Original Paper

Cardioprotective Effects of Combined Therapy with Hyperbaric Oxygen and Diltiazem Pretreatment on Myocardial Ischemia-Reperfusion Injury in Rats

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Key Words

Hyperbaric oxygen • Diltiazem • Myocardial ischemia-reperfusion injury • Nitric oxide • Apoptosis

Abstract

Background/Aims: In this study, we examined whether the combination of hyperbaric oxygen (HBO) and diltiazem therapy provided a cardioprotective effect on myocardial ischemia-reperfusion injury (MIRI) rat model. **Methods:** Sixty healthy Sprague-Dawley rats were randomly divided into sham, IR, diltiazem (5 mg/kg), HBO (0.25 MPa, 60 min) and combination therapy (HBO plus diltiazem) groups. MIRI model was established by ligating the left anterior descending for 30 min, followed by 60 min of reperfusion. **Results:** The results show that HBO and diltiazem preconditioning significantly improves cardiac function and myocardial infarction area, increases nitric oxide, endothelial nitric oxide synthase and ATPase (Na⁺-K⁺-ATPase and Ca²⁺-Mg²⁺-ATPase) activity and decreases levels of oxygen stress, myocardial enzymes and endothelin-1. Notably, HBO and diltiazem preconditioning significantly increased Bcl-2 protein expression and decreased Bax protein and caspase-3 mRNA expression. **Conclusions:** These data indicate that combination therapy protected against heart MIRI by reducing oxygen stress damage, correcting energy metabolism, improving endothelial function and inhibiting cell apoptosis.

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Introduction

Despite the progressive implementation of preventive therapies and better control of risk factors, acute myocardial infarction remains a significant health problem, representing a major contributor to mortality/morbidity worldwide [1]. The pathogenesis of myocardial ischemia-reperfusion injury (MIRI) is complex. Previous studies have shown that oxygen free radicals, calcium overload, vascular endothelial dysfunction, inflammation and apoptosis are all involved in the occurrence of MIRI [2-4]. Sources of reactive oxygen species (ROS) include the mitochondrial electron transport chain (ETC); enzymes such as xanthine oxidase, nitric oxide synthase and nicotinamide adenine dinucleotide phosphate (NADPH) oxidase; and leukocyte infiltration [5, 6]. ROS leads to damage of cell membranes, protein, DNA, and mitochondrial membrane potential ($\Delta\Psi_m$) [7]. The latter causes uncoupling and subsequently leads to hyperactivity of the ETC due to inefficient adenosine triphosphate (ATP) production [8, 9]. Reduced ATP causes inhibition of the ATP-dependent sodium-potassium pump ($\text{Na}^+\text{-K}^+\text{-ATPase}$), which further contributes to myocardial sodium loading [10]. Intracellular calcium overload is also the most important factor in the induction of apoptosis in MIRI by activating calcium-dependent proteases [11, 12]. Additionally, vascular endothelial dysfunction are related to a decrease in the bioavailability of nitric oxide (NO) as well as to augmented endothelin-1 (ET-1) synthesis, release, or activity [13].

Hyperbaric oxygen (HBO) therapy is routinely used to treat various ischemia illnesses, such as stroke, myocardial infarction and neonatal hypoxia ischemia in China. However, the underlying mechanism is under investigation [14, 15] and none of these uses for HBO is widely accepted by major hyperbaric craft groups like the Undersea And Hyperbaric Medical Society or the European Underwater and Baromedical Society. Increasing evidence suggests that HBO preconditioning alleviates ischemic brain injury by upregulating antioxidant enzymes, such as superoxide dismutase (SOD) and glutathione peroxidase (GSH-Px), and inhibiting neuroinflammation factor cyclooxygenase-2 expression [14] and neuronal apoptotic pathways [16]. In addition, HBO therapy may attenuate hypoxia in marginally perfused tissue (sometimes referred to as the “ischemic penumbra”), and this may be useful in avoiding cell death and restoring function [17]. Recent research suggests that HBO prevents myocardium from ischemia injury by suppression of apoptotic pathways (blockage of caspase-3 activity and protection against Bax/Bcl-2-mediated apoptosis) [18] and protects MIRI via PI3K/Akt/Nrf2-dependent antioxidant defensive system in animal models [19]. Moreover, for people with acute coronary syndrome, there is some evidence from clinical trial to suggest that HBO is associated with a reduction in the risk of death, the volume of damaged muscle and the risk of major adverse cardiac events [20]. Additionally, as a calcium channel blocker, diltiazem was used as reference drug. Its beneficial effect included reducing the infarct size and lipid peroxidation, increasing myocardial performance following MIRI [21]. Several clinical trials did demonstrate the cardioprotective effect of diltiazem in the reduction of MIRI [22, 23]. Our previous study exhibited that diltiazem could prevent oxidative stress-related alteration and attenuated apoptosis in MIRI [24]. However, the effect of HBO combined with diltiazem therapy on a MIRI is still understood.

Here, for the first time, we investigated the effect of this combination therapy on a MIRI model that is induced by ligation of the left anterior descending (LAD) artery as well as the potential mechanisms that are involved.

Material and Methods

Drugs and reagents

Diltiazem hydrochloride was purchased from Shanghai Sine Wanxiang Pharmaceutical Co., Ltd. (Shanghai, China). Evans blue and tetrazolium chloride (TTC) were purchased from Shanghai Chemical Reagent Co., Ltd. (Shanghai, China). SOD, GSH-Px, Methane Dicarboxylic Aldehyde (MDA), NO, endothelial nitric oxide synthase (eNOS), $\text{Na}^+\text{-K}^+\text{-ATPase}$ and $\text{Ca}^{2+}\text{-Mg}^{2+}\text{-ATPase}$ activity assay kits were supplied by Nanjing Jianchen Bioengineering Institute (Nanjing, China). Creatine kinase (CK), creatine kinase-MB

isoenzyme (CK-MB), lactate dehydrogenase (LDH) and lactate dehydrogenase-1 isoenzyme (LDH1) were purchased from Randox Laboratories Ltd. (England). Rabbit anti-Bax, rabbit anti-Bcl-2, and biotinylated goat anti-rabbit immunoglobulin G were the products of Beijing Zhongshan Jinqiao Biological Technology Co., Ltd. (Beijing, China).

Animals

The research was conducted according to protocols approved by our institutional ethical committee (approval no.: 20110501202) and US guidelines (NIH publication No. 85-23, revised in 1985) for laboratory animal use and care. Sprague-Dawley (SD) rats of both sexes, weighing 180 - 220 g, were provided by the Experimental Animal Center of Guangxi Medical University (Certificate No. SYXK 2009-0002). The animals were housed under controlled conditions at $25 \pm 2^\circ\text{C}$, with a relative humidity of $60 \pm 10\%$. Food and water were available ad libitum. The investigation conforms to the Guide for the Care and Use of Laboratory Animals published by the US National Institute of Health (NIH Publication No. 85-23, revised 1996).

Experimental design

After 1 week of acclimatization to laboratory conditions, the animals were randomly (random number table method) divided into five groups as follows:

Sham group (n = 23): SD rats were intragastrically administered normal saline with 2 mL/kg body weight for 14 days + subjected to thoracotomy and encircling of the LAD artery with a suture but not ligation;

IR group (n=23): SD rats were intragastrically administered normal saline with 2 mL/kg body weight for 14 days + ligation of the LAD artery for 30 min and reperfusion for 1 h;

DIL group (n=23): SD rats were intragastrically administered diltiazem hydrochloride (diluted with normal saline) with 5 mg/kg body weight for 14 days + ligation of the LAD artery for 30 min and reperfusion for 1 h;

HBO group (n=23): SD rats were administered hyperbaric oxygen + intragastrically administered distilled water with 2 mL/kg body weight for 14 days + ligation of the LAD artery for 30 min and reperfusion for 1 h;

Combination therapy group (n=23): SD rats were administered hyperbaric oxygen + intragastrically administered diltiazem hydrochloride with 5 mg/kg body weight for 14 days + ligation of the LAD artery for 30 min and reperfusion for 1 h.

Blood samples were drawn before sacrifice. Then necrosis size was measured immediately after sacrifice. Meanwhile, myocardial tissue samples were harvested.

General surgical procedure

Ischemia-reperfusion injury was inflicted by occlusion of the left anterior descending coronary artery and then reperfusion as previously described [25]. Briefly, the rats were anesthetized using sodium pentobarbital (30 mg/kg, i.p.) and restrained in the supine position. The animals were intubated and mechanically ventilated (Shanghai Alcott Biotech Co., Ltd., respiration rate 70 min^{-1} , respiration-to-expiration ratio 1:2, and tidal volume 50 mL/kg) during the surgical procedures. The chest was opened through the fourth intercostal space on the left side, and the ribs were gently retracted to expose the heart. After opening the pericardium, the LAD artery was ligated using a 5-0 silk suture for 30 min and released to allow reperfusion for 1 h by cutting the silk suture. In sham-operated rats, the suture was placed encircling the LAD artery, but no ligation occurred. Before and during the operation, the electrocardiogram of each rat was recorded.

Hyperbaric therapies

The animals were placed in hyperbaric chambers (Yantai Hongyuan Co., Ltd.) as previously described [16]. Then, the pressure was increased to 0.25 MPa at a rate of 100 kPa/min, and each treatment lasted 60 min pure oxygen. Decompression was performed at a uniform rate over 10 min. HBO treatment was performed once daily for 14 consecutive days.

Hemodynamics

A polyethylene catheter was passed through the right carotid arteries into the left ventricle. The pressure transducer was connected to an MS 4000 biological signal quantitative analytical system (Longfeida

Technology Co., Ltd.), and the hemodynamic parameters including heart rate (HR), left ventricular systolic pressure (LVSP), left ventricular end-diastolic pressure (LVEDP), maximal rate of the left ventricular systolic pressure (+dp/dtmax) and maximal rate of the left ventricular diastolic pressure (-dp/dtmax) were recorded [26, 27]. Hemodynamic parameters were measured at baseline, after 30 min of ischemia and after 60 min of reperfusion.

Measurement of myocardial infarction area

At the end of 1 h reperfusion, myocardial infarct size of five rats in each group was evaluated by Evans blue-TTC staining [28, 29]. The coronary artery was religated and 0.5 g/L Evans blue solution 1.5 mL was injected via the thoracic aorta. The hearts were washed with saline water and stored at -70°C. Frozen hearts were slice into parallel 1- to 1.5-mm-thick sections. The slices were incubated in TTC (1 % in 0.1 mol/L phosphate buffer, pH 7.4) for 15 min at 37°C. Then the slices were washed with pre-cooled saline and fixed with 10 % formalin solution. The infarction area was not stained (pale), the normal area was stained blue, the ischemic area was stained red, and the risk area exhibited a mix of red staining and unstained tissue. Three regions were cut and weighed (weight of infarct, WI; weight of risk, WR; weight of left ventricular, WLW), and the percentages of WI/WLV and WR/WLV were used to reflect the degree of myocardial injury.

Detection of myocardial enzyme levels, lipid peroxidation, NO and eNOS levels in serum

Blood samples were collected from the abdominal aorta after reperfusion for 60 min and were centrifuged for 3500 rpm at 4°C for 10 min. Heart muscle damage indicators such as CK, CK-MB, LDH and LDH1 were determined by a 200FR NEO automatic biochemical analyzer (Toshiba, Japan). SOD, GSH-Px, MDA, NO and eNOS were measured using commercial kits according to the manufacturer's instructions [30].

Determination of Na⁺-K⁺-ATPase and Ca²⁺-Mg²⁺-ATPase activity in myocardial infarct tissue

At the end of reperfusion, the cardiac lesion tissue was isolated, weighted and homogenized in ice-cold physiological saline (10% w/v) to produce a 5% homogenate. The contents of Na⁺K⁺-ATPase and Ca²⁺Mg²⁺-ATPase were measured using a commercial kit according to the manufacturer's instructions.

Histological analysis

The myocardial infarct tissues were isolated and processed by routine histology procedures [31]. At the end of reperfusion, the myocardial tissue samples were fixed in 4% buffered paraformaldehyde solution and then embedded in paraffin. Next, 4-μm-thick sections were stained with hematoxylin and eosin (HE) or incubated with various antibodies for immunohistochemical examination. The degree of heart damage was analyzed by the method of [32].

Immunohistochemistry of Bax and Bcl-2 expression

Immunohistochemical procedures were conducted according to the method of a previous study [16]. Briefly, the waxed and dehydrated specimens were incubated with primary antibody overnight at 4°C. Following three washes with 0.1 mol/L PBS for 3 min each, the sections were incubated with biotinylated goat anti-rabbit immunoglobulin G at 30°C for 25 min. After washing three times with 0.1 mol/L PBS for 3 min each, the specimens were incubated with a streptavidin-biotin complex at 30°C for 20 min. Then, they were rinsed five times in 0.1 mol/L PBS for 3 min, incubated with diaminobenzidine for 15 min at room temperature, counterstained with hematoxylin, cleared, mounted and examined.

The areas of Bcl-2- and Bax-positive cells were analyzed in five independent sections from all the experimental groups and were quantified using a microscope at a magnification of 400× (Olympus, Germany). The region of interest was captured using a camera and analyzed using Imagepro-Plus software.

Analysis of caspase-3 and ET-1 mRNA by Real Time PCR

At the end of 1 h reperfusion, the heart was promptly removed, washed with cold saline, frozen in liquid nitrogen and preserved at -80°C. PCR was performed by standard methods, as described in a previous report [33, 34]. Total RNA was extracted from the ischemic myocardial tissue using Trizol reagent (Invitrogen). The 25 μL reaction volume included 2 μg of total RNA. cDNA was synthesized from total RNA using reverse transcriptase (Takara) by incubating at 37°C for 1 h and then at 95°C for 10 min. As a template, the cDNA was amplified using the 7300 Real Time PCR System (Applied Biosystems) with primers. Then, the levels

of caspase-3 and ET-1 mRNA expression were determined. The parallel amplification of rat β -actin was performed for reference. The sequences of the primers used in this study were as follows: caspase-3 (191 bp, Forward primer: 5' GAA AGC ATC CAG CAA TAG GC 3', Reverse primer: 5' TGA GTT CCT TCC TTT CTT TGT G C 3'); ET-1 (394 bp, Forward primer: 5' TGG CTT TCC AAG GAG CTC 3', Reverse primer: 5' GCT TGG CAG AAA TTC CAG 3'); and β -actin (240 bp, Forward primer: 5' AAC CCT AAG GCC AAC CGT GAA AAG 3', Reverse primer: 5' TCA TGA GGT AGT CTG TCA GGT 3'). Levels of mRNA expression were determined using the 7300 Real-Time PCR System SDS software (Applied Biosystems) according to the $2^{-\Delta\Delta C_t}$ method.

Statistical analysis

Hemodynamic parameters were performed using two-way analysis of variance (ANOVA), with treatment as the between subject variable and time as the within subject variable. Histological results were analyzed by Kruskal-Wallis H test. The remaining data were analyzed by one-way ANOVA followed by Tukey-Kramer's post hoc test. The level of significance was set at $P < 0.05$.

Results

Effect of combination therapy on cardiac function

No significant differences were observed in the baseline of LVSP, LVEDP, + dp/dtmax and -dp/dtmax between any of the groups ($F = 1.203$, $P = 0.334$; $F = 1.620$, $P = 0.200$; $F = 1.359$, $P = 0.276$; $F = 2.495$, $P = 0.069$). Two-way ANOVA revealed significant effects of time [$F = 425.92$, $P = 0.000$], treatment [$F = 54.642$, $P = 0.000$], and time $\times$ treatment [$F = 19.521$, $P = 0.000$]. The post hoc analysis revealed that precondition with diltiazem, HBO or HBO plus diltiazem partially ameliorated the alterations in LVSP, LVEDP, + dp/dtmax and -dp/dtmax at myocardial ischemia 30 min and reperfusion 60 min (Table 1, $P < 0.05$). Interestingly, pretreatment with diltiazem or HBO plus diltiazem significantly reduced HR at baseline, ischemia 30 min and reperfusion 60 min compared with the IR group ($P < 0.05$). Precondition with HBO significantly reduced HR at reperfusion 60 min ($P < 0.05$).

Table 1. Hemodynamic parameters at baseline, at the end of ischemia and at the end of reperfusion. Note: HR, heart rate; LVSP, left ventricular systolic pressure; LVDEP, left ventricular end-diastolic pressure; +dp/dtmax, maximal rate of left ventricular systolic pressure; -dp/dtmax, maximal rate of left ventricular diastolic pressure. The data are presented as the mean $\pm$ SD ($n = 15$). $\Delta P < 0.05$ compared with the sham group, * $P < 0.05$ compared with the IR group at the same time point

	Baseline	Ischemia 30 min	Reperfusion 60 min
HR (beats/min)			
Sham	407.60 $\pm$ 9.05	400.60 $\pm$ 5.75	397.87 $\pm$ 9.87
IR	396.27 $\pm$ 16.72	346.42 $\pm$ 12.29 Δ	336.53 $\pm$ 14.89 Δ
DIL	357.88 $\pm$ 13.36 *	306.70 $\pm$ 12.93 *	280.72 $\pm$ 15.27 *
HBO	373.32 $\pm$ 13.09	326.52 $\pm$ 10.47	305.47 $\pm$ 12.07 *
HBO + DIL	364.77 $\pm$ 15.32 *	317.72 $\pm$ 13.84 *	296.27 $\pm$ 16.41 *
LVSP (mmHg)			
Sham	405.74 $\pm$ 16.57	402.97 $\pm$ 15.56	398.34 $\pm$ 13.20
IR	384.76 $\pm$ 25.39	302.74 $\pm$ 16.48 Δ	203.07 $\pm$ 20.45 Δ
DIL	387.41 $\pm$ 14.95	330.74 $\pm$ 10.21 *	265.74 $\pm$ 16.46 *
HBO	385.37 $\pm$ 17.74	317.10 $\pm$ 14.13	230.07 $\pm$ 23.46
HBO + DIL	393.06 $\pm$ 21.04	341.10 $\pm$ 11.27 *	296.87 $\pm$ 12.92 *
LVEDP (mmHg)			
Sham	52.14 $\pm$ 7.33	54.01 $\pm$ 9.18	52.21 $\pm$ 8.14
IR	46.89 $\pm$ 7.81	92.61 $\pm$ 5.24 Δ	111.25 $\pm$ 10.87 Δ
DIL	49.68 $\pm$ 6.04	77.61 $\pm$ 8.98 *	83.74 $\pm$ 7.45 *
HBO	55.15 $\pm$ 7.93	68.19 $\pm$ 6.76 *	76.27 $\pm$ 7.31 *
HBO + DIL	46.52 $\pm$ 5.56	63.54 $\pm$ 4.22 *	68.69 $\pm$ 1.89 *
+dp/dtmax (mmHg/s)			
Sham	10648.2 $\pm$ 415.7	10982.0 $\pm$ 789.2	10822.8 $\pm$ 665.0
IR	10271.7 $\pm$ 890.7	6891.2 $\pm$ 706.2 Δ	4588.9 $\pm$ 550.7 Δ
DIL	10913.6 $\pm$ 836.1	8013.9 $\pm$ 643.0 *	5972.6 $\pm$ 550.1 *
HBO	11292.6 $\pm$ 1081.0	7881.9 $\pm$ 751.1	5544.0 $\pm$ 571.9
HBO + DIL	10970.3 $\pm$ 628.3	8781.5 $\pm$ 698.2 *	7475.0 $\pm$ 815.5 *
-dp/dtmax (mmHg/s)			
Sham	-11838.0 $\pm$ 1203.0	-10668.1 $\pm$ 940.1	-10951.8 $\pm$ 747.7
IR	-10753.5 $\pm$ 645.3	-6477.8 $\pm$ 702.2 Δ	-4811.6 $\pm$ 422.9 Δ
DIL	-11229.2 $\pm$ 1014.3	-7815.5 $\pm$ 967.4	-6213.4 $\pm$ 859.2 *
HBO	-11363.4 $\pm$ 930.9	-7387.3 $\pm$ 656.6	-6324.0 $\pm$ 497.1 *
HBO + DIL	-10274.3 $\pm$ 739.2	-7791.7 $\pm$ 499.0 *	-6668.5 $\pm$ 489.3 *

Fig. 1. Effect of HBO pretreatment on myocardial infarct size. Images represent the infarction area and risk area of myocardial tissue in the sham, IR, DIL, HBO and combination therapy (HBO + diltiazem) groups, respectively. Infarction area is pale [present as (WI/WLV) %], risk area is red [present as (WR/WLV) %] and the normal tissue is blue. The results are presented as the mean $\pm$ SD (n = 5). $^{\Delta}P < 0.05$ compared with sham group, $^*P < 0.05$ compared with the IR group, $^{\#}P < 0.05$ compared with the DIL group. In the sham group, no infarction was observed in the myocardium.

Effect of combination therapy on myocardial infarct size

To quantify the results, risk size, defined as a ratio of WR to WL (WR/WL), was assessed first. No significant differences were observed in the risk area between any of the groups (Fig. 1, sham: 95% CI, 35.01-45.39; IR: 95% CI, 35.55-52.64; DIL: 95% CI, 34.86-49.61; HBO: 95% CI, 36.20-51.24; DIL + HBO: 95% CI, 35.90-51.86; $F = 0.378$, $P = 0.822$). The infarction area (presented as WI/WL) was increased in the IR group compared with the sham group (22.80 ± 4.39 % vs. 0.00 ± 0.00 %; sham: 95% CI, 0-0; IR: 95% CI, 17.34-28.26; $F = 42.728$, $P = 0.000$). However, pretreatment with HBO or HBO plus diltiazem reduced the infarction size to 15.12 ± 2.63 % and 12.24 ± 2.42 % (HBO: 95% CI, 11.85-1.39; DIL + HBO: 95% CI, 9.24-15.24; $P = 0.001$, $P = 0.000$, vs. IR).

Effect of combination therapy on serum lipid peroxidation and myocardial enzyme activities in acute MIRI rats

In this study, the content of MDA increased almost 2.1-fold and endogenous antioxidative enzymes, such as SOD and GSH-Px, were markedly decreased in the IR group by 50.33 % and 57.85 % compared with the sham group (MDA: $F = 15.692$, $P = 0.000$; SOD: $F = 35.594$, $P = 0.000$; GSH-Px: $F = 28.531$, $P = 0.000$). However, the MDA concentrations were reduced by 36.08 %, and SOD and GSH-Px activities were increased 1.5 times and 2.05 times in the combination therapy group than in the IR group ($P = 0.000$; $P = 0.000$; $P = 0.000$). Additionally, the levels of SOD and GSH-Px were 1.16 times and 1.1 times higher in the combination therapy group than that in the DIL group (Fig. 2, $P = 0.001$; $P = 0.000$).

To examine how HBO influences myocardial injury induced by MIRI, the activities of CK, CK-MB, LDH and LDH1 were measured at the end of reperfusion. As indicated in Fig.

Fig. 2. Effect of HBO pretreatment on serum lipid peroxidation in MIRI rats. The results are presented as the mean $\pm$ SD ($n = 10$). $\Delta P < 0.05$ compared with sham group, $* P < 0.05$ compared with the IR group, $\# P < 0.05$ compared with the DIL group.

Fig. 3. Effect of HBO pretreatment on myocardial enzyme activities in MIRI rats. The results are presented as the mean $\pm$ SD ($n = 10$). $\Delta P < 0.05$ compared with sham group, $* P < 0.05$ compared with the IR group, $\# P < 0.05$ compared with the DIL group.

3, the serum levels of CK, CK-MB, LDH and LDH1 were significantly higher in the IR group than in the sham group (3.19-fold, 3.13-fold, 2.44-fold and 4.47-fold respectively; $F = 26.838$, $P = 0.000$; $F = 41.545$, $P = 0.000$; $F = 44.642$, $P = 0.000$; $F = 55.299$, $P = 0.000$), which were recovered in the DIL, HBO or combination therapy group (all $P < 0.05$). Moreover, the serum CK, CK-MB, LDH and LDH1 concentrations was lower in combination therapy group than that in the DIL group (27.76 %, 31.73 %, 27.66 % and 21.43 % respectively; $P = 0.000$; $P = 0.000$; $P = 0.000$; $P = 0.001$).

Effect of combination therapy on serum NO and eNOS production during acute MIRI in rats

As shown in Fig. 4, serum NO and eNOS activities were significantly decreased following MIRI surgery, compared with the sham group (NO: 56.36 ± 8.93 vs. 21.91 ± 4.63 , $F = 19.390$,

Fig. 4. Effect of HBO pretreatment on serum NO and eNOS levels in MIRI rats. The results are presented as the mean $\pm$ SD ($n = 10$). $^{\Delta}P < 0.05$ compared with sham group, $*P < 0.05$ compared with the IR group, $^{\#}P < 0.05$ compared with the DIL group.

Fig. 5. Effect of HBO pretreatment on myocardial tissues Na⁺-K⁺-ATPase and Ca²⁺-Mg²⁺-ATPase levels in MIRI rats. The results are presented as the mean $\pm$ SD ($n = 5$). $^{\Delta}P < 0.05$ compared with sham group, $*P < 0.05$ compared with the IR group, $^{\#}P < 0.05$ compared with the DIL group.

$P = 0.000$; eNOS: 15.4 ± 1.37 vs. 6.08 ± 1.04 , $F = 73.622$, $P = 0.000$). By pretreating with HBO plus diltiazem, the NO and eNOS activities were increased compared with the IR group (NO: 43.94 ± 8.92 , $P = 0.000$; eNOS: 11.94 ± 0.61 , $P = 0.000$). The similarly changes of NO and eNOS levels were observed in the HBO group (NO: 32.46 ± 3.89 , $P = 0.024$; eNOS: 10.37 ± 1.18 , $P = 0.000$). However, no significant difference was observed between the DIL group and the IR group with regard to NO level (28.03 ± 9.73 , $P = 0.176$).

Effect of combination therapy on Na⁺-K⁺-ATPase and Ca²⁺-Mg²⁺-ATPase activities

The activities of Na⁺-K⁺-ATPase and Ca²⁺-Mg²⁺-ATPase in myocardial tissues were investigated in response to MIRI. Both of the ATPase activities were significantly reduced in the IR group compared with the sham group (Na⁺-K⁺-ATPase: 2.58 ± 0.43 vs. 5.02 ± 0.57 , $F = 21.832$, $P = 0.000$; Ca²⁺-Mg²⁺-ATPase: 1.71 ± 0.34 vs. 4.12 ± 0.42 , $F = 25.356$, $P = 0.000$). However, these changes were reversed by HBO or HBO plus diltiazem pretreatment (Na⁺-K⁺-ATPase: 3.66 ± 0.58 , $P = 0.004$, Ca²⁺-Mg²⁺-ATPase: 3.55 ± 0.61 , $P = 0.000$; Na⁺-K⁺-ATPase: 4.82 ± 0.72 , $P = 0.000$, Ca²⁺-Mg²⁺-ATPase: 3.88 ± 0.65 , $P = 0.000$, respectively). The DIL group exhibited a significant increase in Ca²⁺-Mg²⁺-ATPase activity (2.52 ± 0.36 , $P = 0.008$) and no significant difference in Na⁺-K⁺-ATPase activity (2.82 ± 0.60 , $P = 0.484$) compared to the IR group (Fig. 5). In addition, the Na⁺-K⁺-ATPase activity was 1.71-fold higher in combination therapy group than that in the DIL group ($P = 0.000$).

Effect of combination therapy on histopathological alterations

As shown in Fig. 6, sham group rats showed a normal structure of the myocardium with uniform color, whereas IR group rats showed significant membrane damage of

Fig. 6. Histologic photographs (400 ×) and pathological grade of rats with myocardial ischemia-reperfusion injury. Grade 0: cardiomyocytes are normal, no abnormal changes including myocardial cell edema (or vacuoles). Grade I: myocardial swelling, limited myocardial cell necrosis with scattered distribution. Grade II: myofiber disruptions, focal necrosis. Grade III: myocardial structural disorder, large scope of myocardial necrosis with edema and infiltration of inflammatory cells. * $P < 0.05$, significantly different from the IR group.

cardiomyocytes with extensive edema, myonecrosis, hemorrhage, and inflammatory cell infiltration. Combination therapy group was significantly different from the IR group through myocardial pathological grades with decreasing myonecrosis, infiltration of inflammatory cells, extravasation of red blood cells and edema ($\chi^2 = 23.486$, $P = 0.012$).

Effect of combination therapy on apoptosis-related protein

Bcl-2 and Bax protein mainly accumulated in the cytoplasm. Immunohistochemical DAB coloration revealed diffuse distribution or brown granules in the cell cytoplasm. Compared to the sham group, IR group showed significantly down-regulated expression of Bcl-2 and up-regulated expression of Bax in the myocardial tissues (Bcl-2: 5.00 ± 0.89 vs. 8.00 ± 0.89 , $F = 6.805$, $P = 0.000$; Bax: 13.33 ± 1.63 vs. 6.50 ± 1.87 , $F = 17.447$, $P = 0.000$). HBO or HBO plus diltiazem pretreatment clearly alleviated these alterations (Bcl-2: 7.17 ± 1.17 , $P = 0.004$, Bax: 10.33 ± 1.21 , $P = 0.003$; Bcl-2: 7.67 ± 1.03 , $P = 0.001$, Bax: 8.17 ± 1.17 , $P = 0.000$, respectively) (Fig. 7). Moreover, the expression of Bax was decreased by 28.96 % in combination therapy group than that in the DIL group ($P = 0.001$).

The ratio of Bcl-2/Bax was lower in the IR group compared with the sham group (0.38 ± 0.07 vs. 1.30 ± 0.30 ; sham: 95% CI, 0.98-1.61; IR: 95% CI, 0.31-0.45; $F = 25.973$, $P = 0.000$). Interestingly, pretreatment with HBO or HBO plus diltiazem led to 1.84 to 2.23-fold increase in the ratio of Bcl-2/Bax (HBO: 0.38 ± 0.07 , 95% CI: 0.56-0.84, $P = 0.004$; HBO + DIL: 0.94 ± 0.07 , 95% CI: 0.87-1.01, $P = 0.000$). The diltiazem treatment was not associated with a significant difference in the Bcl-2 and Bax expression and the ratio of Bcl-2/Bax in comparison with the IR group ($P = 0.151$; $P = 0.056$; $P = 0.130$).

Effect of combination therapy on caspase-3 and ET-1 mRNA expression

The result of the real-time PCR analysis of caspase-3 and ET-1 mRNA in ischemic myocardial tissue of each group is shown in Fig. 8. It was obvious that the level of caspase-3

Fig. 7. Immunohistochemical results of cardiac muscle under a light microscope in rats (400 ×). Left: Representative photographs displaying the number of positive cells (Bax, and Bcl-2). Right: Calculation of positive neurons (Bax, and Bcl-2; cells/mm²). Each column represents the mean ± SD (n = 8). Statistical analyses were performed using Wilcoxon's test.

Fig. 8. Effect of HBO pretreatment on apoptosis-related protein and gene expression in MIRI rats. The results are presented as the mean ± SD (n = 5). ^Δ*P* < 0.05 compared with sham group, **P* < 0.05 compared with the IR group, #*P* < 0.05 compared with the DIL group.

and ET-1 mRNA was higher in the IR group than that in the sham group (caspase-3: 1.90 ± 0.14 vs. 0.79 ± 0.11 , $F = 69.577$, $P = 0.000$; ET-1: 1.45 ± 0.10 vs. 0.87 ± 0.09 , $F = 34.431$, $P = 0.000$), whereas its expression was decreased greatly in rats treated with diltiazem, HBO or HBO plus diltiazem compared with the IR group (caspase-3: 1.23 ± 0.16 , $P = 0.000$, ET-1: 1.18 ± 0.12 , $P = 0.000$; caspase-3: 1.03 ± 0.14 , $P = 0.000$, ET-1: 1.01 ± 0.09 , $P = 0.000$; caspase-3: 0.91 ± 0.07 , $P = 0.000$, ET-1: 0.96 ± 0.12 , $P = 0.000$, respectively). Moreover, the expression of caspase-3 and ET-1 were decreased by 26.01 % and 18.64 % in combination therapy group than that in the DIL group ($P = 0.000$; $P = 0.000$).

Discussion

MIRI can lead to myocardial apoptosis, necrosis, and arrhythmias [35]. In our present experiment, a successful rat model of MIRI was demonstrated by the ST segment, which was raised after the LAD artery ligation. Then, it gradually declined during reperfusion. The risk area in each group showed no significant difference compared with the model group, which could imply that the ligation portion of the procedure was basically consistent across the study group. Our results showed that pretreatment with HBO plus diltiazem improved cardiac function, attenuated levels of ROS, pro-apoptotic protein Bax and ET-1, increased levels of Na⁺-K⁺-ATPase, Ca²⁺-Mg²⁺-ATPase activities and anti-apoptotic protein Bcl-2, which was consistent with the decreasing myocardial infarct size and histopathological injuries.

In the present study, HR, LVSP, +dp/dtmax and -dp/dtmax in the IR group were lower, whereas the LVEDP was higher than those in the sham group, findings that are consistent with previous studies [36]. Nevertheless, except HR, the other hemodynamic parameters were reversed by HBO plus diltiazem pretreatment, indicating that when given prior to an insult resulting in MIRI, the combination therapy of HBO plus diltiazem may maintain better heart function than would otherwise be the case. Notably, both the diltiazem and combination therapy reduced HR at baseline, ischemia 30 min and reperfusion 60 min ($P < 0.05$), indicating that the two treatments reduced myocardial oxygen consumption during MIRI. Additionally, HBO treatment may have an effect on enhancing oxygen storage in myocardial tissues.

The oxidative stress events are recognized to participate in MIRI processes, ultimately leading to cell apoptosis and death, cell damage, and mitochondrial dysfunction [3, 37]. Following a period of ischemia, re-establishment of the oxygen supply will produce an excessive amount of ROS, which triggers oxidative stress and then induces MIRI [38, 39]. SOD and GSH-Px are the main endogenous antioxidant enzymes and are important markers of the scavenging capacity of free radicals. SOD scavenges superoxide radicals (O₂[•]) by accelerating its conversion to hydrogen peroxide (H₂O₂). GSH-Px reduces lipid hydroperoxides to their corresponding alcohols and free H₂O₂ to water. MDA as an indirect marker of injury is an end product of lipid peroxidation. Therefore, inhibition of oxidative stress may contribute to MIRI. Accumulating evidence has shown that HBO preconditioning could protect against various ROS-related injuries [40, 41]. The present results were consistent with these previous studies. HBO or HBO plus diltiazem pretreatment exhibited protective effects in reducing MIRI-mediated oxidative stress through decreased production of radical derivatives, as evidenced by the decreased MDA level. Moreover, HBO or the combination therapy attenuated serum SOD and GSH-Px depletion after MIRI. Furthermore, the combination therapy showed appreciably elevated the levels of SOD and GSH-Px when compared to the DIL group. Similar results were observed in our previous study. Indeed, HBO was capable of inhibiting peroxidation *in vivo* [16]. The results illustrated that HBO combined with diltiazem affected anti-MIRI by enhancing the ability of eliminating oxidant radicals and enhancing antioxidant ability.

During myocardial ischemia, permeability is increased in anaerobic glycolysis and the integrity of the membrane of cardiac muscle cells is damaged, causing the permeability of the cell membrane to be more transparent and myocardial enzymes to be released into the blood, which significantly increases the content of serum enzymes including CK, CK-MB, LDH and LDH1 [42]. Thus, measurements of these enzymes can be used as indexes to observe the effects and prognosis of MIRI [43]. In the present study, we found such enzyme rises in our IR group, and showed that diltiazem, HBO or HBO plus diltiazem pretreatment significantly reduced the activities of CK, CK-MB, LDH and LDH1. Even the combination therapy group showed the lower myocardial enzymes contents than the DIL group. Our results indicating that the cardioprotection of HBO combined with diltiazem may be due to the maintenance of the membrane integrity of myocardial cells. Moreover, our histopathological findings revealed that combination therapy group significantly decreased myonecrosis, infiltration

of inflammatory cells, and extravasation of red blood cells, which supported the myocardial enzyme changes.

The endothelium has important regulatory functions during the MIRI for endothelial cells are quite sensitive in this phase [44]. Endothelial cells can modulate cardiomyocyte releasing of mediators such as NO, endothelin, angiotensin, and so on. NO is usually synthesized by eNOS in endothelium, but is also can be produced by iNOS which is induced by pathological conditions, such as cytokines and lipopolysaccharide stimulation [45]. eNOS-derived NO provides cardioprotective effects including coronary vasodilation, regulation of platelet and neutrophil functions, and tonic inhibition of mitochondrial O₂ consumption [46]. On the other hand, high levels of iNOS-derived NO leads to increase production of peroxynitrite and cause myocardial dysfunction [47]. To observe the endothelial function, in this study, we investigated the eNOS-derived NO parameter. We found that serum NO and eNOS levels were markedly decreased following MIRI, which supported that NO and eNOS participated in the development of MIRI. However, HBO plus diltiazem pretreatment elevated significantly levels of NO and eNOS, suggesting that HBO plus diltiazem improved coronary circulation in MIRI rats due to the increased release of eNOS-derived NO. It has been reported that depletion of circulating eNOS increase infarct size and cardiac dysfunction in a MIRI mice model [48]. Interestingly, we also found that the expression of ET-1 was decreased greatly in rats treated with HBO plus diltiazem, suggesting that HBO combined with diltiazem may modulate the endothelin signaling system by suppressing the synthesis or release of ET-1. These results provided valuable reference for the cardioprotective effects of HBO combined with diltiazem against MIRI.

During ischemia, when ATP is progressively depleted, ion pumps cannot function, resulting in a rise in intracellular and mitochondrial Ca²⁺, which further accelerates ATP depletion and results in mitochondrial dysfunction and rupture of the plasma membrane and cell death [49]. Na⁺-K⁺-ATPase is an integral membrane protein and is achieved through coupling ATP hydrolysis to the transport of Na⁺ and K⁺. Ca²⁺-Mg²⁺-ATPase is the predominant active transport protein that can regulate intracellular calcium levels and maintain the cation gradient for homeostatic and is crucial for the contractility and excitability of muscles [50]. In our study, both Na⁺-K⁺-ATPase and Ca²⁺-Mg²⁺-ATPase activities were significantly reduced in the IR group, but these changes were reversed by HBO or HBO plus diltiazem pretreatment. The results suggested that HBO plus diltiazem may protect the myocardium by increasing ATP enzyme activity and maintaining the homeostasis of the intracellular environment.

Apoptosis is mediated by two different evolutionarily conserved pathways: the intrinsic and extrinsic cell death pathways, which are respectively represented by the Bcl-2 family (including Bax and Bcl-2) and caspase-3 [51, 52]. Here we performed immunohistochemistry assay to investigate the effect of combination therapy on the expression of Bcl-2 and Bax. Measurement of the expression of caspase-3 mRNA in ischemic myocardial tissue by Real Time PCR was then conducted. Bcl-2 is an anti-apoptotic protein expressed on the outer membrane of the mitochondria, which may prevent mitochondrial permeability transition pore (mPTP) opening and Ca²⁺ overload, thereby inhibiting apoptosis. Bax is a pro-apoptotic protein located in the cytoplasm, which has the opposite effect as Bcl-2 [53, 54]. Caspase-3 as the terminal shear enzyme in the caspase cascade reaction plays a pivotal role in apoptosis [51]. Therefore, the Bcl-2/Bax ratio and caspase-3 are usually used to represent the extent of apoptosis. The experimental study found that Bcl-2 expression and the Bcl-2/Bax ratio were significantly increased and Bax and caspase-3 expression were significantly decreased in the HBO or combination therapy group. Importantly, there was a significant lower expression of Bax and caspase-3 in combination therapy group than that in the DIL group. In addition, our previous investigation also found that HBO was capable of significantly attenuating Bax expression and increasing Bcl-2 expression in a rat model of Parkinson's disease [16]. This evidence indicated that inhibition of apoptosis may be one of the mechanisms by which HBO plus diltiazem protects against MIRI.

Our study has certain limitations. First, the MIRI rats' model and drug doses are only very close to human reality. Secondly, it is not clear whether the combination therapy reduces

MIRI is involving in the opening of the mitoK_{ATP} channels. Thirdly, we analyze oxidative stress, energy metabolism, endothelial function and apoptosis indexes to evaluate the effect of HBO combined with diltiazem on MIRI. More comprehensive parameters reflected the regulation network are needed to clarify the mechanism of action behind combination therapy protect against MIRI.

In conclusion, the present study demonstrates for the first time that the myocardial protective effect of HBO combined with diltiazem during MIRI is due to a reduction in oxidative stress, correction of energy metabolism, improvement in endothelial function and inhibition of cell apoptosis. This evidence suggests that HBO combined with diltiazem may be a useful therapy that can be applied clinically to prevent MIRI.

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Disclosure Statement

The authors declare that there are no conflicts of interest.

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