

Thea sinensis melanin prevents cisplatin-induced nephrotoxicity in mice

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Abstract

The preventive effect of *Thea sinensis* melanin (TSM) against cisplatin-induced nephrotoxicity was studied on ICR mice. Animals were given 20 mg/kg i.p. of cisplatin, and TSM was injected i.p. in doses 10–40 mg/kg 2 h before intoxication. The protective effects were evidenced by a complete inhibition of the cisplatin-induced elevation of serum Blood Urea nitrogen (BUN), prevention of oxidative stress, and complete blockade of cisplatin-induced elevation of serum creatinine. TSM by itself, however, did not affect the renal functional parameters, including serum BUN and creatinine. Real-time RT-PCR was applied to quantify mRNA levels of cisplatin-treated mouse kidney compared to normal mouse kidney for selected marker genes. Cisplatin treatment increases mRNA levels 40-fold for glutathione-S-transferases (Gstp2), 15-fold for soluble epoxide hydrolase (Ephx1), 15-fold for lipocalin 2 (Lcn2), 9-fold for lysozyme (Lyz), 5-fold for UDP glycosyltransferase 2 (Utg2b), 30-fold for survival motor neuron (Smn1), 30-fold for guanidinoacetate methyltransferase (Gamt), 80-fold for urine retinol binding protein (Rbp4), 60-fold for aminopeptidase N (Apn), 60-fold for cytochrome P450 (Cyp2d18), and 100-fold for ornithine aminotransferase (Oat). Pre-administration of TSM restored normal expression of marker genes for cisplatin-treated mouse kidneys. TSM by itself, however, did not affect the transcription for marker genes. Results obtained demonstrate that TSM pre-administration can prevent the renal toxic effects of cisplatin.

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1. Introduction

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 (*cis*-dichlorodiammine-platinum (II); *cis*-platinum (II)) 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 damage liver and, especially, renal functions. Protection of normal cells from free radical attack during therapy is the determining factor for the prognosis of cancer treatment (Goldstein and Mayor, 1983; Fillastre and Raguenez-Viotte, 1989).

The major site of renal injury is the S3 segment of the proximal tubule in the outer strip of the outer medulla of kidney (Safirstein et al., 1984). Uptake of cisplatin inhibits

Abbreviations: TSM, *Thea sinensis* melanin; Gstp2, glutathione-S-transferases; GSH, glutathione; Ephx1, epoxide hydrolase; Lcn2, lipocalin 2; Lyz, lysozyme; Utg2b, UDP glycosyltransferase 2; Smn1, survival motor neuron; Gamt, guanidinoacetate methyltransferase; Rbp4, urine retinol binding protein; Apn, aminopeptidase N; Cyp2d18, cytochrome P450; Oat, ornithine aminotransferase; cisplatin, *cis*-dichlorodiammine-platinum (II), *cis*-platinum (II); BUN, blood urea nitrogen; MM, molecular mass; UV, ultraviolet-visible; IR, Infrared; Ct, threshold cycle number.

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protein synthesis, depletes reduced glutathione, and damages mitochondria (Kuhlmann et al., 1997). Cisplatin binds and modifies chromosomal DNA which leads to apoptosis (Fisher, 1994). Genes associated with oxidative stress and cell cycle control are differentially expressed. Systematic studies using microarray technology identified genetic markers that are up- or down-regulated upon the treatment of cisplatin (Huang et al., 2001; Amin et al., 2004; Kramer et al., 2004).

Recently, we have extracted melanin from *Thea sinensis* Linn. (Sava et al., 2001b). Significant properties concerning melanin chelating and free radical properties were disclosed. *T. sinensis* melanin (TSM) represents the high molecular part of tea polyphenols (Sava et al., 2001a). TSM has demonstrated a wide range of biochemical and pharmacological activities in animals including antioxidant, free radical scavenging, and immunomodulatory effects (Hung et al., 2002a,b; Sava et al., 2002). TSM also revealed unexpected protective activity against various toxic substances such as benzidine, hydrazine, snake venoms, and acetaminophen (Hung et al., 2002a, 2003, 2004a,b; Sava et al., 2003).

The purpose of the present work was to examine whether TSM could work against cisplatin-induced nephrotoxicity. The antioxidant properties of TSM were primarily considered as a prerequisite in realization of protective activity. To explore the possible multiple protective effects of TSM, real-time RT-PCR was performed to quantify marker genes associated with a variety of biological functions. Results obtained demonstrate that TSM pre-administration can prevent the multiple toxic effects of cisplatin.

2. Materials and methods

2.1. Materials

Thea sinensis leaves were harvested in Miaoli, Taiwan and were identified in the Institute of Chinese Pharmaceutical Sciences, China Medical University. Cisplatin, EDTA, Tris-HCl, Triton X-100, Sephadex G-75, and molecular size markers were purchased from Sigma Chemical Co. (St. Louis, MO). All other chemicals were of analytical grade or higher from Merck (Darmstadt, Germany).

2.2. Isolation and physico-chemical characterization of TSM

Isolation of TSM was performed according to the previously reported procedure (Sava et al., 2001b) with minor adjustments. Namely, the extraction time was diminished to 12 h avoiding excessive oxidation of TSM. The extracted mixture was filtered and centrifuged at 15,000g for 30 min to obtain TSM extract. This extract was acidified by the addition of 2 N HCl to pH 2.5 and centrifuged at 15,000g for 15 min. The purified product thus obtained was dissolved in 0.2% NH₄OH, and the solution was subjected to repeated precipitations. Four precipitations were employed to sequester TSM from low molecular impurities and to improve its homogeneity. The resultant solutions were filtered through a Nalgene 0.45 μm syringe filter. Finally, TSM was purified on a Sephadex G-75 (the column's dimensions were 1.6 × 40 cm) in a 50 mM phosphate buffer (pH 7.5) at a flow rate of 0.5 mL min⁻¹. Fractions were monitored at 280 nm. To evaluate the molecular mass (MM) of TSM, a Sephadex G-75 column was calibrated

with bovine serum albumin (MM 66,000), carbonic anhydrase (MM 29,000), cytochrome C (MM 12,400), and aprotinin (MM 6500) as size markers.

Physical and chemical characterizations of TSM were performed according to conventional procedures (Nicolaus, 1968; Balentine et al., 1997; Protta, 1998). Ultraviolet–visible (UV) absorption spectra were obtained with a JASCO V-530 UV–visible Spectrophotometer (Jasco Ltd., Great Dunmow, UK). Infrared (IR) spectra were recorded for KBr samples on a Perkin–Elmer spectrometer 1600 FT (Perkin–Elmer Instruments, Norwalk, CT). Solubility in water, aqueous acid, and in common organic solvents; oxidative bleaching by means of KMnO₄, K₂Cr₂O₇, NaOCl, and H₂O₂; and a positive reaction for polyphenols were used as typical tests for melanin.

2.3. Animals and treatment

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 (four groups: 4 h, 8 h, 24 h, and 5 days). For the TSM 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 distilled 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 TSM protection experiment were sacrificed by ether anesthesia 24 h after the cisplatin exposure. Sera were isolated and underwent Blood Urea nitrogen (BUN) and creatinine analysis without delay. Kidneys were removed and snap-frozen in liquid nitrogen for the extraction of total RNA.

2.4. Determination of TBARS

Formation of lipid peroxide derivatives was evaluated by measuring TBARS (Cascio et al., 2000). Briefly, kidney was homogenized in ice-cold 1.15% KCl (w/v); then 0.4 mL of the homogenates was mixed with 1 mL of 0.375% thiobarbituric acid, 15% TCA (w/v), 0.25 N HCl, and 6.8 mM butylated-hydroxytoluene, placed in a boiling water bath for 10 min, removed, and allowed to cool on ice. Absorbance (532 nm) was measured in the supernatants after centrifugation at 3000 rpm for 10 min. The amount of TBARS produced was expressed as nmol TBARS per milligram of protein using malondialdehyde bis(dimethyl acetal) for calibration.

2.5. Superoxide dismutase assay

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 (Eltner and Heupel, 1976). Nitrite was generated in a mixture containing 25 μL xanthine (15 mM), 25 μL hydroxylammonium chloride (10 mM), 250 μL phosphate buffer (65 mM, pH 7.8), 90 μL distilled water, and 100 μL xanthine oxidase (0.1 U/μL). The inhibitory effect of inherent SOD was assayed at 25 °C during 20 min of incubation with 10 μL of brain tissue extracts. Determination of the resulting nitrite was performed on the reaction (20 min at room temperature) with 0.5 mL sulfanilic acid (3.3 mg/mL) and 0.5 mL α-naphthylamine (1 mg/mL). Optical absorbance at 530 nm was measured with Ultrospec III spectrophotometer (Pharmacia, LKB). The results were expressed as units of SOD activity calculated per milligram of protein.

2.6. Real-time reverse transcription polymerase chain reaction

Total RNA was extracted from kidney tissues employing protocol supplied with TRI-reagent (Molecular Research Center, Inc., USA). Quality of RNA was examined by agarose gel electrophoresis and by OD 260/280 ratio (greater than 1.8). Total cellular RNA (2.5 µg per 20 µL reaction) was reverse transcribed using polydT(17) and Superscript RT II (Invitrogen, USA) per manufacturer specified conditions. Control without reverse transcriptase (minus RT) was also generated for each RNA sample.

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) (Morrison et al., 1998). Relative transcript quantities were calculated by the $\Delta\Delta C_t$ method using ribosomal protein L18 as a reference gene amplified from samples. ΔC_t is the difference in threshold cycles of the sample mRNAs relative to ribosomal protein L18 mRNA. $\Delta\Delta C_t$ is the difference between ΔC_t normal control and ΔC_t treated sample. Values for fold-induction varied less than 5% among replicates. Fold change in mRNA expression was expressed as $2^{\Delta\Delta C_t}$.

2.7. Statistical analysis

All data were presented as means \pm SEM. Statistical analysis was performed using a Student's *t*-test. The minimum level of significance was set at $P < 0.05$.

3. Results

3.1. Extraction and characterization of TSM

TSM was extracted from tea (*T. sinensis* Linn.) as previously reported (Sava et al., 2001b) with minor adjustments. In particular, 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). Final separation of TSM using Sephadex G-75 yielded one major (92%) and one minor fraction with molecular masses of 14 ± 3 kDa and 8 ± 3 kDa, respectively. Further study of TSM was carried out on the major fraction.

The purified preparation of TSM exhibited all the physical and chemical properties common to natural melanin previously reported (Paim et al., 1990; Bilinska, 1996). 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. IR-spectroscopy of TSM demonstrated similar structural peculiarities compared to previously studied melanin pigments including synthetic melanin (Safirstein et al., 1984; Hung et al., 2004a). The IR spectrum of TSM showed a broad band at 3450 cm⁻¹, attributed to stretching vibrations of -OH and -NH₂ groups. A strong absorption at 1650 cm⁻¹ was recognized as the vibrations of aromatic C=C or C=O groups. After the acid hydrolysis of TSM the intensity of both bands at 3450 cm⁻¹ and 1650 cm⁻¹ was reduced, a phenomenon caused by the reaction between phenolic and carboxylic groups owing to the formation of lactones (Paim et al., 1990), which likely are involved in various biological effects.

3.2. 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 h of injection and was maintained up to 5 days. We selected a time point at 24 h to measure the potential preventive property of TSM.

We surveyed time interval of TSM injection for protective activity. Intraperitoneal injection of 40 mg/kg TSM was performed at 3 h (-3 h), 2 h (-2 h), and 1 h (-1 h) prior to the injection of cisplatin. TSM injection was also administered simultaneously with cisplatin injection and at 1 h (+1 h), 2 h (+2 h), and 3 h (+3 h) after cisplatin injection. Serum BUN and creatinine levels were measured 24 h after cisplatin injection (Fig. 1). Serum BUN reduced to control level by -2 h, -1 h, 0 h, and +1 h TSM injection. Serum creatinine level, on the other hand, exhibited a broader range of reduction without apparent fluctuation between -3 h and +2 h. Pretreatment of TSM 2 h prior to intoxication was sufficient to prevent cisplatin-induced renal injury.

Table 1
Primer sequences used for real-time RT-PCR

Symbol	Forward sequence ^a	Reverse sequence	Length
Gstp2	CACTTCTCTGACAGC	ATCATTACCATATCCACC	307
Ephx1	CCAACTCATCTCCTATTCC	ATGTAGCTTAACACAGCTATGC	309
Lcn2	CATCTCTGCTCACTGTCC	GCACATTGTAGCTCTGTACC	300
Lyz	CTTCACTTCTCAGTCTCC	CAGTCTCAGTTCTCATCC	300
Utg2b	CACCGTAGATGAGACAATGAGC	TTGACCCAGAGAAAACACC	203
Smn1	TTCAGGACCACCAATAATCC	TGATGACGACACCACTAGC	322
Gamt	CCTCACCTACTGCAACCTCACG	AGAGCTGGAGCCCACAATCC	291
Rbp4	TCACAGACACTGAAGATCC	TCACATCCTAGACGTTGC	340
Apn	CCCTAATAATAACACGATCC	ACAGTTTCTTCCAGTTGC	317
Cyp2d18	CCCATAGCCATATTACG	CAGCCCATTAGTACAACC	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 \pm SEM (mg/dL)	Creatinine \pm SEM (mg/dL)
Control ^a	19.3 \pm 3.1 ^b	0.45 \pm 0.1
4 h	23.4 \pm 4.2	0.44 \pm 0.04
8 h	28.7 \pm 5.3	0.55 \pm 0.06
24 h	35.5 \pm 6.3 ^{*,c}	0.85 \pm 0.08 [*]
5 day	34.1 \pm 4.3 ^{**}	0.6 \pm 0.12

^{*} $P < 0.05$.

^{**} $P < 0.01$.

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

^b Data represent means \pm SEM.

^c Significantly different from the control.

The animals pretreated with TSM 2 h prior to intoxication showed dose-dependent protection against cisplatin (Table 3). 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. TSM caused a dose-dependent effect against cisplatin challenge, with plasma BUN level being reduced 2.0, 6.9, 15.9, and 19.4 mg/dL and serum creatinine level being reduced 0.11, 0.39, 0.57, and 0.54 mg/dL when 10, 20, 30, and 40 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. 2). 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. 3). Significant restoration of SOD activity was

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

Treatment	BUN \pm SEM (mg/dL)	Creatinine \pm SEM (mg/dL)
C ^a	20.2 \pm 3.1 ^b	0.41 \pm 0.03
NC	19.2 \pm 4.2	0.38 \pm 0.04
PC	40.4 \pm 6.0 ^{**,c}	0.90 \pm 0.12 [*]
10	38.4 \pm 5.6	0.79 \pm 0.10
20	33.5 \pm 3.8	0.51 \pm 0.09
30	24.5 \pm 4.8	0.33 \pm 0.07
40	21.0 \pm 4.5	0.36 \pm 0.06

^{*} $P < 0.05$.

^{**} $P < 0.01$.

^a C represents control group not receiving any treatment. NC represents negative control, PC represents positive control, and numbers indicate doses of TSM pretreatment (in mg/kg).

^b Value is expressed as mean \pm SEM of six mice.

^c Asterisks depict significant differences between positive control and joint effect of cisplatin and TSM.

observed when TSM (10–40 mg/kg) was administered 2 h prior to injection of cisplatin. 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.

3.3. Real-time RT-PCR validated molecular markers of cisplatin-induced nephrotoxicity

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

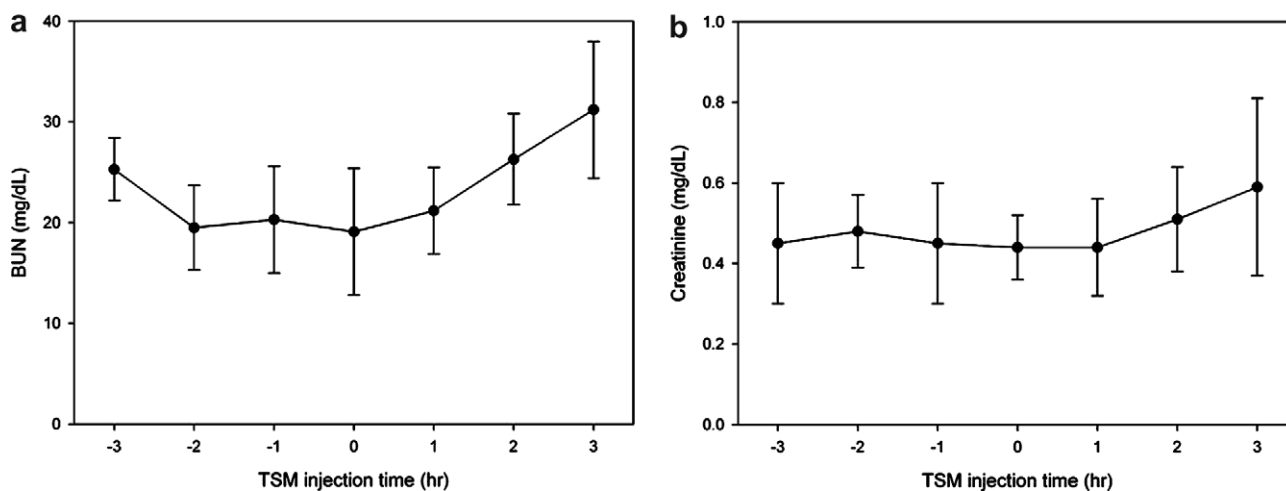


Fig. 1. Protective effect of TSM administered before and after cisplatin injection. Intraperitoneal injection of 40 mg/kg TSM was performed at 3 h (–3 h), 2 h (–2 h), and 1 h (–1 h) prior to the injection of cisplatin. TSM injection was also administered simultaneously with cisplatin injection (0 h) and at 1 h, 2 h, and 3 h after cisplatin injection. Serum BUN (a) and creatinine (b) levels were measured 24 h after cisplatin injection. Results are expressed as means \pm SEM of six experiments.

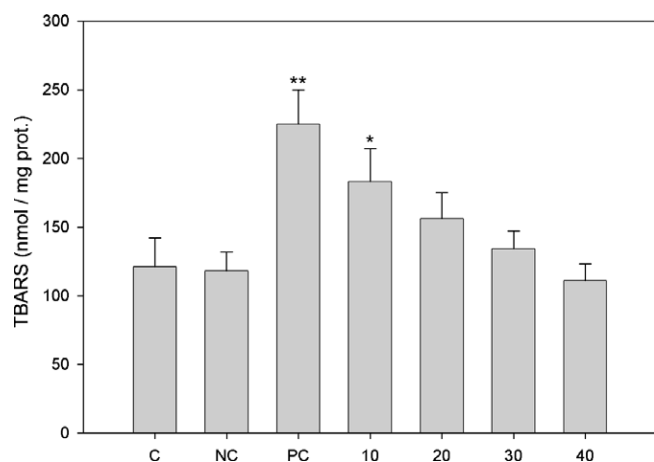


Fig. 2. Effect of various doses of TSM given to mice 2 h prior to cisplatin injection (20 mg/kg) on accumulation of TBARS in kidney tissue. Results are expressed as means \pm SEM of six experiments. C bar depicts TBARS level in control group not receiving any treatment. NC bar represents negative control, 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).

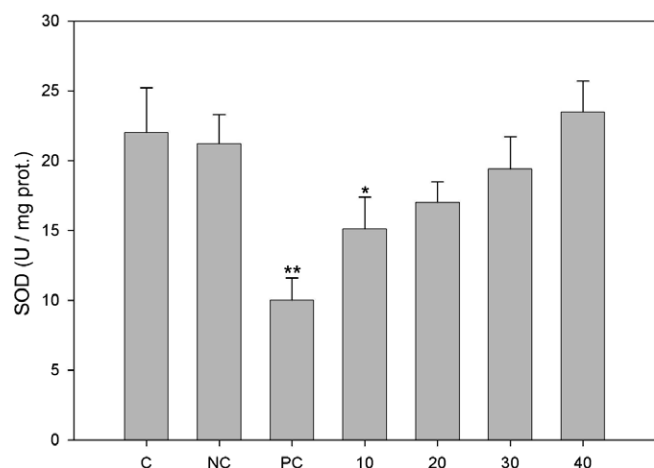


Fig. 3. Effect of protecting doses of TSM on SOD activity in kidney of ICR mice administered with cisplatin. Results are expressed as means \pm SEM of six experiments. C bar depicts SOD activity in control group not receiving any treatment. NC bar represents negative control, PC bar represents positive 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).

(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 h 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. 4). 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.

4. Discussion

The present study reveals that melanin derived from *T. 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. Treatments with some antioxidants like naringenin, erdosteine or α -tocopherol and diphenylphenyl-enediamine (Fetoni et al., 2004; Yilmaz et al., 2004; Badary et al., 2005) have shown to be effective in preventing cisplatin-induced nephrotoxicity. As it seen from our experiments, the antioxidant activity of TSM was involved in protecting animals against cisplatin-induced nephrotoxicity. This can be realized from the dose-dependent suppression of TBARS (Fig. 2) and restoration of SOD activity (Fig. 3).

TSM provides protection by multiple pathways. We previously demonstrated that TSM prevents liver damage induced by acetaminophen, hydrazine, and benzidine (Sava et al., 2002, 2003; Hung et al., 2003, 2004b). In the case of acetaminophen-induced hepatotoxicity, TSM causes a dose-dependent effect against NAPAP challenge, with plasma ALT level being reduced to normal control, depleted glutathione (GSH) level being recovered to a normal level, the activity of hepatic isozymes of cytochrome P450 being effectively inhibited, oxidative stress being suppressed, SOD activity being restored, and normal immunity of the animal being restored to the level of intact mice.

Multiple pathways are also 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.

GSTs catalyze the conjugation of GSH to a wide variety of endogenous and exogenous electrophilic compounds. Gstp2 plays a regulatory role in the MAP kinase pathway that participates in cellular survival and death signals via apoptosis signal-regulating kinase. It is plausible that GSTs serve direct detoxification and an inhibitor of the MAP kinase pathway (Satta et al., 1992). Gstp2 gene expression indicates the cellular state of oxidative stress and apoptosis (Prota, 1998). Transcriptional level of Gstp2 increased 40-fold by cisplatin treatment which indicated enormous oxidative stress introduced to kidney. Pretreatment of

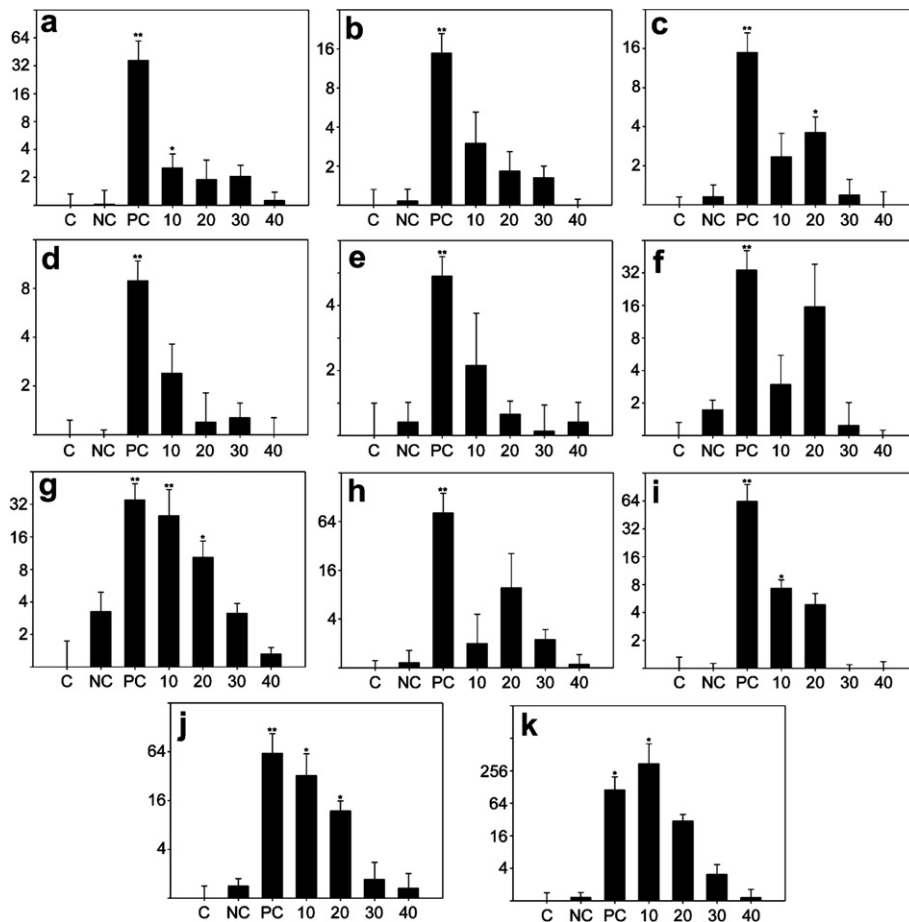


Fig. 4. Effect of various doses of TSM on mRNA levels in kidney of ICR mice poisoned with cisplatin. Results are expressed as means \pm SEM of six experiments. Y-axis depicts fold change in mRNA expression relative to control group not receiving any treatment. The value equals to $2^{\Delta\Delta Ct}$. 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) Snn1, (g) Gamt, (h) Rbp4, (i) Apn, (j) Cyp2d18, and (k) Oat.

20 mg/kg melanin alleviated the abnormal transcription of Gstp2 and provided a recovery to normal stress levels (Fig. 4a).

Ephx1 plays an important role in the regulation of renal eicosanoid levels and systemic blood pressure (Yu et al., 2004; Zhao et al., 2004). Ephx1 mRNA level indicates levels of oxidative stress and vasodilation. A 15-fold induction of Ephx1 transcriptional level upon cisplatin treatment strongly suggested that cisplatin may cause an increase in blood pressure. Pretreatment of 40 mg/kg melanin alleviated the abnormal transcription of Ephx1, which indicated a normal level of blood pressure (Fig. 4b).

Lcn2 is an iron-siderophore-binding protein that converts embryonic kidney mesenchyme to epithelia. Lipocalin 2 could suppress cell invasiveness in vitro and tumor growth and lung metastases in vivo. Lcn2 represents an early and quantitative urinary biomarker for cisplatin nephrotoxicity and renal ischemia (Mishra et al., 2004; Hanai et al., 2005). We found a 15-fold increase of Lcn2

transcript consistent with previous reports. TSM pretreatment significantly decreased the level of Lcn2 expression (Fig. 4c), indicating its protective role.

Lyz is one of the anti-microbial agents found in human milk and is also present in spleen, lung, kidney, white blood cells, plasma, saliva, and tears. Missense mutations in Lyz have been identified in heritable renal amyloidosis. Evidence suggested lysozyme may protect against diabetic renal damage by sequestering sugar-derived proteins or lipids (Nishimura, 1987). A 9-fold increase of expression was induced by cisplatin treatment due to early renal injury. TSM pretreatment restored lysozyme to a normal transcription level indicating a protective effect in a dosage dependent manner (Fig. 4d).

The microsomal UGTs conjugate and eliminate potentially toxic xenobiotics and endogenous compounds. Utg2b has unique specificity for 3,4-catechol estrogens and estriol, suggesting that it may play an important role in regulating the level and activity of these potent estrogen metabolites.

Utg2b is involved in conjugating lipophilic aglycon substrates with glucuronic acid. It is a steroid glucuronidation enzyme involved in termination of steroid signaling (Turgeon et al., 2001). Cisplatin-induced up-regulation of Utg2b indicated interruption of steroid signaling and possibly abnormal growth of kidney. A 5-fold induction of expression was reduced to a normal state by pre-injection of 40 mg/kg TSM (Fig. 4e).

Smn1 is an RNA-binding protein playing parts in the pre-mRNA processing. Smn1 functions in early motor axon development whose defects may lead to subsequent motorneuron loss. The role of Smn1 in cisplatin-induced renal injury is unclear (Coovert et al., 1997). A 30-fold induction of transcription was obtained by cisplatin treatment and was alleviated by TSM pre-injection (Fig. 4f).

Guanidinoacetate methyltransferase (Gamt) is the enzyme that catalyzes the last step of creatine biosynthesis (Komoto et al., 2004). In people with a history of renal disease or those taking nephrotoxic medications, creatine may be associated with an increased risk of renal dysfunction (Yoshizumi and Tsourounis, 2004). A 30-fold induction was obtained by cisplatin treatment consistent with elevated levels of creatinine. Pre-administration of TSM decreased the transcription level and presumably prevented renal injury (Fig. 4g).

Rbp4 excretion is diagnostic for renal tubular dysfunction in diabetic patients (Hosaka et al., 2003). High concentrations of Rbp4 are found in the urine of patients with tubulointerstitial injury (Shimizu et al., 1992). Urinary Rbp4 was reported as a marker for the early assessment of cisplatin-induced nephrotoxicity. An 80-fold increase in Rbp4 activity after cisplatin injection indicated tubulointerstitial injury. Pretreatment of 40 mg/kg TSM restored the expression to a normal level, indicating the salutary effects of TSM (Fig. 4h).

Apn is able to cleave numerous regulatory peptides. Apn could represent a new therapeutic target in pathological processes, such as tumoral proliferation and/or angiogenesis associated with cancer development (Jardinaud et al., 2004). Transcription of Apn increased 60-fold by the treatment of cisplatin. Pretreatment of TSM recovered Apn to normal transcriptional levels (Fig. 4i).

Cyp2d18 enzymes play a key role in the metabolism of drugs and environmental chemicals. Several Cyp2d18 enzymes metabolically activate procarcinogens to genotoxic intermediates. Phenotyping analyses revealed an association between Cyp2d18 enzyme activity and the risk of developing several forms of cancer (Agundez, 2004). Sixty-fold induction of Cyp2d18 indicated cellular damage induced by cisplatin (Bompart, 1989). This abnormal increase of Cyp2d18 transcript was prevented by 40 mg/kg TSM (Fig. 4j).

Oat encodes the mitochondrial enzyme ornithine aminotransferase, which is a key enzyme in the pathway that converts arginine and ornithine into the major excitatory and inhibitory neurotransmitters glutamate and GABA. Transcription of Oat increased 100-fold by the treatment of

cisplatin. Pretreatment of TSM recovered Oat to normal transcriptional levels (Fig. 4k).

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 immunostimulation. 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.

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