international journal of andrology ISSN 0105-6263

ORIGINAL ARTICLE

Oxidative stress biomarkers and hormonal profile in human patients undergoing varicocelectomy

Graciela E. Hurtado de Catalfo,* Adalberto Ranieri-Casilla,† Fernando A. Marra,‡ María J. T. de Alaniz* and Carlos A. Marra*

*INIBIOLP (Instituto de Investigaciones Bioquímicas de La Plata), CONICET-UNLP, Cátedra de Bioquímica de la Facultad de Ciencias Médicas de la Universidad Nacional de La Plata, †Hospital Interzonal General de Agudos José de San Martín, Servicio de Urología, La Plata, and ‡Clínica Vaccarini, Servicio de Medicina General, La Plata, Argentina

Keywords:
antioxidant enzymes, hormonal levels, human varicocelectomy, oligoelements, oxidative stress, semen characteristics

Correspondence:
Carlos Alberto Marra, INIBIOLP, Cátedra de Bioquímica, Facultad de Ciencias Médicas de la Universidad Nacional de La Plata, Calles 60 y 120, 1900 La Plata, Argentina.
E-mail: camarra@atlas.med.unlp.edu.ar

Received 19 September 2006; revised 24 November 2006; accepted 12 December 2006

doi:10.1111/j.1365-2605.2007.00753.x

Summary

The aetiology of varicocele is multifactorial although hormonal imbalance and oxidative stress play a key role in the progression of illness. No conclusive evidence has been presented previously, describing the changes in these two factors and the evolution of patients after varicocelectomy. Semen characteristics and hormonal profile were analysed in 36 infertile men with unilateral left varicocele and 33 age-paired controls (proved to be fertile men), after careful inclusion/exclusion selection criteria. Liposoluble and hydrosoluble antioxidants, oligoelements and enzyme activities of the antioxidant defence system were also determined in plasma and erythrocyte from antecubital and spermatic veins, and in spermatozoa. Data were compared between groups at different times before and after varicocelectomy. Decreased levels of liposoluble and hydrosoluble antioxidants and increased activities of the antioxidant defence system enzymes were observed in patients compared with controls. Varicocelectomy normalized this condition at different post-surgical times. Levels of Zn and Se in seminal plasma, protein carbonyls and fragmented DNA remained elevated up to 1 month after surgery. Luteinizing and follicle stimulating hormone concentrations exhibited a biphasic behaviour while testosterone was diminished in patients but normalized soon after varicocelectomy. The results clearly demonstrate the link between the antioxidant defence system, hormonal status and semen characteristics along the post-varicocelectomy period. We suggest that oxidative biomarkers may be appropriate in controlling the evolution of post-varicocelectomy patients, and antioxidant supplementation may improve the clinical condition of infertile men with varicocele.

Introduction

The aetiology of human varicocele, usually observed in the left testis, is multifactorial and controversial. Nonetheless, hormonal imbalance (Naughton *et al.*, 2001) and oxidative stress (Chen *et al.*, 2001; Naughton *et al.*, 2001) are believed to play an important role in the progression of illness towards infertility. The incidence of varicocele in the population is high (approx. 15%) and unilateral left varicocele accounts for 41% infertility (Naughton *et al.*, 2001). It has been suggested that varicocelectomy improved semen characteristics and pregnancy rates (Marks *et al.*, 1986), although this is a matter of controversy as other reports failed to demonstrate this association (Kamischke & Nieschlag, 1999, 2001). There are additional studies showing that male infertility and varicocele are associated with elevated reactive oxygenated (ROS) or nitrogenated (RNS) species in seminal plasma and/or spermatozoa, with a concomitant reduced antioxidant capacity (Alkan *et al.*, 1997; Hendin *et al.*, 1999b). Moreover, El-Demerdash *et al.* (2004) demonstrated that antioxidants preserve semen quality in Cd-intoxicated male rats under oxidative stress conditions.

In human varicocele, a careful revision of data already reported revealed controversial results between laboratories that could be attributed to different criteria in

selecting patients with clinical diagnoses and/or lifestyles. In addition, there is a lack of evidence on the relationship between hormonal status, semen characteristics and the antioxidant defence system in varicocelectomized patients. For these reasons, the major aim of this investigation was to correlate those parameters in left varicocele patients and healthy donors and to gain an insight into the evolution of the illness along the pre- and post-surgical periods. The purpose was also to obtain information about (i) the convenience of antioxidant supplementation in these patients, and (ii) the utility of oxidative and/or hormonal biomarkers as potential predictive indexes of fertility.

Materials and methods

Study population

This study was approved by the local institutional review ethical board, which follows the Helsinki Declaration (1983 revised). From a large population of patients (approx. 200) treated in local hospitals only 36 infertile males with unilateral left varicocele (VC group) were selected for this study. Another group of 33 age-paired control donors who were fertile men (CO group) was also selected. For both groups, serial semen analyses were performed according to the standard WHO criteria (World Health Organization, 1999). Patients suffering from bilateral or right varicocele, smokers, drug and/or alcohol consumption, ongoing medical treatments for any other illness, diabetes, asthma, hypertension, testicular injury, cord injury, history of venereal disease(s), autoimmune disorders, infectious diseases of any aetiology, azoospermic ejaculate, infertility <1 year, hormonal-associated illness, focal testicular and/or scrotal abnormalities not associated with left varicocele, and particular diets and/or use of diet supplements (before or after varicocelectomy) of any composition were excluded from this study. Patients and controls were 28 ± 3 and 28 ± 4 years old, respectively, and they underwent complete clinical and laboratory examinations, and also ultrasound and colour Doppler scanning between 8:00 and 10:00 h after overnight fasting. Scrotal ultrasound assessment of testicular volume was performed following the procedure of Battaglia *et al.* (2001). The volume was automatically calculated after the ultrasound assessment by means of the software included in the device used (AU-4 Idea; Esaote, Milan, Italy). Doppler flow measurements of transmediastinal arteries (TMAs) were performed in each testis using a trans-scrotal sensor settled at 6.5 mHz and using a 50-Hz filter to eliminate low frequency signal originating from vessel wall movements. Colour flow images of TMAs were obtained in a longitudinal plane at the level of the testicular mediastinum. The angle of insonication was modified to obtain the maximum colour intensity and blood flow velocity waveforms were recovered by placing the Doppler in the pulsed mode. The pulsatility index (PI) was calculated electronically.

Chemicals

Standards for high-performance liquid chromatography (HPLC), cofactors for enzymatic reactions, thiobarbituric acid, deferoxamine mesylate, CoA-SLi, *N*-ethylmaleimide, tetraetoxypropane, Trolox, Tris, sodium deoxycholate (grade II), *N*-acetylcysteine, dithiothreitol and butylated hydroxytoluene were purchased from Sigma Chem. Co., (Buenos Aires, Argentina). Sodium nitrite and nitrate were from Fluka Chemie AG, GmbH & Co. (Buenos Aires, Argentina.). Nitric acid and solvents were HPLC grade and provided by Carlo Erba (Milan, Italy). Organic and inorganic chemicals of analytical grade were obtained from local commercial sources. Luteinizing hormone (LH), follicle-stimulating hormone (FSH) and testosterone were determined by radioimmunoassay (RIA) using commercial kits (Radim, Ponenzia, Italy).

Blood and semen samples

After 3 days of sexual abstinence, semen samples were obtained by masturbation, collected between 8:00 and 10:00 h and allowed to liquefy at 34 °C for 25 min. Computer-assisted semen analysis (CASA) was performed on all samples with a Motion Analysis VP50 Semen Analyzer (Motion Analysis, Co., Santa Rosa, CA, USA). Sperm count was assessed using a counting chamber. In some cases CASA results were confirmed by manual assessment using conventional microscopy examination under a blind code to increase the accuracy of the analyses. Seminal plasma was obtained by centrifugation (350 ***g*** for 10 min) and stored under nitrogen atmosphere at −80 °C. Pelleted spermatozoa were washed twice with cold phosphate-buffered saline (PBS) (pH 7.40), resuspended in the same fresh solution (approx. 20.10^6 spermatozoa/mL), and immediately processed for DNA fragmentation assay based on the diphenylamine reaction (Gil *et al.*, 2003). Whole blood samples from the antecubital vein were collected in 15 IU/mL heparin. Plasmas were obtained by centrifugation (600 ***g*** for 10 min) in the cold and stored under nitrogen atmosphere at −80 °C. An aliquot was supplemented with *N*-ethylmaleimide for reduced (GSH) and oxidized (GSSG) glutathione analyses. Another aliquot was treated with deferoxamine mesylate 0.1 mM (Menditto *et al.*, 1997) and immediately analysed for ascorbate content (Benzie *et al.*, 1999). Erythrocytes were washed twice in cold PBS (pH 7.40) and centrifuged (600 ***g*** for 10 min) to prepare erythrocyte lysates (Berlin

et al., 1989). In the case of the VC group whole blood samples were also collected from the spermatic vein (left-hand side) during varicocelectomy before ligation of varicocele veins, and processed as peripheral blood samples.

Biochemical assays

The presence of leucocytes in semen samples was assessed by myeloperoxidase test (Shekarriz *et al.*, 1995). Thiobarbituric acid reactive substances (TBARS) were measured in blood and seminal plasma fluorometrically (Yagi, 1976). Oxidized glutathione (GSSG) was determined by HPLC (Asensi *et al.*, 1994) while reduced glutathione (GSH) was measured following the glutathione-*S*-transferase assay (Brigelius *et al.*, 1983), α-tocopherol (Buttriss & Diplock, 1984) and retinol (Catigiani & Bieri, 1983) content were determined by HPLC. The following antioxidant enzymes were also determined: catalase (Aebi, 1984), superoxide dismutase (SOD) (Flohé & Ötting, 1984), gutathione peroxidase (GSH-Px) (Wheeler *et al.*, 2001), glutathione transferase (GSH-Tr) (Habib *et al.*, 1984) and glutathione reductase (GSH-Rd) (Callberg & Mannervick, 1985). Protein carbonyls were determined spectrophotometrically (Levine *et al.*, 1990) and quantified using a molar absorption coefficient for 2,4-dinitrophenylhidrazones of 22 000 mol/L/cm (Reznick & Packer, 1994). Total antioxidant capacity (FRAP assay) was performed in peripheral and seminal plasma (Benzie & Strain, 1996). Results were normalized using Trolox as a reference antioxidant. Nitric oxide synthetase (NOS) activity was estimated indirectly by quantification of nitrite plus nitrate ([NOx]) levels (Verdon *et al.*, 1995). For trace element analyses the samples were mineralized overnight with concentrate nitric acid, diluted with bi-distilled water and centrifuged (10 000 ***g***, 15 min) to remove undissolved particulates. The supernatant was used to determine mineral levels by atomic absorption spectrophotometry (Habib *et al.*, 2002). Protein content was determined by the micromethod of Lowry *et al.* (1951). Total lipid content in sperm samples was measured according to Folch *et al.* (1957) and quantified gravimetrically (Marra *et al.*, 1998).

Graphic software and statistical treatment of the data

All values represent the mean of 33 control (healthy) (CO) or 36 (VC) individual determinations (assayed in duplicate) ± 1 standard error of the mean. Binomial Gaussian distribution was checked for both the patients and the control group. Statistical significance of differences was analysed by the Student's *t*-test or by ANOVA, with the aid of Systat (version 8.0 for Windows) from SPSS Science (Chicago, IL, USA), Sigma Scientific Graphing Software (version 8.0) from Sigma Chem. Co. (St Louis, MO, USA), and/or GB-STAT Professional Statistics Program (version 4.0) from Dynamic Microsystems Inc. (Silver Springs, MD, USA).

Results

Testicular and sperm characteristics

Varicocele patients did not present a significant difference in volume of testis with respect to the control group. Moreover, there was no difference between right and left testicles (15.6 ± 1.9 cm^3 and 16.6 ± 2.2 cm^3 respectively), with a 29% variation between extreme values. PI has been shown to reflect blood flow impedance downstream of the point of sampling. Significant differences were found between PI of left and right testicles (0.87 ± 0.07 and 1.33 ± 0.10 respectively). TMA characteristics confirmed in all cases the typical pattern of left varicocele. In all VC patients internal spermatic vein had a diameter larger than 3.5 mm associated with reversal flow. Neither healthy donors nor patients exhibited leucocytospermia defined as the presence of at least 1.10^6 white blood cells/mL as they were negative for myeloperoxidase test (data not shown). Data regarding the evolution of semen parameters along the experimental period were analysed (Fig. 1). Before surgery, all parameters studied except ejaculated volume were significantly lower in the VC group compared with CO; they reached control values between 3 and 6 months after surgery. More relevant from the clinical point of view was the comparison of patients before and after surgery. It evidenced (Fig. 1) that varicocelectomy improved steadily the total sperm number, morphology and sperm concentration, the data being significantly higher 3 months after surgery when compared with the data obtained before surgery. No significant variations were seen in total motility up to 6 months post-correction.

Hormonal profile in CO and VC groups

Table 1 shows testosterone, FSH and LH levels in plasma from peripheral blood samples along the experimental period. VC patients showed a significant decrease in testosterone levels before the surgery that normalized after 1 month. LH and FSH values showed a biphasic pattern. Both hormones were elevated before surgery, normalized during the post-surgical period, and again were elevated 6 months after varicocelectomy. Values remained high at least up to the end of the period studied.

Oxidative stress biomarkers

Thiobarbituric acid reactive substances were significantly elevated in both peripheral and seminal plasma before

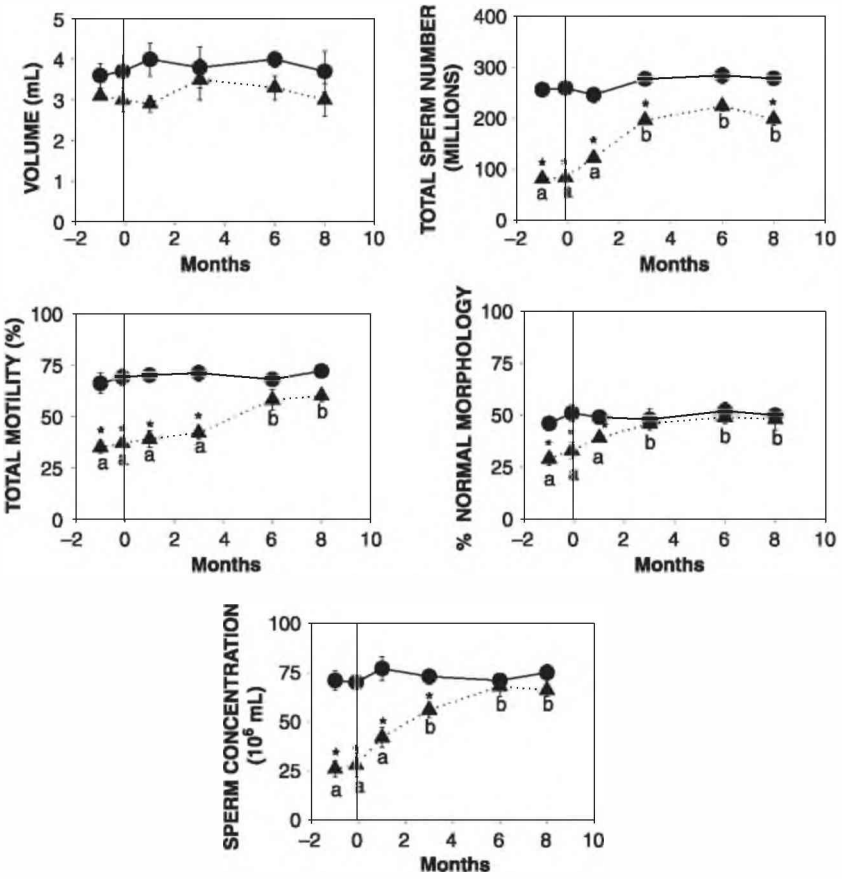

Figure 1 Major sperm characteristics in CO (–●–) and VC (···▲···) groups determined by CASA following the WHO guidelines. Each sample was taken and processed under the same conditions Results are expressed as the mean ± 1 SEM of 33 (CO) or 36 (VC) independent analysis. Statistical differences were tested (ANOVA + Bonferroni test) between control and patients groups (*$p < 0.001$), and patient group before and after surgery (different letters, $p < 0.01$).

Table 1 Hormonal parameters in plasma from control donors (CO) and varicocelectomized (VC) patients

	Testosterone (ng/dL)		LH (ng/dL)		FSH (IU/L)	
Months	CO	VC	CO	VC	CO	VC
–1.0	424 ± 18	298 ± 17*	5.3 ± 0.2	8.1 ± 0.1*	10.7 ± 0.3	17.5 ± 0.2*
–0.1	396 ± 11	301 ± 11*	6.1 ± 0.2	7.8 ± 0.2*	11.3 ± 0.2	18.3 ± 0.3*
1.0	408 ± 15	342 ± 30	5.8 ± 0.1	6.6 ± 0.3	12.0 ± 0.3	15.5 ± 0.4
3.0	420 ± 20	440 ± 18	5.5 ± 0.3	6.8 ± 0.1	10.9 ± 0.4	13.3 ± 0.3
6.0	431 ± 26	398 ± 20	6.3 ± 0.3	7.9 ± 0.2*	11.0 ± 0.1	16.0 ± 0.3*
8.0	411 ± 20	382 ± 15	6.0 ± 0.2	8.0 ± 0.1*	12.1 ± 0.3	16.8 ± 0.1*

Results were obtained by radioimmunoassay as described in Materials and methods. Each value is expressed as the mean ± 1 SEM of 33 or 36 individual determinations assayed in duplicated from plasma of CO or VC groups respectively. LH, luteinizing hormone; FSH, follicle-stimulating hormone. *Significantly different with respect to the corresponding control value ($p < 0.001$).

surgery and remained high up to 1 month post-varicocelectomy (Fig. 2). Values were higher in seminal plasma than in peripheral plasma, notwithstanding the pattern displayed was essentially the same in both kinds of samples. The content of nitrates plus nitrites ([NOx]) in seminal plasma was elevated in the VC group before varicocelectomy. After surgery, it remained significantly higher for 1 month and normalized between 1 and 3 months (Fig. 3). In peripheral blood [NOx] concentration showed a similar pattern to that observed in seminal plasma, although differences were not statistically significant (data not shown). The concentration of [NOx] in the spermatic vein was 63.4 ± 5.8 μM which was significantly higher ($p < 0.01$) with respect to that measured in the antecubital vein of the VC group (mean value during the pre-surgical period: 31.3 ± 4.0 μM). Figure 4 shows the content of liposoluble and hydrosoluble antioxidants determined in total sperm. The α-tocopherol content

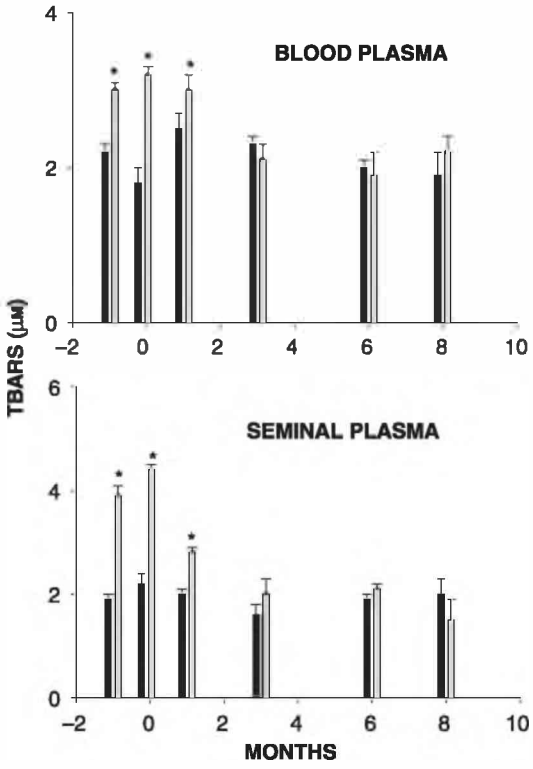

Figure 2 Thiobarbituric acid reactive substances (TBARS) in plasma from peripheral blood and seminal plasma in CO (black bars) or VC (grey bars) groups before and after varicocelectomy. Technical details are described in Materials and methods. Results are expressed as the mean of 33 (CO) or 36 (VC) independent analyses assayed in duplicate (*$p < 0.001$).

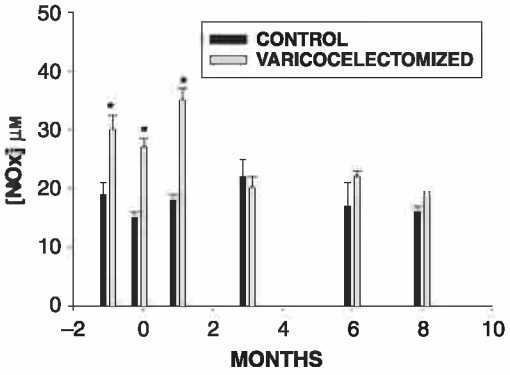

Figure 3 Indirect estimation of the nitric-oxide synthetase activity by determination of nitrate plus nitrite ([NOx]) concentrations in healthy (black bars) or varicocele patients (grey bars) before and after varicocelectomy. Results are expressed as the mean ± 1 SEM of 33 (CO) or 36 (VC) independent analyses assayed in triplicate (*$p < 0.001$).

(γ-isomer amount was negligible) was normalized in respect to the corresponding lipid amount in each sample. The total lipid content in sperm samples within each

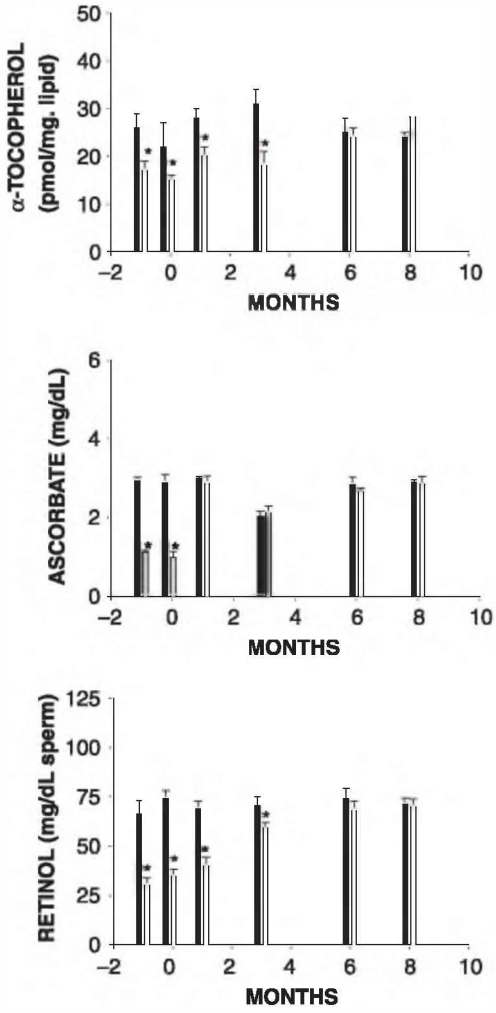

Figure 4 Antioxidant contents in sperm of CO (black bars) or VC (grey bars) individuals before and after varicocelectomy. Methodological procedures are mentioned in Materials and methods. Results are expressed as the mean ± 1 SEM of 33 (CO) or 36 (VC) independent analyses assayed either in duplicate or triplicate (*$p < 0.001$).

group was very similar and constant during the study (never exceeded 9 % of the mean value) while the total lipid concentration in the VC group was slightly reduced (−16 %) compared with CO donors. The α-tocopherol concentration of VC patients was significantly lower than CO values before surgery and up to 3 months after varicocelectomy. Diminished levels of ascorbate and retinol observed in the VC group before surgery normalized 1 or 3 months after the operation, respectively. Reduced (GSH) and oxidized (GSSG) glutathione content in seminal plasma is shown in Table 2. No significant differences were found between CO and VC groups along the period of observation. However, the ratio GSH/GSSG was reduced in the VC group before surgery and up to

Table 2 Reduced (GSH) and oxidized (GSSG) glutathione contents in seminal plasma from control donors (CO) and varicocelectomized (VC) patients

	CO			VC		
Months	GSH	GSSG	GSH/GSSG	GSH	GSSG	GSH/GSSG
−1.0	2.2 ± 0.10	0.11 ± 0.01	20 ± 2	1.7 ± 0.10	0.15 ± 0.02	11 ± 1*
−0.1	1.9 ± 0.06	0.13 ± 0.02	15 ± 3	1.5 ± 0.21	0.19 ± 0.01	8 ± 2*
1.0	2.2 ± 0.20	0.10 ± 0.01	22 ± 1	1.6 ± 0.30	0.17 ± 0.03	9 ± 2*
3.0	2.0 ± 0.03	0.14 ± 0.03	14 ± 4	1.8 ± 0.10	0.20 ± 0.07	9 ± 2*
6.0	1.9 ± 0.02	0.09 ± 0.01	21 ± 1	2.0 ± 0.28	0.10 ± 0.01	20 ± 4
8.0	2.0 ± 0.10	0.10 ± 0.02	20 ± 2	2.2 ± 0.22	0.10 ± 0.06	22 ± 3

Glutathione contents were determined by HPLC as described in the experimental part (blood and semen samples). Data are expressed in μmol/L as the mean ± 1 SEM of 33 or 36 individual determinations assayed in duplicate from CO donors or VC patients, respectively. *Significantly different with respect to the corresponding control value ($p < 0.001$).

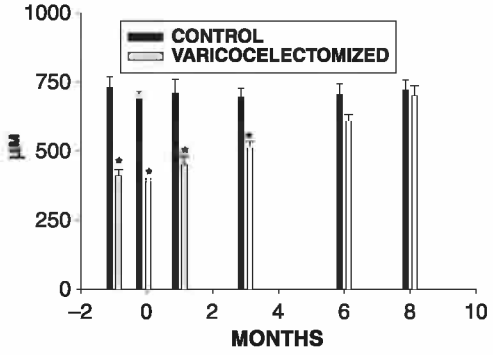

Figure 5 Total antioxidant capacity of seminal plasma determined by the FRAP assay in CO (black bars) or VC (grey bars) samples before and after varicocelectomy. Details of the technical procedure are summarized in Materials and methods. Results are expressed as the mean ± 1 SEM of 33 (CO) or 36 (VC) independent analyses assayed in duplicate (*$p < 0.001$).

3 months post-varicocelectomy. The total antioxidant capacity of seminal plasma estimated by the FRAP assay reflected the changes observed in antioxidant components. Data were relatively constant in the CO group (approx. 750 μM), but exhibited decreased values in the VC group before varicocelectomy and up to 3 months after surgery (Fig. 5). Similar results were obtained in peripheral plasma (data not shown). Unfortunately, blood samples from the spermatic vein of CO donors were not available for the FRAP assay. However, at the time of surgery the results obtained in plasma from the spermatic vein of VC patients (462.8 ± 31.3 μM) were significantly reduced (approx. 30%) compared to those obtained from blood peripheral plasma within the same group (680.1 ± 45.6 μM).

Table 3 shows novel findings in Zn and Se contents of seminal plasma from the CO and VC groups. Both Zn and Se showed increased values before surgery and up to 1 month after varicocelectomy. Results observed in seminal plasma did not correlate with those from peripheral plasma, as no differences were noted between the CO and VC groups in antecubital vein samples along the study (data not shown).

Table 3 Oligoelement concentrations in seminal plasma from control donors (CO) and varicocelectomized (VC) patients

	Zn (mg/dL)		Se (μg/L)	
Months	CO	VC	CO	VC
−1.0	215 ± 11	151 ± 20*	66.1 ± 4.1	46.2 ± 3.0*
−0.1	203 ± 22	145 ± 16*	59.0 ± 3.3	41.5 ± 4.4*
1.0	195 ± 27	167 ± 10*	60.6 ± 2.9	50.3 ± 3.1*
3.0	223 ± 18	218 ± 13	61.7 ± 4.8	65.3 ± 6.2
6.0	211 ± 14	229 ± 22	63.6 ± 5.1	60.8 ± 7.0
8.0	202 ± 12	233 ± 11	60.1 ± 6.0	58.3 ± 6.5

Data were obtained as described in Materials and methods and expressed as the mean ± 1 SEM of 33 and 36 independent determinations from CO and VC groups respectively. *Significantly different ($p < 0.001$) with respect to the corresponding control value.

The activities of several antioxidant enzymes were also measured in erythrocyte lysates from peripheral blood plasma and in sonicated sperm from the CO and VC groups, as well as in erythrocyte lysates from blood of spermatic vein (at the time of surgery) in VC patients. SOD and catalase activities were high in VC peripheral erythrocytes and also in erythrocytes from spermatic vein compared with controls before surgery and after 1 month post-intervention (Table 4). Similar results were obtained in sonicated sperm. Antioxidant enzyme activities involved in glutathione metabolism were significantly modified (Table 5). Glutathione peroxidase activity was increased in erythrocyte lysates from peripheral and spermatic vein, and also in sonicated sperm. Activities returned to control values 1 months after surgery.

Glutathione reductase and transferase showed no significant changes in erythrocyte lysates from peripheral blood, but they were increased in spermatic vein and in

Table 4 Superoxide dismutase and catalase activities in erythrocyte lysates from antecubital and spermatic veins, and sonicated sperm from control donors (CO) or varicocelectomized (VC) patients

	Erythrocyte lysates				
	Antecubital		Spermatic	Sonicated sperm	
Month	CO[a]	VC[a]	VC[a]	CO[b]	VC[b]
Superoxide dismutase					
−1.0	1272 ± 85	1655 ± 121*	n.d.	133 ± 9	186 ± 15*
0.0	1196 ± 108	1703 ± 134*	1933 ± 141*	148 ± 11	207 ± 21*
1.0	1301 ± 90	1549 ± 96*	n.d.	125 ± 7	175 ± 10*
8.0	1273 ± 111	1380 ± 135	n.d.	142 ± 22	154 ± 14
Catalase					
−1.0	115 ± 19	142 ± 16*	n.d.	2.6 ± 0.2	3.6 ± 0.2*
0.0	122 ± 14	157 ± 21*	169 ± 12*	3.0 ± 0.1	4.0 ± 0.3*
1.0	108 ± 21	119 ± 13	n.d.	2.7 ± 0.1	3.9 ± 0.1*
8.0	117 ± 15	124 ± 17	n.d.	2.8 ± 0.2	3.0 ± 0.3

Enzyme activities were determined according to the procedures described in Materials and methods. Data are expressed as the mean ± 1 SEM of 33 (CO) or 36 (VC) individual determinations assayed in duplicate. n.d., not determined. [a]Units/g haemoglobin; [b]Units/mg protein. *Significantly different with respect to the corresponding control value ($p < 0.001$).

Table 5 Glutathione-related enzyme activities in erythrocyte lysates from antecubital and spermatic veins, and sonicated sperm from control donors (CO) or varicocelectomized (VC) patients

	Erythrocyte lysates				
	Antecubital	Spermatic		Sonicated sperm	
Month	CO[a]	VC[a]	VC[a]	CO[b]	VC[b]
Glutathione peroxidase					
−1.0	18.6 ± 1.9	26.6 ± 3.8*	n.d.	9.8 ± 0.7	13.6 ± 0.2*
0.0	21.5 ± 3.3	25.8 ± 3.1*	39.7 ± 4.1*	10.2 ± 0.8	14.0 ± 0.3
1.0	17.3 ± 4.0	18.2 ± 2.9	n.d.	7.3 ± 0.4	13.9 ± 0.1*
8.0	20.1 ± 1.5	19.5 ± 4.4	n.d.	8.8 ± 0.6	13.0 ± 0.3*
Glutathione reductase					
−1.0	13.6 ± 1.1	16.0 ± 0.3	n.d.	113 ± 9	148 ± 12*
0.0	15.0 ± 2.3	15.1 ± 2.2	22.8 ± 2.2*	124 ± 11	173 ± 20*
1.0	14.2 ± 1.8	18.2 ± 3.5	n.d.	131 ± 14	140 ± 11
8.0	14.0 ± 2.0	17.0 ± 2.4	n.d.	118 ± 8	122 ± 17
Glutathione transferase					
−1.0	25.4 ± 2.2	26.8 ± 3.0	n.d.	44.6 ± 3.2	66.3 ± 4.2*
0.0	31.7 ± 2.0	30.1 ± 2.6	38.6 ± 3.0*	50.1 ± 4.3	69.7 ± 3.3*
1.0	28.3 ± 3.1	24.7 ± 3.8	n.d.	47.5 ± 3.8	50.1 ± 4.0
8.0	29.9 ± 1.9	28.6 ± 4.0	n.d.	48.0 ± 4.4	51.3 ± 5.2

Enzyme activities were determined according to the procedures described in Materials and methods. Data are expressed as the mean ± 1 SEM of 33 (CO) or 36 (VC) individual determinations assayed in duplicate. n.d., not determined. [a]Units/g haemoglobin; [b]Units/mg protein. *Significantly different respect to the corresponding control value ($p < 0.001$).

sonicated sperm. These activities normalized almost immediately after varicocelectomy (Table 5). Damages observed in the antioxidant defence system of VC patients were also reflected in the increased protein carbonyl content measured in proteins from seminal plasma (Fig. 6), and in fragmented DNA in isolated spermatozoa (Fig. 7). These values were normalized 1 month after surgery.

Discussion

The main parameters of semen samples determined in our patients with left varicocele (demonstrated by ultrasound and colour Doppler scanning of testis) were in accordance with those reported previously from other laboratories (Pasqualotto *et al.*, 2000; Daitch *et al.*, 2001;

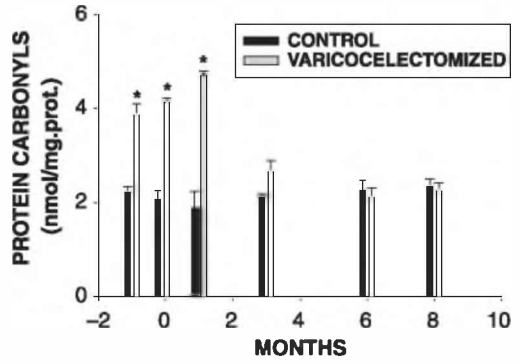

Figure 6 Oxidative damage of proteins estimated by the formation of protein carbonyls in CO (black bars) or VC (grey bars) samples before and after varicocelectomy. Details of the technical procedure are summarized in Materials and methods. Results are expressed as the mean ± 1 SEM of 33 (CO) or 36 (VC) independent analyses assayed in duplicate (*$p < 0.001$).

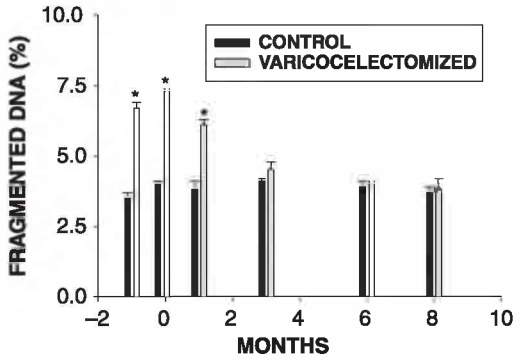

Figure 7 Estimation of the DNA oxidative damage determined by the percentage of fragmented DNA in total sperm from CO (black bars) or VC (grey bars) samples before and after varicocelectomy. Details of the technical procedure are summarized in Materials and methods. Results are expressed as the mean ± 1 SEM of 33 (CO) or 36 (VC) independent analyses assayed in triplicate (*$p < 0.001$).

Xu *et al.*, 2003). In spite of the abundance of data characterizing semen quality and antioxidant status in human cases of varicocele, evidence regarding the post-surgical evolution of left varicocele is extremely scarce. Daitch *et al.* (2001) observed that although varicocelectomy did not improve semen characteristics in all men studied, it appeared to improve pregnancy and live birth rates among couples who underwent intrauterine insemination for male factor infertility. They concluded that men with varicocele should be screened for other functional factors not measured in routine semen analysis. These factors could involve changes in hormonal status and oxidative stress of sperm, among others. In this study, we observed important alterations in both types of parameters when studying infertile left varicocele patients before and after surgical correction. However, the exact mechanism by which varicocele is responsible for men infertility and its real impact on the epidemiology of the illness, is still unknown (Naughton *et al.*, 2001). Several authors have stated that from the development of varicocele at puberty, many factors (hyperthermia, testicular blood flow and pressure changes, reflux of renal/adrenal substances, hormonal dysfunction, autoimmunitive and oxidative stress) could play a role in the evolution of this illness and its consequences (Naughton *et al.*, 2001; Santoro & Romeo, 2001). In this regard, Allamaneni *et al.* (2004) reported that reactive oxygen species (ROS) levels showed a significant correlation with left varicocele grade, and that significantly elevated seminal ROS levels were seen in men with left varicocele grades 2 and 3 compared with grade 1. There is experimental evidence that the normal production of the reactive species ROS and RNS by testicular cells and spermatozoa plays a key role in signal transduction mechanisms involved in fertilization, regulation of sperm capacitation, acrosome reaction and spermatozoa–oocyte attachment (Aitken & Fisher, 1994; De Lamirande & Gagnon, 1995; Özdamar *et al.*, 2004). In healthy men, production and neutralization of ROS and RNS are strictly controlled by a complex antioxidant defence system. Our results clearly indicated that before surgery, VC group exhibited a significant reduction in hydrosoluble and lyposoluble antioxidant contents as well as in GSH/GSSG ratio in seminal plasma compared with CO donors. These observations support the idea of an increased spermatozoa sensitivity to oxidative damage in this kind of patients as suggested by Mancini *et al.* (1998) and Sharma & Agarwal (1996). The patients also had a reduced antioxidant capacity as estimated by the FRAP assay. Similar results were observed by Sharma *et al.* (1999) using TAC, and Hendin *et al.* (1999a) with luminol-dependent chemiluminiscense assay. Augmented levels of [NOx] observed in seminal plasma could be attributed to an incremented NOS activity in varicocele patients. On the other hand, Rosselli *et al.* (1995) reported that elevated nitric oxide concentration decreases sperm motility and induces sperm toxicity. We found that [NOx] was significantly elevated in the spermatic vein when compared with the antecubital vein in the VC group. This result is in agreement with those previously reported by Özbek *et al.* (2000). Levels of [NOx] were inversely correlated with the PI of TMAs suggesting its important role in vascular modulation of testicular vessels and ultimately in sperm output (Battaglia *et al.*, 2000, 2001).

The increased sensitivity of testicular cells to oxidative stress was associated with the high content of polyunsaturated fatty acids (PUFA) of their membrane lipids (Coniglio, 1994). Lenzi *et al.* (1996) demonstrated that PUFA content of sperm plasma membranes was significantly

decreased in varicocele patients compared with normospermic donors. Testicular biopsy of patients with varicocele showed that malondialdehyde (MDA) concentration, which is a useful indicator of ROS-induced lipid peroxidation, was greater in patients with varicocele (Naughton *et al.*, 2001). Moreover, Koksal *et al.* (2000) have proposed a central role of MDA in the pathophysiology of varicocele as this oxidative biomarker was consistently elevated in testicles of patients with mild varicocele and its concentration increased in good correlation with illness progression. Other authors (Aitken *et al.*, 1989; Gomez *et al.*, 1996; Keating *et al.*, 1997) reported a direct correlation between defective sperm function and excessive ROS production in lipid moieties. It is well known that TBARS are directly associated with MDA production (Meagier & Fitzgerald, 2000; Wild *et al.*, 2001). Our results showed elevated TBARS levels in both peripheral and seminal plasma from the VC group that were rapidly normalized after varicocelectomy. However, major sperm characteristics such as sperm number, sperm concentration and total motility remained pathologically altered after normalization of TBARS concentration. This led us to consider that MDA accumulation is not the main determinant in sperm quality. ROS and RNS production were also associated with a high rate of double- and single-strand DNA damage, and to induction of mutations (Lopes *et al.*, 1998; Twigg *et al.*, 1998). In this work we observed that both the level of DNA fragmentation and the formation of protein carbonyls – widely used as biomarkers for ROS-induced damage to proteins – were significantly higher in the VC group than in the CO group. Oxidative modification of DNA and proteins is considered as one of the earliest events caused by oxidative stress. Therefore, protein carbonyl concentration and DNA mutation are not only biomarkers of ROS and RNS-induced damage but also a causal factor for oxidative injury and dysfunction in human testis. These modifications precede loss of cellular ATP, and eventually, cell death (Berlett & Satadtman, 1997; Ciolino & Levine, 1997; Chen *et al.*, 2001). Fortunately, both DNA fragmentation and protein oxidation were rapidly reversed after varicocelectomy. As suggested for the case of the TBARS–time course evolution, these biomarkers seem not to be the key factors in the normalization of sperm quality.

With the aim of seeking a biochemical parameter that correlates with post-surgical evolution of VC patients (especially sperm quality) we studied various enzyme activities, oligoelement concentrations and hormonal status of patients. All antioxidant enzymes tested demonstrated a clear oxidative stress condition in VC patients compared to those of control donors which remained altered for at least 1 month after surgical procedure. Zn concentration is an attractive issue as there have been controversial reports concerning its effect on spermatozoa motility. While Sorensen *et al.* (1999) reported that high seminal Zn concentrations suppress motility, other authors proposed that Zn had a positive effect on spermatogenesis and fertility by improving both motility and density (Chia *et al.*, 2000). In this study, Zn concentration correlated well with SOD activity rather than with sperm motility as this semen parameter remained low in the VC group at least 4 months after surgery. The correlation of Zn concentration with SOD activity was statistically significant ($r_s = 0.96$, $p < 0.01$) as determined by Pearson and Spearman rank test (parametric and nonparametric assays respectively). Gaussian distribution of data, in the present study, made possible both kinds of statistic estimations. However, the conclusions obtained were completely equivalent. Regarding Se, a recent study by Xu *et al.* (2003) indicated that Se concentration was inversely correlated with DNA damage in human spermatozoa. This observation is interesting as the mechanism of DNA fragmentation may be associated with ROS and RNS production and with increased levels of protective antioxidant enzymes such as GSH-Px; one of its isoforms is a selenium-dependent protein. GSH-Tr is another antioxidant enzyme that was related to oxidative damage in testis. We demonstrated that this enzyme activity was elevated during the pre-surgical period but normalized after varicocelectomy. Chen *et al.* (2002) and Rajimakers *et al.* (2003) demonstrated that some isoforms of the superfamily of human GSH-Tr prevent oxidative damage in sperm of VC patients.

We also observed alterations in the hormonal profile of VC patients that were modified by surgery, the chief being the reduced testosterone level in plasma from the VC group that normalized after varicocelectomy. This finding is in agreement with data obtained in animal models (Shafick *et al.*, 1989; Ghosh & York, 1994). Moreover, Naughton *et al.* (2001) reviewed those studies on testosterone levels in plasma from men with this pathology and found that they were significantly lower than those of controls, suggesting a deleterious effect of varicocele on Leydig cell function. Previous evidence demonstrated that the steroidogenic route in Leydig cells is inhibited by oxidative stress condition (Pomerantz & Pitelka, 1998; Murugesam *et al.*, 2005). After varicocelectomy, the enzymes involved in testosterone biosynthesis would be initially restored to control levels. Despite these results, reversibility of hormonal dysfunction after varicocelectomy remains controversial as some authors reported no significant changes in testosterone concentrations before and after surgery (Hudson *et al.*, 1985; Segenreich *et al.*, 1986) while others observed a clear increase in androgen concentration after surgical correction of the illness (Su *et al.*, 1995). Despite this, the real impact of this fact on sperm quality and fertility remains unclear.

Regulation of the hypothalamic-pituitary axis is a very complex mechanism controlled by a feedback system operating from the gonads to the pituitary. To study how this control system may be altered in varicocelectomized patients we measured FSH and LH levels. Both showed a biphasic behaviour that could reflect the adaptation of the pituitary-gonadal axis after varicocele correction. From the data obtained in this experimental design we are not able to propose (without any speculation) a mechanism to explain the increase in these gonadotrophic hormones 6 months after varicocelectomy. However, the fact that both LH and FSH have the same oscillations in the pre and post-surgical periods suggest that they respond to a similar mechanism of adaptation. The physiological significance of these changes remains to be clarified.

In conclusion, we demonstrated that various oxidative stress biomarkers were altered in infertile left varicocele patients. They normalized at different times after varicocelectomy depending on the particular biomarker measured. We also observed that hormonal alterations may be ascribed to an indirect effect of reactive species (ROS, RNS) on Leydig and/or Sertoli cell function. Our study suggests that antioxidant supplements may improve the clinical condition in infertile men with varicocele. We also suggest that some oxidative biomarkers as well as testosterone determination could have potential clinical applications in evaluating the evolution of varicocelectomized patients. More exhaustive investigations in this field could clarify these questions.

Acknowledgements

The authors are grateful to Eva Illara de Bozzolo for excellent technical assistance and to Prof. Enrique Gustavo Bozzarello for data analysis. Language was revised by Norma Tedesco. This study was partially supported by grants from CIC and CONICET, Argentina.

References

Aebi, H. (1984) Catalase in vitro. *Methods in Enzymology* 105, 121–126.

Aitken, R. J. & Fisher, H. (1994) Reactive oxygen species generation and human spermatozoa: the balance of benefits and risk. *Bioassays* 16, 259–267.

Aitken, R. J., Clarkson, J. S. & Hargreave, T. B. (1989) Analysis of the relationship between defective sperm function and generation of reactive oxygen species in cases of oligospermia. *Journal of Andrology* 10, 214–220.

Alkan, I., Simsek, F. & Haklaar, G. (1997) Reactive oxygen species production by spermatozoa of patients with idiopathic infertility: relationship to seminal plasma antioxidants. *The Journal of Urology* 157, 140–143.

Allamaneni, S. S., Naughton, C. K., Sharma, R. K., Thomas, A. J. & Agarwal, A. (2004) Increased seminal reactive oxygen species levels in patients with varicoceles correlate with varicocele grade but not with testis size. *Fertility and Sterility* 82, 1684–1686.

Asensi, M., Sastre, J., Pallardo, F. V., Delaasunción, J. G., Estrela, J. M. & Vina, J. (1994) A high-performance liquid chromatography meted for measurement of oxidized glutathione in biological samples. *Analytical Biochemistry* 217, 323–328.

Battaglia, C., Guilini, S., Gegnani, G., Di Girolamo, R., Paganelli, S., Facchinetti, F. & Volpe, A. (2000) Seminal plasma nitrite/nitrate and intratesticular Doppler flow in fertile and infertile subjects. *Human Reproduction* 15, 2554–2558.

Battaglia, C., Guilini, S., Regnani, G., Madgar, I., Facchinetti, F. & Volpe, A. (2001) Intratesticular Doppler flow, seminal plasma nitrites/nitrates, and non-obstructive sperm extraction from patients with obstructive and non-obstructive azoospermia. *Fertility and Sterility* 75, 1088–1094.

Benzie, I. F. F. & Strain, J. J. (1996) The ferric reducing ability of plasma as a measure of antioxidant power: the FRAP assay. *Analytical Biochemistry* 238, 70–76.

Benzie, I. F. F., Chung, W. Y. & Strain, J. J. (1999) Antioxidant (reducing) efficiency of ascorbate in plasma is not affected by concentration. *Journal of Nutritional Biochemistry* 10, 146–150.

Berlett, B. & Satadtman, E. R. (1997) Protein oxidation in aging, disease, and oxidative stress. *Journal of Biological Chemistry* 272, 20313–20316.

Berlin, E., Bhathera, S. J., Judd, J. T., Nair, P. P., Jones, D. Y. & Tayler, P. R. (1989) Dietary fat and hormonal effects on erythrocyte membrane fluidity and lipid composition in adult women. *Metabolism* 8, 790–796.

Brigelius, R., Muckel, C., Akerboom, T. P. M. & Sies, H. (1983) Identification and quantitation of glutathione in hepatic protein mixed disulfides and its relationship to glutathione disulfide. *Biochemical Pharmacology* 32, 2529–2534.

Buttriss, J. L. & Diplock, A. T. (1984) High-performance liquid chromatography methods for vitamin E in tissues. *Methods in Enzymology* 105, 131–138.

Callberg, E. & Mannervick, A. (1985) Glutathione reductase. *Methods in Enzymology* 113, 484–495.

Catigiani, G. L. & Bieri, J. G. (1983) Simultaneous determination of retinal and alpha-tocopherol in serum or plasma by liquid chromatography. *Clinical Chemistry* 29, 708–712.

Chen, S. S., Chang, L. S. & Whi, Y. H. (2001) Oxidative damage to proteins and decrease of antioxidant capacity in patients with varicocele. *Free Radical Biology Medicine* 30, 1328–1334.

Chen, S. S., Chang, L. S., Chen, H. W. & Wei, Y. H. (2002) Polymorphisms of glutathione *S*-transferase M1 and male infertility in Taiwanese patients with varicocele. *Human Reproduction* 17, 718–725.

Chia, S. E., Ong, C. N., Chua, L. H., Ho, L. M. & Tay, S. K. (2000) Comparison of zinc concentration in blood and seminal plasma and the various sperm parameters between fertile and infertile men. *Journal of Andrology* 21, 53–57.

Ciolino, H. P., & Levine, R. L. (1997) Modification of proteins in endothelial cell death during oxidative stress. *Free Radical Biology Medicine* 22, 1277.

Coniglio, J. G. (1994) Testicular lipids. *Progress in Lipid Research* 33, 387–401.

Daitch, J. A., Bedaiwy, M. A., Pasqualotto, E. B., Hendin, B. N., Hallak, J., Falcone, T., Thomas, A. J., Jr, Nelson, D. R. & Agarwal, A. (2001) Varicocelectomy improves intrauterine insemination success rates in men with varicocele. *Journal of Urology* 165, 1510–1513.

De Lamirande, E. & Gagnon, C. (1995) Impact of reactive species on spermatozoa: a balancing act between beneficial and detrimental effects. *Human Reproduction* 10, 15–21.

El-Demerdash, F. M., Yosouf, M. I., Kedwany, F. S. & Baghdadi, H. H. (2004) Cadmium-induced changes in lipid peroxidation, blood hematology, biochemical parameters and seen quality of male rats:protective effects of vitamin E and β-carotene. *Food Chem Toxicol* 42, 1563–1571.

Flohé, L. & Ötting, F. (1984) Superoxide dismutase assays. *Methods in Enzymology* 105, 93–114.

Folch, J., Lees, M. & SloaneStanley, G. A. (1957) A simple method for the isolation and purification of total lipids from animal tissues. *Journal of Biological Chemistry* 226, 497–509.

Ghosh, P. K. & York, J. P. (1994) Changes in testicular testosterone and acid and alkaline phosphatase activity in testis and accessory sex organs after induction of varicocele in Noble rats. *Journal of Surgical Research* 56, 271–276.

Gil, L., Martínez, G., González, I., Tarinas, A., Alvarez, A., Giuliani, A., Molina, R., Tápanes, R., Pérez, J. & León, O. S. (2003) Contribution to characterization of oxidative stress in HIV/AIDS patients. *Pharmacological Research* 47, 217–224.

Gomez, E., Buckingha, D. W. & Brindle, J. (1996) Development of an image analysis system to monitor the retention of residual cytoplasm by human spermatozoa: correlation with biochemical markers of the cytoplasmic space, oxidative stress, and sperm function. *Journal of Andrology* 17, 276–287.

Habib, W. H., Pabst, M. J. & Jakoby, W. B. (1984) Glutathione-*S*-transferases, the first enzymatic step in mercapturic acid formation. *J Biol Chem* 249, 7130–7139.

Habib, H. T., Chacko, M., Fawzi, A., Hilal, A. S. & Dashti, H. M. (2002) Antioxidant enzyme level in the testes of cirrhotic rats. *Nutrition* 18, 56–59.

Hendin, B. N., Kollettis, P. N. & Sharma, R. K. (1999a) Varicocele is associated with elevated spermatozoal reactive oxygen species production and diminished seminal plasma antioxidant capacity. *The Journal of Urology* 161, 1831–1834.

Hendin, B. N., Kolettis, P. N., Sharma, R. K., Thomas, A. J., Jr & Agarwal, A. (1999b) Varicocele is associated with elevated spermatozoal reactive oxygen species production and diminished seminal plasma antioxidant capacity. *The Journal of Urology* 161, 1831–1834.

Hudson, R. W., Perez-Murero, R. A. & Crawford, V. A. (1985) Hormonal parameters of men with varicocele before and after varicocelectomy. *Fertility and Sterility* 43, 905–910.

Kamischke, A. & Nieschlag, E. (1999) Analysis of medical treatment of male infertility. *Human Reproduction* 14, 1–23.

Kamischke, A. & Nieschlag, E. (2001) Varicocele treatment in the light of evidence-based andrology. *Human Reproduction Update* 7, 65–69.

Keating, J., Grundy, C. E. & Fivey, P. S. (1997) Investigation into the association between the presence of cytoplasmic residues on the human sperm midpiece and defective sperm function. *Journal of Reproduction and Fertility* 110, 71–77.

Koksal, I. T., Tefleki, A. & Usta, M. (2000) The role of reactive oxygen species in testicular dysfunction associated with varicocele. *British Journal of Urology* 86, 549–552.

Lenzi, I. T., Picardo, M. & Gandini, L. (1996) Lipids of the sperm plasma membrane: from polyunsaturated fatty acids considered as markers of sperm function to possible scavenger. *Human Reproduction Update* 2, 246–256.

Levine, R. L., Garland, D. & Oliver, C. N. (1990) Determination of carbonyl content in oxidatively modified proteins. *Methods in Enzymology* 186, 464–478.

Lopes, L. G., Jurisicova, A. & Sun, J. (1998) Reactive oxygen species: potential cause for DNA fragmentation in human spermatozoa. *Human Reproduction* 13, 896–900.

Lowry, O. H., Rosebrough, M. J., Farr, A. J. & Randall, R. J. (1951) Protein measurement with the Folin phenol reagent. *Journal of Biological Chemistry* 193, 275–295.

Mancini, A., Conte, G. & Milardi, D. (1998) Relationship between sperm cell ubiquinone and seminal parameters in subjects with and without varicocele. *Andrologia* 30, 1–4.

Marks, J. J., McMahon, R. & Lipshulz, I. I. (1986) Predictive parameters of successful varicocele repair. *The Journal of Urology* 136, 609–612.

Marra, C. A., Mangioni, J. O., Tavella, M., Alaniz, M. J. T., de Ortiz, D. & Sala, C. (1998) Hormonal-induced changes on the lipid composition and DPH fluorescence anisotropy of erythrocyte ghosts from pre- and post-menopausal women. *Acta Physiologica, Pharmacologica et Therapeutica Latinoamericana* 48, 8–17.

Meagier, E. & Fitzgerald, G. A. (2000) Indices of lipid peroxidation in vivo: strengths and limitations. *Free Radical Biology Medicine* 28, 1745–1750.

Menditto, A., Pietraforte, D. & Minetti, M. (1997) Ascorbic acid in human seminal plasma is protected from iron-mediated oxidation, but is potentially exposed to cooper-induced damage. *Human Reproduction* 12, 1699–1705.

Murugesam, P., Kanagaraj, P., Yuvaraj, S., Balasubramanian, K., Aruldhas, M. M. & Arunakaran, J. (2005) The inhibitory effects of polychlorinated biphenyl Aroclor 1254 on Leydig cell LH receptors, steroidogenic enzymes and antioxidant enzymes in adult rats. *Reproductive Toxicology* 20, 117–126.

Naughton, C., Nangia, A. K. & Agarwal, A. (2001) Varicocele and male infertility: Part II. Pathophysiology of varicocele in male infertility. *Human Reproduction Update* 7, 473–481.

Özbek, E., Turkoz, Y., Gokdeniz, R., Davarci, M. & Ozugurlu, F. (2000) Increased nitric oxide production in the spermatic vein of patients with varicocele. *European Urology* 37, 172–175.

Özdamar, A. S., Soylu, A. G., Culha, M. & Gökalp, A. (2004) Testicular oxidative stress. Effects of experimental varicocele in adolescent rats. *Urologia* 73, 343–347.

Pasqualotto, F., Sharma, R. K., Nelson, D. R., Thomas, A. J. & Agarwal, A. (2000) Relationship between oxidative stress semen characteristics, and clinical diagnosis in men undergoing infertility investigation. *Fertility and Sterility* 73, 459–464.

Pomerantz, D. K. & Pitelka, V. (1998) Nitric oxide is a mediator of the inhibitory effect of activated macrophages on production of androgen by the Leydig cell of the mouse. *Endocrinology* 139, 922–931.

Rajimakers, M. T. M., Roelofs, H. M. J., Steegers, E. A. P., Steegers-Theunissen, R. P. M., Mulder, T. P. J., Knapen, M. F. C. M., Wong, W. Y. & Peters, W. H. M. (2003) Glutathione and glutathione-*S*-transferases A1-1 and P1-1 in seminal plasma may play a role in protecting against oxidative damage to spermatozoa. *Fertility and Sterility* 79, 169–172.

Reznick, A. Z. & Packer, L. (1994) Oxidative damage to proteins: spectrophotometric method for carbonyl assay. *Methods of Enzymology* 233, 357–363.

Rosselli, M., Dubey, R. K., Imthurn, B., Macas, E. & Keller, P. J. (1995) Effects of nitric oxide on human spermatozoa: evidence that nitric oxide decreases sperm motility and induces sperm toxicity. *Human Reproduction* 10, 1786–1790.

Santoro, G. & Romeo, C. (2001) Normal and varicocele testis in adolescents. *Asian Journal of Andrology* 3, 259–262.

Segenreich, E., Shumuely, H. & Singer, R. (1986) Andrological parameters in patients with varicoceles and fertility disorders by high ligation of the left spermatic vein. *International Journal of Fertility* 31, 200–203.

Shafick, A., Wali, M. A. & AbdelAzis, Y. E. (1989) Experimental model of varicocele. *European Urology* 16, 298–303.

Sharma, R. K. & Agarwal, A. (1996) Role of reactive oxygen species in male infertility. *Urology* 48, 835–850.

Sharma, R. K., Pasqualotto, F. F. & Nelson, D. R. (1999) The reactive oxygen species-total antioxidant capacity (ROS-TAC) score is a new measure of oxidative stress to predict male infertility. *Human Reproduction* 14, 2801–2807.

Shekarriz, M., Sharma, R. K., Thomas, A. J., Jr & Agarwal, A. (1995) Positive myeloperoxidase staining (Endtz test) as an indicator of excessive reactive oxygen species formation in semen. *Journal of Assisted Reproduction and Genetics* 12, 70–74.

Sorensen, M. B., Bergdahl, I. A., Hjollund, N. H. I., Bonde, J. P. E., Stoltenberg, M. & Ernst, E. (1999) Zinc, magnesium and calcium in human seminal fluid: relations to other semen parameters and fertility. *Molecular Human Reproduction* 5, 331–337.

Su, I., Goldstein, M. & Schlegel, P. N. (1995) The effects of varicocelectomy on serum testosterone levels in infertile men with varicoceles. *The Journal of Urology* 154, 1752–1755.

Twigg, J., Fulton, N. & Gómez, E. (1998) Analysis of the impact of intracellular reactive oxygen species generation on the structural and functional integrity of human spermatozoa: lipid peroxidation, DNA fragmentation and effective antioxidants. *Human Reproduction* 13, 1429–1436.

Verdon, C. P., Burton, B. A. & Prior, R. L. (1995) Sample pretreatment with nitrate reductase and glucose-6-phosphate dehydrogenase quantitatively reduces nitrate while avoiding interference by NADP+ when the Griess reaction is used to assay for nitrite. *Analytical Biochemistry* 224, 502–508.

Wheeler, M. D., Nakagami, M., Bradford, B. U., Uesugi, T., Mason, R. P., Connor, H. D., Dikalova, A., Kadiiska, M. & Thurman, R. G. (2001) Overexpression of manganese superoxide dismutase prevents alcohol-induced liver injury in the rat. *Journal of Biological Chemistry* 276, 36664–36672.

Wild, C. P., Anderson, C., O'Brien, N. M., Wilson, L. & Woods, J. A. (2001) A critical evaluation of the application of biomarkers in epidemiological studies on diet and health. *British Journal of Nutrition* 86, S37–S53.

World Health Organization (1999) WHO Laboratory manual for the examination of human semen and sperm-cervical mucus interaction. Cambridge University Press, Cambridge, UK.

Xu, D. X., Shen, H. M., Zhu, Q. X., Chua, L., Wang, Q. N., Chia, S. E. & Ong, C. N. (2003) The associations among semen quality, oxidative DNA damage in human spermatozoa and concentrations of cadmium, lead, and selenium in seminal plasma. *Mutation Research* 534, 155–163.

Yagi, K. (1976) A simple fluorimetric assay for lipoperoxide in blood plasma. *Biochemical Medicine* 15, 212–216.