Heme oxygenase-1 alleviates ischemia-reperfusion injury by inhibiting hepatocyte pyroptosis after liver transplantation in rats

Tao Wang  
Kunming Medical University First Affiliated Hospital

Yuan Fang  
Kunming Medical University First Affiliated Hospital

XiaoLi Zhang  
Kunming Medical University First Affiliated Hospital

Yang Yang  
Kunming Medical University

Li Jin  
Kunming Medical University First Affiliated Hospital

ZhiTao Li  
Kunming Medical University First Affiliated Hospital

WeiQiang Tang  
University of South China

Dan Zhao  
Shanghai Jiaotong University: Shanghai Jiao Tong University

YingLei Miao  
Kunming Medical University First Affiliated Hospital

Zhong Zeng  
Kunming Medical University First Affiliated Hospital

HanFei Huang (✉ huanghanfei@kmmu.edu.cn)  
Kunming Medical University First Affiliated Hospital  https://orcid.org/0000-0002-0852-9596

Research Article

Keywords: Heme oxygenase-1, Donors after circulatory death, liver transplantation, ischemia-reperfusion injury, Pyroptosis

Posted Date: April 19th, 2022

DOI: https://doi.org/10.21203/rs.3.rs-1465368/v1
Abstract

Background:

Heme oxygenase-1 (HO-1) is recognized as a key cytoprotective mechanism for inflammation during ischemia-reperfusion injury (IRI). Accumulating evidence suggests that pyroptosis plays an important role in inflammation of IRI. However, the mechanism between HO-1 and pyroptosis in IRI requires further investigation.

Methods:

The "two-cuff" method was performed to establish a model of liver transplantation (LT) from donors after circulatory death. The HO-1 overexpression and shRNA recombinant adeno-associated virus (AAV) were constructed and transfected into rats. Flow cytometry was used to detect the expression of ROS in cells to evaluate the degree of oxidative stress. An automatic biochemical analyzer was performed to detect serum ALT and AST levels and evaluate liver function. Paraffin sections were stained with HE to observe the degree of pathological damage to the tissues. ELISA was applied to detect the levels of IL-1β and IL-18 in the liver tissues. The tissues were analyzed for the expression of HO-1, pro-caspase-1, p22, full-GSDMD, and cleaved-N-GSDMD by western blot. Immunohistochemistry was used to detect the expression of NLRP3.

Results.

The model was successfully constructed, and the difference in survival time obtained was statistically significant after HO-1 overexpression and shRNA recombinant AAV transfected into the donor. The expression of HO-1 was time-dependent with IRI. HE and Suzuki score showed that the short-term groups' tissue necrosis was severer than the control. ROS, ALT, and AST expression in the short-term after IRI was significantly higher than control. The expression of pro-caspase-1, p22, full-GSDMD, and cleaved-N-GSDMD trend was consistent with HO-1, increased significantly in short-term while decreasing in long-term. The expression level of NLRP3 was the lowest in the recent six hours. At six hours after IRI, compared with HO-1-shRNA groups, the ROS, IL-1β, IL-18, ALT, and AST in the HO-1 overexpression were significantly reduced, as well as p22 and cleaved-N-GSDMD, were significantly inhibited. Immunohistochemical revealed that the expression of NLRP3 had a higher level in HO-1 overexpression groups.

Conclusion.

HO-1 improved the survival rate of rats after LT. And HO-1 promoted the recovery of IRI after LT. This study demonstrated that HO-1 could inhibit hepatocyte pyroptosis, thereby reducing IRI after LT.

1. Background
In liver diseases, many factors can cause liver insufficiency, and irreversible damage to organs, as well as endanger life. Liver transplantation (LT) is the only curative treatment for these diseases.\[^1\] Organ shortage is currently the biggest obstacle to the success of treatment, which directly leads to the death of thousands of patients on the waiting list every year.\[^2\] Donors after circulatory death (DCD) have been the main source of organs in recent years. However, DCD livers are inevitably exposed to a longer ischemic period.\[^3\]

Hepatic ischemia-reperfusion injury (IRI) is the main risk factor for early allograft dysfunction, and acute or chronic rejection. It is also an important reason for the shortage of donor organs.\[^4\] The main problem with the donor liver is that it is difficult to assess the quality of the liver after the ischemia-reperfusion (IR) stage; thus primary graft dysfunction, such as abnormal of liver function, is prone to occur after the operation. Since the mechanism underlying liver IRI is not well understood, it is necessary to study the mechanism of graft IRI to improve clinical LT results.

In the liver IR stage, cell death is induced, including apoptosis, necrosis, iron death and pyroptosis.\[^5\] Disturbance of metabolic homeostasis, during the IRI phase, leads to the release of danger-related molecular patterns (DAPMs), and mitochondrial dysfunction triggers graft inflammation. DAMPs that are passively released or actively secreted from immunocompetent cells after stimulation can be recognized by PRRs on the cell membrane surface of Kupffer cells (KCs), thereby activating KCs. A variety of DAMPs promote KCs to form a variety of inflammatory complexes through different PRRs, including NLRP3.\[^6\] NLRP3 recruits pro-caspase-1 and autohydrolyses two adjacent pro-caspase-1 molecules to produce caspase-1 with enzymatic activity, which cleaves the precursors of IL-1\(\beta\) and IL-18. This renders it from the precursor form to the activated form and it is then secreted into the extracellular form to perform biological functions.\[^7\] Pyroptosis is closely associated with the mechanisms underlying hepatic IRI. Pyroptosis relies on inflammasome-mediated caspase-1 activation, and the insertion of gasdermin D (GSDMD) leads to the formation of plasma membrane pores, leading to the release of intracellular proteins, ion decompensation, water influx, and cell swelling.\[^8\] Activated caspase-1 can also cleave GSDMD to produce GSDMD-N and -C.\[^9\] The spliceosomes GSDMD-N and -C mediate cell perforation and rupture, causing caspase-1-dependent cell pyrolysis.\[^10\] In addition, due to the initiation of the oxidative stress response, mitochondria produce excessive oxygen free radicals, among which the increase in reactive oxygen species (ROS) is the most significant.\[^11\] Overexpression of ROS can also promote the maturation of NLRP3, and ultimately can also mediate the occurrence of pyrolysis through caspase-1.\[^12\]

Heme oxygenase-1 (HO-1) is the rate-limiting enzyme for the oxidative degradation of heme, a stress-inducing protein that catalyzes the oxidation and conversion of heme into carbon monoxide, iron, and biliverdin.\[^13\] Moreover, HO-1 is considered to be an antioxidant and cytoprotective agent in various organ damage and disease models (i.e., lung, kidney, heart, liver).\[^14\] Therefore, HO-1 can be used as a pleiotropic regulator of inflammatory signals by removing heme and producing its enzymatic degradation products.
Our previous studies have shown that HO-1 plays a vital role in the process of ischemia reperfusion injury, enhancing the ability of liver cells to resist oxidative stress and stabilizing mitochondrial function. For LT, pretreatment with adenovirus to interfere with the expression of HO-1 can significantly improve IRI after orthotopic IRI in SD rats and promote the recovery of liver function.\(^{11,15,16}\)

Based on the above studies, we aimed to determine whether HO-1 could alleviate hepatic IRI in DCD rats and improve liver function by inhibiting hepatocyte pyroptosis.

2. Methods

2.1 Ethics Statement

Male Sprague-Dawley (SD) rats (150–180 g; 5–6 weeks old; SPF grade) were purchased from Beijing HuaFuKang Bioscience Company (Beijing, China). The animal protocol was approved by the Institutional Animal Care and Use Committee of Kunming Medical University (Kunming, China) (KMMU2020189), and all animal experiments were performed in compliance with the National Institute of Health guidelines for animal experimentation.

2.2 SD Rat Surgery Procedure

In our study, the "two-cuff" technique established by Kamada et al. was used to establish a SD rat liver transplantation model.\(^{17}\) A survival time of more than 24 h was considered a successful model, and a postoperative survival curve was drawn. In brief, all animals were fasted without limiting water for 12 h before the operation. The rats were anesthetized with 5% isoflurane and maintained under anesthesia with 2-3% isoflurane, which was monitored by observing the color of the lip mucosa and the movement of the thorax. After obtaining fresh liver tissue samples, the rats had euthanasia by exsanguination. And the study conformed to the ARRIVE guidelines 2.0.\(^{18}\)

2.3 Adeno Associated Virus Transfection Efficiency Test

To explore the efficiency of adeno-associated virus (AAV) in SD rats, 25 rats were randomly allocated to five groups: shRNA-HO-1 empty-loaded virus (shRNA-NC), HO-1 related shRNA-AAV (shRNA-HO-1), HO-1 related overexpression empty-loaded virus (AAV-NC), HO-1 overexpressing AAV (AAV-HO-1), and normal saline (Normal). Each group injection volume was 200 \(\mu l\) ((Hanbio Biotechnology Co. Ltd., Shanghai, China; AAV titer was 2.5*109 mg/\(\mu l\)). After the injection, the animals were kept in a SPF-class animal room for 21 days to stably express AAV. After we obtained a fresh rat liver tissue specimen, we rinsed it and cut it into 1.0*1.0*0.5 cm pieces and immediately put it in liquid nitrogen to freeze it. Then we stored the cut liver tissue in a liquid nitrogen tank with an EP tube divider.

2.4 Animals Study Design

Eighty-eight SD rats, injected through the tail vein, were randomly divided into the following six groups to plot the survival curve: the sham operation group (Sham, n=8, only laparotomy); the DCD group (DCD,
n=16, the donors were injected with normal saline); the HO-1 interference expression control group (DCD+shRNA-NC, n=16, the donors were transfected with HO-1-shRNA empty-loaded virus); the HO-1 interference expression group (DCD+shRNA-HO-1, the donors were transfected HO-1-shRNA-AAV); the HO-1 overexpression control group (DCD+AAV-NC, the donors were transfected HO-1-overexpressing empty-loaded virus); and the HO-1 overexpression group (DCD+AAV-HO-1, the donors were transfected with HO-1 overexpression AAV).

In addition, to explore whether HO-1 induced pyroptosis with hepatic IRI and its time-dependent manner, 54 rats were randomly allocated to two groups: the normal group (n=6) and the hepatic IR group with different durations of reperfusion: 6 h (DCD+IR6 h), 24 h (DCD+IR24 h), 72 h (DCD+IR72 h), and 168 h (DCD+IR168 h); n=12/group.

Furthermore, 66 SD rats were randomly divided into the following six groups for the detection of 6 h HO-1 related cell pyroptosis (n=12, i.e., 6 pairs). The sham group (n=6, SD rats were only treated with laparotomy); the DCD liver transplantation group (the donor was injected with normal saline through the tail vein), the HO-1 interference expression control group (DCD+shRNA-NC, the donor was transfected with HO-1 shRNA AAV via the tail vein); the HO-1 interference expression group (DCD+shRNA-HO-1, the donor was transfected with HO-1 shRNA AAV through the tail vein); the HO-1 overexpression control group (DCD+AAV-NC, the donor was transfected through the tail vein infection with HO-1 overexpression of AAV empty virus); and the HO-1 overexpression group (DCD+AAV-HO-1, the donor was transfected HO-1 through the tail vein, overexpression of AAV).

2.5 Biochemical Assay and Measurement of cytokines

The serum ALT and AST levels of SD rats were evaluated using a fully automatic biochemical analyzer (BIO-RAD, USA), expressed in U/L. The levels of IL-1β and IL-18 were detected using a commercially available ELISA kit according to the manufacturer's instructions (Neobioscience Technology Company, Shenzhen, China).

2.6 Flow cytometry

We diluted DCFH-DA with serum-free culture medium according to 1:1000 to make a final concentration of 10 μmol/L. We weighed 50 μg of fresh SD rat liver tissue, homogenized and filtered it to make a cell suspension, centrifuged to discard the supernatant, and then added an appropriate volume of diluted DCFH-DA. We incubated medium in a 37°C incubator for 20 min. The cells were washed three times with serum-free cell culture medium to fully remove the DCFH-DA that had not entered the cells. Rosup was added to the ROS-positive control group to stimulate the cells for 20–30 min. For flow cytometry detection we used the 488 nm excitation wavelength, 525nm emission wavelength and detected the fluorescence intensity before and after stimulation.

2.7 Histological and immunohistochemical study
The SD rat liver samples were fixed with 4% paraformaldehyde for 48 h before processing. The fixed liver samples were dehydrated, embedded in paraffin, and cut into small pieces (<3 mm thick, three for each liver). The sections were stained with HE for pathological evaluation. All images shown in the results represent at least three images of each liver. The Liver Suzuki scoring standard was used.\cite{19, 20}

### 2.8 Western-blot analysis

We performed western blotting as described previously.\cite{21} The rabbit anti-HO-1, β-tubulin, and IgG antibodies were purchased from Cell Signaling Technology; Anti-Caspase-1, and Anti-GSDMD were purchased from Abcam; and NLRP3 rabbit polyclonal antibody was purchased from Proteintech. β-Tubulin (Cell Signaling Technology) was used as a loading control.

### 2.9 RT-PCR experiments

We performed RT-PCR as described previously.\cite{22} The primer sequences of mRNAs were detected by PCR using specific primers as follows: HO-1 forward 5’-CCATCCCTTACACACCAGCC-3’, reverse 5’-GGTAGCGGGTATATGCGTGG=3’; β-Tubulin forward 5’-GAGGCAGATGGCAGTGACAG-3’, reverse 5’-TGGTTGGGGAACACCGAGTA-3’.

### 2.10 Statistical analysis

SPSS24.0, was used for data analysis. The measurement data were expressed in the form of ±s. For the comparison of multiple groups of measurement data we used single-factor analysis of variance and an advanced homogeneity of variance test. If the variance was uniform, the least significant difference test was used; if the variance was uneven, Dunnett’s T3 test was used. The Kalplan-Meier method was used for the survival analysis and statistical analysis. Differences were considered statistically significant at $P < 0.05$. The statistical graph was drawn with GraphPad Prism software (version 8.0).

### 3. Results

#### 3.1 Fluorescence results and HO-1 mRNA transcription level with AAV transfection.

The recombinant AAV of HO-1 overexpression, HO-1 shRNA, and the respective blank control virus vectors were transfected into SD rats for 21 days. Fresh liver tissues were taken and embedded in optimum cutting temperature (OCT), frozen, sectioned, and observed under an inverted fluorescence microscope. The fluorescence of the co-expressed EGFP and ZsGreen indicated that 21 days after AAV was transfected into SD rats, the recombinant DNA was stably expressed in the rat liver. (Fig. 1A~1D)

HO-1 transcription levels were substantially decreased in shRNA-HO-1 compared to shRNA-NC. The gene transcription level increased in AAV-HO-1 compared with the AAV-NC, shRNA-HO-1, shRNA-NC, and normal. (Fig. 1E)

#### 3.2 Survival curve of SD rats after DCD liver transplantation.
The SPSS24.0 was used for survival analysis and statistics on SD rats that had been successfully modeled. The overall comparison result was \( x^2 = 14.449 \), the degree of freedom \( \nu = 5 \), and \( P < 0.05 \). There was at least a statistically significant difference between the two groups. Paired comparisons again found \( P < 0.05 \) among the three groups for the sham, DCD+shRNA-HO-1, and DCD+AAV-HO-1. There was no remarkable difference in survival rate among the DCD, DCD+shRNA-NC and DCD+AAV-NC. (Fig. 1F)

3.3 HE staining results and Suzuki score of donor liver after LT.

We then explored the effects of donor liver ischemia followed by different durations of reperfusion (DCD+IR6 h, DCD+IR24 h, DCD+IR72 h, DCD+IR168 h). According to HE, we found that tissue damage of DCD+IR6 h progressed rapidly, and obvious necrosis was visible. As time progressed, the damage to DCD+IR24 h was further aggravated, and extensive necrosis appeared. However, for DCD+IR72 h and DCD+IR168 h, necrosis was not as obvious as DCD+IR24 h, and the degree of damage was gradually reduced. The structure of the normal lobules had been destroyed, and the sinus space was still wider than normal. Compared with the normal group, the DCD+IR6 h, DCD+IR24 h, DCD+IR72 h, and DCD+IR168 h groups showed statistically significant differences (\( P < 0.05 \)) in the degree of damage. Compared with DCD+IR6 h, the difference in the degree of injury for the normal group, DCD+IR24 h and DCD+IR168 h, was statistically significant (\( P < 0.05 \)). Notably, the difference in the DCD+IR72 h group was not remarkable compared with DCD+IR6 h. (Fig. 2)

3.4 The expression of ROS in reperfused tissue of the donor liver after DCD LT.

Over time, the oxidative stress reaction in the tissues of each group gradually decreased after reperfusion. Compared with the normal group, the expression differences of DCD+IR 6 h, DCD+IR24 h, DCD+IR72 h, and DCD+IR168 h groups were statistically significant (\( P < 0.05 \)). Compared with DCD+IR6 h, the normal group, DCD+IR72 h, DCD+IR168 h expression showed a statistically significant difference (\( P < 0.05 \)), but there was no statistically significant difference in the expression of DCD+IR24 h. (Fig. 3)

3.5 Changes in liver function and cytokines of donor liver after LT.

As time goes on, the function and cytokines of donor liver would change accordingly after reperfusion. For function, the expression levels of ALT and AST had increased significantly in the short-term (6 h and 24 h), but gradually decreased in the long-term (72 h and 168 h). Compared with Normal, the expression differences of DCD+IR6 h, DCD+IR24 h, DCD+IR72 h, and DCD+IR168 h were statistically significant (\( P < 0.05 \)), respectively. And compared with DCD+IR6 h expression, the Normal, DCD+IR72 h, DCD+IR168 h difference was statistically significant (\( P < 0.05 \)), respectively. But the DCD+IR6h expression difference with DCD+IR24h was not remarkable. (Fig. 4A,4B)

Over time, the expression of IL-1β and IL-18 in hepatocytes showed a trend from increased to decreased, which was consistent. Compared with normal group, the expression differences of DCD+IR6 h, DCD+IR24 h, DCD+IR72 h, DCD+IR168 h were statistically significant (\( P < 0.05 \)). Compared with DCD+IR6 h, the
normal group, DCD+IR72 h, and DCD+IR168 h expression differences were statistically significant ($P < 0.05$). However, the difference in DCD+IR6h expression with DCD+IR24h was not remarkable. (Fig. 3C,3D)

3.6 The expression of each protein was detected in tissue.

Over time, the proteins showed a trend with the prolongation of the postoperative reperfusion time after LT. Western-blotting was performed to detect the expression of each protein in tissues. (Fig. 4E)

The expression of HO-1 showed a significant increase in the short-term, while the long-term expression gradually decreased as the postoperative recovery time increased. DCD+IR6 h increased remarkably. Compared with the normal group, DCD+IR72 h, and DCD+IR168 h groups, the difference in expression of DCD+IR6 h was statistically significant ($P < 0.05$), while it was not statistically significant compared with DCD+IR24 h. In addition, compared with the normal group, the differences in expressions of the DCD+IR6 h, DCD+IR24 h, and DCD+IR72 h groups were statistically significant ($P < 0.05$). However, for DCD+IR168 h, the expression of HO-1 was higher than the normal group, but the difference was not remarkable. (Fig. 4F)

The spliced body p22 of Pro-caspase1 was significantly increased in the short-term but gradually decreased in the long-term. Compared with the normal group, the expressions of DCD+IR6 h, DCD+IR24 h, and DCD+IR72 h groups was statistically different ($P < 0.05$). Although the expression of p22 in DCD+IR168 h was higher than that in the normal group, the difference was not statistically significant. Compared with DCD+IR6 h, the expressions of the Normal group, DCD+IR72 h, and DCD+IR168 h groups were statistically different ($P < 0.05$), but there was no remarkable in the expression of DCD+IR24 h. (Fig. 4G)

For the cleaved-N-GSDMD spliced body of Full-GSDMD, after IRI, the expression level was the highest in the short-term, and the expression level gradually decreased in the long-term. Compared with the normal group, the expressions of DCD+IR6 h, DCD+IR24 h, and DCD+IR72 h groups showed a statistically significant difference ($P < 0.05$). Although the expression of DCD+IR168 h was higher than the normal group, the difference was not statistically significant. Compared with DCD+IR6 h, the expressions of the normal group, DCD+IR72 h, and DCD+IR168 h were all statistically significant ($P < 0.05$). However, there was no remarkable in the expression of DCD+IR6 h and DCD+IR24 h. (Fig. 4H)

For NLRP3, immunohistochemistry found that the normal group was not expressed. DCD+IR6 h and DCD+IR24 h displayed extensive necrosis. However, the expression level of NLRP3 in DCD+IR6 h was the lowest among the four time points. Over time, the expression of NLRP3 increased in the short-term, and then gradually decreased. Compared with DCD+IR6 h, the expression differences of the normal group, DCD+IR24 h, DCD+IR72 h, and DCD+IR168 h were remarkable ($P < 0.05$). (Fig. 5)

3.7 With AAV treatment, the donor liver of DCD LT with IRI for reperfusion of tissues with HE results, and Suzuki score.
Based on previous experiments, we chose 6h as the time point for reperfusion. After 21 days of AAV pretreatment, the donor liver was reperfused for 6 h after DCD LT. Compared with the control groups, the DCD+shRNA-HO-1 had worse damage in the liver tissue, with large areas of necrosis, severe congestion, and sinus space. The necrotic area of DCD+shRNA-HO-1 was significantly reduced, the hepatic cord was intact, the sinus space was approximately the same as that in the sham, and the degree of congestion was significantly relieved. Compared with DCD, the sham group, DCD+shRNA-HO-1, and DCD+AAV-HO-1 showed statistically significant differences in the degree of damage (P < 0.05). DCD+shRNA-NC and DCD+AAV-NC damage was not significantly different. Compared with DCD+shRNA-HO-1, the damage degree of DCD, DCD+shRNA-NC, DCD+AAV-NC, and DCD+AAV-HO-1 were statistically significant (P < 0.05). Compared with DCD+AAV-NC, the difference in DCD+AAV-HO-1 was statistically significant (P < 0.05). (Fig. 6)

3.8 The expression level of ROS in liver tissues of each group with AAV treatment.

The oxidative stress reactions in the liver tissue of each group were not the same. The expression of ROS in DCD+shRNA-HO-1 was significantly higher than that in other groups in liver tissue, while DCD+AAV-HO-1 expression was significantly lower. Compared with DCD+shRNA-HO-1, the expression of DCD, DCD+shRNA-NC, DCD+AAV-NC, and DCD+AAV-HO-1 were statistically significant (P < 0.05). Compared with DCD+AAV-NC, the expression of DCD+AAV-HO-1 was statistically different (P < 0.05). The DCD, DCD+shRNA-NC, and DCD+AAV-NC were compared with each other, and there was no statistically significant difference in expression. (Fig. 7)

3.9 The changes in liver function and cytokines of each group with AAV treatment.

After 21 days of AAV pretreatment, the donor liver was reperfused for 6 h after DCD LT, and the expression levels of ALT and AST in the serum of DCD+shRNA-HO-1 increased significantly. However, the expression levels of ALT and AST in DCD+AAV-HO-1 serum showed a downward trend compared to the control, but they were still higher than those in the sham group.

For ALT, compared with DCD+shRNA-HO-1, the expression of DCD, DCD+shRNA-NC, DCD+AAV-NC, and DCD+AAV-HO-1 was statistically significant (P < 0.05). Compared with the DCD+AAV-NC, the expression of DCD+AAV-HO-1 was statistically different (P < 0.05). There was no statistical difference in the expression of DCD, DCD+shRNA-NC, and DCD+AAV-NC groups. (Fig. 8A)

For AST, compared with DCD+shRNA-HO-1, the expression differences of DCD, DCD+shRNA-NC, DCD+AAV-NC, and DCD+AAV-HO-1 was statistically significant (P < 0.05). Compared with DCD+AAV-NC, the expression of DCD+AAV-HO-1 was statistically different (P < 0.05). The expression differences between DCD, DCD+shRNA-NC, and DCD+AAV-NC were not remarkable. (Fig. 8B)

The expression levels of IL-1β and IL-18 in DCD+shRNA-HO-1 liver tissues were higher than those in the other groups. The expression levels of IL-1β and IL-18 in DCD+AAV-HO-1 liver tissue showed a downward trend compared with the control group, while compared with the DCD+shRNA-HO-1 group, they were
significantly lower, but still slightly higher in the sham group. At the same time, the expression levels of IL-1β and IL-18 in each group were positively correlated.

For IL-1β, compared with DCD+shRNA-HO-1, the expression differences of DCD, DCD+shRNA-NC, DCD+AAV-NC, and DCD+AAV-HO-1 were statistically significant (P<0.05). Compared with the DCD+AAV-NC, the expression of sham and DCD+AAV-HO-1 was statistically significant (P<0.05). The expressions of DCD, DCD+shRNA-NC, and DCD+AAV-NC were no remarkable among the three groups. (Fig. 8C)

For IL-18, compared with DCD+shRNA-HO-1, the expressions of DCD, DCD+shRNA-NC, DCD+AAV-NC, and DCD+AAV-HO-1 were statistically different (P<0.05). Compared with DCD+AAV-NC, the expression of the sham and DCD+AAV-HO-1 was statistically different (P<0.05). The expressions of DCD, DCD+shRNA-NC, and DCD+AAV-NC were no remarkable among the three groups. (Fig. 8D)

### 3.10 The expression of each protein of each group with AAV treatment.

After 21 days of AAV pretreatment, the donor liver was reperfused for 6 h. The HO-1 mRNA expression level in the transplanted liver in each group was consistent with expectations. The mRNA transcription level of the HO-1 interference expression group was significantly lower than that of the control but compared with the sham group, there was still a slight increasing trend, while the mRNA transcription level of the DCD+AAV-HO-1 was significantly improved. The expression levels of DCD, DCD+shRNA-NC, DCD+shRNA-HO-1, DCD+AAV-NC, and DCD+AAV-HO-1 were compared, and the differences were statistically significant (P<0.05). However, the difference in expression between DCD+shRNA-HO-1 and sham was not statistically significant. At the protein expression level, the expression trend of HO-1 was consistent with the transcription level. Compared with DCD+shRNA-HO-1, except for DCD, the expression of HO-1 in the other groups was statistically different (P<0.05). At the same time, comparing DCD+AAV-HO-1 and DCD+AAV-NC, the expression of HO-1 was statistically different (P<0.05). (Fig. 8E-G)

For the spliced p22 of Pro-caspase1, the expression of DCD+shRNA-HO-1 increased significantly, while the expression of DCD+AAV-HO-1 decreased. Compared with DCD+shRNA-HO-1, the expressions of DCD, DCD+shRNA-NC, DCD+AAV-NC, and DCD+AAV-HO-1 were statistically different (P<0.05). Compared with DCD+AAV-NC, the expression of the DCD+AAV-HO-1 group was statistically different (P<0.05). The expressions of DCD, DCD+shRNA-NC, and DCD+AAV-NC were no remarkable. (Fig. 8H)

For the spliced cleaved-N-GSDMD of Full-GSDMD, the expression level was similar to that of p22. The expression of DCD+shRNA-HO-1 was significantly increased, while that of DCD+AAV-HO-1 was significantly reduced. Compared with DCD+shRNA-HO-1, the expressions of DCD, DCD+shRNA-NC, DCD+AAV-NC, and DCD+AAV-HO-1 were statistically different (P<0.05). The expression between DCD+AAV-NC and DCD+AAV-HO-1 was no remarkable. There was no remarkable difference in expression among the DCD, DCD+shRNA-NC, and DCD+AAV-NC groups. (Fig. 8I)

In addition, intervention in the expression of HO-1 immediately affected the expression of NLRP3, which in turn affected the expression and activation of Caspase-1 and GSDMD.
For the expression of NLRP3, Immunohistochemical detection was used. The expression of NLRP3 in DCD+shRNA-HO-1 was significantly decreased, and there was also a large area of tissue necrosis. The expression differences of DCD, DCD+shRNA-NC, and DCD+AAV-NC were statistically different ($P < 0.05$). There was no remarkable difference in NLRP3 expression between DCD and DCD+shRNA-NC. Compared with DCD+AAV-NC, the expression of NLRP3 in DCD+AAV-HO-1 was significantly reduced, and the expression was statistically different ($P < 0.05$). (Fig. 9)

4. Discussion

It is well known that LT is the most effective curative treatment for end-stage liver disease. Optimizing graft function and reducing IRI is essential to facilitate hepatic DCD, eventually increasing donor liver availability. The come into being ROS, cytokine, and other inflammatory factors were the main stimuli for liver, during liver transplantation.

HO-1 is the rate-limiting enzyme of heme metabolism, and its high expression is currently recognized as a key cytoprotective mechanism against inflammation, hyperthermia, and IRI. After DCD LT, the expression of HO-1 was correlated with the ischemia-reperfusion time, and it increased significantly in the DCD+IR6 h, and then gradually decreased. The trend coincided with the most severe early stress response of IRI, and ROS expression was also consistent with this trend. This is also consistent with previous research. IR6 h and IRI24 h significantly increased the expression of HO-1 in IRI, indicating that the DCD donor liver would induce significant expression of HO-1 after IRI, which may have the potential to resist oxidative stress damage. However, the recent high expression of HO-1 did not immediately relieve liver damage. As shown in Fig. 2, HE showed that there was extensive necrosis at DCD+IR6 h, and DCD+IR24 h had the most severe damage. And according to the suzuki score, the long-term (78 h and 168 h) damage was significantly relieved compared with the short-term (6 h and 24 h) damage. The results of liver function serum ALT and AST biochemical analysis indicated that the recent liver cell damage was severe, and the DCD+IR24 h was slightly higher than the DCD+IR6 h, and then gradually decreased. We speculate that in the near term, although HO-1 was highly expressed during IRI and LT, liver cell damage occurred before its expression. Therefore, high expression of HO-1 could only be seen as a follow-up response to oxidative stress, and it could play a role in anti-inflammatory and anti-injury effects in the future. The reason for the gradual reduction in long-term damage may be because the high expression of HO-1 in the early stage exerted a long-term effect, which enhanced the tolerance of the undamaged liver tissue to oxidative stress and inhibited the combination of DAMPs and PRR. Subsequently, the inflammatory response was inhibited and the damage to long-term liver cells was reduced, which was consistent with previous research by our group.

To further explain the cause of hepatic injury, we tested the expression of NLRP3, cleaved-caspase-1 (p22/p20), and cleaved-N-GSDMD in the donor liver at each IRI time point. The results showed that the expression of p22 and cleaved-N-GSDMD was the highest in the short-term, indicating that pyroptosis occurred when the donor liver underwent IRI. The degree of pyroptosis is the most serious at the
beginning of IRI and then gradually decreases with the prolongation of reperfusion time. However, NLRP3, which is directly related to pro-caspase-1 activation, has the opposite effect. The results of NLRP3 immunohistochemistry showed that the expression level of the IR6 h group was the lowest, which was inconsistent with the most severe scorch death in the IR6 h group. Burdette et al. showed that NLRP3 was expressed in the cytoplasm, thereby mediating pro-caspase-1 splicing activation and functioning\[26\]. Therefore, we speculate that the inconsistent expression trend of NLRP3 was due to serious damage to the tissue cells in the IR6 h group. After cell rupture, NLRP3 is completely released and cannot be color-developed by immunohistochemistry. This speculation was consistent with the most severe pyroptosis observed in the IR6 h group.

At the same time, we also tested the expression of IL-1β and IL-18 in liver tissues, and the expression trend was that the IR24 h group showed the highest expression, followed by a gradual decrease. Zhang et al. revealed that warm ischemia injury could lead to a decrease in the number of KCs in the liver, resulting in a general decrease in Th1 cytokines produced by non-parenchymal cells in the liver.\[27\] However, postoperative ischemia reperfusion sends local inflammatory chemokines of the transplanted liver to the whole body, such as CXC-chemokine ligand 1 (CXCL1), CXCL2, and CXCL8 to attract neutrophil infiltration; CC-chemokine ligand 1 (CCL1), CCL2, CCL25, and CX3CL1 can chemoattract bone marrow mononuclear cell infiltration.\[28\] It was confirmed by HE staining that the number of inflammatory cells that infiltrated in the liver tissue of the IR24 h group were significantly increased compared to that of the IR6 h group, which could explain the upward trend of IL-1β and IL-18.

RT-qPCR detected the overexpression of HO-1, HO-1 shRNA recombinant AAV, and the respective blank control viral vectors were transfected into normal SD rats. This showed that the intervention effect of HO-1 overexpression and HO-1 shRNA recombinant AAV was statistically significant compared with the blank group, and the difference between the two groups was statistically significant. HO-1 overexpression was positively correlated with the survival rate of SD rats after DCD liver transplantation.

After transfection with adeno-associated virus, liver transplantation from a cardiac death donor was performed. We used RT-qPCR and western blotting to detect the mRNA and protein expression levels of HO-1, and the expression of HO-1 was in line with expectations. When HO-1 was overexpressed, the expression level of p22 was significantly suppressed, and the expression of cleaved-N-GSDMD was also significantly reduced. However, when the expression of HO-1 was suppressed, the expression levels of p22 and cleaved-N-GSDMD increased significantly. This showed that under the condition of high HO-1 expression in the donor liver, pyrolysis was inhibited when the cardiac death donor underwent ischemia reperfusion after liver transplantation. This result was consistent with the HE staining results of the transplanted liver. HE staining showed that when HO-1 was overexpressed, tissue cell damage was light, and cell destruction and lysis were not obvious. When the expression of HO-1 was inhibited, the tissue cell damage was aggravated, the necrotic area was increased compared with each control group, cell lysis was severe, and there was no intact and normal hepatic cord nor hepatic sinusoid structure. At the same time, according to the results of AST and ALT, the overexpression of HO-1 could reduce the level of liver damage after liver transplantation from cardiac death donors. This proves that the degree of damage
after liver transplantation was significantly alleviated by overexpression of HO-1 in the donor liver. For the expression of NLRP3, under the condition that the expression of HO-1 was inhibited, the immunohistochemical detection was significantly lower than that of the donor liver with overexpression of HO-1. This could be caused by severe liver tissue necrosis when HO-1 expression was blocked, which made immunohistochemical coloration undetectable. As for the HO-1 overexpression group, because the tissue structure was complete and the immunohistochemical coloration was normal, these data are reliable. Compared with each control group, it was significantly lower, indicating that the activation of NLRP3 was inhibited at that time.

The expression of HO-1 was increased in the early stage of ischemia-reperfusion after liver transplantation from donors of cardiac death, but it was only a follow-up response to oxidative stress and could not immediately alleviate liver injury. However, the early high expression of HO-1 played a role in the recovery of long-term liver function. Oxidative stress was the most serious in the 6-hour ischemia-reperfusion group of donors with cardiac death after liver transplantation, and early pyrolysis was very likely to be one of the main causes of liver damage. Therefore, inhibiting early liver injury could be key to promoting the recovery of transplanted liver function.

The donor was pretreated by HO-1 overexpression and HO-1 shRNA recombinant adeno-associated virus, so that the donor liver could reach a state of stable HO-1 expression before liver transplantation. This proved the effect of preconditioning the donor liver to change the expression of HO-1 in ischemia-reperfusion injury after liver transplantation from a cardiac-death donor. Based on this, we concluded that HO-1 overexpression could inhibit hepatocyte pyrolysis, thereby alleviating ischemia-reperfusion injury after liver transplantation from a cardiac death donor in SD rats.

5. Conclusion

The study selected a cardiac death rat donor model to simulate the acquisition and preservation of a clinically transplanted liver, and the study was conducted by interfering with the expression of HO-1 in the donor liver. The results showed that HO-1 could promote ischemia reperfusion recovery in SD rats after DCD liver transplantation; HO-1 overexpression inhibited hepatocyte pyrolysis, thereby alleviating ischemia-reperfusion injury after liver transplantation from a cardiac death SD rat donor.

Abbreviations

HO-1  Heme oxygenase-1  
SD    Sprague-Dawley
LT    Liver transplantation
DCD   Donors after circulatory death
AAV  adeno-associated virus
IR  ischemia-reperfusion
IRI  ischemia-reperfusion injury
DAPMs  danger-related molecular patterns
GSDMD gasdermin D

Declarations

Ethics approval and consent to participate:
This study was approved by the Institutional Animal Care and Use Committee of the First Affiliated Hospital of Kunming Medical University. (KMMU2020189)

Consent for publication: Not applicable.

Availability of Data and Materials: Not applicable.

Competing Interest:
The authors declare no conflict of interest.

Funding:
The study was financially supported by a grant from National Natural Science Foundation of China (No. 81960123 and 81760124) and Scientific Research Fund of the Education Department of Yunnan Province (2022Y199). The funding body had no role in study design, data collection, analysis, and interpretation of data, and writing the manuscript.

Author Contributions:
Y M, Z Z, and HF H: Conceptualization and financial support.
T W, Y F: Contributed equally to this work. Carried out the main experiments, analyzed the data, and drafted this manuscript;
XL Z, Y Y, L J, Z Tao L, WQ T, D Z: Technical support and manuscript revision. All authors have read and agreed to the published version of the manuscript.

Acknowledgement: Not Applicable
References


Figures
Figure 1

A~D: Fresh liver tissue was embedded in OCT and then frozen sectioned, and the co-expressed EGFP and ZsGreen fluorescence were observed under an inverted fluorescence microscope to observe the transfection effect. 400x.

A: AAV2/9-EGFP interference control;

B: AAV2/9-r-Hmox1 shRNA-EGFP interferes with expression;

C: AAV2/9-ZsGreen overexpression control;
D: AAV2/9-CMV-r-Hmox1-3xflag-ZsGreen is overexpressed.

E: The change of HO-1 gene transcription level mRNA after AAV transfection in SD rats. ∗: Compared with the Normal group, the difference is statistically significant, \( P < 0.05 \); #: Compared with the shRNA-HO-1 group, the difference is statistically significant, \( P < 0.05 \); &: Compared with the AAV-NC group, the difference is statistically significant, \( P < 0.05 \).

F: Analysis of postoperative survival time of SD rats between groups after successful DCD liver transplantation modeling

Figure 2

A~E. Normal(A), DCD+IR6 h(B), DCD+IR24 h(C), DCD+IR72 h(D) and DCD+IR168 h(E), respectively, HE results of liver tissue after LT. F: The histogram based on the Suzuki score, ∗: \( P < 0.05 \) compared with Normal; #: \( P < 0.05 \) compared with DCD+IR6h.
Figure 3

A-E: After DCD LT, the donor liver of ischemia was followed by different durations of reperfusion for 6 h, 24 h, 72 h, and 168 h.

F: Flow cytometry was used to detect the expression level of ROS in the liver tissue. ∗: Compared with Normal group, P<0.05; #: Compared with DCD+IR6h group, P<0.05.
Figure 4

*: Compared with Normal, $P<0.05$; #: Compared with DCD+IR6 h, $P<0.05$.

A. Biochemical analysis results of ALT
B. Biochemical analysis results of AST
C. The expression level of IL-1β in liver tissue.
D. The expression level of IL-18 in liver tissue.
E. Western blot method to detect the protein expression of HO-1, Caspase-1, p22, full-GSDMD and cleaved-N-GSDMD in each group;

F~H: the statistical results of HO-1/β-tubulin, p22/β-tubulin, and cleaved-N-GSDMD/β-tubulin in each group respectively;

Figure 5

A~E. Normal, DCD+IR6 h, DCD+IR24 h, DCD+IR72 h and DCD+IR168 h, respectively, immunohistochemistry of NLRP3 results of liver tissue after LT. F: The expression level of NLRP3 in the liver tissue. ∗: Compared with Normal, $P<0.05$; #: Compared with DCD+IR6 h, $P<0.05$.

Figure 6
HE results and Suzuki score of liver tissue after AAV pretreatment.

A~F: Sham, DCD, DCD+shRNA-NC, DCD+shRNA-HO-1, DCD+AAV-NC, DCD+AAV-HO-1 postoperative liver tissue HE staining results.

G: Draw a histogram based on the Suzuki score, *: compared with the DCD group, \( P \leq 0.05 \); #: compared with the DCD+shRNA-HO-1 group, \( P \leq 0.05 \); &: compared with the DCD+AAV-NC group, \( P < 0.05 \).

![Figure 7](image)

After pretreatment with AAV, the donor liver was reperfused for 6 hours after DCD LT, and the expression level of ROS in liver tissues.

*: Compared with DCD group, \( P \leq 0.05 \); #: Compared with DCD+shRNA-HO-1 group, \( P \leq 0.05 \); &: Compared with DCD+AAV-NC group, \( P \leq 0.05 \).
Figure 8

*: Compared with DCD group, P<0.05; #: Compared with DCD+shRNA-HO-1 group, P<0.05; &: Compared with DCD+AAV-NC group, P<0.05.

With AAV pretreatment, the donor liver was reperfused for 6 hours after DCD LT.

A. Biochemical analysis results of ALT

B. Biochemical analysis results of AST

C. The expression level of IL-1\(\beta\) in liver tissue.

D. The expression level of IL-18 in liver tissue.
E. The mRNA level of HO-1 in the liver tissue.

F. Western blot method to detect the protein expression of HO-1, Caspase-1, p22, full-GSDMD and cleaved-N-GSDMD in each group;

G~I: the statistical results of HO-1/β-tubulin, p22/β-tubulin, and cleaved-N-GSDMD/β-tubulin in each group respectively;

Figure 9

After AAV pretreatment, the donor liver was reperfused for 6 hours after DCD LT, and the expression of NLRP3 was observed. (200X) After immunohistochemical examination, the results of NLRP3 coloration in each group.

A~F: Sham, DCD, DCD+shRNA-NC, DCD+shRNA-HO-1, DCD+AAV-NC, DCD+AAV-HO-1. G: The statistical results of the relative expression levels of NLRP3 in each group relative to the Sham. ∗: Compared with DCD group, $P<0.05$; #: Compared with DCD+shRNA-HO-1 group, $P<0.05$; &: Compared with DCD+AAV-NC group, $P<0.05$.

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- AuthorChecklistE10only.pdf