Original Article
Protective effects of 2-deoxy-D-glucose on nephrotoxicity induced by cyclosporine A in rats

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Abstract: Objective: This study aims to explore the protective effect mechanism of 2-deoxy-D-glucose on nephrotoxicity of cyclosporin A in vivo. Method: Renal toxicity of SD rats model induced by CsA was established. Serum creatinine, blood urea nitrogen, urine NAG, GSH and MDA were determined and the histopathological changes of rat renal cortex were observed to explore the protective effects of 2-DG on CsA-induced nephrotoxicity. Results: Serum creatinine, BUN and urinary NAG of rats were significantly changed in experimental groups. Pathological results showed that there was obvious renal tubular injury in model group, however, the renal injury was significantly reduced in pre-treated with 2-DG. Conclusions: 2-DG had obvious protective effect on nephrotoxicity especially with high dose. This protective effect could be related to the reduction of ROS induced by CsA. However, 2-DG had no effect on the expression of RIP3.

Keywords: Cyclosporin A, 2-deoxy-D-glucose, SD rats model, RIP3, GSH, MDA

Introduction

Cyclosporin A (CsA), a powerful immunosuppressant, is widely used in transplantation of organ and have clinical application in the treatment of various autoimmune disorders [1-3]. However, its application has been limited because of its severe toxicity especially renal injury [4, 5]. The pathogenesis of CsA nephrotoxicity was thought to be secondary to the hemodynamic changes caused by its intense effect on vasoconstriction [6]. Studies found that shallot, black grape and garlic extracts could protect against CsA nephrotoxicity [7, 8], L-arginine, N-acetylcysteine, Vitamin E also had roles against nephrotoxicity induced by CsA [9-12]. Some of them had the glycolytic inhibition roles. 2-deoxy-D-glucose (2-DG) is a kind of deoxy glucose and has been widely studied as adjuvant of anti-tumor drugs [13, 14]. So we explored whether 2-DG, a typical inhibition drug of glycolysis, can protect against nephrotoxicity caused by CsA in vivo.

There are two main methods to establish the animal model of renal toxicity of CsA: low salt diet method and normal diet method. Eleing et al firstly developed CsA chronic nephrotoxicity model rats using a low salt diet in 1993 [15]. This model is mainly used for the study of CsA-induced chronic fibrosis of renal toxicity. Under the normal diet situation, 25-50 mg.kg-1 CsA was given to rats can cause typical toxic kidney damage, such as serum creatinine and BUN increased, renal tubular cellular degeneration and necrosis and infiltration of inflammatory cells in interstitial [16-18].

In this study, we used CsA given to rats by gavage to establish CsA nephrotoxicity model.

Materials and methods

Experimental animals

40 Adult Sprague-Dawley (SD) rats weighing 140±10 g were obtained from the animal exper-

Table 1. Effects of 2-DG on body weight of rats treated with CsA for 0 week~4 weeks

time (week)	Ctrl	2-DG	CsA	CsA+2-DG（H）	CsA+2-DG（L）
W0	227.8±18.9	223.9±18.9	221.6±15.0	222.9±14.4	211.6±8.9
W1	280.9±24.5	285.1±24.9	249.4±14.9[a]	244.1±11.1[a]	231.1±24.7[a]
W2	323.0±26.2	316.0±43.6	271.8±1.0[a]	290.8±12.0[b]	269.8±16.0[a]
W3	359.0±30.5	343.5±47.9	313.0±6.1[a]	329.5±13.1[b]	313.5±17.1[a]
W4	372.8±30.2	358.5±52.0	308.0±9.2[a]	335.8±14.4[b]	312.8±17.1[a]

Mean±SD; n=8 (0~2 weeks) or n=4 (3~4 weeks); [a]: $P < 0.05$, vs control group; [b]: $P < 0.05$, *vs* CsA group.

Table 2. Effects of 2-DG on Kidney weight (right)/Body weight ratio of rat treated with CsA for 2 weeks and 4 weeks

Group	2 week	4 week
control	0.0036±0.0001	0.0035±0.0004
2-DG	0.0033±0.0001	0.0033±0.0002
CsA	0.0039±0.0004[a]	0.0030±0.0002[a]
CsA+2-DG (Low)	0.0040±0.0003[a]	0.0030±0.0001[a]
CsA+2-DG (High)	0.0035±0.0002[b]	0.0031±0.0001[a]

Mean±SD; n=4; [a]: $P < 0.05$, *vs* ctrl group; [b]: $P < 0.05$, vs CsA group.

Table 3. Effect of 2-DG on CsA induced urine changes in control and treated rats

Group	W1	W2	W3	W4
control	18.1±5.4	20.1±5.3	20.6±5.9	21.8±7.3
2-DG	20.17±4.6	24.8±9.6	23.0±9.9	28.0±9.5
CsA	29.6±7.8[a]	37.1±13.9[a]	40.3±11.5[a]	26.8±5.3
CsA+2-DG (Low)	26.8±7.1[a]	32.9±8.7[a]	35.0±9.1	33.8±5.9[a]
CsA+2-DG (High)	25.1±6.5[a]	29.8±10.9[a]	34.5±14.7	29.8±2.8

Mean±SD; n=8 (2 weeks) or n=4 (4 weeks); [a]: $P < 0.05$, vs ctrl group.

imental center of Guangdong province. These SD rats were kept under clean and quiet environment with room temperature of 22±1°C and relative humidity as 40-50%, provided with 12D:12N photoperiod cycle (6:00 AM-6:00 PM). The rats had free access to food and drinking water and were pre feeding for 3 days to adapt to the environment. They were randomly divided into 5 groups (control group, 2-DG (400 mg/kg) group, CsA (50 mg/kg) group, CsA (50 mg/kg)+2-DG (100 mg/kg) group and CsA (50 mg/kg)+2-DG (400 mg/kg) group) according to the random number table. Each group has 8 rats and four rats share a cage. Cages, food and drinking water were regularly changed.

The experiment was divided into 2 batches. The first four rats of each group were killed after being treated by CsA for two weeks, the other four rats of each group were killed after being

treated by CsA for four weeks. In the experimental first week, the rats in 2-DG (400 mg/kg) group and CsA (50 mg/kg)+2-DG (400 mg/kg) group were treated with daily intraperitoneal injection of 2-DG (400 mg/kg), the rats in CsA (50 mg/kg)+2-DG (100 mg/kg) group were treated with daily intraperitoneal injection of 2-DG (100 mg/kg), the rats in CsA and control group were treated with intraperitoneal injection of physiological saline. From the second week, the rats in control group were treated with olive oil by gavage and intraperitoneal injection of physiological saline, the rats in 2-DG group were treated with olive oil by gavage and intraperitoneal injection of 2-DG (400 mg/kg), the rats in CsA group were treated with CsA (50 mg/kg) by gavage and intraperitoneal injection of physiological saline daily, the rats in CsA (50 mg/kg)+2-DG (100 mg/kg) group were treated with CsA (50 mg/kg) by gavage and intraperitoneal injection of 2-DG (100 mg/kg) daily, the rats in CsA (50 mg/kg)+2-DG (400 mg/kg) group were treated with CsA (50 mg/kg) by gavage and intraperitoneal injection of 2-DG (400 mg/kg) daily.

Housing and procedures involving experimental animals were in accordance with the Guide for the Care and Use of Laboratory Animals (eighth edition, published by the National Academies Press). All experimental procedures were approved by the Care of Experimental Animals Committee of our hospital.

General observation and weighing

Every morning the rats were observed for state of mind, body shape, coat color, luster, physical activity and reaction, abnormal situation was
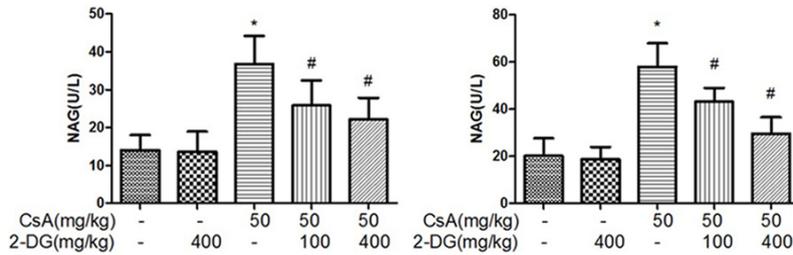
Figure 1. Effect of 2-DG on CsA induced urine NAG changes in control and treated rats. Values are expressed as mean±SD; n=8 (2 weeks) or n=2 (4 weeks); [a]: $P < 0.05$, vs ctrl group; [b]: $P < 0.05$, vs CsA group.
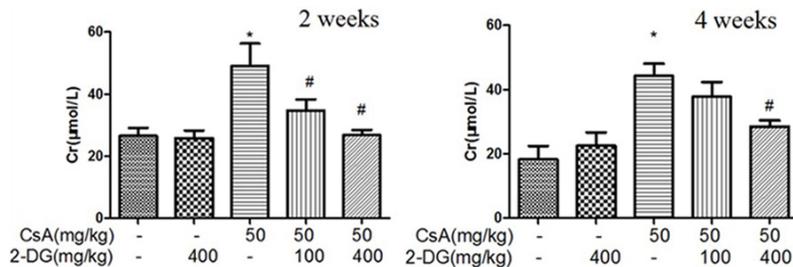
Figure 2. Effect of 2-DG on the levels of serum Creatinine of the rats treated with CsA and 2-DG for 2 weeks and 4 weeks. Values are expressed as mean±SD; n=4; [*]: $P < 0.05$, vs ctrl group; [#]: $P < 0.05$, vs CsA group.

Detection of urine N-acetyl-β-D-glucosaminidase (NAG)

50 μL of rat urine was taken for NAG detection according to the manual of NAG assay kit.

Determination of serum creatinine and blood urea nitrogen

500 μL of rat serum was taken for determining the creatinine and blood urea nitrogen content with automatic biochemical analyzer (Roche).

Determination of the GSH and MDA content in renal cortex

Renal cortex homogenate of rats after being treated by CsA for 4 weeks was centrifuged at 12000 rpm for 10 min. The supernatant was taken to determine the GSH and MDA content using GSH and MDA Assay Kit according to the manual. They were detected on the ELISA reader to determine absorbance and then the contents were calculated.

recorded. The body weights of rats were weight and dosage was calculated according to the body weight.

Urine collection

The 24 h urine samples of rats were collected with metabolic cage at 1, 2, 3 and 4 week after being treated by CsA. Rats were fasted with free drinking. The samples were centrifuged at 3000rpm for 5min and supernatant were taken to determine.

Collection of serum and renal cortical samples

At the end of CsA administration, rats were anesthetized with intraperitoneal injection of urethane. The abdominal cavity was opened and the arterial blood was taken. Serum was reserved for detection after centrifugation at 3000 rpm for 5 min. Kidney of rats was removed and the film was stripped for weighing. The left kidney was placed in liquid nitrogen immediately frozen and reserved in the -80°C refrigerator, the right kidney was fixed with 10% formalin immediately.

Western blotting

The expression levels of RIP3 were detected with western blotting method. Briefly, the supernatant of renal cortex homogenate was taken and the protein concentrations were detected with Bradford protein assay. Total proteins were separated using 12% sodium dodecyl sulfate polyacrylamide gel electrophoresis. Then they were transferred onto polyvinylidene difluoride membranes (Millipore Co, Billerica, MA, USA). The membranes were blocked with 5% non-fat milk in TBST (10 mM Tris-HCl (pH 8.0), 150 mM NaCl, and 0.1% Tween-20) for 2 h, then incubated with the primary antibodies: rabbit anti-RIP3 antibodies (1:1000) or mouse anti- tubulin antibodies (1:10000) at 4°C overnight. Then they were incubated with secondary antibodies conjugated with horseradish peroxidase at room temperature for 1 h. Antibody binding was
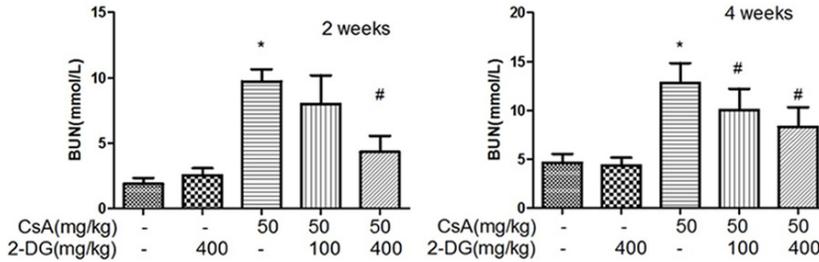
Figure 3. Effect of 2-DG on the levels of serum BUN of the rats treated with CsA and 2-DG for 2 weeks and 4 weeks. Values are expressed as mean±SD; n=4; *: $P <$ 0.05, vs ctrl group; #: $P <$ 0.05, vs CsA group.
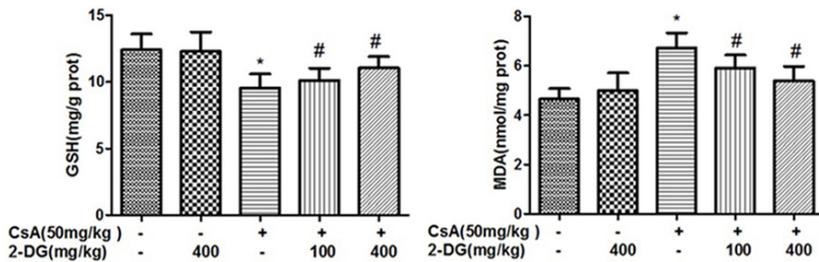
Figure 4. Effects of 2-DG on the levels of GSH and MDA in the kidney of the rats treated with CsA for 4 weeks. Values are expressed as mean±SD; n=4; *: $P <$ 0.05, vs ctrl group; #: $P <$ 0.05, vs CsA group.
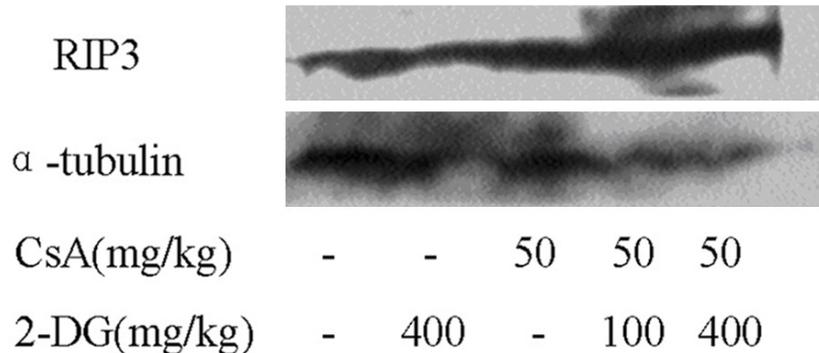
Figure 5. The expression of RIP3 protein in renal cortex.

antigen retrieval, the sections were blocked with 5% BSA and incubated at 37°C for 20 min, and then they were incubated with 1:200 diluted rabbit anti-RIP3 antibodies at 4°C overnight. After that, they were washed with PBS and incubated at 37°C in water bath for 2 h after drop-adding the 2nd antibody and washed with PBS. After treated with the DAB solution, they were flushed completely, counterstained with hematoxylin, washed with water, treated with dehydration and transparency, mounted on slides and observed under microscope.

Histopathological observation of renal cortex

Conventional paraffin sectioning and hematoxylin and eosin (HE) staining methods were adopted. They were observed by ordinary optical microscope and pictures were collected.

Data analysis

All data presented as mean values±standard deviation (SD). Statistical analysis was performed using Statistical Package for the Social Science (SPSS, version 17.0) Differences between experimental groups were analyzed by One-way ANOVA and Kruskal-Wallis tests. $P <$ 0.05 was considered to be significant.

Results

Body weight changes of rats

There was no difference in body weight of rats before being treated with CsA. The body weights of rats in experimental groups were lighter than that of rats in control group after being treated

detected using an Enhanced Chemiluminescence Detection Kit (ECL).

Detection of the expression of RIP-3 in rat cortex with immunohistochemical method

Briefly, the kidney specimens fixed in 10% formaldehyde were taken out and washed, then they were paraffin-embedded with automatic embedding machine. The embedded specimens were sliced at thickness of 4 μm. Following deparaffinization, dehydration, and
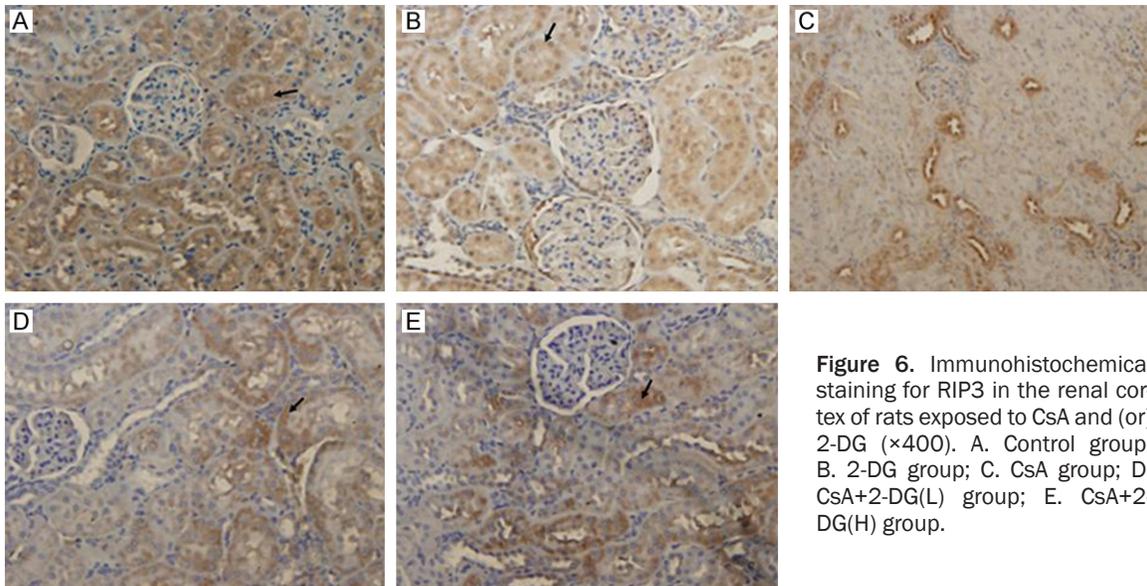
Figure 6. Immunohistochemical staining for RIP3 in the renal cortex of rats exposed to CsA and (or) 2-DG (×400). A. Control group; B. 2-DG group; C. CsA group; D. CsA+2-DG(L) group; E. CsA+2-DG(H) group.

with CsA for one week. The body weights of rats in CsA group and CsA+2-DG (high) group decreased significantly than that of rats in the control group in the second to the fourth week after being treated with CsA. There was no significant difference in body weights of rats between CsA+2-DG (low) group and control group, between CsA+2-DG (high and low) groups and CsA group (**Table 1**).

Kidney weight (right)/Body weight ratio changes of rats

As shown in **Table 2**, the kidney weight (right) / Body weight ratio of rats in CsA group and CsA+2-DG (low) group increased significantly than that of rats in the control group after being treated with CsA for 2 weeks. The kidney weight (right)/Body weight ratio of rats in CsA group and CsA+2-DG (high and low) group decreased significantly than that of rats in the control group after being treated with CsA for 4 weeks.

Effects on rat urine volume

As shown in **Table 3**, the urine volume of rats in CsA group increased significantly than that of rats in the control group after being treated with CsA for 1, 2 and 3 weeks. The urine volume of rats in in CsA+2-DG (low) group increased significantly than that of rats in the control group after being treated with CsA for 4 weeks.

Effects on urine NAG secretion

As shown in **Figure 1**, the urine NAG secretion of rats in CsA group increased significantly than that of rats in the control group and that in CsA+2-DG (high and low) groups decreased significantly than that of rats in CsA group after being treated with CsA for 2-4 weeks.

Effect on serum creatinine and urea nitrogen content

The serum creatinine of rats in CsA group increased significantly than that of rats in the control group after being treated with CsA for 2 weeks. The serum creatinine of rats in CsA+2-DG (high and low) group decreased significantly than that of rats in CsA group after being treated with CsA for 2 weeks. The serum creatinine of rats in CsA+2-DG (high) group decreased significantly than that of rats in CsA group but CsA+2-DG (low) group had no significant effects after being treated with CsA for 4 weeks (**Figure 2**).

The serum urea nitrogen of rats in CsA group increased significantly than that of rats in the control group after being treated with CsA for 2 weeks. The serum urea nitrogen of rats in CsA+2-DG (high) group decreased significantly than that of rats in CsA group after being treated with CsA for 2 weeks. The serum urea nitrogen of rats in CsA+2-DG (high and low) group decreased significantly than that of rats in CsA group after being treated with CsA for 4 weeks (**Figure 3**).
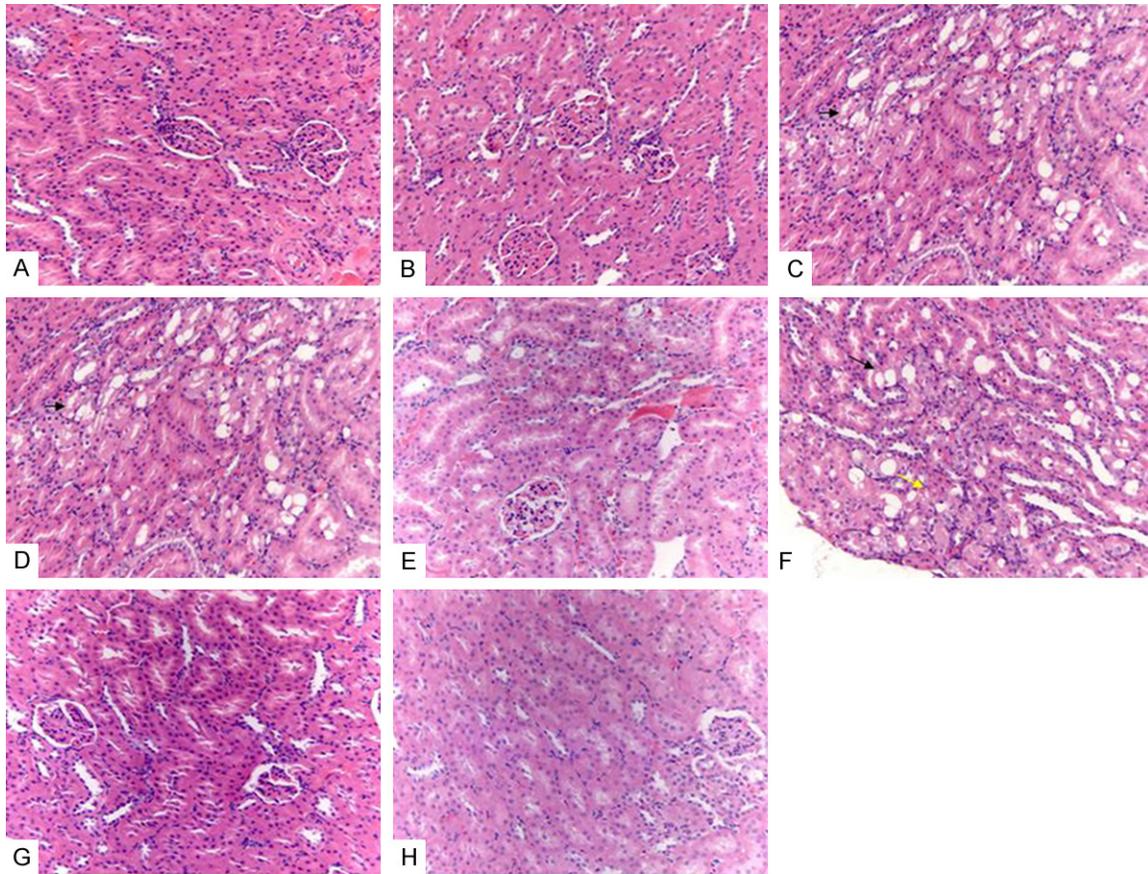
Figure 7. The pathological results after being treated with CsA for 2 weeks. A. There was no abnormal renal glomerulus and tubules and no inflammatory cell infiltration in interstitium (control group, HE×200); B. There was no abnormal renal glomerulus and tubules and no inflammatory cell infiltration in interstitium (2-DG group, HE×200); C. There was vacuolar degeneration in some renal tubular epithelia (CsA group, HE×200); D. There was local fibrosis in renal interstitium and acidophilic degeneration in some renal tubular epithelia (CsA group, HE×200); E. There was no abnormal renal glomerulus and tubules and no inflammatory cell infiltration in interstitium (CsA+2-DG low-dosage group, HE×200); F. There was local fibrosis in renal interstitium (yellow arrow) and vacuolar degeneration in some renal tubular epithelia (black arrow) (CsA+2-DG low-dosage group, HE×200); G. There was no abnormal renal glomerulus and tubules and no inflammatory cell infiltration in interstitium (CsA+2-DG high-dosage group, HE×200); H. There was vacuolar degeneration in some renal tubular epithelia (CsA+2-DG high-dosage group, HE×200).

Effects on the GSH and MDA content in renal cortex

As shown in **Figure 4**, the GSH content in renal cortex of rats in CsA group decreased while the MDA content in renal cortex of rats in CsA group increased significantly than that of rats in the control group after being treated with CsA for 4 weeks. The GSH content in renal cortex of rats increased while the MDA content decreased in CsA+2-DG (high and low) groups significantly than that of rats in CsA group after being treated with CsA for 4 weeks.

Expression of RIP3 in renal cortex of rats

The expression of RIP3 in renal cortex of rats increased however, there was no significant difference among the groups (**Figures 5** and **6**).

The pathological results after being treated with CsA for 2 and 4 weeks

After being treated with CsA for 2 weeks, there was no abnormal renal glomerulus and tubules and no inflammatory cell infiltration in interstitium in control and 2-DG group. There was vacuolar and acidophilic degeneration in some renal tubular epithelia and local fibrosis in renal interstitium in CsA group. There was local fibrosis in renal interstitium and vacuolar degeneration in some renal tubular epithelia in CsA+2-DG (high and low) group (**Figure 7**). After being treated with CsA for 4 weeks, renal cysts disappeared, glomerular atrophy and regeneration of renal tubular after necrosis presented in CsA group, a small amount of renal tubular epithelial cells shed and formed tube, some renal tubu-
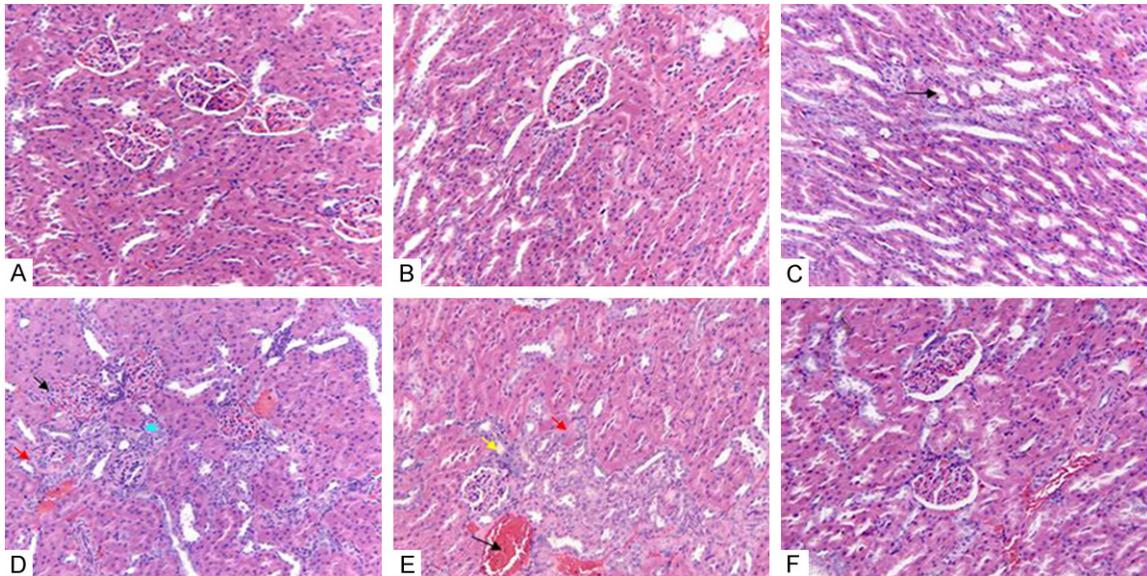
Figure 8. The pathological results after being treated with CsA for 4 weeks. A. There was no abnormal renal glomerulus and tubules and no inflammatory cell infiltration in interstitium (control group, HE×200); B. There was no abnormal renal glomerulus and tubules and no inflammatory cell infiltration in interstitium (2-DG group, HE×200); C. There was vacuolar degeneration in some renal tubular epithelia (black arrow, CsA group, HE×200); D. Renal cysts disappeared (black arrow), glomerular atrophy (red arrow) and regeneration of renal tubular after necrosis (blue arrow) (CsA group, HE×200); E. A small amount of renal tubular epithelial cells shed and formed tube (red arrow), some renal tubular epithelial cells regenerated (yellow arrow) and glomerular congestion (CsA+2-DG low-dosage group, HE×200); F. There was no abnormal renal glomerulus and tubules and no inflammatory cell infiltration in interstitium (CsA+2-DG high-dosage group, HE×200).

lar epithelial cells regenerated and glomerular congestion in CsA+2-DG (low) group, and there was no abnormal renal glomerulus and tubules and no inflammatory cell infiltration in interstitium in CsA+2-DG (high) group (**Figure 8**).

Discussion

In most animal experiments, 2-DG was given once a day with relatively large dose, such as intraperitoneal injection of 500 mg. kg-1.d-1. This dosage was non-toxic to rats in the test period [19-22]. So in this study, we selected 100 and 400 mg. kg-1.d-1 dosage to do the test. We did not find that 2-DG had renal toxicity on rats in the experiment. The nephrotoxic representation of CsA can be divided into two types: functional type and structural type. Functional change is the results of afferent arteriole contraction, structural damage includes the changes of afferent arteriole, glomerular and tubular interstitial.NAG is a lysosomal enzyme in renal tubular cells and is early detection index of renal toxicity [23-26]. Our results showed that urine NAG increased obviously in the first week after being treated by CsA, while it could be significantly inhibited in 2-DG intervention group, which also suggested

that CsA could damage renal tubular epithelial cells in the first week. Urine NAG in CsA group continued to increase in 2-4 weeks after being treated by CsA, which suggested that renal damage of CsA on rats increased with time. The body weight gain of rats decreased significantly and the serum creatinine, BUN, and urine NAG increased significantly after being treated by CsA for 2 weeks, while they were significantly inhibited in 2-DG intervention group. Pathological results showed that in the second week after being treated by CsA, there was vacuolar degeneration in some renal tubular epithelia cells, local necrosis of renal tubules, contraction of glomerulus and local fibrosis in renal interstitium in CsA group, while 2-DG could alleviate these changes, the protective effects were more obvious especially in the high dose group. In the fourth week after being treated by CsA, there were renal cysts and glomerular atrophy, renal tubular vacuoles degeneration and necrosis were more obvious, shedding epithelial cells could be seen in the lumen and the regeneration of renal tubular after necrosis in CsA group, while these phenomena were few in 2-DG intervention group, 2-DG could reduce CsA-induced damage of renal tubules.

The GSH and MDA content in renal cortex were determined in this study. The results showed that GSH decreased and MDA increased in the CsA group 4 weeks after being treated by CsA, while they were significantly inhibited in 2-DG intervention group especially in high dosage group. CsA can increase the ROS of renal cortex, which can decrease GSH and increase MDA, while some antioxidants could recovery renal cortical redox state [27, 28]. Our results suggested that 2-DG can reduce CsA-induced oxidative damage because of the increase of active oxygen.

In previous study we found that RIP3 and ROS are involved in CsA-induced necroptosis [29]. In this study, we detected the expression of RIP3 in rat cortex using the western blotting and immunohistochemical method. The results showed that RIP3 mainly expressed in renal tubular epithelial cells while few expressed in glomerular cells and interstitial cells. We did not find the effects of 2-DG on the expression of RIP3. Whether RIP3 is involved in the renal toxicity of CsA in vivo need further study. The mechanism of protective effect of 2-DG on normal tissue cells also includes the phosphorylation of AKT, increasing the expression of stress proteins such as GRP78 and HSP70 and so on. We only found the effect of 2-DG on ROS, the detailed protective mechanism need further study.

In a word, in this study, we successfully established CsA nephrotoxicity model by CsA50 mg.kg-1.d-1 gavage in rats. Serum creatinine, BUN and urinary NAG of rats were significantly changed. Pathological results showed that there was obvious renal tubular injury in model group, however, the renal injury was significantly reduced in pre-treated with 2-DG or intraperitoneal injection of 100 mg.kg-1.d-1 or 400 mg.kg-1.d-1 2-DG groups and their serum creatinine, BUN and urinary NAG were also significantly reduced. Therefore, 2-DG had obvious protective effect on nephrotoxicity especially with high dose. This protective effect was related to the reduction of ROS induced by CsA. However, 2-DG had no effect on the expression of RIP3. A detailed protective mechanism need to be further studied.

Disclosure of conflict of interest

None.

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