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REVIEW

New Possibilities of the Prevention and Treatment of Cardiovascular Pathologies. The Potential of Molecular Hydrogen in the Reduction of Oxidative Stress and its Consequences

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Summary

Disproportion between reactive oxygen species (ROS) production and the body's antioxidant system can cause oxidative stress, which is considered a common denominator in various pathological conditions, including cardiovascular diseases, aging, and cognitive disorders. The generation of free radicals, which occurs through partial reduction of oxygen, can quickly overwhelm the endogenous antioxidant system capacity of the cell. This causes lipid, protein, DNA and RNA damage, inflammation, and overall cell degeneration, which can be mitigated by various antioxidants. However, their use in human medicine did not bring the expected effect. Molecular hydrogen (H_2), due to its unique physical and chemical properties, provides a number of benefits for alleviating oxidative stress. H_2 is superior to conventional antioxidants as it can selectively reduce $^{\bullet}OH$ radicals while preserving important ROS that are otherwise used for normal cell signaling.

Key words

Oxidative stress • Cardiovascular diseases • Molecular hydrogen • ROS • Inflammation

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Oxidative stress

Oxidative stress arises from a dysregulation between the reactive oxygen species (ROS) generation and the antioxidant system when the number of oxidants and reactive molecules exceeds the body's endogenous enzymatic and non-enzymatic antioxidant protection. This disproportion is known as a common causative factor in many pathological processes because it deteriorates cellular and organ function [1]. ROS are byproducts of oxygen reduction, which occurs during normal cellular metabolism. Primary sources of ROS are mitochondrial respiration, NADH/NADPH oxidase, and xanthine oxidoreductase [2]. Reactive molecules include ROS and reactive nitrogen species (RNS). ROS is a category represented by radicals and by non-radical chemical substances. Free radicals are chemical species that contain an unpaired electron; for example, hydroxyl radicals ($^{\bullet}OH$), superoxide anion radicals ($\bullet O_2^-$), alkoxyl radicals (RO•), peroxyl radicals (R-OO•), carbon radicals (RC•), and various RNS (e.g., nitric oxide, NO•). Non-radical reactive molecules include hydrogen peroxide (H_2O_2), singlet oxygen ($^1O_2$), and various RNS (e.g., peroxynitrite [$ONOO^-$], nitrogen dioxide [NO_2], dinitrogen trioxide [N_2O_3]). As already mentioned, most ROS are produced in the mitochondrial electron transport chain (ETC), primarily at complex 1. Other sites of ROS production include NADPH oxidase, nitric oxide synthase, xanthine oxidase, cytochrome p450, aldehyde oxidase, heme proteins, etc. [3]. Enhanced metabolism and other ways of induced activation of these systems in pathological states result in a significant increase in ROS production (Fig. 1).

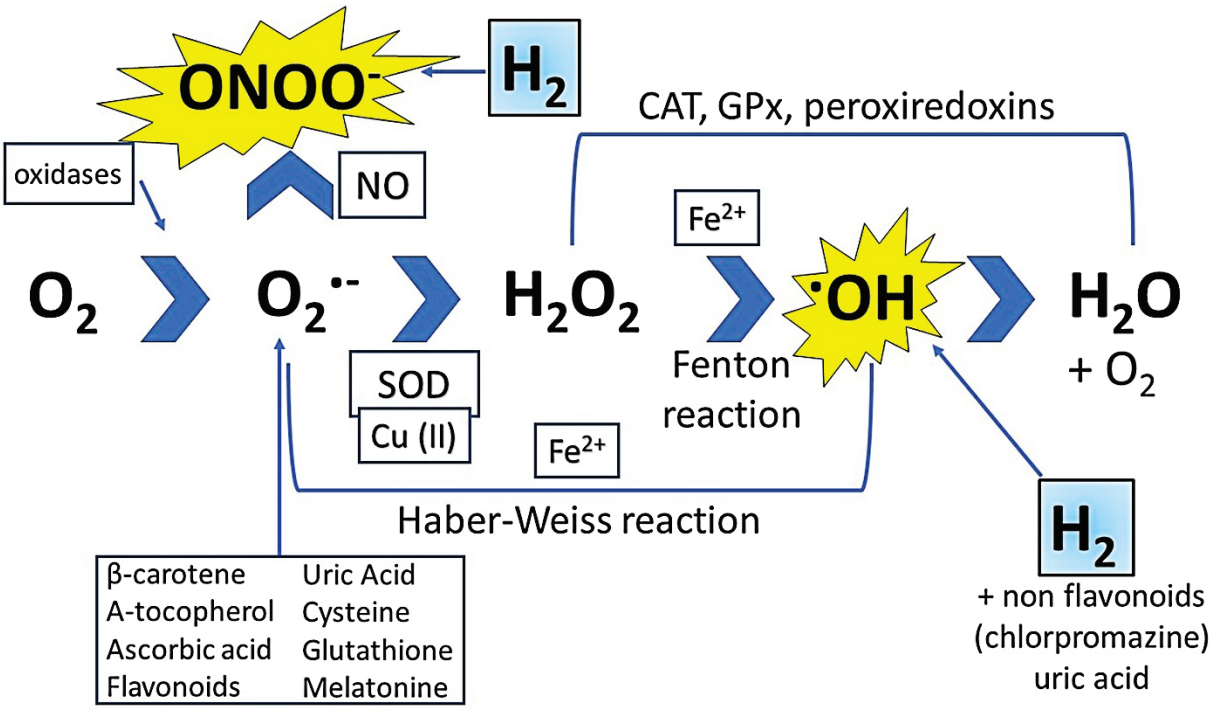

Fig. 1. Superoxide production increases during immune responses and physical exertion due to increased oxygen intake. This induced superoxide acts as an important signaling molecule. The concentration of superoxide is regulated within a narrow range. Superoxide can be dismutated by the body's antioxidant enzyme superoxide dismutase to produce H_2O_2. The produced H_2O_2, as a signaling molecule, can further regulate gene expression and induce favorable cellular changes before it is converted into water and O_2 by catalase. If the concentration of superoxide or H_2O_2 exceeds regulatory systems, oxidative stress occurs. Higher levels can also lead to increased production of toxic hydroxyl radicals (˙OH) through the Haber-Weiss and Fenton reactions. $ONOO^-$ and ˙OH oxidize cellular biomolecules, leading to diseases and various damages. Antioxidants can reduce the concentration of ROS, but molecular hydrogen is considered, due to its relative inertness, a selective scavenger of $ONOO^-$ and ˙OH. Modified according to Slezák *et al.* [4].

The emergence of ROS in the cell and the possibilities of using antioxidants in regulation and dysregulation of ROS

Reactive molecules can have harmful or beneficial effects depending on their origin, concentration, location, and duration of action. For example, under normal metabolic conditions, superoxide radicals are continuously formed by the reduction of molecular oxygen in the mitochondrial ETC, in nuclear and plasma membranes through NADPH oxidases, in the endoplasmic reticulum during protein formation, as well as in the macrophages. Superoxide production increases during immune responses because it is essential for the elimination of pathogens and increasing levels of inflammatory cytokines [5].

Similarly, the formation of superoxide increases during physical exertion due to increased oxygen intake. Such induced superoxide acts as an important signaling molecule, activating various transcription factors, resulting in improved physical capacity. The concentration of superoxide is regulated within a narrow range by controlling its production and clearance. Superoxide can be dismutated by the body's antioxidant enzyme superoxide dismutase to produce H_2O_2. The produced H_2O_2, as another important signaling molecule, can further regulate gene expression and induce favorable cellular changes before it is converted into water and O_2 by catalase [3]. If the concentration of superoxide or H_2O_2 exceeds the capacity of regulatory systems, then oxidative stress occurs. Higher levels can also lead to an increased production of toxic ˙OH through the Haber-Weiss and Fenton reactions. Iron and superoxide can then be regenerated, which further supports the production of hydroxyl radicals as seen from the following equations and Figure 1.

Fenton reaction:

$$Fe^{2+} + H_2O_2 \rightarrow Fe^{3+} + OH^- + {}^{\bullet}OH,$$
$$Fe^{3+} + {}^{\bullet}O_2^- \rightarrow Fe^{2+} + O_2,$$
$$Fe^{3+} + H_2O_2 \rightarrow Fe^{2+} + {}^{\bullet}O_2^- + 2H^+$$

Haber-Weiss reaction:

$$^{\bullet}O_2^- + H_2O_2 \rightarrow O_2 + {}^{\bullet}OH + OH^-$$

Due to its high reactivity, ˙OH reacts with virtually any biomolecule at diffusion-controlled rates

($\approx 10^{10}$ M/s), making them very harmful. The nitric oxide radical (NO•) is also a fundamental signaling molecule and is necessary for endothelial function (e.g., vasodilation), gene expression, and cell proliferation, and it is produced close to its targets. However, just as with superoxide, when its concentration is too high, for example during hyperactivation of iNOS, an excess of NO• can almost instantly combine with superoxide to form harmful peroxynitrite molecules. Peroxynitrite is a stronger oxidant than nitric oxide or superoxide and, in addition to being able to oxidize proteins, lipids, and DNA, it is also a source of toxic hydroxyl radicals not derived from the Fenton reaction [6]. These two oxidants (i.e., $ONOO^-$ and $^{\bullet}OH$) do not have a known physiological benefit and easily oxidize cellular biomolecules leading to various forms of damage. Excessive ROS formation associated with depletion of the body's antioxidant system (e.g., superoxide dismutase [SOD], glutathione peroxidase [GPx], vitamin C, etc.) leads to oxidative stress and its subsequent deleterious consequences [5]. Oxidative stress is considered a common denominator of various pathological conditions including cardiovascular and metabolic diseases, aging, and deterioration of cognitive function. It causes damage to lipids, proteins, DNA, and RNA and overall cell degeneration, which can be alleviated by various antioxidants that can enter the cells and reduce the concentration of ROS. However, their use has not met with the expected results in human medicine. On the other hand, molecular hydrogen (H_2) is considered, due to its relative inertness, a selective scavenger of the most reactive radicals $ONOO^-$ and $^{\bullet}OH$ without affecting their signaling function. H_2 provides, thanks to its unique physical and chemical properties, several advantages in mitigating oxidative stress. H_2 is more beneficial than conventional antioxidants since it can selectively reduce $^{\bullet}OH$ radicals while preserving important ROS that are otherwise used for normal cell signaling.

Formation and physiological role of molecular hydrogen

According to various theories, H_2 is closely involved in the origin and development of life in eukaryotic cells. Molecular hydrogen is formed through various chemical and biological processes. Mammalian and human cells do not produce hydrogen endogenously, as they are lacking enzymes with hydrogenase activity. However, in the large intestine, anaerobic organisms generate hydrogen by breaking down carbohydrates, primarily from undigested polysaccharides in starches and plant cells, through hydrogenase activity.

The primary source of endogenous H_2 in the human body is anaerobic bacteria, such as *Firmicutes* and *Bacteroides*, in the gut and other organs, which produce it through reversible oxidation reactions. H_2 can modulate glucose and lipid metabolism in both animal models and humans [7].

H_2-producing bacteria

Gaseous H_2 in the body is produced by fermentation of carbohydrates such as lactose, lactulose, and fructose by gut bacteria [8]. Bacteria commonly found in the large intestine are mainly the group *Bacteroides fragilis*, *Clostridium perfringens*, and *Pseudomonas*, which have hydrogenases to produce H_2. The largest amount of H_2 is produced by *Blautia coccoides* and *Clostridium leptum*. Depending on the composition of the microflora, however, substantial amounts of produced H_2 are consumed by methanogenic bacteria and sulfate-reducing bacteria. A promising way to improve gut flora might be drinking H_2-rich water (HRW). H_2 dissolved in water significantly improves the gut environment, including microbial composition, which can contribute to improving the diversity of the gut microflora in favor of a composition that through the formation of H_2 contributes to a protective and therapeutic effect. A recent report stated that 70 % of gastrointestinal microbial species of the human microbiome encode the genetic capacity to metabolize H_2, meaning that H_2 may affect the composition of gut microbiome [9].

Although the amount of H_2 produced endogenously is relatively small, it is believed to play a role in maintaining gut health and possibly exert systemic effects by modulating pro-inflammatory cytokines and signaling pathways. H_2 has played and continues to play a significant role in the origin of the universe and life. The presence of basal levels of H_2 in the human body may indicate that it also has a physiological role. Many plants, insects, animals, and also humans have developed a mutual relationship with H_2-producing bacteria, which they use to their advantage.

Physiological roles of molecular hydrogen

H_2 has been shown as a promising agent in protecting brain cells from oxidative stress and inflammation, which could be beneficial in

neurodegenerative diseases like Parkinson's and Alzheimer's. H_2 can reduce the extent of damage caused by heart attacks (myocardial infarction) and improve heart function. It was shown that H_2 may help regulate metabolic processes, potentially aiding in conditions like diabetes and obesity [10]. Preliminary studies suggest H_2 might inhibit cancer cell proliferation and enhance the effectiveness of certain chemotherapy drugs. And finally, by reducing oxidative stress and inflammation, H_2 could potentially slow down aging processes and improve overall health [10].

While the roles of endogenous H_2 in physiology are still being actively studied, current findings are promising for a wide range of applications. Inadequate production of endogenous H_2 may play essential roles in brain, heart, and liver disorders, while enhanced endogenous H_2 production can improve overall health and longevity. However, the physiological significance and potential therapeutic implications of endogenous H_2 production are still under investigation [7,10].

Therapeutic effects of molecular hydrogen

Molecular hydrogen has been studied for its potential therapeutic effects, which include antioxidant properties. H_2 can neutralize harmful ROS, reducing oxidative stress and cellular damage. Moreover, it has been shown that molecular hydrogen exerts anti-inflammatory and antiapoptotic effects by modulating pro-inflammatory cytokines and signaling pathways. H_2 can prevent programmed cell death (apoptosis) in various cell types, which is beneficial in conditions like ischemia-reperfusion injury. In addition, H_2 can protect cells from damage by various stressors, including radiation and toxins [4,11,12].

Pharmacokinetics and application possibilities of molecular hydrogen

Once introduced into the body, molecular hydrogen exhibits several unique effects: Due to its small size and non-polar nature, H_2 can diffuse rapidly through the cell membranes and tissues. H_2 is relatively soluble in both lipids and water, allowing it to penetrate various cellular compartments. Unlike other antioxidants, H_2 selectively reduces highly reactive oxygen species such as hydroxyl radicals (•OH) without affecting beneficial ROS involved in cell signaling. There are several methods of H_2 administration, including inhalation of gaseous H_2, intravenous injection of H_2-rich saline, dialysis solution rich in H_2 for hemodialysis, using a hyperbaric H_2 chamber, bathing in H_2-rich water, increasing the production of H_2 by intestinal bacteria, topical application, oral ingestion of H_2-producing tablets, and simply drinking HRW. HRW can be prepared by bubbling gaseous H_2 into water under pressure, by water electrolysis ($2H_2O = 2H_2 + O_2$), and also by reaction with metallic magnesium ($Mg + 2H_2O = H_2 + Mg(OH)_2$), or some other metals. Various products are commercially available from drinks prepared for consumption in aluminum sachets/cans and electrolytic devices to H_2-producing tablets and inhalation devices. Inhalation of H_2 or administration of HRW increases the concentration of H_2 in arterial and venous blood proportionally to the administered dose. Consumption of H_2 by both methods reaches a maximum concentration in the blood within 5-15 min and returns to baseline levels 45-100 min after administration, depending on the dose [13].

Physical and chemical properties of hydrogen from the perspective of biomedicine

The cellular biological availability of H_2 can be very high due to its unique physical and chemical properties. Its small size, low weight, neutral charge, and nonpolar nature, along with its high diffusion rate, allow it to easily penetrate cellular biomembranes and diffuse into mitochondria and the nucleus [14].

H_2 is the smallest molecule with a covalent radius of 31 pm, which is less than half of the size of an oxygen gas molecule. It is the lightest known molecule (2.0159 g/mol). Since it is electrically neutral and has low polarization, it is relatively insoluble in water. At standard ambient temperature and pressure, water can be saturated with H_2 up to a concentration of 0.8 mM (1.6 mg/l). However, it is 3-5 times more soluble in lipids [15]. Unlike other substances, it is completely non-toxic, making it very safe. H_2 has been used since the 1940s to prevent decompression sickness in deep-sea diving. No toxicity has been found even at concentrations significantly above clinically effective doses. The reactivity of H_2 is so mild that it does not react with physiological levels of ROS, which are involved in cellular signaling. This is a great advantage because ROS play an active role in cellular functions and intercellular communication [16]. No toxic effects have been observed even at 98.87 % H_2 and 1.13 % O_2 at a pressure of 19.1 atm. Moreover, gaseous H_2 is naturally found in our blood and breath due to normal fermentation

of indigestible carbohydrates by intestinal bacteria. This bacterially produced gaseous H_2 has also proven to be therapeutically active. H_2 also has no effect on physiology, temperature, blood pressure, pH, or pO_2 [17].

Understanding the role of ROS and inflammation is crucial in understanding of the potential use of gaseous H_2 as a preventive or therapeutic agent. H_2 also plays a significant role in according to the theory proposed in the 1950s by Denham Harman, which was largely based on the strong correlation between oxidative stress and various diseases [18].

Research on H_2 has significantly increased after 2000, when Ohsawa and colleagues published their findings in Nature Medicine. They stated that inhalation of only 2-4 % gaseous H_2 significantly reduced the extent of brain infarction in a rat model of ischemic-reperfusion (I/R) injury caused by occlusion of the middle cerebral artery. Dissolved H_2 in the medium of cultured cells at biologically acceptable concentrations selectively reduced levels of toxic $^{\bullet}OH$ radicals but did not reduce other physiologically important ROS (such as superoxide, nitric oxide, and hydrogen peroxide) [19].

Currently, there are more than 1800 scientific works repeatedly discussing the possibility of medical applications of H_2. More than 80 clinical studies on humans show the translational potential of using H_2 from animals to humans for a wide range of diseases [20,21]. The positive effect of H_2 was observed, for example, in a preclinical experiment of heart transplantation. In this experiment, the use of H_2 significantly improved markers of heart damage and oxidative stress, proving that its use in clinical practice could significantly help improve heart function after transplantation and extend both the storage time of the heart during transport and the survival time of patients [22,23]. In 2017, H_2 gas inhalation was approved by the Japanese government as an advanced drug for the treatment of post-cardiac arrest syndrome, which is now being clinically administered in a large multicenter study with 360 patients with promising preliminary results [24].

Effects of molecular hydrogen

Unlike conventional antioxidants and anti-inflammatory drugs, the physical and chemical properties and biomedical studies of the effects of H_2 suggest that H_2 has great potential in mitigating the effects of excessive amounts of ROS and inflammation. Compared to traditional antioxidants, H_2 is a small molecule that easily diffuses throughout the body, tissues, organs, and cells without affecting signaling reactive species [25].

H_2 is a relatively stable molecule that does not react with reactive signaling molecules under biological conditions without a catalyst, but can selectively scavenge cytotoxic hydroxyl and nitrozyl radicals, which is exactly what is most needed. H_2 has become an attractive agent in the biomedical field by functioning as a gas signaling modulator that effectively reduces oxidative stress and inflammation. It has been shown that H_2 has therapeutic potential in more than 170 different disease models in animals and humans. Several animal studies have shown that H_2 is effective in increasing resistance and mitigating the negative effects of acute and chronic stress, such as inflammation, as well as increased ROS, anxiety, and depression [26]. H_2 also reduces oxidative stress by regulating gene expression and acts as an anti-inflammatory and antiapoptotic agent. Newer results have demonstrated pleiotropic therapeutic effects of H_2 in various animal disease models, as well as in many human diseases [27]. For example, H_2 has been shown to reduce the expression of several pro-inflammatory mediators and markers of oxidative stress and apoptosis, including tumor necrosis factor alpha (TNF-α), various interleukins (IL-6, IL-1β, IL-10, IL-12), chemokine ligand 2 (CCL2), intercellular adhesion molecule 1, nuclear factor kappa B (NF-κB), nuclear factor of activated T-cells (NFAT), high mobility group protein 1, prostaglandin E2, cyclooxygenase-2 (COX2), serum diamine oxidase, tissue malondialdehyde (MDA), carbonylated protein, thiobarbituric acid reactive substances (TBARS), myeloperoxidase activity, cJun-N-terminal Kinase (JNK), and caspase-3. Antioxidant, anti-inflammatory, and antiapoptotic effects of H_2 appeared to be important in providing cytoprotection against radiotherapy and chemotherapy. It has been shown that H_2 has radioprotective effects on cultured cells and mice [28]. For example, in irradiated animals, the lipid peroxidation marker MDA was significantly increased in the small intestine but was not similarly increased in the H_2-water group [29]. Similarly, increased markers of oxidative stress (MDA) and inflammation (TNF-α) were observed in blood plasma after chest irradiation in an animal model of rats. However, the application of H_2 significantly reduced the levels of these markers of oxidative stress and inflammation [30]. The favorable chemical, physical and biological properties of H_2 qualify it as an excellent candidate for the prevention and treatment of I/R injury. In a rat model of myocardial I/R injury, inhalation of 2 % H_2 at the onset of ischemia

and 60 min after reperfusion reduced infarct size, reduced left ventricular end-diastolic pressure, reduced pathological remodeling, and improved cardiac function 30 days after myocardial I/R injury [31]. In addition, H_2 significantly potentiated antiapoptotic and anti-infarct effect of hypoxic postconditioning in post-I/R rat hearts, attenuated the incidence and duration of ventricular fibrillation and improved the recovery of contractile function and coronary flow [32]. In pigs, inhalation of 4 % H_2 improved myocardial damage and reduced myocardial infarct size [33]. Although nitric oxide also has the ability to reduce infarct size in myocardial I/R injury, it also has toxic effects that are largely attributed to the formation of various reactive nitrogen species, specifically peroxynitrite. Peroxynitrite is a very reactive molecule that reacts with tyrosine. These adverse effects can be conveniently reversed by inhalation of H_2. Breathing NO with H_2 can reduce cardiac damage and improve recovery of left ventricular function by removing toxic byproducts of NO metabolism [34]. Similarly, injection of H_2-rich saline (HRS) before reperfusion significantly reduced myocardial 8-hydroxy-2′-deoxy-guanosine (8-OHdG) and MDA levels in risk zones. As previously mentioned, I/R leads to rapid calcium accumulation and ROS production in mitochondria, which triggers the opening of the mitochondrial transition pore. This results in loss of membrane potential and induction of apoptotic signaling. At the onset of reperfusion, H_2 appears to be able to reduce ROS generation and thereby reduce DNA damage and lipid peroxidation, while preserving mitochondrial membrane potential and ATP. These factors work together to protect the heart by inhibiting mitochondrial pore opening. Importantly, HRS administered 5 min before reperfusion was sufficient to influence these protective effects, including inhibition of caspase 3 signaling activation, which subsequently reduced cardiomyocyte apoptosis [35]. In addition to I/R injury, HRS can protect against high-dose isoproterenol (ISO)-induced acute myocardial infarction in a rat model through its antioxidant and anti-inflammatory activities. H_2 inhibits ISO-induced cardiomyocyte autophagy both *in vivo* and *in vitro*. Accordingly, 2 % H_2 inhalation attenuated myocardial I/R injury by reducing cardiac endoplasmic reticulum stress and autophagy. HRS also protects against doxorubicin-induced myocardial damage in rats and improves their survival. Inhalation of H_2 also improved survival and functional outcomes of rats after cardiac arrest [36]. The protective effects of H_2 on cardiac hypertrophy were also confirmed in spontaneously hypertensive rats. Administration of HRS effectively attenuated left ventricular hypertrophy by suppressing inflammation and oxidative stress, maintaining mitochondrial function, and suppressing angiotensin II levels locally in the left ventricle by reducing angiotensin-converting enzyme (Fig. 2) [37].

Signaling pathways influenced by molecular hydrogen

In addition to the effect of H_2 on gene expression, H_2 can change the expression of various proteins without changing mRNA expression. For example, H_2 has been shown to reduce the level of CD36 proteins but does not change CD36 mRNA expression. This suggests that H_2 may have an impact on post-transcriptional events, such as increasing protein degradation or inhibiting protein translation. Regardless of the method, however, it has been found that H_2 changes the level of proteins of many important biomolecules and regulates signal transduction and protein phosphorylation cascades. Many of the effects of H_2 are indeed mediated by modulating the activities and expressions of many biomolecules, such as AMPK (adenosine monophosphate-activated protein kinase), ASK1 (apoptosis signal-regulating kinase), JNK, Lyn, FGF-21 (fibroblast growth factor 21), FOXO1 (forkhead box protein O1), HMGB1 (high mobility group box 1), mTOR (mammalian target of rapamycin), NLRP3 (NOD-, LRR- and pyrin domain-containing protein 3), NF-κB (nuclear factor kappa B), PGC-1α (peroxisome proliferator-activated receptor gamma coactivator 1-alpha), NFATC1 (nuclear factor of activated T-cells, cytoplasmic 1), mtUPR (mitochondrial unfolded protein response), STAT3 (signal transducer and activator of transcription 3), VEGF (vascular endothelial growth factor), SIRT (sirtuin), Nrf2 (nuclear factor erythroid 2-related factor 2), miRNA (microRNA), and many others. These, however, are considered molecules that are indirectly changed by H_2. The primary major regulators/drivers of these molecular changes require further clarification. Nevertheless, many of these molecules play a crucial role in mediating the beneficial effect of H_2 [39,40].

H_2 activates the Nrf2 pathway. Perhaps the most significant and important effect of H_2 on gene expression is the induction of phase II enzymes through the activation of the Nrf2-antioxidant response element

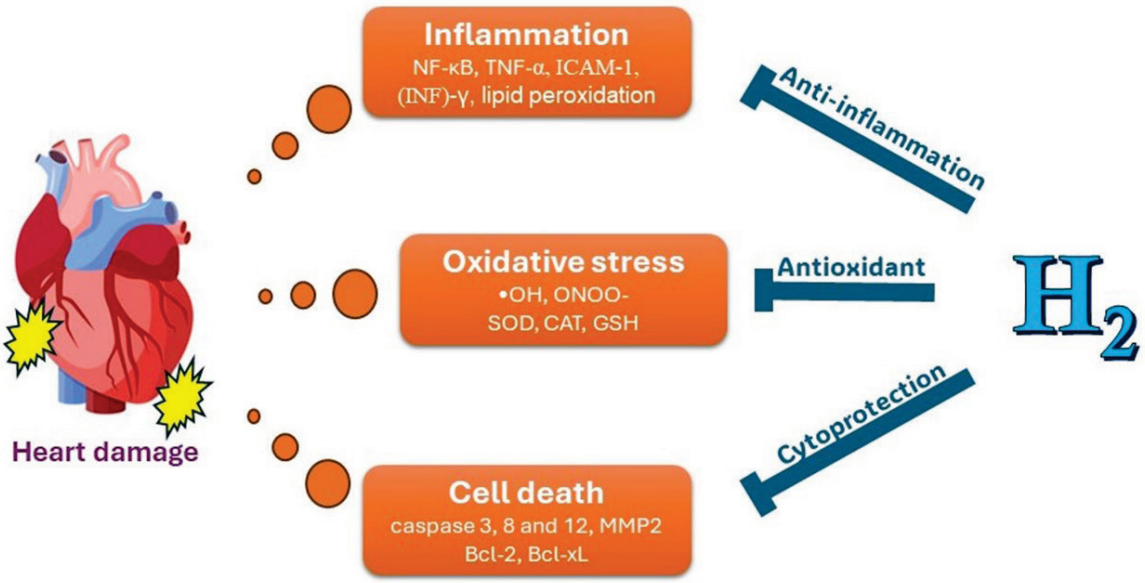

Fig. 2. Mechanisms of action of molecular hydrogen under conditions of increased oxidative stress. It has been shown that molecular hydrogen provides protective effects through several mechanisms including antioxidative, anti-inflammatory, and cytoprotective effects, as well as through signal modulation. NF-κB = nuclear factor kappa B, TNF-α = tumor necrosis factor alpha, ICAM-1 = intercellular adhesion molecule 1, (INF)-γ = inferferon gamma, SOD = superoxide dismutase, CAT = catalase, GSH = glutathione, MMP2 = matrix metalloproteinase 2, Bcl-2 = B-cell lymphoma 2, Bcl-xL = B-cell lymphoma extra-large. Modified according to LeBaron *et al.* [38].

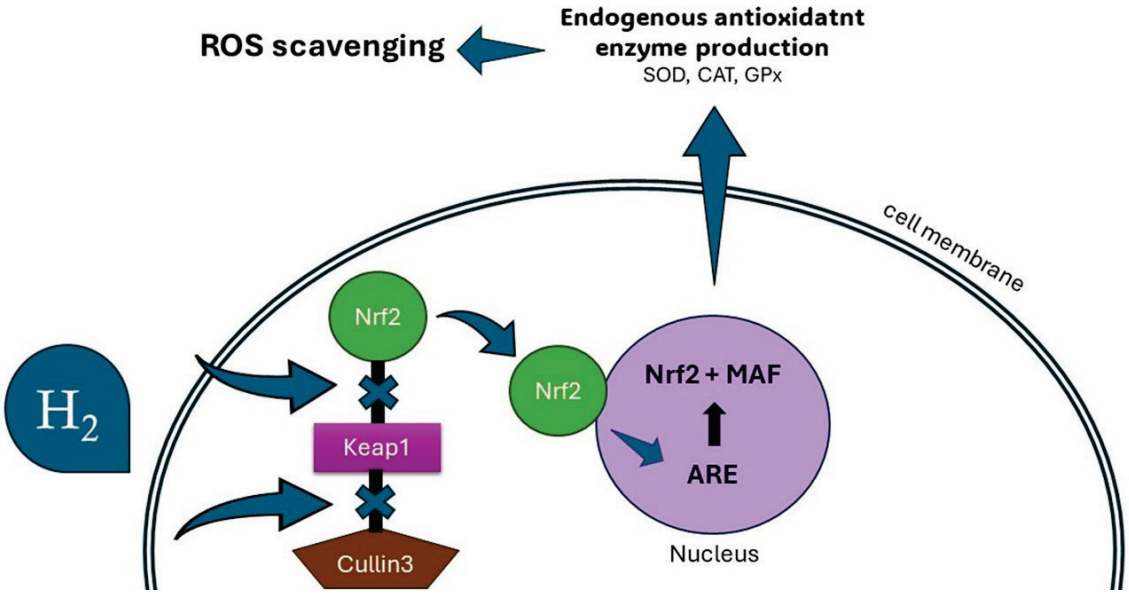

Fig. 3. The mechanism of H_2 action: transcription and production of innate antioxidants after entering the cell cytoplasm, release, and accumulation of Nrf2, and its translocation into the nucleus. CAT = catalase, SOD = superoxide dismutase, GPx = glutathione peroxidase, Nrf2 = nuclear factor erythroid 2-related factor 2, Keap1 = Kelch-like ECH-associated protein 1, ARE = antioxidant response element, MAF = musculoaponeurotic fibrosarcoma. Modified according to LeBaron *et al.* [38].

(ARE) signaling pathway. Nrf2 is considered an important regulator of electrophilic/antioxidant homeostasis that promotes cell functional integrity, especially under conditions of oxidative stress. This pathway regulates the expression of more than 200 genes involved in antioxidation and detoxification. A dysregulated cellular redox state caused by increased levels of ROS or reduced antioxidant status is an important signal for inducing a transcriptional response. This mechanism is illustrated in Figure 3.

Under unstressed conditions, Nrf2 levels in the cytoplasm are regulated by the Kelch-like ECH-associated protein 1 (Keap1), which prevents its release into the nucleus and promotes its degradation. It appears

that the ubiquitination rate of post-translational modification of Nrf2 and its degradation in unstressed cells largely depends on the concentration of Keap1 protein. Keap1 is also a sensor for a wide range of Nrf2 activators/inducers with small molecules. Activation of the Nrf2 pathway in response to stress signals induces dissociation of Nrf2 from the Keap1 protein, allowing the Nrf2 transcription factor to translocate into the nucleus, where it binds to a related DNA regulatory element called ARE or electrophile response element (EpRE). Binding initiates the transcription of antioxidant genes, leading to the production of many cytoprotective proteins. It has been shown that H_2 activates the Nrf2/EpRE signaling pathway and that *in vivo* H_2 activates the Nrf2 pathway, leading to the prevention of radiation-induced lipid peroxidation in the rat heart. Administration of H_2 for 9 days significantly increased SOD-2 and the increase in phosphorylation of Akt kinase on Ser473, a cell survival signaling molecule, which is involved in Nrf2 regulation. It is likely that many of the therapeutic effects of H_2 can be attributed to the activation of the Nrf2 pathway, which stimulates the production of innate antioxidants, as well as the reduction of apoptosis and inflammation [39].

H_2 may act as a hormetic effector

The pleiotropic effects of H_2, given some sporadic and paradoxical findings, may also be explained by the hypothesis that the pleiotropic effect of H_2 can be attributed to its hormetic mechanism of action. Hormesis is a process similar to "preconditioning" or adaptation to mild toxic stress, followed by increased cellular protection. For example, activation of the Nrf2 pathway by H_2 may seem somewhat paradoxical, as the H_2 molecule is considered a reducing agent and it appears that the Nrf2 protein is induced by electrophilic chemicals and suppressed by mild nucleophilic substances [41]. Oxidative stress is indeed a major activator of the Nrf2 pathway. For example, lipid peroxidation product, 4-hydroxy-2-nonenal, in cardiomyocytes mediates Nrf2-dependent upregulation of uncoupling protein 3 (UCP3). This effect may be particularly important in mediating the protective effects of preconditioning, which induces mild oxidative stress and subsequent upregulation of various proteins including cytokines, heat shock proteins, NF-κB, and Nrf2 [42]. According to this hypothesis, H_2 acts as a redox adaptogen in maintaining redox homeostasis by acting hormetically, or through the modulation of redox-sensitive processes.

Cardiovascular diseases and protection by H_2

Ischemia and reperfusion injury

Oxidative and inflammatory stress are fundamental causative factors of myocardial I/R injury. Cardiac myocytes require a large amount of ATP for their physiological function, so a high density of mitochondria is needed to satisfy their high energy demand. Mitochondria filled with reactive intermediates and proapoptotic components are closely involved in I/R injury. The inner mitochondrial membrane is responsible for maintaining the mitochondrial transmembrane potential and is usually impermeable to ions and proteins. Under stress, however, the opening of the mitochondrial permeability pore (mPTP) creates a non-selective channel between the inner membrane of mitochondria and the sarcoplasm, leading to feedback of increased ROS production from mitochondria ("ROS-induced ROS release"). Subsequently, there is a loss of electrochemical gradient, ROS production, Ca^{2+} overload, and formation of apoptosomes [43]. The production of free radicals by partial reduction of oxygen during ischemia followed by reperfusion is well known. These highly reactive ROS can quickly overwhelm the cell's endogenous antioxidant system, subsequently causing damage to lipids, proteins, DNA, and RNA of cells. Substrates of xanthine oxidase, xanthine, and hypoxanthine, accumulate during ischemia, which triggers the activation of xanthine oxidase and subsequently higher ROS production. Accumulation of ROS, proteases, and growth factors induces proliferation of fibroblasts, aberrant accumulation of collagen and fibrosis, causing tissue destruction. In addition, reperfusion further increases tissue damage mediated by inflammation [44].

Recently, researchers and physicians have focused on the effect of H_2 on various diseases, mostly associated with increased production of free radicals, such as I/R injury, myocardial or brain infarction, heart storage for cardiac transplantation, and heart failure, or radiation-induced heart disease and the like [22,38,45,46]. Protective effects of H_2 in pathological situations induced by oxidative stress were demonstrated more than 16 years ago. Since then, it has been experimentally used in many disease models and in several clinical studies with optimistic results. H_2 appears to be an important biological regulatory factor that acts selectively on the most reactive hydroxyl and nitrosyl radicals. In recent years, scientific interest has focused on

its mechanisms of action, which are not yet fully understood. Current knowledge from molecular studies of H_2 suggests that it can be used in the prevention and treatment of cardiovascular diseases associated with oxidative stress [4,38,39,47].

The effect of H_2 administration on aging and cognitive function decline: the phenomenon of global aging

Increased long-term oxidative stress has repeatedly been shown in experiments and clinical studies as a significant factor that accelerates aging. Therefore, H_2 and its selective action in reducing the toxic effects of oxidative stress may significantly prevent and therapeutically influence the signs of population aging. The average human lifespan has been steadily increasing over the past two centuries, leading to an increase in the proportion of older people worldwide. The process of global aging is expected to continue in the future and the proportion of older people will increase. At the same time, structural and functional changes in the brain that may occur with aging will also increase. The volume of gray matter decreases with age, the size of neurons and the number of connections between neurons diminish. There is also a loss of brain white matter with age. Cognitive abilities also change. If cognitive disorders develop that also affect an individual's complex functional activities, there may be a significant decline in cognitive functions such as dementia or other disorders [9]. However, these changes do not affect all cognitive domains and there are large interindividual variations in their trajectories. In addition, the human brain has "reserves" that help it compensate for the negative effects associated with aging. Certain intellectual and physical activities, as well as social and dietary factors, are associated with positive cognitive outcomes in older individuals.

Microbiome, aging, and the microbiota-gut-brain axis and molecular hydrogen

The term microbiome began to be officially spoken of only in the late 1990s when, based on genetic studies of genomes, the significance of the human microbiome for health and various pathologies began to be better understood. We now know that bacteria in the microbiome help digest our food, regulate our immune system, protect against other bacteria that cause diseases, and produce vitamins including vitamins B (thiamine and riboflavin) and vitamin K. The human microbiome is now recognized as an "organ" without which the body would not function properly. An imbalance in gut microbial flora – dysbiosis, is associated with inflammatory bowel diseases, diabetes, obesity, cancer, cardiovascular disorders as well as central nervous system disorders and mental health, which represents the concept of the microbiota-gut-brain axis. This bidirectional axis is gaining more and more attention in the areas of exploring the biological and physiological bases of psychiatric, neurodevelopmental, and age-related neurodegenerative disorders. Signaling runs from the gut microflora to the brain and from the brain to the gut microflora through nervous, endocrine, immune, and humoral ties [48]. Microbiota and the biochemistry of mood changes. Our gut microbes convert food into short-chain fatty acids (SCFA). These SCFAs communicate with neurons that produce serotonin, a neurotransmitter that regulates our mood as well as levels of anxiety and happiness. Another important neurotransmitter, gamma-aminobutyric acid (GABA), regulates and influences mood because it helps to calm the nervous system and to turn off stress responses. Essentially, diet can help bacteria protect mental well-being, because consuming the right foods affects bacterial function [9].

Recent advances in molecular hydrogen research

Research in the field of molecular hydrogen is progressing rapidly. Since our publication in 2016, many new breakthrough findings have been published that open up further dimensions of molecular hydrogen importance including:

- Epigenetic modulation where recent studies have begun to explore how H_2 can influence epigenetic modifications, such as DNA methylation and histone acetylation, which are crucial for gene expression regulation. This could provide novel insights into how H_2 affects long-term cellular function and aging [49]. Emerging research suggests that H_2 may influence the gut microbiome, which in turn can affect systemic oxidative stress and inflammation. This interaction opens new avenues for understanding how H_2 supplementation might benefit gut health and overall metabolic function [50]
- Advances in nanoparticle technology have enabled more efficient delivery of H_2 to specific tissues or cells. These delivery systems can enhance the

bioavailability and therapeutic efficacy of H_2, potentially overcoming some limitations of traditional methods of administration [51]

- Recent studies were focused on the potential of H_2 in treating neurodegenerative diseases like Alzheimer's and Parkinson's. New findings indicate that H_2 can modulate pathways involved in neuroinflammation and protein aggregation offering new hope for therapeutic interventions [52]
- Novel studies have shown that H_2 can enhance the efficacy of conventional cancer therapies, such as chemotherapy and radiotherapy, by reducing oxidative stress and protecting normal cells from damage. This area is gaining traction and could significantly impact cancer treatment protocols [53]
- While earlier publications explored cardiovascular benefits, recent studies have delved deeper into how H_2 affects specific signaling pathways like the Notch and Wnt pathways, which are crucial for cardiac repair and regeneration [54]
- Current research has highlighted the role of H_2 in improving mitochondrial function and biogenesis including its impact on mitochondrial dynamics, such as fission and fusion processes, which are vital for the cellular energy homeostasis and health [55]
- Recent findings suggest that H_2 can influence autophagy and mitophagy, the processes by which cells remove damaged organelles and proteins. This has implications for aging and diseases associated with cellular debris accumulation [56]
- There has been shown an increase in clinical trials investigating the effects of H_2 in various conditions including metabolic syndrome, rheumatoid arthritis, and chronic obstructive pulmonary disease (COPD). These studies provide more robust data on the safety and efficacy of H_2 in human populations [57]
- Novel research is exploring the synergistic effects of H_2 when used in combination with other treatments, such as antioxidants, anti-inflammatory drugs, and lifestyle interventions like exercise and diet. This could lead to more comprehensive treatment strategies [58]

Recent advances in molecular hydrogen research have extended our understanding of its potential therapeutic applications. These include its role in epigenetic modulation, microbiome interaction, advanced delivery systems, neurodegenerative disease and cancer therapy, cardiovascular alterations, mitochondrial function, and planning of clinical trials.

The perspective of using molecular hydrogen in clinical medicine. Summary of potential therapeutic benefits of molecular hydrogen

H_2 may provide important biological benefits for the following medical interventions: a) it has ideal permeability, can quickly reach subcellular compartments through passive diffusion, and protect DNA, RNA, proteins, cell membranes, and mitochondria from damage, b) due to the relative inertness of H_2, it selectively reacts only with the most cytotoxic radicals without eliminating benefitial ROS signaling, c) maintains redox homeostasis by reducing the burden of oxidants through signal modulation (e.g., down-regulation of the NADPH oxidase system), d) activates the Nrf2 pathway with subsequent up-regulation of transcription of endogenous antioxidants (e.g., GSH, CAT, GPx and induction of heme oxygenase-1), and e) reduces the excessive level of pro-inflammatory mediators (e.g., cytokines, COX2, NFAT, etc.) In addition, in some cases, H_2 may increase the production of oxidants (e.g., superoxide), thus potentially providing hormetic benefits. H_2 also increases the expression of Sirt3, maintains the potential of the mitochondrial membrane, increases ATP production, and provides other benefits. H_2 acts as a mitohormetic effector by transiently increasing the production of mitochondrial superoxide, followed by upregulation of Nrf2. It is necessary to continue searching for optimal methods and doses of H_2 administration and more detailed molecular mechanisms of the effect of H_2, some of which are still unclear and require further investigation. The pleiotropic effects of H_2 on various proteins, molecules, and signaling pathways can at least partially explain its almost universal pluripotent preventive and therapeutic potential.

Conflict of Interest

There is no conflict of interest.

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