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Synergistic effects of green tea polyphenols and alphacalcidol on chronic inflammation-induced bone loss in female rats

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Abstract

Summary—Studies suggest that green tea polyphenols (GTP) or alphacalcidol is promising agent for preventing bone loss. Findings that GTP supplementation plus alphacalcidol administration increased bone mass via a decrease of oxidative stress and inflammation suggest a significant role of GTP plus alphacalcidol in bone health of patients with chronic inflammation.

Introduction—Studies have suggested that green tea polyphenols (GTP) or alphacalcidol are promising dietary supplements for preventing bone loss in women. However, the mechanism(s) related to the possible osteo-protective role of GTP plus D_3 in chronic inflammation-induced bone loss is not well understood.

Methods—This study evaluated bioavailability, efficacy, and related mechanisms of GTP in combination with alphacalcidol in conserving bone loss in rats with chronic inflammation. A 12-week study of 2 (no GTP vs. 0.5% GTP in drinking water) × 2 (no alphacalcidol vs. 0.05 μg/kg alphacalcidol, 5×/week) factorial design in lipopolysaccharide-administered female rats was performed. In addition, a group receiving placebo administration was used to compare with a group receiving lipopolysaccharide administration only to evaluate the effect of lipopolysaccharide.

Results—Lipopolysaccharide administration resulted in lower values for bone mass, but higher values for serum tartrate-resistant acid phosphatase (TRAP), urinary 8-hydroxy-2′-deoxyguanosine, and mRNA expression of tumor necrosis factor-*α* and cyclooxygenase-2 in spleen. GTP

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Conflicts of interest None.

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supplementation increased urinary epigallocatechin and epicatechin concentrations. Both GTP supplementation and alphacalcidol administration resulted in a significant increase in bone mass, but a significant decrease in serum TRAP levels, urinary 8-hydroxydeoxyguanosine levels, and mRNA expression of tumor necrosis factor-α and cyclooxygenase-2 in spleen. A synergistic effect of GTP and alphacalcidol was observed in these parameters. Neither GTP nor alphacalcidol affected femoral bone area or serum osteocalcin.

Conclusion—We conclude that a bone-protective role of GTP plus alphacalcidol during chronic inflammation bone loss may be due to a reduction of oxidative stress damage and inflammation.

Keywords

Bone biomarker; Bone mineral density; Inflammation; Oxidative stress; Tea; Vitamin D

Introduction

Osteopenia (also called low bone mass) has been reported in patients with chronic inflammatory diseases such as chronic periodontitis [1] and pancreatitis [2], inflammatory bowel disease [3], rheumatorid arthritis [4], and lupus erythematosus [5]. The pathogenesis of low bone mass in patients with such chronic inflammatory diseases is complex. It involves pro-inflammatory production of cytokine mediators (i.e., interleukin-1β, tumor necrosis factor-α (TNF-α), and cyclooxygenase-2 (COX-2) [4,6–8]), glucocorticoid treatment [9], and reduced muscular function, resulting in suppressed bone formation, elevated bone resorption, increased risk for falls, and therefore, increased risk for bone fracture [10].

Chronic inflammation-induced bone loss has been associated with high levels of oxidative stress in animals [11]. Reactive oxygen species (ROS), i.e., superoxides and hydrogen peroxide, can lead to severe damage to DNA, protein, and lipids [12]. Oxidative stress results from high levels of ROS produced during normal cellular metabolism or from environmental stimuli perturbing the normal redox balance, shifting cells into a state of oxidative stress [13]. Studies show that oxidative stress leads to (a) an increase in osteoblast and osteocyte apoptosis [14], (b) a decrease in osteoblast number via extracellular signal-regulated kinases (ERK) and ERK-dependent nuclear factor- κB signaling pathways [15], (c) a decrease in the rate of bone formation via Wnt/β-catenin signaling [16], and (d) an increase in the differentiation and function of osteoclasts [17]. Recently, Shen et al. [18] reported that oxidative stress (as shown by an increase in urinary 8-hydroxydeoxyguanosine (8-OHdG), an oxidative stress biomarker) is involved in the pathogenesis of bone loss in female rats due to chronic inflammation.

Green tea, one of the most popular beverages in the world, has received considerable attention due to its many scientifically proven beneficial effects on human health, including maintaining bone mass [18–20]. Epidemiological [21–25], animal [11,18], and cellular [26–28] studies strongly suggested that green tea polyphenols (GTP, green tea extract) are a promising dietary supplement for preventing bone loss in women and men with low bone mass [20,29]. In addition, our recent study shows that drinking water supplemented with GTP mitigated bone loss in various models due to an increase of antioxidant capacity [11], a reduction of oxidative stress [11,18], or a decrease in inflammation [11].

Ringe and Schact [29] reported that plain vitamin D can be used as a nutritional supplement for mitigating bone loss in patients with low bone mass. However, such a plain vitamin D is not activated in the kidney in those vitamin D-replete patients [29]. Instead of plain vitamin D, alfacalcidol (1-α-OH-vitamin D_3, a hydroxylated form of vitamin D) was shown to increase available amounts of active D-hormone in different target tissues of patients [29,30]. Thus, alfacalcidol has become more popular in the treatment of bone loss-related diseases [30–34]. Studies have shown that alfacalcidol mitigated bone loss in a model of glucocorticoid/

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inflammation-induced osteoporosis [30] as well as a model of osteoporosis in rheumatoid arthritis [35,36] due to its anti-inflammatory effect. However, the effect of 1-α-OH-vitamin D_3 on chronic inflammation-induced bone loss along with possible mechanism(s) has never been evaluated. In addition, the potential effect of green tea bioactive components plus 1-α-OH-vitamin D_3 on this chronic inflammation-induced bone loss and related molecular mechanism(s) is not well understood. Therefore, the present study was designed to investigate the potential benefit of two dietary supplements, GTP and 1-α-OH-vitamin D_3, in the treatment or prevention of bone loss in female rats with chronic inflammation. We hypothesized that (a) supplementation of GTP in drinking water plus 1-α-OH-vitamin D_3 administration will synergistically mitigate chronic inflammation-induced bone loss in female rats and (b) such changes are related to a reduction of oxidative stress-induced DNA damage along with a reduction of inflammation. Studying the potential effect of GTP plus 1-α-OH-vitamin D_3 on bone remodeling in female rats with chronic inflammation will advance the understanding of their effects on skeletal biology to minimize bone loss in humans with chronic inflammation.

Materials and methods

Experimental design

Virgin 3-month-old CD female rats receiving lipopolysaccharide (LPS) administration is a well-established model of chronic inflammation-induced bone loss [11,37]. Among the LPS-treated rats, a 2 (no GTP vs. 0.5% GTP in drinking water) × 2 (0 vs. 0.05 μg/kg body weight 1-α-OH-vitamin D_3) factorial design enabled evaluation of effects of GTP, 1-α-OH-vitamin D_3, and GTP × 1-α-OH-vitamin D_3 interaction on chronic inflammation-induced bone loss along with related mechanism(s). In addition, a group receiving placebo administration only (the P group) was used to compare with a group receiving LPS administration only (the L group) to evaluate the effect of LPS.

Animals and treatments

Fifty virgin CD female rats (3-month-old, Charles River, Wilmington, MA, USA) were allowed to acclimate for 5 days to a rodent chow diet and distilled water ad libitum. After acclimation, the rats were subjected to the procedures of placebo or LPS administration described previously [11]. In brief, LPS (*Escherichia coli* Serotype 0127:B8, Sigma, St Louis, MO, USA) was incorporated into time-release pellets (Innovative Research of America, Sarasota, FL, USA) and designed to deliver a consistent dose (33 μg/day) for 12 weeks.

For the LPS animals (n=40), the dorsal neck area was shaved and sterile techniques were utilized. A small incision equal in diameter to that of the pellet (2.25 mm) was made at the back of the neck, and a horizontal pocket for LPS pellet administration (about 2 cm beyond the incision site) was formed using forceps. The incision site was closed with surgical glue. The remaining 10 rats in the placebo-operated group (the P group) received a pellet containing matrix only using the same procedures of administration described above. All rats were maintained on a regular rodent chow diet throughout the study period.

After surgery, LPS-treated rats were randomized by weight and assigned to (1) LPS administration (L, n=10), (2) LPS + GTP (LG, n=10), (3) LPS + 1-α-OH-vitamin D_3 (LD, n=10), and (4) LPS + GTP + 1-α-OH-vitamin D_3 (LGD, n=10) throughout the 12-week study period. The rats in the L group were given drinking water only. The rats in the LG group were given a concentration of 0.5% GTP in drinking water daily to mimic human consumption of green tea of four cups a day based on our previous human [38] and animal studies [11,18]. The rats in the LD group were orally administrated with 1-α-OH-vitamin D_3 at 0.05 μg/kg body weight, five times per week. The use of 1-α-OH-vitamin D_3 is based on a previous study that 1-α-OH-vitamin D_3 (0.5 μg/kg, five times a week) increased the number of osteoblasts and

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suppressed trabecular bone resorption and markedly increased periosteal and endocortical bone formation [39]. The rats in the LGD group were given both GTP and 1-α-OH-vitamin D_3. In addition, the rats in the P group were given drinking water only.

Distilled water mixed with GTP was prepared fresh daily. The amount of water consumed was recorded for each rat. GTP was purchased from the same source as that used previously in our studies (Shili Natural Product Company, Inc., Japan), with a purity higher than 98.5%. According to the HPLC-ECD and HPLC-UV analyses, every 1,000 mg of GTP contained 464 mg of (−)-epigallocatechin gallate (EGCG), 112 mg of (−)-epicatechin gallate (ECG), 100 mg of (−)-epicatechin (EC), 78 mg of (−)-epigallocatechin (EGC), 96 mg of (−)-gallocatechin gallate (GCG), and 44 mg of catechin. Rats were housed individually under a controlled temperature of 21±2°C with a 12-h light–dark cycle. Rats were examined daily and weighed weekly. All procedures were approved by the Texas Tech University Health Sciences Center Institutional Animal Care and Use Committee.

Sample preparation

Twenty-four-hour urine samples were collected from metabolic cages at baseline and after 6 (midpoint) and 12 weeks (end point) of study intervention for each animal and stored at −80°C until they were analyzed. After anesthetization, blood samples were drawn from the heart into Vacutainer tubes, and serum samples were isolated and stored at −80°C for later analyses. Final body weights were recorded. Spleen samples were harvested, immediately immersed into liquid nitrogen, and stored at −80°C prior to analysis. Femora were removed and cleaned of adhering soft tissue. The femur samples were stored in 70% ethanol for bone parameter assessments.

Measurement of urinary GTP components

The concentrations of GTP components in urine were determined according to the method described in Shen et al. [11,18]. In brief, thawed 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. The urine samples were extracted twice with ethyl acetate. Organic phases were pooled, dried in vacuo with a Labconco Centrivap concentrator (Kansas City, MO, USA), reconstituted in 15% acetonitrile, and analyzed with the ESA HPLC-CoulArray system (Chelmsford, MA, USA). The 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-C_{18} (5 μm, 4.6×250 mm). The mobile phase included buffer A (30 mM NaH_2PO_4/CAN/THF=98/1.8/0.2, pH 3.36) and buffer B (15 mM NaH_2PO_4/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, and 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 components were generated separately, and ECG, EC, EGCG, and ECG were eluted at 14, 21, 24, and 29 min, respectively. The electrochemical detector was set at −90, −10, 70, and 150 mV potentials, with the main peaks appearing at −10 (EGC), 70 (EC, EGCG), and 150 mV (ECG).

Quality assurance and quality control procedures were taken during analyses, including analysis of authentic standards for every set of five samples and simultaneous analysis of spiked urine sample daily. The 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 to eliminate the variation in the urine volume. The level of urinary creatinine

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was determined colorimetrically using a Diagnostic Creatinine Kit (Sigma) at 500 nm (DU640 VIS/UV spectrophotometer).

Assessment of femur bone mass

Total bone area, bone mineral content (BMC), and bone mineral density (BMD) of the whole left femur of each rat were determined by dual-energy X-ray absorptiometry (DEXA; HOLOGIC QDR-2000 plus DEXA, Waltham, MA, USA) [11,18]. The instrument was set at an ultra-high resolution mode with a line spacing of 0.0254 cm, resolution of 0.0127 cm, and a collimator diameter of 0.9 cm diameter. The bone was placed in a Petri dish, and to simulate soft-tissue density, tap water was poured around the bones to a depth of 1 cm. Total bone area and BMC were measured, and BMD of this area was calculated by dividing BMC by bone area. The coefficient of variation of these measurements at our laboratory was less than 1.0% [40].

Blood and urine analyses

The concentrations of osteocalcin (OC) and tartrate-resistant acid phosphatase (TRAP) in serum were quantitatively measured with commercial kits from Biomedical Technologies, Inc. (Stoughton, MA, USA) and Immunodiagnostic Systems Ltd (Fountain Hills, AZ, USA), respectively, following the manufacturer's instruction.

Measurement of urinary 8-hydroxy-2′-deoxyguanosine (8-OHdG) concentration

The levels of 8-OHdG in urine were determined following a method described in Shen et al. [11,18]. 8-OHdG was extracted from 1 ml urine with the Oasis® HLB 3 cm^3 (60 mg) cartridge. The eluents were dried under an ultra-pure N_2 stream and reconstituted in buffer (10 mM ammonium acetate in 2% methanol, pH 4.3) for analysis with the ESA HPLC-CoulArray system. The HPLC column for 8-OHdG analysis was a Waters YMC basic™ column (S3 μm, 4.6 × 150 mm). The mobile phase consisted of buffer A (10 mM ammonium acetate, pH 4.3) and buffer B (methanol). Flow rate was kept at 0.8 ml/min, and a linear gradient (0–40% methanol in 15 min) was applied for chromatographic separation with the peak of 8-OHdG eluted at around 9.5 min. The CoulArray Detector was set at 270, 300, 330, and 360 mV, with the highest peak appearing at the 330 mV channel. Authentic standard 8-OHdG was used for qualification by retention times and response patterns and quantification by calibration curves. The limit of detection for 8-OHdG was 1 ng/ml. The amount of 8-OHdG was adjusted by urinary creatinine level.

Determination of TNF-*α* and COX-2 mRNA expression in spleen

Administration of LPS to rodents produced a generalized inflammatory response with increased release of TNF-*α* into the circulation and that of mRNA expression in spleen [11, 41]. Total spleen RNA was extracted using TRIzol reagent (Invitrogen Life Science) according to the manufacturer's instructions. One microgram of total RNA was reverse-transcribed into complementary DNA (cDNA) in a 20 μL reverse transcription system (High-Capacity cDNA Reverse Transcription Kits, Applied Biosystems Inc, Foster City, CA, USA) according to the manufacturer's instructions. Aliquot (2 μL) of each diluted cDNA sample was used for polymerase chain reaction amplification in a 25 μL reaction volume. The cDNA samples were amplified using TaqMan® Gene Expression Assays on an ABI GeneAmp PCR system 7000 in the presence of 1× SYBR Green master mix (Applied Biosystems) and a 400-nm concentration of each of the forward and reverse primers. The following commercial available primer pairs were used for the PCR: TNF-*α* (forward primer, 5′-CCC CTT TAT CGT CTA CTC CTC A-3′; reverse primer, 5′-ACT TCA GCA TCT CGT CTG TTT C-3′), COX-2 (forward primer,5′-CGG ACT TGC TCA CTT TGT TG-3′; reverse primer, 5′-GGT ATT TCA TCT CTC TGC TCT GG-3′), and GAPDH (forward primer, 5′-TAT CAC TCT ACC CAC

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GGC AAG-3′; reverse primer, 5′-ATA CTC AGC ACC AGC ATC ACC-3′). The thermal profile of the reaction consisted of a preheating step at 50°C for 2 min, an initial denaturation step at 95°C for 10 min, then followed by 40 cycles consisting of a denaturation step at 95°C for 15 s, and an annealing/extension step at 60°C for 1 min. The amount of mRNA for each gene was calculated using a standard curve generated from 10-fold dilution of control RNA (Applied Biosystems), and expression levels were normalized to GAPDH.

Statistical analysis

Results were expressed as mean ± standard error of the mean (SEM). All data were analyzed using SigmaStat, version 2.03 (Systat Software, Inc., San Jose, CA, USA). Normality of distribution and homogeneity of variance were tested. Difference between the P group and the L group was analyzed by paired *t* test for each parameter to evaluate the effect of LPS administration.

Data of body weight, urinary EGC and EC, and urinary 8-OHdG were analyzed by three-way analysis of variance (ANOVA; GTP levels × 1-α-OH-vitamin D_3 administration × time) followed by Fisher's protected least significant difference (LSD) post hoc test to evaluate the effect of GTP levels, 1-α-OH-vitamin D_3 administration, time, or interaction. Data of total femoral area, BMC, BMD, OC, TRAP, and mRNA expression of TNF-α and COX-2 in spleen were analyzed by two-way ANOVA followed by Fisher's LSD post hoc test to evaluate the effect of GTP levels, 1-α-OH-vitamin D_3 administration, or interaction. Differences among the four LPS-administered groups (L, LD, LG, and LGD) were analyzed by one-way ANOVA followed by Fisher's LSD post hoc test to determine the effect of treatment. The level of significance was set at $P<0.05$ for all statistical tests.

Results

Body weight and water consumption

There was no significant difference in initial body weight among all treatment groups (Fig. 1). There was no difference in body weight between the P and the L group throughout the study period. Over the course of the 12-week study, all LPS-treated animals gained body weights in a time-dependent manner, regardless of treatment groups.

According to the results of two-way ANOVA analysis, neither GTP supplementation nor 1-α-OH-vitamin D_3 administration significantly affected body weights of rats throughout the study period. In terms of water consumption, the animals in the GTP-supplemented groups (the LG group, 25.7 ml/day and the LGD group, 28.6 ml/day) consumed less water than those without GTP in drinking water (the L group, 31.7 ml/day and the LD group, 32.4 ml/day) throughout the study.

Urinary GTP ingredients

The major forms of GTP ingredients in urine are EGC and EC. During the 12-week intervention period, the levels of EGC (Fig. 2a) and EC (Fig. 2b) in the urine of the P, L, and LD groups were undetectable. The results of three-way ANOVA analysis show that (a) GTP supplementation significantly increased the concentrations of urinary EGC and EC in a time-dependent manner ($P<0.001$), (b) throughout study period, 1-α-OH-vitamin D_3 administration did not significantly affect the levels of EGC and EC in urine, and (c) no interaction between GTP levels and 1-α-OH-vitamin D_3 administration was observed.

Bone mass and turnover biomarkers

Table 1 shows that the rats in the L group had significantly lower values for BMC and BMD than those in the P group. However, there was no difference in femoral bone area between the

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L group and the P group (Table 1). The effects of GTP supplementation or 1-α-OH-vitamin D_3 administration on femoral bone area, BMC, and BMD are also presented in Table 1. Based on the results of two-way ANOVA, after 12 weeks of treatment, (a) neither GTP levels nor 1-α-OH-vitamin D_3 administration significantly affected femoral bone area, (b) GTP supplementation significantly increased femur BMC and BMD of rats, (c) 1-α-OH-vitamin D_3 administration also significantly increased both parameters, and (d) there was no interaction between GTP supplementation and 1-α-OH-vitamin D_3 administration in femur bone area, BMC, and BMD of the LPS-treated rats. Among these LPS-treated rats, the rats in the LGD group had the highest values for femoral BMC and BMD compared to those in the other groups (Table 1).

In terms of bone turnover biomarkers, the rats in the L group had a significantly higher value for TRAP (bone resorption biomarker) than those in the P group (Table 1). On the other hand, there was no significant difference in serum OC level (bone formation biomarker) between the L group and the P group (Table 1). The results of two-way ANOVA analysis (Table 1) show that (a) GTP supplementation significantly decreased serum TRAP concentration, but had no effect on serum OC concentration; (b) 1-α-OH-vitamin D_3 administration resulted in a reduction of serum TRAP level, but no change in serum OC level; and (c) there was an interaction between GTP levels and 1-α-OH-vitamin D_3 administration in TRAP (P=0.018), but not in OC (P=0.119). After 12 weeks, the order of serum TRAP in the LPS-treated rats was the following: L group > LG group > LD group = LGD group (Table 1).

Urinary 8-OHdG

The effect of GTP supplementation plus 1-α-OH-vitamin D_3 administration on oxidative stress-induced DNA damage was determined by the level of urinary 8-OHdG (Fig. 3). At baseline, there was no significant difference in urinary 8-OHdG level among all treatment groups. As expected, the rats in the L group had higher urinary 8-OHdG levels at the mid (P=0.04) and the end of study (P= 0.002), when compared to the rats in the P group (Fig. 3).

The results of three-way ANOVA demonstrate that both GTP supplementation and 1-α-OH-vitamin D_3 administration significantly decreased urinary 8-OHdG levels in a time-dependent manner. A significant synergistic interaction between GTP supplementation and 1-α-OH-vitamin D_3 administration was observed at the end of study (P<0.001). After 12 weeks, among all LPS-treated rats, the order of urinary 8-OHdG was the following: L group > LD group > LG group = LGD group.

mRNA expression of TNF-*α* and COX-2 in spleen

After 12 weeks, the rats in the L group had significant higher values for mRNA expression of TNF-α (P<0.001; Fig. 4a) and COX-2 (P=0.025; Fig. 4b) in spleen, when compared to the rats in the P group. Figure 4 also presents the effect of GTP supplementation plus 1-α-OH-vitamin D_3 administration on mRNA expression of TNF-α (Fig. 4a) and COX-2 (Fig. 4b) in spleen. The results of two-way ANOVA analysis show that (a) after the 12-week study period, GTP supplementation significantly suppressed the mRNA expression of TNF-α (P<0.001) and COX-2 (P=0.019) and in spleen; (b) 1-α-OH-vitamin D_3 administration also significantly inhibited that of TNF-α (P=0.05) and COX-2 (P=0.01) in spleen; and (c) interaction between GTP levels and 1-α-OH-vitamin D_3 administration was only observed in COX-2 mRNA expression (P=0.001), but not in that of TNF-α (P=0.551).

Discussion

In the present investigation, a model of LPS administration of female rats was successfully employed to investigate the impact of two dietary supplements, GTP supplementation in

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drinking water and 1-α-OH-vitamin D_3 administered orally, in chronic inflammation-induced bone loss. Our findings demonstrate that LPS-induced chronic inflammation produced a detrimental effect on bone mass compared to a placebo-treated group, a result in agreement with previous studies [42,43]. Compared to those receiving GTP (the LG group) or 1-α-OH-vitamin D_3 (the LD group) alone, the rats receiving GTP supplementation plus 1-α-OH-vitamin D_3 (the LGD group) for 12 weeks had a synergistic effect with the higher values for femur BMC and BMD, without any changes in total femoral bone area (Table 1). In addition to assessing bone mass of femur (probably mainly cortical bone mass) by DEXA, the bone mass of proximal tibia (mainly trabecular bone) was also assessed by histomorphometry [44]. Based on histomorphometric analyses, the results of two-way ANOVA show that both GTP and 1-α-OH-vitamin D_3 treatments independently increased trabecular volume fraction and number in proximal tibia. There was an interaction between GTP and 1-α-OH-vitamin D_3 in trabecular separation in proximal tibia (data not shown) [44].

In the present study, the effect of GTP in mitigating the influence of LPS was modest at only 3% BMC and BMD. However, such a relatively minor increase in BMC and BMD has significantly improved the strength of femur (as shown on a higher level of yield point force by three-point bending test) as well as the microarchitecture of bone (as shown on higher values for trabecular bone volume fraction and number in both proximal tibia and femur, but lower values for trabecular separation in proximal tibia and femur; data not shown) [44].

GTP, as the secondary metabolites in tea plants, accounts for 30% to 36% weight of the water extractable materials in tea leaves. The major GTP components consist of EGCG, EGC, EC, and ECG [38,45]. In the present study, the different urinary profiles of EC (Fig. 2b) and EGC (Fig. 2a) may be caused by the following possible factors. In the case of EC, the excretion rate stabilizes in relation to the dietary intake rate. Unlike EC, urinary EGC substantially increases between 6 and 12 weeks. We suspect that EC and EGC may have different metabolism and accumulation/excretion rate in animals after a constant treatment, which has been proven in a human study [38]. Moreover, extended treatment of GTP and 1-α-OH-vitamin D_3 together may influence metabolism and accumulation/excretion rate of EC and EGC, as the profile was only changed in EC and not in EGC. The response of urinary GTP composition (i.e., EGC and EC) to the GTP supplementation in drinking water in both LG- and LGD-treated rats agrees with our previous studies on GTP supplementation in drinking water in middle-aged intact and ovariectomized rats [11,18]. These findings further corroborated those published in human populations that urinary excretion of EGC and EC can be employed as practical and reliable biomarkers for green tea bioavailability [45,46].

The present study shows that protecting bone loss in LPS-administered rats by GTP, 1-α-OH-vitamin D_3, or a combination of both was due to the suppression of bone resorption (as shown in lower serum TRAP levels), but not due to bone formation (as shown by no change in serum OC levels; Table 1). The order of ratio for bone formation (OC) to resorption (TRAP) is LGD group > LD group > LG group > L group. The net balance of OC and TRAP leads to an increase in the ratio of OC/TRAP in LGD, LD, or LG group. Such an increase ratio appears to benefit bone remodeling (as shown in BMD levels in Table 1). In addition, the present study is the first study to demonstrate an osteo-protective benefit of GTP and 1-α-OH-vitamin D_3. Such an action of preserving bone mass in the LGD group agrees with those data previously reported for GTP supplementation [11,18] or 1-α-OH-vitamin D_3 administration [29,31,35,47,48] in various bone loss models.

The ability of GTP to suppress bone resorption demonstrated in this study can be elaborated by several previous studies in terms of EGCG's effect on osteoclast activity [20,49–58]. In vitro cellular studies showed that EGCG significantly increased the apoptosis of osteoclasts [50–52], inhibited the survival of differentiated osteoclasts [49], or directly suppressed

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osteoclastogenesis [58]. Such inhibitory actions on osteoclastic activity may be involved in the following mechanisms: (a) EGCG inhibited the differentiation of osteoclasts [53] and the formation of osteoclasts by suppressing the expression of matrix metalloproteinase-9 [49,51, 52,54] in osteoblasts or by decreasing nuclear factor-κB activation [53]; (b) EGCG induced cell death of osteoclasts via single-strand DNA damage, without affecting osteoblastic cells [50,51] via Fenton reaction [50,55] and caspase activation [56]; and (c) (+) catechin inhibited bone resorption and prevented osteoclast activation by acting on bone collagen that could well render bone tissue less prone to resorption [57].

Similar to the effect of GTP on osteoclastic activity, the inhibitory effect of bone resorption by 1-α-OH-vitamin D_3 is supported by others in terms of its impact in osteoclastic activity [59,60]. Sakai et al. [59] reported that 1-α-OH-vitamin D_3 suppresses trabecular bone resorption by inhibiting osteoclastogenic potential in bone marrow cells after ovariectomy in mice. Shibata et al. [60] also found that 1-α-OH-vitamin D_3 inhibits osteoclastogenesis in vivo by decreasing the pool of osteoclast exists precursors in bone marrow. On the other hand, anabolic effect of 1-α-OH-vitamin D_3 has been reported by Weber et al. [61] that 1-α-OH-vitamin D_3 prevents cortical bone loss in ovariectomized rats by decreasing marrow area and increasing cortical area, periosteal perimeter, and periosteal and endocortical bone formation rate.

Previous studies indicate an inverse relationship between the bone mass and urinary 8-OHdG concentration (oxidative stress biomarker) [11,18]. In the present study, we found that a combination of these two interventions, GTP supplementation and 1-α-OH-vitamin D_3 administration, in the LGD group appears to preserve more bone mass (as shown higher BMC and BMD levels in Table 1) than any single treatment by further protecting bones from oxidative stress (as shown the concentration of urinary 8-OHdG in Fig. 3). The findings of GTP's impact on such an inverse relationship (bone mass and urinary 8-OHdG) is supported by previous studies indicating that GTP mitigates bone loss in LPS-treated rats due to GTP's antioxidant capacity, as indicated by higher liver GPX activity [18] and lower urinary 8-OHdG level [11,18]. Moreover, the present study is the first study to elucidate that bone conservation ability of 1-α-OH-vitamin D_3 may be mediated by suppressing oxidative stress-induced DNA damage.

In addition to oxidative stress causing bone loss, chronic inflammation by LPS also contributes to bone loss [11,42]. In general, bone formation and resorption occur at the same time in equilibrium, and they are modulated by systemic hormones, including vitamin D or parathyroid hormone [62,63], by the function of immune cells [64], and by bone-derived local factors (such as TNF-α, prostaglandin E_2 (PGE_2) [62], pro-inflammatory cytokines [62–65], or nitric oxide [66]).

Pro-inflammatory cytokine TNF-α has been shown to enhance bone resorption [67] by increasing osteoclast differentiation and activity and to suppress bone formation via inhibiting osteoblast progenitor cell recruitment and stimulating osteoblast apoptosis [68,69]. The present study demonstrates that a bone-conserving effect of GTP or 1-α-OH-vitamin D_3 in the LPS-treated rats may be mediated by a down-regulation of TNF-α (Fig. 4a).

Prostaglandin E_2, produced by osteoblastic lineage [70], is a potent local factor stimulating bone resorption both in vivo [71] and in vitro [71,72] in response to the catabolic effects of vitamin D, parathyroid hormone, and cytokines [73,74]. The impacts of PGE_2 in bone formation are biphasic and concentration dependent [75–77]. Regarding bone resorption, PGE_2 has a stimulatory role in osteoclastogenesis by enhancing expression of nuclear factor-kappa B ligand and by suppressing granulocyte macrophage-colony stimulating factor [78], leading to more mature osteoclasts resorbing bone (as a result of low bone mass) [78–80].

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COX-2, which mediates PGE_2 production, was elevated in the spleen of rats in a model of inflammation [11,81]. Similar to the findings of TNF-α, this study shows a bone-protective effect of GTP, 1-α-OH-vitamin D_3, or that a combination of both in the LPS-treated rats may be mediated by a down-regulation of COX-2 (Fig. 4b). The findings that down-regulation of TNF-α and COX-2 along with a low bone mass due to a dietary intervention agree with those results are reported by Smith et al. [37].

Green tea polyphenols are potent antioxidants. One of the effects of green tea is its anti-inflammatory property [81], suggesting that GTP supplementation may have a protective role in bone mass maintenance through a reduction of inflammation. Furthermore, because 1-α-OH-vitamin D_3 also holds an anti-inflammatory potential [30], 1-α-OH-vitamin D_3 administration may also have an osteoprotective role in such a LPS-induced chronic inflammation model. In this study, we explored the relationship between GTP and inflammation genes as well as the relationship between 1-α-OH-vitamin D_3 and inflammation genes in the spleen using a model of chronic inflammation-induced bone loss. We demonstrate that GTP supplementation, 1-α-OH-vitamin D_3 administration, or a combination of both significantly decreased mRNA levels of inflammation (TNF-α in Fig. 4a and COX-2 in Fig. 4b) in spleen and increased bone mass (Table 1). Such a bone-sparing effect of these experimental treatments on chronic inflammation is consistent with effects of other antioxidants such as dried plum polyphenols [37] or soy isoflavones [43], with a similar model of bone loss.

Conclusion

In the present study, GTP supplementation plus 1-α-OH-vitamin D_3 administration was evaluated as an alternative treatment option for conserving bone mass in a chronic inflammation-induced bone loss model. Our data demonstrate that GTP plus 1-α-OH-vitamin D_3 has a synergistic potent effect on bone mass in female rats during chronic inflammation. These changes may be mediated in part through a decrease in oxidative stress-induced DNA damage in conjunction with a reduction in inflammation. The present study suggests a potentially significant prophylactic role of GTP plus 1-α-OH-vitamin D_3 in bone health of patients with chronic inflammation, in terms of their effects on suppression of bone resorption. Further study should investigate the potential protective effect of GTP plus 1-α-OH-vitamin D_3 on bone structure and mechanical properties in this model to further understand the role of GTP plus 1-α-OH-vitamin D_3 in skeletal health and how to translate the findings from animal studies to human clinical investigation in order to prevent pathological bone loss (osteoporosis) during chronic inflammation.

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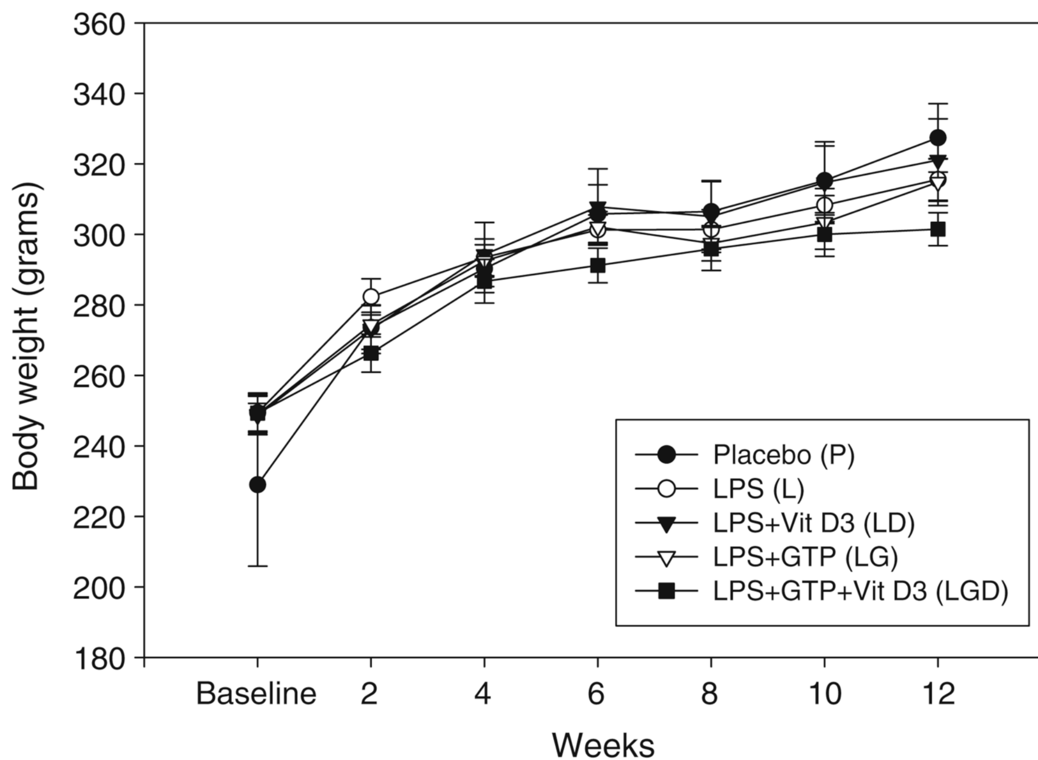

Fig. 1.

Body weight changes in female rats supplemented with green tea polyphenols (*GTP*) in drinking water or 1-α-OH-vitamin D_3 (*D3*) administration for 12 weeks. Values are mean (n=10) with their SEM represented by *vertical bars*. Throughout the study period, no significant difference was observed between the placebo-administered group (the P group) and the LPS-administered only group (the L group). Among the LPS-treated groups (the L, LD, LG, and LGD groups), data were evaluated by three-way ANOVA (GTP levels × D_3 administration × time interaction). Neither GTP supplementation nor D_3 administration affected body weight throughout the study period (P>0.05)

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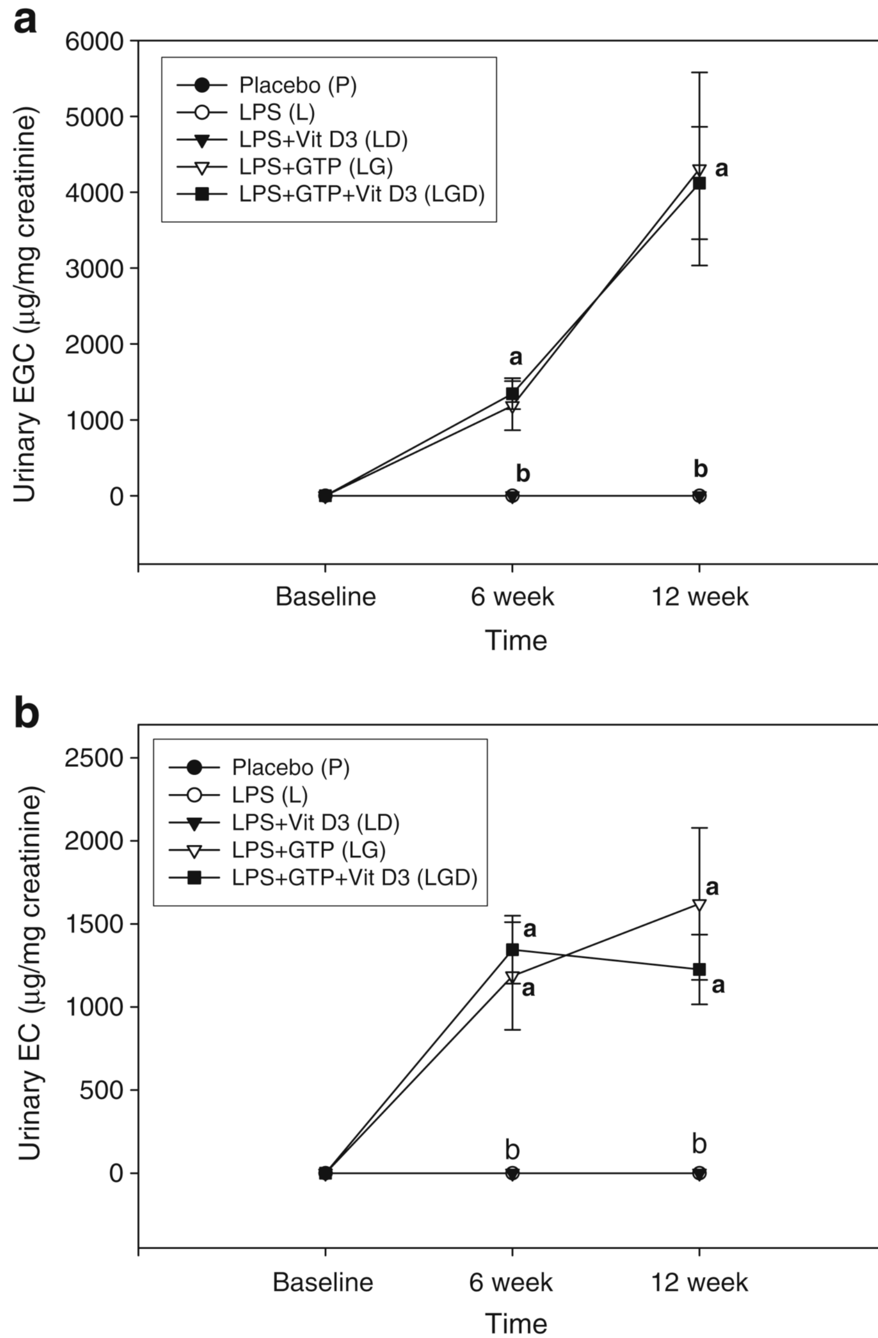

Fig. 2.

Urinary (−)-epigallocatechin (*EGC*; **a**) and (−)-epicatechin (*EC*; **b**) concentrations in female rats supplemented with green tea polyphenols (*GTP*) in drinking water or 1-*α*-OH-vitamin D_3 (*D3*) administration for 12 weeks. Values are mean (*n*=10) with their SEM represented by *vertical bars*. Throughout the study period, no significant difference was observed between the placebo-administered group (the *P* group) and the LPS-administered only group (the *L* group). Among the LPS-treated groups (the *L*, *LD*, *LG*, and *LGD* groups), data were evaluated by three-way ANOVA (GTP levels × D_3 administration × time interaction). Urinary EGC and EC increased significantly in the GTP-supplemented group in a time-dependent manner ($P<0.05$). Urinary EGC and EC concentrations were not affected by D_3 administration

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(P>0.05). No interaction between GTP levels and D_3 administration was observed. Significantly different among the LPS-treated groups (the *L*, *LD*, *LG*, and *LGD* groups) at the same study period, $^{a,b}P<0.05$

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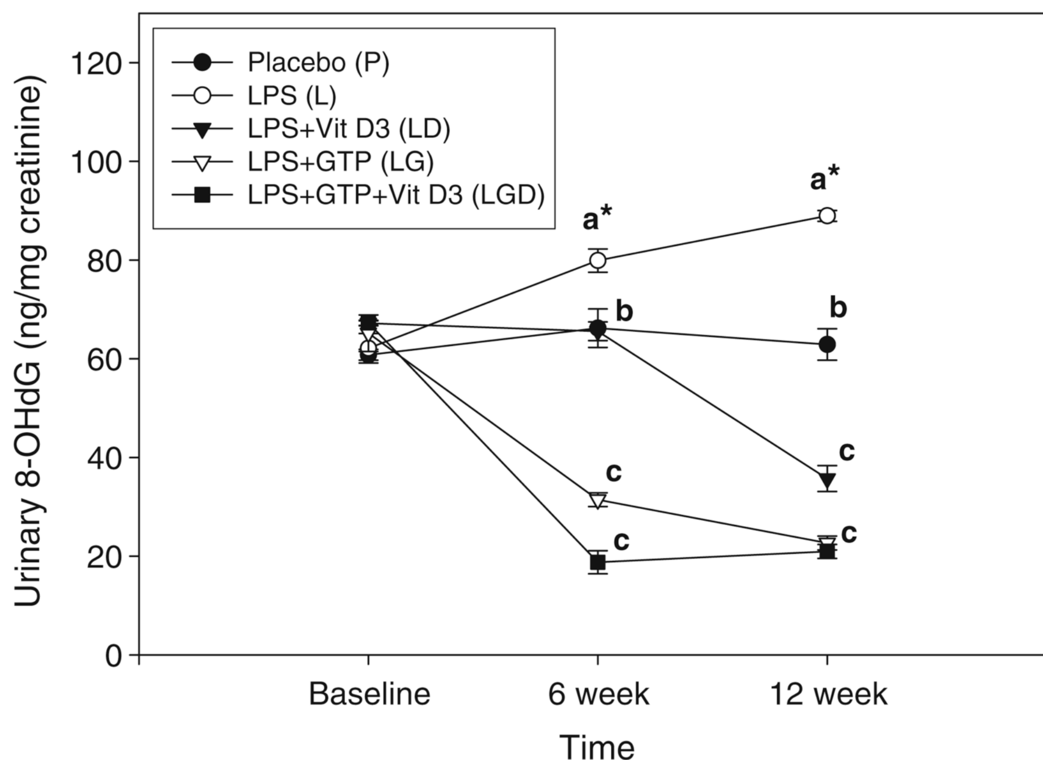

Fig. 3.

Urinary 8-hydroxy-2′-deoxyguanosine (*8-OHdG*) in female rats supplemented with green tea polyphenols (*GTP*) in drinking water or 1-α-OH-vitamin D_3 (*D3*) administration for 12 weeks. Values are mean (n=10) with their SEM represented by *vertical bars*. At the baseline, there was no significant difference between the placebo-administered group (the *P* group) and the LPS-administered only group (the *L* group). After 6 and 12 weeks of study, the L group had significantly higher values for urinary 8-OHdG than those in the P group. Among the LPS-treated groups (the L, LD, LG, and LGD groups), data were evaluated by three-way ANOVA (GTP level × D_3 administration × time interaction). Urinary 8-OHdG decreased significantly in the GTP-supplemented groups in a time-dependent pattern (P<0.001). Urinary 8-OHdG also decreased significantly in the D_3-administered groups in a time-dependent manner (P<0.001). A significant interaction between GTP levels and D_3 administration was observed at the end of study (P<0.001). Significantly different from the P group (between the L group and the P group), *P<0.05. Significantly different among the LPS-treated groups (the L, LD, LG, and LGD groups) at the same study period, $^{a,b,c}P$<0.05

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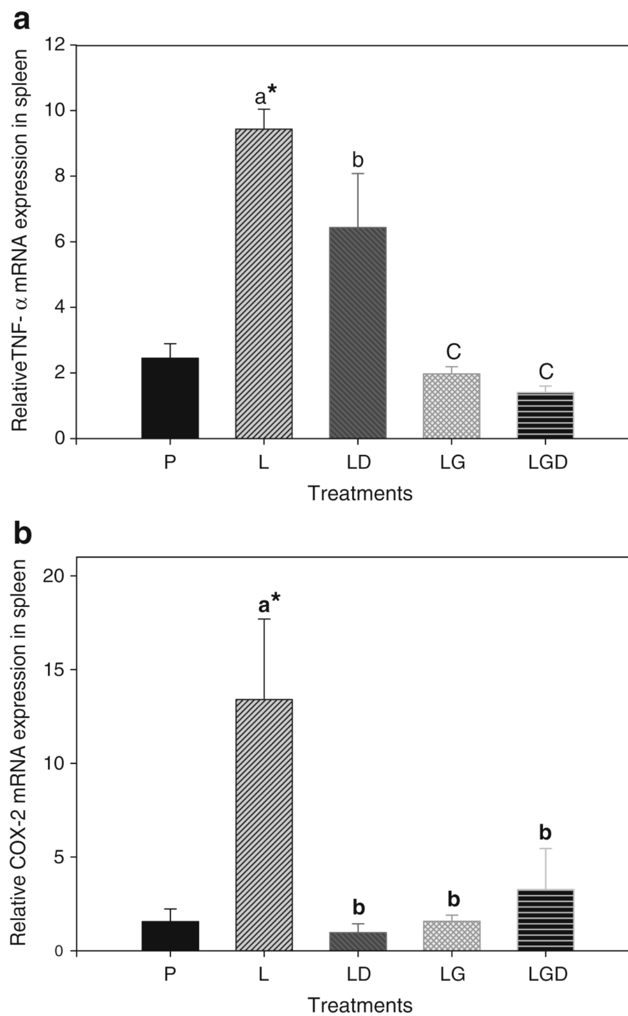

Fig. 4.

Relative mRNA expression of tumor necrosis factor-*α* (*TNF-α*; **a**) and cyclooxygenase-2 (*COX-2*; **b**) in spleen of female rats supplemented with green tea polyphenols (GTP) in drinking water or 1-*α*-OH-vitamin D_3 (D_3) administration for 12 weeks. Values are mean (n=10) with their SEM represented by *vertical bars*. The LPS-administered only group (the *L* group) had significantly higher values for both TNF-*α* and COX-2 mRNA expression in spleen than those in the placebo-administered group (the *P* group). Among the LPS-treated groups (the *L*, *LD*, *LG*, and *LGD* groups), data were evaluated by two-way ANOVA (GTP level × D_3 administration). mRNA expression of TNF-*α* in spleen decreased significantly in the GTP-supplemented groups (P<0.001) and in the D_3-administered groups (P=0.05). mRNA expression of COX-2 in spleen suppressed significantly in the GTP-supplemented groups (P=0.019) and in the D3-administered groups (P=0.01). An interaction between GTP levels and D_3 administration was observed in COX-2 mRNA expression (P= 0.001), but not in TNF-*α* mRNA expression (P=0.551). Significantly different from the P group (between the L group and the P group), *P<0.05. Significantly different among the LPS-administered groups (the *L*, *LD*, *LG*, and *LGD* groups), $^{a,b,c}P<0.05$

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Table 1

Bone mass and turnover biomarkers in female rats supplemented with green tea polyphenols (GTP) in drinking water or 1-α-OH-vitamin D_3 (D_3) administration for 12 weeks

Parameters	**Placebo (P group)**	**−GTP**		**+GTP**		**Two-way ANOVA *P* value**		
		−D_3 (L group)	**+D_3 (LD group)**	**−D_3 (LG group)**	**+D_3 (LGD group)**	**GTP**	**D_3**	**GTP × D_3**
Bone mass by DEXA								
Femur bone area (cm^2)	1.938 ± 0.031	1.931 ± 0.036	1.900 ± 0.021	1.899 ± 0.029	1.937 ± 0.028	0.598	0.884	0.525
Femur BMC (mg)	531 ± 4	492 ± 10c*	524 ± 8b	506 ± 6c	561 ± 14a	0.030	<0.001	0.307
Femur BMD (mg/cm^2)	269 ± 2	258 ± 3d*	273 ± 2b	266 ± 2c	284 ± 2a	<0.001	<0.001	0.535
Bone turnover biomarkers by ELISA								
Serum OC (ng/ml)	12.18 ± 0.53	12.11 ± 0.46	12.00 ± 0.77	11.25 ± 0.54	12.76 ± 0.67	0.821	0.163	0.119
Serum TRAP (U/L)	4.27 ± 0.43	8.71 ± 0.93a*	3.67 ± 0.22c	5.18 ± 0.73b	3.19 ± 0.41c	0.002	<0.001	0.018

Results are expressed as mean values ± SEM. Difference between the placebo-administered group (the P group) and the LPS-administered only group (the L group) was analyzed by paired *t*-test to evaluate the effect of LPS administration. All the LPS-treated groups (the L, LD, LG, and LGD groups) were analyzed by two-way ANOVA (GTP levels × D_3 levels) followed by Fisher's protected least significant difference post hoc test to evaluate the effect of GTP levels, D_3 administration, or interaction. Lowercase letters indicate that they are significantly different among the LPS-administered groups (the L, LD, LG, and LGD groups), $P<0.05$

BMC bone mineral content, *BMD* bone mineral density, *OC* osteocalcin, *TRAP* tartrate resistant acid phosphatase

* $P<0.05$, significantly different from the P group (between the L group and the P group)