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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 ischemia[9-11] 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/100 g 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 medication system, 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 supraventriclar rhythm with mean artery 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.[20] 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.[20] 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.[21] 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 south-west 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).

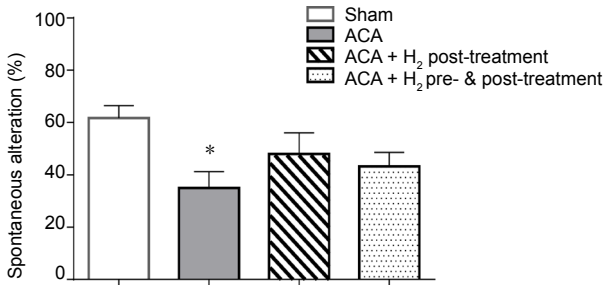

Figure 1: Effect of hydrogen (H_2) treatment on the T-maze test of rats with asphyxia induced-cardiac arrest (ACA) at 7 days post-resusciation.
Note: Rats subjected to ACA had significantly lower rates of spontaneous alteration between the right and left arms than shams. There was a tendency toward improved T-maze performance in ACA rats that received hydrogen gas pre&post-treatment. Data were presented as the mean ± SEM ($n = 6$/group). *$P < 0.05$, *vs*. sham group (one-way analysis of variance followed by Student-Newman-Keuls *post hoc* test).

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.[22] The hippocampal CA1 region has been shown to be highly vulnerable to hypoxia-ischemia.[4,5] In the present study, asphyxia induced-cardiac arrest resulted in global brain injury with significant degenera-

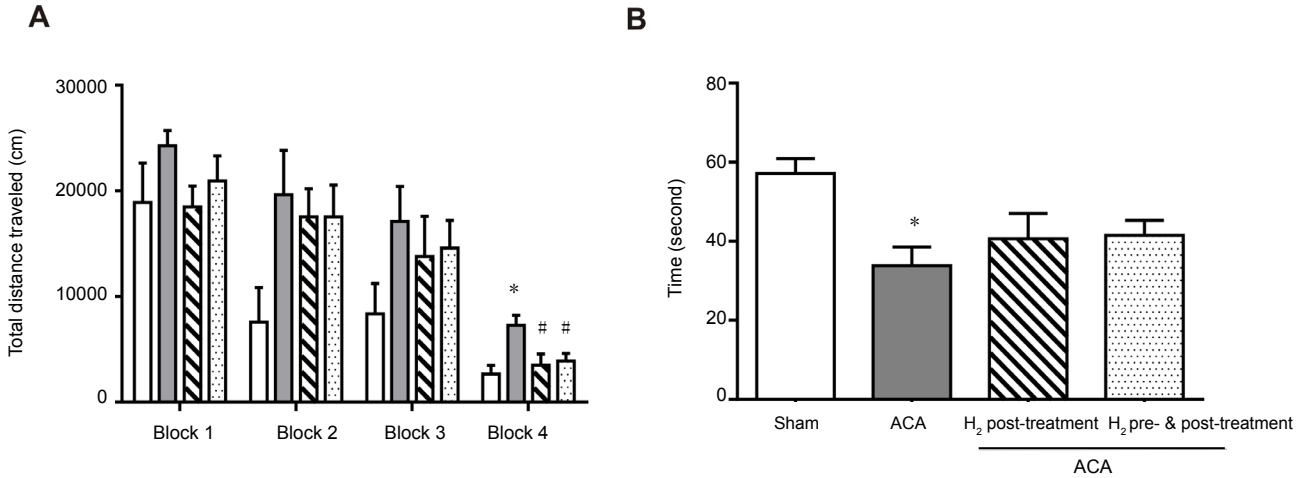

Figure 2: Effect of hydrogen (H_2) treatment on the Morris water maze test of rats with asphyxia induced-cardiac arrest (ACA) starting at 14 days post-resuscitation.
Note: (A) Swimming distance. There were signficant longer swimming distances in rats subjected to ACA at the 4th day of spatial learning testing. H_2 treatment significantly shortened the total swimming distance. (B) Latency. Probe test showed that rats subjected to ACA spent significantly lesser time than shams in the quadrant where the platform was placed during spatial learning testing. This effect was diminished by H_2 treatments. 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).

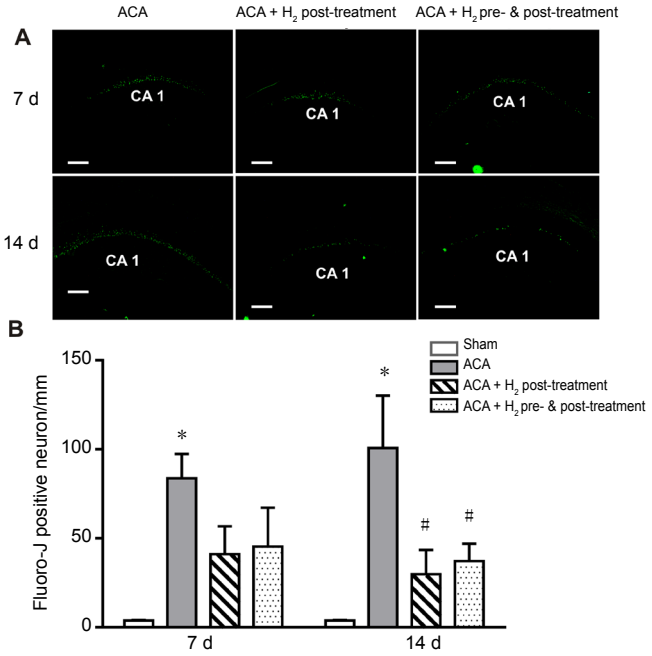

Figure 3: Effect of hydrogen (H_2) treatment on the neuronal survival in the hippocampal CA1 region of rats with asphyxia induced-cardiac arrest (ACA) at 7 and 14 days (d) post-resusciation.

Note: (A) In rats subjected to ACA, there were signifcantly greater number of Fluoro-Jade staining positive neurons, suggesting neuronal degeneration. H_2 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).

tion of neurons within hippocampal CA1 regions at 7 and 18 days post-resuscitation. This neuronal damage was associated with hippocampal dysfunction as detected by T-maze and water maze test. The retired breeder rats used in this study were 8–12 months of age and our findings are consistent to previous report by Cohan et al.[23] In their study, 9-month-old rats demonstrated significant spatial memory impairment and hippocampal CA1 neuron reduction following asphyxia cardiac arrest.[23]

Oxidative stress causes lipid peroxidation and DNA oxidation, contributing to neuronal apoptosis in the setting of ischemia.[24] Low concentration hydrogen gas inhalation at the start of CPR provided similar neuroprotection to therapeutic hypothermia and resulted in additive effects through its unique antioxidant features when applied with hypothermia in a rat model of ventricular fibrillation cardiac arrest.[25,26] Hydrogen-rich saline also reduced the hippocampal tissue damage induced by global cerebral ischemia/reperfusion *via* protection of mitochondrial function.[27] In 2016, Cui et al.[17] demonstrated the neuroprotective efficacy of water electrolysis derived 67% hydrogen inhalation in a rat model of focal brain ischemia. Two hours of high concentration hydrogen treatment decreased brain reactive oxygen species levels, neuronal apoptosis and neuroinflammation within the cerebral cortex and hippocampus in the absence of systemic toxicity.[17] In the present study, a modified T-maze used as a quick test of hippocampal function at 7 days after resuscitation showed water-electrolysis 67% hydrogen tended to improve exploratory behavior although this was not statistically significant. The improved functional outcome correlated with a tendency toward increased hippocampal CA1 neuron survival at 7 days post-resuscitation. Over 4 days beginning 14 days after resuscitation, rats treated with 67% hydrogen had significant improvement of hippocampal spatial learning memory as tested by water maze, a comprehensive behavioral test. Significantly less hippocampal CA1 neuron degeneration was associated with this cognitive improvement. Activation of the phosphoinositide 3-kinase/Akt pathway has been shown to play a role in the protective effects of 67% hydrogen gas in a rat model of liver ischemia-reperfusion.[28] Because of the importance phosphoinositide 3-kinase/Akt signaling in neuronal survival,[29] we speculate that a similar mechanism of protection would underlie the hippocampal neuron protection provided by high concentration hydrogen. Nevertheless, molecular hydrogen has been shown to activate a variety of signaling pathways contributing to its neuroprotection.[30]

Human clinical studies of therapeutic hydrogen gas administration have been undertaken in Japan in 2017.[12,31,32] The feasibility and safety of 2% hydrogen for patients with post cardiac arrest syndrome have been validated in a pilot clinical study.[33] A multicenter randomized trial to confirm the efficacy of low concentration hydrogen gas inhalation on neurological outcomes in comatose survivors of out-of-hospital cardiac arrest is underway.[12] Additionally, 3% hydrogen inhalation administered to patients 1 hour twice a day for 7 days following mild ischemic stroke significantly improved both MRI and neurological outcomes.[32]

If the dose dependent effects of molecular hydrogen therapy that have been demonstrated in animal studies[34,35] translate to human neurological outcomes, then water electrolysis would be a safe way to generate high concentration hydrogen gas and would be feasible to use in clinical management of cardiac arrest survivors.

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 the 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 manscript 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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before publication.

Data sharing statement

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

Plagiarism check

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