Bilateral increased apoptosis and bilateral accumulation of cadmium in infertile men with left varicocele

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BACKGROUND: Varicoceles are associated with venous flux that may cause increased heat and interstitial pressure within the testes, but these effects are variable. Some men with varicocele have infertility, but others do not. We question whether other factors contribute to the infertility, and whether these other factors could be identified by specific molecular/genetic markers. Can such markers predict the outcome of varicocele repair? Can these markers be demonstrated bilaterally in unilateral left varicocele?

METHODS: Limited bilateral testes biopsies were obtained by ultrasonically guided percutaneous aspiration at the time of varicocelectomy. In each specimen, cadmium levels were determined by atomic absorption and the percentage apoptosis within the seminiferous tubules was quantified.

RESULTS: The percentage of apoptotic nuclei and cadmium levels were high in some men with varicocele. There was a concordance of these values in both testes despite the presence of left-sided varicocele only. These values were inversely related to an increase in sperm concentration after varicocelectomy.

CONCLUSIONS: Cadmium, a metal ion inducer of apoptosis, may contribute to this form of male infertility. Apoptosis may deplete the sperm concentration among men with varicocele and infertility. Pre-operative measurements of apoptosis and cadmium content may predict the outcome of varicocelectomy repair.

Key words: apoptosis/cadmium/varicocele/varicocelectomy

Introduction

Varicocele has been associated with male infertility, but the pathophysiology remains unclear. Part of the confusion relates to the clinical diversity that exists among these men. This includes variable semen findings, differences in sizes of the lesions and presence of bilateral versus left-sided varicoceles. In addition, the physical phenomena associated with varicocele may vary and are difficult to determine in a practical way, including increased temperature and increased interstitial pressure within the testes (Harrison et al., 1986; Turner and Lopez, 1990).

Recently, several studies have identified molecular/cellular/genetic changes that may prove more reliable tools for understanding the pathophysiology. These include abnormal karyotypes, Y chromosome deletions and increased oxidative stress (e.g. Cayan et al., 2001; Santoro et al., 2001; Chen et al., 2002). In addition, increased apoptosis has been associated with varicocele (Baccetti et al., 1996; Simsek et al., 1998). At least three pathways leading to apoptosis have been identified in animal models with varicocele: (i) excess heat (Sinha-Hikim and Swerdloff, 1995; Yin et al., 1997); (ii) androgen deprivation at the testicular level (Tapanainen et al., 1993; Sinha-Hikim and Swerdloff, 1995); and (iii) accumulation of toxic agents including the products of cigarette smoke such as cadmium (Chia et al., 1994a,b; Li et al., 1996; Vine et al. 1996; Benoff, 1997; Benoff et al., 1997, 1999; Benoff and Gilbert, 2001). In earlier studies, we observed that cadmium was elevated in seminal plasma from infertile men with varicocele as compared with those without varicocele (Benoff et al., 1997). We have also reported a relationship between seminal plasma cadmium levels, improvement in sperm density post-varicocelectomy and pregnancy outcome (Benoff et al., 1998). In the present report, we will examine cadmium content and percentage apoptosis in seminiferous tissue obtained by percutaneous aspiration biopsy, and we will determine whether these markers may be predictive of varicocelectomy outcome. We believe that an examination of testis tissue can link changes in histology with other factors that are active in men with varicocele.

In the past, testis biopsies for men with varicocele have been used primarily for histological assessment. These studies
Cadmium, apoptosis and varicocele infertility

**Materials and methods**

**Products and reagents**

Optima grade (trace metal ion-free) concentrated HCl and concentrated HNO₃ were obtained from Fisher Scientific Company (Pittsburgh, PA). PCR reagents were purchased from Qiagen (Valencia, CA). All other enzymes were obtained from New England Biolabs (Beverly, MA). Unless otherwise noted, all other reagents were purchased from Sigma Chemical Company (St Louis, MO).

**Human subjects**

All protocols employing human subjects were reviewed and approved by the Institutional Review Boards of North Shore University Hospital and Cooper Hospital.

Testicular biopsies were obtained (with written informed consent from each patient) from 47 infertile men at varicocele repair (by the subinguinal approach; Marmar and Kim, 1994) or testicular sperm retrieval prior to ICSI from men with obstructive azoospermia and normal spermatogenesis [Johnsen (1970) score ≥ 8.0] (n = 16). No patient underwent a biopsy solely for research purposes. Prostate tissue was obtained from men who had transurethral resection of the prostate for benign hypertrophy. At the point of discard, a portion of each tissue specimen was placed in formalin, and examined for cadmium concentration.

**Testis biopsies**

Complete medical histories, including occupational exposures and a drug/medication profile, were obtained for all males evaluated for primary infertility, and comprehensive multisystem physical examinations were performed. Enrolled patients had two naturally descended testes and no other known systemic medical illnesses. Testicular sizes of each patient were measured by a Prader orchidometer. The direction of venous flow was documented by a 10 MHz probe that was attached to a Doppler. The patient stood in an upright position and the arterial beat was localized over the scrotum. Retrograde blood flow was documented by a continuously audible sound of reflux during the Val Salva manoeuvre.

Potentially confounding lifestyle variables were addressed during the initial consultation, including patient’s occupation, smoking habits, alcohol intake, history of urogenital tract infection or testicular injury, and the use of prescription medications or vitamins.

All subjects subsequently chosen for study presented with grade II (audible by Doppler ultrasonography, palpable, not visible) or grade III varicocele (audible, palpable, visible). Subjects with grade I varicocele (‘subclinical‘; audible, not visible, not palpable) were excluded from the study population as there are significant questions remaining unanswered concerning the benefit of varicocele repair in

![Figure 1. Percutaneous aspiration biopsy (Marmar, 1998). (A) Colour Doppler recently has been shown to aid testicular sperm retrieval by fine needle aspiration through identification of testicular arteries (Foresta et al., 1998). This technique is based on the premise that the most viable tubules will be located close to the blood supply. We now employ this technique prior to sperm retrieval by percutaneous testis aspiration biopsy. The white areas in the figure identify arteries and the sites for sperm retrieval. (B) Aspiration biopsy of testis with angiocath and negative pressure. (C) Seminiferous tubules from aspiration biopsy of testis on stretch. This approach allows us to examine a complete length of seminiferous tubule, rather than multiple or random biopsies from several different tubules, for uniformity of cadmium levels and gene expression (see text).](Image)
this group (e.g. Comhaire et al., 1995; Yamamoto et al., 1996). All studies subjects were non-smokers. This was done to avoid cadmium as a confounder as: (i) cigarette smoke is a major source of cadmium (1–4.5 μg/cigarette; Saldivar et al., 1991; Chia et al., 1994a,b); (ii) at least one-tenth of the metal content of a cigarette is inhaled (Elinder et al., 1985); and (iii) cigarette smoking is associated with an increase in frequency of oligozoospermia in varicocele-associated infertility (Klaiber et al., 1987).

At the time of the clinical procedure, the testes were examined with a variable 7–12 MHz ultrasound probe with the aid of colour Doppler. The major intratesticular arteries were documented by power Doppler and by spectral analysis (Figure 1A). The skin was anaesthetized with 1% xylocaine and an 18 gauge angiocath was placed into a region of the seminiferous tissue that avoided arterial injury. With negative pressure, ~200–400 mg of seminiferous tubule tissue was obtained. These tubules could be stretched to ~30 cm and, in a few cases, an entire tubule was cut into contiguous sections for study (Figure 1B and C). This technique is not to be confused with fine needle aspiration that requires only droplets of tissue. The testicular tissue within the angiocath had no histological distortion compared with open biopsy material from the same patient (Marmar, 1998), and follow-up ultrasound studies have shown no demonstrable trauma to the testis after this procedure.

Semen data
The semen data were collected according to the protocol previously described (Marmar and Kim, 1994). Pre-operatively, each patient provided at least three semen specimens within 6 months. Each specimen was collected by masturbation after 48 h of abstinence. The sperm density and percentage motility were determined with a Makler chamber. The morphology was reported according to the criteria established by the World Health Organization (1987). The semen results were averaged for each patient and a single value was computed for each parameter. The patient was considered for surgery as long as the duration of infertility exceeded 12 months and the average value was computed for each parameter for statistical comparison with the pre-operative average value.

Study design
All biopsy material was immediately divided in two, one part being fixed in Bouin’s solution and the other used for clinical diagnosis. The seminiferous tissue placed in Bouin’s solution was used for histology performed at Cooper Hospital. At the point of discard, the remainder of the specimen was placed in formalin and transported to the North Shore-LIJ Research Institute. Formalin-fixed tissues were used in all molecular investigations because formalin both preserves the antigenic character of the tissue and also inactivates RNases (Benoff et al., 2000). In addition, formalin-fixed tissue exhibits considerably less autofluorescence than that fixed in Bouin’s solution (Elliott et al., 1998).

Analysis of apoptosis
A portion of each biopsy was processed, embedded in paraffin and sectioned. Testis sections (9 μm) were collected on adhesive pre-treated slides (in situ PCR glass slides; Perkin-Elmer, Foster City, CA), deparaffinized in xylene, rehydrated in ethanol–water mixtures and heated to 60°C for 1 h.

Apoptosis in testis biopsy sections was quantified in situ by deoxynucleotidyl transferase labelling (TUNEL). All TUNEL assays were performed using a TACS 2 TdT-DAB in situ Apoptosis Detection Kit (Catalogue No. 4810-30-K; Trevigen, Inc., Gaithersburg, MD) according to the manufacturer’s protocol (see Benoff and Gilbert, 2001). All labelled sections were viewed at 600× magnification with an Olympus BX50 microscope (Olympus Corporation, Lake Success, NY) and photographed using an Olympus DP-12 Digital Camera System.

Indirect immunofluorescence histochemistry
Testis sections were prepared as described for quantification of apoptosis and reacted with anti-actin antibodies following established laboratory protocols (Benoff, 1997; Benoff et al., 1997).

The buffer system used in the histochemical labelling procedure contained 0.5% Triton X-100 to allow primary antibody access to internal antigens (Danto and Fishman, 1984). Testis sections were reacted sequentially with primary antibody (rabbit polyclonal sera against muscle actin; Sigma No. A-2668) and human serum protein-pre-absorbed, fluorescein isothiocyanate (FITC)-conjugated sheep anti-rabbit IgG for 1 h each at room temperature. Control reactions employed pre-immune rabbit sera or secondary antibody alone. Slides were stored at 4°C and scored within 2 weeks of staining. Stained testis sections were viewed at 600×. Identical fields were photographed on 35 mm/400 ASA Tmax film using both phase contrast and UV-epifluorescence illumination.

Determination of cadmium in storage vessels and experimental plastic ware
Stringent efforts were made to exclude exogenous heavy metals (e.g. cadmium and lead) during sampling, sample processing and analysis. All sample storage, processing and analysis took place in a closed, dedicated room, with access restricted to persons actively involved in this work. All solutions were prepared using 18 MΩ reagent H2O from a Millipore MilliRO system. Polypropylene and fluorocarbon labware was used to avoid metal ion contamination of our samples through diffusion and ion-exchange of metal ion impurities, as in the case of glass. All labware was soaked sequentially for 24 h in large volumes of 1:4 HCl:H2O, followed by 24 h in 1:4 HNO3:H2O, to leach out metal ions. The polypropylene leaching containers, including their tight-fitting covers, were themselves equilibrated with several volumes of the same acids before being used. Every container was kept closed except during labware transfers. Only fluorocarbon tools (tongs and stirring rods) touched plastic ware during transfer to minimize contamination of leaching solutions. Exhaustive washing (~20 container volumes of H2O) removed traces of leaching acids. Labware was stored under H2O in leached and capped 500 ml polypropylene bottles until used. Samples in all stages of processing were kept closed except during labware transfers. Only fluorocarbon tools (tongs and stirring rods) touched plastic ware during transfer to minimize contamination of leaching solutions. Exhaustive washing (~20 container volumes of H2O) removed traces of leaching acids. Labware was stored under H2O in leached and capped 500 ml polypropylene bottles until used. Samples in all stages of processing were kept closed except during labware transfers. Only fluorocarbon tools (tongs and stirring rods) touched plastic ware during transfer to minimize contamination of leaching solutions. Exhaustive washing (~20 container volumes of H2O) removed traces of leaching acids. Labware was stored under H2O in leached and capped 500 ml polypropylene bottles until used. Samples in all stages of processing were kept closed except during labware transfers. Only fluorocarbon tools (tongs and stirring rods) touched plastic ware during transfer to minimize contamination of leaching solutions. Exhaustive washing (~20 container volumes of H2O) removed traces of leaching acids. Labware was stored under H2O in leached and capped 500 ml polypropylene bottles until used. Samples in all stages of processing were kept closed except during labware transfers. Only fluorocarbon tools (tongs and stirring rods) touched plastic ware during transfer to minimize contamination of leaching solutions. Exhaustive washing (~20 container volumes of H2O) removed traces of leaching acids. Labware was stored under H2O in leached and capped 500 ml polypropylene bottles until used. Samples in all stages of processing were kept closed except during labware transfers. Only fluorocarbon tools (tongs and stirring rods) touched plastic ware during transfer to minimize contamination of leaching solutions. Exhaustive washing (~20 container volumes of H2O) removed traces of leaching acids. Labware was stored under H2O in leached and capped 500 ml polypropylene bottles until used. Samples in all stages of processing were kept closed except during labware transfers. Only fluorocarbon tools (tongs and stirring rods) touched plastic ware during transfer to minimize contamination of leaching solutions. Exhaustive washing (~20 container volumes of H2O) removed traces of leaching acids. Labware was stored under H2O in leached and capped 500 ml polypropylene bottles until used. Samples in all stages of processing were kept closed except during labware transfers. Only fluorocarbon tools (tongs and stirring rods) touched plastic ware during transfer to minimize contamination of leaching solutions. Exhaustive washing (~20 container volumes of H2O) removed traces of leaching acids. Labware was stored under H2O in leached and capped 500 ml polypropylene bottles until used. Samples in all stages of processing were kept closed except during labware transfers. Only fluorocarbon tools (tongs and stirring rods) touched plastic ware during transfer to minimize contamination of leaching solutions. Exhaustive washing (~20 container volumes of H2O) removed traces of leaching acids. Labware was stored under H2O in leached and capped 500 ml polypropylene bottles until used. Samples in all stages of processing were kept closed except during labware transfers. Only fluorocarbon tools (tongs and stirring rods) touched plastic ware during transfer to minimize contamination of leaching solutions. Exhaustive washing (~20 container volumes of H2O) removed traces of leaching acids.

Determination of cadmium levels in testis biopsies
A portion of biopsy was weighed and lyophilized to a constant weight. The dry sample was dispersed in 800 μl of 50% HNO3 and microwave digested in a 2 ml perfluoroalkoxy (PFA) digestion vessel. The effective power of the microwave oven (Model SNAC-70D; Memmert, St Louis, MO) employed was measured to be 130 W at 50% duty cycle. Each dried biopsy portion was digested separately for 1.31 min in the presence of a 1000 ml water ballast. The volume of
the sample was too small to alter microwave power levels significantly. The resulting aqueous supernatants were assayed for cadmium by furnace atomic absorption (SpectraAA 250 Plus spectrometer, GTA 97 furnace; Varian Instruments, Walnut Creek, CA).

Cadmium measurement followed published protocols (Benoff et al., 1997; Hurley et al., 1997). Absorbances of cadmium in each sample were assayed in triplicate at 228.8 nm, using a 0.25–40 µg/l calibration (Inorganic Ventures, Inc., Lakewood, NJ). Replicates matched within 5%. Selected samples were spiked with cadmium. Cadmium levels found in spiked samples matched expected values within 5%. To determine uniformity of cadmium levels along a seminiferous tubule (see Figure 1C), two to three spatially separated fragments from 20 biopsies chosen at random were assayed for cadmium. Less than 10% intra-biopsy variation was observed.

Comparison of our measured cadmium levels in normal human prostate ($n = 4, 0.895 \pm 0.343$ ng/mg dry weight) and in testis biopsies from obstructive azoospermic controls ($n = 16, 0.195 \pm 0.129$ ng/mg dry weight) with reference values (Lahtonen, 1985; Oldereid et al., 1993; Keck et al., 1995) verified our cadmium calibration curves.

**Autometallography**

Autometallography was used to examine the distribution of endogenous metal ions in testis biopsies. Testis biopsy sections were prepared as described for the TUNEL assay.

The autometallographic protocol employed was used previously to demonstrate the topographical association between metal ions and rat and human sperm (Stoltenberg et al., 1997a,b) and generously provided in great detail to us by the originator, Dr. Meredin Stoltenberg (Institute of Anatomy, University of Aarhus, Denmark). This is a highly sensitive technique; after conversion of metal ions to metal sulphides by reaction with sodium sulphide, <10 catalytic atoms of a given metal can be visualized as black silver deposits (‘BSDs’) by physical development with silver lactate (Danscher, 1984).

Although different metals produce different shaped BSDs (Soto et al., 1996), we distinguished between metal ions chemically (Danscher and Moller-Madsen, 1985; Stoltenberg et al., 1996). After development and colloid removal, one slide that had previously been treated with sodium sulphide was incubated in 0.1 M HCl/1% KCN for 1 h at room temperature. This treatment dissolved BSDs resulting from zinc. A second slide that had previously been treated with sodium sulphide was incubated in 1% H$_2$O$_2$ for 1 h at room temperature. This oxidation eliminated BSDs resulting from cadmium sulphides. Slides not treated with sodium sulphide and those treated with sodium sulphide, developed with silver lactate but not further treated served as controls.

**Statistical analyses**

All statistical analyses were performed with the SAS/PC software package (SAS Institute, Inc., Cary, NC). Statistical significance was set at $P < 0.05$.

Data were analysed using two-sample $t$-tests, analysis of variance (ANOVA), Spearman correlations, Cohen’s kappa coefficient and Fisher’s exact tests (two-tail). In addition, the Wilcoxon signed rank test was used to compare left and right testes with respect to mean difference of apoptosis percentages and of cadmium levels. Although the WSR does not specifically compare means, the data are nevertheless summarized using 95% confidence intervals (95% CIs) for mean differences (right testis minus left testis). It should be noted that the results of the paired $t$-test were virtually identical to those of the WSR.

**Results**

**Apoptosis**

Left testis biopsies were obtained from 15 men with left varicocele, 30 men with bilateral varicocele and two men for whom the laterality of varicocele was not recorded. Three 9 µm sections from individual left testis biopsies were scored for the percentage of apoptotic nuclei. Only circular tubular cross-sections cut in bold-face were scored for apoptotic nuclei (Benoff and Gilbert, 2001). Nine to 20 tubules, ranging in diameter from 70 to 120 µm, were scored per testis section. The number of nuclei scored for each testis section totalled between 1000 and 7000. The percentages of apoptotic nuclei in left testis biopsy sections from infertile men with varicocele ranged from 1.3 to 53.9%. This range was considerably wider, and the upper end considerably higher, than that observed for control biopsy sections from men with obstructive azoosperma and normal spermatogenesis ($n = 4, 7.3\text{--}13.9\%$).

Actin loss in somatic cells is associated with an increase in apoptosis (Russo et al., 1982; Tsukidate et al., 1993). Consistent with this, actin loss was also observed in testis biopsy sections from men with high levels of apoptosis (Figure 2C, typical results). The overall fluorescence intensity of the biopsy section was reduced, there was a loss of cell
outline within the seminiferous epithelium and a loss of staining at the level of the basement membrane as compared with biopsy sections from men with obstructive azoospermia (Figure 2A) and from men with varicocele and low levels of apoptosis (Figure 2B).

Apoptosis was assessed in 34 patients where matched left and right testis biopsy sections were available for analysis (Figure 3A). A significant positive relationship was detected (Spearman correlation, $r = 0.697$, $P < 0.0001$). No difference was detected between the percentage apoptosis detected in left and right testes (WSR, $P = 0.35$, not significant; 95% CI $-1.12812$ to $4.6224$). When subdivided by diagnosis, within men with varicocele, no difference was detected between left and right testes (WSR, $P = 0.47$, not significant; 95% CI $-6.71933$ to $3.70433$). Similar findings were obtained for men with bilateral varicocele (WSR, $P = 0.12$, not significant; 95% CI $-0.20093$ to $7.14760$). Thus, the interaction of varicocele group with side (left, bilateral) was not significant.

A significant negative relationship was detected between the percentages of apoptotic nuclei within the seminiferous epithelium and spermatogenic response to varicocele surgery (Figure 4A; Spearman correlation, $n = 32$, $r = -0.475$, $P < 0.006$). A ‘normal’ (i.e. ‘positive’) response to varicocele surgery has > 50% increase in sperm concentration in the ejaculate post-operatively compared with baseline pre-operative levels (Figure 5). Subjects were divided into two groups, i.e. ‘normal’ response or ‘low’ response. Apoptosis in these two groups was compared with that of control specimens from men with obstructive azoospermia and was found to differ significantly (Figure 5A; ANOVA, $P < 0.0001$). Subsequent pair-wise analyses indicated that the percentages of apoptotic nuclei within the seminiferous epithelium of the ‘normal’ group and that of the control group (obstructive azoospermia) were similar ($t$-test, $P = 0.100$, not significant), while the ‘low’ group exhibited significantly higher levels of apoptosis than the controls ($t$-test, $P < 0.001$).

**Cadmium levels in testicular biopsies**

Actin loss by somatic cells can result from elevated cadmium exposures (Wang et al., 1996). Preliminary findings suggested that a relationship existed between actin loss and an increase in testicular cadmium levels in infertile men with varicocele.
were similar (WSR, matched left and right testis biopsies from individual donors to those from men with bilateral varicocele. Cadmium levels in testis of men with left varicocele and compared the data with increased cadmium levels were observed in the contralateral in spermatogenesis (Figure 5). Therefore, we queried whether those who did not respond to varicocele repair with an increase equally distributed among those subjects that responded and cadmium levels (Table I). Men with left varicocele were could not be distinguished on the basis of left testicular weight).

Figure 4. Correlational analyses. Upper panel: a strong negative relationship was detected between the percentages of nuclei within seminiferous epithelium undergoing apoptosis and the fraction increase in sperm count post-operatively. Lower panel: a strong positive relationship was detected between the percentages of nuclei within the seminiferous epithelium undergoing apoptosis and the cadmium concentration in the testis biopsy. The solid line in each figure represents the regression line. See text for results of the statistical analysis.

(Benoff and Gilbert, 2001). To follow up on these observations, cadmium concentrations in left testis biopsies were determined by graphite furnace atomic absorption spectroscopy.

The mean cadmium level for individual biopsies from men with varicocele-associated infertility varied over a wide range (0.058 to >1.5 ng/mg dry weight). Elevated cadmium levels were uniformly observed along the length of seminiferous tubule. The range of testicular cadmium values in subjects with varicocele was considerably wider than that observed in men with obstructive azoospermia (n = 16, 0.091–0.397 ng/mg dry weight).

Men with left varicocele and men with bilateral varicocele could not be distinguished on the basis of left testicular cadmium levels (Table I). Men with left varicocele were equally distributed among those subjects that responded and those who did not respond to varicocele repair with an increase in spermatogenesis (Figure 5). Therefore, we queried whether increased cadmium levels were observed in the contralateral testis of men with left varicocele and compared the data with those from men with bilateral varicocele. Cadmium levels in matched left and right testis biopsies from individual donors were similar (WSR, P = 0.58, not significant; 95% CI –0.22177 to 0.08226), irrespective of whether the subject presented with left varicocele (WSR, P = 0.074, not significant; 95% CI –0.41049 to 0.04984) or with bilateral varicocele (WSR, P = 0.85, not significant; 95% CI –0.24366 to 0.20953) (see Table I and Figure 3B).

The ages of the control subjects with obstructive azoospermia and those of infertile men with varicocele were similar (respectively, 36.25 ± 5.92 years versus 33.93 ± 4.87 years; t-test, P = 0.127, not significant). Elevated testicular cadmium levels in testis biopsies from men with varicocele were unrelated to subject age (Spearman correlation, n = 45, r = 0.001, P = 0.996, not significant). Further, elevated concentrations of cadmium in the testis biopsies samples were not associated with occupation or place of residence. As the subjects were non-smokers, the source(s) of the cadmium remains unexplained.

Figure 5. Infertile men with varicocele were divided into two groups based on spermatogenic response to varicocele repair. In a pilot study, we compared the fractional increase in sperm count after varicocele repair [e.g. (pre-post count minus pre-op count)/post-op count] of men who subsequently initiated a pregnancy (n = 4) with those who failed to get their partners pregnant (n = 3) (respectively, 0.853 versus 0.227). A threshold for a ‘normal’ increase in sperm count post-varicocelectomy was defined according to normal distributions theory (Zar, 1984) by subtracting 2 SDs (e.g. 0.134) from the pregnant group to give a threshold of >0.5, which is similar to thresholds used in studies by other investigators (e.g. Cayan et al., 2000, 2001, 2002). The threshold of >0.5 eliminates all cases where sperm count increases from ~1 × 10⁶/ml to ~2 × 10⁶/ml, an increase which we do not consider significant. As observed in prior studies (Matkov et al., 2001), patients who experienced a return of fecundity also had post-operative sperm concentrations >20 × 10⁶/ml. All subjects studied herein were thus assigned to one of two groups: ‘normal’ response or ‘low’ response. The ‘normal’ group (n = 11) was comprised of four men with left varicocele and seven men with bilateral varicocele, while the ‘low’ group (n = 21) was comprised of five men with left varicocele and 16 men with bilateral varicocele. (A) Apoptosis was studied in four men with obstructive azoospermia and normal spermatogenesis (controls) and 32 infertile men with varicocele. The level of apoptosis was low and similar in controls and subjects displaying a ‘normal’ increase in sperm count, (e.g. ‘normal’) after varicocelectomy. In contrast, apoptosis was markedly elevated in subjects where sperm count was only slightly affected by varicocele repair (e.g. ‘low’). (B) Cadmium levels were determined by atomic absorption in testis biopsies from 16 men with obstructive azoospermia and normal spermatogenesis (controls), and in the 11 infertile men with varicocele defined as ‘normal’ and 21 infertile men with varicocele defined as ‘low’ shown in (A). See text for results of the statistical analyses.
We identified a threshold value for normal cadmium levels based on the results from testis biopsies from men with obstructive azoospermia (see Table I). Testis biopsies from infertile men with varicocele could be divided into two groups (‘normal-Cd’, \( n = 22 \) and ‘high-Cd’, \( n = 25 \)) using this threshold value. Six men in the ‘normal-Cd’ group and nine men in the ‘high-Cd’ group presented with left varicocele. Autometallography was used to assess the distribution of cadmium ions in testis biopsy sections from the ‘normal-Cd’ and ‘high-Cd’ cadmium groups. Testis biopsy sections from men with obstructive azoospermia served as controls. Typical results are shown in Figure 6.

In all cases, when sodium sulphide treatment was omitted, BSDs were absent (Figure 6A). Only the counter-stain was visible. In contrast, following sodium sulphide treatment, BSDs were observed at high density. BSDs were uniformly distributed throughout the seminiferous epithelium in all testis biopsy sections analysed (Figure 6B). The BSDs in biopsy sections from men with obstructive azoospermia and from varicocele defined as ‘normal-Cd’

### Table I. Examination of the agreement of cadmium concentrations in matched biopsies from individual donors

<table>
<thead>
<tr>
<th>Left testis biopsy, testicular Cd concentration</th>
<th>‘Normal-Cd’</th>
<th>‘High-Cd’</th>
<th>Cohen’s ( \kappa ) statistic</th>
<th>95% CI</th>
</tr>
</thead>
<tbody>
<tr>
<td>‘Normal-Cd’</td>
<td>11 (LV = 4)</td>
<td>3 (LV = 3)</td>
<td>0.426</td>
<td>0.069-0.784</td>
</tr>
<tr>
<td>‘High-Cd’</td>
<td>4 (LV = 0)</td>
<td>7 (LV = 2)</td>
<td></td>
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</tbody>
</table>

Parameters were compared in their categorical states in a 2 × 2 matrix in the above contingency table. In this table, agreement is observed on the left to right diagonal while disagreement is found in the right to left diagonal. The kappa (\( \kappa \)) statistic and its 95% CI were employed to examine whether or not agreement was observed between two parameters. Essentially \( \kappa \) measures the percentage of cases for which there is agreement and then adjusts that percentage for the amount of agreement that would have been expected just by chance alone. A \( \kappa \) statistic of <0.4 would indicate weak agreement. A \( \kappa \) statistic between 0.4 and <0.6 would indicate moderate agreement. A \( \kappa \) statistic of >0.6 would indicate strong agreement. However, if the 95% CI contains zero, then \( \kappa \) is not statistically different from zero.

LV = number of subjects in each category with left varicocele; the remainder have bilateral varicocele.

According to normal distribution theory, any value for testicular cadmium content within 2 SDs of the mean of values obtained from testis biopsies from men with obstructive azoospermia and normal spermatogenesis (Johnsen score >8) should be considered ‘normal’ (Zar, 1984). The threshold identified was \( \leq 0.453 \) ng/mg dry weight, based on analysis of 16 testis biopsies with a mean cadmium concentration of 0.195 ± 0.129 ng/mg dry weight. This threshold was used to categorize the 25 testis biopsies from infertile men with varicocele into two groups: ‘normal-Cd’ or ‘high-Cd’.

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**Figure 6.** The distribution of cadmium in testis biopsies from infertile men with varicocele was examined by autometallography. The testis sections shown were taken from a left testicular biopsy from an infertile man with bilateral varicocele. The cadmium level in this biopsy was assessed previously by graphite furnace atomic absorption spectroscopy and was determined to be 1.54 ± 0.18 ng/mg dry weight. (A) Control, not treated with sodium sulphide to convert the tissue metals to metal sulphides. No black silver deposits (BSDs) are seen. Scale bar = 20 μm. (B) Standard autometallography, demonstrating a high concentration of BSDs, indicating the presence of metal ions. Note that the BSDs are particularly enriched in the region of the basement membranes. (C) The density of BSDs is markedly reduced in a section treated with 1% \( \text{H}_2\text{O}_2 \), indicating that a large fraction of the BSDs in (B) resulted from the presence of cadmium.
were unaffected by H₂O₂ treatment, indicating that such BSDs did not result from cadmium deposits. In contrast, the density of BSDs in testis biopsy sections from subjects with ‘high-Cd’ (Figure 6C) was markedly reduced after exposure to H₂O₂, supporting findings by atomic absorption spectroscopy.

Subjects who exhibited a marked improvement in sperm count post-operatively (‘normal’ group) had lower testicular cadmium levels than those of the ‘low’ group (Figure 5B; t-test, \( P < 0.0001 \)). The cadmium levels of these two groups were compared with that of control specimens from men with obstructive azoospermia and were found to differ significantly (ANOVA, \( P < 0.0001 \)). Subsequent pairwise analyses indicated that the cadmium level of the ‘normal’ group and that of ‘low’ group differed significantly from that of the controls (t-test, \( P < 0.0001 \)) and from each other (t-test, \( P < 0.0001 \)). Comparing the association between cadmium levels and spermatogenic response to varicocele surgery in their categorical states (respectively, ‘normal’ versus ‘high’ and ‘normal’ versus ‘low’) in the 32 patients for whom these data were available revealed that eight out of 10 men with normal cadmium levels had a normal spermatogenic response and 18 out of 22 men with high cadmium levels had a low spermatogenic response, which indicated 81% agreement/correct predictions (Fisher’s exact test, \( P < 0.002 \)).

These data indicate that pre-operative testicular cadmium levels can be used to predict a normal spermatogenic response to varicocele repair. We suggest that this is because a positive relationship exists between testicular cadmium levels and the percentages of cells within the seminiferous epithelium undergoing apoptosis (Figure 4B; Spearman correlation, \( n = 47, r = 0.563, P < 0.0001 \)).

Discussion

This project was undertaken because explanations for the bilateral effects of left varicocele cannot account for the highly variable effects of varicocele on fertility, including bilateral retrograde venous flow leading to bilateral loss of thermoregulation (Narayan et al., 1981). We have characterized some molecular parameters of bilateral testis biopsies from men with left-sided varicocele. We did this to test the second hit hypothesis (Marmor, 2001), that the varicocele is a secondary opportunistic lesion that contributes to infertility, the underlying cause being genetic or epigenetic factors expressed in both testes. We chose to monitor factors that are known to affect spermatogenesis, sperm concentration in the ejaculate and sperm morphology.

Apoptosis-related factors seemed a good choice, based on the literature. Spermatogenesis is an ongoing proliferative process that yields 200–400 million mature sperm per day in the rat, but one in which 75% of all pre-leptotene spermatocytes are lost by apoptosis (Huckins, 1978). Apoptosis has also been proposed as the cause of germ cell loss in elderly men (Brinkworth et al., 1997). Lin et al. (1997a,b) reported increased numbers of apoptotic germ cells in testis biopsies for men with hypospermatogenesis and spermatogenetic arrest at the spermatid level. Early histological assessments of testis biopsies from infertile men with varicocele suggested a predominant phenotype of maturation arrest at the round spermatid stage (Eriby et al., 1967; Zukerman et al., 1978). This maturation arrest phenotype is predictive of improvement in semen parameters post-surgery in azoospermic men with varicocele (Kim et al., 1999). Increased apoptosis has been documented in laboratory animals with surgically induced varicocele (Caruso et al., 1999), in human testis tissue from subjects with varicocele (Simsek et al., 1998) and in ejaculated sperm from varicocele patients (Baccetti et al., 1996). To the best of our knowledge, only one group has presented contrary findings (Fujisawa et al., 1999; Tanaka et al., 2002). Therefore, we evaluated apoptosis in bilateral testis biopsies from men with varicocele. We selected specific apoptosis markers for the study based on prior literature. We found a concordance of apoptotic score between left and right testis biopsies from the same varicocele patient in 29 out of 34 (85%) of cases.

We also related the percentage of apoptosis to the toxic accumulation of cadmium. We chose to evaluate cadmium in testis biopsies of non-smokers because cigarette smoking and varicocele have been noted to be interacting co-factors leading to infertility (Klaiber et al., 1987; Peng et al., 1990; Vine et al., 1996). Cadmium, a toxic metal, is one of the major toxicants in cigarette smoke (Chia et al., 1994a,b) and has been suggested as a toxicant that leads to apoptosis (Jones et al., 1997; Yan et al., 1997). Our previous studies indicate that cadmium was elevated in seminal plasma from infertile men with varicocele as compared with those without varicocele (Benoff et al., 1997). We also found previously that a relationship existed between seminal plasma cadmium levels post-varicocelectomy and pregnancy outcome (Benoff et al., 1998). On a dry weight basis, the cadmium concentration in testis biopsies in our control population (men with obstructive azoospermia and normal spermatogenesis) was 0.195 ng/mg (range 0.091–0.397). We established a threshold level for abnormality at 0.45 ng/mg cadmium in testis tissue at 2 SDs from this mean value for the control population. We then determined the cadmium content in the left testis in 47 infertile men with varicocele, finding that 22 (47%) of our varicocele subjects were ‘normal’ and 25 (53%) were ‘high’ for cadmium (\( P < 0.0001 \)). Cadmium concentrations were assessed in matched right testis biopsies from 25 of these men, and cadmium levels (‘high’ or ‘normal’) were concordant in 72% (18/25) of these cases.

Comparing levels of cadmium and apoptosis in the same biopsies, we found a significant correlation between cadmium content and the number of apoptotic cells within the seminiferous tubules (see Figure 4B). As cadmium accumulation may disrupt the actin cytoskeleton, which in fertile men contributes to shaping of the acrosome around the sperm nucleus (e.g. Vogl, 1989), it was not surprising that immunoreactive studies on these biopsies demonstrated a high correlation of actin loss with increased cadmium concentration (Benoff and Gilbert, 2001). A second function of the sperm actin cytoskeleton is to effect acrosome exocytosis (Rogers et al., 1989; Spungin et al., 1995; Liu et al., 1999, 2002). Cadmium in sperm appears to act as an effector that mechanistically accounts simultaneously for the oligosperma
that accompanies varicocele, the ‘stress’ sperm morphology seen in varicocele (e.g. Naftulin et al., 1991) and the acrosome reaction insufficiency (e.g. Benoff, 1997; Benoff et al., 1997) typical among men with varicocele. We have shown that these effects of cadmium can be induced in vitro in sperm from known fertile donors by exposing them to increasing concentrations of cadmium. Sperm lose actin and demonstrate alterations in morphology and the acrosome reaction similar to those observed with varicocele (Benoff, 1997; Benoff et al., 1997).

We have not collected blood from the current patient population as our prior studies indicate that blood plasma cadmium levels are in the normal range in infertile patients with varicocele irrespective of whether or not reproductive tract cadmium concentrations are elevated (e.g. Benoff et al., 1997). Therefore, lesions in the testis must be responsible for cadmium accumulation in these patients. Histological studies of adolescent varicocele show that actin loss by cells near the basement membrane of seminiferous tubules may be a key event in early varicocele. The blood–testis barrier was overcome (Hienz et al., 1980), and the seminiferous endothelium appeared damaged (Hadziselimovic et al., 1989). These structural changes may be the simple result of an increase in local venous blood pressure in the testis (Shafik and Bedeir, 1980; Harrison et al., 1983; Sweeney et al., 1995) after the varicocele begins to form, or it may have a complex molecular origin.

These lesions may provide a route of entry for toxic metals such as cadmium. Data presented here showed that high cadmium levels are present in both testes despite a unilateral varicocele. The early changes in basement membranes may allow increased interstitial pressures to drive cadmium through these barriers. Once accumulated, the cadmium itself can alter the properties of the cell membranes by altering calcium ion channels. The pore region of these channels results from alternate splicing, and microdeletions in the pore region may lead to less selective ion channels which would permit cadmium to pass more easily through cell membranes into the cytoplasm. Our studies indicate that changes in isoform expression of a testis-specific L-type voltage-dependent calcium channel (L-VDCC) produce an altered gate that should facilitate cadmium entry into the cells of the germ cell lineage (Benoff et al., 2000; S.Benoff, unpublished observations). Since the testis lacks an active pump to remove cadmium (Gunn et al., 1961) and resists stress-induced expression of transportable cadmium-sequestering molecules (e.g. metallothioneins) (Abe et al., 2000), testicular cadmium levels must become elevated over time (Oldereid et al., 1993; Yan et al., 1997). Increased cadmium levels on the testes side of the blood–testes barrier may compound the effect of increased venous pressure because cadmium itself alters the permeability of testicular vascular endothelium (Setchell and Waites, 1970) producing oedema (Mason et al., 1964; Gunn et al., 1968; Clegg et al., 1969; Aoki and Hoffer, 1978; Gazid and Kaminski, 1984), rendering the blood–testes barrier more porous, and rendering the testis able to accumulate cadmium more rapidly.

We have looked for alteration in testes L-VDCC isoforms expressed in clinical subjects. We sequenced the mRNA encoding the L-VDCC expressed in human testis using an RNA/RT–PCR assay, with primers for the ‘pore’ region of the channel. About two-thirds of men with varicocele expressed microdeletions in the pore region of the calcium channel. This region modulates entry of calcium and other trace metals such as cadmium, nickel, zinc and lead. We assessed occurrence of these microdeletions as a predictor of the outcome of varicocelectomy (Mayer et al., 2002). The sperm counts of all patients pre-operatively ranged between 10^10 and 12 × 10^10 sperm/ml. The sperm count of patients with intact calcium channels increased to 32 × 10^10 sperm/ml after surgery ($P < 0.0001$). The patients with microdeletions failed to improve. Recently, we documented calcium channel microdeletions in both testes of 19 of 30 men studied (S.Benoff and J.L.Marmar, unpublished observations). While the details of these studies will be the subject of a separate publication, the finding of calcium channel microdeletions in both testes adds additional support to the second hit hypothesis.

Many manuscripts suggest that the retrograde blood flow into the pampiniform plexus noted among varicocele patients leads to an increase in intra-testicular temperature (Goldstein and Eid, 1989; Zorgniotti, 1991) and that varicocele surgery reduces testicular heating (Wright et al., 1997). However, reproducible measurements of intra-testicular temperatures have proven difficult in a clinical setting, and there is often an overlap in temperatures recorded among varicocele patients and controls (Brackin et al., 2003). In separate investigations and as an alternative measure of heat effects, we examined a molecular marker within our testis biopsies that may represent a heat shock effect. We measured levels of mRNA for the heat shock protein 70-1 (HSP 70-1). The data will be reported in a separate correspondence but, briefly, HSP 70-1 expression was highly variable among varicocele patients and was independent of whether the lesion was bilateral or unilateral. On the other side of the argument, preliminary studies suggest that some factor associated with varicocele (including but not limited to elevated temperature) must interact with cadmium to regulate apoptosis. We have found cases of maturation arrest or hypospermatogenesis without varicocele that present with elevated temperature) must interact with cadmium to regulate apoptosis. We have found cases of maturation arrest or hypospermatogenesis without varicocele that present with ‘high’ testicular cadmium concentrations. However, in these cases, the percentages of apoptotic nuclei within the seminiferous epithelium are on average 1.5-fold lower than with varicocele and ‘high’ cadmium (S.Benoff, unpublished observations).

Although varicocele surgery has been used in clinical practice for decades, these procedures have been the source of some controversy and discussion. A large body of literature suggests improved semen parameters and fertility following varicocelectomy (Schlesinger et al., 1994), but some investigators have challenged the benefit of these procedures because these are case-controlled studies rather than prospective randomized trials (Evers and Collins, 2003). As this debate continues, reproductive endocrinologists have been offering IVF/ICSI as another clinical alternative. However, some studies suggest that although IVF/ICSI is no more effective than varicocelectomy, it is clearly more expensive than the
surgical procedure. Penson et al. (2002) reported that the probability of a live birth after varicocelectomy was 29.7%, with 1% having twins. In comparison, the live birth rate after IVF/ICSI was 25.4%, with 39% of the couples achieving multiple gestations. In a separate study on cost analysis, Schlegel (1997) reported that the cost of a delivered baby with IVF/ICSI was $89,091 compared with $26,268 after varicocelectomy. Therefore, varicocelectomy surgery seems desirable especially if these cases can be pre-selected as to varicocelectomy outcome.

If molecular/genetic markers are utilized in the future, there may be more appropriate patient selection for varicocelectomies. In fact, investigators might utilize these screening techniques for constructing prospective randomized trials to include patients that may truly benefit from this type of surgery and avoid procedures on those who have little or no chance of success. Assisted reproduction or IVF/ICSI may be utilized for those identifiable patients who would not benefit from surgery. This approach has already been used for males with azoospermia or severe oligozoospermia. In those men with a histology of hypospermatogenesis as opposed to Sertoli cell-only syndrome or maturation arrest, and without abnormal karyotypes or Y chromosome deletions, varicocelectomy may help some azoospermic men achieve sperm in the ejaculate. In some with severe oligospermia, varicocelectomy may lead to sufficient post-operative sperm in the ejaculate for insemination and/or IVF/ICSI and in rare cases there may be a natural pregnancy (Matthews et al., 1998; Kim et al., 1999; Cayan et al., 2002; Kibar et al., 2002). These findings support other studies that have demonstrated that infertile men with varicocele with Y chromosome microdeletions and abnormal karyotypes do not respond positively to varicocelectomy repair (Pryor et al., 1997; Cayan et al., 2001; Foppiani et al., 2001).

In the future, percutaneous aspiration biopsies can provide some seminiferous tissue with minimal or no trauma to the testis. These procedures may be performed in an office setting with local anaesthesia. Identification of molecular/genetic markers in this tissue, including apoptosis and cadmium levels, may add to the selectivity for surgery. Where patients are selected for surgery on the basis of these markers, the results may present a new challenge to those reports that categorically state that varicocelectomy is of no benefit (Kamischke and Nieschlag, 2001; Silber, 2001; Evers and Collins, 2003). Finally, we believe that molecular/genetic evaluations in these cases should support the second hit hypothesis and help clarify the pathophysiology of varicocele.

Acknowledgements
The authors thank Terry Turner, PhD for guidance in TUNEL protocols, Meredith Stoltenberg, MD for protocols for autotomography, Dorothy Guzowski, PhD for synthesis of PCR primers, Craig Gavel, BSc for performing the fluorescence-based automated DNA sequencing, Martin L. Lesser, PhD for additional statistical input, and Asha Jacob, PhD, Larisa Dubovsky, MA, Stephanie Canaras, MA and Meghan E. Fleming for their technical assistance. This work was supported by Grant No. ES 10496 to S.B. from the National Institute of Environmental Health Sciences, National Institutes of Health, Bethesda, MD and, in part, by grant no. OH 07330 to S.B. from the National Institute for Occupational Safety and Health of the Centers for Disease Control and Prevention, Cincinnati, OH.

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Cadium, apoptosis and varicocele infertility

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Submitted on December 23, 2002; resubmitted on September 22, 2003; accepted on November 24, 2003

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