Value of quantitative ultramorphological sperm analysis in infertile men

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SUMMARY

A specific cause of infertility cannot be identified in at least 25% of men referred to a specialized clinic. Diagnosis of infertile men is based mainly on standard semen analysis and the observation of sperm under light microscope. The aim of our study was to find the subcellular sperm characteristics that could explain infertility in a group of teratozoospermic infertile men. Morphological characteristics of sperm from non–teratozoospermic (control donors) and teratozoospermic infertile men were analyzed by transmission electron microscopy (TEM) and quantified. Our analysis showed that sperm cells from control donors presented a higher number of normal heads and tails
than infertile men. Regarding subcellular characteristics of nucleus and tails, only the percentage of vacuolated nucleus, the absence of at least one pair of microtubules of the axoneme and the total distortion of the tail were statistically higher in infertile men than in control donors. There were no differences in the number of normal acrosomes between the groups. Although the ultrastructural sperm defects overlapped between control donors and infertile men, TEM permits the identification and differentiation of a larger amount of defects than light microscopy. Vacuolated nucleus and gross alterations of the tail are the major sperm defects that seem to have prognostic value in teratozoospermic men. Reproductive Biology, 2010 10 2: 125-139.

Key words: electron microscopy, teratozoospermia, sperm alterations, male infertility, quantification

INTRODUCTION

Between 25 to 50% of men referred to a specialized clinic for the evaluation of fertility have idiopathic infertility meaning that a specific cause for their affection cannot be identified [10, 21]. The imprecision of this diagnosis has spurred the development of numerous semen analysis tests looking for a diagnosis and causes of male infertility [10]. Of all the semen parameters, sperm morphology has consistently been the best indicator of male fertility in vivo and in vitro. Many authors have gone as far as to argue that sperm morphology is a reflection of sperm functional competence [23, 25].

Transmission electron microscopy (TEM) has been advocated as a tool for assessing the structural integrity and the potential effectiveness of sperm [36]. Several studies have found marked ultrastructural differences between the sperm of fertile and infertile subjects including changes in the ultrastructure of the acrosome disparities, in the level of chromatin stability [30] and a consistent reduction in the number and arrangement of axonemal components [19]. When a particular sperm defect predominates in the semen sample such as round-headed [28], large head [16], double heads [20], short tail or dysplasia of the fibrous sheath [4, 11, 12, 13, 14] and abnormal middle piece [29] family associations are usually established and candidate
genes are investigated. The meaning of the ultrastructural alterations found in men without a known genetic cause is more controversial. In spite of the efforts made by several authors to standardize and quantify the ultrastructural defects of sperm from fertile and infertile men, it has been difficult to define a clear limit between both categories.

Here, we present the TEM findings in patients with idiopathic teratozoospermia in pure male-factor infertility with no indication of genetic causes. The aim of this study is to show and quantify the most common ultrastructural characteristics of sperm cells found in teratozoospermic men.

MATERIAL AND METHODS

Subjects

The protocol was approved by the School of Medicine Ethics Committee, Montevideo, Uruguay. Informed written consent was obtained from men prior to collection of biological material. The study’s subjects were 25 men in the age between 21 to 43 years old. The samples were collected at the School of Medicine Montevideo, Uruguay between October 2005 and May 2009. The patients (n=15) were men who consulted in the Unit of Human Reproduction at the Pereira-Rossell Hospital Center (UHR-PRHC) and who had had at least two examinations of semen samples showing abnormal sperm morphology (teratozoospermia, morphologically normal sperm forms ≤14%) [24, 25]. None of them had a child of their own and all of them were classified as having idiopathic infertility. Patients with varicocele or endocrine disorders were specially discarded. No genetic or family history of male infertility was noted. Some patients were excluded because their female partners had possible causes of infertility (ovulatory dysfunctions, anatomic or cervical factors). The major criterion for ordering TEM of the ejaculate was unexplained abnormalities of sperm morphology. Thus, all patients in the study had impaired sperm morphology and nine of them also had motility defects. Control donors (n=10) were men who reported having fathered at least one child of their own. Their semen samples revealed normal sperm morphology.
Semen analysis

Semen samples were obtained by masturbation after 2 to 7 days of sexual abstinence. The ejaculate was allowed to liquefy at 37°C. Sperm concentration and motility were evaluated using MicroCell counting chambers (Conception Technologies, San Diego, California). Normal values for semen analysis were based on the World Health Organization (WHO) standard criteria [34] i.e. seminal volume $\geq 1.5$ ml, sperm concentration $\geq 20\times10^6$/ml, $\geq 50\%$ spermatozoa with rapid (category a) + slow (category b) progressive motility or $\geq 25\%$ male gametes with rapid progressive motility, $>14\%$ morphologically normal sperm forms. The morphology evaluation of Shorr [In Vitro Diagnostic Medical Device (IVD); Merck KGaA, Germany] stained spermatozoa was based on Kruger’s strict criteria-Tygeberg’s strict criteria [24, 26] recommended by WHO [34]. One hundred randomly chosen spermatozoa per smear were classified at 1000× magnification (oil immersion; Nikon ECLIPSE E200 brightfield microscope). Each patient had at least two semen analyses available for review (mean: 2.3, range: 2 to 5). The values of each characteristic were averaged (arithmetic mean). An aliquot of each sample was preserved for TEM examination.

Electron microscopic analysis of sperm

After liquefaction, semen samples were centrifuged at 5000 g for 5 minutes and fixed overnight at 4°C in a mix-solution of 4% (w/v) paraformaldehyde (PAF) in phosphate buffer (PB) with 2.5 % (v/v) glutaraldehyde, pH 7.2 to 7.4. Sperm were subsequently post-fixed in 1% (w/v) osmium tetroxide for 1 hour, dehydrated in ascending graded ethanol solutions (25, 50, 75, 95 and 100% v/v), followed by 100% acetone and embedded in Araldite resin. Ultrathin sections (50-70 nm) were made on a RMC MT-X ultramicrotome (RMC Products, Arizona, USA) with a DIATOME diamond knife and contrasted with uranyl acetate followed by lead citrate, and examined with a JEOL JEM-1010 (Jeol, Japan) transmission electron microscope at 80 kV. The images were obtained with a Hamamatsu C-4742-95 digital camera (Hamamatsu Photonics UK Limited, United Kingdom) and processed with
the Photoimpact program. At least 100 sections of sperm heads and tails were analyzed in each sample. The TEM characteristic of the organelles were classified based on Baccetti et al [2] and Chemes and Rawe [12] counted and expressed as mean±SEM of the percentage of the total sections observed from each individual.

**Statistical analysis**

Statistical analysis was performed using the Sigma Stats package (SigmaStats® Systat Software). The means of sperm characteristics (concentration, morphology and motility) as well as means of percentage of TEM categories from control donors versus infertile men were analyzed. The Mann-Whitney-Rank-Sum-Test was used to compare the means of sperm concentration and morphology, while sperm motility and TEM categories were analyzed by Student’s t-test. All tests were two-tailed with statistical significance assessed at the p<0.05 level.

**RESULTS**

**Light Microscopy and Semen Characteristics**

Sperm characteristics (concentration, morphology and motility) of each control donor and patient are shown in Table 1. Isolated teratozoospermia (T, only sperm morphology defect) was found in five patients, asthenoteratozoospermia (AT, motility and morphology defects) in three patients, oligoteratozoospermia (OT, concentration and morphology defects) in one patient and oligoasthenoteratozoospermia (OAT, concentration, motility and morphology defects) in six out of 15 patients. Nine control donors exhibited normal sperm concentration, motility and morphology (N, normozoospermia). Oligoasthenozoospermia (OA, concentration and motility defects) was noted in one out of ten control donors (tab. 1). As expected, concentration of sperm cells (158±39×10⁶/ml vs. 51.5±19×10⁶/ml) and percentage of normal
sperm forms (24.5±1.9% vs. 5.9±1.0%) were significantly higher in control donors than in patients (p<0.05). No significant difference was found in the rapid+slow progressive motility (a+b) of spermatozoa between control donors and patients (57.1±5.3% vs. 41.9±5.6%).

Electron Microscopic Analysis of the Spermatozoa

A total of 3058 sperm sections from donors and 4254 from patients were evaluated. The number of normal sperm sections from fertile men was 1.6 higher than that from patients (48.5±1.5% vs. 31.9±3.3%; p<0.05). Of the total sperm sections in donors, 1091 corresponded to sections of heads and 1967 of tails. In patients, 1569 sections of heads and 2685 of tails were analyzed.

Heads were considered normal if plasma membrane, nuclear envelope, acrosome and chromatin were regular in form, position and appearance. Mean percentage of normal heads from donors was 2.5 times higher than that from patients (17.5±4.0% vs. 7.5±1.6%; p<0.05). Tail sections were considered normal when axonemal components were well organized and mitochondria and plasma membrane were complete and had not swelled. The mean percentage of normal tails from donors was 1.4 times higher than that from patients (66.6±2.3% vs. 46.6±3.9%; p<0.05).

Figures 1 and 2 show the sperm defects found in both control donors and patients. Head sperm ultrastructural defects (fig. 1) were divided in nuclear and acrosomal abnormalities. In some sections, both organelles were altered (fig. 1a-e). Severely damaged nuclei were observed. The normal compact arrangement of the chromatin was replaced by either granular chromatin (fig. 1a–f) and/or large vacuoles (fig. 1a, d and f). Binucleated sperm with chromatin usually granular or vacuolated were considered as another anomaly of the nucleus (fig. 1c). Acrosome abnormalities were classified as follows: complete absence of the organelle, altered shape (fig. 1a and c), reduced size (miniacrosome) and dispersed acrosomal content (fig. 1d) or in an unusual disposition far from the nucleus (fig. 1b and e).

In the cross sections of tails, the characteristic well organized structure of the flagellum was frequently disrupted (fig. 2). Six types of flagellar
Figure 1. Electron micrographs of sperm head defects; a/ altered shape of the acrosome, granular chromatin; b/ unusual disposition of the acrosome displaced far from the nucleus, granular chromatin; c/ binucleated head, granular chromatin; d/ severe vacuolar defect of the chromatin, granular chromatin, reacted acrosome with dispersion of its content; e/ round-headed spermatozoa with granular chromatin and altered acrosome position, abundant residual cytoplasm around the nucleus; f/ vacuolar defect of the chromatin, granular chromatin. Ac: acrosome, Ax: axoneme, M: mitochondria, V: vacuolar defect of the chromatin, *: residual cytoplasm; scale bars: a, b, c, d, e = 0.1 μm, f = 0.5 μm.
Figure 2. Electron micrographs of sperm tail defects; cross sections through the principal piece (a–d, f) and midpiece (e); a/ supernumerary doublets (arrow) and duplication of the central pair (arrow head); b/ absence of axonemal doublets and microtubular translocation; c/ group of flagella covered by the same plasma membrane, one of them presents an incomplete axoneme; d/ hemiaxoneme. e/ absence of axonemal central structures (9+0). f/ Misassembled axonemal structures; CP: central pair of microtubules, FS: fibrous sheet, MS: mitochondrial sheet, MT: microtubules, ODF: outer dense fibers, PM: peripheral microtubules; scale bars: a, c, d, f = 0.1 μm, b and e = 0.5 μm.
abnormalities were defined: the presence of extra pairs of microtubules (fig. 2a), absence of one or more peripheral microtubular doublets (fig. 2b, c), presence of half of the axoneme – hemiaxoneme (fig. 2d), absence of the central pair of microtubules (9+0; fig. 2e), displacement of the axonemal or periaxonemal structures to one side and total disorganized axonemal or periaxonemal structures (fig. 2f). Residual cytoplasm containing a clump of mitochondria, large vacuoles and axonemal components was observed in some spermatozoa (fig. 1b and e). Mitochondria appeared to be normal in almost all of the sections (data not shown).

Quantification of the sperm characteristics is shown in Table 2. As expected, control donors showed higher values of normal chromatin and axoneme (p<0.05) than infertile men. Patients and control donors do not differ in the number of normal acrosomes. Both, control donors and patients have a great number of nuclei showing granular chromatin but only the percentage of vacuolated nucleus with empty chromatin areas differed statistically between groups (p<0.05). Acrosome alterations tended to be heterogeneous and very abundant in both, control donors and infertile men. None of the analyzed characteristics reached statistically significant differences between groups. Regarding analysis of tails, only total distortion of the tail and absence of at least one pair of microtubules showed significant differences between groups (p<0.05; tab. 2).

DISCUSSION

Sperm morphology assessment has been considered a valuable and stable method for predicting the in vivo and in vitro sperm fertilizing ability [24, 25]. Conventional light microscopy tests cannot identify the entire variety of morphologic defects that may occur in sperm organelles, head structures and tail organization [5, 6, 36]. Transmission electron microscopy has been widely used to analyze the characteristics of sperm cell detecting shape, substructure and location of organelles [2]. Our TEM analysis revealed that semen samples from control donors and infertile men exhibited the same types of sperm abnormalities. In addition, regardless of control
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donor or patient, a great heterogeneity of sperm morphology was observed in each individual indicating a common phenomenon for human sperm. Our data are in agreement with the results previously reported on variations in semen morphology observed in the healthy male population and assessed by TEM [27] or a combination of several techniques [15].

Predisposition to infertility should be considered only when the number of anomalous spermatozoa reaches a large proportion of the total number of cells. Using light microscopy the threshold between fertile and infertile men based on sperm morphology has been controversial. In the absence of an absolute specific value, it is accepted that when sperm-normal morphology falls below 15% of normal sperm forms the rate of fertilization in vitro decreases [24, 25, 26].

At TEM level, the establishment of a limit that defines male pathology has been even more difficult. It is well known that statistics obtained by TEM examination of ultrathin sections are extremely imperfect and questionable, particularly in long and tortuous cells, such as spermatozoa, because of the impossibility of determining whether different sections of one or several tails are observed in the various slices [2]. However, some authors reported the advantage of quantitative ultramorphology analysis of sperm subcellular organelles on the basis of TEM and scanning electron microscopy observations over routine morphological assessment [5, 6]. Other researchers elaborated a mathematical formula based on EM observations for determining natural male fertility potential (fertility index; [2]).

We have been able to establish the alterations of sperm appearance that are significantly more frequent in a population of idiopathic infertile men than in control fertile donors. These data could be useful to patients who are concerned not only about their possibilities to conceive but also about the causes of their infertility. The new concept of sperm pathology tries to characterize structural and functional deficiencies in abnormal spermatozoa seeking an explanation for the causes of male infertility [12]. Applying this concept, Chemes and Rawe [12] distinguished two main groups of abnormal spermatozoa forms. In the first and more frequent variety, a heterogeneous combination of different alterations is found randomly distributed in each individual and among different patients.
These alterations can be referred to as nonspecific or non-systematic sperm defects. The second variety, called systematic, presents a characteristic anomaly that affects the vast majority of spermatozoa in a semen sample. Systematic alterations tend to show family clustering and have proven or suspected genetic origin. As expected, in the studied patients, nonsystematic sperm defects were found in men without genetic history. However, we discovered that these sperm abnormalities were unevenly distributed between fertile and infertile individuals.

Interestingly, the number of vacuolated nuclei was higher in patients than control donors. The functional significance of this alteration is not clear, even though the presence of a large vacuole, single or multiple, empty or filled with membranous materials, has a negative association with natural male fertility potential [8, 11, 35]. Recently, Franco et al [18] established that genomic DNA damage occurs more frequently in spermatozoa with large nuclear vacuoles (LNV) suggesting precocious decondensation and disaggregation of sperm chromatin fibers which probably explains the low rate of fertility in sperm with LNV.

The presence of a high number of sperm with vacuolated nuclei in patient semen may have a practical implication. Sperm-carrying vacuoles can be avoided to be chosen for intracytoplasmic sperm injection (ICSI). Bartoo et al [7] developed a method of sperm selection for ICSI based on high magnification motile sperm organellar morphology examination (MSOME). Although this application is under investigation, the use of MSOME did result in significantly higher pregnancy rates compared with conventional in vitro fertilization – ICSI [8, 9].

There was also an increase in some of the analyzed abnormalities in spermatozoa tails of infertile men compared with control donors. Gross abnormalities, like total distortion of the usually well organized tail structure, tended to be more frequent in patients. Subcellular analysis of other organelles showed fewer differences between healthy and infertile men. The fact that fertile and infertile men display an equal number of absent or dispersed content of the acrosomes could reflect that acrosome reaction has been triggered uniformly in both groups. It might be interpreted more as a physiological than a pathological issue.
In order to identify the molecular basis of abnormal sperms, efforts should be focused on detecting candidate factors that can explain the defects of chromatin condensation and normal tail arrangement. This notion is based on the idea that the high frequency of abnormal heads and gross defective tails in patients may be caused by disturbances during spermatogenesis. Such disturbances may impair production of a sufficient number of normal sperm cells to achieve fertility. It is known that spermatozoa with chromatin abnormalities have diminished fertility potential and could be associated with abortions during the first trimester of pregnancy. Various methods were developed to detect chromatin abnormalities e.g. aniline blue staining of histones, flow cytometry after staining with acridine orange, TUNEL assay and ultrastructural examination of spermatozoa [3, 11, 17, 31, 36]. Integrity and functionality of the sperm tail, especially those of the mitochondrial middle piece have also been evaluated using fluorescent markers like MitoTracker and MitoFluor mitochondrion-selective probes [1, 12, 33]. Although TEM may be considered expensive or time-consuming, is currently the only tool available to analyze the subcellular characteristics of sperm cells [2, 32]. TEM coupled with the above mentioned techniques should be employed to characterize sperm pathologies and establish a diagnosis [12].

Finally, ethnics and epidemiology differences should be also considered in order to find causes of infertility [22]. The prevalence of men infertility in Uruguay seems to be similar to those reported by authors of other countries; 43% to 48% of couples that consulted the UHR-PRHC in 2006-2009 had a male identified cause (unpublished data). However, there is not enough data to discard the notion that ethnic or demographic differences are related to the causes of male infertility. Very few studies were performed concerning this issue in the Latin-American population.

In summary, we propose a straightforward method of classification and quantification of sperm anomalies found by TEM to expand the routine analysis of sperm parameters. Using this approach chromatin vacuolation and gross tail alterations seem to be the best predictive characters of infertility in teratozoospermic men. However, more studies should be completed in healthy fertile men in order to set up limits between fertile and infertile men.
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REFERENCES


