An ultrastructural and immunocytochemical study of a rare genetic sperm tail defect that causes infertility in humans

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Objective: To characterize and describe the ontogenesis of a rare flagellar defect affecting the whole sperm population of a sterile man.

Design: Case report.

Setting: Regional referral center for male infertility in Siena, Italy.

Patient(s): A 28-year-old man with severe asthenozoospermia.

Intervention(s): Physical and hormonal assays, semen analysis, and testicular biopsy.

Main Outcome Measure(s): Semen samples and testicular biopsies were analyzed by light and transmission electron microscopy; immunocytochemical study with anti-β-tubulin and anti-AKAP 82 antibodies was performed to detect the presence and distribution of proteins.

Result(s): Ultrastructural analysis of ejaculated spermatozoa and testicular biopsy revealed absence of the fibrous sheath in the principal-piece region of the tail. Fibrous sheath-like structures were observed in cytoplasmic residues and residual bodies released by spermatids in the seminiferous epithelium. Other anomalies observed were supplementary axonemes and mitochondrial helix elongation. These features were confirmed by immunocytochemical staining.

Conclusion(s): This rare sperm tail defect, characterized by absence of the fibrous sheath, presence of supplementary axonemes, and an abnormally elongated midpiece, originates in the seminiferous tubules during spermiogenesis, as detected in testicular biopsy sections. These defects occur in the whole sperm population, and therefore a genetic origin could be suggested. (Fertil Steril 2004;82:463–8. ©2004 by American Society for Reproductive Medicine.)

Key Words: Genetic infertility, human spermatogenesis, immunocytochemistry, electron microscopy, fibrous sheath

Submicroscopic alterations in the cytoskeletal structure of the sperm flagellum are associated with severely reduced or completely absent motility in subfertile or infertile men. Ultrastructural sperm tail anomalies might be phenotypic or genotypic. The former randomly affects axonemal and periaxonemal structures in a variable percentage of sperm in ejaculate. These alterations might be related to pathologies that respond to drug therapy or surgery, such as infections, varicocele, or local immune disorders. Genotypic sperm alterations are characterized by a precise monomorphic defect, affecting the total sperm population in sterile patients. Such anomalies are significantly more frequent in consanguineous individuals (1).

Genetic sperm defects affecting the tail structures include [1] stump and short tail defects (2), characterized by tails of reduced length in 70%–100% of sperm, with defective axonemes and accessory fiber patterns and marked fibrous sheath dysplasia (3, 4); [2] immotile cilia syndrome (5), characterized by the absence of axonemal dynein arms associated with immotility of respiratory cilia and sperm flagella; [3] detached tails, a primary sperm defect originating during spermiogenesis (6, 7),

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distinguished by headless tails or abnormal head–midpiece attachment (8, 9), and [4] “9+0 axoneme” and “absent axoneme,” very rare defects characterized by the absence of the central pair of microtubules and the total lack of the flagellar axoneme, respectively (10, 11).

The present study concerns absence of the fibrous sheath, a rare flagellar defect affecting the whole sperm population of a sterile man. Other peculiar alterations of the mitochondrial helix and axonemal structures were observed. The defects seem to have a genetic basis and to originate in late spermiogenesis.

**CASE REPORT**

A 28-year-old man came to our center for semen analysis after 1 year of unprotected sexual intercourse without conception. Sexual development, medical history, and physical examination were normal. The hormonal baseline profile of FSH, LH, PRL, androstenedione, DEAS, E2, T, free T, and B-inhibin showed levels within the standard range.

Microbiological investigations did not reveal any urogenital infection. The lymphocytic karyotype showed a normal chromosomal constitution, 46XY.

Semen samples were collected by masturbation after 4 days of sexual abstinence. The ejaculate was fully liquefied and then volume, pH, number, and motility of spermatozoa were evaluated according to World Health Organization guidelines (12). The analysis was repeated three times, each at a 3-month interval, for a total of 9 months.

Fresh semen was stained with 0.5% eosin Y (CI 45380) in 0.9% aqueous sodium chloride solution. Two minutes after staining, the sample was observed with a light microscope, and unstained (live) and stained (dead) sperm were scored.

Semen samples and testicular tissue were fixed for 2 hours at 4°C in Karnovsky fluid, postfixed in osmium tetroxide, dehydrated in a graded ethanol series, and embedded in Epon-Araldite. Ultrathin sections, counterstained with uranyl acetate and lead citrate, were examined with transmission electron microscopy (TEM) (Philips CM10; Philips, Eindhoven, The Netherlands).

Semen samples of the patient, sperm with dysplasia of the fibrous sheath, and sperm of individuals of proven fertility were washed in sperm washing buffer (Sage Biopharma, Bedminster, NJ) and incubated with fluorescent stain (MitoTracker Green FM; Molecular Probes, Eugene, OR) to a final concentration of 5 nmol/L for 30 minutes at 37°C. After washing in phosphate-buffered saline (PBS), the sperm were smeared on glass slides, air-dried, rinsed in PBS, and fixed for 15 minutes in methanol at −20°C. Samples were then treated with blocking solution (PBS −1% bovine serum albumin [BSA] −5% normal goat serum [NGS]) for 20 minutes at room temperature and incubated overnight at 4°C with mouse monoclonal anti-β-tubulin (Sigma Chemical, St. Louis, MO) and mouse monoclonal anti-AKAP 82 (BD Biosciences, Erembodegem, Belgium) antibodies diluted 1:100 and 1:50, respectively, in PBS −0.1% BSA −1% NGS. After three washes in PBS, the samples were treated with goat antimouse IgG-Texas red conjugated antibody (Southern Biotechnology Associated, Birmingham, AL). Finally, the slides were extensively washed in PBS and mounted with Vectashield (Vector Labs, Burlingame, CA). Incubation in primary antibodies was omitted in procedure control samples.

Testicular tissue, cryofixed by buffering in iso-pentane in liquid nitrogen, was cut with a cryostat (Leica CM 1850, Leica, Milan, Italy). Cryosections were treated with anti-β-tubulin and anti-AKAP 82 antibodies, followed by antimouse IgG-Texas red conjugated secondary antibody. The sections were counterstained with eosin 50% solution for 1 minute at room temperature, briefly washed in water, and mounted with Vectashield.

Observations and photographs were made with a light microscope (Leitz Aristoplan, Leica) equipped with fluorescence apparatus.

Analysis of three subsequent sperm samples of the patient showed normal volume, pH (12), and sperm number (83 × 10⁹/mL to 209 × 10⁹/mL). Rapid and slow progressive motility were severely reduced, between 0.6% and 1% and 1.5% and 5%, respectively. Light microscopy showed short tail. Eosin staining revealed that 72% of sperm were alive.

Ultrastructural analysis showed regularly shaped acrosomes and nuclei (Fig. 1a), with well-condensed chromatin in 56% of cells; however, axonemes had regular 9+2 patterns in 63% of cells, mainly when observed in cross-section at the midpiece level. We also observed bundles of one to seven supplementary axonemes showing complete or incomplete (Fig. 1b) sets of microtubules, enveloped in the same membrane. This feature was confirmed by immunocytochemical staining, with monoclonal anti-tubulin antibody showing a bundle of axoneme-like structures emerging at the end of the midpiece region in 19% of sperm (Fig. 2a and b). By this method, 33% of cells showed a disorganized network of microtubular structures emerging randomly at any level of the flagellum (Fig. 2c and d); 28% of sperm had very short tails, a typical feature of “stump tail defect” (Fig. 2e and f [arrows]), whereas 10% had straight tails, and another 10% had rolled-up tails. No flagella with regular structure were observed.

Electron microscopy showed dynein arms, and the accessory fibers were normal in half of the sections analyzed. In a longitudinal section of the midpiece region, we observed abnormal extension of the mitochondrial helix (up to 17 μm; normal length 5 μm) (Fig. 1a). Abnormal length of the midpiece regions in the whole sperm sample was evidenced by double-fluorescence staining (MitoTracker Green FM) and anti-tubulin antibody (Fig. 2e and f).
A peculiar sperm tail defect was absence of the fibrous sheath in the principal-piece region in all sections examined (Fig. 1c). Immunocytochemical labeling with a mouse anti-AKAP82 monoclonal antibody recognizing human AKAP4 protein, a specific marker of fibrous sheath, confirmed absence of the fibrous sheath (all sperm were unstained). The same antibody demonstrated the disorganization or the reduced presence of AKAP4 protein at the tail level of sperm with fibrous sheath dysplasia of another sterile patient (Fig. 2g and h). Fluorescence staining was regularly present at the principal-piece level of sperm from proven-fertile individuals (Fig. 2i).

Immunocytochemical staining of cryo-fixed testicular tissue with anti-AKAP82 monoclonal antibody showed the expected diffuse presence of AKAP4 protein (Fig. 2j). In particular, we observed also clusters of this protein in cytoplasmic regions of developing spermatids and residual bodies, probably corresponding to fibrous sheath remnants detected by TEM. Immunolabeling with anti-tubulin antibody revealed widespread presence of this protein in the cytoplasm of all testicular germinal cells. Abnormally numerous organized microtubular structures were observed throughout the seminiferous epithelium, from the basal to the adluminal region (Fig. 2k).

Ultrastructural analysis of testicular tissue highlighted normal features of early stages of spermatogenesis, from spermatogonia to spermatocytes. Spermatids generally showed well-formed acrosome and nucleus but frequent anomalies of flagellar assembly. The implantation structures (basal plate, striated columns) were sometimes doubled. A centriolar adjunct emerged from the proximal centriole (Fig. 3a and b), and a large granular chromatoid body surrounded the distal centriole (Fig. 3c). The ring-shaped annulus (Fig. 3d) surrounded the complex of microtubules or accessory fibers, both structures involved in axoneme assembly. As observed in ejaculated sperm, bundles of axonemes and disorganized microtubules (Fig. 3e) were often present in the cytoplasm of elongating spermatids. The accessory fibers were usually well organized around the axoneme at the midpiece and principal-piece levels, where the fibrous sheath was completely absent (Fig. 3f).

Fibrous sheath cytoskeletal components, produced during spermiogenesis, were observed in cytoplasmic residues of spermatids but were not organized around the principal piece axoneme (Fig. 3b). Remnants of fibrous sheath remained embedded in residual bodies released by spermatids into the tubular lumen and observed in ejaculate (Fig. 1d).

**DISCUSSION**

In the present study, we analyzed ultrastructural and molecular characteristics of ejaculated spermatozoa and spermatogenetic stages in a sterile patient with severe asthenozoospermia.

The sperm head appeared generally well structured, but the tail had three major defects: absence of the fibrous sheath, extra-axonemal microtubules arranged in supplementary axonemes or isolated doublets, and an abnormally long mitochondrial sheath.
A similar case was described by Ross et al. (13), who did not investigate the nature and structure of the tubulin system, which can only be highlighted by specific immunofluorescent staining. In both cases, the mitochondrial helix was longer than in sperm of normal individuals. Spermatogonia and spermatocytes showed the usual features, with the three sperm defects originating at spermatid stage. These peculiar defects occurred in all germinal cells, and therefore we believe that they have a genetic origin.

AKAP4 protein was present in the testicular tissue of our patient, concentrated in the cytoplasm of spermatids and in residual bodies, where remnants of fibrous sheath were observed by TEM. Miki et al. (14) demonstrated that targeted disruption of the AKAP4 gene in mice caused absence of the fibrous sheath on the principal piece of mature sperm. The interaction between outer dense fibers and microtubules was maintained in the sperm of mutant mice, and middle-piece dimensions of mutant sperm were similar to those of wild-type sperm, but the sperm flagellum was generally shorter. In our patient, the presence of AKAP4 protein was demonstrated by immunofluorescent staining of testicular tissue. Absence of the protein from mature sperm was probably due to failure of association with a pre-existing template to complete formation of the fibrous sheath, as suggested by Miki et al. (14).

The unusual abundance of tubulin, detected by electron microscopy and immunofluorescent staining in our patient, included the ubiquitous presence of abnormally numerous microtubular complexes, supernumerary axonemes, and disordered individual microtubules, frequently grouped in bundles, throughout the seminiferous epithelium, from the basal to the adluminal region. It is difficult to explain this exceptionally disordered abundance of microtubules in mature sperm cells. In entire taxonomical groups of several invertebrates, a similar situation can be found in mature sperm and is interpreted as persistence of the nuclear manchette of the spermatid into mature spermatozoa. This characteristic of invertebrates could be due to a gene that is usually silent at maturity, but it has been never observed in vertebrates, whose spermatids normally have a tubular manchette that surrounds the nucleus during its formation, disappearing in the mature spermatozoon.
Transmission electron microscopy micrographs of longitudinal sections of testicular spermatids. Acrosomes (A) surround nuclei (N) with evidently immature chromatin. (a, b) An emerging, well-developed, centriolar adjunct (CA) is evident. (b) A peculiar abundance of mitochondria (M), assembled around a rolled-up or doubled axoneme at the midpiece level and spread in the upper region of cytoplasmic residue (CR). Fibrous sheath remnants (FSR) are present in the cytoplasm of spermatids, but they are never organized around the principal piece (PP), emerging at the lower region of cytoplasm. (c) A large cromatoid body (CB) envelopes the distal centriole. (d) The ring-shaped annulus (An) surrounding the axoneme (AX) and the accessory fibers. (e) Bundle of axoneme (AX) with complete microtubular set enveloped in the same membrane. (f) Longitudinal section of spermatid with acrosome (A) surrounding a regularly shaped nucleus (N) with almost condensed chromatin and a cross-section of the principal piece (arrow) with well-organized axoneme and accessory fibers but completely lacking in fibrous sheath. Original magnification, ×7000 for A; ×6500 for B; ×9500 for C; ×16,000 for D; ×34,000 for E; ×20,000 for F.

The fibrous sheath is essential for flagellar development and movement, being scaffolding for enzymes with active roles in flagellar activity. This rare combination of human sperm defects could be due to a mutation that cannot be transmitted by the male but arises spontaneously or is transmitted by the female.

References