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 ED$_{50}$ 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 physico-chemical characteristics that are similar to those of typical melanin. TSM has demonstrated a wide range of biochemical
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 (15) 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 μm syringe filter. Filtered TSM was purified on a Sephadex G-75 column (the dimensions were 1.6 x 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), carboanhydrase (MM = 29 000), cytochrome c (MM = 12 400), and aprotinin (MM = 6500) as size markers.

Physical and chemical characteristics 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 ± 1.5 g) were employed for all experiments. Animals were housed under controlled conditions (25 ± 1 °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 distilled 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), 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 Ciacso 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.

Coenzymes Q Assay. Determination of superoxide dismutase activity in mouse liver was based on inhibition of nitrate formation in the reaction of oxidation of hydroxylammonium with superoxide anion radical (27). Nitrate was generated in a mixture containing 25 μL of xanthine (15 mM), 25 μL of hydroxylationammonium chloride (10 mM), 250 μL of phosphate buffer (65 mM, pH 7.8), 90 μL of distilled water, and 100 μL of xantine 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 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 Ultraspec III spectrophotometer (Pharmacia, LKB). The results were expressed as units of SOD activity calculated per milligram of protein.

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 pentoxysresorufin O-alkylase (P450 2B1) were measured according to the method described by Burke and Mayer (29). N-Nitosodimethylamine demethylation (P450 2E1) activity was determined as an index of P450 2E1 activity by the method of Peng et al. (30).
Evaluating the Toxicity of NAPAP.

**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 was performed by fractionation with molecular masses of 14 kDa and 8 kDa, with recovery of 1.9% (dried weight). The purified preparation of TSM exhibited all the physical and chemical properties common to natural melanins 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 (ethanol, hexane, acetone, benzene, and chloroform); dissolved in 0.1 M phosphate buffer (pH 8.0) and exhibited strong optical absorption in the UV region. 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.

**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 × 10⁸ 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 10⁶ 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 reported as means ± 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 melanins 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.
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 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 isoforms 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 prometazine 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, pentoxysresorufin 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; 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

Liver Protective Activity of Tea Melanin


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