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- oxidative injury

Summary

1. Hydrogen is a colourless, odourless, tasteless and flammable gas. Hydrogen is considered a physiologically inert gas and is often used in deep sea diving medicine. In mammals, endogenous hydrogen is produced as a result of the fermentation of non-digestible carbohydrates by intestinal bacteria and it is absorbed into the systemic circulation.

2. Recent evidence indicates that and so may have potential medical application. The present review evaluates the concept of 'hydrogen resuscitation', based on knowledge that hydrogen treatment effectively protects cells, tissues and organs against oxidative injury and helps them recover from dysfunction.

3. Hydrogen therapy can be delivered by inhalation, the administration of hydrogen-enriched fluid or by approaches that affect endogenous hydrogen production.

4. Studies have shown that hydrogen resuscitation has cytoprotective effects in different cell types and disease models, including ischaemia–reperfusion injury, inflammation, toxicity, trauma and metabolic disease. The underlying mechanism may be the selective elimination of hydroxyl radicals, although other mechanisms may also be involved (e.g. hydrogen functioning as a gaseous signalling molecule).

5. Hydrogen resuscitation may have several potential advantages over current pharmacological therapies for oxidative injuries. However, more work is needed to identify the precise mechanism underlying the actions of hydrogen and to validate its therapeutic potential in the clinical setting.

Introduction

Hydrogen is the simplest and most abundant natural chemical element, constituting approximately 75% of the universal elemental mass. It is colourless, odourless, tasteless and highly combustible under standard temperatures and pressures. Hydrogen is a novel energy source and recent attention has focused on hydrogen as an energy storage medium that burns in a less-polluting way than fossil fuels. In the field of biological medicine, hydrogen has been considered a physiologically inert gas and it is often used in diving medicine. However, recent studies have demonstrated that **hydrogen is also a potent anti-oxidative and anti-inflammatory agent with potential for medical application, particularly given that hydrogen treatment effectively protects cells, tissues and organs against oxidative injury.**[1]

Hydrogen Resuscitation

It is generally accepted that hydrogen is a gas with reducing reactivity. In radiochemistry, hydrogen reacts directly with hydroxyl radicals. However, in the field of biological medicine, hydrogen has always been considered to be physiologically inert, like nitrogen, and not to react with anything in the human body under physiological conditions. Kayar *et al.*[2] reported that mammalian tissues did not oxidize hydrogen even under hyperbaric conditions. Thus, hydrogen is often included in the gas mixtures used by divers. For example, high concentrations of hydrogen are present in Hydreliox, a mixture of hydrogen (49%), helium (50%) and oxygen (1%), for very deep diving ventures because it shortens decompression time, prevents decompression sickness and averts

nitrogen narcosis.[3] A mixture of hydrogen, helium and oxygen was used in the deepest recorded diving (701 m).[4,5]

In July 2007, Japanese researchers published a paper in *Nature Medicine* reporting that molecular hydrogen selectively reduced the levels of hydroxyl radicals (·OH) *in vitro* and that the hydrogen molecules exerted a therapeutic anti-oxidant activity in a rat model of middle cerebral artery occlusion (MCAO);[6] this report aroused considerable interest worldwide. Since the publication of this report of the anti-oxidant effect of hydrogen under normal pressures in a model of cerebral ischaemia–reperfusion (I/R) injury, scientists have explored the therapeutic value of hydrogen in various disease models. Accumulating evidence suggests that hydrogen can protect various cells, tissues and organs against oxidative injury.[1]
In fact, molecular hydrogen is produced constantly in the human body under physiological conditions, especially during fermentation of non-digestible carbohydrates, by intestinal bacteria in the large intestine.[7] This physiological production of hydrogen gas may be responsible for the base levels of circulating hydrogen detected in mammals.[8] Hydrogen is excreted as flatus, further metabolized by the gut flora or is exhaled as a natural component of abdominal gas.[9]

The finding of the anti-oxidant effect of hydrogen and its therapeutic value led us to suggest that endogenous hydrogen may play an important physiological role in maintaining homeostasis. Hydrogen may scavenge or mitigate excessive free radicals and maintain them at basal physiological levels, like most other endogenous anti-oxidants. High levels of free radicals are produced in the body under ischaemic, inflammatory or other pathological conditions, resulting in oxidative damage to cells and tissues. The anti-oxidant capacity of tissues is relatively low under these conditions and hydrogen treatment may increase this capacity, balancing out the oxidation state and thus protecting cells, tissues and organs against oxidative injury and restoring physiological function. We refer to this new cytoprotective approach of using hydrogen treatment to restore anti-oxidant capacity and function as 'hydrogen resuscitation'.

Approaches for Hydrogen Resuscitation Therapy

Delivery of exogenous hydrogen

Hydrogen can be delivered via inhalation of gas. There is evidence that inhaled hydrogen gas has anti-oxidant and anti-apoptotic properties to protect organs against I/R-induced injury.[6] Inhalation of hydrogen acts rapidly, because hydrogen diffuses immediately into tissues. Furthermore, the therapeutic value of hydrogen gas has been proved in other animal models of various diseases (see below). Hydrogen can be delivered to the lungs by a ventilator circuit, facemask or nasal cannula.[1]

However, hydrogen gas is highly flammable and burns in the air over a wide concentration range (4–75% by volume in air). Hydrogen gas mixtures can be detonated by sparks, heat or sunlight and hydrogen gas leaking into the air may ignite spontaneously. Moreover, extremely hot hydrogen fire is almost invisible, which may result in accidental burns. Although the concentration of gaseous molecular hydrogen used in the studies (approximately 4%)[6] is lower than the threshold at which it is known to be flammable in air (4.6%), the safety of inhaled molecular hydrogen remains a concern that may limit its use. Inhaled molecular hydrogen is not practical for daily use, nor is it suitable for the continuous administration of hydrogen gas.

The use of hydrogen-rich water is a clearly more convenient method for the delivery of molecular hydrogen. Hydrogen-rich water can be made relatively easily and safely. A Japanese group has reported making hydrogen water by dissolving electrolysed hydrogen into pure water (hydrogen-bubbled water),[10] whereas an American group used an electrochemical reaction between magnesium and water (hydrogen/Mg water) to produce hydrogen water (chemical reaction: $Mg + 2H_2O \rightarrow Mg(OH)_2 + H_2$).[11] The primary advantages of using hydrogen-rich water as a means of is delivering molecular hydrogen are that it is portable, easily administered and safe. In addition, the concentration of hydrogen required in the water is not necessarily high: it has been reported

that concentrations as low as 0.08 p.p.m. hydrogen in water have almost the same effect as saturated hydrogen water (1.5 p.p.m.).[12]

We have developed hydrogen-saturated saline, which can be administered intravascularly[13] or intraperitoneally.[14] Using this route of administration is more likely to deliver accurate concentrations of hydrogen than drinking hydrogen-rich water because some of the hydrogen may be lost in the stomach or intestine after ingestion.

Another method of delivering hydrogen is to use electrolysed-reduced water (ERW), which has a high pH, low dissolved oxygen content, extremely high dissolved molecular hydrogen content and an extremely negative redox potential.[15] This form is considered 'active hydrogen' because it has been demonstrated that ERW scavenges active oxygen species[16] and protects the DNA, RNA and proteins from oxidative damage.[17] In addition, ERW has been reported to have beneficial effects in haemodialysis,[18] diabetes,[19,20] against tumour angiogenesis,[21] liver injury[22] and infection,[23] and following alcohol consumption.[24]
Recently, Ueda *et al.*[25] reported that hydrogen could be generated from coral calcium hydride (CCH) solution. The CCH solution may exert its anti-oxidant effect by significantly enhancing the basal endogenous anti-oxidant capacity of the hippocampus via a synergistic effect with α-tocopherol and ascorbic acid.[25] Thus, using CCH solution may be an easy way by which to activate the brain anti-oxidant system and, at the same time, a safe way to increase hydrogen gas in the central nervous system (CNS).[25]

With regard to hydrogen concentrations in the human body, it has been reported that the hydrogen content of arterial blood increases following the inhalation of hydrogen in proportion to the concentration inhaled (over the range 0–4%) together with O_2 and N_2O. The amount of hydrogen dissolved in venous blood was less than that in artery blood, suggesting that hydrogen is incorporated into tissues.[6] Inhalation of 1% hydrogen gas was sufficient to protect organs against injury,[6,26] under which conditions the hydrogen level in the blood should be 8 μmol/L because the saturated level of hydrogen reached 800 μmol/L under atmospheric pressure. The continuous consumption of hydrogen water may have an effect even at much lower concentrations than 8 μmol/L because continuous exposure to hydrogen may change blood components towards the reductive state, indirectly influencing the oxidative state in tissues. It has been reported that the blood concentration of hydrogen in mice after ingestion of saturated hydrogen water was 5 μmol/L.[27,28] However, changes in hydrogen concentration were not detected by another group after instillation of saturated hydrogen water into the stomach of anaesthetized rats or in free-moving rats that were given hydrogen water to drink.[12] The apparent discrepancy between these studies may be due to technical problems associated with the detection of low levels of hydrogen.

Use of endogenous hydrogen
Current research seeks to clarify the effect of endogenous hydrogen and to make use of it. Physiologically, approximately 150 mL hydrogen gas is produced daily by numerous strains of intestinal bacteria, primarily in the large intestine.[9] The hydrogen gas produced is then excreted as a natural component of abdominal gas, exhaled or further metabolized by colonic flora. Hydrogen is usually produced as a product of anaerobic metabolism via reactions catalysed by iron- or nickel-containing enzymes called hydrogenases.[8] When unabsorbed carbohydrate enters the colon, it is rapidly fermented by anaerobic colonic bacteria to produce short-chain fatty acids, liberating carbon dioxide, hydrogen and methane, which is increasingly considered to have biological effects on gut function.[29] The highest levels of hydrogen occur in the caecum, followed by the small intestine, large intestine, liver, spleen and blood. Only trace levels of hydrogen are detectable in the brain.[30] It has been reported that hydrogen concentrations in the mouse stomach and liver are in the range 20–80 μmol/L.[31]

Antibiotic eradication of bacterial overgrowth in patients with irritable bowel syndrome (IBS) results in symptomatic relief from diarrhoea, suggesting that it is the hydrogen-producing bacteria that induce hypercontractility.[32] The ability of the colonic flora to metabolize hydrogen was considered important for the prevention of untoward effects in divers using Hydreliox.[33] It is widely accepted that breath concentrations of hydrogen reflect carbohydrate fermentation in the colon. Measurement of breath hydrogen can be used as an index of small bowel transit time, colonic fermentation, abnormal fermentation, galactose and/or lactose intolerance, as well as sometimes IBS.[34,35]

Because most mammals lack catabolic enzymes to generate hydrogen, intestinal bacteria are the only possible source of hydrogen in the body. Thus, exogenous factors, such as antibiotics, may affect the functional amount of hydrogen in the body, making the organism susceptible to disease. A study by Kajiya *et al.*[30] indicated that systemic treatment of mice with antibiotics (sulphamethoxazole and trimethoprim) significantly decreased the amount of hydrogen detected in all organs tested. Cultures of fresh faecal matter sampled from mice treated with antibiotics also revealed significantly lower hydrogen production compared with samples collected from control (untreated) mice.[30]

Suppression of intestinal bacterial flora by antibiotics increased the severity of concanavalin A (ConA)-induced hepatitis, whereas reconstitution of intestinal flora with hydrogen-producing *Escherichia coli*, but not hydrogen-deficient mutant *E. coli*, downregulated ConA-induced liver inflammation.[30] However, the anti-inflammatory effect of hydrogen produced by intestinal bacteria was lower than that observed following the ingestion of hydrogen water.[30] This relatively low potency of endogenous hydrogen may be most plausibly attributed to the scavenging of the hydrogen by other bacteria present deep inside the intestinal mucosa or the stomach, such as *Helicobacter hepaticus*, which has been reported to consume significant amounts of hydrogen.[36] In addition, compared with exogenous hydrogen supplementation, endoluminal administration of hydrogen-producing bacteria runs the risk of bacterial overgrowth, causing infectious enteritis.

Suzuki *et al.*[37] reported that acarbose, an α-glucosidase inhibitor, significantly increased hydrogen production, which was tested in healthy volunteers. The cardiovascular benefits of α-glucosidase inhibitors may be due, in part, to their ability to neutralize oxidative stress by increasing hydrogen production in the gastrointestinal tract. Compared with curry that does not contain turmeric, turmeric-containing curry was demonstrated to significantly increase the area under the curve of breath hydrogen and to shorten small bowel transit time.[38] Thus, dietary turmeric may activate carbohydrate colonic fermentation, elevate hydrogen production and enhance bowel motility. The ability of certain foods to enhance hydrogen production in the colon could be a marker of anti-oxidant stress.[38]

A study in five subjects without specific diseases compared the effects of the ingestion of hydrogen water and milk on breath hydrogen. It was found that the ingestion of hydrogen water resulted in a rapid, dose-dependent increase in breath hydrogen, but the rise was not sustained compared with that following the ingestion of milk.[39] Other nutrients have also been observed to impact on hydrogen production (e.g. the ingestion of beans improves hydrogen production), which may be considered a 'food therapy' approach.[40] Liu *et al.*[41] speculated that oral administration of mannitol may be an effective treatment against I/R injury because bacteria in the large intestinal could produce endogenous hydrogen and preliminary experiments had revealed that oral administration of mannitol in humans and animals significantly increased levels of endogenous hydrogen. However, there is considerable progress to be made before a healthy and effective approach is developed to make full use of endogenous hydrogen.

Effects of Hydrogen Resuscitation on Various Diseases

Before the reports in *Nature Medicine* in 2007,[6] some scientists had identified an anti-oxidant effect of hydrogen under hyperbaric conditions. For example, Dole *et al.*[42] reported in *Science* in 1975 that hyperbaric hydrogen treatment resulted in marked aggression of skin tumours in mice. In a later study in 2001, Gharib *et al.*[43] observed that animals maintained in a hydrogen-supplemented hyperbaric chamber were significantly protected from schistosomiasis-associated chronic liver injury, as evidenced by decreased fibrosis, improved haemodynamics, increased nitric oxide synthase II activity, increased anti-oxidant enzyme activity, decreased lipid peroxide levels and decreased circulating tumour necrosis factor (TNF)-α levels. However, these studies did not attract much attention from biologists, probably because the application of hyperbaric hydrogen to clinical medicine was difficult.

Hydrogen resuscitation and I/R injury

In 2007, the results of a study from the Japanese group[6] revealed that hydrogen, at atmospheric pressure, had the potential to ameliorate cellular injury caused by I/R both *in vitro* and *in vivo*. In the *in vivo* experiments, the authors administered four different gas mixtures to four groups of rats subjected to MCAO for 90 min, followed by reperfusion for 30 min; all mixtures contained 30% oxygen and either 0, 1, 2 or 4% hydrogen, with the balance made up by nitrous oxide. Infarct volume was significantly reduced in rats exposed to 2% and 4% hydrogen compared with the other two groups, although, interestingly, the greatest reduction in infarct size was seen in the group receiving 2% hydrogen. Furthermore, hydrogen was effective only when it was administered during reperfusion.[6]

Subsequently, I/R injury in other organs was shown to be alleviated by hydrogen resuscitation. For example, inhalation of 2% hydrogen gas was also found by this group to suppress hepatic injury caused by warm I/R by reducing oxidative stress.[26]

In isolated perfused hearts, hydrogen gas enhanced the recovery of left ventricular function following anoxia–re-oxygenation in rats.[44] Inhalation of 2% hydrogen gas rapidly increased the regional concentration of hydrogen in the area at risk (AAR) of myocardial infarction before coronary blood flow was re-established in the occluded artery and alleviated I/R injury at the time of recanalization of the coronary artery.[44] In addition, we have found that hydrogen-rich saline attenuates myocardial I/R injury.[45] In another study, hydrogen-rich saline treatment attenuated regional myocardial I/R-induced cell apoptosis, as evidenced by significant improvement in heart function parameters.[46] Furthermore, hydrogen-rich saline decreased oxidative stress and inflammation in the AAR in rat hearts.[46] Combination therapy with hydrogen and carbon monoxide in a syngeneic heterotopic heart transplantation model enhanced therapeutic efficacy via both anti-oxidant and anti-inflammatory mechanisms.[47]

Hydrogen-rich saline solution has also been reported to attenuate renal I/R injury in a rodent model.[48] In a rat model of kidney transplantation, Cardinal *et al.*[49] observed that oral administration of molecular hydrogen dissolved in water improved allograft function, slowed the progression of chronic allograft nephropathy and improved overall survival.

Buchholz *et al.*[50] reported that perioperative inhalation of 2% hydrogen increased arterial hydrogen levels by 3.5-old and ameliorated intestinal transplant injury by protecting graft structure and function, as well as by blunting graft and systemic molecular inflammatory responses via anti-oxidant effects. The intravenous administration of hydrogen-rich saline has been shown to protect rats against intestinal I/R injury[13] and hydrogen treatment has been shown to have a protective effect against intestinal contractile dysfunction and damage induced by intestinal I/R by inhibiting I/R-induced oxidative stress and apoptosis, as well as by promoting epithelial cell proliferation.[51] In addition, lung injury induced by intestinal I/R has been shown to be attenuated by hydrogen-rich saline treatment.[52] Kawamura *et al.*[53] found that inhaled hydrogen prevented lung I/R injury and significantly improved the function of lung grafts after extended cold preservation, transplant and reperfusion.

In a model of acute retinal I/R injury, continuous administration of hydrogen-loaded eye drops increased the concentration of hydrogen in the vitreous body, reduced the number of retinal apoptotic and oxidative stress marker-positive cells and reversed retinal thinning, with accompanying activation of Müller glia, astrocytes and microglia.[54]

Hydrogen resuscitation in the CNS

In a neonatal hypoxia–ischaemia rat model, we found that 2% hydrogen gas or hydrogen-rich saline therapy reduced apoptosis.[14,55] However, another group has reported that 2.9% hydrogen gas therapy does not ameliorate moderate-to-severe ischaemic damage in a neonatal hypoxia–ischaemia rat model,[56] although they did find that hydrogen gas reduced the infarction and haemorrhagic transformation and improved neurological functions in a rat model of MCAO.[57] Hyperglycaemia is one of the major factors contributing to haemorrhagic transformation after ischaemic stroke. Chen *et al.*[57] found that the protective effect of hydrogen in the brain is accompanied by a reduction in oxidative stress and blood glucose levels after dextrose injection in rats. In addition, hydrogen-rich pure water has been reported to prevent superoxide formation in brain slices of vitamin C-depleted SMP30/GNL-knockout mice during hypoxia–re-oxygenation.[58] In a model of perinatal asphyxia in newborn pigs, ventilation with 2.1% hydrogen-supplemented room air significantly preserved cerebrovascular reactivity to hypercapnia and reduced neuronal injury induced by asphyxia–reventilation.[59]

Hydrogen resuscitation also protects cells against degeneration and improves brain function. For example, the consumption of hydrogen water prevented stress-induced impairments in hippocampus-dependent learning tasks during chronic physical restraint in mice.[27] In a rat model of Parkinson's disease, half-saturated hydrogen water protected against 6-hydroxydopamine-induced nigrostriatal degeneration and retarded the development and progression of Parkinson's disease.[60] In a 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine mouse model of Parkinson's disease, hydrogen in the drinking water reduced oxidative stress and dopaminergic neuronal loss.[12] Concentration as low as 0.08 p.p.m. hydrogen in water have almost the same effect as saturated (1.5 p.p.m) hydrogen water.[12] In the a rat model of Alzheimer's disease, hydrogen-rich saline prevented β-amyloid-induced neuroinflammation and oxidative stress and improved memory dysfunction.[61] Finally, it has been reported that drinking hydrogen water ameliorated the cognitive impairment in senescence-accelerated mice.[62]

Hydrogen resuscitation and inflammation

The effect of hydrogen resuscitation has been demonstrated in models of hepatitis. First, hydrogen-rich saline was shown to attenuate bile duct ligation-induced liver damage, possibly by reducing inflammation and oxidative stress, as well as by inhibiting the extracellular signal-regulated kinase 1/2 pathway.[63] Second, the administration of hydrogen-producing bacterial or hydrogen water downregulated the ConA-induced mouse liver inflammation. Furthermore, the *in vitro* production of both TNF-α and interferon-γ by ConA-stimulated spleen lymphocytes was significantly inhibited following the introduction of hydrogen.[30] Third, Sun *et al.*[64] found that hydrogen protected against mouse liver injury by d-galactosamine (GalN)/lipopolysaccharide (LPS), CCl_4 and dihydroethidine (DEN) challenge. It also inhibited the processes leading to liver cirrhosis and hepatocyte compensatory proliferation.[64]

Hydrogen-rich saline treatment also significantly attenuates the severity of l-arginine-induced acute pancreatitis in rats by inhibiting oxidative stress, apoptosis and nuclear factor (NF)-κB activation and to promoting acinar cell proliferation.[65] Hydrogen-saturated water has been shown to prevent the development of dextran sodium sulphate-induced colitis in mice, with this effect likely due to suppression by hydrogen of macrophage activation in response to luminal bacterial antigens, such as LPS.[66]

In a mouse model of systematic inflammation, hydrogen resuscitation (inhaltion) significantly improved the survival rate of septic mice in a concentration- and time-dependent

manner.[67] Treatment of mice with 2% hydrogen had beneficial effects on sepsis and sepsis-associated organ damage, as evidenced by decreased levels of oxidative products, increased anti-oxidant enzyme activity and reduced levels of high-mobility group box 1 in serum and tissue.[67] Furthermore, 2% hydrogen treatment has been reported to protect mice against multiple organ damage in a zymosan-induced generalized model of inflammation.[68]

Hydrogen resuscitation and toxicity

Hydrogen resuscitation can attenuate drug- or chemical-induced cell damage. Inhalation of 1% hydrogen gas or drinking hydrogen water *ad libitum* mitigated the nephrotoxicity induced by the anticancer drug cisplatin in mice.[28] Hydrogen also reduced cisplatin-induced oxidative stress, mortality and bodyweight loss. However, hydrogen did not impair the antitumour activity of cisplatin against cancer cell lines *in vitro* or *in vivo* in tumour-bearing mice.[28] Meanwhile, the protective effect of hydrogen-rich water against cisplatin-induced nephrotoxicity was verified in rats using dynamic contrast-enhanced computed tomography.[69]

In a model of antimycin A-induced auditory hair cell damage, incubation of hair cells in a hydrogen-saturated medium significantly reduced the generation of radical oxygen species (ROS) and subsequent lipid peroxidation in auditory epithelia, leading to increased survival of hair cells.[70] Hydrogen gas has also been reported to effectively protect against the morphological and functional vestibular hair cell damage induced by ROS.[71]

Exposure to high concentrations of oxygen may lead to acute lung injury. Zheng *et al.*[72] found that saturated hydrogen saline alleviated hyperoxia-induced pulmonary injury, which was partly responsible for the inhibition of oxidative damage. It was also found that hydrogen-rich saline ameliorated hyperoxia-induced acute lung injury by reducing oxidative stress and inflammatory cascades in lung tissue.[73]

Hydrogen resuscitation and trauma

Hydrogen-rich saline has been reported to reduce acute spinal cord contusion injury, possibly by reducing oxidative stress and increasing levels of brain-derived neurotrophic factor.[74] Ji *et al.*[75] reported that inhalation of 2% hydrogen by rats with traumatic brain injury significantly attenuated the resultant brain injury.

Hydrogen treatment before irradiation significantly inhibited ionizing irradiation-induced injury in human lymphocyte AHH-1 cells and protected the gastrointestinal endothelium of mice against radiation-induced injury.[76] It has also been reported that hydrogen-rich water has a cardioprotective effect against radiation-induced injury.[77]

Lin *et al.*[78] suggested that hydrogen facilitated the recovery of hair cell function and attenuated temporary noise-induced hearing loss in guinea-pigs. Recently, it was shown that irrigation of the cornea with isotonic hydrogen solution significantly reduced angiogenesis after alkali burn injury;[79] hydrogen downregulated ROS production by the cornea, NF-κB phosphorylation and levels of vascular endothelial growth factor (VEGF) and monocyte chemoattractant protein-1 (MCP-1).[79]

Hydrogen resuscitation and tumour

Platinum nanocolloid (Pt-nc)-supplemented hydrogen water has been reported to have a significant inhibitory effect on the growth of human tongue carcinoma HSC-4 cells in preference over normal human tongue DOK cells.[80] This effect has been suggested to be due to hydrogen water-induced enhancement of the anti-oxidant capacity of Pt-nc.[80]

Hydrogen resuscitation and metabolism

Consumption of hydrogen water *ad libitum* prevents the development of atherosclerosis in apolipoprotein E-knockout mice, partly by limiting the degree and deleterious effects of oxidative stress in the blood vessels of these mice.[10]

Furthermore, hydrogen has been able to modulate metabolism in some clinical tests. For example, a randomized double-blind placebo-controlled cross-over study in patients with Type 2 diabetes or impaired glucose tolerance demonstrated that supplemental hydrogen water (900 mL/day for 8 weeks) improved lipid and glucose metabolism.[81] In an open label 8 week study on 20 patients with potential metabolic syndrome, drinking hydrogen-rich water (1.5–2 L/day) was found to be a potentially novel therapeutic and preventive strategy for metabolic syndrome.[11] Furthermore, the addition of hydrogen to haemodialysis solutions has been shown to ameliorate inflammatory reactions and improve blood pressure control in haemodialysis patients.[82]

Hydrogen resuscitation and allergy

Itoh *et al.*[83] found that ingestion of hydrogen-rich water abolished the immediate-type allergic reaction in mice. Using rat RBL-2H3 mast cells, they demonstrated that hydrogen attenuated phosphorylation of FcεRI-associated Lyn and its downstream signal transduction, which subsequently inhibited NADPH oxidase activity and reduced the hydrogen peroxide production. They also found that inhibition of NADPH oxidase attenuated phosphorylation of Lyn in mast cells, indicating the presence of a feed-forward loop that potentiates the allergic responses. Accordingly, hydrogen inhibited all signalling molecules tested in the loop.[83]

Others

Kawasaki *et al.*[84] have demonstrated that hydrogen can prevent the senescence process during the expansion of bone marrow multipotential stromal cells/mesenchymal stem cells (MSC). In that study, the addition of 3% hydrogen gas enhanced preservation of colony forming early progenitor cells within MSC preparations and prolonged the *in vitro* replicative lifespan of MSC without them losing their differentiation potential or paracrine capability.[84]

Overall, more and more results are suggesting that hydrogen has anti-oxidant, anti-inflammatory and anti-apoptotic effects in various models of diseases. The results of *in vitro* and *in vivo* experiments, as well as clinical trials into the effects of hydrogen, are summarized in Tables S1–S3, available as Supplementary Material for this paper. The findings so far indicate that hydrogen resuscitation may be a novel cytoprotective treatment to protect multiple organs, tissues and cells against oxidative injury.

Mechanisms of Hydrogen Resuscitation

Radicals (often referred to as free radicals) are atoms, molecules or ions with unpaired electrons on an open shell configuration. The unpaired electrons make these radicals highly chemically reactive.[85] Reactive oxygen species are free radicals that contain oxygen atoms and are produced as a normal product of cellular metabolism. Reactive oxygen species can be beneficial because they function as necessary signalling molecules, modulate activation of the immune system and participate in antibacterial defence. However, they may become extremely detrimental when they overcome the anti-oxidant capacity of host cells. Excessive ROS can damage cellular macromolecules, including peroxidizing membrane lipids, oxidizing DNA and denaturing proteins. This results in a situation known as oxidative stress.[86] Oxidative stress can be caused by several factors, including inflammation, intense exercise, cardiac infarction, cessation of blood flow and organ transplantation.

Reactive oxygen species include superoxide anion ($\cdot O_2^-$), hydrogen peroxide (H_2O_2), $\cdot OH$ and singlet oxygen ($^1O_2$). The $\cdot O_2^-$ radicals are generated in the mitochondria by electron transport chain leakage and are produced by metabolic oxidases. Excessive generation of $\cdot O_2^-$ radicals drives the production of H_2O_2 and subsequently $\cdot OH$ via the Fenton reaction.[87] $\cdot O_2^-$ and H_2O_2 can be detoxified by endogenous cellular anti-oxidants, such as superoxide dismutase, catalase, peroxidase and glutathione peroxidase. However, $\cdot OH$ is the strongest of the oxidant species and mammal species lack endogenous detoxification systems to neutralize it. Therefore, the therapeutic targeting of $\cdot OH$ could be critical for the amelioration of oxidative injury. Various

substances, including glucose, mannitol, formate, thiourea and dimethylsulphoxide, have been reported as hydroxyl radical scavengers.[88,89]

Many anti-oxidants have been shown to reduce ROS levels in oxidation-related diseases. However, studies have suggested that excessive anti-oxidant treatment is not beneficial and may even be harmful[90] because low levels of ROS, such as $\cdot O_2^-$ and H_2O_2, function as signalling molecules to regulate apoptosis, cell proliferation and differentiation.[91] Strategies that decrease oxidative status intensively may produce unwanted side-effects by interfering with these essential defensive mechanisms.

Because the hydrogen molecule is electronically neutral and much smaller than the oxygen molecule, it should easily penetrate the cellular and intracellular membranes that are normally barriers preventing water-soluble anti-oxidants from entering cells and organelles such as the mitochondria, a major source of ROS production. Ohsawa *et al.*[6] reported that hydrogen selectively inactivated ·OH by forming water, but not $\cdot O_2^-$ and hydrogen peroxide. In culture, hydrogen has been demonstrated to prevent DNA oxidation and to preserve mitochondrial membrane potential and ATP synthesis, thus maintaining cellular morphology.[6] Therefore, hydrogen is thought to be effective in protecting against cerebral I/R injury because of its distinctly selective nature of inactivation.

Owing to its selective ·OH-scavenging ability, hydrogen may exert its cytoprotective effects in several ways. First, hydrogen protects cells by reducing oxidative damage to the DNA, lipids and protein. This has been shown in many studies, as described above, as the attenuation of elevated levels of 8-hydroxydeoxyguanosine (8-OHdG), malondialdehyde, 4-hydroxynonenal, 3-nitrotyrosine, protein carbonyl, etc. In addition, the cytoprotective effects of hydrogen may be due to: (i) its anti-apoptotic effect;[54,55,74] (ii) its inhibition of inflammatory mediators;[30,47,50,66,68] (iii) an increase of endogenous anti-oxidant enzymatic activity;[11,67,75] (iv) its downregulation of the activation of certain inflammatory signalling pathways, such as mitogen-activated protein kinases[49,63] and NF-κB;[65,79] and (v) its stimulation of cell proliferation.[27,51,65]

However, recent studies have indicated that the effects of hydrogen cannot be ascribed solely to the exclusive removal of ·OH. Kawasaki *et al.*[84] have reported that hydrogen gas treatment prolongs the replicative lifespan of bone marrow multipotential stromal cells *in vitro*. However, the same hydrogen gas treatment did not decrease hydroxyl radical, protein carbonyl or 8-OHdG, suggesting that scavenging of hydroxyl radicals may not be responsible for the effects of hydrogen gas in that study. Ueda *et al.*[25] demonstrated that CCH solution, which releases hydrogen in solution, had a synergistic reaction with α-tocopherol and ascorbic acid, thereby enhancing the endogenous anti-oxidant action of these drugs in both *in vitro* and *in vivo* experiments.

Oral administration of CCH solution resulted in a 35% greater anti-oxidant effect that that seen following direct perfusion of the solution into the brain. On the basis of these findings, it was proposed that hydrogen enables the synergistic enhancement of basal endogenous anti-oxidant activity, resulting in increased free radical-scavenging ability by the α-tocopherol and ascorbic acid system, leading to increased elimination of nitroxide radicals. In the immediate-type allergic reaction, Itoh *et al.*[83] found that the beneficial effects of hydrogen were due not to its radical-scavenging activity, but to modulation of a specific signalling pathway, namely FcεRI-mediated signal transduction.

On the basis of their findings, Itoh *et al.*[83] suggested that the effects of hydrogen in other diseases were possibly mediated by modulation of yet unidentified signalling pathways. Both internally produced and exogenously administered hydrogen may serve as a modulator of signal transduction, thereby exerting biological effects under physiological and pathological conditions and implying that hydrogen may be the fourth gaseous signalling molecule after nitric oxide (NO), carbon monoxide (CO) and hydrogen sulphide (H_2S). Therefore, although it is not so that clear of

the mechanism of hydrogen resuscitation so far, it might be divided into two pathways, one dependent on and the other independent of hydroxyl radical neutralization, as illustrated in Fig. 1.

Figure 1. Schematic representation of mechanisms involved in hydrogen resuscitation. Reactive oxygen species (ROS) include superoxide anion ($\cdot O_2^-$), hydrogen peroxide (H_2O_2), hydroxyl radical ($\cdot OH$) and singlet oxygen ($^1O_2$). The mechanisms of action of hydrogen can be divided into two pathways, one dependent on and the other independent of hydroxyl radical neutralization.

Prospects for Research into Hydrogen Resuscitation

Hydrogen has a number of advantages as a potential anti-oxidant. First, it effectively neutralizes $\cdot OH$ in living cells. Unlike most known anti-oxidants, which are unable to successfully target organelles, hydrogen has favourable distribution characteristics: it can penetrate biomembranes and diffuse into the cytosol, mitochondria and nuclei. Despite the moderate reducing activity of hydrogen, its rapid gaseous diffusion may make it highly effective for the reduction of cytotoxic radicals.

Second, hydrogen selectively reduces $\cdot OH$, the most reactive ROS, and does not interact with $\cdot O_2^-$ and hydrogen peroxide, both of which have physiological roles. In addition, the reaction between hydrogen and $\cdot OH$ results in the formulation of water, an essential substance for the body. Moreover, hydrogen is produced continuously in the body by colonic bacteria and normally circulates in the blood. Hydrogen dissolved in the blood is distributed to tissues in proportion to regional blood flow and is rapidly eliminated by the lungs. Inhalation of hydrogen gas does not influence physiological parameters, such as body temperature, blood pressure, pH or the Po_2 of the blood.[6] Hydrogen is already being used to prevent decompression sickness in divers at a level of 2 MPa partial pressure of hydrogen, suggesting that 16 μmol/L hydrogen in the blood should be safe.[4]

Hydrogen inhalation at therapeutic doses has no adverse effects on arterial oxygen saturation (S_po_2) or haemodynamic parameters, including heart rate and left ventricular pressure.[44] In a recent study on the biological safety of neutral pH, hydrogen-enriched electrolysed water (NHE water) in terms of mutagenicity, genotoxicity and subchronic oral toxicity, the level for no observable adverse effect was estimated to be > 20 mL/kg per day NHE water under the conditions examined; on this basis, a 60 kg human can safely drink up to 1.2 L/day NHE water.[92] Thus, we propose that hydrogen could be widely used in clinical settings as a safe and effective anti-oxidant with minimal side-effects.

Third, hydrogen may be applied in the treatment of multiple diseases. In preclinical experimental models of disease, hydrogen resuscitation has been shown to be effective in ischaemia, hypoxia, transplantation, Parkinson's disease, drug intoxication, sepsis, diabetes and cancer.[6,12,50] If confirmed in human trials, hydrogen resuscitation could benefit many patients.

Fourth, the production of hydrogen is not expensive compared with most other drugs. If it could be used in clinical settings, it would prove to be a cost-effective drug.

However, research on the biological use of hydrogen is just beginning. Thus far, the precise mechanism as to how hydrogen exerts its cytoprotective effects is not clear. Furthermore, there is little information as to whether hydrogen shows selective reduction of ROS *in vivo*. Ohsawa *et al.*[6] reported that 4% hydrogen was not as effective as 2% hydrogen in the MCAO model: the effects of hydrogen gas inhalation do not appear to be positively correlated with its concentration. It is possible that high concentrations of hydrogen have a less selective reducing ability. It remains possible that hydrogen also protects cells against stress by directly or indirectly reducing other strong oxidant species in living cells. We hope that further studies will reveal the mechanisms by which hydrogen quenches oxidative stress.

Hydrogen is recognized as a potent anti-oxidant and anti-inflammatory gas. To some extent, hydrogen is similar to other gaseous signalling molecules, such as H_2S. Unlike NO, CO and H_2S, hydrogen was not characterized as a toxic gas, does not react with haemoglobin and cannot be produced by mammalian cells. Effects of hydrogen on signalling transduction have been discounted. However, whether hydrogen is a gaseous signalling molecule remains unknown. Mechanisms underlying the signalling pathways involved in hydrogen-mediated anti-oxidant activity and other effects have yet to be clarified. In addition, the capacity of hydrogen to influence cellular metabolism as a therapeutic strategy remains to be explored.

Most of the results regarding hydrogen resuscitation come from *in vitro* studies and animal experiments, with only a few coming from human trials in a limited number of subjects. Indeed, some therapeutic strategies for scavenging ROS that seemed promising in animal models failed in human clinical trials. In addition, even though we consider hydrogen resuscitation as a safe treatment, 'over resuscitation' may cause worse damage because we know that the balance between oxidation and anti-oxidation is important. Thus, we need to know more about the pharmacokinetics, biology, dose effects and side-effects of hydrogen, especially in humans. Hydrogen resuscitation cannot be used in the clinical setting unless data are collected from large-scale, blinded, randomized, multicentre and adequately powered clinical studies using standardized methods.

In summary, although medical gas therapy is a novel and untapped field of science, never has hydrogen resuscitation attracted so much attention from scientists as it does today. Hydrogen, which selectively reduces levels of detrimental hydroxyl radicals, may have several potential advantages over current pharmacological therapies. Hydrogen gas therapy could be delivered by simple inhalation, via hydrogen-enriched fluid or via an approach that affects endogenous hydrogen production. Hydrogen resuscitation could be an effective anti-oxidant, anti-apoptotic, anti-inflammatory treatment, as demonstrated in a variety of diseases models. More work is needed to identify the precise mechanism underlying the effects of hydrogen and to validate the therapeutic potential of hydrogen resuscitation in the clinical setting.

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