Article

Significance of large nuclear vacuoles in human spermatozoa: implications for ICSI

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Abstract

The aim of this study was to determine the extent of DNA fragmentation and the presence of denatured single-stranded or normal double-stranded DNA in spermatozoa with large nuclear vacuoles (LNV) selected by high magnification. Fresh semen samples from 30 patients were prepared by discontinuous isolate concentration gradient. Spermatozoa with normal nucleus (NN) and LNV 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 in spermatozoa with LNV (29.1%) was significantly higher ($P < 0.001$) than in spermatozoa with NN (15.9%). Therefore, cleavage of genomic DNA in low molecular weight DNA fragments (mono- and oligonucleosomes), and single-strand breaks (nicks) in high molecular weight DNA occur more frequently in spermatozoa with LNV. Similarly, the percentage of denatured-stranded DNA in spermatozoa with LNV (67.9%) was significantly higher ($P < 0.0001$) than in spermatozoa with NN (33.1%). The high level of denatured DNA in spermatozoa with LNV suggests precocious decondensation and disaggregation of sperm chromatin fibres. The results show an association between LNV and DNA damage in spermatozoa, and support the routine morphological selection and injection of motile spermatozoa at high magnification for ICSI.

Keywords: *denatured DNA, DNA fragmentation, human spermatozoa, ICSI, large nuclear vacuoles*

Introduction

It is known that IVF/intracytoplasmic sperm injection (ICSI) is associated with an increased (although relatively low) risk of birth defects and genetic and epigenetic abnormalities in children. To date, it is unclear whether the ICSI procedure or the underlying infertility is responsible for these defects (Cox *et al*., 2002; Hansen *et al*., 2002; Ludwig *et al*., 2005, Varghese *et al*., 2007). The risk of birth defects (major and minor) was found to be significantly higher (odds ratio ~1.5) in children conceived by IVF/ICSI than in those naturally conceived. The prevalence of chromosomal abnormalities (de-novo abnormalities) was found to significantly higher (1.6/0.5%) in children conceived by ICSI than in those conceived naturally (Bonduelle *et al*., 1998). Epigenetic abnormalities such as errors in DNA methylation have been linked to certain rare genetic diseases (Beckwith–Wiedmann and Angelman syndromes), and while still rare, are found to be slightly more prevalent in children conceived by IVF/ICSI than in those conceived naturally (Maher *et al*., 2003). Successful human reproduction depends in part on the inherent integrity of sperm DNA. There appears to be a DNA damage threshold beyond which embryo development and subsequent pregnancy outcome are impaired. Clinical evidence now shows that sperm DNA damage is detrimental to reproductive outcomes and that spermatozoa of infertile men possess substantially more DNA damage than do the spermatozoa of fertile men. However, an understanding of the causes of sperm DNA damage and the full impact of any sperm defect on reproductive outcome in humans remains undeveloped (Zini and Libman, 2006).

One specific sperm alteration is the presence of large nuclear vacuoles (LNV). Ultramorphological investigation has revealed that this sperm malformation has a negative association with natural male fertility potential (Bartoov *et al*., 1994; Mundy *et al*., 1994). LNV cannot be detected at regular magnification (×200–400) used during routine ICSI. According to Bartoov *et al*. (2002) selection of morphological motile spermatozoa at high magnification (motile sperm organellar morphology examination; MSOME) is the only method to precisely detect LNV in human spermatozoa for ICSI. Berkovitz *et al*. (2006a) observed that microinjection of spermatozoa (intracytoplasmic morphologically selected sperm injection; IMSI) with a normal nuclear shape but large vacuoles affects ICSI pregnancy outcome (reduces pregnancy rate and increases early abortion). However, the mechanism underlying why large vacuoles impair late embryonic development is not clear. The aim of this study was to determine the presence or absence of DNA damage in spermatozoa with LNV selected by high magnification. These spermatozoa were submitted to DNA fragmentation analysis. The presence of single-stranded (denatured) DNA was also determined by acridine orange fluorescence (AOF).

Materials and methods

Study participants and sperm preparation

Fresh semen samples (one per subject) from 30 patients in an unselected group of couples undergoing infertility investigation and treatment at the Centre for Human Reproduction Professor Franco Junior were prepared by Isolate (Irvine Scientific, USA) discontinuous concentration gradient. The final pellet was resuspended in 0.2 ml modified human tubal fluid (HTF) medium (Irvine Scientific). An aliquot of 1 µl of sperm cell suspension was transferred to a 5 µl microdroplet of modified HTF medium containing 8% polyvinyl pyrrolidone solution (PVP medium Irvine Scientific). This microdroplet was placed in a sterile glass dish (FluoroDishTM-Word Precision Instrument, 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 way, suspended motile spermatozoa in the observation droplet could be examined at high magnification using an inverted microscope (Eclipse TE 2000 U Nikon, Japan) equipped with high-power differential interference contrast optics (DIC/Nomarski). The total calculated magnification was ×8400. Spermatozoa with normal nucleus (NN) and those with LNV (**Figure 1**) were selected using a micromanipulation system with angled glass micropipettes and placed on different slides. Spermatozoa were smeared 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. LNV spermatozoa were defined (Bartoov modified classification) by the presence of one or more vacuoles occupying ≥50% of the sperm nuclear area (Bartoov *et al*., 2002).

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 tetramethylrhodamine-labelled dUTP (Roche, Monza, Italy). TUNEL identifies single- and double-stranded DNA breaks by labelling free 3-OH termini with modified nucleotides in an enzymatic reaction with terminal deoxynucleotidyl transferase (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 LNV respectively) selected specifically for DNA fragmentation were air-dried and then fixed at 4ºC 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 4ºC for 2 min. After washing with phosphate-buffered saline (PBS), the slides were then 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 stopping the enzyme reaction, 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 fluorescence microscope and the percentage of TUNEL-positive spermatozoa 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 DNase I 1 mg/ml (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 (Vagnini *et al*., 2007).

Determination of single- (denatured) or double-stranded DNA by acridine orange fluorescence

Slides with different spermatozoa (NN and LNV respectively) selected specifically for AOF were air-dried and then fixed overnight at 4ºC 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 0.1 mol/l citric acid and 0.25 ml 0.3 mol/l $Na_2HPO_4.7H_2O$, added to 1 ml 1% AOF stock solution in distilled water. AOF stock solution was stored in the dark at 4ºC for 4 weeks. In order to reduce variation in fluorescence intensity, each stained slide was read immediately after washing. Spermatozoa with single- (denatured) or double- (normal) stranded 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. The same technician, blinded to subject identity, performed all AOF procedures.

Sample size and statistical analysis

Sample size was calculated planning a comparison between two proportions, control and experimental. Usually, DNA fragmentation and AOF assay both had abnormal values ≤30% in the spermatozoa of the fertile male population. Thus a

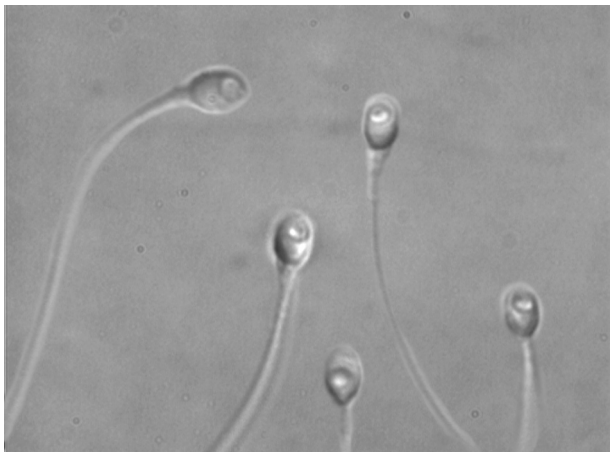

Figure 1. Spermatozoa with large nuclear vacuoles observed at high magnification (×8400).

sample size of 350 spermatozoa in each group has 80% power to detect an increase of 10% with a significance level alpha of 0.05 (two-tailed). Data were analysed using InStat version 3.0 (GraphPad Software, San Diego, CA, USA) on a Macintosh computer (Apple Computer Inc., Cupertino, CA, USA). The Fisher's exact test was used.

Results

Tables 1 and **2** show DNA fragmentation and denatured/double-stranded DNA values in spermatozoa with LNV and NN. The percentage of positive DNA fragmentation in spermatozoa with LNV (111/382; 29.1%) was significantly higher ($P < 0.0001$) than in NN spermatozoa (65/410; 15.9%). In addition, the percentage of single-stranded denatured DNA (252/371; 67.9%) in spermatozoa with LNV was significantly higher ($P < 0.0001$) than in NN spermatozoa (117/354; 33.1%).

Discussion

The accuracy with which morphological normality of spermatozoa for ICSI can be assessed depends on the resolution power of the optical magnification system. Conventionally, ICSI is performed with a ×20/×40 objective, resulting in an overall optical magnification of ×200 to ×400 (De Vos *et al.*, 2003). However, spermatozoa appearing as morphologically normal at this magnification may in fact carry various structural abnormalities that can only be detected at higher optical magnification; spermatozoa with vacuoles would not be detected in conventional ICSI (Hazout *et al.*, 2006). This is a serious disadvantage, because microinjection of spermatozoa with vacuolated nuclei has been shown to be associated with low implantation and pregnancy rates, and with early abortion (Berkovitz *et al.*, 2005, 2006a,b). Berkovitz *et al.* (2006a) suggested that vacuolization of the sperm nucleus reflects some underlying chromosomal or DNA defects, but their study did not provide data confirming this hypothesis. Thundathil *et al.* (1998) found that bovine spermatozoa with multiple nuclear vacuoles are defective in zona pellucida binding. However, vacuolated spermatozoa gaining access to the ooplasm apparently participate normally in fertilization and early embryonic development

Evaluation of DNA damage by TUNEL was introduced by Gorczyca *et al.* (1993) to identify a population of spermatozoa in ejaculate that were believed to be apoptotic. Baccetti *et al.* (1996) observed that apoptosis is abnormally frequent in the spermatozoa in the ejaculate of sterile men. However, numerous other studies using the same technique have followed (Sun *et al.*, 1997; Muratori *et al.*, 2000), demonstrating that DNA fragmentation assessed by the TUNEL method was not associated with an apoptosis-like phenomenon in ejaculated spermatozoa and that DNA fragmentation should be considered a sign of defective sperm maturation, probably originating at the time of DNA packaging. The TUNEL assay is usually described as the method 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 significantly higher in sperm nuclei with LNV ($P < 0.0001$). Therefore, cleavage of genomic DNA in low molecular weight DNA fragments (mono- and oligonucleosomes), and single strand breaks (nicks) in high molecular weight DNA occur more frequently in spermatozoa with LNV. Acridine orange staining is an established cytochemical method for determining sperm DNA integrity, allowing differentiation between normal, double-stranded and abnormal, denatured/single-stranded DNA, using the metachromatic properties of the dye (Tejada *et al.*, 1984). Some studies have shown that sperm denatured/single-

Table 1. DNA fragmentation values in spermatozoa with large nuclear vacuoles and normal nucleus.

DNA fragmentation	*Number of spermatozoa*	
	Large vacuoles	*Normal nucleus*
Positive	111	65
Negative	271	345

$P < 0.0001$.

Table 2. Denatured and double-stranded DNA evaluated by acridine orange fluorescence in spermatozoa with large nuclear vacuoles and normal nucleus.

DNA	*Number of spermatozoa*	
	Large vacuoles	*Normal nucleus*
Denatured	252	117
Double-stranded	119	237

$P < 0.0001$.

stranded DNA, detected by AO staining, negatively affects the fertilization process in a classical IVF programme (Liu *et al*., 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).

The second part of the data shows significantly more denatured DNA in spermatozoa with LNV ($P < 0.0001$). This high level of denatured DNA in LNV could arise from precocious decondensation and disaggregation of sperm chromatin fibres. Kosower *et al*. (1992) showed that the colour of AOF of the sperm nucleus after acetic alcohol treatment is determined by the thiol disulphide status of DNA-associated protamines. An unwanted high degree of sperm decondensation (disruption of disulphide bridges/red acridine orange fluorescence) can result in asynchronous chromosome condensation, and may lead to cytoplasmic fragments in the embryo (Ménézo *et al*., 2007).

In conclusion, the present results support an association between spermatozoa with LNV and DNA damage and the routine use of MSOME/IMSI. This adverse effect (DNA fragmentation or denaturation) leads to concern, particularly about the possibility of iatrogenic transmission of genetic abnormalities.

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