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Hydrogen gas inhalation attenuates sepsis-induced liver injury in a FUNDC1-dependent manner

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ABSTRACT

Sepsis-induced hepatic dysfunction is considered as an independent risk factor of multiple organ dysfunction syndrome (MODS) and death. Mitophagy, a selective form of autophagy, plays a major role in sepsis-induced organ damage. We have demonstrated that hydrogen gas (H_2), a selective antioxidant, exerts protective effects in septic mice. Here, we hypothesize that the therapeutic effects of H_2 on septic animals with liver damages may be exerted through regulation of the Fun14 domain-containing protein 1 (FUDNC1)-induced mitophagy pathway. Male C57BL/6J mice were subjected to sham or cecal ligation and puncture (CLP) operation and treated with 2% H_2 gas inhalation for 3 h starting at 1 h after sham or CLP surgery. To verify the role of FUNDC1, the cell-penetrating peptide P (NH2-GRKKRRQRRRPQDYESDDESYEVLDLTEY-COOH) (1 mg/kg) that functions as a FUNDC1 inhibitor was intraperitoneally injected into mice 24 h before the sham or CLP operation. To evaluate the severity of septic liver injury, the 7-day survival rate, liver histopathologic score, alanine aminotransferase (ALT) and aspartate aminotransferase (AST) levels, respiration control ratio (RCR), and FUDNC1, P-18-FUDNC1, P62, LC3B-II, Tim23, and caspase-1 levels were evaluated after the sham or CLP operation. The results demonstrated that 2% H_2 gas inhalation resulted in an increase in the 7-day survival rate, ALT and AST levels, RCR, and P62 and LC3B-II expression but decreased the histological score and FUDNC1, P-18-FUDNC1, Tim23, and caspase-1 levels after sepsis. However, no significant differences were reported between the CLP + peptide P and CLP + H_2 + peptide P groups. These observations indicate that 2% H_2 gas inhalation for 3 h may serve as an effective therapeutic strategy for sepsis-induced liver injury through the regulation of FUNDC1-dependent mitophagy.

1. Introduction

Sepsis is a well-recognized systemic inflammatory response syndrome (SIRS) induced by invading microorganisms [1]. The incidence rate of sepsis has increased from 1.5% to 8%, and > 18 million people are known to suffer from sepsis each year worldwide [2]. However, no effective therapeutic treatment is available for sepsis [3]. As the largest gland in humans, the liver plays a key role in metabolism and immune homeostasis in sepsis [4]. During sepsis, the invading bacteria and their products are first captured and cleared by hepatocytes. At the same time, the hepatic immunocytes release pro-inflammatory mediators that may cause acute liver injury [5]. Evidence suggests that acute hepatic insufficiency and failure may directly contribute to disease deterioration and death in patients with sepsis, while the amelioration of liver injury or restoration of liver function may reduce morbidity and mortality in these patients [6].

Molecular hydrogen (H_2) acts as a special selective antioxidant and could be effectively used for the treatment of > 70 types of diseases [7]. In comparison with traditional antioxidants such as vitamin E, H_2 possesses two unique properties. First, it has the ability to readily penetrate the cell membrane and rapidly translocate to the nucleus and mitochondria. Second, it selectively neutralizes the more cytotoxic hydroxyl (OH^-) and peroxynitrite ($ONOO^-$) radicals without reacting with the less potent reactive oxygen species (ROS) [8]. Our previous studies have shown that H_2 gas inhalation or drinking of hydrogen-rich saline was effective against sepsis and sepsis-induced damage of organs, including the liver, lung, brain, and kidney [9–11]. We have also shown that 2% H_2 gas inhalation could markedly alleviate liver injury and

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improve the 7-day survival rates of septic mice [9]. However, the underlying mechanism remains unclear.

Mitochondria provides about 90% of energy for organic life and are essential cellular components. Mitochondria also perform several other biological functions, including regulation of ROS production, cellular redox reaction, signal transduction networks, and apoptosis [12]. In patients with sepsis, mitochondrial dysfunction in vital organs may reduce the cellular energy level, resulting in multiple organ failure [13,14]. Clinical studies suggest that treatments focusing on mitochondrial dysfunction, instead of direct inhibition of the inflammatory cascade, may improve the outcomes in patients with sepsis [14].

Mitophagy, as a selective form of autophagy, is a basic process to control the mitochondrial quality at the organelle level. Mitophagy is also necessary for the maintenance of mitochondrial networks and reprogramming of cellular metabolism [15]. Fun14 domain-containing protein 1 (FUNDC1) is one of mitophagy receptors localized at the outer membrane of the mitochondrion, and is involved in the removal of damaged mitochondria in response to mitochondrial stresses and hypoxia [16]. FUNDC1 can interact with LC3B-II and regulate the hypoxia-induced selective mitophagy via reversible phosphorylation at some crucial sites [17,18].

Here, we aimed to reveal the role of FUNDC1-induced mitophagy in the pathogenesis of liver injury caused by sepsis and the protective mechanism of 2% H_2 gas inhalation in a cecal ligation and puncture (CLP) model of sepsis mouse.

2. Materials and methods

2.1. Animals and experiment protocol

The animal protocol was subject to approval by the Institutional Animal Care and Use Committee of Tianjin Medical University General Hospital (Tianjin, China). Adult male C57BL/6J mice (6 weeks old and weighing 20–25 g) were obtained from the Laboratory Animal Center of the Academy of Military Medical Sciences (Beijing, China). All animals were fed with adequate food and water in a stable environment (temperature 22 °C ± 1 °C, humidity 50%–60%, 12:12 h light-dark cycle). Mice were randomly divided into five groups: sham, CLP, CLP + peptide P, CLP + H_2, and CLP + H_2 + peptide P. To verify the role of FUNDC1, the cell-penetrating peptide P (NH2-GRKKRRQRRRPQDYESDDESYEVLDLTEY-COOH) (1 mg/kg, synthesized by Shanghai Qiangyao Company, Shanghai, China), a type of FUNDC1 inhibitor, was intraperitoneally administered to mice 24 h before the CLP or sham operation. The other groups were injected with the same volume of saline. Septic models were generated by CLP. The mice from the sham group underwent the same process except CLP treatment as compared with those from the CLP group. The mice from the CLP + H_2 and CLP + H_2 + peptide P groups inhaled H_2 gas (2%) for 3 h starting at 1 h after the sham or CLP surgery [19], while the mice from the sham, CLP, and CLP + peptide P groups inhaled air (21% O_2) only. At 1 h from the sham or CLP operation, the H_2 gas concentration in the arterial and venous blood of mice from different groups was measured at 0, 10, 20, 30, 45, 60, 120, and 180 min from the start of H_2 gas inhalation as well as at 5, 15, 30, 45, and 60 min after the termination of H_2 gas inhalation. The survival rate in each group was analyzed from day 1 to 7 after the sham or CLP surgery. At 6 h post-surgery, we executed the mice and obtained blood samples and liver tissues. Liver slices were used to perform histological examination. Mitochondria were isolated from liver tissues at 6 h post-operation to analyze respiration control ratio (RCR). The levels of alanine aminotransferase (ALT) and aspartate aminotransferase (AST) in blood samples were measured by enzyme-linked immunosorbent assay (ELISA), while FUNDC1, p-18-FUNDC1, P62, LC3B-II, and caspase-1 levels in liver tissues were determined by western blotting (Fig. 1).

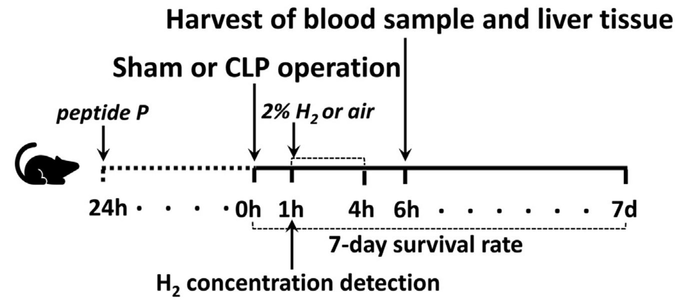

Fig. 1. Experimental design.
Male C57BL/6J mice (6–8 weeks old, weighing 20–25 g) were subjected to sham or CLP operation. We used 2% hydrogen gas (H_2) or fresh air to treat mice for 3 h from 1 h after sham or CLP operation. To verify the key role of FUNDC1, the cell-penetrating peptide P, a FUNDC1 inhibitor, was intraperitoneally administered to mice 24 h before the CLP or sham operation; other groups were injected with the same volume of saline. The serum samples and liver tissues from different groups were collected at 6 h after the sham or CLP operation and used for all the tests described in this research.

2.2. CLP model

The CLP model was generated as described in our previous research [10]. First, 2% sodium pentobarbital (50 mg/kg) dissolved in saline was intraperitoneally injected into mouse as an anesthesia. The mouse was placed on a special animal-use sterile operation table and a midline incision about 1 cm long was made on the abdomen to expose the cecum. The cecum was ligated at one quarter of its distal end and a 20-gauge needle was used to puncture the distal cecum. Some of the excretion was squeezed out through the point of puncture. Finally, the cecum was gently returned to the abdomen and the incision was sutured with a sterile 3–0 silk suture. After operation, 1 mL of normal saline was subcutaneously injected into the mouse for resuscitation.

2.3. H_2 gas treatment

H_2 gas treatment procedure was performed as previously described [19] with some modifications. Mice were put into a sealed plexiglass box with inlet and outlet valves. H_2 gas mixed with fresh air was delivered from a TF-1 gas flowmeter (YUTAKA Engineering Corp) into the box at a rate of 4 L/min. Carbon dioxide exhaled from mice was cleaned away by a bag of baralyme. H_2 gas concentration was monitored by a special handhold detector (HY-ALERTA Handhold Detector Model 500; H_2 Scan). We used 2% H_2 gas to treat mice for 3 h starting from 1 h post-surgery, while mice from the sham or CLP group were placed in the same box in the presence of air (21% O_2) but without 2% H_2 gas.

2.4. Measurement of H_2 gas concentration

A needle-type hydrogen sensor (Unisense A/S, Aarhus, Denmark) was used to detect the concentration of H_2 gas in the arterial and venous blood samples of animals, as previously described [11]. Arterial and venous blood samples were collected at 0, 10, 20, 30, 45, 60, 120, and 180 min after the start of H_2 gas inhalation and 5, 15, 30, 45, and 60 min after the end of H_2 gas inhalation.

2.5. Survival rate determination

Mice survival rates were observed from day 1 to 7 after the sham or CLP surgery. The tests were repeated twice.

2.6. Histological analysis

Liver tissue specimens (5-μm thickness) were obtained at 6 h post-surgery and stained with hematoxylin-eosin to evaluate the degree of

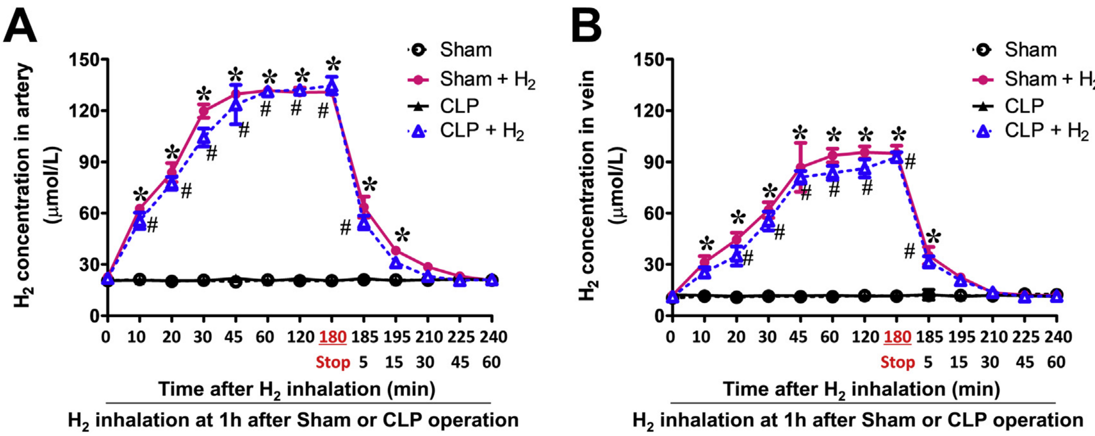

Fig. 2. H_2 concentration in arterial and venous blood of mice after 2% H_2 inhalation.
At 1 h after sham or CLP operation, H_2 concentration in mouse (A) arterial blood and (B) venous blood was measured at 0, 10, 20, 30, 45, 60, 120, and 180 min after the start of H_2 inhalation and at 5, 15, 30, 45, and 60 min after the termination of H_2 inhalation. Data are expressed as mean ± SD ($n = 3$/group). $^{*}P < 0.05$ versus the sham group, $^{\#}P < 0.05$ versus the CLP group.

liver damage. Hepatic injury was observed with light microscopy, and the scoring criteria were as follows: capsular inflammation, portal inflammation, spotty necrosis, ballooning degeneration, and steatosis [20].

2.7. Transaminase analysis

Blood samples were collected from the left ventricle of mice at 6 h post-operation, and serum AST and ALT levels were detected using an automatic biochemical analyzer.

2.8. Isolation of liver mitochondria

Liver mitochondria were obtained through a conventional differential centrifugation method. In brief, the right lobule of the liver was collected and minced in an ice-cold isolation buffer containing 70 mM sucrose, 220 mM mannitol, 5 mM HEPES, 1 mM phenylmethane sulfonyl fluoride (PMSF), and 0.2% defatted bovine serum albumin (BSA), pH 7.4. The blood-free tissue was homogenized with a glass-made homogenizer in the isolation medium (1 g/8 mL), and the homogenate was centrifuged at 1500 ×*g* and 4 °C for 10 min. The supernatant was centrifuged at 12,000 ×*g* and 4 °C for 10 min. After excluding the supernatant, the mitochondrial pellet was resuspended in a medium containing 70 mM sucrose, 220 mM mannitol, and 5 mM HEPES. Mitochondrial protein level was determined by the Coomassie Brilliant blue method using BSA as standard. The freshly obtained mitochondrial suspension (about 30–40 mg protein/mL) was incubated on ice (0–4 °C) during the mitochondrial respiration measurement, which was carried out within 4 h.

2.9. Mitochondrial respiration analysis

Mitochondrial respiration was detected using an Oroboros Oxygraph-2K instrument (Oroboros, Austria) at 25 °C. Liver mitochondria (0.6 mg) were added to the reaction medium (225 mM mannitol, 70 mM sucrose, 1 mM ethylenediaminetetraacetic acid [EDTA], 10 mM Pi-K buffer, and 0.1% defatted BSA, pH 7.4) in each chamber. Approximately 5 min after the introduction of mitochondria to the chamber, succinate (5 mM) was added. After the observation of a steady-state 4 respiration stage, 50 mM ADP was added to achieve the state 3 respiration state. The consumption of total ADP would lead to the reversion to steady-state 4 respiration stage. Between experiments, the chambers were washed twice with water and ethanol. RCR was calculated according to the ratio of state 3/state 4 respiration.

2.10. Western blot analysis

Mice were killed at 6 h post-operation and the liver tissues from different groups were collected and lysed in radioimmunoprecipitation assay (RIPA) buffer (with one tablet of protease inhibitor dissolved in 10 mL of RIPA buffer) to extract proteins. The total amount of proteins was quantified using the bicinchoninic acid protein assay kit (Well-bio, Shanghai, China). The same amount (30 μg) was used to determine the expression of FUNDC1, p-18-FUNDC1, P62, LC3B-II, and caspase-1. The primary antibodies, including anti-FUNDC1 (1:1000, Abgent, SuZhou, China), anti-p-18-FUNDC1 (1:1000, Abgent, SuZhou, China), anti-P62 (1:2000, Abcam, Cambridge, UK), anti-LC3B-II poly-clonal (1:2000, Abcam, Cambridge, UK), anti-caspase-1 (1:1000, Arigo Taiwan, China), and anti-glyceraldehyde 3-phosphate dehydrogenase (anti-GAPDH; 1:10000, Proteintech, Chicago, USA), and specific secondary antibodies (1:5000, KPL, Massachusetts, USA) were used. The protein bands were treated with a chemiluminescence plus reagent and visualized with an enhanced chemiluminescence (ECL) reagent. The intensity of each band was calculated using the GeneTools software (Syngene, Cambridge, UK). The relative levels of FUNDC1, p-18-FUNDC1, P62, LC3B-II, and caspase-1 were normalized to GAPDH levels.

2.11. Statistical analysis

Survival rates are expressed as percentages (%), and other data are reported as mean ± standard deviation (SD). The log-rank (Mantel-Cox) test was used to analyze the 7-day survival rate in different groups. We used unpaired *t*-test (if the values were normally distributed) or Mann–Whitney test (if the values had a non-normal distribution) to analyze differences between two groups (sham versus CLP, CLP versus CLP + H_2, or CLP + peptide P versus CLP + H_2 + peptide P group). We also used one-way analysis of variance (ANOVA) to analyze the interactions among all the groups. A value of $P < 0.05$ was considered statistically significant. Statistical analysis was performed using SPSS (version 21.0, IBM, Inc., US) and GraphPad Prism (version 5.01, GraphPad Software, Inc., US).

3. Results

3.1. H_2 gas concentration in arterial and venous blood of mice

At 1 h post-surgery, the concentration of H_2 gas in mouse arterial and venous blood samples was measured. As described in Fig. 2A and B, H_2 concentration in mice from the sham + H_2 and CLP + H_2 groups gradually increased over time from 0 to 45 min from the inception of H_2

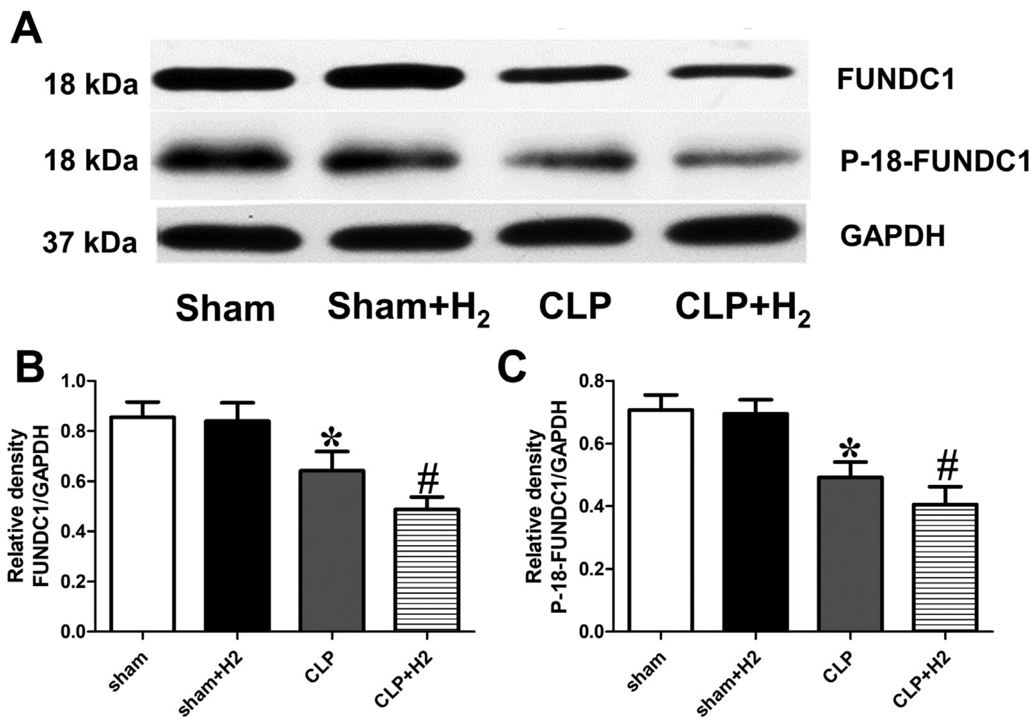

Fig. 3. Effects of H_2 inhalation on FUNDC1 and P-18-FUNDC1 expression levels in mouse liver tissue.
At 6 h after sham or CLP operation, the liver samples were harvested. The levels of FUNDC1 and P-18-FUNDC1 were measured by western blotting (A). Quantitative analysis of (B) FUNDC1 and (C) P-18-FUNDC1 expression is shown as the ratio of protein density relative to GAPDH density. Data are expressed as mean ± SD ($n = 6$/group). $^*P < 0.05$ versus the sham group, $^{\#}P < 0.05$ versus the CLP group.

inhalation both in arterial and venous blood samples. H_2 concentration reached its peak at 45 min, and the maximum level was maintained from 45 to 180 min after the start of H_2 inhalation both in arterial and venous blood samples. After the end of H_2 inhalation, H_2 concentration in the mice from the sham + H_2 and CLP + H_2 groups quickly decreased to the baseline level both in arterial and venous blood (Fig. 2A and B). However, no obvious difference was observed between the CLP + H_2 and sham + H_2 groups ($P > 0.05$ versus sham + H_2 group).

3.2. Expression levels of FUNDC1 and p-18-FUNDC1

At 6 h post-operation, the protein levels of FUNDC1 and p-18-FUNDC1 were evaluated by western blotting (Fig. 3A). The levels of FUNDC1 and p-18-FUNDC1 were lower in mice from the CLP group than in those from the sham group ($P < 0.05$ versus sham group; Fig. 3B–C). However, 2% H_2 treatment decreased the expression levels of FUNDC1 and p-18-FUNDC1 in mice from the CLP + H_2 group compared to that in the CLP group ($P < 0.05$ versus CLP group; Fig. 3B–C).

3.3. Survival rates

During the 7-day experimental observation, only one mouse died in the sham group. Moreover, the 7-day survival rate markedly decreased for mice from the CLP group as compared with that in the sham group ($P < 0.05$ versus sham group). In comparison with the CLP group, CLP + H_2 group showed a marked increase in the 7-day survival rate after 2% H_2 inhalation ($P < 0.05$ versus CLP group). However, no statistical difference was observed between the CLP + peptide P and CLP + H_2 + peptide P groups ($P > 0.05$ versus CLP + peptide P group) (Fig. 4).

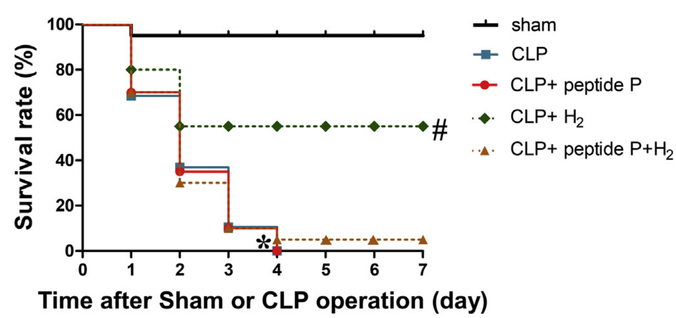

Fig. 4. Effects of H_2 inhalation on the 7-day survival rate of mice.
The survival rates were monitored for 7 days in mice from different treatment groups. Values are expressed as survival percentage ($n = 20$ per group). $^*P < 0.05$ versus the sham group, $^{\#}P < 0.05$ versus the CLP group.

3.4. Liver injury

We used HE staining to assess the histopathological changes in the liver tissue at 6 h after sham or CLP surgery (Fig. 5A). No obvious changes were observed in the histology of the liver tissues from the normal mice. In the CLP group, morphological changes associated with liver injury, including spotty necrosis and capsular inflammation, were reported in a large area of the hepatic lobule. However, the injuries were significantly attenuated in mice from the CLP + H_2 group as compared with those in the CLP group ($P < 0.05$ versus CLP group; Fig. 5B). Moreover, the levels of ALT and AST in serum samples were measured to evaluate the status of liver injury. In comparison with mice from sham group, those from the CLP group showed a marked increase in the levels of ALT and AST ($P < 0.05$ versus sham group; Fig. 5C and D), while 2% H_2 inhalation markedly decreased the levels of ALT and AST ($P < 0.05$ versus CLP group; Fig. 5C and D). However, pathological score and expression of ALT and AST showed no statistical difference between the CLP + peptide P and CLP + H_2 + peptide P groups ($P > 0.05$ versus CLP + peptide P group, Fig. 5B–D). Thus, the peptide P could inhibit the therapeutic effect of H_2 on the liver injury of sepsis mice.

3.5. Mitochondrial respiration

After 6 h from operation, the liver mitochondria were isolated and mitochondrial respiration level was evaluated (Fig. 6A). The RCR level decreased in mice from the CLP group as compared with that in the sham group ($P < 0.05$ versus sham group; Fig. 6B), but 2% H_2 treatment improved RCR level in mice from the CLP + H_2 group ($P < 0.05$

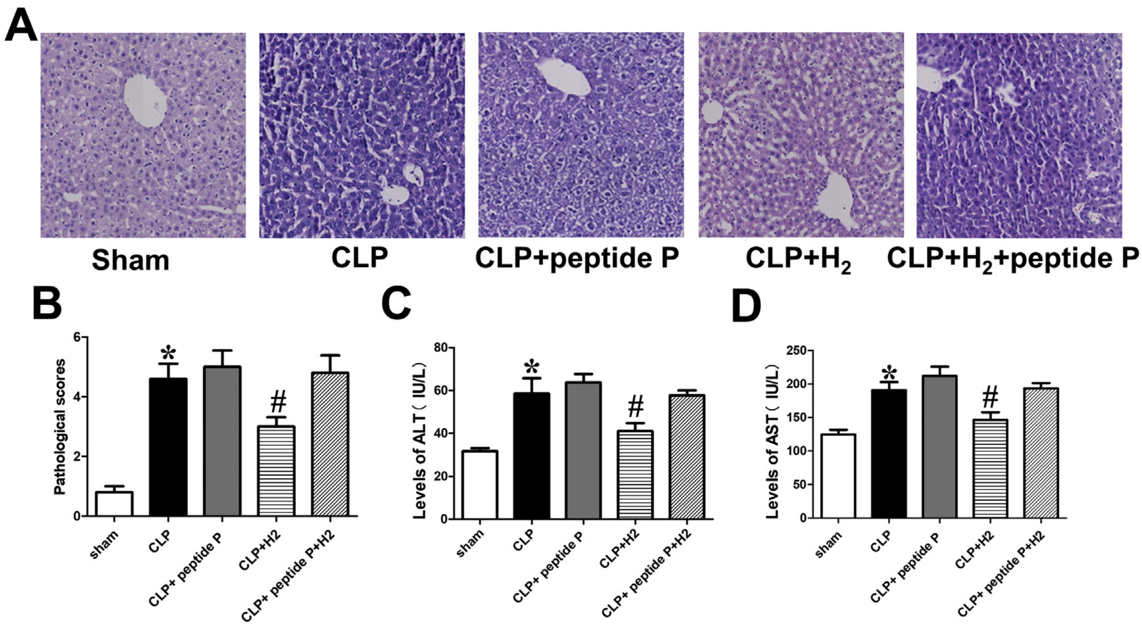

Fig. 5. Effects of H_2 inhalation on liver injury in mouse liver tissues and serum.
Mice were sacrificed at 6 h after sham or CLP operation and the liver tissues were harvested for (A) HE staining (original magnification ×200). Liver injury was scored by observing the morphological structure (B). Mouse serum samples were collected to detect ALT (C) and AST (D) levels. Data are shown as mean ± SD (n = 6/group). *$P < 0.05$ versus the sham group, $^{\#}P < 0.05$ versus the CLP group.

versus CLP group; Fig. 6B). No statistical difference was observed between mice from the CLP + peptide P and CLP + H_2 + peptide P groups ($P > 0.05$ versus CLP + peptide P group; Fig. 6B).

3.6. Protein levels of P62, LC3B-II, Tim23, and caspase-1

The protein levels of autophagy-associated proteins (P62, LC3B-II, Tim23, and caspase-1) were analyzed by western blotting (Fig. 7A). In comparison with samples from the sham group, those from the CLP group showed an increase in the levels of P62 and LC3B-II ($P < 0.05$ versus sham group), while samples from the CLP + H_2 group showed higher levels of these proteins than those from the CLP group ($P < 0.05$ versus CLP group; Fig. 7B and C). Furthermore, samples from the CLP group had lower levels of Tim23 than those from the sham group ($P < 0.05$ versus sham group), while Tim23 expression level further decreased in samples from the CLP + H_2 group ($P < 0.05$ versus CLP group; Fig. 7D). Caspase-1 is an important biomarker of inflammatory response. The level of caspase-1 obviously increased in samples from the CLP group as compared with those from the sham group ($P < 0.05$ versus sham group), while the 2% H_2 treatment resulted in a decrease in caspase-1 expression ($P < 0.05$ versus CLP group; Fig. 7D). No significant difference was observed in all these protein levels between the CLP + peptide P and CLP + H_2 + peptide P groups ($P > 0.05$ versus CLP + peptide P group; Fig. 7B–D). These

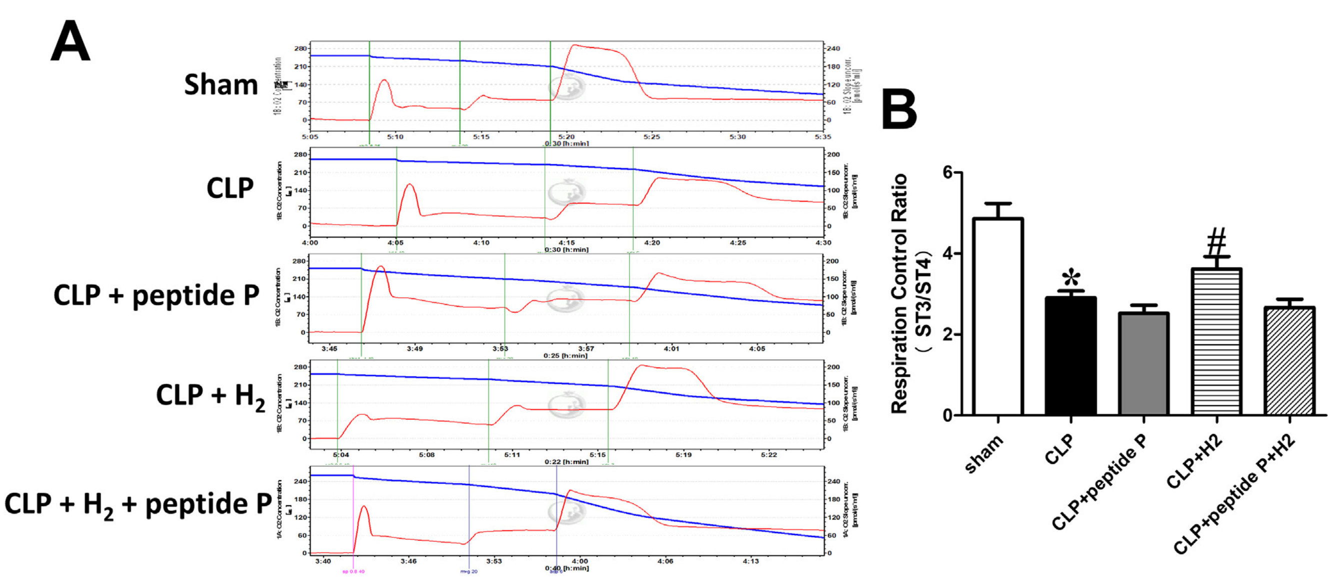

Fig. 6. Effects of H_2 inhalation on mitochondrial respiration in mouse liver tissue.
Liver mitochondria were isolated 6 h post-surgery. (A) Oxygen consumption rates were evaluated via Oroboros Oxygraph-2K instruments, and (B) respiration control ratio (RCR) was calculated as the ratio of state 3 to state 4. Data are expressed as mean ± SD (n = 6/group). *$P < 0.05$ versus the sham group, $^{\#}P < 0.05$ versus the CLP group.

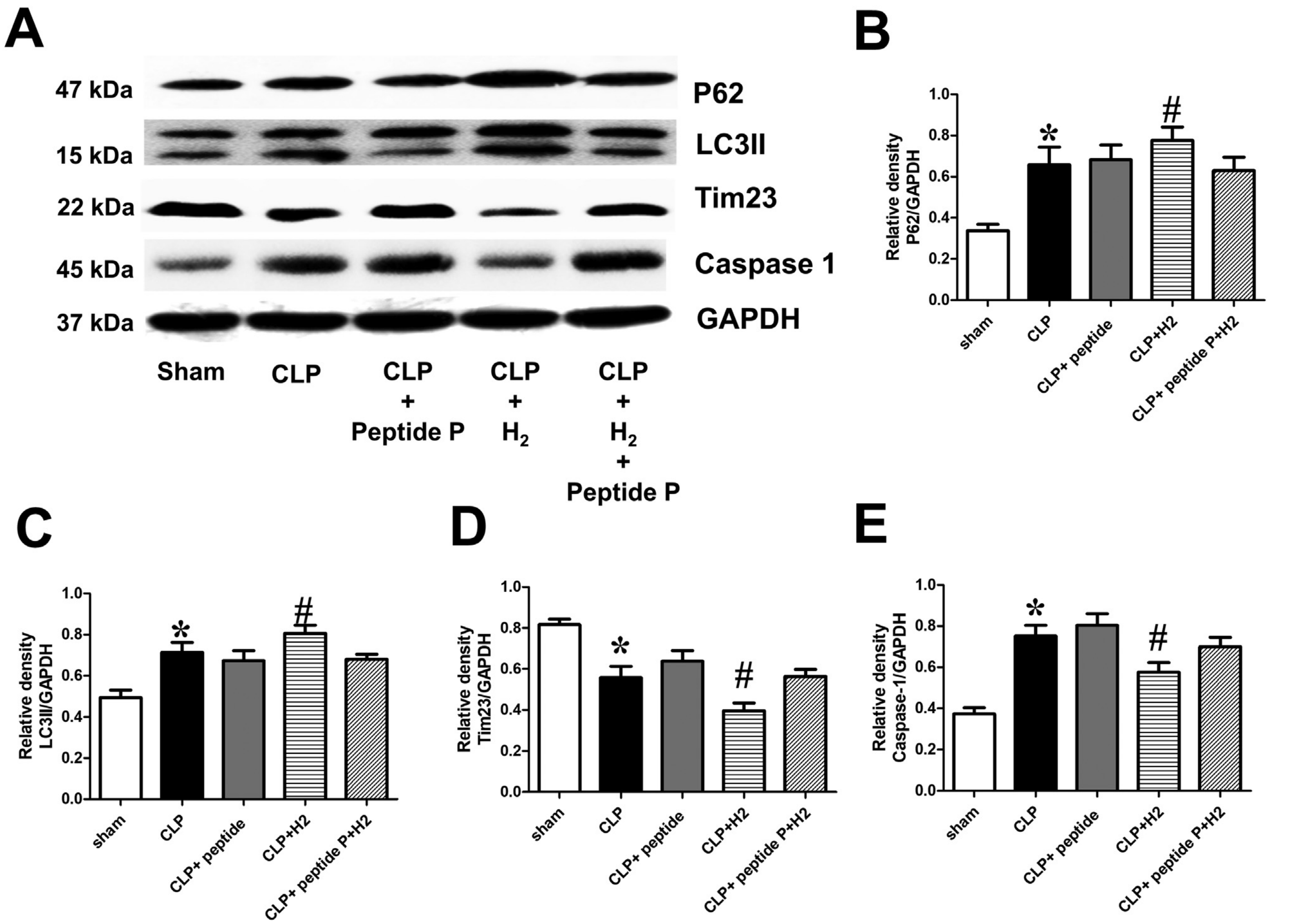

Fig. 7. Effects of H_2 inhalation on P62, LC3B-II, Tim23, and caspase-1 expression levels in mice.
Liver tissues from different groups were collected 6 h after sham or CLP operation for (A) the detection of the expression of mitophagy-related proteins by western blotting. Quantitative analysis of (B) P62, (C) LC3B-II, (D) Tim23, and (E) caspase-1 is shown as the ratio of density of test protein relative to that of GAPDH. Data are expressed as mean ± SD (n = 6/group). $^{*}P < 0.05$ versus the sham group, $^{\#}P < 0.05$ versus the CLP group.

results suggest that sepsis could increase the level of FUNDC1-induced mitophagy, and this effect could be further enhanced after 2% H_2 treatment. Moreover, the FUNDC1 inhibitor peptide P could effectively block the sepsis-induced mitophagy and reverse the protective effect of H_2 on liver injury in sepsis mouse.

4. Discussion

Liver, the largest gland in the human body, plays a crucial role in the clearance of pathogens and in immunological response [15]. Under septic conditions, the liver acts as a core component and may undergo organ dysfunction. Liver injury or dysfunction has recently been recognized as an early event in sepsis that may seriously affect the development of illness [21]. In our recent research, we successfully created an acute sepsis model (4-day mortality rate was 100%, as shown in Fig. 4) and found that sepsis mice showed obvious liver damage at 6 h post-CLP (Fig. 5). In comparison with the sham group, the CLP group showed a dramatic increase in the pathological score and ALT and AST levels in the liver tissue. However, 2% H_2 gas treatment for 3 h starting at 60 min post-CLP decreased the pathological score and increased the expression of ALT and AST in mice from the CLP + H_2 group.

As the “power stations” of eukaryotic cells, mitochondria provide > 90% of the energy needed by the human body [22,23]. Mitochondrial dysfunction owing to mitochondrial oxidative stress, mitochondrial fission, or mitochondrial calcium overload, is known as an important cellular event that leads to MODS, which plays a crucial role in sepsis [14]. To determine the effects of H_2 gas on the sepsis-induced mitochondrial dysfunction, we measured the mitochondrial respiration rate using a commercial kit. At 6 h after sham or CLP operation, the RCR level obviously decreased in mice from the CLP group as compared with those from the sham group, while 2% H_2 inhalation improved the RCR level in mice from the CLP + H_2 group (as shown in Fig. 6).

Previous studies have indicated that selective mitochondrial autophagy (mitophagy) serves as an essential axis for mitochondrial quality control in response to mitochondrial damage [24]. FUNDC1, a protein located on the outer membrane of the mitochondrion, acts as a mitophagy receptor and removes dysfunctional mitochondria in response to mitochondrial stresses and hypoxia [25]. Under normal situations, FUNDC1 is phosphorylated at the Tyr18 position in the LIR motif by Src kinase and CK2 and inhibits the process of mitophagy in cells. However, in response to hypoxia or other hazardous situations, Src is inactivated and FUNDC1 gets dephosphorylated by PGAM family member 5 (PGAM5), resulting in an increase in the co-localization of and interaction between FUNDC1 and LC3-II. The consequence includes the formation of isolation membranes that engulf the damaged mitochondria [26]. In our recent experiment, we found that the expression of FUNDC1 and p-18-FUNDC1 reduced in mice from the CLP group as compared with that in the sham group, and 2% H_2 gas treatment for 3 h significantly reduced the levels of these two proteins in mice from the CLP + H_2 group (Fig. 3). Thus, the inhalation of 2% H_2 gas for 3 h promoted mitophagy and protected mice from the sepsis-induced liver injury.

We also measured the expression levels of mitophagy-related proteins such as P62, LC3B-II, Tim23, and caspase-1. P62 protein is a marker for autophagy flux, and its interaction with LC3B-II was shown to lead to the delivery of P62 and its cargoes to the autophagosome [27]. Moreover, Tim23 is a channel-forming subunit of the translocase of the inner mitochondrial membrane complex and mediates pre-protein translocation across the mitochondrial inner membrane. It also reflects the mitochondrial quantity [28]. Caspase-1 activates various pathways that precipitate in mitochondrial disassembly, eventually resulting in the fragmentation of the mitochondrial network. It may also inhibit mitophagy to amplify mitochondrial damage [29]. In our research, we found that mice from the CLP group exhibited higher expression of P62, LC3-II, and caspase-1 but lower expression of Tim23 than those from the sham group and that 2% H_2 inhalation increased the levels of P62, LC3-II, and caspase-1, but not Tim23, in mice from the CLP + H_2 group (Fig. 7). These data suggest that 2% H_2 inhalation may improve mitophagy and protect septic animals against mitochondrial damage.

The synthetic cell-penetrating peptide P (NH2-GRKKRRQRRRPQDYESDDESYEVLDLTEY-COOH) containing the LIR motif of FUNDC1 was reported to block the interaction between FUNDC1 and LC3, and consequently prevent mitophagy in cells [30]. According to the study published by Zhang et al., pretreatment of mice with peptide P for 24 h could effectively prevent hypoxia-induced mitochondrial dysfunctions [31]. Therefore, to verify the key role of FUNDC1, the cell-penetrating peptide P (1 mg/kg) was intraperitoneally administered to mice 24 h before the CLP or sham operation in this study. However, no statistical difference was observed in the 7-day mortality rate, pathological scores, ALT and AST levels, RCR, and mitophagy-related protein levels (P62, LC3B-II, Tim23, caspase-1) between mice from the CLP + peptide P and CLP + H_2 + peptide P groups (Figs. 3–7). According to our results, peptide P injection could reverse the therapeutic effects of 2% H_2 gas on the sepsis-induced liver injury, mitochondrial dysfunction, and mitophagy.

5. Conclusion

In conclusion, 2% H_2 gas inhalation for 3 h may serve as an effective therapeutic strategy for the treatment of sepsis-induced liver damage via the regulation of FUNDC1-dependent mitophagy.

Conflict of interest statement

All the authors declare no competing financial interests.

Acknowledgement

Mengying Yan and Yang Yu contributed equally to this research.

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