Expression of claudin-11 in a rat model of varicocele and its effects on the blood-testis barrier

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Abstract. Varicocele (VC) is an abnormal tortuosity and venous distension of the pampiniform plexus in the spermatic cord. VC is the most common surgically correctable cause of male infertility. The purpose of the present study was to investigate the effects of VC on the tight junctions and the blood-testis barrier (BTB) of Sertoli cells in the bilateral testes of rats. A model of VC was established by left renal vein narrowing in Sprague-Dawley rats; control rats underwent dissection of the vein without narrowing. The bilateral testes were harvested at 4, 6 and 8 weeks after the operation. The relative expression of claudin-11 and transforming growth factor (TGF)-β in the testis was determined by reverse transcription-polymerase chain reaction analysis and immunohistochemistry (IHC). The expression level of claudin-11 was prominently downregulated in the VC model group compared with the control group, while the level of TGF-β in the testes was higher in the VC group. IHC examination demonstrated that VC led to destruction of the integrity of the BTB, and the degree of destruction increased with time. Furthermore, it was also observed that unilateral VC affected contralateral testicular function. In conclusion, the present study partially explained the molecular mechanisms underlying the pathogenesis of VC and provided grounds for further research into the treatment of male infertility.

Introduction

Varicocele (VC) is considered to be the main cause of male infertility (1). It has been demonstrated that VCs adversely affect sperm quality (2,3), sperm function (4,5), testicular histology (6,7) and reproductive hormones (8). VCs are considered to exert a negative effect on the function of Sertoli and Leydig cells. The imbalanced hypothalamus-pituitary-gonadal axis ultimately affects spermatogenesis due to the decreased testosterone levels (9).

The blood-testis barrier (BTB) consists of junctional and cytoskeletal structures, and it is one of the tightest blood-tissue barriers in mammals (10,11). Tight junctions (TJs), gap junctions (GJs) and desmosomes form this barrier. Claudins are proteins that interact with cytoskeletal proteins and are important components of tight junctions (12). A variety of claudins are expressed in rodent testes (13) and several participate in the formation of the BTB. Claudin-11 is detected at day 12 post-coitus (14), and claudin-11 knockout is not lethal in mice, but they are sterile (13). Claudin-$11^{-/-}$ Sertoli cells exhibit a unique phenotype by loss of tight junction connection integrity, leading to loss of the epithelial phenotype (15). The BTB has a crucial role in the process of spermatogenesis, and claudin-11 is an important tight junction protein expressed by Sertoli cells that is involved in the formation of the BTB. Abnormal expression of claudin-11 affects the function of the BTB; however, precisely how VC affects claudin-11 expression resulting in further alterations of the BTB remains unknown.

Recent studies have demonstrated that transforming growth factor (TGF)-β secreted by testicular cells can alter the tightness of the tight junctions via the p38-mitogen-activated protein kinase pathway (16,17). Therefore, it would be interesting to elucidate whether TGF-β is involved in the pathogenesis of VC, as preventing the testicular cells from synthesizing TGF-β may affect the tightness of the junctions between supporting cells and prevent spermatogenesis. To address these questions, a VC rat model was established and the molecular and histological changes in the testes were observed, in order to provide evidence on the pathogenic mechanisms of VC and determine the potential value in development of non-hormonal male contraceptives.

To the best of our knowledge, this study is the first to measure claudin-11 expression levels in ipsilateral and contralateral testicular Sertoli cells in a VC model, and to investigate its characteristics over time and the effects on BTB function from the perspective of VC-induced male infertility. The research results may further establish the inclusion of claudin-11 in BTB-associated studies and provide a basis for the development of potential non-hormonal male contraceptives.

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Materials and methods

Animals and groups. A total of 32 mature male (12 weeks) Sprague-Dawley rats, weighing 200±20 g, were purchased from the Shanghai Laboratory Animal Centre (Shanghai, China). The animal experiments were performed in accordance with the guidelines of the ethics committee of Zhejiang University, and the animals were handled according to the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (18). The experiment was approved by the Animal Care and Use Committee of Zhejiang University (ZDSX2017-0012).

The rats were randomly divided into the following 7 groups: Normal control group (NC), 4 weeks VC (4W-VC), 4 weeks sham surgery (4W-SHAM), 6 weeks VC (6W-VC), 6 weeks sham surgery (6W-SHAM), 8 weeks VC (8W-VC) and 8 weeks sham surgery (8W-SHAM). The VC model was established by partial ligation of the left renal vein according to the method described by Hurt *et al* (19,20). Briefly, 1% pentobarbital sodium (35 mg/kg) was administered via intraperitoneal injection to induce anaesthesia. The left renal vein was isolated at its junction with the inferior vena cava, and a 0.8-mm metal ligature wire was placed to narrow the left renal vein to half of its original diameter. The branch veins of the spermatic vein were also ligated. Successful modelling criteria included: i) Diameter of the spermatic vein >1 mm; and ii) no difference in size between the left and right kidneys. Isolation of the left renal vein with no ligation was performed in the sham surgery control group. The rats were euthanized by cervical dislocation following anaesthesia with intraperitoneal injection of 10% chloral hydrate (200 mg/kg). The testicles were collected at 4, 6 and 8 weeks after model establishment and weighed. The structure of the spermatic vein was observed under a microscope, and the diameter was measured with scales.

Reverse transcription-quantitative polymerase chain reaction (RT-qPCR) analysis. Total RNA was extracted from testicular tissues using TRIzol® reagent (Invitrogen; Thermo Fisher Scientific Inc., Waltham, MA, USA). RNA quantity and quality were tested with NanoDrop 1000 (NanoDrop; Thermo Fisher Scientific Inc.) and gel electrophoresis. The primers (Table I) for qPCR were designed using Primer Premier 5.0 software and synthesized by Generay Biotech Co., Ltd. (Shanghai, China). cDNA synthesis was performed using the ReverTra Ace® qPCR RT kit (Toyobo Life Science, Osaka, Japan) according to the manufacturer's protocol. The reverse transcription reaction was performed at 65˚C for 5 min, 37˚C for 15 min and 98˚C for 5 min. qPCR was performed using the KAPA SYBR Green Supermix PCR kit (Kapa Biosystems; Roche Diagnostics, Indianapolis, IN, USA) according to the kit protocol, with AriaMx Real-Time PCR System (Agilent Technologies, Inc., Santa Clara, CA, USA). The thermocycling conditions were as follows: 95˚C for 30 sec, followed by 40 cycles of 95˚C for 20 sec and 61˚C for 30 sec. The relative expression of different genes was determined using the $2^{-\Delta\Delta Cq}$ algorithm (21).

Immunohistochemistry (IHC). Testicular tissues were fixed with 4% paraformaldehyde for 24 h at 4˚C, embedded in paraffin and cut into 5 μm thick sections by Servicebio (Shanghai, China). Sections were blocked in 5% BSA for 1.5 h at 37˚C (Beyotime Institute of Biotechnology, Shanghai, China). The antibodies were purchased from Abcam (Cambridge, UK) and included anti-TGF-β1 (cat. no. ab9758; 1:1,000), anti-claudin-11 (cat. no. ab7474; 1:1,000) and horseradish peroxidase-conjugated secondary antibody (cat. no. ab6721; 1:1,000). Detailed experimental steps were performed as described by Jasinski-Bergner *et al* (22). Scoring of the expression of claudin-11 and TGF-β1 was performed by two independent pathologists as described by Sewify *et al* (23).

Statistical analysis. All data are expressed as the mean ± standard deviation unless otherwise specified. GraphPad Prism 5 (GraphPad Software, Inc., La Jolla, CA, USA) was used to perform statistical analysis for all results. Significance between groups was evaluated by one-way analysis of variance. Each experiment of RT-qPCR and IHC was performed three times. $P<0.05$ was considered to indicate a statistically significant difference.

Results

VC modelling. The diameter of the spermatic vein in the VC model group was significantly larger compared with that in the NC group, and further increased with time ($P<0.001$; Fig. 1). The left testicular weight in the 6W-VC group was significantly decreased compared with the right testicular weight (1.36±0.05 vs. 1.46±0.08 g, respectively; $P<0.05$), and the left testicular weight in the 8W-VC group was significantly decreased compared with the right testicular weight (1.35±0.05 vs. 1.50±0.06 g, respectively; $P<0.001$; Fig. 2). These results indicated successful construction of the VC model in rats.

RT-qPCR of claudin-11. There was no statistically significant difference in mRNA levels of claudin-11 between the SHAM and VC groups at 4 weeks. However, the expression of claudin-11 was significantly downregulated in 6W-VC-L compared with 6W-SHAM-L ($P<0.05$). In addition, the expression of the claudin-11 was more significantly downregulated in 8W-VC-L compared with the 8W-SHAM-L ($P<0.001$). Notably, there was a statistically significant difference between 8W-VC-L and 8W-VC-R, and between 8W-SHAM-R and 8W-VC-R ($P<0.01$; Fig. 3).

IHC of claudin-11 and TGF-β. The expression of claudin-11 was further analysed in 32 stained paraffin-embedded tissue samples of rat testicular tissue (Fig. 4). The expression of claudin-11 was downregulated in the VC model compared with the NC group ($P<0.01$), and there was a significant difference between the diseased side (left) and the healthy side (right). The expression level of TGF-β in the VC model group was significantly higher compared with that in the SHAM group at week 6 ($P<0.01$), and more significantly increased at week 8 ($P<0.001$). Compared with the normal side (VC-R), the operated side (VC-L) also exhibited significantly higher expression of TGF-β in all three time points ($P<0.05$; Fig. 5).

Table I. Primer sequences used in the reverse transcription-quantitative polymerase chain reaction.

Primer	Direction	Sequence (5'→3')
Claudin-11	F	TTAGACATGGGCACTCTTGG
	R	ATGGTAGCCACTTGCCTTC
GAPDH	F	CAAGTTCAACGGCACAGTCAAG
	R	ACATACTCAGCACCAGCATCAC

F, forward; R, reverse.

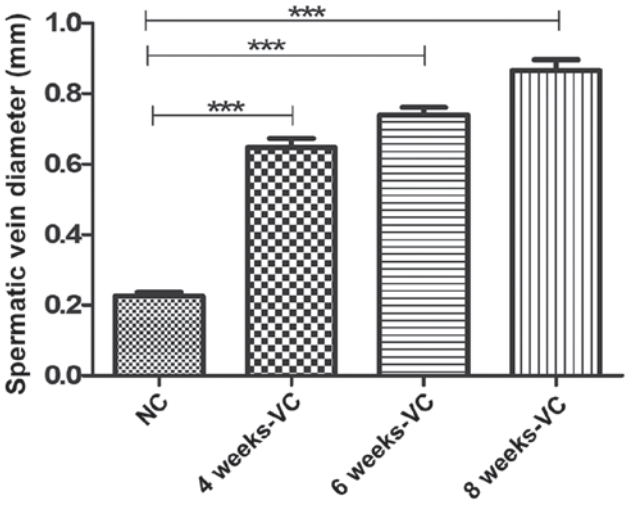

Figure 1. Spermatic vein diameter of different groups. The spermatic vein diameter was significantly higher in the VC model groups compared with the NC group. ***P<0.001. NC, normal control group; VC, varicocele.

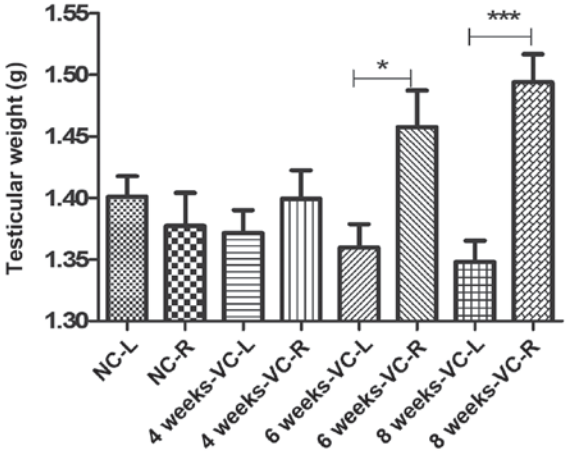

Figure 2. Testicular weight was significantly higher on the right healthy side compared with the left diseased side. *P<0.05; ***P<0.001. L, left; R, right; NC, normal control group; VC, varicocele.

Discussion

In the present study, a VC rat model was established to study the molecular and histological changes in the testes in the early phases of VC development. The pathogenic process was initiated by disruption of the BTB.

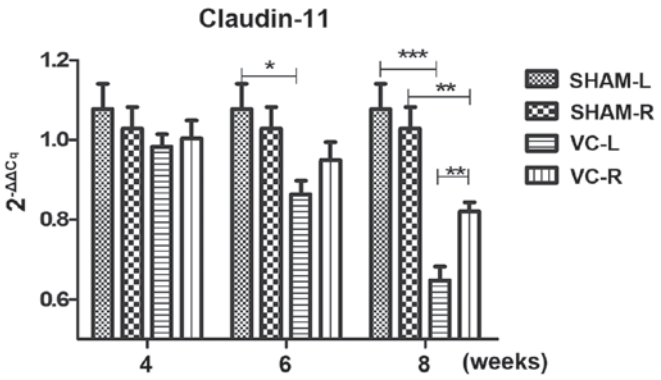

Figure 3. Expression of claudin-11 at the mRNA level. *P<0.05; **P<0.01; ***P<0.001. SHAM, sham surgery control group; L, left diseased side; R, right healthy side; VC, varicocele.

As rats are commonly used in BTB disruption studies (24,25), a classic VC rat model was established to mimic damage to the BTB in humans, and determine the effect in claudin-11 in the variceal and contralateral testis at different time points postoperatively (12).

RT-PCR revealed that the expression of claudin-11 was downregulated in VC rats at 6 and 8 weeks, and the diseased side was more significantly affected compared with the healthy side at week 8. The same trends were observed on IHC examination. In addition, the expression of claudin-11 decreased gradually over time.

The histopathological examination revealed increasing disruption of the integrity of the BTB over time, and unilateral VC also affected contralateral testicular function. Oh *et al* (26) reported that an increase in the levels of pro-inflammatory cytokines may be caused by deregulation of claudin-11 expression in the Sertoli cells of VC testes, leading to alterations in the permeability of the BTB and immunological barriers to normal spermatogenesis. The results of previous studies (24,27) suggest that VC-induced male infertility is mediated by damage to the BTB.

The results of the current study also demonstrated that the expression of TGF-β increased in VC rats, which was not limited to the diseased side, but also gradually affected the healthy side over time. TGF-β was reported to be associated with fibrosis of the seminiferous tubules and disruption of spermatogenesis. In the testis, TGF-β is known to stimulate collagen and fibronectin, further triggering the production of extracellular matrix (28). During puberty, the expression of TGF-β is regulated by hormonal influences and is involved in steroidogenesis and spermatogenesis (29,30).

In conclusion, the present study partially explained the histopathological and molecular mechanisms underlying the pathogenesis of VC, and the findings may be useful in the treatment of male infertility.

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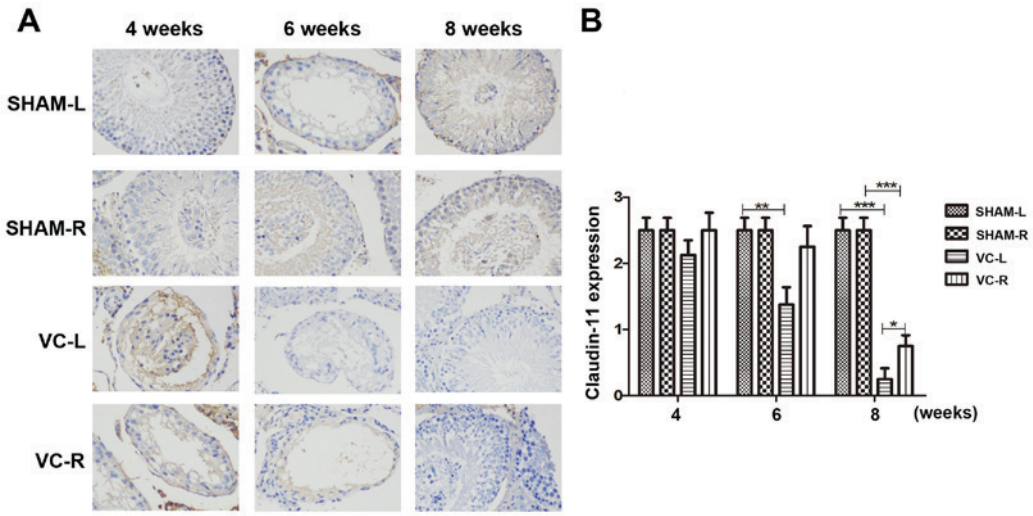

Figure 4. Claudin-11 expression in rat testicular tissue. (A) Immunohistochemical examination of claudin-11 expression in Sprague-Dawley rat testicular tissue. (B) Scoring of the expression of claudin-11. *P<0.05; **P<0.01; ***P<0.001. SHAM, sham surgery control group; L, left diseased side; R, right healthy side; VC, varicocele.

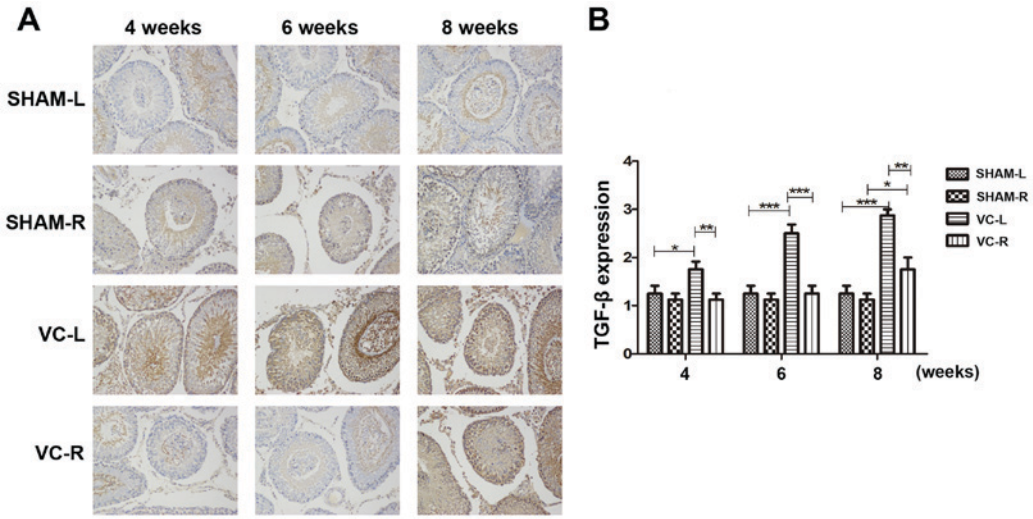

Figure 5. TGF-β expression in rat testicular tissue. (A) Immunohistochemical examination of TGF-β in Sprague-Dawley rat testicular tissue. (B) Scoring of the expression of TGF-β. *P<0.05; **P<0.01; ***P<0.001. TGF-β was more highly expressed in the VC-L compared with all other groups at all three time points. SHAM, sham surgery control group; L, left diseased side; R, right healthy side; VC, varicocele; TGF-β, transforming growth factor-β.

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Availability of data and materials

The datasets used and/or analyzed during the present study are available from the corresponding author on reasonable request.

Authors' contributions

JP was involved in all aspects of the research. ZZ and JY were involved in the animal experiments and data analysis. GX, LN, LY and ZGL were involved in RT-qPCR and IHC. All the authors have read and approved the final version of this manuscript.

Ethics approval and consent to participate

The animal experiments were performed in accordance with the guidelines of the Ethics Committee of Zhejiang University (Shaoxing, China), and the animals were handled according to the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health. The experiment was approved by the Animal Care and Use Committee of Zhejiang University (ZDSX2017-0012).

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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