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Original Article

Ischemic postconditioning provides protection against ischemia-reperfusion injury in intestines of rats

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Abstract: In the present study, we investigated the protective role of ischemic postconditioning (IPOST) against intestine ischemia-reperfusion (I/R) injury in rats. Male Sprague-Dawley rats were divided into sham-operation group (S), I/R group (I/R), ischemic preconditioning group (IPC), ischemic postconditioning group (IPOST). After reperfusion, small intestines were resected for histopathologic evaluations. To evaluate DNA fragmentation, resolving agarose gel electrophoresis was performed. To measure cellular apoptotic rates in intestine tissues, we performed TUNEL staining. To examine lipid peroxidation, production of superoxide radicals and tissue neutrophil infiltration, we tested the content of malondialdehyde and activities of superoxidase dismutase and myeloperoxidase in intestine tissues, respectively. Under light microscope, intestinal mucosal impairment in IPOST and IPC groups was found milder than that in I/R group ($P < 0.05$). The number of apoptosis cells in I/R group was significantly higher than that in IPOST and IPC groups ($P < 0.05$). The content of malondialdehyde and activity of myeloperoxidase were significantly reduced in IPOST group and IPC group compared with I/R group, but the activity of superoxidase dismutase in IPOST group and IPC group was enhanced compared with I/R group ($P < 0.05$). These results suggest that IPOST results in protection against intestine I/R injury, which may be related to reduced production of reactive oxygen species, enhanced activities of antioxidant systems and inhibited apoptosis of intestinal mucosal cells.

Keywords: Intestine, ischemia-reperfusion, ischemic preconditioning, ischemic postconditioning, apoptosis

Introduction

Shock, abdominal aneurysm repair, and small bowel transplantation are clinical conditions that are associated with mesenteric ischemia-reperfusion (I/R) [1]. The intestine is particularly susceptible to I/R injury. I/R injury can result in tissue damages caused by generation of oxygen free radical, neutrophil infiltration, mucosal apoptosis, etc. Subsequently, recovery is delayed, morbidity is increased, and multiple organ failures occur [1-4].

The mechanisms of I/R of intestine and the ways to limit them have been under extensive researches for almost a decade. Several therapeutic modalities have been used successfully to attenuate reperfusion injury in animal models of intestinal I/R. Ischemic preconditioning (IPC) refers to a phenomenon whereby exposure of tissues to brief periods of ischemia protects them from deleterious effects of prolonged I/R injury. This phenomenon is first described by Murry et al. in canine hearts [5]. Subsequently, beneficial effects have been demonstrated in the liver, brain, kidney, and intestine in various animal models. However, clinical application of IPC has been disappointingly limited. The implementation of protective therapy at the time of reperfusion is clinically feasible because the onset of reperfusion is more predictable and under clinician's control.

In 2003, Vinten-Johnsen and colleagues reported that brief episodes of myocardial ischemia and reperfusion employed during reperfusion after a prolonged ischemic insult have been demonstrated to play a cardioprotective role [6]. This phenomenon was termed "ischemic postconditoning (IPOST)" [6]. In a dog model, postconditioning limits infarct size, reduces tissue edema and polymorphonuclear neutrophil accumulation in the area at risk myocardium, and improves endothelial function. It has been suggested that postconditioning is as effective as preconditioning in limiting infarct size and

preserving postischemic endothelial function. Kin et al. [7] proposed that IPOST might be related to the attenuation of oxygen-derived free radical production in the early minutes of reflow. IPOST is an alternative, more amenable approach. However, the mechanism of this new method remains unknown and there are no studies about the protection of IPOST against I/R intestine. In this study, we investigate the protective role and mechanism of IPOST against intestinal I/R injury in rats. This study not only provides a novel approach for clinical practice, but also provides useful theoretical and practical evidences for reducing I/R injury.

Materials and methods

Animals

The study was performed using male Sprague-Dawley (SD) rats weighing 220 to 250 g that were maintained in accordance with the Animal Committee Guidelines for Use and Care. Animals were fasted for 12 h before surgery. The rats were randomly divided into four experimental groups, and anesthetized with intraperitoneal injection of 20% urethane (1 g/kg body weight) for laparotomy. The superior mesenteric artery (SMA) was identified and freed by blunt dissection. A microbulldog clamp was placed at the root of SMA to induce complete cessation of blood flow for 45 min, after which the clamp was loosened. After experiments, the entire small intestine was carefully removed and placed on ice. The oral 10-cm segment was removed, wedges of 1 to 2 cm from the ileum were collected, and the rest of the intestine was divided into four equal segments. Each segment was rinsed thoroughly with physiological saline and opened longitudinally on its antimesenteric border to expose the intestinal mucosa. The mucosal layer was harvested by gentle scraping using a glass slide [8].

The animals were randomly divided into the following groups (n = 8): i) sham-operation group (S), in which SMA was separated, but without occlusion; ii) ischemia-reperfusion group (I/R), for which SMA occlusion time was 45 min and reperfusion time was 1 h; iii) ischemic preconditioning (IPC), in which SMA was occluded by 2 cycles of 5 min reperfusion before another 45 min of prolonged occlusion; iv) ischemic postconditiong group (IPOST), in which 45 min of SMA occlusion preceded reperfusion that was initiated for 30 s before 30 s of reocclusion (a total of 3 cycles). Reperfusion was continued for a total of 1 h in all experiments.

Hematoxylin and eosin staining and mucosal injury scoring

After fixation by immersion in 10% buffered formaldehyde solution, tissues were embedded in paraffin blocks, sectioned in 5-μm slices, placed on glass microscope slides, and stained with hematoxylin and eosin. Light microscopic evaluation of the tissues was performed in a masked fashion by a pathologist who scored the histology using a system described by Chiu et al. [9]: grade 0, normal mucosa; grade 1, subepithelial space development at the tip of the villus, often with capillary congestion; grade 2, lifting of the epithelial layer from the lamina propria and moderate extension of subepithelial space; grade 3, some denuded tips of villi and massive lifting of epithelial layer; grade 4, dilated and exposed capillaries and denuded villi; grade 5, hemorrhage, ulceration, and disintegrated lamina propria.

Agarose gel electrophoresis

Total DNA was extracted from small intestine before removing proteins with a phenol/chloroform/isoamyl alcohol mixture (volume ratio, 25:24:1), followed by purification as described by Wu and colleagues [10]. Resolving agarose gel electrophoresis was performed with 1.5% gel strength containing 1.0 μg/ml ethidium bromide. Depending on experiments, 20 μg DNA per well was loaded. DNA standards (0.5 μg/well) were included to identify the size of DNA fragments. Electrophoresis was performed for 4 h at 30 V, and the DNA was visualized under ultraviolet fluorescent light.

Terminal deoxynucleotidyl transferase-mediated dUDP-biotin nick-end labeling staining (TUNEL) assay

Four-micrometer thick sections were collected on poly-L-lysinecoated glass slides. Nuclear DNA fragmentation of apoptotic cells was labeled in situ by TUNEL assay. After treatment with xylene and rehydration with progressively decreasing alcohol concentrations followed by washing with phosphate-buffered saline (PBS), each section was treated with 20 μg/mL proteinase K in 0.1 mol/L Tris/HCL buffer (pH 7.4)

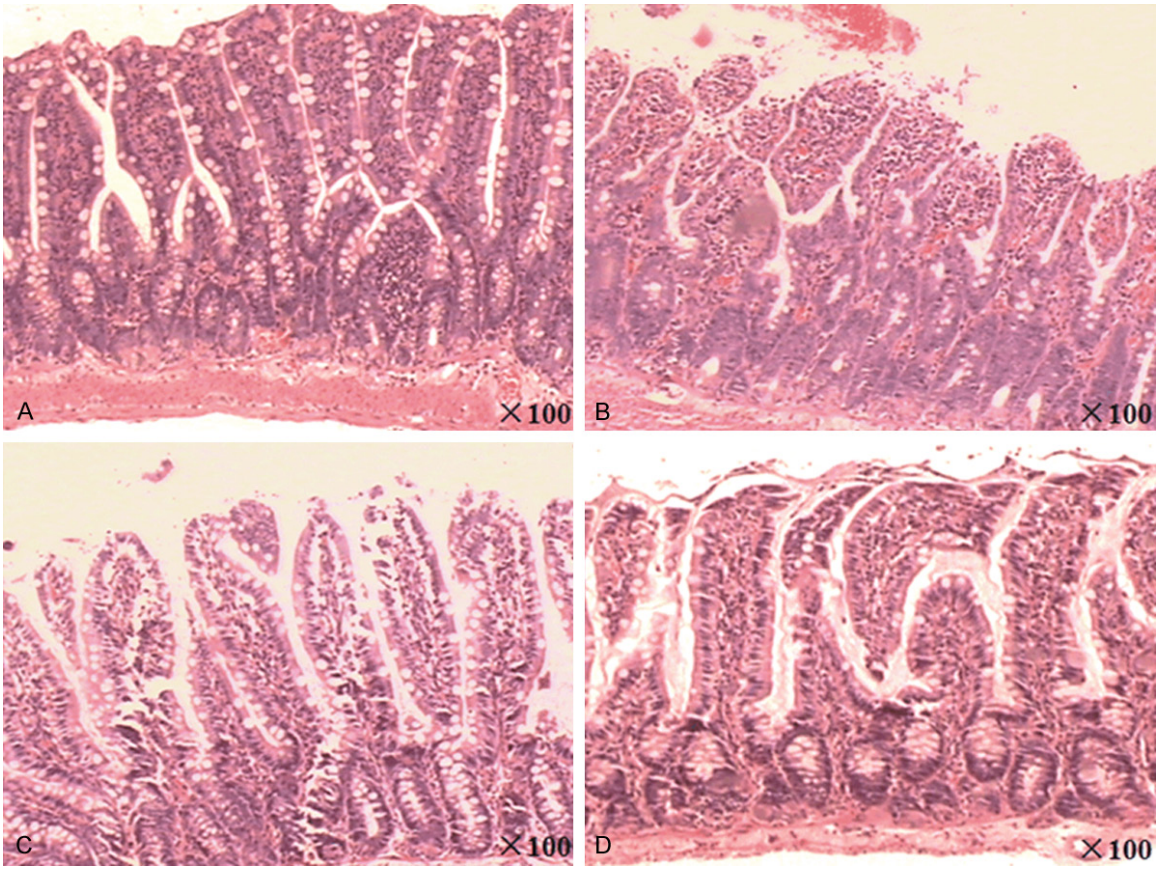

Figure 1. Histopathological changes of rat intestine 1 h after reperfusion. A. S group. B. I/R group. C. IPC group. D. IPOST group. After fixation by immersion in 10% buffered formaldehyde solution, tissues were embedded in paraffin blocks, sectioned in 5-μm slices, placed on glass microscope slides, and stained with hematoxylin and eosin.

for 15 min. After rinsing with PBS, endogenous peroxidase activity was blocked with 3% H_2O_2 for 5 min. After rinsing with PBS, the sections were incubated with 0.5 U/μL terminal deoxynucleotidyl transferase and 0.05 nmol/μL biotinylated deoxyuridine triphosphate in terminal deoxynucleotidyl transferase buffer for 60 min in a humidified chamber at 37°C. Each slide was then observed under a microscope to check the staining quality before image acquisition. For each animal, five sections were analyzed by counting apoptotic bodies in five randomly chosen fields. Apoptotic index was determined by dividing the number of apoptotic cells by the total number of cells in the crypt column and being multiplied by 100.

Measurement of tissue myeloperoxidase (MPO)

MPO enzyme activity in tissue lysates was measured using H_2O_2-dependent oxidation of 3,3',5,5'-tetramethylbenzidine and SureBlue tetramethylbenzidine-component microwell peroxidase. Absorbance changes at 450 nm were measured with a kinetic microplate spectrophotometer (CL-700; Beckman Coulter, Brea, CA, USA). MPO was expressed as units of activity per gram of wet tissue, where 1 unit is defined as the quantity of MPO required to convert 1 μm of hydrogen peroxide to water in 1 minute at 22°C.

Measurement of tissue malondialdehyde (MDA)

MDA levels that reflect lipid peroxidation in intestinal homogenates were determined with spectrophotometer (CL-700; Beckman Coulter, Brea, CA, USA) using thiobarbituric acid. 1,1,3,3-Tetraethoxypropane was used as standard and the results were expressed as nmol MDA/mg protein. Protein concentrations were determined by the method of Lowry.

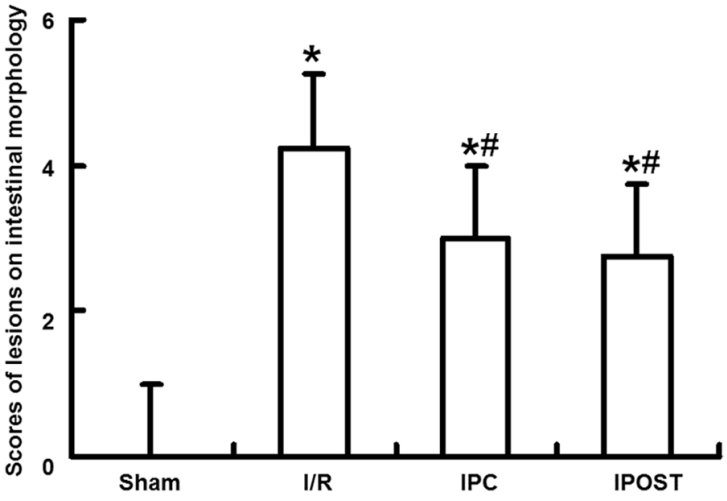

Figure 2. Scores of lesions on intestinal morphology. After staining with hematoxylin and eosin, light microscopic evaluation of the tissues was performed in a masked fashion by a pathologist who scored the histology. Scoring system: grade 0, normal mucosa; grade 1, subepithelial space development at the tip of the villus, often with capillary congestion; grade 2, lifting of the epithelial layer from the lamina propria and moderate extension of subepithelial space; grade 3, some denuded tips of villi and massive lifting of epithelial layer; grade 4, dilated and exposed capillaries and denuded villi; grade 5, hemorrhage, ulceration, and disintegrated lamina propria. *, $P < 0.05$ compared with S group; #, $P < 0.05$ compared with I/R group. Data are presented as means ± SD.

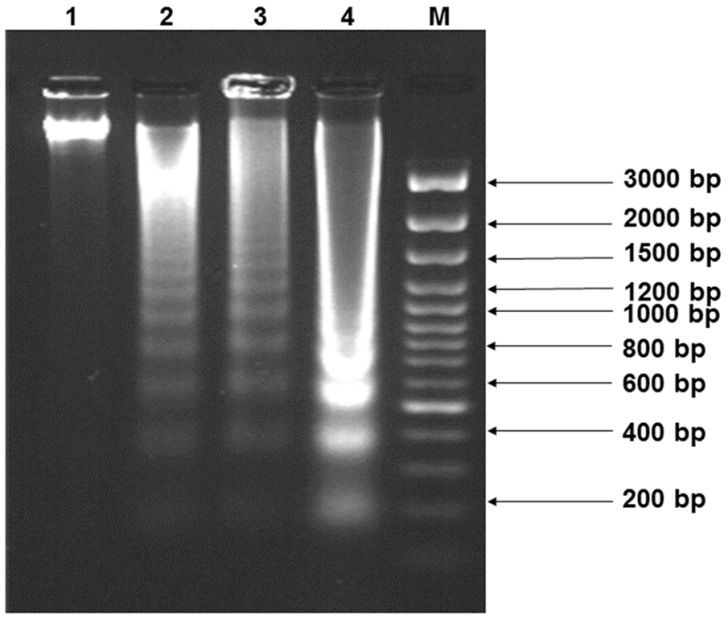

Figure 3. Agarose gel electrophoresis of DNA in intestinal mucosa. Lane 1, Sham-operation group; Lane 2, IPOST group; Lane 3, IPC group; Lane 4, I/R group; M, DNA marker (100 bp). Resolving agarose gel electrophoresis was performed with 1.5% gel strength containing 1.0 μg/ml ethidium bromide. Depending on experiments, 20 μg DNA per well was loaded. DNA standards (0.5 μg/well) were included to identify the size of DNA fragments. Electrophoresis was performed for 4 h at 30 V, and the DNA was visualized under ultraviolet fluorescent light.

Determination of tissue superoxide dismutase (SOD) activity

The activity of SOD in cytosol fractions was determined by means of xanthine and xanthine oxidase system for the production of superoxide radicals and subsequent measurement of cytochrome C as a scavenger of the radicals. SOD activity was expressed as units/mg proteins, where one unit of activity is the amount of enzymes required to inhibit the rate of reduction of cytochrome C by 50%.

Statistical analysis

Data were presented as means ± standard derivation (SD). Statistical significance of differences among groups was determined by analysis of variance (ANONA) followed by Duncan's and Tukey-Kramer multiple comparison tests. The differences were considered significant if $P < 0.05$.

Results

IPOST exerts protection against intestine ischemia-reperfusion injury

To determine mucosal injury, hematoxylin and eosin staining was performed. Intestine in S group exhibited normal mucosal architecture with intact villi (**Figure 1A**). In I/R group, denuded villi, disintegration of lamina propria, and exposed capillaries were observed (**Figure 1B**). However, IPC group and IPOST group only showed capillary congestion and mild epithelial lifting from lamina propria (**Figure 1C** and **1D**). In addition, mucosal injury score for IPC group or IPOST group was dramatically smaller than that in I/R group ($P < 0.05$) (**Figure 2**). These results suggest that IPOST plays a protective role

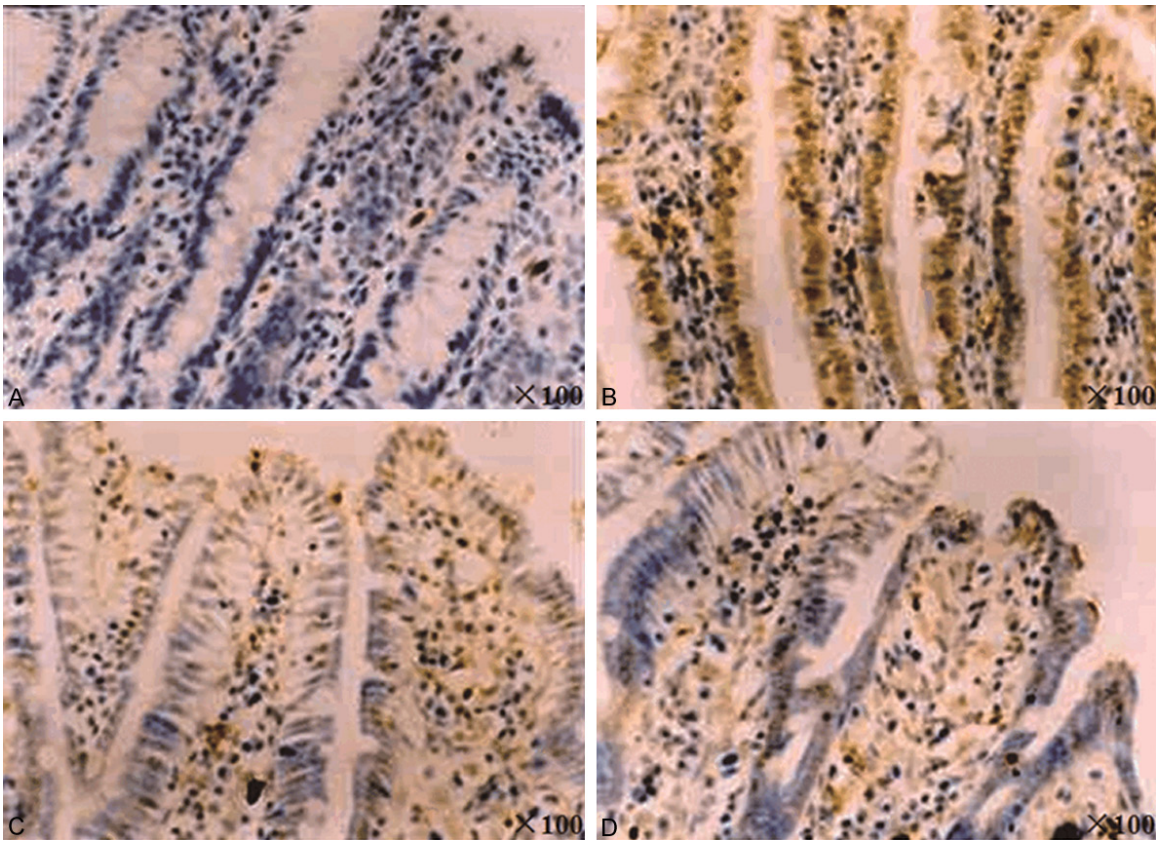

Figure 4. Changes of apoptosis of intestine. A. S group. B. I/R group. C. IPC group. D. IPOST group. After fixation by immersion in 10% buffered formaldehyde solution, tissues were embedded in paraffin blocks, sectioned in 5-μm slices, placed on glass microscope slides, and stained with hematoxylin and eosin.

against intestine injury in rats with ischemia-reperfusion.

IPOST inhibits mucosal apoptosis in small intestine

To evaluate fragmented DNA in small intestinal mucosa, resolving agarose gel electrophoresis was employed. The data showed that I/R significantly induced fragmentation of mucosal DNA, resulting in increased DNA ladder that is characteristic of apoptosis. By contrast, IPOST and IPC reduced ladder formation (**Figure 3**). These results indicate that IPOST, like IPC, inhibits mucosal apoptosis in small intestine.

IPOST decreases apoptotic rate of cells in intestine tissues

To measure cellular apoptotic rates in intestine tissues, we performed TUNEL staining. The data showed that few apoptotic cells were observed in S group (**Figure 4A**). In I/R group, the number of apoptotic cells increased, and these cells were distributed from tip to base of villi. Of note, apoptosis was relatively severe in submucosa and lamina propria (**Figure 4B**). By contrast, the number of apoptotic cells in IPOST group or IPC group was smaller than that of I/R group (**Figure 4C** and **4D**). Consistently, apoptotic indices of IPOST group and IPC group were significantly decreased compared with I/R group ($P < 0.05$) (**Figure 5**). These results suggest that IPOST, like IPC, decreases apoptotic rate of cells in intestine tissues.

IPOST decreases lipid peroxidation and tissue neutrophil infiltration, but enhances the production of superoxide radicals

To examine lipid peroxidation, production of superoxide radicals and tissue neutrophil infiltration, we determined the content of MDA and activities of SOD and MPO in intestine tissues, respectively. The content of MDA and the activity of MPO in I/R group, IPOST group and IPC

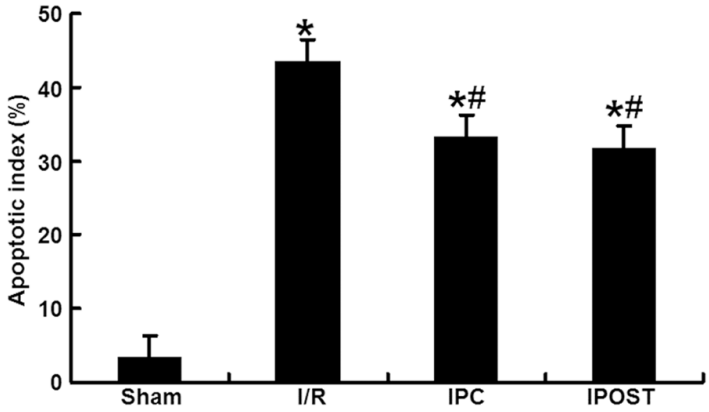

Figure 5. Changes of apoptotic index of cells in intestine tissues. Nuclear DNA fragmentation of apoptotic cells was labeled in situ by TUNEL assay. Apoptotic index was determined by dividing the number of apoptotic cells by the total number of cells in the crypt column and being multiplied by 100. *, $P < 0.05$ compared with S group; #, $P < 0.05$ compared with I/R group. Data are presented as means ± SD.

Table 1. The content of MDA, and the activities of SOD and MPO in intestine tissues (n = 8)

Groups	MDA (nmol/mg)	SOD (U/mg)	MPO (U/g)
S	3.288 ± 0.164	13.45 ± 1.07	0.318 ± 0.024
I/R	7.584 ± 0.891*	5.52 ± 2.08*	2.503 ± 0.335*
IPC	6.143 ± 0.488*,#	7.45 ± 1.59*,#	2.118 ± 0.372*,#
IPOST	6.546 ± 0.774*,#	7.14 ± 1.19*,#	2.080 ± 0.444*,#

Note: *, $P < 0.05$ compared with S group; #, $P < 0.05$ compared with I/R group. Data are presented as means ± standard deviation.

group were significantly higher than those of S group, but the activity of SOD was significantly lower than that in S group. In addition, the content of MDA and the activity of MPO in both IPOST group and IPC group were significantly decreased compared with those of I/R group, while the activity of SOD in both IPOST group and IPC group was increased compared with that of I/R group ($P < 0.05$) (**Table 1**). These results indicate that IPOST decreases lipid peroxidation and tissue neutrophil infiltration, but enhances the production of superoxide radicals.

Discussion

Among all internal organs, the intestine is probably the most sensitive to I/R. Reperfusion of intestine after a period of ischemia is a common situation after events such as shock, vascular surgery, and small bowel transplantation. In addition to damages caused by ischemia, more tissue damages are caused by the generation of oxygen free radical, neutrophil infiltration, mucosal apoptosis and so on following I/R injury, probably as consequences of loss of physical integrity of mucosal barrier, tissue edema, or impaired distant organs [11, 12].

IPC is a mechanical method being studied extensively. It can provide protective roles against I/R injury in multiple organs including intestine. In recent years, another mechanical method, IPOST, is reported by Vinten-Johansen's group. In IPOST, brief episodes of ischemia are performed just at the onset of reperfusion following a prolonged ischemic insult, dramatically reducing infarct size [6]. Subsequently, some studies also demonstrate that IPOST can provide protective roles that are comparable to IPC [7, 13-20]. Kin et al. [7] propose that postconditioning might be related to attenuation of oxygen-derived free radical production in the early minutes of reflow. A recent clinical study demonstrates that IPOST protects human heart [21].

In the present study, we adapt an established protocol of IPOST on the heart to test the hypothesis that postconditioning attenuates intestinal injury resulted from I/R. The brief repetitively interrupted reperfusion with postconditioning applied during the onset of reperfusion significantly reduced intestine injury scores, and inhibited apoptosis. Apoptosis is widely considered to be a distinct entity from necrotic cell death. Many studies have demonstrated that apoptosis is the major form of cell death occurring after short-term ischemia or I/R in rat intestine [22-24]. The present study suggests that apoptosis-positive cells are increased significantly in number, and distribute from tip to base of villi in I/R group. Apoptosis is severe in submucosa and lamina propria. By contrast, apoptosis is reduced in IPOST group and IPC group compared with I/R group. In addition, postconditioning is associated with significantly decreased content of MDA-reactive products of lipid peroxidation,

enhanced activity of SOD and reduced neutrophil accumulation in the reperfusion of intestine. These observations suggest a reduction in the generation of reactive oxygen species and less oxidant-mediated injury by postconditioning.

In conclusion, we demonstrate that IPOST decreases MDA content and MPO activity, increases the activity of SOD, induces less mucosal injury, and significantly inhibited apoptosis in small intestinal mucosa. To our knowledge, this is the first report demonstrating the protective effect of postconditioning on intestine tissues. The IPOST procedure is very simple, and may be clinically applicable. Further studies are necessary to determine the optimal algorithm of perfusion-rocclusion sequences, and other precise mechanisms by which postconditioning provides protection for small intestine.

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Disclosure of conflict of interest

None.

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