

Original Article

Cyclosporine A inhibits renal tubular epithelial cell apoptosis in rats with acute renal failure

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Abstract: Acute renal failure is a common kidney disease in the clinic. Cyclosporine is a commonly used immunosuppressive agent. This study aimed to investigate the effect of cyclosporine on apoptosis of renal tubular epithelial cells in rats with acute renal failure. The rat acute renal failure model was established and defined as model group. Rats in the experimental group were treated with 2 mg/kg cyclosporine A (CsA). Renal morphological changes and function were evaluated. Renal tubular epithelial cell apoptosis was detected by TUNEL assay. Bcl-2 and Bax protein expression in renal tubular epithelial cells was determined by Western blot. The levels of serum creatinine (Scr), urea nitrogen (BUN), and urinary protein (UP) in the experimental group were significantly lower than those in the model group, which were higher than those in the sham group ($P < 0.05$). Creatinine clearance rate (Ccr) and glomerular filtration rate (GFR) in experimental group were obviously higher than those in the model group but lower than those in the sham group ($P < 0.05$). Renal tubular epithelial cell apoptosis in the experimental group was markedly lower than that in the model group while higher than that in the sham group ($P < 0.05$). Bcl-2 protein in the experimental group was apparently higher than that in the model group and lower than the sham group ($P < 0.05$). Bax showed the opposite trend. In summary, CsA restored the structure and function of normal renal cells, inhibited renal tubular epithelial cell apoptosis, increased Bcl-2 expression, and decreased Bax level in rats with acute renal failure, leading to amelioration of renal injury.

Keywords: Cyclosporine A, acute renal failure, renal tubular epithelial cell apoptosis

Introduction

Acute renal failure (ARF) is a clinical crisis that is characterized by rapid decline of renal function [1]. There are many causes of ARF, of which the most important one is ischemia or poisoning induced renal tubular epithelial cell damage. Ischemia and reperfusion injury is inevitable during kidney surgery, kidney transplantation, acute ischemia, and renal hypoperfusion, leading to acute renal tubular epithelial cell death, GFR rapid decrease, and subsequent acute ischemic renal failure [2]. The incidence of acute ischemic renal failure after renal transplantation is 20%-50%. Damage is manifested in two forms: necrosis and apoptosis [3]. It has been confirmed that many ARFs are not caused by necrosis of tubule epithelial cells, but by sub-lethal cell damage and apoptosis [4]. In clinic, renal biopsy of ARF patients showed that most of the renal tubules are normal with limited renal tubular necrosis. It is

demonstrated that apoptosis is the main form of early renal tubular cell death in ARF. Apoptosis and necrosis occurs simultaneously in ischemia-reperfusion injury, while apoptosis occurs earlier, and necrosis occurs after a few days [5].

Cyclosporine (CsA) is an immunosuppressive agent widely used in organ transplantation and autoimmune diseases. It is a potent immunosuppressive agent selectively acting on T lymphocytes, and is mainly used to prevent graft-versus-host (GVH) response after post-transplantation or treat a variety of autoimmune diseases [6]. However, its side effects cannot be ignored. Cell necrosis, inflammatory infiltration, and vascular degeneration in acute kidney damage or fibrosis in chronic injury can cause renal dysfunction and even renal failure. Previous studies have shown that low-dose CsA can effectively reduce renal ischemia-reperfusion injury [7]. In addition, recent studies reported that low-dose CsA can reduce folic acid-

induced acute renal injury in mice [8]. In this study, we established the ARF rat model to investigate the effect of CsA on the apoptosis of renal tubular epithelial cells in ARF rats and explore its specific mechanism.

Materials and methods

Experimental animals

A total of 90 healthy Wistar rats in SPF grade at 8-week old and weighing 180 ± 20 g were provided by the Experimental Animal Center of Harbin Medical University (license number: SCXK2002-001). The rats received routine food and drink.

Rats were used for all experiments, and all procedures were approved by the Animal Ethics Committee of Jiamusi Central Hospital.

Experimental reagents and instruments

CsA (Novartis, Switzerland); rabbit anti-mouse Bcl-2 and Bax monoclonal antibodies, goat anti-rabbit secondary antibody, goat anti-rabbit β -actin monoclonal antibody (Sigma, USA); triphenyltetrachloride (Sigma, USA); weighting instrument, normal saline, forceps, needle holder, ferrule, eye speculum, and suture (Shanghai Yuanhong Medical Appliance co., Ltd); automatic biochemical analyzer (Beckman AU5800, USA); -80°C freezer (SANYO, Japan).

Experimental methods

Rat RF model establishment: The rats were equally and randomly divided into 3 groups ($n = 30$). In the model group rats were anesthetized by 3% pentobarbital sodium (50 mg/kg) intraperitoneal injection. Next, the abdomen was opened to separate renal capsule. Bilateral renal artery was clipped by noninvasive artery clip to induce renal ischemia for 30 min. Then the clip was removed to restore perfusion, and the kidneys changed from dark red gradually back to bright red, indicating successful reperfusion. The rats were sacrificed at 18 hours after reperfusion. In the sham group rats underwent the same procedures in RF modeling but without clipping the renal artery. The experimental group consisted of rats that received 2 mg/kg CsA intraperitoneal injection at 15 min before renal artery clipping.

Specimen collection: The rats were fasted but free to drink water. They were maintained in the

metabolic cage to collect 24 h urine. The total urine output was recorded. The urine was kept at about 5 ml and centrifuged at 3000 rpm/min for 10 min.

The rats were fixed on the edge of the experimental table. The eyelash was cut off using ophthalmic scissors. Next, the capillary tube was inserted vertically into the inner canthus and turned in the direction of the fundus to enter the venous plexus. The blood was collected in the Eppendorf tube and preserved at -80°C .

The kidneys were washed with 0.9% sodium chloride solution at 4°C , followed by separation of kidneys and removal of the capsule. The specimens of the renal tissue were cut into the same size of about $0.5\text{ cm} \times 0.5\text{ cm}$. The tissue specimens were fixed in neutral formalin for H&E staining or preserved at -80°C for the following experiments.

Morphological observation: Renal tissue was fixed in 10% formalin solution. After routine dehydration, waxing, and paraffin embedding, the specimen was sliced at 3-4 μm in thickness. At last, the section was stained by H&E and observed under microscope.

Automatic biochemical analyzer detection of renal function: The urine and blood were collected and centrifuged to measure the level of Scr, BUN, UP, Ccr, and GFR on an automatic biochemical analyzer.

TUNEL assay: The sample was prepared by dehydration, hyalinization, paraffin embedding, and section. Next, the sample was washed by xylene and gradient ethanol. Then the sample was treated with Proteinase K followed by addition of 50 μl TUNEL to count apoptotic cell number. Fifty μl converter-POD and 50-100 μl DAB substrate were then added into the sample. Next, the sample was restained with hematoxylin or methyl green. The nuclei with brown particles were defined as a positive marker. The number of positive cells and the total number of cells in the field of view were counted under a light microscope. The apoptotic rate (AR) was calculated.

Western blot: The cells were washed by PBS three times and treated by 50 μl RIPA (50 mmol/L pH7.4 Tris-HCl, 150 mmol/L NaCl, 1% Triton X-100, 1% sodium deoxycholate, 1% NP-40, 0.1% SDS, sodium orthovanadate, sodi-

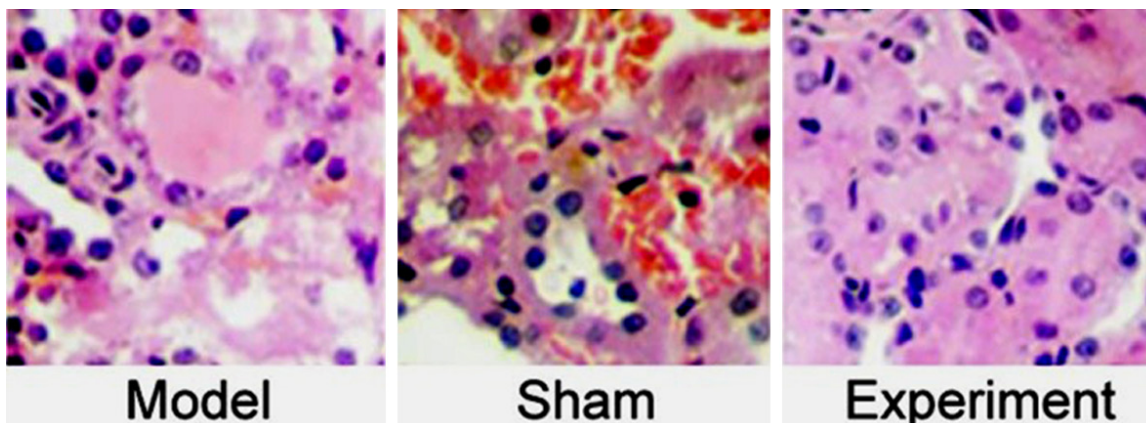


Figure 1. Histopathological changes of renal tissue in rats ($\times 400$).

um fluoride, EDTA, and leupeptin), 0.1 mg/mL PMSF, aprotinin, and phosphotransferase inhibitor on ice for 10 min. Next, the cells were ultrasonic grinded and centrifuged at 12000 rpm for 30 minutes at 4°C. The loading buffer was added into the protein samples and boiled at 100°C for 5 min. Then the protein was separated by electrophoresis on 5% stacking gel and 10% SDS-PAGE gel and transferred to PVDF membrane. After washed by TBST (50 mmol/L pH7.6 Tris-HCl, 150 mmol/L NaCl, and 0.1% Tween 20) at 4°C for 5 min, the membrane was blocked at 4°C overnight. Next, the membrane was incubated in primary antibody (1:1000 dilution) at 37°C for 2 h and secondary antibody (1:2000 dilution) at room temperature for 1 h. At last, the membrane was developed after adding Pierce ECL Western Blotting Substrate (Thermo Fisher Scientific). The protein expression was quantified as ratio to b-actin using Image J software.

Data analysis: All data analyses were performed on SPSS17.0 statistical software. The measurement data were compared by Chi-square test, while the enumeration data (represented as mean \pm standard deviation) were analyzed by one-way ANOVA. $P < 0.05$ was considered as statistical significance.

Results

CsA ameliorates histopathological changes of renal tissue in rats with RF

Model group: the kidney was pale pink or gray in hard texture and adhered with the surrounding organs. Local thrombosis was observed. Glomerular volume reduction, focal segmental sclerosis, mesangial stromal hyperplasia, base-

ment membrane thickening, partial balloon local or extensive adhesions; renal tubular atrophy, cysts shrink or disappear, renal tubular epithelium cell degeneration, luminal dilatation, and protein tube type. A large number of inflammatory cell infiltration and fibrous tissue hyperplasia were found in the renal interstitium.

Sham group: normal kidney shape, color, size, and texture. Clear glomerular and renal tubular structure. No abnormal tube type or inflammatory cell infiltration was observed.

Experimental group: the kidney was dark red in normal size. Glomerular structure was normal, no basement membrane thickening, clear cysts without adhesion, and no significant mesangial proliferation. The renal tubular structure was clear without atrophy. No lumen expansion or protein tube type was observed. There was only a small amount of inflammation cell infiltration (**Figure 1**).

CsA reduces levels of Scr, BUN, UP, and increases Ccr, and GFP levels in RF rats

The levels of Scr, BBUN, and UP in the experimental group were significantly lower than those in the model group but higher than those in the sham group in a time-dependent manner ($P < 0.05$). Ccr and GFR in experimental group were obviously higher than those in the model group but lower than those in the sham group with time dependence ($P < 0.05$) (**Table 1**).

Decreased renal tubular epithelial cell apoptosis in the RF rats treated with CsA

Renal tubular epithelial cell apoptosis in the experimental group was markedly lower than

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Table 1. Scr, BUN, UP, Ccr, and GFR levels comparison

Group	Cases	Scr ($\times 10^9/L$)	BUN (g/L)	UP (mmol/L)	Ccr ($\mu\text{mol/L}$)	GFR (ml/min)
Model	30					
12 h		72.1 \pm 6.2*	10.3 \pm 1.7*	26.6 \pm 3.1*	0.65 \pm 0.1*	17.8 \pm 0.5*
24 h		88.1 \pm 6.4* ^{#,&}	11.8 \pm 1.6* ^{#,&}	37.5 \pm 6.5* ^{#,&}	0.49 \pm 0.1* ^{#,&}	15.1 \pm 0.2* ^{#,&}
Sham	30					
12 h		4.6 \pm 0.2	4.7 \pm 1.1	13.1 \pm 2.2	1.17 \pm 0.2	100.2 \pm 1.3
24 h		4.5 \pm 0.3	4.6 \pm 1.3	14.2 \pm 2.5	1.21 \pm 0.1	100.8 \pm 1.1
Experiment	30					
12 h		17.9 \pm 2.1* [#]	5.7 \pm 1.4* [#]	20.3 \pm 1.1* [#]	0.87 \pm 0.1* [#]	38.7 \pm 0.9* [#]
24 h		26.3 \pm 3.4	7.8 \pm 1.3	19.8 \pm 1.2	0.31 \pm 0.1	29.2 \pm 1.5

*P < 0.05, compared with sham group; #P < 0.05, compared with model group; &P < 0.05, compared with 12 h.

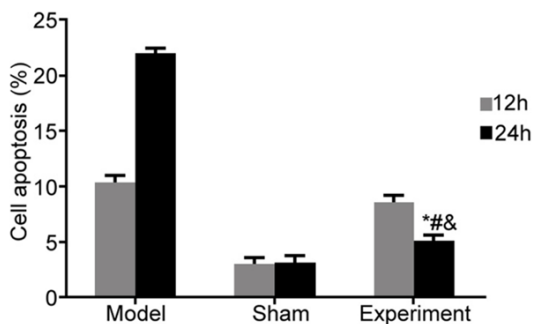


Figure 2. Renal tubular epithelial cell apoptosis in the rat. *P < 0.05, compared with sham group; #P < 0.05, compared with model group; &P < 0.05, compared with 12 h.

Table 2. Bcl-2 and Bax protein expression in the renal tubular epithelial cells of rat

Item	Model	Sham	Experiment
Bcl-2	0.11 \pm 0.03*	0.17 \pm 0.02	0.23 \pm 0.11* [#]
Bax	0.83 \pm 0.15*	0.16 \pm 0.06	0.58 \pm 0.12* [#]

*P < 0.05, compared with sham group; #P < 0.05, compared with model group.

that in the model group while higher than that in the sham group with time dependence (P < 0.05) (Figure 2).

Lower Bcl-2 and higher Bax, cleaved caspase-3 and PARP1, in the renal tubular epithelial cells of RF rat treated with CsA

Bcl-2 protein in the experimental group was apparently higher than that in the model group and lower than that in the sham group (P < 0.05). However, expression of Bax showed the opposite trend (Table 2, Figure 3). In addition, we also found decreased cleaved caspase-3 PARP1 in the experimental group compared

with those in the model group, which was higher than those in the sham group (Figure 4).

Discussion

Ischemia and reperfusion pathology damage in the renal surgery, renal transplantation, acute ischemia, and renal hypoperfusion can induce acute renal tubular epithelial cell death and rapid GFR decrease, leading to ischemic ARF [9]. In recent years, the role of renal tubular cell apoptosis in the development and progression of ARF has been received more attention. CsA is currently the most widely used immunosuppressive agent in clinic that mainly used in the transplant rejection of liver, kidney, and heart. It also can combine with adrenal cortex hormone to treat diffuse connective tissue disease, autoimmune correlation nephritis, and other immune diseases [10]. In this study, we established the ARF rat model to investigate the effect of CsA on the apoptosis of renal tubular epithelial cells in ARF rats and explore its specific mechanism.

This study established rat ARF model and applied CsA for intervention. The kidney in the model group was pale pink or gray in hard texture and adhered with the surrounding organs. Local thrombosis was observed. Glomerular volume reduction, focal segmental sclerosis, mesangial stromal hyperplasia, basement membrane thickening, partial balloon local or extensive adhesions; renal tubular atrophy, cysts shrink or disappear, renal tubular epithelium cell degeneration, luminal dilatation, and protein tube type. A large number of inflammatory cell infiltration and fibrous tissue hyperplasia were found in renal interstitium. The kidney in the experimental group was dark red in

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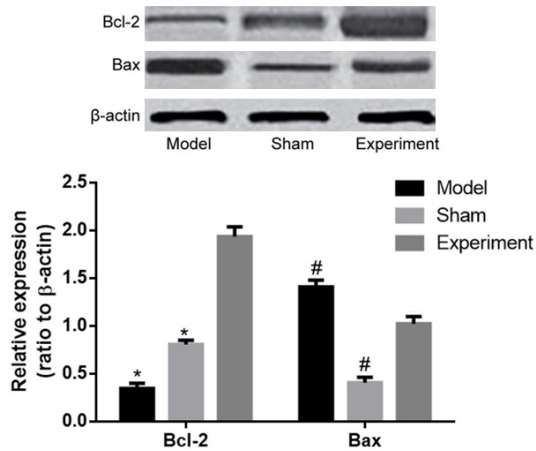


Figure 3. Bcl-2 and Bax protein expressions in the renal tubular epithelial cells of rat. Compared with the Experimental group, *P < 0.05, #P < 0.05.

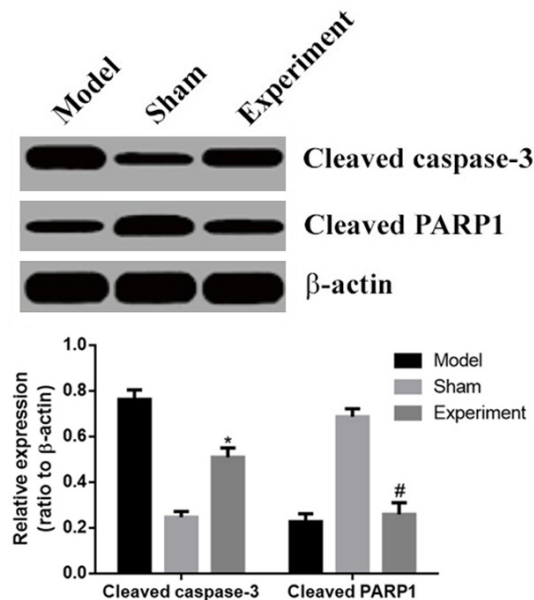


Figure 4. Cleaved caspase-3 and PARP1 protein expressions in the renal tubular epithelial cells of rat. Compared with the Sham group, *P < 0.05, #P < 0.05.

normal size. Glomerular structure was normal, no basement membrane thickening, clear cysts without adhesion, and no significant mesangial proliferation. The renal tubular structure was clear without atrophy. No lumen expansion or protein tube type was observed. There was only a small amount of inflammation cell infiltration. The kidney in the sham group exhibited normal shape, color, size, and texture. Clear glomerular and renal tubular structure. No abnormal tube type or inflammatory cell infiltration was

observed. Further analysis showed that the levels of Scr, BBUN, and UP in the experimental group were significantly lower than those in the model group but higher than those in the sham group with time dependence. Ccr and GFR in the experimental group were obviously higher than those in the model group but lower than those in the sham group with time dependence, indicating that ARF model was successfully established and CsA can improve the renal morphology, alleviate local microstructural destroy, relieve renal tubular epithelial cell injury, and recover normal renal structure.

Under physiological conditions, cells activate apoptosis-inducing genes and/or apoptosis-suppressing genes in an orderly and coordinated manner, which control the cellular metabolic function and maintain the dynamic balance of the intracellular environment [11]. It was found that apoptosis is currently the main cause of renal tubular death in early ARF [12]. In this study, renal tubular epithelial cell apoptosis in the experimental group was markedly lower than that in the model group while higher than that in the sham group with time dependence. CsA is characterized by selective action on T lymphocytes without impacts on bone marrow cells [13]. CsA-induced renal toxicity is due to its accumulation in the formation of vacuoles in renal tubular cells, and then sinks in the lumen and capillary loop, resulting in embolization [14]. However, it was reported that low-dose CsA has a protective effect on renal ischemia-reperfusion injury within 3 days, while its long-term application can cause chronic renal insufficiency [15, 16]. Our results suggest that CsA intervention can inhibit the apoptosis of renal tubular epithelial cells in ARF rats, leading to amelioration of renal injury.

In order to analyze the mechanism of CsA on the apoptosis of ARF renal tubular epithelial cells, we measured expression of Bcl-2 and Bax protein in rat renal tubular epithelial cells. Bcl-2 protein in the experimental group was apparently higher than that in the model group but lower than that in the sham group. However, expression of Bax showed the opposite trend. In the regulation of mitochondria on apoptosis, Bcl-2 family protein plays an important role in the release of some pro-apoptotic factors from mitochondria. Bax family mainly includes Bax, Bcl-XS, and Bad that promote apoptosis [17-19]. Increased Bax/Bcl-2 ratio may promote

apoptosis by accelerating Bax-Bax formation of homodimer. On the other hand, Increased Bcl-2/Bax ratio which forms a heterodimer of Bcl-2 and Bax may inhibit apoptosis [20, 21]. Our results suggest that expression of Bcl-2 and Bax are abnormal in renal tubular epithelial cells of ARF rats. CsA intervention obviously elevated Bcl-2 and reduced Bax level, thus played an inhibitory role on cell apoptosis and improved renal injury.

Conclusion

CsA restored the structure and function of normal renal cells, inhibited renal tubular epithelial cell apoptosis, increased Bcl-2 protein, and decreased Bax level in rats with acute renal failure, leading to alleviation of renal injury.

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Disclosure of conflict of interest

None.

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