## Original Article Postconditioning with inhaled hydrogen attenuates skin ischemia/reperfusion injury through the RIP-MLKL-PGAM5/Drp1 necrotic pathway

Xin-Hang Dong<sup>4\*</sup>, Hao Liu<sup>1\*</sup>, Ming-Zi Zhang<sup>1</sup>, Peng-Xiang Zhao<sup>2</sup>, Shu Liu<sup>3</sup>, Yan Hao<sup>1</sup>, You-Bin Wang<sup>1</sup>

<sup>1</sup>Department of Plastic Surgery, Peking Union Medical College Hospital, Beijing 100730, China; <sup>2</sup>College of Life Science and Bioengineering, Beijing University of Technology, Beijing 100124, China; <sup>3</sup>Department of Plastic Surgery, China Meitan General Hospital, Beijing 100037, China; <sup>4</sup>Department of Plastic Surgery, Peking Union Medical College Hospital, Chinese Academy of Medical Sciences, Peking Union Medical College, Beijing 100730, China. \*Co-first authors.

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**Abstract:** This study explored the flap-protective effects of high concentrations of hydrogen (HCH) inhalation in a rat flap ischemia/reperfusion (I/R) injury model and the potential mechanism of necroptosis. Forty-five male Sprague-Dawley rats were randomly divided into three groups: SH, IR and HCH groups. After undergoing 3 h of I/R management, the surgery groups were treated with ambient air (SH and IR) and high concentrations of hydrogen (HCH). On the third postoperative day, blood perfusion in the flap was measured using Laser Doppler flowmeters. RIP1, RIP3, MLKL, PGAM5 and Drp1 were examined by immunological detection and RT-qPCR. Compared to the IR group, larger areas of the skin flaps from the SH and HCH groups survived and displayed more blood perfusion. RIP1, RIP3, MLKL, PGAM5 and Drp1 were expressed at high levels in the IR group, and their expression was significantly decreased in the HCH group. In the SH and HCH groups, the necrotic factors measured here showed similar expression levels, which were significantly lower than the levels in the IR group, indicating that HCH-mediated protective effects on rat skin I/R necrosis may be associated with the necrotic pathway.

Keywords: Skin flaps, ischemia/reperfusion, high concentrations of hydrogen, protective effects, necrotic pathway

#### Introduction

Surgical operations and various trauma often cause skin defects and deformities, and skin flap necrosis continues to be a significant problem in flap transplantation. Despite improvements in microsurgical techniques and equipment, flap loss remains the major operative complication. Ischemia/reperfusion (I/R) injury in the surgical skin flap is believed to be the pivotal factor that causes harmful changes within the tissue and vasculature, resulting in flap loss [1].

Various factors are involved in the process of I/R injury of the flap, including the activation of reactive oxygen free radicals, as well as the release of inflammatory factors and cell apoptosis. In recent years, necroptosis, an important alternative to cell death, has been increasingly mentioned as an important process in the

cell life cycle. Necroptosis, which is distinct from apoptosis, autophagy, necroptosis and pyroptosis, is a special type of cellular necrosis that is regulated by a particular molecular mechanism and contributes to a range of human diseases, including I/R injury. Necroptosis plays a crucial role in heart [2], brain [3], liver [4] and kidney [5] I/R injury.

Caspase-inhibited cells may be stimulated to undergo necroptosis rather than apoptosis [6] through the initiation of the necrotic pathway. RIP (receptor-interacting protein) kinase is a member of the Ser/Thr protein kinase family and has been identified as a key enzyme that regulates necroptosis [7], particularly RIP1 and 3. The interaction of RIP1 and 3 is required for necroptosis [8]. MLKL was initially identified as an essential mediator of RIP1/RIP3 kinase-initiated necroptosis [9], and its N-terminal CC domain and phosphorylated kinase region play crucial roles in modulating downstream necrotic effectors [10]. PGAM5, a mitochondrial phosphoglycerate mutase, is anchored in the outer mitochondrial membrane [11] and plays a vital role in the transmission of necrotic signals to mitochondria. Upon induction, PGAM5 recruits the mitochondrial fission factor Drp1 and induces its GTPase activity through dephosphorylation. Mitochondrial fission follows Drp1 activation, which is an early and obligatory step for the execution of the necroptosis pathway [12-15].

The protective effect of inhaled hydrogen was initially reported to significantly attenuate cerebral I/R injury by inhibiting oxidative stress. Based on accumulating evidence, hydrogen protects against I/R injury in the liver [16, 17], heart [18, 19], lung [20], skin [21], retina [22] and intestine [23]. Previous studies of the protective effects of hydrogen on I/R injury in flaps have mainly focused on the scavenging of hydroxyl radicals, suppression of inflammation and inhibition of apoptosis; however, researchers have not determined whether necroptosis is involved. Recently, high concentrations of hydrogen (HCH, 66.7% hydrogen and 33.3% oxygen) have been applied to many diseases in animal models [17, 22, 24]. HCH is produced by an AMS-H-01 hydrogen oxygen nebulizer (Asclepius, Shanghai, China), which generates H<sub>2</sub> and O<sub>2</sub> by electrolyzing water. The present study aims to evaluate the inhibitory effect of hydrogen on necroptosis and its ability to regulate the expression of RIP1, RIP3, MLKL, PGAM5 and Drp1 in a rat skin flap I/R injury model following inhalation of HCH.

## Materials and methods

## Animals and grouping

Forty-five male Sprague-Dawley rats aged 6-8 weeks and weighing 280-320 g were housed in comfortable cages at 22-25°C with adequate food and water. All procedures were strictly performed according to the National Institutes of Health Guide for the Care and Use of Laboratory Animals. This study was performed in accordance with the Experimentation Ethics Committee on Animal Rights Protection of Peking Union Medical College Hospital. Before surgery, rats were randomly divided into three groups: SH: sham surgery group treated with ambient air (n=15), IR: 3-h I/R group treated with ambient air (n=15), and HCH: 3-h I/R group treated with HCH (n=15).

## HCH management

Immediately after I/R, animals in the HCH group were placed in a comfortable 40×26×17 cm chamber equipped with a round inlet and an outlet and flushed with HCH (66.7% hydrogen and 33.3% oxygen), which was produced with the AMS-H-01 hydrogen producer (Asclepius, Shanghai, China). The AMS-H-01 hydrogen producer replaced the air inside the chamber for 5 minutes after the rat was placed in it. The HCH treatment lasted for 60 min at normal pressure.

## Surgical procedure

This surgical procedure was based on the method reported by Küntscher [25], with some modifications [21, 26]. Rats were intraperitoneally anesthetized with 10%, 1 ml/300 g chloral hydrate. A 6 cm×9 cm rectangular flap area on the abdomen was marked and well sterilized. In the IR and HCH groups, the right superficial epigastric artery of rats was clamped with a microvascular clamp for three hours, whereas the left superficial epigastric artery was ligated. The SH group was not subjected to ischemia, but the left superficial epigastric artery remained ligated. A 0.1-mm-thick silicone sheet was then placed between the flap and the recipient bed to prevent revascularization. Reperfusion was initiated by removing the clamp and was confirmed by the return of pulsation to the vascular arcade.

## Evaluation of skin flap perfusion

Seventy-two hours after reperfusion, a laser Doppler flowmeter (LDF, Perimed AB, Stockholm, Sweden) and laser speckle contrast analysis (LSCA, Perimed AB, Stockholm, Sweden) were used to measure flap perfusion after the procedures described above. All rats were anesthetized and then fastened to the operating table to expose the entire flap. Then, capillary blood flow in the dermal layer of the rats was measured using the LDF. Perfusion in the necrotic (black areas) and surviving (red, yellow and the adjacent blue areas) areas of the abdominal flap were automatically obtained by delineating the specific area in the image using the LSCA. Vascular flow was measured as perfusion units (PUs) (ml·100 g<sup>-1</sup>·min<sup>-1</sup>).

Target gene	ID	Forward	Reverse	
RIP1	306886	CCAGCCAGCCAAATCAAAGT	TGGCTTACACTTGGCCCATA	
RIP3	246240	CGTGGAGCAGTGTGTAACAG	GGCTCAGAACTCCAGCAATG	
MLKL	690743	TAGTCCTGAGGGCAGCTAGA	CTGCTGATGTTTCCGTGGAG	
PGAM5	288731	AGGCTGGACCACTATAAGGC	TCCTTCTCCATCGAGCCATC	
Drp1	114114	TGATGCCTGTGGGCTAATGA	GTTCCTGACCACCATCTCCA	

Table 1. Sequences of primers used for quantitative real-time PCR

Seventy-two hours after reperfusion, rats were sacrificed by the administration of a high-dose anesthesia, and one piece of the flap tissue  $(1 \times 1 \text{ cm}^2 \text{ in size})$  was removed from the proximal area of the vascular axis of the flap for hematoxylin and eosin staining, immunohistochemical studies, western blot analysis, and RNA extraction.

## Hematoxylin and eosin (H&E) staining

The specimen was embedded in paraffin, sectioned, and mounted on a slide. Then, slides were stained with H&E for histological examinations.

## Immunohistochemical studies

Paraffin-embedded sections were routinely dewaxed, rehydrated and then incubated with 3% H<sub>2</sub>O<sub>2</sub> for 10 min to block endogenous catalase activity. Antigen retrieval was performed by heating the unstained slides in citrate buffer at 95°C for 15 min. An incubation with normal goat serum at 37°C for 30 min blocked nonspecific staining. Sections were placed in a humidified chamber and incubated with anti-RIP1 (1:200, Abcam, Cambridge, UK), anti-RIP3 (1:500, Abcam, Cambridge, UK), anti-MLKL (1:200, Abcam, Cambridge, UK), anti-PGAM5 (1:200, Abcam, Cambridge, UK), or anti-Drp1 (1:100, Abcam, Cambridge, UK) antibodies for 2 h at 37°C. Horseradish peroxidase-conjugated secondary antibodies (ZSGB-BIO, Beijing, China) were used to label the primary antibodies. Then, samples were washed with PBS, stained with DAB, and counterstained with hematoxylin. A brown color implied the presence of an antibody bound to antigen and was detected by light microscopy using a computer-controlled digital camera and imaging software.

## Western blot analysis

Western blotting was used to examine the levels of the RIP1, RIP3, MLKL, PGAM5 and Drp1

proteins 72 h after reperfusion. The tissue was rapidly weighed on ice and then proteins were extracted with a cell lysis kit (BioRad Laboratories, Hercules, CA). Samples were homogenized on ice for 10 min in lysis buffer (246  $\mu$ L of lysis buffer, 1.25  $\mu$ L of

phosphatase inhibitors, 0.25 µL of protease inhibitors, and 2.5 µL of PMSF) and then centrifuged at 14,000 rpm for 15 min. For western blotting, equal amounts of protein supernatants (60 µg) were separated on 10% SDS-PAGE gels and transferred to nitrocellulose membranes for immunoblotting. The membrane was blocked with blocking buffer (LI-COR. Lincoln, NE) for 2 h at room temperature and then incubated with either anti-RIP1, anti-RIP3, anti-MLKL, anti-PGAM5 or anti-Drp1 (1:500, Abcam, Cambridge, UK) antibodies for 12 hours at 4°C. Membranes were then incubated with a 1:10,000 dilution of secondary antibodies (LI-COR, Lincoln, NE) for 1 h at room temperature in the dark and then detected with a double-color infrared laser imaging system (Odyssey, Li-cor, Lincoln, NE).

## RNA isolation and RT-qPCR

Total RNA was extracted using the RNeasy Fibrous Tissue Mini Kit (Qiagen, Düsseldorf, Germany), according to the manufacturer's instructions. The concentration of the extracted RNA was determined using a UV spectrophotometer (Thermo, Waltham, MA, USA) and the integrity of the RNA was visualized by electrophoresis on 1% agarose gels. Reverse transcription of 1 µg total RNA was performed to synthesize cDNAs using the ProtoScript M-MuLV First Strand cDNA Synthesis Kit (New England Biolabs, Ipswich, MA, USA) and an anchored oligo d(T) primer [d(T)23VN]. PCR was performed using a Real-Time qPCR System (Agilent, Santa Clara, CA, USA). Sequences of the specific primer sets for RIP1, RIP3, MLKL, PGAM5, Drp1 and  $\beta$ -actin used in this study are presented in Table 1. The expression of target genes was normalized to the levels of the β-actin gene as an internal housekeeping control. Real-time PCR cycle parameters included UDG pretreatment at 50°C for 2 min, initial denaturation at 95°C for 10 min followed by 40 cycles of denaturation at 95°C for 15 s, anneal-



**Figure 1.** The condition of the abdominal skin flaps 72 h after I/R injury. The black zones represent the areas with the lowest blood perfusion. The red, yellow and adjacent blue areas represent surviving areas with rich blood perfusion. A. Representative photographs of abdominal skin flap microcirculation in the three groups are shown. B. The survival rate of the total flap area. Markedly higher flap survival rates were observed in the SH and HCH groups. C. The average blood perfusion of total skin flaps. A greater average blood perfusion value for the total flap was observed in the HCH group than in the IR group. Values are presented as means  $\pm$  SD (n=15 animals per group; \*\*\*P < 0.001 compared with the SH group; comparison of the HCH and IR groups, ###P < 0.001).



**Figure 2.** Images of H&E staining 72 h after IR injury. Observations  $(400 \times)$  of the morphology of the flap tissue in all groups using H&E staining. Fewer infiltrating cells (red arrow) were observed in the SH and HCH groups than in the IR group.

ing at the Tm for 1 min and extension at  $72^{\circ}$ C for 30 s.

#### Statistical analysis

In this study, all data are reported as means  $\pm$  standard deviation. Significant differences were determined using one-way analysis of variance (ANOVA) followed by the LSD-t test. Statistical significance was set to P < 0.05. All analyses were conducted using SPSS 24.0 software.

#### Results

#### Flap survival rate

Necrotic flap tissues were inelastic and brown, gray, or black in color on the third postoperative day. In contrast, the surviving tissues were pink and elastic (Figure **1A**). Results of the statistical analysis are shown in Figure 1B. In the SH group, the flap survival rate was 90.874 ± 4.520%, which was the highest rate among the three groups. Compared with the IR group (17.580 ± 3.442%), the flap survival rates of the SH and the HCH groups (55.151 ± 4.014%) were significantly increased (IR vs. SH, P < 0.001; IR vs. HCH, P < 0.001; Figure 1B).

#### Flap perfusion

On the third day after surgery, the average blood perfusion in the SH group was  $186.512 \pm 29.340$  PU (mL·100 g<sup>-1</sup>·min<sup>-1</sup>). The average blood perfusion was  $57.040 \pm 8.931$  PU in the IR group and  $108.451 \pm$ 15.348 PU in the HCH group. Significant differences were observed between



**Figure 3.** Representative micrographs (400×) of immunohistochemical staining for RIP1, RIP3, MLKL, PGAM5 and Drp1 in the skin tissues from all groups are presented above. Brown staining indicates areas with positive expression, and the shade of the color represents the expression level of the target protein. Lower levels of RIP1, RIP3, MLKL, PGAM5 and Drp1 were detected in the skin tissues from rats in the HCH group than rats in the IR group.



Figure 4. Levels of the RIP1, RIP3, MLKL, PGAM5 and Drp1 proteins. A. Representative images of western blots for RIP1, RIP3, MLKL, PGAM5 and Drp1 are

shown, B. Densitometry analysis of levels of the RIP1, RIP3, MLKL, PGAM5 and Drp1 proteins; the results are consistent with the immunohistochemical staining. Values are presented as the means ± SD (\*\*\*P < 0.001 and \*P < 0.05 compared with the SH group, ###P < 0.001). Levels of the RIP1, RIP3, MLKL, PGAM5 and Drp1 mRNAs in skin flap samples were analyzed. C. Compared to the IR group, levels of the RIP1, RIP3, MLKL, PGAM5 and Drp1 mRNAs were reduced in the HCH group.

the IR and SH groups (P < 0.001) and between the IR and HCH groups (P < 0.001; Figure 1C).

#### H&E staining

H&E staining was used to assess ischemic injury (**Figure 2**) by evaluating inflammatory infiltration. Inflammatory infiltration was observed in all the three groups, but the number of inflammatory cells was greatest in the IR group skin

Factor —	Percentage of positive cells (%)			Divoluo
	SH	IR	HCH	Pvalue
RIP1	0.2561 ± 0.0219	0.8589 ± 0.0257	0.3716 ± 0.0097	HCH vs. IR, P < 0.001
RIP3	0.4221 ± 0.0206	0.8932 ± 0.0127	0.6068 ± 0.0239	HCH vs. IR, P < 0.001
MLKL	0.1976 ± 0.0212	0.4521 ± 0.0213	0.2261 ± 0.0129	HCH vs. IR, P < 0.001
PGAM5	0.1533 ± 0.0166	0.5968 ± 0.0422	0.1957 ± 0.0193	HCH vs. IR, P < 0.001
Drp1	0.2114 ± 0.0156	0.6157 ± 0.0217	0.2989 ± 0.0279	HCH vs. IR, P < 0.001

Table 2. Percentage of positive cells in immunofluorescence staining analysis

Percentage of immunofluorescence staining positive cells in all groups. The highest percentages of RIP1, RIP3, MLKL, PGAM5, and Drp1 positive cells are shown in the IR group. Values are means ± SD.

Table 3. Protein relative levels in all groups

Factor —		Protein relative level		
	SH	IR	HCH	Pvalue
RIP1	0.1553 ± 0.0116	0.7625 ± 0.0137	0.2835 ± 0.0129	HCH vs. IR, P < 0.001
RIP3	0.5828 ± 0.0358	0.9230 ± 0.0102	0.5403 ± 0.0142	HCH vs. IR, P < 0.001
MLKL	0.1528 ± 0.0180	0.3375 ± 0.0236	0.1873 ± 0.0099	HCH vs. IR, P < 0.001
PGAM5	0.1710 ± 0.0110	0.6365 ± 0.0591	0.1833 ± 0.0240	HCH vs. IR, P < 0.001
Drp1	0.2005 ± 0.0181	0.5465 ± 0.0258	0.1560 ± 0.0250	HCH vs. IR, P < 0.001

Values are presented as the means  $\pm$  SD.

tissue, which indicated that HCH may attenuate the inflammatory response.

## Expression of the RIP1, RIP3, MLKL, PGAM5 and Drp1 proteins after IR injury

Immunological detection studies reflect the expression of target proteins, such as RIP1, RIP3, MLKL, PGAM5 and Drp1, which have been shown to be key proteins involved in necroptosis. The RIP1, RIP3, MLKL, PGAM5 and Drp1 proteins were expressed at higher levels in the IR group than in the SH and HCH groups, as visualized by western blotting (Figure 4; Table 3). Immunohistochemical staining (Figure 3) showed similar results to the western blot assay. Table 2 shows the percentage of positively stained cells as analyzed via immunofluorescence. There is a significant difference for RIP1, RIP3, MLKL, PGAM5 and Drp1 between the HCH and IR groups (P < 0.001).

# Expression of the RIP1, RIP3, MLKL, PGAM5 and Drp1 mRNAs

Seventy-two hours after I/R, levels of the RIP1, RIP3, MLKL, PGAM5 and Drp1 mRNAs in HCH group were  $0.7262 \pm 0.0378$ ,  $0.5378 \pm 0.0540$ ,  $0.7952 \pm 0.1087$ ,  $0.7278 \pm 0.1020$  and  $0.4703 \pm 0.0747$ , respectively, compared with the IR group (Figure 4C).

## Discussion

In this study, we investigated the protective effects of HCH on a rat model. After skin flap I/R injury, inhalation of hydrogen at a high concentration (66.7% H<sub>2</sub>) significantly improved the flap survival rate and inhibited the necroptosis of cells in the skin flap after I/R injury. In addition, the anti-necroptosis effect of HCH was at least partially associated with the decreased levels of RIP1, RIP3, MLKL, PGAM5 and Drp1, based on the results from immunohistochemical staining, western blot and RT-PCR analyses. Therefore, HCH shows promise as a novel anti-necroptosis agent for attenuating skin flap I/R injury.

Hydrogen is the smallest molecule, has a high diffusion speed and is non-toxic; it has also been reported to efficiently protect against I/R injury [17, 21, 27]. In addition, hydrogen can quickly reach relatively high concentrations due to its favorable distribution characteristics, enabling it to easily penetrate biomembranes. Moreover, excess hydrogen is eliminated from the body, leaving no side effects [28]. For security reasons, inhaled hydrogen was typically administered at a low concentration ( $\leq 4\%$ ) in previous studies [16, 29]. The AMS-H-O1 hydrogen producer, a new hydrogen and 33.3%

oxygen) by electrolyzing water and has been verified in many experiments [22, 27], was well applied in this experiment. Postconditioning with inhaled HCH attenuates I/R injury [22, 27], consistent with the results our study; these findings are related to the anti-inflammatory, anti-oxidant, and anti-apoptosis effects of hydrogen. Both low and high concentrations of hydrogen have been confirmed to exert protective effects on I/R injury; however, researchers have not clearly determined whether necroptosis is involved in this process.

Regarding flap ischemia/reperfusion injury, previous studies on the role of hydrogen have mainly focused on of its ability to inhibit oxidative stress, inflammation and apoptosis, whereas necroptosis and the signal transduction pathway have rarely been examined in this field. Necroptosis is related to apoptosis [30-32]. Necroptosis is a form of regulated and programmed necrosis [9] characterized by cellular organelle swelling, a loss of integrity of the cell membrane, and leakage of cell contents. In recent years, considerable research has supported the importance of necroptosis in the development of inflammatory diseases and several forms of cancer [33-35]. The complicated mechanisms by which certain factors, including I/R injury, induce necroptosis have been illustrated in recent studies [10, 12, 30, 31]. Upon the inhibition of caspase-8 activity, RIP3 and RIP1 interact through their respective homotypic interaction motif (RHIM) domains [36], which is located in the C-terminus of RIP1. RIP3 then undergoes auto-phosphorylation at the serine 227 site [10], an event that leads to the recruitment of a pseudokinase, MLKL [31], which interacts with RIP1/RIP3 to form a necrosome. The necrosome binds to PGAM5 and subsequently recruits and activates the mitochondrial splitting factor Drp1 [12], eventually leading to mitochondrial fragmentation and cell death.

RIP1 and 3 are two key proteins that activate necroptosis. After initiation, necroptosis requires a series of signal transmission events and the execution of death signals. RIP1 is viewed as a protein at the top of the pyramid during the process [37], and RIP3 acts as a molecular switch between TNF-induced apoptosis and necroptosis [38]. MLKL activation is mediated by receptor-interacting kinase-3 (RIPK3), a component of the necrosome. Activated MLKL

forms membrane-disrupting pores that cause membrane leakage, thus extending the prototypical concept of morphological and biochemical events [9]. Mitochondria, which are interconnecting cellular organelles that undergo dynamic changes, represent the key organelle in which HCH exerts its protective effect on reducing ischemia/reperfusion injury. The mitochondria are not only a responder but also may be the trigger of many signal transduction pathways, including cell necroptosis [12, 21]. Therefore, the translocation of the necrosome to mitochondria-associated membranes is essential for necroptosis signal transduction. Two isoforms of PGAM5 are located downstream of the necrosome and specifically associate with the necrosome after induction of necroptosis, resulting in PGAM5 phosphorylation and activation [12]. The recruitment of PGAM5 and Drp1 cause mitochondrial fission. Once bound to mitochondria, Drp1 assembles into spirals at division sites around the outer mitochondrial membrane and contributes to the process of mitochondrial fission [39]. As shown in our previous study, Necrostatin-1, a specific inhibitor of necroptosis [3], exerts a protective effect on I/R injury by inhibiting RIP1 in the skin island flap model [40].

Based on the abovementioned theories, our study focused on the application of HCH and revealed its distinctive effect on protecting a rat skin flap from I/R injury by inhibiting necroptosis. Based on our results, the administration of a high hydrogen concentration protected against flap ischemia/reperfusion injury, characterized by reductions in the ischemic area and the alleviation of cell necroptosis. In the presence of HCH postconditioning, the ischemic area was significantly increased, and the expression of the abovementioned factors RIP1, RIP3, MLKL, PGAM5 and Drp1 was significantly decreased, compared with IR group, suggesting that the protective effects of HCH are associated with the activation of the necroptosis signal transduction pathway. In the present study, a new theory was established to explain the mechanism by which HCH treated skin flap ischemia/reperfusion injury. This theory provides a new perspective on methods for alleviating ischemia/reperfusion injury during skin flap transplantation and the development of new treatment strategies. Regarding the limitations of our study, the detailed molecular mechanisms of the flap-protective effects of

HCH on necroptosis have not been fully disclosed in this experiment, and we will explore the detailed mechanism in future studies.

## Conclusions

In the present study, HCH suppressed skin ischemia/reperfusion injury and increased the skin flap survival rate by attenuating cell necroptosis. Furthermore, we provided novel evidence that the anti-necroptosis effect of hydrogen may be associated with regulatory effects on RIP1, RIP3, MLKL, PGAM5 and Drp1 expression. These results may be helpful for elaborating the therapeutic mechanism of hydrogen.

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## Disclosure of conflict of interest

None.

Address correspondence to: You-Bin Wang, Department of Plastic Surgery, Peking Union Medical College Hospital, Shuaifuyuan 1#, Dongcheng District, Beijing 100730, China. Tel: 010-69152712; Fax: 69152712; E-mail: wybenz@sina.com

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