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Evaluation of nuclear DNA damage in spermatozoa from infertile men with varicocele

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Objective: To examine levels of sperm DNA damage and oxidative stress (OS) in infertile men with varicocele.

Design: Prospective controlled study.

Setting: Male infertility clinic, Glickman Urological Institute, Cleveland Clinic Foundation, Cleveland, Ohio.

Patient(s): Thirty-one infertility patients and 16 fertile controls.

Intervention(s): Sperm DNA fragmentation index (DFI), levels of seminal reactive oxygen species (ROS), and total antioxidant capacity (TAC) were assessed using the sperm chromatin structure assay, chemiluminescence assay, and enhanced chemiluminescence assay, respectively. ROS-TAC score was calculated as a measure of OS.

Main Outcome Measure(s): Median (interquartile range) DFI and ROS-TAC scores.

Result(s): Sixteen of the 31 patients had left varicocele [grade I (n = 3), grade II (n = 10), and grade III (n = 3)], and the remaining 15 had normal genital examination. Patients with varicoceles had significantly higher percent DFI than controls (25%, range: 20%–35%; vs. 15%, range: 10%–22%). Patients with varicoceles had significantly lower ROS-TAC scores (21, range: 9.5–31) than the infertile patients with normal genital examination (34, range: 28–42) or the controls (40.3, range: 38–44).

Conclusion(s): Infertile men with varicoceles showed significantly increased spermatozoal DNA damage that appears to be related to high levels of OS in semen. (Fertil Steril® 2003;80:1431–6. ©2003 by American Society for Reproductive Medicine.)

Key Words: Male infertility, nuclear DNA, oxidative stress, spermatozoa, varicocele

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Varicoceles are found in approximately 15% of the general population and in 19%–41% of men presenting for infertility investigations (1–3). The incidence of varicoceles in men with secondary infertility is about 70%–80% (4, 5). These data suggest that varicoceles may cause a progressive decline in fertility, and men with prior fertility may suffer from varicocele-mediated secondary infertility.

Many patients with varicoceles also experience altered spermatogenesis, which has been attributed to many factors, including reflux of toxic metabolites from adrenal or renal origin, disturbed hormone status, spermatic venous hypertension, testicular hypoxia secondary to stasis, and abnormal temperature regulation (6). How these diverse etiologies mediate precisely the detrimental effects of varicoceles on sperm function leading to infertility is not completely understood (7).

Fujisawa et al. (8) found a significant reduction in levels of DNA polymerase (α, β, and γ) in extracts of testicular tissues from infertile men with varicoceles. As a result, they suggested that the decrease in DNA polymerase activities might have deleterious effects on spermatogenesis in patients with varicoceles.

Another factor that may lead to sperm DNA damage in these patients is seminal oxidative stress (OS). A recent study by Hendin et al. (7) found high levels of seminal OS as evidenced by increased levels of reactive oxygen species (ROS) and reduced total antioxidant capacity (TAC) both in fertile and infertile men with a clinical diagnosis of varicoceles. Increased levels of seminal OS have been correlated with

sperm dysfunction through different mechanisms that include lipid peroxidation of sperm plasma membrane and impairment of sperm metabolism, motility, and fertilizing capacity (9). In addition, OS has been shown to affect the integrity of the sperm chromatin and to cause high frequencies of single and double DNA strand breaks (10).

Recent data indicate that increased sperm nuclear DNA damage strongly and negatively affects natural and assisted fertility (11, 12). Furthermore, it has been reported that sperm chromatin/DNA is an independent measure of sperm quality that may have better diagnostic and prognostic capabilities than standard sperm parameters (concentration, motility, and morphology) (13).

We hypothesized that spermatozoal dysfunction in association with varicoceles may be related, at least in part, to increased levels of sperm DNA damage caused by high levels of OS commonly seen in these patients. The objective of this study was to examine and compare levels of sperm nuclear DNA damage using the sperm chromatin structure assay (SCSA) and OS among a group of infertile men clinically diagnosed with varicoceles, a group of infertile men with normal genital examination, and fertile donors.

MATERIALS AND METHODS

The Institutional Review Board of the Cleveland Clinic Foundation approved this study, and written informed consent was obtained from all participants.

Study Groups

The study included 31 men attending the male infertility clinic who had a history of infertility that had persisted for at least 1 year. The diagnosis of varicoceles was assessed in these patients clinically by genital examination, which was performed by an infertility specialist (ATJ) and confirmed by scrotal colored Doppler ultrasound. Sixteen of the 31 patients had a left varicocele (group 1) (grade I, n = 3; grade II, n = 10; and grade III, n = 3), and 15 had a normal genital examination (group 2). A group of healthy fertile volunteers (n = 16) who had initiated a natural pregnancy within the past 12 months and had a normal genital examination was included as a control group.

Standard Semen Analysis

Sperm Concentration, Motility, and Morphology

Semen specimens were collected by masturbation after a period of 48–72 hours of sexual abstinence. After liquefaction, manual semen analysis was performed using a Microcell counting chamber (Conception Technologies, San Diego, CA) to determine sperm concentration and motility. Smears of the raw semen were stained using Diff-Quik kit (Baxter Healthcare Corporation, Inc., McGaw Park, IL) for assessment of sperm morphology using World Health Organization (WHO) criteria. Normal values for sperm parameters were as follows: sperm concentration, $\geq 20 \times 10^6$/mL; forward progressive motility, ≥50%; and normal sperm forms, ≥30% (14).

Quantification of Seminal Leukocytes

Leukocyte concentrations in semen were quantified by a myeloperoxidase-staining test (15). The results were recorded as $\times 10^6$ peroxidase-positive leukocytes/mL of semen. Leukocytospermia was diagnosed if the concentration of peroxidase-positive leukocytes was greater than 1×10^6/mL of semen (14).

Measurement of Seminal ROS

Levels of seminal ROS were measured by a chemiluminescence assay using luminol (5-amino-2, 3, -dihydro-1, 4-phthalazinedione; Sigma, St. Louis, MO) as a probe (16). Liquefied semen was centrifuged at 300 *g* for 7 minutes, and the seminal plasma was separated and stored at −80°C for measurement of total antioxidant capacity (TAC). The pellet was washed with phosphate-buffered saline (PBS) and resuspended in the same media at a concentration of 20×10^6 sperm/mL. Ten microliters of luminol, prepared as 5-mM stock in DMSO, were added to 400-μL aliquots of the resulting cell suspension. Eight microliters of horseradish peroxidase (12.4 U of HRP Type VI, 310 U/mg, Sigma) were added to sensitize the assay for measurement of the extracellular hydrogen peroxide (H_2O_2). A negative control was prepared by adding 10 μL of luminol to 400 μL of PBS.

Measurement was performed using a luminometer (model LKB 953, Wallac Inc., Gaithersburg, MD) in an integrated mode for 15 minutes. The results were expressed as $\times 10^6$ counted photons per minute (cpm)/20 $\times 10^6$ sperm/mL.

Measurement of Total Antioxidant Capacity

Total antioxidant capacity in seminal plasma was measured with an enhanced chemiluminescence assay (17). Frozen samples of seminal plasma were thawed at room temperature and immediately assessed for TAC. Seminal plasma was diluted 1:20 with deionized water (dH_2O) and filtered through a 0.2-μ filter (Allegiance Healthcare Corporation, McGaw Park, IL). Signal reagent was prepared by adding 30 μL H_2O_2 (8.8 mol/L), 10 μL para-iodophenol stock solution (41.72 μM), and 110 μL of luminol stock solution (3.1 mM) to 10 mL of Tris buffer (0.1 M, pH 8.0). Horseradish peroxidase (HRP) working solution was prepared from HRP stock solution by making a dilution of 1:1 with dH_2O to give a chemiluminescence output of 3×10^7 cpm.

Trolox (6-hydroxyl-2, 5, 7, 8-tetramethylchroman-2-carboxylic acid), a water-soluble tocopherol analogue, was prepared as a standard solution (25, 50, and 75 μM) for TAC calibration. With the luminometer in the kinetic mode, 100 μL of signal reagent and 100 μL of HRP working solution were added to 700 μL of dH_2O and mixed. The mixture was equilibrated to the desired level of chemiluminescence output (between 2.8 and 3.2×10^7 cpm) for 100 seconds. One

TABLE 1

Comparison of standard sperm parameters (concentration, motility, and normal sperm forms) among fertile donors, infertile men with varicoceles (group 1), and infertile men with normal genital examination (group 2).

Parameters	Fertile donors (n = 16)	Group 1 (n = 16)	Group 2 (n = 15)	A	B	C
Sperm concentration ($\times 10^6$/mL)	72 (37, 138)	18 (10, 45)	59 (41, 74)	.001	.293	.003
Sperm motility (%)	63 (57, 73)	38 (28, 67)	61 (56, 69)	.04	.84	.08
Normal sperm forms by WHO (%)	36 (32, 41)	19 (12, 32)	35 (30, 37)	.007	.96	.005
Seminal leukocytes ($\times 10^6$/mL)	0 (0, 0)	0 (0, 0.4)	0 (0, 0.4)	.21	.14	.94

Note: Values are median and interquartile range (25%, 75%). A = *P*-value between fertile donors and group 1; B = *P*-value between fertile donors and group 2; C = *P*-value between groups 1 and 2. Wilcoxon rank sum test was used for the analysis and $P<.05$ was considered statistically significant.

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hundred microliters of the prepared seminal plasma was immediately added to the mixture, and the chemiluminescence was measured. Suppression of luminescence and the time from the addition of seminal plasma to 10% recovery of the initial chemiluminescence was recorded. The results were expressed as molar Trolox equivalents.

SCSA

Assessment of SCSA-defined DNA damage parameters was performed using a flow cytometer (Ortho Diagnostic Inc., Westwood, MA) as described elsewhere (18). Five thousand acridine orange stained sperm were measured at a rate of ~250 cells/second for the amount of green (515–530 nm = native DNA) and red (>630 nm = denatured DNA) fluorescence/cell. Computer analysis determined the DNA fragmentation index (DFI = red fluorescence/total [red + green fluorescence]). The DFI was formerly termed as alpha *t*. Computer gating defined the $\bar{\chi}$DFI (mean of DFI ranging from 0 to 1,034 channels), the SD DFI (standard deviation of DFI), the percent DFI (%DFI; percent sperm with DNA fragmentation), and the percent HDS (%HDS; percent sperm with high DNA stainability). Current statistical clinical thresholds have been established for %DFI as [1] excellent ≤15%, [2] good to fair = 15%–30%, and [3] poor = >30% for a significant decrease in fertility potential.

Statistical Analysis

Three-way analysis of variance was used to examine the relationship of varicoceles with DFI, ROS, and TAC after adjusting for standard sperm parameters and infertility status. Continuous variables among the groups were compared using Kruskal-Wallis tests. Pair-wise comparisons among the groups were performed with Wilcoxon rank sum tests. Fisher's exact test was used for the categorical variables. The correlation between variables was calculated using Spearman's nonparametric method. All hypothesis testing was two-tailed. $P<.05$ was considered statistically significant.

As a secondary analysis, the ROS-TAC score was calculated using principal component analysis as described in an earlier study (19). The ROS-TAC score is derived from levels of ROS in washed semen and TAC in seminal plasma. The resulting score minimizes the variability present in the individual parameters of OS (ROS alone or TAC alone). Individuals with ROS-TAC scores below 30, the lower limit of normal, are considered at higher risk for OS.

All analyses were calculated with the SAS statistical software package (version 8.1, SAS Institute Inc., Cary, NC). Summary statistics are presented as median and interquartile range (IQR; [25th, 75th percentiles]).

RESULTS

No statistically significant differences in the median (IQR) value in age were found among the patients with varicoceles (31 years [29, 35 years], patients with normal genital examination (32.5 years [30, 36 years]), or the fertile donors (33 years [31, 34 years])($P=.53$).

Standard Semen Parameters

Comparisons of standard sperm parameters (concentration, motility, and normal sperm forms) among infertile men with varicoceles, infertile men with normal genital examination, and fertile donors are shown in Table 1. Infertile men with varicoceles had significantly lower sperm concentration, motility, and normal sperm forms compared with fertile controls ($P=.001$, .04, and .007, respectively). Moreover, the same group had significantly lower sperm concentration and normal sperm forms compared with infertile men without varicoceles ($P=.003$, and .005, respectively).

Oxidative Stress Indices (ROS, TAC, and ROS-TAC scores)

Comparisons of ROS, TAC, and ROS-TAC scores among infertile men with varicoceles, infertile men with normal genital examination, and fertile donors are shown in Table 2. Using the three-way analysis of variance, varicoceles were significantly correlated with reduced levels of TAC ($P=.03$)

TABLE 2

Comparison of seminal oxidative stress (OS) parameters (ROS, TAC, and ROS-TAC score) among fertile donors, infertile men with varicoceles (group 1), and infertile men with normal genital examination (group 2).

Parameters	Fertile donors (n = 16)	Group 1 (n = 16)	Group 2 (n = 15)	A	B	C
ROS ($\times 10^6$ cpm)	0.36 (0.1, 2)	12 (1.3, 53.4)	1.7 (0.1, 5.4)	.01	.38	.06
TAC (trolox equivalent)	871 (699, 1288)	693 (499, 882)	904 (693, 978)	.03	.74	.08
ROS-TAC score	40.3 (38, 44)	21 (9.5, 31)	34 (28, 42)	.002	.10	.02

Note: ROS = reactive oxygen species; TAC = total antioxidant capacity. Values are median and interquartile range (25%, 75%). A = *P*-value between fertile donors and group 1; B = *P*-value between fertile donors and group 2; C = *P*-value between groups 1 and 2. Wilcoxon rank sum test was used for the analysis, and $P<.05$ was considered statistically significant.

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after adjusting for standard sperm parameters and infertility status.

SCSA-Defined DNA Damage Parameters

Comparisons of SCSA-defined DNA damage parameters among men with varicoceles, infertile men with normal genital examination, and fertile donors are shown in Table 3. Patients with varicoceles had a significantly higher %DFI than the fertile controls (25% [20%, 35%]; vs. 15% [10%, 22%]; $P=.002$). In addition, infertile patients with varicoceles manifested higher values of DFI than infertile patients with normal genital examination, however, the difference between these two groups was not significant (25% [20%, 35%]; vs. 20% [13%, 28%]; $P=.12$). Using the three-way analysis of variance, varicoceles were significantly correlated with %DFI ($P=.04$) after adjusting for standard sperm parameters and infertility status. In patients with varicoceles, $\bar{\chi}$DFI was negatively correlated with sperm concentration ($P=.008$; $r=-.64$), motility ($P=.03$; $r=-.52$), and normal sperm forms ($P=.02$; $r=-.6$). In the same group, SD DFI was negatively correlated with sperm concentration ($P=.001$; $r=-.76$), motility ($P=.001$; $r=-.73$), and normal sperm forms ($P=.002$; $r=-.7$). Also, levels of ROS were positively correlated with SD DFI ($P=.02$; $r=.57$) in patients with varicoceles.

DISCUSSION

Despite the high frequency of men with varicoceles within the infertile population and the documented fact that varicoceles negatively affect sperm function, it is quite surprising and even disconcerting that the exact mechanism(s) of this negative effect has not yet been resolved (20). Our results indicate that infertile men with varicoceles have sperm with significantly high levels of nuclear DNA damage (Table 3).

Varicoceles are strongly associated with OS. This association may be attributed to an increase in nitric oxide (NO) and the release of NO synthase and xanthine oxidase in the dilated spermatic veins of men affected with varicoceles (21, 22). Another factor could be the significant decrease of the antioxidant defenses normally present in seminal and blood plasma (23, 24). Since there is strong evidence suggesting that high levels of OS mediate the DNA fragmentation in the spermatozoa of infertile men (25, 26), it may be concluded that DNA damage in spermatozoa from patients with varicoceles is related to the role of seminal OS in mediating such damage.

A recent report has indicated that exposing spermatozoa to artificially produced ROS significantly increases DNA

TABLE 3

Comparison of sperm chromatin structure assay–defined DNA damage parameters among fertile donors, infertile men with varicoceles (group 1), and infertile men with normal genital examination (group 2).

Parameters	Fertile donors (n = 16)	Group 1 (n = 16)	Group 2 (n = 15)	A	B	C
$\bar{\chi}$DFI (%)	228 (207, 269)	268 (247, 317)	254 (227, 273)	.02	.13	.19
SD DFI (%)	173 (143, 190)	191 (149, 234)	165 (154, 174)	.18	.71	.14
%DFI	15 (10, 22)	25 (20, 35)	20 (13, 28)	.002	.08	.12

Note: $\bar{x}$DFI = mean flow cytometry channel value ranging from 0 to 1,034; SD DFI = the variability of chromatin structure abnormalities within the sperm population; %DFI = % of sperm with DNA fragmentation. Values are median and interquartile range (25%, 75%). A = *P*-value between fertile donors and group 1; B = *P*-value between fertile donors and group 2; C = *P*-value between groups 1 and 2. Wilcoxon rank sum test was used for the analysis, and $P<.05$ was considered statistically significant.

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damage by modifying all bases and producing base-free sites, deletions, frame shifts, and DNA cross-links (27). Our select group of infertile men with varicoceles in this study demonstrated significantly high levels of seminal OS as evidenced by high ROS, low TAC, and low ROS-TAC scores. This finding confirmed the results of a previous study conducted by our group that indicated a significant elevation of ROS and reduction of TAC in 21 infertile patients with varicoceles when compared with 17 fertile controls (7). Therefore, it may be speculated that increased DNA damage in spermatozoa from patients with varicoceles is related, at least in part, to OS.

Another potential explanation for DNA damage in patients with varicoceles may be related to the elevated intratesticular temperature associated with varicoceles affecting testicular function (28). This hypothesis may be explained by the direct thermal damage to nuclear DNA at the level of seminiferous tubules (8). However, the exact mechanism(s) of increased sperm DNA damage in patients with varicoceles warrants further research. Our results also indicate a significant reduction in sperm concentration and normal sperm forms in infertile men with varicoceles compared with infertile men with normal genital examination and normal fertile controls. However, sperm motility in the patients with varicoceles was significantly lower than that of the fertile donors and was not significantly different from men with normal genital examination.

Several studies have shown a significant correlation between varicoceles and poor sperm quality. Some show a correlation with all parameters, and others with certain parameters only. A study conducted by the WHO in 1992, which included 9,034 men from 34 centers in 24 countries who presented as a partner of the infertile couple, showed lower total sperm count per ejaculate in those with varicoceles (29). However, other sperm characteristics of motility and morphology were not influenced by the presence of varicoceles. More recently, all sperm parameters were found to be significantly lower in 40 patients with varicoceles than in 40 fertile subjects, but no correlation was found with the varicocele grade (30).

In a recent study, varicocelectomy was associated with a significant increase in pregnancy and live birth rates for couples who underwent intrauterine insemination, although standard semen parameters were not improved in all patients (31). Therefore, the improvement in pregnancy rates after varicocelectomy may be due to a factor not tested during routine semen analysis, such as sperm DNA damage. In our study, levels of sperm DNA damage in infertile men with normal genital examination who also had normal standard semen parameters and low levels of seminal OS were comparable to those found in the patients with varicoceles, although they were not significantly different than the fertile group.

This finding is consistent with the conclusion of our previous study: increased DNA damage may be, at least in part, responsible for the poor fertility in men who otherwise have normal standard semen parameters on repeated analyses and as a result were diagnosed as unexplained or idiopathic (32). This observation is important and may indicate a higher potential for sperm DNA damage testing in discriminating between fertile and infertile populations. Another important implication for this finding is that varicoceles may escalate already existing DNA damage in spermatozoa from the infertile population.

In conclusion, we report the novel finding that sperm from infertile men with varicoceles have statistically significant high levels of DNA damage. Our results indicate the importance of counseling patients with varicoceles about the potential negative effects of increased sperm DNA damage on their fertility potential. The finding of high seminal OS in patients with varicoceles may indicate that OS plays a role in the pathogenesis of sperm DNA damage in these patients. However, further research is needed to understand the exact mechanism(s) by which DNA damage increases in spermatozoa from infertile men with varicoceles and determine whether varicocele repair can reduce such damage.

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