

## Original Research Article

# Amelioration of Cisplatin-Induced Nephrotoxicity in Rats by Curcumin and $\alpha$ -Tocopherol

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## Abstract

**Purpose:** To investigate the possible protective role of curcumin and  $\alpha$ -tocopherol against cisplatin-induced nephrotoxicity in rat.

**Methods:** Male Wistar rats were divided into five groups. Groups 1 and 2 were intraperitoneally (i.p.) injected with normal saline and cisplatin (20 mg/kg), respectively. Groups 3, 4 and 5 were pre-treated with a single doses of  $\alpha$ -tocopherol (250 mg/kg), curcumin (200 mg/kg) and  $\alpha$ -tocopherol with curcumin, respectively, for 24 h prior to the administration of cisplatin. After 72 h following injection, specimens were collected. Serum blood urea nitrogen (BUN), creatinine and malondialdehyde (MDA) levels, superoxide dismutase (SOD) and catalase activities, kidney histopathological study and gene expressions of nicotinamide adenine dinucleotide phosphate (NADPH) oxidase and p38 mitogen-activated protein kinase (p38-MAPK) were investigated.

**Results:** Pre-treatment with combined curcumin and  $\alpha$ -tocopherol exhibited significantly reduced MDA levels and enhanced activities of SOD and catalase compared with cisplatin-treated group ( $p < 0.05$ ). It also improved BUN as well as creatinine levels and kidney histopathology. Moreover, gene expressions of NADPH oxidase were decreased, whereas p38-MAPK gene expressions were not significant compared with cisplatin-treated group.

**Conclusion:** Combined curcumin and  $\alpha$ -tocopherol are able to reduce cisplatin-induced nephrotoxicity via possible inhibition of NADPH oxidase, resulting in improvement of kidney function and histology.

**Keywords:** Cisplatin, Oxidative stress, Curcumin,  $\alpha$ -Tocopherol, Nephrotoxicity.

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## INTRODUCTION

Cisplatin or *cis*-diamminedichloroplatinum (II) is the chemotherapeutic agent useful in the treatment of various cancers such as ovarian cancer and testicular cancer [1]. Unfortunately, it exerts various side effects in several organs particularly in the kidney [2]. Only days after initiating treatment, approximately one-third of cisplatin-treated patients exhibit reduced

glomerular filtration rate and increased blood urea nitrogen (BUN) and creatinine levels [3]. The underlying mechanism of nephrotoxicity remains incompletely understood. Oxidative stress has been implicated in the pathogenesis of kidney injury induced by cisplatin through increasing reactive oxygen species (ROS) resulting in augmented oxidation reaction of macromolecules especially lipid, protein and nucleic acid and reducing activity of enzymatic

antioxidants such as SOD, glutathione peroxidase (GPX) and catalase [1,4]. ROS particularly superoxide radical ( $O_2^{\cdot-}$ ) and hydroxyl radical ( $\cdot OH$ ) play the important role in cisplatin-induced acute renal failure by reducing renal blood flow and inducing tubular damage associated with increased renal contents of MDA, a biomarker of lipid peroxidation (LPO) [5]. NADPH oxidase is an enzyme which catalyzes the generation of  $O_2^{\cdot-}$  in large amounts.  $O_2^{\cdot-}$  is able to rapidly change into other ROS such as hydrogen peroxide ( $H_2O_2$ ) and  $\cdot OH$  [6]. Moreover, apoptosis also plays key role in cisplatin-induced renal cell injury [7]. Activated p38-MAPK has been implicated as a pro-apoptotic cellular signaling during oxidative stress [8].

Previous study provides evidence which supports that antioxidant supplementation is the important therapeutic strategy to abrogate kidney damage activated by cisplatin [9]. Curcumin, an active phenolic compound derived from the rhizomes of *Curcuma longa* used as a spice and food coloring shows various antioxidant abilities *in vivo* [10]. Treatment with curcumin has been reported to attenuate kidney dysfunction, histopathological alteration, oxidative stress and the reduced activities of enzymatic antioxidants induced by chemical agents [11]. The activity of curcumin alone is lower than that of the two-compound mixture [12].

Vitamin E is a fat-soluble antioxidant and includes tocopherols and tocotrienols. Alpha-tocopherol is the most biologically active form of vitamin E. Curcumin and/or vitamin E have been reported to exhibit effective antioxidant activity against L-thyroxine-induced rat testicular and hepatic oxidative stress [13]. The present study, therefore, aims to investigate the protective role of curcumin and  $\alpha$ -tocopherol against cisplatin-induced oxidative stress in rat kidney.

## EXPERIMENTAL

### Chemicals

*cis*-Diamminedichloroplatinum (II), curcumin from *Curcuma longa*,  $\alpha$ -tocopherol, Bradford assay kits and 10 % neutral buffered formalin solution were purchased from Sigma-Aldrich Chemical Company (USA). Thiobarbituric acid reactive substances (TBARS), SOD and catalase assay kits were obtained from Cell Biolabs, Inc (USA). RNeasy mini kit, Omniscript RT kit and HotStar Taq DNA polymerase were purchased from Qiagen (Germany). All other chemicals were of analytical grade.

### Animals

Twenty-five male Wistar rats (*Rattus norvegicus*), ranging from 180-200 g body weight (BW), were obtained from Division of Animal House, Faculty of Science, Prince of Songkla University, Thailand. All animal procedures were reviewed and approved by Animal Ethics Committee, Walailak University (Protocol no. 004/2012) and were conducted according to the Guide for the Care and Use of Laboratory Animals, National Research Council [14]. The rats were maintained in stainless-steel cages under constant conditions of temperature ( $23 \pm 2$  °C), relative humidity (50 – 60 %) and lighting (12 h light/dark cycles). They were provided with standard commercial rat feed (Division of Animal House, Faculty of Science, Prince of Songkla University, Thailand) and distilled water, and were acclimatized and closely monitored under laboratory conditions for 2 weeks prior to experimentation.

### Experimental design and specimen collection

The rats were divided in five groups. Group 1 was treated with a single i.p. injection of normal saline. Group 2 was injected with a single dose of cisplatin (20 mg/kg, i.p.). Groups 3, 4 and 5 were treated with a single doses of  $\alpha$ -tocopherol (250 mg/kg BW) alone, curcumin (200 mg/kg BW) alone and  $\alpha$ -tocopherol (250 mg/kg BW) plus curcumin (200 mg/kg BW), respectively, via i.p. route, 24 h prior to a single-dose injection of cisplatin (20 mg/kg BW). Seventy two hours (72 h) after the first injection, the rats were anesthetized with thiopental sodium intraperitoneally (50 mg/kg BW). Peripheral blood from heart was collected in clotted tubes and the rats euthanized by anesthetizing with an overdose of thiopental sodium (100 mg/kg BW). The abdominal cavity of rats was opened, the kidneys immediately collected and then washed in ice-cold isotonic saline.

### Determination of serum BUN and creatinine levels

The peripheral heart blood samples were centrifuged at 3000 rpm for 5 min. Sera were collected and the levels of BUN and creatinine measured using a Cobas Mira Plus CC Chemistry Analyzer (Switzerland).

### Determination of MDA levels

The kidney was re-suspended at 50 mg/ml in phosphate buffered saline (PBS) containing 1X butylated hydroxytoluene (BHT), homogenized on ice and centrifuged at 10000 x g for 5 min to

collect supernatant. According to the protocol of OxiSelect™ TBARS Assay Kit (Cell Biolabs, Cat no: STA-330), 100 µl of sample or MDA standard was added to separate in microcentrifuge tubes; 100 µl of the SDS lysis solution was added, mixed thoroughly, incubated for 5 min at room temperature, and 250 µl of thiobarbituric acid (TBA) reagent added. Each tube was closed, incubated at 95 °C for 60 min, removed and then cooled to room temperature in an ice bath for 5 min. All the sample tubes were centrifuged at 3000 rpm for 15 min, the supernatant removed, and 200 µl of which was transferred, along with 200 µl of MDA standard, to a 96-well microplate compatible with a spectrophotometric plate reader (Syngene, USA) and the absorbance read at 532 nm.

#### Determination of SOD activity

The kidney was homogenized to 50 mg/ml in cold 1X lysis buffer (10 mM Tris, pH 7.5, 150 mM NaCl, 0.1 mM EDTA), centrifuged at 12000 x g for 10 min and the supernatant collected for analysis. According to protocol of OxiSelect™ SOD Activity Assay Kit (Cell Biolabs, Cat No: STA-340), 20 µl of the sample, 5 µl of xanthine solution, 5 µl of chromagen solution, 5 µl of 10X SOD assay buffer, 50 µl of deionized water were mixed together to yield a total volume of 90 µl in a 96 well microplate; 10 µl of pre-diluted 1X xanthine oxidase solution was added to each well, mixed well, incubated for 1 h at 37 °C and the absorbance read with a spectrophotometric plate reader at 490 nm.

#### Determination of catalase activity

The kidney was homogenized to 50 mg/ml in cold PBS with 1mM EDTA, centrifuged at 10000 x g, 4 °C for 15 min and the supernatant collected. According to the protocol of OxiSelect™ Catalase Activity Assay Kit (Cell Biolabs, Cat No: STA-341), 20 µl of the diluted catalase standard or sample was added to a 96-well microplate, 50 µl of the H<sub>2</sub>O<sub>2</sub> working solution (12 mM) was added to each well, mixed thoroughly, incubated for 1 min, and the reaction stopped by adding 50 µl of catalase quencher into each well and mixed thoroughly; 5 µl of each reaction well was transferred to a fresh well, 250 µl of the chromogenic working solution added to each well, incubated for 60 min with vigorous mixing and its absorbance read with a spectrophotometric plate reader at 520 nm.

#### Protein determination

Protein content of kidney sample was determined in order to calculate MDA level as

nmol/g protein, SOD and catalase activities as U/mg protein by Bradford assay (Sigma, USA) using bovine serum albumin (BSA) as standard.

#### Histopathological examination of kidney sample

The kidney was preserved in 10 % neutral buffered formalin solution for 24 h and washed with 70 % ethanol. The tissue was then placed in small metal caskets, stirred by a magnetic stirrer, dehydrated using alcohol series from 70 to 100 % and embedded in paraffin using an embedding machine. Paraffin block was sectioned using a rotary ultra microtome, distributed onto glass slides and then dried overnight. The slide was observed under a light microscope after being stained with hematoxylin and eosin (H&E) dyes and mounted. Two pathologists evaluated histopathological grading using a semi-quantitative scale: normal = 0; mild = < 25 %; moderate = 25 – 50 %; severe = > 50 % of affected area.

#### Determination of NADPH oxidase and p38-MAPK gene expressions

Total RNA was extracted from kidney tissue by RNeasy mini kit. RNA content and purity were measured by a UV spectrophotometer. RT-PCR was done using the extracted RNA for detection of NADPH oxidase and p38-MAPK genes. For amplification of the targets genes, reverse transcription and PCR were run in two separate steps. Briefly, Reaction mixture of RT reaction containing 1 µg total RNA, 0.5 µg random primer, 5 × RT buffer, 2.5 mmol/l dNTP, 20 U RNase inhibitor and 200 U MMLV reverse transcriptase in a total volume of 25 µl was incubated at 37 °C for 60 min, then heated to 95 °C for 5 min to inactivate MMLV. PCR was carried out with 1.5 µl RT products, 10 × PCR buffer (without Mg<sup>2+</sup>) 2.5 µl, 2.0 µl dNTP (2.5 mmol/l), 2.0 µl MgCl<sub>2</sub> (25 mmol/l), 0.5 µl each primer (20 µmol/l) of β-actin, 0.5 µl each primer of gene to be tested (20 µmol/l) and 1 U of Taq DNA polymerase, in a final volume of 25 µl. Thermal cycler conditions were as follows: a first denaturing cycle at 97 °C for 5 min, followed by a variable number of cycles of amplification defined by denaturation at 96 °C for 1.5 min, annealing for 1.5 min and extension at 72 °C for 3 min. A final extension cycle of 72 °C for 15 min was included [15]. The primers, including NADPH oxidase, p38-MAPK and β-actin, are illustrated in Table 1.

All PCR products were electrophoresed on 2 % agarose stained with ethidium bromide and visualized by UV transilluminator. The amounts

of PCR products were quantified using the gel and image analysis software (Syngene, USA).

**Table 1:** The primers of NADPH oxidase, p38-MAPK and  $\beta$ -actin

NADPH oxidase	
Forward primer:	5'-GGAAATAGAAAGTTGACTGGCCC -3'
Reverse primer:	5'-GTATGAGTGCCATCCAGAGCAG-3'
p38-MAPK	
Forward primer:	5'TGACTTGCTTCCCTGTTCTTGA-3'
Reverse primer:	5'-TTTGAAATGTGTCCACAGAGG-3'
$\beta$ -actin	
Forward primer:	5'TGTTGTCCTGTATGCCTCT-3'
Reverse primer:	5'-TAATGTCACGCACGATTTC-3'

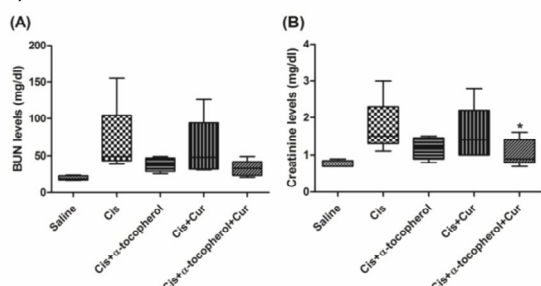
**Statistical analysis**

The results were expressed as mean  $\pm$  standard deviation (SD). Differences between groups were determined by one-way analysis of variance (ANOVA). Post hoc test was performed for group comparisons using the least significant difference (LSD) test and  $p < 0.05$  was considered significant.

**RESULTS**

**Effect of treatments on kidney function biomarkers**

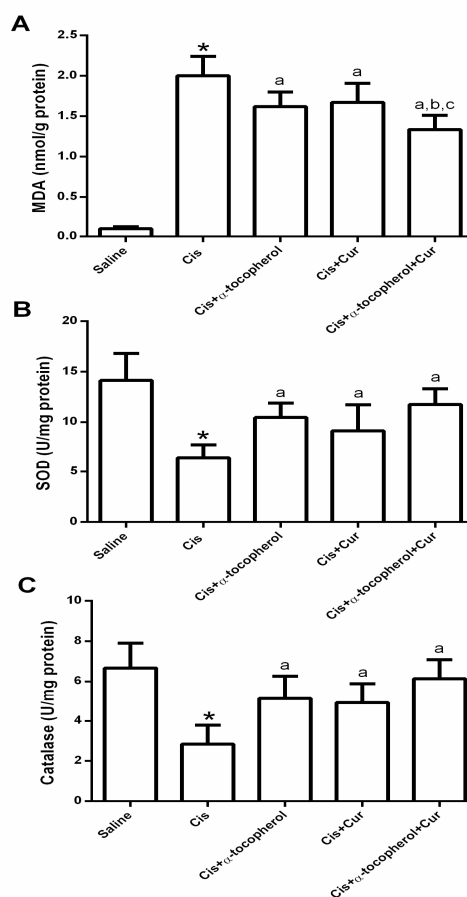
Cisplatin elicited a significantly increased serum BUN and creatinine levels compared with saline group ( $p < 0.05$ ) (Fig 1). Although not significant, curcumin and/or  $\alpha$ -tocopherol were able to reduce BUN levels compared with cisplatin-treated group (Fig 1A). Surprisingly, co-administration of curcumin and  $\alpha$ -tocopherol produced significant reduction of creatinine levels ( $p < 0.05$ ) compared with cisplatin-treated group. Additionally, curcumin or  $\alpha$ -tocopherol lowered creatinine levels but not significantly (Fig 1B).



**Fig 1:** Kidney function test of rat study groups. (A) Serum BUN levels. (B) Serum creatinine levels. Values represent mean  $\pm$  SD (n = 5); \* $p < 0.05$  compared with cisplatin-treated group. Cis: cisplatin, Cur: curcumin

**Effect on lipid peroxidation and enzymatic antioxidant activities**

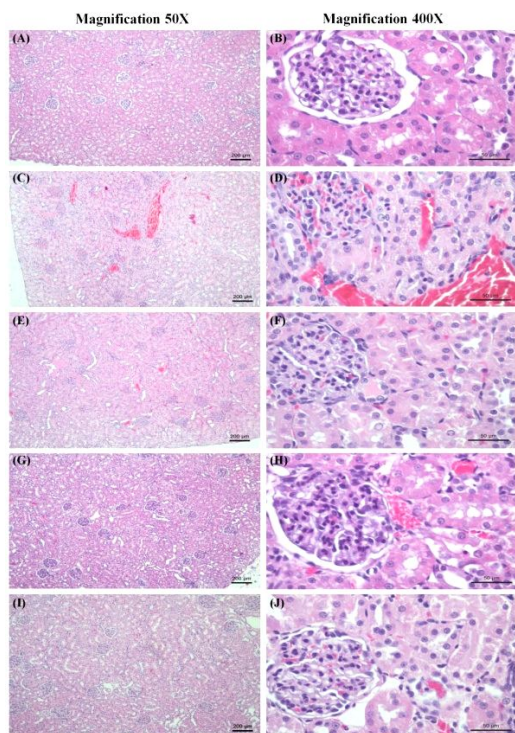
Cisplatin produced a markedly increased MDA levels compared with saline group ( $p < 0.05$ , Fig 2A) which indicate enhancement of lipid peroxidation. Cisplatin pre-treated with curcumin and/or  $\alpha$ -tocopherol-treated groups showed a significant reduction in MDA levels compared with cisplatin-treated group ( $p < 0.05$ ). Surprisingly, cisplatin pre-treated with combined curcumin and  $\alpha$ -tocopherol treated group demonstrated significantly reduced MDA levels compared with cisplatin pre-treated with  $\alpha$ -tocopherol or curcumin ( $p < 0.05$ ). As shown in Fig 2B and 2C, cisplatin demonstrated marked reduction in SOD and catalase activities compared with the saline control group ( $p < 0.05$ ). Cisplatin pre-treated with curcumin and/or  $\alpha$ -tocopherol treated groups showed significantly increased SOD and catalase activities compared with cisplatin-treated group ( $p < 0.05$ ).



**Fig 2:** Lipid peroxidation and enzymatic antioxidant activities of the rats. (A) Renal MDA levels. (B) SOD activity. (C) Catalase activity. Values represent the mean  $\pm$  SD (n = 5); \*  $p < 0.05$  compared with control group; <sup>a,b,c</sup>  $p < 0.05$  compared with cisplatin, cisplatin pre-treated with  $\alpha$ -tocopherol and cisplatin pre-treated with curcumin-treated groups, respectively. Cis: cisplatin, Cur: curcumin.

### Effect of treatments on on kidney histopathology

Cisplatin at 20 mg/kg BW caused various pathologies of kidney especially in tubules including moderate accumulation of proteinaceous casts in tubular lumen, tubular necrosis and congestion (Fig 3C and 3D). Pre-treatment with curcumin and/or  $\alpha$ -tocopherol were able to improve kidney pathology induced by cisplatin (Fig 3E - J) (Table 2).



**Fig 3:** Effect of curcumin and/or  $\alpha$ -tocopherol pre-treatment on histopathological alterations upon cisplatin treatment in Wistar rats (H&E staining): (A, B) Control group. (C, D) Cisplatin-treated group. (E, F) Cisplatin pre-treated with  $\alpha$ -tocopherol-treated group. (G, H) Cisplatin pre-treated with curcumin-treated group. (I, J) Cisplatin pre-treated with combined curcumin and  $\alpha$ -tocopherol-treated group. Scale: 200 and 50  $\mu$ m correspond to magnification at 50 x and 400 x, respectively.

### Effect of treatment on NADPH oxidase and p38-MAPK gene expressions

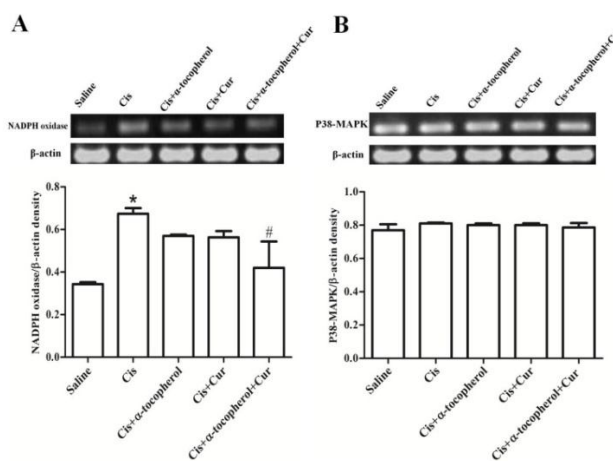
As shown in Fig 4A, treatment with cisplatin exhibited a significant enhancement of mRNA levels of NADPH oxidase compared with saline group ( $p < 0.05$ ). Cisplatin pre-treated with combined curcumin and  $\alpha$ -tocopherol-treated groups showed significantly reduction of mRNA levels of NADPH oxidase compared with cisplatin-treated group ( $p < 0.05$ ). Although the effects were not significant, curcumin or  $\alpha$ -tocopherol reduced BUN levels compared with cisplatin-treated group. Cisplatin exhibited the increased mRNA levels of p38-MAPK but did not

significant compared with saline group (Fig 4B). Additionally, cisplatin pre-treated with curcumin and/or  $\alpha$ -tocopherol-treated groups did not reduce mRNA levels of p38-MAPK compared with cisplatin-treated group.

**Table 2:** Renal histological evaluation of the rats (n = 5).

Histological alteration	Group*				
	1	2	3	4	5
Accumulation of proteinaceous casts in tubular lumen	0	2+ (4/5) 3+ (1/5)	0 (2/5) 1+ (2/5) 2+ (1/5)	0 (3/5) 1+ (1/5) 2+ (1/5)	0 (4/5) 1+ (1/5)
Tubular necrosis	0	1+ (1/5) 2+ (3/5) 3+ (1/5)	0 (3/5) 1+ (1/5) 2+ (1/5)	0 (3/5) 1+ (1/5) 2+ (1/5)	0 (3/5) 1+ (1/5) 2+ (1/5)
Congestion	0	2+ (5/5)	0 (3/5) 1+ (2/5)	0 (3/5) 1+ (2/5)	0 (4/5) 1+ (1/5)

Severity scores: 0 = Not found; 1+ = Mild; 2+ = Moderate; 3+ = Severe. \*1 = Control group; 2 = Cisplatin-treated group; 3 = Cisplatin pre-treated with  $\alpha$ -tocopherol-treated group; 4 = Cisplatin pre-treated with curcumin-treated group; 5 = Cisplatin pre-treated with combined  $\alpha$ -tocopherol and curcumin-treated group.



**Fig 4:** NADPH oxidase and p38-MAPK gene expressions in the rats. (A) Agarose gel electrophoresis showing gene expression of NADPH oxidase by RT-PCR with its density compared with  $\beta$ -actin. (B) Gene expressions of p38-MAPK with its density compared with  $\beta$ -actin. Values represent the mean  $\pm$  SD (n = 5). \*  $p < 0.05$  compared with control group. #  $p < 0.05$  compared with cisplatin. Cis: cisplatin, Cur: curcumin.

### DISCUSSION

Normally, oxidative stress was caused by increasing oxidants and/or reducing antioxidants. Oxidants include ROS and reactive nitrogen species (RNS) which can be produced by both endogenous sources (inflammatory cells, fibroblast, epithelial cells, endothelial cells, respiratory chain, xanthine and NADPH oxidase) and exogenous sources (cigarette smoke, exogenous toxins, pollution, radiation, carcinogens and drugs) [16]. Under normal physiological conditions, oxidants are scavenged

through antioxidant defense mechanism [17]. If incompletely clearance by antioxidants, oxidants will be caused oxidative stress. Inefficiency and insufficiency of antioxidant defense system are concerned in some pathological conditions induced by oxidative stress. Evidence of oxidative stress induced by cisplatin in kidney has been demonstrated in various studies [1,4].

The present study demonstrated that oxidative stress contributes to cisplatin-induced nephrotoxicity by increasing MDA levels and reducing activities of enzymatic antioxidants including SOD and catalase, similarly to previous reports [1,15]. NADPH oxidase is a membrane-bound enzyme complex which donates an electron from NADPH to molecular oxygen ( $O_2$ ) to produce  $O_2^{\cdot-}$ . Thereafter,  $O_2^{\cdot-}$  is converted into  $H_2O_2$  by SOD.  $H_2O_2$  is also converted to  $\cdot OH$  by the Fenton reaction. We suggest that cisplatin play a key role in ROS production by enhancement of NADPH oxidase gene expression causing increased lipid peroxidation, ultimately may caused membrane damage and cell death. Moreover, the present study illustrated that oxidative stress induced by cisplatin caused various kidney pathologies and increased serum BUN and creatinine levels, indicating kidney dysfunction.

The present study demonstrates that pre-treatment with combined curcumin and  $\alpha$ -tocopherol could reduce gene expressions of NADPH oxidase, MDA levels, BUN and creatinine levels, and could increase activities of SOD and catalase. We suggest that curcumin and  $\alpha$ -tocopherol acted as antioxidant to scavenge ROS, eventually abrogating oxidative stress and improve kidney pathology and function. Natural products-derived antioxidants are gradually high interest to investigate for reduce the side effects of cisplatin in various organs. Alpha lipoic acid plays a renoprotective role on cisplatin-induced nephrotoxicity through antioxidant and anti-apoptotic mechanisms in mice [1]. Chrysin or 5, 7-dihydroxyflavone which is present in honey and various plants revealed the protective effect against cisplatin-induced colon toxicity relating with attenuation of oxidative stress and apoptosis in male albino rats [18]. Caffeic acid phenethyl ester which is a flavonoid found in the honeybee propolis extract elicited partial protection against cisplatin-associated biochemical and histopathological alterations in the rabbit [19]. Powder of grain of *Triticum sativum*, Lisosan G, also attenuated the oxidative stress and the preservation in antioxidant enzymes induced by cisplatin in renal and testicular tissues of male Wistar rats [20]. Additionally, cisplatin revealed not significantly

increased p38-MAPK gene expression indicating that occurred cell death may not be associated apoptotic pathway. We suggested that necrotic pathway may be involved.

## CONCLUSION

The present study has shown that oxidative stress plays an important role in nephrotoxicity induced by cisplatin. Pre-treatment with combined curcumin and  $\alpha$ -tocopherol ameliorates cisplatin-induced nephrotoxicity via possible abrogation of oxidative stress. It also decreases gene expression of NADPH oxidase which is considered as one of the important endogenous sources of  $O_2^{\cdot-}$ . Moreover, the antioxidant effect is responsible for the reduction of lipid peroxidation and increased activities of SOD and catalase, resulting in improvement of kidney function and histopathology. Therefore, pre-treatment with combined curcumin and  $\alpha$ -tocopherol may be useful for patients undergoing chemotherapy by cisplatin but further *in vivo* studies and clinical trials are required to ascertain this.

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