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REGULAR ARTICLE

Effect of silencing HIF-1α gene on testicle spermatogenesis function in varicocele rats

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Abstract

This study uses the CRISPR/Cas9 gene editing technique to silence the expression of hypoxia-inducible factor-1α (HIF-1α) gene and investigate its effect on testicle spermatogenesis function in varicocele (VC) rats. Sprague Dawley rats were divided into four groups; the control, VC model, VC+HIF-1α-lentivirus and VC+Luciferase-lentivirus group. The sperm count and survival rate were analyzed using computer-aided sperm analysis. The morphological changes of seminiferous tubules were observed by a microscope. Expressions of HIF-1α, Bax, cleaved caspase-3 and Bcl-2 were detected via Western blot, immunofluorescence and real-time polymerase chain reaction methods. One-way ANOVA was used to analyze the differences between groups. The sperm count and survival rate were significantly lower ($p < 0.05$) and the seminiferous epithelium was more disordered in the VC group than that in the control group. The expression of Bax and cleaved caspase-3 were increased and Bcl-2 was reduced in the VC group than the control group. Compared with the VC group, sperm count and survival rate noticeably increased ($p < 0.05$), seminiferous epithelium was inordered arrangement and fewer spermatogenic cells were injured in the VC+HIF-1α-lentivirus group. Expression of Bax and cleaved caspase-3 were decreased significantly in the VC+HIF-1α-lentivirus group compared with the VC group and VC+Luciferase-lentivirus group ($p < 0.05$), whereas the expression of Bcl-2 was increased ($p < 0.05$). No significant difference was observed between the control group and the VC+HIF-1α-lentivirus group ($p > 0.05$). Results show that the apoptosis of spermatogenic cells was decreased and the testicle spermatogenesis function was significantly improved after silencing HIF-1α gene in testis of VC rats. HIF-1α may play a crucial role during spermatogenesis in VC inducing male infertility.

Keywords HIF-1α · Varicocele · Spermatogenic cell function · Apoptosis

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Introduction

Varicocele (VC) is a vascular disease characterized by varying degrees of dilation and tortuosity of the spermatic veins (Sheehan et al. 2014). Several reports have shown that 20–40% of men have primary infertility problems and those with secondary infertility account for up to 80% (Qin et al. 2017). The present mechanisms of male infertility caused by VC include scrotal hyperthermia, reflux of adrenal and renal metabolite, oxidative stress and hypoxia (Sheehan et al. 2014; Razi and Malekinejad 2015; Alsaikhan et al. 2016). These mechanisms eventually lead to decreased proliferation of germ cells and increased apoptosis (Shiraishi et al. 2012). Cell apoptosis is a type of programmed cell death characterized by gene regulation and energy dependence (Liu et al. 2013). Apoptosis of normal spermatogenic cells plays a key role in spermatogenesis (Schmidt et al. 1995). Many studies have found that VC could induce ascending apoptosis of testicular spermatogenic cells and abnormal increase of apoptosis may lead to lower testicular function and male infertility (Xu et al. 2016; Wang et al. 2015).

Testicular hypoxia is an important pathophysiological feature in VC rats and infertile men with varicocele (Wang et al. 2010; Ghandehari-Alavijeh et al. 2019). Hypoxia-inducible factor-1 (HIF-1) is a highly specific nuclear transcription factor that plays an active role under hypoxia (Cai et al. 2014). HIF-1α is the active subunit of HIF-1 and the physiological activity of HIF-1 mainly depends on the activity and expression of HIF-1α subunit. A positive correlation exists between the expression of HIF-1α and the degree of hypoxia (Krohn et al. 2008; Semenza 2009). Data showed that the expression of HIF-1α increased and the apoptosis of spermatogenic cells in testis of VC rats was significantly higher than that in normal rats (Zhu et al. 2017; Xu et al. 2016). Ghandehari et al. found that high expression of HIF-1α along with DNA damage induces apoptosis in asthenozoospermia with VC patients, which suggested the hypoxia pathway may play a role in asthenozoospermia (Ghandehari-Alavijeh et al. 2019). Therefore, we speculate that HIF-1α may be involved with the apoptosis of germ cells and play an important role in male infertility caused by VC. This study aims to elucidate the role of HIF-1α in modulating the apoptosis of spermatogenic cells to explore its potential biological significance in spermatogenesis by silencing the expression of HIF-1α in testis of VC rats.

Materials and methods

Animals

Thirty male Sprague Dawley rats weighing 250–300 g were used in this study. All rats were fed the same diet and raised in a constant environment with a 12-h light/dark cycle. The rats were randomly divided into two groups: control group (C group; $n = 6$; not treated with initial surgery) and VC model group (V group; $n = 24$). All animals in the VC model group were subdivided randomly into three groups: V group ($n = 6$), VC+HIF-1α-lentivirus group (H group; $n = 9$; 2 months post-surgery of VC for silencing HIF-1α gene) and VC+Luciferase-lentivirus group (L group; $n = 6$, 2 months post-surgery of VC for silencing luciferase gene, this group is the control of the H group). One rat in the V group, two rats in the H group and one rat in the L group were excluded because of death or unsuccessful surgery. All animal experiments conformed with the Guide for Care and Use of Laboratory Animals and were approved by Shanxi Provincial People's Hospital.

Establishment of the VC model

The rats of the VC model were referenced from Turner (Turner 2001). The rats were generally anesthetized with an intraperitoneal injection of pentobarbital sodium 40 mg/kg. An abdominal midline incision was performed. The left renal vein, inferior vena cava, left spermatic vein, left suprarenal vein and left kidney were exposed. Following a careful blunt dissection, a clamp was passed behind the left renal vein just distal to the spermatic vein insertion, a loose ligature of 4-zero silk was placed around the left renal vein at the site and a rigid hydrophilic 0.8-mm-diameter guide wire was placed on the left renal vein. The ligature was tied around the vein over the top of the guide wire and the wire was withdrawn, allowing the vein diameter to be reduced to approximately half of normal. After the injection of penicillin into the abdominal cavity, the midline incision was closed in two layers using 4/0 silk sutures. The model was considered successfully established when the diameters of the left internal spermatic veins were measured with an increase of 2-fold or more. The animals in the C group and V group were sacrificed 60 days after the experimental varicocele was constructed.

Preparation of lentivirus

HIF-1α lentivirus and luciferase lentivirus were prepared from Hesheng Company (Beijing, China). The main processes were as follows: The sgRNA sequence was annealed and phosphorylated to form double-stranded sgRNA, which was ligated to the lentiCRISPR-v2 expression vector that was digested by Bbs 1 and transformed into *E. coli* DH5α-competent cells to proliferate. PCR was used to verify the connection effect of sgRNA. The correctly ligated plasmids were cotransfected into 293 T cells with packaging plasmids pMD2.G and psPAX2. The lentivirus solution was collected after 48 and 72 h, respectively. The titer of the virus was

measured and more than 1×10^9 TU/ml lentivirus solution was selected to infect rat testis.

Infection of lentivirus on testicles of VC rats

The 60 μl lentivirus was incubated at room temperature for 30 min. Rats were anesthetized by intraperitoneal injection of 1% of pentobarbital sodium (40 mg/kg). Next, 75% alcohol was used to sterilize the scrotum, the testicles were exposed by pressing the rat's abdomen lightly and a 1-ml syringe with a needle was used to thrust into the testicles and avoid blood vessels. The HIF-1α and luciferase lentiviruses were injected slowly into the testicles of rats in the H and L groups, respectively. After injection, the rats were awakened at room temperature. The animals in the H group and L group were sacrificed 2 months after the lentivirus was infected.

Sperm count and survival rate

The left cauda epididymis from rats was cut off and the spermatozoa were collected by the diffusion method after rinsing with normal saline. The left cauda epididymis tissues were cut into pieces and were placed in normal saline in a constant-temperature water incubator at 37 °C for 10 min. The 50 μl spermatozoa suspension was added into 3 ml preheated normal saline and 10 μl of diluted spermatozoa suspension was dropped onto the counting plate. Sperm concentration and sperm motility were measured by computer-aided sperm analysis.

Hematoxylin and eosin staining

After fixation in 4% paraformaldehyde, one-third left testicle tissue was embedded in paraffin and was cut into 4 μm sections. After routine dewaxing and hydration, sections were stained with hematoxylin for 5 min and eosin for 2 min at room temperature. The morphology of seminiferous tubules was observed under an optical microscope.

Western blot analysis

The proteins were extracted from the left testicle tissue of rats. In total, 100 μg of protein was subjected to 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis and transferred onto polyvinylidene fluoride membranes (EMD Millipore Corporation, Billerica, MA, USA). Membranes were blocked in tris-buffered saline containing 0.1% Tween 20 and 5% nonfat milk powder (TBST) for 3–4 h at room temperature, then incubated with the primary antibody overnight at 4 °C. Antibodies used were as follows: anti-HIF-1α (1:1000; ab2185; Abcam, Cambridge, UK), anti-Bax (1:500; sc-526; Santa Cruz Biotechnology Inc., CA, USA), anti-cleaved caspase-3 (1:1000; 9661S; Cell Signaling Technology, Inc., USA), anti-Bcl-2 (1:200; sc-7382; Santa Cruz Biotechnology Inc., CA, USA) and anti-β-actin (1:5000; I102; Bioworld Technology, Co. Ltd. USA). Four washes with TBST for 10 min each followed. The membranes were then incubated with HRP-conjugated secondary antibody (1:5000; BA1054; Boster Biotechnology Co., Wuhan, China) for 1 h at room temperature. After washing, the specific proteins were visualized by enhanced chemiluminescence and imaged by Chemi DOC XRS$^+$ (Bio-Rad Laboratories, Inc., Hercules, CA, USA). The relative intensity and density of immunoreactive bands was measured using Image J software (National Institute of Health, MD, USA). The relative expression of goal protein was normalized with β-actin as a control. To obtain values of fold change from the control, we divided the relative expression of the other groups by the relative expression of the corresponding control group. Therefore, the fold-change value for the control group would be one (Afiyani et al. 2014).

Immunofluorescence analysis

Paraffin sections of 2 μm were heated at 50–60 °C for 2 h using dimethyl-benzene dewaxing twice for 10 min each, then placed in absolute ethyl alcohol, gradient alcohol and distilled water for 10 min each. Three washes with phosphate-buffered saline (PBS) for 10 min each followed. Antigen repair was performed by microwave treatment in 0.01 M sodium citrate buffer (pH 6.0), at 95 °C for 15 min and the sections were cooled at room temperature for 30 min. Blocking buffer with 5% goat serum was used to block the nonspecific site. The slides were incubated with anti-HIF-1α antibody (1:50; ab2185; Abcam, Cambridge, UK), anti-Bax antibody (1:200; sc-526; Santa Cruz Biotechnology Inc., CA, USA), anti-Bcl-2 antibody (1:200; A16776; ABclonal, Wuhan, China) and anti-active caspase-3 antibody (1:500; ab49822; Abcam, Cambridge, UK) overnight at 4 °C. As a negative control, PBS was used. After three washes with PBS for 10 min each, slides were incubated with an Alexa Fluor secondary antibody (Life Technologies, Carlsbad, USA) for 1 h at room temperature. The nucleus was stained by 4′,6-diamidino-2-phenylindole for 5 min after three washes with PBS for 10 min each and the sections were examined under a fluorescence microscope. The intensity and density of positive immunostaining cells from different rats in each group was analyzed with Image J software (National Institute of Health, MD, USA). The results were converted to numerical values to compare the relative protein abundance of the immunoreactive bands. To obtain values of fold change from the control, we divided the relative expression of the other groups by the

relative expression of the corresponding control group (mean = 1).

RT-qPCR analysis

Total *RNA* was isolated from left testis tissue using Trizol reagent and the *RNA* concentration was measured by spectrophotometry. Next, 500 ng of the template *RNA* was reverse-transcribed using a reverse transcription kit (Takara Bio Inc., Japanese). The primers were designed as shown in Table 1. Real-time PCR (Applied CFX96™ Real-Time PCR Detection System, Bio-Rad) was initiated by heating at 95 °C for 30 s, followed by 40 cycles (95 °C for 5 s, 60 °C for 30 s). The primer sequences were synthesized by Invitrogen (Carlsbad, USA). The results were obtained using at least three separate samples. β-actin was the housekeeping gene as control. The difference in gene expression was calculated according to the $2^{-\triangle\triangle Ct}$ method (Livak and Schmittgen 2001). All PCR amplifications were performed in duplicate and mean values were calculated. To obtain values of fold change from the control group, we divided the relative expression of the other groups by the relative expression of the corresponding control group (mean = 1).

Statistical analysis

All the statistical analysis and graph preparation were performed using IBM SPSS Statistics version 21.0 (SPSS Inc., Chicago, IL, USA) or Microsoft Excel 2016 (Microsoft Corporation, Redmond, WA, USA) or GraphPad Prism 6 software (GraphPad Software, Inc., La Jolla, CA, USA). All data were presented as the mean ± standard deviation. One-way ANOVA was used to compare the means of the different groups. The LSD test and Dunnett T3 test were applied for homogeneity of variance and a variance heterogeneity, respectively. $p < 0.05$ was considered to indicate a statistically significant difference. All experiments were performed at least three times.

Results

Silence of HIF-1α on testicle of VC rat

The immunofluorescence results show that HIF-1α was mainly expressed in the cytoplasm of spermatogenic cells, especially in spermatocytes (Fig. 1a–l). Western blot and immunofluorescence results show that the expression levels and positive rates of HIF-1α were significantly increased in the V group compared with those in the C group ($p < 0.05$). No significant difference of HIF-1α expression level was observed in the V group and L group ($p > 0.05$) but HIF-1α lentivirus reduced the expression of HIF-1α protein in the H group (Fig. 2a–c). Similar results were obtained when the mRNA level of HIF-1α was measured by RT-PCR ($p < 0.05$). No noticeable difference was observed between the C group and H group ($p > 0.05$) (Fig. 2d). These results indicate that the lentivirus effectively suppressed HIF-1α expression at *mRNA* and protein levels.

Sperm count and survival rate

The mean sperm concentration in the V group was 1.92 ± 0.53, which was significantly lower than that in the C group (4.10 ± 0.75). No noticeable difference was observed between the C group and H group (3.81 ± 0.60). The mean sperm concentrations in the V group and L group (2.05 ± 0.75) were significantly lower than those in the H group ($p < 0.05$). Compared with the C group and H group, the epididymal sperm survival rate was markedly decreased in the V group and in the L group ($p < 0.05$). No significant difference was observed between the C group and H group ($p > 0.05$) (Table 2).

Morphological changes in seminiferous tubules

Histological morphology demonstrated that the germ cells of the seminiferous tubules in the C group were arranged in

Table 1 Summary of RT-PCR primers used in this study

Gene	Primer sequences (5′-3′)	Length (bp)
HIF-1α	F: CGGAAACTGAAGACCAACAAC R: CAGAGGCAGGTAATGGAGACA	114
Bax	F: TTTCCTACTTCGGGACCCCC R: GAAGCCTCAGCCCATCTTCTT	101
Bcl-2	F: GAACTGGGGGAGGATTGTGG R: GGGGTGACATCTCCCTGTTG	80
Cleaved-caspase-3	F: TCTACCGCACCCGGTTACTA R: CGTACAGTTTCAGCATGGGCG	88
β-actin	F: CCCATCTATGAGGGTTACGC R: TTTAATGTCACGCACGATTTC	150

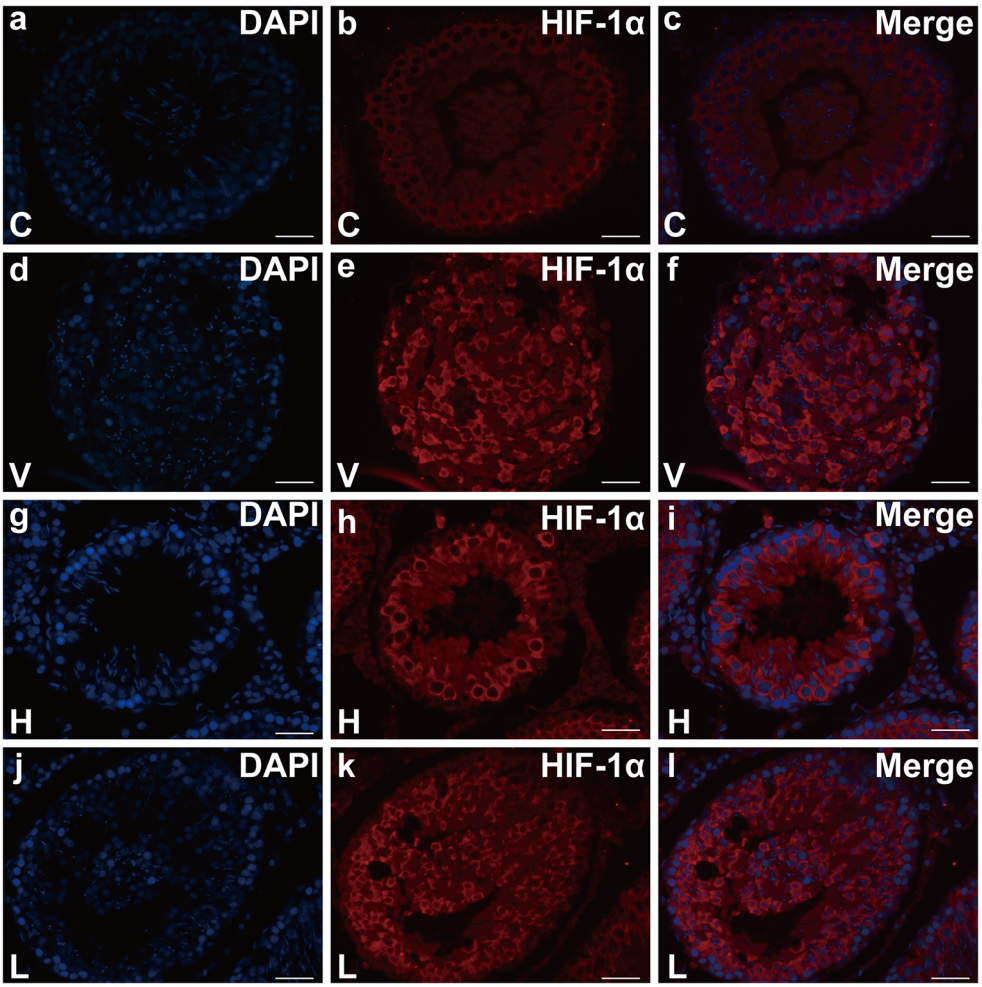

Fig. 1 Immunofluorescence was used to detect the expression and location of HIF-1α protein on seminiferous tubules in the control group (**a**–**c**), varicocele group (**d**–**f**), VC+HIF-1α-lentivirus group (**g**–**i**) and VC+Luciferase-lentivirus group (magnification, × 400) (**j**–**l**), scale bar is 25 μm, DAPI = 4′6-diamidino-2-phenylindole. C, control group; V, varicocele group; H, VC+HIF-1α-lentivirus group; L, VC+Luciferase-lentivirus group

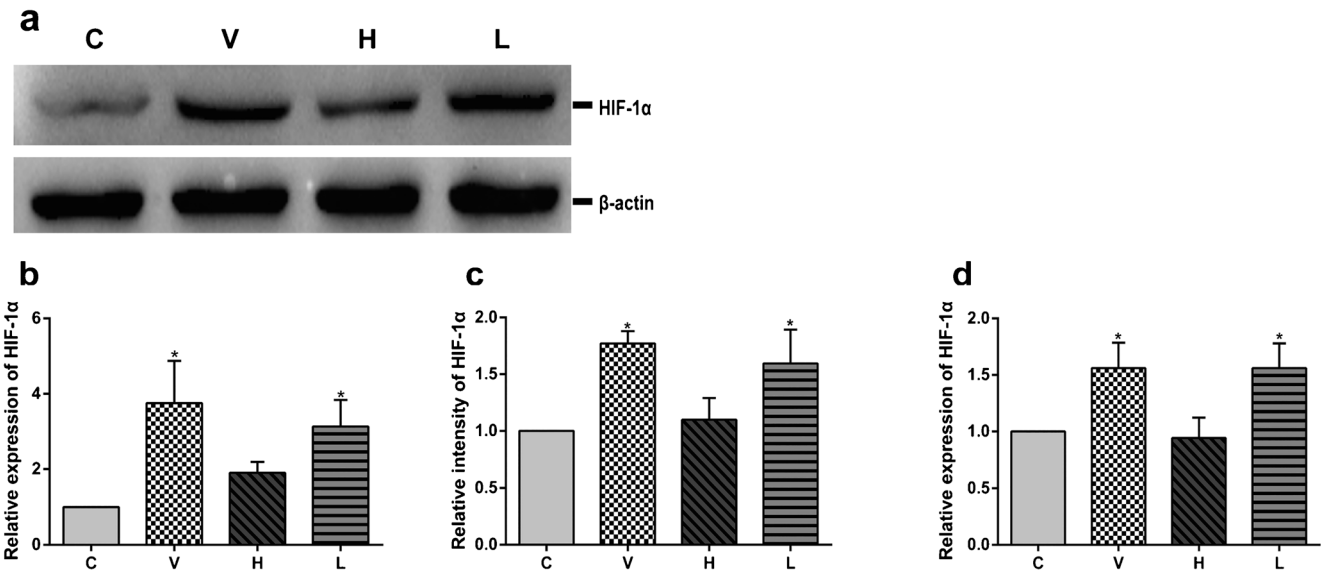

Fig. 2 To detect the effect of HIF-1α gene silence on the testicle of VC rats, Western blot (**a**, **b**), immunofluorescence (**c**) and RT-PCR methods (**d**) were used. **a** Analysis of HIF-1α protein expression by Western blot, β-actin was used as loading control. **b**, **c** The expression of HIF-1α was semiquantified by densitometry (**b**) and was semiquantified by fluorescent intensity of positive cells and densitometry(**c**). **d** mRNA expression levels of HIF-1α relative to β-actin were studied using RT-PCR methods. C, control group; V, varicocele group; H, VC+HIF-1α-lentivirus group; L, VC+Luciferase-lentivirus group. *$p < 0.05$ versus C group and H group

Table 2 Sperm concentration and survival rate in the C, V, H and L groups

Group	Sperm concentration($\times 10^6$/ml)	Sperm survival rate
C	4.10 ± 0.75	0.55 ± 0.04
V	1.92 ± 0.53*	0.35 ± 0.08*
H	3.81 ± 0.60	0.58 ± 0.06
L	2.05 ± 0.75*	0.38 ± 0.05*

C, control group; *V*, varicocele group; *H*, VC+HIF-1α-lentivirus group; *L*, VC+Luciferase-lentivirus group. *$p < 0.05$ versus C group and H group

order, and some sperms dispersed in the lumen (Fig. 3a–c). In the V group, the seminiferous epithelium was extensively injured and the germ cells were disorderly distributed. Germ cells were fewer and the testicular spermatogenic function was significantly damaged (Fig. 3d–f). Fewer seminiferous epithelium and germ cells were changed in the H group than in the V group but no noticeable difference was observed between the C group and H group (Fig. 3a–i). However, the seminiferous epithelium was disorganized and germ cells were seriously reduced in the L group (Fig. 3j–l).

mRNA expression of Bax, cleaved caspase-3 and Bcl-2

The mRNA expression levels of Bax and cleaved caspase-3 were significantly higher in the V group and L group than in the C group and H group ($p < 0.05$) (Fig. 4a, b). However, compared with the C group and H group, the mRNA expression levels of Bcl-2 were decreased in the V group and L group ($p < 0.05$) (Fig. 4c). No significant difference was observed between Bax, cleaved caspase-3 and Bcl-2 expression levels in the C group and H group ($p > 0.05$) (Fig. 4).

Protein expression of Bax, cleaved caspase-3 and Bcl-2

The immunofluorescence results show that Bax and Bcl-2 were mainly expressed in the cytoplasm of all levels of spermatogenic cells (Figs. 5 and 6). Cleaved caspase-3 was mainly expressed in the nucleus of prespermatid (Fig. 7). Western blot and immunofluorescence results show that pro-apoptotic proteins such as Bax and cleaved caspase-3 revealed higher levels in the V group compared with those in the C group ($p < 0.05$).

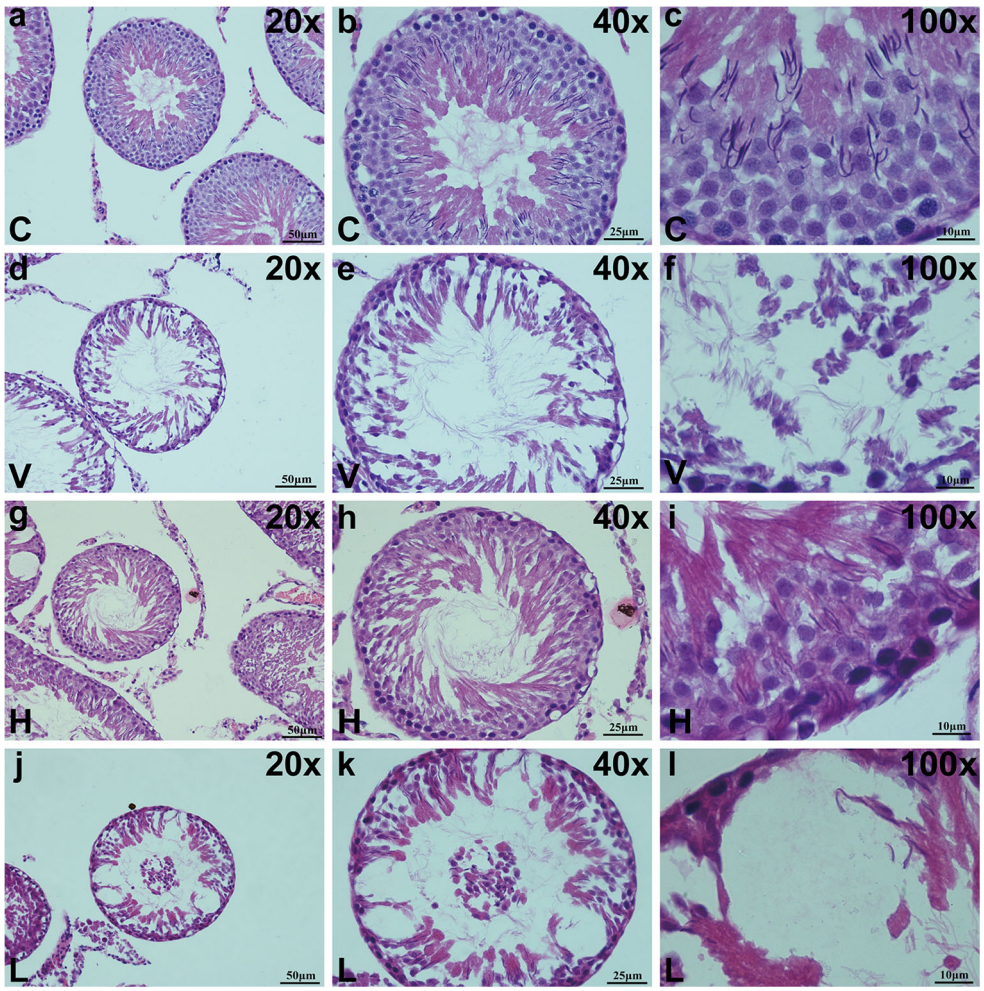

Fig. 3 Upper panel shows the morphology of seminiferous tubules. Testis tissues in the control group (**a–c**), varicocele group (**d–f**), VC+HIF-1α-lentivirus group (**g–i**) and VC+Luciferase-lentivirus group (**j–l**) were analyzed using hematoxylin and eosin stain (magnification, × 200, × 400, × 1000). C, control group; V, varicocele group; H, VC+HIF-1α-lentivirus group; L, VC+Luciferase-lentivirus group

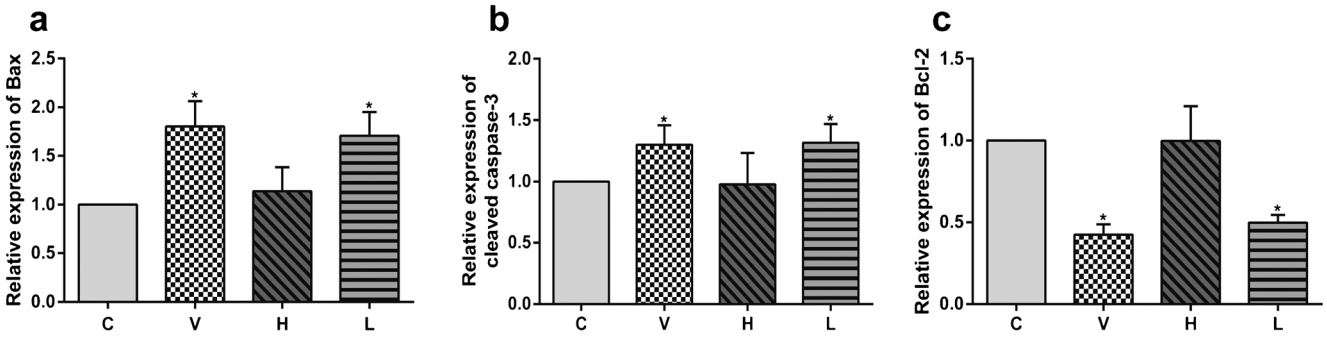

Fig. 4 mRNA expression levels of Bax (**a**), cleaved caspase-3 (**b**) and Bcl-2 (**c**) relative to β-actin were studied using reverse transcription-quantitative polymerase chain reaction. C, control group; V, varicocele group; H, VC+HIF-1α-lentivirus group; L, VC+Luciferase-lentivirus group. *$p < 0.05$ versus C group and H group

However, the relative quantity and positivity of Bax and cleaved caspase-3 in the H group were clearly decreased compared to the L group ($p < 0.05$) (Figs. 8a, b and 9a–c). By contrast, the expression levels of Bcl-2 in the V group and L group were lower than in the C group and H group ($p < 0.05$) (Figs. 8c and 9d). No significant difference was observed in Bax, cleaved caspase-3 and Bcl-2 expression levels between the C group and H group ($p > 0.05$) (Figs. 8 and 9).

Discussion

VC has been shown to cause serious damage of testicular spermatogenesis (Du and Gao 2013). In this study, we found that the seminiferous epithelium of testis was disordered and that the germ cells were seriously decreased. Moreover, the sperm concentrations were clearly reduced and the sperm survival rate was markedly decreased in the V group

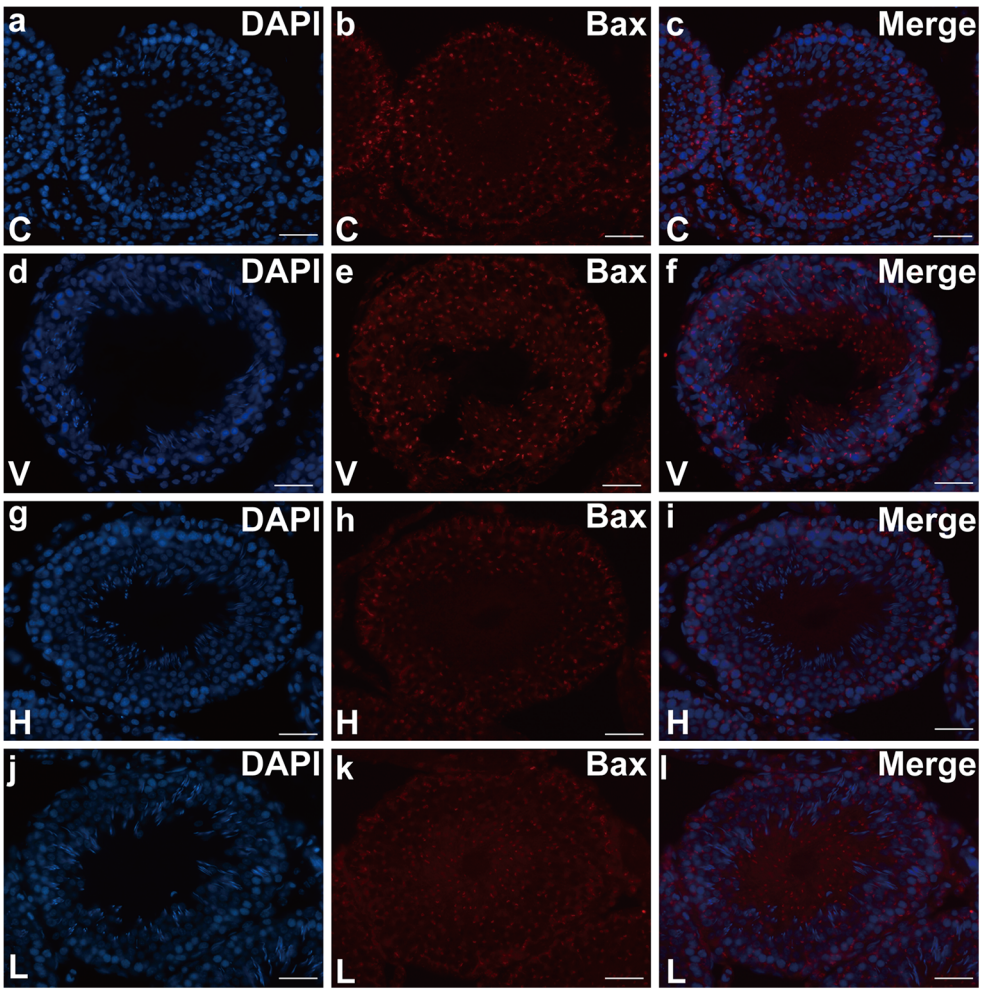

Fig. 5 Immunofluorescence was used to detect the expression and location of Bax protein on seminiferous tubules in the control group (**a**–**c**), varicocele group (**d**–**f**), VC+HIF-1α-lentivirus group (**g**–**i**) and VC+Luciferase-lentivirus group (magnification, × 400) (**j**–**l**), scale bar is 25 μm, DAPI = 4′6-diamidino-2-phenylindole. C, control group; V, varicocele group; H, VC+HIF-1α-lentivirus group; L, VC+Luciferase-lentivirus group

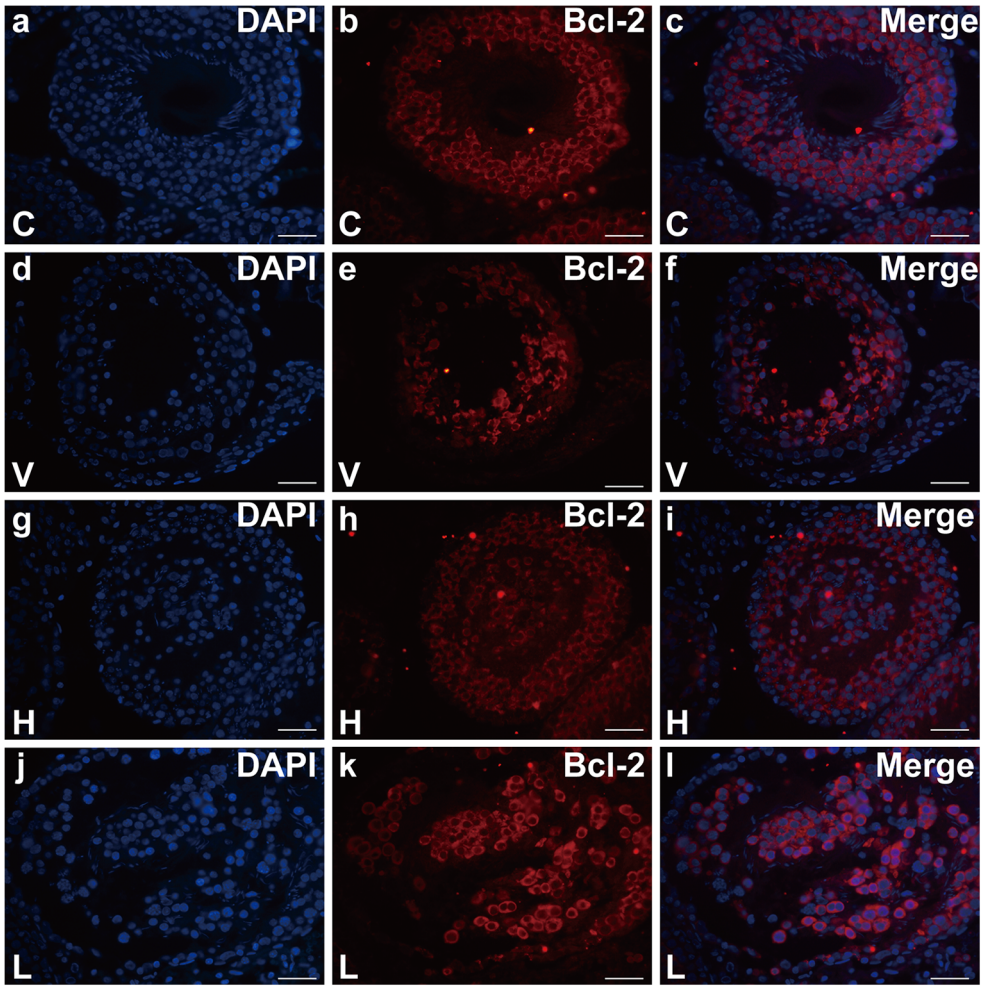

Fig. 6 Immunofluorescence was used to detect the expression and location of Bcl-2 protein on seminiferous tubules in the control group (**a–c**), varicocele group (**d-f**), VC + HIF-1α-lentivirus group (**g-i**) and VC+ Luciferase-lentivirus group (magnification, × 400) (**j–l**), scale bar is 25 μm, DAPI = 4′6-diamidino-2-phenylindole. C, control group; V, varicocele group; H, VC+HIF-1α-lentivirus group; L, VC+Luciferase-lentivirus group

compared with that in the C group, which is consistent with what Qin et al. (2017) reported.

By silencing the expression of HIF-1α gene, the seminiferous epithelium was relatively orderly arranged, the number of germ cells was increased, and the sperm density and motility were recovered in the H group. Therefore, the quality of sperm was improved and the structure and function of testis were restored by reducing the expression of HIF-1α gene. How does HIF-1α gene mediate the testicular spermatogenesis function in VC rats?

Testicular hypoxia was an important factor for VC patients (Lee et al. 2006) and HIF-1α was produced by tissue hypoxia (Hu et al. 2015). During hypoxia, the degradation of HIF-1α protein was blocked, which could activate downstream genes and induce angiogenesis, the expression of glucose transferase and glycolytic enzyme (Semenza 2009). Our study found that the expression of HIF-1α in testis of VC rats was significantly higher than that of normal rats in the mRNA level and protein level ($p < 0.05$), which is consistent with what Goren et al. (2017) reported.

VC was associated with abnormal apoptosis of spermatogenic cells (Wang et al. 2010). As an important transcriptional regulator in cells, HIF-1α had both anti-apoptosis and pro-apoptotic effects in the process of cell apoptosis (Zhang et al. 2016). Overexpression of HIF-1α in rat spinal cord injury model could reduce the apoptosis of spinal cord cells (Chen et al. 2013). However, the expression levels of HIF-1α on hypoxia/reoxygenation-induced apoptosis in primary neonatal rat cardiomyocytes was high. When the RNA interference technique was used to decrease the expression of HIF-1α, cardiomyocyte apoptosis decreased significantly (Wang et al. 2012), which suggested that HIF-1α played an important role in promoting apoptosis. Hypoxia in VC rats was related to the increase of testicular temperature. It was found that hypoxia could significantly increase the expression of heat shock protein A2 (HSPA2) in the testis of VC rats; the mechanism may be that HIF-1 binds to hypoxic response element 1 (HRE1) on the HSPA2 promoter to activate the transcription of the gene, which disrupts the seminiferous epithelium and primarily affects pachytene spermatocytes and round spermatids. This further leads to increased apoptosis of spermatogenic cells and prevents cell maturation and development resulting in azoospermia (Xia et al. 2009; Afiyani et al. 2014). However, Motiei et al. research found that the

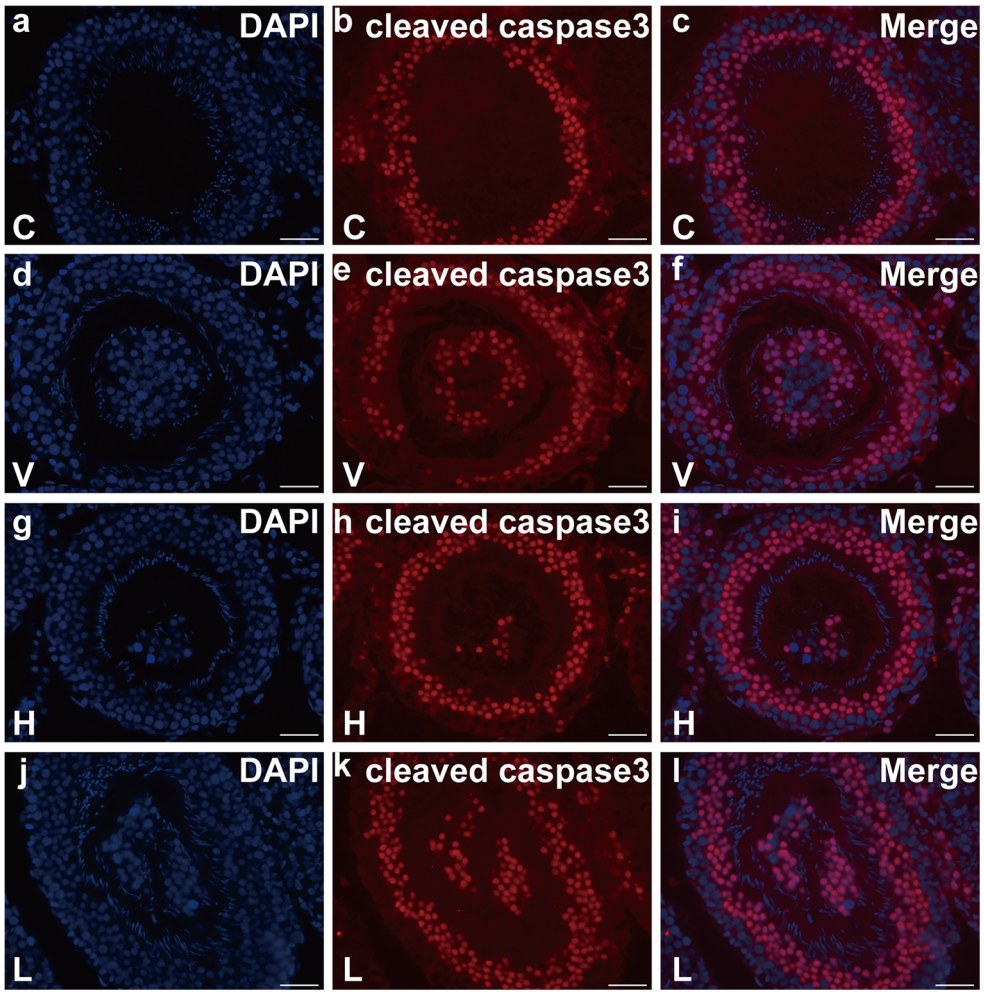

Fig. 7 Immunofluorescence was used to detect the expression and location of cleaved caspase-3 protein on seminiferous tubules in the control group (**a–c**), varicocele group (**d–f**), VC+HIF-1α-lentivirus group (**g–i**) and VC+Luciferase-lentivirus group (magnification, × 400) (**j–l**), scale bar is 25 μm, DAPI = 4′6-diamidino-2-phenylindole. C, control group; V, varicocele group; H, VC+HIF-1α-lentivirus group; L, VC+Luciferase-lentivirus group

expression of HSPA2 in the fertile individuals was higher than infertile individuals and they consider that HSPA2 expression plays an important role in sperm capacitation (Motiei et al. 2013). Therefore, which role HIF-1α played was affected by tissue type and degree of hypoxia (Zhang et al. 2016). Studies have shown that the main role of HIF-1α was harmful rather than beneficial under the hypoxic conditions induced by VC in testicular tissue and the apoptosis index of germ cells in VC rats was positively correlated with the relative intensity of HIF-1α staining in bilateral testis, which indicated that HIF-1α promoted germ cell apoptosis under the condition of testicular hypoxia induced by VC (Wang et al. 2010). Similar studies have found that the expression of HIF-1α was positively correlated with the apoptosis rate of tumor cells and

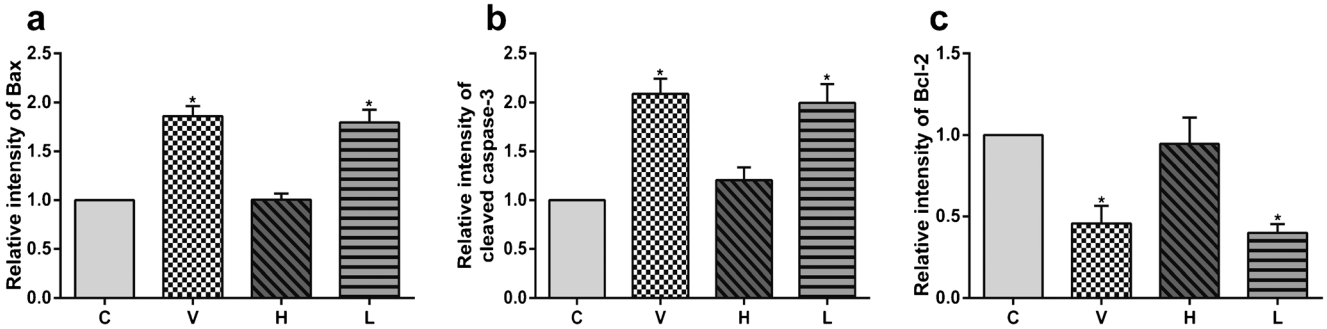

Fig. 8 Expression of Bax, cleaved caspase-3, and Bcl-2 in rat testis tissue were measured with immunofluorescence analysis. The expression of Bax (**a**), cleaved caspase-3 (**b**) and Bcl-2 (**c**) were semiquantified by fluorescent intensity of positive cells. C, control group; V, varicocele group; H, VC+HIF-1α-lentivirus group; L, VC+Luciferase-lentivirus group. *$p < 0.05$ versus C group and H group

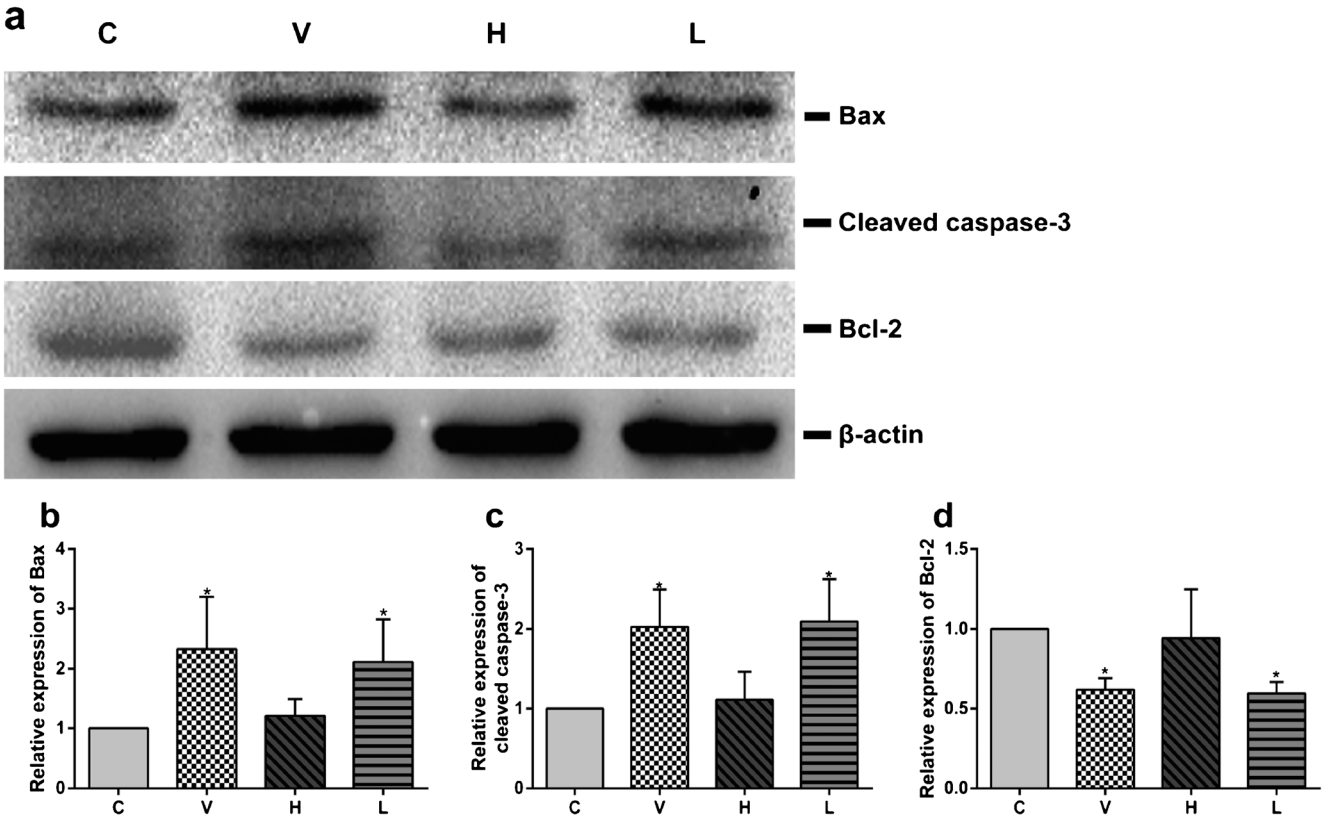

Fig. 9 Protein expression levels of Bax, cleaved caspase-3 and Bcl-2 in rat testis tissue were determined by Western blot. β-actin was used as loading control. **a** Analysis of Bax, cleaved caspase-3 and Bcl-2 protein expression by Western blot. The expression of Bax (**b**), cleaved caspase-3 (**c**) and Bcl-2 (**d**) were semiquantified by densitometry. C, control group; V, varicocele group; H, VC+HIF-1α-lentivirus group; L, VC+Luciferase-lentivirus group. *$p < 0.05$ versus C group and H group

apoptosis promoting factors such as caspase-3 (Volm and Kooma¨gi 2000). In addition, the relationship between autophagy and apoptosis may play an important role in VC infertility. Overexpression of HIF-1α activates the HIF-1α/BNIP3/Beclin1 autophagy signaling pathway during hypoxia, to initiate the autophagy pathway. In the early stages of VC, autophagy can timely remove the damaged organelles and proteins. When cells are seriously damaged, apoptotic pathways are strongly activated to inhibit the autophagy pathway, resulting in increased apoptosis (Zhu et al. 2017). Therefore, HIF-1α was a useful factor in predicting the degree of germ cell apoptosis in rat testis (Wang et al. 2010).

The Bcl-2 family proteins (Bcl-2 and Bax) and caspase family proteins (caspase-3) are important regulatory proteins in cell apoptosis (Liu et al. 2015). The expression of anti-apoptotic protein Bcl-2 was decreased and pro-apoptotic protein Bax was increased in rat testis of VC to activate the expression of caspase-3 (Mostafa et al. 2014). Consistent with this study, we found that the expression of Bax and cleaved caspase-3 in testis of VC rats was significantly higher than those of the control group both at the mRNA level and protein level ($p < 0.05$), when Bcl-2 was reduced. Bcl-2 could inhibit apoptosis by inhibiting the release of cytochrome C from mitochondria outer membrane (Lee et al. 2012). When the expression of Bcl-2 was reduced and the cleaved caspase-3 and Bax were increased, which in turn activated cell apoptosis via a signal transduction pathway and the apoptosis of spermatogenic cells was increased, these eventually lead to spermatogenic dysfunction and male infertility (Ning et al. 2017; Onur et al. 2004; Zhang et al. 2018). In this study, we found that apoptosis-related proteins Bax and cleaved caspase-3 were reduced after the expression of HIF-1α gene was decreased but the expression of anti-apoptotic protein Bcl-2 was increased after the HIF-1α gene expression was reduced.

Through these results, we speculate that VC causes hypoxia in testis, which further induces the expression of HIF-1α and activates downstream signaling molecules, thereby resulting in increased apoptosis of spermatogenic cells, which affects spermatogenesis and leads to male infertility. In our study, by reducing the expression of HIF-1α gene, we found that germ cell apoptosis was decreased, sperm count and sperm motility was significantly increased and testicular spermatogenic function was restored owing to the lower expression of HIF-1α gene that blocked the downstream signaling pathway as well as the apoptosis pathway of Bcl-2 and caspase-3. Therefore, we assert that the overexpression of HIF-

1α can lead to the apoptosis of spermatogenic cells. By decreasing the expression of HIF-1α, the apoptosis of spermatogenic cells was reduced, which is beneficial to the recovery of testicular function and the improvement of fertility. So the decreased expression of HIF-1α may be considered a predictor of the improvement of hypoxia, which provides a new theoretical basis for the treatment of VC infertility.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

Ethical statement All authors declare that this research was done by strictly adhering to the rules of good scientific practice and are responsible for its content. All experiments were performed in a manner that maximized rigor and reproducibility and without bias.

Ethical approval All applicable international, national and/or institutional guidelines for the care and use of animals were followed. All animal experiments conformed with the Guide for Care and Use of Laboratory Animals and were approved by Shanxi Provincial People's Hospital.

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