

# Autophagy may play an important role in varicocele

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Received February 19, 2017; Accepted August 4, 2017

DOI: 10.3892/mmr.2017.7253

**Abstract.** The present study aimed to determine the expression of autophagy and investigate whether the hypoxia-inducible factor 1 $\alpha$  (HIF-1 $\alpha$ )/BCL2 interacting protein (BNIP3)/Beclin-1 autophagy signaling pathway serves an important role in activating autophagy in varicocele (VC) rat testes cells. Furthermore, the current study aimed to explain the possible association between autophagy and apoptosis. A total of 48 adult male Sprague Dawley rats were divided into group A (control), group B (VC 15-day), group C (VC 30-day) and group D (VC 45-day), with 12 rats in each group. The rats in group A did not receive any interventions, and in groups B, C, and D the VC model was established simultaneously. At 0, 15, 30, and 45 days, an orchidectomy on the left testes was performed in groups A-D, each on its respective day. Transmission electron microscopy was used to investigate the expression of autophagy. Compared with groups A and B, it was demonstrated that the expression of autophagy in groups C, and D was significantly increased. Hematoxylin and eosin staining revealed that as the rats survived VC longer, the testicular tissue damage became more serious. Furthermore, the Johnson score revealed that VC impaired the spermeiogenesis function of the male rats. Additionally, it was demonstrated that the apoptosis index of the seminiferous epithelia cells in VC rat testes increased over time, as measured using TUNEL staining. Immunohistochemical analysis revealed that as the VC was prolonged, the expression of HIF-1 $\alpha$  gradually increased while the expression of (apoptosis regulator Bcl-2) Bcl-2 gradually decreased. Furthermore, western blot analysis revealed that the protein expression of Bcl-2 decreased and apoptosis regulator Bax increased. Furthermore, HIF-1 $\alpha$ , BNIP3, Beclin1 and microtubule associated protein 1 light chain 3  $\alpha$  (LC3)II/LC3I expression gradually increased. However, significant increases

in Beclin 1 and LC3II/LC3I were only observed between the day 0 and day 30 groups. In addition, the expression of p62 significantly increased between day 0 and day 15, but gradually decreased between day 15 and day 45. The results of the present study revealed that VC can lead to testicular tissue hypoxia, and that the HIF-1 $\alpha$ /BNIP3/Beclin1 autophagy signaling pathway may upregulate autophagy in VC rats testes. Thus, the association between autophagy and apoptosis may serve an important role in male infertility caused by VC.

## Introduction

Varicocele is an abnormal venous dilatation and/or tortuosity of the pampiniform plexus in the scrotum. VC is one of the main factors causing male infertility, and male patients with VC account for approximately 15% of all healthy male adults and approximately 35% of patients with primary infertility (1). VC causes severe damage to male reproductive health. Although the high ligation of the spermatic vein is a good way to improve a patient's semen quality, there are still some patients unable to restore fertility, and studies have found that the surgery cannot improve the postoperative natural pregnancy rate (2,3). Therefore, it is particularly important to clarify the mechanism of VC leading to male infertility; unfortunately, the mechanism is not yet fully clear.

The hypoxia-inducible transcription factors (HIFs) are induced under hypoxia, and they mainly comprise HIF-1 and Hif-2. HIF-1 comprises two subunits, HIF-1 $\alpha$  and HIF-1 $\beta$ . HIF-1 $\beta$ , as a stable subunit, could express stably in hypoxic cells. HIF-1 $\alpha$  is often selected as a biomarker of hypoxia (4). HIF-1 $\alpha$ , as the activity subunit of HIF-1, is regulated by the oxygen signal, which controls the activity of Hif1. HIF-1 $\alpha$  transfers to the nucleus to activate a series of genes that affect cell metabolism, growth, proliferation, biosynthesis, apoptosis, and autophagy (5,6). One of the important roles of HIF-1 $\alpha$  is to activate the *BNIP3* (Bcl-2 and adenovirus E1B 19-kDa interacting protein 3) genes, which leads to the synthesis of more BNIP3 protein (7). As one of the Bcl-2 family proteins, BNIP3 contains BH3 Domains. The HIF-1 $\alpha$ /beclin-1 signal pathway plays an important role in the activation of autophagy (8,9). Although studies have found that HIF-1 $\alpha$  was significantly increased in VC rat testes (10,11), the role of HIF-1 $\alpha$  in VC is still unclear.

The autophagosome, induced by cell hunger, hypoxia, or infection, endocytoses cytoplasm, damaged organelles, and redundant proteins and then fuses with the lysosome,

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**Key words:** autophagy, varicocele, apoptosis, testis, HIF-1 $\alpha$ , BNIP3, Beclin1

eventually forming an autolysosome. The contents are hydrolyzed in the autolysosome to utilize the energy, amino acids, and other cellular components. Autophagy and apoptosis are often inseparable and highly interactional. In different diseases, the relationship between autophagy and apoptosis is not the same. Wu *et al* (12) found that specifically blocking autophagy enabled ROS to increase significantly in malignant glioma cells; thus, autophagy can aggravate the apoptosis of tumor cells. Another study found that autophagy could improve the ability of cells to fight infection, which reduces the apoptosis of infected cells (13). However, the promotion of autophagy is also increased during cell apoptosis (14). Taking all of the above into consideration, what promotes autophagy in VC that inhibits apoptosis or what promotes apoptosis is unknown.

Apoptosis is the main method of programmed cell death. Apoptosis is mainly mediated by two pathways: the first is via a membrane receptor, such as Fas/FasL; the second is the mitochondrial pathway (15). The latter mainly regulates the MOMP (mitochondrial outer membrane permeabilization) through the Bcl-2 family proteins. The Bcl-2 family contains many proteins, such as the proapoptotic proteins Bax and BAK, the antiapoptotic proteins Bcl-2, Bcl-xL, and BH3-the only protein containing BH3 domains (16). Under normal conditions, the antiapoptotic protein's hydrophobic groove combines with the BH3 domains of BH3s-only protein and antiapoptotic proteins, which can form heterodimers to prevent MOMP. In addition, Bcl-2 can also function as an antioxidant, clearing reactive oxygen species (ROS). Bcl-2 also affects transmembrane transport, changes the distribution of calcium ion, and activates endogenous enzymes (17,18).

When under the condition of hypoxia, infection, or oxidative stress, the apoptosis pathway is activated. Proapoptotic proteins translocate to the mitochondria, causing damage to its structure and function, causing MOMP. In addition, the caspases and other hydrolases are released into the cytoplasm, which causes irreversible damage to the cells. The apoptosis of seminiferous epithelia cells affects the quality and quantity of sperms, and it may be an important reason for male infertility caused by VC. Some studies found that the apoptosis of spermatogenic cells significantly increased in VC rat testes (19,20). In addition, other studies found that the expression of the Bax gene and protein is increased in human semen (21,22).

The present study mainly focuses on the observation of autophagy and apoptosis in VC rat testes, tries to verify whether the HIF-1 $\alpha$ /BNIP3/Beclin1 autophagy signaling pathway is highly expressed, and provides a brief analysis about the possible relation between autophagy and apoptosis in VC rat testes.

## Materials and methods

**Animal study.** The experimental procedures and the animal use and care protocols were approved by the Committee on Ethical Use of Animals of Renmin Hospital of Wuhan University. In all, 48 adult SD male rats (approximately 200–240 g) were purchased from the Experimental Animal Center of The Centers for Disease Control (Wuhan, China) and randomized to 4 groups: Group A (control group/VC 0 days), group B (VC 15 days), group C (VC 30 days), group D

(VC 45 days), with 12 rats in each group. All rats were fed in the same SPF environment, and all operations were performed under aseptic conditions. Each rat was raised for three days in the same environment after purchase to allow adaptation to the new environment. All rats in group A were anesthetized and decapitated to obtain the left testis. Partial ligation of the left renal vein surgery was used to establish the VC model. Each rat in groups B, C, and D underwent this surgery under the same conditions at the same time. The left testis of groups B, C, and D were obtained 15, 30, and 45 days after establishing the VC model, respectively.

**VC model creation.** According to the method of Turner (23), 1% pentobarbital sodium (35 mg/kg) was administered via intraperitoneal injection to induce anesthesia. After anesthesia was achieved, we fixed the limbs and chose an abdominal median incision. We opened the abdominal cavity, moved the viscera around the left kidney to the right, and then completely exposed the left kidney, left renal vein, left spermatic vein and inferior vena cava. We isolated the left renal vein at the junction of the left renal vein and inferior vena cava and then placed a 0.8-mm metal bar under the left renal vein. Then, 3-0 silk was used to ligate the left renal vein and metal bar at the junction. This reduced the diameter of the left renal vein by half. Then, we drew out the metal rods and immediately observed the expansion of the left renal vein. The branch veins of the spermatic vein were also ligated. After peritoneal lavage with normal saline, we sutured the abdomen with 4-0 silk suture. Success criteria: The size was not obviously different between left and right sides of the kidneys. However, 1 rat of group C and 2 rats of group D were found with the left kidney obviously atrophied, therefore they were excluded from the study. Consequently, the number of the samples in each group was 12, 12, 11, 10.

**Antibodies.** Rabbit anti Hif1 $\alpha$  primary antibody, sc-10790, was obtained from Santa Cruz Biotechnology, Inc. (Dallas, TX, USA). Mouse anti BNIP3 primary antibody, Abcam Ab10433, was purchased from Abcam (Abcam, Cambridge, UK). Rabbit anti Beclin1 primary antibody, CST 3738, was obtained from Cell Signaling Technology (Danvers, MA, USA). Rabbit antibody LC3 (CST 2775) rabbit antibody p62 (CST 5114) and mouse anti Bcl-2 primary antibody, (sc-7382). Rabbit anti Bax (CST 14796) and rabbit anti GAPDH, AB-P-R 001, were provided by Hangzhou Goodhere Biotechnology Co. (LTD, Hangzhou, China). Horseradish peroxidase (HRP) labeled sheep anti IgG secondary antibody, BA1054, were obtained from Wuhan Boster Biological Engineering Co. (Wuhan, China).

**Jonhsen score.** H&E staining samples were observed under a 40x10 Olympus microscope (Olympus Corp., Tokyo, Japan) and 10 images in each sample were randomly selected for Jonhsen score (24). To judge spermatogenesis, according to the Jonhsen scores. Johnsen scores ranging between 1 and 10. While a score of 10 represents an excellent spermatogenesis, 1 represents a worst level.

**Transmission electron microscopy (TEM).** A small piece of testicular tissue was cut with a sharp blade in 1 min, with a size

of approximately 0.2 cm<sup>3</sup>, and fixed in 2.5% glutaraldehyde. Then, the tissue was sliced into approximately 1 mm<sup>3</sup> in fixative at 4°C for 12 h. The testicular tissues were washed with 0.1 M phosphate buffer saline buffer, fixed in 1% osmic acid for 1 h, washed in PBS again, and dehydrated in ethanol, acetone and epoxy resin for 4 h. After being embedded and fixed, the prepared tissue was cut to 60-μm sections and stained with uranyl acetate and lead citrate and then observed under an transmission electron microscope (H-7600; HITACHI, Ltd., Tokyo, Japan). Fifteen fields were randomly selected for each sample.

**TUNEL staining.** Terminal-deoxynucleotidyl Transferase Mediated Nick and Labeling (TUNEL) staining was used to detect apoptosis of the spermatogenic cells of the testes. Prepared tissue was fixed with 4% neutral formaldehyde, embedded in paraffin and serially cut. After regular dewaxing, hydration, and serum blocking, we used an In Situ Cell Death Detection kit, POD (REF no. 11684817910, Roche Diagnostics GmbH, Mannheim, Germany) according to the manufacturer's instructions. The cells were observed and photographed under an optical microscope. Ten high image fields of each sample were randomly selected, and 200 cells were counted in each field. The apoptosis index (AI) was calculated according to the number of apoptotic spermatogenic cells.

**Immunohistochemistry.** An immunohistochemistry kit was purchased from Dako Denmark A/S (K5007, Code; Glostrup, Denmark). Briefly, paraffin sections were deparaffinized, dehydrated, and then soaked in boiled 0.01 mol/l citric acid buffer (pH 6.0) in a pressure cooker for 2 min to retrieve the antigen. The sections were incubated with 3% hydrogen peroxide solution at room temperature for 20 min to inhibit endogenous peroxidases and then TBS washed 3 times. Then, 5% goat serum for closing 20 min, directly dripping primary antibody HIF-1α (1:250), Bcl-2 (1:250) at 4°C overnight. The sections were restored for 40 min at room temperature. Then, biotin-labeled secondary antibody was added, hatched for 25 min at 37°C, and followed by DAB for 30 min. Each step was washed with 0.01 mol/l (pH 7.4 slightly) PBS for 5 min three times. Sections were visualized by using horseradish peroxidase, then counterstained with hematoxylin for 3 min.

**Quantitative and qualitative analyses.** Each sample was stained under the same conditions, then scanned under a 40x10 Olympus microscope. Ten image fields were randomly selected from each section and then used the Image J Analysis System (version 1.46, public domain, Image-Processing and Analysis in Java) to measure and analyze the cells. In all, 200 spermatogenic cells were randomly measured in each image. We scored the results according to the grey value of the cells: Grey values of ~80, 80~120, 120~160, and ~160 were scores of 0, 1, 2, and 4, respectively. Then, the average score of each cell was calculated.

**Western-blot.** Testes were washed in precooling normal saline, cut into small pieces, weighed and homogenized with 10 volumes of lysis buffer (containing PMSF) (P0013; Beyotime Biotechnology, Shanghai, China) and then placed on ice for

Table I. Jonhsen score and seminiferous epithelia cells apoptosis index (AI%) in each group.

	Number	Jonhsen score	AI (%)
Group A	12	9.53±0.18	0.732±0.215
Group B	12	7.79±1.20 <sup>b</sup>	1.638±0.640 <sup>b</sup>
Group C	11	5.89±1.38 <sup>b,c</sup>	6.259±2.220 <sup>b,d</sup>
Group D	10	4.00±1.66 <sup>b,d</sup>	10.306±2.565 <sup>b,d,f</sup>

Jonhsen score and Seminiferous epithelia cells apoptosis index (%), AI Significant difference: <sup>a</sup>P<0.05 with respect to group A; <sup>b</sup>P<0.01 with respect to group A; <sup>c</sup>P<0.05 with respect to group B; <sup>d</sup>P<0.01 with respect to group B; <sup>e</sup>P<0.05 with respect to group C; <sup>f</sup>P<0.01 with respect to group C. AI, apoptosis index

30 min. The lysis solution was pureed in a blender, followed by ultrasonication and centrifugation at 4°C for 5 min. The BCA Protein Quantitation kit (P0010; Beyotime Biotechnology) was used to determine the protein concentration. Next, 60 μg of protein was mixed in the loading buffer and heated at 100°C for 5 min. The proteins were separated by 12% sodium dodecyl sulfa-polyacrylamide gel electrophoresis (SDS-PAGE). The proteins were transferred onto nitrocellulose membranes. The membranes were washed with TBST for 5 min 3 times, blocked with a solution of 0.1% Tween-20 containing 5% non-fat dry milk for 2 h, and then incubated with primary antibody at 4°C overnight. The membranes were incubated with IgG secondary antibody (1:10,000) bound horseradish peroxidase for 1 h at room temperature. The proteins of the bands were imaged using the ECL method. The relative density of the bands was normalized with β-actin as a control. Software Image J were used to measure the contents of the proteins.

**Statistical analysis.** SPSS 19.0 (IBM SPSS, Armonk, NY, USA) statistical software was used for statistical analysis of the measurement data, and the data were expressed as the means ± standard error of the means. One-way analysis of variance was applied to analyze the data from two groups, an LSD test applied for homogeneity of variance, and a Dunnett T3 test applied for variance heterogeneity. P<0.05 was considered to indicate a statistically significant difference.

## Results

**Histological changes.** As the VC prolongs, the damage of the seminiferous tubules are more serious. The damage mainly impairs the structure of the seminiferous tubule; There are fewer cell layers of the seminiferous tubules and the distribution of the seminiferous epithelia cells are disorganized. What's more, there are a few sperm in the seminiferous tubules (Fig. 1).

**Jonhsen score.** The scores of group B, C and D are significantly lower than group A (P<0.01); As the VC prolongs, the scores of group B, C and D decrease, but the scores between group C and D have no significant differences (P>0.05) (Table I). All of these indicate that VC impairs the spermatogenesis of the testis.



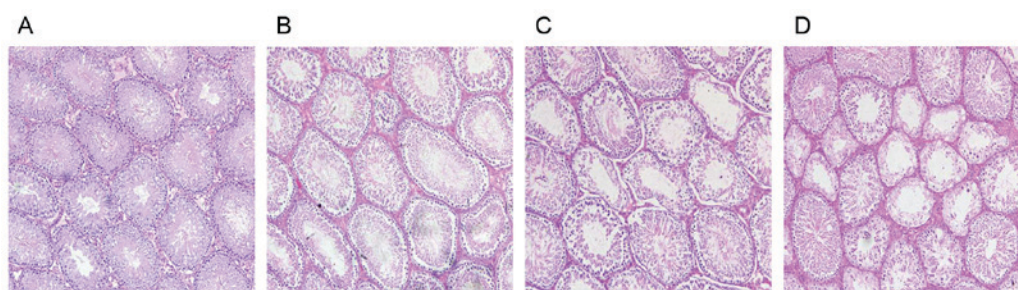


Figure 1. Hematoxylin and eosin staining 10x10 (A) group A (VC 0 day): The basilar membrane of the seminiferous tubule showing the structural integrity and spermatogenic cells distribute from basilar membrane to the lumen. Cells in the seminiferous tubule are hierarchical and orderly, and there are a large number of sperm in the lumen. (B) group B (VC 15 days): The situation is similar to group A. (C) group C (VC 30 days): Partial basilar membrane showing disrupted structural integrity, the number of the cell layers significantly decreased and the cells were disorganized. There are few sperm in the lumen. (D) group D (VC 45 days): The situation is worse than group C. VC, varicocele.

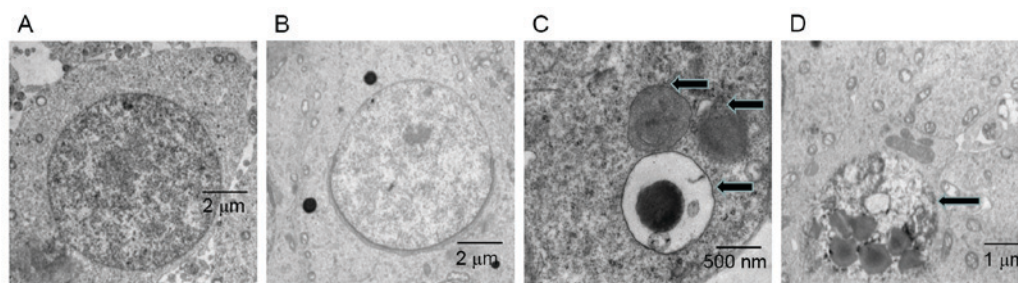


Figure 2. TEM ‘←’ represent lysosomes, autophagosomes or autolysosomes (A) group A (VC 0 day): There are abundant organelles and few autophagosomes, lysosomes, autolysosomes in seminiferous epithelia cells. (B) group B (VC 15 days): Autolysosomes are rare in spermatogenic cells. (C) group C (VC 30 days): Compared to the control group, the number of the autophagosomes, lysosomes, autolysosomes increased significantly in seminiferous epithelia cells. (D) group D (VC 45 days): The situation is similar to group C. TEM, Transmission electron microscopy; VC, varicocele.

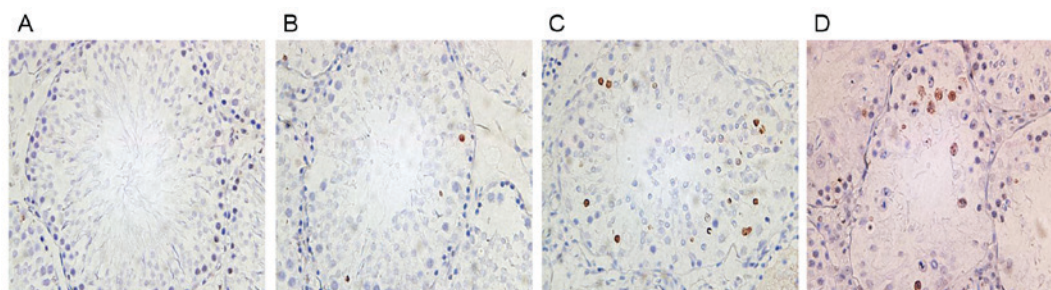


Figure 3. TUNEL staining 40x10 (A) group A (VC 0 day): Apoptotic seminiferous epithelia cells are rarely seen. (B) group B (VC 15 days): There are few apoptotic seminiferous epithelia cells. (C) group C (VC 30 days): The number of the apoptotic seminiferous epithelia cells increased significantly. (D) group D (VC 45 days): The number of the apoptotic seminiferous epithelia cells increased significantly. TUNEL, Terminal-deoxynucleotidyl Transferase Mediated Nick and Labeling; VC, varicocele.

**Transmission electron microscopy.** Seminiferous epithelia cells were observed under transmission electron microscopy. In the control group, we found that there were abundant mitochondria in the spermatogenic cells and sertoli cells, and the morphology of the mitochondria were normal. However, as the VC prolonged, the number of edema mitochondria and autophagosome obviously increased in the spermatogenic cells (Fig. 2).

**TUNEL staining.** We observed the apoptosis of the seminiferous epithelia cells calculated by TUNEL staining. And found that apoptotic seminiferous epithelia cells were rarely seen in the 30-day VC group, and the number of apoptotic

spermatogenic cells increased gradually (Fig. 3). The AI of the control group is approximately 0.73%, but the AI of the 45-day VC group is approximately 10.31% (Table I). As VC was prolonged, AI of the spermatogenic cells increased rapidly ( $P<0.01$ ) (Table I).

**Immunohistochemistry.** HIF-1 $\alpha$  proteins mainly expressed in the cytoplasm and nucleus of the spermatogenic cells (Fig. 4). As VC was prolonged, the expression of HIF-1 $\alpha$  gradually increased, and average scores among the groups showed a statistically significant difference ( $P<0.01$ ) (Table II). Bcl-2 proteins mainly expressed in cytoplasm of the spermatogenic cells (Fig. 5). As VC was prolonged, the expression of Bcl-2

gradually decreased, and the average scores among the groups showed a statistically significant difference ( $P<0.05$ ) (Table III).

**Western blot analysis.** We measured the proteins related to the HIF-1 $\alpha$ /BNIP3/Beclin1 signaling pathways, and the autophagy molecular markers LC3-II, and other proteins p62, Bcl2 and Bax (Figs. 6 and 7). Compared to the control group, the expression of HIF-1 $\alpha$ , BNIP3, Beclin1, and LC3-II/LC3-I increase gradually ( $P<0.05$ ) (Fig. 7A-D). Though the expression of Beclin1 and LC3-II/LC3-I significantly increase from the VC 0-days groups to VC-30 days groups, there are no significant difference between VC-30 and VC-45 ( $P>0.05$ ) (Fig. 7C and D). The expression of p62 proteins sharply increases but begins to decrease at VC 45 days (Fig. 7E). What's more the pro-apoptotic protein Bax increase sharply (Fig. 7G) and the anti-apoptotic protein Bcl-2 decrease gradually (Fig. 7F).

## Discussion

There are many reasons for male infertility caused by VC, such as local temperature rising in testes, hypoxia, oxidative stress and immune abnormalities (25,26). All of these factors will injure the spermatogenesis function of the testis. Through histological observing, as the VC prolongs, the structure of the seminiferous tubules are gradually destroyed; the number of cell layers significantly decreases and the distribution of the seminiferous epithelia cells are disorganized. There are a few sperm in the seminiferous tubule (Fig. 1). Jonhsen score is a good index to evaluate the spermatogenesis function of the testis. We can see, as the VC prolongs, the scores of group A, B, C, D decrease gradually (Table I). These reveal that VC gradually injury the spermatogenesis function of the rats testis.

We examined this tissue through immunohistochemical and Western blot analyses and found that HIF-1 $\alpha$  was positively correlated with VC duration. Other studies (10,27,28) also obtained similar results, indicating that VC can lead to testicular tissue hypoxia, and the degree of hypoxia is highly correlated with VC time. All of these researches indicate that hypoxia may be a important factor for infertility caused by Varicocele. One important role of the hypoxia is to induce HIF-1 $\alpha$ , which activates a series of autophagy-related genes, such as BNIP3 (29). As for physiological conditions, Beclin-1 and Bcl-2 form a complex compound leading to inhibit the activation of the autophagy pathway. Under hypoxia conditions, the expression of HIF-1 $\alpha$  significantly increases, which up-regulates BNIP3. BNIP3 interreacts with Bcl-2 or Bcl-XL and eventually forms heterodimer, which will release Beclin-1 from Beclin-1/Bcl-2 complex compound. Then, the free Beclin-1 will activate the autophagy pathway (30).

As a result, HIF-1 $\alpha$ /BNIP3/Beclin-1 signaling pathway is an important way of inducing autophagy under hypoxic conditions. But there are few researches about autophagy in VC rats testes. Moreover, how does the autophagy be activated still unknown. Therefore, we tested proteins related to this autophagy signaling pathway by Western blot. The expression of HIF-1 $\alpha$ , BNIP3, and Beclin-1 significantly increased in the VC groups compared to the control group ( $P<0.05$ ) (Fig. 7A, B and C). This indicates that VC can lead to testicular tissue hypoxia. What's more, hypoxia induces and

Table II. Immunohistochemistry Hif-1 $\alpha$  average score of each seminiferous epithelia cell.

	Number	Average score
Group A	12	0.645 $\pm$ 0.081
Group B	12	0.809 $\pm$ 0.071 <sup>b</sup>
Group C	11	1.117 $\pm$ 0.098 <sup>b,d</sup>
Group D	10	1.485 $\pm$ 0.120 <sup>b,d,f</sup>

Hif-1 $\alpha$  average score of each seminiferous epithelia cell, Significant difference: <sup>a</sup> $P<0.05$  with respect to group A; <sup>b</sup> $P<0.01$  with respect to group A; <sup>c</sup> $P<0.05$  with respect to group B; <sup>d</sup> $P<0.01$  with respect to group B; <sup>e</sup> $P<0.05$  with respect to group C; <sup>f</sup> $P<0.01$  with respect to group C.

Table III. Immunohistochemistry Bcl-2 average score of each seminiferous epithelia cell.

	Number	Average score
Group A	12	1.963 $\pm$ 0.143
Group B	12	1.799 $\pm$ 0.102 <sup>b</sup>
Group C	11	1.582 $\pm$ 0.108 <sup>b,d</sup>
Group D	10	1.451 $\pm$ 0.177 <sup>b,d,e</sup>

Bcl-2 average score of each seminiferous epithelia cells, Significant difference: <sup>a</sup> $P<0.05$  with respect to group A; <sup>b</sup> $P<0.01$  with respect to group A; <sup>c</sup> $P<0.05$  with respect to group B; <sup>d</sup> $P<0.01$  with respect to group B; <sup>e</sup> $P<0.05$  with respect to group C; <sup>f</sup> $P<0.01$  with respect to group C.

activates the expression of HIF-1 $\alpha$ , which may up-regulate the HIF-1 $\alpha$ /BNIP3/Beclin-1 autophagy signaling pathways.

Autophagy is divided into induction, nucleation, elongation, the formation of autophagosome, the fusion of autophagosome and lysosome, and the degradation of substrate. A series of ATG (autophagy related gene) products are involved in the formation of autophagy. Beclin 1 plays an important role in initiating the process of autophagy. At the stage of nucleation, Beclin1 combines with Vps34 and UVRAG to form class III of phosphoinositide 3-kinase (class III PI3K Complex). Class III PI3K Complex induce the formation of Phosphatidylinositol 3-phosphate (PI3P), which is crucial in the process of autophagy. LC3II distributes inside and outside the surface of the autophagosome membrane as a biomarker for reflecting the level of autophagosome. p62 interacts with LC3II and mediates the ubiquitinated substrates into autophagosome. Moreover, itself and the substrates then degrades in the autophagosome. Therefore, the content of p62 reflects 'autophagic flux'. Through western blot testing, we found that compared with the control group, the expression of Beclin-1 and LC3II is significantly higher in the VC groups ( $P<0.05$ ) (Fig. 7C and D). These results reveal that autophagy expresses increasingly in VC rats testes. Furthermore, the expression of p62 proteins sharply increases but begins to decrease at last (Fig. 7E). This indicates that VC may active the autophagy,



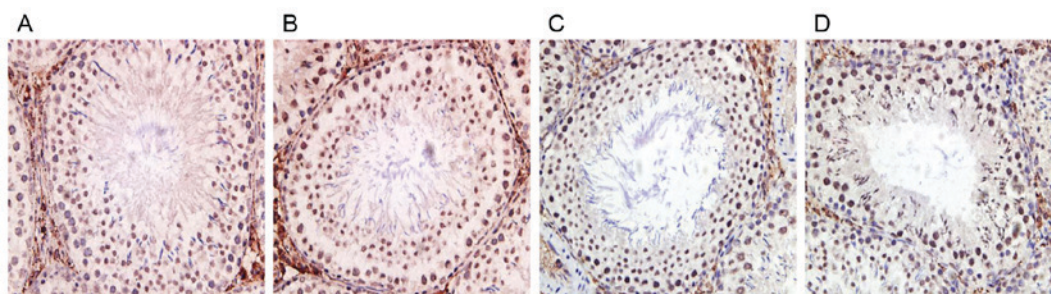


Figure 4. HIF-1 $\alpha$  immunohistochemistry 40x10. HIF-1 $\alpha$  protein is dyed with brown granules, and it is mainly expressed in the nucleus of spermatogenic cells and Sertoli cells. (A) group A (VC 0 day): Brown granules are lowly expressed in the cytoplasm and nuclei of spermatogenic cells. (B) group B (VC 15 days): The expression of brown granules in group B is similar to group A. (C) group C (VC 30 days): Brown granules are highly expressed in nuclei of seminiferous epithelia cells. (D) group D (VC 45 days): Brown granules are highly expressed in the nuclei of spermatogenic cells.

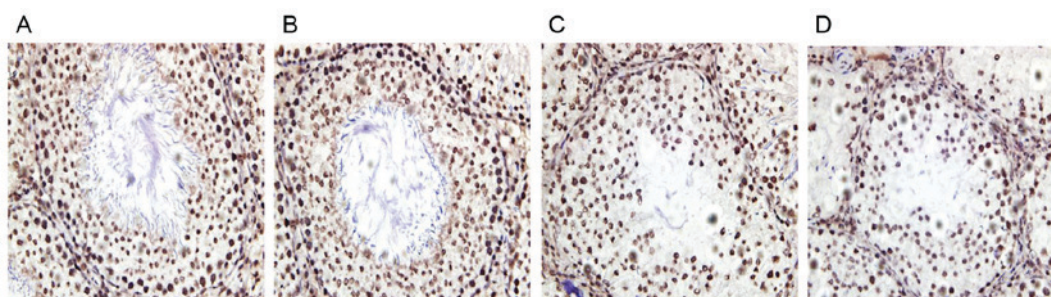


Figure 5. Bcl-2 immunohistochemistry 40x10. Bcl-2 protein is dyed with brown granules, and it is mainly expressed in the nucleus. (A) group A (VC 0 day): Brown granules are highly expressed in the nucleus of seminiferous epithelia cells. (B) group B (VC 15 days): Brown granules are highly expressed in the nucleus. (C) group C (VC 30 days): Brown granules are lowly expressed in the nucleus. (D) group D (VC 45 days): Brown granules are lowly expressed in the cytoplasm of seminiferous epithelia cells.

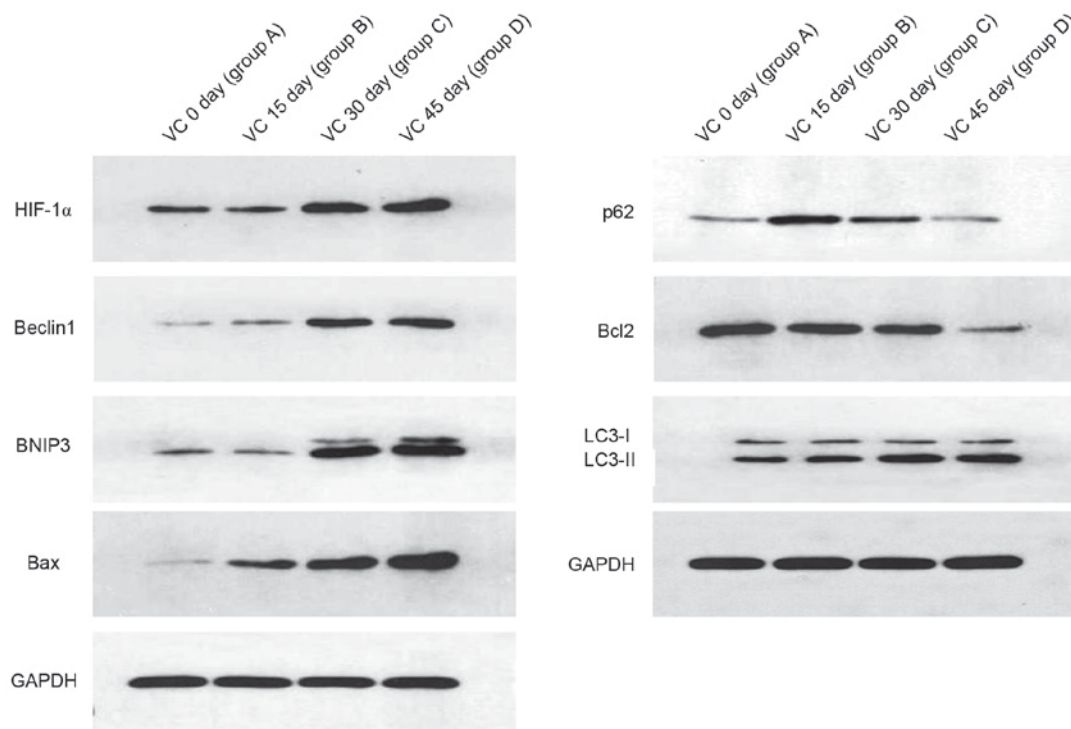


Figure 6. Western blots for HIF-1 $\alpha$ , Beclin1, p62, Bcl2, Bax, BNIP3, LC3-II, LC3-I, and GAPDH. HIF-1 $\alpha$ , hypoxia-inducible factor 1 $\alpha$ ; Beclin1, BCL2 interacting protein.

but as the VC time prolongs, the 'autophagic flux' decreases, something may effect the autophagy at the late stage of VC.

Transmission electron microscopy is one of the most common and precise methods to observe autophagy in tissue. Moreover,

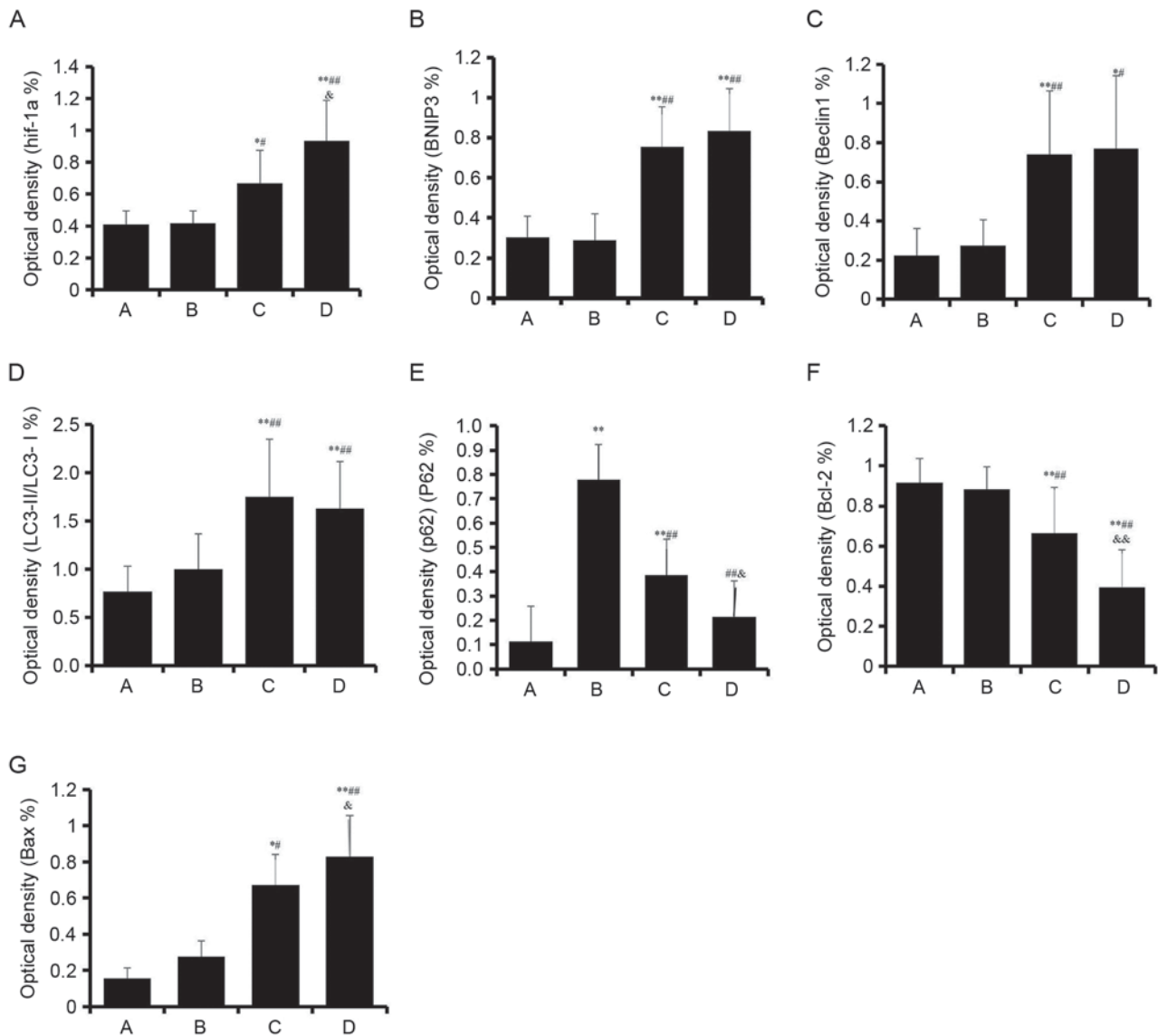


Figure 7. Western blots for (A) HIF-1 $\alpha$ , (B) BNIP3, (C) Beclin1, (D) LC3-II/LC3-I, (E) p62, (F) Bcl2, (G) Bax and GAPDH. The intensity of the protein blots was normalized with GAPDH. The data are expressed as the mean  $\pm$  SEM. Optical density (%), significant difference: \* $P$ <0.05 with respect to group A; \*\* $P$ <0.01 with respect to group A; <sup>#</sup> $P$ <0.05 with respect to group B; <sup>##</sup> $P$ <0.01 with respect to group B; <sup>&</sup> $P$ <0.05 with respect to group C; <sup>&&</sup> $P$ <0.01 with respect to group C. HIF-1 $\alpha$ , hypoxia-inducible factor 1 $\alpha$ ; Beclin1, BCL2 interacting protein.

under a transmission electron microscope, we found that there were rare autophagosomes, lysosomes, and autolysosomes in the control group. However, the autophagy increased obviously in the 30-day and 45-day groups (Fig. 2). The results of the TEM test support the results of the Western blot test, so we can conclude that VC can lead to the high expression of autophagy in seminiferous epithelia cells.

Apoptosis is an important way to maintain tissue stability; it involves a series of gene activation, transcription and regulation processes. Bcl-2 is one of the most important antiapoptotic proteins in the Bcl-2 family; the four domains gather in tridimensional space and form a hydrophobic groove. The groove can combine with the BH3 domain of antiapoptotic proteins or BH3-only proteins, preventing MOMP mediated by the BH3 domain. Bax is one of a important proapoptotic proteins in the Bcl-2 family.

Through western blot analysis we found that rats survived VC longer, Bcl2 decreased more obviously; conversely, the

expression of Bax increased more obviously. Furthermore, we used TUNEL staining (Fig. 3) to observe the apoptosis of seminiferous epithelia cells in VC rats testes and found that seminiferous epithelia cells AI (apoptosis index, AI) in the 30 and 45-day groups were significantly higher than in the control and VC-15 groups ( $P$ <0.01) (Table I). All the above shows that VC leads to seminiferous epithelia cells apoptosis. Some other researches have reported similar results to ours (10). The apoptosis of seminiferous epithelia cells leads to spermatogenesis impairment, eventually leading to poor quality and quantity of sperm.

Relationship between autophagy and apoptosis. In the early phase of apoptosis, antiapoptotic proteins (such as the Bcl-2) decrease and proapoptotic proteins and BH3-only proteins increase, which make the beclin1/Bcl-2 or beclin1/Bcl-XL complex depolymerize. Antiapoptotic proteins and proapoptotic proteins form heterodimers. Furthermore, beclin-1 will be freed from the beclin1/Bcl-2 or beclin1/Bcl-XL

complex, activating autophagy. Under hypoxia conditions, the expression of HIF-1 $\alpha$  increases, which will up-regulate BNIP3 and BNIP3L (BNIP3-like) (31). BNIP3 and BNIP3L are BH3-only proteins that contain BH3 domains. They are located in the endoplasmic reticulum, mitochondria and affect apoptosis by regulating the mitochondrial respiration and ROS level. In addition, BNIP3 contains LIR (LC3 interaction region), so it can mediate mitochondrial the autophagy pathway.

As we describe that LC3-II/LC3-I gradually increase from early phase and medium of VC, but there are no difference between VC 30 days groups and VC 45 days groups (Fig. 7D). Moreover, p62 sharply increases at the early stage of VC, but gradually decrease from VC 15 days, and there are no difference between VC 45 days group and control group (Fig. 7E). It indicates that autophagy is strongly activated at early phase and medium of VC, and may be inhibited by some other factors at the late period. Interestingly, we find that AI and Bax increase gradually, and sharply increase in VC 45 days group (Table I and Fig. 7G). It indicates that the apoptosis of the seminiferous epithelia cells is slightly at early stage of VC, is heavily in the terminal VC. Combined with the changes of LC3-II/LC3-I and p62, we try to hypothesize that early hypoxia does harm to seminiferous epithelia cells at the beginning of VC. The damaged organelles and proteins in the seminiferous epithelia cells activate autophagy. Autophagy can timely remove these damaged substrates to protect cells from secondary lesion. When the cells are severely injured, apoptotic pathways are strongly activated, which will inhibit the autophagy pathway. However, the relationship between apoptosis and autophagy is very complicated and still needs further research.

In conclusion, apoptosis and autophagy were firstly observed in the seminiferous epithelia cells in VC rats testes. HIF-1 $\alpha$ /BNIP3/Beclin1 autophagy signaling pathways may play an important role in the activation of autophagy. In addition, we hypothesize that autophagy may protect seminiferous epithelia cells from apoptosis at the beginning of VC, and apoptosis may inhibit autophagy pathways in later stages. However, the relationship between apoptosis and autophagy is complicated, so the assumption still needs further research. Above all, autophagy may provide a new direction for the diagnosis and treatment of male infertility caused by varicocele.

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