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Beneficial effects of microsurgical varicocelectomy on sperm maturation, DNA fragmentation, and nuclear sulfhydryl groups: a prospective trial

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SUMMARY
There is evidence to show that varicocele repair can improve conventional sperm parameters but the effects on sperm chromatin integrity have not been fully elucidated. We sought to examine the effects of varicocelectomy on sperm maturation, nuclear chromatin integrity and nuclear sulfhydryl groups. We conducted a prospective study of consecutive infertile men (n=29) that underwent a microsurgical sub-inguinal varicocelectomy for treatment of a clinically palpable varicocele and abnormal semen parameters. Six healthy sperm donors served as controls. We evaluated conventional sperm parameters and markers of sperm chromatin and DNA integrity (aniline blue (AB) staining, iodoacetamide fluorescein (IAF) fluorescence and, % DNA fragmentation index (%DFI) and percent high DNA stainability (%HDS) by sperm chromatin structure assay) before and 6 months after surgery. The sperm %DFI, %HDS, % 5-IAF staining (diffuse head staining) and % AB staining (dark blue) were all significantly lower in the control group compared to infertile men with varicocele (8 vs. 20%, 4.0 vs. 9.6%, 1.7 vs. 16.3%, and 2.5 vs. 13.5% respectively). The %HDS and %DFI decreased significantly after surgery (from 10% to 6% and from 20% to 13%, respectively). Similarly, the %5-IAF and %AB staining also decreased significantly after surgery (from 16.3% to 5.4%, and from 13.5% to 5.4%, respectively). We observed significant inversely relationships between sperm progressive motility and both %IAF staining and %DFI (r=−0.44 and −0.43, respectively). The data show that varicocelectomy is associated with an improvement in sperm DNA integrity and chromatin compaction using three different assays of sperm chromatin integrity.

INTRODUCTION
Varicocele is a pathological condition associated with dilatation of veins of the pampiniform plexus within spermatic cord. Varicocele is found in approximately 15% of the general population, but the prevalence of clinical varicocele is approximately 40% in men with a history of infertility (Alsaikhan et al., 2016). Although controversial, it is postulated that varicocele induces sperm dysfunction through increased scrotal temperature, reflux of blood from the spermatic vein, and impaired microcirculation (Benoff et al., 2009). Recent studies indicate that varicocele repair results in improved sperm quality and pregnancy rates in couples with clinical varicocele (Bzaaeez et al., 2011).

During spermatogenesis, spermatid nuclear remodeling and compaction is associated with displacement of nuclear histones by transition proteins and then by protamines (Johnson et al., 2011). Disrupted spermatogenesis may result in the generation of spermatozoa with impaired protamination, poor chromatin compaction, and an increased susceptibility to DNA damage (De Iuliis et al., 2009). There is evidence to suggest that spermatozoa of infertile men possess substantially more chromatin defects and DNA damage than spermatozoa of fertile men (Evenson et al., 1999; Zini et al., 2001b). The etiology of sperm DNA damage is multifactorial and most investigators have proposed that ultimately, oxidative stress, aberrant chromatin remodeling (compaction), and abortive apoptosis can result in sperm DNA damage (Sakkas et al., 2003; Laberge & Boissonneault, 2005; Aitken & De Iuliis, 2007).

Conventional sperm parameters (sperm concentration, motility, and morphology) are generally evaluated in varicocele studies. However, the use of conventional sperm parameters as outcome measures is weakened by virtue of the high degree of biological variability in these parameters and their modest value in predicting male fertility potential (Guzick et al., 2001). Moreover, pregnancy is not an ideal parameter to assess outcomes of varicocele repair as it is highly influenced by female factors. An improvement in sperm DNA integrity following varicocelectomy is more credible than a change in standard sperm.
parameters because tests of sperm DNA damage (particularly, the SCSA) exhibit a lower degree of biologic variability (coefficient of variation – CV in the range of 10–30%) than conventional semen parameters (CV in the range of 25–55%) (Evenson et al., 1991; Erenpreiss et al., 2006; Keel, 2006; Smit et al., 2007; Oleszcuk et al., 2011).

A number of investigators have recently examined the association between varicocele and sperm DNA damage. These studies have shown that varicocele repair is associated with improved sperm DNA integrity (Zini & Dohle, 2011). However, the majority of these studies lack randomization and they rarely evaluate more than one aspect of the sperm chromatin or DNA before and after varicocele repair. As such, the purpose of this study was to prospectively examine the effect of varicocele repair on several sperm chromatin and DNA assays (sperm maturation, sperm DNA fragmentation index, distribution of sperm nuclear sulfhydryl groups) in order to gain further insight into the nature of sperm chromatin and DNA damage in infertile men.

MATERIALS AND METHODS

Materials

Acridine orange (AO) was purchased from PolySciences (Warrington, PA, USA). IAF (5-iodoacetamide fluorescein) was purchased from Invitrogen (Burlington, ON, Canada). Unless otherwise stated, all other chemicals were obtained from Sigma Chemical Co (St. Louis, MO, USA) and were at least of reagent grade.

Patient population

We conducted a prospective study of couples presenting for infertility evaluation at the OVO fertility clinic in Montreal, Canada between January 2008 and June 2010. This cohort of patients includes patients evaluated in a prior varicocelectomy study (Zini et al., 2011). Men presenting to our clinic with one year or more of infertility, a clinically palpable varicocele and abnormal semen parameters (reduced sperm concentration or motility on two or more semen samples) were deemed to be candidates for varicocele repair (Cooper et al., 2010). Morphology scores were not included in this study because criteria for categorizing morphology had changed during the course of the study. Baseline testicular volumes (estimated with an orchidometer) and serum FSH, LH, and testosterone levels were obtained. Men with azoospermia, severe oligozoospermia (<5 million spermatozoa/mL), complete asthenozoospermia, or evidence of genital tract infection were excluded. Men were not selected based on the results of sperm DNA damage. Couples in whom the wife had tubal obstruction or ovarioly failure were not included. All of the operations (microsurgical varicocelectomy) were performed by the same surgeon (AZ), as previously described (Goldstein et al., 1992).

We recruited and evaluated 29 consecutive men who satisfied the inclusion and exclusion criteria. All 29 men underwent microsurgical varicocelectomy during the study period and these men were contacted (by phone) to maximize compliance. The men recruited for the study were asked to submit pre- and post-operative semen samples: a sample at 1–2 months before surgery and another two samples at 4 and 6 months after varicocelectomy for evaluation of standard sperm parameters, sperm DNA and chromatin integrity (assessed by SCSA – sperm chromatin structure assay), and cytochemical tests. However, analysis of the semen sample submitted 4 months after surgery was not performed because many of the patients were not compliant with the post-operative sample at 4 months.

Six healthy sperm donors with normal sperm parameters served as controls. The study was approved by the ethics review board at McGill University and all men signed an informed consent prior to participating. Patient information for this study remained confidential and within the institution.

Semen handling

Samples were obtained by masturbation after 3–7 days of sexual abstinence. After liquefaction of semen, standard semen parameters (volume, concentration, motility) were obtained using a computer-assisted semen analyzer – CASA. All the semen samples had motile spermatozoa and none had significant numbers of round cells or leukocytesperia as per WHO guidelines (<1 million round cells/mL).

Following liquefaction, two 25–100 μL aliquots of semen (containing approximately 2 million spermatozoa) were collected from the original sample and frozen at −70 °C for later evaluation of sperm chromatin structure assay (SCSA) parameters (% DNA fragmentation index – %DFI, % high DNA stainability – %HDS) and cytochemical chromatin tests (%IAF fluorescence and %AB staining).

Sperm DNA fragmentation index and high DNA stainability

Sperm DNA damage was assessed by the sperm chromatin structure assay (SCSA) and the results were expressed as percent sperm DNA fragmentation index (%DFI, an index of DNA damage) and percent sperm high DNA stainability (%HDS, an index of nuclear chromatin compaction) as previously described (Evenson et al., 1999; Zini et al., 2001a). Stored semen samples were thawed on ice and treated for 30 sec with 400 μL of a solution of 0.1% Triton X-100, 0.15 M NaCl, and 0.08 N HCl, pH 1.2. After 30 sec, 1.2 mL of staining buffer (6 μg/mL AO, 37 mm citric acid, 126 mm Na2HPO4, 1 mm disodium EDTA, 0.15 M NaCl, pH 6.0) was admixed to the test tube. The sample was placed into the FACS Calibur flow cytometer (Becton Dickinson, San Jose, CA, USA) with the sample flowing to establish excellent sheath/sample flow, and then measurements were taken at exactly 3 min after AO staining. A minimum of 5000 cells from two aliquots of each sample were analyzed by FACS scan interfaced with a data handler (cellquest 3.1; Becton Dickinson) on a Power Macintosh 7600/132 computer (Cupertino, CA, USA). WinList (Verity Softwarehouse Inc., Topsham, ME, USA) was used to generate the cytogram (red vs. green fluorescence) and histogram (total cells vs. DFI) plots as well as % DFI and % HDS readings. A mean of the two sperm % DFI and % HDS values was reported. The variability of the replicate SCSA measures (% DFI and % HDS) was <5%. Testing of paired samples (pre- and post-surgery) was always carried out on the same run.

We have shown that testing fresh and frozen–thawed samples gives comparable results (<5% variability) and that the inter-assay variability in sperm % DFI is low (<5%) by repeat assessments of reference semen samples, (Evenson et al., 1999; Zini et al., 2001a). 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 previously validated our assay by assessing sperm DNA fragmentation (by terminal
nucleotidyl transferase dUTP nick end labeling – TUNEL assay) in parallel with sperm % DFI and have shown a strong association ($r = 0.71$) between these two measures of DNA damage (Zini et al., 2001a).

Cytochemical tests of sperm chromatin: aniline blue and iodoacetamide-fluorescein

Thawed semen samples were fixed with 70% ethanol and kept at −20 °C before further processing. Smears were prepared from the fixed semen samples, left to air dry at 20 °C for 30 min and immediately stained. For aniline blue (AB) staining (de Lamirande & Gagnon, 1998; de Lamirande et al., 2012), smears are incubated with the dye (5% AB in 4% acetic acid) for 5 min, washed three times with dH2O, and mounted with glycerol. For evaluation of iodoacetamide-fluorescein (IAF) fluorescence (IAF, free for sulfhydryl group) (de Lamirande & Gagnon, 1998; de Lamirande et al., 2012), smears are incubated with 0.1 M Tris (pH 6.8) for 5 min and then with 0.1 mM IAF for 15 min. The IAF-stained smears were rinsed briefly with dH2O, washed with Tris, and then mounted with DABCO.

We counted at least 200 spermatozoa per slide and followed the same grading system as previously reported (de Lamirande & Gagnon, 1998; de Lamirande et al., 2012). For the AB stain, we categorized the spermatozoa into one of the three groups: dark blue (dark blue stain over the whole head), pale (light blue staining of the entire head), or intermediate staining (dark blue staining of the post-acrosomal region only). For IAF grading, we also categorized the spermatozoa into one of the three distinct groups: intense (bright fluorescence over the entire head), pale (whole head pale), and intermediate fluorescence (bright fluorescence of post-acrosomal region only). For this study, we reported positive staining as the % of cells with dark blue staining (AB) or positive fluorescence as the % of cells with intense fluorescence.

Data analysis

Results were expressed as means ± one SD. Differences between the pre- and 6-month post-varicocelectomy parameters were estimated by Wilcoxon signed-ranks test. The calculations of correlation coefficients between parameters (variables) were performed using a non-parametric procedure, the Spearman rank-order correlation. All hypothesis testing was two-sided with a probability value of 0.05 deemed as significant. Analyses were conducted using the sigma stat program (SPSS, Chicago, IL, USA).

RESULTS

We recruited 29 infertile men with clinical varicocele. Each one of the 29 infertile men presented with reduced sperm concentration (<15 million/mL), reduced progressive motility (~32%), or both (mean concentration: 42 million/mL, range: 6–78 million/mL, and mean % progressive motility 25%, range: 10–40%). The baseline (pre-operative) sperm %DFI and %HDS of infertile men with varicocele were significantly higher than that of healthy donors (20 ± 10.6 vs. 7.4 ± 5% and 10.4 ± 6.1 vs. 3.6 ± 3.6%, respectively, see Table 1). Similarly, the baseline (pre-operative) sperm % positive AB staining and % positive 5-IAF fluorescence of infertile men with varicocele were significantly higher than that of healthy donors (13.5 ± 7 vs. 2.5 ± 1% and 16.3 ± 6 vs. 1.7 ± 1%, respectively).

We observed a significant improvement in sperm concentration and % progressive motility 6 months after varicocelectomy (see Table 2). We also observed a significant reduction in %DFI and %HDS following varicocelectomy (20 ± 10.6 vs. 12 ± 5.7% and 10.4 ± 6.1 vs. 6.4 ± 4.6%, respectively, see Table 2). Similarly, we observed a significant reduction in % positive AB staining and % positive 5-IAF fluorescence after varicocelectomy (13.5 ± 7 vs. 5.4 ± 3.4% and 16.3 ± 6 vs. 5.4 ± 2.7%, respectively, see Table 2).

We found no significant relationships between AB staining and %DFI, motility, or concentration. The only notable relationship was between aniline blue staining and %HDS post-varicocelectomy ($r = 0.57, p < 0.05$). In addition, there were no significant relationships between %IAF staining and %DFI, % HDS, or sperm concentration. The only notable relationship was between %IAF staining and sperm motility ($r = -0.44, p < 0.01$).

DISCUSSION

In this study, varicocelectomy repair was associated with a significant decrease in sperm %DFI (a measure of sperm DNA fragmentation) and sperm %HDS (a measure of sperm chromatin compaction). The improvement in sperm DNA integrity and chromatin compaction after varicocelectomy is more credible than changes in conventional sperm parameters because measures of sperm DNA damage exhibit a lower degree of biologic variability than standard semen parameters (Evenson et al., 1991; Zini et al., 2001b; Smit et al., 2007; Oleszczuk et al., 2011). Nonetheless, we recognize that the results of our study would be strengthened had we conducted a controlled or randomized controlled trial (RCT) of varicocelectomy. Unfortunately, the ability to perform a randomized varicocelectomy study is becoming increasingly difficult in the era of assisted reproduction with couples unwilling to be placed in the observation arm (Trussell et al., 2011).

The observed improvement in sperm DNA integrity following varicocele repair further supports the hypothesis that

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Pre-op</th>
<th>Post-op (4 months)</th>
<th>p-value</th>
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</thead>
<tbody>
<tr>
<td>Sperm %DFI</td>
<td>20 ± 10.6</td>
<td>7.4 ± 5</td>
<td>0.018*</td>
</tr>
<tr>
<td>Sperm %HDS</td>
<td>10.4 ± 6.1</td>
<td>3.6 ± 3.6</td>
<td>0.0018*</td>
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<tr>
<td>% positive AB stain</td>
<td>13.5 ± 7</td>
<td>2.5 ± 1</td>
<td>0.0009*</td>
</tr>
<tr>
<td>% positive 5-IAF</td>
<td>16.3 ± 6</td>
<td>1.7 ± 1</td>
<td>0.0001*</td>
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Values are means ± SD. *Wilcoxon signed-rank test.

Table 2 Conventional sperm parameters, positive aniline blue stain (dark staining), %IAF fluorescence (diffuse head fluorescence), sperm DNA fragmentation index (%DFI), and high DNA stainability (%HDS) before, and, 6 months after microsurgical varicocelectomy (n = 29)
varicocelectomy improves spermatogenesis and reduces oxidative stress. A number of investigators have shown that varicocele is associated with an increased seminal oxidative stress level and that varicocele repair may lower the oxidative stress levels (Saleh et al., 2003; Chen et al., 2008). De Iuliis et al. (2009) have proposed a two-step hypothesis to explain the generation of sperm DNA damage. Based on this model, oxidative stress (2nd step) acts on poorly protaminated cells (i.e. cells with incomplete replacement of histones by protamines) that are generated by defective spermiogenesis (1st step) (De Iuliis et al., 2009). We have previously observed a strong relationship between % of HDS and sperm nuclear histone H2B staining, suggesting that % HDS is associated with an incomplete histone to protamine exchange during spermiogenesis (Zini et al., 2008).

We have found that infertile men with varicocele demonstrate a higher % of spermatozoa with intense AB staining compared to fertile men. We have also observed that varicocele repair is associated with a significant reduction in the % of spermatozoa with intense AB staining (13.5% ± 7 pre-operatively vs. 5.4% ± 3.4 post-operatively). Aniline blue (AB) binds to lysinerich nuclear proteins (histones) in spermatozoa. In infertile men, spermatozoa with dark staining are generally morphologically abnormal and/or immature (Erenpreiss et al., 2001; de Lamirande et al., 2012). The higher proportion of AB staining in the spermatozoa of infertile compared to fertile men may reflect a higher level of nuclear histones and/or a different histone orientation allowing the AB to access the histones more readily in the former group of men (Sadek et al., 2011; de Lamirande et al., 2012). The lower % of spermatozoa with diffuse AB staining after surgery suggests that varicocelectomy may lower the proportion of spermatozoa with retained histones and/or favor the production of spermatozoa with a more compact chromatin rendering the histones less accessible to AB staining.

One of the unique aspects of our study is the evaluation of free sperm nuclear sulfhydryl groups by iodoacetamide-fluorescein (IAF) fluorescence. IAF is an excellent sulfhydryl-targeted reactive with proven usefulness to label sperm proteins (head and flagellum) (Chatterjee et al., 2001). We found that the proportion of sperm nuclei with intense IAF fluorescence was significantly higher in the varicocele group compared to controls and that the % of spermatozoa with intense nuclear IAF fluorescence decreased significantly after varicocele repair. We speculate that the high proportion of sperm nuclei with intense IAF fluorescence in infertile men with varicocele may be because of excessive reduction in sperm nuclear disulfide bonds and/or increased accessibility to sulfhydryl groups (de Lamirande & Gagnon, 1998). It has been reported that infertile men can have incomplete thiol oxidation (high levels of free SH groups) and at times thiol oxidation but there are no data specifically regarding men with varicocele (de Mateo et al., 2011). Our data also indicate that varicocelectomy is associated with an oxidation of sperm nuclear sulfhydryl groups (with the formation of disulfide bonds) and/or decreased accessibility to sulfhydryl groups.

The assessment of free sperm nuclear sulfhydryl groups allowed us to validate our findings with the SCSA and AB staining assay, and gain further insight into the sperm nuclear changes that take place after varicocele repair. As with AB staining and %HDS, the proportion of sperm nuclei with intense IAF fluorescence decreased after varicocele repair (compared to pre-operatively). Taken together, the data indicate that the sperm nuclei of infertile men with varicocele are poorly compacted (relative to that of fertile men) and that after varicocele repair, sperm nuclear chromatin compaction is enhanced. We postulate that the improvement in sperm nuclear compaction after varicocele repair is at least in part because of enhanced testicular function based on the observed parallel increase in sperm concentration (a marker of testicular function) after surgery. Enhanced testicular function would allow for a more complete histone to protamine exchange during spermiogenesis. However, we cannot exclude the possibility that the improved sperm nuclear compaction after varicocele repair is also a result of enhanced post-testicular (e.g. epididymal) function with oxidation of sperm nuclear sulfhydryl groups and the formation of disulfide bonds.

The relationship between varicocele and sperm DNA damage has been evaluated but remains to be fully elucidated. Zini and Dohle published a systematic review on studies that have examined the relationship between varicocele and sperm DNA integrity (Zini & Dohle, 2011). In 2011, Zini and Dohle identified 16 evaluable studies that compared the semen parameters of men with and without varicocele: nine of the studies evaluated sperm DNA damage in infertile men and seven studies evaluated sperm DNA damage in non-infertility populations (Zini & Dohle, 2011). They reported that the studies of infertile men did not demonstrate a specific relationship between varicocele (five of the nine studies reported that the level of sperm DNA damage in infertile men with varicocele was similar to that of infertile men without varicocele). On the other hand, in non-infertility populations, a strong association between varicocele and sperm DNA damage was observed (Zini & Dohle, 2011). Zini and Dohle also examined the effect of varicocele repair on sperm DNA damage. They reported that all of the 12 evaluable studies demonstrated that varicocele repair is associated with reduced sperm DNA damage (Zini & Dohle, 2011). Our study results are consistent with these observations. Moreover, to the best of our knowledge, our study is the first to show an improvement in sperm DNA and chromatin integrity after varicocelectomy using three different assays (including the assessment of sperm sulfhydryl groups) on the same cohort of patients. In contrast, previous studies have generally used a single sperm DNA assay (e.g. SCSA, TUNEL, COMET) to assess the effect of varicocele repair on human sperm DNA integrity.

We observed that mean sperm concentration and % sperm progressive motility increased 6 months after varicocele repair. Recent analyses of varicocelectomy studies demonstrate that repair of a clinical varicocele is associated with an improvement in semen parameters and our study findings are in keeping with this. Baazeem et al., have conducted a systematic review and meta-analysis of RCTs and prospective studies using weighted mean difference on repeated measures to estimate the improvement in sperm concentration (22 studies) and motility (17 studies) after varicocele repair (Baazeem et al., 2011). They reported a mean improvement in sperm concentration of 12.3 million spermatozoa per mL (95% CI: [9.5, 15.2]; p < 0.0001) and a mean improvement in % total sperm motility of 10.9% (95% CI: [7.1, 14.7]; p < 0.0001). Similarly, a number of recent reviews (mostly meta-analyses of RCTs) have demonstrated that repair of a clinical varicocele is associated with improved pregnancy outcomes. In 2012, Kroese et al., published an update of their
earlier Cochrane reviews (Kroese et al., 2012). They conducted a sub-analysis of five RCTs that included only studies of infertile couples with clinical varicoceles and abnormal semen parameters, including 505 couples with clinical varicocele and abnormal semen parameters: three of the studies in this analysis had positive results (benefit of treating varicoceles) and two had negative results (no benefit). They reported that varicocele repair was associated with a higher pregnancy rate than observation alone with a combined OR of 2.39 using a fixed effect model (95% CI, 1.56–3.66; p < 0.0001).

In summary, in this prospective study of infertile men with varicocele, we have shown that varicoceolecotomy is associated with a significant improvement in sperm chromatin compaction and DNA integrity, using three different assays. The beneficial effect of varicoceolecotomy on sperm DNA damage further supports the premise that varicocele may impair sperm DNA integrity, and provides an additional mechanism for the reported improvement in pregnancy rates after varicocele repair. We recognize the limited sample size of this study, hence, larger, well-designed studies are needed to better define the relationship between varicocele and sperm DNA damage.

REFERENCES


