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Potential adverse effect of sperm DNA damage on embryo quality after ICSI

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BACKGROUND: Sperm DNA damage is prevalent amongst infertile men and has been shown to strongly impact adversely natural reproduction, intrauterine insemination-assisted reproduction and to a lesser degree IVF/ICSI fertilization. The objective of this study was to examine further the relationship between sperm DNA denaturation (DD) and reproductive outcomes after ICSI. METHODS: We evaluated infertile couples (n = 60) undergoing IVF/ICSI at a single centre. Sperm DD was assessed by flow cytometry analysis of Acridine Orange-treated sperm and expressed as the percentage of sperm with DD. Couples were sub-grouped according to sperm DD results: group 1: 0–15%; group 2: >15–30%; group 3: >30%. RESULTS: There were no differences between the three groups with regard to maternal age, sperm parameters, oocyte maturation, fertilization or pregnancy rates. Group 3 had a significantly higher rate of multinucleation among the embryo cohorts compared to either groups 1 or 2 (20% versus 10% and 8% respectively, P = 0.04). There was a statistically insignificant trend toward an increased spontaneous pregnancy loss rate in group 3 (P =0.50). CONCLUSION: Although we did not observe significant relationships between sperm DNA damage and either fertilization or pregnancy rates, the potential adverse effect of sperm DNA damage on embryo quality and spontaneous pregnancy loss is concerning.

Key words: DNA damage/embryo/male infertility/multinucleation/sperm

Introduction

Mammalian fertilization involves the direct interaction of the sperm and oocyte, fusion of the cell membranes and subsequent union of male and female gamete genomes (Primakoff and Myles, 2002).Animal studies have shown that the completion of this process and subsequent embryo development depend in part on the inherent integrity of the sperm DNA and that there may be a threshold of sperm DNA damage (e.g. DNA fragmentation) beyond which fertilization and embryo development are impaired (Ahmadi and Ng, 1999). However, DNA-damaged sperm can form pronuclei at fertilization (Twigg *et al.*, 1998) and allow for normal embryo development (Bungum *et al.*, 2004), and this has led investigators to recommend assessment of sperm DNA damage as part of the assisted reproduction programme (Perreault *et al.*, 2003).

There is now clear evidence that infertile men possess substantially more sperm DNA damage than do fertile men (Evenson *et al.*, 1980; Irvine *et al.*, 2000; Shen and Ong, 2000; Spano *et al.*, 2000; Zini *et al.*, 2001, 2002). This is clinically relevant given that infertile men (especially those with severe male-factor infertility and with poor sperm DNA integrity) will be seeking treatment with assisted reproductive technologies. We have shown that the mean percentage of sperm with denatured DNA (DD: abnormally high levels of single-stranded portions of DNA) and DNA fragmentation (DF) is 25 and 28% respectively in infertile men and 10 and 13% respectively in fertile men (Zini *et al.*, 2001). We have also reported that ~20% of non-azoospermic, infertile men, but none of the fertile men, have a high percentage (>30%) of sperm with DD (Zini *et al.*, 2002).

The influence of sperm DNA damage on reproductive outcomes (assisted and unassisted) has been the subject of numerous studies. It has been reported that couples in which the husband has a high percentage of sperm with DD have a lower level of natural fertility, with 10% of couples achieving a pregnancy within 1 year when the percentage of sperm with DD is >30% (Evenson *et al.*, 1999; Spano *et al.*, 2000). Moreover, sperm DNA integrity is poorer in those couples whose natural pregnancy resulted in miscarriage as compared to that of the highly fertile couples (Evenson *et al.*, 1999; Carrell and Liu, 2001; Virro *et al.*, 2004). To date, the bulk of the data indicate that the fertilization rate at IVF or ICSI is not influenced by the level of sperm DNA damage (Lopes *et al.*, 1998; Host *et al.*, 2000; Tomlinson *et al.*, 2001; Tomsu *et al.*, 2002; Morris *et al.*, 2002; Benchaib *et al.*, 2003; Larson-Cook *et al.*, 2003; Henkel *et al.*, 2004; Bungum *et al.*, 2004). On the other hand, most

studies have observed that sperm DNA damage is inversely related to pregnancy rates at IVF and ICSI but the threshold level of sperm DNA damage (beyond which no pregnancy is observed) has not been established (Host *et al.*, 2000; Tomlinson *et al.*, 2001; Tomsu *et al.*, 2002; Benchaib *et al.*, 2003; Larson-Cook *et al.*, 2003; Henkel *et al.*, 2004; Bungum *et al.*, 2004). A recent report suggests that human sperm DNA damage may have an adverse effect on embryo development (Seli *et al.*, 2004; Virro *et al.*, 2004) but this subject has not been studied extensively.

As such, we sought to evaluate further the relationship between sperm DNA denaturation measured by the flow cytometry analysis of Acridine Orange (AO)-treated sperm and reproductive outcomes (including embryo quality) after ICSI.

Materials and methods

Materials

Acridine Orange was purchased from PolySciences (Warrington, PA, USA). Unless otherwise stated, all other chemicals were obtained from Sigma Chemical Co. (St Louis, MO, USA) and were reagent grade or higher.

Study subjects and semen handling

The study is based on a cohort of consecutive infertile couples undergoing IVF/ICSI at the LifeQuest Centre for Reproductive Medicine between September 2003 and March 2004 (IUI and standard IVF cycles were not included). Only ICSI cycles with ejaculated sperm were included in the analysis. (Cycles using surgically retrieved sperm were excluded.) The inclusion criteria for female partner was age <40 years.

After informed consent, male partners were asked to submit a semen sample 1–2 weeks prior to planned ICSI for evaluation of sperm DNA denaturation. All semen samples were produced by masturbation after 3–5 days of sexual abstinence. After liquefaction of semen, standard semen parameters (volume, concentration, and motility, morphology) were obtained according to World Health Organization guidelines (WHO, 1999). A portion of the unprocessed semen (~25–100 ml, containing ~2×10^6 sperm) was frozen at –70°C for later analysis of sperm DNA denaturation. None of the semen samples had significant leukocytospermia as per WHO guidelines (WHO, 1999).

This study was approved by the Ethics Board at the Mount Sinai Hospital in Toronto. Patient information for this study remained confidential and within the institution.

DNA denaturation: AO sperm staining and flow cytometry

Sperm DNA integrity was assessed by monitoring sperm DNA denaturation (DD), as previously described (Evenson *et al.*, 1999; Zini *et al.*, 2002). Stored semen samples were thawed on ice and 200 ml of TNE (0.01 mol/l Tris–HCl, 0.15 mol/l NaCl and 1 mmol/l EDTA, pH 7.4) was added to the sample. The samples were treated for 30 s with 400 ml of a solution of 0.1% Triton X-100, 0.15 mol/l NaCl, and 0.08 N HCl, pH 1.2. After 30 s, 1.2 ml of staining buffer (6 mg/ml AO, 37 mmol/l citric acid, 126 mmol/l Na_2HPO_4, 1 mmol/l disodium EDTA, 0.15 mol/l NaCl, pH 6.0) was added to the test tube and 3 min later the sample was analysed by flow cytometry.

Following excitation by a 488 nm wavelength light source, AO bound to double-stranded DNA emits green fluorescence (515–530 nm) and AO bound to single-stranded DNA emits red florescence (≥630 nm). Three minutes after AO staining, the samples were analysed in a FACS Calibur flow cytometer (Becton Dickinson, San José, CA, USA). A minimum of 5000 cells from two aliquots of each sample were analysed by FACS scan interfaced with a data handler (Cellquest 3.1; Becton Dickinson) on a Power Macintosh 7600/132 computer (Cupertino, CA, USA). FCS Express version 2 (De Novo Software, Version 2, Thornhill, ON, Canada) was used to generate the cytogram and histogram plots (see Figure 1) and the sperm DD results (expressed as the percentage of sperm with DD). A mean of the two sperm DD values was reported.

Fresh and frozen–thawed samples have yielded similar results (<5% variability) (Zini *et al.*, 2002). We have shown that inter-assay variability of sperm DD is low (<5%) by repeat assessments of reference semen samples (Zini *et al.*, 2002). More than 300 aliquots of the same semen sample ('reference sample') have been stored at –70°C for ongoing assessment of inter-assay variability. A reference sample is used to set the red and green photomultiplier tube (PMT) voltage gains to give the same means for red and green fluorescence levels (130/1000 and 500/1000 channels + 5). A new reference sample is run

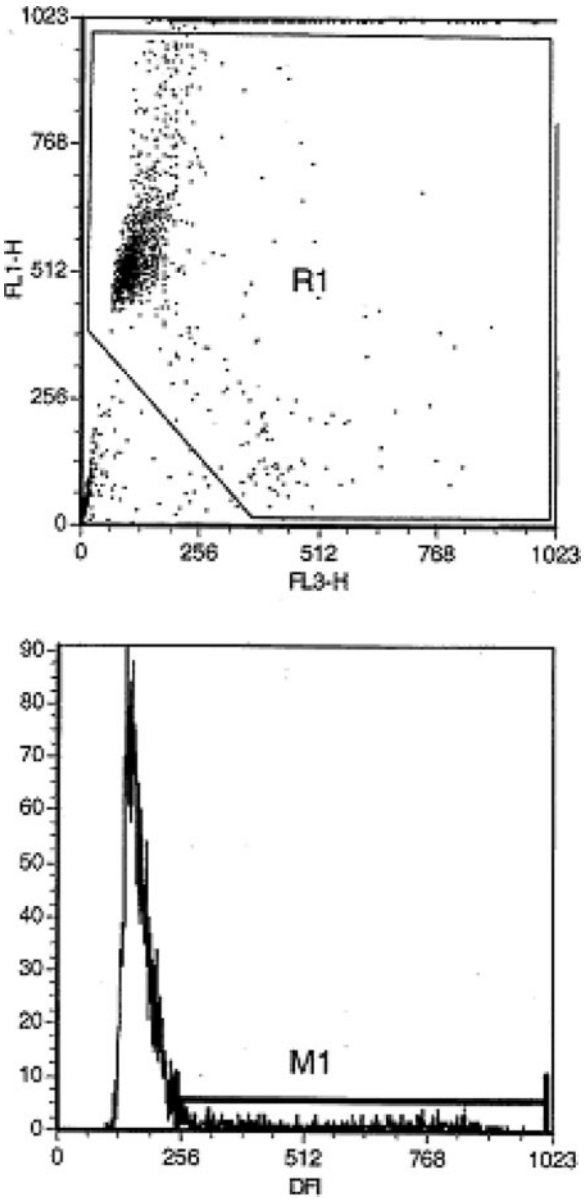

Figure 1. Representative cytogram and corresponding histogram of the sperm DNA denaturation assay.

every 6–10 samples to avoid drift. We have previously validated our assay by assessing sperm DNA fragmentation (DF) in parallel with sperm DD and have shown a strong association between these two measures of DNA integrity (Zini *et al.*, 2001).

Ovarian stimulation

Patients start a low dose oral contraceptive (Marvelon, Organon) within 7 days of beginning their last menstrual period. On the third week of oral contraceptive, GnRH agonist (leuprolide acetate, 0.1 mg) is commenced. After 2 weeks of down-regulation, gonadotrophins (Puregon; Organon, Canada) are administered at a dose appropriate to patient diagnosis and age. Cycle is monitored regularly by a combination of estradiol levels and follicular monitoring. Oocyte maturation is commenced by a timed hCG administration given 36 h prior to oocyte retrieval.

Oocyte retrieval and sperm preparation:

Thirty-six hours after hCG administration, the oocytes were retrieved from the developing follicles. The oocyte–cumulus complexes were found, recorded, washed, labelled and incubated for a minimum of 3 h before insemination. Semen sample was collected by masturbation and allowed to liquefy for ≥0.5 h before processing. The semen and sperm were separated by either density gradient centrifugatioin or swim-up method. The initial specimen and the washed fractions were analysed for count, motility and morphology (WHO, 1999). The washed fraction was placed in a labelled tube at room temperature until 1 h before insemination, at which time it was incubated at 37°C until insemination (ICSI or IVF).

ICSI

The technique for IVF–ICSI has been described previously (Greenblatt *et al.*, 1995). Sperm used for ICSI were chosen based on criteria for normal morphological sperm (WHO, 1999). During ICSI, oocytes were assessed for morphological features (Van Blerkom and Henry, 1992; Meriano *et al*., 2001). The ICSI oocytes were then washed in equilibrated HTF/10% synthetic serum substitute (Irvine Scientific, Santa Anna, CA, USA) and cultured in microdrops of the same media under sterile equilibrated mineral oil in a tri-gas (5% CO_2/5% O_2 90% N_2) environment. Approximately 18 h after injection, oocytes were assessed for signs of fertilization (two distinct pronuclei and two polar bodies) and pronuclear morphology was evaluated based on characteristics described by Tesarik and Greco (1999). At 40–42 and 69–71 h incubation, cleaved embryos were identified and graded based upon blastomere symmetry, degree of fragmentation, and nuclear status. Multinucleation of blastomeres was observed and noted on day 2 of development (Meriano *et al*., 2004). Day 5 classification was performed using the grading system of Gardner *et al.* (2000).

Data analysis

Results are expressed as mean ± SEM. Inter-group (positive and negative pregnancy groups) differences in clinical and semen parameters were assessed by parametric and non-parametric tests as appropriate. The difference in reproductive outcomes (e.g. pregnancy rate, fertilization rate, embryo quality) between couples with a low (0–15%), intermediate (>15 – 30%) and high (>30%) percentage of sperm with DNA denaturation were compared using a χ^2-test (significance set at the $P < 0.05$ level). The relationships between parameters were examined using linear regression techniques with Pearson's correlation coefficient. All hypothesis testing was two-sided with $P = 0.05$ deemed as significant. Statistical analysis was performed using Sigma Stat software (SPSS Inc, Chicago, USA).

Results

Sixty couples were included in this study. These couples were assigned to three groups based upon the male partner's sperm DD score. Twenty-three couples were assigned to group 1 (0–15% DD), 26 to group two (>15–30% DD) and 11 to group three (>30% DD). We did not identify any significant correlations between sperm DD values and conventional semen parameters (concentration, motility and morphology) (data not shown).

There were no significant differences between the three groups with regard to maternal age, mean sperm count, motility and morphology, oocyte maturation, fertilization or day 3 cleavage rates (see Table I). Also, there were no significant differences between the three groups with regard to E_2 and day of hCG injection (data not shown). The pregnancy rates were similar in all groups. There was a trend toward an increased spontaneous pregnancy loss rate in group 3 (P =0.50). All of the five spontaneous abortions (two in group 1, one in group 2 and two in group 3) were late first trimester abortions.

Group 3 (>30% DD) had a significantly higher multinucleation rate among the embryo cohort compared to either group 2 or 1 (20% versus 10% and 8% respectively, $P = 0.04$). Two of the patients in group 3 had levels of sperm DD >40%. In one case (sperm DD = 41% and two of the seven embryos with multinucleation), a clinical pregnancy was achieved but this subsequently resulted in a late first trimester abortion. In the other case (sperm DD = 70% and none of the eight embryos were multinucleated), no clinical pregnancy was achieved.

We did not identify any significant correlations between multinucleation rate and maternal age or oocyte maturation. There were no significant differences in female age, number of mature oocytes, conventional semen parameters and sperm DD values between the couples that achieved and those that did not achieve pregnancy (see Table II).

Table I. Clinical parameters as a function of the percentage of sperm with DNA denaturation

	Group 1 0–15% (n = 23)	Group 2 >15–30% (n = 26)	Group 3 >30% (n = 11)	P
Maternal age	34.7 ± 0.5[a]	35.0 ± 0.5	33.7 ± 1.3	0.52[b]
Sperm concentration ($\times 10^6$/ml)	74.9 ± 13.8	62.9 ± 13.0	70.2 ± 13.8	0.53[c]
Motile sperm (%)	47.3 ± 3.0	42.0 ± 4.0	39.3 ± 3.6	0.36[b]
Normal forms (%)	45.2 ± 2.2	43.4 ± 2.4	47.0 ± 2.5	0.65[b]
Oocytes retrieved	10.9 ± 1.1	14.8 ± 1.8	13.1 ± 1.4	0.19[b]
Metaphase II oocytes	8.1 ± 0.9	12.1 ± 1.5	10.3 ± 1.2	0.09[c]
Oocyte maturation (%)	71 (178/251)	82 (315/385)	78 (113/144)	0.51[d]
Fertilization (%)	82 (146/178)	76 (239/315)	74 (84/113)	0.82[d]
Day 3 cleavage (%)	73 (106/146)	73 (174/239)	73 (61/84)	0.99[d]
Multinucleation (%)	8 (12/146)	10 (25/239)	20 (17/84)	0.04[d]
Mean embryo transfer	1.8 ± 0.2	2.2 ± 0.1	2.0 ± 0.2	0.10[c]
Clinical pregnancy rate (%)	52 (12/23)	50 (13/26)	55 (6/11)	0.99[d]
Spontaneous abortion (%)	17 (2/12)	8 (1/13)	33 (2/6)	0.50[d]

[a]Values are expressed as means ± SEM.
[b]One way ANOVA.
[c]Kruska–Wallis one-way ANOVA on ranks.
[d]χ^2-Test.

Table II. Clinical and semen parameters in those couples who achieved and those who did not achieve a clinical pregnancy

	Pregnancy	No pregnancy	*P*
Female age	34.1 ± 0.4[a]	35.2 ± 0.6	0.17[b]
No. of mature oocytes	11.1 ± 1.2	9.4 ± 1.0	0.28[b]
Sperm with DNA denaturation (%)	20.4 ± 1.7	20.8 ± 2.4	0.80[c]
Sperm concentration ($\times 10^6$/ml)	67.9 ± 13.2	69.9 ± 9.7	0.28[c]
Motile sperm (%)	45.8 ± 2.5	41.1 ± 3.7	0.44[b]
Normal forms (%)	44.3 ± 2.1	45.2 ± 1.9	0.75[b]

[a]Values are expressed as means ± SEM.
[b]*t*-Test.
[c]Mann–Whitney signed rank test.

Discussion

In the present study, we have observed that a high percentage of sperm with DD (>30%) is associated with an increased prevalence of multinucleated blastomeres at ICSI, suggesting that increased chance of sperm DNA damage may have an adverse effect on embryo quality. Multinucleated blastomeres are detected in ~15–35% of ICSI embryos (Hardy *et al.*, 1993; Van Royen *et al.*, 2003), are associated with blastomere fragmentation (Hnide *et al.*, 2004) and are believed to arise from failed cytokinesis. Multinucleated blastomeres can lead to cleavage stage arrest *in vitro* and have been associated with a lower blastocyst formation rate and a lower implantation rate (Van Royen *et al.*, 2003). Taken together, these observations suggest that the reported inverse relationship between sperm DNA damage and male fertility potential *in vivo* (Evenson *et al.*, 1999; Spano *et al.*, 2000; Zini *et al.*, 2001) may in part be explained by the potential adverse effect of sperm DNA damage on embryo development. Indeed, Seli *et al.* (2004) and Virro *et al.* (2004) have also recently observed that sperm DNA damage impacts on blastocyst development after IVF. We cannot exclude the possibility that the development of multinucleated blastomeres may in some cases be due to an oocyte problem rather than sperm DNA damage.

In keeping with previous reports, we have not observed a relationship between sperm DNA damage and fertilization rates with IVF/ICSI (Lopes *et al.*, 1998; Host *et al.*, 2000, Morris *et al.*, 2002; Benchaib *et al.*, 2003; Larson-Cook *et al.*, 2003; Henkel *et al.*, 2004; Bungum *et al.*, 2004).Indeed, neither fertilization nor early embryo development are dependent on sperm DNA integrity since the embryonic genome is not expressed until after the second cleavage division (Braude *et al.*, 1988; Tesarik *et al.*, 2002).

Unlike what has been reported in several clinical studies, we have not observed an inverse relationship between sperm DNA damage and pregnancy rates at IVF/ICSI (Benchaib *et al.*, 2003; Larson-Cook *et al.*, 2003; Henkel *et al.*, 2004). We speculate that the discrepancy between, on the one hand, the observed relationship between sperm DNA damage and embryo quality (multinucleation) and, on the other hand, the lack of any association (in this study) between sperm DNA damage and pregnancy rates, may be a result of the selection of only morphologically normal sperm, and selection of morphologically good quality embryos for transfer at IVF. This selection process may also explain the lack of association between sperm DNA damage and pregnancy rates observed in a recent study (Bungum *et al.*, 2004). However, it is possible that morphologically normal embryos from those couples with high levels of sperm DNA damage and multinucleated blastomeres do not develop as well as those embryos from couples with low levels of sperm DNA damage and without multinucleated blastomeres. Moreover, the loss of embryos (due to multinucleation) may also reduce the total number of available embryos for transfer and, ultimately, pregnancy outcomes.

The conclusions drawn from animal studies on sperm DNA damage and reproductive outcomes may not always apply to humans. In animals with induced sperm DNA damage, there may well be a threshold of DNA injury below which normal fertilization can occur but at which pregnancy outcome is impaired (Ahmadi and Ng, 1999). However, in these animal experiments (where DNA damage is induced artificially) the population of sperm is homogeneous such that each sperm possesses a relatively uniform degree of DNA damage and the relationship between sperm DNA damage and reproductive outcomes is linear. In contrast, the DNA damage within the subpopulation of human sperm selected for ICSI may be different (perhaps with a lower level of DNA damage) than that measured in the entire population of sperm. Indeed, an analogous situation was observed with respect to sperm morphology. It has been reported that the ICSI success rate is related to the morphological characteristics of the sperm specifically selected for ICSI but not to that of the percentage normal morphology in the entire population of sperm (Bartoov *et al.*, 2003; De Vos *et al.*, 2003). Moreover, as discussed earlier, the selection of only high quality embryos for transfer at IVF may reduce the potential adverse effect of sperm DNA damage on pregnancy rates. On the other hand, the selection process itself (resulting in the loss of embryos) may on occasion lead to reduced pregnancy rates due to fewer embryos available for transfer. Although unethical, only randomized studies using both morphologically normal and abnormal embryos (multinucleated) would help to assess the influence of multinucleation on pregnancy outcome.

Sperm DNA integrity may become an important parameter in the setting of ICSI (where natural selection barriers are bypassed and sperm selection is largely a random process) as there is concern that the use of DNA-damaged sperm may result in the iatrogenic transmission of *de novo* genetic abnormalities (Cox *et al.*, 2002; DeBaun *et al.*, 2003; Orstavik *et al.*, 2003; Van Opstal *et al.*, 1997). Studies have shown that fertilization, embryo development and subsequent pregnancy are possible despite a high level of DNA fragmentation in the sperm population (Twigg *et al.*, 1998; Bungum *et al.*, 2004). Although the concept has not been tested in the context of mammalian reproduction, DNA that possesses measurable damage (e.g. DNA oxidation) may cause misreading errors to occur during DNA replication *in vitro* resulting in the generation of *de novo* mutations (Kuchino *et al.*, 1987). As such, we cannot dismiss the possibility that successful fertilization with DNA-damaged sperm may cause *de novo* mutations in the offspring. Oocytes and embryos can repair sperm DNA damage; however, there is a threshold beyond which sperm DNA cannot be repaired (Ahmadi and Ng, 1999).

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