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Original Article

Effects of autophagy on the spermatogonia of chronic hypoxic mice

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Abstract: Varicocele (VC) is a common cause of male infertility. An increasing number of studies have confirmed that chronic hypoxia plays an important role in male infertility caused by VC. Our previous study suggested that autophagy may have a crucial function in VC. Whether autophagy plays an important role in hypoxic spermatogonia remains unknown. We established a new method to study VC by culturing spermatogonia under hypoxic conditions to simulate the chronic hypoxic environment of VC. Moreover, the effects of autophagy on hypoxic spermatogonia were observed by regulating autophagy. Spermatogonia from GC-1 spg mice were cultured in vitro in chronic hypoxic conditions (2% O_2, 93% N_2, 5% CO_2) with a tri-gas incubator. Cells were separately cultured under normal oxygen and hypoxic conditions, and then, the autophagy blocker drug LY294002 was subsequently administered. Cytoactivity and proliferation were tested by CCK8 assays. Cells cultured in hypoxic conditions were divided into the hypoxia for 24 h, 48 h, and 72 h groups and the LY294002+hypoxia for 24 h, 48 h, and 72 h groups. Hoechst staining and flow cytometry/Annexin-V-FITC/PI staining were used to detect cell apoptosis, while transmission electron microscopy was used to observe the structure of the spermatogonia. Western blot analysis was used to observe the expression of Hif-1α, Beclin-1, LC3, SOD2, GPX4, and Cyt C. As the hypoxia time increased, CCK8 detection showed that the cytoactivity of the spermatogonia was suppressed. After administration of the inhibitor LY294002, the inhibition ratio of the spermatogonia increased. As the hypoxia time was prolonged, the apoptosis index of spermatogonia increased. After administration of LY294002, the apoptosis index increased significantly. Transmission electron microscopy showed that there were abundant structurally abnormal mitochondria and autophagosomes and autolysosomes in hypoxic spermatogonia. In addition, LY294002 reduced the expression of autophagosomes and autophagosomes, but the number of abnormal mitochondria and vacuoles in hypoxic cells increased significantly. As the hypoxia time was prolonged, the expression levels of Hif-1α, Beclin-1, LC3 II/LC3-I, and Cyt C increased gradually, but there were no significant changes in SOD2 and GPX4. After administration of LY294002, the expression levels of Beclin-1, LC3 II/LC3-I, and GPX4 significantly decreased, but Cyt C significantly increased. Thus, chronic hypoxia decreases cytoactivity and proliferation of spermatogonia. Moreover, hypoxia damages the mitochondria and other organelles. At the same time, hypoxia activates the autophagic system to clear these damaged organelles. The inhibition of autophagy in spermatogonia under chronic hypoxic conditions prevents the removal of damaged mitochondria and eventually results in a significant decrease in the antioxidant system molecule GPX4, rather than SOD2, and a significant increase in the apoptosis rate.

Keywords: LY294002, Hif-1α, autophagy, varicocele, spermatogonia, hypoxia, GPX4

Introduction

This study is the first to simulate the chronic hypoxic environment of VC by culturing spermatogonia under hypoxia conditions.

Varicocele (VC) is a leading cause of male infertility. Men with VC account for approximately 10%~15% of the male population, and approximately 40% of these men have primary infertility [1-3]. The effects of VC on males include the impairment of spermatogenesis and pain in the perineum. Dysfunction of spermatogenesis is the most serious complication. Surgery is an effective method to treat VC, but there is still controversy regarding the indications and value of surgery. For patients with poor semen quality who have not reached the standard of surgery indication, who are not willing to accept the surgery or who have received the VC surgery but

still show impaired fertility, effective treatment measures are lacking. The mechanism of infertility caused by VC has not been thoroughly elucidated. A series of theories have been proposed, including high temperature in the testicles, hypoxia, oxidative stress, inflammation, and decreased sex hormones [4-6]. Among these theories, hypoxia may be an important reason affecting spermatogenesis [7, 8].

Autophagy can be induced by a variety of factors, such as hunger, oxidative stress and other factors. These stressors can damage the organelles or proteins, and the damaged substrates will undergo ubiquitination by the ubiquitin enzyme system. The ubiquitination of organelles or proteins will be sequestered by a membrane, then the vesicle fused with a lysosome in which the substrate is degraded. However, autophagy plays a different role in different diseases. Several scholars have shown that autophagy can promote tumor cell growth and reduce tumor cell apoptosis in certain tumors [9]. However, in a fraction of tumors (such as gliomas), autophagy can inhibit the evolution and proliferation of tumor cells [10]. Otherwise, a number of scholars believe that promoting autophagy also aggravates cell apoptosis [11].

Studies on autophagy in VC have rarely been reported. Our previous studies [12] showed that Hif-1α was significantly increased in seminiferous epithelial cells from rats with VC, and the expression of autophagy in testicular epithelial cells of VC rats was significantly higher than in normal testicular cells. The current research primarily describes how autophagy affects the hypoxic spermatogonium and whether it promotes growth or promotes apoptosis. These findings will provide direction and a theoretical basis for further research.

Materials and methods

Experimental cell line

The GC-1 spg mouse spermatogonium cell line was purchased from the Beijing BeNa Culture Collection Institute for Biological Research (Beijing, China).

LY294002 affects the inhibition ratio of spermatogonium under hypoxic conditions

Logarithmic-phase mouse spermatogonium GC-1 spg cells were inoculated in 96-well plates at a density of 1.7×10^5/ml. The cells were cultured in a tri-gas incubator (37°C, 5% CO_2, 2% O_2, 93% N_2), and the nutrient solution was refreshed after 24 h. Four groups were established: the medium control group, the medium+different concentrations of LY294002 group, the cell suspension group, and the cell suspension+different concentrations of LY294002 group.

Different concentrations of LY294002 were added to the corresponding wells, and the final drug concentrations in each well were 1 μM (1 μmol/L), 2 μM, 3 μM (3 μmol/L), 5 μM, 10 μM, 15 μM, 20 μM, and 50 μM. Each concentration was replicated in four pores, and the volume of liquid in each pore was 100 μL. Cells were cultured in an oxygen-deficient environment for 24 h, 48 h, and 72 h. Then, 10 μL of the CCK8 kit reagent was added to each well and cultured at 37°C for 80 min. Each sample was assessed at 450 nm 3 times. The optical density (OD) was recorded to calculate the inhibition ratio of each group. Inhibition ratio = [1 - (cell suspension+LY294002 group - medium group)/(cell suspension group - medium group)].

Hoechst staining

The logarithmic-phase spermatogonia were inoculated in 6-well plates (on glass) and covered approximately 60% of the area. Next, the cells were cultured in normal conditions (37°C, 5% CO_2), and the nutrient solution was later refreshed after 24 h. Cells were divided into 6 groups. Group A, B, C, D, E, and F respectively represent the hypoxia for 24 h, 48 h, and 72 h groups and the drugs+hypoxia for 24 h, 48 h, and 72 h groups. The hypoxia for 24 h, 48 h, and 72 h groups represent spermatogonia cultured under hypoxia conditions for 24 h, 48 h, and 72 h, respectively. The drugs+hypoxia for 24 h, 48 h, and 72 h groups represent spermatogonia administered LY294002 (final concentration of 10 μM) and then cultured under hypoxic conditions for 24 h, 48 h, and 72 h, respectively. Cells were incubated in a tri-gas incubator (37°C, 2% O_2, 5% CO_2) to establish hypoxic conditions. At 24 h, 48 h and 72 h, the cells were stained with Hoechst. The number of apoptotic cells was counted, and then, the apoptosis index (AI) was calculated. AI = apoptotic cells/total cells.

For cells grown on slides in 6-well plates, the medium was removed from the plates, and 0.5 mL fixing solution was later added for 10 min.

The fixing solution was removed, and the slides were washed with PBS twice for 3 min each time. Then, 0.5 mL Hoechst 33258 (C1011; Beyotime Biotech, Nantong China) stain was added to the plates for 5 min. The dyeing process was performed on a table concentrator. The staining liquid was discarded, and the cells were washed twice with PBS for 3 min each time. The liquid was discarded, and 1 drop of anti-fluorescence quenching liquid was added to the slides and then covered with cover glasses. The nuclei were observed under a fluorescence microscope, and the excitation wavelength was 350 nm, with the emission wavelength at approximately 460 nm. Normally, little dye attaches to the nucleus, so the nuclei were dyed light blue. In addition, nuclei were regular circular shapes, and the edges were smooth and round. When the cells are apoptotic, the DNA in the nuclei easily fracture, so the dye easily attached to the nuclei. Under excitation of the fluorescence microscope, the nuclei were very bright, shrunken, and irregular.

Flow cytometry/Annexin-V-FITC/PI staining

The logarithmic-phase spermatogonia were inoculated in 6-well plates (on glass slides) at approximately 60% confluency. The grouping and culture conditions were the same as those described in the *Hoechst staining* section. Then, the cells were stained with Annexin-V-FITC/PI (556547, BD; USA).

The media from the 6-well plates were removed, and 1 mL precooled PBS was added to the 6-well plates. Then, the wells were washed twice. The washing liquid was removed, and 1 mL of pancreatin was added to each well for digestion, followed by incubation at 37°C for 1 min and 30 s. Next, 1 mL medium was added to the wells for neutralization. The suspension was collected in a test tube after pipetting, and the cells were mixed in the well. Then, 2 mL precooled PBS was used to wash the cells in the test tubes twice. The liquid in the test tubes was centrifuged at 1000 rp/min for 5 min, and the supernatant was discarded. The cells were resuspended in 3 mL PBS and centrifuged for 5 min. Next, the supernatant was discarded, and the washing procedure was repeated once again.

The supernatant was discarded in the test tube and 100 μL 1× binding buffer was added to the test tube. The liquid in the tube was resuspended, and 5 μL Annexin-V-FITC and PI were added. The samples were later incubated in a 25°C water bath for 15 min. Next, 400 μL 10× binding buffer was added to the test tube, which was mixed for the test.

Transmission electron microscopy

The group establishment and cell culture method are same as those described in *Hoechst staining*, and the extraction method of the cells is same as the step in *Flow cytometry*. After centrifugation, we carefully discarded the supernatant and collected the cell deposits. Then, the fixing solution was added to the cell deposit overnight, which was rinsed, fixed, dehydrated and buried. Eventually, all samples were stained with uranyl acetate and lead citrate and then observed under a transmission electron microscope (H-7600; HITACHI, Ltd., Tokyo, Japan). Fifteen fields were randomly selected for each sample [12].

Western blot

The group settings and cell culture methods were the same as those in *Hoechst staining*. Next, the supernatant from the 6-well plates was removed. Then, 2 mL of precooled PBS at 4°C was used to wash the samples twice, and the washing fluid was removed. Next, 100 μL cell lysis fluid, including 2 μL PMSF and 2 μL phosphorylase inhibitor, was added to each plate and incubated on ice for 15 min. The lysed cells were collected into EP tubes with a scraper. The liquid was centrifuged at 4°C and 12000 rp/min for 15 min, and then, the supernatant was carefully moved to another clean EP tube. The loading buffer was added to the EP tube at a dilution of 1:4 and then boiled at 100°C for 15 min.

The concentration of proteins was determined by the BCA method. Polyacrylamide gel electrophoresis (PAGE) was used to separate proteins. The membranes were blocked with 5% milk for 1 h after transfer. After the membranes were washed, they were incubated in 1:1000-diluted primary antibodies against Hif-1α (1:1000, ab179483; Abcam, Cambridge, UK), Beclin-1 (1:1000, ab210498; Abcam, Cambridge, UK), LC3 (1:1000, ab192890; Abcam, Cambridge, UK), SOD2 (1:2000, 24127-1-AP; Proteintech Group, USA), GPX4 (1:1000, 14432-1-AP; Pro-

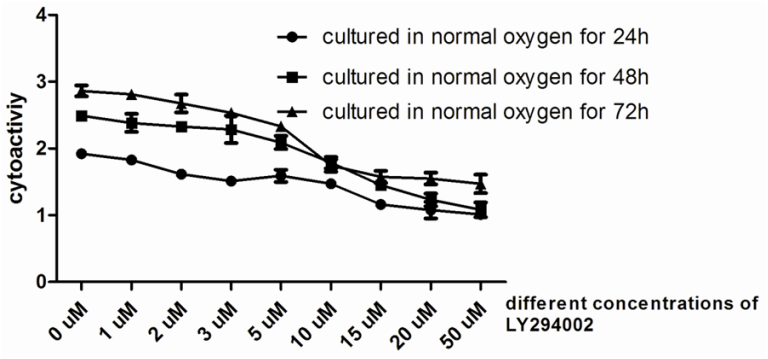

Figure 1. The effect of different concentration of LY294002 on the cytoactivity of spermatogenium. Cells are cultured in normal oxygen. As the concentrate of the LY294002 increases, the cytoactivity of the cells gradually decrease. Moreover, in the same concentrate of LY294002, the cytoactivity of cells cultured in normal oxygen for 72 h is significantly lower than 24 h.

teintech Group, Cambridge USA), Cyt C (1:1000, EPR1327; Abcam, Cambridge, UK) or GAPDH (1:1000, EPR16891; Abcam, Cambridge, UK) at 4°C on a shaking bed for 12 h. After the membranes were washed, they were incubated with HRP-labeled goat anti-rabbit or goat anti-mouse (1:10000) secondary antibodies for 1 h. After three washes, the bands were detected by an Odyssey system.

Statistical analysis

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

Results

Cytoactivity of the spermatogonia under normal oxygen conditions

Under normal oxygen conditions, different concentrations of LY294002 were administered to each group, and the cytoactivity (absorbance value) of each group was measured at 24 h, 48 h and 72 h, as shown in **Figure 1**. The cytoactivity of each group decreased with increasing concentrations of LY294002. In addition, at 10 μM, the cytoactivity showed the fastest decline and was about half of the cytoactivity at 0 μM. Therefore, 10 μM was used for further experiments.

Inhibition ratio of spermatogonia under hypoxic conditions

Cells were cultured under hypoxic conditions, and the cytoactivity was measured at 24 h, 48 h and 72 h. As shown in **Figure 2A**, as the hypoxia time was prolonged, the cytoactivity decreased substantially, and the decreased amplitude was approximately 0.3. Different concentrations of LY294002 were added to each group, and then, the cells were cultured and measured with the same procedure. As shown in **Figure 2B**, the higher the concentration of LY294002, the higher the cell inhibition ratio was. Moreover, the inhibition ratio of hypoxia at 72 h was higher than that at 24 h.

Hoechst staining

Figure 3A-C show the cells cultured under chronic hypoxia for 24 h, 48 h, and 72 h, respectively. **Figure 3D-F** show cells treated with LY294002 (final concentration of 10 μM) and cultured under chronic hypoxic conditions for 24 h, 48 h, and 72 h, respectively. The apoptotic rates of each group were compared. As shown in **Table 1**, in the hypoxic groups, there was no significant difference in the apoptotic rates between the 24 h and 48 h groups ($P=0.977>0.05$). However, the apoptosis index of the 72 h group significantly increased compared with that of the 24 h group ($P<0.001$). In the hypoxia+LY294002 groups, there was no significant difference between the 24 h group and the 48 h group ($P=0.203>0.05$). However, the apoptosis index at 72 h significantly increased compared with that at 24 h ($P<0.001$). In addition, the apoptosis rate of the hypoxia+LY294002 72 h group was significantly higher than that of the 72 h hypoxia group ($P=0.033<0.05$).

Flow cytometry/Annexin-V-FITC/PI staining

The grouping and drug administration were the same as those described in the *Hoechst staining* section. In **Figure 4A-F**, the lower left quad-

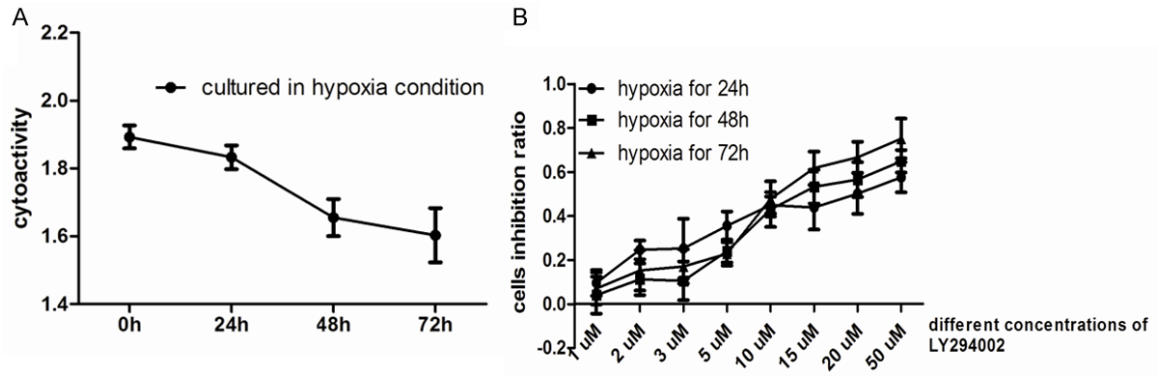

Figure 2. Effects of hypoxia on cytoactivity. A. The cytoactivity of the spermatogenium gradually decrease as the hypoxia time prolonges. B. The inhibition ratio of different concentrations of LY294002 and different hypoxia time. As the concentrate of the LY294002 increases, the inhibition ratio of the cells cultured in hypoxia condition gradually increase. In addition, in the same concentrate of LY294002, the inhibition ratio of the cells cultured in hypoxia condition for 72 h is significantly higher than 24 h.

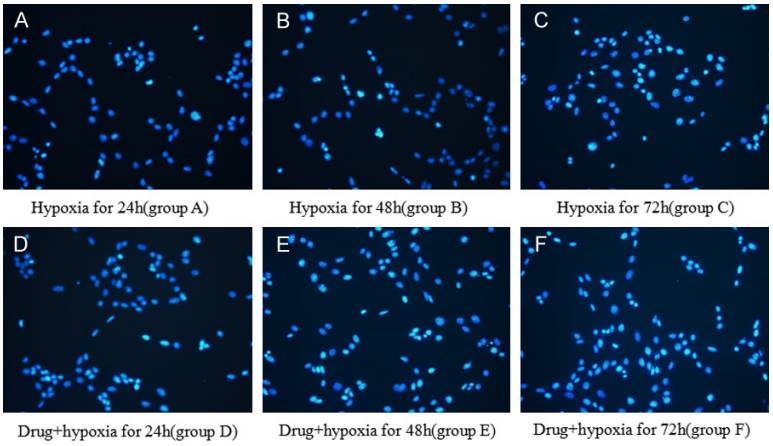

Figure 3. Hoechst staining. The nucleus of normal spermatozoa were round and dyed light blue. But the nucleus of apoptotic spermatozoa were wrinkled, uneven in high density and high brightness. A-C. Cells were cultured in hypoxia condition for 24 h, 48 h, and 72 h. There were few of high brightness nucleus in hypoxia for 24 group, but the high brightness nucleus were more common in hypoxia for 72 h group. D-F. Cells were treated with LY294002 (final concentration of 10 uM) and cultured under chronic hypoxic conditions for 24 h, 48 h, and 72 h, respectively. As the hypoxia time prolonged, the number of hightness and wrinkled nucleus of spermatogenia increased gradually.

rant shows normal cells, the lower right quadrant shows early apoptotic cells, and the upper right quadrant shows late apoptotic cells. The comparison methods were the same as those described in *Hoechst staining*. As shown in **Table 2**, the apoptosis rate of spermatogonia in the hypoxia 72 h group was higher than that of the hypoxia 24 h group (P=0.002<0.01), and the apoptotic cells were mainly in the early stage of apoptosis. In the hypoxia+LY294002 groups, the apoptosis rate of the hypoxia+LY294002 72 h group was higher than that of the hypoxia 72 h group (P<0.001), and there were more late-stage apoptotic cells in the hypoxia+LY294002 72 h group than in the hypoxia 72 h group (**Figure 4C** and **4F**).

Transmission electron microscopy

The grouping and drug administration were the same as those described in *Hoechst staining*. "←" represents autophagosomes or autolysosomes. As shown in **Figure 5A**, the nuclei of the spermatogonia were round, and there were abundant normal mitochondria and few autophagosomes and autolysosomes. However, as shown in **Figure 5C**, as the hypoxia time was prolonged, the number of the swollen, elongated mitochondria, autophagosomes and autolysosomes increased significantly. Furthermore, after administration of 10 μmol/L of LY294002, we observed that the number of autophagosomes and autolysosomes decreased, but the number of structurally abnormal mitochondria increased significantly (**Figure 5D**, **5E**). In addition, few mitochondria but many vacuoles, autophagosomes and autolysosomes were observed in the hypoxia+LY294002 72 h group (**Figure 5F**).

Table 1. Hoechst detects apoptosis cells, AI (apoptosis index)

	Hypoxia for 24 h (group A)	Hypoxia for 48 h (group B)	Hypoxia for 72 h (group C)	Drug+Hypoxia for 24 h (group D)	Drug+Hypoxia for 48 h (group E)	Drug+Hypoxia for 72 h (group F)
No. of visual fields (*n*)	30	30	30	30	30	30
Apoptosis index, AI (%) (mean ± SD)	2.93±1.72	2.47±1.28	5.80±2.55^{aa,bb}	2.40±1.54^{cc}	3.57±2.05^{cc}	7.97±2.70^{aa,bb,c,dd,ee}

AI (apoptosis index) of the 6 groups. Group A, B, C, D, E, F respectively represent hypoxia for 24 h, 48 h, 72 h group and drugs+hypoxia for 24 h, 48 h, 72 h group. Hyoxia for 24 h, 48 h, 72 h group respectively represents spermatogonia cultured under hypoxia condition for 24 h, 48 h, 72 h. Drugs+hypoxia for 24 h, 48 h, 72 h group represents spermatogonia administrated with LY294002 (final concentration of 10 uM) and then cultured under hypoxia conditions for 24 h, 48 h, and 72 h, respectively. Significant difference: ^{aa}P<0.01 with respect to group A; ^{bb}P< 0.01 with respect to group B; ^{c}P<0.05, ^{cc}P<0.01 with respect to group C; ^{dd}P<0.01 with respect to group D; ^{ee}P<0.01 with respect to group E. AI in group C is higher than group A (^{aa}P<0.01), and AI in group F significantly increases with respect to group C (^{c}P<0.05).

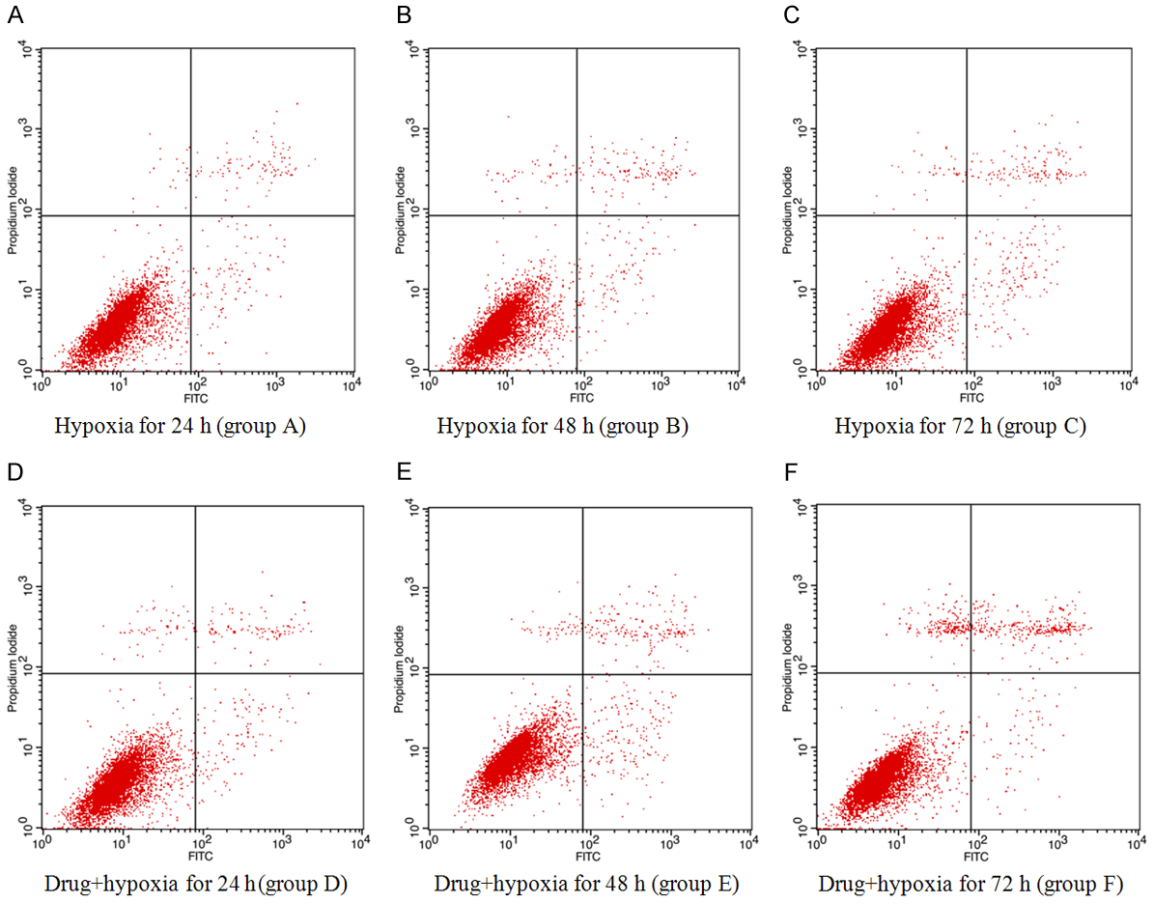

Figure 4. Flow cytometry Annexin-V-FITC/PI staining. The horizontal axis represents FITC staining and the vertical axis represents PI staining. The left lower quadrant represents normal spermatogenia, the right lower quadrant represents early apoptotic spermatogenia, and the upper right quadrant represents advanced apoptotic cells. A-C. Cells were cultured in hypoxia condition for 24 h, 48 h, and 72 h. As the hypxia time prolonged, the spermatogenia in the upper right quadrant and the lower right quadrant gradually increased. D-F. Cells were treated with LY294002 (final concentration of 10 uM) and cultured under chronic hypoxic conditions for 24 h, 48 h, and 72 h, respectively, and the spermatogenia in the upper right quadrant and the lower right quadrant also gradually increased.

Western blot analysis

The grouping and drug administration were the same as those in *Hoechst staining*. Blot bands are shown in **Figure 6A**, **6B**. **Figure 6C-H** shows the diagrams of the content of each protein. As shown in **Figure 6** and **Table 3**, compared with the hypoxia 24 h group, the hypoxia 72 h group had significantly increased levels of the hypoxic marker Hif-1α, the autophagy-related molecules LC3-2/LC3-1 and Beclin-1, and the mitochondrial damage marker Cyt C (P<0.01). However, the antioxidation system enzymes SOD2 and GPX4 showed no significant changes (P>

Table 2. Flow cytometry detects the apoptosis cells

	Hypoxia for 24 h (group A)	Hypoxia for 48 h (group A)	Hypoxia for 72 h (group A)	Drug+Hypoxia for 24 h (group D)	Drug+Hypoxia for 48 h (group D)	Drug+Hypoxia for 72 h (group D)
No. of samples (*n*)	9	9	9	9	9	9
Apoptosis index, AI (%) (mean ± SD)	2.64±0.78	4.27±1.03^{aa}	4.56±1.31^{aa}	3.91±0.70^{a}	6.60±1.52^{aa,bb,c,dd}	9.47±1.67^{aa,bb,cc,dd,ee}

Histogram of the apoptosis index of the 6 groups. Group A, B, C, D, E, F respectively represent hypoxia for 24 h, 48 h, 72 h group and drugs+hypoxia for 24 h, 48 h, 72 h group. Hyoxia for 24 h, 48 h,72 h group respectively represents spermatogonia cultured under hypoxia condition for 24 h, 48 h, 72 h. Drugs+hypoxia for 24 h, 48 h, 72 h group represents spermatogonia administrated with LY294002 (final concentration of 10 uM) and then cultured under hypoxia conditions for 24 h, 48 h, and 72 h, respectively. Significant difference: ^{a}P<0.05, ^{aa}P<0.01 with respect to group A; ^{bb}P<0.01 with respect to group B; ^{c}P<0.05, ^{cc}P<0.01 with respect to group C; ^{dd}P<0.01 with respect to group D; ^{ee}P<0.01 with respect to group E. AI in group C is higher than group A (^{aa}P<0.01), and AI in group F significantly increases with respect to group C (^{cc}P<0.01).

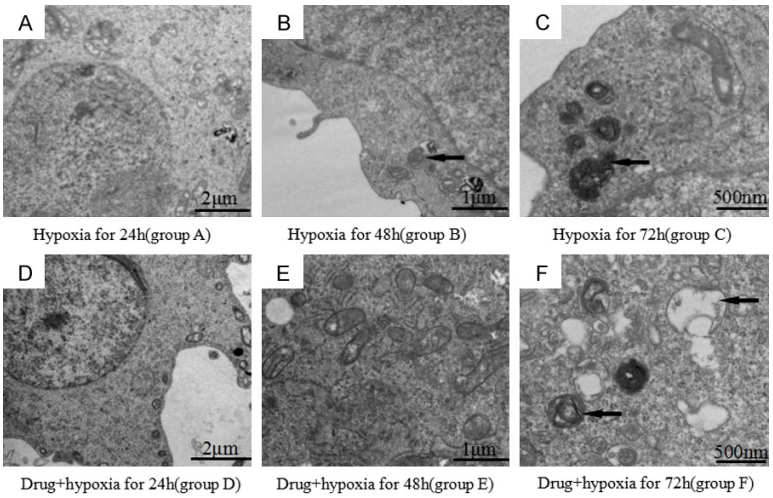

Figure 5. Transmission electron microscopy. "←" represents autophagosomes or autolysosomes. In physiological state, the nucleus of spermatogonia is round, with abundant organelles, and there are few of autophagosomes, autolysosomes. A-C. Cells were cultured in hypoxia condition for 24 h, 48 h, and 72 h. A. There were abundant of normal organelles in spermatogonia. B. A large number of elongated mitochondria and autophagolysosomes were in spermatogonia (indicated by the black arrow), and normal mitochondria were rare. C. There were some structural abnormal mitochondria, such as swelling and elongation, and these mitochondria were being swallowed by autophagosomes (indicated by the black arrow). D-F. Cells were treated with LY294002 (final concentration of 10 uM) and cultured under chronic hypoxic conditions for 24 h, 48 h, and 72 h, respectively. D. There were some swollen mitochondria and few of autophagosomes, autolysosomes. E. There were abundant of abnormal structure mitochondria and some "vacuoles" appeared in spermatogenia in drugs+hypoxia 48 h group. F. A few of mitochondria, but abundant of autophagosomes, autolysosomes, "vacuoles" and other structure appeared in drugs+hypoxia 72 h group. Spermatogenia.

0.05). After administration of LY294002, Beclin-1, GPX4, and LC3-II/LC3-I in the drugs+hypoxia 72 h group were significantly lower than those in the hypoxia 72 h group (P<0.01). However, there was no significant difference in Cyt C between the two groups (P>0.05). Overall, compared with the hypoxia 24 h group, the drug+hypoxia for 72 h group showed significantly increased Hif-1α and Cyt C and significantly decreased Beclin-1, LC3-II/LC3-I, and GPX4. However, there was no significant difference in SOD2 among the groups (P>0.05).

Discussion

Previous studies [8, 13, 14] have found that Hif-1α is significantly higher in testes from animals with VC than in normal testes. This finding indicates that hypoxia may be an important factor in testicular damage. Our previous study found that the expression of Hif-1α in testicular tissue gradually increased as the time the rats suffered from VC increased. Moreover, for the first time, we found that autophagy was significantly enhanced in the spermatogenic epithelium of VC rats.

Autophagy plays different roles in different diseases. Autophagy has been shown to play different roles in different tumor cells. The effects of autophagy, though widely reported in other diseases, are poorly understood in VC. For the first time, we cultured spermatogonia in a tri-gas incubator to simulate the hypoxic environment of VC and found that hypoxia could induce autophagy. However, whether autophagy plays a role in protecting spermatogonia from apoptosis or promoting apoptosis remains unknown.

Autophagy is regulated by a variety of signaling pathways, including the PI3K/AKT/mTORC signaling pathway, the ATP dependent protein

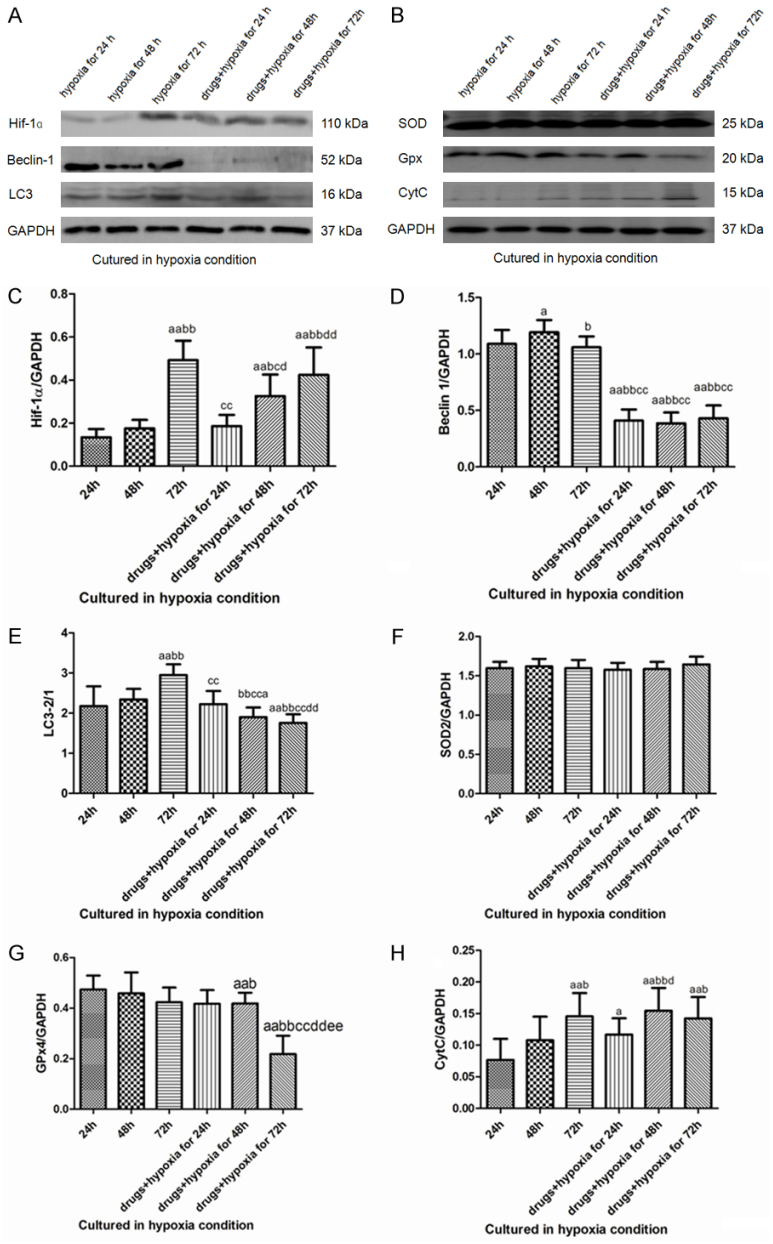

Figure 6. Western blot analysis for hypoxia marks molecular Hif-1α, autophagy related molecular Beclin-1, LC3, antioxidant enzyme system SOD2, GPX4, mitochondrial damage markers cytC. Group A, B, C, D, E, F respectively represent hypoxia for 24 h, 48 h, 72 h group and drugs+hypoxia for 24 h, 48 h, 72 h group. Hyoxia for 24 h, 48 h, 72 h group respectively represents spermatogonia cultured under hypoxia condition for 24 h, 48 h, 72 h. Drugs+hypoxia for 24 h, 48 h, 72 h group represents spermatogonia administrated with LY294002 (final concentration of 10 uM) and then cultured under hypoxia conditions for 24 h, 48 h, and 72 h, respectively. Significant difference: $^{a}P<0.05$, $^{aa}P<0.01$ with respect to group A; $^{b}P<0.01$, $^{bb}P<0.01$ with respect to group B; $^{c}P<0.05$, $^{cc}P<0.01$ with respect to group C; $^{d}P<0.05$, $^{dd}P<0.01$ with respect to group D; $^{e}P<0.05$, $^{ee}P<0.01$ with respect to group E. A, B. Blotting bands of Hif-1α, Beclin-1, LC3, SOD2, GPX4, cytC. C-H. The histograms of each proteins. Hif-1α in group C is higher than group A ($^{aa}P<0.01$), but there is no difference between group C and group F ($P>0.05$). Beclin-1, LC3-II/LC3-I in group D, E, F is significantly lower than in the group A, B, C ($P<0.05$). Moreover, there is no significant difference in SOD2 among the groups, but the level of GPX4 significantly decreases in group F with respect to group A ($^{aa}P<0.01$) and C ($^{cc}P<0.01$). The level of Cyt C in group C is higher than group A ($^{aa}P<0.01$), but there is no significant difference between group C and F ($P>0.05$).

kinase pathway, and the insulin AKI pathway [15]. The regulatory targets are all ATG1 complexes (Autophagy related gene 1 complex, ATG1 complex). The ATG1 complex consists of ATG13, ATG101, FIP200, and ULK1. Activated ULK1 will induce Beclin-1 and other proteins to generate the class III PI3K complex I, which is localized on the autophagic initial membrane, and then, LC3 is catalyzed by a series of enzymes to form LC3-II. All of these proteins play a key role in synthesizing the autophagic initial membrane [16]. LY-294002 is an autophagy blocker and can effectively inhibit the synthesis of class I/II/III PI3K. Furthermore, class III PI3K plays an important role in initiating autophagy [17]. We inhibited the autophagy of hypoxic spermatogonia to observe the effects. The gold standard for autophagy detection is to observe autophagosomes under transmission electron microscopy. In addition, the detection of Beclin-1, LC3 and other autophagy-related molecules also allows for observation of autophagy.

This research established a new way to further study the VC. We established a chronic anoxic environment to culture spermatogonia and to simulate the anoxic environment of VC. Under normal oxygen conditions, blocking autophagy decreased the proliferation of spermatogonia (**Figure**

Table 3. Western-blot detects the expression of the molecules

	Hypoxia for 24 h (group A)	Hypoxia for 48 h (group A)	Hypoxia for 72 h (group A)	Drug+Hypoxia for 24 h (group D)	Drug+Hypoxia for 48 h (group D)	Drug+Hypoxia for 72 h (group D)
No. of samples (*n*)	9	9	9	9	9	9
Hif-1α/GAPDH	0.134±0.039	0.176±0.040	0.492±0.091^{aa,bb}	0.187±0.052^{cc}	0.326±0.100^{aa,b,c,d}	0.424±0.127^{aa,bb,dd}
Beclin 1/GAPDH	1.089±0.124	1.922±0.108	1.059±0.097^{b}	0.409±0.100^{aa,bb,cc}	0.386±0.096^{aa,bb,cc}	0.431±0.114^{aa,bb,cc}
LC3-2/GAPDH	2.174±0.492	2.338±0.266	2.949±0.271^{aa,bb}	2.222±0.331^{cc}	1.899±0.244^{bb,cc,d}	1.754±0.217^{aa,bb,cc,dd}
SOD2/GAPDH	1.600±0.076	1.621±0.089	1.597±0.100	1.577±0.084	1.588±0.085	1.645±0.093
GPx4/GAPDH	0.473±0.052	0.458±0.078	0.423±0.054	0.417±0.051	0.384±0.061^{aa,b}	0.218±0.068^{aa,bb,cc,dd,ee}
CytC/GAPDH	0.077±0.032	0.107±0.035	0.146±0.035^{aa,b}	0.117±0.024^{a}	0.154±0.034^{aa,bb,d}	0.142±0.032^{aa,b}

It is the relative content of each proteins in each group. The grouping and comparisons among the groups are the same as **Tables 1** and **2**. Significant difference: ^{a}P<0.05, ^{aa}P<0.01 with respect to group A; ^{b}P<0.01, ^{bb}P<0.01 with respect to group B; ^{c}P<0.05, ^{cc}P<0.01 with respect to group C; ^{d}P<0.05, ^{dd}P<0.01 with respect to group D; ^{e}P<0.05, ^{ee}P<0.01 with respect to group E.

1). As the hypoxia time was prolonged, the expression of the hypoxic marker Hif-1α gradually increased (**Figure 6A** and **6C**), and the cytoactivity significantly decreased (**Figure 2A**). Furthermore, hypoxia also enhanced the expression of autophagy-related molecules (such as Beclin-1 and LC3) in the spermatogonia (**Figure 6A-D** and **Table 3**). Moreover, the apoptotic spermatogonia gradually increased (**Figure 3A-C**, **Tables 1** and **2**), and these apoptotic cells were mainly in the early phage of apoptosis (**Figure 4A-C**). The above findings indicated that chronic hypoxia not only decreased the cytoactivity of the spermatogonia but also activated autophagy. Furthermore, as the hypoxic time was prolonged, the environmental pressure exacerbated the apoptosis of spermatogonia.

When LY294002 was added to the hypoxic spermatogonia, we found that the inhibition ratio of spermatogonia significantly increased, and the rangeability of the inhibition ratio was largest with 10 μM treatment (**Figure 2B**). After administration of LY294002, the expression of the autophagy-related molecules Beclin-1 and LC3-II/I significantly decreased (**Figure 6C-E**), and autophagosomes also decreased (**Table 3**). Furthermore, in hypoxic conditions, blocking the autophagy of spermatogonia increased the apoptosis rate of the hypoxic spermatogonia (**Figures 3D**, **3E**, **4D**, **4E**, **Tables 1**, **2**). This finding indicated that autophagy may play an important role in spermatogonia against the hypoxic environment.

SOD and glutathione peroxidase (GPX) are important antioxidant enzymes that have several subtypes. When tissues or cells are attacked by oxidative stress, these enzymes can remove free radicals and maintain homeostasis of the cells. Among them, SOD2 and GPX4 play an important role in spermatogenesis of mammalian testis. The expression of SOD2 was reported to decrease with hyperglycemic-induced oxidative stress in human spermatogonia [18]. Exogenous toxic substances may decrease the quality of the sperm by affecting *SOD2*, *GPX4* genes and DNA repair genes [19]. Studies showed that VC can lead to ROS damage in rat sperm, which can significantly reduce the level of SOD2 [20]. GPX4, an important antioxidant enzyme, is the predominant selenoenzyme in testis and plays a vital role in spermatogenesis [21]. A study of rats showed that heat stress-induced testicular injury resulted in a significant decrease in the level of antioxidation systems such as GPX4 in the testicular tissue [22]. Manuela Schneider et al. performed a study of *GPX4* gene knockout rats and discovered that the quality of the sperm seriously declined, and the sperm displayed higher protein thiol content and recapitulated features typical of severe selenodeficiency [21]. Whether SOD2 and GPX4 play an important role in hypoxia spermatogonia remains unknown. Our study found that there was no significant difference in the relative SOD2 content in each group (**Figure 6F**), indicating that SOD2 did not play a major role in combating oxidative stress in hypoxic spermatogonia. GPX4 did not decrease significantly as the hypoxic time was prolonged, but when autophagy was blocked, GPX4 decreased significantly (**Figure 6G**), and the apoptosis index also increased significantly. These findings suggested that GPX4 may play an important role in reducing hypoxia-induced oxidative stress injury and cell apoptosis. However, how GPX4 functions requires further study.

Cyt C is distributed on the outside of the mitochondrial inner membrane and is an important component of the electron transport chain. Cyt C is usually localized in the mitochondria and seldom enters into the cytoplasm. However, when cells are exposed to stimuli such as oxi-

dative stress, mitochondria are damaged, and the permeability of the mitochondria membrane increases, which eventually leads to high levels of Cyt C released into cytoplasm and mediates apoptosis. Therefore, the content of Cyt C reflects the stability of the mitochondrial membrane and cells. Chronic hypoxia and other stimulatory factors can lead to oxidative stress injury, and excessive ROS will damage the mitochondria and other organelles. Moreover, the damaged organelles also activate autophagy to remove themselves in a timely manner and to prevent secondary damage [23, 24]. Our study showed that continuous hypoxia could damage the mitochondria and lead to structural abnormalities (**Figure 5A-C**). Meanwhile, hypoxia also activated autophagy (**Figure 5A-C**). After inhibition of autophagy, the increase of abnormal mitochondria was more obvious (**Figure 5D**, **5F**), the content of Cyt C significantly increased (**Figure 6H**), and the apoptosis rate of cells also significantly increased (**Tables 1** and **2**).

In our previous study, we established the rat VC model through the method of Tuner [25]. We also observed that the expression of Hif-1α significantly increased in seminiferous epithelial cells of VC rats. At the same time, autophagy significantly increased in the seminiferous epithelial cells of VC rats. In this study, we investigated the role of autophagy in chronic hypoxic spermatogonia by blocking the autophagic pathway. We found that hypoxia could inhibit the cytoactivity and cell proliferation. Hypoxia increased structurally abnormal mitochondria and activated autophagy in chronic hypoxic spermatogonia. Structurally abnormal mitochondria and vacuoles significantly increased when autophagy was blocked in chronic hypoxic conditions. The apoptosis rate of these cells also increased significantly compared with that of untreated cells. The antioxidant enzyme SOD2 may not play a major role in hypoxic spermatogonia, but blocking autophagy can inhibit the clearance of damaged mitochondria and significantly reduce the content of GPX4, ultimately aggravating mitochondrial damage and inducing apoptosis of spermatogonia. Overall, in chronic hypoxic conditions, autophagy may play an important role in protecting spermatogonia and reducing apoptosis.

Disclosure of conflict of interest

None.

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