Hydrogen gas therapy improves survival rate and neurological deficits in subarachnoid hemorrhage rats: a pilot study

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Abstract

The high morbidity, high mortality, and significant shortage of effective therapies for subarachnoid hemorrhage (SAH) have created an urgency to discover novel therapies. Human studies in Asia have established the safety of hydrogen gas in the treatment of hepatic, renal, pulmonary, and cardiac diseases. Mechanistically, hydrogen gas has been shown to affect oxidative stress, inflammation, and apoptosis. We hypothesized that hydrogen therapy would improve neurological function and increase survival rate in SAH. High dose hydrogen gas (66% at 3 L/min) was administered for 2 hours at 0.5, 8, and 18 hours after SAH. This treatment increased 72-hour survival rate and provided 24-hour neuroprotection after SAH in rats. To our knowledge, this is the first report demonstrating that high dose hydrogen gas therapy reduces mortality and improves outcome after SAH. Our results correlate well with the proposed mechanisms of hydrogen gas therapy within the literature. We outline four pathways and downstream targets of hydrogen gas potentially responsible for our results. A potentially complex network of pathways responsible for the efficacy of hydrogen gas therapy, along with a limited mechanistic understanding of these pathways, justifies further investigation to provide a basis for clinical trials and the advancement of hydrogen gas therapy in humans. This study was approved by the Institutional Animal Care and Use Committee of Loma Linda University, USA (Approval No. 8160016) in May 2016.

Key words: hydrogen gas therapy; subarachnoid hemorrhage; mortality; survival; early brain injury; high dose hydrogen; oxidative stress; free radicals; reactive oxygen species; hydrogen pathway; cerebral vasospasm

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Introduction

The high morbidity, high mortality, and significant shortage of effective therapies for subarachnoid hemorrhage (SAH) have created an urgency to discover novel therapies.[1,2] Reversal of cerebral vasospasm and subsequent delayed cerebral ischemia has been a long-standing target for the treatment of SAH. Although animal studies have shown promising results for the treatment of cerebral vasospasm, the CONSCIOUS-1 trial showed that even after reversal of cerebral vasospasm, significant improvement in morbidity and mortality could not be achieved.[3-9] Clearly additional mechanisms are at play since nimodipine, a calcium channel blocker, has shown clinical benefit while lacking a significant effect on cerebral vasospasm.[10] From these observations, research has pivoted toward reduction of early brain injury.[11] Specifically, reduction of inflammation and oxidative stress using high dose hydrogen gas shows promise for treatment of the deleterious aftermath of SAH.[12-18]

Human studies in Asia have established the safety of hydrogen gas in the treatment of hepatic, renal, pulmonary, and cardiac diseases.[19] Mechanistically, hydrogen gas has been shown to affect oxidative stress, inflammation, and apoptosis.[20-22] Recent findings in basic science research support the safety and benefit of hydrogen gas in the management of neuro-inflammatory diseases (subarachnoid hemorrhage, ischemic stroke, intracerebral hemorrhage, traumatic brain injury, germinal matrix hemorrhage, and spinal cord injury).[23] Most significantly, hydrogen gas has been shown to neutralize hydroxyl free radicals, a damaging oxidant for which the body has no endogenous defense.[20] Additionally, hydrogen gas provides established protection against ischemic blood-brain barrier damage and likely regulates gene expression to mitigate disease processes.[24]

Materials and Methods

Animals

A total of 35 adult male Sprague-Dawley rats (weighing 300–320 g, 8–10 weeks of age) were used in the proposed study. All experiments were approved by the Institutional Animal Care and Use Committee of Loma Linda University, USA (Approval No. 8160016) in May 2016, complied with the National Institutes of Health's Guide for the Care and Use of Laboratory Animals, and are reported according to the Animal Research: Reporting of *In Vivo* Experiments (ARRIVE) guidelines. Animals were housed in a 12-hour light-dark cycle, temperature-controlled room. Animals were divided into sham, SAH + air, and SAH + hydrogen groups in a randomized fashion and experiments were performed in a blinded manner. Rats subjected to SAH were treated with hydrogen gas (SAH + hydrogen gas group) *versus* room air gas (SAH

+ air group) for 2 hours at 0.5, 8, and 18 hours (**Figure 1**). Neurobehavioral testing (forelimb placement) was performed at 24 or 72 hours before sacrifice. Survival analysis took place at 72 hours between air and hydrogen groups.

Subarachnoid hemorrhage model

The endovascular perforation model was induced as previously described.[25] Briefly, rats were intubated and maintained with 3% isoflurane anesthesia in the air. Rodents were placed in a supine position, and the neck was opened with a scalpel in the midline. After localization of the appropriate vessels, a sharpened 3-cm, 4-0 nylon suture was inserted into the left internal carotid artery through the external carotid artery and the common carotid bifurcation. The suture was advanced until resistance was reached, further advanced to puncture the vessel, and then immediately withdrawn after artery perforation. The sham group underwent the same procedure without an endovascular puncture. After removal of the puncturing suture, the skin incision was sutured, and the rats were housed individually in heated cages until recovery.

Subarachnoid hemorrhage grade

SAH grade scoring was performed at 24 and 72 hours after SAH by an independent, blinded investigator as previously described.[26] Briefly, the basal cistern was divided into six segments (left and right frontal, left and right temporal, and upper and lower brain stem) that were scored from 0 to 3 according to the amounts of subarachnoid blood (Grade 0, no subarachnoid blood clotting; grade 1, minimal subarachnoid blood clotting; grade 2, moderate blood clotting with recognizable arteries; and grade 3, blood clotting obliterating all arteries within the segment). Rats with a grade < 7 at 24 and 72 hours after SAH were excluded from this study.

Hydrogen gas administration

The AMS-H-01, manufactured by Asclepius Meditec Co., Ltd., Shanghai, China, produces a ~66% hydrogen gas mixture at a rate of 2–3 L/min. It is capable of real-time hydrogen production for inhalation through a unique water electrolysis method. Molecularly, the AMS-H-01 splits water into its stoichiometric 2:1 hydrogen to oxygen ratio, explaining the ~66.6% hydrogen and ~33.3% oxygen gas mixture produced by the machine.[27] Inhalation of hydrogen gas started 0.5 hour after SAH. Rats were placed in a transparent chamber that had an inlet connected with 66% hydrogen gas and an outlet to a ventilating hood. A handheld hydrogen detector (H_2 scan, Valencia, CA, USA) was used to confirm the concentration of hydrogen gas intermittently throughout the treatment. The treatment episodes lasted 2 hours. Two additional treatment sessions were performed at 8 and 18 hours after SAH induction. In between and after treatment, animals were housed individually with free access to food and water in the animal facility.

Neurological test

Vibrissae evoked forelimb placing test was assessed by a blinded observer at 24 and 72 hours post-SAH, as previously described.[28] We conducted left/right forelimb testing to assess for asymmetry in the sensorimotor cortex and striatum. The experimenter held the animal so that all four limbs hung freely, and the whiskers were stimulated by sweeping each side against the edge of a table. This elicits an ipsilateral forelimb reflex to place the paw on the table top. Each rat was stimulated 10 times on each side, and the total number of paw placements was recorded.

Statistical analysis

Quantitative data are presented as the mean ± SD. One-way analysis of variance with post-hoc Tukey test was used to determine significant differences among groups at each time point for SAH grade and neurobehavior. The Kaplan-Meier survival analysis was used to determine significant survival rate differences amount groups. $P < 0.05$ was considered statistically significant. GraphPad Prism 6 (GraphPad Software, La Jolla, CA, USA) was used for graphing and analyzing all data.

Results

Effects of hydrogen gas therapy on subarachnoid hemorrhage grade and neurobehavioral function 24 hours after subarachnoid hemorrhage

Our hypothesis is that hydrogen gas will provide therapeutic benefits following SAH in rats and improve neurological outcomes. In SAH + air and SAH + hydrogen groups, there

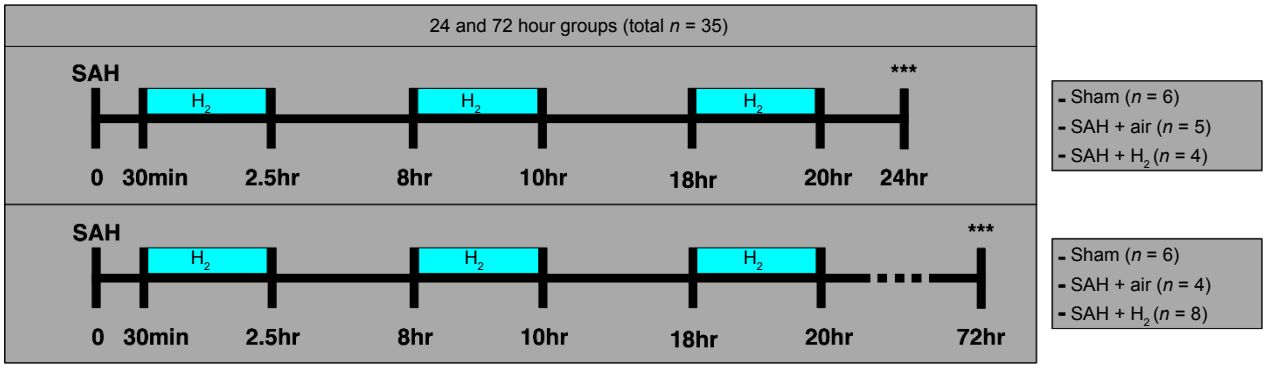

Figure 1: Timeline for experimental procedures.
Note: Diagram showing hydrogen gas therapy duration and administration post-subarachnoid hemorrhage (SAH). Rats were divided into three cohorts: sham, SAH + air, and SAH + hydrogen (H_2) groups. min: Minutes; hr: hours. *** represents the mortality, neurobehavioral test, and SAH grade analysis.

was a significant increase in SAH grade compared to the sham group (one-way analysis of variance followed by Tukey's test; $n = 4–6$, $P < 0.05$; **Figure 2A**). In the SAH + air group, there was a significant reduction in forelimb placement compared to the sham group ($P < 0.05$; **Figure 2B**); however, in the SAH + hydrogen group, neurobehavior scores were restored to sham levels with increased left-forelimb placement scores compared to the SAH + air group ($P < 0.05$; **Figure 2B**).

Effects of hydrogen gas therapy on subarachnoid hemorrhage grade and neurobehavioral function 72 hours after subarachnoid hemorrhage

To evaluate the effects of high dose hydrogen gas treatment (66% hydrogen) on long-term neurological outcomes, SAH grade and neurobehavior were assessed at 72 hours. Consistent with the other results in this study both in the SAH + air and SAH + hydrogen groups, there was a significant increase in SAH grade compared to the sham group (one-way analysis of variance followed by Tukey's test; $n = 4–8$; $P < 0.05$; **Figure 3A**). At 72 hours, no significant difference was observed between groups in right-forelimb placement scores ($P > 0.05$; **Figure 3B**). Since our 24 hours results support a significant benefit from acute hydrogen treatment, while 72 hours results do not, more sensitive neurological testing may expose additional significance. Nonetheless, this treatment shows promise in subarachnoid hemorrhage.

Hydrogen gas improved the survival rate of rats after subarachnoid hemorrhage

Hydrogen gas therapy increased the survival rate in the SAH rat model (**Figure 4**). The Kaplan-Meier survival analysis estimated the rate of death over the course of 72 hours among rats with air *vs*. hydrogen treatment. Two rats (out of 6) and 0 rat (out of 8) died in the SAH + air and SAH + hydrogen groups, respectively. The survival rate of the SAH+ hydrogen group (100%) was significantly higher than that in the SAH + air group (log-rank (Mantel-Cox) test; $n = 6–8$; $P = 0.0115$; **Figure 4**).

Discussion

In the present study, we made the following observations: (1) hydrogen gas therapy had no detrimental effects on SAH grade 24 hours after SAH compared to air; (2) hydrogen gas therapy after SAH improved neurobehavioral function at 24 hours; (3) 72 hours after hydrogen gas therapy, there were no deleterious effects between the SAH + air and SAH + hydrogen groups; (4) hydrogen gas significantly improved survival rate compared to the SAH + air group.

Basic science discoveries will ideally create new clinical treatment opportunities for the currently limited list of effective SAH therapies. Thus, we hypothesized that hydrogen therapy would improve neurological function and increase survival rate in SAH. We observed an increase in left-forelimb placement scores at 24 hours. This may be attributed to the amelioration of oxidative stress associated with SAH. A potentially complex network of pathways responsible for the efficacy of hydrogen gas therapy, along with a limited mechanistic understanding of these pathways, justifies further investigation to provide a basis for clinical trials and the

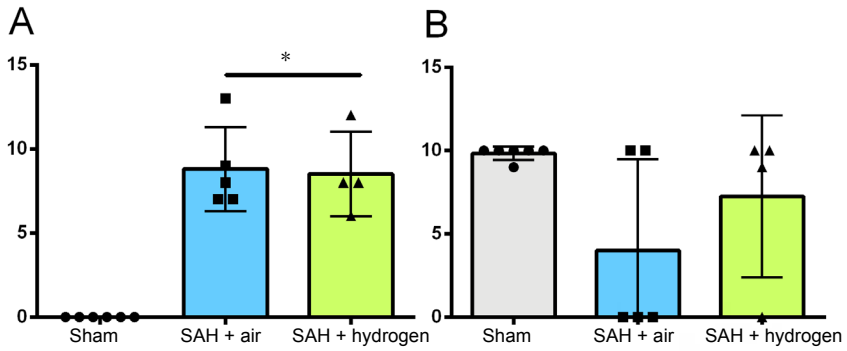

Figure 2: Hydrogen gas therapy impact on subarachnoid hemorrhage (SAH) grade and neurological function 24 hours after SAH.
Note: (A) SAH grade. There was no significant difference between air and hydrogen gas therapy at 24 hours. (B) Neurological test. Left-forelimb placement was improved with hydrogen gas therapy. Data represent the mean ± SD ($n = 6$ in sham group, 5 in SAH + air group, and 4 in SAH + hydrogen group). *$P < 0.05$, *vs*. sham group (one-way analysis of variance followed by *post-hoc* Tukey's test).

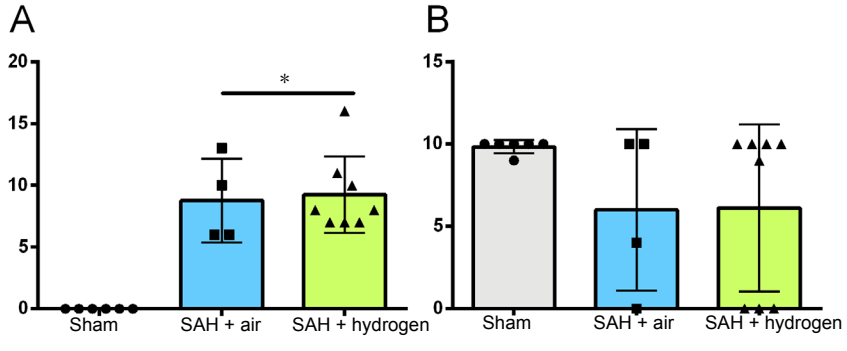

Figure 3: Hydrogen gas had no effect on subarachnoid hemorrhage (SAH) grade and neurological function 72 hours after SAH.
Note: (A) SAH grade. SAH grade quantification was carried out and showed no significant difference between SAH + air and SAH + hydrogen groups; however, both were significant compared to the sham group. (B) Neurological test. Right-forelimb placement scores were restored to sham levels, showing no significant difference between groups ($P > 0.05$). Data represent the mean ± SD ($n = 6$ in sham group, 4 in SAH + air group, and 8 in SAH + hydrogen group). *$P < 0.05$, *vs*. sham group (one-way analysis of variance followed by *post-hoc* Tukey's test).

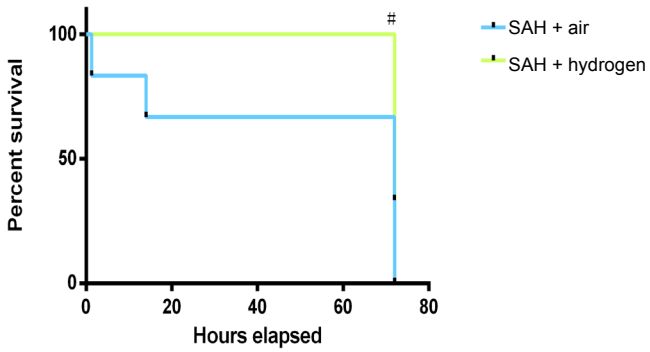

Figure 4: General evaluation of subarachnoid hemorrhage (SAH) models and the effect of hydrogen gas on mortality 72 hours after SAH.
Note: Survival analysis during 72 hours after SAH. *n* = 6 in SAH + air group, 8 in SAH + hydrogen group, #*P* < 0.05, *vs.* SAH + air group (Kaplan-Meier survival analysis).

advancement of hydrogen gas therapy in humans.

To date, the majority of studies have investigated the use of low dose hydrogen therapy, including concentrations of 1%,[29] 1.3%,[30] 2%,[29] 2.1%,[31] and 4%,[29] but using the AMS-H-01 electrolysis method, we were able to achieve a concentration of ~66.6% hydrogen in our therapy. In the present study, we observed a reduction of mortality at 72 hours and neurological deficits at 24 hours with hydrogen gas therapy after SAH induction. To our knowledge, this is the first report demonstrating that high dose hydrogen gas therapy reduces mortality and improves outcomes after SAH. Also, high dose hydrogen therapy is shown to have no deleterious effects on neurological function at both 24 and 72 hours after SAH. Studies report SAH mortality to range from 25% to 35% in high-income countries and up to 48% in low-income countries; however, since the data did not include pre-hospital deaths, the actual rate is likely much higher than these reported percentages.[32] In spite of therapeutic advances in endovascular techniques for the prevention of re-bleeding, mortality caused by SAH has not decreased.[33] The ability of hydrogen gas therapy to reduce mortality rates after SAH in our rats is a tremendous breakthrough, especially if these results can successfully be translated to humans. In parallel, literature has shown efficacy of hydrogen gas therapy through the attenuation of oxidative stress in neurons under oxygen and glucose deprivation,[34] attenuation of infarction volume in rat occlusion models,[31,35,36] and increased survival rate in mice after global ischemia.[30] Our results correlate well with proposed mechanisms of hydrogen gas therapy within the literature. Recent studies exploring downstream targets of hydrogen gas proposed four potential pathways responsible for our results: reduction of hydroxyl radicals in neurons,[37] reduction of inflammatory pathways through the upregulation of miR-199,[38] reduction of cellular death through the activation of an autophagosomal-lysosomal pathway (*via* the Adenosine 5′-monophosphate-activated protein kinase/mammalian target of rapamycin pathway),[39-41] and upregulation of mitochondrial unfolded protein responses.[42,43] Additionally, early brain injury in patients with SAH is linked to a poor neurological grade at the time of hospital admission.[41] Thus, the effects of hydrogen gas observed in the 24-hour neurobehavioral evaluation may occur through the reversal of these acute pathological processes.

We outline several limitations to the current study. First, no significant difference was observed in neurobehavior at 72 hours. One possible reason is that early death within the SAH + air group skewed neurobehavior scores of the surviving rats within this group toward an elevated average and precluded significant differences in neurobehavior scores at 72 hours. In contrast, hydrogen gas may have increased survival of animals that would have died without treatment, thus biasing the neurobehavior scores of the SAH + hydrogen group toward falsely depressed averages and once again providing a possible explanation for the absence of significant differences in neurobehavior scores at 72 hours. Second, this study was designed as a pilot to assess for a potential survival benefit of hydrogen gas after SAH. Although our results have allowed for additional conclusions, the sample size needed to support survival benefit is not optimized to rigorously assess for long term neurological benefits. Furthermore, neurobehavior testing is a surrogate end point for neurological function and may be limited in its ability to detect differences between groups. Finally, downstream pathways, especially within this model, remain unexplored. Although, canonically, hydrogen gas is known to reduce reactive oxygen species (above mentioned pathways), subsequent research has broadened potential mechanisms to include activation of downstream mitogen-activated protein kinase pathways, autophagy, histone modification, mitochondrial unfolded protein response, and upregulation of miR-199.[37] Incomplete understanding of these mechanisms, especially within SAH, supports future investigations into the mechanism of hydrogen gas therapy after SAH.

In the present study, high-dose hydrogen gas increased 72 hours survival rate and provided neuroprotection at 24 hours after SAH in rats. This pilot study is the first to support an increased survival rate due to high dose hydrogen gas in a SAH model. We present our findings from this pilot study to accelerate the dissemination of this novel therapy in a timely manner for further SAH research and application in additional ischemic and inflammatory pathologies. We hypothesize that previously proposed mechanisms for the anti-inflammatory effects of hydrogen gas, although incompletely investigated in SAH, are at least partially responsible for the beneficial results observed within this study. Despite an almost insurmountable mortality rate associated with SAH and the limitations of this study, hydrogen gas therapy shows promise as a novel therapeutic agent to increase survival rate after SAH. Further mechanistic studies and growing translational research will lay a foundation for additional pharmacological targets and better treatment of SAH.

Author contributions
Experimental design: RC, NM, JC, JT, JHZ; Main experiments performing and manuscript writing: RC; manuscript revisions: all authors.
Conflicts of interest
The authors have no conflict of interest.
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Institutional review board statement
The study was approved by the Institutional Animal Care and Use Committee of Loma Linda University, USA (Approval No. 8160016) in May 2016.
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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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