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Effect of varicocele on chromatin condensation and DNA integrity of ejaculated spermatozoa using cytochemical tests

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Summary

Varicocele occurs in approximately 15% to 20% of the general male population and it is the most common cause of poor semen production and decreased semen quality. It has been demonstrated that patients with varicocele have a significantly higher DNA fragmentation index (DFI) and spermatozoa with nuclear anomalies than healthy fertile men. Therefore, the aim of this study was to evaluate sperm chromatin integrity in these patients. Sixty men referring to the andrology laboratory were categorised into three different groups: 20 infertile men with varicocele, 20 infertile men with abnormal semen parameters and 20 fertile men who had normal spermatogram were considered as control group. Semen analysis was performed according to WHO criteria. To evaluate sperm chromatin quality and DNA integrity, after fixation of sperm smears, aniline blue, toluidine blue, chromomycin A_3 and acridine orange staining were applied in three groups. The slides were analysed by light and fluorescent microscopy and to determine the percentage of mature or immature spermatozoa, 200 spermatozoa were counted in each slide. The results showed that the rates of aniline blue-reacted spermatozoa were significantly higher in infertile and varicocele patients than in the normal group ($P < 0.001$). In addition, with regard to chromomycin A_3, acridine orange and toluidine blue staining, there was a significant difference between the three groups ($P < 0.001$). The results showed that the varicocele samples contain a higher proportion of spermatozoa with abnormal DNA and immature chromatin than those from fertile men as well as infertile men without varicocele. Therefore, varicocele results in the production of spermatozoa with less condensed chromatin and this is one of the possible causes of infertility due to varicocele.

Introduction

Varicocele occurs in approximately 15–20% of the general male population. It is one of the main causes of male factor infertility in 40% of infertile men (Nagler *et al.*, 1997). Varicocele usually leads to alterations in sperm characteristics, decrease in sperm parameters and also testicular volume (Lipshultz & Corriere, 1977).

A higher frequency of spermatozoa with damaged DNA has been reported in the ejaculate of patients with varicocele in comparison with fertile men (Saleh *et al.*, 2003; Chen *et al.*, 2004). Recently, it has been reported that infertility because of varicocele may produce spermatozoa with less condensed chromatin (Fuse *et al.*, 2006). Indeed, sperm DNA damage such as DNA fragmentation, abnormal chromatin packaging and protamine deficiency have been demonstrated to be a cause of male factor infertility (Ahmadi & Ng, 1999; Cho *et al.*, 2003). There is clinical evidence to show that sperm chromatin defects have been correlated with the reduced ability of spermatozoa to fertilise oocytes in the context of assisted reproduction techniques and normal fertility (Sakkas *et al.*, 1996; Lopes *et al.*, 1998; Filatov *et al.*, 1999). However, little is known about cellular mechanisms underlying varicocele-associated

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reduction in fertility rates (Marmar, 2001). The results from our previous study failed to show any significant difference in the DNA integrity of the spermatozoa between infertile and fertile men. Also, no correlation was noticed between the DNA abnormality and the semen parameters in the studied samples (Khalili *et al.*, 2006).

Moreover, elevated scrotal temperature caused by impaired circulation and accumulation of toxic metabolites in the testes appear to be the most reproducible defect in subjects with varicocele (Brown *et al.*, 1967). Heat stress apparently plays a key role in the reduction of fertility rates (Mieusset & Bujan, 1995), as it can lead to spermatogenic arrest (Nakai *et al.*, 2000). Varicocele also leads to increased reactive oxygen species (ROS) generation and apoptosis rates, both in the testes and in semen (Simsek *et al.*, 1998; Hendin *et al.*, 1999). Seminal oxidative stress and testicular apoptosis are well documented causes of increased sperm nuclear DNA fragmentation (Sakkas *et al.*, 2002; Saleh *et al.*, 2003). In another study, we found that ROS levels in seminal fluid of infertile men are significantly higher than in fertile donors and also in infertile men with varicocele, it is higher than in patients with unknown cause (Moein *et al.*, 2007).

However, our understanding of the cause of sperm DNA damage and the full impact of this sperm defect on reproductive outcomes remains rudimentary. In fact, new markers are needed that might better discriminate infertile from fertile men and can predict pregnancy outcome and the risk of adverse reproductive events. In this study, the effects of varicocele on sperm DNA integrity, chromatin quality and semen parameters were evaluated in both infertile patients with or without varicocele and fertile population. According to our knowledge, this study is the one of few reports on evaluation of sperm chromatin condensation using cytochemical tests in patients with varicocele compared with infertile men without varicocele.

Materials and methods

Patients

To study sperm chromatin status, semen samples from 60 males attending the Research and Clinical Center for Infertility in Yazd were analysed. These patients were classified into three groups: (i) infertile men without varicocele ($n = 20$); (ii) infertile men with varicocele ($n = 20$); (iii) healthy fertile donors ($n = 20$). Patients who had no child after a period of unprotected intercourse for more than 1 year with one or various abnormal semen parameters according to the recommendations of the World Health Organization (1999) were considered as infertile. The varicocele diagnosis was made, by the same urologist, for the patients in standing position and via scrotal palpation in a temperature-controlled room (~23 °C). Patients with varicocele grade I were excluded from the study. Twenty healthy donors with proven fertility who had a successful pregnancy within the last 12 months and normal spermogram at the time of study were selected as control group. The institutional review board at Yazd University of Medical Sciences approved this prospective study.

Semen analysis

All specimens were collected by masturbation after 2–4 days of abstinence and delivered to the andrology laboratory. After semen liquefaction, routine semen analysis was performed according to WHO criteria (World Health Organization, 1999) and Diff quick staining was applied for sperm morphology evaluation. Azoospermic samples were excluded from the study. Normal values were sperm motility ≥50% (a + b), sperm concentration $\geq 20 \times 10^6$ per ml and normal sperm forms >30%. All analyses were performed by one experienced technician blinded to the study.

Evaluation of sperm nuclear chromatin

To assess sperm nuclear chromatin, four techniques of cytochemical staining were applied.

Aniline blue (AB) staining

Aniline blue selectively stains lysine-rich histones and has been used for distinguishing sperm chromatin condensation anomalies (Auger *et al.*, 1990). For this purpose, air-dried smears were prepared from fresh semen samples of each study participant, fixed in 3% buffered glutaraldehyde in 0.2 M phosphate buffer (pH 7.2) for 30 min at room temperature. Each smear was stained with 5% aqueous AB stain in 4% acetic acid (pH 3.5) for 5 min. In evaluation with light microscopy (Olympus, Tokyo, Japan), 200 spermatozoa were counted in each slide and unstained or pale blue stained were considered as normal spermatozoa while dark blue stained were rated as abnormal spermatozoa.

Chromomycin A_3 (CMA_3) staining

Chromomycin A3 is a fluorochrome specific for guanosine cytosine-rich sequence, which is used for evaluation of the degree of protamination of mature spermatozoa (Nasr-Esfahani *et al.*, 2001). For this purpose, smears were first dried and then fixed in Carnoys solution (methanol/glacial acetic acid, 3 : 1) at 4 °C for 10 min.

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Each slide was treated with 100 μl of CMA_3 (Sigma, St Louis, MO, USA) (0.25 mg ml^{-1} in McIlvain buffer; 7 ml citric acid, 0.1 M + 32.9 ml $Na_2HPO_4{\cdot}7H_2O$, 0.2 M, pH 7.0 containing 10 mM $MgCl_2$) for 20 min. After staining, the slides were washed in buffer and mounted with buffered glycerol (1 : 1). Bright yellow stained chromomycin-reacted spermatozoa (CMA_3+) and yellowish green stained nonreacted spermatozoa (CMA_3−) were viewed under axiplane fluorescent microscope with a 460-nm filter (Zeiss Co., Jena, Germany).

Toluidine blue (TB) staining

Toluidine blue is a metachromatic dye which measures the rate of sperm nuclear chromatin condensation via binding to phosphate groups of DNA strands (Rao *et al.*, 1990). For this staining, air-dried sperm smears were fixed in fresh 96% ethanol–acetone (1 : 1) at 4 °C for 30 min and then hydrolysed in 0.1 N HCl at 4 °C for 5 min. Thereafter, the slides were rinsed thrice in distilled H_2O for 2 min and finally stained with 0.05% TB for 10 min. The component of staining buffer was 50% citrate phosphate (McIlvain buffer, pH 3.5) (Erenpreiss *et al.*, 1997). In light microscopic study using 100× eyepiece magnification, the chromatin quality of spermatozoa was determined according to metachromatic staining of sperm heads in following scores: 0, light blue (good chromatin); 1, dark blue (mild abnormal chromatin); 2, violet and purple (severe chromatin abnormality) (Rao *et al.*, 1990). So, the sum of spermatozoa with score 1 and score 2 were considered as TB+ or abnormal chromatin, score 0 as TB− or spermatozoa with normal chromatin.

Acridine orange (AO) staining

Acridine orange is a fluorescence probe for measurement of the susceptibility of sperm nuclear DNA to *in situ* acid-induced denaturation. Sperm DNA integrity was determined by AO staining. For this purpose, the smears were first air-dried and then fixed overnight in Carnoy's solution. Each sample was stained for 10 min in freshly prepared AO (0.19 mg ml^{-1}) in McIlvain phosphate–citrate buffer (pH 4) for 10 min. Smears were evaluated on the same day with the aid of fluorescent microscope with a 460-nm filter. The duration of illumination was limited to 40 s per field. The percentage of green (normal double-stranded DNA) and orange/red (abnormally denatured DNA) fluorescence spermatozoa per sample were calculated (Talebi *et al.*, 2007).

Statistical analysis

The Statistical Package for the Social Sciences (SPSS) 15.0 software (SPSS Inc., Chicago, IL, USA) was used to analyse data of all patients. Data were expressed in mean ± SD. Differences between variables with normal distribution were analysed using ANOVA test and between groups were assessed using nonparametric Kruskal–Wallis test. A *P*-value of ≤05 was considered statistically significant.

Results

Sperm parameters

The mean age of infertile men without varicocele, infertile men with varicocele and normal subjects was 31.85 ± 3.75,

Table 1 Mean and standard deviation of sperm parameters of three groups under investigation

Variables	Control (n = 20)	Infertile (n = 20)	Varicocele (n = 20)	P-value
Count (mil ml^{-1})	107.10 ± 58.41	69.20 ± 35.74	47.20 ± 34.97	0.123[a] 0.009[b] 0.000[c]
Grade 'a' or rapid motility (%)	27.55 ± 6.23	10.35 ± 10.03	4.10 ± 7.82	0.019[a] 0.000[b,c]
Grade 'b' or slow motility (%)	30.30 ± 3.68	31.80 ± 7.31	31.95 ± 14.56	0.961[a] 0.625[b] 0.591[c]
Grade 'c' or nonprogressive motility (%)	10.70 ± 2.43	19.5 ± 6.76	18.00 ± 7.46	0.431[a] 0.000[b,c]
Normal morphology (%)	41.45 ± 7.81	27.30 ± 15.22	20.50 ± 11.48	0.076[a] 0.000[b,c]

[a]Difference between infertile and varicocele group.
[b]Difference between infertile and control group.
[c]Difference between varicocele and control group.

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32.05 ± 8.39 and 29.30 ± 5.52 respectively ($P = 0.301$). Duration of infertility was 5.30 ± 3.06 and 4.60 ± 2.58 in infertile men without and with varicocele respectively. In the varicocele group, 25% of the patients presented with a grade III left varicocele and in 75% it was defined as grade II left varicocele.

Sperm parameters including sperm count, rapid (grade a), slow (grade b) and nonprogressive (grade c) motility and sperm morphology in three groups are listed in Table 1.

Sperm count was significantly lower in the infertile and varicocele groups than in the fertile group. Rapid motility (grade a) was higher in controls than in the infertile group and also higher in infertile men than in the varicocele group ($P = 0.000$). Slow motility (grade b) was similar in all groups. The rate of normal sperm morphology was different in three groups; the difference was significant between controls and the other two groups ($P = 0.000$), the difference between varicocele and infertile group was notable but insignificant ($P = 0.076$).

Assessment of sperm DNA and chromatin

The sperm nuclear integrity in the three groups of infertile, varicocele and control are shown in Table 2. The rates of AB-reacted spermatozoa (AB+) were similar in both infertile and varicocele groups ($P = 0.995$), but the difference between these two groups and controls was significant ($P = 0.000$).

With regard to CMA_3 staining, there was a significant difference between the three groups. The rate of reacted spermatozoa to CMA_3 in the normal, infertile and varicocele group was 23.4%, 41.45% and 57.15% respectively.

Sperm nuclei from infertile men with varicocele showed a significant evidence of DNA damage with regard to AO test (increase in orange–red fluorescence) when compared with those of infertile men without varicocele and normal subjects ($P = 0.000$). There was also a significant difference between infertile with varicocele ($P = 0.000$) and normal groups ($P = 0.001$).

There was a significant difference between control, infertile and varicocele groups with regard to TB staining. The difference was significant between each group to the others ($P = 0.000$).

Discussion

Varicocele is characterised by stasis and high pressure in the veins which constitute the pampiniform plexus. Little is known about the cellular mechanisms underlying varicocele-associated reduction in fertility rates. One common trait in infertile men is an increased sperm nuclear DNA fragmentation (Sakkas *et al.*, 2002; Saleh *et al.*, 2003). Using the sperm chromatin structure assay (SCSA), Saleh *et al.* (2003) reported that infertile men with varicoceles show significantly increased spermatozoal DNA damage.

The purpose of this study was to compare the DNA chromatin status in infertile patients with varicocele, infertile and fertile donors. On performing chromatin analysis in the different groups of patients, significant differences were found for chromatin damage. The sperm chromatin condensation was shown with application of two assays of AB and CMA_3. Aniline blue is a marker for detection of extra lysine-rich histone proteins, while CMA_3 detects protamine defects during histone–protamine replacement in the testicular phase of sperm chromatin condensation. The rates of AB-reacted spermatozoa (AB+) were similar in both infertile and varicocele groups but the difference between these two groups and the normal donors was significant. In agreement with our findings, Foresta *et al.* (1992) observed a higher percentage of stained spermatozoa after staining with AB in infertile men than in fertile donors. Our results are also similar to those of Salsabili *et al.* (2006), who showed that total staining score in patients with idiopathic infertility was similar to patients with varicocele.

Table 2 Characteristics of sperm nuclear integrity of three groups under investigation

Variables	Control ($n = 20$)	Infertile ($n = 20$)	Varicocele ($n = 20$)	*P*-value
AB+ (%)	15.75 ± 5.44	40.60 ± 14.71	40.95 ± 13.97	0.995[a] 0.000[b,c]
CMA_3+ (%)	23.40 ± 6.84	41.45 ± 10.07	57.15 ± 8.31	0.000[a,b,c]
AO+ (%)	17.30 ± 7.43	33.50 ± 13.84	60.55 ± 15.55	0.000[a,b,c]
TB+ (%)	16.70 ± 8.55	33.50 ± 9.58	60.85 ± 15.61	0.431[a] 0.000[b,c]

Data are presented as Mean ± SD.
[a]Difference between infertile and varicocele group.
[b]Difference between infertile and control group.
[c]Difference between varicocele and control group.

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In many studies, the chromatin packaging quality of spermatozoa in connection with fertility status has been assessed by staining the spermatozoa with CMA_3 fluorochrome. A correlation has been reported to exist between abnormal sperm chromatin packaging as evaluated by CMA_3 staining and the presence of DNA strand breaks, decreased sperm penetration, the absence of sperm decondensation within the oocyte and IVF/ICSI failure (Bianchi *et al.*, 1993; Sailer *et al.*, 1995; Nasr-Esfahani *et al.*, 2001; Razavi *et al.*, 2003).

In this study, the mean percentage of CMA_3-positive spermatozoa within control sample was significantly lower than in the infertile and varicocele groups, which was in concordance with the studies cited above. Our findings are also in agreement with Singleton *et al.* (2007) who used CMA_3 for detecting protamine deficiency in infertile men but not in varicocele patients. The authors cannot find any reports on staining of sperm smears with CMA_3 and also TB in varicocele patients.

There was a significant evidence of increased red fluorescence with AO staining, which is a biomarker of increased susceptibility to acid denaturation. It may result from DNA damage or altered chromatin structure in spermatozoa from the varicocele group compared with those of infertile men without varicocele and normal subjects. In accordance to our findings, Fuse *et al.* (2006) showed that AO staining significantly increased in patients with varicocele. Another study reported a significant decrease in the percentage of DNA denaturation using AO staining after varicocelectomy (Zini *et al.*, 2005). Several studies using the SCSA as a quantitative assessment of susceptibility of DNA to acid-induced denaturation like AO staining (Saleh *et al.*, 2003; Smith *et al.*, 2006; Smit *et al.*, 2007) and have reported a negative effect of high percentage of DNA damaged spermatozoa on pregnancy rates (Larson *et al.*, 2000; Saleh *et al.*, 2003; Tesarik *et al.*, 2004). Two independent studies (Evenson *et al.*, 1999; Spanò *et al.*, 2000) demonstrated that when denaturated DNA was above threshold (>30%), ultimately fertile couples took longer time to conceive. A study by Saleh *et al.* (2003) using the SCSA demonstrated that infertile patients with varicocele possessed a significantly higher DNA fragmentation index (DFI) than healthy fertile controls.

For measuring the rate of DNA abnormalities, toluidine blue staining was applied. Spermatozoa with dark blue and also violet and purple head were significantly higher in the varicocele and infertile groups than in controls. This is in line with Erenpreisa *et al.* (2003), who reported that the proportion of TB dark cells in normal samples did not exceed 35%. Light blue sperm cell heads prevailed in normal samples, whereas dark and blue sperm cell heads dominated in abnormal samples. In contrast with our results, it has been reported in the literature that the difference in the proportion of spermatozoa with abnormal, easily denaturable DNA is not statistically significant between infertile and fertile men (Tejada *et al.*, 1984; Hoshi *et al.*, 1996).

The significant difference observed has many possible explanations including apoptosis, increased ROS and testis temperature.

One of the responsible factors for sperm chromatin disorders in varicocele patients is apoptosis, which is more frequent in the spermatozoa of these patients (Simsek *et al.*, 1998). Varicocele may contribute to an increased sperm programmed cell death, necrosis, degeneration or physiological degeneration as a result of abnormal testicular temperatures (Chen *et al.*, 2004). Heat treatment has been shown to produce fragile DNA in a population of spermatozoa and is associated with poor capacitation characteristics and apoptosis (Mann *et al.*, 2002). One study demonstrated that apoptotic sperm cell count in patients with varicocele is approximately a 100 times higher than in those without varicocele (Baccetti *et al.*, 1996). In addition, another study reported that apoptosis is seven times higher in the testicular tissue of patients with varicocele compared with that of normal men (Simsek *et al.*, 1998).

Another possibility that would explain the increasing nuclear damage is high concentrations of ROS (Koksal *et al.*, 2000) and reduction in antioxidant defences (Barbieri *et al.*, 1999) in higher grades of varicocele, as our previous work showed that ROS levels in infertile patients with varicocele are higher than in patients with unknown cause. At molecular level, ROS would directly affect DNA, and also alter intracellular Ca^{2+} levels, which is shown to be one of the most powerful ways of inducing apoptosis (Cam *et al.*, 2004).

In conclusion, our study with using cytochemical tests indicates a significant increase of sperm DNA chromatin damage in patients with varicocele and these damages may affect the quality of the ejaculated spermatozoa and decrease their fertility potential.

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