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The protective effects of ischemic preconditioning on rats with renal ischemia-reperfusion injury and the effects on the expression of Bcl-2 and Bax

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Abstract. The aim of the present study was to investigate the protective effects of ischemic preconditioning on rats with renal ischemia-reperfusion injury and the effects on the expression of Bcl-2 and Bax. Thirty-six SD rats were randomly divided into three groups (n=12) including sham operation (S) group, ischemia-reperfusion group (I/R) group and ischemic preconditioning (IP) group. After anesthesia with intraperitoneal injection of chloral hydrate, bilateral renal pedicles were clipped for 45 min, followed by perfusion for 6 h to establish the I/R model. Both kidneys in rats of S group were separated and exposed for 45 min, but renal pedicles were not clipped. In IP group, bilateral renal pedicles were clipped for 5 min, followed by perfusion for 5 min, this procedure was repeated 3 times. Then bilateral renal pedicles were clipped for 45 min, followed by perfusion for 6 h. Blood samples were collected and rats were sacrificed to collect renal tissue. Levels of serum creatinine (Cr) and blood urea nitrogen (BUN) were measured. Activity of superoxide dismutase (SOD) was measured by xanthine oxidase assay. Degree of renal injury was evaluated by H&E staining. TUNEL kit was used to detect the number of apoptotic cells in renal tissue. Expression levels of Bcl-2 and Bax were detected by semi-quantitative PCR and western blot analysis at mRNA and protein levels, respectively. Results showed that levels of Cr and BUN in I/R and IP groups were significantly higher than those in S group, and levels of Cr and BUN in I/R group were significantly higher than that in IP group ($P<0.05$). Activity of SOD in I/R group and IP group were significantly lower than those in S group, and activity of SOD in I/R group were significantly lower than those in IP group ($P<0.05$). H&E staining showed that, compared with S group, renal injury in the I/R and IP groups was more serious than that in the S group, and I/R group was more serious than the IP group ($P<0.05$). TUNEL apoptosis assay showed that number of apoptotic cells in IP and I/R groups were significantly higher than that in the S group ($P<0.01$). Semi-quantitative PCR and western blot analysis showed that, compared with the S group, expression levels of Bcl-2 mRNA and protein were significantly decreased, expression levels of Bax mRNA and protein were significantly increased, and the ratio of Bcl-2/Bax was significantly decreased in the IP and I/R groups ($P<0.01$). Compared with the I/R group, expression level of Bcl-2 was significantly increased, the level of Bax was significantly deceased, and the ratio of Bcl-2/Bax was significantly increased in the IP group ($P<0.01$). As a result, ischemic preconditioning can protect rats with renal ischemia-reperfusion injury possibly by increasing the expression level of Bcl-2 and decreasing the expression level of Bax.

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Introduction

As a common renal disease in clinical practice, acute renal injury is caused by renal ischemia-reperfusion. Ischemia/reperfusion is the determinant factor for the early functional recovery of renal transplantation (1,2). As a highly perfused organ, kidney is vulnerable to ischemic injury. Studies have shown that renal ischemia for more than 20 min will cause damage, ischemia for 20-40 min is reversible, but reperfusion after ischemia for more than 40 min can cause permanent damage (3). Studies have shown that the development of tolerance of organs to ischemia can inhibit or reduced the ischemia-reperfusion injury (4). Takahashi-Sato *et al* (5) carried out a study to explore the effects of different preconditioning and postconditioning methods on ischemia/reperfusion in the heart. They found that postconditioning had a protective effect on reperfusion after cardiac ischemia (6-8). However, there is no research on the effect of preconditioning and postconditioning on renal ischemia-reperfusion. It was found (9) that Bcl-2 and Bax were involved in the process of ischemia-reperfusion in the heart, and Bcl-2 and Bax may be related to the protective effect of postconditioning on ischemia-reperfusion injury in the heart. In this study, rat ischemia-reperfusion model was established

to investigate the protective effects of preconditioning on rats with ischemia-reperfusion, and to test whether Bcl-2 and Bax were involved in that process. This study aimed to provide new insights into the pathogenesis and clinical treatment of renal ischemia-reperfusion injury.

Materials and methods

Instruments and materials. Creatinine (Cr) assay kit (R&D Systems, Minneapolis, MN, USA); blood urea nitrogen (BUN) assay kit (R&D Systems); xanthine oxidase assay kit used to determine the activity of superoxide dismutase (SOD; R&D Systems); TUNEL kit (R&D Systems); dimethyl sulfoxide (DMSO; Sigma-Aldrich, St. Louis, MO, USA), TRIzol kit (Invitrogen, Carlsbad, CA, USA), reverse transcription kit (Invitrogen); rabbit anti-Bax-beta, rabbit anti-Bax and rabbit anti-GAPDH primary antibodies, and horseradish peroxidase labeled anti-rabbit secondary antibody (Cell Signaling Technology, Danvers, MA, USA); ECL luminescent substrate (Invitrogen); color developing powder (Invitrogen); pipettes (Eppendorf, Hamburg, Germany); PCR instrument (Applied Biosystems, Inc., Foster City, CA, USA); UV imaging system (Biometra GmbH, Göttingen, Germany); electronic balance (Sartorious BP121S; Sartorius AG, Goettingen, Germany); -80˚C refrigerator (Thermo Fisher Scientific, Schwerte, Germany); low temperature centrifuge (Thermo Fisher Scientific) and frozen slicers (Leica Microsystems, Wetzlar, Germany). The sources of other related instruments and reagents are described in the relevant section.

Experimental animals and grouping. Male Sprague-Dawley (SD) rats (220-250 g) were purchased from the Shanghai Medical Laboratory Animal Center. Laboratory animal certificate number: SCXK (Shanghai) 2013-0015. Rats were raise in quiet environment with free access to food and water following the circadian rhythm for 1 week to adapt to the environment. Thirty-six SD rats were randomly divided into three groups (n=12) including sham operation (S) group, ischemia-reperfusion group (I/R) group and ischemic preconditioning (IP) group.

Establishment of renal ischemia-reperfusion model. Rats were fasted for 12 h before surgery. After anesthesia with intraperitoneal injection of 4% chloral hydrate at a dose of 10 ml/kg, abdominal hair was removed and an incision was made on abdomen along the mediventral line to open abdominal cavity. Renal capsule was bluntly separated to separate the kidneys. Arterial clip was then used to clip bilateral kidney pedicles. The dark red color of kidneys indicated the successfully established ischemia model. In I/R group, bilateral renal pedicles were clipped for 45 min, followed by perfusion for 6 h to establish I/R model. Both kidneys in rats of S group were separated and exposed for 45 min, but renal pedicles were not clipped. In IP group, bilateral renal pedicles were clipped for 5 min, followed by perfusion for 5 min, this procedure was repeated 3 times, after that rats were subjected to the treatment described in the I/R group.

Sample collection and preparation. Peripheral blood was collected after reperfusion for 6 h. Blood samples were centrifuged 2,650 x g for 15 min to collect supernatant. Supernatant was transferred to tubes and stored at -80˚C. After blood sample collection, rats in each group were sacrificed, and both kidneys were separated and fixed in 10% formalin for subsequent pathologic examination and other studies.

Table I. Primers used in PCR.

	Sequences
Bax	Forward: 5'-ATCCAGAGACAAGACATGTAC-3' Reverse: 5'-TTCAGATGTTCTAAGCCTACGG-3'
Bcl-2	Forward: 5'-TGGCGGTTTGCGGTGGAC-3' Reverse: 5'-CCAGTGCAGGGTCCGAGGT-3'
GAPDH	Forward: 5'-GATGATTGGCATGGCTTT-3' Reverse: 5'-CACCTTCCGTTCCAGTTT-3'

Renal tissue injury scoring. After H&E staining, pathological changes were observed under optical microscope (BX-42; Olympus, Tokyo, Japan) and scores of renal pathological damage were evaluated. Scoring was performed according to the standard of renal injury: normal renal tissue, 0 point; area of renal tissue damage in renal tubular area <25%, 1 point; area of renal tissue damage in renal tubular between 25 and 50%, 2 points; area of renal tissue damage in renal tubular between 50 and 75%, 3 points; area of renal tissue damage in renal tubular between >75%, 4 points.

Determination of levels of Cr and BUN and activity of SOD in serum. Contents of Cr and BUN in serum of rats in each group were determined according to the instructions of the Cr assay kit and BUN assay kit. Cr content was expressed as number + μmg/ml and BUN content expressed as number + mmol/ml. SOD activity in serum of rats in each group was determined according to the instructions of SOD activity assay kit and expressed as number + U/mg.

TUNEL to detect the apoptosis of rat renal cells. TUNEL assay was performed according to the instructions of kit and apoptotic cells were observed under an optical microscope. Cells with brown nucleus were positive cells. Five visual fields were randomly selected (magnification, x400) to count the number of apoptotic cells. The proportion of the number apoptotic cells to the number of total cells was used as apoptotic index.

Semi-quantitative PCR to detect the expression of related mRNA. Total RNA was extracted from renal tissue using the TRIzol kit. The quality of total RNA was checked by agarose gel electrophoresis. Only RNA samples with satisfactory qualities were used in subsequent experiments. Reverse transcription was performed using a kit to synthesize cDNA and expression levels of Bax and Bcl-2 were detected by semi-quantitative PCR with GAPDH as endogenous control. Reaction conditions were: 95˚C for 4 min, followed by 35 cycles of 95˚C for 30 sec, 64˚C for 25 sec and 72˚C for 30 sec. Primers were synthesized by Tiangen Biotech Co., Ltd. (Beijing, China). All primers are listed in Table I. PCR products were subjected to agarose gel

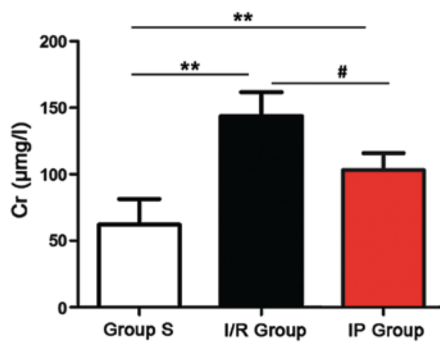

Figure 1. Levels of Cr in peripheral blood of rats in each group. Compared with group S, level of Cr was significantly increased in I/R and IP groups (**P<0.01). Compare with IP group, level of Cr was significantly increased in I/R group (#P<0.01).

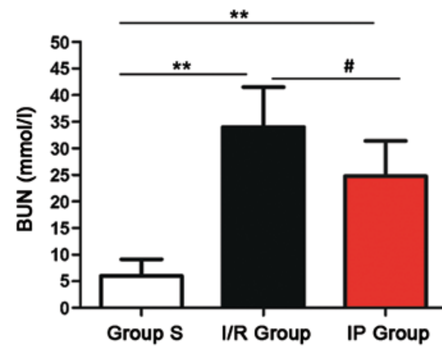

Figure 2. Levels of BUN in peripheral blood of rats in each group. Compared with group S, level of BUN was significantly increased in I/R and IP groups (**P<0.01). Compared with IP group, level of BUN was significantly increased in I/R group (#P<0.01).

electrophoresis and results were observed using a UV imaging system. The relative expression levels of Bax and Bcl-2 were represented by the ratio of Bax/GAPDH and Bcl-2/GAPDH.

Western blot analysis to detect the expression of related proteins. Renal tissue was mixed with RIPA lysate with a ratio of 100 mg:1 ml. The mixture was centrifuged (10,500 x g) at 4˚C for 10 min to collect the supernatant. Protein concentration was measured using the DAB Protein Quantitative kit (Invitrogen). After that, protein samples were subjected to SDS-PAGE gel electrophoresis, followed by transmembrane to PVDF membrane. After blocking with 5% skim milk, membranes were incubated with primary rabbit polyclonal Bax antibody (dilution, 1:500; cat. no. ab53154) and rabbit monoclonal Bcl-2 antibody (dilution, 1:500; cat. no. ab32124) overnight at 4˚C. After washing with TBST 3 times, 5 min for each time, membranes were incubated with secondary goat anti-rabbit (HRP) IgG antibody (dilution, 1:2000; cat. no. ab6721) for 2 h. All antibodies were all purchased from Abcam (Cambridge, MA, USA). After washing with TBST 3 times, proper amount of ECL luminescent substrate (ratio of A and B was 1:1) was added and incubated with the membranes in the dark. The results were scanned and processed by ImageJ to calculate the expression level of each protein.

Statistical analysis. Data were expressed as mean ± standard deviation, and processed using the SPSS 19.0 software (SPSS, Inc., Chicago, IL, USA). Comparisons between 2 groups were performed using t-test, and comparisons among multiple groups were performed using variance analysis. If the variance was homogeneous, the comparisons between two groups were performed using Bonferronic method. If the variance was not homogeneous, Welch method was used. Multiple comparisons were performed using Dunnett's T3 method. P<0.05 was considered to be statistically significant.

Results

Changes in renal function in rats. Levels of Cr and BUN in peripheral blood of rats in each group were detected by Cr and BUN detection kit. As shown in Figs. 1 and 2, compared with group S, levels of Cr and BUN were significantly increased in I/R group and IP group (P<0.01). Compared with IP group, levels of Cr and BUN were significantly increased in the I/R group (P<0.01).

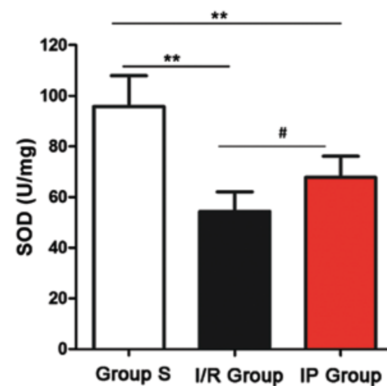

Figure 3. Activity of SOD in rats of each group. Activity of SOD in I/R group and IP group was significantly lower than those in S group (**P<0.01), and activity of SOD in I/R group was significantly lower than those in IP group (#P<0.05).

Changes in SOD activity. Activity of SOD was detected by SOD activity test kit. As shown in Fig. 3, activity of SOD in the I/R and IP groups was significantly lower than in the S group (P<0.01), and activity of SOD in the I/R group was significantly lower than in the IP group (P<0.05).

Pathological changes of the kidneys in rats. Pathological changes of renal tissue in each group after H&E staining were observed under an optical microscope. Structure of renal tissue in group S was clear, renal tubular and glomerular were normal, degeneration and atrophy were not observed in renal tubular epithelial cells, and the cavity was not expanded. In the I/R and IP groups, renal tissue glomerular telangiectasia and congestion, renal tubular epithelial cell edema, and granular degeneration or vacuolar degeneration were observed and the cavity was narrowed. After scoring (Table II), renal injury was found to be more serious in the I/R and IP groups than in the S group (P<0.01), and renal injury was more serious in the I/R group than in the IP group (P<0.05).

Renal cell apoptosis. Apoptotic cells were detected using TUNEL kit. Number of positive cells was counted and the

Table II. Pathological changes of kidney in rats of each group.

Groups	Renal injury score
S group	0.68±0.19
I/R group	2.39±0.29[a]
IP group	1.89±0.33[a,b]

[a]P<0.01 compared with the S group; [b]P<0.05 compared with the I/R group.

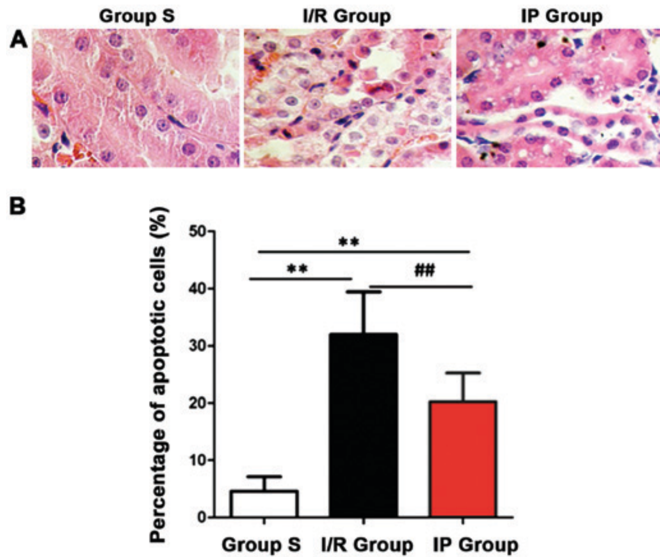

Figure 4. (A and B) Cell apoptosis detected by TUNEL. Compared with S group, number of apoptotic cells in I/R group and IP group was significantly increased (**P<0.01). Compared with IP group, number of apoptotic cells in I/R group was significantly increased (##P<0.01).

apoptotic index was calculated. As shown in Fig. 4, compared with S group, number of apoptotic cells in I/R group and IP group was significantly increased (P<0.01). Compared with IP group, the number of apoptotic cells in I/R group was significantly increased (P<0.01).

mRNA expression detected by semi-quantitative PCR. Expression of Bcl-2 and Bax mRNA was detected by semi-quantitative PCR. As shown in Fig. 5, compared with S group, expression level of Bcl-2 mRNA was significantly decreased, expression level of Bax mRNA was significantly increased, and the ratio of Bcl-2/Bax was significantly decreased in IP group and I/R group (P<0.01). Compared with I/R group, expression level of Bcl-2 mRNA was significantly increased, expression level of Bax mRNA was significantly deceased, and the ratio of Bcl-2/Bax was significantly increased in IP group (P<0.05).

Protein expression detected by western blot analysis. Expression of Bcl-2 and Bax protein was detected by western blot analysis. As shown in Fig. 6, compared with S group, expression level of Bcl-2 protein was significantly decreased, expression level of Bax protein was significantly increased, and the ratio of Bcl-2/Bax was significantly decreased in IP group and I/R group (P<0.01). Compared with I/R group, expression level of Bcl-2 protein was significantly increased, expression level of Bax protein was significantly deceased, and the ratio of Bcl-2/Bax was significantly increased in IP group (P<0.01).

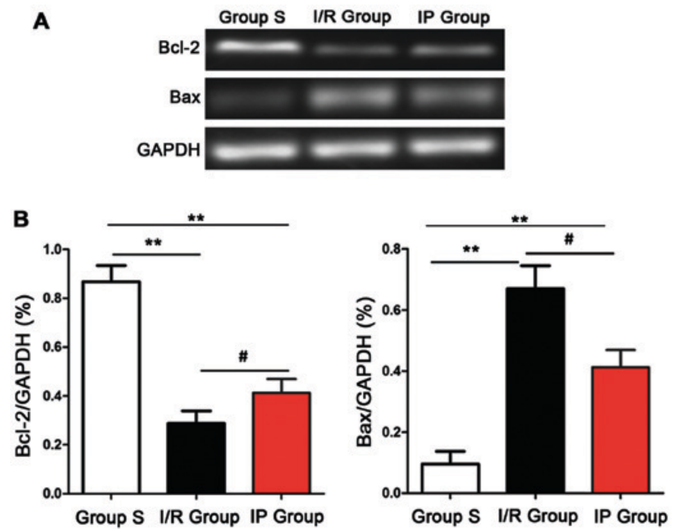

Figure 5. (A and B) Expression of Bcl-2 and Bax mRNA detected by semi-quantitative PCR. Compared with S group, expression level of Bcl-2 mRNA were significantly decreased, expression level of Bax mRNA was significantly increased, and the ratio of Bcl-2/Bax was significantly decreased in IP group and I/R group (**P<0.01). Compared with I/R group, expression level of Bcl-2 mRNA was significantly increased, expression level of Bax mRNA was significantly deceased, and the ratio of Bcl-2/Bax was significantly increased in IP group (#P<0.05).

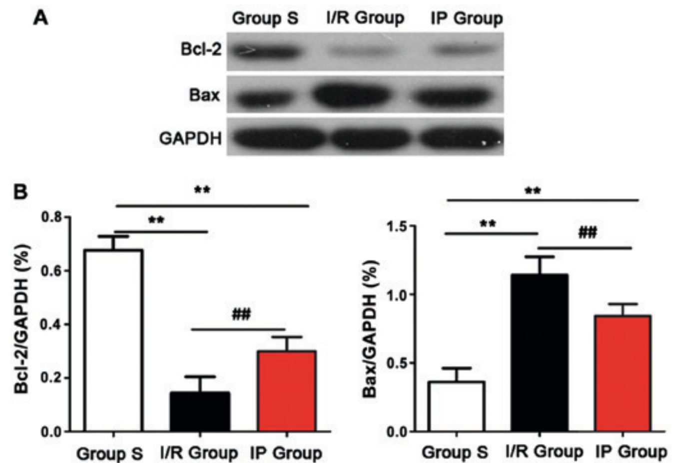

Figure 6. (A and B) Expression of Bcl-2 and Bax protein detected by western blot analysis. Compared with S group, expression level of Bcl-2 protein were significantly decreased, expression level of Bax protein was significantly increased, and the ratio of Bcl-2/Bax was significantly decreased in IP group and I/R group (**P<0.01). Compared with I/R group, expression level of Bcl-2 protein was significantly increased, expression level of Bax protein was significantly deceased, and the ratio of Bcl-2/Bax was significantly increased in IP group (##P<0.01).

Discussion

Renal blood flow needs to be blocked in renal transplantation, nephrolithotomy and surgical resection of renal tumors and other complex surgeries, and renal reperfusion after blood

flow blocking can cause renal damage (10). Numerous studies have shown that ischemia-reperfusion injury may be related to the production of oxygen free radicals, adenine nucleotide metabolic disorders and cell apoptosis (11). Drugs (ulinastatin and propofol), low temperature, preconditioning and postconditioning can play a protective role in ischemia-reperfusion injury, and all these methods can be used to reduce ischemia-reperfusion injury to the kidneys (12-14).

In this study, rat ischemia-reperfusion model was established and levels of Cr and BUN in serum were detected to evaluate the degree of renal injury. Results showed that levels of Cr and BUN in the serum of rats in I/R group were significantly higher than those of S group, and the difference was statistically significant ($P<0.01$), indicating the successful establishment of rat ischemia-reperfusion model. Ischemia-reperfusion model was established by clamping bilateral renal pedicle. Reperfusion time and clamping sites can seriously affect the stability of the model. Preliminary experiment showed that reperfusion could significantly affect renal function and the mode was stable. This study showed that preconditioning performed by clamping bilateral renal pedicle for 5 min and reperfusion for 5 min could improve the renal ischemia-reperfusion injury in rats. Levels of Cr and BUN in IP group were higher than those in the I/R group ($P<0.01$). Ischemic preconditioning can induce the production of endogenous protective substances in the body to resist ischemia and reperfusion injury to organs and tissues, and this protective effect in mulple organs have been proved by various studies (15,16). Li and Liu (17) found that ischemic preconditioning can effectively reduce the effects of ischemia-reperfusion on myocardial function. Lipid peroxidation caused by oxygen free radical is one of the factors that cause ischemia-reperfusion injury. SOD activity can be used to indirectly assess the ability of the body to scavenge oxygen free radicals (18). In this study, compared with S group and IP group, SOD activity in I/R group was significantly reduced, indicating that ischemia-reperfusion could cause renal injury and ischemic preconditioning can protect the body. As the currently most commonly used method to study cell apoptosis, TUNEL can be used to directly observe apoptotic cells, and tissue or cell apoptosis can be evaluated by calculating apoptosis index. Bcl gene family, which contains anti-apoptotic gene Bcl-2 and pro-apoptotic gene Bax, is closely related to cell apoptosis. Bcl-2 can inhibit cell apoptosis by inhibiting the production of free radicals, intracellular calcium overload, reducing the permeability of mitochondrial membranes and maintaining the oxidative function of mitochondria, while Bax has opposite functions (19,20). This study found that, compared with S group, cell apoptosis was significantly increased in I/R group, and results of semi-quantitative PCR and western blot analysis showed that, compared with S group, expression levels of Bcl-2 mRNA and protein were significantly decreased, expression levels of Bax mRNA and protein were significantly increased, and the ratio of Bcl-2/Bax was significantly decreased in IP group and I/R group. Compared with I/R group, expression level of Bcl-2 was significantly increased, expression level of Bax was significantly deceased, and the ratio of Bcl-2/Bax was significantly increased in IP group. Those results suggest that ischemia-reperfusion can lead to increased cell apoptosis, while ischemic preconditioning can increase the membrane stability and protect the kidneys by upregulating the expression of Bcl-2 and downregulating the expression of Bax in renal tissue.

In conclusion, ischemic preconditioning can protect rats with renal ischemia-reperfusion injury possibly by increasing the expression level of Bcl-2 and decreasing the expression level of Bax. The repeated short-term ischemia-reperfusion treatment can be applied in clinical practice to induce the injury to kidneys caused by ischemia-reperfusion.

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