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ORIGINAL ARTICLE

Large nuclear vacuoles are indicative of abnormal chromatin packaging in human spermatozoa

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Summary

The aim of this investigation was to determine the presence of abnormal sperm chromatin packaging in spermatozoa with large nuclear vacuoles (LNV) selected via high magnification by analysing the pattern of chromomycin A3 (CMA3) staining. A prospective observational study was designed to analyse semen samples obtained from 66 men undergoing infertility diagnosis and treatment. The numbers of cells with normal (dull yellow staining of the sperm head/CMA3-negative) and abnormal (bright yellow fluorescence of the sperm head/CMA3-positive) chromatin packaging were determined on slides with normal and LNV spermatozoa. The presence of bright yellow fluorescence (CMA3-positive) was significantly higher ($p < 0.0001$) in spermatozoa with LNV than in normal spermatozoa (719/1351; 53.2% vs. 337/835; 40.3%, respectively), reflecting a higher percentage of abnormal chromatin packaging in spermatozoa with large LNV. Our data support the hypothesis that the presence of LNV reflects the presence of abnormal chromatin packaging, which may facilitate sperm DNA damage. As sperm nuclear vacuoles are evaluated more precisely at high magnifications using motile sperm organelle morphology examination (MSOME), the present results support the use of high-magnification sperm selection for intracytoplasmic sperm injection (ICSI).

Introduction

Sperm DNA damage is detrimental to reproductive outcomes, and there is clinical evidence that the spermatozoa of infertile men have substantially more DNA damage than those of fertile men (Zini & Libman, 2006). A morphological classification system related to DNA fragmentation or denaturation of human spermatozoa would be of great clinical value in improving reproductive outcomes in assisted reproduction technologies. Bartoov *et al.* (2002, 2003) have developed a method called motile sperm organelle morphology examination (MSOME) to evaluate human spermatozoa in real time at high magnification. MSOME is performed using an inverted microscope equipped with Nomarski interferential contrast optics, which enables a magnification of >6000×, compared with 200× to 400× magnification used for conventional intracytoplasmic sperm injection (ICSI) (Bartoov *et al.*, 2002). This method facilitated the development of intracytoplasmic morphologically selected sperm injection (IMSI), which is based on sperm normality as defined by the MSOME classification system and aims to improve conventional ICSI outcomes by focusing on the correlation between DNA damage and sperm morphological abnormalities observed at high magnification (Bartoov *et al.*, 2003; Antinori *et al.*, 2008; Franco *et al.*, 2008; Nadalini *et al.*, 2009; Oliveira *et al.*, 2009, 2010). The mechanisms proposed to explain the aetiology of DNA damage include apoptosis, oxidative stress and abnormal chromatin packaging (Ménézo *et al.*, 2010; Miller *et al.*, 2010). There is evidence that DNA damage may derive from abnormal chromatin packaging due to underprotamination, which induces DNA strand breaks (Sakkas *et al.*, 1998; Esterhuizen *et al.*, 2000; Plastira *et al.*, 2007). Among the nuclear abnormalities related to DNA damage observed during MSOME classification were large nuclear

vacuoles (LNV), especially those occupying more than 50% of the nuclear area (Franco *et al.*, 2008; Oliveira *et al.*, 2010).

Chromomycin A3 (CMA3), a polymerase inhibitor, is used in another indirect approach for the evaluation of normal protaminosis. Based on the in situ competition between protamine and CMA3, this assay is inversely correlated with the protamination state of spermatozoa (Bizzaro *et al.*, 1998). This inverse correlation means that incorporation of CMA3 can prevent the accessibility of DNA polymerase I to the DNA. Almost none of the CMA3-negative spermatozoa present nicked DNA because CMA3 is unable to access their DNA; in addition, there are indirect signs of sufficient protamination and correct disulphide bonds (Manicardi *et al.*, 1995; Bianchi *et al.*, 1996; Lolis *et al.*, 1996). In light of these data, this investigation was designed to use CMA3 staining to determine the presence of normal and abnormal sperm chromatin packaging in spermatozoa with LNV.

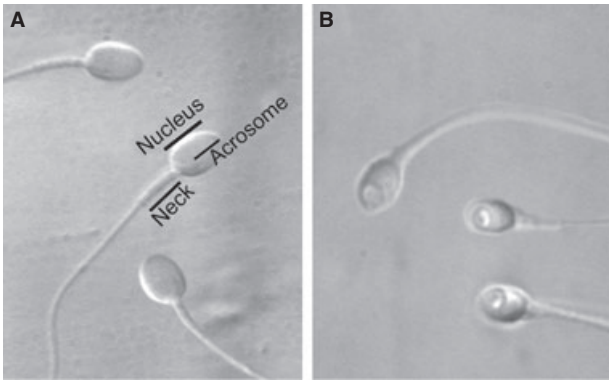

Figure 1 (A) Normal spermatozoa observed at high magnification (8400×); (B) Spermatozoa with large nuclear vacuoles at high magnification (8400×).

Materials and methods

Study participants and sperm preparation

Fresh semen samples (one per patient) from 66 patients in an unselected group of couples undergoing infertility investigation and treatment at the Centre for Human Reproduction Professor Franco Junior were prepared by the Isolate (Irvine Scientific, Santa Ana, CA, USA) discontinuous concentration gradient. The final pellet was resuspended in 0.2 mL of modified human tubal fluid (HTF) medium (Irvine Scientific). A 1-μL aliquot of sperm cell suspension was transferred to a 5-μL microdroplet of modified HTF medium containing 7% polyvinylpyrrolidone solution (PVP medium; Irvine Scientific). This microdroplet was placed in a sterile glass dish (Fluoro-Dish; World Precision Instruments, Sarasota, FL, USA) under sterile paraffin oil (Ovoil-100; VitroLife, Goteborg, Sweden). The sperm cells suspended in the microdroplet were placed on a microscope stage above an Uplan Apox oil/1.35 objective lens previously covered by a droplet of immersion oil. In this manner, motile spermatozoa suspended in the observation droplet could be examined at high magnification using an inverted microscope (Eclipse TE 2000 U Nikon; Nikon Corporation, Tokyo, Japan) equipped with high-power differential interference contrast optics (DIC/Nomarski; Nikon, Nikon Corporation, Tokyo, Japan). The total calculated magnification was 8400× (objective magnification = 100×; magnification selector = 1.0×; video coupler magnification = 1.0×; calculated video magnification = 84.50×). Spermatozoa with normal nuclei (Fig. 1A) and those with large vacuoles (Fig. 1B) were selected by means of a hydraulic micromanipulation system with angled glass micropipettes and placed on slides. Spermatozoa were deposited over a small area that was marked on the back of the slide with a glass pen to help locate the spermatozoa under the microscope. During high-magnification evaluation, a spermatozoon was classified as morphologically normal, when it exhibited a normal nucleus, acrosome, post-acrosomal lamina, neck and tail as well as the absence of a cytoplasmic droplet or cytoplasm around the head (Bartoov *et al.*, 2002). The morphological state of the nucleus was defined by the form and content of the chromatin. The criterion for normality of nuclear form was a smooth, symmetric and oval configuration. Normal means for length and width of the sperm head were estimated to be 4.75 ± 2.8 and 3.28 ± 0.20 μm (Bartoov *et al.*, 2002), respectively. For rapid evaluation of nuclear form, a fixed, transparent, celluloid form of a sperm nucleus that fit the criteria was superimposed on the examined cell (chablon construction based on ASTME1951-02).

Large nuclear vacuoles spermatozoa were defined (Bartoov modified classification) by the presence of one or more vacuoles occupying ≥50% of the sperm nuclear area.

Determination of sperm chromatin packaging by CMA3

The spermatozoa were analysed for chromatin packaging using CMA3 staining. Slides with different spermatozoa (containing normal and LNV) were air-dried and fixed in methanol/glacial acetic acid (3 : 1) for 20 min at 4 °C. These slides were allowed to air dry at room temperature (20 min) and were treated for 20 min with 100 μL CMA3 solution (0.25 mg/mL CMA3 in McIlvaine's buffer, containing 10 μM $MgCl_2$) and then rinsed in PBS. On each slide, the number of cells with normal (dull yellow staining/CMA3-negative) and abnormal (bright yellow

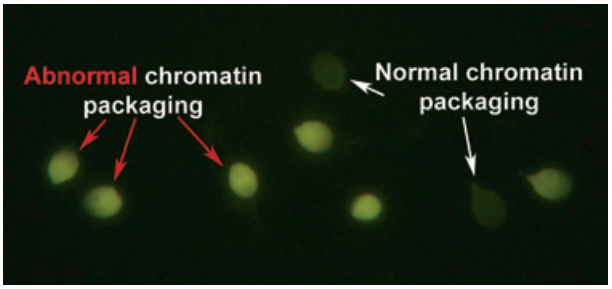

Figure 2 Large nuclear vacuoles with negative (normal) and positive (abnormal) chromomycin A3 staining.

fluorescence of the sperm head/CMA3-positive) chromatin packaging was determined (Fig. 2).

Sample size and statistical analysis

Sample size was calculated by comparing the control and experimental proportions. Thus, a sample size of 400 spermatozoa in each group has 80% power to detect an increase of 10% (normal total sperm values ≤45% of positive sperm chromomycin A3 levels) with a significance level of 0.05 (two-tailed). Only 15–20 normal spermatozoa and 25–40 spermatozoa with LNV were assessed per sample for each test; this may have increased the error. However, the technical difficulties in the selection and evaluation processes should be stressed. First, in our general population, the frequency of normal spermatozoa was relatively low (1.5–2.5%). Second, the spermatozoa from each morphological form in the same patient must be selected one at a time under high magnification, a time-consuming process. Finally, there were losses of spermatozoa during the fixing and staining procedures. These limitations resulted in a small sperm sample for analysis. In contrast, the increase in the number of individuals studied (66 men) diversified the total sperm samples and may have reduced the error. The data were analysed using InStat version 3.0 (GraphPad Software, San Diego, CA, USA) on a Macintosh computer (Apple Computer Inc., Cupertino, CA, USA). A chi-square test was used to determine statistical significance.

Results

The general characteristics of the study population are summarized in Table 1. Table 2 displays the values for abnormal chromatin packaging (seen by CMA3 staining) in spermatozoa with LNV (719/1351; 53.2%). The numbers of spermatozoa with CMA3 staining were significantly higher ($p < 0.0001$) than normal spermatozoa (337/835; 40.3%).

Table 1 General characteristics of the men in the studied population

Characteristics	
Patients	66
Age (years)	37.8 ± 6.5
Duration of infertility (years) (mean ± SD)	4.0 ± 3.9
Aetiology (%)	
Male factor	57
Tubal	11
Idiopathic	12
Endometriosis	14
Ovulatory	6
Infertility (%)	
Primary	60.6
Secondary	39.4
Spermocytogramme	
Motility (% spermatozoa) (rapid + slow progression)	61.3 ± 14.6
Total sperm count (10^6/mL)	65.3 ± 42.6
Morphology*	
Normal forms (%)	1.04 ± 0.96
Spermatozoa with large nuclear vacuoles (occupying ≥50% of the nuclear area) (%)	30.1 ± 17.8
Vitality (%) (mean ± SD)	71.4 ± 12.9
Leucocytes in semen (10^6) (mean ± SD)	0.3 ± 0.4

*Categorized according to motile sperm organelle morphology examination (MSOME) criteria for assessment of morphology.

Table 2 Chromomycin A3 values in normal spermatozoa and in those with large nuclear vacuoles

	Number of spermatozoa	
Chromomycin A3	Normal	Large nuclear vacuoles
Positive (bright yellow)	337	719
Negative (dull yellow)	498	632

$p < 0.0001$.

Discussion

There is much room for improvement in assisted reproductive technologies (ART), considering that 8 in 10 embryos transferred do not implant and two in three IVF/ICSI cycles do not result in pregnancies (Seli *et al.*, 2010). Moreover, there is a concern about higher malformations resulting from ICSI cycles, due to the possibility of iatrogenic transmission of genetic abnormalities to the offspring (Hindryckx *et al.*, 2010). Studies comparing ART cycles and natural births suggest that infants conceived by IVF/ICSI techniques have three times a risk of a congenital heart defect (Wen *et al.*, 2010) as well as a higher risk of autosomal and gonosomal aneuploidies (Alukal & Lamb, 2008), cardiovascular and musculoskeletal

defects (Olson *et al.*, 2005), male reproductive abnormalities such as hypospadia (Funke *et al.*, 2010) and rare epigenetic abnormalities related to imprinting disorders, such as Beckwith-Wiedemann and Angelman syndromes (Maher *et al.*, 2003). Although it is still not clear whether these anomalies are related to the ART procedures or to the underlying infertility population itself, it is evident that gametes that are not only morphologically but also genetically normal should be chosen for fertilization. The introduction of MSOME to select human spermatozoa has brought benefits to ART procedures in the last several years because this method produces better outcomes with regard to fertilization rates (Junca *et al.*, 2004; Yazbeck *et al.*, 2008), embryo quality (Bartoov *et al.*, 2003; Berkovitz *et al.*, 2006a; Hazout *et al.*, 2006; Yazbeck *et al.*, 2008), development to the blastocyst stage (Vanderzwalmen *et al.*, 2008; Yazbeck *et al.*, 2008), implantation and pregnancy rates (Bartoov *et al.*, 2003; Junca *et al.*, 2004; Berkovitz *et al.*, 2005, 2006b; Hazout *et al.*, 2006; Antinori *et al.*, 2008; Vanderzwalmen *et al.*, 2008; Tasaka *et al.*, 2009), chances of having a healthy, normal child (Berkovitz *et al.*, 2007) and lower miscarriage rates (Bartoov *et al.*, 2003; Berkovitz *et al.*, 2005, 2006a; Antinori *et al.*, 2008). These observations may be explained by the possession of hidden anomalies by apparently normal spermatozoa visualized under relatively low magnification in conventional ICSI; these anomalies might be detected with the higher magnification used in MSOME.

Large nuclear vacuoles are specific sperm alterations observed under high magnification, and their presence has been related to poor outcomes in ART (Berkovitz *et al.*, 2006a) and an increase in DNA fragmentation and denaturation (Franco *et al.*, 2008; Garolla *et al.*, 2008). Recently, Perdrix *et al.* (2011) showed that DNA fragmentation was not increased in spermatozoa with LNV. To explain this contradiction with the published data (Franco *et al.*, 2008; Garolla *et al.*, 2008), two hypotheses could be proposed. First of all, the absence of any selection on native spermatozoa before DNA fragmentation (Perdrix *et al.*, 2011) could introduce an important methodology bias: dead spermatozoa were present in original semen samples, but not in vacuolated spermatozoa (selection after sperm preparation) probably affecting terminal deoxyribonucleotidyl transferase)-mediated dUTP nick-end labeling results (Barrat *et al.*, 2010). Second, there are two different criteria for LNV: Franco *et al.* (2008) defined LNV by the presence of one or more vacuoles occupying ≥50% of the sperm nuclear area and Perdrix *et al.* (2011) defined LNV by the presence of a vacuole occupying >13% of the sperm nuclear area. On the other hand, a recent investigation has related LNV to the absence of the acrosome reaction, which could explain the hampering of embryo development when a spermatozoon with this characteristic is chosen for microinjection (Kacem *et al.*, 2010). Nevertheless, it is well established that the capacity of human spermatozoa to fertilize an oocyte and produce an embryo with a high potential for implantation and development depends, among other factors, on DNA integrity (Garolla *et al.*, 2008). Among the mechanisms producing DNA damage, abnormalities in chromatin packaging play an important role. By conferring a highly stable structure on chromatin, the replacement of histones by protamines is one of the major mechanisms of DNA protection in the chromatin of human spermatozoa (Manicardi *et al.*, 1995). Sperm chromatin packaging protects DNA from chemical and physical damage; therefore, abnormal protamination may create a favourable environment for DNA damage (Tarozzi *et al.*, 2009). CMA3 is a fluorochrome specific for guanine–cytosine-rich sequences of DNA and has been shown to be a useful tool to assess chromatin packaging (Bianchi *et al.*, 1996). CMA3 positivity, determined by the presence of bright yellow fluorescence in the sperm head, is indicative of protamine-depleted spermatozoa because CMA3-positive staining does not occur in the presence of protamines and normally formed disulphide bonds (Bianchi *et al.*, 1993, 1996).

This investigation was designed to analyse whether LNV are associated with abnormal chromatin packaging by determining the CMA3 staining status of normal and vacuolated spermatozoa. Our results showed that CMA3-positive staining was significantly higher in spermatozoa with LNV than in normal spermatozoa. More than 50% of vacuolated spermatozoa showed CMA3 positivity, suggesting that in these spermatozoa, there was deficient protamination leading to abnormal chromatin packaging. A greater percentage of spermatozoa with positive staining might be vacuolated if it were possible to make the inverse correlation. DNA damage related to deficient protamination may help explain the poorer outcomes in human reproduction after vacuolization of nuclear spermatozoa is observed (Berkovitz *et al.*, 2006b). Therefore, it is important to look for this type of alteration in semen samples destined for ICSI treatment. Moreover, distinguishing spermatozoa with normal morphology from those with LNV using MSOME could be a new and efficient method of semen analysis and selection of spermatozoa with low DNA damage, as recently suggested by our group (Oliveira *et al.*, 2009, 2010). At the moment, these data support the hypothesis that LNV could reflect the presence of molecular anomalies, such as abnormal chromatin packaging, which would facilitate DNA damage in spermatozoa. This fact may be relevant for ART outcomes because the data indicate that the use of a spermatozoon with LNV should be avoided, whenever possible, to obtain embryos with a higher potential for

implantation and development. As LNV are identified at high magnification by MSOME, the present results support the use of MSOME and injection of motile spermatozoa at high magnification during ICSI, given that spermatozoa with LNV are not identified with precision at the usual magnifications employed.

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