International Immunopharmacology 74 (2019) 105711

Contents lists available at ScienceDirect

International Immunopharmacology

journal homepage: www.elsevier.com/locate/intimp

Combined ischemic and rapamycin preconditioning alleviated liver ischemia and reperfusion injury by restoring autophagy in aged mice

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ARTICLE INFO

Keywords:
Liver ischemia and reperfusion injury
Ischemic preconditioning
Rapamycin preconditioning
Autophagy
Aging

ABSTRACT

Old livers are more damaged by hepatic ischemia and reperfusion (IR) injury than young livers. The aim of this study was to investigate the effects of ischemic and rapamycin preconditioning on IR injury in old livers. Young (8-week-old) and aged (60-week-old) mice were subjected to IR or a sham control procedure. The aged mice were randomly divided into six groups: IR (CON), IR with ischemic preconditioning (IPC), IR with rapamycin preconditioning (RAPA), IR with combined ischemic and rapamycin preconditioning (IPC + RAPA), IR with 3-methyladenine (3-MA), IR with combined ischemic and rapamycin preconditioning with 3-MA pretreatment (IPC + RAPA + 3-MA). Liver injury was evaluated 6 h after reperfusion. Hepatocellular autophagy induction was also analyzed by western blotting. The results revealed that aged mice had aggravated liver IR injury as compared to young mice. In aged mice following IR, IPC + RAPA but not IPC or RAPA alleviated liver injury, as evidenced by lower levels of serum ALT, improved preservation of liver architecture with lower Suzuki scores, and decreased caspase-3 activity compared with CON. In addition, western blot analysis revealed increased LC3B II but decreased p62 protein expression levels in the IPC + RAPA group, indicating that autophagic flux was restored by combined ischemic and rapamycin preconditioning. Furthermore, autophagy inhibition by the inhibitor 3-MA abrogated the protective role in the IPC + RAPA group, while no significant effects were observed in the CON group. In conclusions, our results demonstrated that combined ischemic and rapamycin preconditioning protected old livers against IR injury, which was likely attributed to restored autophagy activation.

1. Introduction

Hepatic ischemia and reperfusion (IR) injury is an important cause of liver injury in patients undergoing partial liver resection and liver transplantation [1]. With the increase of the elderly population, the number of elderly patients with liver cancer has increased. Thus, more and more elderly patients would undergo partial hepatectomy. In addition, due to the critical shortage of organs, transplantation with livers from old donors is continuously increasing. However, increased liver IR injury in aging livers has been reported, which may further impair liver function and patient recovery after surgery. Therefore, investigating strategies to prevent or ameliorate liver IR injury is critical.

Ischemic and pharmacological preconditioning has been shown to effectively protect organs against IR injury in many studies [2]. However, controversial results have been reported regarding the role of ischemic and pharmacological preconditioning in aged animals or humans. Limani P et al. found that remote ischemic preconditioning effectively protected old livers from IR injury [3]. In another study, ischemic preconditioning was shown to be highly protective against liver IR injury in young but not in aged mice [4].

Autophagy is a critical process that is involved in aging and liver IR injury [5]. During liver IR injury, autophagy allows cells to cope with nutrient starvation and anoxia and promotes hepatocellular cell survival and liver function [6]. Interestingly, both ischemic preconditioning and rapamycin preconditioning effectively have been shown to protect livers from IR injury by autophagy induction in young mice [7,8]. Nevertheless, whether ischemic preconditioning and rapamycin preconditioning protect the liver against IR injury in aged mice by modulating autophagy induction remains unclear.

In the present study, we investigated the synergistic effects of

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https://doi.org/10.1016/j.intimp.2019.105711
Received 8 January 2019; Received in revised form 21 April 2019; Accepted 18 June 2019
Available online 11 July 2019

ischemic and rapamycin preconditioning on regulating autophagy and liver IR injury in aged mice.

2. Materials and methods

2.1. Mice

Male C57BL/6 mice aged 8 weeks (young group) and 60 weeks (old group) were purchased from the Laboratory of Animal Resources of Nanjing Medical University. The mice were maintained under specific pathogen-free conditions with free access to tap water and food. The mice received humane care in compliance with a protocol (protocol number NMU08-092) approved by the Institutional Animal Care and Use Committee of Nanjing Medical University.

2.2. Model of warm liver IR

A model of segmental (70%) hepatic warm IR was used, as previously described [9]. Mice were anesthetized with inhaled isoflurane (1.5%). The arterial and portal venous blood supply to the cephalad lobes of the liver was blocked using an atraumatic clip. After 90 min of ischemia, the clip was removed to initiate liver reperfusion. The mice were anesthetized again and sacrificed 6 h post-reperfusion. Sham controls underwent the same procedure but without vascular occlusion.

For the ischemic preconditioning group (IPC), aged mice were subjected to 10 min of ischemia followed by 10 min of reperfusion prior to the standard IR procedure (90 min of ischemia and 6 h of reperfusion). For the rapamycin preconditioning group (RAPA), rapamycin (1 mg/kg, Tocris Bioscience, Cambridge, United Kingdom) was administered intraperitoneally 1 h prior to the onset of liver ischemia. The IPC + RAPA group received both rapamycin and ischemic preconditioning followed by IR. In the 3-MA and IPC + RAPA + 3-MA groups, 3-methyladenine (3-MA, 30 mg/kg, Sigma-Aldrich) was administered intraperitoneally 1 h before the onset of liver ischemia. The same volume of phosphate-buffered saline (PBS) was used for vehicle control. The experimental group and procedure was summarized in Table 1.

2.3. Serum biochemical measurements and liver histopathology

Mice were sacrificed after 6 h of reperfusion. Blood and liver samples were collected. Serum levels of alanine aminotransferase (sALT) were measured using an AU5400 automated chemical analyzer (Olympus, Tokyo, Japan). Portions of the liver specimens were fixed in buffered formalin (10%) and embedded in paraffin. Hematoxylin and eosin staining was performed on 4 μm archived sections. The severity of liver IR injury was graded in a blinded manner using Suzuki's criteria on a scale from 0 to 4.

2.4. Western blotting

Proteins were extracted and subjected to 12% SDS-PAGE electrophoresis and transferred to a PVDF nitrocellulose membrane. Primary antibodies against LC3B, p62 and β-actin (Cell Signaling Technology, MA, USA) were used and incubated overnight at 4 °C. After 2 h of incubation with the appropriate HRP-conjugated secondary antibody, a Clarity™ Western ECL Substrate (Bio-Rad, CA, USA) was used for chemoluminescence development. ImageJ v1.47 software was used to quantify the western blot bands.

2.5. Caspase-3 activity assay

Caspase-3 activity was determined using a commercially available caspase-3 colorimetric activity assay kit (Calbiochem, La Jolla, CA, USA) following the manufacturer's instructions.

2.6. Statistical analysis

The results are shown as the mean ± SEM. Multiple group comparisons were performed using one-way analysis of variance (ANOVA) followed by Bonferroni's post hoc test. All analyses were performed using Stata software (version 11.0). *P*-values < 0.05 (two-tailed) were considered statistically significant.

3. Results

3.1. Aging aggravated liver IR injury

We first confirmed whether liver IR injury was aggravated in aged mice. The young and aged mice were subjected to 90 min of liver ischemia followed by 6 h of reperfusion or the sham procedure. Liver injury was compared between the groups. Indeed, the old group showed significantly higher levels of serum ALT (Fig. 1A) and less preserved liver architectures (Fig. 1B) with higher Suzuki scores (Fig. 1C) compared with the young control group. The old group also demonstrated increased hepatocellular cell death, as evidenced by increased caspase-3 activity (Fig. 1D).

3.2. Combined but not separate treatment of ischemic and rapamycin preconditioning protected livers against IR injury in aged mice

Next, we investigated whether ischemic and rapamycin preconditioning could protect livers against IR injury in aged mice. Unfortunately, no significant effects of ischemic and rapamycin preconditioning were observed in livers with IR injury in aged mice, as shown by similar serum ALT levels, liver pathology, Suzuki scores and caspase-3 activity (Fig. 2A–D, CON vs. IPC; CON vs. RAPA).

Furthermore, we evaluated whether synergistic effects could be achieved by the combined application of ischemic and rapamycin preconditioning in aged IR-stressed mice. Interestingly, the combined

Table 1
Experimental group and procedure.

Group	Procedure
Sham	Laparotomy without vascular occlusion
CON	Standard IR procedure: 90 min of ischemia and 6 h of reperfusion with PBS vehicle control pretreatment
IPC	Ischemic preconditioning: 10 min of ischemia followed by 10 min of reperfusion prior to the standard IR procedure
RAPA	Rapamycin preconditioning: rapamycin (1 mg/kg) administered intraperitoneally 1 h prior to the standard IR procedure
IPC + RAPA	Combined ischemic and rapamycin preconditioning: Rapamycin preconditioning followed by ischemic preconditioning as described above prior to the standard IR procedure
3-MA	3-MA pretreatment: 3-methyladenine (30 mg/kg) administered intraperitoneally 1 h prior to the standard IR procedure
IPC + RAPA + 3-MA	Combined ischemic and rapamycin preconditioning and pretreatment with 3-MA: 3-MA pretreatment, rapamycin preconditioning and ischemic preconditioning as described above prior to the standard IR procedure

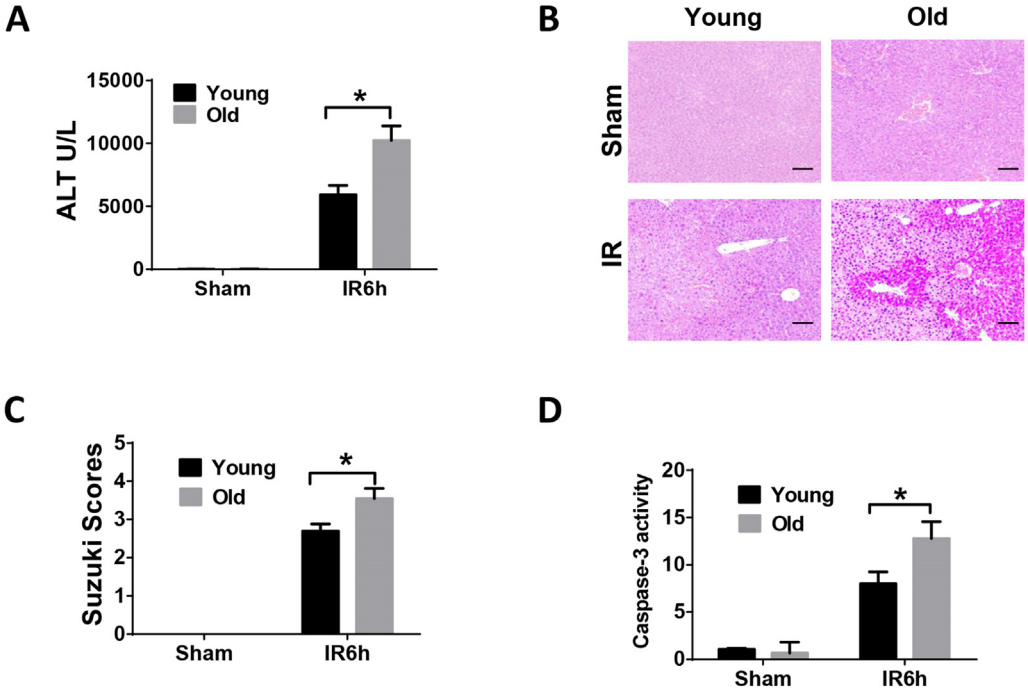

Fig. 1. Aggravated liver IR injury in aged mice. Young and aged mice were subjected to a sham or IR treatment, as described in the Materials and Methods section. After 6 h of reperfusion, blood and liver samples were collected for further analysis. (A) Serum ALT levels. (B) Liver histopathology (H&E staining, 100× magnification, scale bars indicate 100 μm). (C) The severity of liver IRI that was analyzed using Suzuki's histological grading. (D) Caspase-3 activity was determined using a caspase-3 activity assay in liver samples from different groups. $n = 6$ mice/group. Data are expressed as the means ± SEM; $^*p < 0.05$.

application of ischemic and rapamycin preconditioning effectively protected livers against IR injury in aged mice, as evidenced by significantly lower levels of serum ALT, improved preservation of liver architectures, lower Suzuki scores and decreased caspase-3 activity (Fig. 2A-D, CON vs. IPC + RAPA).

3.3. Combined ischemic and rapamycin preconditioning restored autophagy activation in old IR-stressed livers

Both ischemic and rapamycin preconditioning have been reported to protect livers against IR injury in young mice in an autophagy-dependent manner [7,8]. Thus, we evaluated the roles of ischemic and rapamycin preconditioning in modulating hepatocellular autophagy activation in old livers following IR. IR triggered autophagy inhibition

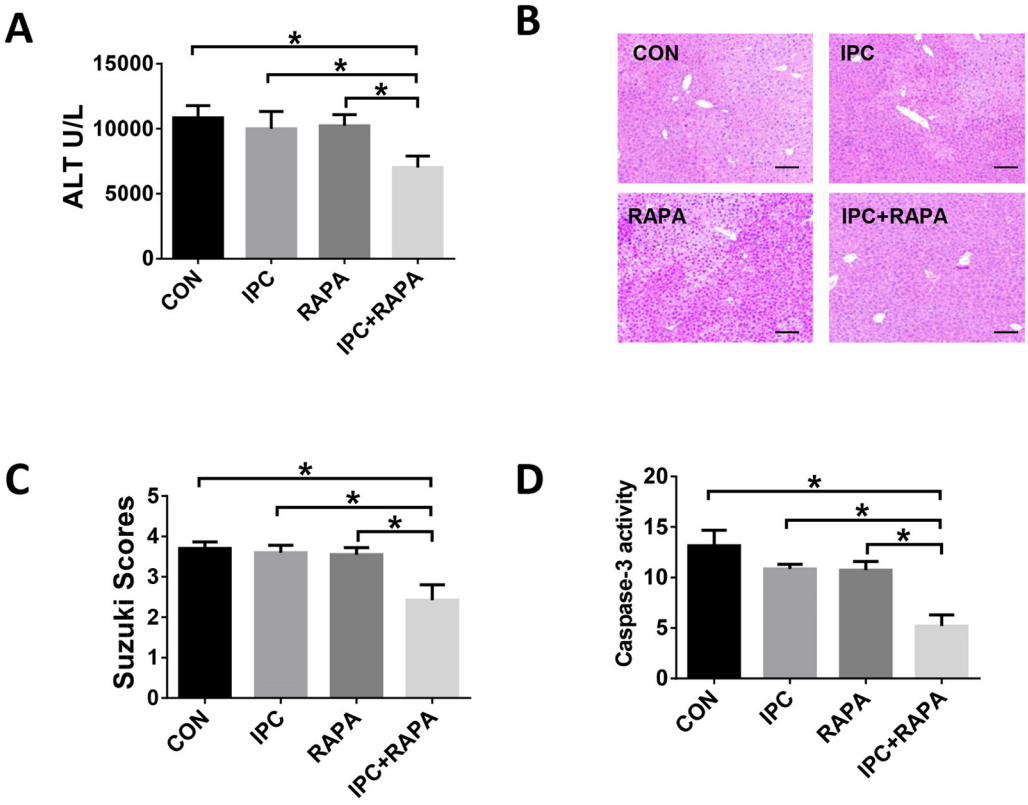

Fig. 2. Combined but not separate treatment by ischemic and rapamycin preconditioning protected livers against IR injury in aged mice. Aged mice were subjected to IR (CON), ischemic preconditioning (IPC), rapamycin preconditioning (RAPA), or combined ischemic and rapamycin preconditioning (IPC + RAPA) followed by IR, as described in the Materials and Methods section. After 6 h of reperfusion, blood and liver samples were collected for further analysis. (A) Serum ALT levels. (B) Liver histopathology (H&E staining, 100× magnification, scale bars indicate 100 μm). (C) The severity of liver IRI was analyzed using Suzuki's histological grading. (D) Caspase-3 activity was determined using a caspase-3 activity assay in liver samples from different groups. $n = 6$ mice/group. Data are expressed as the means ± SEM; $^*p < 0.05$.

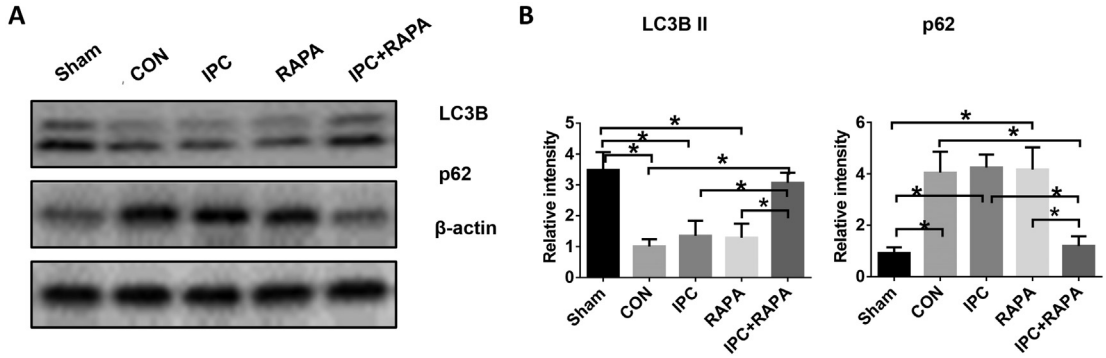

Fig. 3. Combined ischemic and rapamycin preconditioning restored autophagy activation in IR-stressed old livers. Aged mice were subjected to Sham procedure (Sham), IR (CON), ischemic preconditioning (IPC), rapamycin preconditioning (RAPA), or combined ischemic and rapamycin preconditioning (IPC + RAPA) followed by IR. After 6 h of reperfusion, liver tissues were collected for western blot analysis. (A) Protein expression levels of LC3B I/II, p62, and β-actin were analyzed by western blotting. (B) Both the ratios of LC3B II/β-actin and p62/β-actin were calculated in different experimental groups and then compared to the Sham group. $n = 6$ mice/group. Data are expressed as the means ± SEM; $^*p < 0.05$.

in old livers post IR, as evidenced by decreased LC3B II but increased p62 protein expression levels (Fig. 3A&B CON vs. Sham). Neither ischemic nor rapamycin preconditioning alone promoted autophagy activation in old livers following IR, as shown by similar protein expression levels of LC3B II and p62 (Fig. 3A&B, IPC vs. CON; RAPA vs. CON). In contrast, the combined application of ischemic and rapamycin preconditioning restored autophagic flux, as evidenced by increased LC3B II but decreased p62 protein expression levels (Fig. 3A&B IPC + RAPA vs. CON). These results indicated the synergistic effects of ischemic and rapamycin preconditioning on modulating hepatocellular autophagy activation in old livers following IR.

3.4. Combined ischemic and rapamycin preconditioning protected old livers against IR injury by autophagy induction

Finally, we determined whether the protective role of combined ischemic and rapamycin preconditioning in old livers was dependent on autophagy induction. 3-MA, an autophagy inhibitor, was used to inhibit autophagy in the CON and IPC + PARA groups. Indeed, autophagy inhibition by 3-MA abrogated the protective role in the IPC + RAPA group, as evidenced by significantly higher levels of serum ALT, less preserved liver architectures with higher Suzuki scores and increased caspase-3 activity (Fig. 4A–D, IPC + RAPA + 3-MA vs. IPC + RAPA). In contrast, 3-MA pretreatment showed no significant effects on liver IR injury in the CON group (Fig. 4A–D, 3-MA vs. CON).

4. Discussion

To the best of our knowledge, this is the first study to use combined ischemic and rapamycin preconditioning as a treatment for IR injury in aged mice. Combined ischemic and rapamycin preconditioning effectively alleviated liver IR injury by restoring hepatocellular autophagy in old livers, while ischemic or rapamycin preconditioning alone did not demonstrate these effects.

Due to the lack of adequate organs, the number of patients waiting for liver transplantation is constantly increasing. Transplantation of aged liver grafts is an important strategy for increasing the amount of donor organs. However, recipients of organs from old donors showed increased posttransplant morbidity and mortality due to enhanced IR injury. Johnson SR et al. reported in a clinical study of 10,545 liver transplants that donor age was a significant risk factor for primary nonfunction [10]. In 2005, Tomohisa Okaya et al. conducted the first systematic investigation of the age-dependent responses to hepatic IR injury in mice [11]. Significantly increased liver injury and less accumulated but more highly activated neutrophils were found in mature adult mice than in young adult mice. A similar result was observed in older versus young rat livers following IR [12].

However, the mechanisms underlying age-mediated hypersensitivity to IR injury remain poorly understood. Energy metabolism, inflammatory response, and autophagy are potential critical factors that affect liver IR injury in aged mice [5]. Aging enhances the activation of the permeability transition pore in mitochondria [13]. Mitochondria in aged livers produce much less adenosine triphosphate [14]. Mitochondrial dysfunction, decreased intrahepatic energy content and subsequent poor tolerance against ischemic injury have been observed in old livers [4]. Selzner M et al. found that increased TNF-α release was responsible for aggravated liver IR injury in aged mice [15]. Impaired autophagic flux due to the loss of ATG4B has been shown to aggravate liver IR injury in aged livers [16]. Consistent with this finding, our present study showed that autophagic flux was also inhibited. In addition, a recent study has demonstrated that the loss of sirtuin 1 and mitofusin 2 contribute to enhanced IR injury in old livers [17].

Protective roles of autophagy in liver IR injury in young mice have been widely reported [18]. In young mice, strategies used to activate autophagy have been shown to effectively protect livers against IR injury. Everolimus and rapamycin have both been shown to protect the liver against hepatic IRI by activating autophagy [8,19]. In a previous study, we showed that autophagy was impaired by prolonged ischemia, and restoration of autophagic flux by endoplasmic reticulum stress suppression protected livers against IR injury [20]. In another study, we also found that isoflurane preconditioning alleviated liver IR injury by restoring AMPK/mTOR-mediated autophagy [21]. However, rapamycin preconditioning showed no noticeable effects in aged mice in the present study.

Ischemic preconditioning consists of short periods of ischemia followed by reperfusion that provides the organ with improved resistance to prolonged periods of ischemia. However, in old livers, the protective role of ischemic preconditioning has not been satisfactory in clinical and experimental studies. Clavien PA et al. conducted a prospective randomized study and found that ischemic preconditioning protected against hepatic ischemic injury in humans. However, the protective effects were maximal in younger patients and decreased with increasing age of the patients [22]. Accordingly, another recent study also reported that aging attenuated the protective effect of ischemic preconditioning against endothelial IR injury in humans [23]. Selzner M et al. found that ischemic preconditioning was highly protective against liver IR injury in young but not in aged mice [4]. In another study, remote ischemic preconditioning, but not standard ischemic preconditioning, protected old livers from IR injury [3]. In our study, no significant protective effects of ischemic preconditioning were observed in the aged mice.

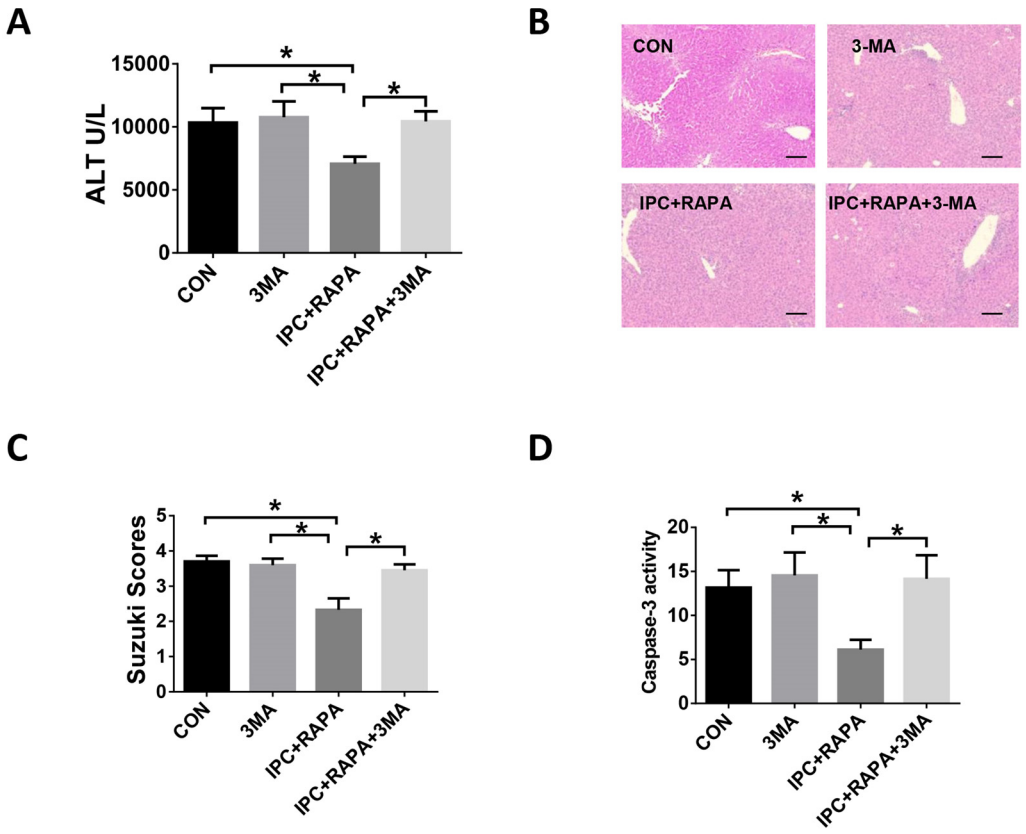

Fig. 4. Combined ischemic and rapamycin preconditioning protected old livers against IR injury by autophagy induction. Aged mice were subjected to IR (CON), IR with 3-MA pretreatment (3-MA), combined ischemic and rapamycin preconditioning (IPC + RAPA), or combined ischemic and rapamycin preconditioning with 3-MA pretreatment (IPC + RAPA + 3-MA) followed by IR. After 6 h of reperfusion, blood and liver samples were collected for further analysis. (A) Serum ALT levels. (B) Liver histopathology (H&E staining, 100× magnification, scale bars indicate 100 μm). (C) The severity of liver IRI was analyzed using Suzuki's histological grading. (D) Caspase-3 activity was determined using a caspase-3 activity assay in liver samples from different groups. $n = 6$ mice/group. Data are expressed as the means ± SEM; $^{*}p < 0.05$.

We speculated why ischemic or rapamycin preconditioning alone failed to protect against IR injury in old livers. The autophagy activation analysis showed that neither ischemic preconditioning nor rapamycin preconditioning restored autophagic flux in old livers following IR. The synergistic protective effects caused by combining ischemic preconditioning with pharmacological preconditioning have been proposed in other studies [4,24]. Interestingly, in our study, combined ischemic and rapamycin preconditioning effectively restored autophagic flux and ameliorated liver IR injury in aged mice. Similarly, Selzner M et al. found that ischemic and glucose preconditioning, but not ischemic preconditioning alone, protected old livers against IR injury [4]. These findings indicate that old livers exhibit more severe impairment of autophagy than young livers. In the present study, we found that autophagy was inhibited by IR in aged mice. Combined ischemic and rapamycin preconditioning inhibited hepatocellular apoptosis and promoted autophagy induction, leading to reduced IR injury in aged mice. Furthermore, autophagy inhibition by its inhibitor 3MA abrogated the protective role of combined ischemic and rapamycin preconditioning in the aged mice post IR. These findings suggested us combined ischemic and rapamycin preconditioning alleviated liver ischemia and reperfusion injury by restoring autophagy in aged mice.

The interplay of autophagy and apoptosis have been reported recently [25]. Many stress pathways could induce autophagy and apoptosis within the same cell. Autophagy generally promotes cell survival and inhibits the induction of apoptosis, while in special cases, autophagy may induce apoptosis/cell death as well. Autophagy induction by overexpression of ATG1 inhibited cell growth and induced apoptosis [26]. In the present study, we found that autophagy was inhibited by IR in aged mice. Combined ischemic and rapamycin preconditioning inhibited hepatocellular apoptosis and promoted autophagy induction. Furthermore, autophagy inhibition by its inhibitor 3MA increased Caspase-3 activity and abrogated the protective role of combined ischemic and rapamycin preconditioning in the aged mice post IR. These findings suggested us that combined ischemic and rapamycin preconditioning inhibited hepatocellular apoptosis by restoring autophagy activation in aged mice post liver IR. However, the precise molecular intersections between the autophagic and apoptotic pathways remains to be further studied.

Direct hepatocellular injury caused by deprivation of oxygen and nutrition and subsequent intrahepatic inflammation are the two characteristic pathogeneses of liver IR injury [1]. Tejima K el al. found that ischemic preconditioning protected liver IR injury by reactive oxygen species produced by Kupffer cells [27]. In a recent study, our group found that inhibition of mTOR signaling by rapamycin ameliorated ATF3 deficiency-mediated liver damage in IR-induced liver injury in young mice (8-week-old) [28]. Thus, ischemic and rapamycin preconditioning could also have potential functions in modulating immune response in aged mice. However, we did not studied the effects of ischemic and rapamycin preconditioning on the immune modulatory ability. Further studies are needed to dissect the exact role of ischemic preconditioning and rapamycin preconditioning in different cell types including hepatocytes and macrophages.

5. Conclusions

In summary, our study demonstrated that combined ischemic and rapamycin preconditioning protected old livers against IR injury, which was likely attributed to restored autophagy activation. These findings warrant efforts toward clinical translation.

Acknowledgement

This study was supported by the National Natural Science Foundation of China (No. 81600450, 81530048 and 81470901), Six Talent Peaks Project in Jiangsu Province (No. 2018-WSN-011), Jiangsu Science and Technology Association Young Science and Technology Talents Lifting Project (No. DG000D4007) and A Project Funded by the PAPD.

Conflicts of interest

The authors declare no conflicts of interest.

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