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Inhalation of high-concentration hydrogen gas attenuates cognitive deficits in a rat model of asphyxia induced-cardiac arrest

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Abstract

Cognitive deficits are a devastating neurological outcome seen in survivors of cardiac arrest. We previously reported water electrolysis derived 67% hydrogen gas inhalation has some beneficial effects on short-term outcomes in a rat model of global brain hypoxia-ischemia induced by asphyxia cardiac arrest. In the present study, we further investigated its protective effects in long-term spatial learning memory function using the same animal model. Water electrolysis derived 67% hydrogen gas was either administered 1 hour prior to cardiac arrest for 1 hour and at 1-hour post-resuscitation for 1 hour (pre- & post-treatment) or at 1-hour post-resuscitation for 2 hours (post-treatment). T-maze and Morris water maze were used for hippocampal memory function evaluation at 7 and 14 days post-resuscitation, respectively. Neuronal degeneration within hippocampal Cornu Ammonis 1 (CA1) regions was examined by Fluoro-Jade staining ex vivo. Hippocampal deficits were detected at 7 and 18 days post-resuscitation, with increased neuronal degeneration within hippocampal CA1 regions. Both hydrogen gas treatment regimens significantly improved spatial learning function and attenuated neuronal degeneration within hippocampal CA1 regions at 18 days post-resuscitation. Our findings suggest that water electrolysis derived 67% hydrogen gas may be an effective therapeutic approach for improving cognitive outcomes associated with global brain hypoxia-ischemia following cardiac arrest. The study was approved by the Animal Health and Safety Committees of Loma Linda University, USA (approval number: IACUC #8170006) on March 2, 2017.

Key words: high concentration hydrogen gas; cardiac arrest; global brain ischemia; brain resuscitation; cognitive deficit; water maze; neuron; rat

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INTRODUCTION

Cerebral hypoxia-ischemia injury is a significant source of morbidity and mortality in cardiac arrest survivors.1 Cardiac arrest disrupts global cerebral circulation, leading to neuronal damage in multiple brain regions including the hippocampus, cortex, cerebellum, thalamus, prefrontal cortex, and putamen.2,3 The Cornu Ammonis 1 (CA1) region of the hippocampus is particularly sensitive to cerebral ischemia and CA1 neuronal degeneration results in hippocampus dysfunction.4,6 Cardiac arrest survivors commonly experience cognitive deficits in memory and executive function.1,3,7,8

Emerging evidence has demonstrated that low concentration hydrogen gas (up to 3%) or hydrogen-rich saline attenuates hippocampal neuronal injury in animal models of global brain ischemia9-11 due to unique antioxidative and antiapoptotic properties. The safety of hydrogen gas therapy at this concentration was reported in several cases of patients with post-resuscitation syndromes.12 It may serves as a novel translational therapeutic approach in addition to neuroprotection strategies previously reported in the setting of experimental ischemic stroke.13-16 Recently, water-electrolysis derived high concentration hydrogen gas has been shown in rats to provide similar neuroprotection against focal brain and retina ischemia-reperfusion injury without systemic adverse effects.17,18 We previously reported a tendency toward improvement in short-term neurological deficit scores provided by 67% hydrogen gas in a rat model of asphyxia-induced cardiac arrest (ACA).19 In the present study, we further investigated the efficacy of this 67% hydrogen gas in improving cognitive outcomes in global brain hypoxia-ischemia induced by ACA in rats.

MATERIALS AND METHODS

All protocols were approved by the Animal Health and Safety Committees of Loma Linda University, USA (approval number: IACUC #8170006) on March 2, 2017) and were in compliance with Federal regulations.

Animal model and groups

A total of 44 male Sprague-Dawley rats (9 months old, body weight 450–550 g) were investigated in the following groups: sham (n = 6), ACA (n = 13), ACA + hydrogen post-treatment (n = 13), ACA + hydrogen pre- & post-treatment (n = 12) groups. ACA was induced as previously described.19 Briefly, rats were anesthetized with pentobarbital (45 mg/kg; Virbac AH, Inc., Fort Worth, TX, USA) and the trachea was orally intubated with a 14-gauge plastic catheter. For monitoring
arterial pressure and administration of resuscitation drugs, two PE 50 catheters (Becton Dickinson, Franklin Lakes, NJ, USA) were inserted into the left femoral artery and vein until reaching the abdominal aorta and the inferior vena cava, respectively. Electrocardiogram (Lead II) was continually recorded. The animals were mechanically ventilated with room air at a rate of 100 breaths/min at a tidal volume of 0.55 mL/kg for 15 minutes. Asphyxia resulted from intravenous injection of a chemical neuromuscular blockade (vecuronium 2 mg/kg intravenously injected, Mylan Institutional LLC., Rockford, IL, USA) followed by ventilator disconnection and endotracheal tube obstruction. After 9 minutes of asphyxia, precordial compression was started using a pneumatically driven mechanical chest compressor coincident mechanically ventilation (100% oxygen) at ratio of 2:1. At the start of precordial compression, a bolus dose of epinephrine (7.5 μg/kg, International medical systems, LLC., South El Monte, CA, USA) with sodium bicarbonate (1 mEq/kg, Hospira, Lake Forest, IL, USA) was injected into the left femoral vein. Resuscitation or return of spontaneous circulation (ROSC) was defined as the return of supraventricular rhythm with mean arterial pressure over 60 mmHg (1 mmHg = 0.1333 kPa) for 5 minutes.

After resuscitation, mechanical ventilation support was continued, and then stopped 1 hour after ROSC. All catheters including endotracheal tube were removed. Animals were closely observed by the investigator for an additional 2 hours. Body temperature was monitored through a rectal temperature sensor (Model BAT-12, Physitemp instrument Inc., Clifton, NJ, USA) and maintained at 36.8 ± 0.2°C. Lead II electrocardiogram, end-tidal carbon dioxide and arterial pressure were continuously recorded beginning 15 minutes before asphyxia induction and continuing to 1 hour after ROSC using a precordial compression-based data-acquisition system supported by WINDAQ software (DATAQ, Akron, OH, USA).

Hydrogen treatment
Hydrogen was delivered through a hydrogen treatment chamber with the inlet connected to a hydrogen nebulizer (AMS-H-01; Asclepius Meditec Co. Ltd., Shanghai, China) that produced a 67% hydrogen and 33% oxygen gas mixture. The hydrogen concentration was monitored by a hydrogen detector during the administration. Hydrogen was administered starting at 1 hour prior to cardiac arrest for 1 hour and then restarted at 1 hour post-resuscitation for 1 hour (pre- & post-treatment group) or starting at 1 hour post-resuscitation for 2 hours (post-treatment).

Cognitive function evaluation
Both T-maze and water maze are tests of cognitive function. The water-maze is a more comprehensive test of cognitive function but requires the rats to swim. At 7 days post-resuscitation, ACA rats did not have sufficient motor strength to swim, which would confound cognitive assessments. Thus at 7 days post-resuscitation, T-maze for spontaneous alternation was used to examine exploratory behavior and working memory of the hippocampus as previously described. Briefly, rats were placed in a T-shaped maze and allowed to freely explore the two arms of the maze throughout 10-trials. The spontaneous alternation rate was calculated as the percentage of the alternating choices out of 10 trials.

Starting on day 14 post-resuscitation, Morris water maze test was used to assess the learning and spatial memory function of rats as previously described. This four-day test consisted of both cued and hidden tests with test duration up to 60 seconds per trial. The activities and the swim paths of the rats were recorded by a video recording system. The total distance of swimming, latency, and swimming speed were measured by the Video Tracking System SMART-2000 (San Diego Instruments Inc., San Diego, CA, USA).

Fluoro-Jade C staining
ACA rats were sacrificed after completion of testing at either 7 days (n = 6/group) or 18 days post-resuscitation (n = 6/group), and sham rats (n = 6) were sacrificed on day 18 by deep anesthesia using 5% isoflurane followed by transcardial perfusion with 10% formaline. Brain frozen coronal slices (10 μm) at the level of the hippocampus were sectioned in a cryostat (CM3050S; Leica Microsystems, Buffalo Grove, IL, USA).

Neuronal degeneration was evaluated by Fluoro-Jade C staining kit (Millipore, Temecula, CA, USA) as previously described. Based on the manufacturer’s instructions, slices were incubated in 1% sodium hydroxide solution for 5 minutes, followed by 2-minute rinses with 70% ethanol then distilled water. Subsequently, the slices were incubated in 0.06% potassium permanganate solution for 10 minutes. Following 2-minute rinse in distilled water, the slices were incubated with 0.0001% solution of Fluoro-Jade C which was dissolved in 0.1% acetic acid. Then the slices were rinsed with distilled water 3 times for 1 minute each time. Slides were dried for 5 minutes, and then immersed in xylene for 1 minute, and then a cover slip with DPX (Sigma-Aldrich, St. Louis, MO, USA) was placed. The sections were reviewed using a Leica DMi8 fluorescence microscope (Leica Microsystems, Buffalo Grove, IL, USA). Image J software (ImageJ 1.5, NIH, Bethesda, MD, USA) was used to evaluate the numbers of Fluoro-Jade C positive neurons within CA1 regions (averaged from three fields of views for each rat).

Statistical analysis
Quantitative data were presented as the mean ± standard error of mean (SEM). SigmaPlot 11.0 (Systat Software, Inc., San Jose, CA, USA) was used for statistical analysis. One-way analysis of variance was applied for multiple comparisons followed by Student-Newman-Keuls post hoc test. A P value less than 0.05 was considered statistically significant.

RESULTS
All animals survived the pre-ACA surgical procedure. One rat in ACA and one rat in ACA + hydrogen post-treatment groups could not be resuscitated.

Hydrogen treatment does not affect the hemodynamic parameters in rats with asphyxia induced-cardiac arrest
There were no significant differences in baseline and post-resuscitation values of heart rate, mean arterial pressure and
end-tidal carbon dioxide among ACA, ACA + hydrogen post-treatment and ACA + hydrogen pre- & post-treatment groups ($P > 0.05$; data not shown).

**Hydrogen treatment improves cognitive function of rats with asphyxia induced-cardiac arrest**

**T-maze**

At 7 days after resuscitation, the ACA rats had significantly worse performance in T-maze test than shams ($P = 0.025$). Although there was not statistical difference (hydrogen post-treatment + ACA vs. ACA: $P = 0.345$; hydrogen pre- & post-treatment +ACA vs. ACA: $P = 0.295$), both hydrogen gas treatment regimens tended to improve the percentage of spontaneous alternations, suggesting better exploratory behavior and working memory (Figure 1).

**Morris water maze**

Starting at 14 days after resuscitation, the Morris water maze test showed memory-learning deficits in ACA rats. On the 4th day of testing (18 days after resuscitation), ACA rats had significantly longer total swimming distances than shams ($P = 0.012$) before reaching the platform placed in the water. In the probe test in which the platform was removed from the southwest quadrant, ACA rats spent significantly lesser time in the southwest quadrant, suggesting spatial memory dysfunction ($P = 0.005$). Hydrogen treatments significantly shortened the total swimming distance in comparison to ACA rats ($P = 0.025$, ACA + hydrogen post-treatment vs. ACA; $P = 0.01$, ACA + hydrogen pre- & post-treatment vs. ACA; Figure 2). There was no significant difference in swimming speed among groups, suggesting the comparable motor function (data not shown).

**Hydrogen treatment does increased the neuronal survival in the hippocampal CA1 region of rats with asphyxia induced-cardiac arrest**

Correlating with the functional outcomes, Fluoro-Jade C staining showed: 1) elevated neuron death at day 7 ($P = 0.016$, vs. sham group) and day 18 ($P = 0.013$, vs. sham group) post-resuscitation within hippocampal CA1 regions in animals subjected to ACA; 2) hydrogen gas treatment improved neuronal survival at 7 days, reaching statistical significance at 18 days ($P = 0.027$, ACA + hydrogen post-treatment vs. ACA; $P = 0.016$, ACA + hydrogen pre- & post-treatment vs. ACA) after resuscitation (Figure 3).

**DISCUSSION**

Using a rat model of asphyxia induced-cardiac arrest, we demonstrated that 67% hydrogen gas administered either as pre- & post-treatment or post-treatment resulted in improved spatial learning function, which was associated with better neuronal survival within hippocampal CA1 regions.

Complex pathophysiologic mechanisms underlie global brain injury following cardiac arrest and cardiopulmonary-cerebral resuscitation, in which neuronal apoptosis is one of the important pathologic changes. The hippocampal CA1 region has been shown to be highly vulnerable to hypoxia-ischemia. In the present study, asphyxia induced-cardiac arrest resulted in global brain injury with significant degenera-
Figure 3: Effect of hydrogen (H2) treatment on the neuronal survival in the hippocampal CA1 region of rats with asphyxia-induced cardiac arrest (ACA) at 7 and 14 days (d) post-resuscitation.

Note: (A) In rats subjected to ACA, there were significantly greater number of Fluoro-Jade staining positive neurons, suggesting neuronal degeneration. H2 treatment improved neuron survival, which was significant at 14 d post-resuscitation. (B) Quantitative result of Fluoro-Jade staining positive neurons. Data were presented as the mean ± SEM (n = 6/group). *P < 0.05, vs. sham group; #P < 0.05, vs. ACA group (one-way analysis of variance followed by Student-Newman-Keuls post hoc test).

The mean ± SEM (n = 6/group). *P < 0.05, vs. sham group; #P < 0.05, vs. ACA group.

There are several limitations in the study. We only evaluated the cognitive benefits of hydrogen up to 18 days after ACA. Future study is necessary to confirm our finding at longer time points. In this study, we demonstrated cognitive benefits associated with the improved neuron survival following hydrogen administration after resuscitation from ACA. However, mechanisms of neuroprotective effects potentially provided by high-concentration hydrogen need to be further elucidated.

In conclusion, our study suggests that high concentration hydrogen derived by water electrolysis could be a promising therapeutic strategy to decrease cognitive deficits in the setting of cardiac arrest.

Author contributions
Study performing, and references gathering: LH; study design: RLA, PMA, JHZ; data collection: LG, UO; manuscript conceiving and drafting: LH, JHZ; manuscript revising: RLA, PMA, WB. All authors approved the final version of manuscript for publication.

Conflicts of interest
None declared.

Financial support
None.

Institutional review board statement
The study was approved by the Animal Health and Safety Committees of Loma Linda University, USA (approval number: IACUC #8170006) on March 2, 2017.

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Data sharing statement
Datasets analyzed during the current study are available from the corresponding author on reasonable request.

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