*PREVENTIVE EFFECT OF *THEA SINENSIS* MELANIN AGAINST CISPLATIN-INDUCED NEPHROTOXICITY IN MICE

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ABSTRACT:

A fundamental goal of cancer treatment is to enhance the therapeutic index of cancer chemotherapy, while toxicity to the dose-limiting normal cells remains minimized. A major limiting factor in successful cancer therapy is the ability of the tumor to develop resistance to the drugs used for treatment. A second fundamental problem faced by the oncologist treating patients with chemotherapy is the toxic effects of the drugs to the normal tissues. Cisplatin is one of the major therapeutic compounds in the treatment of gynecological cancers. Its activity is greatly restricted by bioavailability and toxicity. Cisplatin acts on cancer cells by releasing free radicals, which at the same time damages liver and especially renal functions. Protection of normal cells from free radical attack during therapy is the determining factor for the prognosis of cancer treatments. The major site of renal injury is the S3 segment of the proximal tubule in the outer strip of the outer medulla of kidney. Uptake of cisplatin inhibits protein synthesis, depletes reduced glutathione, and damages mitochondria. Cisplatin binds and modifies chromosomal DNA which leads to apoptosis. Genes associated with oxidative stress and cell cycle control is differentially expressed. Systematic studies using microarray technology identified genetic markers that are up- or down-regulated upon the treatment of cisplatin. Recently, we have extracted melanin from *Thea sinensis Linn*. Significant properties

concerning melanin chelating and free radical properties were disclosed. *Thea sinensis* melanin (TSM) represents the high molecular part of tea polyphenols . TSM has demonstrated a wide range of biochemical and pharmacological activities in animals including antioxidant, free radical scavenging, and immunomodulatory effects. TSM also revealed unexpected protective activity against various toxic substances such as benzidine, hydrazine, snake venoms, and acetaminophen .

MATERIALS & METHODS:

Thea sinensis leaves were harvested in Miaoli, Taiwan and were identified in the Institute of Chinese Pharmaceutical Sciences, China Medical University. Isolation of TSM was performed and the extracted mixture was filtered and centrifuged at 15,000 g for 30 min to obtain TSM extract. The purified product thus obtained was dissolved in 0.2% NH4OH, and the solution was subjected to repeated precipitations. Physical and chemical characterizations of TSM were performed according to conventional procedures. Adult male ICR mice (30±5 g) were employed for all experiments. Animals were housed under controlled conditions 25 ± 2 °C, with 12 h light/dark cycle, and allowed free access to food and water but fasted overnight before treatment. For the time course experiment, animals were divided into several groups including control group (not receiving any treatment), with experimental groups receiving cisplatin (4 groups: 4 hr, 8 hr, 24 hr, and 5 days). For the TSA protection experiment, animals were divided into several groups including control group (not receiving any treatment), negative control (receiving TSM alone), positive control (receiving only cisplatin), and experimental groups receiving cisplatin and TSM together. Each group consisted of 6 mice. Cisplatin was dissolved in normal saline (pH 7.4) and administered intraperitoneally (i.p.) with a dose of 20 mg/kg. TSM was dissolved in distillate water at pH 7.2 and administered i.p. with doses of 10, 20, 30, or 40 mg/kg 2 h before intoxication. Animals in the time course group were sacrificed by ether anesthesia at the designated time point. Animals in the TSA protection experiment were sacrificed by ether anesthesia 24 h after the cisplatin exposure. Sera were isolated and underwent BUN and

creatinine analysis without delay. Kidneys were removed and snap-frozen in liquid nitrogen for the extraction of total RNA. Formation of lipid peroxide derivatives was evaluated by measuring TBARS . The amount of TBARS produced was expressed as nmol TBARS per milligram of protein using malondialdehyde bis(dimethyl acetal) for calibration. Determination of superoxide dismutase activity in mouse kidney was based on inhibition of nitrite formation in reaction of oxidation of hydroxylammonium with superoxide anion radical. The results were expressed as units of SOD activity calculated per milligram of protein. Total RNA was extracted from kidney tissues employing protocol supplied with TRI-reagent . Real-time PCR analysis was performed using iQ™ SYBR green supermix (Bio-Rad) according to manufacture's instructions with the specific primer pairs for selected genes and primer pairs for ribosomal protein L18 as reference gene (Table 1). Threshold cycle number (Ct) was measured using the iCycler and its associated software (Bio-Rad).

Relative transcript quantities were calculated by the $\Delta\Delta$Ct method

using ribosomal protein L18 as a reference gene amplified from samples. Values for fold-induction varied less than 5% among replicates. The final statistical analysis was performed using a Student's t-test and the minimum level of significance was set at $P < 0.05$.

RESULT:

Extraction and characterization of TSM **---TSM was extracted from tea (Thea sinensis Linn.) and extraction time was diminished to 12 h to avoid excessive oxidation of TSM. The average yield of TSM obtained after purification was 1.9 % (dried weight). The purified preparation of TSM exhibited all the physical and chemical properties common to natural melanin. It was insoluble in organic solvents (ethanol, hexane, acetone, benzene, and chloroform); dissolved only in alkali; precipitated below pH 3 and in alkaline FeCl3; was bleached by H2O2, KMnO4, K2Cr2O7, and NaOCl; and produced a blue color with FeSO4/ferricyanide. The solution of TSM in 0.1 M phosphate buffer (pH 8.0) exhibited strong optical absorbance similar to synthetic melanin. The IR spectrum of TSM**

showed a broad band at 3450 cm-1, attributed to stretching vibrations of -OH and -NH2 groups.

TSM prevented cisplatin-induced nephrotoxicity ---Cisplatin treatment induced renal injury evidenced by the elevation of serum BUN and creatinine (Table 2). Renal injury reached a plateau at 24 hr of injection and was maintained up to 5 days. We selected a time point at 24 h to measure the potential preventive property of TSA. Administration of 40 mg/kg TSM alone did not induce any toxicity. The behavior of the animals in experimental group resembled the control group in movement activity. The animals pretreated with TSM 2 h prior to intoxication showed protection against cisplatin (Table 3). TSM caused a dose-dependent effect against cisplatin challenge, with plasma BUN level being reduced to 74, 14 and 3% and serum creatinine level being reduced to 60, 20, and 6% of positive control when 10, 20, and 30 mg/kg doses were given to animals. The highest dose of TSM (40 mg/kg) completely blocked cisplatin-induced nephrotoxicity. Cisplatin also induced oxidative stress. Pretreatment by TSM significantly decreased cisplatin-induced lipid peroxidation in a dose-dependent manner (Fig. 1). Increased dose of TSM suppressed peroxidation with the highest dose causing full blockage of TBARS. Administration of TSM alone did not produce any significant effect as compared to control (no treatments). Introduction of cisplatin caused an almost 2-fold decrease of SOD activity as compared to control (Fig. 2). Significant restoration of SOD activity was observed when TSM (10-40 mg/kg) was administered 2 h prior to injection of NAPAP. Resumption of SOD activity reached a plateau at higher doses of TSM indicating the ability of TSM to maintain SOD activity at the level of negative control. Administration of TSM alone did not affect SOD activity. This suggests an indirect influence of TSM on SOD activation.

Real time RT-PCR validated molecular markers of cisplatin-induced nephrotoxicity ---Real time RT-PCR was applied to evaluate mRNA levels of marker genes with and without the TSM treatment to investigate the possible mechanism of how tea melanin prevented cisplatin-induced renal injury. Marker genes for cisplatin-induced renal toxicity were selected from previous reports (glutathione-S-transferases (Gstp2), soluble epoxide hydrolase (Ephx1), lipocalin 2 (Lcn2), lysozyme (Lyz), UDP glycosyltransferase

2 (Utg2b), survival motor neuron (Smn1), guanidinoacetate methyltransferase (Gamt), urine retinol binding protein (Rbp4), aminopeptidase N (Apn), and cytochrome P450 (Cyp2d18), ornithine aminotransferase (Oat)). Primers were designed based on the published sequences in GenBank (Table 1). Real time RT-PCR was performed on kidneys from individuals 24 hrs after receiving cisplatin injection and compared to control mice. Cisplatin treatment increased mRNA levels 40-fold for Gstp2, 15-fold for Ephx1, 15-fold for Lcn2, 9-fold for Lyz, 5-fold for Utg2b, 30-fold for Smn1, 30-fold for Gamt, 80-fold for Rbp4, 60-fold for Apn, 60-fold for Cyp2d18, and 100-fold for Oat (Fig. 3). Prior administration of tea melanin restored the expression for all marker genes back to normal levels in a dosage dependent manner. The effective dosage ranged from 30 to 40 mg/kg.

Table 1 Primer Sequences Used For Real Time RT -PCR

Symbol	Forward sequence[a]	Reverse sequence	Length
Gstp2	CACTTCTCTCTGCACAGC	ATCATTCACCATATCCACC	307
Ephx1	CCAAACTCATCTCCTATTCC	ATGTAGCTTAACACAGCTATGC	309
Lcn2	CATCTCTGCTCACTGTCC	GCACATTGTAGCTCTGTACC	300
Lyz	CTTTCACTTCTCAGTCTCC	CAGTCTCAGTTCTCATCC	300
Utg2b	CACCGTAGATGAGACAATGAGC	TTGACCCCAGAGAAAACACC	203
Smn1	TTCAGGACCACCAATAATCC	TGATGACGACACCACTTAGC	322
Gamt	CCTCACCTACTGCAACCTCACG	AGAGCTGGAGCCCACAATCC	291
Rbp4	TCACAGACACTGAAGATCC	TCACATCCTAGACGTTGC	340
Apn	CCCTAATAATAACACGATCC	ACAGTTTCTTCCAGTTGC	317
Cyp2d18	CCCATAGCCATATTCACG	CAGCCCATTCAGTACAACC	231
Oat	AACACTATCTGCAATCTCC	CTGACATTCTCATGATCC	312

[a]All sequences were written in 5' to 3' direction.

Table 2 Time-course experiment to evaluate the toxicity of cisplatin administration

Time	BUN±SEM (mg/dL)	Creatinine±SEM (mg/dL)
Control[a]	19.3±3.1[b]	0.45±0.1
4 hr	23.4±4.2	0.44±0.04
8 hr	28.7±5.3	0.55±0.06
24 hr	35.5±6.3*[c]	0.85±0.08*
5 day	34.1±4.3**	0.6±0.12

[a] Control mice were given saline. Experimental animals received 20 mg/kg, i.p. cisplatin.

[b] Data represent means ± SEM.

[c] Significantly different from the control (*P<0.05; **P<0.01).

Table 3 Preventive effect of TSM on cisplatin-induced renal injury

Treatment	BUN±SEM (mg/dL)	Creatinine±SEM (mg/dL)
C[a]	20.2±3.1[b]	0.41±0.03
NC	19.2±4.2	0.38±0.04
PC	40.4±6**[c]	0.9±0.12*
10	38.4±5.6	0.79±0.1
20	33.5±3.8	0.51±0.09
30	24.5±4.8	0.33±0.07
40	21±4.5	0.36±0.06

[a] C represents control group not receiving any treatment. NC represents negative control, PC represents positive control, a nd numbers indicate doses of TSM pre-treatment (in mg/kg).

[b] Value is expressed as mean ± SEM of 6 mice.

[c] Asterisks depict significant differences between positive control and joint effect of cisplatin and TSM (*P<0.05, **P<0.01).

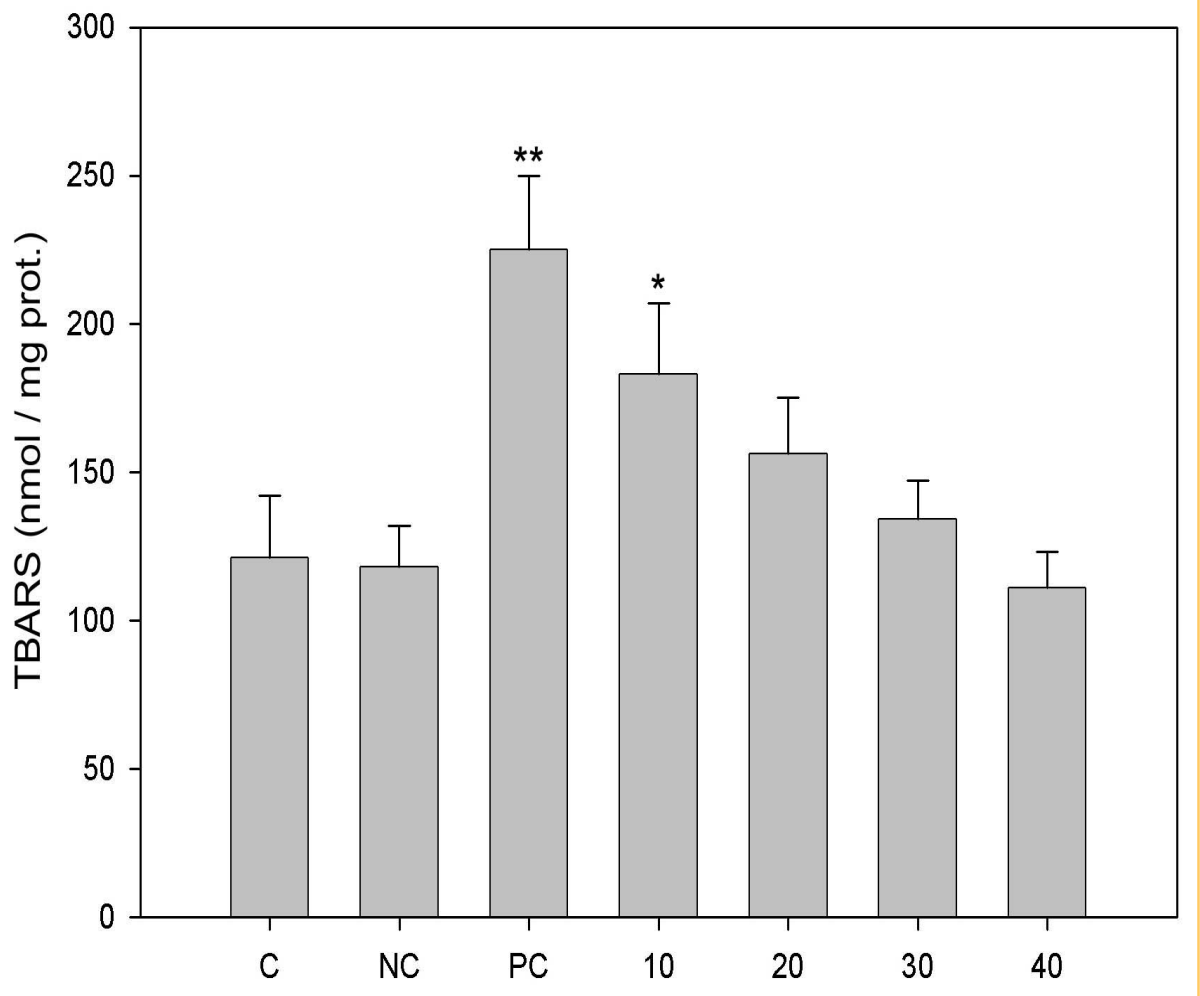

Fig. 1. Effect of various doses of TSM given to mice 2h prior to cisplatin injection (20 mg/kg) on accumulation of TBARS in kidney tissue. Results are expressed as mean ± SEM of 6 experiments. C bar depicts TBARS level in control group not receiving any treatment. NC bar represents negative control, PC bar represents positive control, and numbers indicate doses of TSM (in mg/kg). Asterisks depict significant differences between positive control and joint effect of cisplatin and TSM (*P<0.05, **P<0.01).

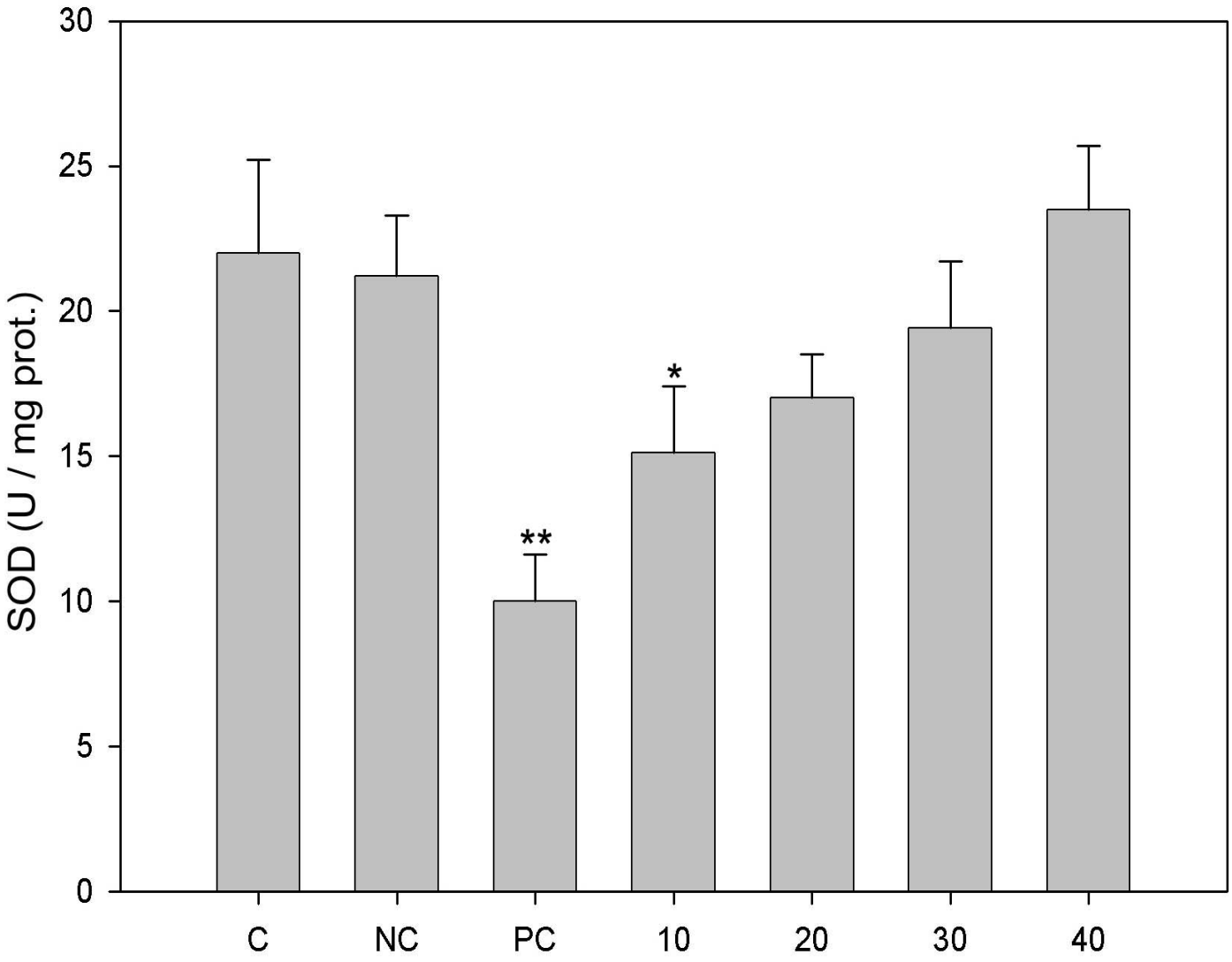

Fig. 2 Effect of protecting doses of TSM on SOD activity in kidney of ICR mice administered with cisplatin. Results are expressed as mean ± SEM of 6 experiments. C bar depicts SOD activity in control group not receiving any treatment. NC bar represents negative control, PC bar represents positive control, and numbers indicate doses of TSM (in mg/kg). Asterisks depict significant differences between positive control and joint effect of cisplatin and TSM (*P<0.05, *P<0.01).

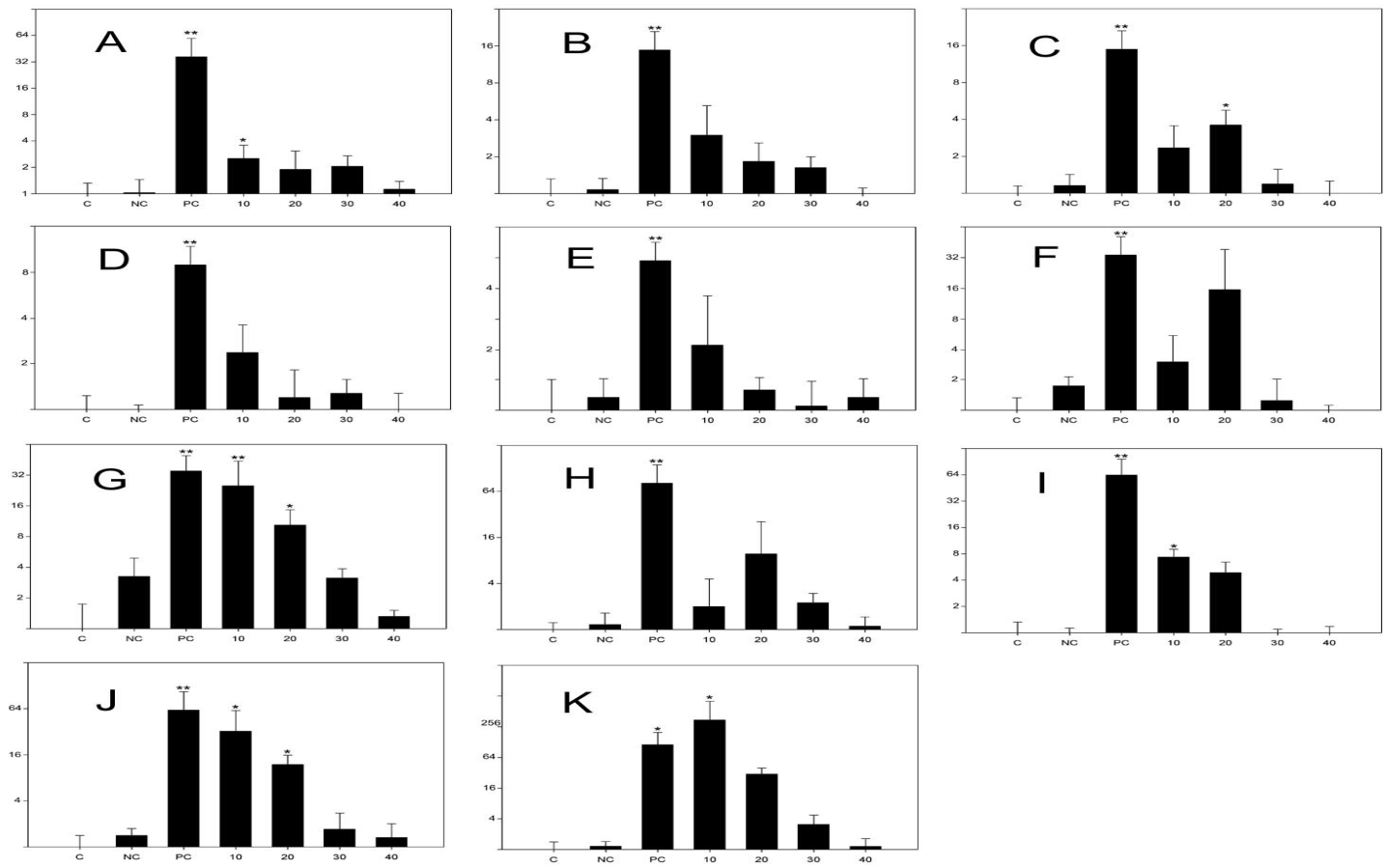

Fig. 3 Effect of Various Doses of TSM on mRNA Levels in Kidney of ICR Mice Poisoned With Cisplatin. Results are expressed as mean ± SEM of 6 experiments. Y-axis depicts fold change in mRNA expression relative to control group not receiving any treatment. **The value equals to $2^{-\Delta\Delta Ct}$.** Relative transcript quantities were calculated by the $\Delta\Delta$Ct method using ribosomal protein L18 as a reference gene amplified from samples. ΔCt is the difference in threshold cycles of the sample mRNAs relative to ribosomal protein L18 mRNA. **$\Delta\Delta$Ct is the difference between ΔCt normal control and ΔCt treated sample. Fold change in mRNA expression was expressed as $2^{-\Delta\Delta Ct}$.** C bar represents control group not receiving any treatment. NC bar represents negative control, PC bar represents positive control, and numbers indicate doses of TSM (in mg/kg). Asterisks depict significant differences between positive control and joint effect of cisplatin and TSM (*P<0.05, **P<0.01). Genes in sequence are (A) Gstp2, (B) Ephx1, (C) Lcn2, (D) Lyz, (E) Utg2b, (F) Smn1, (G) Gamt, (H) Rbp4, (I) Apn, (J) Cyp2d18, and (K) Oat.

CONCLUSION:

The present study reveals that melanin derived from Thea sinensis leaves has protective effects against the renal injury induced by cisplatin. The protective effects were evidenced by a complete blockage of the cisplatin-induced increase in serum BUN, reduction of creatinine to the control level, decrease of TBARS concentration to the control level, restoration of SOD activity, and a complete restoration of the mRNA levels for marker genes tested so far. As it seen from our experiments, the antioxidant activity of TSM was involved in protecting animals against cisplatin-induced nephrototoxicity. Multiple pathways are involved in the cisplatin-induced nephrotoxicity. Marker genes selected in this study were meant to cover the whole spectrum of renal injury, i.e. oxidative stress, apoptosis, cell cycle control, inflammatory response, immune response, renal damage, vasodilation, ischemia, steroid signaling, and renal dysfunction. In addition, cisplatin-induced kidney damage was also ameliorated by TSM treatment as evidenced by the dosage-dependence of SOD activity.

The present work demonstrates that the protective effect of TSM against cisplatin nephrotoxicity is based on a combination of different factors including cytochrome P450 inhibitory activity, antioxidant properties, apoptosis, steroid signaling, inflammatory response, and immuno-stimulation. Such a combination opens the possibility for a comprehensive protection of the kidney against heavy intoxication and may serve as a first approach in developing a natural modulator for chemotherapy. TSM might be considered a new health product that possesses a potential therapeutic value in the prevention of toxic renal injury.