

The protective effects of GYY4137 on ipsilateral testicular injury in experimentally varicocele-induced rats

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Abstract. The aim of the present study was to evaluate whether morpholin-4-ium 4 methoxyphenyl (morpholino) phosphonodithioate (GYY4137) exhibits a protective effect on ipsilateral testicular injury in experimentally varicocele (VC)-induced rats. A total of 48 rats were randomly divided into the following 6 groups (n=8 each): Group A (control group); group B (sham group); group C (VC group); group D (VC group administered 5 mg/kg/day GYY4137); group E (VC group administered 10 mg/kg/day GYY4137) and group F (VC group administered 20 mg/kg/day GYY4137). Indicators of oxidative stress, apoptosis and inflammation were measured to evaluate the effect of GYY4137 on ipsilateral testicular injury. Compared with groups A and B, rats in group C exhibited severe histological changes and an increase in oxidative stress, apoptosis and inflammation. By contrast, amelioration of testicular damage was evident in the group D, E and F that were treated with GYY4137. These results demonstrate that GYY4137 may be a promising therapy to treat VC as it alleviates oxidative stress, apoptosis and inflammation in experimentally VC-induced rats.

Introduction

A varicocele (VC) is defined as the abnormal expansion, elongation and tortuosity of the spermatic vein and is considered to be the primary cause of infertility in males (1). The exact mechanism of testicular dysfunction and infertility induced by VC has not yet been clearly defined. It may be associated with a number of factors, including microcirculation disturbance of the testis, vasoactive substance reflux, oxidative stress, an

increase in nitric oxide (NO) concentration, hypoxia, hyperthermia, inflammation or apoptosis (2). It has been reported that the excessive production of reactive oxygen species (ROS) causes germinal cell necrosis, endothelial damage and DNA damage, leading to sperm dysfunction in VC-induced rats (3). Furthermore, elevated spermatogenic cell apoptosis and testicular inflammation are closely associated with male infertility (4).

Hydrogen sulfide (H₂S) is a colourless, water-soluble, volatile gas with a characteristic smell of rotten eggs and was initially considered to be a toxic gas (5). It has been suggested that H₂S may be a third type of endogenous gaseous transmitter, such as carbon monoxide (CO) and NO, and may induce antioxidant, anti-inflammatory and anti-apoptotic effects in various systems (6,7). NaHS has been widely used in clinical experiments to evaluate the biological effects of H₂S (5,8). However, NaHS releases large amounts of H₂S over a short period of time and cannot effectively imitate the biological course of naturally produced H₂S (9). Morpholin-4-ium 4 methoxyphenyl (morpholino) phosphonodithioate (GYY4137) was one of a series of compounds synthesized that were based on the chemical structure of Lawesson's compound, which releases H₂S in organic solvents (10). GYY4137 is novel H₂S donor that, unlike NaHS, decomposes and over a period of a few hours produces small amounts of H₂S under physiological conditions, thus potentially mimicking H₂S release *in vitro* and *in vivo* (10). Meng *et al* (11) reported that GYY4137 may significantly increase the ventricular ejection fraction and reduce the ischemic area in rats, thus protecting against myocardial ischemia-reperfusion injury by attenuating oxidative stress and apoptosis. Additionally, Tang *et al* (12) demonstrated that GYY4137 serves an anti-inflammatory role in the process of acute lung injury in rats. Therefore, the current study hypothesized that GYY4137 has the ability to ameliorate oxidative stress, inflammation and spermatogenic cell apoptosis.

Materials and methods

Animals. The current study involved 48 adult male Sprague Dawley rats (8-10 weeks), weighing 250-300 g, obtained from the Hubei Center for Disease Control (Hubei, China). Prior to the experiment, rats had free access to food and water in a 12 h light/dark cycle with a constant temperature (22±2°C) and humidity (40-70%). All experimental procedures adhered to

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the National Institutes of Health Guide for the Care and Use of Laboratory Animals (13) and were approved by the Animal Care and Use Committee of Wuhan University (Wuhan, China).

Experimental grouping and surgical procedures. All rats were anaesthetized by intraperitoneal administration of 2% sodium phenobarbital (50 mg/kg; Propbs Biotechnology Co., Ltd., Beijing, China) and were then placed on a homeothermic table to maintain a rectal temperature of 37–38°C. The 48 rats were then randomly divided into 6 different groups (all n=8). The rats in group A (control) did not undergo surgery. The rats in group B (sham group) underwent an abdominal median incision. The left renal vein was separated and there was no additional intervention. The rats in group C (VC group), underwent a procedure in which the left renal vein was carefully separated on the inside of the adrenal vein and the spermatic vein, and a metal probe (0.5–0.85 mm diameter) was placed parallel to the left renal vein. The vein and metal probe were ligated together with 4-0 silk suture, reducing the vein diameter to ~50% of its original diameter. The metal probe was then removed and the left renal vein was reversibly constricted. This procedure followed the procedure previously performed by Turner (14) and induced the following characteristics in the rats: Abnormal expansion, elongation and tortuosity of the left renal and spermatic vein; diameter of the left renal vein >1 mm and no pathological lesion in the left kidney. The rats in groups D, E and F underwent the procedures performed in group C followed by the intraperitoneal administration of 5, 10 or 20 mg/kg GYY4137 (Sigma-Aldrich; Merck KGaA, Darmstadt, Germany), once a day respectively.

A total of 4 weeks following the successful establishment of the VC model, the left testis of the rat from the all groups was removed and divided into two halves. One half was fixed in 4% paraformaldehyde at 4°C for 12 h prior to histological examination. The other half was immediately frozen at -80°C for later analysis.

Hematoxylin and eosin (H&E) staining. Following fixation in 4% paraformaldehyde, the testis tissue was embedded in paraffin and cut into 4- μ m thick sections following standard protocols. Following routine dewaxing and hydrating, sections were stained with hematoxylin for 5 min and eosin for 2 min at the room temperature.

Biochemical evaluation. Frozen testicular tissues were homogenized and centrifuged at 500 x g for 10 min at 4°C, and the supernatants were collected. Testicular Malondialdehyde (MDA) content and superoxide dismutase (SOD) activity were measured spectrophotometrically using thiobarbituric acid (TBA) and xanthine oxidase methods following the manufacturer's protocol (A003-1; Malondialdehyde assay kit; Jiancheng Bioengineering Institute, Nanjing, China). MDA content was detected by measuring absorbance at 532 nm and expressed as nmol/g protein. SOD activity was determined by measuring absorbance at 550 nm using a microplate reader and presented as U/mg protein.

TUNEL assays. A TUNEL assay was performed to detect spermatogenic cell apoptosis in the testes using the

transferase-mediated dUTP nick-end labelling (TUNEL) method with an *in situ* apoptosis detection kit (Roche Diagnostics, Basel, Switzerland) following the manufacturer's protocol. Briefly, the rat testis tissue was fixed in 4% paraformaldehyde/PBS solution (pH 7.4) at 4°C overnight and then placed into 70% ethanol at 20°C for 24 h. Following washing 3 times with PBS, the samples were immersed in a permeabilization buffer for 15 min. Subsequently, they were incubated with 50 ml reaction buffer (TdT Enzyme 5 ml + Labeling Safe Buffer 45 ml) at 37°C for 90 min. Nuclei that stained brown were considered to be TUNEL-positive cells as observed under a Zeiss LSM 510 confocal laser scanning microscope (Zeiss AG, Oberkochen, Germany). Five high-power fields of vision were randomly selected in each slice and the average number of apoptotic cells per 200 cells was calculated. The apoptosis index (AI) was expressed as follows: AI=(positive cells/total cells counted) x 100%.

Immunohistochemistry. Caspase-3 and Bax expression were measured using immunohistochemical staining. Tissues were fixed in 4% paraformaldehyde, embedded in paraffin and then cut in 4 μ m thickness, and endogenous peroxidase activity was blocked with 3% hydrogen peroxide at 37°C for 10 min. The sections were incubated with 10% normal goat serum in Tris-buffered saline (TBS) for 30 min at 37°C. Staining was performed using rabbit polyclonal anti-caspase-3 (sc7148; Santa Cruz Biotechnology, Inc., Dallas, TX, USA) and rabbit polyclonal anti-Bax (sc493; Santa Cruz Biotechnology, Inc.) antibodies. After being washed three times with PBS, all sections were incubated in DAB reagents and counterstained with haematoxylin. All steps were performed following the manufacturer's instructions and the results were evaluated by comparing the staining intensity with an Olympus BX50 light microscope (Olympus Corporation, Tokyo, Japan).

Western blot analysis. All testicular tissue proteins were extracted with radioimmunoprecipitation assay lysis buffer (P0013B; Beyotime Institute of Biotechnology, Haimen, China) then quantified using a bicinchoninic acid assay. Briefly, 40 μ g/lane protein samples were separated by 10% SDS-PAGE and transferred to a polyvinylidene difluoride membrane. The membrane was blocked at 37°C for 2 h with 5% non-fat milk in TBST buffer and then incubated with the following primary antibodies at 4°C overnight: Bax (sc-493; 1:500; Santa Cruz Biotechnology, Inc.), cleaved-caspase-3 (sc7148; 1:1,000; Santa Cruz Biotechnology, Inc.), tumor necrosis factor (TNF)- α (ab6671; 1:500; Abcam, Cambridge, UK) and interleukin (IL)-1 β (ab100768; 1:200; Abcam). Following three washes with TBST buffer, membranes were incubated with secondary goat anti-rabbit antibodies conjugated with horseradish peroxidase (LK2001/LK2003; 1:100; Sungene Biotech, Co., Ltd., Tianjin, China) at room temperature for 1 h. All specific bands were visualized using an enhanced chemiluminescence system (Pierce; Thermo Fisher Scientific, Inc., Waltham, MA, USA). Optical densities were detected using ImageJ software version 1.48 (National Institute of Health, Bethesda, MD, USA).

Reverse transcription-quantitative polymerase chain reaction (RT-qPCR). Total RNA was extracted from the testicular tissue

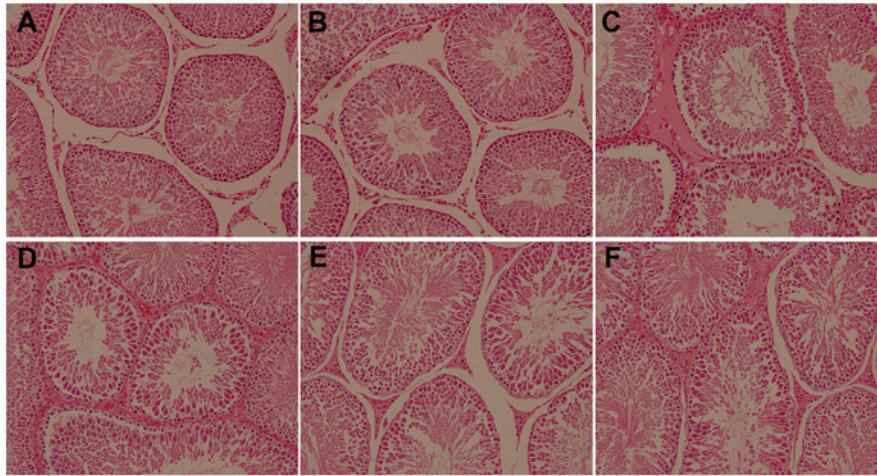


Figure 1. Microphotographs of testes tissues in the 6 groups. (A) Control group, (B) sham group, (C) VC group, (D) VC+5 mg/kg/day GYY4137 group, (E) VC+10 mg/kg/day GYY4137 group and (F) VC+20 mg/kg/day GYY4137 group. Groups A and B exhibited no marked morphological changes. Group C indicated significant pathological and morphological changes. Groups D, E and F exhibited the reversal of these changes. Hematoxylin and eosin staining; magnification, x200. VC, varicocele; GYY4127, Morpholin-4-ium 4 methoxyphenyl (morpholino) phosphonodithioate.

samples from each group using TRIzol reagent (Invitrogen; Thermo Fisher Scientific, Inc.) and RNA concentration was detected using spectrophotometry. First-strand cDNA was synthesized using a cDNA synthesis kit (Promega Corporation, Madison, WI, USA), following the manufacturer's protocol. Subsequently, qPCR was performed using an Applied Biosystems SYBR Green mix kit on an ABI 7900 Real-Time PCR system (Applied Biosystems; Thermo Fisher Scientific, Inc.). The primer sequences for TNF- α and IL-1 β were as follows: TNF- α forward, 5'-ATCCGCGACGTGGAAC TG-3', and reverse, 5'-ACCGCCTGGAGTTCTGGAA-3'; IL-1 β forward, 5'-GAGCACCTTCTTTTCCTTCATCTT-3', and reverse, 5'-TCACACACCAGCAGGTTATCATC-3'. GAPDH was used as a housekeeping gene and the primer sequences for GAPDH were as follows: Forward, 5'-ACAGCAACAGGG TGGTGGAC-3' and reverse, 5'-TTTGAGGGTGCAGCGAAC TT-3'. The data were presented as a ratio to GAPDH mRNA. qPCR was performed with 40 cycles of 94°C for 30 sec, followed by 56°C for 30 sec and 72°C for 25 sec. The quantitative analysis was conducted using the $2^{-\Delta\Delta C_q}$ method (15).

Statistical analysis. All data are presented as the mean \pm standard deviation. Statistical analysis was performed using SPSS 19.0 (IBM Corp, Armonk, NY, USA). The means were compared using one-way analysis of variance followed by the Student-Newman-Keuls test for the different groups. $P < 0.05$ was considered to indicate a statistically significant difference. All experiments were performed at least 3 times.

Results

GYY4137 alleviates VC-induced histopathological damage. H&E staining indicated that there were no marked morphological changes in the left testicular tissue of rats in groups A and B. The spermatogenic cells in the seminiferous tubules were arranged in order, including the primary and secondary spermatocytes, spermatids and spermatozoa. Rats in group C exhibited marked damage in their spermatogenic function, as determined by the detachment of epithelial cells in the

lumen, the disordered arrangement of spermatogenic cells and extensive damage of the seminiferous epithelium. However, treatment with GYY4137 reduced severe testicular damage and there were fewer spermatogenic cells and seminiferous epithelium changes in group C compared with groups D, E and F (Fig. 1).

GYY4137 reduces the increases in MDA content and reduction in SOD activity induced by VC. To evaluate levels of oxidative stress in VC-induced rats, MDA content and SOD activity were measured in testicular tissue. In group C, MDA content was significantly increased and SOD activity was significantly decreased, compared with groups A and B. However, treatment GYY4137 significantly reversed the increase in MDA content and the reduction in SOD activity induced by VC in groups D, E and F (Fig. 2).

GYY4137 inhibits the apoptosis of spermatogenic cells induced by VC. To assess apoptosis and determine the AI, the expression of Bax and caspase-3 in testicular spermatogenic cells was evaluated by TUNEL, immunohistochemistry and Western blot analysis. There was a significant increase in the number of TUNEL-positive spermatogenic cells in group C compared with groups A and B (Fig. 3). Furthermore, immunohistochemistry indicated that caspase-3 and Bax levels were markedly increased in group C compared with groups A and B. However, following treatment with GYY4137, significantly fewer TUNEL-positive spermatogenic cells were detected. Additionally, treatment with GYY4137 decreased caspase-3 and Bax expression. Testis taken from mice in group E that received 10 mg/kg/day GYY4127 experienced an ideal therapeutic effect on damaged testes, evident by a significant reduction of TUNEL-positive spermatogenic cells (Fig. 3) and marked reduction in the expression of caspase-3 and Bax (Fig. 4).

GYY4137 attenuates the VC-induced inflammatory response. The expression of TNF- α , IL-1 β , Bax and cleaved caspase-3 was significantly higher in group C than in groups A and B

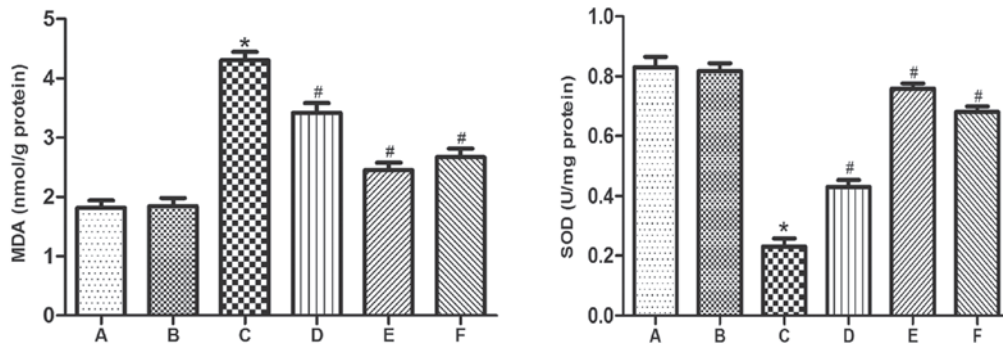


Figure 2. MDA content and SOD activity in the 6 groups. (A) Control group, (B) sham group, (C) VC group, (D) VC+5 mg/kg/day GYY4137 group, (E) VC+10 mg/kg/day GYY4137 group, (F) VC+20 mg/kg/day GYY4137 group. *P<0.05 vs. group A; #P<0.05 vs. group C. VC, varicocele; GYY4127, Morpholin-4-ium 4 methoxyphenyl (morpholino) phosphonodithioate; MDA, malondialdehyde; SOD, superoxide dismutase.

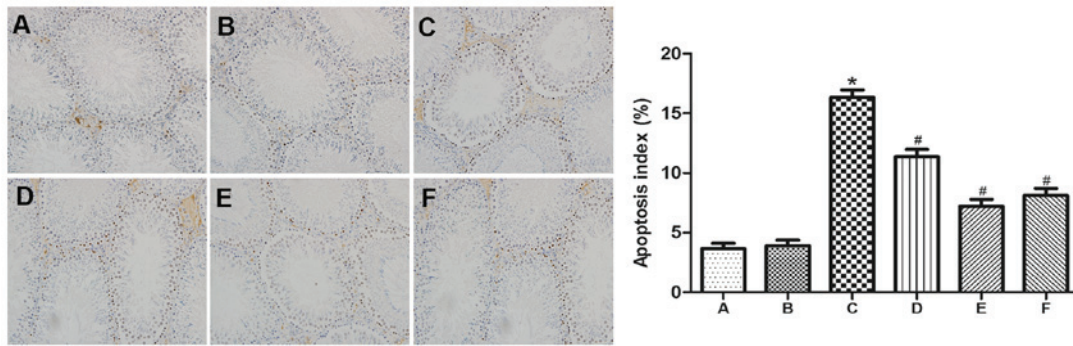


Figure 3. Apoptosis index in the 6 groups. (A) Control group, (B) sham group, (C) VC group, (D) VC+5 mg/kg/day GYY4137 group, (E) VC+10 mg/kg/day GYY4137 group, (F) VC+20 mg/kg/day GYY4137 group. Magnification, x200. *P<0.05 vs. group A; #P<0.05 vs. group C. VC, varicocele; GYY4127, Morpholin-4-ium 4 methoxyphenyl (morpholino) phosphonodithioate.

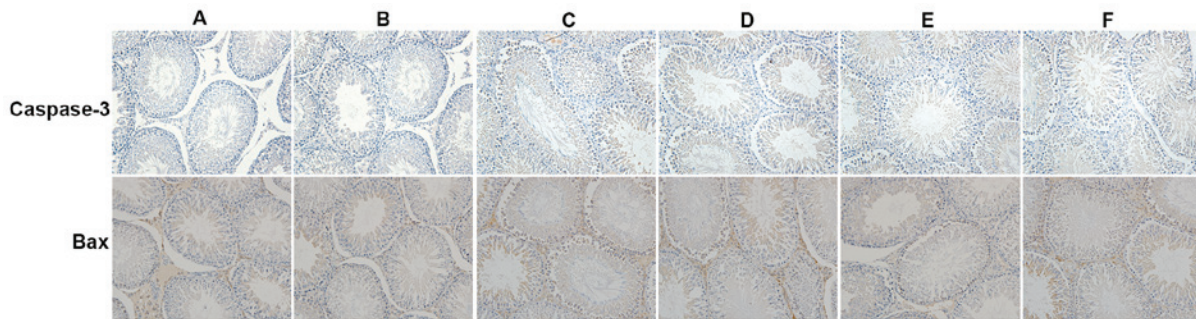


Figure 4. Immunohistochemistry was performed to evaluate the expression of Bax and caspase-3. (A) Control group, (B) sham group, (C) VC group, (D) VC+5 mg/kg/day GYY4137 group, (E) VC+10 mg/kg/day GYY4137 group, (F) VC+20 mg/kg/day GYY4137 group. Magnification, x200. VC, varicocele; GYY4127, Morpholin-4-ium 4 methoxyphenyl (morpholino) phosphonodithioate.

(Figs. 5 and 6). However, administration of GYY4137 significantly decreased the expression of TNF- α , IL-1 β , Bax and cleaved caspase-3 in groups D, E and F compared with group C. The results demonstrated that GYY4137 was most effective at reducing the expression of inflammation-related genes at a dose of 10 mg/kg/day (Figs. 5 and 6).

Discussion

VC is described as the abnormal expansion, elongation and tortuosity of the spermatic vein and is considered to be the primary cause of infertility in adolescent males (1). The

incidence of VC in the general population is ~15%, however, in infertile men it is much higher, at 35% (16). Nevertheless, the mechanism of testicular dysfunction and infertility associated with VC remains unclear, therefore, a number of studies investigating the pathophysiology of this condition have been performed. GYY4137 is a novel H₂S-releasing molecule and induces a wide range of pharmacological effects, including antioxidant, anti-apoptosis, anti-inflammatory and immune-modulating activities, in a wide range of different organs (17-19). The current study initially evaluated the effect of GYY4137 on the histological changes that occur in the testes of rats with a VC. The results demonstrated that VC

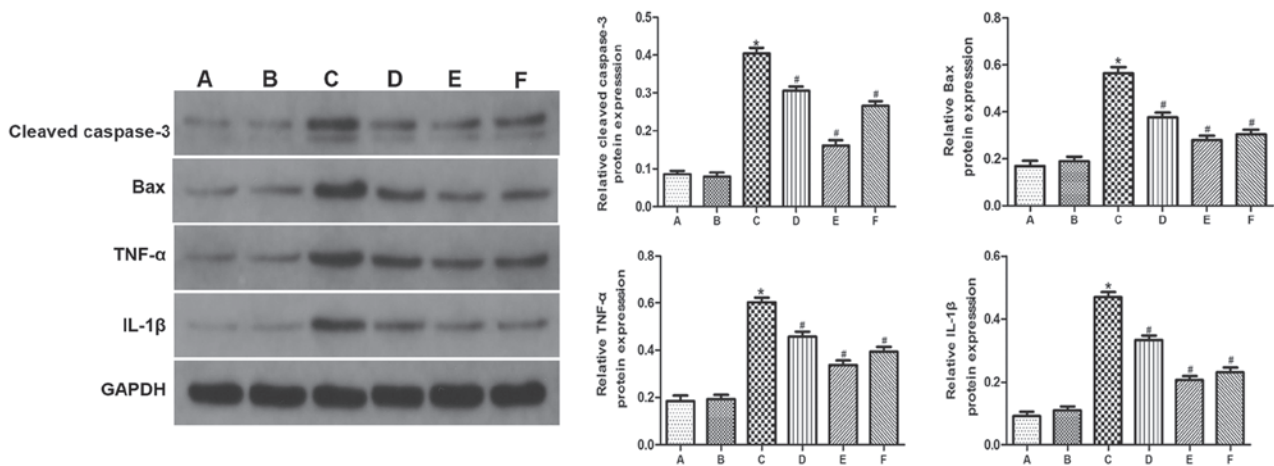


Figure 5. The expression of Bax, cleaved caspase-3, TNF- α and IL-1 β was detected by western blotting. GAPDH was as a loading control. (A) Control group, (B) sham group, (C) VC group, (D) VC+5 mg/kg/day GYY4137 group, (E) VC+10 mg/kg/day GYY4137 group, (F) VC+20 mg/kg/day GYY4137 group. *P<0.05 vs. group A; #P<0.05 vs. group C. VC, varicocele; GYY4127, Morpholin-4-ium 4 methoxyphenyl (morpholino) phosphonodithioate; TNF- α , tumor necrosis factor α ; IL-1 β , interleukin 1 β .

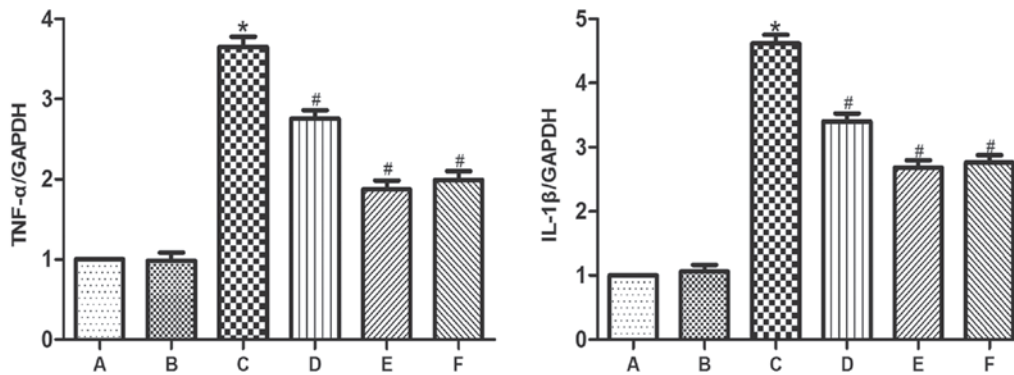


Figure 6. Reverse transcription-quantitative polymerase chain reaction was performed in testes tissues. The expression of TNF- α and IL-1 β relative to GAPDH was determined. (A) Control group, (B) sham group, (C) VC group, (D) VC +5 mg/kg/day GYY4137 group, (E) VC+10 mg/kg/day GYY4137 group, (F) VC+20 mg/kg/day GYY4137 group. *P<0.05 vs. group A; #P<0.05 vs. group C. VC, varicocele; GYY4127, Morpholin-4-ium 4 methoxyphenyl (morpholino) phosphonodithioate; TNF- α , tumor necrosis factor α ; IL-1 β , interleukin 1 β .

severely damages testicular spermatogenic function, in accordance with the results of previous studies (20,21). However, different doses of GYY4137 ameliorated histological injury to different extents and the ideal effect was induced following the administration of 10 mg/kg/day GYY4137 in experimental rats with a VC.

Oxidative stress is a result of the accumulation of ROS in tissues, which leads to the deregulation of antioxidant mechanisms (22). Under physiological situations, the accumulation of ROS is maintained at a low level throughout the antioxidant defence system and has specific regulatory effects on cell growth, development, differentiation and death. However, in pathological circumstances, including ischemia and hypoxia, the overproduction of ROS can result in the oxidation of cellular membrane proteins, lipids and DNA to cause a range of cellular dysfunction and cell death (22,23). Mammalian testes are highly sensitive to oxidative stress and the pathology of VC is closely associated with the overproduction of ROS (24). MDA, a major product of lipid peroxidative decomposition generated by ROS, is typically used to evaluate the extent of cellular damage

under conditions of oxidative stress (25). SOD is a critical component in the processes of cell growth, differentiation and protection, and has the ability to convert a superoxide anion radical into hydrogen peroxide; its activity reflects the scavenging capacity of ROS in organisms (26). It has been demonstrated that H₂S serves a key role in reducing ROS production and has therapeutic potential in preventing oxidative stress damage in different types of organs (27). In the current study, treatment with the novel H₂S donor, GYY4137, alleviated oxidative stress in VC testes by reducing the expression of MDA and increasing the expression of SOD.

Apoptosis is a normal physiological phenomenon that serves an important role in maintaining homeostasis during spermatogenesis (28). However, the pathological process of VC usually leads to widespread spermatogenic cell apoptosis, which may induce further testicular dysfunction and cause infertility (29). Zheng *et al* (30) demonstrated that the incidence of apoptosis in the ipsilateral testis was greatly raised in a rat model of VC and that there was a positive correlation between the extent of apoptosis and injury duration. It has been demonstrated that the process of apoptosis is regulated by gene expression and its

activation may stimulate the degradation of cellular substrates and participate in a variety of pathogenesises, including acting as a functional obstacle of spermatogenesis and decreasing sperm motility and DNA levels (31). Furthermore, Bax may promote the release of cytochrome *c* from the mitochondria to induce apoptosis (32). Mostafa *et al* (33) indicated that the expression of Bax was significantly increased in infertile men with a VC. Additionally, caspase-3 is an inactive zymogen among the cysteine proteases and the convergence point of multiple apoptosis-stimulating signals. Its activation is a sign of an irreversible commitment to cellular apoptosis (30). It has been demonstrated in an androgen-deficiency model that the decrease in testosterone induced by spermatogenic cell apoptosis in testicular tissue is caspase-3 dependent, indicating that caspase-3 activation induces spermatogenic cell apoptosis (34). Furthermore, it has been demonstrated that exogenous H₂S inhibits the activation of caspase-3 to induce an anti-apoptotic effect in renal ischemia-reperfusion injury (35). The damage induced by VC has complicates the pathological course and inflammation has been recognized as an important factor in the onset and development of VC (36). TNF- α and IL-1 β are key inflammatory cytokines involved in the pathological process of VC. Germ cell-derived TNF- α increases tissue damage and the inflammatory response and may control physiological spermatogenic cell apoptosis by regulating Fas ligand levels (37). IL-1 β is an immune-derived cytokine and promotes its own secretion under ischemia and hypoxia and the increased expression of IL-1 β induces detrimental effects in the testes of infertile males with VC (38,39). In the present study, it was hypothesised that GYY4137 inhibits the expression of Bax, caspase-3, TNF- α and IL-1 β , thereby alleviating apoptosis and inflammatory responses in a rat model of VC.

In conclusion, the present study demonstrated that GYY4137 attenuates testicular dysfunction by alleviating oxidative stress, inflammation and apoptosis in a rat model of VC. GYY4137 was most effective at attenuating this dysfunction at a dose of 10 mg/kg/day. However, the specific underlying protective mechanisms of GYY4137 in VC remain unknown and further studies are required.

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