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Hydrogen-rich saline attenuates ischemia–reperfusion injury in skeletal muscle

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ABSTRACT

Background: To investigate the potential beneficial effect of hydrogen-rich saline (HRS) in ischemia–reperfusion (IR) injury of skeletal muscle.

Methods: Three experimental groups were established in male Sprague–Dawley rats: (1) sham group, (2) IR with normal saline group, (3) and IR with HRS group. A rat model of skeletal muscle IR injury was induced by 3-h tourniquet occlusion on its left hind limb and 4-h reperfusion. Normal saline and HRS (1.0 mL/100 g) were administered intraperitoneally at 10 min before reperfusion, respectively. Muscle and serum samples were analyzed for detecting the levels of myeloperoxidase (MPO), superoxide dismutase (SOD), malondialdehyde (MDA), and hydroxyl radical (•OH). Muscle samples were assessed by wet/dry rate, hematoxylin and eosin histologic assessment, Bcl2, Bax, cytochrome C, LC3B, terminal deoxynucleotidyl transferase-mediated dUTP-biotin nick end labeling, and electron microscopy.

Results: The wet/dry ratio increased significantly in the IR group ($P < 0.01$ compared with that in the sham group) and decreased significantly in IR with HRS groups (4.12 ± 0.14 *versus* 4.12 ± 0.14, $P < 0.01$ compared with that in the IR group). Muscle tissues and serum of the IR group had significantly increased levels of MPO, MDA, •OH content, and decreased SOD activities compared with the sham group ($P < 0.01$). The activity of SOD in the IR with HRS group was greatly elevated compared with that in the IR group (295.028 ± 9.288 *versus* 249.190 ± 5.450 in muscle tissues; 91.627 ± 2.604 *versus* 73.4045 ± 6.487 in serum; $P < 0.01$), whereas the levels of MPO, MDA, and •OH content were clearly reduced (MPO: 0.5649 ± 0.0724 *versus* 1.0984 ± 0.0824 in muscle tissues; 0.7257 ± 0.1232 *versus* 1.3147 ± 0.0531 in serum. MDA: 4.457 ± 0.650 *versus* 7.107 ± 0.597 in muscle tissues; 2.531 ± 0.434 *versus* 4.626 ± 0.237 in serum. •OH: 16.451 ± 0.806 *versus* 19.871 ± 0.594 in muscle tissues; 500.212 ± 7.387 *versus* 621.352 ± 7.591 in serum, $P < 0.01$). The integrated optical density of positive amethyst staining increased significantly in the IR group ($P < 0.01$ compared with that in the sham group) and decreased significantly in IR with HRS group (928.79 ± 234.537 *versus* 3005.972 ± 83.567, $P < 0.01$ compared with that in the IR group). Muscle tissues of the IR group had significantly increased levels of Bax, cytochrome C, LC3B

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content, and decreased Bcl2 activities compared with those in the sham group ($P < 0.01$). The activity of Bcl2 in the IR with HRS group was greatly elevated compared with that in the IR group (0.2635 ± 0.0704 *versus* 0.1242 ± 0.0662; $P < 0.01$), whereas the levels of Bax, cytochrome C, and LC3B content were clearly reduced (Bax: 0.3103 ± 0.0506 *versus* 0.5122 ± 0.0148; cytochrome C: 0.4194 ± 0.1116 *versus* 0.8127 ± 0.0166; LC3B: 0.5884 ± 0.0604 *versus* 1.3758 ± 0.0319; respectively, $P < 0.01$).

Conclusions: HRS seems to be effective in attenuating IR injury in skeletal muscle via its antioxidant, anti-apoptosis, and anti-autophagy effect.

1. Introduction

Skeletal muscle has high metabolic activity and therefore is acutely sensitive to reperfusion injury after ischemia. Many clinical events including trauma, primary thrombosis, arterial embolism, limb or flap replantation, arterial grafting, prolonged tourniquet application, and compartment syndrome can cause severe skeletal muscle ischemia and resulting reperfusion injury as blood flows back into the ischemic muscle. Ischemia–reperfusion (IR) injury of skeletal muscle can lead to severe damage to the extremities, including severe necrosis leading to amputation, or even multisystem organ dysfunction syndrome and threats to life. Given the range of events that can cause ischemia and the potential for severe damage from IR injury, it is important to develop some means to minimize limb IR injury and reduce the extent of damage to skeletal muscle to decrease amputation rates and improve the recovery of extremity function. Clinically, a therapeutic intervention, which changes the biochemical environment during the IR period and prevents subsequent damage, would be a significant benefit. Several pharmacologic agents with the potential to reduce IR injury have been evaluated, but none has yet been translated into clinical trials [1–4].

Ischemia can lead to energy depletion, accumulation of toxic metabolic products, activation of phospholipase and lysozymes, and cell damage. Reperfusion can then worsen the injury by inducing cellular infiltration and generating reactive oxygen species (ROS), leading to further cell damage. ROS and activated neutrophils are the most important factors responsible for local and systemic damage caused by IR [5]. Apoptosis is thought to be an inevitable phase in IR-induced cell death [6,7]. In addition, autophagy may play a critical role in the IR-induced cell death because regulation of the autophagic process can have a positive influence on IR injuries [8–13]. However, the evidence of IR-induced autophagy in skeletal muscle is sparse and divided although there is evidence of IR-induced autophagy in many other tissues and organs like the heart [8], the liver [9], the intestine [10], the kidney [11,12], and the brain [13].

It has been demonstrated that hydrogen could selectively reduce cytotoxic ROS and reactive nitrogen species, such as hydroxyl radical (•OH) and ONOO- *in vitro* and thus exert therapeutic antioxidant activity in a rat middle cerebral artery occlusion model [14]. However, to our knowledge, the protective effect of hydrogen on skeletal muscle IR injury has not been reported. Therefore, the present study investigated the possible therapeutic effects and underlying mechanisms of hydrogen-rich saline (HRS) on skeletal muscle IR injury in rats and explored the possible mechanism of the therapeutic effects. Our study sought to determine whether HRS can attenuate IR injury in skeletal muscle through antioxidant, anti-apoptosis, and anti-autophagic mechanism.

2. Materials and methods

2.1. Animals

Eighteen six-wk-old male Sprague–Dawley rats (Animal Department of Xiangya School of Medicine, Central South University, China) weighing 250–300 g were housed in our research facility under standard conditions with a 12-h light–dark cycle and free access to water and food. All animal care and experimental procedures were performed in compliance with the Guide for the Care and Use of Laboratory Animals [15] and were approved by the Institutional Animal Care and Use Committee of Xiangya School of Medicine, Central South University.

2.2. Chemicals

Phosphate-buffered saline (PBS) was purchased from Sigma–Aldrich (St. Louis, MO). Malondialdehyde (MDA), superoxide dismutase (SOD), and •OH assay kit were purchased from Nanjing Jiancheng Bioengineering Institute (Nanjing, China). Rabbit polyclonal antibody to LC3B (ab63817) and terminal deoxynucleotidyl transferase-mediated dUTP-biotin nick end labeling (TUNEL) *in situ* cell death detection kit (ab66108) were purchased from Abcam (Cambridge, MA). Rabbit polyclonal antibodies to Bcl-2 (#2870), Bax (#2772), and cytochrome C (#11940) were purchased from Cell Signaling Technology, Inc (Boston, MA). Rabbit polyclonal antibody to GAPDH and goat anti–rabbit IgG were purchased from Santa Cruz Biotechnology, Inc (Dallas, TX). A total Protein Extraction Kit (SJ-200501) was purchased from ProMab (Richmond, CA), 0.1% triton X-100 was purchased from MP Biomedicals (Solon, OH), and 0.1% citric acid was purchased from Mallinckrodt (Hazelwood, MO). All chemicals and reagents were of analytical grade.

2.3. HRS production

Hydrogen was dissolved in normal saline (NS) for 6 h under high pressure (0.4 MPa) to a supersaturated level using apparatus produced by Beijing Hydrovita Biotechnology Co, Lt (Beijing, China). The saturated HRS was stored under