Protective effect of GYY4137, a water-soluble hydrogen sulfide-releasing molecule, on intestinal ischemia-reperfusion

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Abstract. The present study aimed to clarify the protective effects of p-methoxyphenyl morpholino-phosphinodithioic acid (GYY4137), a water-soluble hydrogen sulfide-releasing molecule, on a rat model of intestinal ischemia-reperfusion (IIR). A total of 40 healthy male Sprague Dawley (SD) rats were randomly divided into four groups (n=10/group): Group A, a sham-surgery group; Group B, the IIR group; group C, rats with IIR that were administered an abdominal injection of low-dose GYY4137 (40 mg/kg); and group D, rats with IIR that were administered high-dose GYY4137 (80 mg/kg). Intestinal histomorphology was observed using hematoxylin and eosin staining, and the concentrations of malondialdehyde (MDA) and superoxide dismutase (SOD) were measured. Apoptotic index (AI) was determined by terminal deoxynucleotidyl-transferase-mediated dUTP nick end labeling. Reverse transcription-quantitative PCR analysis was performed to assess the expression levels of intestinal caspase-3, Bax and Bcl-2. Notably, disordered arrangement of intestinal villi and mucosal necrosis were detected in group B, which was substantially improved by GYY4137 treatment (groups C and D). MDA content (nmol/mg) was 2.83±0.36, 9.23±0.78, 4.97±0.45 and 3.51±1.05 nmol/mg in groups A, B, C and D, respectively. In addition, SOD concentration (U/mg) was 135.37±3.34, 76.45±1.39, 95.13±1.64 and 115.13±2.54 in groups A, B, C and D, respectively. Furthermore, AI in group B (21.73±1.17%) was markedly higher than that in group A (4.53±0.28%) and in the GYY4137 intervention groups (9.53±0.96 and 6.53±0.76% in groups C and D, respectively). Compared with in group A, the mRNA expression levels of Bax and caspase-3 were markedly higher in group B ($P<0.05$), whereas the expression of Bcl-2 was significantly lower ($P<0.05$). Furthermore, compared with in group B, Bcl-2 expression was higher, and Bax and caspase-3 expression was lower in groups C and D ($P<0.05$). In conclusion, GYY4137 may alleviate IIR-induced damage in SD rats.

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Introduction

Intestinal ischemia-reperfusion (IIR) may occur in healthy individuals and clinical patients (1,2). Under physiological conditions, IIR may help induce blood redistribution to important organs and tissues, resulting in intestinal hypoperfusion and decreased mesenteric blood flow (3). However, under pathological conditions, including major surgery, trauma, inflammatory bowel disease or hypovolemia, IIR may lead to severe mesenteric artery embolism or phlebothrombosis (4). A previous study identified that the injury of distant organs and tissues caused by intestinal ischemia does not mainly come from ischemic injury itself, but bacterial translocation, endotoxin translocation and massive release of inflammatory cytokines during the recovery of intestinal blood supply and blood flow. This can result in multiple organ injury to the liver, kidney and lung, which leads to systemic inflammatory reaction, systemic multiple organ dysfunction syndrome and even mortality (5). The clinical classification of IIR comprises acute mesenteric ischemia, chronic mesenteric ischemia and ischemic colitis (6). Patients with a certain specific medical history, including congestive heart failure, arrhythmia, atherosis, sepsis and collagen deposition disease, have an elevated morbidity rate when faced with IIR (6,7). Prompt mesenteric blood flow recovery and ischemic tissue reoxygenation are the dominant treatment principles for IIR; however, they may increase vessel and tissue damage (7,8). Previous studies have indicated that GYY4137, as a novel type of hydrogen sulfide (H_2S) donor, is able to stably induce sustained release of H_2S under physiological pH and temperature, which may effectively simulate the release process of H_2S (9,10). Furthermore, GYY4137 has been demonstrated to protect myocardial cells from damage caused by high glycemia-induced cytotoxicity, and reduce vascular inflammation and oxidative stress (10-12). The effects of GYY4137 on IIR have so far remained elusive; therefore, the present study assessed the effects of GYY4137 on a Sprague Dawley (SD) rat model of IIR and investigated the underlying mechanisms.

Materials and methods

Animals and experimental procedure. The protocols of the present study were approved by the Institutional Animal Care and Use Committee of Wuhan University (approval ID. WHU20110312). A total of 40 male specific pathogen-free SD

rats (aged 30-35 days; weight, 120-150 g) were obtained from the Experimental Animal Center of Wuhan University. All rats were selected and kept under conventional conditions in an environmentally controlled room (temperature, 22-25˚C; humidity, 50-70%; 12-h light/dark cycle) for 7 days prior to the procedure. GYY4137 was obtained from Abcam (cat. no. ab142145). All rats were randomly divided into the following four groups (n=10/group): Group A, a sham-surgery group; group B, an IIR group; group C, an IIR group that was administered an abdominal injection of low-dose GYY4137 (40 mg/kg); and group D, an IIR group that was administered high-dose GYY4137 (80 mg/kg). For groups C and D, abdominal injection of 40 or 80 mg/kg GYY4137, respectively, was administered daily for 3 consecutive days prior to surgery. After 7 days acclimation, sodium pentobarbital (50 mg/kg) was used for anesthesia prior to surgery. For all groups, regular disinfection was conducted and a 2-cm incision was made on the central hypogastrium; layer-by-layer cutting and isolation of the perimesenteric adipose tissue were performed in sequence. Subsequently, for groups B, C and D, a microvascular clip was used to obstruct the superior mesenteric artery (intestinal appearance changed from red to maroon) for 45 min, in order to generate the ischemia model. Conversely, no clipping was performed in group A. Following the removal of the clip (reperfusion) in groups B, C and D, all four groups were euthanized by an overdose of1 anesthesia. For each rat, 5 mm of the jejunum was obtained for biochemical and pathological evaluation. In addition, blood was collected from the aorta abdominalis for further analysis.

Hematoxylin and eosin (H&E) staining. According to standard protocols, tissues were embedded in paraffin (paraffin I, 60˚C, 1 h; paraffin II, 60˚C, 1 h; paraffin III, 60˚C, 1 h). After cutting into 4-μm sections, tissues were dewaxed, hydrated and stained with hematoxylin at 25˚C for 5-7 min. Sections were observed at a magnification of x200 using an Olympus BX53 light microscope (Olympus Corporation).

Biochemical analysis. Tissues were homogenized and centrifuged (1,000 x g at 25˚C for 15 min) prior to analysis. Malondialdehyde (MDA) content (nmol/g protein) and superoxide dismutase (SOD) activity (U/mg protein) were measured spectrophotometrically through thiobarbituric acid and xanthine oxidase methods, respectively, according to the manufacturer's protocols (MDA Assay kit, cat. no. A003-1; SOD Assay kit, cat. no. A001-3-2; Nanjing Jiancheng Bioengineering Institute).

Terminal deoxynucleotidyl-transferase-mediated dUTP nick end labeling (TUNEL) assay. TUNEL staining using the *in situ* Cell Death Detection kit, Fluorescein (Roche Diagnostics) was performed to detect apoptosis of intestinal mucous epithelial cells according to manufacturer's protocol. TUNEL-positive cells (nuclei stained brown) were observed under a Zeiss LSM 510 confocal laser scanning microscope (Zeiss AG). Apoptotic index (AI) was expressed as AI=(TUNEL-positive cells/total cells) x100%.

Immunohistochemistry. Rabbit polyclonal anti-caspase-3 (cat. no. sc-7148; Santa Cruz Biotechnology, Inc.) and rabbit polyclonal anti-Bax (cat. no. sc-493; Santa Cruz Biotechnology, Inc.) antibodies were used for immunohistochemical staining. Tissue treatment prior to staining consisted of several steps, including dehydration (25˚C, 75% ethanol 4 h, 85% ethanol 2 h, 90% ethanol 1.5 h, 100% ethanol I 0.5 h, 100% ethanol II 0.5 h), permeabilization (25˚C,100% ethanol:xylol 1:1, 10 min; xylol I 10 min, xylol II 7 min), paraffin embedding, slicing, and baking and dewaxing. Subsequently, sections (4 μm) underwent antigen retrieval, endogenous peroxidase blocking and serum blocking. For antigen retrieval, sections were boiled with the retrieval solution (0.01 M diluted citric acid buffer solution, pH 6.0) at 100˚C for 10-15 min. The solution was then left to cool at room temperature for 40 min and washed with PBS (pH 7.4) three times (3 min/wash). For peroxidase blocking, sections were incubated with 3% hydrogen peroxide (usually diluted with methanol or distilled water for 3% hydrogen peroxide) at room temperature for 15 min, then washed with PBS three times (3 min/wash). For serum blocking, sections were incubated with 10% normal goat serum (Dako; Agilent Technologies, Inc.) at room temperature for 30 min.

Subsequently, sections were incubated with primary antibodies at 4˚C for 15 h, and with goat anti-rabbit/mouse secondary antibodies (cat. no. k500711-2; Dako; Agilent Technologies, Inc.) at 37˚C for 20 min. Counterstaining was conducted using Harris hematoxylin for 30 sec to 1 min, after which, sections were washed with 1% hydrochloric acid and then with tap water or PBS. Staining evaluation was performed under an Olympus BX50 light microscope (Olympus Corporation). The results were evaluated by comparing staining intensity upon microscopic examination.

Western blot analysis. Proteins were extracted from tissues using RIPA buffer (cat. no. 9806S; Cell Signaling Technology, Inc.) and PMSF (cat. no. 8553; Cell Signaling Technology, Inc.), and quantified using the bicinchoninic acid assay. Briefly, equivalent protein samples (40 μg/lane) were separated by SDS-PAGE on 10% gels and were transferred to nitrocellulose membranes. Subsequently, the membranes were blocked with 5% non-fat milk in Tris-buffered saline with (10%) Tween 20 at 25˚C for 30 min and were then incubated with primary antibodies at 4˚C overnight. After extensive washing with TBST, the membranes were incubated with horseradish peroxidase-conjugated secondary goat anti-rabbit or anti-mouse antibodies (cat. nos. LK2001 and LK2003; 1:100; Tianjin Sungene Biotech, Co., Ltd.) for 1 h at 25˚C. All specific bands were visualized using an ECL system kit (Pierce; Thermo Fisher Scientific, Inc.). Optical densities were detected using ImageJ software (version 1.48; National Institutes of Health). The following primary antibodies were used: Bax (cat. no. sc-493; 1:500; Santa Cruz Biotechnology, Inc.), cleaved-caspase-3 (cat. no. sc-7148; 1:1,000; Santa Cruz Biotechnology, Inc.) Bcl-2 (cat. no. sc-509; 1:100; Santa Cruz Biotechnology, Inc.) and (GAPDH, 37 kDa, anti-rabbit, AB-P-R 001; 1:1,000 Hangzhou Goodhere Biotechnology Co., Ltd.).

Reverse transcription-quantitative PCR (RT-qPCR). Total RNA was extracted from 100 mg intestinal tissue sample using TRIzol® reagent (Invitrogen; Thermo Fisher Scientific, Inc.) and RNA purity was detected by spectrophotometry. Subsequently, first-strand cDNA was synthesized using random primers and M-MLV Reverse Transcriptase (Promega Corporation). RT temperature conditions were as follows:

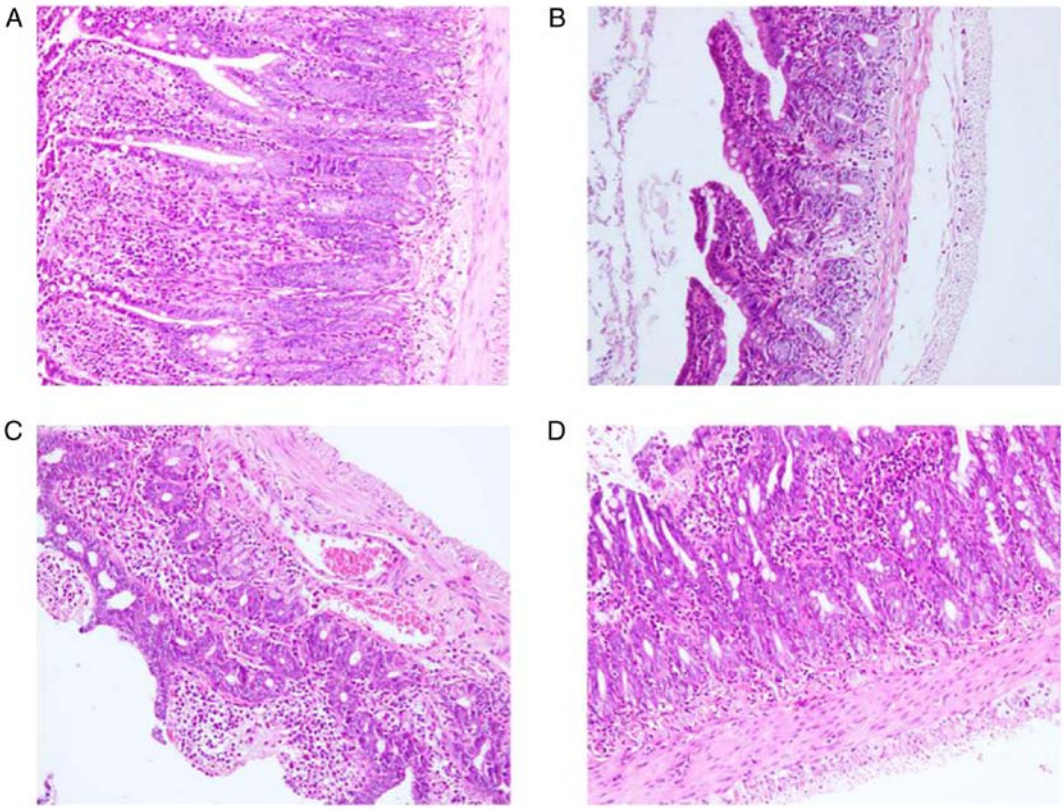

Figure 1. Pathological alterations in intestinal tissues following intestinal ischemia-reperfusion and GYY4137 intervention. Group A, a sham-surgery group; group B, an IIR group; group C, an IIR group that was administered an abdominal injection of low-dose GYY4137 (40 mg/kg); and group D, an IIR group that was administered high-dose GYY4137 (80 mg/kg). Magnification, x100.

25˚C for 5 min, 50˚C for 15 min, 85˚C for 5 min and 4˚C for 10 min. cDNA was amplified by qPCR using an Applied Biosystems SYBR Green mix kit (Applied Biosystems, Inc.) and the ABI 7900 Real-Time PCR system (Applied Biosystems, Inc.). Thermocycling conditions were as follows: 50˚C for 2 min, 95˚C for 10 min; 95˚C for 30 sec, 60˚C for 30 sec all for 40 cycles. mRNA expression was normalized to GAPDH (13). Primer sequences were as follows: Caspase-3, forward 5'-TGGACTGCGGTATTGAGACA-3', reverse 5'-GCGCAAAGTGACTGGATGAA-3'; Bax, forward 5' TGA ACTGGACAACAACATGGAG-3, reverse 5' AGCAAAGTA GAAAAGGGCAACC-3'; Bcl-2, forward 5'-ATGCTTCAG ACCTCCCTT-3', reverse 5'-CTCCACCAACTATCTCCA CT-3'; and GAPDH, forward 5'-ACAGCAACAGGGTGG TGGAC-3' and reverse 5'-TTTGAGGGTGCAGCGAAC TT-3'.

The conditions for RT and qPCR were as follows: i) RT system: RNA, 3.192 μg; Oligo (dT) 15 (10 μM), 2 μl; dNTP (2.5 mM), 4 μl; 5X Hiscript Buffer, 4 μl; Hiscript Reverse Transcriptase, 1 μl; Ribonuclease Inhibitor, 0.5 μl; ddH_2O (RNase-free), ≤20 μl. ii) Semi-quantitative RT-PCR; DNA products were run on a 10% agarose gel containing ethidium bromide at 120V and 100 mA for 30 min. DNA bands were visualized using an ultraviolet analytical instrument (Beijing Junyi-Dongfang Electrophoresis Equipment Co., Ltd., JY02S) and quantified using Image Lab 3.0 (Bio-Rad Laboratories, Inc.) with GAPDH as the internal reference gene: Forward primer (10 μM), 0.5 μl; reverse primer (10 μM), 0.5 μl; dNTP (2.5 mM), 2 μl; Ex Taq, 0.25 μl; 10X Ex Taq E buffer, 2.5 μl; cDNA, 1 μl; ddH_2O, ≥25 μl. Temperature conditions: 94˚C for 4 min; 30 cycles at 94˚C for 30 sec, 56˚C for 30 sec, 72˚C for 25 sec; 72˚C for 4 min and 4˚C for 4 min. iii) RT-qPCR: cDNA, 4 μl; forward primer (100 μM), 0.4 μl; reverse primer (100 μM), 0.4 μl; SYBR Green/Fluorescein qPCR Master Mix (2X), 10 μl; H_2O, 5.2 μl.

Statistical analysis. Experiments were repeated three times. Data are expressed as the mean ± standard deviation. SPSS 19.0 (IBM Corp.) was used for statistical analysis. Comparisons between different groups were performed by one-way analysis followed by the Student-Newman-Keuls test. $P<0.05$ was considered to indicate a statistically significant difference.

Result

Pathological alterations in intestinal tissues. In group A, aligned mucosal epithelium and morphological structural integrity were observed without any histopathological abnormalities. In group B, disordered alignment of the epithelium was detected and villi were obviously swollen; in addition, necrosis of the intestinal mucosa was observed. Conversely, group C exhibited reduced histopathological damage compared with group B (partially swollen villi and partial mucosal necrosis). Compared with group C, group D exhibited reduced histopathological damage, which presented as mild mucosal necrosis without any swollen villi (Fig. 1).

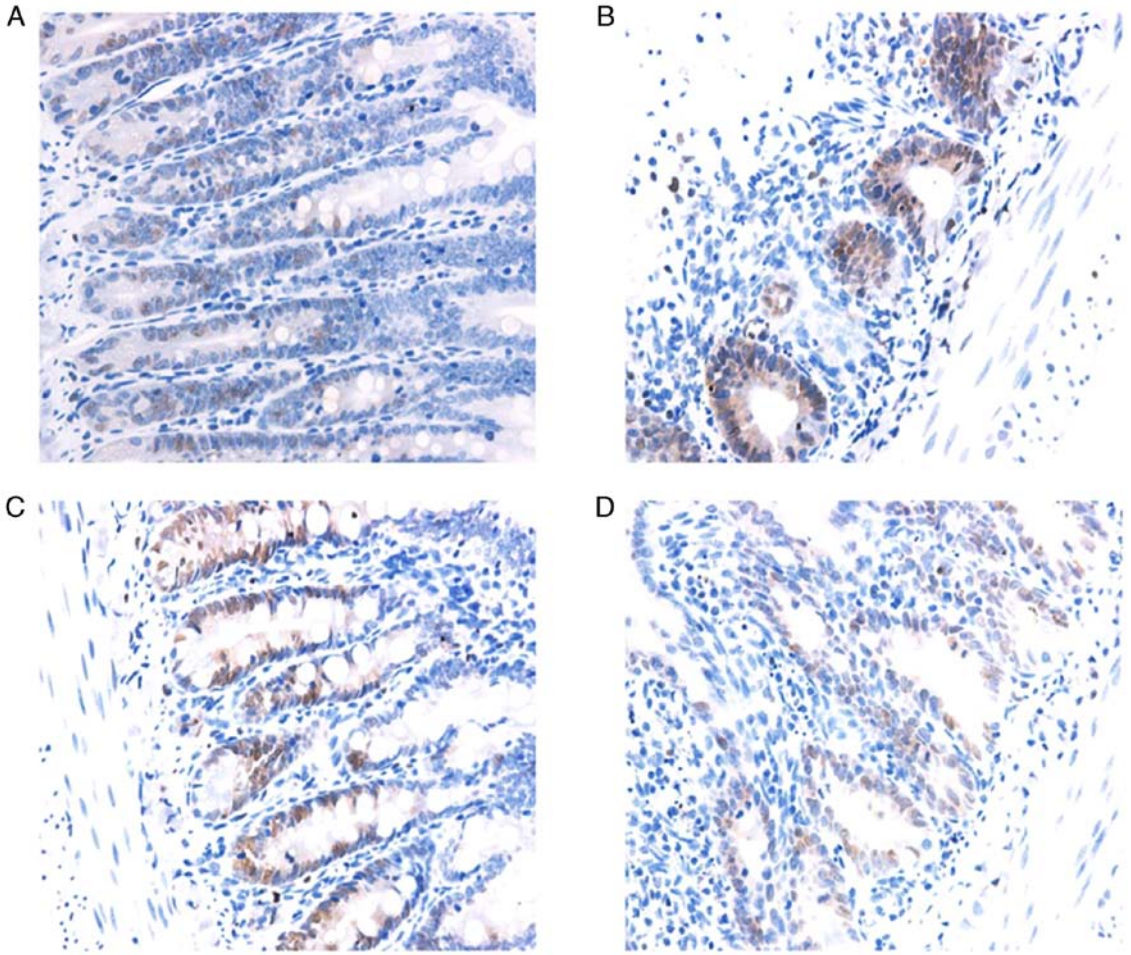

Figure 2. Apoptosis of intestinal mucous epithelial cells following intestinal ischemia-reperfusion and GYY4137 intervention. Group A, sham-surgery group; Group B, the IIR group; Group C, rats with IIR that were administered an abdominal injection of low-dose GYY4137 (40 mg/kg); and Group D, rats with IIR that were administered high-dose GYY4137 (80 mg/kg). Magnification, x400.

Table I. MDA and SOD levels following intestinal ischemia-reperfusion and GYY4137 intervention.

Group	No.	MDA (nmol/mg)	SOD (U/mg)
A	10	2.83±0.36	135.37±3.34
B	10	9.23±0.78[a]	76.45±1.39[a]
C	10	4.97±0.45[b]	95.13±1.64[a,b]
D	10	3.51±1.05[c]	115.13±2.54[a-c]

[a]P<0.05 vs. group A; [b]P<0.05 vs. group B; [c]P<0.05 vs. group C. MDA, malondialdehyde; SOD, superoxide dismutase.

Table II. AI of intestinal mucous epithelial cells.

Group	No.	AI (%)
A	10	4.53±0.28
B	10	21.73±1.17[a]
C	10	9.53±0.96[a,b]
D	10	6.53±0.76[a-c]

[a]P<0.05 vs. group A; [b]P<0.05 vs. group B; [c]P<0.05 vs. group C. AI, apoptotic index.

MDA content and SOD activity. In group B, MDA content was markedly increased compared with in group A, whereas it was markedly decreased in groups C and D compared with in group B (P<0.05). Furthermore, in group B, SOD activity was significantly decreased compared with in group A, whereas it was markedly increased in groups C and D compared with in group B (P<0.05; Table I).

Apoptosis of intestinal mucous epithelial cells. Epithelial cells in group B had significantly increased AI compared with in group A (P<0.05). However, in groups C and D, AI of epithelial cells was significantly decreased compared with in group B (P<0.05; Table II and Fig. 2).

Expression of caspase-3 and Bax, as determined by immunohistochemistry. A marked reduction in caspase-3 and Bax expression was identified in groups C and D compared with in group B (Fig. 3).

Protein expression levels of caspase-3, Bax and Bcl-2, as determined by western blot analysis. Group B exhibited a

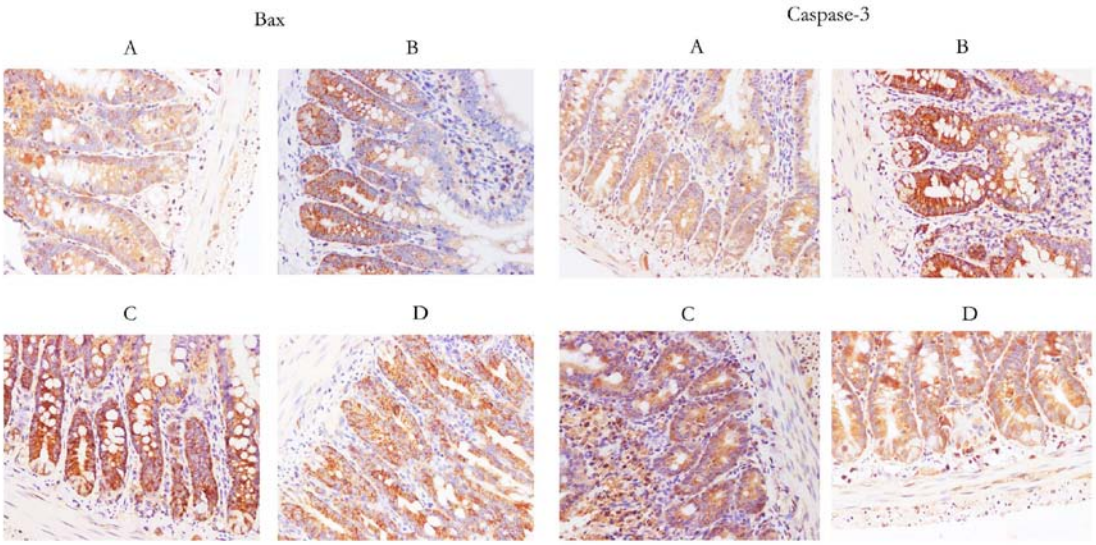

Figure 3. Protein expression of caspase-3, and Bax following intestinal ischemia-reperfusion and GYY4137 intervention, as determined by immunohistochemistry. Group A, sham-surgery group; Group B, the IIR group; Group C, rats with IIR that were administered an abdominal injection of low-dose GYY4137 (40 mg/kg); and Group D, rats with IIR that were administered high-dose GYY4137 (80 mg/kg). Magnification, x100.

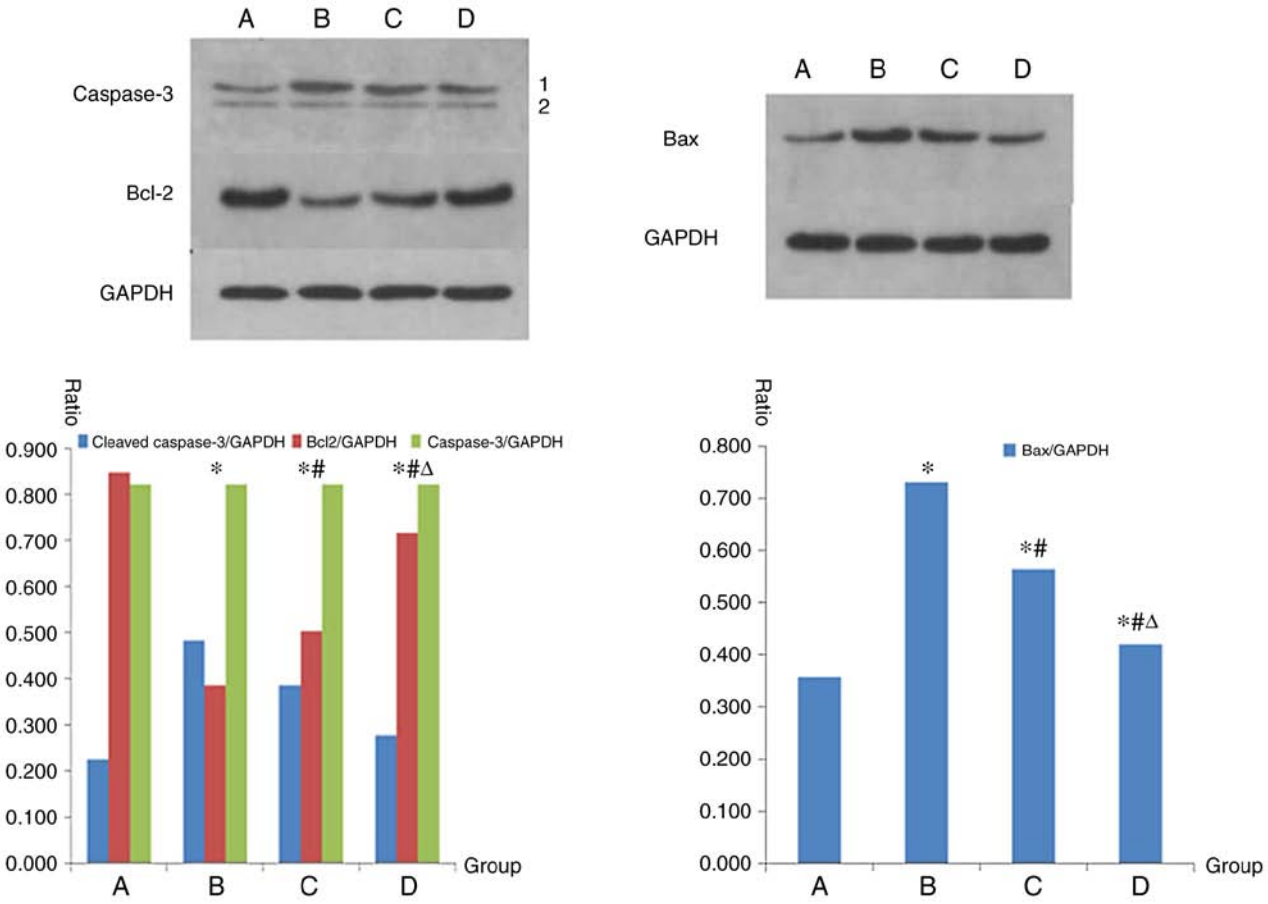

Figure 4. Protein expression of caspase-3, Bax and Bcl-2 following intestinal ischemia-reperfusion and GYY4137 intervention, as determined by western blotting. Band 1, cleaved caspase-3; band 2, total caspase-3. *P<0.05 vs. group A; #P<0.05 vs. group B; ΔP<0.05 vs. group C.

marked increase in the protein expression levels of Bax and cleaved caspase-3 compared with in group A. Compared with in group B, in groups C and D, protein expression levels of Bax and cleaved caspase-3 were decreased. Statistically significant differences were detected between the groups (P<0.05). Conversely, in group B, the protein expression levels of Bcl-2 were markedly decreased compared with in group A, whereas the protein expression levels of Bcl-2 were increased in groups

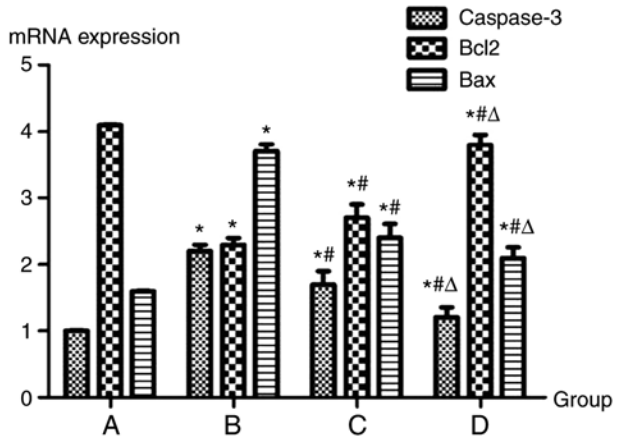

Figure 5. mRNA expression levels of caspase-3, Bax and Bcl-2 after intestinal ischemia-reperfusion and GYY4137 intervention. *P<0.05 vs. group A; #P<0.05 vs. group B; ΔP<0.05 vs. group C.

C and D compared with in group B. Statistically significant differences between the groups were detected (P<0.05; Fig. 4).

mRNA expression levels of caspase-3, Bax and Bcl-2. Group B exhibited a marked increase in the mRNA expression levels of Bax and caspase-3 compared with in group A. Compared with in group B, groups C and D exhibited decreased mRNA expression of Bax and caspase-3. Statistically significant differences between the groups were detected (P<0.05). Conversely, in group B, the mRNA expression levels of Bcl-2 were markedly decreased compared with in group A, whereas in groups C and D, the mRNA expression levels of Bcl-2 were increased compared with in group B. Statistically significant differences were detected between the groups (P<0.05; Fig. 5).

Discussion

GYY4137 is a water-soluble, slow-releasing H_2S donor, which may activate endothelial cells to secrete growth factors, promote the proliferation of smooth muscle cells and reduce oxidative stress. Previous studies have indicated that GYY4137 exerts anti-apoptotic and anti-inflammatory effects that reduce hepatocellular and brain cell injury (12-15). In accordance with previous studies, the present study demonstrated that pretreatment with GYY4137 had a marked protective effect on intestinal tissues subjected to IR.

MDA, as the final product of lipid peroxide decomposition, is frequently used to evaluate the extent of cellular damage under oxidative stress. In addition, SOD has an important role in cell growth and differentiation, and its antioxidant capacity may attenuate the damage associated with IIR (14-17). The results of the present study indicated a decreased MDA content and enhanced SOD activity in the groups with GYY4137 intervention, which may lead to attenuated oxidative stress damage following IIR.

As a normal physiological phenomenon, apoptosis is a cell death program regulated by genetic mechanisms (16,18). Due to consumption of energy, generation of a large number of active metabolites and activation of the mitochondrial signal transduction pathway, IIR may induce massive cell apoptosis and tissue damage (16,17,19). In the process of IIR injury, the increased level of oxidative stress may further induce abnormal apoptosis of mucosal epithelial cells (18,20). Caspase-3 is a key mediator enzyme of apoptosis and a marker of the irreversible phase of apoptosis (18,19,21). The pro-apoptotic factor Bax and anti-apoptotic factor Bcl-2 are a pair of closely linked apoptotic genes (20-23). In the present study, compared with in group A, AI was significantly increased in group B (IIR group), whereas cell apoptosis in groups C and D was significantly reduced compared with in group B. The protein and mRNA expression levels of Bax and caspase-3 were significantly higher in group B than in group A. In the groups with GYY4137 intervention (groups C and D), the mRNA expression levels of Bax and caspase-3 were reduced compared with in group B. The decrease was more obvious in group D than in group C, and a statistically significant difference was identified among the groups. Conversely, the protein and mRNA expression levels of Bcl-2 exhibited the opposite trend to those of caspase-3 and Bax.

Regarding the strengths and limitations of the present study, the experiment focused on the effects of GYY4137 on IIR. It was performed *in vitro* without any *in vivo* studies, and the regulation of upstream and downstream signaling pathways was not studied in depth, which represents a limitation.

In conclusion, the present study indicated that, compared with in the IIR group, GYY4137 intervention significantly decreased the expression of Bax and caspase-3, and AI, whereas the expression of Bcl-2 was significantly increased. It may therefore be suggested that GYY4137 has a protective effect against IIR injury.

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Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Authors' contributions

NC wrote the manuscript and analyzed data. HL performed data analysis. YZ designed the study. All authors read and approved the final manuscript.

Ethics approval and consent to participate

Not applicable.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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