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Safety evaluation of green tea polyphenols consumption in middle-aged ovariectomized rat model

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Abstract

The present work evaluates chronic safety in middle-aged ovariectomized rats supplemented with different dosages of green tea polyphenols (GTP) in drinking water. The experiment used 6-month-old sham (n=39) and ovariectomized (OVX, n=143) female rats. All sham (n=39) and 39 of the OVX animals received no GTP treatment and their samples were collected for outcome measures at baseline, 3 months, and 6 months (n=13 per group for each). The remaining OVX animals were randomized into 4 groups receiving 0.15%, 0.5%, 1%, and 1.5% (n=26 for each) of GTP (wt/vol), respectively, in drinking water for 3 and 6 months. No mortality or abnormal treatment-related findings in clinical observations or ophthalmologic examinations were noted. No treatment-related macroscopic or microscopic findings were noted for animals administered 1.5% GTP supplementation. Throughout the study, there was no difference in the body weight among all OVX groups. In all OVX groups, feed intake and water consumption significantly decreased with GTP dose throughout the study period. At 6 months, GTP intake did not affect hematology, clinical chemistry, and urinalysis, except for phosphorus and blood urea nitrogen (increased), total cholesterol, lactate dehydrogenase, and urine pH (decreased). This study reveals that the no-observed-adverse-effect level (NOAEL) of GTP is 1.5% (wt/vol) in drinking water, the highest dose used in the present study.

Practical Application: The NOAEL of green tea polyphenols will provide a basis to design a future long-term human clinical trial using postmenopausal women.

Conflict of interest

The authors declare that there are no conflicts of interest.

Introduction

Tea, the dried leaves of the plant *Camellia sinensis*, makes a popular beverage consumed worldwide. Green tea polyphenols (GTP), such as flavonols or catechins, are making up 30 to 40% of the extractable solids of dried green tea leaves (Yang and others 2001). The main catechins in green tea are catechins, epicatechin (EC), epicatechin-3-gallate (ECG), epigallocatechin (EGC), and epigallocatechin-3-gallate (EGCG, major) (Yang and others 2001).

Our previous studies demonstrated the osteoprotective benefits of GTP in estrogen-deficient bone loss ovariectomized (OVX) animal model using 0.5% of GTP (weight/volume) in drinking water (Shen, et al. and others 2008; Shen and others 2009b). However, Iwaniec et al. reported that green tea extract supplementation (1% and 2% of diet) to both lean and genetically obese leptin-deficient (ob/ob) wild male mice caused detrimental effects on bone health (Iwaniec and others 2009). The discrepancy between Shen's and Iwaniec's studies may be due to differences in dosage of green tea, bone models, and age of animals, reflecting different stages of bone metabolism. Although green tea has been considered a relatively safe beverage and a rich source of antioxidants, a high dosage of green tea in Iwaniec's study may become a source of prooxidants that has a detrimental impact on bone matrix. Furthermore, there is evidence suggesting that high dose of EGCG, a major ingredient of green tea extract, has potential toxicity to animals (Isbrucker and others 2006a; 2006b; and 2006c) and humans (Chow and others 2003, Crew and others 2012, Dryden and others 2013).

The impacts of long-term GTP consumption on bone health and its potential toxicity in animals and humans are still unknown. A study designed to evaluate the effects of long-term GTP supplementation in OVX animals would allow translation of findings to long-term GTP supplementation in postmenopausal osteopenic women. The current study of long-term GTP supplementation in animals included investigation of both safety and changes of bone properties in middle-aged OVX rats that were selected as an animal model to simulate the estrogen-deficient condition for postmenopausal osteoporosis study (Kimmel 1996). While the effects of GTP on bone properties in OVX rats will be reported in a different publication, the current study focuses on the safety evaluation in these middle-aged OVX rats supplemented with different dosages of GTP in drinking water for 6 months. The objective of the present work is to evaluate chronic toxicity and safety in middle-aged OVX rats supplemented with different dosages of GTP in drinking water. Our hypothesis is that 6 months of GTP supplementation up to 1.5% weight/volume in drinking water would be safe in the OVX animals, in terms of parameters of clinical hematology, clinical chemistry, urine analysis, and histopathology.

Materials and Methods

GTP materials

Green tea polyphenols (decaffeinated) was purchased from Zhejiang Yixin Pharmaceutical Co., Ltd. (Zhejiang, China). The procedure of GTP production involved extracting tea leaves with hot water followed by spray-drying and extraction with ethyl acetate. The resulting

green tea extract was further purified by column chromatography, yielding a final product of higher than 98.5% purity. No excipient materials were added or contained in the final product. An authentication reference of GTP, including EGCG, ECG, EC, and EGC in dry powder form, is commercially available from Sigma Chemical Co. (St. Louis, MO). According to the results of HPLC, the study GTP consisted of 65.37% of EGCG, 19.08% of ECG, 9.87% of EC, 4.14% of EGC, and 1.54% of catechin.

The GTP product was also checked through batch analysis by the FDA-certified laboratory at Zhejiang Standard Bureau of Product Inspection for contaminants, including heavy metals, pesticide residues, and microbes, to ensure meeting the maximum levels of US regulation limits. GTP extract was stored at -80°C , a temperature under which GTP extract is very stable and has at least 2 years of shelf life.

Animal group and experimental design

Six-month-old virgin Sprague Dawley female rats (n=182, Harlan Laboratories, Indianapolis, IN) were used in the present study. Prior to arriving at the experimental site, 143 rats had bilateral OVX through the dorsal approach under anesthesia, and the other 39 rats had sham operation under the same anesthesia condition, all performed by the vendor. After arrival, all animals were acclimated for 7 days to a pelleted AIN-93M diet (Dyets, Bethlehem, PA) and distilled water *ad libitum* prior to GTP treatment. Animals were grouped by body weight (BW) stratification and randomization 1 day prior to the initiation of GTP treatment.

The sham rats (n=39) were assigned to 3 groups (n=13/group), and the OVX rats (n=143) were assigned to 11 groups (n=13/group). There are 6 treatment arms: Sham-control, OVX-control, OVX+0.15%GTP, OVX+0.5%GTP, OVX+1.0%GTP, and OVX+1.5%GTP. Table 1 lists the treatment arms and numbers of animal samples collected at each sample collection time. In this study, we chose to administer GTP via drinking water to be consistent with our previous animal GTP studies (Shen et al., 2008; Shen et al., 2009).

All rats were fed the pelleted AIN-93M diet during the 6-month feeding period. Rats had free access to distilled water containing GTP. Distilled water mixed with GTP was prepared fresh daily. Rats were housed in individual stainless steel cages ($21\pm 2^{\circ}\text{C}$, 12 h light-dark cycle). All procedures were approved by the local Institutional Animal Care and Use Committee. All the non-clinical laboratory tests including hematology, clinical chemistry, urine analysis, and histopathology were conducted by Experimur Co. (Chicago, IL, USA). Experimur Co. is fully compliant with FDA, EPA, OECD and JMHW GLPs to conduct hematology and clinical chemistry measurements of our rat specific QC samples.

Clinical examination, body weight, water consumption, and feed intake

Throughout the study period, all animals were observed once daily for clinical signs of toxicity, mortality, and morbidity. The animals were subjected to detailed clinical examination before initiation of the treatment and weekly thereafter. Clinical examination was conducted outside the cage and included locomotor activity, gait, posture, tumor development, skin and hair coat, stereotypies, eyes, and other observations. Animal BW

were recorded at receipt, the day of treatment initiation, and weekly thereafter. Individual animal feed intake and water consumption were recorded weekly.

Measurement of serum and urinary GTP ingredients

The concentrations of GTP ingredients in serum and urine were determined following a method described in Luo et al. (Luo and others 2005). Thawed serum and urine samples were centrifuged and 1 ml supernatant was taken for a 1 h digestion with 500 U of β -glucuronidase and 2 U of sulfatase (Sigma) to release conjugated tea polyphenols. Urine samples were extracted twice with ethyl acetate. Organic phases were pooled, dried *in vacuo* with a Labconco Centrivap concentrator (Kansas City, MO), reconstituted in 15% acetonitrile, and analyzed with the ESA HPLC-CoulArray system (Chelmsford, MA). System consisted of double Solvent Delivery Modules (Model 582 pump), Autosampler (Model 542) with 4°C cool sample tray and column oven, CoulArray Electrochemical Detector (Model 5600A), and an operating computer. The HPLC column was an Agilent Zorbax reverse-phase column, Eclipse XDB-C₁₈ (5 μ m, 4.6 mm $\times$ 250 mm). Mobile phase included buffer A (30 mM NaH₂PO₄/CAN/THF=98/1.8/0.2, pH 3.36) and buffer B (15 mM NaH₂PO₄/CAN/THF=30/63/7, pH 3.45). Flow rate was set at 1 ml/min and the gradient started from 4.0% buffer B, to 24% B at 24 min, to 95% B at 35 min, kept at 95% to 42 min, dropped to 4% at 50 min, and maintained at 4% to 59 min. Authentic standards were prepared with ascorbic acid, and aliquots of the mixture stock were stored at -80°C. Calibration curves for individual GTP component were generated separately, and ECG, EC, EGCG, and EGC were eluted at 14, 21, 24, and 29 min, respectively. Electrochemical detector was set at -90, -10, 70, and 150 mV potentials, with the main peaks appearing at -10 mV (epigallocatechin, EGC), 70 mV (epicatechin, EC; EGCG), and 150 mV (epicatechin-3-gallate, ECG). Quality assurance and quality control procedures were followed during the analyses, including analysis of authentic standards for every set of five samples and simultaneous analysis of spiked urine sample daily. Limits of detection were 1.0 ng/ml-urine for EC and EGC, and 1.5 ng/ml-urine for EGCG and ECG, respectively. Urinary GTP components were adjusted by creatinine level (Diagnostic Creatinine Kit, Sigma).

Ophthalmoscopic examination

At baseline, 3 and 6 months, the Sham-control, OVX-control, OVX+1% GTP, and OVX +1.5% GTP groups were subjected to ophthalmological examination using a standard indirect ophthalmoscope (Keeler®) and a standard 28 Diopter eye examination lens (Nikon®). For each study animal, the bilateral eye exam included (i) an initial non-dilated examination of the external eye (eyelids and conjunctiva) and the anterior segments (cornea, anterior chamber, iris and lens) using direct illumination and the 28 Diopter lens, and (ii) dilation with one drop of Cyclomyril® (Alcon®) in each eye and a 15-minute pause, followed by direct examination of the lens again using the 28 Diopter lens and then the posterior segments (vitreous, optic nerve, retina vessels, posterior retina and peripheral retina) via the standard indirect eye exam with the indirect ophthalmoscope along with a 28 Diopter lens.

Hematology and clinical chemistry

At baseline, 3 and 6 months, overnight fasting blood samples were collected using K2-EDTA tubes for hematology and clinical chemistry analyses by Advia® 2120 Hematology analyzer (Siemens Corporation, Washington DC, USA) and AU400e® (Beckman Coulter Inc., Brea, CA, USA), respectively. Hematologic parameters included hemoglobin (HGB), hematocrit (HCT), erythrocyte count (RBC), total leukocyte count (WBC), mean corpuscular volume (MCV), mean corpuscular hemoglobin (MCH), mean corpuscular hemoglobin concentration (MCHC) and platelet count (PLT), automated differential leukocyte count [neutrophils (NEUT), lymphocytes (LYMPH), monocytes (MONO), eosinophils (EOS), basophil (BASO), and large unstained cell (LUC)], automated reticulocyte counts (RETIC), automated red cell morphology and blood clotting time [prothrombin time (PT) and activated partial thromboplastin time (aPTT)]. Clinical chemistry parameters included aspartate aminotransferase (AST), alanine aminotransferase (ALT), total bile acid (TBA), alkaline phosphatase (ALKP), total bilirubin (T.BILI), gamma glutamyl transference (GGT), albumin (ALB), globulin (GLB-calculated), total protein (T.PRO), calcium (Ca), chloride (Cl), phosphorus (P), potassium (K), sodium (Na), total cholesterol (CHOL-total), triglycerides (TRIG), glucose (GLU), creatinine (CREAT), blood urea nitrogen (BUN), creatine phosphokinase (CPK) and lactate dehydrogenase (LDH).

Urine analysis

At baseline, 3, and 6 months, urine samples were collected from all groups for urine analyses. Urine parameters included physical properties (color, appearance, volume), chemistry (specific gravity, pH, glucose, protein, ketone bodies, occult blood, bilirubin, urobilinogen, nitrite, and leukocyte esterase) using the Clinitek®Advantus Urine Chemistry Analyzer (Siemens), and sediments (WBC, RBC, renal, transitional, squamous, mucus, bacteria, yeast, and crystals) microscopically.

Gross necropsy, tissue collection, and organ weights

Study animals were subjected to complete gross necropsy, including examination of external surfaces, orifices, cranial, thoracic and abdominal cavities, carcass, and all organs. Organs were carefully dissected and trimmed to remove fat and other contiguous tissue and were weighed immediately to minimize the effects of drying on organ weight. Organs included liver, kidneys, adrenals, spleen, heart, spleen, brain, uterus, and thymus. Paired organs were weighed together.

Histopathological examination

All gross lesions were examined microscopically. All tissues from the animals in the Sham-control, OVX-control, and OVX+1.5% GTP groups were examined histopathologically at the baseline and 6 months. All tissues from animals that died prematurely or were sacrificed during the study were examined microscopically. Collected tissues were fixed in 10% buffered formalin, the tissues were trimmed and paraffin blocks were made. Sections (5 µm) of tissues were prepared, and further stained with hematoxylin and eosin for microscopic examination by a board-certified veterinary pathologist.

The tissues processed for histological examination included digestive system (salivary gland, esophagus, stomach including glandular and non-glandular, duodenum, jejunum, ileum, cecum, colon, rectum, liver including middle, left and triangular lobes, and pancreas), respiratory system (trachea and lung with bronchi), cardiovascular system (heart and aorta), reticulo-endothelial/hematopoietic system (bone-femur, bone marrow-sternum, mandibular lymph node, mesenteric lymph nodes, spleen, and thymus), urogenital system (kidneys, ovaries with oviducts, uterus with cervix, urinary bladder, and vagina), neurologic system (brain with 3 different levels, spinal cord-cervical, spine cord-thoracic, spinal cord-lumbar, and sciatic nerve), glandular system (adrenal gland, Harderian gland, mammary glands, Zymbals gland, pituitary gland, and thyroid/parathyroid glands), and others (eyes with optic nerve, fat, skeletal muscle, skin, tongue, larynx, and gross lesions). Tissues with gross lesions observed during necropsy (in interim sacrifice groups and remaining terminal sacrifice groups) and animals found dead or sacrificed in moribund status were also processed as mentioned above. The resulting tissue sections on glass slides were examined by a board-certified veterinary pathologist and all microscopic pathology findings were entered into a validated pathology computer program (Provantis™ 8.4.2.0, Data Management System).

Statistical analysis

All data analyses were conducted using Statistical Analysis System (SAS, N. Carolina, US). A linear regression model was used to analyze the continuous outcomes (i.e., clinical hematology, clinical chemistry, and urine analyses) including the indicators for Sham-control group at 3 months and 6 months; the interactions between GTP groups at 3 months and 6 months; and the interactions between Sham-control and 3 months and 6 months. The intercept corresponded to the mean for the OVX-control at baseline. This model was equivalent to a linear regression including the means for each group at different time as parameters. An overall test of difference among the treatment by time groups were conducted based on linear regression model. If there was a significant difference, we compared the Sham-control with the OVX-control at baseline (0 month), 3 and 6 months, as well as the OVX-control and GTP-supplemented groups at 3 and 6 months. We also evaluated the effect of 3 and 6 months relative to 0 month for each treatment. Note that the OVX-control worked as 0 month reference for all GTP groups as GTP treatment had not yet started at 0 month. Holm's method was applied to adjust for multiple comparisons of continuous variables.

For longitudinal observations of BW, feed intake, and water consumption, a linear mixed effects model was used to characterize the trajectory for each group starting from week 1 when the intervention started. Since animals in different groups had different follow-up time, it caused a problem of missing data after animals were sacrificed. Mixed effects model can deal with such missing-at-random data. We compared models with linear trajectory, quadratic trajectory and piecewise linear trajectory and various error correlations, and selected the model based on Bayesian information criterion (BIC). Among these models, the piecewise linear trajectory model was selected for BW and the quadratic trajectory model was selected for both feed intake and water consumption, all with AR(1) correlated errors between the longitudinal in each group.

For discrete variables such as urine color and appearance, at 0, 3, and 6 months, the Fisher's exact test was used to determine if there was any overall difference among the groups. If there was a significant difference, a separate Fisher's exact test was conducted to compare each group with the OVX-control group.

Results

Survival clinical observations

One animal in the OVX-control group developed an abdominal peritoneal cystic lesion at 5 weeks. Although the cystic mass was large enough to compress the surrounding organs, no cyst was observed in those organs. This animal was terminated at 9 weeks. One animal in the OVX+0.15%GTP group was found dead at 9 weeks without any negative medical record. Two animals in the Sham-control group developed non-invasive mammary tumor at 22 weeks and 23 weeks, respectively. Both animals were terminated at the scheduled sample collection times. Throughout the study, except for these 4 animals described above, there was no sign of departure of study animals from normal activity, morbidity, and mortality based on daily observation of individual animals. Based on weekly expanded clinical observation, there was no abnormal activity, changes in gait, posture, skin, fur, eyes, response to handling, stereotypic (e.g., excessive grooming, repetitive circling) or bizarre behavior in all animals throughout the study period.

Body weight, feed intake, and water consumption

Figure 1 presents the estimated mean body weight for different treatments. At baseline (i) the OVX-treated groups had significantly larger BWs than the Sham-control rats; and (ii) there was no significant difference in BW among all the OVX treatment groups. Throughout the study period, BW significantly increased in all animals, regardless of treatment. Relative to the Sham-control group, all OVX groups had significantly greater BW. During the first month, mean BW increased at a rate between 12.81 and 9.60 g/week in OVX groups, while that in the Sham-control group was 0.98 g/week. From 5 weeks to the end of the study, the BW increase rate significantly dropped in all treatment groups. There was no significant difference in BW among all OVX groups at the end of the study.

Figure 2 presents estimated mean feed intake of all groups. At week 1, the mean daily feed intake is between 20.11 g and 17.18 g in OVX groups, and 14.83 g in the Sham-control group. In general, starting 1 week to 18 weeks, the mean daily feed intake decreased in all OVX groups, while that increased in the Sham-control group, and the difference in feed intake between the Sham-control group and the OVX groups attenuated. Starting 18 weeks to the end of the study, the trend of feed intake reversed in both OVX groups and the Sham-control group, except for the OVX+1.5%GTP group. At the end of the study, there was no difference among all treatment groups, except for the OVX+1.5%GTP group that showed a smaller feed intake.

Figure 3 shows data of estimated mean water consumption per day of all groups. Water consumption among the study animals was significantly different at 1 week. Throughout the study, there was no significant difference in water consumption between the Sham-control

and OVX-control groups. The water consumption was significantly different among GTP-supplemented groups, probably due to the taste of GTP. At the end of the study, daily water consumption of rats significantly decreased with GTP concentration for each pair of groups with closest GTP supplements, except for no difference between the OVX+0.5%GTP and the OVX+1%GTP groups.

Organ weight

Table 2 lists the final organ weight data. At 0 month, except for brain weight and adrenal weight (Sham-control=OVX-control) and uterus weight (Sham-control >OVX-control), relative to the OVX-control group, the Sham-control group had significantly lower values for other organ weights including liver, kidney, heart, spleen, and thymus. At 3 and 6 months, there were no significant differences in weights of liver, heart, spleen, brain, and adrenal between the Sham-control group and the OVX-control group; compared to the OVX-control group, the Sham-control group had significantly greater uterus weights and significantly smaller kidney and thymus weights.

Among all OVX groups, without adjusting for multiple comparisons, GTP supplementation at 1% and 1.5% dosages significantly decreased weights of liver, heart, spleen, and thymus at 3 and 6 months. However, after adjusting for multiple comparisons, only GTP supplementation at higher dosages (1% and 1.5%) resulted in significantly smaller liver weights of animals.

With regard to time effect, the uterus weights of the Sham-control group at 6 months were significantly greater than those at 0 month. For all GTP-supplemented groups, the thymus and adrenal weights at 3 and 6 months were significantly smaller than those at 0 month. Furthermore, before adjusting for multiple comparisons, relative to 0 month, GTP supplementation resulted in significantly smaller weights of liver (OVX+1.5%GTP), kidney (OVX+0.5%GTP, OVX+1.0%GTP, OVX+1.5%GTP), heart (OVX+1%GTP and OVX+1.5%GTP), and spleen (OVX+1.5%GTP) at 3 months, as well as smaller weights of liver (OVX+1.5%GTP) and spleen (OVX+1.0%GTP, OVX+1.5%GTP) at 6 months. After adjusting for multiple comparisons, such statistical differences were only observed in weights of thymus and adrenal for all OVX-treated groups at 3 and 6 months as well as those of uterus for Sham-control group at 6 months.

Serum and urine GTP concentration

The concentrations of GTP in serum and urine are shown in Figures 4 and 5, respectively. The major free and total forms of GTP ingredients in serum and urine are EGC, EC, EGCG, and ECG. In general, the levels of GTP ingredients in either serum or urine of the Sham-control and the OVX-control groups were undetectable. Throughout the study period, the GTP concentrations in serum and urine significantly increase with the GTP dose, and as expected, the OVX+1.5%GTP group had the highest levels of GTP concentrations at both 3 and 6 months for all GTP ingredients (free and total). Relative to 0 month, the concentrations of free and total GTP ingredients in both urine and serum are significantly higher at 3 and 6 months and the GTP concentrations increase with GTP dosage, even after adjusting for multiple comparisons.

Ophthalmological examinations

Undilated external and anterior segment examinations show consistent results of all eye examination sessions throughout the study. A small portion of the study animals manifested minor ocular surface abnormalities, including two animals in the Sham-control group each with a corneal scar at the right eye, two animals in the OVX-control group each with corneal opacity at the left eye, one animal in the OVX+1%GTP group with corneal opacity at the right eye, and two animals in the OVX+1.5%GTP group with corneal opacity at the left eye. These abnormalities were characterized as mild to moderate level of corneal opacities/scarring, and none of them was progressive, as assessed at 0, 3, and 6 months. Also, none of the abnormalities prohibited examination of the posterior segments of the eye. A couple of the abnormalities regressed/resolved during the course of this study. All the abnormalities were classified as minor ocular surface trauma consistent with study animals' living conditions before arrival at the experimental site. Dilated indirect ophthalmoscope examinations determined that the lenses, and posterior segments including the vitreous, optic nerve, retina vessels, retina and overall fundus of each animal was within normal limits for the respective age of the animals at each of the three study examination time points.

Clinical hematology

Table 3 presents the main findings of clinical hematological results. At 0 month, the OVX-control group had higher MCV, MCH, MCHC, and RETIC, but lower RBC, %MONO, and Abs. MONO. At 3 months, relative to the Sham-control group, the OVX-control group had significantly higher RBC and significantly lower MCH and MCHC. At 6 months, compared to the Sham-control group, the OVX-control group had significantly lower values for MCV and RETIC. After adjusting for multiple comparisons, statistically significant differences were only observed in MCH and MONO at 0 month as well as MCV at 6 months.

Among all OVX-treated groups, at 3 months, a high dose (1.5%) of GTP treatment significantly increased HGB, MCH, and MCHC levels relative to OVX-control. At the end of the study (6 months), the HCT level significantly decreased and the MCHC level significantly increased with GTP dose in OVX groups. After adjusting for multiple comparisons, there was no difference in any hematological parameters among all OVX-treated groups at 3 and 6 months, regardless of GTP doses.

Regarding time effect, data at 3 and 6 months showed that in the Sham-control group the levels of MONO, MCH, MCHC, and aPTT significantly decreased with time, while levels of HCT, MCV, LUC, and Abs. LUC significantly increased with time. In the OVX-control group, the HCT, RBC, and LUC significantly increased with time, while the MCH and MCHC significantly decreased with time. Without adjustment for multiple comparisons, relative to 0 month, GTP supplementation significantly increased levels of HCT, Abs LYMPH at 3 months, MCV and MONO at 6 months, as well as HCT, RBC, and LUC at 3 and 6 months; GTP supplementation also significantly lowered levels of Abs RETIC at 3 months, EOS at 6 months, and MCH, MCHC, PLT, and RETIC at 3 and 6 months. After adjustment for multiple comparisons, significant time effects were only observed in HCT, RBC, MCH, MCHC, RETIC and LUC (Table 3). See more detailed statistical results of clinical hematology in Supplemental I.

Clinical chemistry

Table 4 presents the main findings of clinical chemistry. Relative to the Sham-control group, (a) the OVX-control group had higher K, CHOL-total, and LDH levels, but significant lower CREAT and BUN at 0 month; (b) the OVX-control group had higher ALKP, T.BILI, and CREAT levels, but significant lower ALB, A/G, T.PRO, and Ca levels at 3 months; and (c) the OVX-control group had higher ALKP and CREAT levels, but lower ALB, A/G, and P levels at 6 months. After adjusting for multiple comparisons, difference was statistically significant only in ALB at 6 months.

Prior to adjustment for multiple comparisons, among all OVX-treated groups, GTP treatment at a higher dosage (especially OVX+1.5% GTP) significantly increased Cl and BUN at 3 months as well as P and BUN at 6 months, while it significantly decreased AST, T.BILI, K, CHOL-total, TRIG, and GLU at 3 months as well as CHOL-total, TRIG, and LDH at 6 months, relative to the OVX-control group. After adjusting for multiple comparisons, GTP's effects were only observed in K at 3 months, and P and CHOL-total at 6 months.

Results related to the time effect on clinical chemistry are summarized below with all data adjusted for multiple comparisons (Table 4). In the Sham-control group, the levels of TBA, T.BILI, ALB, GLB-calculated, T.PRO, and TRIG significantly increased with time, while the levels of P and K significantly decreased with time. In the OVX-control group, the levels of ALKP, GLB-calculated, T.PRO, Cl and CHOL-total were significantly elevated with time, while the levels of ALB, A/G, P, and K significantly reduced with time. Among the GTP-supplemented groups, in general, the levels of ALT, GLB-calculated, T.PRO, CL, CHOL, and BUN significantly increased with time, but the levels of A/G, P, and K significantly decreased with time. See more detailed statistical results of clinical chemistry in Supplement II.

Urinalysis measures

At baseline, there was no difference in any parameters of urine analyses between the Sham-control group and the OVX-control group (Table 5). At 3 and 6 months, urine volume significantly decreased with GTP dose, while the darkness and cloudiness of urine significantly increased with GTP dose at 6 months. High GTP doses (1% and 1.5%GTP) significantly increased specific gravity of urine at 3 and 6 months.

At 3 months, relative to the OVX-control group, GTP supplementation at higher doses significantly increased the incidences of protein (OVX+1.0%GTP and OVX+1.5%GTP), ketones (OVX+1.0%GTP and OVX+1.5%GTP), and bilirubin (OVX+1.5%GTP) in urine of OVX animals. Such increasing effect of GTP was also observed at 6 months. There was no difference in occult blood, urobilinogen, nitrate, and leukocyte esterase among all treatment groups at any time points after adjustment for multiple comparisons. It was noted that at 6 months, relative to the OVX control, OVX+1.5%GTP treatment significantly increased the incidences of glucose existence in urine of animals. The results of microscopic examination (WBC, RBC, renal, transitional, squamous, mucus, bacteria, yeast, casts, or crystals

including CaC_2O_4 and TPO_4) did not reveal any significant differences among the treatment groups throughout the study period (data not shown).

Histopathology

One animal in the OVX+0.15%GTP group was found dead with no clinical history. The majority of the organs in this animal were in a state of severe postmortem autolysis. No cause of death was identified in this animal. Based on the results of histopathologic examinations, there were no treatment-related macroscopic or microscopic findings in the examined groups (Sham-control, OVX-control, and OVX+1.5%GTP) at the scheduled necropsy (0 and 6 months).

There was no histopathological abnormality in the organs that could be attributed to GTP supplementation at 1.5% (wt/vol) dose in drinking water. All findings were consistent with normal background lesions in clinically normal rats of the age and strain used in the present study, were considered spontaneous, and/or were incidental in nature and unrelated to GTP treatment for animals administered 1.5% (wt/vol) GTP for at least 180 consecutive days. For example, mammary tumors were observed in two Sham-control rats, which were spontaneous tumors commonly observed in aged rats. Protein casts were observed in renal tubules of controls (Sham-control and OVX-control) and treated animals (OVX+1.5%GTP), which are age related findings commonly observed in aged rats. Relative to the Sham-control group, a decrease or loss of bone trabeculae was observed in the femur section of OVX-control and OVX+1.5%GTP groups. This is a histopathological observation associated with surgical removal of the ovary and an expected change, which is part of the utility of this animal model. The incidence and severity of the change in trabecular bone were comparable between OVX-control group and OVX+1.5%GTP groups; however, a definitive grading was not performed and was beyond the scope of this evaluation. Therefore, under the conditions of the present study, the no-observed-adverse-effect level (NOAEL) for the orally administered GTP in the middle-aged OVX rat was 1.5%GTP, wt/vol in drinking water.

Discussion

This is the first long-term study to comprehensively evaluate the safety of middle-aged OVX Sprague Dawley rats supplemented with GTP in drinking water up to 1.5% (wt/vol) for 6 months. The results support our hypothesis that 1.5% GTP is safe in the OVX animals with regard to clinical hematology, clinical chemistry, urine analysis and histopathology.

In this study, supplementation of GTP in drinking water at high doses tended to lower BW gains and overall BW (Morita and others 2009), while clinical pathology and other observations did not reveal any toxicologically relevant findings. Published works suggest that GTP's weight-reducing effect is associated with increased energy expenditure, based on an *ex vivo* study using brown adipose tissue of male rats treated with green tea extract (50–250 μM in buffer medium) via stimulating lipid peroxidation (Dullo et al., 2000) or suppressing lipid absorption (Janssens et al., 2016).

We noticed that animals in the high GTP dose groups seemed to have lower water consumption, probably due to the unfavorable bitter taste and loss of appetite. Therefore, as

expected, daily intake of GTP increased with both GTP dose and time, as reflected in serum and urine GTP concentrations. EGCG in both serum and urine in the 0.1% and 0.5%GTP-treated groups is significantly lowered at 6 months compared to 3 months. Lower EGCG concentration in the lower-dose groups might reflect fast metabolism/excretion of EGCG as compared to other GTP ingredients, because EGCG is the highest abundant component among GTP ingredients and may serve as the first line to be eliminated or degraded.

At 3 months, there were statistically significant increases in HGB, MCH, and MCHC of the OVX+1.5%GTP group compared with the OVX-control, but these increases were not considered representing any adverse effect due to the GTP treatment, as discussed below. In general, treatment of the OVX animals with GTP at doses up to 1.5% (wt/vol) was well tolerated. Except for MCHC, the changes noted in the HGB and MCH in the OVX +1.5%GTP group were also noted in the OVX rats supplemented with lower GTP doses (0.15% and 0.5%) Detailed data are presented in Supplement I. Such elevated HGB, MCH, and MCHC levels diminished at 6 months (Takami and others 2008). Therefore, these changes at 3 months were considered incidental biological variations, and they did not represent adverse effects related to GTP treatment.

Our findings agree with previous studies in that (a) OVX-control group had larger BW, kidney and thymus weight but lower uterus weight than the Sham-control group (Lin, et al., 2013); (b) OVX-control group had higher serum ALKP at 3 and 6 months than those in the Sham-control group (Liu and others 2012; Erben and others 2003); and (3) GTP supplementation elevated serum P (Takami and others 2008). In addition, the cholesterol-lowering capability of GTP in OVX animals observed in the present study was consistent with that of other animal studies, including fat-enriched, diet-induced hyperlipidemia in rats (Amanolahi and others 2013), cholesterol-fed-induced hypercholesterolaemia in rabbits (Bursill and others 2007), high-fat-diet-induced hyperlipidemia in hamsters (Chan and others 1999), and high-fat-diet-induced hypercholesterolemia in C57BL/6 mice (Kim and others 2012). Such GTP's CHOL-total lowering mechanisms may involve the following: decrease in micellar cholesterol solubility resulting in reduction of intestinal absorption (Raederstorff and others 2003; Ikeda and others 1992) and an increase in fecal cholesterol excretion (Yang and Koo 2000; Yokozawa and others 2002), decrease in intestinal TRIG absorption (Ikeda and others 1992), and reduction of the activity of hepatic fatty acid synthase resulting in TG synthesis (Takami and others 2008).

The change in urine color from yellow to brown was simply due to natural coloring of GTP; water consumption decreased as high GTP concentration made the drinking water not tasty; and low water consumption naturally reduced urine volume. Although we noted significant increases in the urinary ingredients (e.g. glucose, protein, ketones, bilirubin, and nitrate) in high GTP doses groups which might be related to renal failure, there was no evidence of toxicity in the clinical chemistry and histopathology, and there was no clear relationship with dose. Therefore, such urinalysis changes were not considered toxicologically significant. Instead, these changes were considered to be due to the smaller urine volume noted in these groups.

Previous studies have evaluated the EGCG's safety in intact animals. For example, in a 13-week toxicity study, Isbrucker et al. reported that a dose of 2000 mg EGCG/kg BW/day was lethal to rats (Isbrucker and others 2006b). Doses up to 500 mg EGCG/kg BW/day were not toxic in terms of clinical observation, clinical hematology, and clinical chemistry. Similarly, no adverse effects were noted when 500 mg EGCG/kg BW/day was administered to pre-fed dogs in divided doses, suggesting a NOAEL of 500 mg EGCG/kg BW/day for up to 13 weeks (Isbrucker and others 2006b). Morita et al. reported that the NOAEL for decaffeinated green tea catechins was 1200 mg/kg BW/day for male rats. However, a NOAEL for female rats could not be determined but would be lower than 1200 mg/kg BW/day as 1200 mg/kg BW/day was the only dosage tested for female rats (Morita and others 2009). In this 6-month animal study, the NOAEL for GTP supplement in drinking water for middle-aged OVX rats was 633 mg GTP/kg-BW/day. In other words, if we consider 65.37 % of GTP is EGCG, our study suggests a NOAEL of 414 mg EGCG/kg BW/day for up to 6 months, a result corroborated by Morita's study (Morita et al., 2009).

Previous experimental studies suggest that administration of high dose of green tea extract may result in hepatotoxicity (Mazzanti and others 2015, Emoto and others 2014). For example, administration of high-dose green tea extracts to non-fasted dogs induced liver, gastrointestinal, and renal toxicities (Kapetanovic and others 2009, Wu and others 2011). The key findings included necrosis of hepatic cells, gastrointestinal epithelia and renal tubules, atrophy of reproductive organs, atrophy and necrosis of hematopoietic tissues, and associated hematological changes (Kapetanovic and others 2009). Molinair et al. reported a case of acute liver failure in a patient consuming dietary supplement high in green tea extracts (Molinari and others 2006). In our study, administration of GTP to OVX rats at dose levels up to 1.5% for 6 months did not cause hepatotoxicity, as demonstrated by serum markers of liver function (AST, ALT, and TBA) and liver histopathology observation. Such result agrees with Morita's study that green tea extract had no hepatotoxicity in female growing rats for up to 400 mg/kgBW (Morita and others 2009). In the present study, a decreased liver weight in GTP-treated groups did not appear to be related to the GTP treatments because no alterations were found in liver histopathology and there was no difference in relative liver weights (liver weight/100 g BW) among all GTP-treated groups at 6 months (Morita, et al., 2009).

The available evidence from clinical studies in which green tea extract was used as a therapeutic agent also did not reveal any serious adverse events or toxicity greater than grade 2 (moderate adverse event) related to treatment in humans (Chow and others 2003; Dryden and others 2013). In a double-blinded, placebo-controlled pilot study involving initially 20 patients with mild to moderate ulcerative colitis, who received daily doses of oral Polyphenon E (400 mg or 800 mg EGCG daily) or placebo for 56 days, did not reveal any treatment-related, significant adverse effects based on data of clinical chemistry (Dryden and others 2013). Chow's study also concluded that it is safe for healthy individuals to take up to 800 mg EGCG daily for 4 weeks, according to the results of blood chemistry profiles (Chow and others 2003). There is a >60% increase in the systemic availability of free EGCG after chronic GTP administration at a high daily bolus dose (800 mg EGCG or Polyphenon E daily). However, in Chow's study, adverse events reported during the 4-week treatment period include excess gas, upset stomach, nausea, heartburn, stomach ache, abdominal pain,

dizziness, headache, and muscle pain in both placebo and EGCG groups, but all of the reported events were rated as mild. The study by Crew et al. in a Phase IB randomized, double-blinded, placebo-controlled, dose escalation study of Polyphenon E in women with hormone receptor-negative breast cancer suggested the maximum tolerated dose for Polyphenon E to be 600 mg twice daily (1200 mg/day) for 6 months (Crew and others 2012). Dostal et al. recently reported that decaffeinated green tea extract containing 843 mg of EGCG for 12 months was well tolerated by overweight/ obese postmenopausal women after 12 months (Dostal and others 2016). Observations from these clinical studies support the safety of GTP in humans up to 1200 mg EGCG/day for up to 6 months or 843 mg EGCG/day for up to 12 months. The 6-month intervention period was selected for the present study as such period of time in rats approximately equals almost 12 years in humans (Andreollo et al., 2012). The safety results of the current animal study provide a basis for establishing a safe maximum daily dosage for long-term GTP supplement in postmenopausal women.

Conclusion

The results of the present dose-response study demonstrate that daily supplementation of GTP at levels up to 1.5% (wt/vol) in drinking water (approximately 633 mg GTP/kg BW/day) for 6 months were safe to OVX rats as evaluated by clinical condition of the animals, motor activity, organ weights, gross necropsy, hematology, serum chemistry, urinalysis, as well as macroscopic and microscopic evaluations. Based on this study, the NOAEL of GTP supplementation is up to 1.5% (wt/vol) in drinking water, equivalent to approximately 633 mg GTP/kg BW/day, for middle-aged OVX Sprague Dawley rats for 6 months with no sign of toxicity.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Figure 1.

Estimated mean body weights in Sham-control and OVX rats supplemented with GTP at different doses. All OVX groups had significant greater body weights than sham-control group and there was no difference in body weights among all OVX groups at the end of the study.

— Sham-control; OVX-control; - - - - - OVX+0.15%GTP;
 - - - - - OVX+0.5%GTP; — — — OVX+1.0%GTP; — . — . OVX+1.5%GTP

Figure 2.

Estimated mean feed intake in Sham-control and OVX rats supplemented with GTP at different doses. Throughout the first 18 weeks, all OVX groups decreased feed intake, while the sham-control group increased it. After 18 weeks, such trends were reversed and no significant difference among all groups, except for OVX+1.5%GTP group.

———— Sham-control; OVX-control; ----- OVX+0.15%GTP;
 - - - - - OVX+0.5%GTP; — — — OVX+1.0%GTP; — . — . OVX+1.5%GTP

Figure 3.

Estimated mean water consumption in Sham-control and OVX rats supplemented with GTP at different doses. There was no difference in water consumption between the sham-control and OVX-control group. The amount of water intake was negatively associated with GTP dosages.

— Sham-control; OVX-control; ----- OVX+0.15% GTP;
 - - - - - OVX+0.5% GTP; — — — OVX+1.0% GTP; — . — . OVX+1.5% GTP

Figure 4.

Serum GTP concentrations. Data are presented as mean $\pm$ SEM. EC, epicatechin; ECG, epicatechin gallate; EGC, epigallocatechin; EGCG, epigallocatechin gallate. Within a given month, values that share the same superscript letter (x, y, or z) are not statistically different from each other among the OVX groups (OVX-control, OVX+0.15% GTP, OVX+0.5% GTP, OVX+1.0% GTP, OVX+1.5% GTP) after adjustment for multiple comparisons. indicates a difference from 0 month of the control treatment (sham-control or OVX-control respectively) after adjustment of multiple comparison at $p < 0.05$.

Figure 5.

Urine GTP concentrations. Data are presented as mean $\pm$ SEM. EC, epicatechin; ECG, epicatechin gallate; EGC, epigallocatechin; EGCG, epigallocatechin gallate. Within a given month, values that share the same superscript letter (x, y, or z) are not statistically different from each other among the OVX groups (OVX-control, OVX+0.15% GTP, OVX+0.5% GTP, OVX+1.0% GTP, OVX+1.5% GTP) after adjustment for multiple comparisons. Δ indicates a difference from 0 month of the control treatment (sham-control or OVX-control respectively) after adjustment of multiple comparison at $p < 0.05$.

Table 1

Group assignments and number of animal samples collected at each sample collection time

Treatment arm	Sample collection time		
	0 month	3 months	6 months
Sham-control (n = 39)	13	13	13
OVX-control (n = 39)	13	13	13
OVX+0.15% GTP (n = 26)		13	13
OVX+0.5% GTP (n = 26)		13	13
OVX+1.0% GTP (n = 26)		13	13
OVX+1.5% GTP (n = 26)		13	13

Table 2

Final organ weight

	SHAM-control	OVX-control	OVX+0.15%GTP	OVX+0.5%GTP	OVX+1%GTP	OVX+1.5%GTP	p-Value
<i>Liver (g)</i>							<0.001
0 month	5.97 ^a ±0.12	7.22±0.26					
3 month	6.52±0.12	7.49 ^a ±0.25	7.47 ^a ±0.25	6.86 ^a ±0.24	6.67 ^b ±0.17	6.19 ^b ±0.15	
6 month	6.56 ±0.19	7.58 ^a ±0.22	6.86 ^b ±0.18	7.15 ^a ±0.18	6.69 ^b ±0.24	6.49 ^c ±0.18	
<i>Kidney (g)</i>							0.0342
0 month	1.469 ^a ±0.024	1.656±0.035					
3 month	1.522 ^a ±0.028	1.538 ^a ±0.040	1.565 ^a ±0.042	1.545 ^a ±0.023	1.530 ^a ±0.042	1.532 ^a ±0.021	
6 month	1.544 ^a ±0.028	1.528 ^a ±0.026	1.477 ^b ±0.033	1.588 ^a ±0.041	1.584 ^a ±0.040	1.586 ^a ±0.047	
<i>Heart (g)</i>							<0.0001
0 month	0.889 ^a ±0.018	1.050±0.029					
3 month	0.915±0.018	1.040 ^a ±0.031	1.058 ^a ±0.019	1.008 ^a ±0.025	0.955 ^b ±0.037	0.962 ^b ±0.021	
6 month	0.924±0.020	1.023 ^a ±0.020	1.003 ^b ±0.017	1.074 ^a ±0.026	1.004 ^b ±0.025	0.997 ^b ±0.029	
<i>Spleen (g)</i>							<0.0001
0 month	0.543 ^a ±0.014	0.673±0.021					
3 month	0.555±0.010	0.652 ^a ±0.023	0.622 ^a ±0.024	0.625 ^a ±0.022	0.643 ^a ±0.033	0.586 ^b ±0.016	
6 month	0.509±0.021	0.615 ^a ±0.021	0.643 ^a ±0.019	0.652 ^a ±0.026	0.605 ^a ±0.021	0.611 ^a ±0.024	
<i>Brain (g)</i>							0.004
0 month	1.838±0.012	1.877±0.024					
3 month	1.805±0.016	1.877 ^a ±0.019	1.849 ^a ±0.017	1.873 ^a ±0.018	1.848 ^a ±0.024	1.897 ^a ±0.018	
6 month	1.818±0.019	1.905 ^a ±0.021	1.862 ^a ±0.021	1.874 ^a ±0.013	1.869 ^a ±0.016	1.897 ^a ±0.019	
<i>Uterus (g)</i>							<0.0001
0 month	0.670 ^a ±0.056	0.230±0.011					
3 month	0.740 ^a ±0.058	0.154 ^a ±0.010	0.153 ^a ±0.010	0.152 ^a ±0.008	0.152 ^a ±0.006	0.143 ^a ±0.007	
6 month	0.932 ^a ±0.053	0.270 ^a ±0.045	0.246 ^a ±0.038	0.187 ^a ±0.030	0.209 ^a ±0.026	0.181 ^a ±0.011	
<i>Thymus (g)</i>							<0.001

	SHAM-control	OVX-control	OVX+0.15%GTP	OVX+0.5%GTP	OVX+1%GTP	OVX+1.5%GTP	p-Value
0 month	0.239 ^{§*} ±0.029	0.505±0.039					
3 month	0.191 ^{§*} ±0.011	0.335 ^{axy} ±0.019	0.350 ^{ax} ±0.015	0.327 ^{axy} ±0.014	0.260 ^{by} ±0.012	0.303 ^{abxy} ±0.014	
6 month	0.147 ^{§*} ±0.014	0.218 ^{ax} ±0.015	0.214 ^{ax} ±0.021	0.214 ^{ax} ±0.011	0.191 ^{ax} ±0.017	0.193 ^{ax} ±0.011	
<i>Adrenal (g)</i>							<0.0001
0 month	0.066±0.002	0.069±0.003					
3 month	0.057 ±0.001	0.055 ^{ax} ±0.002	0.054 ^{ax} ±0.002	0.050 ^{ax} ±0.003	0.055 ^{ax} ±0.002	0.052 ^{ax} ±0.002	
6 month	0.058 ±0.002	0.056 ^{ax} ±0.002	0.052 ^{ax} ±0.002	0.052 ^{ax} ±0.002	0.056 ^{ax} ±0.002	0.053 ^{ax} ±0.002	

Data are presented as mean ± SEM.

[§] indicates a difference between sham-control and OVX-control, $p < 0.05$, without adjustment for multiple comparisons.

Within a given row, values that share the same superscript letter (a, b, or c) are not statistically different from each other among the OVX groups (OVX-control, OVX+0.15% GTP, OVX+0.5% GTP, OVX+1.0% GTP, OVX+1.5% GTP) without adjustment for multiple comparisons.

Within a given row, values that share the same superscript letter (x, y, or z,) are not statistically different from each other among the OVX groups (OVX-control, OVX+0.15% GTP, OVX+0.5% GTP, OVX+1.0% GTP, OVX+1.5% GTP) after adjustment for multiple comparisons.

Indicates a difference from the 0-month data of the control treatment (sham-control or OVX-control respectively) at $p < 0.05$.

^{*} indicates a difference after adjustment of multiple comparison at $p < 0.05$.

Note that ^{*} implies [§], and ^{§*} implies [§].

Table 3**Main findings of clinical hematology**

	Increase	Decrease
<i>OVX effect (Sham-control vs. OVX-control)</i>		
0 month	MCV, MCH [*] , MCHC, RETIC	RBC, MONO [*] , Abs MONO
3 months	RBC	MCH, MCHC
6 months		MCV [*] , RETIC
<i>GTP effect</i>		
3 months	HGB, MCH, MCHC	
6 months		LYMPH
<i>Time effect</i>		
Sham-control group		
3 months	MCV, LUC [*]	MCHC, RETIC, Abs RETIC, MONO [*] , Abs MONO
6 months	HCT [*] , MCV [*] , LUC [*] , Abs LUC [*]	MCH, MCHC [*] , aPTT
OVX-control group		
3 months	HCT [*] , RBC [*] , WBC, Abs LYMPH, LUC [*]	MCH [*] , MCHC [*] , RETIC, Abs RETIC
6 months	HCT [*] , RBC [*] , MCV, LUC [*]	MCH [*] , MCHC [*] , PLT, RETIC, Abs RETIC, EOS
OVX+0.15%GTP group		
3 months	HGB, HCT [*] , RBC, WBC, LYMPH, Abs LYMPH [*] , LUC [*]	MCH, MCHC [*] , PLT, RETIC [*] , Abs RETIC, NEUT, EOS
6 months	HCT [*] , RBC [*] , MCV [*] , MONO, Abs MONO, LUC [*] , Abs LUC	MCH [*] , MCHC [*] , PLT, RETIC [*] , EOS
OVX+0.5%GTP group		
3 months	HCT, RBC, WBC, Abs LYMPH [*] , LUC [*] , Abs LUC	MCH, MCHC, PLT, RETIC [*] , Abs RETIC
6 months	HCT [*] , RBC [*] , MCV, MONO, Abs MONO, LUC [*] , Abs LUC [*]	MCH [*] , MCHC [*] , PLT, RETIC
OVX+1.0%GTP group		
3 months	HCT [*] , RBC [*] , WBC, Abs LYMPH, LUC [*] , Abs LUC	MCH [*] , MCHC [*] , RETIC [*] , Abs RETIC
6 months	HCT [*] , RBC [*] , MONO, Abs MONO, LUC [*] , Abs LUC	MCH [*] , MCHC [*] , PLT, RETIC [*] , EOS
OVX+1.5%GTP group		
3 months	HCB [*] , HCT, RBC, WBC, Abs LYMPH, LUC [*]	MCH, MCHC, PLT, RETIC [*] , Abs RETIC
6 months	HCT [*] , RBC, MCV, MONO, LUC [*]	MCH [*] , MCHC [*] , PLT, RETIC, EOS, Abs EOS

Abs. absolute; aPTT, activated partial thromboplastin time; EOS, eosinophils; HCT, hematocrit; HGB, hemoglobin; LUC, large unstained cell; LYMPH, lymphocyte; MCH, mean corpuscular hemoglobin; MCHC, mean corpuscular hemoglobin concentration; MCV, mean corpuscular volume; MONO, monocyte; NEUT, neutrophil; PLT, platelet time; RBC, red blood cell; RETIC, reticulocyte; WBC, white blood cell.

* indicates statistically significant differences after adjusting for multiple comparisons.

Table 4**Main findings of clinical chemistry**

	Increase	Decrease
<i>OVX effect (Sham-control vs. OVX-control)</i>		
0 month	K, CHOL, LDH	CREAT, BUN
3 months	ALKP, T.BILI, CREAT	ALB, A/G, T.PRO, Ca
6 months	ALKP, CREAT	ALB [*] , A/G, P
<i>GTP effect</i>		
3 months	Cl, BUN	AST, T.BILI, K [*] , CHOL, TRIG, GLU
6 months	P [*] , BUN	CHOL [*] , TRIG, LDH
<i>Time effect</i>		
Sham-control group		
3 months	GLB, T.PRO, CHOL, GLU, LDH [*]	A/G, P [*] , K, CREAT
6 months	TBA [*] , ALKP, T.BILI [*] , ALB [*] , GLB [*] , T.PRO [*] , Cl, CHOL, TRIG [*]	A/G, P [*] , K [*]
OVX-control group		
3 months	AST, T.BILI [*] , GLB, TRIG, GLU [*] , CPK [*]	ALB [*] , A/G [*] , Ca [*] , P [*]
6 months	AST, ALT, TBA, ALKP [*] , T.BILI, GLB [*] , T.RPO [*] , Cl [*] , CHOL [*]	A/G [*] , Ca, P [*] , K [*]
OVX+0.15%GTP group		
3 months	T.BILI, GLB [*] , GLU	ALB, A/G [*] , Ca, P [*]
6 months	T.BILI, GLB [*] , T.PRO [*] , Cl [*] , Na, CHOL [*] , CREAT	A/G [*] , P [*] , K [*]
OVX+0.5%GTP group		
3 months	T.BIL, GGT, GLU	ALB [*] , A/G [*] , Ca [*] , P [*] , K,
6 months	AST, ALT, TBA [*] , TBIL [*] , GLB [*] , T.PRO [*] , Cl [*] , Na, CHOL [*] , CREAT, BUN	A/G [*] , Ca, P [*] , K [*]
OVX+1.0%GTP group		
3 months	AST, TBA, T.BILI [*] , GLB, GLU, BUN [*] , CPK, LDH	ALB, A/G [*] , Ca, P [*] , K
6 months	ALT, TBA, T.BILI, GLB [*] , T.PRO [*] , Cl [*] , CHOL [*] , CREAT, BUN [*]	A/G [*] , P [*] , K [*]
OVX+1.5%GTP group		
3 months	GLB, Cl, CREAT, BUN [*] , CPK	ALB [*] , A/G [*] , Ca [*] , P [*] , K [*]
6 months	AST, ALT [*] , TBA [*] , ALKP, GLB [*] , T.PRO [*] , Cl [*] , CHOL, CREAT, BUN [*]	A/G [*] , Ca, P [*] , K [*]

A/G: Albumin/Globulin; ALB, albumin; ALKP, alkaline phosphatase; ALT, alanine aminotransferase; AST, aspartate aminotransferase; BUN, blood urea nitrogen; Ca, calcium; CHOL, cholesterol-total; Cl, chloride; CPK, creatine phosphokinase; CREAT, creatinine; GGT, gamma glutamyl transference; GLB, globulin-calculated; GLU, glucose; K, potassium; LDH, lactate dehydrogenase; Na, sodium; P, phosphorus; TBA, total bile acids; T.BILI, total bilirubin; T.PRO, total protein; TRIG, triglycerides.

* indicates statistically significant differences after adjusting for multiple comparisons (p<0.05).

Table 5

Urine analysis

	SHAM-control	OVX-control	OVX+0.15%GTP	OVX+0.5%GTP	OVX+1%GTP	OVX+1.5%GTP	p-Value
<i>Continuous data</i>							
Urine volume (mL)							
0 month	10.2±1.4	9.4±1.0					<0.0001
3 month	14.4 ±0.3	12.9 ^{ax} ±0.9	11.7 ^{abx} ±0.9	9.8 ^{bx} ±1.2	4.0 ^{cy} ±1.0	3.5 ^{cy} ±0.7	
6 month	10.3±1.2	9.8 ^{bx} ±1.1	13.7 ^{ax} ±0.5	10.5 ^{abx} ±1.0	4.0 ^{cy} ±0.7	3.5 ^{cy} ±0.4	
Specific gravity							
0 month	1.014±0.001	1.014±0.001					<0.0001
3 month	1.008 ±0.003	1.012 ^{by} ±0.002	1.015 ^{by} ±0.002	1.016 ^{by} ±0.002	1.027 ^{ax} ±0.002	1.029 ^{ax} ±0.001	
6 month	1.008 ±0.001	1.012 ^{cz} ±0.002	1.014 ^{cz} ±0.001	1.020 ^{byz} ±0.001	1.026 ^{axy} ±0.002	1.026 ^{ax} ±0.001	
pH							
0 month	6.19±0.11	6.46±0.19					0.0696
3 month	6.19±0.12	6.00 ^{bxy} ±0.17	6.27 ^{abxy} ±0.17	6.42 ^{ax} ±0.10	6.15 ^{abxy} ±0.14	5.80 ^{cy} ±0.17	
6 month	5.96±0.11	6.19 ^{ax} ±0.13	6.21 ^{ax} ±0.07	6.08 ^{ax} ±0.05	6.15 ^{ax} ±0.09	6.25 ^{ax} ±0.14	
<i>Discrete data</i>							
Color (# of yellow/# of orange/# of brown, total n=12-14)							
0 month	13/0/0	13/0/0					1.000
3 month	13/0/0	13/0/0	13/0/0	13/0/0	13/0/0	13/0/0	1.000
6 month	13/0/0	13/0/0	12/0/0	12/0/1	4/9/0 [‡]	1/13/0 [‡]	<0.0001
Appearance (# of clear/# of slightly cloudy/# of cloudy/# of turbid, total n=12-14)							
0 month	13/0/0/0	13/0/0/0					1.000
3 month	13/0/0/0	13/0/0/0	13/0/0/0	9/4/0/0	11/1/1/0	10/3/0/0	0.0153
6 month	13/0/0/0	13/0/0/0	11/1/0/0	7/3/3/0 [‡]	2/5/5/1 [‡]	1/2/9/2 [‡]	<0.0001
Glucose (mg/dL) (# of negative/# of trace/# of +1/ # of +2, total n=12-14)							
0 month	13/0/0/0	12/1/0/0					1.000
3 month	13/0/0/0	12/1/0/0	10/2/1/0	6/7/0/0	8/5/0/0	8/5/0/0	0.0072
6 month	10/3/0/0	10/2/1/0	10/2/0/0	8/5/0/0	6/3/4/0	1/3/9/1 [‡]	<0.0001
Protein (mg/dL) (# of negative/# of trace/# of +1/ # of +2/# of +3, total n=12-14)							

	SHAM-control	OVX-control	OVX+0.15%GTP	OVX+0.5%GTP	OVX+1%GTP	OVX+1.5%GTP	p-Value
0 month	10/2/1/0/0	8/2/2/1/0					0.8317
3 month	9/4/0/0/0 [‡]	11/0/2/0/0	9/0/4/0/0	8/3/0/2/0	2/2/3/6/0 [‡]	2/2/4/5/0 [‡]	<0.0001
6 month	12/0/1/0/0 [‡]	6/4/3/0/0	8/1/3/0/0	3/0/3/7/0 [‡]	2/3/1/4/3 [‡]	0/1/4/8/2 [‡]	<0.0001
Ketones (mg/dL) (# of negative/# of trace/# of +1, total n=12-14)							
0 month	13/0/0	13/0/0					1.000
3 month	13/0/0	13/0/0	13/0/0	10/3/0	7/6/0 [‡]	3/9/1 [‡]	<0.0001
6 month	13/0/0	13/0/0	12/0/0	10/3/0	3/10/0 [‡]	0/12/2 [‡]	<0.0001
Occult blood (# of negative/# of trace-intact/# of tract-lysed/ # of +1/# of +2, total n=12-14)							
0 month	13/0/0/0/0	10/1/1/1/0					0.2200
3 month	13/0/0/0/0	13/0/0/0/0	13/0/0/0/0	12/1/0/0/0	13/0/0/0/0	12/1/0/0/0	1.0000
6 month	10/1/0/1/1	11/1/0/0/1	12/0/0/0/0	12/1/0/0/0	12/1/0/0/0	11/1/2/0/0	0.8391
Bilirubin (# of negative/# of +1/# of +2, total n=12-14)							
0 month	13/0/0	13/0/0					1.0000
3 month	13/0/0	12/1/0	13/0/0	11/2/0	8/5/0	5/6/2 [‡]	0.0003
6 month	13/0/0	13/0/0	12/0/0	13/0/0	5/4/4 [‡]	0/3/11 [‡]	<0.0001
Urobilinogen (EU/dL) (# of 0.2/# of 1.0, total n=12-14)							
0 month	13/0	13/0					1.000
3 month	13/0	12/1	13/0	11/2	8/5	7/6	0.0025
6 month	13/0	13/0	12/0	13/0	12/1	13/1	1.000
Nitrate (# of negative/# of positive, total n=12-14)							
0 month	13/0	13/0					1.0000
3 month	13/0	13/0	13/0	13/0	13/0	12/1	1.0000
6 month	11/2	13/0	11/1	8/5	13/0	10/4	0.0204
Leukocyte esterase (# of negative/# of trace/# of +1/# of +2, total n=12-14)							
0 month	12/0/1/0	11/1/1/0					0.3818
3 month	12/1/0/0	12/1/0/0	11/2/0/0	12/1/0/0	13/0/0/0	12/0/1/0	0.0418
6 month	12/1/0/0	8/4/1/0	9/2/1/0	3/5/4/1	12/0/1/0	14/0/0/0	0.1743

Data are presented as mean ± SEM.

[‡] indicates a difference between sham-control and OVX-control, p<0.05, without adjustment for multiple comparisons.

Within a given row, values that share the same superscript letter (a, b, c, or d) are not statistically different from each other among the OVX groups (OVX-control, OVX+0.15% GTP, OVX+0.5% GTP, OVX+1.0% GTP, OVX+1.5% GTP) without adjustment for multiple comparisons.

Within a given row, values that share the same superscript letter (x, y, or z) are not statistically different from each other among the OVX groups (OVX-control, OVX+0.15% GTP, OVX+0.5% GTP, OVX+1.0% GTP, OVX+1.5% GTP) after adjustment for multiple comparisons.

Indicates a difference from the 0-month data of the control treatment (sham-control or OVX-control respectively) at $p<0.05$.

* indicates a difference after adjustment of multiple comparison at $p<0.05$.

Note that * implies , and §* implies §.

‡ indicates a difference from OVX control at $p < 0.05$, after adjustment for multiple comparisons.