Kidney remote ischemic preconditioning as a novel strategy to explore the accurate protective mechanisms underlying remote ischemic preconditioning

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Abstract: *Introduction:* This study reports a novel strategy for investigating the key factors responsible for the protective effect of remote ischemic preconditioning (RIPC) against renal ischemia-reperfusion (IR) injury, which remains the leading cause of the acute kidney injury that increase the morbidity and mortality in patients with renal impairment. *Methods:* The renal blood flow of the right kidneys in kidney remote ischemic preconditioning (KRIPC) group was occluded for 20 min. After 48 h, the renal blood flow of the left kidneys of both KRIPC and IPC groups was occluded for 30 min, and mice were dissected after 7 days of the last surgery. Blood samples were analyzed by an animal blood counter. The levels of creatinine, urea nitrogen, lipid peroxidation, nitric oxide (NO), and high-density lipoproteins (HDLs) were estimated in the plasma of mice. Kidney slices were stained with 2% triphenyltetrazolium chloride (TTC) to estimate the renal infarction. *Results:* Unlike KRIPC group, data from IPC group revealed a massive reduction in neutrophils count, a significant increase in creatinine, urea nitrogen, and HDLs levels, and an increase in the renal infarction compared with control group. *Conclusion:* This is the first study demonstrating KRIPC as a novel and applicable model with the goal of defining the accurate protective mechanisms underlying RIPC against IR injury.

Keywords: acute kidney injury, kidney remote ischemic preconditioning, remote ischemic preconditioning, neutrophils, creatinine

Introduction

Remote ischemic preconditioning (RIPC) is widely defined as a strategy of protection for organs against ischemia-reperfusion (IR) injuries by applying brief episodes of IR in a distant organ [1] or in the same organ. Since brief episodes of IR elicit bursts of oxygen-derived free radicals, which in turn triggers the endogenous and exogenous antioxidants that act as free radical scavengers [2]. Despite the fact that RIPC has been extensively studied, yet such sincere and sustained efforts to understand the mechanism(s) of its protection against tissue damages are met with roadblocks, for many reasons discussed below. Ischemia is a restriction in blood flow to tissue that prevents adequate delivery of oxygen, glucose, and other nutrients to an organ. This initiates hypoxia, which is the lacking of oxygen concentration in the tissues or in the ambient tissue below normal. In fact, the ischemic period associates with an interruption in the distribution of ions across the cell membrane, which increases the accumulation of these ions in the intracellular space. Thus, the extracellular fluid and plasma water will withdraw into the cytosol, causing cell swelling, and edema associated with an increase in the blood viscosity. During ischemia, anaerobic cellular respiration prevails, thus the cells produce lactic acid instead of pyruvate, which is produced under the normal physiological conditions. So, the cellular elasticity decreases due to the elevation of acidosis and changes in pH [3] and may cause capillary flow obstruction. A worth note is that the

formation of lactate adds a further stress on the liver to deter such toxic product. After its formation, lactate is transported to the liver and converted to pyruvate through the pathway of gluconeogenesis in the pretense of oxygen during reperfusion. Furthermore, due to ATP depletion, cells must resort to yet other sources of energy to offer the minimal level of energy for their activities. Purine is one of these alternative sources of energy in the cell, which produce hypoxanthine during its catabolism. When oxygen becomes available during reperfusion, hypoxanthine could be oxidized to xanthine oxidase, which generates superoxides ions (O_2^-) [4]. On the other hand, neutrophils, the most abundant leukocytes in circulation, are the main source of reactive oxygen species (ROS); thus, they play a marginal role in the inflammatory cascade [5]. After their activation, neutrophils adhere to endothelial cells of the blood capillaries with the aid of specific adhesion molecules, thus they could plug capillary, leading to a vascular congestion [4]. In this context, neutrophils migration requires a temporal and spatial regulation of intracellular signaling pathways allowing the neutrophil to detect a gradient of attractant, to polarize [6], and to migrate toward the highest concentration of the chemoattractant at the injured tissue. Infiltration and degranulation of neutrophils trigger release of proteases and myeloperoxidase in addition to the generation of ROS, which can exaggerate damage in the endothelial and epithelial cells of the outer medulla. On the other regard, the inflammatory cascade, triggered by innate and adaptive immune systems, has been speculated as the major pathogenic mechanism of the renal IR injury [7]. The inflammatory immune response mediated by the tubular cell death, associative oxidative stress, and mitochondrial dysfunction has been also identified as key elements driving the pathophysiology of acute kidney injury [8].

In fact, RIPC is widely and extensively studied to investigate the underlying mechanisms of its action with the goal of finding new therapeutic approaches that would imitate ischemic preconditioning. Some studies have applied brief episodes of IR in distant organ(s) (e.g., hind limbs) before induction of ischemia on the target organ(s) (e.g., kidney, heart, and brain) [9, 10]. Other studies have applied cycles of short periods of IR directly on the target organ before applying a prolonged period of sustained occlusion followed by reperfusion [11]. It is believed that neither RIPC in the distant organ(s) nor ischemic preconditioning on the same organ could provide a clear picture about the mechanism(s) of the protection. Unlike other organs, kidneys are sensitive and have their unique and varied structures that aside their complicated microvasculature. Thus, in this study, kidney remote ischemic preconditioning (KRIPC) is used as a novel strategy to better understand the underlying mechanisms of how RIPC offers protection, at least in part, against renal IR injury and to explore the changes in the microenvironment of the injured kidney due to short bouts of IR and thus define factors that may be responsible for the induction of renal IR injury.

Materials and Methods

Animal care

In this study, 30 male albino mice (mass 20 ± 5 g) were acclimated in the laboratory for 2 weeks under the same natural environmental conditions of temperature and photoperiod and with free access to food and water. All procedures along with the surgical protocols were in accordance with the protocol of National Animal Care and Use Committee and Guidelines for the Care and Use of Experimental Animals.

Preparation of mice for surgery

Mice were divided into three groups (10 mice in each group): KRIPC group, IPC group, and a sham group. Mice were anesthetized with an intraperitoneal injection of a fresh combination of ketamine (80 mg/kg) and Xyla-Ject® (xylazine, 12 mg/kg). After 5 min, the depth of anesthesia was determined by touching the medial corner of the eye and by testing the withdrawal response by applying pressure with a fingernail to the back foot of the animal. As the body temperature in mice may decrease by several degrees following administration of ketamine and xylazine [12], the surgery was carried out in a room with hot air condition for the stabilization of the body temperature (36.8–37 °C). Before surgery, the body temperature of each mouse was determined by using a rectal probe to assure that it is stable, and the frequency of breathing rate was monitored to ensure that it is of adequate depth and normal.

Surgery

The right side of the abdomen is depilated and cleaned with 70% ethanol, and fur in the dorsal side of abdomen was removed by using a depilatory agent to avoid dermatitis or chemical irritation (One® cream, Eva Cosmetics Co., Cairo, Egypt). Beneath the right lower rib, the dorsal skin was opened with a paramedian incision, approximately 1 cm, and the renal pedicle was carefully dissected with fine-point tweezers to remove the perihilar adipose tissue. After liberating the kidney from surrounding tissue, it was carefully pushed outside with an elastic forceps along with the adrenal gland, located at the upper pole of the kidney. The fact that adrenal glands have its own blood supply should be considered here, as the mice will die if glands are removed or injured. For KRIPC group, the unilateral renal blood flow of the right kidney

was occluded for 20 min (four cycles of 5 min of occlusion-reperfusion) by clamping a nontraumatic vascular clamp over renal pedicles. The vascular clip was released every 5 min for allowing reperfusion. Occlusion was verified visually by a change in the color of kidney to a paler shade and reperfusion by a blush. After the fourth cycle of ischemia, the vascular clip was released, and the kidney was gently pushed back to its intraperitoneal place, then each of the muscular layer and skin were sutured separately. After 48 h of the first surgery, the second surgery was performed with the same surgical procedure as described above. For the left kidney of KRIPC and IPC groups, the unilateral renal blood flow was occluded for 30 min (six cycles of 5 min of occlusion-reperfusion). A sham group was involved and subjected to the exact same surgical procedure, aside from clamp placement. A non-absorbable, silk black braided 4–0 suture (German Medical Solutions (GMS), Cairo, Egypt) was used for suturing the muscle layer, followed by closing of the skin. A 0.5-mL warm saline (37 °C) was injected intraperitoneally to restore the fluid loss during surgery. Wounds were cleaned with betadine every other day followed by a sufficient cleaning with moist sterile cotton to remove any remnants of betadine, and the mice were dissected after 7 days of the last surgery. *Figure 1* concluded the experimental protocols of this study.

Blood cells count

Freshly blood samples collected by cardiac puncture were used to analyze leukocyte counts using a blood counter at the Center of Excellence for Cancer Research (CECR), Tanta University.

Biochemical analysis

The level of high-density lipoproteins (HDLs) was determined according to the method described by Burstein et al. [13]. The concentration of nitric oxide (NO) was measured with a Griess assay reagent as previously described [14]. The levels of lipid peroxidation, creatinine, and blood urea nitrogen (BUN) were determined by the commercially available kits (Bio-Diagnostic Co., Cairo, Egypt).

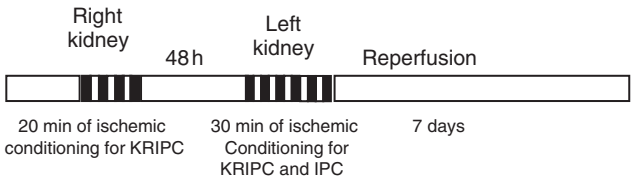

Fig. 1. Experimental protocols. Mice in the KRIPC group underwent a surgical protocol of the unilateral renal IR for the right kidney, a conditioning stimulus comprising four cycles of 5 min of ischemia (black bars) and 5 min reperfusion (white bars). After 48 h, mice in KRIPC and IPC groups were subjected to the unilateral renal IR for the left kidney, a conditioning stimulus comprising six cycles of 5 min of IR

Morphological analysis

The kidneys were cut into 2-mm thick slices and weighed, then incubated with 2% triphenyltetrazolium chloride (TTC) at 37 °C for 15 min, with rotation for every 2 min to allow uniform tissue staining, and each slice was photographed with a digital camera. All slices were photographed from both sides by a color CCD camera (ELPH 100 HS, Canon 12.1, Japan). In each slide, the infarct area (unstained by TTC) was analyzed using ImageJ software (National Institutes of Health, Bethesda, MD, USA) for the planimetry of the kidney and infarct region on TTC images. Infarct volume was calculated as a relative percentage of the total kidney volume.

Statistical and data analysis

The data were analyzed with SigmaPlot 10 software (Systat Software Inc., San Diego, CA, USA) and Prism 3.0 package (GraphPad Software Inc., San Diego, CA, USA). One-way analysis of variance Newman–Keuls multiple test was used as a *post hoc* comparison test. The threshold for statistical significance was set at $P < 0.05$.

Results

Renal infarction

As shown in *Fig. 2*, kidney slices (2 mm thickness) were stained with 2% TTC. The renal infarction volume of the left kidney in the IPC group was $21.83\% \pm 2.16\%$, whereas in the KRIPC group, it was $4.47\% \pm 2.05\%$ *(Fig. 3)*, which was occasionally detected.

Leukocyte counts

The percentage of neutrophils count from the blood samples in the IPC group was significantly declined ($13.8\% \pm 4.63\%$) compared with the control ($34.4\% \pm 6.63\%$) and KRIPC ($40.9\% \pm 3.63\%$) groups *(Fig. 4)*. In contrast, the percentage of basophils count was prominently increased in the IPC group ($33.3\% \pm 6.6\%$) compared with the control ($6.4\% \pm 2.6\%$) and KRIPC ($0.5\% \pm 09\%$) groups.

Biochemical analysis

Data show that the level of HDLs was significantly increased from 17.02 ± 1.25 μmol/L in the control

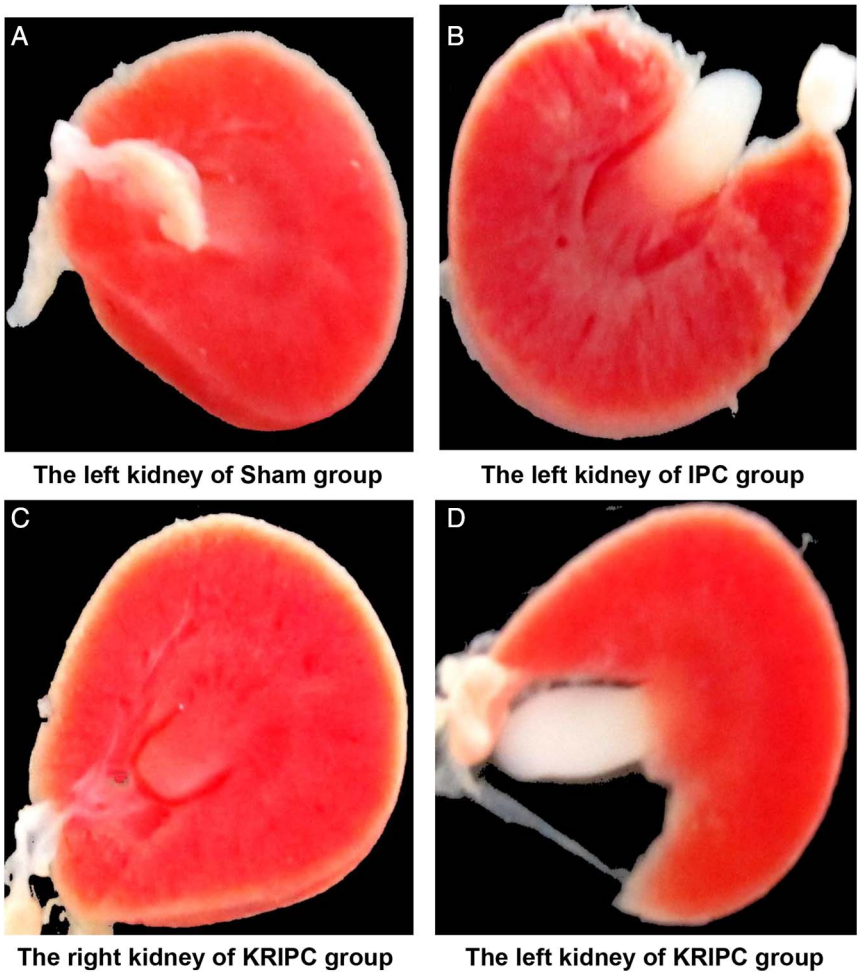

Fig. 2. Slices of the left kidney of the sham (A) and ischemic preconditioning (IPC) (B) groups, and the right (C) and left (D) kidneys of the kidney remote ischemic preconditioning (KRIPC) group. Kidney slices (2 mm thickness) were stained with 2% triphenyltetrazolium chloride (TTC) dissolved in saline solution and incubated for 15 minutes at 37 °C then photos were taken

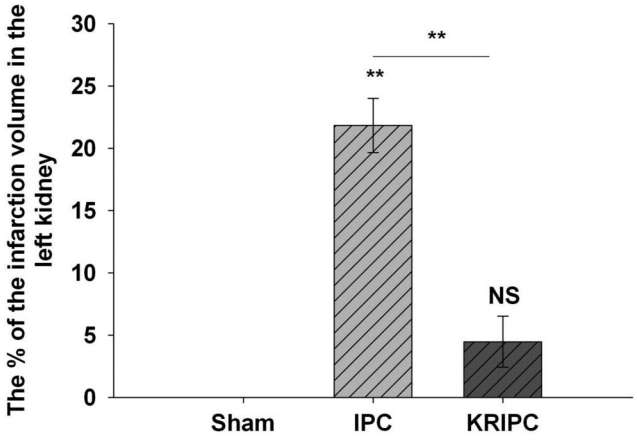

Fig. 3. The percentage of the left kidney infraction volume. The infarct area (unstained by TTC) was analyzed using ImageJ software (National Institutes of Health) for the planimetry of the kidney and infarct region on TTC images. Infarct volume was calculated as a relative percentage of total kidney volume. $^{**}P < 0.01$ and NS: statistically non-significant

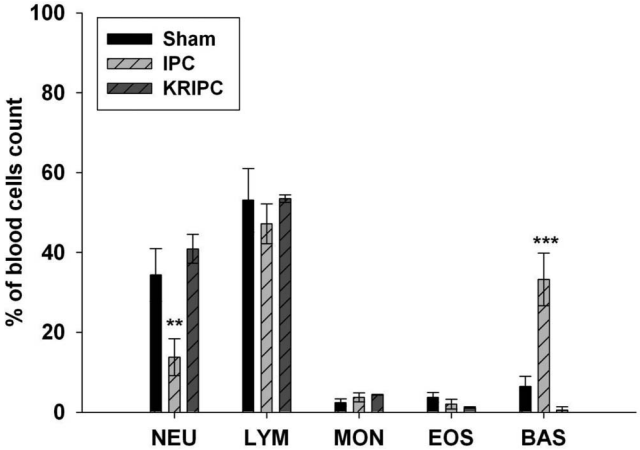

Fig. 4. Leukocyte counts in fresh blood samples collected from different groups involved in this study. $^{**}P < 0.01$ and $^{***}P < 0.001$

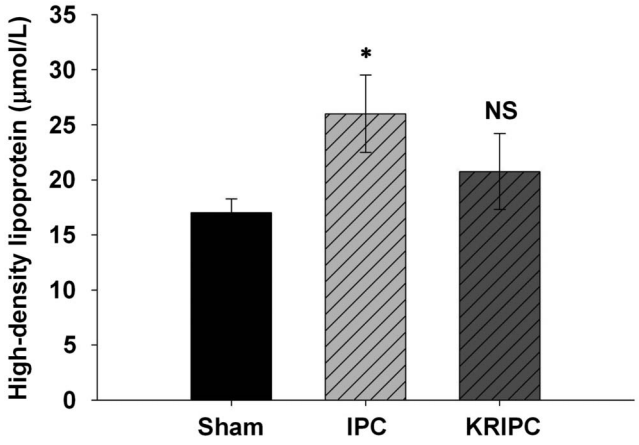

Fig. 5. The level of HDLs in the plasma of mice involved in this study. $*P < 0.05$ and NS: statistically non-significant

group to 26 ± 3.51 µmol/L in the IPC group *(Fig. 5)*. Compared with the control group, the level of HDLs was insignificantly increased in KRIPC group (20.75 ± 3.44 µmol/L). On the other hand, the level of lipid peroxidation was 1.58 ± 0.08, 2 ± 0.12, and 1.79 ± 0.08 nmol/mg protein in the control, IPC, and KRIPC groups, respectively. Data show a significant increase in the level of lipid peroxidation in IPC group compared with the control group *(Fig. 6A)*. In contrast, the level of NO was declined from 202.6 ± 7.52 µmol/L in the control group to 122.8 ± 6.93 in the IPC group *(Fig. 6B)*. Although the level of NO was significantly decreased in KRIPC group (178.4 ± 8.76 µmol/L) compared with the control group, it was significantly increased in KRIPC group compared with IPC group. As shown in *Fig. 7A*, the level of creatinine was prominently increased in the IPC group (0.55 ± 0.06 mg/dL) compared with the control group (0.22 ± 0.07 mg/dL).

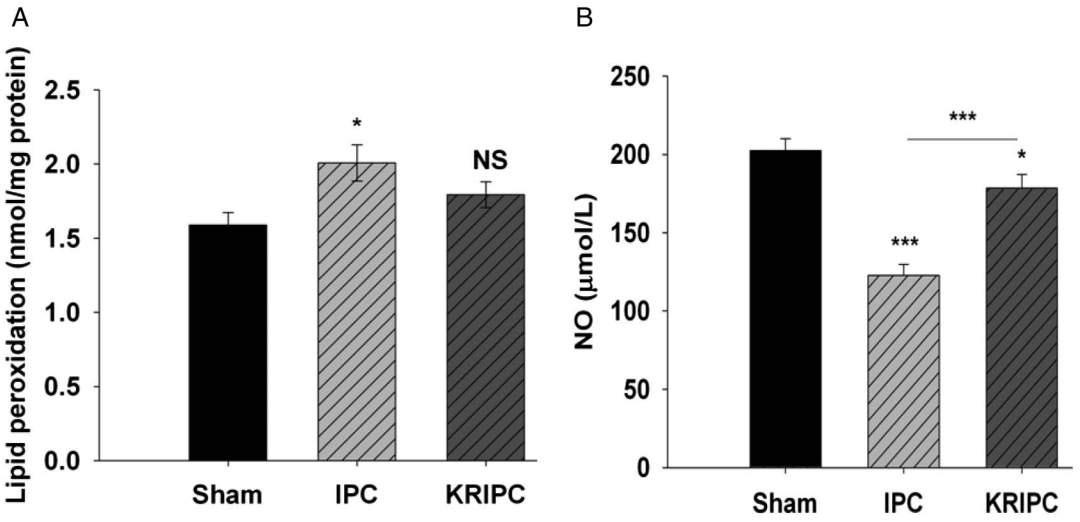

Fig. 6. The level of lipid peroxidation (A) and NO (B) in the plasma of different groups involved in this study. $*P < 0.05$, $**P < 0.01$, and $***P < 0.001$, NS: statistically non-significant

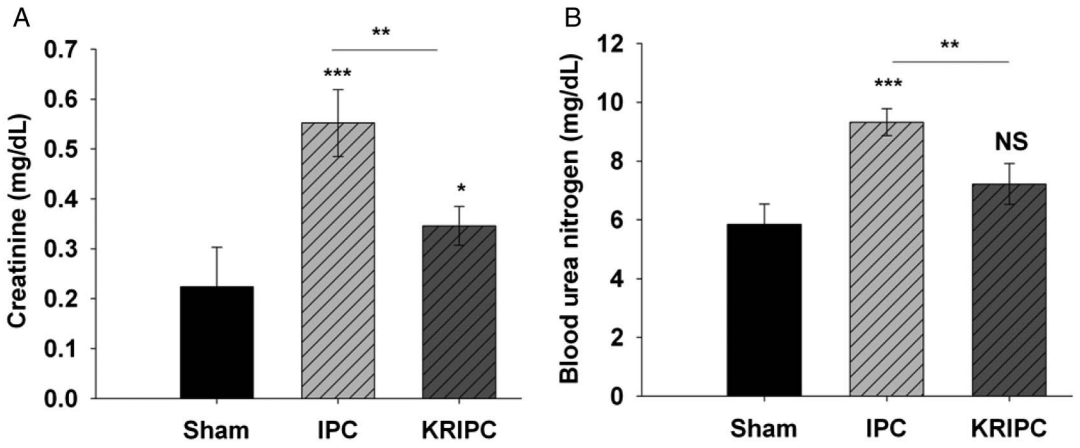

Fig. 7. The level of creatinine (A) and BUN (B) in the plasma of different groups involved in this study. $*P < 0.05$, $**P < 0.01$, and $***P < 0.001$, NS: statistically non-significant

Compared with IPC group, the level of creatinine was significantly declined to 0.34 ± 0.03 mg/dL. The level of BUN was significantly increased in the IPC group (9.32 ± 0.45 mg/dL) compared with the control group (5.85 ± 0.68 mg/dL). Compared with its level in IPC group, the level of BUN was significantly decreased to 7.22 ± 0.69 mg/dL *(Fig. 7B)*.

Discussion

Although the rapid initiation of reperfusion is the most effective way to reduce the cellular death due to ischemia, reperfusion itself can cause a serious cellular damage [15]. Neutrophils have emerged as the main inducer for tissue injuries after reperfusion [16, 17]. Additionally, neutrophils are the main source for ROS that cause irreversible damaging effects, which are amplified upon reperfusion. The generation of ROS impairs each of the mitochondrial and enzymatic activities, and triggers lipid peroxidation, leading to DNA damage finally. Despite that the short bouts of ischemia produce ROS, remains that it can be resolved by free radicals and other antioxidants such as NO [18]. In contrast, the long bouts of ischemia increase the production of ROS [19], which in turn stops NO production and deters antioxidant enzyme activities. In this study, in the contrary of NO, the level of lipid peroxidation was significantly increased in IPC group, but this increase was significantly attenuated in KRIPC group. The elevation of ROS, the decline of NO production, and the reduction of the antioxidant enzyme activities, collectively cause endothelial cells damage, loss of barrier integrity, and enforce the release of ROS into the extracellular matrix, thereafter.

Although the gold standard to assess renal function is creatinine, questions have been raised regarding its reliability as the impact of tubular creatinine excretion on creatinine clearance is even larger in mice than in humans [20]. Therefore, other outcome measures, such as BUN and/or renal histology, may also be of great value when translating animal study results to the human setting [21]. In this study, the level of creatinine was increased in IPC group, reflecting a renal atrophy. As creatinine does not bind to plasma proteins, it is freely filtered by the glomerulus of the kidney [22]. Similarly, the level of BUN was significantly increased in IPC group and declined in KRIPC group. It is noticeable that BUN is a protein metabolism product, which can be used as indicator for the excessive breakdown of protein. These data together reflect that KRIPC effectively provide renal protection against IR injury.

Despite both IPC and KRIPC groups were exposed to the ischemic conditions, the percentage of neutrophils in the peripheral blood was significantly declined in the IPC group. In contrast, in the KRIPC group, neutrophils returned to their normal percentage in the peripheral blood. This reflects the ability of HDLs to prevent neutrophils migration under ischemic conditions, which may attributed to the antielastase activities of HDLs. Elastase is an important mediator of endothelial layer permeability [23–25]. The antielastase function of HDLs was recently reported [26] and pointed as a novel protective effect of these particles in pathologic conditions [27]. As HDLs may be able to transport alpha-1 antitrypsin into the cells, it can frustrate the deleterious effects of intracellular elastase [28]. In contrast to previous findings [29, 30], data of this study are in agreement with a recent study that demonstrated ischemic conditions are not sufficient to induce an increased permeability [27]. Taken together, data show that ischemia triggers neutrophil recruitment cascade as reflected by the massive reduction in neutrophils counts in the peripheral blood, which may due to the massive migration of neutrophil to the injured kidney due to IR.

Conclusion

This is the first study demonstrating KRIPC as a novel and applicable model for the experimental ischemia and stroke studies. In this study, data of the IPC group revealed that ischemic conditions trigger neutrophils recruitment to the injured tissue, but that may not the general phenomenon as reflected by the KRIPC model. As ischemic conditions were not sufficient to increase the vascular permeability and then allow neutrophils to migrate to the injured kidney, this strategy could be useful to define the intrinsic factors responsible for the initiation of the renal IR injury. This could provide new diagnostic and therapeutic approaches, which would be helpful in our battle against chronic kidney disease and renal failure.

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Authors' contribution: MJT designed the study, operated surgery, performed the sequence alignment of biochemical and morphological analysis, the statistical analysis, wrote and revised the manuscript.

Conflict of interest: The author declares no conflict of interest.

Ethics: This article does not contain any studies with human subjects performed by the author. For animal subjects, all the procedures were in accordance with the protocol of National Animal Care and Use Committee and Guidelines for the Care and Use of Experimental Animals.

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