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REVIEW ARTICLE

Hydrogen-induced Neuroprotection in Neonatal Hypoxic-ischemic Encephalopathy

Ferenc Domoki[1,*]

[1]*Department of Physiology, University of Szeged, School of Medicine, Szeged, Hungary*

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Abstract: Hypoxic-ischemic encephalopathy (HIE) remains to be a major cause of morbidity, mortality and severe neurodevelopmental disability in term neonates. Moderate whole body hypothermia is an established, effective neuroprotective therapy to reduce mortality and long-term disability associated with HIE, however, research for adjunct therapies is still warranted to complement the effect of hypothermia. In the last decade, molecular hydrogen emerged as a simple, available, inexpensive substance with advantageous pharmacokinetics to ameliorate hypoxic-ischemic cellular damage. The present review examines the preclinical studies employing hydrogen to combat the deleterious consequences of hypoxic-ischemic insults in rodent and piglet HIE models. Hydrogen exerted unequivocal neuroprotective actions shown by preserved neurovascular function, neuronal viability, and neurocognitive functions in virtually all model species and hypoxic-ischemic insult types tested. Administration of hydrogen started in most studies after the hypoxic-ischemic insult enhancing the translational value of the findings. Among the explored mechanisms of hydrogen-induced neuroprotection, antioxidant, anti-apoptotic and anti-inflammatory effects appeared to be dominant. Unfortunately, the additive neuroprotective effect of hydrogen and therapeutic hypothermia has not yet been demonstrated, thus such studies are warranted to promote the clinical testing of molecular hydrogen as an adjunct neuroprotective treatment of HIE.

Keywords: Asphyxia neonatorum, birth asphyxia, cerebral hypoxia-ischemia, medical gas research, molecular hydrogen, neonatal animals, neuroprotectants, translational research.

1. INTRODUCTION

Hypoxic-ischemic encephalopathy (HIE) is a clinical diagnosis applied to term neonates based on the fulfilment of two sets of criteria: (1) clinical signs of asphyxia at birth, and (2) signs of moderate to severe encephalopathy in the first hours after birth [1]. Birth asphyxia and HIE remain to be major causes of perinatal mortality and long-term disability. The incidence of HIE was estimated to be as low as 1.5/1000 live births in developed countries, but it is assumed to be much higher in low-income settings, the global estimate of HIE incidence is 8.5/1000 live births amounting to 1.15 million babies affected with HIE yearly world-wide. However, the real social impact of HIE may even be better appreciated by the result of the Global Burden of Disease 2010 study, estimating that birth asphyxia and HIE are responsible for 50.2 million disability-adjusted life years alone, 2.4% of the total burden [2, 3]. Moderate whole body hypothermia offers significant neuroprotection and has been shown to reduce mortality and improve both short-term and long-term neurodevelopmental outcomes [4-6]. However, therapeutic hypothermia is unable to save every affected infant, a systematic meta-analysis of 11 cooling trials concluded that the number needed to treat to benefit is 7 (4-17), meaning that on average 7 HIE patients with severe encephalopathy must be cooled to avoid 1 death or major disability [7]. Therefore, adjunct neuroprotective measures are warranted to complement the neuroprotective effect of hypothermia. Application of therapeutic gases appears to be a straightforward method to achieve neuroprotection as babies born with severe asphyxia usually require assisted ventilation. Medical gas research has identified a number of gases showing neuroprotective effects, including administration of xenon, argon, helium, hydrogen, carbon monoxide, and the management of carbon dioxide and oxygen [8]. The major objective of the present review was to critically examine and discuss the results of the preclinical studies on the neuroprotective effects and the mechanisms of neuroprotection by molecular hydrogen-treatment in HIE animal models, as these were not exhausted in a previous study [8].

Preclinical HIE research must rely heavily on animal models that have to balance availability, feasibility, cost, and translational value. Most rodent HIE studies involved in hydrogen research [9-14] used the Rice-Vannucci model to elicit hypoxic-ischemic injury [15, 16]. This model is relatively simple to implement, elicits a robust brain infarct, allows high throughput, but lacks important features of human HIE as hypoxic-ischemic injury is initiated one week after birth and the asphyxia is not present. On the other hand, the newborn pig emerged as the major HIE large animal model in molecular hydrogen research [17-20]. The newborn pig is an accepted model of the term human neonate for HIE research, as piglets possess similar brain structure, brain developmental stage at birth to human babies, and they have a relatively modest cost and easy availability compared with other large animal models such as newborn lambs or primates [21, 22].

Molecular hydrogen, once considered to be an inert gas, was used to measure cerebral blood flow due to its lipid-solubility and rapid crossing through the blood-brain barrier in preclinical animal models since the 1960s, for a comprehensive review on the so-called hydrogen-clearance technique [23]. Since the seminal paper of Ohsawa *et al*. [24], the study of molecular hydrogen witnessed exponential growth, and there are excellent reviews summarizing the disease states where molecular hydrogen showed beneficial neuroprotective effects, and the putative mechanisms of hydrogen-induced protection, namely attenuation of oxidative damage, abrogation of apoptosis and neuroinflammation are discussed [25-27]. These putative mechanisms are in full accordance with the current concepts of the pathophysiology of HIE development. Neuronal injury during HIE is thought to be a continuous process, in which different mechanisms are playing variable roles at different time points: in the acute phase, oxidative stress secondary to reoxygena-

*Address correspondence to this author at the Department of Physiology, University of Szeged, School of Medicine, 10 Dóm tér Szeged, 6720, Hungary; Fax: +36 62-545842; Tel: +36 62-545923;
E-mail: domoki.ferenc@med.u-szeged.hu

tion injury is important, and after the 6-24 hours long latent phase, the so-called secondary energy failure follows that is characterized by further neuronal loss secondary to apoptotic and neuroinflammatory processes [28, 29]. The following sections will first showcase the observations of hydrogen-induced neuroprotection in rodent and piglet HIE models, followed by the analysis of unique or shared mechanisms of neuroprotection among the different models.

2. HYDROGEN-INDUCED NEUROPROTECTION IN RODENT HIE MODELS

The neuroprotective effect of hydrogen to combat HIE was first studied in postnatal day 7 (P7) rat pups [9]. In this study, the Rice-Vannucci model was employed to inflict hypoxic-ischemic injury (left common carotid artery occlusion + 90 min inhalation of 8% oxygen). Hydrogen treatment started immediately after the hypoxia by inhalation of 2% hydrogen gas for 30-60-120 minutes in respective groups. The brains were processed 24 hours after the completion of hydrogen administration. The major findings of the study were that (1) molecular hydrogen significantly reduced infarct size determined by 2,3,5-triphenyltetrazolium chloride (TTC) staining; (2) it decreased neuronal loss in the neocortex and the CA1 region of the hippocampus determined by cell counting of Nissl-stained sections; and (3) hydrogen administration also reduced the number of apoptotic neurons in the same brain regions assessed with terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) staining. Furthermore, the neuroprotective effect of hydrogen was clearly dependent on treatment duration, thus 120 min administration was significantly superior to 30 or 60 min hydrogen therapy. The same research group soon published a follow-up study using the same rat pup HIE model that utilized an alternative route of molecular hydrogen delivery, namely intraperitoneal injection of hydrogen saline [10]. Hydrogen saline was prepared by dissolving molecular hydrogen in physiological saline at high pressure (0.6 MPa) to obtain supersaturated (>0.6 mmol/L) hydrogen concentration. Hydrogen saline was administered twice, immediately and 8 hours after the completion of hypoxia. Three doses of hydrogen saline were employed: 2.5, 5, and 10 mL/kg doses. However, neuronal cell counting indicated that the maximal neuroprotection was elicited by the 5mL/kg dose, the animals treated with a 10 mL/kg dose had a significantly larger loss of neurons. Therefore, for all the other outcome measures, data from only the 5 mL/kg treated group were reported. Similar to the inhalation route of hydrogen delivery, hydrogen saline treatment was also neuroprotective, shown by smaller infarct size determined with TTC staining, and reduced number of TUNEL-positive cells on day 1. Importantly, in this study, a subgroup of animals was maintained for 5 weeks that allowed functional studies. Indeed, compared to the untreated animals, hydrogen saline treatment preserved motor functions shown by postural reflex testing and spontaneous locomotor activity. Also, a clear difference between the two groups was shown in the Morris water maze paradigm, indicating that hydrogen helped to preserve spatial recognition and learning after hypoxic-ischemic injury.

In contrast to the initial two positive studies on hydrogen-induced neuroprotection [9, 10], the study published by Matchett *et al.* disputed the efficacy of hydrogen treatment to ameliorate the consequences of hypoxic-ischemic insult [11]. This study also employed the Rice-Vannucci model, albeit there were two major alterations compared to the studies by Cai *et al.* [9, 10]. Firstly, P10 and not P7 rat pups were used that likely increased neuronal vulnerability to hypoxia as neonatal hypoxic tolerance is maximal at birth [30, 31]. Secondly, instead of 90 min, 120 and 150 min long hypoxic periods were employed that triggered much larger brain injury. Cai *et al.* [9, 10] reported infarct sizes determined by TTC staining were ~10% of forebrain volume, however, in the study by Matchett *et al.* [11], the average infarct sizes were ~30% and ~50% for 120 and 150 min long hypoxia durations, respectively, the latter value meaning that virtually the whole affected hemisphere was infarcted. Under these conditions, inhalation of 2.9% molecular hydrogen initiated either before, during or after the hypoxic stress did not affect the infarct size significantly, and the only qualitatively assessed histopathology yielded similar negative results. Notably, the elongation of hypoxia (from 90 min [9, 10] to 120-150 min [11]) was also accompanied by the reduction of hydrogen inhalation after the stress (from max. 120 min to max 60-90 min, respectively). These differences are likely accountable for the negative outcome of the latter study.

A recent study re-visited the assessment of neuroprotection afforded by the inhalation of molecular hydrogen using the Rice-Vannucci model in P7 rat pups [14]. The study design and the main outcome measures of neuroprotection were essentially the same as in the study by Cai *et al.* [9]. The authors used a slightly longer (100 instead of 90 min) hypoxia period that resulted in slightly larger infarcts (~15% *versus* ~10% of brain volume) in the untreated animals exposed to hypoxic-ischemic stress. Also, the concentration of inhaled molecular hydrogen was higher, 3% instead of 2%. Confirming all previous findings, 30-60-90 min inhalation of molecular hydrogen initiated immediately after the hypoxic insult resulted in the amelioration of neuronal injury in a treatment-duration dependent manner. More specifically, the increased duration of molecular hydrogen treatment progressively decreased infarct size shown by TTC staining at 24 hours after hypoxia, and it also progressively reduced the number of TUNEL+ neurons in the cortex and the CA1 hippocampal region assessed at 72 hours after hypoxia. Furthermore, 5 weeks after the hypoxic stress, the spatial learning of the animals was tested with the Morris water maze paradigm. Similar to the findings obtained with hydrogen saline administration [10], inhalation of hydrogen could also significantly preserve spatial learning and memory function, in fact, by the end of the learning protocol, the escape latency data (time needed to reach the hidden platform) on the 4th day, and the time spent in the correct quadrant (from which the platform was removed) on the 5th day were indistinguishable between the naïve group and the animals receiving the longest (90 min) duration of hydrogen treatment after the hypoxic-ischemic injury [14].

In addition to rats, the neuroprotective effect of molecular hydrogen was also investigated in a murine HIE model [12, 13]. The hypoxic-ischemic injury was initiated using the Rice-Vannucci model (P7 mouse pups, unilateral common carotid artery occlusion, 90 min inhalation of 8% oxygen). Molecular hydrogen treatment was performed with intraperitoneal injections of hydrogen saline. Hydrogen saline was prepared and dosed as in a previous study [10]. However, hydrogen saline (2.5 or 5 mL/kg) was first administered only at 24 hours after completion of hypoxia and then at 24-hour intervals for 3 days. Despite the very delayed initiation of hydrogen treatment, 2.5 and 5 mL/kg hydrogen saline dose-dependently and significantly reduced infarct size assessed with TTC staining, and also attenuated cerebral oedema shown by the reduction of brain water content 3 days after the hypoxic-ischemic insult [12, 13]. In addition, the 5 mL/kg dose of hydrogen saline also preserved short-term and long-term neurological performance. On P10, hydrogen saline treated mice demonstrated better performance on the negative geotaxis test (a postural test) than the untreated HIE animals, although this difference was not present on P12 and P14 any longer, and there was no difference in the performance of the front-limb suspension test on any of the three test days. More importantly, hydrogen saline treated animals achieved better scores on the novel object recognition test on P35, and also demonstrated better spatial learning and memory performance in the Morris water maze paradigm shown by reduced escape latency on days 4 and 5, and by increased time spent in the correct quadrant on day 6 compared to untreated HIE animals [13].

The above-discussed rodent studies utilized the Rice-Vannucci model that results in a robust focal infarct, however, this ischemic stroke-like pathology virtually never occurs in term infants with

HIE. In contrast, Mano *et al.* investigated the neuroprotective effect of maternal hydrogen water administration in an intrauterine asphyxia model [32]. In this model, foetal asphyxia was induced by transient bilateral occlusion of utero-ovarian artery for 30 min on the 16th day of pregnancy. The pregnant dams drank hydrogen water *ad libitum* from day 14 to 22 of pregnancy until delivery. Hydrogen water was prepared similar to hydrogen saline and special care was taken to prevent degassing of the drinking water. The primary outcome measure of the study was the assessment of neuronal injury in the CA1 and the CA3 hippocampal regions by determining the ratio of injured neurons in hematoxylin-eosin stained sections in brain samples obtained on P7. Foetal asphyxia resulted in a similar 34-35% ratio of damaged neurons in both hippocampal regions, however, hydrogen water treatment apparently prevented the neuronal injury as the values from these animals were virtually identical to the normoxic controls, the ratio of damaged neurons was mere 2-5% in these animals. The secondary outcome measure was the assessment of spatial learning and memory function in 8 weeks old animals using the Morris water maze. Indeed, offspring of the hydrogen-treated dams had significantly shorter escape latency on the 4th but not on the 5th day of the trial, indicating a somewhat better learning performance compared to the untreated asphyxia group. Although it does not belong strictly to HIE research, the same research group published a study investigating the neuroprotective effect of maternal hydrogen water on foetal brain injury induced by maternal inflammation elicited with lipopolysaccharide injection [33]. The efficiency of molecular hydrogen to ameliorate foetal brain damage under these conditions has translational value in HIE research, as maternal/foetal inflammation is an established major risk factor of HIE development [34] and may contribute to worse outcomes in HIE patients [35].

3. HYDROGEN-INDUCED NEUROPROTECTION IN PIGLET HIE MODELS

In newborn pigs, the putative neuroprotective effect of molecular hydrogen was first studied in an acute asphyxia model [17]. In that study, asphyxia was elicited with the transient cessation of mechanical ventilation and occlusion of the endotracheal tube to prevent spontaneous breathing in alpha-chloralose anesthetized animals for 10 minutes. Administration of molecular hydrogen started immediately at the onset of reventilation by inhalation of 2.1% hydrogen in the air for four hours when the experiments were terminated. The primary outcome measure of the study was the assessment of cerebrovascular reactivity to graded hypercapnia and to NMDA. These stimuli elicit pial arteriolar vasodilation acting primarily on cerebrovascular or neuronal elements, respectively [36, 37]. Importantly, both responses have been shown to be severely impaired by hypoxic/ischemic stress in this species [38, 39]. These vulnerable, ischemia-sensitive neuronal-vascular responses have been repeatedly utilized as sensitive bioassays to assess putative neuroprotective mechanisms. For instance, using pretreatment experimental protocols, the attenuation of both hypercapnia- and NMDA-induced vasodilation after hypoxic-ischemic stress was prevented by the administration of the mitochondrial ATP-sensitive potassium channel opener diazoxide [40, 41], or the pituitary adenylate cyclase activating polypeptide (PACAP) [42]. In contrast to these studies, molecular hydrogen was delivered only after the asphyxia, along with the restoration of normoxia and normocapnia, thus molecular hydrogen could not exert a preconditioning-like effect [17]. The study showed that in the sham animals not exposed to asphyxia, molecular hydrogen did not affect the cerebrovascular reactivity to either hypercapnia or NMDA. However, one hour after the onset of reventilation, the cerebrovascular reactivity to hypercapnia was significantly attenuated in the untreated controls but was essentially intact in the hydrogen-treated animals. Interestingly, the employed hypoxic/ischemic stress did not elicit significant impairment of NMDA-induced vasodilation in this study, thus the question of whether molecular hydrogen could also preserve this response remained unanswered. The secondary outcome measure of the study was the neuropathological assessment of neuronal injury in several neocortical, hippocampal, and subcortical regions. The relatively short time-span (four hours) of the experiments proved to be suboptimal for detecting asphyxia-induced neuronal injury, for instance, only 5% of neurons showed morphological damage in the CA1 area of the hippocampus in the untreated control group. Still, in most regions, the untreated but not the hydrogen-treated asphyxiated animals showed significantly more damaged neurons compared to the sham, normoxic animals suggesting the neuroprotective potential of molecular hydrogen. In addition to the short observation period after asphyxia, the study had another limitation. The severity of asphyxia was likely too low, despite that severe hypoxia, acidosis, and ~70% decrease in cortical blood flow assessed by laser-Doppler flowmetry were recorded. However, the cerebral temperature may have dropped during asphyxia, providing some neuroprotection for two reasons. Firstly, prior to asphyxia, a stainless steel cranial window was built into the parietal bone to allow the visualization and the assessment of pial arteriolar responses, however, the surgery affected the integrity of the scalp and the skull, and it may have unwantedly affected brain temperatures. Moreover, as the core body temperature was maintained with electrical pads, and a servo-controlled heating device was not employed, therefore, a small spontaneous drop in the core temperature secondary to hypoxic hypometabolism that is well-developed in newborn mammals [31] triggered by asphyxia could also have occurred. These two factors could have reduced the brain temperature and mitigated the deleterious effects of asphyxiation that could have been responsible for the unaltered cerebrovascular reactivity to NMDA.

The follow-up piglet study wished to address these limitations in a comprehensive manner [18]. Most importantly, the observation period has been extended to 24 hours, allowing to follow the development of HIE into the subacute period. In addition, the anesthesia was switched from alpha-chloralose to morphine/ midazolam, intensive monitoring and supportive care measures were implemented to be in compliance with neonatal intensive care unit practices [43, 44]. Importantly, core temperature regulation was achieved with a servo-controlled heating system and there was also no cranial surgery prior to asphyxia. Based on preliminary experiments, the duration of asphyxia (elicited with endotracheal tube occlusion) had to be reduced to 8 min as the 10 min long stress under these conditions was lethal too often. Using laser-speckle contrast imaging and analysis, during asphyxia, cortical blood flow was found to be reduced to 10-20% of baseline within 4-5 minutes, corresponding with very severe ischemic stress [45]. Molecular hydrogen treatment started immediately at the onset of reventilation by inhalation of 2.1% hydrogen in the air for four hours, similar to the previous study [17]. The monitored physiological parameters were not different among the treatment groups, but the recovery of the brain's electrical activity after asphyxia was (in the first four hours) faster in the hydrogen-treated animals shown with amplitude-integrated electroencephalography suggesting some neuroprotection. The primary outcome measure of the study was again the assessment of cerebrovascular reactivity to graded hypercapnia and to NMDA, but in this study, pial arteriolar responses were determined 24 hours after the asphyxia period. The normoxic time control animals showed virtually identical vascular responses to both stimuli compared to those obtained in previous short-term experiments [17, 40, 42], thus establishing that the changes in the length and means of anesthesia did not affect the cerebrovascular reactivity to either stimuli. In the untreated animals exposed to asphyxia, the cerebrovascular reactivity to both graded hypercapnia and NMDA was severely attenuated indicating that HIE development involves neurovascular unit dysfunction stretching into the subacute period. The impairment of these cerebrovascular responses was selective and did not reflect general deterioration of vascular function as the constrictor response to norepinephrine and the

dilator response to the nitric oxide donor sodium nitroprusside remained intact. Importantly, molecular hydrogen administered only in the early (4 hours) reventilation period, alleviated the delayed neurovascular damage triggered to both stimuli. The response to graded hypercapnia was essentially intact in the hydrogen-treated animals, however, NMDA-induced vasodilation remained significantly depressed compared to the normoxic time controls, but nevertheless cerebrovascular reactivity to NMDA was significantly better than in the untreated animals. The secondary outcome measure of the study was also the neuropathological assessment of neuronal injury. Compared to the results of the previous study, the percentage of damaged neurons after asphyxia in most examined regions was higher (*e.g*., in the CA1 hippocampal area, 10-15% of neurons showed morphological signs of damage instead of the 5% in the previous study). Although there was a tendency for reduced neuronal damage in the hydrogen-treated animals in most brain areas, due to the relatively mild neuronal damage and the small differences between the treated and untreated groups, there were no statistically significant differences except in the frontal cortex.

Based on the results of the above discussed studies [17, 18], it became apparent that the asphyxia induced by endotracheal tube occlusion is inadequate to create a hypoxic/ischemic stress that is prolonged/severe enough to elicit such degree of neuronal injury that is sufficient to assess the promising neuroprotective potential of molecular hydrogen. Therefore, we aimed to achieve an asphyxia model that truthfully mimics the hallmark features of spontaneous or experimental intrauterine asphyxiation in terms of hypoxia, hypercapnia, lactic acidosis, and duration in piglets [46, 47]. After a series of preliminary experiments, we established and characterized a new experimental asphyxia model that was induced by ventilating the animals with a gas mixture containing 6% oxygen and 20% carbon dioxide for 20 minutes, meeting all of the criteria mentioned above [19]. Cardiovascular function was less affected by this form of asphyxia, allowing the significant elongation of the duration of stress. The lack of systemic hypotension during this form of experimental asphyxia likely contributed to the fact that severe reductions in cortical blood flow were not detected in this model using laser-speckle contrast imaging [19]. Most importantly, however, the new asphyxia model yielded easily detectable, marked neuronal injury, the percentage of neurons showing damage rose as high as 50-60% in the CA1 hippocampal area. Molecular hydrogen treatment was administered in full compliance with the previous studies (inhalation of 2.1% hydrogen started immediately after reventilation/reoxygenation for 4 hours). The primary outcome measure of the study was to assess the neuroprotection afforded by molecular hydrogen. Indeed, the animals of the hydrogen-treated group had significantly reduced neuronal injury virtually in all examined areas: the frontal, parietal, temporal and occipital lobes, the CA1 and CA3 hippocampal area, the basal ganglia, the thalamus, and the cerebellar Purkinje cells as well [19]. In fact, in most areas, the percentage of injured neurons was similar in the hydrogen-treated animals to the normoxic time controls. The neuropathological findings were also corroborated by electrophysiology, especially EEG power analysis showed significantly better restoration of electrical activity in the hydrogen-treated compared to the untreated asphyxiated animals, and this difference persisted throughout the observation period.

A recently published study addressed the important question of whether the neuroprotective effect of molecular hydrogen could augment the neuroprotection afforded by therapeutic hypothermia [20]. In this study, brain injury was not induced by asphyxia, instead, 30 minutes of normocapnic severe hypoxia was induced by ventilation with 4%, the inspired oxygen fraction was modulated by +/-1% steps to develop and maintain low voltage brain electric activity monitored with amplitude-integrated EEG and to keep mean arterial blood pressure at >70% of its original value. After the hypoxic insult, the animals were ventilated with 100% oxygen for 10 minutes to mimic resuscitation. These features of the employed hypoxic stress do not support the translational value of the model. Firstly, normocapnic hypoxia virtually never occurs in the perinatal situation, as birth asphyxia always involves hypercapnia. The metabolic response of the foetal brain is markedly different to isolated hypoxia *versus* asphyxia [48]. Secondly, resuscitation with 100% oxygen is not recommended in current guidelines of neonatal resuscitation [49], as it elicits reoxygenation injury, increasing mortality [50]. Indeed, both of these challenges should have increased neuronal injury. After the hypoxic injury, the anesthetized animals were randomized to control, therapeutic hypothermia, and therapeutic hypothermia + inhalation of 2.1-2.7% molecular hydrogen for 24 hours. Afterwards, the animals were rewarmed, weaned off the anaesthesia and extubated, then they were further observed for 5 days for the assessment of neurological function every 6 hours. At the end of the observation period, the brains were processed for neuropathology. The study demonstrated that the almost full recovery of neurological function was similar in the animals treated with therapeutic hypothermia alone as compared with those also receiving molecular hydrogen. Thus, although the hypoxia-induced deterioration of neurological function and the neuroprotective effect of hypothermia were both prominent, clear additive neuroprotection afforded by hydrogen could not be demonstrated. Interestingly, the neuropathological examination found a significant difference between the therapeutic hypothermia and the therapeutic hypothermia combined with hydrogen groups only in the dorsal parietal cortex, where the number of TUNEL (+) cells was significantly reduced in the animals also receiving hydrogen. However, there were no significant differences among the three groups in the hematoxylin-eosin stained sections in the neocortex, the hippocampus or the cerebellum, and there was only a tendency for reduced TUNEL (+) cells in the mid-temporal cortex.

4. MECHANISMS OF HYDROGEN-INDUCED NEUROPROTECTION IN HIE MODELS

Molecular hydrogen exerts marked, robust neuroprotective effect, while its administration routes, the timing of the initiation and duration of hydrogen treatments varied considerably among the positive studies that likely determine the major mechanisms by which hydrogen could exert its beneficial effects. Molecular hydrogen is well-known to exert pleiotropic protective effects in different systems; in the following paragraphs, an attempt is made to synthesize the so far revealed mechanisms of hydrogen in HIE models, more specifically antioxidant, anti-apoptotic and anti-inflammatory mechanisms will be discussed in detail. Notably, these mechanisms cannot be considered fully independent of each other, as they all form elements of a vicious circle in which oxidative stress leads to cellular dysfunction that triggers necrotic/apoptotic cell death, the former recruiting a neuroinflammatory response that can in turn result in further oxidative damage *etc*. In fact, the primary cause and the consequence are difficult to identify (the egg-hen problem), thus pinpointing the primary target(s) of the hydrogen molecule is not possible at this time.

The seminal paper by Ohsawa *et al.* [24] set the stage for the study of molecular hydrogen as a therapeutic antioxidant. The imbalance between the generation and the elimination of reactive oxygen and reactive nitrogen species are of major importance in determining cellular injury in virtually all disease models [51]. There are a number of indications for this mechanism to contribute to neuroprotection in all HIE model species. The preservation of cerebrovascular reactivity to hypercapnia and to NMDA by hydrogen in piglets [17, 18] was in concert with hydrogen's presumed antioxidant effect, as the pial arteriolar response to hypercapnia and to NMDA was previously demonstrated to be sensitive to oxidative stress [36, 52]. Indeed, one of the most important sources of reactive oxygen species in the piglet cerebral cortex appears to be cyclooxygenase-2 (COX-2): COX-2 inhibitors were shown to re-

duce cortical superoxide levels and to preserve cerebrovascular reactivity [53, 54]. COX-2 has a prominent, region-dependent neuronal expression in newborn pigs [55], and neuronal COX-2 expression was assessed using immunohistochemistry in brain samples obtained from studies [18, 19] examining hydrogen-induced neuroprotection after asphyxia [56]. The effect of asphyxia on the abundance of neuronal COX-2 immunopositivity was dependent on the length of asphyxia, and showed regional differences as well. A significant increase in COX-2 immunopositive neurons could be demonstrated only after 20 but not after 8 min asphyxia in the parietal and occipital cortices, as well as the CA3 hippocampal area, although there was a tendency for increased COX-2 abundance in the frontal and the temporal cortices as well. However, COX-2 abundance was low in the subcortical areas such as the basal ganglia or the thalamus, and in these regions, asphyxia did not induce COX-2 expression. Importantly, hydrogen treatment fully prevented changes in neuronal COX-2 expression in both the cerebral cortex and the hippocampus, the values were similar to normoxic time-controls. Neuronal COX-2 abundance was correlated with cortical neuropathology scores and it was established that high COX-2 abundance always coincided with severe neuronal injury, although low COX-2 abundance could occur with either mild or severe injury, in fact, in the animals with a severe injury, the low COX-2 abundance may have indicated translation block of the COX-2 protein [56].

Hydrogen treatment also drastically reduced lipid peroxidation induced by hypoxic-ischemic stress assessed with determining cerebral malondialdehyde levels in rats [10]. Furthermore, hydrogen attenuated cortical neuronal ROS production measured with dihydroethidium staining in mice [13]. The antioxidant mechanism of hydrogen's action was also supported by 8-hydroxy-2'-deoxyguanosine (8-OHdG) immunohistochemistry in piglets [19, 56] and rats [32]. 8-OHdG indicates oxidative DNA damage, and the ratio of 8-OHdG-positive nuclei greatly increased 24 hours after asphyxia in the piglet parietal cortex compared to normoxic time controls from ~20% to ~80%, however, hydrogen treatment significantly reduced the ratio of immunoreactive nuclei to ~60% indicating reduced oxidative stress. Similar results were obtained in rat brain samples obtained 2 weeks after intrauterine asphyxia. In addition, hydrogen also effectively blocked asphyxia-induced lipid peroxidation shown by preventing asphyxia-induced increases in 4-hydroxy-2-nonenal modified proteins levels [32]**.** The data from these studies suggest that oxidative stress processes contribute to neuronal injury from the early reoxygenation (1 hour) [17] through the subacute period (1-3 days) [10, 13, 18, 56] to the chronic (2 weeks) phase [32] of HIE development.

Anti-apoptotic effect of hydrogen treatment was reported also in rats [9, 10, 14], mice [12] and piglets [20]. Accordingly, the activation of caspase 3 representing the final common pathway has been blunted by hydrogen treatment in most models [9, 10, 12]. Hydrogen treatment has been shown to modulate multiple signalling pathways promoting neuronal survival. One of these mechanisms is the inhibition of the C-Jun N-terminal kinase (JNK) - p53 transcription factor pathway involved in the induction of apoptosis of the mitochondrial pathway [57]. Hydrogen treatment was demonstrated to reduce the number of phosphorylated JNK and p53 positive neurons in the cortex and the hippocampus of rats after hypoxic-ischemic stress compared to untreated animals [14]. Another important target of the anti-apoptotic effect of hydrogen appears to be the mitigation of endoplasmic reticulum stress. In mice, hypoxic-ischemic stress elevated the mRNA and the protein levels of the endoplasmic reticulum stress markers glucose-regulated protein 78 (GRP78) and C/EBP homologous protein (CHOP) [12, 58]. Hydrogen prevented the increases in GRP and CHOP levels, also reduced the number of GRP and CHOP immunopositive neuronal cell counts in the cortex ipsilateral to hypoxic-ischemic injury, indicating reduced endoplasmic reticulum stress [12]. Furthermore, there was a simultaneous observation of restoration of the proapoptotic Bcl-2-associated X protein (Bax)/ B-cell lymphoma 2 (Bcl-2) ratio in the hydrogen-treated animals confirming inhibition of apoptosis. A similar mechanism of hydrogen-induced prevention of endoplasmic reticulum stress is likely to occur also in rats, as hydrogen prevented the increases in caspase 12 activation in response to hypoxic-ischemic injury [9], and caspase 12 activation is known to be triggered by endoplasmic reticulum stress [59]. Hydrogen may prevent neuronal apoptosis after hypoxic-ischemic stress by preserving autophagy necessary for normal turnover of cellular organelles and self-repair after stress [60]. This possibility has been explored in mice, and multiple mechanisms have been identified [12]. Hypoxic-ischemic stress reduced protein levels of Beclin-1, a necessary component of autophagosome assembly, and hydrogen prevented this reduction. Also, hydrogen counteracted the changes in phosphorylation patterns of signalling molecules important for autophagy control, specifically, hydrogen inhibited the hypoxic-ischemic stress-induced phosphorylation/activation of mammalian target of rapamycin (mTOR) and signal transducer and activator of transcription 3 (Stat3) proteins that inhibit autophagy, and simultaneously prevented the dephosphorylation/inactivation of extracellular signal-regulated kinase ERK promoting autophagy [12]. This latter mechanism may not apply to all species, for instance, in piglets, asphyxia did not elicit dephosphorylation/inactivation of ERK, although P-ERK/ERK ratios were indeed high in virtually all brain areas studied [61].

Hydrogen treatment has been reported to ameliorate some aspects of the neuroinflammatory response in all species studied [10, 13, 14, 56]. Hypoxic-ischemic stress elevated the numbers of cortical Iba-1 immunopositive microglia in rats and mice, and this change has been ameliorated by hydrogen treatment [10, 13, 14]. Moreover, hydrogen reduced the protein levels of the inflammatory markers interleukin-1β (IL-1β) and nuclear factor κB p65 in the cortex, and also the number of Iba-1 positive microglia coexpressing IL-1β and NF- κB p65 in rats [14]. In the mouse, an even more detailed analysis of the neuroinflammatory response was performed [13]. Hypoxic-ischemic stress was found to elevate the number of both classical, proinflammatory M1, and alternative, neuroprotective M2 microglial cells. M1 and M2 cells were identified with co-expression studies of CD16 and CD206 with Iba-1, respectively. The authors reported that hydrogen treatment resulted in decreased number of M1 and increased number of M2 phenotype microglia promoting neuronal survival. Furthermore, hydrogen also attenuated the hypoxic-ischemic stress induced increases in the number of central nervous system associated (CD11b+/ CD45high) phagocytes [13]. In concert with the inhibitory effect on proinflammatory microglia/macrophages, hydrogen also lowered the expression of the inflammatory markers IL-1β, CD86, reduced the proinflammatory pNF-κB/NF-κB ratio, but increased the expression of anti-inflammatory markers like transforming growth factor β (TGF-β) and the YM-1 protein. The authors found that all beneficial effects of hydrogen treatment are dependent on the activation of 5' adenosine monophosphate-activated protein kinase (AMPK), a key regulator of cellular energy homeostasis activated. Hypoxic-ischemic stress *per se* elicited increases in the ratio of phosphorylated/total levels of AMPK (P-AMPK/AMPK) compared to the sham group. However, hydrogen treatment even further increased the P-AMPK/AMPK) ratio [13]. Administration of the AMPK inhibitor (compound C) reversed all neuroprotective and anti-inflammatory effects of hydrogen [13].

In the piglet, the proinflammatory phenotype of the resident Iba-1 immunopositive microglia was assessed by determining the so-called ramification index. The ramification index quantitatively detects the degree of process withdrawal and amoeboid appearance characteristic of activated proinflammatory microglia [62]. Compared to normoxic controls, asphyxia significantly reduced micro-

glial ramification index in the parietal cortex in untreated animals indicating activation of microglia. However, this postasphyxial reduction in microglial ramification index did not occur in hydrogen treated animals indicating a reduced neuroinflammatory response also in this large animal HIE model.

CONCLUSION

Molecular hydrogen exerts a significant neuroprotective effect on virtually all animal HIE models studied so far. The neuroprotective potential of hydrogen has been repeatedly demonstrated by outcome measures of neuronal survival and neurocognitive function as well. The administration of hydrogen in most studies started 0-24h after the hypoxic stress and stopped 4h-3 days afterwards, supporting their translational value. The few negative findings, in fact, indicate that the therapeutic potential of hydrogen is not unlimited: extremely severe hypoxic-ischemic stress [11] or possibly too high dose of hydrogen saline (as 10 mL/kg in [10]) may result in unwanted consequences. All major mechanisms of hydrogen-induced neuroprotection have also been identified in HIE animal models: reduction of oxidative injury, inhibition of pro-apoptotic with simultaneous activation of anti-apoptotic pathways, and inhibition of neuroinflammatory with simultaneous enhancement of neurotrophic actions of microglia. Future studies need to focus on the combination of hydrogen treatment with therapeutic hypothermia. The only published study so far found no additive neuroprotective effect [20], but in this study, therapeutic hypothermia alone afforded full protection against the neurological dysfunction alone. Carefully executed translational studies with sufficiently severe HIE are needed to explore if such an additive neuroprotective effect of hydrogen to hypothermia could exist, thus paving the way for the design of a clinical study.

CONSENT FOR PUBLICATION

Not applicable.

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CONFLICT OF INTEREST

The author declares no conflict of interest, financial or otherwise.

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