IVYSPRING INTERNATIONAL PUBLISHER

International Journal of Biological Sciences

2019; 15(6): 1261-1275. doi: 10.7150/ijbs.30741

Review

Recent Advances in Studies of Molecular Hydrogen against Sepsis

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Received: 2018.11.27; Accepted: 2019.01.09; Published: 2019.05.11

Abstract

Sepsis is a syndrome comprised of a series of life-threatening organ dysfunctions caused by a maladjusted body response to infection with no effective treatment. Molecular hydrogen is a new type of antioxidant with strong free radical scavenging ability, which has been demonstrated to be effective for treating various diseases, such as infection, trauma, poisoning, organ ischemia-reperfusion, metabolic diseases, and tumors. Molecular hydrogen exerts multiple biological effects involving anti-inflammation, anti-oxidation, anti-apoptosis, anti-shock, and autophagy regulation, which may attenuate the organ and barrier damage caused by sepsis. However, the underlying molecular mechanisms remain elusive, but are likely related to the signaling pathways involved. This review focuses on the research progress and potential mechanisms of molecular hydrogen against sepsis to provide a theoretical basis for clinical treatment.

Key words: molecular hydrogen, sepsis, oxidative stress, apoptosis, shock, autophagy

Introduction

Sepsis is a serious systemic inflammatory response syndrome caused by infection, which is characterized by an explosive inflammatory reaction, oxidative stress, and immune system disorder, that eventually leads to septic shock and multiple organ failure[1]. At present, there are several treatments for sepsis, such as anti-inflammation and anti-shock. However, there is no definite therapeutic effect and the mortality rate remains high.

Molecular hydrogen was first reported in *Nature Medicine (2007)* as a natural antioxidant and selective scavenger of oxygen free radicals to treat oxidative stress [2]. A large number of studies have subsequently used it to treat various types of diseases, including infection, trauma, metabolic diseases, organ ischemia-reperfusion injury, and tumors and have achieved satisfactory efficacy[3]. Molecular hydrogen provides a variety of advantages for treating diseases due to its unique physical and chemical properties. There are three main forms of molecular hydrogen for research: hydrogen rich saline/water (HRS/W), inhaled hydrogen and hydrogen rich medium (HRM). Molecular hydrogen is safe, non-toxic and can balance the pH of body fluids[4]. Because of its small molecular weight, molecular hydrogen spreads easily and penetrates membranes into the cytoplasm, mitochondria, and even the nucleus [4]. Molecular hydrogen is non-flammable and non-explosive at the therapeutic concentration. Its effect is moderate and its metabolites are non-toxic. Although it has antioxidant capacity, molecular hydrogen does not interfere with normal metabolism or redox reactions [5]. In addition, molecular hydrogen promotes cell detoxification, increases cell hydration, and strengthens the host immune system[5].

Current studies have indicated that molecular hydrogen exerts its biological effects in two ways, one of which is reacting with hydroxyl radicals and peroxynitrite directly, and the other is modulating specific gene expression or signaling pathways[3] (Fig. 1).

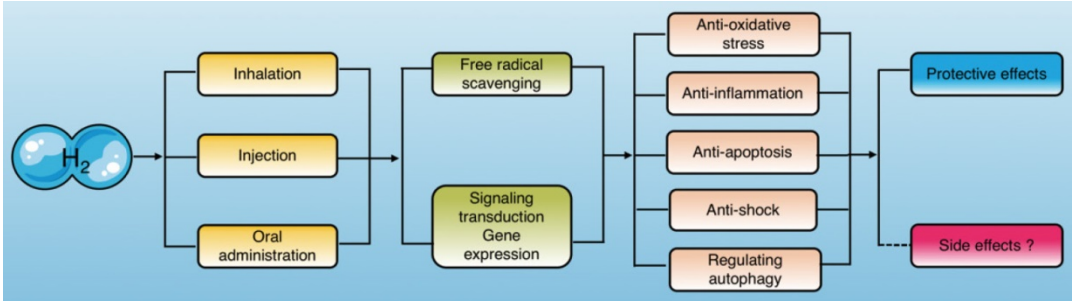

Figure 1. Molecular hydrogen exerts biological effects. Molecular hydrogen can be ingested in a variety of ways and exerts biological effects, including anti-oxidation, anti-inflammation, anti-apoptosis, anti-shock, and autophagy regulation through scavenging free radicals directly and regulating signal transduction and gene expression indirectly.

Based on these therapeutic advantages, molecular hydrogen has been widely used in studies of organ protection during sepsis in recent years, and has yielded ideal results. Molecular hydrogen attenuates the injury and dysfunction of important organs (heart, liver, lung, kidneys, and brain) and physiological barriers (epithelial cell barrier, vascular endothelial cell barrier) by suppressing oxidative stress and inflammation as well as reducing apoptosis [6-10] and regulating sepsis-induced autophagy[11, 12]. However, the underlying molecular mechanisms of these effects have not been elucidated. Therefore, understanding the research status of molecular hydrogen against sepsis and the underlying mechanisms are of great significance for treating sepsis.

Molecular hydrogen against organ injury induced by sepsis

Lipopolysaccharide (LPS) is a component of the cell wall of Gram-negative bacteria and the most important pathogenic factor in sepsis. It has been demonstrated that molecular hydrogen extenuates LPS-induced ALI in rats by reducing the release of inflammatory factors, inhibiting the aggregation of inflammatory cells, reducing oxidative stress and apoptosis[8, 11, 13]. Moreover, molecular hydrogen alleviates pulmonary edema caused by LPS through upregulating the expression of pulmonary aquaporin (AQP)[14]. These lung protective effects are thought to be associated with a reduction in LPS-induced p38 mitogen-activated protein kinase (p38 MAPK) and c-Jun N-terminal kinase (JNK) activation by molecular hydrogen[11, 14, 15]. In addition to protecting the mature lung, HRS also alleviates bronchopulmonary dysplasia (BPD) induced by LPS in neonatal mice[16]. Fibroblast growth factor receptor 4 (FGFR4) and vascular endothelial growth factor receptor 2 (VEGFR2) are important for maintaining alveolar structures and lung development[17, 18]. Oral intake of HRS ameliorates LPS-induced suppression of genes encoding FGFR4, VEGFR2, and heme oxygenase 1 (HO-1) in neonatal mice[16]. In addition, study showed[19] that LPS promotes the alveolar epithelial-mesenchymal transition (EMT) and pulmonary fibrosis by increasing the production of reactive oxygen species (ROS) and transforming growth factor-β (TGF-β). HRS alleviates oxidative stress and pulmonary fibrosis by reducing LPS-induced E-cadherin loss and α-smooth muscle actin production[20].

The liver is the most important organ for removing cytotoxic substances from the body, but may become overloaded by sepsis and exhibit injury and dysfunction[6]. A series of studies have reported that HRS reduces liver damage caused by endotoxin in rats [6, 21, 22]. Iketani et al. found that HRS Alleviates liver injury induced by oxidative stress through further increasing LPS-induced HO-1 expression and decreasing endothelin-1 (ET-1) expression [6]. Xu et al. demonstrated that HRS mitigates the pathological injury of the septic rat liver and improves survival rate by reducing the release of inflammatory cytokines and reducing hepatocyte apoptosis and oxidative stress[22]. Inhibiting signaling pathways, such as p38 MAPK, JNK, extracellular regulated protein kinase (ERK), and nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB), and reducing the second mitochondria-derived activator of caspase (Smac) level contribute to HRS-mediated liver protection[22].

Multiple studies[23-25] have demonstrated that HRS protects renal function by reducing serum creatinine and urea nitrogen levels and relieving renal structural damage caused by sepsis. Liu et al. reported that inhaling molecular hydrogen alleviates brain damage and cognitive dysfunction in septic rats by inhibiting the neuronal inflammatory response and oxidative stress, while neuronal apoptosis is reduced by increasing the expression of nuclear factor erythroid 2-related factor 2 (Nrf2) and HO-1[26]. In the septic pancreatitis rat model, Zhang et al. found that HRS exerts anti-inflammation, anti-oxidant, and antibacterial effects by inhibiting the NF-κB signaling pathway[27]. It has been demonstrated that

myocarditis and insufficient energy production in the myocardium are the main causes of heart dysfunction caused by sepsis[28]. Tao et al. reported that LPS challenge increases left ventricular diameter and reduces fractional shortening and fatty acid oxidation (FAO)-related gene expression in rats, which can be attenuated by HRS[7]. However, HRS has little effect on myocardial glucose metabolism in septic rats[7]. Part of the protective mechanism of HRS on LPS-induced myocardial injury might be that HRS inhibits activation of the JNK signaling pathway, restores FAO, and increases myocardial energy production[7]. Moreover, one study showed that HRS reduces endotoxin-induced uveitis by inhibiting the aqueous humor protein[10]. However, HRS could neither effectively reduce iridocyclitis infiltration nor restore retinal function[10].

Molecular hydrogen reduces the physiological barrier dysfunction caused by sepsis

Physiological barrier dysfunction is one of the pathological manifestations of sepsis [9, 29, 30]. Vascular endothelial cells, epithelial cells, and intercellular junctions are important components of physiological barrier[31, 32]. Adherens junctions (AJ), represented by cadherin, and tight junctions (TJ), represented by occluding, are the main cell junctions involved in formation of barriers[31, 32]. Components of the barrier become damaged during sepsis and normal intercellular junctions are destroyed, which leads to increased barrier permeability[29, 30, 33]. Subsequently, proteins or liquids leak out of the blood vessels into tissues, leading to edema and hypoproteinemia, which aggravate endotoxin shock[34].

Many studies have investigated how molecular hydrogen alleviates the physiological barrier damage caused by sepsis, including protective effects on vascular endothelial cells[30, 35, 36], epithelial cells (e.g., alveoli and gastrointestinal epithelium)[9, 11, 29, 37] and intercellular junctions[9, 30, 33, 38], which are characterized by decreased permeability, increased transmembrane resistance, and regulation of expression and distribution of intercellular junction-related proteins.

Molecular hydrogen improves LPS-induced hyperpermeability of the vascular endothelium, represented by an increase in trans-endothelial electrical resistance (TEER)[30, 38] and a decrease in fluorescein isothiocyanate-dextran (FITC-dextran) flux of endothelial cells[38]. Molecular hydrogen stabilizes the AJ between endothelial cells and reduces barrier permeability by reducing internalization of VE-cadherin[38] and increasing the expression of VE-cadherin *in vivo*[35] and *in vitro*[36, 38].

Similar to vascular endothelial cells, HRS has a protective effect on epithelial cells. Studies by Zhang et al.[11] and Liu et al.[37] have demonstrated that HRS attenuates the alveolar epithelial barrier damage caused by LPS, improves alveolar gas exchange, and reduces cell damage caused by alveolar epithelial cell apoptosis and excessive autophagy. Yang et al.[9] and Ikeda et al.[29] reported that molecular hydrogen also protects the gastrointestinal epithelial barrier during sepsis. LPS can disrupt barrier integrity by reducing trans-epithelial resistance [9, 33] and increasing FITC-dextran flux in a concentration-dependent manner[9], which can be attenuated by molecular hydrogen through elevating LPS-induced down-regulated expression of occludin and E-cadherin[9].

According to current research results[9, 30, 35, 36, 38], molecular hydrogen protects physiological barriers mainly through the following mechanisms: regulating signaling of the Ras homolog gene family member A (RhoA) and its effector protein kinases involving mammalian diaphanous-related formin 1 (mDia1) and Rho-associated coiled-coil protein kinase (ROCK); and by reducing adhesion of inflammatory cells.

The Rho GTPases are a family of small signaling G proteins and a subfamily of the Ras superfamily. Members of the Rho GTPase family (Cdc42, Rac1, and RhoA) regulate many aspects of intracellular actin dynamics, which are essential for the formation of AJ and TJ[39]. It has been demonstrated that the Rho GTPase family has an important effect on stability and integrity of the physiological barrier by regulating cell junctions[40](Fig. 2). The baseline activities of Rac1, Cdc42, and RhoA are required to maintain the integrity of epithelial and endothelial barriers[30, 40]. However, additional activation of Rac1 and Cdc42 can exert a role in barrier stabilization[40], while increased stimulation of RhoA has a dual effect on barrier permeability[40-42] (Fig. 2).

RhoA is the most studied molecule in the Rho family and its main effectors are ROCK and mDia1. ROCK promotes the contraction of myosin and aggregation of actin, while mDia1 promotes aggregation of actin and organization of microtubules [41, 43]. Actin aggregation is important for correct localization of AJ components, and microtubule organization stabilizes the cell periphery[41]. RhoA/ROCK regulates cell adhesion, migration, proliferation, and apoptosis by controlling the arrangement of the actin skeleton and cell contraction[44]. RhoA/mDia plays an important role in the localization of E-cadherin at the cell junction and the formation of AJ[45, 46]. Gavard et al. reported that both intensity and space distribution of active RhoA impact the downstream signaling options [42]. Moderate activation of RhoA

selectively activates mDia signaling to stabilize cell junctions and the periphery, whereas excessive activation tends to cause ROCK-dependent myosin contraction and disrupt cell junctions[40, 41] (Fig. 2).

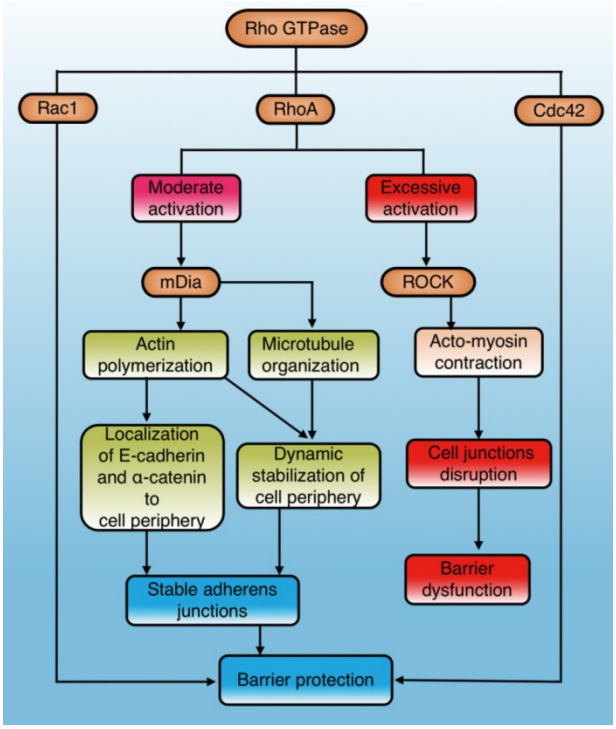

Figure 2. The role and mechanisms of Rho GTPase in regulating physiological barriers. Cdc42 and Rac1 in the Rho GTPase family stabilize physiological barriers after activation, while RhoA has dual effects on the barrier. Moderate RhoA activation selectively activates mDia1 signaling to protect the barrier, while excessive activation leads to ROCK-mediated barrier disruption. Cdc42: cell division control protein 42 homolog; Rac1: Ras-related C3 botulinum toxin substrate 1; RhoA: Ras homolog gene family member A; mDia1: mammalian diaphanous-related formin 1; ROCK: Rho-associated coiled-coil protein kinase.

Studies have demonstrated that LPS leads to endothelial hyperpermeability by decreasing VE-cadherin expression[47] and increasing phosphorylation of myosin light chain [48-50] by activating the RhoA/ROCK signaling pathway. In contrast, HRM has been demonstrated to reduce LPS-induced neutrophil/polymorphonuclear cell (PMN) adhesion to endothelial cells, increase the expression of vascular cell adhesion molecule-1 (VCAM-1) and E-selectin, and improve the decreased TEER and VE-cadherin expression in endothelial cells by inhibiting the Rho/ROCK signaling pathway[30]. In addition, molecular hydrogen improves the decreased expression of mDia induced by LPS in epithelial cells[9]. Inhibiting RhoA or knockdown of mDia eliminates the molecular hydrogen-induced benefits of occludin, E-cadherin, and AJ[9], indicating that molecular hydrogen exerts a protective effect on the epithelial barrier through RhoA or mDia signaling. As strong proof, researchers have revealed that molecular hydrogen reduces LPS-induced excessive activation of RhoA to a moderate level and increases the expression of mDia; thus, preventing destruction of TJ and AJ and alleviating intestinal epithelial barrier dysfunction[9].

Excessive numbers of inflammatory cells during sepsis activate and adhere to endothelial cells through adhesion molecules, which subsequently leads to endothelial cell apoptosis, disruption of cell junctions, cell remodeling, and other damage[30]. Studies have shown that LPS increases the production of multiple adhesion molecules, including VCAM-1, intercellular adhesion molecule-1 (ICAM-1) and E-selectin, which increase adhesion and aggregation of monocytes to vascular endothelial cells and induce decomposition of TJ or redistribution of VE-cadherin[30, 36, 38]. Instead, HRM reduces the damage of endothelial cells and AJ by downregulating the expression of adhesion molecules; thus, reducing permeability of the endothelial barrier[30, 36, 38].

Notably, the inhibition of adhesion molecules by molecular hydrogen is associated with the RhoA/mDia/ROCK signaling pathway. RhoA mediates LPS-induced ICAM-1 expression in endothelial cells by activating p38 and NF-κB[51]. The absence of mDia results in more adhesion of neutrophils to endothelial cells in sepsis[52], and the ROCK inhibitors Y-27632 or fasudi reduce the expression of adhesion molecules[30]. In addition, some researchers believe that HRS may reduce the production of adhesion molecules by activating the Nrf2-mediated HO-1 signaling pathway[36].

The biological effects and mechanisms of molecular hydrogen against sepsis

Anti-oxidative stress

Studies have shown that excessive production or insufficient elimination of free radicals, such as ROS and reactive nitrogen species (RNS), is one of the most important pathogenic mechanisms of sepsis[53]. ROS mainly include superoxide anions ($\cdot O_2^-$), hydroxyl radicals ($\cdot OH$), and hydrogen peroxide (H_2O_2), and RNS include nitric oxide ($\cdot NO$), nitrogen dioxide ($\cdot NO_2$), and peroxynitrite anion ($\cdot ONOO^-$).

Molecular hydrogen is a natural antioxidant that antagonizes oxidative stress in several ways (Fig. 3): (1) by neutralizing $\cdot OH$[54], (2) reducing $\cdot ONOO^-$ and its gene expression directly[55], and inhibiting the production of nitro-tyrosine indirectly, which is an indicator of $\cdot ONOO^-$ generation[13], (3) by inducing antioxidant gene expression and increasing antioxidant enzyme activity, including superoxide dismutase (SOD), HO-1, catalase (CAT), and myeloperoxidase (MPO) [56], (4) by reducing the

levels of oxidative stress indicators, such as 8-iso-prostaglandin F2α [57], and the lipid peroxidation marker malondialdehyde (MDA)[13], (5) by reducing ·NO production through inhibiting inducible nitric oxide synthase (iNOS)[29, 58] and endothelial nitric oxide synthase (eNOS)[59], and (6) by inhibiting NADPH oxidase activity, which is the main source of free radicals in sepsis[59-61].

Numerous studies have demonstrated that molecular hydrogen attenuates LPS-induced tissue damage by reducing ROS, increasing antioxidant enzyme activities, and inhibiting pro-oxidant enzyme activities (Fig. 4) (Table 1). HRS reduces oxidative stress in LPS-induced BPD neonatal mice by reducing ROS production in alveolar epithelial cells[16]. In addition, molecular hydrogen reduces the LPS-induced alveolar EMT and pulmonary fibrosis by inhibiting ROS-mediated TGF-β production[20]. Iketani et al. reported that HRS pretreatment further increases the expression of HO-1 induced by LPS, while reducing the expression of ET-1[6], which is a potential endogenous vasoconstrictor that stimulates ROS production dominated by superoxide anions and aggravates oxidative stress associated with lipid peroxidation[62]. Similarly, Chen et al. found that molecular hydrogen increases Nrf2-mediated HO-1 expression, and reduces the endothelial cell injury caused by sepsis[36]. Moreover, studies have shown that HRS inhibits activation of the MAPK and NF-κB signaling pathways, thereby significantly reducing MDA levels in liver tissues of septic rats[22] and reducing oxidative stress in rats with septic peritonitis [27].

NADPH oxidase is an important pro-oxidative enzyme that catalyzes the production of superoxide free radicals by transferring electrons from NADPH to oxygen[63]. NADPH oxidase is dormant under physiological conditions but can be rapidly activated by bacterial products and cytokines during sepsis and become the main source of free radicals[63]. Studies have demonstrated that HRS reduces ROS production and attenuates mitochondrial dysfunction by inhibiting NADPH oxidase activity in rat cardiomyocytes [59, 61]. In addition, researchers have found that molecular hydrogen reduces the levels of the p40 phox, p47 phox, and p67 phox subunits of NADPH oxidase in the cell membrane, while increasing their levels in the cytoplasm, suggesting that molecular hydrogen reduces NADPH oxidase activity by limiting the translocation of these molecules to the cell membrane[60].

Anti-inflammation

An excessive inflammatory response is the most significant pathological process occurring in sepsis[1]. Numerous studies have shown that molecular hydrogen effectively alleviates the inflammatory response and plays a protective role in animal models of sepsis[8, 10, 11, 13, 14, 16, 21-24, 27, 36, 38, 64] (Table 2).

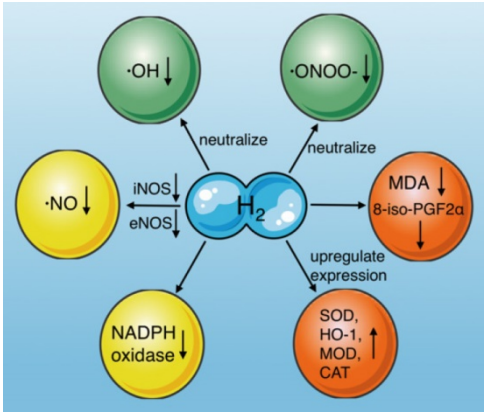

Figure 3. Effects of molecular hydrogen on oxidative stress. Molecular hydrogen antagonizes oxidative stress through multiple pathways, including neutralizing ·OH and ·ONOO-, reducing ·NO production, upregulating antioxidant gene expression (SOD, HO-1, CAT, and MOD), suppressing NADPH oxidase activity, and reducing 8-iso-PGF2α and MDA. ·OH: hydroxyl radicals; ·ONOO-: peroxynitrite anions; SOD: superoxide dismutase; CAT: catalase, MOD: myeloperoxidase; MDA: malondialdehyde; iNOS: inducible nitric oxide synthase; eNOS: endothelial nitric oxide synthase; NADPH oxidase: nicotinamide adenine dinucleotide phosphate oxidase.

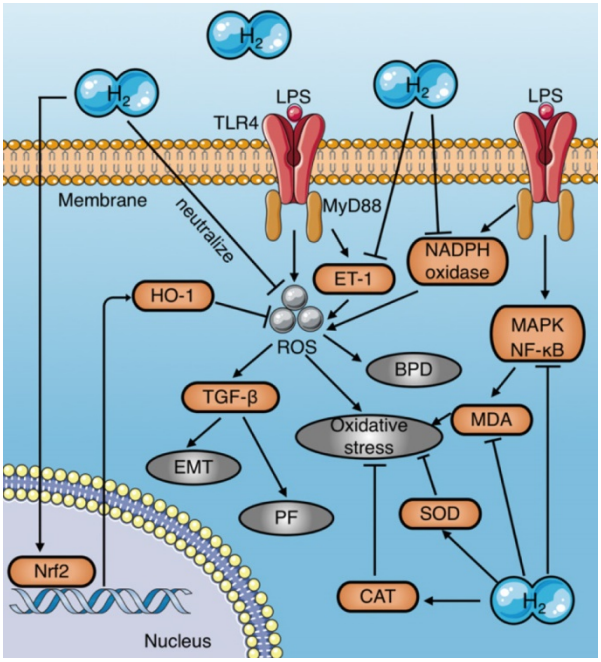

Figure 4. Mechanisms of molecular hydrogen against oxidative stress in sepsis. (a) Molecular hydrogen eliminates LPS-induced ROS by direct neutralization, promoting HO-1 expression mediated by Nrf2, and inhibiting LPS-induced ET-1 activation. (b) Molecular hydrogen reduces MDA by inhibiting activation of MAPK and NF-κB signaling induced by LPS. (c) Molecular hydrogen increases antioxidant enzyme activities, such as CAT and SOD. (d) Molecular hydrogen suppresses NADPH oxidase activity. EMT: epithelial-mesenchymal transition; PF: pulmonary fibrosis; BPD: bronchopulmonary dysplasia; Nrf2: nuclear factor erythroid 2-related factor 2; TLR4: toll-like receptor 4; MyD88: myeloid differentiation primary response 88; MDA: malondialdehyde; ROS: reactive oxygen species; HO-1: heme oxygenase 1; CAT: catalase; SOD: superoxide dismutase

Table 1. Studies of molecular hydrogen against sepsis through an anti-oxidative stress effect

Drug	Authors	Animal/cell	Disease model	Administration	Effects	Signaling pathways
HRW	Iketani et. al 2017[6]	C57BL/6 mice	Septic liver injury (LPS)	HRW po. × 3d	4-HNE ↓, 8-OHdG ↓	HO-1 ↑
2% H_2	Qiu et. al 2011[13]	C57BL/6 mice	Septic lung injury (LPS)	2% H_2 inhalation× 2h	MDA ↓, SOD ↑	Nitro tyrosine (NT) ↓
HRW	Muramatsu et. al 2016[16]	Sprague-Dawley rats, A549	Bronchopulmonary Dysplasia (LPS)	HRW po. × 10d	8-OHdG ↓, ROS ↓	HO-1 ↑
HRS	Dong et. al 2017[20]	ICR mice	Pulmonary Fibrosis (LPS)	HRS 2.5, 5 or 10 ml/kg/d× 8d i.p.	MDA ↓, T-AOC ↑, SOD ↑, CAT ↑	N/A
HRS	Xu et. al 2013[22]	Sprague-Dawley rats	Septic liver injury (LPS)	HRS 8 ml/kg/h i.v.× 6h	MDA ↓, MPO ↓	NF-κB ↓, Smac ↓, MAPK(JNK/P38) ↓
HRS	Li et. al 2013[23]	Sprague-Dawley rats	Sepsis (CLP)	HRS 5 ml/kg/h i.p. (0, 6, 18h after CLP)	MDA ↓, SOD ↑	N/A
2% H_2	Xie et. al 2010[24]	C57BL/6 mice	Sepsis (CLP)	2% H_2 inhalation (4L/min)	SOD ↑, CAT↑	N/A
2% H_2	Liu et. al 2014[25]	Wistar rats	Septic shock (LPS)	2% H_2 inhalation× 4h	MDA ↓, MPO ↓, SOD ↑	N/A
HRW	Zhang et. al 2014[27]	Sprague-Dawley rats	Septic peritonitis injury (LPS/CLP)	HRW 6 ml/kg/d po. × 10d	MDA ↓, MPO ↓	NF-κB ↓

HRS/W/M: hydrogen rich saline/water/media, LPS: Lipopolysaccharide, CLP: cecal ligation and puncture, MDA: malondialdehyde, SOD: superoxide dismutase, MPO: myeloperoxidase, CAT: catalase, 4-NHE: 4-hydroxy-2-nonenal, 8-OHdG: 8-hydroxy-2′-deoxyguanosine, ROS: reactive oxygen species, i.p.: Intraperitoneal injection, i.v.: Intravenous injection, po.: oral administration.

Table 2. Studies of molecular hydrogen against sepsis through an anti-inflammation effect

Drug	Authors	Animal/cell	Disease model	Administration	Effects	Signaling pathways
HRS 2% H_2	Xie et. al 2012[8]	C57BL/6 mice	Septic lung injury (LPS)	2% H_2 inhalation/HRS 10 ml/kg i.p.	PMNs↓, TNF-α↓, IL-6↓ HMGB1↓, IL-1β↓, IL-10↑	NF-κB ↓
HRS	Yan et. al 2017[10]	Sprague-Dawley rats	Endotoxin-induced uveitis (LPS)	HRS 10 ml/kg i.p.	Inflammatory cells infiltration ↓	AqH ↓
HRS	Zhang et. al 2015[11]	Sprague-Dawley rats	Septic lung injury (LPS)	HRS 8 ml/kg/h i.v.× 6h	TNF-α↓, IL-6↓	P38 MAPK ↓
2% H_2	Qiu et. al 2011[13]	C57BL/6 mice	Septic lung injury (LPS)	2% H_2 inhalation× 2h	TNF-α↓, IL-6↓ IL-1β↓, MPO↓	JNK ↓
HRS	Tao et. al 2016[14]	Sprague-Dawley rats	Septic lung injury (LPS)	HRS 10 ml/kg i.p. (1h,4h after LPS)	PMNs ↓	P38 MAPK ↓ JNK ↓
HRW	Muramatsu et. al 2016[16]	Sprague-Dawley rats, A549	Bronchopulmonary Dysplasia (LPS)	HRW po. × 10d	TNF-α↓, IL-6↓	HO-1 ↑
HRS	Sun et. al 2011[21]	C57BL/6 mice	Septic liver injury (LPS/GaIN)	HRS 8ml/kg/3h × 3 i.p.	TNF-α↓, IL-6↓	JNK ↓
HRS	Xu et. al 2013[22]	Sprague-Dawley rats	Septic liver injury (LPS)	HRS 8 ml/kg/h i.v.× 6h	TNF-α↓, IL-6↓	NF-κB ↓ P38 MAPK ↓, JNK ↓
HRS	Li et. al 2013[23]	Sprague-Dawley rats	Sepsis (CLP)	HRS 5 ml/kg i.p. (0,6,18h after CLP)	MPO↓, IL-6↓ HMGB1↓	N/A
2% H_2	Xie et. al 2010[24]	C57BL/6 mice	Sepsis (CLP)	2% H_2 inhalation (4L/min)	HMGB1↓	N/A
2% H_2	Liu et. al 2014[25]	Wistar rats	Septic shock (LPS)	2% H_2 inhalation× 4h	TNF-α↓, IL-6↓ IL-10↑	N/A
HRW	Zhang et. al 2014[27]	Sprague-Dawley rats	Septic peritonitis injury (LPS/CLP)	HRW 6 ml/kg/d po. × 10d	WBCs↓, endotoxin↓ TNF-α↓, IL-6↓	NF-κB ↓
HRS HRM	Chen et. al 2015[36]	ICR mice, HUVECs-12	Sepsis (CLP) HUVECs (LPS)	HRS 5 ml/kg i.p. HRM 0.6 mmol/L	TNF-α↓, IL-1β↓ HMGB1↓, IL-10↑	Nrf2/HO-1 ↑
HRM	Yu et. al 2015[38]	HUVECs-12	HUVECs (LPS)	HRM 0.6 mmol/L	Leukocyte coagulation ↓	N/A
HRM	Chen et. al 2103[64]	RAW264.7	Macrophages (LPS)	HRM 0.6 mmol/L	TNF-α↓, IL-1β↓ HMGB1↓, IL-10↑	HO-1 ↑

HRS/W/M: hydrogen rich saline/water/media, LPS: Lipopolysaccharide, CLP: cecal ligation and puncture, i.p.: Intraperitoneal injection, i.v.: Intravenous injection, po.: oral administration, HUVECs: human umbilical vein endothelial cells, HMGB1: high mobility group box 1, PMNs: polymorphonuclear neutrophils.

The anti-inflammatory effects of molecular hydrogen in sepsis are mainly represented by the followings: (1) reducing the release of LPS-induced pro-inflammatory cytokines, including TNF-α, IL-1β, IL-6, and HMGB1[8, 16, 35, 36], and increasing the level of the anti-inflammatory cytokine IL-10[36]; (2) reducing the release of chemokines, such as macrophage inflammatory protein 1 (MIP1) and MIP2[8]; (3) reducing the aggregation and infiltration of neutrophils and macrophages[8, 13, 20, 22]; (4) alleviating vascular endothelial injury induced by inflammatory cells adhesion through reducing LPS-induced adhesion molecules production[38].

These anti-inflammatory effects of molecular

hydrogen may be related to the following pathways (Fig. 5):

(1) NF-κB: HRS has been demonstrated to play a protective role by inhibiting activation of the NF-κB pathway during sepsis[8, 27], which may be achieved by inhibiting IκBα phosphorylation or suppressing ROS and its downstream related signaling pathways [8].

(2) MAPK (p38, ERK, and JNK): Tao et al. reported that HRS reduces LPS-induced neutrophil aggregation by inhibiting activation of the p38 MAPK and JNK pathways [14]. Similarly, Liang et al.[15] and Xu et al.[22] demonstrated the inhibitory effect of HRS on the p38 MAPK pathway. Activation of ERK promotes separation of IκBα from NF-κB complexes in an acute liver injury model of septic rats, subsequently activating NF-κB and promoting the release of inflammatory factors[65]. However, HRS reduces LPS-induced activation of the ERK pathway [65]. Itoh et al. found that HRS inhibits activation of apoptotic signal-regulated kinase 1 (ASK1) and its downstream signaling pathways p38 MAPK, JNK, and IκBα[58]. (3) HO-1: Chen et al. reported that HRS attenuates the inflammatory response during sepsis by activating the Nrf2-mediated HO-1 signaling pathway[36]. Similarly, the HO-1 pathway mediates the anti-inflammatory effects of molecular hydrogen in LPS-challenged RAW264.7 macrophages[64].

Anti-apoptosis

Apoptosis induced by sepsis aggravates tissue damage and organ dysfunction[8, 26]. In particular, immunosuppression caused by apoptosis of immune cells, such as macrophages, is a major cause of deterioration in sepsis[66]. Several studies have demonstrated that molecular hydrogen alleviates septic injury in rats by reducing apoptosis (Table 3). The last common pathway of apoptosis is activation of a series of proteases called caspases. Studies have shown that molecular hydrogen suppresses activation of caspases-3, 8, and 9 and inhibits apoptosis through multiple signaling pathways[67]. The inhibitory mechanisms of molecular hydrogen on caspase activation are very complex and may be involved multiple upstream pathways (Fig. 6): (1) Molecular hydrogen Inhibits the activation of ASK1-MAPKs (p38/JNK/ERK)-Bcl-2 signaling pathways network [65, 68, 69] by ROS neutralization[13, 21, 22] or NF-κB suppression[8, 22, 68]. However, study also found HRS reduces LPS-induced hepatocyte apoptosis by inhibiting ERK-mediated activation of NF-κB[65]; (2) Molecular hydrogen Inhibits the ROS-p53-caspase-3 signaling pathway[70]. It has been proved that ROS inhibit p53 phosphorylation, and lead to cleavage of cytochrome *c* and caspase-3 in mitochondria, which finally promote apoptosis[70]; (3) Molecular hydrogen activates the PI3K/Aκt/GSK3β signaling pathway[71, 72], which is essential for cell survival[73]. GSK3β phosphorylation occurs after activation of PI3K/Aκt, and subsequently regulates the endogenous apoptotic pathway and its downstream molecules involving MCL-1 (Bcl-2 family member) and BAD (Bcl-Xl/Bcl-2 related death promoting factors)[74, 75].

Anti-shock

Endotoxin shock is one of the most important causes of death in septic patients[76]. A large quantity of NO is an important cause of septic shock[77, 78]. Carnio et al. reported that LPS-induced hypotension decreases significantly in iNOS knockout mice[78].

Table 3. Studies of molecular hydrogen against sepsis through an anti-apoptosis effect

Drug	Authors	Animal/cell	Disease model	Administration	Effects	Signaling pathways
HRS	Sun et. al 2011[21]	C57BL/6 mice	Septic liver injury (GalN/LPS)	HRS 8ml/kg/3h × 3 i.p.	TUNEL (+) ↓ , caspase-3 ↓, cPARP ↓	ROS-JNK-Caspase3 ↓
2% H_2	Qiu et. al 2011[13]	C57BL/6 mice	Septic lung injury (LPS)	2% H_2 inhalation	TUNEL (+) ↓, Bax ↓, caspase-3↓, Bcl-xl ↑	ROS-JNK-Caspase3 ↓
HRS 2% H_2	Xie et. al 2012[8]	C57BL/6 mice	Septic lung injury (LPS)	2% H_2 inhalation/HRS 10 ml/kg i.p.	TUNEL (+) ↓, caspase-3 ↓	NF-κB ↓
HRS	Zhou et. al 2012[113]	Wistar rats	Sepsis (CLP)	HRS 2.5 or 10 ml/kg i.p.	TUNEL (+) ↓, caspase-3 ↓	N/A
HRS	Li et. al 2013[23]	Sprague-Dawley rats	Sepsis (CLP)	HRS 5 ml/kg i.p.	TUNEL (+) ↓	N/A
HRS	Xu et. al 2013[22]	Sprague-Dawley rats	Septic liver injury (LPS)	HRS 8 ml/kg/h i.v.× 6h	TUNEL (+) ↓, Smac ↓, caspase-3 ↓	NF-κB ↓, Smac ↓, MAPK (JNK/P38) ↓
2% H_2	Liu et. al 2014[26]	ICR mice	Septic brain injury (CLP)	Inhale 2% H_2 1h (post-operation 1h and 6h)	TUNEL (+) ↓	Nrf2/HO-1 ↑
HRS HRM	Chen et. al 2015[36]	ICR mice, UVECs-12	Sepsis (CLP) HUVECs (LPS)	HRS 5 ml/kg i.p., HRM 0.6 mmol/L	Annexin-V/PI ↓, caspase-3 ↓	Nrf2/HO-1 ↑
HRS	Zhang et. al 2015[11]	Sprague-Dawley rats	Septic lung injury (LPS)	HRS 8 ml/kg/h i.v.× 6h	TUNEL (+) ↓, caspase-3 ↓	P38 MAPK ↓
HW	Iketani et. al 2017[6]	C57BL/6 mice	Sepsis (LPS)	HW oral (72h before and after LPS)	TUNEL (+) ↓	HO-1 ↑, ET-1 ↑

HRS: hydrogen rich saline, HRM: hydrogen rich medium, HW: hydrogen water, LPS: Lipopolysaccharide, CLP: cecal ligation and puncture, GalN: D-galactosamine, i.p.: Intraperitoneal injection, i.v.: Intravenous injection, TUNEL: terminal deoxynucleotidyl transferase mediated dUTP nick end labeling, cPARP: cytoplasm poly ADP-ribose polymerase, ROS: reactive oxygen species, Smac: second mitochondria-derived activator of caspases.

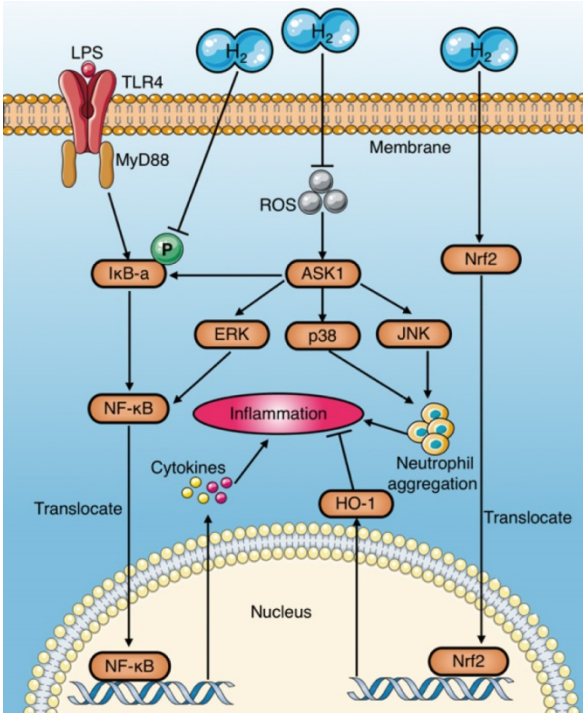

Figure 5 Anti-inflammation mechanisms of molecular hydrogen during sepsis. (a) Molecular hydrogen suppresses the NF-κB pathway to reduce pro-inflammatory cytokines by inhibiting IκB-a phosphorylation or suppressing ERK activation mediated by upstream and ASK1. (b) Molecular hydrogen reduces neutrophil aggregation by inhibiting activation of ROS-ASK1-MAPK. (c) Molecular hydrogen attenuates the inflammatory response by activating the Nrf2-mediated HO-1 signaling pathway. ASK1: apoptotic signal-regulated kinase 1; ROS: reactive oxygen species; ERK extracellular regulated kinase; MAPK: mitogen activated protein kinase.

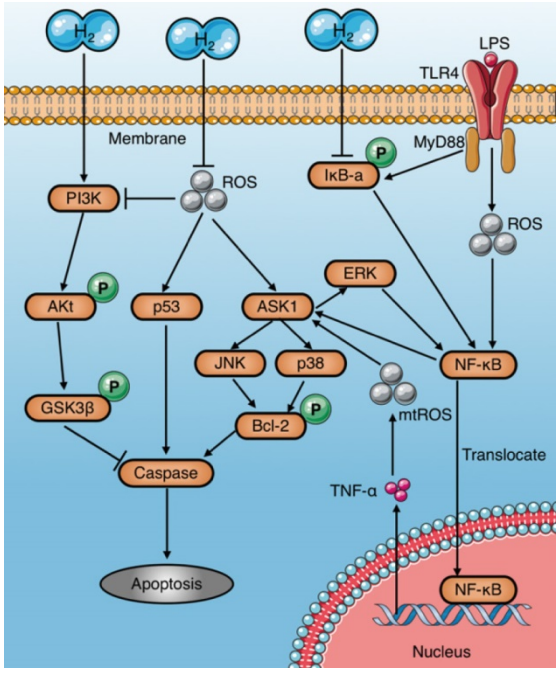

Figure 6. Anti-apoptosis mechanisms of molecular hydrogen in sepsis. Molecular hydrogen suppresses caspases through several pathways: (a) inhibiting ASK1-MAPKs(p38/JNK/ERK)-Bcl-2 activation by neutralizing ROS or suppressing NF-κB; (b) inhibiting p53 activation induced by ROS; (c) activating the PI3K-AKt-GSK3β signaling pathway. PI3K: phosphatidylinositide 3-kinase; AKt: protein kinase B; GSK3β: glycogen synthase kinase 3 beta; ROS: reactive oxygen species.

As a natural antioxidant, molecular hydrogen selectively scavenges ROS[2]. Although it does not react directly with NO [2], molecular hydrogen plays an anti-shock role by indirectly eliminating NO through various mechanisms[23, 29, 58, 59, 79-81] (Table 4) Saramago et al. determined that LPS leads to severe hypotension, which coincides with a sharp increase in NO production during sepsis[79]. In contrast, molecular hydrogen alleviates LPS-induced hypotension by significantly reducing NO production[79]. Up to now, the mechanism of NO removal by molecular hydrogen can be summarized in two ways (Fig. 7A): (1) Reduced expression of iNOS and eNOS, which are key NO production enzymes. LPS-induced phosphorylation of p38 MAPK, JNK, and IκBα, leading to activation of transcription factors involving AP1, ELK and NF-KB, which bind to the promoter region of iNOS and increase its expression [58]. Molecular hydrogen counteracts the effect of LPS on iNOS and reduces NO production[58]. Ikeda et al. demonstrated that HRS reduces iNOS expression in intestinal epithelial cells of septic rats[29]. In addition, Zheng et al. reported that HRS reduces eNOS expression in vascular endothelial cells of spontaneously hypertensive rats and alleviates vascular dysfunction [59]. (2) Positive feedback consumption of NO by eliminating NO-derived peroxynitrite: Under various stress conditions, NADPH oxidase is rapidly activated and becomes the main source of ROS[63]. Superoxide ($\cdot O_2^-$) produced by catalysis of NADPH oxidase reacts with NO to form peroxynitrite ($\cdot ONOO^-$). Molecular hydrogen selectively scavenges $ONOO^-$, and may consume NO in a positive feedback manner[81].

In addition to NO-induced hypotension, the decline in cardiac pump function caused by insufficient ATP production in cardiomyocytes is an important reason for endotoxic shock[28]. Mitochondria are the main organelles of ATP production. Several studies have proved that molecular hydrogen improves mitochondrial function through preserving the mitochondrial membrane potential ($\Delta\Psi$)[82] and alleviating mitochondrial swelling[59, 61] (Table 4). Tao et al. demonstrated that HRS reverses LPS-induced phosphocreatine (PCr)/ATP decline and improves the myocardial energy supply[7]. In addition, multiple studies reported HRS increases ATP production in cardiomyocytes by restoring electron transport chain enzyme activity[59, 61, 83]. The increase of ATP production by molecular hydrogen is achieved by activating mitochondrial oxidative phosphorylation (OXPHOS)[84], and the mechanisms include upregulating the expression of a growth hormone

releasing peptide (ghrelin) and fibroblast growth factor 21 (FGF21) and increasing glucose metabolism [85]. Moreover, the regulation of molecular hydrogen on glucose metabolism can be divided into the insulin-dependent pathway (glucose transporter 4 [GLUT4]) and the insulin-independent pathway (GLUT1) (Fig. 7B). In addition, crosstalk occurs in the regulatory mechanism of molecular hydrogen on ghrelin and glucose metabolism (Fig. 7B). Ghrelin has recently been acknowledged as a major modulator of mitochondrial bioenergetics, as it increases mitochondrial energy production by increasing protein complexes III and IV[86]. GLUT4 is an insulin-regulated glucose transporter that plays a carrier role during cellular uptake of glucose. FGF21 is a hepatic hormone that enhances utilization of fatty acids and glucose. Therefore, positive regulation of GLUT4 and FGF21 by molecular hydrogen may play a role in enhancing energy metabolism.

Moreover, as previously mentioned, hyperpermeability of the vascular endothelium may aggravate endotoxin shock[34] (Table 4). However, molecular hydrogen can alleviate LPS-induced vascular endothelial injury by regulating the RhoA/mDia/ROCK signaling pathway and down-regulating the expression of adhesion molecules (Fig. 7C).

In conclusion, molecular hydrogen alleviates LPS-induced hypotension or shock by eliminating NO, increasing ATP production in cardiomyocytes, and decreasing vascular endothelium permeability.

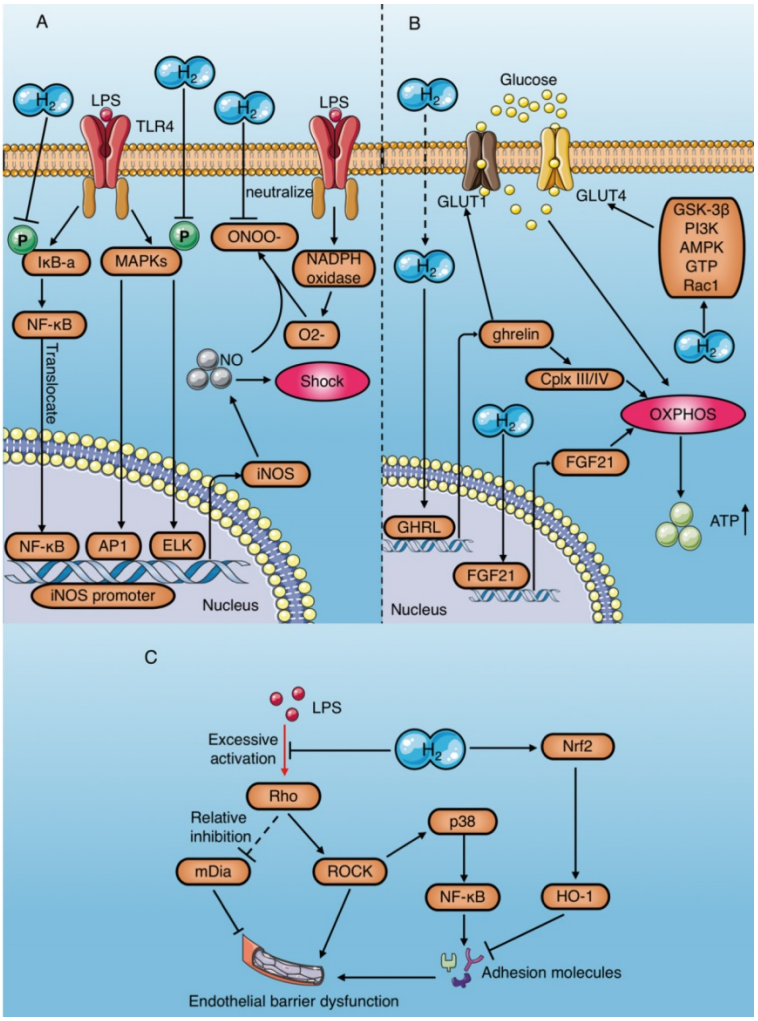

Figure 7. Anti-shock mechanisms of molecular hydrogen during sepsis. (A) Molecular hydrogen inhibits production of the vasodilator nitric oxide and promotes its consumption. (B) Molecular hydrogen increases ATP production mediated by OXPHOS activation in cardiomyocytes. (C) Molecular hydrogen alleviates vascular endothelial barrier damage mediated by the RhoA/ROCK/mDia signaling pathway and adhesion molecules. AP1: activator protein 1; ELK1: ETS domain-containing protein; OXPHOS: oxidative phosphorylation; Cplx: mitochondrial redox carrier (complex); GLUT: Glucose transporters; ghrelin: growth hormone releasing peptide; FGF21: fibroblast growth factor 21.

Regulation of autophagy

Autophagy is one of the most important functions of eukaryotic cells, which encapsulates substrates within a double-membrane-bound vesicle, termed an autophagosome that fuses with lysosomes for degradation and recycling of the sequestered substrates[87]. Cells clear damaged organelles, abnormal proteins, and extracellular pathogens in the cytoplasm by autophagy, which is essential for maintaining cell homeostasis [87]. Autophagy is involved in the regulation of immunity and inflammation [87-91], and plays an important role in sepsis [92-95].

Studies have demonstrated that molecular hydrogen plays a protective role by modulating autophagy in multiple diseases and conditions, including sepsis [11, 12, 20], ischemia- reperfusion injury[96-98], organ transplantation [70, 99-102], and pathological neuralgia [103-105] (Table 5). However, the regulation of autophagy by molecular hydrogen remains controversial, and the regulatory mechanism is very complex (Fig. 8).

Autophagy is a double-edged sword. Moderate autophagy helps cells survive harsh environments by reducing apoptosis and necrosis, while excessive autophagy leads to autophagic death[106]. Zhang et al. reported that HRS attenuates lung injury by inhibiting autophagy in alveolar epithelial cells of septic rats, which might be related to inhibition of the p38 MAPK signaling pathway[11] (Fig. 8A). Similarly, other studies have found that molecular hydrogen inhibits excessive autophagy to attenuate the ALI induced by sepsis by activating HO-1 and the mammalian target of rapamycin (mTOR) pathway, and inhibiting the NF-κB pathway[12, 107] (Fig. 8A). In contrast, Dong

et al. demonstrated that inhaling molecular hydrogen enhances the autophagy level of lung tissue in septic mice, which improves mitochondrial function, and protects the lungs [20] (Fig. 8A).

The same controversy has also occurred in studies of animal organ ischemia-anoxia reperfusion injury models[96-98] and orthotopic liver transplantation (OLT) models[70, 99-102]. Bai et al. reported that molecular hydrogen enhances autophagy in neurons and attenuates brain damage by suppressing mTOR and signal transducer and activator of transcription 3 (Stat3) phosphorylation, and preventing the activation of ERK1/2 induced by hypoxia-ischemia [96] (Fig. 8B). A study by Chen et al. showed that HRS reduces renal fibrosis after ischemia-reperfusion injury by increasing autophagy, which may be related to the retention of Klotho gene expression [98] (Fig. 8B). However, in a rat model of myocardial ischemia-reperfusion injury, HRS was demonstrated to reduce myocardial injury by inhibiting cardiomyocyte autophagy[97], the mechanism of which was reduced suppression of the mTOR signaling pathway mediated by HRS-induced inhibition of AMP-activated protein kinase activation[97] (Fig. 8B).

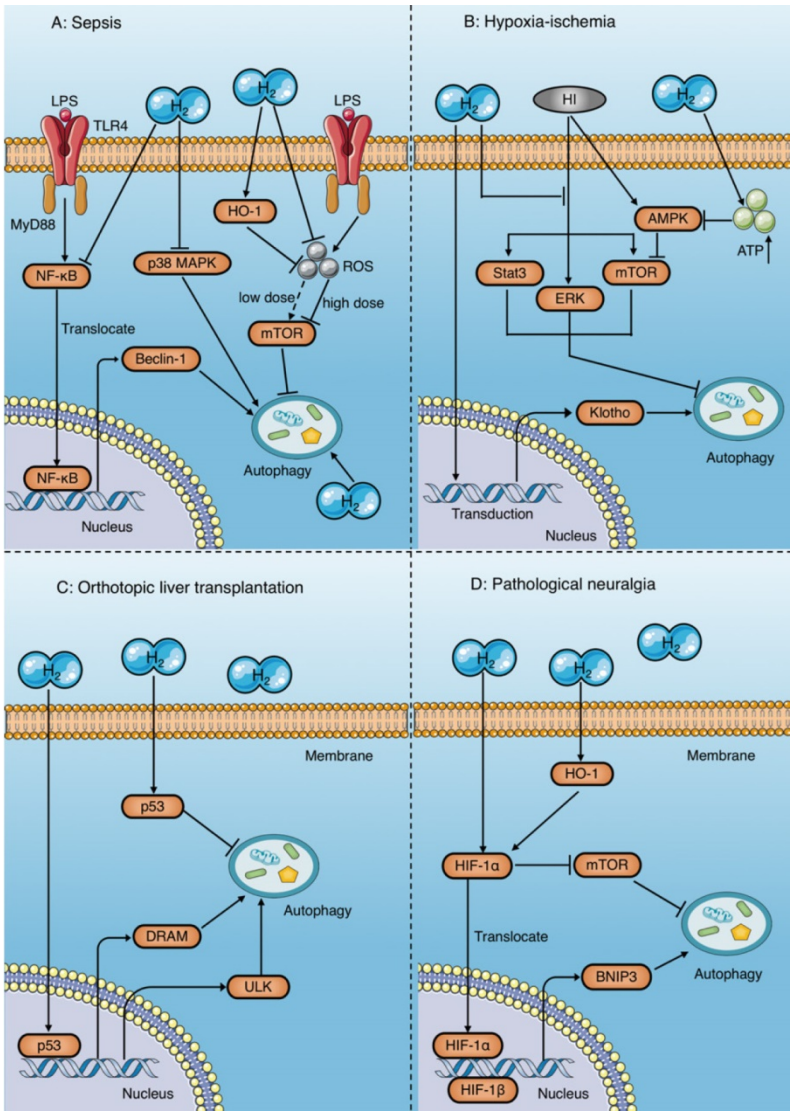

Figure 8. Autophagy regulatory mechanisms of molecular hydrogen. (A) Molecular hydrogen has a dual effect on the regulation of autophagy during sepsis. On the one hand, molecular hydrogen suppresses excessive autophagy by inhibiting NF-κB and p38 MAPK, enhancing HO-1, and reducing mTOR suppression induced by reactive oxygen species. On the other hand, molecular hydrogen may enhance autophagy through an unknown mechanism. (B) In studies of ischemia-anoxia reperfusion injury, molecular hydrogen enhances autophagy by inhibiting activation of ERK, mTOR, and Stat3, or by restraining Klotho gene expression. However, molecular hydrogen inhibits AMPK activation by increasing ATP production, which subsequently reduces mTOR suppression and inhibits autophagy. (C) Molecular hydrogen promotes autophagy in orthotopic liver transplantation by activating p53 in the nucleus and increasing target gene expression involving DRAM and ULK. In contrast, molecular hydrogen suppresses autophagy by activating p53 in the cytoplasm. (D) In pathological neuralgia, molecular hydrogen activates HIF-1α and subsequently increases BNIP3 expression or inhibits mTOR, both of which promote autophagy. HI: hypoxia-ischemia; OLT: orthotopic liver transplantation; HIF-1α: hypoxia inducing factor-1; BNIP3: Bcl-2 19 kilodalton interacting protein 3; mTOR: mammalian target of rapamycin; Stat3: signal transducer and activator of transcription 3; AMPK: AMP-activated protein kinase; DRAM: damage regulated autophagy modulator; ULK: Unc-51 like autophagy activating kinase.

Results in a rat model of OLT show that HRS exerts a protective effect by inhibiting autophagy in orthotopic liver tissue[99, 100], but enhances autophagy in other distant tissues, such as the brain and kidney[70, 101, 102], both of which are associated with p53 activation (Fig. 8C). Tumor suppressor gene p53 is widely involved in regulation of the cell cycle, apoptosis, and autophagy induction. However, the regulatory mechanism of p53 in autophagy is very complex and not fully understood. Researchers believe that the effect of p53 on autophagy may depend on its location within the cell[108]. p53 upregulates autophagy levels by acting as a transcriptional activator in the nucleus, while inhibiting autophagy in the cytoplasm[108]. This viewpoint has been validated in several studies. Shi et al.[100] reported that HRS inhibits autophagy by increasing p53 phosphorylation in the hepatic cytoplasm after OLT, and reduces hepatocyte damage after transplantation (Fig. 8C). In contrast, the results of Du et al.[70], Wu et al.[101], and Chen et al.[102] suggest that HRS promotes autophagy to protect the kidney and brain after OLT by activating the p53 signaling pathway (Fig. 8C).

In addition, a number of studies have shown that HRS promotes autophagy in rats with neuropathic pain and plays a protective role[103-105]; the underlying mechanism of which is activation of

hypoxia inducing factor-1 (HIF-1α) by HRS[105] (Fig. 8D). HIF-1α induces autophagy by increasing the expression of its target gene BNIP3[105] and inhibiting mTOR[109]. Wang et al. also showed that HRS promotes autophagy in CCI rats by further increasing activation of HIF-1α and expression of BNIP3[105]. In addition, studies have shown that HRS increases HIF-1α expression by promoting HO-1 activation[110, 111], which may also be involved in the regulation of autophagy by HRS (Fig. 8D).

Notably, there is crosstalk between the regulatory mechanisms of molecular hydrogen on autophagy and apoptosis, which involve MAPK[11, 65, 69, 96] and p53[70, 100-102]. Therefore, we can infer that HRS may change the fate of cells and the prognosis of diseases by regulating the balance of apoptosis and autophagy, which requires further research.

Table 4. Studies of molecular hydrogen against sepsis through an anti-shock effect

Drug	Authors	Animal/cell	Disease model	Administration	Effects	Signaling pathways
HRS	Li et. al 2013[23]	Sprague-Dawley rats	Sepsis (CLP)	HRS 5 ml/kg i.p. (0,6,18h after CLP)	NO ↓ ,	N/A
HRS	Ikeda et. al 2018[29]	C57BL/6 mice	Intestinal barrier dysfunction (CLP)	HRS 8 ml/kg/d × 7d gavage	Permeability ↓,	N/A
HRM	Itoh et. al 2011[58]	RAW264	Macrophages (LPS)	HRM 0.3 mmol/L	NO ↓	iNOS ↓
2% H_2	Saramago et. al 2019[79]	Wistar rats	Sepsis (LPS)	2% H_2 inhalation× 6h	MAP↑, HR ↓,NO ↓	PGE2 ↓
HRS	Tao et. al 2015[7]	Sprague-Dawley rats	Heart dysfunction (LPS)	HRS 10 ml/kg i.p. (1h,4h after LPS)	ATP ↑	PGC-1α↑, PPARα↑, ERRα↑, JNK↓
HRM	Xie et. al 2015[30]	HUVECs U937	Endothelial dysfunction (LPS)	HRM 0.6 mmol/L	VCAM-1↓, E-selectin ↓, TEER ↑, E-cadherin ↑	Rho/ROCK ↓
HRM	Wang et. al 2013[35]	HUVEC-12 U937	Endothelial dysfunction (LPS)	HRM 0.6 mmol/L	VCAM-1↓, E-selectin ↓, VE-cadherin ↑	N/A
HRS HRM	Chen et. al 2015[36]	ICR mice, HUVECs-12	Sepsis (CLP), HUVECs (LPS)	HRS 5 ml/kg i.p., HRM 0.6 mmol/L	ICAM-1 ↓,VCAM-1 ↓	Nrf2/HO-1 ↑
HRM	Yu et. al 2015[38]	HUVECs-12	HUVECs (LPS)	HRM 0.6 mmol/L	VCAM-1↓, ICAM-1↓, E-selectin ↓, TEER ↑, VE-cadherin ↑	N/A

HRS/M: hydrogen rich saline/media, LPS: Lipopolysaccharide, CLP: cecal ligation and puncture, i.p.: Intraperitoneal injection, HUVECs: human umbilical vein endothelial cells, VCAM-1: vascular cell adhesion molecule -1, ICAM-1: intercellular adhesion molecule-1, TEER: trans-endothelial electrical resistance, PGE2: Prostaglandin E2, PGC-1α: Peroxisome proliferator-activated receptor gamma coactivator 1-alpha, PPARα: Peroxisome proliferator-activated receptor alpha, ERRα: Estrogen-related receptor alpha, Rho: Ras homolog gene, ROCK: Rho-associated coiled-coil protein kinase.

Table 5. Studies and mechanisms of molecular hydrogen modulating autophagy

Drug	Authors	Animal/cell	Disease model	Administration	Effects	Signaling pathways
HRS	Zhang et. al 2015[11]	Sprague-Dawley rats	Septic lung injury (LPS)	HRS 8 ml/kg/h i.v. × 6h	Autophagosome ↓, LC3II/I ↓	P38 MAPK ↓
2% H_2	Dong et. al 2017[20]	ICR mice	Sepsis (CLP)	2% H_2 inhalation	LC3II/I ↑	N/A
HRS	Bai et. al 2016[96]	BALB/c mice	HIBD	HRS 5 ml/kg/d i.p. × 3d	LC3II/I ↑, Beclin1 ↑	mTOR ↓, stat3 ↓, ERK1/2 ↓
HRS	Pan et. al 2015[97]	Sprague-Dawley rats	Myocardial injury after IR	HRS 10 ml/kg i.p.	LC3II/I ↓, Beclin1 ↓	AMPK ↓, mTOR ↑
HRS	Chen et. al 2017[98]	C57BL/6 mice	CKD after IR	HRS 1 ml/kg i.p.	LC3II/I ↑, Beclin1 ↑	Klotho
HRS	Du et. al 2016[70]	Sprague-Dawley rats	AKI after OLT	HRS 6 ml/kg i.v.	LC3II/I ↑, Autophagosome ↑, Beclin1 ↑, P62 ↓	p53 ↑
HRS	Shi et. al 2016[99]	Sprague-Dawley rats	IRI after OLT	HRS 6 ml/kg i.v.	LC3II ↓, Beclin1 ↓	N/A
HRS	Shi et. al 2016[100]	Sprague-Dawley rats	OLT	HRS 6 ml/kg i.v.	LC3II ↓, Beclin1 ↓	p53 ↑
HRS	Wu et. al 2015[101]	Sprague-Dawley rats	AKI after OLT	HRS 6 ml/kg i.v.	LC3II ↑, Beclin1 ↑	p53 ↑
HRS	Chen et. al 2017[102]	Sprague-Dawley rats	HNA after OLT	HRS 6 ml/kg i.v.	LC3II ↑, Beclin1 ↑	p53 ↑
HRS	Wang et. al 2016[103]	Sprague-Dawley rats	Neuropathic pain (CCI)	HRS 5 ml/kg/d i.p. × 7d	LC3II/I ↑, Beclin1 ↑, P62 ↓	N/A
HRS	Ma et. al 2017[104]	Sprague-Dawley rats	PHN	HRS 10 ml/kg/12h i.p. × 14d	LC3II ↑, Beclin1 ↑, P62 ↓	N/A
HRS	Wang et. al 2018[105]	Sprague-Dawley rats	Neuropathic pain (CCI)	HRS 10 ml/kg/d i.p.× 15d	Beclin1 ↑, P62 ↓, Autophagosome ↑	HIF-1α ↑

HRS: hydrogen rich saline, LPS: Lipopolysaccharide, CLP: cecal ligation and puncture, HIBD: hypoxic-ischemic brain damage, IR: ischemia-reperfusion, IRI: ischemia-reperfusion injury, CKD: chronic kidney disease, AKI: acute kidney injury, OLT: orthotropic liver transplantation, HNA: hippocampal neuron apoptosis, CCI: chronic constriction injury, PHN: post-herpetic neuralgia, i.p.: Intraperitoneal injection, i.v.: Intravenous injection.

Effective concentration and dose of molecular hydrogen against sepsis

HRS/HRW was generally prepared as the method originally described by Ohsawa et. al[112]. Briefly, hydrogen gas was dissolved in NS or water under 0.4 MPa pressure to be oversaturated (>0.6 mmol/L) and then stored in a sealed container without dead volume at 4°C under atmospheric pressure followed by γ-radiation sterilization before use. According to the method described by Ohsawa et. al[2], HRM was prepared through dissolving hydrogen gas into medium under 0.4 MPa pressure to achieve supersaturated status (>0.6 mmol/L) and stored in a hydrogen-rich closed culture flask. Moreover, the method of inhaling 2% hydrogen gas referred to the research of Xie et. al[8]. The various concentrations and doses of molecular hydrogen, which have been proved effective in organs and cells protection in sepsis, were summarized in Table 6.

Table 6. Effective concentration and dose of molecular hydrogen against sepsis

Organ/cell	Administration	Effective concentration	References
Lung	H_2 inhalation	2%	[8, 13, 15, 20]
	HRS 8 ml/kg i.v.	> 0.6 mmol/L	[11]
	HRS 10 ml/kg i.p.	> 0.6 mmol/L	[14]
	HRW po.	0.25-1.6 mmol/L	[16]
Liver	HRW po.	0.6 mmol/L	[6]
	HRS 8 ml/kg i.p.	> 0.6 mmol/L	[21]
	HRS 8 ml/kg i.v.	> 0.6 mmol/L	[22]
Kidney	H_2 inhalation	2%	[24, 25]
	HRS 5 ml/kg i.p.	> 0.6 mmol/L	[23]
Brain	H_2 inhalation	2%	[26]
Pancreas	HRW 6 ml/kg po.	0.62-0.82 mmol/L	[27]
Heart	HRS 10 ml/kg i.p.	> 0.6 mmol/L	[7]
Eye	HRS 10 ml/kg i.p.	> 0.6 mmol/L	[10]
Endothelial cell	HRM incubation	0.6 mmol/L	[30, 35, 36, 38]
	HRS 5 ml/kg i.p.	> 0.6 mmol/L	[36]
Epithelial cell	HRS 8 ml/kg gavage	0.7 mmol/L	[29]
	HRS 8 ml/kg i.v.	> 0.6 mmol/L	[11]
	HRM incubation	0.6 mmol/L	[9]

Conclusion and perspectives

In summary, molecular hydrogen exhibits multiple advantages in the treatment of sepsis due to its unique physicochemical properties. Molecular hydrogen scavenges free radicals selectively, modulates signaling transduction, and enters the nucleus to regulate transcription. Recent studies have shown that molecular hydrogen has a significant protective effect on multiple organs and physiological barriers in septic animal models. In addition to the well-known anti-oxidative stress effects, the mechanisms of molecular hydrogen against sepsis include anti-inflammation, anti-apoptosis, anti-shock and regulation of autophagy, each of which involves multiple signaling pathways and crosstalk. However, the potential molecular mechanisms are still not completely clear, and some results remain controversial, which need further research. Moreover, the current research results are mainly based on animal experiments. Whether these findings are equally applicable to humans is not yet known, which also requires further clinical studies to validate. Nevertheless, the advantages of molecular hydrogen have provided important means and optimistic prospects for treating sepsis.

Abbreviations

HRS: hydrogen-rich saline; HRW: hydrogen-rich water; HRM: hydrogen-rich medium; LPS: lipopolysaccharide; ALI: acute lung injury; BPD: bronchopulmonary dysplasia; FGFR4: fibroblast growth factor receptor 4; VEGFR2: vascular endothelial growth factor receptor 2; HO-1: heme oxygenase 1; EMT: epithelial-mesenchymal transition; ROS: reactive oxygen species; TGF-β: transforming growth factor-β; EVLW: extravascular lung water; AQP1,5: Aquaporin 1,5; MAPK: mitogen-activated protein kinase; JNK: c-Jun N-terminal kinase; ERK: extracellular regulated protein kinases; NF-κB: nuclear factor kappa-light-chain-enhancer of activated B cells; Smac: second mitochondria-derived activator of caspase; ET-1: endothelin-1; Nrf2: nuclear factor erythroid 2-related factor 2; LVD: left ventricular diameter; FS: fractional shortening; FAO: fatty acid oxidation; EIU: endotoxin-induced uveitis; AqH: aqueous humor; VCAM-1: vascular cell adhesion molecule -1; ICAM-1: intercellular adhesion molecule-1; TEER: trans-endothelial electrical resistance; TER: trans-epithelial resistance; AJ: adherens junction; TJ: tight junction; FITC-dextran: fluorescein isothiocyanate-dextran; MLC: myosin light chain; TNF-α: tumor necrosis factor alpha; IL-1β: interleukin 1 beta; IL-10: interleukin 10; HMGB1: high mobility group box 1; HUVECs: human umbilical vein endothelial cells; CLP: cecal ligation and puncture; Cdc42: Cell division control protein 42 homolog; Rac1: Ras-related C3 botulinum toxin substrate 1; RhoA: Ras homolog gene family member A; mDia1: mammalian diaphanous-related formin 1; ROCK: Rho-associated coiled-coil protein kinase; RNS: reactive nitrogen species; ·O2-: superoxide anion; ·OH: hydroxyl radical; H_2O_2: hydrogen peroxide; ·NO: RNS include nitric oxide; ·NO2: nitrogen dioxide; ·ONOO-: peroxynitrite anion; SOD: superoxide dismutase; CAT: catalase; MOD: myeloperoxidase; 8-iso-PGF2α: 8-iso-prostaglandinF2α; MDA: malondialdehyde; iNOS: inducible nitric oxide synthase; eNOS: endothelial nitric oxide synthase; NADPH oxidase: nicotinamide adenine dinucleotide phosphate oxidase; BPD: bronchopulmonary dys-

plasia; EMT: epithelial-mesenchymal transition; PF: pulmonary fibrosis; TGF-β: transforming growth factor-β; MPO: myeloperoxidase; PMNs: polymorphonuclear neutrophils; BALF: bronchoalveolar lavage fluid; AP1: activator protein 1; ELK1: ETS domain-containing protein; OXPHOS: oxidative phosphorylation; Cplx: mitochondrial redox carrier (complex); GLUT: Glucose transporters; ghrelin: growth hormone releasing peptide; FGF21: fibroblast growth factor 21; MIP: macrophage inflammatory protein; IκBα: nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor alpha; ASK1: apoptotic signal-regulated kinase 1; GSK3β: Glycogen synthase kinase 3 beta; HI: hypoxia-ischemia; OLT: orthotopic liver transplantation; HIF-1α: hypoxia inducing factor-1; BNIP3: Bcl-2 nineteen kilodalton interacting protein 3; mTOR: mammalian target of rapamycin; Stat3: signal transducer and activator of transcription 3; AMPK: AMP-activated protein kinase; DRAM: damage regulated autophagy modulator; ULK: Unc-51 like autophagy activating kinase.

Acknowledgements

This study was supported by the Shenyang Municipal Science and Technology Commission (Grant Number 17-230-9-45); the Liaoning Education Department (Grant Number LK201634); and the National Natural Science Foundation of China (Grant Number 81701951).

Competing Interests

The authors have declared that no competing interest exists.

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