



Effects of intraperitoneal hydrogen injection on nitric oxide synthase mRNA and malondialdehyde following limb ischemia-reperfusion in rabbits

Dai-Jun WANG¹, Hua TIAN², Bao-Xiang ZHUANG³, Hong-Juan WU³

¹Weifang Medical University, Department of Human Anatomy, Weifang, Shandong, China

²Weifang Medical University, School Hospital, Weifang, Shandong, China

³Weifang Medical University, Department of Morphological Laboratory, Weifang, Shandong, China

Objective: The purpose of this study was to investigate the effects of intraperitoneal hydrogen (H₂) injection on the mRNA expression levels of inducible nitric oxide synthase (iNOS) and endothelial nitric oxide synthase (eNOS) as well as the serum malondialdehyde (MDA) level in a rabbit model of limb ischemia-reperfusion (I/R)-induced skeletal muscle injury.

Methods: To establish the hind limb I/R animal model, 30 rabbits were randomly assigned to one of 3 groups: sham, I/R, and ischemia-reperfusion + H₂ (IRH). An intraperitoneal injection of H₂ was given to the IRH group, while an equivalent amount of air was given to the sham and I/R groups. At 3, 6, 12, and 24 h after reperfusion, serum MDA level as well as skeletal muscle iNOS and eNOS mRNA expression levels were determined.

Results: Both iNOS mRNA expression and serum MDA levels were higher in the I/R group than the sham group ($p < 0.01$) and lower in the IRH group than the I/R group ($p < 0.01$, $p < 0.05$, respectively) at various time points after reperfusion. The eNOS mRNA expression level exhibited no significant difference between the I/R and sham groups after reperfusion but was significantly higher in the IRH group than in the sham group ($p < 0.01$, $p < 0.05$, respectively).

Conclusion: During the I/R process, the expression of iNOS mRNA was up-regulated along with an increase in MDA. Intraperitoneal injection of H₂ can down-regulate iNOS mRNA expression and up-regulate eNOS mRNA expression in the I/R process, suggesting a protective effect of H₂ in I/R-induced skeletal muscle injury.

Keywords: Endothelial nitric oxide synthase mRNA; hydrogen; inducible nitric oxide synthase mRNA; ischemia-reperfusion; malondialdehyde.

Limb ischemia-reperfusion (I/R) injury is commonly observed in surgeries involving the bones or vasculature and those resulting from trauma. These processes result in a series of physiological reactions that eventually cause injury or necrosis to histiocytes. Furthermore, remote

organs can also be impaired, and severe life-threatening complications occasionally occur following limb I/R.^[1–3] Tremendous effort has been focused on the study of I/R, but the mechanism still is not fully understood. Currently, no effective treatment exists for this condition.

Correspondence: Dai-Jun Wang, MD. Weifang Medical University, Department of Human Anatomy, Weifang, Shandong, China.

Tel: +86 536 8462023 e-mail: daijunwang@yahoo.com

Submitted: February 14, 2015 **Accepted:** May 17, 2015

©2015 Turkish Association of Orthopaedics and Traumatology

Available online at
www.aott.org.tr
doi: 10.3944/AOTT.2015.14.0296
QR (Quick Response) Code



In the 1980s, Palmer^[4] and Moncada et al.^[5] discovered that nitrogen monoxide (NO) shared similar characteristics with endothelium-derived relaxing factor (EDRF) and eventually proved that they were in fact the same. EDRF is a novel carrier for intercellular communication, and the decreased release of EDRF is an important factor in I/R injury. NO is produced from nitric oxide synthase (NOS)-catalyzed L-arginine (L-Arg) *in vivo*. NOS thereby plays an important regulatory role in NO production. The two isoforms of NOS include constitutive NOS (cNOS) and inducible NOS (iNOS); cNOS can be further categorized as endothelial NOS (eNOS) and neuronal NOS (nNOS). The process of cNOS-catalyzed NO formation provides functions such as the dilation of blood vessels and the inhibition of thrombosis. However, excessive NO produced by iNOS and related metabolites could lead to severe histiocyte injury.^[6–8]

In recent years, the biological application of hydrogen (H₂) has been studied extensively.^[9] Hydrogen is the simplest element in nature; H₂ is a colorless, odorless, tasteless diatomic gas of hydrogen with certain reducibility. In the past, many scholars suggested that H₂ is a physiologically inert gas. In 2007, the Japanese researchers Ohsawa et al.^[10] discovered that the inhalation of 2% H₂ could effectively reduce the area of cerebral infarction and improve prognosis of stroke in a mouse model. H₂ was suggested to have a selective antioxidation effect. Subsequently, various animal experiments confirmed the protective effect of H₂ on various tissues and organs subjected to I/R-induced injury.^[11–13] Primary H₂ administration methods include inhalation,^[10] intravenous or intraperitoneal injection of hydrogen-rich saline,^[14,15] and consumption of hydrogen water.^[12,16] In addition, intraperitoneal injections of H₂ have been performed successfully by a few researchers.^[8] To date, the protective effect of H₂ on I/R-induced limb injury has not been adequately studied. In this study, we applied the intraperitoneal injection of H₂ to an I/R rabbit model; the mRNA expression levels of iNOS and eNOS in skeletal muscles as well as serum malondialdehyde (MDA) levels were determined to investigate the protective effect of H₂ on I/R-induced limb injury and the potential underlying mechanisms.

Materials and methods

The primary kits and equipment used in this study are as follows: an *in situ* molecular hybridization kit for iNOS and eNOS mRNA determination (Wuhan Boster Bioengineering Co., Ltd., Wuhan, China); an MDA test kit (Nanjing Jiancheng Bioengineering Institute, Nanjing, China); an M177021 hydrogen gas generator (Beijing

West Broad Technology Co., Ltd., Beijing, China); a TDZ5-WS centrifuge (Changsha Xiangzhi Centrifuge Instrument Co., Ltd., Changsha, China); a TU-1810 UV-Vis spectrophotometer (Beijing Purkinje General Instruments, Beijing, China); and a MetaMorph/DP10/BX41 microscopy image analysis system (UIC/Olympus, USA/Japan).

H₂ of 99.999% purity was produced by the electrolysis of water using a hydrogen gas generator. The H₂ produced was stored in aseptic soft plastic infusion bags and was used within 12 h.

This study was carried out in strict accordance with the recommendations of the Provisions and General Recommendations of Chinese Experimental Animal Administration Legislation. The protocol was approved by the Weifang Medical University Local Ethics Committee. All surgery was performed under 20% urethane anesthesia, and all efforts were made to minimize suffering.

Thirty healthy male adult New Zealand white rabbits weighing 2.21±0.13 kg were supplied by the Experimental Animal Center of Shandong University. The rabbits were fed in an SPF-grade animal house in separate cages for a week after purchase. The feeding environment was set at 25 °C and 40–60% humidity. Prior to the experiment, the animals fasted for 12 h but had free access to drinking water.

Thirty rabbits were randomly assigned to 3 groups (10 animals/group): the sham surgery group (sham), the ischemia-reperfusion group (I/R), and the ischemia-reperfusion + H₂ group (IRH). The hind limb I/R injury animal model was established according to the method described by Nanobashvili et al.,^[17] which has also been described in our previous studies.^[18] Rabbits were anesthetized with a 0.20% urethane injection at 5 mL/kg through the auricular vein. The right inguinal region was carefully sheared without harming the skin, sterilized, and covered with an aseptic hole towel. A skin incision was performed along the femoral vascular nerves, and the exposed femoral artery, veins, and femoral nerves were separated under a surgical microscope. A micro vessel clamp was used to block the femoral artery for animals in the I/R and IRH groups. A cerclage procedure was performed with a rubber compression cord to block collateral circulation at the hind limb proximal end, thereby ensuring complete devascularization of the right hind limb. The micro vessel clamp and the rubber compression cord were removed after 2 h to restore blood flow. The animals in the sham group received only the skin incisions at the right inguinal region to expose the femoral vessels; there was no femoral artery clamping or cerclage of the hind limb.

Ten minutes before blood flow was restored by removing the vascular clamp, an intraperitoneal injection of H_2 was given at 10 mL/kg to the animals in the IRH group within 60 s. An equivalent amount of air was given to the animals in the other groups.

Femoral vein blood (0.5 mL) was drawn from each animal in the 3 groups at 3, 6, 12, and, 24 h after reperfusion. Serum was separated from the blood by centrifugation at 2,500 rpm for 10 min and stored in test tubes at -20°C for subsequent MDA measurement.

Skeletal muscle tissue with an area of $2 \times 1 \times 0.5 \text{ cm}^3$ was excised from the anterior tibialis muscle at 3, 6, 12, and 24 h following reperfusion. The tissue samples were fixed in 0.4% paraformaldehyde containing 1/1,000 diethylpyrocarbonate (DEPC) for the determination of iNOS and eNOS mRNA levels.

Serum MDA level was determined using the thiobarbituric acid (TBA) assay.

Routine dehydration, wax impregnation, embedding, and slicing were performed on the fixed skeletal muscle samples. Two sequential slices were placed onto the same glass slide for the measurement of iNOS and eNOS mRNA. The manufacturer's detailed in situ hybridization procedure was strictly followed. A brownish-yellow color represented a positive hybridization signal. Microscopic image analysis was performed on positive stains. The intensity of the hybridization signal is expressed as average optical density (OD).

Measurement data are expressed as mean \pm standard deviation. The software package SPSS 16.0 for Windows (SPSS Inc., Chicago, IL, USA) was used for statistical

analysis. Comparative analyses of sample averages across multiple groups were performed using the one-way analysis of variance (ANOVA) method. Pairwise comparisons were analyzed with the Newman-Keuls test (q test). $p < 0.05$ was considered statistically significant.

Results

No significant difference was observed in the serum MDA levels of the sham group at various time points ($p > 0.05$). MDA levels in the I/R group were higher than those of the sham group ($p < 0.01$). MDA levels in the I/R group gradually increased over the 3–24 h time period after reperfusion; the differences between 24 h and 3 or 6 h were statistically significant ($p < 0.05$). MDA levels in the IRH group were lower than those of the I/R group ($p < 0.01$) at various time points after reperfusion and showed no significant difference from those of the sham group (Figure 1).

The results of the in situ hybridization assay demonstrated positive iNOS mRNA expression at various time points, as indicated by brownish-yellow spots in the skeletal muscle tissue. The primary intracellular expression location was the cytoplasm. The signal intensities varied between samples collected at different time points. The average OD values of the positive signals were calculated following image analysis. The results depicted low-level iNOS mRNA expression at various time points after reperfusion in the sham group, and no significant difference was observed between these time points ($p > 0.05$). The level of iNOS mRNA expression in the I/R group increased after 3 h after reperfusion and peaked at 6 h,

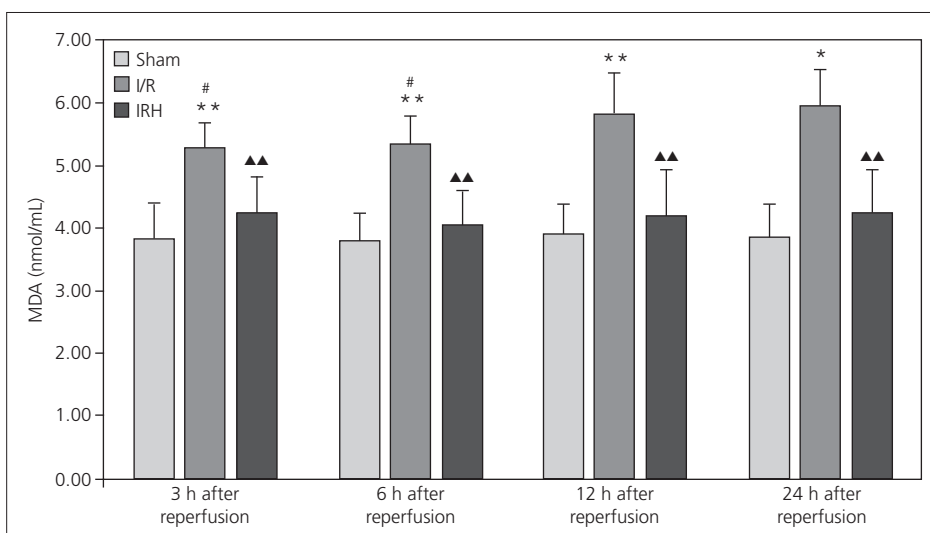


Fig. 1. Comparison of serum MDA levels among groups. Values are expressed as mean \pm standard deviation. $n=10$ per group. $**p < 0.01$ vs. sham group; $*p < 0.05$ vs. 24 h after reperfusion of I/R group; $▲▲p < 0.01$ vs. I/R group.

after which it decreased gradually. Data collected after 6 h showed a statistically significant difference compared to data from other time points ($p < 0.01$, $p < 0.05$, respectively). The iNOS mRNA expression levels at various time points in the I/R group were all significantly higher than those of the sham group at each corresponding time point ($p < 0.01$). At each time point, the iNOS mRNA expression level in the IRH group was significantly decreased relative to the I/R group ($p < 0.01$, $p < 0.05$, respectively) (Figure 2).

eNOS mRNA expression was observed at each time point after reperfusion in the sham group. No significant difference was observed for eNOS mRNA expres-

sion level between the sham and I/R groups. At each time point, eNOS mRNA expression levels in the IRH group were significantly increased compared to those of the corresponding time point in the I/R group ($p < 0.01$, $p < 0.05$, respectively) (Figure 3).

Discussion

This study demonstrated considerably up-regulated iNOS mRNA expression in limb skeletal muscles after I/R. An excessive amount of reactive oxygen species (ROS) and inflammatory mediators are produced and released after limb I/R. A significant increase in tissue peroxidation induced and activated iNOS in the skel-

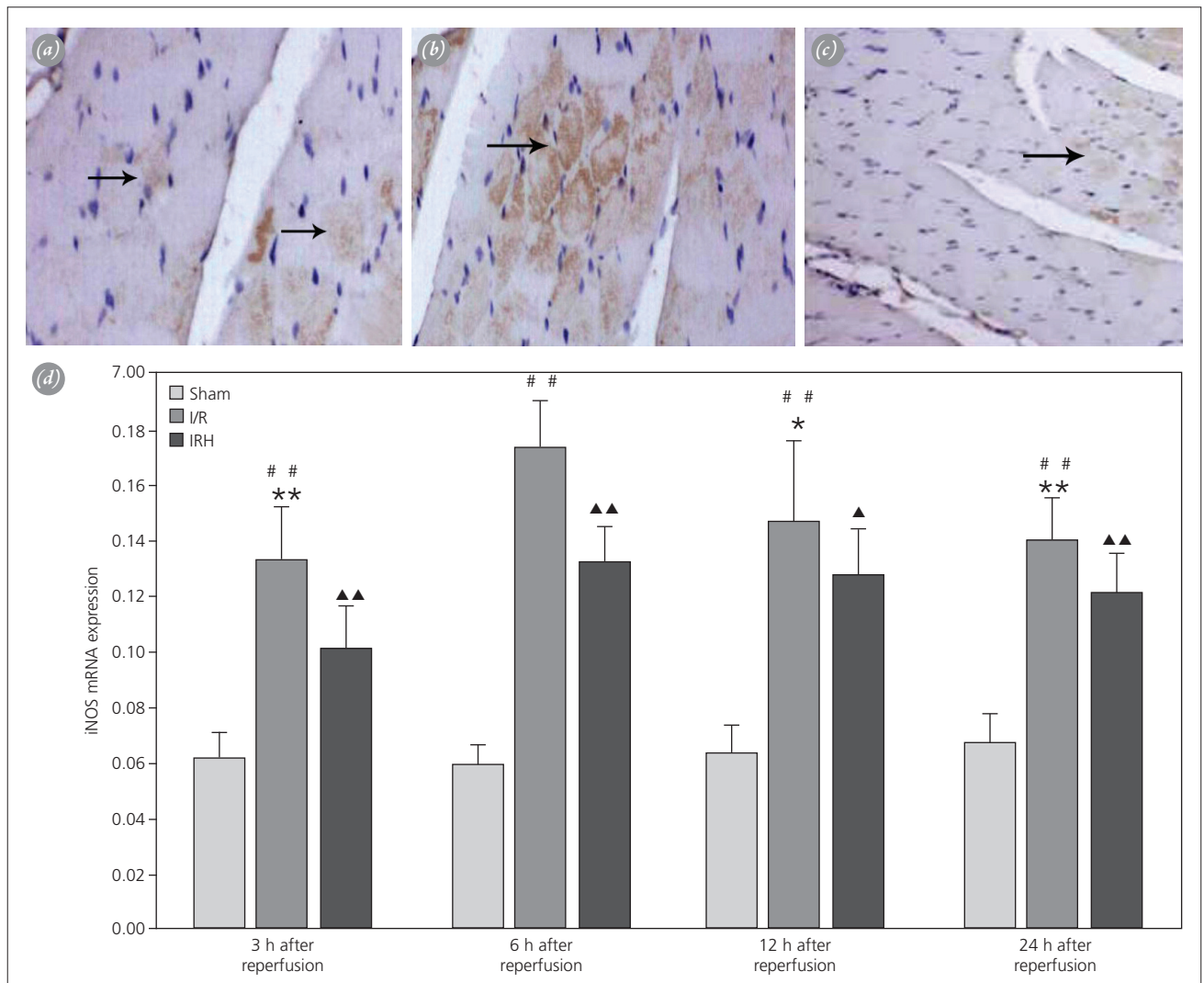


Fig. 2. Levels of iNOS mRNA expression. **(a)** 6 h after reperfusion of sham group, showing the low-level of iNOS mRNA expression. **(b)** 6 h after reperfusion of I/R group, showing the significantly higher level of iNOS mRNA expression. **(c)** 6 h after reperfusion of IRH group, showing that the level of iNOS mRNA expression is significantly decreased relative to I/R group. **(d)** Comparison of the levels of iNOS mRNA expression among groups. Values are expressed as mean ± standard deviation. $n = 10$ per group. * $p < 0.05$, ** $p < 0.01$ vs. 6 h after reperfusion of I/R group; # $p < 0.01$ vs. sham group; ▲ $p < 0.05$, ▲▲ $p < 0.01$ vs. I/R group. [Color figure can be viewed in the online issue, which is available at www.aott.org.tr]

etal muscle cells, thereby increasing tissue NO levels and mediating I/R injury. Our results demonstrated a significant increase in iNOS mRNA starting at 3 h following reperfusion, which peaked at 6 h, suggesting that a large amount of stimulating factors were produced in the first 3–6 h following limb I/R. The decrease in iNOS mRNA expression 6 h after reperfusion may be the result of decreased gene transcription due to the histiocyte damage associated with the condition. This may also be a feedback regulatory effect of excessive NO production on iNOS gene expression. MDA is a typical product of lipid peroxidation and reflects the degree of cell damage. The MDA measurement results revealed that MDA lev-

el was not proportional to the iNOS mRNA level: the MDA level started to increase soon after reperfusion, and this increase continued for 24 h. This phenomenon may be attributed to the oxygen free radical-generating activity of iNOS, which could induce O_2^- production and thereby increase MDA levels. The iNOS mRNA expression level started to decrease 6 h after reperfusion. However, oxygen free radicals could stimulate the aggregation of leukocytes and secretion of inflammatory mediators. This peroxidation reaction cascade could continue and cause more oxygen free radical production, thus maintaining the increase in MDA levels. Following the administration of H_2 , both iNOS mRNA expres-

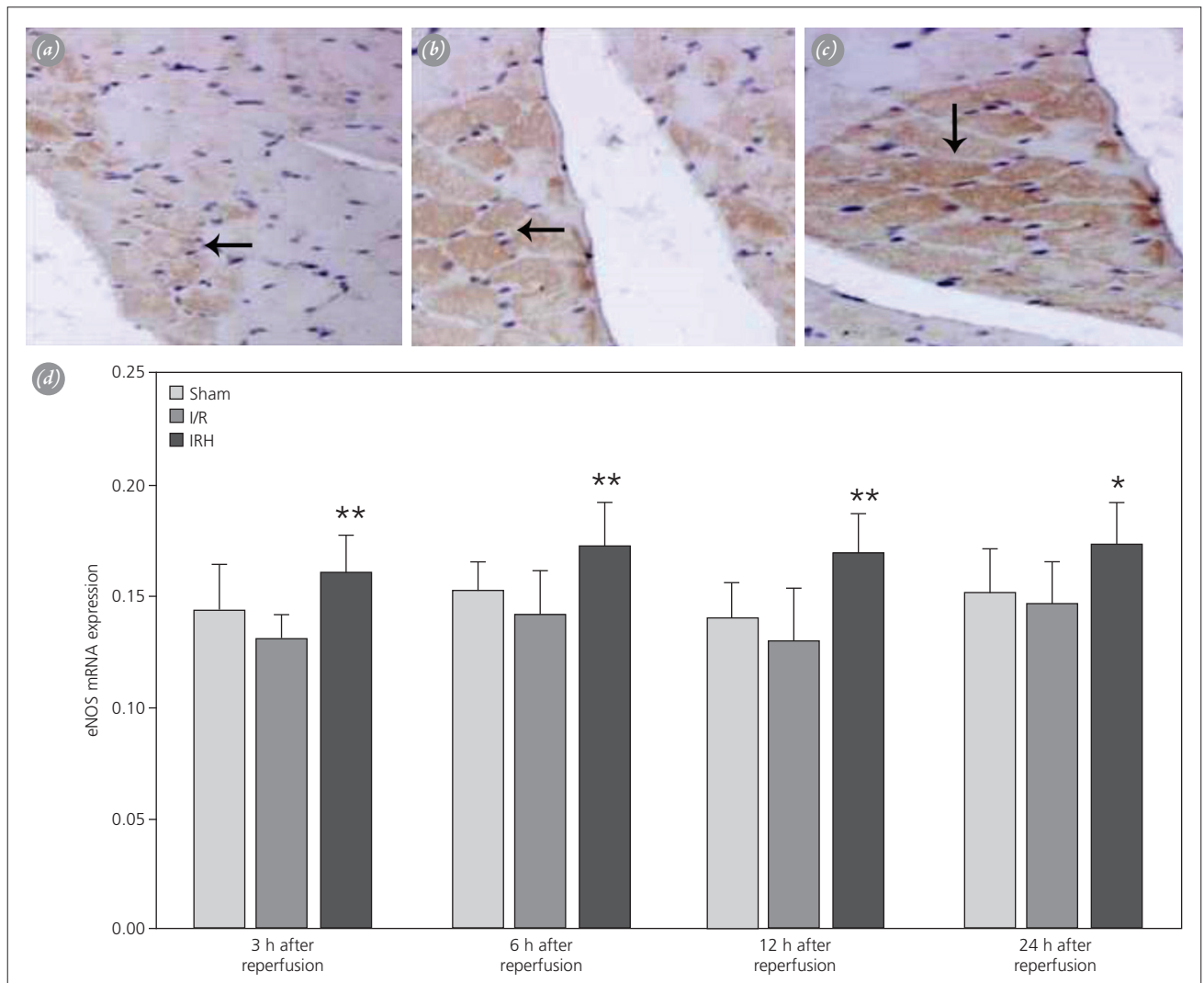


Fig. 3. Levels of eNOS mRNA expression. **(a)** 6 h after reperfusion of sham group, showing the level of eNOS mRNA expression. **(b)** 6 h after reperfusion of I/R group, showing the level of eNOS mRNA expression. No significant difference is observed for the level of eNOS mRNA expression between sham and I/R groups. **(c)** 6 h after reperfusion of IRH group, showing that the level of eNOS mRNA expression is significantly increased compared to that in I/R group. **(d)** Comparison of the eNOS mRNA expression levels among groups. Values are expressed as mean \pm standard deviation. $n=10$ per group. * $p<0.05$, ** $p<0.01$ vs. I/R group. [Color figure can be viewed in the online issue, which is available at www.aott.org.tr]

sion levels and MDA levels were significantly decreased, suggesting that H₂ could inhibit the I/R-induced up-regulation of iNOS mRNA to prevent high level NO production and tissue injury. Whether eNOS mRNA and protein expression is enhanced or reduced remains controversial. Most researchers have claimed that I/R-induced endothelial injury could cause endothelial dysfunction and reduce eNOS mRNA expression. However, a small number of studies have reported no significant decrease in eNOS mRNA and protein expression after I/R^[19] or even an increased expression of eNOS mRNA.^[20] Our study demonstrated no significant variation of eNOS mRNA expression levels at various time points between the I/R and sham groups, supporting the latter findings. However, following the administration of H₂, eNOS mRNA expression increased significantly, suggesting that H₂ could up-regulate eNOS mRNA expression and exert various functions such as protection of the endothelium, reduction of adhesion molecules, and prevention of neutrophil granulocyte migration.

In conclusion, this study investigated the effect of H₂ on the mRNA expression levels of iNOS and eNOS at the genetic level following limb I/R. The results revealed an up-regulated expression of iNOS mRNA, accompanied by increased MDA (a peroxidation product) in the I/R process. H₂ could down-regulate the expression of iNOS mRNA, up-regulate the expression of eNOS mRNA, and provide a protective effect to the limb during I/R injury.

Additional studies are required to confirm this conclusion, as the pathological and physiological mechanisms of NOS remain unclear. This study was limited to the examination of iNOS and eNOS mRNA expression patterns. Future research should focus on a comparative study including an investigation of protein expression.

Acknowledgements

This work was supported by the Shandong Provincial Natural Science Foundation, China (Grant ZR2010HL047). The authors are grateful to Qian-Qian Liang, Zhi-Xin Wei, and Xiao-Gang Wei for their excellent technical assistance.

Conflicts of Interest: No conflicts declared.

References

- Baue AE. The horror autotoxicus and multiple-organ failure. *Arch Surg* 1992;127:1451–62.
- Nanobashvili J, Neumayer C, Fuegl A, Sporn E, Prager M, Polterauer P, et al. Ischaemia/reperfusion injury of skeletal muscle: Mechanisms, morphology, treatment strategies, and clinical applications. *European Surgery* 2002;34:83–9.
- Guillot M, Charles AL, Chamaraux-Tran TN, Bouitbir J, Meyer A, Zoll J, et al. Oxidative stress precedes skeletal muscle mitochondrial dysfunction during experimental aortic cross-clamping but is not associated with early lung, heart, brain, liver, or kidney mitochondrial impairment. *J Vasc Surg* 2014;60:1043–51.e5.
- Palmer RM, Ferrige AG, Moncada S. Nitric oxide release accounts for the biological activity of endothelium-derived relaxing factor. *Nature* 1987;327:524–6.
- Moncada S, Palmer RM, Higgs EA. Nitric oxide: physiology, pathophysiology, and pharmacology. *Pharmacol Rev* 1991;43:109–42.
- Rochette L, Lorin J, Zeller M, Guillard JC, Lorgis L, Cottin Y, et al. Nitric oxide synthase inhibition and oxidative stress in cardiovascular diseases: possible therapeutic targets? *Pharmacol Ther* 2013;140:239–57.
- Korkmaz A, Kolankaya D. Inhibiting inducible nitric oxide synthase with rutin reduces renal ischemia/reperfusion injury. *Can J Surg* 2013;56:6–14.
- Huang G, Zhou J, Zhan W, Xiong Y, Hu C, Li X, et al. The neuroprotective effects of intraperitoneal injection of hydrogen in rabbits with cardiac arrest. *Resuscitation* 2013;84:690–5.
- George JF, Agarwal A. Hydrogen: another gas with therapeutic potential. *Kidney Int* 2010;77:85–7.
- Ohsawa I, Ishikawa M, Takahashi K, Watanabe M, Nishimaki K, Yamagata K, et al. Hydrogen acts as a therapeutic antioxidant by selectively reducing cytotoxic oxygen radicals. *Nat Med* 2007;13:688–94.
- Dixon BJ, Tang J, Zhang JH. The evolution of molecular hydrogen: a noteworthy potential therapy with clinical significance. *Med Gas Res* 2013;3:10.
- Ohsawa I, Nishimaki K, Yamagata K, Ishikawa M, Ohta S. Consumption of hydrogen water prevents atherosclerosis in apolipoprotein E knockout mice. *Biochem Biophys Res Commun* 2008;377:1195–8.
- Hayashida K, Sano M, Ohsawa I, Shimura K, Tamaki K, Kimura K, et al. Inhalation of hydrogen gas reduces infarct size in the rat model of myocardial ischemia-reperfusion injury. *Biochem Biophys Res Commun* 2008;373:30–5.
- Shingu C, Koga H, Hagiwara S, Matsumoto S, Goto K, Yokoi I, et al. Hydrogen-rich saline solution attenuates renal ischemia-reperfusion injury. *J Anesth* 2010;24:569–74.
- Fang Y, Fu XJ, Gu C, Xu P, Wang Y, Yu WR, et al. Hydrogen-rich saline protects against acute lung injury induced by extensive burn in rat model. *J Burn Care Res* 2011;32:e82–91.
- Fujita K, Seike T, Yutsudo N, Ohno M, Yamada H, Yamaguchi H, et al. Hydrogen in drinking water reduces dopaminergic neuronal loss in the 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine mouse model of Parkinson's disease. *PLoS One* 2009;4:e7247.

17. Nanobashvili J, Neumayer C, Fügl A, Punz A, Blumer R, Prager M, et al. Ischemia/reperfusion injury of skeletal muscle: plasma taurine as a measure of tissue damage. *Surgery* 2003;133:91–100.
18. Wang DJ, Tian H. Effect of Mailuoning injection on 8-iso-prostaglandin F2 alpha and superoxide dismutase in rabbits with extremity ischemia-reperfusion injury. *J Surg Res* 2014;192:464–70.
19. Wan LL, Xia J, Ye D, Liu J, Chen J, Wang G. Effects of quercetin on gene and protein expression of NOX and NOS after myocardial ischemia and reperfusion in rabbit. *Cardiovasc Ther* 2009;27:28–33.
20. Küçük A, Yucel M, Erkasap N, Tosun M, Koken T, Ozkurt M, et al. The effects of PDE5 inhibitory drugs on renal ischemia/reperfusion injury in rats. *Mol Biol Rep* 2012;39:9775–82.