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Hydrogen gas treatment improves survival in a rat model of crush syndrome by ameliorating rhabdomyolysis

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Abstract

Objectives: Crush syndrome (CS) is characterized by a systemic manifestation of traumatic rhabdomyolysis, leading to multiple organ dysfunction and death. Ischemia-reperfusion (IR) injury is commonly responsible for systemic response. Extending studies have shown that hydrogen gas treatment ameliorated IR injury in numerous experimental models; however, its effect on CS has not been well examined. This study aimed to investigate the effects of hydrogen gas inhalation following crush injury in an experimental model of CS.

Methods: Male Sprague-Dawley rats were subjected to experimental CS by applying a total of 3.0 kg weight to both hindlimb under general anesthesia for 6 h. Immediately after decompression, the animals were randomly placed in a gas chamber filled with either air or 1.3% hydrogen gas. Animals were sacrificed 18 h or 24 h following gas exposure for non-survival studies or for survival study, respectively.

Results: The rats with hydrogen treatment (n = 6) had a higher 24-h survival than the rats with air treatment (n = 9) (100% vs. 44%, p = 0.035). Lactate concentrations (2.9 ± 0.2 vs. 2.2 ± 0.2 mmol/L, p = 0.040) and creatine kinase (34,178 ± 13,580 vs. 5005 ± 842 IU/L, p = 0.016) were lower in the hydrogen group compared with the air group 18 h after decompression (n = 4 in the air group, and n = 5 in the H_2 group). Histological analysis revealed that the damage to the rectus femoris muscle and kidney appeared to be ameliorated by hydrogen treatment.

Conclusion: Hydrogen gas inhalation may be a promising therapeutic approach in the treatment of CS.

Keywords

Crush syndrome, experimental model, hydrogen, ischemia, reperfusion injury

Introduction

Crush syndrome (CS) is characterized by a systemic manifestation of traumatic rhabdomyolysis, leading to multiple organ dysfunction and death. The condition commonly occurs after crush injury due to prolonged limb compression during natural disasters such as a devastating earthquake.[1] Systemic response in CS is most often attributed to ischemia-reperfusion (IR) injury as well as direct trauma to the tissue.[2] Basically, ischemia drives

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mitochondrial dysfunction as a result from excessive reactive oxygen species (ROS) production. Cumulative damage to mitochondria is exacerbated by long-lasting opening of mitochondria permeability transition pores during the reperfusion phase, leading to mitochondrial swelling and subsequent cell death.[3,4] To date, specific targeted therapies are lacking in this aberrant response other than supportive care.

Indeed, numerous literature have shown that hydrogen gas treatment ameliorated IR injury in experimental models, including cardiac ischemia, intestinal or lung transplantation, as well as hemorrhagic shock.[5,6] Although the precise mechanisms are not fully understood, hydrogen gas exhibits anti-oxidative effects partly through eliminating ROS. In addition, hydrogen gas exerts anti-inflammatory and anti-apoptotic effects under various physiological and pathological conditions. Scavenging properties for reactive oxygen species are unlikely to be the only explanation and undefined biological mechanisms of hydrogen as a signaling molecule may be involved. Mechanism underlying the cellular protection afforded by hydrogen may be an increase in antioxidant enzymes such as catalase, superoxide dismutase or heme oxygenase-1.[7,8] Given its beneficial implications, we hypothesized that hydrogen gas serves as a therapeutic target of CS. As inhaled hydrogen has a low chemical toxicity,[9] this gaseous therapy has good clinical feasibility, as long as its flammability can be controlled. Therefore, the present study was aimed to explore the effects of hydrogen gas inhalation following crush injury in an experimental model of CS.

Methods

Animals

Specific pathogen free male Sprague-Dawley rats weighting 230–300 g were obtained from CLEA Japan Inc. (Tokyo, Japan) and were kept at a temperature of 23 ± 1°C on a 12-h light/dark cycle, with free access to food and water. All animal experiments were conducted following the national guidelines and the relevant national laws on the protection of animals. All experimental procedures were approved by the Animal Care and Use Committee, Okayama University, and conducted in accordance with the Policy on the Care and Use of the Laboratory Animals, Okayama University (OKU-2022275).

Crush syndrome model

The CS model was adopted from previously described procedures with some modifications.[10,11] The experimental procedure is described in elsewhere (Figure 1). Briefly, the anesthesia was induced and maintained with isoflurane gas. A polyurethane tube was surgically inserted into right jugular vein for fluid resuscitation. Then, the animals were placed in supine position on an apparatus which was specifically designed for inducing crush injury (A-tech Co, Okayama, Japan). A total of 3.0 kg weight was applied to both hindlimb with a plastic cylinder 3.0 cm in diameter

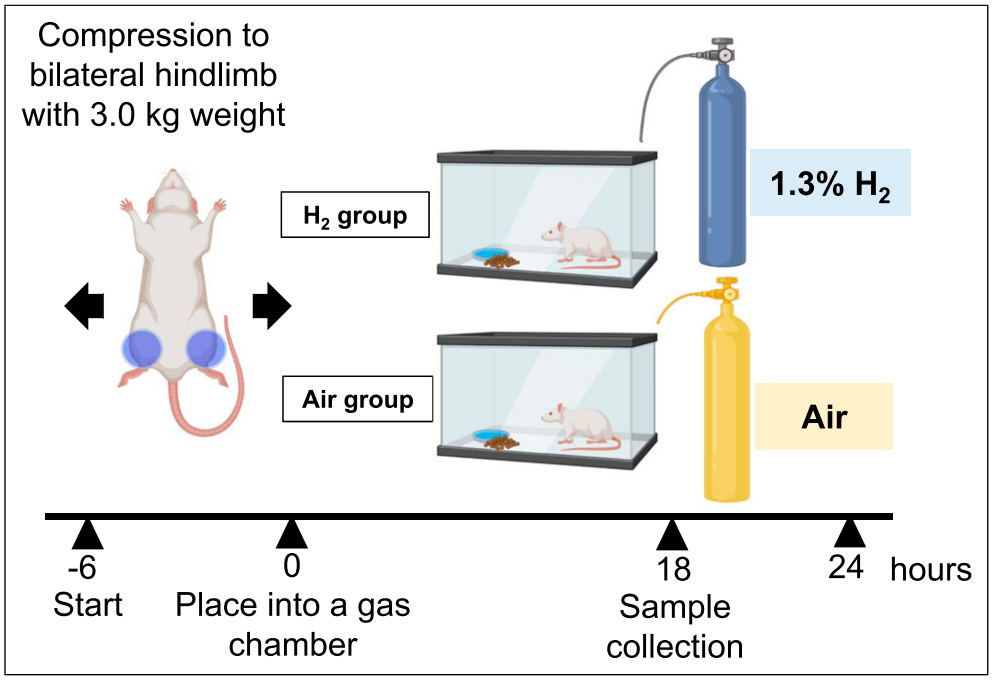

Figure 1. Experimental procedure.

along the bilateral inguinal ligament for 6 h. Immediately after decompression of the hindlimb, the animals were placed in a 20-L air-tight chamber to be exposed to either hydrogen gas or air in a random manner. All animals received normal saline via the jugular venous catheter at 5 mL/kg per hour for the first 6 h of compression and bolus infusion of 10 mL/kg normal saline immediately after decompression. Animals were sacrificed 18 h or 24 h following gas exposure by exsanguination under deep isoflurane anesthesia for non-survival studies or for survival study, respectively. The animals used for survival study were exclusively observed up to 24 h (i.e., blood sampling was not performed at 18 h). For non-survival studies, three animals in the air group died before sample collection.

Hydrogen gas exposure

The animals were exposed to 1.3% hydrogen gas in a chamber at a flow rate of 1.0 L/min for as long as 24 h with regular diet and water ad libitum. Animals in the control group were exposed to air instead of hydrogen gas.

Blood gas analysis

Blood sample was collected 18 h after decompression via abdominal aorta puncture. Blood gas analysis was performed to determine pH, partial pressure of arterial carbon dioxide ($PaCO_2$), partial pressure of arterial oxygen (PaO_2), base deficit, and lactate concentrations (ABL800, RADIOMETER, Tokyo, Japan).

Serum biochemistry

The remaining part of the collected blood sample was centrifuged at 3,000 rpm for 10 min at 4°C. Serum potassium, creatine kinase (CK), aspartate aminotransferase (AST), alanine aminotransferase (ALT), and creatinine levels were measured (Oriental Yeast Co., Ltd, Tokyo, Japan).

Histological assessment

At sacrifice, animals were transcardially perfused with PBS followed by 4% paraformaldehyde (PFA). Left rectus femoris muscle and left kidney were removed and fixed with 4% PFA for 48 h. The tissue was then processed for paraffin embedding. Each block was sectioned into 4 μm-thick slices and stained with hematoxylin and eosin (H&E). Muscle damage was assessed by grading histopathological changes of muscle fibers and inflammation following the methods described previously.[12] Briefly, histological scores were based on the sum of the two grades; disorganization and degeneration of the muscle fibers (0: normal, 1: mild, 2: moderate, 3: severe) and inflammatory cell infiltration (0: normal, 1: mild, 2: moderate, 3: severe). Renal injuries were scored by evaluating tubular necrosis, interstitial edema, loss of brush border and casts formation according to a previously described method.[13] Severity of change for each parameter was graded as following: 0, absent; 1, minimal changes; 2, moderate changes; 3, marked changes). The sum of each score was obtained as a total histological score of renal injury. The evaluation was done by two members (TH and TN) who were unaware of the experimental procedure.

Statistical analysis

Data are presented as mean ± SEM. Mann-Whitney test was used for two-group comparison, and Log-rank test was performed to analyze survival study. A p value of <0.05 was considered to be statistically significant. Statistical analyses and generation of summary graphs were performed in Prism 9.0 (GraphPad, San Diego, CA).

Results

Survival analysis

The rats with hydrogen treatment had a higher 24-h survival after inducing CS than the rats with air treatment, (100% vs. 44%, $p = 0.035$, $n = 6$ in the H_2 group and $n = 9$ in the air group, Figure 2).

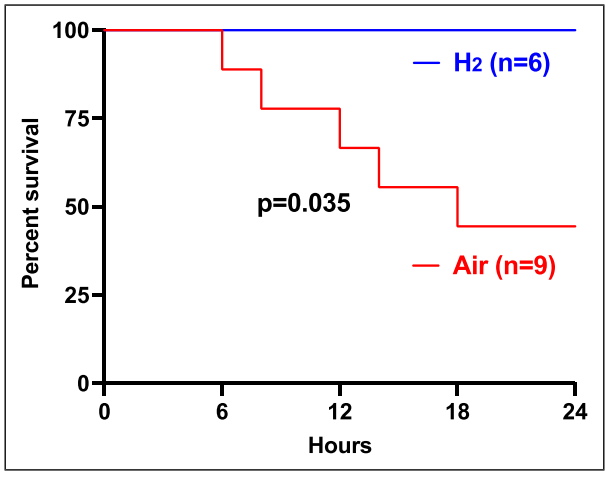

Figure 2. Effect of hydrogen gas inhalation on survival from CS. Hydrogen gas-inhaled rats had significantly higher 24-h survival than air-inhaled rats ($n = 9$ in the air group and $n = 9$ in the H_2 group, $p = 0.035$).

Blood gas analysis

Lower lactate concentrations were observed in hydrogen group compared with air group 18 h after decompression (2.9 ± 0.2 vs. 2.2 ± 0.2 mmol/L, $p = 0.040$, $n = 4$ in the air group, and $n = 5$ in the H_2 group, Figure 3(a)). There were no significant differences in pH, $PaCO_2$, PaO_2, and base deficit between the hydrogen group and the air group 18 h after decompression (Figures 3(b) to (e)).

Serum biochemistry

Serum concentrations of CK were lower in the hydrogen group compared with the air group at 18 h (34,178 ± 13,580 vs. 5005 ± 842 IU/L, $p = 0.016$, $n = 4$ in the air group, and $n = 5$ in the H_2 group, Figure 3(f)). In contrast, no differences were seen in serum potassium, AST, ALT, and creatinine levels at 18 h between the two groups (Figures 3(g) to (j)).

Histological analysis

H&E staining of the rectus femoris muscle 18 h after decompression revealed interstitial edema and hemorrhage accompanied with infiltration of neutrophils in the air-treated rat, while these changes were mitigated by hydrogen treatment (Figure 4(a)). Muscle damage score was higher in the air group compared with the hydrogen group (5.3 ± 0.25 vs. 2.5 ± 0.29, $p = 0.029$, $n = 4$ per group, Figure 4(b)).

Kidney damages including tubular degeneration, interstitial edema, brush border loss and tubular luminal occlusion with casts appeared to be ameliorated by hydrogen gas treatment (Figure 4(c)); however, the difference in a total histological score of renal injury was not statistically significant (8.0 ± 0.58 vs. 5.5 ± 0.65, $n = 4$ per group, $p = 0.09$, Figure 4(d)).

Discussion

This study demonstrated that survival was markedly increased in hydrogen gas-inhaled rats following CS induction. This survival benefit could be attributed to an amelioration of direct damage to a muscle, as shown by a decrease in serum CK levels and histology. We observed 24 h survival rate in the control group of 44%, which was in line with previous studies using the same rat model of CS.[11,14] As shown in these literature, systemic inflammation induced by IR injury might be responsible for hypovolemic shock, leading to early death. It was speculated that hydrogen treatment ameliorated this process and showed survival benefit, as evident by lower serum lactate levels in the hydrogen group. Meanwhile, it is well-known that acute kidney injury is one the most serious complications of CS;[2] however, it is unclear why serum creatinine levels and potassium were similar between the hydrogen group and the air group. As elevated creatinine is a by-product of impaired clearance, it may take more time to increase significantly after an acute kidney injury. This may explain the lack of difference in creatinine values between the groups. In addition, no differences in pH, $PaCO_2$, PaO_2, base deficit, AST, ALT, and a total histological score of renal injury were detected between the groups. Further evaluation at later timepoint as well as larger sample size would tell us more about the mechanism of survival benefit of hydrogen gas.

A former study has shown that intravenous administration of vitamin E derivative anti-oxidant, known as ETS-GS, alleviated muscle damage by suppressing ROS generation using a rat model of crush injury.[15] Another

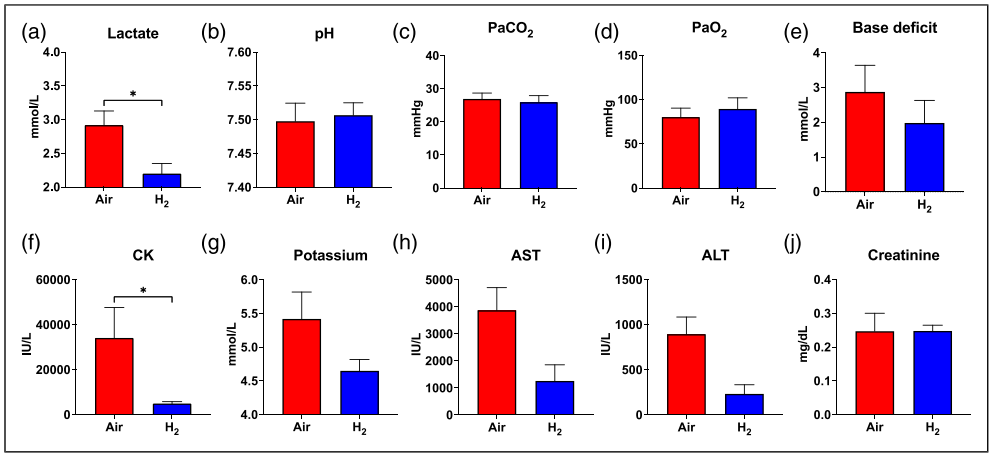

Figure 3. Blood gas analysis and serum biochemistry 18 h after decompression. Lactate and CK levels were lower in hydrogen group compared with air group, while no differences were seen in pH, $PaCO_2$, PaO_2, base deficit, potassium, AST, ALT, creatinine between the groups ($n = 4$ in the air group, and $n = 5$ in the H_2 group). Data are shown as mean ± SEM.

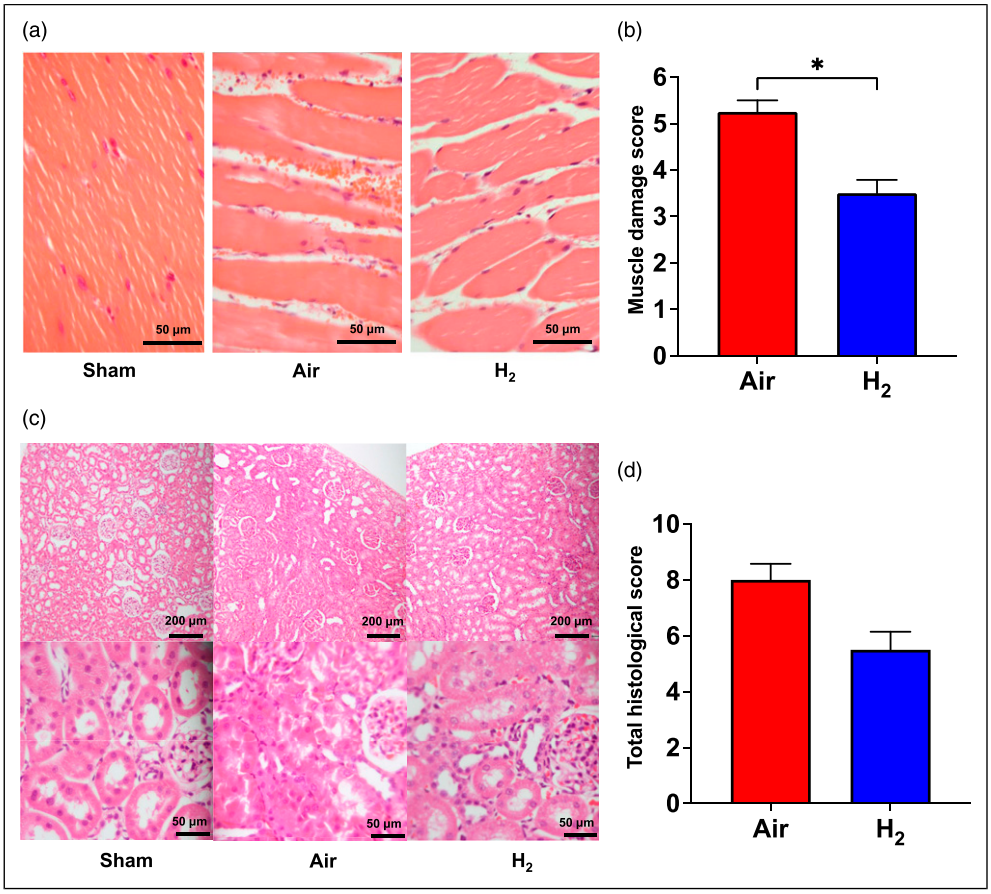

Figure 4. Histological analysis of the rectus femoris muscle and kidney 18 h after reperfusion. Representative histomicrograph of the rectus femoris muscle shows interstitial edema and hemorrhage accompanied with infiltration of neutrophils in rat treated with air, which were ameliorated by hydrogen treatment (A). Muscle damage score was higher in the air group compared with the H_2 group ($n = 4$ per group, $p = 0.029$) (B). Representative histological micrographs of renal tissue are characterized by tubular degeneration, interstitial edema, brush border loss and tubular luminal occlusion with casts (C). Hydrogen gas treatment trended toward decreasing a total histological score of renal injury, but statistical significance was not reached ($p = 0.09$). Data are shown as mean ± SEM.

study has demonstrated that intraperitoneally administration of sodium hydrogen sulfide, which is a donor of hydrogen sulfide as an antioxidant, decreased CK levels following crush injury in rats.[16] Our findings presented herein yield new insights into the specific therapy for CS by inhalation of hydrogen gas. Since the pathophysiology of CS is complicated, our model may not be appropriate to determine detailed protective mechanisms of the actions provided by hydrogen inhalation. However, we still believe that our study is an important step toward clinical application of hydrogen inhalation therapy.

This study was limited by a single timepoint assessment and minimum mechanistic study, as the pathophysiology of CS includes multiple complicated processes. We could have missed some important changes that may explain the survival benefit after hydrogen gas treatment. Further research is warranted to offer additional mechanistic insights. Another limitation includes that we did not perform power calculation for estimation of sample size selected for the study. Recently, the therapeutic effects of hydrogen in a rhabdomyolysis model have been reported.[17] However, we believe that our study may make an important step toward expanding hydrogen has therapy. For clinical application, minimum duration of treatment, timing and effectiveness of inhalation after reperfusion, and whether it is beneficial in a mechanically ventilated setting need to be explored.

Conclusions

This study demonstrates that hydrogen gas treatment results in the mitigation of rhabdomyolysis and lowered lactate levels, leading to improved survival after induction of CS. Hydrogen gas inhalation may represent a potential novel therapeutic agent against CS.

Author Contributions

TY and AN designed research. TY performed most experiments with the assistance of TA, TH, HY, TO, TN, and HN. TY, TA, and AN analyzed and interpreted the data. TY and AN wrote the paper. All authors approved the final version of the manuscript.

Declaration of Conflicting Interests

The author(s) declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

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Ethical approval

Ethical approval for this study was obtained from the Animal Care and Use Committee, Okayama University (OKU-2022275). The present study followed international, national, and/or institutional guidelines for humane animal treatment and complied with relevant legislation.

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