Inhibitory effect of tea polyphenals on transforming growth factor-β1 expression in rat with cyclosporine A-induced chronic nephrotoxicity

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KEY WORDS tea; cyclosporine A; transforming growth factor-β; kidney; polymerase chain reaction; immunohistochemistry; Western blotting

ABSTRACT

AIM: To investigate the inhibitory effect of tea polyphenols (TP) on the transforming growth factor-β1 (TGF-β1) expression in rat model of cyclosporine A (CsA)-induced chronic nephrotoxicity. **METHODS**: The rat model of CsA-induced chronic nephrotoxicity was used, 4 groups of rats were respectively treated with vehicle (0.1 mL $\cdot kg^{-1} \cdot d^{-1}$ sc), TP (80 $mg \cdot kg^{-1} \cdot d^{-1}$ ig), CsA (15 $mg \cdot kg^{-1} \cdot d^{-1}$sc) and TP plus CsA (CsA 15 $mg \cdot kg^{-1} \cdot d^{-1}$ sc+TP 80 $mg \cdot kg^{-1} \cdot d^{-1}$ ig). At the end of day 28 of treatment, serum and urine are analyzed for creatinine clearance, kidney tissue for pathologic analysis. The TGF-β1 mRNA and its protein expression were detected by RT-PCR, immunohistochemistry, and Western blot. **RESULTS**: CsA-treated rats had increased renal expression of TGF-β1 mRNA and its protein, compared with the vehicle- or TP- treated controls. The renal function and interstitial fibrosis were ameliorated and renal expression of TGF-β1 mRNA and its protein was decreased in animals treated with CsA plus TP, compared with aminals treated with CsA alone ($P<0.05$). **CONCLUSION**: TP significantly inhibits renal expression of TGF-β1 in rat model of cyclosporine-induced chronic nephrotoxicity, suggesting that the decreased renal expression of TGF-β1 exerted by TP is one of mechanisms to protect renal function and tissue structure.

INTRODUCTION

Cyclosporine A (CsA) is the main immunosuppressive agent in the organ transplantation, and its clinical impact is much enormous. Short-term (ex:1-year) kidney, heart, liver, lung, and pancreas allograft survival rates have been greatly improved since the clinical use of CsA. One-year hepatic allograft survival rates now approach or surpass 80 %, when compared with the survival rates of 30 %-50 % before clinical CsA use. However, the long-term viability of both renal and nonrenal allograft has not shown similar improvement. At present five-year liver allograft survival rates are 60 % or less[1-3]. Clinical retrospective analysis has indicated that calcineurin inhibitor-based immunosuppressant such as CsA and FK506 treatment is associated with a variety of side effects, especially their chronic nephrotoxicity that can shorten the long-term survival rates of allografts and patients[1,2]. The mechanisms leading to chronic nephrotoxicity of calcineurin inhibitor are not clear. The following factors are associated with chronic nephrotoxicity: transforming growth factor-β1 (TGF-

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β1), angiotensin II, nitric oxide (NO), reactive oxygen species (ROS), thromboxane A_2 (TXA_2), and leukotriene (LT). TGF-β1 plays an important role in the progressive interstitial fibrosis in the chronic CsA nephrotoxicity[2]. CsA increases free radical formation in the process of CsA nephrotoxicity. Vitamin E significantly suppresses CsA-induced lipid peroxidation and improves glomerular filtration rate and reduces renal fibrosis[4]. The treatment of adenovirus to express superoxide dismutases significantly blocks formation of ROS and minimizes pathological alterations and inhibition of renal function caused by CsA[5]. Tea polyphenals (TP), as potent antioxidant, can neutralize free oxygen radicals such as O_2^-, OH· *in vitro* and *in vivo*[6]. TP effectively attenuates the acute nephrotoxicity induced by CsA, which is contributed to the antioxidant properties of TP[7]. The purpose of this study was to determine whether TP could reduce the chronic CsA nephrotoxicity, and the expression of TGF-β1 in tubular and interstitial cells in the rat model of CsA-induced chronic nephrotoxicity.

MATERIALS AND METHODS

Experimental design Forty adult male Sprague-Dawley rats weighing (200-230 g) were obtained from the Center of Experimental Animals, Zhejiang University. They were housed in individual cages and received a low-salt diet (0.049 % sodium, measured by flame photometer in the Zhejiang Povincial Center of Disease Control and Prevention). After one week on the low-salt diet, weight-matched pairs of rats were randomly divided into 4 groups. Group 1: rats (n=10) were administered vehicle olive oil 1.5 mL·kg^{-1}·d^{-1} sc (subcutaneous injection). Group 2: rats (n=10) were gavaged with TP 80 mg·kg^{-1}·d^{-1}. Group 3: rats (n=10) were received CsA 15 mg·kg^{-1}·d^{-1} sc. Group 4: rats (n=10) were treated with CsA 15 mg·kg^{-1}·d^{-1} sc plus TP 80 mg·kg^{-1}·d^{-1} ig. TP was gifted by YANG Xian-Qiang (Institute of Tea Scence, Zhejiang University). CsA was purchased from Sandoz Company. Body weight was recorded daily. Ten rats of each group were treated for 28 d. At the end of treatment period, 24-h urine samples were collected in metabolic cages. After opening the abdomen through a middle incision under the anesthesia with ketamine, 2 mL blood was taken from inferior vein cava and serum was harvested immediately by centrifugation at 4 ºC and stored at -70 ºC until use. The aorta was cannulated retrogradely below the renal arteries with a 9-gauge needle. After the left renal aorta was occluded by ligation and the right renal veins were opened by a small incision for outflow, the kidneys were perfused with 20 mL of cold and heparinized saline (contained heparin 500 IU). The kidneys were removed. The cortex was carefully dissected from the medulla. The TGF-β1 mRNA and its protein expression was assayed by RT-PCR, immunohistochemistry, and Western blot.

Functional studies Creatinine of urinary and serum, were measured by Biochemical Autoanalyzer (model 7170, HITACHI). The creatinine clearance (Ccr) was calculated using standard formulae. CsA blood levels after 4-h administration were determined in whole blood by monoclonal radioimmunoassay.

Morphology Tissue samples were fixed in 10 % buffered formalin and embedded in paraffin. Sections at 4 μm were stained with hematoxylin and eosin, and Masson trichrome. Findings ascribed to tubular injury included cellular vacuolization, tubular collapse, tubular distention, and hyalinization of afferent arterioles. For tubular injury, the following semiquantitative score was utilized[8]: 0=no tubular injury, 0.5=<5% of tubules injured, 1=5 % to 20 %, 1.5=21 % to 35 %, 2=36 % to 50 %, 2.5=51 % to 65 %, and 3=>65 %. The findings of interstitial fibrosis consisted of matrix-rich expansion of the interstitium with distortion, collapse of the tubules, and thickening of the tubular basement membranes. Interstitial fibrosis was estimated by counting the percentage of injured areas per field and was scored semiquantitatively using the following[8]: 0=normal interstitium, 0.5=<5 % of areas injured, 1=5 % to 15 %, 1.5=16 % to 25 %, 2=26 % to 35 %, 2.5=36 % to 45 %, and 3=>45 %.

TGF-β1 mRNA expression by RT-PCR The primers were designed by computer assistance according to the gene bank. TGF-β1: forward, 5′-ACTGATACGCCTGAGTGGCTGT-3′; reverse, 5′-CTCTGTGGAGCTGAAGCAGTAG-3′, the size of amplified fragment is 303 bp. Internal control GAPDH: forward, TGGCACAGTCAAGGCTGAGA; reverse, CTTCTGAGTGGCAGTGATGG; the size of amplified fragment is 387 bp. Total RNA from each sample was isolated using TRIzol solution (Gibco Inc, USA). Total RNA was quantified with the ratio of absorption values of RNA samples at 260 nm and 280 nm. For each sample, 4 μg of total RNA was reverse transcribed into first strand of cDNA in 20-μL reaction system at 37 ºC for 50 min. Then polymerase chain reaction was performed from the synthesized cDNA in a 50 μL solution containing 3

μL of cDNA, 1 μL of 25 mmol primers (up-stream and down-stream) of TGF-β1, 0.5 μL of 25 mmol primers (up-stream and down-stream) of GAPDH, 10 mmol dNTP 1 μL, 25 mmol $MgCl_2$ 4 μL, 10×buffer 5 μL, 0.3 μL of *Taq* DNA polymerase (Promega). Amplification was performed in a thermal cycler (MJ Research Co, USA) under the following conditions: 26 cycles of denaturation at 94 ºC for 50 s, annealing at 59 ºC for 45 s, extension at 70 ºC for 40 s, followed by a final extension for 5 min. PCR product 10 μL was electrophoresed on a 1.5 % agarose gel, and stained with EB. The TGF-β1mRNA level in each sample was semi-quantified by comparing the intensities of TGF-β1mRNA band with those of the internal control GAPDH band.

Immunohistochemistry Formalin-fixed tissues were paraffin-embedded and cut into 4-μm sections. The sections were deparaffinized in xylene and rehydrated in graded ethanol to PBS. After blocking endogenous peroxidase activity with 3 % H_2O_2/methanol (1/32 vol/vol) for 10 min. Tissue sections was boiling for the antigen epitope retrieval in 0.1 mol/L citrate buffer (pH 6.0) for 10 min, the tissue sections were then interacted overnight at 4 ºC with the 1:200 diluted primary rabbit anti-rat TGF-β1 antibodies (Santa Cruz Biotechnology), controls were incubated with the normal rabbit serum and the PBS instead of primary antibodies. After ample washings with PBS, the slides were incubated for 30 min with EnVision+horseradish peroxidase-labeled secondary antibody goat anti-rabbit IgG (Dako Company) at room temperature, again washed extensively in PBS. The slides were then developed in diaminobenzidine for 2-5min and rinsed with water. The slides were then counterstained with haematoxylin. A minimum of 20 randomly selected areas per sample were observed at ×400 magnification. The intensity of staining for the glomerular, tubulointerstitial, and vascular compartments was evaluated by an observer blinded to the treatment groups using the following semiquantitative scale: 0=diffuse, very weak, or absent staining, 1=staining involving less than 25 %, 2=25 %-50 %, 3= 50 %-75 %, 4=70 %-100 %. Photographs were obtained at identical exposure and development time intervals.

Western blot analysis Protein extracts were prepared by homogenizing the 500 mg renal cortex or 500 mg medulla respectively in cold 1.5 mL RIPA buffer containing 1 % Nonidet P-40 (Amaresco), 0.5 % sodiumdeoxycholate (Sigma), 0.1 % sodium dodecyl sulfate (Amaresco), 15 μg phenylmethylsulfonyl fluoride (Sigma), and 45 U aprotinin (Sigma) with a glass tissue homogenizer. The tissue homogenates were centrifuged at at 10000×*g* 4 ºC for 10 min. The supernatants were collected and centrifuged at at 10000×*g*,4 ºC for 10 min again. Protein concentration in the supernatant was determined by Brandford method. 2×Loading buffer (Tris HCl 42 mmol/L, 10 % glycerol, 2.3 % sodium dodecyl sulfate, 5 % 2-mercaptoethanol, and 0.002 % bromophenol blue) was then added to each supernatant with equal volume containing equal amounts of protein, which was subsequently boiled for 3 min and then electrophoresed on a SDS-polyacrylaminde gel. Proteins were transferred to nitrocellulse and incubated sequentially with rabbit anti-rat TGF-β1 (Santa Cruz Biotechnology), and then with peroxidase-conjugated secondary antibody (Santa Cruz Biotechnology) in the second reaction. Detection was performed with enhanced chemiluminescence reagent (Santa Cruz Biotechnology).

Statistical analysis Results are presented as mean±SD. Analysis of variance (ANOVA), Kruska-Wallis test, and Tukey method were used by SAS8.0 statistical software. The level of statistical significance was chosen as $P<0.05$.

RESULTS

Physiologic studies Animals treated with CsA and CsA plus TP failed to gain body weight when compared with those receiving vehicle and TP at d 28 ($P<0.05$). The glomerular filtration rate (C_{cr}) was lower in the CsA-treated rats compared with (CsA+TP)-treated group at d 28 ($P<0.05$, Tab 1). There was no statistical difference in whole blood CsA level between CsA-treated animals and (CsA+TP)-treated animals (Tab 1).

Histological changes CsA-treated rats had characteristic morphologic changes similar to the chronic human lesion, mostly evident at 15 $mg \cdot kg^{-1} \cdot d^{-1}$ for 28 d. Tubular injury consisted of cellular and intercellular vacuolization, tubular collapse, and tubular dilation. Striped interstistial fibrosis was present with thickening of the tubular basement membranes and Bowman's caspsule. Both tubular injury and tubulointerstitial fibrosis were improved in (CsA+TP)-treated group when compared with CsA-treated group at d 28 ($P<0.05$, Tab1).

Expression of TGF-β1 mRNA by RT-PCR TGF-β1 mRNA was markedly increased by d 28 ($P<0.05$) in the CsA-treated rats when compared with con-

Tab 1. Changes in cyclosporine A (CsA) whole blood level, serum creatinine clearance, and semiquantitative scores of tubular injury and tubulointerstitial fibrosis in the experimental groups at d 28. $^{b}P<0.05$ *vs* (CsA+TP)-treated group.

Groups	CsA whole blood level/ $\mu g \cdot L^{-1}$	Creatinine clearance/ $mL \cdot min^{-1} \cdot kg^{-1}$	Tubular injury (0–3+)	Interstitial fibrosis (0–3+)
VH		3.0 ±0.8	0.55±0.21	0.30±0.11
TP		2.9 ±0.5	0.45±0.21	0.35±0.14
CsA	2418 ±210	1.2 ±0.3^{b}	2.3±0.4^{b}	2.83±0.20^{b}
CsA+TP	2370 ±245	2.2 ±0.2	1.42±0.26	1.46±0.19

VH: vehicle olive oil 1.5 $mL \cdot kg^{-1} \cdot d^{-1}$ sc; TP: tea polyphenols 80 $mg \cdot kg^{-1} \cdot d^{-1}$ ig; CsA: cyclosporine A 15 $mg \cdot kg^{-1} \cdot d^{-1}$ sc; CsA+TP: CsA 15 $mg \cdot kg^{-1} \cdot d^{-1}$ sc+TP 80 $mg \cdot kg^{-1} \cdot d^{-1}$ ig.

trol vehicle and TP-treated rats. But expression of TGF-β1 mRNA in both cortex and medulla in (CsA+TP) group was lower than that in CsA-treated group by d 28 (P <0.05, Fig 1).

Expression of TGF-β1 by immunohistochemistry Expression of TGF-β1 by immunohistochemistry was mainly present at tubular cells, interstitial cells, and Bowman's caspsule. The total amount of TGF-β1 expression was reduced in the kidneys of (CsA+TP)-treated rats as compared to CsA-treated rats at d 28 (P<0.05, Fig 2). In the control groups the expression of TGF-β1 protein was very weak.

Expression of TGF-β1 by Western blot analysis The total amount of TGF-β1 protein expression in both cortex and medulla was inhibited in CsA plus TP-treated group by 28 d (P<0.05), when compared with the CsA-treated group. In the control group the expression of TGF-β1 protein was very weak (Fig 3).

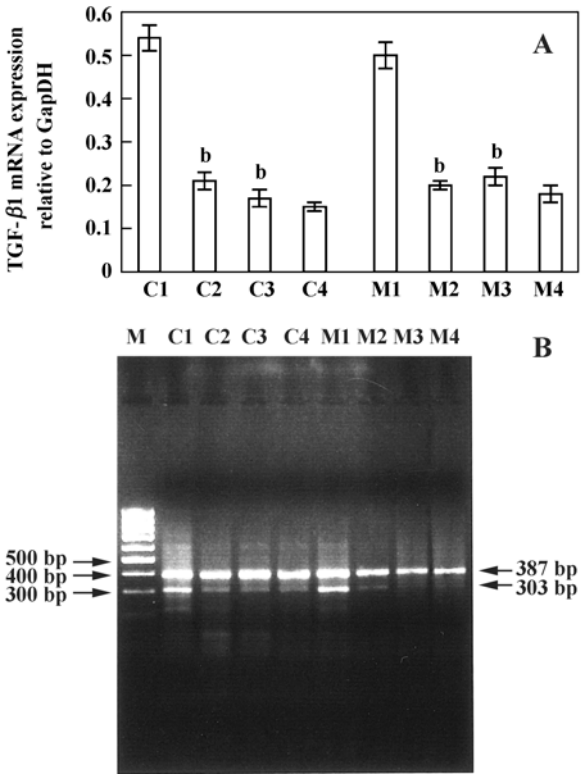

Fig 1. TGF-β1 mRNA expression in the kidney. A) The expression of TGF-β1 mRNA relative to GAPDH detected by RT-PCR in vehicle, tea polyphenals (TP), cyclosporine A (CsA), and (CsA+TP)-treated rats. B) Quantitation of mRNA expression of TGF-β1. C and M are representative of cortex and medulla, respectively. C1-C4 indicates tissues taken from cortex of rats treated by CsA, CsA+TP, TP, and vehicle respectively. M1-M4 indicates tissues taken from medulla of rats treated by CsA, CsA+TP, TP, and vehicle, respectively. The expression of mRNA was significantly increased in both cortex and medulla in the CsA group by d 28. $^{b}P<0.05$ *vs* CsA+TP group.

DISCUSSION

We examined an experimental model of chronic CsA nephrotoxicity with pathologic features similar to the human lesion characterized by tubular atrophy, progressive striped interstitial fibrosis with loss of cellularity in areas of fibrosis, afferent arteriole hyalinization and glomerulosclerosis[2,8]. In our experiment CsA promoted the total protein expression of renal tissue TGF-β1 which was mainly expressed in tubular and interstitial cells. Antioxidant TP inhibited the CsA-induced TGF-β1 expression and interstitial fibrosis. Bioptic tissues taken from renal allografts with CsA nephrotoxicity are detected by immunohistochemistry that TGF-β1 expression is also mostly concentrated in tubular cells[9]. CsA in a dose-dependent manner stimulates expression of TGF-β1 protein and steady-state mRNA levels in both proximal tubular cells and tubulointerstitial fibroblasts[10]. TGF-β1 mimics and anti-TGF-β1 antiboby or soluble TGF-β II receptor abrogates the chronic nephrotoxicity of CsA *in vivo*. TGF-β1 promotes fibronectin and collagen synthesis[11,12] . These data suggest that TGF-β1 play an important role in mediating CsA chronic nephrotoxicity.

The mechanisms by which antioxidant TP inhibits the TGF-β1 expression in CsA-induced chronic nephrotoxicity may be the following: (1) TP increases tissue

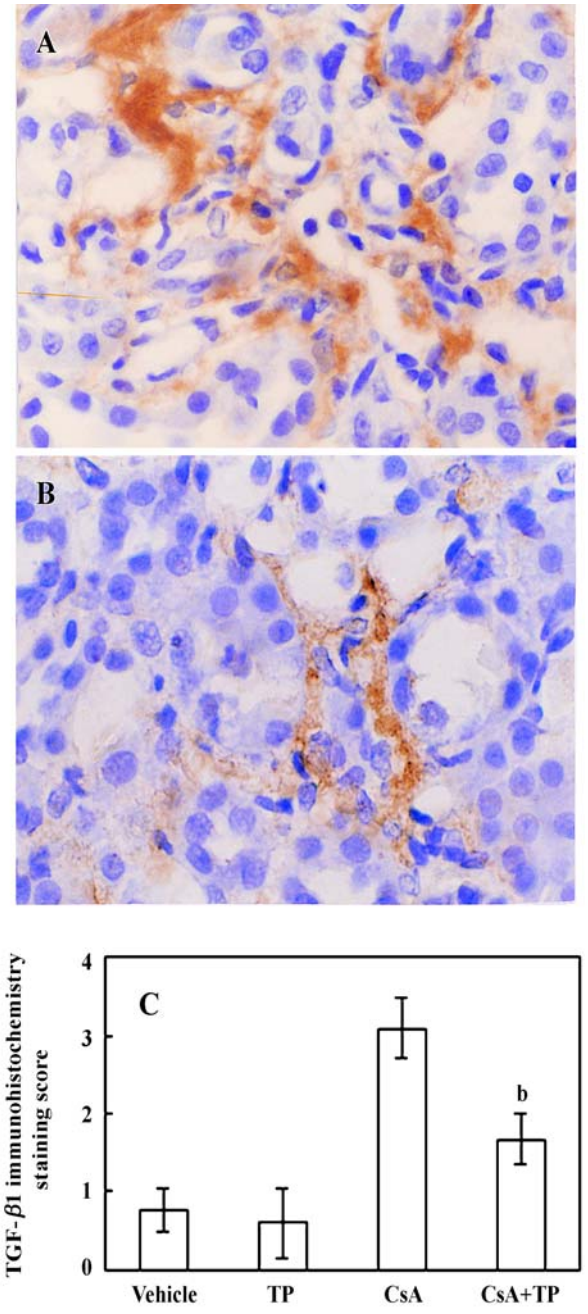

Fig 2. Expression of transforming growth factor-β1 (TGF-β1) by immunohistochemistry assay. Photomicrograph (A) showed that cyclosporine A (CsA) 15 mg·kg^{-1}·d^{-1} sc induced great expression of TGF-β1. (B) In rats treated with CsA 15 mg·kg^{-1}·d^{-1} sc+TP 80 mg·kg^{-1}·d^{-1} ig showed mild to moderate staining (×600). (C) Pathological semiquantitative scores for TGF-β1 expression. ^{b}P<0.05 *vs* the CsA-treated group.

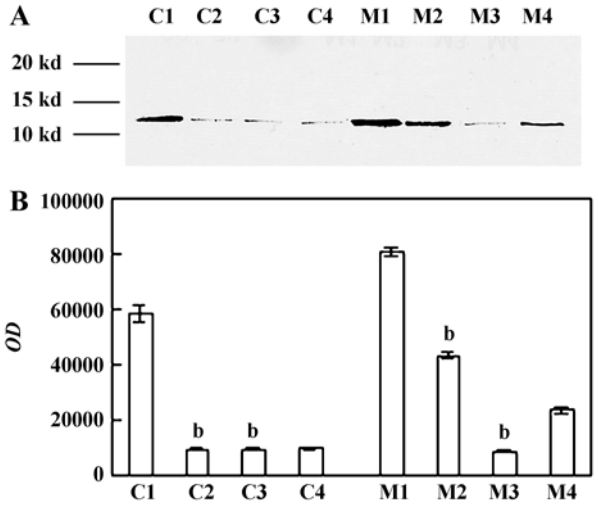

Fig 3. TGF-β1 expression in the kidney by Western blot. A) TGF-β1 expression in cortex and medulla of rat kidneys. B) Quantitative scoring of TGF-β1 expression. C and M are representative of cortex and medulla, respectively. C1-C4 indicates tissues taken from cortex of rats treated by CsA, CsA+TP, TP, and vehicle, respectively. M1-M4 indicates tissues taken from medulla of rats treated by CsA, CsA+TP, TP, and vehicle, respectively. ^{b}P<0.05 *vs* the CsA-treated group.

bioactivity of superoxide dismutase and glutathione peroxidase which can inactivate free oxygen radicals such as O_2^-. Because O_2^- can react with NO by forming $ONOO_2^-$ to loose bioactivity of NO, TP may reduce nitric oxide (NO) loss and maitain NO physical function[7,13]. In the experiment of chronic CsA nephrotoxicity, *N*-nitro-*L*-arginine-methyl ester (*L*-NAME) as the NO synthase inhibitor, strikingly worsens the glomerular filtration rate and the CsA-induced fibrosis, and upregulates expression of TGF-β1, plasminogen activator inhibitor-1 (PAI-1); *L*-arginine, as the substrate for NO synthase, has the opposite beneficial effect[14]. (2) TP may inhibit the synthesis of TXA_2 and LT which are mediated by CsA-induced lipid peroxidation. CsA induces the glomerular synthesis of O_2^-, H_2O_2, malondialdehyde. Vitamin E minimizes the adverse effects of CsA on kidney function and the glomerular synthesis of these compounds[15]. The treatment of adenovirus to express superoxide dismutase significantly blocks formation of ROS and minimizes pathological alterations and inhibition of renal function caused by CsA[5]. CsA can promote the expression of cycloxy-genase I (COX I), which was completely inhibited by vitamin E in chronic CsA nephrotoxicity[16]. COX I can promote the synthesis of TXA_2. F_2-isoprostane produced by a noncyclooxygenase mechanism involving lipid peroxidation, as a potent preglomerular vasoconstrictor acting principally through the interaction with TXA_2 receptor, also may be involved in the CsA nephrotoxicity by CsA lipid peroxidation[17]. The synthesis of leukotrienes (LT)

which are produced by the 5-lipoxygenase pathway is caused by the CsA-induced lipid peroxidation. Peptidoleukotriene as one kind of LT can stimulate fibroblast proliferation and extracellular matrix synthesis. LT receptor antagonist can largely block the CsA-induced renal vasoconstriction and morphological impairment[18]. Treatment with TXA_2 receptor antagonist GR32191 has a similar effect as thromboxane inhibitors in CsA nephrotoxicity[19]. (3) The potent vasoconstriction effects of TXA_2 and LT contribute to the activation of the renin-angiotensin system. Angiotensin II can cause the excessive expression of TGF-β1 and osteopontin which are associated with renal interstitial fibrosis. Osteopontin is also a macrophage chemoattract[20]. There are intrarenal angiotensin II deposits and excessive expression of angiotensin II type I receptor in renal medulla[21]. Angiotensin II type 1 receptor antagonist losartan can markedly reduce the degree of CsA-induced tubulointerstitial fibrosis, and the expression of TGF-β1 and osteopontin[22] .

These data suggested that antioxidant TP significantly ameliorated CsA-induced chronic nephrotoxicity and inhibited the expression of TGF-β1 in CsA-induced chronic nephrotoxicity.

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