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Preconditioning with one-time hydrogen gas does not attenuate skin flap ischemia-reperfusion injury in rat models

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Summary *Background:* Hydrogen gas exists in the atmosphere and was previously considered an inert gas. It has been reported to have protective effects on tissue ischemia-reperfusion (IR) injuries in animal models. The protective mechanism of hydrogen molecules is based on selectively reducing highly strong oxidants in cells, thereby reducing inflammation and decreasing the contents of MDA, FOXO3a, and other pathways that result in flap necrosis. Previous studies were conducted with postconditioning with hydrogen. In this article, we want to investigate whether inhalation of hydrogen has a preventive effect on IR injury.
Methods: Forty-five adult male Sprague Dawley rats (body weight 220–250 g) were randomly divided into three groups: (1) Sham operation group (SH), (2) Ischemia-reperfusion injury group (IR), and (3) Ischemia-reperfusion injury with preconditioning hydrogen group (PRH). IR injury was induced by clamping the right superficial epigastric artery for 3 h. Before undergoing 3 h of IR management, the PRH group was treated with hydrogen inhalation for 1 h. On the third postoperative day, survival area and blood perfusion of the flap were assessed using laser Doppler flowmetry. RIP1 and RIP3 were examined by immunological detection and western blot analysis.
Results: Both the IR and PRH groups had less skin flap survival area and less blood perfusion than the sham group ($P < 0.05$). RIP1 and RIP3 were highly expressed in the IR and PRH groups when compared with those in the SH group ($P < 0.05$). There were no significant differences in flap survival rate (32.34 ± 2.19% and 33.09 ± 1.64%), average blood perfusion (41.66 ± 3.53 pu, 48.57 ± 2.83 pu), and expression of RIP1 and RIP3 (0.5167 ± 0.1409 and 0.4693 ± 0.1454) between the IR and PRH groups.

[1] Yan Hao and Xinhang Dong contributed equal to this study, they are co-first authors.

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Conclusions: Preconditioning with one-time inhaled hydrogen does not attenuate skin flap IR injuries in rat models.

Introduction

Ischemia-reperfusion (IR) injury is the major cause of skin flap necrosis in various flap transplantations. Events that occur are neutrophil-mediated endothelial cytotoxicity, activation of free radicals, triggering of cytokines and chemokines, and activation of adhesion molecules.[1]

Hydrogen gas exists in the atmosphere and was previously considered an inert gas. It is a small molecule that diffuses into tissues rapidly and disturbs metabolic redox reactions.[2] Studies in recent years have shown that hydrogen molecules are potential therapeutic agents; this finding has been confirmed in many animal experiments. Hydrogen can attenuate IR injury in the intestine, heart, brain, kidneys, lungs, spinal cord, and other structures.[3-12] In these studies, hydrogen was used either after ischemia and before reperfusion or after IR injury. The protective mechanism of hydrogen molecules is based on selectively reducing highly strong oxidants in cells, reducing inflammation, decreasing the contents of MDA and FOXO3a, and affecting other pathways that result in flap necrosis.[2,10,15] Currently, there are many easily obtained products containing hydrogen by inhalation of low- or high-dose hydrogen gas, drinking hydrogen-rich water, intraperitoneal injection, and infusion of hydrogen-rich saline.[13] In our previous experiments, the use of hydrogen-rich water after flap transplantation could effectively reduce IR injury in a rat flap model.[14] In this study, we aimed to investigate whether hydrogen can be protective before IR injury.

In recent studies, it has been confirmed that IR injury can lead to necroptosis, ultimately causing organ failure.[16] The role of necroptosis in the neuroprotection of hydrogen in a mouse model of cerebral IR injury was revealed by Jun-Long Huang.[17] The purpose of this study is to investigate whether preconditioning with hydrogen gas can improve the rat skin flap outcome against IR injury and to determine whether necroptosis is involved in IR injury Table 1.

Materials and methods

Experimental animals

All procedures were approved by the Ethics Committee for Animal Rights Protection at Peking Union Medical College Hospital and were performed in accordance with the National Institutes of Health (NIH) guidelines for the care and use of laboratory animals. Adult male Sprague Dawley (SD) rats weighing 220–250 g were obtained from Huafang Co., China. The rats were housed alone in cages (15 × 20 × 35 cm) under standard conditions at 22 °C to 25 °C with a 12-h light/dark cycle.

Table 1 Primary antibodies used for western blotting.

Antibody	Manufacturer	Product code	Species
RIP1	Abcam, Cambridge, UK	ab72139	Mouse
RIP3	Abcam, Cambridge, UK	ab56164	Rabbit

Primary antibodies were raised against the following proteins:RIP1, receptor interacting protein 1; RIP3, receptor interacting protein kinase 3.

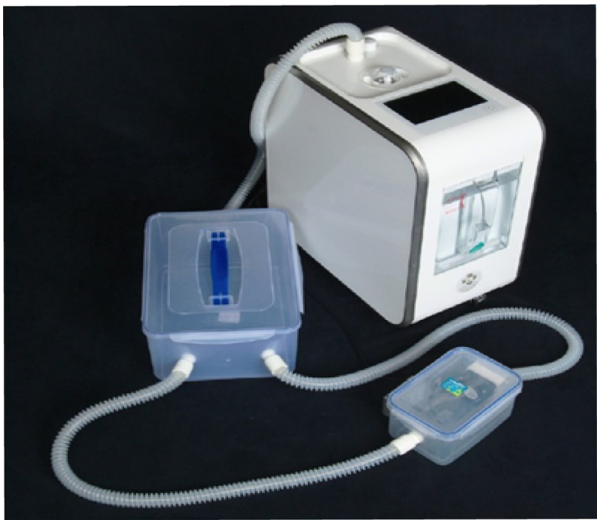

Figure 1 The AMS-H-01 hydrogen producer (Asclepius, Shanghai, China).Animals were held in the larger container(30 cm × 20 cm × 15 cm); the smaller box (18 cm × 12 cm × 7 cm) was used for moisture and carbon dioxide removal.

Study design

Forty-five male SD rats were divided randomly into three groups: (1) Sham (SH, $n = 15$), the left superficial epigastric artery was only ligated; (2) IR ($n = 15$): 3-h ischemia induced by clamping the right superficial epigastric artery, with the left superficial epigastric artery also ligated; and (3) preconditioning with inhaled hydrogen +IR (PRH=15), animals were treated with hydrogen gas for 1 h before the IR management.

Hydrogen gas was produced by an AMS-H-01 hydrogen producer (Asclepius, Shanghai, China). This machine produces hydrogen gas (66.7%) by ionizing water molecules (Figure 1).

Surgical procedures

Rats were anesthetized intraperitoneally with 10%, 1 ml/300 g chloral hydrate. A 5 cm x 7 cm rectangular

flap area on the abdomen was marked and well sterilized. In the IR and PRH groups, the right superficial epigastric artery of each rat was occluded with a microvascular clamp for three hours, while the left superficial epigastric artery was ligated. The SH group was free from IR induction, but the left superficial epigastric artery remained ligated. A silicone sheet of 0.1 mm thickness was then placed between the flap and the recipient bed to prevent revascularization.

Skin flap survival rates and blood perfusion measurements

Seventy-two hours after reperfusion, each rat was anesthetized and fastened to the operating table to expose the entire flap. A laser Doppler flowmeter (LDF, Perimed AB, Stockholm, Sweden) and a laser speckle contrast analysis system (LSCA, Perimed AB, Stockholm, Sweden) were used to measure the flap survival rates and the blood perfusion after the above management. Then, one piece of the flap tissue was collected from the proximal area of the vascular axis for hematoxylin and eosin staining, immunohistochemical studies, and western blot analysis.

Hematoxylin-eosin (HE) staining

The flap samples were paraffin-embedded, sectioned, and mounted onto slides for hematoxylin-eosin (HE) staining.

Immunohistochemical studies

Paraffin-embedded sections were routinely deparaffinized and rehydrated in xylene and different concentrations of ethanol. The sections were then incubated for 10 min with 3% H_2O_2 to inhibit endogenous catalase. The sections underwent antigen retrieval with sodium citrate buffer for 15 min and were then incubated with anti-RIP1 (1:200, Abcam, Cambridge, Britain) and anti-RIP3 (1:500, Abcam, Cambridge, Britain) antibodies at room temperature for 1 h. The sections were rinsed with PBS and incubated with an appropriate amount of horseradish peroxidase-conjugated secondary antibodies (ZSGB-BIO, Beijing, China) at 37 °C for 30 min. Then, the sections were rinsed with PBS. DAB was used as the chromogen. The slides were detected under 400x magnification by light microscopy with a digital camera and imaging software. The positive cells were colored brown. The number of positive cells was counted in each image. The data were calculated as the percentage of immunohistochemistry-positive cells of the total number of cells per image.

Western blot analysis

Tissue (30 mg) was rapidly sampled from the harvest flap on ice. Protein was extracted through the tissue with a cell lysis kit (Bio-Rad Laboratories, Hercules, CA). The protein samples were electrophoresed using 10% SDS-PAGE for 1.5 h at 100 V. Following transfer to nitrocellulose membranes, blots were blocked with BSA for 1.5 h. Beta Actin antibody (1:1000, ZSGB-BIO, Beijing, China), RIP1 antibody (1:1000, Abcam, Cambridge, Britain), and RIP3 antibody (1:1000, Abcam, Cambridge, Britain) were incubated overnight at 4 °C in blocking buffer. Blots were washed three times in TBS+0.05% Tween, followed by incubation with secondary antibodies (1:10000, LI-COR, Lincoln, NE) in the dark for 1 h at room temperature. Immunoblots were read on an Odyssey Imager (Odyssey, Lincoln, NE).

Statistical analysis

All data are presented as the means± standard deviations. Values between groups were determined by one-way analysis of variance (ANOVA). $P < 0.05$ was considered statistically significant. All analyses were performed using SPSS software (version 22; IBM Corp., Armonk, NY, USA) and GraphPad Prism (version 7; GraphPad Software Inc., La Jolla, California, USA).

Results

Flap survival rate

Seventy-two hours after reperfusion, necrotic tissues were inelastic and colored brown, gray, or black. In contrast, the survival areas were pink and very flexible. Both the IR and PRH groups showed high loss in total skin flap area. The mean flap survival rates in the SH, IR, and PRH groups were **72.09 ± 2.92%, 32.34 ± 2.19%, and 33.09 ± 1.64%,** respectively. There was no significant difference between the IR and PRH groups **($P = 0.818$)** (Figure 2, Table 2).

Average blood perfusion

The average blood perfusion amounts in the SH, IR, and PRH groups were **131.62 ± 8.13 pu, 41.66 ± 3.53 pu, and 48.57 ± 2.83 pu,** respectively. There was no significant difference between the IR and PRH groups **($P = 0.368$)** (Figure 3, Table 2).

Hematoxylin-eosin (HE) staining

Both the IR and PRH groups showed more shrunken and condensed cell nuclei and inflammatory cells than the SH group (Figure 4).

Immunohistochemical studies

The proportions of RIP1-positive cells in both the PRH **(65.89 ± 14.42%)** and IR groups **(81.8 ± 10.56%)** were significantly higher than that of the cells in the SH group **(27.33 ± 8.63%)**. There was no significant difference between the PRH and IR groups **($P = 0.0715$).** The proportion of RIP3-positive cells in the IR group **(83.9 ± 11.95%)**

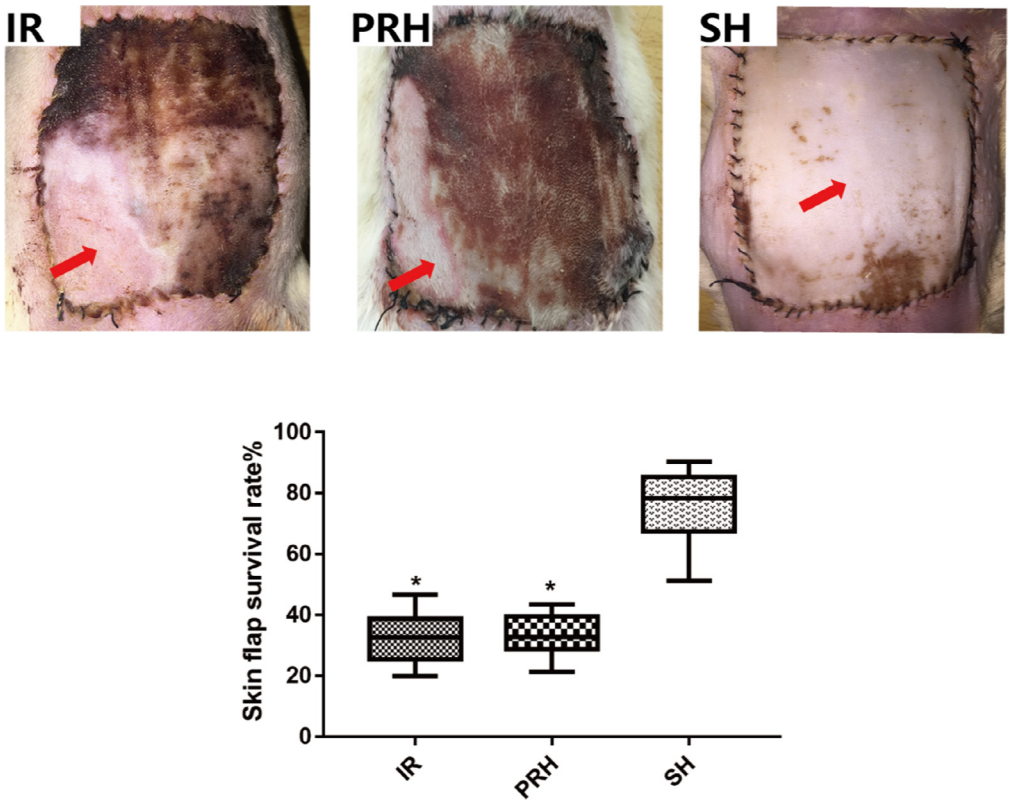

Figure 2 The representative pictures of skin flap survival area in three groups are shown respectively. The fields indicated by red arrows represent the survival areas of the flap. There was no significant difference between IR and PRH group. Both IR and PRH groups had less flap survival rate than SH group(*$P < 0.05$).

Table 2 Results in all groups.

		SH	IR	PRH	*P* value
FSR (%)		72.09 ± 2.92	32.34 ± 2.19*	33.09 ± 1.64*	IR vs PRH, $P = 0.818$
BP(pu)		131.62 ± 8.13	41.66 ± 3.53*	48.57 ± 2.83*	IR vs PRH, $P = 0.368$
PC (%)					
	RIP1	27.33 ± 8.63	81.8 ± 10.56***	65.89 ± 14.42***	IR vs PRH, $P = 0.0715$
	RIP3	15.11 ± 8.53	83.9 ± 11.95***	63.39 ± 16.3***	IR vs PRH, $P = 0.0337$
PRV					
	RIP1	0.1920 ± 0.0235	0.5167 ± 0.1409****	0.4693 ± 0.1454***	IR vs PRH, $P = 0.318$
	RIP3	0.3000 ± 0.1215	0.6827 ± 0.1333***	0.6580 ± 0.1706***	IR vs PRH, $P = 0.688$

Values are presented as means ± SEM. Group means were compared using one-way analysis of variance. FSR, flap survival rate; BP, blood perfusion; PC, positive cells; PRV, protein relative value.
* $P < 0.05$ vs. sham,.
*** $P < 0.001$ vs. sham.

was greater than that in the PRH group **(63.39 ± 16.3%, $P < 0.05$)**. The RIP3-positive levels were significantly higher in both the IR and PRH groups than in the SH group **(15.11 ± 8.53%, $P < 0.001$)** (Figure 5, Table 2).

Expression of the RIP1 and RIP3 proteins

RIP1 and RIP3 expression levels were examined by western blot. The relative protein levels of RIP1 in the SH, IR, and PRH groups were 0.2 ± 0.02, 0.5 ± 0.14, and 0.5 ± 0.15, respectively. The relative protein levels of RIP3 in the SH, IR, and PRH groups were 0.3 ± 0.12, 0.7 ± 0.13, and 0.7 ± 0.17, respectively. There were no significant differences in the levels of RIP1 **($P = 0.318$)** and RIP3 **($P = 0.688$)** between the IR and PRH groups. The RIP1 and RIP3 expression levels in both the IR and PRH groups were significantly greater than those in the SH group **($P < 0.001$)** (Figure 6, Table 2).

Discussion

This study demonstrated no benefit with inhaled hydrogen before IR injury in a rat skin flap model.

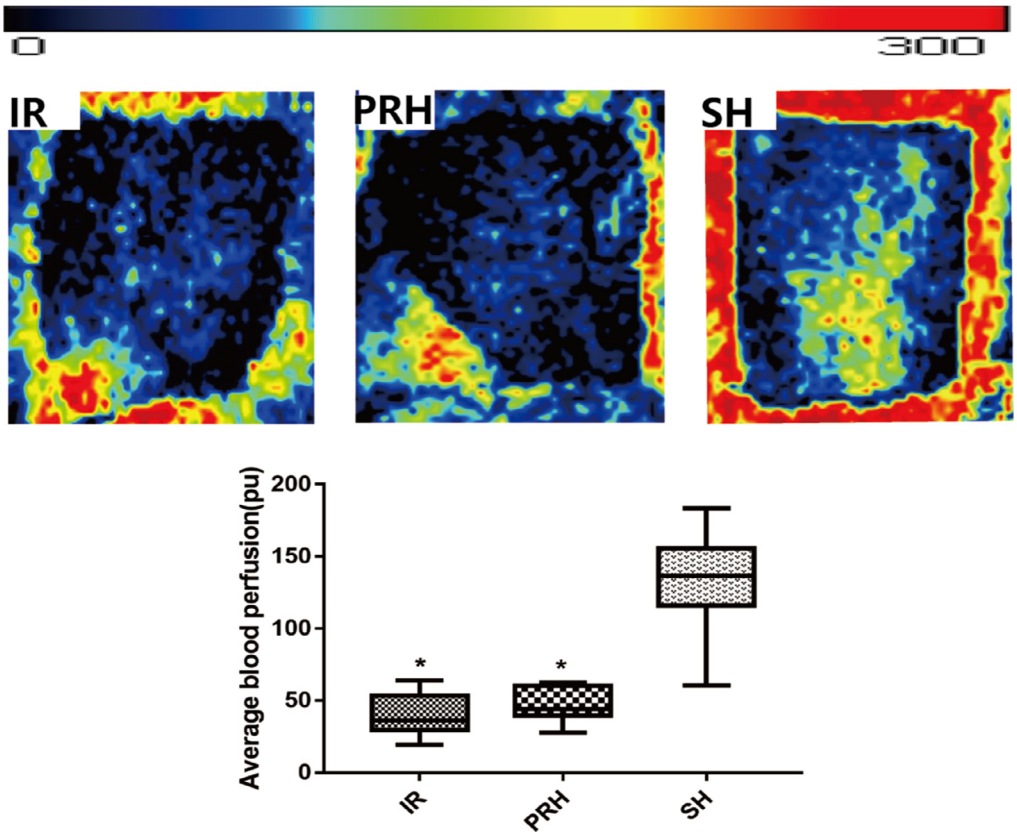

Figure 3 The representative pictures of average blood perfusion in three groups are shown respectively. There was no significant difference between IR and PRH group. Both IR and PRH groups had less blood perfusion than SH group($^{*}P < 0.05$).

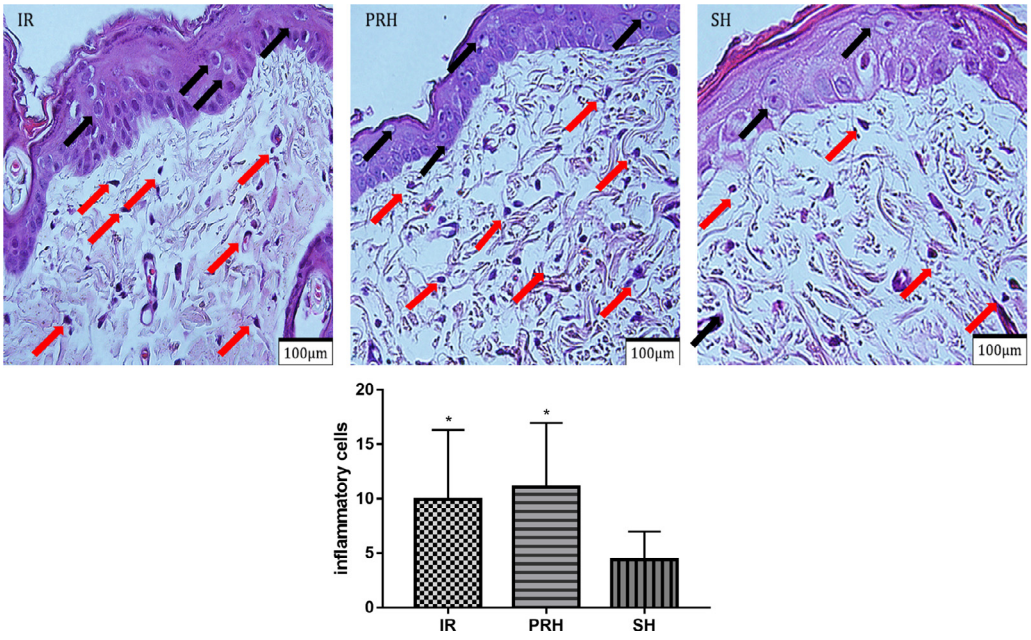

Figure 4 Morphologic observation (400×) of skin tissue with HE staining in all groups. Red arrow indicates inflammatory cells. Black arrow indicates condensed or fragmented cell nucleus. IR and PRH group showed more condensed, fragmented cell nucleus and inflammatory cells than SH group.

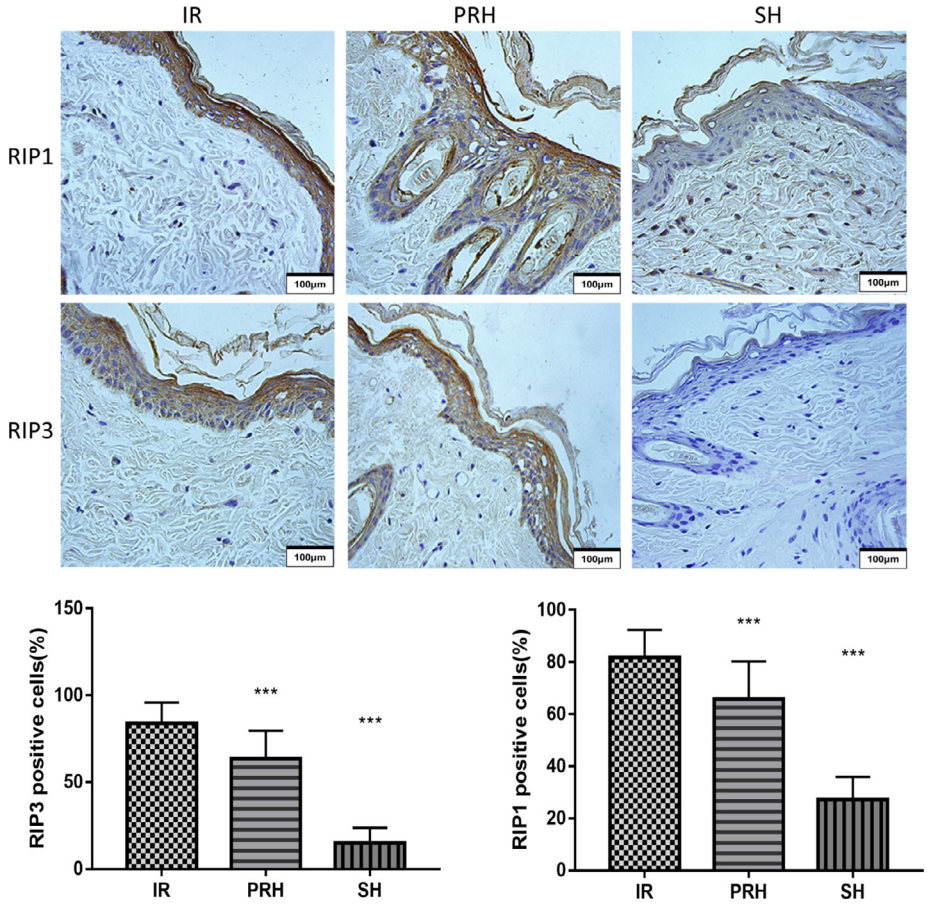

Figure 5 Representative micrographs (400X) of immunohistochemistry for RIP1and RIP3 of skin tissue from the three groups. Positive cells were colored in brown. The results showed that the levels of RIP1 and RIP3 in IR and PRH groups were higher than in the SH group($^{***}P < 0.001$).

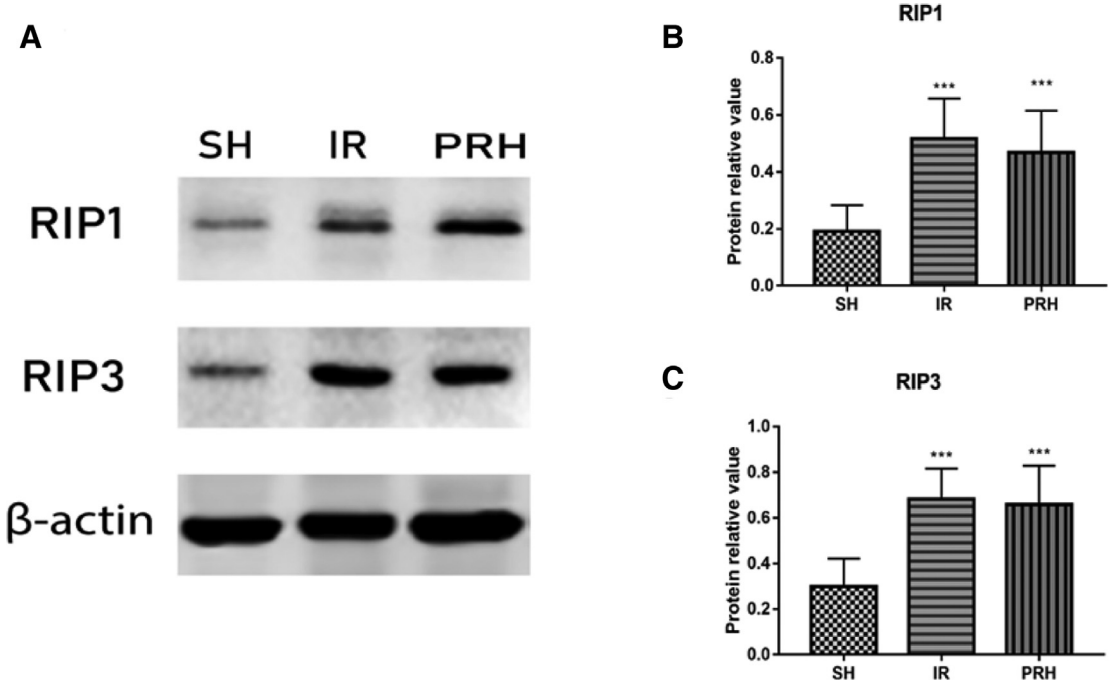

Figure 6 (A)Representative images of western blots for RIP1 and RIP3 from three groups. (B, C) Densitometry analysis of RIP1 and RIP3 protein levels based on the western blot results are showed. All values are expressed as mean ± SEM. ($n = 15$ for each group; $^{***}P < 0.001$ versus SH).

IR injuries on tissues and organs are common in clinical practice, especially in various transplant operations, such as kidney, lung, and liver, and in cardiac operations after cardiopulmonary bypass. In plastic and reconstructive surgery, flap grafting is essential for the coverage of large tissue defects, and with the development of microvascular anastomosis techniques, more flap transplantation surgeries can be performed. However, the failure and necrosis rates of postoperative skin flaps are still very high. Partial flap necrosis may be encountered in 7–20% of free flaps and in 20–33% pedicle flaps.[18] IR injury has been shown to play an important role in skin flap damage.[19] Current studies have reported that necroptosis is involved in the process of cardiac IR injury,[20,21] retina,[22] acute kidney injury,[23] and other injuries. Necroptosis is a form of regulated necrosis that leads to rapid plasma membrane permeabilization and to the release of cell contents and exposure to damage-associated molecular patterns (DAMPs).[16,24] RIP1 and RIP3 are two key factors that activate necroptosis.[25]

Previous studies have demonstrated that hydrogen molecules have a protective effect against IR injury[3–12] by antagonizing oxygen free radicals and reducing inflammatory factors to achieve the purpose of reducing tissue damage. Previous studies have focused on postconditioning with hydrogen after IR injury in the tissue. For the first time, this study discusses whether preconditioning with hydrogen has a protective effect against IR injury. In our experiment, hydrogen gas was produced from ionized water using an AMS-H-01 hydrogen producer, and the concentration of hydrogen in the container where the rats had been held was approximately 66.7%.[26] In previous studies, the inhaled hydrogen concentrations were 2%,[4] 1.3%,[27] and 3%.[28] The pretreatment time with hydrogen was set to 1 h. Considering an actual clinical situation, patients may not have enough time to be treated with inhaled hydrogen before undergoing skin flap transplantation.

Our results showed that there was no significant difference between the IR group and the hydrogen pretreatment group in terms of survival area rate and mean blood flow, which suggested that the pretreatment has no benefit on rat skin flap IR injury. We suspected that this lack of benefit may be related to the following reasons. First, hydrogen gas can permeate and escape tissues quickly; therefore, it has a very short retention time in the body and is present at extremely low concentrations when IR injuries occur. Second, hydrogen gas may be a short-acting agent with a short half-life, meaning it is easily metabolized; thus, its delaying effect against IR injury is limited. Apart from these considerations, the higher expression levels of RIP1 and RIP3 in the IR and PRH groups than in the SH group suggested that necroptosis was induced by IR. In addition, in our previous study, we found out that the tissue structure had been changed and that there were more capillaries after pretreatment with hyperbaric oxygen. However, we did not find the same phenomenon as that in the hydrogen-treated groups.

There were several limitations in this study. The negative outcome with hydrogen preconditioning may due to insufficient treatment time, suggesting that the rats did not have enough reaction time. In addition, the hydrogen concentration may be different in different routes of use, such as inhalation, ingestion, or injection. The accuracy of the half-life for in vivo research, the proper time for use, and the best mode of administration will require further study. Thus, we should seek an optimal solution for hydrogen application in clinical practice.

Conclusions

In summary, no protective effects were observed in response to preconditioning with hydrogen gas in a rat skin flap model against IR injury. These results will contribute to further studies of hydrogen application in the future, with the aim of preventing skin flap IR injury.

Acknowledgment

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Conflicts of interest

There are no conflicts of interest.

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