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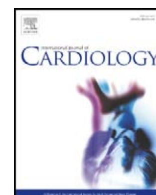

Anti-inflammatory effect of hydrogen-rich saline in a rat model of regional myocardial ischemia and reperfusion

Yufeng Zhang [a,1], Qiang Sun [b,1], Bin He [c], Jian Xiao [a], Zhinong Wang [a,*], Xuejun Sun [b,*]

[a] Department of Cardiothoracic Surgery, Changhai Hospital, Second Military Medical University, Shanghai 200433, PR China
[b] Department of Diving Medicine, Faculty of Naval Medicine, Second Military Medical University, Shanghai 200433, PR China
[c] Department of Anesthesiology, Shanghai Jiaotong University School of Medicine, Shanghai 200092, PR China

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ABSTRACT

Introduction: To study the possible anti-inflammatory effect of hydrogen-rich saline (H_2 saline) on rat hearts with regional myocardial ischemia and reperfusion (I/R).
Methods: Sixty-six rats were equally randomized to three groups: sham-operated group, I/R group (control group) and I/R plus H_2 saline treatment group. Myocardial I/R was established by occlusion of the left anterior descending (LAD) coronary artery for 30 min and reperfusion for 24 h.
Results: H_2 saline treatment attenuated I/R-induced cardiac cell apoptosis, presenting as significant improvement of heart function parameters 24 h after reperfusion, including left ventricular systolic pressure (LVSP), left ventricular diastolic pressure (LVEDP), +(dP/dt)max and −(dP/dt)max. It also decreased neutrophil infiltration, 3-nitrotyrosine level, expression of intercellular adhesion molecule 1(ICAM-1) and myeloperoxidase (MPO) activity in the area at risk zones (AAR) of rat hearts subjected to regional myocardial I/R, and attenuated the increase of I/R induced proinflammatory cytokine tumor necrosis factor-α (TNF-a) and interleukin-1β (IL-1b) levels in the AAR.
Conclusion: H_2 saline has an anti-inflammatory effect on rat hearts with regional myocardial I/R.
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1. Introduction

Acute myocardial infarction (AMI) is a potentially fatal event. Restoring blood flow is the most effective and important therapy for AMI at present [1]. Although blood flow restoration is critical, reintroduction of molecule oxygen often triggers a cytotoxic cascade, during which reactive oxygen species drives downstream signal networks leading to both cell death (or apoptosis) and inflammation [2–4]. As the human heart has a low regenerative ability, the inflammatory response and cytokines released from the myocardium play an important role in cardiac repair and the pathophysiological response to I/R injury [5]. In many studies [6–9], acute inflammatory reaction is regard as a mediator of ischemia-reperfusion (I/R) injury. It is known that methods that reduce levels of inflammatory cytokines [10] or infiltration of leukocytes [11] can attenuate reperfusion-induced myocardial damage. However, there is a lack of well recognized methods to ameliorate the inflammatory response during reperfusion [12].

Hydrogen, a highly flammable gas, has proved to be protective against I/R injury to various organs including the brain [13,14], intestine [15], liver [16] and heart [17] through inhibition of oxidant stress. A recent study [18] reported that ingestion of H_2-water also has an anti-inflammatory effect and could efficiently suppress dextran sodium sulfate (DSS)-induced colitis through its anti-inflammatory effect. In our previous study [19], we found that hydrogen-rich saline, which is safe, economical and easily available, had a cardioprotective effect against myocardial I/R injury in rats through the anti-oxidative stress and apoptotic pathways. However, it is unclear whether H_2 saline could efficiently decrease the inflammatory response in myocardial I/R injury. To test this, we measured a variety of parameters related to inflammatory response, infarct size and heart function in a regional myocardial I/R rat model after administration of H_2 saline, and demonstrated that it had a potent anti-inflammatory effect.

2. Materials and methods

2.1. Animals

Adult male Sprague–Dawley rats weighing 250–280 g (Experimental Animal Center of the Second Military Medical University, Shanghai, China) were housed with free access to food and water under a natural day/night cycle, and acclimated for 7 days before any experimental procedure. All experimental procedures were approved by the Institutional Animal Care and Use Committee of the Second Military Medical University.

* Corresponding author. Tel./fax: +86 21 81873327.
E-mail address: zyf19810824@163.com (Z. Wang).
[1] Yufeng Zhang and Qiang Sun contributed equally to this work.

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2.2. H_2 saline production

Hydrogen was dissolved in 0.9% saline for 6 h under a high pressure (0.4 MPa) to a supersaturated level. H_2 saline was stored under an atmospheric pressure at 4 °C in an aluminum bag with no dead volume, sterilized by gamma radiation, and freshly prepared once a week to ensure that the concentration was maintained at 0.6 mmol/L. Gas chromatography was performed to confirm the content of hydrogen in saline by the method described by Ohsawa et al. [13].

2.3. Experimental protocols

The rats were anesthetized intraperitoneally with chloral hydrate (300 mg/kg), intubated and ventilated with a small-animal ventilator. The heart was exposed through a left thoracotomy in the fourth intercostal space. After the pericardium was opened, a 5.0 prolene suture was tightened around the proximal left anterior descending (LAD) coronary artery. Sixty-six rats were equally randomized to a sham-operated group, where the rats underwent surgical preparation without occlusion; an I/R group (control group), where the rats underwent surgical preparation, 30-min LAD occlusion and 24-h reperfusion, and then were treated with intraperitoneal injection of saline 5 min before LAD reperfusion at 10 ml/ kg; and an H_2 group, where the rats underwent surgical preparation, 30-min LAD occlusion and 24-h reperfusion, and then were treated intraperitoneal injection of H_2 saline 5 min before LAD reperfusion at 10 mg/ kg. There were 22 animals in sham, control, and H_2 group: 8 for the measurement of infarct size, 6 for histological and immunohistochemical study, and 8 for the measurement of TNF-a, IL-1b and MPO concentrations.

2.4. Hemodynamic measurements

Hemodynamic measurements were adapted from our previous study [19]. The rats undergoing I/R were intraperitoneally anesthetized with chloral hydrate (300 mg/kg) 24 h after the I/R procedure. A PE 50 catheter was introduced into the right carotid artery. The proximal end of the catheter was connected to a low pressure transducer. The inserted tip of the catheter was advanced downward until it reached the left ventricular lumen, where left ventricular pressure (LVP) signals were obtained, monitored, analyzed and recorded in real time. Heart rate (HR), LVSP, LVDP and ± (dP/dt)max were calculated from the continuously recorded LVP signals. As we used a fluid-filled catheter to get the signals, it was difficult to measure the end-diastolic pressure accurately, and therefore LVDP represented the minimum diastolic pressure in the present study.

2.5. Quantification of myocardial tissue injury

By the end of the 24 h reperfusion period, the LAD was re-occluded, and 1 ml Evans blue dye (2% wt/vol) was injected into the animal via the jugular vein. The heart was harvested and stored in a −20°C freezer for 30 min, and then sliced into 2 mm thick sections 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 2 mm thick uniform by placing them between two glass plates separated by a 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).

2.6. Immunohistochemical localization of intercellular adhesion molecule 1 and 3-nitrotyrosine

By the end of the experiment, the heart was 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. The paraffin sections were deparaffinized in xylene, rehydrated using various grades of ethanol, and pretreated with 10 μg/ml proteinase K (to permeabilize the nucleus) for 30 min at 37 °C. Nonspecific binding of immunoglobulin was blocked by incubating the sections in 10% BSA for 20 min. Then the sections were incubated overnight with (1) purified antimouse intercellular adhesion molecule 1 (ICAM-1; CD54; 1:500 vol/vol in PBS; DBA R&D Systems) or (2) antinitrotyrosine mouse monoclonal antibody (1:400 vol/vol in PBS abcam). Sections were washed with PBS and incubated with secondary antibody. Specific labeling was detected with a biotin-conjugated goat antirabbit immunoglobulin G (R&D Systems) and avidin–biotin peroxidase complex (DBA). Four slide fields were randomly examined using a defined rectangular field area with magnification (×20). ICAM-1 positive cells were counted in each field. The data were represented as the number of ICAM-1 positive cells per field.

2.7. PMN leukocyte influx into cardiac tissue

The total number of infiltrating leukocytes into the cardiac tissue was assessed quantitatively by counting the number of PMNs in 20 high-power fields. Neutrophil count was measured as the number of neutrophils per square millimeter of tissue.

Table 1
Hemodynamic parameters 24 h after reperfusion.

Groups	LVSP (mm Hg)	LVEDP (mm Hg)	+dP/dtmax (mm Hg/s)	−dP/dtmax (mm Hg/s)
Sham	128 ± 3	3 ± 2.3	9037 ± 597	−6555 ± 507
Con	83 ± 3$^{\#}$	11 ± 4.1$^{\#}$	3776 ± 242$^{\#}$	−2285 ± 504$^{\#}$
H_2	105 ± 3$^{*\#}$	6 ± 5.8$^{*\#}$	6041 ± 1284$^{*\#}$	−4877 ± 720$^{*\#}$

LVDP, LVSP, +(dP/dt)max and −(dP/dt)max changes 24 h after I/R. Results are expressed as mean ± S.E.M. (n = 8, $^*P<0.05$ relative to sham group, $^{\#}P<0.05$ relative to control group).

2.8. TNF-a, IL-1b and MPO levels in cardiac tissue

Cardiac tissue was washed in normal saline and then homogenized immediately on ice in 1 ml normal saline (4°C). The homogenate was centrifuged at 3000 g at 4°C for 15 min. TNF-a, IL-1b and MPO levels were measured with a commercial ELISA kit following the instructions of the manufacturer. The absorbance was read on a microplate reader and the concentration was calculated according to the standard curve. Protein content in the sample was calculated by Coomassie blue assay, and the results were corrected for protein content.

2.9. Statistical analysis

Quantitative data were expressed as mean ± SD. Differences between groups were determined with a one-way ANOVA followed by a Student–Newman–Keuls test. A value of $P<0.05$ was considered to denote statistical significance.

3. Result

3.1. Hemodynamic measurements

As shown in Table 1, intraperitoneal injection of H_2 saline decreased I/R-induced degradation of the hemodynamic parameters including LVSP, LVEDP, +(dP/dt)max and −(dP/dt)max. All the hemodynamic parameters in both control group and H_2 group were significantly lower than those in the sham group. No significant difference was seen in the heart rate after 24-h I/R between 3 groups.

3.2. Measurement of infarct size

The infarct size in H_2 group was significantly smaller than that in control group ($^*P<0.05$) (Fig. 1). There was no significant difference in AAR/LV between the three groups.

3.3. Effect of H_2 saline on the increase of ICAM-1 and 3-nitrotyrosine caused by regional myocardial I/R

Immunohistochemical analysis for ICAM-1 and 3-nitrotyrosine staining was performed on biopsies obtained from the AAR. Compared with the hearts obtained from sham group rats, there was a significant increase in ICAM-1 and 3-nitrotyrosine staining in the hearts obtained from the rats subjected to regional myocardial I/R, suggesting nitrosative stress within the AAR. H_2 significantly reduced the number of ICAM-1 positive cells induced by I/R ($^*P<0.05$) and alleviated the increase in I/R-induced nitrosative stress (Figs. 2, 3).

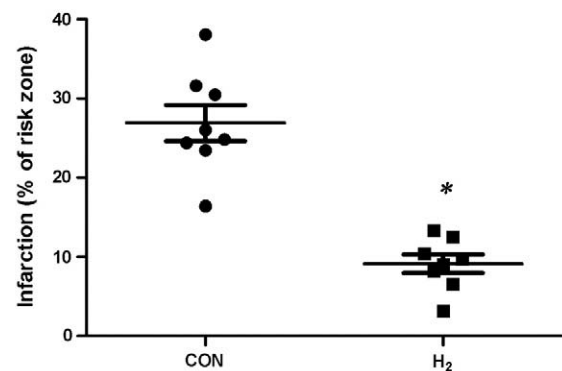

Fig. 1. Infarct size as percentage of AAR. Individual points are represented by the dark spots or squares, and the group mean values are represented by the level lines. H_2-dependent reduction in infarct size is expressed as the percentage of the infarct area versus AAR (n = 8 for each group, $^*P<0.05$ compared with control group).

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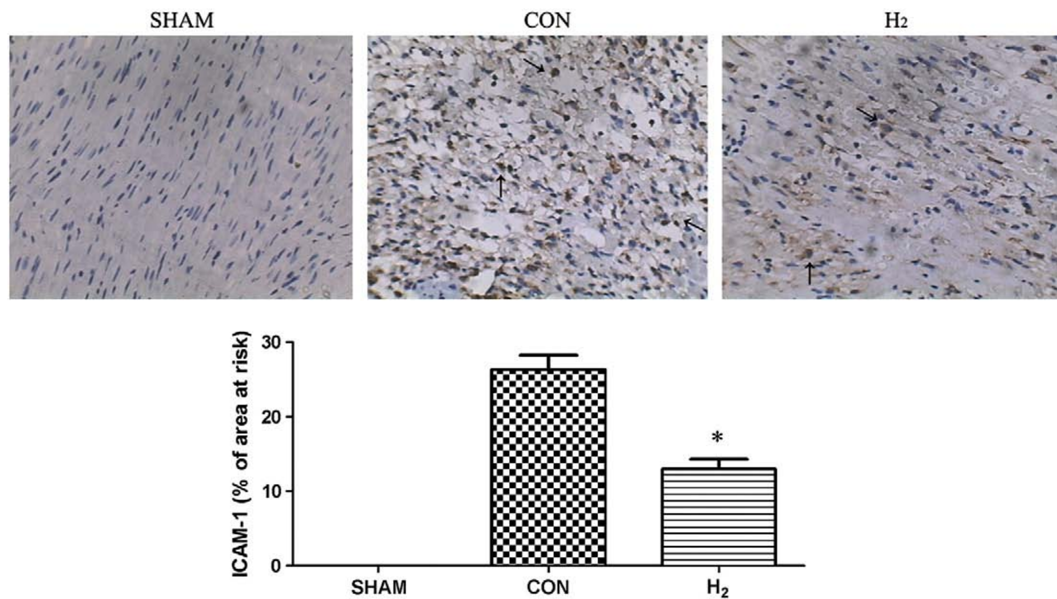

Fig. 2. ICAM-1 positive cells in sham, control and H_2 groups by the end of 24-h reperfusion. Compared with the control group, H_2 significantly reduced the number of ICAM-1 positive cells (brown staining) per field. Values are mean ± SEM; (n = 6, $^*P<0.05$ compared with control group; $^\#P<0.05$ compared with sham group). For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.

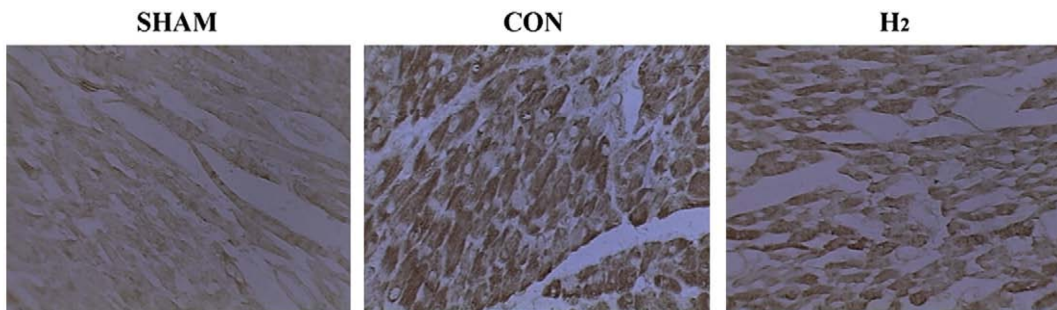

Fig. 3. 3-nitrotyrosine positive cells in sham, control and H_2 groups by the end of 24-h reperfusion. Compared with the control group, H_2 significantly reduced the expression of 3-nitrotyrosine in myocardial AAR.

3.4. Effect of H_2 saline on regional myocardial I/R-induced PMN accumulation in the myocardium

Polymorphonuclear neutrophil (PMN) accumulation was measured histologically in biopsies obtained from the AAR. Compared with the sham group, PMN accumulation in the control group increased ($^*P<0.05$), which was significantly attenuated by H_2 saline treatment ($^*P<0.05$) (Fig. 4).

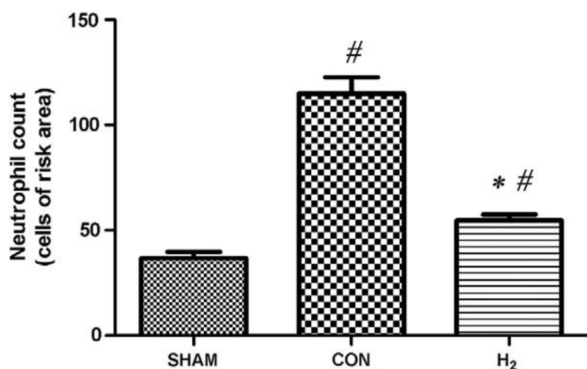

Fig. 4. Neutrophil count in sham, control and H_2 groups by the end of 24-h reperfusion. Compared with control group, H_2 significantly reduced the number of neutrophils per field. Values are mean ± SEM; (n = 6, $^*P<0.05$ compared with the control group; $^\#P<0.05$ compared with the sham group).

3.5. Effect of H_2 saline on TNF-a, IL-1b and MPO levels in cardiac tissue

The results of ELISA showed that TNF-a, IL-1b and MPO levels in the myocardium increased markedly after I/R injury. H_2 saline treatment reduced the elevation of TNF-a ($^*P<0.05$), IL-1b ($^*P<0.05$) and MPO ($^*P<0.05$) in the myocardium (Figs. 5, 6, 7).

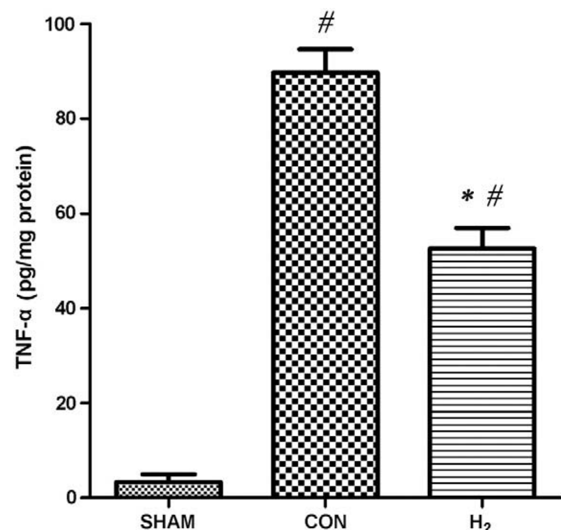

Fig. 5. Myocardial TNF-a concentration by the end of 24-h reperfusion. H_2 significantly reduced TNF-a concentration (n = 8, $^*P<0.05$ compared with the control group; $^\#P<0.05$ compared with the sham group).

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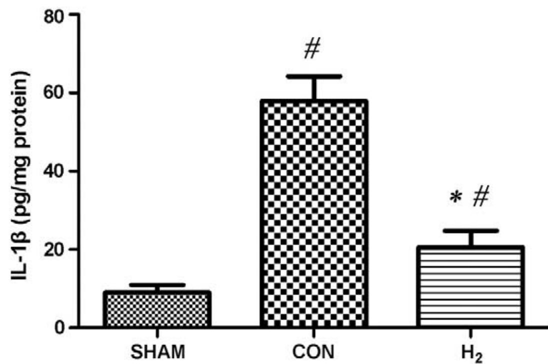

Fig. 6. Myocardial IL-1b concentration by the end of 24-h reperfusion. H_2 significantly reduced IL-1b concentration (n = 8, *$P<0.05$ compared with the control group; #$P<0.05$ compared with the sham group).

4. Dicussion

This is the first study demonstrating that H_2 saline had an anti-inflammatory effect in a rat model subjected to regional myocardial I/R injury, and this anti-inflammatory effect may play an important role in the cardioprotection of H_2 saline. In the present study, we detected the levels of infarct size and inflammatory cytokines after 24-h reperfusion, because they are relatively stable at this time point and 24-h after reperfusion is the peak of inflammatory reaction according to our previous work [19]. It was found that the improvement in post-I/R functional recovery was parallel to reduction of the infarct size and attenuation of increased 3-nitrotyrosine, ICAM-1, MPO activity, PMN accumulation and decreased cytokines (TNF-a, IL-1b).

Acute inflammatory reaction plays an important role in I/R injury through leukocyte activation and expression of adhesion molecules and cytokines. ROS is closely related to leukocyte activation in I/R injury [10]. Methods of scavenging ROS could alleviate the inflammatory reaction. Since the hydrogen molecule is electrically neutral and much smaller than the oxygen molecule, it can easily penetrate membranes and enter cells and organelles such as the nucleus and mitochondria, where most antioxidant cannot arrive [20]. Furthermore, hydrogen can react with ROS and scavenge them effectively and selectively [13]. Peroxynitrite (OONO−), whose metabolic end-product is 3-nitrotyrosine, plays a key role in I/R injury and no enzyme could detoxify effectively [21,22]. It was found in the present study that the level of 3-nitrotyrosine was significantly decreased by H_2 saline, indicating that it is an effective antioxidant that can reduce the ONOO−, thus further ameliorating the leukocyte activation. In addition, the administration of H_2 saline seemed to attenuate the increase in ICAM-1 expression and PMN infiltration in the myocardium subjected to I/R injury. ICAM-1, which is one of the most important adhesion molecules, is involved in the recruitment of circulating leukocytes into the myocardium and also in the development of subsequent myocardial cell damage after I/R injury [23,24]. ICAM-1-mediated leukocyte adhesion and subsequent infiltration into the infarct area could be responsible for cardiomyocyte damage via free radicals released [25]. Reduction in ICAM-1 transcription decreases PMN infiltration and inflammation. It was found in this study that H_2 saline alleviated the increase in ICAM-1 expression and PMN infiltration effectively, which further proves the anti-inflammatory effect of the H_2 saline. In addition, in this study we found that MPO activity was enhanced in the cardiac tissue subjected to I/R injury, and H_2 saline treatment decreased this activity effectively. MPO accounts for 5% of the dry weight of the neutrophil, so the number of neutrophils in the myocardium could also be estimated by MPO activity. Therefore, in this study, the decrease in MPO activity indicates the reduction of the neutrophils and attenuation of inflammation, which is consistent with our previous results.

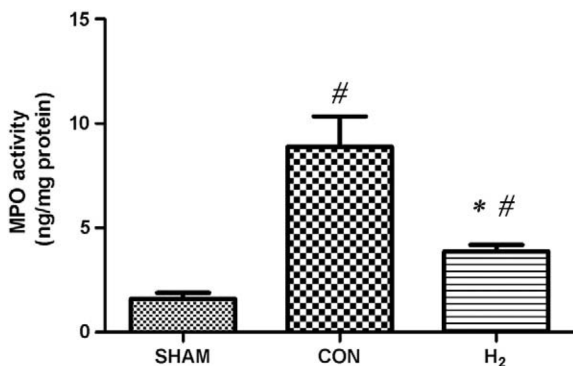

Fig. 7. Myocardial MPO activity by the end of 24-h reperfusion. H_2 significantly reduced MPO activity (n = 8, *$P<0.05$ compared with the control group; #$P<0.05$ compared with the sham group).

In addition, the levels of proinflammatory cytokines TNF-a and IL-b in the AAR could also be decreased by H_2 saline in the present study. Myocardial necrosis after AMI induces complement activation and free radical generation, triggering a cytokine cascade initiated by tumor necrosis factor-alpha (TNF-a) release. TNF-a and IL-1b, which are discharged from activated macrophages and neutrophils, both depress the cardiac function in dose-dependent fashions and synergistically reduce myocardial contraction [26]. So proinflammatory cytokines, which can regulate myocyte survival and induce additional cellular inflammatory responses, are implicated in the pathogenesis of myocardial dysfunction in I/R injury [27,28]. The severity of cardiac injury has been shown to correlate with TNF-a and IL-1b activity [29,30]. In the present study, H_2 saline significantly decreased them in the cardiac tissue, suggesting that the anti-inflammatory effect of H_2 saline on cardiac injury could be mediated by depression of TNF-a and IL-1b.

Together, these findings confirmed that H_2 saline has a cardioprotective effect against I/R injury and further suggest that H_2 saline exerts beneficial effects on the suppression of organ inflammation, which is parallel to the recovery of cardiac function. The anti-inflammatory effect of H_2 saline observed in other organs [18] also supports our finding. Whether the anti-inflammatory effect of H_2 saline is only secondary to its anti-oxidative effect needs to be further investigated.

Our present study mainly focused on the beneficial effects of H_2 saline on I/R injury. However, its effect on permanent myocardial infarction hasn't been identified which has the same importance. Theoretically it may be difficult for a single dose of H_2 saline to induce potent cardioprotection in animals who developed myocardial infarction and could not be reperfused. Since permanent myocardial infarction is a long course and needs additional time to recover, H_2 saline will provide protection only if stable H_2 concentration is provided in the blood.

The present study didn't include sacrifice of animals at different times after administration of H_2 saline, which needs further investigation. In addition, the hemodynamic effects of H_2 saline administration after ischemia reperfusion injury needs to be better described (e.g., cardiac index, ejection fraction), this is our study limitation.

However, this study shows that H_2 saline induced cardioprotection through its anti-inflammatory effect. Due to its efficacy, convenience and low cost, H_2 saline may prove to be a potential therapy for cardiac I/R injury in the future.

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