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An invited review following *the Soujinkai Fujiu Memorial Award*: Involvement of Oxidative Stress in The Pathophysiology of Varicocele

Koji Shiraishi

Department of Urology, Yamaguchi University School of Medicine, 1-1-1 Minami-Kogushi, Ube, Yamaguchi 755-8505, Japan
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Correspondence to Koji Shiraishi, MD, PhD. E-mail: shirak@yamaguchi-u.ac.jp

Abstract A varicocele is an abnormal dilatation of the pampiniform plexus within the spermatic cord and is the most common and treatable cause of male infertility, with a variety of etiologies. Varicocelectomy increases the fertilization, pregnancy and live birth rates, indicating improved sperm function; it is therefore important even in couples undergoing intracytoplasmic sperm injection. However, despite ongoing extensive research on varicoceles, the exact mechanisms by which varicocele influences male infertility is not fully understood. Recent studies have shown that infertile men with varicocele have higher levels of oxidative stress (OS), and/or lower seminal antioxidant levels, than do fertile men and infertile men without varicocele. Heat stress has been shown as one of the most possible causes. The abnormally high levels of seminal OS biomarkers in infertile men with varicocele is clinically relevant as these markers have been associated with poor sperm function and reduced fertility potential. In addition, infertile patients with varicocele possess high levels of sperm DNA damage is believed to be at least in part due to OS. The observed improvement in seminal OS, spermatogenesis and sperm DNA damage after varicocelecotmy supports the premise that varicocele can induce seminal OS.

Key words: Varicocele, male infertility, oxidative stress

Introduction

A varicocele is an abnormal dilatation of the pampiniform plexus within the spermatic cord and is the most common surgically treatable cause of male infertility. Varicocele is found in approximately 15-20% of healthy men, but the prevalence is extremely high, up to 40%, in men with abnormal semen parameters. Several hypotheses on the pathophysiology of varicocele have been proposed, including endocrine and testicular paracrine disturbances, heat stress, hypoxia, oxidative stress (OS), accumulation of toxic substances, genetic disturbances, and autoimmunity, leading to decreased proliferation of germ cells and apoptosis and also development of interstitial lesions. Autophagic cell death has been shown to play a role, at least in part, in the impairment of spermatogenesis.[1] In 1980's, retrograde flow of adrenal metabolites, such as, catecholamines cortisol, prostaglandins E and F, and serotonin had been considered to be causable factors, but these metabolites do not appear to be associated with the effects of varicocele. The mechanisms to disturb the spermatogenesis is multifactorial, in other words, combinations of several factors affects spermatogenesis and sperm function, and the relative involvement of these factors is different in each patient. On the other hand, a majority of men with varicocele are infertile. Varicocele accelerates spermatogenic injury along with other

underlying cofactors and/or intrinsic testicular under development. Several studies of human seminal plasma and sperm have shown the involvement of oxidative stress (OS), the most plausible factor impairing spermatogenesis and sperm function. In the present review, we summarize the intratesticular and seminal events, especially regarding to the association between varicocele, oxidative stress, and sperm DNA damage, on the basis of our studies of men with varicocele.

Varicocele and oxidative stress

The exact mechanisms by which varicocele induces testicular failure and sperm dysfunction is still under debate; however, OS seems to play a principal role in reproductive dysfunction. In addition, the oxidative cellular and molecular pathways, which may be abnormally activated in men with varicocele, are not completely understood.

Seminal OS results from an imbalance between reactive oxygen species (ROS) production (superoxide anions, hydrogen peroxide, hydroxyl radical, and nitric oxide) and ROS scavenging by seminal antioxidants (superoxide dismutase, glutathione peroxidase, catalase, uric acid, vitamins C and E, and albumin). Alternative of the reflux of toxic substance through internal spermatic vein, oxidative stress has been the most investigated factor involved in the pathophysiology of varicocele in the past 10 years. Because of the high cellular turnover in normal spermatogenesis, ROS and reactive nitrogen species (RNS), such as superoxide, hydroxyl, peroxyl, hydroperoxyl, NO and nitrogen dioxide, which are produced by the peroxidation and oxidation of many cellular lipids, proteins, carbohydrates and nucleic acids, are generated. OS is essential to maintain cellular homeostasis, but excess oxidative stress leads to cellular dysfunction. Agarwal *et al.* reported that the level of seminal ROS correlates with the varicocele grade in men with varicocele.[2] Specifically, men with grade 2 and 3 varicocele have greater levels of ROS in the seminal plasma than men with grade 1 varicocele.[3] Furthermore, we have shown that the effectiveness of varicocelectomy greatly depends on the preoperative testicular oxidative stress level.[4] If OS has only a minor role in the pathophysiology of varicocele, no association should exist between the outcomes of varicocelectomy and the level of testicular oxidative stress.

When we consider the pathophysiology of varicocele, it is easy to understand that the events in the testis and semen are completely different stories. Investigation using semen sample is easy to perform whereas obtaining human testicular tissues is extremely difficult, inhibiting the promotion of investigations in this field. The plasma membrane of testicular cells is rich in polyunsaturated fatty acids, and is therefore vulnerable to OS. Koksal *et al.* reported that the levels of lipid peroxidation and malondialdehyde (MDA) increase depending on the varicocele grade in men with varicocele.[5,6] One possible mechanisms is the compensatory release of nitric oxide (NO), in response to testicular venous congestion, with associated elevation in MDA that together contribute to excessive lipid peroxidation and defective spermatogenesis.[7,8] We focused on a specific and stable end product of lipid peroxidation, the aldehyde 4-HNE, which can diffuse within, or even escape from, the cells and attack targets far from the site of the original free radical event. It is a potent alkylating agent that reacts with DNA and proteins, generating various forms of adducts (cysteine, lysine and histidine residues) capable of inducing specific cellular stress responses, such as signaling and apoptosis.[9] Analysis of testicular biopsy samples has shown that the expressions of 4-HNE-modified proteins increase with the varicocele grade and correlate with increasing patient age, evidencing the progressive effects of varicocele.[10] Localization of 4-HNE-modified proteins have been observed in almost all the testicular components, especially in Sertoli cells and spermatocytes. Testicular 4-HNE-modified p53 expression is also greater in men with varicocele than in fertile men.[11] Another investigator has shown increased expression of 8-hydroxy-2′-deoxyguanosine (8-OHdG), a product of oxidative DNA damage, in the testicular tissue of men with varicocele, which localized mainly in spermatocytes.[12] Generally cadmium deposition in the testes induces OS and apoptosis by induction of the Fas ligand to trigger apoptosis; by

indirect generation of the hydroxyl radical, $O2^-$, hydrogen peroxide and/or NO; and/or by decreased concentration, which acts as an anti-oxidant. Benoff *et al.* reported elevated apoptosis levels and cadmium accumulation in the bilateral testes of infertile men with left varicocele.[13] Cadmium could also affect sperm quality directly by affecting spermatogenesis or indirectly by increasing oxidative stress. As direct action of cadmium in testicular components, increased cadmium concentration could disrupt Sertoli-cell actin, block spermiation, disrupts membrane integrity by causing loss of sperm-head actin and damage the actin cytoskeleton. To evaluate testicular oxidative stress, stable products generated by oxidative stress (e.g. MDA, 4-HNE-modified proteins, 8-OHdG and nitrite/nitrate) have been used as surrogate markers. In an experimental left varicocele (ELV)-based study, testicular $O2^-$ was found to be threefold higher in the ELV group than in the control group.[14]

Another hypothesis is cytokine-mediated oxidative stress. The expression of IL-1 and IL-6, both potent proinflammatory activators, has been shown to be upregulated in experimental and clinical varicocele, respectively.[8,15] In many testicular disorders causing male infertility, extracellular ROS are produced by leukocytes, mainly neutrophils. Leukocytes are normally present in the prostate and seminal vesicles, and are, at least in part, involved in OS in the ejaculates. However, given that few inflammatory cells are observable in testes with varicocele, heat stress and tissue hypoxia, toxic substances can be considered the major sources of OS in men with varicocele. Infiltration of mast cells might also be partly involved in the generation of oxidative stress in such patients.[16]

Fertile men with and without varicocele have comparable testicular volume and oxidative stress, indicating increased ROS production or impaired ROS-scavenging activity in infertile men with varicocele.[17] The testes contain several anti-oxidants that protect germ cells from oxidative damage. Two types of mechanisms are responsible for such protection: enzymatic and nonenzymatic. The testicular antioxidant enzymes in mammals are SOD, GSR, GPX, GST, catalase and HO-1.[18,19] Germ cells, in general, do not appear to use the enzymatic antioxidant system as the major defense against ROS damage, and are consequently more susceptible to oxidative stress than somatic cells.[18] A reduced expression of HO-isoenzyme 1 may result in an increased vulnerability of testicular germ cell to oxidative injury in the presence of varicocele.[10]

Heat induces oxidative stress in the testis

Heat stress is the most plausible cause of the impairment of spermatogenesis in men with varicocele, and has been investigated since 1941.[20] Spermatogenesis is temperature sensitive,[21,22] and proceeds optimally at approximately 36℃ in men. The internal spermatic artery (surrounded by the pampiniform plexus) maintains the testes at 35-36℃, 1-2℃ lower than the core temperature, by the countercurrent heat-exchange system. The involvement of heat stress on pathophysiology of varicocele is one of the most causable factors and OS is provoked by heat stress. *In vitro* and *in vivo* experiments suggest that heat stress usually produces ROS, such as superoxide radicals, hydroxyl radicals, and hydrogen peroxide. The generation of ROS occurs constantly, even under physiological conditions, in all living cells, and the rate of free radical generation in the testis or spermatozoa appears to be temperature dependent.[23] For example, the level of spontaneous lipid peroxidation by cultured mouse spermatozoa, as measured by the generation of MDA, increase with temperature elevation.[24] In addition, activities of several scavenging enzymes in the testes of rats with experimentally induced cryptorchidism were impaired and were accompanied by increased peroxidation of cellular lipids,[25-27] indicating that testicular oxidative stress is determined by the balance between the generation of ROS and their scavenging systems. Ultimately heat induce cell fate is categorized in Figure 1. The common cell fates are apoptosis and cell cycle arrest of the germ cells. Autophagy[1] and thermotolerance are indicated as possible mechanisms, however, these phenomena have not fully investigated. Necrosis of the testicular components is merely seen in testis with varicocele. Effects of heat stress on cellular components are listed as follows: Cell

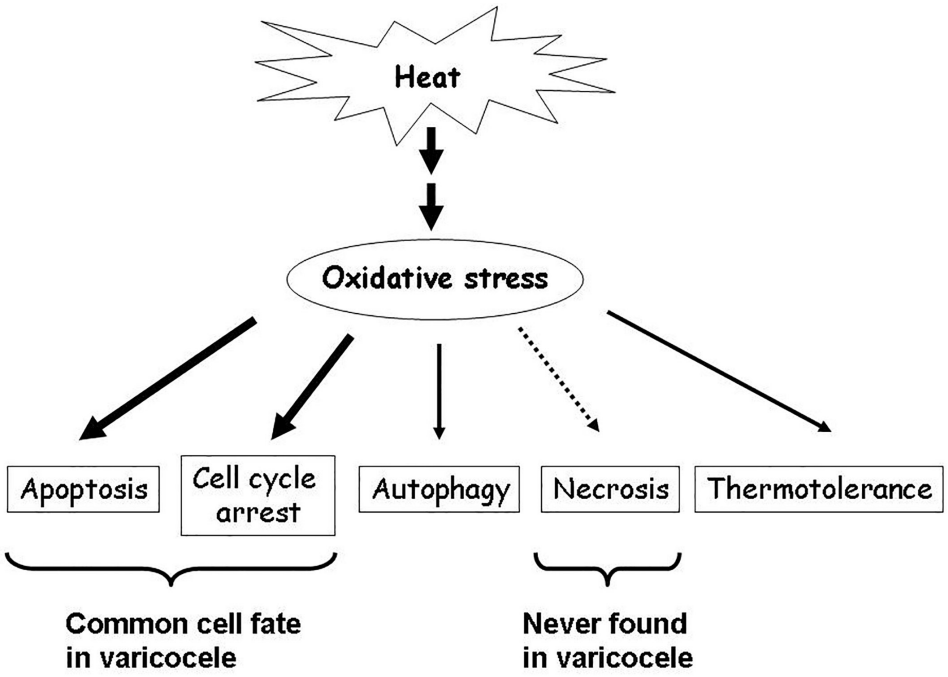

Fig. 1
Behavioral patterns of testicular cells, mainly germ cells, exposed to heat-induced oxidative stress. Thin lines indicate possible pathways. These phenomena usually occur concurrently but not alone.

membrane—changes in fluidity/stability, alteration in structure, impairment of ion transport, and modulation of the transmembrane efflux pump; cytoplasm—impairment of protein synthesis, denaturation of protein structure and function, aggregation of proteins, and induction of heat shock protein synthesis; mitochondria—depolarization of mitochondrial membrane potential, depletion of ATP production, production of ROS, and disruption of Ca^{2+} transport across the mitochondrial membrane; endoplasmic reticulum (ER)—ER stress from excessive accumulation of misfolded proteins; nucleus—impairment of DNA synthesis, inhibition of DNA repair enzymes, alteration of DNA conformation, and changes in gene expression and signal transduction. In particular, plasma membranes are known to be extremely sensitive to heat stress because of their complex molecular composition of lipids and proteins. Upon temperature elevation, the physical state of lipids change from a tightly packed gel to a less tightly packed crystalline structure, and the permeability of the cell membrane increases, followed by alteration of the cellular content of several ions (Na^+, Mg^{2+}, K^+, and Ca^{2+}). For example, influxes of extracellular Ca^{2+} stimulates the activity of calmodulin-dependent protein kinases and inositol triphosphate production,[28] resulting in the alteration of intracellular signal transduction cascades. These changes are universal effects observed in all mammalian cells and likely occur in the germ cells. The plasma membrane of testicular cells is rich in polyunsaturated fatty acids and is therefore vulnerable to oxidation by H_2O_2 and other ROS.

Sperm DNA damage and effect of varicocceletomy on infertility treatment

Sperms of infertile men possess substantially more chromatin defects and DNA damage than those of fertile men, suggesting that sperm DNA damage is predictive of male fertility potential.[29-31] The etiology of sperm DNA damage is multifactorial and many studies have proposed that OS can result in sperm DNA damage.[32] Varicocelectomy improves pregnancy and live birth rates even after assisted reproduction technologies (ART) such as *in vitro* fertilization (IVF) or

intracytoplasmic sperm injection (ICSI) but not natural pregnancy or assisted insemination (Table). To explain this phenomenon, two published prospective studies of varicocelectomy on sperm DNA damage demonstrates that varicocele repair is associated with a significant decrease in sperm DNA damage.[33,34] These data further support the premise that varicocele reduces testicular OS if we consider that the sperm DNA damage in these men is likely due to OS. As discussed earlier, seminal ROS levels are higher in semen samples from infertile men with varicocele,[33,35,36] including men with normozoospermia,[35,37] than in controls and there are now several reports showing that varicocelectomy is generally associated with a reduction in seminal OS. However, the effect of varicocelecotmy on seminal antioxidant capacity and seminal antioxidant enzyme activity (e.g., SOD) is mixed with some studies reporting lower and others higher antioxidant activity after varicocelectomy.[38-43] Nevertheless, the true effect of varicocelectomy on seminal OS is not proven as most of the studies are retrospective and none are randomized, controlled trials.

Mostafa *et al.*[38] reported a significant reduction in seminal plasma oxidants (NO, H_2O_2, MDA) and a significant increase in seminal plasma antioxidant levels (SOD, catalase, glutathione peroxidase, and vitamin C) at 3 and 6 months after varicocele repair. Hurtado de Catalfo *et al.*[40] demonstrated a shift in the seminal glutathione status (with increased reduced glutathione, GSH and decreased oxidized glutathione, GSSG), an increase in the levels of nonenzymatic antioxidants (Zn, Se), and a paradoxical decrease in the antioxidant enzyme levels (to levels comparable to that of fertile controls) as early as 1 month after varicocele repair. Chen *et al* evaluated the effect of varicocelecotmy on sperm mitochondrial DNA deletions, DNA oxidation (8-OHdG), and seminal plasma protein thiols and ascorbic acid levels at 6 months after varicocelecotmy compared to preoperatively.[42] Sakamoto *et al.* evaluated the effect of varicocelectomy on markers of seminal OS (nitric oxide, 8-OHdG, hexanoyl-lysine) and seminal SOD activity and reported lower levels of semen OS markers levels and lower seminal SOD activity after surgery. In contrast to otherwise largely positive studies, Mancini *et al.* evaluated the effect of varicocelectomy on seminal plasma total antioxidative capacity (TAC) and reported no significant reduction in seminal TAC after varicocele repair.[39] One report evaluated the effect of varicocelectomy on peripheral blood plasma OS markers (C-reactive protein, thiobarbituric acid reactive substances: TBARS, and plasma lipid peroxidation susceptibility) in adolescents with

Table ICSI outcomes in infertile couples in whom the male partner had treated or untreated clinical varicocele

		With varicocelectomy	Without varicocelectomy	p-value
Esteves et al. (2010)		(n=80)	(n=162)	
_	% 2PN fertilization	78.0	66.0	0.04
_	% clinical pregnancy	60.0	45.0	0.04
_	% live birth	46.2	31.4	0.03
Pasqualotto et al. (2011)		(n=169)	(n=79)	
_	% 2PN fertilization	64.9	73.2	0.04
_	No. clinical pregnancy	30.9	31.1	0.98
Shiraishi et al. (2012)		(n=21)	(n=53)	
_	% 2PN fertilization	70.3	68.8	0.93
_	% clinical pregnancy	61.9	28.3	0.02
_	% live birth	52.3	24.5	0.04
Gokce et al. (2013)		(n=105)	(n=65)	
_	% clinical pregnancy	62.5	47.1	0.001
_	% live birth	47.6	29.0	0.0002

varicocele and ipsilateral testicular hypotrophy.[41] They reported higher levels of TBARS and a higher mean plasma peroxidation susceptibility in adolescents with varicocele compared to controls. They also observed a significant reduction in plasma TBARS levels and in the plasma peroxidation susceptibility 1 year after varicocelectomy. Regarding to the events in testis, we examined the effect of varicocelecotmy on the levels of testicular 4-HNE-modified proteins, an OS marker. We found higher levels of testicular 4-HNE-modified proteins in those men who responded to varicocelectomy suggesting that varicocelectomy reduces OS in those infertile men who exhibit a high level of baseline testicular OS and that varicocelectomy is not as effective in men without OS.[4] These findings also support that OS plays a major role in the pathophysiology of varicocele.

Conclusion

OS has been shown as the most causable factor to deteriorate spermatogenesis in men with varicocele, however, the involvement might differ among each patient. We need to evaluate how OS is involved in the deterioration of spermatogenesis in each patients. Then, we will be able to establish etiology-based managements (i.e., varicocelectomy, anti-oxidant therapy, testicular cooling etc.) (Fig. 2).

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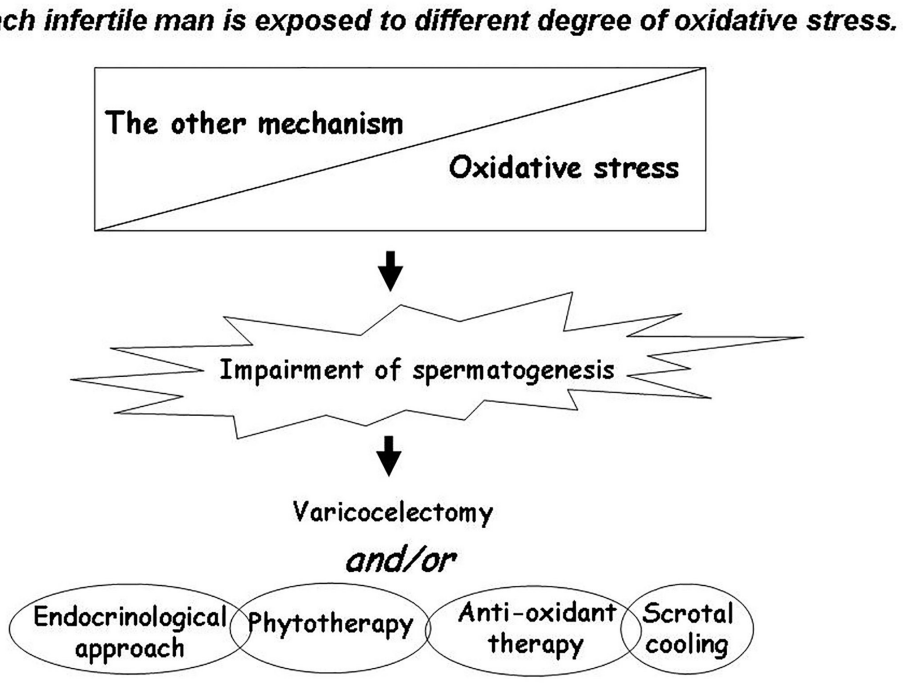

Fig. 2
Relationships between the etiologies of varicocele and the involvement of oxidative stress. Note that various etiologies are overlapped in each patient. Among them, oxidative stress plays a major role in causing the impairment of spermatogenesis by heat and hypoxic stress. Current established management for varicocele is varicocelectomy, but etiology-based management is carried out if the pathophysiology of each patient can be evaluated.

technologies.

Conflict of Interest

The author states no conflict of interest.

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