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Review

Hydrogen: A Rising Star in Gas Medicine as a Mitochondria-Targeting Nutrient via Activating Keap1-Nrf2 Antioxidant System

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Abstract: The gas molecules O_2, NO, H_2S, CO, CH_4, have been increasingly used for medical purposes. Beside these gas molecules, H_2, the smallest diatomic molecule in nature, has become a rising star in gas medicine in the past few decades. As a non-toxic and easily accessible gas, H_2 has shown preventive and therapeutic effects on various diseases of the respiratory, cardiovascular, central nervous and other systems, but the mechanisms are still unclear and even controversial, especially the mechanism of H_2 as a selective radical scavenger. Mitochondria are the main organelles regulating energy metabolism in living organisms, as well as the main organelle of reactive oxygen species generation and target. We propose that the protective role of H_2 may be mainly dependent on its unique penetrating ability to everywhere of the cells to regulate mitochondrial homeostasis by activating the Keap1-Nrf2 phase II antioxidant system, rather than its direct free radical scavenging activity. In this review, we summarize the protective effects and focus on the mechanism of H_2 as a mitochondria-targeting nutrient by activating the Keap1-Nrf2 system in different disease models, and wish to provide a more rational theoretical support for the medical applications of hydrogen.

Keywords: hydrogen; gas medicine; antioxidant; mitochondria; Keap1-Nrf2; Nrf2 activator

1. Introduction

Gas molecules are increasingly being used for medical purposes and have developed into a separate field of medicine. The gases most widely used in medicine include oxygen (O_2), nitric oxide (NO), methane (CH_4). carbon monoxide (CO), hydrogen sulfide (H_2S) and hydrogen (H_2). As shown in Figure 1, the number of articles related to medical gas molecules has grown substantially from 1998 to 2022, especially O_2 and H_2.

O_2 and NO are the first two medical gas molecules that attract the researchers' attention, with tens of thousands of studies focus on these two gases as early as the 1990s. O_2 is the most crucial gas for all living organisms on earth which accounts for around 1/5 of the volume of air. As an important gas to maintain human respiration, O_2 is mainly used to provide supplemental respiration for the sick, astronauts traveling in space, and mountaineers, etc. In addition, it has the function of destroying bacteria. Due to the importance of O_2, the 2019 Nobel Prize in Physiology or Medicine was awarded to Willianm G. Kaelin Jr., Sir Peter J. Ratcliffe and Gregg L. Semenza who discovered how cells sense and adapt to the availability of O_2[1]. NO, a free-radical gas named "laughing gas". At the end of the last century, NO was found to work as a mediator of cell-to-cell communication in vasodilatation, inflammation, and neurotransmission. Robert F. Furchgott, Louis J. Ignarro, and Ferid Murad et al. demonstrated that NO is an important signaling molecule in the cardiovascular system, and this discovery won the 1998 Nobel Prize in Physics or Medicine[2].

CH_4 is the simplest of the organic compounds. For decades, CH_4 was thought to have almost no physiological role, while in the last few years, scientists have realized that CH_4 can play important

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biological roles such as anti-inflammatory, antioxidant, and anti-apoptotic. As a result, CH_4 has been used as a gastric decontaminant in emergency clinical settings of poisoning or drug overdose, and also serves as a passive indicator of colonic function[3].

CO and H_2S have long been known as hazardous factors. Long-term exposure to environments which rich with CO may be fatal. However, a growing amount of research suggests that CO is an important gaseous mediator along with NO and H_2S. Endogenously produced or inhaled CO has important physiological functions in regulating vascular function, inflammation, apoptosis, cell proliferation, and signaling pathways. Studies have shown that inhaled CO suppresses chronic inflammation in patients with stable chronic obstructive pulmonary disease (COPD)[4]. Same as CO, Scientists simply regard H_2S as a harmful gas initially since exposure to H_2S may irritate the eyes and respiratory system. However, scientists have now shown that H_2S is an essential physiological factor as it is produced by bacteria in the human oral cavity and gastrointestinal tract. Being the least appreciated of the three gaseous mediators (gas transport mediators), it is now considered to be an important gas transport mediator after NO and CO. H_2S has been shown to modulate many physiological processes such as vasodilation, anti-inflammation, resistance to oxidative stress, and protection against ischemia-reperfusion injury, etc.[5]

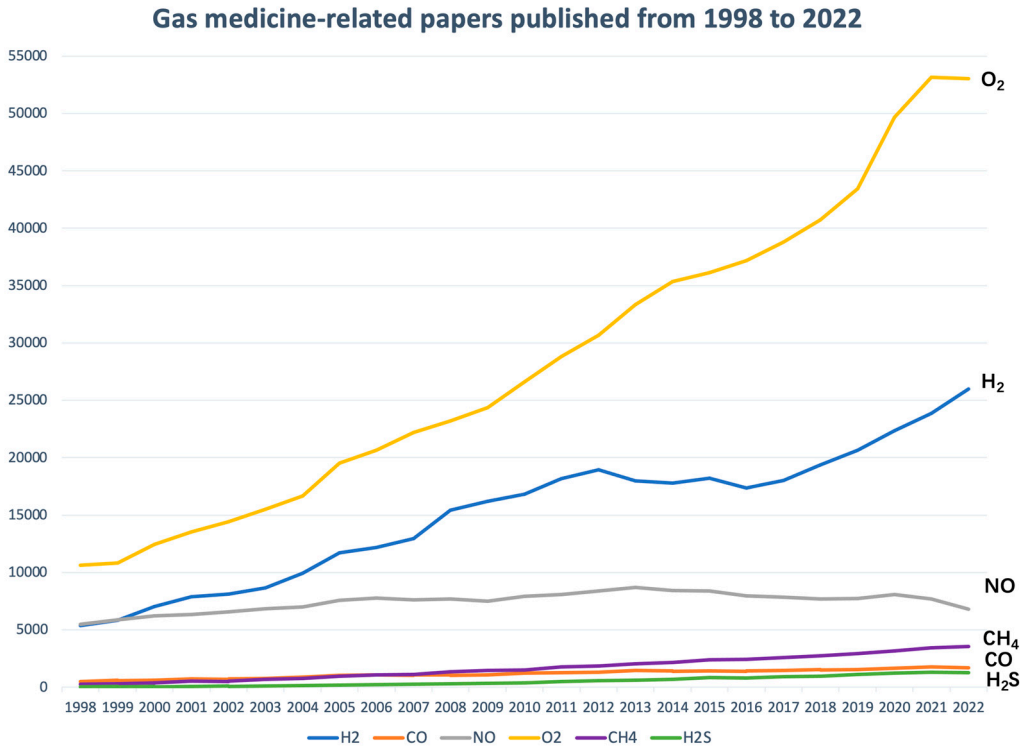

Figure 1. The papers on the most widely used gases (H_2, CO, NO, O2,CH_4,H_2S) for medicine published from 1998 to 2022.

As the smallest of all molecules, the functions of H_2 have also caught the eye of scientists in the field of biomedicine. As early as the beginning of the last century, H_2 was first tested as a diving gas, proving that it is the best breathing medium for medium and deep diving, and is safe for the organism, with no toxic side effects found. Up to now, H_2 biomedicine has investigated the effect and mechanism by which H_2 molecules, including H_2 positive and negative ions and heavy H_2 (deuterium and tritium), act in various diseases[6].

2. History of H_2 medicine

H_2 is known to be a colorless, odorless, and tasteless gas with chemically stable[7]. In general, around 35 mL to 321 mL of H_2 is produced and released through bacterial fermentation by the human digestive system per day[8]. Several ways are used to ingest or consume H_2, such as drinking or

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injecting H_2 water (HW), inhalation of H_2, bathing in HW, dropping H_2 saline into the eyes, etc. H_2 plays an anti-inflammatory and anti-apoptotic effect through its selective antioxidant properties and has become a unique cytoprotective agent[9].

H_2 used to be considered an inert gas, not involved in any life activity. It was not until 1975 that Dole et al. found significant regression of mouse skin tumors in squamous cell carcinoma mice exposed for a fortnight into a mixture of 97.5% H_2 and 2.5% O_2 at a total pressure of eight atmospheres, confirming the medical usefulness of H_2 firstly[10]. Unfortunately, this study has not attracted academic attention due to the technical difficulties of applying hyperbaric H_2 therapy in a clinical application.

Until 1996, Chinese scientist Yuanwei Du noticed the significance of H_2 for life[11]. Dr. Du believes that excessive accumulation of peroxides produced in the metabolic process is the root cause of various diseases and aging, the organism must have a certain mechanism to fight against these peroxides. H_2 is a reducing agent, which can eliminate peroxides naturally without side effects, making creatures achieve a balance in the sense of redox balance. In Du's experiment, tritium gas was produced by electrolysis of tritium water, the tritium gas was then fed instead of H_2 into the mouse living environment. He found that tritium was present in all tissues and organs of mice, which means that tritium gas is involved in the life activities of living organisms by transforming into tritium ions prevalent in living organisms, indirectly proving that the air H_2 is both a constituent substance and an energetic substance of life. This experiment also proves the basic mechanism of H_2 metabolism. A number of H_2 medicine-related papers published by Yuanwei Du at the end of the 20th century further confirmed that H_2 produced by water electrolysis has a pronounced effect on the vital activities of plants (lilac branches), animals (mice), as well as humans[12]. Du's work creatively combines the physiological effects of H_2 with the free radical aging theory, explains the antioxidant activity of H_2 molecules, as well as confirms that H_2 may have an immeasurable effect on a wide range of diseases.

In 2007, Ohsawa et al. from the Nippon Medical School published an important article on H_2 medicine in the journal Nature Medicine[13]. This study used a low concentration of H_2 (1-4%) for inhalation over a short period (35 minutes) to mice and found positive effects in the treatment of the cerebral ischemia-reperfusion injury, showing that short-term inhalation of a low concentration of H_2 for the treatment of the disease is feasible. They proposed a mechanism that H_2 could act as a therapeutic antioxidant, selectively reducing cytotoxic oxygen radicals (•OH and $ONOO^-$), leading to inhibition of cerebral ischemia-reperfusion injury. Because this study was published in the top journal of Nature Medicine, it provides a broad prospect for both basic and clinical research on H_2 and has brought H_2 medicine to the attention of a wide range of academic cycles. Since then, more and more scholars have joined the research on H_2 medicine to explore its effects on various diseases such as inflammation, drug toxicity, and obesity. More than a thousand peer-reviewed research papers have been published to date.

In the beginning, scientists focused mainly on acute and chronic organ injuries related to oxidative stress, such as the animal experiment of drug toxic injury or ischemia-reperfusion injury in vital organs such as the heart and liver. During this period, researchers mostly used diverse injury models to validate the therapeutical effects of H_2 inhalation. Between 2009 and 2012, more research began to appear on drinking H_2-enrich water (HRW)[14] and injecting H_2-enrich saline (HRS)[15], as well as studies on boosting H_2 replenishment through gut bacteria[16]. Meanwhile, a number of clinical studies have used HRW in the treatment of diseases including metabolic syndrome, Parkinson's disease, hemodialysis, sports injuries, and rheumatoid arthritis[9]. For the past few years, on the foundation of previous studies, H_2 medicine research has studied the molecular mechanisms, especially focusing on the molecular pathways of inflammation and oxidative stress mediated by H_2. However, regarding the molecular mechanism of H_2, most scholars have followed the view of Ohsawa et al. in Nature Medicine paper that H_2 is a selective hydroxyl radical (•OH) scavenger, and focused on the antioxidant mechanisms of H_2 mainly based on it[17,18]. Nevertheless, some scholars have proposed that H_2 plays a signaling role that may be involved in metabolic processes and may even provide energy for cells, which is subversive to the development of H_2 research[19,20].

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A number of Chinese researchers have devoted themselves to developing H_2 medicine, which has received more than 80 grants from the National Natural Science Foundation of China and has published hundreds of basic and clinical academic papers. Prof. Xuejun Sun of the Second Military Medical University is one of the leading figures in H_2 medicine in China. Prof. Sun's group engaged in the research of diving hyperbaric medicine for a long time, the most important research object of diving hyperbaric medicine is all kinds of gases that can be breathed by human beings, with H_2 being one of the key types of gases in the field of diving hyperbaric medicine. Sun's group focuses on the biological effects of H_2 and its application in medicine for the first time revealing the value of H_2 in medicine in China. Moreover, Prof. Sun participated in organizing several international symposiums on H_2 medicine, inviting experts from all over the world to discuss the future of H_2 medicine. His team collaborates with medical organizations around the world to carry out research on the application of H_2 medicine and to expand the scope of H_2 applications in the medical field.

Prof. Shucun Qin of Shandong First Medical University is another key promoter of H_2 molecular medicine in China. Prof. Qin established the first H_2 Biomedical Research Institute at the university in 2015, training a number of key researchers in H_2 medicine. He established the standardized laboratory for H_2 molecular biology that has published multiple placebo-controlled population trials, providing important clinical evidence for the translation of H_2 into medicine. Qin's recent review summarizes 51 clinical trials involving 1,213 subjects in four areas of H_2 biomedicine: basic research, exercise, dermatology and healthcare[21]. The results showed that H_2 can reduce oxidative stress damage caused by strenuous exercise, reduce lactic acid build-up after exercise, prevent exercise acidosis, and reduce exercise fatigue. In addition, H_2 intervention can play a positive role in skin beauty, and improve cardiovascular health.

Prof. Xuemei Ma's team at the Beijing Institute of Technology is also an early group of H_2 medicine researchers in China. Prof. Ma is committed to elucidating the biological basis of H_2 medicine at the molecular, cellular and holistic levels, conducting in-depth basic research and clinical translational research, especially in the mechanism of H_2 molecules on tumor prevention. Her team has verified that H_2 can inhibit the proliferation of gliomas (Gliomas) by inducing glial stem cell differentiation in vitro and in vivo experiments[22].

Besides these key researchers, there are hundreds of scientists are doing work on H_2 medicine, including the Chinese academicians of Prof. Nanshan Zhong, Zhaofen Xia, Hongyang Wang, and young scientists like Prof. Qianjun He who proposed the concept of H_2 nanomedicine to address the issues of H_2 medicine by using functional micro/nanomaterials for augmented H_2 therapy in cancer, and Wenbiao Shen who is devoted in the application of H_2 in agriculture. An academic association of H_2 medicine with more than 400 members has been formed. As of today, current clinical studies on H_2 are still continuously emerging and the scale of the studies is gradually expanding. With its favorable biosafety and the convenience of safe low-dose use, the H_2 inhalation device has been included in the Chinese Food and Drug Administration's new medical device development process. Moreover, in Japan, H_2 has been approved as a food supplement.

3. H_2: a mitochondria-targeting molecule/nutrient, rather than a selective •OH scavenger

Sustained oxidative stress leads to the onset and progression of many common diseases. Up to now, little achievement has been gained, although a large number of studies have attempted to develop an effective antioxidant without side effects. Mitochondria, as a major source of oxidative stress, is considered a new therapeutic target for small molecule interventions[23]. H_2 suppresses reactive oxygen species (ROS) accumulation, inhibits the cell death program, maintains the mitochondrial structure and function[24,25]. Preliminary clinical trials suggest that drinking H_2-dissolved water appears to improve the pathology of mitochondrial disease[26,27].

Mitochondria have a double membrane structure that forms the difference in potentials between the inner and outer membranes and controls the movement of diverse molecules and factors (e.g., ions) in and out of the organelle while affecting mitochondrial stability. Although the outer membrane is comparatively permeable to the small molecules and large proteins (which are transported by diffusion or transposases), the inner mitochondrial membrane is highly impermeable

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to most molecules[28]. Nearly special membrane transport proteins (e.g., TIM-TOM (preprotein translocase of the inner membrane of mitochondria-preprotein translocase of the outer membrane of mitochondria) complex, etc.) are needed for all ions and molecules to enter or leave the mitochondrial matrix. This makes most antioxidants cannot enter the mitochondria to effectively scavenge •OH[29,30]. The difference with other antioxidants is as the smallest molecule in nature, H_2 can easily spread and penetrate into the cell membrane to react with organelles such as mitochondria and the nucleus[31].

While the idea that H_2 is a selective antioxidant is popularized[7], it is still not known whether the effects of H_2 are from the direct reaction with •OH or from the inhibition of •OH production. Let's first give a little basic information on free radicals.

As we know, •OH is generated by the Haber−Weiss reaction:

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

This reaction is thermodynamically feasible but kinetically too slow. So, •OH is mainly generated by the Fenton reaction:

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

The three main properties of •OH are below: 1. SHORT LIFE: •OH has a very short half-life (10−9 s, or 1 ns whereas the half-life of superoxide is 15 sec), no time to diffuse (no more than 50 molecular diameters from the site of formation), so the reaction is local with antioxidant at where •OH is produced; 2. HIGH REACTIVITY: •OH is the most ROS with high reduction potential, compared to other oxygen species, it reacts with extremely high rate constants (high reactivity) that approach diffusion-limited, with rate constants of 10^9–10^{10} M−1 s−1. So, •OH is the strongest (most powerful) oxidant of the oxyradicals; 3. UNSELECTIVE and INDISCRIMINATE: •OH reacts unselectively and indiscriminately with almost every type of molecule found in living cells, including lipids, proteins, amino acids, DNA, RNA and sugars. Therefore, the best antioxidant is not a •OH scavenger, but rather an iron chelate to prevent the generation of •OH.

The reaction with many substances in the body at a rate that exceeds that of H_2, which means H_2 is difficult to compete with these molecules effectively in the body, especially when H_2 is at a relatively lower concentration than other endogenous substances. Biokinetic analyses of the intracellular reactions of •OH /$ONOO^-$ show that intracellular molecules, such as nucleic acids and amino acids, react with •OH more readily at a significantly faster rate than H_2[32,33], which implies that H_2 can hardly act as an •OH scavenger or barely direct react with •OH.

In 2005, we first proposed the new concept of "mitochondrial nutrient". The so-called "Mitochondrial nutrients" refer to any compound that can protect mitochondria from damage, repair mitochondria injury, and promote mitochondrial function. Their mechanisms of action may include (1) protect mitochondrial enzymes and / or stimulate enzyme activity by increasing the levels of substrate and cofactors; (2) induce the activation of endogenous antioxidant systems such as phase II enzymes to enhance antioxidant defense; (3) prevent mitochondria from producing ROS and removing ROS in mitochondria, and (4) protect and repair mitochondrial damage, including energy promoters[34-36].

Researchers in our lab reported that in the LPS-induced lung injury mouse model, hyperoxic HRS effectively reduced mitochondrial swelling and cristae breaks, as well as reversed the reduction of mitochondrial complex I, IV, and V activities significantly[37,38]. Not coincidentally, in the high-fat diet (HFD)-induced liver injury model, coral calcium hydride (CCH, a solid form of molecular H_2 carrier made from coral calcium) treatment improved glucose and lipid metabolism, ameliorated hepatic mitochondria abnormalities, restored the protein expression and the activity of complex II, while also activated phase II enzymes[37,38]. These studies imply that H_2 is able to target mitochondria, as a highly promising mitochondrial nutrient.

Ohsawa et al.[13] used antimycin A (an inhibitor of mitochondrial respiratory complex III) to induce excess $O_2^{\bullet-}$ production. In this model, $O_2^{\bullet-}$ rapidly converted to H_2O_2, which was further converted to •OH. Their result showed that H_2 treatment prevented the decrease in mitochondrial membrane potential caused by antimycin A treatment, believing that H_2 protects mitochondria from •OH damage. The researchers hypothesized that H_2 enters the mitochondria and acts on the

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mitochondrial respiratory chain, weakening the Fenton reaction by inhibiting transition metal activity and ultimately inhibiting •OH production but not scavenging •OH directly[39]. Accordingly, H_2 is considered as a potential and promising mitochondria-targeting molecule or nutrient that acts as a redox homeostasis regulator[40].

As is well known, H_2 is a moderate/mild reducing agent (The standard reduction potential of H^+/H_2 at PH7 is -0.42, stronger than NAD^+/NADH -0.32 but weaker than acetate/acetaldehyde -0.60), barely able to scavenge •OH directly in a living body (Fig 2). Because mitochondria are the main sites of ROS generation and the targets of ROS, we suggest that the more important mechanism of H_2 molecule maybe that can easily enter cells and subcellular organelles, including mitochondria, to play a protective role through their strong penetration ability, then activating the Keap1-Nrf2 (Kelch-1ike ECH-associated protein l, NF-E2-related factor 2) antioxidant defense system, to inhibit oxidative damage and improve the mitochondrial function, finally prevent and improve a various of disease. H_2 has been shown to significantly activate the Keap1-Nrf2 system, regulate the activities of endogenous antioxidants, and enhance the ability of cells to fight against damage[41].

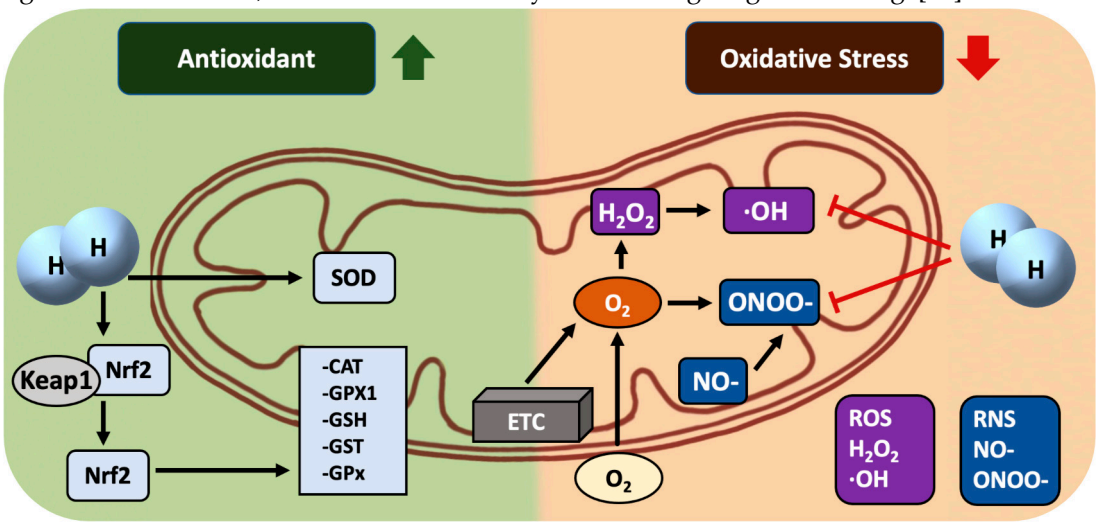

Figure 2. Mechanisms of mitochondria-targeting by molecular H_2: 1) Barely reacting with •OH and $ONOO^-$ directly; 2) Mainly activating Keap1-Nrf2 antioxidant systems indirectly.

4. The mechanisms of H_2 as an Nrf2 activator

Nrf2 is a key factor in the regulation of oxidative stress which belongs to the CNC-BZIP transcription factor family. Upon normal physiological conditions, Nrf2 binds to Keap1 to form a complex present in the cytoplasm in a low-activity state[42]. When the organism is stimulated by oxidative stress or other pathological conditions, the cysteine residue of Keap1 is modified or Nrf2 is phosphorylated, then, Nrf2 is released from the complex and translocated to the nucleus where it binds to the antioxidant response elements (AREs) sequence in the nucleus, initiating NRF2-mediated transcriptional processes to activate a series of phase II antioxidant enzymes to generate antioxidants to scavenge ROS and other harmful substances.

It is reported that various ways can activate Nrf2, among which the Keap1-Nrf2 pathway is the most classical Nrf2 activation pathway. Keap1 contains multiple oxidative stress response sensor proteins which have different physiological functions in response to different forms of stress. Up to now, several studies have demonstrated that H_2 activated Nrf2 through the Keap1-Nrf2 system[43,44], but the clear mechanism of the activation is not known.

Nrf2 inducers are diverse, of which most are electrophilic and readily react with Keap1 by the cysteine thiol groups. Among them, Cys151/Cys273/Cys288 plays a fundamental role in the perception of electrophilic Nrf2-inducing chemicals. Therefore, the Nrf2 inducer has been divided into different categories based on the different cysteine residues of Keap1 they react with (Table 1). The first class specifically targets the Cys151 sensor, such as medically relevant bardoxolone methyl. Bardoxolone methyl acts as an electrophilic inducer of Nrf2 that forms a covalent interaction with the

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Cys151 residue of Keap1, thereby inhibiting Nrf2 ubiquitination. In mice, the Cys151 point mutation in Keap1 eliminated Nrf2 signaling and hepatoprotective effect of bardoxolone methyl in vivo[45]. The second class of inducer targets Cys288, and 15-deoxy-prostaglandin J2 (15d-PGJ2) has been identified in this group. 15d-PGJ2, one of the endogenous Nrf2 inducers synthesized from arachidonic acid, forms a covalent compound with Keap1 to compete for the Keap1-Nrf2 binding. Class III targets Cys151/Cys273/Cys288, such as 4-hydroxynonenal (4-HNE). Mass spectrometry analysis revealed that 4-HNE directly modifies cysteine residues on Keap1 and deregulates its inhibition of Nrf2 by inhibiting Keap1, further increasing the expression levels of Nrf2 target genes (e.g. TXNRD1, thioredoxin reductase-1)[46]. Indeed, Nrf2 activation was significantly reduced when Cys151 was mutated, whereas Nrf2-induced target gene activation was only slightly affected when Cys273 and Cys288 residues were mutated[47,48].

Table 1. Classification of Nrf2 inducers targeting Keap1-Nrf2.

		Mechanism	Example
Class I	Elec-trophilic	Cys151-dependent compounds	Bardoxolone methyl Sulforaphane, Dimethyl-fumarate
Class II		Target Cys288	15d-PGJ_2
Class III		React with any of the three sensor cysteines Cys151/Cys273/Cys288	4-HNE, $NaAsO^2$, 9-nitro-octadec-9-enoic acid
Class IV		Target cysteines Cys77/ Cys434	Pubescenoside A
Class V	Non-Electrophilic	Target Cys226/Cys613/Cys622/Cys624	H_2O_2, Cadmium chloride, Zinc chloride, Prostaglandin A2
Class VI		Protein-protein interaction inhibitors (PPI)	CPUY192018

In addition, the electrophilic compound that activated Nrf2 on the cysteine residues other than Cys151/Cys273/Cys288, we classify as Class IV. The compounds of this group include, for example, Pubescenoside A, which acts on Cys77/ Cys434.

Moreover, several inducers activate Nrf2 in a more complex way than previously identified electrophilic sensors that bind to Cys226, Cys613, Cys622, and Cys624. We classify them as Class V. Hydrogen peroxide (H_2O_2), a key ROS molecule important in cellular physiology, is representative of this classification. Suzuki et al. revealed that Keap1 uses cysteine residues to create a special mechanism to make a disulfide bond between any combination of Cys226, Cys613, Cys622 and Cys624 to sense H_2O_2[49]. This sensing mechanism is different from that used by the electrophilic Nrf2 inducer.

There is also a kind of inducers that do not act through the cysteine of Keap1 which have been classified as a Class VI, they directly inhibit the interaction between Keap1 and Nrf2, such as non-electrophilic protein-protein interaction inhibitors (PPIs)[50]. Horie et al. suggested that Keap1 binding to Nrf2 is a 'hinge and latch model', with PPIs actively using a hinge-locking mechanism, whereas electrophilic Nrf2 activators do not use this mechanism when activating Nrf2[51].

As the smallest and one of the simplest molecules, H_2 molecules have the capacity to pass through the Keap1 and Nrf2 binding structure and play the role of an activator, the mechanism of Nrf2 activation by H_2 seems different from the mechanism of perception of electrophilic Nrf2 inducers but may be closer to the mechanism of class V and VI to inhibit the interaction between Keap1 and Nrf2 (Fig.3)[49]. Up to now, the activation of Nrf2 and its mediated antioxidant enzyme system by H_2 has been reported in a variety of tissue-associated diseases, including brain, lung, liver, heart, ovary and kidney[43,52,53].

A result in neuroblastoma cells showed that exposure of SH-SY5Y cells to H_2 increased the production of mitochondrial superoxide. This process was accompanied by Nrf2 nucleus translocation, as well as increased expression of Nrf2-regulated antioxidant enzymes, suggesting that H_2 alleviates mitochondrial oxidative stress through activating Nrf2[54]. Inhaled H_2 also reduces neuroinflammation in memory-related regions through increasing Nrf2 protein expression in a

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sepsis-induced blood-brain barrier impairment and memory dysfunction[55,56]. Interestingly, one of the studies we were involved in reported that H_2 (2%-4%) protected against delayed encephalopathy after acute carbon monoxide poisoning and this protective effect was related to the involvement of Nrf2 and its mediated phase II enzyme system[57].

Similar results were obtained in the lung from seawater instillation-induced acute lung injury rabbit or cecal ligation and puncture-induced sepsis mice, which proved that H_2 could regulate the expression of heme oxygenase-1 (HO-1), the Nrf2 downstream antioxidant protein[58,59]. Inhaled H_2 significantly alleviated the drop in blood O_2 during hyperoxic exposure, remitted lung inflammation, and upregulated the HO-1 expression. However, H_2 could not attenuate hyperoxic lung injury or induce HO-1 in Nrf2-KO mice, suggesting that H_2 could improve the hyperoxic lung injury through the Nrf2-HO-1 pathway[60]. In the sepsis-induced acute lung injury model, H_2 molecules inhibited high mobility group protein1 (HMGB1) expression by activating the Nrf2-HO-1 pathway [61,62]. The latest research has revealed that H_2 also affected COVID-19-induced lung injury via Nrf2[63].

Sun et al.[44] demonstrated that H_2 administration reduced oxidative stress in the LPS-treated mice livers through activation of the Keap1-Nrf2 system. Moreover, Liu et al.[52] reported that H_2 improved lipid accumulation by modulating the miR-136/MEG3 /Nrf2 pathway in non-alcoholic fatty liver disease.

In the ischemia model induced in the H9C2 cell line, the H_2 gas-rich medium reduced the production of •OH, promoted Nrf2 nuclear translocation and regulated the Nrf2-HO-1 pathway, suggesting that H_2 can preserve ischemic cardiomyocytes by stimulating the Nrf2 pathway[64]. H_2 amelioration of LPS-injured HUVECs injured and inflammatory responses through Nrf2 and its downstream protein HO-1[65].

In the long-term cyclosporine A (CsA) induced nephrotoxicity model, HRW reduced the ROS and MDA levels, increased the activities of GSH and SOD, then improved the vascular and renal functions of rats with renal damage. Meanwhile, HRW significantly decreased the level of Keap1 while increasing the expression of Nrf2, NADPH dehydrogenase quinone1, and HO-1. Suggesting that HRW restored the balance of the redox state and improved CsA-induced renal function suggesting that HRW restored the balance of the redox state and improved CsA-induced renal function by activating the Keap1-Nrf2 signaling pathway[43].

In the ovarian injury rat model induced by cisplatin, HRS recovered the activity of SOD and catalase, reduced MDA levels in serum and ovarian tissues, as well as increased ovarian Nrf2 expression[66]. Inhalation of 2% H_2 also attenuated severe sepsis-induced intestinal injury by modulating HO-1 and HMGB1 release mice[67].

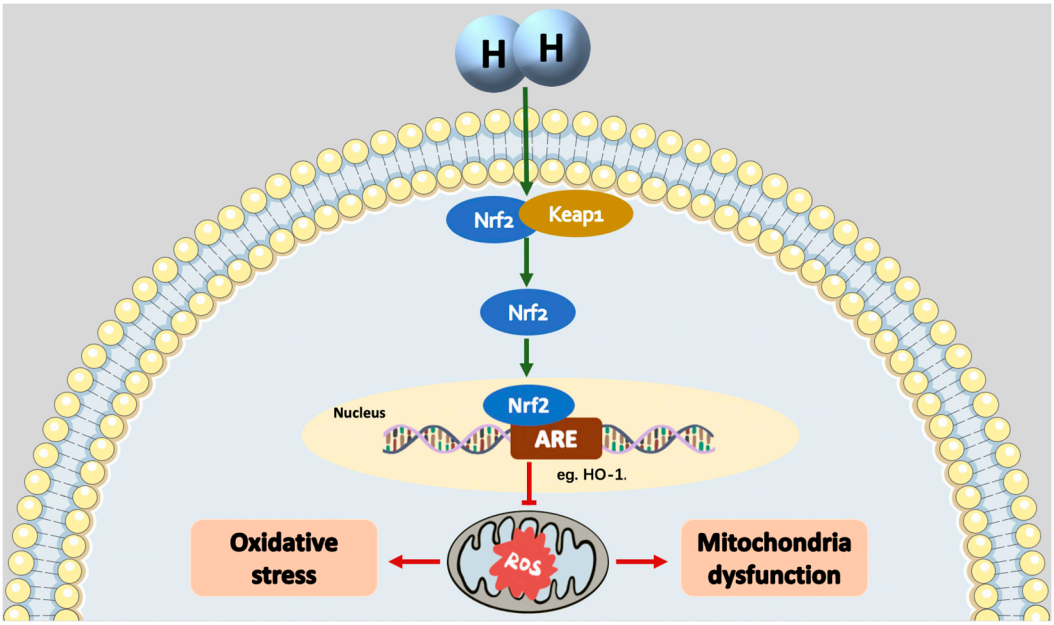

Figure 3. H_2 may activate Nrf2 and its mediated phase II enzyme system via non-electrophilic protein-protein interactions.

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5. The Medical effects of H_2: Focus on the effect on mitochondria

A great number of basic and clinical studies have found that H_2 is an important physiological regulator that protects against tissue-related diseases such as lung, heart, central nervous system, renal, pancreas, etc., through protective effects such as antioxidant, anti-inflammatory, and anti-apoptotic effects. Mitochondrial dysfunction is closely related to disease development[35]. In this section, we focus on the effects of H_2 on mitochondrial function in different diseases.

5.1. Effects of H_2 on Respiratory System Diseases

To date, molecular H_2 has been reported to have positive effects in the prevention and treatment of acute lung injury, chronic obstructive pulmonary disease, asthma, and pulmonary hypertension[63]. Of interest, the National Health Commission of China (NHC 7th Edition Trial: Beijing, 2020) and the Chinese Centre for Disease Control and Prevention (CDCP 6th Edition Trial: Beijing, 2020) recommend effective O_2 therapy as one of the modalities for the general treatment of patients with COVID-19. They also noted that inhalation of a mixture of molecular H_2 and O_2 (66.6% H_2 - 33.3% O_2) is more effective than inhalation of O_2 alone[68]. The research in our lab showed that H_2-enriched and oxygenated saline inhibited LPS-induced lung injury in C57BL/6 mice through the NF-κB/NLRP3 signaling pathway. H_2 performed a more significant effect in inflammatory and anti-apoptotic mechanisms, while O_2 enhanced the hypoxic of the organism, with the combined protective effect of the two gases being better than their respective effects[69].

2% H_2 inhalation improves mitochondria function through increased mitochondrial-membrane potential and ATP levels, as well as promotes the activity of mitochondrial-respiration complex I and complex II. H_2 also regulates mitochondria dynamics which decreases the expression of mitochondria fission protein Drp1 but increases the expression of mitochondria fusion protein mitofusin-2 (MFN2)[70].

Post-transplant morbidities such as graft ischemia-reperfusion damage and graft-versus-host disease are key challenges in transplantation. H_2 acts as a prophylactic agent against post-transplant complications in several animal models of organ transplantation[71]. In the rat lung transplantation model, the combination of mechanical ventilation and prolonged cold ischemia resulted in a significant reduction of gas exchange in rat lung tissue (treatment with 98% O_2 plus 2% nitrogen), while treatment with 98% O_2 plus 2% H_2 inhibited the increased tendency of pro-inflammatory cytokines and apoptotic molecules, upregulate the expression of HO-1 in the lung grafts[72]. Not only that, H_2 molecules inhibited the levels of pro-apoptotic proteins caspase-3 and caspase-8 in lung grafts, activated the expression of anti-apoptotic proteins Bcl-2 and Bcl-xL, and stabilized the mitochondrial outer membrane, prevented the cytochrome c release into the cytosol[73]. In addition, advanced treatment of rat lung donors with H_2 induces the gene expression of stress response and ATP synthesis[74].

H_2 is considered as a potential radioprotective agent[75]. In radiation-injured lung epithelial cell line A549, H_2 down-regulates the gene expression of pro-apoptotic Bax and inhibits its translocation to mitochondria through an unknown mechanism[76].

5.2. Effects of H_2 on Cardiovascular System Diseases

Molecular H_2 has shown many benefits in cardiovascular disease (CVD) applications and can be used to treat a wide range of CVD that cover ischemia-reperfusion injury, atherosclerosis, cardiac hypertrophy, radiation-induced cardiac damage, chemotherapy-induced cardiotoxicity[77-79]. We evaluated the influence of inhaled H_2 on heart and nerve function after cardiopulmonary resuscitation by comparing the effects of H_2 inhalation in the rat cardiac arrest asphyxiation model. The results showed that compared with O_2, serum troponin T and S100B were significantly reduced after inhaling H_2. In the meanwhile, left ventricular ejection fraction, cardiac function and neurological function were significantly improved after H_2 inhalation[79].

H_2 increases autophagy by promoting autophagic flow thereby alleviating harmful stress[80]. HRS was found to promote PINK1/Parkin-mediated autophagy, activate mitochondrial autophagy,

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cause damaged mitochondria to be engaged by lysosomes, further ameliorate the inflammatory response and apoptosis induced by myocardial Ischemia/reperfusion (MI/R)[81]. Feng et al. reported that HRS combined with early aerobic exercise enhances acute myocardial infarction-induced superoxide dismutase levels and total antioxidant capacity, promotes mitochondrial and DNA repair by partially regulating the expression of antioxidant-associated proteins and mitochondria-associated proteins and protects against myocardial injury after MI[82].

HRW protects cardiac and aortic graft recipients from inflammation-related deterioration and improves allograft survival by decreasing endothelial cell proliferation, inhibiting T-cell proliferation, and reducing oxidative stress, in the heterotopic heart transplantation rat model[83]. This protection mechanism also correlates with ATP levels, increases enzyme activity of complex II, III, and V on the mitochondrial respiratory chain.

Sepsis is associated with systemic infections and inflammatory responses induced by the cardiovascular system[84]. In the sepsis-induced myocardial injury mice model, molecules H_2 promote protein increase of HO-1, MFN2, and PGC1-1α expression, inhibited sepsis-induced mitochondrial dysfunction, and remodeled fatty acid oxidation of the heart in the sepsis model by increasing myocardial energy[85,86].

Oxidative stress is a major risk factor for worsening LV hypertrophy. Yu et al. found that H_2 saline water improves mitochondria function by restoring electron transport chain enzyme activity, inhibiting ROS formation, and increasing ATP production in spontaneously hypertensive rats with LV hypertrophy. H_2 saline water also inhibits oxidative stress, inflammatory processes, and angiotensin II[87].

Zhang et al. found that HRS treatment ameliorates vascular functional abnormalities such as aortic hypertrophy and endothelial dysfunction in spontaneously hypertensive rats by alleviating oxidative stress, restoring pressure receptor function, preserving mitochondrial function and increasing NO bioavailability[88].

5.3. Effects of H_2 on Nervous System Diseases

H_2 is engaged in the restoration of neurodegenerative diseases[89,90]. Research in our laboratory administered HRW to Alzheimer's Disease (AD) mice for 3 consecutive months to study its effect on cognitive function. The result showed that HRW significantly improved cognitive behaviors, also ameliorated oxidative stress and inflammatory responses in the brains of female AD mice. Moreover, estrogen levels are closely related to mitochondrial function, such as 17β-estradiol enhances mitochondrial signaling clusters. Our results suggest that the effects of molecular H_2 in female AD mice were most likely attributable to estrogen ERβ signaling[91].

Chen et al. reported that H_2 treatment blocks the opening of the mitochondrial permeability transition pore in neurons. 75% H_2 inhalation ameliorates mechanical damage to spinal cord neurons in a dose-dependent manner, significantly inhibits the production of ROS and oxidative stress markers, inhibits neuronal apoptosis, restores mitochondrial function[24].

The results of a clinical trial on Parkinson's disease showed that H_2 significantly improved neurodegenerative symptoms with a therapeutic effect comparable to non-ergot dopamine treatment. Researchers hypothesized that this may be achieved by H_2 improving cellular energy metabolism by targeting mitochondria[92]. In another experiment, H_2 treatment significantly increased the levels of ATP and $\Delta\psi m$ in neuroblastoma[53], further confirming the role of H_2 in activating oxidative phospho-rylation and mitochondrial energy.

HRS improves neuronal ischemia-reperfusion by improving mitochondrial function and reducing oxidative stress[93]. Earlier studies have found that H_2 restores mitochondrial structural damage while reducing microRNA-210 in hypoxia-reperfusion neural model[94]. HRS also ameliorated the activation of caspase-3, attenuated ROS accumulation, closed mitochondrial permeability transition pores and restored mitochondrial membrane potential in isoflurane-induced cognitive impairment mice. This suggests that HRS has the potential to attenuate anesthetic neurotoxicity[95].

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5.4. Effects of H_2 on Digestive System Diseases

Majority of gastrointestinal microbial species show a genetic ability to metabolize H_2, which means that H_2 may influence the composition of gut bacteria and modulate digestive-related diseases[96,97]. It was found that HW inhibited rat intestinal I/R-induced oxidative stress, apoptosis and inflammation[98].

Clinical data suggest that H_2 may improve glucose metabolism by interfering with the gut microbiota of impaired fasting glucose patients[99]. Another study in patients with clinical stage IV colorectal cancer found that H_2 inhalation activated PGC-1α expression and enhanced mitochondrial activity, thereby reducing the proportion of PD-1 and $CD8^+$ T cells. The reduction of these cells was associated with improved cancer prognosis[100].

5.5. Effects of H_2 on Metabolic Syndrome

Mitochondrial dysfunction results in reduced mitochondrial biogenesis and increased ROS, which has been involved in the pathogenesis of a number of metabolic diseases including diabetes and obesity. It has been widely demonstrated that H_2 can scavenge ROS directly by inhibiting ROS production or indirectly by enhancing antioxidant enzyme activity, suggesting that this may be contributing to the improved mitochondrial function in metabolic disorders. Numerous studies have proven the protective effects of H_2 on metabolic syndrome, which include lowering total cholesterol, total triglycerides (TG) and low-density lipoprotein (LDL)[101], reducing serum glucose and insulin levels in mice[102], as well as modifying adiposity and body weight in db/db obese mice[103]. The protective effect of H_2 on diabetes and its complications may be associated with the inhibition of oxidative stress, inflammation, apoptosis, activation of the mitochondrial ATP-sensitive potassium (Mito-K-ATP) pathway etc[104].

Ma et al. proved that H_2 promotes fatty acid oxidation by transporting fatty acids to mitochondria and subsequent catabolism to ketone bodies in rats[105]. A clinical study evaluated the effects of H_2 supplementation in ten middle-aged overweight women on the indicators such as hormonal status and mitochondrial function. The results showed a significant decrease in body fat, arm fat index, serum TG and insulin levels after 4 weeks of oral administration of H_2-generating minerals. Fasting blood lactate accumulation reflects mitochondrial dysfunction, which in turn affects the risk of metabolic diseases. After H_2 intervention 4 weeks, blood lactate levels were significantly lower than those in the placebo group, implying that the improvement in mitochondrial function may be related to the anti-obesity effect of H_2[106]. However, due to the small number of subjects in this study, the reliability of this result is limited, a long-term large-scale trial is needed to further verify the improvement of H_2 on obesity.

Another clinical research in our lab suggests that H_2 may have a potentially beneficial effect on glucose metabolism by interfering with the gut microbiota of individuals with impaired fasting glucose. Not only that, HRW may play an important role in reducing body fat and reducing fatty liver. This suggests its potential as a therapeutic intervention to improve lipid metabolism and liver health[99].

5.6. The Others

H_2 restores mitochondrial oxidoreductase activity while preventing the downward trend of mitochondrial membrane potential. It ameliorated tertbutyl hydroperoxide-induced THP-1 (human acute monocytic leukemia cell line) cytotoxicity by inhibiting fatty acid peroxidation and mitochondrial dysfunction[107]

Mikako et al. reported a 12-week double-blind trial of five patients with progressive muscular dystrophy (PMD), four patients with polymyositis/dermatomyositis (PM/DM), and five patients with mitochondrial myopathy (MM), in which the patients consumed 1.0 liters of HRW per day, and 18 serum markers were measured every four weeks. The results showed a significant improvement in lactate levels in the MM patients after drinking HRW. The lactate-to-pyruvate ratio in patients with DM also showed a favorable response[108].

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4. Conclusions and perspectives

In conclusion, H_2 medicine has risen as a bright star in gas medicine, but it faces a few problems. Firstly, in H_2 basic research area, although a large number of H_2 medicine-related studies have been carried out, the mechanisms of H_2 effects are quite controversial. People do not have a high level of awareness of H_2, doubts still exist about the efficacy and safety of H_2. Therefore, more specific and clear mechanisms need to be clarified. This requires more outstanding scientists to join and make more efforts. This review attempts to challenge the view that H_2 is a selective •OH scavenger by proposing that H_2 is a mitochondria-targeting molecule/nutrient via activating the Keap1-Nrf2 antioxidant system. Of course, this is a quite premature idea and needs more and further investigations to test and challenge.

Secondly, in H_2 industry, the market demand for H_2 health products is insufficient. There are still many technical bottlenecks in the H_2 medicine industry, such as low efficiency of H_2 preparation, high storage and transport costs. In addition, the industrial chain of H_2 medicine is incomplete, as well as lacks the development of relevant standards. The H_2 health industry involves a number of links, such as H_2 preparation, storage and transport, H_2 generators, H_2 testing, and so on. At present, these links have not formed a complete industrial chain, the connection between the links is not smooth enough. Due to the lack of complete and well-defined standards, the H_2 industry chain is difficult to regulate with high quality.

Thirdly, insufficient policy support for H_2 medicine. While the H_2 health industry has a great potential for development, the current government support for the H_2 health industry is insufficient and there are some deficiencies in the policy support, in terms of there is a lack of clear policy planning and support measures. Therefore, the market prospect of the H_2 medicine industry is promising, which urgently needs to be promoted.

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References

1. Wilson, J.W.; Shakir, D.; Batie, M.; Frost, M.; Rocha, S. Oxygen-sensing mechanisms in cells. *The FEBS journal* **2020**, *287*, 3888-3906, doi:10.1111/febs.15374.
2. Yu, B.; Ichinose, F.; Bloch, D.B.; Zapol, W.M. Inhaled nitric oxide. *Br J Pharmacol* **2019**, *176*, 246-255, doi:10.1111/bph.14512.
3. Roccarina, D.; Lauritano, E.C.; Gabrielli, M.; Franceschi, F.; Ojetti, V.; Gasbarrini, A. The role of methane in intestinal diseases. *Am J Gastroenterol* **2010**, *105*, 1250-1256, doi:10.1038/ajg.2009.744.
4. Morse, D.; Sethi, J.; Choi, A.M. Carbon monoxide-dependent signaling. *Crit Care Med* **2002**, *30*, S12-17.
5. Kabil, O.; Motl, N.; Banerjee, R. H2S and its role in redox signaling. *Biochimica et biophysica acta* **2014**, *1844*, 1355-1366, doi:10.1016/j.bbapap.2014.01.002.
6. Zheng, X.F.; Sun, X.J.; Xia, Z.F. Hydrogen resuscitation, a new cytoprotective approach. *Clinical and experimental pharmacology & physiology* **2011**, *38*, 155-163, doi:10.1111/j.1440-1681.2011.05479.x.
7. Hong, Y.; Chen, S.; Zhang, J.M. Hydrogen as a selective antioxidant: a review of clinical and experimental studies. *The Journal of international medical research* **2010**, *38*, 1893-1903, doi:10.1177/147323001003800602.
8. Levitt, M.D. Production and excretion of hydrogen gas in man. *The New England journal of medicine* **1969**, *281*, 122-127, doi:10.1056/nejm196907172810303.
9. Du, D.; Zhao, L.; Shen, M.; Noda, M.; Qin, S.; Long, J.; Sun, X.; Liu, J. Hydrogen medicine: A rising star in gas medicine. **2020**, *03*, 153-161, doi:10.1142/s2575900020300052.

Preprints (www.preprints.org) | NOT PEER-REVIEWED | Posted: 31 October 2023 doi:10.20944/preprints202310.1953.v1

10. Dole, M.; Wilson, F.R.; Fife, W.P. Hyperbaric hydrogen therapy: a possible treatment for cancer. *Science (New York, N.Y.)* **1975**, *190*, 152-154, doi:10.1126/science.1166304.
11. Du, Y.; Wei, C. The reflection on the materiality of qi and its application prospect. *Zibo Keji Bao (Zibo Sci & Tech News)* **1996**.
12. Du, Y.; Wei, C. New scientific topics — a preliminary study on the significance of hydrogen in life activities.ity of qi and its application prospect. *Journal of Shandong Normal University* **1999**, *2*, 196–197
13. Ohsawa, I.; Ishikawa, M.; Takahashi, K.; Watanabe, M.; Nishimaki, K.; Yamagata, K.; Katsura, K.; Katayama, Y.; Asoh, S.; Ohta, S. Hydrogen acts as a therapeutic antioxidant by selectively reducing cytotoxic oxygen radicals. *Nature medicine* **2007**, *13*, 688-694, doi:10.1038/nm1577.
14. Sato, Y.; Kajiyama, S.; Amano, A.; Kondo, Y.; Sasaki, T.; Handa, S.; Takahashi, R.; Fukui, M.; Hasegawa, G.; Nakamura, N.; et al. Hydrogen-rich pure water prevents superoxide formation in brain slices of vitamin C-depleted SMP30/GNL knockout mice. *Biochemical and biophysical research communications* **2008**, *375*, 346-350, doi:10.1016/j.bbrc.2008.08.020.
15. Sun, Q.; Kang, Z.; Cai, J.; Liu, W.; Liu, Y.; Zhang, J.H.; Denoble, P.J.; Tao, H.; Sun, X. Hydrogen-rich saline protects myocardium against ischemia/reperfusion injury in rats. *Experimental biology and medicine (Maywood, N.J.)* **2009**, *234*, 1212-1219, doi:10.3181/0812-rm-349.
16. Kajiya, M.; Sato, K.; Silva, M.J.; Ouhara, K.; Do, P.M.; Shanmugam, K.T.; Kawai, T. Hydrogen from intestinal bacteria is protective for Concanavalin A-induced hepatitis. *Biochemical and biophysical research communications* **2009**, *386*, 316-321, doi:10.1016/j.bbrc.2009.06.024.
17. Penders, J.; Kissner, R.; Koppenol, W.H. ONOOH does not react with H2: Potential beneficial effects of H2 as an antioxidant by selective reaction with hydroxyl radicals and peroxynitrite. *Free radical biology & medicine* **2014**, *75*, 191-194, doi:10.1016/j.freeradbiomed.2014.07.025.
18. Yu, Y.; Yang, Y.; Bian, Y.; Li, Y.; Liu, L.; Zhang, H.; Xie, K.; Wang, G.; Yu, Y. Hydrogen Gas Protects Against Intestinal Injury in Wild Type But Not NRF2 Knockout Mice With Severe Sepsis by Regulating HO-1 and HMGB1 Release. *Shock (Augusta, Ga.)* **2017**, *48*, 364-370, doi:10.1097/shk.0000000000000856.
19. Hyspler, R.; Ticha, A.; Schierbeek, H.; Galkin, A.; Zadak, Z. The Evaluation and Quantitation of Dihydrogen Metabolism Using Deuterium Isotope in Rats. *PLoS One* **2015**, *10*, e0130687, doi:10.1371/journal.pone.0130687.
20. Itoh, T.; Fujita, Y.; Ito, M.; Masuda, A.; Ohno, K.; Ichihara, M.; Kojima, T.; Nozawa, Y.; Ito, M. Molecular hydrogen suppresses FcepsilonRI-mediated signal transduction and prevents degranulation of mast cells. *Biochemical and biophysical research communications* **2009**, *389*, 651-656, doi:10.1016/j.bbrc.2009.09.047.
21. Jiang Xue; Liu Boyan; Wu Fenglin; Xue Yazhuo; Shucun, Q. Progress in clinical research on the use of hydrogen molecules in preventive health care. *Chinese Journal of Geriatric Care* **2023**, 117-122.
22. Liu, M.Y.; Xie, F.; Zhang, Y.; Wang, T.T.; Ma, S.N.; Zhao, P.X.; Zhang, X.; Lebaron, T.W.; Yan, X.L.; Ma, X.M. Molecular hydrogen suppresses glioblastoma growth via inducing the glioma stem-like cell differentiation. *Stem Cell Res Ther* **2019**, *10*, 145, doi:10.1186/s13287-019-1241-x.
23. Smith, R.A.J.; Hartley, R.C.; Cochemé, H.M.; Murphy, M.P. Mitochondrial pharmacology. *Trends in Pharmacological Sciences* **2012**, *33*, 341-352, doi:https://doi.org/10.1016/j.tips.2012.03.010.
24. Chen, X.; Cui, J.; Zhai, X.; Zhang, J.; Gu, Z.; Zhi, X.; Weng, W.; Pan, P.; Cao, L.; Ji, F.; et al. Inhalation of Hydrogen of Different Concentrations Ameliorates Spinal Cord Injury in Mice by Protecting Spinal Cord Neurons from Apoptosis, Oxidative Injury and Mitochondrial Structure Damages. *Cell Physiol Biochem* **2018**, *47*, 176-190, doi:10.1159/000489764.
25. Zhong, H.; Song, R.; Pang, Q.; Liu, Y.; Zhuang, J.; Chen, Y.; Hu, J.; Hu, J.; Liu, Y.; Liu, Z.; et al. Propofol inhibits parthanatos via ROS-ER-calcium-mitochondria signal pathway in vivo and vitro. *Cell Death Dis* **2018**, *9*, 932, doi:10.1038/s41419-018-0996-9.
26. Ostojic, S.M. Targeting molecular hydrogen to mitochondria: barriers and gateways. *Pharmacological research* **2015**, *94*, 51-53, doi:10.1016/j.phrs.2015.02.004.
27. Ito, M.; Ibi, T.; Sahashi, K.; Ichihara, M.; Ito, M.; Ohno, K. Open-label trial and randomized, double-blind, placebo-controlled, crossover trial of hydrogen-enriched water for mitochondrial and inflammatory myopathies. *Medical gas research* **2011**, *1*, 24, doi:10.1186/2045-9912-1-24.
28. Murphy, M.P.; Smith, R.A. Drug delivery to mitochondria: the key to mitochondrial medicine. *Advanced drug delivery reviews* **2000**, *41*, 235-250, doi:10.1016/s0169-409x(99)00069-1.
29. Herrmann, J.M.; Neupert, W. Protein transport into mitochondria. *Current Opinion in Microbiology* **2000**, *3*, 210-214, doi:https://doi.org/10.1016/S1369-5274(00)00077-1.
30. Ohta, S. Recent progress toward hydrogen medicine: potential of molecular hydrogen for preventive and therapeutic applications. *Current pharmaceutical design* **2011**, *17*, 2241-2252, doi:10.2174/138161211797052664.
31. Ohta, S. Molecular hydrogen is a novel antioxidant to efficiently reduce oxidative stress with potential for the improvement of mitochondrial diseases. *Biochimica et Biophysica Acta (BBA) - General Subjects* **2012**, *1820*, 586-594, doi:https://doi.org/10.1016/j.bbagen.2011.05.006.

Preprints (www.preprints.org) | NOT PEER-REVIEWED | Posted: 31 October 2023 doi:10.20944/preprints202310.1953.v1

32. Zhai, X.; Chen, X.; Ohta, S.; Sun, X. Review and prospect of the biomedical effects of hydrogen. *Medical gas research* **2014**, *4*, 19, doi:10.1186/s13618-014-0019-6.
33. Sun, X.; Ohta, S.; Zhang, J.H. Discovery of a hydrogen molecular target. *Medical gas research* **2023**, *13*, 41-42, doi:10.4103/2045-9912.356472.
34. Liu, J.; Ames, B.N. Reducing mitochondrial decay with mitochondrial nutrients to delay and treat cognitive dysfunction, Alzheimer's disease, and Parkinson's disease. *Nutritional neuroscience* **2005**, *8*, 67-89, doi:10.1080/10284150500047161.
35. Liu, J.; Shen, W.; Zhao, B.; Wang, Y.; Wertz, K.; Weber, P.; Zhang, P. Targeting mitochondrial biogenesis for preventing and treating insulin resistance in diabetes and obesity: Hope from natural mitochondrial nutrients. *Advanced drug delivery reviews* **2009**, *61*, 1343-1352, doi:10.1016/j.addr.2009.06.007.
36. Peng, Y.; Gao, P.; Shi, L.; Chen, L.; Liu, J.; Long, J. Central and Peripheral Metabolic Defects Contribute to the Pathogenesis of Alzheimer's Disease: Targeting Mitochondria for Diagnosis and Prevention. *Antioxidants & redox signaling* **2020**, *32*, 1188-1236, doi:10.1089/ars.2019.7763.
37. Hou, C.; Wang, Y.; Zhu, E.; Yan, C.; Zhao, L.; Wang, X.; Qiu, Y.; Shen, H.; Sun, X.; Feng, Z.; et al. Coral calcium hydride prevents hepatic steatosis in high fat diet-induced obese rats: A potent mitochondrial nutrient and phase II enzyme inducer. *Biochem Pharmacol* **2016**, *103*, 85-97, doi:10.1016/j.bcp.2015.12.020.
38. Fan, Y.; Wang, J.; Feng, Z.; Cao, K.; Liu, J.; Xu, H. Hydrogen-rich and hyperoxygenate saline inhibits lipopolysaccharide-induced lung injury through mediating NF-κB/NLRP3 signaling pathway in C57BL/6 mice. *Environ Toxicol* **2022**, *37*, 1575-1586, doi:10.1002/tox.23507.
39. Zhang, J.Y.; Liu, C.; Zhou, L.; Qu, K.; Wang, R.; Tai, M.H.; Lei Lei, J.C.; Wu, Q.F.; Wang, Z.X. A review of hydrogen as a new medical therapy. *Hepato-gastroenterology* **2012**, *59*, 1026-1032, doi:10.5754/hge11883.
40. Hirano, S.I.; Ichikawa, Y.; Kurokawa, R.; Takefuji, Y.; Satoh, F. A "philosophical molecule," hydrogen may overcome senescence and intractable diseases. *Medical gas research* **2020**, *10*, 47-49, doi:10.4103/2045-9912.279983.
41. Sies, H. Hydrogen peroxide as a central redox signaling molecule in physiological oxidative stress: Oxidative eustress. *Redox biology* **2017**, *11*, 613-619, doi:10.1016/j.redox.2016.12.035.
42. Bellezza, I.; Giambanco, I.; Minelli, A.; Donato, R. Nrf2-Keap1 signaling in oxidative and reductive stress. *Biochimica et biophysica acta. Molecular cell research* **2018**, *1865*, 721-733, doi:10.1016/j.bbamcr.2018.02.010.
43. Lu, Y.; Li, C.F.; Ping, N.N.; Sun, Y.Y.; Wang, Z.; Zhao, G.X.; Yuan, S.H.; Zibrila, A.I.; Soong, L.; Liu, J.J. Hydrogen-rich water alleviates cyclosporine A-induced nephrotoxicity via the Keap1/Nrf2 signaling pathway. *Journal of biochemical and molecular toxicology* **2020**, *34*, e22467, doi:10.1002/jbt.22467.
44. Sun, R.; Zhao, N.; Wang, Y.; Su, Y.; Zhang, J.; Wang, Y.; Yu, Y.; Wang, G.; Wang, Z.; Xie, K. High concentration of hydrogen gas alleviates Lipopolysaccharide-induced lung injury via activating Nrf2 signaling pathway in mice. *International immunopharmacology* **2021**, *101*, 108198, doi:10.1016/j.intimp.2021.108198.
45. Gatbonton-Schwager, T.; Yagishita, Y.; Joshi, T.; Wakabayashi, N.; Srinivasan, H.; Suzuki, T.; Yamamoto, M.; Kensler, T.W. A Point Mutation at C151 of Keap1 of Mice Abrogates NRF2 Signaling, Cytoprotection in Vitro, and Hepatoprotection in Vivo by Bardoxolone Methyl (CDDO-Me). *Molecular pharmacology* **2023**, *104*, 51-61, doi:10.1124/molpharm.123.000671.
46. Gao, Q.; Zhang, G.; Zheng, Y.; Yang, Y.; Chen, C.; Xia, J.; Liang, L.; Lei, C.; Hu, Y.; Cai, X.; et al. SLC27A5 deficiency activates NRF2/TXNRD1 pathway by increased lipid peroxidation in HCC. *Cell death and differentiation* **2020**, *27*, 1086-1104, doi:10.1038/s41418-019-0399-1.
47. Zhang, D.D.; Hannink, M. Distinct cysteine residues in Keap1 are required for Keap1-dependent ubiquitination of Nrf2 and for stabilization of Nrf2 by chemopreventive agents and oxidative stress. *Mol Cell Biol* **2003**, *23*, 8137-8151, doi:10.1128/mcb.23.22.8137-8151.2003.
48. Shin, J.W.; Chun, K.S.; Kim, D.H.; Kim, S.J.; Kim, S.H.; Cho, N.C.; Na, H.K.; Surh, Y.J. Curcumin induces stabilization of Nrf2 protein through Keap1 cysteine modification. *Biochem Pharmacol* **2020**, *173*, 113820, doi:10.1016/j.bcp.2020.113820.
49. Suzuki, T.; Muramatsu, A.; Saito, R.; Iso, T.; Shibata, T.; Kuwata, K.; Kawaguchi, S.I.; Iwawaki, T.; Adachi, S.; Suda, H.; et al. Molecular Mechanism of Cellular Oxidative Stress Sensing by Keap1. *Cell reports* **2019**, *28*, 746-758.e744, doi:10.1016/j.celrep.2019.06.047.
50. Baird, L.; Yamamoto, M. The Molecular Mechanisms Regulating the KEAP1-NRF2 Pathway. *Mol Cell Biol* **2020**, *40*, doi:10.1128/mcb.00099-20.
51. Horie, Y.; Suzuki, T.; Inoue, J.; Iso, T.; Wells, G.; Moore, T.W.; Mizushima, T.; Dinkova-Kostova, A.T.; Kasai, T.; Kamei, T.; et al. Molecular basis for the disruption of Keap1-Nrf2 interaction via Hinge & Latch mechanism. *Commun Biol* **2021**, *4*, 576, doi:10.1038/s42003-021-02100-6.
52. Liu, B.; Xue, J.; Zhang, M.; Wang, M.; Ma, T.; Zhao, M.; Gu, Q.; Qin, S. Hydrogen inhalation alleviates nonalcoholic fatty liver disease in metabolic syndrome rats. *Mol Med Rep* **2020**, *22*, 2860-2868, doi:10.3892/mmr.2020.11364.

Preprints (www.preprints.org) | NOT PEER-REVIEWED | Posted: 31 October 2023 doi:10.20944/preprints202310.1953.v1

53. Murakami, Y.; Ito, M.; Ohsawa, I. Molecular hydrogen protects against oxidative stress-induced SH-SY5Y neuroblastoma cell death through the process of mitohormesis. *PLoS One* **2017**, *12*, e0176992, doi:10.1371/journal.pone.0176992.
54. Iketani, M.; Sakane, I.; Fujita, Y.; Ito, M.; Ohsawa, I. H(2)-induced transient upregulation of phospholipids with suppression of energy metabolism. *Medical gas research* **2023**, *13*, 133-141, doi:10.4103/2045-9912.344973.
55. Jesus, A.A.; Passaglia, P.; Santos, B.M.; Rodrigues-Santos, I.; Flores, R.A.; Batalhão, M.E.; Stabile, A.M.; Cárnio, E.C. Chronic molecular hydrogen inhalation mitigates short and long-term memory loss in polymicrobial sepsis. *Brain research* **2020**, *1739*, 146857, doi:10.1016/j.brainres.2020.146857.
56. Yu, Y.; Feng, J.; Lian, N.; Yang, M.; Xie, K.; Wang, G.; Wang, C.; Yu, Y. Hydrogen gas alleviates blood-brain barrier impairment and cognitive dysfunction of septic mice in an Nrf2-dependent pathway. *International immunopharmacology* **2020**, *85*, 106585, doi:10.1016/j.intimp.2020.106585.
57. Shen, M.; Zheng, Y.; Zhu, K.; Cai, Z.; Liu, W.; Sun, X.; Liu, J.; Zhu, D. Hydrogen gas protects against delayed encephalopathy after acute carbon monoxide poisoning in a rat model. *Neurol Res* **2020**, *42*, 22-30, doi:10.1080/01616412.2019.1685064.
58. Diao, M.; Zhang, S.; Wu, L.; Huan, L.; Huang, F.; Cui, Y.; Lin, Z. Hydrogen Gas Inhalation Attenuates Seawater Instillation-Induced Acute Lung Injury via the Nrf2 Pathway in Rabbits. *Inflammation* **2016**, *39*, 2029-2039, doi:10.1007/s10753-016-0440-1.
59. Chen, H.G.; Xie, K.L.; Han, H.Z.; Wang, W.N.; Liu, D.Q.; Wang, G.L.; Yu, Y.H. Heme oxygenase-1 mediates the anti-inflammatory effect of molecular hydrogen in LPS-stimulated RAW 264.7 macrophages. *International journal of surgery (London, England)* **2013**, *11*, 1060-1066, doi:10.1016/j.ijsu.2013.10.007.
60. Kawamura, T.; Wakabayashi, N.; Shigemura, N.; Huang, C.S.; Masutani, K.; Tanaka, Y.; Noda, K.; Peng, X.; Takahashi, T.; Billiar, T.R.; et al. Hydrogen gas reduces hyperoxic lung injury via the Nrf2 pathway in vivo. *Am J Physiol Lung Cell Mol Physiol* **2013**, *304*, L646-656, doi:10.1152/ajplung.00164.2012.
61. Xie, K.; Yu, Y.; Pei, Y.; Hou, L.; Chen, S.; Xiong, L.; Wang, G. Protective effects of hydrogen gas on murine polymicrobial sepsis via reducing oxidative stress and HMGB1 release. *Shock (Augusta, Ga.)* **2010**, *34*, 90-97, doi:10.1097/SHK.0b013e3181cdc4ae.
62. Li, Y.; Xie, K.; Chen, H.; Wang, G.; Yu, Y. Hydrogen gas inhibits high-mobility group box 1 release in septic mice by upregulation of heme oxygenase 1. *The Journal of surgical research* **2015**, *196*, 136-148, doi:10.1016/j.jss.2015.02.042.
63. Zhang, Y.; Zhang, J.; Fu, Z. Molecular hydrogen is a potential protective agent in the management of acute lung injury. *Molecular medicine (Cambridge, Mass.)* **2022**, *28*, 27, doi:10.1186/s10020-022-00455-y.
64. Xie, Q.; Li, X.X.; Zhang, P.; Li, J.C.; Cheng, Y.; Feng, Y.L.; Huang, B.S.; Zhuo, Y.F.; Xu, G.H. Hydrogen gas protects against serum and glucose deprivation-induced myocardial injury in H9c2 cells through activation of the NF-E2-related factor 2/heme oxygenase 1 signaling pathway. *Mol Med Rep* **2014**, *10*, 1143-1149, doi:10.3892/mmr.2014.2283.
65. Chen, H.; Xie, K.; Han, H.; Li, Y.; Liu, L.; Yang, T.; Yu, Y. Molecular hydrogen protects mice against polymicrobial sepsis by ameliorating endothelial dysfunction via an Nrf2/HO-1 signaling pathway. *International immunopharmacology* **2015**, *28*, 643-654, doi:10.1016/j.intimp.2015.07.034.
66. Meng, X.; Chen, H.; Wang, G.; Yu, Y.; Xie, K. Hydrogen-rich saline attenuates chemotherapy-induced ovarian injury via regulation of oxidative stress. *Exp Ther Med* **2015**, *10*, 2277-2282, doi:10.3892/etm.2015.2787.
67. Harris, S.C.; Devendran, S.; Méndez-García, C.; Mythen, S.M.; Wright, C.L.; Fields, C.J.; Hernandez, A.G.; Cann, I.; Hylemon, P.B.; Ridlon, J.M. Bile acid oxidation by Eggerthella lenta strains C592 and DSM 2243(T). *Gut microbes* **2018**, *9*, 523-539, doi:10.1080/19490976.2018.1458180.
68. Conti, P.; Gallenga, C.E.; Tetè, G.; Caraffa, A.; Ronconi, G.; Younes, A.; Toniato, E.; Ross, R.; Kritas, S.K. How to reduce the likelihood of coronavirus-19 (CoV-19 or SARS-CoV-2) infection and lung inflammation mediated by IL-1. *Journal of biological regulators and homeostatic agents* **2020**, *34*, 333-338, doi:10.23812/Editorial-Conti-2.
69. Fan, Y.; Wang, J.; Feng, Z.; Cao, K.; Liu, J.; Xu, H. Hydrogen-rich and hyperoxygenate saline inhibits lipopolysaccharide-induced lung injury through mediating NF-kappaB/NLRP3 signaling pathway in C57BL/6 mice. *Environ Toxicol* **2022**, *37*, 1575-1586, doi:10.1002/tox.23507.
70. Dong, A.; Yu, Y.; Wang, Y.; Li, C.; Chen, H.; Bian, Y.; Zhang, P.; Zhao, Y.; Yu, Y.; Xie, K. Protective effects of hydrogen gas against sepsis-induced acute lung injury via regulation of mitochondrial function and dynamics. *International immunopharmacology* **2018**, *65*, 366-372, doi:10.1016/j.intimp.2018.10.012.
71. Yuan, L.; Shen, J. Hydrogen, a potential safeguard for graft-versus-host disease and graft ischemia-reperfusion injury? *Clinics (Sao Paulo, Brazil)* **2016**, *71*, 544-549, doi:10.6061/clinics/2016(09)10.
72. Kawamura, T.; Huang, C.S.; Tochigi, N.; Lee, S.; Shigemura, N.; Billiar, T.R.; Okumura, M.; Nakao, A.; Toyoda, Y. Inhaled hydrogen gas therapy for prevention of lung transplant-induced ischemia/reperfusion injury in rats. *Transplantation* **2010**, *90*, 1344-1351, doi:10.1097/TP.0b013e3181fe1357.

Preprints (www.preprints.org) | NOT PEER-REVIEWED | Posted: 31 October 2023 doi:10.20944/preprints202310.1953.v1

73. Liu, R.; Fang, X.; Meng, C.; Xing, J.; Liu, J.; Yang, W.; Li, W.; Zhou, H. Lung inflation with hydrogen during the cold ischemia phase decreases lung graft injury in rats. *Experimental biology and medicine (Maywood, N.J.)* **2015**, *240*, 1214-1222, doi:10.1177/1535370214563895.
74. Tanaka, Y.; Shigemura, N.; Kawamura, T.; Noda, K.; Isse, K.; Stolz, D.B.; Billiar, T.R.; Toyoda, Y.; Bermudez, C.A.; Lyons-Weiler, J.; et al. Profiling molecular changes induced by hydrogen treatment of lung allografts prior to procurement. *Biochemical and biophysical research communications* **2012**, *425*, 873-879, doi:10.1016/j.bbrc.2012.08.005.
75. Hu, Q.; Zhou, Y.; Wu, S.; Wu, W.; Deng, Y.; Shao, A. Molecular hydrogen: A potential radioprotective agent. *Biomedicine & pharmacotherapy = Biomedecine & pharmacotherapie* **2020**, *130*, 110589, doi:10.1016/j.biopha.2020.110589.
76. Terasaki, Y.; Ohsawa, I.; Terasaki, M.; Takahashi, M.; Kunugi, S.; Dedong, K.; Urushiyama, H.; Amenomori, S.; Kaneko-Togashi, M.; Kuwahara, N.; et al. Hydrogen therapy attenuates irradiation-induced lung damage by reducing oxidative stress. *Am J Physiol Lung Cell Mol Physiol* **2011**, *301*, L415-426, doi:10.1152/ajplung.00008.2011.
77. Saengsin, K.; Sittiwangkul, R.; Chattipakorn, S.C.; Chattipakorn, N. Hydrogen therapy as a potential therapeutic intervention in heart disease: from the past evidence to future application. *Cellular and molecular life sciences : CMLS* **2023**, *80*, 174, doi:10.1007/s00018-023-04818-4.
78. Li, L.; Li, X.; Zhang, Z.; Liu, L.; Zhou, Y.; Liu, F. Protective Mechanism and Clinical Application of Hydrogen in Myocardial Ischemia-reperfusion Injury. *Pakistan journal of biological sciences : PJBS* **2020**, *23*, 103-112, doi:10.3923/pjbs.2020.103.112.
79. Wang, P.; Jia, L.; Chen, B.; Zhang, L.; Liu, J.; Long, J.; Li, Y. Hydrogen Inhalation is Superior to Mild Hypothermia in Improving Cardiac Function and Neurological Outcome in an Asphyxial Cardiac Arrest Model of Rats. *Shock (Augusta, Ga.)* **2016**, *46*, 312-318, doi:10.1097/shk.0000000000000585.
80. Luo, Z.L.; Cheng, L.; Ren, J.D.; Fang, C.; Xiang, K.; Xu, H.T.; Tang, L.J.; Wang, T.; Tian, F.Z. Hydrogen-rich saline protects against ischemia/reperfusion injury in grafts after pancreas transplantations by reducing oxidative stress in rats. *Mediators of inflammation* **2015**, *2015*, 281985, doi:10.1155/2015/281985.
81. Yao, L.; Chen, H.; Wu, Q.; Xie, K. Hydrogen-rich saline alleviates inflammation and apoptosis in myocardial I/R injury via PINK-mediated autophagy. *International journal of molecular medicine* **2019**, *44*, 1048-1062, doi:10.3892/ijmm.2019.4264.
82. Feng, R.; Cai, M.; Wang, X.; Zhang, J.; Tian, Z. Early Aerobic Exercise Combined with Hydrogen-Rich Saline as Preconditioning Protects Myocardial Injury Induced by Acute Myocardial Infarction in Rats. *Applied biochemistry and biotechnology* **2019**, *187*, 663-676, doi:10.1007/s12010-018-2841-0.
83. Noda, K.; Tanaka, Y.; Shigemura, N.; Kawamura, T.; Wang, Y.; Masutani, K.; Sun, X.; Toyoda, Y.; Bermudez, C.A.; Nakao, A. Hydrogen-supplemented drinking water protects cardiac allografts from inflammation-associated deterioration. *Transplant international : official journal of the European Society for Organ Transplantation* **2012**, *25*, 1213-1222, doi:10.1111/j.1432-2277.2012.01542.x.
84. Qiu, P.; Liu, Y.; Zhang, J. Recent Advances in Studies of Molecular Hydrogen against Sepsis. *International journal of biological sciences* **2019**, *15*, 1261-1275, doi:10.7150/ijbs.30741.
85. Zhang, Y.; Dong, A.; Xie, K.; Yu, Y. Protective Effects of Hydrogen on Myocardial Mitochondrial Functions in Septic Mice. *Biomed Res Int* **2020**, *2020*, 1568209, doi:10.1155/2020/1568209.
86. Tao, B.; Liu, L.; Wang, N.; Tong, D.; Wang, W.; Zhang, J. Hydrogen-Rich Saline Attenuates Lipopolysaccharide-Induced Heart Dysfunction by Restoring Fatty Acid Oxidation in Rats by Mitigating C-Jun N-Terminal Kinase Activation. *Shock (Augusta, Ga.)* **2015**, *44*, 593-600, doi:10.1097/shk.0000000000000467.
87. Yu, Y.S.; Zheng, H. Chronic hydrogen-rich saline treatment reduces oxidative stress and attenuates left ventricular hypertrophy in spontaneous hypertensive rats. *Molecular and cellular biochemistry* **2012**, *365*, 233-242, doi:10.1007/s11010-012-1264-4.
88. Zhang, Y.; Sun, Q.; He, B.; Xiao, J.; Wang, Z.; Sun, X. Anti-inflammatory effect of hydrogen-rich saline in a rat model of regional myocardial ischemia and reperfusion. *International journal of cardiology* **2011**, *148*, 91-95, doi:10.1016/j.ijcard.2010.08.058.
89. Htun, Y.; Nakamura, S.; Kusaka, T. Hydrogen and therapeutic gases for neonatal hypoxic-ischemic encephalopathy: potential neuroprotective adjuncts in translational research. *Pediatric research* **2021**, *89*, 753-759, doi:10.1038/s41390-020-0998-z.
90. Noda, M.; Liu, J.; Long, J. Neuroprotective and Preventative Effects of Molecular Hydrogen. *Current pharmaceutical design* **2021**, *27*, 585-591, doi:10.2174/1381612826666201019103020.
91. Hou, C.; Peng, Y.; Qin, C.; Fan, F.; Liu, J.; Long, J. Hydrogen-rich water improves cognitive impairment gender-dependently in APP/PS1 mice without affecting Aβ clearance. *Free radical research* **2018**, *52*, 1311-1322, doi:10.1080/10715762.2018.1460749.
92. Yoritaka, A.; Takanashi, M.; Hirayama, M.; Nakahara, T.; Ohta, S.; Hattori, N. Pilot study of H_2 therapy in Parkinson's disease: a randomized double-blind placebo-controlled trial. *Movement disorders : official journal of the Movement Disorder Society* **2013**, *28*, 836-839, doi:10.1002/mds.25375.

Preprints (www.preprints.org) | NOT PEER-REVIEWED | Posted: 31 October 2023 doi:10.20944/preprints202310.1953.v1

93. Cui, Y.; Zhang, H.; Ji, M.; Jia, M.; Chen, H.; Yang, J.; Duan, M. Hydrogen-rich saline attenuates neuronal ischemia--reperfusion injury by protecting mitochondrial function in rats. *The Journal of surgical research* **2014**, *192*, 564-572, doi:10.1016/j.jss.2014.05.060.
94. Li, Q.; Yu, P.; Zeng, Q.; Luo, B.; Cai, S.; Hui, K.; Yu, G.; Zhu, C.; Chen, X.; Duan, M.; et al. Neuroprotective Effect of Hydrogen-Rich Saline in Global Cerebral Ischemia/Reperfusion Rats: Up-Regulated Tregs and Down-Regulated miR-21, miR-210 and NF-κB Expression. *Neurochemical research* **2016**, *41*, 2655-2665, doi:10.1007/s11064-016-1978-x.
95. Li, C.; Hou, L.; Chen, D.; Lin, F.; Chang, T.; Li, M.; Zhang, L.; Niu, X.; Wang, H.; Fu, S.; et al. Hydrogen-rich saline attenuates isoflurane-induced caspase-3 activation and cognitive impairment via inhibition of isoflurane-induced oxidative stress, mitochondrial dysfunction, and reduction in ATP levels. *American journal of translational research* **2017**, *9*, 1162-1172.
96. Xia, C.; Liu, W.; Zeng, D.; Zhu, L.; Sun, X.; Sun, X. Effect of hydrogen-rich water on oxidative stress, liver function, and viral load in patients with chronic hepatitis B. *Clinical and translational science* **2013**, *6*, 372-375, doi:10.1111/cts.12076.
97. Li, T.; Chiang, J.Y. Bile acid signaling in metabolic disease and drug therapy. *Pharmacological reviews* **2014**, *66*, 948-983, doi:10.1124/pr.113.008201.
98. Hu, H.L.; Gao, J.; Guo, W.J.; Zhou, F.H.; Liu, H.Y.; Su, C.C. Anti-injury effect of hydrogen-enriched water in a rat model of liver injury induced by aflatoxin B(1). *Sheng Li Xue Bao* **2019**, *71*, 725-731.
99. Liang, B.; Shi, L.; Du, D.; Li, H.; Yi, N.; Xi, Y.; Cui, J.; Li, P.; Kang, H.; Noda, M.; et al. Hydrogen-Rich Water Ameliorates Metabolic Disorder via Modifying Gut Microbiota in Impaired Fasting Glucose Patients: A Randomized Controlled Study. *Antioxidants (Basel, Switzerland)* **2023**, *12*, doi:10.3390/antiox12061245.
100. Xu, F.; Yu, S.; Qin, M.; Mao, Y.; Jin, L.; Che, N.; Liu, S.; Ge, R. Hydrogen-Rich Saline Ameliorates Allergic Rhinitis by Reversing the Imbalance of Th1/Th2 and Up-Regulation of CD4+CD25+Foxp3+Regulatory T Cells, Interleukin-10, and Membrane-Bound Transforming Growth Factor-β in Guinea Pigs. *Inflammation* **2018**, *41*, 81-92, doi:10.1007/s10753-017-0666-6.
101. Yao, W.; Guo, A.; Han, X.; Wu, S.; Chen, C.; Luo, C.; Li, H.; Li, S.; Hei, Z. Aerosol inhalation of a hydrogen-rich solution restored septic renal function. *Aging* **2019**, *11*, 12097-12113, doi:10.18632/aging.102542.
102. Chen, J.; Zhang, H.; Hu, J.; Gu, Y.; Shen, Z.; Xu, L.; Jia, X.; Zhang, X.; Ding, X. Hydrogen-Rich Saline Alleviates Kidney Fibrosis Following AKI and Retains Klotho Expression. *Frontiers in pharmacology* **2017**, *8*, 499, doi:10.3389/fphar.2017.00499.
103. Shi, Q.; Liao, K.S.; Zhao, K.L.; Wang, W.X.; Zuo, T.; Deng, W.H.; Chen, C.; Yu, J.; Guo, W.Y.; He, X.B.; et al. Hydrogen-rich saline attenuates acute renal injury in sodium taurocholate-induced severe acute pancreatitis by inhibiting ROS and NF-κB pathway. *Mediators of inflammation* **2015**, *2015*, 685043, doi:10.1155/2015/685043.
104. Xie, F.; Song, Y.; Yi, Y.; Jiang, X.; Ma, S.; Ma, C.; Li, J.; Zhanghuang, Z.; Liu, M.; Zhao, P.; et al. Therapeutic Potential of Molecular Hydrogen in Metabolic Diseases from Bench to Bedside. *Pharmaceuticals (Basel)* **2023**, *16*, doi:10.3390/ph16040541.
105. Adzavon, Y.M.; Xie, F.; Yi, Y.; Jiang, X.; Zhang, X.; He, J.; Zhao, P.; Liu, M.; Ma, S.; Ma, X. Long-term and daily use of molecular hydrogen induces reprogramming of liver metabolism in rats by modulating NADP/NADPH redox pathways. *Scientific reports* **2022**, *12*, 3904, doi:10.1038/s41598-022-07710-6.
106. Korovljev, D.; Trivic, T.; Drid, P.; Ostojic, S.M. Molecular hydrogen affects body composition, metabolic profiles, and mitochondrial function in middle-aged overweight women. *Irish journal of medical science* **2018**, *187*, 85-89, doi:10.1007/s11845-017-1638-4.
107. Iuchi, K.; Nishimaki, K.; Kamimura, N.; Ohta, S. Molecular hydrogen suppresses free-radical-induced cell death by mitigating fatty acid peroxidation and mitochondrial dysfunction. *Canadian journal of physiology and pharmacology* **2019**, *97*, 999-1005, doi:10.1139/cjpp-2018-0741.
108. Ito, M.; Ibi, T.; Sahashi, K.; Ichihara, M.; Ito, M.; Ohno, K. Open-label trial and randomized, double-blind, placebo-controlled, crossover trial of hydrogen-enriched water for mitochondrial and inflammatory myopathies. *Medical gas research* **2011**, *1*, 24, doi:10.1186/2045-9912-1-24.

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