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ORIGINAL ARTICLE

Significance of extruded nuclear chromatin (regional nuclear shape malformation) in human spermatozoa: implications for ICSI

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Summary

The aim of this study was to determine the extent of DNA fragmentation and the presence of denatured single-strand or normal double-strand DNA in spermatozoa with extruded nuclear chromatin (ENC) selected by high magnification. Fresh semen samples from 55 patients were prepared by discontinuous isolate concentration gradient. Spermatozoa with normal nucleus (NN) and ENC were selected at 8400× magnification and placed on different slides. DNA fragmentation was determined by TUNEL assay. Denatured and double-stranded DNA was identified by the acridine orange fluorescence method. DNA fragmentation was not significantly different ($p = 0.86$) between spermatozoa with ENC (19.6%) and those with NN (20%). However, the percentage of spermatozoa with detectable denatured-stranded DNA in the ENC spermatozoon group (59.1%) was significantly higher ($p < 0.0001$) than in the NN group (44.9%). The high level of denatured DNA in spermatozoa with ENC suggests premature decondensation and disaggregation of sperm chromatin fibres. The results show an association between ENC and DNA damage in spermatozoa, and support the routine morphological selection and injection of motile spermatozoa at high-magnification intracytoplasmic sperm injection.

Introduction

It has been reported that using intracytoplasmic morphologically selected sperm injection (IMSI), based on sperm normality as determined at high magnification (≥6000×) (Bartoov *et al.*, 2002), resulted in significant increases in the fertilization rate (Junca *et al.*, 2004), embryo quality (Bartoov *et al.*, 2003; Berkovitz *et al.*, 2006a; Hazout *et al.*, 2006), development rate through the blastocyst stage (Vanderzwalmen *et al.*, 2008), rates of implantation and pregnancy after transference on days 2 and/or 3 (Bartoov *et al.*, 2001, 2003; Junca *et al.*, 2004; Berkovitz *et al.*, 2005, 2006a,b; Hazout *et al.*, 2006; Antinori *et al.*, 2008) and/or blastocyst stage (Vanderzwalmen *et al.*, 2008) and the chance of having a healthy normal child (Berkovitz *et al.*, 2007), while the miscarriage rate was significantly decreased (Bartoov *et al.*, 2003; Berkovitz *et al.*, 2006a,b; Antinori *et al.*, 2008). Nevertheless, it is not unusual that during the IMSI procedure, morphologically normal spermatozoa are either not found or are less numerous than the number of oocytes being injected. Thus, the necessity of using sperm with some nuclear morphological alteration raises the importance, in clinical prognosis, of understanding the implications of the dimorphisms evidenced at high magnification. Nevertheless, with the exception of large nuclear vacuoles (Franco *et al.*, 2008; Garolla *et al.*, 2008), the specific significance of each particular nuclear sperm malformation for embryonic development remains undefined.

To comprehend better the clinical value of high-magnification sperm selection, we aimed at determining the presence or absence of DNA damage in spermatozoa with extruded nuclear chromatin (regional nuclear shape malformation) as selected by high magnification. These spermatozoa were submitted to DNA fragmentation analysis. The presence of single-stranded (denatured)

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DNA was also determined by acridine orange fluorescence (AOF).

Materials and methods

Study participants and sperm preparation

Fresh semen samples (one per subject) from 55 patients in an unselected group of couples undergoing infertility investigation and treatment at the Centre for Human Reproduction Professor Franco Jr. were prepared by 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% polyvinyl pyrrolidone solution (PVP medium; Irvine Scientific). This microdroplet was placed in a sterile glass dish (FluoroDish, Word Precision Instrument, 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 Apo 100× oil/1.35 objective lens previously covered by a droplet of immersion oil. In this manner, suspended motile spermatozoa 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). The total calculated magnification was 8400× (total magnification: objective magnification = 100 × magnification selector = 1.0 × video coupler magnification = 1.0 × calculated video magnification = 84.50). Spermatozoa with normal nucleus (NN/control group; Fig. 1A) and those with extruded nuclear chromatin (ENC; Fig. 1B) were selected by means of a hydraulic micromanipulation system with angled glass micropipettes and placed on different slides. Spermatozoa were deposited over a very small area, which was marked on the back of the slides with a glass pen to help locate the spermatozoa under the microscope.

At high-magnification evaluation, a spermatozoon was classified as morphologically normal (NN) when it exhibited an NN, acrosome, post-acrosomal lamina, neck, tail, besides not presenting a cytoplasmic droplet or cytoplasm around the head (Bartoov *et al.*, 2002). For the nucleus, the morphological state 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 were estimated as 4.75 ± 2.8 and 3.28 ± 0.20 μm (Bartoov *et al.*, 2002), respectively. In the same manner, the form of the nucleus was considered abnormal if extrusion or invagination of the nuclear chromatin had been detected (regional disorders of nuclear form). For rapid evaluation of nuclear form, a fixed, transparent, celluloid form of sperm nucleus fitting the criteria was superimposed on the examined cell (chablon construction based on ASTM E1951-02). Chromatin content was considered abnormal if one or more vacuoles were observed to occupy >4% of the nuclear area. A nucleus was considered normal if both nuclear form and chromatin content presented normality.

The only alteration in ENC was extrusion (the presence of one or more vacuoles that occupy ≤4% of the nuclear area was not considered an abnormality). The same technician performed all sperm selection.

Determination of DNA fragmentation

DNA fragmentation in spermatozoa was measured using the TdT (terminal deoxyribonucleotidyl transferase)-mediated dUTP nick-end labelling (TUNEL) assay, which was performed using an in situ cell death detection kit with

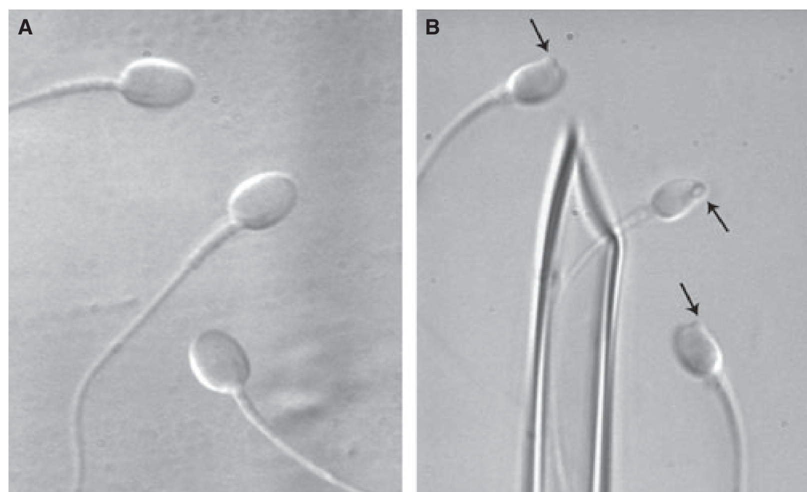

Figure 1 (A) Normal spermatozoa observed at high magnification (8400×); (B) spermatozoa with extruded nuclear chromatin (arrows) at high magnification (8400×).

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tetramethylrhodamine-labelled dUTP (Roche, Monza, Italy). TUNEL identifies DNA breaks by labelling free 3′-OH termini with modified nucleotides in an enzymatic reaction with TdT. TdT catalyses the polymerization of labelled nucleotides to free 3′-OH DNA ends in a template-independent manner. Slides with different spermatozoa (NN and ENC, respectively) selected specifically for DNA fragmentation were air-dried and then fixed at 4 °C in Carnoy's solution (methanol/glacial acetic acid, 3 : 1), and permeabilized with 0.1% Triton X-100 (VETEC Química Fina Ltd, Duque de Caxias, Brazil) in 0.1% sodium citrate at 4 °C for 2 min. After being washed with phosphate-buffered saline (PBS), the slides were processed for TUNEL assay. The TdT-labelled nucleotide mix was added to each slide and incubated in the dark in a humidified atmosphere for 2 h at 37 °C. After the enzyme reaction was stopped, slides were rinsed twice in PBS and then counterstained with Vectashield Mounting Medium with DAPI (4,6-diamidino-2-phenylindole, 1.5 μg/mL; Vector Laboratories, Burlingame, CA, USA). The final evaluation was achieved using a fluorescent microscope and the percentage of TUNEL-positive spermatozoa was determined. The number of cells per field stained with DAPI (blue) was first counted; in the same field, the number of cells with red fluorescence (TUNEL-positive) was expressed as a percentage of DNA fragmentation. Controls were included in every experiment: for negative control, TdT was omitted in the nucleotide mix. Positive controls were generated by pre-incubating the fixed and permeabilized sperm cells using 1 mg/mL of DNase I (New England Biolabs Inc., Ipswich, MA, USA) for 30 min at 37 °C. TUNEL labelling of positive controls varied between 89% and 98% of cells. The same technician, blinded to subject identity, performed all examinations.

Determination of single-stranded (denatured) or double-stranded DNA by AOF

Slides with different spermatozoa (NN and ENC, respectively) selected specifically for AOF were air-dried and then fixed overnight at 4 °C in Carnoy's solution (methanol/glacial acetic acid, 3 : 1). After fixation, the slides were air-dried, stained with AOF staining solution for 5 min and then gently rinsed with distilled water. The AOF staining solution was prepared daily as follows: a mixture of 4 mL of 0.1 mol/L citric acid and 0.25 mL of 0.3 mol/L $Na_2HPO_4 \cdot 7H_2O$ was added to 1 mL of 1% AOF stock solution in distilled water. AOF stock solution was stored in the dark at 4 °C for 4 weeks. To reduce variation in fluorescence intensity, each stained slide was read immediately after washing. Spermatozoa with single-stranded (denatured) or double-stranded (normal) DNA were identified under a fluorescence microscope at 400× and 1000× magnification with 450–490 nm excitation. Spermatozoa with double-stranded DNA were fluorescent green and those with denatured DNA were fluorescent red or yellow. Because of the low sample size per slide, all AO fluorescence results were obtained using dark-field fluorescence microscopy. A single observer, blinded to subject identity, interpreted the fluorescent colour shades to rule out inter-technician variability. To determine intra-technician and intra-assay variabilities for the AO test, before each series of analyses, duplicate slides were made from at least two randomly selected donors to analyse the percentage of spermatozoa with double-stranded DNA and percentage of spermatozoa with denatured DNA. Intra-individual variability for the percentages of green-fluorescing sperm and of red/yellow sperm was ≈5% (for both), comparable with that of classical sperm quality parameters (Auger *et al.*, 2000).

Sample size and statistical analysis

Sample size was calculated by making a comparison between two proportions, control and experimental. Thus, a sample size of 350 spermatozoa in each group has 80% power to detect an increase of 10% with an α significance level of 0.05 (two-tailed). Only 15–20 normal and 15–20 abnormal spermatozoa were actually assessed per sample for each test and this may increase the error. However, the technical difficulties in the selection and evaluation processes should be stressed. First, in our general population, the frequencies of the normal sperm form and spermatozoa with chromatin extrusion are relatively low (1.5% and 3.1%, respectively). Second, the spermatozoa from each morphological form have to be selected one by one using high magnification, a time-consuming process. Finally, there are losses of spermatozoa during the fixing and staining procedures. These points led to working with a small sperm sample. In contrast, the increase in the number of individuals included (55 men) diversifies the total sperm sample and can reduce the error. Data were analysed using InStat version 3.0 (GraphPad Software, San Diego, CA, USA) on a Macintosh computer (Apple Computer Inc., Cupertino, CA, USA). Fisher's exact test was used.

Results

Tables 1 and 2 display values for DNA fragmentation and denatured DNA in spermatozoa with ENC and NN. The percentage of positive DNA fragmentation in ENC spermatozoa (157/801, 19.6%) did not differ significantly ($p = 0.86$) from the NN group (216/1079, 20%). However, the percentage of denatured/single-stranded DNA (516/873, 59.1%) in ENC spermatozoa was significantly higher ($p < 0.0001$) than in NN (441/983, 44.9%).

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Table 1 DNA fragmentation values in spermatozoa with extruded nuclear chromatin (ENC) and normal nucleus (NN)

	Number of spermatozoa	
DNA fragmentation	ENC	NN
Positive	157	216
Negative	644	863

$p = 0.86$.

Table 2 Denatured and double-stranded DNA evaluated by acridine orange fluorescence in spermatozoa with extruded nuclear chromatin (ENC) and normal nucleus (NN)

	Number of spermatozoa	
DNA	ENC	NN
Denatured	516	441
Double-stranded	357	542

$p < 0.0001$.

Discussion

Disturbances in the quality of genetic material from spermatozoids can lead to compromise of male fertility potential. In fact, the level of DNA damage has been correlated negatively with rates of fertilization, embryonic cleavage, implantation and live birth and positively with miscarriage rates (Larson *et al.*, 2000; Evenson *et al.*, 2002; Morris *et al.*, 2002; Benchaib *et al.*, 2003; Carrell *et al.*, 2003; Saleh *et al.*, 2003; Agarwal & Allameneni, 2004; Evenson & Wixon, 2005; Zini *et al.*, 2005, 2008; Agarwal *et al.*, 2008). Given that sperm DNA damage can, at least potentially, account for repeated failure of assisted reproduction treatment (Tesarik *et al.*, 2004), the improvement in the clinical laboratory result observed when utilizing high-magnification sperm selection, in part, may be a result of the existence of a correlation between some morphological parameters and DNA damage (Cohen-Bacrie *et al.*, 2009). Recent studies have shown a correlation between chromatin defects and the presence of vacuoles in the spermatic heads. Berkovitz *et al.* (2005, 2006a), who graded the severity of nuclear morphological alterations by focusing on the presence of large vacuoles, suggested that vacuolization of the sperm nucleus reflects some underlying chromosomal or DNA defects, but did not show data confirming this hypothesis. Franco *et al.* (2008) demonstrated an association between large nuclear vacuoles and both the presence of DNA fragmentation and denaturation in the spermatozoa. In addition, Garolla *et al.* (2008) showed that the presence of nuclear vacuoles affects mitochondrial function, chromatin status and aneuploidy rate. However, to the best of our knowledge, until now, no other sperm nuclear malformation identified at high magnification has been analysed. It is not common to find information on frequencies of different sperm forms observed at high magnification because this criterion was developed only for sperm selection for intracytoplasmic sperm injection (ICSI). However, as our group has been testing the application of high magnification analysis as a method for classifying sperm morphology (Oliveira *et al.*, 2009, 2010) we found, in 690 semen samples, the mean percentage of spermatozoa carrying the anomaly of extruded nuclear chromatin to be 3.1 ± 2.6% (range: 0–17%). Until now, there was no information about association between presence of spermatozoa with chromatin extrusion in any special group of patients or pathologies. In spite of this, as stated previously, the necessity of using sperm with some nuclear morphological alteration in ICSI procedures raises the importance of understanding the implications of the dimorphisms evidenced at high magnification.

The TUNEL assay is usually described as the method of choice for detecting real DNA damage and providing a direct measurement of DNA breaks in spermatozoa (Li *et al.*, 2006). The first part of the data reported in the present study shows that DNA fragmentation values were not significantly different in sperm nuclei with ENC ($p = 0.86$). Therefore, cleavage of genomic DNA in low molecular weight DNA fragments (mononucleosomes and oligonucleosomes) and single-strand breaks (nicks) in high molecular weight DNA occur in an equal frequency in spermatozoa with ENC and in those with NN.

However, the second part of the data shows significantly more denatured DNA in spermatozoa with ENC ($p < 0.0001$) compared with ones with NN. AO staining is an established cytochemical method for determining sperm DNA integrity, which allows differentiation between normal, double-stranded and abnormal, denatured/single-stranded DNA by using the metachromatic properties of the dye (Tejada *et al.*, 1984). In samples with high levels of DNA stainability by AO, there is higher acceptability of sperm DNA upon staining, which suggests that the chromatin may be less compact and, consequently, more vulnerable (Evenson *et al.*, 1986). Despite not indicating real DNA damage as found by TUNEL, some studies have shown that sperm-denatured/single-stranded DNA, detected by AO staining, negatively affects the fertilization process in a classic in vitro fertilization programme (Liu & Baker, 1994; Hoshi *et al.*, 1996). Increased denatured/single-stranded DNA in spermatozoa of infertile men after density gradient preparation is linked to results showing fewer embryos suitable for transfer or cryopreservation (Virant-Klun *et al.*, 2002).

This high level of denatured DNA in ENC could arise from premature decondensation and disaggregation of

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sperm chromatin fibres. Kosower *et al.* (1992) showed that the colour of AOF in the sperm nucleus after acetic alcohol treatment is determined by the thiol disulphide status of DNA-associated protamines. Zini *et al.* (2009) reported that treatment of spermatozoa with dithiothreitol (to induce decondensation) resulted in a substantial decrease in nuclear chromatin compaction, but no measurable change in the DNA fragmentation index (sperm chromatin structure assay/AO). An unwanted high degree of sperm decondensation (disruption of disulphide bridges/red AOF) can result in asynchronous chromosome condensation, and may lead to cytoplasmic fragments in the embryo (Ménézo *et al.*, 2007).

In summary, there was significantly more denatured DNA in spermatozoa with ENC than in those with NN. The high level of denatured DNA suggests premature decondensation and disaggregation of sperm chromatin fibres. The results show an association between ENC and DNA damage in spermatozoa, and support the routine morphological selection (MSOME) and injection of motile spermatozoa at high magnification in ICSI given that the sperm with ENC are not identified with precision at the usual magnifications employed.

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