Homogeneous Microscopic Abnormalities in Sperm Morphology and Immotility as A Cause of Male Infertility

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Objective To study the identification of the cause of specific sperm abnormalities.

Methods Two adult men with specific alterations in sperm morphology causing 100% immobility were included in this study. The study of sperm used: transmission electron microscopy (both patients); apoptotic markers, DNA fragmentation test and fluorescence in-situ hybridization (patient 1) and immunocytochemistry study of sperm flagellum using anti-β tubulin antibodies and ciliary activity test (patient 2).

Results Increased DNA fragmentation (52.6%) and apoptosis biomarkers were detected in patient 1, and loss of the central pair of microtubules in patient 2 (‘9+0’ axoneme); the nasal ciliary activity was normal.

Conclusion Results suggest an apoptotic origin of the abnormalities in the sperm from patient 1 and dysplasia of the fibrous sheath in patient 2.

Key words: apoptotic changes; dysplasia of fibrous sheath; midpiece thickening; necrospermia; sperm immotility

Sperm abnormalities such as morphological and motility disorders are associated with male infertility. The two main causes of sperm immotility are necrozoospermia (the presence of non-viable spermatozoa) and ultrastructural abnormalities of sperm tails. The causes of necrospermia are varied, including testicular disorders, endocrinopathies, defects in androgen
biosynthesis, toxins, drugs, chronic systemic diseases, infections and epididymal function or storage defect\[1,2\].

With regard to the morphological alterations, there are two main types of teratozoospermia: heterogeneous non-specific anomalies in different sperm components (the most frequent type) or a very homogeneous microscopic systematic sperm defect pattern which is present in most spermatozoa\[3\]. The latter can affect the sperm head or flagellum, with abnormalities in the head-neck region including varying degrees of misalignment, up to the most extreme defect resulting in acephalic spermatozoa\[4\].

In this paper, we present two cases of sperm immotility, one with thickening of the midpiece and anomalous head-neck attachment causing some headless sperm with severe necrospermia features we had not previously seen (patient 1), and another with short and thick tails, findings compatible with previously reported cases of dysplasia of the fibrous sheath (DFS) and/or primary ciliary dyskinesia (PCD) (patient 2). To reach the diagnosis of both cases, basic semen analysis and biochemical study of the seminal plama were insufficient. For this reason, ultrastructural studies and other techniques as appropriate were added:

In case 1, special stainings, fluorescence in situ hybridisation (FISH) and apoptosis markers on sperm were performed. FISH was carried out to estimate the proportion of aneuploidy and structural aberrations present in his ejaculate\[5\]. DNA fragmentation is the most characteristic feature of apoptosis, which is caused by the endogenous DNA degrading endonucleases activated by apoptotic responses, for example, caspase-associated cell signalling\[6\]. The DNA damage correlates well with the reproductive potential of sperm\[7\].

In case 2, to confirm the diagnosis of DFS and/or PCD, immunocytochemistry study of sperm flagellum and nasal ciliary beat and cellular rotation test were performed. Regarding flagellar structure, all eukaryotic cilia and flagella possess a central bundle of microtubules (formed by the polymerization of \( \alpha \)- and \( \beta \)-tubulin), called the axoneme, which consists of nine doublet microtubules surrounding a central pair of singlet microtubules (the distinctive ‘9+2’ microtubule arrangement). The fibrous sheath is a cytoskeletal structure encasing the axoneme and other dense fibres in the principal piece of sperm tail. It consists of two longitudinal columns linked to axonemal doublets 3 and 8\[8\].

PCD is a congenital disease in which the respiratory cilia are immotile, dysmotile, or both\[9\]; resulting in a range of chronic clinical manifestations such as bronchitis, rhino-sinusitis and otitis media, situs inversus\[10\]. These anomalies are observed in the flagella of spermatozoa of most patients, causing male infertility. Among the systematic sperm tail defects, DFS has been particularly well characterised\[11,12\]. It is a disorder related to PCD in which there is both widespread disarray of the sperm fibrous sheath and axonemal ciliary defects. In these cases the majority of spermatozoa are rigid, short, thick, and immotile and the fibrous sheath
appears hypertrophic, discontinuous, and disorganised. Affected sperm also have perturbed axonemal structures which might include part or complete absence of dynein arms, lack the central microtubule pair or radial spokes, or disorganised microtubules[13].

**Materials & Methods**

**Patients**

Two adult men with total sperm immotility and characteristic microscopic abnormalities were included in this study. Both patients were referred to the fertility laboratory after 2 and 3 years of primary infertility history respectively. They had not undergone any medical or surgical treatments in the three months prior to semen examination. A signed informed-consent form was obtained from the two patients included in the study, and the project was approved by the ethics committee at the University Hospital Dr. Peset before starting.

**Patient 1**

A 37-year-old man who had a previous genitourinary infection caused by *Enterococcus faecalis* was treated with Ciprofloxacin, which resolved three months later. Hormonal studies were normal except for slightly increased follicle-stimulating hormone (FSH) levels. He underwent scrotal ultrasound and a small cyst in the left testicle was identified. Family history: the patient has a brother with diagnosed azoospermia who was not included in this study.

**Patient 2**

A 39-year-old man with no history of genitourinary infections and negative semen bacteriological cultures. Hormonal serum screening and his somatic phenotype were normal. The patient had chronic secretory otitis media in his left middle ear and he underwent myringoplasty in 2010. Given this history, a nasal ciliary activity test was performed in order to check the PCD diagnosis.

**Semen analysis in both patients**

Semen samples were collected by masturbation after 4 d of sexual abstinence. For light microscopy five independent samples from each patient were studied over a 6–12-month period and analysed within 60 min of collection. Semen parameters were evaluated according to World Health Organization guidelines[14]. A Makler® (Sefi-Medical Instruments, Haifa, Israel) chamber was used to determine the sperm concentration and motility by direct observation with a phase contrast microscope Eclipse E400 Nikon® (Tokyo, Japan). Semen samples were stained with Sperm VitalStain® (Nidacon International AB, Mölndal, Sweden) and the percentage of the corresponding dead (stained) and living (unstained) cells was calculated. The morphological study was performed after methanol fixation and subsequent staining with modified Giemsa (Quick Panoptic®, QCA, Amposta, Spain), classifying the sperm as normal or abnormal.
As part of the biochemical analysis, fructose and citric acid concentrations in the seminal plasma were determined enzymatically (BioSystems S.A., Barcelona, Spain). Control reference values were: citrate greater than 24 μmol/ejaculate; fructose greater than 13 μmol/ejaculate. For the ultrastructural studies an aliquot from the same samples used in the biochemical analysis was studied using transmission electron microscopy (TEM). The semen samples were fixed in 2.5% glutaraldehyde (buffered in pH 7.4, 0.1 mol/L Sorensen phosphate solution). The tissue was then post-fixed for 2 h in 1% osmium tetroxide in buffer, and after dehydration with graded concentrations of acetone, the tissue blocks were embedded in Epon 812® (TAAB Lab, England). Semi-thin Epon sections (1 μm) were stained with toluidine blue as a light microscope control and were trimmed for ultrastructural study. Ultra-thin sections were cut with a diamond knife using a Reichert-Jung ultramicrotome® (Leica, Illinois, USA), contrasted with uranyl acetate and lead citrate, and examined in a Jeol Jem 1010® (Tokyo, Japan) electron microscope operated at 60 kV.

**Patient 1**

Meiotic segregation was explored by FISH using a mix of DNA probes for chromosomes 13, 18, 21, X, and Y. The studies were performed using alpha-satellite probes for the chromosome centromeres and were labelled as follows: 18p11.1–q11 with the aqua fluorochrome, Xp11.1 Q11.1 with the green fluorochrome, Q11.1 Yp11.1 with the red fluorochrome, and unique sequence probes for identifying the 13q14 and 21q22.13–q22.2 loci with green and red fluorochromes. Automatic study of 3 808 spermatozoa was performed with the Metafer MetaCyte system® (MetaSystems, Altlussheim, Germany). Reference values from the control population: nullisomy less than 3%; disomy and diploidy less than 2%. For DNA fragmentation analysis, terminal dUTP nick-end labelling (TUNEL), which labels breaks in ssDNA and dsDNA using an in situ cell death fluorescein detection kit® (Roche Applied Science, Basel, Switzerland), was performed. The DNA fragmentation index from 1 266 spermatozoa was obtained. Patient sperm was also used for TUNEL positive and negative hybridization controls which were achieved following in situ cell death fluorescein detection kit® instructions (1 000 spermatozoa from each control). Reference value of the fertile population was < 20% positive.

For analysis of apoptosis markers, cell pellets were lysed on ice in Laemmli’s buffer (62.5 mmol/L Tris-HCl pH 6.8, 2% sodium dodecyl sulphate, 5% β-mercaptoethanol, 10% glycerol, and 0.01% bromophenol blue) for 30 min in presence of complete protease inhibitor cocktail (Roche Applied Science, Basel, Switzerland), they were then boiled for 5–7 min at 100 °C and subjected to Western blotting analysis. Crude extracts (10 μg) were separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE; 12% polyacrylamide gel), using human fibroblasts treated for 5 min with ultraviolet light to induce apoptosis.
as positive control. Polyvinylidene difluoride (PVDF) membranes were blocked with 5% dry milk powder dissolved in tris-buffered saline (10 mmol/L Tris-HCl, pH 7.4, 150 mmol/L NaCl) for 1 h at room temperature and then incubated with different primary antibodies: Annexin V (Abcam, Cambridge, UK), Poly (ADP-ribose) polymerase (PARP1), and Caspase-3 (Cell Signalling, Danvers, USA), all at a 1:1 000 dilution, overnight at 4 °C, followed by incubation with the corresponding IgG-horseradish peroxidase (HRP) conjugated secondary antibody (at a 1:3 000 dilution) for 2 h at room temperature. Blots were visualised using a Lumi-Light Western blotting detection kit (Roche Applied Science, Basel, Switzerland).

In order to determine the composition of the material deposited on the midpiece, Oil Red O (an oil-soluble dye for staining lipids) and periodic acid-schiff (to detect polysaccharides such as glycogen, and mucosubstances such as glycolipids, glycoproteins, and mucins) were used. The Papanicolaou stain was also used to complement the morphological study. A karyotype and Y-chromosome study were also performed on whole blood to search for microdeletions in the azoospermia factor region Yq11 (AZF) and a, b, and c loci were tested.

**Patient 2**

An immunocytochemistry study of sperm flagellum using mouse monoclonal anti-β tubulin antibodies was performed. Cells on glass slides were fixed for 5 min with methanol at −20 °C. After this, the cells were washed twice with phosphate buffered saline (PBS) and blocked with 4% foetal bovine serum/PBS for 30 min. Immunostaining was subsequently carried out, by incubating the cells overnight at 4 °C with mouse monoclonal anti-β tubulin antibodies (at a 1:200 dilution; ab6046, Abcam, Boston, USA). The next day, the cells were washed twice with PBS and incubated in the dark with goat anti-rabbit secondary antibody Alexa Fluor 488 (at a 1:500 dilution; A11078, Invitrogen, Carlsbad, USA) for 1 h at room temperature. The slides were mounted and observed using an Observer Z.1® fluorescence microscope (Carl Zeiss, Jena, Germany).

To analyse β tubulin, cell pellets were lysed on ice in Laemmli’s buffer for 30 min in the presence of a mixture of several protease inhibitors with broad inhibitory specificity (complete protease inhibitor cocktail, Roche Applied Science, Basel, Switzerland), boiled for 5–7 min at 100 °C and subjected to Western blotting. Mouse monoclonal antibodies which recognise β tubulin (1:1 000 dilution; ab6046, Abcam, Cambridge, UK) and anti-actin (1:10 000 dilution; A-2066, Sigma-Aldrich, St. Louis, USA) were used. Blots using anti-rabbit (1:5 000 dilution) or anti-mouse (1:10 000 dilution) primary antibodies were probed with IgG-HRP conjugated secondary antibody, and visualised using a Lumi-Light Western blotting detection kit (Roche Applied Science, Basel, Switzerland).

To check for the presence of impaired respiratory ciliary motility and characteristic of PCD, nasal ciliary beat frequency and beat pattern were tested, and cellular rotation test
was performed using high resolution digital high-speed video imaging (DHSV), as previously described\textsuperscript{[15]}. Samples of ciliary airway epithelial cells were obtained from the middle nasal concha using curettage without local anaesthesia, in a period during which acute infection was absent. The tissue sample was immersed in 1 ml of Dulbecco’s modified Eagle’s medium (DMEM, Cambrex, Walkersville, USA) supplemented with 10% fetal calf serum (FCS), 2 mmol/L glutamine, penicillin (100 U/ml), and streptomycin (100 μg/ml), and turned over until partial dissolution occurred. Then, 150 μl of the nasal epithelium biopsy cells were plated onto tissue culture plates (12-well Corning\textsuperscript{®} Costar\textsuperscript{®} 3513, New York, USA), previously coated with 0.5% gelatin to promote adherence of the ciliated cells. The ciliary beat frequency was measured at room temperature (23–27 °C) within 30 min of biopsy.

Samples were tested using the high resolution digital high-speed video imaging (DHSV) technique with a Nikon Eclipse TS100\textsuperscript{®} (Tokyo, Japan) microscope equipped with a 40 × Nikon phase-contrast objective, providing a final optical gain of 400 × . A Multimetrix\textsuperscript{®} XA3051 power source was used with transillumination provided by visible and red-filtered 150 W halogen lamps. The light path was directed through the multi-image module to a charge-coupled device (CCD) camera (JAI CV-A33 CL\textsuperscript{®} digital quad high-speed progressive scan camera) with a resolution of 649 (horizontal) × 494 (vertical) pixels and a maximum resolution velocity of 120 frames per second. Video signals were digitised and processed with an HP Workstation (xw6200, Xeon 3.4 GHz). The normal range of nasal cilia beat frequency is between 9 and 13 Hz. Cellular rotation test: the test is positive when an isolated nasal ciliary cell spontaneously turns on itself quickly and continuously with a regular pattern and ciliary beat frequency. If the movement is dyskinetic, the ciliary cell does not rotate (negative test).

**Results**

Table 1 summarises the main features of the semen samples from both patients included in this study.

**Patient 1**

The morphological study revealed thickening of the midpiece (Figure 1A), more evident in the vitality stain (Figure 1B), indicating that 85% of spermatozoa were affected, with 12% being decapitated forms (Figure 1B). Most spermatozoa were dead (99%). The ultrastructural TEM study showed many headless spermatozoa (Figure 2A), high density deposits (Figure 2B), and disorganisation in the midpiece. Normal mitochondria could not be distinguished (Figure 2C). The FISH assay on semen samples from patient 1 (Tables 2,3) showed an abnormally increased percentage of nullisomic spermatozoa (above 3%), affecting the five chromosomes studied. Diploidy 18/X/Y was identified in more than 2% of spermatozoa.
Table 1 Semen characteristics and biochemical analysis from both patients studied

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Patient 1</th>
<th>Patient 2</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ejaculate volume (ml)</td>
<td>1.4</td>
<td>3.6</td>
</tr>
<tr>
<td>pH</td>
<td>8.7</td>
<td>8.5</td>
</tr>
<tr>
<td>Viscosity</td>
<td>Normal</td>
<td>Normal</td>
</tr>
<tr>
<td>Spermatozoa count (×10⁶/ml)</td>
<td>20.6</td>
<td>0.9</td>
</tr>
<tr>
<td>Normal spermatozoa (%)</td>
<td>13.0</td>
<td>3.0</td>
</tr>
<tr>
<td>Spermatozoa viability (%)</td>
<td>1.0</td>
<td>40.0</td>
</tr>
<tr>
<td>Spermatozoa immotility (%)</td>
<td>100.0</td>
<td>100.0</td>
</tr>
<tr>
<td>Biochemistry</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Citric acid (μmol/ejaculate)</td>
<td>42.3</td>
<td>98.7</td>
</tr>
<tr>
<td>Fructose (μmol/ejaculate)</td>
<td>24.3</td>
<td>44.6</td>
</tr>
</tbody>
</table>

a: Mean of 5 independent samples

b: One sample analysed

The ratio of sexual chromosomes was 1 : 1 as expected. The percentage of sperm DNA damage was 52.6%, well above the normal range in the fertile population. The constitutional karyotype was normal and no microdeletions were found on chromosome Y in whole blood analysis. Apoptosis biomarkers were detected at higher levels in this patient and positive
controls than in negative controls by Western blotting analysis (Figure 3A). Microscopic fat deposits (Red Oil O) or polysaccharides (PAS) were not observed. Papanicolaou staining showed discrete thickening in the midpiece (images not shown).

Table 2 Aneuploidy studies by FISH: probes for chromosomes 13, 18, 21, X and Y

<table>
<thead>
<tr>
<th>Aneuploidy</th>
<th>Total analysed cells</th>
<th>Total altered cells</th>
<th>Alteration rate (%)</th>
<th>Above reference value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nullisomic</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>13</td>
<td>1 346</td>
<td>59</td>
<td>4.4</td>
<td>✓</td>
</tr>
<tr>
<td>18</td>
<td>2 462</td>
<td>112</td>
<td>4.5</td>
<td>✓</td>
</tr>
<tr>
<td>21</td>
<td>1 346</td>
<td>64</td>
<td>4.8</td>
<td>✓</td>
</tr>
<tr>
<td>X/Y</td>
<td>2 462</td>
<td>37</td>
<td>3.5</td>
<td>✓</td>
</tr>
<tr>
<td>Disomic</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>13</td>
<td>1 346</td>
<td>10</td>
<td>0.7</td>
<td></td>
</tr>
<tr>
<td>18</td>
<td>2 462</td>
<td>20</td>
<td>0.8</td>
<td></td>
</tr>
<tr>
<td>21</td>
<td>1 346</td>
<td>6</td>
<td>0.4</td>
<td></td>
</tr>
<tr>
<td>XX</td>
<td>2 462</td>
<td>20</td>
<td>0.8</td>
<td></td>
</tr>
<tr>
<td>XY</td>
<td>2 462</td>
<td>37</td>
<td>1.5</td>
<td></td>
</tr>
<tr>
<td>YY</td>
<td>2 462</td>
<td>5</td>
<td>0.2</td>
<td></td>
</tr>
</tbody>
</table>

Figure 2 Transmission electron microscopy (TEM) on spermatozoa from patient 1
Patient 2

The light microscopy morphological (Figure 4A) and vitality (Figure 4B) studies showed that 85% of spermatozoa had short flagella. Of those, 65% had thickened rigid flagella and 35% had rudimentary flagella which were not thickened. Only 3% were normal spermatozoa. The unstructured and thickened fibrous sheath together with the hypoplastic and disorganized tail midpiece (Figure 5A) were observed by TEM: the normal central pair of microtubules was absent, leaving the structure known as the ‘9+0’ axoneme (Figure 5B,C), compared
with the normal flagellar structure in the middle piece (Figure 6). Phase contrast and immunofluorescence images of the fibrous sheath showed a hypertrophy of the fibrous sheath in this patient’s samples (Figure 7). Western blotting for β-tubulin also revealed an abnormal microtubular pattern in our patient versus the normal pattern of human sperm (Figure 3B). A ciliary motility test on nose epithelium showed the two characteristic phases and the beat frequency was 9 Hz (normal) and the cellular rotation test showed normal epithelial cell rotation motion, thus a diagnosis of PCD was discarded.

**Discussion**

Here, we present two clinical cases of specific alterations in sperm morphology which result in 100% immobility, and which are likely of a genetic origin: one with thickening of the midpiece and a high rate of DNA fragmentation, and the other with loss of the central pair of microtubules and fibrous sheath dysplasia. We found the low spermatozoa viability of the sample from patient 1, the severe oligozoospermia in patient 2, and the full immotility of the sperm from both patients particularly striking. Citrate and fructose levels in seminal plasma were within the non-pathological range in both patients, which ruled out pathology of the accessory sex glands (prostate and seminal glands).

**Patient 1**

Semen stains did not identify the nature of the abnormal deposit of material in the midpiece so further investigation of the possible origin of this abnormal feature using different methods was required. The apoptotic marker study was the most informative in this sense, showing an increase in typical apoptotic proteins.
Some studies have shown that sperm DNA damage is higher in ejaculated sperm compared with testicular sperm, which reinforces the idea that sperm DNA damage increases in transit from the testis to the epididymis and into the ejaculate\textsuperscript{[16]}. At present we do not know what determines a given cell’s apoptotic potential, and it is not clear whether the apoptotic markers detected in spermatozoa are produced by apoptotic processes starting before or after the ejaculation\textsuperscript{[17]}. However, it is thought that endogenous caspases (cysteinyl-aspartate-specific proteinases) cause apoptosis by inducing DNA fragmentation during spermatogenesis.
This patient had almost absolute necrozoospermia, the aetiology of which is a poorly studied cause of male infertility; hence we found very little literature which could help to explain the pathology we have described. In this case, given that the patient had an infertile brother (who could not be studied) we postulate that the necrozoospermia probably has a genetic origin. The presence of stationary sperms, some with normal and others with abnormal morphology, may indicate that they had undergone apoptotic changes. Furthermore, Burrello et al. suggest that genomic germ cell quality and the sperm remodelling that occurs during
spermatogenesis are not associated, i.e. a germ cell may be aneuploid or have its nucleus altered by apoptosis, whereas a spermatozoa could have a normal morphology. The origin of sperm DNA damage is multifactorial and can be attributed to internal and external testicular factors: abnormal DNA packing during spermatogenesis, environmental toxins, oxidative stress during sperm migration from the seminiferous tubules to the epididymis, DNA fragmentation induced by endogenous caspases/endonucleases. This latter process occurs during spermatogenesis and is caused by an error in the germ cell selection process by Sertoli cells, thus allowing defective germ cells enter meiosis I and appear in the ejaculate, as indicated by the expression of apoptotic spermatozoa\textsuperscript{[19]}. Caspase activation plays an important role in the initiation and activation of sequential apoptotic processes, causing the cleavage of critical cellular substrates, including PARP1, a protein with molecular weight of 116 000 involved in DNA repair which can be cleaved by Caspase-3 \textit{in vivo}\textsuperscript{[20]}. PARP1 specifically binds at DNA strand breaks and helps to maintain cell viability and so its cleavage facilitates cellular disassembly and serves as a cell apoptosis marker. Caspase-3 is activated by cleavage, generating two subunits of 35 000 and 20 000 each. Annexin V, a protein with molecular weight of 30 000, binds specifically to phosphatidylserine whose presence is also related to apoptotic events\textsuperscript{[21]}. Inactive Caspase-3 (35 000) was detected in our patient’s sperm and in positive controls, but was absent in negative sperm controls. Active Caspase-3 (20 000) was also detected in low quantities in the patient sperm but was not in negative controls. Additionally, the presence of Annexin V was more pronounced in the patient than in negative semen controls. Taken together, the presence of apoptosis-related proteins in this patient’s sperm suggests an apoptotic origin for the abnormal deposits of material in the midpiece. Moreover, the preservation of sperm morphology supports an apoptotic origin for the changes observed in this case. Apoptosis, unlike necrosis, is characterised by conservation of the cell structure.

Seminal FISH from the patient 1 showed abnormal meiotic recombination, presence of aneuploidy (nullisomy) and diploidy, so a normal constitutional karyotype does not exclude the presence of anomalies in sperm chromosomes; increase in the frequency of aneuploidy in semen chromosomes of the patients with poor semen quality has been observed\textsuperscript{[22]}. The chromosomic study of the spermatozoa is of great interest and should be seriously considered in infertile couples, mainly in the case of repeated \textit{in vitro} fertilization failure.

Our patient had large numbers of thickened midpiece cells (85%), consistent with the high rate of sperm DNA fragmentation observed (52.6%). Despite the necrozoospermia and morphological alterations, his sperm did not present teratozoospermia: the change did not affect the total sperm population and there was a high percentage of normal forms (13%). This patient was remarkable for this singular accumulation of material in the midpiece of his
sperm, which we observed in the vitality study but which was less evident in the morphological and Papanicolaou stainings. The other cell elements were quite well preserved and the good shape of the head was outstanding.

Proximal centriole abnormalities and degeneration of the midpiece, producing headless sperm, has been described as ‘easily decapitated spermatozoa’ defect[23]. Head separation appears to be the result of a specific morphogenetic process although a patient with head separation from the tail in 100% of their spermatozoa has been described[24], although other authors claim that loose heads are rarely found in semen[25]. The significant number of detached heads found in our patient’s sample, associated with acephalic sperm, is also interesting. We think that sperm head loss in this patient does not represent the extreme phase of head-midpiece disjunction due to misalignment in neck area, but instead that the deposition of abnormal material in the head-tail junctions makes the sperm unusually fragile and frequently results in head separation. This is caused and enhanced by mechanical phenomena when handling these particularly delicate samples in the laboratory.

**Patient 2**

In this case the total sperm immobility and short, rudimentary or rigid and thickened forms of the flagellum drew our attention. Anomalies present in the samples included the absence of central pair doublets and the presence of coiled, short/stump tails but not all the spermatozoa were affected (85%). This incomplete form of flagellar pathologies has already been described by other authors[26,27].

Given the morphological findings described above, and the clinical history (previous surgical intervention on the tympanum), we chose to first check the diagnosis of PCD. Expression of axonemal alterations in sperm tails and respiratory cilia is variable: Chemes et al.[28] described two cases of concomitant ciliary dyskinesia and hyperplasia of the fibrous sheath; Neugebauer et al.[13] reported a patient with 100% immotile spermatozoa in which the central pair of microtubules was missing, a change which was also reflected in 15% of nasal cilia. This defect, called the ‘9+0’ axoneme, is associated with a thickened fibrous sheath.

As Armengot et al.[15] pointed out, video analysis is probably more useful than studying the ultrastructure of cilia when attempting to screen for PCD. Escudier et al.[29] found a normal nasal cilia beat frequency in 84 % of infertile men (11/13), while significant ciliary ultrastructural abnormalities were observed in the nasal cilia of 92% of infertile male patients (12/13), with only one patient in the cohort also suffering severe respiratory disease. However, PCD can only be definitively rejected by findings related to ciliary function, because 30% of PCD patients show normal ultrastructural respiratory cilia[30]. There are also reported cases of discordance between flagella and cilia: immotile sperm with ultrastructural axoneme alterations but normal cilia (motility and morphology) and vice versa[9,31]. The nasal cilia
function in our patient showed a normal ciliary beat frequency and pattern, and no clinical significance in terms of pulmonary involvement was found. Although he had repeated ear infections that required a surgery, together these clinical findings ruled out PCD.

The sperm head-tail attachment in our patient was normal and was not associated with acephalic sperm, unlike observations from similar cases\(^4,32\). Patients with totally immotile spermatozoa due to ‘9+0’ ultrastructural flagellar anomalies have been associated with polycystic kidney disease in approximately 25% of cases\(^33\), although we did not find such an association in our patient. In this case, the lack of central microtubules and hyperplasic extension of the dense outer fibres suggests that the normal fibrous sheath structure may help to maintain the integrity of the axonemal microtubules\(^8\). The flagella morphological findings were compatible with DFS.

Chemes et al. suggested a genetic origin for this DFS\(^25\). Several human flagellar defects have been found in brothers of consanguineous parents, such as the absence of dynein arms, detached tail, ‘9+0’ flagella syndrome, DFS, and short/stump tail, indicating a genetic basis for this particular fibrous sheath anomaly\(^134\). Most sperm anomalies with a genetic basis are determined by recessive autosomal mutations, resulting in abnormal or the complete lack of motility\(^32\), which unfortunately can be transmitted by assisted reproduction techniques\(^12,35\). In our patient’s case, follow up studies following the patient’s genealogical family tree could help to identify inheritance patterns which might associate the abnormal morphology with a genetic cause.

**Conclusions**

As discussed in this paper, the high level of DNA fragmentation and the presence of apoptosis-related proteins in sperm from patient 1 suggest an apoptotic origin for the abnormal deposits of material in the midpiece. On the other hand, the ultrastructural study of the sperm tail showing the absence of the central pair of microtubules together with the normal nasal ciliary test performed in patient 2 ruled out PCD, being these findings consistent with DFS. These features cannot be identified with a basic spermiogram nor with routine biochemical analysis, and so promoting close collaboration between clinical andrology laboratories and biological research centres in order to achieve better diagnoses of these alterations is essential.

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