

Evaluation of Oxidative Stress in Testis and Sperm of Rat Following Induced Varicocele

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Purpose: Oxidative stress (OS) plays a central role in the pathophysiology of varicocele (VC), however, comprehensive studies concomitantly assessing semen parameter along with chromatin status, oxidative stress, and enzymatic antioxidants in both testis and sperm are limited. Therefore, this study aims to assess these parameters in varicocele rats.

Materials and Methods: For this study, 30 Wistar rats were randomly divided into three groups: Control group (I); sham-operated group (II) and left varicocele group (III). Left varicocele was induced and two months after surgery, we evaluated sperm parameters, persistent histone, DNA integrity and lipid peroxidation in sperm and also oxidant/antioxidant markers in testis.

Results: The results showed that sperm concentration, motility, and normal morphology significantly decreased in varicocele group compared to other groups ($P < 0.001$). Also, we observed a significant increase in persistent histone and DNA damage of sperm cells in varicocele rats ($P < 0.05$). In addition, oxidant assessment analysis showed that ROS level was higher in testis tissue and sperm cells from the left varicocele rats compared to the control group ($P < 0.05$).

Conclusion: This results show that varicocele has a negative effect on spermatogenesis and increased oxidative stress and reduce in antioxidant capacity hand in hand lead to the production of sperm with damaged chromatin which reduces the fertility potential and may jeopardize the future health of the progeny.

Keywords: DNA damage; oxidative stress; rat; varicocele

INTRODUCTION

Varicocele is one of the most common causes of male infertility which occurs in 4.4% to 22% of the male population and is described as the dilation and tortuosity of plexus pampiniformis (especially in the left testis) and leads to some pathological problems in the testis tissue.⁽¹⁻³⁾ The pathogenesis of varicocele-related infertility is not completely defined, although there are numerous hypothesis such as scrotal hyperthermia, oxidative stress, hypoxia, hormonal disturbances, testicular hypo-perfusion, testicular hypoxia, and backflow of toxic metabolites to explain the correlation of varicocele and infertility. But the most confirmed mechanism is the testicular hyperthermia.^(4,5) Indeed, the male scrotum temperature is kept 1–2°C lower than core body temperature. Reduced scrotal temperature compared to body temperature is necessary for proper testicular function. But it is still obscure why most of the mam-

als have evolved to maintain their testes at low temperatures.⁽⁶⁾ In varicocele, dilation of the pampiniform plexus leads to backflow of warm blood into the internal spermatic vein which impairs testicular temperature exchange system. So, failure to adjust scrotal temperature and increased testicular temperature of around 2.5°C, result in testicular heat stress which affects spermatogenesis, and subsequently increase of oxidative stress (OS), accumulation of reactive oxygen species (ROS), induction of germ cell apoptosis and DNA fragmentation (both germ cell and epididymal sperm) as well as hormonal imbalance.⁽⁷⁻⁹⁾

Generally, OS is due to an imbalance of ROS and protective antioxidant system. Interestingly, testis tissue is extremely vulnerable to oxidative stress because of two main reasons: (I) the existence of abundant unsaturated fatty acids in the plasma membrane of cells (II) the presence of potential reactive oxygen species (ROS)-generating systems with low enzymatic anti-

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oxidant capacity due to low cytoplasm of sperm.⁽¹⁰⁾ Indeed, a physiological level of ROS usually produces by some of the cells like somatic cells and spermatozoa as a byproduct of the electron transfer chain in mitochondria. The main types of ROS are H₂O₂ and O₂ and antioxidants like glutathione peroxidase (GPx), catalase (CAT), and superoxide dismutase (SOD) present in mitochondria help to balance ROS. Both oxidants and antioxidants are also present within the reproductive tract.⁽¹¹⁾ Although the normal physiological level of ROS is essential for a successful fertilization such as regulation of sperm hyperactivation, capacitation, acrosome reaction and sperm-oocyte fusion, numerous studies have demonstrated the harmful effects of excess ROS on sperm function.^(4,12) Leukocytes and abnormal spermatozoa are considered as important sources of ROS generation. In addition, oxidative stress is able to disrupt the steroidogenic ability of Leydig cells and also impairing the germinal epithelium to differentiate into normal spermatozoa.⁽¹⁰⁾

In view of the fact that oxidative stress is known as the main factor in the pathophysiology of varicocele and in the most cases, increase of ROS level and decrease antioxidant capacity have been observed.^(4,13-15) To the best of our knowledge, this is the first study to evaluate sperm parameters, chromatin status and also ROS level in both testis and sperm of rats with experimentally induced varicocele.

MATERIAL AND METHODS

Study population and Study design

30 mature, male Wistar rats, aged 4 weeks and weighing 150- 200 gram were used in this study. The rats were obtained from the Institute for Biotechnology (Isfahan, Iran). This study received approval of the Institutional Review Board from the Royan Institute (NO: 97000110), and all the experiments conducted on the animals were in accordance with Royan institute guidance from the Ethical Committee for Research on Laboratory Animals. Rats were maintained and housed in a controlled environment (12 hours light, 12 hours dark at 24°C) with free access to the standard food and water. Rats were randomly divided into 3 groups of 10. In the first group, according to Ko, left-side varicocele was induced surgically (varicocele group).⁽¹⁶⁾ Rats in group 1 underwent a sham laparotomy (sham group). And the third group consists of untreated rats (control group). Surgical technique and Outcome assessment

Each rat was anesthetized by an intraperitoneal injection of ketamine and xylazine mixture. After induction of standardized anesthesia, the left renal vein was exposed and identified through a midline abdominal incision. In order to reduce the renal vein diameter to 1 mm, a 4.0 silk suture was tied around the left renal vein medial to the adrenal and spermatic veins. This occlusion led to increased intravenous pressure lateral and then the pressure is transmitted to the left spermatic vein. This causes a varicocele to develop.

At the end of the second month, all rats in the control, sham, and varicocele groups were sacrificed, and their genital systems were collected. At first, the length, width, thickness, and weight of the left testes were assessed using caliper. Then, the dissected testes were used for oxidant/antioxidant assessment. Samples were weighed and placed phosphate-buffered saline (PBS; Gibco-Europe, Uxbridge, Middlesex, UK) and homog-

enized by a homogenizer. Subsequently, homogenized tissues were centrifuged (three-time 10 min at 12000 rpm) and the supernatant was collected and stored at -70°C until assay. In addition, the left epididymis was separated from the testis and after removing the blood vessels, the epididymis was divided into three distinct segments: caput, corpus, and caudal.⁽¹⁷⁾ The caudal segment was isolated and placed in a petri dish that contained 5 mL of sperm washing media (VitaSperm, Inoclon, Tehran, Iran). The sperm retrieved from the caudal epididymis were evaluated for sperm concentration, morphology, motility, DNA damage, and protamine deficiency.

Oxidant/antioxidant assessment in testis

For evaluation of enzymatic oxidants, total protein was determined in tissue samples by Biuret method (Parsazmoon kit, Iran) and using BT-1500 autoanalyzer. The activity of catalase (CAT) was measured at 37°C by following the rate of disappearance of H₂O₂ at 240 nm.⁽¹⁸⁾ The activity of glutathione peroxidase (GPX) was measured using a commercial kit (ZellBio GmbH, Germany). Superoxide dismutase (SOD) activity was determined using a commercial kit (Ransod-Randox Lab, Antrim, UK). In addition, Malondialdehyde (MDA) which is an important indicator of oxidative damage was calculated in homogenized tissue by detecting the absorbance of thiobarbituric acid reactive substances at 532 nm as an index of lipid peroxidation.⁽¹⁹⁾

Sperm parameters assessment

Sperm concentration and motility was determined with the aid of a sperm counting chamber (Sperm meter; Sperm Processor, Aurangabad, India) and light microscopy using prewarmed slides and also eosin/nigrosin staining was used for morphology assessment. In brief, sperm samples were washed in PBS afterward 30 µl of the sperm suspension was mixed with 60 µl of eosin (Merck, Darmstadt, Germany) for 3 min. Then, 90 µl of nigrosin (Merck) was added to this mixture and used to prepare the smears. We observed 200 sperm under a light microscope and the percentage of abnormalities visualized in the head, neck, and tail were determined for each sample.⁽²⁰⁾

Evaluation of sperm chromatin condensation and DNA damage using aniline blue and Acridine Orange staining

Histones during the spermatogenesis are replaced by protamines. Defected spermatozoa with poor protamination are rich in lysine and stained by aniline blue (AB) while normal spermatozoa with protamine-rich nuclei are contained arginine and cysteine and cannot be stained by this staining.⁽²¹⁾ So, for assessment of sperm maturation, a drop of sperm sample was smeared, air-dried and fixed for 120 min in 3% glutaraldehyde in 0.2 M phosphate buffer (pH = 7.2). when the smear was dried, each sample was stained with 5% aqueous aniline blue mixed with 4% acetic acid (pH 3.5) for 90 minutes (pH = 3.5). Subsequently, for each sample, at least 200 sperm were counted per slide by light microscopy and the rate of sperm with persistent histones was determined.⁽²⁰⁾

For evaluation of sperm DNA damage, sperm samples were washed with PBS buffer and fixed with 4% methanol-free formaldehyde. Afterward, slides were stained with acridine orange stain (Merck) according to Afyani⁽²⁰⁾. Consequently, microscopic analyses of

Table 1. Comparison of mean weight, volume, length, width, and thickness of left testis between control, sham and varicocele groups.

Variable ^c	Control	Sham	Varicocele	P-value
Testis weight, gr; mean ± SD	1.66 ± 0.08	1.63 ± 0.13	1.5 ± 0.16	> 0.05
Testis volume, mL; mean ± SD	1.59 ± 0.15 ^a	1.66 ± 0.23 ^a	1.12 ± 0.30 ^b	< 0.001 *
Testis length, mm; mean ± SD	1.71 ± 0.07 ^a	1.78 ± 0.07 ^a	1.54 ± 0.12 ^b	.04 **
Testis width, mm; mean ± SD	0.89 ± 0.08 ^a	0.89 ± 0.05 ^a	0.72 ± 0.07 ^b	< 0.001 *
Testis thickness, mm; mean ± SD	0.70 ± 0.08 ^a	0.68 ± 0.07 ^a	0.48 ± 0.06 ^b	< 0.001 **
				< 0.001 *
				< 0.001 **

Different letters indicate significant differences between groups at $p < 0.05$.

^c Continuous variables were compared by One-way analysis of variance (ANOVA)

*Varicocele to Control

**Varicocele to Sham

^c Continuous variables were compared by One-way analysis of variance (ANOVA)

each slide were done using an Olympus fluorescent microscope (BX51, Tokyo, Japan) by the appropriate filters (460–470 nm). The percentage of green (normal double-stranded DNA) and orange/red (abnormal/denatured DNA) fluorescence sperm per sample was counted.⁽²⁰⁾

Oxidant assessment in sperm

Assessment of sperm lipid peroxidation was done according to Aitken protocol.⁽²¹⁾ BODIPY C11 loading BODIPY w 581/591 C11 (D3861, Molecular Probes) was added to 2×10^6 spermatozoa at a final concentration of 5 mM and allowed to incubate for 30 min at 37°C. Then, samples were washed twice with PBS buffer at 650g for 5 min and evaluated by a FACSCalibur flow cytometer (Becton Dickinson, San Jose, CA, USA). Positive controls were obtained after the addition of H₂O₂ to sperm suspensions.

Statistical Analysis

Data analysis was done using the Statistical Package for the Social Sciences for Windows, version 18.0. The Shapiro-Wilk test was performed to evaluate the normal distribution and Levene's test for equality of variances. All results are presented as mean ± SD. Differences among groups were analyzed by One-way analysis of variance (ANOVA) followed by Tukey HSD test to identify significant differences among the three groups. Means were considered statistically significant when $P < .05$.

RESULTS

Comparison of morphometric parameters of testis

Measurement of testis length, width, thickness, weight, and volume of each group are presented in **Table 1**.

These parameters significantly reduced in varicocele group compared to control and sham groups ($P < .05$) except testis weight. Comparison of the aforementioned parameters showed no significant difference between sham and control groups.

Comparison of sperm parameters

Mean of sperm concentration were 115.2 ± 8.28 , 99.3 ± 9.49 and 62.8 ± 12.84 in control, sham and varicocele groups, respectively and a significant reduction was observed between the varicocele compared to the control ($P < 0.001$) and sham ($P = 0.005$) groups (**Table 2**).

Mean percentage of sperm motility were 85.2 ± 3.7 , 78.8 ± 5.51 and 42.0 ± 7.03 in control, sham and varicocele groups, respectively and a significant reduction was observed in varicocele group compared to the control ($P < .001$) and sham ($P = .04$) groups (**Table 2**).

Mean percentage of abnormal sperm morphology were 6.0 ± 1.82 , 8.0 ± 1.33 and 9.6 ± 1.07 in control, sham and varicocele groups, respectively and a significant reduction was observed between the varicocele compared to the control ($P < .001$) and sham ($P = .012$) groups (**Table 2**).

For the aforementioned parameters, no significant difference was observed between the control and sham groups.

Comparison of sperm chromatin condensation, DNA damage, and lipid peroxidation

The result of aniline blue staining in **Table 3** showed that the percentage of sperm with persistent histone was significantly ($P < .05$) higher in varicocele group (9.1 ± 4.69) compared to control (4.2 ± 1.27) and sham (5.8 ± 2.37) groups while no difference was observed between the control and sham groups ($P > .05$).

Table 2. Comparison of sperm parameters between control, sham and varicocele groups.

Variable ^c	Control	Sham	Varicocele	P-value
Concentration, 10 ⁶ /mL; mean ± SD	115.2 ± 8.28 ^a	99.3 ± 9.49 ^a	62.8 ± 12.84 ^b	< 0.001 *
Motility, %; mean ± SD	85.2 ± 3.70 ^a	78.8 ± 5.51 ^a	42.0 ± 7.03 ^b	.005 **
Abnormal morphology, %; mean ± SD	6.0 ± 1.82 ^a	8.0 ± 1.33 ^a	9.6 ± 1.07 ^b	< 0.001 *
				.04 **
				< 0.001 *
				.012 **

Different letters indicate significant differences between groups at $p < 0.05$.

^c Continuous variables were compared by One-way analysis of variance (ANOVA)

*Varicocele to Control

**Varicocele to Sham

Table 3. Comparison of mean percentage sperm with persistent histone, DNA damage, and lipid peroxidation between groups.

Variable ^c	Control	Sham	Varicocele	P-value
persistent histone, %; mean ± SD	4.2 ± 1.27 ^a	5.8 ± 2.37 ^a	9.1 ± 4.69 ^b	.02 *
DNA damage, %; mean ± SD	40.4 ± 22.55 ^a	39.4 ± 12.45 ^a	52.7 ± 18.77 ^b	.04 **
lipid peroxidation, %; mean ± SD	6.7 ± 4.62 ^a	7.9 ± 5.3 ^a	39.7 ± 4.78 ^b	.02 *
				.03 **
				< 0.001 *
				< 0.001 **

Different letters indicate significant differences between groups at $p < 0.05$.

^c Continuous variables were compared by One-way analysis of variance (ANOVA)

*Varicocele to Control

**Varicocele to Sham

Assessment of DNA damage by acridine orange staining in **Table 3** showed that percentage of sperm DNA damage was significantly ($P < .05$) higher in varicocele induction (52.7 ± 18.77) group compared to control (40.4 ± 22.55) and sham (39.4 ± 12.45) while no difference was observed between the control and sham groups.

Assessment of lipid peroxidation level by BODIPY C11 in **Table 3** showed that the percentage of Bodipy C11 positive spermatozoa for lipid peroxidation was significantly ($P < 0.05$) increased in varicocele group (39.7 ± 4.78) compared to control (6.7 ± 4.62) and sham (7.9 ± 5.3) groups while no difference was observed between the control and sham groups.

Comparison of antioxidant status

The results of testicular antioxidants assessment in this study are shown in **Table 4**. The level of CAT was significantly ($P < .05$) lower in varicocele group (7.24 ± 1.23) compared to control (15.67 ± 3.36) and sham (15.64 ± 3.61) groups while such difference was not observed between the control and sham groups. In addition, the level of SOD was significantly ($P < .05$) lower in varicocele group (0.22 ± 0.03) compared to control (0.5 ± 0.11) and sham (0.55 ± 0.05) groups while such difference was not observed between the control and sham groups. Similar to CAT and SOD, the level of GPX was significantly ($P < .05$) lower in varicocele group (1.89 ± 0.72) compared to control (4.86 ± 0.64) and sham (5.8 ± 0.05) groups while such difference was not observed between the control and sham groups. Furthermore, the level of MDA as a marker of lipid peroxidation was significantly ($P < .05$) higher in varicocele group (0.83 ± 0.15) compared to control (0.26 ± 0.08) and sham (0.23 ± 0.04) groups while such difference was not observed between the control and sham groups.

DISCUSSION

In accordance with the results of the current study and previous studies, increased testicular temperature due to varicocele results in reduction of testis weight and testicular volume and these reductions can be related to loss of germ cells by apoptosis with the high mitotic activity which is highly sensitive to hyperthermia.^(23, 24) Germ cells can incur damage by main mediators such as apoptosis, autophagy and, oxidative stress. In view of the fact that heat stress is involved in the induction of oxidative stress within the testis and the close relationship between heat exposure and ROS generation has been confirmed in various studies.^(15, 25) Therefore, increased lipid peroxidation observed by Bodipy staining and malondialdehyde formation is consistent with the result of this and previous studies.^(9, 26, 27) Over production or under production of these ROS, termed “oxidative” or “reductive” stress, respectively, are both considered to negatively influence normal sperm physiological functions.^(28, 29) However, cells to contest with overproduction of ROS has become highly equipped with different forms of antioxidants.⁽³⁰⁾ SOD, catalase and, GPX are three enzymatic antioxidants which protect cells from oxidative stress. SOD is present both in mitochondria and cytoplasm and its main role is to reduce superoxide, the main ROS produced through cellular oxygen metabolisms to hydrogen peroxide which in turn is reduced to O₂ and water by either catalase or GPX to protect cells from damaging effects of these oxygen species. Therefore, a balance between the oxidant-antioxidant system permits the beneficial oxidants to perform their normal cellular functions and concurrently limits the detrimental effects of excess oxidative stress.⁽³¹⁾ However, the condition gets aggravated in presence of traces of metals (such as iron, copper or zinc), superoxide and hydrogen peroxide which work hand in hand to form hydroxyl radicals (OH⁻) in a reaction called Haber-

Table 4. Comparison mean of CAT, SOD, and GPX activity, and also MDA level between groups

Variable ^c	Control	Sham	Varicocele	P-value
Catalase, μ/mg Protein; mean ± SD	15.67 ± 3.36 ^a	15.64±3.61 ^a	7.24±1.23 ^b	.004 *
SOD, μ/mg Protein; mean ± SD	0.5 ± 0.11 ^a	0.55±0.05 ^a	0.22±0.03 ^b	.002**
GPX, IU/mg Protein; mean ± SD	4.86 ± 0.64 ^a	5.8 ± 0.05 ^a	1.89±0.72 ^b	.019 *
MDA, nmol/mg Protein; mean ± SD	0.26 ± 0.08 ^a	0.23 ± 0.04 ^a	0.83±0.15 ^b	.015 **
				.004 *
				.002 **
				.004 *
				.005 **

Different letters indicate significant differences between groups at $p < 0.05$.

^c Continuous variables were compared by One-way analysis of variance (ANOVA)

*Varicocele to Control

**Varicocele to Sham

Weiss. These radicals are considered to be highly toxic and damaging to cellular functions. An imbalance in the chain of reduction of superoxide, to hydrogen peroxide and consequently to oxygen and water may prone cells to oxidative stress which as stated, the damage becomes intensified in presence of traces of metals.⁽³²⁾ To observed the status of these enzymes in testis of a rat with varicocele, the content or activities of SOD, catalase, and GPX enzymes were assessed in the left testis of these rats and the results were compared with corresponding controls. The results revealed that the antioxidant activity or the content of the three antioxidant enzymes significantly reduced in the varicocele group compared to sham or control groups. Taken together the results of this study concomitant with other studies in the literature^(33,34) demonstrate that the content or the activity of these enzymes (SOD, catalase, and GPX) are reduced in state of varicocele which may account for increased rate of lipid peroxidation assessed by Bodipy and malondialdehyde formation and increased DNA damage assessed by acridine orange staining.

In addition, oxidative stress and elevated temperature hinder the rate of histone/protamine exchange which makes sperm nuclear DNA more sensitivity to ROS damages.⁽³⁵⁻³⁷⁾ Indeed, observed increase in the percentage of aniline blue-positive spermatozoa, indicating the excessive presence of histones, and increased rate of DNA damage strengthens the association between hinder histone/protamine exchange and DNA damage in the state of varicocele which is in line with previous reports.⁽³⁸⁻⁴⁰⁾ To reduce the chance of fertilization by DNA damaged sperm, epididymis has become equipped by a system called "ubiquitin-proteasome system" that remove damaged sperm. Miss-folded or damaged structures and proteins present on the sperm surface are recognized and become ubiquitinated which prone the labeled sperm to phagocytosis by epididymis.⁽⁴¹⁾ The process by which damaged sperm bypass these barriers is called abortive apoptosis and this process appear to be active in the state of varicocele.⁽⁴²⁾ The main reason for ROS associated sperm DNA damage might be the activation of mitochondria in caudal epididymis which derives both ATP and ROS production. Production of the excessive amount of superoxide by mitochondria become converted to hydrogen peroxide by both mitochondrial and cytoplasmic SOD, H₂O₂ can freely leave these structure and become entangled with lipids within and between spermatozoa to induce lipid peroxidation and the formation of lipid adducts such as 4 hydroxynonenal, acrolein, and malondialdehyde. These adducts interact with nucleophilic sensitive proteins especially succinic acid dehydrogenase within the mitochondria which further aggravate ROS production and subsequently induce a vicious cycle for production ROS.⁽³⁶⁾ The vicious cycle of ROS production become detrimental in presence of traces of metal elements and reduce the enzymatic antioxidant capacity of sperm which is intensified in state varicocele, and eventually lead to the execution of apoptosis and cell death. Therefore, increase rate of malondialdehyde formation and lipid peroxidation, reduced enzymatic antioxidant capacity in the state of varicocele in our model, intensifies the possible activity of this vicious cycle of ROS production in the state of varicocele. Lipids, in the form of polyunsaturated fatty acids (PUFAs), are considered as a central part of the fluidity of membrane layers, es-

pecially in sperm as a highly motile cell.⁽³⁶⁾ Approximately 60% of the fatty acid is lost from the membrane while the lipid peroxidation cascade occurs in the sperm and affects sperm function by reduction of its fluidity, mobility, increasing non-specific permeability to ions, and inactivating membrane-bound receptors and enzymes.⁽¹²⁾ Therefore, increased in LPO assessment by BODIPY and MDA formation (**Table 3**) may account for the notable decrease in motility due to alterations in the membrane and also increases of sperm DNA fragmentation.

So judging by the recent studies and our results, the relationship between varicocele and OS are proven by detecting the higher ROS and lipid peroxidation product like MDA in varicocele status.^(15,26) Moreover, the negative effects of lipid peroxidation in membrane structure can influence the ability of spermatozoa to take place in the membrane fusion events relevant to fertilization. Overall, there is evidence in the literature that defective sperm function is commonly induced by oxidative stress, affect sperm motility by lipid peroxidation, DNA integrity by base oxidation and capability for sperm-oocyte to fuse by the formation of adducts.^(36, 43)

In conclusion based on literature background study, varicocele through different route including testicular hypoxia, increase of scrotal temperature and reflex of metabolites can lead to elevated levels of ROS which through lipid peroxidation, DNA fragmentation and induction of apoptosis can as well as reduced antioxidant capacity impair sperm functional characteristics, seminal parameters and consequently decrease of male reproductive potential. Therefore, concomitant assessment of these parameters may provide more insight in mechanism involved in varicocele male infertility and may help the specialist in this filed to provide therapeutic approach based on molecular mechanism defined in these models.

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CONFLICT ON INTEREST

The authors have no conflict of interest or financial disclosures to declare.

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