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ORIGINAL ARTICLE

Involved intrinsic apoptotic pathway of testicular tissues in varicocele-induced rats

Jane-Dar Lee · Tsung-Han Lee · Wen-Hsuan Cheng · Shaw-Yeu Jeng

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Abstract
Introduction Increased testicular germ cell apoptosis has been reported in varicocele-induced rats. We studied intrinsic or extrinsic pathway of apoptosis by detecting Bcl-2, caspase-9, caspase-8, and activated caspase-3 expressions in the bilateral testes of experimental varicocele-induced rats.
Materials and methods Experimental left varicocele (ELV) was created by partial ligation of left renal vein in a study group of 24 adult male Sprague–Dawley rats. The other 24 rats were as control group. Eight rats from each group were killed at 4, 8, and 12 weeks following varicocele creation. Testicular tissues of both groups were sampled for TUNEL assay and immunoblotting.
Results Increased apoptotic germ cell was found in the ipsilateral testis of varicocele group at 8 and 12 weeks after operation ($P < 0.05$). Increased activated caspase-3 expression in the contralateral (right) testis was noted at 12 weeks following varicocele creation ($P < 0.05$).
Conclusions Our study demonstrates down-regulation of Bcl-2 expression and increased expressions of caspase-9 and activated caspase-3 in the ipsilateral testis of ELV rats at 8 and 12 weeks, indicating gradually increased testicular tissues apoptosis through the intrinsic pathway in varicocele-induced rats. Simultaneously, increased apoptosis in the contralateral testis was observed at 12 weeks ($P < 0.05$) following varicocele creation also.

Keywords Intrinsic apoptotic pathway · Testis · Varicocele induced · Rat

J.-D. Lee · W.-H. Cheng
Division of Urology, Department of Surgery,
Armed Forces Taichung General Hospital,
Taichung, Taiwan, Republic of China

T.-H. Lee
Department of Life Sciences,
National Chung-Hsing University,
Taichung, Taiwan, Republic of China

S.-Y. Jeng (✉)
Department of Surgery, Yuanshan Veterans Hospital,
No. 386, Rongguang Road, Yuanshan Township Yilan
County 264, Yuanshan, Taiwan, Republic of China
e-mail: jsy202@mail.ysvh.gov.tw

J.-D. Lee · S.-Y. Jeng
Central Taiwan University of Science and Technology,
Taichung, Taiwan, Republic of China

Introduction

Varicocele consists of an engorgement and dilatation of the pampiniform plexus above the testes. It has long been recognized as the most common cause of male infertility in adults [1, 2]. The incidence of varicocele in the general population is estimated to be 9.5–15% [3, 4]. Nevertheless, the effects of varicocele on testicles remain unclear. Kilinç et al. [3] had reported that experimental varicocele-induced hypoxia inducible factor-1α expression in rat testes and Barqawi et al. [5] had reported that increased testicular germ cell apoptosis in varicocele-induced rats. These results demonstrated that hypoxia and increased cell apoptosis occurred in the testes of rats with ELV.

Our understanding of how a cell undergoes apoptosis centers on the activation of caspases in vertebrates [6]. Two major pathways (intrinsic and extrinsic) are known to induce apoptotic cell death; these pathways differ in how the death signal is transduced [7–9]. The intrinsic (or mitochondrial) pathway is induced by cellular stress; this involves Bcl-2, mitochondrial outer-membrane permeability, and caspase-9 [7–9]. The extrinsic (or death receptor)

pathway is induced by specific ligands that engage death receptors; this involves Fas, the bindings and activation of caspase-8 [7–9]. Caspase-3 is considered to be a major executioner protease in cell apoptosis.

In this study, we examined Bcl-2, initiator proteins (caspase-9, caspase-8), and activated caspase-3 (effector protein) expressions in bilateral testes of all rats to differentiate intrinsic or extrinsic apoptotic pathways.

Materials and methods

Tissue samples

The study included 48 adult male Sprague-Dawley rats of the same weaning age (10 weeks), each weighing about 300 gm. All rats were fed the same food and maintained in a constant environment with a 12:12-h light–dark cycle. Rats were assigned to two groups. Group 1 rats (sham control, $n = 24$) underwent laparotomy except that ligatures were only placed in position and not tied. Group 2 rats (study group, n = 24) underwent varicocele-induced surgery. Eight rats of the study group and eight rats of the control group were sacrificed 4, 8 and 12 weeks following varicocele creation. Two of the initial 24 rats in Group 2 were excluded due to unsuccessful surgery. In both groups, each rat's bilateral testes were harvested for TUNEL assay, immunohistochemical stain for activated caspase-3 and immunoblotting analyses including Bcl-2, caspase-9, caspase-8 and activated caspase-3. This study was approved by the Institute of Ethical Committee of our hospital.

Technique of experimental left varicocele

In this study, we created an experimental left varicocele (ELV) in Sprague-Dawley rats according to the method of Saypol et al. [10] with some modifications. Each animal was anesthetized with an intraperitoneal injection of sodium phenobarbital (50 mg/kg) [5]. An abdominal midline incision was made. The left renal vein, inferior vena cava, and left spermatic vein were identified and a clamp was passed behind the left renal vein just distal to spermatic vein insertion. A 4-0 silk ligature was loosely placed around the left renal vein at this site and a rigid hydrophilic guide wire of 0.64 mm in diameter was placed on the left renal vein [3, 11]. The ligature was tied around the vein over the top of the guide wire. The guide wire was then withdrawn and the vein was allowed to expand to the limits of the ligature, which decreased the vein diameter to approximately half of its original diameter [3, 5, 11]. Finally, the midline incision was closed in two layers with 4-0 silk suture. When rats were sacrificed, the external diameter of the spermatic vein was measured by a ruler as it crossed anterior to the iliolumbar veins. Varicocele creation was considered to be successful when the diameter was greater than 1 mm [12].

TUNEL assays

Testes from rats were fixed in 10% formaldehyde for terminal deoxynucleotidyltransferase-mediated dUTP nick-end labeling staining (TUNEL) assays. To use 5-μm thick paraffin sections that were deparaffinized by immersing in xylene, rehydrated, and incubated in phosphate-buffered saline (PBS) with 2% H_2O_2 to inactivate endogenous peroxidases. Next, the sections were incubated with proteinase K (200 μg/ml), washed in PBS, and incubated with terminal deoxynucleotidyl transferase for 90 min and fluorescein isothiocyanate-dUTP for 30 min at 37°C using an apoptosis detection kit (Roche, Indianapolis, IN, USA). At this state, samples can be analyzed in a drop of PBS under a fluorescence microscope using an excitation wavelength in the range of 450–500 nm and detection in the range of 515–565 nm. After incubating slides with Converter-POD in a humidified chamber for 30 min at 37°C, the sections were stained with diaminobenzine for 10 min, and washed in PBS. Apoptotic germ cells were quantified by counting the number of TUNEL stained nuclei per seminiferous tubular cross section [5]. Cross sections of 100 tubules per specimen were assessed and the mean number of apoptotic nuclei per cross section was calculated.

Antibodies

Five primary antibodies were used in the present study: (1) Bcl-2, a mouse monoclonal antibody (sc-7382, Santa Cruz, Santa Cruz, CA, USA); (2) caspase-8, a goat polyclonal antibody (sc-6134, Santa Cruz); (3) caspase-9, a rabbit polyclonal antibody (sc-8355, Santa Cruz); (4) activated caspase-3, a rabbit monoclonal antibody (9664, Cell Signaling, Beverly, MA, USA); (5) α-tubulin, a mouse monoclonal antibody (sc-5286, Santa Cruz). The secondary antibodies were horseradish peroxidase (HRP)-conjugated goat anti-mouse IgG (0031430, Pierce, Rockford, IL, USA), goat anti-rabbit IgG (0031460, Pierce), or rabbit anti-goat IgG (74131, Jackson, West Grove, PA, USA) for immunoblotting and a commercial kit (PicTure™, Zymed, South San Francisco, CA) for immunohistochemistry.

Immunoblotting

The method was modified from the previous study [4]. The proteins of testicular tissues from both groups were separated by electrophoresis on sodium dodecyl sulfate (SDS)-containing 10–14% polyacrylamide gels. The immunoreactive bands were analyzed using MCID 7.0 (Imaging Research, Ontario, Canada) to compare the relative proteins abundance.

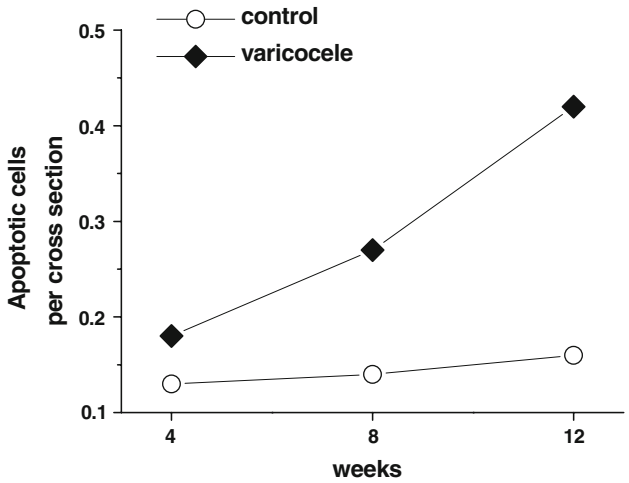

Fig. 1 Apoptotic germ cells per tubular cross section at 4, 8, and 12 weeks after varicocele creation of both groups

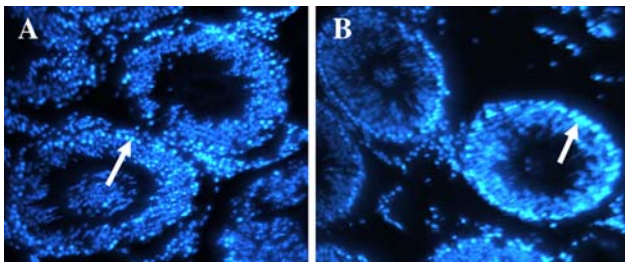

Fig. 2 Representative micrograph of TUNEL stain in testicular section of control group (**a**) and varicocele group (**b**) at 8 weeks after operation. *White arrow* apoptotic spermatogonia (×20)

Immunohistochemistry for activated caspase-3

The method of staining and microscopy were modified from previous study [4]. Negative control experiments, in which goat serum was used instead of the primary antibody, were conducted (data not shown) to confirm the positive results.

Statistical analysis

Data were analyzed with the Student t test with $P < 0.05$ considered statistically significantly different from corresponding controls.

Results

In seven, seven, and six rats varicocele had been successfully induced at 4, 8, and 12 weeks after surgery (varicocele creation), respectively. The success rate of varicocele creation was 83.33% (20/24) in this study. Rats sacrificed at 4, 8 and 12 weeks after varicocele creation had 0.18, 0.27 and 0.42 apoptotic germ cell per tubular cross section in the ipsilateral testis of varicocele group and 0.13, 0.14 and 0.16 apoptotic germ cells per tubular cross section in control group, respectively (Fig. 1). A statistically significant increase was noted in the number of apoptotic germ cells per tubule in the ipsilateral testis 8 and 12 weeks after left varicocele

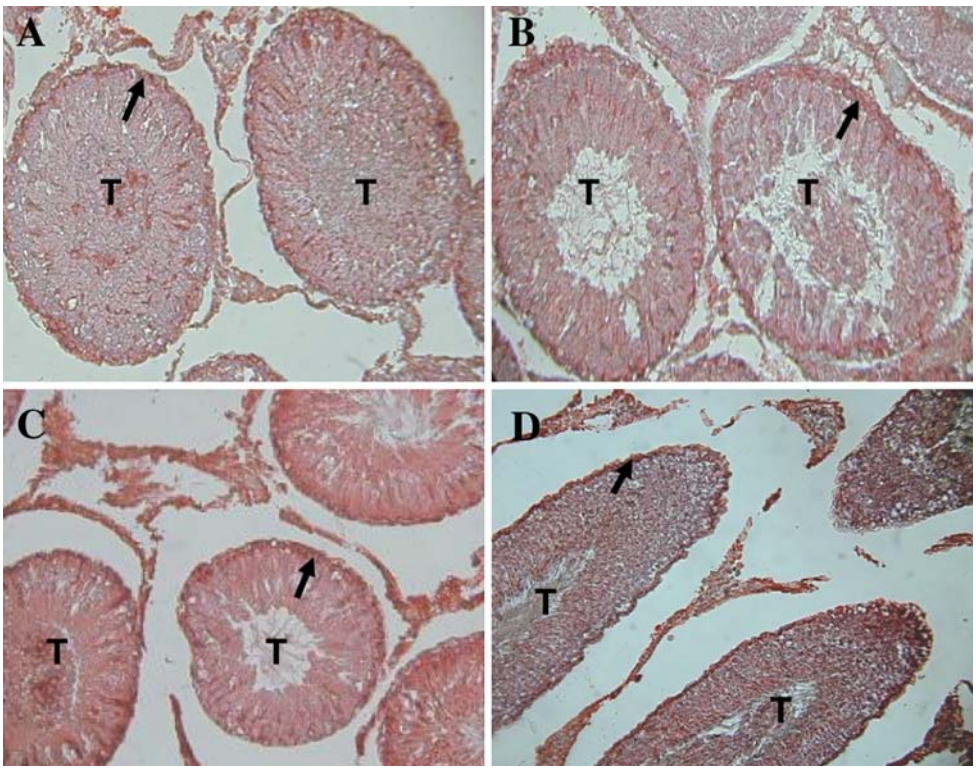

Fig. 3 Representative micrographs of immunohistochemical staining of activated caspase-3 in rat testis sections. *Panels* **a** and **c**, control group at 4 and 12 weeks; *panels* **b** and **d**, varicocele group at 4 and 12 weeks. *Arrows* the intensity of activated caspase-3 immunostaining (*deep pink*) is predominant on spermatogonia. *T* seminiferous tubules (×200)

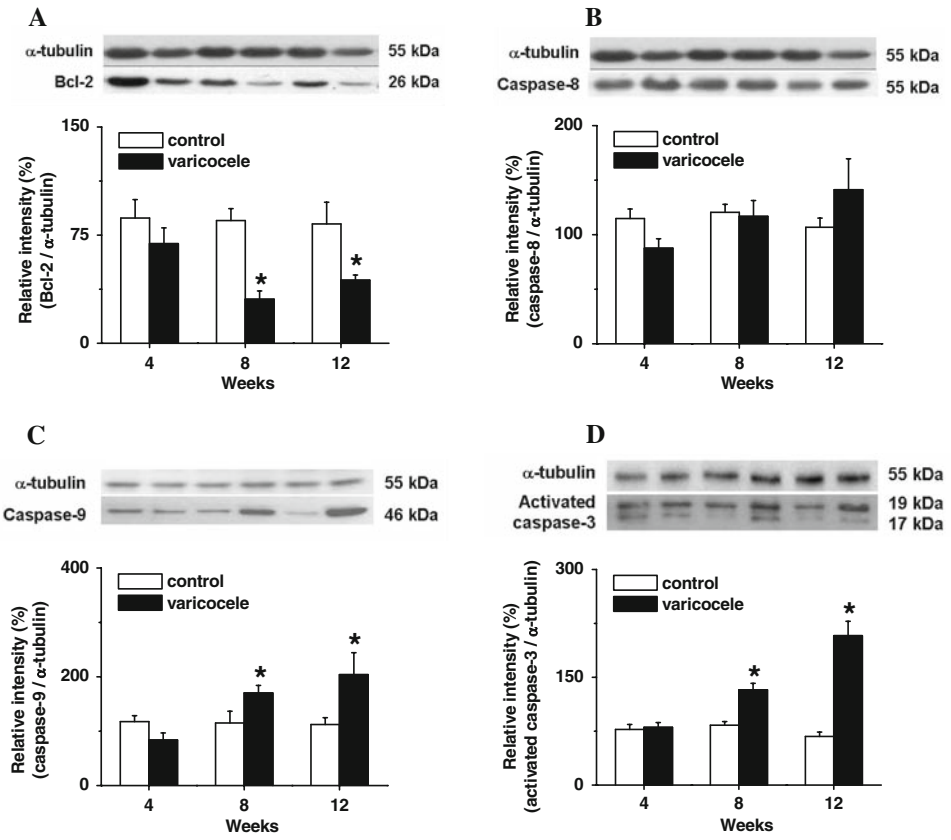

Fig. 4 Representative immunoblot and relative intensity of **a** Bcl-2, **b** caspase-8, **c** caspase-9, and **d** activated caspase-3 proteins in the varicocele and control groups of left testis. A-tubulin was used as loading control

*P<0.05, Value is mean ±S.E.

creation compared with controls ($P < 0.05$). Figure 2 showed representative micrograph of apoptotic germ cell in testicular section of both groups by TUNEL stain at 8 weeks after operation ($P < 0.05$). IHC stain of activated caspase-3 in the varicocele group is stronger than the control group at 8 and 12 weeks, respectively, after varicocele creation (Fig. 3). This result is compatible with TUNEL stain which means more apoptotic cells occurrence in spermatogonia of the varicocele group. Expression of Bcl-2 (anti-apoptotic protein) in varicocele group was significantly lower than in control group at 8 and 12 weeks (Fig. 4a; $P < 0.05$). The relative intensity of caspase-8 has no statistical difference in either group at 4, 8, and 12 weeks (Fig. 4b). Conversely, the expressions of caspase-9 and activated caspase-3 in varicocele group were significantly higher than in control group at 8 and 12 weeks, respectively (Fig. 4c, d; $P < 0.05$). The apoptotic executioner of activated caspase-3 significantly increased in contralateral (right) testis at 12 weeks after varicocele creation (Fig. 5d; $P < 0.05$). These results demonstrate that increased testicular tissues apoptosis gradually through intrinsic pathway in the testes of varicocele group.

Discussion

Varicocele, described as abnormal tortuosity and dilatation of gonadal veins within the spermatic cord [1, 2, 4], is the most common etiologic factor associated with male infertility [1–4]. Previous studies of experimental varicocele models in rats documented that increased hypoxia inducible factor-1α [3] and germ cell apoptosis in the testes [5]. This means that varicocele can lead to testicular tissue hypoxia and cause germ cell apoptosis [13–15]. It has been theorized that abnormally high levels of germ cell apoptosis may contribute to testicular failure and male infertility [5].

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 [16]. Briefly, two major pathways can induce apoptotic cell death: the intrinsic (or mitochondrial) pathway is involved

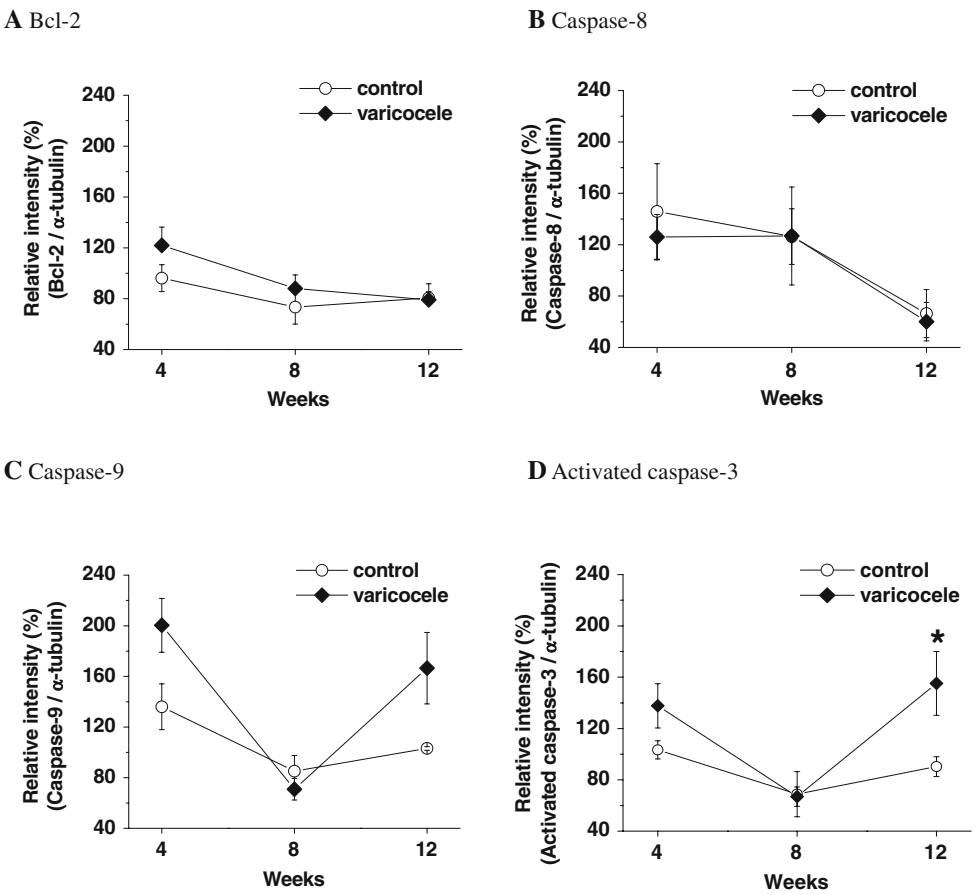

Fig. 5 Relative intensity of **a** Bcl-2, **b** caspase-8, **c** caspase-9, and **d** activated caspase-3 proteins in the varicocele and control groups of right testis

*P<0.05, Value is mean ±S.E.

Bcl-2, and caspase-9 protein [7–9]; and the extrinsic (or death receptor) pathway is involved Fas and caspase-8 protein [7–9]. In the human apoptotic pathway cascade, 14 caspases (cysteinyl 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 [6].

The oncogenic properties of Bcl-2 have been attributed mainly to its ability to inhibit apoptosis by interfering with activation of cytochrome c/Apaf-1 (apoptosome) pathway through stabilization of the mitochondrial outer membrane [17–19]. The presence of these enzymes will stimulate caspase-9 activation, leading to caspase-3 execution of cell apoptosis [7, 8]. Studies on venous stasis due to blood stagnation of ISV have involved testicular tissue hypoxia and increased apoptosis has been documented in human testis tissue of varicocele patients [3, 13, 20]. Under hypoxic stress, testicular cell apoptosis can increase and may also be a factor of varicocele-caused male infertility.

Barqawi et al. [5] reported experimental varicocele induces ipsilateral testicular germ cell apoptosis. In advance, we analyzed the apoptotic pathway of this study. It 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. These findings demonstrate that testicular tissue apoptosis increases through the intrinsic pathway in rats with ELV 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. Previous studies showed that testicular damage appears progressive and generally is observed on both sides 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 [21, 22].

This animal study demonstrates down-regulation of Bcl-2 expression and increased expressions of caspase-9 and activated caspase-3 in the testes of rats with ELV at 8 and 12 weeks. A trend was revealed of increasing apoptosis in both testes with a longer duration through the intrinsic

pathway of varicocele-induced rats. But these results were not compatible with some previous studies about apoptosis-related proteins in infertile men with varicocele [23–26]. In several respects, the animal model has some differences from the clinical varicocele of humans. Additionally, the obvious differences between the anatomy and physiology, varicocele disease is a long-standing lesion, to which the response may differ from that seen actually following experimental varicocele creation [5]. Moreover, it is known that apoptosis is a time limited process and in response to a noxious stimulus, a wave of apoptosis may be generated, after which only low levels may be seen [5]. So the duration and severity of apoptosis in germ cell after varicocele-induced rats for 12 weeks may be different from in human diseases.

However, our results from this animal model indicate that varicocele-induced rat is associated with increased testicular tissues apoptosis through the intrinsic pathway. We believe a better understanding of the factors, mediators and apoptotic pathway may bring a new insight into the treatment of varicocele and male infertility.

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Conflict of interest statement There is no conflict of interest.

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