

# Increased sperm DNA damage in patients with varicocele: relationship with seminal oxidative stress

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R.Smith<sup>1,4</sup>, H.Kaune<sup>1</sup>, D.Parodi<sup>1</sup>, M.Madariaga<sup>1</sup>, R.Rios<sup>2</sup>, I.Morales<sup>3</sup> and A.Castro<sup>1</sup>

<sup>1</sup>Institute of Maternal and Child Research, School of Medicine, University of Chile and San Borja-Arriarán Clinical Hospital,

<sup>2</sup>Department of Endocrinology, San Borja-Arriarán Clinical Hospital, National Health Service and <sup>3</sup>Department of Urology, Barros-Luco Trudeau Hospital, National Health Service, Santiago, Chile

<sup>4</sup>To whom correspondence should be addressed at: Institute of Maternal and Child Research, School of Medicine, University of Chile, PO Box 226-3, Santiago, Chile. E-mail: rsmith@med.uchile.cl

**BACKGROUND:** The pathophysiology of the testicular damage in varicocele has not been completely understood. Oxidative stress and related sperm DNA damage have been identified as significant causes of male infertility. The current study was designed to determine the extent of sperm nuclear DNA damage in patients with varicocele and to examine its relationship with oxidative stress. **METHODS:** Semen samples from 55 patients with clinical varicocele and 25 normozoospermic donors were examined. Varicocele sperm samples were classified as normal or abnormal according to World Health Organization guidelines. Sperm DNA damage was evaluated by the sperm chromatin structure assay/flow cytometry and by the terminal deoxyribonucleotidyl transferase-mediated dUTP nick-end labeling (TUNEL) assay. Levels of reactive oxygen species (ROS) and total antioxidant capacity were assessed by a chemiluminescence assay. **RESULTS:** DNA fragmentation index (DFI) (percentage of sperm with denatured DNA) values and the percentage of TUNEL-positive cells were significantly greater in patients with varicocele, either with normal (DFI,  $20.7 \pm 4.0$ ; TUNEL positive,  $26.1 \pm 3.2$ ) or with abnormal (DFI,  $35.5 \pm 9.0$ ; TUNEL positive,  $32.2 \pm 4.1$ ) semen profile, compared with controls (DFI,  $7.1 \pm 0.9$ ; TUNEL positive,  $14.2 \pm 1.2$ ). Similarly, ROS levels were significantly higher ( $P < 0.01$ ) in both groups of patients with varicocele. **CONCLUSIONS:** The presence of a varicocele is associated with high levels of DNA-damage spermatozoa even in the presence of normal semen profile. The results also indicate that oxidative damage is associated with sperm DNA damage in these patients.

*Key words:* apoptosis/DNA damage/oxidative stress/spermatozoa/varicocele

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## Introduction

Varicocele has long been implicated as a major cause of male infertility (Schlesinger *et al.*, 1994; Benoff and Gilbert, 2001; Hauser *et al.*, 2001), but the pathophysiology remains unclear. Clinical varicocele is found in about 15% of the general population including adolescents and adults: in 35% of men with primary infertility and in up to 80% of men with secondary infertility (WHO, 1992; Belloli *et al.*, 1993). Although the varicocele is clinically evident, not all men with varicocele are infertile. Recently, several studies suggest that an individual with varicocele even with a normal semen analysis or documentation of previous fertility is at risk of subsequent loss of testicular function and fertility (Cozzolino and Lipshultz, 2001; Marmar, 2001). At present, despite numerous reports, the pathogenetic mechanisms by which varicocele induces testicular dysfunction and infertility are not completely understood. The proposed mechanisms include reflux of toxic metabolites from adrenal or renal origin, impairment of the hypothalamic-gonadal axis, venous stasis leading to testicular hypoxia and elevation of testicular temperature (Fujisawa *et al.*, 1989; Comhaire, 1991;

Sweeney *et al.*, 1995; Benoff *et al.*, 2004). Recently, studies evaluating the role of oxidative stress in male infertility have shown that infertile men with varicocele have elevated levels of sperm-derived reactive oxygen species (ROS).

A number of studies have demonstrated high levels of seminal oxidative stress, as evidenced by increased levels of ROS and reduced total antioxidant capacity (TAC) in men with a clinical diagnosis of varicocele, suggesting that sperm dysfunction in varicocele patients may be in part related to oxidative stress (Barbieri *et al.*, 1999; Hendin *et al.*, 1999; Pasqualotto *et al.*, 2000; Zini *et al.*, 2000; Agarwal *et al.*, 2003; Meucci *et al.*, 2003). In addition, oxidative stress has been shown to affect the integrity of the sperm genome by causing high frequencies of single- and double-strand DNA breaks which are often detected in the ejaculates of infertile men (Fraga *et al.*, 1996; Irvine *et al.*, 2000; Saleh and Agarwal, 2002; Wang *et al.*, 2003; Moustafa *et al.*, 2004). The increased levels of a specific form of oxidative damage such as 8-hydroxy-2-deoxyguanosine in sperm support such a theory (Shen and Ong, 2000; Loft *et al.*, 2003).

Different hypotheses have been proposed to explain the origin of DNA damage in mature spermatozoa from infertile men, including abnormalities in chromatin packing, microdeletions, aneuploidy, chromosomal rearrangements, DNA strand breaks and apoptosis (Gorczyca *et al.*, 1993; Manicardi *et al.*, 1995; Sakkas *et al.*, 1995, 1999a; Barroso *et al.*, 2000; Cho *et al.*, 2001). Presently, DNA damage in spermatozoa is considered as an important cause of male infertility, and the presence of sperm with DNA fragmentation and chromatin abnormalities in human ejaculates is well documented (Sun *et al.*, 1997; Spano *et al.*, 2000; Saleh *et al.*, 2002; Agarwal and Said, 2003; Sakkas *et al.*, 2003a), in particular in men with poor semen quality.

Oxidative stress has also been correlated with apoptosis (Jabs, 1999; Higuchi, 2003; Kwon *et al.*, 2003; Barzilai and Yamamoto, 2004). Animal studies have suggested that apoptosis is a key regulator of spermatogenesis in normal and pathological conditions (Rodriguez *et al.*, 1997; Tesarik *et al.*, 1998; Lin *et al.*, 1999; Pentikainen *et al.*, 1999; Print and Loveland, 2000; Hikim *et al.*, 2003). Spontaneous germ cell apoptosis has been identified in spermatogonia, spermatocytes and spermatids in the testis of normal men and in patients with non-obstructive azoospermia (Tesarik *et al.*, 2002).

Recently, attention has been focused on the role of apoptosis in ejaculated sperm (Sakkas *et al.*, 2002; Shen *et al.*, 2002; Wang *et al.*, 2003; Moustafa *et al.*, 2004). Although mature sperm have been reported to present distinct signs of apoptosis-related cell damage, the existing knowledge of the apoptotic signalling pathways in ejaculated sperm is still confusing (Tesarik *et al.*, 2004a). Evidence suggests that in subfertile patients, cells destined to undergo apoptosis may escape the clearance mechanism during spermatogenesis, and their presence in the ejaculate thus contributes to poor sperm quality (Sakkas *et al.*, 1999b, 2003b). Whether apoptosis accounts for a significant proportion of DNA damage in patients with varicocele that may explain the patient's subfertility status is not completely known. Increased apoptosis has been associated with varicocele (Tanaka *et al.*, 2002; Saleh *et al.*, 2003a; Cam *et al.*, 2004; Chen *et al.*, 2004). Recently, a relationship between apoptotic nuclei and cadmium levels has been reported in testicular biopsies from men with varicocele (Benoff *et al.*, 2004).

Sperm DNA integrity is essential for the accurate transmission of genetic information. To determine the clinical significance (diagnostic and prognostic role) of sperm nuclear DNA fragmentation, it is critical to understand the impact of DNA damage in ejaculated sperm on preimplantation and post-implantation embryonic development. Several studies have examined the relationship of sperm nuclear DNA fragmentation [as measured by the sperm chromatin structure assay (SCSA) and the terminal deoxyribonucleotidyl transferase-mediated dUTP nick-end labelling (TUNEL) assay] with cleavage rates, embryo quality and pregnancy rates for naturally initiated (Evenson *et al.*, 1999; Spano *et al.*, 2000) and assisted reproduction technique (ART)-initiated pregnancies (Morris *et al.*, 2002; Benchaib *et al.*, 2003; Larson-Cook *et al.*, 2003; Loft *et al.*, 2003; Bungum *et al.*, 2004; Gandini *et al.*, 2004; Seli *et al.*, 2004; Tesarik *et al.*, 2004b). Overall, these

studies have suggested that high levels of DNA fragmentation may have a negative influence on embryo development and pregnancy rates.

To clarify the pathophysiological mechanisms of sperm dysfunction that might account for the infertility in patients affected by varicocele, the purposes of the present study were (i) to assess the levels of sperm nuclear DNA damage in ejaculated spermatozoa of patients clinically diagnosed with varicocele and (ii) to determine the relationship of sperm DNA damage with seminal oxidative stress.

## Materials and methods

### Patients

The study included 55 patients who consulted for testicular discomfort at Unit of Andrology and Department of Urology. All patients exhibited grade II or grade III clinical varicocele (Hargreave and Liakatas, 1991) that was diagnosed by palpation as well as by Doppler ultrasound examination. The control group included 25 normozoospermic healthy donors, with unknown fertility, who had a normal genital examination and normal standard semen parameters according to World Health Organization (WHO) criteria (WHO, 2001). In all patients and volunteers, a detailed medical history, including men's occupation, smoking habits, alcohol intake and the use of prescription medications, was obtained. All subjects with evidence of urogenital infections, leukocytospermia, hypogonadism (testicular volume <15 ml), a history of smoking and excessive alcohol and drug consumption were excluded from the study. All subjects were assessed for serum gonadotropin and testosterone levels and underwent semen analysis. Written informed consent for participation was obtained, and the study was approved by the ethics committee for research involving human subjects at the Faculty of Medicine, University of Chile.

### Sperm collection and semen analysis

Semen samples were obtained after 72 h of sexual abstinence and were analysed within 1 h of collection. In all patients, a standard semen analysis was performed, assessing semen parameters including sperm concentration, motility, viability, morphology and leukocyte concentrations according to the WHO guidelines. Sperm morphology was assessed using the WHO classification and Kruger's strict criteria (Kruger *et al.*, 1986). Sperm parameters were considered normal when sperm concentration was  $\geq 20 \times 10^6$ /ml of semen, motility was  $\geq 40\%$ , and normal sperm forms were  $\geq 30\%$  by WHO (2001) criteria and  $>14\%$  by Kruger's strict criteria. Semen samples from patients were classified as samples with normal or abnormal semen parameters on the basis of the results of semen analysis.

### Measurement of ROS

Basal or unstimulated ROS levels were measured by a chemiluminescence assay using luminol (5-amino-2,3-dihydro-1,4-phthalazinedione; Sigma, St Louis, MO, USA) as a probe (Kobayashi *et al.*, 2001). Liquefied semen samples were centrifuged at 300g for 10 min, and the sperm pellet was resuspended in phosphate-buffered saline (PBS) (pH 7.4) at a concentration of  $3 \times 10^6$  sperm/ml. Ten microlitres of luminol, prepared as a 5 mM stock in dimethylsulphoxide, was added to 500  $\mu$ l of the sperm suspension. A negative control was used by adding 10  $\mu$ l of luminol to 500  $\mu$ l of PBS. The ROS levels were assessed by measuring the luminol-dependent chemiluminescence with a luminometer (Triathler Multilabel Tester; Hidex, Turku, Finland). The results are expressed as counted photons per minute, counted in 20 s, per  $3 \times 10^6$  sperm.

### Total antioxidant capacity

Total non-enzymatic antioxidant capacity was measured in the seminal plasma by using a commercially available kit (Total Antioxidant Status Assay Kit, Calbiochem, La Jolla, CA, USA). The assay is based on the ability of the antioxidants present in the seminal plasma sample to inhibit the oxidation of 2,2'-azino-di-(3-ethylbenzthiazoline sulphonate) (ABTS) to ABTS radical (ABTS<sup>•+</sup>) in the presence of a peroxidase (metmyoglobin) and hydrogen peroxide (H<sub>2</sub>O<sub>2</sub>). The assay was performed according to the manufacturer's recommended protocol. Liquefied semen samples were centrifuged at 300g for 10 min, and seminal plasma was aliquoted and stored at -80°C. After thawing at room temperature, 10 µl samples were tested immediately. Trolox (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid), a water-soluble tocopherol analogue, was used as a reference standard for TAC calibration. The amount of ABTS<sup>•+</sup> produced was measured by reading the absorbance at 600 nm with a spectrophotometer (Jenway 6405, Barloworld Scientific Ltd., UK). The results are expressed as antioxidant concentration (mM).

### Sperm chromatin structure assay

Sperm DNA damage was measured by SCSA following the procedure as described elsewhere (Evenson and Jost, 2000; Evenson *et al.*, 2002). SCSA measures the susceptibility of sperm nuclear DNA to acid-induced DNA denaturation *in situ*. Briefly, frozen semen samples containing  $1 \times 10^6$  to  $2 \times 10^6$  sperm were thawed and immediately treated with a low-pH (pH 1.2) detergent solution containing 0.1% Triton X-100, 0.15 M NaCl and 0.08 M HCl. After 30 s, sperm were stained with a staining solution containing 6.0 µg/ml of acridine orange (AO) in a phosphate citrate buffer (pH 6.0). Cells were analysed using a FACScan flow cytometer (Becton Dickinson, San Jose, CA, USA). A total of 50 000 events were accumulated for each measurement. When cells were excited with a 488-nm light source, AO intercalated to double-stranded DNA fluoresces green and AO associated with single-stranded (denatured) DNA emits red fluorescence. Scattergram analysis of raw data, with each point representing the coordinates of red and green fluorescence intensity value for every sperm, was carried out using standard Becton Dickinson software. We expressed the extent of DNA denaturation in terms of the DNA fragmentation index (DFI), which was previously known as COMP $\alpha_1$ , and represents the percentage of sperm with abnormal chromatin structure.  $\alpha_1$  was calculated as the ratio of red fluorescence to the total of red and green fluorescence of an individual sperm cell [ $\alpha_1$  = red/total (red + green) fluorescence].  $X\alpha_1$  represents the mean population of  $\alpha_1$ . SCSA inter-assay variability was less than 4%.

### TUNEL assay

Sperm DNA fragmentation related to the presence of apoptosis was evaluated by the TUNEL assay using an In-situ Cell Death Detection Kit with fluorescein isothiocyanate-labelled dUTP (Roche Diagnostics

GmbH, Mannheim, Germany). The sperm suspension was centrifuged for 10 min at 800g; the supernatant was discarded and the remaining pellet resuspended with PBS (pH 7.4). Sperm samples were smeared on microscope slides, fixed with 3.5% formaldehyde in PBS for 1 h at 4°C and permeabilized with 0.1% Triton X-100 in 0.1% sodium citrate. After washing with PBS, the slides were incubated in the dark at 37°C for 1 h in TUNEL reaction mixture containing labelled nucleotides and enzyme terminal transferase. After stopping the enzyme reaction, the slides were washed in PBS and counterstained with propidium iodide at 1 µg/ml in PBS. The slides were mounted with DABCO (Sigma) and observed in a fluorescence microscope (Nikon, Optiphot-2, Japan) with a 100× oil-immersion objective. For each slide, 500 spermatozoa were evaluated. The intra-assay coefficient of variation was <7%. Negative and positive controls were performed, respectively, by omitting the enzyme terminal transferase and by pre-incubating fixed and permeabilized sperm samples with DNase I (1 mg/ml) for 20 min at room temperature. Each sperm was assigned to contain fragmented DNA (intense green nuclear fluorescence) or normal DNA (red nuclear fluorescence due to propidium iodide).

### Statistical analysis

Data were analysed using the SAS statistical software package (SAS Institute, version 6.12, Cary, NC, USA). Data distribution was evaluated by the Kolmogorov-Smirnov test. Normally distributed data were analysed with Tukey's multiple comparison test. Non-parametric variables were compared using the Mann-Whitney *U*-test. Correlation between variables was calculated using Spearman's non-parametric methods. Statistical significance was set at  $P < 0.05$ .

### Results

Varicocele was detected by physical examination and confirmed by Doppler ultrasound in the 55 patients who entered the study. Nine of 55 (16%) patients showed bilateral varicocele. In the others, varicocele was present in the left testis. The median age was  $26.2 \pm 6$  years (18–37) for patients and  $30.2 \pm 3.2$  years (21–37) for controls.

The main sperm characteristics of controls and patients are shown in Table I. Of the 55 patients, 37 (67%) had normal sperm parameters, while 18 (33%) had an abnormality in one or more of these parameters. In this latter group, the most frequent disorder was teratozoospermia, either alone or associated with oligoasthenozoospermia (25%). No significant differences were observed between controls and the subgroup of patients with normal semen parameters regarding normal sperm morphology as assessed by the WHO guidelines and Kruger's strict criteria. However, sperm concentration and

**Table I.** Sperm characteristics in control subjects and patients with varicocele

Parameters	Controls ( <i>n</i> = 25)	Patients	
		Normal SP ( <i>n</i> = 37)	Abnormal SP ( <i>n</i> = 18)
Volume (ml)	2.9 ± 1.6 (0.7–7.3)	2.8 ± 1.5 (1.0–6.4)	2.8 ± 1.9 (0.7–6.0)
Concentration (10 <sup>6</sup> /ml)	102.3 ± 53.9 (31.4–215.0)	79.2 ± 58.5 (21.4–245.3)*	45.1 ± 88.6 (0–348.0)**
Progressive motility (%)	77.7 ± 8.4 (59.0–89.0)	67.7 ± 1.7 (40.0–88.0)**	36.3 ± 26.3 (2.0–71.0)**
Normal sperm morphology			
WHO (%)	56.7 ± 12.4 (36.0–77.0)	52.5 ± 11.3 (31.0–78.0)	30.5 ± 14.3 (7.0–66.0)**
Kruger's (%)	16.6 ± 6.1 (7.0–28.0)	13.8 ± 5.8 (7.0–28.0)	11.5 ± 6.3 (2.0–23.0)

Values are mean ± SD; values in parenthesis are ranges; SP, semen parameters; WHO, World Health Organization.

\* $P < 0.05$  versus control; \*\* $P < 0.001$  versus control.

**Table II.** Reproductive basal hormones in control subjects and patients with varicocele

Hormones	Reference values	Controls	Patients	
			Normal SP	Abnormal SP
FSH (mIU/ml)	1.5–7.0	2.4 ± 0.5 (1.5–2.9)	3.6 ± 1.7 (1.6–6.5)	2.8 ± 2.5 (1.5–8.5)
LH (mIU/ml)	1.0–8.0	2.7 ± 1.6 (1.0–6.0)	3.8 ± 1.9 (1.3–7.4)	3.7 ± 1.7 (2.2–5.4)
Testosterone (ng/ml)	2.8–8.0	4.5 ± 1.6 (3.0–7.8)	6.8 ± 2.14 (4.2–8.7)	5.9 ± 3.4 (3.3–10.5)

Values are mean ± SD; values in parenthesis are ranges; SP, semen parameters.

percentage of progressive sperm motility (moderate and very active forward progression), although within normal values (WHO criteria), were significantly lower compared with the control group (Table I). Hormone values of controls and patients, classified according to seminal data, are reported in Table II. All plasma hormone concentrations were within the normal range, and no significant differences were observed between all the evaluated groups.

### Oxidative stress markers

Comparisons of sperm ROS production and TAC in seminal plasma between normal donors and patients with varicocele are shown in Table III. Significant differences were seen in ROS production between controls and patients with normal or abnormal semen parameters. Mean (±SD) adjusted ROS levels [ $\log(\text{ROS} + 1)$ ] were significantly higher in the varicocele groups with abnormal ( $4.3 \pm 1.1$ ;  $P < 0.01$ ) or normal ( $3.3 \pm 1.2$ ;  $P < 0.05$ ) semen profile compared with the control group ( $2.8 \pm 0.9$ ). Despite the fact that semen parameters were not significantly altered in the varicocele patients with normal semen profile, evidence of oxidative stress was seen in this group of patients.

A significant negative correlation was found between endogenous generation of ROS and sperm concentration ( $r = -0.48$ ;  $P < 0.05$ ) and progressive motility ( $r = -0.79$ ;  $P < 0.01$ ). No statistically significant differences were found in the status of non-enzymatic antioxidant defences (TAC) of human seminal plasma between the control group ( $1.2 \pm 0.1$  mM) and the groups of patients affected by varicocele, either with normal ( $1.1 \pm 0.4$ ) or with abnormal ( $1.1 \pm 0.5$ ) semen parameters (Table III). When the 55 patients with varicocele were considered collectively, 17 (30%) had TAC values lower than the normal range of the controls (0.9–1.5 mM). TAC mean value (±SD) in blood plasma of control subjects was  $1.0 \pm 0.1$  mM.

**Table III.** Comparison of seminal oxidative stress markers (ROS and TAC) between controls and patients with varicocele

	Controls	Patients	
		Normal SP	Abnormal SP
Log (ROS+1)	2.8 ± 0.9 (1.2–4.6)	3.3 ± 1.2 (1.5–5.9)*	4.3 ± 1.1 (2.5–5.9)**
TAC (mM)	1.2 ± 0.1 (0.9–1.5)	1.1 ± 0.4 (0.2–2.0)	1.1 ± 0.5 (0.3–2.2)

Values are mean ± SD; values in parenthesis are ranges; SP, semen parameters. \* $P < 0.05$  versus control; \*\* $P < 0.01$  versus control.

### Sperm DNA fragmentation measured by the TUNEL assay

The TUNEL assay results are summarized in Table IV. The percentage of TUNEL-positive cells ( $14.2 \pm 1.2\%$ ) in control subjects increased up to  $26.1 \pm 3.2$  and  $32.2 \pm 4.1$  in patients with normal and abnormal semen profile, respectively. Interestingly, about 50% of the patients with normal seminal profile showed DNA fragmentation values higher than the upper limit exhibited by the control group.

### Sperm DNA damage determined by SCSA

Comparisons of SCSA results between controls and patients with varicocele are shown in Table IV. The mean value for DFI (percentage of sperm with denatured DNA) in the control group was 7.1%, with a lower and upper limit of 1.5 and 17.4%, respectively. This value was significantly greater in both groups of varicocele patients either with normal ( $20.7 \pm 4.0$ ) or with abnormal ( $35.5 \pm 9.0$ ) semen profile. Similarly,  $X\alpha_1$  in both groups of patients with varicocele was significantly higher than in the control group. SCSA results in both groups of patients were highly variable; DFI ranged from 2.7 to 59.6 in the patients with normal seminal profile and from 6.9 to 99.8 in the group with abnormal seminal parameters. In the group of patients with varicocele, increased DNA damage (defined as the mean of the control group plus 2SD) was seen in 18 of 37 (49%) patients with normal semen profile and in 58% of patients with abnormal semen parameters (data not shown).

A positive correlation ( $r = 0.50$ ;  $P = 0.001$ ) was found between DNA fragmentation as assessed by the TUNEL assay and chromatin alterations as measured by SCSA (Figure 1B).

### Relationship between DNA damage and ROS

Figure 1A illustrates the relationship between DNA damage, as assessed by the SCSA, and ROS levels. DFI was significantly correlated ( $r = 0.30$ ;  $P = 0.025$ ) with ROS levels within patients. DNA damage and ROS were not significantly correlated within the controls.

### Relationship between DNA fragmentation and conventional sperm parameters

In patients with varicocele, SCSA parameters were negatively correlated with sperm concentration, progressive motility and normal sperm forms. We found negative correlations between DFI and sperm concentration ( $r = -0.35$ ;  $P < 0.01$ ), progressive motility ( $r = -0.38$ ;  $P < 0.01$ ) and normal morphology ( $r = -0.23$ ;  $P < 0.01$ ). A weak positive correlation ( $r = 0.17$ ;  $P < 0.05$ ) was observed

**Table IV.** Comparison of sperm DNA fragmentation (TUNEL) and chromatin alterations (SCSA) in controls and patients with varicocele

Groups	TUNEL (%)	SCSA	
		DFI (%)	X $\alpha_1$
Control	14.2 $\pm$ 1.2 (4.5–24.8)	7.1 $\pm$ 0.9 (1.5–17.4)	303.6 $\pm$ 5.2 (243–360)
Patients			
Normal SP	26.1 $\pm$ 3.2 (6.5–58.3)*	20.7 $\pm$ 4.0 (2.7–59.6)**	331.8 $\pm$ 12.9 (282–448)*
Abnormal SP	32.2 $\pm$ 4.1 (13.1–50.6)**	35.5 $\pm$ 9.0 (6.9–99.8)**	391.9 $\pm$ 30.7 (279–565)**

Values are mean  $\pm$  SE; values in parenthesis are ranges; SP, semen parameters; TUNEL, terminal deoxyribonucleotidyl transferase-mediated dUTP nick-end labelling.

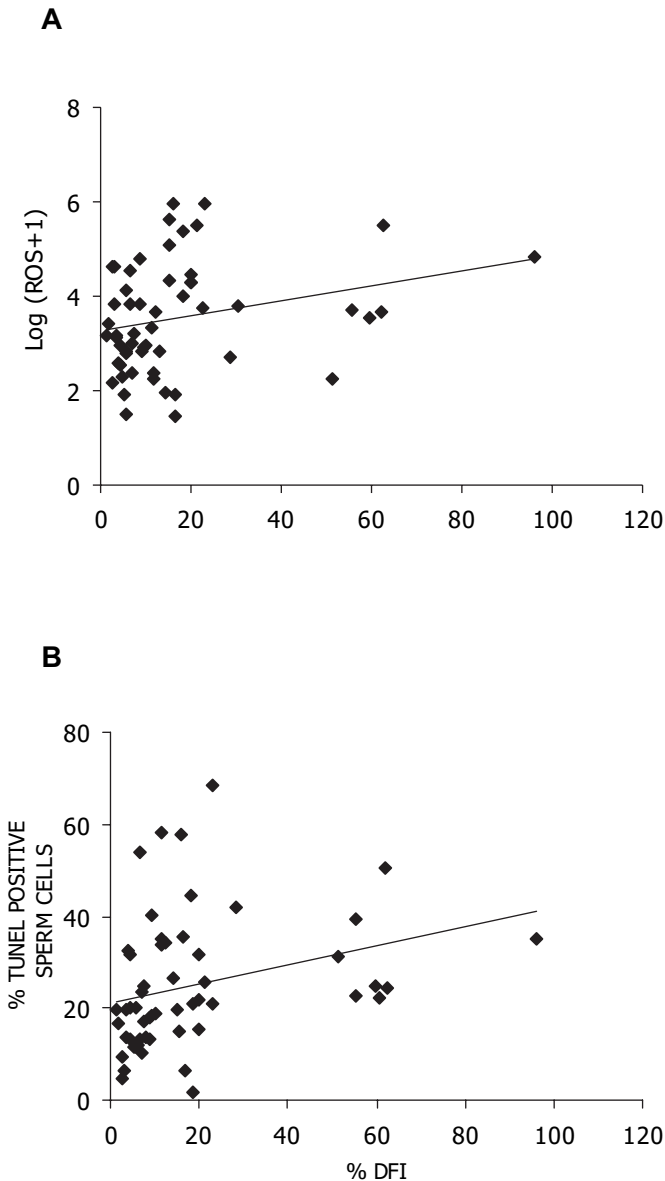
\* $P < 0.05$  versus control; \*\* $P < 0.01$  versus control.

**Table V.** Correlation between SCSA parameters (DFI and X $\alpha_1$ ) and sperm concentration, motility and morphology

	DFI (%)		X $\alpha_1$	
	<i>r</i>	<i>P</i>	<i>r</i>	<i>P</i>
Sperm concentration (10 <sup>6</sup> /ml)	-0.35	<0.01	-0.29	<0.01
Progressive motility (%)	-0.38	<0.01	-0.25	<0.01
Normal morphology (%)	-0.23	<0.01	-0.11	NS
Head abnormality (%)	-0.17	<0.05	0.10	NS
Neck abnormality (%)	-0.02	NS	0.04	NS
Tail abnormality (%)	0.16	NS	0.05	NS

DFI, percentage of sperm with DNA fragmentation; X $\alpha_1$ , mean population of  $\alpha_1$  [ $\alpha_1$  = red/total (red + green) fluorescence].

NS, not statistically significant.

**Figure 1.** Correlation between DNA fragmentation index (DFI) and levels of ROS (A) and terminal deoxyribonucleotidyl transferase-mediated dUTP nick-end labelling (TUNEL)-positive spermatozoa (B) in patients with varicocele.

between DFI and head abnormality. There was also a negative correlation between X $\alpha_1$ , sperm concentration ( $r = 0.29$ ;  $P < 0.01$ ) and sperm progressive motility ( $r = 0.25$ ;  $P < 0.01$ ) (Table V).

## Discussion

Varicocele is the andrological pathology of greatest incidence (15%) in the general population (Belloli *et al.*, 1993) and is commonly diagnosed among men with infertility (WHO, 1992; Benoff and Gilbert, 2001; Hauser *et al.*, 2001). However, as it also occurs in fertile individuals, its association with male infertility is still under debate. Varicocele may be associated with a variety of spermatogenetic conditions, ranging from normozoospermia to moderate oligoasthenoteratozoospermia or azoospermia. We studied a select group of young patients with clinical varicocele, over 50% of whom showed normal routine semen parameters. Varicolectomy is the most common practice to correct this lesion. A number of studies have suggested an improvement in semen quality and pregnancy rates after varicocele repair (Schlesinger *et al.*, 1994; Kim *et al.*, 1999), whilst other studies fail to demonstrate a significant improvement after varicolectomy (Nieschlag *et al.*, 1998).

Several factors associated with varicocele may induce pathways that lead to DNA damage and apoptosis, including heat stress (Mieusset and Bujan, 1995; Wright *et al.*, 1997), androgen deprivation (WHO, 1992; Fujisawa *et al.*, 1994), exposure to toxic agents (Hikim and Swerdloff, 1995; Benoff *et al.*, 1997, 2004), testicular hypoxia (Hsu *et al.*, 1994; Li *et al.*, 1999) and increased oxidative stress (Hendin *et al.*, 1999). In recent years, we have seen an increasing number of molecular/genetic studies that are helping us to better understand the mechanism responsible for the pathophysiology of varicocele (Marmar, 2001; Naughton *et al.*, 2001). Our goal was to examine

the levels of sperm DNA damage in ejaculated spermatozoa from young varicocele patients either with normal or with abnormal semen parameters. In particular, it was considered important to establish whether oxidative stress indicators are modified and whether non-enzymatic antioxidants are depleted in the seminal plasma of varicocele patients.

Oxidative stress is considered an important key factor in the aetiology of male infertility regardless of its origin, seminal or testicular, and its negative effect on fertility is well documented (Hauser *et al.*, 2001; Jarow, 2001). Increased ROS levels have also been implicated in reduced fertility in patients with varicocele. Previous studies have demonstrated that ROS concentrations are higher in semen samples from men with varicocele, suggesting that sperm dysfunction in varicocele is in part related to oxidative stress (Barbieri *et al.*, 1999; Hendin *et al.*, 1999; Pasqualotto *et al.*, 2000; Agarwal *et al.*, 2003). In this study, we demonstrated that the spermatozoa from patients with varicocele produce significantly higher ROS levels, even in the group of patients with completely normal standard semen parameters, in agreement with several previous reports (Barbieri *et al.*, 1999; Saleh *et al.*, 2003b).

In the present study, we also assessed the status of the total non-enzymatic antioxidant defences in seminal plasma. A number of studies have proposed that the increased sensitivity to oxidative damage of the spermatozoa from varicocele men may, in part, be due to an impaired seminal plasma antioxidant capacity, suggesting an association between decreased TAC and male infertility (Lewis *et al.*, 1995; Smith *et al.*, 1996). In contrast to our previous study in which we observed a significant reduction in the antioxidant defences of both seminal and blood plasma in patients with varicocele (Barbieri *et al.*, 1999), in the present study, we showed that the antioxidant levels (mean  $\pm$  SD) exhibited by normozoospermic or non-normozoospermic varicocele patients were not significantly different from those found in controls. The differences observed between our previous and present studies may be due to the better-defined composition of the study groups: in the present study, we used a younger group of patients and stricter exclusion criteria. Consequently, the pathological levels of ROS detected in the semen of men with varicocele are most likely caused by increased ROS production on its own, rather than by reduced antioxidant capacity of seminal plasma. Normal TAC levels in seminal plasma associated with increased levels of ROS production have been reported in patients examined for infertility (Moustafa *et al.*, 2004). A number of studies have proposed that the presence of spermatozoa with damaged DNA may be the result of an impaired chromatin packing or may be indicative of apoptosis (Sakkas *et al.*, 2002). Apoptosis is a dominant process occurring during spermatogenesis that controls the overproduction of male gametes to numbers that can be supported by the Sertoli cells (Lee *et al.*, 1997) and ensures the selection of sperm cells with undamaged DNA. Several authors have described the presence of apoptotic markers including phosphatidylserine, DNA fragmentation, caspase and Fas expression in ejaculated spermatozoa (Shen *et al.*, 2002; Paasch *et al.*, 2003). These are present to a greater extent in spermatozoa from men with deranged spermatogenesis (Weng *et al.*, 2002). However, apoptotic markers and DNA

fragmentation did not necessarily exist in unison. In fact, Sakkas *et al.* (2002) showed that TUNEL positivity and apoptotic markers such as Fas, Bcl-x and p53 are not expressed in the same spermatozoa. Further studies have found that there was no relationship between samples displaying Fas positivity and double-strand breaks as assessed by the Comet assay (McVicar *et al.*, 2004). It is not clear whether the apoptotic markers detected in spermatozoa are residues of an abortive apoptotic process started before ejaculation (Sakkas *et al.*, 1999b, 2003b) or whether they result from apoptosis initiated at the post-testicular level. In our study, DNA fragmentation occurred at increased levels in spermatozoa from patients with either normal or abnormal semen parameters. On the other hand, DNA damage was detected only in a small percentage of ejaculated spermatozoa from normozoospermic controls. Using the TUNEL assay, a negative association was found between the percentage of sperm with DNA fragmentation and fertilization and embryo cleavage rates after IVF (Sun *et al.*, 1997; Host *et al.*, 2000) or ICSI (Lopes *et al.*, 1998). When 20% is used as a threshold for TUNEL-positive sperm samples, embryo development and pregnancy rates appear to be severely affected (Benchaib *et al.*, 2003; Seli *et al.*, 2004).

SCSA is a quantitative assessment of sperm chromatin integrity defined as susceptibility of DNA to acid-induced denaturation *in situ*. The predictive value of SCSA in relation to the outcome of IUI, IVF and ICSI has been widely reported by several authors. Most of these studies have shown that SCSA has a strong prognostic power in predicting negative pregnancy outcomes in couples attempting pregnancy via ART. Two independent studies (Evenson *et al.*, 1999; Spano *et al.*, 2000) demonstrated that when denatured DNA was above threshold (>30%), ultimately fertile couples took longer to conceive. Several other recent studies have evaluated DNA structure and integrity in human ejaculated sperm samples and have reported a negative effect of high percentages of DNA-damaged spermatozoa on pregnancy rates (Larson *et al.*, 2000; Larson-Cook *et al.*, 2003; Saleh *et al.*, 2003b; Tesarik *et al.*, 2004b). In these studies, there seems to be a consensus that no pregnancies resulted after ART if >27% of sperm in the net sample showed DNA denaturation by SCSA. Contrary to previous reports, Gandini *et al.* (2004) demonstrated ICSI term pregnancies even with semen samples characterized by a high SCSA DFI (>30%); however, in this study, the total number of patients studied was very small. In a larger study on the predictive value of SCSA in relation to the outcome of IVF and ICSI, Bungum *et al.* (2004) reported that a DFI level >27% is not incompatible with pregnancy following IVF and ICSI.

A recent study by Saleh *et al.* (2003a), using the SCSA, has demonstrated a significant increase in the extent of DNA damage in infertile patients with varicocele. In the present study, the presence of varicocele was associated with high levels of DNA-damaged spermatozoa as assessed by SCSA and TUNEL assay even in the presence of normal sperm parameters. Therefore, the finding of normal conventional sperm parameters does not guarantee the absence of DNA damage in the sperm population from the ejaculate of varicocele patients. On the other hand, the finding of a significant positive correlation between levels of ROS and DFI is indicative

that sperm nuclear DNA damage may be mediated by seminal oxidative stress.

In conclusion, our study indicates a significant increase of DNA-damaged spermatozoa in young patients with varicocele even in the presence of normal sperm parameters. The finding of increased ROS levels may indicate that seminal oxidative stress may be involved in the pathogenesis of sperm DNA damage in these patients. Sperm DNA damage, independent of its cause, may affect the quality of the ejaculated spermatozoa and may have implications on their fertility potential.

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