

# Evaluating the effect of remote ischemic preconditioning on kidney ischemia–reperfusion injury

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**Background:** Acute kidney injury is a high-risk complication in a variety of clinical situations mostly due to ischemia–reperfusion (IR) injuries. The novel idea of remote ischemic preconditioning (rIPC) was proposed to prevent serious ischemia sequels. To address the controversy of previous reports, the current study was performed to assess the effect of rIPC on kidney IR injury. **Materials and Methods:** Male BALB/c mice were exposed to either rIPC or sham intervention, 24 h before kidney IR. In two independent sets of experiments, rIPC was accomplished by inducing three cycles of 5 min ischemia with 5 min reperfusion intervals through the ligation of the left external iliac artery or infrarenal abdominal aorta. Kidney IR injury was performed by left renal pedicle occlusion for 35 min and simultaneous right nephrectomy. After 48 h, mice were sacrificed for the assessment of kidney function and structure. **Results:** According to the serum urea and creatinine, as well as histopathological measures, none of the exploited rIPC procedures could significantly protect against kidney IR injury. **Conclusion:** Based on our findings and the divergent results of previous animal and human studies, it can be concluded that the renoprotective effects of rIPC are minimal, if any, and are not robustly detectable.

**Key words:** Acute kidney injury, ischemic preconditioning, reperfusion injury

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## INTRODUCTION

Acute kidney injury (AKI) is a common medical problem associated with increased risk of mortality and morbidity, and hence, is of great concern all around the world. Despite considerable attention and effort accorded to finding effective management, the treatments to date are all conservative rather than being curative; thus, the preventive actions are critically important.

Tissue hypoxia is a leading underlying mechanism not only for AKI but also for a variety of other clinical conditions. In 1986, using a canine model of myocardial infarction, Murry *et al.* proposed a novel preventive technique for the ischemic injuries. They claimed that

applying brief episodes of ischemia could protect the tissue from a subsequent sustained insult.<sup>[1]</sup> Since then, the so-called Ischemic Preconditioning (IPC) method is investigated in several other settings and promising outcomes are achieved.<sup>[2–8]</sup> However, its clinical application remains limited due to the unavailability of internal organs for such a procedure.

Interestingly, it was later found that the IPC may be beneficial even when performed on a distant site of the body.<sup>[9]</sup> Although the mechanisms of this effect are largely unknown, it is suggested that this remote IPC (rIPC) may activate hypoxia signaling pathway in target organs via the humoral or neural signaling.<sup>[10]</sup> This feasible, inexpensive, and noninvasive approach has been assessed for the protection from ischemic

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injuries in some medical states such as open cardiac surgeries,<sup>[11]</sup> ischemic strokes in the brain,<sup>[12]</sup> liver surgeries, and transplantations.<sup>[13]</sup> However, the findings regarding the efficiency of this method for kidney protection remain inconclusive; using a rat model, it has been shown that brief small intestinal ischemia episodes can reduce the subsequent kidney ischemia injury.<sup>[14]</sup> Similarly, Zimmerman *et al.* found that rIPC is significantly effective on the incidence of AKI after cardiac bypass surgery.<sup>[15]</sup> Conversely, creatinine clearance and tubular damage were not different between rIPC and sham group in another rat model.<sup>[16]</sup> Furthermore, in a randomized clinical trial by Rahman *et al.*, rIPC did not change the renal function after cardiac bypass surgery.<sup>[17]</sup> To approach this controversial issue, this study is designed to assess the protective effect of rIPC for AKI using a mouse model of ischemia–reperfusion (IR) injury.

## MATERIALS AND METHODS

### Animals

Male BALB/c mice at the age of 6–8 weeks were obtained from Pasteur Institute of Iran. The study was accepted by the Ethical Committee of Isfahan University of Medical Sciences. It was conducted based on the institutional guides for the care and use of laboratory animals. The animals had free access to food and water with normal light cycle. Pain and distress were controlled before surgical procedures and scarifications through appropriate administration of anesthetics and analgesics.

### Kidney ischemia–reperfusion model

Following anesthetization by intraperitoneal injection of 115 mg/kg ketamine and 11.5 mg/kg xylazine (Alfasan, Woerden, Netherlands), the mice were put supine on a warm stage at a temperature of 37.5°C. After covering the eyes with tetracycline ointment, the kidneys were exposed via a mid-abdominal incision in a sterile condition. The left kidney pedicle was occluded for 35 min with an atraumatic vascular clamp (Medicon, Tuttlingen, Baden-Württemberg, Germany), after which the clamp was removed and reperfusion was confirmed by observing tissue color change. During left kidney ischemia time, right nephrectomy was done following double ligation of the pedicle and ureter with a 4/0 silk. Finally, abdominal muscles and skin were sutured and 1 ml of dextrose saline serum was injected subcutaneously to avoid dehydration. For sham operation, the same procedure was followed except that the left pedicle was not occluded, and instead, the clamp was adjusted under the pedicle for 35 min.

### Remote ischemic preconditioning

The mice were anesthetized and operated on the warm stage, as described above. Two different techniques were used for rIPC. In the first method, after prepping and

draping, the left inguinal skin was incised longitudinally and the iliac artery was isolated from the surrounding soft tissues just above the inguinal ligament. Then, the external iliac artery was clamped intermittently for 3 episodes of 5 min, each time followed by a period of 5 min reperfusion. The arterial occlusion was confirmed by visual inspection of limb muscle color change. The sham surgery was done in the same way, but the artery was just touched by the clamp instead of being occluded. Finally, the skin was sutured using 4/0 silk, 1 ml of dextrose saline serum was injected subcutaneously, and the mice were allowed to recover. For the second rIPC technique, abdominal aorta was accessed via mid-abdominal incision and was clamped below the renal arteries derivation. The time schedule for ischemia and reperfusion episodes was the same as the first technique.

Twenty-four hours after rIPC operation, mice were subjected to kidney IR procedure, as described above. Forty-eight hours after kidney IR, the animals were anesthetized and blood samples were collected from orbital sinus, as described previously.<sup>[18]</sup> Next, they were sacrificed by cervical dislocation, and the left kidneys were harvested and kept in buffered formaldehyde solution. After blood coagulation at room temperature, two rounds of centrifugation at 6000 rpm for 6 min were performed and the isolated serum samples were kept at –20°C until biochemical analyses.

### Biochemical measurements

Urea measurement was performed using a urea kit (Pars-Azmun, Tehran, Iran) according to the manufacturer instructions. Optical densities were measured at 578 nm using a spectrophotometer (UNICO, Dayton, Ohio, USA). Creatinine concentration was measured via enzymatic procedure by the Cobas Integra Analyzer (Roche, Indianapolis, Indiana, USA).

### Histopathological investigations

Five micrometer thick sections were prepared from formalin-fixed paraffin-embedded kidneys and hematoxylin and eosin staining was performed. The slides were assessed in a blinded manner and the average number of hyaline casts per high-power field was determined by inspecting 40 random cortical fields. Furthermore, based on the previous studies,<sup>[19]</sup> with some modifications, an injury score ranging between 0 and 300 was assigned to each kidney section; 100 tubules in cortical field were randomly examined using ×40 objective and a number between 0 and 3 was assigned to each tubule (0: normal histology, 1: tubular cell swelling, brush border loss, and nuclear condensation, with loss of up to one third of the tubule nuclei, 2: same as for score 1, but more than one-third and less than two-thirds of the tubular profile showing nuclear loss, and 3: more than two-thirds of the tubular profile showing nuclear loss).

**Statistical analysis**

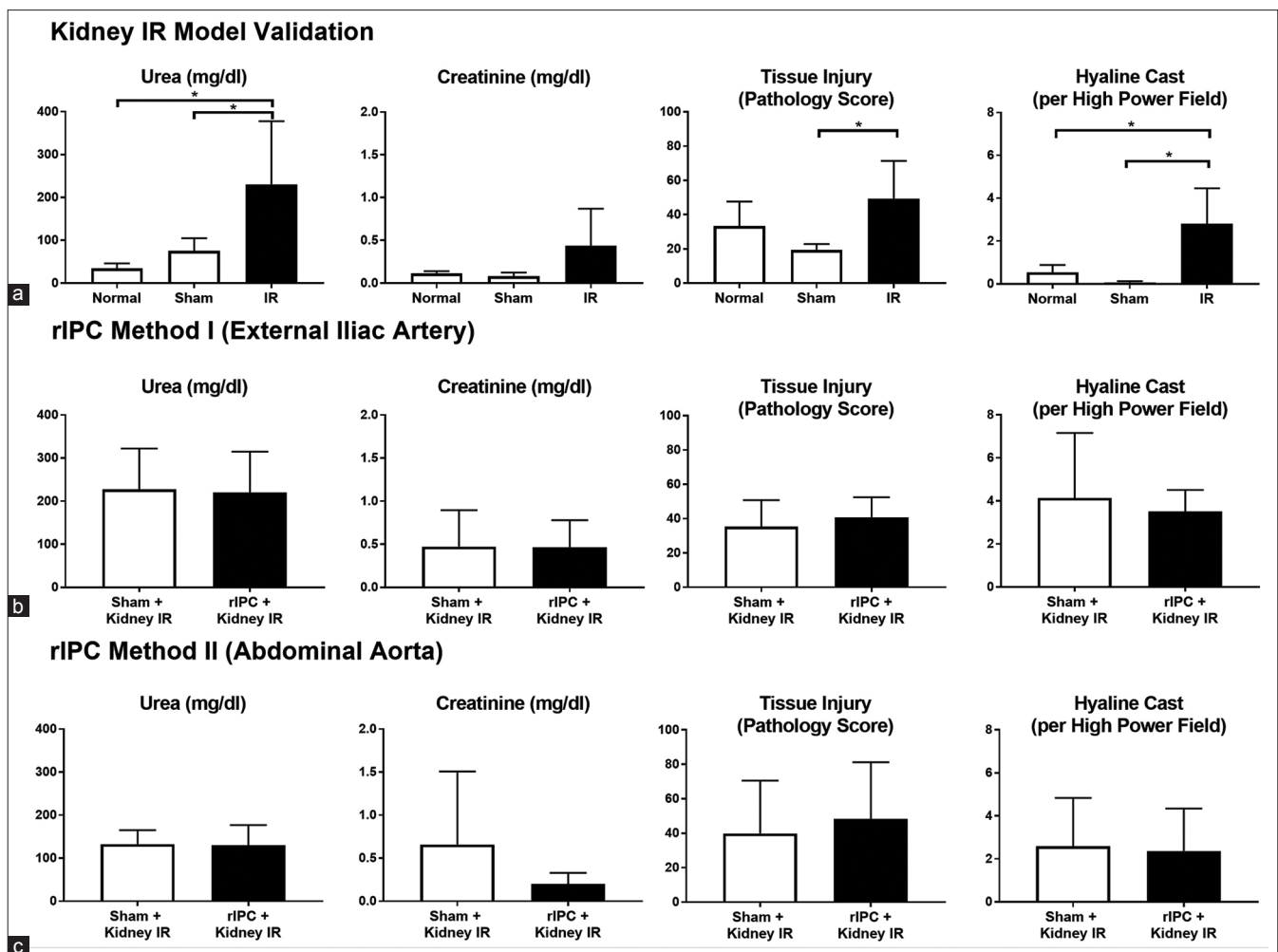
Data are described as mean ± standard error of the mean (SEM). For the statistical analysis, GraphPad Prism 5.01 (GraphPad Software, San Diego, USA) was used. Mann–Whitney U-test was applied to compare the groups. The statistical significance level was considered 0.05.

**RESULTS**

Temperature and ischemia time are the two determining parameters in tissue IR injury.<sup>[20]</sup> According to our previous works,<sup>[21]</sup> the mouse model of kidney IR was established by inducing 35 min of left kidney warm ischemia, with right nephrectomy being simultaneously conducted to reduce the variations. Six mice were subjected to either kidney IR or sham surgery and were compared to six untreated normal animals. Subsequent biochemical and histopathological assessments confirmed the validity of the model [Figure 1a].

To assess the protective effect of rIPC procedure, 16 mice were randomized to be subjected to either rIPC or sham surgery, followed by kidney IR injury 24 h later. The rIPC operation was performed by applying three cycles of 5 min external iliac artery ligation with 5 min of reperfusion intervals. The animals were sacrificed 48 h after kidney IR surgery. rIPC did not improve kidney function according to the serum urea, creatinine, pathology score, and the number of hyaline casts [Figure 1b].

To assess whether the above unsuccessful observations are due to the technical issues, another rIPC procedure was employed. Hence, 20 mice were exposed to either abdominal aorta rIPC or sham interventions, and both the groups were subjected to kidney IR 24 h later. Although serum creatinine showed a nonsignificant decline in rIPC compared to sham, the other biochemical and histopathological parameters were almost the same in both the groups [Figure 1c]. Therefore, none of the rIPC methods assessed in this study were shown to be renoprotective.



**Figure 1:** Remote ischemic preconditioning did not protect the kidney against ischemia–reperfusion injury. The kidney ischemia–reperfusion model was validated by performing biochemical and histopathological measurements in untreated normal (n = 5), sham (n = 3), and ischemia–reperfusion operated (n = 3) mice (a). Twenty-four hours before kidney ischemia–reperfusion, rIPC procedure was carried out by inducing three cycles of 5 min intermittent ischemia and reperfusion to either external iliac artery (b) or the infrarenal abdominal aorta (c). The asterisks indicate P < 0.05. Data are mean ± standard error of mean

## DISCUSSION

AKI is a critical medical complication affecting a large number of in-hospital patients. Its occurrence is highly predictable since it is mostly followed by some certain medical interventions such as coronary artery bypass graft surgery and contrast agent administration. Therefore, the preventive strategies have been given considerable attention in recent years. rIPC is a simple, noninvasive, and inexpensive method that was first introduced to protect tissues against ischemic injuries. However, subsequent studies could not provide satisfying evidence on its efficacy, resulting in a call for further investigations.

In the current study, rIPC did not confer protection against kidney ischemic injury. Given their accepted vulnerability to ischemic injuries,<sup>[22]</sup> we conducted kidney IR surgery using male BALB/c mice by clamping the renal artery for 35 min at 37.5°C. This warm ischemia could be a proper simulation for the acute hypoxia followed by the on-pump cardiovascular surgeries. Our timing program in the rIPC procedure was derived from the previous successful studies.<sup>[23,24]</sup> First, we have tried rIPC on the left external iliac artery, and then, following the negative results, we wondered whether enlarging the territory of the ischemic region could provide more protective factors against the subsequent kidney injury. Therefore, we repeated the experiments by performing rIPC on the abdominal aorta. However, the postoperative measurements did not reveal any protection even with this Protocol.

Our data are just a small part of the controversy on rIPC efficacy. The diversity in the rIPC protocols can describe part of the divergent reported data regarding this procedure; The type of ischemia, which can be continuous or intermittent, the number and duration of the ischemia episodes in the intermittent type, and the period between the rIPC and IR surgery varies highly among the studies.<sup>[25]</sup> Moreover, the animal strain and gender, as well as the site of preconditioning, are influential factors. It is shown that male animals compared to females and mice compared to rats benefit more from rIPC.<sup>[23,26]</sup> These conflicting reports are not limited to animal studies; in two recent multicentric, sham-controlled, randomized clinical trials, which were both conducted on large numbers of coronary artery bypass graft patients, the postoperative kidney outcomes were controversial in spite of very similar experiment protocols.<sup>[27,28]</sup> Both the studies assessed the occurrence and severity of AKI based on the same criteria 72 h after the cardiac surgery. Therefore, it is wise to assume that the putative beneficial effects of rIPC are not profound and robust and so can be detected only in certain conditions leaving it a nonpromising preventive strategy.

A key to the riddle of rIPC is to distinguish the molecular mechanisms of this phenomenon. In the previous studies, several molecules have been proposed to mediate the rIPC effects via humoral and neural pathways.<sup>[26]</sup> Furthermore, kidney transcriptome<sup>[29]</sup> and plasma proteome<sup>[30]</sup> have been profiled to identify differentially expressed genes in response to rIPC. In spite of these efforts, the elements which mediated the protection of the remote tissue remain largely unknown. Identifying these molecules may pave the way toward optimizing rIPC protocols and also to discovering novel therapeutic small molecules.

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### Conflicts of interest

There are no conflicts of interest.

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