



# Diabetes induces hepatocyte pyroptosis by promoting oxidative stress-mediated NLRP3 inflammasome activation during liver ischaemia and reperfusion injury

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**Background:** Although diabetes mellitus has been reported to aggravate liver ischaemia and reperfusion (IR) injury, the basic mechanism remains largely unknown. The object of the present study was to determine the role of oxidative stress and hepatocellular pyroptosis in liver IR injury in diabetic mice.

**Methods:** Db/db and C57BL/6 mice at 8 weeks of age were subjected to liver IR injury. Liver injury and hepatocyte cell death were analyzed. A NOD-like receptor family pyrin domain-containing 3 protein (NLRP3) inflammasome antagonist (CY09) and a reactive oxygen species (ROS) antagonist (N-Acetyl-L-cysteine, NAC) were used to determine the role of ROS-mediated hepatocellular pyroptosis in diabetic mice post-IR.

**Results:** Aggravated liver IR injury was found in db/db mice compared to C57BL/6 control mice, as demonstrated by increased serum alanine aminotransaminase (ALT) and aspartate aminotransaminase (AST) levels, liver architecture damage and Suzuki scores. Interestingly, IR induces the pyroptosis of hepatocytes in db/db mice, as evidenced by enhanced NLRP3 inflammasome activation, increased numbers of terminal deoxynucleotidyl transferase dUTP nick end labelling (TUNEL)-positive hepatocytes and increased gene expression of interleukin-1 $\beta$  (IL-1 $\beta$ ) and IL-18 in livers post-IR. The inhibitory effect of CY09, an NLRP3 antagonist, efficiently abrogated the exacerbation effects of diabetes on liver IR injury in db/db mice. Furthermore, increased ROS expression was detected in db/db mice compared to control mice after IR. ROS scavenging by NAC pretreatment markedly inhibited hepatocellular NLRP3 inflammasome activation and pyroptosis in the db/db mice post-IR, indicating that ROS play an essential role in mediating hepatocyte pyroptosis in the setting of diabetes mellitus.

**Conclusions:** Our results demonstrate that diabetes induces hepatocyte pyroptosis by promoting oxidative stress-mediated NLRP3 inflammasome activation during liver IR injury. Strategies targeting ROS and NLRP3 inflammasome activation would be beneficial for preventing liver IR injury in diabetic patients.

**Keywords:** Diabetes; hepatic ischaemia and reperfusion injury (hepatic IR injury); NOD-like receptor family pyrin domain-containing 3 protein inflammasome (NLRP3 inflammasome); reactive oxygen species (ROS); pyroptosis

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## Introduction

Hepatic ischaemia and reperfusion (IR) injury has been classified into cold and warm ischaemia. The occurrence of cold ischaemia is associated with the preservation and storage of organs prior to transplantation (1,2). Warm ischaemia is associated with shock, trauma, transplantation, and robotic liver surgery, during which a temporary interruption of the blood supply may occur. The mechanism of warm hepatic IR injury is complex. Some studies suggest that tissue inflammatory immune response and oxidative stress play key roles in the pathogenesis of warm liver IR injury (3,4).

Diabetes is a universal disease, and almost a quarter of patients undergoing liver transplantation have pre-existing diabetes mellitus (5). Pre-transplant diabetes can predict poor prognosis after transplant (6). Higher risk of rejection has been found in diabetic patients post liver transplant (7). Diabetes is also a major risk factor associated with the heart (8), brain (9), kidney (10), and liver ischemic injury (11,12). The inflammatory response caused by immune activation plays a major role in transplant rejection. The most significant sign of diabetes is hyperglycaemia and it has been proved to cause chronic inflammation (13). Markers of diabetes, such as hyperlipidaemia and hyperglycaemia, have been shown to promote the phenotype of inflammatory macrophages (14). In addition, under inflammatory conditions, hyperglycaemic mice have exhibited a growing number of macrophage in the liver, kidney, intestine, and peritoneal cavity (15). Studies have indicated that hepatic IR injury increases NOD-like receptor family pyrin domain-containing 3 protein (NLRP3) inflammasome activation (16,17). Our previous research proved that hyperglycaemia promotes the activation of NLRP3 inflammasome in liver-resident macrophage and then aggravates acute liver injury. Furthermore, the excessive reactive oxygen species (ROS) and following disorder of redox balance also play an important role in liver ischemic injury. Various liver cells can produce ROS post IR-stress (16). Whether diabetes

increases NLRP3 inflammasome activation during warm hepatic IR injury and the effects of ROS on murine hepatic IR injury remain unknown.

Herein, we found that diabetes induces hepatocyte pyroptosis by promoting oxidative stress-mediated NLRP3 inflammasome activation during liver IR injury. The ROS antagonist NAC could mitigate hepatic IR injury by suppressing NLRP3 inflammasome activation. Strategies targeting ROS and NLRP3 inflammasome activation would be beneficial for preventing liver IR injury in diabetic patients. We present the following article in accordance with the ARRIVE reporting checklist (available at <http://dx.doi.org/10.21037/atm-20-1839>).

## Methods

### *Animals*

Male C57BL/KsJ-leprdb/leprdb (db/db) and C57BL/6 control mice (eight-week-old) were provided by the Model Animal Research Center of Nanjing University. The Institutional Animal Care and Use Committee of Nanjing Medical University approved this animal experiment protocol (protocol number NMU08-092).

### *Mouse liver partial warm IR model*

Mice were randomly separated into two groups: sham group and IR group (n=6 mice/group). Inhaled isoflurane (1.5%) was used to anesthetize mice. A midline laparotomy was performed on the mice. Seventy percent of the hepatic portal blood supply was blocked for 90 min by an atraumatic clip. The clip was then removed to allow hepatic reperfusion. The same procedure was performed in sham controls but without the step of vascular occlusion. Serum and liver tissues were collected for further analysis (18). CY09 (2.5 mg/kg), N-Acetyl-L-cysteine (NAC, 150 mg/kg, MedChemExpress, Monmouth Junction, MN, USA), or the vehicle control, was intraperitoneally administered 1 h prior

to ischaemia.

### **Serum biochemical measurements**

Serum alanine aminotransaminase (sALT) and aspartate aminotransaminase (sAST) were detected using an AU5400 automated chemical analyser (Olympus, Tokyo, Japan).

### **Histopathology**

Liver sections (4  $\mu\text{m}$ ) were stained with haematoxylin and eosin (H&E), oil red o, and Masson. The severity of liver injury was scored according to Suzuki's criteria as described previously (19).

### **Transferase dUTP nick end labelling (TUNEL) staining**

A fluorescence detection kit (Roche, Basel, Switzerland) were used for terminal deoxynucleotidyl TUNEL staining according to the manufacturer's protocols.

### **Measurement of measurement of malondialdehyde (MDA), glutathione (GSH), superoxide dismutase (SOD) and ROS levels**

MDA, GSH, SOD and ROS were conducted by using commercial kits. Liver tissues were washed with PBS, homogenized in lysis buffer and sonicated. After being sonicated, the lysed tissue was centrifuged (10,000  $\times g$ , 10 min) to remove debris and retain the supernatant. A microplate reader was used to measure the levels of MDA, GSH, SOD and ROS in the supernatant. In addition, MDA, GSH, SOD and ROS levels were normalized according to the protein concentration. Two-step collagenase perfusion was used to obtain mouse primary hepatocytes (20). With reference to the specification, the DHR123 fluorescent probe was used to detect the content of ROS-producing cells by flow cytometry.

### **Western blot analysis**

The protein (20  $\mu\text{g}$  per sample) extracted from liver tissues. Next liver proteins were electrophoresed. Primary antibodies directed against NLRP3, pro-caspasae-1, caspase-3, cleaved caspase-1/3/7/9, p-p65, p-I $\kappa$ B $\alpha$  and GAPDH (Cell Signaling Technology, San Diego, CA, USA) were used.

### **Quantitative RT-PCR analysis (qRT-PCR)**

Total RNA extract from liver tissue. RNA from each sample was reversetranscribed into first-strand cDNA by using a Prime Script RT reagent Kit (Takara Bio). Quantitative PCR was performed using the StepOnePlus Real-Time PCR System (Applied Biosystems, Foster City, CA, USA) (21). Each experiment was repeated independently three times. Target gene expression was analyzed by the ratio to the hypoxanthine phosphoribosyl transferase (HPRT).

### **ELISA**

Interleukin-1 $\beta$  (IL-1 $\beta$ ), TNF- $\alpha$  and IL-6 levels in sera were measured using an ELISA kit (eBiosciences, San Diego, CA, USA) according to the manufacturer's protocols.

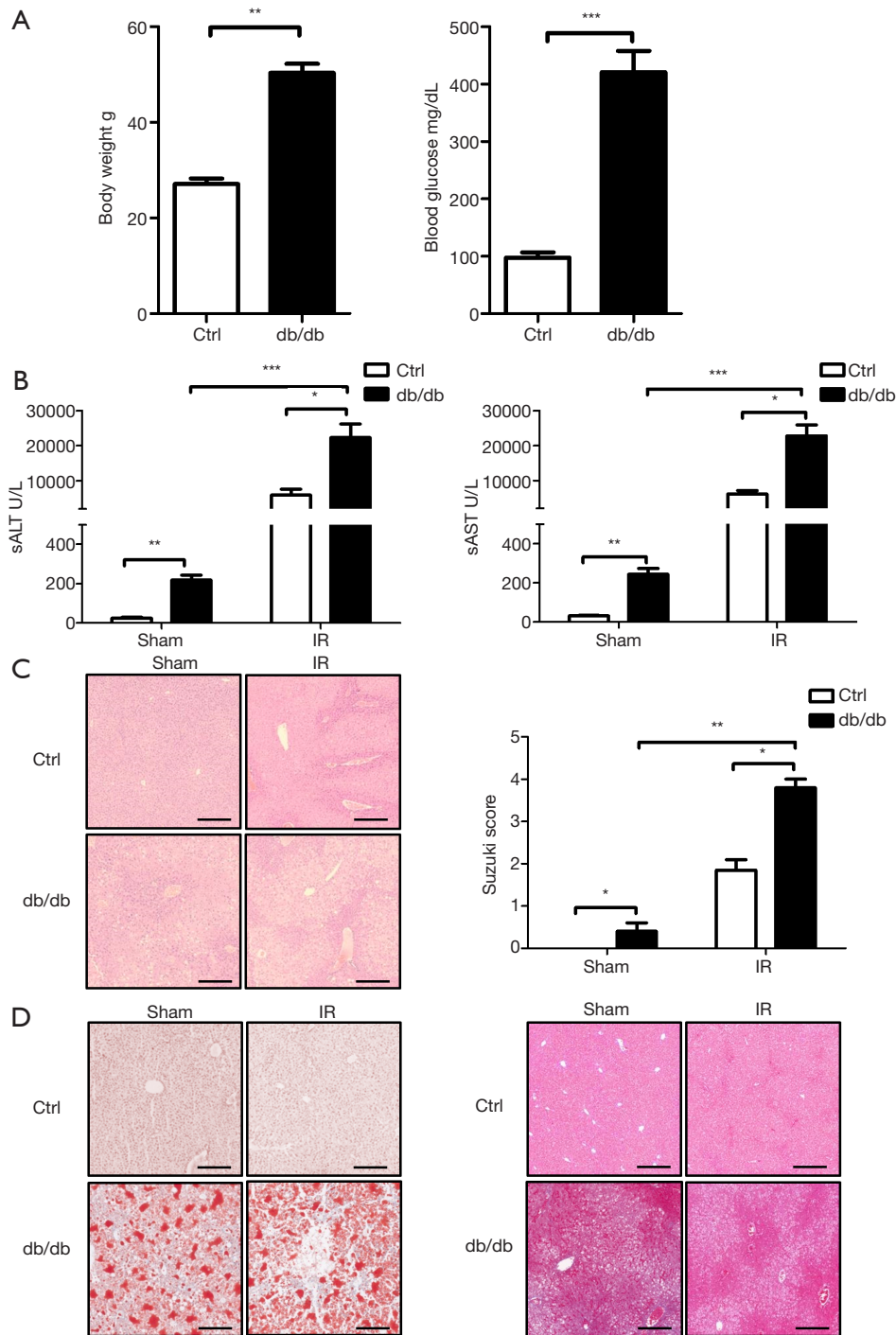
### **Statistical analysis**

Data are expressed as the mean  $\pm$  SEM. Two-group comparisons were performed using a *t*-test. And for multiple group comparisons, ANOVA with Bonferroni corrections using STAT software, version 11.0 was used. P values <0.05 were considered statistically significant.

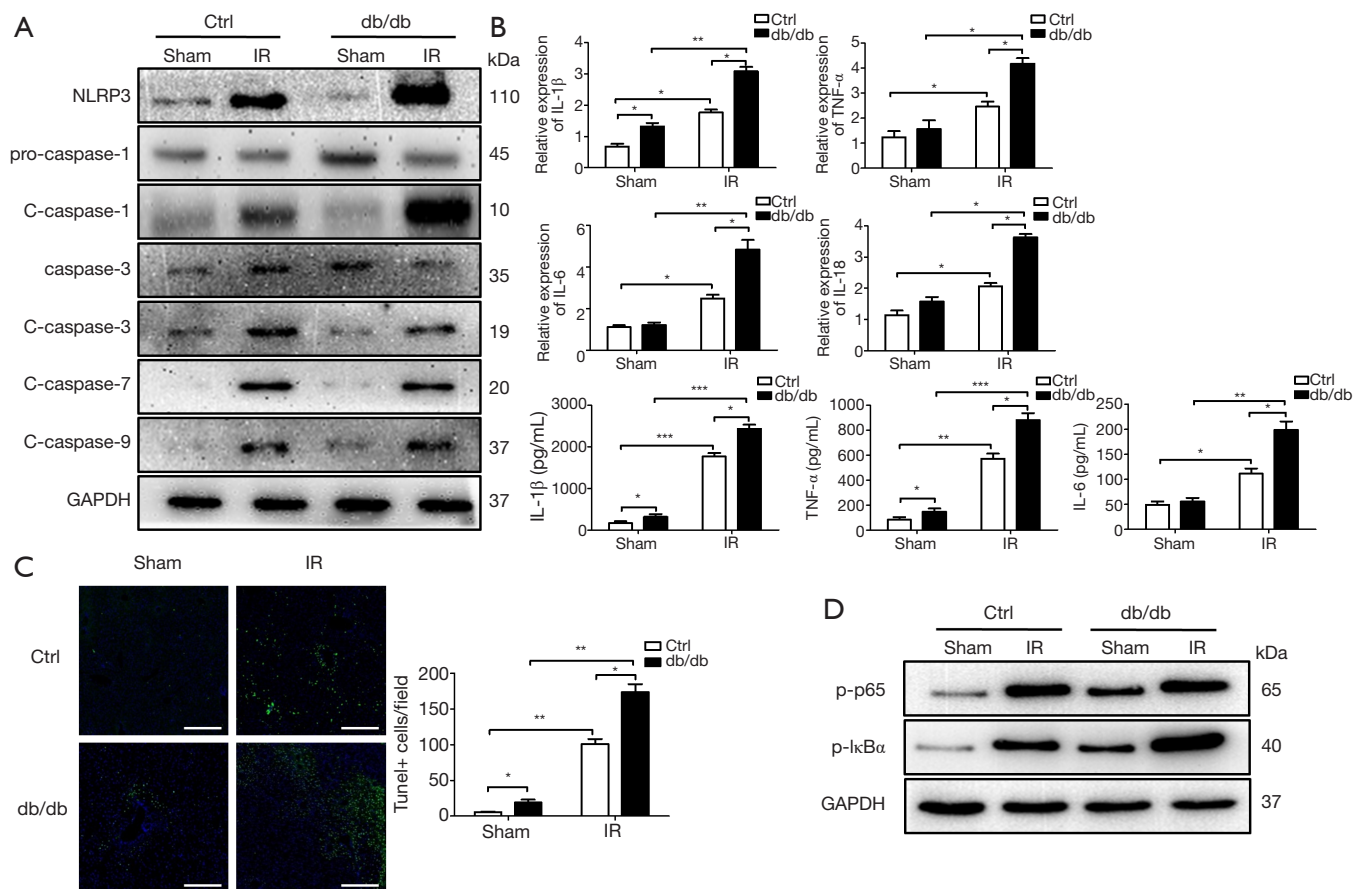
## **Results**

### **Diabetes exacerbates liver IR injury**

Db/db mice at 8 weeks of age displayed obesity and had an increased body weight compared with C57BL/6 mice (50.3 $\pm$ 1.9 *vs.* 27.1 $\pm$ 1.1 g). Blood glucose was higher in db/db than C57BL/6 mice (420 $\pm$ 37 *vs.* 98 $\pm$ 9 mg/dL) (Figure 1A). Firstly we assessed whether liver IR injury is exacerbated in db/db mice. A 90-min liver ischaemia or sham procedure was performed on db/db and C57BL/6 (control) mice, and then reperused for 6 h. We compared liver injury among these groups. Certainly, the db/db mouse group showed meaningfully higher sALT and sAST levels (Figure 1B) and less-well preserved liver architecture with higher Suzuki scores than the sham mouse group (Figure 1C) than the control group. Through oil red o and Masson staining, the fat accumulation and fibrosis levels in liver tissues of db/db mice were significantly higher than those of the control group, but IR injury did not affect this difference (Figure 1D). These results demonstrate that diabetes exacerbates liver IR injury.



**Figure 1** Exacerbated liver IR injury in db/db mice. Db/db and control mice underwent a sham or IR operation as described in the section of Methods. Blood and liver samples were collected at 6 h post reperfusion. (A) Body weight and blood glucose. (B) sALT and sAST levels. (C) H&E staining (original magnification  $\times 100$ ) and Suzuki's histological score. (D) Oil red o and Masson staining. Scale bars represent 100  $\mu\text{m}$ . n=6 samples/group. \*,  $P < 0.05$ ; \*\*,  $P < 0.01$ ; \*\*\*,  $P < 0.001$ . IR, ischaemia and reperfusion; sALT, serum alanine aminotransaminase; sAST, serum aspartate aminotransaminase; H&E, haematoxylin and eosin.



**Figure 2** Increased NLRP3 inflammasome activation and hepatocyte pyroptosis in liver IR injury of db/db mice. Db/db and control mice underwent a sham or IR operation. The liver parenchyma was isolated at 6 h post reperfusion. (A) Immunoblotting of NLRP3, pro-caspase-1, caspase-3, cleaved caspase-1/3/7/9 protein expression in liver tissue. (B) qRT-PCR analysis of IL-1 $\beta$ , IL-6, TNF- $\alpha$ , IL-18 and ELISA test of IL-1 $\beta$ , IL-6, TNF- $\alpha$  in serum. (C) TUNEL staining of the liver tissues (original magnification  $\times 100$ ) and the relative proportion of TUNEL-positive cells. Scale bars represent 100  $\mu$ m. (D) Immunoblotting of p-p65, p-I $\kappa$ B $\alpha$  protein expression in liver tissue. n=6 samples/group. \*, P<0.05; \*\*, P<0.01; \*\*\*, P<0.001. NLRP3, NOD-like receptor family pyrin domain-containing 3 protein; IR, ischaemia and reperfusion; qRT-PCR, quantitative RT-PCR; IL-1 $\beta$ , interleukin-1 $\beta$ ; TUNEL, transferase dUTP nick end labelling.

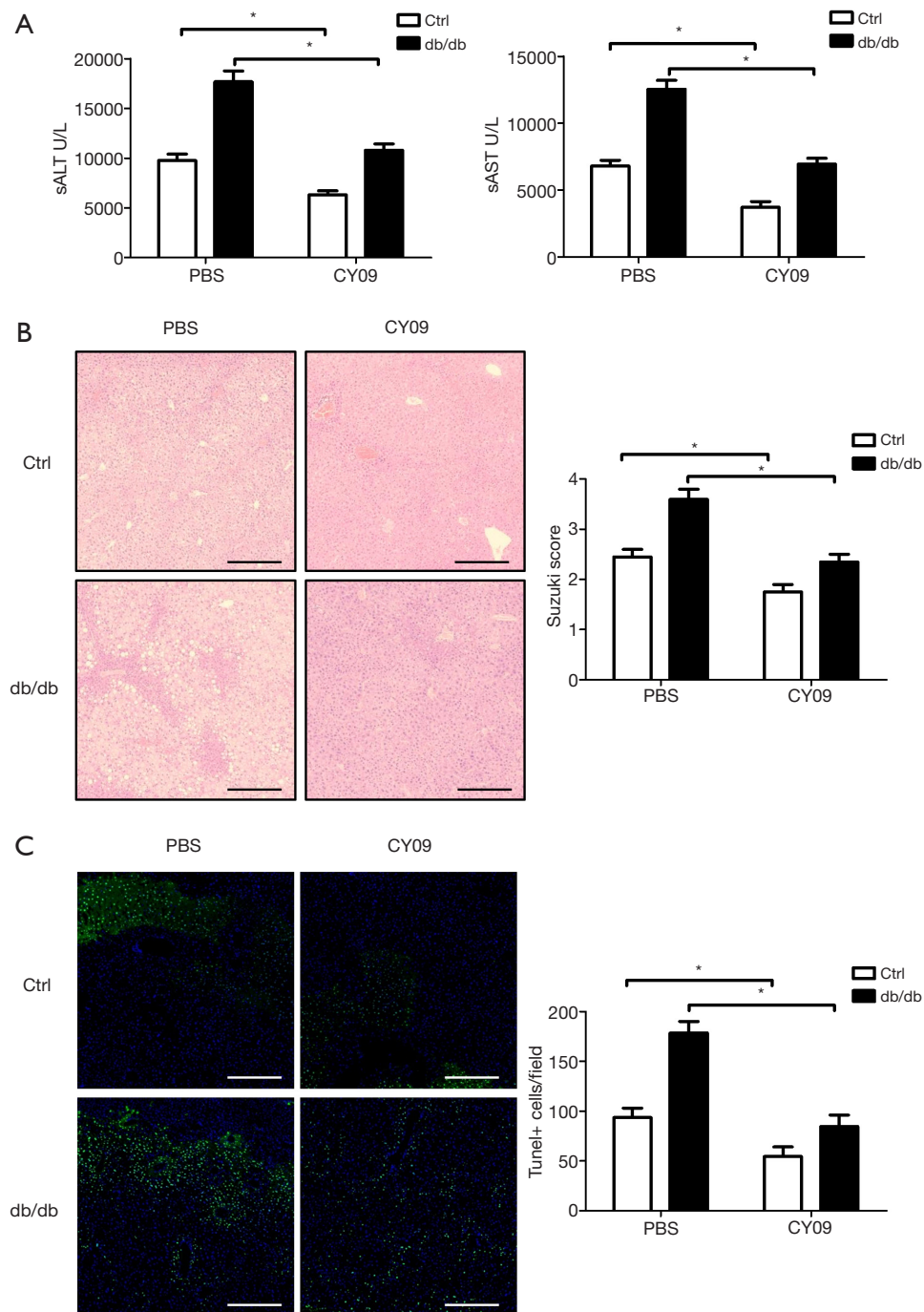
### ***Diabetes exacerbates liver IR damage by activating NLRP3 inflammasome and hepatocyte pyroptosis***

To assess the effect of diabetes associated with liver IR injury, we compared activation of NLRP3 inflammasome in different groups. Interestingly, protein levels of NLRP3 and cleaved caspase-1 were fairly increased in liver tissue isolated from db/db mice after 6 h of IR injury (Figure 2A). At the same time, the activation of caspase-3/7/9 was also detected and found to increase after IR, but no significantly differences were observed between db/db and control mice. The livers of db/db mice also had increased IL-1 $\beta$ , TNF- $\alpha$ , IL-6 and IL-18 gene induction and TUNEL-

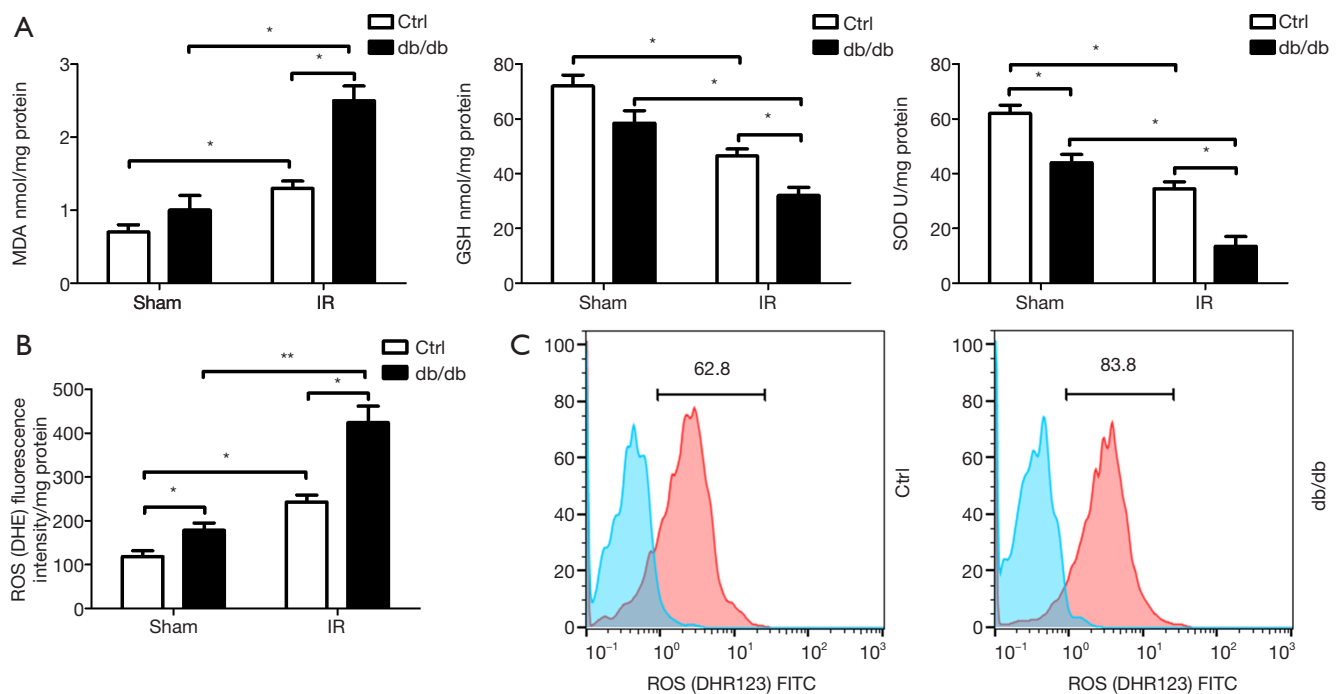
positive hepatocytes (Figure 2B,C). In addition we analyzed the activation of NF- $\kappa$ B by western blot and found that increased NF- $\kappa$ B activation in db/db diabetic mice (Figure 2D). These results suggest that there is an increase in apoptosis in both db/db and control mice, but NF- $\kappa$ B-mediated activation of NLRP3 and cell pyrolysis play a more important role. Thus, diabetes induces activation of NLRP3 inflammasome and hepatocyte pyroptosis in liver IR injury.

### ***Diabetes induces hepatocyte pyroptosis by promoting NLRP3 inflammasome activation during liver IR injury***

CY09 was used to obstruct NLRP3 inflammasome



**Figure 3** NLRP3 inhibition alleviates liver IR injury and pyroptosis in db/db mice. PBS or CY09 was intraperitoneally administered 1 h prior to the IR injury of control and db/db mice. (A) sALT and sAST levels. (B) H&E staining (original magnification  $\times 100$ , scale bars indicate  $100\ \mu\text{m}$ ) and Suzuki's histological score. (C) TUNEL staining of the liver tissues (original magnification  $\times 100$ ) and the relative proportion of TUNEL-positive cells. Scale bars represent  $100\ \mu\text{m}$ .  $n=6$  samples/group. \*,  $P<0.05$ . NLRP3, NOD-like receptor family pyrin domain-containing 3 protein; IR, ischaemia and reperfusion; sALT, serum alanine aminotransaminase; sAST, serum aspartate aminotransaminase; H&E, haematoxylin and eosin; TUNEL, transferase dUTP nick end labelling.



**Figure 4** Increased ROS expression in db/db mice after IR. Db/db and control mice underwent a sham or IR operation. (A) MDA and GSH levels and SOD activity in the liver tissues. (B) ROS levels in the liver tissues of each group of mice were determined using a tissue ROS test kit (DHE). (C) Proportion of ROS-producing cells of each group of mice was determined using DHR123 fluorescence probe. n=6 samples/group. \*, P<0.05; \*\*, P<0.01. ROS, reactive oxygen species; IR, ischaemia and reperfusion; MDA, measurement of malondialdehyde; GSH, glutathione; SOD, superoxide dismutase.

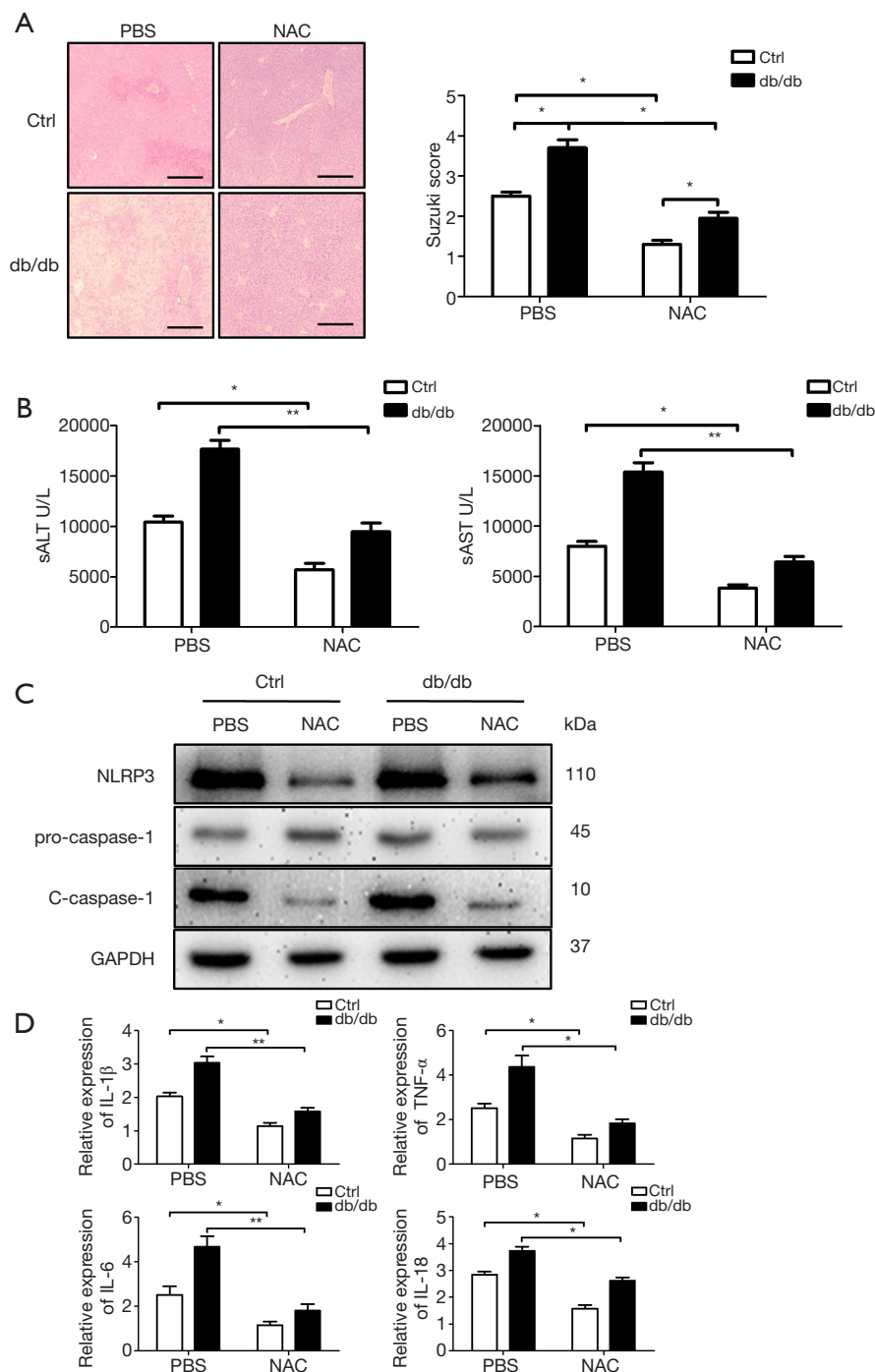
activation in order to research the function of enhanced activating NLRP3 inflammasome in diabetes during liver IR injury. Compared to the control treatment, NLRP3 inhibition prevented liver IR injury in db/db mice, as illustrated by decreased sALT and sAST levels (Figure 3A) and reduced liver structural damage (Figure 3B). CY09 pretreatment also reduced the quantity of TUNEL-positive hepatocytes (Figure 3C). These results suggest that diabetes induces pyroptosis in hepatocytes by promoting NLRP3 inflammasome activation during liver IR injury.

#### ***Oxidative stress induces hepatocyte pyrolysis by promoting NLRP3 inflammasome activation during diabetic mouse liver IR injury***

Considering oxidative stress is the essential mechanism of liver IR injury, we studied the function of oxidative stress during liver IR injury in db/db mouse. Liver IR injury induced significant increases in the MDA level and reduced the GSH content and SOD activity in db/db mouse livers

compared to the control mouse livers (Figure 4A). Then, we further detected ROS levels in liver tissue. Through DHE fluorescence and DHR123 fluorescence probe, the ROS level and the proportion of ROS-producing cells in the liver tissue of db/db mice were significantly increased (Figure 4B,C). Liver IR injury significantly elevated the ROS content in the db/db mouse groups.

To explore the functional role of ROS in liver IR injury in diabetic mice, NAC was utilized to protect against oxidative stress. NAC pretreatment significantly alleviated liver injury in db/db mice, as indicated by the reduced liver architecture damage and Suzuki scores (Figure 5A) and decreased sALT and sAST levels (Figure 5B). Moreover, activation of the NLRP3 inflammasome was decreased in liver tissue of db/db mice after liver IR injury due to NAC pretreatment, as shown by the decreased protein expression levels of NLRP3 and cleaved caspase-1 (Figure 5C), and gene expression levels of IL-1 $\beta$ , TNF- $\alpha$ , IL-6 and IL-18 (Figure 5D). These results demonstrate that diabetes induces pyroptosis in hepatocytes by promoting oxidative stress-mediated NLRP3 inflammasome activation during liver IR injury.



**Figure 5** Inhibition of ROS reduces liver IR injury and pyroptosis in db/db mice. PBS or NAC was intraperitoneally administered 1 h prior to the induction of ischaemia in control and db/db mice. (A) H&E staining (original magnification  $\times 100$ ) and Suzuki's histological score. (B) sALT and sAST levels. (C) Immunoblotting of NLRP3 and cleaved caspase-1 protein expression in liver tissue. (D) qRT-PCR analysis of IL-1 $\beta$ , IL-6, TNF- $\alpha$  and IL-18. Scale bars represent 100  $\mu$ m. n=6 samples/group. \*, P<0.05; \*\*, P<0.01. ROS, reactive oxygen species; IR, ischaemia and reperfusion; H&E, haematoxylin and eosin; sALT, serum alanine aminotransaminase; sAST, serum aspartate aminotransaminase; NLRP3, NOD-like receptor family pyrin domain-containing 3 protein; qRT-PCR, quantitative RT-PCR; IL-1 $\beta$ , interleukin-1 $\beta$ .



## Discussion

Diabetes mellitus is a significant public health problem that threatens people around the world (22,23). Diabetes is a chronic condition that causes various complications, including atherosclerosis (24), diabetic nephropathy (25), diabetic retinopathy (26), and neural damage (22). Some studies have suggested that diabetes aggravates myocardial IR injury (27,28). Other studies have demonstrated that diabetes enhances renal IR injury (29-31). Our study addressed the questions of whether and how diabetes affects warm hepatic IR injury.

Two different stages of tissue injury have been defined in liver IR injury. Oxygen and nutrition depletion caused by ischemia result in direct injury of hepatocytes, which triggers activation of various immune cells and subsequent inflammatory tissue injury. In previous studies, we have reported strategies to protect against liver IR injury by targeting both the parenchymal liver cells (32) and proinflammatory immune activation (19,33-35).

ROS play a critical role in maintaining normal cellular physiological functions, including cellular development, growth, and differentiation. ROS are maintained in cells at baseline levels that maintain cell proliferation and metabolism. Meanwhile ROS also play the key role to regulate many significant regulatory and metabolic pathways as signal transduction molecules in cells (36). Essential roles of oxidative stress in the occurrence of diabetes complications have been reported in recent studies (37,38). Studies have shown that hyperglycaemia triggers ROS formation in macrophages (22,39,40). Under diabetic conditions, the endothelium secretes monocyte chemoattractant protein-1 (MCP-1) and attracts monocytes (39). Migrating monocytes differentiate into macrophages and produce high levels of ROS, exacerbating inflammation and tissue damage (39). Other studies have also shown that macrophage mitochondrial dysfunction and abnormal activation of cytoplasmic NADPH oxidase (NOX) can increase ROS production under hyperglycemic conditions (41,42). Contrary to the regulation of ROS in the antimicrobial response, the metabolic ROS production in diabetics is more unstable and maladjusted (43). Studies have indicated that hepatic IR injury increases ROS production (16,44). Our study focused on the role of ROS in murine hepatic IR injury accompanied by diabetes.

Various pathogen-associated molecular patterns (PAMPs) and danger-associated molecular patterns (DAMPs) released from injured cells could activate NLRP3 and promote caspase-1 and IL-1 $\beta$  activation (16,45). Studies have shown

that hepatic IR injury activates the NLRP3 inflammasome (16,17,46). Additionally, one study demonstrated that the production of intracellular ROS induces NLRP3 translocation to the cytoplasm from the nucleus in LPS-treated neonatal rat cardiomyocytes. NLRP3 cytoplasmic translocation is prevented by elimination of ROS (47). In a previous study, we demonstrated that hyperglycemia aggravated acute liver injury by promoting NLRP3 inflammasome activation in liver-resident macrophages (48). NLRP3 inflammasome inhibition protected the liver from liver damage, inflammation and steatosis of experimental steatohepatitis with diabetes (49). Although increasing evidence indicates that NLRP3 inflammasome activation plays an important role in liver IR injury in the setting of hyperglycemia/diabetes, the precise effects of NLRP3 regulation by hyperglycemia/diabetes on hepatocellular pyroptosis remains largely unclear.

According to the above studies, we investigated how diabetes aggravates hepatic IR injury. We first performed liver IR experiments. The results showed that diabetes exacerbated hepatic IR injury. It has been reported that the perioperative hyperglycemia/diabetes resulted in a poor organ function and increased the rate of liver graft rejection in patients post liver transplantation, which could be improved by intensive insulin treatment (50-52). However, we did not analyze the impact of hyperglycemia/diabetes on liver IR injury in humans. The results also suggested that NLRP3 inflammasome activation and hepatocyte pyroptosis were increased in diabetic livers post IR. The NLRP3 inflammasome antagonist CY09 alleviated hepatic injury. Further study demonstrated a significant increase in ROS expression in livers from db/db mice at 6 h after reperfusion. The ROS antagonist NAC suppressed the activation of NLRP3 inflammasome and hepatocyte pyroptosis and attenuated hepatic IR injury. These results show that diabetes aggravates hepatic IR injury by enhancing ROS expression and increasing NLRP3 inflammasome activation.

In conclusion, our results demonstrate that diabetes induces hepatocyte pyroptosis by promoting oxidative stress-mediated NLRP3 inflammasome activation during liver IR injury, providing a promising strategy for attenuating liver injury in patients with diabetes undergoing hepatic trauma, resection and transplantation.

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## Footnote

*Reporting Checklist:* The authors have completed the ARRIVE reporting checklist. Available at <http://dx.doi.org/10.21037/atm-20-1839>

*Data Sharing Statement:* Available at <http://dx.doi.org/10.21037/atm-20-1839>

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*Ethical Statement:* The authors are accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved. All animals received humane care, and all animal procedures met the relevant legal and ethical requirements according to a protocol (number NMU08-092) approved by the Institutional Animal Care and Use Committee of Nanjing Medical University.

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