Renal ischemia-reperfusion injury attenuated by splenic ischemic preconditioning

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Abstract. – OBJECTIVE: To investigate the therapeutic effect of splenic ischemic preconditioning (sIPC) on renal ischemia-reperfusion (IR) injury.

MATERIALS AND METHODS: A total of 18 adult male Sprague Dawley (SD) rats were treated by 45 min renal ischemia and followed by 24 h reperfusion. In the sIPC group, three cycles of splenic ischemic preconditioning including 5 min ischemia and 5 min reperfusion were carried out before renal ischemia. The blood samples and kidney tissues were collected after 24 h. The levels of Cr and BUN in serum were measured to evaluate the kidney function. The morphological changes in ischemia-reperfusion kidneys were determined by hematoxylin-eosin (HE) staining. The levels of pro-inflammatory cytokines including TNF-α and IL-6 in serum, and renal tissues, were measured by ELISA and Western Blotting. Furthermore, the levels of IKK-β, intra-nuclear NF-κB, p65, and IL-10 in renal tissues were also measured.

RESULTS: The results demonstrated that the level of Cr and BUN in the IR group were increased while decreased in the sIPC group. HE staining showed that the damage caused by renal ischemia-reperfusion was attenuated by sIPC with a low renal injury score in the sIPC group. ELISA and Western Blotting results showed that the production and secretion of TNF-α and IL-6 induced by IR were inhibited by sIPC. The expression level of IKK-β and intranuclear p65 in renal tissues were increased in the IR group while sIPC had exhibited the function of depressing the increased expression levels of IKK-β and intranuclear p65. Compared with the IR group, the expression level of IL-10 in serum and renal tissues in the sIPC group were increased.

CONCLUSIONS: sIPC exhibited a potent anti-inflammatory capacity to attenuated renal IR injury.

Key Words: Renal ischemia-reperfusion, Splenic ischemic preconditioning, Inflammation, NF-κB, IL-10.

Introduction

Ischemia-reperfusion (IR) injury is very common in urology surgeries, such as renal allograft transplantation and partial nephrectomy. Delayed graft function (DGF) and poor graft survival rate can be attributed to acute renal injury induced by IR in renal allograft transplantation. Adverse events can be generated by IR, which includes inflammation, oxygen free radical, necrosis, apoptosis, and thrombosis. These adverse events can aggravate the renal injury by activating the adaptive immune system and inducing graft vs. host disease (GVHD) followed by endothelial cell necrosis and apoptosis.

Some studies have demonstrated that the risk of DGF was associated with the duration of renal ischemia both in the adult and infant recipients. A previous literature reported that the duration time of ischemia affected the renal function post partial nephrectomy.

Ischemic preconditioning refers to the transient and repeated ischemic conditioning before ischemia events which occur in a certain organ or tissue. Previous reports have confirmed the protective effect of ischemic preconditioning against IR injury in animal models. Chen et al. found that renal ischemic preconditioning has a protective effect on inhibiting cell apoptosis, limiting lipid peroxidation and improving the function of ischemic kidneys. Remote ischemic preconditioning (RIPC) is one kind of therapeutic strategy for repeated transient ischemia in many organs and tissues such as heart, liver, small intestine, skeletal muscle, and so on. Compared with local ischemic preconditioning, RIPC has the advantage of no fatal injury to the important target organs during surgeries. However, the views of RIPC on the protection of IR damage have not been reached. The results of a retrospective analysis indicated
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It has been proved that RIPC of the lower limb fails to improve the renal function of recipients who receive living renal transplantation\textsuperscript{11}. Moreover, the mechanisms, the effectiveness and the potentially involved signal pathways of RIPC are different from those of local ischemic preconditioning, which need further study\textsuperscript{22}. The spleen also is a source of inflammatory cells apart from the bone marrow and the marginal cell pool\textsuperscript{13}. IR can induce the production and release of oxygen free radicals and inflammatory cytokines, which further induce the recruitment of inflammatory cells and the generation of sterile inflammation\textsuperscript{14}. This sterile inflammation shares many similarities, including the signal transduction of Toll-like receptors (TLR) and the activation of innate and adaptive immune system\textsuperscript{15}. The spleen, as part of the immune system, may play an important role in mediating the effect of IR injury. A previous study\textsuperscript{16} has reported that splenectomy preserves renal function after IR and reduces mortality of the rats. However, the effect of splenic ischemic preconditioning on renal ischemia-reperfusion has not been investigated yet.

In this work, a randomized controlled animal trial was conducted. The rat model of renal ischemia-reperfusion injury was established, and the spleen of model rats was pretreated after ischemia-reperfusion. The effect of spleen preconditioning on renal IR injury was assessed, and the possible mechanism was explored.

Materials and Methods

Animals

Eighteen adult male Sprague Dawley rats weighting 200 g-300 g were provided by the animal experiment center of Wuhan University to establish animal models. The animal study protocol was in accordance with the Guide on Care and Use of Laboratory Animals published by the National Institute of Health and approved by the Ethnic Committee of the People’s Hospital of Wuhan University. All the rats were administered with free access to food, and water in a clean environment at room temperature of 20-23°C and fasted for one day before the operation.

Surgery Procedure

The rats were randomly divided into three groups (n=6 for each group): the sham operation group (sham), the renal ischemia-reperfusion group (IR) and the splenic ischemic preconditioning group (sIPC). For all the groups, the animals were anesthetized with 1.5% sodium pentobarbital intraperitoneally in a dose of 40 mg/kg. The abdominal cavity was opened and the right kidney was removed. The abdominal cavity was closed immediately after the right nephrectomy in the sham group. For the IR group, the left kidney was clamped for 45 min with a non-traumatic vascular clip following the right nephrectomy and the clip was removed to allow the blood flow to return the left renal vessel. The reperfusion continued for 24 h. For the sIPC group, after the right nephrectomy, three cycles of splenic ischemic preconditioning were firstly performed by using non-traumatic vascular clips before the renal ischemia. The blood vessels in the splenic pedicle were dissected and, then, were clamped to ensure the blockage of blood flow. Each cycle of the splenic ischemic preconditioning was 5 min of ischemia followed by 5 min of reperfusion. Following three cycles of splenic ischemic preconditioning, the renal ischemia was performed for 45 min and, then, the reperfusion was performed for 24 h. For all the animals of the IR group and the sIPC group, the abdominal cavity was closed immediately after the start of renal reperfusion. The rats were reared in individual cages and fed with food and water at will after the operation. All the rats underwent another anesthesia with the anesthetic protocol above after 24 h. The blood samples were collected from a puncture to the inferior vena cava and the left kidney was harvested for histological analysis and Western blot. Subsequently, a lethal dose of anesthetic was used for euthanasia of the rats.

SCr and BUN Levels Measurement

The blood samples collected from rats were centrifuged at 3000 rpm for 10 min after coagulation and the serum of the upper layer was collected. The concentrations of serum Cr (SCr) and BUN were determined by commercial kits (Jiancheng Bioengineering Institute, Nanjing, China), according to the manuscript’s instructions.

Serum TNF-\(\alpha\), IL-6, IL-10 Levels Measurement

The blood samples were centrifuged at 3000 rpm for 10 min after coagulation and the serum of the upper layer was collected. The levels of serum cytokines including TNF-\(\alpha\), IL-6, and IL-10 were measured by utilizing commercially avai-
lable ELISA kits (Elabscience, Wuhan, China), according to the manuscript’s instructions. The concentrations of cytokines were calculated by the standard curve.

**Histological Analysis**

The collected kidney tissues were fixed in 10% formalin, embedded in paraffin and sliced. To analyze the histopathological changes, the sections were stained with hematoxylin-eosin. A semi-quantitative pathological grading method described by Erdogan et al. was used to assess the degree of the renal damage. Twenty-four images under the high-power lens (10×20) were randomly acquired in a blinded manner in each section. Under the criteria for renal injury, including tubular dilation, tubular necrosis, cast formation, and immune cell infiltration, the histopathological changes on each image were determined by two pathologists who were blinded to the treatment. The percentage of images with pathological changes in all 24 images of one section was calculated according to the renal injury score (0 for 0%, 1 for less than 5%, 2 for 5% to 25%, 3 for 25% to 75%, 4 for 75% to 100%). Under the criteria of kidney injury, two cases of undifferentiated pathologists were considered, including tubular dilatation, tubular necrosis, cast formation, and immune cell infiltration and histopathological changes. In all 24 images, the percentage of pathological changes in each section was calculated, according to the score of kidney injury (0, 0%, 1, less than 5%, 2 from 5% to 25%, 3 from 25% to 75%, and 75% to 75%).

**Measurement of TNF-alpha, IL-6, IL-10, IKK-beta, NF-kappaB p65 in Kidney Tissues**

Kidney tissues were collected one-day post-operation from rats. Total proteins for TNF-α, IL-6, IL-10, IKK-β, β-actin and nuclear proteins for NF-κB p65 were extracted and the concentrations were determined by using bicinchoninic acid assay (n=6 for each group). Proteins of 40 μg per lane were separated on 12% sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) gel and transferred onto polyvinylidene difluoride (PVDF) membranes. Then, the membranes were blocked with 5% nonfat milk in the Tris-buffered saline and Tween (TBST) buffer solution for 2 h and incubated overnight at 4°C with anti-rat polyclonal primary antibodies to TNF-α (1:300, Bioser, Wuhan, China), IL-6 (1:500, Elabscience, Wuhan, China), IL-10 (1:500, Bioss, Woburn, MA, USA), NF-kB p65 (1:500, Proteintech, Rosemont, IL, USA), IKK-β (1:2000, Abcam, Cambridge, MA, USA), β-actin (1:200, Boster, Wuhan, China) and LaminB (1:300, Boster, Wuhan, China), respectively. After washed with TBST for 2 times, the membranes were incubated with goat anti-rabbit or goat anti-mouse secondary antibodies for conjugating with horseradish peroxidase (HRP) for 2 h. Specific bands were visualized by using ECL chemiluminescent detection system and the expression levels were quantized by Bandscan software.

**Statistical Analysis**

All data were presented as mean ± SD. The statistical analysis was performed with SPSS 19.0 (SPSS Inc. IBM, Armonk, NY, USA). The results were evaluated in the Kolmogorov-Smirnov (KS) test of normality and analyzed with one-way ANOVA-Student-Newman-Keuls test in different groups. Significant differences were considered when p-values < 0.05.

**Results**

**Protective Effect of Splenic Ischemic Preconditioning Against Renal Ischemia Reperfusion Injury**

The levels of serum Cr and BUN were significantly increased in the IR group compared with the Sham group (p<0.05) (Figure 1). In addition, there was a decrease in the levels of SCr and BUN of the sIPC group (p<0.05), indicating that the renal function depressed by IR was improved by sIPC. The HE staining sections showed severe renal injury in the IR group. In the sIPC group, sIPC attenuated renal ischemic damage and there were less tubular necrosis, tubular dilation, and immune cell infiltration in the sIPC group. According to the grade system described above, the semi-quantitative histopathological score of the IR group was dramatically increased (p<0.05). The score of the sIPC group showed a significant reduction compared with that of the IR group (p<0.05). The histological results suggest that sIPC can reduce renal injury induced by renal IR (Figure 2).

**Inhibition of Splenic Ischemic Preconditioning on TNF-alpha and IL-6 Release and Expression**

ELISA assay and Western blot were used to detect the releases and expressions of TNF-α and IL-6. Serum TNF-α and IL-6 levels of the IR group were increased compared with those in the
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Figure 1. Effect of splenic ischemic preconditioning on ischemia-reperfusion renal function: the level of SCr (A) and BUN (B) in three groups. The values are presented in Mean±SD. Statistical analysis was carried out by one-way ANOVA Student-Newman-Keuls test, *p<0.05 vs. sham group; #p<0.05 vs. IR group.

Figure 2. Effect of splenic ischemic preconditioning on histological injury of ischemia-reperfusion kidneys: The HE staining sections of sham group (A), IR group (B) and sIPC group (C) and the renal injury score of three groups (D). The values are presented in Mean±SD, statistical analysis was carried out by one-way ANOVA Student-Newman-Keuls test, *p<0.05 vs. sham group; #p<0.05 vs. IR group.

Sham group (p<0.05), while there was a significant decrease of serum TNF-α and IL-6 levels of the sIPC group compared with those in the IR group (p<0.05). In consistent with the serological results of cytokines, the levels of TNF-α and IL-6 expressions of kidney tissues were up-regulated.
The effects of sIPC on the NF-κB pathway were detected, and the expression level of IKK-β and NF-κB p65 in different groups was measured. The results showed that the expression level of IKK-β and NF-κB p65 in the IR group was significantly higher than that in the sham group \((p < 0.05)\). However, sIPC depressed IKK-β and nuclear NF-κB p65 expression levels at 24 h after renal IR compared with IR group \((p < 0.05)\) (Figure 4).

The serum concentration and renal expression level of IL-10 in the IR group both increased significantly compared with the sham group \((p < 0.05)\). The serum IL-10 concentration in the sIPC group showed a significant increase \((p < 0.05)\). In agreement with the serological results, the level of renal IL-10 expression was up-regulated in the sIPC group when compared with the IR group \((p < 0.05)\) (Figure 5).

**Figure 3.** Effect of splenic preconditioning on TNF-α and IL-6 release and expression: the serum TNF-α and IL-6 levels of three groups (A), the relative protein expression of TNF-α in three groups (B), the relative protein expression of IL-6 in three groups (C). The values are presented in Mean±SD; statistical analysis was carried out by one-way ANOVA Student-Newman-Keuls test, \(p < 0.05\) vs. sham group; \#\(p < 0.05\) vs. IR group.
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**Discussion**

Previous studies\(^{18,19}\) showed that RIPC attenuated renal ischemia-reperfusion injury in animal models. However, the potential mechanisms were not clear yet. This investigation established a kind of rat model of renal IR injury. The spleen ischemic preconditioning was performed by the intermittent clamping of the splenic pedicle to form the ischemic cycles. Previous researches\(^{20,21}\) have demonstrated that the degree of cytoprotection of ischemic preconditioning depends on the dura-
tion time of ischemia and reperfusion cycles of preconditioning. Kapitsinou et al.²² demonstrate that endothelial hypoxia-inducible factor-2 (HIF-2) induced by ischemic preconditioning plays an important role in inducing the inflammatory cells infiltration and adhesion by mediating expressions of cytokines and chemokines like VCAM-1 to suppress IR injury. The duration time of HIF-2 activation and generation determined the protective effect of ischemic preconditioning²³. The cycles of RIPC usually contain 5 min of ischemia and 5 min of reperfusion in previous studies.²⁴⁻²⁶ Nevertheless, there were no standards for the cycle strategy of splenic preconditioning yet.

In this investigation, the spleen was treated by three ischemic preconditioning cycles of 5 min ischemia and 5 min reperfusion. Renal function was assessed serologically and histologically. The results demonstrated that the serum levels of Cr and BUN in the IR group increased significantly compared with the sham group, suggesting that IR caused loss to renal function. The decreased serum levels of Cr and BUN in the sIPC group indicated a protective effect of the ischemic kidneys on renal IR injury.

The typical light microscopic features of renal IR injury are tubular necrosis, tubular dilation, and interstitial cellular infiltration. The degrees of the lesion in different parts are different on one section. In this work, we applied the semi-quantitative score method, which was described by Erdogan et al.,²⁷ to evaluate the histological performance of each group. The pathological fields, including tubular cell necrosis, tubular dilation, and interstitial cellular infiltration were counted. The percentage of the pathological areas, representing for the extent of the renal lesion of each section, was recorded as the injury score. The results showed that the IR group had a higher pathological score than the sham group. However, the pathological score of sIPC group was lower significantly than the sham group, indicating sIPC reduced the damage to the ischemic kidneys. Ischemia-reperfusion can induce inflammatory injury through cytokines and chemokines. Tumor necrosis factor-α (TNF-α), Interleukin-6 (IL-6) are pro-inflammatory mediators which are proved to be closely associated with renal IR.²⁷ TNF-α is one kind of pro-inflammatory mediators induced by ischemia. Moreover, TNF-α has been proved to act as a “dominant regulator” of the cytokine cascade.²⁸

TNF-α, combined with IL-17A, participates in the transcriptional level of IL-6, which is another pro-inflammatory cytokine involved in the immune and inflammatory response to renal IR injury.²⁹ Donnahoo et al.³⁰ have demonstrated that TNF-α expressing in renal tubular cells contributes to the infiltration of various immune cells. IL-6, as was mentioned above, is another pro-inflammatory cytokine and is regarded as a multi-functional mediator. IL-6 is responsible for regulating the expressions of endothelium adhesion molecules and cytokines including TNF-α, with which IL-6 forms a positive feedback cycle of inflammation.¹¹ In our work, the levels of serum TNF-α and IL-6 in the sIPC group were significantly lower than those in the IR group. Meanwhile, the renal expressions of TNF-α and IL-6 in the sIPC group were apparently down-regulated, indicating that sIPC has the capacity to inhibit the secretions and productions of TNF-α and IL-6.

Nuclear factor of kappa B (NF-κB) pathway has been considered as one kind of mechanisms for inflammation-involved lesions. NF-κB is a transcription factor expressed in almost every tissue, regulating the expressions of many pro-inflammatory cytokines including TNF-α and IL-6. Recent studies³²,³³ have demonstrated that NF-κB pathway is responsible for inflammatory damage to the ischemic kidney and the suppression of NF-κB pathway can reduce renal IR injury at the acute stage. In general, NF-κB is located in the cytoplasm in an inactive form of p50-p65 dimer combined with IκB.

When multiple stimulations occur, IκB kinase (IKK) complex is activated and IκB begins to be degraded. IKK complex consists of three sub-units: IKK-α, IKK-β, and IKK-γ (also known as NEMO, NF-κB essential modifier). IKK-α and IKK-β catalyze the phosphorylation of serine residue in IκB and induce the degradation of IκB while IKK-γ works as a regulatory subunit. Without IκB, NF-κB p50-p65 dimer translocated into the nucleus and mediated the expressions of the target genes including pro-inflammatory cytokines.³⁵ Previous studies³⁶,³⁷ have revealed that the activity of NF-κB pathway is labeled as the recruitment of NF-κB subunit p65 to the NF-κB regulating gene promoters.

Interestingly, anti-inflammatory cytokines also mediate the expressions of pro-inflammatory cytokines through the NF-κB pathway. IL-10 is one of the most important anti-inflammatory cytokines secreted by tubular epithelium, monocytes/macrophages and regulatory T (Treg) cells. The expression of IL-10, which was dependent on Treg cells, could decrease TNF-α secretion.³⁸
Wan et al. reported that IL-10/-/- mice endured severer renal ischemia-reperfusion injury with higher levels of TNF-α and IL-6, indicating that IL-10 could depress the expressions of inflammatory cytokines.

The involved mechanism of IL-10 down-regulating pro-inflammatory cytokines is that IL-10 can inhibit IKK activity and delay the degradation of IκB to suppress translocation of p50-p65 dimer in NF-κB pathway with the presence of stimulus from TNF-α. Our results demonstrated that the expressions of IKK and nuclear NF-κB p65 were down-regulated with the increased level of serum IL-10 and the advanced expression of renal IL-10 in the sIPC group. It suggests that sIPC might inhibit the NF-κB pathway activity, and the depression of NF-κB could be associated with the up-regulation of IL-10 expression.

Conclusions

We have demonstrated that splenic ischemic preconditioning can protect kidneys against IR injury by suppressing inflammation. The possible mechanism of this protective effect is generated by inhibiting NF-κB pathway to decrease the expression of pro-inflammatory cytokines. In addition, an up-regulation of IL-10 may be involved in the inhibition to NF-κB pathway. However, there were some limitations in this study. The long-term effects of sIPC are not mentioned, and the details of splenic regulating IL-10 expression are not investigated. These problems need to be solved in the further studies.

Acknowledgements

This work was supported by the National Natural Science Foundation of China (81400753).

Conflict of Interest

The Authors declare that they have no conflict of interest.

References


