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Original Article

The protective effects of GYY4137 on testicular torsion/detorsion injury in rats

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Abstract: The aim of this study was to verify the protective effect of GYY4137 on testicular torsion/detorsion (T/D) injury involves Hsp70. Forty rats were randomly divided into 4 groups of 10 each: control group, T/D group, T/D + GYY4137 group and T/D + GYY4137 + quercetin group. To establish a model of testicular T/D, the left testis was rotated at 720 degrees clockwise for 2 hours before reperfusion. GYY4137 was administered intraperitoneally at the beginning of the ischaemia phase, and quercetin was injected intraperitoneally 1 hour prior to ischaemia. After 6 hours of reperfusion, the testis was removed for subsequent assessment. The T/D group resulted in severe histological changes and an increase in oxidative stress and apoptosis. Meanwhile, the expression of Hsp70 was increased by T/D and further elevated by GYY4137. GYY4137 ameliorated these observed changes. Quercetin attenuated the protective effects of GYY4137. Our data demonstrated that GYY4137 protects against ischaemia–reperfusion injury (IRI) following testicular T/D, which may be due to the induction of Hsp70.

Keywords: GYY4137, testicular torsion/detorsion, reactive oxygen species, apoptosis, Hsp70

Introduction

Testicular torsion is the most primary cause of urological emergency in the prepubertal and adolescent period [1, 2]. Once diagnosed, manipulative reduction and surgical treatment should be adopted to recover blood flow to the testis as early as possible [3]. The process displays many features of ischaemia-reperfusion injury (IRI). Thus, testicular torsion/detorsion (T/D) is considered a procedure of ischaemia followed by reperfusion [4]. According to previous studies, some agents have been applied to reduce testicular IRI in experimental animal models [5-8].

Hydrogen sulfide (H_2S) is a colourless, water-soluble,volatile gas, with a rotten egg odour. It was initially thought to be a toxic agent [9]. Recently, H_2S has been considered the third kind of endogenous gaseous transmitter, together with carbon monoxide (CO) and nitric oxide (NO), and can produces obvious effects on cell growth regulation [10, 11]. Until now, H_2S-releasing "drugs" used in clinical experiments have largely relied on sulfide salts to evaluate its biological effects, sodium hydrosulfide (NaHS) is most commonly used, as it releases vast amounts of H_2S in a short time (seconds) and cannot effectively imitate the biological course of naturally produced H_2S [12]. Morpholin-4-ium 4 methoxyphenyl (morpholino) phosphinodithioate (GYY4137) was synthesized in 2008 by Moore et al. for the first time, and was based on the chemical structure of Lawesson's compound. As a novel H_2S donor that, unlike NaHS, decomposes and releases H_2S slowly (hours) under physiological pH and temperature, GYY4137 may effectively mimic the process of H_2S release in vitro and in vivo [13].

Heat shock proteins (HSPs) are a group of highly conserved protein molecules, that exist in almost all organisms from bacteria to mammals [14]. These proteins are categorized into various subfamilies basing on their molecular weight [15]. Hsp70 is involved in protein folding, conformation, and repair in a physiological manner. Under stress conditions, Hsp70 can be

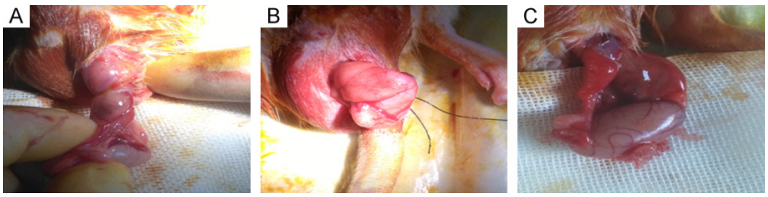

Figure 1. Testicular T/D in rat models. (A) Testicular torsion (B) Fixing the testis to the scrotal skin (C) Testicular detorsion.

induced to participate in the different stages of spermatogenesis and to have anti-apoptotic effects [16-18].

We hypothesized that GYY4137 had the ability to ameliorate oxidative stress and spermatogenic cell apoptosis and that such protective effects against IRI following testicular T/D may be ascribed to the induction of Hsp70.

Materials and methods

Animals

This study involved 40 adult male Wistar rats, weighing 250-300 g, that were obtained from the Hubei Center for Disease Control. Before the experiment, all rats received the same food and water in a 12:12 hour light-dark room with a constant temperature (22 ± 2°C). All experimental procedures were approved by the committee for experimental animals of Wuhan University and followed the Guide for the Care and Use of Laboratory Animals (1996).

Experimental grouping and surgical procedures

Forty rats were randomly divided into 4 groups (n=10, each): control group, T/D group, T/D + GYY4137 group and T/D + GYY4137 + quercetin (an inhibitor of HSPs) group. All rats were anesthetized by the intraperitoneal administration of 2% sodium phenobarbital (50 mg/kg) and were maintained at a temperature of 37°C.

For group A (control), the testis was localized through a left-sided scrotal incision, and then the incision was sutured with 5/0 silk without additional intervention.

For group B (T/D), was designed to investigate the effects of testicular IRI on the ipsilateral testis. The left testis was accessed by scrotal incision. Torsion was achieved by rotating the testis 720 degrees clockwise and fixing it to skin of scrotum with 5/0 silk. The testis was detorsioned to the natural position after 2 hours of torsion, and the scrotal skin was closed with 5/0 silk (**Figure 1**).

For group C (T/D + 100 μmol/kg GYY4137), in addition to the procedures performed in group B, GYY4137 (Sigma-Aldrich, St. Louis, MO) was administered intraperitoneally to each rat at the beginning of the ischaemia phase.

For group D (T/D + 100 μmol/kg GYY4137 + 100 mg/kg quercetin), in addition to the procedures performed in group C, we injected quercetin (Sigma-Aldrich, St. Louis, MO) intraperitoneally 1 hour prior to ischaemia.

After 6 hours of reperfusion, the left testis of each rat in the above 4 groups was removed and divided into two halves, with one half fixed in 4% paraformaldehyde and the other half immediately frozen and transferred to -80°C for later analysis.

HE staining

Testis tissues were fixed in 4% paraformaldehyde, and the half testis was paraffin-embedded and cut into 5 μm sections after routine procedures, such as dewaxing and hydration. The sections were then stained with haematoxylin and eosin (HE).

Biochemical evaluation

Testicular MDA content and SOD activity were detected spectrophotometrically using MDA and SOD Assay kits, following the manufacturer's instructions (Nanjing Jiancheng Bioengineering Institute, China). The MDA content was determined according to the absorbance at 532 nm, and presented as nmol/g protein. SOD activity was measured by checking the absorbance of 550 nm. SOD activity was presented as U/mg protein.

TUNEL assays

Spermatogenic cell apoptosis in the testis was analysed using a transferase-mediated dUTP nick-end labelling (TUNEL) method with a detection kit (Roche, Mannheim, Germany). The nuclei that stained brown were considered as

TUNEL-positive cells. Approximately 200 cells were counted per field; five fields were randomly selected from each slide. The apoptosis index (AI) was determined as follows: AI = positive cells/total cells counted) × 100%.

Immunohistochemistry

Antibodies for immunohistochemistry were bought from Santa Cruz Biotechnology Co. Ltd (Santa Cruz Biotechnology, Santa Cruz, CA): Hsp70 (sc-32239, a mouse monoclonal antibody) and Caspase-3 (sc-7148, a rabbit polyclonal antibody). All steps were performed following the manufacturer's recommendations, and the results were analysed by comparing the staining intensity with microscopic examination.

Western blotting

All testis tissue proteins were extracted, then quantified via the bicinchoninic acid assay. In brief, Equivalents of protein samples (40 μg/lane) were prepared for gel electrophoresis, separated by electrophoresis on sodium dodecyl sulfate-polyacrylamide gel (SDS-PAGE), and transferred to a polyvinylidene difluoride membrane (Millipore, Billerica, MS). The membrane was blocked by usage of 5% non-fat milk in TBST buffer and then hatched with the following major antibodies at 4°C overnight:Bax (1:500 dilution; Santa Cruz Biotechnology, Santa Cruz, CA), Bcl-2 (1:1000 dilution; Santa Cruz Biotechnology, Santa Cruz, CA), Hsp-70 (1:1000 dilution; Santa Cruz Biotechnology, Santa Cruz, CA) and Caspase-3 (1:1000 dilution; Santa Cruz Biotechnology, Santa Cruz, CA). After rinsing twice with TBST buffer, the membranes were hatched with secondary antibodies for 1 hour. All specific bands were visualized by usage of an ECL system kit (Pierce Biotechnology, Beijing, China). Optical densities were estimated by ImageJ software (NIH, Bethesda, MD, USA).

Reverse transcription-polymerase chain reaction (RT-qPCR)

Total RNA was extracted from the testis tissue samples using TRIzol reagent (Invitrogen Life Technologies, Carlsbad, CA, USA), and the RNA purity was measured by spectrophotometry. First-strand cDNA was synthesized through the use of a cDNA synthesis kit (Promega Corporation, WI, USA) following the manufacturer's instructions. Subsequently, the cDNA was amplified by qPCR via an Applied Biosystems SYBR Green mix kit (Applied Biosystems, CA, USA) and the ABI 7900 Real-Time PCR system (Applied Biosystems Life Technologies, Foster City, CA, USA). The primers for Hsp70 and caspase-3 were designed as follows: Hsp70 forward, 5'-ATGCTTCAGACCTCCCTT-3', and reverse, 5'-CTCCACCAACTATCTCCACT-3'; and Caspase-3 forward, 5'-TGGACTGCGGTATTGAGACA-3', and reverse, 5'-GCGCAAAGTGACTGGATGAA-3'. GAPDH was taked as a housekeeping gene. The data are measured as a proportion of gene to GAPDH mRNA (forward, 5'-ACAGCAACAGGGTGGTGGAC-3', and reverse, 5'-TTTGAGGGTGCAGCGAACTT-3'). PCR was performed with 40 cycles of 94°C for 30 sec, 56°C for 30 sec and 72°C for 25 sec, using the ABI 7900 Real-Time PCR system (Applied Biosystems Life Technologies, Foster City, CA, USA).

Statistical analysis

All data were presented as the mean±SD. Statistical analysis were executed using SPSS 17.0 (SPSS Inc, Chicago, IL, USA). The means were compared using one-way analysis of variance followed by the Student-Newman-Keuls test for the different groups. *P*-values < 0.05 were considered as statistically significant.

Results

GYY4137 alleviates histopathological changes after testicular T/D

The HE-stained left testis tissue sections showed that there were no obvious morphological changes in group A. Testicular T/D resulted in significant damage of spermatogenic function, as evidenced by detachment of epithelial cells in the lumen, disordered arrangement of spermatogenic cells, and extensive damage of the seminiferous epithelium. However, treatment with GYY4137 reduced severe testicular damage, while quercetin inhibited the protective effects of GYY4137 significantly (**Figure 2**).

GYY4137 mediates a decrease in MDA content and increase in SOD activity after testicular T/D

To evaluate the levels of oxidative stress associated with testicular T/D, the MDA content and SOD activity in testis tissue were measured. Compared with group A, the MDA content was

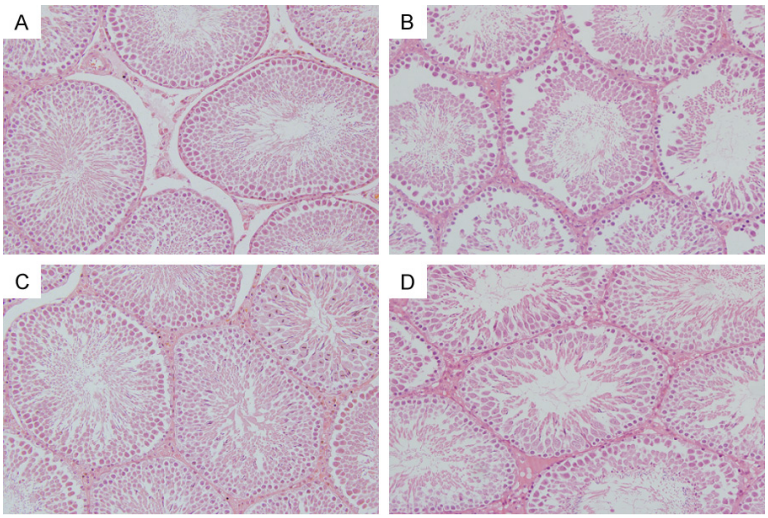

Figure 2. Microphotographs of testis tissues in four groups (hematoxylin and eosin stain; magnification, ×200). (A) Control group: no obvious morphological changes. (B) T/D group: significant damage of spermatogenic function, such as extensive seminiferous epithelium injury, the appearance of vacuoles, and a disorderly distribution of spermatogenic cells. (C) T/D + GYY4137 group: there were fewer spermatogenic cells and seminiferous epithelium changes than there were in the T/D group. (D) T/D + GYY4137 + quercetin group: suppressed the protective effects of GYY4137.

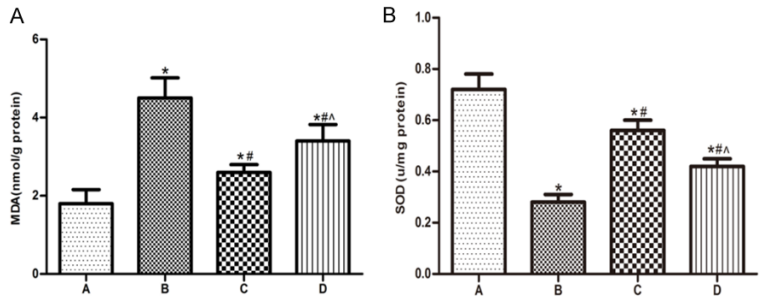

Figure 3. MDA content and SOD activity in four groups. Bars represent the mean ± standard error of the mean (n=10). One-way ANOVA analysis of variance was used to evaluate statistical significance. Compared with group A, the MDA content was obvious increased in group B, while the SOD activity was decreased. GYY4137 ameliorated the levels of oxidative stress in testicular T/D. This effect was reversed by quercetin. (A) Control group, (B) T/D group, (C) T/D + GYY4137 group, (D) T/D + GYY4137 + quercetin group. (*p < 0.05 vs. group A, #p < 0.05 vs. group B, ^P < 0.05 vs. group C).

marked increased in group B, while the SOD activity was obvious decreased. GYY4137 ameliorated the pathology associated with oxidative stress of testicular T/D. This effect was reversed by quercetin (**Figure 3A**, **3B**).

GYY4137 reduces spermatogenic cell apoptosis after testicular T/D

To observe apoptosis and assess the AI, the expression of Bax, Bcl-2 and Caspase3 in testicular spermatogenic cells was evaluated by immunohistochemistry, Western blotting, RT-qPCR analyses and TUNEL assays. In group B, the expression of caspase-3 and Bax was increased obviously (**Figures 4C**, **4D**, **5A**, **5C**, **5E**). An increase in TUNEL-positive spermatogenic cells was also observed. GYY4137 decreased the above expression, and fewer TUNEL-positive spermatogenic cells were detected. The decrease in apoptosis in group C was ameliorated by quercetin (**Figure 4A**, **4B**). Meanwhile, compared with group B, the expression of Bcl-2 was increased in group C, basing on the results of Western blotting, and the protective effects of GYY4137 were obvious attenuated by quercetin (**Figure 4C**, **4E**).

GYY4137 increases the expression level of Hsp 70

The expression level of Hsp70 in testicular T/D was induced at the protein and mRNA level. The expression level of Hsp70 was significantly higher in group B than in group A and further elevated by GYY4137 according to the immunohistochemistry results and RT-qPCR analyses. Quercetin inhibited the GYY4137-mediated increases of Hsp70 (**Figure 5A**, **5B**, **5D**).

Discussion

Testicular T/D is considered to be the primary pathophysiological event of IRI [4]. Previous studies have shown that 30 min to 1 hour of torsion, and then 1 to 4 hours of detorsion is enough to successfully form an IRI model [19, 20]. Therefore, we established a model in which a 2 hour testicular torsion model with 720 degrees clockwise rotation of the left testis was followed by a 6 hour detorsion. In our study, we evaluated the effect of testicular IRI on the

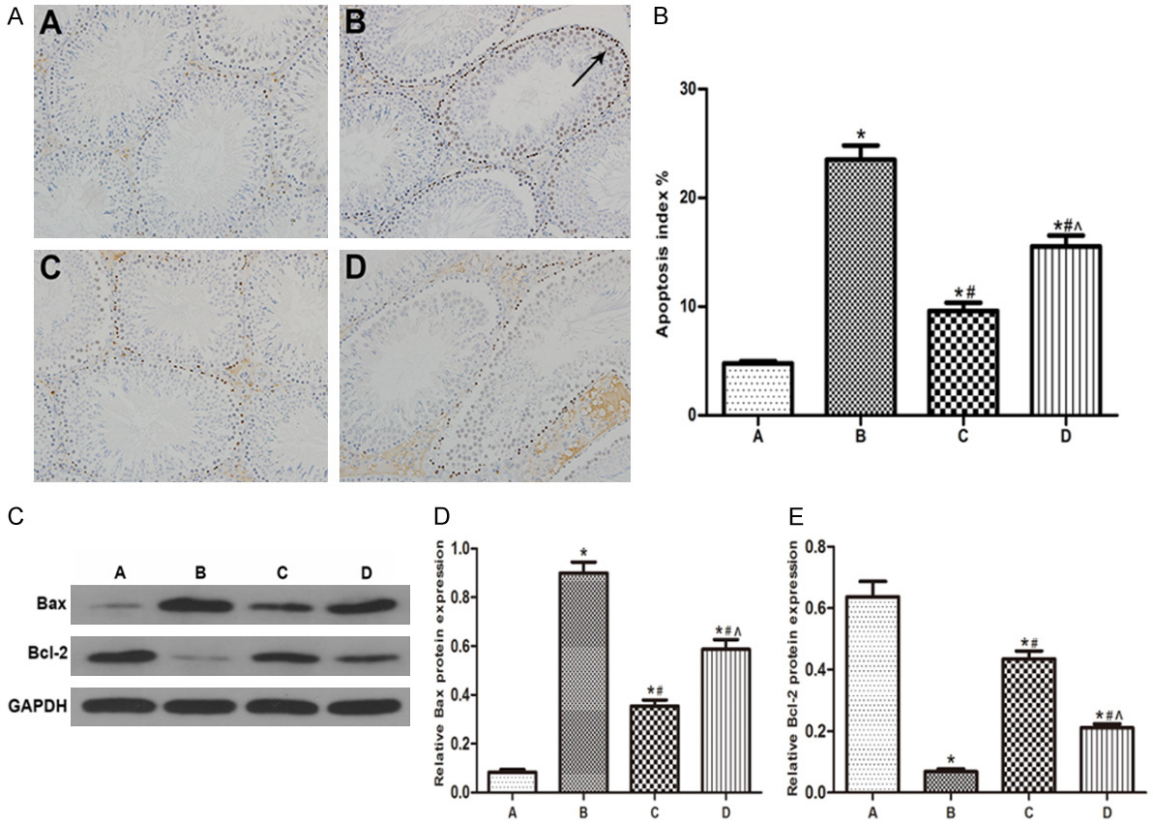

Figure 4. Apoptosis index (AI) in four groups (magnification, ×200) and the expression of Bax and Bcl-2 was detected by western blot. GAPDH was used to show equal amouts of protein loading in each lane. One-way ANOVA analysis of variance was used to evaluate statistical significance. In group B, TUNEL-positive cells were widely observed, and there was an increase compared with the group A. GYY4137 decreased the number of TUNEL-positive spermatogenic cells compared with the group B. However, this effect was reversed by Quercetin. The expression of Bax was increased obviously in group B, and GYY4137 reduced the above expression, the decrease in the expression of Bax was ameliorated by quercetin. Meanwhile, compared with group B, the expression of Bcl-2 was increased in group C, and the protective effects of GYY4137 were obvious attenuated by quercetin. (A) Control group, (B) T/D group, (C) T/D + GYY4137 group group, (D) T/D + GYY4137 + quercetin group. (*$p < 0.05$ vs. group A, #$p < 0.05$ vs. group B, ^P < 0.05 vs. group C).

histological changes in the testes of rats. Our results showed that testicular IRI severely damages testicular spermatogenic function, as demonstrated by other studies [21, 22]. However, GYY4137 markedly ameliorated the above histological injury after testicular T/D.

IRI is due to an overproduction of reactive oxygen species (ROS), and testes are extremely sensitive to oxidative stress in mammals [23, 24]. Under physiological situations, the accumulation of ROS is maintained at a low level through the antioxidant defence system. In pathological circumstances, such as reperfusion of ischaemia, the overproduction of ROS can oxidize cell membrane proteins, lipids, and DNA, leading to a range of cellular dysfunction or death [25, 26]. Malondialdehyde (MDA), an end product of lipid peroxidative decomposition generated by ROS, is typically used as a reliable marker of oxidative stress in many pathological situations, including IRI. Superoxide dismutase (SOD) is a critical component in the process of cell growth, differentiation and protection, and the ability of antioxidants to protect testes against IRI following testicular torsion has been investigated [27, 28]. In this study, treatment with GYY4137 alleviated the oxidative stress in T/D testes by reducing the expression of MDA and increasing the expression of SOD; as such, our data are consistent with that of other investigations [29, 30].

Apoptosis plays a significant role in maintaining homeostasis in spermatogenesis [31]. However, testicular IRI usually leads to wide-spread sper-

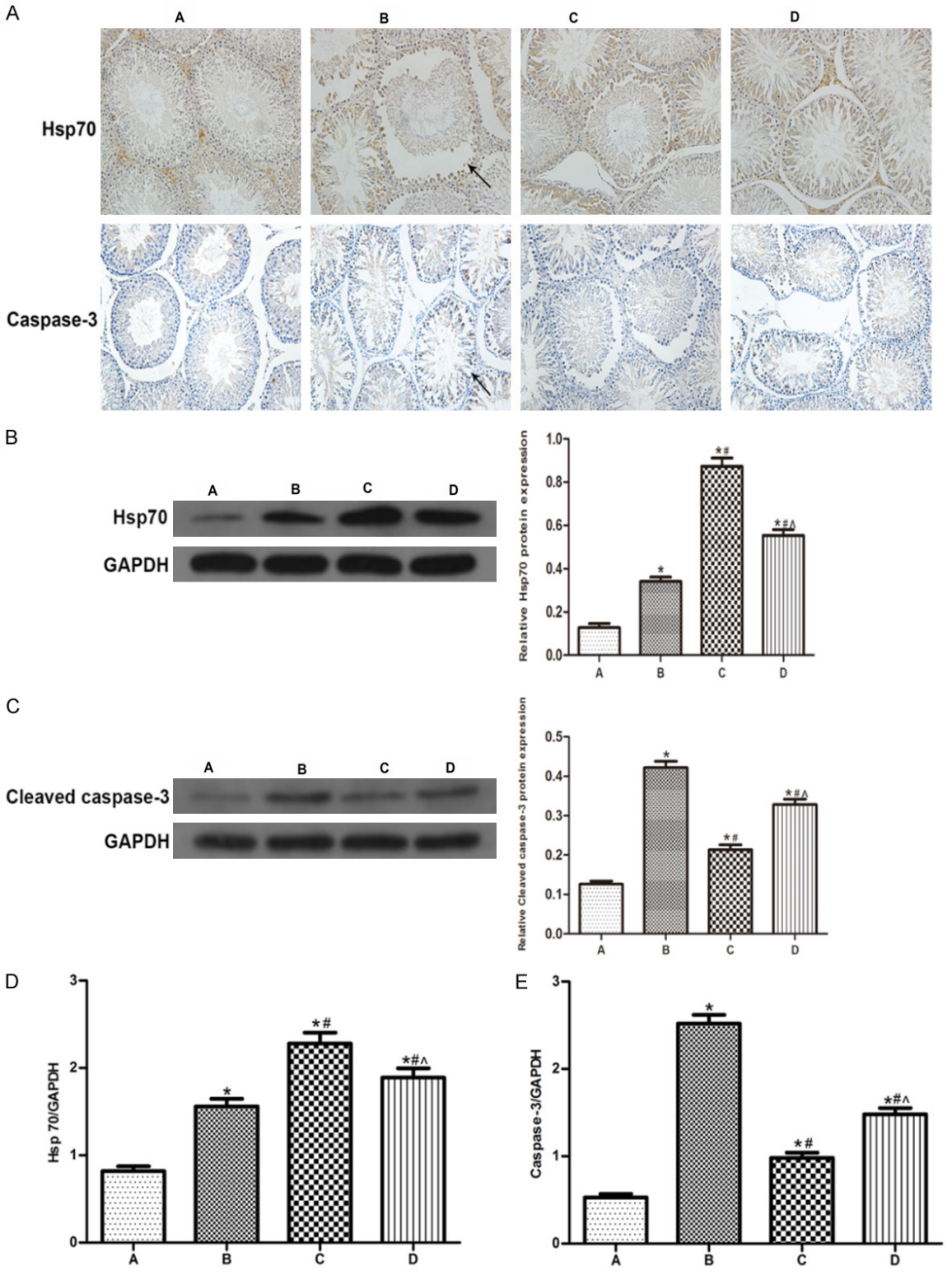

Figure 5. Immunohistochemistry, western blot and RT-qPCR analyses were performed to evaluate the expression of Hsp70 and Caspase-3 (magnification, ×200). The expression of Hsp70 and Caspase-3 relative to GAPDH was determined. The means were compared using one-way ANOVA analysis of variance. Hsp70 and caspase3-positive cells were rarely observed in group A. However, the expression of these proteins was significantly increased in group B, which appeared as yellow or brown granules. Compared with group B, the expression of caspase3 was reduced in group C, in contrast to the expression of Hsp70. However, this effect was reversed by Quercetin. (A) Control group, (B) T/D group, (C) T/D + GYY4137 group, (D) T/D + GYY4137 + quercetin group. (*$p < 0.05$ vs. group A, #$p < 0.05$ vs. group B, ^P < 0.05 vs. group C).

matogenic cell apoptosis, which can cause impaired testicular function and male infertility [32]. In a recent study, Hadziselimovic et al. identified that the incidence of apoptosis in the ipsilateral testis raised, and the extent of apoptosis was related to the duration of the ischaemia [33]. The results of the present study indicated that, compared with the control group, apoptosis of spermatogenic cells increased in T/D testes, and the number of apoptotic cells was significantly reduced following treatment with GYY4137, as demonstrated by TUNEL assays. In addition, it has been generally believed that the mitochondrial signal pathway (or intrinsic signal pathway) is the main channel of apoptosis, which is precisely regulated by gene expression [34]. The Bcl-2 family consist of anti-apoptotic factors (e.g., Bcl2) and pro-apoptotic factors (e.g., Bax) [35]. Recently, these two proteins have been found to be closely related apoptotic proteins. The results of previous studies have demonstrated that the ratio of Bcl2/Bax is crucial for normal spermatogenesis and determines whether apoptosis happens in cells exposed to damage [36, 37]. Moreover, Caspase-3 is an inactive zymogen in the cytoplasm and the convergence point of multiple apoptotic pathways. Its activation is an irreversible sign to induce cell apoptosis, leading to changes in cell shrinkage, chromatin condensation and DNA degradation [38, 39]. To obtain a better understanding of the apoptosis pathway in testicular T/D, we investigated the above apoptosis-related factors. We observed that the expression of Caspase-3 and Bax were up-regulated and the expression of Bcl-2 was down-regulated in group B, as measured by immunohistochemistry, Western blotting and RT-qPCR analyses. Previous studies reported that a decrease in the Bcl-2/Bax ratio transforms into an apoptotic signal after acting on cells, and then opens the mitochondrial permeability transition pore (MPTP), which further activates Caspase-3 and performs the apoptotic program, this sequence of events may contribute to spermatogenic cell apoptosis following IRI in the rat. All of the above showed the involvement of the mitochondrial signalling pathway in GYY4137-induced apoptosis [40, 41].

Hsp70 is a type of non-specific protective protein that is involved in the regulation of intracellular antioxidant enzyme activities and decreases ROS damage in the IRI process [42]. In our study, the expression level of Hsp70 was upregulated following IRI, which means the introduction of Hsp70 could be regarded as a protective mechanism against IRI, consistent with the study of Zhang et al. [10]. Quercetin, as an inhibitor of HSPs, can prevent protein expression by interfering with activities and impacting the transcript pattern [43]. In this study, we injected quercetin (100 mg/kg) intraperitoneally 1 hour prior to ischaemia as Yang et al. [44] and Yao et al. [45] described in previous studies. The expression of Hsp70 in group D was higher than group B, but lower than group C. These results indicate that quercetin inhibited GYY4137-induced Hsp70 expression.

In conclusion, our present study demonstrated that GYY4137 attenuated oxidative stress and spermatogenic cell apoptosis and that such protective effects against IRI following testicular T/D may be attributed to the induction of Hsp70.

Disclosure of conflict of interest

None.

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