Original Article

External pressure induces the dysfunction of spermatogonia via triggering the intrinsic pathway of apoptosis

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Background: Cryptorchidism, the failure of testes to descend into the scrotum, exposes the testes to higher temperature and external pressure. Scholars from Razi University found through research conducted at different pressure gradients (0, 25, 50, and 100 mmHg) and time gradients (2 and 4 h) that high hydrostatic pressure may lead to sperm apoptosis. In this work, we investigated the effect of external pressure on spermatogonia, exploring a new mechanism of male infertility caused by cryptorchidism.

Methods: Various pressure gradients (0, 25, 50, and 100 mmHg) were applied to spermatogonia for different durations (0, 2, and 4 h) in the Cell Counting Kit-8 (CCK8) experiment. Morphological changes, cell ultrastructure, apoptosis rates, and the expression of apoptosis-related proteins (bax, bcl-2, caspase-3, and caspase-9) were assessed through immunofluorescence, electron microscopy, terminal deoxynucleotidyl transferase-mediated dUTP-biotin nick end labeling (TUNEL) assay, flow cytometry, immunohistochemistry, real-time quantitative polymerase chain reaction (qPCR), and western blot.

Results: The cell viability assay showed that higher external pressure had a greater negative time-dependent impact on cell viability. Immunofluorescence results indicated that external pressure stimuli altered the morphology of spermatogonia. The results of TUNEL assay and flow cytometry demonstrated that external pressure stimuli induced apoptosis in spermatogonia. Transmission electron microscopy (TEM) observations showed the generation of apoptotic bodies, mitochondrial swelling, vacuolization, and mitochondrial cristae fusion. The results of immunohistochemistry indicated that pressure induced the expression of caspase-3 and caspase-9 proteins. qPCR and western blot analyses revealed an increased ratio of bax/bcl-2 and expression of caspase-3 and caspase-9. Methazolamide (cytochrome C inhibitor) blocked the pressure-induced cell apoptosis and inhibited the activation of caspase-3 while Z-IETD-FMK (caspase-8 inhibitor) did not.

Conclusions: External pressure promotes spermatogonia apoptosis through the intrinsic apoptosis pathway, which may be one of the mechanisms of male infertility induced by cryptorchidism.

Keywords: Apoptosis; cryptorchidism; external pressure; spermatogonia

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Introduction

During the process of reproductive development, cryptorchidism refers to the failure of the testes to descend into the scrotum, including incomplete descent and ectopic testes (1). The incidence of this condition in full-term male infants is approximately 1% to 4.6% (2-4). There are various factors associated with cryptorchidism and male infertility, including genetic variations, oxidative stress, production of anti-sperm antibodies, increased apoptosis, the influence of the genitofemoral nerve, and hormonal imbalances (5,6). The production of anti-sperm antibodies may trigger an autoimmune reaction, leading to infertility (7). Furthermore, damage to the genitofemoral nerve leads to an increase in calcitonin gene-related peptide released by the genitofemoral nerve and its terminals (8), as well as a decrease in testosterone, which cause changes in testicular blood flow and local temperature, resulting in testicular damage and infertility (9). In addition, increased cell apoptosis is considered one of the key factors leading to a decrease in sperm count (6). Genetic variations affect spermatogonia apoptosis through multiple pathways and oxidative stress and high levels of nitric oxide generate excessive reactive oxygen species, potentially leading to an increase in testicular germ cell apoptosis, while high temperature has been shown to induce spermatogonia apoptosis (10-12).

In clinical practice, undescended testes that are surgically removed generally have a smaller volume, soft texture, or are underdeveloped. Conventional wisdom holds that many factors, including hormonal balance, age, health status, stress levels, lifestyle choices, environmental influences, genetic predispositions, and conditions like varicocele, can impact sperm production. Cryptorchidism can elevate testicular temperature, disrupt hormone equilibrium, and induce genetic alterations. Additionally, research indicates that excessive external pressure can alter sperm morphology, reduce motility, increase abnormal sperm production, cause sperm DNA damage, and impair male fertility in mice (13).

Research indicated that the pressure within the inguinal canal exceeds normal atmospheric pressure by approximately 28.15 cmH_2O (14), while the pressure within the scrotum typically remains at atmospheric levels. Moreover, abdominal pressure plays a crucial role in facilitating testicular descent (15). Therefore, it is hypothesized that when the testes fail to descend smoothly into the scrotum and remain in the abdominal cavity and inguinal canal, the elevated pressure behind the peritoneum and in the inguinal canal may also contribute to germ cell apoptosis. This study aimed to evaluate the impact of external pressure on spermatogonia. We present this article in accordance with the MDAR reporting checklist (available at https://tau.amegroups.com/article/view/10.21037/tau-24-158/rc).

Highlight box

Key findings

- In this experiment, we established a clear link between heightened external pressure and apoptosis of spermatogenic cells.

What is known and what is new?

- Cryptorchidism is a known factor contributing to male infertility. In clinical practice, undescended testes that are surgically removed typically exhibit characteristics such as reduced volume, soft texture, or underdevelopment. In cases where the testicles remain undescended, the external pressure exceeds that within the scrotum.
- The escalation of external pressure leads to intrinsic apoptosis of spermatogenic cells.

What are the implications, and what needs to change?

- The surge in exogenous pressure triggering spermatogenic cell apoptosis offers a fresh perspective for investigating the mechanisms behind male infertility associated with cryptorchidism.

Methods

Materials

The spermatogonia (cell line GC-1) were purchased from Wanwu Biotechnology, Hefei, China. The cell pressure culture instrument was from the Naturethink Company, Shanghai, China. The CCK8 assay kit (BS350B, Bioharp Biotechnology, Hefei, China), phalloidin fluorescent dye (CA1610, Solaibao, Beijing, China), terminal deoxynucleotidyl transferase-mediated dUTP-biotin nick end labeling (TUNEL) assay kit (E-CK-A321, Elabscience Company, Wuhan, China), flow cytometry assay kit (AP101-100, Union-Biotech, Hangzhou, China), RNA extraction kit (220010, Feijie Biotechnology, Shanghai, China), Evo M-MLV reverse transcription kit (AG11707, Accurate Biology, Changsha, China), Evo M-MLV one-step reverse transcriptase quantitative polymerase chain reaction (RT-qPCR) kit (AG11732, Accurate Biology), Z-IETD-FMK (ab141382, Abcam, Cambridge, UK), SuperSignal™ West Atto Kit (A38555, Thermo Scientific, Waltham, MA, USA), Pierce™ enhanced chemiluminescence (ECL) western

blot substrate (32209, Thermo Scientific), bicinchoninic acid protein assay kit (23227, Thermo Scientific), β-actin monoclonal antibody (66009, Proteintech, Wuhan, China), rabbit anti-bax (ab32503, Abcam), bcl-2 rabbit polyclonal antibody (A0208, Abclonal, Wuhan, China), caspase-3 rabbit polyclonal antibody (A16793, Abclonal), caspase-9 rabbit polyclonal antibody (A2636, Abclonal), and rabbit anti-survivin (sc-10811, Santa Cruz Biotechnology, Dallas, TX, USA) were purchased.

Cell culture and cell viability assay

The cells were cultured in a six-well plate in a constant temperature incubator at 37 ℃ with a 5% carbon dioxide concentration. The complete culture medium was prepared using Dulbecco's modified Eagle medium, fetal bovine serum, and a mixture of penicillin-streptomycin, and the medium was changed every day. Cell passages were performed every 2 days. Once the cells adhered to the plate, they were incubated for 48 h. Cells exhibiting approximately 80% confluence and in robust condition were subjected to pressure treatment. In Karimi's study, cells were exposed to various pressure gradients (0, 25, 50, and 100 mmHg) using a pressure incubator (13). Each pressure level was applied for 2 and 4 h to assess its impact on cellular responses. Our study draws upon this research, employing identical pressure gradients and exposure times to simulate cellular pressures. According to the instructions of the Cell Counting Kit-8 (CCK8) kit, the spermatogonia were prepared as a cell suspension, and approximately 2×10^3 cells were counted. After continued cultivation in a 96-well plate for 24 h, the cells were subjected to pressure stimulation by the gauge from Naturethink Company. Pressure-dependent viability assay was examined at various pressure gradients (0, 25, 50, and 100 mmHg) for 4 h. Time-dependent viability assay was assessed for different time durations (0, 2, and 4 h) with 50 mmHg. Finally, the absorbance of each well was measured at 450 nm.

Cell morphology

The cells were pretreated with 4% paraformaldehyde and 0.5% triton. Subsequently, the cells were incubated with phalloidin dye in the dark for 30 minutes. The nuclei were stained with 4',6-diamidino-2-phenylindole (DAPI) for 5 minutes and mounted with an anti-fade mounting reagent. The cells' morphological changes were examined using the fluorescence microscope (ZEIZZ Axioscope 5, Oberkochen, Badenweg, Germany) at magnifications of 200× and 400×. Three high-power fields were assessed for each section, and each measurement was conducted in triplicate.

TUNEL assay

According to the instructions of the TUNEL assay kit, cells were treated with 4% paraformaldehyde and 0.5% triton in advance. After thorough washing, cells were incubated with TdT equilibrium buffer in the incubator at 37 ℃ for 30 minutes. Then, cells were incubated with labeled working solution containing TdT enzyme for 60 minutes at 37 ℃. DAPI staining was performed for 5 minutes. Finally, the results were observed under the fluorescence microscope (ZEIZZ Axioscope 5, GER) at magnifications of 100×. Three high-power fields were assessed for each section, and each measurement was conducted in triplicate. TUNEL positive cells were counted by Image J.

Flow cytometry

According to the flow cytometry instructions, approximately 3×10^5 cells were counted by the flow cytometer, and 5 μL of Annexin V-fluorescein isothiocyanate (FITC) and 10 μL of propidium iodide (PI) were added. The cells were incubated in the dark for 5 minutes and the analysis was performed by Agilent NovoCyte flow cytometer (Agilent Technologies, Palo Alto, CA, USA). The raw data were further analyzed with the use of FlowJo software (Tree Star Inc., Ashland, OR, USA). The proportion of apoptotic cells was the proportion of cells in the first and fourth quadrant of flow cytometry.

Transmission electron microscopy (TEM)

The cells under 50 mmHg for 0, 2, and 4 h were fixed with 3% glutaraldehyde and after thorough fixation, frozen cell blocks were cut into 60 nm ultrathin sections. The sections were stained with 2% uranyl acetate and lead citrate and then examined using a TEM system (TECNAI 10, Phillips, Eindhoven, The Netherlands) at an accelerating voltage of 80 kV.

Immunohistochemistry

The cells were pretreated with 4% paraformaldehyde and 0.5% triton and then blocked with normal goat serum at room temperature for 30 minutes. After blocking, the

cells were incubated with primary antibodies for caspase-3 (dilution ratio 1:200) and caspase-9 (dilution ratio 1:200) at 4 ℃ for 16 h. Subsequently, the cells were exposed to mouse horseradish peroxidase at room temperature for 50 minutes and stained with 3,3'-diaminobenzidine (DAB) for 5 minutes. After restaining, bluing, and differentiation, the cells were dehydrated and sealed. The results were observed with the optical microscope (ZEIZZ Axioscope 5, GER) at magnifications of 100×. Three high-power fields were assessed for each section, and each measurement was conducted in triplicate.

Real-time qPCR

The total cellular RNA was extracted using an RNA extraction kit. According to the instructions, complementary DNA (cDNA) was obtained through reverse transcription using a cDNA reverse transcription kit.

The following primers were used for qPCR: bax forward, 5'-TCCACAAGCTGAGCGAG-3' and reverse, 5'-GTCAGCCATGATGGTTCT-3'; bcl-2 forward, 5'-GCGGGATATGGCCTTTTTG-3' and reverse, 5'-GCGGGATATGTTGACTTCA CTTG-3'; caspase-9 forward, 5'-GCGTTACGGCACAGATGCAT-3' and reverse, 5'-TGCTCGCGTTCTTGCGAGG-3'; caspase-3 forward, 5'-ATGACAACGAACTCCG TG-3' and reverse, 5'-CCACTCTCTCATTCCTTAGTG-3'; caspase-8 forward, 5'-TG CTTGGACTACATCCCACAC-3' and reverse, 5'-GTTGCAGTCTAGGAAGTTGA CC-3'; β-actin forward, 5'-GGCTGTATTCCCCTCCATCG-3' and reverse, 5'-CCAG TTGGTAACAATGCCATGT-3'. Real-time qPCR detection was performed using the QuantStudio™ 1 System (Thermo Scientific).

Western blot

Cellular proteins were extracted with radioimmunoprecipitation assay lysis buffer. The protein concentration was determined by the bicinchoninic acid kit. A total of 20 μg of proteins were isolated using polyacrylamide gel electrophoresis and transferred to a polyvinylidene fluoride (PVDF) membrane. The PVDF membrane was blocked with a blocking solution prepared from skim milk powder at room temperature for 2 h. Then the PVDF membrane was incubated with the antibody working solution at 4 ℃ overnight. After incubation, the sample was labeled with mouse horseradish peroxidase for 2 h at room temperature. Finally, the ECL kit was used for exposure of caspase-3, caspase-8, caspase-9, bax, bcl-2, and β-actin antibodies. The obtained images were analyzed using Image J software to determine the grayscale value of each protein band.

For the blocking experiment, Z-IETD-FMK (caspase-8 inhibitor, 20 μM) and methazolamide (cytochrome C inhibitor, 2.5 μM) were added to the complete cell culture medium and spermatogonia were subjected to pressure (50 mmHg) for 2 and 4 h. Then the cells were collected and the above western blot assay was repeated to detect the expressions of caspase-3 and caspase-9.

Statistical analysis

Each experiment was conducted in triplicate to ensure statistical significance. All data were analyzed using GraphPad software and represented as the mean ± standard deviation (SD) of continuous variables. Statistical differences between the two groups were analyzed using analysis one-way of variance. The Tukey Kramer test was used for post hoc comparison. The statistical significance was set to $P<0.05$. The apoptotic pathway diagram was drawn by Figraw software.

Results

Changes in the morphology of spermatogonia under external pressure

Immunofluorescence was used to observe the morphological changes of the cells. Under increasing pressure, the cell cytoskeleton became loose and the cell nuclei fragmented (*Figure 1*).

External pressure inhibited the activity of spermatogonia

The CCK8 experiment results (*Figure 2A,2B*) reflected the impact of external pressure on the activity of spermatogonia, showing a decrease in cell activity with increasing pressure gradients and time durations ($P<0.05$).

External pressure enhanced the apoptosis of spermatogonia

TUNEL assay and flow cytometry were used to identify the proportion of apoptotic cells. The results indicated that with the prolonged duration of external pressure, the rate of apoptotic cells significantly increased ($P<0.01$) (*Figure 2C-2F*).

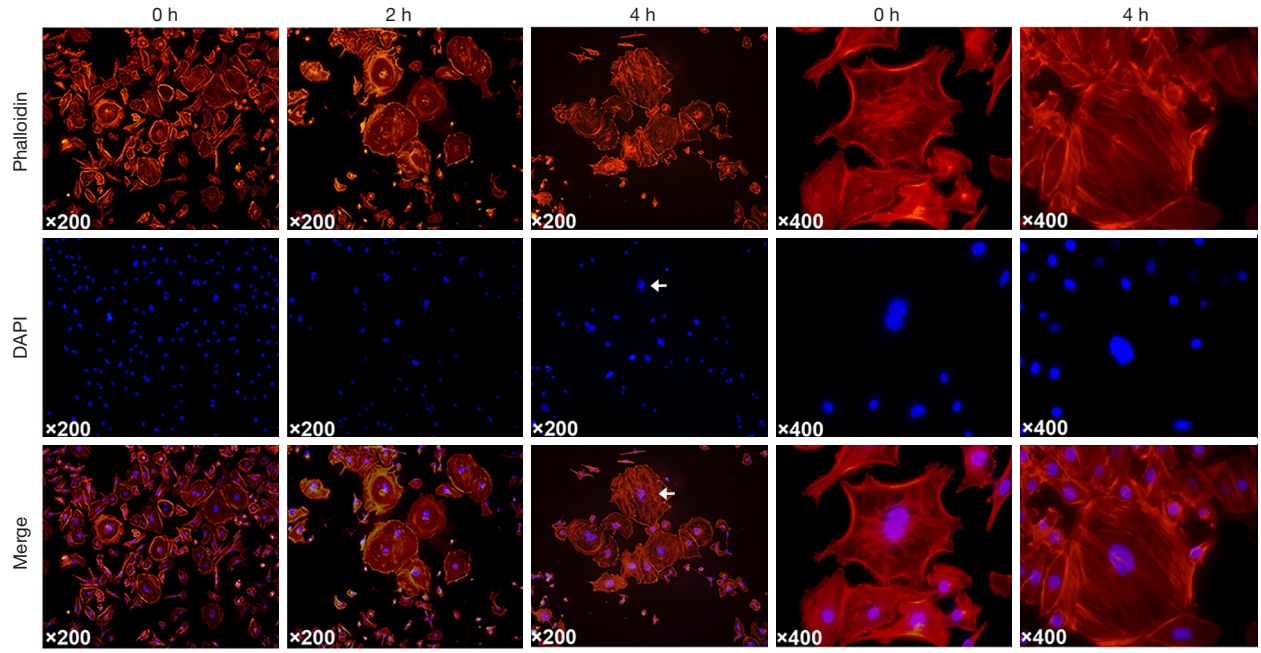

Figure 1 Immunofluorescence staining demonstrated changes in cell morphology with increased external pressure. At 0 h of pressure application, the morphology of the spermatogonia was irregular. As the duration of pressure application increased, it was observed that the spermatogonia became rounder, the cellular framework loosened, and the cell nucleus fragmented. DAPI, 4',6-diamidino-2-phenylindole.

Changes in the ultrastructure of spermatogonia under external pressure

TEM was used to observe the ultrastructure of the cells (*Figure 3*). Cells subjected to pressure showed changes in ultrastructure, with early apoptotic cells (2 h) exhibiting cell shrinkage, and chromatin condensation, while late apoptotic cells (4 h) showed nuclear condensation and fragmentation, cytoplasmic vacuolization, and the formation of apoptotic bodies, along with mitochondrial swelling and fusion of mitochondrial cristae.

Modulation of apoptosis-related protein expressions under external pressure

Immunohistochemistry was used to observe the expressions of caspase-3 and caspase-9 (*Figure 4A*,*4B*). The proportion of cells expressing positive caspase-3 and caspase-9 increased with prolonged exposure to pressure ($P<0.05$). (*Figure 4C*) The qPCR showed significantly increased messenger RNA (mRNA) expressions of bax, caspase-3, and caspase-9 ($P<0.05$). The mRNA expression of bcl-2 was inhibited ($P<0.05$), with no remarkable effect on caspase-8 ($P>0.05$). Western blot analysis showed higher bax/bcl-2 ratio, as well as a higher expression levels of caspase-3 and caspase-9 with increasing time under pressure (50 mmHg) ($P<0.05$) (*Figure 4D*,*4E*). Cytochrome C inhibitor, methazolamide, blocked the activation of caspase-9 and caspase-3 ($P>0.05$) (*Figure 4F*). The caspase-8 inhibitor, Z-IETD-FMK, did not block the expression of caspase-3 ($P<0.05$) (*Figure 4G*).

Discussion

Cryptorchidism is a congenital developmental abnormality in which one or both testes fail to descend into the scrotum during fetal development (16). It may be caused by factors such as gubernaculum issues, hormonal imbalances, and genetic predisposition (17). The incidence in full-term newborns is 1–4.6% and even higher in preterm infants (4). Cryptorchidism impacts testicular germ cell development, increasing the risk of infertility and malignant tumors (18).

Cryptorchidism is implicated in male infertility through a multitude of factors, including oxidative-reduction mechanisms, anti-sperm antibody production, increased cell apoptosis, genitofemoral nerve influence, and endocrine disturbances (2,19). Elevated levels of nitric oxide have been associated with an augmented rate of testicular germ

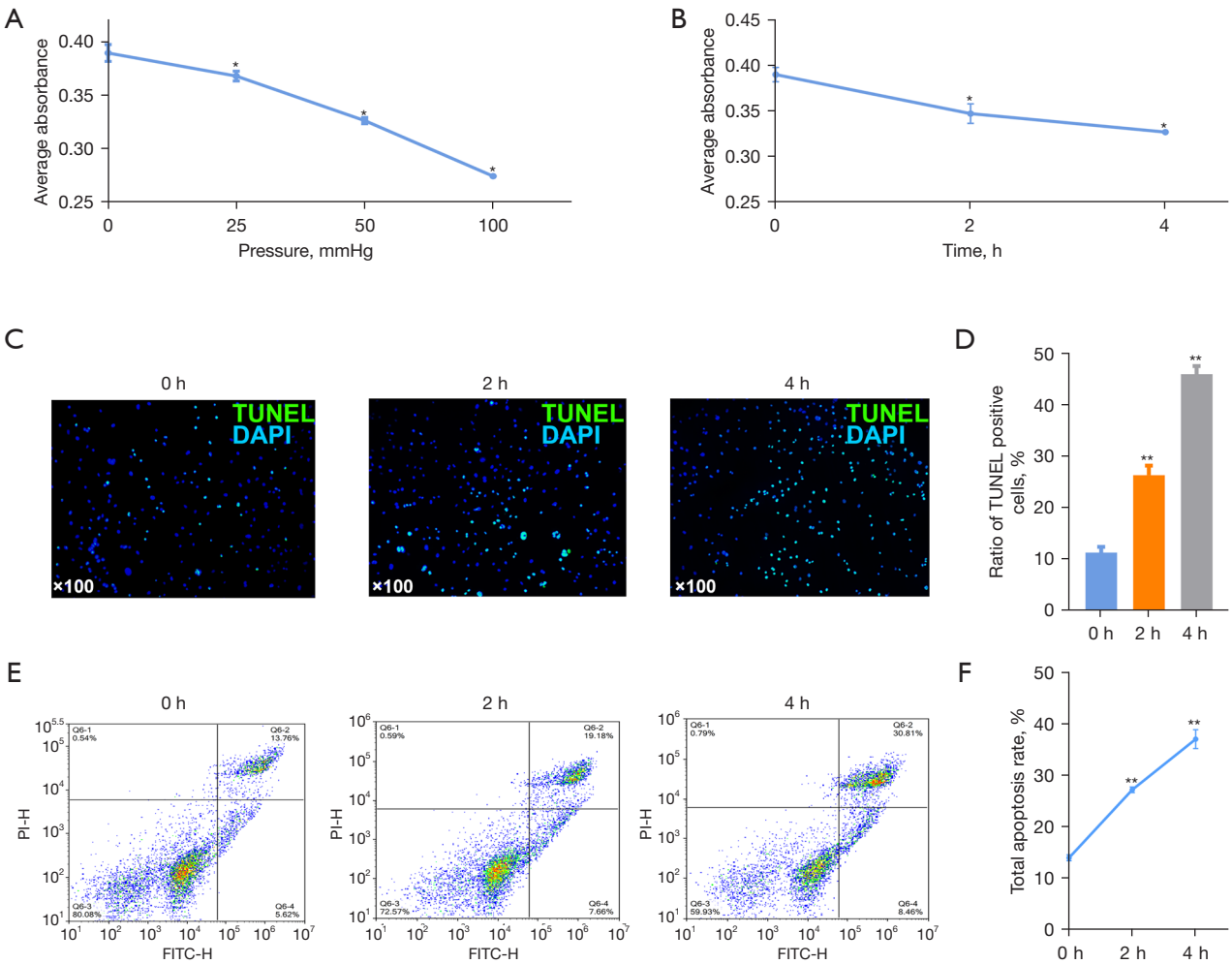

Figure 2 Detection of apoptosis in spermatogonia. (A) The impact of external pressure on spermatogonia viability was assessed by the CCK8 experiment. Spermatogonia were subjected to 0, 25, 50, and 100 mmHg pressures for 4 h, with respective absorbance as follows: 0.389±0.008, 0.367±0.004, 0.326±0.003, 0.274±0.003. *, P<0.05 *vs.* 0 mmHg. (B) When spermatogonia were subjected to an external pressure of 50 mmHg, the absorbance at 0, 2, and 4 h were: 0.389±0.008, 0.346±0.011, and 0.326±0.003. *, P<0.05 *vs.* 0 h. (C,D) TUNEL and DAPI staining were used to identify apoptotic cells. When 50 mmHg external pressure was applied to spermatogonia, TUNEL-positive cells were detected at 0, 2, and 4 h as follows: 11.37±1.11, 26.36±1.83, and 45.95±1.56. **, P<0.01 *vs.* 0 h. (E,F) The apoptosis rates of spermatogonia measured by flow cytometry at 0, 2, and 4 h after hyperbaric treatment were as follows: 19.64±0.62, 27.34±0.56, and 37.17±1.85. **, P<0.01 *vs.* 0 h. TUNEL, terminal deoxynucleotidyl transferase-mediated dUTP-biotin nick end labeling; DAPI, 4',6-diamidino-2-phenylindole; CCK8, Cell Counting Kit-8.

cell apoptosis, ultimately leading to reduced male fertility (11,20). Furthermore, the presence of anti-sperm antibodies may contribute to infertility (21). Additionally, the elevated temperature environment associated with cryptorchidism not only directly triggers apoptosis of spermatogonia but also disrupts the blood-testis barrier, potentially allowing sperm leakage and immune cell infiltration, leading to an immune response and the production of anti-sperm antibodies, thus impacting fertility (20,22-24). The genitofemoral nerve and the release of calcitonin gene-related peptides from its terminals also impact male fertility by influencing testicular blood flow and temperature, ultimately causing testicular damage (7). Moreover, reduced serum testosterone levels may lead to germ cell DNA

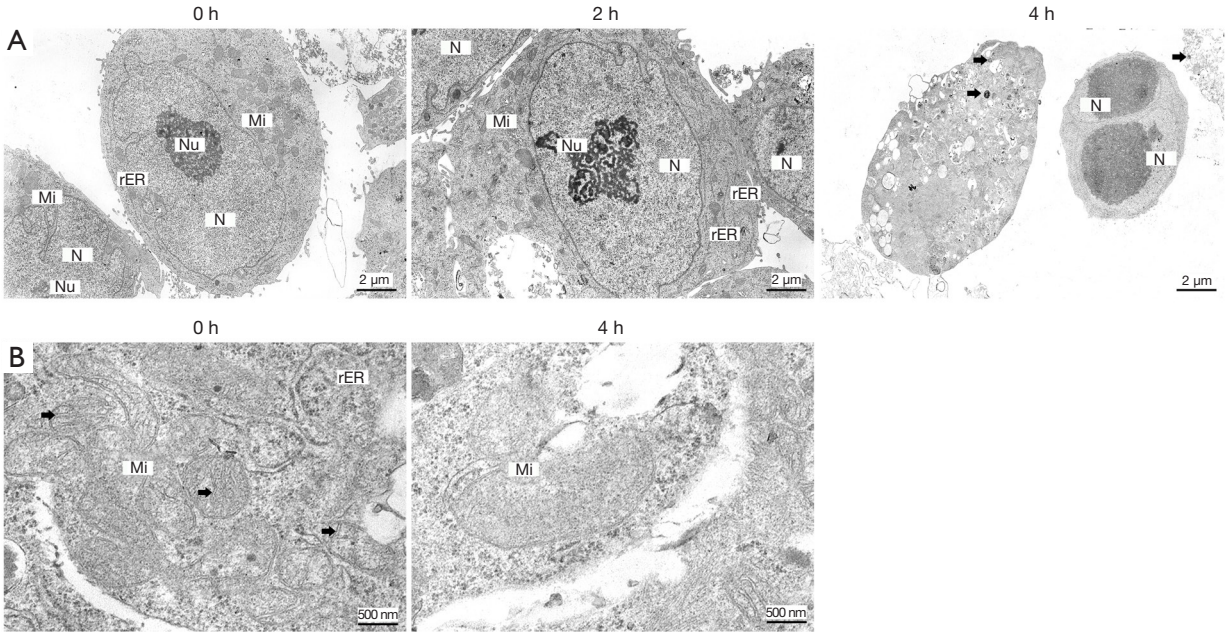

Figure 3 Under an electron microscope, the ultrastructure changes of cells were observed. (A) The chromatin cyclized and marginalized, and apoptotic bodies formed (as shown by arrows). (B) Mitochondrial swelling, vacuolation, and fusion of mitochondrial cristae were observed during spermatogonia apoptosis (4 h). The areas indicated by the arrows represent normal mitochondrial cristae. Mi, mitochondria; Nu, nucleolus; rER, rough endoplasmic reticulum; N, nucleus.

degradation and an increase in apoptotic cells, thereby affecting sperm production and quantity (9).

In clinical practice, surgically removed undescended testes typically exhibit reduced volume, a softer texture, or are underdeveloped. We hypothesize that the pressure in the retroperitoneum and inguinal canal affects testicular development and thus impacts sperm quality. A team from Razi University studied the effect of hydrostatic pressure on mouse sperm and the results indicated that excessive hydrostatic pressure reduced sperm motility, increased the rate of abnormal sperm, damaged sperm DNA, and thus reduced sperm fertilization ability (13). Spinal surgery research indicated that increased hydrostatic pressure of 30 atm induces apoptosis and decreases the viability of human nucleus pulposus cells by activating the Wnt/β-catenin pathway (25). In addition, higher external pressure can cause apoptosis of retinal ganglion cells (26,27). Therefore, elevated external pressure may be one of the causes of apoptosis in spermatogonia and this was further explored in this study.

In this study, we observed the inhibition of spermatogonia activity and changes in cell morphology under elevated pressure, as well as an increase in the rate of apoptosis of spermatogonia with increasing external pressure. There are mainly two classic pathways of apoptosis (*Figure 5*). The first is intrinsic apoptosis. Apoptotic stimuli trigger the opening of the non-selective channel mitochondrial permeability transition pore, causing mitochondrial swelling and increased membrane permeability, leading to cytochrome C release into the cytosol. Cytochrome C combines with Apaf-1 and pro-caspase-9 to form the apoptosome. Activated caspase-9 initiates apoptotic pathways and activates caspase-3, which cleaves substrate proteins, resulting in cellular apoptosis (28). The second is extrinsic apoptosis, in which death receptors bind to their ligands, such as Fas/FasL, leading to activation of caspase-8 and then caspase-3 to induce apoptosis (29). To further explore the pathways through which pressure-induced apoptosis in spermatogonia, TEM was used to observe mitochondrial swelling and vacuolization in apoptotic cells, suggesting the involvement of the mitochondrial pathway.

The activation and aggregation of bax protein lead to changes in mitochondrial membrane permeability and the release of apoptotic molecules from the mitochondria (30).

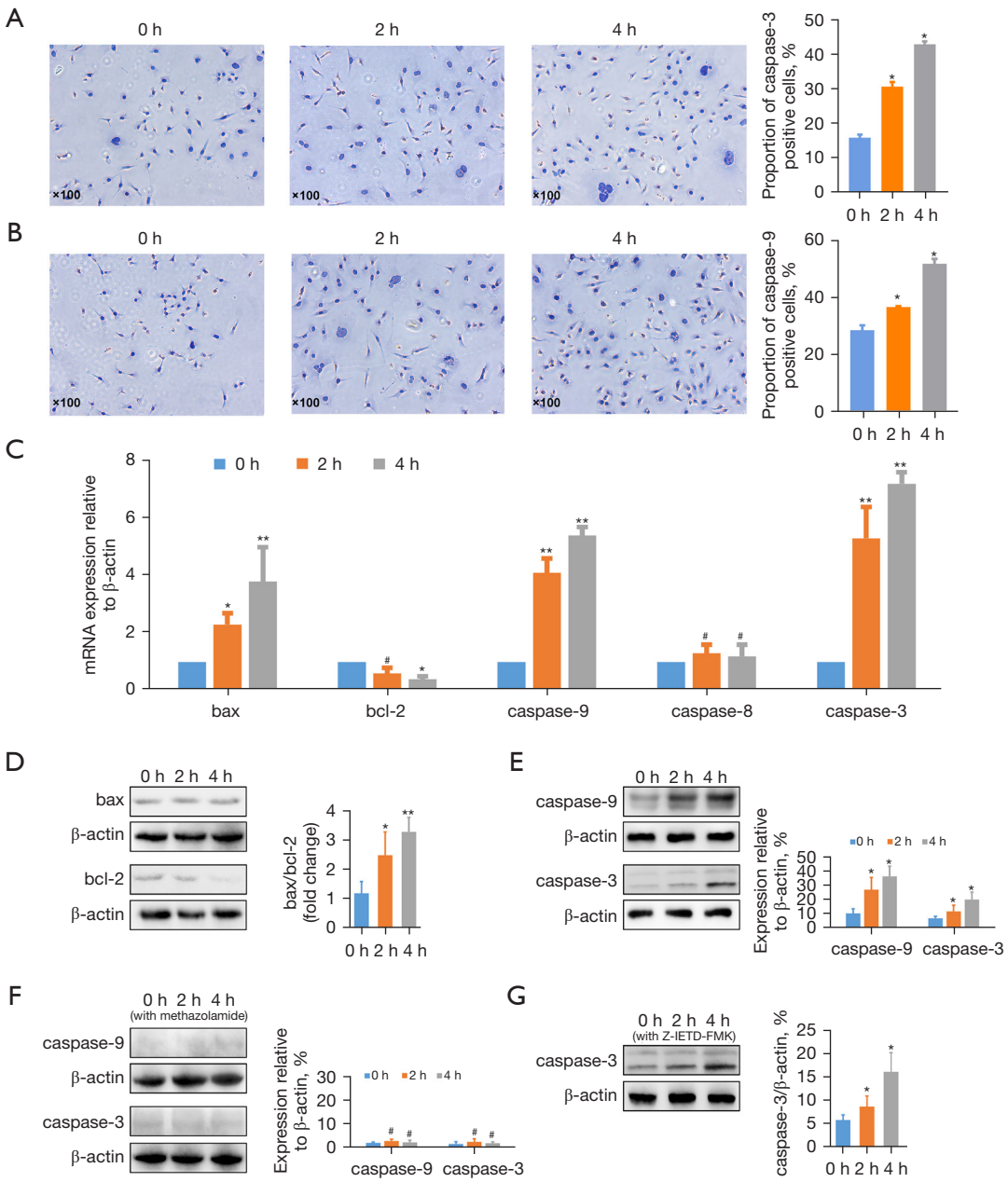

Figure 4 Detection of apoptotic proteins (bax, bcl-2, caspase-9, caspase-8, and caspase-3). (A) After incubation with the primary antibody, followed by DAB and hematoxylin staining, the percentages of caspase-3 positive cells measured at 0, 2, and 4 h were as follows: 15.6±0.92, 30.43±1.39, and 42.68±0.88. *, P<0.05 *vs.* 0 h. (B) After incubation with the primary antibody, followed by DAB and hematoxylin staining, the percentages of caspase-9 positive cells measured at 0, 2, and 4 h were as follows: 28.57±1.71, 36.67±0.29, and 51.86±1.73. *, P<0.05 *vs.* 0 h. (C) Expressions of bax, bcl-2, caspase-9, caspase-3, and caspase-8 in cultured spermatogonia treated by external pressure in a time course were analyzed with qPCR. *, P<0.05; **, P<0.01; #, P>0.05 *vs.* 0 h. (D) Western blot analysis of bax and bcl-2 expression, β-actin served as an internal control. Analysis of western blot bands gray value, determination of bax/bcl-2 ratio. *, P<0.05; **, P<0.01 *vs.* 0 h. (E) Western blot analysis of caspase-9 and caspase-3 expression and caspase-9/β-actin and caspase-3/β-actin ratios in the absence of methazolamide and Z-IETD-FMK. *, P<0.05 *vs.* 0 h. (F) Western blot analysis of caspase-9 and caspase-3 expression and caspase-9/β-actin and caspase-3/β-actin ratios after treatment with methazolamide. #, P>0.05 *vs.* 0 h. (G) Western blot analysis of caspase-3 expression and caspase-3/beta-actin ratio after treatment with Z-IETD-FMK. *, P<0.05 *vs.* 0 h. mRNA, messenger RNA; DAB, 3,3'-diaminobenzidine; qPCR, quantitative polymerase chain reaction.

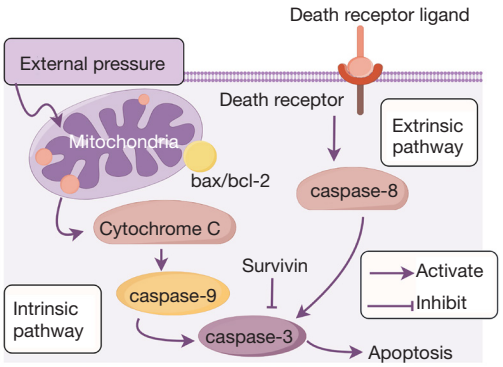

Figure 5 Basic molecular mechanisms in two apoptotic pathways.

The role of the bcl-2 protein is to inhibit the activity of the bax protein, preventing changes in mitochondrial membrane permeability and the occurrence of apoptosis (30,31). Therefore, changes in the ratio of bax/bcl-2 proteins in cells affect the sensitivity of apoptosis. This study found that pressure increased the expression of bax and decreased the expression of bcl-2, leading to an increased bax/bcl-2 ratio and an increased tendency for cell apoptosis (30,31). Caspase-3 is the execution of cell apoptosis and can be activated by intrinsic and extrinsic apoptotic pathways (30). The results of the study showed that pressure increased the expressions of caspase-9 and caspase-3, without remarkable effect on caspase-8. The cytochrome C inhibitor, methazolamide, effectively inhibited the activation of caspase-9 and inhibited the activation of caspase-3. Conversely, the caspase-8 inhibitor, Z-IETD-FMK, failed to demonstrate the same inhibitory effect on caspase-3. These findings suggested that external pressure may induce apoptosis of spermatogonia through the intrinsic apoptotic pathway.

This study only explored the effect of pressure on the intrinsic apoptotic pathway of spermatogonia *in vitro* and did not conduct *in vivo* experiments. We are developing animal models of cryptorchidism and examining the effects of external pressure on testicular tissue and spermatogenesis. This is an aspect of our research that we plan to improve in future studies.

Conclusions

External pressure induces apoptosis of spermatogonia through intrinsic apoptotic pathways, which may be one of the reasons for impaired sperm quality caused by cryptorchidism.

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Footnote

Reporting Checklist: The authors have completed the MDAR reporting checklist. Available at https://tau.amegroups.com/article/view/10.21037/tau-24-158/rc

Data Sharing Statement: Available at https://tau.amegroups.com/article/view/10.21037/tau-24-158/dss

Peer Review File: Available at https://tau.amegroups.com/article/view/10.21037/tau-24-158/prf

Conflicts of Interest: All authors have completed the ICMJE uniform disclosure form (available at https://tau.amegroups.com/article/view/10.21037/tau-24-158/coif). The authors have no conflicts of interest to declare.

Ethical Statement: The authors are accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved.

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