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Anti-Apoptotic Effect of Hyperbaric Oxygen Preconditioning on a Rat Model of Myocardial Infarction

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Background. **In our previous study, it was indicated that hyperbaric oxygen preconditioning (HOP) could induce a preconditioning against myocardial infarction and promote neovascularization. In this study, attempts were made to investigate whether a modified short-term pre-exposure protocol could also induce cardioprotection, and its potential mechanisms.**

Materials and methods. **Adult male Sprague Dawley rats were divided into seven groups; group 1 was sham surgery (SHAM) and group 2 was pre-exposed to normal air (CTL), and the other groups to HOP 1, 6, 24, 48, and 72 h (H1, H6, H24, H48, H72 groups) before permanent ischemia. The infarct size was measured by triphenyltetrazolium chloride staining, and left ventricular function parameters were recorded. The extent of apoptosis was determined by terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL)-positive staining. Caspase-3 activity, Bcl-2, and Bax expression were also measured.**

Results. **Compared with CTL group, myocardial infarct size was significantly decreased as well as cardiac cell apoptosis in area at risk zones (AAR) in H48 group. Meanwhile, the activity of caspase 3 was reduced, the ratio of Bcl-2/Bax expression was up-regulated, and the heart function parameters, including left ventricular systolic pressure (LVSP), left ventricular diastolic pressure (LVEDP), $+(dP/dt)_{max}$, and $-(dP/dt)_{max}$ were also significantly improved after preconditioning in H48 group.**

Conclusion. **The results indicate that short-term HOP could induce cardioprotection and may not last for more than 24 h. HOP prevents myocardium from permanent ischemia injury by suppression of apoptotic pathways.**

Key Words: **myocardial infarction; hyperbaric oxygen preconditioning; apoptosis.**

INTRODUCTION

Myocardial infarction is among the leading causes of death in clinical settings. Although myocardial protection has improved during the last decades, effective treatments are still lacking. Ischemia-preconditioning, which was first reported by Murry *et al.* [1], is so far the most powerful cardioprotective intervention in experimental studies reducing infarct size, reperfusion arrhythmias and microvascular stunning; furthermore, it is also effective for cardiac surgery patients [2, 3]. However, the safety concerns and practical feasibility have limited the applications of ischemia preconditioning in practice.

Hyperbaric oxygen preconditioning (HOP) has been tested to induce ischemic tolerance in heart models [4–6] and in organs such as spinal cord [7, 8], brain [9–11], and liver [12, 13], suggesting that HOP produces a wide-scale protective effect, and may be a safer preconditioning stimulus compared with other stimuli involved, e.g., hypoxia [14]. Our previous findings suggest HOP could alleviate myocardial ischemia through promotion of neovascularization [6]. Yet the intensive mechanisms underlying its myocardial protective effects remained poorly defined. Therefore, we examined the cardioprotective effect and mechanisms of a short-term intermittence HOP in an established rat model of myocardial infarction. We evaluated specifically the reduction of infarction, improvement of cardiac functions, inhibition of apoptosis, and the expression of anti-apoptotic proteins Bcl-2 and Bax by HOP.

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MATERIALS AND METHODS

Animals

Adult male Sprague-Dawley rats weighing 250–280 g were used in all experiments. The animals were housed in individual cages in a temperature-controlled room with a 12 h light/dark cycle and free access to food and distilled water. All the protocols were approved by the Second Military Medical University, China, in accordance with the Guide for Care and Use of Laboratory Animals published by the US NIH (publication no. 96-01).

Experimental Protocol

Animals were randomized into the following seven groups and then they were allocated to the following protocols: (1) SHAM group: normoxia + sham surgery ($n = 18$); (2) CTL group: normobaric air preconditioning 48 h before infarction ($n = 18$); (3) H1 group: HBO preconditioning 1 h before infarction ($n = 12$); (4) H6 group: HBO preconditioning 6 h before infarction ($n = 12$); (5) H24 group: HBO preconditioning 24 h before infarction ($n = 12$); (6) H48 group: HBO preconditioning 48 h before infarction ($n = 18$); (7) H72 group: HBO preconditioning 72 h before infarction ($n = 12$).

We used a short-term intermittence hyperbaric oxygen preexposing protocol first reported by Choi's group [5]. Rats in HOP groups were exposed to 100% O_2 at 3 atm for 20 min; four consecutive cycles with 20 min of normobaric air intermission in a hyperbaric chamber. Compression and decompression were carried out at a rate of 0.2 atm/min. The gas in the chamber was continuously ventilated to minimize $PpCO_2$ changes, and the temperature of the chamber was maintained at a range of 22–25°C. After the exposure to HBO, the rats were maintained in a normoxic environment until the operation of myocardial infarction or the sham operation. Rats under normobaric room air served as control.

Surgical Preparation

Rats were intraperitoneally anesthetized with chloral hydrate (300 mg/kg). The rats were intubated and ventilated with a small-animal ventilator. Briefly, the hearts were exposed through a left thoracotomy in the fourth intercostal space. The pericardium was opened, and a 5.0 prolene suture was tightened around the proximal left anterior descending (LAD) coronary artery (before the first branch of diagonal artery). Positive end-expiratory pressure was applied to fully inflate the lungs. Then the muscle layer and the skin were closed separately and the animals were allowed to recover.

Hemodynamic Measurements

Hemodynamic measurements were adapted from our previous study [15]. Rats were intraperitoneally anesthetized with chloral hydrate (300 mg/kg) 3 d after myocardial infarction. A small incision was made to the right of the midline in the neck. The right carotid artery was identified and a PE 50 catheter was introduced into the artery. The proximal end of the catheter was connected to a low pressure transducer. The inserted tip of this catheter was advanced down until it reached the left ventricular lumen and the left ventricular pressure (LVP) signal was obtained. The pressure signals were monitored, analyzed, and recorded in real time. Heart rate (HR), left ventricular systolic pressure (LVSP), left ventricular diastolic pressure (LVEDP), and $\pm(dP/dt)_{max}$ were all calculated from the continuously obtained LVP signal.

Since we used fluid-filled catheter to get the signal, end-diastolic pressure was difficult to measure accurately; LVEDP represented minimum diastolic pressure here.

Infarct Size Determination

Infarct size determination was adapted from our previous study [6]. Three days after infarction (when the infarct size was relatively stable) 2% Evans blue dye was infused into the heart through the apex to mark the AAR as unstained (not blue) tissue. The hearts were excised and placed in a −20°C freezer for 30 min. Frozen hearts were then cut into 2 mm thick slices parallel to the atrioventricular groove. Sections were thawed and incubated in a 1% tetrazolium chloride (TTC) phosphate buffered solution (pH 7.4) at 37°C for 15 min and fixed in 10% formalin to increase the contrast of the Evan's blue and TTC staining. Tissue sections were compressed to a uniform 2 mm thickness by placing them between two glass plates separated by 2 mm space. The viable tissue was stained red with TTC, while the dead tissue (infarcted tissue) was unstained. The infarct size was calculated as a percentage volume of the infarct area (white area) *versus* the AAR (blue area).

Histologic and Immunohistochemical Studies

Rats were sacrificed after 3 d of LAD occlusion. The hearts were harvested, sectioned, and immersion-fixed in 4% buffered paraformaldehyde. The paraffin was cut into 4 μm thick serial sections. The standard deparaffinization protocol was used.

In Situ Apoptosis Assay

TUNEL staining was performed on paraffin-embedded sections by using the *in situ* cell death detection kit (Roche, Indianapolis, IN, USA). According to standard protocols, the sections were dewaxed and rehydrated by heating the slides at 60°C. Then these sections were incubated in a 20 μg/mL proteinase K working solution for 15 min at room temperature. The slides were rinsed three times with PBS before they were incubated in TUNEL reaction mixture for 1 h at 37°C. Area around sample was dried by filter paper and converter-AP was added on samples for 1 h at 37°C. After rinsing with PBS (5 min, three times), sections were colored in dark incubated with nitroblue tetrazolium (NBT) and 5-bromo-4-chloro-3-indolylphosphate (BCIP). Four slide fields were randomly examined using a defined rectangular field area with magnification (×40). One hundred cells were counted in each field. The data were represented as the percentage of TUNEL-positive cells of total cell nuclei per field.

Caspase-3 Activity

Caspase-3 activity was determined 24 h after reperfusion using a Fluorometric Assay Kit (Bivision Research Products, Mountain View, CA), according to the manufacturer's instructions. In brief, 20–200 μg cell lysates of AAR were incubated in a 96-well plate with 2× reaction buffer (50 μL). The reaction was started by adding 1 mM DEVD-APC substrate (5 μL). After incubation in the dark at 37°C, the plate was read in a fluorometer equipped with a 400-nm excitation filter and a 505-nm emission filter. Fold-increase in caspase-3 activity was determined by comparing these results with the level of the uninduced control.

Western Blot Analysis

After 24 h reperfusion, total cell lysates for Bcl-2 and Bax analyses were obtained from AAR of the left ventricles, using a nuclear extraction kit (Active Motif, Carlsbad, CA, USA) and following the manufacturer's protocol. Equal amounts of the samples containing 40 μg proteins were loaded per lane, separated on 12% dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred electrophoretically to nitrocellulose membranes. Membrane were blocked with 2% BSA in Tris-buffered saline containing 0.1% Tween20 (TBST) at room temperature for 1 h and then incubated overnight at 4°C with

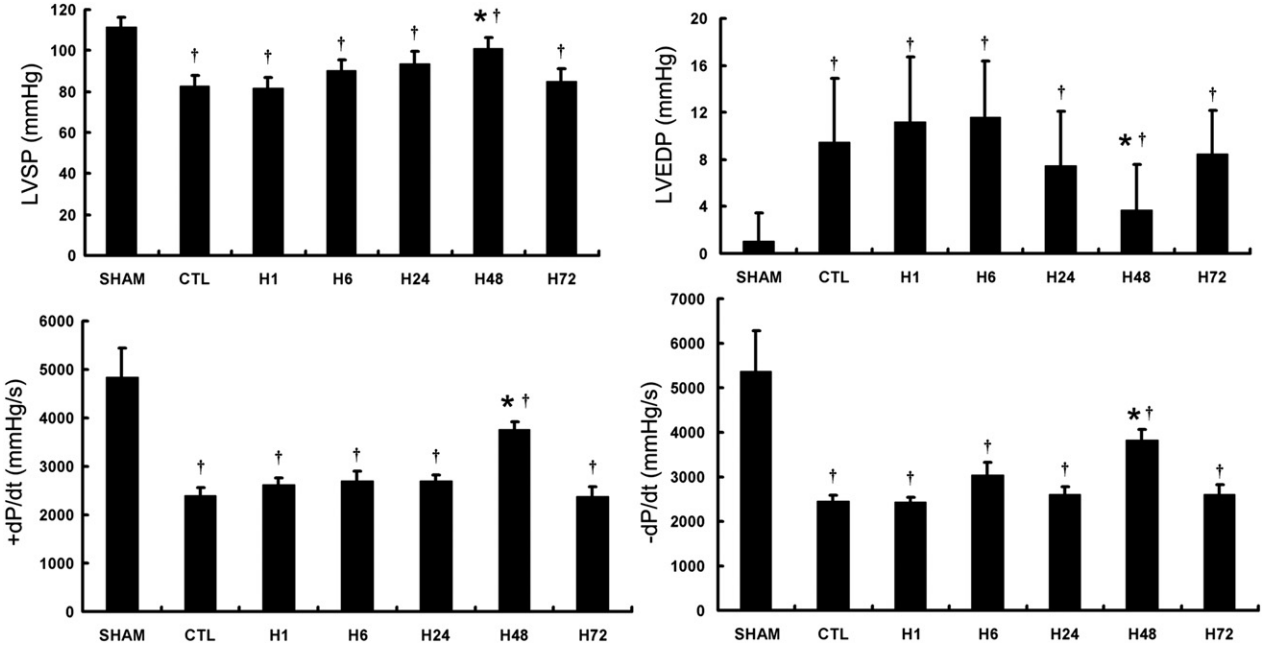

FIG. 1. The changes of LVEDP, LVSP, $+(dP/dt)_{max}$ and $(dP/dt)_{max}$ 3 d after myocardial infarction. Results are expressed as means ± SEM; ($n = 6$, $^{*}P < 0.05$ relative to CTL group, $^{\dagger}P < 0.05$ relative to SHAM group).

anti-Bcl-2 polyclonal antibody (1:1000 dilution; Chemicon International, Inc.,), rabbit anti-Bax polyclonal antibody (1:1000 dilution, Stressgen Bioreagents Corp., Ann Arbor, MI, USA), and β-actin antibody (1:1000 dilution, Sigma, St. Louis, MO, USA). Then, horseradish peroxidase-conjugated anti-rabbit IgG (Santa Cruz Biotechnology, Santa Cruz, CA, USA) was used as the secondary antibody (1:700 dilution in 2% BSA in TBST, 1 h incubation) and the antigen–antibody complexes were visualized using an enhanced chemiluminescence reagent (Pierce Biotechnology, Inc., Rockford, IL). The amount of Bcl-2 and Bax were quantitated by densitometry scanning using a densitometer (GS-700; Bio-Rad Laboratories, Inc., Hercules, CA), analyzed with Quantity One 4.5.2 software (Bio-Rad Laboratories, Inc.) and normalized to β-actin, an internal standard.

Statistical Analysis

All results were expressed as means ± SEM. Differences between groups were determined with a one-way ANOVA followed by least significant difference tests. A value of $P < 0.05$ was considered to denote statistical significance.

RESULTS

Hemodynamic Measurements

Hemodynamic data for LVSP, LVEDP, $+(dP/dt)_{max}$, and $-(dP/dt)_{max}$ in the seven groups are shown in Fig. 1. All the hemodynamic parameters in five preconditioning groups and CTL group were significantly lower than those in the SHAM group. However, preconditioning of hyperbaric oxygen 48 h before surgery significantly decreased the myocardial ischemia-induced degrading of hemodynamic parameters, including LVSP, LVEDP, $+(dP/dt)_{max}$, and $-(dP/dt)_{max}$. No statistical difference was found for heart rate among groups (data not shown).

Measurement of Infarct Size

The infarct size in the H48 group was significantly smaller than in the CTL group (12.7% ± 3.9% *versus* 26.7% ± 5%, $^{*}P < 0.05$), as shown in Fig. 2. In the other four preconditioning groups infarct size was similar ($P =$ NS *versus* each other). Infarct size was also similar to that of the CTL Group ($P =$ NS for all), but significantly ($P < 0.05$) greater than that of the H48 group. There was no significant difference in AAR/LV among groups.

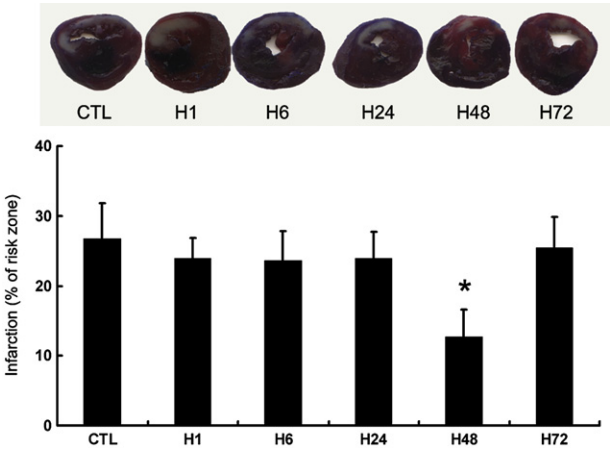

FIG. 2. Representative photographs of serial heart sections obtained from rats subjected to myocardial infarction in the presence or absence of hydrogen-rich saline injection. Bar = 2 mm. Infarct size as a % of AAR and it is expressed as a percentage volume of the infarct area *versus* the AAR; ($n = 6$ for each group, $^{*}P < 0.05$ compared with CTL group). (Color version of figure is available online.)

Detection of Apoptotic Cell Death

As shown in Fig. 3, the number of TUNEL-positive cells were increased in the AAR myocardium. Preconditioning of hyperbaric oxygen 48 h before ischemia comparably decreased the percent of TUNEL-positive cells relative to the CTL group (19.8% ± 1.9% *versus* 29.8% ± 6.8%, * P <0.05 *versus* CTL group).

Caspase-3 Activity

The caspase-3 activity is shown in Fig. 4. Myocardial infarction significantly increased caspase-3 activity relative to the nonischemic myocardium. Preconditioning of hyperbaric oxygen 48 h before ischemia comparably reduced caspase-3 activity relative to the CTL group.

Expression in Bcl-2 and Bax Protein After Infarction

The protein expression of Bcl-2 and Bax proteins in AAR myocardium was visualized by Western blot analysis as shown in Fig. 5. Bcl-2 was clearly expressed in AAR myocardium zones after ischemia. Preconditioning of hyperbaric oxygen 48 h before ischemia was associated with greater expression of Bcl-2 relative to the CTL group. There was no significant difference of the expression of Bax among groups.

DISCUSSION

Our present study demonstrated pre-exposed to short-term hyperbaric oxygen 48 h before permanent ischemia significantly improved postischemic functional recovery of rat hearts. The improvement in post-ischemic functional recovery was parallel to a significant reduction in infarct size, decreased caspase-3 activity, attenuation of cardiac cell apoptosis, and the up-regulation of the ratio of apoptotic related protein Bcl-2/Bax expression. This cardiac improvement may result from anti-apoptotic effect of hyperbaric oxygen.

Since the preconditioning phenomenon was first identified in the heart by Murry *et al.* [1], the same effect could be induced by several other stimuli such as hyperbaric oxygen, chemical agents, sleep deprivation, dietary restriction, and both hyperthermia and hypothermia. Among these stimuli, hyperbaric oxygen is one outstanding candidate for clinical application

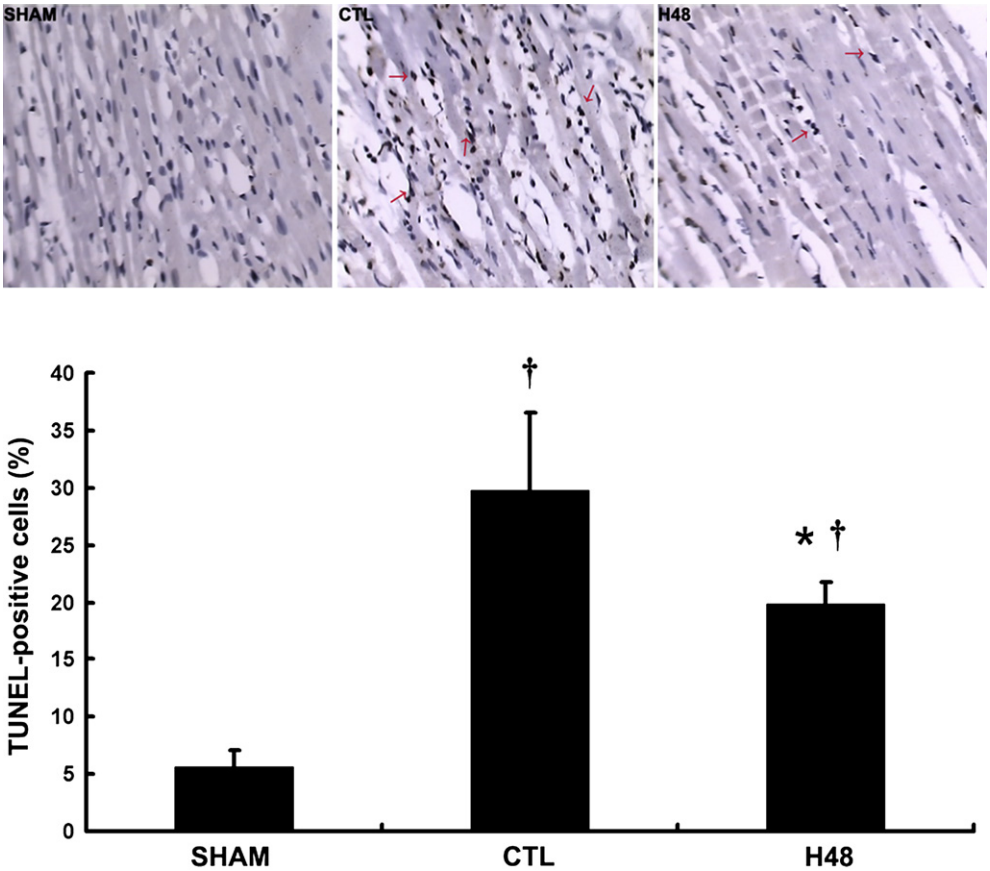

FIG. 3. Detection of apoptotic cell death by TUNEL staining in the SHAM, CTL, and H48 groups 3 d after myocardial infarction. Relative to the CTL, hyperbaric oxygen preconditioning significantly reduced the number of TUNEL-positive cells (brown staining). Values are mean ± SEM; *$P < 0.05$ compared with CTL group; $n = 6$ for each group. †$P < 0.05$ relative to SHAM group. (Color version of figure is available online.)

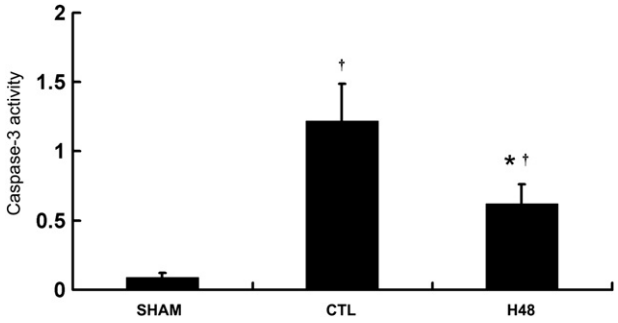

FIG. 4. Caspase-3 activity in the SHAM, CTL and H48 groups 24 h after myocardial infarction. Hyperbaric oxygen preconditioning significantly reduced caspase-3 activity compared with CTL group; $n = 6$, $^{*}P < 0.05$ compared with CTL group; $^{\dagger}P < 0.05$ relative to SHAM group).

because of its property of lower side effect under proper management. Our previous findings have shown that intermittently exposed to 100% O_2 at 2.5 atmosphere absolute (ATA) for 60 min, twice daily for 2 d could alleviate myocardial ischemic injury and this may be associated with accelerated angiogenesis [6]. Previous studies also suggest HOP-induced protection is associated with enhanced expression of antioxidant enzymes [4], and suppressed apoptosis due to improved balance between anti- and pro-apoptosis proteins [4, 16, 17]. These findings of anti-apoptotic effect of hyperbaric oxygen were performed in model organs such as brain [10], and spinal cord [7], as well as Langendorff heart ischemia-reperfusion injury [5]. Whereas no study has been conducted *in situ*, myocardium and the mechanism underlyng in HBO-induced protection is far from discriminated. For a long time, necrosis has been thought to be the mode of myocardial cell death. Apoptosis as a pathogenetic factor in heart disease was introduced relatively late compared with other fields of medicine [18]. Unlike necrosis, the step-by-step nature in development of apoptosis is thought to be an essentially reversible process, independently contribute to irreversible myocardial damage [19], and it may be amenable to therapeutic intervention [20]. In the present study, inhibition of apoptosis by HOP significantly improved left ventricular function following 3 d infarction, suggesting that apoptotic cell death detected from the AAR of left ventricle may in part participate in subsequent development of intractable left ventricular dysfunction. These results confirmed previous animal and clinical studies that showed the correlation between apoptosis and cardiac function as well as the recovery in dysfunction by inhibition of apoptosis [21–23]. Associated with these changes, expression of anti-apoptotic protein Bcl-2 was found up-regulated, and caspase-3 activity increased. Bcl-2 is a mitochondria-anchored anti-apoptotic protein originally identified as a proto-oncogene. The anti-apoptotic bcl-2 proteins exert cardioprotection by multiple mechanisms such as a direct anti-oxidant effect [24] and mitochondrial membrane stabilization especially the inhibition of the open of mitochondrial permeability transition pore [25]. The up-regulation of bcl-2 associated apoptosis reduction was already documented in ischemic preconditioning [26]. In this regard, we postulate the cardioprotection effect of HOP may partially due to inhibition of apoptosis. Since a different HBO protocol was applied in this research, the results cannot be combined with our previous findings [6]. It is necessary to confirm the pro- angiogenesis effect of HBO using the new protocol.

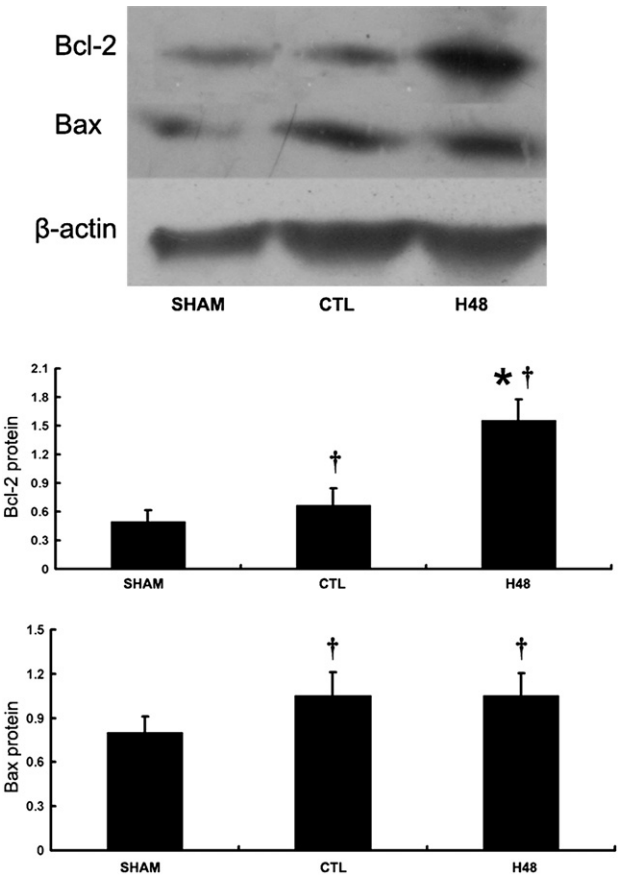

FIG. 5. Protein levels of Bcl-2 and Bax in the hearts of CTL and preconditioning groups. Upper panel: representative western blot image for Bcl-2, Bax and β-actin. Lower panel: densitometry analyses of Bcl-2 and Bax levels. Values are means ± SEM; ($n = 6$ for each group; $^{*}P < 0.05$ *versus* CTL group; $^{\dagger}P < 0.05$ *versus* SHAM group).

As was reported, ischemia preconditioning induces a biphasic tolerance against subsequent insult, with an early phase that lasts for 2 to 3 h and a delayed phase that initiates about 24 h later and continues up to 72 h [27, 28]. Choi's group, using HBO in the isolated rat hearts [5], also showed a similar biphasic effect. Our results only confirmed the late phase of HOP in H48 group. One possibility that no early phase of HOP was found in our experiments is that HBO may have an early preconditioning phase in less than 1 h, since we did not induce

myocardial infarction right after HOP (because we need about 15 min to decompress and some time to prepare for surgery, LAD ligation could not be performed right after HBO preconditioning). According to the ischemia preconditioning, the early phase last shorter and is less potent than the late phase, so we doubt if the early phase of HBO preconditioning exists at a relatively earlier stage and vanish sooner before we test it. The other possibility may be the early phase of HOP is not potent enough to induce obvious cardioprotection. As it was noted in the previous section, the delayed phase of preconditioning lasts for up to 72 h (*in vivo*), and the potent late phase of HOP might cause a great interest in clinical practice. Furthermore, this short-term protocol of HBO, preexposing is more convenient and applicable than the long-term repetition HOP protocol (2 to 5 d consecutively exposing in HBO, 1 to 2 h each time).

In summary, short-term intermittence exposure to HBO 48 h before myocardial infarction may induce a late preconditioning that lasts for about 24 h, and this preconditioning effect may be induced by inhibition of myocardial apoptosis.

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