Acute kidney injury (AKI) is the major complication of rhabdomyolysis (RM) clinically, which is usually mimicked by glycerol injection in basic research. Oxidative stress, inflammatory response and apoptosis are recognized to play important roles in development of this disease. Recently, numerous studies have reported the therapeutic effects of molecular hydrogen (H₂) on oxidative stress and inflammation-related diseases. Here, the effects of H₂ against glycerol-induced AKI and the underlying mechanisms were explored in rats. Low (4%) and high (67%) concentrations of H₂ were prepared using a self-made device to investigate the dose-response. After 72 hours of glycerol injection (8 mL/kg), we found that glycerol triggered oxidative stress, inflammatory reactions, and apoptotic events. These caused subsequent renal damage, evidenced by a significant reduction of antioxidases and up-regulation of the relevant damaged biomarkers. H₂ inhalation reversed the above alterations and exerted renoprotective effects. Interestingly, for RM/AKI-related factors, no consistent dose-response benefits of H₂ were observed. However, higher concentration of H₂ inhalation improved histological and morphological changes better. This study suggests that H₂ is a potential alternative therapy to prevent or minimize RM induced AKI possibly via its antioxidant, anti-inflammatory, anti-apoptotic and anti-necroptotic properties.

Key words: acute kidney injury; apoptosis; glycerol; hydrogen gas; inflammation; necroptosis; oxidative stress; rats

Abstract

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INTRODUCTION

Acute kidney injury (AKI) is a heterogeneous group of conditions mainly manifested as a sudden kidney dysfunction accompanied by increase of serum creatinine and urea nitrogen due to the decrease of glomerular filtration rate. Many factors contribute to the pathology of AKI including acute illness, complications of medications and medical procedures. Recently, a few studies have reported the association with coronavirus disease 2019 (COVID-19), and early investigations from China indicated that 25% of patients with COVID-19 in critical care have AKI. AKI is one of the most severe complications of rhabdomyolysis (RM), which can cause internal environment disorders and damages to various organs due to the leakage of toxic muscle-cell contents including myoglobin, electrolytes, and other sarcoplasmic proteins (e.g., creatinine kinase and lactate dehydrogenase) into the systemic circulation. RM-induced AKI accounts for 10% to 40% of all diagnosed AKI cases. In clinical practice, the actual incidence rate is 13% to 50%. The mortality rate is estimated to be 20% for patients who do not develop AKI from RM, however, for patients who develop AKI, the mortality rate increases to as high as 59%. Although RM-induced AKI has been extensively studied over a long time, the exact pathogenesis involved has not been elucidated. Several factors including oxidative stress, inflammatory response, apoptosis, vasoconstriction, and tubular obstruction are recognized to play important roles. Traditionally, the initial conservative measure is usually fluid resuscitation, which is a ubiquitous intervention in critical care medicine. However, the treatment is ineffective in the case of severe oliguria or anuria that may lead to interstitial and pulmonary edema, secondary abdominal cavity syndrome, renal perfusion pressure reduction, or even acute respiratory distress syndrome. Recently, numerous studies have focused on various compounds or molecules, such as vitamin c, L-carnitine, and oleuropein, in preventing RM-induced
AKI and most of them attributed the mitigating effects to their antioxidant properties.\textsuperscript{8,14,15} Antioxidants inhibit lipid peroxidation in proximal tubular cells and balance the redox cycles of myoglobin.\textsuperscript{16} Although several drugs can alleviate the disease, the majority of them are bio-macromolecules with poor penetrability, hence difficult to enter the reactive oxygen production site. Moreover, their long-term safety remains to be verified, hence the need to develop better antioxidants with minimal or no side effects.

Molecular hydrogen (H\textsubscript{2}) is a small molecule with the capacity to efficiently enter the plasma membrane and other cell organelles. Numerous studies have reported the therapeutic effects of H\textsubscript{2} on oxidative stress and inflammation-related diseases such as ischemia and reperfusion injuries,\textsuperscript{17-19} atherosclerosis,\textsuperscript{20-22} metabolic diseases,\textsuperscript{23-25} neurodegenerative disorders,\textsuperscript{26,27} and cancer.\textsuperscript{28-31} These effects of H\textsubscript{2} are usually attributed to its anti-oxidative, anti-inflammatory, and anti-apoptotic capabilities. Recently, H\textsubscript{2} supplements have been found to play a role in glycerol-induced nephrotoxicity is attributed to reactive oxygen metabolites, especially the hydroxyl radicals (•OH) mimics the RM induced AKI in humans.\textsuperscript{36} The most important role in glycerol-induced nephrotoxicity is attributed to reactive oxygen metabolites, especially the hydroxyl radicals (•OH) which causes myoglobin-induced AKI.\textsuperscript{37}

In this study, glycerol was administered to induce the AKI model of Sprague-Dawley rats, and H\textsubscript{2} inhalation was used to evaluate the potential renoprotective effects and possible mechanisms against RM-induced AKI. For the first time, low (4\%) and high (67\%) concentrations of H\textsubscript{2} were provided using our self-made device to investigate the dose-dependent response of H\textsubscript{2} for this model.

**Materials and Methods**

**Animals**

Forty 8-week old specific pathogen free level male Sprague-Dawley rats weighing 200–230 g were purchased from Jinan Pengyue Experimental Animal Breeding Co., Ltd. (Jinan, China; licence No. SCXK (Lu) 20190003). The sex selection of rats is based on most literature reports.\textsuperscript{37-39} All rats were housed in cages under standard conditions (22 ± 1°C and 50–60\% relative humidity with a 12/12 hours light/dark cycle) with ad libitum access to water and food before the experiment. All experiments were approved by the Laboratory Animal Ethics Committee of Shandong First Medical University & Shandong Academy of Medical Sciences (No. 2020-1033) on March 18, 2020. All experiments were designed and reported according to the Animal Research: Reporting of In Vivo Experiments (ARRIVE) guidelines.

After 1-week acclimation, the rats were randomly divided into four groups (n = 10 per group) including: (1) control group (Con): air inhalation and saline intramuscularly (IM); (2) model group (AKI): air inhalation and glycerol IM; (3) low H\textsubscript{2} group (AKI + LH\textsubscript{2}): 4\% H\textsubscript{2} + 21\% O\textsubscript{2} + 75\% N\textsubscript{2} inhalation and glycerol IM; and (4) high H\textsubscript{2} group (AKI + HH\textsubscript{2}): 67\% H\textsubscript{2} + 21\% O\textsubscript{2} + 12\% N\textsubscript{2} inhalation and intramuscular injection of glycerol.

**H\textsubscript{2} inhalation**

Different concentrations of H\textsubscript{2} (4\% and 67\%, vol/vol) were prepared using a self-made device as previously reported.\textsuperscript{23} Different gases were controlled by adjusting the rotor flow meter connected to the H\textsubscript{2} cylinder, O\textsubscript{2} cylinder, and air generator. The gases were mixed in a box and then pumped into the sealed animal chamber at a total rate of 3 L/min for 2 hours once daily. Concentrations of H\textsubscript{2} and O\textsubscript{2} (about 21\%) were monitored using gas detectors XP-3140 (New Cosmos Electric Co., Ltd., Japan) and JR2000-O2 (JingRuiBo Technology Co., Ltd., Beijing, China), respectively, to confirm the stability of each gas component. The gas intervention continued throughout the entire experimental period (9 days).

**AKI model**

The AKI model was constructed through a single intramuscular injection of 50\% (vol/vol) in sterile saline) glycerol. After six days of gas inhalation (see the above “Animals” part for details), the rats were dehydrated for 16 hours as in many other studies,\textsuperscript{36,39} after which an 8 mL/kg dose of glycerol (99\% purity, Shanghai Aladdin Biochemical Technology Co., Ltd., Shanghai, China) was injected intramuscularly into the bilateral hindlimbs. The control group was administered with equal normal saline intramuscularly. Immediately after the saline or glycerol injection, drinking water was resumed. Meanwhile, clinical signs were recorded for all animals during the whole period of the experiment.

**H\textsubscript{2} concentration monitoring in the kidney in vivo**

The real-time concentration of H\textsubscript{2} was monitored using a miniaturized Clark-type hydrogen microsensor (Unisense, Aarhus, Denmark). 4\% and 67\% H\textsubscript{2} were prepared as the H\textsubscript{2} inhalation” part mentioned above. Rats were sedated by intraperitoneal injection of 20\% urethane (7 mL/kg) mainly due to its properties including stable anesthetic effect and a long duration of anesthesia (AVMA euthanasia guidelines 2020). After losing consciousness and breathing steadily, the rat was dissected to expose the kidney and then the microsensor tip (diameter 40–60 μm) was inserted into the tissue at a depth about 1 mm. Initially, the pure air was administrated to get a stable baseline. Then 4\% or 67\% concentration of H\textsubscript{2} was delivered continuously until the recording H\textsubscript{2} concentration approaching saturation point. And then, the mixed gas was replaced by the pure air and the monitoring was continued until the H\textsubscript{2} concentration returned to the baseline. At the end of the experiment, rats were euthanized by injecting excessive amounts of anesthetics to reduce the subsequent pain and other distress. Three rats were used for each H\textsubscript{2} concentration.
Sampling
All rats were weighed at the beginning and end of the experiment. The rats were anesthetized by an intraperitoneal injection of 2% pentobarbital sodium (50 mg/kg body mass, Merck, Darmstadt, Germany) after 72 hours of intramuscular injection. Blood samples (15 mL/kg body mass, about 270 g/rat) were collected from the inferior vena cava, centrifuged, and stored at −80°C for further biochemical analysis. The left ventricle was perfused using a peristaltic pump BT100-2J (Longer Precision Pump Co., Ltd., Baoding, China) to expel residual blood from the body. Animal death was defined as mydriasis, respiratory arrest and cardiac arrest for a period of >5 minutes. Both kidneys were quickly dissected, rinsed in cold phosphate-buffered saline, and weighed to estimate the renal somatic index, which was calculated by dividing the kidney weight (g) by final body mass (g) and multiplying by 100. The left kidneys were photographed, and the appearance and color were recorded. Then they were bisected longitudinally, photographed, and fixed with 4% paraformaldehyde for histopathological examinations. The appearance was scored mainly according to the color of the longitudinal section of the kidney and whether the boundary between cortex and medulla was clear. The score was from 1 to 5, and the lower scores represented more severe damage. The right kidney was sliced, flash-frozen in liquid nitrogen, and then stored at −80°C for total RNA extraction and biochemical analysis.

Determination of superoxide dismutase & catalase activities, glutathione content and malondialdehyde level
The sliced kidney was homogenized in 0.1 M phosphate-buffered saline and centrifuged at 3000 × g for 15 minutes at 4°C. The supernatant was used to determine the biochemical parameters including superoxide dismutase (SOD), glutathione (GSH) and catalase (CAT). Malondialdehyde (MDA) content was measured to determine the level of lipid peroxidation. All procedures were performed using commercially available kits according to the manufacturer’s instructions (Nanjing Jiancheng Bioengineering Institute, Nanjing, China).

Assessment of renal and muscle injuries
Urea (UA), creatinine (Cr), blood urea nitrogen (BUN), lactate dehydrogenase 1, creatinine kinase (CK), and creatinine kinase isoenzyme concentrations in plasma were measured using an autoanalyzer Chemray-240 (Rayto Life and Analytical Sciences, Shenzhen, China) to determine the renal and muscular dysfunctions, respectively.

Histopathological observation
The paraformaldehyde-fixed kidney was washed with running tap water for 12 hours, dehydrated in serial gradient concentrations of ethyl alcohol, and then embedded in paraffin blocks. Paraffin sections were cut at 5 μm thick, dewaxed, and rehydrated for hematoxylin and eosin staining. All the sections were observed using an Olympus BX53 light microscope (Olympus Corporation, Tokyo, Japan) and photographed at 400× magnification. The kidney pathology score was used for evaluating the severity of the renal injury. For each group, three samples and at least three different views were analyzed. Histological slides were evaluated according to glomerular atrophy, tubular casts and shedding of tubular epithelial cells (score 5 < 5%; 4 = 6–25%; 3 = 26–55%; 2 = 56–80%; 1 > 80%).

Immunohistochemical staining
Immunohistochemical staining was performed to evaluate the expression of oxidative stress (heme oxygenase-1 (HO-1)), inflammatory responses (tumor necrosis factor-alpha (TNF-α)) and apoptotic proteins (Bax). Paraffin-embedded sections were dewaxed and hydrated. After antigen repair in the citric acid solution, the sections were incubated in 3% hydrogen peroxide for 25 minutes to neutralize the endogenous peroxidase activity. Then sections were treated with goat serum containing 3% bovine serum albumin for 30 minutes and further incubated with primary antibodies (anti-HO-1 rabbit polyclonal antibody, dilution of 1:100, Cat# GB11104; anti-TNF-α rabbit polyclonal antibody, dilution of 1:500, Cat# GB11188; anti-Bax rabbit polyclonal antibody, dilution of 1:100, Cat# GB11007-1; all provide by Servicebio, Wuhan, China) at 4°C overnight. This was followed by incubation with a specific secondary antibody (goat anti-rabbit, Cat# GB23303, dilution of 1:200, Servicebio) at room temperature for 50 minutes. Finally, the sections were visualized using dianimobenzidine substrate liquid and the chromogenic reaction observed under a microscope. Images were recorded at an original magnification of 400× using an Olympus BX53 light microscope.

Terminal deoxynucleotidyl transferase dUTP nick end labeling staining
Cell apoptosis in kidney tissue was detected by terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) assay using an in-situ apoptosis detection kit (11684817910, Roche, Mannheim, Germany) according to the manufacturer’s instructions. Kidney tissue was fixed with 4% paraformaldehyde overnight, dehydrated, embedded in paraffin, cut into 5 μm-thick sections, and placed on a polylysine-coated glass slide to stain TUNEL positive cells (green), and the nuclei were stained with 4′,6-diamidino-2-phenylindole (blue). All stained sections were photographed at 400× magnification.

Real-time quantitative polymerase chain reaction
Total RNA was extracted from kidney samples using cold Trizol reagent according to the manufacturer’s instructions (Invitrogen, Carlsbad, CA, USA). RNA quality and concentration were measured using an ultraviolet-visible spectrophotometer (DeNovix DS-11, Wilmington, DE, USA). The extracted RNA was reverse transcribed into complementary DNA using a reverse transcription kit (Cat# CW2020, CoWin Biosciences, Beijing, China). Subsequently, semiquantitative real-time quantitative polymerase chain reaction (RT-PCR) was performed using a SYBR Green I PCR kit in a total volume of 25 μL prepared according to manufacturer’s instructions (Cat# CW2601, CoWin Biosciences). Expression levels of oxidation-, inflammation-, apoptosis- and kidney injury-related genes were measured, and β-actin was used as a housekeeping gene. The thermocycling reactions were performed using specific primers (Additional Table 1) under the following conditions: Pre-denatured at 95°C for 10 minutes, followed by 40 cycles of 95°C for 10 seconds, 61°C for 32 seconds
and 72°C for 30 seconds. Relative expressions of target genes were calculated using the 2^(-ΔΔCt) method upon normalizing to β-actin mRNA level.

Statistical analysis

Results are presented as mean ± standard error of the mean (SEM). Sample group data conforming to normal distribution were analyzed by one-way analysis of variance followed by least significant difference post hoc test (homogeneity of variance) or Tamhane’s T2 test (heterogeneity of variance). Otherwise, Kruskal-Wallis multiple tests were performed. All statistical analyses were performed using SPSS version 26.0 (IBM, Armonk, NY, USA) and Graphpad Prism version 8.0.1 (GraphPad Prism, SanDiego, CA, USA). P < 0.05 was considered statistically significant.

RESULTS

Clinical signs and general records

After injection, rats in the control group moved freely and were able to feed and drink normally. However, rats in the AKI group appeared to have little activity and hardly drank any water. H₂ inhalation improved the mental state of rats and showed gradual recovery of activity and drinking of some water.

General records including body mass, kidney index and length of rats in the AKI group showed significant alterations compared with the control group (P < 0.05), whereas H₂ inhalation had little influence on these parameters (Additional Figure 1).

H₂ concentration in the kidney after 4% and 67% H₂ inhalation

After inhalation of different concentrations of H₂, the real-time changes of H₂ concentration and saturation concentration in the kidney are shown in Figure 1. H₂ concentration in rat kidneys increased gradually with the continuous H₂ delivery and then approached saturation state (20.521 ± 2.857 μM) at about 300 seconds for 4% H₂ inhalation (Figure 1A and C), whereas saturation concentration (411.376 ± 29.363 μM) at about 800 seconds for 67% H₂ inhalation (Figure 1B and C). The ratio of saturated H₂ concentration measured (about 20 times) was similar with the ratio of inhaled H₂ concentration (about 17 times). When exogenous H₂ supply was withdrawn, H₂ concentration in the kidney started to decrease gradually until dropping to the baseline. It needed more time for 67% H₂ than 4% H₂ (Additional Figure 2).

Renal histopathology

Kidneys in the control group displayed normal morphology as well as plasma membrane with a smooth texture. The contour of the kidney was intact with distinct cortex and medulla boundaries. After glycerol administration, the surface color of the kidney turned lighter and was covered with strawberry-like spots. The longitudinal section displayed dark red brown to black color, and the boundary between the cortex and medulla was mingled (Figure 2A). Result of scores for kidney injury, RM- and AKI-related biomarkers in renal tissue homogenate

Hematoxylin and eosin staining showed that rat kidneys from the control group exhibited normal structures with regular renal tubular epithelial cells and without inflammatory cell infiltration in the stroma (Figure 2C). However, in the AKI group displayed numerous red blood cell casts in the tubule lumen, marked atrophy of glomerular tufts and broken deciduous renal tubular epithelial cells. H₂ inhalation significantly relieved the lesions with an obvious reduction of tubular casts and integral structures of renal tubular epithelial cells (Figure 2C). The kidney pathology scores of slices by hematoxylin and eosin staining among groups supported the above pathological changes, in which AKI rats presented significant lower scores than the Con rats (P < 0.001), whereas H₂ inhalation could better mitigate the deterioration trend by increased scores, especially for the high concentration of H₂ (P < 0.001; Figure 2B).

RM- and AKI-related biomarkers in plasma

Expression levels of kidney function parameters including BUN, lactate dehydrogenase 1, Cr and UA (P < 0.001 or P < 0.05; Figure 3A–D), and RM-related parameters CK (P < 0.01; Figure 3E) in the AKI group were significantly increased compared with those in the control group. The expression of another biomarker for muscle injury of creatinine kinase iso-enzyme was also increased, although no significant difference was found between the groups (Figure 3F). Treatment with H₂ significantly attenuated changes in these plasma indices. However, 67% hydrogen failed to further improve these parameters (Figure 3).

AKI-related biomarkers in renal tissue homogenate

Kidney injury molecule 1 and neutrophil gelatinase-associated lipocalin (NGAL), which are emerging and early indicators for kidney injury, 12,42 were quantified using RT-PCR. Results
displayed a significant increase ($P < 0.001$) in both genes in the AKI group compared to the control group. $H_2$ administration resulted in marked downregulation for both genes, and low-$H_2$ inhalation showed more powerful improved effects ($P < 0.001$; Figure 4).

**Oxidative status**

Oxidative stress plays a significant role in the development and progression of AKI. Biochemical parameters associated with oxidative stress including SOD, CAT, GSH, and MDA were measured in kidney tissue homogenate. The results showed a significant decrease in SOD, GSH, and CAT levels ($P < 0.001$), and a slight increase in MDA content in the AKI group compared to the control group. $H_2$ inhalation alleviated oxidative stress induced by an imbalance between the antioxidant level and free radical production. This was evidenced by a significant reduction in MDA levels, increased SOD activity, and GSH content compared to the AKI group. CAT activity was also improved slightly after $H_2$ inhalation though not statistically significant (Figure 5A–D).

To further gain insights into the potential molecular mechanisms underlying the anti-oxidative effect, HO-1 mRNA expressions were investigated by RT-PCR. Rats in the AKI group showed significant upregulation in mRNA expression levels compared to the control group. After $H_2$ inhalation, HO-1 mRNA expression in the renal tissue was markedly downregulated compared to the AKI group, especially for the
high H₂ concentration group (Figure 5E).

Immunohistochemical staining provided a more direct visualization of HO-1 distribution (Figure 5F). Kidney sections from the control group showed normal HO-1 expression. In contrast, sections from the glycerol-administered group showed numerous HO-1 positive staining, indicating overexpression of the protein. H₂ inhalation significantly downregulated the expression of HO-1 and reduced the severity of the renal injury (Figure 5F).

Inflammatory responses

Interleukin 1β (IL-1β), TNF-α, nuclear factor kappaB (NF-κB), and monocyte chemoattractant protein 1 (MCP-1) levels were quantified using RT-PCR. Compared to the control group, rats administered with glycerol exhibited high levels of these inflammatory mediators in renal tissue. However, H₂ treatment significantly suppressed mRNA expressions of the four inflammatory cytokines (P < 0.05) except for NF-κB under low H₂ inhalation (Figure 6A–D).

Meanwhile, expression of TNF-α was also detected via immunohistochemistry. The result exhibited significant positive staining in the AKI group compared to the control group. Interestingly, after H₂ inhalation, the distribution of renal tubular TNF-α was significantly decreased, indicating the anti-inflammatory activity of H₂ (Figure 6E).

Apoptosis and necroptosis

Apoptotic and necroptosis events following glycerol injection were assessed by determining the transcriptional levels and distributions of pro-apoptotic proteins including Bax and/or Caspase-3 in the kidney tissue. RT-PCR results revealed significant upregulation in mRNA expression levels of Bax, Caspase-3, receptor-interacting serine-threonine kinase 3 (RIPK3), and mixed lineage kinase domain-like protein (MLKL) in the AKI group compared to the control group (P < 0.05). For H₂-treated rats, significant reductions of all the above genes were revealed (P < 0.05) except for RIPK3 under low concentration of H₂ inhalation, which may be associated with the within-group variance (Figure 7A–D).

As shown in Figure 7E, the distribution of Bax was extended in the kidney in the AKI group and was mainly localized in the damaged proximal tubular epithelial cells and renal tubular lumen. Following H₂ administration, apoptotic reactions were markedly alleviated and only weak Bax signal was observed around glomerulus (Figure 7E).

Tubular cell apoptosis was also analyzed by TUNEL staining. Consistent with RT-PCR and immunohistochemical staining results, a large number of TUNEL-positive cells were observed mainly in the renal cortex of the AKI group, whereas H₂ treatment significantly decreased the number of TUNEL-positive cells. Besides, fewer apoptotic cells were observed in the H₂ inhalation groups compared with the AKI group (Figure 8).
Discussion

Extensive studies have explored the pathophysiology in an animal model of myoglobinuric AKI induced by glycerol injection,\textsuperscript{12,37,41,44} which is the most popular model to study RM induced AKI. However, due to the inherently high osmotic pressure of glycerol, local body fluid accumulation leads to decreased blood volume and renal blood flow, increased blood viscosity and renal vascular impedance, decreased glomerular filtration rate, and even dissolution of muscle, release of myoglobin, hemoglobin, and potassium, resulting in nephrotoxicity and ultimately to glomerular and tubular damages.\textsuperscript{45} Glycerol-induced AKI has dual effects of renal ischemia and endogenous...
toxicity on the renal injury. The intramuscular injection of a single dose of 8 mL/kg of 50% glycerol (v/v) is regarded as the most appropriate for animal models to mimic the AKI in humans. Therefore, glycerol with injection volumes of 8 mL/kg was divided equally between the two hindlimbs to construct the AKI model of rats.

Similar to H$_2$S, NO, and CO, H$_2$ is a new emerging gaseous signaling molecule which has been demonstrated to protect and treat a variety of diseases. A previous study reported the protective effect of H$_2$-rich saline on kidney injury, and a high concentration of H$_2$-rich saline performed better than the low dose. However, H$_2$ is not easily dissolved in water, and 100%-$\cdot$saturated hydrogen water only contains 1.6 ppm or 0.8 mM H$_2$ under atmospheric pressure and room temperature. Nevertheless, inhalation provides a higher concentration of H$_2$ than the injection of H$_2$-rich saline. Liu et al. reported that H$_2$ inhalation significantly induced higher H$_2$ concentration in the muscle compared to the other modes of H$_2$ administration. Therefore, H$_2$ inhalation may exert a more powerful protective effect against RM induced AKI. Peng et al. demonstrated the therapeutic effect of a high concentration of H$_2$ (about 67%) inhalation on glycerol-induced AKI. However, the O$_2$ concentration (about 33%) will be higher than that in the atmosphere (about 21%) when a high concentration of H$_2$ is produced by electrolyzing water. Besides, the dose-effect of different concentrations of H$_2$ inhalation also requires further investigations. In this study, we provided low (4%) and high (67%) concentrations of H$_2$ (21% O$_2$) to explore the effects and mechanisms of H$_2$ against glycerol-induced AKI and the dose-effects.

Glycerol is a nephrotoxic agent that can cause kidney damage, which is marked by elevated kidney index associated with swelling of stromal and epithelial cells and an increase of glomerular volume. The increased Cr, BUN, lactate dehydrogenase 1, and UA levels in plasma are important renal injury markers used as indicators in almost all renal injury studies. RM induced AKI often leads to a more rapid increase in plasma Cr than other forms of AKI. Increased CK and creatinine kinase isoenzyme levels were observed in the AKI group in this study, which may result from damaged skeletal muscle fibers caused by the toxic effects of glycerol. Kidney injury molecule 1 is a molecular marker of tubular injury released into circulation after kidney proximal tubule injury and provides a more sensitive indication of kidney damage. Recent studies have demonstrated that NGAL is strongly associated with AKI and can be detected earlier than other AKI markers. Besides, it can be overexpressed by reactive oxygen species and NF-$\kappa$B. Therefore, NGAL has shown the potential to serve as a new effective biochemical marker of AKI. All these products induce oxidative stress, inflammation, apoptosis, vasoconstriction, and tubular obstruction, and then further aggravate AKI. Changes in the content of these products are inhibited by low and high concentrations of H$_2$ inhalation, thus reducing renal injury.

The therapeutic effect of H$_2$ is mainly attributed to its selective antioxidant effect, namely scavenging hydroxyl and peroxyl radicals without affecting the beneficial radicals. An experiment has supported that increasing oxidative stress is involved in glycerol-induced renal damage. Glycerol injection increases hydrogen peroxide generation, which is a substrate for hydroxyl radical formation via the iron-catalyzed Fenton and Haber-Weiss reactions. As a heme protein, myoglobin released from the muscle cell contains iron (Fe$^{2+}$), which is important in binding with molecular oxygen. Molecular oxygen promotes the oxidation of Fe$^{2+}$ to Fe$^{3+}$ and generates hydroxyl radicals. Under normal body conditions, the antioxidant molecules in the body maintain a stable oxidation potential. However, upon stimulation by external drugs such as glycerol, a large release of myoglobin leads to uncontrolled leakage of free radicals and causes renal cellular injury. SOD and CAT are important antioxidases, which can effectively eliminate reactive oxygen species from the body and maintain a balance between oxidation and anti-oxidation. GSH content is also an important indicator of antioxidant capacity. In this study, H$_2$ inhalation increased the antioxidant capacity of the kidney by regulating the endogenous antioxidant defense system, which was evidenced by the increase in SOD, GSH, and CAT. Recent studies have shown that myoglobin can exhibit peroxidase-like enzyme activity and leads to uncontrolled oxidation of biomolecules, lipid peroxidation, and production of isoprostanes. MDA is a major product of lipid peroxidation. This study showed an increase of MDA in the kidney tissue after glycerol injection and a significant decrease after H$_2$ inhalation. HO-1 plays pivotal roles in the maintenance of renal functions and the protection of renal structures under oxidative stress, especially within the renal tubular epithelial cells. Glycerol releases pro-oxidant heme (myoglobin/hemoglobin) into the systemic circulation, from whence it might increase intrarenal HO-1 gene expression. In the present study, mRNA expression of HO-1 was significantly upregulated in the renal cortex in the glycerol group, which strongly implied that the kidney may be the site of HO-1 release. All the above factors were improved by H$_2$ inhalation, which indicated inhibition of oxidative stress progression.

Pro-inflammatory cytokines are produced in response to a variety of stimuli such as pro-oxidant and certain chemical agents, and they participate in both physiological and pathological processes and result in tissue damage. In this study, inflammatory responses were recorded following glycerol injection in the renal tissue, which was evidenced by the excessive release of IL-1$\beta$ and TNF-$\alpha$. The upregulation of both in AKI rats may be attributed to the uncontrolled release of myoglobin, reactive oxygen species overproduction, and the activation of NF-$\kappa$B. Here, H$_2$ inhalation decreased the expression of NF-$\kappa$B, IL-1$\beta$, and TNF-$\alpha$ at the transcriptional level. The anti-inflammatory effect may be attributed to the suppression of NF-$\kappa$B production. MCP-1 is a critical molecule in chemotaxis and activation of macrophages, which has attracted increasing attention as a renal biomarker expressed in injured and inflammatory sites such as in progressive kidney disease. The results showed that a significant increase in MCP-1 was inhibited by pre- and post-treatment with H$_2$, which was consistent with the results from a previous study. During AKI, various forms of cell death including apoptosis and necrosis are reported. Apoptotic pathways are active in the tubular epithelium induced by caspase cascade activation, mitochondrial membrane, and so on, and these promote renal epithelial cell loss and lead to AKI disease. Caspase-3 is
the key executive factor in the modification of cell apoptotic proteins. Accumulating evidence demonstrates that Caspase-3 activation is the most predominant mechanism responsible for the apoptosis of renal tubular cells in AKI. In this study, \( H_2 \) inhalation blocked the cleavage of Caspase-3 in the injured kidney, suggesting anti-apoptosis effects. Another crucial regulatory factor in apoptosis is the pro-apoptotic protein. In this study, the impact of \( H_2 \) on the level of apoptotic Bax was evaluated by RT-PCR and immunohistochemical staining. The results showed that \( H_2 \) inhalation significantly suppressed Bax expression in the kidney tissues of the AKI group. The result of the TUNEL assay also confirmed the anti-apoptotic effect of \( H_2 \) inhalation against this disease.

Necrosis, another form of cell death that contributes to the pathogenesis of AKI, is considered as one of the important mechanisms involved in glycerol-induced AKI. A previous study revealed that blocking the necrotic pathways could mitigate renal injury, indicating a promising outlook in the management of AKI. TNF-\( \alpha \) has been documented to be an upstream molecule and mediates necroptosis, which is also the main component of AKI in the glycerol model. Necroptosis can be triggered by various events including engagement of death receptors in the presence of caspase inhibition, stimulation of Toll-like receptors, signaling through interferons, and so on. Any of the initial triggers utilizes the receptor-interacting protein-homotypic interacting motif domain to activate the kinase RIP3, an essential mediator of necroptosis. RIPK3 activation induces the phosphorylation, oligomerization, and translocation of MLKL, the downstream substrates, to membranes and leads to permeabilization of the plasma membrane and cell death. RT-PCR results in this study showed upregulation in TNF-\( \alpha \) and the associated RIPK3 and MLKL expression in the AKI group compared with the control group. This indicates that TNF-\( \alpha \) and RIPK3/MLKL axis are the predominant mediators in necroptosis of tubular injury in glycerol-induced AKI. \( H_2 \) inhalation reversed the situation and decreased the expression of RIPK3 and MLKL.

Therefore, \( H_2 \) possesses inhibitory effects on tubular epithelial cell apoptosis and necrosis in the pathogenesis of AKI. The involved mechanism of \( H_2 \) against AKI has been summarized in Figure 9. The present study demonstrated the protective effects of \( H_2 \) inhalation on RM induced AKI and provided new evidence regarding the potential treatment effects of RM induced AKI in clinical practice.

The dose-effect of \( H_2 \) has been reported in several studies, especially the intraperitoneal injection of \( H_2 \)-rich saline, but only a few by \( H_2 \) inhalation. Liu et al. reported that inhalation of 22% and 41.6% \( H_2 \) showed better outcomes compared to 2% inhalation of \( H_2 \) in the treatment and prevention of chronic obstructive pulmonary disease in rats. However, for several parameters detected, there was no significant difference between the therapeutic effects of 22% and 41.6% \( H_2 \). Liu et al. reported a dosage effect of \( H_2 \) inhalation in the rat model of metabolic syndrome with nonalcoholic fatty liver disease and the results showed a better effect of low concentration \( H_2 \) (4%) than high concentration (67%) for most detected indicators. In this study, low (4%) and high (67%) \( H_2 \) concentrations were used. For nearly half of the parameters including Cr, UA, kidney injury molecule 1, NGAL, SOD, IL-1\( \beta \), MCP-1, and MLKL, the low \( H_2 \) performed better than the high. For about a third of the parameters like BUN, GSH, CAT, TNF-\( \alpha \), Bax, and Caspase-3, the effect of low and high \( H_2 \) was not significantly different. The high \( H_2 \) concentration resulted in greater improvement mainly based on histological and morphological analysis.

There are several limitations regarding our study. First, only RIPK3-MLKL-mediated necroptosis was explored, and execution of necroptosis including the interaction between RIP1 and RIP3 needs to be better evaluated. Second, more intermediate concentrations of \( H_2 \) such as 42% can be considered since the large variation between 4% and 67% concentrations to further explore the dose-effect or optimal concentration of \( H_2 \) inhalation that is most effective in preventing and treating AKI.

**Figure 9:** A schematic illustration of the potential protective effects of \( H_2 \) against glycerol-induced AKI of rats, including anti-oxidation, anti-inflammation, anti-apoptosis and anti-necroptosis.

**Note:** AKI: Acute kidney injury; Cyt c: cytochrome c; IL: interleukin; MCP-1: monocyte chemoattractant protein 1; MDA: malondialdehyde; MLKL: mixed lineage kinase domain-like protein; NF-kB: nuclear factor kappaB; RIPK3: receptor-interacting serine-threonine kinase 3; TNF-\( \alpha \): tumor necrosis factor-alpha.

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### Author contributions
Design of the study: JLX and SCQ; data collection: MZ, MYZ and MYW; data analysis: BYL, QQG and XYZ; manuscript writing: JLX and BYL.

### Conflicts of interest
The authors declare no conflicts of interest.

### Availability of data and materials
All data generated or analyzed during this study are included in this published article and its supplementary information files.

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### Additional files

**Additional Figure 1:** Effects of \( H_2 \) inhalation on body mass (A), kidney index (B) and kidney length (C) of rats in different groups.

**Additional Table 1:** The primers used for the reverse transcription polymerase chain reaction in this study.

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Additional Figure 1: Effects of H₂ inhalation on body weight (A), kidney index (B) and kidney length (C) of rats in different groups.

Note: Data are expressed as mean ± SEM (n = 9-10). *P < 0.05, **P < 0.05, ***P < 0.001, vs. Con group (one-way analysis of variance followed by least significant difference post hoc test). AKI: acute kidney injury group; AKI+H₂: 67% H₂ inhalation group; AKI+LH₂: 4% H₂ inhalation group; Con: control group; H₂: Hydrogen.
Additional Figure 2: Real-time curve of H$_2$ concentration in kidney over time after stopping inhaling different concentrations of H$_2$.

Note: (A) H$_2$ concentration curve after stopping 4% H$_2$ inhalation. (B) H$_2$ concentration curve after stopping 67% H$_2$ inhalation. The red areas represent SEM values, and the blue line represents mean values ($n = 3$). H$_2$: Hydrogen.
### Additional Table 1: The primers used for the reverse transcription polymerase chain reaction in this study

| Gene     | Primer sequence (5’-3’)                        |
|----------|-----------------------------------------------|
| **RIPK3**| F: CTG TCG CCT GCT AGA GGA AG                  |
|          | R: TCT GCT AAC TTG GCG TGG AG                  |
|          | F: CCC GAG TTG TTG CAG GAG AT                  |
|          | R: TCT CCA AGA TTC CAT CCG CAG                 |
|          | F: GTG AGT GGA CCA GGC ACA CA                  |
|          | R: AAT CCC TTG ATC CAT TGT TTT CTT             |
| **MLKL** | F: TCT GGG CCT CAA GGA TAA CAA C               |
|          | R: AGA CAG GTG GGA CCT GAA CCA                 |
| **β-Actin**| F: TTC CTT CCT GGG TAT GGA AT                 |
|          | R: GAG GAG CAA TGA TCT TGA TC                  |
| **KIM-1**| F: CTG GAA GAG GAG ATA GAG C                   |
|          | R: CTG GTG TGT AAG GGA TGG                     |
| **IL-1β**| F: CCT TCC AGG ATG AGG ACA TGA                 |
|          | R: TGA GTC ACA GAG GAT GGG CTC                 |
| **TNF-α**| F: ACC CTC ACA CTC ACA AAC CA                  |
|          | R: ACC CTG AGC CAT AAT CCC CT                  |
| **Bax**  | F: CTG AGC TGA CCT TGG AGC                     |
|          | R: GAC TCC AGC CAC AAA GAT G                   |
| **Caspase-3**| F: GAG CTT GGA ACG GTA CGC TA               |
|          | R: CCG TAC CAG AGC GAG ATG AC                  |
| **NF-κB**| F: ACG ATC TGT TTC CCC TCA TCT                 |
|          | R: TGC TTC TCT CCC CAG GAA TA                  |
| **MCP-1**| F: AGC CCA GAA ACC AGC CAA CTC                 |
|          | R: GCC GAC TCA TTG GGA TCA TCT T               |

Note: F: Forward; HO-1: heme oxygenase-1; IL-1β: interleukin 1β; KIM-1: kidney injury molecule 1; MCP-1: monocyte chemoattractant protein 1; MLKL: mixed lineage kinase domain-like protein; NF-κB: nuclear factor kappaB; NGAL: neutrophil gelatinase-associated lipocalin; R: reverse; RIPK3: receptor-interacting serine-threonine kinase 3; TNF-α: tumor necrosis factor-alpha.