

RESEARCH ARTICLE

# Mitochondrial Modulation by Epigallocatechin 3-Gallate Ameliorates Cisplatin Induced Renal Injury through Decreasing Oxidative/Nitrative Stress, Inflammation and NF- $\kappa$ B in Mice

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## Abstract

Cancer chemotherapy drug cisplatin is known for its nephrotoxicity. The aim of this study is to investigate whether Epigallocatechin 3-Gallate (EGCG) can reduce cisplatin mediated side effect in kidney and to understand its mechanism of protection against tissue injury. We used a well-established 3-day cisplatin induced nephrotoxicity mice model where EGCG were administered. EGCG is a major active compound in Green Tea and have strong anti-oxidant and anti-inflammatory properties. EGCG protected against cisplatin induced renal dysfunction as measured by serum creatinine and blood urea nitrogen (BUN). EGCG improved cisplatin induced kidney structural damages such as tubular dilatation, cast formation, granulovacuolar degeneration and tubular cell necrosis as evident by PAS staining. Cisplatin induced kidney specific mitochondrial oxidative stress, impaired activities of mitochondrial electron transport chain enzyme complexes, impaired anti-oxidant defense enzyme activities such as glutathione peroxidase (GPX) and manganese superoxide dismutase (MnSOD) in mitochondria, inflammation (tumor necrosis factor  $\alpha$  and interleukin 1 $\beta$ ), increased accumulation of NF- $\kappa$ B in nuclear fraction, p53 induction, and apoptotic cell death (caspase 3 activity and DNA fragmentation). Treatment of mice with EGCG markedly attenuated cisplatin induced mitochondrial oxidative/nitrative stress, mitochondrial damages to electron transport chain activities and antioxidant defense enzyme activities in mitochondria. These mitochondrial modulations by EGCG led to protection mechanism against cisplatin induced inflammation and apoptotic cell death in mice kidney. As a result, EGCG improved renal function in cisplatin mediated kidney damage. In addition to that, EGCG attenuated cisplatin induced apoptotic cell death and mitochondrial reactive oxygen species

**Competing Interests:** The authors have declared that no competing interests exist.

(ROS) generation in human kidney tubular cell line HK-2. Thus, our data suggest that EGCG may represent new promising adjunct candidate for cisplatin.

## Introduction

Cisplatin is a highly prescribed drug for cancer. Cisplatin cause cell death to cancer cells after binding to DNA. However, its chemotherapeutic efficacy is severely limited by its nephrotoxicity. The toxicity is mainly due to high amount of cisplatin accumulation in the kidneys with involvement of a specific renal transport systems [1]. Production of reactive oxygen species (ROS) by cisplatin in kidney is crucial to the progression of nephrotoxicity [1,2]. The major sources of ROS production in cisplatin nephrotoxicity are mitochondria and nicotinamide adenine dinucleotide phosphate (NADPH) oxidases [3,4].

Epigallocatechin-3-gallate (EGCG) is a type of catechin and most abundant in Green Tea (*Camellia sinensis Theaceae*). Green Tea has many health benefits such as cancer, inflammation, neuro-protection, cardiovascular disease etc. and is traditionally used as medicine in China and India [5]. Several studies on the beneficial effect of active compound EGCG of Green Tea is mostly focused in cancer [6–8].

Several plant derived phenolic compounds such as Broccoli derived sulforaphane, asian spice derived curcumin, capsaicin, rosemary derived Carnosic acid and arabic gum prevents cisplatin induced nephropathy [9–16]. Transcriptional regulators NF- $\kappa$ B, p53 and NRF2 play important roles as protection mechanism [4,17,18]. Recently, we have demonstrated that metalloporphyrin protects against cisplatin induced renal injury in mice by reducing peroxynitrite formation [19]. Mitochondria targeted antioxidants and PARP pathway inhibitors also demonstrate protection against cisplatin nephrotoxicity in mice [3,20]. One of the key component of cisplatin nephrotoxicity is inflammation followed by recruitment of neutrophils/other leukocytes [4,9,20]. Thus, most cell culture based in vitro studies lack this important component (renal circulation) and limit the understanding of mechanistic aspect.

EGCG mediated protection against cisplatin nephrotoxicity has been reported in rat and mouse model [21,22]. In rat model, Nrf2/HO-1 signaling pathway plays role in protection mechanism by EGCG [22]. In a recent study on mouse model of cisplatin nephrotoxicity, EGCG ameliorate cisplatin induced apoptosis by modulating death receptor Fas, Bax and Bcl-2, those factors involved in extrinsic pathway of apoptosis [21]. However, little is known about detail upstream events of apoptosis which leads to renal protection by EGCG.

In this study we aimed to understand the mechanism of protection by EGCG against cisplatin administration in mice. We used a well-studied 3-day cisplatin induced kidney injury model. EGCG attenuated cisplatin induced serum BUN and creatinine by modulating apoptotic cell death. The mechanism of preventing apoptotic cell death of EGCG was mediated by specific mitochondria mediated protection. EGCG ameliorated cisplatin induced mitochondrial oxidative/nitrative stress and improved cisplatin induced damages of mitochondrial electron transport chain complexes. EGCG also improved functions of anti-oxidant defense enzymes glutathione peroxidase and manganese superoxide dismutase, which lost activity with cisplatin administration in mouse kidney. Thus, EGCG regulated the apoptotic trigger by these combined mitochondrial modulation and also reduced cisplatin induced inflammatory response. In a human tubular cell line HK-2, EGCG reduced cisplatin-induced mitochondrial ROS production and apoptotic cell death.

## Materials and Methods

### Ethics Statement of the study

This study was performed under “Guide for the Care and Use of Laboratory Animals” of the National Institutes of Health. All protocols were approved by the Committee on the Ethics of Animal Experiments of First Affiliated Hospital, College of Medicine, Zhejiang University (Permit Number: 09–028) at the Chinese Academy of Sciences. All efforts were made to minimize suffering.

### Animal Experiments

The mouse strain C57BL/6 was used. Male mice of ~8 weeks age with weights of 18–22 g were used in all experiments. Each experimental group was composed of 6–8 mice. Animals were kept under constant temperature (25°C) and humidity and had access to food and water *ad libitum* throughout the study. Mice were sacrificed under deep anesthesia with 5% isoflurane followed by cervical dislocation on third day (72 hours) after a single injection of cisplatin (cis-diammine platinum (II) dichloride, Sigma) at dose 20 mg/kg i.p. in DMSO/saline vehicle. EGCG was purchased from Sigma Chemical. Drug was dissolved in saline and administered at 100 mg/kg (or as described in text), i.p. for two days, starting 2h before the cisplatin administration. EGCG and vehicle were administered alone (without cisplatin treatment) as separate group.

### Cell culture, treatment and flow cytometry

HK-2 cells (ATCC, VA, USA) were cultured in a Keratinocyte Serum Free Medium and supplements (Life Technologies Inc, CA, USA). Cells were grown under conditions of 95% air and 5% CO<sub>2</sub> at 37°C. HK-2 cells were seeded onto 12 well cell culture plates. Cells were treated with a final concentration of EGCG at 10 μM. Following a 2h pretreatment with vehicle (saline) or EGCG, cells were exposed to cisplatin at concentration 60 μM for 24h. Cells were incubated with MitoSOX red at 5 μM for 15 mins. Cells were analyzed for simultaneous detection of apoptosis and mitochondrial ROS generation as described earlier in details [23,24]. Flow cytometry was performed with FACS Calibur flow cytometer (BD Biosciences, CA, USA). Early apoptotic cell death was characterized with Annexin V-Allophycocyanin (APC) staining and late apoptosis were determined with Sytox Green staining. Mitochondrial ROS was measured with MitoSOX Red dye and mean intensity was calculated for each samples. Total Cell death was determined for each samples by adding early and late apoptotic cell numbers (Quadrant Q3 and Q2).

### Measurements of serum Creatinine and BUN

On the day of the sacrifice, blood was immediately collected by cardiac puncture and processed. Serum levels of blood urea nitrogen (BUN) and creatinine were measured as described earlier [19].

### Histological evaluation of kidney damage

Kidneys were fixed with 10% formalin for 24 hours in a shaker and embedded in paraffin. Kidneys were sectioned with a microtome, deparaffinized and stained with periodic acid–Schiff (PAS) reagents for histological examination as described earlier [19]. Tubular damage in PAS-stained sections was examined under the microscope and scored based on the percentage of cortical tubules showing epithelial damage: 0-normal; 1<10%; 2–10–25%; 3–26–75%; 4>75%. In details, scoring was carried out based on tubular dilatation, cast formation, granulovacuolar

degeneration and tubular cell necrosis. The morphometric examinations were performed in a blinded manner.

### Isolation of Mitochondria from fresh tissue

All manipulations were performed at 4°C or on ice to minimize mitochondrial-membrane and protein degradation. Whole kidney from experimental animals were harvested and immersed in isotonic homogenization buffer (225 mM mannitol/75 mM sucrose/10 mM MOPS/1 mM EGTA/0.5% BSA, pH 7.2). Mitochondria were isolated by differential centrifugation as described earlier [25]. In briefly, kidney tissues were homogenized isotonic solution described above. The homogenate was centrifuged at 1500g for 5min at 4°C. The supernatant was centrifuged again at 8000g for 15mins at 4°C and the step was repeated one more time. After final centrifugation, mitochondrial pellets were suspended in 150–600 µl of RIPA buffer (Sigma, Hong Kong, China), and protein concentration of mitochondrial fraction lysate was determined by using Bio Rad protein assay kit (BioRad Laboratories, Shanghai, China). The values obtained were corrected for BSA.

### Isolation of nuclear fraction from tissue

Nuclear fractions were prepared using commercial nuclear fractionation kit (Pierce, IL, USA) and followed manufacturer's instruction.

### Estimation of mitochondrial respiratory enzyme activities

The mitochondrial respiratory enzyme activities such as NADH dehydrogenase (Complex I Enzyme Activity Microplate Assay Kit, Abcam Trading Company Ltd. Shanghai, China.), succinate dehydrogenase (Complex II Enzyme Activity Microplate Assay Kit, Abcam Trading Company Ltd. Shanghai, China) Enzyme Activity Microplate Assay Kit, Abcam Trading Company Ltd. Shanghai, China) and cytochrome oxidase (cytochrome c oxidase assay kit, Sigma-Aldrich Co, St Louis, MO, USA) were estimated according to manufacturer's instruction.

### Quantitative measurement of renal mitochondrial protein nitration in mice

Nitrotyrosine content was evaluated by ELISA. Briefly, an identical amount of protein from mitochondrial fraction was applied to a Maxisorp ELISA plate together with nitrated BSA (Bovine serum albumin) standard and allowed to bind overnight at 4°C. After blocking, wells were incubated at 37°C for 2 h with a mouse monoclonal antibody anti-nitrotyrosine (Upstate Biotechnology, Lake Placid) and then for 1h at 37°C with a peroxidase conjugated goat anti-mouse IgG secondary antibody. After washing, peroxidase reaction product was generated using 3, 3', 5 5'-tetramethylbenzidine (TMB) peroxidase substrate.

### Quantitative measurement of renal mitochondrial HNE protein adducts

HNE adducts from mitochondrial fraction were determined using OxiSelect™ HNE Adduct ELISA Kit according to manufacturer's instruction (Cell Biolabs, Genetimes Technology, Inc, Shanghai, China).

### Quantitative determination of MnSOD activity

MnSOD activity was determined from mitochondrial fraction using SOD activity kit (Enzo Life Sciences International, Inc., Plymouth Meeting, PA, USA). This colorimetric assay evaluates the ability of SOD to reduce the superoxide ion concentration generated from the conversion of

xanthine and oxygen to uric acid and hydrogen peroxide by xanthine oxidase. SOD activity was determined from percent inhibition of the rate of WST-1-formazan formation with readout at 450 nm. Each sample was loaded in a 96 well plate to the final amount of 10 $\mu$ g/well. A kinetic assay was performed according to manufacturer's instruction. SOD activity was expressed as fold change compared to vehicle control.

### Quantitative Glutathione Peroxidase Assay from mitochondrial fraction

Mitochondrial glutathione peroxidase was measured using Glutathione Peroxidase (GPX) Assay Kit (Abcam Trading Company Ltd. Shanghai, China) according to manufacturer's instruction. Glutathione peroxidase reduces cumene hydroperoxide while oxidizing GSH to GSSG. The generated GSSG is reduced to GSH with consumption of NADPH by GR. The decrease of NADPH (measured at 340 nm) is proportional to GPX activity. The GPX activity was displayed as fold change compared to vehicle.

### Quantitative measurement of renal apoptosis in mice

Caspase-3/7 activity of the lysate was measured using Apo-One Homogenous caspase-3/7 Assay Kit (Promega Corp., Madison, WI, USA). An aliquot of 100 $\mu$ l caspase assay agent was added to equal volume tissue lysate in each well, mixed on a plate shaker for 2 h at 37°C in dark and the fluorescence was measured [19].

### Quantitative measurement of renal DNA fragmentation in mice

The DNA fragmentation assay was carried out in tissue extracts using a commercially available kit (Cell Death Detection ELISA Kit, Roche China Ltd., Shanghai) according to manufacturer's instructions [19].

### Real-time PCR

Isolation of RNA and Real-time PCR were carried out as described earlier [19]. The primer sets for TNF $\alpha$ , IL1 $\beta$  and  $\beta$ -actin were purchased from Qiagen (Pudong, Shanghai, China).

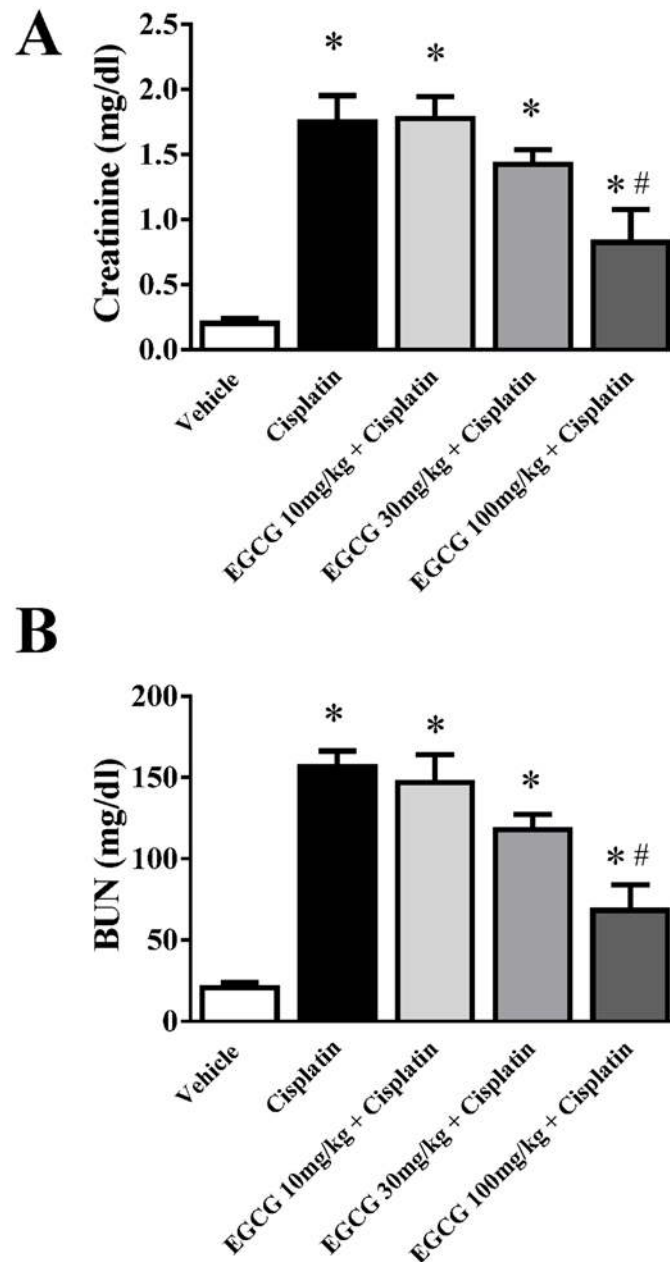
### Statistical Analysis

All data were presented as the means  $\pm$  SEMs. Multiple comparisons (Tukeys) were performed using one way ANOVA. The analyses were performed with Graph Pad Prism software Version 6.01 (GraphPad Software, Inc, CA, USA). A p value <0.05 was considered statistically significant.

## Results and Discussions

### Effect of EGCG in cisplatin induced kidney dysfunction and tubular damage

Cisplatin caused significant renal dysfunction at 72 hours as observed in kidney injury parameters creatinine and BUN (Fig 1). Cisplatin administration resulted 8.75 and 7.6-fold increase in Creatinine and BUN respectively. EGCG attenuated both kidney injury markers in a dose dependent manner. EGCG at 100mg/kg reduced significantly kidney injury as shown by decrease in serum level of BUN (156.5 to 68.2) and creatinine (1.7 to 0.8). EGCG at 100mg/kg (also designated as EGCG 100) was most protective and used for all other experiments described in this study. Dose dependent studies of EGCG in mice demonstrated a linear dose relationship up to 500mg/kg and plateaued between 500 and 2000 mg/kg [26]. Relative bioavailability was between 1.6% at a low dose (75 mg/kg body weight) and 13.9% at higher doses (250 mg/kg and

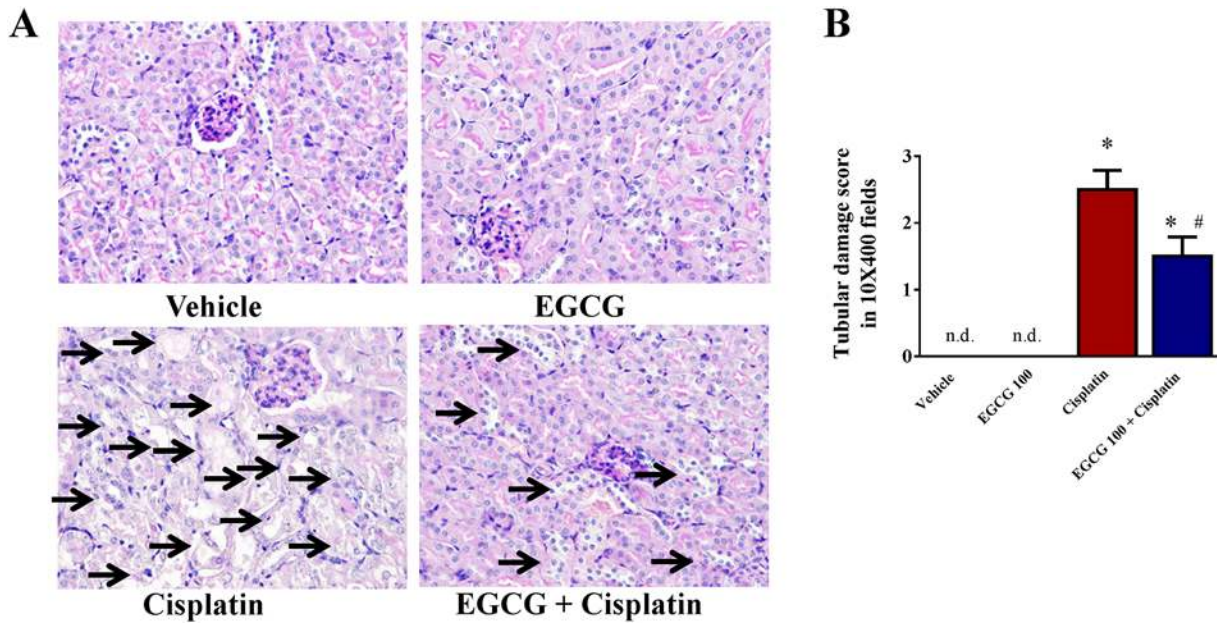


**Fig 1. Effect of EGCG on cisplatin-induced renal dysfunction in mice.** Cisplatin caused significant renal dysfunction as measured by the levels of creatinine (Panel A) and BUN at 72 hours (Panel B). Cisplatin administration resulted in severe kidney injury which was attenuated by EGCG treatment (at dose 100mg/kg). Results are mean  $\pm$  S.E.M. n = 4/group. \* p<0.05 versus vehicle; and # p<0.05 versus cisplatin.

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400 mg/kg) when provided orally to healthy volunteers [27]. EGCG does not have any effect on other organs in mice at dose provided but a single high oral dose of 1500mg/kg in mice resulted hepatotoxicity [28].

Cisplatin also induced significant tubular damages in kidney and the damage was examined by PAS staining. Histological examination and quantification revealed necrosis, vacuolation, and desquamation of epithelial cells in the renal tubules indicated by arrow (Fig 2). The



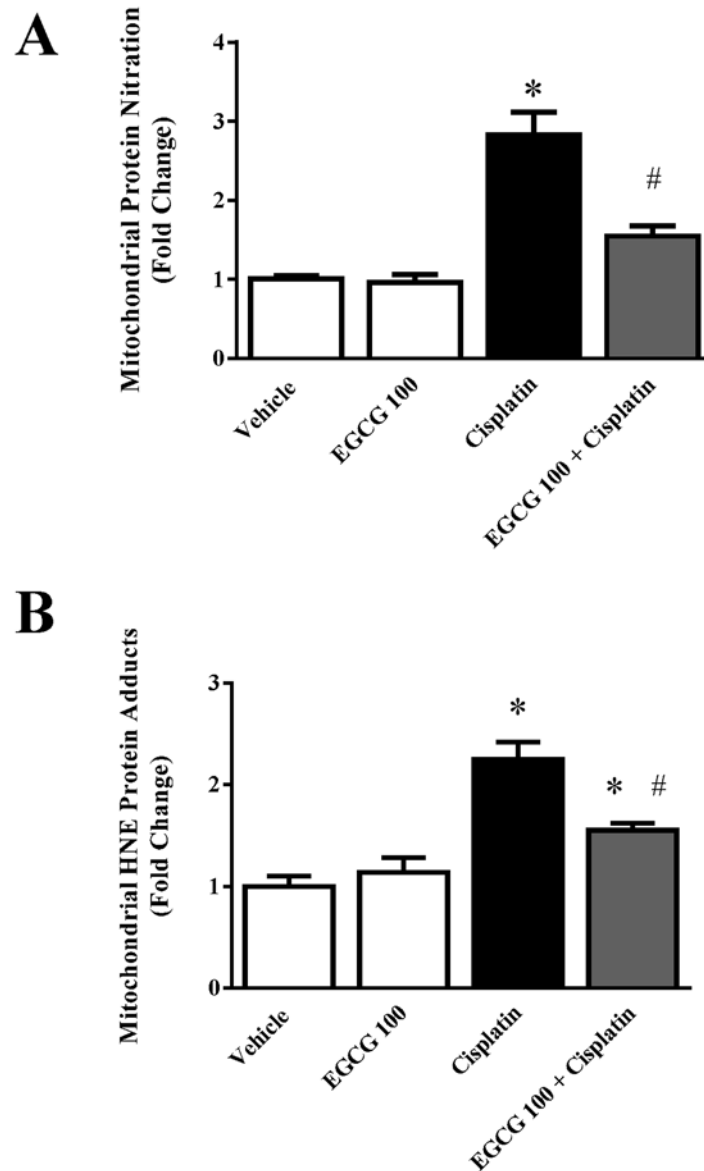
**Fig 2. Effect of EGCG on cisplatin-induced kidney tubular damage in mice.** Cisplatin administration resulted in severe tubular damage as shown by histological examination using PAS staining. The damage was attenuated by EGCG treatment at dose 100mg/kg (designated as EGCG 100). Results are mean  $\pm$  S.E.M., n = 4/group. \* p<0.05 versus vehicle; and # p<0.05 versus cisplatin.

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damage was significantly attenuated by EGCG 100 treatment in mice. The primary target of cisplatin is tubular epithelial cells as reported earlier [29].

### Effect of EGCG on cisplatin induced mitochondrial oxidative/nitrative stress in kidney

It has been reported earlier that EGCG accumulates in mitochondria and modulate oxidative stress [30]. Based on those finding, we examined the effect of EGCG in cisplatin induced mitochondrial oxidative footprints such as HNE protein adducts and protein nitration from isolated mitochondria. Two oxidative/nitrative stress markers HNE and protein nitration were increased 2.2 and 2.8 fold in cisplatin treated mice (Fig 3). Treatment with EGCG reduced mitochondrial oxidative stress significantly (45% and 30% for HNE and nitration respectively). EGCG alone did not produce any significant changes in values. Both oxidative markers are widely used in the literature [31–33]. It is well known that mitochondria are the major source for reactive oxygen species (ROS) generation and the sources include electron transport chain and NOX4 [34,35]. EGCG is a very potent antioxidant and its accumulation in mitochondria might play significant role in reducing mitochondrial oxidative stress [30,36]. We used another marker of oxidative stress which is protein nitration, a product of peroxynitrite formation [33]. Peroxynitrite is formed by superoxide (from mitochondria) and nitric oxide (from cytosolic iNOS) in a diffusion controlled reaction. It might suggest that the ameliorative effect of EGCG on cisplatin induced protein nitration was less pronounced compared to HNE adduct formation due to cytoplasmic component of the reaction. EGCG demonstrated to reduce cisplatin induced mitochondrial oxidative and nitrative stress and thus modulated the trigger of apoptotic cell death. To address this issue further, we examined the role of EGCG on mitochondrial sources of ROS namely electron transport chain enzyme complexes.



**Fig 3. Effect of EGCG on cisplatin-induced oxidative/nitrative stress in mitochondria.** Panel A. Quantitative measurement of protein nitration from mitochondrial fraction by ELISA demonstrated cisplatin induced content of protein nitration. EGCG attenuated cisplatin induced mitochondrial protein nitration. Panel B. Quantitative measurement of HNE adducts from mitochondrial fraction by ELISA. A trend similar to protein nitration was also observed. Results are mean  $\pm$  S.E.M. n = 4/group. \* p<0.05 versus vehicle; and # p<0.05 versus cisplatin.

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### Effect of EGCG on cisplatin induced mitochondrial respiratory enzyme complex activities in kidney

Mitochondrial electron transport chain complexes have been shown to play crucial role in cisplatin induced nephrotoxicity [3,4]. We also examined the effect of EGCG on three active mitochondrial complexes activities namely NADH dehydrogenase Activity (Complex I), Succinate Dehydrogenase Activity (Complex II) and Cytochrome Oxidase Activity (Complex IV). Cisplatin significantly reduced all three enzyme activities namely complex I, complex II



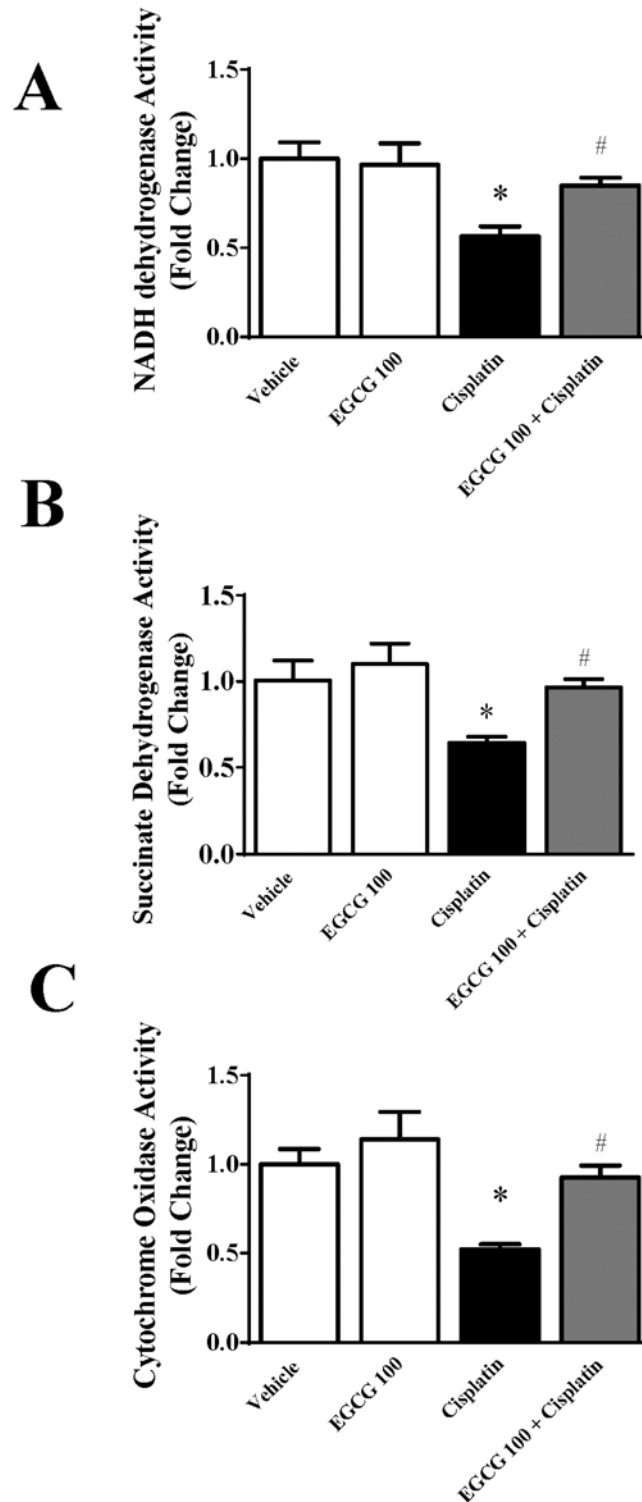
and Complex IV to 44%, 36% and 47.5% respectively (Fig 4). Treatment with EGCG at 100mg/kg improved those activities to normal level. EGCG alone did not produce any significant changes. The specific effect of EGCG on mitochondrial complex has two aspects. First, the antioxidant property of EGCG reduce the oxidative stress in mitochondrial from cytosolic ROS sources and thus preventing production of more ROS from mitochondrial enzyme complex sources [4]. The other aspect is the role of EGCG in mitochondrial antioxidant defense mechanisms such as MnSOD and Glutathione peroxidase activity. We also carried out those enzyme activities in mitochondrial fraction from kidney tissue suspension. Indeed, both antioxidant defense enzymes reduced in the presence of cisplatin as reported earlier [30,37]. EGCG restored both activities to normal level (Fig 5). Similar renal mitochondrial protection against oxidative stress was observed in epicatechin, a close family member of EGCG [38]. EGCG improved cisplatin induced damages to mitochondrial electron transport chain complexes in mouse kidney by increasing their activities to normal level.

### Effect of EGCG on cisplatin induced inflammation in kidney

Inflammation plays a critical role in cisplatin induced nephrotoxicity [9,39,40]. In order to evaluate the role of EGCG in cisplatin induced renal inflammation, we analyzed two pro-inflammatory cytokines (TNF $\alpha$  and IL1 $\beta$ ). As reported earlier, both pro-inflammatory cytokines TNF $\alpha$  and IL1 $\beta$  increased at mRNA level to 4.5 and 4.15 fold respectively in response to cisplatin toxicity. As shown in Fig 6, EGCG attenuates cisplatin induced TNF $\alpha$  and IL1 $\beta$  mRNA to 30% and 41% respectively. EGCG alone did not change those cytokine mRNA level significantly. The inflammatory response followed by infiltration of neutrophil supports its important role and has been reported earlier [4,9,19]. TNF $\alpha$  is important in cisplatin mediated toxicity and key player in kidney injury. Studies with TNF $\alpha$  antibody treatment or TNF $\alpha$  knock out mice are resistance to cisplatin-induced nephrotoxicity [40,41]. Although anti-inflammatory role of EGCG has been reported earlier in various immunology models [42–44], this study is first report of its anti-inflammatory role in a drug induced tissue injury model. Anti-inflammatory role of EGCG was reported in a surgical obstructive nephropathy model earlier [45,46]. Here, EGCG demonstrated its anti-inflammatory role in response to cisplatin induced inflammatory process.

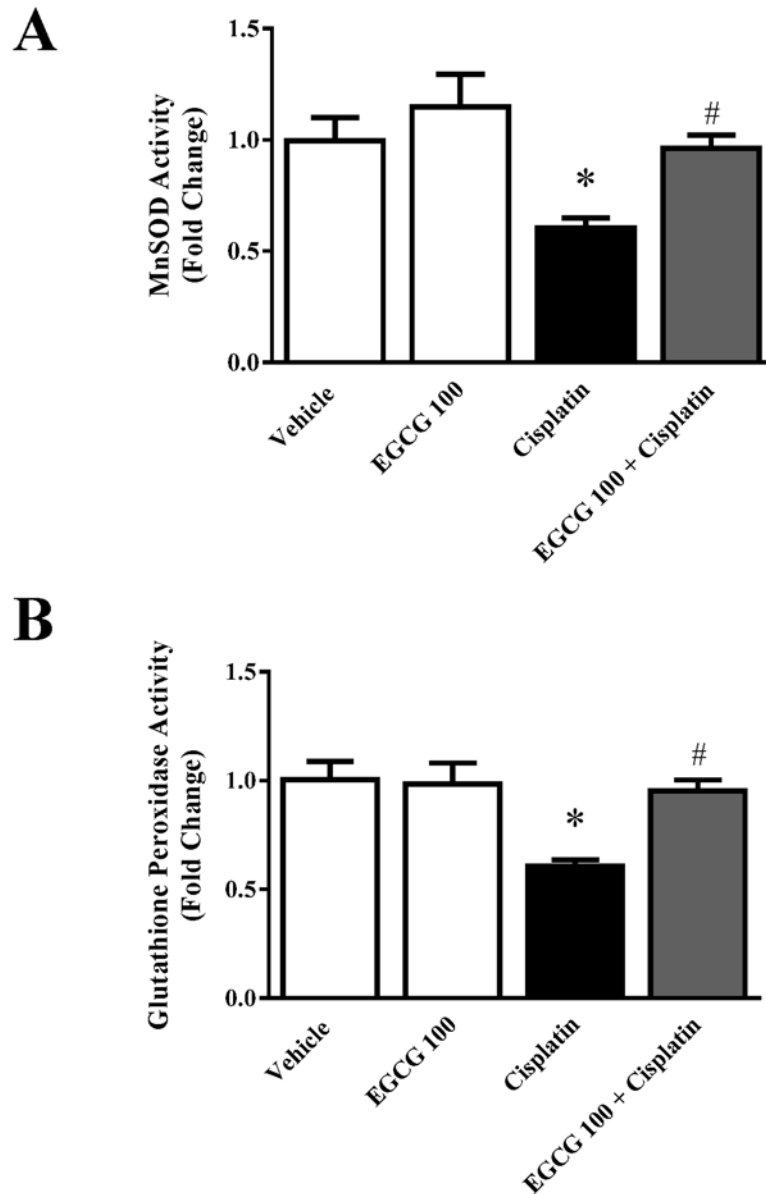
### Effect of EGCG on cisplatin induced modulation of transcription factor NF $\kappa$ B and p53 in kidney

NF $\kappa$ B contributes to oxidative stress, inflammation and mitochondrial gene regulation. We examined the effect of EGCG on NF- $\kappa$ B (p65) protein expression in nuclear fraction. Western blot analyses demonstrated significant increase in NF- $\kappa$ B (p65) in nuclear fraction from cisplatin administered mice when compared to vehicle or EGCG treated groups (Fig 7A). EGCG administration significantly decreased the amount of p65 in nuclear fraction compared to cisplatin treated group. In recent study the important role of NF- $\kappa$ B (p65) has been demonstrated in cisplatin induced kidney injury [4]. Another important transcription regulator in cisplatin nephrotoxicity is p53 and is also modulated by EGCG. Cisplatin significantly increase p53 protein level and the induction is attenuated by EGCG treatment (Fig 7B). There is no significant change with EGCG treatment alone. It has been demonstrated earlier that cisplatin induced tubular cell apoptosis and renal failure were ameliorated by p53 inhibitor pifithrin or p53knock out mice [47]. The mechanism of p53 is not clear. However, it is believed that cisplatin upon entering tubular cell, crosslink with DNA in its active form and that cause DNA damage [48]. EGCG either acted directly as antioxidant or indirectly via other mechanisms which reduced p53 induced DNA damage. We demonstrated here that EGCG modulated cisplatin induced



**Fig 4. Effect of EGCG on cisplatin-induced changes in mitochondrial enzyme complex activities in mice.** Cisplatin reduced the enzyme activities of mitochondrial electron transport chain namely (Panel A) NADH dehydrogenase Activity (Complex I), (Panel B) Succinate Dehydrogenase Activity (Complex II) and (Panel C) Cytochrome Oxidase Activity (Complex IV). EGCG administration protected mitochondrial enzyme against cisplatin induced damages. Results are mean  $\pm$  S.E.M.  $n = 4/\text{group}$ . \*  $p < 0.05$  versus vehicle; and #  $p < 0.05$  versus cisplatin.

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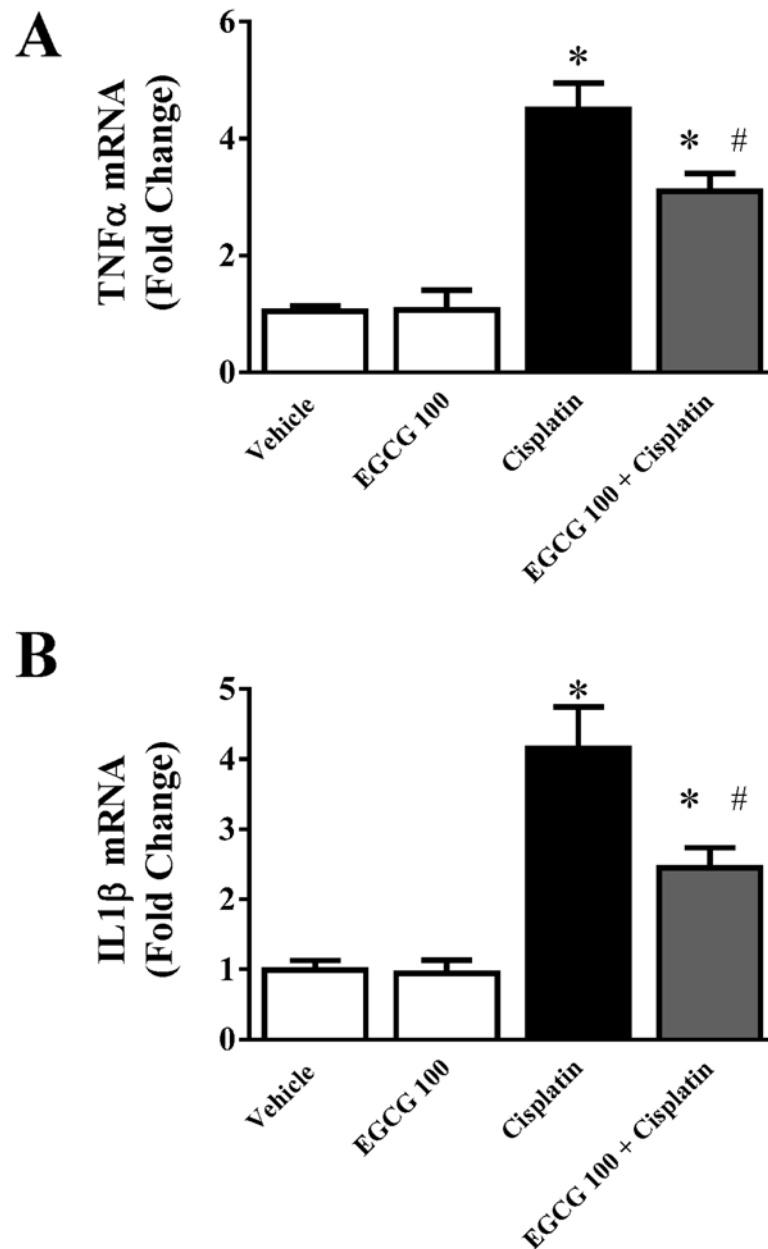


**Fig 5. Effect of EGCG on cisplatin-induced nitrate stress in mice.** Panel A. Histological examination revealed significant protein nitration in the renal tubules of the cisplatin-treated group. EGCG treatment reduced the level of protein nitration similar to vehicle level. Panel B. Quantitative measurement of 3-nitrotyrosine adducts in protein also demonstrated similar trend. Results are mean  $\pm$  S.E.M. n = 4/group. \* p<0.05 versus vehicle; and # p<0.05 versus cisplatin.

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NF- $\kappa$ B nuclear accumulation and p53 induction. EGCG might influence both inflammatory response and apoptotic cell death through these transcriptional regulators.

It has been proposed in obstructive nephropathy study that both NF- $\kappa$ B and NRF-2 are induced by EGCG and translocate to nucleus. They promote heme oxygenase 1(HO-1) production and modulate inflammation [46]. We also observed the importance of EGCG induced NF- $\kappa$ B in cisplatin induced nephropathy. Interestingly, Nrf-2/HO-1 pathway is a key antioxidant defense system inside cells which modulates both antioxidant and anti-inflammatory response and they are also linked to NF- $\kappa$ B. Thus, EGCG modulation of NF- $\kappa$ B and Nrf-2 is a



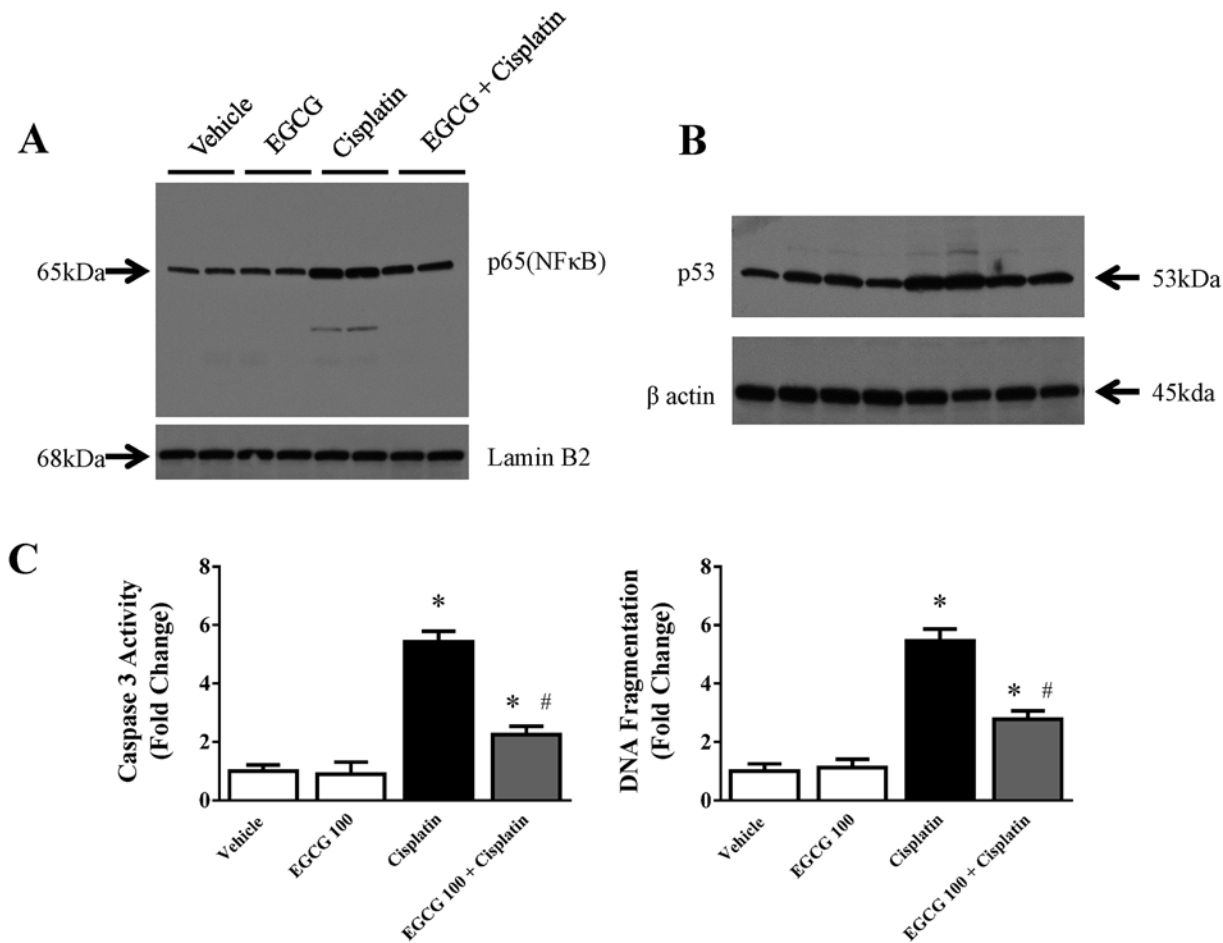
**Fig 6. Effect of EGCG on cisplatin-induced pro-inflammatory cytokines in mice.** Real-time PCR based analyses of pro-inflammatory cytokines TNF $\alpha$  and IL1 $\beta$  indicated profound increase in cisplatin treated mice. EGCG treatment attenuated cisplatin induced TNF $\alpha$  and IL1 $\beta$  production. Results are mean  $\pm$  S.E.M. n = 4/group. \* p<0.05 versus vehicle; and # p<0.05 versus cisplatin.

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critical element for oxidative stress induced cell death and inflammation or both in acute kidney injury. Green tea extract also reduce contrast media induced acute kidney injury in rats [49]. Thus, EGCG is beneficial in a variety of acute kidney injury models.

### Effect of EGCG on cisplatin induced apoptotic cell death in kidney

We also measure DNA fragmentation in our study and EGCG indeed reduced cisplatin induced DNA fragmentation (Fig 7C) to from 5.5 fold increase to 2.8 fold (compared to vehicle



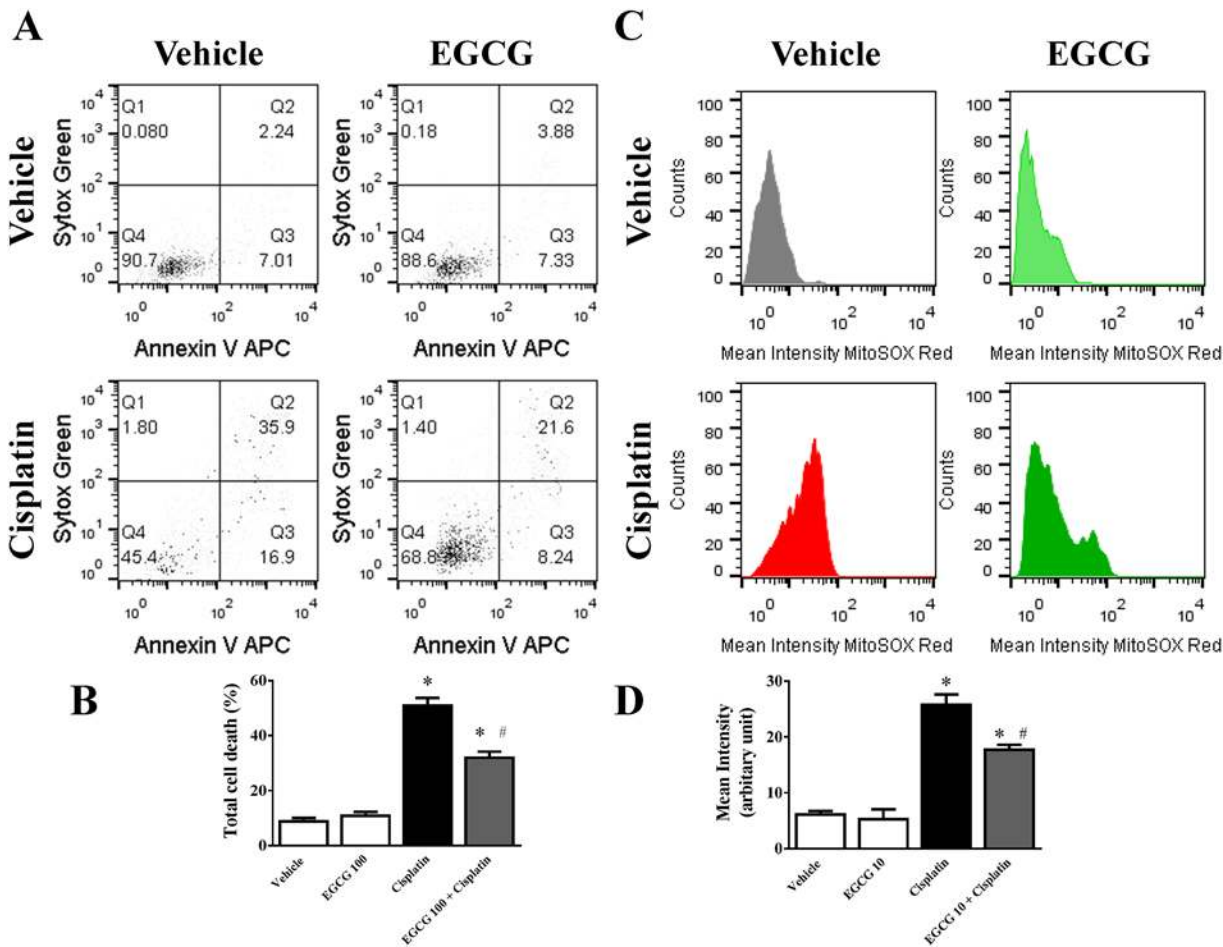
**Fig 7. Effect of EGCG on cisplatin-induced nuclear translocation of NFκB, induction of p53 and apoptosis.** Panel A. Immuno-blot analyses of nuclear fraction from kidney demonstrated increase in p65 in cisplatin treated mice. The level is reduced in EGCG and cisplatin treated group. Panel B. Immuno-blot analyses of total lysate from kidney demonstrated increase in p53 in cisplatin treated mice. Panel C. Quantitative measurement of Caspase 3 activity and DNA fragmentation. Results are mean ± S.E.M. n = 4/group.\* p<0.05 versus vehicle; and # p<0.05 versus cisplatin

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control). An early marker of apoptosis, caspase 3/7 activity, also demonstrated EGCG attenuates cisplatin induced apoptosis to 58% (5.4 fold to 2.25 fold, Fig 7D). It is well known that cisplatin leads to apoptosis in mouse kidney tubular cells followed by renal failure and thus increase in serum BUN and creatinine [21,39]. Both DNA fragmentation and caspase 3/7 activity presented here are markers for apoptotic cell death and has been used earlier in cisplatin induced nephrotoxicity [50,51]. Several mechanisms have been proposed for the trigger for apoptotic cell death [52–54]. However, recent studies demonstrated that mitochondria plays a key role in cisplatin induced renal cell death [3,55–57]. In addition to that, mitochondria are functional regulator of apoptosis [58]. In this study, we demonstrated that EGCG targets mitochondria and thus modulate apoptotic cell death.

### Effect of EGCG on cisplatin induced apoptotic cell death and mitochondrial ROS generation in human tubular epithelial cells HK-2

Simultaneous detection of apoptotic cell death and mitochondrial ROS production in cisplatin treated (60μM) HK-2 cells for 24 hours results significant cell death (5.8 fold) and increase in mitochondrial ROS production (4.2 fold) as measured by mean intensity of MitoSOX RED



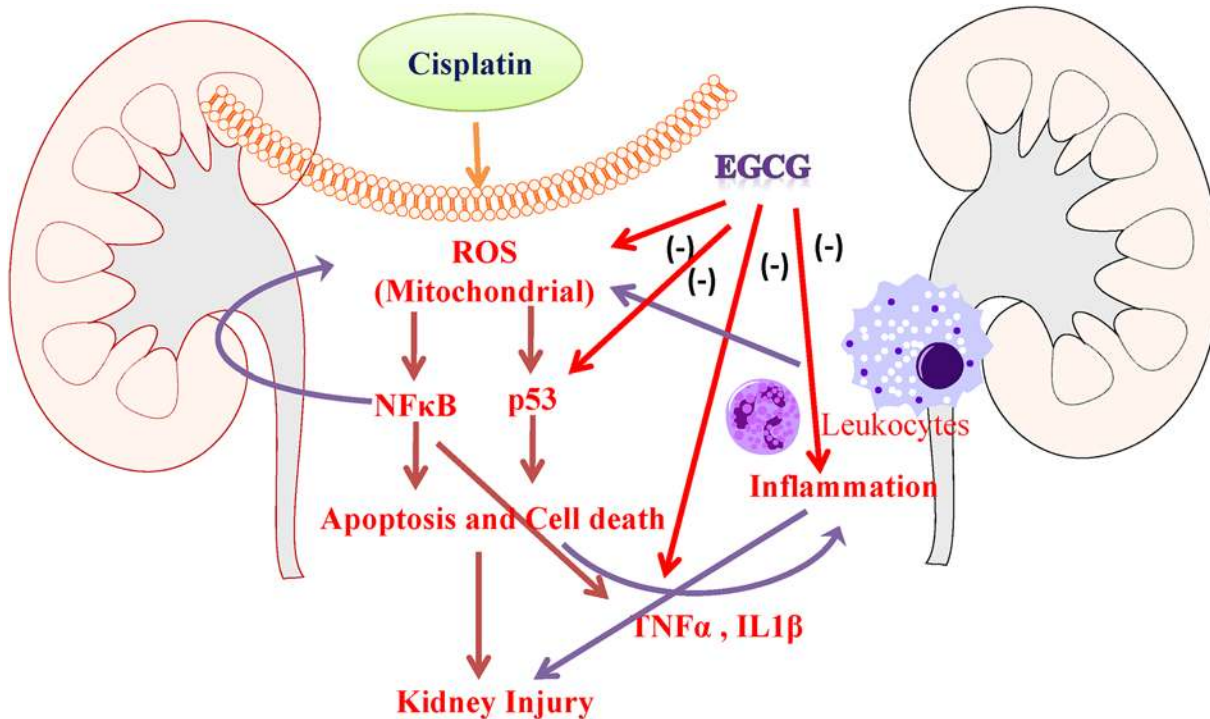
**Fig 8. Effect of EGCG on cisplatin-induced apoptosis and mitochondrial ROS generation in HK-2 cells.** Panel A: Representative dot plot data showing early apoptosis (AnnexinV-APC staining) and late apoptosis (with Sytox Green) as measured by flow cytometry. Cisplatin was used at 60 $\mu$ M and EGCG was added at 10 $\mu$ M 2h prior to cisplatin addition. Panel B: Quantitative determinations of total cell death including early and late apoptosis were presented. Results are mean  $\pm$  S.E.M. n = 4/group. \* p<0.05 versus vehicle; and # p<0.05 versus cisplatin. Panel C: Representative histogram analyses of MitoSOX Red intensities from the same samples were presented. Panel D: Quantitative determinations of MitoSOX Red mean intensities. Results are mean  $\pm$  S.E.M. n = 4/group. \* p<0.05 versus vehicle; and # p<0.05 versus cisplatin.

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using flow cytometry (Fig 8). Treatment with EGCG at 10 $\mu$ M reduced both apoptosis (37%) and mitochondrial ROS production (31.3%). These data demonstrated that EGCG contributed mitochondrial ROS mediated protection in cisplatin induced cell death for human kidney tubular cell line. EGCG is implicated in reducing ROS production and mitochondrial integrity earlier in fibroblast [59]. We would like to caution that cell line data might be different compared to cisplatin induced tissue injury situation where the process is more complex. However, EGCG demonstrated direct role by modulating cisplatin induced mitochondrial ROS in live kidney tubular cells.

## Conclusion

Briefly, in this study we demonstrated EGCG protects against cisplatin induced renal injury through mitochondrial protection in three ways (1) by improving cisplatin induced mitochondrial electron chain complexes, (2) by improving mitochondrial antioxidant function in enzymes MnSOD and GPX, (3) improving cisplatin induced mitochondrial oxidative/nitrate



**Fig 9. Schematic diagram of protection mechanism of EGCG in cisplatin induced kidney injury.** EGCG inhibit cisplatin induced mitochondrial ROS (Reactive Oxygen Species) in the renal tubular cells which caused cell death. Cell death in the tubular cells leads to pro-inflammatory response with cytokines (TNF $\alpha$  and IL1 $\beta$ ). These process leads to leukocytes infiltration with additional burst of oxidative stress. EGCG also neutralize these pro-inflammatory cytokines. All combinatorial effects leads to reduced inflammation and cell death, thus protecting against cisplatin induced kidney injury.

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damage and (4) anti-inflammatory effect (Fig 9). Here, we also demonstrated that EGCG reduced cisplatin induced inflammatory response by lowering pro-inflammatory cytokines and neutrophil infiltration. EGCG also modulated cisplatin induced nuclear translocation of NF $\kappa$ B (p65), which has role in inflammation and oxidative stress. Another transcription factor p53, DNA damage response factor, also modulated by EGCG in cisplatin affected tubular cells. This modulation lead to improvement of cisplatin induced DNA fragmentation and Caspase activity or apoptosis by EGCG in renal tissue. These protective effects of EGCG, also a natural ingredient of Green Tea, will be very useful for therapeutic purpose in cisplatin induced kidney injury or other kidney injury models where mitochondria/oxidative stress/inflammation is involved. It is important to note that EGCG is reported to be effective against various cancer [60–64] and this will make is ideal adjunct candidate for cisplatin chemotherapy.

### Author Contributions

Conceived and designed the experiments: HP JC KS XW PW GF HM YW BJ. Performed the experiments: HP KS XW PW GF. Analyzed the data: HP JC HM YW BJ. Contributed reagents/materials/analysis tools: HP KS. Wrote the paper: HP BJ.

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