

Green tea polyphenols mitigate bone loss of female rats in a chronic inflammation-induced bone loss model^{☆,☆☆}

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Abstract

The purpose of this study was to explore the bioavailability, efficacy and molecular mechanisms of green tea polyphenols (GTP) related to preventing bone loss in rats with chronic inflammation. A 2 [placebo vs. lipopolysaccharide (LPS)] \times 2 (no GTP vs. 0.5% GTP in drinking water) factorial design enabled the evaluation of effects of LPS administration, GTP levels, and LPS \times GTP interaction. Urinary GTP components and 8-hydroxy-2'-deoxyguanosine (8-OHdG) levels were determined by high-pressure liquid chromatography for bioavailability and molecular mechanism, respectively. Efficacy was evaluated by examining changes in femoral mineral content (BMC) and density (BMD) using dual-energy X-ray absorptiometry, and bone turnover biomarkers [osteocalcin (OC) and tartrate-resistant acid phosphatase (TRAP)] using respective ELISA kits. The mRNA expression of tumor necrosis factor- α (TNF- α) and cyclooxygenase-2 (COX-2) in spleen was determined by real-time RT-PCR. Neither LPS administration nor GTP levels affected body weight and femoral bone area throughout the study period. Only GTP supplementation resulted in increased urinary epigallocatechin and epicatechin concentrations. LPS administration led to a decrease in femur BMC and BMD, and serum OC levels, but an increase in serum TRAP, urinary 8-OHdG and spleen mRNA expression of TNF- α and COX-2 levels. GTP supplementation resulted in higher values for femur BMC, BMD and serum OC, but lower values for serum TRAP, urinary 8-OHdG and spleen mRNA expression of TNF- α and COX-2 levels. We conclude that GTP mitigates bone loss in a chronic inflammation-induced bone loss model by reducing oxidative stress-induced damage and inflammation.

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1. Introduction

Low bone mass (also called osteopenia) has been reported in patients with a variety of chronic inflammatory diseases, including chronic periodontitis [1] and pancreatitis [2], inflammatory bowel disease [3], rheumatoid arthritis [4] and lupus erythematosus [5]. The pathogenesis of low bone mass in patients with such chronic inflammatory diseases is complex and involves pro-inflammatory production of cytokine mediators (i.e., tumor necrosis factor- α (TNF-

α), cyclooxygenase-2 (COX-2) and interleukin-1 β [4,6,7]), glucocorticoid treatment [8] and decreased muscular function, resulting in decreased bone formation, increased bone resorption, increased risk for falls and, therefore, increased risk for bone fracture [9].

Bone loss has been associated with high levels of oxidative stress in animal [10,11] and human epidemiologic studies [12,13]. Reactive oxygen species (ROS), such as superoxides and hydrogen peroxide, can cause severe damage to DNA, protein and lipids [14]. Oxidative stress results from high levels of ROS produced during normal cellular metabolism (e.g., mitochondrial electron transport) or from environmental stimuli (e.g., cytokines, UV radiation) perturbing the normal redox balance, shifting cells into a state of oxidative stress [15]. Recently, Shen et al. [10] demonstrated that oxidative stress [as shown by an increase in urinary 8-hydroxy-2'-deoxyguanosine (8-OHdG), an oxidative stress biomarker] is involved in the pathogenesis of bone loss in middle-aged female rats due to aging as well as aging plus estrogen deficiency. Oxidative stress leads to (i) an increase in osteoblast and osteocyte apoptosis [16], (ii) a decrease in osteoblast number via extracellular signal-regulated kinases (ERK) and ERK-dependent nuclear factor- κ B signaling pathways [17], (iii) a decrease

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in the rate of bone formation via Wnt/ β -catenin signaling [18] and (iv) an increase in the differentiation and function of osteoclasts [19]. On the other hand, there is mounting evidence suggesting that oxidative stress may also contribute to bone loss due to chronic inflammation [9,20]. However, no data are available showing a relationship between oxidative stress and chronic inflammation-induced bone loss.

Green tea is one of the most popular beverages in the world, and it has received considerable attention because of its many scientifically proven beneficial effects on human health, including maintaining bone mass [10,11,21]. Both epidemiological [22–25], animal [10,11] and cellular [26–28] studies strongly suggested that green tea polyphenols (GTP, green tea extract) are a promising dietary antioxidant for preventing bone loss in women and men with low bone mass [21]. In addition, our recent study shows that drinking water supplemented with GTP mitigated bone loss due to an increase of antioxidant capacity in conjunction with a decrease in oxidative stress damage [10]. However, the effect of green tea or green tea bioactive components on chronic inflammation-induced bone loss and related molecular mechanism(s) is unclear. Therefore, the present study was designed to investigate the potential benefit of dietary antioxidants, GTP, in the treatment or prevention of bone loss in female rats with chronic inflammation. We hypothesized that (i) supplementation of GTP in drinking water will mitigate chronic inflammation-induced bone loss in female rats and (ii) such changes are related to a reduction of oxidative stress-induced damage in conjunction with a reduction of inflammation. Studying the effect of GTP on bone remodeling in female rats with chronic inflammation will advance the understanding of their effects on skeletal biology to minimize bone loss in human with chronic inflammation.

2. Materials and methods

2.1. Animals and GTP treatments

Forty virgin CD female rats (3 months 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, rats were randomized by weight and assigned to placebo implantation (P), lipopolysaccharide (LPS) administration (L), P+0.5% GTP (PG) and LPS+0.5% GTP (LG) for 12 weeks. This 2 (placebo vs. LPS administration) \times 2 (no GTP vs. 0.5% GTP in drinking water) factorial design enabled the evaluation of effects of LPS administration, GTP levels and LPS \times GTP interaction.

Twenty rats in LPS-operated groups were subjected to the following procedures modified from Smith et al. [29]: LPS (*E. coli* Serotype 0127:B8, Sigma, St. Louis, MO, USA) was incorporated into time-release pellets (Innovative Research of America, Sarasota, FL, USA), designed to deliver a consistent dose for 12 weeks. For LPS animals, the dorsal neck area was shaved and sterile techniques 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 (33.3 μ g/day) implantation (approximately 2 cm beyond the incision site) was formed using forceps. The incision site was closed with surgical glue. Rats were maintained on a regular rodent chow diet with free access to no-GTP (L group) or 0.5% GTP drinking water (LG group) throughout the 12-week study period. The remaining 20 rats in the placebo-operated group received a pellet containing matrix only using the same procedures of administration described above. The placebo rats were also maintained on a regular rodent chow diet with free access to no-GTP (P group) or 0.5% GTP drinking water (PG group) throughout the study period. Rats in the GTP treatment were given 0.5% concentration of GTP in drinking water daily to mimic human consumption of green tea of four cups a day [30,31].

Distilled water mixed with GTP was prepared fresh daily and the amount of water consumed was recorded for each rat. GTP was purchased from the same source as that used in our previous studies (Shili Natural Product Company, Japan), with a purity higher than 98.5%. Every 1000 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 (–)-gallicocatechin gallate (GCG) and 44 mg of catechin according to the HPLC-ECD and HPLC-UV analyses. Rats were housed individually under a controlled temperature of 21 \pm 2°C with a 12-h light–dark cycle. Rats were weighed weekly and examined daily. All procedures were approved by the local institutional animal care and use committee.

2.2. Sample preparation

Twenty-four-hour urine samples were collected from metabolic cages at baseline and after 6 (midpoint) and 12 weeks (end point) of intervention for each animal and stored at –80°C until 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. Tissue samples were harvested, immediately immersed into liquid nitrogen and stored at –80°C prior to analysis. Femora were harvested and cleaned of adhering soft tissue. The femur samples were stored in 70% ethanol for bone parameter assessments.

2.3. Measurement of urinary GTP components

The concentrations of GTP components in urine were determined following a method described in Shen et al. [10]. Thawed urine samples were centrifuged and 1 ml supernatant 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-C₁₈ (5 μ m, 4.6 \times 250 mm). The 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 components were generated separately, and ECG, EC, EGCG and EGC 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 mV (EGC), 70 mV (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. Urinary creatinine level was determined colorimetrically with a Diagnostic Creatinine Kit (Sigma) at 500 nm (DU640 VIS/UV spectrophotometer).

2.4. 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, Hologic, Waltham, MA, USA) [10]. The instrument was set at an ultrahigh-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. BMC and bone area 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% [32].

2.5. Blood and urine analyses

The concentrations of osteocalcin (OC) and tartrate-resistant acid phosphatase (TRAP) in serum were quantified by using commercial kits from Biomedical Technologies (Stoughton, MA, USA) and Immunodiagnostic System (Fountain Hills, AZ, USA), respectively, following the manufacturers' instruction.

2.6. Measurement of urinary 8-OHdG concentration

The levels of 8-OHdG in urine were determined following a method described in Shen et al. [10]. 8-OHdG was extracted from 1 ml urine with the Oasis HLB 3 ml (60 mg) cartridge. The eluents were dried under an ultra-pure N₂ 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 (53 μ m, 4.6 \times 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 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.

2.7. 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 [33]. Total spleen RNA was extracted using TRIzol reagent (Invitrogen Life Science) according to the manufacturer's instruction. One microgram of total RNA was reverse transcribed into complementary DNA (cDNA) in a 20- μ l reverse transcription

system (High-Capacity cDNA Reverse Transcription Kits, Applied Biosystems, Foster City, CA, USA) according to the manufacturer's instructions. A 2- μ l aliquot of each diluted cDNA sample was used for polymerase chain reaction amplification in a 25- μ l reaction volume. The cDNA samples were amplified using TaqMan Gene Expression Assays on an ABI GeneAmp PCR system 7000 in the presence of 1 \times 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 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.

2.8. Statistical analysis

Results were expressed as mean \pm S.E.M. All data were analyzed using SigmaStat, version 2.03 (Systat Software, San Jose, CA, USA). Normality of distribution and homogeneity of variance were tested. Data of body weight, urinary EGC and EC, and urinary 8-OHdG were analyzed by three-way analysis of variance (ANOVA) (LPS administration \times GTP levels \times Time) followed by Fisher's protected least significant difference (Fisher's LSD) *post hoc* test to evaluate the effect of LPS administration, GTP levels, time (week) or interaction. Data of bone mass, 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 LPS administration, GTP levels or interaction. Differences among the four dietary treatment groups (P, L, PG and LG) 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 $P < .05$ for all statistical tests, and statistical trend ($P < .10$) was also indicated.

3. Results

3.1. Body weight and water consumption

There was no significant difference in initial body weight among all treatment groups (Fig. 1). Over the course of the 12-week study, all animals gained body weights in a time-dependent manner, regardless of treatment groups. Neither LPS administration nor GTP supplementation significantly affected the body weights of rats throughout the study period. In terms of water consumption, the animals in GTP-supplemented groups (G group: 25.7 ml/day; LG group: 25.8 ml/day)

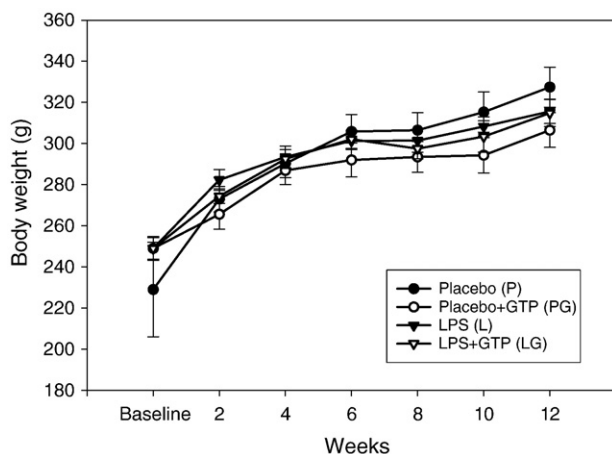


Fig. 1. Body weight changes in placebo- and LPS-administrated female rats supplemented with GTP in drinking water for 12 weeks. Values are mean ($n=10$) with their S.E.M. represented by vertical bars. No differences were observed between groups for all treatments at baseline ($P > .05$). Data was evaluated by three-way ANOVA (LPS administration \times GTP level \times Time interaction). Neither LPS administration nor GTP supplementation affected body weight throughout the study period ($P < .05$).

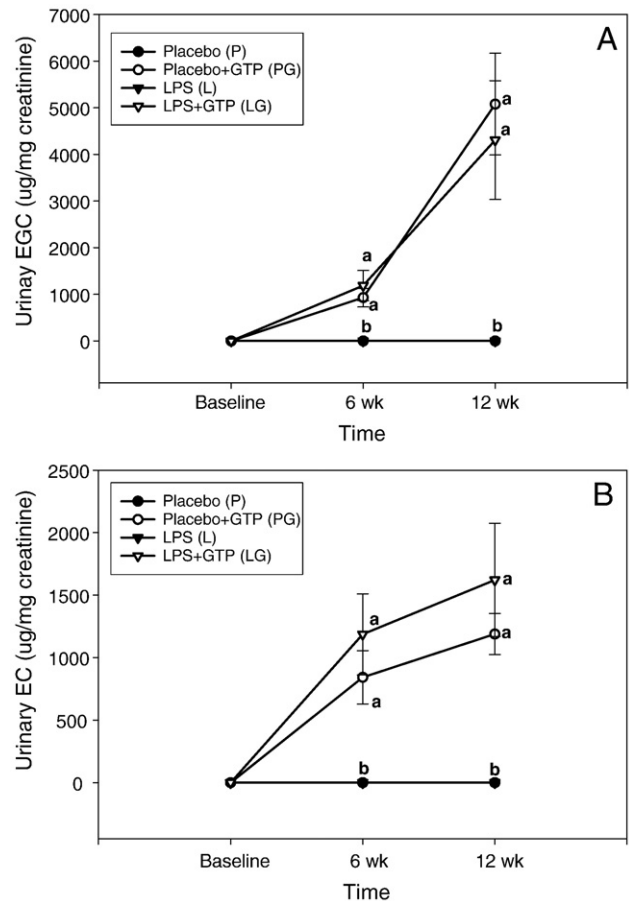


Fig. 2. Urinary EGC (A) and EC (B) concentrations in placebo- and LPS-administrated female rats supplemented with GTP in drinking water for 12 weeks. Values are mean ($n=10$) with their S.E.M. represented by vertical bars. Data was evaluated by three-way ANOVA (LPS administration \times GTP level \times Time interaction). Urinary EGC and EC concentration was not affected by LPS administration ($P > .05$). Urinary EGC and EC increased significantly in GTP-supplemented group in a time-dependent manner ($P < .05$). No interaction between LPS administration and GTP levels was observed.

consumed less water than those without GTP in drinking water (P group: 33.7 ml/day; L group: 31.7 ml/day) throughout the study.

3.2. Urinary GTP ingredients

The major forms of GTP ingredients in urine are EGC and EC. The levels of EGC (Fig. 2A) and EC (Fig. 2B) in the urine of the P and L group were undetectable during the intervention. The results of two-way ANOVA analysis show that, throughout the 12-week study period, (i) LPS administration did not significantly affect the levels of EGC and EC in urine; (ii) GTP supplementation significantly increased the concentrations of urinary EGC and EC in a time-dependent manner; and (iii) no interaction between LPS administration and GTP levels was observed.

3.3. Bone mass and turnover biomarkers

The effects of LPS administration or GTP supplementation on femoral bone area, BMC, BMD and turnover biomarkers are described in Table 1. Neither LPS administration nor GTP levels significantly affected femoral bone area after 12 weeks. Based on the results of two-way ANOVA, after 12 weeks of treatment, LPS administration resulted in a decrease in the values for femur BMC and BMD of rats,

Table 1
Bone mass and turnover biomarkers in placebo- and LPS-administrated female rats supplemented with GTP in drinking water for 12 weeks

Parameters	– LPS		+ LPS		Two-way ANOVA <i>P</i> value		
	No GTP (P group)	0.5% GTP (PG group)	No GTP (L group)	0.5% GTP (LG group)	LPS	GTP	LPS×GTP
Bone mass by DEXA							
Femur bone area (cm ²)	1.938±0.031	1.931±0.036	1.916±0.035	1.899±0.029	.804	.536	.818
Femur BMC (mg)	505±13 ^{a,b}	533±9 ^a	488±11 ^b	506±6 ^{a,b}	.044	.034	.596
Femur BMD (mg/cm ²)	265±3 ^{a,b}	270±2 ^a	258±2 ^b	265±2 ^{a,b}	.039	.036	.606
Bone turnover biomarkers by ELISA							
Serum OC (ng/ml)	12.18±0.54 ^b	13.67±0.58 ^a	12.11±0.46 ^b	11.25±0.54 ^b	.028	.563	.037
Serum TRAP (U/L)	4.27±0.43 ^b	4.66±0.46 ^b	8.71±0.94 ^a	5.19±0.73 ^b	<.001	.044	.011

^{a,b}Mean values within a row with unlike superscript letters differing significantly among dietary treatments by one-way ANOVA followed by Fisher's LSD test ($P<.05$). Results are expressed as mean values±S.E.M. All dietary treatment groups were analyzed by two-way ANOVA (LPS administration×GTP levels) followed by Fisher's LSD *post hoc* test to evaluate the effect of LPS implementation, GTP levels or interaction.

BMC, Bone mineral content; BMD, bone mineral density; OC, osteocalcin; TRAP, tartrate-resistant acid phosphatase.

while GTP supplementation led to an increase in the values for both parameters. There is no interaction between LPS administration and GTP level in femur BMC and BMD of rats.

In terms of bone turnover biomarkers, the results of two-way ANOVA analysis (Table 1) show that, throughout the 12-week study period, (i) LPS administration significantly resulted in a reduction of bone formation (OC) level, but an elevation of bone resorption (TRAP) level in serum; (ii) GTP supplementation significantly decreased the concentrations of serum TRAP, but had no effect on serum OC; and (iii) there was interaction between LPS administration and GTP levels in both serum OC ($P=.011$) and TRAP ($P=.037$). Rats in the PG group had the highest value for serum OC compared to those in the other groups. There was no significant difference in serum OC among the P, L and LG groups. On the other hand, the rats in the L group had the highest value for serum TRAP than those in other groups; and there was no significant difference in serum TRAP among the P, PG and LG groups.

3.4. Urinary 8-OHdG

The effect of LPS administration or GTP supplementation 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, LPS administration significantly increased urinary 8-OHdG level at a time-dependent pattern ($P<.001$), (ii) GTP supplementation significantly decreased urinary 8-OHdG level at a time-dependent manner ($P<.001$) and (iii) interaction between LPS administration and GTP levels was observed at the end of study ($P<.001$). After 12 weeks, the order of urinary 8-OHdG is the following: L group>P group=PG group=LG group.

3.5. Messenger RNA expression of TNF- α and COX-2 in spleen

Fig. 4 shows the impact of LPS administration or GTP supplementation on mRNA expression of TNF- α (A) and COX-2 (B) in spleen. The results of two-way ANOVA analysis show that (i) after the 12-week study period LPS administration significantly induced the mRNA expression of TNF- α and COX-2 in spleen, (ii) GTP supplementation significantly suppressed those of TNF- α and COX-2 in spleen, and (iii) interaction between LPS administration and GTP levels was observed in TNF- α ($P<.001$), likely in COX-2 ($P=.098$). The order of TNF- α and COX-2 mRNA expression in spleen is the following: L group>P group=PG group=LG group.

4. Discussion

In the present investigation, a model of LPS administration of female rats was successfully employed to investigate the impact of

GTP supplementation in drinking water in chronic inflammation-induced bone loss. Compared with the rats receiving placebo administration (P and PG groups), the rats receiving LPS administration (L and LG groups) for 12 weeks had lower values for femur BMC (F value=4.51) and BMD (F value=4.71). The results of histomorphometric analyses show that LPS lowered trabecular volume fraction, number and thickness in proximal tibia, whereas GTP supplementation increased these parameters (data not shown) [34]. Such findings demonstrate that chronic inflammation produced a detrimental effect on bone mass, a result in agreement with a previous study [29,35]. As expected, supplementation of GTP in drinking water given to the rats (PG and LG groups) for 12 weeks resulted in higher values for femur BMC (F values=5.03) and BMD (F values=8.63), compared to those without GTP supplementation (P and L groups) (Table 1). It should be noted that chronic LPS administration in the present study did not compromise animal growth (as shown in no change in body weight throughout the study) [29].

Green tea polyphenols are the secondary metabolites in tea plants and accounts for 30% to 36% weight of the water-extractable materials in tea leaves. The major GTP components include EGCG, EGC, EC and ECG [36,37]. The response of urinary GTP composition (viz. EGC and EC) to the GTP supplementation in drinking water in both placebo- and LPS-treated rats is consistent with our previous study on GTP supplementation in drinking water in middle-aged intact and ovariectomized female rats [10]. Such findings also support those

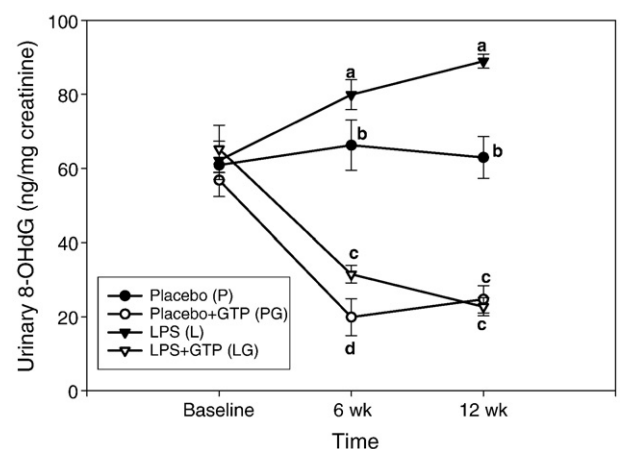


Fig. 3. Urinary 8-OHdG in placebo- and LPS-administrated female rats supplemented with GTP in drinking water for 12 weeks. Values are mean ($n=10$) with their S.E.M. represented by vertical bars. Data was evaluated by three-way ANOVA (LPS administration×GTP level×Time interaction). Urinary 8-OHdG increased significantly in LPS-administrated groups ($P<.001$). Urinary 8-OHdG decreased significantly in GTP-supplemented groups ($P<.001$). An interaction between LPS administration and GTP levels was observed at the end of study ($P<.001$).

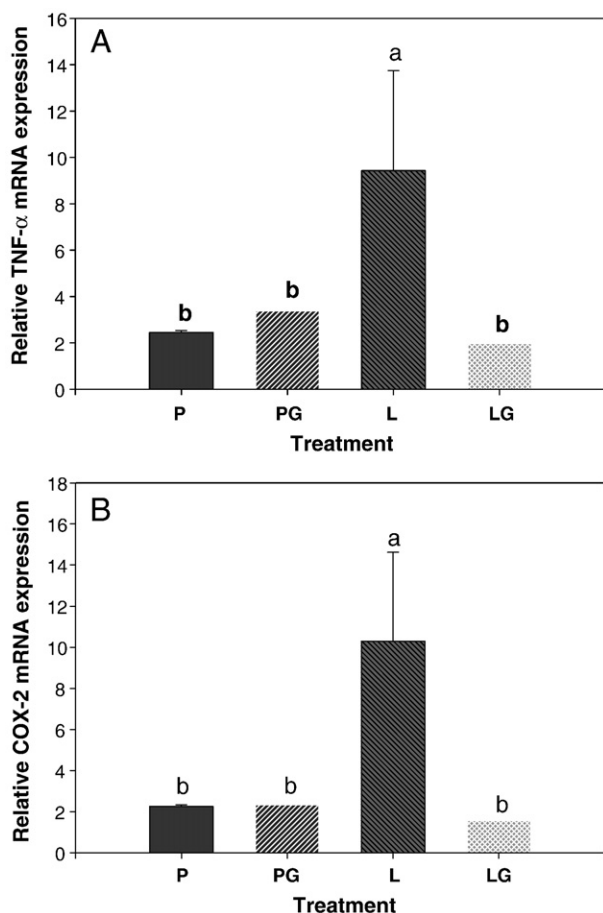


Fig. 4. Relative mRNA expression of TNF- α and COX-2 (B) in spleen of placebo- and LPS-administrated female rats supplemented with GTP in drinking water for 12 weeks. Values are mean ($n=10$) with their S.E.M. represented by vertical bars. Data was evaluated by two-way ANOVA (LPS administration \times GTP level). mRNA expression of TNF- α and COX-2 in spleen increased significantly in LPS-administrated groups ($P<.05$). mRNA expression of TNF- α and COX-2 in spleen decreased significantly in GTP-supplemented groups ($P<.05$). An interaction between LPS administration and GTP levels was observed.

published in human populations [37,38] that urinary excretion of GTP components (i.e., EGC, EC) can be used as practical and reliable biomarkers for green tea bioavailability.

The present study shows that mitigating bone loss in LPS-treated rats by GTP supplementation in drinking water was due to the suppression of bone resorption (as shown in lower serum TRAP), but not due to bone formation (as shown by no change in serum OC). The net balance of OC and TRAP leads to a higher ratio of bone formation to resorption in GTP-supplemented groups to benefit bone remodeling. The finding that preserving bone mass in the rats by GTP supplementation in drinking water corroborates those reported in our previous study using intact and ovariectomized rats [10] as well as in several cross-sectional human studies [22–25].

The ability of GTP to inhibit bone resorption demonstrated in the presented study can be explained by several previous studies in terms of EGCG's effect on osteoclast activity. Cellular studies demonstrated that EGCG (i) significantly inhibited the survival of differentiated osteoclasts [39] and increased the apoptosis of osteoclasts [40–42]; (ii) inhibited the differentiation of osteoclasts [43] and the formation of osteoclasts by inhibiting the expression of matrix metalloproteinase-9 [39,41,42,44] in osteoblasts or via decreasing nuclear factor- κ B activation [43]; (iii) induced cell death of osteoclasts in terms of single-strand DNA damage, without affecting osteoblastic cells in a cocultured system of osteoblasts and osteoclasts [40,41] via Fenton

reaction [40,45] and caspase activation [46]; and (iv) (+) catechin inhibited bone resorption and prevented osteoclast activation by acting on bone collagen that could well render bone tissue less prone to resorption [47].

Previous studies indicate that increased ROS production may exacerbate the chronic inflammation-induced bone loss process by elevating oxidative stress [9,20]. In the present study, we found an inverse relationship between bone mass and urinary 8-OHdG concentration (oxidative stress biomarker) in LPS-treated rats. We further found that the rats receiving GTP supplementation had higher femur bone mass (BMC and BMD) (Table 1) along with lower urinary 8-OHdG levels (Fig. 3). These findings are supported by previous results indicating that GTP mitigates bone loss in both middle-aged intact and ovariectomized rats due to GTP's antioxidant capacity, as indicated by higher liver GPX activity and lower urinary 8-OHdG level [10].

In addition to oxidative stress, chronic inflammation also contributes to systemic bone loss [29]. In general, bone formation and bone resorption occur simultaneously in equilibrium, and they are regulated by systemic hormones (such as vitamin D and parathyroid hormone) [48,49], bone-derived local factors including prostaglandins [48], proinflammatory cytokines [48–50], nitric oxide [51] and the function of immune cells [52]. Among the prostaglandin E_2 (PGE_2) produced by osteoblastic lineage [53] is a potent local factor stimulating bone resorption both *in vivo* [54] and *in vitro* [54,55] in response to the catabolic effects of vitamin D, parathyroid hormone and cytokines [56,57]. *In vitro* studies [58–60] show that effects of PGE_2 on bone formation are biphasic and concentration dependent. At low concentrations, PGE_2 supplementation stimulates bone formation [58,61,62], while at higher concentrations, PGE_2 inhibits bone formation [58,63].

In terms of bone resorption, PGE_2 has a stimulatory role in osteoclastogenesis via enhancing expression of nuclear factor- κ B ligand (RANKL) and via suppressing granulocyte macrophage-colony stimulating factor [54], leading to more mature osteoclasts resorbing bone (as a result of low bone mass) [64–66]. In the present study, COX-2, which mediates PGE_2 production, was elevated in the spleen of rats receiving chronic LPS administration (Fig. 4B).

Similarly, pro-inflammatory cytokine mRNA TNF- α expression in spleen was also increased in the LPS-treated rats (Fig. 4A). TNF- α has been shown to enhance bone resorption [67] via increasing osteoclast differentiation and activity as well as to inhibit bone formation via suppressing osteoblast progenitor cell recruitment and stimulating osteoblast apoptosis [68,69]. The finding that up-regulation of COX-2 and TNF- α in spleen along with a low bone mass agrees with those results reported by Smith et al. [10].

Green tea polyphenols are potent antioxidants. One of the effects of green tea is its anti-inflammatory property [70], suggesting GTP supplementation in drinking water may have a protective role in bone mass through a reduction of inflammation. In this study, we explored the relationship between GTP and inflammation genes in the spleen in a model of chronic inflammation-induced bone loss. We demonstrated that GTP supplementation significantly decreased mRNA levels of inflammation (i.e., COX-2, TNF- α) in spleen (Fig. 4) and increased bone mass (Table 1). Such a bone-sparing effect of GTP due to chronic inflammation is consistent with other antioxidants such as soy isoflavones [35], with the same model of bone loss. Our results show that by down-regulating inflammatory mediators such as COX-2 and TNF- α , GTP supplementation may reduce the risk of osteoporosis (severe bone loss).

5. Conclusion

In the present study, GTP was evaluated as an alternative treatment option for mitigating reduced bone mass due to chronic

inflammation. Our data demonstrate that GTP supplementation has potent effects on BMD 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 in bone health of human with chronic inflammation-induced bone loss in terms of their effects on suppression of bone resorption. The 0.5% GTP concentration employed in the current study is commensurate with a feasible dose for human consumption (four cups a day) [10,31,71]. Such a dose is feasible in a human clinical investigation on bone health [71], while larger doses may be required in order for other dietary supplements to substantiate benefits to human bone [72]. Further study should investigate the potential protective effect of GTP on bone structure and mechanical properties in this model of chronic inflammation-induced bone loss to further understand the role of GTP supplementation 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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