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

Richard Camara¹, Nathanael Matei¹, Justin Camara¹, Budbazar Enkhjargal¹, Jiping Tang¹, John H. Zhang¹,2,3,7
1 Department of Physiology and Pharmacology, Loma Linda University, Loma Linda, CA, USA
2 Department of Anesthesiology, Loma Linda University, Loma Linda, CA, USA
3 Department of Neurosurgery, Loma Linda University, Loma Linda, CA, USA

*Correspondence to: John H. Zhang, MD, Johnzhang3910@yahoo.com.
oricid: 0000-0002-4319-4285 (John H. Zhang)

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 (60% 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

doi: 10.4103/2045-9912.260648
How to cite this article: Camara R, Matei N, Camara J, Enkhjargal B, Tang J, Zhang JH. Hydrogen gas therapy improves survival rate and neurological deficits in subarachnoid hemorrhage rats: a pilot study. Med Gas Res. 2019;9(2):74-79.
Funding: This work was supported by the National Institutes of Health grant (NS081740 to JHZ).

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 ⁴ Clearly additional mechanisms are at play since nimodipine, a calcium channel blocker, has shown clinical benefit while lacking a significant effect on cerebral vasospasm.⁵ From these observations, research has pivoted toward reduction of early brain injury.⁶ 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.⁹ Mechanistically, hydrogen gas has been shown to affect oxidative stress, inflammation, and apoptosis.⁰ ¹² 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 (AARIVE) 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...
Subarachnoid hemorrhage grade
SAH grade scoring was performed at 24 and 72 hours after SAH by an independent, blinded investigator as previously described. 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 individually in heated cages until recovery. 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.

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

Subarachnoid hemorrhage model
The endovascular perforation model was induced as previously described. 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 individually in heated cages until recovery. 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.
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.](image)

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.](image)

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). \( \ast P < 0.05 \), vs. sham group (one-way analysis of variance followed by post-hoc Tukey’s test).
and glucose deprivation, the attenuation of oxidative stress in neurons under oxygen results can successfully be translated to humans. In parallel, hydrogen gas therapy to reduce mortality rates after SAH mice after global ischemia. 30 Adenosine 5'-monophosphate-activated protein kinase/mammalian target of rapamycin pathway), and upregulation of mitochondrial unfolded protein responses. 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.

Financial support
This work was supported by the National Institutes of Health grant (NS081740 to JHZ).

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.

Copyright license agreement
The Copyright License Agreement has been signed by all authors before publication.

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

Plagiarism check
Checked twice by iThenticate.

Peer review
Externally peer reviewed.

Open access statement
This is an open access journal, and articles are distributed under the terms of the Creative Commons Attribution-NonCommercial-ShareAlike 4.0 License, which allows others to remix, tweak, and build upon the work non-commercially, as long as appropriate credit is given and the new creations are licensed under the identical terms.

REFERENCES
1. King JT, Jr. Epidemiology of aneurysmal subarachnoid hemorrhage. Neuroimaging Clin N Am. 1997;7:659-668.
2. van Gijn J, Kerr RS, Rinkel GJ. Subarachnoid hemorrhage. Lancet. 2007;369:306-318.
3. Wilkins RH. Cerebral vasospasm. Crit Rev Neurobiol. 1990;6:51-77.
4. Ferguson S, Macdonald RL. Predictors of cerebral infarction in patients with aneurysmal subarachnoid hemorrhage. Neurosurgery. 2007;60:658-667; discussion 667.
5. Fisher CM, Roberson GH, Ojemann RG. Cerebral vasospasm with ruptured saccular aneurysms–the clinical manifestations. Neurosurgery. 1977;1:245-248.
6. Rabinstein AA, Friedman JA, Weigand SD, et al. Predictors of cerebral infarction in aneurysmal subarachnoid hemorrhage. Stroke. 2004;35:1862-1866.
7. Pluta RM, Hansen-Schwartz J, Dreier J, et al. Cerebral vasospasm following subarachnoid hemorrhage: time for a new world of thought. Neurrol Res. 2009;31:151-158.
8. Zhou Y, Martin RD, Zhang JH. Advances in experimental subarachnoid hemorrhage. Acta Neurochir Suppl. 2011;110:15-21.
9. Macdonald RL, Kassell NF, Mayer S, et al. Clazosentan to overcome neurological ischemia and infarction occurring after subarachnoid hemorrhage (CONSCIOUS-1): randomized, double-blind, placebo-controlled phase 2 dose-finding trial. Stroke. 2008;39:3015-3021.
10. Bederson JB, Connolly ES, Jr., Batjer HH, et al. Guidelines for the management of aneurysmal subarachnoid hemorrhage: a statement for healthcare professionals from a special writing group of the Stroke Council, American Heart Association. Stroke. 2009;40:994-1025.
11. Fujii M, Yan J, Rolland WB, Soejima Y, Caner B, Zhang JH. Early brain injury, an evolving frontier in subarachnoid hemorrhage research. Transl Stroke Res. 2013;4:432-446.
12. Ayer RE, Zhang JH. Oxidative stress in subarachnoid haemorrhage: significance in acute brain injury and vasospasm. Acta Neurochir Suppl. 2008;104:33-41.
13. Endo H, Nito C, Kamada H, Yu F, Chan PH. Reduction in oxidative stress by superoxide dismutase overexpression attenuates acute brain injury after subarachnoid hemorrhage via activation of Akt/glycogen synthase kinase-3beta survival signaling. J Cereb Blood Flow Metab. 2007;27:975-982.
14. Ersahin T, Toklu HZ, Cetineli S, Yulkes M, Yegen BC, Sener G. Melatonin reduces experimental subarachnoid hemorrhage-induced oxidative brain damage and neurological symptoms. J Pineal Res. 2009;46:324-332.
15. Kusaka G, Ishikawa M, Nanda A, Granger DN, Zhang JH. Signaling pathways for early brain injury after subarachnoid hemorrhage. J Cereb Blood Flow Metab. 2004;24:916-925.
16. Cahill J, Calvert JW, Zhang JH. Mechanisms of early brain injury after subarachnoid hemorrhage. J Cereb Blood Flow Metab. 2006;26:1341-1353.
17. Cheng G, Wei L, Zhi-Dan S, Shi-Guang Z, Xiang-Zhen X. Atorvastatin ameliorates cerebral vasospasm and early brain injury after subarachnoid hemorrhage and inhibits caspase-dependent apoptosis pathway. BMC Neurosci. 2009;10:7.
18. Sugawara T, Jadhav V, Ayer R, Chen W, Suzuki H, Zhang JH. Thrombin inhibition by argatroban ameliorates early brain injury and improves neurological outcomes after experimental subarachnoid hemorrhage in rats. Stroke. 2009;40:1530-1532.
19. Shen M, Zhang H, Yu C, Wang F, Sun X. A review of experimental studies of hydrogen as a new therapeutic agent in emergency and critical care medicine. Med Gas Res. 2014;4:17.
20. Reeves MJ, Trotter GW. Reciprocal apparatus dysfunction as a cause of severe hind limb lameness in a horse. J Am Vet Med Assoc. 1991;199:1047-1048.
21. Hayashida K, Sano M, Ohsawa I, et al. Inhalation of hydrogen gas reduces infarct size in the rat model of myocardial ischemia-reperfusion injury. Biochem Biophys Res Commun. 2008;373:30-35.
22. Fujii Y, Shirai M, Inamori S, et al. Insufflation of hydrogen gas restrains the inflammatory response of cardiopulmonary bypass in a rat model. Artif Organs. 2013;37:136-141.
23. Liu L, Xie K, Chen H, et al. Inhalation of hydrogen gas attenuates brain injury in mice with cecal ligation and puncture via inhibiting neuroinflammation, oxidative stress and neuronal apoptosis. Brain Res. 2014;1589:78-92.
24. Zhan Y, Chen C, Suzuki H, Hu Q, Zhi X, Zhang JH. Hydrogen gas ameliorates oxidative stress in early brain injury after subarachnoid hemorrhage in rats. Crit Care Med. 2012;40:1291-1296.
25. Xie Z, Enkjhargal B, Reis C, et al. Neriin-1 preserves blood-brain barrier integrity through deleted in colorectal cancer/focal adhesion kinase/RhoA signaling pathway following subarachnoid hemorrhage in rats. J Am Heart Assoc. 2017;6:e005198.
26. Zhu Q, Enkjhargal B, Huang L, et al. AggF1 attenuates neuroinflammation and BBB disruption via PI3K/Akt/NF-kappaB pathway after subarachnoid hemorrhage in rats. J Neuroinflammation. 2018;15:178.
27. Asclepius Meditec Co., Ltd. Product. http://www.ascleway.org/. Accessed by Apr 20, 2016.
28. Matei N, Camara J, McBride D, et al. Intranasal wnt3a attenuates neuronal apoptosis through Fzr1/PIWIL1a/FOXM1 pathway in MCAO rats. J Neurosci. 2018;38:6787-6801.
29. Ohsawa I, Ishikawa M, Takahashi K, et al. Hydrogen acts as a therapeutic antioxidant by selectively reducing cytotoxic oxygen radicals. *Nat Med.* 2007;13:688-694.

30. Nagatani K, Wada K, Takeuchi S, et al. Effect of hydrogen gas on the survival rate of mice following global cerebral ischemia. *Shock.* 2012;37:645-652.

31. Hugyecz M, Mracskó E, Hertelendy P, Farkas E, Domoki F, Bari F. Hydrogen supplemented air inhalation reduces changes of prooxidant enzyme and gap junction protein levels after transient global cerebral ischemia in the rat hippocampus. *Brain Res.* 2011;1404:31-38.

32. Feigin VL, Lawes CM, Bennett DA, Barker-Collo SL, Parag V. Worldwide stroke incidence and early case fatality reported in 56 population-based studies: a systematic review. *Lancet Neurol.* 2009;8:355-369.

33. Terpolilli NA, Feiler S, Dienel A, et al. Nitric oxide inhalation reduces brain damage, prevents mortality, and improves neurological outcome after subarachnoid hemorrhage by resolving early pial microvasospasms. *J Cereb Blood Flow Metab.* 2016;36:2096-2107.

34. Wu X, Li X, Liu Y, et al. Hydrogen exerts neuroprotective effects on OGD/R damaged neurons in rat hippocampal by protecting mitochondrial function via regulating mitophagy mediated by PINK1/Parkin signaling pathway. *Brain Res.* 2018;1698:89-98.

35. Ji Q, Hui K, Zhang L, Sun X, Li W, Duan M. The effect of hydrogen-rich saline on the brain of rats with transient ischemia. *J Surg Res.* 2011;168:e95-101.

36. Zhai X, Chen X, Shi J, et al. Lactulose ameliorates cerebral ischemia-reperfusion injury in rats by inducing hydrogen by activating Nrf2 expression. *Free Radic Biol Med.* 2013;65:731-741.

37. Matei N, Camara R, Zhang JH. Emerging mechanisms and novel applications of hydrogen gas therapy. *Med Gas Res.* 2018;8:98-102.

38. Liu GD, Zhang H, Wang L, Han Q, Zhou SF, Liu P. Molecular hydrogen regulates the expression of miR-9, miR-21 and miR-199 in LPS-activated retinal microglia cells. *Int J Ophthalmol.* 2013;6:280-285.

39. Gao Y, Yang H, Chi J, et al. Hydrogen gas attenuates myocardial ischemia reperfusion injury independent of postconditioning in rats by attenuating endoplasmic reticulum stress-induced autophagy. *Cell Physiol Biochem.* 2017;43:1503-1514.

40. Yang F, Zhang L, Gao Z, et al. Exogenous H2S protects against diabetic cardiomyopathy by activating autophagy via the AMPK/mTOR pathway. *Cell Physiol Biochem.* 2017;43:1168-1187.

41. Sehba FA, Hou J, Pluta RM, Zhang JH. The importance of early brain injury after subarachnoid hemorrhage. *Prog Neurobiol.* 2012;97:14-37.

42. Chen S, Wu H, Tang J, Zhang J, Zhang JH. Neurovascular events after subarachnoid hemorrhage: focusing on subcellular organelles. *Acta Neurochir Suppl.* 2015;120:39-46.

43. Sobue S, Inoue C, Hori F, Qiao S, Murate T, Ichihara M. Molecular hydrogen modulates gene expression via histone modification and induces the mitochondrial unfolded protein response. *Biochem Biophys Res Commun.* 2017;493:318-324.

Received: January 22, 2019
Accepted: March 21, 2019