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ORIGINAL ARTICLE

The combination of hydrogen gas and hydrogen-rich solution does not protect against ischemic spinal cord injury in rabbits

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Abstract

Purpose This study aimed to determine whether the combination of H_2 gas inhalation and administration of hydrogen-rich acetated Ringer's solution (HS) could protect against ischemic spinal cord injury in rabbits.

Methods In Experiment 1, rabbits were randomly assigned to a 1.2% H_2 gas group, HS group, 1.2% H_2 gas + HS group (combination group), or control group ($n=6$ per group). The H_2 concentration of HS was 0.65 mM. H_2 was inhaled for 60 min, starting 5 min before reperfusion. HS (20 mL/kg) was divided into six bolus injections at 10-min intervals, starting 5 min before reperfusion. Spinal cord ischemia was produced by occluding the abdominal aorta for 15 min. Neurologic and histopathologic evaluations were performed 7 days after reperfusion. In Experiment 2, H_2 concentrations in spinal cord tissue according to the administration of 1.2% H_2 gas or HS were compared by measuring the electric current through a platinum needle electrode ($n=2$). In Experiment 3, rabbits were assigned to a 2% H_2 gas group or control group ($n=6$ per group). Spinal cord ischemia was produced and neurologic and histopathologic evaluations were performed as in Experiment 1.

Results There were no significant differences among the groups in the neurologic and histopathologic outcomes in Experiments 1 and 3. Bolus administration of HS (10 mL) transiently increased the current to only 1/30th and 1/27th of the plateau current with 1.2% H_2 gas inhalation in two animals.

Conclusion These results suggest that the combination of 1.2% H_2 gas inhalation and administration of a hydrogen-rich solution does not protect against ischemic spinal cord injury and that the increase in H_2 concentration in spinal cord tissue after administration of HS is very low compared to 1.2% H_2 gas inhalation.

Keywords Hydrogen gas · Hydrogen-rich solution · Rabbit · Spinal cord ischemia

Introduction

Paraplegia is one of the most devastating complications after surgical repair of thoracic aortic aneurysm (TAA) or thoracoabdominal aortic aneurysm (TAAA). However, no protective strategy has been established, and there is a need for a method that is safe and easy to implement in most hospitals.

The use of molecular hydrogen (H_2) might be a promising strategy that meets the above requirement. Although H_2 had been thought to be biologically inert, recent studies have demonstrated that it has biological effects against oxidative stress, including selective reduction of hydroxyl radical and peroxynitrite without adverse effects [1, 2]. H_2 can be administered by either inhalation of H_2 gas or intravenous administration of a hydrogen-rich solution [3, 4].

Inhalation of 2–4% H_2 gas, but not 1% H_2 gas, has been shown to protect against ischemic spinal cord injury in rabbits [3] and rats [5]. However, administration of 2% H_2

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gas inhalation for spinal cord protection is difficult during surgical repair of TAA or TAAA. A H_2 gas concentration exceeding 4% is an explosion risk [2], and thus, regulations in Japan strictly limit the maximum H_2 gas concentration to 1.3% when it is stored together with 30% oxygen in a gas cylinder and to 4% when it is stored with 96% nitrogen gas. Thus, when using 2% H_2 gas (4% H_2:100% $O_2 = 1:1$), the maximal oxygen concentration is 50%, which might not be enough to maintain adequate PaO_2 during the surgical repair of TAA or TAAA because one-lung ventilation is often necessary. Increasing the oxygen concentration requires a corresponding decrease in the H_2 concentration. For example, when the oxygen concentration is increased to 70%, the maximum H_2 gas concentration becomes 1.2%.

Administration of hydrogen-rich solution does not have a risk of explosion. However, only one preclinical study has provided evidence for the effectiveness of hydrogen-rich solution for spinal cord protection [4]. In that study, 5 or 10 mL/kg of hydrogen-rich saline was administered 5 min before reperfusion [4]. This means that large amounts of hydrogen-rich saline were administered very rapidly, which does not seem to be clinically relevant, particularly with 10 mL/kg of hydrogen-rich saline.

In the present study, we sought to investigate whether a more clinically applicable method of hydrogen administration, namely, the combination of 1.2% H_2 gas inhalation and slower administration of a hydrogen-rich solution, could protect against ischemic spinal cord injury in rabbits. We also compared the H_2 concentrations in spinal cord tissue between 1.2% H_2 gas inhalation and intravenous administration of a hydrogen-rich solution because no study to our knowledge has examined the difference in H_2 concentration in spinal cord tissue between these modes of administration. We expected that the difference in spinal cord H_2 concentration could explain the results of the outcome study. In addition, because Experiment 1 showed no protective effect even with the combination of 1.2% H_2 gas inhalation and administration of a hydrogen-rich solution, we performed a replication study on whether 2% H_2 gas inhalation showed protective effects against ischemic spinal cord injury in our model.

Materials and methods

This study was approved by the Animal Care and Use Committee at Yamaguchi University Graduate School of Medicine and used 38 male New Zealand white rabbits weighing 2.8 ± 0.2 kg (mean ± standard deviation).

Experiment 1

Surgical preparation was performed as previously reported [6, 7]. Briefly, after overnight fasting with unrestricted access to water, the rabbits were anesthetized with 2–3% isoflurane in 30% oxygen/70% nitrogen and fentanyl under mechanical ventilation. A catheter was inserted into an ear vein to administer fluid (lactated Ringer's solution, 10 mL/kg/h) and drugs, and another catheter was inserted into an ear artery to measure blood pressure. Esophageal and paravertebral muscle tissue temperatures were monitored, and the paravertebral muscle tissue temperature was maintained at approximately 38.0 °C throughout the study. A PE-50 catheter was inserted into the right femoral artery to measure blood pressure below the aortic occlusion to be created later. Mean arterial pressure and heart rate were monitored until the catheters were removed.

Spinal cord ischemia was also produced as described previously [8]. Briefly, with the rabbit in the right lateral decubitus position, a PE-50 catheter was placed retroperitoneally around the aorta immediately distal to the left renal artery for later occlusion of the aorta. After completion of the surgery, the end-tidal isoflurane concentration was maintained at 2%.

The experimental protocols are presented schematically in Fig. 1. Twenty-four rabbits were randomly assigned to a H_2 gas group, a hydrogen-rich acetated Ringer's solution (HS) group (HS group), a H_2 gas + HS group (combination group), or a control group ($n = 6$ per group). The H_2 concentration of H_2 gas was 1.2% and that of HS was 0.65 mM. H_2 gas (stored in a gas cylinder containing 1.2% hydrogen/30% oxygen/68.8% nitrogen) and HS were supplied by Iwatani Co. (Tokyo, Japan). The HS and combination groups received 20 mL/kg of HS and the H_2 gas and control groups received 20 mL/kg of an acetated Ringer's solution, divided into six bolus injections at 10-min intervals, starting 5 min before reperfusion. We did not infuse HS because H_2 is permeable through ordinary infusion tubes, resulting in a decrease in the amount of H_2 administered. HS and the acetated Ringer's solution were administered over 1 min each time. In the H_2 gas and combination groups, the inspired gas (30% oxygen/70% nitrogen) was changed to 1.2% hydrogen/30% oxygen/68.8% nitrogen from 5 min before reperfusion to 55 min after reperfusion. If the proximal mean arterial blood pressure dropped below 55 mmHg, phenylephrine was infused to maintain blood pressure at 55 mmHg or higher.

Immediately before occlusion of the aorta, 400 U of heparin was administered. Ischemia was induced by occluding the abdominal aorta for 15 min. The mean arterial pressure, heart rate, and esophageal and paravertebral muscle temperatures were recorded immediately before

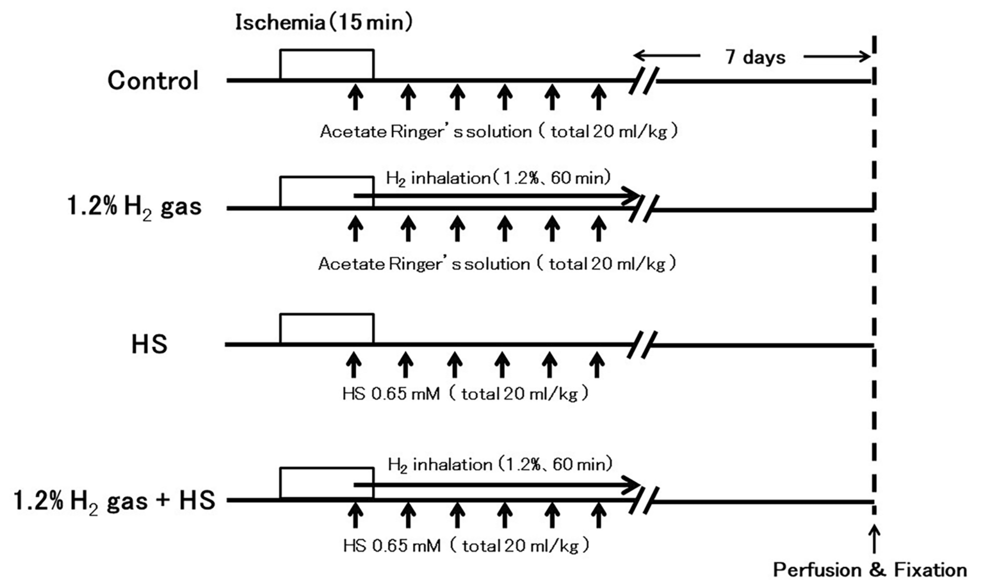


Fig. 1 Experimental protocol in Experiment 1. Abbreviation: *HS* hydrogen-rich acetated Ringer's solution

ischemia, at 7.5 min during ischemia, and 15 min after reperfusion. Arterial blood was sampled to determine PaO_2, $PaCO_2$, pH, hematocrit, and plasma glucose before the onset of ischemia and 15 min after reperfusion. All catheters were then removed and the incisions were sutured. An antibiotic (gentamicin, 4 mg/kg) was administered intramuscularly. Extubation of the trachea was performed when vigorous spontaneous ventilation and movement occurred. The animals were allowed to recover in a warmed plastic box that contained supplemental oxygen for 3 h. The bladder contents were expressed manually as required.

The animals were neurologically assessed daily for 7 days after reperfusion by an observer blinded to the treatment groups using the 5-point scoring system proposed by Drummond and Moore [9]: 4, normal motor function; 3, ability to draw legs under body and hop but not normally; 2, some lower extremity function with good antigravity strength but inability to draw legs under body and/or hop; 1, poor lower extremity function but weak antigravity movement only; 0, paraplegic with no lower extremity function.

After the final neurological assessment (7 days after reperfusion), transcardiac perfusion and fixation were performed with 10% phosphate-buffered formalin under isoflurane anesthesia. Coronal sections of the spinal cord (8 µm thick) were obtained at the L5 level and stained with hematoxylin and eosin. Normal neurons in the anterior spinal cord (anterior to a line drawn through the central canal perpendicular to the vertical axis) were counted in two sections under a light microscope and averaged for each rabbit by an observer blinded to the treatment groups. Damaged neurons were identified by cytoplasmic eosinophilia with loss of Nissl substances and the presence of pyknotic homogenous nuclei as reported previously [8].

Experiment 2

Experiment 2 was performed to compare H_2 concentrations in spinal cord tissue according to the administration of H_2 gas or HS in New Zealand white rabbits ($n=2$). Anesthesia was induced as in Experiment 1. With the animals in the prone position, the midline skin and subcutaneous fascia were incised between the third lumbar and the first sacral spinous process. After dissection of the muscles, the third to seventh spinous processes, ligamentum flavum, and epidural fat were sequentially removed, and the underlying dura was exposed. A platinum needle electrode of 0.3 mm in diameter (Unique Medical, Tokyo, Japan) was inserted vertically to a depth of 3 mm, 1 mm lateral to the mid-vertical axis line at the level of the L5 process using a micromanipulator and placed in the gray matter of the lumbar spinal cord. An Ag–AgCl reference electrode was placed in the musculature of the midback. In this method, the electric current through the electrode is proportional to the H_2 concentration in the tissue, enabling a comparison of the relative H_2 concentration in spinal cord tissue between H_2 gas inhalation and HS administration.

During the measurement, the animals were anesthetized using the same conditions as in Experiment 1. We first administered 1.2% H_2 gas for 8 min. After the H_2 level returned to the baseline, a 10 mL bolus of HS was administered. The volume of 10 mL was almost the same as the single bolus dose in Experiment 1. Before Experiment 2, we planned to perform the measurement in four animals. However, we observed large differences in the spinal cord H_2 concentrations between H_2 gas and HS in two animals and small differences in the results for the two animals. Therefore, to minimize the number of experimental animals used,

we did not perform the measurement in the additional two animals.

Experiment 3

Experiment 3 was a replication study performed to investigate whether 2% H_2 gas inhalation could protect against ischemic spinal cord injury. Rabbits were assigned to a 2% H_2 gas group or control group ($n=6$ per group). The methods for spinal cord ischemia, H_2 gas inhalation, and neurologic and histopathologic evaluations were the same as in Experiment 1.

Statistical analysis

The physiological variables are presented as the mean ± standard deviation and were analyzed by repeated-measures analysis of variance. Statistical analysis of the hindlimb motor function data and the number of normal-appearing neurons in the anterior spinal cord was performed using a nonparametric method (the Kruskal–Wallis test or Mann–Whitney U test). A *P*-value of less than 0.05 was considered to be statistically significant.

Results

The physiological variables in Experiment 1 are shown in Table 1. There were no significant differences in the physiological variables before ischemia, at 7.5 min during ischemia, or 15 min after reperfusion among the four groups in Experiment 1. All the rabbits in this experiment survived until the final neurological assessment (7 days after reperfusion).

Figure 2 shows the time course of changes in motor function score for each group in Experiment 1. Motor function scores were not significantly different among the four groups.

Figure 3a shows the number of normal-appearing neurons in each group, and no significant difference was observed among the four groups. Figure 3b, c shows representative microphotographs of the lumbar spinal cord (at the L5 level) of a paraplegic animal (score = 0) in the control group (Fig. 3b) and a normal animal in the HS group (Fig. 3c). In animals with severe motor dysfunction (score ≤ 1), the structure of the gray matter in the spinal cord was destroyed, most motor neurons had disappeared, and prominent inflammatory cell infiltration was observed. Vacuolation was also observed in the white matter of these animals. In contrast, in the animals with normal motor function (score = 4), the structure of the gray and white matter in the spinal cord was mostly intact and motor neurons retained a normal appearance.

Figure 4 shows the representative data for the change in electric current through the electrode placed in the anterior gray matter of the lumbar spinal cord (at the L5 level). After the start of 1.2% H_2 gas inhalation, the current rapidly increased and plateaued in 5 min. When H_2 gas inhalation was discontinued, the current returned to the baseline value in 5 min. Bolus administration of HS (10 mL) transiently increased the current to only 1/30th and 1/27th of the plateau current with 1.2% H_2 inhalation in two animals, respectively.

The physiological variables in Experiment 3 are shown in Table 2. There were no significant differences in the physiological variables before ischemia, at 7.5 min during ischemia, or 15 min after reperfusion between the two groups in Experiment 3. One animal that showed acute severe paraplegia after emergence from anesthesia died on day 1. Therefore, the final neurologic and histopathologic evaluations were done in 11 animals.

Figure 5 shows the time course of changes in motor function score for each group in Experiment 3. Motor function scores were not significantly different among the two groups.

Figure 6 shows the number of normal-appearing neurons in each group, and no significant difference was observed between the two groups.

Discussion

We chose 1.2% H_2 in the present study, assuming the use of H_2 gas to protect the spinal cord during surgical repair of TAA or TAAA. The concentration of 1.2% H_2 was selected so that the inspired O_2 concentration could be increased up to 70%, which might help maintain PaO_2 under one-lung ventilation during the surgery. We also planned to administer HS in a more clinically relevant way, namely 20 mL/kg HS divided into six boluses administered at 10-min intervals, with the aim of comparing this method with rapid administration of 10 mL/kg H_2-rich saline in a study by Zhou et al. [4]. For 1.2% H_2 and divided administration of HS, we expected that the protective effect of each alone would not be as strong as that seen in the previous studies [3–5]. However, we expected that the combination of 1.2% H_2 gas and HS could protect against ischemic spinal cord injury if together they could increase the spinal cord H_2 concentration to the same level as 2% H_2. However, no protective effect was observed even with the combination of 1.2% H_2 gas and HS.

The negative results of Experiment 1 prompted us to conduct two additional studies. We compared the H_2 concentrations in spinal cord tissue between 1.2% H_2 gas inhalation and HS administration. Also, we performed a replication study on whether 2% H_2 gas inhalation showed protective

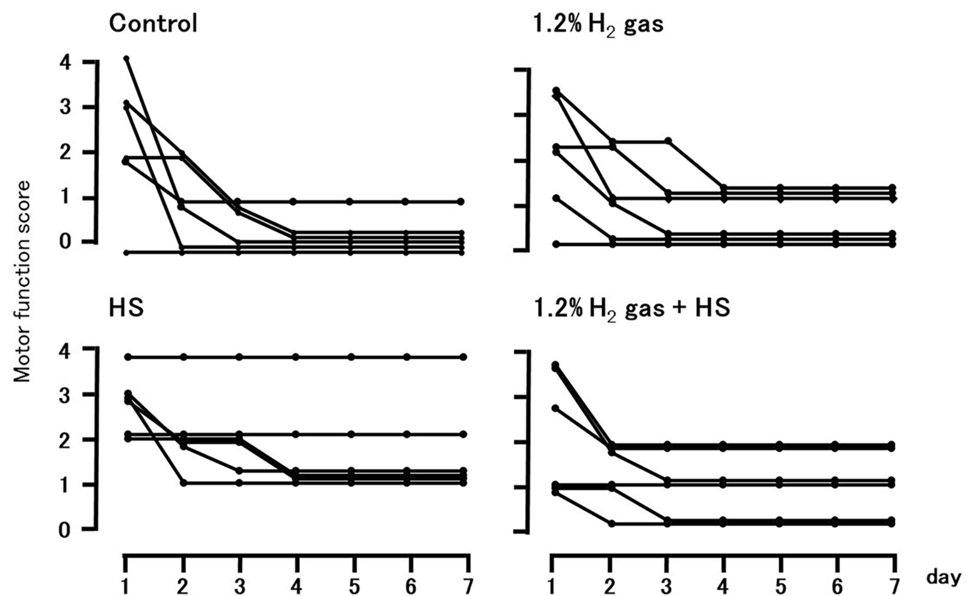
Table 1 Physiological variables (Experiment 1)

	MAP (mmHg)		HR (bpm)	Temperature (°C)		pH	PaO_2 (mmHg)	$PaCO_2$ (mmHg)	Glucose (mg/dL)	Hematocrit (%)
	Proximal	Distal		Esophageal	Paravertebral					
Control ($n=6$)										
Preischemia	59 ± 2	60 ± 5	297 ± 17	38.1 ± 0.3	38.0 ± 0.1	7.40 ± 0.06	120 ± 4	39 ± 1	141 ± 16	37 ± 2
Ischemia 7.5 min	50 ± 8	7 ± 1	275 ± 24	38.0 ± 0.3	37.8 ± 0.3					
Reperfusion 15 min	63 ± 4	64 ± 3	275 ± 20	38.1 ± 0.2	37.8 ± 0.1	7.38 ± 0.04	126 ± 2	39 ± 1	143 ± 25	34 ± 2
1.2% H_2 gas ($n=6$)										
Preischemia	59 ± 2	59 ± 2	305 ± 34	38.3 ± 0.3	38.0 ± 0.1	7.36 ± 0.04	123 ± 4	39 ± 2	147 ± 9	40 ± 1
Ischemia 7.5 min	49 ± 3	8 ± 1	288 ± 44	38.3 ± 0.4	37.6 ± 0.3					
Reperfusion 15 min	65 ± 4	66 ± 3	275 ± 20	38.2 ± 0.2	38.0 ± 0.1	7.33 ± 0.06	122 ± 10	38 ± 2	140 ± 9	40 ± 1
HS ($n=6$)										
Preischemia	58 ± 3	60 ± 2	320 ± 12	38.5 ± 0.2	38.0 ± 0.1	7.39 ± 0.05	117 ± 7	38 ± 1	132 ± 10	40 ± 1
Ischemia 7.5 min	55 ± 5	9 ± 1	280 ± 22	38.4 ± 0.2	37.4 ± 0.5					
Reperfusion 15 min	64 ± 5	67 ± 7	285 ± 16	38.2 ± 0.2	38.0 ± 0.2	7.37 ± 0.07	116 ± 7	36 ± 3	126 ± 15	38 ± 1
1.2% H_2 gas + HS ($n=6$)										
Preischemia	60 ± 6	59 ± 3	330 ± 10	38.5 ± 0.2	38.0 ± 0.1	7.37 ± 0.05	136 ± 7	37 ± 1	135 ± 10	39 ± 1
Ischemia 7.5 min	54 ± 8	8 ± 1	297 ± 24	38.4 ± 0.3	37.4 ± 0.2					
Reperfusion 15 min	67 ± 3	67 ± 5	297 ± 14	38.3 ± 0.3	38.0 ± 0.1	7.34 ± 0.04	128 ± 13	35 ± 1	136 ± 21	39 ± 1

Data are mean ± SD

MAP mean arterial blood pressure, *HR* heart rate

Fig. 2 Changes in individual motor function scores from 1 to 7 days after reperfusion in Experiment 1. Motor function scores range from 0 (paraplegia) to 4 (normal). Each symbol represents data for one animal. Abbreviation: *HS* hydrogen-rich acetated Ringer's solution



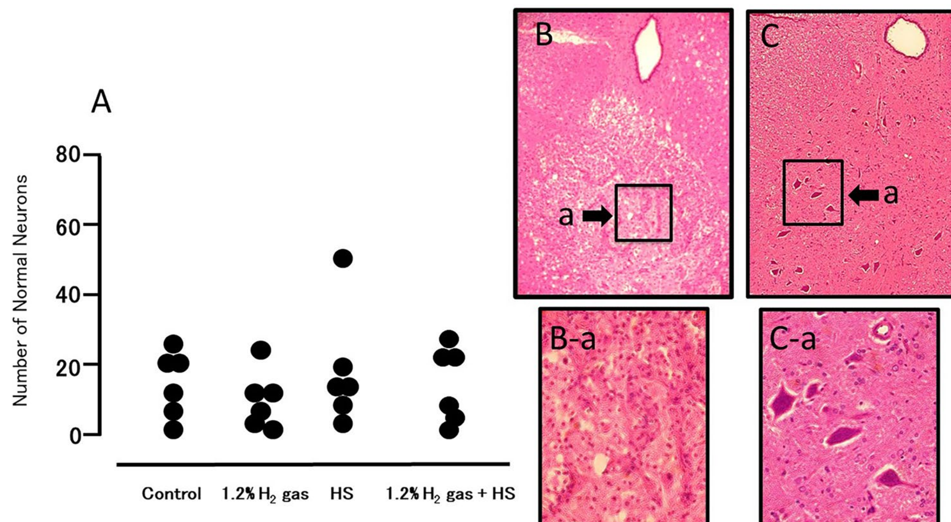


Fig. 3 Number of normal neurons in the anterior spinal cord (at the L5 level) 7 days after reperfusion in Experiment 1. Each symbol represents data for one animal (**a**). Representative light microphotographs of the lumbar spinal cord of a paraplegic animal (motor function score = 0) in the control group (**b**, **b**-a) and normal animal (motor function score = 4) in the HS group (**c**, **c**-a) in Experiment 1 (at the L5 level, hematoxylin–eosin stain). Few normal neurons and complete destruction of the entire gray matter with inflammatory changes are seen in the paraplegic animal (**b**, **b**-a). In contrast, the structure of the gray matter in the spinal cord is mostly intact and motor neurons are preserved in the normal animal (**c**, **c**-a). B and C, original magnification 40 X; **b**-a and **c**-a, original magnification 400 X. Abbreviation: *HS*, hydrogen-rich acetated Ringer's solution

effects against ischemic spinal cord injury in our model. To our surprise, the peak H_2 concentrations in spinal cord tissue after 10 mL bolus administration of HS (approximately 3.6 mL/kg) were far below than those in the plateau phase of 1.2% H_2 gas inhalation. No protective effect was observed with 2% H_2 gas inhalation.

The present results suggest two possibilities. The first is that 2% H_2 gas inhalation may not have strong protective effects against ischemic spinal cord injury, even though treatment with 2% H_2 gas inhalation has been reported to protect against ischemic spinal cord injury in rabbits [3] and rats [5]. It might be argued that our ischemic insult was too severe to see any protective effect. However, the aortic occlusion time in our study was 15 min, compared with 20 min in Huang's study [3]. Also, we believe that our model can detect efficacy of protective measures as we have used

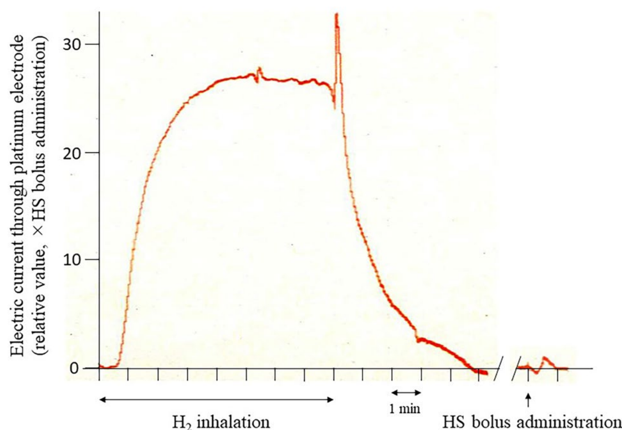


Fig. 4 Electric current through the platinum electrode placed in the anterior gray matter of the spinal cord (at the L5 level) after H_2 gas inhalation and HS administration. In this method, the electric current through the electrode is proportional to the H_2 concentration in the tissue, enabling relative comparison of H_2 concentrations in spinal cord tissue between H_2 gas inhalation and HS administration. The peak current after H_2 gas inhalation is approximately 27 times greater than that after HS administration. (The artifact at the end of the plateau phase of H_2 inhalation indicates the end of H_2 inhalation.) Abbreviation: *HS*, hydrogen-rich acetated Ringer's solution

it to demonstrate the strong protective effects of ischemic preconditioning [7]. The second possibility is that hydrogen-rich solutions may not protect against ischemic spinal cord injury because of an insufficient increase in H_2 concentration in spinal cord tissue. The H_2 concentration in HS was 0.65 mM in the present study and our bolus dose of HS was approximately 3.6 mL/kg in Experiment 2. Even if we had tried the same regimen as in Zhou's study (H_2 concentration 0.85 mM, 10 mL/kg bolus) [4], the H_2 concentration in the spinal cord still would have been far below than that with 1.2% H_2 gas inhalation.

H_2 is not highly soluble in aqueous solutions, with maximum H_2 concentration thought to be 0.8 mM under 1 atm at room temperature. Considering that the comparison of the concentrations of H_2 in the spinal cord tissue showed a great difference between 1.2% H_2 gas inhalation and bolus administration of HS in the present study, it seems difficult to deliver sufficient amount of H_2 to the spinal cord tissue by intravenous administration of a hydrogen-rich solution. Indeed, in humans, the blood concentration of H_2 was reported to be more than 10 times higher when H_2 was given by 2% H_2 gas inhalation compared with intravenous administration of a hydrogen-rich solution (0.8 mM) [10]. Taken together, it seems unlikely that the combination of 1.2% H_2 gas inhalation and administration of a hydrogen-rich solution can deliver a greater amount of H_2 to the spinal cord than 2% H_2 gas inhalation. As 2% H_2 gas inhalation did not have protective effects in the present study, we do not think that the combination of 1.2% H_2 gas inhalation and administration

Table 2 Physiological variables (Experiment 3)

	MAP (mmHg)		HR (bpm)	Temperature (°C)		pH	PaO_2 (mmHg)	$PaCO_2$ (mmHg)	Glucose (mg/dL)	Hematocrit (%)
	Proximal	Distal		Esophageal	Paravertebral					
Control ($n=5$)										
Preischemia	63 ± 5	64 ± 6	279 ± 36	38.3 ± 0.3	37.9 ± 0.1	7.41 ± 0.04	136 ± 3	41 ± 1	134 ± 20	36 ± 1
Ischemia 7.5 min	57 ± 13	7 ± 2	270 ± 32	38.3 ± 0.3	37.6 ± 0.3					
Reperfusion 15 min	61 ± 2	60 ± 3	279 ± 25	38.3 ± 0.4	38.0 ± 0.1	7.41 ± 0.06	140 ± 4	40 ± 1	123 ± 16	36 ± 1
2% H_2 gas ($n=6$)										
Preischemia	66 ± 6	68 ± 6	303 ± 22	38.1 ± 0.3	38.0 ± 0.1	7.42 ± 0.03	137 ± 9	41 ± 1	135 ± 15	37 ± 2
Ischemia 7.5 min	61 ± 5	8 ± 1	288 ± 33	38.1 ± 0.4	37.5 ± 0.3					
Reperfusion 15 min	59 ± 2	60 ± 4	285 ± 33	38.3 ± 0.3	38.0 ± 0.1	7.42 ± 0.04	137 ± 6	41 ± 1	133 ± 20	37 ± 1

Data are mean ± SD

MAP mean arterial blood pressure, *HR* heart rate

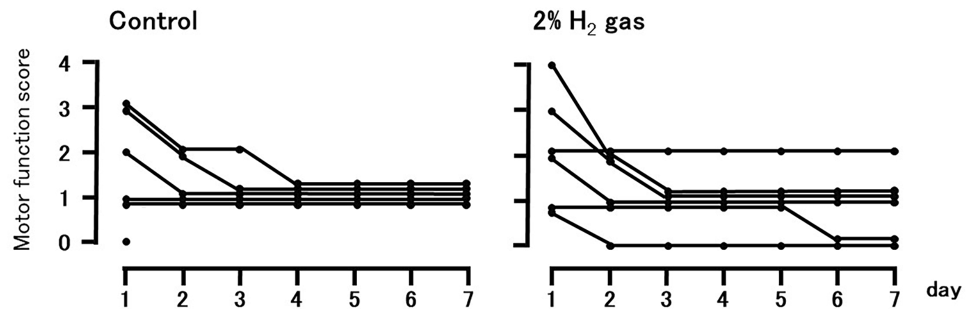


Fig. 5 Changes in individual motor function scores from 1 to 7 days after reperfusion in Experiment 3. Motor function scores range from 0 (paraplegia) to 4 (normal). Each symbol represents data for one animal. One animal in the control group died on day 1

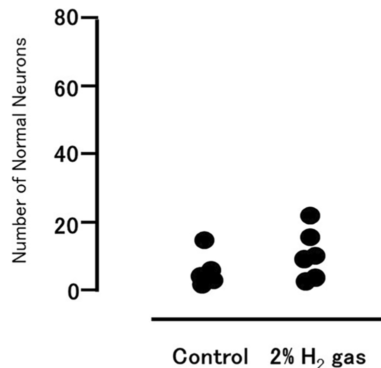


Fig. 6 Number of normal neurons in the anterior spinal cord (at the L5 level) 7 days after reperfusion in Experiment 3. Each symbol represents data for one animal

of a hydrogen-rich solution can be effective in protecting against ischemic spinal cord injury. Because the maximum H_2 gas concentration when H_2 gas is used with oxygen is strictly regulated in Japan, it remains challenging to use H_2 for spinal cord protection in the repair of TAA or TAAA.

There are several limitations in the present study. First, we did not measure absolute concentrations of H_2 in spinal cord tissue. However, the technique we used in the present study was able to measure electric current through the electrode that was proportional to the H_2 concentration in spinal cord tissue. Therefore, we were able to compare the concentrations of H_2 between H_2 gas inhalation and HS administration. Second, we started H_2 gas inhalation 5 min before reperfusion, whereas Huang et al. [3] started H_2 gas inhalation 10 min before reperfusion. However, after the start of 1.2% H_2 gas inhalation, the electric current rapidly increased and plateaued in 5 min in Experiment 2. Thus, H_2 concentration in spinal cord tissue in the H_2 gas inhalation groups was thought to be stable at the beginning of reperfusion.

In conclusion, we investigated whether the combination of 1.2% H_2 gas inhalation and a hydrogen-rich solution could protect against ischemic spinal cord injury in rabbits. The results suggest that the combination strategy tested in the present study does not protect against ischemic spinal cord injury.

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Data availability Data used in this paper may be submitted upon request.

Declarations

Conflict of interest The authors declare no conflict of interest.

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