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INHALATION OF WATER ELECTROLYSIS-DERIVED HYDROGEN AMELIORATES CEREBRAL ISCHEMIA–REPERFUSION INJURY IN RATS – A POSSIBLE NEW HYDROGEN RESOURCE FOR CLINICAL USE

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Abstract—Hydrogen is a kind of noble gas with the character to selectively neutralize reactive oxygen species. Former researches proved that low-concentration of hydrogen can be used to ameliorating cerebral ischemia/reperfusion injury. Hydrogen electrolyzed from water has a hydrogen concentration of 66.7%, which is much higher than that used in previous studies. And water electrolysis is a potential new hydrogen resource for regular clinical use. This study was designed and carried out for the determination of safety and neuroprotective effects of water electrolysis-derived hydrogen. Sprague–Dawley rats were used as experimental animals, and middle cerebral artery occlusion was used to make cerebral ischemia/reperfusion model. Pathologically, tissues from rats in hydrogen inhalation group showed no significant difference compared with the control group in HE staining pictures. The blood biochemical findings matched the HE staining result. TTC, Nissl, and TUNEL staining showed the significant improvement of infarction volume, neuron morphology, and neuron apoptosis in rat with hydrogen treatment. Biochemically, hydrogen inhalation decreased brain caspase-3, 3-nitrotyrosine and 8-hydr oxy-2-deoxyguanosine-positive cells and inflammation factors concentration. Water electrolysis-derived hydrogen inhalation had neuroprotective effects on cerebral ischemia/reperfusion injury in rats with the effect of suppressing oxidative stress and inflammation, and it is a possible new hydrogen resource to electrolyze water at the bedside clinically. © 2016 Published by Elsevier Ltd on behalf of IBRO.

Key words: water electrolysis, hydrogen gas, ischemia–reperfusion injury, oxidative stress, inflammation, new hydrogen resource.

INTRODUCTION

Cerebral ischemia/reperfusion (I/R) injury is closely related to stroke, which is a serious disease with poor blood flow to the brain. A large number of deaths result from stroke (Ono et al., 2012). I/R injuries may happen when stroke patients are treated with rapid brain–blood reperfusion (Srivastava et al., 2008; Huang et al., 2010). As brain–blood reperfusion is the standard treatment for stroke with no other effective replacement, it is very important to find effective therapies for IR injury.

It's not easy to figure out the exact mechanisms of cerebral I/R injury, because it is really complex and there are many factors involved, such as oxidative stress, inflammation, immunity, apoptosis, and autophagy. It is believed that the effects of oxidative stress including the increase of reactive oxygen species (ROS) are related to I/R injury pathophysiology. One of the effects of ROS is to modify the chemical structure of protein, lipid, and DNA, if the ROS cannot be cleaned up effectively, it causes damages. On the other hand, ROS can also trigger chain reactivity of cell apoptosis after ischemic attack (Broughton et al., 2009). The expression of inflammatory cytokines may be elevated by ROS, and inflammation bursts subsequently (Lakhan et al., 2009). In summary, oxidative stress causes a complicated series of biochemical cascades after ischemic/reperfusion insult, which will ultimately aggravate the damage of stroke (Piotrowska et al., 2012).

In recent years, hydrogen is considered as a novel antioxidant, and it has been proven that molecular hydrogen (H_2) can be effective to protect various ischemia/reperfusion injuries (Ohsawa et al., 2007). It is demonstrated by many researches that hydrogen selectively neutralizes several certain ROS, which are $ONOO^-$ and $^{\cdot}OH$ (Yu et al., 2011; Hong et al., 2012), and protects the tissue from oxidative stress and inflammatory

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Abbreviations: 3-NT, 3-nitrotyrosine; 8-OHdG, 8-hydroxy-2-deoxyguanosine; ALT, alanine aminotransferase; AST, aspartate transaminase; BUN, blood urea nitrogen; Cr, Creatinine; ELISA, enzyme-linked immuno sorbent assay; I/R injury, ischemia–reperfusion injury; MCAO, middle cerebral artery occlusion; ROS, reactive oxygen species; TTC, 2,3,5-Triphenyltetrazoliumchloride; TUNEL, terminal deoxynucleotidyl transferase dUTP nick-end labeling.

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cascades injury (Ono et al., 2012; Kawamura et al., 2013). Both inhalation of hydrogen (2%, 4%) and administration of hydrogen-rich saline are effective.

However, in daily practice, water electrolysis is the most convenient means to obtain hydrogen and theoretically the concentration is much higher than that previously used. We hypothesized that water electrolysis-derived hydrogen could ameliorate cerebral ischemia/reperfusion injury in rats through inhibition of oxidative stress and inflammation.

EXPERIMENTAL PROCEDURES

Animal preparation

The animal model protocols were approved by the Institutional Animal Care and Use committees of the Second Military Medical University and conformed to the U.S. National Institutes of Health guidelines for use of animals in research. Male Sprague–Dawley rats (180–220 g) were all offered by the Experimental Animal Center of Second Military Medical University (certification: SCXK (Shanghai) 2007-0003). Animals were kept in relatively comfortable environment with free access to food and water in a climate controlled vivarium with a 12-h light–dark cycle. We provided 7 days for the rats acclimating to the environment before the experiment.

Sample size calculation

The sample size calculation is based on our preliminary experiments with 90% power and a 5% risk of type 1 error. Five rats per experimental group would be enough to detect among-group differences. Thirty percent death and exclusion rate is allowed among a total of 80 rats. The formula was as follows:

$$n = \frac{\varphi^2\left[\sum_{i=1}^{k}\frac{s_i^2}{k}\right]}{\left[\frac{\sum_{i=1}^{k}(\bar{x}_i^2-\bar{x}^2)}{(k-1)}\right]}$$

Study groups design

We divided the eighty male Sprague–Dawley rats randomly into following four groups of 20 rats each: Sham operation group (Sham, $n = 20$), I/R group (MCAO, $n = 20$), hydrogen–oxygen gas group (HO, $n = 20$), nitrogen–oxygen gas group (NO, $n = 20$). The gas was inhaled immediately after reperfusion: rats in HO group inhaled mixed gas consisting of 66.7% H_2 and 33.3% O_2 (vol/vol) for 2 h; rats in NO group inhaled mixed gas consisting of 66.7% N_2 and 33.3% O_2 (vol/vol) for 2 h.

Gas administration

The mixed gas consisting of 66.7% N_2 and 33.3% O_2 was stored in the steel gas cylinder beforehand. The mixed gas consisting 66.7% H_2 and 33.3% O_2 was produced by the AMS-H-01 hydrogen oxygen nebulizer (Asclepius, Shanghai, China), which was designed to produce hydrogen by electrolyzing water. The capacity to produce mixed gas is 3.0 L/min according to the instructions of the machine. Without a high-pressure cylinder for temporary storage, the water electrolysis-derived gas was directly leading in a closed room for inhalation. A transparent closed box (20 × 18 × 15-cm, length × width × height) was used as the gas inhalation room for the rats. Before the experiment, we flushed the box with mix gas for 30 min to replace the air in the box. During each experiment, Thermal trace GC ultra-gas chromatography (Thermo Fisher, MA, USA) was used to monitor the concentration of hydrogen gas in the box.

Safety evaluation and hydrogen concentration test

Before the dominant research, we carried out a safety study for the hydrogen–oxygen gas. Twenty male Sprague–Dawley rats were randomly divided into two groups, the experimental group (Hydrogen, $n = 10$) and control group (Control, $n = 10$). Rats in Hydrogen group inhaled hydrogen–oxygen gas for 2 h. Rats in Control group inhaled normal air in the same environment. Blood was collected for ALT (alanine aminotransferase), AST (Aspartate transaminase), BUN (blood urea nitrogen) and Cr (Creatinine) examination. Brain, liver and one of the kidneys were collected after paraformaldehyde perfusion for pathological examination. For hydrogen concentration test, rats are pretreated with heparin (250 U/ml) in a dose of 100 U/100 g. Immediately after the inhalation, blood, brain and liver were taken to test the hydrogen concentration.

Middle cerebral artery occlusion model establishment

Chloralhydrate (Fortuneibo-Tech Co. Ltd., Shanghai, China) as 10% solution was used for experimental animals anesthesia. An intraluminal monofilament was used to conduct the MCAO and the following reperfusion operation (Li et al., 2008). The occlusion of middle cerebral artery was conducted though the internal carotid artery (ICA). First pterygopalatine artery of the ICA was identified and occluded with an artery clamp. Then a small incision is created on the right external carotid artery (ECA), through which a monofilament suture was inserted into the ICA for about 18–20 mm from the bifurcation. During the process, the right common carotid artery was occluded, and was perfused along with the pterygopalatine artery of the ICA at the end of the surgery. A thermal insulation pad (TC-1000, Man Pu Biotechnology Co. Ltd., Shanghai, China) was used to maintain the animals at a body temperature of 39 °C during and after the surgery.

A Doppler laser blood flow meter (Periflux 5010, Perimed, Stockholm, Sweden) was used to detect the occlusion and reperfusion condition.