Beneficial effect of microsurgical varicocelectomy on human sperm DNA integrity

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BACKGROUND: Human sperm DNA damage may adversely affect reproductive outcomes, and the spermatozoa of infertile men possess substantially more DNA damage than that of fertile men. To date, there is no available treatment for men with high levels of sperm DNA damage. The objective of this study was to examine the effect of varicocelectomy on sperm DNA denaturation (DD, an index of sperm DNA damage) in infertile men with a clinical varicocele. METHODS. We reviewed the reports of 37 men who underwent microsurgical varicocelectomy at our institution from September 2001 to July 2002. Standard semen parameters and the percentage of spermatozoa with DD (monitored by flow cytometry analysis of acridine orange-treated spermatozoa) were assessed before and 6 months after varicocelectomy. RESULTS. The percentage of spermatozoa with DD decreased following varicocelectomy compared with pre-operatively (27.7 versus 24.6%, respectively, P < 0.05). Sperm concentration and the percentages of motile sperm and normal forms (WHO criteria) increased following varicocelectomy, but the difference did not reach statistical significance. CONCLUSIONS. Our data suggest that varicocelectomy can improve human sperm DNA integrity in infertile men with varicocele. These data represent the first report of improved sperm DNA integrity after therapy and further support the beneficial effect of varicocelectomy on human spermatogenesis.

Key words: DNA denaturation/flow cytometry/ICSI/spermatozoa/varicocele

Introduction

Spermatogenesis is an organized process of germ cell differentiation and maturation in the testis (Clermont, 1972). During spermiogenesis (the last stage of spermatogenesis), spermatid nuclear remodelling and condensation is associated with the displacement of nuclear histones by transition proteins and then by protamines (Mills and Means, 1977; Balhorn 1982). Inter- and intramolecular disulphide cross-links between the cysteine-rich protamines are responsible for the compaction and stabilization of the sperm nucleus. It is thought that this nuclear compaction is important to protect the sperm genome from external stresses such as oxidation or temperature elevation (Kosower et al., 1992; Said et al., 1999). A disruption in the process of spermatogenesis may result in the generation of spermatozoa with high levels of DNA damage (Evenson and Jost, 1993).

There is now evidence to show that spermatozoa of fertile men possess substantially more DNA damage than spermatozoa of fertile men. Indeed, ~10% of the spermatozoa from fertile men and a higher percentage (~20–25%) of the spermatozoa from infertile men possess measurable levels of DNA damage (Zini et al., 2001a). We have reported recently that ~20% of non-azoospermic, infertile men have a high percentage (>30%) of spermatozoa with DNA denaturation (DD) (Zini et al., 2002).

The aetiology of sperm DNA damage is multi-factorial. An important subset of infertile men (~5–15%), but not of fertile men, possesses a complete sperm protamine deficiency (Belokopytova et al., 1993; De Yebra et al., 1998; Carrell and Liu, 2001). Sperm protamine deficiency will affect sperm nuclear compaction, and this may result in sperm DNA damage, as well as impaired sperm decondensation during fertilization (Said et al., 1999). Sperm DNA damage may be due to apoptosis during spermatogenesis (Sakkas et al., 2003). It has been proposed that spermatozoa with fragmented DNA may have initiated and then escaped apoptosis (‘abortive apoptosis’) (Sakkas et al., 2003). Sperm DNA damage can also be caused by post-testicular factors such as systemic fever and chemotherapy (Evenson et al., 2000; Morris, 2002). Recently, studies have shown that varicoceles are associated with human sperm DNA damage (Saleh et al., 2003).

It is reported that overall, varicocele repair results in improved semen quality in 60–80% of infertile men (Schlesinger et al., 1994). However, the true effect of adult varicocelectomy on male fertility remains controversial...
largely because of the paucity of randomized and controlled trials. Also, using the improvement in conventional semen parameters as an outcome measure after varicocelectomy is limited by virtue of the high degree of biological variability of these parameters. An improvement in sperm DNA integrity would provide more credibility as to the therapeutic effect of varicocelectomy because, unlike standard semen parameters, measures of sperm DNA damage (particularly, sperm DD) exhibit a low degree of biological variability (Evenson et al., 1991; Zini et al., 2001b).

As such, the purpose of this study was to examine the effect, if any, of varicocelectomy on sperm DNA damage in infertile men with a clinical varicocele.

### Materials and methods

#### Patient population and semen samples

We performed a retrospective review of consecutive couples who presented for infertility evaluation at the Mount Sinai Hospital Andrology Clinic in Toronto, Canada, between May 2001 and July 2002. We identified 37 consecutive men who underwent varicocelectomy (from September 2001 to July 2002) for clinically detectable varicocele. Men presenting to our clinic with 1 year or more of infertility, a clinically palpable varicocele and abnormal semen parameters (reduced sperm concentration, motility or morphology on two or more semen samples) were deemed to be candidates for varicocele repair. None of the patients were offered varicocelectomy based on the levels of sperm DNA damage. None of the patients were azoospermic or had evidence of genital infection. Couples where the wife had tubal obstruction or ovulatory failure were not included. All microsurgical varicocelectomies were performed by the same surgeon (A.Z.) as previously described (Goldstein et al., 1992).

At initial presentation (for consideration of varicocelectomy), most men had two or more semen analyses. However, because many of the semen analyses were performed at outside laboratories (with some having limited experience in semen analysis), we insisted that all patients submit at least one additional semen sample for testing at our laboratory in order to have reliable and comparable data. Sperm DD was assessed on the same sample. Similarly, we insisted that all men provide at least one sample (the 6 month post-operative sample) for analysis at our laboratory. Again, sperm DD was assessed on the same sample. Therefore, in order to be consistent, we only used the two semen analyses performed at our institution (pre- and post-operative) for data analysis.

Samples were obtained by masturbation after 3–5 days of sexual abstinence. After liquefaction of semen, standard semen parameters (volume, concentration, motility, morphology and viability) were obtained according to WHO guidelines (World Health Organization, 1999).

During the period of May 2001 to May 2003, two aliquots of raw semen (~25 to 100 µl each, containing 1 × 10^6 spermatozoa) from men presenting for infertility evaluation at our Andrology Clinic were routinely snap-frozen and stored at −70°C for later assessment of sperm DNA damage. The sperm DD analysis was performed in duplicate (expressed as an average of two values) and on the same test run (after the 6 month sample had been submitted). We have used the sperm DD assay (flow cytometry-based assay) because, in our experience, it has proven to be an objective and reproducible test of sperm DNA damage. However, we recognize that a limitation of the sperm DD assay is that it is not universally available because it requires a flow cytometer with supporting software and the standardization protocol can be challenging (Zini et al., 2001a). None of the semen samples had significant leukocytospermia as per WHO guidelines (World Health Organization, 1999).

This study was undertaken under ongoing internal review board approval. Patient information for this study remained confidential and within the institution.

#### DNA denaturation: acridine orange (AO) sperm staining and flow cytometry

Sperm DD was assessed as previously described (Evenson et al., 1991; Zini et al., 2001a). Stored sperm samples were thawed on ice, fixed in 70% ethanol for 30 min and then re-hydrated in TNE (0.01 mol/l Tris–HCl, 150 mmol/l NaCl and 1 mmol/l EDTA, pH 7.4) at room temperature. The fixed samples were treated for 30 s with 400 µl of a solution of 0.1% Triton X-100, 150 mmol/l NaCl and 0.08 mol/l HCl, pH 1.2. After 30 s, 1.2 ml of staining buffer (6 µg/ml AO, 37 mmol/l citric acid, 126 mmol/l Na2HPO4, 1 mmol/l disodium EDTA, 150 mmol/l NaCl, pH 6.0) was admixed to the test tube and, exactly 3 min later, analysed by flow cytometry.

Following excitation by a 488 nm wavelength light source, AO bound to double-stranded DNA fluoresces green (515–530 nm) and AO bound to single-stranded DNA fluoresces red (≥630 nm). Three minutes after AO staining, the samples were analysed in a FACS Calibur flow cytometer (Becton Dickenson, San Jose, CA). A minimum of 5000 cells was analysed by fluorescence-activated cell sorting (FACS) scan interfaced with a data handler (CELLQUEST 3.1, Becton Dickinson) on a Power Macintosh 7600/132 computer (Cupertino, CA). A reference sample is used to set the red and green photomultiplier tube 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 every 6–10 samples to avoid drift.

The proportion of cells exhibiting abnormal emission of red fluorescence (reflecting the percentage of sperm with denatured DNA) was recorded. Fresh and frozen-thawed samples yielded similar results (<5% variability) (Zini et al., 2002). We have shown that the inter-assay variability of sperm DD is low (<5%) by repeat assessments of reference semen samples (Zini et al., 2002). Over 300 aliquots of the same semen sample (‘reference sample’) have been stored at −70°C for ongoing assessment of inter-assay variability. We have also shown that inter-laboratory variability is low (<5%) by testing a series of duplicate samples (with low, intermediate and high levels of sperm DD) in our laboratory and in the laboratory of Dr Donald Evenson in Brookings, South Dakota.

#### Data analysis

Results are expressed as means ± SEM. Differences between the pre- and post-varicocelectomy parameters were estimated by parametric and non-parametric tests as appropriate. The relationships between sperm parameters were examined using linear regression techniques with Pearson’s correlation coefficient. All hypothesis testing was two-sided with a probability value of 0.05 deemed as significant. Analyses were conducted with the Sigma Stat program (SPSS, Chicago, IL).

#### Results

Of the 37 men who underwent varicocelectomy, 26 underwent left and 11 bilateral varicocelectomy. The mean age of the 37 men was 35.7 years (range 28–54) with a mean duration of infertility of 3.5 years (range 1–9). Twenty-eight
men presented with primary infertility and nine had secondary infertility.

The mean (±SE) percentages of spermatozoa with DD before and at a mean of 6 months following microsurgical varicocelectomy were 27.7 ± 2.9% versus 24.6 ± 2.7%, respectively (P = 0.04). The improvement in sperm concentration, motility and morphology following varicocelectomy did not reach statistical significance (see Table 1).

We observed significant negative correlations between the percentage of sperm with DD and both sperm motility (r = −0.36, P < 0.05) and sperm viability (r = −0.59, P < 0.05).

Discussion

The association between a clinical varicocele and impaired spermatogenesis is well described (Dubin and Hotchkiss, 1969; Johnsen and Agger, 1978; Terquem and Dadoune, 1981). Most studies have reported varying degrees of hypospermatogenesis, as well as Sertoli cell changes and premature sloughing of germ cells into the seminiferous tubule lumen. Recently, varicoceles have been associated with high levels of sperm DNA damage (Saleh et al., 2003). These investigators have implicated both elevated temperature and the elaboration of reactive oxygen species as potential mechanisms responsible for varicocele-mediated sperm dysfunction and DNA damage (Saleh et al., 2003).

Although several studies have reported improved semen parameters and pregnancy rates after varicocelectomy repair (Stewart et al., 1974; Dubin and Amelar, 1977; Newton et al., 1980; Rageth et al., 1992; Madgar et al., 1995), the true effect of adult varicocelectomy on male fertility remains controversial (Nieschlag et al., 1998; Evers and Collins, 2003). In the present study, the decrease in sperm DNA damage is a more credible outcome measure owing to the low degree of biological variability associated with this parameter (Evenson et al., 1991; Zini et al., 2001b). However, the clinical impact of a 3% reduction in the percentage of spermatozoa with DD is hard to gauge in view of the limited data on the influence of sperm DNA damage on reproduction. We suspect that the observed reduction in sperm DD represents a relatively modest improvement. Prospective studies (with pregnancy outcome data) are needed to assess the clinical importance of our findings.

Sperm DNA integrity correlates with male fertility potential in vivo and may help predict failed pregnancies in couples with unknown fertility potential (Evenson et al., 1999; Spano et al., 2000). Couples where the husband has a high percentage of spermatozoa with DD (>30%) have very low potential for natural fertility (Evenson et al., 1999; Spano et al., 2000). Moreover, sperm DNA integrity is poorer in those couples whose pregnancy resulted in miscarriage as compared with that of the highly fertile couples (Evenson et al., 1999; Carrell et al., 2003). Taken together with the demonstration that sperm DD is correlated with male fertility potential, the results of this study provide a possible mechanism for the observed improvement in spontaneous pregnancy rates reported in most controlled varicocelectomy series (Schlesinger et al., 1994).

Numerous studies have examined the possible influence of sperm DNA integrity on reproductive outcomes after standard IVF and IVF/ICSI. As expected, there is no consistent relationship between sperm DNA damage and fertilization rates with IVF or IVF/ICSI (Sun et al., 1997; Lopes et al., 1998; Host et al., 2000; Tomlinson et al., 2001; Morris et al., 2002; Tomsu et al., 2002; Benchab et al., 2003; Henkel et al., 2003; Larson-Cook et al., 2003; Razavi et al., 2003). Indeed, neither fertilization nor early embryo development (up to the 4-cell stage) 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). However, sperm DNA damage is inversely related to pregnancy rates at IVF and IVF/ICSI (this inverse relationship is observed in most studies) and the exact threshold DNA damage varies depending on the assay used (Host et al., 2000; Tomlinson et al., 2001; Tomsu et al., 2002; Benchab et al., 2003; Henkel et al., 2003; Larson-Cook et al., 2003). These observations are very much in keeping with animal studies indicating that there may be a threshold of DNA injury below which normal fertilization can occur but at which pregnancy outcome is impaired (Ahmadi and Ng, 1999).

Our data would suggest that varicocelectomy may be of benefit in those couples who plan to undergo ICSI and/or have previously failed ICSI and have both a clinical varicocele and measurable sperm DNA damage.

To date, the short- and long-term ramifications of successful fertilization and development with DNA-damaged spermatozoa are unknown. DNA that possesses measurable damage (specifically, DNA oxidation) may cause misreading errors to occur during DNA replication (Kuchino et al., 1987). Although the concept has not been tested in the context of mammalian reproduction, we cannot dismiss the possibility that successful fertilization with DNA-damaged sperm can cause de novo mutations in the offspring (Kuchino et al., 1987). Animal studies suggest that the repair capacity of the oocyte will allow for normal development when spermatozoa possess mild to moderate degrees of DNA damage (Ahmadi and Ng, 1999).

Clearly, the knowledge that sperm DNA damage is common in infertile men (Zini et al., 2002) together with the unknown consequences of iatrogenic transmission of abnormal genetic material during assisted conception (e.g. IVF/ICSI) urge us to explore therapies that may potentially reduce sperm DNA damage.
In summary, we have shown that in infertile men with varicoceles, varicocelectomy can reduce the percentage of spermatozoa with DD. These data suggest that varicocelectomy improves spermatogenesis and sperm function and provide additional mechanisms for the reported improvement in pregnancy rates after varicocelectomy repair.

References


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