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Ellagic acid protects against cisplatin-induced nephrotoxicity in rats: a dose-dependent study

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Abstract. – BACKGROUND: The anticancer-drug cisplatin (CP) causes nephrotoxicity through different mechanisms, including generation of free radicals. Ellagic acid (EA) is a polyphenolic antioxidant found in fruits and nuts.

AIM: This study aimed to investigate the ability of different doses of EA to ameliorate CP nephrotoxicity in rats.

MATERIALS AND METHODS: Animals were randomly divided into six groups and treated with saline; CP alone (6 mg/kg); two doses of EA, both alone (10 and 30 mg/kg) or with CP.

RESULTS: Treatment with CP alone reduced body weight, water intake, urine output, and renal total antioxidant and reduced glutathione (GSH) concentrations ($p < 0.01$). In addition, it increased relative kidney weight, plasma creatinine, and blood urea nitrogen (BUN) concentrations ($p < 0.01$). However, a dose of 30 mg/kg EA mitigated most of the CP-induced actions, but no effect was seen for the 10 mg/kg dose. Histopathologically, rats given CP+EA30 showed < 25% necrotic lesions in the renal cortical area compared with > 60% in rats treated with CP alone. Molecular analysis showed that clusterin (Clu) mRNA and protein were expressed in all treated groups, meanwhile kidney injury molecule-1 (Kim-1) mRNA and protein were only expressed in the CP and CP+EA treated rats.

CONCLUSIONS: EA (30 mg/kg) ameliorated most of the physiological, histological, and biochemical markers of CP nephrotoxicity. The molecular findings in this work did not completely tally with the conventional method used. The overexpression of the molecular markers may be related to the EA induced repair mechanism.

Key Words:
Ellagic acid, Cisplatin, Nephrotoxicity, Rats.

Introduction

Cisplatin (CP) or cis-diamminedichloroplatinum II is an inorganic platinum-based antineoplastic drug used to treat solid tumors such as head and neck, testicular, ovarian, small cell and non-small cell cancers[1]. However, its effectiveness against tumors is limited by its neurotoxicity, ototoxicity, nephrotoxicity and bone marrow suppression. The nephrotoxic effect of CP is considered to be one of its major limitations, where it arises in 20-30% of the treated patients, one third of which end up with irreversible kidney damage[2].

Different mechanisms have been reported describing the pathogenesis of CP nephrotoxicity, including the production of nephrotoxic metabolites, vascular injury, inflammation, generation of free radicals and apoptotic pathways[1-4]. In general, CP damages the S3 segment of the proximal tubules, where the expression of copper transporter receptor1 (Ctr1) and organic cation receptor2 (Oct2) is high. These two receptors actively transport CP into the kidney tubules contributing to the fact that the kidney has the highest concentration of CP compared to any other organ in the body[4,5]. The accumulated CP enters the cell and causes disturbance to the redox status either by reducing the endogenous antioxidant, such as GSH and NADPH, or by damaging the mitochondrial inner membrane which releases free radicals such as reactive oxygen species (ROS) and reactive nitrogen species (RNS). The released ROS and RNS induce intrinsic cell death leading to apoptosis[6,7].

Ellagic acid (EA) is a natural polyphenolic compound found in nuts and a wide range of vegetables and fruits[8]. EA has an antioxidant[9], anticancer[10], antimutagenic[11], and anti-inflammatory activity[12,13]. The antioxidant effect of EA on CP-induced cytotoxicity was evaluated by different studies. For example, it was reported that EA (2 mg/kg) can ameliorate CP-induced testicular dam-

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age by preventing lipid peroxidation and reducing apoptosis through scavenging ROS[14]. In a different study, the effect of EA at a single dose (10 mg/kg) on CP-induced nephrotoxicity in rats was investigated and it was found that EA can correct creatinine and blood urea nitrogen (BUN) concentrations, elevate the non-enzymatic antioxidant reduced glutathione (GSH), and reduce the necrotic lesions in the renal tubular cells[15].

The aim of this study was to verify and extend the previous work of Atessahin et al, in which a single dose of EA was used[15], by using two doses of EA (10 and 30 mg/kg) in CP-induced nephrotoxicity. We assessed the impact of EA by examining an array of biochemical, histopathological and molecular markers.

Materials and Methods

Animals

Female Sprague-Dawley (SD) rats, weighing 250 g ± 20 g, were used in this investigation. The rats were housed in individual polypropylene cages and were provided with standard laboratory chow diet containing normal sodium (Oman Flour Mills, Muscat, Oman) and normal tap water *ad libitum*. Rats were housed under standard conditions of temperature (22±2°C), humidity (60%) and a 12hr light: dark cycle. The study was approved by Sultan Qaboos University Animal Ethical Committee, and was conducted in accordance to international laws and policies (EEC Council directives 86/609, OJL 358, 1 December, 12, 1987; NIH Guide for the Care and Use of Laboratory Animals, NIH Publication No. 85-23, 1985).

Experimental Design

The CP dose used in this experiment (6 mg/kg) was similar to that previously described[16]. The rats were randomly divided into six groups with a minimum of six rats in each group. The first group (control) received corn oil orally by gavage for 9 consecutive days, and a single injection of normal saline (0.9% NaCl) on the fifth day. The second group received corn oil orally for 9 consecutive days, and a single CP injection (6 mg/kg) on the fifth day. Groups 3 and 4 received different doses of EA alone (10 mg/kg and 30 mg/kg) dissolved in corn oil orally for 9 consecutive days, and a single injection of normal saline (0.9% NaCl) on the fifth day. Groups 5 and 6 received the same doses of EA at (10 mg/kg and 30 mg/kg) dissolved in corn oil orally for 9 consecutive days, and a single CP injection on the fifth day. All injections were given intraperitoneally (i.p.).

The experiment lasted for 10 days. On day nine, the rats were kept in metabolic cages for 24hr urine collection and volume measurement. The amount of water intake was also recorded. On day 10, the rats were dosed with 0.2 ml of (100 mg/ml) ketamine and 0.1ml of (20 mg/ml) xylazine, to facilitate blood collection in heparinized tubes from the inferior vena cava (5 ml). The blood samples were centrifuged at 900 x*g* for 10min at 4°C and plasma was then separated and stored at –80°C pending analysis. The kidneys were also collected, cleaned of fats, and weighed immediately; two small portions there from were taken for histology (stored in 10% neutral buffered formalin) and for molecular analysis (stored in RNA later solution (Ambion) at 4°C for 24hr, and then transferred to –80°C). The rest of the kidney was then wrapped in foil, frozen immediately in liquid nitrogen and transferred to a –80°C freezer.

Cisplatin Concentration Measurement

The concentration of CP in the kidney was measured as platinum by dissolving a piece of renal tissue (0.5 g) of CP treated rats into 15.7 M nitric acid (3 ml) and 11.5 M hydrochloric acid (2 ml). The samples were then kept in a water bath at 100°C for 2 hr. Finally, platinum concentration was estimated using an inductively coupled plasma (ICP) machine, as described previously[17].

Histopathology

The kidney samples stored in 10% formalin were processed through increasing concentration of ethanol for dehydration, cleared in xylene and blocked in paraffin wax. Haematoxylin and eosin staining was performed on 5 µm thick sections by a histopathologist who was unaware of either the treatments or the arrangement of the groups. The necrotic lesion of the proximal tubules was scored as described previously[18]. The scoring was on a scale 0-4 where 0 indicates no necrosis; 1 indicates a few focal necrotic spots; 2 indicates 50 % necrosis; 3 indicates 60% necrosis; and 4 indicates necrotic lesions in almost all the area. The size of necrosis was then estimated and presented as mean ± SD.

Staining for apoptosis was performed using a signal stain cleaved caspase-3 immunohistochemical detection Kit (Cell Signaling Technology, Boston, MA, USA). This kit was used to detect the activation of caspase-3 using an avidin-

biotin immunoperoxidase method to detect intracellular cleaved caspase-3 protein. Staining was performed on 5 μm paraffin sections from the right kidney by a standard technique using rabbit anti-cleaved caspase3 (clone Asp175, 1:50)[19]. Known positive control sections for apoptosis were used. For a negative control, primary antibody was replaced with normal rabbit serum.

Biochemical Markers

Osmolality

Urine osmolality was determined by using freezing point depression (–70°C) on a Freezing Point Osmometer machine (Ultrasound Technology, Teltow, Germany). The machine determines the osmotic concentration of the biological fluid by super cooling the sample to a crystallized state, which causes the release of heat of fusion of the sample's water. The released heat of fusion warms up the sample to an equilibrium state, between its solid and ice status. The equilibrium state differs according to solute concentration in the sample where samples with high solute concentration require more energy to reach the equilibrium state[20].

Plasma Urea and Creatinine

Human GmbH kit (Wiesbaden, Germany) was used to measure plasma creatinine based on the Jaffé reaction, and urea based ona modified Berthelot reaction.

Antioxidant Analysis (TAS, GSH)

The medullary region was removed from the kidney and a part of the cortical area (0.5 g) was weighed and homogenized in phosphate buffered saline (PBS) buffer (5 ml). The homogenate was then centrifuged at 6000 x *g* for 1hr at 4°C. The supernatant was then collected and stored at –80°C.

Total antioxidant concentration was measured spectrophotometrically in the kidney homogenate, as described previously using Total Antioxidant Status Assay Kit (Calbiochem, Darmstadt, Germany)[21]. Reduced glutathione (GSH) was also measured spectrophotometrically in the kidney homogenate using a Glutathione Assay Kit (BioVision, Milpitas, CA, USA)[22,23]. Total protein concentration was estimated in the homogenate using the Lowry et al method.

Molecular Markers

Western Blot For Detection of Kim-1 and Clu Proteins

The expression of clusterin (*Clu*) and kidney injury molecule-1 (*Kim-1*) proteins in the proximal tubules of the kidney were detected through Western blot. *β-Actin* was used to normalize the obtained data. The cortical area of the kidney was homogenized in 1 ml (T-PER) Tissue Protein Extraction Reagent and Halt Protease Cocktail Inhibitor (1X) (ThermoScientific, Rockford, IL, USA), and centrifuged at 10,000 × *g* for 5 min at 4°C. Total protein concentration was estimated using Bradford's method[24]. About 10 μg of protein was loaded onto a 10% acrylamide gel for electrophoresis. The protein was then blotted and transferred to a nitrocellulose membrane for 1 hr; the membrane was then blocked with 5% low fat milk for 2hr at room temperature, and probed with the appropriate primary antibody overnight at 4°C (Table I). Membranes used for *Kim-1* and *β-Actin* detection were then washed four times,

Table I. Primary and secondary antibodies used for detection of kidney injury molecule-1 (*Kim-1*), Clusterin (*Clu*) and β-actin proteins using Western Blot

Protein	Antibody	Type	Concentration	Source
Kim-1	Rat TIM-1/KIM-1/HAVCR Affinity Purified Polyclonal Antibody, Goat IgG	Primary	1:1000	R & D System
	Rabbit Anti-Goat IgG HRP Affinity Purified PAb, Rabbit IgG	Secondary	1:5000	R & D System
Clusterin	Clusterin (CLU) Mouse anti-Rat Monoclonal Antibody	Primary	1:500	LifeSpanBioScience
	Goat Anti-Mouse IgG HRP Affinity Purified PAb, Goat IgG	Secondary	1:2000	R & D System
β-Actin	Rabit Anti-Human Monoclonal IgG	Primary	1:500	Cell Signaling
	Goat Anti-RabitIgG HRP G Affinity Purified PAb, Goat Ig	Secondary	1:2000	Cell Signaling

Table II. Sequence of primers and probes used in real-time PCR for quantification of kidney injury molecule-1 (Kim-1) and clusterin mRNA expression.

Gene	Type	Sequence	Product Size
Kim-1	Primer-F	5'-GAGTTCATTAGAGCCATTTCCACTCC-3' (26)	146bp
	Primer-R	5'-GAAAGCCTGTGTCCTGCTCTCTCT-3' (24)	
	Probe	5'-AACTCACCCACTGAGCTCTGAATTAGGTGCAG-3' (32)	
Clusterin	Primer-F	5'-GAGAGGCTGACCCAGCAGTACAA-3' (23)	124bp
	Primer-R	5'-TGAGGTTAGCCAGCTGGGACA-3' (21)	
	Probe	5'-CTCCAGTCCAAGATGCTCAACACCTCATCC-3' (30)	
GAPDH	Primer-F	5'-TCCTGCACCACCAACTGCTT-3' (20)	125bp
	Primer-R	5'-GAGGGGCCATCCACAGTCTT-3' (20)	
	Probe	5'-CACTCATGACCACAGTCCATGCCATCAC-3' (28)	

for 10 min each. However, the membrane used for *Clu* detection was washed two times, for 5 min each. The membranes were then probed with secondary antibody (Table I) for 2hr and washed, as described above. Finally, the protein bands were detected by ECL Advance Western Blotting Detection reagent (Amersham, Buckinghamshire, UK) and visualized using CHEMI GENIUS Bio Imaging System (Syngene, Cambridge, UK).

Expression Levels of Kim-1 and Clu genes

Reverse transcriptase (RT) followed by real-time PCR was used for the quantification assay of *Kim-1* and clusterin mRNA expression. The primers and probes used in qPCR are listed in Table II. RNA was extracted from 30 mg renal tissue using RNeasy Mini kit (Qiagen, Milan, Italy). The tissue was homogenized using a needle and syringe, and RNA extraction was performed as described by the manufacturer (Qiagen, Milan, Italy). A NanoDrop1000 Spectrophotometer (Thermo Fisher Scientific, Wilmington, DE 19801, USA) was used to estimate the quantity of extracted RNA, and 0.25 µg of the RNA was converted to cDNA by using a High Capacity cDNA Reverse Transcription kit (Applied Biosystem, Monza, Italy). For real time PCR, 1 µl of the cDNA was used in a total reaction volume of 20 µl, which contained 0.35 µM forward primer, 0.35 µM reverse primer, 100 µM probe and 1x Taqman Universal PCR master mix (ABI, Monza, Italy). The reaction took place in an ABI 7500 fast real time machine on standard mode. The reaction conditions were as follows: 10min at 95°C to activate the polymerase enzyme; 40 cycles of denaturation at 95°C for 15s, then annealing and extension at 60°C for 1min. A comparative C_T method was employed, and *GAPDH* was used as the endogenous control to obtain the relative quantification (RQ).

Statistical Analysis

Statistical analysis was performed using Microsoft excel sheet and GraphPad Prism 4. Data are expressed as mean ± SEM. Paired *t*-test was used to compare water intake and urine output before and after treatment, and the ANOVA test was used to compare the six groups for the other biochemical and molecular parameters. Statistical significance was considered at $p < 0.05$. In real time RT-PCR, relative quantification (RQ) was done automatically by the ABI machine using the C_T value ($2^{-\Delta\Delta CT}$), as described by the manufacturer (Applied Biosystems, Warrington, UK).

Results

CP Distribution in the Rats Kidney

Platinum concentration was measured in rats treated with either CP alone or CP and EA at different doses. There was no significant difference in the platinum concentration between the groups (Table III).

Table III. Platinum (Pt) concentration in kidneys of rats treated with either cisplatin (CP) alone or CP combined with different doses of EA.

No.	Treatment	Pt (mg/l)*
1	CP	0.286 ± 0.01
2	CP + EA10	0.297 ± 0.01
3	CP + EA30	0.325 ± 0.01

*Values in the table are mean ± SEM from at least 6 rats. There were no significant differences between the groups.

Table IV. The effect of saline, cisplatin (CP) or CP plus ellagic acid (EA) given at doses of 10 mg/Kg and 30 mg/Kg on body weight and relative kidney weight of rats.

Group	N	Initial weight*	Final weight*	Change in body weight (%)	Relative kidney weight*
Saline (control)	6	247 ± 7	259 ± 8	4.8	0.71 ± 0.04
CP	6	242 ± 5	212 ± 5	-12.1**	1.12 ± 0.12**
EA10	7	244 ± 6	256 ± 6	5.2	0.68 ± 0.04
CP+EA10	7	250 ± 6	221 ± 7	-11.7**	0.94 ± 0.07**
EA30	6	241 ± 9	250 ± 7	3.8	0.72 ± 0.02
CP+EA30	9	244 ± 7	242 ± 6	-2.8◊	0.94 ± 0.05**

*Values in the table are mean ± SEM from at least 6 rats. **ANOVA significantly different from control rats, significant at $p < 0.05$. ◊ANOVA significantly different from CP rats, significant at $p < 0.05$.

Kidney Function and Biochemical Markers

CP-treated rats had significantly reduced body weight, while the kidney size of the rats increased significantly (Table IV). Treatment with EA alone at different doses did not affect either the body or kidney weight. However, all rats treated with CP+EA lost weight and the kidney weight increased, but the weight loss in CP+EA30 was not significant compared with the control rats.

Paired *t*-test was used to compare the amount of water intake before and after treatment. There was no significant difference in the amount of water consumed by the controls and rats treated with EA alone before and after treatment (Table V). However, water consumption decreased sharply following CP and CP+EA10 treatment ($p < 0.01$), while it increased dramatically following CP+EA30 treatment ($p < 0.05$). The osmolality of urine decreased significantly in all CP treated rats (Table V).

The creatinine (Cr) and BUN concentrations increased significantly in rats treated with CP and CP+EA10 ($p < 0.01$) (Figure 1 A and B). However, Cr and BUN concentrations in rats treated with CP+EA30 showed no significant difference when compared with the control rats, but there was a significant difference in Cr concentration ($p < 0.01$) and BUN ($p < 0.05$) between this group and the CP alone treated rats. The BUN concentration of EA30 treated rats was higher than that of the control rats, but the increase was not significant.

Histopathology

Rats treated with saline (control) and with EA alone at different doses showed normal kidney architecture and histology, and a score of 0 (Figure 2 A, C and E). There were no apoptotic bodies found in these groups (Figure 3 A, C and E). However, CP and CP+EA10 treated rats showed diffuse acute tubular necrosis in > 60% of the examined tissue areas, with a score of 3 (Table VI), and showed tubular distention with necrotic material involving two thirds of the ex-

Table V. The effect of saline, cisplatin (CP) or CP plus ellagic acid (EA) given at doses of 10 mg/Kg and 30 mg/Kg on water intake, urine output and urine osmolality in control rats, and rats treated with either cisplatin (CP) or ellagic acid (EA) alone or CP combined with different doses of EA.

Treatment	Water intake*		Urine Output*		Urine osmolality*
	Before treatment	After treatment	Before treatment	After treatment	
Saline (control)	17 ± 1	15 ± 1	6 ± 0.6	6 ± 1.0	2119 ± 205
CP	17 ± 2	2 ± 0.4◊	6 ± 0.5	3 ± 0.8◊	1098 ± 143**
EA10	19 ± 2	17 ± 2	9 ± 0.7	9 ±1.4	2431 ± 330
CP+EA10	20 ± 3	3 ± 2◊	7 ± 1.0	2 ± 0.5◊	1038 ± 199**
EA30	15 ± 2	18 ± 3	7 ± 0.5	9 ± 2.2	1959 ± 233
CP+EA30	19 ± 1	31 ± 4◊	10 ± 1.1	23 ± 3.2◊	793 ± 123**

*Values in the table are mean ± SEM from at least 6 rats. **ANOVA significantly different from control rats, significant at $p < 0.05$. ◊Paired *t*-test, significant at ($p < 0.05$).

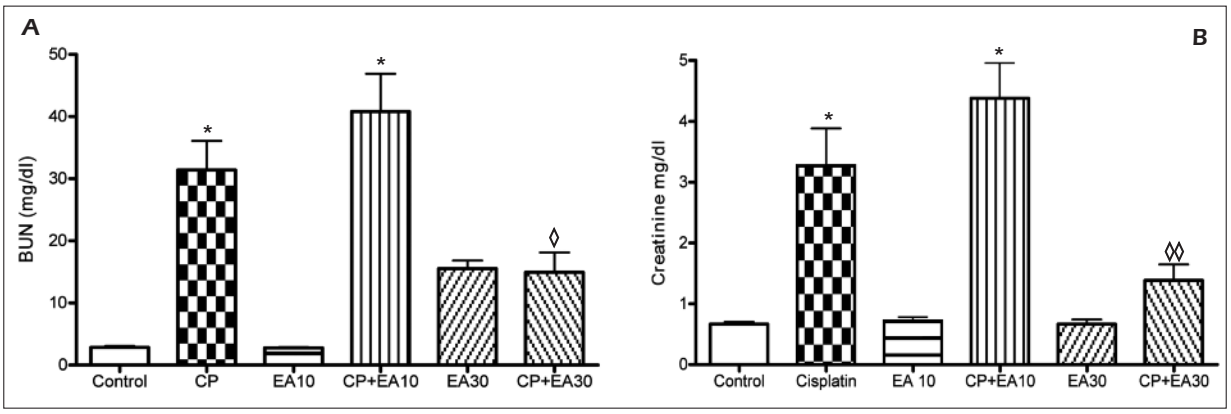

Figure 1. Blood urea nitrogen (BUN) ***(A)*** and creatinine ***(B)*** in control rats, and rats treated with either cisplatin (CP) or ellagic acid (EA) alone or CP combined with different doses of EA. *ANOVA, target compared to control: *($p < 0.01$), **($p < 0.05$); ◊ANOVA, target compared to CP: ◊($p < 0.05$), ◊◊($p < 0.01$).

amined tissue (Figure 2 B and D), and many apoptotic cells (Figure 3 B and D). The CP+EA30 rats showed dramatic improvement in the histological appearance when compared with the CP treated group (Table VI). There were a few focal areas of acute tubular necrosis involving < 25% of the examined areas (score 1) (Figure 2F). Apoptosis staining showed a marked reduction in the apoptotic cells in the examined sections (Figure 3F).

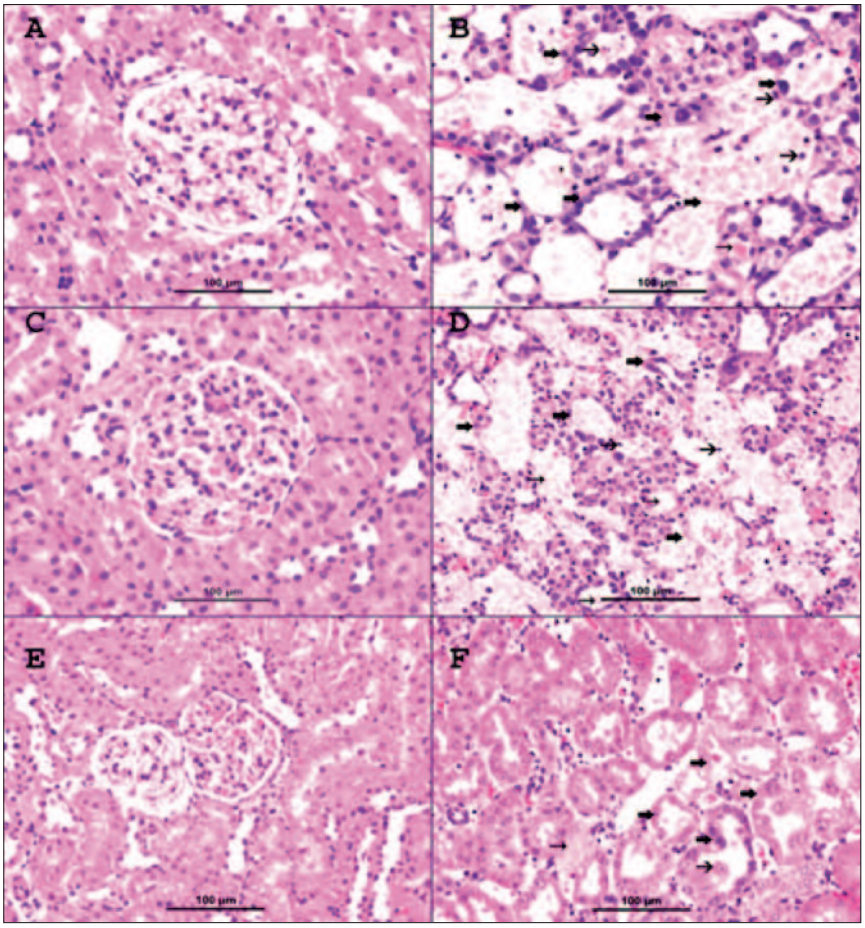

Figure 2. Representative section of kidney architecture histology, H&E, in control and treated rats. ***A,*** Control rats with normal kidney architecture. ***B,*** CP treated rats with acute tubular necrosis in nearly two thirds of the examined area (*thick arrow*) and many apoptotic cells involving two thirds of the examined tissue (*thin arrows*). ***C,*** EA10 rats with normal kidney architecture. ***D,*** CP+EA10 treated rats with histological appearance similar to the CP alone treated rats. ***E,*** EA30 rats with normal kidney architecture. ***F,*** CP+EA30 treated rats showed dramatic improvement in the histologic appearance with few focal areas of acute tubular necrosis (*thick arrows*) involving less than 25% of the examined areas, few apoptotic bodies (*thin arrow*) and intratubular eosinophilic material (*thin arrow*).

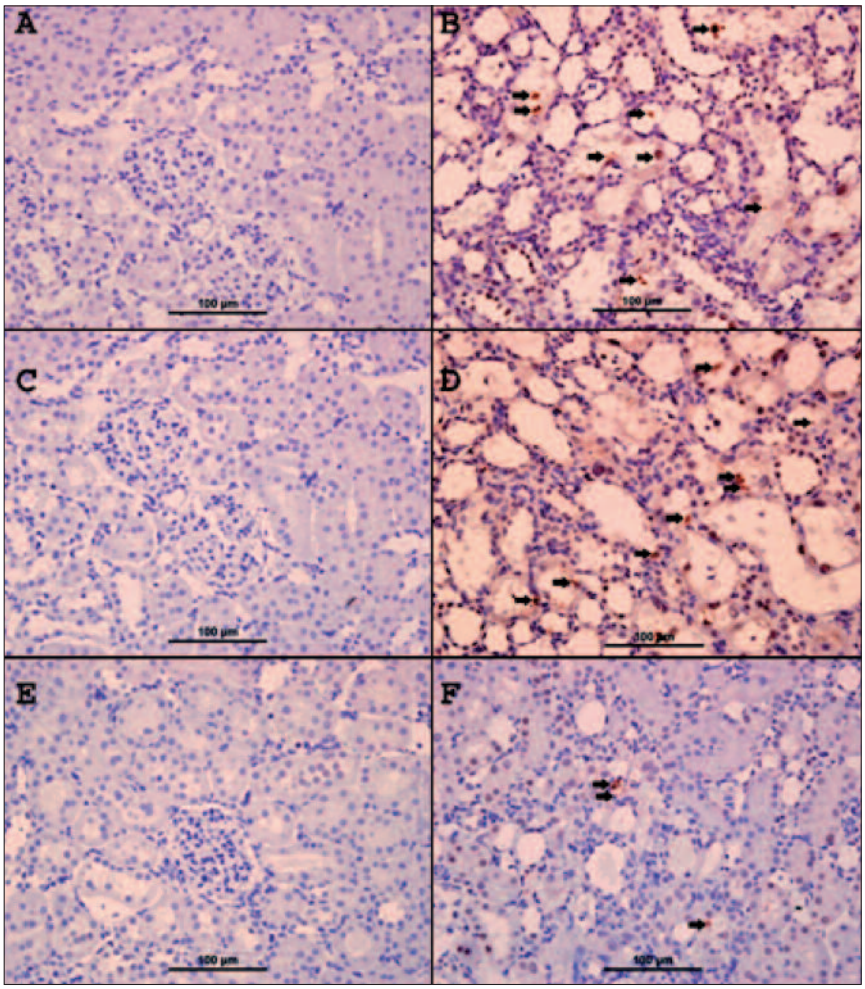

Figure 3. Representative section of kidney architecture histology and apoptotic body formation, Streptavidin-biotin immunoperoxidase method, in control and treated rats. ***A,*** Control rats with normal kidney architecture with no apoptotic bodies. ***B,*** CP treated rats with acute tubular necrosis in nearly two thirds of examined tissue areas with tubular distention, necrotic material and many apoptotic cell showing brown cytoplasmic staining (*thick arrows*). ***C,*** EA10 rats with normal kidney architecture. ***D,*** CP+EA10 treated rats with histological appearance similar to the CP treated rats. ***E,*** EA30 rats with normal kidney architecture. ***F,*** CP+EA30 treated rats showed dramatic improvement in the histologic appearance with few focal areas of acute tubular necrosis and few apoptotic bodies (*thick arrow*).

Total Antioxidant Status (TAS)

Both total antioxidant and GSH concentrations decreased significantly following CP treatment. However, the concentrations of total antioxidant and GSH were similar to those in the control when CP was combined with 30 mg/kg of EA (Table VII).

Table VI. Microscopic scoring of kidney section for necrosis in control rats, and treated rats either with cisplatin (CP) or ellagic acid (EA) alone or CP combined with different doses of EA.

No	Treatment	Score	Necrosis (%)*
1	Saline	0	0
2	CP	3	63.2 ± 2.7
3	EA10	0	0
4	CP + EA10	3	62.7 ± 2.4
5	EA30	0	0
6	CP + EA30	1	17.5 ± 3.4

*Values in the table are mean ± SD, from at least 6 rats.

Molecular Markers

Kim-1 and Clusterin Proteins

Western blot was performed to examine the expression level of *Kim-1* and *Clu* proteins in all rats. *β-Actin*, a house keeping gene, was used to

Table VII. The effect of saline, cisplatin (CP) or CP plus ellagic acid (EA) given at doses of 10 mg/Kg and 30 mg/Kg on total antioxidant (TAS) and reduced glutathione (GSH) concentrations in controls, rats treated cisplatin (CP) or ellagic acid (EA30) alone, or CP combined with 30 mg/Kg EA.

Treatment	N	TAS*	GSH*
Saline (control)	6	2.85 ± 0.01	68 ± 2
CP	6	2.59 ± 0.06**	49 ± 2
EA30	6	2.74 ± 0.06	62 ± 4
CP + EA30	9	2.74 ± 0.02	66 ± 2

*Values in the table are mean ± SEM from at least 6 rats. **ANOVA significantly different from control rats. Significant at $p < 0.05$.

normalize the target proteins. The expression of *β-actin* protein was similar in the controls and different treated rats (Figure 4 A). A similar pattern was seen for *Clu* protein; there was no apparent variation in levels of expression in all treatment groups, including the control (Figure 4 B). However, the *Kim-1* protein was detected in the CP alone and CP+EA treated rats, regardless of the concentration of EA dose (Figure 4 C).

mRNA Expression

The level of mRNA expression of *Clu* and *Kim-1* genes was examined using a Taqman gene expression mix (ABI) and normalized with that of the housekeeping gene glyceraldehydes 3-phosphate dehydrogenase (*GAPDH)*. The expression levels of both genes were not affected by different doses of EA alone (Figure 5). However, *Clu* and *Kim-1* expression levels increased significantly when either CP alone or CP+EA at different doses were used ($p < 0.001$). The expression levels of *Clu* and *Kim-1* in CP+EA30 were higher than those in CP alone, but the increase was not statistically significant (Figure 5).

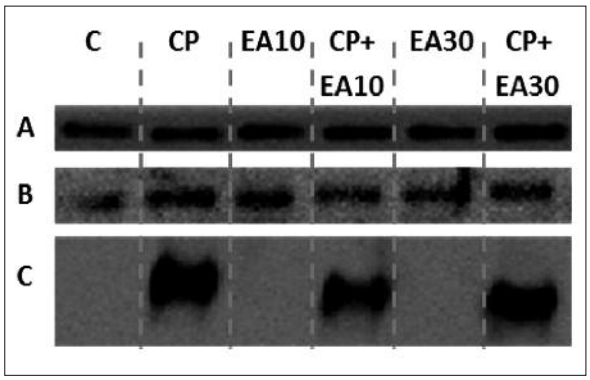

Figure 4. Kidney protein expression detected by western blot in control rats, rats treated with either cisplatin (CP) or ellagic acid (EA) alone or CP combined with different doses of EA. *Panel A*: expression of β-Actin; *Panel B*: expression of Clusterin; *Panel C*: expression of Kim-1.

Discussion

A significant loss in body weight (BW) was observed in rats treated with CP. This reduction may be due to either tubular injury, which affects water reabsorption leading to dehydration and loss of BW[16], or to CP cytotoxic effects on the gastrointestinal tract, which affects rats' eating behavior[25], alters gastrointestinal rhythm, and delays gastric emptying, which subsequently causes BW loss. Rats treated with either CP alone or CP together with different doses of EA showed significant increase in relative kidney weight (RKW). The increase in RKW is suggested to be an indication of kidney damage caused by an increase in the glomerular volume and cellular degenerative changes, including cytoplasmic vacuolization of the proximal tubular cells, and tubular dilation[26].

Histopathological examination revealed acute tubular necrosis and many apoptotic cells in 60% of the examined sections of the cortical area of the kidney in rats treated with CP. Similar results were obtained in rats treated with CP combined with EA at doses of 10 mg/kg. However, less than 25% of the tubular necrosis was observed when CP was combined with 30 mg/kg EA. Histopathological examination of the kidney is

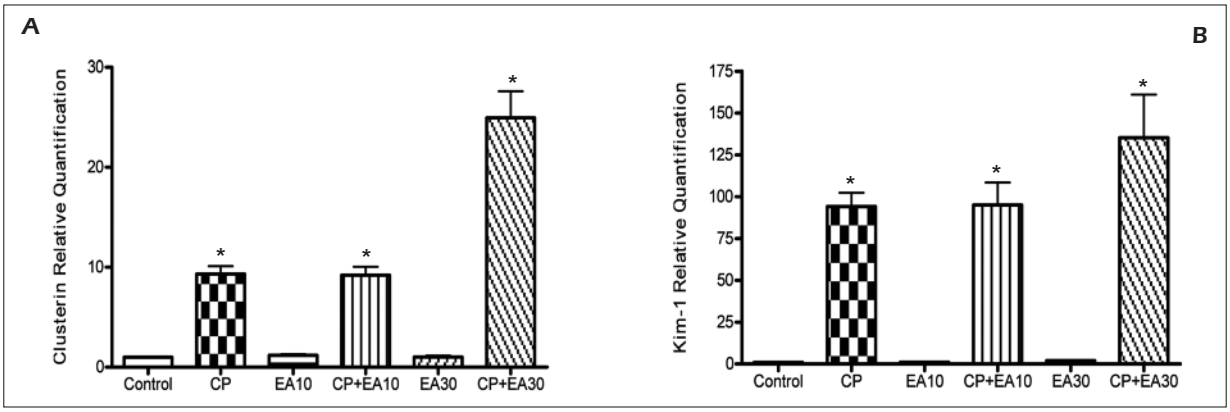

Figure 5. Clusterin ***(A)*** and kidney injury molecule-1 (Kim-1) ***(B)*** mRNA expression in control rats, and rats treated with either cisplatin (CP) or ellagic acid (EA) alone or CP combined with different doses of EA. Data are shown as kidney Kim-1 and *Clu* mRNA expression relative to expression of the house keeping gene GAPDH. *ANOVA, target compared to control: *($p < 0.001$).

considered to be the golden standard method to detect renal injury[27]. It has been reported that CP can induce epithelial cell atrophy and degeneration, loss of brush border, tubular dilation, necrosis and apoptosis, leading to severe renal injury through increased expression of intercellular adhesion molecule 1 (ICAM-1) in renal proximal tubules, macrophage infiltration, nuclear factor-kappa B (NF-κB) activation and ROS production[28-30]. However, Atessahin et al[15] reported that EA, at a dose of 10 mg/kg, ameliorated the histopathological changes induced by CP in the kidney. They reported that EA at 10 mg/kg protected the kidney from tubular necrosis, degeneration, desquamation and tubular dilatation. This finding differs from our results where a dose of 10 mg/kg of EA was found to provide no salutary effect on the histological architecture of the kidney. Ameliorative effects were only seen when a dose of 30 mg/kg was used, and the histological improvement of the kidney at this dose of EA was consistent with that seen in the biochemical parameters.

Rats treated with CP showed significant reduction in water intake and urine output post CP treatment. However, it has been reported that CP administration damages the renal tubule and distorts its ability to reabsorb water and cause polyuria, which can also lead to polydipsia[31]. Recent studies reported that CP administration leads to increase urine output[16,32]. These findings differ from those found in the present study. The significant reduction in urine volume may be due to either significant reduction in water intake or to severe kidney injury. However, the reduction in urine volume was also accompanied by low urine osmolality (Table V). The reduction in urine osmolality is thought to be caused by distort reabsorption of water by the kidney cells, as mentioned above[31]. Administration of EA at a dose of 30 mg/kg prior to and post CP treatment increased water intake and urine output, which suggests improved kidney function.

Additional conventional biochemical markers used in the present study to detect kidney injury were plasma creatinine (Cr) and BUN. In the present study, plasma Cr and BUN increased more than 4- and 10-fold, respectively, following either CP treatment or CP combined with 10 mg/kg EA. However, Cr and BUN concentrations decreased by at least half (from 3.3 mg/dl to 1.4 mg/dl and from 31 mg/dl to 16 mg/dl, respectively) when 30 mg/kg EA was administered prior to and post CP treatment.

The total antioxidant and GSH concentrations were reduced significantly in CP treated rats. The level of cellular antioxidants, such as GSH, catalase and SOD, decreases after CP treatment[6]. The introduction of an exogenous antioxidant is thought to help the endogenous antioxidant system in scavenging the ROS produced during an imbalance in redox status induced by CP[7]. In the present study, the combined treatment of CP 30 mg/kg seems to maintain the total antioxidant and GSH concentrations at a level similar to those in the control rats. It was reported that when 10 mg/kg AE is combined with CP treatment, the level of GSH is improved[15]. However, in our investigation, the improvement was observed at 30 mg/kg EA. The reason for this discrepancy is not certain, but it may indicate that the low dose of EA was not sufficient to ameliorate the negative effect of CP treatment and correct the redox imbalance in the cell. The discrepancy may also be due to the difference in the gender of animals used in the two experiments. Female SD rats were used in our study, where male SD rats were used in the other report[15]. Other parameters used beside the antioxidant assay showed that EA at doses of 10 mg/kg did not improve either the kidney architecture or function. The effect of graded dosed of EA (30, 60 and 90 mg/kg) on alcohol induced oxidative stress was investigated and it was reported that low and high doses of EA did not have an effect on improving the imbalance in redox status[8]. It was suggested that a high dose of EA (90 mg/kg) was not effective due to non-specific binding to non-ROS molecules[8]. In addition, it has been reported that antioxidants, such as polyphenols, when administered at high concentration, may induce ROS in the cell, cause DNA double strand break, apoptosis and cytotoxicity[33]. The effective dose found in the alcohol induced oxidative stress study was 60 mg/kg instead of 30 mg/kg in the current study. The discrepancy may be due to the difference in the studied organ and the cytotoxic agent used[8].

At a dose of 30 mg/kg, EA treatment improved most of the biochemical parameters and the kidney histology. The consistency of EA at this dose in improving the kidney status may be due to either the ability to scavenge the ROS as antioxidant and protect the kidney from oxidative stress or EA may affect CP uptake by the kidney cells. The latter possibility is unlikely, is CP concentration was not significantly different between kidneys of rats treated with CP and those treated with CP+EA. It is known that the kidneys accu-

mulate CP more than other organs[5], resulting in necrosis in the terminal portion of the proximal renal tubules and apoptosis in the distal nephron[16]. In this work, EA was found not to alter significantly the concentration of CP in the renal cortex of CP-treated rats. This strongly implies that the nephroprotective action of EA is not mediated thorough a reduction in the accumulation of the drug in the renal cortex, and further suggests that the antitumor activity of CP may not be adversely affected by the concomitant administration of EA.

Biochemical markers such as Cr and BUN elevate significantly following administration of nephrotoxic drugs. However, these markers, though reliable for detection of renal injury, lack the sensitivity and specificity for early kidney damage detection[34]. Therefore, highly sensitive molecular markers, such as *Kim-1* and *Clu* genes and their proteins, have been deployed to detect early signs of CP-induced nephrotoxicity and access the ameliorative effect of EA. In the present study, *Clu* protein was detected in all treated groups, including the control. This was surprising as *Clu* is not expected to be expressed in high quantities in normal kidneys[35]. However, the protein is expressed in all tissues and organs and it has a role in cell remodeling and repair, lipid recycling and cell aggregation[36]. The method we used for detection of *Clu* (Western blot) is not a quantitative assay and the level seen in untreated control animals probably reflects normal cellular levels. On the contrary, *Kim-1* protein was detected in all CP treated rats, but not in either the untreated control rats or rats treated with EA alone. Kim-1 can be found in different tissues in rats, but when a nephrotoxic drug such as CP is introduced, its expression is up-regulated in the kidney only[37]. It was reported that *Kim-1* and *Clu* are very sensitive (95%) to early kidney injury detection and their expression persists until kidney recovery[34]. In the current study, the histopathological analysis showed that there was less than 25% damage in the kidney of rats treated with CP and 30 mg/kg EA. This agrees with the restoration of levels of the conventional biochemical markers. However, *Clu* and *Kim-1* were still elevated, and their levels were higher in rats treated with CP plus 30 mg/kg EA compared with those treated with either CP alone or CP plus 10 mg/kg EA. This could be due to reflection of the role of both *Clu* and *Kim-1* in cell remodeling and repair at the dose of 30 mg/kg EA in the regenerative area after kidney injury[38,39]. EA may have driven the process of repair (elevating remodeling and repair molecules such as *Clu* and *Kim-1*) in a dose dependent manner. In addition, it has been postulated that *Kim-1* can be involved in cell debris removal of damaged renal tissue and participates in epithelial cell growth and differentiation[38]. Therefore, we hypothesize that the increased level of *Kim-1* and *Clu* in the kidney tissue of rats treated with CP and 30 mg/kg EA is due to kidney repair encouraged by EA administration and regulated by tissue repair proteins, including *Kim-1* and *Clu*. Since the study was conducted for 10 days only and the recovery state of the kidney was not complete, it was expected that both *Clu* and *Kim-1* would be high. Thus, the levels of *Kim-1* expression are in agreement with the histopathological results and levels of biochemical markers at the dose of 30 mg/kg EA compared with 10 mg/kg EA.

Conclusions

This work has shown that EA, at a dose of 30 mg/kg (but not 10 mg/kg), had an ameliorative effect against CP nephrotoxicity.

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References

1) Miller RP, Tadagavadi RK, Ramesh G, Reeves WB. Mechanisms of cisplatin nephrotoxicity. Toxins 2010; 2: 2490-2518.
2) Ali BH, Al Moundhri MS. Agents ameliorating or augmenting the nephrotoxicity of cisplatin and other platinum compounds: a review of some recent research. Food Chem Toxicol 2006; 44: 1173-1183.
3) Hanigan MH, Devarajan P. Cisplatin nephrotoxicity: molecular mechanisms. Cancer Ther 2003; 1: 47-61.
4) Pabla N, Murphy RF, Liu K, Dong Z. The copper transporter Ctr1 contributes to cisplatin uptake by renal tubular cells during cisplatin nephrotoxicity. Am J Physiol Renal Physiol 2009; 296: F505-511.

5) Yonezawa A, Inui K. Organic cation transporter OCT/SLC22A and H(+)/organic cation antiporter MATE/SLC47A are key molecules for nephrotoxicity of platinum agents. Biochem Pharmacol 2011; 81: 563-568.

6) Santos NA, Catao CS, Martins NM, Curti C, Bianchi ML, Santos AC. Cisplatin-induced nephrotoxicity is associated with oxidative stress, redox state unbalance, impairment of energetic metabolism and apoptosis in rat kidney mitochondria. Arch Toxicol 2007; 81: 495-504.

7) Chirino YI, Pedraza-Chaverri J. Role of oxidative and nitrosative stress in cisplatin-induced nephrotoxicity. Exp Toxicol Pathol 2009; 61: 223-242.

8) Devipriya N, Srinivasan M, Sudheer AR, Menon VP. Effect of ellagic acid, a natural polyphenol, on alcohol-induced prooxidant and antioxidant imbalance: a drug dose dependent study. Singapore Med J 2007; 48: 311-318.

9) Kannan MM, Quine SD. Ellagic acid ameliorates isoproterenol induced oxidative stress: Evidence from electrocardiological, biochemical and histological study. Eur J Pharmacol 2011; 659: 45-52.

10) Umesalma S, Sudhandiran G. Ellagic acid prevents rat colon carcinogenesis induced by 1, 2 dimethyl hydrazine through inhibition of AKT-phosphoinositide-3 kinase pathway. Eur J Pharmacol 2011; 660: 249-258.

11) Romert L, Jansson T, Curvall M, Jenssen D. Screening for agents inhibiting the mutagenicity of extracts and constituents of tobacco products. Mutat Res 1994; 322: 97-110.

12) Kassim M, Achoui M, Mustafa MR, Mohd MA, Yusoff KM. Ellagic acid, phenolic acids, and flavonoids in Malaysian honey extracts demonstrate in vitro anti-inflammatory activity. Nutr Res 2010; 30: 650-659.

13) Rosillo MA, Sanchez-Hidalgo M, Cardeno A, de la Lastra CA. Protective effect of ellagic acid, a natural polyphenolic compound, in a murine model of Crohn's disease. Biochem Pharmacol 2011; 82: 737-745.

14) Turk G, Ceribasi AO, Sahna E, Atessahin A. Lycopene and ellagic acid prevent testicular apoptosis induced by cisplatin. Phytomedicine 2011; 18: 356-361.

15) Atessahin A, Ceribasi AO, Yuce A, Bulmus O, Cikim G. Role of ellagic acid against cisplatin-induced nephrotoxicity and oxidative stress in rats. Basic Clin Pharmacol Toxicol 2007; 100: 121-126.

16) Ali BH, Al-Moundhri M, Tageldin M, Al Husseini IS, Mansour MA, Nemmar A, Tanira MO. Ontogenic aspects of cisplatin-induced nephrotoxicity in rats. Food Chem Toxicol 2008; 46: 3355-3359.

17) Batista BL, Grotto D, Rodrigues JL, Souza VC, Barbosa F, Jr. Determination of trace elements in biological samples by inductively coupled plasma mass spectrometry with tetramethylammonium hydroxide solubilization at room temperature. Anal Chim Acta 2009; 646: 23-29.

18) Mohan IK, Khan M, Shobha JC, Naidu MU, Prayag A, Kuppusamy P, Kutala VK. Protection against cisplatin-induced nephrotoxicity by Spirulina in rats. Cancer Chemother Pharmacol 2006; 58: 802-808.

19) Vielhauer V, Stavrakis G, Mayadas TN. Renal cell-expressed TNF receptor 2, not receptor 1, is essential for the development of glomerulonephritis. J Clin Invest 2005; 115: 1199-1209.

20) Er TK. Effect of sample volume for the measurement of osmolality by using the Advanced 3250 osmometer. Am J Emerg Med 2008; 26: 1060-1061.

21) Miller NJ, Rice-Evans C, Davies MJ, Gopinathan V, Milner A. A novel method for measuring antioxidant capacity and its application to monitoring the antioxidant status in premature neonates. Clin Sci (Lond) 1993; 84: 407-412.

22) Guntherberg H, Rost J. The true oxidized glutathione content of red blood cells obtained by new enzymic and paper chromatographic methods. Anal Biochem 1966; 15: 205-210.

23) Griffith OW. Determination of glutathione and glutathione disulfide using glutathione reductase and 2-vinylpyridine. Anal Biochem 1980; 106: 207-212.

24) Bradford MM. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal Biochem 1976; 72: 248-254.

25) Vera G, Chiarlone A, Martin MI, Abalo R. Altered feeding behaviour induced by long-term cisplatin in rats. Auton Neurosci 2006; 126-127: 81-92.

26) Saad SY, Najjar TA, Al-Sohaibani MO. The effect of rebamipide on cisplatin-induced nephrotoxicity in rats. Pharmacol Res 2000; 42: 81-86.

27) Haase M, Mertens PR. Urinary biomarkers–silver bullets to faster drug development and nephron protection. Nephrol Dial Transplant 2010; 25: 3167-3169.

28) Sung MJ, Kim DH, Jung YJ, Kang KP, Lee AS, Lee S, Kim W, Davaatseren M, Hwang JT, Kim HJ, Kim MS, Kwon DY, Park SK. Genistein protects the kidney from cisplatin-induced injury. Kidney Int 2008; 74: 1538-1547.

29) An Y, Xin H, Yan W, Zhou X. Amelioration of cisplatin-induced nephrotoxicity by pravastatin in mice. Exp Toxicol Pathol 2011; 63: 215-219.

30) Ahmad ST, Sultana S. Tannic acid mitigates cisplatin-induced nephrotoxicity in mice. Human Exp Toxicol 2012; 31: 145-156.

31) Wong NL, Walker VR, Wong EF, Sutton RA. Mechanism of polyuria after cisplatin therapy. Nephron 1993; 65: 623-627.

32) Abdelrahman AM, Al Salam S, AlMahruqi AS, Al husseni IS, Mansour MA, Ali BH. N-acetylcysteine improves renal hemodynamics in rats with cisplatin-induced nephrotoxicity. J Appli Toxicol 2010; 30: 15-21.

33) Bouayed J, Bohn T. Exogenous antioxidants–Double-edged swords in cellular redox state: Health beneficial effects at physiologic doses versus deleterious effects at high doses. Oxid Med Cell Longev 2010; 3: 228-237.

34) Ozer JS, Dieterle F, Troth S, Perentes E, Cordier A, Verdes P, Staedtler F, Mahl A, Grenet O, Roth DR, Wahl D, Legay F, Holder D, Erdos Z, Vlasakova K, Jin H, Yu Y, Muniappa N, Forest T, Clouse HK, Reynolds S, Bailey WJ, Thudium DT, Topper MJ, Skopek TR, Sina JF, Glaab WE, Vonderscher J, Maurer G, Chibout SD, Sistare FD, Gerhold DL. A panel of urinary biomarkers to monitor reversibility of renal injury and a serum marker with improved potential to assess renal function. Nat Biotechnol 2010; 28: 486-494.

35) Rosenberg ME, Silkensen J. Clusterin: physiologic and pathophysiologic considerations. Int J Biochem Cell Biol 1995; 27: 633-645.

36) Dieterle F, Perentes E, Cordier A, Roth DR, Verdes P, Grenet O, Pantano S, Moulin P, Wahl D, Mahl A, End P, Staedtler F, Legay F, Carl K, Laurie D, Chibout SD, Vonderscher J, Maurer G. Urinary clusterin, cystatin C, beta2-microglobulin and total protein as markers to detect drug-induced kidney injury. Nat Biotechnol 2010; 28: 463-469.

37) Vaidya VS, Ozer JS, Dieterle F, Collings FB, Ramirez V, Troth S, Muniappa N, Thudium D, Gerhold D, Holder DJ, Bobadilla NA, Marrer E, Perentes E, Cordier A, Vonderscher J, Maurer G, Goering PL, Sistare FD, Bonventre JV. Kidney injury molecule-1 outperforms traditional biomarkers of kidney injury in preclinical biomarker qualification studies. Nat Biotechnol 2010; 28: 478-485.

38) Malyszko J, Koc-Zorawska E, Malyszko JS, Mysliwiec M. Kidney injury molecule-1 correlates with kidney function in renal allograft recipients. Transplantat Proc 2010; 42: 3957-3959.

39) Harpur E, Ennulat D, Hoffman D, Betton G, Gautier JC, Riefke B, Bounous D, Schuster K, Beushausen S, Guffroy M, Shaw M, Lock E, Pettit S; HESI Committee on Biomarkers of Nephrotoxicity. Biological qualification of biomarkers of chemical-induced renal toxicity in two strains of male rat. Toxicol Sci 2011; 122: 235-252.