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Apoptosis and Kinematics of Ejaculated Spermatozoa in Patients With Varicocele

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ABSTRACT: Increased DNA fragmentation is found in sperm from infertile men. Varicocele is an important cause of male infertility, even though it is present in 15% of men who father children. Semen analysis does not always identify infertility in these patients. Sperm motility is strongly correlated with male fertility potential. The goal of this study was to determine the correlation between apoptosis and kinematics in the ejaculated spermatozoa of patients affected by varicocele. Fresh semen samples were obtained from 30 patients with varicocele and 15 fertile controls. These samples were compared using computer-assisted semen analysis and were assayed to determine the degree of sperm apoptosis. The apoptotic index (AI) was calculated by dividing the number of terminal deoxynucleotidyl transferase–mediated deoxyuridine-5′-triphosphate nick end labeling (TUNEL) stained spermatozoa by the total number of Hoechst 33258–stained sperm cells for 300 sperm. Five microscopic fields were analyzed to obtain 5 AIs for each individual. Results demonstrated no significant difference in semen quality and sperm motion characteristics; however, a significantly higher AI (23.05% ± 4.07%: mean difference ± SE, 95% CI, 15.06%–31.03%, $P < .0001$) was identified in the varicocele group than in the fertile controls. We concluded that sperm apoptosis does not seem to correlate with semen quality and sperm kinematics and that apoptosis is increased in ejaculated spermatozoa in patients with varicocele compared to normal fertile men.

Key words: Computer-assisted semen analysis, terminal deoxynucleotidyl transferase–mediated deoxyuridine-5′-triphosphate nick end labeling, Hoechst 33258.

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The World Health Organization (1971) has clearly identified varicocele as an important cause of male infertility, although the role of varicocele in male infertility has been the subject of much controversy. Routine semen analysis has often been criticized for its subjectivity, technician dependence, and intersubject variability. Sperm motility has been thought to correlate strongly with male fertility potential (Krause, 1995; Joshi et al, 1996; Seftel et al, 1997; Hirano et al, 2001). Computer-assisted semen analysis (CASA), a tool used for providing quantitative data on sperm motility, gives more objective and detailed spermatozoa kinematics information than do traditional methods (ESHRE Andrology Special Interest Group, 1998). The biologic relevance of various CASA parameters to the prediction of male fertility potential requires elucidation (ESHRE Andrology Special Interest Group, 1996). Impaired motility may account for reproductive failure in certain couples, but other, less obvious abnormalities of sperm function in men can account for subfertility, such as the strong association between the presence of nuclear DNA damage in the mature spermatozoa of men and poor semen parameters (Sun et al, 1997; Lopes et al, 1998; Irvine et al, 2000). Apoptosis, characterized by distinct ultrastructural changes (Kerr et al, 1972; Wyllie et al, 1980) and biochemical changes (Wyllie et al, 1980; Williams and Smith, 1993) in cells such as chromatin aggregation, cytoplasmic condensation, and indentation of nuclear and cytoplasmic membranes, has been reported in a wide variety of cells (Wyllie et al, 1980; Wyllie, 1997). Extensive investigation of spermatogonia, spermatocytes, and spermatids in the testis has identified many apoptotic factors (Jurisicova et al, 1999; Sinha Kikim and Swerdloff, 1999; Pentikanen et al, 2000). Since a link between apoptosis and a variety of human diseases has been suggested (Barr and Tomei, 1994), spermatogenic dysfunction may in some cases be related to abnormal control of apoptosis. Terminal deoxynucleotidyl transferase–mediated deoxyuridine-5′-triphosphate nick end labeling (TUNEL) investigations have found increased DNA fragmentation in ejaculated spermatozoa from infertile men (Baccetti et al, 1996; Gandini et al, 2000). The percentage of sperm containing fragmented DNA has been found to negatively correlate with fertilization in IVF (in vitro fertilization) (Sun et al, 1997) and intracytoplasmic sperm injection (Lopes et al, 1998),

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and the percentage of ejaculated sperm with endogenous DNA nicks has been found to correlate with reduced fertility (Manicardi et al, 1995; Bianchi et al, 1996; Sakkas et al, 1996; Donnelly et al, 2000). The influence of apoptosis on semen quality in humans is still controversial because of the lack of control studies and clear patient categorization. The goal of this study was to investigate the nature of DNA damage in ejaculated human spermatozoa from patients with varicocele and, by comparing normal men and patients with varicocele, to establish what, if any, association exists between observed semen quality and apoptosis in spermatozoa.

Materials and Methods

Patients

A total of 45 adult men ranging in age from 20 to 40 years were recruited; this group consisted of 30 patients with varicocele and 15 fertile controls. Varicocele was diagnosed by a medical history and physical examination, the Valsalva maneuver, and Doppler auscultation. Informed consent was obtained from all volunteers, and semen samples were obtained by masturbation after 3 days of sexual abstinence. All experiments were approved the by the ethics committee for research involving human subjects at the Tri-Service General Hospital, Taiwan, and were conducted under the rules of the Declaration of Helsinki.

Semen Analysis

Semen was allowed to liquefy at 37°C before use. Conventional parameters (volume, motility, concentration, progression, and morphology) were assayed. A 6-μL aliquot of each specimen was loaded into a 20-μm microcell slide and subjected to CASA (Model 2030, version 7.1; Hamilton Thorne Research, Beverly, Calif) with the parameters that are used in Setup-A. All analyses were conducted at room temperature, with the stage of the CASA analyzer set to 36°C. The concentration of spermatozoa, percentage of motile and progressively motile spermatozoa, smooth path velocity (VAP), straight-line velocity (VSL), track velocity (VCL), linearity (LIN), straightness (STR), wobble (WOB), amplitude of lateral head displacement (ALH), and beat cross frequency (BCF) were measured. Sperm morphology was assessed by light microscopy for 200 sperm with Eosin Y and Nigrosin stains at 1000× magnification.

Assessment of the Percentage of Ejaculated Spermatozoa Exhibiting DNA Damage Using the TUNEL Assay and the Hoechst 33258 Dye Apoptosis Assay

TUNEL Assay—Supernatant with floating cells was transferred to a 15-mL conical tube; then, the monolayer was washed in phosphate-buffered saline (PBS). After the appropriate amount of Trypsin-EDTA was added, cells were collected and transferred for centrifugation (200 × *g*, room temperature, 10 minutes). Supernatant was discarded, and cells were washed with 1 mL of 1× PBS and centrifuged (200 × *g*, room temperature, 5 minutes); then, again, the supernatant was discarded. Semen samples were fixed with 4% (wt/vol) paraformaldehyde for 60 minutes at room temperature. Sperm cells were then washed with PBS. After permeabilization with 0.1% Triton X-100 for 10 minutes on ice, the samples were washed twice with PBS. The positive control samples for DNA fragmentation in individual apoptotic spermatozoa were detected by the TUNEL technique for DNA strand breaks (*In Situ* Cell Death Detection Kit, fluorescein; Roche Diagnostics, Indianapolis, Ind) according to the instructions of the manufacturer. For the negative control, no deoxyuridine-5′-triphosphate was added. The samples were incubated for 1 hour at 37°C and then washed with PBS and analyzed using fluorescence microscopy.

Hoechst 33258 Dye Apoptosis Assay—Slides assayed by TUNEL were washed with 1× PBS by putting the slide into a slide chamber for 5 minutes; they were then stained by adding 2.5 μg/mL of Hoechst 33258 in PBS for 5 minutes at room temperature. Following the staining procedure, cells were washed once again with PBS before coverslips were mounted with glycerol–phosphate-buffered saline (9:1 [vol/vol]) on glass slides. Nuclear shape and chromosomal structure can be visualized and counted by staining nuclear DNA with Hoechst 33258 in live, apoptotic, and necrotic sperm cells by fluorescence microscopy. Fluorescence microscopic fields at a magnification of 40× were selected at random to count 300 sperm cells in each field. The apoptotic index (AI) was calculated as a percentage by dividing the number of spermatozoa labeled in green using the TUNEL technique by the total number of sperm cells stained blue by the Hoechst 33258 dye, namely, AI = the number of apoptotic spermatozoa/total number of sperm cells. Five fluorescence microscopic fields were analyzed to obtain 5 AIs for each individual.

Statistical Analysis

The generalized estimating equation in Statistical Analysis Systems version 8.2 (SAS Institute, Cary, NC) was used to assess the correlated data arising from the same sample of 5 AIs at 5 different microscopic fields. In total, there were 150 observations in the varicocele group (30 patients × 5 microscopic fields) and 75 observations in the fertile control group (15 patients × 5 microscopic fields). The statistical model was started with group (varicocele vs fertile controls), microscopic field (different field), and group × microscopic fields and was stopped with the *P*-value <.05. Sperm quality and kinematics were determined by the Student's *t* test. $P < .05$ was considered statistically significant.

Results

After adjusting for the microscopic field and group-by-microscopic field interaction, we found that there was a statistically significant AI difference in the varicocele group vs the fertile control group (23.05% ± 4.07%: mean difference ± SE, 95% CI, 15.06%–31.03%, $P < .0001$, Table 1; Figures 1 through 4).

All semen analyses in both groups were normal according to World Health Organization criteria. Data were presented as the mean plus or minus the standard devia-

Table 1. *Comparison of sperm apoptotic index between patients with varicocele and fertile controls by group, different microscopic field, and group × different microscopic fields*

Parameter	Estimate (mean difference)	Standard Error	95% Confidence Limits	*z* Score	*P*-Value
Intercept	0.0982	0.0095	0.0795–0.1168	10.31	<.0001
Patients with varicocele vs fertile controls	0.2305	0.0407	0.1506–0.3103	5.66	<.0001

tion. Semen volume, sperm concentration, and morphology did not differ significantly between the control group and the patients with varicocele. The CASA sperm motility characteristics for both groups did not differ significantly, either (Table 2).

Discussion

Abnormal testicular thermoregulation secondary to a disrupted thermal exchange apparatus is currently believed to be the mechanism underlying sperm pathophysiology in the presence of varicocele. Varicocele may contribute to an increased sperm programmed cell death, necrosis, degeneration, or physiologic degeneration as a result of abnormal testicular temperatures. Heat treatment has been shown to produce fragile DNA in a population of sperm and is associated with poor capacitation characteristics and apoptosis (Mann et al, 2002). In the current study, patients with varicocele had a significantly higher AI than did fertile controls at the end point of spermatogenesis in ejaculated spermatozoa. It seems plausible that exposure to poorly regulated epididymal temperatures may cause spermatozoa to undergo significant apoptosis or necrosis during spermatogenesis. Crucially regulated apoptosis plays a role in the production of male gametes (Print and Loveland, 2000) and in spermatogenesis (Baccetti et al, 1996). However, decreased apoptosis of germ cells in the testes of infertile men with varicocele has been previously reported (Fujisawa et al, 1999). Our study presents evidence that increased apoptosis of ejaculated spermatozoa occurs in patients with varicocele. The mechanisms responsible for producing abnormal spermatozoa in the ejaculate of patients with varicocele have been poorly understood. It is unclear whether a modulating mechanism of one kind or another acts to decrease the apoptosis of germ cells in the testes in response to increased apoptosis of ejaculated spermatozoa.

The presence of apoptotic spermatozoa in fresh breeding bull semen has been suggested as one of the causes of poor fertility (Anzar et al, 2002). Lower concentrations of spermatozoa have been reported in men with a greater proportion of apoptotic spermatotozoa, with around 20% of ejaculated spermatozoa being apoptotic (Oosterhuis et al, 2000), but these results seem to depend on the inclusion criteria used for the subjects. The present study included groups that were clearly categorized with respect to patients with varicocele and fertile controls. Despite significant AI mean differences (23.05% ± 4.07%, $P < .0001$) between the varicocele group and the fertile controls, there was no significant difference in sperm concentration and morphology between the 2 groups.

There is ample evidence that CASA parameters can be used to predict fertility (Macleod and Irvine, 1995). CASA is thought to be the most appropriate technique for

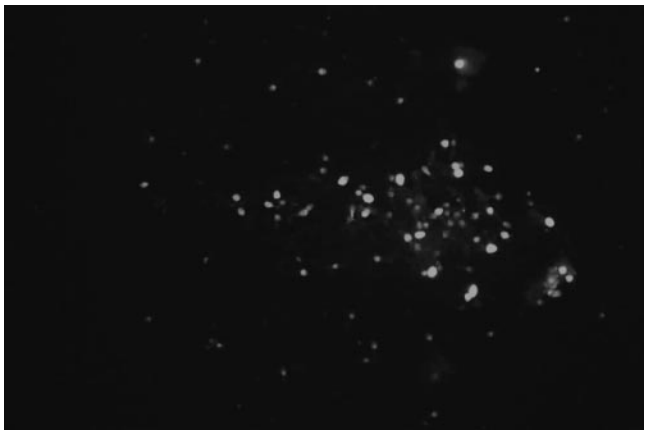

Figure 1. Spermatozoa labeled with terminal deoxynucleotidyl transferase–mediated deoxyuridine-5′-triphosphate nick end labeling (TUNEL) are stained green (apoptotic spermatozoa) in patients with varicocele. Fluorescence microscopic fields are at a magnification of 40×.

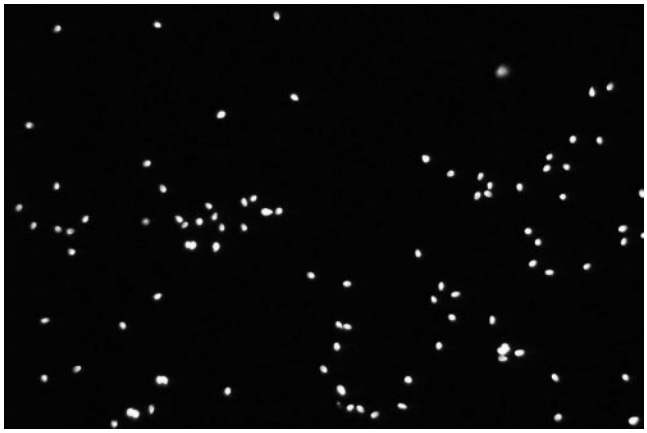

Figure 2. Sperm cells stained blue by Hoechst 33258 in patients with varicocele. Fluorescence microscopic fields are at a magnification of 40×.

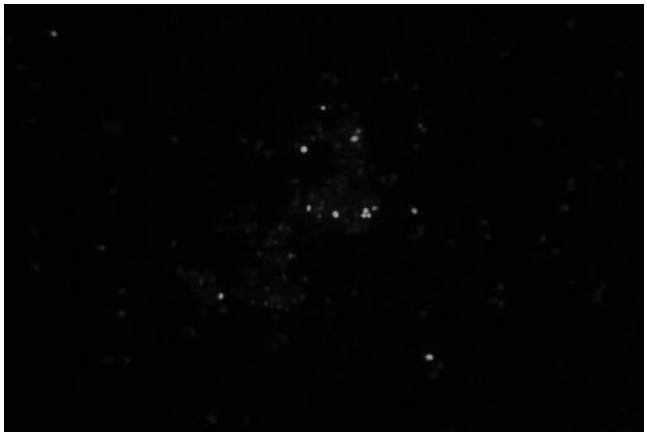

Figure 3. Spermatozoa labeled with terminal deoxynucleotidyl transferase–mediated deoxyuridine-5′-triphosphate nick end labeling (TUNEL) are stained green (apoptotic spermatozoa) in fertile controls. Fluorescence microscopic fields are at a magnification of 40×.

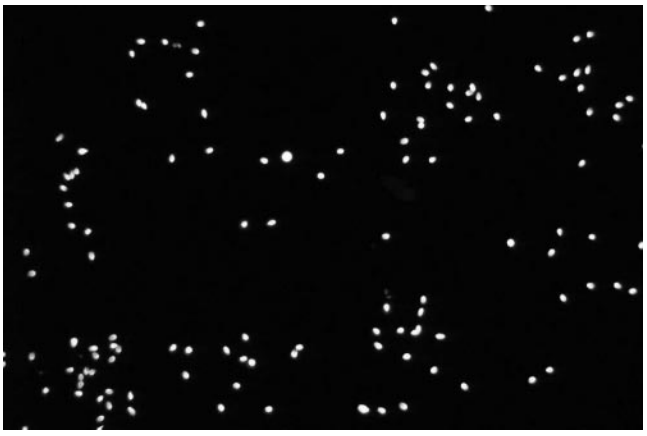

Figure 4. Sperm cells stained blue by Hoechst 33258 in fertile controls. Fluorescence microscopic fields are at a magnification of 40×.

the measurement of sperm motion rather than for the measurement of total sperm concentration and motility (ESHRE Andrology Special Interest Group, 1996). ALH is a nonsense value for hyperactivated spermatozoa, but it remains a very useful concept because it reflects the amplitude of the proximal flagellar wave (ESHRE Andrology Special Interest Group, 1996). ALH is an important value when attempts are made to predict IVF success in the cat (Stachecki et al, 1993). Fertilization rates are related to ALH, VCL, VSL, and rapid sperm movement, with motility (rapid) and VCL being the 2 most important parameters (Hirano et al, 2001). Motility, LIN, curvilinear velocity, and average path velocity—but not VSL—may serve as prognostic indicators for the fertilization potential of sperm (Joshi et al, 1996). A strong correlation exists between morphology and VCL, VSL, ALH, and motility but not LIN (Stachecki et al, 1993). In combination,

Table 2. *Comparison of sperm count, morphology, and CASA motility parameters between patients with varicocele and fertile controls**

	Patients With Varicocele (n = 30) (mean ± SD)	Fertile Controls (n = 15) (mean ± SD)	*P*-Value
Semen quality, sperm count, and morphology			
Volume (mL)	3.9 ± 1.3	4.0 ± 0.8	.8
Concentration (10^6/mL)	109.5 ± 102.9	135.2 ± 81.6	.25
Morphology (%)	42.2 ± 10.5	41.9 ± 6.5	.46
pH	7.9 ± 0.2	8.0 ± 0.1	.15
RBC	1.2 ± 0.5	1.0 ± 0	.09
WBC	1.8 ± 1.2	1.3 ± 0.5	.84
CASA parameter			
Motility (%) (Gr, 3 + 4; VAP, >25)	55.5 ± 23.9	70.4 ± 21.2	.07
Local motility (%) (Gr, 2; VAP, 5–25)	20.4 ± 9.3	15.6 ± 8.0	.1
Immotile (%) (Gr, 1; VAP, <5)	24.1 ± 18.2	14.1 ± 14.3	.08
Progression (%) (Gr, 4; VAP, >25; STR, >80%)	30.8 ± 5.6	32.3 ± 6.2	.29
Pattern of sperm movement			
VCL motile	75.0 ± 22.8	75.9 ± 18.3	.46
VSL motile	25.1 ± 2.1	24.3 ± 4.5	.36
VAP motile	43.4 ± 17.7	45.8 ± 9.6	.34
LIN motile	36.7 ± 12.3	34.2 ± 10.2	.3
STR motile	56.6 ± 12.3	54.9 ± 12.0	.37
WOB motile	65.9 ± 9.7	63.6 ± 6.2	.24
ALH motile	1.9 ± 0.5	2.0 ± 0.4	.33
BCF motile	9.0 ± 1.6	9.8 ± 1.2	.09

* ALH indicates amplitude of lateral head displacement; BCF, beat cross frequency; CASA, computer-assisted semen analysis; GR, group; LIN, linearity; RBC, red blood cell count; STR, straightness; VAP, smooth path velocity; VCL, track velocity; VSL, straight-line velocity; WBC, white blood cell count; and WOB, wobble.

the concentration of spermatozoa and a VCL greater than 25 μm/s was reported to be the most significant and independent CASA parameter for the prediction of male fertility potential (Larsen et al, 2000). In our study, ejaculated spermatozoa in both groups had a similar ALH and a high VCL (75 μm/s) measurement and concentration despite a significant difference in the AI. None of the motion characteristics showed statistical differences between the 2 groups. In agreement with the findings of Ricci et al (1996), sperm apoptosis did not seem to correlate with semen quality and spermatozoa kinematics. Although major DNA fragmentation is a late event in apoptosis (Collins et al, 1997), the modification of the sperm's integrity that occurs during apoptosis seems to bear no relationship to sperm kinematics, which are dominated by events that take place in the sperm's midpiece and mitochondria. Varicocele has been suggested as a potential cause for the impaired fertility potential observed in patients with normal or nearly normal semen parameters (Cockett et al, 1984). The increased AI in ejaculated spermatozoa that is observed in patients with varicocele, despite a seemingly normal semen analysis and sperm kinematics, may compromise events that take place during the acrosome reaction and chromatin condensation during the process of fertilization. However, our study was not designed to investigate this matter. Further studies such as penetration and fecundity assays, despite being time-consuming, will be enlightening.

The findings of this study are in keeping with previous reports that semen analysis alone does not predict fertility in patients with varicocele. Clinically, the diagnostic value of spermatozoa apoptosis may lie in allowing a more precise estimation to be made for fertility when examining patients. The drawbacks of this study are the small sample size, the lack of examination for the outer leaflet of the cell membrane, and the lack of sperm subjection to a functional test. Without doubt, determination of whether improved thermal regulation after varicelectomy could alter sperm quality and apoptosis is deserving of further study.

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