

Preventive Effect of *Thea sinensis* Melanin against Acetaminophen-Induced Hepatic Injury in Mice

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The preventive effect of *Thea sinensis* melanin (TSM) against overdoses of *N*-acetyl-*p*-aminophenol (NAPAP) was studied on ICR mice. Animals were given 400 mg/kg intraperitoneally (i.p.) of NAPAP, and TSM was injected i.p. in doses 10–40 mg/kg 2 h before intoxication. The protective effects were evidenced by a complete blockage of the NAPAP-induced elevation of plasma alanine aminotransferase (ALT) activity, decreased concentration of thiobarbituric acid reactive substances (TBARS) to the control level, and a partial prevention of reduced glutathione (GSH) depletion in the liver tissue. Preadministration of TSM also caused restoration of superoxide dismutase (SOD) activity and resumed content of coenzymes Q9 and Q10. TSM by itself, however, did not affect the hepatic functional parameters, including serum ALT, TBARS, GSH, SOD, or coenzymes Q in the liver. Administration of TSM caused a dose-dependent inhibition of *N*-nitrosodimethylamine demethylase activity with ED₅₀ of 15.8 mg/kg. Activities of ethoxyresorufin *O*-dealkylase and pentoxyresorufin *O*-alkylase isozymes were changed insignificantly. The immune suppressive effect of NAPAP on the in vivo antibody-forming cell responses was demonstrated using ICR-sensitized mice with sheep red blood cells. The joint effect of TSM and NAPAP indicated the capability of TSM to recover immunity of the animals to the level of intact mice. Results obtained demonstrate that TSM preadministration can prevent the multiple toxic effects of NAPAP.

KEYWORDS: *Thea sinensis* melanin; acetaminophen-induced liver injury; liver protection

INTRODUCTION

N-Acetyl-*p*-aminophenol (NAPAP) is a common analgesic and antipyretic drug also known as acetaminophen, paracetamol, or Tylenol. NAPAP is safe in therapeutic doses; however, overdosing can produce hepatic injury causing centrilobular hepatic necrosis, liver failure, and death (1). Severe poisoning with NAPAP often requires liver transplantation (2).

Hepatotoxicity of NAPAP is greatly associated with accumulation of the highly reactive metabolite *N*-acetyl-*p*-benzoquinoneimine (NAPQI) generated by cytochrome monooxygenase P450 (1, 3). NAPQI is normally detoxified by conjugation with reduced glutathione (GSH). However, when the capacity of GSH is exhausted, NAPQI covalently binds to other cellular structures resulting in hepatic injury (4). Suppression of P450 can decrease the formation of reactive metabolite and, therefore, may be useful for protection against NAPAP hepatotoxicity (5, 6).

Oxidative stress and, particularly, lipid peroxidation also contribute in the progression of hepatic injury induced by NAPAP (7). It was demonstrated that the loss of endogenous antioxidants played a critical role in NAPAP-induced hepatic injury (8). Therefore, replenishing antioxidants can serve as a preventive measure against the hepatotoxicity of NAPAP. From this point, the natural antioxidants presented in food might be of particular interest (9). For example, the antioxidants contained in tea have been found to be promising natural protectors (10, 11). Tea was the oldest folk medicine known in China 5000 years ago due to its detoxifying properties. The major composition and properties of tea antioxidants are well documented, but scarce information is available concerning their polymeric forms (12).

Recently, we have extracted melanin from *Thea sinensis* Linn (13). Similar melanin pigments derived from various sources were intensively studied earlier (14, 15), and the most significant properties concerning melanin-chelating and free radical properties were disclosed. *Thea sinensis* melanin (TSM) represents the high molecular part of tea polyphenols (16) with physicochemical characteristics that are similar to those of typical melanin. TSM has demonstrated a wide range of biochemical

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and pharmacological activities in animals including antioxidant, free radical scavenging, and immunomodulatory effects (13, 17, 18). TSM also revealed unexpected protective activity against various toxic substances such as benzidine, hydrazine, and snake venoms (19–22).

The purpose of the present work was to examine whether TSM could work against overdoses of NAPAP. The antioxidant properties of TSM were primarily considered as a prerequisite in realization of protective activity. Additionally, we have discovered the inhibitory effect of TSM on cytochrome P450 that was considered to be especially beneficial against hepatotoxicity of NAPAP. Therefore, evaluation of its capability in prevention of liver injury included both antioxidant and inhibitory effects of TSM. The immunostimulating properties disclosed earlier in melanin (17) were also considered in regard to their possible effect on the reticuloendothelial system of the liver that was achieved in the case of immune-stimulants (23). We attempted to test the ability of TSM in enhancing production of antibodies that normally is inhibited by NAPAP (24). Results obtained demonstrate that TSM preadministration can prevent the multiple toxic effects of NAPAP.

MATERIALS AND METHODS

Materials. *Thea sinensis* leaves were harvested in Miaoli, Taiwan, and were identified in the Institute of Chinese Pharmaceutical Sciences, China Medical University, and a voucher specimen (GSH-001) was deposited at the Herbarium of this Institute. NAPAP, EDTA, Tris-HCl, Triton X-100, Sephadex G-75, molecular size markers, and a kit for serum alanine aminotransferase activity were purchased from Sigma Chemical Co. (St. Louis, MO). All other chemicals were of analytical grade or higher from Merck (Darmstadt, Germany).

Isolation and Physicochemical Characterization of TSM. Isolation of TSM was performed according to the previously reported procedure (13) employed with minor adjustments. Namely, the extraction time was diminished to 12 h, avoiding excessive oxidation of TSM. After extraction, the mixture was filtered and centrifuged at 15000g 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 15000g for 15 min to pellet form. Acid hydrolysis was employed for purification of TSM. The purified product thus obtained was dissolved in 0.2% NH₄OH, and the solution was subjected to repeated precipitations. The precipitation procedure was repeated three more times to sequester TSM from low molecular impurities and to improve its homogeneity. The solutions thus obtained were filtered through a Nalgene 0.45 μ m syringe filter. Finally, TSM was purified on a Sephadex G-75 column (the dimensions were 1.6 \times 40 cm) in a 50 mM phosphate buffer (pH 7.5) at a flow rate of 0.5 mL \cdot 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 apotinin (MM = 6500) as size markers.

Physical and chemical characterizations of TSM were performed according to conventional procedures (14, 15). Ultraviolet–visible (UV) absorption spectra were obtained with a JASCO V-530 UV–visible spectrophotometer (Jasco Ltd., Great Dunmow, U.K.). 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 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.

Animals and Treatment. Adult male ICR mice (30 \times b1 5 g) were employed for all experiments. Animals were housed under controlled conditions (25 \times b1 2 °C) and a 12 h light/dark cycle; they were allowed free access to food and water but fasted overnight before treatment. Animals were divided into several groups including a control group (not receiving any treatment), a negative control (receiving TSM alone), a positive control (receiving only NAPAP), and experimental groups receiving NAPAP and TSM together. Each experimental group consisted of six mice. NAPAP was dissolved in normal saline (pH 7.4)

and administered intraperitoneally (i.p.) with a dose of 400 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. All animals were sacrificed by ether anesthesia 24 h after the NAPAP exposure. Blood samples were withdrawn by cardiac puncture for determination of alanine aminotransferase (ALT) activity using a commercially available kit (Sigma 505-P). Liver was isolated and perfused with normal saline to wash out the blood. It was then used for determination of GSH, oxidized glutathione (GSSG), superoxide dismutase (SOD), thiobarbituric acid reactive substances (TBARS), and reduced forms of coenzymes Q9 (CoQ9) and Q10 (CoQ10).

GSH and GSSG Assays. Frozen liver tissue was homogenized in 5% trichloroacetic acid (TCA) supplemented with 5 mM EDTA under a stream of nitrogen gas and then centrifuged at 20000g for 10 min at 4 °C. TCA was removed from the supernatant by extracting three times with diethyl ether. The total hepatic glutathione level representing the sum of GSH and GSSG was determined using dithiobis-2-nitrobenzoic acid (25). The GSSG level was determined after elimination of GSH by reaction with 2-vinylpyridine, and the actual GSH level was calculated by subtracting the GSSG level from the total glutathione.

Determination of TBARS. Formation of lipid peroxide derivatives was evaluated by measuring TBARS according to Cascio et al. (26). Briefly, liver 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 nanomoles of TBARS per milligram of protein using malondialdehyde bis(dimethyl acetal) for calibration.

Superoxide Dismutase Assay. Determination of superoxide dismutase activity in mouse liver was based on inhibition of nitrite formation in the reaction of oxidation of hydroxylammonium with superoxide anion radical (27). Nitrite was generated in a mixture containing 25 μ L of xanthine (15 mM), 25 μ L of hydroxylammonium chloride (10 mM), 250 μ L of phosphate buffer (65 mM, pH 7.8), 90 μ L of distilled water, and 100 μ L of xanthine oxidase (0.1 U/ μ L). The inhibitory effect of inherent SOD was assayed at 25 °C during 20 min of incubation with 10 μ L of brain tissue extracts. Determination of the resulting nitrite was performed on the reaction (20 min at room temperature) with 0.5 mL of sulfanilic acid (3.3 mg/mL) and 0.5 mL of α -naphthylamine (1 mg/mL). The optical absorbance at 530 nm was measured with an Ultrospec III spectrophotometer (Pharmacia, LKB). The results were expressed as units of SOD activity calculated per milligram of protein.

Coenzymes Q Assay. Determination of the reduced forms of CoQ9 and CoQ10 was carried out by the method of Ikenoya et al. (28). Liver tissue was homogenized in ice-cold water under a stream of nitrogen gas. Coenzymes Q were extracted with a mixture of ethanol/*n*-hexane (2:5 v/v), and the *n*-hexane layer was collected. The solvent was evaporated using a rotary evaporator and redissolved in the ethanol. The extract was analyzed using HPLC with a Jasco 840 EC detector and a Chemosorb ODS-H column (4.6 \times 250 mm). The mobile phase consisted of ethanol/methanol/70% HClO₄ (700:300:1 v/v) supplemented with 0.7% NaClO₄ H₂O.

Evaluation of the Activities of P450 Isozymes. Experiments were performed on five groups of intact ICR mice consisting of six animals each. Different doses of TSM (0, 10, 20, 30, and 40 mg/kg) were given to mice i.p. Animals were sacrificed 24 h after injection; the livers were removed, perfused with chilled KCl (154 mM), and homogenized in buffer containing 50 mM Tris-HCl and 154 mM KCl (pH 7.4). The homogenates were centrifuged at 9000g for 20 min at 4 °C, and the microsomal fraction was separated from the supernatant by centrifugation at 105000g for 90 min at 4 °C. The microsomal pellet was washed with homogenizing buffer, centrifuged again at 105000g for 90 min at 4 °C, and then suspended in 250 mM sucrose. The activities of ethoxyresorufin *O*-dealkylase (P450 2A1) and pentoxyresorufin *O*-alkylase (P450 2B1) were measured according to the method described by Burke and Mayer (29). *N*-Nitrosodimethylamine demethylase (P450 2E1) activity was determined as an index of P450 2E1 activity by the method of Peng et al. (30).

Table 1. Effect of TSM on the Toxicity of NAPAP

animal group ^a	mortality (dead/total)	ALT (U/L)	GSH (nmol/mg protein)	GSSG (nmol/mg protein)
control	0/6	42 ± 4 ^b	39 ± 4	3.5 ± 0.4
TSM (40 mg/kg)	0/6	40 ± 5	40 ± 3	3.6 ± 0.3
NAPAP (400 mg/kg)	2/6	2043 ± 231 ^{***c}	13 ± 1 ^{**}	3.2 ± 0.4
TSM (10 mg/kg) + NAPAP (400 mg/kg)	0/6	1528 ± 142 ^{**}	15 ± 2 ^{**}	3.5 ± 0.3
TSM (20 mg/kg) + NAPAP (400 mg/kg)	0/6	298 ± 30 ^{**}	22 ± 2 [*]	2.9 ± 0.2
TSM (30 mg/kg) + NAPAP (400 mg/kg)	0/6	63 ± 5 [*]	28 ± 3 [*]	3.3 ± 0.2
TSM (40 mg/kg) + NAPAP (400 mg/kg)	0/6	43 ± 5	28 ± 2 [*]	3.1 ± 0.3

^a Control mice were given saline. Experimental animals received TSM (10, 20, 30, or 40 mg/kg, i.p.) and NAPAP (400 mg/kg, i.p.) that was injected 2 h after administration of TSM. ^b The toxic effect was evaluated at 24 h of NAPAP exposure, and the data represent means ± SEM. ^c Significantly different from the control [(*) $P < 0.05$, (***) $P < 0.01$].

Evaluation of Antibody-Producing Response. Experiments were performed on 10 groups of intact ICR mice consisting of four animals each. The first 5 groups were injected i.p. with the following single doses of TSM: 0, 10, 20, 30, and 40 mg/kg. The second 5 groups were injected similarly to the first groups with TSM, but after 2 h the animals were again injected (i.p.) with 400 mg/kg NAPAP. One day after administration of TSM or TSM + NAPAP, mice were injected into the tail vein with 1×10^8 sheep red blood cells (SRBC) prepared in 0.2 mL of saline. An additional four animals received only SRBC (antigen control). Four days after their sensitization with SRBC, the animals were sacrificed, and their spleens were removed. Single splenocytes were prepared from each spleen in 5 mL of RPMI-1640 media. Enumeration of the antibody-forming cells (AFC) was performed using a plaque assay (31). AFC values were calculated for 10^6 splenocytes.

Protein Assay. The amount of protein in the samples was determined using bicinchoninic acid (32) and bovine serum albumin as a standard.

Statistical Analysis. All data were presented as means ± SEM standard error of the mean (SEM). Statistical analysis was performed using a Student's *t*-test. The minimum level of significance was set at $P < 0.05$.

RESULTS

TSM was extracted from tea (*Thea sinensis* Linn.) as previously reported (13) 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 MLP 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 (14, 15, 33, 34). 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. A 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 (33, 34). 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 and 1650 cm⁻¹ was reduced, a phenomenon caused by the reaction between phenolic and carboxylic groups owing to the formation of lactones (13), which likely are involved in various biological effects.

Table 2. Effect of TSM on the Activity of Hepatic Isozymes of Cytochrome P450 Monooxygenase

exptl conditions	activity of P450 isozymes ^a		
	2A1	2B1	2E1
control (vehicle)	7.35 ± 1.23	1.4 ± 0.13	2.85 ± 0.33
TSM 10 mg/kg	7.5 ± 1.41	1.6 ± 0.12	2.52 ± 0.25
TSM 20 mg/kg	7.28 ± 1.11	1.4 ± 0.15	1.51 ± 0.19 ^b
TSM 30 mg/kg	7.44 ± 1.25	1.3 ± 0.13	1.1 ± 0.09 ^b
TSM 40 mg/kg	7.64 ± 1.52	1.5 ± 0.16	0.93 ± 0.1 ^b

^a Activity is expressed as mean ± SEM of six mice. Enzyme activities are expressed as follows: 2A1 and 2B1, from amount of resorufin (pmol/min-mg protein); 2E1, from amount of formaldehyde (nmol/min-mg protein) ^b Value is significantly different from that of control ($P < 0.01$).

Administration of 40 mg/kg TSM alone did not induce any toxicity. The behavior of the animals in the experimental group resembled that of the control group by movement activity. In contrary, all animals treated with NAPAP alone (400 mg/kg) were sick as compared to the control group and were unable to move around the cages. Such treatment caused hepatocellular damage in mice, as indicated by a drastic elevation of ALT level (Table 1). The animals pretreated with TSM 2 h prior to intoxication showed protection against NAPAP. TSM caused a dose-dependent effect against NAPAP challenge, with the plasma ALT level being reduced to 74, 14, and 3% of the positive control when 10, 20, and 30 mg/kg doses were given to animals. The highest dose of TSM (40 mg/kg) completely blocked hepatotoxicity of NAPAP. Animal lethality observed for 400 mg/kg NAPAP administered alone was 66% (Table 1). Administration of 40 mg/kg of TSM by itself did not give animal death. TSM (10–40 mg/kg) administered prior to NAPAP also prevented lethality of animals.

Hepatic GSH was determined 24 h after the NAPAP administration. TSM, by itself, did not affect the hepatic GSH level (Table 1). Administrations of NAPAP alone significantly depleted the GSH level (2.6-fold as compared to the control). Pretreatment with TSM decreased GSH depletion, demonstrating a dose-dependent protective effect. Concentration of GSSG was restored to about the same level, suggesting that GSH loss might not have resulted from reaction with glutathione peroxidase but was due to conjugation of glutathione with NAPQI.

Preadministration of TSM caused an inhibitory effect on the activity of hepatic isozymes of cytochrome P450 (Table 2). To determine the relative inhibitory effect on various P450 isozymes, different substrates were used for incubation with liver microsomes. TSM caused a dose-dependent inhibition of P450 2E1 specific *N*-nitrosodimethylamine demethylase activity with an ED₅₀ value of 15.8 mg/kg body weight. Activities of P450 2A1 and P450 2B1 were changed insignificantly, consistent with the

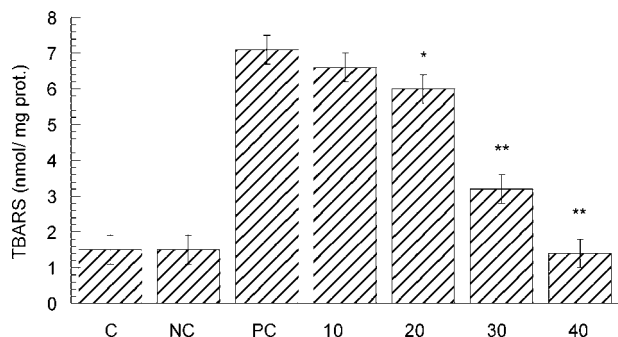


Figure 1. Effect of various doses of TSM given to mice 2 h prior to NAPAP administration (400 mg/kg) on accumulation of TBARS in liver tissue. Results are expressed as mean \pm SEM of six experiments. The C bar depicts the TBARS level in the control group not receiving any treatment. The NC bar represents the negative control, the PC bar represents the positive control, and numbers indicate doses of TSM (in mg/kg). Asterisks depict significant differences between the positive control and the joint effect of NAPAP and TSM [(*) $P < 0.05$, (**) $P < 0.01$].

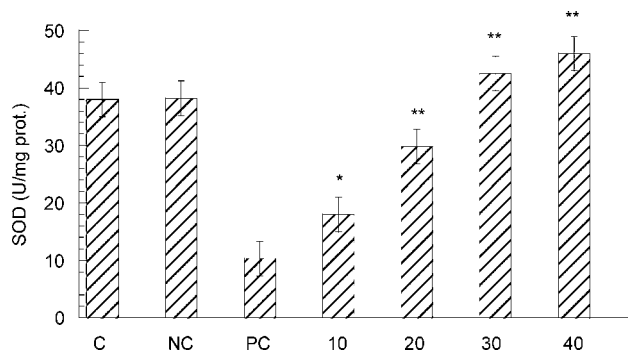


Figure 2. Effect of various doses of TSM on SOD activity in liver of ICR mice poisoned with NAPAP. Results are expressed as mean \pm SEM of six experiments. The C bar depicts SOD activity in the control group not receiving any treatment. The NC bar represents the negative control, the PC bar represents the positive control, and numbers indicate doses of TSM (in mg/kg). Asterisks depict significant differences between the positive control and the joint effect of NAPAP and TSM [(*) $P < 0.05$, (**) $P < 0.01$].

normalization trends of ALT and GSH. Pretreatment by TSM significantly decreased NAPAP-induced lipid peroxidation in a dose-dependent manner (Figure 1). An increased dose of TSM suppressed peroxidation with the highest dose causing full blockage of TBARS. Administration of TSM alone (negative control) did not produce any significant effect as compared to the control (no treatments).

Introduction of NAPAP caused an almost 4-fold decrease of SOD activity as compared to the control (Figure 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 the negative control. Administration of TSM alone did not affect SOD activity. This suggests an indirect influence of TSM on SOD activation.

NAPAP itself caused a significant decrease in the quantity of reduced forms of antioxidant enzymes CoQ9 and CoQ10 in animal liver (Figure 3). Namely, coenzyme Q10 content was diminished 2.2-fold as compared to the negative control. The content of CoQ9 was 2.5 times below that of the control. Administration of TSM 2 h before NAPAP allowed an increasing quantity of both antioxidant coenzymes in a dose-dependent manner. The highest dose of TSM (40 mg/kg) reduced the

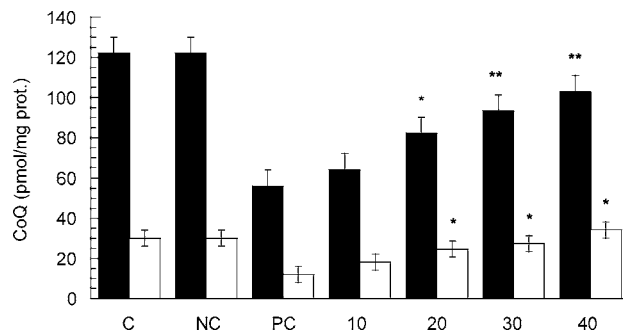


Figure 3. Effect of various doses of TSM on the content of endogenous CoQ9 (filled bars) and CoQ10 (open bars) in the livers of mice administered with 400 mg/kg of NAPAP. Results are expressed as mean \pm SEM of six experiments. The C bars depict coenzyme Q9 and Q10 levels in the control group not receiving any treatment. The NC bars represent the negative control, the PC bars represent the positive control, and numbers indicate doses of TSM (in mg/kg). Asterisks depict significant differences between the positive control and the joint effect of NAPAP and TSM [(*) $P < 0.05$, (**) $P < 0.01$].

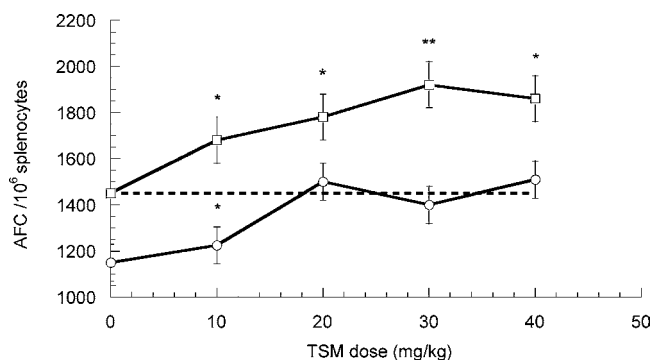


Figure 4. Effect of TSM on the antibody-producing response of splenocytes of ICR mice. Squares depict the effect of TSM itself, and circles represent the joint effect of TSM + NAPAP. The dashed line represents the antigen control. Mice were sensitized with SRBC 1 day after administration of TSM and/or NAPAP. The mean and SEM (error bars) were obtained from four animals. Asterisks depict significant differences between the antigen control and the effects of NAPAP and TSM [(*) $P < 0.05$, (**) $P < 0.01$].

content of coenzyme Q9 to 83% but increased Q10 to 113% of the control level. However, administering the same dose of TSM alone did not produce any significant difference as compared to the control, suggesting an indirect effect on coenzymes Q.

The in vivo antibody-producing responses were employed to evaluate the effect of TSM alone and the joint effect of TSM + NAPAP on the humoral immunity of ICR mice. Introduction of TSM demonstrated a dose-dependent immunostimulating effect (Figure 4) similar to that obtained earlier on BALB/C mice (17). At doses of 30–40 mg/kg of TSM, the antibody-secreting cells produced significantly ($P < 0.05$) more antibodies (26–28%) than did the antigen control. Administration of NAPAP caused a suppression of AFC of 26% against the antigen control level. However, preadministration of TSM before NAPAP intoxication increased AFC in a dose-dependent manner and restored TSM immunity to the level of the antigen control at doses starting from 20 mg/kg (Figure 4).

DISCUSSION

The present study reveals that melanin derived from *Thea sinensis* leaves has protective effects against the hepatic injury induced by NAPAP. The protective effects were evidenced by

a complete blockage of the acetaminophen-induced increase in serum ALT activity, decrease of TBARS concentration to the control level, and a partial prevention of GSH depletion in the liver tissues of ICR mice. TSM by itself, however, did not affect the hepatic functional parameters, including the serum ALT activity, TBARS, or GSH level in the liver.

Hepatic injury induced by NAPAP involves GSH depletion and formation of the toxic metabolite NAPQI (35, 36). Cytochrome P450 isozymes 2E1 and 1A2 are the major enzymes involved in the activation of NAPAP in animals or humans (37). Therefore, the suppression of P450 activity could result in the decreased formation of the reactive metabolites causing liver injury. It was demonstrated earlier that hepatotoxicity of NAPAP can be modulated through the activity of P450. For example, ethanol and isoniazid potentiate toxicity of NAPAP through the induction of P450 2E1 (38, 39). Other compounds that inhibit P450 enzymes, such as disulfiram, 4-methylpyrazole, and cimetidine, protect against NAPAP-induced toxicity (5, 6, 40). In our experiment, TSM caused an inhibitory effect mostly on P450 2E1 but not P450 2A1 or P450 2B1. Thus, inhibition of P450 2E1 by TSM contributes in metabolic detoxification of NAPAP. Among the multiple cytochrome enzymes, P450 2E1 has been reported to play a significant role in bioactivation of NAPAP. Studies on P450 2E1 knockout mice evidenced that P450 2E1 is the most important factor in acetaminophen bioactivation (37). P450 2E1 inhibitors such as diallyl sulfide and related organosulfur compounds protect the liver against NAPAP-induced injury (41), evidencing the crucial role of the P450 2E1 enzyme in mediating toxicity of NAPAP.

Alternatively, another detoxification pathway of NAPAP is based on the conjugation of NAPQI with GSH. NAPAP was demonstrated to play a key role in relieving the toxic metabolites of GSH (35, 36). Our results show that pretreatment of mice with TSM significantly reduces NAPAP-induced hepatic GSH depletion (Table 1). However, TSM itself did not affect GSH. This is probably due to the inhibitory effect on P450 that decreased formation of NAPQI and, therefore, prevented depletion of GSH.

Some results suggest that hepatotoxicity of NAPAP is also caused by cellular oxidative stress (7, 8). Several antioxidants and antioxidative enzymes, such as α -tocopherol, ascorbic acid, superoxide dismutase, and coenzymes Q have been tested as protectors against NAPAP-induced liver injury (8). Treatments with some antioxidants such as promethazine or α -tocopherol and diphenylphenyl-enediamine (42) have been shown to be effective in protecting the liver from NAPAP toxicity. As seen from our experiments, the antioxidant activity of TSM was involved in protecting animals against NAPAP hepatotoxicity. This can be realized from the dose-dependent suppression of TBARS (Figure 1). TSM also caused activation of SOD (Figure 2) and recovered the content of endogenous coenzymes Q9 and Q10 (Figure 3).

The immune suppressive effect of NAPAP on the in vivo AFC responses was demonstrated (Figure 4) using ICR mice sensitized with SRBC. Administration of TSM alone gave a 26–28% rise in antibody-producing response, agreeing with our previous results (17). The joint effect of TSM and NAPAP indicated the capability of TSM to restore the immunity of the animals to the level of intact mice.

The present work demonstrates that the protective effect of TSM against NAPAP hepatotoxicity is based on a combination of different factors including cytochrome P450 inhibitory activity, antioxidant properties, and immunostimulation. Such a combination opens the possibility for a comprehensive

protection of the liver against heavy intoxication and may serve as a first approach in developing natural hepatoprotectors.

ABBREVIATIONS USED

ALT, alanine aminotransferase; AFC, antibody-forming cells; P450 2A1, ethoxyresorufin *O*-dealkylase; P450 2B1, pentoxresorufin *O*-alkylase; P450 2E1, *N*-nitrosodimethylamine demethylase; IR, infrared; i.p., intraperitoneally; MM, molecular mass; NAPAP, *N*-acetyl-*p*-aminophenol; NAPQI, *N*-acetyl-*p*-benzoquinoneimine; GSSG, oxidized glutathione; GSH, reduced glutathione; CoQ10, reduced forms of coenzymes Q10; CoQ9, reduced forms of coenzymes Q9; SRBC, sheep red blood cells; SOD, superoxide dismutase; TSM, *Thea sinensis* melanin; TBARS, thiobarbituric acid reactive substances; TCA, trichloroacetic acid; UV, ultraviolet–visible.

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LITERATURE CITED

- Ray, S. D.; Mumaw, V. R.; Raje, R. R.; Fariss, M. W. *J. Pharmacol. Exp. Ther.* **1996**, *279*, 1470–1483.
- Koivusalo, A. M.; Isoniemi, H.; Vakkuri, A.; Hockerstedt, K.; Nuutinen, H. Without liver transplantation paracetamol intoxication is often be lethal, in spite of *N*-acetylcysteine. *Duodecim* **2002**, *118*, 649–650.
- Esterline, R. L.; Ray, S. D.; Ji, S. Reversible and irreversible inhibition of hepatic mitochondrial respiration by acetaminophen and its toxic metabolite, *N*-acetyl-*p*-benzoquinoneimine (NAPQI). *Biochem. Pharmacol.* **1989**, *38*, 2387–2390.
- Albano, E.; Rundgren, M.; Harvison, P. I.; Nelson, S. D.; Moideus, P. Mechanisms of *N*-acetyl-*p*-benzoquinone imine cytotoxicity. *Mol. Pharmacol.* **1985**, *28*, 306–322.
- Jeong, H. G.; Park, H. Y. The prevention of carbon tetrachloride-induced hepatotoxicity in mice by α -hederin: inhibition of cytochrome P450 2E1 expression. *Biochem. Mol. Biol. Int.* **1998**, *45*, 163–170.
- Jorgensen, L.; Thomsen, P.; Poulsen, H. E. Disulfiram prevents acetaminophen hepatotoxicity in rats. *Pharmacol. Toxicol.* **1988**, *62*, 267–271.
- Wendel, A.; Feuerstein, S.; Konz, K. H. Acute paracetamol intoxication of starved mice leads to lipid peroxidation in vivo. *Biochem. Pharmacol.* **1979**, *28*, 2051–2055.
- Amimoto, T.; Matura, T.; Koyama, S. Y.; Nakanishi, T.; Yamada, K.; Kajiyama, G. Acetaminophen-induced hepatic injury in mice: the role of lipid peroxidation and effects of pretreatment with coenzyme Q10 and α -tocopherol. *Free Radical Biol. Med.* **1995**, *19*, 169–176.
- Stavric, B. Role of chemopreventers in human diet. *Clin. Biochem.* **1994**, *27*, 319–332.
- Katiyar, S. K.; Agarwai, R.; Mukhtar, H. Protective effects of green tea polyphenols administered by oral intubation against chemical carcinogen-induced forestomach and pulmonary neoplasia in A/J mice. *Cancer Lett.* **1993**, *73*, 167–172.
- Wang, Z. Y.; Khan, W. A.; Bickers, D. K.; Mukhtar, H. Protection against polycyclic aromatic hydrocarbon-induced skin tumor initiation in mice by green tea polyphenols. *Carcinogenesis* **1989**, *10*, 411–415.
- Balentine, D. A.; Wiseman, S. A.; Bouwens, I. C. The chemistry of tea flavonoids. *Crit. Rev. Food Sci. Nutr.* **1997**, *37*, 693–704.
- Sava, V.; Yang, S.-M.; Hong, M.-Y.; Yang, P.-C.; Huang, G. S. Isolation and characterization of melanic pigments derived from tea and tea polyphenols. *Food Chem.* **2001**, *73*, 177–184.
- Nicolaus, R. *Melanins*; Hermann: Paris, 1968.
- Prota, G. *Melanins and melanogenesis*; Academic Press: San Diego, 1998.

- (16) Hung, Y.-C.; Sava, V. M.; Juan, C.-L.; Yeh, T.-C.; Shen, W.-C.; Huang, G. S. Gastrointestinal enhancement of MRI with melanin derived from tea leaves (*Thea sinensis* Linn.). *J. Ethnopharmacol.* **2002**, *79*, 75–79.
- (17) Sava, V. M.; Galkin, B.; Hong, M.-Y.; Yang, P.-C.; Huang, G. S. A novel melanin-like pigment derived from black tea leaves with immuno-stimulating activity. *Food Res. Int.* **2001**, *34*, 337–343.
- (18) Hung, Y.-C.; Sava, V. M.; Mekan, S. Y.; Chen, J. T.-S.; Hong, M.-Y.; Huang, G. S. Antioxidant activity of melanins derived from tea: comparison between different oxidative states. *Food Chem.* **2002**, *78*, 233–240.
- (19) Sava, V. M.; Hung, Y.-C.; Golkin, B. N.; Hong, M.-Y.; Huang, G. S. Protective activity of melanin-like pigment derived from tea on *Drosophila Melanogaster* against the toxic effects of benzidine. *Food Res. Int.* **2002**, *35*, 619–626.
- (20) Sava, V. M.; Hung, Y.-C.; Blagodarsky, V. A.; Hong, M.-Y.; Huang, G. S. The liver-protecting activity of melanin-like pigment derived from black tea. *Food Res. Intl.* **2003**, *36*, 505–511.
- (21) Hung, Y.-C.; Sava, V. M.; Blagodarsky, V. A.; Hong, M.-Y.; Huang, G. S. Protection of tea melanin on hydrazine-induced liver injury. *Life Sci.* **2003**, *72*, 1061–1071.
- (22) Hung, Y.-C.; Sava, V. M.; Hong, M.-Y.; Huang, G. S. Inhibitory effects on phospholipase A2 and antivenin activity of melanin extracted from *Thea sinensis* Linn. *Life Sci.* **2004**, *74*, 2037–2047.
- (23) Kate, K.; Onodera, K.; Kato, J.; Kasai, S.; Mite, M. The immune-stimulant OK432 enhances liver regeneration hepatectomy. *J. Hepatol.* **1995**, *23*, 81–94.
- (24) Yamaura, K.; Ogawa, K.; Yonekawa, T.; Nakamura, T.; Yano, S.; Ueno, K. Inhibition of the Antibody Production by Acetaminophen Independent of Liver Injury in Mice. *Biol. Pharm. Bull.* **2002**, *25*, 201–205.
- (25) Griffith, O. W. Determination of glutathione and glutathione disulfide using glutathione reductase and 2-vinylpyridine. *Anal. Biochem.* **1980**, *106*, 207–212.
- (26) Cascio, C.; Guarneri, R.; Russo, D.; De Leo, G.; Guarneri, M.; Piccoli, F.; Guarneri, P. Pregnenolone sulfate, a naturally occurring excitotoxin involved in delayed retinal cell death. *J. Neurochem.* **2000**, *74*, 2380–2391.
- (27) Elstner, E. F.; Heupel, A. Inhibition of nitrite formation from hydroxylammoniumchloride: a simple assay for superoxide dismutase. *Anal. Biochem.* **1976**, *70*, 616–620.
- (28) Ikenoya, S.; Takada, M.; Yuzuriha, T.; Abe, K.; Katayama, K. Studies on reduced and oxidized ubiquinones. I. Simultaneous determination of reduced and oxidized ubiquinones in tissues and mitochondria by high performance liquid chromatography. *Chem. Pharm. Bull.* **1981**, *29*, 158–164.
- (29) Burke, M. D.; Thompson, S.; Weaver, R. J.; Wolf, C. R.; Mayer, R. T. Cytochrome P450 specificities of alkoxyresorufin O-dealkylation in human and rat liver. *Biochem. Pharmacol.* **1994**, *48*, 923–936.
- (30) Peng, R.; Tu, Y. Y.; Yang, C. S. The induction and competitive inhibition of a high affinity microsomal nitrosodimethylamine demethylase by ethanol. *Carcinogenesis* **1982**, *3*, 1457–1461.
- (31) Jerne, N. K.; Nordin, A. A. Plaque formation in agar by single antibody-producing cells. *Science* **1963**, *140*, 405.
- (32) Smith, P. K.; Krohn, R. I.; Hermanson, G. T.; Mallia, A. K.; Gartner, F. H.; Provenzano, M. D.; Fujimoto, E. K.; Goeke, N. M.; Olson, B. J.; Klenk, D. C. Measurement of protein using bicinchoninic acid. *Anal. Biochem.* **1985**, *150*, 76–85.
- (33) Bilinska, B. Progress of infrared investigations of melanin structures. *Spectrochim. Acta, Part A* **1996**, *52*, 1157–1162.
- (34) Paim, S.; Linhares, L. F.; Magrich, A. S.; Martin, J. P. Characterization of fungal melanins and soil humic acids by chemical analysis and infrared spectroscopy. *Biol. Fert. Soils* **1990**, *10*, 72–76.
- (35) Vermeulen, N. P. E.; Bessems, J. G. M.; van de Straat, R. Molecular aspects of paracetamol-induced hepatotoxicity and its mechanism-based prevention. *Drug Metab. Rev.* **1992**, *24*, 367–407.
- (36) Cohen, S. D.; Khairallah, E. A. Selective protein covalent binding and acetaminophen-induced hepatotoxicity. *Drug Metab. Rev.* **1997**, *29*, 59–77.
- (37) Zaher, J. T.; Buters, M.; Ward, J. M.; Bruno, M. K.; Lucas, A. M.; Stern, S. T.; Cohen S. D.; Gonzalez, F. J. Protection against acetaminophen toxicity in CYP1A2 and CYP2E1 double-null mice. *Toxicol. Appl. Pharmacol.* **1998**, *152*, 193–199.
- (38) Peterson, F. J.; Holloway, D. E.; Erickson, R. R.; Duquette, P. H.; McClain, C. L.; Holtzman, J. L. Ethanol induction of acetaminophen toxicity and metabolism. *Life Sci.* **1980**, *27*, 1705–1711.
- (39) Burk, R. F.; Hill, K. E.; Hunt, R. W.; Martin, J.; Martin, A. E. Isoniazid potentiation of acetaminophen hepatotoxicity in the rat and 4-methylpyrazole inhibition of it. *Res. Commun. Chem. Pathol. Pharmacol.* **1990**, *69*, 115–118.
- (40) Kadri, A. Z.; Fisher, R.; Winterton, M. C. Cimetidine and paracetamol hepatotoxicity. *Human Toxicol.* **1988**, *7*, 205–211.
- (41) Wang, E. J.; Li, Y.; Lin, M.; Chen, L.; Stein, A. P.; Reuhl, K. R.; Yang, C. S. Protective effects of garlic and related organo-sulfur compounds on acetaminophen-induced hepatotoxicity in mice. *Toxicol. Appl. Pharmacol.* **1996**, *136*, 146–154.
- (42) Albano, E.; Poli, G.; Chiarpotto, E.; Biasi, F.; Dianzani, M. U. Paracetamol-stimulated lipid peroxidation in isolated rat and mouse hepatocytes. *Chem. Biol. Interactions* **1983**, *47*, 249–263.

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