Green tea extract synergistically enhances the effectiveness of an antiresorptive drug on management of osteoporosis induced by ovariectomy in a rat model

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Abstract. Antiresorptive drugs are effective for reducing bone loss in postmenopausal women, but their long-term application may be associated with adverse effects. The present study aimed to investigate the potential *in vivo* synergistic effects of green tea extract (GTE) and alendronate or raloxifene on the management of osteoporosis. Ovariectomized rats were fed orally with GTE, alendronate and raloxifene at different concentrations and various combinations for 4 weeks. Bone mineral density (BMD) at the lumbar spine, femur and tibia was monitored weekly using peripheral quantitative computed tomography. Bone microarchitecture in the left distal femur was analyzed using micro-CT, while serum biochemical levels were measured using ELISA kits at the end of the study. GTE alone effectively mitigated BMD loss and improved bone microarchitecture in rats. The co-administration of GTE and alendronate increased total BMD in the lumbar spine, femur and tibia. Particularly, GTE synergistically enhanced the effect of alendronate at a low dose on bone microarchitecture and decreased serum tartrate-resistant acid phosphatase. These findings imply that the dosage of certain antiresorptive agents could be reduced when they are administrated simultaneously with GTE, so that their adverse effects are minimized. The findings may be used to support the development of a new synergistic intervention between food therapy and pharmacotherapy on the management of osteoporosis in a long-term basis.

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Introduction

Osteoporosis is a major health concern throughout the world. It primarily affects the elderly, particularly postmenopausal women (1). Osteoporosis is characterized by low bone mass and deterioration of bone microarchitecture (2). In addition, it contributes to an increase in bone fragility, resulting in disability and mortality of the elderly (3). The worldwide incidence of osteoporotic hip fracture is ~1.6 million each year and the incidence is predicted to increase to 6.3 million by 2050 (4). With the increasing trend in life expectancy, the prevalence of osteoporotic hip fractures is expected to increase. To reduce the onset of severe osteoporosis, various antiresorptive drugs are currently used, including alendronate and raloxifene (5). However, their osteo-protective actions were not conclusive. In a review, alendronate was reported to be effective in reducing both vertebral and non-vertebral fractures in postmenopausal women as a secondary prevention, but it did not exhibit a significant effect on primary prevention (6). On the other hand, raloxifene was found to be effective in reducing vertebral fracture but not non-vertebral fractures (7). Nonetheless, it was recommended to patients only if bisphosphonates (such as alendronate) were not suitable for them. Notably, prolonged use of these antiresorptive drugs may be associated with various adverse effects. For instance, bisphosphonates may cause odd-fracture and osteonecrosis in jaw (8), while raloxifene may increase the risk of deep vein thrombosis (9).

Public attention regarding the use of herbal medicines is increasing. Numerous studies have explored their pharmacological functions to prevent or treat osteoporosis. In our previous studies, various Chinese medicines were found to be effective in the prevention and treatment of osteoporosis, including *Epimedii Herba, Ligustri Lucidi Fructus* and *Psoraleae Fructus* (10-12). Besides herbal medicinal products, green tea may represent another possible phyto-candidate

for maintaining bone health. It is one of the most extensively studied plants with well-regarded health benefits and has a long history of consumption with wide safety margins (13). Common green tea polyphenols include (-)-epigallocatechin-3-gallate (EGCG), (-)-epigallocatechin (EGC), (-)-epicatechin-3-gallate (ECG) and (-)-epicatechin (EC) (14). EGCG constitutes >50% of the total ingredients in green tea, followed by EGC and ECG. Epidemiological evidence has demonstrated an association between tea consumption and prevention of age-related bone loss (15). Our group reported that green tea extract (GTE) and common tea polyphenols such as EGC and EGCG showed positive effects on bone metabolism through a dual process of promoting osteoblastic activity and inhibiting osteoclast differentiation using cultured rat osteoblast-like osteosarcoma UMR-106 and mice monocyte/macrophage-like RAW 264.7 cell lines, respectively (16). Furthermore, green tea polyphenols also promoted osteogenesis and inhibited adipocyte formation in human and rat mesenchymal stem cells (17,18). Shen *et al* (19,20) reported that green tea polyphenols mitigated bone loss in ovariectomized (OVX) and chronic inflammation-induced animal models via increasing antioxidant capacity, and decreasing oxidative stress damage and inflammation. Recently, the authors also found that green tea polyphenols at higher doses suppressed bone turnover in the trabecular and cortical bone of OVX rats and resulted in improved cortical bone structural and biomechanical properties, although it could not prevent the notable cancellous bone loss induced by OVX (21). Nonetheless, a previous randomized clinical trial reported that 1-year supplementation of GTE daily did not modify bone mineral density (BMD) or adiposity in overweight/obese postmenopausal women with apparently normal bone mass (22). The aforementioned reports revealed that GTE supplement itself does not effectively prevent the development of osteoporosis compared with antiresorptive drugs, even though positive results had been demonstrated in animal models.

Both phytochemicals and pharmaceutical agents exert individual pharmacological properties. Their combination may complement one another and maximize the ultimate therapeutic effect, while minimizing the adverse effects of pharmaceutical agents by reducing their dosages. Previously, a group found that a Chinese herbal formula (containing *Epimedii Herba*, *Ligustri Lucidi Fructus* and *Psoraleae Fructus*, named ELP) synergistically enhanced the therapeutic effect of raloxifene, but not that of alendronate, in rats with osteoporosis (23). The different responses indicated that the interaction between each of the herb-drug combinations is specific and more complex than simply complementing one another. Considering that GTE and its bioactive polyphenols can promote bone formation while antiresorptive drugs (alendronate and raloxifene) can inhibit bone resorption, it was hypothesized that the combination of green tea and antiresorptive drugs may exert synergistic effects on inhibiting osteoporosis onset. Given the increasing popularity of consuming green tea as a health supplement, information on the efficacy and safety of its interaction with various pharmaceuticals is essential.

In the present study, it was hypothesized that GTE could synergistically enhance the efficacy of antiresorptive drugs at a low dose, and therefore their clinical dosage could be eventually reduced. The information generated from this project will provide novel insights for osteoporosis management through a synergistic intervention between herbal health supplements and conventional pharmacotherapy. The present study aimed to investigate the synergistic effects of GTE and antiresorptive drugs on bone protection in an OVX rat model in relation to BMD and bone microarchitecture.

Materials and methods

Chemicals. All chemicals were purchased from Sigma-Aldrich (Merck KGaA) unless otherwise specified. Alendronate sodium and raloxifene hydrochloride were purchased from Merck KGaA and Eli Lilly and Company, respectively.

Preparation and characterization of GTE. Raw green tea leaves materials 'E Mei Xue Ya' were obtained from E Mei Mountain (Sichuan, China). The herbarium voucher specimen (reference no. GTE-1001) of the tested herb was deposited in the Institute of Chinese Medicine, The Chinese University of Hong Kong. For GTE preparation, the tea leaves (100 g) were brewed with 1 l hot distilled water (80˚C) 3 times (15 min each). The infusion was then cooled to room temperature and filtered through cellulose filter paper (0.45 μm; EMD Millipore). The filtrate was concentrated using a vacuum rotary evaporator, followed by freeze-drying at -50˚C overnight to produce the GTE powder. The chemical composition of GTE was analyzed using high-performance liquid chromatography (Fig. S1) and the method is described in Data S1.

Model establishment and treatment. Three-month-old female Sprague-Dawley rats were used and housed (n=3/cage) in a room at 22˚C with a 12-h light-dark cycle. They were maintained on standard rodent chow that contained 0.9% calcium and 0.7% phosphate, and distilled water was available *ad libitum*. After 1-week acclimation, the rats were anaesthetized intraperitoneally using a cocktail of ketamine (70 mg/kg) and xylazine (10 mg/kg) and then OVX bilaterally. Animals in the sham group underwent the same surgical procedure but without the ligation of the oviducts or excision of the ovaries. Animal experimentation ethics approval for this study was obtained from the Animal Experimental Ethics Committee of The Chinese University of Hong Kong (approval no. 13/032/MIS-5).

Three weeks after the surgical operation, osteoporosis was developed in this animal model according to our previous study, in which a significant decrease in total BMD in lumbar spine, femur and tibia was induced (23). Next, animals received GTE and two antiresorptive drugs [alendronate (A) and raloxifene (R)] via oral administration daily using gavage for 4 weeks (Table I). GTE and both drugs were dissolved in distilled water. The OVX and sham groups were administered the same volume (2 ml) of distilled water. The treatment period was designed based on our previous study, which revealed that a significant difference in BMD between the OVX and the alendronate/raloxifene treatment groups was identified after 4 weeks of treatment (23). Body weight and BMD of the animals were measured weekly. At the end of study, blood samples were obtained from the abdominal inferior vena cava of the animals after they had been anaesthetized as mentioned above, and the animals were then euthanized immediately via cervical dislocation. The confirmation of death was assessed

Table I. Grouping of the animals in the present study (n=8/group).

Group number	Group name	Treatment received
1	Sham	Sham-operated group
2	OVX	OVX only group
3	GTE (L)	OVX treated with 400 mg/kg GTE
4	GTE (M)	OVX treated with 800 mg/kg GTE
5	GTE (H)	OVX treated with 1,600 mg/kg GTE
6	A (L)	OVX treated with 0.05 mg/kg/day alendronate
7	A (H)	OVX treated with 0.5 mg/kg/day alendronate
8	GTE (M) + A (L)	OVX treated with GTE (800 mg/kg) + 0.05 mg/kg/day alendronate
9	GTE (M) + A (H)	OVX treated with GTE (800 mg/kg) + 0.5 mg/kg/day alendronate
10	R (L)	OVX treated with 0.62 mg/kg/day raloxifene
11	R (H)	OVX treated with 6.2 mg/kg/day raloxifene
12	GTE (M) + R (L)	OVX treated with GTE (800 mg/kg) + 0.62 mg/kg/day raloxifene
13	GTE (M) + R (H)	OVX treated with GTE (800 mg/kg) + 6.2 mg/kg/day raloxifene

GTE, green tea extract; OVX, ovariectomized; A, alendronate; R, raloxifene; L, low dose; M, medium dose; H, high dose.

via direct cardiac palpation to confirm lack of cardiac activity. Femora were then harvested for microarchitectural analyses. Uteruses were also harvested and weighed immediately (data not shown). Completed ovariectomy was confirmed at necropsy by marked atrophy of the uterine horns and absence of ovarian tissue.

The animals were divided into 13 groups (n=8/group) as shown in Table I. Group 1 was sham operated. The other 12 groups of rats were OVX. Three doses of GTE (group 3, 400 mg/kg; group 4, 800 mg/kg; and group 5, 1,600 mg/kg) were tested, according to a previous study (24), and then the optimal dose was selected for combination studies. High dose of alendronate [group 7, A (H), 0.5 mg/kg/day] and raloxifene [group 11, R (H), 6.2 mg/kg/day] were equivalent to clinical dose [calculated from the human equivalent dose table (25)]. Low dose (1/10 of the high dose) of alendronate [group 6, A (L)] and raloxifene [group 10, R (L)] were also tested to determine the dose-dependent effect of each drug. For the remaining groups, combined treatment of GTE with high or low dose of alendronate or raloxifene was administered to study the interactions between GTE and various drug combinations.

Monitoring changes in BMD. From the start of the treatment (day 0), changes in BMD at lumbar vertebra (L5), proximal tibial metaphyses and distal femoral metaphyses of the rats were monitored weekly for 4 weeks using peripheral quantitative computed tomography (pQCT; XCT2000; Stratec Medizintechnik GmbH). Briefly, the animals were anesthetized as described in the ovariectomy section. They were then placed and secured on a custom-made translucent plastic holder. Lumbar spine (L5), right proximal tibia and distal femurs were scanned under the built-in research mode of pQCT. The scan speed was 25 mm/sec with a voxel resolution of 0.2 mm. Total BMD (BMD including both cortical and trabecular areas) was generated and presented. The coefficient of variation of standard measurements was <4%.

Bone microarchitecture analysis. The microarchitecture of the left distal femur was analyzed using micro-CT (Micro CT 40; Scanco Medical AG). Briefly, the femur was aligned perpendicularly to the scanning axis. The scanning was conducted at 55 kVp and 144 μA with a resolution of 16 μm per voxel. The trabecular bone in the distal femur was identified using drawn contour at each two-dimensional section semi-automatically. Segmentation parameters were fixed as follows: σ=0.5, support=1.0 and threshold=245. The volume of interest (VOI) was determined within 50 continuous slices. The microarchitectural parameters of the VOI were obtained via three-dimensional reconstructed images using the built-in software of the micro-CT workstation. Parameters from the direct model [bone volume fraction (BV/TV), trabecular number (Tb.N), trabecular thickness (Tb.Th) and trabecular plate separation (Tb.Sp)] were analyzed.

Assessment of serum bone turnover markers. Serum was obtained by centrifuging the blood samples at 1,630 x g for 20 min at 4˚C, and was stored at -80˚C until analysis. Osteocalcin (OC) is secreted solely by osteoblasts, and its concentration in serum is often used as a measure of bone formation. C-terminal telopeptide (CTX) is released into the bloodstream during bone resorption and hence can serve as a specific marker for the degradation of mature type I collagen in bone. In addition, tartrate-resistant acid phosphatase 5b (TRAcP 5b) is a specific marker of osteoclasts, which are known to mediate bone resorption (26). Hence, TRAcP 5b can also serve as an indicator of the extent of bone resorption.

To elucidate the mechanism by which bone metabolism is involved in the potential synergistic effect between GTE and alendronate or raloxifene, the serum concentrations of OC, TRAcP 5b and CTX in the groups treated with GTE in combination with drugs exhibiting a synergistic bone protective effect at various concentrations were measured using ELISA kits [Rat-MID™ Osteocalcin EIA (cat. no. AC-12F1), RatTRAP™ (TRAcP 5b) ELISA (cat. no. SB-TR102) and

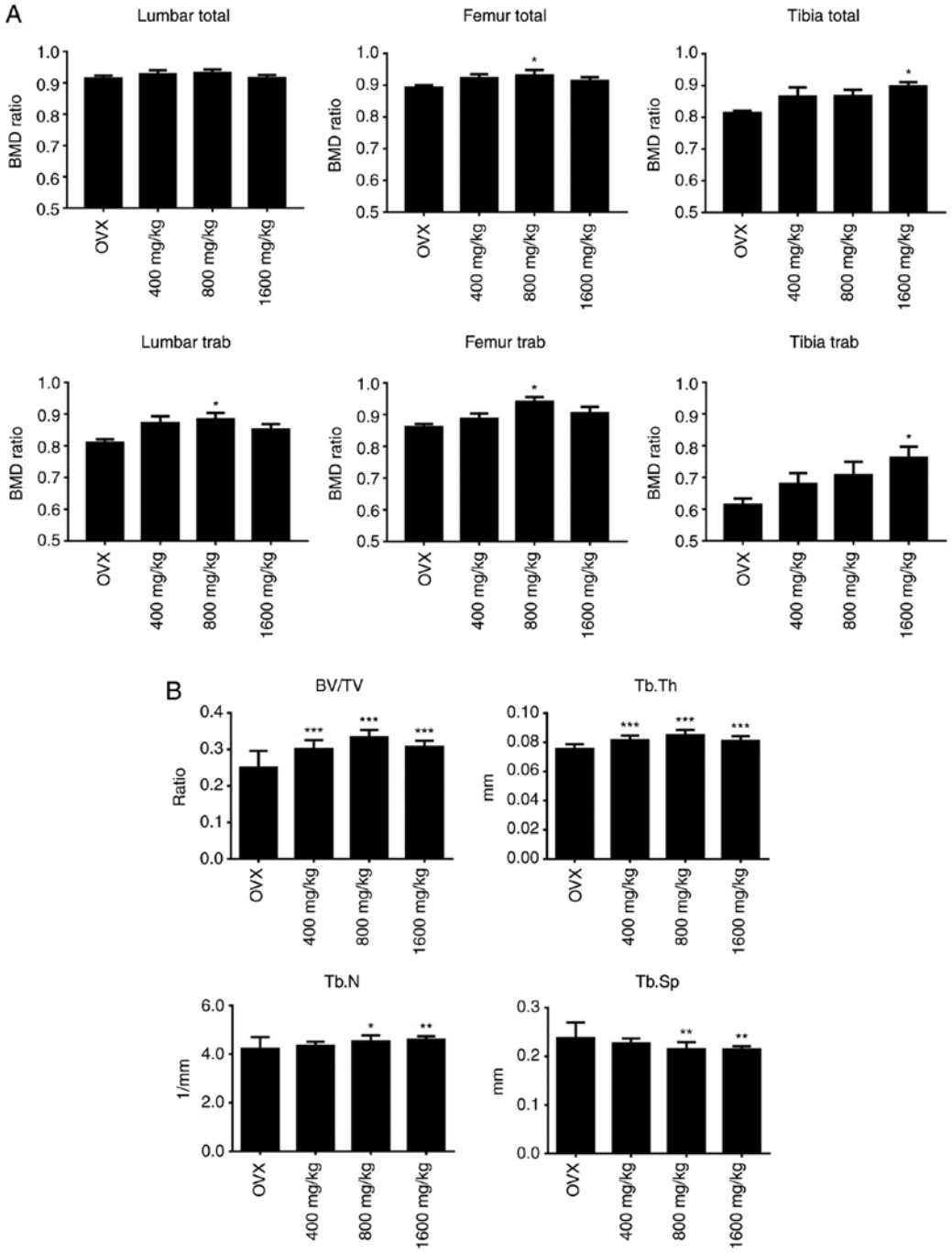
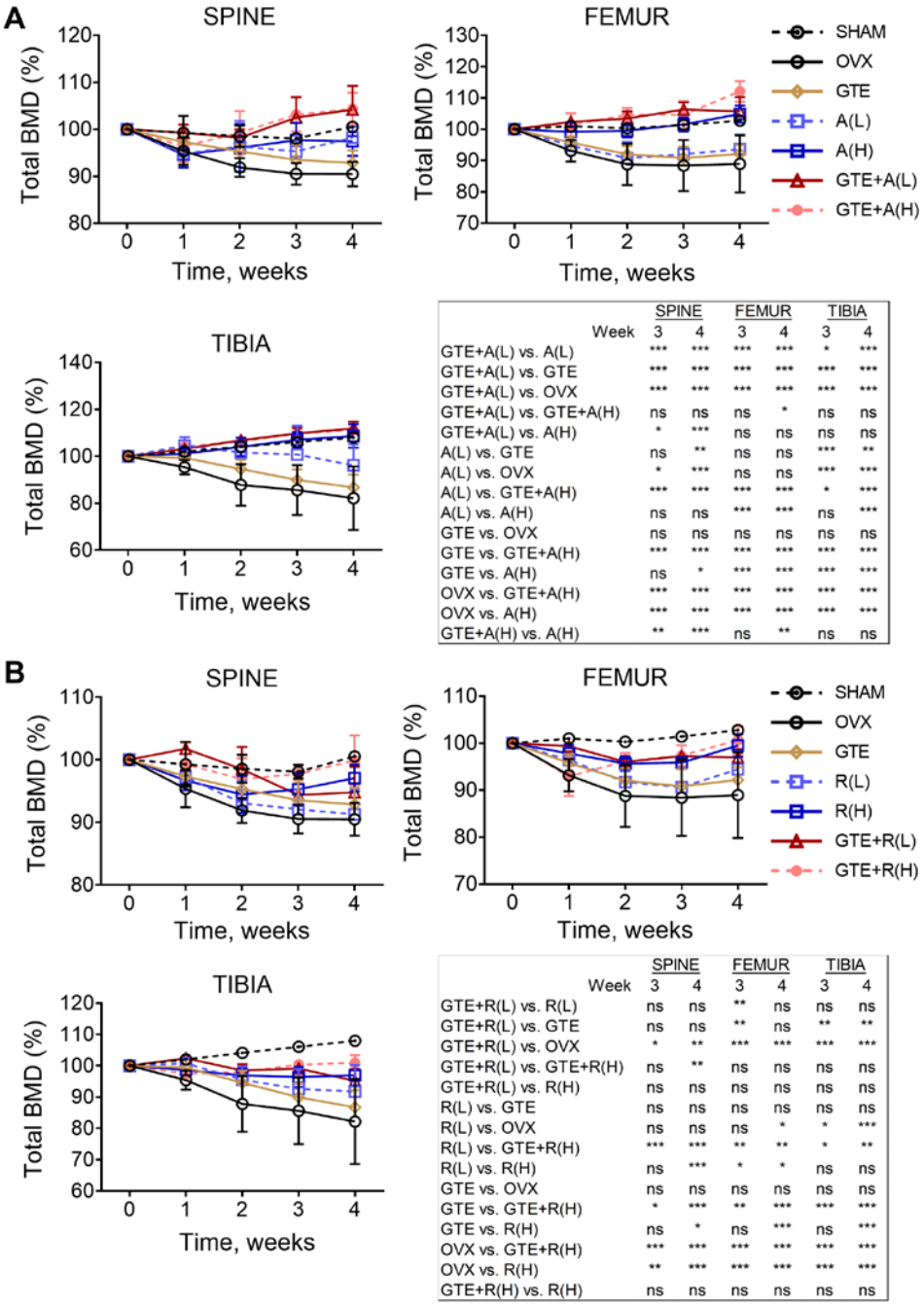


Figure 1. Effect of GTE at different concentrations on the bone of osteoporotic rat after 4 weeks of treatment. (A) Mean of the ratio from baseline (Day 0) of total and Trab BMD in lumbar spine, distal femur and proximal tibia measured by pQCT; (B) Differences in BV/TV, Tb.N, Tb.Th and Tb.Sp at the metaphysis of the distal femur measured by micro-CT. The error bar represents the + SD. *P<0.05; **P<0.01; ***P<0.001 vs. OVX without treatment. GTE, green tea extract; OVX, ovariectomized; BV/TV, trabecular bone volume; Tb.N, trabecular number; Tb.Th, trabecular thickness; Tb.Sp, trabecular separation; Trab, trabecular; BMD, bone mineral density.

Serum CrossLaps® (CTX-I) (cat. no. AC-02F1), respectively, Immunodiagnostic Systems Holdings], according to the manufacturer's instructions. A standard curve was generated from each kit, and the concentration of each bone turnover marker was calculated from the corresponding standard curve.

Statistical analysis. To determine whether BMD changes according to time and/or different treatments, a mixed two-way ANOVA was conducted, followed by Bonferroni's correction (n=8/group at each time point). For other measuring parameters, the differences between treatment and control groups were evaluated using one-way ANOVA followed by Bonferroni's correction (n=8/group). The groups with alendronate and raloxifene were compared separately. All the covariates were adjusted for statistical analysis, which was performed using the GraphPad Prism version 6.0 for Windows (GraphPad Software, Inc.). Data are expressed as the mean ± standard deviation. P<0.05 was considered to indicate a statistically significant difference.



	SPINE		FEMUR		TIBIA	
Week	3	4	3	4	3	4
GTE+A(L) vs. A(L)	***	***	***	***	*	***
GTE+A(L) vs. GTE	***	***	***	***	***	***
GTE+A(L) vs. OVX	***	***	***	***	***	***
GTE+A(L) vs. GTE+A(H)	ns	ns	ns	*	ns	ns
GTE+A(L) vs. A(H)	*	***	ns	ns	ns	ns
A(L) vs. GTE	ns	**	ns	ns	***	**
A(L) vs. OVX	*	***	ns	ns	***	***
A(L) vs. GTE+A(H)	***	***	***	***	*	***
A(L) vs. A(H)	ns	ns	***	***	ns	***
GTE vs. OVX	ns	ns	ns	ns	ns	ns
GTE vs. GTE+A(H)	***	***	***	***	***	***
GTE vs. A(H)	ns	*	***	***	***	***
OVX vs. GTE+A(H)	***	***	***	***	***	***
OVX vs. A(H)	***	***	***	***	***	***
GTE+A(H) vs. A(H)	**	***	ns	**	ns	ns

	SPINE		FEMUR		TIBIA	
Week	3	4	3	4	3	4
GTE+R(L) vs. R(L)	ns	ns	**	ns	ns	ns
GTE+R(L) vs. GTE	ns	ns	**	ns	**	**
GTE+R(L) vs. OVX	*	**	***	***	***	***
GTE+R(L) vs. GTE+R(H)	ns	**	ns	ns	ns	ns
GTE+R(L) vs. R(H)	ns	ns	ns	ns	ns	ns
R(L) vs. GTE	ns	ns	ns	ns	ns	ns
R(L) vs. OVX	ns	ns	ns	*	*	***
R(L) vs. GTE+R(H)	***	***	**	**	*	**
R(L) vs. R(H)	ns	***	*	*	ns	ns
GTE vs. OVX	ns	ns	ns	ns	ns	ns
GTE vs. GTE+R(H)	*	***	**	***	***	***
GTE vs. R(H)	ns	*	ns	***	ns	***
OVX vs. GTE+R(H)	***	***	***	***	***	***
OVX vs. R(H)	**	***	***	***	***	***
GTE+R(H) vs. R(H)	ns	ns	ns	ns	ns	ns

Figure 2. Changes of total BMD at different regions of rats co-treated with GTE and antiresorptive drugs. Mean of percentage changes from baseline (Week 0) of the total BMD at lumbar spine, proximal tibia and distal femur co-treated with (A) A and (B) R. The error bar represents the ±SD. Two-way ANOVA followed by Bonferroni's correction. *P<0.05; **P<0.01; ***P<0.001. BMD, bone mineral density; GTE, green tea extract; OVC, ovariectomized; ns, not significant; L, low dose; H, high dose; A, alendronate; R, raloxifene.

Results

Changes in BMD. BMD has been long regarded as a surrogate measure of bone strength. The present study revealed that oral GTE treatment for 4 weeks resulted in a higher BMD on the bone compared with that of OVX (Fig. 1A). Rats treated with 800 mg/kg GTE exhibited significantly higher total BMD (4.24%) and trabecular BMD (9.62%) in the distal femur compared with OVX. Those treated with 1,600 mg/kg GTE significantly increased their total and trabecular BMD by 10.16 and 23.99%, respectively, in the proximal tibia compared with the findings in OVX rats. In the lumbar spine, trabecular BMD in the 800 mg/kg GTE-treated group was also 7.48% higher than that of OVX, and the difference was significant. GTE oral administration increased BMD in a dose-dependent manner in proximal tibia. These results suggested that GTE reduced the BMD loss of lumbar spine, distal femur and proximal tibia starting at doses of ≥800 mg/kg.

In the sham group, an overall increase in total BMD was observed in lumbar spine, distal femur and proximal tibia (Fig. 2). The effect of ovariectomy on the reduction of total BMD was prominent in these regions of the OVX group. Total BMD of the OVX group continued to decrease throughout the 4 weeks of treatment, particularly in proximal tibia (Fig. 2).

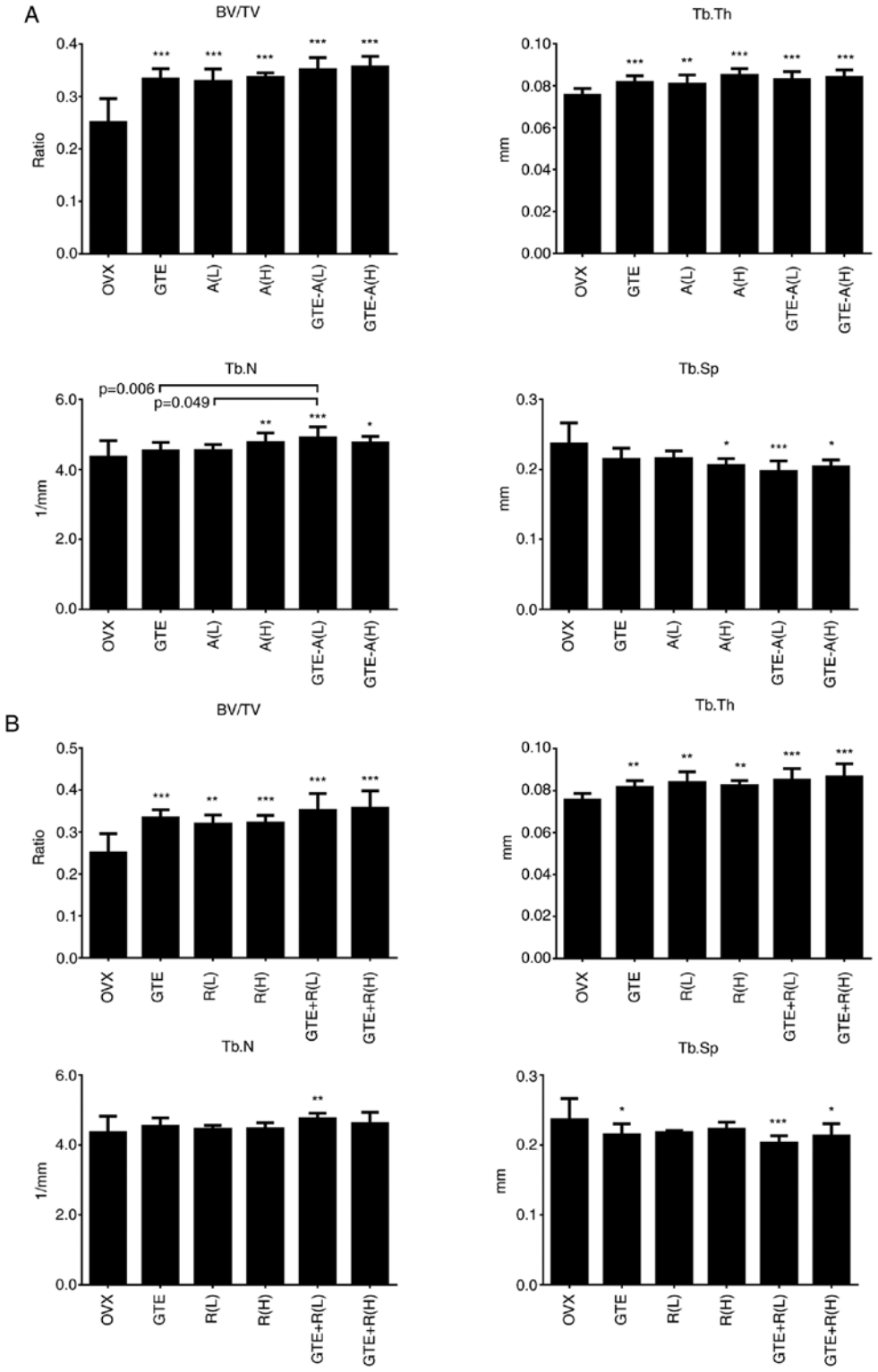


Figure 3. Difference in microarchitectural properties at the distal femur of rats co-treated with GTE and antiresorptive drugs. Mean of trabecular bone volume (BV/TV), trabecular number (Tb.N), trabecular thickness (Tb.Th), and trabecular separation (Tb.Sp) after 4 weeks of co-treatment with (A) A and (B) R. The error bar represents the + SD. *P<0.05; **P<0.01; ***P<0.001 vs. OVX without treatment. GTE, green tea extract; OVX, ovariectomized; BV/TV, trabecular bone volume; Tb.N, trabecular number; Tb.Th, trabecular thickness; Tb.Sp, trabecular separation; L, low dose; H, high dose; A, alendronate; R, raloxifene.

Those 11 treatment groups had a significantly higher BMD at the lumbar spine than the OVX group from week 2 onwards. Regarding treatment with the antiresorptive drug alone, alendronate exhibited a dose-dependent protective effect on BMD in both femur and tibia (Fig. 2A), in contrast to raloxifene (Fig. 2B). Both A (H) and R (H) significantly increased total BMD in all bone regions compared with the findings in the OVX group. The protective effect of alendronate was higher than that of raloxifene. Compared with the baseline value, total BMD in femur and tibia of rats treated with A (H) was significantly higher at weeks 3 and 4 [104.88% (P<0.001) and 108.59% (P=0.004) for femur and tibia, respectively], but these

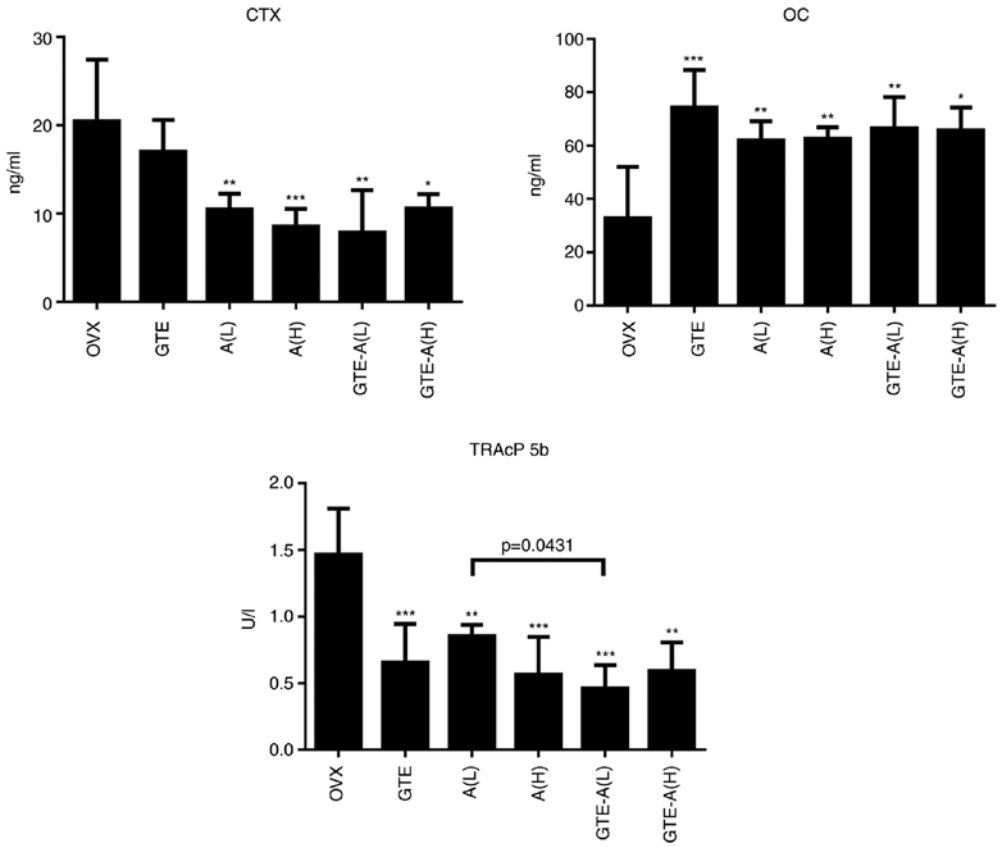


Figure 4. Difference in the concentration of bone turnover markers in the serum of rats treated with GTE and/or combinations of A. Mean of CTX, OC and TRAcP 5b concentration after 4 weeks of treatment with GTE and/or A. Error bar represents the + SD. *P<0.05; **P<0.01; ***P<0.001 vs. OVX without treatment. GTE, green tea extract; OVX, ovariectomized; L, low dose; H, high dose; A, alendronate; OC, osteocalcin CTX, C-terminal telopeptide; TRAcP 5b, tartrate-resistant acid phosphatase 5b.

significant differences were not found in rats treated with R (H) [99.52 and 96.94% for femur and tibia, respectively, at week 4 (P>0.05 for both)].

In the combination studies, the data demonstrated that GTE worked synergistically with alendronate in increasing BMD. Compared with the findings in the OVX group, co-treatment with GTE and A (L) significantly increased total BMD in all bone regions at weeks 3 and 4. However, A (L) alone did not exhibit a significant effect in reducing total BMD in distal femur. GTE was also found to enhance the effect of A (H) on total BMD compared with that of A (H), and significant differences were found in spine (weeks 3 and 4) and femur (week 4) (Fig. 2A). Overall, co-treatment of GTE and A (L) was found to be the most effective combination to reduce total BMD loss among the groups. Notably, the combination of GTE and raloxifene did not result in any synergistic effect on the reduction of total BMD loss, regardless of the concentrations of raloxifene (Fig. 2B).

Differences in bone microarchitecture. To further evaluate the impact of different GTE-drug combinations on the quality of trabecular bone, bone microarchitectural properties of the distal femur were analyzed. All the GTE treatment groups showed improvements in the microarchitectural properties of the trabecular bone over the OVX group. Treatment with 400 mg/kg GTE significantly increased the BV/TV and Tb.Th compared with the OVX group. Treatment of 800 and 1,600 mg/kg GTE significantly increased all the BV/TV, Tb.N and Tb.Th, whereas it decreased the Tb.Sp, compared with the OVX group (Fig. 1B).

Both alendronate and raloxifene treatment resulted in an improvement in the bone microarchitectural properties at the distal femur (Fig. 3). Similar to the total BMD analysis, alendronate treatment resulted in increasing trends in BV/TV, Tb.N and Tb.Th, and a decreasing trend in Tb.Sp, as the dosage of alendronate increased [A(H) compared with A(L)] (Fig. 3A), while raloxifene treatment did not (Fig. 3B). Notably, the combination of GTE and A (L) or R (L) significantly increased Tb.N compared with the findings in the OVX group. This osteo-protective effect could not be observed either in GTE, A (L) or R (L) alone. Rats treated with low-dose alendronate and GTE simultaneously further increased their Tb.N compared with that of rats treated with low-dose alendronate alone. The effect of low-dose alendronate plus GTE on bone microarchitecture was comparable to that of treatment with high-dose alendronate alone, but no statistically significant difference was observed.

Differences in serum biochemical markers. A prominent synergistic protective effect on bones was observed following treatment with GTE and alendronate. Therefore, measurement of serum biochemical markers in the groups treated with GTE and alendronate (alone or in combination) at various concentrations was conducted.

After 4 weeks of treatment, the serum CTX concentration was reduced effectively by alendronate at both low (0.05 mg/kg/day) and high (0.5 mg/kg/day) concentrations (Fig. 4). GTE alone significantly increased the serum OC level, which was similar to the effect of alendronate at both low and high concentrations. Nevertheless, no synergistic effect was observed when GTE was co-administered with alendronate. GTE nor alendronate (at both concentrations) alone could significantly reduce the serum TRAcP 5b level compared with the findings in the OVX group. Notably, the combination of GTE and alendronate at low concentration synergistically decreased the TRAcP 5b level significantly when compared with the effect of alendronate at low level alone. Collectively, these findings indicated an important role of GTE in reinforcing the effects of alendronate on enhancing bone formation and inhibiting bone resorption.

Discussion

In the present study, a synergistic effect between GTE and alendronate on reduction of osteoporotic bone loss caused by ovariectomy was identified. Particularly, GTE was demonstrated to enhance the effect of a low dose of alendronate on inhibiting bone resorption, as indicated by a decrease in TRAcP 5b level. The combination of GTE and alendronate at low doses improved the bone microarchitectural properties (BV/TV, Tb.Th and Tb.N of the trabecular bone in distal femur) as well as BMD, and significant differences were found between GTE + A (L) and either A (L) or GTE alone.

Regarding the antiresorptive drug raloxifene, it was observed that the addition of GTE to raloxifene at all concentrations prevented BMD loss at lumbar spine, distal femur and proximal tibia, and improved the bone microarchitectural properties in the femur compared with the findings in the OVX group. Compared with the effects of treatment with raloxifene alone, however, the presence of GTE did not result in significant differences in any of the parameters evaluated. Notably, our group previously observed that the extract of a Chinese herbal formula (ELP) worked synergistically with raloxifene in increasing the BMD of osteopenic bone in an OVX rat model (23). This synergistic effect was further substantiated by bone microarchitecture analysis. The discrepancy between the results of the two studies may be due to the different compositions of the two herbal extracts. The main composition of green tea is tea polyphenols, which is absent in ELP. In the present study, the composition of the tea polyphenols in the GTE was similar to that of the green tea polyphenols in previous studies conducted by Shen *et al* (20,21). In all of these studies, the most abundant catechin was EGCG, followed by ECG and EGC. A small quantity of catechin was also identified. On the other hand, some estrogenic compounds in ELP may work synergistically with raloxifene to result in osteo-protection. Raloxifene is an oral selective estrogen receptor modulator that has estrogenic actions on inhibiting bone resorption (5,27). When ELP is combined with raloxifene, the stimulation of osteoblasts by ELP may have an additive effect in the anti-osteoclastic action of raloxifene. Besides, the difference in treatment period and osteoporotic conditions between the two studies may also attribute to the discrepancy in the results. The treatment period of the ELP study was 8 weeks, compared with the 4-week GTE treatment period in the current study.

A study by Wu *et al* (24) reported that the highest dose of GTE selected was 370 mg/kg. It was also the effective dose to improve femoral BMD of OVX rats. However, in the current study, the lowest dose selected was 400 mg/kg and effective dose was 800 mg/kg. Considering that BMD was the primary outcome of our study and with reference to the study by Wu *et al*, it was hypothesized that 370 mg/kg as the minimum effective dose. To make the calculation simple, our team started the minimum dose from 400 mg/kg, and then doubled the concentration in order to see if there will be a dose-dependent effect of the GTE on improving BMD. The difference in the effective dose between the two studies might be due to the differences in: i) Species of green tea-Wu *et al* studied 'Yunnan Daye' (*Camellia sinensis* [Linn.] var. assamica [Masters] Kitamura) from the Yunnan province of China while our team studied 'E Mei Xue Ya' from the Sichuan province of China. They may have different chemical composition (different concentrations of alkaloids and catechins); ii) Extraction method: Wu *et al* extracted 100 g of green tea twice using 1,200 ml of water each time (1.5 h) under reflux (100˚C) while our team brewed 100 g of green tea with 1,000 ml hot distilled water (80˚C) 3 times. This difference may alter the concentrations of chemical composition of GTE; iii) species of animal model; and iv) treatment protocol-Wu *et al* started the GTE treatment after 2 weeks of the OVX and the treatment this lasted for 13 weeks. However, the present study started the GTE treatment after 3 weeks of the OVX and the treatment period was only 4 weeks. This meant that osteoporosis had reached a more severe condition than Wu *et al* while the treatment period was shorted than the duration selected. A significant difference in femoral BMD may have been observed at 400 mg/kg of GTE in the current study, if the treatment period was extended to 13 weeks.

Raloxifene is not considered the first-line preventive measure against osteoporosis, compared with bisphosphonates (28). The current data supported this clinical observation. As a preventive measure, alendronate is more effective than raloxifene, with or without GTE. To the best of our knowledge, the present study was the first to demonstrate that GTE and alendronate have a synergistic effect on preventing the reduction in total BMD, suggesting the adjuvant use of this combination in the prevention of osteoporosis. Although the present findings demonstrated that the synergistic effect of GTE and raloxifene on osteo-protection was lower than that of GTE and alendronate, it was confirmed that the combination treatment (GTE + R) has beneficial effects compared with R treatment alone.

It is well documented that green tea exhibits antiobese (29), hypolipidemic and hypoglycaemic properties, hence ameliorating cardiovascular diseases (CVD) (30). However, the negative effects of caffeine in green tea on human behaviors and sleep deprivation (31) are concerned by the majority of people. The present study demonstrated that green tea is beneficial for maintaining bone health in a rat model. Despite this, a high dose of green tea should still be avoided for individuals with multiple chronic conditions. The primary goal of this study was to examine potential drug interactions with

GTE. It was found that GTE could synergistically enhance the osteo-protective effects of alendronate and reduce the dose of alendronate required to achieve its biological effects. Therefore, GTE may be employed in a novel combination with bisphosphonate agents for the management of osteoporotic progression in osteopenic individuals. The current findings justify clinical studies using GTE and standard antiresorptive agents together in an attempt to counteract osteoporosis.

It is generally considered that green tea is safe, based on its nature and long dietary history. However, previous studies indicated that green tea may reduce the anti-coagulant effect of warfarin, and the absorption of folic acid and statin (32,33). The present study indicated that the adverse events caused by green tea to the interaction with antiresorptive drugs were minimal, based on observation no loss of body weight or abnormal behavior (Fig. S2). From the measurement of the body weight within the 4 weeks of the treatment period, the results indicated that various doses of GTE (Fig. S2A) and the combinations of the medium dose of GTE with alendronate or raloxifene (Fig. S2B) did not cause significant change in body weight when compared with OVX without treatment. Further *in vivo* studies are still required to evaluate the effect of GTE on the pharmacokinetics of alendronate and raloxifene, and on the activities of drug metabolizing enzymes (such as CYP3A) and uptake of efflux transporters (such as P-glycoprotein).

Due to the limited sample size, histomorphometrical analysis or molecular assessment to support the results of the changes in bone remodeling markers and to elucidate the molecular pathway of bone turnover could not be conducted.

In conclusion, to the best of our knowledge, this was the first comprehensive study to demonstrate the synergistic effect of GTE and anti-osteoporosis drugs on preserving BMD, improving bone microarchitecture and ultimately preventing osteoporosis in a rat model. The findings of the present study may justify the initiation of clinical trials on supplementing green tea in addition to standard antiresorptive agents to promote bone health. With the new synergistic intervention between food therapy and pharmacotherapy, these trials are expected to have a long-term impact on the elderly by alleviating the increasing trend in the incidence of osteoporosis.

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Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Authors' contributions

WSS verified the methodology, established the animal model, analyzed and interpreted the data and was a major contributor in writing, revision and editing of the manuscript. CHK designed the experiments, analyzed the data and contributed in the writing of the original manuscript. HTS performed the assessment of the serum bone turnover markers, analyzed the data and contributed in the writing of the original manuscript. KKL sourced the green tea, prepared and characterized the green tea extract, performed the animal experiment and BMD measurement. WTS performed measurement and analyzed the bone microarchitecture. PCL formed the concept and idea of the study, acquired funding source and supervised the overall study. JFZ formed the concept and idea of the study and help in acquisition of funding source. All authors read and approved the final manuscript.

Ethics approval and consent to participate

Animal Experimentation Ethics Approval was obtained from the Animal Experimental Ethics Committee of The Chinese University of Hong Kong (ref no. 13/032/MIS-5) for the present study. All the animal experiments complied with the ARRIVE guidelines and were carried out in accordance with the U.K. Animals (Scientific Procedures) Act, 1986 and associated guidelines.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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