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# Review Article
# Protective Mechanism and Clinical Application of Hydrogen in Myocardial Ischemia-reperfusion Injury

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## Abstract

Cardiovascular disease accounts for one-third of all deaths, with ischemic heart disease as the main cause of death. Under pathological conditions, ischemia-reperfusion injury (IRI) often occurs in tissues. Ischemic injury is mainly caused by anaerobic cell death and reperfusion which results in a wide range of inflammatory responses. These responses are able to increase tissue damage and even damage to the whole body. IRI can also aggravate the original cardiovascular disease during the treatment of cardiovascular disease. Therefore, it is particularly important to understand the mechanism of myocardial ischemia-reperfusion injury (MIRI) for clinical treatment and application. At the same time, it is necessary to find a safe, reliable and feasible method for treating MIRI to reduce the incidence of complications and mortality as well as improve the prognosis and quality of life of patients. As a selective antioxidant, hydrogen can neutralize excessive free radicals, has certain anti-apoptotic and anti-inflammatory effects and it has gradually become a focus and hotspot of preclinical and clinical research. Hydrogen has been shown to have a certain therapeutic effect on MIRI, which can provide a new therapeutic direction for the clinical treatment of myocardial ischemia-reperfusion injury. In this review, the protective mechanism and clinical application of hydrogen in myocardial ischemia-reperfusion injury is discussed.

**Key words:** Myocardial ischemia-reperfusion injury, cardiovascular disease, inflammation, hydrogen, therapeutic effect, clinical treatment, clinical application

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**Data Availability:** All relevant data are within the paper and its supporting information files.

## INTRODUCTION

The latest study shows that MIRI caused by acute myocardial infarction in coronary heart disease will cause 10 of patients to die and the incidence of cardiac dysfunction exceeds 25%[1]. Ischemia-reperfusion injury (IRI) refers to tissue damage caused by inadequate oxygen supply in experimental and clinical settings, accompanied by a series of extensive and complex inflammatory reactions caused by successful reperfusion, which may exacerbate the local tissue damage and damage the function of long-distance organs[2-4]. This outcome is also the feature of IRI. The theoretical basis of IRI is based on 2 hypotheses. First, a large number of cells are irreversibly destroyed during tissue ischemia and will eventually perish, while other cells will remain in a clearly viable state. Second, although many cells are at risk of death, a large number of putative cells are regarded as potentially viable[2,5]. Therefore, there are 2 situations in which IRI may occur: acute myocardial infarction and non-acute revascularization. Acute myocardial infarction is myocardial necrosis caused by acute, persistent ischemia and hypoxia in the coronary arteries[6]. Non-acute revascularization includes percutaneous coronary intervention (PCI) and coronary artery bypass surgery (CABG)[7]. PCI refers to the treatment of myocardial perfusion by transcatheter catheterization to clear the stenosis or even occlusion of the coronary lumen[8]. The main principle of CABG is to establish a bypass ("bridge") between the aorta and the diseased coronary artery using the autologous blood vessels (the internal mammary artery, the radial artery, the right gastric artery, the great saphenous vein) to make the aorta [9]. The blood inside is directly perfused to the stenotic distal end across the stenosis of the blood vessel, thereby restoring myocardial blood supply.

Hydrogen is widely distributed in nature and is a colorless and odorless reducing gas. Due to the small mass of hydrogen molecules, it can penetrate the blood-brain barrier and metabolize without residue in the body[10]. The current study found that hydrogen has a certain inhibitory effect on oxidative stress[11], inflammation[12], apoptosis[13] and autophagy[14]. Recent studies have confirmed that hydrogen has certain protective effects for various tissues and organs including the brain[15], kidney[16,17], heart[18], diabetes[19] and multiple organs damage[20]. Hydrogen is expected to be the 4th most important gas signal molecule[21] after nitric oxide (NO), carbon monoxide (CO) and hydrogen sulfide ($H_2S$). In the precious study, the GSR-CAA-67 protein chip from RayBiotech was used, the results of KEGG pathway enrichment analysis demonstrate that the JAK-STAT pathway may be involved in the mechanism of action of hydrogen-rich water. The JAK-STAT signaling pathway has been studied in recent years and it is widely involved in biological processes such as cell proliferation[22], differentiation[23] and the inflammatory response[24]. And the study has shown that hydrogen-rich water can inhibit the apoptosis of myocardial tissue after the ischemia-reperfusion and alleviate ischemia-reperfusion injury by up regulating the expression of the JAK-STAT signaling pathway[25-30].

So far, no effective way has been found to reduce myocardial ischemia-reperfusion injury. Compared with NO, CO and $H_2S$, hydrogen has smaller molecular weight and is easier to enter the biological membrane. Hydrogen can also be taken up by exogenous sources, independent of the body's own production, which has the advantage of being completely cytotoxic compared to other medical gases. In summary, hydrogen has strong potential clinical value in alleviate MIRI. In this review, we demonstrate the protective mechanism of hydrogen in myocardial ischemia-reperfusion injury and hydrogen's advantages compared with other three gases. Thus, we hope that this review will impel scholars study in this field to elucidate the specific target of hydrogen in MIRI in order to put it into clinical application.

### Mechanism of myocardial ischemia-reperfusion injury

**Reactive oxygen species (ROS) production:** Xanthine oxidase (XO) and xanthine dehydrogenase (XD) are presented in the cardiomyocytes. In the myocardial ischemic state, the body's antioxidant defense system is damaged[31]. On the one hand, the uptake of $Ca^{2+}$ by myocardial cells is able to activate a $Ca^{2+}$-dependent proteolytic enzyme to convert XD into XO. On the other hand, due to a decrease in oxygen partial pressure, ATP is degraded into ADP, AMP and hypoxanthine, resulting in a large amount of hypoxanthine accumulation in the ischemic issue. During reperfusion, a large amount of molecular oxygen enters the ischemic tissue with the blood and in the two-step reaction of XO catalyzes the conversion of hypoxanthine to xanthine and further catalyzes the conversion of jaundice into uric acid, both molecular oxygen and electron acceptor are simultaneously used, resulting in a significant increase in ROS production, such as highly activated superoxide ($O_2^-$) and highly destructive hydroxyl radicals (•OH)[32]. Increased hydrogen peroxide ($H_2O_2$) is produced steadily, which leads to direct damage of cell membranes and proteins and induces lipid peroxidation[33] to inhibit the normal function of mitochondria. Cellular lipids, proteins and DNA react directly with ROS to cause cell damage and even death, at the same time, the NF-κB signaling pathway is also activated[34].

**Endothelial cell dysfunction and leukocyte adhesion:** P-selectin, which is expressed on endothelial cells, initiates the

procedure of leukocyte adhesion. P-selectin is mainly responsible for mediating the foremost aggregation of neutrophils (PMN) in myocardial microvessels. After approximately 20 min, L-selectin becomes the primary mediator of leukocyte rolling by binding with sialylated Lewis antigens. The rolling neutrophils are more active and sturdier when attached to the endothelium through protein interactions of the integrin family, LFA-1 (CD11/18), with its corresponding binding proteins ICAM-1 and 2 on endothelial cells[35].

In experimental I/R injury, neutrophils (PMN) are the key leukocyte populations and their effects are mostly releasing oxygen-derived cytotoxic products, superoxide anion and hypochlorous acid as well as superactive cytotoxic substance and matrix metallopeptidase (MMP-9)[36]. I/R injury can cause endothelial cell dysfunction, likely by oxidative damage to the membrane, ion homeostasis and osmotic pressure. When the function of endothelial cells is impaired, the neutrophils and the damaged vascular endothelium will adhere and gather together, which will aggravate the degree of tissue damage. Therefore, in reperfusion injury, animal studies have revealed a reduction in tissue injury after blocking PMN adhesion or PMN consumption in the myocardium[37], intestine[38] and lung tissue[39].

Conclusive evidence has shown that proinflammatory cytokines including tumor necrosis factor (TNF)-α, interleukin-1β (IL-1β) and interleukin-6 (IL-6) are involved in postischemic responses[40]. The decrease in IL-1β and TNF-α signaling defect was found to be associated with attenuation of chemokines upregulation and neutrophil infiltration[41].

IL-8, a member of the CXC chemokine family, has potent chemotactic activity against neutrophils[42]. The results of a focused study of the roles played by IL-8 receptors (CXC-R1 and -R2) are unclear as both active and defective CXC-R2 were found to have protective effects on myocardium[43,44]. In addition, a large amount of experimental research has shown that IRI leads to activation of the complement system in some organs and that both activation pathways (classical/substitution) are involved, moreover, 2 products of complement activation, C5a and C5b-9 are considered to be involved and mainly responsible for IRI[45]. C5a increases the chemotaxis of neutrophils, the release of proteases and many pro-inflammatory effects of oxygen free radical production, C5a may also possibly through the production of TNF-α, interleukin-1, leukocyte-6 and MCP-1, further exacerbate the inflammatory response[46,47]. It has also been demonstrated that C5b-9 significantly contributes to the release of tissue-induced complement, the activation of the NF-κB signaling pathway and the release of induced chemotactic mediators (IL-8, MCP-1)[48].

**$Ca^{2+}$ overload and the opening of MPTP:** An important feature of IRI is that an imbalance in cell ion homeostasis leads to ischemic acidosis and calcium overload. The pivotal mechanism of cell hydrogen ion concentration recovery is the activation of the sodium ($Na^+$)-hydrogen ($H^+$)-exchanger (NHE)[49]. The main function of the NHE as a pH-regulating protein is to promote excessive proton efflux combined with sodium ions influx to maintain electrical neutrality. Sodium-($Na^+$)-potassium ($K^+$)-ATPase is effective in removing sodium from the cytoplasm under physiological conditions, however, it is inhibited during ischemia-induced ATP depletion[50]. Consequently, the sodium ($Na^+$)-calcium ($Ca^{2+}$)-exchanger is activated to avoid intracellular sodium accumulation. By transferring sodium out of the cell, extracellular calcium is transported into the cell and causes intracellular calcium overload[51]. A sudden increase in intracellular $Ca^{2+}$ may result in cell death. During this period, biofilms (cell membranes, mitochondrial membranes, lysosomal membranes, especially mitochondrial membranes) were damaged and their permeability was increased, which aggravated cell dysfunction and structural damage. Reperfusion opens the mitochondrial permeability transition pore (MPTP), which can inhibit the mitochondrial respiratory function and also cause the release of cytochrome c (Cyt c) and activation of apoptosis protease and initiate apoptosis[52,53]. Proton accumulation may also result in reduced efficiency of contractile proteins, impairing recovery function during reperfusion and reducing cardiac function (heart function/myocardial $O_2$ consumption)[54].

During tissue ischemia, when ATP resynthesis is blocked and a large amount of energy is consumed when ATP is sequentially degraded into ADP, AMP, adenosine and eventually hypoxanthine, the internal metabolism changes. In addition, a decrease in ATP in cells leads to mitochondrial dysfunction and promotes the expression of Bax, the most important apoptotic gene in the human body, from the cell matrix to the mitochondrial outer membrane. This mechanism leads to mitochondrial swelling and causes cytochrome c to flow into the cell matrix via the open pores of the osmotic transition pore, the cytochrome c-activated receptor protease in the cell matrix is then activated, triggering apoptosis[2].

## Source of hydrogen

**Synthesis of endogenous hydrogen:** Some intestinal bacteria, such as *E. coli*, can produce large amounts of hydrogen

through the fermentation of undigested sugars in intestines[55], which can be detected *in vivo* in animals. In addition, studies have shown that the content of endogenous hydrogen must be significantly higher than the lowest concentration of exogenous hydrogen to exert antioxidant capacity[56]. However, when oxidative stress or inflammation occurs in the body, endogenous hydrogen no longer plays a significant role[21]. This result may be because the endogenous hydrogen is cleared by the colonic mucosa or other commensal bacteria in the stomach, such as Helicobacter. Moreover, most mammals lack an anabolic enzyme and cannot reuse bacterial metabolites to produce hydrogen. Furthermore, the hydrogen produced by the fermentation of intestinal bacteria cannot be used by the body and is discharged through feces or flatulence, absorbed by methanogens or discharged through the lungs. Therefore, improvement of the utilization of endogenous hydrogen has become a breakthrough in clinical experiments, which encourages researchers and clinical staff. For example, the production of endogenous hydrogen can be promoted by ingesting certain foods such as lactose and turmeric[57].

**Exogenous hydrogen intake:** Exogenous hydrogen is acquired by hydrogen inhalation, drinking hydrogen-rich water and injecting hydrogen rich saline solution[58,59]. Most antioxidants do not reach the infarcted area in time before reperfusion begins, while inhaled hydrogen works quickly and does not affect the patient's hemodynamic parameters, including heart rate and left ventricular blood pressure[60]. Studies have shown that hydrogen can quickly spread to hazardous areas even in the absence of blood flow. There are also clinical studies indicating that inhalation of 3-4% hydrogen has no effect on the physiological parameters of patients with acute ischemic cerebrovascular disease[61]. Drinking hydrogen-rich water in daily life is also a good approach for exogenous hydrogen intake, especially for some chronic diseases[62]. At room temperature, when the solubility of hydrogen in water reaches 0.8 mmol $L^{-1}$, the pH and other properties of water are not affected. In a study conducted by Nagatani[63], it was found that intravenous injection of hydrogen saline was safe and effective in 38 patients with acute ischemic stroke. In addition, hydrogen eye drops or external products that can generate hydrogen can be absorbed into the blood through the skin, which it can be regarded as a feasible method for treating diseases by using hydrogen[64]. It can be seen that the intake of exogenous hydrogen is more effective for the body.

## Protective mechanism of hydrogen in myocardial ischemia-reperfusion injury

**Selective antioxidant effect:** When myocardial tissue is damaged by ischemia-reperfusion, free radicals are generated. When excess free radicals cannot be eliminated by the body, new free radicals are generated through multiple pathways and the accumulation of free radicals will cause structural damage to cell and dysfunction[65]. Ohsawa *et al.*[66] found that hydrogen can selectively reduce hydroxyl radicals and cytotoxic ROS and effectively protect cells but hydrogen does not reduce free radicals by interacting with excess reactive oxygen species. This finding provides a new idea for the study of myocardial IRI and clinical treatment[66]. In recent years, it has Nrf2-ARE pathway has been shown to be one of the most important signaling pathways in the endogenous antioxidant response mechanism[67].

Nuclear factor-related factor 2 (Nrf2) is a transcription factor closely related to antioxidative stress. Normally, Nrf2 is located in the cytoplasm and binds to its retinoic protein cytoplasmic junction protein (Kelch-like ECH-associated protein-1, Keap1). Nrf2, in conjunction with Keap1, is anchored to the cytoskeleton[68]. Keap1 modulates Nrf2 ubiquitination degradation to maintain, a normal level of Nrf2, thereby inhibiting the expression of downstream genes. When an organ or tissue is in an oxidative stress state, Nrf2 dissociates from Keap1 and forms a heterodimer with other proteins, recognizes and binds to the binding site on the antioxidant response element ARE and up-regulates its downstream antioxidant protein and NAD (P) H: quinone oxidoreductase 1 (NQO1) gene expression to reduce oxidative damage[69], on the other hand, oxidative stress accelerates Nrf2 mRNA transcription and increases Nrf2 protein synthesis[70]. Thus, the Nrf2-ARE pathway plays an important role in selective antioxidant action. When myocardial IRI occurs, a large number of cardiomyocytes undergo apoptosis, free radicals are generated during reperfusion. This study indicated that hydrogen can protect the myocardium by activating the Nrf2-ARE signaling pathway. H9c2 cardiomyocytes were treated with serum and glucose deprivation (SGD) methods to simulate ischemic conditions by Xie *et al.*[71] and were cultured in medium rich in $H_2$ (purity 99.999%). The expression of Nrf2 protein was detected by Western blotting and the level of 8-hydroxydeoxyguanosine (8-OHdG) was detected by enzyme-linked immunosorbent assay (ELISA). The survival time of $H_2$-rich SGD cells was prolonged, the production of •OH radicals was decreased and the expression level of Nrf2 was significantly increased, indicating that hydrogen can protect ischemic cardiomyocytes by eliminating •OH radicals

and activating the Nrf2 pathway. This finding suggests that hydrogen may also have a protective effect on myocardial IRI. In addition, there are other signaling pathways involved in the regulation of oxidative stress by hydrogen. For example, ROS can activate the NF-κB signaling pathway and promote TNF-α expression[72], while TNF-α activates the expression of GAPDH oxidase (NOX). This process will produce ROS[73]. Hydrogen molecules can also reduce the inflammatory response by inhibiting the activation of NF-κB[74].

**Anti-apoptotic effect:** According to the literature apoptosis is closely related to many pathophysiological processes and has a great impact on the occurrence and development of disease. Similarly, apoptosis plays an important role in myocardial IRI[75]. During ischemia-reperfusion, oxygen free radicals, calcium overload and MPTP opening cause mitochondrial swelling and rupture, release apoptosis-inducing factors and apoptosis-related proteins such as cytochrome c (Cyt c), further initiate the Caspase cascade and induce programmed apoptosis[76,77]. Recent studies have found that the PI3K/AKT pathway is critical for cardiomyocyte apoptosis[78]. Phosphatidylinositol-3-kinase (PI3K) is a class of protein with enzymatic activity and is an important transduction signal. Protein kinase B (PKB), also known as AKT, is a very important downstream target of the PI3K signaling pathway. When the PI3K/AKT signaling pathway is activated, AKT is transferred to p-AKT. This transfer regulates downstream target proteins, such as the apoptosis-related proteins Bcl-2 and Bax[79] and the forkhead box O protein family (FoxOs)[80]. In the heart of adults, FoxOs play an important role in maintaining myocardial growth, contraction, metabolism, cell cycle transition[81], etc. The PI3K/AKT signaling pathway is an upstream regulatory pathway that inhibits forkhead box protein O. Generally, after the activation of forkhead box protein O, G1/S cell cycle transition is blocked and expression of the cycle-dependent kinase[82] (CDK inhibitor) p27 and the apoptotic factor Bim[83] and apoptosis, aging, autophagy and the stress response are promoted. In addition, forkhead box protein O activation can downregulate peroxisome proliferation activator receptor γ (PPAP-γ) expression levels, inhibit the formation of adipocytes by adipose-derived stem cells, promote protein breakdown and inhibit the development of myoblasts[84]. Therefore, hydrogen may regulate the expression level of FoxO through the PI3K/AKT signaling pathway and thereby exert its anti-apoptotic function.

**Anti-inflammatory effect:** Experimental studies and clinical observations have shown a significant increase in white blood cells (especially neutrophils) in the presence of ischemia-reperfusion[85]. Nuclear factor-κB is a generic term for proteins that specifically bind to the κB site of many gene promoters to promote transcription[86]. Many cytokine and adhesion molecule genes including those involved in inflammation and the immune response, contain a κB site. At the same time, *in vitro* and *in vivo* experiments have shown that NF-κB activation is associated with overexpression of TNF-α, IL-1β and other factors[87]. Myocardial IRI is a complex process involving neutrophil activation, multiple factors and adhesion molecule over expression, accompanied by a variety of inflammatory mediators and signal transduction molecule that play an important role in myocardial IRI[88]. NF-κB undergoes positive feedback regulation outside the cell: NF-κB activation can enhance the transcription of TNF-α and IL-2 and with the increase in cytokine release, NF-κB is further activated. The increase in the production and release of pro-inflammatory factors such as IL-8 leads to further amplification of the inflammatory signal and aggravation of the damage to the body[89]. At the same time, negative feedback regulation of NF-κB is also present inside and outside the cell. In addition to the initiation of inflammatory mediator gene expression by NF-κB activation, IKBα, Bcl-3, p100 and p105 precursor proteins are also up-regulates. These nascent inhibitory proteins rapidly inactivate the activated NF-κB in the nucleus, thereby terminating the transcription of inflammatory mediators and limiting the progression of inflammation. Simple IKBα synthesis is not regulated by NF-κB and thus, IKBα phosphorylation causes long-term activation of NF-κB. NF-κB competitively binds to the κB sequence and inhibits the function of NF-κB. The cytokine IL-10 has an anti-inflammatory effect and inhibits NF-κB activation. LPS, LNF and IL-1 can stimulate IL-10 production, thereby limiting inflammation and alleviating IRI[90]. In recent years, studies have shown that hydrogen-rich water can significantly improve liver IRI. It was found that the activity of myeloperoxidase (MPO) in liver tissue was significantly decreased after intraperitoneal injection of normal saline in rats[91]. Thus, it can be concluded that hydrogen-rich water can inhibit neutrophil infiltration and reduce inflammation after hepatic IRI via a mechanism closely related to inhibition of the NF-κB signaling pathway.

**Progress in the clinical application of hydrogen:** Chen *et al*.[92] reported that high concentrations of respiratory hydrogen have protective effects on myocardial IRI in mice, which may depend on the PI3K-Akt1 signaling pathway. Yue *et al*.[93] reported that intraperitoneal injection of hydrogen-rich saline may protect the rat heart from IRI through the Akt/GSK3β

signaling pathway. Sun *et al.*[94] and Zhang *et al.*[95] demonstrated that hydrogen-rich physiological saline protects against myocardial IRI through antioxidation and anti-inflammatory effects. Pan *et al.*[96] established a model of myocardial ischemia in aged rats and injected hydrogen-rich water into the peritoneal cavity before reperfusion, showing that hydrogen-rich water can alleviate myocardial IRI by inhibiting the autophagy of cardiomyocytes. He *et al.*[97] found in a rat model that the load of hydrogen in the microsphere, which is capable of releasing hydrogen at an effective location, can be visually tracked by an ultra-micro imaging system. Zhang *et al.*[98] used hydrogen in combination with lactic acid, in which hydrogen acts as an antioxidant and lactic acid was shown to simulate tissue acidosis after ischemia. Under these conditions, the body can be posttreated. This finding suggested that the combination of lactic acid and hydrogen exerts the same protection as tissue post treatment. We found that hydrogen-rich water can up-regulate the JAK-STAT signaling pathway in rats with myocardial ischemia-reperfusion injury, inhibit cardiomyocyte apoptosis and reduce ischemia-reperfusion injury[99]. Most of these studies are based on animal models and are rarely used in clinical practice. However, these studies and methods provide a theoretical basis and new ideas for the clinical application of hydrogen, laying a foundation for the clinical application of hydrogen.

## CONCLUSION

In recent years, the main clinical methods against myocardial IRI have mainly targeted these mechanisms, including drug treatment, pretreatment and post treatment. However, these methods have not achieved desirable therapeutic effects and new treatment methods are urgently needed. Nowadays, hydrogen has been increasingly used in animal experiments and has been shown to be associated with ischemia-reperfusion, showing a certain therapeutic effect. It is important and urgent to elucidate the specific target of hydrogen in MIRI in order to put it into clinical application.

## SIGNIFICANCE STATEMENT

This study discovered hydrogen can protect cardiomyocytes against MIRI. Hydrogen molecules are small in mass and easily penetrate the cell membrane and blood-brain barrier and it has effects on myocardial apoptosis, inflammation and oxidative stress. This study will help the researchers to uncover the critical areas of the specific target of hydrogen in MIRI that many researchers were not able to explore. Thus a new theory on the clinical application of hydrogen may be arrived at.

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