Original Article

Correlation of Sperm Associated Antigen 11 (SPAG11) and its Isoforms with Varicocele in Rats

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Abstract

Background: We undertook this study to investigate the variation relationship of sperm associated antigen 11 (Spag11) mRNA expression and SPAG11E protein in the epididymis and spermatozoa of experimental left varicocele (ELV) rats. These findings could contribute to the understanding of the role of epididymal proteins in sperm functions and the mechanism of male infertility induced by varicocele.

Methods: The ELV model was established in adolescent male Sprague-Dawley rats. Four weeks after the operation, tissue distribution and changes in the expressions of Spag11 mRNA and SPAG11E protein caused by ELV in the whole of left epididymis and spermatozoa were studied using quantitative reverse transcription-polymerase chain reaction (RT-QPCR), immunohistochemistry and immunofluorescence. Significant differences were identified using one-way ANOVA followed by Student-Newman-Keuls test. Significance level (p) was fixed at 0.05.

Results: The expected product of Spag11 was 96 *bp* that amplified by RT-QPCR was detected in the epididymal tissue and spermatozoa. SPAG11E protein was confined mainly to the supranuclear region of the principal cells and the stereocilium of the epididymal epithelium, it was concentrated on the acrosome and the tail of spermatozoa except the terminal piece. Statistical analyses of the images and the data indicated that Spag11 mRNA and SPAG11E protein expressions in the left epididymis and spermatozoa of ELV rats presented a considerable decrease ($p<0.001$) compared with that of the corresponding control group.

Conclusion: The expressions of Spag11 mRNA and SPAG11E protein declined markedly in ELV rats, which suggest that SPAG11E may not only play an important role in sperm maturation, but it may also be influenced by varicocele.

Keywords: Epididymis, Rat, Sperm associated antigen 11 protein, Spermatozoa, Varicocele.
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Introduction

Spermatozoa leaving the testis undergo post-testicular maturation and acquire fertilizing ability and forward motility during their passage through the epididymis that provides the microenvironment for their maturation. Numerous proteins synthesized and secreted by the epididymal epithelia are thought to be considerable members of epididymal microenvironment and be involved directly or indirectly in male reproductive activities including the initiation of sperm maturation, sperm-oocyte recognition and the acrosome reaction (1). A family of these proteins includes SPAG11E coded by Spag11 gene in various species (2).

The Spag11 gene, also known as epididymal protein 2 (EP2), formed by the fusion of two ances-

trally independent β-defensin genes (3), generates at least 19 alternative mRNA spliced transcripts initiated at A and B promoters (1, 4–6). These transcripts are transcribed and translated in different portions of the male reproductive tract and in different species, to produce a complex group of small androgen-dependent secretary proteins in which different exon-encoded modules are variously combined (3). Most primate SPAG11 protein isoforms consist of N-terminal common region and C-terminal peptides, which are encoded by different combinations of exons (7). SPAG11 is detected in the epithelial cells of male reproductive organs, predominantly in epididymis. It is believed to play an important role in epididymal immunity in addition to their role in sperm maturation on the basis of their structural features and specific expression pattern. The Spag11 gene in rodents is similar to that in primates but it possesses fewer splicing variants, whose intimate expression pattern and function are less well documented.

Spag11 (known in rat and mouse as Bin1b) is an androgen-dependent gene cloned from the rat epididymis by Peng Li et al. using differential display analysis of mRNA. However, only one rodent isoform, SPAG11E (EP2E), has been well-characterized and its full-length cDNA is composed of 385 base pairs (*bp*), with an open reading frame of 204 *bp* nucleotides, encoding a 68-amino acid protein (8). SPAG11E is exclusively expressed in rat epididymis, strongly in the distal caput and proximal corpus (4, 8), and it can bind the sperm head in all epididymal regions except the initial segment (9). SPAG11E exhibits a structural and antimicrobial similarity to β-defensins, and has become one member of the innate defensive system in epididymal epithelia. Moreover, as an essential component in the epididymal environment, SPAG11E is believed to be relevant to the acquisition and maintenance of progressive motility in sperm (9).

Varicocele (VC), a tortuosity and dilation of the pampiniform plexus in the spermatic cord, has been identified to be one of the main causes of male infertility. However, the detailed mechanisms of varicocele-induced male subfertility and infertility are not well-elucidated. It was reported that varicocele might cause a series of pathophysiological changes in the testis and epididymis, with an adverse effect on the environment for spermatogenesis and sperm maturation. Therefore, we designed our study to observe the variation in the expression of Spag11 mRNA and SPAG11E protein in the epididymis and spermatozoa of the experimental left varicocele (ELV) in a rat model created via partial ligation of the left renal vein, revealing the correlation of Spag11 and its transcripts with VC. This study was also intended to provide the basis for understanding the mechanism of VC-induced infertility.

Methods

Animals: In total, 70 male 6–7 week-old Sprague-Dawley (SD) rats, weighing 130–150 *g*, were obtained from the Laboratory Animal Center of the School China. This study was approved by ethical commitee of Science and Technology Department of Shaanxi Province of China in 2008 and Medical School of Xi'an Jiaotong University in 2010.

Groups and Treatments: The animals were randomly divided into the experimental left varicocele (ELV) group (n=35) and the sham-operated control group (n=35). The rats were sacrificed four weeks after the operation for various experiments.

Procedure for partial left renal vein ligation: The rats were anesthetized with 20% ethylurethanm (5 *ml/kg*) *i.p.* and a midline incision was made to disconnect and expose the left renal vein. A 4–0 silk ligature was made around the vein with a metal probe interposed lateral to inferior vena cava and medial to the entrance of the adrenal and spermatic vein. The diameter of the metal probe was selected in a way to reduce the diameter of the renal vein by approximately 50%. Then the probe was removed and the renal vein could be recanalized via bypass circuit. The incision was sutured with 3–0 ligature. Every operated rat received an intraperitoneal injection of penicillin, 20 million units daily, for three consecutive days to prevent infection. Sham-operated animals, which served as the controls, were exposed to the medication only but no ligation of the left renal vein was made.

Thirty-five male rats were operated successfully and subdivided into three groups, for immunehistochemistry (n=5), for immunofluorescence (n=10) and the remaining (n=20) for real-time quantitative reverse transcriptase polymerase chain reaction (RT-qPCR).

Total RNA extraction: The whole epididymis and sperm mass gained from the cauda epididymis

were immediately snap-frozen in liquid nitrogen, respectively. Total RNA of each sample was extracted by Trizol reagent. All the procedures were carried out according to the manufacture's protocol (Invitrogen, Carlsbad, CA, USA). RNA concentration and quality were determined by measuring the absorbance at 260 *nm* and 280 *nm*.

RT-qPCR: The RT-qPCR protocol was as follows: DNase-treated total RNA (3 *μg*) was reverse transcribed in a volume of 20 *μl* with Moloney Murine Leukemia Virus reverse transcriptase (Fermentas, Lithuania) and random hexamer primer (Fermentas) at 42 ℃ for 60 *min*. Subsequently, the amplifications of Spag11 (GenBank accession NM 145087) and Gapdh (GenBank accession 017008) used as an internal control, were carried out by qPCR (TaqMan). Primers and probes were designed according to their mRNA sequences: Spag 11 forward primer 5'-CTGCTTGGTCCAAA GA AA CTCA-3', reverse primer 5'-CGGCACA TGA AGA GCCTACA-3', probe FCACCGTGTG CTT CATG CAGCGGP. Gapdh forward primer 5'-CA AGTTC AACGGCACAGTCAA-3', reverse primer 5'-TG GTGAAGACGCCAGTAGACTC-3', probe FTCT TCCAGGAGCGAGATCCCGC TA ACP.

QPCR was conducted using PRISM 7000 Sequence Detector (ABI, Foster, USA). Each reaction mixture contained 12.5 *μl* 2×AmpliQ Real Time PCR Master Mix with 10 *mM* $MgCl_2$ (Lifeson, Danmark), 0.9 *μM* forward primer, 0.9 *μM* reverse primer, 0.5 *μM* probe, 1 *μl* cDNA, and deionized water was added to reach a total volume of 25 *μl*. Unless indicated, all reagents used in qPCR were purchased from Genecore Biotechnologies Company (Genecore, Shanghai, China). Cycling parameters were kept at 50 ℃ for 2 *min*, at 95 ℃ for 5 *min* to initially denature and hold, then at 95 ℃ for 30 *s* and at 61 ℃ for 1 *min* for 45 cycles. All reactions including No Template Controls were repeated twice.

Immunohistochemistry: Immunohistochemistry was performed as previously described (10). All the epididymides were fixed in freshly prepared Bouin's solution, then embedded in paraffin wax and cut in 5 *μm* sections. Slices were stained with SPAG11E antibody raised against the peptide ERKGDISSDPWNRC. For control staining, normal rabbit serum was incubated with the sections (data not shown). To demonstrate immunoreactive SPAG11E a Streptavidin/ Peroxidase (SP) Histostain™-Plus Kit (ZSJB, Beijing, China) was employed with diaminobenzidine as chromogen, resulting in brown products. Sections were counterstained with Harris hematoxylin. Photographs were taken by digital microscopy system (Olympus BX-1, Tokyo, Japan). The corrected gray value of immunopositive areas in 5 fields per slide at ×400 magnification was detected by an image analysis software (Image Pro Plus 5.1, Bethesda, MD, USA). The corrected gray value is proportional to the intensity of positive staining.

Immunofluorescence: Sperm smears were produced on the basis of the protocol of Hamil KG et al. (11). The caudae were cut up, and were then placed in 0.01 *M* phosphate-buffered saline (PBS, pH=7.4) for 15 *min*. The supernate was centrifuged at 2,200 ×*g* for 15 *min*. Depositions (including sperm) were washed twice with 0.01 *M* PBS (pH=7.4) and were fixed in 4% paraformaldehyde for 30 *min*. Spermatozoa were washed with 50 *mM* glycine in 0.01 *M* PBS (pH=7.4), smeared on slides after adjusting sperm density, and air-dried. We used 1% Triton X-100 to increase the penetration of primary antibody. After washing twice in 0.01 *M* PBS (pH=7.4), nonspecific binding sites were blocked with normal goat serum for 25 *min* at 37 ℃. The primary antibody, rabbit anti-mouse SPAG11E antibody (1:600) was incubated with spermatozoa overnight at 4 ℃ in 1% BSA, and 0.4% Triton X-100 in PBS. Unbound antibody was removed by three washes with 0.01 *M* PBS (pH=7.4), and FITC-conjugated AffiniPure goat anti-rabbit secondary antibody was added at 37 ℃ for 1 *hr*. The unbound secondary antibody was removed by three washes with 0.01 *M* PBS (pH=7.4).

The sections were mounted by glycerin-bicarbonate buffer, and observed using a digital microscopy system (Olympus BX-1, Tokyo, Japan) with a green filter before taking photos. For control staining, normal rabbit serum was incubated with the sections (data not shown). The corrected average fluorescence intensity of each picture at ×1000 magnification was examined by image analysis software as mentioned above. The optical density indirectly reflects the amount of antigens that can combine with homologous antibodies. The higher the corrected optical density is, the higher binding of the proteins and antibodies will be.

Statistical analysis: The target gene expression difference was shown as a multiple of Spag11 mRNA quantifiability in ELV group relative to the

normal control after homogenization by the reference gene. The relative expression of Spag11 in ELV groups was described using the equation: $R= 2^{-\triangle\triangle CT}$, $\triangle\triangle CT=(CT_{SPAG11}-CT_{GAPDH})_{ELV}-(CT_{SPAG11}-CT_{GAPDH})_{CONTROL}$. Where R is the differences in gene expression level, CT is the threshold cycle.

The data related to immunohistochemical staining was shown as mean±SD. The changes in expression between the cases and controls were evaluated using One-way ANOVA with SPSS, version 13.0, statistical software (SPSS Inc., Chicago, IL, USA). The $p<0.05$ was considered statistically significant.

Results

Animal Model: There was no death among the rats following the surgery. Four weeks after the operation, the left spermatic vein of model rats showed expansion, even beyond twice in diameter than before but the weights of the two kidneys were similar (Figure 1).

Effects of ELV on the Spag11 gene expression: We performed RT-qPCR to measure Spag11 gene expression levels and compared the corrected relative quantification between ELV and control groups. The results are shown in figure 2, table 1 and table 2.

96 *bp* PCR products of Spag11 in the epididymis and spermatozoa were observed on 2% agarose gel electrophoresis in 1×TAE buffer (data not shown). According to the definition of the equation: $R=2^{-\triangle\triangle CT}$, the multiple variance of control group is 1. As expected, ELV caused the down-regulation of Spag11 mRNA expressions of left epididymis and spermatozoa.

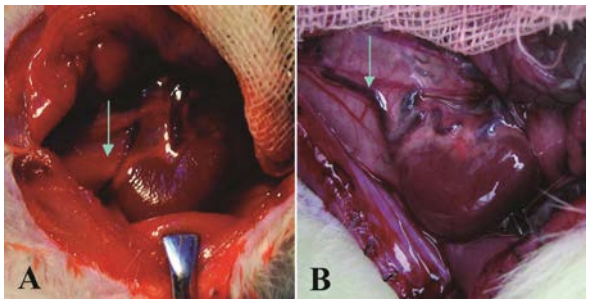

Figure 1. The photograph of cirsoid left vena spermatica vein in ELV model rat. A: control group, normal vena spermatica interna; B: ELV group, varicose vena

Table 1. CT value of target amplification

Group	CT value of target amplification	
	Gapdh	Spag11
Control		
epididymis	24.32±3.15	25.25±2.37
spermatozoa	28.14±1.89	31.62±0.33
ELV		
epididymis	24.91±3.48	27.22±1.39
	30.00±2.09	34.71±0.49

Effect of ELV on the SPAG11E protein expression: In the ductal epithelium of epididymis, SPAG11E protein was expressed mainly in the supranuclear region of principal cells and it was associated with the apical stereocilia. There was no immunostaining in the clear, halo or basal cells. Maximum levels of SPAG11E expression were detected in the proximal, middle corpus, and the cauda, and a weak immunopositive staining was observed in the distal caput, while negative staining occurred

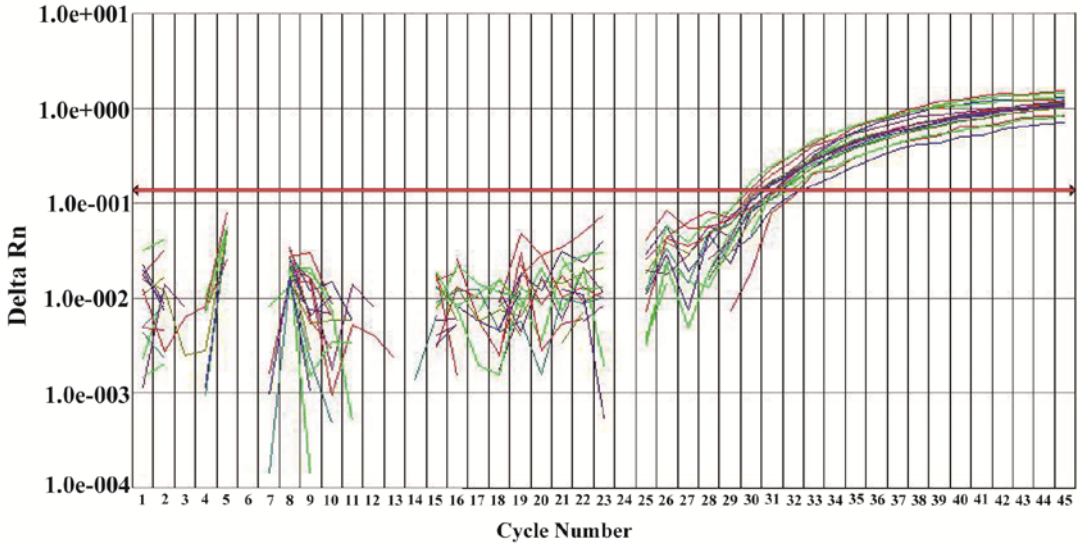

Figure 2. Amplification plot of RT-QPCR.

Table 2. The differences in the expression levels of Spag11 mRNA in the left epididymis and spermatozoa of ELV group compared with that of the corresponding control group

Group	Fold-change of ELV group	Fold-change of control group
Left epididymis	0.3842	1
Left spermatozoa	0.4273	1

Table 3. The analytical results of corrected gray value of SPAG11E by immunohistochemical staining in the epididymis of the ELV rats (Mean±SD)

Group	Corpus	Cauda
Control	0.2901±0.0231	0.2801±0.0087
ELV left	0.2388±0.0156 [a,d]	0.2314±0.0017 [a]
ELV right	0.2617±0.0105 [b]	0.2791±0.0038 [c]

Vs. corresponding control group in ELV group, a: p<0.001; *vs.* corresponding control group in ELV group; b: p<0.05; *vs.* right side in ELV group, c: p>0.05, *vs.* right side in ELV group, d: p>0.05

Table 4. The analytical results of corrected optical density of SPAG11E by immunofluorescence on the spermatozoa from the epididymal cauda in the ELV rats (Mean±SD)

Group	Spermatozoa
Control	
	1.2517±0.2679
ELV	
from left epididymis	0.9241±0.2274 [a,c]
from right epididymis	1.2683±0.1302 [b]

Vs. the corresponding control group in ELV group, a: p<0.05; *vs.* the corresponding control group in ELV group; b: p>0.5; *vs.* right side in ELV group, c: p<0.05

in the initial segment (Figure 3). The protein was concentrated in the acrosome of the sperm. SPAG11E was less abundant on the tail (Figure 3).

Consistent with changes in gene expression, immunohistochemistry showed a considerable de crease in left epididymal SPAG11E expressions in ELV groups (p<0.001) compared with that of the control group (Table 3). Moreover, lower binding of SPAG11E proteins and corresponding antibodies occurred in the spermatozoa from left epididymis of ELV groups (p<0.001) by optical density analysis (Table 4).

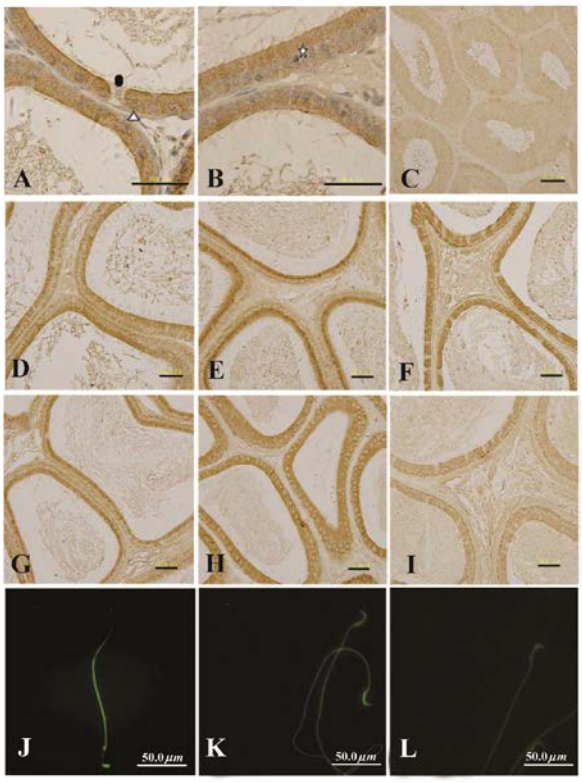

Figure 3. The expressions of SPAG11E protein in the epididymis and spermatozoa of ELV adolescent rats and corresponding control group. Original magnification ×40 (C, D, E, F, G, H and I) and ×100 (A, B, J, K and L). A, B: the cell-specific pattern of localization of SPAG11E in the epididymal epithelium (hematoxylin couterstaining). The supranuclear cytoplasm of principle cells exhibited immunopositive staining, the clear cells, halo cells and basal cells showed immunonegative. ●: clear cell; ☆: halo cell; ∆: basal cell. C, D, E, F: the region-specific expression pattern of SPAG11E in the epididymis. Except for the initial segment (C), the remaining parts were immumopositive, weak in the distal caput (D), strong in the proximal, middle corpus (E) and the cauda (F). G, H, I: The expressions of SP AG11E in the distal caput, proximal corpus and the cauda of ELV rats. J: the localization of SPAG11E in spermatozoa of control rats. Anti-SPAG11E immunofluorescent staining was most intense in the acrosome of head and neck segment, middle segment and principal segment of spermatozoa tail. K: the expression of SPAG11E in spermatozoa from the right epididymis of ELV rats. L: the expression of SPAG11E in spermatozoa from the left epididymis of ELV rats, the intensity was obviously decreased when compared with J

Discussion

The epididymis plays a crucial role in regulating spermiotelcosis (12). Region-specific, as well as cell-specific patterns of gene expression within its epithelium contribute to the spermiotelcosis microenvironment of the epididymis, in which there are rich specialized proteins interacting with spermatozoa. SPAG11, an androgen-dependent and epididymis-specific expressed gene, gives rise to multiple mRNAs that encode a group of small secretary peptides (2). The SPAG11 gene codes for at least five different message variants in Chimpanzees and for at least six different message variants in humans (13). However, Spag11 codes for fewer message variants in rodents, which the

predominant one of is SPAG11E homologous to Bin-1b. Localization of Spag11 mRNA within the defensin gene cluster and identification of the specifically expressed SPAG11E protein in the male reproductive tract suggest that this androgen-dependent secreted protein is likely involved in sperm maturation as a component of the innate antimicrobial peptides in epididymal microenvironment (14).

Varicocele, a common disorder in the field of andriatria, is present in approximately 15% of the general male population. Varicocele accounts for up to 40% of male factor infertility; therefore World Health Organization (WHO) has listed it as the first item for investigation in male infertility. Varicocele frequently occurs in the left side, often involving the contralateral side, and damages the function of reproductive system in a time-progressive pattern. It should be noted that adolescent varicocele is especially serious. Many studies have demonstrated that experimental left varicocele could result in atrophy of the epididymal ducts, degeneration of the epididymal epithelium, apoptosis within principal cells and edema of the interstitial tissue of rats (15, 16).

To confirm the effects of varicocele on SPAG11 protein and its transcript expressions and to study their correlation with VC and infertility caused by VC, we used ELV rats. We investigated the effects of varicocele on the expression of Spag11 and its isomers in the epididymis and spermatozoa at both nucleic acid and protein levels.

In 35 rats subjected to varicocele surgery in this study left spermatic vein showed the expected dilation, which testified that incomplete ligation of the left renal vein was a classic and effective method for inducing ELV and performing the follow-up experiments.

We had reported that Spag11 mRNA expression was epididymis-specific. Among six rat tissues tested by RT-PCR, only the epididymis provided a detectable 376 *bp* PCR product (10). In this study, 96 *bp* specific strip of Spag11 gene was obtained from the frozen spermatozoa mass by RT-qPCR in addition to the same band in the epididymis. This was confirmed by the results of RT-qPCR in which the Spag11 mRNA expressions in the left epididymis and spermatozoa of ELV groups presented remarkable decreases compared with that of the corresponding control groups.

The above-mentioned data illustrate that ELV may have an early impact on the Spag11 gene expression in the left side of the epididymis of the experimental groups. The direct damage to the epididymal epithelium caused by ELV may lead to the decline in Spag11 expression. In parts these changes are likely, to be the result of secondary decline in androgen secretion by varices.

Although the expression of Spag11 variants in rodents is less well-understood, only a single rodent isoform, SPAG11E has been characterized. In rats, SPAG11E was reported to be regionally- and cell-specifically expressed in the epididymis and to be an androgen dependent secretary peptide containing the defensin-like six-cysteine motif. Recent research suggests that SPAG11E could bind sperm within different regions of epididymis (16). Our immunohistochemistry and immunofluorescence findings prove that SPAG11E possesses distinct cell- and region-specific expression patterns in the epididymis and sperm binding, which reveals that SPAG11E may play an essential role in post-testicular sperm maturation as a specific molecule of epididymal origin.

After inducing varicocele in the hibateral epididymis, the expression of SPAG11E protein exhibited a sharp reduction. These changes in the expression rate were more obvious in the left than the right epididymis following ELV. No statistical differences in SPAG11E expression were found in right cauda epididymis of ELV rats, which indicates that the damage caused by varicocele on the left side of epididymis is more serious than that on the right side. These changes are in accordance with the results of its mRNA study.

Conclusion

According to our findings, varicocele may affect the synthesis of some specific proteins with anti-inflammatory properties within the epididymal epithelium. This effect evokes an early but a continuous change in the environment for post-testicular sperm maturation, which may later result in male subfertility or infertility. As defensin-like peptides, Spag11 mRNA and SPAG11E protein expressions showed a considerable decline in left epididymis at an early stage of ELV, which might be one of the reasons why varicoceles are prone to cause genital infection and then male infertility. However, we still need to do further research in order to fully understand how Spag11 and its isomers are involved in the mechanism of VC-induced infertility.

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Conflict of Interest

Authors declare no conflict of interest.

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