1987a, 1998).

DFS spermatozoa display sperm tails that are characteristically short, thick and of irregular profile because of the overabundance and disorganization of the fibrous sheath (FS, Fig. 1a–c). The key component of the DFS phenotype is a redundant and haphazardly arranged FS that forms thick rings or broad meshes without the orderly disposition into longitudinal columns and transversal ribs. The axoneme, embedded in these hyperplastic fibres shows a variable degree of distortion ranging from well-formed axonemes to almost complete obliteration (Fig. 1d–j). Microtubular doublets frequently display partial or total lack of inner/outer dynein arms, with the central pair being absent in about half of the cases. The outer dense fibres 3 and 8, normally restricted to the mid piece, may extend to the principal piece. The annulus fails to migrate caudally, remaining just beneath the connecting piece, and mitochondria do not assemble in a normal mid piece. As a consequence, the hypertrophic FS extends up to the sperm neck, as can be well appreciated in longitudinal sections (Fig. 1c). Studies on testicular biopsies have demonstrated that the DFS phenotype arises during late spermiogenesis as a failure of the fibrous sheath and most cytoskeletal components of the sperm tail to assemble properly (Ross et al. 1973; Barthelemy et al. 1990; Rawe et al. 2002). The coexistence of abnormalities in various cytoskeletal components in addition to the fibrous sheath (dynein arms, central pairs, peripheral doublets and outer dense fibres) indicates that the DFS is actually a dysplasia of the whole flagellar cytoskeleton. This explains why approximately 20% of DFS patients suffer from chronic respiratory disease attributable to the lack of dynein arms in the peripheral doublets of axonemes in respiratory cilia (Chemes et al. 1990). These patients probably share abnormalities in genes coding for dynein arm proteins in axonemes of sperm flagella and respiratory cilia. The multigenic nature of primary ciliary diskinesia has previously been noted (for a review, see Chemes and Rawe 2003).

In recent years, extensive work has identified numerous proteins of the FS with specific functions in signal transduction (AKAP3 and 4; members of the A-kinase anchor protein family), ATP generation in the sperm flagellum (glyceraldehyde dehydrogenase) and disulphide bond reduction of cytoskeletal proteins (the sperm thioredoxin family) being the most significant (for a review, see Eddy et al. 2003). AKAP3 and 4 are the most abundant structural proteins of the FS. They bind to one another providing the structural framework for docking of cAMP-dependent protein kinase A to the fibrous sheath, thereby localizing phosphorylation of target proteins and initiating cAMP-dependent signal transduction (Carrera et al. 1994). In addition to the above-mentioned factors, various other proteins have also been isolated from the FS. The existence of such a variety of proteins indicates that the FS is involved in a series of functions far beyond the mere physical support of the axoneme as has formerly been attributed to it. Immunocytochemical localization of some of these proteins in DFS spermatozoa reveals thick and short flagellar profiles demonstrating that the FS is the main component of these grossly distorted tails, as has also been confirmed by ultrastructural immunolabelling of AKAP4, which specifically localizes over the redundant fibres of the

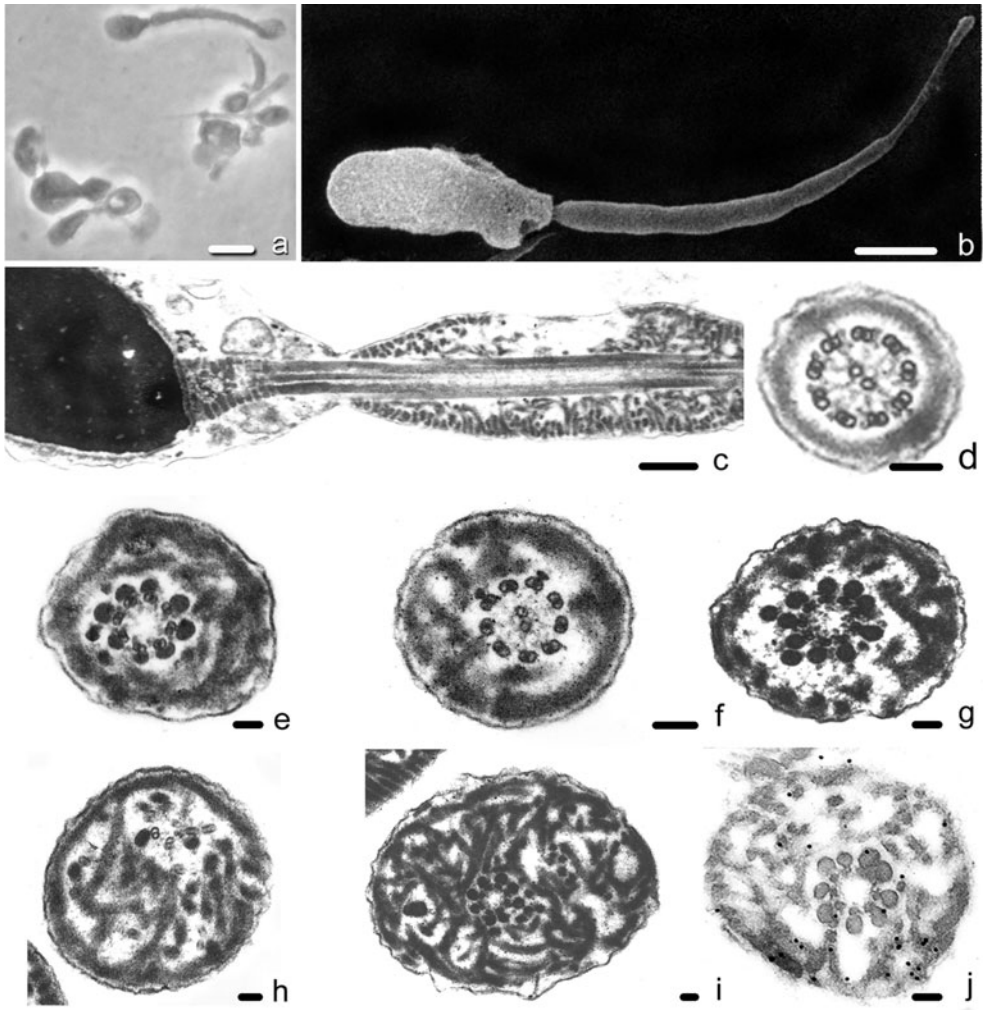


Fig. 1 Dysplasia of the fibrous sheath. Phase-contrast (**a**) and scanning electron microscopy (**b**) of DFS spermatozoa. Sperm tails are short, thick and of irregular profile. Transmission electron microscopy (**c**) of a longitudinal section of a DFS spermatozoon. Note the absence of a mitochondrial sheath and isolated mitochondria at the neck region. The annulus and beginning of the principal piece almost reach the sperm neck. Redundant and randomly oriented fibres of the fibrous sheath occur around the central axoneme. Flagellar diameter at the principal piece is 0.9 μm (normal flagellar diameter 0.4–0.5 μm). Cross section (**d**) of a normal sperm flagellum at the principal piece. The nine peripheral doublets of the axoneme, central pair, dynein arms and radial spokes are clearly seen. The fibrous sheath is composed of two lateral columns inserted in doublets 3 and 8 and semi-circumferential ribs. Flagellar diameter is 0.4 μm. Five transverse sections (**e**–**i**) of DFS spermatozoa at the principal piece. In all, the thickening and disorganization of the fibrous sheath are evident. Various axonemal anomalies can be observed: the central pair is missing (**e**, **g**, **i**), dynein arms are absent from the peripheral doublets (**f**) and redundant outer dense fibres are present (**e**, **g**). Complete axonemal distortion is seen in **h**. Flagellar diameter oscillates between 0.6 μm and 1.6 μm. Immunogold localization of AKAP4 (member of the A-kinase anchor protein family) with gold particles overlying the redundant fibrous sheath (**j**). The axoneme is free of labelling. The central pair is missing and an abnormal extension of outer dense fibres 3 and 8 to the principal piece is present. *Bars* 5 μm (**a**), 2 μm (**b**), 0.5 μm (**c**), 0.1 μm (**d**–**j**). **a** Reproduced from Platts et al. (2007) with permission. **f**–**h** Reproduced from Chemes et al. (1998) with permission. **j** Reproduced from Turner et al. (2001) with permission

FS and not over the axoneme (Figs. 1j, 2a, b). In most cases, the FS labelling extends right up to the nucleus and a mid piece is either absent or represented by only a few mitochondria (Figs. 1c, 2e, f). Axonemal microtubules, immunolabelled with anti-tubulin antibodies, are either discontinuous along the principal piece or are restricted to the end piece (Fig. 2g). Co-localization of several of these proteins demonstrates the complex pattern of flagellar aberrations in DFS spermatozoa (Fig. 2h). The use of molecular techniques in semen from DFS men has demonstrated that AKAP3 and AKAP4 are present in equal amounts and have similar molecular weights in DFS or normal spermatozoa (Fig. 2c, d). This suggests that the increase of FS material around the axonemes is only relative and is attributable to the crowding of normal amounts of FS material in the much shorter DFS tails. No

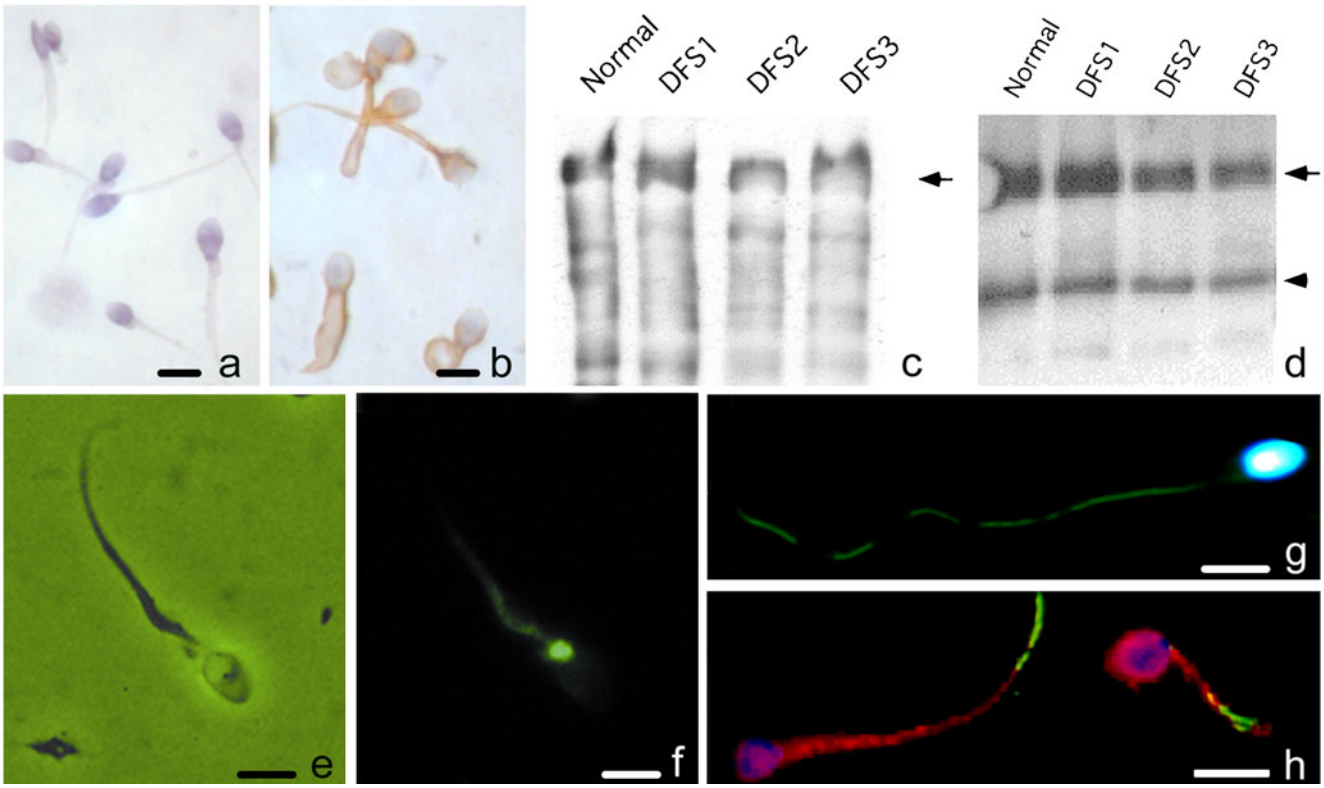


Fig. 2 Immunohistochemical localization of AKAP4 in thick sperm tails (**b**); negative control (**a**). Immunoblots of AKAP3 (**c**) and AKAP4 (**d**); the bands in the fertile control and three DFS patients show similar molecular weights and amounts of protein (*arrows*). Note the precursor (pro-AKAP4; *arrowhead*) and the mature form (AKAP4; *arrow*) in **d**. Phase-contrast (**e**) and fluorescence microscopy (**f**) of a DFS spermatozoon treated with MitoTracker Green. A single mitochondrion can be observed at the sperm neck (*green* in **f**). Immunofluorescence of the sperm tail (**g**) shows discontinuity of axonemal microtubules labelled with anti-tubulin antibodies. The topographical distortion of the sperm cytoskeleton in DFS is seen in **h** (double labelling of DFS spermatozoa with antibodies against AKAP4 in *red* and tubulin in *green*). *Bars* 5 μm (**a**, **b**, **e**-**g**), 10 μm (**h**). **c**, **d** Reproduced from Turner et al. (2001) with permission. **e**–**g** Reproduced from Rawe et al. (2001) with permission

mutations/deletions have been found in discrete sequences of the AKAP3 and 4 genes from affected individuals (Turner et al. 2001). These findings suggested that the AKAP proteins were not involved in the pathogenesis of the DFS phenotype. However, more recently, deletions of the AKAP4 gene and protein have been documented in one DFS patient and a DFS-like phenotype has been reported in a mouse model with selective knockout of the AKAP4 gene (Baccetti et al. 2005; Miki et al. 2002). In the search for additional candidate genes for human DFS, the axonemal dynein heavy chain gene Dnahc 8 has been studied but no mutations or deletions have been found in exons containing sequences known to be important for dynein function or in the intron/exon junctions corresponding to the 5′ and 3′ ends (S. Pilder, unpublished personal communication). Nevertheless, these results are not conclusive since the regions sequenced represent only about 2% of the total exonic DNA from Dnah8 (>14 kb).

In the last decade, evidence has accumulated concerning the presence of RNA transcripts in mature spermatozoa. It is now clear that, after fertilization, the spermatozoon brings to the nascent embryo not only the haploid paternal genome, but also a complement of proteins and a group of paternally transcribed RNAs (Ostermeier et al. 2002; Wang et al. 2004; Krawetz 2005; Gur and Breitbart 2006). Recent evidence suggests that these RNAs regulate development during sperm transit, at fertilization and during early zygote development (Barroso et al. 2009; Avendaño et al. 2009; Ostermeier et al. 2004). Platts et al. (2007) have reported the RNA transcript profiles of normozoospermic and teratozoospermic individuals; their results indicate that proteasomal and ubiquitin transcripts are broadly under-represented in teratozoospermia, which in part agrees with previous reports of decreased enzyme proteasome activity in spermatozoa with defective head-tail attachment and supports the notion that many teratozoospermic phenotypes represent forms of arrested spermiogenic development (Platts et al. 2007; Chemes and Rawe 2003; Rawe et al. 2008). RNA fingerprints from normozoospermic and teratozoospermic semen samples have been found to be substantially different. This includes both non-systematic teratozoospermia and DFS thus indicating that RNA sperm

profiles offer a window to developmental and differentiation events during spermiogenesis under normal conditions and in teratozoospermia.

The DFS phenotype is not modifiable by any therapeutic procedures. This, together with the reported family incidence and its association with dynein deficiency in respiratory cilia, indicates that DFS is a genetic condition of possible autosomic recessive inheritance (Baccetti et al. 1975, 1993, 2001; Alexandre et al. 1978; Bisson et al. 1979; Chemes et al. 1998; Moretti and Collodel 2006). Unquestionable evidence for the involvement of specific genes in the pathogenesis of DFS is still not available and whether DFS is monogenic or polygenic remains unknown. Candidate genes might be found in those coding for the structural proteins of the axoneme or the FS, transcription factors, protein chaperones, regulators of FS or of axonemal assembly or upstream signalling elements.

Abnormal head-tail attachment and centriolar dysfunction

Spermatozoa without heads ("acephalic", "decapitated", "pin heads") can be detected in extremely small numbers in the semen of fertile men and can increase up to 10%–20% in subfertile patients (Chemes et al. 1987b; Panidis et al. 2001). They have been reported as the main seminal abnormality in some sterile men in whom 80%–100% of the sperm population is composed of acephalic forms and a few loose heads (LeLannou 1979; Perotti et al. 1981; Baccetti et al. 1984; Chemes et al. 1987b). When studied with transmission and scanning electron microscopy acephalic spermatozoa have small cranial ends devoid of any nuclear material (Fig. 3a). Loose sperm heads are not frequent since they are usually phagocytosed by Sertoli cells (Chemes et al. 1987b, 1999). Some patients present an interesting mixture of acephalic tails and spermatozoa with misaligned head-mid piece junctions (Fig. 3b). These two variants are related and express a different degree of abnormality of the head-mid piece attachment. Acephalic forms represent the most extreme situation of sperm neck instability, which is also evident as increased fragility in spermatozoa with misaligned head-mid piece junctions that fracture easily when subjected to mechanical stress (centrifugation or micromanipulation; Chemes et al. 1999; Kamal et al. 1999).

Early during spermiogenesis as the proximal centriole migrates towards the spermatid nucleus and attaches to it, microtubules are recruited to the distal centriole forming the sperm tail axoneme. The alterations of head-neck attachment here described are attributable to the lack or abnormal implantation of the centrioles. If they fail to establish physical contact with the nucleus, the sperm head and tail develop independently and separate at spermiation giving rise to acephalic tails and loose heads. Spermatozoa with abnormal head-tail alignment arise when the proximal centriole attaches to the nucleus away from the longitudinal axis that extends from the centre of the acrosome to the caudal pole of the spermatid nucleus (abaxial implantation). When this happens, heads and tails are not aligned on the longitudinal axis of the spermatozoon. We have previously noted that this longitudinal axis runs between the two opposed "poles" of the spermatid nucleus; these poles are defined by the attachment sites of the acrosome and the tail anlagen (Chemes et al. 1979, 1999). Whereas acephalic spermatozoa are derived from the failure of the caudal migration of centrioles, some acrosomeless spermatozoa

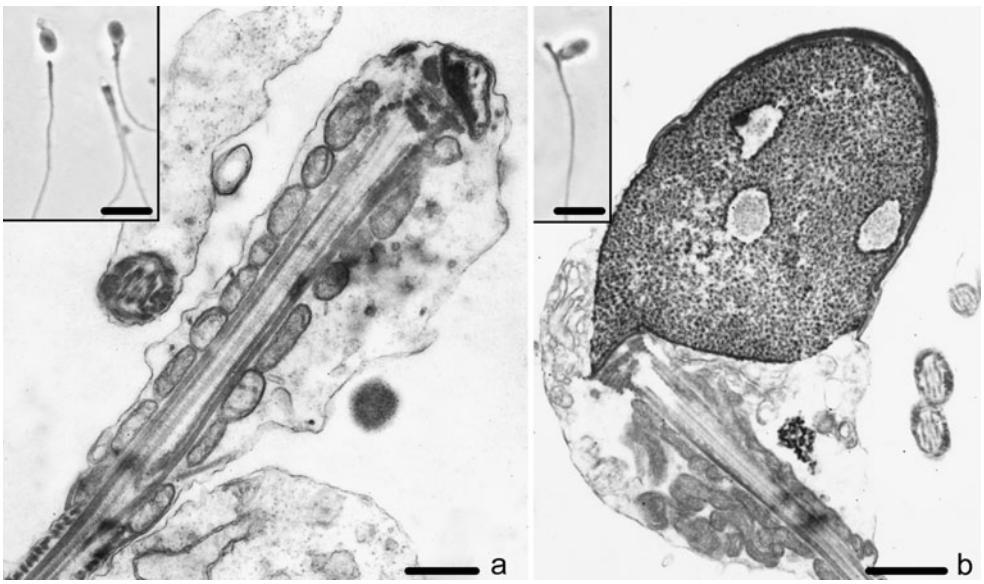
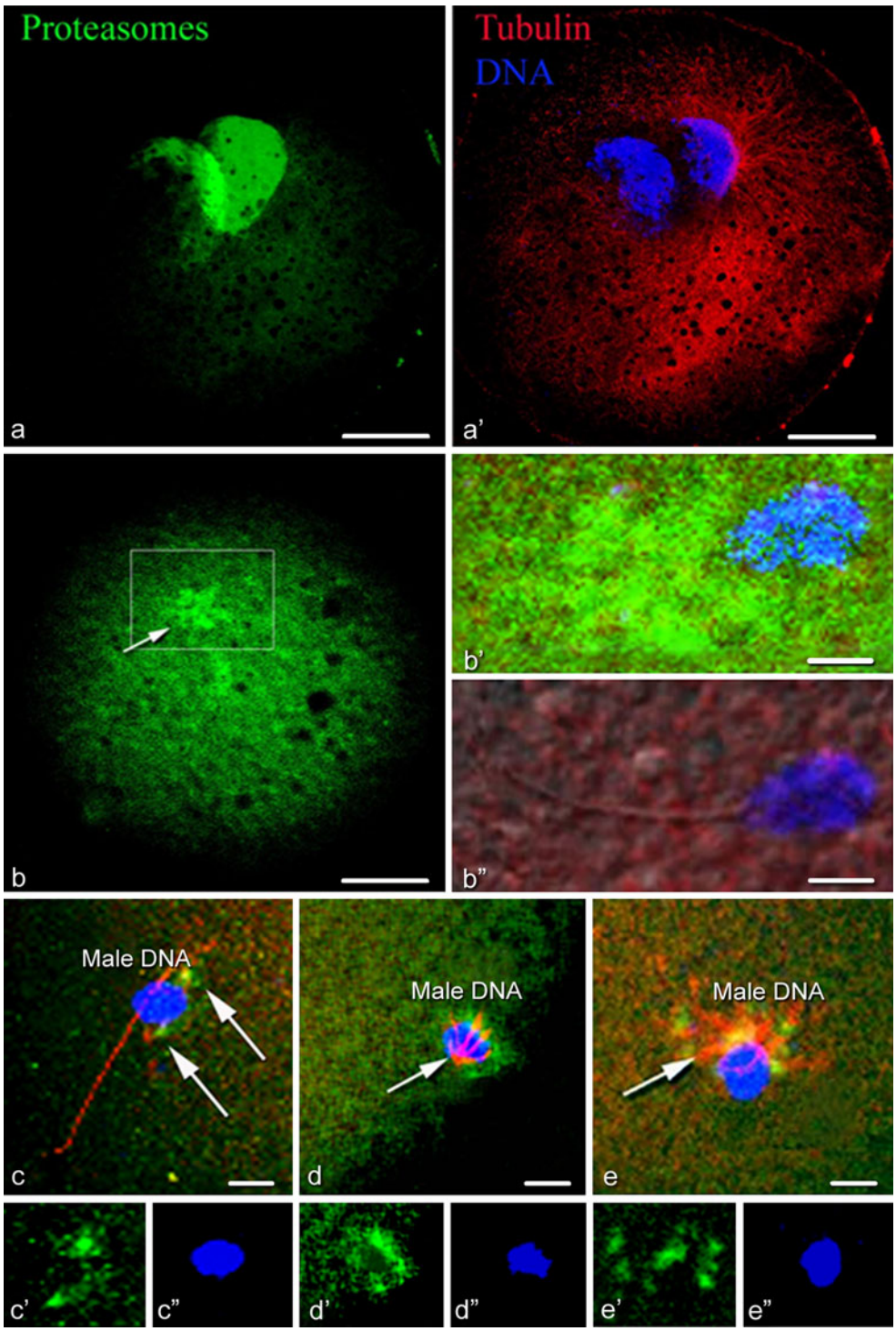


Fig. 3 **a** Acephalic spermatozoon. The plasma membrane covers the connecting piece. The mid piece is well formed. Cranial to the proximal centriole lies a nuclear-envelope-derived vesicular structure. *Inset* Two acephalic spermatozoa (the one *right* with two tails) and a loose head (*upper left corner*). **b** Abnormal head-mid piece connection. Head and tail are misaligned and form a 90° angle. *Inset* Spermatozoon with lateral insertion of the head in the mid piece. *Bars* 1 μm (**a**, **b**), 10 μm (*insets*)



result from the lack of proper attachment of the Golgi-derived proacrosomic vesicles to the cranial aspect of the spermatid nucleus (Zamboni 1987, 1992). The combination of these two abnormal mechanisms in the unusual patient described by Aughey and Orr (1978) with the round acrosomeless heads and acephalic tails in the same patient suggest that these are pathologies derived from an abnormal organization of the bipolar nature of the spermatid nucleus.

We have hypothesized that the inability of centrioles to attach normally to the spermatid nucleus derives from a centriolar dysfunction difficult to characterize because of the ultrastructural integrity of proximal centrioles in

◀ **Fig. 4** Inhibition of proteasomal activity by immunological neutralization during bovine in vitro fertilization (IVF) and in human fertilization failures after in vitro fertilization by sperm microinjection into the oocyte (ICSI). **a** Normal pronuclear development after the incubation of bovine zygotes without anti-proteasome antibodies (control). Proteasomes (*green*) are assembled inside male and female pronuclei. **a'** Normal sperm aster formation is seen (*red*, tubulin) coinciding with fully apposed pronuclei. **b** Bovine zygote obtained by IVF. Inhibition of proteasomal activity using Chariot-E446 antibody transfection (see Rawe et al. 2008). There is no sperm aster formation and a failure of pronuclear apposition. A diffuse *green* labelling of proteasomes is observed throughout the cytoplasm with less intense signal at the oocyte cortex. An area of dense proteasome recruitment is found around the sperm tail connecting piece (*arrow* in *boxed area*). **b'** Detail of the condensed sperm nucleus (*blue*) and proteasome recruitment over the sperm tail connecting piece (*green*). **b"** The same area explored by diffraction microscopy/immunofluorescence depicting the head-connecting piece. The sperm flagellum is clearly seen. **c** ICSI zygote obtained by injection of a streptolysin-O-permeabilized human spermatozoon loaded with p31 anti-proteasomal antibody. Note proteasome accumulation (*green*, *arrows*) to both sides of the sperm pronucleus (*blue*). The sperm aster is not formed (*red*). **c'**, **c"** Proteasome accumulations (*green*) around paternal DNA (*blue*). **d**, **e** After spontaneous human ICSI failure, proteasomes (*green*, *arrow*) accumulate near the sperm nucleus (*blue*). Note the complete lack of sperm aster formation (*red*). **d'**, **d"**, **e'**, **e"** Areas of proteasomal accumulation (*green*) next to the sperm nucleus (*blue*). *Bars* 20 μm (**a**, **a'**, **b**), 5 μm (**b'**, **b"**, **c–e**). Magnifications of **c'–e"** (*bottom row*) is the same as that of **c–e**. Partially modified from Rawe et al. (2008) with permission

acephalic spermatozoa (Chemes et al. 1999; Baccetti et al. 1989). This dysfunction is evidenced by the failures of ICSI after microinjection of abnormally aligned spermatozoa (Chemes et al. 1999; Saias-Magnan et al. 1999; Rawe et al. 2002). In these cases, syngamy and sperm aster development do not occur suggesting a functional failure of the sperm-derived centrosome, the organelle that acts as the microtubular organizing centre (MTOC) of the zygote. In bovines and humans, the MTOC derives from the sperm proximal centriole, which is an essential part of the sperm centrosome. Wójcik et al. (1996) and Fabunmi et al. (2000) have reported that centrosomes of various cell types are rich in proteasomes and ubiqitinated proteins and propose that they constitute proteolysis centres. The sperm proximal centriole is surrounded by the dense capitulum and segmented columns that form the sperm connecting piece from which it should first disengage to be able to nucleate tubulins as the MTOC centre. This process has been hypothesized to depend on the proteolytic activity of proteasomes localized in the sperm connecting piece (Wójcik et al. 2000; Sutovsky et al. 2004; Rawe 2005; Rawe et al. 2008), an interpretation supported by in vitro experiments in human/bovine spermatozoa and oocytes and the study of numerous discarded human post-ICSI fertilization failures. We have used pharmacological inhibitors such as MG132 or specific anti-proteasome antibodies that neutralize proteasomes and disrupt sperm aster formation and pronuclear development/apposition in bovine and human zygotes (Fig. 4). Furthermore, we have also reported decreased proteasome enzymatic activity in sperm from patients with abnormally aligned/acephalic spermatozoa (Morales et al. 2004; Rawe et al. 2008). These findings support our contention that sperm and zygote proteasome proteolytic activity plays a central role in centrosome formation in the zygote by means of releasing a functional sperm centriole inside the oocyte cytoplasm.

In summary, spermatozoa lacking heads or with abnormal head-mid piece alignment originate in dysfunctional sperm centrioles that are unable to migrate and attach normally to the caudal pole of the spermatid nucleus. These spermatozoa have decreased proteasome activity, which probably impairs normal centriolar release and centrosome formation after fertilization determining insufficient nucleation of the zygote sperm aster, lack of syngamy and embryo cleavage.

Concluding remarks

DFS and the anomalies of the head-mid piece attachment represent two excellent models for the study of the cell and molecular biology of sperm differentiation. Characterization of the abnormalities of the tail cytoskeleton and centrioles has uncovered aspects of the subcellular basis of pathological spermiogenesis, has suggested experimental approaches to explore the nature of these anomalies and has opened the way for genetic studies that will ultimately lead to the design of the therapeutic tools of the future.

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