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The safety of ultrasonically guided testis aspiration biopsies and efficacy of use to predict varicocelectomy outcome

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BACKGROUND: We hypothesized that infertile men with varicoceles have molecular/genetic defects that interact with varicoceles to induce infertility. Studies directly on testis tissue appeared to be a way to link histology, markers for molecular/genetic defects and spermatogenesis, but testis biopsies may induce morbidity. In this report, we present safety and efficacy data on ultrasonically guided, single stick, percutaneous aspiration. METHODS: Biopsies were performed on 115 infertile men with varicoceles and five men with obstructive azoospermia. Morbidity was examined by pre- and post-biopsy ultrasound, efficacy by ability of two markers to predict >50% increase in sperm density post-operatively. All patients had three pre- and three post-operative semen analyses. RESULTS: 78.3% of patients had no ultrasonic testicular defects immediately post-biopsy. By 2 months, 100% had no defects. Biopsy markers [testicular cadmium (<0.453 ng/mg tissue) and an intact calcium channel mRNA sequence] predicted >50% increase in sperm density with 82.9 and 90.5% accuracy, respectively. CONCLUSIONS: Ultrasonically controlled, percutaneous aspiration testis biopsies are safe. Specimens so acquired can assist study of molecular/genetic markers associated with spermatogenesis in infertile men with varicoceles. Tissue cadmium level, calcium channel sequence and other markers may predict outcome of varicocele surgery.

Key words: biopsy/percutaneous aspiration/testis/ultrasound/varicocele

Introduction

The mechanisms underlying the effect of varicoceles on semen quality are poorly characterized. The most widely accepted explanation is elevated testicular temperature due to altered testicular blood flow (Comhaire, 1991). This is unsatisfactory. Scrotal temperatures of fertile and infertile men largely overlap (Mieusset and Bujan, 1995). Varicocele repair usually reduces testicular temperature (Agger, 1971; Yamaguchi *et al.*, 1989; Wright *et al.*, 1997), but varicocele correction returns the fertility of only 35–46% of patients (Schlesinger *et al.*, 1994).

The varicocele alone may not be the primary cause of infertility. An understanding of spermatogenic defects in varicoceles seems a prerequisite for treatment, as Holstein *et al.*, (2003) suggested. The interaction of varicoceles with other molecular and genetic factors may produce the infertile state (2nd Hit Hypothesis). In considering this, we have, among others, examined panels of molecular and genetic markers as predictors of varicocele surgery outcome (Benoff and Gilbert, 2001; Marmar, 2001; Benoff and Marmar, 2004). For example, Steger *et al.*, (2001) related decreased protamine-1 and -2 mRNA content of round spermatids seen in testis biopsies to decreased fecundity of ejaculated sperm. Others have related a variety of semen markers to spermatogenesis (Behr and Weinbauer, 2000; Francavilla *et al.*, 2000; Kimmins *et al.*, 2004).

We have chosen to work with testes tissue instead of semen because testis histology has been of use in varicocele assessment. The Johnsen (1970) score of testis biopsies correlates with ejaculate sperm density (Johnsen and Agger, 1978; Abdelrahim *et al.*, 1993; Uygur *et al.*, 1999). However, the prognostic value of histological studies in isolation is equivocal. Premature sloughing of immature germ cells and maturation arrest is the predominant pathology reported, but these findings are not uniform (review: Benoff and Gilbert, 2001). For example, McFadden and Mehan (1978) reported that tubular basement membrane thickness was indicative of poor surgical outcome, while Abdelrahim *et al.*, (1993) found that varicocelectomy did not decrease tubular basement membrane width in matched pre-operative and post-operative bilateral testicular biopsies, a finding consistent with our own (Benoff *et al.*, 2003).

In connection with our studies, we were concerned about potential morbidity. This is a serious problem for open biopsies. Dardashti *et al.* (2000) reported a 3.4% complication rate for open testis biopsies (including scrotal haematomas requiring surgical drainage and testicular atrophy). Post-open-biopsy ultrasound studies revealed persistent hypoechoic testicular lesions for up to 6 months (Schlegel and Su, 1997; Ron-El *et al.*, 1998). Percutaneous needle biopsies have fewer poor outcomes. Coviello *et al.*, (2004) reported using a narrow 19 gauge needle for repeated percutaneous biopsies in the same patient without tissue trauma. But where multiple percutaneous procedures per patient were performed, Harrington *et al.*, (1996) detected hypoechoic intratesticular lesions in 7% of patients at 6 months. In contrast, when one percutaneous stick was performed, Jarow *et al.*, (2001) found no intratesticular lesions by ultrasound 2 months post. These were relatively atraumatic because they utilized fine-needle aspirations to acquire cells, but they were blind procedures that did not preserve the tissue for histology. We therefore considered single stick procedures to study testis with modifications to ensure that the biopsy would be informative. Others have added real-time ultrasound to testes biopsies (Foresta *et al.*, 1998; Ron-El *et al.*, 1998; Belenky *et al.*, 2001; Raviv *et al.*, 2004) to increase safety by identifying areas of reduced and increased vascularity, similar to techniques that assessed ovarian vascularity during transvaginal puncture (Chui *et al.*, 1997; Van Blerkom *et al.*, 1997; Van Blerkom, 2000). We therefore modified our percutaneous aspiration biopsy technique (Marmar, 1996) to include ultrasonic guidance. We have reported the histology of testis biopsies so obtained at the time of varicocelectomy (e.g. Benoff *et al.*, 2004).

This report has two goals: (i) to document the safety of the single stick, ultrasonically guided percutaneous testis deep-aspiration biopsy technique; and (ii) to assess the efficacy of two examples of molecular markers in bilateral versus unilateral testis biopsies to predict the outcome of varicocele surgery [tissue cadmium concentration, and alterations in the L-type voltage-dependent calcium channel (L-VDCC) mRNA sequence]. Testicular cadmium concentration was chosen as an initial test marker as high tissue levels are associated with loss of actin and increased apoptosis that determines final sperm density (Benoff and Gilbert, 2001; Benoff *et al.*, 2004). It is likely that cadmium enters cells of the seminiferous epithelium via L-VDCC containing the α1C subunit (Benoff *et al.*, 2005). Alterations in the L-VDCC were chosen as an initial test marker as variant L-VDCC α1C mRNA with deletions in exons 7 and/or 8 (controlling, respectively, ion-selection properties and voltage-dependent channel inactivation) correlate with increased cadmium and apoptosis, and with poor response to varicocelectomy (Benoff *et al.*, 2005).

Materials and methods

Products and reagents

Optima grade (trace metal ion-free) concentrated HCl and concentrated HNO_3 were obtained from Fisher Scientific Company (USA). Human testis poly(A) + RNA was purchased from Clontech (USA). All PCR reagents were purchased from Qiagen (USA). All other enzymes were obtained from New England Biolabs (USA). Unless otherwise noted, all other reagents were purchased from Sigma Chemical Company (USA).

Human subjects

All protocols employing human subjects were reviewed and approved by the Institutional Review Boards of North Shore University Hospital and Cooper Hospital. Percutaneous testis aspiration biopsies were obtained (with written informed consent) with a single stick and ultrasonic guidance either from 115 infertile men at varicocele repair (by the subinguinal microsurgical approach; Marmar and Kim, 1994). The control group consisted of five men with proven fertility and obstructive azoospermia who required testicular sperm for IVF/ICSI because of a prior vasectomy. Although in animal models it has been shown that obstructive azoospermia is associated with important deterioration of spermatogenesis, including apoptosis (Lohiya *et al.*, 1987), the biopsies from control subjects all had normal histology, normal cadmium levels (0.194 ± 0.104 ng/mg dry weight; see Benoff *et al.*, 2004) and expressed full-length L-VDCC amplicons (see Benoff *et al.*, 2005). Hence, these men were considered as suitable controls. No patient had a biopsy solely for research purposes and no patient had been previously studied. All specimens were anonymized prior to transfer to the laboratories at the North Shore–LIJ Research Institute.

Testis biopsies

Prior to the testis biopsies, complete medical histories, including occupational exposures and a drug/medication profile, were obtained for all males evaluated for primary infertility. Potentially confounding lifestyle variables were addressed during this initial consultation, including smoking habits, alcohol intake and the use of vitamins or dietary supplements.

Comprehensive multi-system physical examinations were performed. Testicular sizes of each patient were measured by a Prader orchidometer. The patients were examined in the upright position by palpation and with a pencil Doppler during a Valsalva manoeuvre. A varicocele was considered significant only when the reflux was continuous during the val-salva. The classification of size was consistent with Marmar and Kim (1994): grade 1, audible; grade 2, audible and palpable; and grade 3, audible, palpable and visible.

Subinguinal microsurgical varicocelectomies were performed on patients with Doppler positive lesions and at least one semen parameter below World Health Organization standards. These patients had bilateral biopsies at the time of surgery with ultrasonic guidance using the following protocol. The testis was grasped and immobilized with gauze at its base. A 5–10 MHz ultrasound probe was used to capture the gray scale image, colour Doppler and power Doppler images. The initial gray scale image demonstrated the homogeneous seminiferous tissue, whereas the latter studies localized major intratesticular vessels. Xylocaine (1%) was administered to the scrotal skin over an area away from major vessels. An 18 gauge, $1\frac{1}{4}$ inch angiocath and stylet were inserted at the anaesthetized site through the skin, tunic albuginea and seminiferous tissue away from major vessels. The stylet was removed. The tip from a section of intravenous extension tubing attached to a 20 ml syringe was inserted into the hub of the angiocath. Negative pressure was created by a pistol grip. The seminiferous tissue was drawn into the angiocath and tubing with repeated in-and-out movements of the hub of the angiocath. Approximately 100–200 mg of tissue were obtained. The specimen was consistent with a thin segment of one

to three seminiferous tubules that were drawn up into the 0.2 mm lumen of the angiocath.

Similar procedures were performed on men who required testis biopsies as a source of sperm in association with IVF/ICSI. However, these men received ~8 ml of Xylocaine (1%) into the spermatic cord prior to the procedure as a local anaesthetic. The men were told to wear support underwear for 5 days post-biopsy, apply ice for 2–3 h daily for 2 days and use acetaminophen (paracetamol) for discomfort.

Repeat grey scale images documented hypoechoic areas within the testis as ultrasonic defects. Colour Doppler and power Doppler ultrasound images were obtained immediately after the biopsy and again at 1–2 months post-biopsy to determine the position and perfusion of the intratesticular vessels.

Study design

Following the suggestion of Steger (2002), all biopsy material was immediately divided in two. One part was fixed in Bouin's solution and was used for histology performed at Cooper Hospital. The remainder was placed in formalin and transported to the North Shore–LIJ Research Institute. Formalin-fixed tissues were used in all molecular investigations because formalin has no effect on atomic absorption analyses (Benoff *et al.*, 2004) and also inactivates RNases (Benoff *et al.*, 2005).

Analyses of testicular cadmium levels, L-VDCC α1C mRNA sequence and apoptosis were performed as part of a larger prospective study examining parameters potentially predictive of the outcome of varicocele repair (e.g. Benoff and Marmar, 2004). Not all assays were performed on all patients because of small biopsy size.

Pre-operative and post-operative semen data were collected according to the protocol previously described (Marmar and Kim, 1994; Benoff *et al.*, 2004, 2005). 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 so long as the duration of infertility was >12 months and the average value for any single semen parameter was less than a specified threshold ($<20 \times 10^6$ sperm/ml, <50% motility and/or <30% normal morphology by modified strict criteria). Post-operatively, two or three additional semen samples were obtained over a 3–12 month period. The average value was computed for each parameter for statistical comparison to the pre-operative average value.

In previous studies (Marmar and Kim, 1994), we considered post-operative pregnancy data for statistical analysis, but these earlier studies had follow-up of ≥18 months. We needed a parameter with which to evaluate the male in his own right, as with infertile couples one can never be completely confident that all female factors have been eliminated. A panel of experts has reported that the likelihood of fecundity decreases with decreasing sperm concentration (Guzick *et al.*, 2001). We took our cue from this study. In the present report, patients were followed for <4 months and spermatogenic response to varicocele repair was used as a surrogate for collection of data on pregnancy outcomes in the varicocele population. A 'normal' (= 'positive') response to varicocele surgery was defined as >50% increase in sperm density [(average post-operative sperm count minus average pre-operative sperm count)/average post-operative sperm count]. This value was chosen based on our previous study showing >50% increase in sperm density in patients who achieved pregnancy after varicocelectomy (Benoff *et al.*, 2004). This statistical technique has been used in other studies (e.g. Cayan *et al.*, 2001, 2002).

Determination of cadmium levels in testis biopsies

Testicular cadmium concentrations were determined using established laboratory protocols. In brief, cadmium levels in individual testis biopsy fragments that had been lyophilized to a constant weight and microwave digested in 50% HNO_3 were assessed by graphite furnace atomic absorption spectroscopy as previously described (Benoff *et al.*, 2004).

Based on studies of men with non-obstructive azoospermia and normal spermatogenesis, testicular cadmium levels ≤0.453 ng/mg dry weight were considered 'normal' whereas those >0.453 ng/mg dry weight were classified as 'abnormal' or 'high' (Benoff *et al.*, 2004).

Examination of L-type voltage-dependent calcium channel (L-VDCC) α1C splice variant expression

RNA was isolated from portions of formalin-fixed human testis biopsies (7–50 mg) using a Purescript RNA Isolation kit (Gentra Systems Inc., USA) as previously described (Benoff *et al.*, 2005).

Oligonucleotide primers (synthesized on an Applied Biosystems Model 394 DNA Synthesizer, USA) were designed to cross exon–intron boundaries and thereby detect spliced mature mRNA sequences in exons 6–9 in the L-VDCC α1C subunit (HUCH 2F and HUCH 1611R; Benoff *et al.*, 2005), and to amplify control mRNA (containing exons 8 and 9 of human glyceraldehyde-3-phosphate dehydrogenase (GAPDH; HG 690F and HG 984R; Goodwin *et al.*, 2000). RT–PCR amplification followed our established laboratory protocols (Goodwin *et al.*, 2000; Benoff *et al.*, 2005). Lengths of PCR products from GAPDH primers were estimated by electrophoresis with 1 kb (Gibco-BRL, USA; cat. no. 5615SB) and 100 bp (Invitrogen, USA; cat. no. 15628-019) ladders on a 1.2% agarose gel (Shelton Scientific, USA). L-VDCC primer product sizes were estimated on 2% low melting point agarose gel (Biorad, USA; cat. no. 162-0019) using the same standards. Size-separated nucleic acids were visualized following ethidium bromide staining and photographed using a Gel Doc 1000 video camera (Bio-Rad Laboratories, USA). PCR products were gel-purified (Wizard PCR Preps; Promega, USA) and sequenced (DNA Sequencing System Model 373A; Applied Biosystems, USA) following manufacturer's protocols. Amplicons were compared with target sequences using the MacVector 5.0 Program (Kodak, USA).

Previous studies have demonstrated consistent expression of one full-length (532 bp) L-VDCC α1C amplicon and variable co-expression of four smaller splice variants (232–520 bp) in testis biopsies from men with obstructive azoospermia (Benoff *et al.*, 2005). In contrast, the full-length (532 bp) L-VDCC α1C amplicon was detected in fewer than half of testis biopsies from infertile men with varicoceles. Its absence has been associated with poor varicocelectomy outcome (Benoff *et al.*, 2005).

Analysis of apoptosis

Apoptosis in testis biopsy sections was quantified by *in situ* by deoxynucleotidyl transferase labelling (TUNEL) of testis biopsy sections using TACS 2 TdT-DAB In Situ Apoptosis Detection Kit (cat. no. 4810-30-K; Trevigen, Inc., USA) as previously described (Benoff *et al.*, 2004).

Table Ia. Testis biopsy safety data: semen data from 60 infertile men with varicoceles who had surgery and biopsies[a]

Median	Pre-operative, pre-biopsy	Post-operative, post-biopsy	P^b
Sperm concentration ($\times 10^6$/ml)	11.92 ± 8.80	19.93 ± 12.00	<0.001
% motility	30.00 ± 12.10	44.02 ± 9.51	<0.001
% normal morphology	14.65 ± 6.71	25.28 ± 6.27	<0.001

[a]Each patient at least three semen specimens within the 6 month period prior to varicocelectomy and two or three additional semen samples were obtained ≤6 months post-surgery. Following our standard protocol (Marmar and Kim, 1994; Benoff *et al.*, 2004), pre-operative and post-operative data were respectively averaged and then the means for all subjects combined were obtained.
[b]Wilcoxon signed rank test.

Statistical analyses

All statistical analyses were performed with the SigmaStat v.3.0 software package (SPSS, Inc., USA). Statistical significance was set at $P < 0.01$.

Results

Examination of the safety of the testis biopsy protocol

Pre-operative and post-operative semen data from 60 infertile men with varicoceles who had single stick testis aspiration biopsies at the time of subinguinal microsurgical varicocelectomies was reviewed (Table Ia and Figure 1). Semen data represent a form of safety information. The semen parameters significantly increased following surgery and biopsy (e.g. the mean fraction increase in sperm count was 0.404; based on the paired data used to construct Table Ib). Twenty-four of the 60 infertile men with varicoceles (40.0%) exhibited a 'normal' spermatogenic response to varicocele surgery. These values were consistent with semen data from previous studies on men who had varicocele surgery but no biopsies (Marmar and Kim, 1994).

After the biopsy, there were occasional reports of minor bruising to the scrotal skin, but there was virtually no swelling and only minimal discomfort. The discomfort was managed with support, ice and acetaminophen. There were no permanent nodules in the testes, even among control men with up to four multiple biopsies that were obtained in connection with repetitive IVF/ISCI cycles. After microsurgical varicocelectomy, they returned to work within 72 h. After IVF/ICSI, control subjects returned to work either later the same day or the following day.

Pre- and post-biopsy ultrasounds were performed on a subgroup (Figure 1; $n = 51$) of the subjects with varicoceles (Figure 2, typical results). Post-biopsy ultrasounds were obtained immediately after the procedure and again at 2 months. Review of these patients' charts revealed that the majority of patients (40/51 = 78.3%) exhibited no ultrasound defects immediately after the biopsy on the grey scale image (Table Ia). When hypoechoic defects were seen post biopsy, they averaged 2–3 mm and never exceeded 5 mm. By 2 months, 100% of the study group had no demonstrable ultrasonic defect on the grey scale study (Table Ib). Follow-up colour and power Doppler studies indicated that the intratesticular vasculature remained in the same position

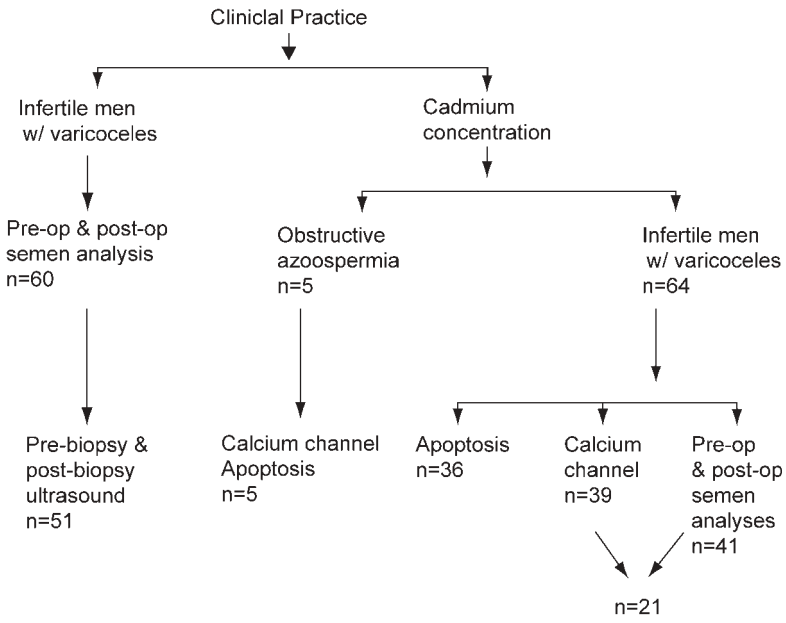

Figure 1. Study flow and subject allocation. All subjects were obtained from a single clinical practice. One group had both pre- and post-operative semen analysis. A subgroup of these subjects also pre- and post-post biopsy ultrasound analyses. A second group of subjects had pre-operative testis biopsies that were analysed for cadmium content. This group was comprised of men with vasectomies, who served as controls, and infertile men with varicoceles. The testis biopsies from all control subjects and subgroups of the varicocele subjects were analysed for L-type voltage-dependent calcium channel (L-VDCC) α1C mRNA structure and apoptosis. Semen data were also obtained from some of the men with varicoceles. Note that there is no overlap between study subjects undergoing pre-biopsy and post-biopsy ultrasound analyses and those whose testis biopsies were subjected to marker analysis.

Table Ib. Testis biopsy safety data: ultrasonic findings of the testes on 51 patients with varicoceles after percutaneous biopsy[a]

No. of patients	No. without defects immediately post-biopsy	No. studied 2 months post-biopsy	No. with normal vasculature post-biopsy
51	40 (78.3)	11 (21.7)	51 (100)

Values in parentheses are percentages.
[a]Testicular structure was analysed using grey scale, colour and power Doppler images (see Figure 1).

and the perfusion was unchanged in all patients (Figure 2, typical results).

Use of testis biopsy cadmium levels to predict varicocelectomy outcome

Bilateral biopsies from an additional 64 patients were subjected to molecular analyses (Figure 1). A mild positive relationship was detected between cadmium in the left testis with cadmium in the right testis (Spearman correlation, $n = 64$, $r = 0.458$, $P < 0.0001$). Cadmium levels were analysed in matched left and right biopsies from individual patients. Three groups resulted from the analysis: group 1, $n = 20$ (31.3%) had neither testis normal for cadmium (≤ 0.453 ng/mg); group 2, $n = 21$ (32.8%) had one testis normal; and group 3, $n = 23$ (35.9%) had both testes with normal cadmium levels (Table IIa). In one-third of the cases (21/64), cadmium values were discordant.

Among the patients studied for testicular cadmium, 41 had three pre- and three post-operative semen analyses (Table I). The pre- and post-operative values of each semen parameter were averages of the replicate measures. The fraction increase post-operatively in sperm density in the ejaculate was calculated from them (Table III). The mean increase in sperm count post-operatively in these 41 patients was 0.435 with 18 of the 41 subjects exhibiting a 'normal' response, similar to that of the patients undergoing ultrasound analyses (*t*-test, $P = 0.61$, not significant). However, these mean values differed significantly between the three groups of patients described above (Table III). As observed in our previous study (Benoff *et al.*, 2004), as mean cadmium levels rose, apoptosis within the seminiferous epithelium increased (Spearman correlation, $n = 36$, $r = 0.426$, $P < 0.009$) and seminal improvement after varicocelectomy decreased (Spearman correlation, $n = 41$, $r = -0.325$, $P < 0.04$) (also see Table III). Note also that the levels of apoptosis in all three groups were significantly higher than in control testis biopsies ($5.12 \pm 1.91\%$; $P < 0.02$).

Using these findings, we compared unilateral (left) testis cadmium measurements (Table IVa) with bilateral mean cadmium as a predictor of seminal improvement measurements (Table IVb) using contingency tables. In both cases abnormal cadmium predicted poor outcome, but bilateral analyses resolved four of the 11 cases with discordant unilateral measurements. Unilateral cadmium assays predicted that 33% (6/24) of men with abnormal cadmium values would improve, while bilateral assays reduced this number to 13.6% (3/22). Similarly, while unilateral assays predicted that 70.5% (12/17) subjects with normal cadmium values would improve, bilateral analysis predicted improvement in 78.9% (15/19) cases.

We used calculations described by Muller (2000) for interpretation and validation of tests for male fertility potential to determine how powerful these tests might be clinically (Table V). This analysis confirmed that bilateral cadmium measurements were more efficacious than unilateral with an overall accuracy of 82.9%. To make comparisons with other

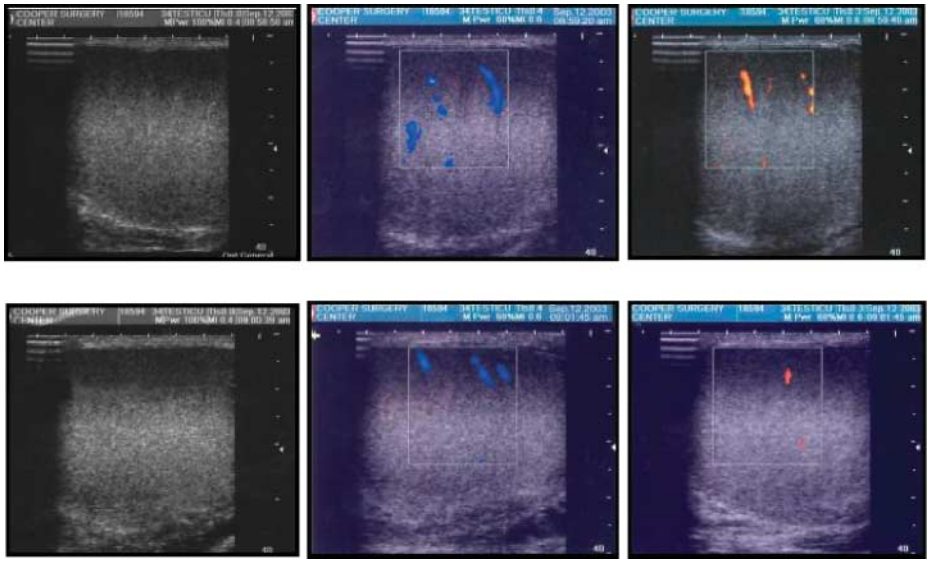

Figure 2. Analysis of testicular structure using Doppler ultrasonography. Typical paired pre- and post-operative images are shown. Grey scale study demonstrates the homogeneous pattern pre-biopsy (**A**) and the unchanged pattern post-biopsy (**D**), colour Doppler demonstrates the position of the major intratesticular vessels before biopsy (**B**) which are unchanged post-biopsy (**E**), and the power Doppler images demonstrate tissue perfusion pre-biopsy (**C**) which is unchanged post-biopsy (**F**). (**A**–**C**) Ultrasound appearance pre-biopsy. (**D**–**F**) Ultrasound appearance 2 months after biopsy.

Tables IIa and IIb. Molecular parameters in matched left and right testis biopsies from individual patients

a

	Left testis biopsy cadmium		Fisher's exact test
	'Abnormal'	'Normal'	
Right testis biopsy cadmium			
'Abnormal'	20 (LV = 6)	9 (LV = 2)	$P < 0.01$
'Normal'	12 (LV = 5)	23 (LV = 7)	

b

	Left testis biopsy L-VDCC α1C full-length amplicon		Fisher's exact test
	Absent	Present	
Right testis biopsy L-VDCC α1C full-length amplicon			
Absent	18 (LV = 6)	6 (LV = 3)	$P < 0.007$
Present	4 (LV = 3)	11 (LV = 2)	

LV = the number of subjects presenting with left varicoceles. The remainder had bilateral varicoceles.
The concordance (left to right diagonal) and discordance (right to left diagonal) in cadmium concentrations or L-type voltage-dependent calcium channel (L-VDCC) α1C full-length amplicon expression in matched left and right testis biopsies from individual study subjects was examined using Fisher's exact test (two-tailed). The results indicate that the proportion of observations in the different categories that define the above contingency tables was significantly different than was expected from random occurrence.

diagnostic tests, we calculated the likelihood ratio of a positive test (that abnormal cadmium levels would predict low spermatogenic response) and the likelihood ratio of a negative test (that normal cadmium levels would predict normal seminal improvement) (Table V). Comparing the values we obtained for the likelihood ratios with the target values described by Muller (2000), these findings suggest that bilateral measurement of testicular cadmium levels might be useful in the evaluation of treatment options for infertile men with varicoceles.

Use of L-VDCC α1C splice variants to predict seminal improvement after surgery

L-VDCC α1C mRNA structure was assessed in a subgroup of subjects examined for testicular cadmium levels. As observed for cadmium, L-VDCC α1C expression in matched left and right biopsies from individual patients divided into three groups: group 1, $n = 18$ (46.1%) had neither testis with a full-length amplicon; group 2, $n = 10$ (25.6%) had one testis with a full-length amplicon; and group 3, $n = 11$ (28.2%) had both testes with full-length amplicons (Table IIb). In one-quarter of the cases (10/39), amplicon expression was discordant.

Mean pre-operative and post-operative semen parameters were available for 21 of these subjects. Seminal improvement post-surgery differed between the three groups described above [analysis of variance (ANOVA), $P < 0.001$]. Applying *post hoc* pairwise comparisons by the Holm–Sidak method, group 1 exhibited an abnormal response (fraction increase in sperm density post-operatively = 0.274 ± 0.212) that differed from groups 2 and 3 ($P < 0.0001$). In contrast, groups 2 and 3 exhibited normal response (respectively, 0.655 ± 0.235 and 0.891 ± 0.050; not significant). Therefore, groups 2 and 3 were combined for the purposes of bilateral L-VDCC α1C amplicon analyses (Table IVd).

Although unilateral or bilateral absence of full-length L-VDCC α1C amplicons predicted low abnormal response to varicocele surgery, the bilateral analyses resolved two of the four cases with discordance on unilateral measurements (Tables IVc and IVd). As observed for cadmium, bilateral L-VDCC α1C analyses was more powerful than unilateral, with an overall accuracy of 90.5% (Table V). These results suggested that bilateral examination of L-VDCC α1C splice variant expression might aid in the evaluation of infertile men with varicoceles.

Discussion

Clinicians deciding how to treat patients with varicoceles know that some may father children and have normal semen analysis, that others may fail surgical correction and that still others will respond favourably to varicocelectomy. Recently, the American Urologic Association and the American Society of Reproductive Medicine jointly convened 'Best Practice Policy Groups for Male Infertility' and stated 'Varicocele repair may be considered the primary treatment option when a man with a varicocele has sub-optimal semen quality and a normal female partner' (Jarow *et al.*, 2002). The panel

Table III. Examination of the relationship between cadmium concordance or discordance and apoptosis and post-operative seminal improvement

Parameter	Group 1 0 testes, cadmium normal		Group 2 1 testis, cadmium normal		Group 3 2 testes, cadmium normal	
	n	Mean ± SD	*N*	Mean ± SD	*n*	Mean ± SD
Cadmium (ng/mg dry wt)[a]	16	0.888 ± 0.070	11	0.488 ± 0.037	14	0.295 ± 0.025
% apoptosis[b]	15	20.35 ± 3.94	10	9.45 ± 1.57	11	8.60 ± 1.50
Fraction post-operative increase in sperm density[c]	16	0.285 ± 0.068	11	0.488 ± 0.098	14	0.597 ± 0.093

[a]One way analysis of variance (ANOVA) with post-hoc pair-wise comparisons (Holm–Sidak method) indicated that the testicular cadmium levels differed significantly among the three groups (ANOVA, $P < 0.001$) and that each group differed significantly from the other (Holm–Sidak, $P < 0.0001$).
[b]The percentage of apoptotic cells within the seminferous epithelium differed significantly among the three groups (ANOVA, $P < 0.003$). Groups 2 and 3 differed significantly from group 1 (Holm–Sidak, $P < 0.0001$) but groups 2 and 3 were similar (Holm–Sidak, $P = 0.495$, not significant).
[c]The fraction post-operative increase in sperm density in the ejaculate differed significantly among the three groups (ANOVA, $P < 0.031$) and each group differed significantly from the other (Holm–Sidak respectively: group 1 versus group 2, $P < 0.0001$; group 1 versus group 3, $P < 0.0001$; and group 2 versus group 3, $P < 0.003$).

Table IV. Examination of the relationship between seminal improvement after varicocelectomy and unilateral versus bilateral testis molecular marker analyses

	Fraction change in sperm count post-operatively		Fisher's exact test (two-tailed)
	≤0.5 'Abnormal'	>0.5 'Normal' r	
a. Using unilateral testis biopsy cadmium concentration			
Cadmium concentration (ng/mg dry wt)			
> 0.453 'Abnormal'	18 (LV = 3) True positive	6 (LV = 2) False positive	$P < 0.001$
≤ 0.453 'Normal'	5 (LV = 0) False negative	12 (LV = 2) True negative	
	Fraction change in sperm count post-operatively		
	≤0.5 'Abnormal'	>0.5 'Normal'	
b. Using mean bilateral testis biopsy cadmium levels			
Cadmium concentration (ng/mg dry wt)			
> 0.453 'Abnormal'	19 (LV = 3) True positive	3 (LV = 2) False positive	$P < 0.001$
≤ 0.453 'Normal'	4 (LV = 0) False negative	15 (LV = 2) True negative	
	Fraction change in sperm count post-operatively		
	≤0.5 'Low' response	>0.5 'Normal' response	
c. Using unilateral L-VDDC α1C RT–PCR analysis			
Testis biopsy full-length L-VDCC amplicon			$P < 0.016$
Absent	11 (LV = 3) True positive	3 (LV = 2) False positive	
Present	1 (LV = 0) False negative	6 (LV = 2) True negative	
	Fraction change in sperm count post-operatively		
	≤0.5 'Low' response	>0.5 'Normal' response	
d. Using bilateral L-VDDC α1C RT–PCR analysis			
Bilateral testis biopsy full-length L-VDCC amplicon			
Absent in both testes	13 (LV = 5) True positive	1 (LV = 0) False positive	$P < 0.001$
Present in at least one testis	1 (LV = 0) False negative	6 (LV = 2) True negative	

LV = the number of subjects presenting with left varicoceles. The remainder had bilateral varicoceles. See legend to Table II for further information on the statistical analysis and Table V for establishment of assay validity.
L-VDCC = L-type voltage-dependent calcium channel.

considered percutaneous embolization and surgery, and noted that most experts performed inguinal or subinguinal microsurgical repairs as these maximize preservation of arterial and lymphatic vessels while reducing persistent recurrence. Although these comments represented the considered opinion of 12 experts out of 125 male infertility consultants, it is still an opinion. More research on varicocele pathophysiology is needed before any indvidual non-selective varicocele repair

Table V. Establishment of the clinical assay validity of the use of unilateral versus bilateral testicular molecular markers to predict post-operative improvement in sperm density

Calculations[a]	Cadmium data		L-VDCC amplicon expression	
	Unilateral[b]	Mean bilateral[c]	Unilateral[d]	Bilateral[e]
Positive predictive value[f]	18/24 (75.0)	19/22 (86.4)	11/14 (78.6)	13/14 (92.8)
Negative predictive value[g]	12/17 (70.6)	15/19 (78.9)	6/7 (85.7)	6/7 (85.7)
Overall accuracy[h]	30/41 (73.2)	34/41 (82.9)	17/21 (80.9)	19/21 (90.5)
False positive rate[i]	6/18 (33.3)	3/18 (16.7)	3/9 (33.3)	1/7 (14.3)
Specificity[j]	12/18 (66.7)	15/18 (83.3)	6/9 (66.7)	6/7 (85.7)
Sensitivity[k]	18/23 (78.3)	19/23 (82.6)	11/12 (91.6)	13/14 (92.8)
Likelihood ratio of a positive test[l]	0.783/0.333 = 2.35	0.826/0.167 = 4.95	0.916/0.333 = 2.75	0.928/0.143 = 6.49
Likelihood ratio of a negative test[m]	0.217/0.667 = 0.325	0.174/0.833 = 0.208	0.084/0.667 = 0.126	0.072/0.857 = 0.084

Values in parentheses are percentages.
[a]Based on the formulas from Muller (2000). Note that, to agree with clinical texts, Muller (2000) defined a good or normal test as a negative result, that is, the ability of an abnormal test to predict an abnormal outcome.
[b]Data from Table IVA.
[c]Data from Table IVB.
[d]Data from Table IVC.
[e]Data from Table IVD.
[f]Positive predictive value = true positives/(true positives + false positives).
[g]Negative predictive value = true negatives/(true negatives + false negatives).
[h]Overall accuracy = (true positives + true negatives)/total subjects.
[i]False positive rate = false positives/(false positives + true negatives).
[j]Specificity = true negatives/(false positives + true negatives).
[k]Sensitivity = (true positive rate) = true positives/(false negatives + true positives).
[l]Likelihood ratio of a positive test = sensitivity/(1–specificity); the likelihood ratio for a positive test can range from 1.0 to infinity, with higher ratios being better.
[m]Likelihood ratio of a negative test = (1–sensitivity)/specificity; the likelihood ratio of a negative test can range from 1.0 to 0.0, with lower being better.
L-VDCC = L-type voltage-dependent calcium channel.

technique can be considered to be validated. Therefore, we studied testicular tissue to determine if molecular/genetic markers could predict improvement in sperm parameters following varicocelectomy.

Our data indicate that testis tissue can be acquired without testicular damage by single stick, ultrasonically guided percutaneous aspiration biopsies. When the angiocath puncture is made away from major intratesticular vessels, there was no evidence of intratesticular defect by grey scale at 2 months, and no vascular changes by colour/power Doppler. This is in agreement with observations of investigators who performed percutaneous biopsies under ultrasound (Belenky *et al.*, 2001). The soft angiocath may help to minimize testicular defects because the biopsy is limited to a few stringy seminiferous tubules with a diameter of the angiocath's lumen. This biopsy technique has been used in >700 cases, including diagnostic biopsies of azoospermic men and sperm acquisition biopsies for IVF/ICSI. By this percutaneous procedure, the patients experienced less pain and swelling than with open biopsies. Although skin ecchymoses occasionally occurred, none required drainage and none of the patients had haematomas or permanent nodules. These findings contrast with open biopsies that may have a 3.4% complication rate (Dardashti *et al.*, 2000).

This biopsy technique preserved histology for study of varicoceles. Presently, some histological findings are being correlated with specific medical therapies in ongoing studies, including: (i) supplemental zinc (Takihara *et al.*, 1987; Ando *et al.*, 1989, 1990; Benoff, 1997; Benoff *et al.*, 1997, 2000), (ii) antioxidants (Barbieri *et al.*, 1999; Tripodi *et al.*, 2003; Onur *et al.*, 2004), and (iii) hormonal stimulation with Clomid (Bandhauer and Meili, 1977; Check, 1980; Unal *et al.*, 2001), tamoxifen (Kadioglu *et al.*, 1999) or hCG (Dubin and Amelar, 1977; Yamamoto *et al.*, 1995; Yan *et al.*, 2004). We here demonstrate the usefulness of molecular data from these biopsies both in investigation of the mechanism of bilateral effects of left varicoceles, and in treatment planning.

Spermatogenesis is decreased bilaterally in testis biopsies both in cases with unilateral and with bilateral varicoceles (Etriby *et al.*, 1975), and increased following varicocele repair (Charny, 1962; Agger and Johnsen, 1978; Johnsen and Agger, 1978; Abdelrahim *et al.*, 1993). These authors proposed a hypothetical anastomosis between the left and right spermatic vein plexuses to explain the bilateral effects of left varicoceles. This was unsatisfying as this anatomical structure has never been adequately documented and led our group to propose the 2nd Hit Hypothesis (Benoff and Gilbert, 2001; Marmar, 2001).

Implicit in our 2nd Hit Hypothesis is the argument that factors intrinsic to varicoceles will be expressed at a higher level in subjects with bilateral varicoceles than those with left varicoceles, and that factors that are extrinsic will be expressed equally in both subject groups. Both testicular cadmium levels and L-VDCC microdeletions are extrinsic factors (Benoff *et al.*, 2004, 2005). This is supported by this report. Cadmium levels are discordant in about one-third of matched biopsies examined, and L-VDCC amplicon expression is discordant in about one-fourth of cases. Consequently, markers from a biopsy of one testis may be insufficient because both testes contribute to semen production. Therefore, we performed biopsies on both testes, even in cases with unilateral varicoceles. Although testicular damage as assessed by histology is often less severe in the contralateral testis (Etriby *et al.*, 1967; Ibrahim *et al.*, 1977; Hadziselimovic, 1995), the molecular studies in matched left and right testis biopsies from individual patients with unilateral varicoceles showed unexpected concordances (Benoff *et al.*, 2004, 2005). Therefore, bilateral biopsies seemed worth exploring by this low-morbidity procedure. For example, we found that apoptosis was elevated bilaterally in infertile men with varicoceles irrespective of whether the patient presented with a left varicocele or with bilateral lesions (Benoff *et al.*, 2004).

The measure of success for varicocelectomy used in this report was >50% increase in sperm density compared to preoperative values, because this was the mean increase demonstrated by the patients that achieved pregnancy after surgery (Benoff *et al.*, 2004). In addition, the patients were stratified by the expression of specific markers in the testis tissue (cadmium levels and L-VDCC α1C splice variants). However, we recognize that use of positive changes in sperm density as a surrogate for reproductive success has been challenged (Vigil *et al.*, 1994). Nevertheless, recent reports on the predictive value of the sperm density have supported its use. For example, Guzick *et al.* (2001) compared sperm densities of fertile and subfertile populations. They demonstrated that the chances of subfertility increased as the sperm density decreased. Those within the range of $13.5–48 \times 10^6$ sperm/ml had an odds ratio of being in the subfertile population of 1.5 (1.2–2.2), whereas those with $<13.5 \times 10^6$ sperm/ml had an odds ratio of 5.3 (3.3–8.3). In a separate report comparing fertile and subfertile populations, Ombelet *et al.*, (1997) reported that the mean sperm densities for these groups were 19.5 versus 8.5×10^6/ml respectively ($P < 0.001$). These data suggest that measurements of sperm densities may help to define the fertility status.

Since pregnancy rates after varicocele surgery are only 35–40%, these molecular markers should help to pre-select patients who would benefit most. In this report, when we stratified patients by normal and abnormal tissue cadmium and microdeletions in L-VDCC, those with normal markers had likelihood ratios of >50% improvement of sperm density of 4.95 and 6.49 respectively. In contrast, those with abnormal markers had only a ratio of 0.28 and 0.08 for improvement. We intend to survey and update the pregnancy data among these patients (couples). With these data, we hope to add additional outcome information to validate this diagnostic approach.

Since this study shows that percutaneous testis biopsies with ultrasonic control are safe, urologists may consider using these biopsies and these markers as part of their pre-operative work-up. Although cadmium measurements can be performed by most hospital laboratories, analysis of L-VDCC α1C splice variant expression may require a more sophisticated setting. Nevertheless, we hope that reference laboratories will make this test available in the future.

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