Regulatory effect of immunosuppressive agents in mice with renal ischemia reperfusion injury

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Received February 6, 2018; Accepted August 6, 2018

DOI: 10.3892/etm.2018.6642

Abstract. This study was designed to investigate the regulatory effect of rapamycin in mice with renal ischemia-reperfusion injury. A total of 100 mice were randomly divided into normal control, sham operation, model and experimental groups with 25 rats in each group. Mice in the experimental group were subjected to rapamycin gavage. Mice in each group were sacrificed 24 h after operation. Then, blood, spleen and left kidney were collected. PAS staining was used for semi-quantitative analysis of renal pathological injury. Serum creatinine (SCr) and blood urea nitrogen (BUN) levels were measured. TUNEL method was used to detect cell apoptosis. Flow cytometry was used to detect the percentage of NKT cells. The expression of CXC chemokine ligand 10 (CXCL10) and vascular endothelial growth factor (VEGF) mRNA were detected by RT-qPCR. Semi-quantitative scoring of renal pathological injury showed that pathological injury score of the experimental group was significantly lower than that of the model group (p<0.05). Serum levels of SCr and BUN in the experimental group was compared to those in the model group (p<0.05). The number of apoptotic cells in the experimental group was compared to that of the model group (p<0.05). The percentage of NKT cells in the experimental group was compared to that of the model group (p<0.05). The percentage of NKT cells was significantly higher in the kidney and peripheral blood of the experimental group than that in the model group (p<0.05). The expression levels of CXCL10 mRNA in the model and experimental groups were significantly higher in the experimental group than those in the model group (p<0.05). The results indicated that rapamycin can significantly upregulate the expression level of CXCL9 and promote the accumulation of NKT cells in kidney from spleen through peripheral blood. Rapamycin can also inhibit the HIF-1α expression level and protect renal ischemia-reperfusion injury.

Introduction

Temporary blocking of renal blood flow is required in some complex renal surgeries, such as renal parenchymal lithotomy, renal transplantation and renal tumor resection. Postoperative reperfusion may lead to increased injury of ischemic renal tissue, which is called renal ischemia-reperfusion injury (RIRI) (1). RIRI can lead to renal failure and acute renal insufficiency, causing high morbidity and mortality. A study has shown that the mechanism of RIRI is very complicated, and many internal factors, such as vascular obstruction, inflammatory mediators, enhanced acidic environment interstitial edema, as well as calcium and oxygen-free radical damage, were involved (2). A study showed that adaptive immune system and innate immunity play an important role in RIRI. Immune system intervention may have a protective effect on RIRI (3). Rapamycin, as a novel immunosuppressive agent, can bind mammalian target of rapamycin (mTOR) via receptor FK50-binding protein-12 to inhibit its signal transduction, thereby reducing the arrest of immune cells in late G1 and suppressing immune response (4). The protective mechanism of rapamycin on ischemia-reperfusion injury is still unclear. In view of this, our study investigated the protective effects of rapamycin on RIRI, and explored the mechanism, so as to find a way to reduce renal ischemia-reperfusion injury.

Materials and methods

Experimental animals and grouping. A total of 100 SPF C57BL/6 male mice weighing 20-25 g (8 weeks old) were purchased from Shanghai Laboratory Animal Center, Chinese Academy of Sciences (Shanghai, China). The mice were raised in cages at 20-25°C, with free access to food and water. Mice
were divided randomly into the normal control, sham operation, model and experimental groups with 25 mice in each group. This study was approved by the Ethics Committee of North China University of Science and Technology (Tangshan, China).

**Main reagents.** Rapamycin was from Wyeth: Pfizer (Collegeville, PA, USA); TUNEL Apoptosis Detection kit was purchased from Wuhan Boster Biological Technology, Ltd. (Wuhan, China); RT-qPCR kit was purchased from Takara Biotechnology Co., Ltd. (Dalian, China); rat anti-mouse NK.1.1 monoclonal antibody was purchased from R&D Systems, Inc. (1:300; cat. no. 694370, Minneapolis, MN, USA).

**Main equipment.** Ultra-clean workbench (AIRTECH; Suzhou Purification Equipment Co., Ltd., Suzhou, China); precision electronic balance (Mettler-Toledo GmbH, Greifensee, Switzerland); UV spectrophotometer (Shanghai Third Analytical Instrument Factory, Shanghai, China); PCR instrument (Bio-Rad Laboratories, Inc., Hercules, CA, USA); Cytomics® FC 500 series flow cytometer and AU680 automatic biochemical analyzer (both from Beckman Coulter, Inc., Brea, CA, USA).

**Animal model construction.** RIRI model was constructed according to the methods described in a previous study (5). Mice were fasted for 12 h before operation but were allowed to drink water. After anesthesia (injected with 10% chloral hydrate), the mice were fixed in supine position. Skin was disinfected with 75% ethanol, and an incision was made along the middle line of the abdomen. Left renal pedicle was separated and clipped with non-invasive microvascular clip. Kidneys gradually changed from bright red to dark purple. Clips were removed 30 min later, and the rapid recovery of kidney color indicated that the model was successfully established. The skin was closed and incision was coated with bupivacaine for analgesia after operation. Bilateral renal pedicle was not clipped in the sham operation group and other steps were the same as the model group. Mice in the control group were not treated.

**Animal treatment.** Rapamycin (3 mg/kg/day) gavage was performed to mice in the experimental group 2 days and 1 day before surgical operation and after successful establishment of the model. Mice in each group were sacrificed at 24 h after operation, and blood, spleen and left kidney were collected. The left kidney was cut into two halves, one half was fixed in formaldehyde, and the other half was stored in liquid nitrogen for RT-qPCR detection.

**Semi-quantitative analysis of renal pathological injury.** After fixation, embedding, slicing and PAS staining, semi-quantitative analysis of renal pathological injury was performed. Ten visual fields of renal plexus junction were randomly selected under high magnification microscope (Olympus, Tokyo, Japan) (x400) to observe the degree of injury. Scoring standards were: 0 for normal, lesion <25% for 1 point, 25-50% for 2 points, 51-75% for 3 points, and >75% for 4 points.

**Renal function.** Serum creatinine (SCr) and blood urea nitrogen (BUN) were detected using Beckman Coulter AU680 automatic biochemical analyzer. Cell apoptosis test. Kidneys were fixed overnight in 4% paraformaldehyde solution, and embedded in paraffin. TUNEL method was used to detect cell apoptosis according to the instructions. Red fluorescence under microscope (Olympus) indicated TUNEL-positive cells. Six non-repetitive visual fields (x200) were randomly selected to calculate the positive apoptotic index (AI) according to the formula: AI = (apoptotic cells/total cells) x 100%.

**Flow cytometry.** Blood, spleen and kidney tissues of mice were used to make single cell suspension. After incubation with rat anti-mouse CD3 and NK.1.1 monoclonal antibodies (1:300; cat. nos. 17A2 and 694370, R&D Systems, Inc., at 4˚C for 1 h, flow cytometry was used to detect the ratio of NKT cells. CD3 and NK.1.1 double-positive cells were defined as NKT cells.

**RT-qPCR detection of expression levels of CXC chemokine ligand 10 (CXCL10), hypoxia-inducible factor-1α (HIF-1α) and vascular endothelial growth factor (VEGF) mRNA.** Total RNA was extracted from kidneys using Trizol. After purification, RNA samples were used in reverse transcription to synthesize cDNA. Primers used in PCR reactions were: β-actin forward, 5'-AGGCATCTGCACCTGAGTA-3' and reverse, 5'-AGGCGGACAGGACACACAGC-3'; CXCL10 forward, 5'-ACTGCTACTCATATCGATGAC-3' and reverse, 5'-TTC ATCCTGCAATGATCTC -3'; HIF-1α forward, 5'-AAGTCTAGGGATGCAGCAC-3' and reverse, 5'-CAAGATCACCAGCATCTAG-3'; VEGF forward, 5'-ATTGAGACCTGGTG GACATC-3' and reverse, 5'-TCTTTCCTCGAATCTGATT-3'. Expression level of each gene was normalized to endogenous control β-actin using 2-ΔΔCT method (6).

**Statistical analysis.** Statistical analyses were performed using SPSS 18.0. (SPSS, Inc., Chicago, IL, USA). Count data were processed by χ² test. Measurement data were expressed as (X ±SD). ANOVA was used for comparison between multiple groups and the post hoc test was SNK test. were processed using parallel t-test. P<0.05 was considered to be statistically significant.

**Results**

**Semi-quantitative analysis of renal pathological injury.** Clear renal tubule and glomeruli structure, and no obvious pathological changes were found in the control group. In the model group, renal tubular structure was damaged, epithelial cells were exfoliated and necrotic, basement membrane was exposed, and vacuolar degeneration was also observed. Only a small number of epithelial cells were exfoliated in the experimental group, and proximal renal tubule swelling was also observed. Kidney injury was significantly lower in the experimental group than in the model group. Renal injury score was higher in the model and experimental groups than in the sham operation group (p<0.05), and the score of the experimental group was significantly lower than that of the model group (p<0.05) (Fig. I).

**Renal function.** Serum levels of SCr and BUN in the model and experimental groups were significantly higher than those
in the sham operation group (p<0.05), and were significantly lower in the experimental group than those in the model group (p<0.05) (Table I).

**Cell apoptosis.** The number of apoptotic cells in the model and experimental groups was significantly higher than that in sham operation group (p<0.05), and was significantly lower in the experimental than in the model group (p<0.05) (Fig. 2).

**Percentage of NKT cells.** Percentage of NKT cells in spleen of the model and experimental groups was significantly lower than that in the sham operation group (p<0.05), and was significantly lower in the experimental than in the model group (p<0.05) (Fig. 2). Percentage of NKT cells in kidney and peripheral blood of the model and experimental groups was significantly higher than that in the sham operation group (p<0.05), and was significantly higher in the experimental than that in the model group (p<0.05) (Table II).

**Expression levels of CXCL10, HIF-1α and VEGF mRNA in different groups.** The expression levels of HIF-1α and VEGF mRNAs in the model and experimental groups were significantly higher than those in the sham-operated group (p<0.05), and were significantly lower in the experimental group than those in the model group (p<0.05). The expression level of CXCL10 mRNA in the model and experimental groups was significantly lower than that in the sham operation group (p<0.05), and was significantly higher in the experimental group than that in the model group (p<0.05) (Table II).

**Discussion**

As a hyperperfusion organ, kidney is sensitive to ischemia reperfusion. Pathogenesis of RIRI is very complex, and the factors which affect RIRI can interact with each other. So the mechanism of RIRI is still unclear. How to reduce RIRI is now a hot research field. As a novel immunosuppressive agent, the effects of rapamycin on kidney transplantation have been confirmed (7). However, the mechanism of the protective effects of rapamycin on ischemia-reperfusion injury remains controversial.

Results of this study showed that rapamycin could significantly reduce the levels of SCr and BUN after renal ischemia-reperfusion in mice to improve renal function, so as to reduce the rate of tubular apoptosis. Histopathological examination also confirmed that rapamycin has a protective
effect on RIRI. Khan et al (8) reported that rapamycin had a protective effect on liver ischemia reperfusion injury in mice, which is consistent with the results of our study. Lui et al (9) reported that in the early stage of RIRI, rapamycin could reduce the degree of renal damage through its anti-apoptotic effect, and in the late stage of RIRI, rapamycin could aggravate ischemia-reperfusion injury by inhibiting tissue repair. Ischemia-reperfusion injury refers to the increased degree of ischemic tissue injury after reperfusion. Rapamycin is a macrolide antibiotic isolated from the culture medium of actinomycetes. Besides the application in the treatment of fungal infections and tumors, rapamycin has also been used in organ transplantation (10). Rapamycin blocks various signaling pathways through different cytokine receptors and blocks the progression of lymphocytes from the G1 phase to the S phase, thereby producing an immunosuppressive effect (11,12).

NKT cell is a kind of immune cell. In recent years, studies on NKT cell have attracted increasing attention. A previous study showed that NKT cells play an important role in ischemia-reperfusion injury, in which NKT cells can be recruited from spleen through peripheral blood to target organ thereby producing an immunosuppressive effect (11,12).

In summary, rapamycin can significantly upregulate the expression of CXCL9 and promote recruitment of NKT cells in kidney after ischemia-reperfusion injury (16).

Acknowledgements

Not applicable.

Funding

No funding was received.

Availability of data and materials

The datasets used and/or analyzed during the present study are available from the corresponding author on reasonable request.

Authors’ contributions

WZ and SL conceived and designed the study. JZ collected the data. FC was responsible for the analysis and interpretation of the data. WZ drafted this manuscript. SL revised the manuscript critically for important intellectual content. All authors have read and approved the final manuscript.
Ethics approval and consent to participate

The study was approved by the Ethics Committee of North China University of Science and Technology (Tangshan, China).

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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