



# Protective effect of ischemic postconditioning on ischemia reperfusion injury in steatotic rat livers

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**Background:** Hepatic steatosis creates a significant risk of liver resection and transplantation and is extremely susceptible to ischemia/reperfusion (I/R) injury. Ischemic postconditioning (IPostC) has been shown to attenuate I/R injury in normal livers; however, its role in steatotic livers remains unknown. The current study sought to explore whether IPostC could attenuate normothermic I/R injury in rats with steatotic livers and to investigate potential protective measures.

**Methods:** Hepatic steatosis was triggered in Wistar rats fed high-fat diets. The role of IPostC was detected in normal and steatotic livers with 30 min of ischemia and 6 h of reperfusion. Blood and liver tissues were collected to assess hepatocyte damage, lipid peroxidation, inflammatory factors, neutrophil accumulation, and adenosine triphosphate (ATP) content.

**Results:** Compared to normal livers, steatotic livers were more susceptible to I/R damage, as evidenced by incremental concentrations of liver enzymes in the blood and more severe pathological changes in the liver. Hepatic I/R injury was significantly reduced by IPostC in both normal and steatotic livers. We further found that endogenous protective measures moderated lipid peroxidation, inflammatory cytokine expression and neutrophil accumulation, and reduced follow-up hepatic injury. The ATP content of steatotic livers was also significantly lower than that of Normal livers before and after I/R injury. IPostC greatly preserved the ATP content of normal and steatotic livers with I/R injury.

**Conclusions:** IPostC appears to provide important protection against hepatic I/R injury in normal and steatotic livers under normothermic conditions. These data have important clinical implications for liver surgery and transplantation.

**Keywords:** Steatosis; ischemia reperfusion injury; ischemic postconditioning (IPostC); liver

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## Introduction

Ischemia/reperfusion (I/R) injury is a significant factor in liver damage that occurs during hepatic inflow occlusion reperfusion via portal triad clamping (the Pringle maneuver) after a hepatectomy and also after a liver transplantation (1). Hepatic I/R injury could result in local and remote multi-

organ dysfunction (2), and unfortunately, is inevitable in many situations. Steatotic livers are more susceptible to I/R injury than normal livers (3). Multiple studies have reported that steatosis is a significant risk factor for postoperative liver failure after a hepatectomy, and a causative factor for primary graft incompetence or dysfunction during liver transplantation (4-7). Additionally, hepatic steatosis is the

most common metabolic disorder with an incidence of 20–30% in Western countries (8). Due to the increasing number of orthotopic liver transplantations and the scarcity of fit donor livers, many transplant centers are using “marginal” livers, such as steatotic livers, in liver transplantations (6). These realities emphasize the necessity of steatotic liver protection during liver surgery or transplantation.

It has been hypothesized that steatotic livers have an increased susceptibility to I/R injury. The hypothesis may manifest as lipid peroxidation, neutrophil infiltration, microcirculatory alterations, decreased intracellular energy levels, and the release of proinflammatory mediators [e.g., tumor necrosis factor (TNF)- $\alpha$ ] (1,9,10). However, the specific roles of these different mechanisms have not yet been elucidated.

Previous research has shown that ischemic preconditioning (IPreC) is an endogenous process that protects multiple organs from I/R injury, including the normal liver, through transient vascular occlusion before sustained ischemia (11–14). Additionally, IPreC has been shown to protect steatotic livers from I/R injury in the course of warm ischemia and transplantation (15,16). The underlying mechanisms of IPreC are not yet known. IPreC protects against I/R injury; however, its use as a clinical strategy is greatly limited by the fact that it is sometimes difficult to implement before I/R injury occurs (17). Ischemic postconditioning (IPostC), defined as several brief cycles of ischemia and reperfusion performed immediately in the initial reperfusion period after ischemia, has been proven to increase several organs' ability to tolerate I/R injury (11,18–21). A previous study has indicated that IPostC can suppress the apoptosis of liver cells and reduce reperfusion injury of liver tissue. However, no studies appear to have been conducted on whether IPostC also has a protective effect on steatotic livers. This study sought to investigate the effects of IPostC on the maintenance of liver function in normal and steatotic livers during the acute phase of I/R injury. We present the following article in accordance with the ARRIVE reporting checklist (available at <https://dx.doi.org/10.21037/atm-21-2275>).

## Methods

### *Animal model, anesthesia, and surgical procedure*

Male Wistar rats, weighing 230–280 g, were used in

this study. To induce steatotic livers, the test animals were fed a commercial high-fat choline-deficient diet (TP36002, Trophic Animal Feed High-tech Co. Ltd., China) for 6 weeks. The progression in steatosis was examined macroscopically and confirmed by a histological examination. The control animals were fed a standard laboratory diet (TP 0010M, Trophic Animal Feed High-tech Co. Ltd., China). All animals were kept in a temperature-controlled environment with 12 h light/dark cycles and with water and food ad libitum until use. The animal experiments were performed under a project license granted by institutional Animal Care and Use Committee of the Air Force Medical University, in compliance with institutional guidelines for the care and use of animals.

Ketamine (50 mg/kg) and chlorpromazine (50 mg/kg) were used to anesthetize normal and steatotic animals. Established segmental hepatic I/R models were used (22). After a midline laparotomy, the ligamentous adhesions of the liver to the diaphragm were severed, and the liver was exposed. Using an atraumatic microvascular clip, the hepatic artery and portal vein of the left and median liver lobes (approx. 70% of the liver) were occluded during the ischemic period of the study. Removed the clamp and began reperfusion. This method of partial hepatic ischemia avoided mesenteric venous congestion by allowing outflow of the splanchnic circulation through the right and caudate lobes. SHAM group was the control group, and the rats were anesthetized and laparotomized, and the hepatic hilum vessels were dissected without the induction of ischemia. Rats in the I/R group received 30 min of clamping followed by 2 or 6 hours of reperfusion in left hepatic artery and left portal vein. IPostC + I/R group, received the same treatment as in the I/R group, but followed by 3 cycles of 30-s reperfusion and 30-s ischemia immediately after 30 min of ischemia. The animals were actively warmed with heat lamps to conserve body heat. Their abdomens were lightly packed with wet gauze to prevent fluid evaporation, and normal saline (1 mL) was administered intraperitoneally to compensate for intraoperative fluid loss before the incision was closed. At the end of the trial, the animals were killed by exsanguination. Blood samples were obtained from the inferior vena cava, centrifuged at 4,000 rpm for 3 min at 4 °C, and preserved at –80 °C for use. Liver samples were taken from the ischemic liver lobes and stored in 10% phosphate-buffered formalin for fixation or immediately frozen at –80 °C for future use.

### **Experimental design**

To evaluate the effect of IPostC on steatotic livers subjected to hepatic I/R, the normal and obese (Ob) animals were randomly divided into the following three groups: (I) the control group (n=12) in which 6 normal and 6 Ob animals were anesthetized and laparotomized, and the hepatic hilum vessels were dissected without the induction of ischemia; (II) the I/R group (n=24) in which the animals were divided into one subgroup of 6 normal and 6 Ob animals that were operated on for 30 min of partial ischemia and then 2 h of reperfusion, and a second subgroup of 6 normal and 6 Ob animals that were operated on for 30 min of partial ischemia and then 6 h of perfusion; and (III) the IPostC plus I/R (IPostC + I/R) group (n=24) in which the animals were subdivided into 2 subgroups (n=12) of 6 normal and 6 Ob animals that received the same treatment as in the I/R group, but with 3 cycles of 30-s reperfusion and 30-s ischemia immediately after 30 min of ischemia.

To explore the mechanisms potentially involved in the protective effects of IPostC, serum samples and hepatic tissues (ischemic lobes) were harvested at predetermined times to evaluate lipid peroxidation, neutrophil infiltration, inflammatory factor releasing, and intracellular energy loss. For these purposes, malondialdehyde (MDA) was measured in liver tissues as an indicator of lipid peroxidation and myeloperoxidase (MPO) activity was measured as an indicator of neutrophil accumulation. TNF- $\alpha$  levels were evaluated in plasma samples, and the content of adenosine triphosphate (ATP) in liver tissues was also detected.

### **Biochemical determinations**

#### **Concentrations of liver marker enzymes**

Hepatocellular injury was determined by measuring the serum levels of liver marker enzymes. Blood was taken in the presence of anticoagulants. Plasma concentrations of alanine transaminase (ALT) and aspartate transaminase (AST) were measured spectrophotometrically using an automated biochemical analyzer (Hitachi 7160, Hitachi Incorporated, Tokyo, Japan).

#### **Inflammatory factor assay**

Plasma TNF- $\alpha$  was measured using a specific rat TNF- $\alpha$  (R&D Systems, Minneapolis, MN) immunoassay. All assays used in this study have been verified in rats and were conducted in accordance with the manufacturer's instructions.

### **Lipid peroxidation and antioxidant enzyme activity assay**

Lipid peroxidation was used as an indirect method to measure oxidative damage induced by reactive oxygen species (ROS), and the content of lipid peroxidation in liver tissues was measured by the thiobarbiturate reaction to determine MDA formation (21). The concentrations of MDA, superoxide dismutase (SOD) and glutathione peroxidase (GSH-PX) activities in liver tissues were determined using assay kits (Nanjing Jiancheng Bioengineering Institute, Nanjing, China) in accordance with each manufacturer's protocol, respectively.

#### **MPO assay**

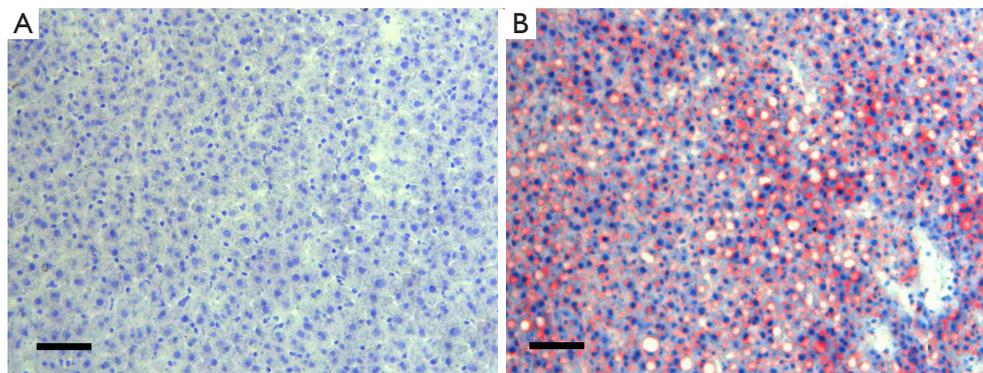
As a marker of polymorphonuclear neutrophil (PMN) infiltration, MPO activity in hepatic tissues was determined using the MPO assay kit (Nanjing Jiancheng Bioengineering Institute) in accordance with the manufacturer's protocol. MPO activity was measured photometrically using 3,3',5,5'-tetramethylbenzidine as a substrate. Frozen liver tissues were macerated, homogenized, sonicated, and centrifuged at 4,000 g for 12 min at 4 °C. MPO activity in the supernatant was measured and calculated based on the change in absorbance at 460 nm. MPO activity was shown as units of MPO activity per gram of wet liver.

#### **Measurement of hepatic tissue ATP**

Immediately after 6 h of reperfusion, the liver samples were frozen in liquid nitrogen for ATP determination. ATP levels in ischemic liver tissues were assayed spectrophotometrically using an assay kit (Nanjing Jiancheng Bioengineering Institute, Nanjing, China) in accordance with the manufacturer's protocol.

### **Histology**

The steatosis of each liver was assessed using red oil staining on frozen specimens following standard procedures. To estimate the extent of hepatic injury, hematoxylin and eosin stained sections were evaluated using an established point-counting method in a blinded fashion (22). The following grades were employed: grade 0: minimal or no proof of injury; grade 1: mild injury including cytoplasmic vacuolization and focal nuclear pyknosis; grade 2: moderate to severe injury with extensive nuclear pyknosis, hypereosinophilic cytoplasm, and loss of intercellular boundaries; and grade 3: severe necrosis, hepatic cord disintegration, hemorrhage, and neutrophil infiltration.



**Figure 1** Differences in steatosis between Ob and normal Wistar rats were determined using red oil staining (n=6). (A) normal rats showed no evidence of steatosis (scale bar, 50  $\mu$ m). (B) Ob rats showed steatotic infiltration of hepatocytes (scale bar, 50  $\mu$ m). Ob, obese.

### Statistical analysis

Data are presented as mean  $\pm$  standard deviation. Differences were analyzed by conducting Student's *t*-tests between two groups, and one-way analyses of variance (ANOVAs) or Fisher exact tests among three groups. A *P* value less than 0.05 was regarded as statistically significant

## Results

### Induction of steatotic livers

All of the animals fed the high-fat diet developed moderate (30% to 60%) steatotic livers. Conversely, none of the rats in the normal groups developed hepatic steatosis. There was no mortality in our model 6 weeks after the induction of steatosis. The animals maintained a normal body weight during the high-fat diet periods, and there was no significant difference between the groups. Liver histological examinations showed moderate macro-vesicular steatosis under light microscopy (see *Figure 1*).

### Hepatocellular injury

AST and ALT, which are established markers of hepatocellular injury, were measured in the normal and steatotic livers of rats after 2–6 h of reperfusion. I/R caused a significant rise in AST and ALT levels in the serum of both the normal and Ob rats. The AST and ALT levels were significantly higher in the Ob rats than the normal rats, indicating that I/R caused more severe liver damage in steatotic livers. IPostC of both steatotic and normal livers was effective at attenuating the damage caused by I/R, as

evidenced by the decrease in serum AST and ALT levels (see *Figure 2*).

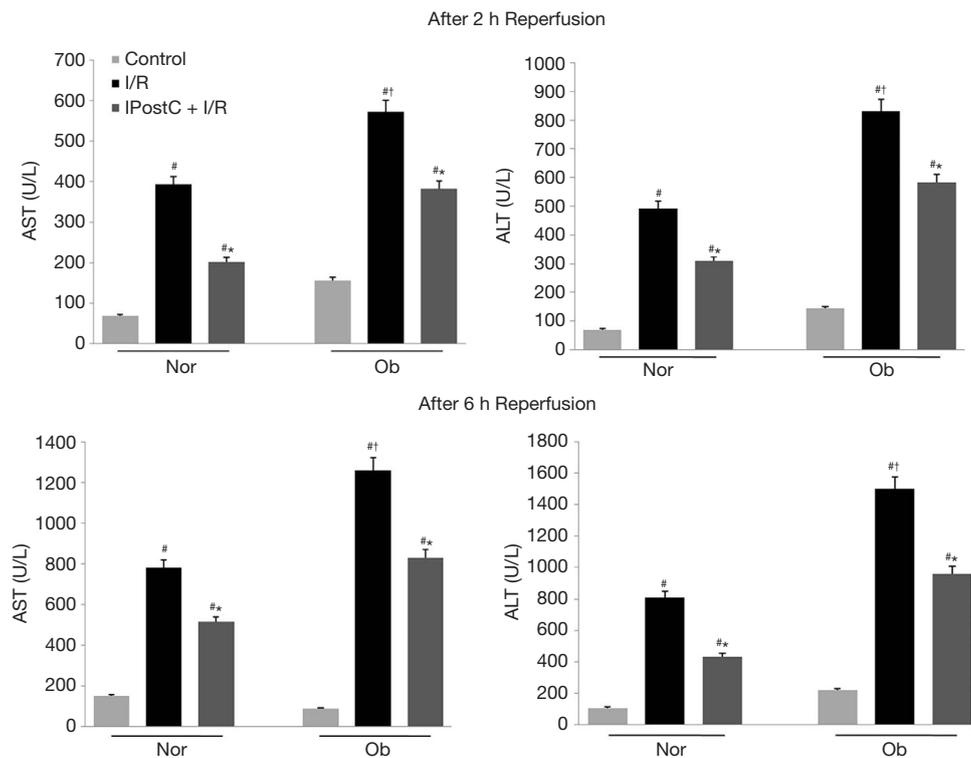
### Inflammatory mediator and neutrophil infiltration

In relation to the beneficial effects of IPostC on hepatic I/R injury, the effects of TNF- $\alpha$  were also examined after 2 h of reperfusion. As *Figure 3* shows, the increased TNF- $\alpha$  levels in I/R groups of both normal and Ob animals were reduced when IPostC was applied. However, no significant difference in TNF- $\alpha$  levels was observed between normal and Ob animals before or after reperfusion.

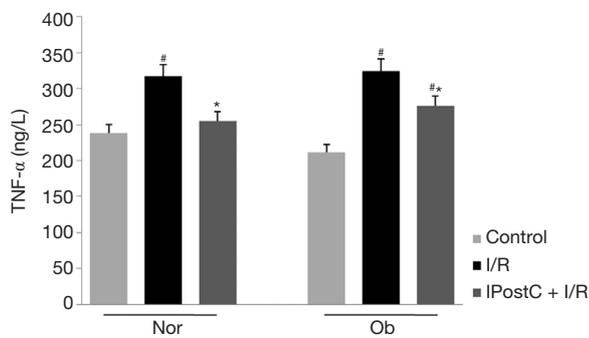
Neutrophil accumulation after liver reperfusion was determined by measuring MPO levels 6 h after liver reperfusion (see *Figure 4*). An increase in neutrophil accumulation was seen in both normal and steatotic livers. When IPostC was exerted after ischemia, hepatic MPO values were significantly lowered.

### Lipid peroxidation and antioxidant enzyme activity

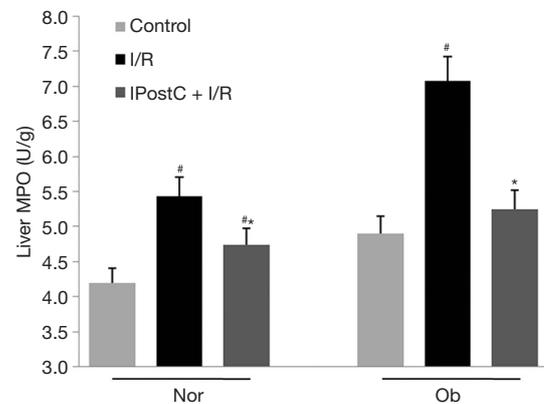
The effects of IPostC on lipid peroxidation and the antioxidant mechanism were evaluated after 6 h of reperfusion. As *Figure 5* shows, high MDA levels were observed in I/R groups of normal and Ob animals. Further, the MDA levels in steatotic livers subjected to I/R were higher than those in normal livers. Conversely, MDA levels were reduced when IPostC was applied. As *Figure 6* shows, the levels of SOD and GSH-PX activities in the I/R group were lower than those in the control group. However, when IPostC was applied, the levels of SOD and GSH-PX activities increased, and the SOD levels in the steatotic



**Figure 2** Effect of IPostC on plasma levels of AST and ALT in normal and Ob Wistar rats re-perfused for 2 and 6 h after 30 min of ischemia. (<sup>#</sup>,  $P < 0.05$  vs. control; <sup>\*</sup>,  $P < 0.05$  vs. I/R; <sup>†</sup>,  $P < 0.05$  vs. I/R of normal). (n=6). IPostC, ischemic postconditioning; AST, aspartate transaminase; ALT, alanine transaminase; Ob, obese; I/R, ischemia/reperfusion.



**Figure 3** Effects of IPostC on plasma levels of TNF- $\alpha$  in normal and Ob Wistar rats preceded by 30 min of ischemia and then 2 h of perfusion (<sup>#</sup>,  $P < 0.05$  vs. control; <sup>\*</sup>,  $P < 0.05$  vs. I/R). (n=6). IPostC, ischemic postconditioning; TNF- $\alpha$ , tumor necrosis factor- $\alpha$ ; Ob, obese; I/R, ischemia/reperfusion.



**Figure 4** MPO in liver tissues of normal and Ob Wistar rats subjected to 30 min of ischemia and then 6 h of reperfusion (<sup>#</sup>,  $P < 0.05$  vs. control; <sup>\*</sup>,  $P < 0.05$  vs. I/R). (n=6). MPO, myeloperoxidase; Ob, obese; I/R, ischemia/reperfusion.

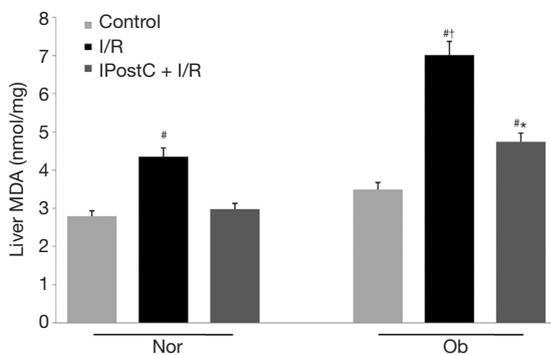
animals were similar to those in the control animals.

### Hepatic tissue ATP

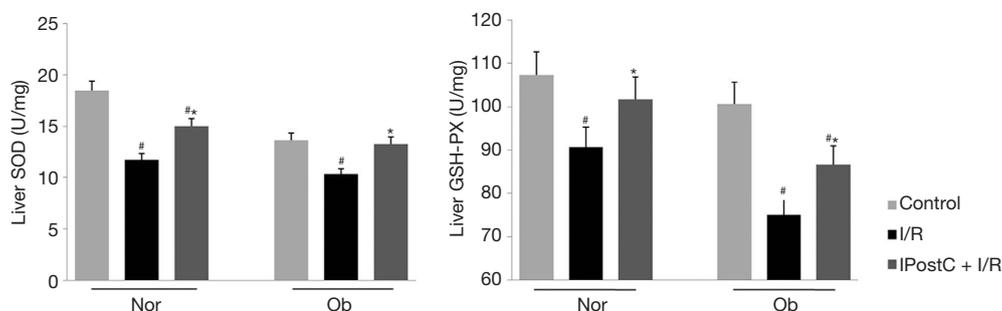
The effects of IPostC on tissue energy preservation were also investigated. ATP levels were measured in liver tissues after 6 h of reperfusion. As *Figure 7* shows, compared with the control groups, I/R significantly decreased ATP levels in both normal and Ob animals, and ATP levels were significantly lower in steatotic livers than in normal livers before and after I/R injury. However, IPostC significantly preserved ATP levels, resulting in increased ATP levels in IPostC + I/R Group comparison to I/R group.

### Histopathology

Parenchymal morphological changes were determined in



**Figure 5** MDA in the livers of normal and Ob Wistar rats after 30 min of ischemia and then 6 h of reperfusion (<sup>#</sup>,  $P < 0.05$  vs. control; <sup>\*</sup>,  $P < 0.05$  vs. I/R; <sup>†</sup>,  $P < 0.05$  vs. I/R of normal). (n=6). MDA, malondialdehyde; Ob, obese; I/R, ischemia/reperfusion.

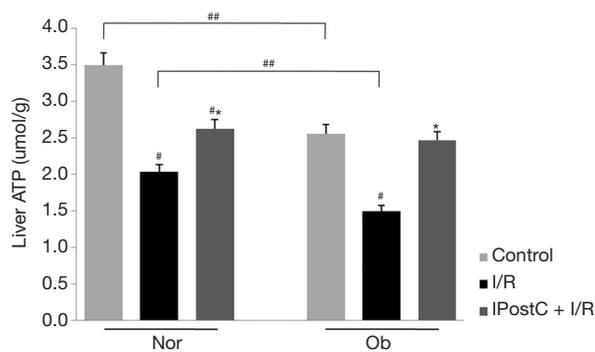


**Figure 6** SOD and GSH-PX levels in the livers of normal and Ob Wistar rats subjected to 30 min of ischemia and then 6 h of reperfusion (<sup>#</sup>,  $P < 0.05$  vs. control; <sup>\*</sup>,  $P < 0.05$  vs. I/R). (n=6). SOD, superoxide dismutase; GSH-PX, glutathione peroxidase; Ob, obese; I/R, ischemia/reperfusion.

liver sections stained with hematoxylin and eosin 6 h after reperfusion (see *Figure 8*). The results of the histological studies in the control group revealed no apparent lesions in either the normal or Ob animals (see *Figure 8A,8B*). However, at 6 h of reperfusion, the histological studies of normal livers undergoing I/R showed swelling and slight coagulative necrosis of hepatocytes with neutrophil infiltration, which were distributed over the entire hepatic parenchyma (see *Figure 8C*). Histological studies of steatotic livers showed severe multifocal areas of coagulative necrosis with neutrophil infiltration, randomly distributed in the liver parenchyma (see *Figure 8D*). In accordance with the biochemical test results, the range of necrosis and the number of necrotic areas were reduced in both normal and steatotic livers after performing IPostC (see *Figure 8E,8F*).

### Discussion

Steatotic livers appear to have increased vulnerability to ischemic injury, and are considered a major risk factor for liver resection and transplantation. In previous studies, the biochemical and histological results indicated that compared to steatotic livers, normal livers tolerated more severe hepatic damage induced by hepatic ischemia (15,22-24). In addition, steatotic animals have shown decreased survival rates after hepatic I/R injury compared to normal animals (15). Due to the high prevalence of steatotic livers, it is essential to develop effective strategies to protect steatotic livers from hepatic I/R injury. The results of this study showed that IPostC, 3 cycles with 30 s of reperfusion and 30 s of ischemia conducted immediately after ischemia, provided protection against hepatic I/R injury upon 30 min of partial ischemia in normal and steatotic livers.



**Figure 7** Hepatic tissue ATP levels from normal and Ob Wistar rats subjected to 30 min of ischemia and then 6 h of reperfusion (#,  $P < 0.05$  vs. control; \*,  $P < 0.05$  vs. I/R; ##,  $P < 0.05$  vs. normal). (n=6). ATP, adenosine triphosphate; Ob, obese; I/R, ischemia/reperfusion.

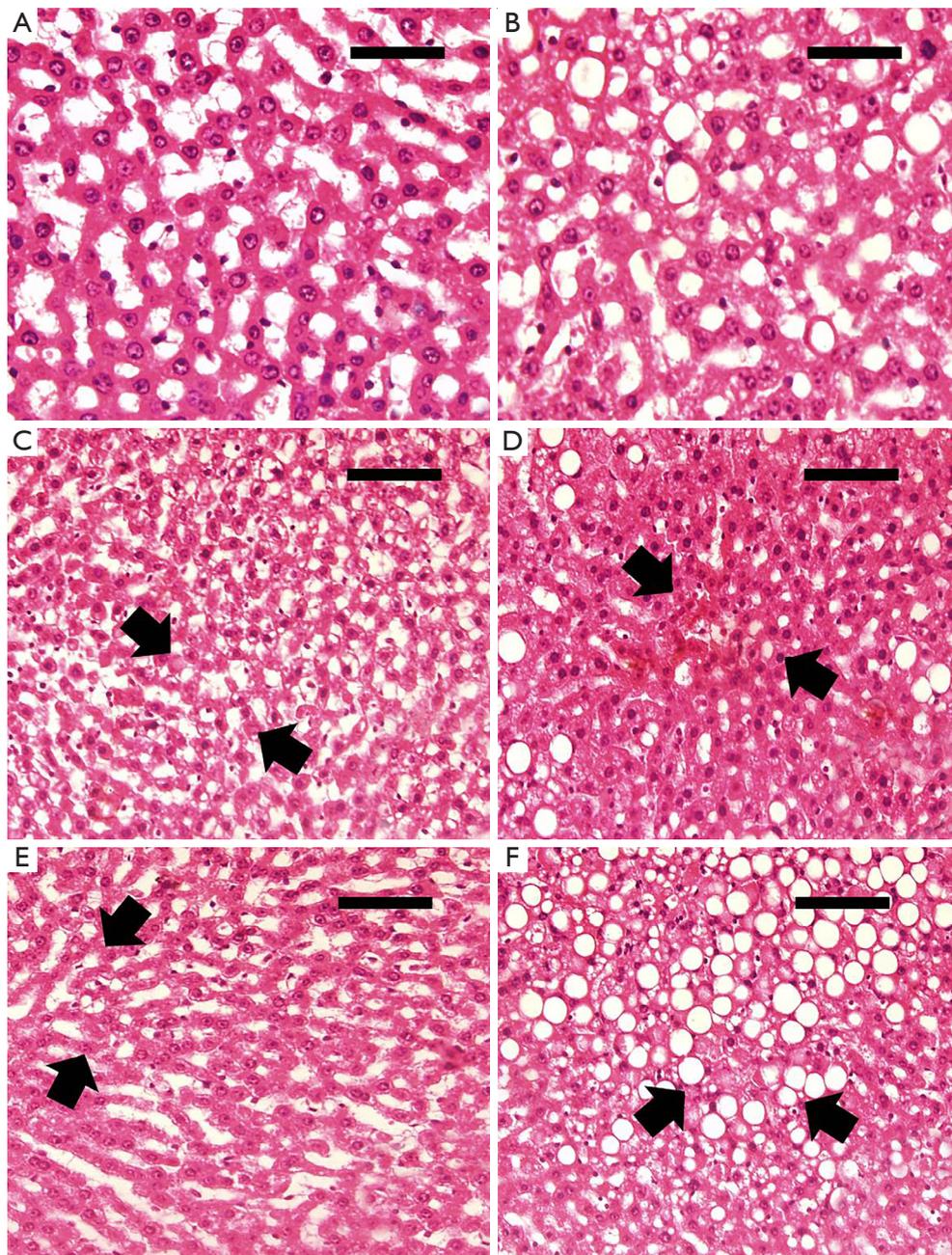
ROS production has been used as a key mechanism for I/R injury in many organs, including the liver (25). After warm ischemia, ROS were produced at the point of reperfusion, promoting leukocyte adhesion to the microvascular endothelium (22). Another important effect of uncontrolled ROS production is the peroxidation of cell membranes and other cellular lipids and proteins that can cause severe cellular injury (26). MDA is a product of free radical lipid peroxidation, and MDA levels may be used to measure the extent of lipid peroxidation in tissues (21). This study showed that lipid peroxidation increased in both normal and steatotic livers under 30 min of normothermic ischemia, and the increase was more prominent in steatotic livers. Thus, steatotic livers were found to be more susceptible to lipid peroxidation, which might be due to excessive fat or the generation of more ROS.

Our study also showed that IPostC inhibits the rise in MDA levels to a considerable extent; thus, IPostC has an antioxidant role in I/R. Given that the animals were randomly assigned to the groups, the livers of each Ob animal group should have shown a similar degree of hepatic steatosis. Thus, the effect of IPostC on lipid peroxidation can be interpreted in terms of reduced ROS production. SOD and GSH have multiple roles in cells, including in the maintenance of the oxidative antioxidant balance and the formation of conjugates with free radicals (27), and their activities indicate the body's ability to scavenge free radicals. Our results showed that the activity of antioxidant enzymes decreased in both normal and steatotic livers after I/R injury, while the activity was significantly higher in the

IPostC + I/R group. Thus, the IPostC-induced increase in antioxidant enzyme activity appears to contribute to the reduction of superoxide radicals after I/R injury in the liver.

TNF- $\alpha$  was shown to increase neutrophil aggregation, activate neutrophils to release inflammatory mediators, and lead to the overexpression of adhesion molecules on endothelial cells and leukocytes (28). Additionally, anti-TNF- $\alpha$  therapy could provide protection against hepatic I/R injury subjected to hepatic normothermic ischemia in steatotic livers (29). In the present study, increases of TNF- $\alpha$  were inhibited by IPostC in both normal and steatotic livers; however, no significant difference in the TNF- $\alpha$  levels was observed between the normal and Ob animals. Thus, in the present experimental model (30 min of 70% liver tissue ischemia), TNF- $\alpha$  did not appear to make steatotic livers more susceptible to hepatic I/R injury than normal livers. PMNs accumulated in the liver sinusoids may be essential effector cells in the pathogenesis of hepatic I/R injury, and they are the main source of ROS, which can cause cellular damage (10). The results of the present study showed I/R injury led to increases in MPO activity in both normal and steatotic livers; however, the increase was more prominent in steatotic livers. Additionally, our results suggest that IPostC could suppress an increase of MPO activity. Thus, the inhibition of TNF- $\alpha$  production may inhibit subsequent neutrophil activation. However, in IPreC studies, hepatic I/R injury was not found to be correlated with TNF- $\alpha$  production, and the advantage of IPreC could not be explained by a decrease in the release of TNF- $\alpha$  (15). In addition, microcirculatory disturbance appears to be one of the mechanisms by which steatotic livers are susceptible to I/R injury. Steatotic livers have reduced sinusoidal space due to fat infiltration, and inflammatory mediators, such as TNF- $\alpha$  and leukocytes, further reduce the sinusoidal space and impair microcirculation, resulting in reduced blood flow and prolonged ischemia (30).

The mechanisms of I/R injury in steatotic livers have not yet been thoroughly explored; a significant decrease in energy status may play a central role. ATP preservation during ischemia and ATP recovery during reperfusion may partially define hepatocyte viability and liver function (31). In addition, the existence of non-esterified fatty acids in steatotic livers is connected with a reduced capacity for ATP production (31). In the current study, the outcomes showed that ATP levels were lower in steatotic livers before or after reperfusion compared to normal livers. Thus, impaired hepatic energy metabolism already appears to be present in steatotic livers, but I/R injury is still not maintained. Our



**Figure 8** Histological lesions in normal and Ob Wistar rats after 6 h of reperfusion (stained with hematoxylin and eosin). (A) Control (normal): absence of necrosis (scale bar, 25  $\mu$ m). (B) Control (Ob): showed fatty infiltration in hepatocytes (scale bar, 25  $\mu$ m). (C) I/R (normal): hepatocyte swelling and slight coagulative necrosis (arrows) with neutrophil infiltration (Scale bar, 50  $\mu$ m). (D) I/R (Ob): severe and multifocal areas of coagulative necrosis (arrows) with neutrophilic infiltration (scale bar, 50  $\mu$ m). (E) IPostC + I/R (normal): irregular area of incipient necrosis (arrows) (scale bar, 50  $\mu$ m). (F) IPostC + I/R (Ob): small area of coagulative hepatic necrosis (arrows) (Scale bar, 50  $\mu$ m). Ob, obese; I/R, ischemia/reperfusion; IPostC, ischemic postconditioning.

results demonstrated that IPostC significantly improved cellular bioenergetics in both normal and steatotic livers. Further, as ATP is essential for apoptosis, an increased susceptibility to I/R injury in the steatotic liver was related to the change from apoptosis, which is predominant in normal livers, to necrosis due to the depletion of intracellular ATP (32). In our study, necrosis was not a remarkable finding, as our protocol included a short ischemia period, and partial pedicle clamping to avoid intestinal blood flow stasis.

The underlying mechanisms by which IPostC could provide protective effects on the hepatic I/R injury in steatotic livers are largely unclear. We speculate that similar protective mechanisms to the hepatic I/R injury in normal livers could be also involved in steatotic livers, mainly including reduced oxygen free radicals, decreased neutrophil accumulation, increased antioxidant activity and enhanced mitochondrial function, during controlled reperfusion. In addition, several studies have also suggested that IPostC may increase nitric oxide (NO) concentrations and produce a cytoprotective environment, thereby reducing cell death and restoring hepatic function after reperfusion (28). Moreover, it has been shown that IPreC and IPostC had pronounced effects on gene expression during early reperfusion (33,34). IPreC and IPostC appear to mediate their protective roles by moderating the same genes and gene networks that are believed to be involved in sustaining cellular homeostasis (33).

In conclusion, IPostC could provide protection against normothermic I/R injury in normal and steatotic livers. ATP preservation and restoration may be one of the mechanisms of protection. These observations could have important clinical implications for the protection of normal and steatotic livers from hepatic I/R injury. Future studies should be conducted to examine the underlying biological mechanisms that may be involved in IPostC.

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### Footnote

*Reporting Checklist:* The authors have completed the

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*Data Sharing Statement:* Available at <https://dx.doi.org/10.21037/atm-21-2275>

*Conflicts of Interest:* All authors have completed the ICMJE uniform disclosure form (available at <https://dx.doi.org/10.21037/atm-21-2275>). The authors have no conflicts of interest to declare.

*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. The animal experiments were performed under a project license granted by institutional Animal Care and Use Committee of the Air Force Medical University, in compliance with institutional guidelines for the care and use of animals.

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