

The Role of Apoptosis and Reactive Oxygen Species in Varicocele-Associated Azoospermia

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Abstract

Background: The exact mechanism of impaired testicular function in patients with varicocele is not yet known. There is an increasing body of evidence pointing towards the role of reactive oxygen species (ROS) and apoptosis in the pathogenesis of varicocele related subfertility.

Objective: The aim of the study is to study the role of apoptosis and reactive oxygen species in varicocele-associated azoospermia.

Methods: Testicular tissue biopsy was obtained from 40 azoospermic infertile patients with varicocele and 40 infertile obstructive azoospermic patients. Caspase 3 level was determined by ELISA and malondialdehyde (MDA) level by colorimetry.

Results: Testicular MDA and caspase 3 level were significantly higher in the varicocele group compared to the control group. Increasing levels of MDA and caspase 3 were associated with higher varicocele grades. There was a statistically positive correlation between testicular caspase 3 and MDA levels in the varicocele group.

Conclusion: The present study documented the role of reactive oxygen species (ROS) and regulators of apoptosis in the pathophysiology of infertility in patients with varicocele-associated azoospermia.

INTRODUCTION

Varicocele is the most common surgically correctable cause of male infertility. (1) Varicoceles are found in approximately 15% of the general population, including adolescents and adults (2), the incidence rises to 35% of men with primary infertility (reported range: 19%-41%), (3) and to 80% of men with secondary infertility. (4)

A number of theories have been proposed to explain the pathophysiology of varicocele-induced male infertility including hyperthermia, reflux of renal and adrenal metabolites into the internal spermatic vein, hormonal dysfunction, hypoxia, abnormal blood flow and antisperm antibodies. (5) Reactive oxygen species (ROS) and oxidative stress were suggested to play a role in the pathogenesis of varicocele related subfertility (6), based on the findings of an increase in oxidative stress markers (e.g. superoxide and nitric oxide anions, peroxinitrite), in the serum, semen and testicular tissues of varicocele patients. (7, 8)

(9) More recently, ROS have often been suggested to play roles in the apoptotic death. Furthermore, activation of apoptotic cascades has been shown to occur as a consequence of increased generation of ROS or treatment with exogenous oxidants. Various antioxidants and antioxidative enzymes are able to delay or block apoptosis. (10)

AIM OF THE WORK

We aimed to measure testicular tissue malondi aldehyde (MDA) a marker of lipid peroxidation, and testicular tissue caspase 3 as a marker of apoptosis in testicular tissue biopsies of patients with varicocele-associated azoospermia.

PATIENTS AND METHODS

Patients

This study is done on eighty infertile male patients selected from the Andrology out-patient clinic of Alexandria Main University Hospital divided into two groups.

Study Group

Forty azoospermic infertile men with varicocele as the sole cause of infertility.

Control Group

Forty infertile obstructive azoospermic patients proved by testicular biopsy and normal hormonal assays.

Inclusion Criteria Were

1. Age 20-45 years, non-smokers.
2. No history of any medication influencing spermatogenesis.
3. No history or clinical manifestations of chronic systemic disease or any endocrinopathy.
4. No evidence of urogenital infections, or leukocytospermia or hypogonadism (testicular volume < 15ml).

Exclusion Criteria Were

1. Patients with recent hormonal therapy in the last six weeks before enrollment into the study.
2. Patients with abnormal epididymal consistency which may indicate previous or present epididymal infection.
3. Patients with vas deferens induration or beading which may suggest non-specific or specific infections (TB).
4. Cases with any urinary changes which may indicate urinary tract infections.
5. Control cases with lower limb varicose veins.

Methods

An informed consent was obtained from all study subjects. The study protocol was approved by the local institutional ethics committee, and followed the International Ethical Guidelines of the 1975 Declaration of Helsinki. A general physical examination including examination of both thighs and legs to detect varicose veins if any. Local genital examination, clinical grading of varicocele according to Dubin and Amelar (11, 12) were done. At least two samples were obtained by masturbation after 2-7 days of sexual abstinence and a morning urine analysis was obtained to rule out infection. FSH hormonal assay by ELISA (13) was

performed. An open testicular biopsy was performed under spinal analgesia by "window technique". (14) Testicular tissue samples with known weight were washed in phosphate-buffered saline (PBS) and centrifuged 1000 x g for 5 minutes. Supernatant was discarded and the pellets were diluted in 1 ml PBS, aliquots were created for the following assays.

A- Human Caspase-3 ELISA assay (15, 16, 17): An anti-human Caspase-3 monoclonal coating antibody is adsorbed onto microwells. Following incubation unbound anti-human Caspase-3 detection antibody and antirabbit- IgG-HRP is removed during a wash out step, and substrate solution reactive with HRP is added to the wells.

A coloured product is formed in proportion to the amount of soluble human Caspase-3 present in the sample. The reaction is terminated by addition of acid and absorbance is measured at 450 nm. A standard curve is prepared from seven human Caspase-3 standard dilutions and human Caspase-3 sample concentration determined. The values were expressed as ng/mg protein.

B- Colorimetric method for determination of malondialdehyde concentration: 2.5ml of 0.02% trichloroacetic acid and 1ml 0.67% thiobarbituric acid (TBA) are added to the tissue homogenate, and the mixture is heated in boiling water bath for ten minutes. The resulting chromogen is extracted with 4ml of n-butyl alcohol and the absorbance of the organic phase is determined at a wavelength of 530 nm. The results were expressed in nmol/gram tissue. (18)

RESULTS

The patient group included 10 patients (25%) with grade I varicocele, 7 patients (45%) with grade II varicocele, and 12 patients (30%) with grade III varicocele. Out of the fifteen patients, 14 patients (35%) had unilateral left sided varicocele, and 26 patients (65%) had bilateral varicocele. The duration of infertility in the patient group ranged from 1-9 years with a mean value of 2.23 ± 2.21 years, and it ranged from 1.5-4 years with a mean value of 2.55 ± 0.98 years in the control group. The age in the patients group ranged from 24-45 years with a mean value of 33.33 ± 5.11 , and it ranged from 24-41 years with a mean value of 31.6 ± 6.48 years in the control group.

The Role of Apoptosis and Reactive Oxygen Species in Varicocele-Associated Azoospermia

In the patient group, the testicular size ranged from 15-20 ml (on both sides) with a mean value of 18.91±3.25 ml on the right side and 18.19±3.52 ml on the left side. In the control group, the testicular size ranged from 15-25 ml (on both sides) with a mean value of 20.9±3.41 ml on the right side and 20.3±3.99 ml on the left side. 11 of the varicocele patients (73.3%) had normal FSH and 4 patients (26.6%) had elevated FSH levels. FSH was found normal in all control group cases (100%).

Testicular Tissue MDA Level (nmol/gram tissue)

In the right side testes of the patient's group, the testicular tissue MDA level ranged from 51.01-155.02 nmol/gram tissue, with a mean value of 108.07±34.85. On the other side (left side), its range was found to be 53.01-155.22 nmol/gram tissue, with a mean value of 107.35±35.42. Within this group, there was no statistically significant difference between the right and left testicular tissue MDA (P2=0.32). In the control group, the right sided testicular tissue MDA ranged from 50.1-74.2 nmol/gram tissue, with a mean value of 56.22±7.62. On the other (left) side; it ranged 50.3-76.92 nmol/gram tissue, with a mean value of 56.94±8.11. Within the control group, there was no statistically significant difference between the right and left testicular tissue MDA (P2=0.41). On the other hand, there was a statistically significant difference between the two studied groups in both right and left testes (P1=0.0001 for each side).

Testicular Tissue Caspase 3 Level (ng/mg protein)

In the right side testes of the patient's group, the testicular tissue caspase 3 level ranged from 0.82-12.3 ng/mg protein, with a mean value of 6.25±3.17. On the other side (left side), its range was found to be 0.55-12.78 ng/mg protein, with a mean value of 6.18±3.44. Within this group, there was no statistically significant difference between right and left testicular tissue caspase 3 level (P2=0.41). In the control group, the right sided testicular tissue caspase 3 level was found with a range from 4.1-4.76 ng/gram protein, with a mean value of 4.30±0.20. On the other (left) side; the range was from 4.2-4.8 ng/mg protein, with a mean value of 4.51±0.21. Also within the control group, there was no statistically significant difference between right and left testicular tissue caspase 3 level (P2=0.52). However, there was a statistically significant difference between the two studied groups in both testes (P1=0.013 as regards the right testes, P1=0.041 as regards the left testes).

Relation between the Grade of Varicocele and MDA (nmol/gram tissue) level

There was a statistically positive significant relation between the grade of varicocele and MDA level on the right and left testicular sides (P=0.001 on the right side and 0.0026 on the left side).

Table 1. Relation between grade of varicocele and MDA (nmol/gram tissue) level.

MDA level	Varicocele grading			
	Right testes	Grade I (no=2)	Grade II (no=6)	Grade III (no=2)
Range		53.01-76.33	95.69-118.61	154-155.02
Mean		68.18	104.7	154.51
SD		13.15	11.3	0.72
F		10.11		
P		0.001*		
Left testes	Grade I	Grade II	Grade III	
Range	53.01-77.34	77.34-155.05	119.75-155.22	
Mean	65.05	110.6	143.94	
S.D.	13.62	24.4	16.72	
F	9.89			
P	0.0026*			

* significant

The Role of Apoptosis and Reactive Oxygen Species in Varicocele-Associated Azoospermia

Relation between the Grade of Varicocele and Caspase 3 Level

There was a statistically positive significant relation

between the grade of varicocele and Caspase 3 level on the right and left testicular sides (P=0.021 on the right side and 0.018 on the left side).

Table 2. Relation between grade of varicocele and caspase 3 (ng/mg protein) level.

Caspase level	Varicocele grading		
	Grade I	Grade II	Grade III
Right testes			
Range	0.82-5.66	5.19-10.29	5.19-12.3
Mean	2.02	7.1	7.67
S.D.	2.26	2.2	3.16
F	8.25		
P	0.021*		
Left testes	Grade I (no=4)	Grade II (no=7)	Grade III (no=4)
Range	0.857-5.66	0.55-10.31	5.21-12.78
Mean	2.10	6.4	7.88
S.D.	2.38	3.3	3.35
F	9.25		
P	0.018*		

*Significant.

Correlation of Testicular Tissue MDA and Caspase 3 Levels

There was a statistically significant positive correlation between the testicular Caspase 3 and MDA levels in the patients group in both testicular sides (P=0.013

on the right side and 0.005 on the left side).

On the other hand, there was no statistically significant correlation between the testicular Caspase 3 and MDA levels in the control group in both testicular sides (P=0.14 on the right side and 0.21 on the left side).

Table 3. Correlation between MDA level (nmol/gram tissue) and Caspase 3 level (ng/mg protein) in patients and control group at both sides.

Testicular side		MDA	
		Patients group	Control group
Right side	r	0.49	0.38
	p	0.013*	0.14
Left side	r	0.51	0.31
	p	0.005*	0.21

*Significant.

DISCUSSION

The pathophysiology of varicocele remains controversial, with various studies suggesting the involvement of hyperthermia, hypoxia, reflux of adrenal and renal metabolites, and hormonal dysfunction. More recently, ROS and regulators of apoptosis have been suggested to play a role in varicocele-induced male infertility. (19)

Varicoceles have been evaluated as one of the potential causes of increased ROS and oxidative stress. (20) High levels of ROS are harmful as they lead to lipid peroxidation of sperm plasma membrane and DNA fragmentation. Increased lipid peroxidation of the sperm plasma membrane is associated with impaired sperm motility and diminished capacity for sperm-oocyte fusion. (21)

Apoptosis is a distinctive form of eukaryotic cell death, occurring in both physiological and pathological tissue. (22) The central component of the apoptotic machinery comprises a family of proteases termed caspases. (23) Caspase 3 is considered to have the best correlation with apoptosis, among identified caspases. (24)

The results of the present study revealed a statistically significant difference between the varicocele patients and the control group as regards the testicular tissue MDA. The result of this study regarding the correlation of testicular MDA and the presence of varicocele came in accordance with Ozdamar et al (25) who measured MDA and nitric oxide (NO) levels in testes of adolescent rats with experimental bilateral varicocele where the mean MDA level was higher than in the control group; this difference was statistically significant ($p < 0.05$). However, Koksal et al (26) reported that the level of MDA (measured by thiobarbituric acid reaction) in testicular biopsy specimens obtained from infertile patients with varicocele and infertile patients without varicocele (patients with testicular failure, idiopathic infertility and obstruction) was not statistically significant. This could be attributed to his choice of the control group which included patients with impaired spermatogenesis. The increased levels of MDA demonstrated in patients with a clinical diagnosis of varicocele, suggests that sperm dysfunction in varicocele patients may be in part related to oxidative stress. ROS may act through impairment of sperm motility, decreased sperm count, decreased sperm-oocyte fusion and sperm DNA damage.

In the present work, there was no statistically significant difference between the right and left testicular tissue MDA in the patients group as well as in the control group ($P = 0.41$). This came in accordance with other authors (27) who observed no statistically significant difference in testicular ROS levels measured by chemiluminescence in bilateral testicular biopsies in a group of rats where experimental left varicocele was induced. This may be attributed to the facts that both testicles are commonly exposed to the same circumstances as regards embryological, environmental and systemic factors, although in some males both testicles may be different.

Regarding testicular tissue caspase 3 levels, the results of the present work revealed a statistically significant increased level of testicular caspase 3 and hence apoptosis among the varicocele patients, compared to the controls. These results of increased apoptosis

in varicocele patients came in agreement with Chen et al (28) who measured the apoptotic index (AI) in 30 varicocele patients and 15 fertile controls using Terminal deoxynucleotidyl transferase-mediated deoxyuridine-5'-triphosphate nick end labeling (TUNEL) assay for DNA strand breaks

On the other hand, there was no statistically significant difference between right and left testicular tissue caspase 3 level within the patients group ($P = 0.41$), as well as within the control group ($P = 0.52$) in this study. This was in agreement with Cam et al (27) who utilized in the in-situ end end-labelling technique to investigate apoptosis in an experimental model of varicocele in rats. They observed no statistically significant difference in the apoptotic index in right and left testicular biopsies. Similar results were demonstrated in human model of varicocele by Benoff et al (29) and Tanaka et al. (30)

Correlations of MDA level and testicular size in this study were statistically insignificant. This was in accordance with Allamaneni et al (31) who assessed seminal ROS levels in 46 men diagnosed with a unilateral left varicocele during an infertility evaluation. They reported a statistically non-significant difference in ROS levels in men with left testicular volume ≤ 19 mL compared to men with testis volume of ≥ 19 mL. The lack of ROS level correlation with the testicular size may be a reflection of the time required to cause testicular size changes, as well as the multifactorial nature of varicocele pathophysiology.

The current study demonstrated no statistically significant correlation between the testicular size and caspase 3 levels in both the patient and control groups, in both the right and left testicular biopsies. These results did not coincide with Xia et al (32) who observed that the incidence of apoptosis was remarkably more frequent in the varicocele group than the control group coupled with a slower testicular growth in the varicocele group in an animal study in Wistar rats. This could be attributed to the differences in the animal varicocele models from clinical varicoceles in humans

The present study revealed a statistically significant relationship between varicocele grade and testicular biopsy MDA levels. This came in agreement with Allamaneni et al (31) who demonstrated that increasing levels of seminal ROS measured in 46 men with unilateral left varicocele was associated with higher grades of varicocele.

A significant relation between the testicular biopsy caspase 3 level and the grade of varicocele was observed in this work. This disagreed with the Tanaka et al (30) who immunohistochemically (avidin-biotin peroxidase-complex method) assessed the expression of caspase 3 in bilateral testicular biopsies from 26 infertile men with varicoceles and 6 normal testicular specimens. He reported that caspase 3 tended to be lower in the varicocele group, with a higher expression in grade I than grade II or III varicocele. Technical reasons (using formalin instead of Bouin's solutions for fixation) may be responsible for the different results reported in that study.

The present study demonstrated a statistically significant correlation between the testicular Caspase 3 and MDA levels in the patients group, whereas the difference was not statistically significant among the control group.

This relationship was similarly demonstrated by Saleh et al (33) who assessed apoptosis by measuring apoptotic DNA damage due to activation of caspase activated DNAase and reported that infertile men with varicoceles had significant increase in spermatozoal apoptotic DNA damage (due to activation of caspase activated DNAase), which appeared to be associated with high ROS levels in the semen. This finding might indicate that ROS plays a role in the pathogenesis of apoptotic sperm DNA damage in such patients.

CONCLUSION

The present study documented the role of reactive oxygen species (ROS) and regulators of apoptosis in the pathophysiology of infertility in patients with varicocele. The increased levels of MDA and Caspase 3 in testicular tissues of infertile patients with varicocele are considered as important molecular causes of oligospermia or azoospermia in cases of varicocele.

As MDA and caspase 3 levels were positively correlated, we therefore believe that an association between varicocele and infertility exists at a molecular level through stimulation of ROS and apoptosis, where the increased rate of apoptosis with varicocele may involve ROS overproduction as the triggering mechanism.

REFERENCES

- [1] Shefi S, Turek PJ. Definition and current evaluation of subfertile men. *Int Braz J Urol* 2006;32:385-97.
- [2] Belloli G, Diágostino S, Pesce C, Fantuz E. Varicocele in childhood and adolescence and

- other testicular anomalies: An epidemiological study. *Pediatr. Med. Chir.*1993;15:159.
- [3] Pryor JL, Howards SS. Varicocele. *Urol. Clin. North Am.*1987;14:499.
- [4] Gorelick JL, Goldstein M. Loss of infertility in men with varicocele. *Fertil Steril* 1993;59, 613-616.
- [5] Benoff S, Goodwin LO, Hurler IR, Pergolizzi RG. Variation in the region IS6 of the L-type voltage-gated calcium (Ca²⁺) channel (L-VDCC) alpha-1 subunit in testis and sperm: implications for role of cadmium in varicocele-associated infertility (VAI). *Fertil. Steril.* 2000;74:555.
- [6] Sharma RK, Agarwal A. Role of ROS in male infertility. *Urology* 1996;48:835.
- [7] Mostafa T, Anis TH, El-Nashar A, Imam H, Othman IA. Varicolectomy reduces reactive oxygen species levels and increases antioxidant activity of seminal plasma from infertile men with varicocele. *Int J Androl* 2001; 24: 261-5.
- [8] Halliwell B. Free radicals, antioxidants, and human disease: curiosity, cause or consequence? *Lancet* 1994;344:721-4.
- [9] Agarwal A, Saleh RA. Role of oxidants in male infertility: rationale, significance, and treatment. *Urol. Clin. North Am.* 2002;29:817-27.
- [10] Andrieu-Abadie N, Gouaze V, Salvayre R, Levade T. Ceramide in apoptosis signaling: relationship with oxidative stress. *Free.Radic.Biol.Med.* 2001; 31:717-28.
- [11] Marsam JW, Schats R. The subclinical varicocele debate. *Hum Reprod* 1994;9:1-8.
- [12] Dubin L, Amelar RD. Varicocele size and results of varicolectomy in selected subfertile men with varicocele. *Fertil Steril* 1970;21:606-9.
- [13] Veldhuis JD. Male hypothalamic-pituitary-gonadal axis. In: Lipshultz L and Howards SS, editors. *Infertility in the male*. Philadelphia: Mosby Year Book;1996.p.23-58.
- [14] Coburn M, Wheeler T, Lipschultz LI. Testicular biopsy, its uses and indications. *Urol Clin Am* 1987;14:551-64.
- [15] Slee EA, Harte MT, Kluck RM, Wolf BB, Casiano CA, Newmeyer DD, Gang Wang H, Reed JC. Ordering the Cytochrome c-initiated Caspase Cascade: Hierarchical activation of Caspases-2,

The Role of Apoptosis and Reactive Oxygen Species in Varicocele-Associated Azoospermia

- 3, -6, -7, -8, and -10 in a Caspase-9-dependent Manner. *J Cell Biol* 1999; 144:281-292
- [16] Wolf Bb, Schuler M, Echeverri F, Green DR. Caspase-3 is the Primary Activator of Apoptotic DNA Fragmentation via DNA Fragmentation Factor-45/Inhibitor of Caspase-activated DNase Inactivation. *J Biol Chem* 1999; 274:30651-6.
- [17] Lowry OH, Rosebrough NJ, Farr L, Randall RJ . Protein measurement with the Folin Phenol Reagent. *J. Biol. Chem* 1951;193:267-75.
- [18] Ohkawa H, Ohishini N, Yagi K. Assay for lipid peroxides in animal tissues by thiobarbituric acid reaction. *Anal Biochem* 1979;95:351-8.
- [19] Baccetti B, Collodel G, Piomboni P: Apoptosis in human ejaculated cells. *J Submicrosc Cytol Pathol* 1996;28:587.
- [20] Rajeev K, Rupin S. Varicocele and male infertility: current status. *The Journal of Obstetrics and Gynecology of India* 2005;55:505-16.
- [21] Agarwal A, Saleh RA, Bedaiwy MA. Role of reactive oxygen species in the pathophysiology of human reproduction. *Fertil Steril* 2003; 79: 829-43.
- [22] Wyllie AH. Glucocorticoid - induced thymocyte apoptosis is associated with endogenous endonuclease activation. *Nature* 1980;284:555-6.
- [23] Cohen GM. Caspases: the executioners of apoptosis. *Biochem. J.* 1997; 326:1-16.
- [24] Krajewski M, Wang HG, Reed JC et al. Immunohistochemical analysis of in vivo patterns of expression of CPP32 (Caspase-3), a cell death protease. *Cancer Res* 1997; 57: 1605-13.
- [25] Ozdamar AS, Soyulu AG, Culha M, Ozden M, Gokalp A. Testicular Oxidative Stress: Effects of Experimental Varicocele in Adolescent Rat. *Urologia Internationalis* 2004;73:343-7.
- [26] Koksall IT, Tefekli A, Usta M, Erol H, Abbasoqlu S, Kadioqlu A. The role of reactive oxygen species in testicular dysfunction associated with varicocele. *BJU International* 2000;86:549-52.
- [27] Cam K, Simsek F, Yuksel M, Turker L, Haklar G, Yalcin S. The role of reactive oxygen species and apoptosis in the pathogenesis of varicocele in the rat model and efficiency of vitamin E treatment. *Int J Androl* 2004;27:228-33.
- [28] Chen CH, Lee SS, Chen DC, Chien HH, Chen IC, Chu YN, et al. Apoptosis and kinematics of ejaculated spermatozoa in patients with varicocele. *Journal of Andrology* 2004;25:348-53.
- [29] Benoff SH, Millan C, Hurley IR, Napolitano B, Marmar JL. Bilateral increased apoptosis and bilateral accumulation of cadmium in infertile men with left varicocele. *Hum Reprod* 2004;19:616-27.
- [30] Tanaka H, Fujisawa M, Tanaka H, Okada H, Kamidono S. Apoptosis-related proteins in the testes of infertile men with varicocele. *BJU International* 2002;89: 905-9.
- [31] Kadioqlu A. The role of reactive oxygen species in testicular dysfunction associated with varicocele. *BJU International* 2000;86:549-52.
- [32] Allamaneni SS, Naughton CK, Sharma CK, Thomas AJ, Agarwal A. Increased seminal reactive oxygen species levels in patients with varicoceles correlate with varicocele grade but not with testis size. *Fertil Steril* 2004;82:1684-6.
- [33] Xia Q, Zhang XB, Zhang J. The apoptosis in damaged testicular tissue caused by varicocele. *Zhonghua Nan Ke Xue* 2002 ;8:414-5.
- [34] Saleh RA, Agarwal A, Sharma RK, Said TM, Sikka SC, Thomas AJ Jr. Evaluation of nuclear DNA damage in spermatozoa from infertile men with varicocele. *Fertil Steril* 2003; 80: 1431-6.

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