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A subacute toxicity evaluation of green tea (*Camellia sinensis*) extract in mice

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ABSTRACT

Green tea is believed to be beneficial to health because it possesses antioxidant, antiviral and anticancer properties. The potential toxicity of green tea when administered at high doses via concentrated extracts, however, has not been completely investigated. The objective of the present study was to evaluate the safety of green tea extract in ICR mice using a subacute exposure paradigm. In this study, mice were orally administered (gavage) green tea extract at doses of 0 (as normal group), 625, 1250 and 2500 mg/kg body weight/day for 28 days. The results showed that oral administration of green tea extract did not cause adverse effects on body weight, organ weights, hematology, serum biochemistry, urinalysis or histopathology. Additionally, administering green tea extract via gavage significantly reduced triglyceride and cholesterol levels. These observed effects could be attributed to the high levels of catechins present in green tea as these compounds have been reported to have beneficial health effects. The no-observed-adverse-effect level for green tea extract derived from the results of the present study was 2500 mg/kg body weight/day.

1. Introduction

Green tea (*Camellia sinensis*, Theaceae) is one of the most popular beverages in the world and is deeply rooted in the cultures of China and Japan. Due to the widespread consumption of green tea, the potential biological effects have been studied both *in vitro* and *in vivo*. Most of the beneficial effects of green tea are attributed to the presence of polyphenols. These polyphenols are mainly comprised of catechins and catechin derivatives, including (−)-epigallocatechin-3-gallate (EGCG), (−)-epicatechin (EC), (−)-epigallocatechin (EGC), (−)-epicatechin gallate (ECG) and (−)-gallocatechin gallate (GCG) (Wang et al., 2003). Green tea catechins have been the subject of a considerable amount of research as they are believed to have beneficial effects on health due to their antioxidant (Higdon and Frei, 2003), antifungal, antibacterial (Friedman et al., 2006) and anticancer properties (Bode and Dong, 2009). Green tea catechins have also been shown to protect against 2-nitropropane-induced hepatotoxicity and cisplatin-induced nephrotoxicity in mice (Khan et al., 2009a; Sai et al., 1998).

In particular, EGCG has been the focus of research in recent years due to its relatively high levels in green tea and higher antioxidant activity. Indeed, a considerable body of literature has shown that EGCG arrests the progression of hepatic fibrosis (Zhen et al., 2007) and prevents carbon tetrachloride (CCl_4)-induced liver injury in animal models by inhibiting oxidative damage (Chen et al., 2004). EGCG has also been shown to inhibit lipopolysaccharide-induced tumor necrosis factor-α and inducible nitric oxide synthase production in mice (Yang et al., 1998; Lin and Lin, 1997). Although EGCG is the most plentiful of the green tea catechins and exhibits a high level of antioxidant activity, preventive effects appear to be stronger when a mixture of tea catechins, such as polyphenon E, a decaffeinated green tea catechin mixture, or a green tea extract, are administered (Bode and Dong, 2009; Fu et al., 2009).

A recent study has suggested that green tea extract is safe as a dietary supplement and has many properties that are beneficial for human health (Frank et al., 2009). However, laboratory studies of

Abbreviations: ALP, alkaline phosphatase; ALT, alanine aminotransferase; ANOVA, analysis of variance; AST, aspartate aminotransferase; BUN, blood urea nitrogen; C, (+)-catechin; CCl_4, carbon tetrachloride; EC, (−)-epicatechin; ECG, (−)-epicatechin gallate; EGC, (−)-epigallocatechin; EGCG, (−)-epigallocatechin gallate; GA, gallic acid; GC, (+)-gallocatechin; GCG, (−)-gallocatechin gallate; Hb, hemoglobin; Ht, hematocrit; MCH, mean corpuscular hemoglobin; MCHC, mean corpuscular hemoglobin concentration; MCV, mean corpuscular volume; MPV, mean platelet volume; OECD, Organization for Economic Cooperation and Development; RBC, red blood cell; SD, standard deviation; WBC, white blood cell.

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green tea-derived preparations such as Teavigo, a commercially available green tea polyphenol preparation containing greater than 90% EGCG and isolating from the initial hot water extract with ethyl acetate and subjected to chromatographic separation of EGCG followed by spray drying, in rodents have revealed toxic effects when high doses (2000 mg/kg) were administered intragastrically (*i.g.*) (Isbrucker et al., 2006a). Additionally, *in vitro* studies reported that administration of rat hepatocytes with high concentrations of EGCG resulted in reduced cell viability (Schmidt et al., 2005; Galati et al., 2006). *In vivo* studies also suggest that administration with a single dose of 1500 mg/kg, *i.g.* EGCG in mice may result in hepatotoxicity (Lambert et al., 2010). Due to numerous interactions and synergisms, it is difficult to study the effects of natural dietary supplements on human health when administered in complex mixtures as opposed to a purified compound (Vitaglione et al., 2004). Thus, a conscientious and careful safety evaluation of green tea extract is necessary. The aim of the present study was to evaluate the safety of green tea extract using a subacute toxicity study design. Female and male ICR mice were administered green tea extract orally at doses of 625, 1250 or 2500 mg/kg/day for 28 consecutive days. Clinical observations, including survival, urinalysis, hematology and serum biochemistry, were measured to monitor treatment-related adverse effects in mice. The extent of treatment-related changes in organ tissues was assessed with histopathology.

2. Materials and methods

2.1. Materials and dosing

Gallic acid (GA), (+)-gallocatechin (GC), (−)-epigallocatechin (EGC), (+)-catechin (C), (−)-epigallocatechin gallate (EGCG), (−)-epicatechin (EC), (−)-gallocatechin gallate (GCG) and (−)-epicatechin gallate (ECG) standards were purchased from Sigma Chemical Company (St. Louis, MO, USA) and the purity of all standards are greater than 95%. Orthophosphoric acid and methanol (MeOH) were analytical grade and purchased from Merck (Darmstadt, Germany). Deionized water was prepared using a Mill-RQ and Milli Q-UV water purification system (Millipore Co. Ltd., Taipei City, Taiwan).

Aqueous green tea extract made from natural tea leaves (*C. sinensis*) was obtained from AGV Co. Ltd., Chiayi City, Taiwan. According to the manufacturer's information, green tea extract was prepared by adding tea leaves 5 g to 500 mL of boiling water, steeped for 30 min. The extraction solution was cooled to room temperature and then filtered. The tea leaves were extracted a second time with 500 mL of boiling water and filtered, and the two extraction solution were combined to obtain the green tea extract solution. The green tea extracts solution preparation currently used in fresh green tea drinks in Taiwan and similar to tea brews consumed by humans. In accordance with the company-provided general analysis, the green tea extract was comprised of 70.05% water, 0.84% protein, 0.36% lipid, 28.26% carbohydrate and 0.49% ash.

The dosages selecting in this study were in accordance with the Guidelines of Health Food Safety Assessment set forth by the Health Food Control Act (Department of Health of the Executive Yuan of the Republic of China, 1999). These regulations conform to the OECD Guidelines for Testing of Chemicals, Section 407 (1995). Generally, at least three test groups and a control group should be used. The high dose was selected with the expectation that it would induce observable toxicity but not death or severe suffering. Thereafter, the moderate and low doses were selected to elucidate dose response effects. Two- to fourfold intervals are frequently optimal for setting the descending dose levels. According to the rationale and desirable green tea intake from previous studies, the highest dose level was 2500 mg/kg body weight/day and a descending sequence of dose levels should be selected at 1250 and 625 mg/kg body weight/day. The dose volume for all treatment groups was 1 mL/100 g body weight. The commercial extract was stored at 4 °C and dosing solutions were freshly prepared with distilled water prior to administration. Dosing solutions were prepared based on the most recently recorded body weights to provide an accurate dosage.

2.2. Animals

Male and female ICR mice (20 ± 2 g; 5 weeks old) were obtained from the Animal Department of BioLASCO Taiwan Co. Ltd., Taipei City, Taiwan. Animals were quarantined and allowed to acclimate for 1 week prior to beginning experimentation. Animals were separated by sex and housed 3–4 per cage under standard laboratory conditions with a 12 h light/dark cycle. The animal room temperature was maintained at 25 ± 2 °C with a relative humidity of 55 ± 5%. Air handling units in the animal rooms were set to provide approximately 12 fresh air changes per hour. A standard rodent diet (Rodent LabDiet 5001; PMI Nutrition International, LLC, Richmond, IN, USA) was used for these studies. Appropriate analyses for the constituents and nutrients were performed by the manufacturer and provided to Laboratory Animal Center, Chung Shan Medical University (Taichung City, Taiwan). Food and water were provided *ad libitum*. The experimental protocols for this study were approved by the Institutional Animal Care and Use Committee and the animals were cared for in accordance with the institutional ethical guidelines.

2.3. Experimental design

Animals were randomly divided into four groups consisting of 10 mice of each gender. Group I animals (control) were administered distilled water by gavage throughout the course of the study. Animals in Groups II (625 mg/kg body weight/day), III (1250 mg/kg body weight/day) and IV (2500 mg/kg body weight/day) were orally administered green tea extract dissolved in deionized water by gastric intubation for a period of 28 days. Urinalyses were conducted during the last 4 days of the administration period. Each group animals were collected urine for 24 h and the volume of urine was measured. Animals were individually placed in metabolic cages in batches for a period of 24 h and provided with water but not food. The animals were fasted only in metabolic cages for a period of 24 h. Food and water were provided *ad libitum* during the other 3 days of other animal groups sampling. At the end of the experiment, animals were anesthetized with phenobarbital sodium (6.0 mg/100 g body weight, intraperitoneal injection) and then cut open for blood sampling from the abdominal aorta. After animals had been cut open and the blood was already withdrawn, animals were put in a CO_2 box for euthanasia. This study was in accordance with the Guidelines of Health Food Safety Assessment set forth by the Health Food Control Act (Department of Health of the Executive Yuan of the Republic of China, 1999). These regulations conform to the OECD Guidelines for Testing of Chemicals, Section 407 (1995).

2.4. Clinical observations and survival

Animals were observed twice daily (morning and afternoon) for signs of clinical toxicity and mortality. Body weights were recorded weekly throughout the study period. Mean daily food consumption was calculated twice a week by subtracting the weight of the remaining food from the weight of the supplied food. Clinical examinations were performed twice daily; first at the time of dose administration and approximately 1–2 h following dose adminis-

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tration. Observations included, but were not limited to, changes in skin, fur, eyes, oral mucosa, respiration, circulation and behavior. The circulation was measured for heartbeat, diastolic pressure and systolic pressure with an indirect blood pressure meter (BP-98A, Softron Co. Ltd., Tokyo, Japan). For the detailed physical examinations, animals were moved from their home cage to a standard arena and observed for permanent or semi-permanent changes in gait, posture, and other behaviors. These examinations were conducted weekly beginning 1 week prior to dose administration and continuing throughout the course of the study.

2.5. Urinalysis

Changes in pH, protein, glucose, specific gravity, uric acid and the presence of occult blood or ketones were assessed with urinary test papers (Uropaper III, Eiken Chemical Co. Ltd., Tokyo, Japan). Samples were also examined microscopically for the presence of urinary sediments, including pus, epithelial cells, red and white blood cells and calcium oxalate crystals.

2.6. Hematology and serum biochemistry

Blood samples were measured for clotting time, red blood cell (RBC) and white blood cell (WBC) counts, hemoglobin (Hb), hematocrit (Ht), lymphocytes, platelet count, mean corpuscular volume (MCV), mean corpuscular hemoglobin (MCH), mean corpuscular hemoglobin concentration (MCHC), mean platelet volume (MPV) and lymphocytes with an automatic hematology analyzer (Sysmex KX-21, Sysmex Co. Ltd., Japan) according to the manufacturer's operator's manual. Serum from blood samples collected in separator tubes was analyzed for changes in biochemistry, including aspartate aminotransferase (AST) (AS1267, Randox), alanine aminotransferase (ALT) (AL1268, Randox), alkaline phosphatase (ALP) (AP502, Randox), total bilirubin (BR243, Randox), total protein (TP245, Randox), albumin (AB360, Randox), glucose (GL2623, Randox), blood urea nitrogen (BUN) (UR107, Randox), total cholesterol (CH201, Randox) and triglycerides (TG213, Randox), with commercially available test kits from Randox Laboratories Ltd. (Crumlin Co., Antrim, United Kingdom). Serum electrolytes, such as calcium (DICA-01K, Orgenics), potassium (RCC0059, Orgenics), chloride (DICL-500, Orgenics) and phosphate (POPB-01K, Orgenics), were measured with commercially available test kits from Orgenics Ltd., Yavne, Israel. These test kits have enough sensitive and accurate to allow the determination of all parameters. The sensitivity value of serum biochemical test kit of aspartate aminotransferase, alanine aminotransferase, alkaline phosphatase, total bilirubin, total protein, albumin, glucose, blood urea nitrogen, total cholesterol and triglycerides is 9.3 U/L, 7.99 U/L, 49.9 U/L, 2.95 μmol/l, 0.476 g/dL, 0.444 g/dL, 0.06 mmol/l, 3.00 mg/dL, 13.7 mg/dL, 22.9 mg/dL, respectively. The limit of detection of phosphate, calcium, chloride and potassium is 0.4 μM, 0.08 mg/dL, 0.7 mg/dL and 0.006 mEq/L, respectively.

About 0.9–1.2 mL blood was obtained from each animal to allow the determination of all parameters in this study. For hematological analysis, there were needed about 260 μL blood samples. Serum biochemical analysis of aspartate aminotransferase, alanine aminotransferase, alkaline phosphatase, total bilirubin, total protein, albumin, glucose, blood urea nitrogen, total cholesterol and triglycerides needed 100, 100, 10, 20, 20, 10, 10, 10, 10 and 20 μL serum samples, respectively. Serum electrolytes analysis, such as calcium, potassium, chloride and phosphate, needed total volume of 20 μL serum samples.

2.7. Histopathological assessment

When animals were sacrificed with CO_2, the vital organs, such as brain, heart, lungs, liver, kidneys, stomach, spleen, testes/uterus and bladder, were removed, weighed and fixed in 10% neutral buffered formalin. Tissue slices were embedded in paraffin, sectioned and stained with hematoxylin and eosin. Histopathological assessments were initially performed only on tissues obtained from the control and 2500 mg/kg dose groups. Relevant tissues from the lower dose groups were examined only if treatment-related changes were identified in highest dose group. If no treatment-related changes were identified in highest dose group, tissues from the lower dose groups were needless to examine.

2.8. Determination of catechin

The catechin contents of the green tea extract were analyzed with a high performance liquid chromatography system (Waters e2695, Waters Co., Milford, MA, USA) fitted with a vacuum degasser, quaternary pump, autosampler, thermostatted column compartment, photodiode array detector and a C18 reversed phase column (250 × 4.6 mm, 5-μm particle size; Gemini 5μ C18 110Å, Phenomenex®) (Torrance, CA, USA) as described previously (Wang et al., 2003). The mobile phases consisted of 0.1% orthophosphoric acid in deionized water (v/v; eluent A) and 0.1% orthophosphoric acid in methanol (v/v; eluent B). The mobile phase gradient was as follows: 0–5 min, 20% eluent B; 5–7 min, linear gradient from 20% to 24% eluent B; 7–10 min, 24% eluent B; 10–20 min, linear gradient from 24% to 40% eluent B; 20–25 min, linear gradient from 40% to 50% eluent B. The post-run time was 5 min. Elution was performed at a solvent flow rate of 1 mL/min. Catechins were detected with a diode array detector and chromatograms were recorded at 280 nm. The column temperature was maintained at 30 °C. Samples were injected using a manual injection valve (10 μL injection volume). Peaks were identified by comparing their retention times and UV spectra in the 200–400 nm range with authentic standards.

2.9. Statistical analysis

All values are expressed as the mean ± SD. Comparisons between groups were performed using a one way analysis of variance (ANOVA) followed by Dunnett multiple comparison tests using the statistical software SPSS (Drmarketing Co. Ltd., New Taipei City, Taiwan). Statistically significant differences between groups were defined as $p < 0.05$.

3. Results

3.1. Determination of catechins in the green tea extract

Table 1 shows the catechin composition in the green tea extract used for this study. The amount of total catechins in the green tea extract was 14524.18 μg/mL. EGCG, EGC and ECG were the major catechins identified, comprising 81.03% of total catechins. Other significant catechins identified in the green tea extract were (+)-

Table 1
Catechin contents in green tea extract used on subacute toxicity studies in mice.

Compound	Content (μg/mL)
Total catechin (sum of below mentioned eight catechins)	14524.18
Gallic acid (GA)	74.47 ± 0.57
(+)-Gallocatechin (GC)	630.75 ± 24.75
(−)-Epigallocatechin (EGC)	2879.48 ± 438.45
(+)-Catechin (C)	251.11 ± 43.54
(−)-Epigallocatechin gallate (EGCG)	7254.69 ± 19.94
(−)-Epicatechin (EC)	1050.78 ± 39.02
(−)-Gallocatechin gallate (GCG)	748.53 ± 10.31
(−)-Epicatechin gallate (ECG)	1634.37 ± 4.67

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gallocatechin (GC), (+)-catechin (C), EC and GCG and a phenolic acid, gallic acid was also identified (Table 1).

3.2. Clinical observations and survival

No treatment-related mortality or clinical signs of toxicity, including hair loss, scabbing, soft or mucoid feces, decreased defecation or feces smaller than normal, wet yellow material in the urogenital area or vocalization upon handling, were observed. Animals from all treatment groups appeared healthy at the conclusion of the study period. In general, there were no statistically significant changes in body or organ weights, regardless of sex or treatment group. There were no significant differences in food or water consumption between the control and treated animals (data not shown).

3.3. Urinalysis

Semi-quantitative urinary examinations, such as urine volume, specific gravity, pH, protein, glucose, urea acid, ketone and occult blood, did not reveal any relevant changes following subacute administration of green tea extract. Also, green tea extract treatment did not cause any significant changes in the presence of urinary sediments (data not shown).

3.4. Hematology

There were no test article-related effects of green tea extract on hematology parameters, including the values of RBC, WBC, lymphocytes and platelet counts. Similarly, there were no significant changes in clotting time, Hb, Ht, MCV, MCH, MCHC and MPV values between the control and treated animals (data not shown).

3.5. Serum biochemistry

Serum biochemistry data are summarized in Table 2. There were no significant changes in the levels of serum total protein, albumin, glucose or BUN. No statistically significant differences in serum electrolytes such as calcium, potassium, chloride or phosphate were noted. The effects of green tea extract on liver function parameters such as ALT, AST, ALP and total bilirubin in serum were also investigated. Female mice treated with 625 mg/kg/day green tea extract exhibited significantly decreased serum ALT (24%) and AST (18%). The 1250 and 2500 mg/kg/day exposure groups, however, did not exhibit significant changes in serum ALT, AST, ALP or total bilirubin.

The effects of green tea extract on triglyceride and cholesterol levels are shown in Table 2. Male mice dosed with 2500 mg/kg/day showed significant decreases in triglyceride (41%) and cholesterol (27%) levels. Similarly, female mice treated with the same dose exhibited a 40% decrease in triglycerides and 19% decrease in cholesterol. Similar effects were observed in both sexes at the 625 and 1250 mg/kg/day exposures.

3.6. Histopathology

Histopathological examinations are an important aspect of safety assessments. Macroscopic examination of vital organs found no abnormalities. Histological evaluation of brain, heart, liver, spleen, adrenal, kidney, stomach, intestine, epididymis, uterus, testes and ovaries did not reveal any pathological changes in highest

Table 2
Serum biochemical data for male and female mice orally administered green tea extract for 28 days.

Parameter	Sex	Normal	Green tea 625 mg/kg body weight	Green tea 1250 mg/kg body weight	Green tea 2500 mg/kg body weight
Alanine aminotransferase (U/L)	Male	30.33 ± 4.60	26.47 ± 2.57	33.64 ± 2.09	31.99 ± 3.47
	Female	32.24 ± 3.13	24.46 ± 1.01*	32.91 ± 1.86	34.05 ± 1.80
Aspartate aminotransferase (U/L)	Male	38.57 ± 3.67	30.06 ± 3.69	33.47 ± 2.31	44.24 ± 4.54
	Female	40.89 ± 1.88	33.55 ± 2.37*	40.37 ± 1.92	43.51 ± 2.60
Alkaline phosphatase (U/L)	Male	50.37 ± 6.45	54.97 ± 7.67	55.47 ± 4.80	58.46 ± 6.41
	Female	61.28 ± 5.51	63.22 ± 4.36	65.08 ± 9.04	62.70 ± 3.95
Total bilirubin (mg/dL)	Male	0.62 ± 0.01	0.61 ± 0.01	0.64 ± 0.01	0.63 ± 0.01
	Female	0.72 ± 0.02	0.72 ± 0.03	0.68 ± 0.01	0.70 ± 0.01
Total protein (g/L)	Male	45.93 ± 2.58	43.81 ± 4.37	43.23 ± 3.12	45.61 ± 2.56
	Female	41.36 ± 3.77	42.88 ± 6.15	44.59 ± 3.76	42.13 ± 5.06
Albumin (g/L)	Male	22.20 ± 1.08	20.26 ± 2.13	21.95 ± 2.37	20.65 ± 1.76
	Female	23.31 ± 2.17	23.65 ± 3.22	22.48 ± 1.79	21.58 ± 3.27
Glucose (mg/dL)	Male	78.52 ± 7.38	80.05 ± 10.63	82.49 ± 7.84	79.66 ± 8.15
	Female	75.18 ± 6.49	72.27 ± 7.31	76.25 ± 5.54	74.63 ± 7.10
Cholesterol (mg/dL)	Male	88.31 ± 4.06	72.00 ± 3.83*	67.54 ± 2.37*	64.54 ± 2.50*
	Female	55.17 ± 3.66	54.17 ± 2.30	44.08 ± 1.08*	44.58 ± 1.38*
Triglyceride (mg/dL)	Male	72.54 ± 11.04	56.31 ± 13.08*	43.38 ± 6.34*	42.85 ± 7.39*
	Female	54.42 ± 4.92	43.00 ± 4.93*	37.92 ± 1.80*	32.92 ± 0.98*
Blood urea nitrogen (mg/dL)	Male	53.94 ± 0.65	52.25 ± 0.58	53.51 ± 0.88	52.77 ± 0.86
	Female	52.71 ± 0.73	52.19 ± 0.49	53.46 ± 0.63	52.11 ± 0.91
Calcium (mg/dL)	Male	3.27 ± 0.09	3.20 ± 0.08	3.22 ± 0.07	3.08 ± 0.14
	Female	3.15 ± 0.07	3.09 ± 0.06	3.12 ± 0.09	3.11 ± 0.05
Potassium (mEq/L)	Male	4.88 ± 0.13	4.85 ± 0.09	4.95 ± 0.09	5.04 ± 0.12
	Female	4.63 ± 0.21	4.57 ± 0.07	4.68 ± 0.15	4.71 ± 0.08
Chloride (mEq/L)	Male	98.52 ± 0.96	98.71 ± 1.23	99.41 ± 0.57	101.57 ± 0.97
	Female	95.27 ± 0.84	95.82 ± 0.93	96.78 ± 1.07	96.45 ± 1.55
Phosphorus (mg/dL)	Male	3.75 ± 0.18	3.56 ± 0.26	3.91 ± 0.25	3.90 ± 0.44
	Female	3.65 ± 0.42	3.71 ± 0.19	3.77 ± 0.29	3.63 ± 0.81

Values are mean ± SD (n = 10/sex/dose).
* $p < 0.05$ compare with normal group.

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dose group (data not shown). Relevant tissues from the lower dose groups were needless to examine because there were no identified treatment-related changes in highest dose group.

4. Discussion

Green tea has been reported to possess various physiological and pharmacological properties. Most of the beneficial effects of green tea are attributed to the presence of polyphenols. These polyphenols are mainly comprised of catechins and catechin derivatives, including EGCG, EC, EGC, ECG and GCG (Chengelis et al., 2008). In the present study we identified EGCG, EGC and ECG as the major catechins, comprising 81.03% of total catechins in the green tea extract. Similarly, Wang et al. (2003) observed that green tea leaf extracts were rich in catechins, with EGCG, EGC and ECG accounting for 80.9–87.7% of the total catechins.

The OECD Guidelines for Testing of Chemicals, Section 407 (1995) and United States Environmental Protection Agency Health Effects Test Guidelines, OPPTS 870.3050 (2000) are preferred the rat as the standard rodent species, but are not limited. Furthermore, before the subacute toxicity study, we demonstrated that green tea extract played a protective role in the reduction of oxidative stress and protected the liver against carbon tetrachloride challenge in mice (unpublished data). Based on the excellent hepatoprotective effects of green tea extract, we need to evaluate the safety of green tea extract in same species. Therefore, a conscientious and careful safety evaluation of green tea extract in mice is necessary. Additionally, there is no subacute oral toxicity report to show that green tea has been studied in mice. The mouse has been one of the main mammalian species used in preclinical studies ranging from pharmacology and safety assessment. The use of mice as models in safety evaluations is currently required in international guidelines for both chemicals and pharmaceuticals (Hedrich and Bullock, 2004). FDA Guidelines for Toxicological Principles for the Safety Assessment of Food Ingredients, Redbook 2000, Chapter IV.C.3.a (2003) indicated that short-term toxicity studies are with rats and mice. Therefore, both male and female mice, which are healthy and have not been subjected to previous experimental procedures, should be used in subacute toxicity study.

Repeat dose mouse studies are most often carried out relatively late in the development or safety testing of a drug or chemical and then usually as dose range finding studies for the oncogenicity studies (Hedrich and Bullock, 2004). The results of the present study not only provide scientific evidences to evaluate the safety of green tea extract using a subacute toxicity study design in mice, but also to find the dose range of green tea extract for the subsequent studies in mice, such as carcinogenicity study and anti-senescence study. Furthermore, this is the first time green tea has been studied in subacute oral toxicity in mice.

In addition, the advantages for using mice for research purposes are numerous. First, despite their obvious physical differences, genes from mice and humans are approximately 99% identical. Genes in the mouse and humans function in virtually the same way in a biological context. Second, the mouse genome is easily manipulated through various genetic engineering technologies for various experiments. Third, they are relatively small in size and ease of maintenance reduces the costs of research. Fourth, their accelerated lifespan (1 mouse year = ~30 human years) allows all life stages to be studied. Fifth, their short gestation time (~3 weeks) and large litter size quickly provide a large sample population and enable rapid genetic and pathophysiologic characterizations. Finally, they can be easily handled with practice (Hedrich and Bullock, 2004). Therefore, the mouse is an ideal model organism for various experiments.

Toxicologists usually divide the exposure of animals to chemicals into four categories: acute, subacute, subchronic and chronic. Acute exposure is defined as exposure to a chemical for less than 24 h. Subacute exposure refers to repeated exposure to a chemical for 1 month or less, subchronic for 1–3 months and chronic for more than 3 months. In rodents, a subacute toxicity study is performed to obtain information on the toxicity of a chemical after 30 days or less of repeated administration. For rodents 10 animals per sex per dose are often used and a typical protocol is to give three to four different dosages of the chemicals to the animals (Klaassen, 2001). In the present study, the period of exposure was 28 days so this model we were using is a subacute model. In this subacute toxicity study, male and female ICR mice were orally administered green tea extract at doses of 625, 1250 and 2500 mg/kg/day for 28 days. In fact, studies have suggested that desirable green tea intake is at least 3 cups per day, providing a minimum of 250 mg/day catechins that have benefit for human health (Kono et al., 1992; Jian et al., 2004; Boehm et al., 2009). Thus the doses used in the present study corresponds to 5–20 times the desirable daily dose. Throughout the study, green tea extracts did not result in mortality or toxicity in mice regardless of gender. No treatment-related adverse effects were found for body weights, organ weights, food and water consumption, urinalysis, hematology or serum biochemistry.

Before our study design, the work of paper research for green tea study in subacute oral toxicity had found a considerable body of literature reported by Chengelis et al. (2008). They has reported that rats treated once daily with up to 2000 mg/kg/day of green tea catechins preparation, that has undergone heat sterilization to mimic the catechins composition in marketed beverages, for 28 days did not show mortality or toxicity. The composition of catechin isomers in green tea beverages can be different according to the heat-sterilization conditions as epimerization of tea catechins occurs under heating conditions (Seto et al., 1997). As a result of the heat sterilization process, the test article of Chengelis et al. (2008) report that has undergone heat treatment contains the most epimerized catechins, such as GC, C, (−)-catechin gallate and GCG. However, the green tea extract in the present study was isolated from green tea leaves with boiling water and currently used in fresh green tea drinks in Taiwan, which similar to tea brews consumed by peoples. The composition of catechins in green tea extract of the present study has no epimerization. Therefore, the literature reported by Chengelis et al. (2008) already includes a good rat study, but the objective is difference between the literature and our study.

Morita et al. (2009) reported that daily oral administration of green tea catechins preparations in rats at doses of up to 400 mg/kg/day for 6 months did not result in any adverse effects. The similar results also in clinical study confirmed that two healthy human males were administered aqueous green tea extract (6 capsules/day for a total of 714 mg green tea polyphenols/day) for 3 weeks with no adverse effects (Frank et al., 2009). A randomized, placebo-controlled study in healthy volunteers reported that EGCG or polyphenon E (a decaffeinated extract of green tea containing 60% EGCG) administered at 800 mg/day for 4 weeks did not result in any adverse effects and well tolerated (Chow et al., 2006; Sarma et al., 2008). Our results are in agreement with previous studies Q1 that green tea extracts at dose up to 2500 mg/kg/day for 28 days did not result in mortality or toxicity in mice regardless of gender.

A few safety assessment studies of green tea catechins, however, have been reported to elicit toxic effects in experimental models. Isbrucker et al. (2006a) indicated that *i.g.* administration of 2000 mg/kg Teavigo in rats resulted in 80% mortality. Lambert et al. (2010) also reported that treatment with a single *i.g.* dose of 1500 mg/kg EGCG in mice resulted in 85% mortality and elicited hepatotoxic responses as evidenced by increased hepatic lipid

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peroxidation as well as elevated levels of plasma 8-isoprostane and ALT. EGCG, the major constituent of polyphenol in green tea, was found to elicit cytotoxicity in rat hepatocytes *in vitro* (Schmidt et al., 2005). Galati et al. (2006) observed that EGCG was the most effective at collapsing the mitochondrial membrane potential and inducing ROS formation that was the major cytotoxic mechanism found with hepatocytes. They also reported that liver injury was also observed *in vivo* when EGCG was administered ip to mice, as plasma ALT levels were significantly increased. The serious adverse effects observed in the above studies may be due to the exposure to very high levels of a single catechin compound such as EGCG that is not part of the normal diet, which tea brews consumed by humans usually contain low to moderate levels of complex catechins not a single catechin compound.

In addition, those different toxicological results reported for green tea extracts might, in part, be due to the extraction procedures employed by the investigators. The extraction procedures of green tea had been effect the composition in the extracts. In recent years, EGCG has been the focus of considerable research due to reports of multiple biological effects and the high levels found in green tea. Many human intervention and bioavailability studies using low to moderate doses of EGCG have reported no serious adverse effects (Lee et al., 2002; Bettuzzi et al., 2006; Chow et al., 2006). Furthermore, Isbrucker et al. (2006b) found that EGCG was not mutagenic in a bacterial reverse mutation assay using *Salmonella typhimurium* and dietary administration of 400, 800 or 1200 mg EGCG/kg/day in mice for 10 days did not induce formation of bone marrow cell micronuclei. Isbrucker et al. (2006a) also reported that dietary administration of an EGCG preparation to rats for 13 weeks did not elicit toxicity at doses up to 500 mg/kg/day. Similarly, no adverse effects were noted when twice-daily of administrations 50, 125 and 250 mg EGCG/kg/day were treated to provide 1 h after feeding Beagle dogs. Vitaglione et al. (2004) reported that, when ingested with food, complex mixtures of natural products have more complicated effects on human health than pure compounds. In the present study, ICR mice administered at up to 2500 mg green tea extract/kg/day for 28 days did not cause mortality or hepatotoxicity, regardless of gender.

Additionally, our study has shown that the only significant reduction in serum ALT and AST activity occurred in the female mice that ingested 625 mg/kg. Similar results have been reported in other studies: Almurshed (2006) found that green tea extract significantly reduced the serum ALT levels and Yasuda et al. (2009) reported that drinking water supplemented with 0.1% EGCG significantly decreased both AST and ALT serum levels of in rat livers exposed to CCl_4. Furthermore, green tea catechins also have been shown to play a hepatoprotective role by reducing serum ALT and AST levels following 2-nitropropane-induced liver damage (Hasegawa et al., 1995). EGCG also has been reported to prevent liver injury induced by a number of hepatotoxicants in animal models (Sai et al., 1998; Chen et al., 2004).

Oral administration of green tea extract significantly reduced levels of triglycerides ($\geqslant$40%) and cholesterol ($\geqslant$17%) in serum. Indeed, a considerable body of experimental work has reported that treatment with green tea catechins can reduce serum cholesterol and triglyceride levels in rodents fed with either a normal or high fat diet (Chan et al., 1999; Ito et al., 2008). Chaudhari and Hatwalne (1977) reported that green tea catechins inhibited liver fat accumulation in rats and several clinical studies have associated daily consumption of a green tea catechins supplement with decreased body fat accumulation (Nagao et al., 2001, 2005). Löest et al. (2002) demonstrated that that green tea extracts markedly inhibited the lymphatic absorption of dietary lipids, such as cholesterol and alpha-tocopherol, which may partly explain the observed lipid-lowering properties. Additionally, green tea catechins have been reported to reduce gentamicin- and cisplatin-induced serum cholesterol and triglyceride levels in rats (Khan et al., 2009a,b). Based on these findings, reductions in serum cholesterol and triglyceride levels in mice treated with green tea extract could be attributed to the high concentrations of catechins.

Green tea, including catechins such as EGCG, EGC and ECG or phenolic acid such as gallic acid, plays an important role in protecting cells and organisms against the harmful effects of light, air and chemicals. The primary mechanism of action of this phenomenon appears to be the ability of green tea to quench excited sensitizer molecules and singlet oxygen (Higdon and Frei, 2003). Recently, many studies demonstrated that green tea extract, which contains abundant catechins, were found to take precautions against various cancer and no serious adverse effects (Sarma et al., 2008; Clement, 2009; Boehm et al., 2009). Therefore, drinking the catechins enriched green tea did not result in any adverse at moderate, regular and habitual use.

In conclusion, the results of the present study clearly show that oral administration of green tea extract at up to 2500 mg/kg body weight/day for 28 days did not cause either mortality or toxicity mice, regardless of gender. Therefore, the no observed adverse effect level for green tea extract derived from our results was 2500 mg/kg body weight/day.

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