Life Sciences 194 (2018) 7–14

Contents lists available at ScienceDirect

Life Sciences

journal homepage: www.elsevier.com/locate/lifescie

Hydrogen-rich saline protects against small-scale liver ischemia-reperfusion injury by inhibiting endoplasmic reticulum stress☆

Hui Li, Ge Bai, Yansong Ge, Qianzhen Zhang, Xiangdong Kong, Weijing Meng, Hongbin Wang*

College of Veterinary Medicine, Northeast Agricultural University, Harbin 150030, PR China
Heilongjiang Key Laboratory for Laboratory Animals and Comparative Medicine, Harbin 150030, PR China

ARTICLE INFO

Keywords:
Hydrogen-rich saline
Ischemia-reperfusion
Pig
Liver
Endoplasmic reticulum stress

ABSTRACT

Aim: Our research investigated the role of Hydrogen-rich saline (HRS) on the Endoplasmic reticulum stress (ERS) pathway and the effect of HRS on tissue injury in small Bama pig model of hepatic ischemia-reperfusion combined with partial hepatectomy.

Main methods: Eighteen healthy Bama miniature pigs were randomly divided equally into three groups: Sham, IRI, and HRS. Laparoscopic technique was employed to establish the model of hepatic ischemia-reperfusion combined with partial hepatectomy. HRS (10 mL/kg) was injected into the portal vein 10 min before perfusion. Histological examinations of the liver tissues were performed after HE staining. Additionally, transmission electron microscopy was performed to detect liver cell microstructure. Real-time PCR, Western blotting, and immunohistochemical staining were performed to analyze various ERS molecules including GRP78, p-eIF2α, XBP-1s, Full-length ATF6α, p-JNK, ATF4, and CHOP.

Key findings: We observed that HRS visibly improved ischemia-reperfusion injury (IRI) by reducing various parameters of ERS stress as evidenced by down-regulation of the mRNA as well as protein levels of GRP78, p-eIF2α, XBP-1s, p-JNK, and CHOP, and reducing the cleavage of Full-length ATF6α.

Significance: Our study demonstrates that HRS protects the liver from IRI by inhibiting ERS.

1. Introduction

Complex liver surgeries such as liver transplantation, resection, and liver pathologies such as circulatory shock and hepatic trauma, will inevitably lead to ischemia and reperfusion phenomenon and can cause serious ischemia-reperfusion injury (IRI). IRI is an important contributor to liver damage in surgery. Finding strategies to reduce liver injury caused by surgery is a clinically urgent problem [1,2]. ER, as an intracellular organelle, plays a pivotal role in the translocation and integration of secretory and membrane proteins respectively, facilitating their folding and transport (to the extracellular environment or the cell membrane), lipid biosynthesis, and maintenance of calcium homeostasis [3]. Liver IRI causes changes in cellular energy levels, $Ca2+$ overload, oxidative stress, and elevates protein synthesis, thereby interfering with ER homeostasis and resulting in Endoplasmic reticulum stress (ERS) [4]. Several studies have demonstrated that ERS plays a key role in the increase in apoptosis and exacerbation of cell damage following liver IRI [5,6]. ERS is thought to be an early response to cellular damage and occurs widely in liver IRI pathophysiology [7,8]. Thus, inhibition or remodeling of the ERS response pathways may provide a new therapeutic intervention strategy for the liver IRI.

Medicinal attributes of molecular hydrogen for animal disease models and human diseases have been explored since 2007 [9]. Ohsawa and colleagues first reported the anti-oxidative effect of hydrogen by directly eliminating hydroxyl radical and peroxynitrite [10]. Hydrogen-rich Saline (HRS) is a derivative of molecular hydrogen and is a known biochemical antioxidant. Many studies have shown that the mechanism of hydrogen action mostly involves antioxidant stress, and anti-inflammatory, and anti-apoptotic effects in different organ systems including the lung, liver, heart, kidneys, intestines, and brain [11–16]. Recent studies have shown that HRS can effectively inhibit ERS [17,18]. However, the underlying mechanisms of HRS remain unclear. In this study, we investigate whether HRS protects against liver IRI by inhibiting ERS in small Bama pig model of hepatic ischemia-reperfusion combined with partial hepatectomy.

☆ All other authors have read the manuscript and have agreed to submit it in its current form for consideration for publication in this journal.

* Corresponding author at: College of Veterinary Medicine, Northeast Agricultural University, Harbin 150030, PR China.
E-mail address: hbwang1940@neau.edu.cn (H. Wang).

https://doi.org/10.1016/j.lfs.2017.12.022
Received 2 October 2017; Received in revised form 13 December 2017; Accepted 14 December 2017
Available online 15 December 2017

2. Materials and methods

2.1. Animals

A total of 18 Bama miniature pigs in good health with an average body weight of 30.57 ± 2.5 kg were obtained from the laboratory animal center of Harbin veterinary research institute (animal license number: SYXK 2011-0039). The experimental protocol was approved by the Northeast Agricultural University Ethical Committee (China). Care and handling of the animals were in accordance with the national animal research guidelines (Approved by the State Council on October 31, 1988, and promulgated by Decree No. 2 of the State Science and Technology Commission on November 14, 1988). These pigs were fed free piglet diet (Shenzhen Jinxinnong Feed, China) and tap water ad libitum with 12 h light–dark cycles at a steady temperature (around 20 °C). These pigs were randomly divided equally into sham group, IRI group, and HRS group.

2.2. Preparation and determination of HRS

Hydrogen generated by the hydrogen generator (QL-500, Shandong Success Hydrogen Energy Co., Shandong, China) was dissolved in physiological saline at 0.4 MPa pressure for 4 h to obtain a supersaturated solution (0.6 mmol/L). A portable dissolved hydrogen meter (ENH-1000, Trustlex Inc., Osaka, Japan) was employed to measure hydrogen concentration.

2.3. Surgical procedure and experimental design

After fasting overnight, 1% to 3% isoflurane gas (Lishi; Jiupai Pharmaceutical, China) was used to induce general anesthesia in the pigs. The pigs were then placed in a supine position, and a carbon dioxide pneumoperitoneum of 10 mm Hg was established. A four-port, purely laparoscopic technique employed in this study. The operation was performed as follows: casing needle was used for puncture. The first tube (laparoscopic access pathway), was positioned at the bottom of the third nipple, 3–5 cm from the left side of the abdominal line, with a casing of diameter 10 mm at an angle of 30 to the laparoscope. The second tube was positioned about at 5 cm to the right of the penultimate nipple, with a casing diameter of 10 mm. The third tube was inserted at 3–5 cm from the right side of the rib margin with a casing diameter of 5 mm. The fourth tube was placed toward the left of the abdominal line and the third casing with a casing diameter of 5 mm. In the sham group, only the pneumoperitoneum was established, and the liver lobe was flipped over. The IRI model was established in the IRI and HRS groups by laparoscopic minimally invasive surgery: The right half of the liver was occluded with a tourniquet for 60 min. The tourniquet was then removed to allow reperfusion. At the same time, the left half of the liver was ligatured with a continuous 1–0 silk suture and immediately resected with a high-frequency electrosurgical generator. Physiological saline was injected into the portal vein at 10 min before perfusion in sham and IRI groups; HRS (10 mL/kg) was injected into the portal vein at 10 min before perfusion in the HRS group. Liver tissue samples were collected from day 1 of laparoscopic surgery from the right outer lobe of the liver.

2.4. Histopathological study

The liver tissues were fixed with 10% formaldehyde. After routine paraffin embedding, HE staining was performed, and the histological changes of the liver were observed under a microscope. Histopathologists with no prior knowledge of the experiment evaluated the extent of tissue damage of the samples. Liver injury was scored on a scale of 0 to 4 for sinusoidal congestion, vacuolization of hepatocyte cytoplasm, and parenchyma, as described by Suzuki et al. [19].

2.5. Immunohistochemistry

Formalin-fixed and paraffin-embedded liver sections of thickness of 4 μm were dewaxed in xylene and graded alcohols, hydrated, and washed in PBS. The samples were pretreated with citric acid in a microwave oven and then cooled to room temperature for antigen recovery. A solution of hydrogen peroxide (3% H 2 O 2) was added to block endogenous peroxidase activity. Following overnight incubation with the primary antibody (GRP78,WL0781,Wanleibio,Shenyang China) at 4 °C, the slides were washed in PBS, HRP-conjugated goat anti-ribbit secondary antibody was added, and the slides were further incubated at room temperature for 30 min. DAB was used for development and counterstained with hematoxylin. A negative control without primary antibody was included in the experiment to verify antibody specificity. The sections were observed by light microscopy and quantified using Image-Pro Plus 6.0 software.

2.6. Transmission electron microscopy

Liver tissues were cut into small cubes, treated with 2% glutaraldehyde buffer and then fixed in osmium tetroxide. The treated sections were observed by electron microscopy.

2.7. Quantitative real-time RT-PCR

Total RNA was extracted from liver tissue using Trizol reagent. The total RNA was reverse transcribed into cDNA using the ReverTra Ace qPCR RT Master Mix along with gDNA Remover Kit (TOYOBO, Bie Jing, China). Real-time qPCR was performed by using FastStart Universal SYBR Green Master (Rox) (Roche, USA); PCR primers were synthesized by Sangon Biotech, Shanghai, China. The primer sequences are listed in Table 1. PCR conditions were as follows: 94 °C for 20 s, followed by 40 cycles of 95 °C for 15 s and 60 °C for 1 min. Comparative and relative quantifications of the gene products were normalized to β-actin and the control group by the $2^{-\triangle\triangle Ct}$ method.

2.8. Western blot analysis

Liver tissues were homogenized using Tissue Protein Extraction Reagent with 1 mM PMSF and phosphatase inhibitor cocktail (Roche, USA) according to the manufacturer's instructions. The supernatants of liver homogenates were collected by centrifugation at 10000 × *g* for 5 min. Total protein concentration was determined using the BCA Protein Assay Kit (Beyotime Biotechnology, China). The proteins were electrophoresed on 12% SDS-PAGE gels and transferred to NC membranes. The membranes were then incubated with 5% bovine serum albumin (BSA) for 2 h at room temperature to block non-specific binding. These membranes were then incubated overnight at 4 °C with the primary antibodies to ATF4, CHOP, XBP1s,β-actin (Wanleibio, Shenyang, China), JNK, P-JNK (Cell Signaling Technology, Boston, MA, USA), Full-length ATF6α, P-eIF2α, and eIF2α (Abcam, Cambridge, UK). The membranes were then incubated at room temperature for 2 h with goat anti-ribbit secondary antibody (Wanleibio, Shengyan, China) at

Table 1
Gene-specific primers used in the qPCR.

Gen	Number	Primer sequences (5′-3′)
GRP78	XM-001927795.4	Forward: TCGGCGATGCAGCCAAGAAC Reverse: CGGGTCATTCCATGTCCGGC
ATF4	NM-001123078.1	Forward: TCAGTGCCTCAGACAACAGC Reverse: GCATGGTTTCCAGGTCATCT
CHOP	NM-001144845.1	Forward: AAGACCCAGGAAACGGAAAC Reverse: GAGCCGTTCGTTCTCTTCAG
β-Actin	XM-021086047.1	Forward: TCTGGCAACCACACCTTCT Reverse: TGATCTGGGTCATCTTCTCAC