Brain Research Bulletin 142 (2018) 122–128

Contents lists available at ScienceDirect

Brain Research Bulletin

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

Research report

Neuroprotective effects of hydrogen inhalation in an experimental rat intracerebral hemorrhage model

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ARTICLE INFO

Keywords:
Key words
Hydrogen
Neuroprotection
Intracerebral hemorrhage
Oxidative stress
Rat
Apoptosis

ABSTRACT

Objective: Hydrogen inhalation has been found to be neuroprotective and anti-oxidative in several brain injury models. Building on these studies, we investigated potential neuroprotective effects of hydrogen inhalation in a rat model of intracerebral hemorrhage (ICH), focusing on apoptosis and inflammation.
Methods: Forty-five 8-week-old male Sprague-Dawley rats were randomly divided into three groups (n = 15 per each group): a sham group, ICH group, and ICH + hydrogen group. Induction of ICH was performed via injection of 0.23 U of bacterial collagenase type IV into the left striatum. Hydrogen was administered via spontaneous inhalation. Mortality and neurologic deficits were investigated at 6, 24, and 48 h after ICH. To investigate the antioxidative activity of hydrogen gas, the expression of malondialdehyde was measured. Real-time polymerase chain reaction analyses of TNF-a, IL-1b, BDNF, and caspase-3 expression were used to detect anti-inflammatory and anti-apoptotic effects. Neuroprotective effect was evaluated by immunohistochemical and TUNEL staining.
Result: At 6, 24 and 48 h post-intracerebral hemorrhage, animals showed brain edema and neurologic deficits, accompanied by up-regulation of TNF-a, IL-b, BDNF, and caspase-3, which is indicative of neuroinflammation, neuroprotection, and apoptosis. Hydrogen treatment significantly reduced the level of oxidative stress, neuroinflammation, neuronal damage, and apoptosis-related genes. This was accompanied by increased neurogenesis and expression of growth factor-related genes at < 24 h, but not 48 h, after ICH.
Conclusion: H_2 gas administration exerted a neuroprotective effect against early brain injury after ICH through anti-inflammatory, neuroprotective, anti-apoptotic, and antioxidative activity.

1. Introduction

Intracerebral hemorrhage (ICH) is a lethal subtype of stroke associated with poor functional disability and a high mortality rate, accounting for 10% to 15% of deaths from stroke (Sacco et al., 2009). The initial mechanisms of injury after ICH involve mechanical destruction, hematoma expansion, and/or herniation caused by the hematoma itself (Xue and Del Bigio, 2000). Subsequently, inflammation, oxidative stress, and impairment of blood flow around the hematoma contribute to edema formation and delayed cell death (Xue and Del Bigio, 2000; Xi et al., 2006).

In experimental ICH models, neurologic deficits are associated with an increase in production of reactive oxygen species (ROS) and reactive nitrogen species (RNS), which damage the blood–brain barrier (BBB) and increase cerebral edema (Wu et al., 2011; Hall et al., 2000). Hydrogen therapy has been found to neutralize ROS and reduce oxidative stress in several brain injury models (Chen et al., 2010; Cai et al., 2008; Ohsawa et al., 2007). Ohsawa et al. reported that hydrogen inhalation markedly decreased oxidative stress and inflammation, and protected brain against ischemia/reperfusion injury and stroke in rats (Ohsawa et al., 2007). In addition. Hydrogen treatment may attenuate neuronal apoptosis by reducing vascular inflammation and oxidative stress (Cai et al., 2008). However, the exact mechanisms of the beneficial effects of hydrogen therapy remain unclear. We investigated whether the neuroprotective effect of hydrogen inhalation therapy in a rat model of ICH operates through effects on inflammation and apoptosis.

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https://doi.org/10.1016/j.brainresbull.2018.07.006
Received 26 September 2017; Received in revised form 9 July 2018; Accepted 11 July 2018

2. Materials and methods

2.1. Induction of ICH

All protocols were approved by Institutional Animal Care and Use Committee of the author's institute. ICH was induced by stereotactic infusion of bacterial collagenase type IV (Sigma-Aldrich, St. Louis, MO, USA) into the left striatum as we previously described (Song et al., 2003; Jwa et al., 2011). Experimental ICH was induced in male Sprague-Dawley rats (230–260 grams; Koatech, Seoul, Korea). Forty-five 8-week-old male Sprague-Dawley rats were randomly divided into three groups (n = 15 per each group): sham-operated, ICH, and ICH-treated with 1.3% hydrogen (ICH + H_2) for 2 h. After an intraperitoneal injection of tiletamine/zolazepam 27.78 mg/kg (Zoletil®; Virbac Laboratories, Carros, France) and 2% xylazine hydrochloride 0.647 mg/kg (2% Rompun®; Bayer Korea, Seoul, Korea), rats were placed prone in a stereotactic frame (Stoleting Co, Wood Dale, IL, USA). During the surgical procedure, body temperature was maintained at 36.5–37.5 °C using a thermistor-controlled heating blanket (Warkentin et al., 2010). After a midline incision, a 1-mm burr hole on the skull was made and a 30-gauge Hamilton syringe needle was inserted into the left striatum (location: 3.0 mm left lateral to the midline, 0.2 mm posterior to the bregma, 6 mm in depth below the skull). The administration of 1 μl of saline containing 0.23 U of collagenase was carried out over 3 min. Then, the Hamilton syringe needle was slowly withdrawn 5 min after infusion. The burr hole was sealed with bone wax, and the scalp wound was sutured. The rats were placed in cages with free access to food and water.

2.2. Hydrogen inhalation treatment

Inhalation of hydrogen gas started 1 h after ICH induction. Rats were placed in a transparent chamber connected to a hydrogen gas generator (H2 Vision, Seoul, Korea). The hydrogen treatment chamber was continually flushed with gas mix 1.3% hydrogen and 21% oxygen balanced with nitrogen. At the beginning of treatment, flow rate of mixed gas was 8 L/min for 5 min, which was followed by a maintenance flow rate of 6 L/min. Hydrogen concentration was confirmed intermittently using a hydrogen meter (H2 Scan, Valencia, CA). No obvious toxicity was observed during the experimental period. 1.3% Hydrogen gas inhalation lasted 2 h followed by daily 1 h inhalation. ICH-induced and sham-operated (needle injury) rats were placed in a chamber, which was continually flushed with medical air (21% oxygen balanced with nitrogen).

2.3. Neurobehavioral test

At 24 and 48 h after ICH, a neurobehavioral test was performed in all rats with the modified Garcia's method (Garcia et al., 1995). An 18-point system was used to evaluate animals' neurological deficits from six aspects, including spontaneous activity (0–3), symmetry in the movement of all four limbs (0–3), forepaw outstretching (0–3), climbing (Sacco et al., 2009; Xue and Del Bigio, 2000; Xi et al., 2006), body proprioception (Sacco et al., 2009; Xue and Del Bigio, 2000; Xi et al., 2006), and response to vibrissae touch (Sacco et al., 2009; Xue and Del Bigio, 2000; Xi et al., 2006). The score given to each animal was summation of all six individual test scores. A minimum neurologic score is 3 and a maximum is 18. A lower score represents a more severe neurological deficit. This test was performed in a blinded manner to avoid bias.

2.4. Tissue processing

The rats were sacrificed at 6, 24, and 48 h after ICH induction by decapitation, and their brains were rapidly dissected. The left (injured side) basal cortical samples were obtained for assessment, frozen on dry ice, and stored at −70 °C until use for biochemical analyses.

2.5. Malondialdehyde (MDA) detection

MDA is a presumptive marker of oxidant-mediated lipid peroxidation, and it was quantified to estimate the extent of lipid peroxidation (Hou et al., 2012). MDA content was measured using commercially available kits (Nanjing Jiancheng Biochemistry Co., Nanjing, China), according to the manufacturers methods. The absorbance of the supernatant was measured by spectrophotometry at 532 nm. All tests were conducted in triplicate. The MDA concentration was calculated from the standard curve and expressed as nmol/mg protein

2.6. Real-time polymerase Chain reaction analysis

The levels of IL-1β, TNF-α, BDNF, and caspase-3 mRNA expression in each group for detection of anti-inflammatory, neuronal survival, or anti-apoptotic effects were determined by real-time polymerase chain reaction (real-time PCR) as we previously described (Lee et al., 2017). Total RNA from the electrode implantation site (n = 3 per each group and time point) was isolated using Qiazol lysis reagent (Qiagen, CA, USA) according to the manufacturer's instructions. One microgram of total RNA was transcribed into cDNA with a QuantiTect Reverse Transcription Kit (Qiagen). The primers of the measured mRNA genes are listed in Table 1. Real-time PCR was analyzed using a Rotor-Gene SYBR Green PCR Kit (Qiagen). The real-time PCR amplifications were conducted for 5 s at 95 °C and 10 s at 60 °C for 45 cycles after the initial denaturation step of 5 min at 95 °C. All the mRNA levels were normalized to the values of reference genes, and the results were expressed as fold changes of the threshold cycle (Ct) value relative to controls (2 weeks post-implantation) using the $2-\Delta\Delta Ct$ method. The average expression level of the reference genes glyceraldehyde 3-phosphate dehydrogenase (GAPDH), 18 s ribosomal RNA (18 s rRNA), beta actin, and hypoxanthine phosphoribosyltransferase 1 (HPRT1) were used for the normalization of quantitative expression data.

2.7. Histological analysis

For the histopathological examination, the brain was fixed with 10% neutral buffered formalin (BBC Biochemical, WA, USA), processed

Table 1
Real-Time PCR primer.

Gene	Forward	Reverse
TNF-a	CCCCGACTATGTGCTCCTCAC	AGGGCTCTTGATGGCGGA
IL-1β	CCTCAAGGGGAAGAATCTAT	GAGGTGCTGATGTACCAGTT
BDNF	CAAAAGGCCAACTGAAGC	CGCCAGCCAATTCTCTTT
Caspase-3	CTGGACTGCGGTATTGAGAC	CCGGGTGCGGTAGAGTAAGC
18 s rRNA	ACGGACCAGAGCGAAAGCAT	TGTCAATCCTGTCCGTGTCC
HPRT1	GCCCCAAAATGGTTAAGGTTGCAAG	ATCCAACAAAGTCTGGCCTGTATCC
β actin	AGGCCAACCGTGAAAAGATG	ACCAGAGGCATACAGGGACAA
GAPDH	AACTCCCTCAAGATTGTCAGCAA	GGCTAAGCAGTTGGTGGTGC