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REVIEW ARTICLE

Molecular Hydrogen as a Neuroprotective Agent

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Abstract: Oxidative stress and neuroinflammation cause many neurological disorders. Recently, it has been reported that molecular hydrogen (H_2) functions as an antioxidant and anti-inflammatory agent. The routes of H_2 administration in animal model and human clinical studies are roughly classified into three types, inhalation of H_2 gas, drinking H_2-dissolved water, and injection of H_2-dissolved saline. This review discusses some of the remarkable progress that has been made in the research of H_2 use for neurological disorders, such as cerebrovascular diseases, neurodegenerative disorders, and neonatal brain disorders. Although most neurological disorders are currently incurable, these studies suggest the clinical potential of H_2 administration for their prevention, treatment, and mitigation. Several of the potential effectors of H_2 will also be discussed, including cell signaling molecules and hormones that are responsible for preventing oxidative stress and inflammation. Nevertheless, further investigation will be required to determine the direct target molecule of H_2.

Keywords: Brain injury, cerebrovascular disease, inflammation, molecular hydrogen, neonatal brain disorder, neurodegenerative disorder, oxidative stress.

1. INTRODUCTION

Oxidative stress and neuroinflammation are known to cause neurological disorders such as those following brain injury, neurodegenerative disorders, and other diseases [1]. Oxidative stress is derived from reactive oxygen species (ROS), such as superoxide anion radical ($O_2^{-}\cdot$), hydrogen peroxide (H_2O_2), nitric oxide, and hydroxyl radical ($OH\cdot$). Recently, it was reported that molecular hydrogen (H_2) selectively reduces the highly toxic ROS $OH\cdot$ and peroxynitrite, but not $O_2^{-}\cdot$, H_2O_2, or nitric oxide. Moreover, inhalation of H_2 gas markedly suppresses ischemia-reperfusion injury (IRI) of the brain by buffering oxidative stress [2]. Subsequent studies further reported the protective effect of H_2 on both heart and liver IRI [3, 4]. The results of these studies show that H_2 has the potential to be a more efficacious antioxidant therapy than conventional choices. Because H_2 rapidly diffuses across cell membranes, it can reach and react with cytotoxic ROS. Indeed, the therapeutic efficacy of H_2 against oxidative damage has been reported in a number of studies since 2007 [5, 6]. Many studies have also shown that H_2 suppresses inflammation in animal models of disease induced by lipopolysaccharide (LPS) [7-9], concanavalin A [10], dextran sodium sulfate [11], or others [5], with decreases in the levels of pro-inflammatory cytokines, such as tumor necrosis factor (TNF)-α, interleukin (IL)-1β, and IL-6. However, many of the anti-inflammatory efficacies and other therapeutic effects of H_2 cannot be completely explained by its ROS scavenging capacity.

Most $O_2^{-}\cdot$ is generated in mitochondria and converted into H_2O_2 by superoxide dismutase. The detoxifying enzymes glutathione peroxidase and catalase convert H_2O_2 into H_2O. ROS plays physiologically important roles in signal transduction cascades and biological processes, such as apoptosis, cell proliferation, and differentiation [12]. Nitric oxide functions as a neurotransmitter for dilation of blood vessels [13]. However, $OH\cdot$, the strongest ROS, reacts indiscriminately with nucleic acids, lipids, and proteins [14]. The $OH\cdot$ is generated by the Fenton reaction [15] and becomes a major cause of the oxidation and destruction of molecules by direct reactions or by triggering the chain reaction of free radicals [16]. Thus, antioxidant therapy only for $OH\cdot$-dependent injuries is required to cure the diseases derived from oxidative stress, indicating that H_2 is an ideal reductant for this purpose. The brain is more vulnerable to oxidative stress than are other organs. Antioxidants are relatively low in the brain despite its high oxygen consumption (20% of the total at rest) [17, 18]. Moreover, neurons are characterized as postmitotic cells and thus accumulate oxidative damage over the years. Indeed, ROS is suspected to be important in neurodegenerative diseases such as Parkinson's disease (PD), Alzheimer's disease (AD), Huntington's disease, and multiple sclerosis. ROS-induced mitochondrial damage in particular is associated with the onset of PD, AD, and other neurodegenerative diseases [19].

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Neuroinflammation is initiated in response to a variety of physiological neuronal changes, such as those brought about by infection, traumatic brain injury, toxic metabolites, and autoimmunity [20]. Immune responses cause widespread neuroinflammation, whereas they are also a major means to protect the central nervous system (CNS) from neural disorders. Microglia, resident innate immune cells in the CNS, are activated in response to these physiological changes and generate cytokines and chemokines. Although the CNS has been believed to be an immune-privileged site protected by the blood-brain barrier (BBB) [21], some cytokines and chemokines, such as IL-6, TNF-α, and chemokine (C-C motif) ligand 2 (CCL2), are known to be transport across the BBB, from the blood into the brain [22]. Moreover, recent studies have shown that circulating peripheral immune cells called macrophages invade the CNS [23, 24]. The crosstalk between the signaling cascades underlying oxidative stress and the inflammatory responses may be a critical factor in neurodegenerative disorders [25, 26]. Thus, both the neuroimmunological interactions and crosstalk with oxidative stress need to be considered when contemplating mechanisms for the anti-inflammatory effects of H_2 in the brain.

2. ROUTES OF HYDROGEN ADMINISTRATION

The routes by which H_2 can be administered or taken into the body are numerous. They are roughly classified into three types: inhalation of H_2 gas, drinking H_2-dissolved water (HW), and injection of H_2-dissolved saline (HS). Administration of H_2 varies depending on the disease [6].

Inhalation of H_2 gas is the simplest method and has been widely used since the first report [2, 6]. Inhaled H_2 diffuses into the alveoli of the lung and is transport throughout the body in the blood. However, this route of administration can be inconvenient and even dangerous because H_2 gas is explosive when the concentration in air is greater than 4%. Thus, the concentration of H_2 in mixed gas is usually maintained between 1% and 4%. Inhalation of H_2 gas ameliorates acute diseases such as IRI and graft injury of several organs.

Drinking water saturated with H_2 (1.6 ppm) is safer and more convenient than inhaling H_2 gas. The first report showed that ad libitum drinking of HW prevents arteriosclerosis in apolipoprotein E knockout mice, a model of the spontaneous development of atherosclerosis. The oxidative stress in the aorta is decreased [27]. Nagata *et al*. reported that continuous drinking of HW ameliorates the stress-induced impairments in learning tasks during chronic physical restraint in mice [28]. Many studies have shown that drinking HW can reduce oxidative stress in several organs, including the brain [5, 6], indicating that HW is useful in daily life to prevent or minimize the risk associated with lifestyle. However, the efficacy of HW administration is different from that of H_2 gas. A recent study has shown that inhaling H_2 gas and drinking HW differentially regulate signaling pathways and gene expression in mice [29].

Injection of HS is also safe, without risk of explosion, and allows for the direct application of HS to the affected area. Although the method is invasive, intraperitoneal injection of HS has shown neuroprotective efficacy against IRI in the brain similar to that following inhalation of H_2 gas [30].

Additionally, utilization of hydrogen-producing bacteria in the gut may be effective. For example, ingestion of lactulose produces a marked increase in the amount of H_2 gas through fermentation by the bacteria in the gastrointestinal tract [31].

3. CEREBROVASCULAR DISEASES

IRI and subarachnoid hemorrhage (SAH) cause severe cerebrovascular diseases through oxidative stress and inflammation [32]. Many studies suggest that administration of H_2 is efficacious in the treatments of cerebrovascular diseases.

Globally, age-standard death rate of ischemic stroke is 57.3 per 100,000 persons in 2013 [33]. However, reperfusion to the ischemic brain region causes additional damage that can be more harmful than the ischemic stroke [34]. The IRI in the brain is a serious medical problem. To date, reports concerning the therapeutic efficacy of H_2 for IRI are the most common among all the H_2 studies [6]. Ohsawa *et al*. were the first to report that inhalation of H_2 gas markedly suppresses rat brain injury induced by focal ischemia-reperfusion by buffering oxidative stress, as assessed by the accumulation of its markers 8-hydroxyl-2′-deoxyguanosine (8-OHdG) and 4-hydroxynonenal (4-HNE) [2]. Surprisingly, treatment with H_2 gas is more effective than that with edaravone, which is an ROS scavenger and used in the treatment of cerebral infarction [35, 36]. Nagatani *et al*. reported that inhalation of H_2 gas improves the survival rate of mice from 8.3% to 50% following global cerebral IRI by bilateral common carotid artery occlusion [37]. These benefits of H_2 are associated with significantly lower levels of 8-OHdG, 4-HNE, and malondialdehyde in brain tissue. The administration of H_2 gas attenuates brain edema with less extravasation of serum albumin in the striatum and parietal cortex, indicating that disruption of the BBB is suppressed by H_2. However, Huang *et al*. reported that systemic intraperitoneal injection of HS improves both 72 hour survival rates and neurological scores, reduces neuronal injury, and inhibits neuronal apoptosis in a rabbit model of cardiac arrest [38]. Administration of HS also reduces 8-OHdG and malondialdehyde both in plasma and hippocampal tissues, with an increase in superoxide dismutase and catalase activities. Importantly, injection of HS significantly reduces the increase in the inflammatory cytokines IL-1β and TNF-α in a rat model of IRI [30]. Acute neuronal death during IRI has been attributed to the loss of mitochondrial permeability transition coupled with mitochondrial dysfunction [39]. Injection of HS reduces not only tissue damage but also the degree of mitochondrial swelling and the loss of the mitochondrial membrane potential [40]. Clinical studies have indicated that intravenous administration of an H_2-enriched solution is safe for patients with acute cerebral infarction, including patients treated with tissue plasminogen activator [41], and combined administration of edaravone and HS in patients with acute brainstem infarction improves MRI indices [42].

SAH is a severe disease that is associated with significant morbidity and mortality. The mortality is approximately 50%, with 30% of survivors having significant morbidity. Moreover, autopsy studies have shown that approximately 5% of the patients harbored intracranial aneurisms and 10 per 100,000 had aneurismal SAH [32]. In a rat model of SAH, inhalation of H_2 gas palliates brain edema and BBB disruption, reduces neuronal apoptosis, and improves neurologic function [43]. These effects are associated with the amelioration of oxidative injury. Moreover, injection of HS attenuates SAH-induced brain injury [44-46]. Shao *et al*. reported that HS improves the neurobehavioral outcome after SAH, in part *via* inactivation of the inflammation pathways, including the nuclear factor-kappa B (NF-κB) pathway and the nucleotide-binding domain, leucine-rich repeat-containing receptor family, pyrin domain-containing 3 inflammasome [44].

4. NEURODEGENERATIVE DISORDERS

Aging is associated with an increase in the incidence of neurodegenerative disorders, creating a large problem that needs to be solved in our aging society. Oxidative stress and inflammation are leading causes of PD [47-49], AD [50, 51], and other neurodegenerative disorders [52]. Many studies indicate that H_2 treatment is potentially useful for alleviating neurodegenerative disorders and improving the quality of life in senior people.

Pathological changes of dopaminergic neurons in the substantia nigra and nigrostriatal pathway are characteristic of PD and mainly disturb motor systems. The substantia nigra in PD exhibits increased oxidized proteins, lipids, and DNA, and decreased endogenous antioxidants, such as glutathione, whereas effective antioxidant therapy has not been established. The incidence of PD rises steeply with age, from 17.4 per 100,000 person-years between 50 and 59 years of age to 93.1 per 100,000 person-years between 70 and 79 years, with a lifetime risk of developing the disease of 1.5%. The neurotoxins 6-hydroxydopamine (6-OHDA) and 1-methyl-4-phenyl-1,2,3,6-tetrahydropyrine (MPTP) destroy neurons by generating ROS and are used to generate animal models of PD [53, 54]. Fu *et al*. reported that drinking 50%-saturated HW before or after injection of 6-OHDA by stereotactic surgery attenuates nigrostriatal degeneration in a rat model of PD [55]. In that study, methamphetamine-induced behavioral analysis and tyrosine hydroxylase staining of the substantia nigra and striatum revealed that HW significantly prevents the reduction of dopaminergic neurons. Moreover, Fujita *et al*. reported that drinking HW suppresses the loss of dopaminergic neurons in a mouse model of PD using both acute and chronic administration of MPTP. The MPTP-induced accumulation of 8-OHdG and 4-hydroxynonenal is decreased in the nigrostriatal pathway of HW-drinking mice, whereas production of $O_2^{-}\cdot$, which was detected by intra-vascular injection of dihydroethidium, is not reduced [56]. Recently, a randomized double-blind placebo-controlled pilot study was conducted to investigate the therapeutic efficacy of HW in patients with PD [57]. Participants drank 1 liter per day of HW or pseudo-water for 48 weeks. The Total Unified Parkinson's Disease Rating Scale scores in the HW group improved, whereas scores in the placebo group worsened.

Aging is recognized as being the principal risk factor for AD. Accumulation of misfolded proteins, such as amyloid β and tau, in the aging brain results in oxidative and inflammatory damage, which in turn leads to energy failure and synaptic dysfunction. The incidence of the disease doubles every 5 years after the age of 65 years, with a diagnosis of 1,275 per 100,000 person-years in those over 65. The odds of receiving a diagnosis of AD after the age of 85 years exceeds one in three [58]. Sun's laboratory reported that intracerebroventricular injection of HS prevents amyloid β-induced neuroinflammation and oxidative stress, which may contribute to the improvement of memory dysfunction in a rat model. After inoculation with amyloid β in the brain, intracerebroventricular injection of HS improved learning and memory, enhanced long-term potentiation *in vivo*, prevented an increase in oxidative stress (malondialdehyde and 8-OHdG) and inflammatory cytokines (IL-6, TNF-α and IL-1β), and suppressed the activation of c-Jun N-terminal kinase and NF-κB [59, 60].

5. NEONATAL BRAIN DISORDERS

Brain disorders in neonates and infants are an important cause of mortality and morbidity, and contribute to the onset of autism, cerebral palsy, mental retardation, and a variety of other neurological and cognitive disabilities. Perinatal asphyxia is a significant cause of brain injury, leading on to neonatal encephalopathy [61]. Even in countries with low neonatal mortality rates (5 < 1000 births), the incidence of neonatal encephalopathy is 1.6 per 1000 births [62]. Thus, it is important to seek suitable therapies that can increase survival and attenuate the neurological deficits. Inflammation and oxidative stress are the major causes of hypoxia-ischemia injury through neuronal apoptosis [63]. Cai *et al*. reported that inhalation of H_2 gas reduces neuronal apoptosis in rat model of neonatal hypoxia-ischemia [64]. They further demonstrated that abnormal behavior at 5 weeks after hypoxia-ischemia is improved by administration of HS [65]. Domoki *et al*. reported that H_2 gas reduces neuronal injury induced by hypoxia-ischemia-reventilation in the cerebral cortex, hippocampus, basal ganglia, and cerebellum of newborn pigs [66]. They further demonstrated that the inhalation of H_2 gas extends the survival period after asphyxia from 4 h to 24 h in newborn pig model, emphasizing the translational potential of H_2 gas [67]. Many studies in laboratory animals have shown that the immature brain responds differently to treatment than does the mature brain. However, administration of H_2 to neonates with ischemic brain injury is highly effective for improving the prognosis. Moreover, Mano *et al*. reported that maternal administration of HW improves hippocampal damage caused by in utero IRI at day 7 after birth *via* reducing 4-hydro-xynonenal and 8-OHdG [68].

Recent findings indicate that administration of anesthetics to immature brains causes pathological changes and long-term behavioral abnormalities [69-71]. Thus, the US Food and Drug Administration launched The Safety of Key Inhaled and Intravenous Drugs in Pediatrics (SAFEKIDS) initiative to promote academic and clinical research [72, 73]. A fundamental study showed that exposure to sevoflurane induces abnormal social behaviors resembling autism

spectrum in mice [74]. However, no efficacious treatment has been found. Yonamine *et al*. reported that co-administration of H_2 gas with sevoflurane in neonatal mice suppresses the increase in oxidative stress, neuronal apoptosis, and subsequent neurobehavioral deficits caused by the exposure to sevoflurane [75]. Moreover, co-administration of H_2 gas prevents the abnormal maternal behavior later in adulthood that was derived from neonatal exposure to sevoflurane [76], suggesting a remarkable potential of H_2 gas for reducing adverse effects caused by anesthetic exposure.

Fetal inflammatory response syndrome (FIRS) is the fetal counterpart of the systemic inflammatory response syndrome. FIRS causes severe brain injury through neuroinflammation [77]. Recently, Nakano *et al*. reported that maternal administration of HW ameliorates mouse fetal brain injury induced with maternal exposure to LPS. Expression of pro-inflammatory cytokines, oxidative damage, and microglial activation caused by intrauterine inflammation are suppressed by HW [78, 79]. These data suggest that prenatal administration of H_2 can be an effective therapeutic approach for FIRS.

6. OTHER NEUROLOGICAL DISORDERS

Retinal ischemia is a common cause of visual impairment and blindness. At the cellular level, ischemic retinal injury consists of a self-reinforcing destructive cascade involving neuronal depolarization, calcium influx, and oxidative stress initiated by energy failure and increased glutamatergic stimulation [80]. Oharazawa *et al*. reported that HS eye drops ameliorate the rat retinal IRI that was induced by raising intraocular pressure [81]. Topical HS eye drops were continuously administered during the ischemia-reperfusion periods, and they found that the drops suppress the increase in $OH\cdot$, reduce the number of retinal apoptotic and oxidative stress marker-positive cells, and prevent retinal thinning with the accompanying activation of Müller glia, astrocytes, and microglia.

Kikkawa *et al*. reported that H_2 protects against antimycin A- and cisplatin-induced oxidative stress in explant cultures of auditory tissues [82, 83], suggesting that H_2 can protect against drug-induced inner ear damage. Noise-induced hearing loss occurs by transmission of too much sound intensity *via* the auditory system. When the ear is exposed to loud sounds for long time, the overstimulation of the hair cells leads to production of ROS and causes oxidative cell death [84]. A recent study has shown that intraperitoneal injection of HS protects against noise-induced hearing loss in guinea pigs. H_2 ameliorates destruction of hair cells in part by reducing the generation of ROS [85].

Traumatic brain injury (TBI) and spinal cord injury cause the majority of death and disability, especially in low- and middle-income countries. The incidence of CNS injuries varies between regions, with estimates of 200–600 injuries per 100,000 people [86]. Ji *et al*. reported that administration of H_2 protects against neuronal cell death in an animal model of TBI. Inhalation of H_2 gas suppresses the increase in oxidative products and enhances the enzymatic activities of endogenous antioxidants (superoxide dismutase and catalase) in brain tissue, leading to the protective efficacy of H_2 treatment in a rat model of TBI [87]. Injection of HS was also shown to be effective against TBI *via* reducing oxidative stress [88]. Dohi *et al*. reported that drinking HW inhibits the edema induced by TBI and completely blocks pathological tau expression in mice [89]. Moreover, it was reported that inhaling H_2 gas and drinking HW protected against spinal cord IRI in rabbits, with a decrease observed in oxidative products and inflammatory cytokines and an increase in endogenous antioxidant enzymatic activities [90, 91]. Similar results have been reported in a rat model of spinal cord IRI, in which injection of HS suppresses reactive astrogliosis and improves locomotor functions [92, 93].

In addition, H_2 treatments were reported to protect against sepsis and LPS-induced brain inflammation and carbon monoxide intoxication in rodents [94-96].

7. MOLECULAR MECHANISMS OF H_2 EFFICACY

Several lines of evidence indicate that H_2 directly reduces highly reactive ROS at the affected area in acute diseases. Detectable amounts of H_2 can be supplied to the brain by both inhalation of H_2 gas and injection of HS. By contrast, after drinking HW, the H_2 concentration in the rodent brain is too low to be detected using a conventional hydrogen sensor. Curiously, drinking HW is more effective than inhaling H_2 gas in an animal model of PD [97]. Recently, Matsumoto *et al*. reported that drinking HW increases gastric expression and secretion of ghrelin in a mouse model of PD. Interestingly, the neuroprotective efficacy of HW is abolished by an antagonist of the ghrelin receptor, the growth hormone secretagogue receptor (GHSR), and by a ghrelin-secretion antagonist [98]. Ghrelin is a hormone that was discovered based on its ability to stimulate growth hormone release and food intake, and GHSRs are expressed in dopaminergic neurons of the substantia nigra. Ghrelin exerts neuroprotective efficacy in PD by inhibiting the neuroinflammation derived from microglia [99]. Based on these results, one can speculate that higher concentration of H_2 in HW acts directly on ghrelin-producing gastric cells and regulates secretion of ghrelin through intracellular signaling.

Sato *et al*. reported previously that administration of HW prevents $O_2^{-}\cdot$ formation in brain slices of vitamin C-depleted senescence marker protein-30/gluconolactonase knockout mice, which cannot synthesize vitamin C [100]. After these knockout mice drank HW for 33 days, $O_2^{-}\cdot$ formation during hypoxia-reoxygenation treatment of live brain slices from the mice was estimated using a real-time biography imaging system with a chemiluminescence probe for $O_2^{-}\cdot$. They found that $O_2^{-}\cdot$ formation is 27.2% lower in slices from H_2-treated mice than in control slices. Because no trace amount of H_2 existed in the slice, H_2 does not reduce ROS directly; however, drinking HW may trigger a protective adaptive response in the brain against oxidative stress.

Kawamura *et al*. reported that inhalation of H_2 gas during exposure to hyperoxia improves blood oxygenation, reduces inflammation, and induces heme oxygenase-1 (HO-1) expression in the lung [101]. HO-1 enzymatically functions in heme catabolism to yield carbon monoxide, free ferrous

ions, and biliverdin, and its transcription is regulated by nuclear factor erythroid 2-related factor 2 (Nrf2). Therefore, HO-1 participates in the cell defense against oxidative stress, and it has been speculated that HO-1 may be a therapeutic target for neuroprotection. Mutations of HO-1 have been associated with high risk for AD and PD. Moreover, several studies have shown that HO-1 and its enzymatic products are associated with ischemic brain injury [25]. In Nrf2-knockout mice, inhalation of H_2 gas does not improve hyperoxic lung injury and does not induce the expression of HO-1 [101]. Moreover, it was reported that the H_2 gas generated from bacteria in the intestine and the administration of HS ameliorates IRI by activating Nrf2 in rats [31].

A recent study has shown that drinking HW prevents LPS or cytokine-induced generation of ROS by microglia, and reduces LPS-induced microglial neurotoxicity [79]. In most inflammatory disease models, the administered H_2 functions as an anti-inflammatory by decreasing the expression of pro-inflammatory factors, such as NF-κB [102], TNF-α, IL-1β, IL-6, IL-10, IL-12, CCL2, interferon-γ, and intercellular adhesion molecule-1 [5, 103]. In the brain, neuroinflammation is likely induced by microglia. Activated microglia generate pro-inflammatory cytokines and ROS [104]. The authors of a previous report speculated that HO-1 and its product carbon monoxide suppress microglia activity and the generation of inflammatory cytokines and ROS [25].

At present, we consider the crosstalk between anti-oxidative stress pathways, such as Nrf2–HO-1, and the anti-inflammatory response to be the most important molecular mechanism for the protective function of H_2 and that regulating microglia is a key mechanism for H_2 efficacy in the brain. Recently, Iuchi *et al.* demonstrated that low concentrations (approximately 1%, v/v) of H_2 modulate Ca^{2+} signal transduction and regulate gene expression by modifying the production of oxidized phospholipid species [105]. Because H_2 is the smallest as well as a nonpolar molecule, it is unlikely that H_2 could bind to some protein mediators. Further investigation will be required to determine the direct target molecule of H_2.

CONCLUSION

Although many neurological disorders are currently incurable, numerous studies suggest the clinical potential of H_2 administration for the prevention, treatment, and mitigation of these disorders. To our knowledge, no adverse effects of H_2 have been reported, and H_2 is relatively easy to use, inexpensive, and effective in daily medical practice. However, the optimal route and dose of H_2 administration for each disease remains to be established. To do so, elucidation of the molecular mechanisms underlying the biological effects of H_2 will be essential for developing H_2 as an effective neuroprotective medicine.

CONFLICT OF INTEREST

The authors confirm that this article content has no conflict of interest.

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