

Original Article

Green Tea Extract-induced Acute Hepatotoxicity in Rats

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Abstract: Although green tea is considered to be a healthy beverage, hepatotoxicity associated with the consumption of green tea extract has been reported. In the present study, we characterized the hepatotoxicity of green tea extract in rats and explored the responsible mechanism. Six-week-old IGS rats received a single intraperitoneal (ip) injection of 200 mg/kg green tea extract (THEA-FLAN 90S). At 8, 24, 48 and 72 hrs and 1 and 3 months after exposure, liver damage was assessed by using blood-chemistry, histopathology, and immunohistochemistry to detect cell death (TUNEL and caspase-3) and proliferative activity (PCNA). Analyses of malondialdehyde (MDA) in serum and the liver and of MDA and thymidine glycol (TG) by immunohistochemistry, as oxidative stress markers, were performed. Placental glutathione S-transferase (GST-P), which is a marker of hepatocarcinogenesis, was also immunohistochemically stained. To examine toxicity at older ages, 200 mg/kg green tea extract was administered to 18-wk-old female rats. In 6-wk-old rats, 12% of males and 50% of females died within 72 hrs. In 18-wk-old rats, 88% died within 72 hrs. The serum levels of aspartate aminotransferase, alanine aminotransferase and/or total bilirubin increased in both males and females. Single-cell necrosis with positive signs of TUNEL and caspase-3 was seen in perilobular hepatocytes from 8 hrs onward in all lobular areas. PCNA-positive hepatocytes increased at 48 hrs. MDA levels in the serum and liver tended to increase, and MDA- and TG-positive hepatocytes were seen immunohistochemically. GST-P-positive hepatocellular altered foci were detected in one female rat at the 3-month time point. In conclusion, a single injection of green tea extract induced acute and severe hepatotoxicity, which might be associated with lipid peroxidation and DNA oxidative stress in hepatocytes. (DOI: 10.1293/tox.2014-0007; J Toxicol Pathol 2014; 27: 163–174)

Key words: apoptosis, green tea, hepatotoxicity, oxidative stress, rats

Introduction

Tea (*Camellia sinensis*) is a widely consumed beverage worldwide. Green tea, which is popular in Japan and China, accounts for about 20% of worldwide tea consumption. About 78% of tea consumed is black tea, which is the main tea beverage in the United States, Europe and Western Asia. The remaining 2% of tea consumed is oolong tea, which is mainly consumed in southeastern China and Taiwan¹. Green tea includes polyphenolic catechins, such as epicatechin, epicatechin gallate, epigallocatechin and epigallocatechin gallate (EGCG), the most abundant and probably the most pharmacologically active polyphenolic catechin^{2,3}. There is wide interest in the medical benefits of green tea,

including the prevention and treatment of cancers such as upper gastrointestinal tract, breast and prostate cancers and oral leukoplakia^{1,2,4}, and non-cancer health benefits, such as improved cardiac function, decreased atherosclerosis, cholesterol-lowering activity, and obesity prevention⁵⁻⁷. Although normal consumption of green tea seems safe, the consumption of concentrated green tea extract (GTE) has been associated with clinical cases of hepatotoxicity, and it can induce hepatocellular damage in rodents (Table 1). Since 1966, at least 216 cases of toxicities attributed to GTE have been reported; most of these cases presented with an acute hepatocellular injury pattern, and most patients recovered after stopping the use of GTE^{8,9}. In 2003, regulatory agencies in France and Spain suspended market authorization for a weight-loss product containing a hydroalcoholic GTE because of hepatotoxicity concerns¹⁰⁻¹². In a recent clinical study of chronic lymphocyte leukemia, Polyphenon E including 400 mg/kg EGCG increased the levels of liver transaminase, suggesting severe liver damage¹³. However, green tea or GTE effectively inhibited the hepatotoxicities induced by 2-nitropropane, galactosamine, carbon tetrachloride, pentachlorophenol and acetaminophen when administered prior to chemical exposure in rats or mice^{14,15}. In

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Table 1. Selected Literature Information about Hepatotoxicity and Carcinogenicity Induced by Green Tea, Green Tea Extract or Catechin in Humans and Rodents

Species	Patients or strains	Exposure	Hepatotoxicity or carcinogenicity	References
Humans	44 years old, ♀	GTE 720 mg/ day, po, >6 months	Jaundice, ALT↑, AST↑, TBIL↑, hepatic necrosis	Molinari et al., 2006 ²⁶
	81 years old, ♀	GTE tablet (90% catechin), po, one tablet/day, 1 month	Jaundice, ALT↑, AST↑, TBIL↑, etc	Mazzanti et al., 2009 ¹¹
	72 years old, ♀	GTE tablet (90% catechin), po, two tablets/day, 3 month	Jaundice, ALT↑, AST↑, TBIL↑, cholestatic hepatitis, granulomatous cholangitis	
	34 cases (19-69 years old), ♂ ♀	Green tea or GTE, po, 2-260 treatments in week	Necrosis, hepatitis, fibrosis, and/or inflammation, etc.	
	42 years old, ♀	Green tea (1 g catechin), po, every other day, 10 days	Multifocal necrosis, hepatocellular apoptosis, mononuclear inflammation	Gallo et al., 2013 ²⁵
	16 years old, ♂	EGCG 400 mg/kg tablet, po, two tablets/day, 2 months	Jaundice, ALT↑, AST↑, hepatic necrosis, inflammation, etc.	Patel et al., 2013 ²⁷
	Patients with chronic lymphocyte leukemia	Polyphenon E 2000 mg/kg (EGCG 400 mg/kg), twice daily, up to 6 months	Increased levels of transaminase (6 of 42 patients)	Shanafelt et al., 2013 ¹³
Mice	BALB/c, Swiss Webster, ♂ ♀	EGCG 50 mg/kg, ip, 7 days	Death, ALT↑, hepatic necrosis	Goodin, 2006 ³³
	CD-1, ♂	EGCG 100, 150, 300 mg/kg, ip, single	Death (150 and 300 mg/kg), ALT↑ (100 mg/kg)	Galati et al., 2006 ³¹
	B6C3F1, ♂ ♀	GTE 1000 mg/kg, po, 14 weeks	Hepatocytic necrosis, inflammation, mitosis, bile duct hyperplasia, glycogen depletion, etc.	Chan P et al, 2010 ³²
	CF-1, ♂	EGCG 750, 1500 mg/kg, po, single	Death, ALT↑, hepatocytic necrosis, apoptosis, liver caspase-3↑, liver MDA↑	Lambert et al., 2010 ⁴¹
	B6C3F1, ♂	GTE 2000 mg/kg, po, single	ALT↑, AST↑, centrilobular necrosis/degeneration	Salminen et al., 2012 ¹⁵
	B6C3F1, ♂ ♀	GTE 300 mg/kg, po, 2 years	No increased number of hepatic tumors	NTP website, 2014 ⁴⁰
Rats	F344, ♂	Multiorgan carcinogenesis model GTE 0.1 and 1 % in diet, 4 weeks	Increased number of GST-P-positive foci	Hirose et al., 1993 ³⁹
	F344, ♂ ♀	GTE 1000 mg/kg, po, 14 weeks	Hepatocytic necrosis, periportal hypertrophy, mitosis, bile duct hyperplasia, etc.	Chan et al., 2010 ³²
	Wistar Han, ♂ ♀	GTE 1000 mg/kg, po, 2 years	No increased number of hepatic tumors	NTP website, 2014 ⁴⁰

GTE, green tea extracts produced by several companies; EGCG, epigallocatechin gallate; ALT, alanine aminotransferase; AST, aspartate aminotransferase; TBIL, total bilirubin; MDA, malonyldialdehyde; GST-P, placental glutathione S-transferase. ↑: increased levels.

our preliminary pharmacological studies of a rat model of retinal degeneration, GTE induced death due to acute hepatotoxicity. The purpose of this study was to characterize and clarify the mechanism of GTE hepatotoxicity in rats following a single intraperitoneal (ip) exposure.

Materials and Methods

Chemical and dose formulation

The GTE used was THEA-FLAN 90S (Ito En, Ltd., Tokyo, Japan; GTE), a decaffeinated product sold as a commercial food additive. GTE is mainly comprised of polyphenols (about 90%) that include tea catechins with a galloyl moiety (70.0%)⁶; epigallocatechin gallate (54%, EGCG), epicatechin (12.4%), gallic acid (2.8%), catechin gallate (0.4%), epigallocatechin (0.3%), gallic acid (0.1%), caffeine (0.0%) and other catechins (0.3%). (THEA-FLAN 90S content information was kindly provided by Ito En, Ltd.) GTE was dissolved in physiologic saline just prior to use.

Animal procedures

The study protocol and all animal procedures were approved by the Animal Care and Use Committee of Kansai

Medical University and were in accordance with the guidelines for animal experimentation at Kansai Medical University. Male and female SPF rats [CrI:CD(SD)] were purchased from Charles River Laboratories Japan (Yokohama, Japan). To examine the sequential changes in hepatotoxicity, at six weeks of age, 33 males and 42 females received a single intraperitoneal (ip) injection of 200 mg/kg GTE dissolved in physiologic saline. Three to six surviving rats of each sex were selected for analysis at 8, 24, 48, and 72 hrs, and 1 and 3 months after GTE injections (Fig. 1). In addition, to confirm the susceptibility to toxicity in older animals, eight female rats were injected with GTE at 18 wks of age and analyzed 7 days after treatment. In our preliminary study, ip injection of 200 mg/kg GTE once or twice induced severe degrees of hepatocellular necrosis one day later, as shown by a histopathological examination; however 100 mg/kg GTE did not induce lesions in the liver. Therefore, in this study, we selected 200 mg/kg as the dosage that can induce hepatotoxicity by single ip exposure. Rats were maintained under specific pathogen-free conditions and had free access to water and a CMF diet (Oriental Yeast, Chiba, Japan). They were housed in plastic cages with paper-chip bedding (Paper Clean, SLC, Hamamatsu, Japan) in an air-condi-

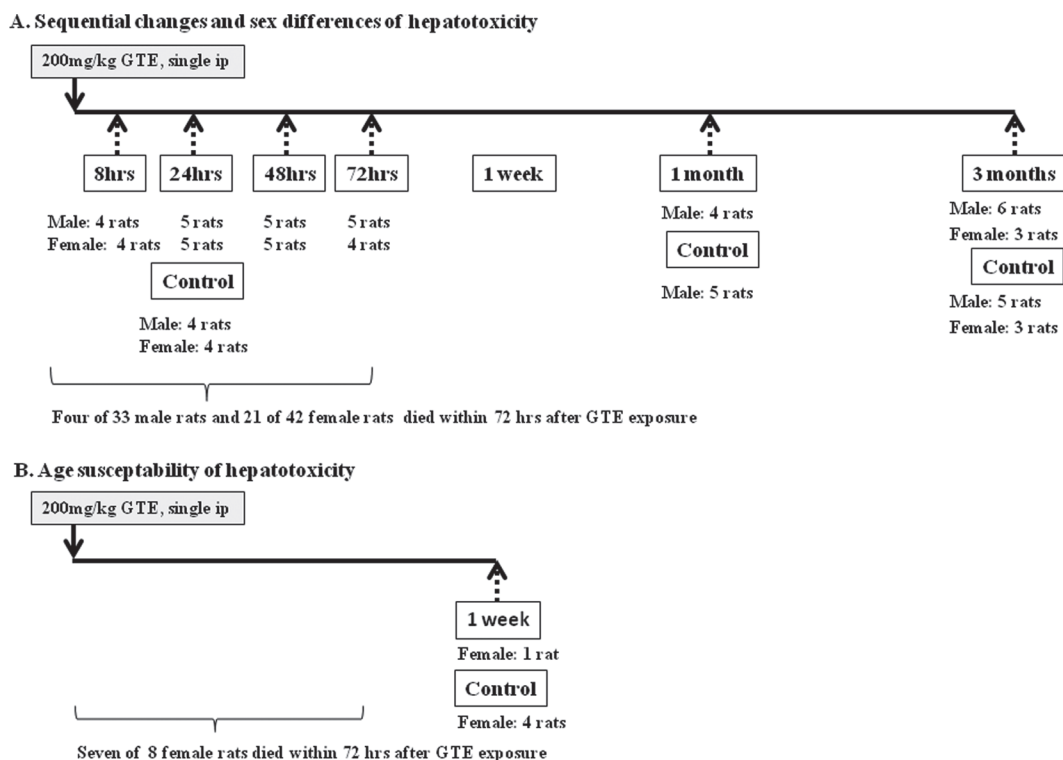


Fig. 1. Experimental protocol. Solid arrows, injection of green tea extract (GTE); broken arrows, time points of necropsy.

tioned room at $22^{\circ}\text{C} \pm 2^{\circ}\text{C}$ and $60\% \pm 10\%$ relative humidity with a 12-hr light/dark cycle. The age-matched control groups consisted of 4 to 5 male and/or female rats that were treated with vehicle (physiological saline) only. Each day, the rats were weighed and observed for clinical signs of toxicity. On the day of sacrifice, GTE- and saline-treated rats were anesthetized with isoflurane (Forane^R; Abbott Japan, Tokyo, Japan) and sacrificed by exsanguination from aortic transection.

Blood chemical analysis to detect hepatotoxicity

At the time of sacrifice, blood samples of all surviving rats were withdrawn from the abdominal aorta under anesthesia. The serum concentrations of aspartate aminotransferase (AST), alanine aminotransferase (ALT), total bilirubin (TBIL) and alkaline phosphatase (ALP) were detected with a Fuji DRI-CHEM 3500V analyzer (Fujifilm Corporation, Tokyo, Japan). AST and ALT levels reflect damage to hepatocytes and are therefore valuable biomarkers of hepatotoxicity induced by chemicals¹⁶, while TBIL and ALP are markers of bile procession alterations and bile duct injury¹⁵.

EGCG concentrations in serum and the liver

Serum and liver samples were collected from male and female rats, two control rats and two GTE-treated rats 48 hrs after the administration of GTE. The serum and hepatic levels of EGCG were determined by HPLC with an electrochemical detector (Coulochem III electrochemical detector, ESA Inc., USA) (HPLC-EC). To determine the total

amount of EGCG, including free and conjugated forms, a plasma sample (200 μL) was incubated with a mixture of β -glucuronidase (500 units) at 37°C for 45 min. Immediately after incubation, 400 μL of acetonitrile and 100 μL of ethyl gallate solution (1 $\mu\text{g}/\text{mL}$) in methanol as internal standard were added to the reaction mixture and vigorously mixed for 5 min, followed by centrifugation at $10,000 \times g$ for 10 min at 4°C to precipitate protein. The resulting supernatant was transferred to another tube and then evaporated to dryness in a vacuum. Then a liver sample (0.2 g) was homogenized with 0.8 mL of saline and centrifuged at $10,000 \times g$ for 10 min at 4°C . The resulting supernatant (400 μL) was treated in the same manner as the plasma sample. The residues were redissolved in 1 mL of 5% methanol aqueous solution and then applied to a Bond Elute cartridge (C18, size 50 mg/1 mL). EGCG in the cartridge was eluted with 1 mL of 50% methanol-50% acetonitrile. The resulting eluents were evaporated to dryness in a vacuum. The residue was redissolved in 200 μL of mobile phase for HPLC (10% methanol, 8% acetonitrile, 0.1% folic acid), filtered through a 0.45- μm filter and then applied to the HPLC-EC system. The analytical conditions were as follows: HPLC column, Inertsil ODS-3 (3 x 150 mm, 5 μm , GL Sciences Inc., Tokyo, Japan); mobile phase, 10% methanol, 8% acetonitrile and 0.1% folic acid; flow rate, 0.4 ml/min; column temperature, 40°C ; detector, ESA Coulochem III, 150 mV.

Malondialdehyde analyses in serum and the liver

The malondialdehyde (MDA) levels, an indicator of free-radical generation that increases at the end of the lipid peroxides¹⁷, were measured in serum and liver tissue of the control and 8, 24, 48 and 72 hrs GTE groups by using an NWLSS kit (Northwest Life Sciences Specialties, Vancouver, Canada). Centrifugation of 10% w/v liver homogenates in cold assay buffer was conducted at 1600 g for 10 minutes at 4°C, and the assay was performed according to the manufacturer's instructions. This assay is based on the reaction of MDA with thiobarbituric acid to form MDA-TBA adducts. The absorbance of reaction mixtures was recorded as spectra from 400-700 nm by using a spectrophotometer, and a 3rd derivative analysis was performed. The results were expressed as micromoles of MDA per gram of wet liver. Serum MDA levels were expressed as micromoles of MDA per liter of the sample.

Histopathological examination of liver

Liver tissues were fixed overnight in 10% neutral buffered formalin, and the right, medial and left lobes were embedded in paraffin, sectioned at a thickness of 4 µm, and stained with hematoxylin and eosin (H&E). Partial left lobes were fixed in Bouin fixative, and paraffin blocks were prepared for immunohistochemical staining of MDA. The histopathological terminology for nonneoplastic hepatic lesions is in accordance with the International Harmonization Nomenclature and Diagnostic Criteria for Lesions in Rats and Mice Project¹⁸.

Immunohistochemical examination of the liver

Sequential sections of livers of the control, 8, 24, 48 and 72 hrs GTE groups were used for immunohistochemical analyses. Cell death was observed by terminal deoxynucleotidyl transferase (TdT)-mediated dUTP digoxigenin nick end-labeling (TUNEL) by using an in situ apoptosis detection kit (Apop-Tag; Millipore, Billerica, MA, USA) according to a previous report¹⁹. Sequential sections were also immunohistochemically stained with anti-active caspase 3 polyclonal antibody (×200; Promega Corporation, Madison, USA) by the modified NIEHS method²⁰. To detect the proliferative activity of hepatocytes in all groups including the 1- and 3-month groups, the monoclonal antibody to proliferating cell nuclear antigen (PCNA) (clone PC10, ×100; Leica Biosystems, Newcastle Upon Tyne, UK) was used²¹. In the control and 8, 24, 48 and 72 hrs GTE groups, immunohistochemistry for malondialdehyde (MDA) (clone 1F83, ×40; Japan Institute for the Control of Aging, Shizuoka, Japan) and thymidine glycol (TG) (clone 2E8, ×20; Japan Institute for the Control of Aging) was conducted to detect the oxidative stress of cytoplasmic lipids and DNA, respectively^{22, 23}. Placental glutathione S-transferase (GST-P) is specifically expressed during rat hepatocarcinogenesis, and it has been used as a reliable tumor marker for experimental rat carcinogenesis²⁴. The liver sections of the control and 1 and 3 months GTE groups were stained with anti-GST-P polyclonal antibody (prediluted; Medical & Biological

Laboratories, Tokyo, Japan). Liver sections fixed in Bouin solution were used for MDA, and liver sections fixed in formalin were used for the other immunostainings. In brief, the staining protocols were as follows: sections were deparaffinized, hydrated and blocked for endogenous peroxidase. Heat-induced epitope retrieval was performed for caspase 3 and PCNA. All primary antibodies were reacted at room temperature for one hour. The antigen-antibody complexes were identified by using a streptavidin-biotin (LSAB) staining kit (Dako, Carpinteria, CA, USA) according to the manufacturer's instructions. The reaction products were visualized with 3-3'-diaminobenzidine tetrahydrochloride. Histopathological and immunohistochemical examinations were conducted by two toxicologic pathologists certified by the Japanese Society of Toxicologic Pathology (K.Y., A.T.).

Statistical analysis

All discrete values except for histopathological and immunohistochemical results were expressed as the mean ± standard error (SE) and analyzed by using the two-tailed independent Student's *t*-test for unpaired samples after confirming the homogeneity of variances. The results presented below include comparisons between GTE-treated rats and saline-treated controls. P-values < 0.05 were considered statistically significant.

Results

General remarks

During the experimental period, the mortality after administration of 200 mg/kg GTE was higher in female rats than in male rats. Acute death within 72 hrs occurred in 4 of 33 males (12% in mortality) and in 21 of 42 females (50% in mortality) that received GTE at the age of 6 weeks (Fig. 1). A 1 week GTE exposure group could not set up due to the large number of dead animals at the age of 6 wks. Because so many 6-wk-old rats died soon after GTE exposure, there were not enough surviving animals to study one week after exposure. Similarly, 7 of 8 females that received GTE at the age of 18 wks died within 72 hrs (88% mortality). The cause of death in these rats was severe liver toxicity, as confirmed by histopathological analysis (data not shown). Susceptibility to GTE-induced toxicity seemed to be higher in older rats.

The body weights in the GTE groups significantly decreased at maximum 12 g and 8.4 g within 24 hrs in males and females, respectively (data not shown). Thereafter, the body weights returned to control levels. At 1 and 3 months after GTE exposure, their weights were comparable to those of the age-matched control rats.

Blood chemistry

The AST levels in the GTE-treated male rats tended to increase with time (Fig. 2). They peaked at 72 hrs in male rats (1268 U/L) and at 48 hrs in female rats (1570 U/L), while they remained low in control rats (male control, 86 U/L; female control, 69 U/L). The AST levels in male rats were

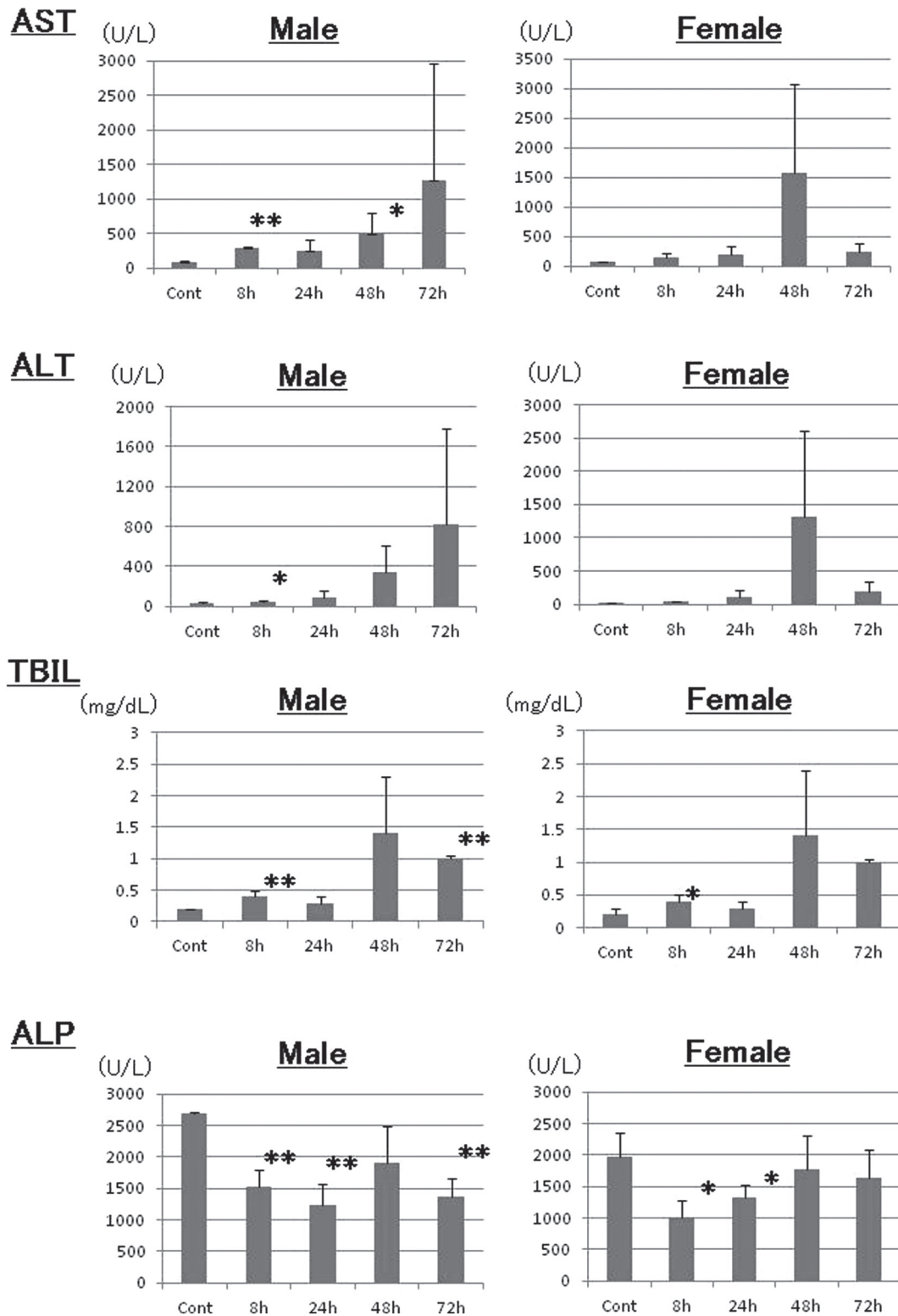


Fig. 2. Blood chemistry. The serum concentrations of aspartate aminotransferase (AST), alanine aminotransferase (ALT), alkaline phosphatase (ALP) and total bilirubin (TBIL) were analyzed. *: $p < 0.05$; **: $p < 0.01$.

significantly increased at 8 and 48 hrs. The ALT levels exhibited the same increasing trend as the AST levels (Fig. 2); the ALT levels peaked at 72 hrs in male rats (818 U/L) and at 48 hrs in female rats (1315 U/L), while the levels remained

low in control rats (male control, 35 U/L; female control, 29 U/L). The ALT levels were significantly increased at 8 hrs in the male GTE-treated rats.

As a marker of bile production alterations and bile duct

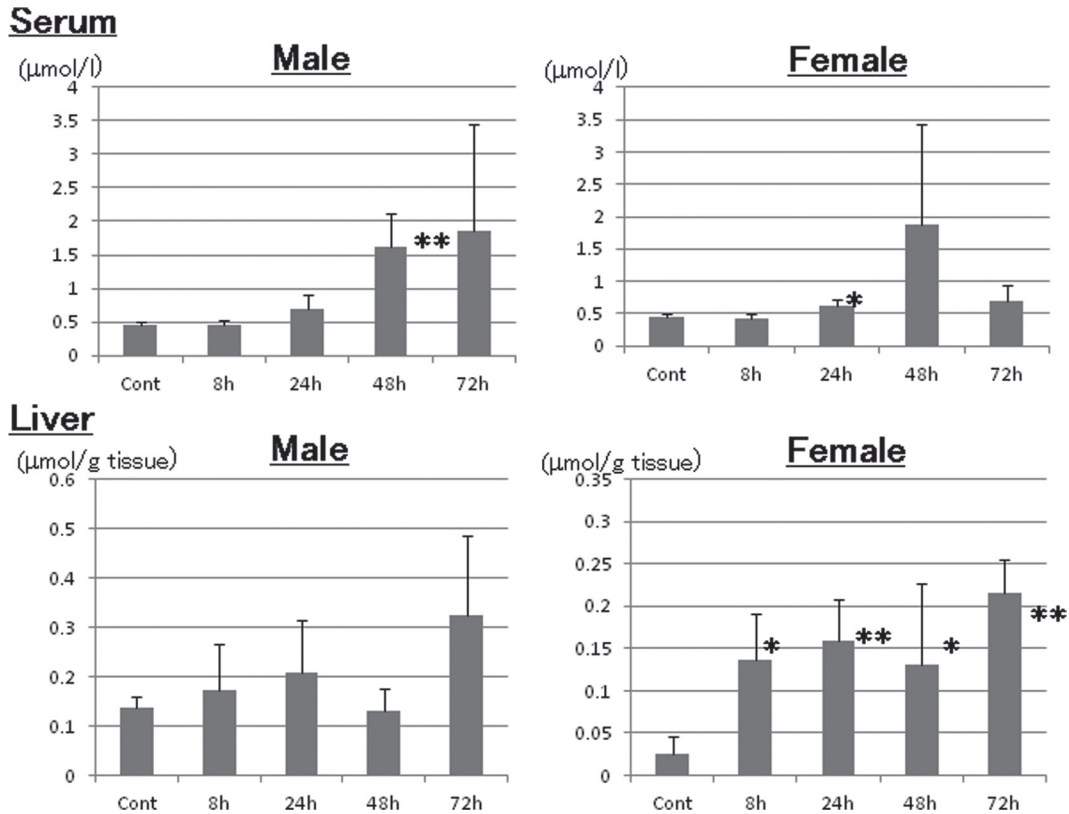


Fig. 3. Analysis of malondialdehyde in serum and the liver. *: $p < 0.05$; **: $p < 0.01$.

injury, there was a trend for increased TBIL levels with time in male and female rats (Fig. 2), and both sexes reached a peak level of 1.4 mg/dL at 48 hrs (male control, 0.2 mg/dL; female control, 0.2 mg/dL). In contrast, the ALP levels decreased to 1530 U/L in males at 24 hrs and 1002 U/L in females at 8 hrs (male control, 2698 U/L; female control, 1970 U/L). The ALP levels returned to control levels in the female rats at 72 hrs, but the decreased levels persisted in males even at 72 hrs (Fig. 2).

At 1 and 3 months after GTE exposure, none of the blood chemistry parameters were different from the levels in the age-matched control rats (data not shown).

EGCG concentrations in serum and liver

EGCG was not detected in the serum or liver of male and female control rats. In the serum of GTE-exposed rats, the EGCG levels reached 0.388 µg/mL (0.880 µM) in males and 0.276 µg/mL (0.602 µM) in females at 48 hrs after GTE exposure. The EGCG levels in the livers of GTE-exposed rats were lower than in the serum; the hepatic levels were 0.107 µg/g tissue (0.232 µM) in males and 0.084 µg/g tissue (0.182 µM) in females.

MDA values in serum and the liver

The serum MDA levels increased beginning a 24 hrs (Fig. 3) and peaked at 72 hrs in males (1.85 µmol/L) and at 48 hrs (1.87 µmol/L) in females (0.44 µmol/L in both male and female controls). There was a trend for increased he-

patic MDA levels beginning at 8 hrs, and the levels peaked at 0.33 and 0.22 µmol/g tissue at 72 hrs in male and female rats, respectively (0.14 and 0.03 µmol/g tissue in male and female controls, respectively). The hepatic MDA levels were significantly increased at 8 to 72 hrs in female rats (Fig. 3).

Liver morphology

The absolute and relative liver weights decreased in males and females at 8, 24 and 48 hrs after exposure (data not shown). The change might be related to the severely decreased body weights. At 1 and 3 months after exposure, the weights in the male and female GTE groups were similar to those in the age-matched control groups.

The histopathological changes induced by GTE exposure are summarized in Table 2. Beginning at 8 hrs after exposure, the levels of glycogen in the hepatocellular cytoplasm of males and females were decreased as compared with the controls (Fig. 4a and b). Single-cell necrosis of hepatocytes appeared in perilobular areas 8 hrs after exposure (Fig. 4b, c and d) and thereafter progressed to all lobular areas as massive necrosis (Fig. 4e). At 72 hrs, the average score of necrosis was 3.0 in both males and females. Inflammatory reactions in the parenchyma and capsules of livers were detected beginning at 24 hrs and 8 hrs after exposure, respectively (Fig. 4c, d, and e). The inflammatory reactions, which were composed mainly of mononuclear cells, suggested acute inflammation. At 72 hrs, the average inflammation scores in the parenchyma were 2.4 and 1.2 in males and

Table 2. Histopathological Scoring of Liver

Group		Hepatocellular single-cell necrosis/hepatocellular necrosis	Inflammatory cell infiltration	Mitosis	Hepatocellular hypertrophy	Bile duct proliferation	Decrease of glycogen in hepatocytes	Capsulitis	Lobular adhesion
Control	Male	0	0	0	0	0	0	0	0
	Female	0	0	0	0	0	0	0	0
GTE 8 hrs	Male	0.8	0	0	0	0	2.0	0.4	0
	Female	0.3	0	0	0	0	1.5	0.5	0
GTE 24 hrs	Male	1.2	0.8	0	0.2	0	1.2	0.6	0
	Female	1.0	0.4	0	0	0	0.8	0.4	0
GTE 48 hrs	Male	2.2	1.2	0.8	0.8	0.4	1.6	0.8	0
	Female	2.4	1.2	0.4	0.4	0.4	0.8	0.6	0
GTE 72 hrs	Male	3.0	2.4	0.4	0.8	0.4	1.2	0.8	0
	Female	3.0	1.2	0.5	0.4	0.3	0.8	0.5	0
GTE 1 month	Male	0	0	0	0	0	0	0	1.0
GTE 3 months	Male	0	0	0	0	0	0	0	1.6
	Female	0	0	0	0	0	0	0	1.3

GTE, green tea extract (THEA-FLAN 90S); 0 = no change, 1 = minimal change, 2 = mild change, 3 = moderate change, and 4 = severe change.

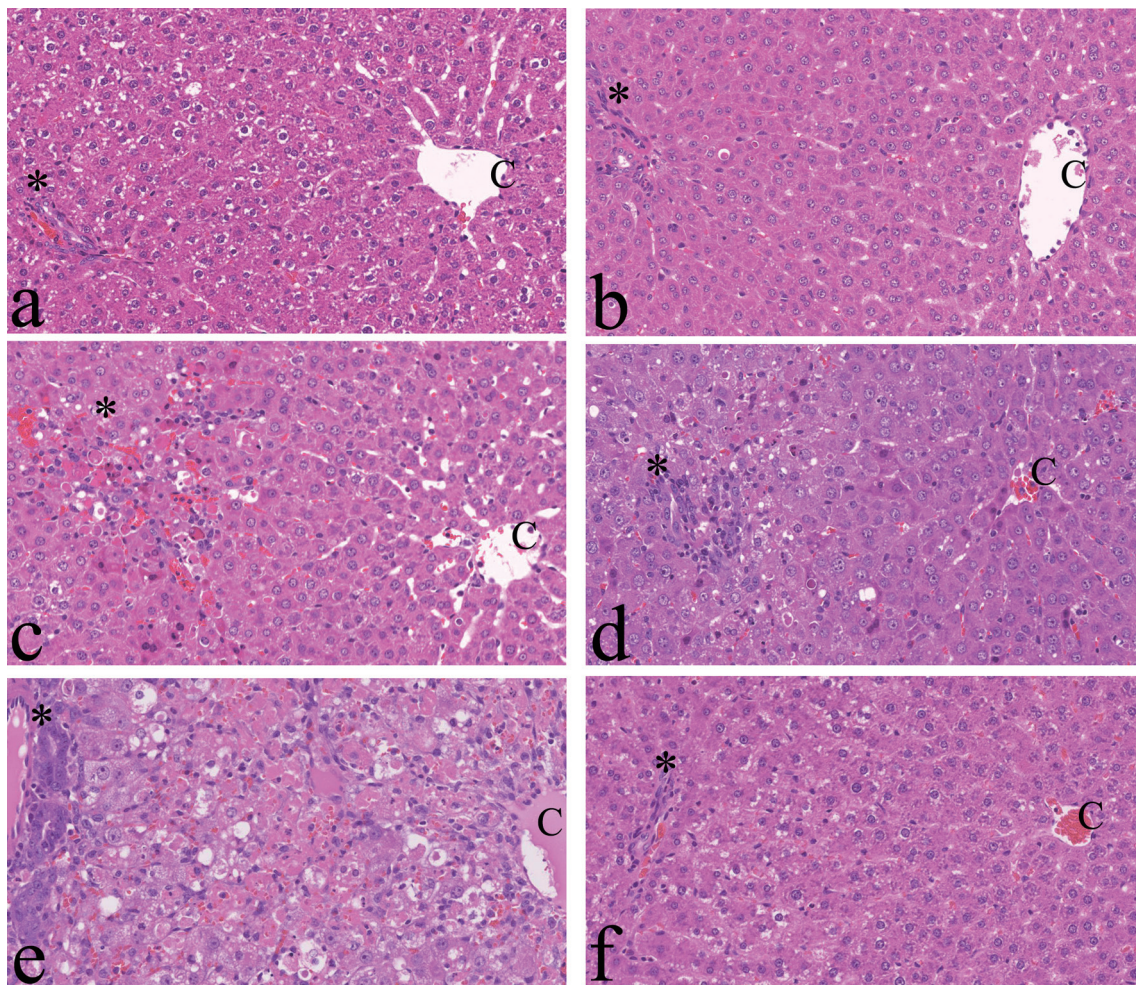


Fig. 4. Histopathological findings. a, Control; b–f, 8 hrs, 24 hrs, 48 hrs, 72 hrs and 1 month after green tea extract (GTE) exposure, respectively. Note that hepatocellular damage progresses gradually from the perilobular (c) to centrilobular area after GTE exposure (e). C: central vein. *Glisson's sheath. H & E, $\times 200$.

females, respectively. Hepatocellular mitosis, hypertrophy (Fig. 4c) and bile duct proliferation (Fig. 4e) were also seen at mainly 48 and 72 hrs after exposure. In some rats of the 1 and 3 months GTE groups, although lobular adhesion related to capsular inflammation was seen, the liver structure was similar to that of the age-matched controls (Fig. 4f).

Immunohistochemistry of the liver

Cell death expression

In control and 8 hrs groups, there were few positive hepatocytes for TUNEL and caspase 3 antibodies (Fig. 5a, b, e, and f). Twenty-four hours after exposure, TUNEL- and caspase 3-positive hepatocytes were seen in the perilobular area of males and females (Fig. 5c and g). Positive cells were scattered to all lobular areas at 48 and 72 hrs (Fig. 5d and h). Single-cell necrosis was correlated with positive results from TUNEL and caspase-3 immunohistochemistry. One and 3 months after exposure, there were a few TUNEL- and caspase 3-positive cells in the GTE-treated rats, which was similar to the age-matched controls.

Proliferative activity

PCNA-positive cells were scattered in the perilobular area of male and female control rats (Fig. 6a). In contrast, at 48 and 72 hrs after GTE exposure, more positive cells were seen in all lobular areas of the male and female GTE-treated rats as compared with the controls (Fig. 6b). In the 1- and 3-month groups, PCNA-positive cells were scattered in the liver of GTE-exposed rats, which was similar to the age-matched controls.

Oxidative stress

In the control group, hepatocytes were negative for TG (Fig. 6c). In the GTE groups, at 48 and 72 hrs after exposure, TG signals were seen in the nuclei of hepatocytes of males and females (Fig. 6d). There was a negative reaction with MDA in control hepatocytes (Fig. 6e), while a positive reaction was detected in the hepatocellular cytoplasm of all lobular areas of males and females at 48 and 72 hrs after exposure (Fig. 6f).

GST-P positivity

In the control female rats at three months, only a few small GST-P-positive foci were seen in GST-P-immunostained slides, and the foci were composed of a small number of hepatocytes (Fig. 7a). In one of three female rats at three months after exposure, large positive foci were scattered throughout the slides and were composed of more than one hundred hepatocytes (Fig. 7b). Positive foci were not seen in any rats one month after exposure, in males three months after exposure, or in the control group at 3 months.

Discussion

In the present study, the toxicological effects of GTE in rats included death, weight loss, and blood chemical and histopathological changes related to hepatocellular damage. Histopathologically, single-cell necrosis with positive results for TUNEL and caspase-3 was seen in perilobular hepatocytes in the early phase and thereafter in all lobular

areas. Cellular hypertrophy and mitoses of hepatocytes, inflammation and bile duct hyperplasia were observed.

Information about hepatotoxicity and carcinogenicity induced by green tea, GTE or catechins in humans and rodents is summarized in Table 1. In some human cases, hepatic necrosis, apoptosis and inflammation were detected histopathologically, as well as elevated levels of transaminases^{11, 25-27}. In rodents, oral or ip exposure to green tea, GTE or catechins also induced severe acute hepatotoxicity as evaluated by blood chemistry and histopathology. General toxicity studies of several types of GTE in rodents have been conducted. In 4 and 26 wk oral studies of GTE-exposed rats conducted by Kao Corporation (Tokyo, Japan), the no-observed-adverse-effect doses were 1000 mg/kg and 400 mg/kg, respectively^{28, 29}, and no hepatic damage could be detected clinicopathologically or histopathologically. Intraperitoneal injection of 82 mg/kg EGCG for 7 days into SD rats did not change biochemical markers of liver damage, such as AST, ALT or ALP³⁰. However, a single ip injection of 150 mg/kg EGCG induced death in mice within one day, while 100 mg/kg elevated the ALP level to 170 U/L, as compared with 49 U/L in controls³¹. Recently, in 14-wk oral toxicity studies of GTE (Amax NutraSource, Inc., Eugene, OR, USA) in rats and mice that were conducted by the National Toxicology Program (NTP)³², 1000 mg/kg GTE induced hepatotoxicity, such as hepatocyte necrosis, hypertrophy, mitosis, glycogen deposition, and bile duct hyperplasia. These changes are similar to our results obtained after a single ip exposure to GTE.

In the present study, mortality after administration of 200 mg/kg GTE was higher in female rats than in male rats. The susceptibility to GTE-induced toxicity seemed to be higher in older rats. From the results of previous studies, female rodents seem to have higher susceptibility to hepatotoxicity induced by GTE or EGCG; ip exposure to 50 mg/kg EGCG for 7 days induced higher mortality due to hepatotoxicity in female BALB/c mice³³, and oral exposure to 1000 mg/kg GTE for 14 weeks induced hepatotoxicity only in female rats. The reasons for the sex- and age-based differences in susceptibility in the present study are not clear, and further *in vivo* research regarding GTE metabolism in the case of ip exposure is required.

Catechins are chemical antioxidants that quench free-radical species. There is evidence that some effects of catechins, such as EGCG, are related to the induction of reactive oxidative stress; these prooxidant effects appear to be responsible for the induction of apoptosis in tumor cells^{3, 34}. GTEs were negative for toxicity in *in vivo* genotoxic studies, such as a bone marrow micronucleus assay, although *in vitro* studies (chromosomal aberration assay or mouse lymphoma assay) revealed a positive trend³⁵. The *in vitro* genotoxic effects of green tea are likely due to H₂O₂, while its genoprotective effects could be due to the direct antioxidant action of green tea or to cellular adaptations to subtle changes in the cellular redox balance induced by the low concentrations of catechins achieved *in vivo*³⁶. *In vitro*, GTE promotes the prooxidant property of catechins to generate active oxygen

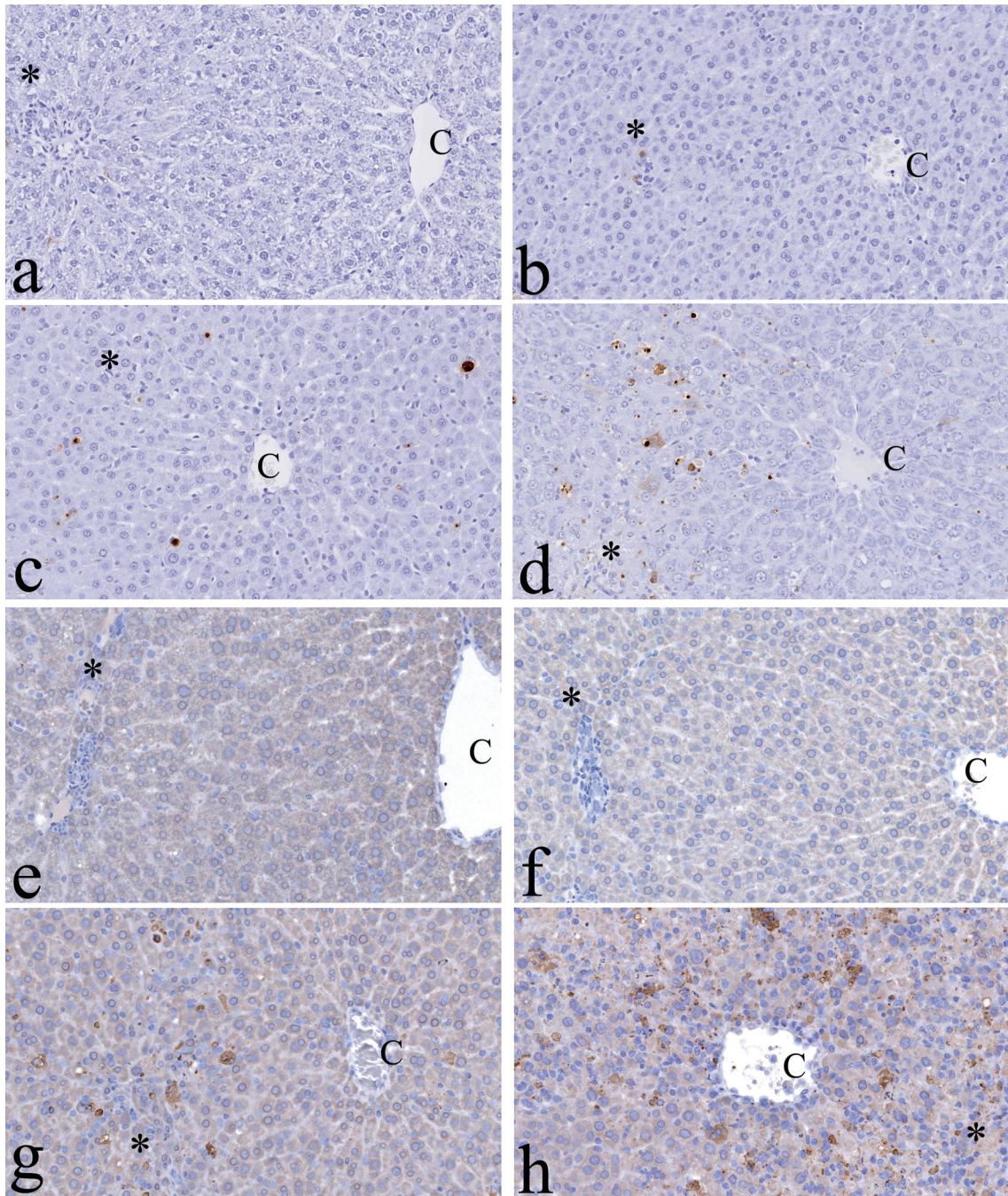


Fig. 5. Immunohistochemistry for cell death in the liver. The signals of terminal deoxynucleotidyl transferase-mediated d UTP digoxigenin nick and end-labeling (TUNEL) can be in the nuclei of hepatocytes of green tea extract (GTE)-exposed rats. a, Control; b–d, 8 hrs, 24 hrs and 48 hrs after GTE exposures, respectively. Caspase 3 signals can also be seen in the nuclei of hepatocytes of GTE-exposed rats. e, Control; f–h, 8 hrs, 24 hrs and 48 hrs after GTE exposure, respectively. C: central vein. *Glisson's sheath. $\times 200$.

species that cause DNA damage^{35,37}.

In the present study, GTE induced hepatocellular apoptosis, as reflected by TUNEL and caspase-3 positivity. Moreover, 48 hrs after treatment, serum and hepatic MDA levels tended to increase and MDA- and TG-positive hepatocytes increased. The most widely used index of lipid peroxidation is MDA formation, often assayed with the thiobarbituric acid (TBA) assay, and TG is one of the DNA hydroxylation

products that acts as an *in vivo* biomarker for oxidative DNA damage¹⁷. Oxidative base modifications may result in mutations, whereas oxidation of deoxyribose moieties may induce base release or DNA strand breaks. Our results suggest that GTE induced oxidative stress in the liver, followed by DNA double-strand breaks and apoptosis in cells.

Seven GTEs significantly enhanced resazurin reduction in an alamarBlue assay at 1-3 mg/ml medium in prima-

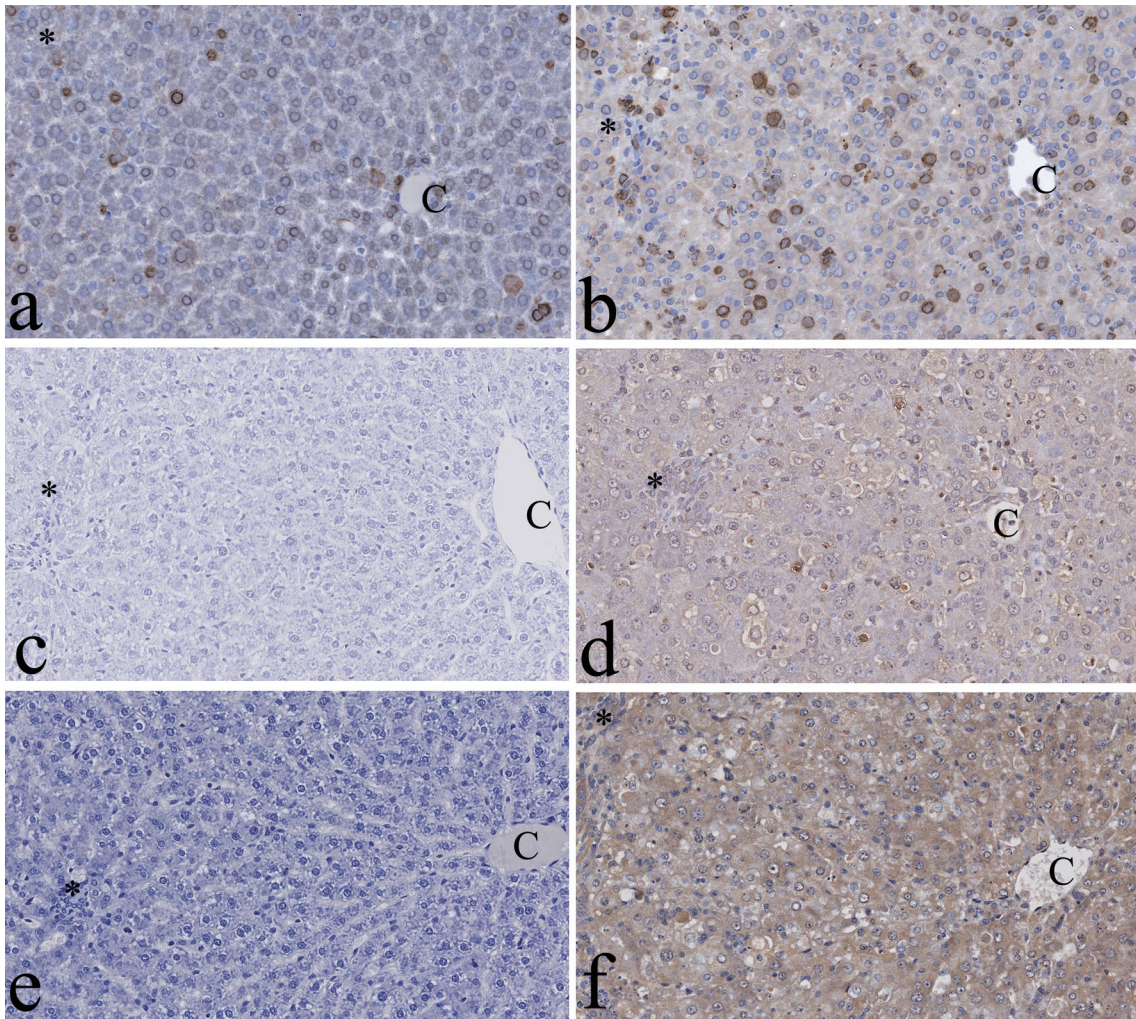


Fig. 6. Immunohistochemistry for proliferative activity and oxidative stress in the liver. Proliferating cell nuclear antigen (PCNA) signals can be seen in the nuclei of hepatocytes of control (a) and green tea extract (GTE)-exposed rats (b). Note that more positive cells can be seen in the rats 48 hrs after GTE exposure (b). No thymidine glycol (TG) signals can be seen in control rats (c), while TG signals in the nuclei of hepatocytes were detected in the rats 48 hrs after GTE exposure (d). No malondialdehyde (MDA) signals can be seen in control rats (e), while positivity for MDA in the hepatocellular cytoplasm was detected in the rats 48 hrs after GTE exposure (f). C: central vein. *Glisson's sheath. $\times 200$.

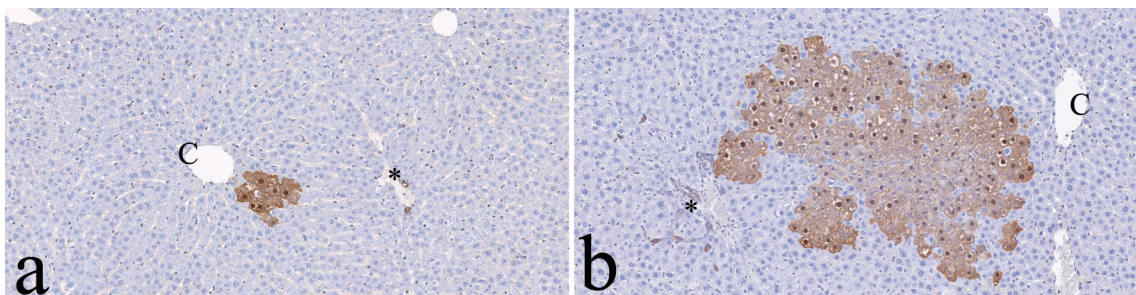


Fig. 7. Immunohistochemistry for placental glutathione S-transferase (GST-P). Note that the larger GST-P-positive foci are found 3 months after green tea extract (GTE) exposure (b) compared with the age-matched controls (a). Bile duct epithelial cells are normally positive for GST-P in both groups. C: central vein. *Glisson's sheath. $\times 100$.

ry culture of rat hepatocytes, and among EGCG, (-)-EC, caffeine and theanine, cytotoxicity was found with EGCG only, calculated 400–1200 μM of EGCG in medium¹². Among four catechins, EGCG, epicatechin-3-gallate, epigallocatechin and epicatechin, EGCG was the most cytotoxic toward isolated rat hepatocytes as it collapsed the mitochondrial membrane and induced reactive oxidative species formation³¹. The LD₅₀ of EGCG cytotoxicity was 200 μM . There is little information *in vivo* about the blood concentration of catechins in the case of intraperitoneal exposure as compared with oral exposures³⁸. After a single ip injection of 100 mg/kg EGCG in SD rats, the plasma EGCG levels were 24, 2, 4, 1 and 1 μM at 0.5, 1, 2, 5 and 24 hrs, respectively. A plasma EGCG concentration of 1 μM would be similar to the levels in a 70-kg human 1 hr after drinking 6–12 cups (200 mL/cup) of tea³⁰. In the present study, the serum and hepatic EGCG concentrations 48 hrs after exposure were 0.880 μM and 0.232 μM in males and 0.602 μM and 0.182 μM in females, respectively. The data revealed high concentrations of EGCG distributed in the livers of rats in the present study. The serum EGCG levels in the early phase after GTE exposure in our study, such as after a few hours, would be much higher than human exposure levels. Further studies are necessary to explain the discrepancy in EGCG concentrations that induce hepatocellular damage between *in vitro* and *in vivo* studies.

In rats, GST-P has been shown to be the most effective marker for altered hepatocellular foci. Single hepatocytes immunoreactive to GST-P that appear early in hepatocarcinogenesis may sometimes represent initiated cells²⁴. The foci may regress by remodeling of the liver. In a rat multiorgan carcinogenesis model, 1% green tea catechins in the diet increased the numbers of altered hepatocellular foci positive for GST-P, suggesting slightly enhanced hepatocarcinogenesis³⁹. However, in the recent NTP rodent carcinogenesis studies, there was no increased occurrence of liver tumors even at 2 years after treatment with 1000 mg/kg GTE administered orally⁴⁰. In the present study, an increased number and area of GST-P–positive foci were seen in one of the three female rats 3 months after exposure to 200 mg/kg ip GTE. The hepatocarcinogenesis of GTE might not occur in humans, as 200 mg/kg GTE is a lethal dose in rats, and ip injection is not the route of exposure in humans.

In conclusion, a single injection of GTE induced severe acute hepatotoxicity in rats via oxidative stress to hepatocellular lipids and DNA. Although GTEs are widely used for their supposed health benefits, their use is associated with adverse events, particularly hepatotoxic reactions. Further studies may be required to elucidate the dose-dependent hepatotoxic effects and underlying molecular mechanism of GTE in our rat model.

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