

Original Article



# Possible role of glutathione peroxidase-3 and organic cation transporter-2 gene expressions in mediating protective effects of curcumin on cisplatin-induced nephrotoxicity in rats

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## Abstract

**Introduction:** Cisplatin induced nephrotoxicity may limit the clinical use of this drug. The aim of this study was to investigate the mechanism of the possible renoprotective effect of curcumin in cisplatin nephrotoxicity.

**Methods:** Thirty male Wistar rats were randomly divided into five groups: 1- control (0.5ml normal saline ip, daily for 10 constitutive days); 2- cisplatin (10mg/kg ip, single dose at the first day); 3- cisplatin + curcumin (10mg/kg ip, dissolved in 5% DMSO, daily for 10 constitutive days); 4- cisplatin + vehicle (5% DMSO, 0.3ml ip) and 5- curcumin (10mg/kg ip). At the end of the study, urine and blood samples were obtained for biochemical (BUN, creatinine, sodium and potassium) analysis. The right kidneys were kept in 10% formalin for H&E and TUNEL staining, and the left kidneys were used for type 2 organic cation transporter (OCT2) and type 3 glutathione peroxidase (GPx3) gene expression and malondialdehyde measurements.

**Results:** Cisplatin significantly increased serum creatinine, BUN, potassium and kidney lipid peroxidation. However, the effect of cisplatin on Gpx3 and OCT2 gene expression was not statistically significant. Curcumin treatment decreased serum creatinine, BUN, and kidney lipid peroxidation, but increased GPx3 and OCT2 gene expression. Moreover, curcumin significantly reversed the cisplatin-induced kidney tissue injury and decreased apoptosis.

**Conclusion:** It is concluded that the ameliorative effect of curcumin in cisplatin nephrotoxicity was assumed to be due to antioxidant effect of this agent. The role of curcumin in mediating uptake of cisplatin is still unclear.

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## Keywords:

Cisplatin;  
Glutathione peroxidase;  
Organic cation transporter;  
Curcumin;  
Nephrotoxicity

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## Introduction

Cisplatin (cis-diamminedichloroplatinum II) is one of the most widely used and potent chemotherapy drugs (Abdollahzade Fard et al., 2013; Pabla and Dong, 2008). It is used to treat a wide variety of cancers including testicular, ovarian, cervical, head and neck tumors, and small cell carcinoma (Reck et al., 2010). The mechanism of cisplatin anticancer action is still not well known. However, the prevailing opinion is that cisplatin is bonded with DNA and leads to cross-linking inside and outside the chain in DNA (Ciccarelli et al., 1985). The cross-binding causes defects in DNA and inhibits its duplication and replication (Reck et al., 2010). Cisplatin is a potent toxic agent for the cell, and renal toxicity is one of the most important side effects of this drug in clinical and laboratory models. In 50% of cases, this complication can be progressive (Pabla and Dong, 2008). Therefore, cisplatin induced nephrotoxicity may limit the clinical use of this drug (Zhou et al., 2004). Renal toxicity is induced by cisplatin due to proximal tubular damage and glomerular filtration reduction. This alteration has been revealed by evaluation of both apoptosis and necrosis (Abdollahzade Fard et al., 2013; Pabla and Dong, 2008).

In the kidney, organic cation transporters (OCTs) are involved in the cisplatin uptake (Filipski et al., 2008; Ludwig et al., 2004), and also in the transport of several cationic compounds from the basolateral to the apical membrane in renal tubule cells. Ludwig et al. showed that cisplatin, when applied to the lateral membrane, produces more toxicity in cells compared with the application to the apical side of the cell. They suggested that toxic damage to tubular cells was related to OCTs (Ludwig et al., 2004; Yonezawa et al., 2005). The three isoforms of organic cations are expressed in the proximal tubule of the kidney and are mostly on the basolateral side. The results of the study by Ciarimboli et al. (2005) provided evidence suggesting that type 2 organic cation transporter (OCT2) is the major transporter for cisplatin uptake in the kidney.

Reactive oxygen species (ROS) production (Nieskens et al., 2018) and kidney micro-vascular contraction are responsible for cisplatin-induced renal injury. Therefore, oxidative stress is known to be a major contributor to cisplatin-induced nephrotoxicity (Pabla and Dong, 2008). Researchers have found that increase in lipid peroxidation markers such as

malondialdehyde (MDA) and 4-hydroxy-2-nonenol is closely related to the severity of renal toxicity caused by cisplatin (Chirino et al., 2008). Glutathione peroxidases (GPx) are primary antioxidant enzymes that collect hydrogen peroxide or organic hydroperoxidases. This action protects the biological membranes and cellular compositions against oxidative stress (Brigelius-Flohé, 1999). GPx isoforms are different in terms of their initial structure and location. Extracellular glutathione peroxidase (pGPx or GPx3) is expressed mainly in the kidney, which is then released into the bloodstream (Brigelius-Flohé, 1999; Brigelius-Flohé and Flohé, 2003).

Curcumin is a substance extracted from turmeric or *Curcuma longa*, which has antioxidant effect due to the phenolic group in its molecular structure. Curcumin is widely used throughout the world as a herbal remedy (Khalaji et al., 2018). The results of a study by Palipoch et al. (2013) showed that the combination of curcumin and alpha tocopherol reduced the nephrotoxicity of cisplatin by inhibiting NADPH oxidase and subsequently improving the renal function and tissue damage. Another study by Ugur et al. (2015) revealed that curcumin potentially had protective effects on cisplatin induced renal damage and could increase the cisplatin treatment window. Their studies showed that this protective effect of curcumin could be resulted by modifying the organic cation as well as organic anion transporters (OCTs and OATs) and membrane proteins from the multi-drug resistance protein (MRP) family (Ulu et al., 2016).

The main objective of this study was to evaluate the effect of curcumin on kidney transport and antioxidant system in cisplatin-induced nephrotoxicity. Considering the potential role of the organic cation transporter and glutathione peroxidase, it emphasizes the effect of curcumin on OCT2 and type3 glutathione peroxidase (GPx3) gene expression in cisplatin nephrotoxicity.

## Materials and methods

### Study design

Thirty male 8-week-old Wistar rats (weighing 200-250g) were purchased from the animal house of the Urmia University of Medical Sciences. The animals were kept under standard conditions with proper ventilation, temperature (22±2°C) and 12:12h light-

dark cycles. The animals had free access to water and food. The entire process was carried out in accordance with the instructions of the Ethics Committee of the Urmia University of Medical Sciences (ethical code, IR.UMSU.REC.1396.399) and the Declaration of Helsinki.

After a week of adaptation, the rats were randomly divided into five groups (6 per each group): group1: control (0.5ml normal saline ip, daily for 10 constitutive days); group2: cisplatin (10mg/kg ip, single dose at the first day [Noruzi and Zare, 2011]); group3: cisplatin + curcumin (10mg/kg ip, dissolved in 5% DMSO diluted in normal saline, daily for 10 constitutive days); group4: cisplatin + vehicle (5% DMSO, 0.3ml ip) and group5: curcumin (10mg/kg ip [Noorafshan et al., 2011]). Both cisplatin and curcumin were purchased from the Merck Company (CAS 1566-7-1, Millipore Sigma, Germany, CAS 458-37-7, Merck, Germany, respectively).

At the end of the study (the 10<sup>th</sup> day), the animals were placed in a metabolic cage for 6h (8am- 2pm) and urine samples were collected. The animals were anesthetized with ether and then the blood sample was obtained by cardiac puncture. Serum was collected after centrifugation (10min at 3000rpm) and kept frozen until assayed for biochemical analysis.

The right kidneys were kept in 10% formalin for histological studies while the left kidneys (n=5) were kept at -80°C for gene expression tests.

### Biochemical analysis

Serum and urine BUN and creatinine (Cr) were measured by an autoanalyzer. Sodium (Na<sup>+</sup>) and potassium (K<sup>+</sup>) were also measured with a flame photometer. To measure lipid peroxidation, the MDA amount of the homogenized kidney tissue was measured. Malondialdehyde-thiobarbituric (MDA-TBA) acid reactive substance levels were determined following the manufacturer's protocol (Zist Koshan TBARS Assay kit, Iran).

### Total RNA extraction and quantitative RT-PCR

Total RNA was isolated from each frozen kidney tissue sample using a kit (GeneAll; Cat no. 305-101), in accordance with the manufacturer's instruction. RNA concentration was determined spectrophotometrically. The sample supernatant was checked for absorbance at 280 and 260nm and determined by TAE (50mM Tris-base, 30mM sodium

acetate, 3mM EDTA) agarose gel electrophoresis. First-strand cDNA was synthesized using a high-capacity cDNA reverse transcription kit (Gene All, South Korea). Reverse transcriptase was measured using Hyper-script™ reverse transcriptase (Gene All, South Korea). Quantitative RT-PCR was performed using an amplification reagent kit (Ampliqon, Denmark) by the XP-cycler instrument (TCXPD, Bioer, USA) with OCT2 (NCBI Reference Sequence: NC\_005119.4), GPx3 (NCBI Reference Sequence: AC\_000078.1) and the rats' glyceraldehyde-3-phosphate dehydrogenase (GAPDH) primers. To amplify the cDNA, the 5' and 3' primer sequences (forward and reverse) of OCT2 and GPx3 genes designed by Primer3 available at <http://primer3.ut.ee>. Moreover, all the primers were verified by the Gene Runner software (Table 1).

The primers (forward and reverse) were also synthesized to amplify the cDNA encoding GAPDH as a house-keeping gene. The sequences of the related primers are also provided in Table 1. Real-time PCR master mix green kit (Ampliqon, Denmark) was used for the real-time quantification of the target genes in a total volume of 25µl, in accordance with the manufacturer's instructions. Furthermore, expression of the mentioned genes was analyzed using an iQ5 real-time PCR detection system (Bio-Rad, CA, USA). The reactions were then prepared for 10min at 95°C in a 48-well optical plate followed by 40 cycles of 60s, each at 58°C. The melting curve was recorded to confirm the specificity of the amplification reactions. Each sample was replicated three times. The value of the threshold cycle (Ct) was the same as that of the corresponding mean. The relative fold expression of each mRNA was calculated by conducting the  $2^{-\Delta\Delta Ct}$  method ( $-\Delta\Delta Ct = \Delta Ct$  test sample  $-\Delta Ct$  calibrator sample), with Ct being the threshold cycle. Next, the calculated levels were normalized to GAPDH and were then analyzed to observe any significant differences using the one-way ANOVA test.

### Histopathological analysis

The paraffin-embedded right kidney tissue samples were cut into 5µm thick sections and then used for hematoxylin-eosin (H&E) and TUNEL (terminal deoxynucleotidyl transferase dUTP nick end labeling) staining. Deparaffinization of sections and H&E staining was done according to routine and standard

**Table 1:** Sequences of primers used to evaluate expressions of GAPDH, OCT2 and GPx3.

Target gene	Primer sequence		Product size (bp)
GAPDH	Forward	5'-AGAACATCATCCCTGCATCC-3'	207
	Reverse	5'-GGTCCTCAGTGTAGCCCAAG-3'	
OCT2	Forward	5'-CCTTTACTGTTGGGCTCCTG-3'	125
	Reverse	5'-CTCCGGTATGCACCAGAAAT-3'	
GPx3	Forward	5'-CAGAACTCCTGGGCTCACCT-3'	151
	Reverse	5'-TCCATCTTGACGTTGCTGAC-3'	

GAPDH: Glyceraldehyde-3-phosphate dehydrogenase; OCT2: Type 2 organic cation transporter; GPx3: Type 3 glutathione peroxidase.

**Table 2:** Plasma BUN, creatinine, sodium and potassium concentrations of each group.

Group	BUN (mg/dl)	Creatinine (mg/dl)	Na (meq/l)	K (meq/l)
Control	20.40 ± 2.35	0.59 ± 0.03	145.98 ± 1.19	4.88 ± 0.73
Cisplatin	41.06 ± 2.01*	1.28 ± 0.18*	143.63 ± 0.77	5.67 ± 0.27*
Cis + Veh	40.77 ± 4.75*	1.23 ± 0.31*	143.97 ± 1.00	5.61 ± 0.11*
Cis+ Cur	22.95 ± 3.71†	0.61 ± 0.05†	145.45 ± 2.07	5.82 ± 0.36*
Curcumin	21.69 ± 1.96	0.55 ± 0.04	145.82 ± 1.31	6.20 ± 0.32*

Data show as mean±SD, values are significantly different (\*compared to control and †compared to cisplatin group;  $P < 0.01$ ) within the same column. Cis=cisplatin, Veh=vehicle, Cur=curcumin.

procedures.

DNA fragmentation was labeled *in situ* using an *in situ* cell death detection kit (Roche Diagnostics, Mannheim, Germany). The paraffin-embedded tissues were cut into 5µm thick sections and after deparaffinization and dehydration, digested with proteinase K and treated according to the protocol provided with the kit. According to TUNEL-reaction bases, the labeled nucleotides were catalytically added to 3'-OH ends of DNA by terminal deoxynucleotidyl trans-ferase in a template-independent manner. The sections were then reacted with anti-fluorescein antibody conjugated with horse radish peroxidase as a reporter enzyme. The *in situ* cell death detection kit provided diaminobenzidine to produce a brown reaction product that marked the nuclei of the apoptotic cells. The sections were counterstained with hematoxylin. The apoptosis positive cells were evaluated by a light microscope. The number of the apoptotic cells in each studied groups was calculated by counting the number of the TUNEL positive cells in 10 field of randomly selected slides at 400× magnification. The counts were averaged and expressed as mean number of TUNEL

positive cells.

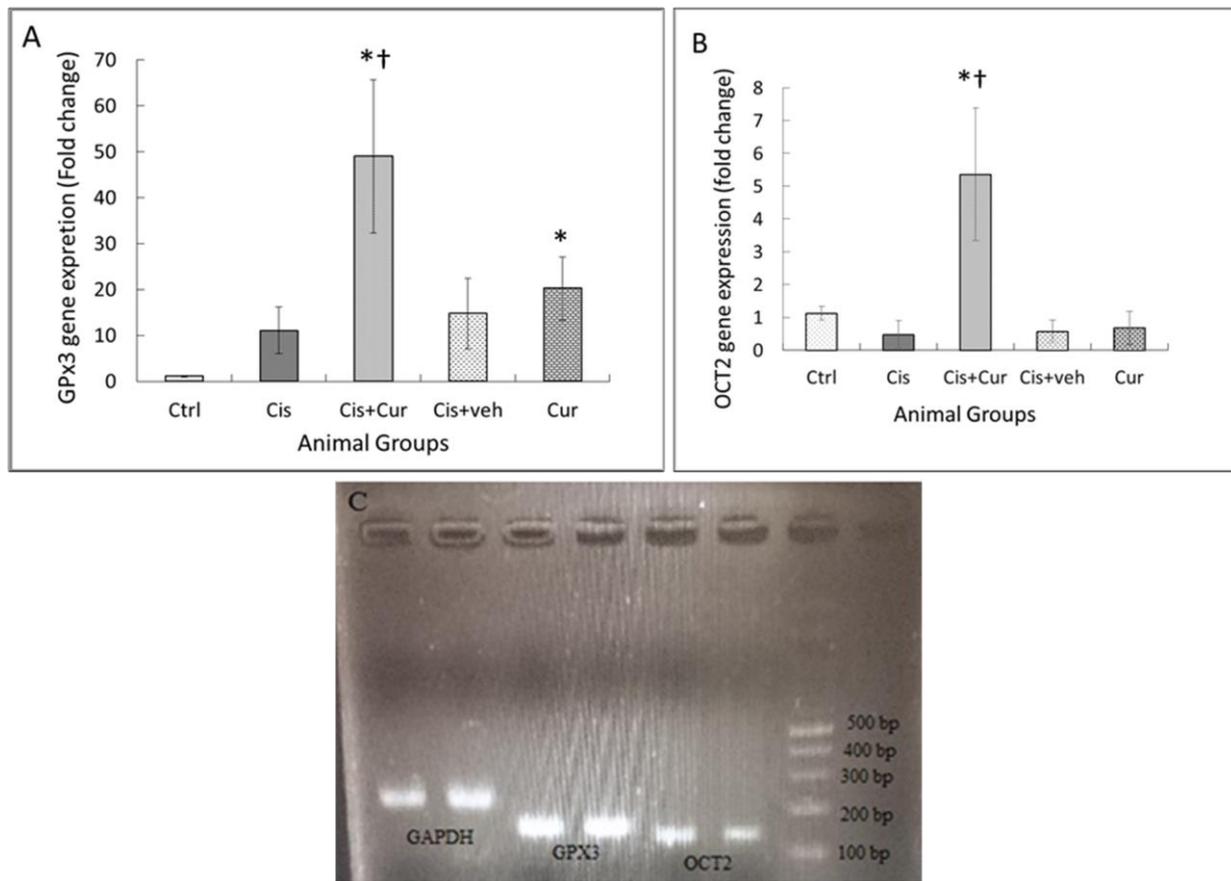
### Statistical analysis

The Kolmogorov–Smirnov test was used to test the normality of the data. The statistical differences between the groups were tested by conducting one-way ANOVA with post-hoc Tukey test. The data obtained from each test were presented as mean±SD and  $P < 0.05$  was considered as statistically significant.

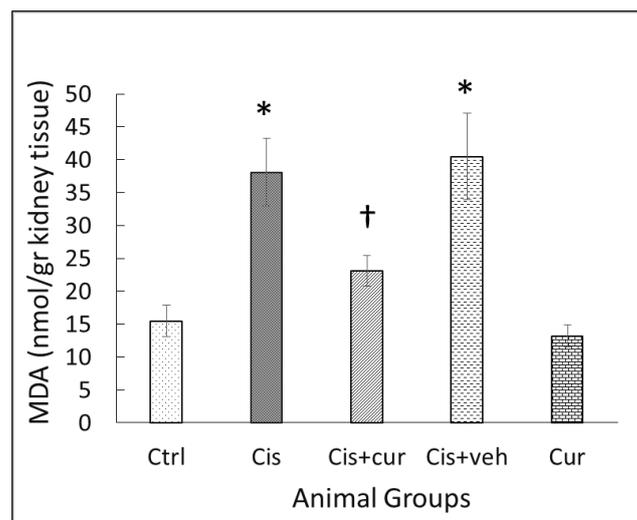
## Results

### Effects of curcumin on serum BUN, Cr, K<sup>+</sup> and Na<sup>+</sup>

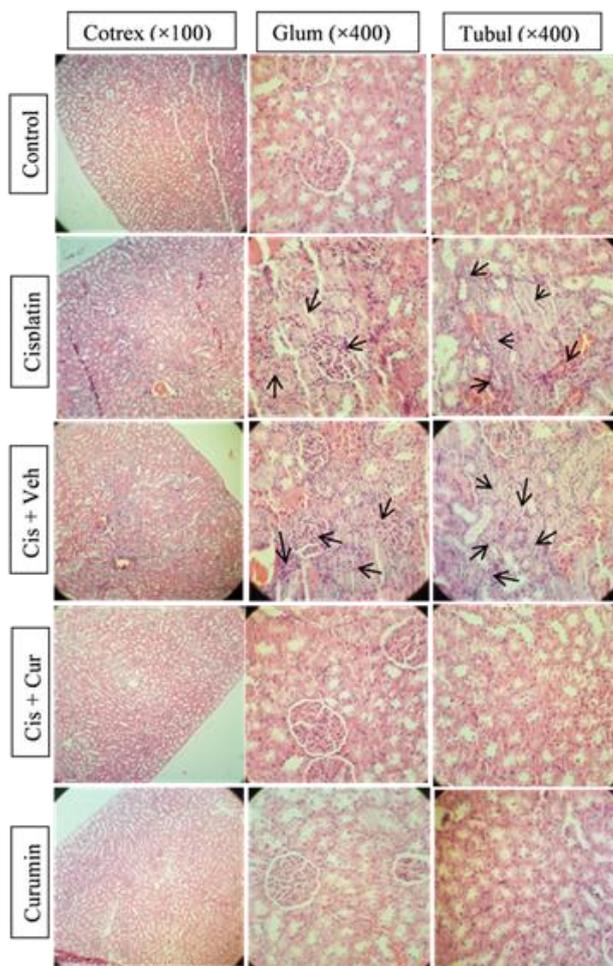
As shown in Table 2, a significant increase could be noted in the serum BUN, Cr and K<sup>+</sup> levels of the cisplatin-induced nephrotoxic rats in comparison with the control ( $P < 0.01$ ,  $P < 0.01$  and  $P < 0.05$ , respectively). However, there was no significant difference in the serum concentration of Na<sup>+</sup> between the groups. Moreover, the administration of curcumin in the cisplatin nephrotoxicity reduced serum BUN and Cr, which were statistically significant ( $P < 0.01$  and  $P < 0.01$ , respectively). Curcumin alone and following the cisplatin increased serum K<sup>+</sup> in comparison with the control group ( $P < 0.01$ ).



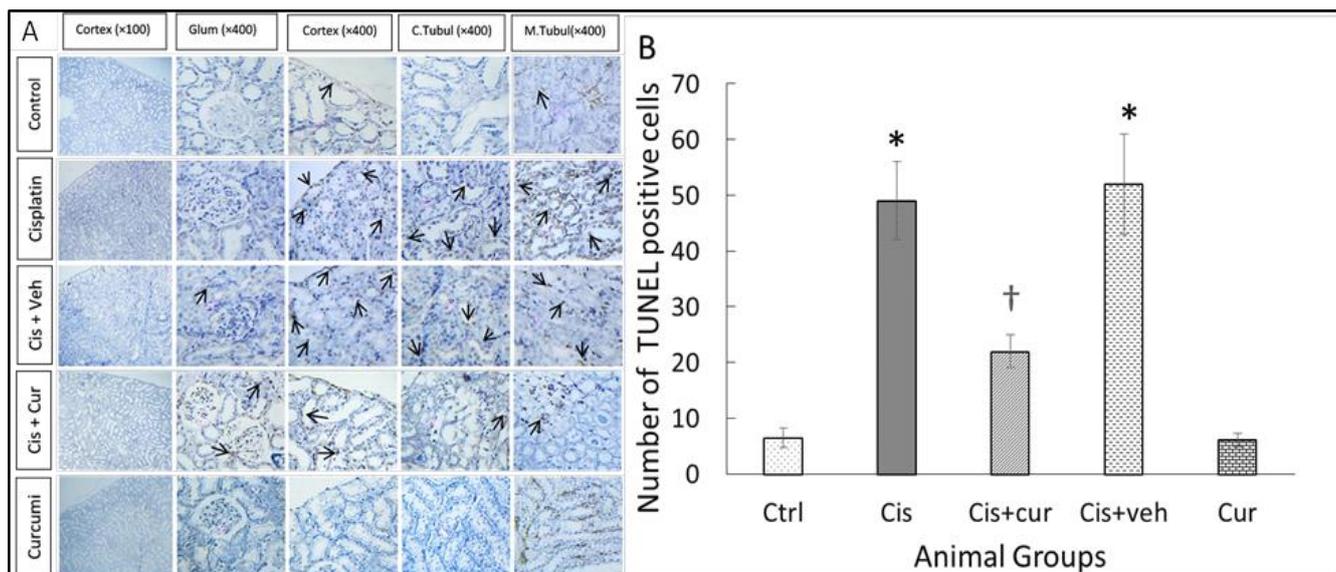
**Fig.1.** A) GPx3 gene expression; B) OCT2 gene expression; C) Agarose gel electrophoresis of reference (Glyceraldehyde-3-phosphate dehydrogenase = GAPDH) and target genes. Data are shown as mean±SD. Values are significantly different (<sup>†</sup>compared to the control and <sup>\*</sup>compared to the cisplatin group,  $P<0.05$ ). Ctrl=control, Cis=cisplatin, Veh=vehicle and Cur=curcumin.



**Fig.2.** Kidney tissue lipid peroxidation (MDA content). Data are shown as mean±SD. Values are significantly different (<sup>†</sup>compared to the control and <sup>\*</sup>compared to the cisplatin group,  $P<0.01$ ). Ctrl=control, Cis=cisplatin, Veh=vehicle and Cur=curcumin.



**Fig.3.** Photomicrographs of renal tissues H&E staining. Curcumin attenuates the cisplatin-induced histopathological damage. Arrows show tubular damages. Glum=glomeruli, Tubul= tubule, Cis=cisplatin, Veh=vehicle and Cur=curcumin.



**Fig.4.** A) Photomicrographs of renal tissues TUNEL staining by the in situ cell death detection kit. Arrows show TUNEL positive cells. B) Number of apoptotic cells. Data are show shown as mean±SD. Values are significantly different (\*compared to the control and †compared to the cisplatin group,  $P<0.001$ ). Curcumin decreases cell apoptosis. Glum=glomeruli, C.Tubul= cortical tubule, M=medullary, Cis=cisplatin, Veh=vehicle and Cur=curcumin.

### Effects of curcumin on GPx3 and OCT2 gene expression

Cisplatin increased GPx3 gene expression in comparison with the healthy control group, but the change was not statistically significant ( $P=0.38$ ). The curcumin treatment significantly increased GPx3 gene expression in comparison with the control and cisplatin-induced nephrotoxic animals ( $P<0.001$ ). Similarly, curcumin alone, without the presence of cisplatin, also significantly increased GPx3 gene expression in comparison with the healthy control group ( $P<0.05$ , Fig. 1A).

As shown in Figure 1B, at the end of the 10-day study period, relative expression of OCT2 gene was  $1.13\pm 0.21$  and  $0.48\pm 0.14$  for the healthy control and cisplatin-induced nephrotoxic animals, respectively, although the change was not statistically significant ( $P=0.78$ ). The administration of curcumin in the cisplatin-induced nephrotoxic animals significantly increased OCT2 gene expression in comparison with the control and cisplatin treated rats ( $P<0.001$ ).

### Effects of curcumin on kidney tissue lipid peroxidation

Kidney tissue lipid peroxidation, as shown with MDA content in the cisplatin nephrotoxic rats, was significantly higher in comparison to the control group ( $P<0.01$ ). However, curcumin administration significantly reversed this increase ( $P<0.01$ , Fig. 2).

### Effects of curcumin on morphological change and cell apoptosis in cisplatin-induced nephrotoxicity

As shown in Figure 3, the histopathology (H&E) analysis demonstrated that the cisplatin-induced nephrotoxicity led to tubular injury characterized by pronounced renal tubular detachment, tubular cell necrosis and loss of brush border. These tissue injuries were mitigated with the curcumin treatment.

Moreover, cisplatin significantly increased cell apoptosis (TUNEL positive cell) in glomerulus, and particularly in proximal tubules, in comparison with the healthy control group (number of positive cells:  $6.5\pm 1.8$  vs  $49\pm 7$ ,  $P<0.001$ ). However, curcumin significantly decreased apoptosis in the cisplatin-induced nephrotoxic rats ( $6.1\pm 1.2$  vs  $49\pm 7$ ,  $P<0.001$ , Fig. 4).

As shown in Figure 3 and 4, the aforementioned pathological changes were significantly alleviated in

the curcumin administered cisplatin-induced nephrotoxic rats.

## Discussion

Cisplatin accumulation in renal cells causes nephrotoxicity. Multiple mechanisms, such as DNA damage, oxidative stress and apoptosis contribute to the pathogenesis of cisplatin nephrotoxicity (Pabla and Dong, 2008). In line with previous studies, the results of the present study also showed that cisplatin induced dysfunctions in the kidney and treatment with curcumin reduced cisplatin-induced renal injury, as assessed by functional (serum BUN and Cr) and histological examinations. An interesting finding of this study was that curcumin did not reduce serum potassium. Although curcumin did not reduce serum potassium level, it exerted a renal protective effect. Therefore, this increase in potassium probably does not have a renal origin. The reason is that serum potassium was expected to decrease with improved kidney tubules, which causes the finding to be still ambiguous.

The precise mechanisms of ameliorative effects of curcumin in cisplatin-induced nephrotoxicity are not fully comprehended. Previous studies have demonstrated that curcumin down-regulates the transcription factor NF $\kappa$ B and suppresses various inflammatory mediators (Nieskens et al., 2018). Numerous antioxidants including curcumin have been used against nephrotoxicity of cisplatin (Palipoch et al., 2013; Ugur et al., 2015). However, there are controversies about the effect of curcumin. For example, oral pretreatment with the natural antioxidant curcumin (8mg/kg, twice) before cisplatin treatment was ineffective in protecting rats from nephrotoxicity (Antunes et al., 2001). Therefore, the effects of curcumin and its mechanism of action are still unclear.

It is evident that cisplatin nephrotoxicity occurs as a result of oxidative stress (Chirino and Pedraza-Chaverri, 2009; Yonezawa et al., 2005) that leads to change in the activity of GPx, depletion of glutathione and enhancement of MDA production in renal tissues (Ali et al., 2007; Yonezawa et al., 2005). All these findings are consistent with the results obtained from the present study. Accordingly, the results of the present study showed that curcumin increased GPx3 gene expression but decreased lipid peroxidation (Malondialdehyde) of the kidney tissue following the

cisplatin-induced nephrotoxicity. Our results about MDA levels are consistent with the previous studies (Antunes et al., 2001; Sahin et al., 2014). According to our searches, we did not find studies on GPx3 gene expression in cisplatin nephrotoxicity. Therefore, the results of the present study suggested that curcumin not only enhanced GPx activity (Song et al., 2015), but also increased its gene expression. Finally, these results confirmed that curcumin enhanced the kidney antioxidant system.

The exact mechanisms of the cisplatin-induced changes in renal glutathione concentrations are not completely elucidated. Results of Bräunlich et al. (1997) study showed that the kidney damage caused by cisplatin was not associated with decrease in renal glutathione. Tian et al. (1997) suggested that under oxidative stress conditions or oscillations in the glutathione level, positive regulation may occur in the biosynthesis of glutathione, contributing to the increase in its intracellular contents. In consistent with Tian et al. the result of the present study also showed that in presence of cisplatin, there was an increase in GPx3 gene expression, although was not significant. Increased concentrations of thiol-containing molecules, including glutathione are known to induce resistance against cisplatin, and cisplatin is detoxified by glutathione through adduct formation (Rudin et al., 2003; Siddik, 2003).

It is obvious that the transport story for platinating agents has yet to be clearly elucidated; however, some transporters may be cell type dependent and each cell type may have multiple transporters, further complicating the situation. The results of Ciarimboli et al. (2005) provided evidence suggesting that OCT2 is the major transporters for cisplatin uptake in the kidney. However, there are many conflicting results about the mechanism of cisplatin influx/efflux and the changes of transporters such as OCT, copper transporters and MRP in cisplatin induced nephrotoxicity (Rudin et al., 2003; Schrenk et al., 2001); therefore, there is no comprehensive agreement on this issue.

The results of the present study showed that cisplatin reduced the expression of the OCT2 gene, although it was not statistically significant. Decrease in the OCT2 expression was observed in some previous studies (Ulu et al., 2016; Yonezawa et al., 2005), suggesting that Nrf2-mediated signaling reduces expression of OCT2 in the rat cortical tissue and Madin-Darby

canine kidney cells (Nieskens et al., 2018; Schrenk et al., 2001; Shen et al., 2000). The results of most recent studies by Nieskens et al. (2018) confirmed these changes in the level of gene expression. The result of the present study also showed that curcumin treatment increased OCT2 gene expression, which finding was not expected. This result is consistent with the western blot analysis of OCT2 protein performed by Ulu et al. (2016). Therefore, the results are similar at the level of gene expression and protein expression.

The results of the study by Kyung Song et al. (2015) contradicted the results of the present study as they suggested that OCT-2 protein expression in the kidney tissue slightly increased after cisplatin treatment but decreased by tetrahydrocurcumin treatment; however, the differences were not statistically significant. Considering the variation of cisplatin uptake mechanisms by cells, according to the results of present study, it is unclear whether curcumin has an effect of another transport pathway. Regarding the results of the effect of curcumin on the expression of GPx3 and OCT2 genes, it was concluded that ameliorative effects of curcumin on cisplatin-induced nephrotoxicity were due to its effect on strengthening the kidney antioxidant system and not due to the effect on the cisplatin transport system in the kidney.

Cisplatin causes renal tubule damage through various mechanisms, such as free radicals, inflammation and apoptosis (Sheth et al., 2017). In the present study, similar results were observed. According to the previous studies, nephrotoxicity related to cisplatin treatment is due to the production of ROS. ROS are only thought to mediate the apoptosis pathway, and are not involved in the necrotic death pathway (Lieberthal et al., 1996). Curcumin, due to its anti-oxidant effect, reduces kidney tubular injury and cell nucleus damage as it is characterized by reduction of apoptotic cells.

## Conclusion

In this study, we investigated the effect of curcumin on two genes involved in cisplatin-induced nephrotoxicity. The role of curcumin on the cisplatin transport system in cisplatin nephrotoxicity is still unclear. In conclusion, the ameliorative effect of curcumin in this model of nephrotoxicity was assumed to be due to antioxidant effect of this agent.

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## Conflict of interest

The authors declare that they have no conflict of interest.

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