Original Article
Cistanoside of *Cistanche Herba* ameliorates hypoxia-induced male reproductive damage via suppression of oxidative stress

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Abstract: Increasing evidence shows that hypoxia is a cause of male infertility, and hypoxia may be related to oxidative stress (OS). Cistanoside (Cis) is a phenylethanoid glycoside compound that can be extracted from *Cistanches Herba* and possesses various biological functions. This study aimed to investigate the protective effects of Cis on reproductive damage induced by hypoxia and explore the specific underlying mechanisms. Cell and animal hypoxia experimental models were constructed, and the protective effects of different subtypes of Cis on the male reproductive system were assessed both *in vitro* and *in vivo*. The results indicated that hypoxia significantly reduced the viability of GC-1 cells through cell cycle arrest and apoptosis activation, which were associated with increased OS. Moreover, Cis showed strong antioxidative effects both *in vitro* and *in vivo*, significantly restoring antioxidant enzyme activities and downregulating reactive oxygen species (ROS) levels while increasing cell viability and decreasing apoptosis. Importantly, the Cis subtypes (Cis-A, Cis-B, Cis-C and Cis-H) studied herein all showed certain antioxidant effects, among which the effects of Cis-B were the most significant. This study demonstrates that Cis markedly attenuates the harmful effects of hypoxia-induced OS by affecting antioxidant enzyme activities in testes and GC-1 cells.

Keywords: Cistanoside, hypobaric hypoxia, male infertility, oxidative stress, reproductive protection

Introduction

Currently, approximately 48.5 million (15%) couples of reproductive age worldwide are affected by infertility [1], among which 40-50% of the cases are ascribed to male infertility [2], a condition that is strongly associated with environmental and lifestyle factors. Evidence suggests that susceptibility of the mammalian testis to low oxygen pressure is a causative factor of some forms of male infertility [3]. As demonstrated in previous studies, spermatogenesis is impaired and reduced upon exposure to hypobaric hypoxia [4-7].

To explore the underlying mechanism, studies have shown that exposure to hypobaric hypoxia increases reactive oxygen species (ROS) pro-duction [8, 9]. ROS play an important role in the male reproductive system. At low levels, they are required for sperm capacitation, the acrosome reaction and spermatozoa-oocyte fusion [10]. However, excessive ROS can induce sperm nuclear/mitochondrial DNA damage and plasma membrane peroxidative damage, which in turn are major etiological factors for the increased risk of male infertility [11, 12]. Thus, the accumulation of hypoxia-induced ROS might be one cause of male infertility.

Cistanches Herba, a perennial parasitic medicinal plant, is widely distributed in arid areas [13] and used widely due to its pharmacological activities [14-17]. Among all the effective contents of *Cistanches Herba*, PhGs have been regarded as the main active component. To

date, 34 PhGs have been isolated from *Cistanches* plants [13]. Cistanoside (Cis), an active PhG isolated from *Cistanches Herba*, has received attention for its antioxidant effects.

Considering the antioxidant effects of Cis and the role of ROS in hypoxia-induced male infertility, Cis is considered a potential drug candidate for the treatment of hypoxia-induced male infertility. However, few reports have addressed the antioxidant effects of Cis extracted from *Cistanches Herba* in the treatment of hypoxia-induced male infertility or the signaling pathways involved. In this study, *in vitro* and *in vivo* hypoxia experimental models were constructed and the effects components of different Cis were assessed.

Materials and methods

Cell culture and reagent

The mouse spermatogonia cell line GC-1spg (GC-1) was purchased from the American Type Culture Collection (ATCC) and cultured in DMEM (Invitrogen, USA) supplemented with 10% fetal bovine serum, 1% L-glutamine (100 mM), penicillin (100 U/mL), and streptomycin (100 µg/mL) at 37°C in a humidified incubator with 5% CO_2. Cis (Cis-A, B, C, H) was purchased from Chengdu Gelipu Biotechnology Co., Ltd. (China).

Cell viability assay

Cell viability was tested by cell counting kit-8 assay (CCK-8). In brief, GC-1 cells were seeded into 96-well plates at 1.5×10^3 per well, and cultured at 37°C for 24 h. Then, the cells were treated with different concentrations (20%, 15%, 10%, 5%) of oxygen or different concentrations (2 µM, 0.2 µM, 0.02 µM) of Cis (Cis-A, Cis-B, Cis-C, Cis-H) for the required time. Then, the supernatant was discarded, and cell viabilities were detected using a CCK-8 kit (Dojindo Japan). The absorbance of each well was measured at 450 nm using a microplate reader (BioRad USA). Finally, the cell viabilities were calculated according to the following formula: Cell viability = $(OD_{experimental\ group} - OD_{blank\ group})/(OD_{control\ group} - OD_{blank\ group}) \times 100\%$.

Western blot analysis

Harvested cells or tissues were homogenized in RIPA buffer to extract proteins (RIPA Beyotime

China; Cocktail Roche Switzerland). Supernatants were collected, and the concentration of proteins was tested by the BCA method (Beyotime). Approximately 40 µg of the extracted proteins from each sample was separated by SDS-PAGE and electrotransferred onto a nitrocellulose (NC) filter membrane (Beyotime China). NC filter membranes were blocked with 5% nonfat milk for 1.5 h and incubated with specific antibodies (anti-PARP 1:1000, anti-Caspase-3 1:1000, anti-Bcl-2 1:1000, anti-Bax 1:1000, anti-GAPDH 1:1000; all antibodies were purchased from Cell Signaling Technology, USA) overnight at 4°C. Then, all NC membranes were incubated with the corresponding horseradish peroxidase-conjugated secondary antibody for 1.5 h at room temperature and imaged with an imaging system (Tannon, China).

Cell cycle detection

A flow cytometric assay (FCM) was performed to analyze the cell cycle. Cells were treated under different conditions for 72 h and harvested. The cells were then washed with PBS, fixed in 75% ethanol and stained with propidium iodide (PI). For each sample, 1×10^4 cells were collected and analyzed by flow cytometry (FACS Calibur, BD Biosciences). Then, the proportion of G1/S/G2 phase cells and the proliferation index [$Phase_{(S+G2)}/Phase_{(G1+S+G2)} \times 100\%$] were calculated.

Ki-67 staining

Cells were cultured in a confocal dish and treated with hypoxia or different subtypes of Cis for 72 h. After fixing all cells with 4% paraformaldehyde, they were incubated with anti-Ki-67 antibody (1:200, Cell Signaling Technology). Then, all the cells were incubated with a corresponding CY-3-conjugated anti-rabbit IgG antibody (1:200, Boster, China) and DAPI solution (1.0 µg/mL, Beyotime). Fluorescence was observed with a Fluoview FV1000 confocal microscope (Olympus, Japan).

Detection of ROS

FCM was introduced to measure intracellular ROS levels using DCFH-DA. Suspended cells were seeded in 6-well plates and subjected to different treatments. After 72 h of treatment, cells were suspended in serum-free DMEM with 10 µM DCFH-DA (Beyotime). ROS contents were then determined by fluorescence-activated cell

sorting on a Beckman Coulter Flow Cytometry System with an excitation wavelength of 488 nm and emission wavelength of 525 nm [18].

Determination of Lipid Peroxidation (LPO)

The thiobarbituric acid reactive substances (TBARS) assay was performed to detect LPO. All operating steps were performed in accordance with the instructions (Sigma USA). The concentrations were calculated using a molar extinction coefficient of $1.56 \times 10^5 /(M \cdot cm)$, which was obtained utilizing malondialdehyde as a standard. The results are expressed as nmol of MDA equivalents/mg of protein.

Determination of enzyme activities

The enzyme activities were tested using assay kits including glutathione reductase (GR), glutathione peroxidase (GPx) and superoxide dismutase (SOD). All operating steps were performed in accordance with the instructions supplied (Nan Jing Jian Cheng Bioengineering Institute China).

Animals and experimental protocol

Mature male Wistar rats (180-220 g, 8 w old) were obtained from the animal center of the Fourth Military Medical University, Xi'an, China. Permission for use of the animals was obtained from the University Ethics Committee (Reference number: 20190506). The animal experiment was carried out according to university guidelines for the care and use of laboratory animals.

All rats were allowed to adapt for approximately 1 week prior to commencement of the experiment. After acclimation, the rats were randomly distributed into 6 groups with 5 animals in each group. The rats in the control group were raised under normal pressure (PO_2: 20%; air pressure: 101.3 kPa), while the rats in the model and Cis treated groups were raised in a low-pressure oxygen chamber (internal pressure of 61.6 kPa, equivalent to a height of 4000 meters above sea level; PO_2: 14.55%) to simulate a high-altitude hypoxic environment. All rats had free access to food and water in plastic cages at 22 ± 2°C and humidity conditions with an automatic 12-h light/dark cycle. The rats in the model and Cis treated groups remained under hypobaric conditions but were transferred to normobaric conditions every 96 h, at which time food and water were provided to them and the cages cleaned. The duration of transition from hypobaric to hypobaric was approximately 2 h. All treatment groups were treated with the corresponding Cis (8 mg/kg/d) via oral gavage for 8 weeks, whereas the control and model rats were treated with an equal volume of water.

After 8 weeks, rats were sacrificed under anesthesia. Testes, epididymis and seminal vesicles were separated and weighed, and the organ index was calculated according to the following formula: (weight of organ/animal weight) × 100%. Subsequently, sperm in the epididymis were collected, and their motility and acrosome enzyme activity were tested. Similarly, testicular tissues were collected for histopathologic studies and ROS, LPO, and antioxidant enzyme activity detection.

Evaluation of live sperm rate

The epididymis was incised with surgical scissors, and the sperm suspension was prepared in normal saline. To evaluate the live sperm rate, 5 μL of the sperm suspension was carefully mixed with an equal volume of eosin-Y stain. The sperm were then counted under a light microscope. Live sperm rates were assessed by calculating both stained (dead sperm) and unstained sperm (live sperm).

Determination of sperm acrosome enzyme activity

The sperm acrosome enzyme activity assay was utilized to evaluate sperm acrosome enzymes [19]. The results in each group were calculated using the following formula: Acrosome enzyme activity (μIU) = ($OD_{Experiment\ group}$ - $OD_{Blank\ group}$)/(247.5×10) × 10^6.

Statistical analysis

All quantitative data are expressed as the mean ± SD and were analyzed using SPSS 22.0 software. Independent Student's *t-test* was used to compare the data between two groups. $^*P < 0.05$ and $^{**}P < 0.01$ were considered statistically significant differences.

Results

Effects of hypoxia on GC-1 cells

To determine the effects of hypoxia on germ cells, we first examined the changes in cell via-

bility after hypoxia treatment with different oxygen concentrations (20%, 15%, 10%, 5%) for 1, 3, 5, and 7 days, respectively. The CCK-8 assay results showed that, compared with the control group (20% oxygen concentration), cells exposed to hypoxia exhibited a significant decrease in viability ($P < 0.01$; **Figure 1A**). Moreover, their survival rate was inversely proportional to the oxygen concentration and further decreased with induction time. To avoid an excessive cytotoxic effect, a 10% oxygen concentration and 3-day induction time were selected as the hypoxic model criteria for subsequent *in vitro* experiments.

Subsequently, FCM and immunofluorescence staining were performed to further evaluate the proliferation alteration of GC-1 cells under hypoxia treatment. The results showed that hypoxia could induce GC-1 cell arrest in G1 phase, thereby reducing cell entry into S phase and inhibiting DNA replication. Thus, hypoxia significantly reduced the proliferation index of GC-1 cells ($P < 0.01$; **Figure 1B**). Positive Ki-67 staining is another specific biomarker of proliferating cells. Therefore, we also examined the ratio of Ki-67-positive cells with or without hypoxia treatment. Compared with the control group, hypoxia treatment remarkably reduced Ki-67-positive cells, as shown in **Figure 1C**.

Next, we aimed to investigate the mode of GC-1 cell viability inhibition induced by hypoxia. As reported in the literature, the level of ROS increased as rats were exposed to a hypobaric hypoxic environment [8, 20]. Hence, endogenous ROS levels in GC-1 cells were measured using the FCM assay. The results showed higher ROS levels under hypoxia in comparison to the normal oxygen group ($P < 0.01$; **Figure 1D**). Accumulated ROS cause marked DNA impairment, which in turn causes cell apoptosis pathway activation and might be the major etiological factor for the increasing risk of male infertility [21, 22]. Next, we detected the apoptotic activation effect of hypoxia on GC-1 cells by TUNEL staining. As shown in **Figure 1E**, treatment with hypoxia resulted in an increase in TUNEL fluorescence compared with the control group, indicating an increase in apoptosis in the model group.

Since ROS-induced cell damage is usually caused by OS, we further tested the OS of GC-1 cells. As presented in **Figure 1F**, the LPO levels of GC-1 cells in the model group were markedly increased compared to the LPO levels of GC-1 cells in the control group. These findings suggested that hypoxia-induced GC-1 cell injury might be related to OS, which is induced by ROS accumulation.

*Effects of Cis on hypoxia-induced GC-1 cell viability in vitro.*

To investigate whether Cis can prevent the inhibitory effects of hypoxia on GC-1 cell viability, a CCK-8 assay was performed. GC-1 cells were treated with different subtypes (Cis-A, B, C, H) and concentration ranges (0.02 µM, 0.2 µM, 2 µM) of Cis for 72 h. Comparison of the model group with the DMSO group showed that DMSO did not directly promote GC-1 cell viability (**Figure 2A**). However, cell viabilities were markedly restored ($P < 0.05$) with Cis treatments. Compared with the model group, Cis-A, Cis-B, Cis-C and Cis-H all showed certain protective effects on hypoxia-induced damage to GC-1 cell viability, and Cis-B showed the most significant effect (**Figure 2A**). The protective effects of Cis at 0.2 µM were significantly higher than the protective effects of Cis at 0.02 µM, while the difference between 2 µM and 0.2 µM was not obvious, indicating that the restored GC-1 cell viability induced by Cis demonstrated a dose-dependent increase in the concentration range from 0.02-0.2 µM (**Figure 2A**). Therefore, according to the experimental needs, 0.2 µM Cis was selected as the optimal concentration in the following *in vitro* experiments. To further confirm whether germ cells were indeed protected by Cis, FCM and Ki-67 staining were performed to assess the alteration of the proliferation of GC-1 cells after treatment with Cis. Upon Cis treatment, the proportion of GC-1 cells in G1 phase was reduced. In contrast, more cells entered S phase, suggesting that Cis-treatment could increase the germ cell proliferation index ($P < 0.01$; **Figure 2B**). The statistics for the GC-1 cell cycle are shown in **Figure 2Bb**. The Ki-67 staining results also showed that Cis-A, Cis-B, Cis-C and Cis-H treatment significantly improved the Ki-67-positive cell ratio of hypoxia-induced GC-1 cells *in vitro* (**Figure 2C**).

The mechanism of Cis protects germ cells from hypoxia in vitro.

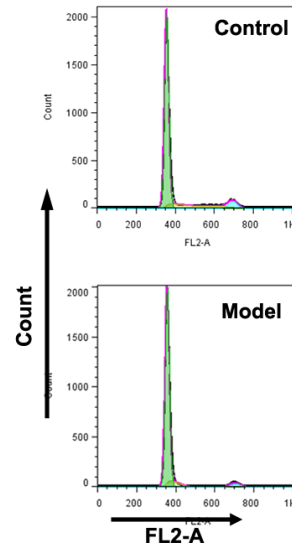To investigate whether the protective effects of Cis on GC-1 cells were related to the removal of excessive ROS, the fluorescent dye DCFH-DA
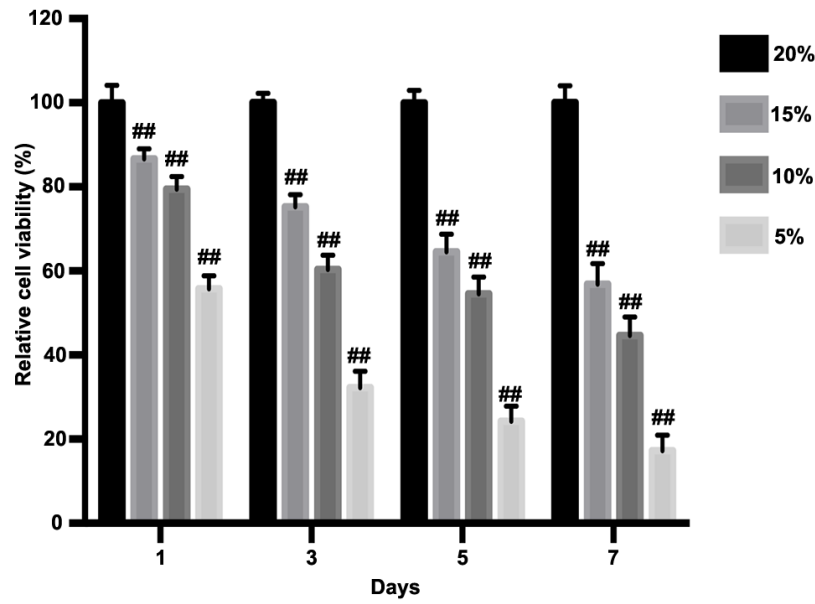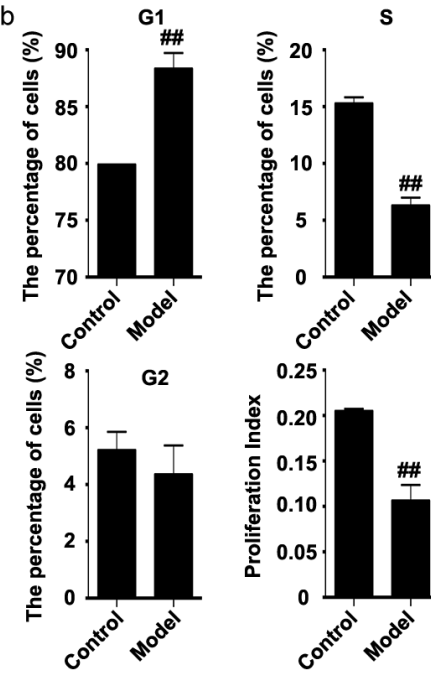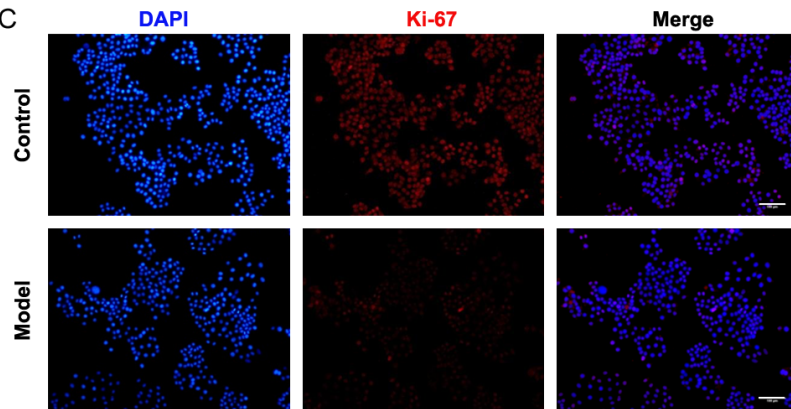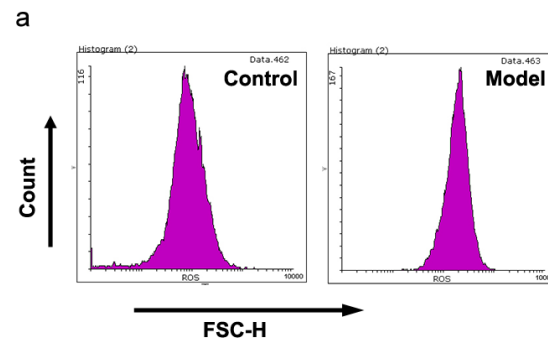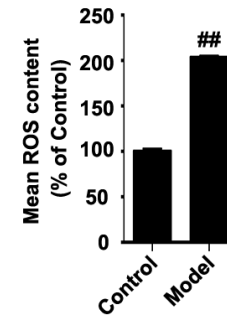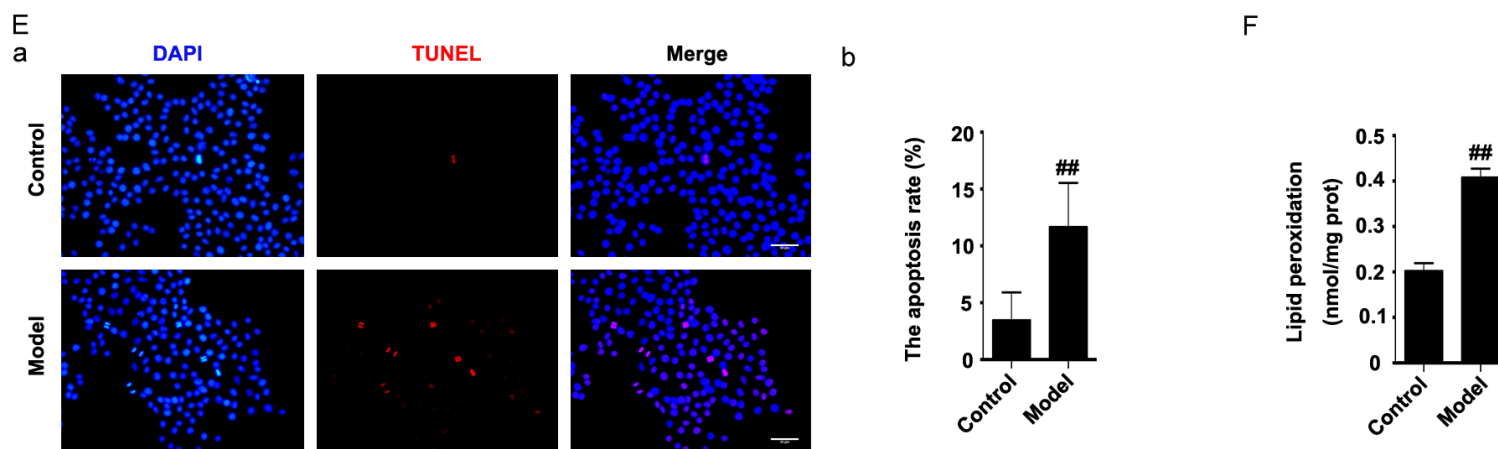
Figure 1. Effects of hypoxia on GC-1 cells. (A) GC-1 cells were treated with a range of concentrations of oxygen (20%, 15%, 10%, 5%; 20%) for 1, 3, 5 and 7 d, respectively. Then, the viability of GC-1 cells was calculated by the CCK-8 assay. Experiment in (B-F): GC-1 cells were subjected to 10% oxygen content (hypoxia model group) or normal oxygen (control group, 20% oxygen content) conditions with or without treatment for 72 h. (B) The cell cycle of GC-1 cells was determined by the FCM assay, and the proportion of G1/S/G2 phase cells and the proliferation index [(S+G2)/(G1+S+G2) × 100%] were calculated. (C) Ki-67 expression in GC-1 cells was tested by immunofluorescence staining assay (Bar = 100 μm). (D) ROS levels in GC-1 cells were tested by FCM assay. (E) Apoptosis of GC-1 cells was tested by TUNEL staining, and the apoptosis rates were calculated (Bar = 20 μm). (F) LPO levels in GC-1 cells were measured by the TBARS assay. Bars indicate the mean ± SD (n = 3). $^{##}P < 0.01$, $^{#}P < 0.05$ (versus the control group).
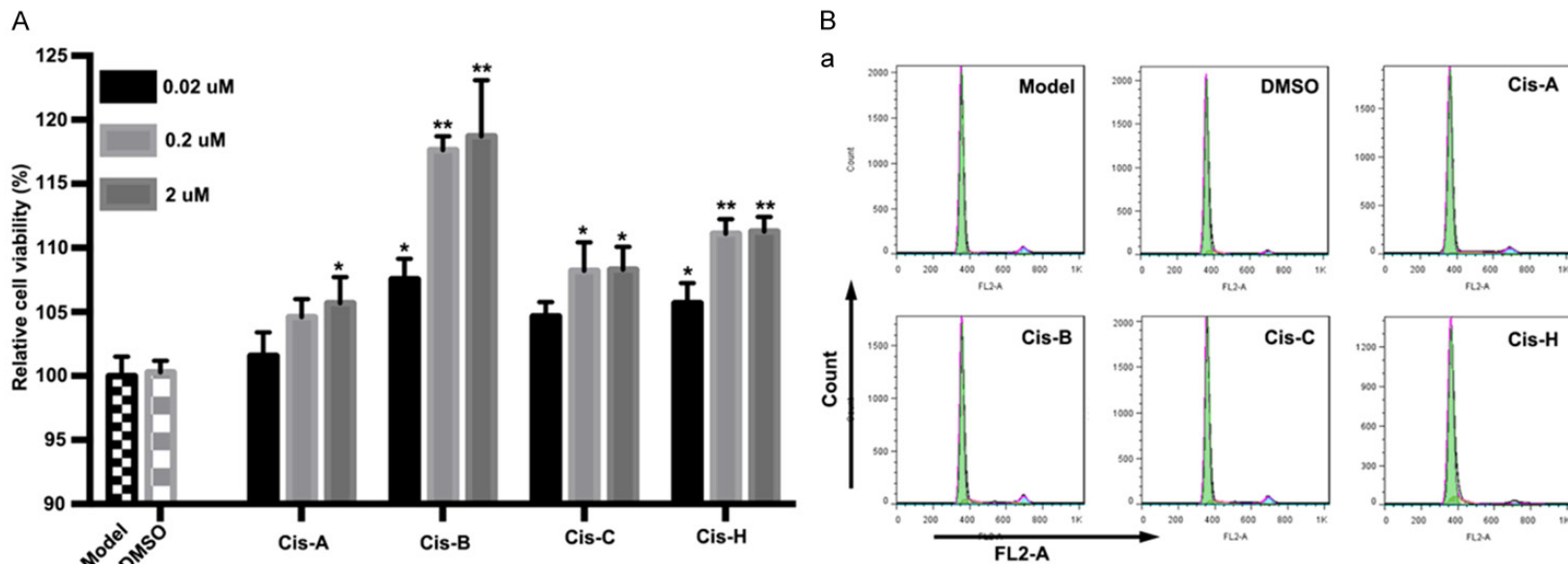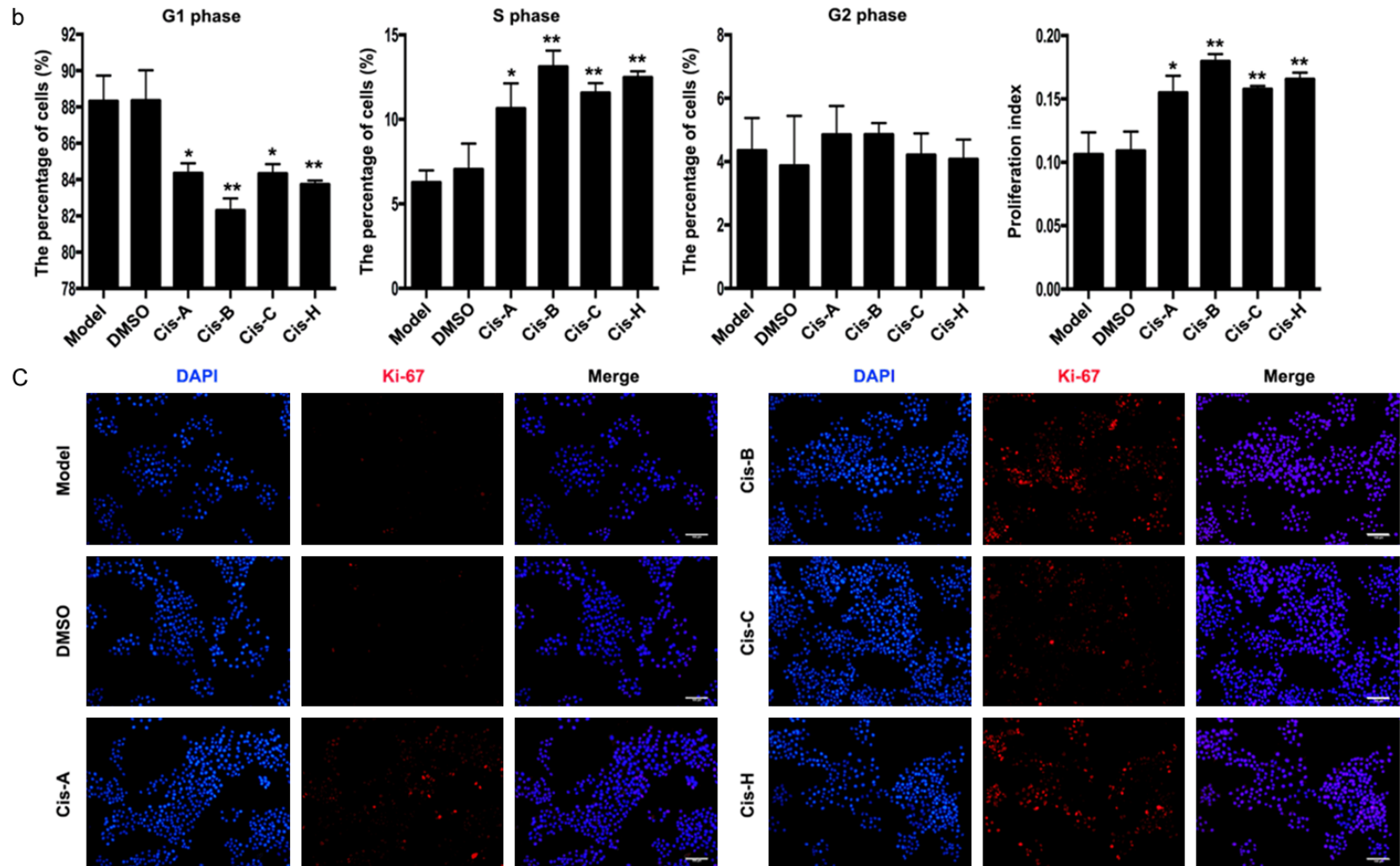
Figure 2. Cis restored hypoxia-induced GC-1 cell viability. (A) GC-1 cells were treated with different concentrations (0.02 μM, 0.2 μM, 2 μM) or subtypes (Cis-A, B, C, H) of Cis for 72 h, and then, the viability of GC-1 cells was calculated by the CCK-8 assay. Experiment in (B, C): GC-1 cells were subjected to hypoxic conditions (10% oxygen content) with or without Cis treatment (0.2 μM Cis-A, B, C, H) for 72 h. (B) The cell cycle of GC-1 cells was tested by the FCM assay, and the proportion of G1/S/G2 phase cells and proliferation index [(S+G2)/(G1+S+G2) × 100%] were calculated. (C) Ki-67 expression in GC-1 cells was tested by immunofluorescence staining assay (Bar = 100 μm). Bars indicate the mean ± SD ($n = 3$). $^{**}P < 0.01$, $^{*}P < 0.05$ (versus the model group).

was used to detect ROS levels in each group. As shown in **Figure 3A**, **3B**, treatment with DMSO did not change the intracellular ROS content or LPO level compared with the model group. However, ROS levels in GC-1 cells were markedly reduced in the Cis-treated groups (**Figure 3A**). Furthermore, a decrease in LPO was also observed in GC-1 cells subjected to Cis (**Figure 3B**).

To further explore the mechanism by which Cis protects germ cells from hypoxic injury, TUNEL staining and Western blot analyses were performed to evaluate apoptosis. TUNEL staining (**Figure 3C**) showed significant apoptosis in the model and DMSO groups. However, fewer apoptotic cells were observed with Cis treatment, which indicated that Cis treatment reduced GC-1 cell apoptosis. Additionally, the expression of PARP, Caspase-3, Bax and Bcl-2 was measured to corroborate the molecular mechanism. As presented in **Figure 3D**, Caspase-3 and PARP were activated in GC-1 cells under hypoxia, and this activation was inhibited by Cis treatment. In addition, the ratio of Bax/Bcl-2 was higher in the model group than in the control group, and Cis treatment reduced the ratio of Bax/Bcl-2 (**Figure 3D**). These data indicated that Cis had a potential capacity to attenuate hypoxia-induced oxidant damage, and this protective effect might be achieved by reducing ROS accumulation and inhibiting Caspase-related apoptosis pathway activation.

The enzymatic mechanism inhibiting OS involves free radical scavengers such as glutathione reductase (GR), glutathione peroxidase (GPx) and superoxide dismutase (SOD) [23]. The enzymatic mechanism inhibiting OS plays an essential role in preventing oxidative damage in cells and tissues [23]. To further validate the potential mechanism of Cis inhibition of hypoxia-induced OS in GC-1 cells, the activities of GR, GPx and SOD were measured. The results revealed that GR, GPx and SOD activities all significantly ($P < 0.01$, **Figure 3E**) decreased under hypoxia when compared to the control groups, and Cis treatment markedly restored their activities in GC-1 cells exposed to hypoxia ($P < 0.05$, **Figure 3E**), suggesting that these compounds could activate the powerful endogenous antioxidant system.

Effects of Cis on reproduction in hypobaric hypoxia-induced rats.

To determine the effects of hypobaric hypoxia on male rats, we first tested morphological

alterations of testes in hypobaric hypoxia-induced rats. The results of HE staining showed that in the control group, normal spermatogenic cells at various stages were arranged in an orderly manner from the basement membrane to the lumen, and mature sperm were visible in tubule lumens (**Figure 4A**). Compared with the controls, pathological alterations of testicular tissue were observed in the model group, the basement membrane of testicular epithelial cells was arranged loosely, the spermatogenic epithelium was extremely thin, and the level and number of germ cells were markedly reduced (**Figure 4A**). However, treatment with Cis remarkably improved the histology of hypobaric hypoxia-induced testicular damage *in vivo* (**Figure 4A**). We also measured body weight, testes weight, epididymis weight and seminal vesicle gland weight, which led to the reproductive organ index (the reproductive organ/body weight ratio) being calculated. As shown in **Figure 4B-D**, the reproductive organ index (testes, epididymis and seminal vesicle gland) was markedly lower in the model group ($P < 0.01$) than in the control group. However, the effect of hypobaric hypoxia on the reproductive organ index of rats was reversed with Cis treatment (**Figure 4B-D**).

Next, the acrosome enzyme activity and the live sperm rate of male rat sperm were also measured to elucidate testicular function damage. As shown in **Figure 4E**, **4F**, acrosome enzyme activity and sperm motility were lower in the model group rats than that in the control group ($P < 0.01$). However, compared with rats in the model group, acrosome enzyme activity was restored in rats treated with 8 mg/kg/d Cis ($P < 0.05$) (**Figure 4D**). Moreover, as shown in **Figure 4F**, treatment with Cis also enhanced the live sperm rate; the rats treated with 8 mg/kg/d Cis all showed a significantly increased live sperm rate (55.83 ± 6.03%, $P < 0.05$; 69.00 ± 2.29%, $P < 0.01$; 52.33 ± 3.40%, $P < 0.05$; and 53.67 ± 2.25%, $P < 0.05$ respectively) when compared with the model rats (43.83 ± 4.01%).
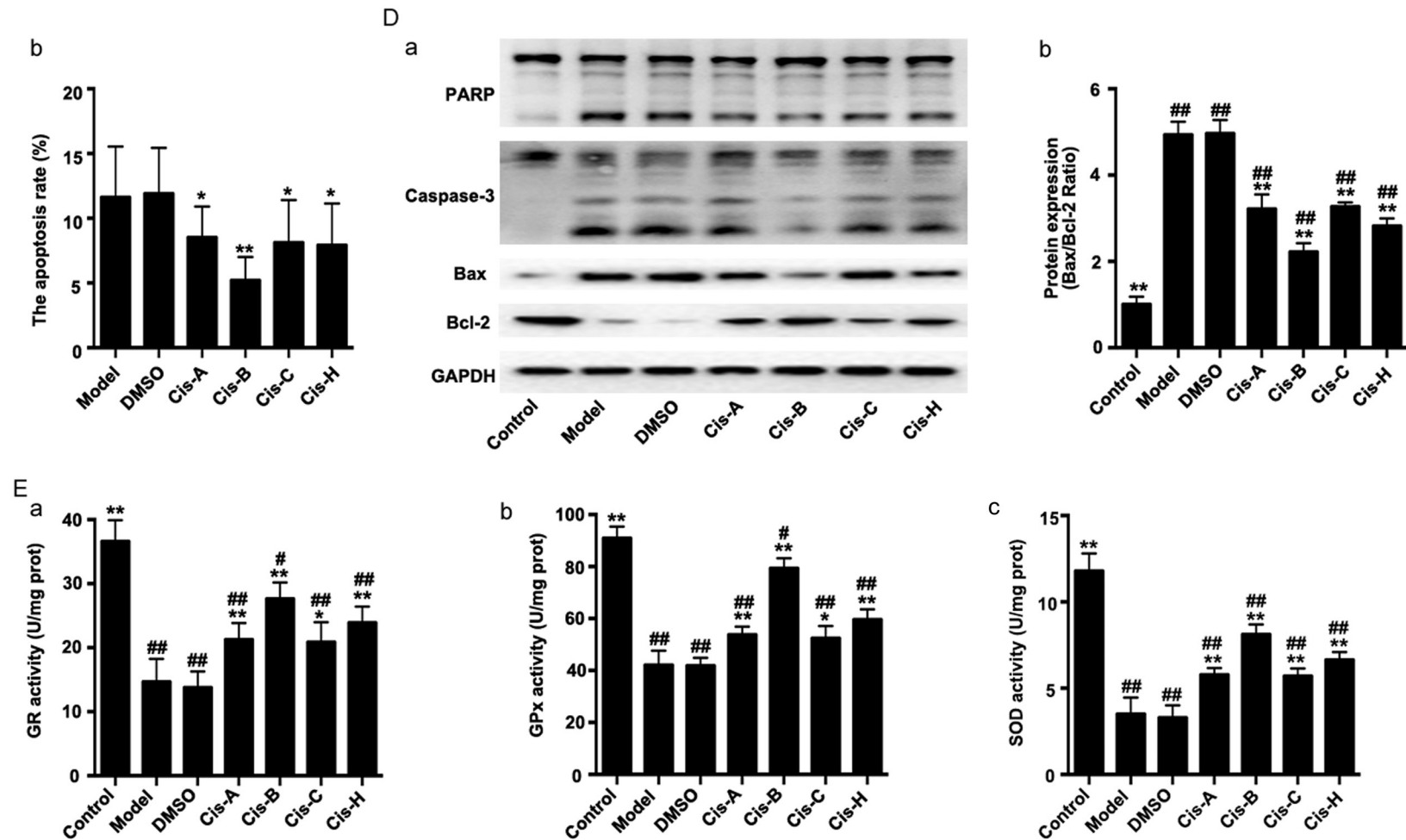
Taken together, these results suggested that the hypobaric hypoxic environment led to testicular morphological alterations, reproductive organ weight loss and testicular function damage in male rats, and Cis could effectively protect the reproductive organs from hypoxia-induced damage.
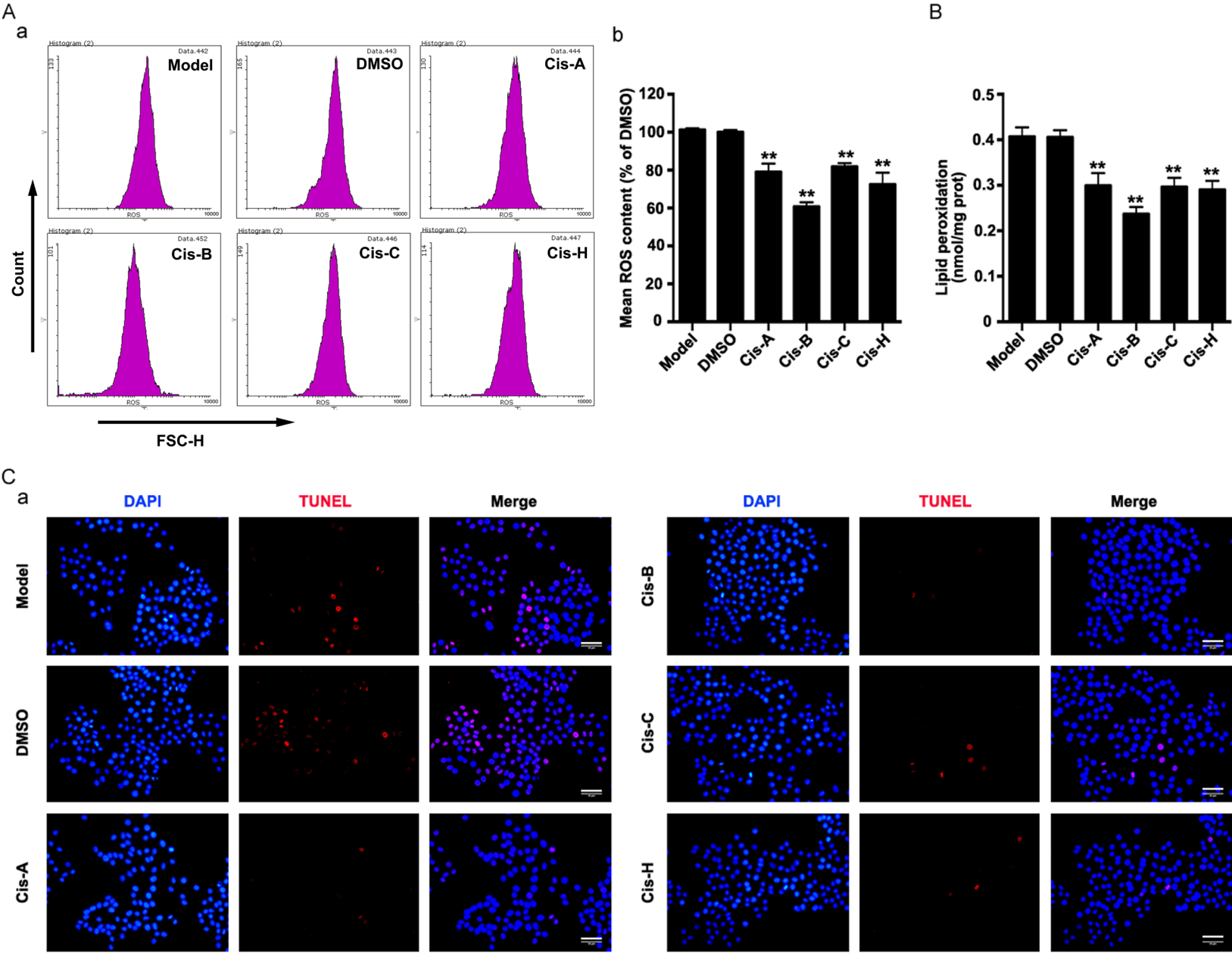
Figure 3. Mechanism by which Cis protects GC-1 cells from hypoxia-induced damage. Experiment: GC-1 cells were subjected to normal oxygen or hypoxic conditions with or without Cis treatment for 72 h. (A) ROS levels in GC-1 cells were tested by FCM assay. (B) LPO levels in GC-1 cells were measured by the TBARS assay. (C) Apoptosis of GC-1 cells was tested by TUNEL staining, and apoptosis rates were calculated (Bar = 20 μm). (D) The levels of PARP, Caspase-3, Bax and Bcl-2 in GC-1 cells were tested by Western blot analysis, and the relative expression intensity of the Bax/Bcl-2 ratio was calculated. (E) GR, GPx, and SOD activities in GC-1 cells were tested by assay kits. Bars indicate the mean ± SD ($n = 3$). $^{**}P < 0.01$, $^{*}P < 0.05$ (versus the model group); $^{##}P < 0.01$, $^{#}P < 0.05$ (versus the control group).
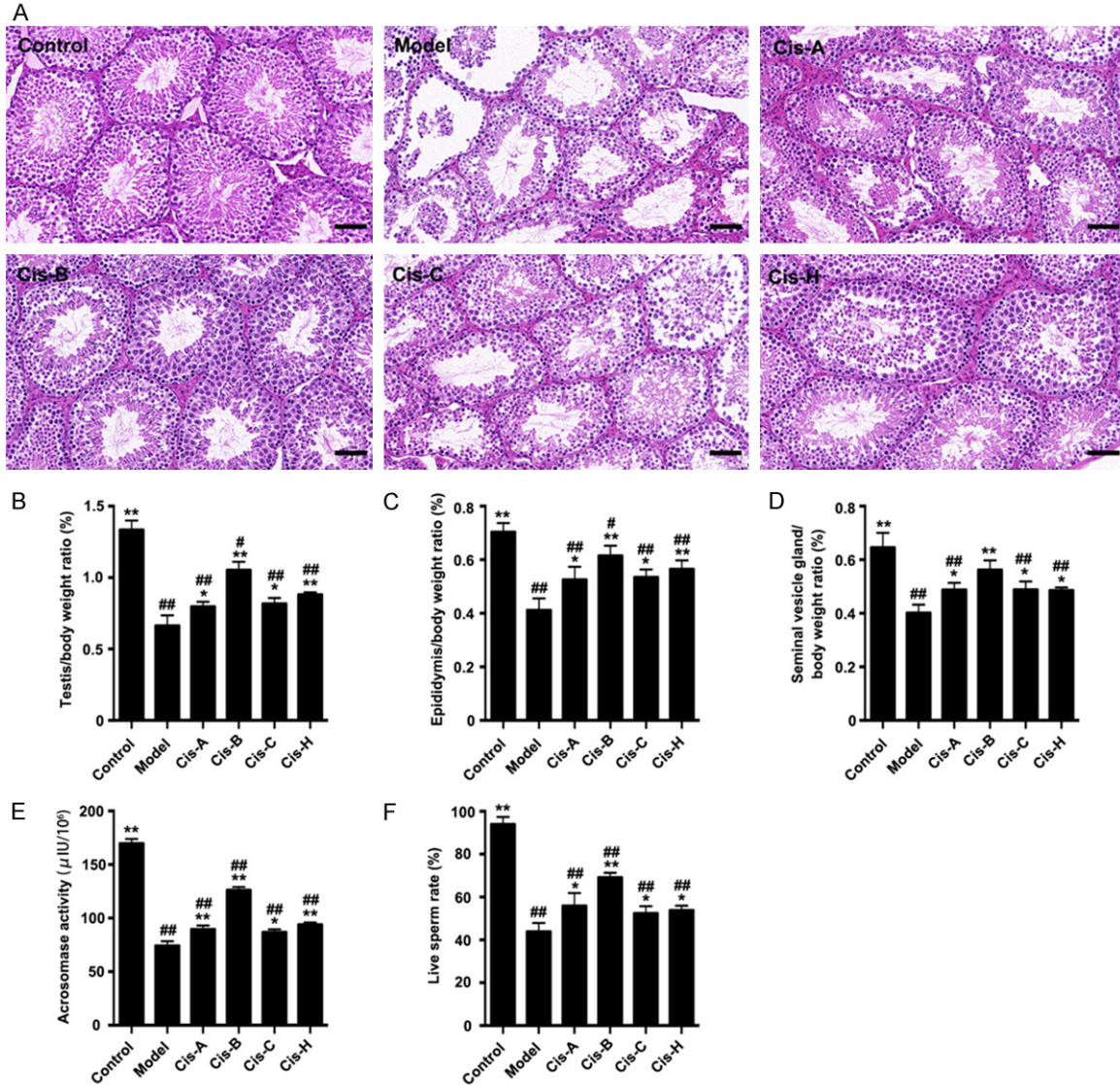
Figure 4. Effects of Cis on the reproductive system of rats exposed to hypobaric hypoxia. Experiment: Rats were subjected to hypobaric hypoxia or normobaric and normal oxygen conditions with or without Cis treatment for 8 weeks. (A) HE staining of testicular tissue from each group (Bar = 60 µm). (B) Measurement of testis/body weight ratios. (C) Measurement of epididymis/body weight ratios. (D) Measurement of (seminal vesicle gland)/body weight ratios. (E) Measurement of acrosome enzyme activity in sperm. (F) Measurement of the live sperm rate. Bars indicate the mean ± SD (n = 5). $^{**}P < 0.01$, $^{*}P < 0.05$ (versus the model group); $^{##}P < 0.01$, $^{#}P < 0.05$ (versus the control group).
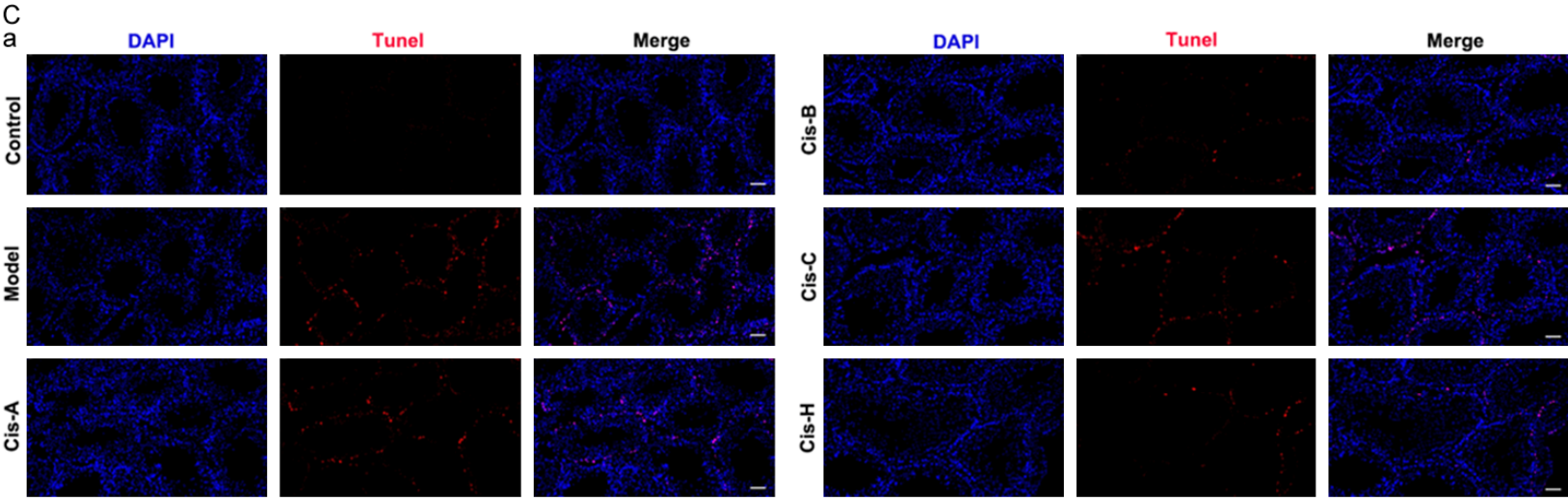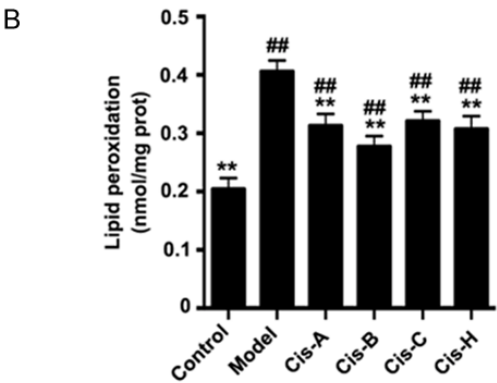
Effects of Cis on OS in the testes of hypobaric hypoxia-induced rats.

The ROS and LPO levels in the testes of rats were measured to analyze the effects of Cis on hypobaric hypoxia-induced OS. ROS analysis revealed that compared to the control group, ROS levels in the testes in the model group were significantly increased ($P < 0.01$ **Figure 5A**). Conversely, LPO was dramatically elevated in the testes ($P < 0.01$) under hypobaric hypoxia compared with normoxic conditions (**Figure**

5B). However, Cis treatment altered the above changes ($P < 0.05$), in which Cis-B exerted better effects than other Cis (**Figure 5A**, **5B**). Cis seemed to protect the testes by reducing OS under hypobaric hypoxic conditions *in vivo*.

Additionally, apoptosis analyses were performed to further evaluate the mechanism by which Cis protected against hypobaric hypoxia-induced testicular function injury. The results of TUNEL staining (**Figure 5C**) showed that, significant apoptosis existed in the model group com-
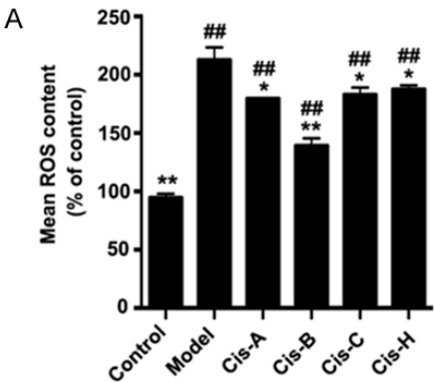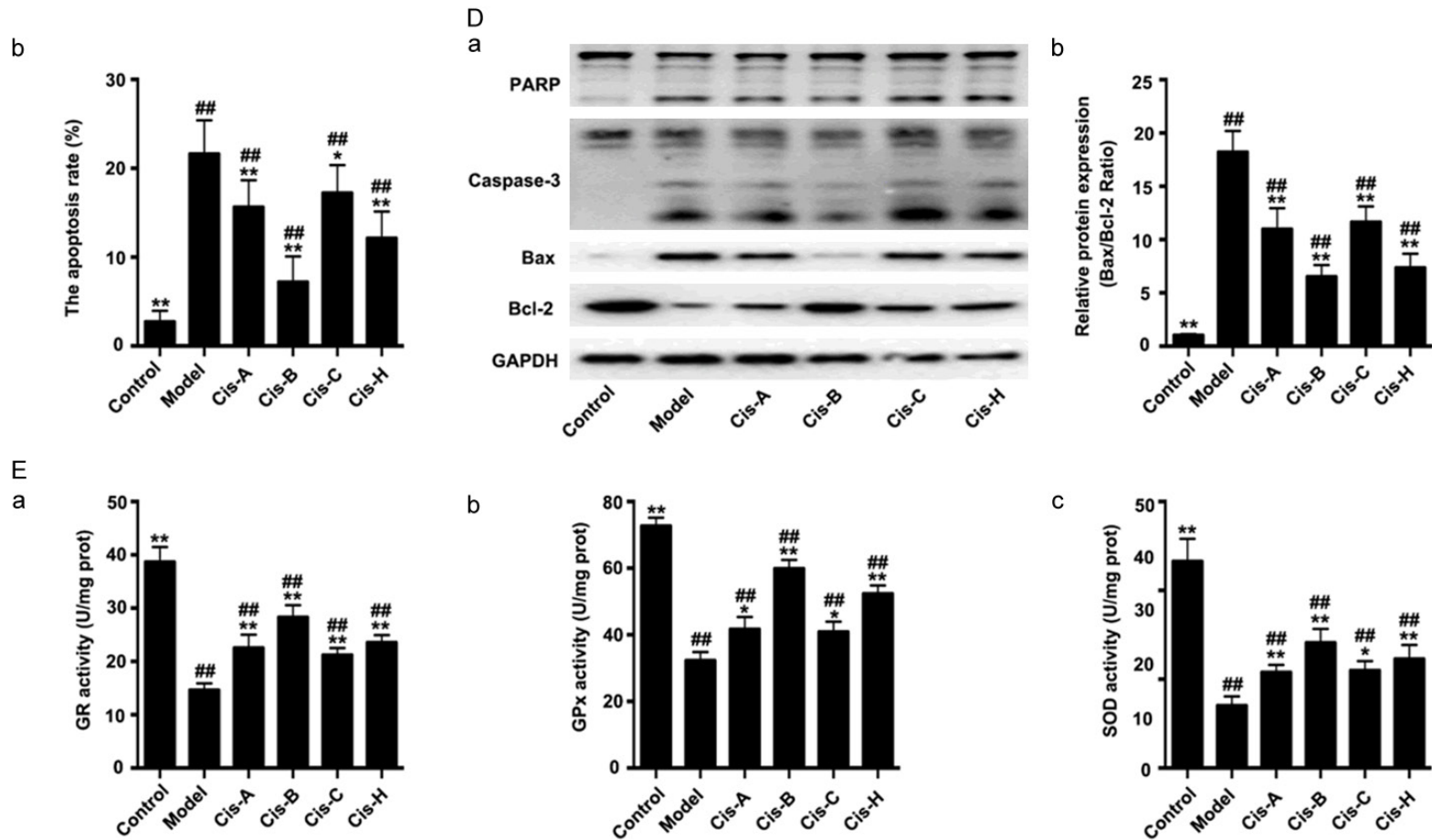
Figure 5. The Effect of Cis on OS in the testes of hypobaric hypoxia-induced rats. Experiment: Rats were subjected to hypobaric hypoxia or normobaric and normal oxygen conditions with or without Cis treatment for 8 weeks. (A) Measurement of relative ROS levels in testis tissues. (B) Measurement of relative LPO levels in testis tissues. (C) Apoptosis of testis tissues was tested by TUNEL staining, and apoptosis rates were calculated (Bar = 100 μm). (D) The expression levels of PARP, Caspase-3, Bax and Bcl-2 in testis tissues were tested by Western blot analysis, and the relative expression intensities of the Bax/Bcl-2 ratio were calculated. (E) Measurement of GR, GPx and SOD activities in testis tissues. Bars indicate the mean ± SD (n = 5). **P < 0.01, *P < 0.05 (versus the model group); ##P < 0.01, #P < 0.05 (versus the control group).

pared to the control group. However, after Cis (8 mg/kg/d) treatment, fewer apoptotic cells occurred ($P < 0.05$) (**Figure 5C**). The Western blot data also showed that, hypoxia and hypobaric treatment resulted in activation of Caspase-3 and PARP and an increased Bax/Bcl-2 ratio in testicular tissue, indicating an increase in apoptosis (**Figure 5D**). In addition, different types of Cis treatment significantly reduced apoptosis in testicular tissue (**Figure 5D**). Similarly, the IHC analysis of testicular tissue showed similar results (Supplementary Figure 1).

To verify the mechanism of Cis-reduced OS triggered by hypobaric hypoxia, we further tested the activities of GR, GPx and SOD in testicular tissue. As shown in **Figure 5E**, compared with the control group, hypobaric hypoxia treatment significantly reduced GR, GPx and SOD activities ($P < 0.01$). However, Cis treatment restored the enzyme activities (GR, GPx and SOD) of testes tissue in rats treated with hypobaric hypoxia ($P < 0.05$). In conclusion, Cis seemed to protect the testes by activating a powerful endogenous antioxidant enzyme defense mechanism under hypobaric hypoxia conditions.

Discussion

In high altitude areas, hypobaric hypoxia is known to affect multiple systems in humans, including the male reproductive system [4, 20]. Recent experimental investigations are geared towards understanding the mechanisms of how hypobaric hypoxia impairs the male reproductive system. In this study, the therapeutic effect of Cis extract from *Cistanches Herba* on hypoxia-induced reproductive damage was investigated. The results demonstrated that Cis may protect the male reproductive system from hypoxic damage by reducing hypoxia-induced ROS accumulation and OS through enhancing the activity of endogenous antioxidant enzymes.

ROS are oxygen-derived free radicals that play a vital role in human physiology and pathology. Low doses of ROS are essential for sperm capacitation, the acrosome reaction and spermatozoa-oocyte fusion [24, 25]. However, excessive accumulation of ROS often leads to damage to germ cells and stromal cells, resulting in male infertility [26]. ROS can easily damage cell membranes, nucleic acids, proteins, enzymes and other biological macromolecules

through peroxidation. Moreover, they also lead to potential cellular and DNA damage when they exceed the antioxidant carrying capacity. Accumulated evidence supports the pivotal role of ROS in the pathogenesis of male fertility [27, 28]. The production of ROS is regulated by oxygen tension. Under hypoxic conditions, the available oxygen in the environment decreases and the blood viscosity increases, thereby affecting many oxygen-dependent metabolic processes in the organism [29, 30]. However, the lower atmospheric pressure at high altitudes causes poor venous return and a decrease in the quantity of oxygen transported by the bloodstream to all cells of the organism, which further increases the hypoxia of organs and cells [29, 30]. Thus, exposure to a high altitude gives rise to a series of hypoxic physiological responses, including the production and accumulation of ROS, when the demand for oxygen exceeds the vascular supply. As mentioned previously, the accumulation of ROS leads to a variety of intracellular effects, the most critical of which is to cause OS in cells.

OS refers to an imbalance between oxidation and reduction reactions, leading to the generation of excess oxidants or molecules that accept an electron from another reactant, which in turn produces ROS [31, 32]. OS is well understood to be able to be triggered by a series of endogenous and exogenous factors, including exposure to high altitude. Spermatozoa are cells that are particularly susceptible to OS given their inadequate cell repair systems and high plasma membrane content of polyunsaturated fatty acids [33]. Testicular and epididymal tissues are not the exception, as the presence of severe OS has been observed in round spermatids in rats subjected to hypoxia [4]. OS affects the stability of DNA, thereby jeopardizing the integrity of the gamete genetic material [34-36]. However a high level of DNA damage in male gametes has been confirmed to lead to activation of apoptosis signaling, which results in a reduction of epididymal sperm count and an increase in the percentage of defective cells [28, 37]. In the present study, hypoxia significantly reduced the viability of GC-1 cells through the induction of apoptosis and cell cycle arrest. More importantly, significantly increased ROS levels were shown by FCM analysis after hypoxia stimulation, with an increased apoptosis rate and higher activation of Caspase-3, PARP and Bax/Bcl-2 ratio, indi-

cating that ROS could activate apoptosis by activating the Caspase signaling pathway during hypoxia-induced fertility damage. The present findings demonstrated that hypoxia led to excessive ROS accumulation, causing oxidative damage to reproductive cells. Thus, it is meaningful to identify new antioxidants that can serve as an effective approach to alleviate hypoxia-induced fertility injury.

To protect against OS, a complex antioxidant system exists in the body, mainly composed of enzymatic factors. Under physiological conditions, the ROS contents and antioxidant system maintain a certain balance. However, ROS overproduction depletes the sperm antioxidant system, leading to OS, which causes sperm DNA damage and results in lower fertility and pregnancy rates [23]. Thus, to address ROS overproduction and related deleterious effects at the cellular level in the male reproductive system, different antioxidant strategies have been tested [23]. Currently, the literature concerning the use of compounds with antioxidant activity and improvement of sperm function is extensive. Importantly, most reports describe an improvement in sperm parameters after oral antioxidant intake, including improvements in sperm concentration and motility or decreases in DNA damage [38]. Thus, a growing number of urologists are prescribing oral antioxidants for infertility due to OS-related problems [39]. These antioxidants include mainly carnitines, vitamins, zinc, melatonin and natural compounds [23, 40]. Presently, with the development of drug extraction technology, an increasing number of TCM extracts are also being considered to mitigate male infertility because these antioxidants can reduce the destructive effects of OS [41]. Yüce A. et al. reported in 2013 that cinnamon has beneficial effects on the oxidative and antioxidant balance in testes and sperm quality [42]. Zhang L et al. showed that curcumin significantly improves sperm motility in patients and decreases H_2O_2 [43]. In addition, a variety of other plant extracts such as blueberry, crocus sativus, pomegranate seeds and green tea have also been shown to protect the reproductive system via antioxidant mechanisms [27, 44-47]. *Cistanches Herba* is an important TCM that possesses a favorable safety profile and broad medicinal functions for the treatment of infertility, among other conditions [13]. Modern pharmacological studies have shown that *Cistanches Herba* possesses various activities, such as antioxidative, anti-inflammatory, hepatoprotective and anti-neurodegenerative disease activities [13, 48]. Therefore, extracts, fractions or compounds from *Cistanches Herba* may have potential antioxidant features for the treatment of infertility.

The active substances in plants that improve fertility include various chemical groups such as PhGs, saponins, oxygenated volatile compounds, and alkaloids [41]. Pharmacological activity studies of PhGs have demonstrated that PhGs exhibit a wide range of bioactivities, such as antioxidation, antiradiation neuroprotection, and sexual function enhancement [49, 50]. Among these activities, antioxidation is gradually attracting attention. Some single components or fractions of PhGs have been reported to inhibit germ cell apoptosis induced by various chemicals, and their antioxidation capabilities *in vitro* have also been demonstrated *in vivo* in several animal models [51, 52]. These results indicate that PhGs could be an attractive candidate for the treatment of male infertility. Cis is an active PhG that can be isolated from *Cistanches Herba*. In the present study, we explored the effects of Cis on hypoxia treated cells or a rat model and investigated the underlying molecular mechanisms. Cis exhibited protective activities on decreases in hypoxia-induced viability and increases in apoptosis in GC-1 cells, and it also showed a protective effect on hypoxia-induced damage in the reproductive system of rats *in vivo*. A significant decrease in GR, GPx and SOD activities under hypoxia in comparison to normoxic groups was observed, while the specific activities of GR, GPx and SOD significantly increased in testes or GC-1 cells treated with Cis. Cis seemed to protect the testes and GC-1 cells under hypoxic conditions by enhancing the activities of antioxidant enzymes.

Enzyme antioxidants function mainly by scavenging superoxide anions, thus preventing lipid peroxidation and DNA damage to prevent infertility. Enzymatic antioxidant mechanisms play a crucial role in preventing oxidative damage [23]. The enzymatic mechanism against OS comprises free radical scavengers and glutathione-dependent enzymes including GR, GPx and SOD [12]. Antioxidant enzymes are well understood to be essential for the male reproductive system. In the current study, the effect of

reduced antioxidant enzyme activities under hypobaric hypoxia was accompanied by increased ROS and LPO in the model group, which is consistent with previous reports [12]. However, Cis administration led to a recovery of antioxidant enzyme activities in GC-1 cells and the testes of rats, making it possible to generate strategies for administering *Cistanches Herba* to prevent hypobaric hypoxia-induced damage, as previously suggested. Although the present results showed that treatment with Cis partly decreased hypoxia-induced germ cell damage in rats, further investigations are needed to unravel the full picture of its reproductive protective effects. For example, the specific mechanism of Cis affects the activity of antioxidant enzymes. In addition, there is a question whether any other mechanisms could also be pertinent as Cis only partially recovered the reproductive damage caused by hypoxia. Finally, whether Cis has a direct growth-promoting effect on germ cells should also be considered.

Conclusions

In general, the findings of this study emphasize the potential of Cis as an antioxidant for the treatment of hypoxia-induced male reproductive damage. Cis can protect against hypoxia-induced male reproductive damage by restoring antioxidant enzyme activity, reducing ROS-induced OS, simultaneously increasing cell viability and decreasing apoptosis. Importantly, the Cis subtypes (Cis-A, Cis-B, Cis-C and Cis-H) studied in this study all showed a certain protective effect on the reproductive system, and Cis-B showed the most significant effect. Therefore, we speculate that Cis might be a good candidate antioxidant for the treatment of hypoxia-induced male reproductive damage, although the precise underlying mechanism requires further investigation.

Acknowledgements

Disclosure of conflict of interest

None.

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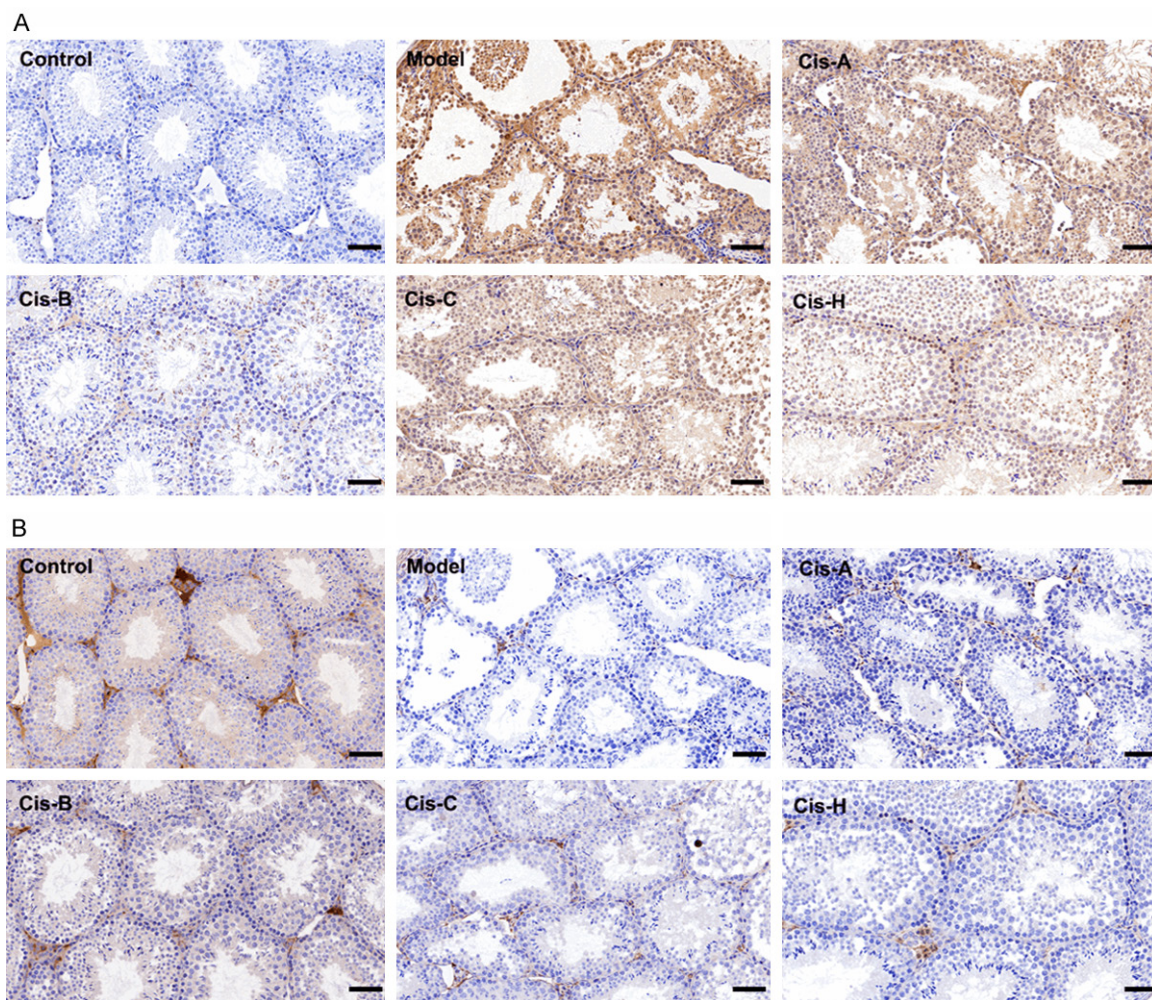
Supplementary Figure 1. The Effect of Cis on apoptosis in the testes of hypobaric hypoxia-induced rats. Experiment: Rats were subjected to hypobaric hypoxia or normobaric and normal oxygen conditions with or without Cis treatment for 8 weeks. A. The expression levels of Bax in testis tissues were tested by IHC analysis (Bar = 60 μm). B. The expression levels of Bax in testis tissues were tested by IHC analysis (Bar = 60 μm).