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Protective actions of green tea polyphenols and alfacalcidol on bone microstructure in female rats with chronic inflammation☆

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Abstract

This study investigated the effects of green tea polyphenols (GTP) and alfacalcidol on bone microstructure and strength along with possible mechanisms in rats with chronic inflammation. A 12-week study using a 2 (no GTP vs. 0.5%, w/v GTP in drinking water)×2 (no alfacalcidol vs. 0.05 μg/kg alfacalcidol orally, 5×/week) factorial design was employed in lipopolysaccharide (LPS)-administered female rats. A group receiving placebo administration was used to compare with a group receiving LPS administration only to evaluate the effect of LPS. Changes in tibial and femoral microarchitecture and strength of femur were evaluated. Difference in expression of tumor necrosis factor-α (TNF-α) in proximal tibia using immunohistochemistry was examined. Compared to the placebo group, the LPS-administered-only group had significantly lower femoral mass, trabecular volume, thickness and number in proximal tibia and femur, and lower periosteal bone formation rate in tibial shafts but had significantly higher trabecular separation and osteoclast number in proximal tibia and eroded surface in endocortical tibial shafts. Both GTP and alfacalcidol reversed these LPS-induced detrimental changes in femur, proximal tibia and endocortical tibial shaft. Both GTP and alfacalcidol also significantly improved femoral strength, while significantly suppressed TNF-α expression in proximal tibia. There were significant interactions in femoral mass and strength, trabecular separation, osteoclast number and TNF-α expression in proximal tibia. A combination of both showed to sustain bone microarchitecture and strength. We conclude that a protective impact of GTP and alfacalcidol in bone microarchitecture during chronic inflammation may be due to a suppression of TNF-α.

Keywords: Tea; Alfacalcidol; Dietary supplement; Inflammation; Histomorphometry; Micro-CT; Bone quality

1. Introduction

Chronic inflammation has been associated with progression of bone loss and microarchitecture deterioration [1–3] through excessive production of pro-inflammatory cytokine mediators (e.g. tumor necrosis factor-α (TNF-α) [4], cyclooxygenase-2 [5] or interleukin-1β [6] and oxidative stress [7]. Agents that inhibit inflammation may have potential therapeutic value for the prevention and/or treatment of chronic inflammation-induced bone loss and microstructure deterioration. A number of agents have been shown to suppress TNF-α expression level in various cells and tissues [8–11]. These inhibitory agents include anti-oxidant and/or anti-inflammatory agents, such as green tea [2,8], alfacalcidol [9], soy isoflavones [10] or dried plum polyphenols [11].

Green tea (*Camellia sinensis*), a popular beverage worldwide, was found to cause a wide range of effects on animal and human health due to its anti-oxidant and/or anti-inflammatory properties [12]. Tea has been reported to have beneficial effects in various inflammatory conditions, such as carrageenan-induced paw edema [13], inflammatory bowel disease [14], collagen-induced arthritis [15] or lipopolysaccharide (LPS)-induced gingival inflammation [16]. On the other hand, alfacalcidol (1-α-OH-vitamin D_3, a hydroxylated form of vitamin D) was also shown to mitigate bone loss in a model of glucocorticoid/inflammation-induced [17,18] or rheumatoid

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arthritis-induced bone loss [9,19] due to its anti-inflammatory property. However, the molecular mechanism(s) of green tea's or alfacalcidol's anti-inflammatory property in protecting deterioration of bone microstructure due to chronic inflammation has not been completely elucidated.

An approach of LPS administration has been used as a model for studying deterioration of bone microstructure with chronic inflammation [1,2]. TNF-α expression is increased in the proximal tibia of rat with LPS administration [1]. Such an excessive expression of TNF-α in bone induced by LPS is associated with deterioration of bone microarchitecture in rats [1]. Therefore, in the present study, we employed the same model to investigate the potential benefit of two dietary supplements, green tea polyphenols (GTP, green tea extract) and 1-α-OH-vitamin D_3, in mitigating bone deterioration in female rats with chronic inflammation. We hypothesized that GTP plus 1-α-OH-vitamin D_3 would improve bone microarchitecture in rats with chronic inflammation. To address the study objectives, we evaluated bone mass and bone structural parameters with bone histomorphometry and micro-computed tomography (μCT). Furthermore, we also examined the expression of pro-inflammatory cytokine mediators, TNF-α, in proximal tibia of rats to investigate a possible mechanism showing how GTP supplementation or 1-α-OH-vitamin D_3 administration may attenuate LPS-deteriorated bone microstructure of rats with chronic inflammation. Studying the potential effect of GTP, 1-α-OH-vitamin D_3, or a combination of both on bone microstructure in female rats with chronic inflammation will advance the understanding of their effects on skeletal biology in humans with chronic inflammation.

2. Materials and methods

2.1. Animals and GTP treatments

Fifty virgin CD female rats (3 months old, from Charles River, Wilmington, MA, USA) were allowed to acclimate for 5 days to a rodent chow diet and distilled water ad libitum. After acclimation, 40 rats were administered LPS pellets (Innovative Research of America, Sarasota, FL, USA) and other 10 rats were administered placebo pellets according to the procedures described previously [2].

After surgery, LPS-administered rats were randomized by body weight and assigned to (1) LPS administration (L, n=10), (2) LPS + 1-α-OH-vitamin D_3 (LD, n=10) (3) LPS + GTP (LG, n=10) and (4) LPS + GTP + 1-α-OH-vitamin D_3 (LGD, n=10) for 12 weeks. The rats in the L group were given drinking water with no GTP. The rats in the LD group were orally given 1-α-OH-vitamin D_3 at 0.05 μg/kg body weight, 5 times per week. Such a dosage of 1-α-OH-vitamin D_3 has shown a decrease in trabecular bone resorption, but an increase in the number osteoblasts, and periosteal and endocortical bone formation of rats [20]. The rats in the LG group were provided with GTP supplementation (0.5%, w/v) in drinking water daily to mimic human consumption of green tea of 4 cups a day based on our previous human [21] and animal studies [2,22]. The rats in the LGD group were given both GTP and 1-α-OH-vitamin D_3. The rats receiving the placebo pellets only (the P group) were given drinking water with no GTP and also were given no 1-α-OH-vitamin D_3. All rats were fed a rodent chow diet ad libitum during the 12-week feeding period.

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, Guangxi, China), with a purity higher than 98.5%. Every 1000 mg of GTP contained 464 mg of (−)−epigallocatechin gallate, 112 mg of (−)−epicatechin gallate, 100 mg of (−)−epicatechin (EC), 78 mg of (−)−epigallocatechin, 96 mg of (−)−gallocatechin gallate and 44 mg of catechin according to the high performance liquid chromatography-electrochemical detection (HPLC-ECD) and high performance liquid chromatography-ultraviolet detection (HPLC-UV) analyses. Rats were housed in individual stainless steel cages under a controlled temperature of 21±2°C with a 12-h light–dark cycle. Rats were weighed weekly and examined daily. All procedures were approved by Texas Tech University Health Sciences Center Institutional Animal Care and Use Committee.

2.2. Sample preparation

Each animal was given an intraperitoneal injection of calcein green (10 mg/kg of body weight; Sigma, St. Louis, MO, USA) at 14 days and 4 days before euthanasia. The final body weight was recorded. After animals were anesthetized and euthanized, femora and tibiae were harvested and cleaned of adhering soft tissues. The right tibia samples were kept in 70% ethanol and then processed for histomorphometric assays. The left tibia samples were kept in 10% formalin and then processed for immunohistochemistry. Left femur samples were stored in phosphate-buffered saline (PBS) solution at 4°C for bone scan and bone strength test.

2.3. Bone mass assessment

Left femur bone mass including bone mineral content (BMC) and bone mineral density (BMD) was determined by dual-energy X-ray absorptiometry (HOLOGIC QDR-2000 plus DXA, Waltham, MA, USA) [22]. The machine was set at an ultra-high resolution mode with line spacing of 0.0254 cm, resolution of 0.0127 cm, and a collimator diameter of 0.9 cm.

2.4. Histomorphometric analysis

Preparation of right tibiae for the static and dynamic bone histomorphometric analysis was described previously [8]. Undecalficied frontal sections of proximal tibia were embedded in methylmethacrylate (Eastman Organic Chemicals, Rochester, NY, USA) and cut (5-μm thickness) using a microtome (Leica RM 2155, Germany) for metaphyseal bone histomorphometric analysis. The adjacent section was stained with Goldner's Trichrome for the measurement of osteoclast surface and osteoclast cell number [23].

Undecalcified tibial shaft was embedded and a cross section of the proximal of the tibiofibular junction (8 μm thickness) was cut using microtome for cortical bone histomorphometric analysis. All sections were coverslipped with Eukitt (Calibrated Instruments, Hawthorne, NY, USA) for static and dynamic histomorphometric analysis using a semiautomatic image analysis system (Osteomeasure Histomorphometry System, OsteoMetrics, Atlanta, GA, USA).

A digitizing morphometric system was used to measure bone histomorphometric parameters. The system consisted of an epifluorescence microscope (Nikon E-400, OsteoMetrics, Atlanta, GA, USA), an Osteomeasure High Resolution Color Subsystem (OsteoMetrics) coupled to an IBM computer and a morphometry program (OsteoMetrics). The measured parameters for cancellous bone included total tissue volume (TV), bone volume (BV), bone surface (BS), single- and double-labeled surfaces, interlabel width, osteoclast surface, osteoclast cell number and eroded surface. These data were used to calculate standard morphometric parameters analyzed in bone studies, including percent cancellous bone volume (BV/TV, %), trabecular thickness (Tb.Th, μm), trabecular number (Tb.N, n/mm), trabecular separation (Tb.Sp, μm) and trabecular bone formation rate (BFR/BS, $\mu m^3/\mu m^2$ per day), osteoclast cell number per bone surface (N.Oc/B.Pm, n/mm), and osteoclast surface per bone surface (Oc.Pm/B.Pm, %) according to the standard nomenclature recommended by the American Society for Bone and Mineral Research Nomenclature Committee [24]. The region of bone measured in all groups is 1–4 mm from the growth plate in the proximal tibia.

Measurements in cortical bone included periosteal mineral total bone area, periosteal perimeter, marrow area, endocortical perimeter, periosteal and endocortical single- and double-labeled perimeters, interlabeled widths and endocortical eroded surface. These measures were then used to calculate percent cortical bone area (Ct.Ar, %), percent marrow area (Ma.Ar, %), percent periosteal mineralized surface/bone surface (Ps-MS/BS, %), periosteal mineral apposition rate (Ps-MAR, μm/day), periosteal bone formation rate (Ps-BFR/BS, $\mu m^3/\mu m^2$ per day), percent endocortical mineralized surface/bone surface (Ec-MS/BS, %), endocortical mineral apposition rate (Ec-MAR, μm/day), endocortical bone formation rate (Ec-BFR/BS, $\mu m^3/\mu m^2$ per day) and endocortical eroded surface/bone surface (Ec-ES/BS, %) [24].

2.5. Bone microarchitecture assessment by μCT

Bone microarchitecture in femur was assessed using μCT (MicroCT40, SCANCO Medical, Switzerland) according to the procedure of Shen et al. [8].

Trabecular bone of the femur was scanned so that 250 images were acquired. The volume of interest comprised the secondary spongiosa in 100 cross-sectional slices of the distal femur beginning 25 slices from the growth plate region. All scans were performed in a 1024×1024 matrix resulting in an isotropic voxel resolution of 16 μm^3. An integration time of 150 milliseconds per projection was used. Trabecular parameters in femur included trabecular BV fraction (BV/TV, %), number (Tb.N, n/mm), thickness (Tb.Th, μm) and separation (Tb.Sp, μm). Coefficients of variation were 2.0% (BV/TV), 1.1% (Tb.N), 0.66% (Tb.Th) and 1.30% (Tb.Sp) for morphometric parameters.

2.6. Bone quality assessment

The strength of femur was determined by a three-point bending test using a custom-designed and built apparatus according to the procedures of Nielsen [25]. Descriptions of the terms used for the assessment of bone strength have been described previously [26]. Maximum force (N) and yield point force (N) to break bones and modulus of elasticity were assessed.

2.7. Expression of TNF-α in proximal tibia

Seven-micron tissue sections were cut from decalcified (Immunocal, American Master*Tech Scientific, Lodi, CA, USA), formalin-fixed, paraffin-embedded blocks to Superfrost/Plus slide (Fisher Scientific, Fair Lwan, NJ, USA); deparaffinized in xylene;

rinsed in 100%, 95% and 70% ethanol and rehydrated in distilled water. Tissue sections were treated with peroxidase blocking reagent (DAKO, Carpenteria, CA, USA); blocked with normal serum (Vector Laboratories, Burlingame, CA, USA) at room temperature; incubated with primary antibody TNF-α (AbD Serotec., Raleigh, NC, USA) at dilution of 1:100; washed with PBS; treated with biotin-labeled secondary antibody (Vector Laboratories) treated with ABC solution for 30 min followed by incubation with NovaRd (Vector Laboratories). Counterstaining was performed with Immuno*Master Hematoxylin (Zymed Laboratories Inc., South San Francisco, CA, USA). Expression of TNF-α was confirmed by comparing control tissue section performed following the same procedures with the omission of the primary antibody. All slides were evaluated by the study pathologist for intensity in a blinded manner according to the following scoring system: normal (0), low (1), medium (2) and high (3).

2.8. Statistical analysis

Data are expressed as mean±standard error of the means(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. Difference between the P group and the L group was analyzed by *t*-test for each parameter to evaluate the effect of LPS administration. Data for body weights were analyzed by three-way analysis of variance (ANOVA) to evaluate the effect of GTP supplementation, 1-α-OH-vitamin D_3 administration, time, or interaction. Data of bone mass, microstructure, dynamics, osteoclast parameters, quality and TNF-α expression were analyzed by two-way ANOVA to evaluate the effect of GTP supplementation, 1-α-OH-vitamin D_3 administration, or interaction. Significant interactions between GTP and 1-α-OH-vitamin D_3 were tested using Fisher's LSD tests to further define treatment effects (the L, LD, LG and LGD groups). The level of significance was set at $P<.05$ for all statistical tests, and statistical trends ($P<.10$) were also indicated.

3. Results

3.1. Body weight

There was no significant difference in initial body weight among all treatment groups (data not shown). After 12 weeks, there was no difference in body weight between the P and the L group. Neither GTP supplementation nor 1-α-OH-vitamin D_3 administration significantly influenced body weights of rats throughout the study period. All animals gained body weight in a time-dependent manner, regardless of treatment groups (data not shown).

3.2. Bone mass

There was no significant difference in femoral bone area between the P and the L group (data not shown). As expected, the rats in the L group had lower values for BMC (Fig. 1A) and BMD (Fig. 1B) than those in the P group. Based on the results of two-way ANOVA analysis, after 12 weeks of treatment, (a) neither GTP supplementation nor 1-α-OH-vitamin D_3 affected femoral bone area; (b) there was a significant interaction between GTP supplementation and 1-α-OH-vitamin D_3 administration such that BMC was enhanced ($P=.029$) in the LGD group and BMD tended to be higher ($P=.095$) in this group and (c) both GTP supplementation and 1-α-OH-vitamin D_3 increased femoral BMC (Fig. 1A) and BMD of rats (Fig. 1B).

3.3. Histomorphometric changes in proximal tibia

When compared to the rats in the P group, the rats in the L group had significantly lower values for BV/TV, thickness (Tb.Th) and number (Tb.N), higher values for Tb.Sp, but no change in bone formation (BFR/BS) (Table 1). The effects of GTP supplementation or 1-α-OH-vitamin D_3 administration on histomorphometric changes in proximal tibia are also presented in Table 1. After 12 weeks of study period, supplementation of GTP in drinking water significantly increased BV/TV and Tb.N, decreased BFR/BS and had no effect on Tb.Th in proximal tibia of rats. 1-α-OH-vitamin D_3 administration significantly increased BV/TV, Tb.Th and Tb.N, but significantly suppressed BFR/BS of rats. A significant interaction between GTP supplementation and 1-α-OH-vitamin D_3 administration was observed in Tb.Sp; and Tb.Sp in proximal tibia was significantly higher in the L group than in groups supplemented with GTP, alfacalcidiol, or their combination (Table 1).

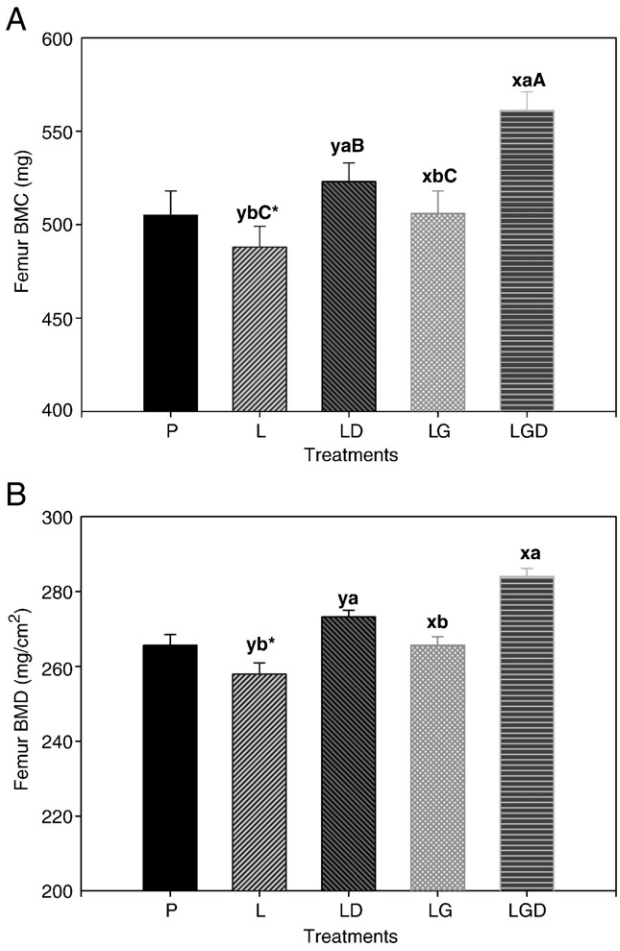

Fig. 1. BMC and BMD in femur of LPS-administered female rats supplemented with GTP in drinking water or 1-α-OH vitamin D_3 (D_3) administration for 12 weeks. Values are mean ($n=10$) with their standard error of mean (S.E.M.) represented by vertical bars. Having different letters (x and y for GTP effect; a and b for D_3 effect; capital letters for interaction effect) are significantly different by two-way ANOVA and Fisher's LSD test ($P<.05$). *Significantly different from the P group (between the L group and the P group) based on *t* test, $P<.05$.

3.4. Alteration in dynamic parameters in cortical bone of tibia shaft

Table 2 shows that relative to the placebo-administered group (the P group), the L-administered only group (the L group) had lower values for percent periosteal mineralized surface/bone surface (Ps-MS/BS), Ps-MAR, Ps-BFR/BS, higher values for endocortical mineralized surface/bone surface (Ec-MS/BS) and endocortial eroded surface/bone surface (Ec-ES/BS), but no difference in total area (T.Ar), percent cortical bone area (Ct.Ar), percent marrow area (Ma.Ar), endocortical mineral apposition rate (Ec-MAR) and endocortical bone formation rate (Ec-BFR/BS) at tibial shaft.

After 12 weeks, neither GTP supplementation nor 1-α-OH-vitamin D_3 administration affected T.Ar, Ct.Ar, Ma.Ar, Ec-MAR and Ec-BFR/BS (Table 2). GTP supplementation significantly enhanced Ps-MS/BS and Ps-BFR/BS, tended to suppress Ec-MS/BS ($P=.055$) and significantly depressed Ec-ES/BS, but did not influence Ps-MAR at tibial shaft (Table 2). On the other hand, 1-α-OH-vitamin D_3 administration significantly increased Ps-MS/BS, Ps-MAR and Ps-BFR/BS, but significantly inhibited Ec-MS/BS and Ec-ES/BS at tibial shaft (Table 2).

Table 1
Bone measurement in trabecular bone of proximal tibia in LPS-administered female rats supplemented with GTP in drinking water or 1-α-OH vitamin D_3 (D_3) administration for 12 weeks [1,2]

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
Histmorphometric analysis of proximal tibia								
BV/TV (%)	23.82±0.90	17.54±1.21 ^{*yb}	28.16±1.06^{ya}	23.82±1.08^{xb}	32.45±1.59^{xa}	<.001	<.001	.443
Tb.Th (μm)	57.05±1.23	51.97±1.34 ^{*b}	62.86±1.92^{a}	53.83±1.14^{b}	65.72±1.65^{a}	.150	<.001	.757
Tb.N (n/mm)	4.17±0.13	3.37±0.21 ^{*yb}	4.49±0.15^{ya}	4.42±0.16^{xb}	4.93±0.17^{xa}	<.001	<.001	.101
Tb.Sp (μm)	265±11	368±30 ^{*xaA}	232±9^{xbB}	251±14^{yaB}	200±11^{ybB}	<.001	<.001	.031
BFR/BS ($\mu m^3/\mu m^2$ / day)	11.97±1.23	12.15±1.23^{xa}	7.61±1.17^{xb}	8.01±1.14^{ya}	5.86±1.05^{yb}	.016	.007	.313

* Significantly different from the P group (between the L group and the P group), $P<.05$.

[1] Results are expressed as mean values±S.E.M. Difference between the placebo-administered group (the P group) and the LPS-administered only group (the L group) was analyzed by *t*-test to evaluate the effect of LPS administration. All the LPS-administered groups (the L, LD, LG and LGD groups) were analyzed by two-way ANOVA to evaluate the effect of GTP supplementation, D_3 administration, or interaction. Significant interactions between GTP and D_3 were tested using Fisher's LSD tests to further define treatment effects (the L, LD, LG and LGD groups). The level of significance was set at $P<.05$ for all statistical tests.

[2] Means within a row having different superscripts (x and y for GTP effect; a and b for D_3 effect; capital letters for interaction effect) are significantly different by two-way ANOVA and Fisher's LSD test ($P<.05$).

There was no significant interaction between GTP levels and 1-α-OH-vitamin D_3 administration in any parameters (Table 2).

3.5. Changes in osteoclast number

The effects of GTP supplementation or 1-α-OH-vitamin D_3 administration on osteoclast parameters in proximal tibia are shown in Fig. 2. Rats receiving LPS administration only (the L group) significantly increased osteoclast parameters, in terms of numbers of osteoclasts per bone perimeter (N.Oc/B.Pm) ($P<.001$) (Fig. 2A) and percentage of bone surface occupied by osteoclasts (Oc.Pm/B.Pm) ($P<.001$) (Fig. 2B) compared to those receiving placebo administration (the P group). The results of two-way ANOVA analysis show (a) supplementation of GTP in the drinking water significantly suppressed N.Oc/B.Pm and Oc.Pm/B.Pm; (b) 1-α-OH-vitamin D_3 administration also significantly suppressed both osteoclast parameters; and (c) significant interactions between GTP supplementation and 1-α-OH-vitamin D_3 administration were found in both parameters ($P=.007$ for both parameters). Among the LPS-administered groups (the L, LD, LG and LGD groups), the L group had the highest values for N.Oc/B.Pm and Oc.Pm/B.Pm, while the LGD group had the lowest values for both parameters (Fig. 2A for N.Oc/B.Pm and 2b for Oc.Pm/B.Pm).

3.6. Microarchitectural parameters of femur

Microarchitectural data of trabecular bone in femur are presented in Table 3. After 12 weeks, the L group had the lower values for BV/TV, Tb.Th and Tb.N, but no difference in Tb.Sp than those in the P group. Both GTP supplementation and 1-α-OH-vitamin D_3 administration significantly increased BV/TV, Tb.Th and Tb.N, while significantly decreased Tb.Sp. There was no significant interaction between GTP supplementation and 1-α-OH-vitamin D_3 administration in any parameters ($P>.05$) (Table 3).

3.7. Bone strength

Fig. 3 shows the impact of GTP supplementation or 1-α-OH-vitamin D_3 administration in bone strength parameters of femur, as determined by a 3-point bending test. The LPS-administered group (the L group) significantly deteriorated bone strength in terms of maximum force (Fig. 3A) and yield point force (Fig. 3B) compared to the placebo-administered group (the P group). After 12 weeks, there were significant interactions affecting maximum force ($P=.05$) and yield point force ($P=.025$) such that either GTP, alfacalcidol, or their combination increased maximum force (Fig. 3A) and yield point force (Fig. 3B) to similar values.

Table 2
Bone histomorphometric measurement in cortical bone of tibia shaft in LPS-administered female rats supplemented with GTP in drinking water or 1-α-OH vitamin D_3 (D_3) administration for 12 weeks [1,2]

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
T.Ar (mm^2)	5.33±0.08	5.33±0.07	5.34±0.06	5.22±0.05	5.41±0.07	.851	.147	.195
Ct.Ar (%)	85.22±0.58	84.04±0.55	85.64±0.61	84.87±0.66	85.10±0.61	.819	.145	.270
Ma.Ar (%)	14.78±0.58	15.96±0.55	14.36±0.61	15.13±0.66	14.90±0.61	.819	.145	.270
Ps-MS/BS (%)	18.20±1.09	14.39±1.33 ^{*yb}	27.56±2.07^{ya}	19.66±1.93^{xb}	30.29±1.06^{xa}	.029	<.001	.474
Ps-MAR (μm/day)	1.039±0.045	0.886±0.079 ^{*b}	1.276±0.071^{a}	0.959±0.063^{b}	1.463±0.084^{a}	.102	<.001	.465
Ps-BFR/BS ($\mu m^3/\mu m^2$/day)	19.05±1.66	12.82±1.64 ^{*yb}	31.62±3.46^{ya}	18.95±2.27^{xb}	45.62±3.35^{xa}	.001	<.001	.178
Ec-MS/BS (%)	2.46±0.98	5.37±1.01 ^{*a}	1.90±0.74^{b}	2.51±0.98^{a}	1.45±0.58^{b}	.055	.01	.156
Ec-MAR (μm/day)	0.140±0.00	0.259±0.082	0.238±0.098	0.214±0.048	0.409±0.182	.608	.478	.386
Ec-BFR/BS ($\mu m^3/\mu m^2$/day)	0.352±0.140	1.591±0.795	1.020±0.843	0.797±0.461	1.293±0.807	.739	.961	.495
Ec-ES/BS (%)	7.82±0.88	14.62±0.97 ^{*xa}	8.19±1.04^{xb}	11.27±0.88^{ya}	7.70±0.55^{yb}	.039	<.001	.117

* Significantly different from the P group (between the L group and the P group), $P<.05$.

[1] Results are expressed as mean values±standard error of the mean (S.E.M.). Difference between the placebo-administered group (the P group) and the LPS-administered only group (the L group) was analyzed by *t*-test to evaluate the effect of LPS administration. All the LPS-administered groups (the L, LD, LG and LGD groups) were analyzed by two-way ANOVA to evaluate the effect of GTP supplementation, D_3 administration, or interaction. Significant interactions between GTP and D_3 were tested using Fisher's LSD tests to further define treatment effects (the L, LD, LG and LGD groups). The level of significance was set at $P<.05$ for all statistical tests.

[2] Means within a row having different superscripts (x and y for GTP effect; a and b for D_3 effect; capital letters for interaction effect) are significantly different by two-way ANOVA and Fisher's LSD test ($P<.05$).

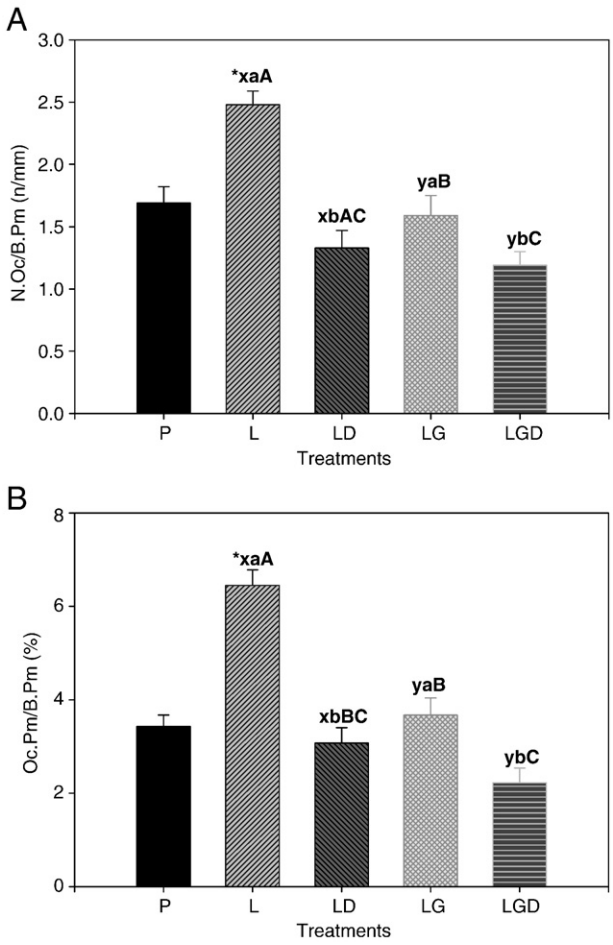

Fig. 2. Osteoclast parameters measured at proximal tibia in LPS-administered female rats supplemented with GTP in drinking water or 1-α-OH vitamin D_3 (D_3) administration for 12 weeks. Values are mean (n=10) with their standard error of mean (S.E.M.) represented by vertical bars. N.Oc/B.Pm, numbers of osteoclasts per bone perimeter; Oc.Pm/B.Pm, percentage of bone surface occupied by osteoclasts. Having different letters (x and y for GTP effect; a and b for D_3 effect; capital letters for interaction effect) are significantly different by two-way ANOVA and Fisher's LSD test (P<.05). *Significantly different from the P group (between the L group and the P group) based on t test, P<.05.

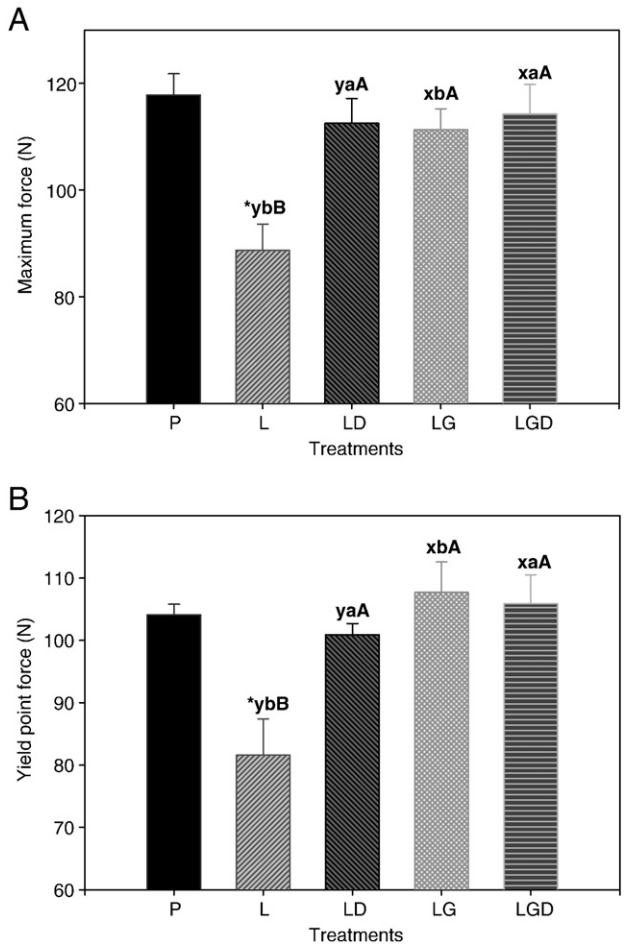

Fig. 3. Maximum force (A) and yield point force (B) of femur in LPS-administered female rats supplemented with GTP in drinking water or 1-α-OH vitamin D_3 (D_3) administration for 12 weeks. Values are mean (n=10) with their standard error of mean (S.E.M.) represented by vertical bars. Bars having different letters (x and y for GTP effect; a and b for D_3 effect; capital letters for interaction effect) are significantly different by two-way ANOVA and Fisher's LSD test (P<.05). *Significantly different from the P group (between the L group and the P group) based on t-test, P<.05.

3.8. Expression of TNF-α in proximal tibia

Relative to the rats receiving the placebo treatment (the P group), the rats receiving LPS treatment (the L group) significantly induced the expression of TNF-α in proximal tibia with the greatest extent in the growth plate region of the proximal tibia of rats (Fig. 4). The results of two-way ANOVA analysis show that both GTP supplementation (P=.017) and 1-α-OH-vitamin D_3 administration (P=.028) significantly suppressed the expression of TNF-α in proximal tibia of rats. In addition, a significant interaction between GTP supplementation and 1-α-OH-vitamin D_3 administration in TNF-α expression in proximal tibia was observed (P=.021).

Table 3
Bone microarchitectural properties of femur in LPS-administered female rats supplemented with GTP in drinking water or 1-α-OH vitamin D_3 (D_3) administration for 12 weeks[1,2]

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
Trabecular bone								
BV/TV (%)	35.80±2.2	27.30±1.66^{*yb}	44.79±6.25^{ya}	44.23±1.85^{xb}	50.76±3.09^{xa}	<0.001	0.001	0.104
Tb.Th (mm)	0.081±0.003	0.070±0.001^{*yb}	0.089±0.002^{ya}	0.093±0.009^{xb}	0.103±0.005^{xa}	0.001	0.008	0.331
Tb.N (n/mm)	6.12±0.16	5.53±0.19^{*yb}	6.33±0.12^{ya}	6.20±0.31^{xb}	6.49±0.18^{xa}	0.048	0.012	0.220
Tb.Sp (mm)	0.153±0.008	0.169±0.007^{xa}	0.136±0.004^{xb}	0.143±0.011^{ya}	0.126±0.005^{yb}	0.018	0.002	0.246

* Significantly different from the P group (between the L group and the P group), P<.05.

[1] Results are expressed as mean values±standard error of the mean (S.E.M.), n=8–10. Difference between the placebo-administered group (the P group) and the LPS-administered only group (the L group) was analyzed by t-test to evaluate the effect of LPS administration. All the LPS-administered groups (the L, LD, LG and LGD groups) were analyzed by two-way ANOVA to evaluate the effect of GTP supplementation, D_3 administration, or interaction. Significant interactions between GTP and D_3 were tested using Fisher's LSD tests to further define treatment effects (the L, LD, LG and LGD groups).

[2] Means within a row having different superscripts (x and y for GTP effect; a and b for D_3 effect; capital letters for interaction effect) are significantly different by two-way ANOVA and Fisher's LSD test (P<.05).

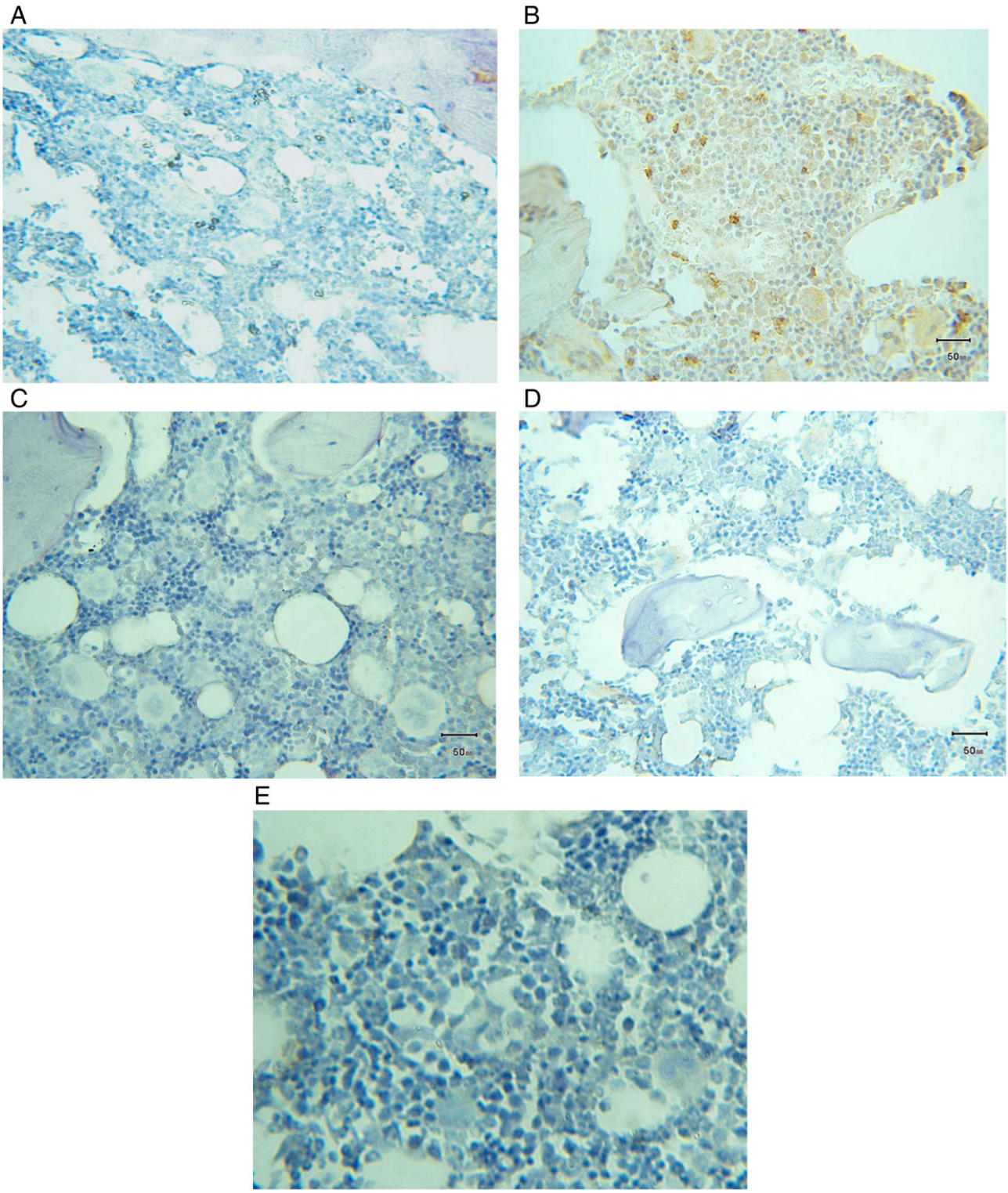

Fig. 4. Immunohistochemical staining for TNF-α in the tibia of LPS-administered female rats supplemented with GTP in drinking water or 1-α-OH vitamin D_3 (D_3) administration for 12 weeks. Section obtained from the proximal tibia metaphysic area (40×) showing the P group (A), the L group (B), the LD group (C), the LG group (D) and the LGD group (E). Both GTP supplementation and D_3 administration suppressed LPS-induced expression of TNF-α.

4. Discussion

In the present study, a model of systemic LPS administration of female rats was successfully employed to demonstrate a beneficial effect of two dietary supplements, GTP supplementation in drinking water and 1-α-OH-vitamin D_3 administration orally, on sustaining bone mass and mitigating the deterioration of bone microstructure during chronic inflammation. The findings show that LPS-induced chronic inflammation (the L group) produced a detrimental effect on bone mass and microarchitecture, in terms of a decrease in trabecular

volume and thickness of femur and proximal tibia, and an increase in Tb.Sp in proximal tibia, compared to a placebo-treated group (the P group). Such a detrimental effect on bone mass and microarchitecture by LPS agrees with a previous study as measured by μCT [20]. Compared to those receiving GTP (the LG group) or 1-α-OH-vitamin D_3 alone (the LD group), the rats receiving both treatments (the LGD group) for 12 weeks had a synergistic effect with the highest values for bone mass of femur (Fig. 1).

Administration of LPS to the rats decreased bone modeling (shown by lowered Ps-BFR/BS in tibial shaft, Table 2) and increased bone remodeling (shown by elevated BFR/BS of trabecular tibia and enhanced Ec-ES/BS of tibial shaft, Table 2) resulting in an increase in bone turnover rate with a net bone loss. Supplementation of GTP in the drinking water suppressed such a high turnover rate induced by LPS. Additionally, GTP supplementation also enhanced bone modeling process by stimulating Ps-BFR/BS in tibial shaft (Table 2). Such a suppression of turnover rate along with stimulating bone modeling appears to benefit bone microstructure, leading to a larger net bone mass (Fig. 1). Similar to the impact of GTP supplementation in bone microstructure, 1-α-OH-vitamin D_3 administration orally was also found to suppress LPS-induced high turnover rate in trabecular bone of proximal tibia (Tables 1 and 2), enhance Ps-MAR and inhibit Ec-MS/BS at tibial shaft of rats (Table 2) resulting in a larger net bone mass (Fig. 1).

The ability of GTP to increase indices of bone formation (osteoblastogenesis) and to decrease indices of bone resorption (osteoclastogenesis) has been suggested by previous studies, in terms of GTP's impact in osteoblastic and osteoclastic activity, respectively [12]. Evidence demonstrates that the active components in green tea stimulate osteoblastogenesis by (a) increasing osteoblastic survival through an inhibition of TNF-α and interleukin-6 production [27], (b) elevating proliferation and differentiation of osteoblasts via Wnt signaling pathway [28], (c) enhancing bone formation through vascular endothelial growth factor-mediated mechanism [29] and (d) eventually promoting mineralization via Runt-related transcription factor-2-mediated mechanism [12,30]. On the other hand, the inhibitory action of GTP on bone resorption shown in this study is supported by previous studies, in terms of an inhibitory effect of epigallocatechin-3-gallate (an abundant GTP in green tea) on osteoclastogenesis [12,16,31–35]. Epigallocatechin-3-gallate has been shown (a) to increase apoptosis of osteoclasts through caspase activation-dependent mechanism [31] or Fenton reaction mechanism [32], (b) to reduce the formation of osteoclasts through suppressing matrix metalloproteinases pathway [33], (c) to suppress the differentiation of osteoclasts through receptor activator of NF-kappa B ligand signaling pathway [16] or JNK/c-Jun signaling pathway [34] and (d) possibly to modulate the production of cytokines by immune cells [35].

The observations that 1-α-OH-vitamin D_3 administration inhibits bone resorption along with stimulating bone formation are supported by reported studies [36–38]. With regard to bone resorption, 1-α-OH-vitamin D_3 has been shown to suppress osteoclastogenesis by decreasing the pool of osteoclast precursors or osteoclastogenic potential in bone marrow [37,38]. Regarding bone formation, 1-α-OH-vitamin D_3 was found to prevent cortical bone loss in ovariectomized rats by decreasing marrow area as well as by increasing cortical area, periosteal perimeters and periosteal and endocortical bone formation rate [36].

Intriguingly, compared to GTP supplementation alone (the LG group) or 1-α-OH-vitamin D_3-administered alone (the LD group), a combination of GTP and 1-α-OH-vitamin D_3 (the LGD group) favors bone remodeling/modeling process (Table 2). These changes in bone microarchitecture may be mediated in part through suppressing bone resorption (as shown by lowered osteoclastic activity in proximal tibia and less bone erosion at endocortical surface of tibial shaft) in conjunction with enhancing bone formation at endocortical bone of tibia, resulting in a larger net bone mass. This study is the first study to report a bone-protective benefit of GTP and 1-α-OH-vitamin D_3 on bone microarchitecture. An action of maintaining bone microstructure in the LGD group is consistent with those data previously reported for GTP supplementation [8] or 1-α-OH-vitamin D_3 administration [33,39–41] in various bone loss models.

In addition to the impact of GTP plus 1-α-OH-vitamin D_3 in sustaining bone mass and microarchitecture, our data demonstrate that a combination of two improved femoral strength of rats during systemic chronic inflammation, as shown by an increase in maximum force and yield point force to break the femoral bone of rats (Fig. 3). We have noticed that either GTP supplementation or 1-α-OH-vitamin D_3 administration alone also imposes such a breaking-resistant capacity; and there was no significant difference in femoral strength among the LG, LD and LGD groups. These results confirm our hypothesis that chronic inflammation-induced deterioration in bone mechanical properties of female rats can be mitigated by GTP supplementation, 1-α-OH-vitamin D_3 administration, or both.

Tumor necrosis factor-α, a pro-inflammatory cytokine, has been shown to depress bone formation through inhibiting osteoblast progenitor cell recruitment and enhancing osteoblast apoptosis [42,43] as well as to stimulate bone resorption by increasing osteoclast differentiation and activity [44,45]. One of the aims of the present study was to elucidate the mechanism of GTP or 1-α-OH-vitamin D_3 in mitigating the deterioration of bone microstructure in rats during systemic chronic inflammation. Both GTP [12,46] and 1-α-OH-vitamin D_3 [13–16] have been characterized as anti-inflammatory agents suggesting GTP supplementation in drinking water or 1-α-OH-vitamin D_3 administration orally may have an osteo-protective role in bone microarchitecture through a reduction of inflammation in bone locally. Therefore, in the present investigation, we explored the relationship between GTP and bone TNF-α expression and the relationship between 1-α-OH-vitamin D_3 and bone TNF-α expression using a model of chronic inflammation-induced deterioration in bone microstructure.

The present study demonstrates that 1-α-OH-vitamin D_3 administration (Fig. 4C), GTP supplementation (Fig. 4D) and a combination of both (Fig. 4E) significantly down-regulated TNF-α expression in proximal tibia induced by chronic LPS stimulation. Such an osteo-protective effect of the studied anti-inflammatory agents (GTP or 1-α-OH-vitamin D_3) on bone microarchitceture due to chronic inflammation is consistent with other antioxidants, for example, soy isoflavones [10] or dried plum polyphenols [11] using the same model of bone deterioration.

This study also shows that the improvement in bone microarchitecture and quality along with the down-regulation bone TNF-α expression mechanism in the LGD group further corroborate the anti-inflammatory role of GTP plus 1-α-OH-vitamin D_3 in skeletal health which may reduce the risk of osteoporosis (severe bone loss). Future study should address the mechanistic profiles to clarify the preventive role of GTP and alfacalcidol in process of bone remodeling under chronic inflammation.

5. Conclusions

In a model of LPS-induced deterioration of bone microarchitecture, this study demonstrates beneficial effects of GTP supplementation and 1-α-OH-vitamin D_3 administration on skeletal bone modeling/remodeling process, in terms of maintaining cancellous and cortical bone compartment and improving bone strength via suppressing bone erosion along with enhancing bone formation. Such an osteo-protective role of GTP plus 1-α-OH-vitamin D_3 may, in part, be attributed to a suppression of pro-inflammatory cytokine mediator, TNF-α.

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