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Ischemic preconditioning and remote ischemic preconditioning provide combined protective effect against ischemia/reperfusion injury

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ABSTRACT

Aims: Our objective was to compare the protective efficacy of ischemic preconditioning (IPC) and remote ischemic preconditioning (RIPC) against liver ischemia/reperfusion injury (IRI) and to evaluate their combined protective effect in mouse liver transplantation (MLT).
Materials and methods: Mice were randomly allocated to sham, IPC, RIPC, or IPC + RIPC groups. The animals were sacrificed at 2 h, 24 h, and 3 days after reperfusion. Blood samples were collected to evaluate alanine aminotransferase, TNF-α, and innate immune response. Liver tissue samples were obtained for histological evaluation, terminal deoxynucleotidyltransferased UTP nick end labeling, malondialdehyde (MDA) assay.
Key findings: Mice given preconditioning measures had significantly lower increase in transaminase, TNF-α expression, MDA formation, liver injury scores, and apoptosis index at 2 h, 24 h and 3 days after liver transplantation. The percentages of $CD11b^+$, $CD11b^+$ $CD16/32^+$ and $CD11b^+$ $CD16/32^{high}$ in white blood cells at 3 days after MLT were significantly lower than in the sham group. The results of factorial analysis demonstrated no synergistic effect for IPC and RIPC, except for MDA formation 2 h after reperfusion ($p = 0.038$).
Significance: Based on the synergistic and addictive effect on liver IRI induced by MLT between IPC and RIPC, the study suggested ways in which combined preconditionings could be elicited in patients undergoing planned procedures complicated by IRI to support better outcomes.

1. Introduction

For end-stage liver diseases, liver transplantation has become a sole effective therapeutic approach. However, hepatic ischemia/reperfusion injury (IRI) is a serious concern because it increases the risk of primary graft nonfunction or dysfunction, which occur in about 5% and 10% of transplants, respectively [1,2]. In addition, IRI can cause the release of endogenous toxic molecules that promote graft rejection, allograft vasculopathy, and activation of innate immune responses [3,4]. Therefore, researchers turned to the protective strategies in order to enable the graft to enhance its tolerance against the prolonged or lethal ischemia.

Ischemic preconditioning (IPC), a well-known method induced by several brief periods of ischemia and reperfusion, increases organ tolerance to subsequent prolonged ischemia [5,6]. Although numerous studies have shown that IPC was effective in attenuating IRI and improving systemic complications, its clinical applications have been limited because of direct stress to the target organ and mechanical trauma to major vascular structures [7]. Another method, remote ischemia preconditioning (RIPC), in which brief ischemia followed by reperfusion of one organ confers protection of distant organs, is considered more practical for clinical use [8–10]. RIPC is now thought to be a complex neuro-humoral process, which includes the release of blood-borne protective factors dependent on prior activation of sensory afferent nerves, the transfer of the biochemical messengers through blood circulation and the exertion of the protective effect in the target organ [11–14].

Ischemic tolerance is an adaptive process mainly induced by regulation of endothelial function, energy metabolism, microcirculation, and reduced macrophage and neutrophil activity, resulting in decreased endothelial and parenchymal injury. There are evidences that multiple cellular signaling pathways are associated with the ischemic tolerance induced by IPC and RIPC. Endothelial NO, free radicals, adenosine, kinases, opioids, catecholamines and ATP-sensitive potassium (K_{ATP}) channels may be involved, but the mechanism is not fully understood [15–17].

Many studies have shown that both IPC and RIPC not only protect the liver against IRI, but also improve liver regeneration [18–20]. However, to our knowledge, there are few reports on the effect of limb RIPC on IRI induced by mouse liver transplantation, and the comparison with IPC. We developed a mouse model of IPC and RIPC to compare the

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protective efficacy of IPC and RIPC on transplanted livers. In addition, we investigated whether the combined efficacy of these two methods is additive or synergistic.

2. Methods

2.1. Animals

It's known that estrogen and its derivatives could protect against liver IRI, therefore, all experiments were performed in 6- to 8-week-old male C57BL/6 mice (Vital River Laboratories, Beijing, China) weighing 25–30 g. Mice were housed under standard conditions with a 12 h dark: light cycle and free access to water and food. All mice were fasted without water deprivation for 12 h before mouse liver transplantation (MLT). One hundred and forty couples of mice were assigned randomly to four groups of equal size that received no treatment, IPC, RIPC, or IPC + RIPC before graft retrieval. All animal experiments were reviewed and approved by the Jilin University Animal Care and Use Committee, and all mice were treated in accordance with were performed in accordance with the guidelines for the care and use of laboratory animals from the National Institutes of Health (US).

2.2. Surgical procedures

All surgical procedures were performed under clean, but not sterile conditions using inhalation anesthesia with isoflurane. A surgical microscope (Olympus SZX 3.0, Japan) was used, and the procedure was a slight modification of techniques described by Qian et al. [21].

Briefly, for IPC + RIPC group, RIPC was induced through six cycles of 4 min of limb ischemia followed by 4 min of reperfusion, which were carried out by repeated occlusion and opening of the femoral vascular bundle, as described by Abu-Amara et al. [20]. While RIPC was administered, the ligaments of the liver and separating the right adrenal vessels, right renal arteries and veins, the portal vein, hepatic artery, and common bile duct were separated, and after completing the first three cycles of RIPC, IPC was induced by occlusion and opening of hepatic inflow for 10 and 15 min, respectively. When RIPC was close to completion, the right adrenal vessels were dissected by electrocoagulation, a polyethylene tube was inserted into the lumen of the common bile duct and the pyloric vein, right renal vein renal and artery were ligated and divided. After heparinization, the liver was perfused with cold University of Washington (UW) solution through the abdominal aorta, freed and then placed into a container of cold UW solution for further preparation. The suprahepatic vena cava anastomosis was constructed with a continuous microsuture using 10–0 prolene (Ethicon, USA). Reconstruction of the portal vein and infrahepatic vena cava was performed using the cuff technique. Biliary continuity was restored by inserting a stent into the common bile duct of recipient. In each procedure, the liver was exposed to cold ischemia for about 2 h.

Recipient blood and hepatic tissue were sampled under anesthesia at 2 h, 24 h, and 3 days after portal reperfusion. Blood was used for serum assays and flow cytometric analysis. Each liver sample was divided into two sections. One piece was fixed in 10% formaldehyde and embedded in paraffin and the other was snap-frozen in liquid nitrogen at −80 °C for molecular analyses. A total of 20 mice were followed for 30 days after surgery. Mice that died or were euthanized (for presumed surgical complications) within 24 h after transplantation were excluded from analysis.

2.3. Serum assays

After centrifugation of blood samples at 4000 rpm for 15 min at 4 °C, the serum supernatant was collected for serological testing. Serum alanine aminotransferase (ALT) was assayed with a standard automatic biochemistry analyzer (Vitros 350, Johnson & Johnson, USA). Cytokine (TNF-α) secretion was measured by enzyme-linked immunosorbent assay (ELISA) following the kit manufacturer's protocol (eBioscience, San Diego, USA).

2.4. Flow cytometric analysis

White blood cells were separated from the blood following treatment with red blood cell lysis buffer (Beyotime, China). The following antibodies were used in the flow cytometric analysis of innate immune response: anti-mouse CD11b-APC (eBioscience, USA); anti-mouse CD16/32-PE (Biolegend, USA); rat immunoglobulin G2a was used as an isotype control (BD Pharmingen, USA). Labeled cells were analyzed using a FACSCalibur (BD LSRFortessa, USA).

2.5. Histological examination

Harvested livers were fixed in 10% formalin solution, embedded in paraffin, sectioned at 4 μm thickness, and stained with hematoxylin and eosin (HE) for general histopathologic evaluation of IRI according to Suzuki's criteria, in which sinusoidal congestion, hepatocyte necrosis, and ballooning degeneration are graded from 0 to 4 [22].

Apoptosis of hepatocytes was detected by terminal deoxynucleotidyltransferase-mediated dUTP nick end-labeling (TUNEL) using a TransDetectIn Situ Fluorescein TUNEL Cell Apoptosis Detection Kit (Transgen Biotech, China). Only hepatocytes were included in this analysis. The apoptotic index of the TUNEL assay was determined as the percentage of apoptotic events per hepatic cell population in five random fields at ×400 magnification (Olympus IX51, Japan).

2.6. Molecular analysis of liver tissue

Liver tissue was centrifuged at 3000 rpm for 15 min, malondialdehyde (MDA), a biomarker of lipid peroxidation, was assayed in the supernatant using a colorimetric/fluorometric method according to the kit manufacturer's protocol (Biovision, Milpitas, CA, USA), the absorbance level of the complex formed by MDA and thiobarbituric acid at a wave length of 532 nm was measured, the data were expressed in terms of nmol/mg protein weight.

2.7. Statistical analysis

Results were presented as means ± SEM. Differences between two dependent groups were evaluated with the paired Student's *t*-test. Comparisons among multiple groups were performed with one-way ANOVA followed by Bonferroni post-hoc tests. Animal survival analysis was performed with the Kaplan-Meier analysis and the log-rank test. All analyses were conducted with SPSS® version 17.0 (SPSS, Chicago, Illinois, USA). All *p*-values were two-tailed, and $p < 0.05$ was accepted as being statistically significant. GraphPad Prism 6.0 (La Jolla, CA, USA) was used to generate the graphs.

3. Results

3.1. Survival

A total of 143 MLT were performed. Three mice were excluded because their proper hepatic arteries arose from the esophageal arteries, which encircle the esophagus. In addition, four MLT were excluded from the analysis because of death within 24 h post-transplantation from suprahepatic vena cava bleeding or an unknown cause. Five recipients died from bile duct complications or an unknown cause between 8 and 30 days post transplantation. The anhepatic and total cold ischemia times were 17.5 ± 1.5 min and 117.2 ± 16.9 min respectively, and there were no significant differences among the four groups ($p > 0.05$). The 30 day graft survival in the sham, IPC, RIPC, and IPC + RIPC groups