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Protective Effects of Hydrogen on Fetal Brain Injury During Maternal Hypoxia

Wenwu Liu,

Department of Physiology and Pharmacology, Loma Linda University, School of Medicine, Loma Linda, CA, USA and Department of Diving Medicine, The Second Military Medical University, Shanghai, People's Republic of China

Oumei Chen,

Department of Physiology and Pharmacology, Loma Linda University, School of Medicine, Loma Linda, CA, USA

Chunhua Chen,

Department of Physiology and Pharmacology, Loma Linda University, School of Medicine, Loma Linda, CA, USA

Bihua Wu,

Department of Physiology and Pharmacology, Loma Linda University, School of Medicine, Loma Linda, CA, USA

Jiping Tang, and

Department of Physiology and Pharmacology, Loma Linda University, School of Medicine, Loma Linda, CA, USA

John H. Zhang

Department of Physiology and Pharmacology, Loma Linda University, School of Medicine, Loma Linda, CA, USA and Department of Physiology, Loma Linda University, School of Medicine, Risley Hall, Room 223, Loma Linda, 92354, CA, USA johnzhang3910@yahoo.com

Abstract

This study aimed to investigate the effects of hydrogen on fetal brain injury during maternal hypoxia. Pregnant rats ($n = 12$, at gestational day 17) were randomly assigned into three groups; air, hypoxia, and hypoxia plus hydrogen groups were put into a chamber and flushed with room air (21% O₂ and 79% N₂), hypoxia (8% O₂ and 92% N₂), and hypoxia with hydrogen mixture (2% H₂, 8% O₂ and 90% N₂), respectively, for 4 consecutive hours. After birth, body and brain weights, body-righting reflex, and negative geotropism of neonates were measured, and then pups were killed at days 1 and 7. Oligodendrocytes were studied at post-natal day 1 by immunohistochemistry. We found significant decreases in body weight in the hypoxia group ($P < 0.05$ vs. room air group), but not in the hypoxia plus hydrogen group ($P > 0.05$ vs. room air group). Even though brain weight was not different among groups, the brain weight to body weight ratio in the room air group was significantly ($P < 0.05$) lower than that in the hypoxia alone or hypoxia plus hydrogen groups. Body-righting reflex at day 1 and negative geotropism at days 3–4 showed deficiency in hypoxia animals when compared with the room air group ($P < 0.05$). Hydrogen treatment improved the body-righting reflex and negative geotropism ($P < 0.05$ vs. room air group). The above-mentioned functional changes caused by hypoxia were not associated

with morphology and cell death of oligodendrocytes. Therefore, the maternal hypoxia-induced body weight loss, and functional abnormalities and hydrogen treatment during hypoxia offered a protective effect and improved functions in neonates.

Keywords

Maternal hypoxia; Neonates; Hydrogen; Functional evaluation

Introduction

Pathological conditions during pregnancy, such as hypoxia, seizures, and infection, are known to cause fetal brain damage [1]. Among these factors, low brain oxygenation is associated with an increased risk of cerebral palsy and periventricular leukomalacia (PVL) in newborns [2]. Depending on the type, the severity, and the duration, hypoxia produces either temporary brain dysfunction or permanent brain injury [1]. During hypoxia, excessive production of reactive oxygen species (ROS) actively oxidizes protein, DNA, and lipid, and results in cellular injury. Among these ROSs, the hydroxyl radical ($\cdot\text{OH}$) and peroxynitrite (ONOO^-) are more noticeable because there is not a known detoxification system in human body for $\cdot\text{OH}$ and ONOO^- [3].

Recently, the protective effects of hydrogen have attracted attention in ischemic and hypoxic insults because of its electric neutrality, cellular permeability, harmless metabolite (H_2O), and non-interference with metabolic oxidation-reduction reactions [4]. Hydrogen seems to selectively scavenge $\cdot\text{OH}$ and ONOO^- during ischemia and reperfusion injury [4] in focal ischemia, neonatal hypoxia-ischemia, Parkinson's, and stress-induced impairment rat models [4–7]. Therefore, the aim of this study is to investigate the effect of hydrogen on fetal neurodevelopmental brain damage in an established maternal hypoxia rat model.

Materials and Methods

Experimental Regimen

Pregnant Sprague-Dawley (SD) rats at gestational day 17 were assigned randomly to the following groups: room air group ($n = 4$), hypoxia group ($n = 4$), and hypoxia plus hydrogen group ($n = 4$). The rats in the room air, hypoxia, and hypoxia plus hydrogen group were put into a chamber and flushed with room air (21% O_2 and 79% N_2), hypoxia (8% O_2 and 92% N_2), and hypoxia with a hydrogen mixture (2% H_2 , 8% O_2 and 90% N_2), respectively, for 4 consecutive hours. Then, the mother rats were taken out, and maintained in a normal atmosphere and humidity-controlled condition with a 12:12-h light-dark cycle. The Animal and Ethics Review Committee at the Loma Linda University evaluated and approved the protocol used in this study.

Negative Geotropism

Pups were placed on a sloping rough surface (at an angle of 20° , size 300×330 mm) head downwards. The magnitude of rotation of the animal to reach the normal head-upwards position was assessed during a 1-min period using a four-point scale: 0 = no reaction; 1 = weak rotation (up to 90°); 2 = incomplete rotation (from 90°); 3 = complete rotation (180°) [8]. Negative geotropism was tested daily from 3 days after delivery.

Body Righting

Pups were gently placed on their backs, and a score was given according to the following criteria: 0 = no response; 1 = weak response; 2 = incomplete response (e.g., a leg remaining

beneath the animal's body); 3 = complete response [8]. In addition, the time taken before the animal showed the complete righting response was measured (cutoff: 60 s). Body-righting was tested daily.

Body and Brain Weight

Animals were weighed daily and killed at postnatal day 1 (P1) and day 7 (P7). The brains were removed and weighed. The ratio of brain weight to body weight was obtained.

Immunohistochemistry

Brains were obtained and fixed in formaldehyde followed by frozen sectioning. Then sections were washed with PBS three times and treated with 0.3% Triton $\times$ 100 for 15 min. After washing with PBS three times, antigen retrieval was performed in sodium citrate solution. Then, sections were washed with PBS three times and treated with 3% hydrogen peroxide. After washing with PBS three times, sections were blocked with 3% donkey serum in PBS for 1 h at room temperature followed by incubation with primary antibody (Anti-CNPase, 1:200; Millipore, USA) overnight. Then, sections were washed with PBS three times and treated with secondary antibody (1:200) for 2 h. Color development was performed according to the manufacturer's instructions (Santa Cruz, USA). The number of oligodendrocytes was counted under a light microscope.

Results

Body and Brain Weights

The mean body weight of neonates in the room air group was higher at days 1, 6, and 7 than in the hypoxia group. Hydrogen treatment increased body weight even though statistical significance was not obtained ($P > 0.05$ vs. hypoxia group, Fig. 1a). Daily body weight gain was calculated, and animals in the room air group had significant gains at days 5 and 7 when compared to the hypoxia groups ($P < 0.05$ vs. hypoxia, hypoxia + hydrogen groups). No statistical significance was obtained between the hypoxia and hypoxia plus hydrogen groups (Fig. 1b).

Brain weights were obtained at days 1 and 7, and no differences were observed among these three groups ($P > 0.05$), although there was a trend that the brain weight in room air and hypoxia plus hydrogen groups were higher than in the hypoxia group at day 7 (Fig. 1c). Brain to body weight ratio was calculated, and a significantly higher ratio was observed in the hypoxia, but not in the hypoxia plus hydrogen groups when compared with room air animals ($P < 0.05$, Fig. 1d).

Functional Evaluation

A transient but marked deficiency of body-righting reflex was observed at day 1 in the hypoxia group when compared with room air animals ($P < 0.05$), and hydrogen treatment abolished the deficiency of body-righting reflex ($P < 0.05$ vs. hypoxia, Fig. 2a). Similarly, a marked deficiency of negative geotropism was observed at days 3–4 in the hypoxia group when compared with room air animals ($P < 0.05$). Again, hydrogen treatment improved significantly the deficiencies ($P < 0.05$ vs. hypoxia group, Fig. 2b).

Oligodendrocytes Morphology

Oligodendrocytes and their precursors begin to arise in significant numbers in the developing rodent forebrain around embryonic day 18, and they are sensitive to hypoxia [9]. Therefore, we killed pups on day 1 (E18) to observe oligodendrocytes. The number of oligodendrocytes was counted at a magnification of 100. We did not observe morphological

abnormalities in this animal model ($P > 0.05$, Fig. 3a). The cell numbers of oligodendrocytes tended to be lower in the hypoxia group without statistical significance ($P > 0.05$ vs. room air). Hydrogen treatment failed to affect the number of oligodendrocytes ($P > 0.05$ vs. hypoxia or room air groups, Fig. 3b).

Discussion

In this study, a mild and transient maternal hypoxia retarded brain and body development slightly and reversibly, similar to the low brain oxygenation-caused perinatal brain injury in human [10]. This mild developmental retardation is accompanied by an abnormal body-righting reflex observed at day 1 and abnormal negative geotropism noted at days 3 and 4. Both sensory reflexes returned to normal level rapidly after birth. Oligodendrocytes and their precursors begin to arise in significant numbers in the developing rodent forebrain around embryonic day 18 (E18) [9]. Oligodendrocytes are particularly vulnerable to neonatal hypoxia/ischemia insults and are preferentially lost following neonatal unilateral carotid ligation combined with hypoxia in rodents. This mild and transient maternal hypoxia does not produce morphological brain abnormalities or oligodendrocyte cell death.

Mouse and rat brains at the last trimester of gestation paralleled the developmental stage of a human fetal brain at the first-second trimester. Study showed delays in the development of various sensory and motor reflexes were observed during the first month of rodent life after maternal hypoxia exposure [11]. Negative geotropism is essential for adaptation to the environment and is developed during the first postnatal week in the rat and mouse. Zhuravin et al. [12] indicated acute prenatal hypoxia delayed the development of this ability. Any one of the neuromuscular responses aims to restore the body to its normal upright position when it has been displaced, which is called the righting reflex and represents a sensory reflex. A similar trend of slower development in newborns exposed to prenatal or neonatal hypoxia/ischemia, as compared to control groups, has been reported for other sensorimotor reflexes, including the righting reflex [13]. Despite the delay in reflex development, it is important to note that newborns exposed to hypoxia/ischemia pre- or neonatally do complete the development of all the sensorimotor reflexes tested [11]. Our results were consistent with previous study in which researchers showed abnormal sensory reflexes could resolve [11–13].

We tested the potential therapeutic effect of hydrogen in this mild and transient maternal hypoxic pup model, because a recent study reported that inhalation of hydrogen gas could selectively neutralize $\cdot\text{OH}$ and peroxynitrite (ONOO^-) and protect the brain against ischemia/reperfusion injury [4]. Even though the results of hydrogen on body weight, body weight gain, and brain weight to body weight ratio are inconsistent, hydrogen treatment during hypoxia improved the body-righting reflex and negative geotropism. In summary, mild and transient maternal hypoxia caused neurological functional abnormalities in this animal model, and hydrogen may be an alternative treatment to prevent subtle brain injury and especially improve functional outcomes.

Acknowledgments

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Fig 1.

(a) Body weight among groups (* $P < 0.05$, air group vs. hydrogen group or hypoxia group). (b) Body weight gain among groups (* $P < 0.05$, air group vs. hydrogen group or hypoxia group). (c) Brain weight among groups. (d) Brain weight to body weight ratio among groups (* $P < 0.05$, air group vs. hydrogen group or hypoxia group)

Fig 2.

(a) Body-righting reflex among groups (* $P < 0.05$, air group vs. hydrogen group or hypoxia group). (b) Negative geotropism in different groups (* $P < 0.05$, air group vs. hydrogen group or hypoxia group; # $P < 0.05$ hydrogen group vs. hypoxia group)

Fig 3.

(a) Representative photographs of oligodendrocytes in the cortex. No marked morphological changes were noted in oligodendrocytes in any groups. (b) The number of oligodendrocytes was counted, and no significant difference was observed among three groups