

Review Article

Oxidative Stress, Apoptosis and Varicocele-Related Infertility

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Citation: Bolat D (2018) Oxidative Stress, Apoptosis and Varicocele-Related Infertility. J Urol Ren Dis: JURD-193. DOI: 10.29011/2575-7903.000093

Received Date: 14 May, 2018; **Accepted Date:** 25 May, 2018; **Published Date:** 08 June, 2018

Abstract

Varicocele is the most commonly seen and correctable cause of male factor infertility. The relationship between varicocele and infertility has not been fully elucidated due to the unclear pathophysiology of varicocele. Several hypotheses on the impairment of spermatogenesis have been proposed, including endocrine and testicular paracrine disturbances, increased temperature and heat stress, testicular hypoxia, oxidative stress, accumulation of toxic substances, genetic disturbances, and autoimmunity, leading to decreased proliferation of germ cells and apoptosis. In this review, apoptotic mechanism of the varicocele-related infertility was discussed.

Keywords: Apoptosis; Hypoxia; Germ Cell; Infertility; Pathophysiology; Oxygen Species; Oxidative Stress; Reactive Varicocele

Introduction

Infertility is defined as the inability of a sexually active, non-contracepting couple to achieve spontaneous pregnancy in one year [1]. About 15% of couples do not achieve pregnancy within one year and seek medical treatment for infertility [2]. In 50% of involuntarily childless couples, a male-infertility-associated factor is found together with abnormal semen parameters [1]. Varicocele is the most commonly seen and correctable cause of male factor infertility [3]. Varicoceles have an incidence of 4.4-22.6% in the general population, 21-41% in men with primary infertility, and 75-81% in men with secondary infertility [4,5]. Despite the fact that, the relationship between varicocele and infertility has not been fully elucidated. All patients with varicocele are not infertile and all patients who undergo varicocelectomy do not become fertile. This explains why nearly two-thirds of men with varicocele remain fertile, and why controversy continues to surround the clinical utility of varicocele treatment [6].

Although it has been known for many years that varicocele may impair spermatogenesis [5], this information is not enough to explain the association between varicocele and infertility [7]. According to the comprehensive reviews published in the past 10 years regarding the pathophysiology of varicocele, several

hypotheses on the impairment of spermatogenesis have been proposed, including endocrine and testicular paracrine disturbances, increased temperature and heat stress, testicular hypoxia, oxidative stress, accumulation of toxic substances, genetic disturbances, and autoimmunity, leading to decreased proliferation of germ cells and apoptosis [8]. Varicocele is also associated with increased sperm DNA damage, and this sperm pathology may be secondary to varicocele mediated oxidative stress [8].

Effects of Varicocele on Testicular Morphology

The high incidence of varicocele among infertile men is usually associated with histological changes, testicular hypotrophy and impairment in semen parameters [9,10]. Varicocele-bearing testis showed depressed spermatogenesis with a predominant picture of maturation arrest, sloughing, increased Leydig cells, thickened tubular basement membranes and interstitial blood vessel walls with narrowing with their lumina, and increased deposition of interstitial fibrous tissue [11]. In addition, varicocele-bearing testis had significantly more peritubular fibrosis and tubular hyalinisation [12]. Previous studies have suggested that altered germ cell apoptosis may have a role in varicocele related infertility in human [13-15]. It has been established that germinal epithelium is highly sensitive to various noxious stimuli, responding with an increase in germ cell apoptosis. Factors such as increased temperature [16], cryptorchidism [17], exposure to radiation and chemotherapeutic agents [18], and androgen deprivation trigger increased germ cell apoptosis within the testis.

Hypoxia And Varicocele

Previous studies of experimental varicocele models in rats documented that increased hypoxia inducible factor-1 α and germ cell apoptosis in testes [19,20]. This means that varicocele can lead to testicular tissue hypoxia and cause germ cell apoptosis [21-23]. It has been theorized that abnormally high levels of germ cell apoptosis may contribute to testicular failure and male infertility [5,19]. The hypoxia is thought to be caused by increased hydrostatic pressure in the venous drainage system, which exceeds the pressure in the testicular arterial microcirculation due to venous stasis produced in the disease [24,25]. Wang et al. detected the higher levels of HIF-1 α expression, which is an intrinsic marker of tissue hypoxia [26] in the right and left testes of rats with left-sided varicoceles than sham and control groups. Additionally, they showed that HIF-1 α expression in the right and left testes of rats in the experimental group did not differ [24].

HIF-1 α is constitutively expressed and stabilized in the testis, where it may play a role in testicular homeostasis. Additionally, it may affect male reproductive function by regulating Hsd3b1 transcription [27]. Under hypoxic conditions, stabilized HIF-1 α can activate the transcription of several genes, including angiogenic factors (vascular endothelial growth-factor), growth factors (insulin-like growth-factor-II), glucose transporters (GLUT-1), and glycolytic enzymes (Aldolase A/C). Thus, HIF-1 α appears to play an important role in protecting solid tumors from hypoxia by promoting angiogenesis, inducing the expression of growth factors, preventing apoptosis, or increasing anaerobic metabolism [28]. Kilinc, et al. documented increased VEGF expression, angiogenesis, and HIF-1 α in the testicular tissue of rats with varicocele [29]. This means that HIF-1 α is beneficial for tissue survival under hypoxic conditions, which are induced by the presence of varicocele. Wang et al. showed that there was a positive correlation between the apoptotic index of germ cells and the relative intensity of HIF-1 α staining in the bilateral testes of rats in the experimental group. This indicated that, under conditions of testicular hypoxia induced by varicocele, HIF-1 α promoted germ cell apoptosis [24]. Manfred and Reet reported that HIF-1 α expression was found to be positively correlated with the apoptotic rate of tumor cells and the presence of pro-apoptotic factors including caspase-3, Fas, and Fas ligand [30]. Thus, HIF-1 α can promote both cell survival and apoptosis under hypoxic conditions, and the dominant effect that is exerted by HIF-1 α depends on the type of tissues and degree of hypoxia. Wang et al. showed that, under the hypoxic conditions induced by varicocele in testicular tissue, HIF-1 α 's primary effect was harmful rather than beneficial, as it was found to be associated with increased rates of apoptosis [24]. In addition, Reactive Oxygen Species (ROS), which can be created during an intermediate event between hypoxia and the induction of apoptosis, also play an important role in apoptosis induced by hypoxia. ROS are oxygen metabolites, and include

superoxide anions, hydrogen peroxide, hydroxyl radical, and nitric oxide. Excess production of ROS could damage spermatozoa by inducing oxidative stress and subsequently damaging cellular lipids, proteins and DNA [31,32].

The dilated and thickened Internal Spermatic Vein (ISV) wall in varicocele was defined similar to that of varicose veins. The venous stasis due to blood stagnation has involved in the development of varicose veins as poor venous return of varicocele [33,34]. Studies on varicose veins showed that many neutrophils adhered to the endothelium of vein incubated in hypoxic conditions rather than in normoxia [34]. Hypoxia-activated endothelial cells secreted growth factors which triggered proliferation of smooth muscle cells [35-38]. Hypoxia was also a stimulus to vascular smooth muscle cell proliferation that occurred in a rat aorta via increasing fms-like tyrosine kinase (Flt-1) activity [39]. Higher HIF-1 α expression in the ISV of patients with varicocele was reported recently, which indicated that hypoxia-related pathophysiologic changes have occurred in the ISV of patients with varicocele [40]. In low or zero oxygen concentrations, mammalian cells undergo cell death through apoptosis, but not necrosis [41]. Apoptotic signaling during oxygen deprivation occurs through the release of cytochrome c and Apaf-1 mediated caspase-9 activation leading to cell apoptosis [41,42].

Hence, hypoxia might be one of the factors responsible for Bcl-2 (anti-apoptotic protein) regulation, as Bcl-2 protein expression was increased in different cells under hypoxic conditions for survival and proliferation [41,43-47]. Lee, et al. demonstrated that Bcl-2 expression in the varicocele group increased significantly compared to that in control group. Bcl-2 overexpression may decrease vascular cell apoptosis in the hypoxic condition and lead to vascular cell proliferation (predominant in muscular layer) causing dilated and thickened ISV wall in patients with varicocele [33].

Reactive Oxygen Species, Oxidative Stress and Varicocele

It was shown that ROS can initiate apoptosis [48]. At molecular level, ROS would directly affect DNA, and also alter intracellular Ca²⁺ levels which is shown to be one of the most powerful ways of inducing apoptosis. Moreover, O₂ alone was shown to stimulate apoptosis in several studies [49]. Cam et al. demonstrated that vitamin E administration lowered O₂ and also apoptosis [50]. Although pathophysiology of the varicocele has not yet been completely determined, elevated testicular temperature or exposure to increased blood flow in the testes was shown to increase Reactive Oxygen Species (ROS) and to suppress the activity of antioxidant enzymes [51,52]. Ikeda et al. investigated the role of ROS in testicular germ cell apoptosis induced by heat stress, and administered the radical oxygen molecules exogenously to culture media containing testicular cells isolated from immature

rats [52]. Treatment with catalase, an antioxidant defense enzyme, significantly attenuated heat or stress-induced apoptosis in their study [52]. Similarly, a role for oxidative stress in the induction of apoptosis was also shown in studies in which the addition of low levels of ROS induced apoptosis, and the observation that various antioxidants such as N-acetylcysteine can inhibit cell death [53,54]. Semercioz et al. showed the relationship between experimental varicocele and degenerative changes in the germinal epithelium as well as atrophy of the seminiferous tubules in rats, and revealed the role of reactive nitrogen species and ROS in the disease status as well as the protective effects of melatonin as an antioxidant [55]. Further assessment of the protective effects of other antioxidants on varicocele rats revealed that exogenously supplied taurine, catalase, or SOD led to significant protection of the testicular functions and epididymal sperm maturation [56]. These results support the potential role of ROS and antioxidants in the pathophysiology of heat induced apoptosis.

The role of apoptosis, also referred to as programmed cell death, in male infertility has also received a good deal of recent attention. First described in 1972 by Kerr et al., apoptosis is a process by which a specific and highly regulated cascade of intracellular events leads to involution and elimination of a cell from its environment [57]. Apoptosis is a normal physiological phenomenon in most tissues and it serves a critical regulatory role. It has been observed that apoptosis is an important aspect of normal spermatogenesis. The overall process of spermatogenesis involves 8 sequential steps in the human and 14 steps in the rat [58]. Sertoli's cells, which are the supportive cells in the seminiferous epithelium, facilitate spermatogenesis by providing structural and nutritional support to germ cells. As germ cells mature, they migrate within the Sertoli's cell cytoplasm from the basement membrane of the seminiferous tubules to the adluminal compartment. The rate of this development is constant, synchronous and species specific. Notably spermatogenesis is accompanied by extensive germ cell apoptosis. This cellular attrition appears to be regulated by multiple signals and it may reflect adjustment in the number of germ cells to what can be maintained by available Sertoli's cells [59].

It has been theorized that abnormally high levels of germ cell apoptosis may contribute to testicular failure and male infertility. Increased apoptosis is found in all of the following conditions: hormonal insufficiency, cryptorchidism, and increase in blood flow to the testis, local temperature increase in testis, and hypoxia due to venous stasis [14,60]. High apoptotic activity has been found in testicular dysfunction, and it has been reported that deterioration of spermatogenesis and hypospermatogenesis may be related to uncontrolled apoptosis [14]. Lin et al. provided support for this hypothesis when they noted increased germ cell apoptosis in biopsy specimens from the testes of men with idiopathic testicular failure and they suggested the hypothesis that the increase of apoptotic germ cells might be the cause of testicular dysfunction

and infertility [61]. To evaluate the possible relationship between varicocele and germ cell apoptosis Baccetti et al. quantified the percent of apoptotic sperm cells found in the ejaculate of men noted to have varicocele [62]. They found that such individuals had a significant increase in apoptotic sperm cells compared with fertile controls. In a study done by Simşek and coworkers, testicular biopsies were obtained from patients undergoing varicocelectomy and from healthy males. They proposed that apoptosis should also be expected in patients with varicocele, and determined the mean number of apoptotic cells in varicocele patients to be seven times higher than in a control group [63]. Lue, et al. [64] demonstrated that a single, transient testicular hyperthermia (43° for 15 min) induces the activation of germ cell apoptosis. Onur et al. assessed the Bax/Bcl-2 ratio in order to evaluate the effects of experimental varicocele, and showed a significantly increased expression of pro-apoptotic Bax protein in experimental left varicocele created group [65].

Cam et al. observed that the increase in testicular tissues of varicocele group was four times of the control group in their experimental varicocele model, which was statistically significant [50]. Conversely Fujisawa et al. noted a decrease in the number of apoptotic germ cells found in testis biopsy material from subfertile men with varicocele compared with biopsies from normal men [14]. Although such results appear contradictory, various factors, including patient clinical status, apoptosis quantifying methodologies and the cell type assessed (ejaculatory sperm versus biopsy material) almost certainly had a role.

Apoptosis and Varicocele

Programmed cell death is a process by which developmental or environmental stimuli activate a genetic programme to implement specific series of events that culminate in the death and efficient disposal of a cell [9, 66,67]. Apoptosis of germ cells is required for normal spermatogenesis through highly conserved events following death of the neighbouring cells. It also functions in diverse processes including removal of abnormal or superfluous cells at specific checkpoints establishment of individualisation of gametes [14]. Apoptotic cell morphology has certain microscopic criteria of cytoplasmic and nuclear condensation, fragmentation and formation of apoptotic bodies phagocytosed by adjacent cells that could be demonstrated by in situ end labeling technique. These changes are associated with intracellular mechanism such as intranucleosomal DNA cleavage [68-70]. Apoptosis has been observed in testicular germ cells as a factor in regulating sperm production suggesting that spermatogenic dysfunction may be related to abnormal control of apoptosis [71,72]. It has been shown that in men with abnormal sperm parameters, nuclear DNA damage in mature spermatozoa may be attributed to apoptosis [72].

Apoptosis is often a genetically encoded process of self-destruction. However, for the initiation of this destructive pathway, several physiological and non-physiological stimuli are required.

Elevated temperature of the testis due to various disease, such as varicocele, was reported to be one of the causes of increased programmed cellular death [56].

Apoptotic Mechanisms In Varicocele

First, the proven deterioration of DNA synthesis and deficiency of essential enzymes involved in its metabolism and nuclear instability in varicocele-associated conditions [71,72]. Second, testicular hyperthermia including germ cell loss by apoptosis that was proved experimentally [73]. Third, FSH hormone and testicular androgen disturbance were aroused as regulating factors of germ-cell apoptosis [74]. Fourth, the release of apoptotic activating factors like cytokines due to disturbed blood-testis barrier [75]. Fifth, increased apoptosis of epididymal epithelium affecting the secretory function of the epididymis [76]. Sixth, increased caspase-3 protein expression in germ cells and Bcl-2 overexpression in the ISV of patients with varicocele may be one of the molecular mechanisms related to excessive testicular germ cell apoptosis in varicocele-associated testis [77].

Basically, there are many pathways leading to apoptosis, and these processes seem to be regulated at three levels. At the cell membrane, there are specific membrane receptors mediating death signals of the tumor necrosis factor receptor family known as Fas and Fas-ligand. At the cytoplasmic level, signal transduction pathways involving cysteine proteases called caspases are also involved. Finally, at the nuclear level, specific apoptotic regulatory genes including p53 and Bcl-2 also exert regulatory effects on apoptosis [78]. Briefly, two major pathways can induce apoptotic cell death: the intrinsic (or mitochondrial) pathway is involved Bcl-2 and caspase-9 protein; and the extrinsic (or death receptor) pathway is involved Fas and caspase-8 protein [79-81]. In the human apoptotic pathway cascade, 14 caspases (cysteine aspartate-specific proteinases) have been found to date. Among them, caspase-3 is considered to be a major executioner protease because it is essential for apoptotic death in mammalian cells [82].

Lee et al. showed down-regulation of Bcl-2 expression and higher expressions of caspase-9 and activated caspase-3 in the ipsilateral testis of varicocele group than in the control group at 8 and 12 weeks, respectively, after varicocele creation [19]. These findings demonstrate that testicular tissue apoptosis increases through the intrinsic pathway in rats with experimental left varicocele and the spermatogonia were more sensitive to hypoxia causing apoptosis proved by TUNEL and IHC stains of activated caspase-3. The same apoptotic proteins (caspase-9 and activated caspase-3) were increased in expression in the contralateral testis of varicocele rats at 12 weeks after operation, and had a statistical difference of activated caspase-3 expression in varicocele group than control group [19]. But these results were not compatible with the some previous studies about apoptosis related proteins in infertile men with varicocele [14,83-85]. In several respects, the

animal model has some differences from the clinical varicocele of humans. In another study, Lee et al. investigated expression of Bcl-2, Fas, caspase-8 and caspase-9 in the Internal Spermatic Vein (ISV) of the patients with varicocele and they showed overexpression of Bcl-2 and downregulation of caspase-9 expression in the ISV under hypoxic stress [86]. The oncogenic properties of Bcl-2 have been attributed mainly to its ability to inhibit apoptosis by interfering with the activation of the cytochrome c/Apaf-1 (apoptotic protease activating factor-1; apoptosome) pathway, through stabilization of the mitochondrial outer membrane [87]. The presence of these enzymes stimulates caspase-9 activation, leading to caspase-3 execution of cell apoptosis [35,87]. Previous studies showed that testicular damage appears progressive and generally observed on both testis even with unilateral varicocele because two factors of cadmium and hypoxia could damage the blood-testis barrier and alter the permeability of testicular vascular endothelium [88,89]. Barqawi, et al. demonstrated rats that underwent experimental varicocele creation showed significantly increased levels of germ cell apoptosis in the ipsilateral testis 14 days following varicocele creation, and this reached a maximum level on day 28 [13].

Fazlioglu et al. observed that the apoptotic index in the testes of the rats following the creation of varicocele almost doubled on the 14th day and continued to increase slowly until day 28 [90]. Fujisawa et al. suggested that there is a decrease in the level of apoptosis in varicocele. These investigators compared testicular biopsies obtained from subfertile patients with varicocele those obtained from healthy individuals and found fewer apoptotic cells in the group with varicocele [14]. Various technical reasons (using formalin instead of Bouin solutions for fixation) were held responsible for the different results reported in this study. Fujisawa and coworkers compared the level of S-Fas in the semen of oligospermic patients with varicocele, oligospermic patients without varicocele, and healthy individuals. The level of S-Fas was low only in the group with varicocele. This shows that Fas/Fas-L system, which is the major regulatory system on apoptosis in testes, is activated; in other words, apoptosis has increased. In the same study, S-Fs levels increased, which indicates that apoptosis had decreased [91].

Apoptosis and Sperm Morphology

It is well known that varicocele is associated with significant volume changes in adolescents, even down to testicular atrophy in severe cases, and spermatogenetic arrest [92]. McLeod has described the changes in testicular tissue seen in varicocele patients as “stress pattern”. Stress pattern in varicocele consists of immature, amorphous, and smaller cells [93]. Interestingly, these changes resemble apoptotic cells [94]. Cytoplasmic condensation, nuclear condensation, nuclear fragmentation and apoptotic bodies (nuclear fragments with cell wall) are the characteristics of apoptosis under light microscopy, and all of these properties are

comparative morphological changes observed in varicocele [93]. Additionally, apoptosis involves active processes as mRNA and protein synthesis, and results in DNA fragmentation [94]. Foresta et al. proposed increased nuclear instability in sperm cells of varicocele group compared with control group [95]. They indicated that several proteins including protamins may alter localization and function of histones of DNA chain [95]. A defective DNA synthesis in varicocele was also proposed by Nakamura et al. and Fujisawa, et al. [14,96]. All of these studies that proposed molecular alterations in varicocele at DNA level suggested that apoptosis may play a role in the pathogenesis of varicocele.

Hassan et al. detected apoptotic cells in all types of spermatogenic cells in the seminiferous tubules without selective sites [9]. This observation differs from that given by Fujisawa et al. who showed that spontaneous apoptosis in normal human testes occurred primarily in germ cells near the basement membrane; in spermatocytes and spermatogonia [14]. Lin et al. detected apoptotic cells centrally located within the seminiferous tubules of infertile men [97]. Moreover, Hassan detected apoptosis of spermatids in both controls and varicocele-bearing testicular specimens [9]. Tesarik et al. indicated that men with complete spermiogenesis failure had significantly higher frequencies of primary spermatocytes and round spermatids carrying the apoptotic-specific DNA damage compared with incomplete spermiogenesis failure [98].

Unilateral Varicocele And Bilateral Testicular Damage

Experimental models with surgically created a unilateral varicocele in animals have showed detrimental effects on both testes. These studies have presented bilateral changes in the form of presence of seminal stress pattern, impairment of spermatogenesis on testicular biopsy, and increase in testicular temperature. The precise underlying mechanism that causes bilateral testicular damage in unilateral varicocele has not been defined. Bilateral increase in both testicular flow and temperature, a stretch receptor in spermatic vein causing bilateral response to unilateral lesion, refluxing down of vasoactive substances from adrenal gland or kidney are some of the argued hypothesis to explain bilateral damage [50,99,100]. Barqawi et al. did not detect a significant difference in contralateral (right) testis compared with the control group [13]. Fazlioglu et al. observed that after the creation of unilateral varicocele, the level of apoptosis increased in both the left and right testes [90]. They also showed that apoptosis in both testes decreased after surgical treatment on day 21, and on day 28 the results were similar to those of the control group [90]. Turner and Lopez have observed destruction and an increase of temperature in both testes of rats on day 30 after the induction of varicocele, whereas following treatment the temperature fell and healing occurred [101].

Detection Of Apoptosis

Various methods can be used in the evaluation of apoptosis. Although each method has certain limitations, TUNEL method has some advantages over others, such as being able to be applicable in tissue culture and paraffinated blocks and being more sensitive due to its ability to stain even preapoptotic cells TUNEL is the standard method in evaluating apoptosis for these reason [102]. However, it is necessary to note that cleaning the tissue of blood cells by perfusion before sampling the tissue, fixating it with 10% formalin, and application of the stain by an experienced team with care, are the factors that will increase the success rate of this sensitive method.

Antiapoptotic Treatments

Because the current information regarding varicocele pathophysiology does not apply to all clinical situations because varicocele patients are not treated etiologically, it is baffling why some men with varicoceles have normal semen and can father children, while others fail to regain fertility despite corrective surgery [23]. Therefore, the identification of the mechanism by which varicocele induces fertility is a crucial step in treating this pathology. Fazlioglu et al. showed that after varicocelectomy, apoptosis decreased significantly on day 21 and on day 28 it was almost equal to the level of control groups in both testes [90]. Mostafa et al. showed that the level of testicular superoxide increased in varicocele and that vitamin E and melatonin, which decrease superoxide levels, decrease testicular germ cell apoptosis [103]. Onur et al. demonstrated experimentally induced left varicocele revealed an increased ratio of pro-apoptotic Bax protein in testicular germ cells, whereas this effect was antagonized by using the potent endogenous hormone, melatonin at a dose of 10 mg/kg/day. The reversal of this effect was attributed to the prevention of damage induced by oxidative stress [65]. Cam et al. reported varicocele increased tissue levels of oxygen (O_2) and apoptosis in both testicles in their experimental varicocele model in rats. In contrast, they showed vitamin E administration lowered O_2 and possibly decreased apoptosis to some degree which in turn suggests a potential induction of apoptosis by O_2 in conjugation with several other factors. They suggested that apoptosis would be the responsible mechanism of tissue damage observed in varicocele [50,104].

Duarte et al. evaluated the effects of NAC which is the most powerful and traditional antioxidant, and is possibly the most widely studied in the field of oxidative stress, but they stated that germ cell apoptosis was not influenced by the administration of NAC in the experimental rat varicocele model [105] In a recent study, Bolat et al. investigated the potential protective effects of losartan which is an angiotensin receptor blocker on varicocele-induced germ cell apoptosis. They found that after losartan

administration, Johnsen score significantly increased and H-score and apoptotic index were significantly decreased in experimental varicocele-created rats when compared with only varicocele group [106]. In another study, Hajipour et al. showed that resveratrol decreased the gene expression levels of ASC, NLRP3, caspase-1 and Bax and increased Bcl-2 gene expression [107]. Recently, Mazhari et al. demonstrated that silymarin decreased the oxidative stress markers and fairly ameliorated the varicocele-decreased spermatogenesis [108].

Conclusion

The high incidence of varicocele among infertile men is usually associated with testicular hypotrophy and impairment in semen parameters. These histological changes are associated with varicocele-induced hypoxia, increased ROS and apoptosis. Although varicocelectomy is the gold standard treatment of varicocele, in order to increase the sperm quality, perhaps in the future it will be necessary to consider the use of these treatments as adjuvants. However, further and more detailed studies at molecular level are needed to explore the regenerative mechanisms of various molecules in varicocele-induced germ cell apoptosis.

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