

Original Article

Epigallocatechin-3-gallate protects against cisplatin nephrotoxicity by inhibiting the apoptosis in mouse

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Abstract: Cisplatin (CP) is a commonly used anticancer drug, but its notable side effect of nephrotoxicity limits its use in clinic. Epigallocatechin-3-gallate (EGCG), an anti-oxidant, anti-inflammatory, and anti-tumorigenic green tea polyphenol, has been available on the market for its beneficial effects. The aim of this study was to investigate whether EGCG can prevent the nephrotoxic effect of CP and the involved mechanisms. Male C57/BL6 mice were randomly divided into four groups: control group, EGCG group, CP group, and CP+EGCG group. On day 5, mice were sacrificed. Our results showed that EGCG treatment significantly ameliorated the histopathological changes and the increased serum creatinine and blood urea nitrogen (BUN) induced by CP. TUNEL-positive cells significantly reduced in the CP+EGCG group compared with CP group. EGCG also inhibited the expression of the ligand of death receptor Fas (Fas-L), apoptosis regulator BAX (Bax) and tumor-suppressor protein p53, and increased the expression of B-cell lymphoma 2 (Bcl-2). These findings suggest that EGCG can ameliorate CP-induced apoptosis in the kidney by regulating death receptor Fas conducted extrinsic pathway, and the expression of Bax and Bcl-2.

Keywords: Cisplatin (CP), Epigallocatechin-3-gallate (EGCG), renal, apoptosis, mouse

Introduction

Cisplatin or *cis*-diamine-dichloroplatinum (II) (CDDP) is a platinum-containing anti-cancer drug widely used against multiple solid tumors. Many side effects such as ototoxicity, gastrotoxicity, myelosuppression, and allergic reactions [1, 2], especially the nephrotoxicity [3, 4], limits its use in cancer therapy. The mechanisms underlying nephrotoxicity induced by CP are not clearly established. Recently many studies have identified that apoptosis plays a pivotal role in renal tubulointerstitial fibrosis [5, 6], and the apoptosis of tubular epithelial cells is a major cause [7]. Several cellular signaling pathways include the intrinsic mitochondrial pathway, the extrinsic death-receptor pathway such as TNF- α /TNFR1 and Fas/Fas-L pathway, and endoplasmic reticulum stress related mechanisms have been reported to be involved in regulation of apoptosis in tubular epithelial cells [8-11]. It has been shown that CP-induced nephrotoxicity associated with increased pro-

apoptotic protein Bax and decreased anti-apoptotic protein Bcl-2 [11]. Fas is expressed on renal tubular cells (RTCs), and its upregulation during acute and chronic renal failure has been documented by many reports [12, 13]. However, whether apoptosis of RTC depends on the Fas/FasL pathway remains controversial [14].

Numerous compounds, such as tomato lycopene complex, grape seed proanthocyanidin extract, extract of Ginkgo biloba, Rosmarinus acid, and cilastatin, can ameliorate cisplatin-induced nephrotoxicity [15-19]. EGCG, the most abundant catechin in tea, has anti-oxidant, anti-inflammatory, and anti-tumorigenic properties. Many studies have showed its effects to blood diseases, cardiovascular diseases, respiratory diseases, eye diseases, and cancers [20-24]. Some studies also demonstrated the renal protect effect EGCG [25-27], leading us to study whether EGCG can ameliorate the acute kidney injury induced by CP. The nephrotoxicity of CP involves Nrf2/HO-1 signaling pathway [28],

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Table 1. The changes in relative kidney weight and the levels of Creatinine and BUN

	Relative kidney weight	Creatinine ($\mu\text{mol/L}$)	BUN (mmol/L)
Control	8.32 ± 0.71	30.57 ± 6.95	9.99 ± 0.97
EGCG	8.46 ± 0.85	32.00 ± 4.43	10.16 ± 1.84
CP	8.85 ± 0.45^a	178.86 ± 19.58^a	82.76 ± 7.57^a
CP+EGCG	8.42 ± 1.08^b	63.71 ± 6.65^b	29.29 ± 3.84^b

Data are presented as means \pm SD. Relative kidney weight is expressed as: left kidney weight/body weight \times 1000. ^a $p < 0.05$ versus control group; ^b $p < 0.05$ versus CP group.

renal proinflammatory (TNF- α) and oxidant stress signals [29]. The protect mechanism of EGCG on CP-induced nephrotoxicity is still uncertain. So, in this study, we treated mouse model of CP-induced nephropathy with EGCG, and study of whether it has the protect effects to cisplatin nephrotoxicity and the mechanisms.

Materials and methods

Reagents and materials

Cisplatin and Epigallocatechin-3-gallate (EGCG) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Primary antibodies were provided as follows: Fas-L, Bax, Bcl-2, p53 and β -actin antibody (Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA). Antimouse and antirabbit secondary antibodies were obtained from Jackson ImmunoResearch Laboratories Inc. (West Grove, PA, USA). TUNEL staining kit (the In Situ Cell Death Detection kit) was purchased from Roche Diagnostics (Indianapolis, IN, USA).

Animals

Twenty-eight 6-8 week-old adult male C57/BL6 mice (20-25 g) were obtained from the Beijing Vital River Laboratory Animal Technology Co., Ltd. (Beijing, China). Mice were housed separately in metal cages. The cages were placed in a room with controlled temperature ($22 \pm 0.5^\circ\text{C}$), humidity ($60 \pm 10\%$), and a 12-h light-dark cycle. Food and water were available ad libitum. The animal experiments conformed to the Animal Management Rules of the Chinese Ministry of Health (document No. 55, 2001) and were approved by the Animal Care Committee of Shandong University.

Experimental design

A total of 28 mice were divided randomly in the following five groups: (1) control group (NC; $n = 7$), only received intraperitoneal (i.p.) injection

of vehicle solution (0.9% saline; 10 ml/kg); (2) EGCG group (EGCG; $n = 7$), received a single i.p. injection of 100 mg/kg EGCG (dissolved in 0.9% saline to reach 20 mg/ml); (3) CP group (CP; $n = 7$), only received an i.p. injection of 20.0 mg/kg CP (dissolved in 0.9% saline to reach a concentration of 2.0 mg/ml); and (4) CP+EGCG 100 mg/kg group (CP+EGCG 100 mg/kg; $n = 7$), which successively received i.p. injection of 100 mg/kg EGCG at 30 min before i.p. injection of CP, and i.p. administration of 100 mg/kg EGCG after i.p. injection of CP 48 h.

Five days after i.p. injection of CP, all mice were sacrificed by cervical dislocation, and all of them were weighed and blood was collected from the endocanthion before the sacrifice. Blood samples were centrifuged at 1,500 g at 4°C for 15 min, and sera were collected. Blood urea nitrogen (BUN) and serum creatinine (Scr) levels were measured in a Cobas® 8000 modular analyser (Roche Diagnostics) in Qilu Hospital, Shandong University. Both kidneys were immediately excised, and then cut in half by coronal position after weighed. Half of each excised kidney was stored at -80°C , and the remaining sections were fixed in 4% buffered paraformaldehyde at 4°C and embedded in paraffin.

Histopathologic observation

Histopathological changes in the kidney were examined by periodic acid-Schiff (PAS) staining. Kidneys embedded in paraffin were cut into 4- μm thick sections. Deparaffinized sections were stained with PAS reagent. Tubular damage was assessed by an index of renal tubular necrosis in 10 different fields: 0 = no damage, 1 = less than 25% damage, 2 = 25-50% damage, 3 = 50-75% damage, and 4 = more than 75% damage.

Immunohistochemical study

For immunohistochemical analysis, deparaffinized tissue slices underwent antigen retrieval by microwaved for 10-15 min in 0.01% sodium citrate buffer (pH 6.0). 3% hydrogen peroxide was used to immerse the tissue slices for 10 min in dark to block endogenous peroxidase. The tissue slices were incubated with the primary antibody (anti-Fas-L 1:100, anti-Bax

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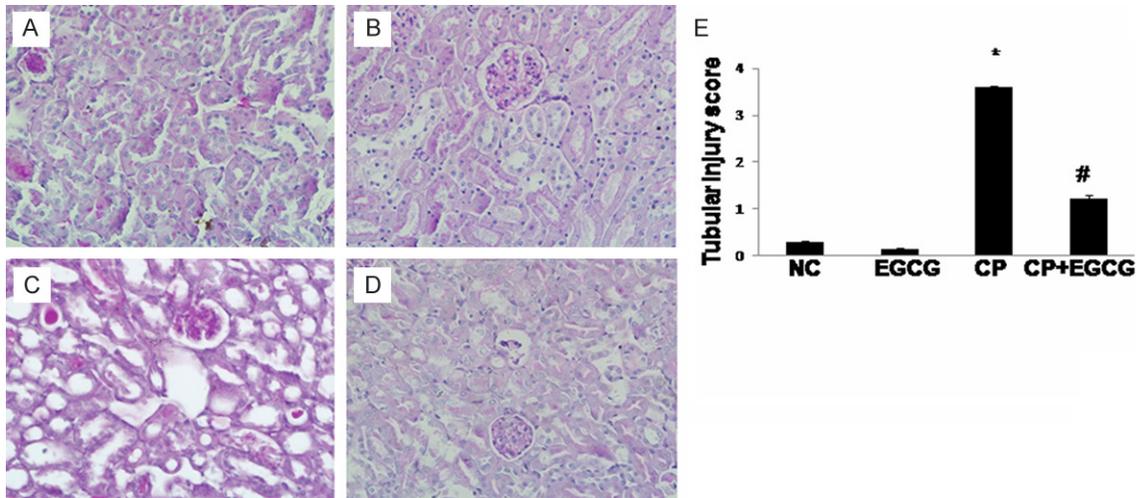


Figure 1. EGCG attenuated the kidney histological abnormalities induced by CP in mice. Mice were treated with vehicle (A), EGCG (B), CP (C), and CP+EGCG (D), separately. In CP group, renal tubular atrophy and dilation, necrosis and desquamation of renal tubular epithelial cells, and intratubular cast formation in the proximal tubules of kidney were obvious. Tubular damage was greatly improved in CP+EGCG group. Tubular injury score (E). Data are presented as means \pm SD. * p <0.05 versus control group; # p <0.05 versus CP group. Original magnification, $\times 400$.

1:100 and anti-Bcl-2 1:100) at 4°C overnight, while negative controls were incubated with PBS. After washing three times, slices were incubated with secondary antibodies for 60 min at 37°C, and then stained with 3, 3'-diaminobenzidine (DAB) and hematoxylin. Stained slides were analyzed by light microscopy. Brown areas were identified as positive. Semi-quantitative analysis was performed on the colored sections using Image-Pro Plus 5.0.

TUNEL assay

The terminal deoxynucleotidyltransferase-mediated nick end labeling (TUNEL) method was performed to evaluate the *in situ* apoptosis in kidney tubular cells. The TUNEL staining was conducted following the manufacturer's instructions. A DAPI filter was used to detect DAPI staining (blue color), and an FITC filter was used to detect TUNEL staining (red color). TUNEL-positive cells were counted in 10 high-power ($\times 400$) fields per section in the cortex.

Western blot analysis

Concentration of the protein extraction of the mouse kidney tissue samples was determined according to the procedure described by the Pierce BCA Protein Assay kit (Thermo Fisher Scientific Inc., Rockford, IL, USA). Equal amounts of protein (30 μ g) were electrophoresed and subsequently transferred to cellulose acetate membranes. The membranes

were blocked in TBS buffer containing nonfat milk for 1 h and then incubated with primary antibodies (anti-Fas-L 1:200, anti-Bax 1:200, antiBcl-2 1:200, anti-p53 1:500 and anti β -actin 1:2,500) at 4°C overnight. The membranes were then washed and incubated with secondary antibodies for 1 h. Finally the membranes were developed with enhanced chemiluminescence (ECL) reagent (Thermo Fisher Scientific Inc., Rockford, IL, USA) and exposed to an X-ray film. Band intensity was measured using Quantity One software (Bio-Rad, Hercules, CA, USA). Fas-L, Bax, Bcl-2 and p53 relative quantities were expressed as a ratio of luminosity of the respective sample to that of the normal control group.

Statistical analysis

Data are given as means \pm standard deviation (S.D.). The intergroup variation between groups was evaluated using one-way analysis variance (ANOVA) followed by Dunnett's multiple comparison test, and the comparisons between two groups were conducted by unpaired Student's *t*-test. P <0.05 was considered statistically significant.

Results

EGCG protects against CP-induced renal injury in mouse

In mice, treatment with CP induced significant increase both in the relative kidney weight and

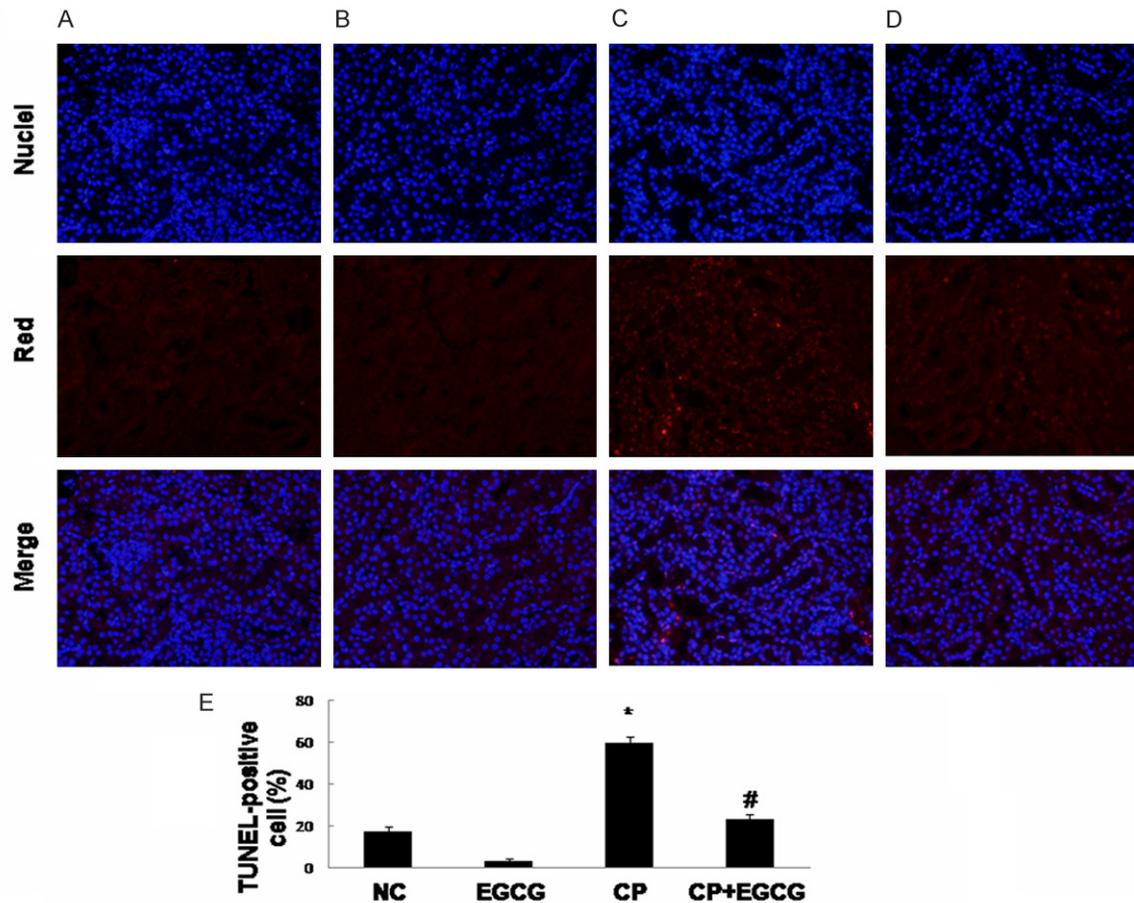


Figure 2. EGCG inhibits the apoptosis of renal tubular epithelial cells induced by CP. Mice were treated with vehicle (A), EGCG (B), CP (C), and CP+EGCG (D), separately. Red staining represents TUNEL-positive cells. Original magnification, $\times 400$. The percentage of TUNEL-positive cells in different groups (E). Data are presented as means \pm SD. * $p < 0.05$ versus control group; # $p < 0.05$ versus CP group.

in the levels of serum creatinine and BUN (Table 1, compare CP group with control). Co-treatment of EGCG together with CP suppressed the increased relative kidney weight and creatinine and BUN levels caused by CP treatment alone (Table 1, compare CP+EGCG group with CP group), while treatment with EGCG alone had no effect on these parameters.

The PAS staining of kidney tissues was conducted to evaluate whether EGCG can ameliorate CP-induced renal tubular damage. Normal tubular morphology was presented in control group (Figure 1A) and EGCG group (Figure 1B). Renal tubular atrophy and dilation, necrosis and desquamation of renal tubular epithelial cells, and intratubular cast formation in the proximal tubules of kidney were observed in CP

group (Figure 1C), while the tubular damage was greatly improved by EGCG (Figure 1D). EGCG dramatically reduced the tubular injury scores after CP treatment, and the reduction was statistically significant ($P < 0.05$). These data indicated EGCG suppressed renal injury caused by CP.

EGCG blocks apoptosis of tubular epithelial cells caused by CP

In order to assess whether EGCG can protect against CP-induced renal tubular epithelial cell apoptosis, TUNEL assay was conducted. A large number of TUNEL-positive renal tubular epithelial cells were detected in CP group (Figure 2C), whereas co-treatment with EGCG strongly reduced the percentage of TUNEL-positive cells (Figure 2D). Limited apoptosis was detected in

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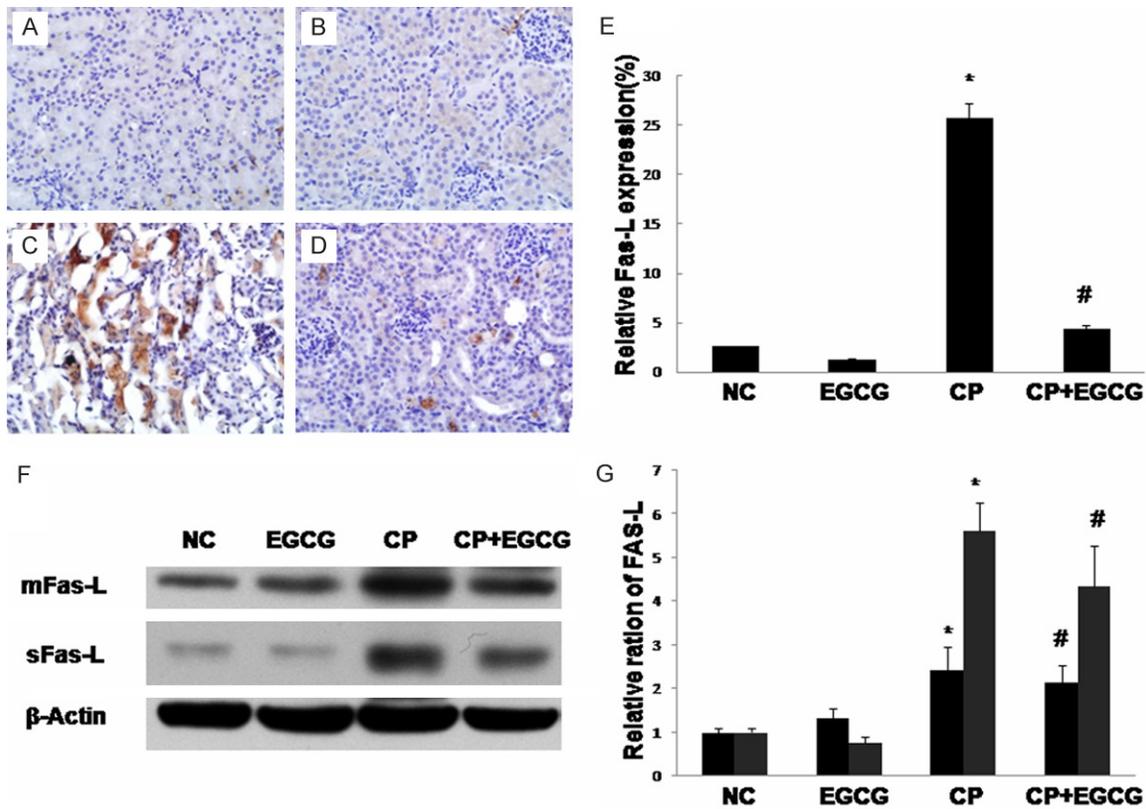


Figure 3. Immunohistochemical of Fas-L in the kidney of mouse. Mice were treated with vehicle (A), EGCG (B), CP (C), and CP+EGCG (D), separately. The brown granules represent positively stained cells. Original magnification, $\times 400$. Measurement of the intensity of Fas-L immunostaining (E). Western blot analysis of Fas-L (F), and quantification of corresponding protein level (G). Data are presented as means \pm SD. * $p < 0.05$ versus control group; # $p < 0.05$ versus CP group.

control group (Figure 2A) and the EGCG group (Figure 2B). Thus, EGCG abrogated CP-induced apoptosis.

The mechanism of the protective effect of EGCG to the nephrotoxicity induced by CP in mouse

Death receptor Fas has been implicated in CP-induced renal epithelial cell death. Marker proteins in this pathway such as Fas-L, Bax, and Bcl-2 have been examined by immunohistochemistry and western blotting. As shown in Figures 3-5, both two approaches exhibited that Fas-L and Bax were highly expressed in CP group while the expression of Bcl-2 was limited. The antiapoptotic activity of EGCG was evident by elevated Bcl-2, coincided with decreased expression of Fas-L and Bax. In the control and EGCG groups, Fas-L and Bax showed limited expression, and Bcl-2 showed high expression.

p53 was reported to be involved in Fas/Fas-L-induced apoptosis, and a possible transcrip-

tional regulator of Fas and Bax. Our results showed that the expression of p53 was high in CP group, and inhibited in CP+EGCG group (Figure 6).

Discussion

CP is a simple platinum-containing inorganic molecule. It is one of the most remarkable successes in cancer therapy and has been widely used for chemotherapy since the accidental discovery over four decades ago [3, 30, 31]. Nephrotoxicity, now recognized as the most prevalent side effect, represents a dose and time-related toxicity, occurring in about one-third of patient undergoing CP treatment [3, 32]. The most severe and common outcome of nephrotoxicity of CP is acute kidney injury (AKI) [32]. It develops primarily in the proximal tubule [33]. CP-induced AKI involves enhanced oxidative stress, inflammatory reactions, and tubular cell apoptosis. Renal tubular apoptosis has been considered as a key mechanism [34, 35]. EGCG is the most abundant catechin in green

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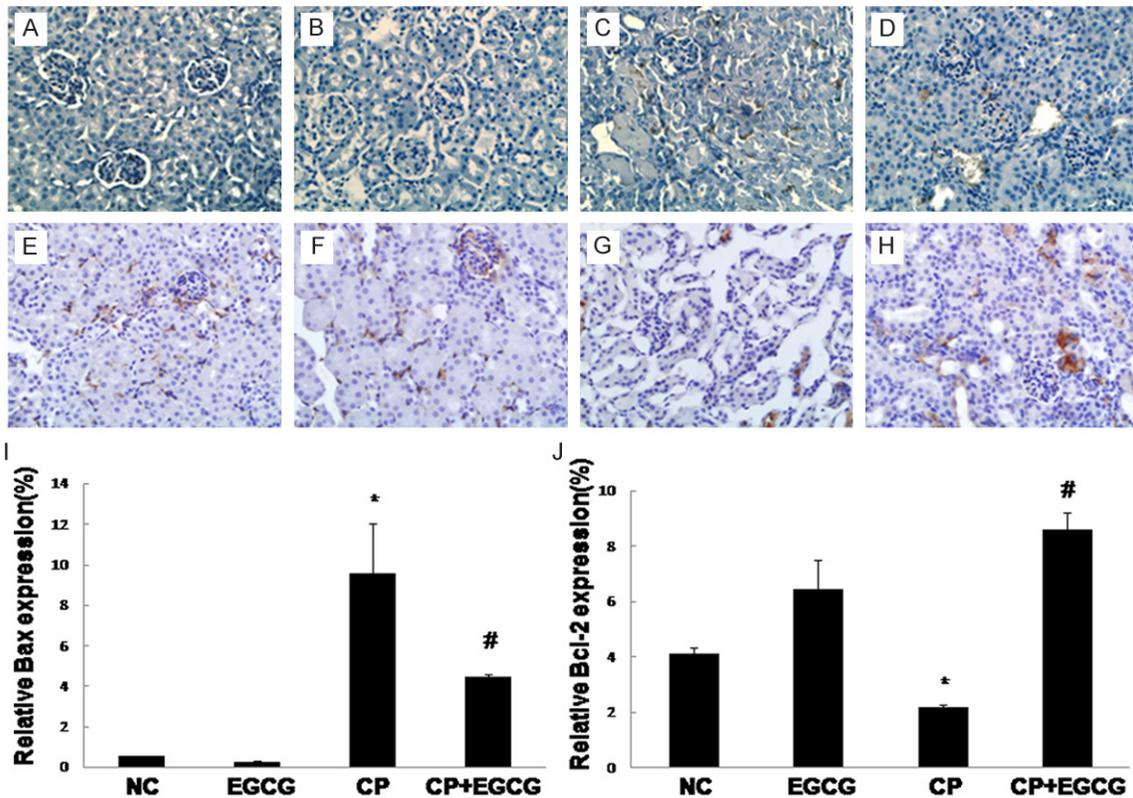


Figure 4. Immunohistochemical detection of Bax and Bcl-2: Mice were treated with vehicle (A, E), EGCG (B, F), CP (C, G), and CP+EGCG (D, H), separately. The brown granules represent positively stained cells. Original magnification, $\times 400$. Measurement of the intensity of Bax (I) and Bcl-2 (J) immunostaining. Data are presented as means \pm SD. * $p < 0.05$ versus control group; # $p < 0.05$ versus CP group.

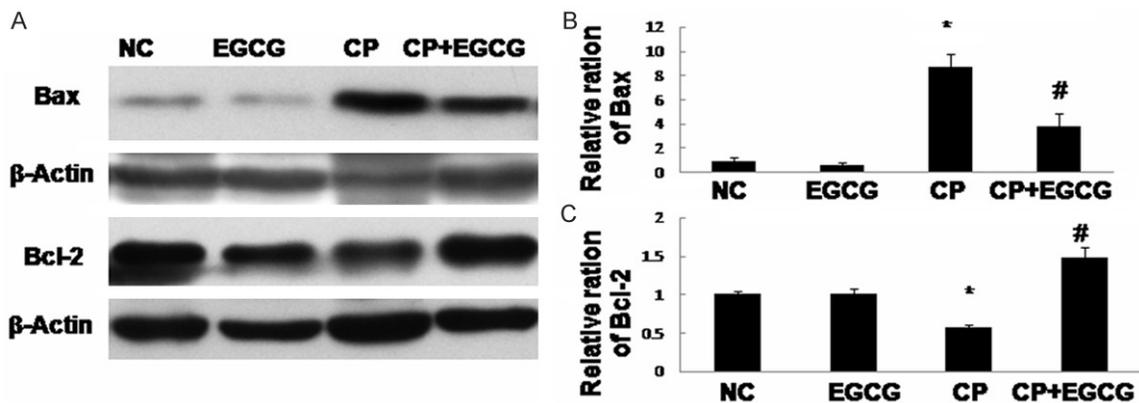


Figure 5. Western blot analysis of Bax and Bcl-2 (A), and quantification of the protein levels (B, C). Mice treated with vehicle, EGCG, CP, and CP+EGCG. Data are expressed as mean \pm SD. * $p < 0.05$ versus control group; # $p < 0.05$ versus CP group.

tea, and it has been shown to reduce and inhibit the growth of various tumors [24]. Based on the antioxidant, anti-inflammatory and anti-apoptotic properties of EGCG, the present study was undertaken to examine the protec-

tive effects of EGCG against CP-induced nephrotoxicity. Results in this study showed that EGCG effectively ameliorate acute kidney injury in CP-treated mice by suppressing the Fas/Fas-L pathway and regulating expressions of

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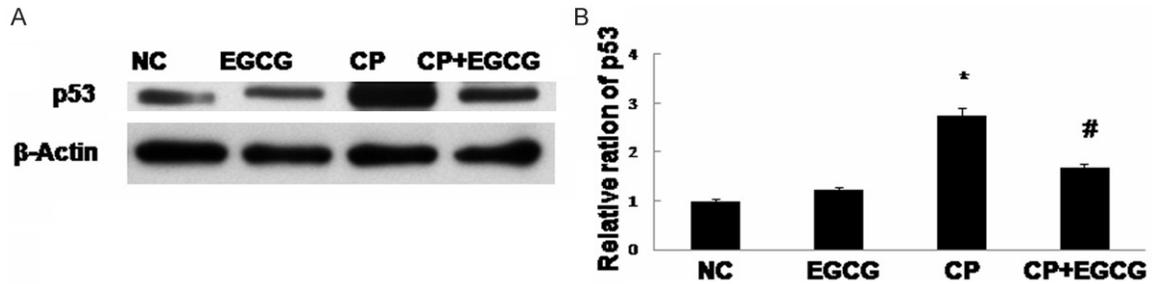


Figure 6. Western blot analysis of p53 (A) and quantification of the protein level (B). Mice treated with vehicle, EGCG, CP, and CP+EGCG. Data are expressed as mean \pm SD. * p <0.05 versus control group; # p <0.05 versus CP group.

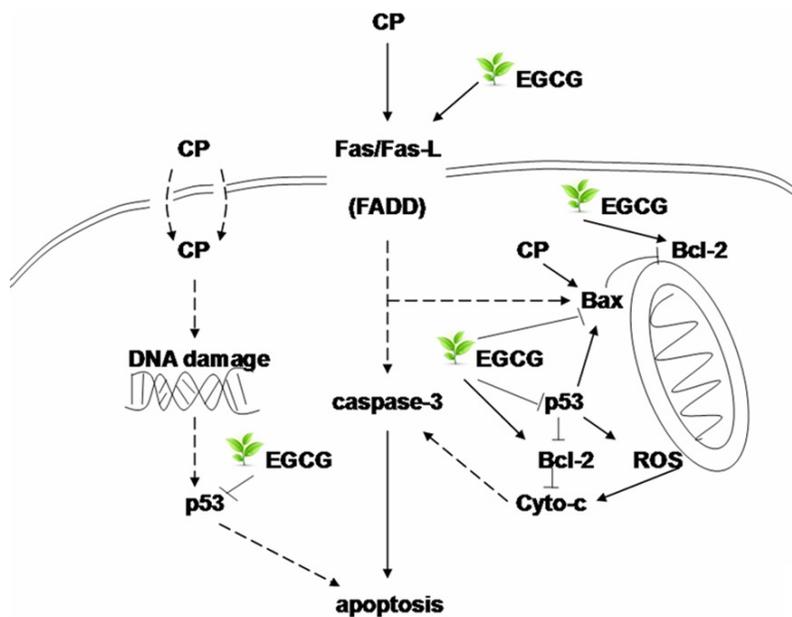


Figure 7. The possible mechanism of the protect effect of EGCG to the nephrotoxicity induced by CP in mouse.

Bcl-2 family members of renal tubular cells. In this study, CP resulted in a severe nephropathy. In CP group, the relative kidney weight, the levels of serum creatinine and BUN, were significantly increased. Apoptosis of renal tubular epithelial cells was also significantly increased. Both the functional defects and apoptosis were markedly ameliorated by EGCG treatment, indicating the renal protective effect of EGCG on CP-induced nephrotoxicity.

Tubulointerstitial inflammation and tubular epithelial cell apoptosis have been demonstrated as the key leading to AKI in CP chemotherapy [14]. Several apoptotic pathways include the extrinsic pathway activated through death receptors, such as TNF receptors or Fas, the

intrinsic mitochondrial pathway and the endoplasmic reticulum stress related mechanisms, have been implicated in CP-induced renal tubular epithelial cell death [32]. In the murine kidneys injured by CP administration, Fas expression markedly induced in cultured proximal tubular epithelial cells, while absence of Fas protected them from undergoing apoptosis [11]. Recently studies reported that CP-induced nephropathy is mediated through upregulation of Fas/Fas-L system [12, 36]. During the intracellular cascade of caspase activation, activation of caspase-8 can activate

the pro-apoptotic protein Bax, and then following the apoptosis in the end.

The intrinsic mitochondrial pathway by which CP activates remains unknown [10]. CP generates reactive oxygen species, which activate the pro-apoptotic Bcl-2 family member Bax, then the activation of Bax induces mitochondrial permeability transition, leading to release of cytochrome c, finally activates caspase-3 and induces the apoptosis [10]. The anti-apoptotic Bcl-2 family members such as Bcl-2, plays a pivotal protective role in preserving mitochondrial structure and function, preventing onset of mitochondrial permeability transition, and finally inhibiting the apoptosis [37, 38]. Over-expression of Bcl-2 obviously ameliorated

CP-induced apoptosis of renal tubular epithelial cells [39]. The integration of diverse pro- and anti-apoptotic signals occurring at the mitochondria may decide cell fate [40]. Several studies have demonstrated that CP can directly activate Bax, thus down-regulate the expression of Bcl-2 [8, 10, 41].

Tumor suppressor p53 is activated by CP and pharmacological or genetic inhibition of p53 suppresses CP-induced apoptosis in RTC in vitro and nephrotoxicity in vivo [42-44]. Several studies have emphasized the involvement of p53 in Fas/Fas-L-induced apoptosis [45, 46]. In addition, oxidant stress can activate p53 [47], and then p53 can directly activate Bax [48]. p53 may be a transcriptional regulator of Fas and Bax [14, 48].

Consistently with previous findings, in this study, the expression of Fas-L, Bax and p53 increased, and the expression of Bcl-2 decreased in the kidneys of CP group. Administration of EGCG before CP treated significantly reduced the overexpression of Fas-L, Bax and p53 and rescued the downregulation of Bcl-2, suggesting the inhibition of tubular apoptosis (**Figure 7**).

In conclusion, the results of this study showed EGCG can effectively ameliorate CP-induced tubular apoptosis, and the inhibition on CP-induced apoptosis in tubular epithelial cells by EGCG may be through the blockage of the Fas/Fas-L pathway, and regulation of the expression of Bcl-2 family members. The suppression of apoptosis in renal tubular epithelial cells by EGCG could be an effective strategy for the treatment of CP-induced nephropathy. EGCG may have potential value in clinical CP chemotherapy while the therapeutic activity of it still needs to be further confirmed.

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Disclosure of conflict of interest

None.

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