ABSTRACT

AIM: Increasing evidence suggests that reactive oxygen species damage the blood-brain barrier and increase brain edema after intracerebral hemorrhage (ICH). Recently, strong clinical and experimental evidence has shown that hydrogen has potent protective cellular effects in various diseases. However, the effect of hydrogen on ICH remains unclear. The present study investigates whether hydrogen has neuroprotective effects and improves functional outcome in the rat ICH model.

MATERIAL and METHODS: ICH model was generated by injecting 50 μl autologous tail artery blood stereotactically into the right caudate nucleus of Sprague-Dawley rats. Rats were randomly divided into four groups: sham, ICH/vehicle, ICH/hydrogen gas, and ICH/hydrogen-rich saline groups. Hydrogen treatment was performed for 3 days. The evaluation of functional outcome was done before, and at 24 and 72 hours after ICH. Hemorrhage volume, immunohistochemistry for 8-hydroxy-2’-deoxyguanosine (8-OhdG), and brain water content were evaluated at 72 hours after ICH.

RESULTS: Hydrogen administration reduced the expression of 8-OhdG in the brain, but did not attenuate brain water content or improve functional outcome, regardless of administration route.

CONCLUSION: Hydrogen administration without surgery has no neuroprotective effect in the blood injection rat ICH model.

KEYWORDS: Hydrogen, Intracerebral hemorrhage, Rat

INTRODUCTION

Hypertensive intracerebral hemorrhage (ICH) accounts for 10–20% of strokes (2). ICH can be devastating with high mortality rates ranging from 30% to 50% at 30 days, and many survivors remain severely disabled (1,3,7,28). The International Surgical Trial in Intracerebral Haemorrhage (STICH) study, a landmark trial of over 1,000 ICH patients, showed that emergent surgical hematoma evacuation via craniotomy within 72 hours of onset failed to improve outcome compared to a policy of initial medical management (19). In addition, no specific medical therapy is available, so the optimal management of ICH has not been definitively established.

Increasing evidence suggests that reactive oxygen species (ROS) damage the blood-brain barrier and increase brain edema (8,22,30). Hydroxyl radicals and peroxynitrites are very strong ROS that react indiscriminately with nucleic acids, lipids, and proteins, resulting in DNA fragmentation, lipid peroxidation, and protein inactivation (4,5,10,20,21,24,26,27). Edaravone (3-methyl-1-phenyl-2-pyrazolin-5-one) is a potent scavenger of hydroxyl radicals and is used for the treatment of ischemic stroke in Japan. Edaravone attenuates the edema and ischemic damage after ICH by reducing oxidative damage in a rat model of ICH (23), and so has increasingly been investigated for use in ICH. However, a review of 10 randomized controlled studies failed to find any beneficial effect of edaravone for the treatment of ICH (31). Therefore, more potent free radical scavengers may be needed.
Recently, strong clinical and experimental evidence has shown that hydrogen, administered by gas inhalation or ingestion of an aqueous hydrogen-containing solution, has potent protective cellular effects in various diseases (4, 5, 10, 20, 21, 24, 26, 27), but without major adverse effects (5). Previous studies have shown that hydrogen has antioxidant, anti-apoptotic, anti-inflammatory, and cytoprotective properties that are beneficial to the cell (5). However, only a few experimental studies have investigated the effect of hydrogen on ICH (17, 18).

The present study investigated whether hydrogen can exert neuroprotective effects and improve functional outcome in the blood injection rat ICH model.

**MATERIAL and METHODS**

**Animal Preparation and Intracerebral Infusion**

This study was carried out in strict accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health. All experimental procedures were approved by the Animal Care and Use Committee of the National Defense Medical College. All efforts were made to minimize suffering. Sixty-three Sprague-Dawley rats (male, 330–400 g, 9–10 weeks of age) were used. The rats were housed in individual cages under controlled environmental conditions (12/12 hour light/dark cycle, 20–22°c) with food and water freely available, for 1 week before experimental procedure. General anesthesia was induced with 3% isoflurane. The rats were intubated, and maintained on a mechanical ventilator after infusion of pancuronium bromide (0.1 mg/kg; tidal volume: 2.5–3.0 ml/kg; respiratory rate: 60/min). The tail artery was cannulated with a polyethylene catheter. Blood pressure was monitored throughout the procedure, and arterial blood samples were intermittently analyzed (PaCO₂ was controlled at 30–40 mmHg). Isoflurane was titrated between 0.5% and 3% to maintain a mean arterial pressure of 80 to 130 mmHg. Rectal temperature was measured with a rectal probe and maintained strictly at 37.0°C with a heating pad or heating lamp. The rats were positioned using a stereotaxic frame, and a cranial burr hole (1 mm) was drilled in the skull (1 mm anterior and 4 mm lateral to bregma). Next, a 27-gauge needle was inserted stereotactically into the right caudate nucleus (5 mm ventral from the skull surface). Autologous whole blood (50 μl with no anticoagulants) was injected at 10 μl/min using a microinfusion pump. The needle was maintained in place for 10 minutes after injection to prevent back-leakage. After needle removal, the burr hole was sealed with bone wax. Sham operation was performed with needle insertion only.

**Production of Hydrogen-Rich Saline**

Hydrogen-rich saline was produced with a non-destructive hydrogen adding apparatus (Miz Co., Fujisawa, Kanagawa, Japan; Patent No. 4486157, Patent Gazette of Japan 2010) (21, 27, 29). Bags of physiological saline solution (500 ml; Terumo Co., Tokyo, Japan) were immersed, without opening or altering the bag, in a water tank in which water was electrolyzed periodically to produce water with hydrogen concentrations of up to 1.6 ppm. The concentration of hydrogen in the bag reaches saturation point, at more than 1.0 ppm, because of diffusion through the wall of the bag. Further information can be obtained using the following link: http://www.e-miz.co.jp/english/technology.html#non_destructive.

**Hydrogen Treatment**

Rats were randomly divided into four groups: sham (n = 12), ICH/vehicle (n = 17), ICH/hydrogen gas (n = 17), and ICH/hydrogen-rich saline group (n = 17). Thirteen minutes after operation, hydrogen-rich saline group rats were administered hydrogen-rich saline (5 ml/kg) via the femoral vein, whereas other group rats were administered normal saline (5 ml/kg) (27). Soon after administration of hydrogen-rich saline or normal saline, rats were placed individually in a sealed Plexiglas box with inflow and outflow outlets. Hydrogen gas group rats were exposed to nitrogen-based standard mixed gas with 1.3% hydrogen and 30% oxygen (Saisan Co., Ltd., Saitama, Saitama, Japan) for 3 hours daily for 3 days, whereas other group rats were exposed to nitrogen-based high pressure mixed gas with 30% oxygen (without hydrogen) (Saisan Co., Ltd.) (20). The hydrogen concentration of 1.3% was applied as a safe level in high pressure mixed gas containing 30% oxygen.

**Morphometric Measurement of Hemorrhage Volume**

Rats were decapitated, and the brains were rapidly removed and sectioned coronally at 2-mm intervals at 72 hours after ICH (n = 5 per group except for the sham group). The hemorrhage area for each section was measured by an observer unaware of the experimental groups using the NIH ImageJ software program (http://rsb.info.nih.gov/ij/). The total hematoma volume was calculated by summing the clot area in each section and multiplying by the distance between sections (13).

**Tissue Preparation**

For the immunohistochemical studies, animals were perfused transcardially with normal saline, followed by 4% buffered paraformaldehyde under intraperitoneal anesthesia at 72 hours after ICH (n = 5 per group). The brain was removed and embedded in paraffin after fixation in 4% buffered paraformaldehyde, followed by 0.1 mmol/l phosphate-buffered saline (pH 7.4) for 24 hours at 4°C. Serial coronal sections (5 μm) were prepared, and a single coronal section through the center of the hemorrhagic lesion was used for 8-hydroxy-2‘-deoxyguanosine (8-OHdG) staining.

**Immunohistochemistry**

Serial coronal sections were stained overnight at 4°C with a mouse monoclonal antibody against 8-OHdG (I:1000; Japan Institute for the Control of Aging, Fukuroi, Shizuoka, Japan). Immunoreactivity was detected using a diaminobenzidine method. Images were observed and captured at magnification 400× with a microscope (Axio Imager.A1, Carl Zeiss) equipped with a digital camera system (Axio Cam MRC 5, Carl Zeiss). For quantitative analysis, the number of 8-OHdG-positive cells was counted in the perihematoma region by an observer unaware of the experimental groups as described previously (20). Eight sampling regions were randomly placed along the perihematoma region, and the average count was calculated for analysis.
Behavioral Tests

Neurological scores were assessed by an observer unaware of the experimental groups before, and at 24 and 72 hours after ICH (n = 7 per group). Corner turn and forelimb placing tests were used in this study (9). The vibrissae-elicited forelimb placing test was performed as follows. Rats were held by their bodies to allow their forelimbs to hang freely. Independent testing of each forelimb was induced by brushing the respective vibrissae on the corner of a tabletop once per trial for trials. A score of 1 was given each time the rat placed its forelimb onto the edge of the table in response to the vibrissae stimulation. Percentage successful placing responses were determined for the impaired and nonimpaired forelimbs. For the corner turn test, rats were allowed to proceed into a corner, the angle of which was 30 degrees. To exit the corner, the rat could turn either to the left or right, and this direction was recorded. This test was repeated 15 times, and the percentage of right turns was calculated.

Brain Water Content

The brain water content was measured using the wet/dry method. Briefly, rats were euthanized under deep anesthesia and decapitated 72 hours after operation (n = 7 per group). Brains were removed immediately, and a 3-mm thick coronal brain slice was then cut at 4 mm from the frontal pole. The brain slice was divided into five parts: ipsilateral and contralateral basal ganglia, ipsilateral and contralateral cortices, and cerebellum. Tissue samples were weighed, after which the tissues were placed in an oven at 110°C for 48 hours and then reweighed. The brain water content was calculated using the following formula: (wet weight - dry weight)/wet weight ×100%.

Statistical Analyses

All assays and measurements in this study were performed by investigators unaware of the experimental groups. The data are presented as means ± standard deviation (SD). Comparisons between multiple groups were performed with analysis of variance, followed by Turkey’s test. A value of P < 0.05 was considered to be significant. The Prism 6.0 software program (GraphPad Software, Inc., San Diego, CA) was used for all statistical tests.

RESULTS

Hematoma Volume

The hematoma volume was 35.53 ± 4.93 mm³ in the ICH/vehicle group, 34.31 ± 3.73 mm³ in the ICH/hydrogen gas group, and 35.32 ± 4.40 mm³ in the ICH/hydrogen-rich saline group. There was no significant difference between the three groups (P > 0.05).

Effect of Hydrogen on 8-OHdG Immunoreactivity

Oxidative DNA damage was assessed in the perihematoma regions using an 8-OHdG antibody at 72 hours after treatment (Figure 1A,B). Faint 8-OHdG immunoreactivity was observed in the sham group (0.08 /0.091 ± 0.10 mm²). However, strong 8-OHdG immunoreactivity was observed in the ICH/vehicle group (16.08 /0.091 ± 0.73 mm²), with significantly lower numbers of 8-OHdG-positive cells in the ICH/hydrogen gas group (6.33 /0.091 ± 0.50 mm²) and the ICH/hydrogen-rich saline group (7.20 /0.091 ± 0.61 mm²) (P < 0.05).

Effect of Hydrogen on Neurological Behaviors

Neurological behaviors were assessed by the forelimb placing and corner turn tests before, and at 24 and 72 hours after ICH (Figure 2A,B). Compared with the sham group, statistically significant neurological deficits were detected in the ICH/vehicle, ICH/hydrogen gas, and ICH/hydrogen-rich saline groups (P < 0.05). However, neither test showed significant differences between the ICH/vehicle, ICH/hydrogen gas, and ICH/hydrogen-rich saline groups (P > 0.05).

Effect of Hydrogen on Brain Edema

Brain edema was assessed by measuring brain water content at 72 hours after treatment (Figure 3). Compared with the sham group, the ICH/vehicle group showed a significant increase in brain water content in the ipsilateral cortex (81.40 ± 0.39 % vs. 78.17 ± 0.30 %, P < 0.05) and ipsilateral basal ganglia (82.96 ± 0.50 % vs. 77.22 ± 0.53 %, P < 0.05). However, the ICH/vehicle, ICH/hydrogen gas, and ICH/hydrogen-rich saline groups showed no significant differences in brain water content in the ipsilateral cortex and basal ganglia (cortex: 81.40 ± 0.39 % vs. 81.65 ± 0.78 % vs. 81.78 ± 0.80 %, basal ganglia: 82.96 ± 0.50 % vs. 82.61 ± 0.70 % vs. 82.47 ± 0.51 %, P > 0.05).

DISCUSSION

The present study showed that hydrogen reduced the expression of 8-OHdG in the brain but did not attenuate brain water content or improve functional outcome in the blood injection rat model, regardless of administration route. In contrast, previous studies have demonstrated that hydrogen gas inhalation treatment attenuated blood-brain barrier disruption, and improved neurobehavioral function via prevention of mast cell activation in collagenase-induced ICH mouse (CD-1) model (17,18). These studies show important discrepancies for several possible reasons. Firstly, these studies used different experimental models, the blood injection rat model or the collagenase injection mouse model. The blood injection and collagenase injection models are most often used but differ in many aspects (12,14-16). Hematoma rapidly accumulates in the brain parenchyma in the clinical setting. In addition, hematoma expansion occurs in 14-40% of ICH patients (6,11,25). Therefore, rapid ICH accumulation and hematoma expansion should be simulated, but do not appear together in the two animal models: the blood injection model cannot reproduce the hematoma expansion, and the collagen injection model cannot simulate the rapid ICH accumulation. Further studies are required to investigate the effect of hydrogen in more realistic ICH models. Secondly, these studies used different methods of hydrogen administration. A concentration of less than 4% by volume hydrogen gas in air is reported to be safe (5). The present study used a concentration of hydrogen...
Figure 1: Effect of hydrogen on oxidative DNA damage in the brain. A) Representative photomicrographs at 72 hours after treatment showing immunostaining for 8-hydroxy-2'-deoxyguanosine (8-OHdG). B) Quantification of 8-OHdG-positive cells in perihematoma regions. Faint 8-OHdG immunoreactivity was observed in the sham group. However, strong 8-OHdG immunoreactivity was observed in the Ich/vehicle group, with significantly lower numbers of 8-OHdG-positive cells in the Ich/hydrogen gas and the Ich/hydrogen-rich saline groups. Values are expressed as mean ± SD. n = 5 in each group. Scale bars = 50 μm. *p < 0.05 vs. sham group, #p < 0.05 vs. Ich/vehicle group.

Figure 2: Effect of hydrogen on neurological behaviors. Corner turn test (A) and forelimb placing test (B) were assessed before, and at 24 and 72 hours after treatment. Compared with the sham group, statistically significant neurological deficits were detected in the Ich/vehicle, Ich/hydrogen gas, and Ich/hydrogen-rich saline groups at 24 and 72 hours after treatment. However, neither test showed significant differences between the Ich/vehicle, Ich/hydrogen gas, and Ich/hydrogen-rich saline groups. Values are expressed as mean ± SD. n = 7 in each group. *p < 0.05 vs. sham group.
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cisternal magnesium infusion in patients with subarachnoid hemorrhage (29). In the present study, hydrogen-rich saline injection as well as hydrogen gas inhalation reduced oxidative DNA damage in the brain, but unexpectedly had no beneficial effects on the brain edema and outcome, indicating that the involvement of ROS in ICH may be more complicated than previously believed. Therefore, we consider that hydrogen administration requires more experimental justification before introduction as clinical therapy. In addition to the limitations described above, there are several other possible limitations of our study. We failed to assess the chronic effects of hydrogen on ICH. In addition, the present study focused on the effects of only medical treatment using hydrogen (without surgery) on ICH, and therefore further studies are required to investigate whether hydrogen administration is effective after surgery for ICH.

CONCLUSION

Hydrogen treatment without surgery has no neuroprotective effects in the blood injection rat ICH model.

REFERENCES


Figure 3: Effect of hydrogen on brain edema. Brain edema was assessed by measuring brain water content at 72 hours after treatment. Compared with the sham group, ICH/vehicle group showed a significant increase in brain water content in the ipsilateral cortex and ipsilateral basal ganglia. However, the ICH/vehicle, ICH/hydrogen gas, and ICH/hydrogen-rich saline groups showed no significant differences in brain water content in the ipsilateral cortex and basal ganglia. Values are expressed as mean ± SD. *p < 0.05 vs. sham group.

Gas of 1.3% because this is the highest concentration that can be mixed and bottled under high pressure with 30% oxygen for clinical use in Japan (20,27). In contrast, 2.9% hydrogen gas was used in other studies (17,18). Hydrogen gas inhalation commonly uses concentrations of 1-4% (5). Furthermore, the effects of hydrogen gas inhalation do not appear to be positively correlated with concentration (24). Therefore, we used the standard mixed concentration of 1.3% to estimate the effects in the clinical setting. However, further dose escalation studies are required to elucidate the effect of hydrogen gas inhalation on ICH.

In addition, we investigated the effect of hydrogen-rich saline injection on ICH using the blood injection rat model. Hydrogen-rich saline injection has recently been used in many experimental studies and in many clinical studies (5,21,27) because of its ease of use. We previously reported administration of intravenous infusion of hydrogen-rich fluid in 38 patients with acute ischemic stroke, and adverse events included diarrhea in one patient and heart failure in one (21). We also started a randomized, placebo-controlled study of intravenous hydrogen-rich fluid therapy with intra-
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