Neuroscience Letters 441 (2008) 167–172

Contents lists available at ScienceDirect

Neuroscience Letters

journal homepage: www.elsevier.com/locate/neulet

Hydrogen therapy reduces apoptosis in neonatal hypoxia–ischemia rat model

Jianmei Cai [a,b,1], Zhimin Kang [a,1], Wen Wu Liu [a], Xu Luo [b], Sun Qiang [a], John H. Zhang [c], Shigeo Ohta [d], Xuejun Sun [a,*], Weigang Xu [a], Hengyi Tao [a], Runping Li [a]

[a] Department of Diving Medicine, Faculty of Naval Medicine, Second Military Medical University, Shanghai 200433, PR China
[b] Department of pathology, Qingdao Medical University, Shandong 266021, PR China
[c] Department of Neurosurgery, Loma Linda University, Loma Linda, California, USA
[d] Department of Biochemistry and Cell Biology, Institute of Development and Aging Sciences, Graduate School of Medicine, Nippon Medical School, 1-396 Kosugi-cho, Nakahara-ku, Kawasaki City 211-8533, Japan

ARTICLE INFO

Article history:
Received 24 April 2008
Received in revised form 20 May 2008
Accepted 20 May 2008

Keywords:
Hydrogen
Hydroxyl radical
Hypoxic ischemia
Apoptosis

ABSTRACT

Hypoxia–ischemia (HI) brain injury is a major cause of neuronal cell death especially apoptosis in the perinatal period. This study was designated to examine the effect of hydrogen therapy on apoptosis in an established neonatal HI rat pup model. Seven-day-old rat pups were subjected to left common carotid artery ligation and then 90 min hypoxia (8% oxygen at 37 °C). Immediately after HI insult, pups were placed into a chamber filled with 2% H_2 for 30 min, 60 min, or 120 min, respectively. 24 h after 2% H_2 therapy, the pups were decapitated and brain injury was assessed by 2,3,5-triphenyltetrazoliumchloride (TTC), Nissl, and TUNEL staining, as well as caspase-3, caspase-12 activities in the cortex and hippocampus. H_2 treatment in a duration-dependent manner significantly reduced the number of positive TUNEL cells and suppressed caspase-3 and -12 activities. These results indicated H_2 administration after HI appeared to provide brain protection via inhibition of neuronal apoptosis.

Hypoxia–ischemia (HI) insult is a relatively frequent occurrence in the perinatal period and can lead to neuron death [17]. In the complex factors involved in the neuronal death, reactive oxygen species (ROS) or reactive nitrogen species (RNS) such as the hydroxyl radical ($^\bullet OH$), superoxide anion (O_2^-), hydrogen dioxide (H_2O_2), nitric oxide (NO), peroxynitrite ($ONOO^-$), appear to play a critical role. The brain has potent defenses including dietary free-radical scavengers (ascorbate, α-tocopherol), the endogenous tripeptide glutathione, and enzymatic antioxidants against ROS. Although increased expression of these enzymes can occur in response to ischemia [6], endogenous antioxidant capacity can be overwhelmed after HI insult, leading to increased ROS concentrations. Excessive ROS can result in DNA fragment, lipid peroxidation, and inactivation of protein [9] leading to apoptosis or necrosis depending on the severity of oxidative stress. Among the ROS, OH and $ONOO^-$ are much more reactive and react indiscriminately with nucleic acids, lipids and proteins. Neuron membranes are rich in polyunsaturated fatty acids, and neonatal brain is more susceptible to oxidative damage [8]. There is not known detoxification system for $^\bullet OH$ and $ONOO^-$; therefore, scavenging $^\bullet OH$ and $ONOO^-$ is a critical antioxidant process [15].

HI injury to the brain has been shown to result in rapid cell death with features of both acute necrosis and delayed apoptotic cell death [11,13]. Apoptosis is a programmed cell death that is characterized by specific ultrastructural changes that include cell shrinkage, nuclear condensation and DNA fragmentation. At the molecular level, apoptosis is activated by the aspartate-specific cysteineprotease (caspase) cascade, including caspase-12 and -3. Caspase-12 is localized to the ER and specifically activated by ER stress, and caspase-3 is considered to be the most important of the executioner caspases and is activated by any of the initiator caspases [4].

Hydrogen gas has been used in medical applications to prevent decompression sickness (DCS) in deep divers for safety profiles [5]. Recently, Ohsawa et al. found that molecular hydrogen can selectively reduce $^\bullet OH$ and $ONOO^-$ in cell-free systems and exert a therapeutic antioxidant activity, in a rat middle cerebral artery occlusion model [14]. But the mechanism involved in the protective effects of H_2 therapy was unclear. In this study, we examined whether H_2 therapy offers neuroprotection by reducing HI-induced caspase-dependent apoptosis.Seven-day-old Sprague–Dawley rat pups were randomly assigned to the following five groups: (1) control group (no carotid ligation, hypoxia) ($n = 20$), (2) HI group

* Corresponding author. Fax: +86 21 65492382.
E-mail address: sunxjk@hotmail.com (X. Sun).
[1] Both contributed equally to this work.

0304-3940/$ – see front matter
doi:10.1016/j.neulet.2008.05.077

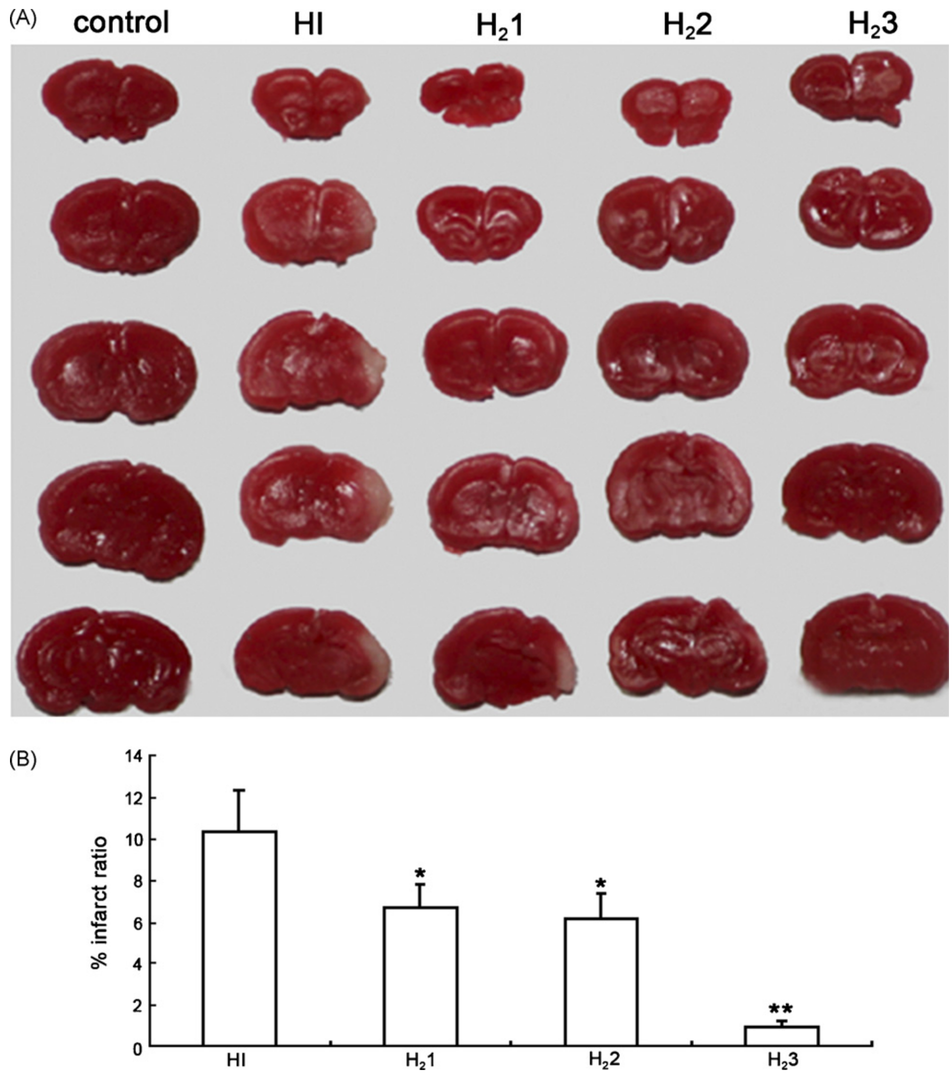

Fig. 1. TTC staining of damaged brains and infarct ratio. (A) Representative samples of TTC-stained coronal sections were derived from 8-day-old neonatal rats after H_2 therapy. Marked cerebral infarction was observed in the HI group. (B) Infarct ratio of each group. The infarct ratio was 10.4% in HI group, 6.77% in $H_2$1, 6.24% in $H_2$2 and 1.04% in $H_2$3 group. The results indicated that H_2 therapy decreased the volume of infarction, especially in $H_2$3 group.

(carotid ligation and hypoxia) (n = 60), (3) HI + $H_2$1 group (30 min 2% H_2 therapy) (n = 60), (4) HI + $H_2$2 group (60 min 2% H_2 therapy) (n = 60), (5) HI + $H_2$3 group (120 min 2% H_2 therapy) (n = 60). Each group was composed of pups from each litter to obtain parity within the groups. The Animal and Ethics Review Committee at the Second Military Medical University evaluated and approved the protocol used in this study.

The model used in this study was based on the Rice–Vannucci model [17]. Pups were housed with the dam under a 12:12 h light-dark cycle, with food and water available ad libitum throughout the studies. These neonatal rats were anesthetized by inhalation with diethyl ether. The rats were kept at a temperature of 37 °C as the left common carotid artery was exposed and ligated with 5–0 surgical sutures. After operation, the pups were returned to the holding container. Anesthesia and surgery time averaged 5 min per pup. Surgery was completed for an entire little, and the pups were allowed to recover with their dams for 1 h (for rehydration via nursing). Then they were placed in a jar perfused with a humidified gas mixture (8% oxygen balanced nitrogen) for 90 min. Both the jar and mixture were kept at 37 °C to maintain a constant thermal environment. All surviving pups were returned to their dams after hypoxia exposure.

The pups were placed into chamber (2% hydrogen; 1.0 atmosphere absolute, ATA) for 30 min, 60 min or 120 min immediately after HI insult. The chamber was flushed with mixed gases for 5 min to replace the air in the chamber. Continuous temperature monitoring was executed to avoid temperature changes. Fresh gas ventilation was maintained throughout treatments.

24 h after 2% H_2 therapy, the pups were decapitated and the left brain hemispheric volumes were measured. Briefly, the brains were quickly removed after decapitation and placed in cold saline for 5 min, cut at 2-mm intervals from the frontal pole into 5 coronal sections. After incubated in 1% 2,3,5-triphenyltetrazolium chloride (TTC) for 8 min at 37 °C, the brain slices were fixed in 4% formalin for 24 h. The volumes of each of the sections were summed by an image analysis system (ImageJ, a public domain image analysis program, developed at the National Institutes of Health). The percentage of infarction (infarct ratio) was calculated by dividing the infarct volume by the total volume of the slices.

For Nissl staining, the 4-μm sections were hydrated in 1% toluidine blue at 50 °C for 20 min. After rinsing with double distilled water, they were dehydrated and mounted with permount. The cortex and the CA1 area of hippocampus from each animal were

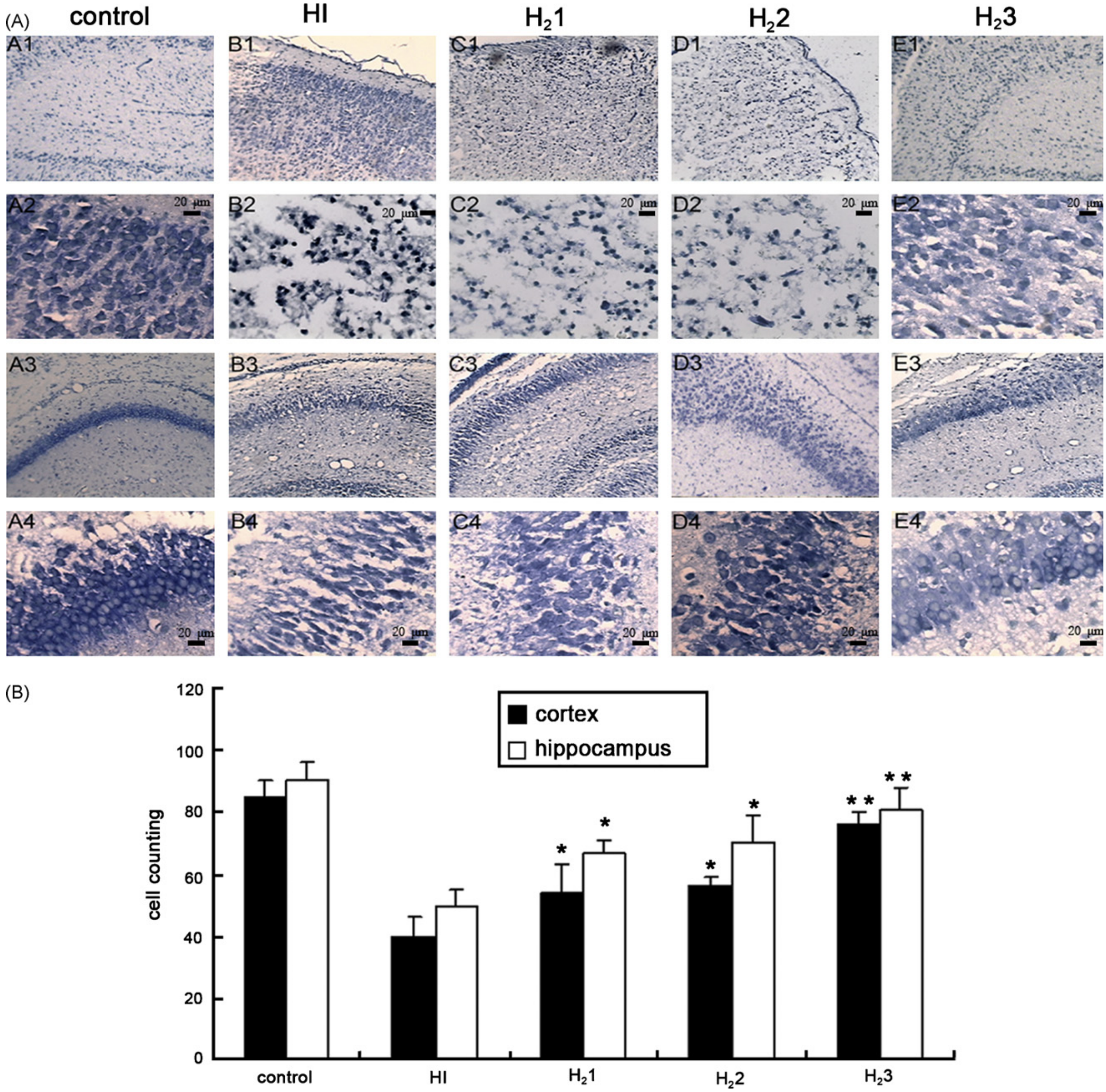

Fig. 2. Nissl staining of damaged cortex (A1-E2) and hippocampus (A3-E4) and cell counting. (A) Nissl staining. Cortex and hippocampus in each group after H_2 therapy are shown at two different magnifications (A1-E1, A3-E3: ×10, A2-E2, A4-E4: ×40). More neuronal loss and dead cells appeared in the HI group after injury. In CA1 sector of control and $H_2$3 group, the cell outline was clear and the structure was compact. Cells were big and have abundant cytoplasm and Nissl body. In HI group, cells arranged sparsely and the cell outline was fuzzy. The cells with eumorphism were significantly reduced. (B) Cell counting. The number of Nissl staining cells in cortex and hippocampus of HI group was lower than that of $H_2$1, $H_2$2 ($P < 0.05$) and $H_2$3 groups ($P < 0.01$).

captured and Imaging-Pro-Plus (LEIKA DMLB) was used to perform quantitative analysis of cell numbers.

TUNEL staining was performed on paraffin-embedded sections by using the in situ cell death detection kit (Roche). According to standard protocols, the sections were dewaxed and rehydrated by heating the slides at 60 °C. Then these sections were incubated in a 20 μg/ml proteinase K working solution for 15 min at room temperature. The slides were rinsed three times with PBS before they were incubated in TUNEL reaction mixture for 1 h at 37 °C. Dried area around sample and added Converter-AP on samples for 1 h at 37 °C. After rinsing with PBS (5 min, three times), sections were coloured in dark with nitroblue tetrazolium (NBT) and 5-bromo-4-chloro-3-indolylphosphate (BCIP).

Six visual fields (0.6 mm^2) of the cerebral cortex and CA1 were photographed in each section. The number of staining cells in each field was counted at higher magnification (×40). The data were represented as the number of cells per high-power field.

Brain samples from the cortex and hippocampus were taken from the impaired hemispheres of neonatal rats at 24 h after H_2 administration. The activities of caspase-3 and -12 were measured with caspase-3/CPP32 Fluorometric Assay Kit and caspase-12/CPP32 Fluorometric Assay Kit (BIOVISION Research Products 980 Linda Vista Avenue, Mountain View, CA 94043 USA).

Briefly, brain samples were homogenized in ice-cold cell lysis buffer and kept at 4 °C for 1 h. Brain homogenate was centrifuged (Eppendorf, 5810R) at 12,000 × g for 15 min at 4 °C. The supernatant

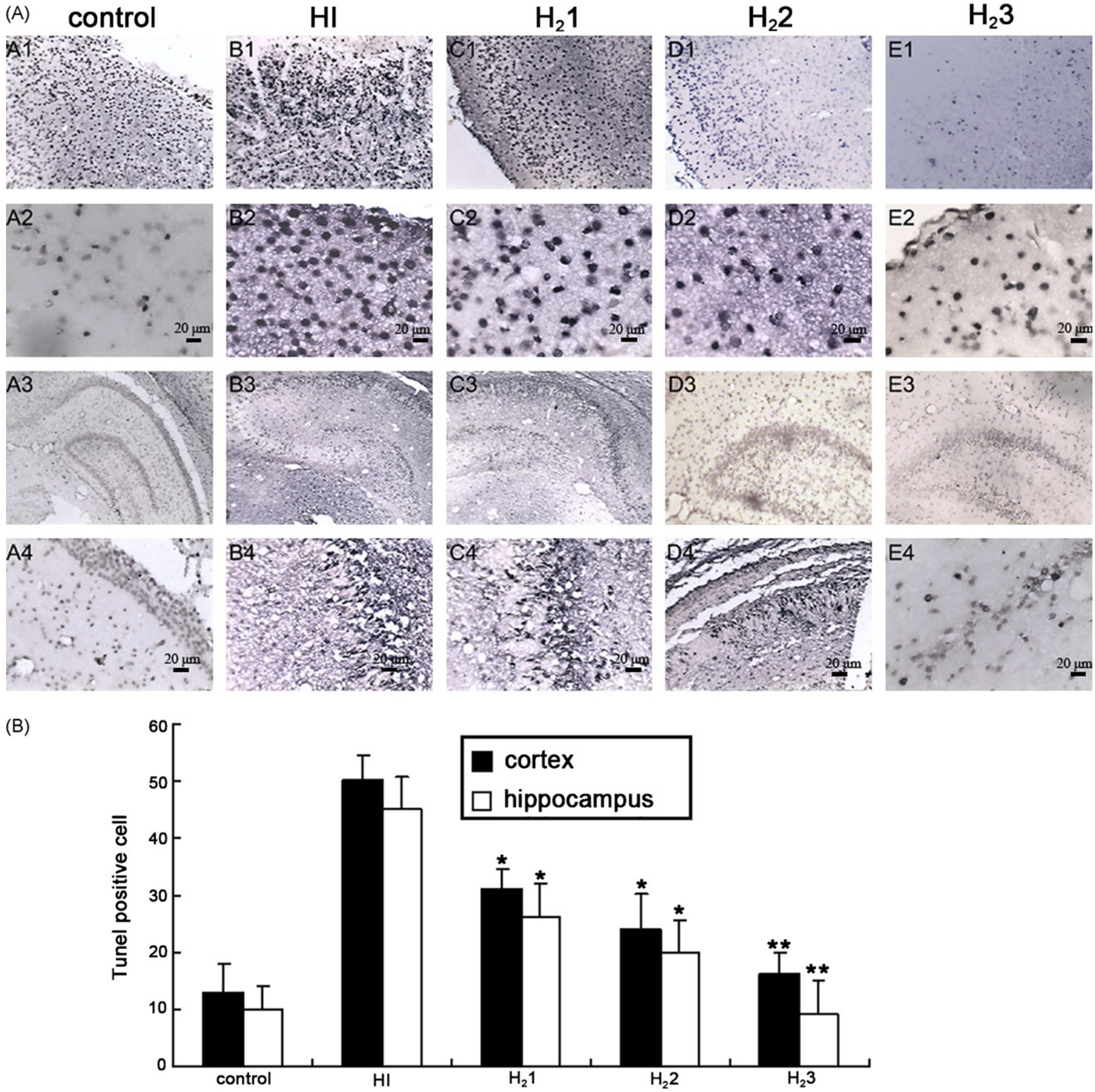

Fig. 3. TUNEL staining of damaged cortex (A1-E2) and hippocampus (A3-E4) and TUNEL-positive cell counting. (A) TUNEL staining. Cortex and hippocampus in each group after H_2 administration are shown at two different magnifications (A1-E1, A3-E3: ×10, A2-E2, A4-E4: ×40). The TUNEL-positive material was localized in the nuclei of the neurons. In samples collected from the HI group, the damaged cells were characterized by a round and shrunken morphology. The processes disappeared and the neuronal body became rounded with strong TUNEL staining in the nucleus. An occasional TUNEL-positive cell was found in control and $H_2$3 group. (B) Cell counting. The cortex and hippocampus of HI group had a higher proportion of TUNEL-positive cells than that of $H_2$1, $H_2$2 ($P<0.05$) and $H_2$3 groups ($P<0.01$). H_2 therapy reduced the number of TUNEL-positive cells, and prevented neurons from apoptosis after HI.

was removed and stored at −80 °C until use. Protein content was measured by using the Enhanced BCA Protein Assay Kit. 20–200 μg cell lysates were incubated in a 96-well plate with 2 × Reaction Buffer (50 μl). The reaction was started by adding 1 mM DEVD–APC substrate (5 μl). After incubation in the dark at 37 °C, the plate was read in a fluorometer equipped with a 400-nm excitation filter and 505-nm emission filter.

All quantitative data are expressed as mean ± S.D. The significance of differences between means was verified by ANOVA followed by Tukey test. For analyzing the results of cell counting, a non-parametric Kruskal–Wallis ANOVA was used followed by Dunn's test. $P<0.05$ was considered significant.

Fig. 1 shows representative photographs of TTC-stained sections from rat pups in each group, at 24 h after 2% H_2 therapy. The infarct ratio in HI group (10.4%) was markedly higher than that in $H_2$1 group (6.77%, 30 min 2% H_2), $H_2$2 group (6.24%, 60 min 2% H_2) and $H_2$3 group (1.04%, 120 min 2% H_2). The results indicated that H_2 therapy dramatically decreased the volume of infarction, especially in $H_2$3 group. However, there was not significantly different in infarct ratio between $H_2$1 group and $H_2$2 group.

Fig. 2 shows representative samples of Nissl staining from the cerebral cortex and hippocampus of pups at 24 h after 2% H_2 therapy. Extensive neuronal changes in the cortex and CA1 sector of the hippocampus were noticed with features of considerable dark,

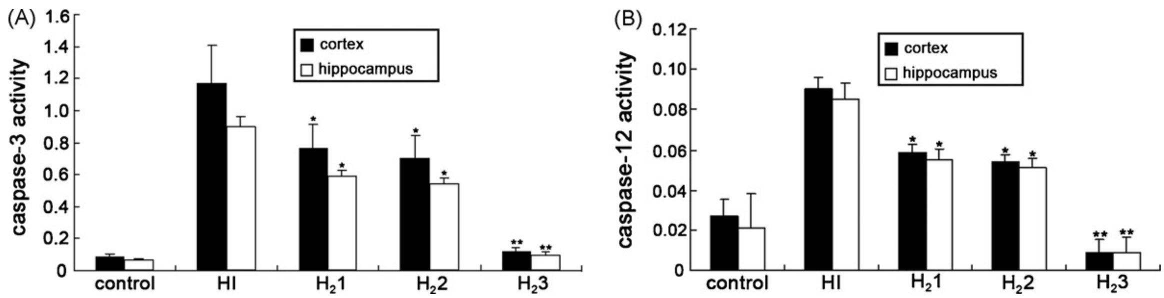

Fig. 4. The activities of caspase-3 (A) and -12 (B) in the impaired cortex and hippocampus. After H_2 therapy, the activities of caspase-3 and -12 were dramatically reduced after HI insult. The activity of caspase-12 in $H_2$3 group was lower than that in control group, while the activity of caspase-3 in $H_2$3 group was higher than that in control group. A single administration of 2% H_2 reduced apotosis after HI insult via suppressing the activities of caspase-3 and -12.

pyknotic neurons in HI group (B1–4). More Nissl-stained cells (E1–4) were observed in $H_2$3 group than that in HI group ($P < 0.01$).

Fig. 3 shows that TUNEL-positive cells were significantly increased in cortex and hippocampus of HI group (B1, B3). 120 min 2% H_2 therapy markedly reduced the number of TUNEL-positive cells (E1, E3). At higher magnification, the nuclei of cells were clearly stained in both hippocampus and cortex (B2, B4, E2, E4). A few TUNEL-positive cells were identified in samples from normal control pups (A1-4). And there was no difference in cell counting between $H_2$1 and $H_2$2 group.

The activities of caspase-3 and -12 were measured at 24 h after HI insult as shown in Fig. 4. The activity of caspase-3 was 1.17 ± 0.23 in cortex and 0.9 ± 0.06 in hippocampus in HI group. 120 min 2% H_2 administration significantly reduced the activity of caspase-3 in the cortex (0.117 ± 0.02) and hippocampus (0.09 ± 0.16) ($P < 0.01$ vs. HI). Similarly, higher caspase-12 activity was obtained in cortex and hippocampus in HI group which was reduced by 2% H_2 treatment ($P < 0.01$ vs. $H_2$3).

In this study, 2% H_2 administration immediately after HI insult significantly reduced the infarct ratio, in a duration-dependent manner. This result is consistent with the observation by Ohsawa et al. [14] in adult focal ischemia. The protective effects of H_2 on HI brain injury seem related to its anti-apoptotic actions because hydrogen increased the number of survival neurons, decreased the number of apoptotic cells, and reduced the activities of caspase-3 and -12. These observations indicate a single and short term 2% H_2 administration may have clinical potentials in the management of HI brain injury in neonates. We are not aware that hydrogen was used previously as a therapy either in animal models of neonatal brain injury or in clinical practice.

The brain consumes a large quantity of oxygen, making it particularly susceptible to oxidative stress [10]. Oxidative stress is a major contributor to ischemic brain injury especially in neonatal brain [2]. An excellent antioxidant for clinical intervention should be easily available, permeable into cytoplasm or nucleus, and without toxicity. Hydrogen is one of the most plentiful gases in the universe. The two most common methods for producing hydrogen are steam reforming and electrolysis (water splitting). It has been established that some algae and bacteria produce hydrogen [3]. Hydrogen molecule is electronically neutral and is expected to easily penetrate the cellular and intracellular membranes. It is oxidized into water in the body which is not harmful to cells. Hydrogen does not disturb metabolic oxidation–reduction reactions nor does it disrupt ROS involved in cell signaling [14]. As a physiological inert gas, hydrogen is less narcotic than nitrogen, and nitrogen easily develops bubbles than hydrogen in the decompression [1]. Therefore, H_2 has been used for deep diving for the above mentioned safety consideration.

Ohsawa et al. found that molecular hydrogen can selectively reduce $^{\bullet}OH$ and $ONOO^-$ in vitro and exert a therapeutic antioxidant activity in a rat middle cerebral artery occlusion model [14]. $^{\bullet}OH$ and $ONOO^-$ are the strongest oxidants and react indiscriminately with nucleic acids, lipids and proteins resulting in DNA fragment, lipid peroxidation, and inactivation of protein. $O_2{}^-$ and H_2O_2 are detoxified by antioxidant defense enzymes, superoxide dismutase, and peroxidase or glutathione-peroxidase, respectively; however, no enzyme detoxifies $^{\bullet}OH$ and $ONOO^-$. Therefore, the ability of hydrogen to reduce or eliminate $^{\bullet}OH$ and $ONOO^-$ may be responsible for the neuroprotective effect especially anti-apoptotic effect observed in this study.

The reason we studied the activity of caspase-12 is that procaspase-12 is predominantly localized at the endoplasmic reticulum (ER) and is specifically activated by disturbances to ER homeostasis such as ER stress and mobilization of intracellular calcium ion store [12]. Studies have shown that the change of Ca^{2+} influx, efflux, release from intracellular Ca^{2+} stores and Ca^{2+} buffering contribute to the HI-induced Ca^{2+} ion disturbances [18]. Elevated intracellular calcium may activate calpain, a noncaspase protease, and induce the translocation of calpain from the cytosol to the membrane [16] where it may cleave procaspase-12 resulting in caspase-12 activation. Then caspase-12 activated caspase-3 which leads to apoptosis [7]. Hydrogen application reduced the activities of caspase-12 and -3 in this study. Apparently hydrogen therapy by quenching free-radicals may inhibit a variety of pathways that lead to caspase-3 activation which may involve caspase-12 and -9.

Hydrogen's neuroprotective effect is time dependent in this study. While the infarct volume was not significantly different between $H_2$1 group (30 min hydrogen) and $H_2$2 group (60 min hydrogen), much less infarction was observed in the $H_2$3 group (120 min hydrogen). Similar morphological observations was obtained in Nissl staining that more Nissl positive cells were observed in $H_2$3 group than that in HI group. In TUNEL staining, again, 120 min 2% H_2 therapy markedly reduced the number of TUNEL-positive cells, while there was no difference in cell counting between $H_2$1 and $H_2$2 group. Finally, only 120 min 2% H_2 administration significantly reduced the activity of caspase-3 and -12.

We conclude that given the easiness of administrating of hydrogen and the safety of 2% hydrogen, hydrogen may be a good candidate in the management of HI brain injury as a safe and effective antioxidant with minimal side effects.

References

[1] A.O. Brubakk, T.S. Neuman, Physiology and Medicine of Diving, 5th ed., Saunders, London, 2003.
[2] J.W. Calvert, J.H. Zhang, Pathophysiology of a hypoxic-ischemic insult during the perinatal period, Neurol. Res. 27 (2005) 246–260.
[3] D. Das, T.N. Veziroglu, Hydrogen production by biological processes: a survey of literature, Int. J. Hydrogen Energ. 26 (2001) 13–28.
[4] S. Elmore, Apoptosis: a review of programmed cell death, Toxicol. Pathol. 35 (2007) 495–516.

[5] P. Fontanari, M. Badier, C. Guillot, C. Tomei, H. Burnet, B. Gardette, Y. Jammes, Changes in maximal performance of inspiratory and skeletal muscles during and after the 7.1-MPa Hydra 10 record human dive, Eur. J. Appl. Physiol. 81 (2000) 325–328.
[6] S. Fukui, T. Ookawara, H. Nawashiro, K. Suzuki, K. Shima, Post-ischemic transcriptional and translational responses of EC-SOD in mouse brain and serum, Free Rad. Biol. Med. 32 (2002) 289–298.
[7] J. Hitomi, T. Katayama, M. Taniguchi, A. Honda, K. Imaizumi, M. Tohyama, Apoptosis induced by endoplasmic reticulum stress depends on activation of caspase-3 via caspase-12, Neurosci. Lett. 357 (2004) 127–130.
[8] T. Ikeda, Y.X. Xia, M. Kaneko, H. Sameshima, T. Ikenoue, Effect of the free radical scavenger, 3-methyl-1-phenyl-2-pyrazolin-5-one (MCI-186), on hypoxia–ischemia-induced brain injury in neonatal rats, Neurosci. Lett. 329 (2002) 33–36.
[9] S. Kuroda, B.K. Siesjö, Reperfusion damage following focal ischemia: pathophysiology and therapeutic windows, Clin. Neurosci. 4 (1997) 199–212.
[10] S. Love, Oxidative stress in brain ischemia, Brain Pathol. 9 (1999) 119–131.
[11] L.J. Martin, M.E. Blue, M.V. Johnston, Apoptosis has a prolonged role in the neurodegeneration after hypoxic ischemia in the newborn rat, J. Neurosci. 20 (2000) 7994–8004.
[12] T. Nakagawa, H. Zhu, N. Morishima, E. Li, J. Xu, B.A. Yankner, J. Yuan, Caspase-12 mediates endoplasmic-reticulum-specific apoptosis and cytotoxicity by amyloid β, Nature 403 (2000) 98–103.
[13] F.J. Northington, D.M. Ferriero, D.L. Flock, L.J. Martin, Delayed neurodegeneration in neonatal rat thalamus after hypoxia–ischemia is apoptosis, J. Neurosci. 21 (2001) 1931–1938.
[14] I. Ohsawa, M. Ishikawa, K. Takahashi, M. Watanabe, K. Nishimaki, K. Yamagata, K. Katsura, Y. Katayama, S. Asoh, S. Ohta, Hydrogen acts as a therapeutic antioxidant by selectively reducing cytotoxic oxygen radicals, Nat. Med. 13 (2007) 688–694.
[15] S.S. Sheu, D. Nauduri, M.W. Anders, Targeting antioxidants to mitochondria: a new therapeutic direction, Biochim. Biophys. Acta 1762 (2006) 256–265.
[16] K. Suzuki, S. Imajoh, Y. Emori, H. Kawasaki, Y. Minami, S. Ohno, Calcium-activated neutral protease and its endogenous inhibitor. Activation at the cell membrane and biological function, FEBS Lett. 220 (1987) 271–277.
[17] R.C. Vannucci, J.R. Connor, D.T. Mauger, C. Palmer, M.B. Smith, J. Towfighi, S.J. Vannucci, Rat model of perinatal hypoxic-ischemic brain damage, J. Neurosci. Res. 55 (1999) 158–163.
[18] H. Yao, G.G. Haddad, Calcium and pH homeostasis in neurons during hypoxia and ischemia, Cell Calcium 36 (2004) 247–255.