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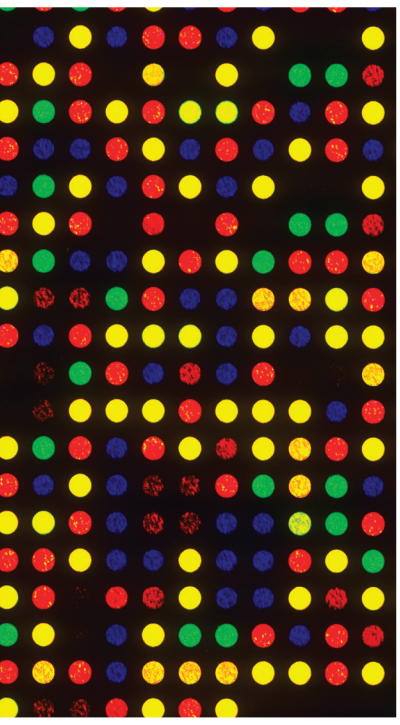
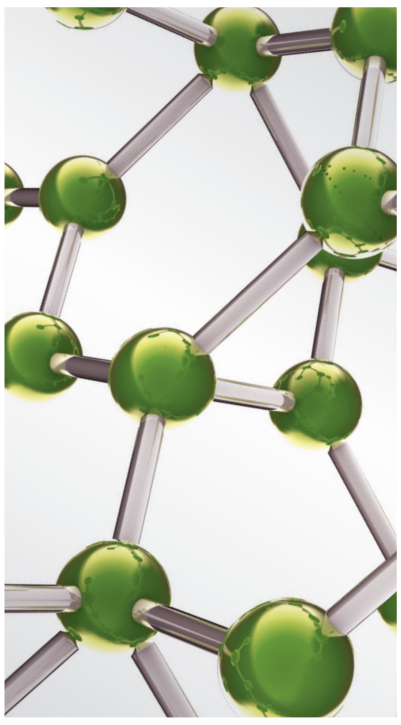
Guest Editors: Ima Nirwana Soelaiman, Srijit Das, Ahmad Nazrun Shuid, Huanbiao Mo, and Norazlina Mohamed



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Editorial Board

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Editorial

Use of Medicinal Plants and Natural Products for Treatment of Osteoporosis and Its Complications

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Osteoporosis is characterized by low bone mass with susceptibility to fracture. It may only be discovered when fractures of the osteoporotic bone have occurred. Therefore, prevention and early treatment of osteoporosis are important to avoid its complications. Some of the commercially available antiosteoporotic drugs are associated with serious side effects. Research is ongoing to find alternative antiosteoporotic agents with comparable effectiveness but minimal side effects. It is hoped that the increased ethnobotanical and ethnopharmacological research may pave the way to the development of novel drug candidates. In this special issue, 28 papers were published addressing various aspects of natural products on osteoporosis and its complications. Of these, 7 were review articles and the rest were original articles. All the published papers contribute significantly towards providing scientific evidence for future use in research and clinical settings.

Amongst the natural products, vitamin E, in particular tocotrienol, was the most studied, whereby 7 papers (4 original articles and 3 review articles) were published. The topics discussed were wide-ranging, including effects on bone structure and strength, mechanisms of action, and effects on osteoporotic fracture healing. S. Abdul-Majeed et al. reported on the effects of a combination of tocotrienol derived from annatto beans and lovastatin on bone resorption and formation indices. N. A. Manan et al. reported an *in vivo* study on the effects of γ-tocotrienol on osteoblasts. In conclusion, vitamin E of the tocotrienol type was shown to protect against experimental osteoporosis, whereby the mechanisms of these effects may be via its antioxidant or mevalonate suppressor properties, or both.

Other natural herbal products discussed were *Eurycoma longifola, Labisia pumila*, virgin coconut oil, *Cosmos caudatus,* curcumin, *Nigella sativa*, *Sambucus nigra,* and *Trifolium sp.* These were mainly extracts from the whole plant or specific parts of the plants, such as the fruit, leaves, or roots. However, S.-W. Choi et al. reported on fisetin, an alkaloid found in several different plants, where they described the mechanisms of the osteoclast inhibitory effects of fisetin. In general, these studies found encouraging effects of these natural products on bone metabolism, structure, and strength, as well as in improving the rate of osteoporotic fracture healing. However, further studies are needed to confirm these effects as well as to elucidate the precise mechanisms of action.

The effects of Chinese herbal products on osteoporosis were well represented in four papers. These studies included *in vivo* animal studies, *in vitro* cell culture studies, as well as a population-based study on usage of Chinese herbal medication in Taiwan. One study used the fermented product from Chinese herbal medication. In general, the animal studies showed that the Chinese medications studied had positive effects on bone. The population based-study identified patterns of Chinese herbal products used for the treatment of osteoporosis. However, further research is required to fully elucidate the efficacy and safety of these Chinese herbal products.

Overall, this special issue described studies on the effects of a plethora of natural products on different aspects of

osteoporosis and osteoporotic fracture healing. These studies contribute greatly to the existing knowledge regarding prevention and treatment options for osteoporosis. It is anticipated that some of these products may be further developed into pharmacotherapy for osteoporosis and osteoporotic fracture healing.

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Research Article

Antiresorptive Activity of *Bacillus*-Fermented Antler Extracts: Inhibition of Osteoclast Differentiation

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Antlers have been traditionally used for thousands of years as a natural product with medicinal and pharmaceutical properties. In developing healthy foods, *Bacillus*-mediated fermentation is widely used to enhance the biological activity of nutrients in foods. Recently, fermentation was shown to enhance the osteogenic activity of antlers. This study aimed to elucidate the antiresorptive activity of *Bacillus*-fermented antler and its mode of action. We found that *Bacillus*-fermented antler extract strongly inhibited osteoclast differentiation by downregulating the expression and activity of nuclear factor of activated T cells, cytoplasmic 1 (NFATc1). This extract also inhibited the activation of phospholipase Cγ2 (PLCγ2), a signaling molecule that could regulate NFATc1 transcriptional activity. This suggested that *Bacillus*-fermented antler extract could inhibit PLCγ2-NFATc1 signaling required for bone resorption and cell fusion. Consequently, *Bacillus*-fermented antler extract might benefit osteoclast-related disorders, including osteoporosis; furthermore, it may improve gastrointestinal activity.

1. Introduction

Bone homeostasis is maintained by a tight balance between osteoclast-mediated bone resorption (or destruction) and osteoblast-mediated bone formation. An imbalance induced by the overactivation of osteoclast differentiation (or osteoclastogenesis) can lead to a variety of bone metabolic diseases, including osteoporosis. Therefore, a promising strategy for treating patients with osteoporosis is to inhibit osteoclast differentiation.

For thousands of years, antlers have been traditionally used in Asian countries, due to their many medicinal and pharmaceutical properties. The combination of beneficial factors such as several amino acids, sulfated-glycosaminoglycan, sialic acid, gangliosides, neutral lipids including cholesterol ester, phospholipids including phosphatidylcholine, lecithin, and polyamines in antler extracts has been suggested to exhibit the several salutary activities in humans. Apparently, the antler is dried and is used powdered or in tea form for a wide variety of health remedy and health maintenance purposes in Asian countries such as China and Korea. Additionally, antler extracts are sold in Asian countries as the type of functional food.

The beneficial role of antler extracts in the prevention and/or treatment of osteoporosis has not been studied well, but several studies have reported their beneficial action

in bone metabolism and in the experimental condition of osteoporosis; antler extracts have exhibited antiosteoporotic activity by increasing the proliferation of osteoblasts and the expression of bone matrix protein [1]. Chondroitin sulfate isolated from deer antler tips has been shown to upregulate the gene expression of bone-specific proteins in a human osteoblastic cell line [2]. Furthermore, anti-resorptive activities of antler extracts, antler blood, collagen, and glue were also reported in several studies [3–5]. These results indicated the dual antiosteoporotic activity of antler to stimulate bone formation and inhibit bone resorption.

Moreover, fermentation caused an enhancement of antler osteogenic activity [1]. Among bacteria used for fermentation, *Bacillus* strains are wildly used to develop healthy foods, because they enhance the biological activity of nutrients in foods. The representative *Bacillus*-fermented healthy foods are Korean food "Cheonggukjang" and Japanese food "Natto." Indeed, *Bacillus subtilis* has been sold commercially around the world as a nutritional supplement in healthy foods [6]. Fermented antler exhibited bone anabolic activity, but it has not been elucidated whether antler fermented with *Bacillus subtilis* has antiresorptive activity. Therefore, this study aimed to evaluate the anti-resorptive activity of antler fermented with *Bacillus subtilis* and its mode of action in osteoclast differentiation.

2. Materials and Methods

2.1. Antlers. Antlers (*Cervus canadensis E.*) and fermented antlers were provided by Korean Medicine Biofermentation Co. Ltd. (Seoul, Korea). Briefly, antlers were purchased from the DaeSeong Elk Farm (Kangwon, Korea). Samples comprised a combination of three sections (40% top; 30% middle; 30% base). The samples were homogenized and stored at −20°C.

2.2. Bacteria and Culture Conditions. *Bacillus subtilis* strain K-11 was isolated from soybeans by Professor Dong-Hyun Kim (College of Pharmacy, Kyung Hee University, Seoul, Korea). Bacteria were cultured in Tryptic Soy Agar or Broth (Difco, KS). Strains were inoculated in Tryptic Soy Broth and grown at 37°C for 1 day.

2.3. Preparation of Antlers and Fermented Antler Extracts. The nonfermented antler extract was prepared by soaking antlers in distilled water (1.5 kg/30 L), followed by autoclaving at 121°C for 20 min, refluxing at 100°C for 3 h, filtering twice with nonwoven fabric, and lyophilizing at −55°C for 4 days. Fermented antler extract was prepared by inoculating the antler sample with 1% (v/v) *Bacillus subtilis* strain K-11 after the antler sample had been soaked and autoclaved in distilled water (800 g/30 L). The mixture was incubated in a fermenter (Seobong Biogen, Kangwon, Korea) at 37°C for 5 days. Then, the fermented antler extract was filtered twice with nonwoven fabric and lyophilized at −55°C for 4 days.

2.4. Osteoclast Differentiation. Bone marrow cells were obtained from 5- to 8-week-old male ICR mice by flushing femurs and tibias in α-MEM supplemented with antibiotics (100 units/mL penicillin, 100 μg/mL streptomycin; Hyclone, UT). Bone marrow cells were cultured for 1 day in 10-cm culture dishes with α-MEM that contained 10% fetal bovine serum (FBS; Gibco, Paisley, UK), antibiotics, and macrophage colony stimulating factor (M-CSF; 10 ng/mL; Peprotech, NJ). Nonadherent bone marrow cells were plated on 9-cm petri dishes and cultured for 3 days in the presence of M-CSF (30 ng/mL). After nonadherent cells were washed out, adherent cells comprised bone marrow-derived macrophages (BMMs). BMMs were induced to differentiate into osteoclasts by culturing (1×10^4 cells/well in a 96-well plate or 3×10^5 cells/well in a 6-well plate) for 4 days in the presence of M-CSF (30 ng/mL) and the receptor activator of nuclear factor kappa-B ligand (RANKL; 5 ng/mL; R&D Systems, MN).

2.5. TRAP Staining and Activity Assay. Mature osteoclasts were visualized by staining tartrate-resistant acid phosphatase (TRAP), a biomarker of osteoclast differentiation. Briefly, cells were fixed with 10% formalin for 10 min and 0.1% Triton X-100 for 10 min, and then stained by using the Leukocyte Acid Phosphatase Kit 387-A kit (Sigma, MO). Images were captured under a microscope equipped with a DP Controller (Olympus Optical, Tokyo, Japan). The number of TRAP-positive osteoclasts was counted on the whole area in a well under a microscope. For measuring TRAP activity, cells were fixed with 10% formalin for 10 min and 0.1% Triton X-100 for 10 min. Then, we added 100 μL of citrate buffer (100 mM, pH 5) that contained 50 mM sodium tartrate and 3 mM *p*-nitrophenylphosphate (Sigma) to the fixed cells. After incubation for 1 h, the enzyme reaction mixtures were transferred into new plates containing an equal volume of 0.1 N NaOH. Absorbance was measured at 405 nm with a Wallac EnVision HTS microplate reader (Perkin Elmer, MA). The experiment was performed in triplicate.

2.6. Cell Viability Assay. BMMs were suspended in α-MEM with 10% FBS and plated in a 96-well plate at a density 1×10^4 cells/well. BMM cells were treated with the fermented antler extract in the presence of M-CSF (30 ng/mL) and incubated for 1 or 3 days. Cell viability was then measured with the Cell Counting Kit-8 (Dojindo Moleculer Technologies, MD) according to the manufacturer's protocol. Measured absorbance was converted to cell number with a standard curve.

2.7. Real-Time PCR. Primers were chosen with an online primer 3 design program [7]. The primer sets used in this study are shown in Table 1. Total RNA was isolated with TRIzol reagent (Invitrogen, NY) according to the manufacturer's protocol. First-strand cDNA was synthesized with the Omniscript RT kit (Qiagen, CA) and 1 μg of total RNA, 1 μM of oligo-dT_{18} primer, 10 units of the RNase inhibitor, RNasin (Promega, WI), according to the manufacturer's protocol. Then, quantitative PCR was performed with the Stratagene Mx3000P real-time PCR system and Brilliant SYBR Green Master Mix (Stratagene, CA). The first-strand cDNA was diluted 1 : 10 and 10 pmol of primers were added

Table 1: Primer sequences used in this study.

Target gene	Forward (5′–3′)	Reverse (5′–3′)
c-Fos	CCAGTCAAGAGCATCAGCAA	AAGTAGTGCAGCCCGGAGTA
NFATc1	GGGTCAGTGTGACCGAAGAT	GGAAGTCAGAAGTGGGTGGA
Cathepsin K	GGCCAACTCAAGAAGAAAAC	GTGCTTGCTTCCCTTCTGG
DC-STAMP	CCAAGGAGTCGTCCATGATT	GGCTGCTTTGATCGTTTCTC
GAPDH	AACTTTGGCATTGTGGAAGG	ACACATTGGGGGTAGGAACA

into each reaction, according to the manufacturer's protocol. The thermocycling protocol consisted of 3 segments. The first segment comprised incubation at 95°C for 10 min to activate the polymerase; the second segment comprised 40 cycles of 94°C for 40 s (denaturation), 53°C for 40 s (annealing), and 72°C for 1 min (extension); the third segment was an incubation at 95°C for 1 min, 55°C for 30 s, and 95°C for 30 s to generate PCR product temperature-dissociation curves (also called "melting curves"). All reactions were run in triplicate, and the data were analyzed with the $2^{-\Delta\Delta C_T}$ method [8]. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as an internal standard. The statistical significance was determined by the Student's t-test, with GAPDH-normalized $2^{-\Delta\Delta C_T}$ values.

2.8. Western Blot Analysis. Briefly, cells were homogenized and centrifuged at 10,000 ×g for 15 min. The supernatant was collected to isolate cytoplasmic proteins. Denatured proteins were separated on the SDS-PAGE gels and transferred onto PVDF membranes (Millipore, CA). The membrane was then washed with TBST (10 mM Tris-HCl pH 7.5, 150 mM NaCl, and 0.1% Tween 20) and incubated in the blocking solution, TBST with 5% skim milk. The membrane was probed with the indicated primary antibody, washed three times for 30 min, incubated with secondary antibody (Santa Cruz Biotechnology) conjugated to horseradish peroxidase for 2 h, and washed three times for 30 min. The membranes were developed with SuperSignal West Femto Maximum Sensitivity Substrate (Pierce) and signals were detected in the LAS-3000 luminescent image analyzer (Fuji Photo Film Co., Ltd., Japan). Antibodies against NFATc1 and Actin were purchased from Santa Cruz biotechnology (CA). Antibodies against (p)-ERK, (p)-JNK, (p)-p38, (p)-Plcγ2, ERK, JNK, p38, and Plcγ2 were obtained from Cell Signaling Technology. All antibodies were diluted 1 : 1000 in TBST with 1% BSA.

2.9. Luciferase Activity Assay. Human embryonic kidney 293T cells were plated in a 24-well plate in triplicate. Cells were then transfected with the following reporter plasmids: (1) the plasmid (100 ng/well) with the DNA-binding sequence for the nuclear factor of activated T-cells, cytoplasmic 1 (NFAT) fused to the firefly-luciferase sequence, (2) the plasmid (100 ng/well) with the receptor activator of nuclear factor κB (RANK) sequence, and (3) the plasmid (20 ng/well) with the renilla luciferase sequence in the pGL4 vector. After 6 hrs, the transfected cells were co-treated with RANKL (50 ng/mL) and the fermented antler extract. After 48 h, the transfected cells were lysed with lysis buffer (Promega, Madison, WI, USA), and luciferase activity was measured with a dual-luciferase assay system (Promega). Luciferase activity was normalized to renilla luciferase activity for each sample.

2.10. Retrovirus Preparation and Infection. Retrovirus packaging was described previously [9]. In brief, to isolate the retroviral particles, we transiently transfected Plat-E cells (platinum-E retrovirus packaging cell line, Ecotropic, Cell Biolabs, Inc.) with pMX-IRES-GFP, a retrovirus vector with green fluorescence protein (GFP), and the pMX vector with a constitutively active (CA) NFATc1 gene. At 48 h after transfection with Lipofectamine 2000 (Invitrogen, NY), the viral supernatants were collected from the culture media according to the manufacturer's protocol. Next, BMMs were incubated with the viral supernatants in the presence of polybrene (10 μg/mL) for 8 h. The infection efficiency was determined by GFP expression, which was always greater than 80%. After infection, BMMs were induced to differentiate in the presence of M-CSF (30 ng/mL) and RANKL (5 ng/mL) for 4 days.

2.11. Statistical Analysis. Each experiment was performed in triplicate and repeated three to five times to confirm the reproducibility of data, and results from one among repeated experiments were analyzed and shown in the figures. Statistical differences were analyzed with the Student's t-test and all quantitative values were presented as mean ± SD. A value of $P < 0.05$ was considered significant.

3. Results

3.1. Fermented Antler Extract Inhibited RANKL-Induced Osteoclast Differentiation. Osteoclast differentiation was evaluated with the RANKL-induced osteoclast differentiation model of primary mouse BMMs. The effect of fermented antler extract was compared to the effect of non-fermented antler extract. We found that, during BMM differentiation into osteoclasts, both antler and fermented antler extracts dose-dependently inhibited the formation of TRAP-positive multinucleated osteoclasts (Figure 1(a)). The inhibitory effect of fermented antler extract on osteoclast differentiation was confirmed by counting the number of TRAP-positive osteoclasts and by evaluating TRAP activity (Figures 1(b) and 1(c)); in a dose-dependent manner, the fermented antler extract significantly inhibited the RANKL-induced formation of TRAP-positive osteoclasts and activation of TRAP. To ascertain that the inhibitory effect of fermented antler extract on RANKL-induced osteoclast differentiation

was not due to cytotoxicity *per se*, its effect on cell viability was evaluated. At the concentrations used in this study, the fermented antler extract did not show any cytotoxicity in BMMs (Figure 1(d)).

3.2. Fermented Antler Extract Inhibited RANKL-Induced NFATc1 Expression and Its Transcriptional Activity. The inhibitory effect of fermented antler extract on osteoclast differentiation was further explored by evaluating the expression of transcription factors. As shown in Figure 2(a), the mRNA expression levels of both c-Fos and NFATc1 were strongly induced by RANKL, but the induction of NFATc1 was significantly inhibited by the fermented antler extract in the early stage of osteoclast differentiation. The expression levels of cathepsin K and DC-STAMP were also elevated with RANKL, but these inductions were significantly inhibited by the fermented antler extract. Furthermore, the RANKL-induced mRNA expression of TRAP was also significantly inhibited by the fermented antler extract (data not shown).

Western blot analysis revealed that the RANKL-mediated induction of NFATc1 protein was completely inhibited by the fermented antler extract (Figure 2(b)). The NFATc1-luciferase reporter activity assay showed that the RANKL-mediated activation of NFATc1 was dose-dependently inhibited by the fermented antler extract (Figure 2(c)). These results suggested that NFATc1 could be involved in the anti-resorptive activity of fermented antler extract.

3.3. Fermented Antler Extract Inhibited NFATc1-Induced Osteoclast Differentiation. We hypothesized that the anti-resorptive activity of fermented antler extract might result from its potential to completely inhibit the expression of NFATc1. We tested this hypothesis by overexpressing NFATc1 in mouse BMMs with a retroviral vector. The infection rates of the retroviral GFP control and the retroviral constitutively active (CA) NFATc1-GFP were similar in either BMMs treated or untreated with the fermented antler extract (Figure 3(a)). TRAP-positive multinucleated osteoclasts were significantly more abundant in BMMs over-expressing NFATc1 than in BMMs treated with the GFP control. However, in the presence of fermented antler extract, the over-expression of NFATc1 could not induce BMM differentiation into osteoclasts (Figure 3(b)). This strong inhibitory activity of fermented antler extract on the NFATc1-mediated formation of TRAP-positive osteoclasts was confirmed by counting the number of osteoclasts (Figure 3(c)) and measuring the activity of TRAP (Figure 3(d)).

3.4. Fermented Antler Extract Inhibited RANKL-Induced PLCγ2 Activation. To gain insight into the mechanism by which the fermented antler extract might block the NFATc1-related process of osteoclast differentiation, we investigated the effect of fermented antler extract on the activation of signaling molecules involved in osteoclast differentiation. First, we considered MAP kinases, because they are well known to be involved in osteoclast differentiation [10]. As shown in Figure 4, RANKL strongly induced the activation of ERK, JNK, and p38, but those inductions were not inhibited by the fermented antler extract. Next, we focused on the involvement of PLCγ2 activation in the anti-resorptive activity of fermented antler extract because PLCγ2 activation can evoke a Ca^{2+} signal required for the activation and induction of NFATc1 in osteoclast differentiation [11]. Interestingly, the fermented antler extract inhibited the RANKL-induced phosphorylation of PLCγ2. These results suggested that the PLCγ2-NFAT signaling axis could be involved in the anti-resorptive activity of fermented antler extract.

4. Discussion

There are two main treatment strategies for reducing the incidence of osteoporotic fracture and the reduction of bone resorption with anti-resorptive agents, like bisphosphonates, and the induction of bone formation with anabolic agents, like parathyroid hormone (PTH). PTH is a currently available for stimulating bone formation, but its use is limited by cost and concerns regarding its long-term safety. Thus, anti-resorptive agents have become the therapeutic mainstay for treating osteoporosis. However, the most common anti-resorptive agent like bisphosphonates also carries the risk of side effects such as bisphosphonate-related osteonecrosis of the jaws [12] and atypical femoral fractures [13]. Therefore, there is a strong need for new anti-resorptive agents.

Natural products have historically yielded a variety of therapeutic agents. Generally, healthy nutrients or foods with medicinal properties are both effective and safe for the long-term management of disorders. Recent studies have aimed to identify natural products or healthy foods that can prevent and/or treat osteoporosis, with minimal adverse effects [14, 15].

Due to many medicinal and pharmaceutical properties, antlers have been recognized in traditional medicines. Recently, several studies have shown that antler extract and its components exhibited anti-osteoporotic activity. Moreover, fermentation of antlers enhanced their osteogenic activity [1]. However, the anti-resorptive activity of antlers fermented with *Bacillus subtilis* and the mode of action have not been elucidated.

In this study, we found that the antler extract fermented with *Bacillus subtilis* strain K-11 significantly inhibited the RANKL-induced differentiation of BMMs into osteoclasts in a dose-dependent manner. In a previous study, a chloroform extract of deer antler inhibited osteoclast differentiation via suppressing the RANKL-induced activation of ERK [16], but in this study, the fermented antler extract inhibited the RANKL-induced phosphorylation of PLCγ2, not those of MAP kinases.

Transcription factors, c-Fos and NFATc1, were known to play a critical role in the regulation of genes required for osteoclast differentiation. The c-Fos transcription factor, an AP-1 family member, was suggested to be essential for osteoclast differentiation [17], and NFATc1 was shown to rescue osteoclastogenesis in cells that lacked c-Fos [18–20]. Furthermore, the coordinating activation between AP-1 and NFATc1 may play an important role in the regulation of osteoclast-specific genes, like TRAP and cathepsin K [21]. Additionally,

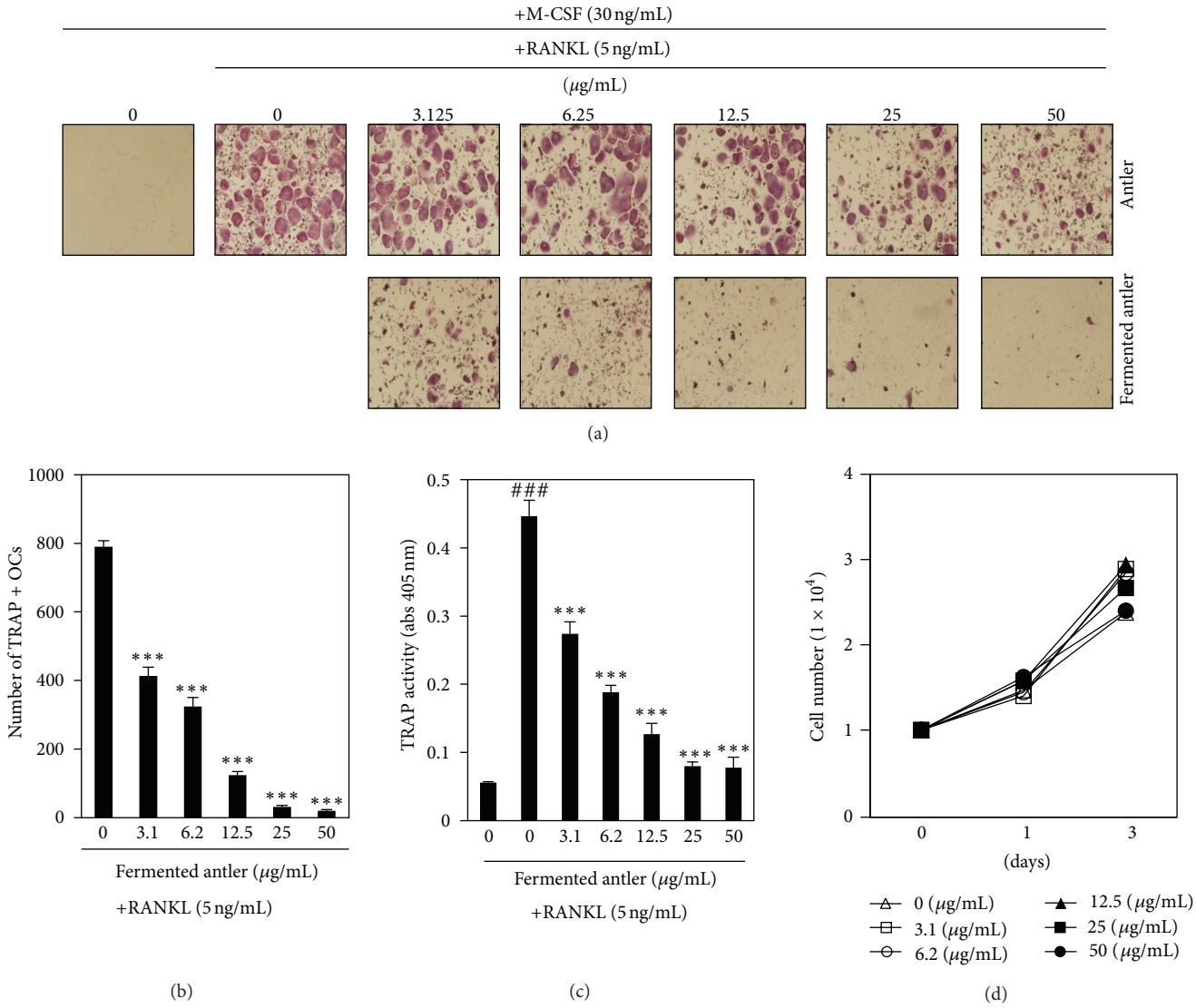

Figure 1: Fermented antler extract inhibits RANKL-induced osteoclast differentiation. (a) BMM cells were cultured for 4 days in the presence of RANKL (5 ng/mL) and M-CSF (30 ng/mL) with antler or fermented antler extract. Osteoclasts were visualized by TRAP staining. (b) The number of TRAP-positive osteoclasts (TRAP+OCs) was counted. $^{***}P < 0.001$ (versus "the control"). (c) TRAP activity was measured. $^{\#\#\#}P < 0.001$ (versus "the negative control"); $^{***}P < 0.001$ (versus "the group treated with RANKL alone"). (d) The effect of fermented antler extract on the viability of BMMs was evaluated by CCK-8 assay. Each experiment was performed in triplicate. Statistical differences were analyzed with the Student's *t*-test and all quantitative values were presented as mean ± SD.

the NFATc1-induced dendrite cell-specific transmembrane protein (DC-STAMP) was reported to be essential for osteoclast fusion [22–24]. In this study, fermented antler extract strongly inhibited RANKL-induced NFATc1 expression and its transcriptional activity and furthermore, it also inhibited NFATc1-induced osteoclast differentiation.

It is known that the activation of NFATc1 requires assembly of the RANKL-RANK-MAP kinases and PLC γ-Ca^{2+} signaling. Previous studies showed that, in osteoclast precursors, RANKL triggered the activations of MAP kinases and PLCγ, and those activations consequently induced the activation of transcription factors [10]. In the present study, we suggested that the fermented antler extract could inhibit the signaling axis of PLCγ2-NFATc1 during osteoclast differentiation. This suggestion was consistent with findings that the PLCγ2-NFATc1 signaling axis could positively regulate RANKL-induced osteoclast differentiation in mice; in that study, PLCγ2-deficient mice had an osteopetrotic phenotype and exhibited reduced NFATc1 expression [25]. Furthermore, in this study, the fermented antler extract strongly inhibited the NFATc1-mediated formation of TRAP-positive multinucleated osteoclasts even in BMMs overexpressing

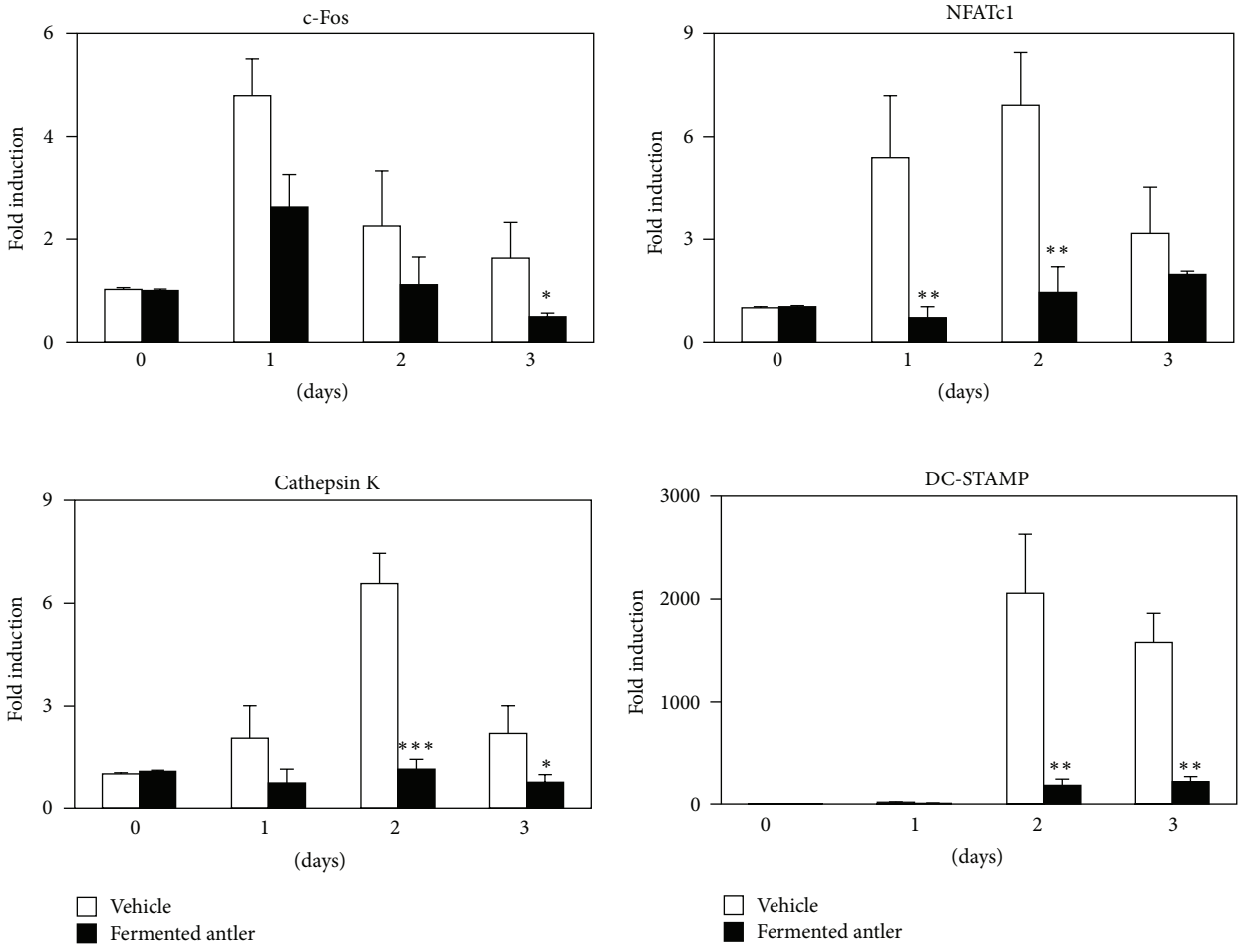

Figure 2: Fermented antler extract suppresses RANKL-induced NFATc1. (a) After pretreated with vehicle or fermented antler extract (25 μg/mL) for 1 hr, BMMs were treated with RANKL (5 ng/mL) for the indicated day, and then the mRNA expression levels were analyzed by the real-time PCR. $^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$ (versus "the vehicle control"). (b) The effect of fermented antler extract on the protein expression of NFATc1 was evaluated by the Western blot analysis. Actin was used as the internal control. (c) The effect of fermented antler extract on the transcriptional activity of NFATc1 was evaluated by luciferase activity assay. Activity was expressed as fold induction compared with the activity of NFAT luciferase only. pGL4 renilla luciferase activity was used to normalize the transfection efficiency and luciferase activity. $^{\#\#}P < 0.01$ (versus "the RANK"); $^{**}P < 0.01$ (versus "the RANK plus RANKL"). Each experiment was performed in triplicate. Statistical differences were analyzed with the Student's t-test and all quantitative values were presented as mean ± SD.

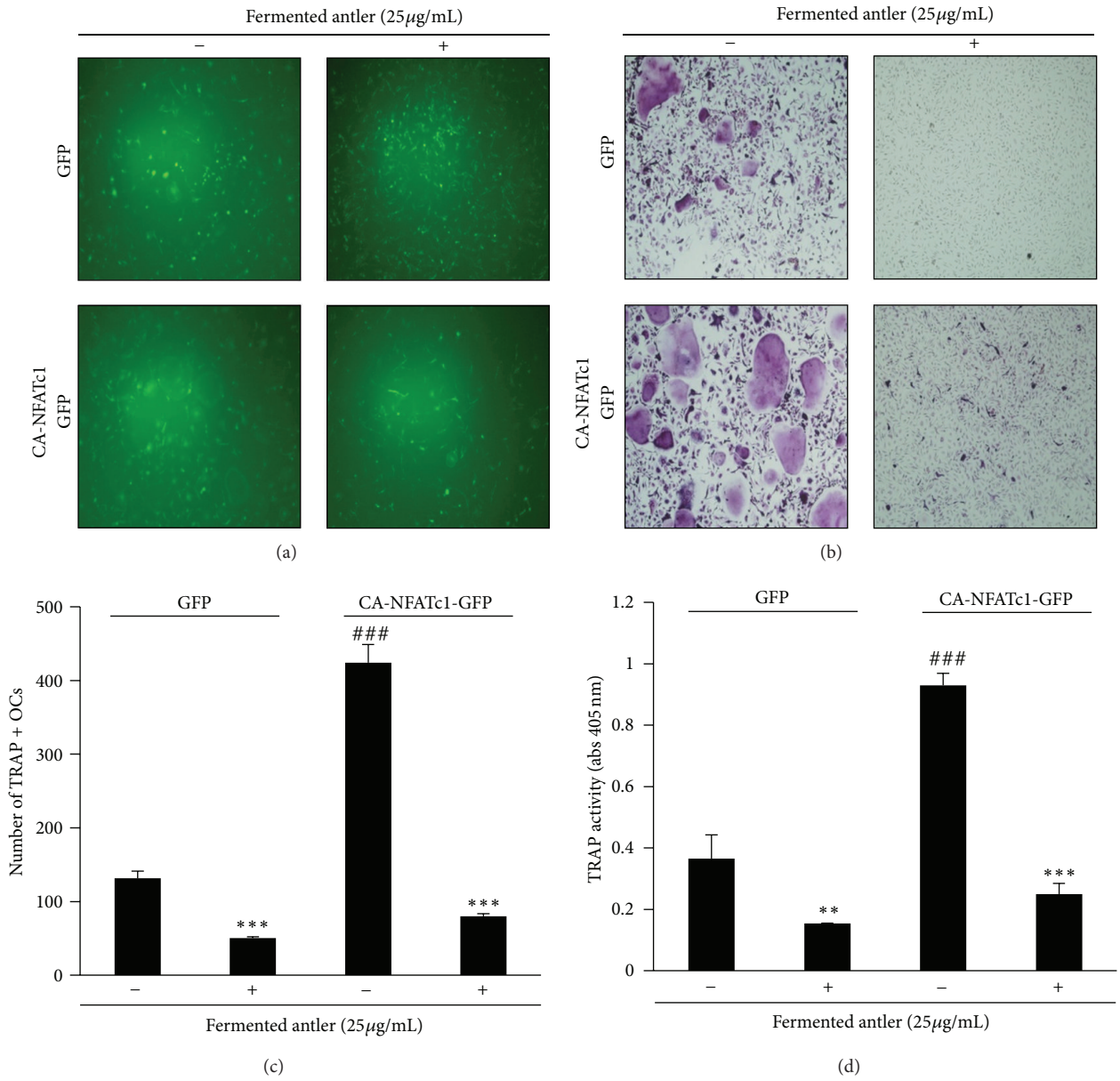

Figure 3: Fermented antler extract suppresses NFATc1-induced osteoclast differentiation. (a) BMMs were infected with pMX-IRES-GFP (GFP) or pMX-IRES-CA-NFATc1-GFP (CA-NFATc1-GFP) for 8 hrs with polybrene (10 μg/mL). The infected BMMs were cultured with M-CSF (30 ng/mL) and RANKL (5 ng) for 4 days in the presence or absence of fermented antler extract (25 μg/mL). After 4 days, cells were fixed, and the GFP expression was visualized under a fluorescence microscope. (b) BMMs were infected with GFP or CA-NFATc1-GFP and then cultured as described in (a). After 4-day culture, mature TRAP-positive osteoclasts were visualized by TRAP staining. (c) TRAP-positive cells (TRAP+OCs) were counted as osteoclasts. $^{\#\#\#}P < 0.001$ (versus "the GFP control"); $^{**}P < 0.01$; $^{***}P < 0.001$ (versus "the fermented antler-nontreated group"). (d) TRAP activity was measured at 405 nm. $^{\#\#\#}P < 0.001$ (versus "the GFP control"); $^{**}P < 0.01$; $^{***}P < 0.001$ (versus "the fermented antler-nontreated group"). Each experiment was performed in triplicate. Statistical differences were analyzed with the Student's t-test and all quantitative values were presented as mean ± SD.

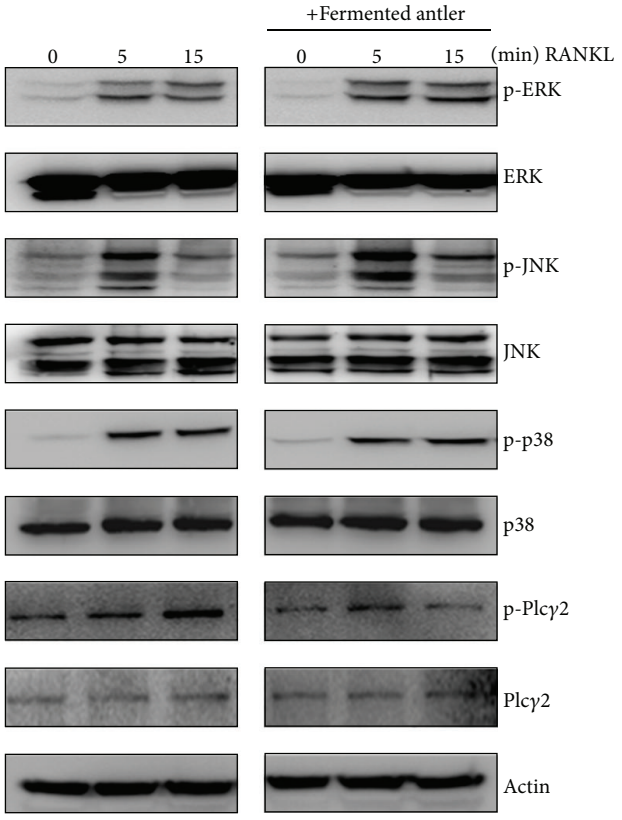

Figure 4: Fermented antler extract inhibits RANKL-induced phosphorylation of PLCγ2. BMMs were pretreated with or without fermented antler extract (25 μg/mL) for 1 hr prior to RANKL stimulation (5 ng/mL) at indicated time periods. Then, the protein expression levels were evaluated by Western blot analysis.

a constitutively active NFATc1. These results suggested the possible involvement of PLCγ2-NFAT signaling axis in the anti-resorptive activity of fermented antler extract.

Moreover, the inhibition of NFATc1 expression was expected to reduce NFATc1-regulated gene expression. Previous studies showed that proximal NFAT binding sites played a significant role in the NFATc1-induced gene expression of cathepsin K in response to RANKL [26] and that NFATc1 induced osteoclast fusion by upregulating DC-STAMP [24]. In this study, we found that the fermented antler extract inhibited the RANKL-induced gene expression of cathepsin K and DC-STAMP; these effects were most likely due to the inhibition of NFATc1 expression.

This study showed that the fermented antler extract strongly inhibited osteoclast differentiation by down-regulating NFATc1 expression and activity. Our results suggested that the mechanism of this inhibition involved the inactivation of PLCγ2. The consequence of down-regulating NFATc1 expression was a decrease in the transcription of cathepsin K and DC-STAMP, essential factors for bone resorption and cell fusion, respectively. Further study will be required to identify the components in the fermented antler extract that confer anti-resorptive activity and the detailed *in vivo* experiment should be carried out before its application to humans. Our results finally suggested that *Bacillus*-fermented antler extract might be developed to functional foods or pharmacological agents for preventing and treating osteoclast-related disorders as well as improving gastrointestinal activity [27].

Conflict of Interests

The authors declare that they have no conflict of interests.

Acknowledgments

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Review Article

Chinese Herbal Medicine for Osteoporosis: A Systematic Review of Randomized Controlled Trails

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Background. Osteoporosis is a major health problem for the elderly population. Chinese herb may be beneficial to osteoporosis due to its capability. *Objectives*. This study was designed to evaluate the effectiveness of Chinese medicine treatment on the patients with osteoporosis. *Search Methods*. Randomized controlled trials were retrieved from different 9 databases. *Results*. This meta analysis included 12 RCTs involving 1816 patients to compare Chinese herbs with placebo or standard anti-osteoporotic therapy in the treatment of bone loss. The pooled data showed that the percent change of increased BMD in the spine is higher with Chinese herb compared to placebo (lumber spine: WMD = 0.07, 95% CI: 0.01–0.04). In the femoral, Chinese herb showed significantly higher increments of BMD compared to placebo (femoral neck: WMD = 0.06, 95% CI: −0.02–0.13). Compared to the other standard anti-osteoporotic drugs, Chinese herbs also show advantage in BMD change (lumber spine: WMD = 0.03, 95% CI: −0.01–0.08; femoral: WMD = 0.01, 95% CI: −0.01–0.02). *Conclusions*. Our results demonstrated that Chinese herb significantly increased lumbar spine BMD as compared to the placebo or other standard anti-osteoporotic drugs.

1. Introduction

Osteoporosis, the thinning of bone due to the net loss of calcium and bone structure, occurs primarily with ageing. It results in a great cost both to the individual suffering from bone fractures and society burdened with the extreme financial costs [1]. In the last decade, pharmaceutical companies developed a rich collection of new drugs that offer specific and clearly targeted therapeutic effects on the improvement of bone quality. The new drugs have been developed based on the understanding of the metabolism of the bone tissue so that building it up could work through the stimulation of the anabolic side or suppression of the catabolic side. Teriparatide and strontium products represent the former and the bisphosphonates the latter [2, 3]. While therapeutic drugs created for the treatment of osteoporosis still show some side effects. Firstly, because the therapeutic agents offer an unbalanced effect through a specific artificial influence on the end of the metabolic cycle of bone physiology, the structural changes of bones from long-term treatment remains unknown. Secondly, adverse reactions of the therapeutic agents may yet appear minor; uncertainty exists with longer uses [4, 5]. Indeed, odd fractures were already reported with prolonged treatment, and large doses of bisphosphonates could induce osteonecrosis of the jaw bone.

Medical scientists in China have studied many medicinal herbs and found that they have antiosteoporotic effects in the laboratory and subsequently in clinical trials [6]. But its effects on bone metabolism and calcium homeostasis are still not clear. During the last 10 years, some unique clinical trials focused on Chinese herbs in the treatment of osteoporosis have been conducted.

It would be valuable to evaluate the quality of these trials and assess the efficacy and safety data provided by the trials in terms of the principles and measurements of evidence-based medicine. In this study, a systematic review and meta-analysis of randomized controlled trail (RCTs) were performed to compare Chinese herbs with standard anti-osteoporotic drugs or placebo and to identify herbs commonly used in the clinical treatment of bone loss. We hypothesized that the eligible trials would provide evidence of the effect of Chinese herbs on bone mineral density (BMD)

Table 1: Assessing the quality of reports of randomized clinical trials.

Item	Score
Randomization	
Was the study described as randomized (this includes the use of words such as randomly, random, and randomization)?	1
If the first answer is yes, the method to generate the sequence of randomization was described and it was appropriate (table of random numbers, computer generated, etc.)	1
If the first answer is yes, the method to generate the sequence of randomization was described and it was inappropriate (patients were allocated alternately, or according to date of birth, hospital number, etc.)	−1
Double blind	
Was the study described as double blind?	1
If the first answer is yes, the method of double blinding was described and it was appropriate (identical placebo, active placebo, dummy, etc.)	1
If the first answer is yes, the method of blinding was inappropriate (e.g., comparison of tablet versus injection with no double dummy)	−1
Description of withdrawals and dropouts?	
Was there a description of withdrawals and dropouts?	1

and the therapeutic benefits of Chinese medicine treatment in patients with bone loss.

2. Materials and Methods

2.1. Eligibility Criteria. All RCTs comparing the efficacy of Chinese herbs for the treatment of osteoporosis were included.

The diagnostic criteria for osteoporosis or osteopenia in the trials were required to be in accordance with the criteria of the World Health Organization (WHO 1994) [7]. Participant selection excluded those receiving any medications known to affect bone or calcium metabolism, including current use or a history of 3-month (or more) use of exogenous estrogens, corticosteroids, and thiazine; those with any systemic, endocrine disease, or any surgery known to affect bone health (i.e., ovariectomy, excision of thyroid gland or intestinal tract, etc.), as well as cancer.

The intervention was required to be oral administration of any kind of herbal preparation used alone or in combination with other herbs for subjects in treatment groups and placebo or other standard anti-osteoporotic drugs for subjects in the control groups. Concurrent administration of calcium and/or vitamin D was acceptable if both groups received the same dose and formulation.

The outcome measures included changes in lumbar, femoral, and forearm BMD values and also the followup of the patients must be at least for six months.

2.2. Search Methods for Identification of Studies. A literature search was performed using the phrase "chinese herb AND herbal AND osteoporosis AND fracture" with the limits "randomized controlled trail" and regardless of language or publication status.

A total of 9 electronic databases were searched including MEDLINE (1966 to February 2012), EMbase (1974 to February 2012), Chinese Biomedical Literature Database (CBM, 1978 to February 2012), China National Knowledge Infrastructure (CNKI, 1994 to February 2012), the Chinese Scientific and Technical Journals database (VIP, 1989 to February 2012), Wanfang Data (1995 to February 2012), China Doctoral Dissertations Full-text Database (CDFD, 1984 to February 2012), China Master's Theses Full-text Database (CMFD, 1984 to February 2012), and China Proceedings of Conference Full-text Database (CPFD, 2000 to February 2012).

At the same time, we searched references of the included studies for any possible titles matching the inclusion criteria. We also wrote emails to the author to request for the data of the trials which did not report the original data.

2.3. Selection of Studies. Two reviewers independently selected articles. The titles and abstracts of articles found in the search were screened by ZQW and JLL, who discarded trials that were clearly not eligible. Full article was selected by two review authors independently (ZQW and JLL).

2.4. Assessments Bias Risk. Risk of bias was assessed independently by two review authors (Z.-Q. Wang and JLL) with the criteria in the Cochrane Handbook for Systematic Reviews of Interventions 5.1.0 [8]. Sequence generation, allocation concealment, blinding (or masking), incomplete data assessment, selective outcome reporting, and other sources of bias were assessed with three potential responses: yes, no, and unclear. Disagreements between review authors were resolved by discussion or with a third author (X.-J. Cui).

2.5. Data Extraction and Management. Information was carefully extracted from all eligible publications independently by two of the authors of the present study (Z.-Q. Wang and WZQ). Two review authors (Y.-L. Sun and X.-J. Cui) checked and entered data into Review Manager (RevMan 5.1). We two graded the methodology quality of all included trails by JADAD [9] (Table 1) and "Risk of Bias table" (Table 2) which recommended by Cochrane handbook

Table 2: The Cochrane Collaboration's tool for assessing risk of bias.

Random sequence generation	
Low risk of bias	The investigators describe a random component in the sequence generation process such as: referring to a random number table; using a computer random number generator.
High risk of bias	The investigators describe a nonrandom component in the sequence generation process. Usually, the description would involve some systematic, nonrandom approach, for example, sequence generated by odd or even date of birth; sequence generated by some rule based on date (or day) of admission.
Unclear risk of bias	Insufficient information about the sequence generation process to permit judgement of "Low risk" or "High risk."
Allocation concealment	
Low risk of bias	Participants and investigators enrolling participants could not foresee assignment because one of the following, or an equivalent method, was used to conceal allocation: central allocation (including telephone, web-based and pharmacy-controlled randomization); sequentially numbered drug containers of identical appearance.
High risk of bias	Participants or investigators enrolling participants could possibly foresee assignments and thus introduce selection bias, such as allocation based on using an open random allocation schedule (e.g., a list of random numbers); assignment envelopes were used without appropriate safeguards (e.g., if envelopes were unsealed or nonopaque or not sequentially numbered).
Blinding of participants and personnel	
Low risk of bias	Any one of the following: no blinding or incomplete blinding, but the review authors judge that the outcome is not likely to be influenced by lack of blinding; blinding of participants and key study personnel ensured, and unlikely that the blinding could have been broken.
High risk of bias	No blinding or incomplete blinding, and the outcome is likely to be influenced by lack of blinding; blinding of key study participants and personnel attempted, but likely that the blinding could have been broken, and the outcome is likely to be influenced by lack of blinding.
Unclear risk of bias	Any one of the following: insufficient information to permit judgement of "Low risk" or "High risk"; the study did not address this outcome.
Blinding of outcome assessment	
Low risk of bias	Any one of the following: no blinding of outcome assessment, but the review authors judge that the outcome measurement is not likely to be influenced by lack of blinding; blinding of outcome assessment ensured, and unlikely that the blinding could have been broken.
High risk of bias	Any one of the following: no blinding of outcome assessment, and the outcome measurement is likely to be influenced by lack of blinding; blinding of outcome assessment, but likely that the blinding could have been broken, and the outcome measurement is likely to be influenced by lack of blinding.
Unclear risk of bias	Any one of the following: insufficient information to permit judgement of "Low risk" or "High risk"; the study did not address this outcome.
Incomplete outcome data	
Low risk of bias	Any one of the following: no missing outcome data; reasons for missing outcome data unlikely to be related to true outcome (for survival data, censoring unlikely to be introducing bias).
High risk of bias	Any one of the following: reason for missing outcome data likely to be related to true outcome, with either imbalance in numbers or reasons for missing data across intervention groups; for dichotomous outcome data, the proportion of missing outcomes compared with observed event risk enough to induce clinically relevant bias in intervention effect estimate.
Unclear risk of bias	Any one of the following: insufficient reporting of attrition/exclusions to permit judgement of "Low risk" or "High risk" (e.g., number randomized not stated, no reasons for missing data provided); the study did not address this outcome.
Selective reporting	
Low risk of bias	Any of the following: the study protocol is available and all of the study's pre-specified (primary and secondary) outcomes that are of interest in the review have been reported in the prespecified way; the study protocol is not available but it is clear that the published reports include all expected outcomes, including those that were pre-specified (convincing text of this nature may be uncommon).
High risk of bias	Any one of the following: not all of the study's prespecified primary outcomes have been reported; one or more primary outcomes is reported using measurements, analysis methods, or subsets of the data (e.g., subscales) that were not prespecified.
Unclear risk of bias	Insufficient information to permit judgement of "Low risk" or "High risk." It is likely that the majority of studies will fall into this category.

Table 2: Continued.

Other bias	
Low risk of bias	The study appears to be free of other sources of bias.
High risk of bias	There is at least one important risk of bias. For example, the study had a potential source of bias related to the specific study design used, or has been claimed to have been fraudulent; or had some other problem.
Unclear risk of bias	There may be a risk of bias, but there is either insufficient information to assess whether an important risk of bias exists or insufficient rationale or evidence that an identified problem will introduce bias.

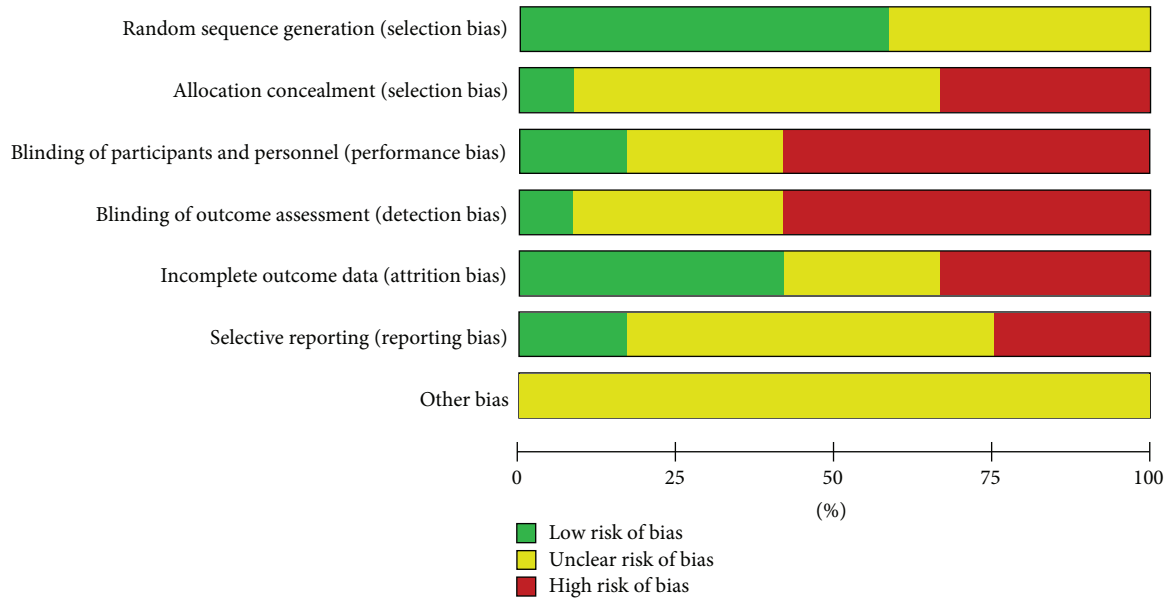

Figure 1: Risk of bias graph: review authors' judgments about each risk of bias item presented as percentages across all included studies.

5.0. Risk of bias was assessed independently by two review authors (Z.-Q. Wang and J.-L. Li) with the criteria in the Cochrane Handbook for Systematic Reviews of Interventions 5.1.0 [8]. Sequence generation, allocation concealment, blinding (or masking), incomplete data assessment, selective outcome reporting, and other sources of bias were assessed with three potential responses: yes, no, and unclear. Disagreements between review authors were resolved by discussion or with a third author (X.-J. Cui). The data extracted consisted of number of patients with lumbar spine and hip areal BMD during follow-up.

2.6. Measures of Treatment Effect. Statistical analysis was performed with RevMan 5.1 software. We expressed continuous data as weighted mean differences (WMD) with 95% CI, or as standardized weighted mean differences (SMD) if outcomes were conceptually the same but measured in different ways in the different trials. Statistical heterogeneity was investigated using the x2 test and I2 statistic (I2 represents the percentage of variability due to between-study variability). We tested heterogeneity among trial results using the I2 statistic. We considered a value greater than 50% as substantial heterogeneity. We calculated an estimate of the treatment effect across trials with the random-effect model if I2 >50%, and with the fixed-effect model if I2 >50%.

3. Results

3.1. Description of Included Studies. The search produced 131 trials from all of the databases searched. Among these 131 studies, 49 either did not include Chinese herb, or were not randomised controlled trials, or were reviews, or did not report original data. Through reading the full text, 56 studies' level of evidence was graded scores lower than 3 according to the Jadad quality score. At the end, 12 published RCTs were included. Table 3 shows baseline characteristics of the 12 studies.

3.2. Risk of Bias in Included Studies. The reports of all trials mentioned randomization, but only seven described the method of randomization [10–16]. In addition, the reports of seven trials mentioned double blinding [11–13, 15, 17–19], and the report of one trial mentioned single blinding in their methodological design [14]. We also grade all included studies by (Table 2) which was recommended by Cochrane Handbook 5.0. Figures 1 and 2 are made to show the results

Table 3: Characteristics of clinical trials of Chinese herbs for osteoporosis.

Trial year prevention/treatment (reference)	Number of patients (treatment/control)	Intervention	Duration	Outcomes measured
Dai and Shen 2007 [10]	107 versus 85	MGT 1.5 g/day\XLGB 1.5 g/day versus placebo, all: (calcium 1000 mg/day)	6 M	BMD: lumber spine and femoral neck
Leung et al. 2011 [17]	75 versus 75	ELP 2.28 g/day versus placebo	12 M	BMD: lumber spine and femoral neck
Wu et al. 2009 [20]	25 versus 25	XLGB 1.5 g/day versus Calcitriol 0.25 μg + calcium 700 mg/day + vitamin D400 IU/day	6 M	BMD: lumber spine and femoral neck
Zhang et al. 2005 [11]	67 versus 66 versus 60	YGC 120 g/day versus Calcitriol 0.25 μg versus placebo, all: (calcium 510 mg/day)	6 M	BMD: lumber spine and femoral neck
Ruan et al. 2006 [21]	48 versus 42	QGJN 0.75 g/day versus oral estradiol valerate 0.5–1.5 mg/day	6 M	BMD: lumber spine
Zhu et al. 2012 [12]	109 versus 61	XLGB 6 g/day versus placebo	12 M	BMD: lumber spine and femoral neck
Xiong et al. 2008 [13]	73 versus 35	JGKL 10 g/day versus placebo, all: (calcium 510 mg/day)	6 M	BMD: lumber spine and femoral neck
Zhou et al. 2009 [14]	355 versus 119	MGT 1.5 g/day versus XLGB 1.5 g/day	6 M	BMD: lumber spine and femoral
Liao et al. 2004 [15]	32 versus 34	BSSS soup 200 mL/day versus oral conjugated estrogen 0.5 mg/d and medroxyprogesterone 2.5 mg/d	6 M	BMD: lumber spine and femoral
Yang et al. 2007 [16]	120 versus 120	XLGB 1.5 g/day versus GKKFY 20 mL/day	6 M	BMD: lumber spine and femoral neck
Wang et al. 2006 [18]	105 versus 105	GSKC 2.56 g/day versus GSKT 20 g/day	6 M	BMD: femoral neck
Zheng et al. 2007 [19]	55 versus 54	JWGT 3 g/day versus placebo, all: (calcium 510 mg/day)	6 M	BMD: lumber spine and hip

MGT: Migu tablet; XLGB: Xian ling Gubao capsule; ELP: Bo-gu Ling capsules; YGC: Yigu capsule; QGJN: Qiang-Gu capsule; JGKL: Jiangu granule; BSSS: Bu Shen Sheng Sui soup; GKKFY: Gu Kang Oral liquid; GSKC: Gushukang capsule; GSKT: Gushukang granules; JWGT: Jinwugutong capsule; BMD: bone mineral density.

of the author's judgment about each methodological quality item for each included studies.

3.3. Effects of Interventions

3.3.1. Effects of Chinese Herbs versus Placebo on Spine BMD. 7 of all included RCTs studied effects of Chinese herbs versus placebo on lumbar spine areal BMD. The pooled data showed that the percent change of increased BMD in the spine is higher with Chinese herbs in comparison to treatment with placebo after 6 months (WMD = 0.07, 95% CI: 0.01–0.04, $n = 892$) (Figure 3).

3.3.2. Effects of Chinese Herbs versus Placebo on Femoral Neck BMD. 7 RCTs reported the femoral neck BMD. In the femoral, Chinese herb showed no significant increments of BMD compared to placebo (WMD = 0.06, 95% CI: −0.02–0.13, $n = 842$) (Figure 4).

3.3.3. Effects of Chinese Herbs versus Standard Antiosteoporotic Drugs on BMD. To compare the efficacy of the Chinese herbs with the standard anti-osteoporotic drugs, a subgroup analysis has been made. 2 of the included RCTs used calcitriol for subjects in the control groups, 2 used HRT treatment for subjects in the control groups and 4 of all included RCTs used another phytotherapy as the control groups. Chinese herbs also show advantage in lumber spine BMD change (WMD = 0.03, 95% CI: −0.01–0.08, $n = 1109$) (Figure 5). In this pooled analysis, the increases in the femoral neck BMD values were not significant between Chinese herbs and standard anti-osteoporotic drugs treatments (WMD = 0.01, 95% CI: −0.01–0.02, $n = 1076$) (Figure 6).

4. Discussion

4.1. Summary of Main Results. Although there are many reports in China endorsing the therapeutic value of various herbs originally used as "Kidney tonics" against "Kidney deficiencies," the claims have not been based on reliable clinical trials. This systematic review shows a trend for traditional Chinese medicine to increase spine BMD in Chinese patients with osteoporosis. Due to the data of 12 included trials (1816 patients involved), our analysis is strengthened with acceptable standard and methodology.

4.2. Overall Completeness and Applicability of Evidence. The reports of 4 of the 12 included trials provided data on the changes in lumbar and femoral BMD in anti-osteoporotic drugs treatment groups. The anti-osteoporotic drugs resulted in an increase of $0.0897 \pm 0.0241\ g/cm^2$ in lumber BMD and $0.0757 \pm 0.0167\ g/cm^2$ in femoral BMD compared with

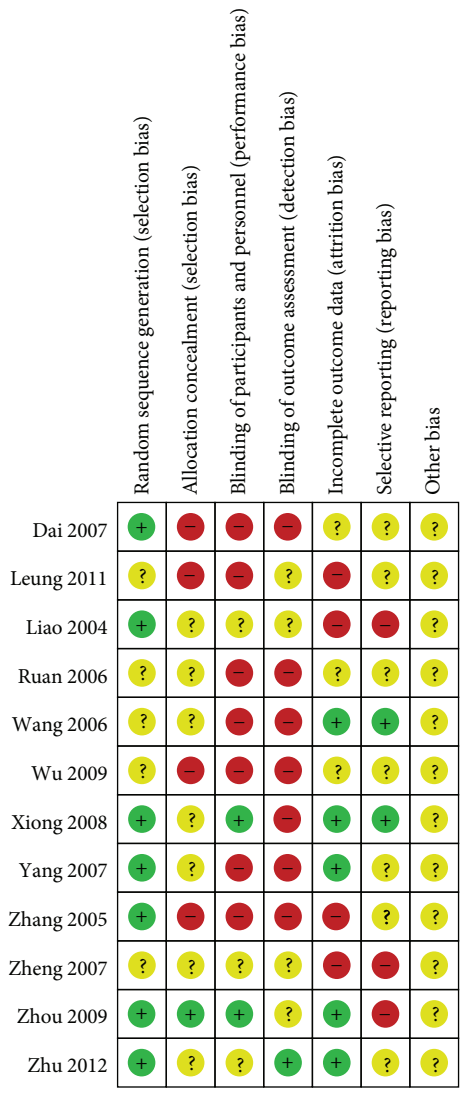

Figure 2: Risk of bias summary: review authors' judgments about each risk of bias item for each included study.

baseline values. The changes in BMD caused by the drugs in the included RCTs were in accordance with the findings of other RCTs conducted in different countries [19]. We systematically reviewed clinical trials and synthesized data from 12 trials in this study. The results showed similar pharmacological effects between Chinese herbs and standard anti-osteoporotic drugs in the regulation of bone turnover. The review indicated the potential of Chinese herbs for the treatment of osteoporosis. It is postulated that some effective components in these herbs were responsible for the anti-osteoporosis drugs like activities in clinical treatment. But there is still no specific target of pharmacological action to indicate that the efficacy would be inferior to target orientated pharmaceuticals. A longer period of observation and a large sample size might be mandatory for a more scientific revelation of the result of treatment.

4.3. Quality of the Evidence. The quality of the study designs and descriptions of the original trials are critical issues for meta-analyses. All of the 12 RCTs included in this study were assessed by the Jadad scale, and all the 12 studies were accepted as high quality trials. Although we included RCTs in high quality for data analysis in this study only, selection and detection biases would have existed if blinding had failed.

4.4. Potential Biases in the Review Process. Similar to other meta-analyses, our study has some limitations. First, the analysis is only based on published data, and no unpublished data are found. Second, differences in treatment length and design as well as in the severity of disease of the participants are also factors that potentially introduce difficulties in the analysis, as large differences in treatment effects can be expected. Moreover, the presented analysis was not designed to assess incident fractures. Finally, we suggest a longer duration of future studies as a 6-month study may not be long enough to reveal BMD changes, especially when the sample size is relatively small.

4.5. Potential Mechanism of Action. Chinese herbal medicine (CHM), a pharmaceutical part of TCM, has a long history of use, with extensive literature and clinical applications covering thousands of years. Chinese herbal formulae lack a well-defined mechanism of action. Plants are rich in a variety of compounds. It may therefore be necessary to identify the active ingredient(s) from a herbal extract for mechanistic investigations [22, 23]. Up till now, many active ingredients have been isolated from commonly used CHM [24].

The chemical composition of naturally grown herb may vary according to climatic conditions, harvest time, storage condition, and so on. These variabilities can result in significant differences in pharmacological activity, making standardization of botanical difficult [25]. At the present time, there are 15 major categories of active ingredients in CHM, including flavones, alkaloids, glucides, glycosides, volatile oils, resins, phytochromes, organic acids, amino acids, tannins, proteins, enzymes, trace elements, polysaccharides, and mineral salts [23].

Compounds that are identified by activity-guided fractionation must be tested in appropriate animal models to confirm in vivo activity [23, 26].

Curculigo orchioides (CO), which belongs to the Amaryllidaceae family, is mainly distributed in the subtropical regions of Asia, especially in southern China and India. Previous phytochemical investigations into rhizomes of this species revealed the presence of phenols and phenolic glycosides, including curculigoside, curculigoside B, curculigoside C, the triterpene saponins curculigosaponins A–M, and other compounds, including 1,3,7-trimethylxanthine, daucosterol, and aliphatic long-chain ketones [27, 28], of which phenols and phenolic glycosides have potential antioxidant activities [29, 30] and immunostimulatory effects [31, 32]. Administration of CO extract prevented bone loss in the trabecular bone of the tibia in ovariectomized rats without affecting the weight of the body and the uterus, increased serum phosphorus, calcium, and OPG levels, and decreased serum DPD/Cr, TRAP, ACTH, and corticosterone levels, but did not alter serum TNF-1, IL-6, and ALP levels in ovariectomized

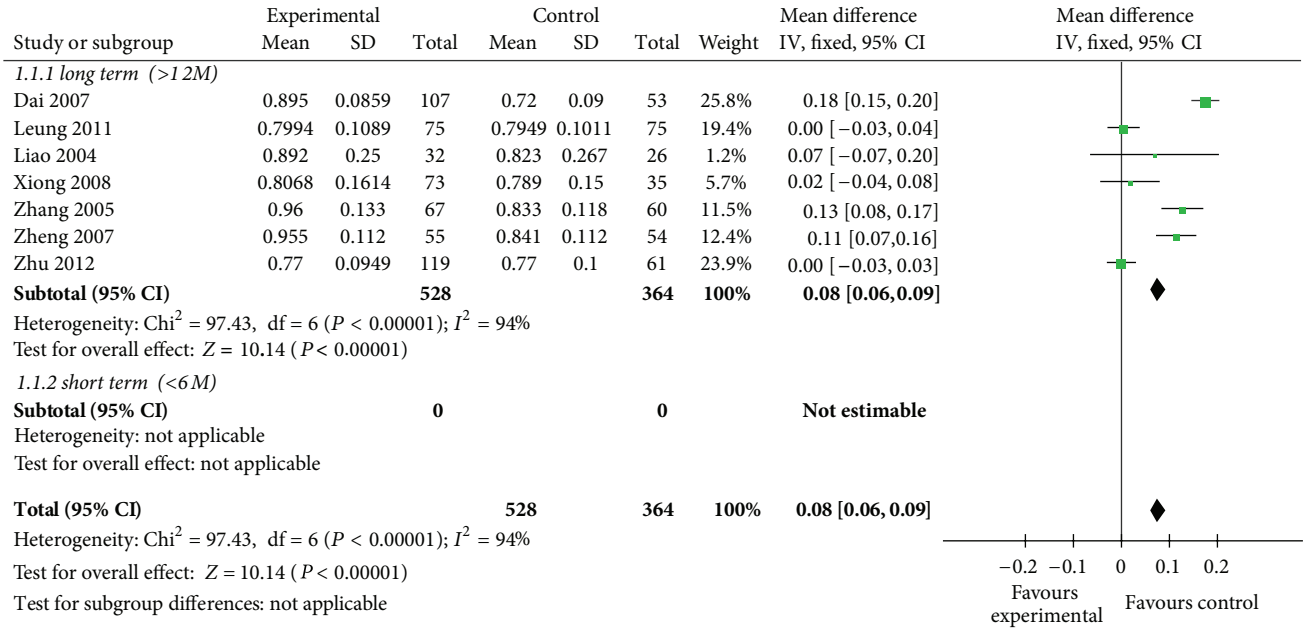

Figure 3: Chinese herbs versus placebo on spine BMD.

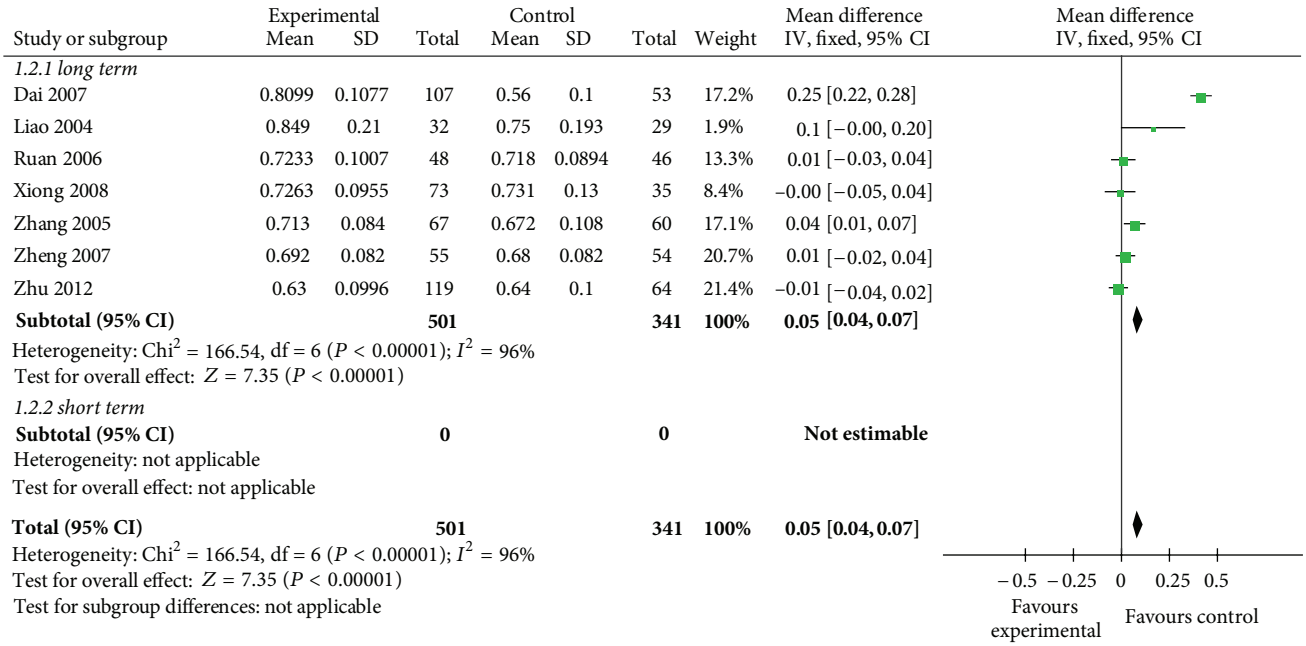

Figure 4: Chinese herbs versus placebo on femoral neck BMD.

rats. CO ethanol extract has a definite protective effect on bone loss in ovariectomized rats by inhibiting bone resorption and increasing serum phosphorus and calcium levels, without affecting bone formation [33]. In traditional Chinese medicine, CO rhizomes are considered to have the effects of maintaining healthy energy and nourishing the liver and kidneys [34, 35], and are thus widely used to treat diseases and disorders of bone metabolism. In most Chinese herbal formulae, they are also used to treat osteoporosis [36]. Herbaepimedii which belongs to the Berberidaceae family, contains a plenty of Isoflavone [37]. Isoflavone is also one of the determined substance in most of the recipes. Pharmacological studies, either on murine models of osteoporosis or in vitro, have provided some convincing evidence of positive effects of soya and isoflavones on bone health [38, 39]. Rutin, the glycosylated form of quercetin, is abundant in onion. It was proposed that at 200–600 mg/kg it was the pharmacologically active compound, although few studies

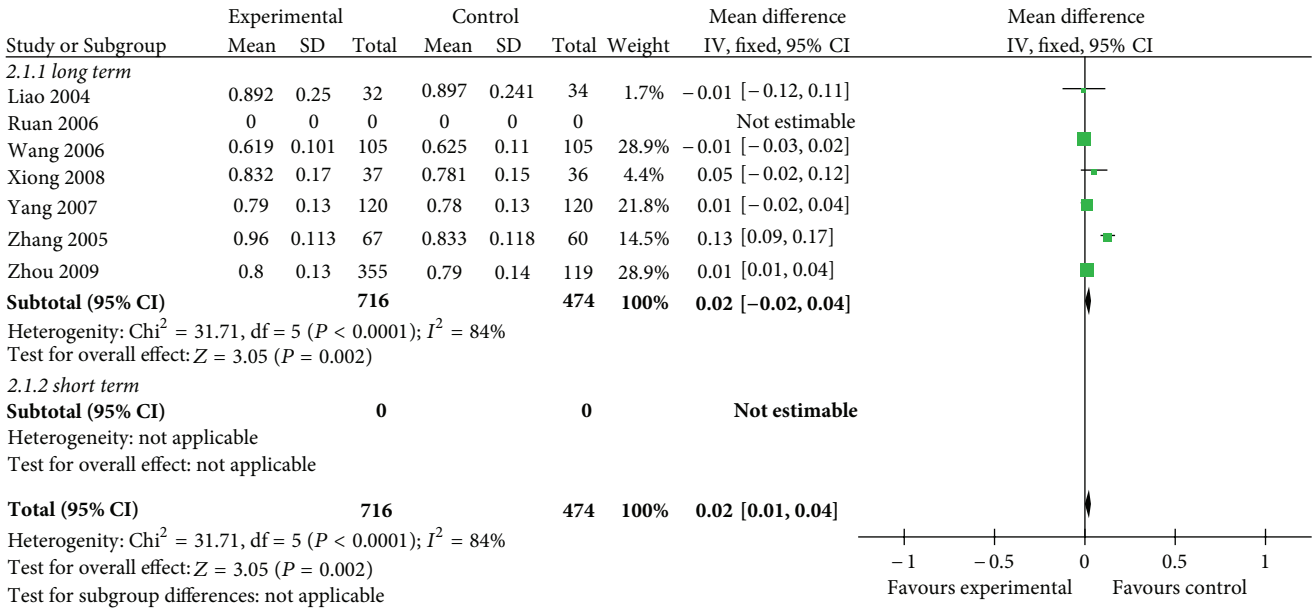

Figure 5: Chinese herbs versus standard anti-osteoporotic drugs on lumber spine BMD.

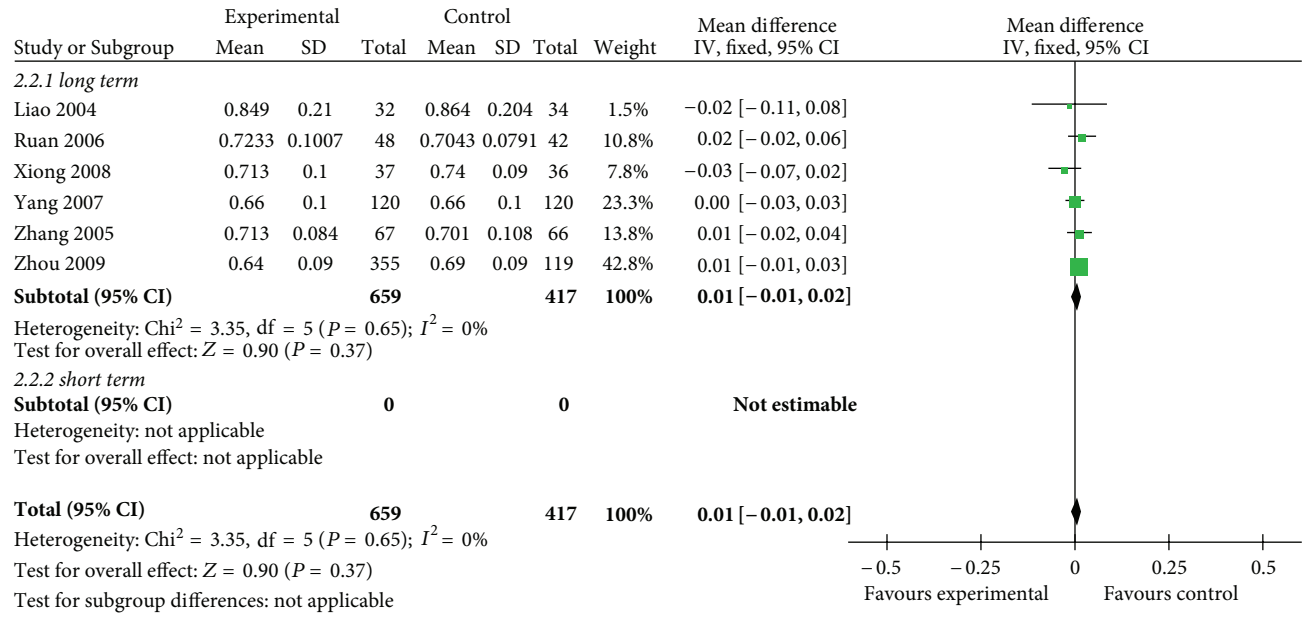

Figure 6: Chinese herbs versus standard anti-osteoporotic drugs on the femoral neck BMD.

have confirmed this. The vitro trails showed that rutin consumption increased femoral strength and trabecular bone density by decreasing bone resorption, although cortical bone density was unchanged. Rats supplemented with rutin also had higher plasma osteocalcin concentrations, indicating an increase in bone formation [40]. Cistanche salsa or the Chineses name of Rou Cong Rong, has been shown to have antiosteoporotic effects in ovariectomized mice. It has been reported by Yamaguchi et al. [41] that 16 mg/day of C. salsa significantly suppressed the femoral bone weight loss caused by ovariectomy; in fact femoral weight increased to 109% of the control in this study. Subsequently, the active compound was isolated and the structure elucidated as (2E,6R)-8-hydroxy-2,6-dimethyl-2-octenoic acid, a novel monoterpene which was found to be antiosteoporotic. This compound significantly suppressed femoral bone loss in ovariectomized mice at a concentration of 1.6–8 μg/kg. It was concluded that the mechanism of action was different to that

of antiosteoporotic agents such as 17β-estradiol since uterine weight was not affected. Drynariae rhizoma is a major component in 56 of 73 fracture prescriptions in traditional Chinese and is also used in Korean medicine. Its effects on protease activity involved in the initiation of bone loss in rats and mice were tested [42]. Both ethanol and aqueous extracts were potent inhibitors of cathepsins K and L, which denature the collagen in bone, with the ethanol extract being more potent. Later a study showed the beneficial effects of D. rhizoma on the proliferation of human bone cells, and immunomodulatory activity in vitro [43]. Human osteoprecursor cells (OPC-1) were cultured with differing concentrations of D. rhizoma and their proliferation was studied. Concentration of $\leq$120 μg/mL enhanced proliferation, whereas >250 μg/mL was inhibitory. Recent in vivo work has confirmed these results [44]. A study investigated Puerariae radix effects on bone loss in castrated mice, as it had previously been shown to exhibit an effect in ovariectomized mice. Male mice were given a dose of either different concentrations in the diet, and another group were given 17β-estradiol at a dose of 0.03 μg/day for comparison. It was shown that even at low doses. Puerariae radix reversed the bone loss induced by castration, with femur BMD and trabecular number increasing, and trabecular separation decreasing [45]. Onobrychis ebenoides is well known as having oestrogenic activity in vitro. This, along with its bone-sparing effect, was described in a study. OVX rats were given 300 mg/kg body weight of extract of OE every day for 6 months which resulted in an increase of tibial BMD from that of the OVX control [46]. Du-Zhong cortex extract, which is rich in polyphenolic compounds such as lignans, phenolic acid, and flavonoids. Dose-dependently inhibited total BMD decrease in the femur caused by OVX, which was accompanied by a significant decrease in skeletal remodeling, as was evidenced by the decreased levels of the bone turnover markers osteocalcin (OC), alkaline phosphatase (ALP), deoxypyridinoline (DPD), and urinary Ca and P excretions. Analysis of the femoral metaphysis showed that DZCE at the highest doses (500 mg/kg/day) significantly prevents decrease in bone volume/tissue volume (BV/TV), connect density (Conn.D), trabecula number (Tb.N), and trabecula thickness (Tb.Th), and increase in trabecula separation (Tb.Sp) and structure model index (SMI) in OVX rats [47]. Treatment of OVX rats with Fructus Ligustri Lucidi extract could prevent OVX-induced increase in bone turnover by suppression of both serum osteocalcin and urinary deoxypyridinoline levels. In addition, Fructus Ligustri Lucidi extract could prevent OVX-induced loss of calcium in rats by increasing the intestinal calcium absorption rate, suppressing urinary Ca excretion as well as increasing bone calcium content [48]. Obvious separation trend between control and model group was found in principal component analysis score plot, the anti-osteoporosis effect of Rhizoma Drynariae can be indicated in partial least squares discriminant analysis score plot among these three groups. Six potential metabolite biomarkers, lysophosphatidylcholines, tryptophane, and phenylalanine, which were proved to be related with osteoporosis, were identified in the rats plasma. Compared with control group, level of all biomarkers increased significantly in model group, while that was much closer to normal in treatment group [49]. Achyranthes bidentata Blume is rich in active phytochemical compounds such as saponins, ketosteroids and flavonoids. The study demonstrated that five new oleanolic acid glycosides from Achyranthes bidentata could inhibit the formation of osteoclast, ecdysterone from Achyranthes bidentata increased osteoblastic activity, and flavonoid quercetin decreased osteoclastic differentiation. 16 weeks treatment of Achyranthes bidentata root extract slowed down the body weight gain and prevented the loss of bone mass induced by the OVX. The prevention effect on bone loss was due to altering the rate of bone remodeling, which could be inferred from the decreased level of bone turnover markers, such as serum alkaline phosphatase (ALP), osteocalcin (OC), and urinary deoxypyridinoline (DPD). The changes of urinary calcium and phosphorus excretion provided the same evidence. The treatment could also enhance the bone strength and prevent the deterioration of trabecular microarchitecture [50]. Cibotium barometz is a kind of usual herbs to treat osteoporosis, its extract prevented total BMD decrease in the femur induced by OVX, which was accompanied by a significant decrease in skeletal remodeling, as was evidenced by the decreased levels of the bone turnover markers, such as osteocalcin (OC), alkaline phosphatase (ALP), deoxypyridinoline (DPD), and urinary Ca and P excretions. The treatment could also enhance the bone strength and prevent the deterioration of trabecular microarchitecture [51].

5. Conclusions

We conclude that Chinese herbs substantially increased BMD of the lumbar spine compared to placebo or anti-osteoporotic drugs as indicated in the current clinical reports on osteoporosis treatment. Long term of Chinese herbs over 12 months of treatment duration may increase BMD in the hip more effectively. However, further studies are needed to corroborate the positive effect of increasing the duration of Chinese herbs on outcome as the results in this analysis are based on indirect comparisons. To date there are no studies available that compare Chinese herbs, Chinese herbs plus anti-osteoporotic drugs, and anti-osteoporotic drug versus placebo in a factorial design. Consequently, we are unable to draw any conclusions on the possible superiority of Chinese herbs plus anti-osteoporotic drug versus anti-osteoporotic drug or Chinese herb alone in the context of BMD.

Conflict of Interests

The authors declare that they have no conflict of interests.

Acknowledgments

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Review Article

Vitamin E and the Healing of Bone Fracture: The Current State of Evidence

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Background. The effect of vitamin E on health-related conditions has been extensively researched, with varied results. However, to date, there was no published review of the effect of vitamin E on bone fracture healing. *Purpose.* This paper systematically audited past studies of the effect of vitamin E on bone fracture healing. *Methods.* Related articles were identified from Medline, CINAHL, and Scopus databases. Screenings were performed based on the criteria that the study must be an original study that investigated the independent effect of vitamin E on bone fracture healing. Data were extracted using standardised forms, followed by evaluation of quality of reporting using ARRIVE Guidelines, plus recalculation procedure for the effect size and statistical power of the results. *Results.* Six animal studies fulfilled the selection criteria. The study methods were heterogeneous with mediocre reporting quality and focused on the antioxidant-related mechanism of vitamin E. The metasynthesis showed α-tocopherol may have a significant effect on bone formation during the normal bone remodeling phase of secondary bone healing. *Conclusion.* In general, the effect of vitamin E on bone fracture healing remained inconclusive due to the small number of heterogeneous and mediocre studies included in this paper.

1. Introduction

According to Castellini et al., vitamin E has been regarded as "the most potent, lipid-soluble, chain-breaking antioxidant in nature" [1]. There are eight distinct, natural forms of vitamin E: four tocopherol isomers (α, β, γ, and δ isomers) and four tocotrienol isomers (α, β, γ, and δ isomers) [2]. Of all these isomers, α-tocopherol has been the most studied because it has the highest bioavailability in human tissues [3]. It is seen as an antioxidant that is required for the protection of cell membranes, in which it reacts with free radicals (i.e., peroxyl and alkoxyl radicals) to preserve polyunsaturated fatty acids in the membrane structure [4]. Besides that, α-tocopherol has also been investigated for its role in the activation of several genes [5].

Nevertheless, there is a dearth of investigation on the biological activity of other isomers of vitamin E, especially in humans. Moreover, the studies of the effect of vitamin E on health-related conditions have been vast and with varied results [2]. Recently, a new controversy has entered into the picture, whereby α-tocopherol was purported to decrease bone mass, through the simulation of osteoclast fusion [6]. This study has prompted us to reexamine the relationship between vitamin E and bone-related conditions. Our focus is on the effect of vitamin E on bone fracture healing. Based upon the initial background search on the issue at hand, the most popular proposed mechanism of action for the effect of vitamin E on bone fracture healing is based upon the cellular-protective property of antioxidant. In bone fracture, oxygen free radicals are produced by the activation of polymorphonuclear neutrophils in the inflammatory phase of bone fracture healing [7], as well as by the impairment of blood supply to the bone ends [8]. These free radicals have been shown to inhibit bone fracture healing [9, 10] by initiating a chain reaction that will cause lipid peroxidation that leads to cell membrane damage and eventually cell lysis [7]. The protective role of vitamin E in this situation may be seen in past studies, where α-tocopherol in the cell membranes was shown to act as an antioxidant that inhibits lipid peroxidation by scavenging the free radicals and thus

breaks the chain reaction [11, 12]. However, there was at least one study that reported α-tocopherol did not have any effects on the lipid peroxidation and antioxidant enzyme activities [13].

Preliminary search in the major databases (MEDLINE, CINAHL, and Scopus) in early March 2012 yielded no result for any narrative reviews, systematic reviews, or meta-analyses of the effect of vitamin E on bone fracture healing. Hence, a proper systematic review will provide a critical evaluation of the current state of evidence for this topic. Additionally, this paper will also serve as a methodological guideline for other researchers who would like to pursue this line of investigation. Generally written in a methodological auditing manner, this systematic review has attempted to fulfil the following seven specific objectives:

(i) brief description of general study designs of past studies of the effect of vitamin E on bone fracture healing;

(ii) brief description of the results from past studies of the effect of vitamin E on bone fracture healing;

(iii) brief description of the suggested mechanism of action for the effect of vitamin E on bone fracture healing in past studies;

(iv) evaluation of the quality of the reported methods and results from past studies of the effect of vitamin E on bone fracture healing;

(v) evaluation of the effect size and statistical power of the effect of vitamin E on bone fracture healing in past studies;

(vi) calculation of optimum sample size for replication of past studies of the effect of vitamin E on bone fracture healing;

(vii) metasynthesis of specific patterns about the effect of vitamin E on bone fracture healing.

2. Methods

In line with the current emphasis on the reduction of bias in concluding the evidence in studies [14], the recommended steps for a systematic review were followed, with a few minor adjustments. An extra step was added in this paper, whereby the data in the included studies were reanalysed, if necessary and possible. This is to provide a better picture of the conclusion derived from the studies.

2.1. Study Selection. A systematic electronic search was conducted in three databases (MEDLINE, CINAHL, and Scopus), encompassing a publication timeline from 1946 to the end of March 2012. The search strategy included the following terms and Boolean operators: (1) vitamin E OR Vit* E OR tocotrienol OR tocopherol; (2) fracture* OR bone* healing; (3) 1 and 2 (all fields; limit to English language only). The search strategy was created by a panel of two researchers with mutual consensus. Its focus was more on sensitivity, rather than specificity, in order to identify as many articles as possible, regardless of the type of study. Due to the lack of resources, there was no effort to include non-English language studies, or to attempt hand-searching the related abstracts. Via this strategy, a total of 109 individual hits were identified (Figure 1). All abstracts from the hits were then downloaded, while 18 duplicates were removed in the process. The pooled 91 abstracts were then screened using a standardised electronic form by two researchers independently. At this stage, abstracts were included in the review based upon these following criteria: (a) the study reported the effect or association between vitamin E isomer and the healing of bone fracture-related conditions; (b) the fracture-related condition was related to lifestyle variables, aging or experimentally-induced conditions. At the same time, abstracts were excluded from the review, if (a) the study was not an original study (e.g., review, letter, or editorial), or (b) the study was related to bone fracture secondary to other pathologies (besides the ones stated in the inclusion criteria). Ambiguous abstracts were excluded only after their full papers were traced and screened in the next phase. Any discrepancies in the results of the screening were resolved through a consensus process. The abstract screening yielded 11 abstracts that fulfilled the selection criteria. This was followed by tracing the full articles for each abstract electronically and manually. These articles were then subjected to the final round of screening by two researchers using a standardised electronic form independently. In this phase, the study would be rejected from the review, if the effect of the vitamin E on bone fracture healing could not be assessed independently (i.e., the vitamin E was mixed with other active compounds). Any disagreements in the screening results were settled by mutual consensus. Via this process, only six full articles were finally included in the review, of which all were animal studies. Due to the small number of the pooled full articles, all studies identified in this phase were included in this review, regardless of their quality of evidence. Nevertheless, to reduce the chance of making a flawed conclusion, the quality of each article was evaluated as a part of the data extraction process.

2.2. Data Extraction. Data extraction procedure was performed by a single researcher, followed by foolproofing by another researcher. Data extraction for each included studies was performed using three different electronic forms—the study design form, the study results form, and the study report quality form. In the study design form, the following information was recorded: (a) sample characteristics; (b) affected bone(s); (c) fracture production procedure; (d) fracture type; (e) fracture fixation/distraction procedure; (f) dose of vitamin E isomer(s) used in the treatment group; (g) duration of treatment; (h) intervention in the control and other comparison group(s); (i) main outcome measure(s). On the other hand, the study results form required the researcher to record the following information: (a) reported descriptive values of main outcome measure(s); (b) reported inferential statistical analysis; (c) result summary; (d) model proposed for the mechanism of action of vitamin E in the study; (e) conclusion of the effect of vitamin E on the phase of fracture healing reported in the study. Finally, the quality of the study report was assessed using a checklist based upon

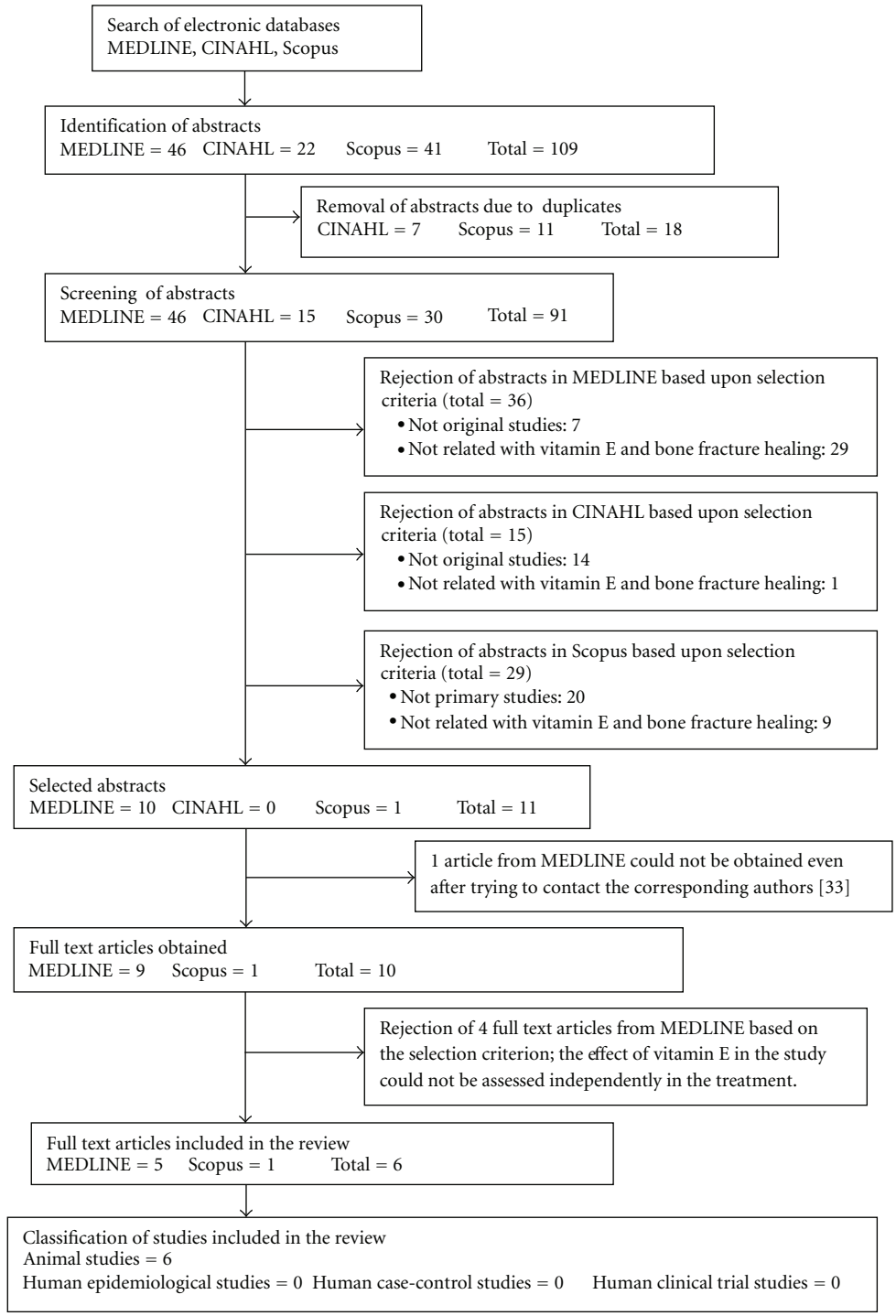

Figure 1: Flowchart to show the selection process of the articles in this review.

the Animal in Research: Reporting In Vivo Experiments (ARRIVE) Guidelines. For this paper, only 22 individual items in Section 2 (item 5 through item 13c) and 6 items in Section 3 (item 14 through item 17b) were used. The ARRIVE Guidelines were specifically developed to provide an outline to optimise the information provided in publications of animal studies, through high-quality and comprehensive reporting [26].

2.3. Reanalysis of Study Results. To reduce the element of subjective judgement in drawing the conclusion, an extra phase was included in the review process. In this phase, the reported results focusing on the comparison of outcome parameters between the vitamin E treatment group and the control group would undergo statistical reevaluation by an assigned researcher and later would be confirmed by another researcher. Using an electronic form, specific

commentaries were given on each relevant statistical analysis reported in the articles. This was followed by a reanalysis of the relevant reported data, as well as the calculation of effect size, statistical power, and sample size for replication studies. A reanalysis of the data was performed using the software Statistical Package for the Social Sciences (SPSS) Version 16.0. In this paper, this had been done by reanalysing the cross-tabulated count-based outcomes reported in the articles via Mann-Whitney *U* test, when it was necessary and possible. Meanwhile, the effect size, statistical power, and sample size calculation were performed using G* Power Version 3.1.2. These calculations were only performed if the mean and standard deviations of the outcome parameters were available, either in the report or through the reanalysis procedure. The standardised effect size (Cohen's *d*) and statistical power calculations were performed based on the alpha value set at 0.05 (two tailed) with minimum asymptomatic relative efficiency (ARE) distribution (i.e., a type of distribution for nonparametric test) [27]. The sample size calculation for replication study was also performed based on the previous alpha value and type of distribution, as well as the statistical power set at 0.80.

3. Results

3.1. Brief Description of General Study Designs of Past Studies of the Effect of Vitamin E on Bone Fracture Healing. One of the most prominent findings in this systematic review was the absence of any published human studies, neither epidemiological observation, case-control study, nor clinical trial, on the association or effect of vitamin E on bone fracture healing. Besides that, another salient observation was the extreme heterogeneity of the study methods employed in the articles included in this paper. Of the six experimental-based animal studies published (Table 1), three studies were based upon the rat model [18, 21, 24], two were based upon the rabbit model [15, 23], and only one was based upon the dog model [8]. The bone model being investigated in the studies was primarily normal bone, except one study that focused on osteoporotic bone model [18]. One of three types of fracture production procedure was generally used in these studies: osteotomy [8, 15], manual fracture [21, 23, 24], or 3-point bending method using a guillotine device [18]. Nearly all of the studies were based upon a secondary bone healing model promoted by either internal fixation using Kirschner wires [18, 21], external fixation [15, 23], or left *in situ* [24]. Only one study was based upon primary bone healing model promoted by internal fixation using plates [8]. To date, there was no published study of the effect of tocotrienol analog on bone fracture healing. All these studies used α-tocopherol isomer for their treatment group, with the dose ranging from 20 to 100 mg/kg/day for the duration between 5 to 60 days. Only one study administered the α-tocopherol treatment for a short duration, before stopping it while the experiment was still going on [24]. Other studies administered the α-tocopherol treatment continuously until the end of their experiments. The α-tocopherol treatment group would generally be compared to a control group that received no treatment at all or only received normal saline/vehicle. Only two studies deviated from this general design. One study observed the effect of the treatment of α-tocopherol on osteoporotic bone fracture compared to normal bone and osteoporotic bone control groups [18]. The other study compared the α-tocopherol treatment group against a control group, a vitamin C treatment group and a combination treatment (α-tocopherol and vitamin C) group [24]. Besides that, there was also heterogeneity in the main outcome measures of each study. These outcomes may be clustered into five major groupings: (a) radiological-based assessment of bone formation [8, 15, 18, 21]; (b) histological-based assessment of bone formation [15, 21, 23, 24]; (c) radiological-based assessment of callous volume/index [18, 24]; (d) radiological-based assessment of callous formation [8, 18]; (e) osteoblastic activity assessment [15].

3.2. Brief Description of the Results from Past Studies of the Effect of Vitamin E on Bone Fracture Healing. Based on Tables 2 and 4, nearly all the studies reported their inferential results based on nonparametric statistical analyses, using either Mann-Whitney *U* test, Kruskal-Wallis test, or chi-square test. There was only one study that did not report the results of the statistical analysis, although the overall conclusion of the α-tocopherol treatment effect was reported [8]. Disregarding the heterogeneity of the studies, a significant superior effect of the α-tocopherol treatment group compared to the control was seen in the radiological-based bone formation parameter [8, 15, 18, 21] and in the histological-based bone formation parameter [15, 21, 23]. In terms of the assessment of callous-related parameters, most of the related studies showed no significant difference between the α-tocopherol treatment group compared to the control group [18, 24], except in one study [8]. A significant superior effect of the α-tocopherol treatment group compared to the control group was also seen in the osteoblastic activity parameter in one study [15]. However, one study [24] demonstrated that there was a significant inferior effect of the α-tocopherol-only treatment group compared to the vitamin C-only treatment group, in terms of the bone formation parameters and callous-related parameters. In the same study, the α-tocopherol-only treatment group was also significantly inferior to the vitamin C and α-tocopherol combination group, which in turn was significantly inferior to the vitamin C-only group, in terms of the aforementioned parameters.

At face value, it is tempting to generalise that most of the evidence showed that α-tocopherol is beneficial for bone fracture healing. However, this would not take into account the quality and the weight of evidence in each study. A better look at these issues would be done later on in this section. Additionally, the significant inferior results of α-tocopherol when compared to vitamin C, as well as the combination of vitamin C and α-tocopherol, will only be touched in Section 4.

3.3. Brief Description of the Suggested Mechanism of Action for the Effect of Vitamin E on Bone Fracture Healing in Past Studies. Out of the six studies included in this paper, only four studies reported the testing of their proposed mechanism of action for vitamin E effect on bone fracture

Table 1: Evidence table for past animal studies of the effect of vitamin E on bone fracture healing.

Study	Sample (size of study groups)	Affected bone(s)	Fracture production	Fracture type	Fracture fixation/distraction	Dose of vitamin E analog used in treatment group	Study design Duration of treatment	Intervention in control and other comparison group(s)	Main outcome measure(s)
Kurklu et al. [15]	Adult New Zealand white rabbits (control = 15; treatment = 15)	Middle third of right tibia (normal bone model)	Osteotomy	Open fracture	Distraction using circular external fixator	20 mg/kg/day α-tocopherol (intramuscular)	30 days	Control group did not receive any treatment.	Radiological evaluation of bone formation (based upon the grading system by Lane and Sandhu [16]) on day 20, day 30, and day 40 of study Scintigraphical assessments to calculate the osteoblastic activity ratio (distracted tibia/contralateral normal tibia) on day 5 and day 20 of study Histopathological evaluation of bone formation of the distracted tibial segments (based upon the grading system by Huddleston et al. [17]) on day 40 of study
Shuid et al. [18]	Female Sprague-Dawley rats (sham = 8; control = 8; treatment = 8)	Mid-diaphysis of right femur (osteoporotic bone model)	Three-point bending method using a guillotine device	Closed fracture	Internal fixation using Kirschner wires	60 mg/kg/day of α-tocopherol (per oral gavage) (ovariectomized treatment group)	14 days	Sham-operated and ovariectomized control groups received vehicle only (oral gavage)	CT-scan measurement of axial callous volume of the dissected femora after day 14 of study Radiological evaluation of callous staging (based upon the grading system by Shuid et al. [19]) of the dissected femora after day 14 of study Radiological evaluation of fracture healing (based upon grading system by Warden et al. [20]) of the dissected femora after day 14 of study
Paskalev et al. [8]	Mixed-breed adult male dogs (control = 6; treatment = 6)	Diaphyses of right tibia and fibula (normal bone model)	Osteotomy	Open fracture	Internal fixation using plates	100 mg/day α-tocopherol (per oral)	30 days	Control group did not receive any treatment	Radiological evaluation of callous formation and bone remodeling staging of the operated limb on day 1, week 2, month 1, month 2, month 3, and month 4 after the surgery (the grading system was not stated)
Turk et al. [21]	Male Sprague-Dawley rats (control = 30; treatment = 30)	Right tibia (normal bone model)	Unspecified manual fracture procedure	Closed fracture	Internal fixation using Kirschner wires	20 mg/kg/day α-tocopherol (intraperitoneal)	60 days	Control groups received saline (intraperitoneal)	Radiological evaluation of the bone formation (based upon the grading system by Lane and Sandhu [16]) of the right tibia on day 60 of study Histopathological evaluation of the fracture healing (based upon the grading system by Allen et al. [22]) of the dissected right tibia after day 60 of study

Table 1: Continued.

Study	Sample (size of study groups)	Affected bone(s)	Fracture production	Fracture type	Fracture fixation/distraction	Dose of vitamin E analog used in treatment group	Study design Duration of treatment	Intervention in control and other comparison group(s)	Main outcome measure(s)
Durak et al. [23]	New Zealand male white rabbits (Control = 10; Treatment = 10)	Right femur (Normal bone model)	Manual stress angulation	Closed fracture	External fixation using cast immobilization.	20 mg/kg α-tocopherol (intramuscular) 1 hour before and at the time of the procedure. 20 mg/kg/day α-tocopherol (intramuscular) after the procedure	5 days	Control group did not receive any treatment.	Histopathological evaluation of the fracture healing (based upon the grading system by Allen et al. [22]) of the dissected right tibia after day 21 of study.
Sarisözen et al. [24]	Adult Sprague-Dawley rats (control = 12; Vit. E only treatment = 12; Vit. C only treatment = 12; Vit. E and Vit. C combination treatment = 12)	Right femur (normal bone model)	Manual stress angulation	Closed fracture	Fracture left *in situ*	40 mg/kg α-tocopherol (intraperitoneal) daily from day 1 to day 3 and three times a week from day 4 onward	14 days 21 days	Control group did not receive any treatment Vit. C only group received 200 mg/kg vit. C daily (intraperitoneal) from day 1 to day 3 and three times a week from day 4 onward Vit. C and Vit. E combination group received 40 mg/kg vit. E and 200 mg/kg Vit. C daily (intraperitoneal) from day 1 to day 3 and three times a week from day 4 onward	Radiological evaluation of fracture healing (based upon the callous index calculation by Oni et al. [25]) of the right tibias from the rats sacrificed on day 14 and 21 Histopathological evaluation of fracture healing (based upon the grading system by Allen et al. [22]) of the dissected right tibias from the rats sacrificed on day 14 and 21

Table 2: Results of past animal studies on the effect of vitamin E on healing of bone fracture.

Study	Reported results				
	Reported descriptive values of main outcome measure(s)	Reported inferential statistical analysis	Reported conclusion	Model proposed for mechanism of action of vitamin E in study	Conclusion of effect of vitamin E based on the phase of fracture healing examined in study
Kurklu et al. [15]	(A) *Radiological-based grade of bone formation* [16]				
	(1) Day 20 Control group ($n = 15$): mean = 1.46, s.d. = 0.51 Treatment group ($n = 15$): mean = 1.73, s.d. = 0.45	Mann-Whitney U test: $P > 0.05$	The radiological-based bone formation grade of the treatment group was significantly higher than the control group on day 30 and day 40. There was no significant difference on day 20	The positive effect of antioxidant property of α-tocopherol (measured based on Total Antioxidant Capacity level) on secondary bone healing (intramembranous ossification) in normal bone	Favourable effect seen during ischemic phase (day 30), but not inflammatory phase of healing (day 5) in rabbits The mechanism model was reported to be supported by the results in the study
	(2) Day 30 Control group: mean ($n = 15$) = 3.46, s.d. = 0.83 Treatment group: mean ($n = 15$) = 4.60, s.d. = 0.91	Mann-Whitney U test: $P < 0.01$			
	(3) Day 40 Control group: mean ($n = 15$) = 5.46, s.d. = 0.83 Treatment group: mean ($n = 15$) = 7.73, s.d. = 1.09	Mann-Whitney U test: $P < 0.01$			
	(B) *Ratio of osteoblastic activity (distracted/normal)*				
	(1) Day 5 Control group: mean ($n = 15$) = 0.97, s.d. = 0.18 Treatment group: mean ($n = 15$) = 1.06, s.d. = 0.20	Mann-Whitney U test: $P > 0.05$	The osteoblastic activity of the treatment group was significantly higher than the control group on day 20. There was no significant difference on day 5		
	(2) Day 20 Control group: mean ($n = 15$) = 1.65, s.d. = 0.54 Treatment group: mean ($n = 15$) = 3.63, s.d. = 1.06	Mann-Whitney U test: $P < 0.01$			
	(C) *Histological-based grade of bone formation* [17]				
	(1) Day 40 Control group: mean ($n = 15$) = 8.00, s.d. = 0.92 Treatment group: mean ($n = 15$) = 9.86, s.d. = 0.35	Mann-Whitney U test: $P < 0.01$	The histological-based bone formation grade of the treatment group was significantly higher than the control group and showed mature bone formation		

Table 2: Continued.

Study	Reported results		Reported conclusion	Model proposed for mechanism of action of vitamin E in study	Conclusion of effect of vitamin E based on the phase of fracture healing examined in study
	Reported descriptive values of main outcome measure(s)	Reported inferential statistical analysis			
Shuid et al. (2011) [18]	(A) *Callous volume* (1) Day 14 sham group ($n = 8$): mean = ±0.15, variability = ? (graph form only) Ovariectomized control group ($n = 8$): mean = ±0.17, variability = ? (graph form only) Ovariectomized treatment group ($n = 8$): mean = ±0.20, variability = ? (graph form only)	$P > 0.05$ (however, it is not known whether ANOVA or Kruskal-Wallis tests was used)	There was no significant difference in the callous volume between the three groups	The boosting-up effect of α-tocopherol on antioxidant enzymes activities (measured based on Bone Superoxide Dismutase and Bone Glutathione Peroxidase levels) on secondary bone healing in osteoporotic bone	Favourable effect seen during early phase of healing (day 14) in rats The mechanism model was reported to be supported by the results in the study (only for bone superoxide dismutase)
	(B) *Radiological-based score of callous staging* [19] (1) Day 14 Sham group ($n = 8$): Score 1 ($n = 1$), Score 2 ($n = 5$), Score 3 ($n = 2$) Ovariectomized control group ($n = 8$): Score 2 ($n = 2$), Score 3 ($n = 4$), Score 4 ($n = 2$) Ovariectomized treatment group ($n = 8$): Score 2 ($n = 2$), Score 3 ($n = 3$), Score 4 ($n = 3$)	Pearson chi-square test: $P > 0.05$	There was no significant difference of callous staging grades between the three groups		
	(C) *Radiological-based score of fracture healing staging* [20] (1) Day 14 Sham-operated group ($n = 8$): Score 2 ($n = 1$), Score 3 ($n = 7$) Ovariectomized control group ($n = 8$): Score 2 ($n = 5$), Score 3 ($n = 3$) Ovariectomized treatment group ($n = 8$): Score 2 ($n = 1$), Score 3 ($n = 7$)	Pearson chi-square test: $P < 0.05$ (difference between ovariectomized control group against sham-operated and ovariectomized treatment groups in terms of score)	Ovariectomized control group has significantly more Score 2 and less Score 3 of the fracture healing grade than both sham-operated and ovariectomized treatment group The fracture healing score of the ovariectomized treatment group was similar with the sham-operated group		

Table 2: Continued.

Study	Reported results		Reported conclusion	Model proposed for mechanism of action of vitamin E in study	Conclusion of effect of vitamin E based on the phase of fracture healing examined in study
	Reported descriptive values of main outcome measure(s)	Reported inferential statistical analysis			
Paskalev et al. [8]	(A) *Radiological-based evaluation of callous formation staging (unspecified)* (1) Day 1, week 2, month 1, month 2, month 3, and month 4 Control group ($n = 6$): no descriptive value reported Treatment group ($n = 6$): no descriptive value reported	No statistical analysis was reported	It was stated that callous formation stage is better in the treatment group compared to the control one	The boosting-up effect of α-tocopherol on antioxidant enzymes activities (measured based on Serum Catalase level) on primary bone healing in normal bone	Favourable effect of α-tocopherol given for 30 days postosteotomy seen from week 1 to the end of the third month of the study (i.e., from early to the late phase of healing) in dogs The mechanism model was reported to be supported by the results in the study
	(B) *Radiological-based evaluation of bone remodelling staging (unspecified)* (1) Day 1, week 2, month 1, month 2, month 3, and month 4 Control group ($n = 6$): no descriptive value reported Treatment group ($n = 6$): no descriptive value reported	No statistical analysis was reported	It was stated that bone remodeling stage is better in the treatment group compared to the control		
Turk et al. [21]	(A) *Radiological-based grade of bone formation* [16] (1) Day 60: Control group ($n = 10$): Grade 3 ($n = 9$), Grade 4 ($n = 1$) Treatment group ($n = 10$) = Grade 3 ($n = 1$), Grade 4 ($n = 9$)	Pearson chi-square test: $P < 0.05$ (difference between control group and treatment group in terms of Grade 3 and Grade 4)	The radiological-based bone formation grade was significantly superior in the treatment group compared to the control group	The positive effect of antioxidant property of α-tocopherol (measured based on Erythrocyte Malondialdehyde level) on secondary bone healing in normal bone	Favourable effect seen during early phase (day 15) up to late phase (day 60) of healing in rats The mechanism model was reported to be supported by the results in the study
	(B) *Histological-based grade of fracture healing* [22] (1) Day 60: Control group ($n = 10$): Grade 2 ($n = 1$), Grade 3 ($n = 7$), Grade 4 ($n = 2$) Treatment group ($n = 10$) = Grade 3 ($n = 1$), Grade 4 ($n = 9$)	Pearson chi-square test: $P < 0.05$ (difference between control group and treatment group in terms of Grade 3 and Grade 4)	The histological-based bone formation grade was significantly superior in the treatment group compared to the control group		
Durak et al. [23]	(A) *Histological-based grade of fracture healing* [22] (1) Day 21 (16 days after the last treatment): Control group ($n = 10$): Grade 2 ($n = 6$), Grade 3 ($n = 4$) [mean = 2.4, s.d. = 0.5] Treatment group ($n = 10$) = Grade 2 ($n = 1$), Grade 3 ($n = 5$), Grade 4 ($n = 4$) [mean = 1.46, s.d. = 0.51]	Mann-Whitney U test: $P < 0.01$	The histological-based bone formation grade of the treatment group was significantly higher than the control group	No specific mechanism model was tested for the effect of α-tocopherol on secondary bone healing in normal bone	Favourable effect of α-tocopherol given for 5 days postfracture seen up to day 21 of study in rabbits (actual healing phase not stated)

Table 2: Continued.

Study	Reported results				
	Reported descriptive values of main outcome measure(s)	Reported inferential statistical analysis	Reported conclusion	Model proposed for mechanism of action of vitamin E in study	Conclusion of effect of vitamin E based on the phase of fracture healing examined in study
Sarisözen et al. [24]	(A) *Callous index* (1) Day 14 Control group ($n = 6$): mean = 1.64, variability = not reported Vit. E only treatment group ($n = 6$): mean = 1.49, variability = not reported Vit. C only treatment group ($n = 6$): mean = 2.17, variability = not reported Vit. E and C combination treatment group ($n = 6$): mean = 1.88, variability = not reported (2) Day 21 Control group ($n = 6$): mean = 1.90, variability = not reported Vit. E only treatment group ($n = 6$): mean = 2.37, variability = not reported Vit. C only treatment group ($n = 6$): mean = 2.54, variability = not reported Vit. E and C combination treatment group ($n = 6$): mean = 1.93, variability = not reported (B) *Histological-based grade of fracture healing* [22] (1) Day 14 Control group ($n = 6$): mean = 1.67, variability = not reported Vit. E only treatment group ($n = 6$): mean = 1.50, variability = not reported Vit. C only treatment group ($n = 6$): mean = 2.67, variability = not reported Vit. E and C combination treatment group ($n = 6$): mean = 2.16, variability = not reported	Kruskal-Wallis test: $P < 0.05$ Post-hoc test using Mann-Whitney U test: (a) $P < 0.05$ for comparison between Vit. C only group and combination group Kruskal-Wallis test: $P < 0.05$ Posthoc test using Mann-Whitney U test: (a) $P < 0.05$—Vit. C only group versus control group (b) $P < 0.05$—Vit. C only group vs. Vit. E only group (c) $P < 0.05$—Vit. C only group versus combination group Kruskal-Wallis test: $P < 0.05$ Posthoc test using Mann-Whitney U test: (a) $P < 0.05$—Vit. C only group versus control group. (b) $P < 0.05$—Vit. C only group versus Vit. E only group. (c) $P < 0.05$—Combination group versus control group. (d) $P < 0.05$—Combination group versus Vit. E only group	On day 14, the mean callous index for the vitamin C only group was significantly higher than the combination group, whereas the vitamin E only group was the lowest and was similar to the control group On day 21, the mean callous index for the vitamin C only group was significantly higher than all groups, whereas the vitamin E only group was higher (although not significant) than the combination and control groups The mean grade for the vitamin C only group and combination group was significantly higher than the vitamin E only group and control group on day 14 and day 21. On day 14, the mean grade for the vitamin E only group was lower (although not significant) than the control group, whereas, on day 21, the mean grade for the vitamin E only group was higher (although not significant) than the control group	No specific mechanism model was tested for the effect of α-tocopherol on secondary bone healing in normal bone	No favourable effect seen during the early phase (day 14) of healing in rats

Table 2: Continued.

Study	Reported results		Reported conclusion	Model proposed for mechanism of action of vitamin E in study	Conclusion of effect of vitamin E based on the phase of fracture healing examined in study
	Reported descriptive values of main outcome measure(s)	Reported inferential statistical analysis			
Sarisözen et al. [24]	(2) Day 21 Control group ($n = 6$): mean = 2.00, variability = not reported Vit. E only treatment group ($n = 6$): mean = 2.33, variability = not reported Vit. C only treatment group ($n = 6$): mean = 3.17, variability = not reported Vit. E and C combination treatment group ($n = 6$): mean = 3.00, variability = not reported	Kruskal-Wallis test: $P < 0.05$ Posthoc test using Mann-Whitney U test: (a) $P < 0.05$—Vit. C only group versus control group. (b) $P < 0.05$—Vit. C only group versus Vit. E only group (c) $P < 0.05$—Combination group versus control group (d) $P < 0.05$—Combination group versus Vit. E only group			

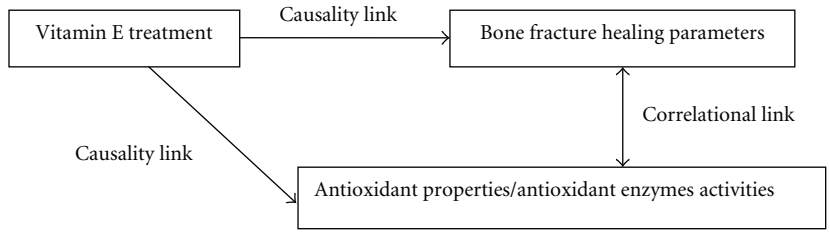

Figure 2: The framework of the general study design of the proposed mechanism of action of vitamin E on bone fracture healing in the included studies.

healing [8, 15, 18, 21] (Table 2). In general, two suggested mechanisms were tested in these studies: (a) the positive effect of antioxidant property of vitamin E on bone fracture healing [15, 21] and (b) the positive effect of vitamin E on antioxidant enzymes activities, which in turn will have a positive effect on bone fracture healing [8, 18]. The parameter of the antioxidant property of vitamin E was measured as Total Antioxidant Capacity level [15] or Erythrocyte Malondialdehyde level [21]. On the other hand, the parameter of the antioxidant enzymes activities was measured via Bone Superoxide Dismutase level [18], Bone Glutathione Peroxidase level [18], or Serum Catalase level [8]. All four studies reported that their proposed mechanism was supported by their findings, through the concurrent increase of the previously mentioned parameters during the improvement of bone fracture healing. However, this conclusion is debatable as the experimental design in these studies did not support a causality link between the increase of the antioxidant-related parameters with the improvement of bone fracture healing. This is clearly illustrated in Figure 2, whereby the proposed mechanism was tested through a quasi-experiment design that would only support a correlational link between the mechanism and the improvement.

3.4. Evaluation of the Quality of the Reported Methods and Results from Past Studies of the Effect of Vitamin E on Bone Fracture Healing. Based upon the ARRIVE Guidelines, the total items that were described satisfactorily in the reviewed articles ranged between 8 to 13 per 22 items in Section 2 and between 0 to 3 per 6 items in Section 3 (Table 3). All included articles fulfilled less than half of the recommendations in Sections 2 and 3. Of particular interest, only three studies mentioned the use of randomisation during the allocation of experimental groups [8, 15, 18] and only two studies reported the use of blinding during the assessment of results [15, 18]. The absence of these components may reflect the probability of subjective bias in the study design itself [28]. Besides that, unsatisfactory description of the characteristics of the animal subjects as well as their housing was present in nearly all the reviewed articles. For example, some studies failed to report the baseline data, sex, age, and source of the animal subjects. Additionally, none of the reviewed articles reported any sample size calculations for their experiments or any attempts of independent replications of the results. As can be seen in Table 4, two studies did not use the appropriate inferential analysis that may yield a more conclusive result [18, 21]. In these studies, the chi-square test was used instead of the Kruskal-Wallis test or Mann-Whitney U test. Besides that, one study did not even bother to report the quantitative results (both descriptive and inferential), aside from a highly questionable statement that the results were significant [8].

3.5. Evaluation of Effect Size and Statistical Power of the Effect of Vitamin E on Bone Fracture Healing in Past Studies. Referring to Table 4, reanalysis was performed (where necessary and possible) primarily to provide the needed descriptive data for the effect size and statistical power calculation using G* Power software. Among the significant results, only one radiological-based assessment of bone formation from one study [18] had a statistical power value of 0.49. This value was far below the accepted conventional value of 0.80 [29]. On the other hand, the effect size of α-tocopherol treatment for (a) radiological-based assessment of bone formation ranged from 0.56 to 2.50 [15, 18, 21]; (b) histological-based assessment of bone formation ranged from 1.49 to 2.46 [15, 21, 23]; (c) radiological-based assessment of callous formation ranged around 0.15 [18]; (d) osteoblastic activity assessment ranged around 0.47 to 2.35 [15]. Conventionally, a Cohen's d value of 0.20 denotes a small effect size; a value of 0.50 denotes a medium effect size, whereas a value of 0.80 denotes a large effect size [30]. Therefore, the wide range of effect sizes of α-tocopherol implied that it is too simplistic (and misleading) to summarise the vitamin E effect from a single angle. It would be more prudent to examine the effects separately in different situations, procedures, or even models. The identification of the common core elements and patterns was attempted later, through metasynthesis [31].

3.6. Calculation of Optimum Sample Size for Replication of Past Studies of the Effect of Vitamin E on Bone Fracture Healing. The calculated optimum sample size for each significant result ranged between 5 to 16 subjects for each experimental group (Table 4). Generally, most of the studies used enough subjects for the testing of each main outcome measure [15, 21, 23]. However, one study did not have enough sample size for a good statistical analysis with proper statistical power [18]. In this study, the sample size for each group was five subjects, rather than 16 subjects as calculated. This indicates a higher chance for a nonsignificant result, if this study is replicated in the future.

3.7. Metasynthesis of Specific Patterns about the Effect of Vitamin E on Bone Fracture Healing. The conclusion about

Table 3: Quality of methods and results reported in animal studies included in the review (based upon ARRIVE Guidelines).

Section	Item in ARRIVE	ARRIVE Guidelines recommendation	Status in article (if described: +; if not described satisfactorily: commentary was given)					
			Kurklu et al. [15]	Shuid et al. [18]	Paskalev et al. [8]	Turk et al. [21]	Durak et al. [23]	Sarisözen et al. [24]
Methods								
Ethical statement	5	Indicate the nature of the ethical review permissions, relevant licences (e.g., Animal (Scientific Procedures) Act 1986), and national or institutional guidelines for the care and use of animals, that cover the research	+	+	+	+	+	+
Study design	6	For each experiment, give brief details of the study design including the following						
	6a	The number of experimental and control groups	+	+	+	+	+	+
	6b	Any steps taken to minimise the effects of subjective bias when allocating animals to treatment (e.g., randomisation procedure) and when assessing results (e.g., if performed, describe who was blinded and when)	+	Randomization was not described for the allocation of sham group	Not described	No blinding procedure was described	Not described	Not described
	6c	The experimental unit (e.g., a single animal, group, or cage of animals)	+	+	+	+	+	+
Experimental procedures	7	For each experiment and each experimental group, including controls, provide precise details of all procedures carried out. For example:						
	7a	How (e.g., drug formulation and dose, site and route of administration, anaesthesia and analgesia used (including monitoring), surgical procedure, method of euthanasia). Provide details of any specialist equipment used, including supplier(s)	The full formulation of α-tocopherol was not described	Method of euthanasia was not described The supplier of the fracture device was not reported	+	Method of euthanasia was not described	+	Methods of analgesia and euthanasia were not described
	7b	When (e.g., time of day)	Not described	Not described	Not described	Not described	Not described	Not described
	7c	Where (e.g., home cage, laboratory, water maze)	+	+	Not described	+	+	Not described
	7d	Why (e.g., rationale for choice of specific anaesthetic, route of administration, drug dose used)	Not described	Not described	+	Not described	Not described	Not described

Table 3: Continued.

Section	Item in ARRIVE	ARRIVE Guidelines recommendation	Status in article (if described: +; if not described satisfactorily: commentary was given)					
			Kurklu et al. [15]	Shuid et al. [18]	Paskalev et al. [8]	Turk et al. [21]	Durak et al. [23]	Sarisözen et al. [24]
Experimental animals	8							
	8a	Provide details of the animals used, including species, strain, sex, developmental stage (e.g., mean or median age plus age range), and weight (e.g. mean or median weight plus weight range)	Sex and age were not described	Age was not described	+	Age was not described	Age was not described	Sex was not described
	8b	Provide further relevant information such as the source of animals, international strain nomenclature, genetic modification status (e.g., knock-out or transgenic), genotype, health/immune status, drug or test naïve, previous procedures, and so forth	Not described	Not described	+	Not described	Not described	Not described
Housing and husbandry	9	Provide details of						
	9a	Housing (type of facility e.g., specific pathogen free (SPF); type of cage or housing; bedding material; number of cage companions; tank shape and material, etc., for fish).	Cage type was not fully described	Cage type was not fully described	Cage type was not fully described	Cage type was not fully described	Cage type was not fully described	Cage type was not fully described
	9b	Husbandry conditions (e.g., breeding programme, light/dark cycle, temperature, quality of water, etc., for fish, type of food, access to food, and water, environmental enrichment)	Specific type of food given was not described	+	Light/dark cycle and temperature were not described	Specific type of food given was not described	Not described (only access to food was described)	Not described (only access to food and water was described)
	9c	Welfare-related assessments and interventions that were carried out prior to, during, or after the experiment	Not described	+	+	Not described	Analgesia was not given after the fracture procedure (just depended on cast immobilization)	Not described
Sample size	10							
	10a	Specify the total number of animals used in each experiment, and the number of animals in each experimental group	+	+	+	+	+	+
	10b	Explain how the number of animals was arrived at. Provide details of any sample size calculation used	Not described	Not described	Not described	Not described	Not described	Not described
	10c	Indicate the number of independent replications of each experiment, if relevant	Not described	Not described	Not described	Not described	Not described	Not described

Table 3: Continued.

Section	Item in ARRIVE	ARRIVE Guidelines recommendation	Status in article (if described: +; if not described satisfactorily: commentary was given)					
			Kurklu et al. [15]	Shuid et al. [18]	Paskalev et al. [8]	Turk et al. [21]	Durak et al. [23]	Sarisözen et al. [24]
Allocating animals to experimental groups	11							
	11a	Give full details of how animals were allocated to experimental groups, including randomisation or matching if performed	Randomization procedure was not fully explained	Randomization procedure was not fully explained for the allocation of treatment groups	Not explained	Randomization procedure was not fully explained	Not explained	Not explained
	11b	Describe the order in which the animals in the different experimental groups were treated and assessed	Not described	Not described	Not described	Not described	Not described	Not described
Experimental outcomes	12	Clearly define the primary and secondary experimental outcomes assessed (e.g., cell death, molecular markers, behavioural changes)	+	+	+	+	+	+
Statistical methods	13							
	13a	Provide details of the statistical methods used for each analysis	+	+	+	+	+	+
	13b	Specify the unit of analysis for each dataset (e.g., single animal, group of animals, single neuron)	+	+	+	+	+	+
	13c	Describe any methods used to assess whether the data met the assumptions of the statistical approach	+ Not relevant as nonparametric test was used	+ Not relevant as nonparametric test was used	+ Not relevant as nonparametric test was used	+ Not relevant as nonparametric test was used	+ Not relevant as nonparametric test was used	+ Not relevant as nonparametric test was used

Table 3: Continued.

Section	Item in ARRIVE	ARRIVE Guidelines recommendation	Status in article (if described: +; if not described satisfactorily: commentary was given)					
			Kurklu et al. [15]	Shuid et al. [18]	Paskalev et al. [8]	Turk et al. [21]	Durak et al. [23]	Sarisözen et al. [24]
Results								
Baseline data	14	For each experimental group, report relevant characteristics and health status of animals (e.g., weight, microbiological status and drug or test naive) prior to treatment or testing (this information may often be tabulated)	Not fully described (i.e., only baseline weight was reported)	Not fully described (i.e., only baseline weight was reported)	Not fully described (i.e., only baseline weight was reported)	Not fully described (i.e., only baseline weight was reported)	Not fully described (i.e., only baseline weight was reported)	Not fully described (i.e., only baseline weight was reported)
Numbers analysed	15							
	15a	Report the number of animals in each group included in each analysis. Report absolute numbers (e.g., 10/20, not 50%)	+	+	Not described	+	+	Not described
	15b	If any animals or data were not included in the analysis, explain why	? (no exclusion was reported)	? (no exclusion was reported)	? (no exclusion was reported)	? (no exclusion was reported)	? (no exclusion was reported)	? (no exclusion was reported)
Outcomes and estimation	16	Report the results for each analysis carried out, with a measure of precision (e.g., standard error or confidence interval)	+ (see full commentary in reanalysis table)	Some statistical analyses were not reported fully (see full commentary in reanalysis table)	Not described. (see full commentary in reanalysis table)	+ (see full commentary in reanalysis table)	+ (see full commentary in reanalysis table)	The measure of precision was not reported in the result (see full commentary in reanalysis table)
Adverse events	17							
	17a	Give details of all important adverse events in each experimental group	+ (no complication reported)	Not described	Not described	Not described	Not described	Not described
	17b	Describe any modifications to the experimental protocols made to reduce adverse events	Not described	Not described	Not described	Not described	Not described	Not described
Total items described satisfactorily		Methods section (per 22 items) Results section (per 6 items)	10 3	11 1	13 0	9 2	10 2	8 0

Table 4: Commentary of statistical analysis and reanalysis of results from past animal studies of the effect of vitamin E on bone fracture healing.

Study	Result	Reanalysed result (where necessary or possible) and final conclusion (focusing on the vitamin E group versus control group)	Effect size and power of the result via G*Power (vitamin E group versus control group)	Sample size for future replication of results via G*Power
Kurklu et al. [15]	(A) *Radiological-based grade of bone formation*			
	(1) Day 20 analysis	*Reanalysis was unnecessary for conclusion* Mann-Whitney U test: $P > 0.05$ Final conclusion: there was no significant difference between the treatment group and control group in terms of radiological-based grade of bone formation	If $\alpha = 0.05$, 2-tailed; distribution: minimum ARE Effect size, $d = 0.56$ Power = 0.28	If $\alpha = 0.05$, 2-tailed; power = 0.80; distribution: minimum ARE Sample size for each group (equal number) = 59
	(2) Day 30 analysis	*Reanalysis was unnecessary for conclusion* Mann-Whitney U test: $P < 0.01$ Final conclusion: the treatment group has significantly higher radiological-based grade of bone formation compared to control group	If $\alpha = 0.05$, 2-tailed; distribution: minimum ARE Effect size, $d = 1.31$ Power = 0.89	If $\alpha = 0.05$, 2-tailed; power = 0.80; distribution: minimum ARE Sample size for each group (equal number) = 12
	(3) Day 40 analysis	*Reanalysis was unnecessary for conclusion* Mann-Whitney U test: $P < 0.01$ Final conclusion: the treatment group has significantly higher radiological-based grade of bone formation compared to control group	If $\alpha = 0.05$, 2-tailed; distribution: minimum ARE Effect size, $d = 2.34$ Power ≈ 1.00	If $\alpha = 0.05$, 2-tailed; power = 0.80; distribution: minimum ARE Sample size for each group (equal number) = 5
	(B) *Ratio of osteoblastic activity*			
	(1) Day 5 analysis	*Reanalysis was unnecessary for conclusion* Mann-Whitney U test: $P > 0.05$ Final conclusion: there was no significant difference between the treatment group and control group in terms of osteoblastic activity	If $\alpha = 0.05$, 2-tailed; distribution: minimum ARE Effect size, $d = 0.47$ Power = 0.21	If $\alpha = 0.05$, 2-tailed; power = 0.80; distribution: minimum ARE Sample size for each group (equal number) = 83
	(2) Day 20 analysis	*Reanalysis was unnecessary for conclusion* Mann-Whitney U test: $P < 0.01$ Final conclusion: the treatment group has significantly higher osteoblastic activity compared to control group	If $\alpha = 0.05$, 2-tailed; distribution: minimum ARE Effect size, $d = 2.35$ Power ≈ 1	If $\alpha = 0.05$, 2-tailed; power = 0.80; distribution: minimum ARE Sample size for each group (equal number) = 5
	(C) *Histological-based grade of bone formation*			
	(1) Day 40 analysis	*Reanalysis was unnecessary for conclusion* Mann-Whitney U test: $P < 0.01$ Final conclusion: the treatment group has significantly higher histological-based grade of bone formation compared to control group	If $\alpha = 0.05$, 2-tailed; distribution: minimum ARE Effect size, $d = 2.67$ Power ≈ 1	If $\alpha = 0.05$, 2-tailed; power = 0.80; distribution: minimum ARE Sample size for each group (equal number) = 5

Table 4: Continued.

Study	Result	Reanalysed result (where necessary or possible) and final conclusion (focusing on the vitamin E group versus control group)	Effect size and power of the result via G*Power (vitamin E group versus control group)	Sample size for future replication of results via G*Power
Shuid et al. [18]	(A) *Callous volume*			
	(1) Day 14 analysis	*Not enough information from the article for reanalysis* Unknown statistical test: $P > 0.05$ Final conclusion: There was no significant difference of callous volume between the ovariectomized treatment group and ovariectomized control group	Not enough information from the article for calculation	Not enough information from the article for calculation
	(B) *Radiological-basedscore of callous staging*			
	(1) Day 14 analysis	*Reanalysis was performed using the tabulated data reported for the chi-square test in the article* Descriptive results: (1) Sham group ($n = 8$): median = 2 (mean = 2.12, s.d. = 0.64) (2) Ovariectomized control group ($n = 8$): median = 3 (mean = 3.00, s.d. = 0.76) (3) Ovariectomized treatment group ($n = 8$): median = 3 (mean = 3.12, s.d. = 0.84) Kruskal-Wallis test: $H(\text{df} = 2) = 6.5$, $P < 0.05$ Posthoc Mann-Whitney U test focused on the comparison between ovariectomized treatment group and ovariectomized control group (Bonferroni's correction was not necessary in this case): $U = 29$, $P > 0.05$ Final conclusion: there was no significant difference between the treatment group and control group in terms of radiological-based callous staging	If $\alpha = 0.05$, 2-tailed; distribution: minimum ARE Effect size, $d = 0.15$ Power = 0.06	If $\alpha = 0.05$, 2-tailed; power = 0.80; distribution: minimum ARE Sample size for each group (equal number) = 811
	(C) *Radiological-based score of fracture healing staging*			
	(1) Day 14 analysis	*Reanalysis was performed using the tabulated data reported for the chi-square test in the article* Descriptive results (1) Sham group ($n = 8$): median = 3 (mean = 2.88, s.d. = 0.35) (2) Ovariectomized control group ($n = 8$): median = 2 (mean = 2.38, s.d. = 0.52) (3) Overaiectomized treatment group ($n = 8$): median = 3 (mean = 2.88, s.d. = 0.35) Kruskal-Wallis test: $H(\text{df} = 2) = 6.2$, $P < 0.05$ Post-hoc Mann-Whitney U test focused on the comparison between ovariectomized treatment group and ovariectomized control group (Bonferroni's correction was not necessary in this case): $U = 16$, $P < 0.05$ Final conclusion: the treatment group had significantly higher radiological-based fracture healing staging than control group	If $\alpha = 0.05$, 2-tailed; distribution: minimum ARE Effect size, $d = 1.13$ Power = 0.49	If $\alpha = 0.05$, 2-tailed; power = 0.80; distribution: minimum ARE Sample size for each group (equal number) = 16

Table 4: Continued.

Study	Result	Reanalysed result (where necessary or possible) and final conclusion (focusing on the vitamin E group versus control group)	Effect size and power of the result via G*Power (vitamin E group versus control group)	Sample size for future replication of results via G*Power
Paskalev et al. [8]	(A) *Radiological-based evaluation of callous formation staging (unspecified)* (1) Day 1, week 2, month 1, month 2, month 3, and month 4 analysis (B) *Radiological-based evaluation of bone remodeling staging (unspecified)* (1) Day 1, week 2, month 1, month 2, month 3, and month 4 analysis	*Not enough information from the article for reanalysis* No statistical test result reported Final conclusion: it was stated that callous formation stage was better in the treatment group compared to the control *Not enough information from the article for reanalysis* No statistical test result reported Final conclusion: it was stated that bone remodeling stage was better in the treatment group compared to the control	Not enough information from the article for calculation Not enough information from the article for calculation	Not enough information from the article for calculation Not enough information from the article for calculation
Turk et al. [21]	(A) *Radiological-based grade of bone formation* (1) Day 60 analysis (B) *Histological-based grade of fracture healing* (1) Day 60 analysis	*Reanalysis was performed using the tabulated data reported for the chi-square test in the article* Descriptive results Control group ($n = 10$): median = 3 (mean = 3.10, s.d. = 0.32) Treatment group ($n = 10$): median = 4 (mean = 3.90, s.d. = 0.32) Mann-Whitney U test: $U = 10.0$, $P < 0.01$ Final conclusion: the treatment group had significantly higher radiological-based grade of bone formation than control group *Reanalysis was performed using the tabulated data reported for the chi-square test in the article* Descriptive results Control group ($n = 10$): median = 3 (mean = 3.10, s.d. = 0.57) Treatment group ($n = 10$): median = 4 (mean = 3.90, s.d. = 0.32) Mann-Whitney U test: $U = 14.5$, $P < 0.01$ Final conclusion: the treatment group had significantly higher histological-based grade of bone formation than control group	If $\alpha = 0.05$, 2-tailed; distribution: minimum ARE Effect size, $d = 2.5$ Power ≈ 1 If $\alpha = 0.05$, 2-tailed; distribution: minimum ARE Effect size, $d = 2.5$ Power ≈ 1	If $\alpha = 0.05$, 2-tailed; power = 0.80; distribution: minimum ARE Sample size for each group (equal number) = 5 If $\alpha = 0.05$, 2-tailed; power = 0.80; distribution: minimum ARE Sample size for each group (equal number) = 5

Table 4: Continued.

Study	Result	Reanalysed result (where necessary or possible) and final conclusion (focusing on the vitamin E group versus control group)	Effect size and power of the result via G*Power (vitamin E group versus control group)	Sample size for future replication of results via G*Power
Durak et al. [23]	(A) *Histological-based grade of fracture healing* (1) Day 21 (16 days after the last treatment) analysis	*Reanalysis was performed using the tabulated data reported for the chi-square test in the article* Descriptive results: Control group ($n = 10$): median = 2 (mean = 2.40, s.d. = 0.52) Treatment group ($n = 10$): median = 3 (mean = 3.30, s.d. = 0.68) Mann-Whitney U test: $U = 17$, $P < 0.01$ Final conclusion: the treatment group had significantly higher histological-based grade of fracture healing than control group	If $\alpha = 0.05$, 2-tailed; distribution: minimum ARE Effect size, $d = 1.49$ Power = 0.82	If $\alpha = 0.05$, 2-tailed; power = 0.80; distribution: minimum ARE Sample size for each group (equal number) = 10
Sarisözen et al. [24]	(A) *Callous index* (1) Day 14 analysis	*Reanalysis was unnecessary for conclusion* Posthoc Mann-Whitney U test: $P > 0.05$ Final conclusion: there was no significant difference between the Vit. E only treatment group and control group in terms of callous index	Not enough information from the article for calculation (absence of standard deviation value)	Not enough information from the article for calculation (absence of standard deviation value)
	(2) Day 21 analysis	*Reanalysis was unnecessary for conclusion* Post-hoc Mann-Whitney U test: $P > 0.05$ Final conclusion: there was no significant difference between the Vit. E only treatment group and control group in terms of callous index	Not enough information from the article for calculation (absence of standard deviation value)	Not enough information from the article for calculation (absence of standard deviation value)
	(B) *Histological-based grade of fracture healing* (1) Day 14 analysis	*Reanalysis was unnecessary for conclusion* Post-hoc Mann-Whitney U test: $P > 0.05$ Final conclusion: there was no significant difference between the Vit. E only treatment group and control group in terms of histological-based grade of fracture healing	Not enough information from the article for calculation (absence of standard deviation value)	Not enough information from the article for calculation (absence of standard deviation value)
	(2) Day 21 analysis	*Reanalysis was unnecessary for conclusion* Post-hoc Mann-Whitney U test: $P > 0.05$ Final conclusion: there was no significant difference between the Vit. E only treatment group and control group in terms of histological-based grade of fracture healing	Not enough information from the article for calculation (absence of standard deviation value)	Not enough information from the article for calculation (absence of standard deviation value)

the effect of vitamin E would be incomplete, if there was no dissection on the results based upon the various study methods, models, or phase of bone healing. After several exploratory cross-tabulations of the results according to different sets of categories, several significant patterns can be seen, as shown in Table 5. Only the results of the comparison between the vitamin E treatment group and the control group were included in this table. One study [8] was omitted because of the lack of any quantitative results. Based on the table, the significant and positive effect of α-tocopherol was consistently observed after day 30 post-fracture, for all types of assessment of bone formation. These results had a large effect size and acceptable statistical power. However, there were mixed results between day 20 to day 29 postfracture for all types of assessment of bone formation. During this phase, the significant and positive effect of α-tocopherol was only observed in the histological-based assessment of fractures stabilized by cast immobilization (with a large effect size and acceptable statistical power) [23]. There were also mixed results for all types of assessment of bone formation between day 10 to day 19 post-fracture. During this phase, the significant and positive effect of α-tocopherol was only observed in the radiological-based assessment of fractures stabilized by internal fixation using the Kirschner wire [18]. Although this result showed a large effect size, the statistical power was unacceptable. It is also of interest to note that this study used a much higher dose of α-tocopherol (i.e., 60 mg/kg/day per oral), if compared to other studies that typically used a dose of 20 mg/kg/day intraperitoneally or intramuscularly. A rather startling pattern was seen, in terms of the effect of α-tocopherol on the callous-related assessments; virtually none of the results was significant, regardless of the day of observation [18, 24]. Likewise, none of the results related to the study where the fracture was left *in situ* was significant, either via bone formation assessments or callous-related assessments [24]. However, it must be remembered that this particular study administered the α-tocopherol treatment only in the early phase of the experiment and discontinued it for the rest of the study period. Besides that, the significant and positive effect of α-tocopherol on osteoblastic activity was seen between day 20 to day 29 post-fracture, but not earlier on [15].

In this metasynthesis, the noncommittal terms of "early phase" or "late phase" of bone healing reported in most of the reviewed articles will be ignored. The absence of a definite cut-off point for the bone healing timeline will hamper the understanding of the underlying mechanism of action. Besides that, the timeline of the phase of bone healing in rabbits was assumed to be nearly similar to rats, for the sake of simplicity. For secondary bone healing, callous formation is at the maximum around day 14 post-fracture, whereas the peak of bone remodeling phase occurs around day 28 to day 35 post-fracture in rats [32]. As the callous lasted from day 3 to day 30 post-fracture, there is an overlap with the bone remodeling phase that started as early as day 14 post-fracture. Once the recategorisation of the bone healing phase was done based on these cut-off points, the whole picture becomes clearer. Four specific patterns may be derived from the evidence in Table 5: (a) there was a significant and positive effect of α-tocopherol on bone formation during the peak of bone remodeling phase of secondary bone healing in animal models; (b) there were mixed results on the effect of α-tocopherol on bone formation during the callous formation phase of secondary bone healing in animal models; (c) there was no significant effect of α-tocopherol on callous formation itself during secondary bone healing in animal models; (d) there was a significant effect of α-tocopherol on the osteoblastic activity starting from the later stage of callous formation (which overlaps with the beginning of bone remodeling phase) of secondary bone healing, but not in the earlier stage of callous formation in animal models.

4. Discussion

4.1. The Issue of the Lack of Human Studies in Investigating the Effect of Vitamin E on Bone Fracture Healing. To date, there was no published human study of the effect of vitamin E on bone fracture healing. This is perplexing, considering that the first related animal study was published back in 1996 by Durak et al., who reported a favourable α-tocopherol effect on fracture haematoma [33]. This lack of interest might be partially fuelled by the damaging evidence shown by one of the early animal study papers on this topic. The study by Sarisözen et al. gave a rather convincing evidence that α-tocopherol has a significantly inferior effect on bone fracture healing, if compared to vitamin C and the combination of vitamin C and α-tocopherol [24]. As the effect of vitamin E compared to control was also not significant in this study, these results might cause a dilemma in potential researchers. It seemed to be a safer bet to pursue vitamin C studies for the promotion of bone fracture healing. Furthermore, the fact that the combination group had significantly inferior effect to vitamin C, suggested a form of negative interaction between the two vitamins. Apparently, the dominant effect of vitamin C was reduced by the addition of α-tocopherol. However, we have to bear in mind that this was the result of only a single study, without any following attempts for independent replications. The total reliance on a popularised single study to reflect "the truth" may lead to "herding" behaviour among researchers [34]. They might decide to follow the path of investigations of popularised publications, while neglecting novel and independent research that may bear better fruits.

The importance of human studies in determining the effect of vitamin E on bone fracture healing cannot be denied. According to the 2009 classification by the Oxford Centre for Evidence-based Medicine, the highest level in the hierarchy of evidence is reserved for systematic reviews of randomised controlled clinical trial in humans [35]. According to one current view, animal studies lie at the lowest rung in the hierarchy of evidence for therapeutic intervention [36], at the same level with unsystematic anecdotal information or expert opinion. Therefore, if there was a lack of studies in the lowest level in the hierarchy, the decision to proceed to the next level might be affected. For example, researchers in the United Kingdom who are planning clinical trials are required to show that there are systematic reviews of previous related studies [37]. Besides

Table 5: Meta-synthesis based upon the cross-tabulation of individual parameter results from the included studies in the review according to type of procedure, type of parameter observed and day of post-fracture.

Type of parameter observed	Type of procedure	The result showed that the vitamin E treatment group was significantly superior to the control group	Postfracture day					
			Below day 10	Day 10–19	Day 20–29	Day 30–39	Day 40–49	Day 50 and above
Radiological-based assessment of bone formation	Internal fixation with Kirschner wire	Yes		$1^{B}d = 1.13^{*}$				$1^{C}d = 2.50$
		No						
	Distraction using external fixator	Yes				$1^{A}d = 1.31$	$1^{A}d = 2.34$	
		No			$1^{A}d = 0.56$			
Histological-based assessment of bone formation	Internal fixation with Kirschner wire	Yes						$1^{C}d = 2.5$
		No						
	Distraction using external fixator	Yes					$1^{A}d = 2.67$	
		No						
	External fixation using cast immobilization	Yes			$1^{D}d = 1.49$			
		No						
	Fracture left *in situ*	Yes						
		No		$1^{E}d =?$	$1^{E}d =?$			
Radiological-based assessment of callous volume/callous index	Internal fixation with Kirschner wire	Yes						
		No		$1^{B}d =?$				
	Fracture left *in situ*	Yes						
		No		$1^{E}d =?$	$1^{E}d =?$			
Radiological-based assessment of callous formation	Internal fixation with Kirschner wire	Yes						
		No		$1^{B}d = 0.15$				
Osteoblastic activity	Distraction using external fixator	Yes			$1^{A}d = 2.35$			
		No	$1^{A}d = 0.47$					

[A] Result from Kurklu et al. [15]: normal bone, rabbit model, 20 mg/kg/day (intramuscular).
[B] Result from Shuid et al. [18]: osteoprotic bone, rat model, 60 mg/kg/day (per oral).
[C] Result from Turk et al. [21]: normal bone, rat model, 20 mg/kg/day (intraperitoneal).
[D] Result from Durak et al. [23]: normal bone, rabbit model, 20 mg/kg/day (intramuscular) for the initial 5 days only.
[E] Result from Sarisözen et al. [24]: normal bone, rat model, 40 mg/kg (intraperitoneal) (daily: day 1 to 3; 3x per week: day 4 onward).
* Significant result which has power less than 0.8.

that, scepticism has risen about the contribution of animal studies towards beneficial findings in humans. According to Pound et al., methodological flaws that were rampant in many animal studies would render the findings inconclusive [37]. They called for an urgent formal evaluation to address the actual contribution of animal studies to clinical medicine. According to them, it is still uncertain whether even valid results from animal experiments may be generalised to human beings.

4.2. The Issue of Reporting Quality of Reviewed Articles. As reported in Section 3, the quality of reporting of all the reviewed papers was mediocre, if the ARRIVE Guidelines were meticulously followed. Of specific interest is the lack of blinding and randomisation procedures reported in the majority of these articles. It should be noted that animal studies that did not report randomisation and blinding were more prone to report significant difference of their study groups, if compared to studies that used these bias-reducing methods [38]. The main purposes of the guidelines were to guarantee that the article included all relevant information for in-depth critique via the peer-review process [26], as well as to allow the animal experiment to be replicated [39]. Nevertheless, the quality assessment performed on the report of a study might not actually reflect the quality of the study *per se*. There was a possibility that the authors neglect to report the important information, due to ignorance or carelessness. Notwithstanding of this apologetic stand on behalf of the affected authors, it is predicted that there might be some problems in independently replicating the studies in this current paper. Difficulties might arise, if independent researchers totally rely on the reported version without knowing the exact methods through contact with the authors of the studies. In a recent scathing commentary on the state of preclinical cancer research, Begley and Ellis reported that only 11 percent of "landmark" studies (6 out 53 studies) could be reproduced, in terms of their findings [40]. These failures of study replication were partially blamed on technical differences and difficulties, as well as the pressure to publish a "perfect story" for the studies. It was stated that some of these nonreproducible papers had "spawned entire fields" of study. The materials of these papers were expanded by hundreds of subsequent secondary publications, without any attempts to confirm the original observations. Another prominent issue in the current reviewed articles was the absence of any attempts to describe how the sample size was actually derived. The rational selection of group size via pilot study or power analysis has been suggested by the Institutional Animal Care and Use Committees in the United States as a way to ensure researchers have enough subjects to reach their study objectives without wasting animals [41]. This is in conjunction with the use of appropriate statistical tests to generate the maximum information from the minimum number of animal subjects. This optimal use is performed in the spirit of the Reduction principle of "3 Rs" tenets of humane treatment of experimental animals, as originally proposed by Russell and Burch in 1959 [42]. The other 2 Rs are Replacement and Refinement. For the reviewed studies, it was unknown whether the sample size determination was performed using *a priori* power analysis, or based upon some pilot test results. There is also a possibility that they just depended on some fixed, arbitrary rule of accepted sample size number in their respective institutions. It should be stressed here that a reduction of animal sample size might not be justified, if the experiment does not have enough statistical power to detect any meaningful effect.

4.3. The Issue of Explaining the Actual Effect of Vitamin E on Bone Fracture Healing. There are several problems of drawing a valid conclusion on the effect of vitamin E on bone fracture healing based upon the heterogeneous findings of the reviewed articles. These difficulties could be summarised by the list of methodological problems of animal experiments as highlighted by Pound et al. [37]. Among their exhaustive list, four problems essentially present in this current systematic review: (a) dissimilar animal species with different metabolic pathways, which may lead to variations in efficacy; (b) dissimilar models for inducing the injury or illness, which may vary in human condition; (c) variability in the selection process, randomisation procedure, and choice of comparison treatment(s); (d) obscurity of the laboratory techniques that may affect results, such as blinding procedures. In spite of this, the four patterns of results showed in the meta-synthesis could be further simplified into a single statement: α-tocopherol may promote bone formation during the peak of the bone remodeling phase of secondary bone healing in animal models, but not callous formation. The α -tocopherol's effect on bone formation during the late callous formation phase remained inconclusive, due to the mixed results that were observed during this phase. These inconsistencies might be related to its overlap with the start of the bone remodeling phase. Besides that, the single result that showed a significant effect for bone formation during the early callous formation phase [18] might be disregarded as an outlier. This is due to its low statistical power, which reflects lower reproducibility likelihood.

Does this generalisation on the α-tocopherol's effect give any credibility to the postulated antioxidant-based mechanism of action described earlier on? None of the reviewed studies was properly designed and analysed to test the causality link of the antioxidant-based mechanism of action of vitamin E on bone fracture healing (Figure 2). This weakness may be solved, if the researchers actually used a more sophisticated statistical analysis called mediation analysis with the proper use of dummy variables [43–45]. Through this analysis, it is possible to test the statistical causality link between the vitamin E treatment and bone fracture healing parameters, with the postulated mechanism parameter as a mediator (Figure 3). If the mediation is significant, the researchers may confidently suggest that mechanism of action is likely to be the cause of the change in the bone fracture healing parameter. However, the failure of the reviewed studies to employ this test has jeopardised the chance of testing the mechanism theory directly in the studies. This failure is eerily reminiscent of the situation warned by Begley and Ellis, whereby many secondary investigations were performed based upon expansion of the materials of unsubstantiated original observation [40].

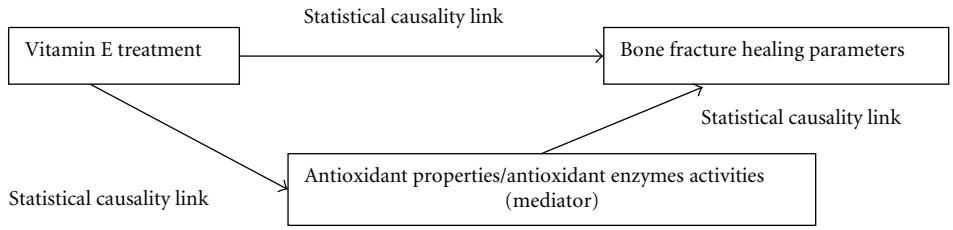

Figure 3: The framework of meditation analysis for the proposed mechanism of action of vitamin E on bone fracture healing in the included studies.

4.4. Implications and Suggestions for Future Studies. Based upon the fact that there were only six related published animal studies in major databases on the topic of bone fracture healing and vitamin E, it is much too early to endorse a more general conclusion. Nevertheless, this limited evidence looked favourable for vitamin E, at least for the promotion bone formation. There are still plenty of rooms for new studies to fill in the various gaps in the evidence as seen in the meta-synthesis shown in Table 5. These new studies may be designed according to other experimental conditions that have not yet been studied, such as the testing of the tocotrienols. There is also merit in designing proper studies to describe conclusively the biochemical mechanism behind the action of vitamin E in bone fracture healing. Some efforts should also be put to replicate the original studies and the subsequent secondary studies to reduce the chance of making a premature false conclusion based on irreproducible studies. In view of the state of evidence in this paper, it is cautiously recommended that investigators begin venturing into observational studies in humans. It is important to see whether the positive findings seen in animal studies might be also true for human beings. For example, cross-sectional studies may be performed on the correlation between overall vitamin E intake in the daily diet and the speed of bone fracture healing among orthopaedic patients. Any positive findings in observational studies might then hasten the progress for proper clinical trials. This methodological upgrade will determine the actual causal effect of vitamin E on human bone fracture healing. Besides that, future studies should take note on the current guidelines of quality reporting to ensure that their results would be reproducible by other independent researchers. On top of that, the issue of sample size justification must not be taken for granted, as there are now established methods or how to handle this optimally. These methods may also be applied to human studies. On the other hand, any attempts to confirm the mechanism of action of vitamin E effect should either use the proper experimental designs or at least employ the proper mediation analysis method.

4.5. Limitation of This Systematic Review. As in many other systematic reviews, this current paper was not immune to the effect of publication bias [28]. This bias happened because there is a systematic exclusion of many studies with nonsignificant results that were not published in peer-reviewed journals. Besides that, the absence of hand-searching for original studies not included in the searched databases, as well as the exclusion of non-English language articles, might have caused some studies to be systematically left out [14, 46].

5. Conclusion

Based on the small number of heterogeneous and mediocre animal studies included in this systematic review, the effect of vitamin E on bone fracture healing remained inconclusive. Despite this, the effect of α-tocopherol on bone formation during the normal bone remodeling phase of secondary bone healing in the reviewed studies was promising; although it has not been shown be effective for callous formation It is recommended that more original studies (experimental animal studies, especially basic mechanism research, and human observational studies) to be properly performed on this topic. These efforts will help in the formulation of a more comprehensive generalisation on the status of vitamin E in bone fracture healing, particularly for humans.

List of Abbreviations

MEDLINE:	Medical Literature Analysis and Retrieval System Online
CINAHL:	Cumulative Index to Nursing and Allied Health Literature
ARRIVE:	Animal in Research: Reporting In Vivo Experiments
SPSS:	Statistical Package for the Social Sciences
ARE:	Minimum Asymptomatic Relative Efficiency
P:	The calculated probability value of type 1 error
α:	The probability value of type 1 error where the significance level is set
d:	Cohen's standardized effect size, d
Kg:	kilogram
mg:	Milligram
n:	Sample size
vit.:	Vitamin.

Conflict of Interests

The corresponding author on behalf of all authors declares that there are no competing interests.

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Research Article

Effects of Extracts from *Trifolium medium* L. and *Trifolium pratense* L. on Development of Estrogen Deficiency-Induced Osteoporosis in Rats

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Some plant species belonging to *Trifolium* L. genus are a source of isoflavones considered to exert phytoestrogenic activities. The aim of the present study was to examine the effects of standardized extract obtained from aerial parts of *Trifolium medium* L., in comparison with the extract of *Trifolium pratense* L., on the development of estrogen deficiency-induced osteoporosis in rats. Both *Trifolium* extracts, at doses corresponding to 10 and 20 mg/kg of isoflavone aglycones daily, or estradiol (0.2 mg/kg daily), were administered orally to ovariectomized (OVX) rats for 4 weeks. Serum bone turnover markers, bone mass, mineralization, and mechanical properties were studied. In OVX control rats, mechanical properties of the tibial metaphysis and femoral neck were strongly worsened in comparison with sham-operated control rats, and those of femoral diaphysis were unaffected. Estradiol counteracted the worsening of the tibial strength and increases in bone turnover markers. Both extracts significantly increased the strength of the femoral diaphysis and calcium and phosphorus content in the bone mineral, but only *T. pratense* extract increased the strength of the tibial metaphysis. In conclusion, effects of both *Trifolium* extracts differed from those of estradiol. It is possible that other than isoflavone extract constituents contributed to their skeletal effects.

1. Introduction

Phytoestrogens are plant derived substances with estrogenic activity, which, binding to estrogen receptors, may exert agonistic, antagonistic, or partial agonistic/antagonistic effects [1]. They are sometimes considered natural selective estrogen receptor modulators (SERMs); however there is only a weak association between the action of phytoestrogens and SERMs [2]. Due to the side effects of hormonal replacement therapy, there is an interest in using phytoestrogens to alleviate the menopausal symptoms, including development of osteoporosis. It is generally accepted that estrogen deficiency contributes to development of osteoporosis in postmenopausal women.

Red clover (*Trifolium pratense* L.) is a well-known source of phytoestrogenic isoflavones. It contains mainly formononetin and biochanin A, and much smaller amounts of daidzein and genistein, mostly in glycosidic form [3, 4].

Although some efficacy of red clover extracts in the preventing of vasomotor symptoms was demonstrated in a meta-analysis of 5 clinical trials [5], more recently both lack of effects [6, 7] and favorable effects [8] were reported. The data on the long-term effects on the cardiovascular, breast, endometrial, and urogenital health, as well as bone health, are much scarcer [9]. There are clinical reports both on lack of significant effects [10, 11], and some favorable effects [12, 13] of red clover extracts on the skeletal system. Nevertheless, beneficial effects of red clover extracts on bones

have been demonstrated in the experimental settings [14–16].

Previous phytochemical studies done by Zgórka [17] revealed that dried, aerial parts of zigzag clover (*Trifolium medium* L.) might constitute quite new and rich source of isoflavone phytoestrogens (especially formononetin and biochanin A). Total content of aglycone forms of isoflavones (native and those released from glycosides by hydrolysis), calculated for this species, was about threefold higher than in red clover and comparable with amounts reported in soybeans.

T. medium is a rhizomatous perennial plant belonging to the Fabaceae (Leguminosae) family. Its trivial name is zigzag clover because of the tendency for the ascending stems to grow in zigzagged (curved) manner. As a representative of the clover genus, *T. medium* distinguishes by characteristic spherical flower heads composed of reddish purple flowers. In general appearance, *T. medium* is similar to commonly known red clover; however it possesses longer stalk, darker flower heads, and longer, lanceolate-elliptic leaflets without the noticeable pale "V" mark, occurring in the latter species. *T. medium* grows wild throughout Eurasia, especially in sunny grasslands, roadsides, waste grounds, and forest margins, often with poor soil. It is listed as a fodder crop in the Mediterranean region; however it is much less common than red clover [18, 19].

The effects of *T. medium* isoflavones on the skeletal system have not been studied so far. The aim of the present study was to examine the effects of the standardized extract obtained from aerial parts of *T. medium*, in comparison with the extract of *T. pratense*, on development of estrogen deficiency-induced osteoporosis in ovariectomized rats. Moreover, ovariectomized rats receiving estradiol served as a positive control.

2. Methods

Trifolium extracts were used. The standardized plant extracts were obtained from dried aerial parts of *T. medium* L. and *T. pratense* L. by ultrasound-assisted extraction with 50% (v/v) ethanol, followed by lyophilization. The standardization procedure, comprising the quantitation of bioactive isoflavones (aglycone forms) in hydrolysed clover extracts, was performed using reversed-phase high-performance liquid chromatography (RP-HPLC) combined with photodiode array detection (PDA). The mean isoflavone content, calculated as the sum of four aglycones: formononetin (F), biochanin A (B), genistein (G), and daidzein (D), was 7.2 and 2.8% of dry weight, for *T. medium* and *T. pratense* extracts, respectively. The percent concentrations of aglycones, in isoflavone fractions, were as followed: 46.3% (F), 41.8% (B), 11.2% (G), and 0.7% (D) for *T. medium*, and 49.0% (B), 44.9% (F), 5.3% (G), and 0.8% (D) for *T. pratense*. Both extracts were used at doses corresponding to 10 and 20 mg/kg of total isoflavone aglycones/day, p.o.

Other drugs used included estradiol hemihydrate (Estrofem, Novo Nordisk A/S, 0.2 mg of estradiol/kg/day, p.o.), ketamine—Bioketan (Vetoquinol Biowet) and xylazine—Xylapan (Vetoquinol Biowet).

The study was carried out with consent of the Local Ethics Commission in Lublin, on 3-month-old female Wistar rats obtained from the Center of Experimental Medicine, Medical University of Silesia. The initial body mass of rats was 210–240 g. The rats were fed a soy-free diet *ad libitum*. The animals were switched from the standard laboratory diet to the soy-free diet on the day before the beginning of the extract or estradiol administration.

Bilateral ovariectomy with the access to the ovaries from the dorsal side was performed in general anesthesia induced by intraperitoneal injections of ketamine with xylazine. During sham operation, the ovaries were exteriorized only. The operations took place 7-8 days before the start of the drug administration.

The rats were divided into the following groups (n = 9-10 per group):

(I) sham-operated control rats,

(II) ovariectomized (OVX) control rats,

(III) ovariectomized rats receiving *T. medium* extract (10 mg isoflavone aglycones /kg p.o. daily),

(IV) ovariectomized rats receiving *T. medium* extract (20 mg isoflavone aglycones /kg p.o. daily),

(V) ovariectomized rats receiving *T. pratense* extract (10 mg isoflavone aglycones /kg p.o. daily),

(VI) ovariectomized rats receiving *T. pratense* extract (20 mg isoflavone aglycones/kg p.o. daily),

(VII) ovariectomized rats receiving estradiol (0.2 mg/kg p.o. daily).

Estradiol or the extracts were administered by a gastric tube (p.o.) for 28 days, at a volume of 2 mL/kg. Control rats were administered the vehicle - tap water at a volume of 2 mL/kg p.o. Moreover, to mark the calcification front, the animals were given tetracycline hydrochloride (20 mg/kg, i.p.) one day before the start of drug or vehicle administration, and calcein (10 mg/kg, i.p.), after 3 weeks.

The next day after the last drug administration, after 24-h fasting, the animals were killed by cardiac exsanguination, in full ketamine-xylazine anesthesia. From the sacrificed animals, estrogen-dependent organs (the uterus and thymus) and bones, the left and right femur, left and right tibia, and L-4 vertebra, were isolated. Immediately after isolation, the organs were weighed (with 0.1 mg accuracy). The length and diameter of left bones were measured with a caliper (with 0.01 mm accuracy). The bones were wrapped in gauze soaked in 0.9% NaCl solution and kept below -20°C until the mechanical tests were performed on thawed bones [20].

2.1. Bone Mechanical Properties Studies. Mechanical properties of the left femoral diaphysis, left tibial metaphysis, and the neck of the right femur were assessed using Instron 3342 500N apparatus with Bluehill 2 version 2.14 software. Mechanical properties of the left femoral diaphysis and

left tibial metaphysis were studied using bending tests with three-point loading, as previously described [20–22]. The load was applied perpendicularly to the long axis of the femur in the midlength of the bone (distance between the supporting points: 16 mm) or to the proximal tibial metaphysis. The displacement rate was 0.01 mm/s. The load-displacement curves, obtained for each bone, representing the relationships between load applied to the bone and displacement in response to the load, were analyzed. Maximum load and displacement, energy, and stress for the maximum load, as well as fracture load and displacement, energy, and stress for the fracture load were assessed. Young's modulus was also determined. To determine moment of inertia in the cross-section, necessary for the calculations of the intrinsic bone mechanical parameters, it was assumed that the femoral diaphysis was an elliptical pipe, and the tibial metaphysis was a circular beam. For the femoral diaphysis, the transverse cross-sections of the right diaphysis were made in the midlength, and the inside and outside diameters were measured, according to [23], using Osteomeasure software (Osteometrics) for histomorphometric measurements. The mean diameter of the tibial metaphysis was measured using a caliper.

Mechanical properties of the femoral neck were studied using a compression test [22, 24]. The load was applied to the head of the femur along the long axis of the femur (displacement rate of 0.01 mm/s) and the maximum load (load causing the fracture of the femoral neck) was determined.

2.2. Bone Histomorphometric Studies. The right femurs were used to prepare histological specimens, as previously described [22, 25, 26]. Histomorphometric measurements were made using an Optiphot-2 microscope (Nikon), connected through an RGB camera (Cohu) to a computer, using Lucia G 4.51 software (Laboratory Imaging), with final magnifications of 200 and 500 times, or using Osteomeasure software (magnification 70 times).

The width of trabeculae in the distal epiphysis and metaphysis was measured in the longitudinal preparation from the femur. The area of the transverse cross-section of the cortical bone and the area of the transverse cross-section of the marrow cavity were determined in transverse cross-sections made from the femoral diaphysis in midlength of the femur. The periosteal and endosteal transverse growth of the femur was also measured.

2.3. Bone Mineralization Studies. To determine the mass of bone mineral (ash), the bones were mineralized at the temperature of 640°C for 48 h in the muffle furnace and weighed. The ratio of the mass of bone mineral to the bone mass was also determined as a substitute for bone mineral density measurements.

Calcium and phosphorus content in the mineralized bones (dissolved in 6 M HCl and then diluted in distilled water) was determined colorimetrically, using a calcium reagent set and a phosphorus reagent set, both produced by Pointe Scientific.

2.4. Biochemical Studies. Serum estradiol concentrations were studied by an ELISA method (Mouse/Rat Estradiol ELISA, Calbiotech, Inc.). Serum osteocalcin levels were determined using an enzyme immunoassay (Rat-MID Osteocalcin EIA, Immunodiagnostic Systems Ltd.). Serum levels of type I collagen fragments released from bone during bone resorption were determined by an enzyme immunoassay (RatLaps EIA, Immunodiagnostic Systems Ltd.). Moreover, serum concentrations of calcium, phosphorus, and total cholesterol were assayed colorimetrically, using Pointe Scientific reagent sets.

2.5. Statistical Analysis. The results are presented as arithmetical means ± SEM. Statistical estimation was carried out based on the analysis of variance. After confirmation of statistically significant differences in one-way ANOVA ($P < 0.05$), further analysis was carried out by means of Duncan's *post hoc* test. In case of a lack of normality (Shapiro-Wilk's test) or of homogeneity of variance (Levene's test), nonparametric tests were used: Kruskal-Wallis ANOVA and Mann-Whitney U test. The results obtained in each experimental group were compared with those of the sham-operated control rats and ovariectomized control rats.

3. Results

3.1. Body Mass Gain, Mass of Estrogen-Dependent Organs, and Serum Levels of Estradiol and Total Cholesterol. The ovariectomized control rats had significantly decreased serum estradiol level and the uterus mass. The mass of thymus, as well as the body mass gain and serum cholesterol level, were significantly increased (Table 1). Neither of the investigated *Trifolium* extracts significantly affected the abovementioned estradiol-dependent parameters. The changes in the uterus mass, body mass gain and serum cholesterol level were partially counteracted by administration of estradiol (0.2 mg/kg daily).

There were no effects of the treatments on serum calcium and phosphorus levels (not shown).

3.2. Bone Mass and Mineralization. Estrogen deficiency in the ovariectomized control rats caused significant decreases in the mass of the tibia, femur, and L-4 vertebra, expressed as the ratio to the body mass, in comparison with the sham-operated rats (Table 2). The ratio of the mass of bone mineral to the bone mass was significantly decreased in the long bones. There was no effect of estrogen deficiency on the calcium and phosphorus content in the bone mineral.

Extracts of *Trifolium* species did not affect the bone mass expressed as the ratio to the body mass nor the content of bone mineral in the bone (the ratio of mass of bone mineral to bone mass) in comparison with the ovariectomized control rats. Both *T. medium* and *T. pratense* extracts at two isoflavone doses significantly increased the content of calcium and phosphorus in the bone mineral of the tibia and L-4 vertebra, but not of the femur.

Administration of estradiol to the ovariectomized rats did not significantly affect the ratio of bone mass to body

mass, nor the ratio of the mass of bone mineral to the bone mass. Administration of estradiol also did not significantly affect the content of calcium and phosphorus in the bone mineral.

3.3. Mechanical Properties of Cancellous Bone (Tibial Metaphysis). Estrogen deficiency very strongly worsened mechanical properties of the cancellous bone (Table 3). In the ovariectomized control rats, the maximum load and energy accumulated for the maximum load, as well as maximum stress and Young's modulus, were significantly decreased in relation to the sham-operated control rats. The mechanical parameters for the fracture point also significantly decreased (not shown).

The extract of *T. medium* in both doses did not affect the mechanical properties of the tibial metaphysis, whereas extract of *T. pratense* significantly increased the maximum load sustained by the bone in the ovariectomized rats. However, energy for maximum load and the intrinsic mechanical parameters (maximum stress and Young's modulus) remained unchanged.

The supplementation of estradiol significantly increased both the maximum load and maximum stress. Although the accumulated energy for the maximum load remained unchanged, Young's modulus did not significantly differ from the sham-operated control rats.

3.4. Mechanical Properties of Cortical Bone (Femoral Diaphysis). Mechanical properties of the femoral diaphysis were not affected by estrogen deficiency. Young's modulus, as well as extrinsic and intrinsic parameters determined for the maximum load (Figure 1) and the fracture point load (Table 4) in ovariectomized control rats, did not differ from those of the sham-operated control rats.

Administration of the extracts from both *Trifolium* species increased the strength of the femoral diaphysis in the ovariectomized rats. The maximum load and the energy for the maximum load tended to increase after administration of both isoflavone doses, and the maximum stress was significantly increased after administration of *T. medium* at both doses and *T. pratense* at the higher dose, when compared with the ovariectomized control rats (Figure 1). Similarly, the load and stress for the fracture point significantly increased, in relation to the ovariectomized control rats, after administration of the higher isoflavone dose. There was no significant effect of the extracts on the Young's modulus of the femoral diaphysis (Table 4).

After administration of estradiol, the stress for the maximum load and for the fracture load significantly increased, in comparison with the ovariectomized control rats; however the effect was slighter than that observed after administration of the higher *Trifolium* extract doses.

3.5. Mechanical Properties of the Femoral Neck. Estrogen deficiency caused a statistically significant decrease in the strength of the femoral neck in the ovariectomized control rats in relation to the sham-operated control rats (Figure 2).

Administration of the *Trifolium* extracts at both isoflavone doses did not affect the maximum load sustained by the femoral neck of the ovariectomized rats. Also supplementation of estradiol to the ovariectomized rats did not significantly affect the strength of the femoral neck.

3.6. Histomorphometric Parameters of the Femur. Although the transverse growth in the femur of the ovariectomized control rats was slightly increased in comparison with the sham-operated rats, the transverse cross-section area of the cortical bone and of the whole diaphysis were not significantly affected (Table 5). However, there was a significant increase in the ratio of the transverse cross-section area of the marrow cavity to the area of the whole diaphysis, indicating the increased cortical bone resorption. The width of trabeculae in the femoral epiphysis and metaphysis significantly decreased in the ovariectomized control rats, indicating a possibility of increased cancellous bone resorption.

The *Trifolium* extracts did not affect the histomorphometric parameters of the femoral diaphysis of the ovariectomized rats but increased the width of epiphyseal trabeculae. Only *T. pratense* extract significantly increased the width of metaphyseal trabeculae.

Administration of estradiol significantly counteracted the effect of estrogen deficiency on the transverse cross-section marrow cavity/diaphysis area ratio in the diaphysis and the width of trabeculae in epiphysis and metaphysis of the femur.

3.7. Serum Biochemical Bone Turnover Markers. Estrogen deficiency increased the serum level of the biochemical marker of bone resorption (RatLaps) and tended to increase the marker of bone formation (osteocalcin) in comparison with the sham-operated controls (Table 6).

The *Trifolium* extracts did not affect the levels of the serum markers of bone turnover in ovariectomized rats.

Supplementation of the ovariectomized rats with estradiol normalized the levels of bone turnover markers.

4. Discussion

Administration of the *Trifolium* extracts significantly affected the skeletal system of ovariectomized rats, counteracting some changes induced by estrogen deficiency, and also inducing changes which seemed to be unrelated to typical estrogenic effects in rats with decreased estrogen levels. There were some differences between the effects of *Trifolium* extracts and those of estradiol, and also differences between the effects of *T. medium* and *T. pratense*.

The data on the skeletal effects of *T. pratense* extracts are very limited, but there are numerous reports on skeletal effects of soy isoflavones. The most abundant soy isoflavones (aglycones) are genistein and daidzein, and the main *Trifolium* isoflavones, biochanin A and formononetin, are metabolized to genistein and daidzein, respectively, in mammalian organisms [27]. Nevertheless, the results of studies on soy product effects on the skeletal system in

Table 1: Effects of *Trifolium medium* L. (TM) and *Trifolium pratense* L. (TP) extracts on the body mass gain, mass of estrogen-dependent organs, and serum levels of estradiol and total cholesterol in ovariectomized (OVX) rats.

Parameters	Sham-operated rats	OVX rats					
		Control	TM10	TM20	TP10	TP20	Estradiol
Final body mass (g)	234.8 ± 3.6	263.1 ± 4.5***	268.3 ± 4.2***	274.8 ± 4.8***	263.9 ± 3.9***	265.2 ± 4.6***	250.6 ± 5.0*
Body mass gain after 28 days (g)	16.5 ± 2.4	39.0 ± 2.5***	42.1 ± 2.3***	43.8 ± 3.6***	38.3 ± 2.0***	40.7 ± 3.3***	25.6 ± 2.9*••
Uterus mass (mg/100 g of body mass)	235.66 ± 27.70	35.09 ± 1.81***	35.64 ± 1.60***	36.03 ± 1.41***	39.39 ± 1.67***	37.19 ± 1.79***	67.31 ± 4.89***•••
Thymus mass (mg/100 g of body mass)	130.07 ± 11.12	196.83 ± 9.25**	203.63 ± 21.41**	199.37 ± 9.66***	201.27 ± 13.81**	196.57 ± 11.35*	181.74 ± 15.74**
Total cholesterol (mg/100 mL)	49.93 ± 2.90	69.11 ± 2.83***	68.88 ± 3.61***	65.66 ± 2.62**	68.23 ± 3.17***	66.13 ± 3.30**	56.19 ± 3.81•
Estradiol (pg/mL)	20.64 ± 3.05	10.37 ± 0.80*	9.83 ± 0.63**	11.68 ± 0.61	9.34 ± 0.44**	10.95 ± 1.07*	13.84 ± 1.26

The lyophilized extracts from TM or TP at doses corresponding to 10 and 20 mg/kg of isoflavone aglycones daily (TM10, TP10 and TM20, TP20, resp.), or estradiol (0.2 mg/kg daily), were administered orally to OVX rats for 4 weeks. Results are presented as the mean ± SEM (n = 9-10). One-way ANOVA followed by Duncan's test or, when appropriate, Kruskal-Wallis ANOVA followed by Mann-Whitney U test was used for evaluation of the significance of the results. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$—significantly different from sham-operated control rats. $^{\bullet}P < 0.05$, $^{\bullet\bullet}P < 0.01$, $^{\bullet\bullet\bullet}P < 0.001$—significantly different from OVX control rats.

Table 2: Effects of *Trifolium medium* L. (TM) and *Trifolium pratense* L. (TP) extracts on bone mass and mineralization in ovariectomized (OVX) rats.

Parameters		Sham-operated rats	OVX rats					
			Control	TM10	TM20	TP10	TP20	Estradiol
Bone mass/body mass ratio (mg/100 g of body mass)	Femur	296.04 ± 3.89	259.58 ± 4.14***	262.10 ± 4.81***	254.99 ± 5.24***	258.66 ± 4.24***	267.53 ± 6.82***	271.58 ± 4.11***
	Tibia	225.87 ± 3.06	198.61 ± 2.43***	193.37 ± 3.14***	192.83 ± 2.74***	200.52 ± 2.80***	200.79 ± 3.72***	202.79 ± 3.40***
	L-4 vertebra	80.48 ± 1.46	72.80 ± 2.18**	68.43 ± 1.04***	68.52 ± 1.11***	73.18 ± 0.94**	72.46 ± 1.65**	75.65 ± 2.03
Mineral mass/bone mass ratio (mg/100 mg of bone mass)	Femur	45.74 ± 0.34	43.92 ± 0.41**	42.59 ± 0.47***	43.17 ± 0.38***	44.42 ± 0.43	43.47 ± 0.56**	44.48 ± 0.47*
	Tibia	46.38 ± 0.56	44.37 ± 0.41**	43.99 ± 0.27***	44.38 ± 0.39**	44.42 ± 0.42**	45.21 ± 0.36	45.45 ± 0.55
	L-4 vertebra	44.32 ± 0.48	42.29 ± 0.77	42.80 ± 0.51	42.64 ± 0.35	43.11 ± 0.45	42.30 ± 0.56	42.92 ± 0.62
Calcium content (mg/g of mineral mass)	Femur	382.79 ± 4.77	379.61 ± 4.73	395.65 ± 5.20	396.39 ± 5.25	394.26 ± 4.06	395.70 ± 6.08	391.82 ± 5.48
	Tibia	397.83 ± 3.93	406.06 ± 9.05	443.56 ± 10.99**•	445.64 ± 9.88***••	439.05 ± 9.18**•	446.67 ± 11.49**•	425.57 ± 14.43
	L-4 vertebra	409.72 ± 7.67	409.35 ± 5.82	446.48 ± 6.59*•	444.44 ± 12.09*•	451.76 ± 9.76**••	453.69 ± 12.65**••	427.06 ± 12.12
Phosphorus content (mg/g of mineral mass)	Femur	161.50 ± 1.73	160.89 ± 1.60	163.82 ± 1.29	163.92 ± 1.51	164.47 ± 1.38	164.41 ± 1.32	162.48 ± 3.25
	Tibia	154.36 ± 2.17	153.32 ± 2.65	168.77 ± 5.30*•	169.92 ± 5.29*•	168.61 ± 3.99*•	168.28 ± 4.36*•	153.65 ± 3.31
	L-4 vertebra	165.01 ± 2.07	163.93 ± 2.91	178.55 ± 4.93*•	177.09 ± 3.77*•	176.66 ± 2.97*•	178.18 ± 5.12*•	171.48 ± 3.45

The lyophilized extracts from TM or TP at doses corresponding to 10 and 20 mg/kg of isoflavone aglycones daily (TM10, TP10 and TM20, TP20, resp.), or estradiol (0.2 mg/kg daily), were administered orally to OVX rats for 4 weeks. Results are presented as the mean ± SEM (n = 9-10). One-way ANOVA followed by Duncan's test or, when appropriate, Kruskal-Wallis ANOVA followed by Mann-Whitney U test was used for evaluation of the significance of the results. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$—significantly different from sham-operated control rats. $^{\bullet}P < 0.05$, $^{\bullet\bullet}P < 0.01$—significantly different from OVX control rats.

Table 3: Effects of *Trifolium medium* L. (TM) and *Trifolium pratense* L. (TP) extracts on mechanical properties of the tibial metaphysis in ovariectomized (OVX) rats.

Parameters	Sham-operated rats	OVX rats					
		Control	TM10	TM20	TP10	TP20	Estradiol
Maximum load (N)	120.63 ± 3.95	66.85 ± 3.56***	70.02 ± 2.53***	69.76 ± 2.41***	79.66 ± 2.86***••	77.12 ± 2.86***•	90.38 ± 3.08***•••
Displacement for maximum load (mm)	0.84 ± 0.06	0.90 ± 0.07	0.83 ± 0.04	0.88 ± 0.02	0.82 ± 0.03	0.89 ± 0.06	0.82 ± 0.05
Energy for maximum load (mJ)	52.02 ± 4.36	39.20 ± 4.61**	33.52 ± 2.33***	36.49 ± 1.61**	36.58 ± 2.67**	37.77 ± 2.86**	39.93 ± 2.91**
Maximum stress (MPa)	103.82 ± 4.23	54.82 ± 4.38***	56.34 ± 3.61***	55.81 ± 2.22***	60.05 ± 2.69***	59.65 ± 2.77***	73.74 ± 4.07***•••
Young's modulus (MPa)	3410.78 ± 237.74	2318.16 ± 182.21**	2246.02 ± 147.56**	2248.36 ± 117.87***	2149.64 ± 178.89***	2417.34 ± 140.06***	2770.31 ± 286.73

The lyophilized extracts from TM or TP at doses corresponding to 10 and 20 mg/kg of isoflavone aglycones daily (TM10, TP10 and TM20, TP20, resp.), or estradiol (0.2 mg/kg daily), were administered orally to OVX rats for 4 weeks. Results are presented as the mean ± SEM (n = 9-10). One-way ANOVA followed by Duncan's test or, when appropriate, Kruskal-Wallis ANOVA followed by Mann-Whitney U test was used for evaluation of the significance of the results. $^{**}P < 0.01$, $^{***}P < 0.001$—significantly different from sham-operated control rats. $^{\bullet}P < 0.05$, $^{\bullet\bullet}P < 0.01$, $^{\bullet\bullet\bullet}P < 0.001$—significantly different from OVX control rats.

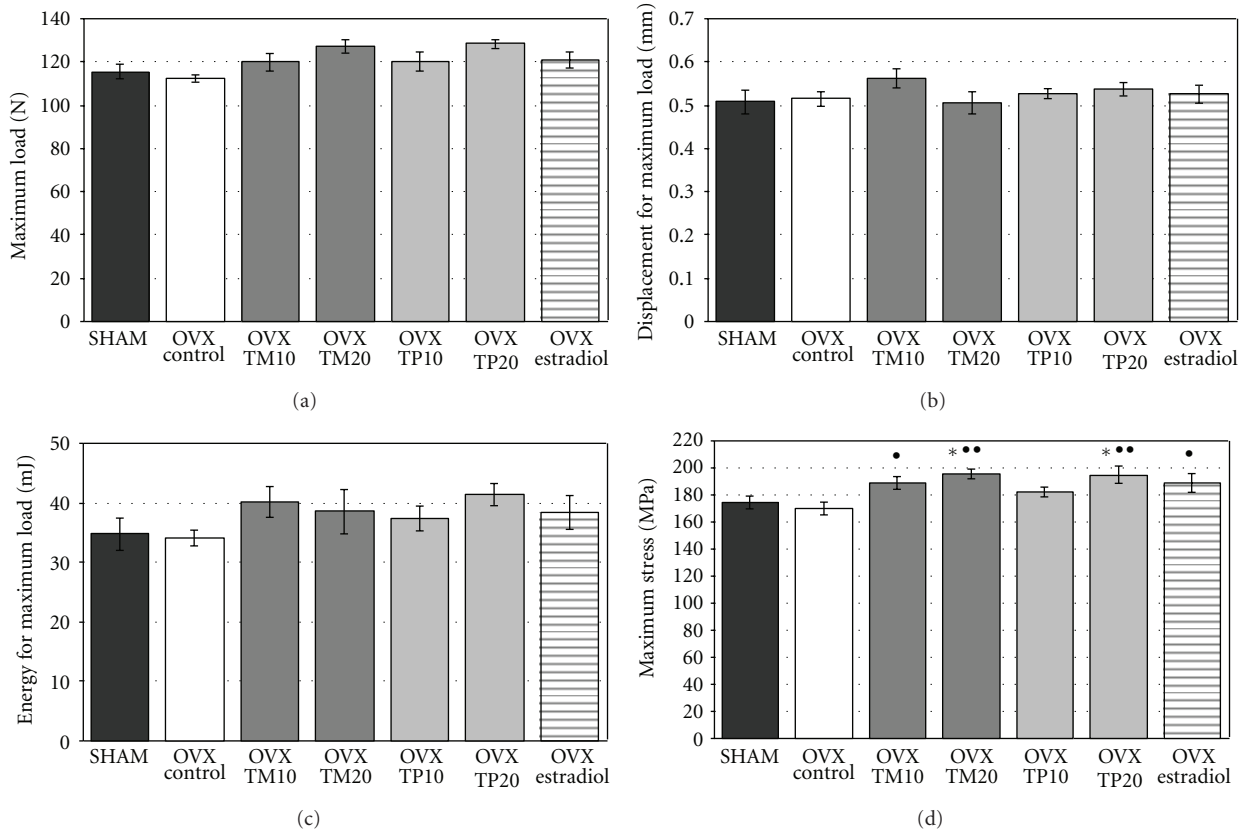

Figure 1: Effects of *Trifolium medium* L. (TM) and *Trifolium pratense* L. (TP) extracts on mechanical properties of the femoral diaphysis (the maximum load point) in ovariectomized (OVX) rats. The lyophilized extracts from TM or TP at doses corresponding to 10 and 20 mg/kg of isoflavone aglycones daily (TM10, TP10 and TM20, TP20, resp.), or estradiol (0.2 mg/kg daily), were administered orally to OVX rats for 4 weeks. Results are presented as the mean ± SEM (n = 9-10). One-way ANOVA followed by Duncan's test was used for evaluation of the significance of the results. $^{*}P < 0.05$—significantly different from sham-operated control rats (SHAM). $^{\bullet}P < 0.05$,$^{\bullet\bullet}P < 0.01$—significantly different from OVX control rats.

Table 4: Effects of *Trifolium medium* L. (TM) and *Trifolium pratense* L. (TP) extracts on mechanical properties of the femoral diaphysis (parameters for the fracture point and Young's modulus) in ovariectomized (OVX) rats.

Parameters	Sham-operated rats	OVX rats					
		Control	TM10	TM20	TP10	TP20	Estradiol
Fracture load (N)	114.56 ± 3.94	111.98 ± 2.33	116.01 ± 4.44	125.82 ± 3.44$^{\bullet}$	116.84 ± 4.00	127.66 ± 2.66$^{*\bullet\bullet}$	120.15 ± 3.80
Displacement for fracture load (mm)	0.52 ± 0.03	0.52 ± 0.02	0.61 ± 0.04	0.52 ± 0.03	0.55 ± 0.02	0.56 ± 0.02	0.54 ± 0.02
Energy for fracture load (mJ)	35.69 ± 2.75	34.71 ± 1.55	46.36 ± 5.00	40.07 ± 4.29	40.04 ± 2.89	43.73 ± 2.66	40.56 ± 3.58
Stress for fracture load (MPa)	172.68 ± 5.76	169.16 ± 5.34	182.65 ± 6.06	193.14 ± 4.41$^{*\bullet\bullet}$	178.01 ± 3.63	193.34 ± 6.89$^{*\bullet\bullet}$	187.79 ± 7.14$^{\bullet}$
Young's modulus (MPa)	9368.24 ± 430.74	8679.70 ± 689.37	9612.18 ± 499.99	9651.65 ± 412.61	9356.92 ± 148.39	8759.22 ± 1028.53	9687.52 ± 654.88

The lyophilized extracts from TM or TP at doses corresponding to 10 and 20 mg/kg of isoflavone aglycones daily (TM10, TP10 and TM20, TP20, resp.), or estradiol (0.2 mg/kg daily), were administered orally to OVX rats for 4 weeks. Results are presented as the mean ± SEM (n = 9-10). One-way ANOVA followed by Duncan's test or, when appropriate, Kruskal-Wallis ANOVA followed by Mann-Whitney U test was used for evaluation of the significance of the results. $^{*}P < 0.05$—significantly different from sham-operated control rats. $^{\bullet}P < 0.05$. $^{\bullet\bullet}P < 0.01$—significantly different from OVX control rats.

Table 5: Effects of *Trifolium medium* L. (TM) and *Trifolium pratense* L. (TP) extracts on histomorphometric parameters of the femur in ovariectomized (OVX) rats.

Parameters		Sham-operated rats	OVX rats					
			Control	TM10	TM20	TP10	TP20	Estradiol
Transverse cross-section area (mm^2)	Cortical bone	5.25 ± 0.08	5.13 ± 0.08	5.12 ± 0.10	5.31 ± 0.09	5.22 ± 0.06	5.28 ± 0.10	5.26 ± 0.11
	Marrow cavity	2.65 ± 0.08	2.82 ± 0.05	2.82 ± 0.08	2.75 ± 0.14	2.86 ± 0.06	2.77 ± 0.07	2.55 ± 0.10
	Whole diaphysis	7.90 ± 0.10	7.96 ± 0.12	7.94 ± 0.16	8.06 ± 0.15	8.08 ± 0.10	8.05 ± 0.10	7.82 ± 0.19
Transverse cross-section marrow cavity/diaphysis area ratio		0.335 ± 0.008	0.355 ± 0.003*	0.355 ± 0.006	0.339 ± 0.012	0.354 ± 0.005*	0.344 ± 0.009	0.326 ± 0.007$^{\bullet\bullet}$
Transverse growth (μm)	Periosteal	38.68 ± 2.50	43.70 ± 2.99	40.14 ± 2.84	40.01 ± 1.98	39.65 ± 0.88	37.75 ± 2.24	38.37 ± 3.91
	Endosteal	28.54 ± 2.99	33.62 ± 2.22	31.47 ± 2.45	29.70 ± 2.90	31.89 ± 1.81	29.75 ± 1.08	29.74 ± 1.67
Width of trabeculae (μm)	Epiphysis	58.76 ± 0.79	53.47 ± 1.09**	57.03 ± 0.73$^{\bullet}$	57.28 ± 0.67$^{\bullet}$	57.85 ± 1.26$^{\bullet}$	59.42 ± 1.35$^{\bullet\bullet}$	60.53 ± 1.49$^{\bullet\bullet}$
	Metaphysis	36.04 ± 0.59	32.98 ± 0.86**	34.54 ± 1.03	35.00 ± 0.80	36.23 ± 0.70$^{\bullet}$	37.46 ± 1.35$^{\bullet\bullet}$	38.49 ± 1.78$^{\bullet}$

The lyophilized extracts from TM or TP at doses corresponding to 10 and 20 mg/kg of isoflavone aglycones daily (TM10, TP10 and TM20, TP20, resp.), or estradiol (0.2 mg/kg daily), were administered orally to OVX rats for 4 weeks. Results are presented as the mean ± SEM (n = 9-10). One-way ANOVA followed by Duncan's test or, when appropriate, Kruskal-Wallis ANOVA followed by Mann-Whitney U test was used for evaluation of the significance of the results. $^{*}P < 0.05$, $^{**}P < 0.01$—significantly different from sham-operated control rats. $^{\bullet}P < 0.05$, $^{\bullet\bullet}P < 0.01$—significantly different from OVX control rats.

Table 6: Effects of *Trifolium medium* L. (TM) and *Trifolium pratense* L. (TP) extracts on the serum bone turnover markers in ovariectomized (OVX) rats.

Parameters	Sham-operated rats	OVX rats					
		Control	TM10	TM20	TP10	TP20	Estradiol
Osteocalcin (ng/mL)	238.30 ± 27.70	325.47 ± 26.72	327.36 ± 28.13	344.84 ± 25.61	344.84 ± 38.73	332.50 ± 39.91	254.33 ± 17.13
RatLaps (ng/mL)	20.88 ± 1.82	30.89 ± 1.44**	30.79 ± 1.99**	30.74 ± 1.93**	32.99 ± 1.78***	28.59 ± 2.09*	23.39 ± 2.80$^{\bullet}$

The lyophilized extracts from TM or TP at doses corresponding to 10 and 20 mg/kg of isoflavone aglycones daily (TM10, TP10 and TM20, TP20, resp.), or estradiol (0.2 mg/kg daily), were administered orally to OVX rats for 4 weeks. Results are presented as the mean ± SEM (n = 9-10). One-way ANOVA followed by Duncan's test was used for evaluation of the significance of the results. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$—significantly different from sham-operated control rats. $^{\bullet}P < 0.05$—significantly different from OVX control rats.

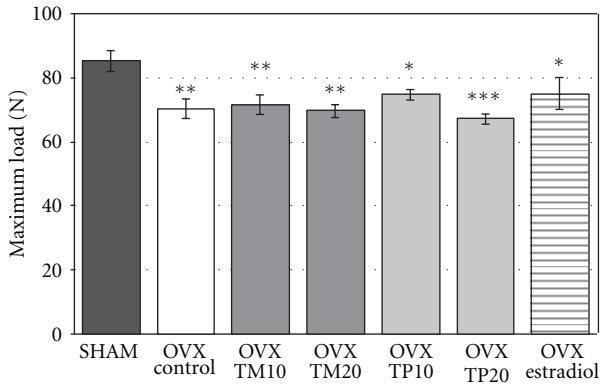

Figure 2: Effects of *Trifolium medium* L. (TM) and *Trifolium pratense* L. (TP) extracts on the strength of the femoral neck in ovariectomized (OVX) rats. The lyophilized extracts from TM or TP at doses corresponding to 10 and 20 mg/kg of isoflavone aglycones daily (TM10, TP10 and TM20, TP20, resp.), or estradiol (0.2 mg/kg daily), were administered orally to OVX rats for 4 weeks. Results are presented as the mean ± SEM (n = 9-10). One-way ANOVA followed by Duncan's test was used for evaluation of the significance of the results, $^{*}P < 0,05.^{**}P < 0.01$, $^{***}P < 0.001$—significantly different from sham-operated control rats (SHAM).

humans are inconsistent [28–32]. Interestingly, a recent study on the effects of dietary phytoestrogens on bone density performed in a European population indicated an independent association between bone density and formononetin in postmenopausal women and biochanin A in men [33]. However, the most important data on the isoflavone effects on fracture risk are still missing [1, 34].

So far, differential effects of particular red clover isoflavones on the skeletal system were demonstrated in experimental *in vivo* studies on biochanin A [35], formononetin [36, 37], genistein [35, 38–46], daidzein [35, 46], and an active metabolite of daidzein—equol [43–46]. However, in aforementioned studies, free isoflavone aglycones were used, whereas in the plants, and in plant extracts (also those examined in our study), isoflavones occur mainly as glycosides.

The present study was carried out using a model of 3-month-old ovariectomized rats, developing osteoporotic changes due to estrogen deficiency. *Trifolium* extracts were administered to the rats for 4 weeks, starting a week after the ovariectomy. A 4-week period of administration was long enough to observe skeletal effects of substances of plant origin in our previous studies [25, 41, 47, 48].

Estrogen deficiency induced characteristic osteoporotic changes in the ovariectomized control rats. Supplementation with estradiol, in most parameters, counteracted the effects of estrogen deficiency: the worsening of the tibial strength, the changes in bone histomorphometric parameters, and increases in bone turnover markers. Moreover, some parameters of the femoral diaphysis improved.

The *Trifolium* extracts did not counteract the effects of estrogen deficiency so consistently as did estradiol. Only *T. pratense* extract increased the strength of the tibial metaphysis (this effect was weaker than that of estradiol). *T. pratense* extract also exerted stronger effect on a cancellous bone histomorphometric parameter (metaphyseal trabeculae width). The reason for the stronger estrogenic activity of the *T. pratense* extract may be speculated. It has been proposed that the estrogenic activity of isoflavones is connected with their metabolism to equol [46, 49, 50]. For the formation of equol, exclusively intestinal microflora is responsible [49]. From among the *Trifolium* isoflavones, formononetin and daidzein can be metabolized to equol, and biochanin A and genistein cannot [49, 50]. Total content of formononetin and daidzein in *T. medium* and *T. pratense* extracts was very similar. However, the *T. pratense* extract, containing smaller amount of isoflavone aglycones, had to be administered at higher doses in order to secure the same isoflavone aglycone intake. Bigger amount of the administered *T. pratense* extract, characteristic of high carbohydrate content, might have contributed to the creation of better environment that stimulated the growth of bacteria responsible for the metabolism of isoflavone glycosides, followed by higher equol production. It has been observed that bioavailability of soy isoflavones depends, among others, on the activity of intestinal bacteria, and a diet rich in carbohydrates may stimulate equol production [50].

Apart from the effects on the cancellous bone, similar to those observed for estradiol, both *Trifolium* extracts exerted other skeletal effects in estrogen-deficient rats: a significant increase in the strength of the compact bone of the femoral diaphysis and increases in the calcium and phosphorus content in the bone mineral of the vertebra and tibia, but not of the femur. The improvement in the strength of the cortical bone of the femoral diaphysis, induced by the *Trifolium* extracts, is consistent with a recent peripheral quantitative computed tomography study of Shedd-Wise et al. [51], who reported that, in postmenopausal women treated with soy isoflavones, some protective effect on cortical bone was observed.

It may be speculated that some skeletal effects of the *Trifolium* extracts may be attributed, at least in part, to other plant constituents, like other flavonoids and phenolic acids; some of phenolic acids have been reported to favorably affect the skeletal system of ovariectomized rats [47, 52]. In our recent study we have observed that caffeic and chlorogenic acids at high doses slightly increased the strength of cortical bone of the femoral diaphysis only [53]. The mechanism of action of the extracts may be connected with antioxidant activity of polyphenolic compounds, including isoflavones. The role of oxidative stress in the development of osteoporosis gains growing attention [54].

The increasing effects of the *Trifolium* extracts on the calcium and phosphorus content in the bone mineral of the tibia and vertebra are surprising. The changes were not accompanied with the increase of the mineral substances (ash) content in the bones. The exact character of changes in the mineral chemical structure, as well as the meaning of the changes for the bone quality, is not known. Significant increases in the calcium and phosphorus content were not observed in the femurs in which thick diaphysis (compact bone) strongly contributes to the bone mass. The *Trifolium* extracts improved mechanical quality of the femoral diaphysis. On the other hand, there was much less effect or

no effect of the extracts on the mechanical properties of cancellous bone of the tibia. It is possible that antiresorptive effect in cancellous bone (increases in the trabeculae width observed in the femur) is balanced by in fact unfavorable effect of the structural changes in bone mineral, which in consequence does not allow improving bone strength. The issue needs further investigations.

It may only be speculated that the increase in the calcium content may be the result of a high intake of sugars present in the *Trifolium* extracts. Some of sugars might have reached the colon and have been fermented by intestinal bacteria, reducing pH of the environment and increasing calcium absorption. So far, such mechanism was proposed for inulin-type fructans, which were reported to increase calcium absorption [55]. Increased calcium absorption was accompanied with increased mineral concentrations in bones of rats fed a diet containing fructooligosaccharides [56]. *Trifolium pratense* does not contain fructans but is rich in other nonstructural carbohydrates [57].

Contrary to estradiol, the extracts did not affect the uterus mass. Moreover, there were no effects on the body mass gain and total cholesterol level, indicating the possibility that the *Trifolium* phytoestrogens did not affect all estrogenic targets in the body. The lack of significant effect on uterine mass is consistent with an experimental study on rabbits [15]. In fact, relative binding affinity of particular *Trifolium* isoflavones to the rat uterine estrogen receptors was much lower than that of estradiol [58]. The lack of significant effects on the uterus has been attributed to their preferential binding to estrogen receptor β, which is less expressed in the uterus [15]. Taking into consideration estrogenic effects on bones, results of the present study support the notion that *Trifolium* phytoestrogens may act as SERMs. It should be stated, however, that higher doses of red clover extract resulted in estrogenic effects, increasing the uterus mass and differentiated vaginal cells in ovariectomized rats [59].

Some skeletal effects of the extracts observed in the present study in rats may not be observed in humans due to differential pattern of isoflavone metabolism by intestinal bacterial flora. Probably due to differences in the intestinal bacterial flora, humans divide into equol producers, and equol nonproducers [50, 60]. Rats, with their large cecum and abundant microflora [49], are much more capable to produce equol than humans [61]. Moreover, different compounds of phytoestrogenic plant extracts undergo metabolism dependent on gut microflora and human enzymes, and there is great interindividual variation concerning the metabolism [27, 50]. Little is known on the extent to which different metabolites, exerting agonistic or antagonistic activity towards α or β estrogen receptors, are formed after consumption of isoflavone-rich foods [27]. Intake of phytoestrogens may be connected not only with health benefits. Except affecting estrogen receptors, they may interact with other biological systems, as comprehensively reviewed by Leclercq et al. [62], and exert serious adverse effects [63]. Estrogenic effects of the *Trifolium* extracts on different targets, including those connected with blood coagulation and risk of stroke or venous thromboembolism, are possible and need further studies.

The present study has some limitations. One of them is a single study period (4 weeks), which may be considered as a relatively short one for testing the effects of dietary supplements on estrogen-deficiency model in rats. It is possible that longer time of the *Trifolium* extract administration would allow to observe more of their estrogenic effects, including those on bone turnover markers. Another limitation of the study is that it concerns only the effects of the extracts; parallel examination of their main components would enable pointing the compounds responsible for their skeletal activity. This issue requires further studies.

In conclusion, the effects of *T. medium* and *T. pratense* extracts on the skeletal system of estrogen-deficient rats differed from those exerted by estradiol, indicating more complex mechanism of action. Skeletal effects of *T. medium* extract seemed to be less similar to those exerted by estradiol than the effects of *T. pratense* extract. It is possible that other than isoflavone constituents of extracts contributed to their effects on the skeletal system.

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Research Article

Comparative Effects of Er-Xian Decoction, *Epimedium* Herbs, and Icariin with Estrogen on Bone and Reproductive Tissue in Ovariectomized Rats

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Er-Xian Decoction (EXD), *Epimedium* herbs (herbs of *Epimedium brevicornum* Maxim, EBH), and icariin (ICA) have been proven to have estrogen-like and antiosteoporotic activity and are used for the treatment of osteoporosis, menopausal syndrome, and age-associated diseases. The present study found that EXD, EBH, and ICA treatments, emulating estrogen, significantly contributed to bone density and architecture in OVX rats and that EXD is similar to estrogen and exerts a concomitant effect on bone formation and bone resorption at the tissue level, while EBH and ICA produced bone-protective effects mainly by inhibiting bone resorption. Nevertheless, EXD, EBH, and ICA treatments manifested a fewer adverse effects on the uterus, mammary gland, and vagina compared to estrogen administrations. Among the EXD, EBH, and ICA, EXD was found to have superior efficacy and safety profile.

1. Introduction

Postmenopausal osteoporosis is a common disease characterized by a systemic impairment of bone mass and microarchitecture that result in fragility fractures [1]. As gonadal hormone deficiency plays a key role in the pathogenesis of postmenopausal osteoporosis, estrogen replacement therapy was regarded in past decades as the most effective and the first choice for the treatment of this disease [2]. Estrogen regulates bone remodeling via estrogen receptor alpha (ERα) and estrogen receptor beta (ERβ). The uterus, vagina, and mammary gland express estrogen receptor and are targets for estrogen. Therefore estrogen may exert stimulatory effects on these estrogen-sensitive organs. Also, estrogen exposure enhances the expression of progesterone receptor (PR), through which progesterone antagonizes the stimulatory effects of estrogen on endometrial tissue [3]. The Women's Health Initiative enrolled more than 150,000 women for a comprehensive trial regarding the effect of combined estrogen and progestin. The risk-benefit index highlighted coronary heart disease, invasive breast cancer, pulmonary embolism, endometrial cancer, and colorectal cancer as side effects [4, 5]. Since the overall health risks exceeded benefits, the trial was stopped. Therefore, the interest to find effective and safe alternatives for the treatment of osteoporosis has grown in recent years [4].

Traditional Chinese medicines containing multi-interactive compounds, which have been used for centuries in China for treatment of bone disorders, have attracted the attention of researchers for their effects on the management of menopausal and related medical conditions [6]. Er-Xian Decoction (EXD), a popular Chinese medicinal formula comprised of *Epimedium brevicornum* Maxim (Berberidaceae, whole herb, EBH), *Curculigo orchioides* Gaertn. (Amaryllidaceae, rhizome, COR), *Anemarrhena asphodeloides* Bge. (Liliaceae, rhizome, AAR), *Phellodendron chinense* Schneid (Rutaceae, bark, PCB), *Morinda officinalis* How. (Rubiaceae, root, MOR), and *Angelica sinensis* (Oliv.) Diels

(Umbelliferae, root, ASR) in a compositional ratio of 9 : 9 : 6 : 6 : 9 : 9, has been used for the treatment of osteoporosis, menopausal syndrome, and age-associated diseases over the past 60 years [7]. A systemic review and meta-analysis of 677 participants involved in 5 clinical investigations indicated that EXD was clinically effective in relieving menopausal syndrome via increasing circulatory estradiol levels [8, 9]. Both animal experiments and clinical practices proved that EXD had definite protective effects on bone loss induced by estrogen deficiency [7, 10, 11].

According to traditional Chinese medicine, *Epimedium brevicornum* and *Phellodendron chinense* are essential ingredients of EXD and appear to play important roles in pmeliorating signs and symptoms of the menopausal syndrome and osteoporosis. *Curculigo orchioides* and *Morinda officinalis* help strengthen the curative effect of the *Epimedium brevicornum*, and *Anemarrhena asphodeloides* helps *Phellodendron chinense* to play a fuller role. *Angelica sinensis* is another ingredient that cooperates with the above five medications to strengthen the therapeutic effects and treat the accompanying disease or syndromes. *Epimedium* herb, as an essential ingredient of EXD, has been reported to possess phytoestrogen-like flavonoids including icariin, incaritin, and baohuoside. These phytoestrogen flavonoids have been shown to exert protective effects on bone loss in OVX rats and postmenopausal women [12, 13]. However, it has been demonstrated that *Epimedium* flavonoids are weak estrogen agonists that act via the ERα and ERβ [14]. Phytoestrogens have the ability to interfere with estrogen action either by interacting directly with the estrogen receptor (ER) or indirectly by modulating endogenous estrogen concentrations [15, 16]. Accumulating evidence from *in vitro* experiments, animal studies, and human clinical trials suggests that in addition to their beneficial effects, phytoestrogens may also increase the risk for several hormone-dependent diseases in humans [17]. Therefore, systemic treatment with EXD, *Epimedium* herbs, or icariin can be expected to exert weak estrogen agonistic effects on estrogen-receptor-positive tissues and may produce unwanted hormone-related side effects such as endometrial hyperplasia and endometrial carcinomas.

The aim of the present study was to compare with estrogen the bone protective effects of EXD, *Epimedium* herbs (EBH), and icariin (ICA) in ovariectomized rats. The safety of EXD, EBH, and ICA was also determined by observing the histologic structure changes and measuring the ERα, ERβ, and PR expressions in reproductive tissues.

2. Materials and Methods

2.1. Materials. The assay kits for alkaline phosphatase (ALP), tartrate resistant acid phosphatase (TRAP), serum or urine calcium (Ca), inorganic phosphorus (P), and urine creatinine (Cr) were purchased from Nanjing Jiancheng Bioengineering Institute (Nanjing, China). Radioimmunoassay (RIA) kits for measurement of estradiol levels were purchased from China Institute of Atomic Energy (Beijing, China). Nylestriol was purchased from Hualian Pharmaceutical Company (Shanghai, China,). Icariin (ICA) and extracts of *Epimedium* herbs (EBH) were purchased from Huike Biopharmaceutical Company (Xi'an, China). The six plant materials in EXD were obtained from Hua Yu Pharmaceutical Company (Shanghai, China) and identified by Professor H.C. Zheng; the voucher specimen numbers for EBH (2010110601), COR (2010110602), AAR (2010110603), PCB (2010110604), MOR (2010110605), and ASR (20101106) are available at the herbarium of the Department of Pharmacognosy, School of Pharmacy of the Second Military Medical University (Shanghai, China). The six plant materials were mixed in a compositional ratio of 9: 9: 6: 6: 9: 9 according to the compatible theory in traditional Chinese medicine (EBH: 281.25 g, COR: 281.25 g, AAR: 187.5 g, PCB: 187.5 g, MOR: 281.25 g, and ASR: 281.25 g; dried materials) and extracted by decocting the mixed herbs with 10x (v/w) distilled water at 100°C for 2 hours. After filtration, the residue was boiled for an additional 1 hour. Filtrates were mixed together and lyophilized with a freeze drier (Labconco, FreeZone), and the resulting 150 g of dry powder were kept at 4°C.

2.2. Animals and Treatments. Forty-eight female Sprague-Dawley (SD) rats aged 12 weeks were purchased from Slacom Experimental Animal Company (Shanghai, China, SCXK: 20070003) and acclimated to conditions for 1 week before the experiment. The experimental animals were housed in an air-conditioned room with 12 h/12 h light-dark illumination cycles at constant temperature (25 ± 2°C) and humidity (50%± 10%). Food and drinking water were supplied ad libitum. The rats were weighed weekly during the experimental period. The osteoporotic model was established 12 weeks after bilateral ovariectomy. Of the 48 female SD rats, 8 were sham operated and treated with deionized water as the control group (sham). The remaining rats were bilaterally ovariectomized and equally randomized into five groups. Rats were treated with deionized water (OVX model control), NYL (Nylestriol, 1 mg/kg, weekly), ICA (Icariin, 20 mg/kg, daily), EBH (extracts of *Epimedium* herbs, 100 mg/kg, daily), or EXD (Er-Xian Decoction; 600 mg/kg, daily) by intragastric administration for 12 weeks. This experiment was approved by the Bioethics Committee of the Second Military Medical University (Shanghai, China), and the procedures of the experiment strictly adhered to generally accepted international rules and regulations.

2.3. Sample Collection. At the end of treatment, the rats were housed individually in metabolic cages without food for 1 day. Urine samples were collected from 20:00 pm to 8: 00 am the next day. Blood samples were collected via the abdominal artery, stabilized with sodium heparin, and then centrifuged at 5500 ×g for 10 min. Urine and serum samples were then stored at −80°C for biochemical determinations. The uterus was removed from each rat, cleaned of adhering soft tissues, and immediately weighed. The left femurs were dissected and placed in physiologic saline and stored at −20°C for measurement of bone mineral density (BMD). Right tibias were removed and fixed in 10% neutral-buffered formalin. Mammary glands, vagina, and uteri were removed and each was cut into two parts equally, one part was stored in liquid

nitrogen for western blotting analysis, and the other was fixed in 10% neutral-buffered formalin and stored at 4°C.

2.4. Assay for Serum and Urine Biochemistry. Serum or urine calcium (Ca), inorganic phosphorus (Pi) concentration, serum alkaline phosphates (ALPs), tartrate resistant acid phosphatase (TRAP), and urine creatinine (Cr) were measured on an automatic analyzer (Ciba-Corning 550, USA) using a diagnostic reagent kit for *in vitro* determination. The levels of estradiol were determined using a specific and sensitive double-antibody RIA kit on a gamma-ray counter.

2.5. Bone Mineral Density Analysis and Bone Histologic Evaluation. The bone mineral density (BMD, g/cm^2) of the left femur was measured by dual-energy X-ray absorptiometry (LUNAR Co. Ltd., USA) using the small-animal scan mode. Right tibias were then dehydrated in ethanol, defatted in xylene, and embedded and undecalcified in methyl methacrylate. The frontal sections were cut at 4 and 10 μm thicknesses using a microtome (Leica RM 2155, Germany). The 4 μm sections were stained with Goldner's trichrome staining for static histomorphometric measurements; unstained 10 μm sections were used for dynamic histomorphometric analyses by morphometry, which were performed with light and fluorescence microscopes (KSS Computer Engineers, Magna, UT).

2.6. Histologic Evaluation of Reproductive Tissues. At the end of treatment, the animals were sacrificed; the uterus, mammary gland, and vagina of each animal were removed. Half of the tissues were collected and immediately fixed in 10% buffered formalin for 48 h. Tissues were then routinely processed in a tissue processor and 5 μm thick sections were prepared and stained with hematoxylin eosin. Histopathologic examination was performed under a light microscope (Leica DMI3000, Germany) and images were analyzed with Image-Pro Plus Software (Media Cybernetics Ltd., USA).

2.7. Western Blotting Analysis for ERα, ERβ, and PR Expression. Tissues were homogenized in 50 mL lysis buffer (600 mM Tris-HCl, 1 mM EDTA, pH 7.4, and two protease-inhibitor tablets) with a polytron homogenizer (Kinematics, Luzern, Switzerland). These suspensions were centrifuged for 30 min at 105,00 ×g and the pellet was discarded. The protein concentrations in the supernatants were measured by the method of Bradford. Aliquots containing 50 μg of each sample were precipitated with trichloroacetic acid (TCA, 10% final) and resuspended in 100% methanol. Samples were placed on ice for 20 min and the protein was recovered by centrifugation. Precipitates were dissolved in 1x SDS sample buffer and used for SDS-PAGE. Equal amounts of protein (50 μg) from each sample were loaded per well onto preformed gradient gels, 4–20% acrylamide (NOVEX, San Diego, CA, USA) with a Tris-glycine buffer system. Material was transferred to ProBlot membranes (Applied Biosystems, Foster City, CA, USA), and electroblotting was done in a Tris-glycine buffer. Prestained precision protein standards (Bio-Rad) were used as molecular weight markers. After 60 min of incubation in blocking buffer (10% skim milk in PBS with 0.1% NP-40) at room temperature, membranes were incubated in 1/75 dilution of ERα6F11, 1/2500 dilution of ERβ LBD, or 1/500 dilution of PRβ2 antibodies in blocking buffer at 4°C, overnight. This was followed by 60 min of washing in blocking buffer and then incubation in 1/7500 dilution of HRP-conjugated secondary antibodies for each species in blocking buffer for 60 min at room temperature. After sequential washing with blocking buffer, PBS with 0.1% NP-40, and PBS, signals were developed using ECL Plus (Amersham Pharmacia Biotech, Uppsala, Sweden). Bands on the exposed film were captured with a Fuji Image Analyzer LAS-1000 and densitometric values were measured with Fuji Image Garge (Fuji Photo Film Co., Japan).

2.8. Statistical Analysis. All numerical data were presented as mean ± SD from replicate experiments. One-way analysis of variance followed by Dunnett's t-test was used for statistical analysis (PASW 18.0 software; SPSS Inc., Chicago, IL, USA). The significance level was set at $P < 0.05$ for all tests.

3. Results

3.1. Body and Uterine Weights. The rats from all six groups had similar initial mean body weights. At the end of the study, the mean body weight of rats in the OVX group was significantly higher than that of the sham group. NYL treatment completely prevented the increase in body weight associated with E_2 deficiency, and EXD, EBH, or ICA treatment did not affect the body weight of OVX rats (Figure 1(a)). As expected, the mean uterine weight of OVX animals was significantly lower than that of sham controls, indicating the success of the surgical procedure. Administering NYL significantly increased the uterine weight compared to the OVX group; ICA, EBH, or EXD treatment did not significantly affect the uterine weight in ovariectomized rats (Figure 1(b)).

3.2. Bone Mineral Density and Bone Histological Examination. As shown in Table 1, ovariectomy induced bone loss, as the femoral BMDs of sham and OVX rats were 0.26 ± 0.01 and 0.25 ± 0.01, respectively, indicating that ovariectomy significantly decreased BMD by 5.7% in 12 weeks. The administration of NYL (1 mg/kg), ICA (20 mg/kg), EBH (100 mg/kg), or EXD (600 mg/kg) to the OVX rats significantly increased femoral BMD. The morphologic observations were quantified by histomorphometric analysis of longitudinal cross-sections obtained from the proximal tibiae. Compared with the sham group, there were significant decreases in the percent of trabecular area, trabecular thickness, and trabecular numbers; a significant increase in trabecular separation. The dynamic parameters of mineral rate (MAR), bone formation rate (BFR), and osteoclast number were also significantly increased in OVX rats. The administration of EXD, EBH, ICA, or NYL increased the trabecular area, trabecular thickness, and trabecular numbers in the tibia of OVX rats. However, there were differences in the effects of EXD, EBH, ICA, or NYL on

Table 1: Mineral density and bone histomorphometric parmeters of proximal tibial cancellous bone.

Groups	Sham	OVX	NYL	ICA	EBH	EXD
BMD (g/cm^2)	0.26 ± 0.01*	0.25 ± 0.01	0.26 ± 0.01*	0.26 ± 0.01*	0.26 ± 0.01*	0.26 ± 0.01*
Trabecular area ratio (%)	21.37 ± 3.42**	5.32 ± 1.09	19.04 ± 3.28**	10.69 ± 2.22**	13.82 ± 3.71**	18.33 ± 2.47**
Trabecular thickness (μm)	62.42 ± 6.06*	43.57 ± 3.40	63.75 ± 6.39*	56.54 ± 4.72*	54.71 ± 5.16*	60.78 ± 7.02*
Trabecular number (#/mm)	3.17 ± 0.47**	1.31 ± 0.22	2.79 ± 0.31**	1.84 ± 0.24	2.34 ± 0.41**	2.67 ± 0.28**
Trabecular separation (μm)	209.73 ± 40.40**	1007.36 ± 127.63	342.57 ± 37.33**	631.77 ± 58.61*	573.75 ± 62.31*	441.81 ± 51.26*
MAR (μm/d)	0.59 ± 0.19**	1.18 ± 0.24	0.77 ± 0.11*	1.26 ± 0.19	1.08 ± 0.22	0.81 ± 0.19*
BFR/BS (mcm/d*100)	14.62 ± 1.85**	36.88 ± 4.71	17.62 ± 2.02**	31.65 ± 8.76	33.82 ± 4.71	18.23 ± 2.57*
BFR/BV (%/year)	139.62 ± 24.86**	302.55 ± 46.37	163.18 ± 25.72**	314.82 ± 28.66	317.65 ± 47.51	208.39 ± 26.12*
Osteoclast number (#/mm)	7.47 ± 1.25**	18.84 ± 3.67	8.84 ± 2.24**	9.96 ± 2.42**	9.21 ± 1.68**	7.96 ± 2.19**

The data represent the mean ± standard deviation ($n = 8$), $^*P < 0.05$, $^{**}P < 0.01$ compared with OVX group.

Table 2: Osteoporosis-related biochemical indices in different treatment groups.

Groups	Sham	OVX	NYL	ICA	EBH	EXD
S-Ca (mmol/L)	2.80 ± 0.25	2.68 ± 0.21	2.70 ± 0.07	2.56 ± 0.22	2.59 ± 0.18	2.54 ± 0.12
S-P (mmol/L)	2.88 ± 0.55	2.99 ± 0.64	2.61 ± 0.43	3.12 ± 0.74	2.85 ± 0.78	2.61 ± 0.63
U-Ca/Cr	0.40 ± 0.06**	1.29 ± 0.21	0.65 ± 0.11**	0.43 ± 0.05**	0.39 ± 0.06**	0.66 ± 0.08**
U-P /Cr	4.19 ± 0.92*	5.65 ± 1.02	6.03 ± 1.08	3.73 ± 0.90*	3.09 ± 0.87*	2.81 ± 0.90**
Estradiol (pg/mL)	35.71 ± 2.64**	16.73 ± 1.78	28.38 ± 2.23**	20.24 ± 2.12	21.73 ± 2.82*	23.37 ± 1.92*
TRAP (IU/L)	7.22 ± 0.91**	15.01 ± 2.62	3.33 ± 0.54**	4.11 ± 0.63**	3.43 ± 0.84**	3.08 ± 0.73**
ALP (IU/L)	80.59 ± 11.12*	109.02 ± 17.24	85.33 ± 14.61*	104.04 ± 18.71	112.32 ± 19.42	91.82 ± 12.24*

The data represent the mean ± standard deviation ($n = 8$), $^*P < 0.05$, $^{**}P < 0.01$ compared with OVX group.

dynamic parameters. NYL and EXD decreased MAR, bone formation rate, and osteoclast number, but ICA and EBH only reduced the osteoclast number and had no effects on bone formation parameters in OVX rats.

3.3. Serum and Urine Biochemical Parameters. As shown in Table 2, the measured values for serum calcium (S-Ca) and serum phosphorus (S-P) in rats did not show significant differences among groups, while the ratio of urinary calcium to creatinine (U-Ca/Cr) and the ratio of urinary phosphorus to creatinine (U-P/Cr) levels in the OVX group were significantly higher compared with the sham group, and NYL, EXD, EBH, and ICA significantly prevented the increase in urinary-Ca levels and urinary-P levels in OVX rats. NYL, EXD, EBH, and ICA increased serum estradiol levels in OVX rats; NYL and EXD decreased both ALP and TRAP activities in the serum of OVX rats; only ICA and EBH reduced the TRAP activity, but did not affect the ALP activity in OVX rats.

3.4. Histologic Parameters of Uterus, Vagina, and Mammary Gland. As shown in Figure 2 and Table 3, uterine lumen in sham-operated rats showed normal endometrial proliferation. In OVX rats, there was a marked decrease in uterine diameter and luminal epithelial cell height. Endometrial stroma was atrophied with a minimal number of glands, the thickness of endometrium and the ratio in uterine thickness were dramatically decreased, and the columnar epithelium of endometrium was fragmented, loose, and thinner. NYL treatment to OVX rats increased markedly uterus, endometrial, luminal epithelium thickness, and endometrial glands numbers; ICA, EBH, or EXD treatment to OVX rats slightly increased the above indices of uterus.

Figure 3 shows photomicrographs of rat uterine epithelium after treatment. The uteri of sham controls rats were characterized by large cells that formed a tall cuboidal-to-columnar epithelium. That of the OVX control was lined by a low cuboidal epithelium. The uteri of NYL-treated rats demonstrated increased thickness in the endometrial and marked degeneration in the connective tissues, a dramatic increase in myometrial thickness, and a substantial change in the area of the lumen with respect to the entire histologic section. Treatment of OVX rats with ICA, EBH, or EXD slightly increased the uterine epithelial heights.

Figure 4 shows the photomicrographs of the vaginal epithelium of experimental animals after treatment. Compared with sham rats, the vaginal epithelium of OVX rats was atrophic: only two-to-three cell layers were seen, and these were composed of flattened cells with no cornification. Following NYL administration, the diminished vaginal epithelia were reversed, and three layers were observed: the basal stratum germinativum, the intermediate stratum granulosum, and the superficial shedding stratum corneum. Treatment with EXD, EBH, or ICA did not significantly alter vaginal epithelia in OVX rats. NYL treatment increased vaginal epithelial height and cell layers; and treatment with either EXD, EBH, or ICA did not affect the vaginal epithelial heights and cell layers in OVX rats (Table 3).

Table 3: Quantitative data of histological feature in uterus, mammary gland and vagina.

Groups	Sham	OVX	NYL	ICA	EBH	EXD
Uterus thickness (μM)	361.27 ± 38.78***	202.16 ± 16.04	377.41 ± 47.17***	220.31 ± 27.22	248.41 ± 37.35*	253.19 ± 32.23*
Uterus endometrial thickness (μM)	259.15 ± 43.41***	111.42 ± 19.26	290.83 ± 47.11***	128.82 ± 28.17	143.35 ± 41.27	148.45 ± 37.30
Uterus endometrial glands numbers	25.73 ± 2.62***	10.39 ± 1.62	32.14 ± 5.41***	15.83 ± 2.42*	13.14 ± 2.32	16.38 ± 2.71*
Uterus luminal epithelium thickness (μM)	10.62 ± 1.73**	3.94 ± 0.73	9.37 ± 1.92**	3.44 ± 0.61	4.94 ± 0.63	5.25 ± 0.74
Vaginal epithelium cell layers	7.11 ± 2.64***	1.52 ± 0.63	10.28 ± 3.12***	2.09 ± 0.91	2.64 ± 0.72	1.67 ± 0.65
Vaginal epithelium thickness (μM)	23.63 ± 0.81***	4.24 ± 0.41	26.17 ± 1.14***	5.31 ± 0.30	6.12 ± 0.64	4.54 ± 0.83
Mammary gland epithelium thickness (μM)	3.63 ± 0.34**	1.25 ± 0.15	4.64 ± 0.44**	1.14 ± 0.12	1.52 ± 0.21	1.51 ± 0.20

The data represent the mean ± standard deviation ($n = 8$), *P< 0.05, **P < 0.01 compared with OVX group.

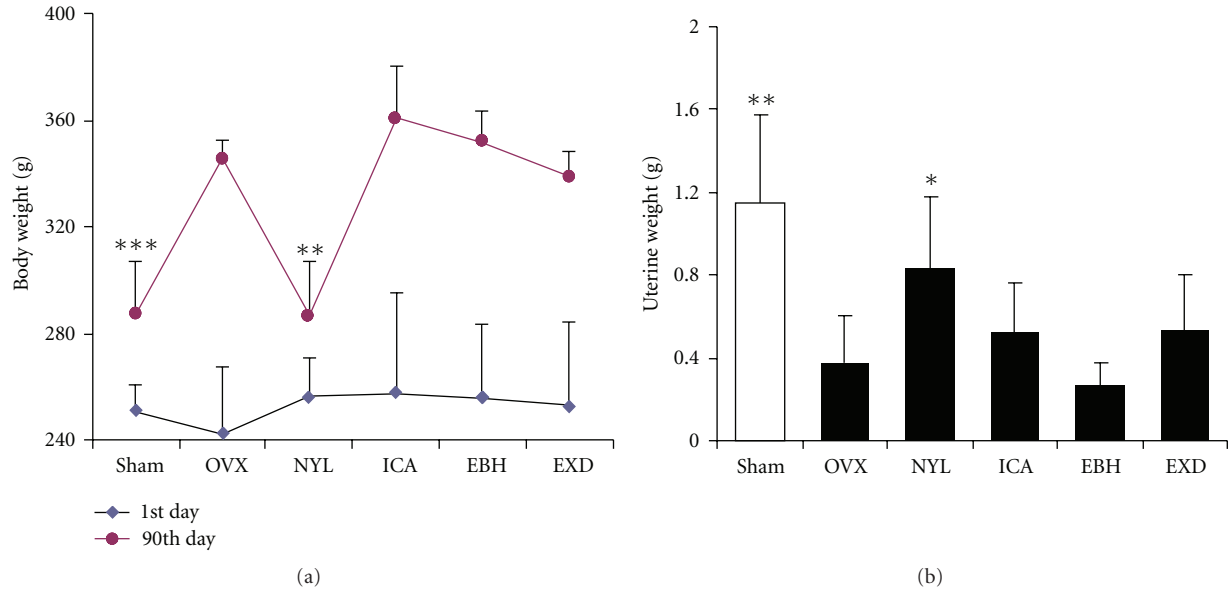

Figure 1: Effects of ICA, EBH, EXD, and NYL on body and uterine weight in ovariectomized osteoporotic rats. Ovariectomized rats were administered NYL (1 mg/kg), ICA (20 mg/kg), EBH (100 mg/kg), or EXD (600 mg/kg) for 12 weeks. The uterine and body weights were weighed. (a): Body weight; (b): uterine weight. Data were presented as mean ± standard deviation, ($n = 8$). *$P < 0.05$, **$P < 0.01$, and ***$P < 0.001$ compared with OVX control.

As shown in Figure 5, the mammary glands of rats are composed of connective tissue, acini, and ducts, with the epithelial cells of the acini and ducts manifesting cubic or low columns. The epithelial structures of mammary glands in OVX rats were atrophic, deep in the fat pad, with scarce clusters of densely packed terminal structures, many of which did not show clear luminal formation. Treatment with NYL resulted in abundant terminal epithelial structures. Mammary glands from animals treated with EXD, EBH, or ICA did not differ from those of the OVX controls with respect to the above-mentioned structures. NYL treatment significantly increased mammary gland epithelium thickness; and treatment with either EXD, EBH, or ICA did not affect the mammary gland epithelium thickness in OVX rats (Table 3).

3.5. Expression of ERα, ERβ, and PR in Uterus, Vagina, and Mammary Gland. As shown in Figure 6(a), compared with the sham group, the expression of uterine PR was not significantly different from OVX rats. NYL slightly increased the expression of PR; ICA and EBH slightly decreased the expression of PR; EXD significantly decreased the expression of PR in uteri of OVX rats. Compared with the sham group, the expression of ERα was not significantly changed in the uteri of OVX rats; NYL did not change the expression of ERα; ICA, EBH, and EXD significantly decreased the expression of ERα in the uteri of OVX rats. Compared with the sham group, the expression of ERβ was significantly increased in the uteri of OVX rats; NYL, ICA, EBH, and EXD significantly decreased the expression of ERβ in the uteri of OVX rats.

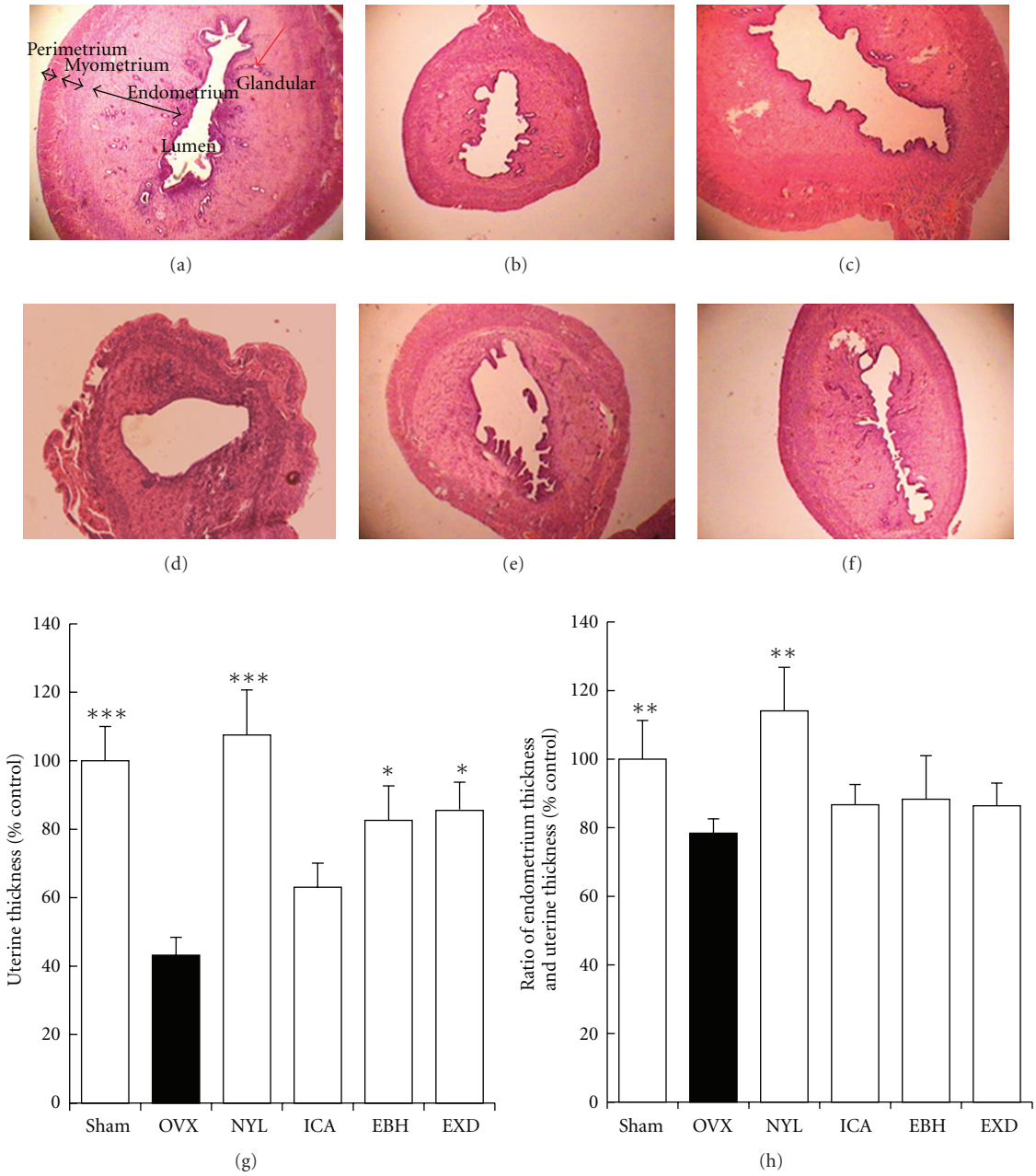

Figure 2: Effects of NYL, ICA, EBH, or EXD on uterine histology in the OVX rats (hematoxylin-eosin staining, magnification ×40). (a): Sham control; (b): OVX control; (c): OVX + NYL treatment; (d): OVX + ICA treatment; (e): OVX + EBH treatment; (f): OVX + EXD treatment; (g): uterine thickness; (h): ratio of endometrial to uterine thickness; the data represent the mean ± standard deviation ($n = 8$); $^{*}P < 0.05$; $^{**}P < 0.01$ compared with the OVX group.

As shown in Figure 6(b), compared with the sham group, the expression of PR in vagina was significantly increased in OVX rats. NYL significantly increased the expression of PR; ICA had no significant effects on the expression of PR; EXD and EBH significantly decreased the expression of PR in the vagina of OVX rats. Compared with the sham group, the expression of ERα and ERβ was not significantly changed; neither NYL, ICA, EBH, nor EXD had any effects on the expression of ERα and ERβ in the vagina of OVX rats.

As shown in Figure 6(c), compared with the sham group, the expression of PR was not significantly changed in the mammary gland of OVX rats. NYL and EBH had no effects on the expression of PR; ICA and EXD significantly decreased the expression of PR in the mammary gland of OVX rats. Compared with the sham group, the expression of ERα was slightly increased; NYL significantly increased the expression of ERα; ICA, EBH, and EXD significantly decreased the expression of ERα in the mammary gland of

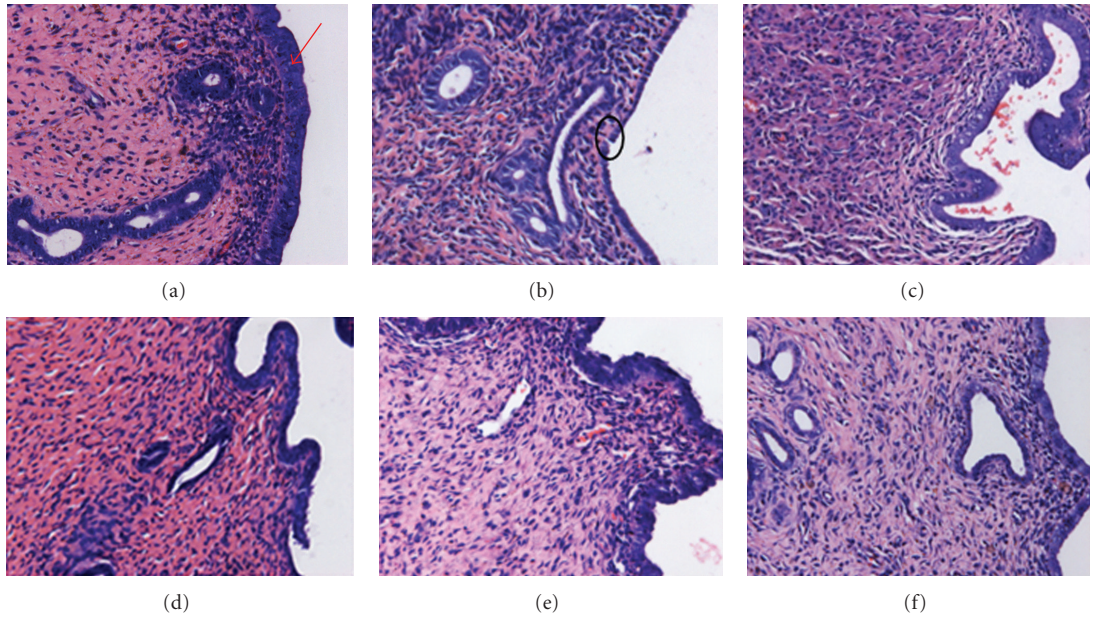

Figure 3: Histologic analysis of uterus (hematoxylin-eosin staining, magnification ×200). (a): Sham group; (b): OVX group; (c): OVX + NYL treatment group; (d): OVX + ICA treatment group; (e): OVX + EBH treatment group; (f): OVX + EXD treatment group.

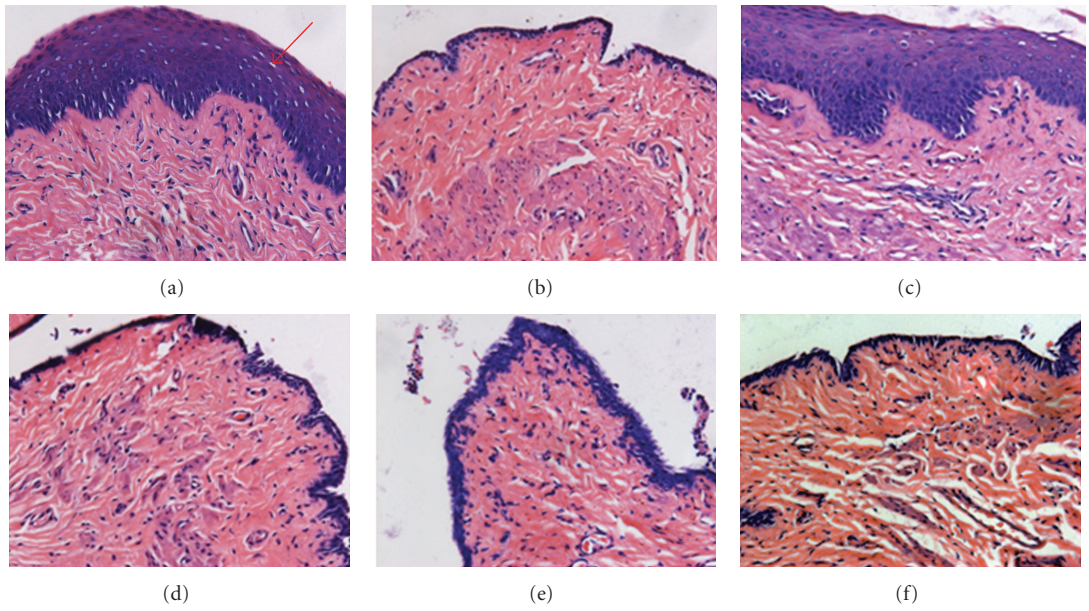

Figure 4: Histologic analysis of vagina (hematoxylin-eosin staining, magnification ×200). (a): Sham group; (b): OVX group; (c): OVX + NYL treatment group; (d): OVX + ICA treatment group; (e): OVX + EBH treatment group; (f): OVX + EXD treatment group.

OVX rats. Compared with the sham group, the expression of ERβ was significantly increased; NYL slightly decreased the expression of ERβ; ICA, EBH, and EXD significantly decreased the expression of ERβ in the mammary gland of OVX rats.

4. Discussions

A key goal in therapeutics is to develop clinically effective medicines with superior safety profiles. This study was designed to investigate the potentials of EXD and its major ingredients EBH and ICA on osteoporotic bone and reproductive tissues by comparison with estrogen. The evaluation of the treatments was done with histologic, biochemical, and analyses of ERα, ERβ, and PR expression of uterus, vagina, and mammary gland, in addition to bone mineral density and weight measurements.

Rapid postmenopausal bone loss, which occurs in female rats following ovariectomy, is characterized by a decrease in trabecular bone density and a deterioration of the bone

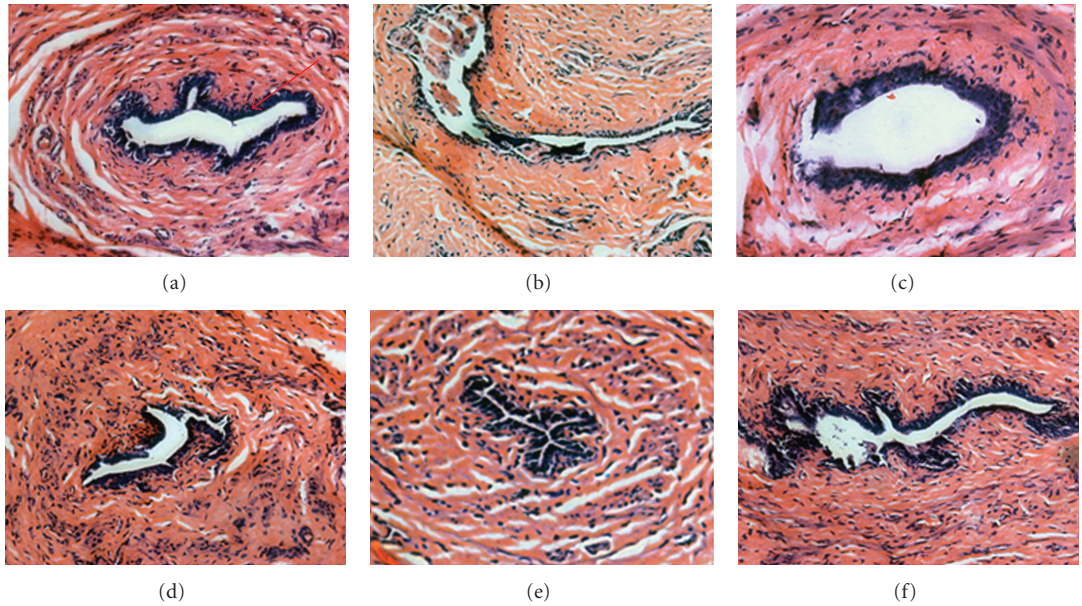

Figure 5: Histologic analysis of mammary gland (hematoxylin-eosin staining, magnification ×200). (a): Sham group; (b): OVX group; (c): OVX + NYL treatment group; (d): OVX + ICA treatment group; (e): OVX + EBH treatment group; (f): OVX + EXD treatment group.

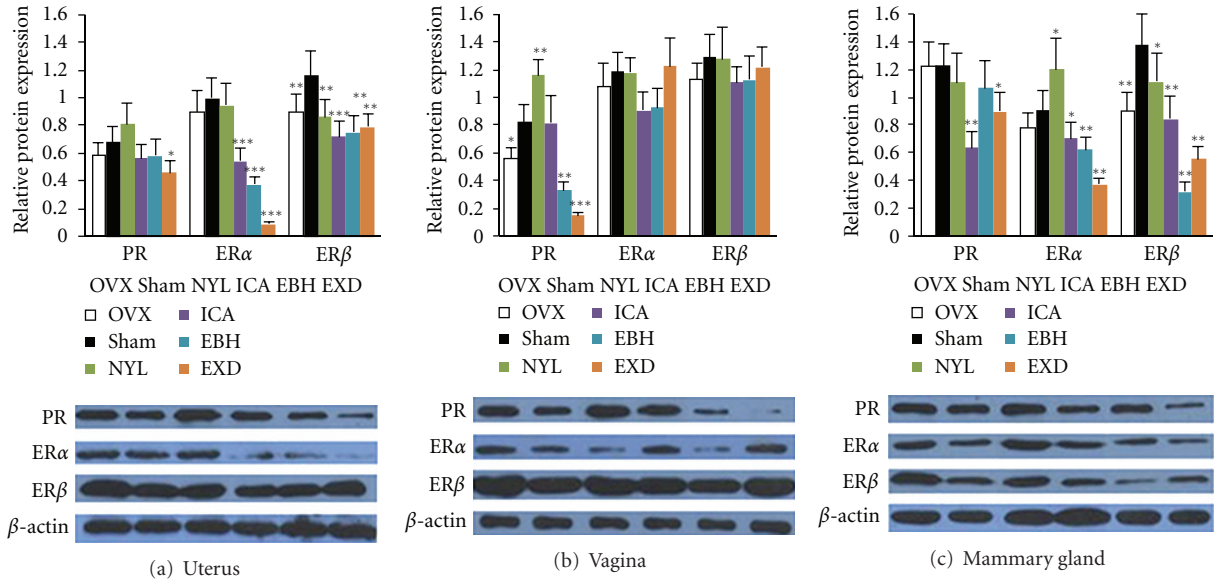

Figure 6: Effects of 12-week ovariectomy treatment and coadministration of NYL, ICA, EBH, and EXD on expression of ERα, ERβ, and PR at protein levels in uterus (a), vagina (b), and mammary gland of rats (c). The protein levels were normalized by β-actin. Values are mean ± standard deviation ($n = 8$). $^{*}P < 0.05$, $^{**}P < 0.01$, and $^{**}P < 0.001$ compared with the OVX group.

architecture, with a particular diminution in the total number of trabecular and an increase in the number of their perforations. Based on the values of histomorphometric parameters, we found that bone turnover in these OVX rats was increased; EXD, EBH, ICA, or NYL treatment attenuated the deterioration of trabecular bone in the OVX rats. The percentages of trabecular area, thickness, and number were increased, and trabecular separation was decreased in the EXD-, EBH-, ICA-, and NYL-treated OVX rats. The greater bone mineral density and percentage of trabecular area might be due to the increase in both trabecular number and trabecular thickness. EXD or NYL treatment in OVX rats resulted in a decrease in the dynamic parameters for bone resorption and bone formation; EBH or ICA treatment in the OVX rats only led to a significant decrease in the dynamic histomorphometric indices for bone resorption, but not other bone formation parameters. This result suggests that EXD is similar to NYL and exerts a concomitant effect on

bone formation and bone resorption at the tissue level, while EBH and ICA produced bone-protective effects mainly by inhibiting bone resorption.

Ovariectomy induces bone loss in rats, leading to an increase in loss of calcium, phosphorous, and creatinine through excretion in urine. Similarly, the increase in levels of ALP and TRAP was observed in the control OVX rats. A reduction in the calcium absorption observed with OVX rats is similar to that for postmenopausal women, and the serum ovarian hormone deficiency can further decrease intestinal calcium absorption [18]. In the present study, EBH and ICA prevented the OVX-induced increase in TRAP activity and urinary calcium and did not affect ALP activity, indicating that EBH and ICA decreased bone loss by inhibiting bone resorption. The characteristics of EXD on bone metabolism are similar to that of Nylestriol. Nylestriol and EXD both significantly decreased serum ALP, TRAP, and urine Ca/Cr levels of OVX rats, indicating that NYL and EXD prevent osteoporosis by inhibiting the high bone turnover through modulating bone formation and bone resorption. The results of our biochemical analyses were found to corroborate the bone histomorphometric results in assessing bone turnover.

The OVX-induced estrogen deprivation was characterized by a significantly increased body weight and decreased uterine wet weight compared to the intact animals. NYL inhibited body weight gain and increased the weight of uteri in OVX rats. EXD, EBH, and ICA did not produce any effects on the treated animals with respect to body and uterine weight, even though EXD, EBH, and ICA were shown to possess estrogenic activity. This lack of uterotrophic activity of EXD, EBH, and ICA could be beneficial in reducing the risk of endometrial, breast, or ovarian cancer associated with estrogen treatment.

The uterus, vagina, and mammary glands are very sensitive to estrogen and represent key organs in clinical safety testing of estrogenic agents. Qualitative analysis of uteri demonstrated increased thickness in the endometrial and marked degeneration in the connective tissues from the NYL-treated rats. Moreover, we observed in this group a dramatic increase in myometrial thickness and a substantial change in the area of the lumen with respect to the entire histologic section, and these uterine phenomena have been reported as typical adverse effects of hormone replacement therapies in women [19]. Thus, the results of the present study substantiate estrogen's stimulatory role in the uterus, vagina, and mammary gland, since there was a significant atrophy in the OVX group. The rats in EXD-, EBH-, and ICA-treated groups maintained a healthy epithelial layer, unlike that of the NYL group.

The endometrial is an exquisitely hormone-sensitive tissue and a principal target of estrogen and progesterone. The coordinated actions of estrogen and progesterone are essential so as to maintain cellular replicative homeostasis. In particular, uterine cell proliferation is augmented by estrogen, which, in turn, is antagonized by progesterone [20, 21]. The effects of estrogen and progesterone on epithelial and endometrial proliferation and differentiation are mediated directly or indirectly through their cognate receptors, namely, estrogen receptor alpha (ERα), estrogen receptor beta (ERβ), and the progesterone receptor (PR) [22]. ERα and ERβ have distinct or even opposing biologic effects in certain cells, where the action of estrogen ligands depends on a balance between ERα and ERβ. In contrast to ERα-promoted cancer cell growth, ERβ inhibits cancer cell proliferation [23, 24]. In endometrial tissue, the expression of PR is known to be ER dependent. In proliferative endometrial and endometrioid carcinoma, PR expression was correlated with ERα, but not with ERβ [25]. ERα promotes proliferation, while ERβ has proapoptotic and prodifferentiating functions. When ERα and ERβ are coexpressed in cells, ERβ can inhibit the proliferative response of ERα on the cells, acting as a dominant inhibitor of ERα [24]. In comparison to normal rats producing endogenous estrogen, the expression levels of both estrogen receptor subtypes ERα and ERβ were significantly higher in the OVX rats. This effect described in the literature is a consequence of a negative feedback mechanism [26]. Phytoestrogens interfere with endogenous estrogen action either by acting as agonists when endogenous estrogen levels are low or by acting as antagonists when endogenous estrogen levels are high [27]. The *Epimedium* flavonoids have the peculiar characteristic of a stronger binding action to ERα, but with a higher affinity for ERβ [28]. In our study, there were decreased serum estradiol levels and slightly increased ERα and ERβ expression in uterus, vagina, and mammary gland of OVX rats. NYL treatment induced higher ERα and PR expression in uterus, vagina and mammary gland, which caused significant epithelial hyperplasia. The ICA, EBH, and EXD treatments had more potentially inhibitory effects on the expression of ERα than on PR and ERβ expression in uterus and mammary gland of OVX rats, leading to an increase in the ratio of ERβ to ERα. The ratio of ERβ to ERα is associated with decreased stimulatory effects on the endometrium [24]. Therefore, EXD, EBH, and ICA, which have estrogen-like activity, could reduce the bone loss induced by estrogen deficiency and maintain a healthy histomorphology in uterus, vagina, and mammary gland.

EXD is composed of six herbal medicines, with *Epimedium* herbs being its major ingredient. *Epimedium* herbs have been reported to possess abundant flavonoids, including icariin (ICA), epimedin A, B, and C, and hyperin. In animal studies, administering ICA and *Epimedium* flavonoids inhibit bone resorption, prevent osteoporosis in OVX rats [13, 29], and improve erectile function in aged male rats [30]. Icaritin, the aglycon of ICA, has estrogenic properties and stimulates estrogen-driven cells [31]. *Curculigo* rhizomes have been reported to have phenolic glycosides, including curculigoside, curculigoside B and curculigine, and have estrogen-like, antiosteoporotic and antioxidant activity [32]. *Anemarrhena* rhizomes contain steroidal saponin, which are shown to possess estrogenic activity and increase bone formation [33, 34]. *Phellodendron* barks have been shown to have alkaloids, such as berberine and palmatine, which attenuate osteoclast differentiation and function through inhibition of receptor activator nuclear factor-κB ligand in osteoblast cells [35]. *Morinda* root has been reported to have anthraquinone compounds that decrease bone resorption by inhibiting formation and differentiation of osteoclasts

induced from bone-marrow cells [36]. The extracts of *Angelica* roots exhibited a stimulation of the uterine histoarchitecture, a significant cornification in the vaginal epithelium, and a reduction in serum luteinizing hormone concentration, thereby exhibiting estrogenic activity [37]. In general, the multiple ingredients of EXD exerted comprehensive effects on bone and estrogen-related organs compared to those of estrogen, but with a fewer adverse effects on the reproductive tissues.

5. Conclusions

EXD, EBH, or ICA was as effective as estrogen in restoring the bone density and architecture of ovariectomized rats but caused fewer adverse effects on reproductive tissues. Among the EXD, EBH, and ICA, EXD was found to have superior efficacy and safety profile.

Conflict of Interests

The authors declare that they have no conflict of interests.

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Research Article

Effects and Interaction of Icariin, Curculigoside, and Berberine in Er-Xian Decoction, a Traditional Chinese Medicinal Formula, on Osteoclastic Bone Resorption

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Er-Xian decoction (EXD), a traditional Chinese medicine, has been reported to have a protective effect against bone loss in ovariectomized osteoporotic rats, and the inclusion of icariin (I), curculigoside (C), and berberine (B) in EXD displays inhibitory effects on osteoclastic bone resorption. In the present paper, we investigated the interaction and effects of I, C, B, and their combination on bone resorption activity *in vitro* on osteoclasts derived from rat bone marrow cells. ICB synergistically decreased the formation of bone resorption pits, the number of multinucleated osteoclasts, and the activity of tartrate-resistant acid phosphatase (TRAP) and showed antagonistic or additive effects on cathepsin K activity in the coculture system of osteoblasts and bone marrow cells in the presence of 1, 25-dihydroxyvitamin D_3 and dexamethasone. The combination of ICB also enhanced the inhibitory effects on the formation of F-actin ring, a cytoskeleton structure of osteoclasts induced from bone marrow cells with macrophage colony stimulation factor (M-CSF) and receptor activator of NF-κB ligand (RANKL). In addition, ICB synergistically improved the ratio of protein expression of osteoprotegerin (OPG) and RANKL in osteoblasts and interfered with the mitogen-activated protein kinases (MAPKs) pathway in osteoclast. These results clearly show that I, C, B, and their combination in EXD exert effects of mutual reinforcement. However, IBC does not show an intensified adverse effect in the ovariectomized murine model, as revealed by change in body and uterine weight, confirming the safety of EXD. These observations are in agreement with the rationality of the formula used in this paper.

1. Introduction

Traditional Chinese medicine (TCM), an empirical system of multicomponent therapeutics, potentially meets the demands of treating a number of complex diseases in an integrated manner, in particular chronic diseases and metabolic syndromes [1]. Naturally occurring herbs and herbal ingredients organized into certain formula have been shown to have potential interaction effects, including mutual enhancement, mutual assistance, mutual restraint, and mutual antagonism. For example, synergistic interactions occur when the efficacy of combinations of herbs (or ingredients) is greater than the summed responses of each individual herb or ingredient [2, 3]. Scientific evidence for TCM is generally achieved through rigorous experimental design, which has been dominated by the search for its biological basis, identification of active substances, and the investigation of the mechanisms of its action. In contrast, research in the role and interaction of active ingredients in the formulas is still scarce, thus hampering the understanding of the rationality of formula design in TCM.

Postmenopausal osteoporosis is believed to be associated with ovarian hormone deficiency and is by far the most common cause of age-related bone loss [4]; and with the reduction in estrogen levels, there is an increase in bone breakdown relative to bone formation, microarchitectural

deterioration, and decreased bone mass [4, 5]. The excessive bone breakdown is caused by an increased number of osteoclast and enhanced ability of bone resorption [6]. Osteoclasts are multinucleated cells (3–10 nuclei per cell) differentiated from hematopoietic precursors and are involved in bone remodeling. Through osteoclastic bone resorption, osteoclasts play both a crucial physiological role in bone remodeling and a pathological role in osteoporosis as characterized by excess activity of osteoclasts [6, 7].

The osteoclasts are formed and differentiated under the control of some cytokines, such as macrophage colony stimulation factor (M-CSF), receptor activator of NF-κB ligand (RANKL), and osteoprotegerin (OPG) [7, 8]. The binding of RANKL to its receptor RANK leads to recruitment of TNF receptor-associated factor 6 (TRAF6) to the cytoplasmic domain of RANK. This then activates the downstream targets of TRAF6 including transcription factors such as nuclear factor kappa B (NF-κB), activator protein-1 (AP-1), and nuclear factor of activated T cells (NFAT), as well as the mitogen-activated protein kinases (MAPKs) including p38 MAP kinases, c-Jun N-terminal kinases (JNK/SAPKs), extracellular signal-regulated kinases (ERKs), and phosphatidyl-inositol-3-kinase (PI3 K)/Akt [9–12]. Osteoprotegerin (OPG), a soluble decoy receptor of RANKL, negatively regulates osteoclast differentiation and bone resorption. Mature osteoclast formation is also associated with the expression of differentiation markers, including tartrate resistant acid phosphatase (TRAP) and cathepsin K. The modulation of these differentiation-related signaling pathways has the potential of being considered as a therapeutic strategy for the treatment of skeletal diseases [13].

Over the past 50 years, Er-Xian decoction (EXD), a traditional Chinese medicinal formula, has been used for the treatment of osteoporosis disorders, menopausal syndrome, and aging diseases [14, 15]. The components of EXD are Epimedii Folium (Yinyanghuo), Curculiginis Rhizoma (Xianmao), Anemarrhenae Rhizoma (Zhimu), Phellodendri Chinensis Cortex (Huangbo), Morindae Officinalis Radix (Bajitian), and Angelicae Sinensis Radix (Danggui), with icariin, curculigoside, timosaponin BII, berberin, nystose, and ferulic acid as major active ingredients [16]. According to traditional Chinese medicine, Epimedium Folium and Phellodendri Chinensis Cortex are essential ingredients of EXD and appear to play important roles in ameliorating signs and symptoms of the menopausal syndrome and osteoporosis. Curculiginis Rhizoma and Morindae Officinalis Radix help strengthen the curative effect of the Epimedium Folium, and Anemarrhenae Rhizoma helps Phellodendri Chinensis Cortex to play a fuller role. Angelicae Sinensis Radix cooperates with the above five medications to strengthen the therapeutic effects and treat the accompanying disease or syndromes [17]. We tested the effects of major active ingredients on osteoblast and osteoclast and found that icariin, timosaponin BII, nystose, and ferulic acid could increase the proliferation and alkaline phosphatase (ALP) activity of osteoblast, and icariin (I), curculigoside (C), and berberine (B) could decrease the number of TRAP positive multinucleated osteoclasts and TRAP-activity of osteoclast, as evidenced by their inhibitory effects on bone resorption [16].

Therefore, in the present study, we firstly proved that the combination of ICB has more potent antiosteoporotic activity than individual compounds on ovariectomized osteoporotic mice, and furthermore, we investigated the inhibitory effects of I, C, B, and their combination on bone resorption of octeoclast to evaluate the interaction between them so as to obtain an understanding of their mechanism of action and to provide an insight into the rationality of formula design for potential use in TCM.

2. Materials and Methods

2.1. Reagents. Reagents used in this study included α-modified minimum essential medium (α-MEM) and fetal bovine serum (FBS) (Gibco, US); 1, 25-dihydroxyvitamin D_3, dexamethasone, trypsin, and coomassie brilliant blue G-250 (Sigma, US); recombinant rat M-CSF (400-28) and RANKL (400-30) (Peprotech EC, US); OPG (sc11383), RANKL (sc9073), and β-actin (sc81178) antibody (Santa Cruz, USA); anti-phospho-Akt (9275), anti-phospho-ERK (3371), anti-phospho-JNK (9251), anti-phospho-p38 (9211), and anti-phospho-I-κB (9245) (Cell Signaling Technology, Beverly, MA); NucBuster Protein Extraction kit (Merck, Novagen, Germany); Cathepsin K activity kit (Biovision, USA); pararosaniline, Hoechst 33258, and rhodamine-conjugated phalloidin (Sigma, USA). Diolamine, potassium sodium tartrate, disodium 4-nitrophenylphosphate, Triton X-100, and 4-nitrophenol were of domestic AR grade.

Icariin (I), curculigoside (C), and berberine (B) were isolated from Epimedii Folium, Curculiginis Rhizoma, and Phellodendri Chinensis Cortex, respectively, and were identified by spectrum analysis. The data of proton nuclear magnetic resonance spectroscopy (^{1}H NMR) and mass spectrometry (MS) were coincidence with previous studies. The purities of icariin, curculigoside, and berberine were up to 99% according to high-performance liquid chromatography (HPLC) analysis.

2.2. Animal Experimental Protocol. Seventy 3-month-old female ICR mice were purchased from SLACOM experimental animal company (Shanghai, China) and acclimatized to laboratory conditions for 1 week before commencing the experiment. Food and drinking water were supplied *ad libitum*. The mice were weighed weekly during the experimental period. An osteoporotic model was established 12 weeks after bilateral ovariectomy. Knowing that the osteoporotic model induced by ovariectomy is associated with estrogen deficiency, nylestriol (an estrogenic substance) was used as the positive drug. Of the 70 female mice, 10 mice were sham-operated and treated with deionized water as the control group. The remaining 60 mice were bilaterally ovariectomized and equally randomized into six groups, with intragastric administration of either deionized water as the OVX model control, nylestriol (1 mg/kg) weekly as the positive control, icariin (40 mg/kg) daily, curculigoside

(20 mg/kg) daily, berberine (120 mg/kg) daily, or a combination of icariin (40 mg/kg), curculigoside (20 mg/kg), and berberine (120 mg/kg) daily as the treatment groups, all for 12 weeks. Mice received treatments starting from day one after surgeries and the success of ovariectomy was confirmed at necropsy by failure to detect ovarian tissue and by observation of marked atrophy of uterine horns. At the end of the treatment, blood samples from all the mice were withdrawn by orbital sinus method and centrifuged to collect serum for the measurement of biochemical parameters. The uterine was removed and immediately weighed.

The tibia was cleaned by removing adhering soft tissues and stored in 75% ethanol for a week prior to analysis. The bone mineral density (BMD) and bone mineral content (BMC) were measured at 3 mm from the proximal epiphysis of right tibia with a peripheral quantitative computed tomography (*p*QCT) densitometry (Stratec XCT Research SA, Germany). Serum alkaline phosphatase (ALP), tartrate-resistant acid phosphatase (TRAP), and serum creatinine (Cr) were measured on an automatic analyzer (Ciba-Corning 550, USA) using diagnostic reagent kit determination. Serum osteoprotegerin (OPG) and deoxypyridinoline cross-links (DPD) were estimated using an Elisa kit according to manufacturer instructions. The experimental protocol used in this study was approved by the Bioethic Committee of the Second Military Medical University, and the procedures employed were strictly according to generally accepted international rules and regulations.

2.3. The Assay of Formation, Differentiation, and Bone Resorption of Osteoclast

2.3.1. Osteoclasts Induced with 1, 25-Dihydroxyvitamin D_3 and Dexamethasone from Bone Marrow Cells in a Coculture System with Osteoblasts. Primary osteoblastic cells were prepared from neonatal rat calvarial osteoblasts according to the literature [18]. Induction and culture of osteoclasts were as follows. Briefly, the femur was disarticulated from Wistar rats aged 3 days, and the ends were removed, and the bone marrow cells were flushed out using a 1 mL syringe. Primary osteoblastic cells (1×10^5/mL) and bone marrow cells (1×10^6/mL) were cocultured in α-MEM medium containing 10% FBS, 1, 25-dihydroxyvitamin D_3 (10 nmol/L) and dexamethasone (100 nmol/L) at 37°C in a humidified atmosphere of 5% CO_2. Prior to plating the cells, cover glasses (5 × 5 mm) or dental slices (40 μm thick) were placed into culture dishes. The formations of multinucleated osteoclasts (MNCs) were confirmed by TRAP staining and resorption pits formed on dental slices. Osteoclastic cells were seeded onto culture plates in α-MEM medium. The medium was replaced the following day with fresh medium containing 1.0 μM icariin, 1.0 μM curculigoside, 1.0 μM berberine, or their combination (including 1.0 μM icariin, 1.0 μM curculigoside, and 1.0 μM berberine) for indicated time. All cultures were maintained at 37°C in a humidified atmosphere of 5% CO_2 throughout. The osteoclasts were used to investigate formation of osteoclast, TRAP and Cathepsin K activity, and bone resorption activity.

2.3.2. Counting of TRAP-Positive Multinucleated Osteoclasts. Primary osteoblasts and bone marrow cells were placed on 96-well plates containing cover glasses and cultured in α-MEM medium containing 10% FBS, 1, 25-dihydroxyvitamin D_3 (10 nM) and dexamethasone (100 nM), for 24 h. Cells were then treated with or without I, C, B, and their combination for 10 days and stained for TRAP with a TRAP staining kit (no. 387A-1KT; Sigma-Aldrich). Cells with three or more nuclei were counted as osteoclast-like multinucleated osteoclasts under a microscope.

2.3.3. Assay for TRAP Activity. Primary osteoblasts and bone marrow cells in α-MEM medium containing 10% FBS, 1, 25-dihydroxyvitamin D_3 (10 nM), and dexamethasone (100 nM) were placed in a 96-well culture dishes, cultured for 6 days, and then treated with or without I, C, B, and their combination for 48 h. TRAP was determined as follows: cells were washed twice with PBS, and then 20 μL 0.1% Triton X-100 was added to the cells to induce lysis at room temperature. After 15 min, 100 μL substrate solution (0.4 g disodium 4-nitrophenylphosphate and 2.0 g potassium tartrate dissolved in 200 mL of deionized water, with a pH adjusted to 3.5 with 1 mol/L HCl) was added to the lysed cells and incubated at 37°C for 30 min, the reaction was terminated by the addition of 100 μL 1 mol/L NaOH to each well, and the absorbance was measured at 405 nm. At the same time, positive cells for TRAP were counted and the TRAP activity was expressed as nanomoles *p*-nitrophenol per minute per 100 osteoclasts.

2.3.4. Determination of Cathepsin K Activity. Primary osteoblasts (2×10^6) and bone marrow cells (2×10^7) in α-MEM medium containing 10% FBS, 1, 25-dihydroxyvitamin D_3 (10 nM), and dexamethasone (100 nM) were placed in a 24-well culture plate, cultured for 7 days, and then treated with or without I, C, B, and their combination for 48 h. Cells were collected by centrifugation, lysed in 50 μL chilled CK cell lysis buffer, incubated on ice for 10 min, and then vortexed for 5 min. CK reaction buffer (50 μL) and 10 mM CK substrate Ac-LR-AFC (2 μL) were then added to the sample and the mixture solution was incubated at 37°C for 2 h. The samples were then transferred onto a 96-well plate; the intensity of fluoresce was measured in a fluorometer equipped with a 400 nm excitation filter and a 505 nm emission filter.

2.3.5. Determination of Bone Resorption Pit. The primary osteoblast and bone marrow cell suspension were seeded into the wells of 96-well culture plates with a sterilized bone slice (40 μm thick, 5 mm × 5 mm). After 24 h culture in α-MEM medium containing 10% FBS, 1, 25-dihydroxyvitamin D_3 (10 nM), and dexamethasone (100 nM), cells were treated with or without I, C, B, and their combination for 12 days. Dental slices were treated with ultrasonic waves in 1 mol/L NH_4OH to remove adherent cells and stained with 0.1% toluidine blue solution. Resorption pits were observed

Table 1: Effects of I, C, B, and their combination on bone mineral content (BMC) and bone mineral density (BMD) in OVX mice ($n = 10$).

Groups	BMC (mg/mm)			BMD (mg/cm^3)		
	Total	Trabecular	Cortical	Total	Trabecular	Cortical
Sham control	1.77 ± 0.27	0.45 ± 0.13	0.89 ± 0.17	500 ± 23	220 ± 25	898 ± 19
OVX control	1.56 ± 0.13	0.33 ±0.08$^{\Delta\Delta\Delta}$	0.82 ± 0.13	426 ±20$^{\Delta\Delta\Delta}$	114 ±16$^{\Delta\Delta\Delta}$	910 ± 19
Nylestriol	1.86 ± 0.21*	0.43 ± 0.04**	0.78 ± 0.14	508 ± 29***	205 ± 20***	916 ± 28
OVX + I (40 mg/kg)	1.67 ± 0.09	0.40 ± 0.08**	0.81 ± 0.16	449 ± 31*	157 ± 25**	906 ± 20
OVX + C (20 mg/kg)	1.62 ± 0.07	0.37 ± 0.09*	0.79 ± 0.12	466 ± 34**	162 ± 30**	885 ± 19
OVX + B (120 mg/kg)	1.56 ± 0.12	0.38 ± 0.11*	0.86 ± 0.15	437 ± 45	149 ± 27**	881 ± 26
OVX + I (40 mg/kg) + C (20 mg/kg) + B (120 mg/kg)	1.52 ± 0.20	0.42 ± 0.05**	0.92 ± 0.17	482 ± 25**	195 ± 22**	889 ± 19

All values are expressed as mean ± SD, $^{\Delta\Delta\Delta}P < 0.001$ versus sham group; $^*P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ versus OVX group.

under a microscope; 20 vision fields of each dental slice were randomly chosen to measure the pit area with image analysis software (Leica Q550IW, Germany). The sum of the resorption pit area of 20 random vision fields was calculated as resorption pit area of each dental slice. The inhibitory effects of the tested compounds on bone resorption were expressed as the resorption pit area of dental slice treated with test compounds/resorption pit area of control × 100%.

2.4. Assay for Cytoskeleton of Osteoclasts

2.4.1. Osteoclasts Induced with M-CSF and RANKL from Bone Marrow Cells. 5×10^7 bone marrow cells were cultured in a 6-well plate containing α-MEM supplemented with 10% FBS and 5 ng/mL M-CSF in a humidified atmosphere of 5% CO_2 for 24 hours. Nonadherent cells were collected and cultured in α-MEM medium containing 10% FBS and 50 ng/mL M-CSF for 3 days. Cells remaining on the bottom of the wells were considered as bone marrow-derived macrophages and cultured in α-MEM containing 10% FBS, 50 ng/mL M-CSF, and 100 ng/mL RANKL. The culture medium was replaced with fresh medium every 3 days, and after 6 days, the cells differentiated into mature osteoclasts. The osteoclasts were used to investigate the formation of actin ring and to analyze the expression of regulating proteins.

2.4.2. Assay for Formation of Actin Ring. Osteoclastic cells (1×10^6) induced with M-CSF and RANKL were seeded onto the 10 × 10 mm glass coverslips and treated with or without I, C, B, and their combination for 4 h. The osteoclasts were then fixed with 4% fresh paraformaldehyde in PBS for 15 min, washed three times with PBS, and then permeabilized with 0.1% Triton X-100 in PBS for 10 min. F-actin ring in the cells was labeled with rhodamine-conjugated phalloidin by incubating for 30 min in darkness. Nuclei were stained at 37°C for 10 min with 5 μg/mL Hoechst 33258 solution, and after further washes in PBS, the cells were mounted in glycerol. Specimens were observed using a confocal laser scanning microscope (LEICA TCS-SP5, Germany) with appropriate combinations of filters and mirrors.

2.5. Western Blot. Cells from neonatal rat cavarial osteoblasts or osteoclasts induced with M-CSF and RANKL from bone marrow cells (as described in Section 2.4.1) were treated with or without I, C, B, and their combination for 24 h. Cells were lysed in a buffer containing 20 mM Tris–HCl, 150 mM NaCl, 1% Triton X-100, and protease and phosphatase inhibitors. The lysates (30–40 mg) were separated by 10% SDS PAGE and transferred to a polyvinylidene difluoride membrane. After blocking with 5% skim milk, the membrane was probed with anti-OPG and RANKL for osteoblasts or anti-phospho Akt, ERK, JNK, p38, and Iκ-B for osteoclasts. The same membrane was stripped and reprobed and chemiluminescent signals were detected with a Gel Doc 2000 luminescent image analyzer (Wealtec Dolphin-Doc, USA).

2.6. Statistical Analysis. The experiments were repeated three times in five replicate samples. Data were expressed as mean ± standard deviation and one-way ANOVA, followed by Dunnett's t-test which was used for statistical analysis (PASW 18.0 software; SPSS Inc., Chicago, USA), and the level of significance was set at $P < 0.05$ (*), $P < 0.01$ (**), $P < 0.001$ (***).

To determine if the compounds were acting synergistically, we used the probability sum test (q test) [19, 20]. The formula used is as follows: $q = E_{A+B}/(E_A + E_B - E_A \times E_B)$. Here, A and B indicate compound A and compound B; E is the rate of change in the treated group compared with the mean values in the control group. E_{A+B} is the real percentage of responders and $(E_A + E_B - E_A \times E_B)$ is the expected response rate. $(E_A + E_B)$ is the sum of the probabilities when compound A and compound B are used alone. $(E_A \times E_B)$ is the probability of cells responding to both compounds when they were used alone. When q was <0.85, the combination was thought to be antagonistic; when $q > 1.15$, the combination was thought to be synergistic; when q was between 0.85 and 1.15, the combination was thought to be additive.

3. Results

3.1. ICB Decrease Bone Loss in Ovariectomized Osteoporotic Mice. As shown in Table 1, 12 weeks after ovariectomy, tibia bone mineral content (BMC) and bone mineral density (BMD) significantly decreased in total and trabecular bone compared with sham mice, but did not change in cortical bone. Administration of nylestriol significantly increased

total and trabecular bone BMC and BMD in tibia compared to the OVX control. Administration of I, C, B, and their combination significantly increased BMC and BMD of trabecular bone and total BMD, but did not change total BMC in tibia. The effects of their combination were more potent than the individual compounds. These results indicate that I, C, B, and their combination improve BMC and BMD of trabecular bone and decrease bone loss induced by ovariectomy.

As shown in Figure 1, administration of I, C, B and their combination did not cause the increase of uterine weights and inhibit weight gain of ovariectomized mice, except for icariin which inhibited the body weights gain. Serum deoxypyridinoline cross-links to creatinine ratio (DPD/Cr), TRAP levels are biochemical markers of bone resorption, and ALP is a marker for bone formation. Ovariectomy induced high bone turnover in mice as evidenced by a significant increase in serum DPD/Cr, TRAP, and ALP levels. Nylestriol decreased serum DPD/Cr, TRAP, and ALP levels and inhibited high bone turnover in OVX mice. Administration of I, C, B, and their combination decreased serum DPD/Cr and TRAP levels, but no decrease in serum ALP levels. OPG (osteoprotegerin) is an endogenous protein produced by osteoblastic cells, which inhibits osteoclast formation and activation. Ovariectomy decreased serum OPG levels and in contrast, nylestriol increased serum OPG. The I, C, B, and their combination significantly increased OPG levels in ovariectomized mice. These results show that I, C, B, and their combination increased bone density by inhibiting bone resorption, and their combination showed higher activity than the individual compounds. Therefore, the effects and interaction of I, C, and B were further investigated for their mechanism of action and mutual interaction on osteoclastic bone resorption.

3.2. ICB Synergistically Inhibits Osteoclast Formation and Differentiation. To clarify the effect of I, C, and B alone or their combination on osteoclast formation, bone marrow cells were cocultured with osteoblasts derived from rat calvaria in the presence of 10^{-8} M 1, 25-dihydroxyvitamin D_3. Many TRAP-positive osteoclasts were formed in the coculture system within 6 days in response to 1α, $25(OH)_2D_3$. As shown in Figures 2(a) and 2(b), the numbers of TRAP-positive multinucleated cells were significantly reduced by I, C, and B or their combinations. In treatment of osteoclast with I, C, or B alone, the numbers of TRAP-positive multinucleated cells were reduced to 68.1%, 70.7%, and 63.5% of control, respectively. The combination of IB, IC, BC, and IBC decreased the number of osteoclasts to 37.1%, 53.0%, 46.0%, and 21.4% of control, respectively. According to the q value, the combination of IC, BC, and IB exerted additive inhibitory effects, and the combination of ICB exerted somewhat synergistic inhibitory effects on osteoclast formation.

TRAP activity was directly related with osteoclastic bone resorption and as shown in Figure 2(c), after 48-h treatment, TRAP activity was significantly suppressed by I, B, C, and their combination. In treatment of osteoclast with I, B, and C alone, the TRAP activity decreased to 89.4%, 80.9% and 90.4% of control, respectively. The combination of IB, IC, BC, and IBC reduced the TRAP activity to 74.8%, 77.6%, 74.9%, and 60.2% of control, respectively. According to the q value, the combination of IB and BC exerted additive inhibitory effects; the combination of IC and ICB exerted synergistic inhibitory effects on the TRAP activity of osteoclast.

Another possible mechanism of the inhibitory effect on bone resorption of mature osteoclasts is that I, B, C, and their combination could reduce bone matrix degradation by inhibiting cathepsin K (CK) activity [21]; hence, we further evaluated the effects on cathepsin K activity of osteoclasts in a cell-free enzyme assay using synthetic substrate Ac-LR-AFC and recombinant cathepsin K. As shown in Figure 2(d), I, B, and C inhibited the osteoclastic CK activity to 86.4%, 88.9%, and 86.6% of control, respectively. Although the treatment of combinations of I, C, and B performed more potential suppressive effects than individual compounds, these combinations exerted antagonistic or additive, but not synergistic, inhibitory effects on cathepsin K activity of osteoclast according to the q value.

3.3. ICB Synergistically Suppresses Osteoclastic Bone Resorption. The dental slices were cocultured with osteoclast for 12 days. Untreated dental slices possess a very homogenous surface. Mature osteoclasts erode this homogenous surface and form resorption pits. After staining with toluidine blue, the resorption pits can be identified easily by their blue color. Under the experimental condition, a statistically significant reduction of number and area of bone resorption pit were observed on the dental slices treated with I, C, B, and their combination compared with control. In the mono-treatment group, I, C, or B alone decreased the area of bone resorption pit to 65.2%, 67.5%, and 62.4% of control. Bitreatment of I, C, and B decreased the area of bone resorption pit to 33.9%, 36.8%, and 39.4% of control. The ICB combination treatment decreased the area of bone resorption pit to 12.8% of control (Figures 3(a) and 3(b)). According to the q value, the combination of I, C, and B exerted slightly synergistic inhibitory effects on the bone resorption of osteoclast. These results suggested that ICB combination intensifies the inhibitory effect on osteoclastic bone resorption.

3.4. ICB Synergistically Blocks RANKL-Induced Osteoclasts Cytoskeletal Organization. Osteoclastic bone resorption is initiated by the attachment of osteoclasts to the bone surface. Upon attachment, the osteoclasts form characteristic actin rings, a cytoskeletal structure essential for optimal osteoclastic bone resorption [22]. To determine if ICB affects cytoskeletal organization in osteoclasts, bone marrow cells were cultured on glass coverlips in the presence of M-CSF and RANKL with or without I, C, B, and their combination. The actin rings were visualized by staining with rhodamine-conjugated phalloidin to assess the activity in the early phase of bone resorption. As shown in Figure 4, confocal laser scanning microscopy revealed that actin rings were dense and had some pseudopodium lying in their periphery. Treatment of osteoclasts with I, C, B, and their combination caused

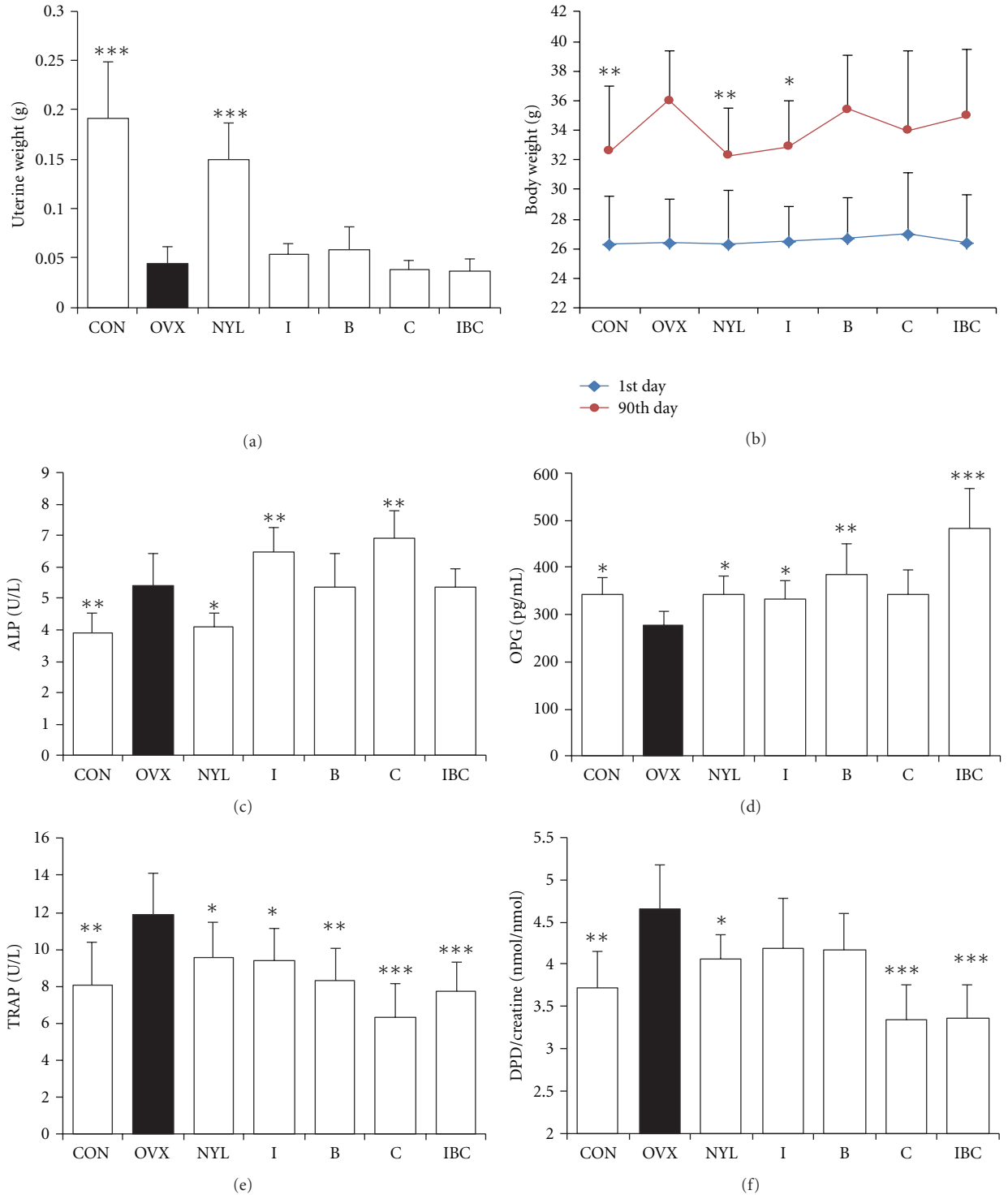

Figure 1: Effects of I, B, C, and their combination on ovariectomized osteoporotic mice. Ovariectomized mice were administered with I (40 mg/kg), B (120 mg/kg), C (20 mg/kg), and their combination (I 40 mg/kg + C 20 mg/kg + B 120 mg/kg) for 12 weeks. Then, the uterine and body weight were weighed, the serum biochemical parameters were assayed. (a) uterine weight; (b) body weight; (c) ALP activity; (d) OPG content; (e) TRAP activity; (f) DPD/creatine. Data were presented as mean ± standard deviation, ($n = 10$). $^{*}P < 0.05$, and $^{**}P < 0.01$, $^{***}P < 0.001$ compared with OVX control.

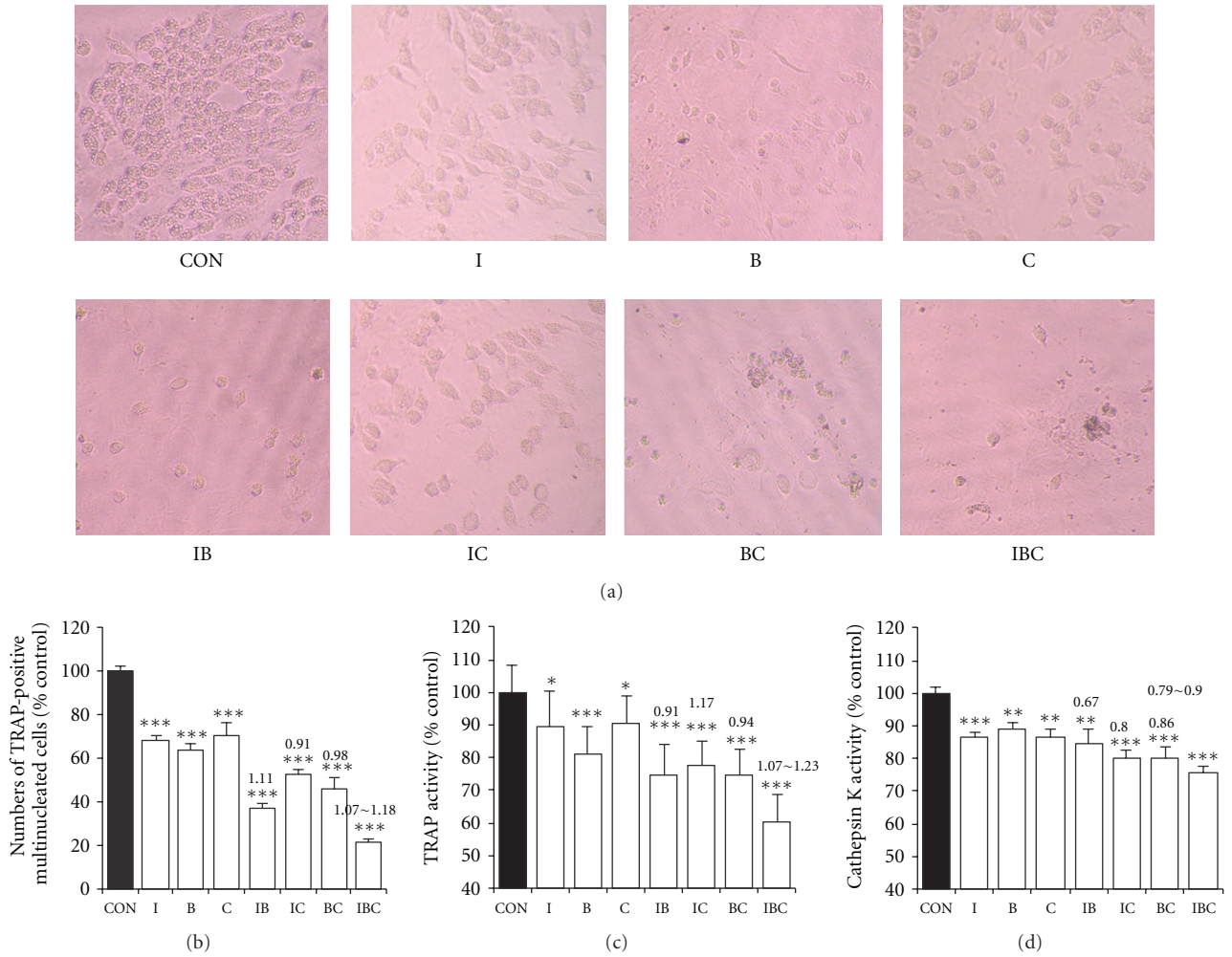

Figure 2: Inhibitory effects of I, B, C, and their combination on osteoclasts. (a) The morphological change of osteoclast (×200). Osteoclasts in the coculture system of primary osteoblasts and bone marrow cells were cultured for 24 h and then treated with or without the I, B, C, and their combination for 10 days. (b) Osteoclasts were treated for 10 days and then stained for TRAP, and those possessing three or more nuclei were counted as osteoclast-like multinucleated osteoclasts under a microscope. (c) Osteoclasts in the coculture system of primary osteoblasts and bone marrow cells were cultured for 6 days and then treated with or without I, B, C, and their combination for 48 hours. TRAP activity was measured by *p*-nitrophenyl sodium phosphate assay. (d) Cells were treated as described in TRAP assay, and the activity of cathepsin K was measured by a fluorometer equipped with a 400 nm excitation filter and 505 nm emission filter. Data were presented as mean ± standard deviation. The experiments were repeated 3 times in five replicate samples ($n = 5$) $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ compared with control. The number on the column is *q* value, indicating the interaction between I, B, and C.

pseudopodium to vanish; F-actin ring was disrupted and was loose and thin in the cytoplasmic area of osteoclasts. The effects of the combination of I, C, and B on the formation of F-actin ring are more potent than that of individual compounds, indicating that I, C, and B could synergistically affect cytoskeletal organization, which is necessary for bone resorption by osteoclasts.

3.5. ICB Elevates the Ratio of OPG and RANKL Expression in Osteoblast. In coculture system, osteoblasts support the differentiation of osteoclast progenitors by expressing RANKL in response to vitamin D_3 and dexamethasone [23]. Thus, it is necessary to determine whether these antiosteoclastogenic compounds were directly affecting osteoclast precursor cells or indirectly targeting to osteoblast and thus modulating the expression of OPG and RANKL, which are critical factors for osteoclast differentiation and activity. In treatment of osteoblast with I, C, B, and their combination for 24 h, the relative protein expressions of OPG in osteoblast were enhanced to 2.38-, 2.42-, 4.13-, 3.29-, 2.11-, 1.18-, and 1.32-fold of control, respectively; the relative protein expressions of RANKL were 1.09-, 1.06-, 1.14-, 0.72-, 0.53-, 0.22-, and 0.39-fold of control, respectively. Therefore, the ratios of the protein expression levels of OPG and RANKL were up to 2.17-, 2.29-, 3.62-, 4.56-, 3.98-, 5.37-, and 3.36-fold

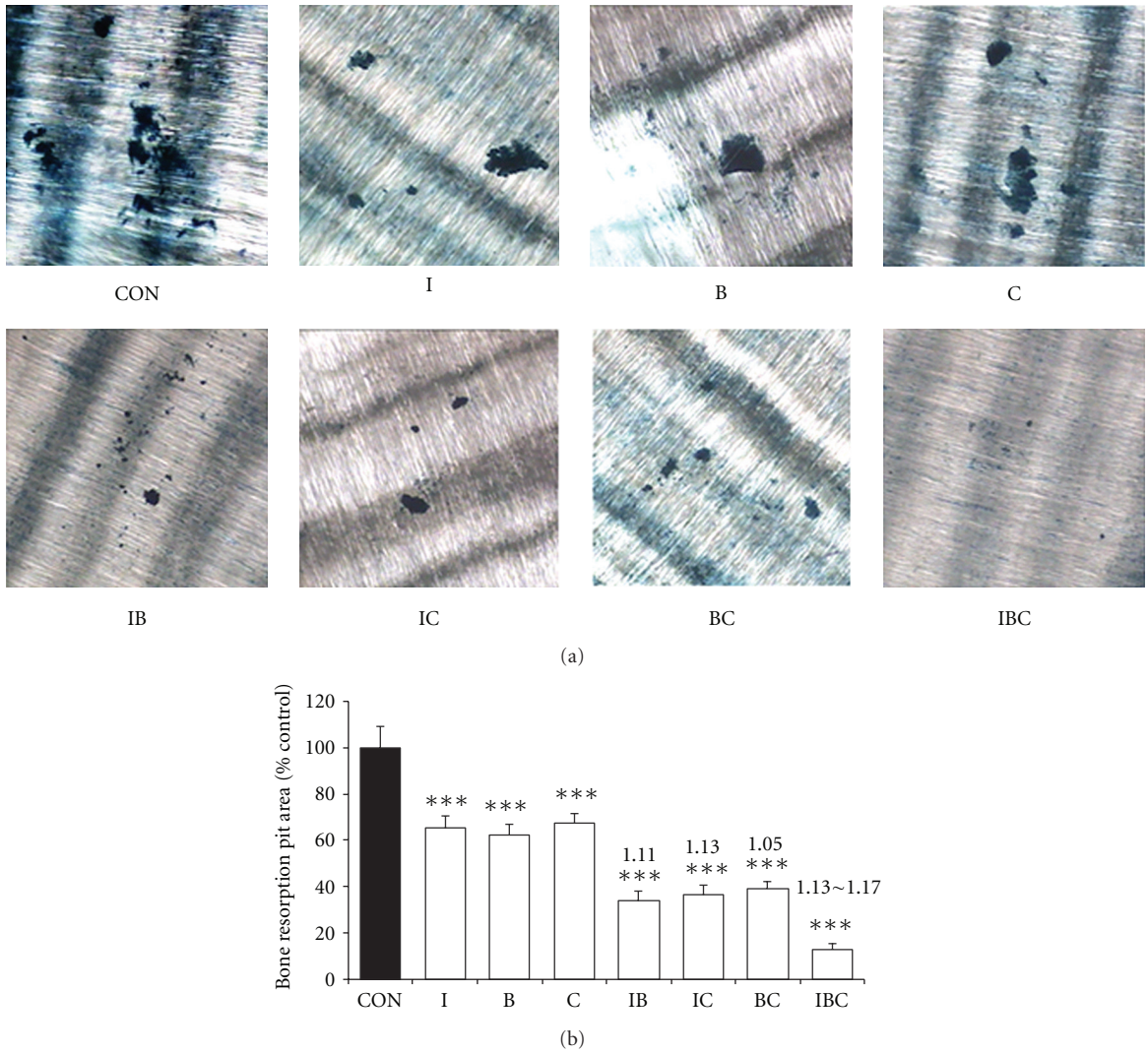

Figure 3: Inhibitory effects of I, B, C, and their combination on osteoclastic bone resorption. Primary osteoblasts and bone marrow cells were cocultured with a sterilized dental slice in α-MEM medium in the presence of 1, 25-dihydroxyvitamin D_3 (10 nM) and dexamethasone (100 nM) and treated with or without I, B, C, and their combination for 12 days. Resorption pits were observed under a microscope, and the pit area was quantitated with image analysis software. (a) Bone resorption pit on dental slices ($\times$100); (b) the change of bone resorption pit area under the treatment of I, B, C, and their combination. Data were presented as mean $\pm$ standard deviation. The experiments were repeated 3 times in five replicate samples ($n = 5$). $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ compared with control. The number on the column is q value, indicating the interaction between I, B, and C.

of control, respectively (Figure 5). These results indicated that I, B, C, and their combinations could regulate the OPG/RANKL system in osteoblast, resulting in the inhibition of osteoclast differentiation in a coculture model system.

3.6. ICB Causes the Decrease of Phosphorylation of RANK Signaling to MAPKs Pathways. RANKL stimulation increases phosphorylation of Akt, ERK, JNK, and p38 in osteoclasts, and activation of the NF-κB pathway requires the phosphorylation of I-κB [24]. Thus, we next determined whether I, C, B, and their combination could affect the signaling pathways involving these kinases. As shown in Figure 6, treatment of osteoclasts with I, C, and B alone, the phosphorylation of IκB and ERK were downregulated, and their combination showed more potent inhibitory effects than the individual compounds. Use of I and C alone upregulated the phosphorylation of p38, and the combination of IB, BC, and IBC significantly decreased this kinase; B increased the phosphorylation of Akt and JNK; all the combination of B with I and C reduced the phosphorylation of Akt and JNK. These results indicated that ICB synergistically decrease the phosphorylation of RANKL signaling in MAPK pathways, leading to the reduction of osteoclastic bone resorption. Taken together, our findings suggested the rationality of EXD in treating osteoporosis.

4. Discussions

In this study, we found that, in ovariectomized mice, combined use of the active components of EXD, namely,

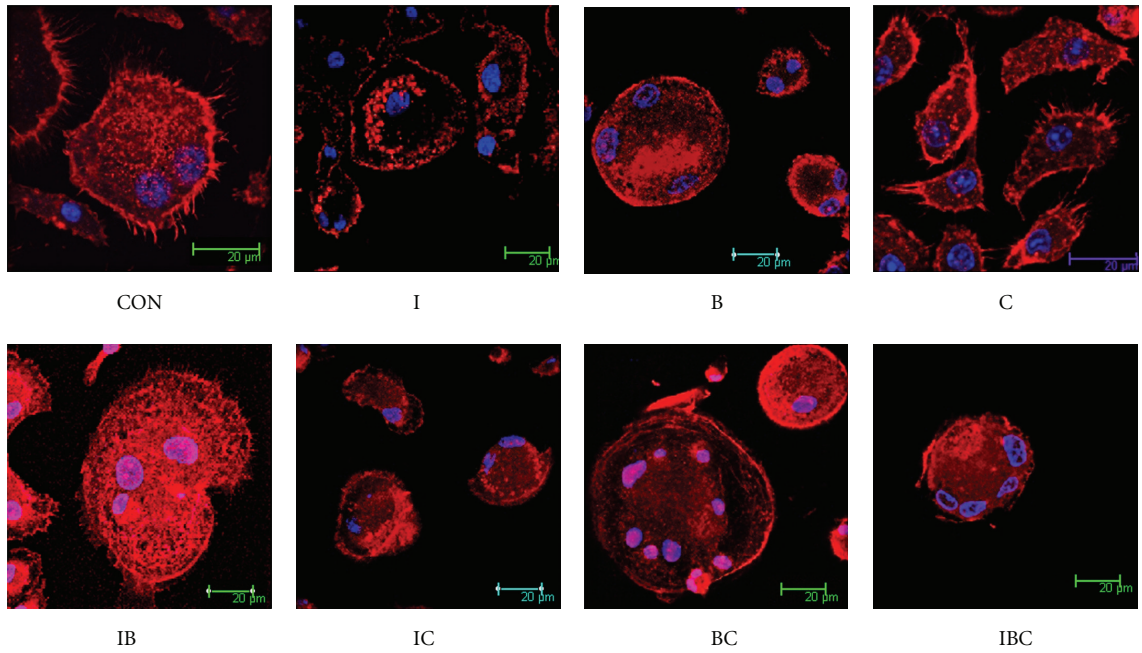

Figure 4: Effects of I, B, C, and their combination on F-actin rings. Osteoclasts were cultured on glass coverslip for 4 h in the absence (control) or presence of I, B, C, and their combination. The cells were then fixed and labeled with rhodamine-conjugated phalloidin and Hoechst 33258 and observed under confocal laser scanning microscope.

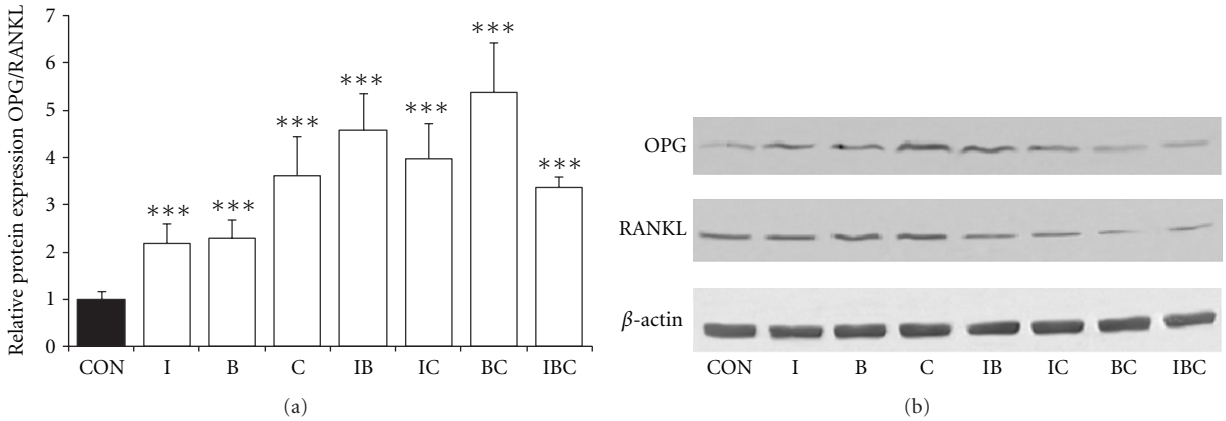

Figure 5: Effects of I, B, C, and their combination on expression of OPG and RANKL of osteoblasts. Primary osteoblastic cells from neonatal rat calvaria were treated with or without I, B, C, and their combination for 24 h. (a) The ratio of protein expression of OPG and RANKL. (b) Protein expression of OPG and RANKL was determined by Western blotting. β-actin was used as an internal reference. Data were presented as mean ± standard deviation. The experiments were repeated 3 times in five replicate samples ($n = 5$), $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ compared with control.

icariin (I), berberine (B), and curculigoside (C), significantly increased the bone mineral density and bone mineral content and regulated the serum biochemical parameters. At the cellular level, the IBC inhibited the formation, differentiation, and bone resorption of osteoclast through modulating OPG/RANKL system in osteoblast and MAPKs pathways in osteoclast. Assessment of the q value by the probability sum test directly demonstrated the synergic effect of I, B, and C on osteoclastic cells to some extent. These results clearly show that components of EXD exert effects of mutual reinforcement. However, IBC does not show an intensified adverse effect in the ovariectomized murine model, as revealed by change in body and uterine weight, consistent with the safety of EXD. These observations are in agreement with the rationality of the formula used in this study, mutual reinforcement of the compounds, and reduction of adverse effects.

Osteoclastic bone resorption is mediated by the formation of new osteoclasts and the bone-resorbing activity of osteoclasts. The mature osteoclasts are characterized by multinuclearity, TRAP staining, cathepsin K activity, an actin ring structure, ruffled border, and acidic cell condition

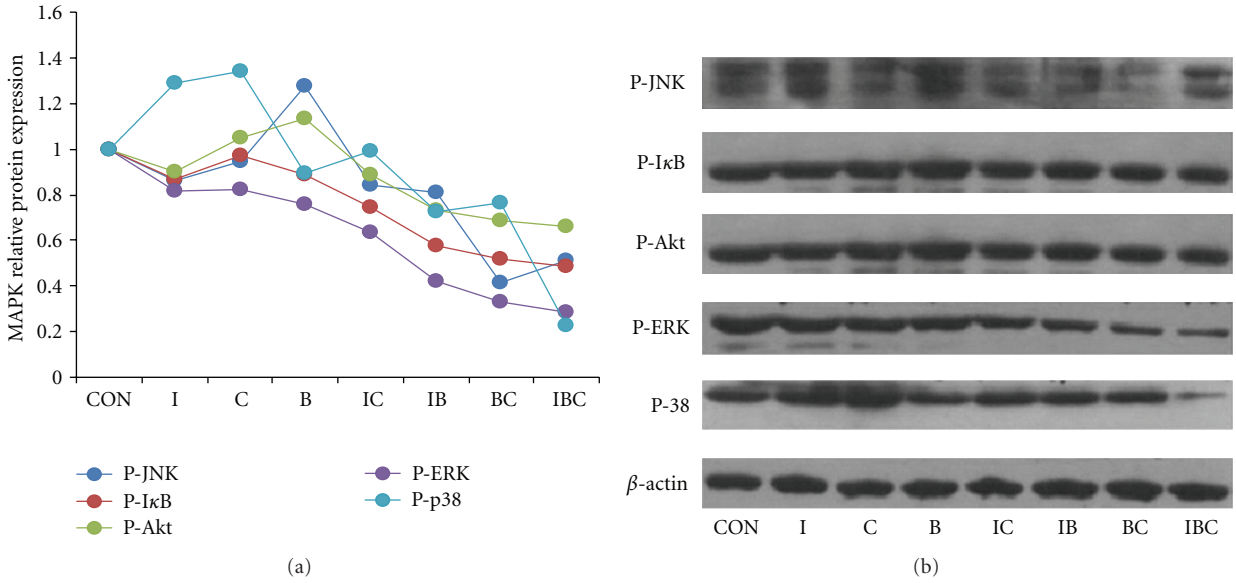

Figure 6: Effects of I, C, B, and their combination on RANKL-induced signaling pathways. Osteoclasts induced from bone marrow cells by M-CSF and RANKL were treated for 24 h and assessed for phosphorylation of JNK, ERK, p38 MAPK, and Akt and the degradation of IκBα by Western blot. β-actin was used as an internal reference.

during resorption [13]. As depicted in Figure 2, exposure of osteoclasts to I, B, C, and their combination significantly reduced the number of TRAP-positive multinucleated cells and the activity of TRAP and cathepsin K and induced the disruption of actin rings, indicating that I, B, C, and their combinations reduced the pit-forming activity of osteoclasts, both by triggering the direct disruption of actin rings and by inhibiting osteoclast formation and the survival of mature osteoclasts. Icariin, which exhibits the strongest therapeutic effects in the ovariectomized osteoporotic rats, has also been shown to be the principal ingredient of EXD in targeting osteoclastic bone resorption [16]; the B and C enhance the inhibitory effects of I on osteoclastic formation, differentiation, and bone resorption, augmenting the effects of I. Thus, the EXD formula proves its rational by modern biochemical analysis.

The receptor activators of NF-κB ligand (RANKL) and osteoprotegerin (OPG) synthesized by osteoblast plays an essential role in the osteoclastic formation, differentiation, and bone resorbing activity [7]. RANKL provides a signal to osteoclast progenitors through the receptor activator of NF-κB (RANK) to activate osteoclast differentiation and function. OPG blocks the interaction between RANKL and the RANK receptor. In other words, OPG inhibits osteoclastogenesis while RANKL supports bone resorption of osteoclast [25]. Therefore, bone remodeling can be assessed by the relative ratio of OPG to RANKL. In the present study, I, B, C, and their combinations significantly increased the relative ratio of OPG to RANKL, indicating that they inhibited the bone resorption by modulating the expression of OPG and RANKL of osteoblast.

The RANK signaling by the interaction with RANKL induces recruitment and activation of tumor necrosis factor receptor-associated factors (TRAFs), leading to the activation of MAPK pathway, including ERK, p38, and JNK [9]. The ERK pathway is involved in the negative regulation of osteoclastogenesis [6]. However, the p38 and JNK pathways have been shown to play a critical role during RANKL-induced osteoclast differentiation [26]. Osteoclast apoptosis is controlled by several signaling molecules, including TRAF6, Src, PI3 K/Akt, ERK, and NF-κB pathways [27]. It was found in our study that the phosporylation of ERK, Akt, JNK, and IκB was decreased slightly and that of osteoclast p38 was increased after I administration; the phosphorylation of ERK was downregulated after C or B administration alone; the phosphorylation of ERK, Akt, p38, JNK, and I-κB of osteoclasts was downregulated significantly after combined administration of I with C or/and B. These results suggest that I, C, and B had different modulating mechanisms through MAPK pathways and their combinations had more potent inhibitory effects on the phosphorylation of these signaling protein of osteoclast.

It is worth pointing out that Chinese medicinal formula may have complicated changes by combining various herbs or their constituents. Some may reinforce or decrease their effects, moderate or eliminate their original toxic side effects; some may facilitate the delivery of the principle element to the disease site in the body [28]. In EXD, Epimedii Folium and Curculiginis Rhizoma have similar properties and effects their combination could reinforce each other's action. Phellodendri Chinensis Cortex has different pharmacological action from Epimedii Folium and Curculiginis Rhizoma but could assist in raising their therapeutic effects [17]. I, C, and B are major active constituents from Epimedii Folium, Curculiginis Rhizoma, and Phellodendri Chinensis Cortex,

respectively. It may be an interesting observation in this work that ICB treatment decreases the osteoclastic bone resorption compared with I treatment alone, whereas I combined with C and/or B significantly upregulates OPG, and downregulates RANKL. Because OPG was upregulated, RANKL was downregulated 24 h after ICB treatment; some other events might also be involved in the enhancement of I action by C and B at the earlier stage. Interestingly, the same results were also observed in the MAPKs pathways. The phosporylation of P38, ERK, Akt, JNK, and IκB was decreased slightly after I administration, while the phosphorylation of these signaling proteins was downregulated significantly after combined administration of I with C or/and B. These results suggest that C and B might assist I in the EXD formula to modulate these key pathways.

The antiosteoporotic activity and mechanism of icariin, curculigoside, and berberine have been extensively studied. Icariin enhances the differentiation and proliferation of osteoblasts and facilitates matrix calcification through the induction of BMP-2, BMP-4, and NO synthesis, subsequently regulating Cbfα1/Runx2, OPG, and RANKL gene expressions and activating BMP signaling [29–32]. Icariin also inhibited osteoclastic differentiation and reduced the motility and osteoclastic bone resorption [29, 33]. These regulatory effects of icariin on bone remodeling are also related with estrogen-like activity [30, 31]. Curculigoside has definite effects on osteoblast and osteoclast, enhances the expression of vascular endothelial growth factor and bone morphogenetic protein-2 in osteoblastic MC3T3, and prevents hydrogen peroxide-induced dysfunction and oxidative damage in calvaria osteoblasts [34–36]. Berberine inhibits RANKL-mediated osteoclast formation and survival through suppression of NF-κB and Akt activation [37]. In osteoblastic cells, berberine enhanced the expression of osteogenic marker genes including osteopontin and osteocalcin through activation of Runx2 by p38 MAPK [38]. These findings indicated that icariin, curculigoside, and berberine modulate the bone metabolism through multitargets and pathways. Furthermore, EXD contains various kinds of active components, including flavonoids, phenolic glycoside, alkaloids, anthraquinone, organic acid, and polysaccharide. It is likely that a complicated interaction of active components exists on multiple targets. Therefore, the protective effects of EXD on bone loss are achieved in a similar manner in that multiactive components are exerting their effects through multitargets and pathways; however, these need to be investigated in more depth.

In conclusion, the present study clarifies the interaction relationship on osteoclast between icariin, curculigoside, and berberine. This supports the theory that in TCM formula, the use of multiherbs or their ingredients can provide mutual reinforcement and assistance thus enhancing the therapeutic effects when compared to individual ingredients.

Conflict of Interests

There is no conflict of interests among the authors.

Authors' Contribution

L. Xue and L. Jiao contributed equally to this work.

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Research Article

In Vivo Study on the Pharmacological Interactions between a Chinese Herbal Formula ELP and Antiresorptive Drugs to Counteract Osteoporosis

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Antiresorptive drugs, alendronate and raloxifene, are effective in lowering bone mineral density (BMD) loss in postmenopausal women. However, long-term treatment may be associated with serious side effects. Our research group has recently discovered that a Chinese herbal formula, ELP, could significantly reduce BMD loss in animal and human studies. Therefore, the present study aimed to investigate the potential synergistic bone-protective effects of different herb-drug combinations using ovariectomized rats. To assess the efficacy of different combinations, the total BMD was monitored biweekly in the 8-week course of daily oral treatment. Bone microarchitecture, bone strength, and deoxypyridinoline level were also determined after 8 weeks. From our results, coadministration of ELP and raloxifene increased the total tibial BMD by 5.26% (2.5 mg/kg/day of raloxifene; $P = 0.014$) and 5.94% (0.25 mg/kg/day of raloxifene; $P = 0.026$) when compared with the respective dosage groups with raloxifene alone. Similar synergistic effects were also observed in BMD increase at distal femur (0.25 mg/kg/day; $P = 0.001$) and reduction in urinary deoxypyridinoline crosslink excretion (2.5 and 0.25 mg/kg/day; both $P = 0.02$). However, such interactions could not be observed in all alendronate-treated groups. Our data provide first evidence that ELP could synergistically enhance the therapeutic effects of raloxifene, so that the clinical dosage of raloxifene could be reduced.

1. Introduction

Osteoporosis is a degenerative disease characterized by low bone mass and deterioration of microarchitecture of bone, which increases bone fragility and susceptibility to fractures [1]. These fractures incur morbidity and mortality to the elderly. Osteoporosis is one of the most serious geriatric health problems in Europe and America [2, 3]. Approximately 30% of all postmenopausal women have osteoporosis in Europe and America. It is estimated that over 200 million people worldwide suffer from this disease [4]. By 2050, the worldwide incidence of osteoporotic hip fracture is projected to increase by 240% in women and 310% in men [5]. The economic costs due to osteoporotic fractures have increased tremendously in the past decade and are predicted to grow. Measures are needed to reduce the prevalence of osteoporosis and incidence of osteoporotic fractures. A number of drugs are currently thought to be effective for the prevention or treatment of osteoporosis. Alendronate and raloxifene are two of the drugs that have been extensively

used. Both are effective in lowering bone mineral density (BMD) loss and reducing the risk of fractures [6]. Their antiosteoporotic actions are quite similar in inhibiting bone resorption process, resulting in the increase of the overall bone formation. However, there is increasing evidence that both drugs have the potential in causing a number of adverse effects upon long-term administration. For instance, long-term bisphosphonates treatments would lead to the osteonecrosis of the jaw [7]. Treatments with raloxifene may increase the risk of venous thrombosis [8]. Although these side effects are not common, there is still concern regarding the long-term use against the use of these antiresorptive agents.

In the past few years, our research group discovered a number of Chinese medicines that can effectively prevent and treat osteoporosis. The herbal formula ELP, which contains three "kidney-tonifying" herbs: Epimedii Herba (E), Ligustri Lucidi Fructus (L), and Psoraleae Fructus (P) with a weight ratio of 5 : 4 : 1, has been shown to be prominent in promoting the osteogenic differentiation in rat mesenchymal stem cells by enhancing bone activities such as alkaline phosphatase activity and matrix calcium deposition [9]. In addition, this formula can inhibit the spinal BMD loss in aged ovariectomized osteopenic rats without any adverse effect [10]. Furthermore, we have completed a clinical trial and found that ELP could reduce both bone loss and hip fractures in post-menopausal women. This randomized controlled clinical trial illustrated that 44 mg/kg per day of ELP extract significantly inhibited the progressive bone loss in the spine of postmenopausal women ($n = 75$) after 12 months of herbal treatment [11]. There are also reports showing that one active compound of ELP, icariin can alleviate the bone loss in ovariectomized animals and promote the bone formation in osteoblastic UMR-106 cells [12]. Mechanistic studies proved that icariin acted with helioxanthin derivatives (other bone anabolic agents) could synergistically enhance bone formation [13]. Therefore, the antiosteoporotic actions of ELP are quite different from bisphosphonates and raloxifene. It is postulated that it stimulates bone formation rather than inhibiting bone resorption in the balance of bone metabolism.

Osteoporosis is the result of an imbalance in bone remodeling, with higher bone resorption rate than bone formation rate. Enhancing the activity of bone-forming osteoblasts, plus reducing that of the bone-breaking osteoclasts, may help restoring the balance in bone metabolism and limiting bone loss in the development of osteoporosis [14]. Since ELP and western conventional drugs act on different cellular/molecular targets in bone metabolism, we hypothesized that synergistic effects would be generated when patients take both together. These medicines might be well combined to complement, or compensate for respective defects in each other. To that end, extensive study of the interactions between Chinese medicines and Western medicines is needed, and predictable adverse effects should be avoided. This herbal combination may enhance the overall osteoprotective effects so that the dosage of these antiresorptive drugs can be minimized. Using an ovariectomized rat model, we here investigated whether there are synergistic effects between ELP and antiresorptive drugs (which are greater than the effects of antiresorptive drugs alone) on increasing bone formation and decreasing bone resorption.

2. Materials and Methods

2.1. Chemicals and Reagents. All chemicals and reagents were purchased from Sigma (USA) unless otherwise specified. Alendronate sodium and raloxifene hydrochloride were obtained from Merck (GmbH) and Eli Lilly (USA), respectively.

2.2. Herbal Extraction and Characterization. Raw herbal materials were purchased from a single renowned supplier in Hong Kong. Morphological, microscopic, and chemical authentications were performed in accordance to Chinese Pharmacopoeia [15]. Herbarium voucher specimens of the tested herbs were deposited at the museum of the Institute of Chinese Medicine, the Chinese University of Hong Kong, with voucher specimen numbers as follows: 2004–2547 (E), 2004–2566 (L), and 2004–2568 (P). Raw herbal materials of Epimedii Herba, Ligustri Lucidi Fructus and Psoraleae Fructus, with a weight ratio of 5 : 4 : 1, were extracted under reflux in boiling water for 1 hour and the extraction was repeated twice. The aqueous extracts were collected and filtered. The filtrate was then concentrated under reduced pressure at 50°C and lyophilized into powder. The formula was subjected to standardization. The chemical profile was examined using the representative markers such as icariin (for Epimedii Herba), salidroside (for Ligustri Lucidi Fructus), and psoralen and isopsoralen (for Psoraleae Fructus) using liquid chromatography-mass spectrometry (LC-MS) (6530 accurate-mass Q-TOF LC/MS, Agilent Technologies, USA). ELP aqueous extract (1 mg/mL) was injected into an ACQUITY UPLC C18 column (2.1 × 100 mm id, particle size 1.7 μm) (Waters, USA). A gradient elution was carried out using the following solvent systems: mobile phase A- double distilled water/formic acid (99.9/0.1; v/v); mobile phase B- acetonitrile. The linear gradient elution system was 100% A to 100% B for 30 min, following by standing at 100% A for 10 min. The flow rate was set at 0.3 mL/min. Identification of the chemical markers was carried out by comparing the retention times of unknown peaks to those of the standards with matched ionization products' size. Detection of ionization products was performed by monitoring positive ions of the combined parent and product compounds in multiple reaction monitoring mode (MRM). The theoretical m/z values of the parent and product ions $[M+H]^+$ were set at 677.66 for icariin, $[M+Na]^+$ 323.304 for salidroside, and $[M+H]^+$ 187.16 for both psoralen and its isomer isopsoralen. The abundance of each marker in ELP extract was determined quantitatively.

2.3. Model Establishment and Treatment Protocol. An Animal Experimentation Ethics Approval had been obtained from the Animal Experimental Ethics Committee of the Chinese University of Hong Kong (Ref No. 09/068/MIS). Eighty-eight 3-month old female Sprague-Dawley (SD) rats were used and

housed four per cage in a room maintained at 22°C with a 12-hour light-dark cycle. The rats were equally divided into groups of 8 individuals. Rats in OVX were ovariectomized bilaterally whereas those in Sham experienced sham surgery. During the experimental period, the rats were maintained on standard rodent chow that contained 0.9% calcium and 0.7% phosphate, and distilled water was available *ad libitum*. After three weeks of ovariectomy operation, ELP extracts and two antiresorptive drugs (alendronate (A) and raloxifene (R)) were orally administered to each rat intragastrically for 8 weeks, as shown in Table 1. Rats' body weight was recorded every week to assess the changes. Fasting 24-hour urine samples were collected by placing the animals in individual metabolic cages for one day before sacrifice. Urine samples were acidified with 2 mL of 1 M hydrochloric acid and centrifuged at 1200 ×g for 10 minutes at 4°C to remove contaminants, and aliquots were stored at −20°C until assayed. After sacrifice, blood samples were taken from the abdominal vena cava. Then serum samples were obtained by centrifuged at 3000 ×g for 20 minutes at 4°C and stored at −80°C before assessment of biochemical parameters. The uteruses were removed and weighed immediately. The success of ovariectomy was confirmed at necropsy by failure to detect ovarian tissue and by observation of marked atrophy of the uterine horns.

The animals were divided into 11 groups as shown in Table 1. Group 1 was sham operated, while the remaining 10 groups of rats were ovariectomized (OVX). The dose of ELP used in the experiment (0.35 g/kg/day; groups 3 and 8–11) was proven to be effective in our previous study [10]. The dose (equivalent to an adult human intake of 6–12 g crude herb) was calculated from the human equivalent dose table [16], multiplied by a factor of extraction yield. Similar justifications were also applied for A and R. Our pilot studies indicated that the optimal concentrations for A and R were 0.5 (group 4) and 2.5 mg/kg/day (group 6), respectively. Low doses of each of the antiresorptive drugs (1/10 of optimal concentration), A (Low; group 5) and R (Low; group 7), were also tested to establish the dose-dependent effect for each drug. For groups 8 to 11, the combined treatment of ELP with high or low dose of A or R was set up in order to study the interactions between each selected herb-drug pair.

2.4. Bone Mineral Density Determination. Changes in bone mineral density (BMD) at lumbar vertebra (L5), proximal tibial metaphyses, and distal femoral metaphyses of the rats were monitored using a peripheral quantitative computed tomography (pQCT) (XCT2000, Stratec Medizintechnik, GmbH) bi-weekly within the eight weeks of the experimental period. The day on which treatment started was Day 0. The coefficient of variation (CV%) of standard measurements was less than 3%. First, the rat was anesthetized using a cocktail of ketamine and xylazine (100 mg/kg body weight and 10 mg/kg body weight, respectively) intramuscularly. It was then fixed 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 the pQCT. The scan speed was 25 mm/sec with voxel resolution 0.2 mm.

Table 1: Grouping and treatment protocol.

Group	Description
(1) Sham	Sham-operated
(2) OVX	Ovariectomized (OVX)
(3) OVX + ELP	OVX treated with 0.35 g/kg/day ELP
(4) OVX + A	OVX treated with 0.5 mg/kg/day alendronate
(5) OVX + A (Low)	OVX treated with 0.05 mg/kg/day alendronate
(6) OVX + R	OVX treated with 2.5 mg/kg/day raloxifene
(7) OVX + R (Low)	OVX treated with 0.25 mg/kg/day raloxifene
(8) OVX + ELP + A	OVX treated with 0.35 g/kg/day ELP + 0.5 mg/kg/day alendronate
(9) OVX + ELP + A (Low)	OVX treated with 0.35 g/kg/day ELP + 0.05 mg/kg/day alendronate
(10) OVX + ELP + R	OVX treated with 0.35 g/kg/day ELP + 2.5 mg/kg/day raloxifene
(11) OVX + ELP + R (Low)	OVX treated with 0.35 g/kg/day ELP + 0.25 mg/kg/day raloxifene

Total BMD (BMD including both cortical and trabecular areas) was generated and presented.

2.5. Bone Microarchitectural Analysis. The microarchitecture of the left distal femur was analyzed using a microCT (Micro CT 40, Scanco Medical, Switzerland) after the rats had been euthanized. 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 within the distal femur was identified with semiautomatically drawn contour at each two-dimensional (2D) sections. Segmentation parameters were fixed at: Sigma = 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 through three-dimensional reconstructed images with the image analysis program of the micro-CT workstation. Parameters from direct model (bone volume density (BV/TV), trabecular number (Tb.N), trabecular thickness (Tb.Th), and trabecular plate separation (Tb.Sp)) were analyzed.

2.6. Biomechanical Test. Right femur was harvested after the rat had been sacrificed. It was wrapped with 0.9% saline-soaked gauze and placed in a resealable plastic bag immediately. All samples were stored at −20°C. Overnight thawing at room temperature (24°C) of the specimen was allowed before biomechanical test. Three-point bending test was performed using Hounsfield material testing machine (KM25, Redhill, UK). A load cell with maximum 250 N was mounted. The span of the lower supports was 20 mm. The midshaft of the bone was loaded at a constant speed of 5 mm/min in medial-lateral approach until failure. Strengths at yield, maximum, and break were recorded for analysis.

2.7. Serum and Urinary Biochemical Markers Determination. Serum osteocalcin (OC) concentration was assayed using rat OC ELISA kit from Biomedical Technologies (USA). Urinary level of deoxypyridinoline cross-links (DPD) was determined by ELISA kit (Quidel, USA). A standard curve was generated from each kit and the concentrations were calculated from the standard curves.

2.8. Statistical Analysis. The differences between treatments and control groups were tested with either (i) one-way analysis of variance (ANOVA) or (ii) Kruskal-Wallis test, followed by the Posthoc Dunnet's or Dunn's test, respectively, depending on the data distribution. The groups with alendronate and raloxifene were compared separately. All the covariates were adjusted for the statistical analysis. All statistical analyses were performed by using the Statistical Package of Social Science (SPSS) version 15.0 for Windows and carried out at the 5% level of significance ($P < 0.05$). Data were expressed as mean ± standard error of the mean (SEM).

3. Results

3.1. Chemical Characterization of ELP Extract. The compositions of marker compounds in ELP aqueous extracts as determined by LC-MS were shown in Figure 1. The retention time of salidroside, icariin, psoralen, and isopsoralen were 4.68, 10.99, 11.45, and 11.81 min, respectively (Figure 1(a)). Isomers psoralen and isopsoralen were well separated using this elution condition. The presence of all marker compounds revealed the presence of Epimedii Herba, Ligustri Lucidi Fructus, and Psoraleae Fructus in ELP extract after a series of preparation processes. In the extract sample, salidroside was the most abundant, followed by icariin, and isopsoralen and psoralen (Figure 1(b)).

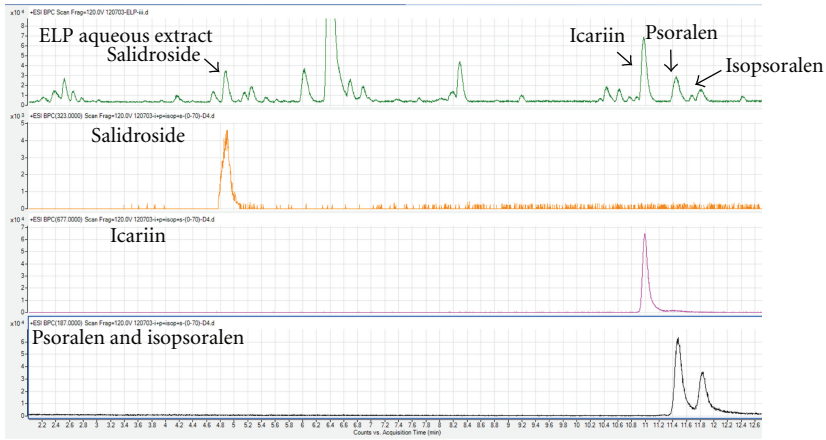
3.2. Model Establishment and Body Weight. For the model establishment, ovariectomy caused a significant decrease of total BMD in lumbar spine, femur, and tibia by 6.5 ± 2.7%, 9.65 ± 3.0%, and 14.37 ± 3.4%, respectively, after three weeks of pretreatment period. During the next 8 weeks of treatment, we found that different combinations of ELP, A, and R did not cause significant change of rat body weight when compared with the OVX without treatment as shown in Figures 2(a) and 2(b).

3.3. Total BMD Analysis. At lumbar spine of OVX group, the effect of ovariectomy in lowering the total BMD was prominent. The total BMD decreased continuously from the baseline (Day 0) to 8.2% during the 8 weeks of treatment period (Figure 3(a)). However, the total BMD of distal femur and proximal tibia was not varied from the baseline (Figures 3(c) and 3(e)). This observation may indicate that ovariectomy results in a longer BMD reduction period in nonweight bearing bone (i.e., lumbar spine) than in weight bearing bones (i.e., femur and tibia) in ovariectomized rats. For the sham group, overall increase in total BMD was observed in all studied regions from baseline to 6.0% (lumbar spine), 11.07% (distal femur), and 14.16 (proximal tibia) at week 8 (data not shown).

All the 9 treatment groups had a higher BMD than OVX significantly at the lumbar spine from week 6. ELP significantly reduced the BMD loss by 5.3% (at week 6; $P < 0.001$) and 3.4% (at week 8; $P = 0.011$) when compared with corresponding OVX groups (Figure 3(a)). This finding was in line with our previous report [10]. However, no significant difference was observed throughout the experiment when ELP was compared with OVX at both distal femur and proximal tibia (Figures 3(c) and 3(e)). This observation reveals that ELP was more effective in prevention of bone loss in nonweight bearing bones than in increasing bone gain in weight bearing bones. For the antiresorptive drugs, dose-dependent effects of A and R were observed at all lumbar spine, distal femur, and proximal tibia. This effect has also been reported in other animal studies [17–19]. Both A (Figures 3(a), 3(c), and 3(e)) and R (Figures 3(b), 3(d), and 3(f)) at their optimal dose could raise the total BMD of the osteopenic rats significantly in all the 3 regions when compared with OVX. The protection efficacies of A were always higher than those of R in all studied regions at optimal dose.

For the combination studies, our data demonstrated that ELP extract could work synergistically with raloxifene in increasing BMD of osteopenic bone. When the osteopenic rats were treated with 0.25 mg/kg/day raloxifene, R (Low), a significant reduction of total BMD loss in lumbar spine and distal femur was observed, but with the lessened efficacies to its optimal dose from week 4 onwards. However, R (Low) had no significant effect in reducing total BMD loss at proximal tibia, similar to ELP group. When the osteopenic rats were cotreated with ELP and R (Low), significant increase in total BMD was observed at femur (Figure 3(d)) and tibia (Figure 3(f)) starting from week 2 to week 8, versus OVX. Interestingly, the bone protective effect of ELP + R (Low) was better than that of R alone at optimal dose from week 2 onwards. Similar observation was found in the lumbar spine, ELP + R (Low) totally abolished the decrease of BMD due to ovariectomy (Figure 3(b)). Cotreatment of ELP and R (Low) even led to higher BMD than R in the weight bearing bones throughout the experiment. Besides, dose-dependent effects of ELP + R and ELP + R (Low) were observed at lumbar spine and tibia. Coadministration of ELP and *R* increased the total tibial BMD by 5.26% (2.5 mg/kg/day of R; $P = 0.014$) and 5.94% (0.25 mg/kg/day of R; $P = 0.026$) when compared with the respective dosage groups with R alone. ELP + R was the most effective treatment group among those groups with R or ELP extract alone in spine and proximal tibia. However, such synergistic effect was absent in all the groups cotreated with ELP and alendronate.

3.4. Bone Microarchitecture Analysis. Although total BMD has been long regarded as a surrogate measure of bone strength, microarchitectural properties provide more comprehensive information to evaluate the impact of different combinations on the quality of femoral trabecular bone. All the treatment groups showed a trend of improvement

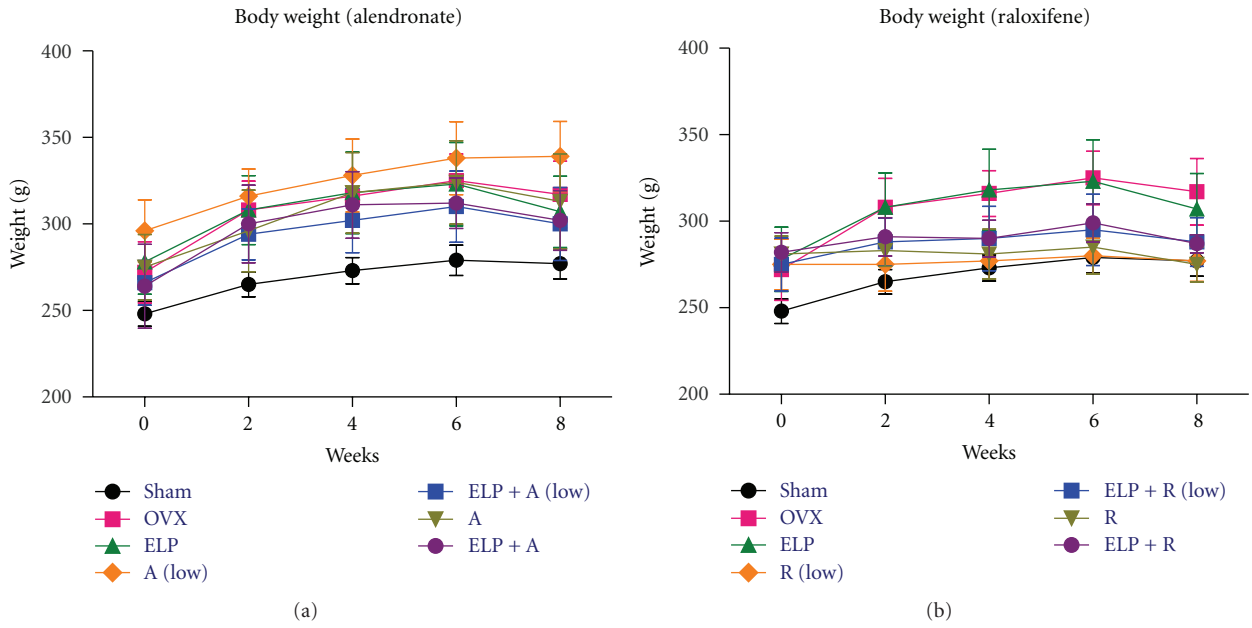


(a)

	Icariin (μg)	Salidroside (μg)	Psoralen (μg)	Isopsoralen (μg)
Abundance per mg	2.7 ± 0.01	3 ± 0.03	0.3 ± 0.02	0.94 ± 0.01

(b)

Figure 1: (a) Comparison of LC-MS base peak chromatograms from ELP aqueous extract and standard chemical markers, salidroside, icariin, psoralen, and isopsoralen; (b) Quantitative analysis of each marker in ELP aqueous extract.



(a) (b)

Figure 2: Mean of rat body weight between week 0 (baseline) and week 8. Rat body weight with different treatment was illustrated: (a) with alendronate; and (b) with raloxifene. The error bar represents the SEM for each treatment group ($n = 8$ per group).

in the microarchitectural properties of the trabecular bone, including higher bone volume fraction (BV/TV), trabecular number (Tb.N), and trabecular thickness (Tb.Th), but lower trabecular separation (Tb.Sp) than OVX. ELP treatment showed a mild, but insignificant increase in BV/TV, Tb.N, and Tb.Th and decrease in Tb.Sp, which was directly correlated with the insignificant increase of total BMD in femoral region, as shown in Figure 3(c). In contrast, a dose-dependent effect of the A and R in increasing bone volume at distal femur of the osteopenic rats was observed. The rats treated with A had a higher BV/TV ($P = 0.007$; Figure 4(a)), Tb.N ($P = 0.004$; Figure 4(c)) but lower Tb.Sp ($P = 0.002$; Figure 4(g)) than OVX. For Tb.N and Tb.Sp, significant differences were not observed in R-treated groups (Figures 4(d) and 4(h)).

Similar to the total BMD analysis, ELP extract could synergistically enhance the efficacy of R at all doses in ovariectomized rats. However, such synergistic effect was

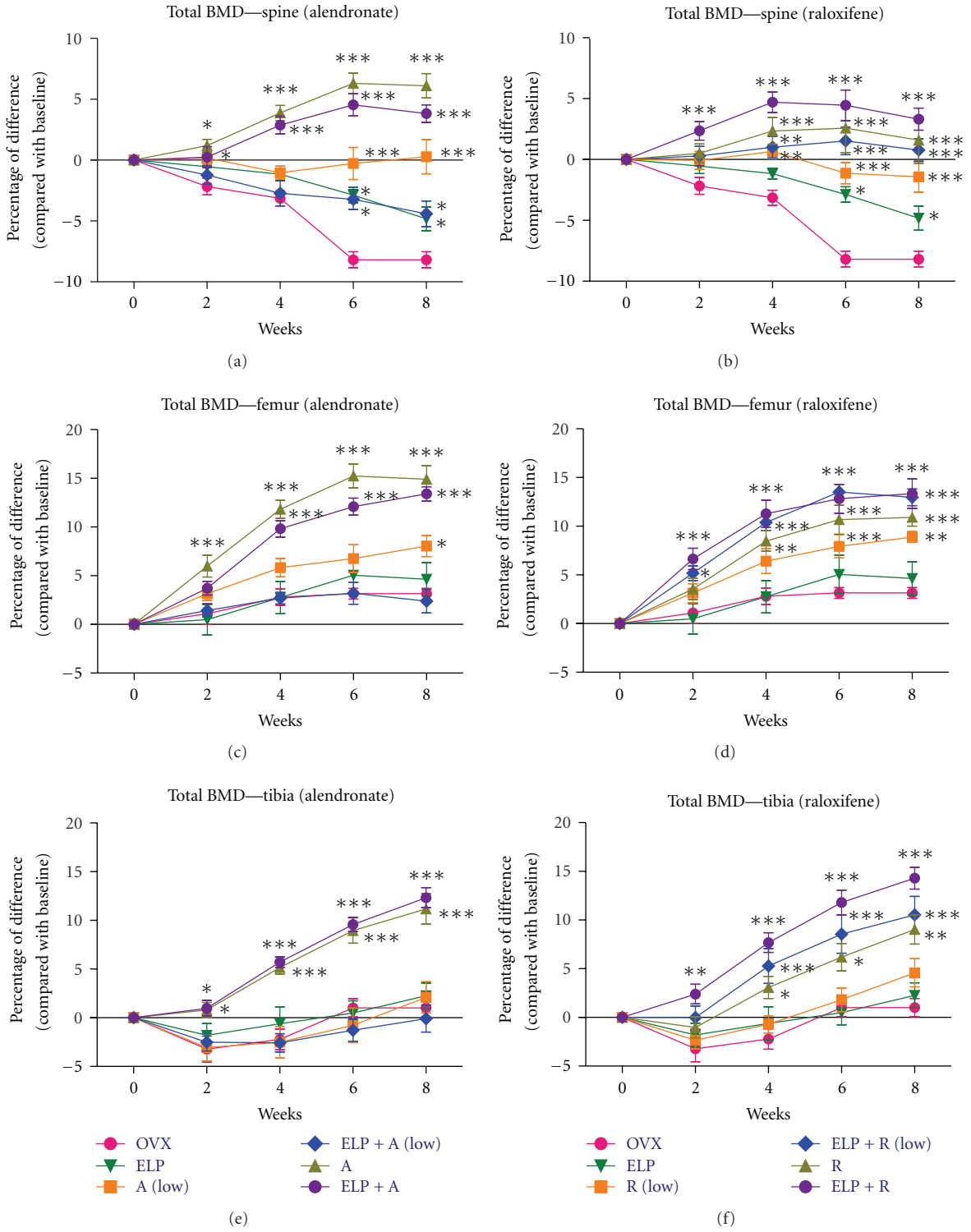


Figure 3: Mean of percentage difference of total BMD in lumbar spine, distal femur, and proximal tibia between week 0 (baseline) and week 8. BMD changes at different regions with different treatment, were illustrated: (a) lumbar spine with alendronate; (b) lumbar spine with raloxifene; (c) distal femur with alendronate; (d) distal femur with raloxifene; (e) proximal tibia with alendronate; (f) proximal tibia with raloxifene. The error bar represents the SEM. Significant difference: $^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$ for difference from OVX group without treatment at the corresponding time point.

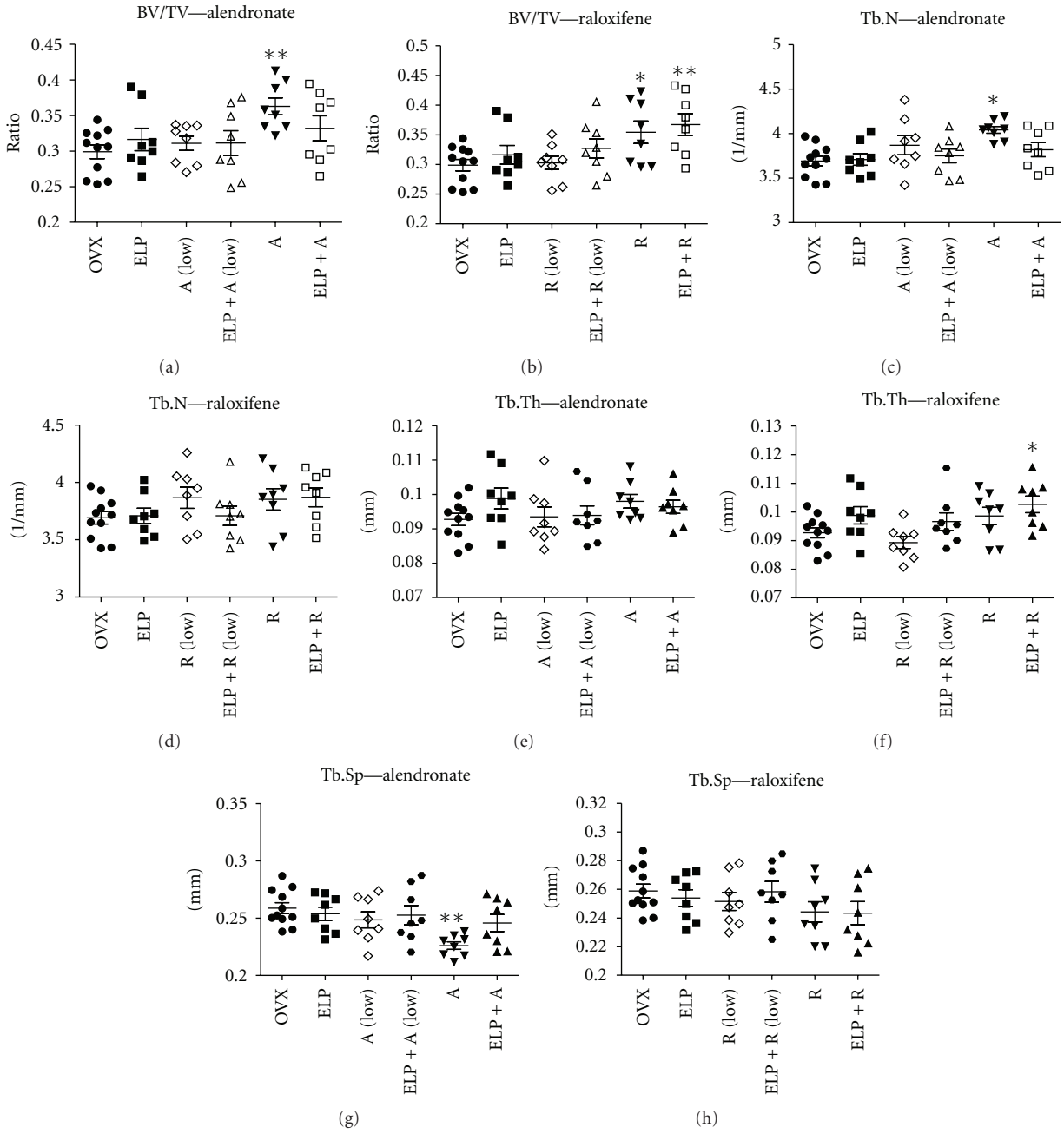


Figure 4: Alterations in trabecular bone volume (BV/TV), trabecular number (Tb.N), trabecular thickness (Tb.Th), and trabecular separation (Tb.Sp) at the distal femur metaphysis following 8 weeks of treatment with different combinations of alendronate (a, c, e, and g) and raloxifene (b, d, f, and h). Bars represent the mean ± SEM for each treatment group ($n = 8$ per group). Significant difference: $^{*}P < 0.05$; $^{**}P < 0.01$ for difference from OVX group without treatment.

absent in all groups cotreated with A. When the rats were cotreated with ELP and R (Low), there was an increasing trend in BV/TV (Figure 4(b)) and Tb.Th (Figure 4(f)), but statistical significance was not achieved, likely due to lower magnitude of changes and limited repetitive tests performed. At higher concentrations of R, ELP + R simultaneously had a further improvement in the microarchitectural properties compared with those treated with R alone. Significant difference was found in BV/TV ($P = 0.007$; Figure 4(b)) and Tb.Th ($P = 0.038$; Figure 4(f)), when compared with OVX groups.

3.5. Biomechanical Testing. Our data on mechanical testing of bones showed that all groups treated with ELP, alendronate, or raloxifene at optimal dose increased the biomechanical properties of the mid-shaft femur of osteopenic rats (Figure 5). Both ELP and R increased the failure strength, ultimate strength, and stiffness significantly compared with

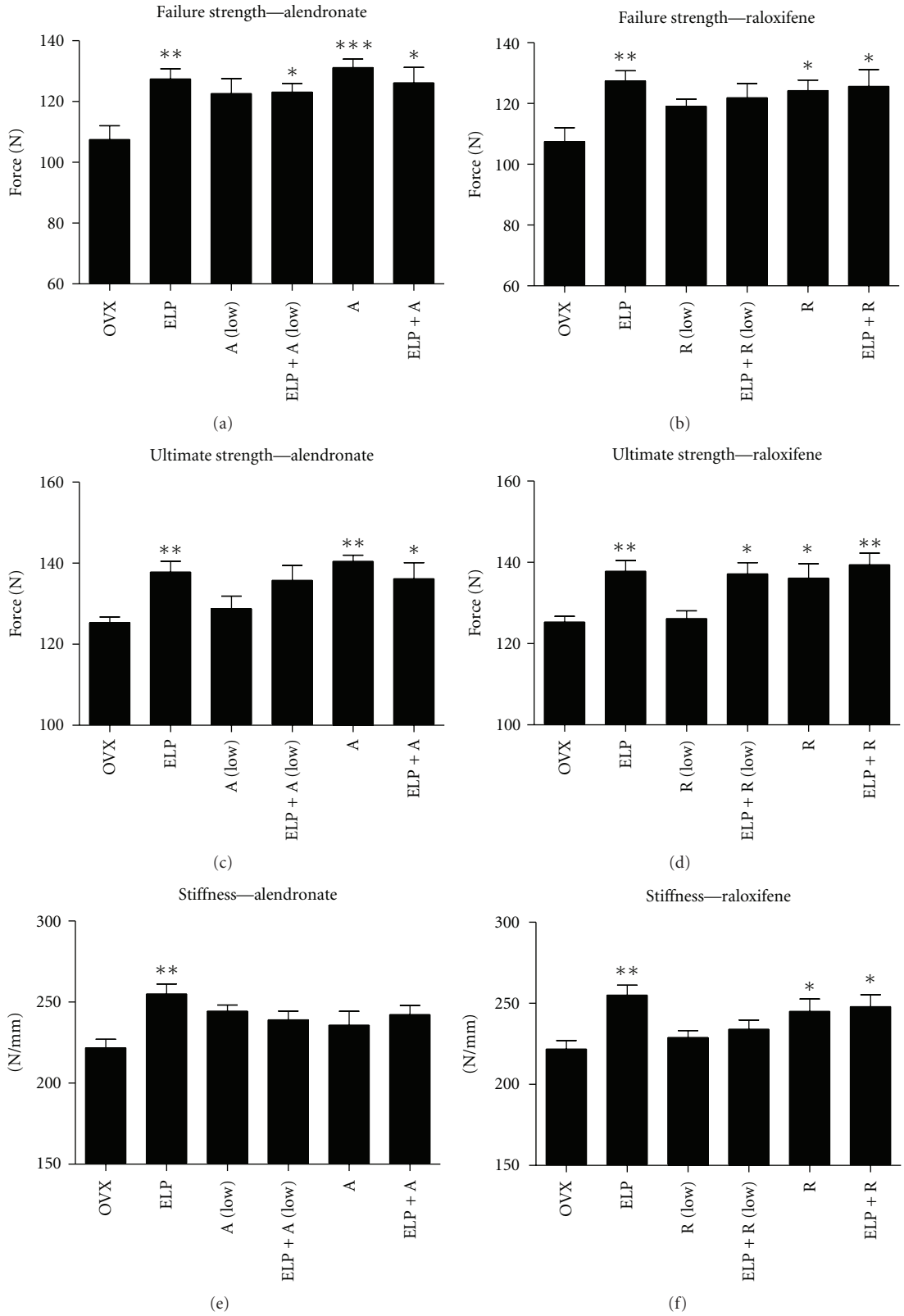


Figure 5: Alterations in failure strength, ultimate strength and stiffness at the femoral midshaft following 8 weeks of treatment with different combinations of alendronate (a, c, and e) and raloxifene (b, d, and f). Bars represent the mean ± SEM for each treatment group ($n = 8$ per group). Significant difference: $^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$ for difference from OVX group without treatment.

OVX, while A increased failure and ultimate strengths significantly. Dose-dependent effect of the two antiosteoporotic drugs on the biomechanical properties was also observed (except the stiffness by A).

For the combination studies, ELP extract could enhance the efficacy of R at all dose levels. On the contrary, such improvement only presented for the groups cotreated with low dose of A for the parameters in failure strength and ultimate strength. When the rats were cotreated with ELP and R (Low), there was an increasing tendency of failure strength (Figure 5(b)), ultimate strength (Figure 5(d)), and stiffness (Figure 5(f)). Similar observations were also found in those groups cotreated with ELP and R. These findings gave further evidence to support that ELP could enhance the function of R in a dose-dependent manner. However, the interaction between ELP and R is not considered as synergistic since the combined effects of any dose of R were weaker than ELP alone. This interaction is regarded as a simple additive.

3.6. Serum and Urinary Biochemical Markers Determination. In order to elucidate the synergistic effect of ELP and R on bone metabolism, the bone formation marker serum osteocalcin level and bone degradation marker urinary DPD level were analyzed. Figure 6(a) showed the corresponding changes in serum osteocalcin level after 8 weeks of treatment. A significant increase in osteocalcin levels was observed in ELP treatment group ($P = 0.031$), but not in both R groups. Similar to the biomechanical test, ELP extract appears to enhance the efficacy of R at all dose levels, although did not reach the level of significance. Figure 6(b) showed the corresponding changes in urinary DPD level after 8 weeks of treatment. Deoxypyridinoline crosslink (DPD), which represents structure degradation of bone by osteoclastic resorption, was effectively regulated by R at 0.25 mg/kg/day ($P = 0.006$) and 2.5 mg/kg/day ($P < 0.001$). There was a trend of decrease in DPD level in ELP treatment group. Interestingly, the coadministration of ELP with R could synergistically decrease the DPD level at both dosages significantly (R (Low), $P = 0.0258$; R, $P = 0.0202$), when compared to R alone. Collectively, these findings provided a proof of an important role for ELP in reinforcing the effects of R mainly through bone resorption inhibition and partially from bone formation enhancement.

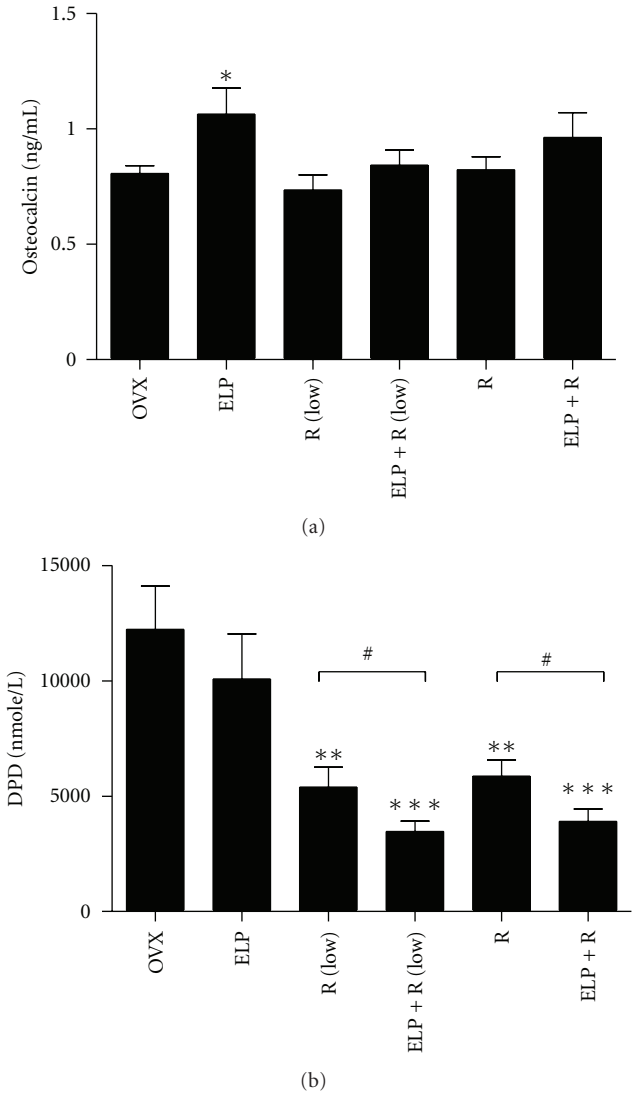


Figure 6: Effects of different combinations of raloxifene-related treatment in serum osteocalcin levels and excretory DPD levels after 8 weeks. Bars represent the mean ± SEM for each treatment group ($n = 8$ per group). Significant difference: $^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$ for difference from OVX group without treatment at the corresponding time point. $^{\#}P < 0.05$ for difference from corresponding raloxifene group (at the same dosage) without ELP cotreatment.

4. Discussion

In our present study, composition of marker compounds in ELP aqueous extracts was determined by LC-MS. We showed that salidroside was the most abundant, followed by icariin, isopsoralen, and psoralen. It may be due to the fact that salidroside is highly soluble in water, while icariin, psoralen, and isopsoralen are water-insoluble in nature.

Comparing two antiresorptive drugs we used in the experiments, we found that alendronate was more effective than raloxifene in reducing overall total BMD loss. As shown in total BMD analysis, alendronate started to have the significant difference from OVX group from week 2, whereas raloxifene started from week 4. These findings were in line with those reported previously that alendronate has a higher efficacy than raloxifene in reducing the risk for osteoporotic fracture [20]. This finding also echoed with the results of bone microarchitecture analysis, which illustrated that the efficacy of alendronate to increase trabecular bone formation of osteopenic bone was better than that of raloxifene. It could also support a current clinical review which reported that onset of efficacy for nonvertebral fracture was reduced by 12 months by alendronate but was reduced by 36 months by raloxifene [21]. Significant reduction on hip fracture rate by alendronate, but not raloxifene, was also reported by

Hopkins et al. [22]. However, for the combination studies, we found that ELP extract could work synergistically with raloxifene in increasing BMD of osteopenic bone. These findings were further substantiated by bone microarchitecture analysis and revealed that ELP had a synergistic effect with raloxifene, but not alendronate, in increasing the new bone deposition on trabecular surface. This observation will be further confirmed by histological analysis.

Interestingly, although ELP extract itself did not cause significant osteo-protective effect on material (BMD) and architectural (microarchitecture) properties of femur, it improved femoral cortical bone strength significantly as shown in the biomechanical test. This unmatched observation might be due to the fact that biomechanical properties of bone depend not only on material and architectural factors, but also the geometric parameters of cortical bone on mid-shaft of long bone [23]. Fracture load was correlated better with BSI (CSA) (a bone strength index including the cortical BMD and the cross-sectional area) of the bone than cortical BMD or total BMD alone in mid-shaft femur and humerus of goats. ELP might affect the geometry of the long bone cortex. Having not measured the geometric parameters was a limitation of this study.

Previously, it has already been demonstrated that ELP extract possessed beneficial effects in promoting bone health in aged ovariectomized rats [10], tail-suspended rats [9], and also in postmenopausal osteopenic women [11]. For the purpose of health supplement and/or pharmaceutical products development, the information related to the herb-drug interactions between ELP and standard antiresorptive drugs is necessary, since some of the osteopenic individuals may consume them simultaneously. In the present study, we have demonstrated that ELP extract itself was able to significantly reduce total BMD loss in lumbar spine and increase biomechanical strength of the bone and serum osteocalcin levels. Provided that ELP and antiresorptive drugs act on different molecular targets in bone metabolism, we postulate that synergistic effects could be generated when individuals take both together, so that the dosage of these antiresorptive drugs can be reduced. In this study, we found that ELP could selectively enhance the therapeutic effects of raloxifene, but not alendronate. In addition, ELP could enhance the effects of raloxifene even at 1/10 of optimal dosage.

To elaborate on the selectivity of ELP actions, we need to look into the characteristics of the animal models and also the fundamental working mechanisms of alendronate and raloxifene. An estrogen deficiency caused by ovariectomy results in (i) inhibition of mature osteoblasts and promotes faster osteoblastic apoptosis; and (ii) stimulation of osteoclast formation and bone resorption; eventually causes a net effect of reduced bone mass [24, 25]. The antiresorptive mechanism of alendronate and raloxifene is not the same. Alendronate is an inorganic pyrophosphate, which preferentially inhibits osteoclast-mediated bone resorption without affecting bone formation [26]. Raloxifene is an oral selective estrogen receptor modulator (SERM) that has estrogenic actions on bone and anti-estrogenic actions on the uterus and breast. It plays an analogous role to estrogens on bone tissue, and its osteoblastic action has recently been shown [27]. It was reported that raloxifene required osteoblastic cells to achieve its anti-osteoclastic action [28]. Previously, we found that ELP not only suppressed the osteoclast formation, but also enhanced the bone formation by increasing the osteogenesis of mesenchymal stem cells [9] and the proliferation of osteoblast as well (unpublished data). The additional osteoblastic actions from ELP may favor the anti-osteoclastic activities of raloxifene in inhibiting bone resorption, as supported by the decrease in DPD level (Figure 6(b)). It may offer a possible explanation as to why the presence of ELP always synergistically enhances the osteoprotective effects of raloxifene against estrogen deficient bone loss in all studied parameters. This suggestion will be verified using different other known anabolic agents, such as strontium ranelate or parathyroid hormone, in combination with raloxifene on the ovariectomized rats. Further experiments are also required to understand the basic underlying mechanisms behind the synergistic actions of ELP on raloxifene using monocytes/macrophages and osteoblast coculture system [28].

5. Conclusions

The primary goal of this work is to examine the drug interaction potential arising from an osteoprotective herbal formula, ELP. We found that ELP extract could (i) synergistically enhance the bone protective effects of raloxifene; and (ii) reduce the dose of raloxifene to achieve its biological effects. The coadministration of ELP may also minimize the adverse effects of raloxifene. The action of ELP was specific to raloxifene but not to alendronate. ELP may be developed as a novel complementary agent to those osteopenic individuals who are receiving raloxifene treatment. To the best of our knowledge, this is the first comprehensive study on the herb-drug interaction in osteoporosis management. These findings justify clinical studies using ELP and standard antiresorptive agents together in an attempt to counteract osteoporosis.

Abbreviations

ANOVA: One-way analysis of variance
R: Raloxifene
A: Alendronate
pQCT: Peripheral quantitative computed tomography
μCT: Micro-computed tomography
LC/MS: Liquid chromatography-mass spectrometry
BMD: Bone Mineral density
BV/TV: Bone volume-to-tissue volume ratio
DPD: Deoxypyridinoline.

Authors' Contribution

C.-H. Ko and W.-S. Siu equally contributed in this work.

Conflict of Interests

The authors declare that they have no conflict of interests in the research.

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Review Article

Vitamin E and Bone Structural Changes: An Evidence-Based Review

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Purpose. This paper explores the effects of vitamin E on bone structural changes. *Methods*. A systematic review of the literature was conducted to identify relevant studies about vitamin E and osteoporosis/bone structural changes. A comprehensive search in Medline and CINAHL for relevant studies published between the years 1946 and 2012 was conducted. The main inclusion criteria were published in English, studies had to report the association or effect of vitamin E and osteoporosis-related bone changes, and the osteoporosis-related bone changes should be related to lifestyle variables, aging, or experimentally-induced conditions. *Results*. The literature search identified 561 potentially relevant articles, whereby 11 studies met the inclusion criteria. There were three human epidemiological studies and eight animal experimental studies included in this paper. Four animal studies reported positive bone structural changes with vitamin E supplementation. The rest of the studies had negative changes or no effect. Studies with positive changes reported better effects with tocotrienol vitamin E isomer supplementation. *Conclusions*. This evidence-based review underscores the potential of vitamin E being used for osteoporosis. The effect of one of the vitamin E isomers, tocotrienols, on bone structural changes warrants further exploration. Controlled human observational studies should be conducted to provide stronger evidence.

1. Introduction

Osteoporosis is a progressive medical condition in which bone density slowly decreases with advancing age. Osteoporosis is often called "silent" because bone loss occurs without symptoms. An estimated 10 million men and women in the United States have osteoporosis and a further 34 million are at risk [1]. Bone is a highly vascularized connective tissue that contains haematopoietic bone marrow, calcium, and phosphate [2]. Calcium is the most abundant mineral found in bone with approximately 98% of human body calcium stored in bone. There are two principal cell types in bone: osteoclast and osteoblast. A balance between bone formation by osteoblasts and bone resorption by osteoclasts is needed for bone remodeling and bone development [3]. Osteoporosis is defined as a bone mineral density (BMD) that lies 2.5 standard deviations or more below the average value for young healthy women. Osteoporosis is a progressive systematic skeletal disorder characterized by low bone mineral density (BMD), deterioration of microarchitecture of bone tissue, and susceptibility to fracture caused by bone resorption [4, 5]. Osteoclast cells have the ability to release free radicals, such as reactive oxygen species (ROS) that will destroy calcified bone tissue and therefore play an integral part in bone remodeling when coupled with osteoblast cells [6–10]. Recent epidemiological studies have also indicated a relationship between oxidative stress and osteoporosis [11, 12].

Vitamin E occurs naturally in eight isoforms: α, β, γ, and δ isomers of tocopherols and tocotrienols. Each isomer of vitamin E consists of an aromatic chromanol ring and a side chain. Tocotrienols possess an unsaturated farnesyl (isoprenoid) side chain compared to tocopherols, which have a saturated phytyl side chain [13]. The unsaturated

side chains of tocotrienols allow them to penetrate more efficiently into the membrane lipid bilayer. Vitamin E has good antioxidant activity, which varies among the different isomers [14]. Vitamin E is a strong antioxidant that plays a vital role in the endogenous defense against peroxidation of membrane lipids [15]. Tocopherols are abundant in polyunsaturated vegetable oils and in the germ of cereal seed [16]. Tocotrienols are abundant in palm oil, cereal grains, and rice bran [17]. Commercial availability of vitamin E is mostly in the form of α-tocopherol, which is taken as an antioxidant supplement [18]. α-Tocopherol has the highest biological activity and is the most abundant form of vitamin E in human tissues and serum [19, 20] as it is selectively retained in the body [21, 22]. Palm olein (refined, deodorized, and bleached palm cooking oil) contains 196 ppm α-tocopherol, 201 ppm α-tocotrienol, 372 ppm β-tocotrienol, and 96 ppm γ-tocotrienol [23]. As described, tocotrienols are found abundant in palm oil and have been reported to be better antioxidants compared to tocopherols [24, 25].

Evidence of vitamin E having a beneficial effect against osteoporosis has been actively researched only recently. Vitamin E has been reported to play a role in increasing bone density. According to researcher NS Ahmad and colleagues in their research on nicotine-treated rats, vitamin E increases the trabecular bone (a spongy-looking bone), prevents bone calcium loss by neutralizing antioxidants, and decreases bone loss of calcium in rats without ovaries [26]. Vitamin E supplementation was able to protect bones from oxidative damage by scavenging free radicals [27, 28] and was able to maintain bone matrix trophysm and stimulate trabecular bone formation [29, 30]. Previous studies reported that vitamin E supplementation protects against bone loss and damage caused by oxidative stress, which is induced by sex hormones deficiency [31, 32] or oxygen-derived free radicals [33–35]. Postovariectomised rats have similar bone changes to those of postmenopausal women [36]. Vitamin E supplementation protects against bone loss and restores bone strength in the aged mouse [37] and ovariectomized rat [32, 38]— both are accepted osteopenic models.

Nicotine has been reported to increase proinflammatory mediators (through oxidative stress), resulting in bone loss and reduced bone mechanical strength (through inhibition of osteoblasts) in rats [39–41]. In humans, smoking is a recognized risk factor for osteoporosis [42, 43]. Recent studies utilising nicotine-induced rat model have reported that vitamin E was able to prevent the increment of bone-resorbing cytokines [26] and reverse the damage on bone histomorphometry [44].

Calcium plays an integral part in bone metabolism and remodeling [45]. A vitamin E-deficient diet will result in bone damage, probably due to impaired calcium absorption [46, 47] that leads to a state of calcium deficiency [48] and increased free radical activity [49].

Elevated levels of bone-resorbing cytokines, mainly interleukin 1 and 6 (IL-1 and IL-6), are known to be associated with accelerated bone resorption after menopause [50–52]. IL-1 is secreted by monocytes in an estrogen deficiency state that will induce osteoblast to secrete IL-6 [50]. IL-6 will stimulate osteoclast proliferation and subsequently increase bone resorption [52, 53]. Vitamin E, especially tocotrienol that was reported to be more potent compared to tocopherol, was reported to be able to prevent the rise of serum IL-1 and adverse effects of free radicals on trabecular bone structure [33, 34].

The increasing number of studies, especially in the past decade, which focused on the role of vitamin E in the prevention or treatment for osteoporosis warrants a review. The aim of this evidence-based review is therefore to explore original research articles in order to determine the effects of vitamin E on bone structural changes.

2. Methods

2.1. Literature Review. A systematic review of the literature was conducted to identify relevant studies about vitamin E and osteoporosis/bone structural changes. To conduct a comprehensive search of health science journals, we used Medline via Ovid Medline (published between 1946 and March 2012) and CINAHL via Ebscohost (published between 1946 and 2012). The search strategy involved a combination of the following two sets of key words (1) vitamin e OR vit* e OR tocotrienol OR tocopherol; (2) bone* OR bone metabolis* OR bone mineral* OR osteoblast* OR osteoclast* OR osteoporo* OR osteopen* OR osteogen*.

2.2. Selection of Research Articles. The results were limited to studies that were published in English language that included abstracts. To be included, studies had to (1) report the association or effect of vitamin E and osteoporosis-related bone changes and (2) the osteoporosis-related bone changes should be related to lifestyle variables, aging, or experimentally-induced conditions. Papers were excluded if the studies were related to (1) osteoporosis that is related to other pathological changes; (2) reviews, news, letter, editorials, or case studies; (3) bone fracture healing; or (4) fetal bone or bone marrow formation.

2.3. Data Extraction and Management. We selected papers to be included in the review in three phases. First, we excluded any paper that did not match the inclusion criteria based solely on the title. Second, we screened all the abstracts of the remaining papers and then excluded a second group of papers that did not meet our inclusion criteria. Lastly, we read the remaining papers from the second phase to exclude any paper that did not meet our inclusion criteria.

After the initial screening of the titles and abstracts, duplicates were removed and the remaining papers were again screened by at least two reviewers. The inclusion of the full papers into the review had to be agreed by at least two reviewers before the data extraction phase. Any discrepancies were resolved through discussion between the reviewers. Data extraction was performed independently and in a standardized manner with the use of a data collection form. We recorded the following data from the studies: (1) the type of study and vitamin E analog studied; (2) a brief description of the sample/population of the study; (3) a brief

description of the methods used in the study; (4) the brief description of the results of the study; (5) our comments and conclusion of the study.

3. Results

3.1. Search Results. The literature searches identified 561 potentially relevant articles. Two reviewers independently assessed all articles for inclusion or exclusion based on the title and abstract. A total of 42 articles were retrieved for further assessment and data extraction. Fifteen of these articles were excluded because they did not focus on primary studies ($n = 8$) or because they were not related with vitamin E and osteoporosis.

Differences of opinion between the reviewers regarding the inclusion or exclusion of the full articles were resolved by discussion. 16 articles from the remaining 27 articles were excluded based on the inclusion and exclusion criteria. A total of 11 articles were included for the purpose of this review. A flow chart of the selection and paper process, including reasons for exclusion, is shown in Figure 1.

3.2. Study Characteristics. The summary of the characteristics of all studies is displayed in Table 1 (animal studies) and Table 2 (human studies). All studies were conducted after the year 2000 with the majority conducted in the past five years. There were three human studies and eight animal studies that used rats as their study model. Although only three of human studies were included in this paper, each of these studies by Macdonald et al. [60], Maggio et al. [59], and Wolf et al. [61] had a large population sample size of at least 150 women. The study by Wolf et al. [61] had a sample of 11,068 participants and hence provided ample power and confidence for the generated results. All human studies involved women only, with bone mineral density (BMD) measurement being the main measured outcome, thus providing a highly reliable indicator for osteoporosis. Studies by Macdonald HM and Wolf RL collected information on vitamin E consumption through the use of questionnaires. It was not mentioned whether the questionnaires were validated. Vitamin E consumption from diet and supplements were then correlated with BMD measurements to determine the effect of vitamin E consumption on BMD. Maggio compared the plasma vitamin E level of osteoporotic participants versus nonosteoporotic participants to determine if there are any differences in vitamin E levels of women suffering from osteoporosis.

One animal study used Winstar rats with the remaining seven studies using Sprague-Dawley rats. Rats were of various ages, with the number of rats for each study (ranging from 24 to 96 rats) kept to a minimal number due to animal ethics requirements. Seven studies [32, 34, 44, 54, 55, 57, 58] assessed trabecular and cortical bone structure using bone histomorphometry analysis of the rat bones after treatment with vitamin E. Three studies [32, 55, 56] conducted various blood biochemical tests, three studies [32, 54, 56] conducted BMD scans, one study [57] conducted a biomechanical test to assess bone strength, and one study [56] used Micro-CT to assess trabecular and cortical bone structure. All animal studies were conducted using experimental design which compared the outcome of vitamin E treated groups (tocopherol, tocotrienol, or combination of various amounts) with control or sham group.

4. Effects of Vitamin E on Osteoporosis for Human Epidemiological Study

There were three human studies included in this paper. The study by Maggio et al. [59], was conducted to determine whether antioxidant defense (including vitamin E) decreased in osteoporotic elderly women by measuring antioxidant plasma levels of participants. There were a total of 150 women participants, whereby 75 women with diagnosed osteoporosis were compared with 75 women with normal BMD (control). The sample characteristics were as follows:mean age: 70.4 years; mean BMI: 25.3; mean years since menopause: 22.8 years. The reported results indicated that the mean plasma levels of vitamin E were significantly lower in osteoporotic women, if compared to control participants ($P < 0.001$). The authors suggested the need of further research to determine the relevance and the mechanism of action between low levels of vitamin E and osteoporosis.

Macdonald et al. [60], conducted a large longitudinal study with 891 women participants to determine the association between antioxidants (including vitamin E) intake and BMD. The characteristics of study participants were as follows: mean age: 53.9 years; mean BMI: 26.1; total vitamin E intake: 13.3 mg. Macdonald concluded that there was no evidence of any association between nutrient intake and BMD change. Total vitamin E intake (dietary plus supplementation) correlated positively with BMD, but was not significant. Dietary vitamin E intake was significantly correlated with BMD ($P < 0.01$), but was negatively correlated.

The largest human epidemiological study included in this review was conducted by Wolf et al. [61], involving 11,068 women participants (mean age: 63.2 years; mean BMI: 28.3; mean total vitamin E intake: 28.9 mg). Due to the high number of participants, the regression analysis done in this study had significant power, with multiple covariate adjustments performed. Wolf reported positive correlation between dietary vitamin E and femoral neck BMD when adjusted for age only. After adjusting for multiple BMD related covariates, no significant association was found between vitamin E consumption and all BMD parameters.

5. Effects of Vitamin E on Osteoporosis for Human Animal Study

A total of 10 animal studies were included in this paper. Ahmed et al. have two studies included in this paper. Shuid et al. used bone histomorphometry and biomechanical strength model, whereby γ-tocotrienol supplementation produced greater trabecular volume and number compared to control rats and rats supplemented with α-tocopherol [57]. Using Ferric nitrilotriacetate (FeNTA) to induce diabetes in rats and subsequently bone damage, N.S. Ahmad et al. reported

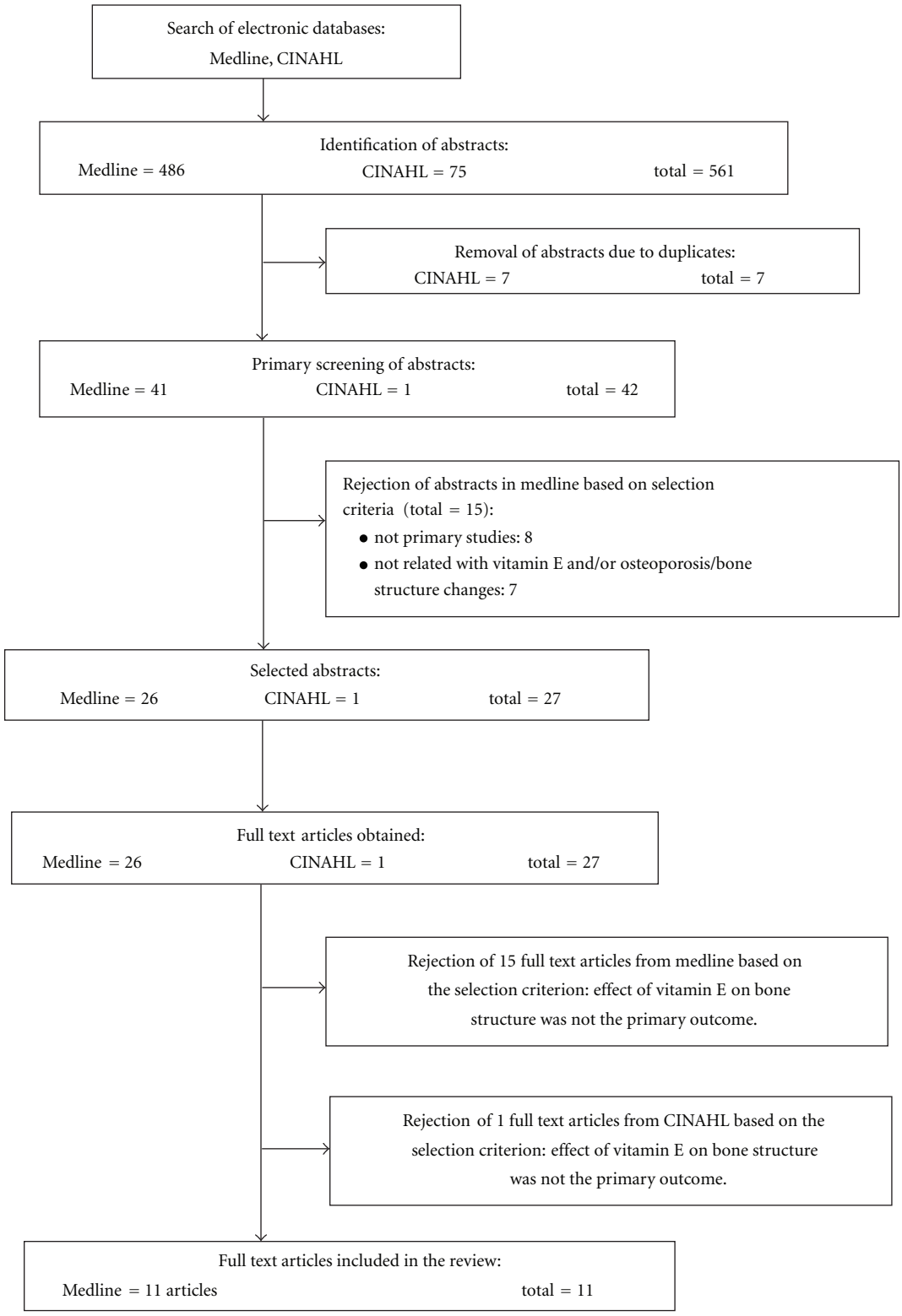

Figure 1: Flow chart to show the selection process of the articles in this review.

Table 1: Characteristics of animal studies included in the review.

STUDY	Type of study/vit E analog	Sample/ population	Methodology	Results (vitamin E and osteoporosis only)	Comments or outcomes
Norazlina et al. 2000 [32]	Animal study Bone mineral density α-Tocopherol Tocotrienol	3-month-old female Sprague-Dawley rats ($n = 80$) Half of rat were ovariectomised and half were left intact Rats were assigned into four group (10 rats for each groups) Duration: 10-month treatment	Three of the four groups were supplemented with palm vitamin E 30 mg/kg (PVE30), palm vitamin E 60 mg/kg (PVE60), or α-tocopherol 30mg/kg (ATF). The other group was supplemented with normal rat chow (RC). After 8 months of treatment, rats were sacrificed. The left femur and lumbar vertebra were dissected out and cleansed of all soft tissue for bone histomorphometry measurements: (a) bone mineral density measurements of the left femur and vertebra were obtained using the Dual-Energy A-ray Absorptiometer (b) bone calcium content (c) serum biomarkers of bone metabolism Serum alkaline phosphatase and serum tartrate resistant acid phosphatase were assayed and measured using a spectrophotometer at 405 nm.	(a) Bone mineral density Bone mineral density did not show any significant difference between all treatment groups. Palm vitamin E 30 mg/kg group of intact rats had higher bone mineral density in the distal part of femur compared to the ovariectomised. (b) Bone calcium content Both intact and ovariectomised rats supplemented with palm vitamin E 30 mg/kg weight rat had lower bone calcium content in femoral and vertebral bone. However, PVE60 and ATF groups were able to maintain bone calcium content. (c) Bone biomarkers The activity of ALP enzyme did not differ between all treatment groups for both intact and ovariectomised rats. The ALP enzyme of ovariectomised rats for group PVE30 and ATF was higher than intact control. The TRAP enzyme activity, PVE60 supplemented rat, was significantly lower compared to intact ATF group. The enzyme activity is significantly lower in ATF ovariectomised group, if compared to intact group.	Both tocopherol and tocotrienol act on bone, but in different mechanism of action. Alpha tocopherol reduced the activity of TRAP that maintains bone mineral density.
Ima-Nirwanaet and Suhaniza 2004 [54]	Animal study Histomorphometry analysis α-Tocopherol γ-Tocotrienol	4-months-old male Sprague-Dawley rats ($n = 42$) Rats were adrenalectomized after two-day receipt Rats were randomly assigned into six groups. 7 rats for each group Duration: 8-week treatment	Rats randomly divided into six group as follows: (a) Group A—dexamethasone 120 μg/kg + vehicle olive oil (b) Group B—dexamethasone 240 μg/kg + vehicle olive oil (c) Group C—dexamethasone 120 μg/kg + α-tocopherol 60 mg/kg (d) Group D—dexamethasone 240 μg/kg +α-tocopherol 60 mg/kg (e) Group E—dexamethasone 120 μg/kg + γ-tocotrienol 60 mg/kg (f) Group F—dexamethasone 240 μg/kg + γ-tocotrienol 60 mg/kg Dexamethasone was dissolved in olive oils and given intramuscularly daily except on Sunday. Parameters: (a) body composition measurement were done using a dual-energy X-ray absorptiometer for bone mineral density for: (i) left femur and fourth lumbar vertebra (ii) whole body fat mass (iii) whole body lean soft tissue mass (b) bone calcium content.	(a) Bone mineral density Whole body bone mineral density was increased after treatment. However, there was no significant difference between groups at the beginning and at the end of the treatment. (b) Lean soft tissue Whole body lean soft tissue mass was increased after the treatment. There was no significance difference between the groups at the beginning and at the end of the treatment. (c) Whole body fat mass Whole body fat mass was higher in group A compared to before treatment. There was no significant difference between the groups at the beginning of treatment. However, group A, C, and D had higher body fat mass, if compared with group F at the end of treatment. (d) Bone calcium content Fourth lumbar vertebral calcium content was higher in group E and F, if compared with group A and B. No significant difference was seen in left femoral calcium content between groups.	Supplementation with γ-tocotrienol was effective in preventing the increase in body fat mass and has the best effect on body composition, while supplementation of α-tocopherol was not beneficial at all.

Table 1: Continued.

STUDY	Type of study/vit E analog	Sample/ population	Methodology	Results (vitamin E and osteoporosis only)	Comments or outcomes
Ahmad et al., 2005 [34]	Animal study Bone histomorphometry analysis Tocotrienol and α-tocopherol	32 male Wistar rats (4 weeks old) Rats divided randomly into four groups (8 rats each) Rats were given daily treatment for 8 weeks	First group (control group) was injected intraperitoneally with saline. 2nd group was injected with 2 mg/kg Fe of ferric nitrilotriacetate (FeNTA) which was used to induce diabetes in rats. Group 3 was injected with FeNTA and was given oral doses of 100 mg/kg bodyweight α-tocopherol acetate (AT). Group 4 was injected with FeNTA and was given oral doses of 100 mg/kg bodyweight of palm tocotrienol (TT) mixture (α-TT 30.7%, γ-TT 55.2%, δ-TT 14.1%). After 8 weeks of treatment, femurs of the rats were removed for bone histomorphometry measurements. Measurements include: (1) trabecular bone volume (BV/TV) (2) trabecular thickness (TbTh) (3) trabecular number (TbN) (4) mean osteoclast number (OcN) (5) mean osteoblast number (ObN) (6) eroded surface/bone surface (ES/BS) (7) bone formation rate (BFR).	FeNTA injection significantly reduced BV/TV and TbTh of FeNTA and FeNTA + AT groups ($P < 0.001$). TT was able to prevent FeNTA-induced reduction of BV/TV and TbTh. FeNTA + TT group had a higher BV/TV and TbTh, if compared to FeNTA + AT group ($P < 0.02$). Supplementation with TT was able to prevent the increase of ES/BS and prevent the decrease of ObN and ES/BS due to FeNTA administration.	FeNTA generates free radicals which damage bone cells and activate osteoclasts which mimic osteoporotic bone structure. Only palm TT mixture was found to be able to prevent bone damage by FeNTA and is superior to AT in protecting bone against FeNTA toxicity.
Smith et al., 2005 [55]	Animal Study Serum α-Tocopherol analysis, serum biochemical marker, oxidative status, bone histology, and bone histomorphometry α-Tocopherol	96 Sprague-Dawley rats (8.5 months old) Randomly assigned into 6 groups (16 rats each) Total 13 weeks study period	3 dietary treatments of α-tocopherol acetate (AT): (1) low dose (LD)—15 IU/kg (2) adequate dose (AD)—75 IU/kg (3) high dose (HD)—500 IU/kg. AD is the recommended dose and functions as control group. Rats were fed one of 3 diets for 13 weeks. After 9 weeks, rats were either hindlimb unloaded (HU) or maintained ambulatory (AMB) for the final 4 weeks. End of treatment period: (1) whole body dual energy X-ray absorptiometry (DXA) scan was performed (2) blood was taken for serum alkaline phosphatise (ALP) and tartrate resistant acid phosphatise (TRAP) level and activity (3) serum AT level determined using HPLC (4) oxidative status evaluated using plasma ferric-reducing ability (FRAP) and liver thiobarbituric acid reactive substances (TBARS) (5) tibias of the rats were harvested for bone histology and the distal third of femur was harvested for bone histomorphometry. Parameters are as previously described.	FRAP improved with HD treatment group compared to LD and AD groups ($P < 0.05$). Biochemical markers did not result in significant findings. HU group had significantly lower bone histomorphometry results compared to AMB group but AT did not have any effect on bone histomorphometry. Concentrating on HU group, BV/TV increased in AD and HD diets compared to LD diet. ($P < 0.05$). AT helped to maintain TbN during unloading.	α-Tocopherol supplementation may provide some protection during hindlimb unloading conditions. However, results were not consistent for all parameters measured.

Table 1: Continued.

STUDY	Type of study/vit E analog	Sample/ population	Methodology	Results (vitamin E and osteoporosis only)	Comments or outcomes
Chai et al., 2008 [56]	Animal study Bone density assessment of aged osteopenic-orchidectomized male rats; control versus high-dose α-tocopherol (AT) supplementation α-Tocopherol	40 Sprague-Dawley rats (12 months old) Randomly assigned into 4 groups (10 rats each) Total 210 days study period	12-month-old rats were fed AIN-93 M casein-based control diet for 120 days to establish bone loss. Rats were then assigned into 4 groups and given treatment for 90 days: (1) sham operated (Sham) (2) orchidectomized + 75 IU AT (3) orchidectomized + 250 IU AT (4) orchidectomized + 550 IU AT. Analysis: (1) whole body scanning done using DXA at baseline (before surgery), 120 days after surgery and 90 days after dietary treatment. Assessment of bone mineral content (BMC), density (BMD), and area (BMA) (2) assessment of trabecular and cortical bone structures of distal femoral metaphysic and femoral midshaft using Micro-CT. Parameters assessed; BV/TV, TbN, TbTh, structural model index (SMI), connectivity density (Conn.D), cortical bone area (CoArea), thickness (CoTh), porosity (CoP), and medullary area (MArea) (3) measurements of bone biochemical markers; serum osteocalcin, urinary deoxypyridinoline (Dpd), and urinary creatinine concentration.	Mean BMD values of Orx animals were significantly different (lower) compared with sham animals at 120 days ($P = 0.009$) and at 210 days ($P = 0.001$). End of dietary treatment: (1) mean BMD values of AT supplemented groups were not different than Sham group (2) BMC and BMA were not affected with Orx or AT supplementation (3) no significant difference of serum osteocalcin and urinary Dpd noted among the four treatment groups (4) AT supplementation had no effect in preventing Orx-induced unfavourable alterations of trabecular bone parameters (5) AT treatment had no significant effect on CoP, CoTh and CoArea.	Supplement doses of AT do not increase BMD values in male rat model of osteoporosis – unable to reverse bone loss due to gonadal hormone deficiency.
Hermizi et al., 2009 [44]	Animal study Bone histomorphometry Tocotrienol-enhanced fraction α-Tocopherol γ-Tocotrienol	3-month-old male Sprague-Dawley rats ($n = 49$) Duration: 4-month treatment Rats were randomly assigned to seven groups with seven rats in each group	Group 1 was the baseline. (B) was killed at the commencement of the study. Group 2 and 3 were control (c) and nicotine (N) groups. The C group was treated with normal saline for 4 months and the N group was treated with nicotine for 2 months. The other four groups were nicotine cessation (NC), tocotrienol-enhanced fraction (TEF), GTT, and ATF. Treatment for these groups was performed in two phase. In the first 2 months they were given nicotine (7 mg/kg) and in the following 2 months treatment with vitamin E preparation (60 mg/kg). Rats were sacrificed after 4 months of treatment for bone histomorphometry measurements. Measurement include: (a) structural measurements: (i) trabecular bone volume (BV/TV) (ii) trabecular thickness (Tb.Th) (iii) trabecular number (Tb.N) (b) cellular measurements: (i) osteoclast surface (Oc.S/BS) (ii) eroded surface (ES/BS) (c) dynamic measurements: (i) single-labelled surface/bone surface (sLS/BS) (ii) mineral apposition rate (MAR) (iii) bone formation rate/bone surface (BFR/BS).	All vitamin E treated groups showed significant increase in BV/TV, MAR, and BFS/BS, but there was reduction in sLS/BS and Oc.S/BC compared to the C, N and NC groups. (a) Structural measurements TEF and GTT groups had a significantly higher trabecular thickness, but lower eroded surface (ES/BS) than the C group. (b) Cellular measurements The TEF group had lower ES/BS than the ATF group. (c) Dynamic measurements GTT improved trabecular bone histomorphometric parameter better than TEF and ATF after nicotine administration, by increasing MAR and BFR/BS.	All vitamin E treated group showed significant increase of bone formation and decrease bone resorption. Palm oil tocotrienol mixture was more potent than α-tocopherol at reversing the deleterious effects of nicotine on BV/TB and Tb.Th.

Table 1: Continued.

STUDY	Type of study/vit E analog	Sample/ population	Methodology	Results (vitamin E and osteoporosis only)	Comments or outcomes
Shuid et al., 2010 [57]	Animal study γ-Tocotrienol α-Tocopherol	3-month-old male Sprague-Dawley rats ($n = 24$) Divided into 3 groups Duration: 4-month treatment	Rats randomly divided into three groups as follows: (a) normal control (NC) Rats were given oral gavage of olive oil (vehicles) (b) α-tocopherol (ATF) Rats were given 60 mg/kg ATF body weight orally (c) γ-tocotrienol (GTT) Rats were given 60 mg/kg GTT body weight orally. At the end of the treatment rats were killed and both femurs of each rat were dissected free of soft tissue for: (a) bone histomorphometry (b) bone biomechanical test.	The GTT group had significantly higher trabecular bone volume, trabecular number, and trabecular thickness, but significantly lower trabecular separation than ATF group. The GTT group have significantly greater load, higher stiffness, higher stress, higher strain, and higher modulus elasticity, if compared to other group.	Vitamin E supplementation produced greater trabecular volume and number, if compared to control rat. GTT supplementation improves both extrinsic and intrinsic parameters.
Mehat et al., 2010 [58]	Animal study Bone histomorphometry analysis Tocotrienol Tocopherol	3-month-old Sprague-Dawley male rats ($n = 32$) Duration: 4 months Rats were randomly assigned into four groups.	The control group was supplemented with oral gavage vehicle olive oil. The treatment group given orally of 60 mg/kg α-tocopherol, δ-tocotrienol, and γ-tocotrienol. After 4 months of treatment, the rats bone was fluorochrome-labeled with intraperitoneal injection of 20 mg/kg calcein at days 9 and 2 days before the rats were killed. The rats were killed and the left femurs were dissected out and fixed with 70% alcohol. After 1 week the femurs were cut for histology slide samples for: (a) bone static (i) osteoclast number (N.Oc) (ii) osteoblast number (N.Ob) (iii) eroded surface/bone surface (ES/BS) (iv) osteoid surface/bone surface (OS/BS) (v) osteoid volume/bone volume (OV/BV) (b) bone dynamic (i) single-labelled surface/bone surface (sLS/BS) (ii) mineral apposition rate (MAR) (iii) bone formation rate/bone surface (BFR/BS) (iv) double labelled surface/bone surface (dLS/BS) (v) mineralizing surface/bone surface (MS/BS).	(a) Bone static All vitamin E treated group had significantly higher in N.Ob, OV/BV, and OS/BS but lower N.Oc and ES/BS. GTT group had increased availability of osteoclast for new bone formation (significant increase in N.Ob. OV/BV, and OS/BS). (b) Bone dynamic The percentages of dLS/BS, BFR/BS, MAR, and MS/BS of rat femora are higher in the vitamin E supplemented groups especially in γ-tocotrienol group compared to normal control group.	Vitamin E may be able to promote bone growth in rats by increasing trabecular bone volume and osteoid volume but reduce in N.Oc and Es/BS (bone resorption). γ-Tocotrienol group demonstrated the best effect in bone static and bone dynamic measurements.

Table 2: Characteristics of human studies included in the review.

Study	Type of study/Vit. E analog	Sample/population	Methodology	Results (Vitamin E and osteoporosis only)	Comments or outcomes
Maggio et al., 2003 [59]	Human observational study Vitamin E	1,100 women recruited for instrumental screening for osteoporosis to the Geriatric Division of Perugia University Hospital 150 women (75 osteoporotic and 75 control) gave their consent form and finally enrolled Duration: 12 months Final analysis: 150 women	Study variable included age years, body mass index, self-reported fractures, smoking habits, and other related variables. All pertinent information was collected through a questionnaire that was administrated by trained interviewer. The bone mineral density was measured using dual energy X-ray absorptiometry densitometer. Study subjects underwent a fasting blood withdraw in 20 ml heparin tubes on the day of the bone scan. Blood was kept on ice and centrifuged within 30 min. Plasma aliquot was frozen at -80°C until analysis.	150 women Mean Value: Age: 70.4 ± 8.5 BMI: 25.3 ± 2.9 Years since menopause: 22.8 ± 9 Mean plasma levels of vitamin E was significantly lower in osteoporotic than controls ($P < 0.001$). Antioxidant and MDA plasma level: Plasma vit E (μmol/liter): 46.7 ± 5 Plasma MDA (μmol/liter): 0.34 ± 0.13 MDA did not show any significant difference between osteoporotic and control subjects.	MDA result did not differ between groups. Low antioxidant levels cause antioxidant deficiency and give negative impact on bone mass. Role of vitamin E and osteoporosis needs further investigation.
Macdonald et al., 2004 [60]	Human epidemiological longitudinal study Vitamin E	1064 healthy premenopausal women aged 45–54 who took part in the Aberdeen Prospective Osteoporosis Screening Study. 896 responded to 2nd bone scan and completed questionnaire. 5 excluded (3 women had bisphosphonate therapy, 1 was on wheelchair and another had an outlier dietary calcium intake) Final analysis: 891 women	Participants chosen from women who took part in the Aberdeen Prospective Osteoporosis Screening Study conducted from 1990 to 1993 and had a bone scan done and a completed food-frequency questionnaire (FFQ). This study was a population-based osteoporotic fracture screening program within a 40 km radius of Aberdeen city, Scotland. Between 1997 and 1999, participants were recalled and had a second bone scan done and again completed the FFQ. Inclusion criteria: women who did not have any conditions or taking any medications that might affect their bone metabolism. Anthropometric measurements were taken. Bone mineral density (BMD) measurements of left proximal femur or femoral neck (FN) and lumbar spine (LS) were measured and compared between the first and second measurement. Dietary intake was assessed using the FFQ. The FFQ contained 98 foods or food groups intake of participants recorded over 7 days. Alcohol intake and dietary supplements were also measured. Physical activity level was obtained using the Scottish heart health Study questionnaire.	891 women participants. Mean values: Age (y): 53.9 ±1.6 BMI (kg/m^2): 26.1 ± 4.4 Total Vitamin E intake (mg): 13.3 ± 32.0 BMD (g/cm^2): Lumbar spine: 0.998 ± 0.17 Femoral neck: 0.833 ± 0.12 BMD results: No evidence of an association between nutrient intake and BMD change. Vitamin E intake (diet only, not including supplements) was negatively correlated with change in both BMD measurements ($P < 0.01$). Total Vitamin E intake was positively correlated with both BMD measurements but was not significant. Regression analysis: Vitamin E (diet only, not total) accounts for 0.4% of change on femoral neck BMD ($P = 0.018$).	Dietary vitamin E appeared to be a negative predictor for FN BMD change. The authors postulated that this may be due to vit E being a surrogate marker for polyunsaturated fatty acids (PUFA). PUFA anditamin E are highly correlated with PUFA having a negative correlation with FN BMD ($P < 0.01$).

Table 2: Continued.

Study	Type of study/Vit. E analog	Sample/population	Methodology	Results (Vitamin E and osteoporosis only)	Comments or outcomes
Wolf et al., 2005 [61]	Human epidemiological cross-sectional study Vitamin E	11,393 women aged 50–79 yrs were recruited between 1993 and 1997 to participate in the Women's Health Initiative (WHI) observational study and clinical trial at 3 clinics. BMD was measured and antioxidant intakes were estimated using self-reported food-frequency questionnaire. Women taking oral glucocorticoids, bisphosphonates, calcitonin or tamoxifen were excluded. Final analysis: 11,068 women.	All participants underwent the following data collection. Questionnaire data: (1) demographic data (2) smoking status (3) alcohol intake (4) medication history (5) use of hormone therapy (6) frequency, duration and intensity (strenuous, moderate and mild) of physical activity. (7) diet intake. Nutrients were calculated using Minnesota Nutrition Coding Center database (8) dietary supplements Clinical measurements: (1) weight and height. body mass index (BMI) (2) BMD measurement using dual X-ray absorptiometry; total body, lumbar spine, total hip, femoral neck, and trochanter. Blood measurements: (1) serum antioxidant concentrations; retinol, α and β carotene, α and γ-tocopherol, β-cryptoxanthin, lycopene, lutein, and zeaxanthin. (2) total cholesterol and triacylglycerols.	11,068 participants, 4.8% had osteoporosis Mean values: Age (y): 63.2 BMI (kg/m^2): 28.3 ± 5.9 Diet vitamin E intake (mg): 7.8 ± 3.8 Total vitamin E intake (mg): 28.9 ± 49.4 Serum total tocopherols (μg/mL): 18.0 ± 6.0 BMD (g/cm^2): Whole body: 1.0 ± 0.1 Lumbar spine: 1.0 ± 0.2 Total hip: 0.9 ± 0.1 Femoral neck: 0.7 ± 0.1 Hip trochanter 0.6 ± 0.1 BMD results: Age-adjusted regression analysis resulted in positive association for dietary vitamin E and femoral neck BMD ($P = 0.002$), but negative association for total vitamin E ($P < 0.0001$). However, results after adjusting for multiple important BMD-related covariates (age, BMI, waist circumference, race, education, income, physical activity, etc.) showed no significant association at any BMD sites with vitamin E. No significant association for serum concentration of tocopherols with any BMD sites.	Authors concluded no significant association between vitamin E and BMD. Authors noted that most participants had normal range BMD which may influence association between low BMD and antioxidants.

that palm tocotrienol mixture (consisting of α-TT 30.7%, γ-TT 55.2%, δ-TT 14.1%) supplementation was able to prevent bone damage [34]. Palm tocotrienol was also reported to have superior protection properties compared to α-tocopherol.

Ima-Nirwana and Suhaniza reported that supplementation with γ-tocotrienol or palm vitamin E successfully prevented osteoporosis in adrenalectomised rats replaced with dexamethasone, if compared to control and rats supplemented with α-tocopherol [54]. Norazlina et al. in their study (published in the year 2000) compared ovariectomized rats and nonovariectomized rats supplemented with palm vitamin E or α-tocopherol. There was no significant change between the treatment groups, in terms of BMD obtained from histomorphometry [32]. Hermizi et al., in a 2009 study using nicotine-induced rat bone damage model, reported that vitamin E was able to reverse the effect of nicotine damage on bone structure and noted γ-tocotrienol supplementation caused significantly higher mineral appositional rate and bone formation rate, if compared to tocotrienol-enhanced fraction and α-tocopherol treated-group [44].

In a recent study by Mehat et al. 2010, rats supplemented with oral vitamin E for four months had increased trabecular and osteoid bone volume, and reduction of bone resorption parameters [58]. γ-tocotrienol supplementation produced the best bone measurements improvement, if compared to δ-tocotrienol and α-tocopherol [58]. Smith et al. reported that α-tocopherol did not have any effects on bone histomorphometry parameters when supplemented at varying doses in hindlimb unloaded rats, if compared to ambulatory rats [55]. Chai et al. reported similar negative findings on varying doses of α-tocopherol supplementation in male orchidectomized rats [56]. No significant BMD parameters were observed and Chai et al. concluded that α-tocopherol supplementation was not able to reverse bone loss due to gonadal hormone deficiency [56].

6. Discussion

This paper has mixed main findings. There was strong evidence of the benefit of vitamin E supplementation in rats, whereby positive changes in bone structure were demonstrated for four of the eight animal studies [34, 44, 57, 58]. Three out of the four remaining animal studies [32, 54, 57] reported positive findings of vitamin E supplementation for biochemical results, although not for bone structure parameters. Only Chai et al. reported no positive finding for vitamin E supplementation in rats. Rat animal model has become acceptable for human bone studies due to the similar mechanism of control gain and loss of bone mass compared to humans. All the three human studies reported no association between vitamin E consumption and BMD changes [59–61].

Nicotine causes an increase of proinflammatory mediators (oxidative stress) that results in bone loss and reduction of bone mechanical strength (through the inhibition of osteoblasts) in rats [39–41]. In humans, smoking is a recognized risk factor for osteoporosis [42, 43]. Hermizi et al. reported that vitamin E treated rats were able to recover from nicotine-induced bone damage, where tocotrienol mixture was more potent compared to tocopherol [44]. Postulated mechanisms were antioxidative effects of vitamin E and the reduction of free radicals.

Five studies denoted a differing effect towards bone structure between vitamin E isomers, mainly between tocotrienol and tocopherol [34, 44, 54, 57, 58]. It has been postulated that tocotrienol has better activity compared to α-tocopherol due to the higher mobility of polyenoic lipids of tocotrienol in the membrane bilayer and therefore is more mobile and less restricted in its interaction with lipid radicals in the membranes [24]. Both of Ahmad et al. studies employed histomorphometry technique to view changes of rat bone structures [34, 57]. In their 2005 study, vitamin E treated rats were able to maintain their bone structure, resisting FeNTA-induced bone damage. Ahmad et al. supplemented α-tocopherol and tocotrienol mixture on different groups of rats and noted that only rats treated with tocotrienol mixture was able to effectively resist bone damage induced by FeNTA [34]. In a recent study by shuid et al. (2010), they reported that vitamin E supplementation had positive anabolic changes on bone structure (i.e., increased trabecular volume and number) compared to control rats [57]. Both studies concluded that improvement was superior among tocotrienol treated-rats compared to α-tocopherol-treated rats. Mehat et al. conducted a study specifically to determine bone structural changes of rats supplemented with different isomers of vitamin E (α-tocopherol versus δ-tocotrienol versus γ-tocotrienol) [58]. Bone histomorphometry analysis revealed that all isoforms promote bone growth in rats with γ-tocotrienol, producing the best effect in both static and dynamic measurements in bones. Mehat et al. did not suggest a mechanism or reason to explain the differences observed between the isomers of vitamin E.

Three out of four remaining animal studies reported positive results for positive biochemical changes, but without any significant bone structural changes. Among the biochemical changes reported were the increase of bone calcium content [32, 54], the reduction in oxidative status measured using plasma ferric-reducing ability [55], the improvement of serum alkaline phosphatase (ALP) and the reduction of serum tartrate-resistant acid phosphatase (TRAP) [32], and the improvement of whole body fat mass [54]. Serum ALP is a measurement of osteoblastic activity, while serum TRAP is specific for osteoclastic activity [62]. Ima-Nirwana and Suhaniza reported γ-tocotrienol-treated rats had the best effect on body composition and postulated that the effect could be due to tocotrienol's antioxidant effect or improvement of calcium transport and utilization [54]. Improvements of either bone structure parameters or biochemical parameters were mostly attributed to vitamin E through its antioxidant effects, free-radical scavenging capability, protection from cellular lipid peroxidation, improvement of calcium transport and utilization, and the suppression of bone resorbing cytokines, IL-1 and IL-6.

Maggio et al. in his study confirmed that plasma concentration of vitamin E is lower in osteoporotic women when compared to nonosteoporotic women [59]. However, all the

three human epidemiological studies in this paper reported no significant association between vitamin E consumption and BMD improvement [59–61]. The largest study, conducted by Wolf et al. with 11,068 participants, had positive age-adjusted regression analysis for dietary Vitamin E and femoral neck BMD ($P = 0.002$) association, but negative association for total vitamin E ($P < 0.0001$) [61]. Due to the large number of participants and sufficient statistical power, Wolf et al. were able to perform regression analysis that adjusted for more than 50 important BMD-related covariates. However, no significant association was reported. Wolf et al. offered several possible explanations of the mixed results: the variety in exposure measurement, outcome measurement and BMD site measurement, and other confounding factors. Among the three human epidemiological reviews, Macdonald et al. had reported negative correlation between dietary vitamin E and BMD measurements ($P < 0.01$), although this association was not persistent for the total vitamin E consumption, suggesting a possible opposite outcome if vitamin E was taken in larger doses [60].

Comparing human epidemiological studies with animal studies outcome is often difficult, as translation of results from animals to humans is hampered by various differences. The mechanisms of which vitamin E exerts its effects could be different in humans. Controlling environmental factors, food intake, and confounding factors is often possible in animal studies, but impossible in human epidemiological studies. Interactions between vitamin E and other antioxidants, nutrients, chemicals, and other food sources have to be explored. The stark differences, with almost opposite outcomes between animal studies and human epidemiological studies in this review, are therefore not surprising. All human epidemiological studies in this review reported consumption of vitamin E as a study variable, but the distinction between the different vitamin E isomers (tocopherols and tocotrienols) was not made. The majority of animal studies actually reported a positive outcome, or at least a superior effect for tocotrienols compared to tocopherols. This could offer a possible explanation for the discrepancy of the study outcomes. We were unable to provide a definitive answer whether vitamin E has any significant effects on bone structure, either positive or negative. The outcomes of studies included in this review were mixed. Half of the animal studies had positive changes on bone structure, whereas none of the human epidemiological studies had positive effects. Tocotrienols seemed to exert superior effects on bone structure and further studies concentrating on this isomer would need to be explored. Empirical human case-control studies or randomized control trials with vitamin E or its isomer derivatives would help ascertain the effect of vitamin E on bone structure.

Strength and Limitation of This Review. The research on the effects of vitamin E on osteoporosis is a promising field with important findings published in the last decade and a critical review is therefore highly relevant. Our search identified 11 research articles that were included in this paper and we believe that this is the first critical review on this subject matter that focuses on vitamin E and bone structural changes. We have also included both animal studies and human studies in this review, providing a better overview of the most recent and reliable evidence available.

This review has several limitations. Many studies did not differentiate the many isomers of vitamin E in their study. Due to the difference in the effect and activity of the various isomers, generalization of results and outcomes for the effect of vitamin E had to be further scrutinized. Many of the original research articles included in this review had other parameters included, especially blood biochemical parameters. The human studies focused on all antioxidants and mostly concentrating on vitamin C, instead of vitamin E. Nevertheless, results were thoroughly screened to avoid misrepresentation of the results of other antioxidants. All the human studies included in the review were epidemiological in nature, which presents with its own inherent weaknesses. Despite this limitation, the number of participants for each human epidemiological study was large. For example, the study by Wolf et al. had over 11,000 participants, providing ample statistical power and the inclusion of various adjusted covariates in the multiple-regression analysis.

Recommendations. Based on the heterogeneity of the study methods, especially among the animal studies, it is crucial that future studies use a standardized protocol to examine the effect of vitamin E various isomers on a consensual gold-standard parameter that assesses the osteoporotic state. Besides that, more effort should be made in designing controlled human observational studies that will help in reducing the numbers of potential confounders in the analysis of the result. These measures will ensure that proper meta-analysis could be conducted in the future to give us a clearer picture about the actual effect of vitamin E on osteoporosis.

7. Conclusion

This evidence-based review underscores the potential of vitamin E being used for osteoporosis, and sought to look into vitamin E and bone structure changes. The effect of vitamin E isomers, especially tocotrienols, on bone structural changes warrants further exploration. Additionally, controlled human observational studies should be conducted to provide stronger evidence. Due to the mix outcomes of the studies included in this paper, it is currently premature to state that vitamin E has positive, negative, or no effect on bone structure, at least until more studies are conducted.

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Research Article

Two Different Isomers of Vitamin E Prevent Bone Loss in Postmenopausal Osteoporosis Rat Model

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Postmenopausal osteoporotic bone loss occurs mainly due to cessation of ovarian function, a condition associated with increased free radicals. Vitamin E, a lipid-soluble vitamin, is a potent antioxidant which can scavenge free radicals in the body. In this study, we investigated the effects of alpha-tocopherol and pure tocotrienol on bone microarchitecture and cellular parameters in ovariectomized rats. Three-month-old female Wistar rats were randomly divided into ovariectomized control, sham-operated, and ovariectomized rats treated with either alpha-tocopherol or tocotrienol. Their femurs were taken at the end of the four-week study period for bone histomorphometric analysis. Ovariectomy causes bone loss in the control group as shown by reduction in both trabecular volume (BV/TV) and trabecular number (Tb.N) and an increase in trabecular separation (Tb.S). The increase in osteoclast surface (Oc.S) and osteoblast surface (Ob.S) in ovariectomy indicates an increase in bone turnover rate. Treatment with either alpha-tocopherol or tocotrienol prevents the reduction in BV/TV and Tb.N as well as the increase in Tb.S, while reducing the Oc.S and increasing the Ob.S. In conclusion, the two forms of vitamin E were able to prevent bone loss due to ovariectomy. Both tocotrienol and alpha-tocopherol exert similar effects in preserving bone microarchitecture in estrogen-deficient rat model.

1. Introduction

Osteoporosis is a disabling and painful condition whereby bone loss predominates, making the bone highly susceptible to fractures [1]. Osteoporosis takes place when bone resorption by osteoclasts far exceeds bone formation by osteoblasts. Abnormalities in endocrine function and metabolism are the most common causes for osteoporosis. In women, estrogen deficiency due to cessation of ovarian function is an important contributing factor for bone loss with advancing age. Other implicated factors in the pathogenesis of osteoporosis include an increase in osteoclast function, inhibition of osteoblast activity, and imbalance in calcium metabolism [2].

Reactive oxygen species (ROS), the radical forms of oxygen, have been linked to many disease processes including osteoporosis. Excessive accumulation of ROS leads to oxidative stress which in turn will cause cellular damage via peroxidation of lipid membrane, proteins, and nucleic acids. Oxidative stress occurs when the body antioxidant defence fails to overcome the generation of ROS. Recent biochemical and genetic studies have provided the evidence to support the link between osteoporosis and oxidative stress [3–5]. Perhaps the most convincing evidence is the study by Muthusami et al. in a postmenopausal osteoporosis rat model, whereby it is shown that the absence of estrogen causes an increase in lipid peroxidation index with a corresponding reduction in the endogenous antioxidant enzymes [6]. Moreover, free radicals are responsible for causing osteoblast apoptosis and reducing osteoblastogenesis. Hydrogen peroxide, the most stable ROS with the highest oxidative activity, has been reported to be involved in the formation and activation of osteoclasts which precede bone resorption [7].

Antioxidant vitamins can potentially be used to treat and prevent the progress of osteoporosis. At the moment, the approach to osteoporosis management is aimed at

preventing fractures from taking place (primary prevention), avoiding further fractures (secondary prevention), stabilizing bone metabolism, and relieving the pain. Nonetheless, not a single agent is able to maintain bone mass and density without exerting undesirable and mostly inconvenient adverse effects. This study was carried out in search for an alternative treatment of osteoporosis using two isoforms of vitamin E. This powerful, lipid-soluble antioxidant vitamin is a collective name for tocochromanols, that is, tocopherols and tocotrienols. A previous study showed that alpha-tocopherol and palm vitamin E (which is rich in tocotrienol) maintained bone mineral density (BMD) in an osteoporosis model [8]. The mechanisms through which vitamin E exerts its effect in preventing bone loss and maintaining BMD are still unclear. In the present study, we report the effects of alpha-tocopherol and tocotrienol on bone microarchitecture in ovariectomized rats, a well-established animal model for postmenopausal osteoporosis.

2. Materials and Method

2.1. Animals. Three-month-old female Wistar rats weighing 200–250 g were randomly divided into five groups with eight rats in each group. The baseline group was killed at the start of the experiment. Another group of rats was the sham-operated and given olive oil (SHAM) which acted as vehicle. The remaining rats were ovariectomized and treated with vehicle (OVX), tocotrienol (OVX + PTT) at a dose of 60 mg/kg body weight or similar doses of alpha-tocopherol (OVX + ATF). Treatment commenced two weeks after ovariectomy to allow the rats to recuperate. The olive oil, tocotrienol, or tocopherol was given orally to the rats using an oral gavage needle six days a week for four weeks. Rats were housed in standard cages in groups of three at room temperature with a 12 h light-dark cycle. They were fed with commercial rat chow diet (Gold Coin, Klang, Selangor, Malaysia). Tap water was given *ad libitum*. The study was conducted with the approval from the Universiti Kebangsaan Malaysia Animal Ethics Committee (approval number FAR/IMA/23-JULY/075).

2.2. Tocotrienol and Alpha-Tocopherol. Alpha-tocopherol was purchased from Sigma Chemical Company (USA). Pure tocotrienol was prepared from palm oil by the Palm Oil Research Institute of Malaysia (PORIM; Selangor, Malaysia) and had the following composition: 37.2% alpha-tocotrienol, 39.1% gamma-tocotrienol, and 22.6% delta-tocotrienol. The total tocotrienol composition was 98.79%. The analysis of the palm tocotrienol was done using HPLC on Hewlett Packard HP 1100 with 0.5% IPA/Hexane as mobile phase and detected by a fluorescence detector. No alpha-tocopherol detected on HPLC.

Alpha-tocopherol and tocotrienol were diluted separately in olive oil (Bertolli Classico, Italy) to obtain the concentration of 60 mg/kg body weight.

2.3. Bone Histomorphometry. At necropsy, the femora were taken and fixed in 4% formaldehyde solution for 24 hours before further processing. Undecalcified bone sections from left femurs were prepared according to the procedure as described by Difford [9]. The distal halves of the femurs were cut in saggital plane using a rotary electronic saw (Black & Decker, USA) and then embedded in methyl methacrylate polymer. A heavy duty microtome (Model 2135; Leica, Germany) was used to cut serial bone sections at 8 microns thick. For structural histomorphometry, the undecalcified bone sections were stained with Von Kossa. Histomorphometric measurements were carried out on the secondary spongiosa of the distal femoral metaphysic at distances between 3 mm to 7 mm from the lowest point of the growth plate and from 1 mm of the bilateral cortices. Total tissue area, cancellous bone area, bone surface, and perimeter data were obtained under light microscope (Leica, Germany) at 4x objective magnification with the aid of an image analyzer (VideoTest-Master, Russia). The structural parameters were as follows: trabecular bone volume (BV/TV)—the amount of trabecular bone within the cancellous space, expressed as %—trabecular thickness (Tb.Th)—the mean thickness of trabecular, expressed as μm—trabecular number (Tb.N)—the mean number of trabeculae expressed as per mm—and Trabecular separation (Tb.S)—the distance between two trabecular edges, expressed in μm.

Cellular parameters were obtained from decalcified sections of right femoral bones. The bones were decalcified in EDTA solution for five weeks and then dehydrated in graded concentrations of ethanol before being embedded in paraffin wax. The decalcified femur bones were sectioned at 5 microns thick using a microtome and later the sections were stained with Hematoxylin and Eosin (H&E). The parameters were osteoclast surface (Oc.S) and osteoblast surface (Ob.S). These parameters were calculated as the percentage of the total bone surface as seen under a light microscope (Olympus BX50, USA) interfaced with an image analyzer (Image Pro-Express, Media Cybernetics, USA).

All the formula, nomenclature, symbols, and units used in this study are those recommended by the American Society for Bone and Mineral Research (ASBMR) Nomenclature Committee [10].

3. Statistical Analysis

Statistical tests showed that all the data were normally distributed. ANOVA test was carried out followed by Tukey's HSD with $P < 0.05$ considered as significantly different.

4. Results

4.1. Body Weight. After four weeks of treatment, all groups of rats showed a consistent increase in body weight throughout the study period. However, the ovariectomized rats had a significant increase in body weight at the end of study compared to the sham and treated rats (Table 1).

4.2. Bone Histomorphometric Parameters. Ovariectomized rats had a significantly reduced bone volume (BV/TV) and trabecular number (Tb.N) compared to baseline, sham,

Table 1: Mean body weight.

	Baseline	Sham	Ovx	Ovx + ATF	Ovx + PTT
Week 0	171 ± 0.11	178 ± 0.09	173 ± 0.1	177 ± 0.12	175 ± 0.08
Week 6	—	269 ± 0.12[a]	293 ± 0.11	261 ± 0.13[a]	263 ± 1.12[a]

Data are mean ± S.E.M.
[a]Indicates significant difference compared to ovariectomy (Ovx) group ($P < 0.05$).
Ovx + ATF: ovariectomy + alpha-tocopherol; Ovx + PTT: ovariectomy + pure tocotrienol.

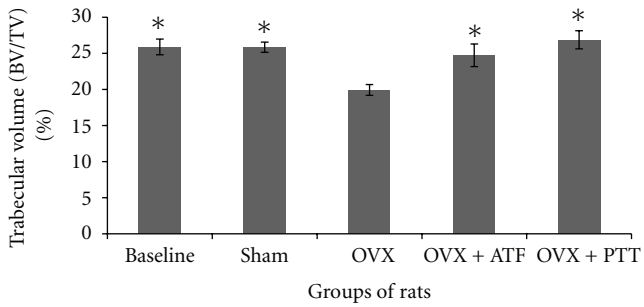


Figure 1: Mean percentage of bone volume to tissue volume. *Indicates significant difference from ovariectomized group ($P < 0.05$). Data are mean ± SEM. OVX: ovariectomy; OVX + ATF: ovariectomy treated with alpha-tocopherol; OVX + PTT: ovariectomy treated with pure tocotrienol.

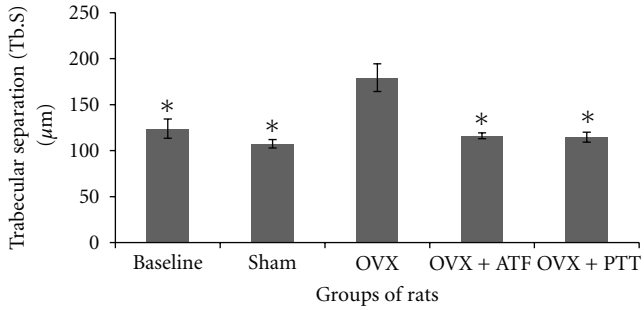


Figure 3: Trabecular separation in different groups of rats. *Indicates significant difference from ovariectomized group ($P < 0.05$). Data are mean ± SEM. OVX: ovariectomy; OVX + ATF: ovariectomy treated with alpha-tocopherol; OVX + PTT: ovariectomy treated with pure tocotrienol.

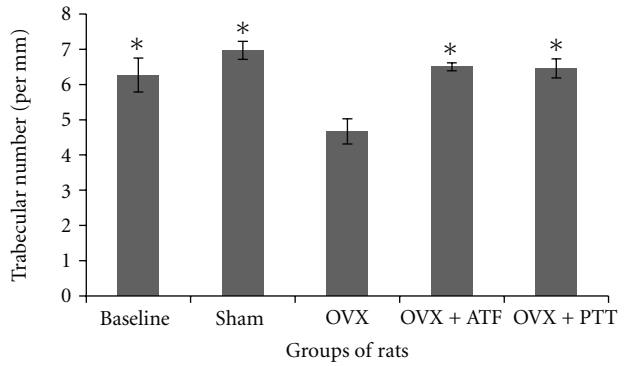


Figure 2: Trabecular number in different groups of rats. *Indicates significant difference from ovariectomized group ($P < 0.05$). Data are mean ± SEM. OVX: ovariectomy; OVX + ATF: ovariectomy treated with alpha-tocopherol; OVX + PTT: ovariectomy treated with pure tocotrienol.

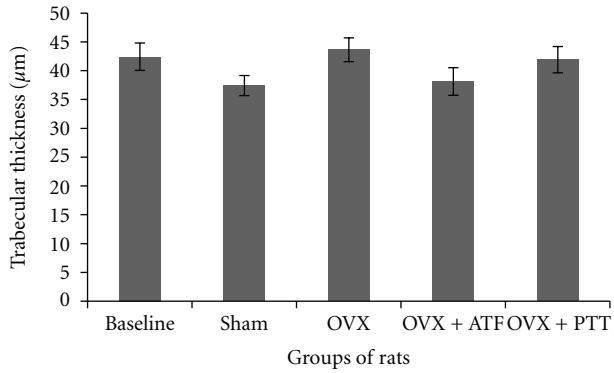


Figure 4: Trabecular thickness in different groups of rats. OVX + PTT: ovariectomy + pure tocotrienol. Data are mean ± SEM. OVX: ovariectomy; OVX + ATF: ovariectomy treated with alpha-tocopherol; OVX + PTT: ovariectomy treated with pure tocotrienol.

and treated groups, while trabecular separation (Tb.S) was increased significantly (Figures 1, 2, and 3). Ovariectomy also caused significant increases in both Osteoclast Surface (Oc.S) and Osteoblast Surface (Ob.S) compared to the other three groups (Table 2). Ovariectomy did not cause any change to trabecular thickness (Tb.Th) parameter (Figure 4).

Treatment of ovariectomized rats with either alpha-tocopherol or tocotrienol prevented the reduction in trabecular bone volume and trabecular number and prevented the increase in trabecular separation. Rats treated with both forms of vitamin E had significantly higher BV/TV and Tb.N while Tb.S was significantly lower than the OVX group (Figures 1, 2, and 3). There were no significant changes seen in trabecular thickness parameter (Figure 4). Treatment with palm tocotrienol or alpha-tocopherol also prevented the increase in osteoclast surface. The rats in OVX + PTT and OVX + ATF groups had significantly lower osteoclast surface than the OVX rats (Table 2). Treatment with either forms of vitamin E did not result in any difference compared to the baseline and sham groups in terms of BV/TV, Tb.N, Tb.S, Tb.Th, and Oc.S. However, ovariectomized rats treated with the two types of vitamin E had high osteoblast surface compared to the rats with intact ovaries (Table 2).

4.3. Bone Histology. Figure 5 shows photomicrographs of distal femur metaphyses taken from a rat representing each

Table 2: Cellular parameters.

	Baseline	Sham	Ovx	Ovx + ATF	Ovx + PTT
Oc.S (%)	2.7 ± 0.35^{a}	3.08 ± 0.26^{a}	7 ± 0.88	3.2 ± 0.35^{a}	2.95 ± 0.25^{a}
Ob.S (%)	9.9 ± 0.66^{a}	9.49 ± 0.38^{a}	21.54 ± 1.47	18.15 ± 2.3^{b}	19.7 ± 1.12^{b}

Data are mean ± S.E.M.
[a]Indicates significant difference compared to ovariectomy (Ovx) group ($P < 0.05$).
[b]Indicates significant difference compared to baseline and sham ($P < 0.05$).
Ovx + ATF: ovariectomy + alpha tocopherol; Ovx + PTT: ovariectomy + pure tocotrienol; Oc.S: osteoclast surface; Ob.S: osteoblast surface.

group. Loss of trabecular bone is apparent in those who were ovariectomized (Figure 5(a)) while treatment with either forms of vitamin E prevented bone loss in ovariectomized rats (Figures 5(c) and 5(d)).

5. Discussion

The effects of ovariectomy on weight gain have long been established. Ovariectomized rats had increased food intake as their appetite was increased [11, 12]. This change in appetite is partly due to low levels of leptin released by adipose tissue when estrogen is deficient. Leptin works on hypothalamus to control food intake and energy expenditure [13, 14].

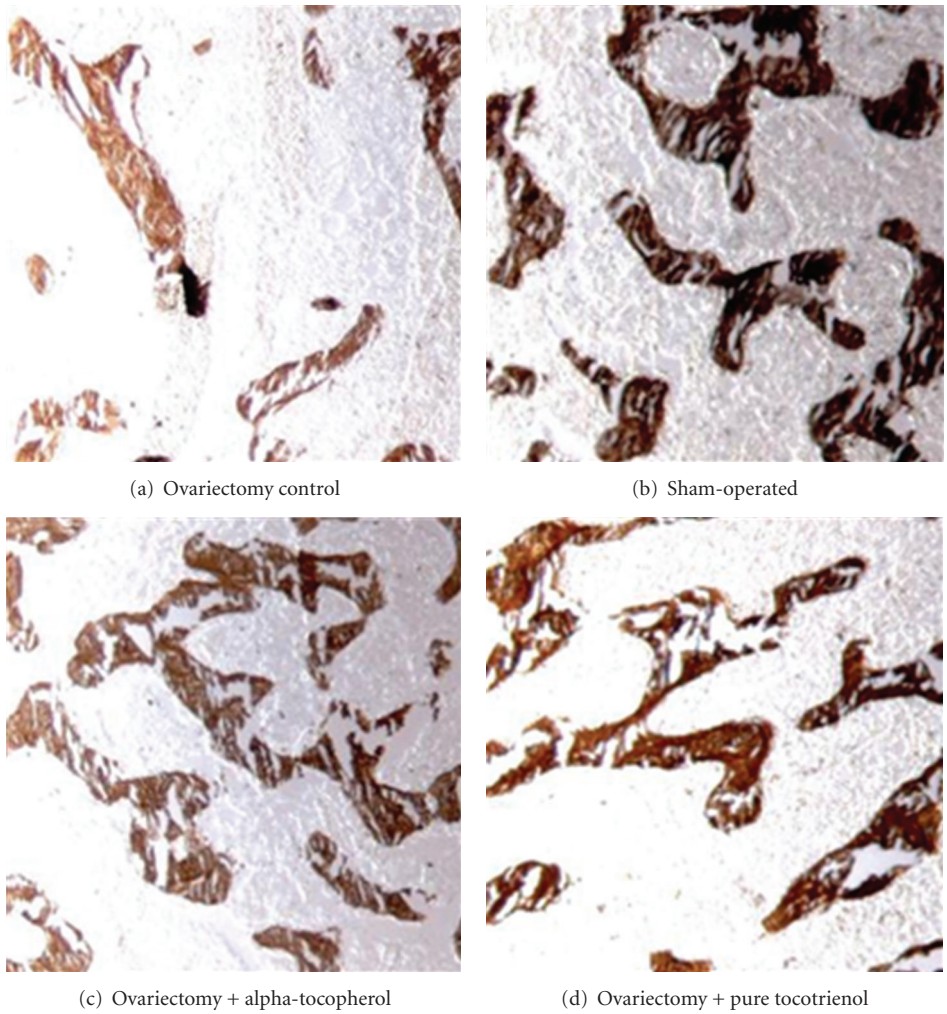
Removal of ovaries causes osteopenia in rats and ovariectomized animals have been used as a model for postmenopausal bone loss [15–18]. In our study, loss of bone with an increase in resorption and formation indices was seen in ovariectomized rats. Structural changes were evident in these rats whereby their trabecular bone volume was significantly lower than the control groups. Total number of trabecular bones was reduced while the bones were widely separated from one another, as seen in the high value of trabecular separation index. The resorption index which is the osteoclast surface (Oc.S) was increased by two fold compared to the sham rats. Ovariectomized rats also showed an increase in the formation index, the osteoblast surface (Ob.S). The increase in both resorption and formation indices showed an increase in bone turnover rate due to estrogen deficiency. Bone resorption and osteoclastic activities have to be greater than the bone formation by osteoblast in order to account for the net loss of bone. The bone loss is reflected in the photomicrograph of the trabecular bone whereby the bones of the ovariectomized rats showed perforations and discontinued trabeculae compared to the sham-operated group (Figures 5(a) and 5(b), resp.). These findings in postmenopausal rat model are consistent with previous studies [19, 20].

The present study also showed a decrease in trabecular number together with an increase in trabecular surface, along with a reduction in trabecular volume without any changes in trabecular thickness. These observations are consistent with previous reports [12, 21]. They proved that the main mechanism of ovariectomy-induced bone loss is due to perforation and loss of trabecular as a result of osteoclast resorption. The loss of bone is not accompanied by thinning of bone plates. This osteoclastic resorption eventually leads to total loss of trabecular bone. Perforation is the main mechanism of bone loss at the early stage of estrogen deficiency. This is the rapid phase of bone loss which happens only transiently, giving rise to the name "remodelling transient" [20]. The subsequent loss of bone is accompanied by thinning of trabeculae, as seen in long-term studies involving ovariectomized rats [22].

The cellular and molecular mechanisms through which estrogen deficiency stimulates bone resorption are increasingly well understood. Estrogen deficiency upregulates RANKL which leads to an increase in osteoclast recruitment and activation as well as a decrease in osteoclast apoptosis. Lack of estrogen also reduces OPG production by osteoblastic cells causing an increase in the RANKL/OPG ratio that favors bone resorption. In addition, there were reports that estrogen suppresses the expression of bone-resorbing cytokines like M-CSF, TNF-α, IL-1, and IL-6 and that lack of estrogen increases these factors. Estrogen also has direct effects on osteoclast and deficiency in this hormone will directly inhibit apoptosis of osteoclast precursor cells and increase osteoclast precursor differentiation into mature osteoclasts [23].

The effects of estrogen deficiency on the skeletal system may be caused by the increase in free radical activities. Recently, there have been a lot of studies that show the link between reactive oxygen species, estrogen deficiency, and bone loss. Several of the intracellular signals essential for osteoclast formation such as nuclear factor-kappa B (NF-⟨kappa⟩B), c-Jun amino-terminal kinase, and phosphatidylinositol 3-kinase are sensitive to reactive oxygen species [24]. Acute loss of estrogens increases the levels of ROS and activates NF-κB. It also enhances the phosphorylation of $p66^{shc}$, a redox enzyme which amplifies ROS generation and stimulates osteoblast apoptosis [25]. Another study showed that estrogen deficiency lowered antioxidant defences in osteoclasts resulting in increased osteoclastic resorption [7].

Administration of either alpha-tocopherol or pure tocotrienol prevents ovariectomy-induced bone loss. This is evident in the present study by the increase in trabecular bone volume with a corresponding increase in trabecular number together with a decrease in trabecular separation. Photomicrographs of the trabecular bone of rats given alpha-tocopherol and palm tocotrienol appear similar to the sham rats (Figures 5(c) and 5(d)). This result is consistent with another study which also used vitamin E to prevent osteopenia induced by nicotine administration [26]. The bone loss protecting effect by vitamin E also corresponds to the reduction in osteoclast surface with a parallel increase in osteoblast surface as shown in the current study. The findings of the present study strongly indicate that vitamin



(a) Ovariectomy control (b) Sham-operated

(c) Ovariectomy + alpha-tocopherol (d) Ovariectomy + pure tocotrienol

Figure 5: Photomicrographs of distal femur metaphyses from ovariectomized (a) and sham-operated (b) rats, as well as ovariectomized rats treated with alpha-tocopherol (c) and tocotrienol (d). Undecalcified histological bone sections stained with Von Kossa. Trabecular bones appear dark by Von Kossa staining. Loss of trabecular bone is apparent in A while treatment with either forms of Vitamin E prevented bone loss in ovariectomized rats ((c) and (d)). Light microscopy at magnification ×40.

E may preserve the bone microarchitecture by inhibiting osteoclastogenesis and stimulating osteoblasts to synthesis more bone. The anabolic property of vitamin E has been proven in a previous study whereby it increases bone volume in male rats supplemented with either alpha-tocopherol or tocotrienol [27]. In addition, vitamin E may prevent trabecular bone loss by enhancing bone calcification and mineralization [8, 28].

The primary role of antioxidant vitamins in scavenging the ROS in oxidative stress is already well established. The vitamin E family consists of eight naturally occurring isomers which are α-, β-, γ-, and δ-tocopherols as well as α-, β-, γ-, and δ- tocotrienols. Most of the studies involving vitamin E reported on the alpha-tocopherol isomer since it is widely available in the market. Tocotrienols are similar to tocopherols except that they have three double bonds in the hydrocarbon tail instead of a saturated tail as found in tocopherols [29, 30]. Our test compound consisted of pure tocotrienols without any tocopherols detected on HPLC. The tocotrienols are made up mainly of the gamma isomer, followed closely by the alpha and the remaining is the delta tocotrienol. The effects of alpha-tocopherol in this study are comparable to those of pure tocotrienols. However, the majority of studies investigating the effects of vitamin E on bone indicated that tocotrienols were better than tocopherols [26, 31–33].

The tocotrienol dose used in the present study was based on the previous studies by Ahmad et al. [33] which showed that, at the dose of 60 mg/kg body weight, tocotrienol was able to prevent the increase of bone-resorbing cytokines in a free-radical-induced rat model. This dose was proven to be safe as toxicity studies in rats showed there was no adverse effect observed even at an extremely high dose of 2500 mg/kg body weight [34].

The positive effects of vitamin E on bone by preventing oxidative stress could be mediated via similar pathway

involving the RANK/RANKL. Lee et al. [35] and Ha et al. [36] showed that Vitamin E prevented osteoclastogenesis and bone resorption by suppressing RANKL expression and signalling without affecting OPG expression. Vitamin E has also been shown to inhibit the release and expression of bone-resorbing cytokines [36]. These limited studies on the action of vitamin E could suggest that it might exert its effects directly on osteoclast recruitment and osteoclastogenesis. Further studies are of course warranted in order to ascertain its exact mechanism of action on bone metabolism.

In conclusion, supplementation with vitamin E either in the form of alpha-tocopherol or tocotrienol prevented bone loss and maintained the bone microarchitecture in osteopenic rats induced by ovariectomy. Further studies are required to explore the potential of different vitamin E isomers in metabolic bone diseases.

Conflict of Interests

The authors declare that they have no conflict of interests.

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Research Article

Prescription Patterns of Chinese Herbal Products for Osteoporosis in Taiwan: A Population-Based Study

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Background. Traditional Chinese medicine (TCM) includes Chinese herbal products (CHPs), acupuncture, and traumatology manipulative therapies. TCM physicians often prescribe CHP to treat patients with osteoporosis; however, the drugs used and their patterns of prescriptions have yet to be characterized. This study, therefore, aimed to evaluate the CHP used for the treatment of osteoporosis in Taiwan and their prescription patterns. *Methods*. A cohort of one million randomly sampled cases from the National Health Insurance Research Database (NHIRD) was analyzed to evaluate the frequencies and percentages of herbal formula and single herb prescriptions for osteoporosis. Association rules were then applied to evaluate the CHP coprescription patterns and the prevalence of osteoporosis. *Results*. The osteoporosis cohort included 16 544 patients, of whom more than 70% had used TCM on one or more occasion. Of these patients, 4 292 (25.9%) had been hospitalized at least once because of fracture. Du-Huo-Ji-Sheng-Tang and Du Zhong (Cortex Eucommiae) were the most frequently prescribed herbal formula and single herb, respectively, for the treatment of osteoporosis. *Conclusion*. This study identified patterns of CHP use for the treatment of osteoporosis. However, further research is required to fully elucidate the efficacy and safety of these CHP.

1. Introduction

Osteoporosis is a major public health problem and characterized by low bone mass, microarchitectural disruption, and skeletal fragility [1], resulting in increased risk of fracture and potentially increased patient morbidity [2, 3]. The prevalence of osteoporosis is higher in women than in men, and increasing prevalence is associated with increasing age in both sexes [4]. Treatment of osteoporosis consists of pharmacotherapy and lifestyle measures, including dietary changes, mineral supplementation, and exercise programs [5, 6]. Traditional Chinese medicine (TCM) is a major component of health care in Taiwan and provides one treatment option for osteoporosis.

TCM includes decoctions, acupuncture, and traumatology manipulative therapies. Chinese herbal products (CHP) represent the modern form of decoctions, with more consistent quality [7] and modern technology for patient use. In Taiwan, the National Health Insurance (NHI) program fully reimburses for single herb or herbal formula CHP. Because of their quality, convenience, and NHI reimbursement, TCM physicians currently prescribe CHP widely. TCM physicians typically prescribe one or more herbal formulae combined with several single herbs for each prescription, depending on the patient's constitution. However, the drugs used for treatment of osteoporosis and their prescription patterns have yet to be fully elucidated.

The computerized reimbursement database of the NHI, the National Health Insurance Research Database (NHIRD), stores longitudinal data on Western and Chinese medicines. It thus, provides an optimal platform for pharmacoepidemiological research on drugs used and prescription patterns in

TCM. Using the NHRID, this study aimed to evaluate the CHP used for the treatment of osteoporosis in Taiwan and their prescription patterns.

2. Materials and Methods

2.1. Data Source. In March, 1995, the Taiwanese government implemented the NHI program, which provides general health insurance coverage to almost the entire Taiwanese population. In this study, a cohort of one million patients, who were beneficiaries of the NHI program from January 1 to December 31, 2005, was randomly sampled. Data were extracted from all medical records of the cohort from 1995 to 2009. Prior to 2002, 2 disease coding schemes, the International Classification of Diseases, 9th Revision, Clinical Modification (ICD-9CM), and the ICD-9 basic tabulation list, were used simultaneously for diagnoses. Data from 2003 to 2009 were, thus, used for the purposes of this study. The cohort of one million randomly sampled patients has been demonstrated to be representative of all NHI beneficiaries. Sex distribution, age distribution, and average insured payroll-related amount showed nonsignificant differences between the cohort and the entire NHIRD. The NHIRD contains medical information including data on medical care facilities and specialties, drugs, treatment and management, patient sex and date of birth, date of visit or hospitalization, transferred identification number, and diagnoses coded in the ICD-9-CM format.

2.2. Study Subjects. Using the diagnostic variables in the outpatient visit database from the NHIRD, the osteoporosis cohort was selected from the outpatient department records. The ICD-9-CM codes 733.0 (osteoporosis), 733.00 (osteoporosis, unspecified), 733.01 (senile osteoporosis), 733.02 (idiopathic osteoporosis), 733.03 (disuse osteoporosis), and 733.09 (other osteoporosis) were used for identification of the cohort. All osteoporosis-related medical records during the study period were analyzed. Patients who visited the outpatient department for osteoporosis fewer than 6 times during the study period received less osteoporosis-related therapy, and had average prescription durations approximately 70% shorter, than patients who visited the outpatient department for osteoporosis 6 times or more during the study period. Despite the lack of guidelines for followup after the diagnosis of osteoporosis and treatment initiation, the followup of patient responses to therapies is recommended [8]. In this study, patients who were lost to followup and those who visited the outpatient department for osteoporosis fewer than 6 times during the study period were excluded from the analyses. Patients who visited the outpatient department for osteoporosis 6 times or more during the study period were included in the evaluations.

2.3. TCM. The list of reimbursed CHP was downloaded from the website of the Bureau of NHI. Corresponding drug information on a specific mixture or name was then obtained from the Committee on Chinese Medicine and Pharmacy website, including the proportions of each constituent, date and period of drug approval, drug names, and manufacturers' codes.

2.4. Statistical Analysis. Drug registration numbers from the Committee on Chinese Medicine and Pharmacy website were linked to the outpatient visit records of the osteoporosis cohort. The frequencies and percentages of herbal formula and single-herb prescriptions were then analyzed. Daily doses and average prescription durations were calculated for each prescription.

TCM physicians typically treat patients with one or more herbal formulae combined with several single herbs. In this study, association rules were applied to evaluate coprescription patterns of CHP for osteoporosis. The support factor (the proportion of coprescriptions of medications A and B amongst all prescriptions) was first determined. The confidence factor (the proportion of coprescriptions of medications A and B amongst all prescriptions containing medication A) was then determined. The prevalence of osteoporosis in the study cohort was also estimated. SAS version 9.1 software (SAS Institute Inc., Cary, NC, USA) was used for data linkage and descriptive statistics on patterns of drug use.

3. Results

Among the cohort of one million randomly sampled patients, we identified 16 544 patients who had visited the outpatient department for osteoporosis 6 times or more during the study period. Table 1 summarizes the characteristics of the study patients. Women accounted for approximately 82% of the osteoporosis cohort. More than 70% of the osteoporosis cohort had used TCM more than once, and 4 292 (25.9%) study patients had been hospitalized at least once because of fracture, during the 7-year study period. Femoral neck fractures (ICD-9-CM 820) were the most common type of fracture in the osteoporosis cohort. As shown in Table 2, the estimated prevalence of osteoporosis in women and men was 2.7% and 0.6%, respectively, which increased with age in both sexes. The prevalence of osteoporosis was 0.2%, 5.8%, and 16.7% in women younger than 45, aged 45–65, and older than 65, respectively. These percentages were higher than those for men in the same age groups. Women older than 65 displayed the highest prevalence of osteoporosis among all patient groups.

Table 3 displays the most commonly prescribed herbal formulae and single herbs for osteoporosis, including the frequency of prescriptions, average daily doses, and average prescription durations. The most commonly prescribed herbal formula for osteoporosis was Du-Huo-Ji-Sheng-Tang (21.4%), followed by Zuo-Gui-Wan (17.4%), Shu-Jing-Huo-Xie-Tang (14.4%), Jia-Wei-Xiao-Yao-San (12.2%), and Gui-Lu-Er-Xian-Jiao (11.0%). Du Zhong (Cortex Eucommiae; 18.4%) was the most commonly prescribed single herb for patients with osteoporosis, followed by Xu Duan (Radix Dipsaci; 13.5%), Dan Shen (Radix Salvia Miltiorrhizae; 11.5%), Niu Xi (Radix Achyranthis Bidentatae; 11.1%), and Yan Hu Suo (Rhizoma Corydalis; 9.5%).

Table 1: Characteristics of osteoporosis population from one million random sampling cohort of the NHIR database.

Characteristics	No.	%
Age		
<45	772	4.7%
45–65	6,836	41.3%
>65	8,936	54.0%
Sex		
Female	13,560	82.0%
Male	2,984	18.0%
TCM use during study period		
Yes	12,007	72.6%
No	4,537	27.4%
Fracture during study period		
Yes	4,292	25.9%
Fracture of neck of femur (ICD-9-CM: 820)	1,355	8.2%
Pathologic fracture (ICD-9-CM: 733.1)	1,043	6.3%
Fracture of vertebral column without mention of spinal cord injury (ICD-9-CM: 805)	876	5.3%
Fracture of radius and ulna (ICD-9-CM: 813)	633	3.8%
No	12,252	74.1%
Total	16,544	100.0%

Table 2: Patient number and estimated prevalence of osteoporosis in Taiwan from one million random sampling cohort of the NHIR database 2003–2009.

Age/sex	All		Female		Male	
All	16,544	1.7%	13,560	2.7%	2,984	0.6%
<45	772	0.1%	571	0.2%	201	0.1%
45–65	6,836	3.3%	6,037	5.8%	799	0.8%
>65	8,936	10.7%	6,952	16.7%	1,984	4.7%

According to the association rules in Table 4, the most commonly prescribed combination of 2-herbal formulae for osteoporosis was Du-Huo-Ji-Sheng-Tang and Shu-Jing-Huo-Xie-Tang, with a support factor of 4.42%. The most commonly prescribed combination of a herbal formula and a single herb for the treatment of osteoporosis was Du-Huo-Ji-Sheng-Tang and Du Zhong, with a support factor of 4.77%, and the most commonly prescribed combination of 2-single herbs was Xu Duan and Du Zhong, with a support factor of 5.87%. Physicians frequently supplemented Du-Huo-Ji-Sheng-Tang and Zuo-Gui-Wan, the 2 most commonly prescribed herbal formulae for osteoporosis, with single herbs, such as Du Zhong and Xu Duan, or another herbal formula.

4. Discussion

To our knowledge, this study is the first to use a random nationwide sample to evaluate prescription patterns of CHP for osteoporosis. Ancient and modern TCM physicians have prescribed a variety of herbal formulae or single herbs to treat osteoporosis. However, prior to this study, the CHP most commonly used for the treatment of osteoporosis in clinical practice had yet to be identified. Using the NHIRD, this study analyzed the drugs used to treat osteoporosis and their prescription patterns and identified potentially effective TCM prescription patterns for osteoporosis treatment. Further clinical trials to evaluate the safety and efficacy of these TCM prescription patterns are warranted.

TCM physicians frequently used Du-Huo-Ji-Sheng-Tang, the most commonly prescribed herbal formula for osteoporosis, to treat a combination of symptoms including knee pain, stiffness, flaccidity, and aversion to cold. According to ancient TCM literature, these symptoms indicate disharmony caused by "wind," "cold," and "dampness," and Du-Huo-Ji-Sheng-Tang can improve these. A 4-week outcome study has shown its effectiveness at reducing pain and stiffness of the knee joint and improving physical function in patients with osteoarthritis [9]. The authors observed no adverse events or drug reactions during the study period [10]. Zuo-Gui-Wan is the second most commonly prescribed herbal formula for osteoporosis. According to the ancient TCM literature, Zuo-Gui-Wan can reduce dizziness, weakness, and soreness in the lumbar and knee regions. Using an animal model, Liu et al. further identified that Zuo-Gui-Wan can prevent and treat glucocorticoid-induced osteoporosis in rats [11].

As shown in Table 4, modern TCM physicians often use Du-Huo-Ji-Sheng-Tang and Zuo-Gui-Wan, with appropriate modifications, to treat soreness in the lower back and osteoporosis accompanied by other symptoms. However, the support factors for coprescriptions were all less than 6%, which might have caused TCM physicians to treat patients according to a syndrome differentiation theory rather than a specific disease [12]. TCM physicians also use differing coprescription patterns to treat individual patients with the same diagnosis but different conditions.

Shu-Jing-Huo-Xie-Tang, the third most commonly prescribed herbal formula for osteoporosis, can reduce blood stasis and dispel "wind dampness" in the meridian to promote blood circulation. Shu-Jing-Huo-Xie-Tang is able to relieve both arthralgia and muscle pain. In previous studies, Shu-Jing-Huo-Xie-Tang was the most commonly prescribed herbal formula for diseases of the musculoskeletal system and connective tissue in climacteric women [13] and the second most commonly used herbal formula in Taiwan during 2004 [14]. Although TCM physicians in Taiwan frequently prescribe Shu-Jing-Huo-Xie-Tang, its effectiveness and safety is uncertified based on clinical trials.

Jia-Wei-Xiao-Yao-San, the fourth most commonly prescribed herbal formula for osteoporosis, was also the most commonly used CHP for the relief of menopausal symptoms [13]. According to previous studies, Jia-Wei-Xiao-Yao-San can be prescribed to relieve hot flushes and other menopausal

Table 3: Top ten herbal formulae and single herb prescribed by traditional Chinese medicine doctors for osteoporosis ($n = 8,369$).

	Frequency of prescription n (%)		Average daily dose (g)	Average duration for prescription (days)
Herbal formulae				
Du-Huo-Ji-Sheng-Tang	1,792	21.4%	11.4	11.8
Zuo-Gui-Wan	1,456	17.4%	7.0	9.8
Shu-Jing-Huo-Xie-Tang	1,204	14.4%	5.0	11.3
Jia-Wei-Xiao-Yao-San	1,019	12.2%	4.1	14.1
Gui-Lu-Er-Xian-Jiao	918	11.0%	6.0	15.0
You-Gui-Wan	589	7.0%	6.0	12.5
Ji-Sheng-Shen-Qi-Wan	572	6.8%	5.3	14.6
Shao-Yao-Gan-Cao-Tang	549	6.6%	3.5	10.0
Zhi-Bo-Di-Huang-Wan	532	6.4%	7.8	14.7
Liu-Wei-Di-Huang-Wan	527	6.3%	6.4	13.6
Single herb				
Du Zhong (Cortex Eucommiae)	1,539	18.4%	1.9	12.6
Xu Duan (Radix Dipsaci)	1,128	13.5%	1.1	12.4
Dan Shen (Radix Salvia Miltiorrhizae)	966	11.5%	1.6	14.0
Niu Xi (Radix Achyranthis Bidentatae)	933	11.1%	1.3	12.2
Yan Hu Suo (Rhizoma Corydalis)	796	9.5%	1.5	11.0
Gu Sui Bu (Rhizoma Drynariae)	658	7.9%	1.3	11.4
Wei Ling Xian (Radix Clematidis)	651	7.8%	1.1	11.8
Mu Gua (Fructus Chaenomilis)	630	7.5%	1.1	12.1
Ji Xue Teng (Caulis Spatholobi)	549	6.6%	1.2	12.5
Chuan Qi (Radix Notoginseng)	494	5.9%	1.2	11.8

Table 4: Coprescriptions (one to one association) of single herbs and herbal formulae for osteoporosis population.

Herbal associations	Support (%)	Confidence (%)	Transaction count	Association rule
Single herb to single herb	5.87	31.95	491	Du Zhong (Cortex Eucommiae) ⇒ Xu Duan (Radix Dipsaci)
	3.94	21.47	333	Du Zhong (Cortex Eucommiae) ⇒ Niu Xi (Radix Achyranthis Bidentatae)
	3.90	21.21	326	Du Zhong (Cortex Eucommiae) ⇒ Dan Shen (Radix Salvia Miltiorrhizae)
	3.62	26.91	303	Xu Duan (Radix Dipsaci) ⇒ Gu Sui Bu (Rhizoma Drynariae)
	3.14	27.42	263	Dan Shen (Radix Salvia Miltiorrhizae) ⇒ Wei Ling Xian (Radix Clematidis)
Herbal formula to herbal formula	4.42	20.65	370	Du-huo-ji-sheng-tang ⇒ Shu-jing-huo-xie-tang
	2.86	16.41	239	Zuo-Gui-Wan ⇒ Si-Jun-Zi-Tang
	2.64	12.33	221	Du-huo-ji-sheng-tang ⇒ Gui-Lu-Er-Xian-Jiao
	2.56	14.70	214	Zuo-Gui-Wan ⇒ Jia-Wei-Xiao-Yao-San
	2.52	39.66	211	zhi-bo-di-huang-wan ⇒ Jia-Wei-Xiao-Yao-San
Herbal formula to single herb	4.77	22.27	399	Du-huo-ji-sheng-tang ⇒ Du Zhong (Cortex Eucommiae)
	4.11	19.20	344	Du-huo-ji-sheng-tang ⇒ Xu Duan (Radix Dipsaci)
	3.69	17.24	309	Du-huo-ji-sheng-tang ⇒ Niu Xi (Radix Achyranthis Bidentatae)
	3.50	20.12	293	Zuo-Gui-Wan ⇒ Yan hu suo (Rhizoma Corydalis)
	3.06	17.58	256	Zuo-Gui-Wan ⇒ Xu Duan (Radix Dipsaci)

symptoms, including insomnia and emotional disturbance [15, 16]. TCM physicians rarely prescribe Jia-Wei-Xiao-Yao-San in isolation for the treatment of osteoporosis, typically combining it with another herbal formula and/or single herb depending on the patient's symptoms. Women with climacteric symptoms have a higher osteoporosis incidence rate than other patient groups. Their high incidence rates might be the reason for Jia-Wei-Xiao-Yao-San being frequently prescribed CHP for osteoporosis.

As shown in Table 3, Du Zhong and Xu Duan were the 2 most frequently prescribed single herbs for osteoporosis. According to the ancient TCM literature, Du Zhong strengthens muscles and bones, and TCM physicians often prescribe it to patients with chronic pain in the lower back and knees, weakness, dizziness, and impotence. Previous studies have shown that extracts of Du Zhong can promote collagen synthesis and suppress osteoclast activity to inhibit osteolysis [17, 18]. TCM physicians have also used Xu Duan to treat patients with pain in the lower back and knee, inflammation and traumatic injuries of the leg joints and bones, and general weakness. In a previous study conducted on mice, Xu Duan increased bone density and altered bone histomorphology [19]. Ancient TCM texts recommended the prescribing of 2-single herbs as a herb pair for 2 specific purposes: to increase the efficacy of treatment and to compensate for the insufficiency of another single herb. One example of a herb pair is Du Zhong and Xu Duan. Du Zhong and Xu Duan exert similar effects in skeletal and muscular systems, and their combination might increase their efficacies. Their use as a herb pair might be the reason for Du Zhong and Xu Duan being the most frequently prescribed combination of 2-single herbs for osteoporosis.

The proportion of a single herb in a herbal formula is changeless; therefore, it is prescribed with another single herb to alter its proportions to suit a patient's condition. Du-Huo-Ji-Sheng-Tang and Du Zhong was the most commonly prescribed combination of a herbal formula and a single herb for the treatment of osteoporosis, even though Du Zhong is one of 15 single herbs contained in Du-Huo-Ji-Sheng-Tang.

In this study, the prevalence of osteoporosis was higher in women than in men, and increased with age. These findings corresponded with those of previous epidemiological studies [4]. However, our osteoporosis cohort included patients who had visited the outpatient department because of osteoporosis 6 times or more during the study period (from 2003 to 2009) only. The omission of other patients might, therefore, have led to the underestimation of the prevalence of osteoporosis in Taiwan. Among the patients, femoral neck fracture (ICD-9-CM 820; 8.2%) was the most common type of fracture, followed by pathologic fracture (ICD-9-CM 733.1; 6.3%), fracture of the vertebral column without spinal cord injury (ICD-9-CM 805; 5.3%), and fracture of the radius and ulna (ICD-9-CM 813; 3.8%). All osteoporotic fractures were associated with increased mortality [2, 3]. Greater attention is, therefore, needed to reduce the risk of such fractures in osteoporosis patients.

Our study suffers from 2 limitations. First, the NHI program only reimburses for CHP prescribed by TCM physicians. Our analyses did not, therefore, include CHP or decoctions purchased directly from pharmacies. This omission might have led to the underestimation of the frequency of CHP use. However, because the NHI program comprehensively covers CHP prescribed by TCM physicians, which generally cost less than CHP or decoctions sold in TCM pharmacies, this underestimation might be of small magnitude. Second, no suitable disease coding system exists for TCM. In Taiwan, TCM physicians use the ICD-9-CM alone for diagnosis. The therapeutic principle and methods of TCM are based on the results of "syndrome differentiation"; therefore, the same disease can be classified into different TCM syndrome types and treated differently, which might have caused the variations in prescription patterns for the treatment of osteoporosis patients. The development of a coding system for future TCM diagnostic classifications could increase the efficacy of TCM evaluations and greatly facilitate TCM research [20, 21].

5. Conclusions

This study analyzed a cohort of one million randomly sampled patients from the NHIRD from 2003 to 2009 and identified the most commonly used herbal formula and single herb for the treatment of osteoporosis in clinical practice and their prescription patterns. However, further research is needed to determine the safety and efficacy of these CHP.

Conflict of Interests

The authors declare that there is no conflict of interests.

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Research Article

Hwangryun-Haedok-Tang Fermented with *Lactobacillus casei* Suppresses Ovariectomy-Induced Bone Loss

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Hwangryun-haedok-tang (HRT) is the common recipe in traditional Asian medicine, and microbial fermentation is used for the conventional methods for processing traditional medicine. We investigated the inhibitory effect of the *n*-butanol fraction of HRT (HRT-BU) and fHRT (fHRT-BU) on the RANKL-induced osteoclastogenesis in bone-marrow-derived macrophages. mRNA expression of osteoclastogenesis-related genes were evaluated by real-time QPCR. The activation of signaling pathways was determined by western blot analysis. The marker compounds of HRT-BU and fHRT-BU were analyzed by HPLC. The inhibitory effect of HRT or fHRT on ovariectomy-induced bone loss were evaluated using OVX rats with orally administered HRT, fHRT (300, 1000 mg/kg), or its vehicle for 12 weeks. fHRT-BU significantly inhibited RANKL-induced osteoclastogenesis, and phosphorylation of p38, IKKα/β, and NF-κBp65 compared to HRT-BU. In addition, fHRT-BU also significantly inhibited the mRNA expression of Nfκb2, TNF-α, NFATc1, TRAP, ATPv0d2, and cathepsin K. Furthermore, administration of fHRT had a greater effect on the increase of BMD, and greater improved bone microstructure of the femora than that of HRT in ovariectomy rats. This study demonstrated that bacterial fermentation enhances the inhibitory effect of HRT on osteoclastogenesis and bone loss. These results suggest that fermented HRT might have the beneficial effects on bone disease by inhibiting osteoclastogenesis.

1. Introduction

Osteoclastic bone resorption and osteoblastic bone formation comprise a coupled process, known as bone remodeling [1]. It is necessary for maintenance of mineral homeostasis, bone composition, and bone microstructure in adults and the elderly. Increase of osteoclastogenesis and/or osteoclastic activity results in an increase of bone resorption over bone formation leading to an imbalance of bone remodeling, which is associated with reduction of bone mass and disruption of the bone microstructure in pathological bone diseases, including osteoporosis [2, 3]. Osteoclasts originating from the monocyte/macrophage lineage of hematopoietic stem cells differentiate into bone-resorbing multinuclear cells in the presence of the receptor activator for nuclear factor-κB (NF-κB) ligand (RANKL), and macrophage colony-stimulating factor (M-CSF) [4]. RANKL, a member of the TNF superfamily, is the key osteoclastogenic cytokine and M-CSF contributes to the proliferation, survival, and differentiation of osteoclast precursor cells. When RANKL interacts with its cell surface receptor, RANK, it in turn triggers the recruitment of tumor necrosis factor receptor-associated factor (TRAF) family members which serve as adapter molecules. Sequential recruitment of TRAF6 to the cytoplasmic domain of RANK leads to activation of NF-κB and mitogen-activated protein (MAP) kinases, including extracellular signal-regulated kinase (ERK), c-jun-N-terminal kinase (JNK), and p38, which stimulates activated protein-1 (AP-1) activation [2, 4]. As a result of RANKL-induced activation, NF-κB and AP-1 stimulate an initial expression of the key transcription factor nuclear factor of activated T cells, cytoplasmic 1 (NFATc1), which

regulates gene transcription for osteoclastogenesis [5, 6]. Ectopic expression of NFATc1 causes osteoclast precursor cells to undergo efficient osteoclastogenesis in the absence of RANKL and restores osteoclastogenesis in the c-fos knockout mouse, suggesting that osteoclastogenesis signals converge on NFATc1 [7, 8].

Hwangryun-haedok-tang (HRT), also known as huanglian-jie-du-tang or Orengedokuto, is a traditional herbal medicine prescribed for "heat clearing" from the internal organs. According to a theory in oriental medicine, HRT has been used for treating inflammatory, ulcerative disease or hypertension. The pharmacological effect of HRT is known to suppress eicosanoid biosynthesis, which results in the inhibition of inflammatory responses in carrageenan-injected air pouches model [9]. HRT also decreases intestinal injury in indomethacin-induced animal model of enteropathy through modulation of the adenosine system [10]. In addition, HRT decreases blood flow in the auricles of rats and prevents the increment of blood pressure and auricular blood flow due to theophylline [11]. However, there have been no studies done to examine the inhibitory effect of HRT on osteoclast differentiation.

Fermentation has a way to improve the therapeutic effect of traditional herbal medicine [12]. Among traditional fermentation with different sources, the beneficial effect of bacterial or fungus fermentation on medicinal herb has been suggested. *Anoectochilus formosanus* Hayata fermented with *Lactobacillus acidophilus* causes an enhancement of the antioxidant effect accompanied with an increase in total phenols [13]. In addition, anabolic activity of antlers on osteoblast differentiation is enhanced by fermentation with *Cordyceps militaris*, which increases the production of the active component, a sialic acid [14]. Interestingly, bacterial fermentation of carbohydrates or milk could have a positive effect on bone mineral content through increase of mineral solubility, expression of calcium-binding protein, and generation of bone-modulating factors, which prevents bone loss in animal models [15, 16]. Based on these studies, it could be suggested that fermentation stimulates the production or conversion of the primary active components to its metabolite in medicinal herb, which may increase the therapeutic effect of herbal medicine. However, no research has been conducted to study whether bacterial fermentation could improve the pharmacological effect of traditional herbal medicine on bone disease.

In a preliminary experiment, we found that the fermented HRT by lactobacteria (fHRT) has significantly more inhibitory effect on osteoclastogenesis than non-fermented HRT (HRT). Furthermore, we selected *n*-butanol as the organic solvent for maximizing the inhibitory effect of fHRT on osteoclastogenesis. In this study, we investigated the inhibitory effect and the molecular mechanism of *n*-butanol extract of fHRT (fHRT-BU) on RANKL-induced osteoclastogenesis in mouse bone-marrow-derived macrophages (BMMs). Ovariectomy (OVX) rat is a typical animal model of bone loss which has similar characteristics of postmenopausal osteoporosis: reduction in bone mineral density (BMD) and an increased rate of bone turnover with osteoclast bone resorption exceeding formation [17, 18]. To evaluate the therapeutic potential of fHRT for treating bone loss, we examined the inhibitory effect of HRT and fHRT in OVX-induced bone loss.

2. Materials and Methods

2.1. Chemicals and Reagents. *Coptis japonica* Makino, *Scutellaria baicalensis* Georgi, *Phellodendron chinense* Schneider, and *Gardenia jasminoides fructus* were purchased from the Korea Medicine Herbs Association (Yeongcheon, Gyeongsangbuk-do, Republic of Korea). General aerobic medium (de Man, Rogosa and Sharpe broth: MRS) and MRS agar were purchased from Difco Co. (Detroit, MI, USA). α-modified minimal essential medium (α-MEM), fetal bovine serum (FBS), antibiotics (100 U/mL penicillin and 100 μg/mL streptomycin), BCA protein assay kit, and Super-Signal West Femto Maximum Sensitivity Substrate were purchased from Thermo Fisher Scientific Inc. (Rockford, IL, USA). A Cell Counting Kit-8 (CCK-8) was obtained from Dojindo Molecular Technologies (Rockville, MD, USA). RNA-spin total RNA extraction kit was purchased from Intron (Daejeon, Republic of Korea). AccuPower RT PreMix and AccuPower GreenStar qPCR Master Mix were obtained from Bioneer (Daejeon, Republic of Korea). Baicalin, berberine, $1,25(OH)_2D_3$, palmatine, polybrene, puromycin, *q*-nitrophenyl phosphate, and trifluoroacetic acid (TFA) were purchased from Sigma Chemical Co. (St. Louis, MO, USA). Antibodies specific for phospho-ERK1/2 (Thr202/Tyr204), ERK, phospho-JNK1/2 (Thr183/Tyr185), JNK, phospho-p38 (Thr180/Tyr182), p38, phospho-IKKα/β, I-κBα, phospho-p65 (Ser536), and p65 were purchased from Cell Signaling Technology (Danvers, MA, USA). Antibodies against NFATc1 (7A6) and c-fos (H-125) were purchased from Santa Cruz Biotechnology (CA, USA). A PVDF membrane was purchased from Millipore (Darmstadt, Germany). Geniposide was purchased from Wako Pure Chemical Industries, Ltd. (Japan). HPLC grade water and methanol were purchased from J. T. Baker (Phillipsburg, NJ, USA).

2.2. Animals. Specific pathogen-free (SPF) Sprague Dawley female rats, 10 weeks of age, were purchased from Samtako Bio Inc. (Seoul, Republic of Korea). All rats were housed at 22 ± 1°C and 55 ± 10% humidity on a 12-h light/dark cycle with free access to food and water. Animal experiments were carried out in accordance with the National Institute of Health's Guidelines for the Care and Use of Laboratory Animals. The experiments were approved by the Institutional Animal Care and Use Committee at the Korea Institute of Oriental Medicine.

2.3. Preparation of HRT. All four constituents of HRT, *Coptis japonica* Makino 250 g, *Scutellaria baicalensis* Georgi 250 g, *Phellodendron chinense* Schneider 250 g, and *Gardenia jasminoides fructus* 250 g were used in this study. All voucher specimens were deposited in the herbal bank of the Center for Herbal Medicine Improvement Research, Korea Institute of Oriental Medicine. HRT was prepared by using a water extraction method (Gyeongseo Extractor Cosmos-600, Inchon, Republic of Korea). The total quantity

of four herbs was placed in 10 L of distilled water for 1 h, and then extracted by heating for 3 h at 115°C. After extraction, HRT was filtered out using standard testing sieves (106 μm) (Retsch, Haan, Germany) and stored at 4°C before use.

2.4. Fermentation and Fractionation of HRT. *Lactobacillus casei* KFRI-127 used in this study was derived from Korea Food Research Institute (KFRI, Seongnam-si, Republic of Korea). After two successive transfers of the test organisms in MRS broth at 37°C for 24 h, the activated cultures were again inoculated into broth. It was then properly diluted to obtain an initial population of 1–5 $\times$ 10^6 CFU/mL and served as the inoculum. Viable cell counts of the strains were determined in duplicate by using the pour-plate method on MRS agar. For fermentation, 5 mL of HRT in a test tube with cap was inoculated with 0.05 mL of the inoculums as described above. This was incubated at 37°C for a period of 48 h. HRT or fHRT was fractionated by successive solvent extraction with *n*-hexane, ethylacetate, and *n*-butanol. Each fraction was then evaporated to dryness under vacuo and stored in desiccators at −20°C before use. The *n*-butanol-soluble fraction of HRT and fHRT was designated as HRT-BU (yield about 16.45%) and fHRT-BU (yield about 17.72%), respectively.

2.5. Cell Culture and Cell Viability Assay. Murine osteoclasts were prepared from bone marrow cells as described previously [2]. Bone marrow cells (BMCs) were cultured in α-MEM containing 10% FBS with M-CSF (60 ng/mL) and antibiotics (100 U/mL penicillin and 100 μg/mL streptomycin) for 3 days, and the attached BMMs were used as osteoclast precursors. To evaluate cell viability, cells (1×10^4 cells/well) were incubated with different concentrations of sample (12.5–200 μg/mL) in the presence of M-CSF (60 ng/mL) for 3 days on 96-well plates. A CCK-8 was used to examine cell viability according to the manufacturer's protocol (Dojindo Molecular Technologies). Data represented the mean ± SD of triplicate.

2.6. TRAP Assay and Osteoclast Formation. To generate osteoclasts, BMMs were cultured with M-CSF (60 ng/mL) and RANKL (150 ng/mL) for 4 days. Total TRAP activity was measured at an absorbance of 405 nm after treatment with substrate (*p*-nitrophenyl phosphate) as described previously [19]. TRAP-positive multinucleated cells (TRAP(+)MNCs) containing more than three nuclei and larger than 100 μm in diameter were counted.

2.7. Real-Time Quantitative PCR (QPCR). To evaluate NF-κB-regulated gene expression, cells (3×10^5 cells/well in a 6-well plate) were preincubated with sample for 2 h in the presence of M-CSF (60 ng/mL) and then stimulated with RANKL (150 ng/mL) for indicated times. To evaluate NFATc1-regulated gene expression, cells (3×10^5 cells/well in a 6-well plate) were coincubated with sample for indicated times in the presence of M-CSF and RANKL. Total RNA was isolated with an RNA-spin total RNA extraction kit according to the manufacturer's protocol. First-strand cDNA was synthesized from 1 μg of total RNA in AccuPower RT PreMix according to the manufacturer's protocol. Subsequently, SYBR green-based QPCR amplification was performed using cDNA diluted to 1 : 3, 10 pmol of primers, and AccuPower GreenStar qPCR Master Mix in the Applied Biosystems 7500 Real-Time PCR System (Applied Biosystems, Foster City, CA, USA) according to the manufacturer's protocol. The PCR reaction consisted of three segments. In the first segment, the polymerase was activated by heating at 95°C for 10 min. The second segment consisted of 40 cycles of 94°C for 30 s, and 60°C for 1 min. The third segment, designed to generate PCR product-temperature dissociation curves (melting curves), consisted of 95°C for 1 min, 60°C for 30 s, and 95°C for 30 s. All reactions were run in triplicate, and data were analyzed using the $2^{-\Delta\Delta CT}$ method. HPRT was used as the internal standard.

2.8. Western Blot Analysis. BMMs were incubated with sample for indicated time, washed with ice-cold phosphate-buffered saline (PBS), and lysed in protein extraction buffer consisting of 50 mM Tris-HCl (pH 8.0), 5 mM EDTA, 150 mM NaCl, 1% NP-40, 0.1% SDS, 1 mM PMSF, one protease-inhibitor cocktail tablet, and one phosphatase-inhibitor cocktail tablet. Cell lysates were centrifuged at 10 000 ×g for 15 min at 4°C. Protein concentration was determined with a BCA protein assay kit. Protein samples were mixed with sample buffer (100 mM Tris-HCl (pH 7.6), 2% SDS, 1% 2-mercaptoethanol, 2% glycerol, and 0.01% bromophenol blue), incubated at 95°C for 5 min, and loaded onto 10% polyacrylamide gels. Electrophoresis was performed using a Mini-PROTEAN 3 Cell (Bio-Rad, Hercules, CA, USA). Resolved proteins were transferred onto a PVDF membrane. The membrane was first incubated in blocking buffer (10 mM Tris-HCl (pH 7.5), 150 mM NaCl, 0.1% Tween 20, and 5% non-fat dry milk) and then incubated for 2 h at room temperature with 1000 diluted primary antibodies. After washing with a washing buffer (10 mM Tris-HCl (pH 7.5), 150 mM NaCl, and 0.1% Tween 20) three times for 10 min each, the membrane was probed with 2000 diluted secondary antibody for 1 h. The membrane was then washed with a washing buffer three times for 10 min each and developed with SuperSignal West Femto Maximum Sensitivity Substrate. Chemiluminescent signals were detected with a LAS-4000 luminescent image analyzer (Fuji Photo Film Co., Japan).

2.9. High-Performance Liquid Chromatography (HPLC) Analysis. The high-performance liquid chromatography (HPLC) DAD system (Hitachi Co., Japan) consisted of a pump (L-2130), autosampler (L-2200), column oven (L-2350), and diode array UV/VIS detector (L-2455). The output signal of the detector was recorded using an EZChrom Elite software by Hitachi. Chromatographic separation was achieved in a Luna C18 column (4.6 mm $\times$ 250 mm, 5 μm, Phenomenex Co., USA) at 254 and 380 nm. The mobile phase was 100% methanol (A) and water-containing 0.1% trifluoroacetic acid

(B) with a step gradient elution (0–5 min, 5% A; 5–70 min, 100% A, 70–80 min, 100% A). The column temperature was maintained at 40°C. Each standard solution was prepared by dissolving each marker components, geniposide, berberine, palmatine, and baicalin, with 100% methanol at the concentration of 100 μg/mL. Prior to analysis, the sample preparation was dissolved with 100% methanol at the concentration of 10 mg/mL and filtered through a 0.45 μm filter. 3 μL of samples was injected for the HPLC analysis.

2.10. Animal Model of Ovariectomized Rats. Female rats were either sham operated (sham, $n = 8$) or surgically OVX (OVX, $n = 40$) after acclimatization in the laboratory environment for one week. One week after OVX surgery, the OVX rats were randomly divided into five groups with eight rats each: (1) OVX: bilaterally OVX; (2) HRT-0.3: bilaterally OVX followed by 0.3 g/kg of HRT administration; (3) HRT-1.0: bilaterally OVX followed by 1.0 g/kg of HRT administration; (4) fHRT-0.3: bilaterally OVX followed by 0.3 g/kg of fHRT administration; and (5) fHRT-1.0: bilaterally OVX followed by 1.0 g/kg of fHRT administration. The administration of samples began 1 week and finished 3 months after OVX surgery. The same amount of saline was orally administered to the sham and OVX groups. Animal experiments were carried out in accordance with the National Institute of Health's Guidelines for the Care and Use of Laboratory Animals. The experiments were approved by the Institutional Animal Care and Use Committee at the Korea Institute of Oriental Medicine.

2.11. Microcomputed Tomography (Micro-CT) Analysis. To determine 3-dimensional bone structure *in vivo*, we performed histomorphometric analysis. Computed tomographic images of the femur of each rat were acquired 3 months after OVX surgery, using the *In-Vivo* Micro-CT (SkyScan 1076, SkyScan N.V., Belgium) at a resolution of 18 μm, with the following parameters: 100 kV, 100 mA, 1,770 ms. The beam-hardening errors were corrected to improve the quality of the micro-CT images by flat-field correction before scanning and beam-hardening correction during reconstruction. Three-dimensional models of the trabecular bones of the femur were reconstructed using SkyScan CT Analyzer version 1.11 to evaluate the alteration of bone. In addition, the structural parameters were measured on the trabecular bone of the femora.

2.12. Statistical Analysis. SPSS software (SPSS Inc., Chicago, IL, USA) was used for all statistical analyses. The significance of difference in TRAP activity and the mRNA expression levels of osteoclast differentiation-related genes were determined using Student's t-test using HPRT-normalized $2^{-\Delta\Delta CT}$ values. For the statistical analysis of the results of the animal experiment, a parametric one-way analysis of variance was used to test for any differences among the groups. Duncan's multiple comparison test was used to confirm significant differences in the mean value between the groups. A P-value less than 0.05 was considered significant.

3. Results

3.1. Effect of HRT and fHRT on Osteoclastogenesis in BMMs. We first evaluated the effect of HRT or fHRT on the cell viability of BMMs to examine any potential cytotoxic effect. This result showed that HRT or fHRT had no cytotoxic effect on mouse BMMs in a wide range of concentrations (12.5–200 μg/mL) (Figure 1(a)). To evaluate the inhibitory effect of HRT or fHRT on RANKL-induced osteoclast differentiation, we examined TRAP activity and the number of TRAP-positive multinuclear cells. HRT or fHRT significantly decreased TRAP activity in a dose-dependent manner (Figure 1(b)). In particular, 100 μg/mL concentration of fHRT had a greater inhibitory effect on TRAP activity (15%) than that of HRT (Figures 1(c) and 1(d)). Furthermore, to maximize the inhibitory effect of fHRT on osteoclastogenesis, we made extracts by fractionating components according to their polarity and compared their effect on osteoclastogenesis. *n*-butanol extracts of fHRT proved to be highly effective in reducing formation of TRAP-positive multinuclear osteoclasts while the other extracts had lower activity or cytotoxicity (data not shown). After fractionation with *n*-butanol, the inhibitory effect of fHRT increased more than 10-fold. We next compared the inhibitory effect of HRT-BU and fHRT-BU on osteoclastogenesis in mouse BMMs. HRT-BU (3–30 μg/mL) significantly decreased RANKL-induced TRAP activity without cytotoxicity in a dose-dependent manner (Figures 2(a) and 2(b)). Interestingly, fHRT-BU at all concentration had about 20% greater inhibitory effects on TRAP activity than HRT-BU. In addition, 3 μg/mL concentration of HRT-BU markedly suppressed about 70% of TRAP-positive multinuclear osteoclast formation induced by RANKL (Figures 2(c) and 2(d)). fHRT-BU completely inhibited multinuclear osteoclast formation at 3 μg/mL. We subsequently used 10 μg/mL of HRT-BU and fHRT-BU in the following study.

3.2. Effect of HRT-BU and fHRT-BU on NFATc1 and c-fos Expression. NFATc1 and c-fos are essential transcription factors for osteoclast differentiation. To determine the inhibitory mechanism of fHRT-BU on osteoclast differentiation, we explored whether fHRT-BU inhibits RANKL-induced NFATc1 and c-fos expression. As compared to the vehicle, HRT-BU and fHRT-BU significantly decreased RANKL-induced mRNA level of NFATc1 and c-fos (Figure 3(a)). We also analyzed mRNA expression of osteoclastogenesis-related genes (TRAP, ATPv0d2, and cathepsin K) regulated by NFATc1. HRT-BU and fHRT-BU significantly decreased RANKL-induced mRNA level of these genes. Specifically, fHRT-BU had a greater inhibitory effect on mRNA level of TRAP, ATPv0d2, and cathepsin K compared with HRT-BU (Figure 3(a)). To confirm these inhibitory effects of HRT-BU and fHRT-BU on NFATc1 and c-fos expression, the protein expression of NFATc1 and c-fos was determined by western blot analysis. HRT-BU and fHRT-BU inhibited RANKL-induced NFATc1 protein expression, but not c-fos (Figure 3(b)).

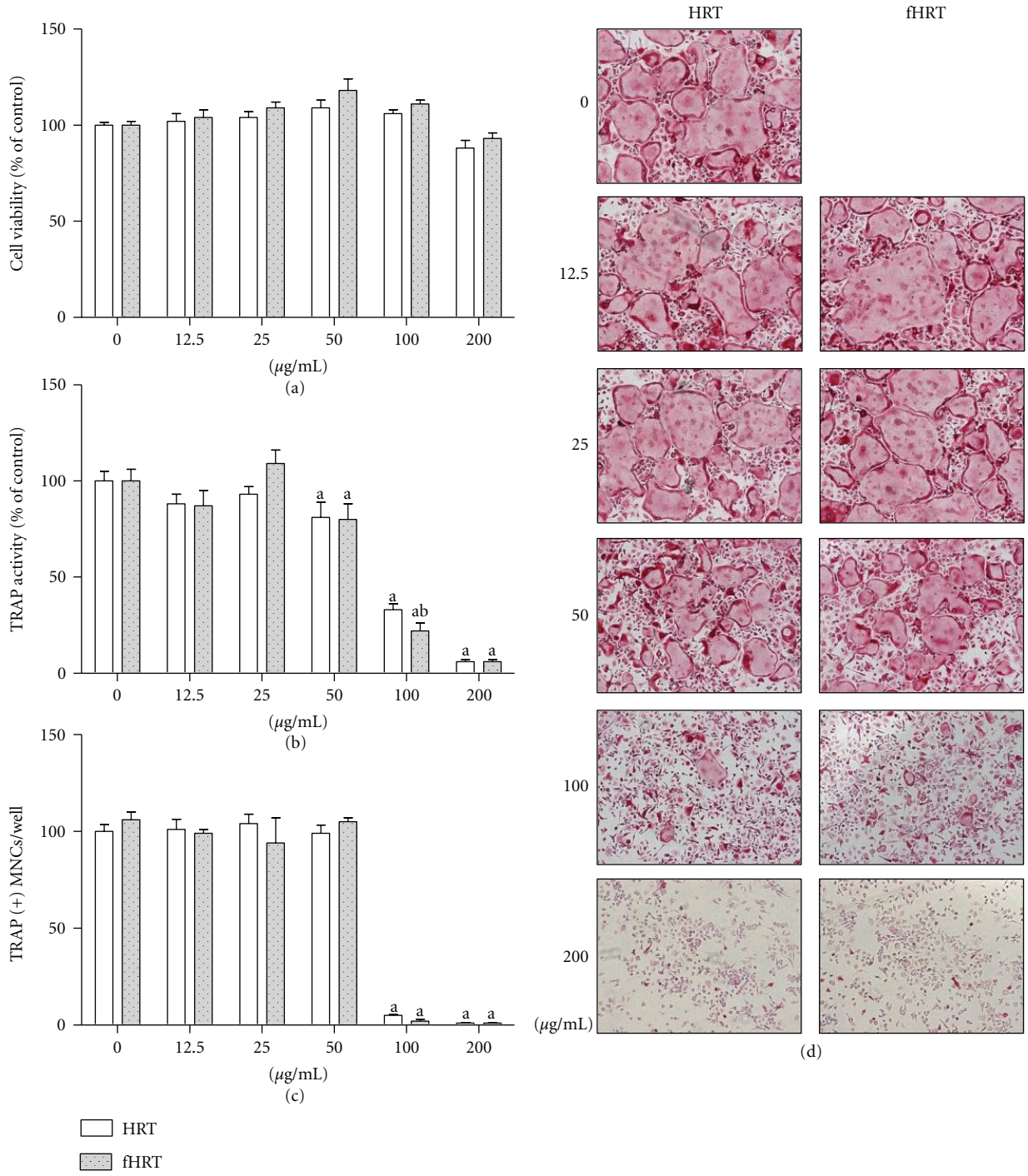

Figure 1: HRT and fHRT inhibit RANKL-induced osteoclast formation in BMMs. BMMs (1×10^4 cells/well in a 96-well plate) were incubated with M-CSF (60 ng/mL), RANKL (150 ng/mL), and the indicated concentrations of HRT or fHRT for 4 days. Cell viability was measured by CCK-8 assay (a). The cells were fixed and stained for TRAP activity (b), and then the number of TRAP-positive multinuclear osteoclast cells (TRAP(+)MNCs) (c) with more than 3 nuclei and larger than 100 μm in diameter were counted. Representative microscopic pictures of multinucleated osteoclasts (d) were shown at a magnification of 100x. Data represents mean ± SD of three independent experiments. [a]$P < 0.05$, versus vehicle treated control. [b]$P < 0.05$, versus HRT.

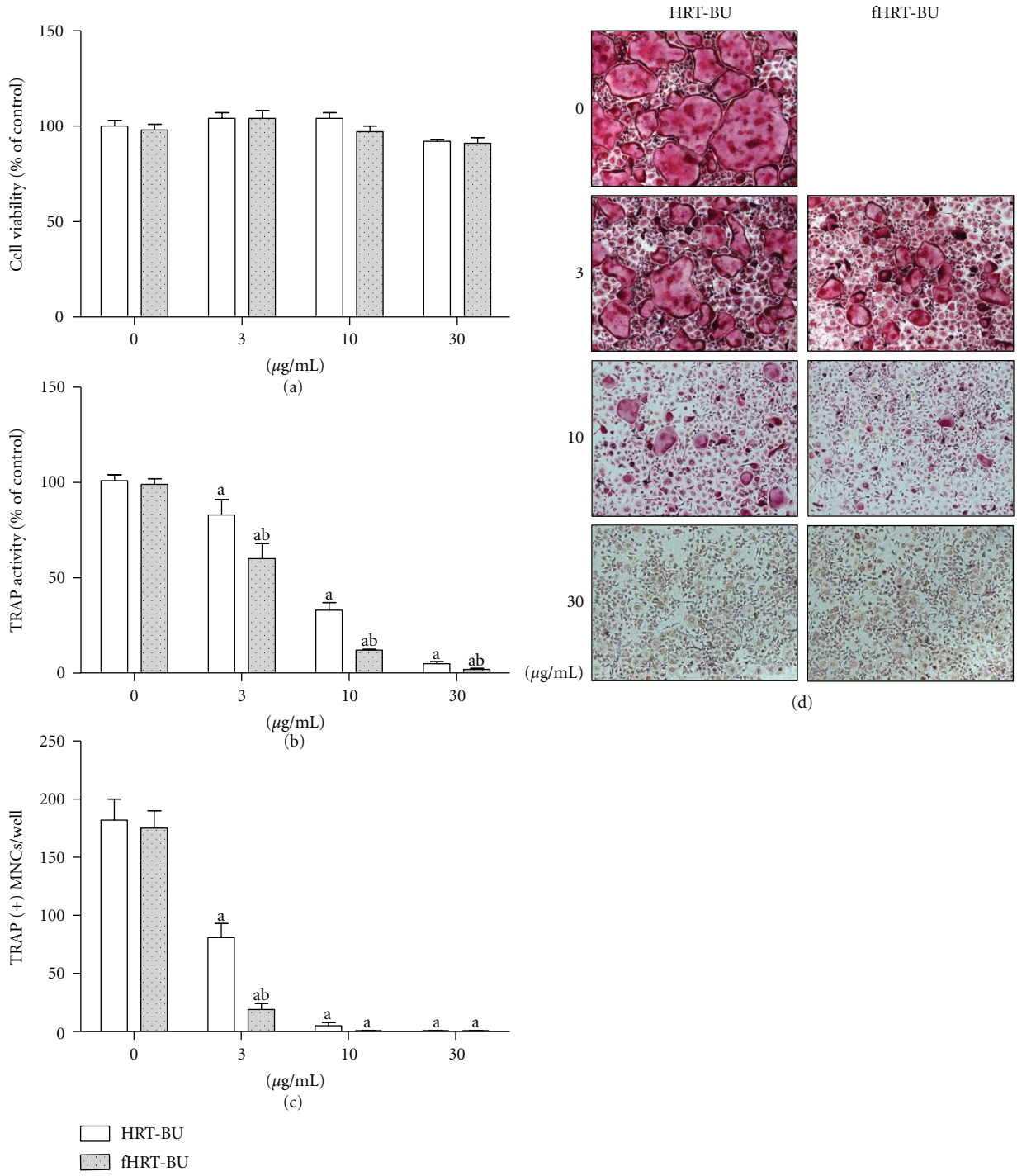

Figure 2: HRT-BU and fHRT-BU inhibit RANKL-induced osteoclast formation in BMMs. BMMs (1×10^4 cells/well in a 96-well plate) were incubated with M-CSF (60 ng/mL), RANKL (150 ng/mL), and the indicated concentrations of HRT-BU or fHRT-BU for 4 days. Cell viability was measured by CCK-8 assay (a). The cells were fixed and stained for TRAP activity (b) and then the number of TRAP-positive multinuclear osteoclast cells (TRAP(+)MNCs) (c) with more than 3 nuclei and larger than 100 μm in diameter were counted. Representative microscopic pictures of multinucleated osteoclasts (d) were shown at a magnification of 100x. Data represents mean ± SD of three independent experiments. [a]$P < 0.05$, versus vehicle treated control. [b]$P < 0.05$, versus HRT.

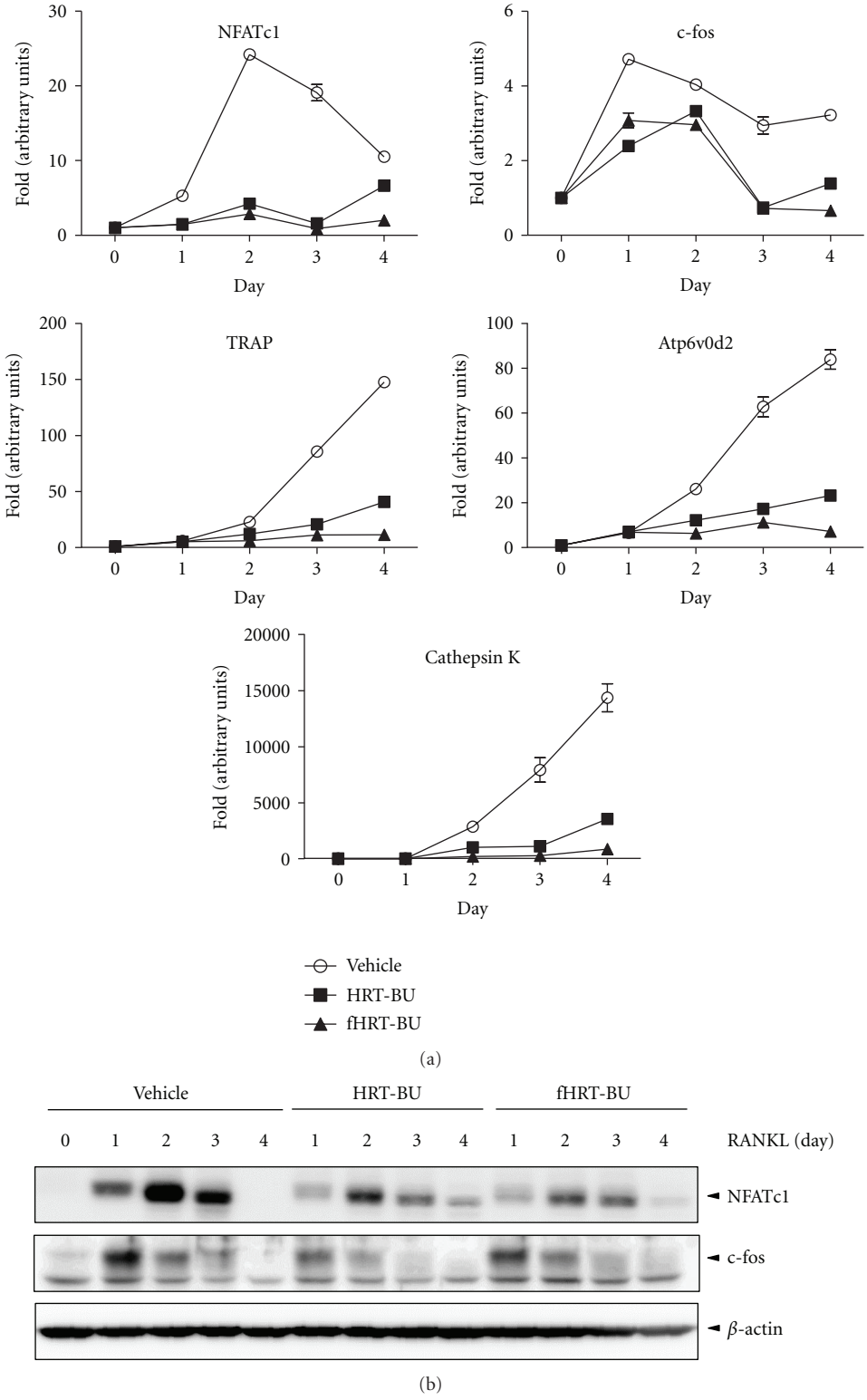

Figure 3: fHRT-BU inhibits RANKL-induced NFATc1 expression in BMM cells. (a) BMMs (3×10^5 cells/well in a 6-well plate) were incubated with HRT-BU (10 μg/mL) and RANKL (150 ng/mL) or fHRT-BU and RANKL for the indicated times. Total RNA was isolated on the indicated time and mRNA expression of NFATc1, c-fos, TRAP, ATPv0d2, and cathepsin K was analyzed by QPCR. (b) Whole cell lysates (30 μg) were analyzed by western blot analysis with antibody specific for NFATc1 and c-fos. β-actin was used as loading control. Data are representative of three independent experiments.

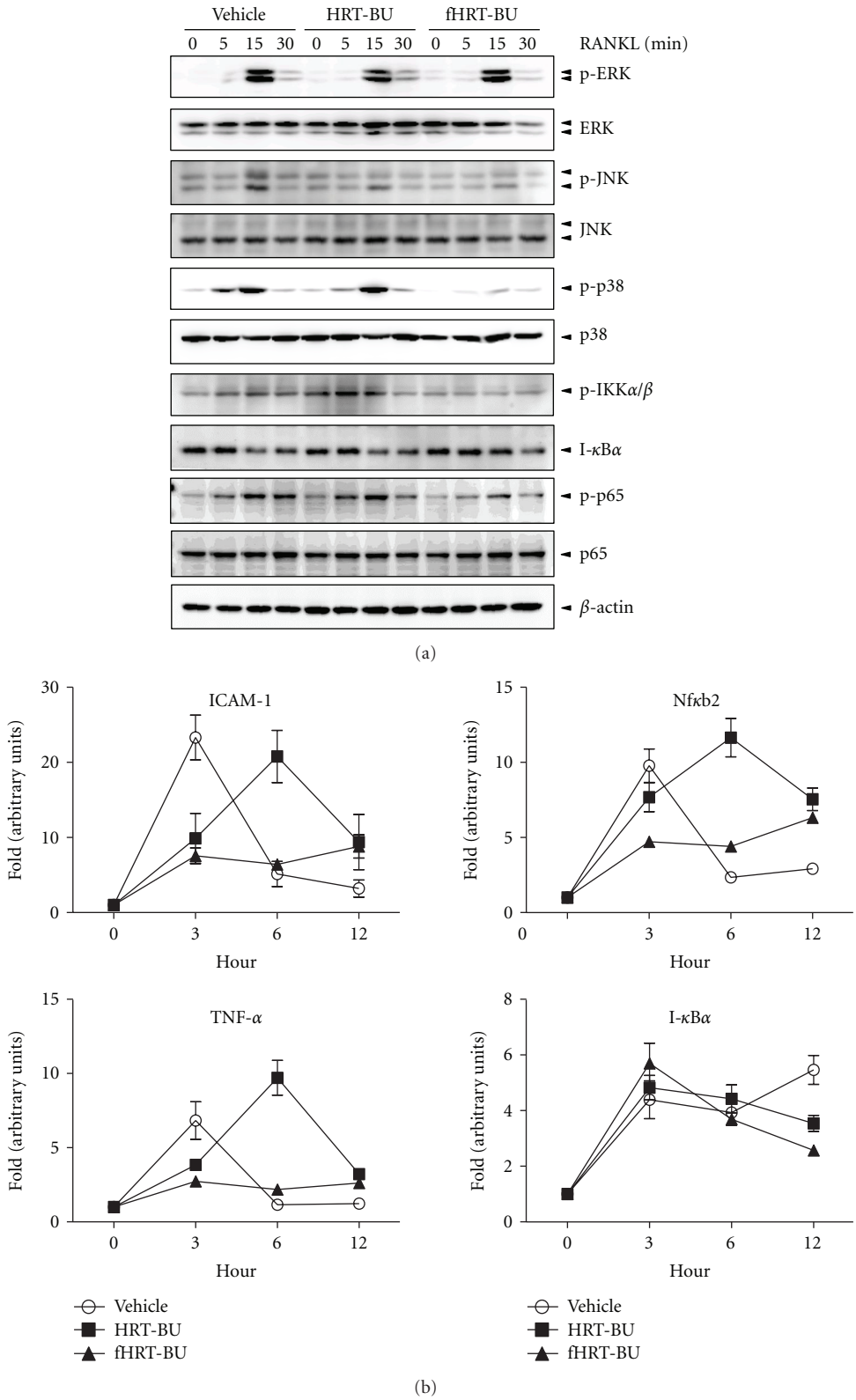

Figure 4: fHRT-BU inhibits RANKL-induced p38, IKKα/β, and p65 phosphorylation in BMM cells. (a) BMMs (3×10^5 cells/well in a 6-well plate) were pretreated with HRT-BU or fHRT-BU (10 μg/mL) for 2 h and then stimulated with RANKL (150 ng/mL) for the indicated times. Whole cell lysates (10 μg) were analyzed by western blot analysis with indicated antibodies. MAP kinases, IKKα/β, and p65 activation was represented by the levels of protein phosphorylation. β-actin was used as loading control. (b) BMMs were pretreated with HRT-BU or fHRT-BU (10 μg/mL) for 2 h and then stimulated with RANKL (150 ng/mL) for 3, 6, and 12 h. Total RNA was isolated on the indicated time and mRNA expression of ICAM-1, TNF-α, Nfκb2, and I-κBα was analyzed by QPCR. Data represents mean ± SD of three independent experiments.

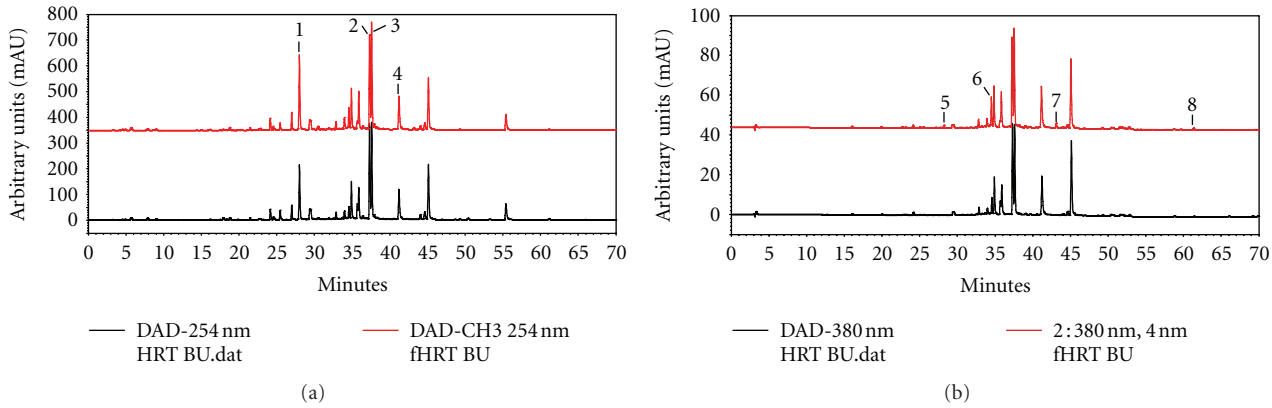

Figure 5: The HPLC analysis chromatograms of HRT-BU and fHRT-BU at 254 nm (a) and 380 nm (b). (1) Geniposide t_R 27.96 min; (2) berberine t_R 37.27 min; (3) palmatine t_R 37.54 min; (4) baicalin t_R 41.16 min; (5) t_R 28.18 min; (6) t_R 34.50 min; (7) t_R 43.08 min; (8) t_R 61.30 min.

3.3. Effect of HRT-BU and fHRT-BU on RANKL-Induced MAP Kinases and NF-κB Signaling. MAP kinases (ERK, JNK, and p38), IKKα/β, I-κBα, and p65 (Ser-536) mediating the signaling pathways play an important role in RANKL-induced osteoclastogenesis. To examine whether HRT-BU and fHRT-BU affect immediate early signaling pathways, BMMs were treated with HRT-BU or fHRT-BU and then were stimulated with RANKL for the indicated periods of time. Both HRT-BU and fHRT-BU decreased the RANKL-induced activation of JNK. Furthermore, fHRT-BU markedly suppressed p38, IKKα/β, and p65 phosphorylation compared with RANKL and HRT-BU treatment (Figure 4(a)).

p38 participates in NF-κB transactivation by mediating p65 phosphorylation [20]. Since fHRT-BU inhibited p38 and p65 (Ser-536) phosphorylation, we explored whether fHRT-BU inhibits NF-κB-dependent transcription in BMMs. QPCR was applied to measure mRNA expression level of four genes (ICAM-1, Nfκb2, TNF-α, and I-κBα) regulated by NF-κB. As compared to HRT-BU, fHRT-BU had a greater inhibitory effect on RANKL-induced mRNA level of Nfκb2, and TNF-α of about 1.5-fold at 3 h after RANKL treatment (Figure 4(b)).

3.4. HPLC Analysis of HRT-BU and fHRT-BU. HRT-BU and fHRT-BU significantly inhibited RANKL-induced osteoclastogenesis and NFATc1 expression in BMMs. Flavonoids have been suggested as potential components related to the therapeutic effect of the medicinal herbs. Therefore, we performed HPLC analysis to characterize HRT-BU and fHRT-BU by fingerprinting the marker components of medicinal herbs in HRT. HPLC analysis successfully separated the major peaks and the change of peaks in HRT-BU and fHRT-BU (Figure 5). HPLC analysis chromatograms simultaneously identified major components of both samples that were geniposide (1, t_R 27.96 min), berberine (2, t_R 37.27 min), palmatine (3, t_R 37.54 min), and baicalin (4, t_R 41.16 min). There was no significant change of major components between HRT-BU and fHRT-BU. However, the intensity of four unidentified compounds, the peak numbers 5 to 8, was increased in fHRT-BU compared to HRT-BU.

3.5. The Quantification of Bone Mass by Micro-CT in fHRT-Treated OVX Rats. To examine the potential inhibitory effect of HRT and fHRT on OVX-induced bone loss, bone mass of femur was analyzed by micro-CT. In comparison with the sham group, the OVX group exhibited significant decrease in BMD of about 70% (Figure 6(a)). Total BMD in fHRT-1.0 groups was significantly higher than that of the OVX and HRT-1.0 group. Particularly, fHRT-1.0 groups showed a 55% and 54% greater BMD than the OVX and HRT-1.0 groups, respectively. The structural parameters of trabecular bone for bone volume/trabecular volume (BV/TV), bone surface/bone volume (BS/BV), trabecular thickness (Tb.Th), trabecular separation (Tb.Sp), and trabecular number (Tb.N) were shown in Figure 6. Compared to the sham group, the OVX group showed significant change in BV/TV (−65%), BS/BV (+29%), Tb.Th (−14%), Tb.Sp (+169%), and Tb.N (−60%). There was no significant change of these parameters in the HRT-0.3 and HRT-1.0 groups. Interestingly, the fHRT-1.0 group showed significant improvement of the decreasing structural parameters induced by OVX. Particularly, fHRT-1.0 group showed significant improvement of all structural parameters over the HRT-1.0 group, recording a 41% greater BV/TV, 23% less BS/BV, 15% greater Tb.Th, but no significant differences in Tb.Sp and Tb.N. Furthermore, the femur of the fHRT-1.0 group formed a tight and dense structure compared with that of the OVX and HRT-1.0 group (Figure 6(g)).

4. Discussion

The interaction of RANKL with its receptor, RANK, initiates early signaling pathways including MAP kinases and the NF-κB pathway through the adaptor protein TRAF in osteoclastogenesis. In this study, we found that fHRT markedly decreased the RANKL-induced phosphorylation of p38, IKKα/β, and NF-κB p65 (Ser-536) (Figure 4(a)). NF-κB p65 and p50 are recruited to the NFATc1 promoter with NFATc2 for autoamplification of NFATc1 expression in RANKL-induced osteoclast differentiation [21]. IKKα/β and p38 are both necessary for enhanced NF-κB p65

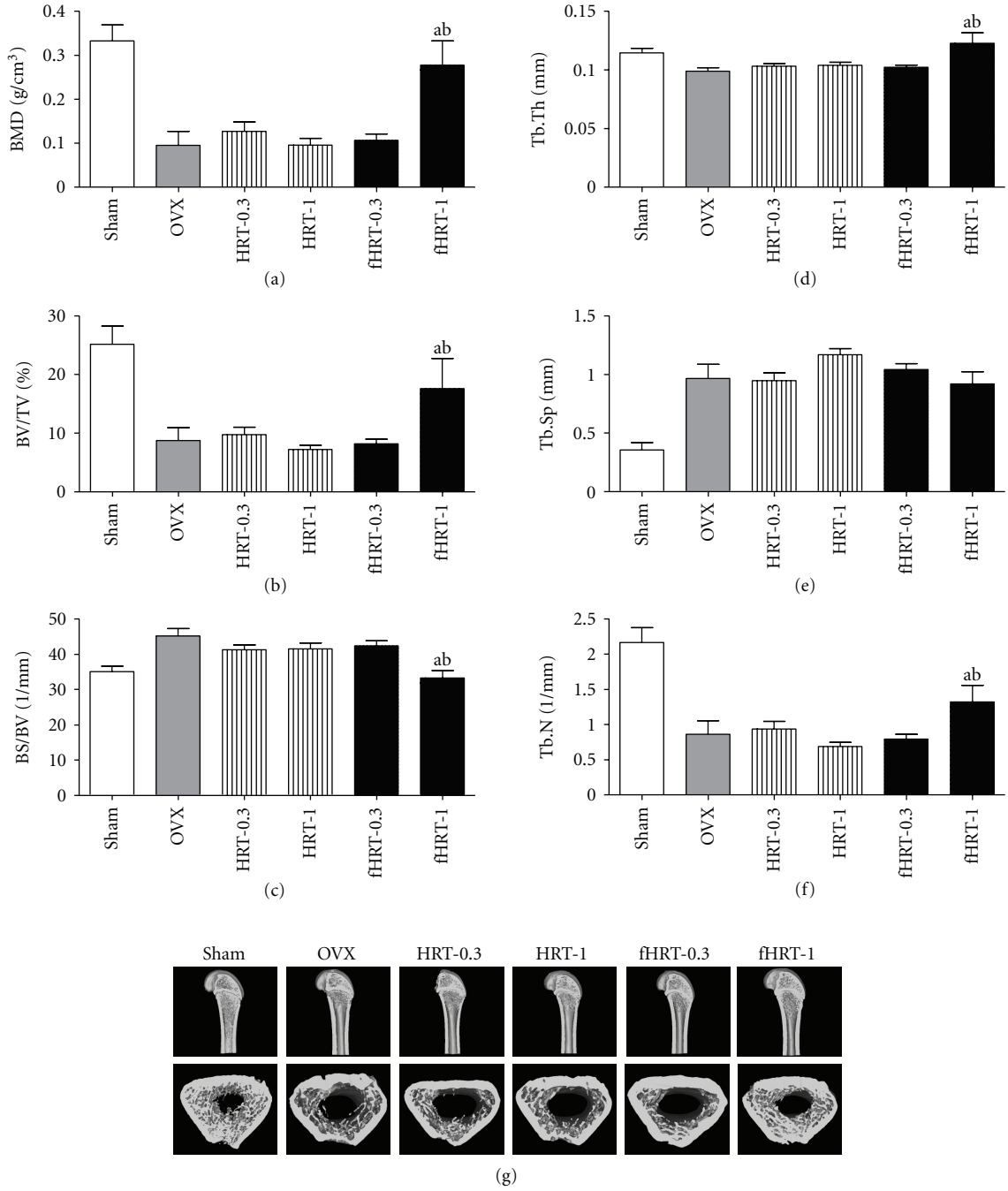

Figure 6: fHRT increases bone density in OVX rats. Bone mineral density (BMD) and structural parameter of trabecular bone of femur were analyzed by micro-CT after 12 weeks of HRT and fHRT administration. Graphs represented BMD (a), bone volume (BV/TV, (b)), bone surface (BS/BV, (c)), trabecular thickness (Tb.Th, (d)), trabecular separation (Tb.Sp, (e)), and trabecular number (Tb.N, (f)). Representative micro-CT images of distal metaphysic femur of sham, OVX, HRT-0.3, HRT-1.0, fHRT-0.3, and fHRT-1.0 group (g). [a]$P < 0.05$, versus OVX. [b]$P < 0.05$, versus HRT-1.0.

transactivation, whereas mutation of either Ser-529 or Ser-536 of p65 abolishes this effect [22]. In particular, p38 participates in the RANKL-stimulated NFATc1 induction, which is mediated by NF-κB p65 activation on Ser-536 [20]. NFATc1 is a master transcription factor regulating the expression of osteoclastogenesis-related gene in osteoclasts [4, 8]. Because fHRT significantly decreased RANKL-induced p38 and NF-κB p65 (Ser-536) activation, and mRNA expression of NF-κB regulated genes (Figure 4), fHRT might inhibit NF-κB activity in osteoclast precursor cells, resulting in the inhibition of osteoclastogenesis.

Consistent with the inhibitory effect of fHRT on MAP kinase (JNK and p38) and NF-κB (IKKα/β and NF-κB p65) pathway (Figure 4), we also found that fHRT-BU significantly augmented the suppressive effect of HRT-BU on NFATc1 expression, both in mRNA and on the protein

level (Figure 3). In addition, fHRT significantly inhibited NFATc1-regulated and osteoclast-specific genes (Figure 3(a)) including TRAP, Atp6v0d2, and cathepsin K that modulate osteoclast fusion, activation, and function [23–25]. ERK activates c-fos, while JNK increases AP-1 transcriptional activity through the phosphorylation of c-Jun [26]. p38 induces NF-κB transactivation for NFATc1 expression [20]. In response to RANKL and TNF, c-fos which is downstream of NF-κB transcriptionally regulates NFATc1 expression [6]. Therefore, as fHRT significantly suppressed mRNA and protein expression of NFATc1, fHRT might target NFATc1 expression and NFATc1-dependent gene expression through inhibition of p38 and NF-κB p65 signaling in RANKL-induced osteoclastogenesis.

Bacterial fermentation results in not only the structure change of flavonoids, stimulating its bioavailability and metabolism [27], but also the generation of components having bone-preserving potential. In this study, we found that the bacterial fermentation changes the contents of flavonoid in fHRT-BU (Figure 5) and increases the inhibitory effect of HRT on bone loss (Figure 6). Our finding is consistent with those of previous studies and indicates that bacterial fermentation produces the inhibitory molecules on bone loss. 1,4-dihydroxy-2-naphthoic acid or 6-*O*-succinylated isoflavone glycoside generated by bacterial fermentation improves bone mass reduction through inhibition of bone resorption and/or bone-resorption-related cytokine levels [28, 29]. The flavonoids undergo a cleavage of the glycosides by bacterial metabolism in the small intestine, which is followed by absorption and metabolism of the aglycone [30]; they may contribute to the beneficial effect on bone health [16]. Thus, it could be suggested that lactic fermentation might increase the generation and bioavailability of the inhibitory components in HRT on osteoclastogenesis, which consequently contributes to enhanced characteristics of bone structure and an increase of BMD in OVX rats.

The present study evaluated whether the bacterial fermentation affects the inhibitory effect of HRT on OVX-induced bone loss. We found that lactic fermented HRT, but not HRT, significantly inhibited the OVX-induced decrease of BMD, BV/TV, Tb.Th, and Tb.N (Figure 6). In general, a significant loss of BV/TV is associated with a loss of Tb.N and Tb.Th in OVX rats, indicating the loss of a finer network of the numerous trabeculae in the metaphyseal region, and the increase of osteoclastic bone resorption [31]. Estrogen maintains trabecular bone volume by suppression of bone resorption and stimulation of bone formation, which consequently suppresses OVX-induced increase of bone turnover [32, 33]. Traditional herbs are known to have some flavonoid with phytoestrogenic activity that is structurally similar to estrogen and interacts with estrogen receptor. Phytoestrogen directly reduces osteoclast activity but stimulates osteoblastogenesis, and dose-dependently decreases bone turnover in the OVX model [34]. In addition, phytoestrogen reduces the postmenopausal characteristics associated with an increase in obesity and vaginal atrophy due to estrogen deficiency. However, fHRT did not reverse estrogen-deficiency-induced obesity and vaginal atrophy (data not shown). Thus, it would be possible that bioactive compounds in fHRT might have positive estrogenic effects on bone without a uterotrophic effect as reported in previous studies [28, 35].

In conclusion, we first found that lactic bacterial fermentation of HRT inhibits OVX-induced bone loss by enhancing bone mineral density and bone microstructure. It might result from the inhibitory effect of fHRT on osteoclastogenesis by downregulating NFATc1 expression. The results of this study suggest that bacteria-fermented HRT could have therapeutic potential for the treatment of postmenopausal osteoporosis and other bone diseases. Further studies would have to be carried out in order to determine the active components in fHRT.

Authors' Contribution

K. Shim and T. Kim equally contributed to this study.

Conflict of Interests

The authors declare no conflict of interests.

Acknowledgment

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Research Article

Curcumin Protects against Ovariectomy-Induced Bone Changes in Rat Model

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Osteoporosis is a metabolic disease affecting both men and women especially in postmenopausal women. Curcumin possesses many medicinal properties. In this study, thirty two female Sprague-Dawley rats were used to determine the potential effect of curcumin in prevention of bone loss following ovariectomy. The animals were divided into Sham group, ovariectomised control, ovariectomised treated with curcumin 110 mg/kg and ovariectomised treated with Premarin 100 μg/kg. The treatments were given via daily oral gavages for 60 days. The structural parameters such as bone volume, trabecular number, trabecular thickness and trabecular separation were found to be deteriorated in ovariectomised rats compared to Sham group. Moreover, the reduced osteoblast count, the increased osteoclast count and increased eroded surface were found in ovariectomised groups. Treatment with curcumin was able to reverse all these ovariectomy-induced deteriorations. Curcumin treatment was as effective as Premarin in most parameters except the bone volume and eroded surface, which were better than Premarin. The high dose of curcumin treatment was not only able to reduce the osteoclast number but also increase the osteoblast count. Therefore, the potential effect of curcumin can be applied as an alternative to oestrogen for prevention of postmenopausal osteoporosis.

1. Introduction

Osteoporosis is a metabolic bone disorder that causes fracture in 40% of white women [1]. Bone metabolism is influenced by several factors. Bone fragility is based on the genetic and environmental factors [2]. After menopause, bone formation rate is less than bone resorption rate leading to the bone-remodelling imbalance which is associated with increased risk of fracture [3, 4]. Thus, oestrogen plays a role in bone metabolism. It controls bone resorption by reducing the osteoclast number [5]. Lack of oestrogen accelerates bone loss causing trabecular thinning and discontinuation as well as cortical thinning and porosity [2]. Estai et al. [6] found a significant reduction in trabecular number and widening of trabecular spaces in the distal portion of rats' femur 6 weeks after bilateral ovariectomy. The possible mechanism of bone loss in ovariectomised rats might be due to oxidative stress produced by the high hydrogen peroxide and lipid peroxidation levels and reduced antioxidant enzyme activities [7].

Hormone replacement therapy (HRT) reduces the fracture risk during menopause. HRT by oestrogen alone increases the risk of endometrial and breast cancer [8, 9]. The doses required for HRT in the prevention of bone loss are higher than those required in treating menopausal symptoms [3], thus the physician should outweigh the benefits and risks of HRT. Therefore, many researchers are interested in medicinal properties of natural herbs with fewer side effects.

Turmeric is a rhizome of *Curcuma longa* plant belonging to the Zingiberaceae family and Curcuma genus [10]. It is used as a spice and flavouring agent in the preparation of Asian cuisine. Curcumin ($C_{21}H_{20}O_6$) is generally regarded as the most active constituent of polyphenolic phytochemicals, comprising 2–8% of most turmeric preparations

[11]. Curcumin is a lipid-soluble active constituent which possesses varieties of potential benefits such as inhibition of lipid peroxidation in renal cell line [12], liver microsomes, erythrocyte membranes, and brain homogenates [13]. It also has anti-inflammatory [14], antimutagenic, and antihypercholesterolemia activities [15]. It has a potential protective medroxyprogesterone acetate- (MPA-) induced mammary tumours by inhibiting the expression of vascular endothelium growth factor (VEGF), *in vitro* and *in vivo* studies [16, 17]. However, it did not alter the oestrogen and progesterone receptors [16, 17]. It is able to inhibit bone resorption by stimulating the osteoclast apoptosis *in vitro* as a nuclear factor-κB (NF-κB) binding site competitor [18]. It also inhibited the osteoclast differentiation and function by inhibiting the signalosome-associated kinase IκB in a dose-dependent response [19]. Curcumin was found to produce beneficial changes in bone turnover and bone strength [20]. The earlier study done by Anna et al. [21] found that an oral dose of 110 mg/kg body weight of curcumin protected against collagen-induced arthritis. Considering the beneficial effects of curcumin, this study was aimed to highlight the protective effect of curcumin with the dose of 110 mg/kg on bone loss due to oestrogen deficiency following ovariectomy.

2. Material and Methods

2.1. Experimental Protocol. A total of 32 three-month-old female Sprague-Dawley rats (200–250 gm) were obtained from the Animal House after getting approval from the Animal Ethics Committee of the university. The rats were kept one rat per cage at room temperature with 12-hour light and dark cycle. The rats were allowed to access water *ad libitum* and standard rat chow (Gold Coin, Selangor, Malaysia). After one week of acclimatization, the rats were randomly divided into four groups with eight rats in each group, namely, sham-operated and given vehicle (sham), ovariectomised-control (OVXC), ovariectomised and treated with curcumin (110 mg/kg) (OVX + CL) [21], and ovariectomised and treated with Premarin (100 μg/kg) (OVX + E_2) [6]. Both curcumin and Premarin were given via oral gavage daily for 60 days. Curcumin was purchased from herbal supplier Sabinsa Company Malaysia. Palm oil without vitamin E (Merck, Germany) was used as vehicle. The 110 mg/kg dose of curcumin was freshly prepared in 1.0 mL of palm oil [21]. The treatment was started two weeks after ovariectomy. Body weights were recorded twice weekly.

2.2. Preparation of Oestrogen Deficient Animal Model. The rats were ovariectomised according to Estai et al. [6] under anaesthesia with intramuscular injection of combination of xylazil (0.03 mL), ketapex (0.1 mL), and zoletil 50 (0.1 mL) (Troy Laboratories, Australia). Both ovaries were removed through midline abdominal incision. Proper postoperative care was given by daily dressing with povidone iodine and treated with antibiotic enrofloxacin (Bayer, Korea) 5% intramuscularly for 7 days [6]. The same procedure was performed on the sham animals by gentle manipulation of ovaries in situ. Histology of excised tissue and marked atrophy of the uterine horns at the end of experiment confirmed the success of the surgery.

2.3. Bone Histomorphometry. After 60 days of treatment, the rats were euthanized and the femora dissected out. The distal portions of the femur were kept in 10% formalin for 48 hours. The samples for structural histomorphometric analysis such as bone volume per tissue volume (BV/TV), trabecular thickness (TbTh), trabecular number (TbN), and trabecular separation (TbSp) were dehydrated and embedded in methyl methacrylate according to Difford (1974) [22]. The tissue was sectioned at 10 μm with a microtome (Leica, Wetzlar, Germany) and stained with Von Kossa stain. For the static parameters such as osteoclast number (OcN), osteoblast number (ObN), eroded surface (ES), osteoid surface (OS), and osteoid volume (OV), bone samples were decalcified with EDTA for 4 weeks and embedded in paraffin wax. The samples were sectioned at 8 μm with a microtome (Leica, Wetzlar, Germany) and stained with hematoxylin and eosin. The measurement for static parameters was done by using the Weibel Technique, a quantitative stereological technique for histological sections [23]. The analysis of all parameters was performed at the metaphyseal region, where the trabeculae are found abundantly [24]. True colour Windows image processing analysis system (R&M Biometrics, BQTCW98, and Version 3.50.6) interfaced with a light microscope (Olympus, Leeds Instruments, USA) was used to analyse the samples. The structural and static parameters were measured in accordance with the guidelines of the American Society of Bone Mineral Research Histomorphometry Nomenclature Committee [25]. Double-blinded assessment was done on three sections per specimen with an interval of 10 serial sections.

2.4. Statistical Analysis. The results were expressed as mean values ± SD. Data analysis was performed using SPSS version 12.0. Statistical test ANOVA followed by Tukey's HSD (Honestly Significantly Different) was used for normally distributed data and Kruskal-Wallis and Mann-Whitney tests for data that was not normally distributed. The significant value was set at $P < 0.05$.

3. Results

3.1. Structural Parameters. The percentages of bone volume per tissue volume (BV/TV %) in curcumin-treated ovariectomised rats (OVX + CL) and the oestrogen replacement groups (OVX + E_2) were significantly higher than the untreated ovariectomised groups (OVXC) ($P < 0.05$). When the two treatment groups were compared, the BV/TV of the OVX + CL group was significantly higher than that of the OVX + E_2 (Figure 1(a)). The TbN and TbTh of OVX + CL and OVX + E_2 groups were significantly higher than the OVXC group but were not significantly different from each other (Figures 1(b) and 1(c)). The trabecular separation (TbSp, μm) was significantly higher in the OVXC group compared to the rest (Figure 1(d)). The trabeculae bony thickness of the Von Kossa stain in the OVX + CL and OVX +

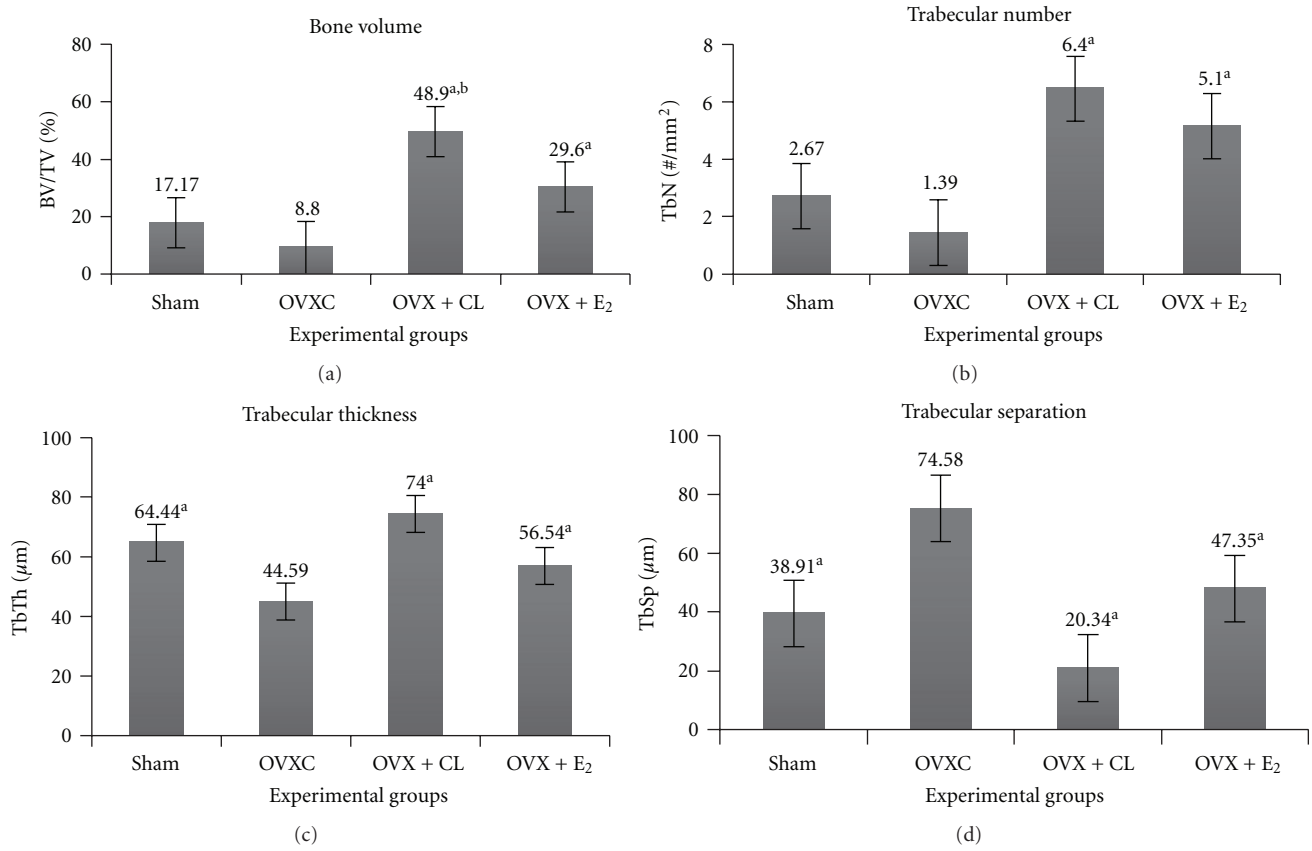

Figure 1: Effect of curcumin on the (a) bone volume, (b) trabecular number, (c) trabecular thickness, and (d) trabecular separation. [a]Significant difference compared to ovariectomised (OVXC) group ($P < 0.05$). [b]Significant difference compared to OVX + E_2 ($P < 0.05$).

E_2 (Figures 2(c) and 2(d)) were more pronounced compared to the OVXC group (Figure 2(b)). This accounted for the trabecular thinning and widening of the trabecular space in the OVXC group (Figure 2(b)) which was due to the process of osteoclastosis resulting in bone resorption.

3.2. Static Parameters. As for the static parameters of the OVXC group, the ES/BS (Figure 3(a)) and OcN (Figure 3(b)) were significantly higher while the ObN (Figure 3(c)) was significantly lower than the sham group. Both the CL and oestrogen treatments were able to significantly reduce the OcN (Figure 3(b)) and increase the ObN (Figure 3(c)) compared to sham group. Only treatment with CL was able to reduce the ES/BS (Figure 3(a)) until it was significantly lower than the OVXC group. The OS and OV of all the groups were not significantly different from one another (data not shown). In the H&E stain, indentations of the Howship's lacunae with numerous multinucleated osteoclasts were observed in the OVXC and OVX + E_2 group (Figures 4(b) and 4(d)). These were less obvious in the sham and OVX + CL groups (Figures 4(a) and 4(c)). Flattened-looking osteoblasts were observed laid on the bony surfaces of the sham and the OVX + CL groups indicating some form of ossification (Figures 4(a) and 4(c)).

4. Discussion

World Health Organisation (WHO) study group [3] suggested that the definition of osteoporosis should be retained as "A disease characterised by low bone mass and microarchitectural deterioration of bone tissue, leading to enhanced bone fragility and a consequent increase in fracture risk." This definition was developed during the Consensus Development Conference in 1991 [26]. Skeletal fragility and fracture risk are associated with low bone mineral density (BMD) [27]. Therefore, BMD is used as an indicator to measure bone mass and serves as a diagnostic parameter for osteoporosis [28]. A study group of WHO [3] reported that the increased fracture risk is associated with BMD as low as the T-score of ≤ 2.5 SD.

In normal individuals, bone mass increases during skeletal growth and reaches the peak bone mass between the ages of 20 to 40 years. Postmenopausal osteoporosis occurs due to low peak bone mass or accelerated bone loss due to hormone deficiency or aging or both factors. However, the mineral content of the remaining bone could be normal and thus there is no shift in the ratio of minerals to protein matrix [29]. The most important microscopic features of bone loss include widening of Haversian canals and thinning of the trabeculae that could be due to increased bone resorption and perforation of trabecular plates [30].

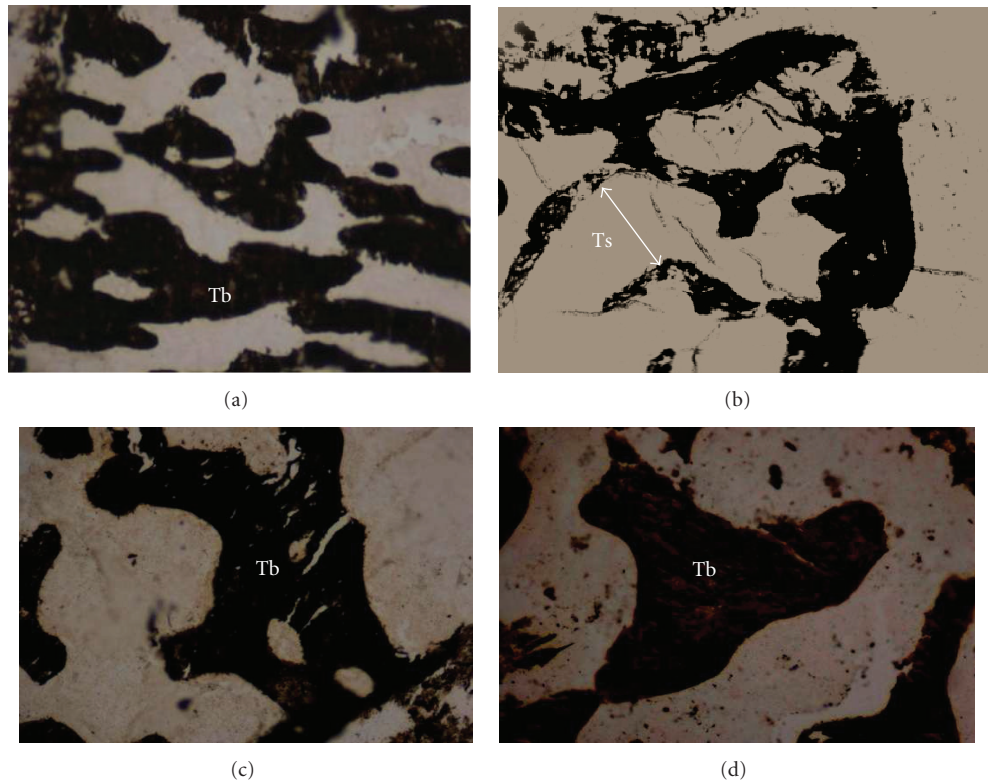

Figure 2: Bone trabeculae (Tb) of rat femur with Von Kossa stain 100x. (a) Sham group without treatment, (b) ovariectomised (OVX) control group—trabecular thinning and widening of trabecular space 60 days after ovariectomy, (c) OVX with curcumin—restoration of the trabecular thickness in ovariectomised rats, and (d) OVX with Premarin. Tb: trabeculae; Ts: trabecular separation.

In oestrogen deficiency state, there were reductions in trabecular number (TbN) and bone volume (BV/TV) and increased trabecular separation (TbSp) which are attributed by the higher bone resorption than bone formation [31]. Estai et al. [6] also mentioned that the trabecular numbers were reduced as early as the 6th week after ovariectomy in young female Sprague Dawley rats. The present study revealed similar findings in ovariectomised control rats by the 10th week after ovariectomy. This was in accordance with the observation by Baldock et al. [24] which found that the trabecular bone loss in ovariectomised rat was mainly due to the decrease in trabecular number.

Oestrogen deficiency induces bone loss causing trabecular thinning [2]. This is explained by the fact that lack of oestrogen stimulates the differentiation and the proliferation of osteoclasts [32]. However, the previous study [6] showed no significant change in trabecular thickness till the 6th week following ovariectomy. In the present study, the trabecular thickness was significantly reduced by the 10th week after ovariectomy. The present finding supported the fact that oestrogen deficiency accelerates bone resorption [5].

However, these ovariectomy-induced bone changes were reversed with curcumin treatment. The present study found that the bone structural changes were significantly reversed in curcumin-treated ovariectomised group compared to ovariectomised-control group. Our results showed that curcumin extract was able to protect the trabecular bone volume against the effect of ovariectomy. These were in agreement with Hie et al. [33], who found that curcumin reduced diabetes-associated bone resorption. Tuba and Gülçin [34] reported that curcumin possesses free radical scavenging activity. This beneficial property may apply to its protective effect against bone loss due to oestrogen deficiency that induces oxidative stress which stimulates the differentiation and proliferation of osteoclasts via cytokine release [32].

The increased osteoclast count in ovariectomised group of the present study supported the findings of Parhami [32]. Ozaki et al. [18] had examined the action of curcumin on rabbit osteoclast apoptosis and suggested that curcumin may be useful in the treatment of osteoporosis as it drastically inhibited bone resorption in parallel with stimulation of apoptosis of the cells. At the molecular level, macrophage colony-stimulating factor (M-CSF) and RANKL, a receptor activator of NF-κB ligand, are essential for differentiation of osteoclasts, and responsible for bone resorption [35]. Kim et al. [36] recently reported that curcumin inhibits osteoclastogenesis by impairing the signalling of RANKL. Therefore, curcumin may have affected the activity and number of osteoclasts rather than osteoblasts in the bone of diabetic rats [33]. This was in agreement with the

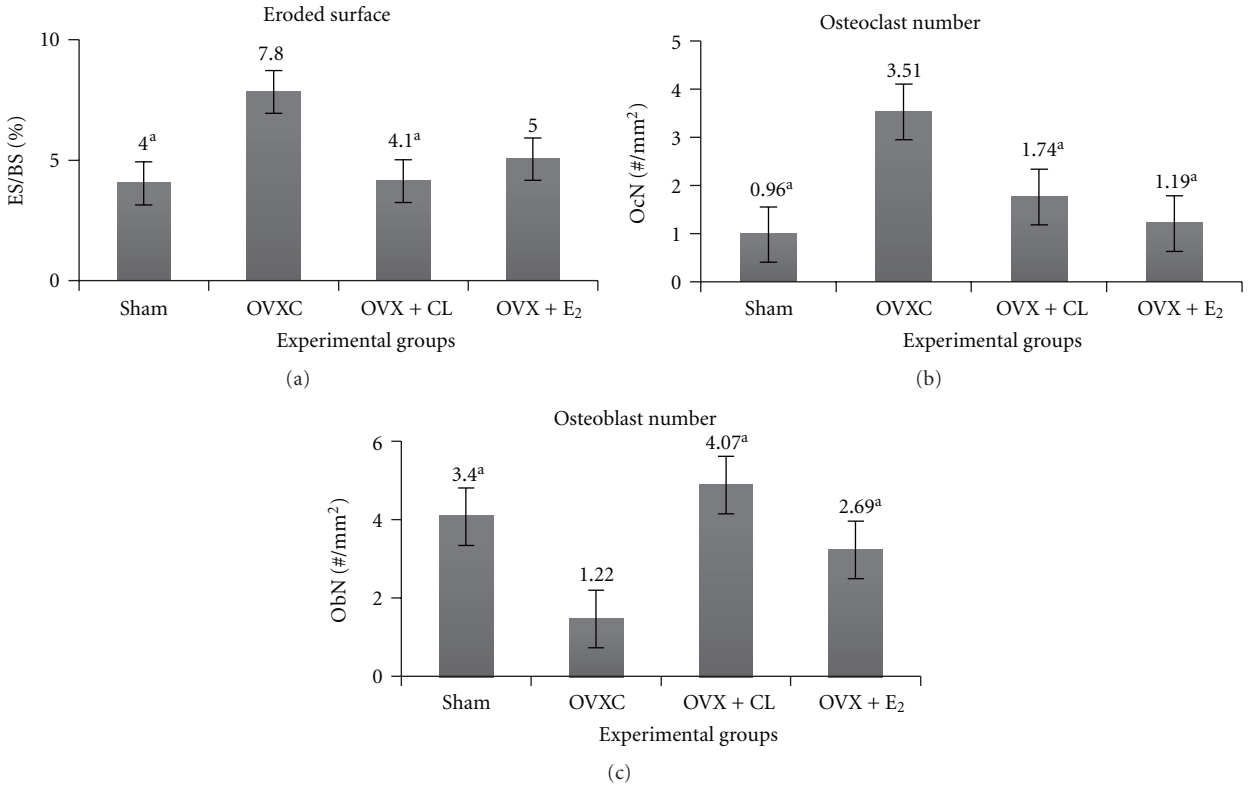

Figure 3: Effect of curcumin on the (a) Eroded surface, (b) osteoclast number, and (c) osteoblast number. [a]Significant difference compared to ovariectomised (OVXC) group ($P < 0.05$).

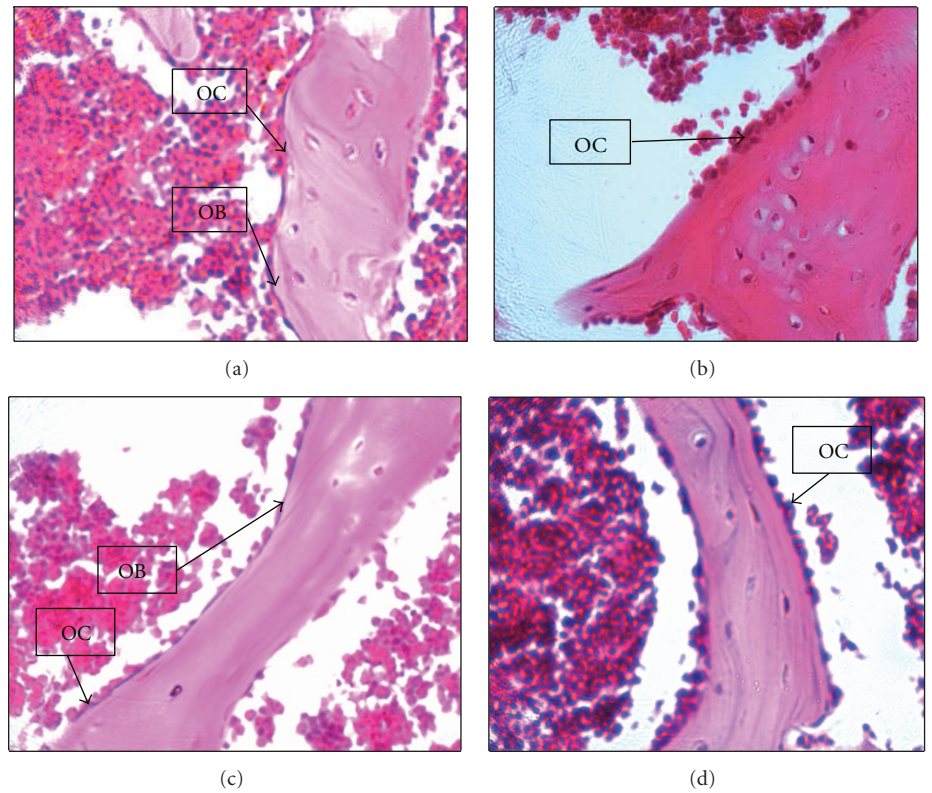

Figure 4: Effect of curcumin on the histological changes of bone in ovariectomised rats H&E stain 40x. (a) Sham group without treatment, (b) ovariectomised (OVX) control group, (c) OVX with curcumin, and (d) OVX with Premarin. OB: osteoblast; OC: osteoclast.

present study which found reduction in osteoclast number of ovariectomised rats with curcumin treatment.

Another important consideration for curcumin treatment is the dosage, duration, and mode of administration. Curcumin was found to induce bone changes after ovariectomy in a dose-dependent manner [20]. The dose of 110 mg/kg body weight used in the present study which was administered by oral gavage is considered high compared to previous studies [20, 33]. However, the dose used was claimed to be safe as there have been no reports of significant adverse effects with the consumption of 500 to 8,000 mg turmeric powder per day in human [37, 38]. Our findings suggested that higher dose of curcumin not only inhibited the osteoclast activity but was also able to stimulate the activity of osteoblast, the cells responsible for osteoid synthesis and mineralisation of matrix [39].

Oestrogen is important in maintaining bone metabolism by inhibiting the osteoclast activity. Premarin, a conjugated oestrogen, is used for the prevention of postmenopausal osteoporosis. Although it is effective in alleviating the postmenopausal syndrome, there are higher risks of endometrial and breast cancers to women with strong family histories. The Woman Health Initiative Study Group [40] reported that it may also increase the risk of stroke and deep vein thrombosis following long-term use because oestrogen induces clotting factors release from the liver. However, curcumin delays breast cancer development related with combined hormonal therapy [17]. It was reported to inhibit the tissue factor activity implicated in thrombotic disorders [41] and decreased platelet adhesion and activation [42]. These actions were achieved through its anti-inflammatory, anticarcinogenic, and antiproliferative effects [41]. Therefore, curcumin reduced the risk of adverse effects of oestrogen and, at the same time, revealed the inhibitory effect on the bone changes following ovariectomy. Its protection on bone changes was comparable to the oestrogen as proven in the present study.

5. Conclusion

Curcumin treatment reversed the bone changes following ovariectomy and it was as effective as oestrogen therapy. Further studies are warranted to explore the potential of curcumin as an alternative agent for oestrogen in postmenopausal osteoporosis.

Conflict of Interests

The test agents such as curcumin and Premarin were purchased from the local distributors. Therefore, the authors declare that there is no conflict of interests on the trademark of these agents.

Acknowledgments

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Research Article

Virgin Coconut Oil Supplementation Prevents Bone Loss in Osteoporosis Rat Model

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Oxidative stress and free radicals have been implicated in the pathogenesis of osteoporosis. Therefore, antioxidant compounds have the potential to be used in the prevention and treatment of the disease. In this study, we investigated the effects of virgin coconut oil (VCO) on bone microarchitecture in a postmenopausal osteoporosis rat model. VCO is a different form of coconut oil as it is rich with antioxidants. Three-month-old female rats were randomly grouped into baseline, sham-operated, ovariectomized control (Ovx), and ovariectomized rats fed with 8% VCO in their diet for six weeks (Ovx+VCO). Bone histomorphometry of the right femora was carried out at the end of the study. Rats supplemented with VCO had a significantly greater bone volume and trabecular number while trabecular separation was lower than the Ovx group. In conclusion, VCO was effective in maintaining bone structure and preventing bone loss in estrogen-deficient rat model.

1. Introduction

Osteoporosis is a metabolic disorder which is characterized by deterioration of bone tissue and loss of bone mass with a consequent increased risk of fracture. It is due to decreased activities of osteoblasts and increased activities of osteoclasts [1]. In both sexes, estrogens and testosterones play a vital role in the pathophysiology of osteoporosis. Postmenopausal osteoporosis which occurs in aging women is usually associated with estrogen deficiency [2]. The absence of ovarian hormone will result in accelerated bone resorption by osteoclasts and reduced bone formation by osteoblasts [3]. Oxidative stress has been implicated in the pathogenesis of osteoporosis as evidenced by numerous *in vitro* and *in vivo* studies [4, 5]. Oxidative stress occurs when the body's antioxidant defense is unable to overcome the cellular damage caused by free radical molecules [6]. Supplementation with antioxidants like vitamins C [7] and E [8, 9] has been shown to prevent bone loss in osteoporosis. On the other hand, deficiency in vitamins E and D may cause a decrease in cartilage cells and osteocytes, as well as thinning of the cortex and trabecular in mice [10].

Virgin coconut oil (VCO) is obtained from fresh, mature coconut kernel without the use of heat and without undergoing refining process [11]. This retains the important biologically active components in the oil such as antioxidant vitamins and phenolic compounds. VCO supplementation in diet has been shown to reduce the cholesterol and triglyceride levels, maintain blood coagulation factors, and prevent oxidation of low-density lipoprotein lipids [12, 13]. Besides, VCO has been reported to have anticancer, antimicrobial, and anti-inflammatory properties [14–16]. Another study showed that virgin coconut oil lowered alcohol-induced oxidative stress by reducing testicular malondialdehyde level (tMDA) and ameliorated the deleterious effect of alcohol on serum testosterone level in rats [17]. Diet supplemented with virgin coconut oil was shown to increase the antioxidant status in rats [18]. To date, there has been no research done on the effects of VCO on bone metabolism.

Rats are widely accepted animal model for studying bone diseases as the bone remodelling and resorption processes in young and adult rats resemble those of human [19]. The responses of bone towards mechanical stress, hormones, and drugs are similar in rat and human [20]. Therefore, ovariectomized rat is a useful model for osteoporosis since the progressive loss of bone matrix is similar to that in postmenopausal women with osteoporosis [21]. In the present study, we investigated the effects of virgin

coconut oil supplementation on bone microarchitecture of ovariectomized rats. We carried out bone histomorphometry analysis on undecalcified bone sections in order to obtain information about the trabecular structure.

2. Materials and Methods

2.1. Preparation of Virgin Coconut Oil (VCO). The preparation of virgin coconut oil was modified from previous studies by Nevin and Rajamohan 2004, 2006 [12, 18]. To summarize the procedure, mature coconuts were bought from a local market and the fresh coconut meat was grated using an electric grater. A 100 g of grated coconut flesh was mixed with 200 g of natural coconut water. The coconut mixture was squeezed into viscous slurry until all the creamy milk was obtained. The coconut milk was left in room temperature for 48 hours to allow fermentation process to take place. At the end of the second day, there would be three layers seen with the virgin coconut oil forming the second layer. The oil was then gently scooped out and filtered to remove the coconut residue. Lastly, the oil was separated from the bottommost aqueous layer using a separatory funnel.

2.2. Experimental Design. A total of 40 female Wistar rats aged 3 months old, weighing between 200–250 g, were obtained from Universiti Kebangsaan Malaysia Animal Centre. The animals were allowed a two-week acclimatization period during which they were fed on commercial rat chow (Gold Coin, Klang Selangor, Malaysia) and deionized water given *ad libitum*. Rats were accommodated two per cage at room temperature with a 12 hours light/dark cycle. The rats were randomly divided into five groups with eight rats in each group: Baseline, Sham, ovariectomized control (Ovx), ovariectomized and treated with virgin coconut oil (Ovx+VCO), and ovariectomized and given calcium (Ovx+Ca). Rats in the baseline group were euthanised at the start of the experiment. The ovariectomized rats had their ovaries removed under anaesthesia. The abdomen of the sham rats were operated on and their ovaries were manipulated but were left intact. Treatment commenced two weeks after ovariectomy to allow ample time for healing. The Ovx+Ca rats received 1% calcium in their drinking water. All the rats except the Ovx+VCO group were fed with standard rat chow diet. The diet for Ovx+VCO rats contained 8% virgin coconut oil mixed with the standard rat chow [18]. The diet was prepared by mixing 8 g VCO into 100 g standard rat chow. Gain in body weight was recorded weekly. Treatment lasted for six weeks after which the rats were euthanised by overdoses of Phenobarbital (400 mg/kg) and their right femora removed for bone histomorphometry analysis. This study was approved by the Animal Ethics Committee of Universiti Kebangsaan Malaysia (UKMAEC: PP/FAR/2009/NORLIZA/24-FEBRUARY/250-MARCH-2009-JULY-2010).

2.3. Bone Histomorphometry. At sacrifice, the right femora were fixed in 10% formalin. After fixation, the bones were cut at the midshaft using a rotary electronic saw (Black & Decker, USA). The distal femora were then halved longitudinally and subsequently dehydrated in graded concentrations of ethanol. They were then further processed for embedding in methyl methacrylate polymer according to the manufacturer's instructions (Osteo-Bed Bone Embedding Kit; Polysciences, USA). Following that, the femora were sectioned using a Manual Rotary Microtome (Model 2235, Leica, Germany) and serial bone sections at 7 microns thick were obtained. The undecalcified bones were stained using von Kossa method [22]. Histomorphometric measurements were carried out on the secondary spongiosa of the distal femoral metaphysis at a distance between 3 mm and 7 mm from the lowest point of growth plate and from 1 mm of the bilateral cortices. Measurements were made at 4x objective magnification using a light microscope (Leica, Germany) connected to an image analyzer (Image Pro-Express, Media Cybernatics, USA). The parameters measured in this study were trabecular bone volume, trabecular thickness, trabecular number, and trabecular separation. Trabecular bone volume (BV/TV) is the amount of trabecular bone within the spongy space (expressed as a percentage). BV/TV is derived from measurements of bone area (B.Ar) and cancellous tissue area (T.Ar) and calculated as BV/TV = 100 $\times$ B.Ar/T.Ar. Trabecular thickness (Tb.Th, in micrometers) was derived from trabecular perimeter (B.Pm) and B.Ar (Tb.Th = 1.99 $\times$ B.Ar/2/B.Pm). Trabecular number (Tb.N, expressed per millimeter) and trabecular separation (Tb.Sp, expressed per micrometer) were calculated assuming that trabecular bone can be modeled by the parallel plates and bar model (Tb.N = Tb.Ar $\times$ 10/Tb.Th; Tb.Sp = 1000/Tb.N $-$ Tb.Th). All the formula, symbols, units, and nomenclature used for bone histomorphometry in this study were in accordance to the recommendation by the American Society for Bone and Mineral Research (ASBMR) Histomorphometry Nomenclature Committee [23].

2.4. Statistical Analysis. Statistical analysis was performed using Statistical Package for Social Science software (SPSS version 19.0; SPSS). Results were expressed as mean $\pm$ standard error of the mean (SEM) with significance defined as P value of 0.05 or less. Data were tested for normality using the Kolmogorov-Smirnov test. For normally distributed data, analysis of variance (ANOVA) followed by Tukey's HSD test was carried out. Kruskal-Wallis and Mann-Whitney tests were used for data that were not normally distributed.

3. Results

3.1. Body Weight. The body weight gain pattern is shown in Figure 1. By the end of the sixth week, the ovariectomized control rats gained significant weight compared to all other groups. The ovariectomized rats treated with virgin coconut oil and calcium had significantly higher body weight than Sham rats (Figure 1).

3.2. Trabecular Bone Volume (TV/BV). Ovariectomized control rats had a reduced trabecular bone volume compared to the Baseline, Sham, and Ovx+VCO groups. The Ovx+VCO

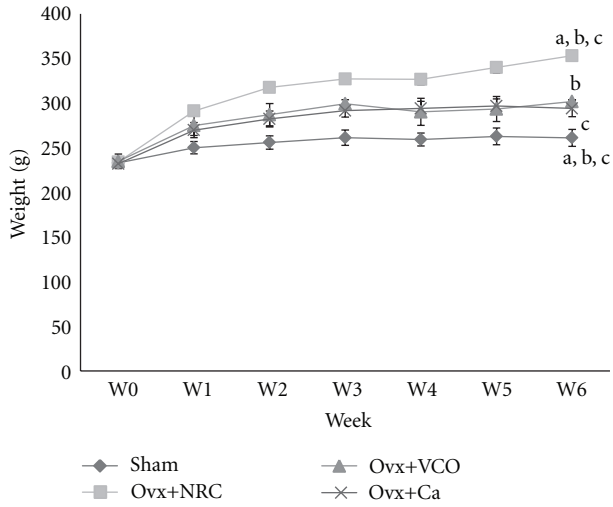

Figure 1: Weight of the rats throughout six-week study period. Same letters indicate significant difference between groups ($P < 0.05$). Data are expressed as mean ± SEM. Sham: sham-operated; Ovx: ovariectomized control rats; Ovx+VCO: ovariectomized rats with virgin coconut oil; Ovx+Ca: ovariectomized rats with calcium.

group had a significantly higher TV/BV than the Ovx group whilst remained comparable with the Sham group (Figure 2). However, there was no significant difference between Ovx group and Ovx+Ca rats. Treatment with calcium did not have any effect on trabecular volume (Figure 2).

3.3. Trabecular Number (Tb.N). The Ovx group had reduced Tb.N compared to Baseline, Sham and Ovx+VCO groups. Treatment with VCO significantly prevented the reduction in Tb.N. There was no significant difference in Tb.N between the Ovx+VCO, Baseline and Sham groups. However, treatment of Ovx rats with calcium failed to prevent the reduction in Tb.N as there was no significant difference in Tb.N between the Ovx and Ovx+Ca groups (Figure 3).

3.4. Trabecular Thickness (Tb.Th). Ovariectomy did not reduce the value of Tb.Th. There was no significant difference noted between Ovx and the other four groups of rats. There was no difference either among the Ovx+VCO, Ovx+Ca, Baseline, and Sham groups (Figure 4).

3.5. Trabecular Separation (Tb.Sp). Rats in the Baseline, Sham, Ovx+VCO, and Ovx+Ca groups had significantly lower Tb.S compared to Ovx group. There was no significant difference between the Baseline, Sham, Ovx+VCO, and Ovx+Ca rats (Figure 5).

3.6. Bone Histology. The trabecular bones of the Ovx group appeared to be sparser compared to the other groups. The bones of Ovx+VCO group appeared thicker and denser than the Ovx group and comparable to the Sham.

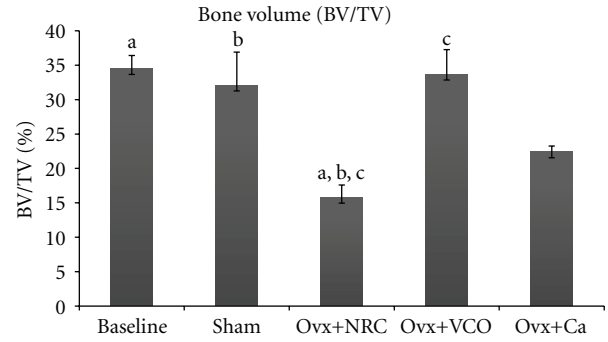

Figure 2: Bone volume (BV/TV). Same letters indicate significant difference between groups ($P < 0.05$). Data are expressed as mean ± SEM. Sham: sham-operated rats; Ovx: ovariectomized control rats; Ovx+VCO: ovariectomized rats with virgin coconut oil; Ovx+Ca: ovariectomized rats with calcium.

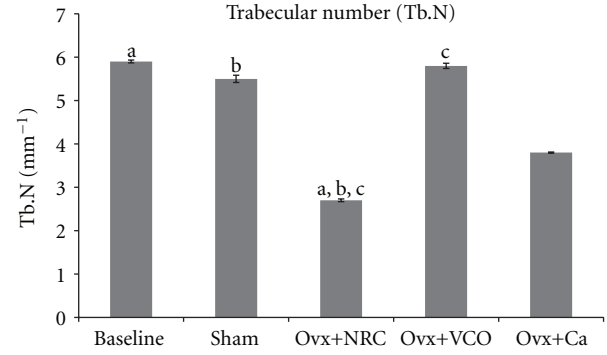

Figure 3: Trabecular number (Tb.N). Same letters indicate significant difference between groups ($P < 0.05$). Data are expressed as mean ± SEM. Sham: sham-operated; Ovx: ovariectomized control rats; Ovx+VCO: ovariectomized rats with virgin coconut oil; Ovx+Ca: ovariectomized rats with calcium.

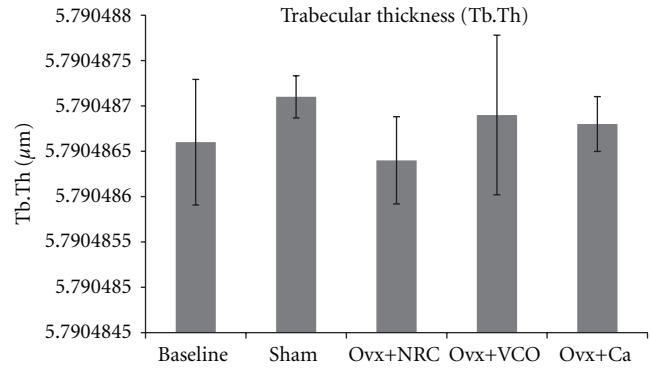

Figure 4: Trabecular thickness (Tb.Th). Same letters indicate significant difference between groups ($P < 0.05$). Data are expressed as mean ± SEM. Sham: sham-operated; Ovx: ovariectomized control rats; Ovx+VCO: ovariectomized rats with virgin coconut oil; Ovx+Ca: ovariectomized rats with calcium.

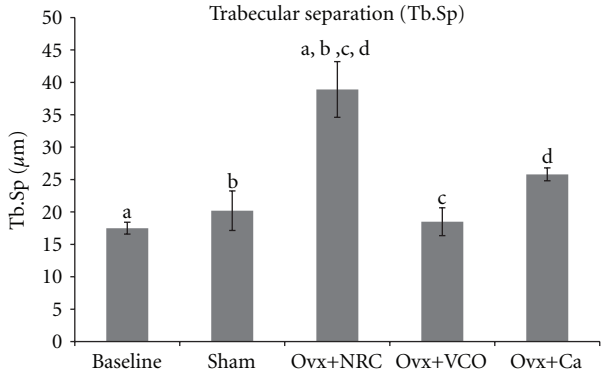

Figure 5: Trabecular separation (Tb. Sp). Same letters indicate significant difference between groups ($P < 0.05$). Data are expressed as mean ± SEM. Sham: sham-operated; Ovx+NRC: ovariectomized control rats; Ovx+VCO: ovariectomized rats with virgin coconut oil; Ovx+Ca: ovariectomized rats with calcium.

4. Discussion

Loss of estrogen contributes to increased body weight in ovariectomized rats (Figure 1). Estrogen deficiency induces hyperphagia in rats [24] as lack of estrogen leads to reduce leptin secretion from the adipose tissues. Leptin, a 14 kDa protein, acts on the hypothalamus to regulate food intake and a low level of it will send signals to the body to increase intake of food [25]. Treatment with VCO and calcium also contributes to weight gain when compared to the sham operated rats. However, the rats in these two groups did not gain as much weight as rat in the Ovx group. Studies involving male rats fed with VCO did not show any significant weight gain compared to control rats [12, 13]. To date, there have not been any studies investigating the effect of VCO on change of weight in estrogen-deficient animals. The effects of calcium on body weight have been so far inconsistent. Our previous study [26] showed that ovariectomized rats receiving calcium in drinking water had a much higher weight gain similar to the Ovx group. The slight increase in rats' body weight in the VCO and calcium groups in the present study could still be due to increased food intake as a result of lower leptin secretion though the impact is less severe compared to Ovx rats.

In the present study we evaluated the effects of six-week virgin coconut oil (VCO) supplementation on bone microarchitecture in ovariectomized rats based on bone histomorphometry. Histomorphometry is an important technique for evaluating the rate of bone turnover and for examining bone quality and architecture [27]. Bone histomorphometry is also used diagnostically in metabolic bone diseases such as hyperparathyroidism, Paget's disease, and osteoporosis [28]. The six-week study period was chosen based on our previous studies which showed that the changes in bone turnover markers were detected as early as the fourth week after exposure to oxidative stress [9] and that histomorphometric changes in ovariectomized rats were already evidenced six weeks postovariectomy [29].

The results of our study revealed that the bone volume and trabecular number in ovariectomized control rats were significantly lower than those in the Baseline, Sham and Ovx+VCO groups. Ovariectomized rats also had higher trabecular separation compared to the other rats (Figures 2, 3, and 5). Consistent with the previous report on changes in lumbar vertebral bodies of ovariectomized rats [30], the trabecular bones in Ovx group were less in number and arranged in a more spaced out fashion with clear evidence of bone loss (Figure 6(c)). In addition, our study did not show any changes in the value of trabecular thickness in Ovx group (Figure 4). This meant that the loss of bone was mainly due to trabecular perforation and loss of trabecular connectivity; not due to trabecular thinning. Trabecular perforation may therefore pose an increased bone fragility [31] associated with an increase in systemic bone turnover markers at the tissue level [30]. Our observation is slightly different from that of Bagi et al. [32] whereby in aged ovariectomized rats, the bone loss was associated with trabecular thinning, reduction in trabecular number and increase in trabecular separation. Our results however were consistent with previous studies performed in mature ovariectomized rat model [33, 34] whereby trabecular perforation is the main mechanism of bone loss at the early stage of oestrogen deficiency [35].

Estrogen is essential for normal skeletal development and maintenance of bone health in both men and women. Estrogen deficiency causes osteoblasts to release interleukin-1 (IL-1) and interleukin-6 (IL-6) which stimulate the differentiation and activation of osteoclasts, leading to increased bone resorption [36–38]. Other than exerting its endocrine function, estrogen has been found to have antioxidant property [39, 40]. As a consequence, lack of estrogen will increase lipid peroxidation and free radical formation [41], which will hinder the functions of bone cells particularly the osteoblasts and the osteoclasts. Oxidative stress damages the osteoblasts and accelerates bone loss by increasing osteoclastogenesis [42]. Bone loss and osteoporosis can be prevented if oxidative stress is minimized. Antioxidants such as vitamin E can protect bone cells from the damaging effect of oxidative stress as shown by *in vivo* studies using estrogen-deficient laboratory animals [8, 41]. Another study using vitamin C revealed that daily intake of this antioxidant vitamin increased the bone mineral density (BMD) of postmenopausal women [43].

Treatment of ovariectomized rats with VCO seems to reverse the effects of estrogen deficiency on the bone structure. The bone volume and trabecular number were significantly higher than the ovariectomized control group (Figures 2 and 3). The six-week treatment of VCO in ovariectomized rats completely restored trabecular bone volume back to the Sham group level. The trabecular bones of the VCO group were more closely connected to each other as the trabecular separation value was lower compared to the ovariectomized control rats. Photomicrograph of the trabecular bone in VCO-treated rats appeared similar to the Sham rats (Figure 5). This indicates that the administration of virgin coconut oil prevented bone loss and maintained bone microarchitecture of estrogen-depleted rats. Virgin coconut oil completely prevented the bone loss by preserving

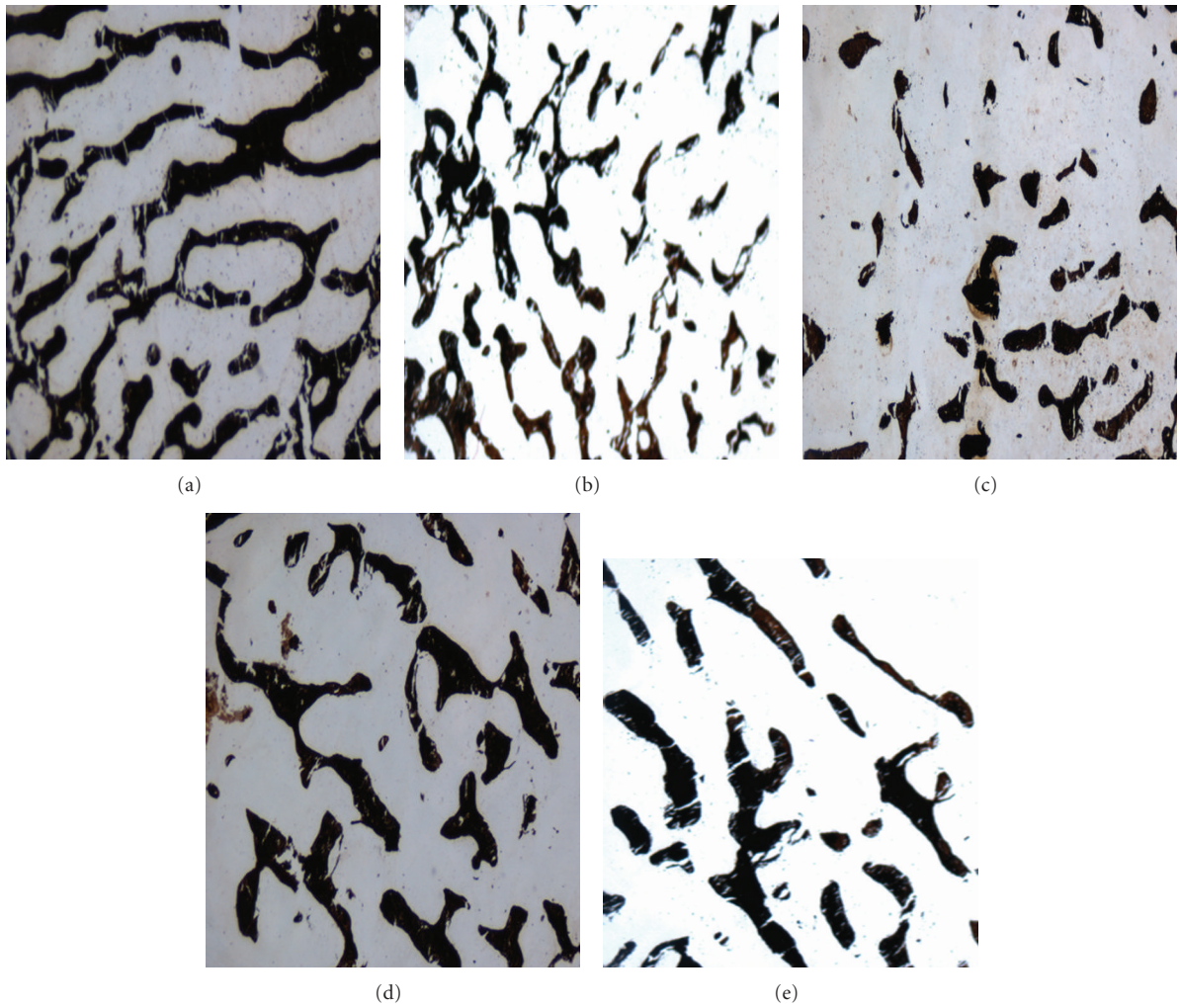

Figure 6: Photomicrographs of undecalcified bone sections stained with von Kossa. (a) Baseline; (b) Sham; (c) ovariectomized control rats (Ovx); (d) ovariectomized rats with virgin coconut oil (Ovx+VCO); (e) ovariectomized rats with calcium (Ovx+Ca). (20X magnification.) The trabecular bones of Ovx+NRC appeared to be sparser compared to the other groups. The bones of Ovx+VCO group appeared thicker and denser than Ovx group and comparable to Sham group.

trabecular bone mass and trabecular network connectivity in the metaphyseal region.

The effects of VCO on bone microarchitecture was much better than treatment with calcium as the latter only prevented the reduction in trabecular separation (Figure 5) but failed to increase the bone volume and trabecular number (Figures 2 and 3). The superior effect of VCO over calcium was probably because VCO contains high amount of saturated fats in the form of medium chain triglycerides (MCTs) [44]. Saturated fats are important for calcium absorption from the intestines [45]. Our findings supported the recommendation that calcium therapy alone is not sufficient for the treatment of osteoporosis [46]. Other researchers also observed that calcium supplementation given to ovariectomized rats still resulted in lower bone volume and trabecular number as compared to sham-operated rats [47]. In yet another study using ovariectomized rats, calcium supplementation was shown to improve fracture healing but failed to improve bone strength [48].

VCO and coconut oil obtained from copra (dried kernel meat of coconut) contain the same composition of triglycerides with more than 90% being short- and medium-chain saturated fatty acids while the remaining are the unsaturated fats [44, 49]. However, VCO is different from coconut oil as the former contains a lot more biologically active components like vitamins and polyphenols [12]. Marina et al. showed that VCO has higher antioxidant capacity than copra oil and alpha-tocopherol [50]. This corresponds to the higher amount of polyphenols in VCO. Phenolic compounds are found to be associated with many health benefits [51, 52]. They exhibit a wide range of physiological properties such as antiallergenic, antiaftherogenic, anti-inflammatory, antimicrobial, antithrombotic, cardioprotective, and vasodilatory effects [53–57].

To the best of our knowledge, we are the first to report the ability of VCO in preventing osteopenia in postmenopausal osteoporosis rat model. The beneficial effects of virgin coconut oil on bone microarchitecture may be due to its antioxidant property. We propose that the antioxidant components in VCO prevented the free-radical-induced bone loss associated with estrogen deficiency. The preventive effects of VCO could be explained by the various bioactivities of the many phytochemicals found in the oil, particularly the polyphenols. Further studies are required to determine the exact mechanism on how VCO and polyphenols provide beneficial effects on bone health. It could be due to its direct antioxidant activity or combination with other mechanisms involving osteoclastogenesis and modulation of osteoblast function. Transcription factors such as NF-κB and activator protein-1 (AP-1) as well as cellular signalling pathways such as mitogen-activated protein kinase (MAPK), bone morphogenetic protein (BMP), oestrogen receptor and osteoprotegerin/receptor activator of NF-κB ligand (OPG/RANKL) may be implicated [58]. The effects of VCO on bone formation rate, other dynamic and cellular parameters as well as on bone turnover markers, would be helpful to provide insights to the mechanisms involved. Information on how VCO affect bone strength, bone density and mineral composition should also be sought after.

The effects of VCO in preventing bone loss were similar to those seen in other experimental osteoporosis studies using other plants which are rich in polyphenols. Phloridzin, an apple polyphenol, green tea polyphenol, and oleuropein, a polyphenol found in olive oil, are few examples of plant polyphenols which exert bone sparing activity ranging from increasing bone mineral density, increasing osteoblast function, improving bone mechanical strength to reducing bone resorption markers [59–61].

Due to the many benefits of VCO, researchers are yet to determine accurately how much coconut oil is needed daily to gain optimal health. The dose that we chose to use in this study is equivalent to the human dose (3.5 tablespoons daily of VCO for a 72 kg man) as recommended by Isaacs and Thormar [62]. This was derived from the amount of medium chain fatty acid (MCFAs) present in human breast milk which is the perfect formula that gives infants protective shield from infections and other illnesses. The 60% composition of MCFAs in virgin coconut oil is almost similar to that in human breast milk [62].

To summarise our findings, virgin coconut oil effectively improved bone structure and prevented bone loss in osteoporosis animal model. The beneficial effects of VCO on bone microarchitecture may be due to the high polyphenols which exert antioxidant property. Virgin coconut oil could offer an interesting approach to prevent accelerated bone loss in osteoporosis especially in postmenopausal women.

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Research Article

Mechanism by *Sambucus nigra* Extract Improves Bone Mineral Density in Experimental Diabetes

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The effects of polyphenols extracted from *Sambucus nigra* fruit were studied in streptozotocin- (STZ-) induced hyperglycemic rats to evaluate its possible antioxidant, anti-inflammatory, antiglycosylation activity, and antiosteoporosis effects in diabetes. DEXA bone mineral density tests were performed in order to determine bone mineral density (BMD), bone mineral content (BMC), and fat (%Fat) in control and diabetic animals, before and after polyphenol delivery. As compared to the normoglycemic group, the rats treated with STZ (60 mg/kg body weight) revealed a significant malondialdehyde (MDA) increase, as an index of the lipid peroxidation level, by 69%, while the total antioxidant activity (TAS) dropped by 36%, with a consistently significant decrease ($P < 0.05$) in the activity of superoxide dismutase (SOD) and glutathione peroxidase (GPX). Also, the treatment of rats with STZ revealed a significant increase of IL-6, glycosylated haemoglobin (HbA_{1c}), and osteopenia detected by DEXA bone mineral density tests. The recorded results highlight a significant improvement ($P < 0.001$) in the antioxidative capacity of the serum in diabetic rats treated with natural polyphenols, bringing back to normal the concentration of reduced glutathione (GSH), as well as an important decrease in the serum concentration of MDA, with improved osteoporosis status. Knowing the effects of polyphenols could lead to the use of the polyphenolic extract of *Sambucus nigra* as a dietary supplement in diabetic osteoporosis.

1. Introduction

Observational studies and animal models suggest that decreased bone strength in diabetes may contribute to fracture risk but this remains a controversial issue [1]. Diabetes can impact bone through multiple pathways including changes in insulin levels, higher concentrations of advanced glycation end products in collagen, hypercalciuria associated with glycosuria, oxidative stress, lower insulin-like growth factor-I, and inflammation [2]. Epidemiologic studies suggest a protective role of dietary flavonoids against coronary heart disease [3]. Duration of diabetes seems to play a key role given the lower bone mineral density found among patients who have had diabetes for over 5 years [4]. In our study, a 12-week hyperglycemia in rats is equivalent to a 6-7-year diabetes mellitus (DM) in humans. A better understanding of these mechanisms may help implement fracture prevention measures in the growing population of adult diabetics.

It is known that natural polyphenols possess physical and chemical properties that contribute to the proper and efficient protection against oxidation of important biomolecules such as lipids, proteins, and nucleic acids [5]. Flavonoids are a major class of active antioxidant principles.

The polyphenolic extract from the isolated and purified vegetable material, which is the mature *Sambucus nigra* (*Caprifoliaceae*, elderberry) fruit, can constitute an important source of antioxidants. DEXA bone mineral density tests were performed in order to determine bone mineral density (BMD), bone mineral content (BMC), and fat (%Fat) in control and diabetic animals, before and after polyphenol delivery. Our goal is to develop a new alternative treatment

that includes a dietary supplement designed to reduce bone loss in diabetic patients.

2. Material and Methods

2.1. Preparation of Extract and Chemical Determinations. Elder fruits (*Sambucus nigra* L.) were washed and shade dried. Dried and powdered fruits (50 g) were extracted with 2×250 mL acidulated methanol (0.5% HCl) using a magnetic stirrer, each time for one hour. The amount of vegetable extract obtained from *Sambucus nigra* fruit was 42.31 g/100 g dry vegetable material.

The total phenolic content in elder fruit extract was determined by the Singleton and Rossi method. The amount of total phenolic content was expressed as gallic acid equivalents (GAE)/100 g extract. The absorbencies of all the solutions were determined by using a V-550 Able Jasco UV-VIS spectrophotometer. The result is the mean of triplicates ± standard deviation. The content in total polyphenols from the *Sambucus nigra* extract was $7,63 \pm 0,12$ mg GAE/100 g extract.

Recent studies have revealed a total phenolic content of 97.26 ± 17.04 mg GAE/100 g in dried Coriander seeds (*Coriandrum sativum*), of 63.51 ± 3.67 mg GAE/100 g in garlic (*Allium sativum*), and of 16.20 ± 0.96 mg GAE/100 g in potatoes (*Solanum tuberosum*) [6].

The dry polyphenol extract was diluted in DMSO, 100 mL polyphenolic solution containing 840 mg natural polyphenols, 95 mL distilled water, and 5 mL DMSO. After repeated testing, it was found that the dose of polyphenols extracted from the fruits of *Sambucus nigra* to be administered as enteral solution (by tube feeding) is 0.040 g/Kg body every two days. The experiment used active therapeutic doses, well-determined fractions of DL50 on an experimental model of diabetes mellitus.

2.2. Experimental Model. The research was carried out on Wistar white male rats, aged 28 weeks at the beginning of the study, weighing 250–280 g on the average, which were divided into four 12-rat groups, namely, W Group: control, normal animals, that were not given natural polyphenols; DM Group: rats with diabetes caused by the rapid intraperitoneal injection of a single dose of streptozotocin (STZ); PGroup: rats who were administered polyphenolic extract for a period of 16 weeks; DM + P Group: rats who were administered a polyphenolic extract for 3 weeks before and 13 weeks after diabetes mellitus induction.

Diabetes was induced by STZ [2-deoxi-2(3-metil-nitrozo-ureido)-p-glucopiranoza] (cytotoxical antibiotic synthesized of *Streptomyces achromonogenes*) obtained from SIGMA S-0130, Batch 31K1379, and delivered in a single dose of 60 mg/Kg body mass, as 1% intraperitoneal (i.p.) solution, after 18 hours of fasting. Streptozotocin delivery causes insulin-deficiency diabetes through selective endocrino-chemical pancreatectomy, destroying only the cells of Langerhans' islets [7]. The onset of permanent diabetic hyperglycemia occurs after 24–48 hours from the diabetogenic injection and it may either be definitive or regress spontaneously after a few days, depending on the animal's diet and specific characteristics.

The animals were kept in normal microclimate conditions. The clinical state of the animals was observed daily, their water and food ingestion, diuresis, glycosuria, and the possible presence of ketone bodies. The animals were fed using a daily intake calculated according to the standard norms for the species. The diet consisted of carbohydrates 59.12%, raw proteins 21.10%, raw lipids 5.08%, raw fibers 4%, minerals 5.14%, humidity of 7.98%.

The experimental study fulfils all the requirements of the guide regarding the use of laboratory animals and biological preparations issued by the International Society of Pain Study (IASP) and the European Council Committee (86/609/EEC). This study was approved by the Laboratory Animal Care Committee of "Gr. T. Popa" University of Medicine and Pharmacy and the rats were kept in accordance with the general guidelines for the care and use of laboratory animals recommended by the Council of European Communities. At the end of the experiment, the 44-week-old animals were killed by cardiac puncture under ketamine anaesthesia (100 mg/kg body weight). Blood samples were collected using sodium citrate as anticoagulant buffer, at a 9:1 blood/citrate ratio, or without anticoagulant. Aliquots of plasma and serum were frozen and kept at -80°C for later analysis.

2.3. Biochemical Methodology. Fasting blood glucose was measured by routine autoanalyzer methods (Synchron CX 7, Beckman), using dedicated kits. TAS was measured in heparinized plasma samples by the method of Miller et al., using the Trolox equivalent antioxidant capacity (TEAC) assay (Randox Laboratories, San Francisco, CA). Both water- and lipid-soluble antioxidants contained in the biological sample under investigation inhibit the 2,2′-azinobis (3-ethylbenzothiazoline 6-sulfonate) radical cation production. Radical cation production was measured by spectrophotometry.

The overall blood glutathione peroxidase (GPX) activity was determined using the method of Paglia and Valentine. Kits for TAS and GPX activity analysis were purchased from Randox (United Kingdom). Blood superoxide-dismutase (SOD) was determined by an adapted method from Minami.

Malondialdehyde (MDA) was determined by measuring thiobarbituric reactive species using the method of Ruiz-Larrea et al. [8] in which the thiobarbituric acid reactive substances react with thiobarbituric acid to produce a red-colored complex having peak absorbance at 532 nm.

Bio-Rad's Micromat II Hemoglobin Instrument was used for HbA1c testing, as it provides results in less than 5 minutes. Based on borate affinity chromatography, there is no interference from abnormal haemoglobins, which makes it very reliable.

IL-6 serum levels were measured by enzyme-linked immunosorbent assay.

The *DEXA* bone mineral density test was performed in an Endocrinology Clinic on HOLOGIC 100 equipment, using special software for small animals, Dynamic evolution of

Table 1: The glycaemia evolution at the studied groups.

	Average glycaemia (mg/dL) ± SD		
Group	Week 2	Week 10	Week 16
W	73.20 ± 4.30	72.80 ± 4.10	70.80 ± 3.80
DM	376.55 ±116.82	551.55 ± 166.66	658.44 ± 199.26
DM + P	329.66 ± 112.07	501.66 ± 172.10	529.44 ± 177.74
P	73.40 ± 4.40	71.80 ± 3.90	70.60 ± 3.70

bone mineral density was analyzed, namely the fat percentage in the entire body.

2.4. Statistical Data Interpretation. Data were expressed as means ± standard deviation (SD). Statistical significance was determined by variance analysis and one-way ANOVA followed by a post hoc Tukey's test using Statistical Software Package SPSS, version 13 (SPSS Incorporation, Chicago, IL, USA). Unpaired Student's t-tests were performed to determine whether there were significant ($P < 0.05$) differences between groups. The statistical interpretation of the data considered the corresponding differences for a significance threshold: $P > 0.05$ statistically insignificant; $P < 0.01$ strong statistical significance; $P < 0.001$ very strong statistical significance.

3. Results

Table 1 shows the dynamic evolution of glycaemia in the diabetic rats under polyphenolic protection as compared to the unprotected diabetic rats.

10 weeks after the beginning of the experiment, the hyperglycemia of the DM + P group compared with the DM group was insignificantly low ($P > 0.05$). Thus, polyphenol delivery did not provide protection against disease onset, yet it reduced glycaemia evolution insignificantly. Our study revealed significant hyperglycemia reduction in the DM+P group compared with the DM group, 16 weeks after the beginning of the experiment ($P < 0.01$).

Serum TAS, GPX, and SOD activities were significantly decreased in the DM group. There is evidence to suggest that oxidative damage is increased in diabetes. Antioxidant activity was improved by polyphenol addition in comparison with the unsupplemented groups. Also, the MDA values revealed the antioxidant effect of polyphenols on diabetic animals (Table 2). There were associations between TAS and HbA1c ($r = -0.43$; $P = 0.0026$).

Bone mineral density tests were performed on laboratory animals at the beginning and at the end of the experiment, respectively. Three parameters were analyzed: BMD (bone mineral density expressed in g/cm^2); BMC (bone mineral content expressed in g), and Body fat (%Fat).

The BMD evolution compared with the area measured in control males throughout the experiment was not statistically significant in any of the bone mineral density tests: overall $\chi^2 = 0.03$; GL = 1; $P = 0.85$; spine $\chi^2 = 0.30$; GL = 1; $P = 0.58$; cervical spine $\chi^2 = 0.11$; GL = 1; $P = 0.74$; femur $\chi^2 = 0.80$; GL = 1; $P = 0.37$. As for the BMD evolution compared with the area measured in diabetic males throughout the experiment, significant statistical differences were noticed: overall $\chi^2 = 23.42$; GL = 1; $P < 0.001$; spine $\chi^2 = 11.26$; GL = 1; $P = 0.0008$; cervical spine $\chi^2 = 9.42$; GL = 1; $P = 0.002$; femur $\chi^2 = 27.85$; GL = 1; $P < 0.001$; the highest being at the femur level. In diabetic males receiving polyphenol treatment, studies showed significant statistical differences in the evolution of their BMD throughout the experiment: overall $\chi^2 = 34.60$; GL = 1; $P < 0.001$; spine $\chi^2 = 56.09$; GL = 1; $P < 0.001$; cervical spine $\chi^2 = 45.78$; GL = 1; $P < 0.001$; femur $\chi^2 = 29.07$; GL = 1; $P < 0.001$.

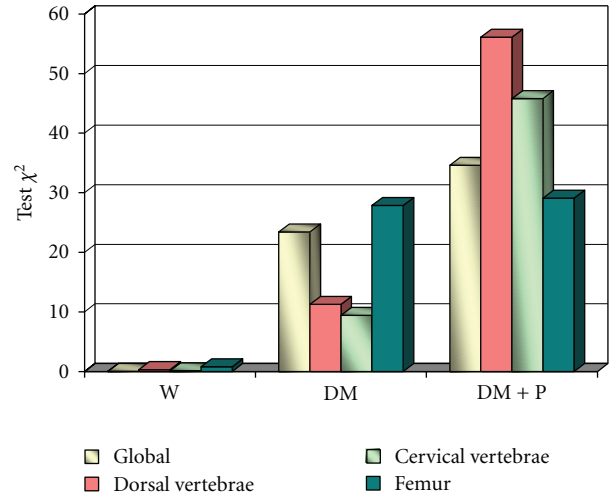

Figure 1: Values of the χ^2 BMD test for each group.

Depending on the examined area, the BMC also showed strong statistical differences throughout the surveyed period and in all the bone mineral density tests performed; the highest increase was recorded in the cervical spine, while both femur and overall BMCs were lower at the end than at the beginning of the experiment: overall $\chi^2 = 134.81$; GL = 1; $P < 0.001$; spine $\chi^2 = 809.02$; GL = 1; $P < 0.001$; cervical $\chi^2 = 1679.20$; GL = 1; $P < 0.001$; femur $\chi^2 = 52.45$; GL = 1; $P < 0.001$.

The comparison between BMC and BMD in diabetic males undergoing polyphenol treatment revealed significant statistical differences both globally and in all the analyzed samples (Figure 1).

The body fat of control males had a slight increase (0.4–1.8%) at the end of the experiment, in all analyzed samples. A 7–11% body fat decrease was noticed in all analyzed samples of diabetic males from the beginning to the end of the experiment. The body fat percentage had the most dramatic decrease in the diabetic male group (Figure 2).

4. Discussion

The connection between DM and bone damage associated with mineral metabolism dysfunction is a common phenomenon found in both humans and rats. Insulin is a potential bone growth regulator, since osteoblasts have insulin receptors [9] as well as IGF1 receptors, which can also

Table 2: TAS, blood GPX activity, SOD activity, and IL-6 values in the studied group.

Experimental groups	W	P	DM	DM + P
TAS (mmoli/L)	1.2148 ± 0.0122	1.3313 ± 0.0339*	1.0464 ± 0.0526***	1.308 ± 0.0224$^{\#\#}$
GPX (nmol/mL)	7.60 ± 0.20	7.84 ± 0.46*	5.16 ± 0.54***	6.84 ± 0.62$^{\#\#}$
SOD (U/mL)	3.60 ± 0.38	4.92 ± 0.60***	2.86 ± 0.24*	3.28 ± 0.18$^{\#}$
MDA (μmoli/mL)	0.068 ± 0.002	0.064 ± 0.004*	1.213 ± 0.072***	0.847 ± 0.36$^{\#\#}$
IL-6 (ng/mL)	8.35 ± 0.323	8.52 ± 0.32*	27.19 ± 3.482***	13.90 ± 3.013$^{\#\#\#}$

Results are mean ± SEM ($n = 12$).
$^{*}P < 0.05$, $^{**}P < 0.01$ $^{***}P < 0.001$ versus W group.
$^{\#}P < 0.05$, $^{\#\#}P < 0.01$, $^{\#\#\#}P < 0.001$ versus DM group.

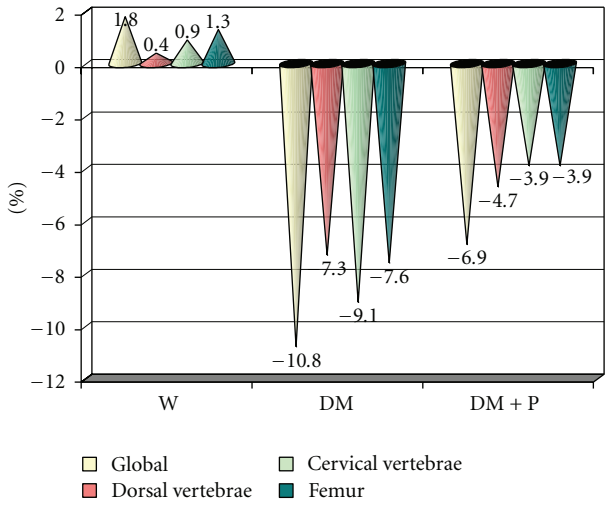

Figure 2: Body fat percentage differences between groups.

mediate the effects of insulin. *In vitro* insulin directly stimulates osteoblast proliferation [10] and when administered locally, over the calvariae of adult male mice, it produces two- to three-fold increases in the histomorphometric indices of bone formation [11]. It is also known that insulin promotes amino acid cell intake in cultured bone. Collagen synthesis is increased too, due to a possible direct effect of insulin on the osteoblasts. Insulin also regulates the activity of glycogen synthase in cultured osteoblast-like cells [12].

Some researchers have reported that a longer history of type 1 diabetes is correlated with decreasing bone mass [13, 14]. The mechanisms that lead to a decreased bone biomechanical competence, besides the decreases in BMD, are collagen glycosylation alterations generated by hyperglycaemia, in the same way as increased haemoglobin glycosylation, which is expressed as HbA1C. These advanced glycation end products (AGE) and their receptors (RAGE) play an important role in bone metabolism and bone strength [2].

Our study results are in agreement with the results of other experimental *in vivo* studies conducted on diabetic rats that were treated with vegetal polyphenols found in red wine or green tea [15, 16]. The increased levels of reactive oxygen species (ROS) and decreased antioxidant defense can cause DNA damage and direct protein inhibition. The main substrates targeted by free oxygen radical activity are polyunsaturated fatty acids in membrane phospholipids, the modification of which results in cell framework and function disorganization. The end product of these reactions is MDA. SOD is the first line of defense against ROS and is active in catalyzing superoxide radical detoxification [17]. SOD is the most abundant antioxidant enzyme in animals. A study assessing the systemic oxidant-antioxidant status throughout the evolution of the disease was carried out. SOD decomposes superoxide anion into hydrogen peroxide and oxygen at almost the highest possible reaction rate. The superoxide radical is involved in various physiological and pathophysiological processes. It is produced in respiratory and cytochome P450 electron transport chain reactions as a by-product. A high amount of this product is also generated by activated neutrophils and macrophages during oxidative burst.

Numerous experimental studies have proven the ability of vegetal polyphenols to diminish lipid peroxidation and to reduce LDL oxidation, probably through the uptake of lipid-peroxil radicals and through lipooxygenases activity reduction, thus delaying atheroma plate formation [18, 19]. Polyphenols are able to penetrate tissues, particularly those in which they are metabolized, but their ability to accumulate in specific target tissues needs to be further investigated. Despite the increasing amount of available data, final conclusions on the bioavailability of most polyphenols are difficult to draw and further studies are necessary [20, 21].

In drug-induced diabetes mellitus with polyphenol protection, the antioxidant quality of the serum increases, the lipid profile is improved and hyperglycemia is significantly ameliorated [22–24]. Two mechanisms were suggested by means of which vegetal polyphenols act as antioxidants within a biological system under oxidative stress conditions [25, 26]: a mechanism according to which polyphenols act as reducing agents or electron donors, converting lipid peroxides in lipid hydroxides or phospholipid peroxides in phospholipid hydroxides, inhibiting lipid peroxidation; another mechanism through which vegetal polyphenols may chelatise transitional metallic ions.

The polyphenolic extract obtained from the *Sambucus nigra* fruit improves the lipid profile significantly ($P < 0.001$) and reduces the atherogenic risk significantly ($P < 0.001$). Hyperglycemia is significantly improved ($P < 0.01$). Aqueous elder (*Sambucus nigra*) extracts have been shown to have an

insulin-like effect on *in vitro* glucose uptake [27]. Whereas hyperglycemia can result in the generation of free radicals through several biochemical pathways (nonenzymatic glycation, the polyol pathway, and glucose autoxidation), the mechanisms underlying oxidative damage in diabetes are still unclear. Free radicals can result in antioxidant defense consumption and enhanced susceptibility to lipid peroxidation. The study showed reduced TAS in type 1 diabetic against nondiabetic control subjects. These results indicate that the alterations in the glucose utilizing system and oxidation status in rats increased by streptozotocin were partially reversed by polyphenolic extract delivery.

The TAS reduction in type 1 diabetic rats was associated with increasing HbA_{1c} and diabetes duration. Thus, increased serum levels of the total antioxidant status and HbA_{1c} were associated with osteoporotic risk reduction in diabetic rats.

In diabetic males, the evolution of bone mineral density compared with the area, measured throughout the experiment, suffered a significant decrease, especially in the femur. In diabetic male rats that were given polyphenolic extract, these differences were statistically minimum.

The highest BMD difference compared with the area was recorded in the spine, followed by the cervical spine. As for the comparison between the BMD of the two groups of males, the most statistically significant difference compared with the area was noticed in diabetic males undergoing polyphenol treatment.

The BMC in the groups of males had the most statistically significant difference compared with the area in the femur of diabetic males, followed by the cervical spine of males undergoing polyphenol treatment. As for the spine, the highest BMC difference compared with the area was found in diabetic males with polyphenol intake, whereas the highest overall significant difference was noticed in diabetic males.

Except for the overall comparison, comparisons between the BMC and BMD of diabetic males revealed significant statistical differences in the spine, lumbar, and femur areas. In diabetic males that were given polyphenol treatment, a 4–7% decrease of their body fat was noticed in all analyzed sampled, at the end of the experiment.

The study revealed reduced TAS in type 1 diabetic against nondiabetic control subjects. The understanding of bone biology may open the way for new osteoporosis treatments. The positive skeletal effects of polyphenols at dietary achievable levels are interleukin-6 mediated [28–30].

The experiment shows the benefits of natural polyphenols extracted from the Elder (*Sambucus nigra*) fruit on osteoporosis regression in diabetic male rats. Extremely low bone mineral density in diabetic rats is improved by polyphenol delivery. Significantly low body fat percentages in diabetic rats are also improved by polyphenol intake. By carefully extending these results to humans, one may conclude that a dietary intake rich in natural polyphenols would lead to the regression of diabetes complications in general and osteoporosis in particular, in diabetic human patients. Osteoporosis regression due to the *Sambucus nigra* extract proves the benefits of polyphenols used to treat chronic diabetes mellitus complications.

Conflict of Interests

No competing financial interests exist.

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Research Article

Fisetin Inhibits Osteoclast Differentiation via Downregulation of p38 and c-Fos-NFATc1 Signaling Pathways

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The prevention or therapeutic treatment of loss of bone mass is an important means of improving the quality of life for patients with disorders related to osteoclast-mediated bone loss. Fisetin, a flavonoid dietary ingredient found in the smoke tree (*Continus coggygria*), exhibits various biological activities, but its effect on osteoclast differentiation is unknown. In this study, fisetin dose-dependently inhibited the RANKL-induced osteoclast differentiation with downregulation of the activity or expression of p38, c-Fos, and NFATc1 signaling molecules. The p38/c-Fos/NFATc1-regulated expression of genes required for cell fusion and bone resorption, such as DC-STAMP and cathepsin K, was also inhibited by fisetin. Considering the rescue of fisetin's inhibitory action by NFATc1 over-expression, the cascade of p38-c-Fos-NFATc1 could be strongly involved in the inhibitory effect of fisetin on osteoclast differentiation. Furthermore, fisetin inhibited the bone-resorbing activity of mature osteoclasts. In conclusion, fisetin may be of use in the treatment of osteoclast-related disorders, including osteoporosis.

1. Introduction

The prevalence of osteoporosis is expected to increase as the population of elderly people increases. Osteoporosis is a common, systemic, degenerative skeletal disorder that is characterized by low bone mass (or bone mineral density) that can lead to an increased risk of fracture. Bone fractures result in serious problems including skeletal deformity, pain, increased mortality, and severe economic burden [1]; thus, the prevention or treatment of loss of bone mass and lifetime fracture is an important means of improving the quality of life of patients with disorders related to bone loss.

Bone homeostasis is maintained by the balance between osteoclast-mediated bone resorption and osteoblast-mediated bone formation. Imbalances, caused most often by over-activated osteoclasts, can lead to loss of bone mass; this suggests that inhibiting osteoclast differentiation, resorptive activity, or both could be a promising strategy for treating patients with disorders such as osteoporosis. Current treatments for osteoporosis inhibit bone degradation through the use of bisphosphonates, but they are associated with unpleasant gastric side effects and a complicated dosing schedule. New antiresorptive agents should be both effective and safe for the long-term management of bone resorption-related disorders.

Osteoclasts are bone-resorbing multinucleated cells derived from hematopoietic cells. Specifically, precursor cells of the monocyte macrophage lineage are differentiated into tartrate-resistant acid phosphatase (TRAP)-positive multinucleated osteoclasts in response to macrophage-colony stimulating factor (M-CSF) and receptor activator of nuclear factor-κB ligand (RANKL). Especially, after RANKL binds to RANK, it stimulates the trimerization of its receptor, resulting in the activation of downstream signaling molecules such as MAP kinases [2]. RANKL-induced activation of MAP kinases further leads to the activation of transcription

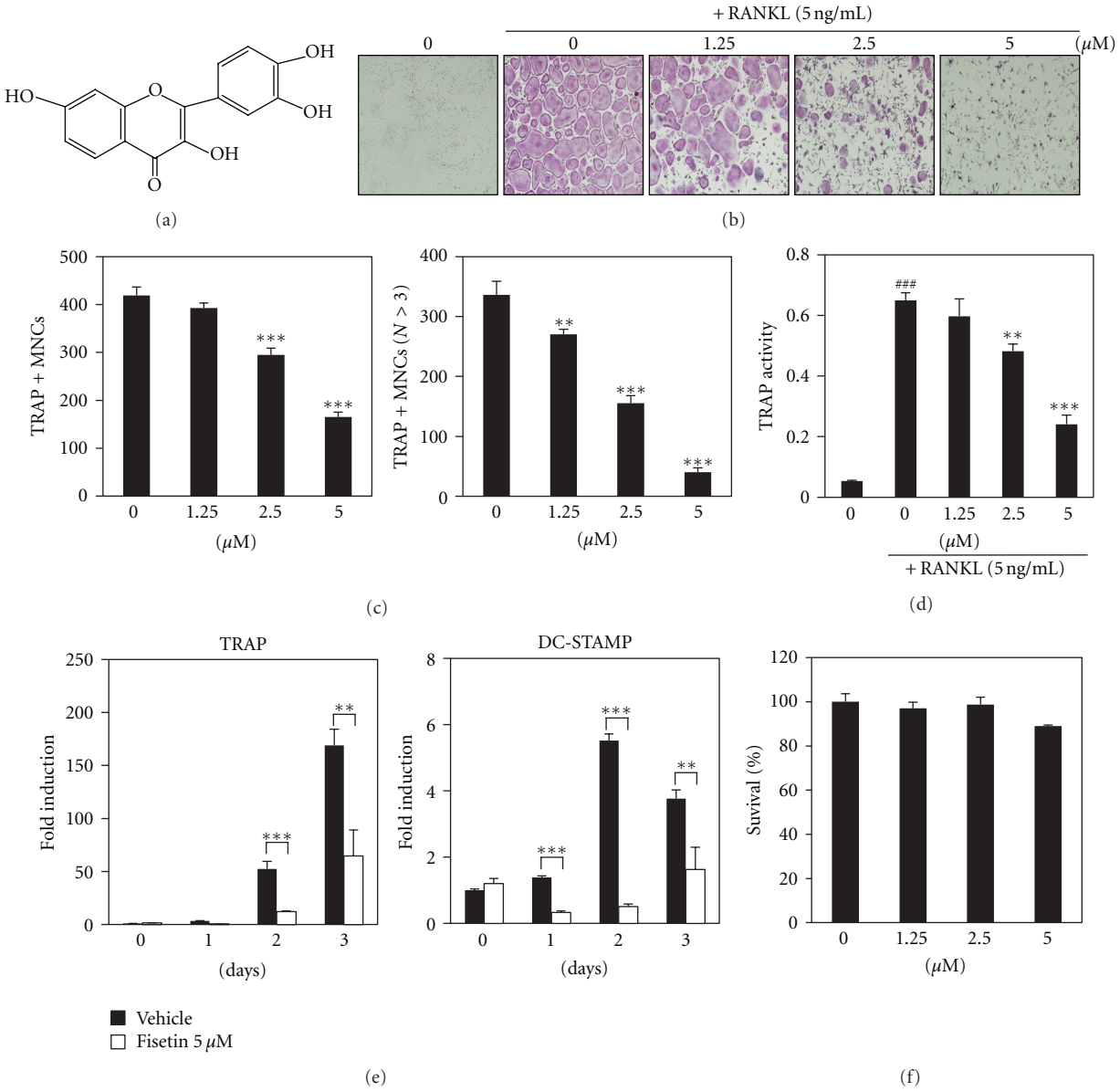

Figure 1: Fisetin inhibits RANKL-mediated osteoclast differentiation. (a) Structure of fisetin. (b) BMM cells were cultured for 4 days in the presence of RANKL (5 ng/mL) and M-CSF (30 ng/mL) with the vehicle (DMSO) or fisetin. Multinucleated osteoclasts were visualized to red-colored giant cells by TRAP staining. (c) Total TRAP-positive multinucleated osteoclasts (TRAP + MNCs; left graph) and TRAP + MNCs with more than 3 nuclei ($N > 3$; right graph) were counted. $^{**}P < 0.01$; $^{***}P < 0.001$ (versus "the control"). (d) TRAP activity was measured. $^{\#\#\#}P < 0.001$ (versus "the negative control"). $^{**}P < 0.01$; $^{***}P < 0.001$ (versus "the positive control"). (e) After pretreatment with the vehicle (DMSO) or fisetin (5 μM) for 1 h, BMMs were treated with RANKL (5 ng/mL) for the indicated number of days, and then mRNA expression levels were analyzed by real-time PCR. $^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$ (versus "the vehicle control"). (f) Effect of fisetin on the viability of BMMs was evaluated by CCK-8 assay.

factors. RANKL strongly triggers the activation of two major transcription factors required for osteoclast differentiation, c-Fos and nuclear factor of activated T cells c1 (NFATc1), and these transcription factors play a critical role in the regulation of genes for osteoclast differentiation [3].

Natural products have historically yielded a variety of therapeutic agents. There have been many efforts to find natural products, including flavonoids that can prevent and treat osteoporosis while minimizing adverse side effects [4, 5]. Due to the abundance of flavonoids in dietary products and their beneficial pharmacological properties, flavonoids are of considerable interest as therapeutic agents as well as health food supplements. Fisetin (3,7,3′,4′-tetrahydroxyflavone; Figure 1(a)) is a flavonoid dietary ingredient found in the smoke tree (*Continus coggygria*); it is also widely distributed in fruits and vegetables, such as strawberries, apples, persimmons, grapes, onions, and cucumbers. A number of studies have investigated whether

Table 1: Primer sequences used in this study.

Target gene	Forward primer (5′–3′)	Reverse primer (5′–3′)
TRAP	GATGACTTTGCCAGTCAGCA	ACATAGCCCACACCGTTCTC
DC-STAMP	CCAAGGAGTCGTCCATGATT	GGCTGCTTTGATCGTTTCTC
c-Fos	CCAGTCAAGAGCATCAGCAA	AAGTAGTGCAGCCCGGAGTA
NFATc1	GGGTCAGTGTGACCGAAGAT	GGAAGTCAGAAGTGGGTGGA
Cathepsin K	GGCCAACTCAAGAAGAAAAC	GTGCTTGCTTCCCTTCTGG
GAPDH	AACTTTGGCATTGTGGAAGG	ACACATTGGGGGTAGGAACA

fisetin has various biological properties, such as antioxidant, antiangiogenic, antiinflammatory, antiaging, antiproliferative, antiapoptotic and antitumor activities [6–8]. To date, the effect of fisetin on osteoclast differentiation has not been studied, but its biological properties such as antiinflammatory activity might affect the osteoclast differentiation [9, 10]. Therefore, in this study, we investigated the effect of fisetin on osteoclast differentiation and possible molecular mechanisms of its action.

2. Materials and Methods

2.1. Reagents and Antibodies. Penicillin, streptomycin, cell culture medium, and fetal bovine serum (FBS) were purchased from Invitrogen Life Technologies. Mouse soluble M-CSF and RANKL were purchased from R&D Systems. The CCK-8 assay kit was purchased from Dojindo Molecular Technologies. Antibodies against NFATc1, c-Fos, and actin were purchased from Santa Cruz Biotechnology. Antibodies against MAP kinases were purchased from Cell Signaling Technology. Fisetin was purchased from Sigma-Aldrich and dissolved in DMSO (Sigma-Aldrich).

2.2. Preparation of Osteoclast Precursor Cells. Bone marrow cells were obtained from 5-week-old male ICR mice by flushing femurs and tibias with α-MEM supplemented with antibiotics (100 units/mL penicillin and 100 μg/mL streptomycin). Bone marrow cells were cultured in α-MEM supplemented with 10% FBS and M-CSF (10 ng/mL) for 1 day on culture dishes. Nonadherent bone marrow cells were plated on petri dishes and cultured for 3 days in the presence of M-CSF (30 ng/mL). After nonadherent cells were washed out, adherent cells were used as bone-marrow-derived macrophages (BMMs).

2.3. Osteoclast Cell Culture and Osteoclast Differentiation. BMMs were maintained in α-MEM supplemented with 10% FBS, 100 units/mL penicillin, and 100 μg/mL streptomycin. The medium was changed every 3 days in a humidified atmosphere of 5% CO_2 at 37°C. To differentiate osteoclasts from BMMs, the BMMs (1×10^4 cells/well in a 96-well plate) were cultured for 3-4 days with M-CSF (30 ng/mL) and RANKL (5 ng/mL). After 3-4 days, multinucleated osteoclasts were observed.

2.4. Cell Viability Assay. BMMs were plated in 96-well plates at a density of 1×10^4 cells/well in triplicate. After treatment with M-CSF (30 ng/mL) and fisetin, cells were incubated for 3 days. Afterwards, cell viability was measured with the CCK-8 kit according to the manufacturer's protocol.

2.5. Western Blot Analysis. Cultured cells were washed with ice-cold phosphate-buffered saline (PBS) and lysed in lysis buffer (50 mM Tris-HCl, 150 mM NaCl, 5 mM ethylenediaminetetraacetic acid (EDTA), 1% Triton X-100, 1 mM sodium fluoride, 1 mM sodium vanadate, and 1% deoxycholate) supplemented with protease inhibitors. Lysates were boiled in sodium dodecyl sulfate (SDS) sample buffer for 5 min and subjected to 10% or 12% SDS-polyacrylamide gel electrophoresis (PAGE) gels. After proteins were separated into gels, they transferred to a polyvinylidene difluoride membrane (Millipore). The membrane was then washed with TBST (10 mM Tris-HCl pH 7.5, 150 mM NaCl, and 0.1% Tween 20) and incubated in the blocking solution, TBST with 5% skim milk. The membrane was probed with the indicated primary antibody, washed three times for 30 min, incubated with secondary antibody conjugated to horseradish peroxidase for 2 h, and washed three times for 30 min. The membranes were then developed with Super-Signal West Femto Maximum Sensitivity Substrate (Pierce) using the LAS-3000 luminescent image analyzer (Fuji Photo Film Co., Ltd.).

2.6. Real-Time PCR. Primers were chosen with the online Primer3 design program [11]. The primer sets used in this study are shown in Table 1. Total RNA was isolated with TRIzol reagent according to the manufacturer's protocol. First-strand cDNA was synthesized with 1 μg of total RNA, 1 μM oligo-dT_{18} primer, and 10 units of RNase inhibitor RNasin (Promega, WI) with the Omniscript RT kit (Qiagen) according to the manufacturer's protocol. SYBR green-based QPCR was performed with the Stratagene Mx3000P Real-Time PCR system and Brilliant SYBR Green Master Mix (Stratagene, CA), with the first-strand cDNA diluted 1 : 10 and 10 pmol of primers according to the manufacturer's protocol. The polymerase was activated at 95°C for 10 minutes, followed by 40 cycles of 94°C for 40 s (denaturation), 53°C for 40 s (annealing), and 72°C for 1 min (extension). This was followed by the generation of PCR-product-temperature-dissociation curves (also called melting curves) at 95°C for 1 min, 55°C for 30 s, and 95°C for 30 s. All reactions were run in triplicate, and data were analyzed by the $2^{-\Delta\Delta C_T}$ method [12]. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as an internal standard

gene. The statistical significance was determined by Student's t-test with GAPDH-normalized $2^{-\Delta\Delta C_T}$ values; differences were considered significant at $P < 0.05$.

2.7. TRAP Staining and Activity Assay. Multinucleated osteoclasts were fixed with 3.7% formalin for 10 min and permeabilized with 0.1% Triton X-100 for 10 min. The cells were stained with TRAP solution (Sigma-Aldrich). TRAP-positive cells were counted as multinucleated osteoclasts (nuclei ≥ 3) or TRAP-positive osteoclasts. To measure TRAP activity, multinucleated osteoclasts were fixed in 3.7% formalin for 5 min and permeabilized with 0.1% Triton X-100 for 10 min. The osteoclasts were treated with TRAP buffer (100 mM sodium citrate pH 5.0, 50 mM sodium tartrate) containing 3 mM p-nitrophenyl phosphate (Sigma-Aldrich) at 37°C for 5 min. Reaction mixtures in the wells were transferred into new plates containing an equal volume of 0.1 N NaOH, and optical density (OD) values were determined at 405 nm.

2.8. Retrovirus Preparation and Infection. Retrovirus packaging was described previously [13]. In brief, to isolate retrovirus, pMX-IRES-GFP (retrovirus vector, GFP; green fluorescent protein) and pMX containing constitutively active (CA)-NFATc1 were transiently transfected into Plat-E cells (platinum-E retrovirus packaging cell line; Cell Biolabs, Inc.) with Lipofectamine 2000 (Invitrogen) according to the manufacturer's protocol. Viral supernatant was collected from the culture medium 48 h after transfection. BMMs were incubated with viral supernatant in the presence of polybrene (10 μg/mL) for 8 h. The infection efficiency of the retrovirus was determined by GFP expression and was always > 80%. After infection, BMMs were induced to differentiate in the presence of M-CSF (30 ng/mL) and RANKL (5 ng/mL) for 4 days.

2.9. Bone-Pit Formation Analysis. Mature osteoclasts were prepared by isolating osteoblasts from the calvariae of newborn mice by serial digestion in collagenase (Gibco), as previously described [14]. Bone marrow cells were isolated as described above. Osteoblasts and bone marrow cells were co-cultured on a collagen-coated 90-mm dish in the presence of 1α, 25-dihydroxyvitamin D_3 ($VitD_3$) and prostaglandin E_2 (PGE_2) for 6 days. Co-cultured cells were detached from the collagen-coated dishes, replated on BioCoat Osteologic MultiTest slides in 96-well plates, and treated with fisetin for 24 h. The cells on the BioCoat slides were stained for TRAP, and photographs were taken under a light microscope at 40x magnification. To observe resorption pits, cells on the BioCoat slides were washed with PBS and treated with 5% sodium hypochlorite for 5 min. After washing the plate with PBS buffer and drying, it was photographed under a light microscope. Quantification of resorbed areas was performed with the ImageJ program.

2.10. Statistical Analysis. All quantitative values are presented as mean ± SD. Each experiment was performed 3–5 times, and results from one representative experiment are shown. Statistical differences were analyzed using Student's t-test. A value of $P < 0.05$ was considered significant.

3. Results

3.1. Fisetin Suppresses RANKL-Induced Osteoclast Differentiation. To determine the effect of fisetin on RANKL-induced osteoclast differentiation, varying concentrations of fisetin were added to primary mouse BMM cultures in the presence of M-CSF (30 ng/mL) and RANKL (5 ng/mL) for 4 days. In the absence of fisetin, BMMs were shown to differentiate into mature TRAP-positive multinucleated osteoclasts, but in the presence of fisetin, the formation and number of TRAP-positive multinucleated cells were inhibited in a dose-dependent manner (Figures 1(b) and 1(c)); BMM differentiated into TRAP-positive multinucleated cells (red-color-stained giant cells in Figure 1(b)), but its formation was inhibited by fisetin. TRAP-positive multinucleated osteoclasts were counted in Figure 1(c). In addition, TRAP activity and mRNA expression were inhibited in the presence of fisetin (Figures 1(d) and 1(e)). Furthermore, the inhibitory effect of fisetin on osteoclast differentiation was confirmed by evaluating the mRNA expression level of DC-STAMP, which plays a role in cell fusion (Figure 1(e)); fisetin significantly inhibited the RANKL-induced mRNA expression of DC-STAMP. The presence of fisetin did not affect the survival of BMMs, indicating that the inhibitory effect of fisetin on osteoclast differentiation was not due to its cytotoxicity (Figure 1(f)).

3.2. Fisetin Inhibits RANKL-Induced Phosphorylation of p38 and Expression of c-Fos and NFATc1. To elucidate the mechanism underlying the inhibition of RANKL-induced osteoclast differentiation by fisetin, we investigated the effect of fisetin on RANKL-induced early signaling pathways, including p38, JNK, and ERK. We found that fisetin only inhibited RANKL-induced phosphorylation of p38 (Figure 2(a)). In the process of osteoclast differentiation, RANKL-induced phosphorylation of p38 subsequently leads to the activation of early-stage and late-stage transcription factors, c-Fos and NFATc1, respectively [3, 15]. Therefore, we further examined the expression levels of c-Fos and NFATc1. Real-time PCR analysis revealed that fisetin strongly inhibited the RANKL-induced mRNA expression of both c-Fos and NFATc1 (Figure 2(b)). Additionally, western blot analysis showed that RANKL-induced protein expressions of c-Fos and NFATc1 were significantly suppressed by fisetin (Figure 2(c)).

3.3. Ectopic Expression of NFATc1 Rescues Fisetin-Mediated Inhibition of Osteoclast Differentiation. When osteoclast differentiation is inhibited by downregulation of the p38-c-Fos-NFATc1 signaling axis, over-expression of NFATc1 restores osteoclast differentiation [13, 16]. Therefore, we evaluated whether the over-expression of NFATc1 could restore osteoclast differentiation that had been inhibited by fisetin. Considering GFP signaling, there was no difference in the infection yield between the control GFP and constitutively active (CA)-NFATc1-GFP plasmid (Figure 3(a)). Consistent with

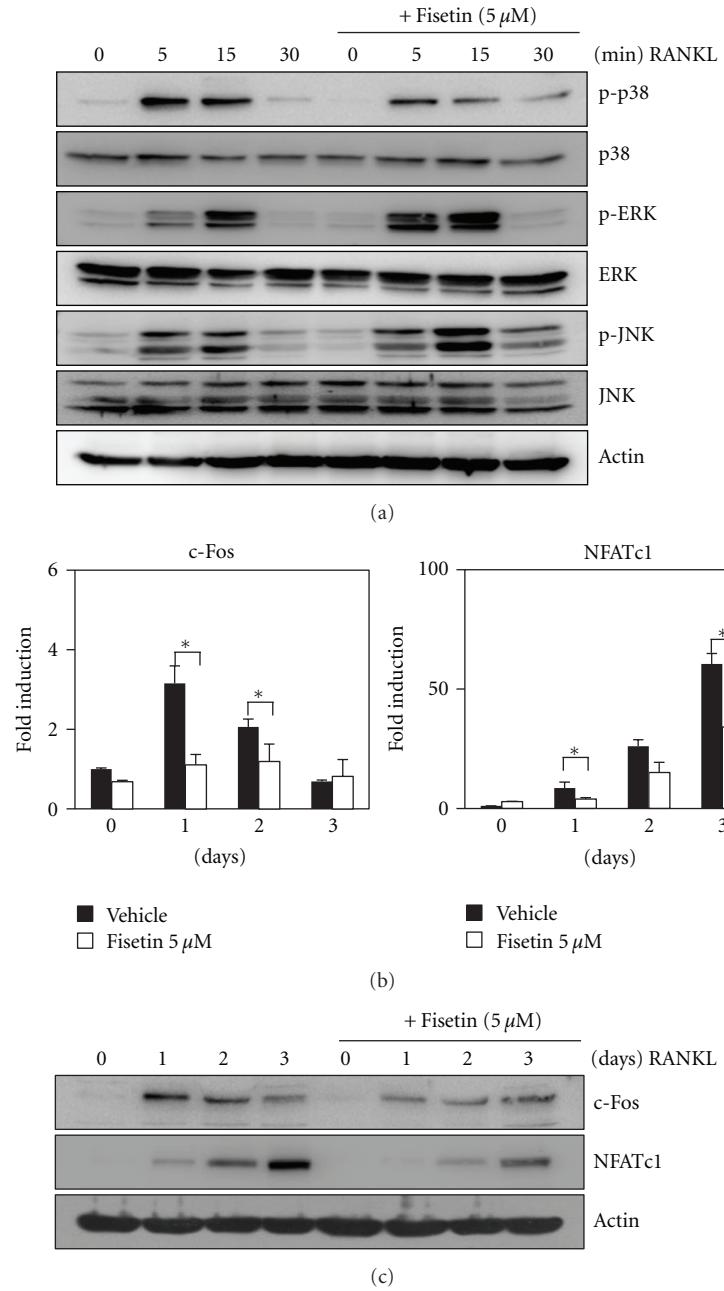

Figure 2: Fisetin inhibits RANKL-induced phosphorylation of p38 and expression of c-Fos and NFATc1. (a) BMMs were pretreated with vehicle or fisetin (5 μM) for 1 h prior to RANKL stimulation (5 ng/mL) at indicated time periods. Then, protein expression levels were evaluated by western blot analysis. Actin was used as the internal control. (b) BMMs were stimulated with RANKL (5 ng/mL) and M-CSF (30 ng/mL) in the presence or absence of fisetin (5 μM) for the indicated times. Then, total RNA was isolated from cells using TRIzol reagent, and mRNA expression levels were evaluated by real-time PCR. $^*P < 0.05$ (versus "the vehicle control"). (c) Effects of fisetin on protein expression levels of c-Fos and NFATc1 were evaluated by western blot analysis. Actin was used as the internal control.

the aforementioned result, the formation of TRAP-positive multinucleated osteoclasts from BMM expressing the control GFP was strongly inhibited by fisetin (upper images in Figure 3(b)). However, even in the presence of fisetin, TRAP-positive multinucleated osteoclasts were formed from BMMs over-expressing NFATc1 (bottom images in Figure 3(b)), unlike those expressing the control GFP. The ameliorating effect of NFATc1 on the fisetin-mediated inhibition of osteoclast differentiation was also confirmed by counting the number of multinucleated osteoclasts (Figure 3(c)) and by measuring the activity of TRAP (Figure 3(d)).

3.4. Fisetin Inhibits the Bone-Resorbing Activity of Mature Osteoclasts. Several natural products with inhibitory activity on osteoclast differentiation also inhibit the bone-resorbing activity of mature osteoclasts [17–19]. Therefore,

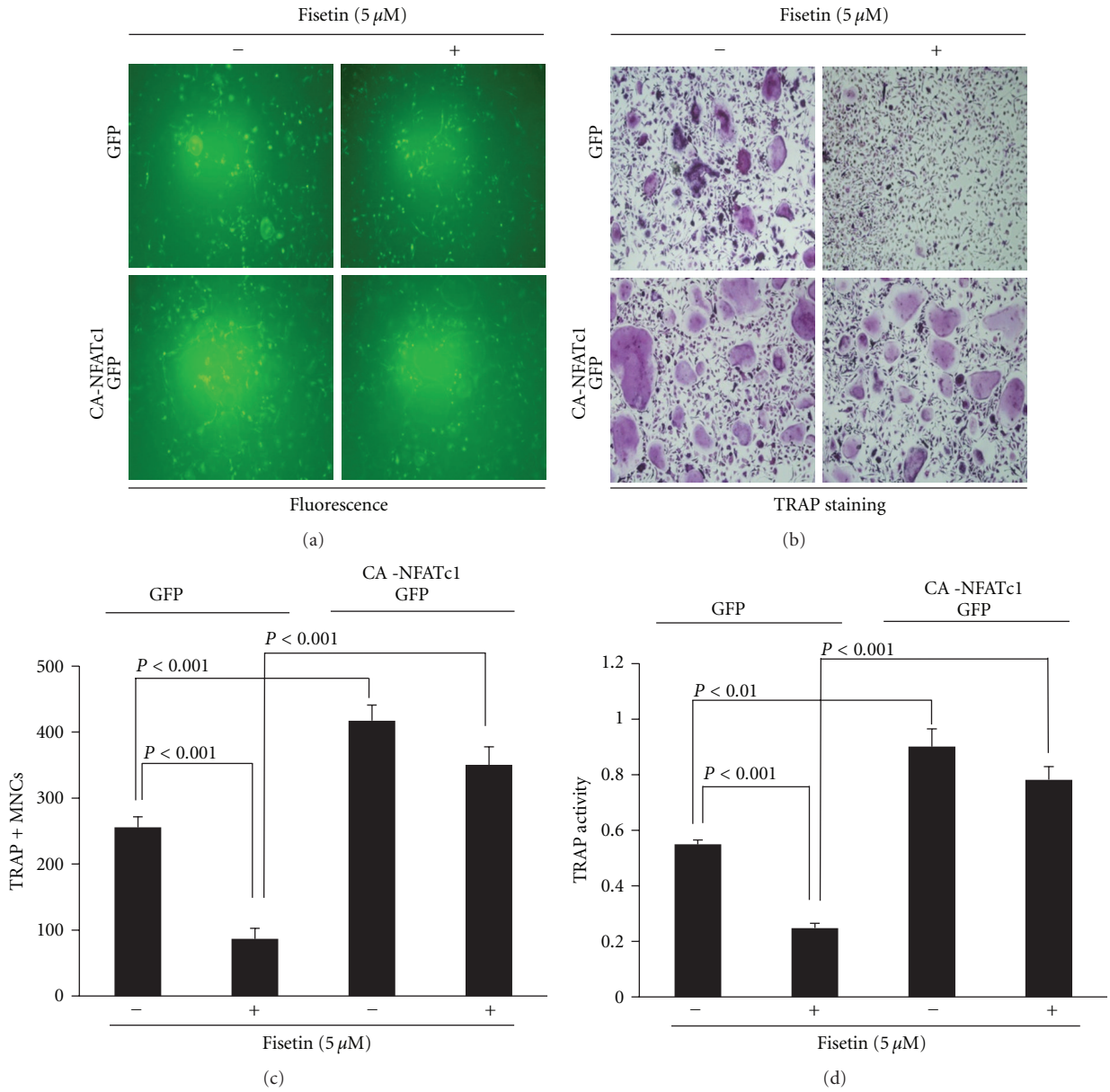

Figure 3: NFATc1 over-expression restores fisetin-mediated inhibition of osteoclast differentiation. (a) BMMs were infected with pMX-IRES-GFP (GFP) or pMX-IRES-CA-NFATc1-GFP (CA-NFATc1-GFP) for 8 h with polybrene (10 μg/mL). Infected BMMs were cultured with M-CSF (30 ng/mL) and RANKL (5 ng) for 4 days in the presence or absence of fisetin (5 μM). After 4 days, cells were fixed and GFP expression was visualized under a fluorescence microscope. (b) BMMs were infected with GFP or CA-NFATc1-GFP and then cells were cultured as described in (a). After 4 days, mature TRAP-positive multinucleated osteoclasts were visualized by TRAP staining. (c) TRAP-positive cells were counted as osteoclasts. (d) TRAP activity was measured at 405 nm.

we further evaluated whether fisetin has the potential to inhibit the bone-resorbing activity of mature osteoclasts. Fisetin inhibited the RANKL-induced mRNA expression of cathepsin K, which is an essential factor for bone resorption (Figure 4(a)). We next examined the antiresorptive activity of fisetin on synthetic carbonate apatite-coated plates. When mature osteoclasts were placed on carbonate apatite-coated plates and cultured in the presence or absence of various concentrations of fisetin for 24 h, fisetin dose-dependently inhibited the bone-resorbing activity of mature osteoclasts (Figure 4(b)); the resorbed areas (bright areas) on the slides were observed under a microscope (bottom images in Figure 4(b)) and measured as described in the Section 2 (upper graph in Figure 4(b)). To determine whether the inhibitory activity of fisetin on bone resorption might result from its potential to trigger the death of mature osteoclasts [9, 20], we counted the number of TRAP-positive multinucleated osteoclasts. As shown in Figure 4(c), fisetin did not affect the number of TRAP-positive multinucleated osteoclasts; there was no difference of the number of the

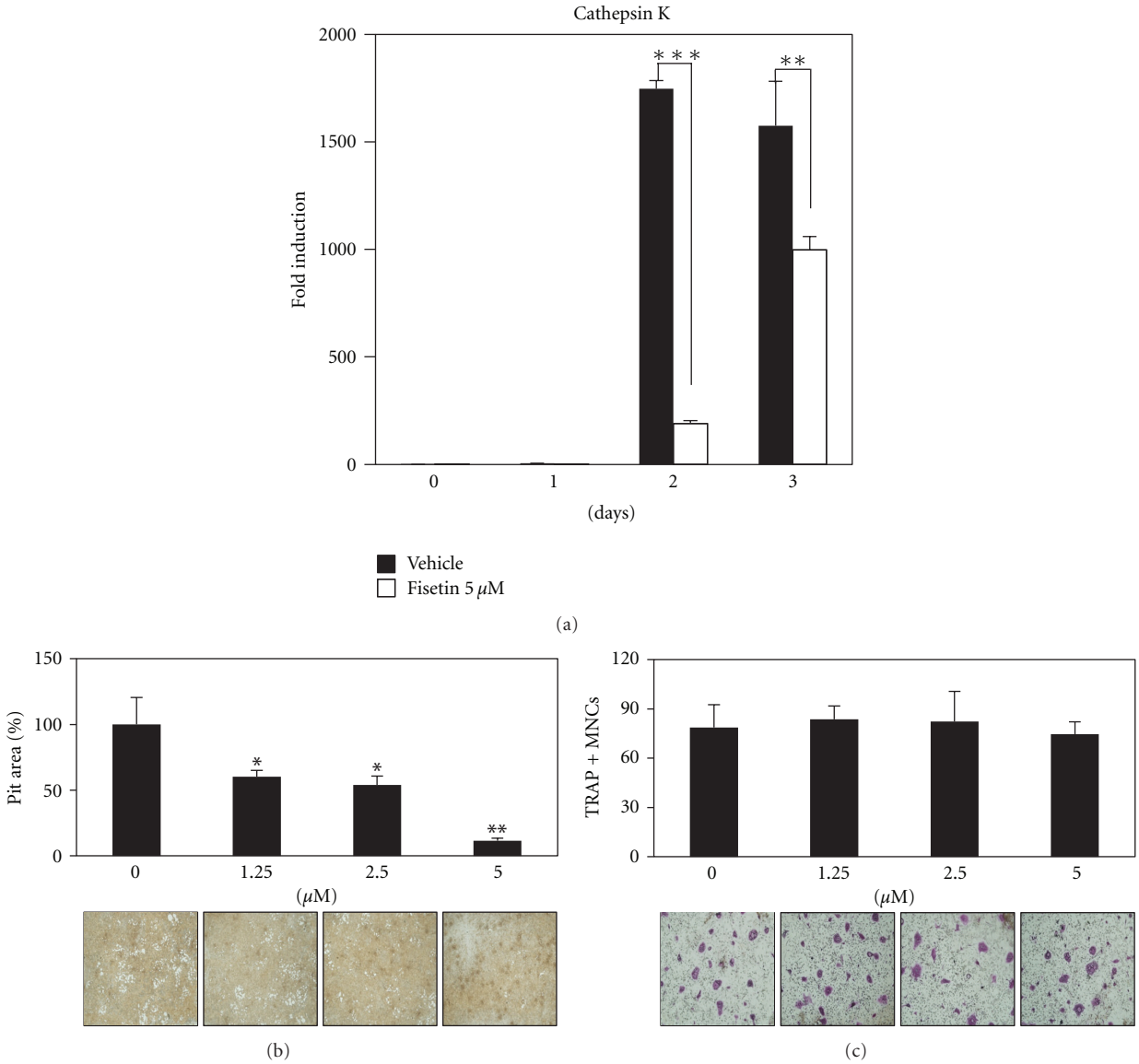

Figure 4: Fisetin inhibits osteoclastic bone resorption. (a) Effect of fisetin on mRNA expression of cathepsin K was analyzed by real-time PCR as described in Figure 1(e). $^{**}P < 0.01$; $^{***}P < 0.001$ (versus "the vehicle control"). (b) Attached cells on BioCoat Osteologic MultiTest slides were removed and photographed under a light microscope (bottom images). Pit areas were quantified using ImageJ program (upper graph). $^{**}P < 0.01$; $^{***}P < 0.001$ (versus "the control"). (c) Mature osteoclasts were plated on BioCoat slides and treated for 24 h with the indicated concentrations of fisetin. Cells were fixed, permeabilized, and stained with TRAP. TRAP-positive multinucleated cells were counted (upper graph) and photographed under a light microscope (bottom images).

red color TRAP-positive multinucleated osteoclasts between the control and fisetin-treated groups (bottom images in Figure 4(c)).

4. Discussion

In this study, fisetin was shown to dose-dependently inhibit osteoclast differentiation. The inhibitory effect of fisetin on osteoclast differentiation was also confirmed by evaluating the mRNA expression levels of TRAP and DC-STAMP. Considering that DC-STAMP has been shown to be essential for osteoclast fusion [21–23], fisetin might have the potential to inhibit this cell fusion. Cell fusion is a necessary event in the maturation of cells so that they can perform specific functions, such as bone resorption in the case of osteoclasts.

The activation of MAP kinases is essential for osteoclast differentiation. Among MAP kinases, fisetin inhibited the RANKL-induced phosphorylation of p38. The involvement of the p38 signaling pathway in RANKL-induced osteoclast differentiation has been reported in several studies [24, 25]. Furthermore, the importance of p38 in inflammatory bone destruction has been suggested in several reports [26, 27], and it is considered to be a potential therapeutic target for

inflammatory osteolysis [28]. Considering the antiinflammatory activity of fisetin [29] and its activity in preventing oxidative damage in osteoblasts [30], the potential antiresorptive property of fisetin could provide benefits for bone health.

RANKL-induced activation of MAP kinases further leads to the activation of transcription factors such as c-Fos and NFATc1. Apparently, c-Fos and NFATc1 play a critical role in the regulation of genes for osteoclast differentiation. An important role for c-Fos in the process of osteoclast differentiation has been clarified in c-Fos knockout mice [31]; these mice had osteopetrosis due to osteoclast deficiency. Furthermore, NFATc1 has been shown to rescue osteoclastogenesis in cells lacking c-Fos [32–34]. These two transcription factors are also functionally linked together; c-Fos is essential for RANKL-mediated induction of NFATc1. c-Fos is expressed in the early stages of osteoclast differentiation, and it further regulates NFATc1 gene expression by binding to the promoter region of NFATc1. After NFATc1 is expressed in the middle or late stages of osteoclast differentiation, it subsequently regulates a number of osteoclast-specific genes, such as cathepsin K.

In this study, fisetin inhibited the expression of c-Fos and NFATc1 at the transcriptional and translational levels. The induction of c-Fos and NFATc1 during RANKL-induced osteoclast differentiation is mediated by the p38 signaling pathway [3]; the inhibitory effect of fisetin on osteoclast differentiation could result from its potential ability to inhibit the p38-c-Fos-NFATc1 signaling axis. The recruitment of p38 and NFATc1 to target genes during osteoclast differentiation has been also reported [15], and the involvement of NFATc1 in fisetin-inhibited osteoclast differentiation was confirmed by the NFATc1 over-expression experiment performed in this study; the fisetin-induced inhibition of osteoclast differentiation was almost entirely rescued by NFATc1 induction. These results suggest that p38-c-Fos-NFATc1 signaling axis is involved in the inhibitory effect of fisetin on osteoclast differentiation.

Cathepsin K is highly expressed in osteoclasts, and it is a well-known proteolytic enzyme that degrades the bone matrix [35, 36]. RANKL-induced cathepsin K gene expression has been shown to be cooperatively regulated by a combination of transcription factors, such as NFATc1 and p38 MAP kinase [37]. In this study, the presence of fisetin was associated with inhibition of the RANKL-induced mRNA expression of cathepsin K; furthermore, fisetin appeared to dose-dependently inhibit the bone-resorbing activity of mature osteoclasts.

5. Conclusions

To our knowledge, this is the first study to report that fisetin has the potential to inhibit RANKL-induced osteoclast differentiation via attenuation of the RANKL-induced activation or expression of p38/c-Fos/NFATc1 signaling molecules. The decrease in signaling by p38/c-Fos/NFATc1 could consequently lead to a decrease in the expression of responsive genes required for cell fusion and bone resorption, such as DC-STAMP and cathepsin K. Furthermore, fisetin appeared to inhibit the bone-resorbing activity of mature osteoclasts. The potential antiresorptive property of fisetin could provide benefits for bone health and it may be of use in the treatment of osteoclast-related disorders including osteoporosis.

Acknowledgments

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Research Article

Labisia pumila Prevents Complications of Osteoporosis by Increasing Bone Strength in a Rat Model of Postmenopausal Osteoporosis

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Estrogen replacement therapy (ERT) is the main treatment postmenopausal osteoporosis. However, ERT causes serious side effects, such as cancers and thromboembolic problems. *Labisia pumila var. alata* (LPva) is a herb with potential as an alternative to ERT to prevent complications of osteoporosis, especially fragility fractures. This study was conducted to determine the effects of LPva on the biomechanical strength of femora exposed to osteoporosis due to estrogen deficiency, using the postmenopausal rat model. Thirty-two female rats were randomly divided into four groups: Sham-operated (Sham), ovariectomized control (OVXC), ovariectomized with *Labisia pumila var. alata* (LP), and ovariectomized with ERT (Premarin) (ERT). The LPva and ERT were administered via oral gavage daily at doses of 17.5 mg/kg and 64.5 μg/kg, respectively. Following two months of treatment, the rats were euthanized, and their right femora were prepared for bone biomechanical testing. The results showed that ovariectomy compromised the femoral strength, while LPva supplementation to the ovariectomized rats improved the femoral strength. Therefore, LPva may be as effective as ERT in preventing fractures due to estrogen-deficient osteoporosis.

1. Introduction

Osteoporosis is defined as a systemic skeletal disease that is characterized by low bone mass and microarchitectural deterioration of bone tissue, with a consequent increase in bone fragility and susceptibility to fracture [1]. According to the World Health Organization [2], osteoporosis occurs when the bone mineral density falls more than 2.5 standard deviations (SD) below the standard reference for maximum bone mineral density of young adult females. After the age of 35 to 40, the bone mass in females begins to decline slowly, but the rate of bone loss increases dramatically after menopause or ovariectomy due to estrogen deficiency.

By the age of 50, the bone mass in women is only two-thirds of that in men [3]. The combination of lower initial adult bone mass and faster rate of bone loss produce a higher incidence of osteoporosis in elderly women compared with men [4]. About one in three women aged more than 50 experienced an osteoporotic fracture in their lifetime [5].

Estrogen replacement therapy (ERT) is the main form of treatment and prevention of postmenopausal osteoporosis. Estrogen given alone or in combination with progesterone is able to prevent postmenopausal osteoporosis effectively [6]. Estrogen binds to estrogen receptors on the osteoclast surface, which causes the release of chemical mediators and reduction of osteoclastic activity, and therefore inhibits bone resorption [7].

The Women's Health Initiative study found that women who took ERT have slightly higher rates of breast cancer, ovarian cancer, heart attack, stroke, thromboembolism, and

Alzheimer's disease [8–10]. Due to the numerous side effects of ERT, alternative antiosteoporotic agents that are comparable in effectiveness to estrogen but with minimal side effect are being investigated. These include soy [11], blueberry [12], and *Achyranthes bidentata* [13]. A histomorphometric study by Fathilah et al. (2012) [14] found that *Labisia pumila var. alata* has potential as an alternative to ERT for the prevention of postmenopausal osteoporosis. In a different study by Nazrun et al. (2010) [15], *Labisia pumila var. alata* was found to produce beneficial effects similar to estrogen on bone biomarkers in the postmenopausal osteoporosis animal model.

Labisia pumila (LP), a herbal plant from the family of *Myrsinaceae,* is a popular herb among women folk in Malaysia and is known locally as "Kacip Fatimah." There are three types of *Labisia pumila*: *Labisia pumila var. alata* (LPva), *Labisia pumila var. pumila* (LPvp), and *Labisia pumila var. lanceolata* (LPvl) [16]. Traditionally, *Labisia pumila* extract is prepared by boiling the roots, leaves, or the whole plant in water, whereby the extract is then taken orally [17, 18]. It is used to facilitate labour, shrink the uterus, and improve menstrual irregularities and as postpartum medicine [17, 19]. Its exclusive use in women has led to the belief that it is a phytoestrogen, a compound with similar chemical structure to estrogen [20]. Therefore it is able to relieve menopausal symptoms. Several studies have demonstrated the estrogenic properties of LPva. It was found to inhibit estradiol binding to antibodies against estradiol [21], increase the uterine weight of ovariectomized rats [22], exert a specific estrogenic effect on human endometrial adenocarcinoma cells (Ishikawa-Var I line) [23], and initiate lipolysis in adipose tissue in a manner similar to estrogen [24]. In ovariectomized rats, LPva was also found to downregulate 11-β hydroxysteroid dehydrogenase1expression in adipose and liver tissues and decrease serum corticosterone levels [25].

Based on the possible estrogenic activities of LPva, it may be a suitable alternative to replace estrogen for the treatment and prevention of postmenopausal osteoporosis. Thus, it may also be effective in preventing complications of osteoporosis, especially fractures, by decreasing bone fragility.

Bone strength is the best and true indicator of bone function. However, it can only be directly assessed in animal models because the bone has to be tested until it fractures. In humans, it can only be indirectly assessed by using computer softwares [26]. The bone biomechanical test is the best method to measure bone strength directly, but it requires exerting a load to the bone until it fractures, which is impossible to be conducted in humans. The strength and stiffness of a bone are important parameters to determine its ability to resist fracture. Thus, improvement in these parameters will be beneficial in preventing fragility fractures [27]. A previous study demonstrated that supplementation of vitamin E, especially GTT, can improve bone structural and biomechanical properties of normal male rats [28]. The present study aimed to study in detail the effects of LPva on bone biomechanical strength in ovariectomized rats.

2. Materials and Methods

2.1. Animal and Treatment. Thirty-two female *Wistar* rats, with the average age of three months and weighing between 200 to 250 g, were used in this study. The rats were allowed to acclimatize for a week before being used for the study. The rats were housed two per cage, at normal room temperature with adequate ventilation and normal 12-hour light-dark cycle. All rats were allowed free access to water and food (commercial laboratory rat's food containing 0.97% calcium, 0.85% phosphorus, and 1.05 IU/g of Vitamin D3) (Gold Coin, Selangor, Malaysia). They were equally divided into four main groups. The sham-operated group (Sham) and the ovariectomized control group (OVXC) were given oral gavages of deionized water (vehicle). The treatment groups were given *Labisia pumila var. alata* at 17.5 mg/kg/day (LPva) and Premarin at 64.5 μg/kg/day (ERT) daily for 8 weeks via oral gavages. The ERT group acted as positive control. After 8 weeks of treatments, the rats were euthanised. The right femora were dissected out and cleaned of any tissues. The distal femora were divided sagittally into two halves and wrapped with gauze dipped in phosphate-buffered saline. The approval for this study was obtained from the University Animal Ethic Committee of Universiti Kebangsaan Malaysia (PP/FAR/2009/NAZRUN/14 JULY/267-JULY 2009-MAY-2010).

2.2. Labisia pumila var. alata (LP) Extract. The LPva extract was supplied by Phytes Biotek Sdn Bhd. (Malaysia), a Good Manufacturing Practice (GMP) licensed manufacturer of herbal products, in the form of a freeze-dried standardized extract (Batch no: KF071107). The extraction was done at a factory in Shah Alam, Selangor, Malaysia, using a patented high-pressure water extraction process (US 7,132,117 B2), filtered at 1–4 mm and freeze-dried without maltodextrin or lactose. The extract was obtained from the root of the LPva plant and was the same extract that had been used previously by Fathilah et al., 2012 [14] and Nazrun et al., 2010 [15]. This extract was also the same form used for human consumption as health supplements. The extract was sent to the Forest Research Institute Malaysia (FRIM) for phytochemical testing. Based on the phytochemical test, the LPva extract that was used in this study contained flavonoids, saponins, and tritepenes.

The brownish powdered extract was dissolved in deionised water and given to the LPva treatment group via oral gavage at the dose of 17.5 mg/kg rat weight daily at 9 am for 8 weeks [14, 15]. The Premarin (Wyeth-Ayerst, Canada) tablet containing 0.625 mg of conjugated estrogen was crushed, dissolved in deionised water, and given to the ERT group via oral gavages at the dose of 64.5 μg/kg rat weight daily at 9 am for 8 weeks [14, 15]. These doses were chosen based on our previous studies, which have demonstrated that LPva has the potential to be used as an alternative to ERT for the prevention of postmenopausal osteoporosis [5, 14]. In order to reduce the number of rats used in this study, we have followed the recommendation by the Animal Ethics Committee to use only one dose of LPva and Premarin, respectively.

2.3. Bone Biomechanical Test. Each right femur was wrapped with gauze dipped in phosphate-buffered saline, rewrapped with aluminum foil, and tested within two hours after dissection. Samples were kept moist at all times during the preparation procedure. The biomechanical properties of the femoral bones were assessed using an Instron Universal Testing Machine (model 5848; Microtester; Instron, Canton, MA, USA) that was equipped with Bluehill 2 software. Each femur was placed in a three-point bending configuration, whereby it was placed on two lower supports that were 5 mm apart. Force was applied at the middiaphysis on the anterior surface of the bone, causing the anterior surface to be in compression and the posterior surface in tension until it fractured. The load, stress, and strain parameters were recorded by the software. Graphs of stress against strain were also plotted. The slope value of the stress-strain curve in the elastic deformation region represents the modulus of elasticity (Young's modulus) of the femur. The main parameters of the bone mechanical test may be divided into extrinsic and intrinsic parameters; the extrinsic parameters (load, displacement, and stiffness) measure the properties of the whole bone, whereas the intrinsic parameters (stress, strain, and modulus of elasticity) measure the material of the bone.

2.4. Statistical Analysis. The results were expressed as mean ± standard error of the mean (SEM). The data analysis was performed using the Statistical Package for Social Sciences software (SPSS 17; SPSS, Chicago, IL, USA). The data were tested for normality using the Kolmogorov-Smirnov test. For normally distributed data, the statistical tests used were the analysis of variance (ANOVA), followed by Tukey's Honestly Significant Difference (HSD) test. For data that were not normally distributed, Kruskal-Wallis and Mann-Whitney tests were used.

3. Results

Femoral strength was evaluated using biomechanical tests (Figures 1–4). The load parameter measured the force received by the femur before it fractured (Figure 1). The ERT group received significantly greater load compared to the Sham and OVXC groups. The LPva group received comparable load to ERT and significantly greater load as compared to OVXC group.

The stress parameter measured the load per unit area received by the femur before it fractured (Figure 2). The ERT group received significantly higher stress than the Sham and OVXC groups. The LPva group received higher stress than the OVXC group and was comparable to the ERT group. The OVXC group received the lowest stress compared to other groups. The strain parameter measured the relative deformation of the femur caused by the stress before it fractured (Figure 3). The ERT and LPva groups had significantly higher strain than the Sham and OVXC groups. The OVXC group had the lowest strain compared to other groups. The modulus of elasticity (Young's modulus) measured the tendency of the femur to be deformed elastically when force is applied to it (Figure 4). The ERT and LPva groups had a significantly higher modulus of elasticity compared to other groups. The OVXC group had the lowest modulus of elasticity compared to the other groups.

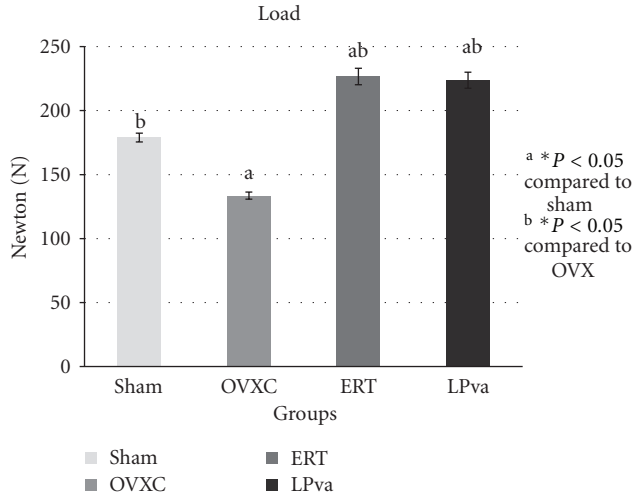

Figure 1: Load values: the load parameter measured the force received by the femur before it fractured. Sham (water vehicle), OVXC (water vehicle), LPva (*Labisia pumila var. alata* 17.5 mg/kg/day), and ERT (Premarin 64.5 μg/kg/day). Value expressed as mean ± SEM; $P < 0.05$ is considered significant. [a]Significantly different from Sham group; [b]significantly different from OVXC group.

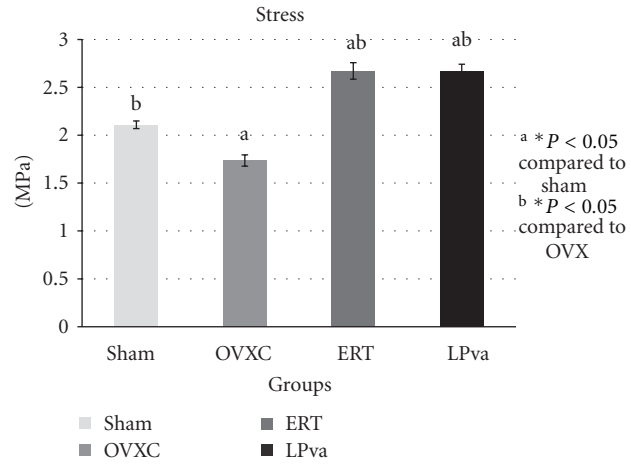

Figure 2: Stress values: the stress parameter measured the load per unit area received by the femur before it fractured. Sham (water vehicle), OVXC (water vehicle), LPva (*Labisia pumila var. alata* 17.5 mg/kg/day), and ERT (Premarin 64.5 μg/kg/day). Value expressed as mean ± SEM; $P < 0.05$ is considered significant. [a]Significantly different from Sham group; [b]significantly different from OVXC group.

4. Discussion

Hormone or estrogen replacement therapy (HRT/ERT) has been used for the prevention and treatment of postmenopausal osteoporosis, but it may cause serious side-effects

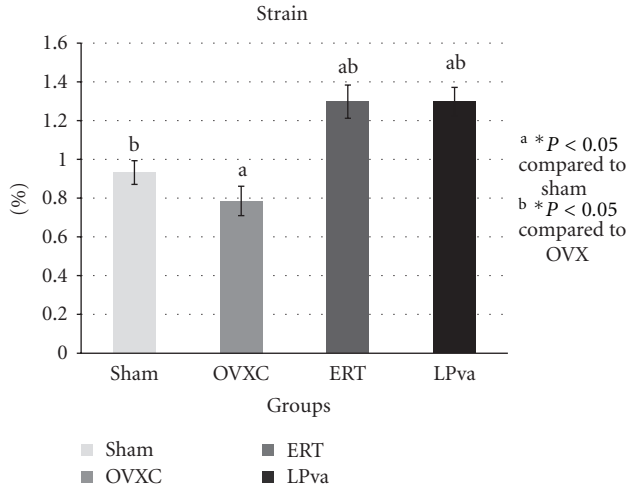

Figure 3: Strain values: the strain parameter measured the relative deformation of the femur caused by the stress before it fractured. Sham (water vehicle), OVXC (water vehicle), LPva (*Labisia pumila var. alata* 17.5 mg/kg/day), and ERT (Premarin 64.5 μg/kg/day). Value expressed as mean ± SEM; $P < 0.05$ is considered significant. [a]Significantly different from Sham group; [b]significantly different from OVXC group.

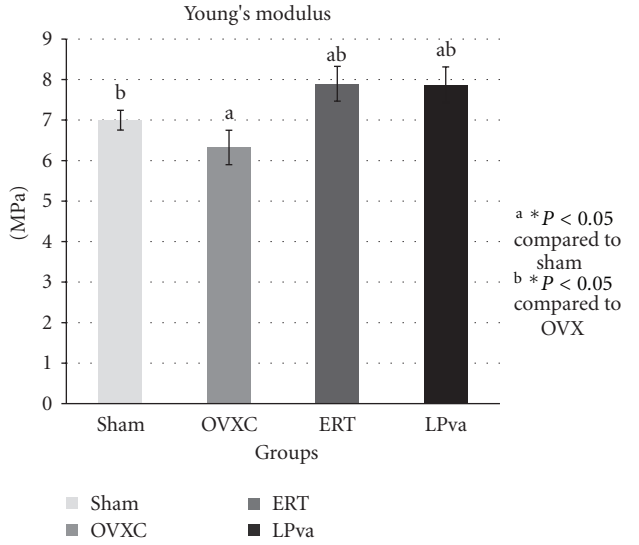

Figure 4: Young's modulus values: the modulus of elasticity (Young's modulus) measured the tendency of the femur to be deformed elastically when a force was applied to it. Sham (water vehicle), OVXC (water vehicle), LPva (*Labisia pumila var. alata* 17.5 mg/kg/day), and ERT (Premarin 64.5 μg/kg/day). Value expressed as mean ± SEM. $P < 0.05$ is considered significant. [a]Significantly different from Sham group; [b]significantly different from OVXC group.

(Ferguson, 2004) [29]. It was reported that women who took HRT have slightly higher rates of breast cancer, ovarian cancer, heart attack, stroke, thromboembolism, and Alzheimer's disease [8–10]. Due to the numerous side effects of ERT, we have investigated the potential of LPva as an alternative treatment for postmenopausal osteoporosis in terms of enhancing the bone resistance to fracture. This herbal plant was selected due to its phytoestrogenic properties [20, 21]. LPva has been found to protect the bone of estrogen-deficient rat in a histomorphometric study [14].

To the best of our knowledge, this is the first report on the effects of LPva on the bone biomechanical strength in an ovariectomized rat model. The effects of LPva on the biomechanical parameters of postmenopausal osteoporosis rat model were compared to ERT, the gold standard treatment for postmenopausal osteoporosis. Rats have become a widely accepted model of human bone disease because their mechanism of controlling the gain and loss of bone mass are similar to humans. An increase in bone mass was observed in longitudinal bone growth and modelling drifts with bone loss related to bone remodelling. Furthermore, the response to mechanical influences, hormones, drugs, and other agents in rats are similar to humans [30]. Young adult rats were selected as the animal model in this study for their dynamic bone growth, which represents the critical bone growth phase of the young adult humans in their twenties. This phase requires an optimum bone growth to achieve the peak bone mass. During this phase, more bones are formed than resorbed in each remodelling cycle. If the peak bone mass is not fully optimized or is disturbed by factors such as unstable hormones, the risk of developing osteoporosis in the elderly years is higher [31].

Similar doses of 17.5 mg/kg of LPva and 0.0645 mg/kg of ERT were used in the present study as those that were used in the study by Fathilah et al. (2012) [14], which found that these doses were effective in the prevention of osteoporosis in the ovariectomised rat model. It was shown that the supplementation of 17.5 mg/kg of LPva to ovariectomized rats for 8 weeks was able to prevent osteoporotic changes that were reflected in the bone biochemical markers [15]. In terms of safety, the LPva extract was found to exhibit no-adverse-effect level (NOAEL) at the dose of 50 mg/kg in a subacute toxicity study [32], 1000 mg/kg in a subchronic toxicity study [33], and 800 mg/kg in a reproductive toxicity study [34]. Fazliana et al. (2009) [22] had used several doses of LPva ranging from 10 to 50 mg/kg, but found that only the 50 mg/kg dose was able to suppress weight gain in ovariectomized rats. The dose of estrogen used in the same study was 0.625 mg/kg, which was higher than the estrogen dose used in our study. However, our lower estrogen dose was effective as this dose was able to prevent bone changes induced by ovariectomy as evidenced by the improvement seen in all the bone biomechanical parameters. In fact, these bone biomechanical parameters were significantly better than the sham group.

The bone loss associated with estrogen deficiency is generally attributed to increased bone resorption and increased bone turnover. There was evidence to suggest that estrogen may exert anabolic effects on bone. Estrogen has been shown to stimulate the differentiation and activity of osteoblasts [35, 36] and increase bone formation and bone mass in animal models [37, 38]. More interestingly, the LPva group also

demonstrated this anabolic effect similar to ERT. The structural improvement by ERT and LPva should lead to stronger bones as bone structure determines their strength. Since no study has been done on the effect of LPva on bone strength, we have carried out bone biomechanical testing and found that ERT and LPva supplementation significantly improved both extrinsic parameters (load) and intrinsic parameters. The findings of the bone biomechanical test suggested that ERT and LPva enhanced the bone biomechanical properties of ovariectomized rats.

A recent study by Fathilah et al. (2012) [14] on bone histomorphometric analysis demonstrated that supplementation of LPva in ovariectomized rats was as effective as ERT in preventing osteoporotic changes. Based on previous studies, there are several possible mechanisms of LPva in protecting the bone against estrogen deficiency. The most likely mechanism is due to its phytoestrogenic actions [23, 39]. LPva contains triterpene saponins, including the compound ardisiacrispin A, which were thought to interact with estrogen receptors [40].

LPva was also found to have similar antioxidative properties as those exhibited by beta carotene, flavonoids, vitamin C, total anthocyanins, and phenolics [41]. LPva extract demonstrated a potent antioxidant activity comparable to that of ascorbic acid, one of the strongest known antioxidants [42]. The other possible mechanism of action of LPva against osteoporosis is via its antioxidative properties as demonstrated by tocotrienols, another potent antioxidant [28]. The antioxidant activity of LPva is contributed by its flavanoids, ascorbic acid, beta carotene, anthocyanin, and phenolic compounds [43]. Tumour necrosis factor-α (TNF-α) is a bone-resorbing cytokine that promotes bone resorption by activating mature osteoclasts or by stimulating proliferation and differentiation of osteoclasts [44]. Inhibition of this cytokine may be another possible mechanism of action that LPva exhibits against osteoporosis. It has been shown that blocking the effect of TNF-α prevented postovariectomy bone loss [45]. LPva was found to suppress the TNF-α level to below the baseline level in cultured HaCat cells [46].

As a conclusion, based on the comparable effects of LPva to ERT on bone biomechanical testing and its safety profile, LPva has the potential to prevent osteoporotic fractures in the postmenopausal or estrogen-deficient state. It may be taken as supplements by postmenopausal women who are afraid of the side effects of estrogen [46]. LPva seemed to be safer than Premarin as it exhibited no reproductive toxicity in animal at forty-five times higher than the dose used in the present study [33]. Further studies are required to determine its antiosteoporotic mechanism of action for the prevention of complications of osteoporosis.

Conflict of Interests

The authors declare that they have no conflict of interest whatsoever. The authors alone are responsible for the content and writing of this paper.

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Review Article

Nigella sativa: A Potential Antiosteoporotic Agent

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Nigella sativa seeds (NS) has been used traditionally for various illnesses. The most abundant and active component of NS is thymoquinone (TQ). Animal studies have shown that NS and TQ may be used for the treatment of diabetes-induced osteoporosis and for the promotion of fracture healing. The mechanism involved is unclear, but it was postulated that the antioxidative, and anti-inflammatory activities may play some roles in the treatment of osteoporosis as this bone disease has been linked to oxidative stress and inflammation. This paper highlights studies on the antiosteoporotic effects of NS and TQ, the mechanisms behind these effects and their safety profiles. NS and TQ were shown to inhibit inflammatory cytokines such as interleukin-1 and 6 and the transcription factor, nuclear factor κB. NS and TQ were found to be safe at the current dosage for supplementation in human with precautions in children and pregnant women. Both NS and TQ have shown potential as antiosteoporotic agent but more animal and clinical studies are required to further assess their antiosteoporotic efficacies.

1. *Nigella sativa*

Nigella sativa is a herbal plant which belongs to *Ranunculaceae* family. It is also known as black cumin or *habatus sauda*, and has a rich historical and religious background. It is found in the southern region of Asia. It can grow up to 30 cm and produces pale blue flowers. The fruit is composed of follicles which contain the seeds, the most valuable part of the plant. The seeds of *Nigella sativa* (NS), which have a pungent bitter taste, are used in confectionery and liquors. The seed is the source of the active ingredients of this plant and has been used in Islamic medicine for its healing powers [1]. Studies have revealed various therapeutic values of NS such as anticancer, antioxidant, antibacterial, antifungal, antiparasitic and antiasthmatic [2–7]. NS may also inhibit renal calculi and improve poultry quality [8, 9]. NS contains 36–38% fixed oils, proteins, alkaloids, saponin, and 0.4–2.5% essential oil [10]. High-performance liquid chromatography (HPLC) analysis of NS essential oil revealed that the main active ingredients were thymoquinone, dithymoquinone, thymohydroquinone, and thymol [11]. Among the compounds identified, thymoquinone (TQ) is the most abundant, which makes up 30–48% of the total compounds. This quinine constituent is the most potent and pharmacologically active compound in NS. There were several studies showing that NS and TQ have beneficial effects on bone and joint diseases.

2. Osteoporosis

The major bone disease is osteoporosis, a systemic skeletal disease characterized by low bone mass and microarchitectural deterioration of bone tissue, with a consequent increase in bone fragility and susceptibility to fracture. According to World Health Organization (WHO), osteoporosis is defined as a bone mineral density that lays 2.5 standard deviations or more below the average value for young healthy women. In osteoporosis, bone loss occurs especially at the trabecular

area when the balance of bone remodeling is tipped towards bone resorption. The bone loss is associated with bone biochemical marker changes such as reduction in osteocalcin level, the marker for bone formation and elevation in cross-link C-telopeptide, the marker for bone resorption. The diagnosis of osteoporosis is made using dual emission X-ray absorptiometry (DEXA) machine but more sophisticated three-dimensional micro-computed tomography (micro-CT) is making way for a better diagnosis.

This paper attempts to discuss the beneficial effects of NS and TQ, its active compound, on osteoporosis and fracture healing.

3. Postmenopausal Osteoporosis

The main cause of osteoporosis is menopause or estrogen-deficiency. Several medicinal plants have been studied using postmenopausal osteoporosis animal model such as soy, blueberry, *achyranthes bidentata,* and *labisia pumila* [12–15]. To date, there is no study of NS or TQ on postmenopausal osteoporosis animal model. There was a human study on the effects of NS supplements on the bone markers of postmenopausal women [16]. It was found that NS supplementation for 3 months to these postmenopausal women failed to cause any significant changes in the bone markers levels. The authors concluded that NS was not recommended for the treatment of postmenopausal osteoporosis. However, there were several weaknesses in the study which may account for the nonsignificant results. The sample size of only 15 postmenopausal women was too small. The duration of study should be longer to obtain bone markers readings at several time points and to register any changes in the bone mineral density. Noncompliant in taking NS oil was also a problem due to the nonfavorable greasy taste. In the future, a long term study with larger sample size should be planned. NS in the form of capsules should be given to the study participants to improve compliance. Before that, the effects of NS should be tested in animal osteoporosis models. *In vitro* studies are also required to determine the effects of NS on osteoblasts and osteoclasts.

4. Diabetes-Induced Osteoporosis

Currently, NS has only been tested in animal osteoporosis model of diabetes-induced osteoporosis. No study has been conducted on other osteoporotic animal models. Osteoporosis is now accepted as a major complication of patients with diabetes mellitus. Diabetes could affect bone through multiple mechanisms such as insulin deficiency, insulin resistance, hyperglycemia, or atherosclerosis. However, the exact mechanism responsible for osteopenia in diabetes is still unknown [17]. Insulin and insulin-like growth factors (IGF-1) may have some roles to play in the pathogenesis of diabetic-induced bone loss due to their anabolic effects [18].

A study found that combined treatment of NS and parathyroid hormone was more effective in reversing the osteoporotic changes and improving the bone strength of steptozocin-induced diabetic rats than either treatment alone [19]. In another study using the same model, histological assessments found that NS was able to ameliorate diabetic changes of the bone [20]. These findings suggested that NS has potential to be used for the treatment of diabetic osteoporosis.

The mechanisms behind the bone protective effects of NS against diabetes induced-osteoporosis are still unclear. NS may have improved the bone metabolism by improving the blood sugar levels. In certain parts of the world, NS is frequently used as the traditional treatment of diabetes [21]. Studies on streptozotocin-induced diabetic rats have shown that NS may reduce hyperglycaemia, increase serum insulin concentrations, and promote partial regeneration or proliferation of pancreatic beta cells, causing an increase in insulin secretion [22–25]. In other study, NS treatments of diabetic rats have been shown to increase the area of insulin immunoreactive beta-cells. These results have shown that NS may be used as an effective antidiabetic therapy [19]. There is a possibility that NS may have exerted its antiosteoporotic effects in diabetes by improving the blood sugar profile, but further studies are required to confirm this.

Besides that, previous literatures on NS and TQ have highlighted two properties that may be responsible for their antiosteoporotic effects, that is, antoxidative and antiinflammatory properties.

5. The Antioxidant Role of NS and TQ against Osteoporosis

Osteoporotic patients were found to be under oxidative stress as their lipid peroxidation levels were elevated and antioxidant enzymes reduced [26, 27]. Most risk factors for osteoporosis were associated with oxidative stress such as hypertension [28], diabetes mellitus [29], and smoking [30]. Exposure to oxidative stress would result in reduction of bone-mineral density [31]. In bone studies, ferric nitrilo-triacetate (FeNTA) was used to induce osteoporosis via oxidative stress [32]. The ferric ions (Fe3+) in FeNTA generate reactive oxygen species through Fenton reaction [33], which may damage bone cells by lipid peroxidation [34, 35]. They could also stimulate osteoclast formation and activity [32, 34, 36], impair osteoblastic function [34, 36], and decrease osteoblast recruitment and collagen synthesis [35]. Free radicals have also been shown to activate nuclear factor-kappa B (NFκB) and raised the levels of bone-resorbing cytokines, interleukin-1(IL-1), and interleukin-6 (IL-6) [32, 37].

Since it is apparent that oxidative stress may lead to osteoporosis, antioxidants may play a role in protecting bone against the damaging effects of free-radicals. Studies have shown that potent anti-oxidants such as tocotrienol and tocopherol, were able to protect bone against FeNTA (oxidative-stress-) induced osteoporosis [32].

It is interesting to find that the most significant property of TQ, the active compound of NS, is its antioxidative activities. It has been reported that the freeradical scavenging capability of TQ is as effective as superoxide dismutase [38]. It is most effective in scavenging superoxides, the reactive oxygen species which plays an important role in the activation of osteoclasts [31]. Since TQ is a potent antioxidant,

it is expected that it may be able to protect bone against osteoporosis due to oxidative stress. In a cancer study, TQ has been proven to suppress the FeNTA-induced oxidative stress, hyperproliferative response and renal carcinogenesis in rats [39]. In studies using rheumatoid arthritis model, TQ was reported to reduce the serum levels of IL-1 and Tumour Necrosis Factor-α [40, 41]. The bone turnover markers, alkaline phosphatase and tartrate-resistant acid phosphatase were also reduced, indicating stable bone formation and resorption activities. The NFκB activation was also blocked by TQ in time-dependent manner [41].

We suspect that the potent antioxidative properties of NS or TQ may account for the antiosteoporotic effects. Furthermore, the antiosteoporotic effects of tocotrienol, another potent antioxidant, have been established in several studies [32, 42].

6. Anti-Inflammatory Role of NS and TQ in Protecting against Osteoporosis

Inflammation is mediated by two enzymes, cyclooxygenase and lipoxygenase, which generates prostaglandins and leukotrienes from arachidonic acid, respectively [43]. Therefore, both prostaglandins and leukotrienes are the main mediators of inflammation [44]. TQ was believed to exert anti-inflammatory effects by inhibiting the synthesis of prostaglandins and leukotrienes [45, 46]. It was found to inhibit in a dose-dependent manner the cyclooxygenase and lipoxygenase pathways of rat peritoneal leukocytes that were stimulated with calcium ionophore A23187 [47].

The antiinflammatory activities of NS were investigated *in vitro*, using the cyclooxygenase (COX) assay. TQ was one of the compounds in NS that was able to inhibit the COX activity at concentrations comparable to indomethacin, a nonsteroidal antiinflammatory drug. Therefore, TQ contributed significantly to the anti-inflammatory activities of NS and has potential to be used as an alternative to nonsteroidal antiinflammatory drugs [48]. Another possible antiinflammatory mechanism of TQ might be suppression of nitric oxide production by macrophages [49].

The anti-inflammatory activity of NS was also demonstrated in studies using adjuvant-induced arthritis rat model. Intraperitoneal injections of NS-inhibited carrageenan-induced paw edema in a dose-dependent manner [50]. Similar reduction of formalin-induced paw edema was also observed with oral treatment of NS [51].

Osteoporosis is known to be caused by various endocrine, metabolic, and mechanical factors. Recently, plenty of evidences had surfaced, linking inflammation to osteoporosis. This has led to the opinions that inflammation may contribute to osteoporosis [52, 53]. Inflammatory conditions such as ankylosing spondylitis, rheumatoid arthritis and systemic lupus erythematosus were associated with higher incidence of osteoporosis [54–57]. The level of C-reactive protein, a marker of systemic inflammation was also found to be negatively associated with bone mineral density [58]. The extend of osteoporosis is directly related to the degree of inflammation, whereby systemic inflammation resulted in general bone loss, while for local inflammation, bone loss is restricted to the site of inflammation [52]. Elevations of proinflammatory cytokines with aging, gouty arthritis, rheumatoid arthritis and psoriatic arthritis may also contribute to osteoporosis [59–62].

Periodontitis is defined as the inflammation and infection of the ligaments and bones that support the teeth. A study has shown that the alveolar bone loss due to periodontitis was reduced by gastric feeding of TQ to rats. This was accompanied by reduction in osteoclast number and raised osteoblastic activity in TQ-treated rats [63]. The protective effects of TQ on the alveolar bone were thought to be contributed by it antioxidative and anti-inflammatory effects (Figure 1).

7. Effects of NS or TQ on Bone Fracture Healing

To date, there is no study on the effects of NS or TQ on the complication of osteoporosis, namely osteoporotic fracture. There is however, an animal study on fracture healing of nonosteoporotic bone, which resembled traumatic fracture healing [64]. In this study, anatomical observations indicated better healing pattern in rats with sustained delivery of TQ. It was concluded that the sustained levels of TQ may enhance bone fracture healing. More studies on fracture healing are required before the effectiveness of NS or TQ in promoting fracture healing can be concluded. In the most recent study, TQ was found to accelerate bone formation and shorten the retention period in rapid maxillary expansion procedure [65].

8. Toxicity of NS

As in other herbal preparations, safety is one of the important criteria before NS can be considered for medicinal use. In an acute toxicity study of NS in mice, toxicity signs were first noticed after 4 to 6 hours of NS extract administration. The median lethal dose (LD_{50}) was about 470 mg/kg body weight. The toxic signs observed were decreased locomotor activity, decreased sensitivity to touch, and jerking. After 10 hours of administration, the mice exhibited tachypnoea, prostration, and reduced food intake [51]. In other acute and chronic toxicity studies in mice and rats, NS was found to have a wide margin of safety at therapeutic doses. However, attention should be paid to the rise in hematocrit and hemoglobin levels and decrease in white blood cells and platelet counts [66].

Sustained delivery of TQ for 30 days using Tri-Calcium Phosphate Lysine (TCPL) capsule loaded with 0.02 grams of TQ to adult male rats have shown little or no side effects on the major vital and reproductive organs [64]. In an *in vitro* study, TQ at the concentration of 0 to 10 μM was not cytotoxic to isolated fibroblast-like synoviocytes [41].

The suitable dose for NS extract in human can be extrapolated from animal studies based on the human equivalent dose of no observed adverse effect level (NOAEL) [67]. The human dose is approximately 0.6 mg/kg/day per oral of TQ [41] or 0.05 mL/kg/day per oral of NS extract, which was well received by postmenopausal women [16]. NS extract should be used with precaution in pregnant ladies

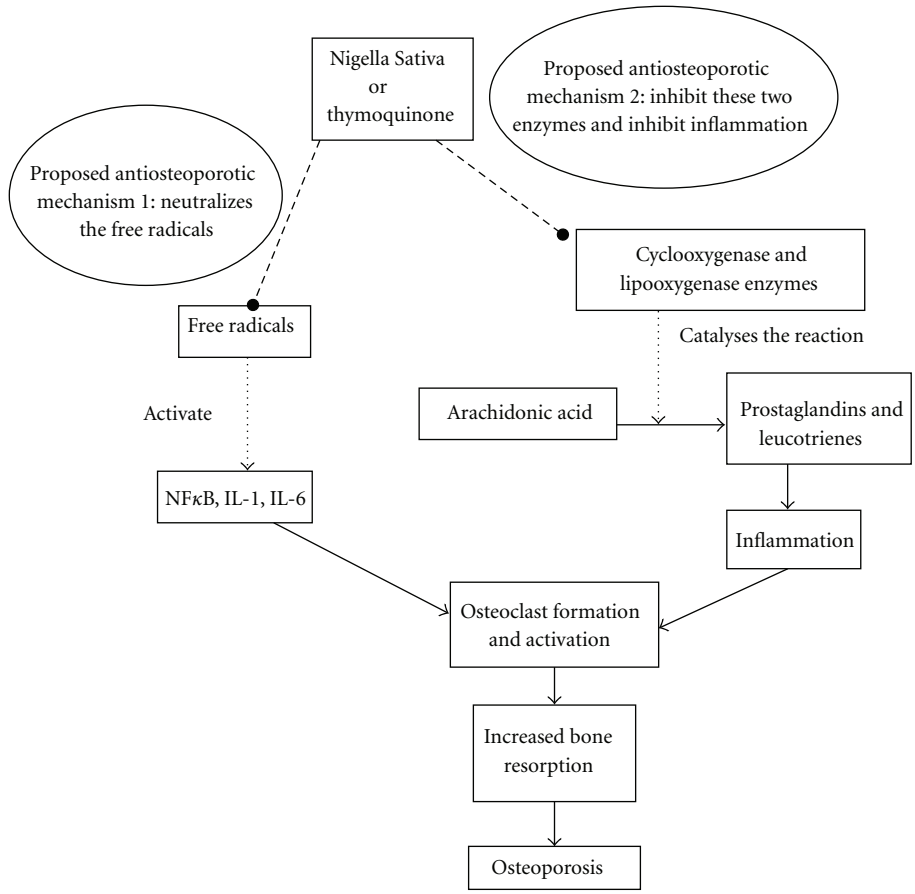

Figure 1: The two pathways which may lead to osteoporosis are shown, that is, activation of osteclastic bone resorption activity by free radicals and by inflammation. The inhibition of these two pathways by *Nigella sativa* or Thymoquinone, its active component, may account for the mechanisms involved in prevention of osteoporosis.

and children due to its well-known hypoglycemic properties. Diabetic patients should consult with their physicians before taking NS [19, 68]. In children, it was recommended that NS should be administered in weight-adapted doses and given after meals [69]. NS at doses below 80 mg/kg was considered safe in children as there were no adverse effects being reported [70].

9. Conclusion

NS or TQ has shown potential as a safe and effective antiosteoporotic agent. However, more studies on the effects of NS or TQ using various animal osteoporotic models are required. Once the antiosteoporotic effectiveness of NS or TQ has been established, human studies can be carried out.

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Review Article

Role of Medicinal Plants and Natural Products on Osteoporotic Fracture Healing

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Popularly known as "the silent disease" since early symptoms are usually absent, osteoporosis causes progressive bone loss, which renders the bones susceptible to fractures. Bone fracture healing is a complex process consisting of four overlapping phases—hematoma formation, inflammation, repair, and remodeling. The traditional use of natural products in bone fractures means that phytochemicals can be developed as potential therapy for reducing fracture healing period. Located closely near the equator, Malaysia has one of the world's largest rainforests, which are homes to exotic herbs and medicinal plants. *Eurycoma longifolia* (Tongkat Ali), *Labisia pumila* (Kacip Fatimah), and *Piper sarmentosum* (Kaduk) are some examples of the popular ethnic herbs, which have been used in the Malay traditional medicine. This paper focuses on the use of natural products for treating fracture as a result of osteoporosis and expediting its healing.

1. Fracture

Fracture is defined as a complete or incomplete separation in the continuity of the bone [1]. Fracture healing is a complex physiological process that involves the coordinated participation of hematopoietic and immune cells within the bone marrow. In conjunction with vascular and skeletal cell precursors, it also includes mesenchymal stem cells (MSCs), which are recruited from the circulation and the surrounding tissues [2, 3].

The two basic types of fracture healing are the primary or direct fracture healing and the secondary or indirect fracture healing. Primary (direct) fracture healing occurs with very minimal callus formation. It is a direct attempt of bone to reestablish its continuity and thus requires direct contact of cells in the cortex [4]. Primary healing occurs rarely as the majority of fracture repairs undergo secondary or indirect healing [5]. Based on the histological observations, secondary fracture healing occurs in four overlapping phases, which are haematoma formation, early inflammatory (two to four weeks), repair (proliferation and differentiation, within a month or two), and late remodelling phase (lasting for months or years) [6].

The clinical impact of fractures is substantial. Significant pain, disability, and deformity will trail following a fragility fracture. If the fracture union is not achieved, the patient may suffer long-term disability. Degenerative joint disease distal to the fracture and reflex sympathetic dystrophy are other recognized complications [7]. Hip fractures, even less common than vertebral fractures, contribute to the majority of the mortality, morbidity, and costs associated with osteoporosis [8]. Osteoporotic fractures are still an unsolved problem to the surgeon as well as for the patient. There are two ways of improving the fracture healing process: first, the developments of special implants to avoid implant failure; second, the improvement of bone quality to speed up and improve callus formation and otherwise to biologically advance implant fixation [9].

2. Osteoporosis

Osteoporosis is a heterogeneous cluster of abnormal processes characterized by the net loss of bone. It results in a decrease in total mineralized bone without a decrease in the ratio of bone mineral to the organic matrix [10, 11]. As a result, there is a decrease in the overall amount of bone. The bone loss affects both cortical and trabecular bone, with trabecular bone loss more predominant in postmenopausal osteoporosis. Consequently, osteoporosis would lead to a bone with less tensile strength and significantly more susceptibility to fracture with less force [10]. This syndrome is clinically silent but progressive, usually only noted when a fracture occurs [12]. It is one of the most major public health problems with a mortality of 30% in the first year following the osteoporotic hip fracture [13].

During the early menopausal years in women, there is a dramatic reduction in circulating estrogen. As a result, there is an increase in the rate of bone resorption, but not reformation. This creates an imbalance and sets the stage for osteoporosis [14, 15]. Although bone loss in women slows after the early postmenopausal years, loss continues through the latter decades of life, and in very old age the rate of loss increases again [16, 17]. In addition to hormonal changes, age-related bone loss is also due to reduced ability to utilize calcium [18], decreased vitamin D supply due to lower production and reduced absorption, and decreased activation of vitamin D by the kidneys [19, 20]. All of these factors contribute to the increase with age in another hormone—parathyroid hormone [21, 22]. When there is too much parathyroid hormone released in the body (hyperparathyroidism), bones release excessive calcium into the blood stream. As a direct result, bones lose their density and hardness.

3. Osteoporosis in Male

Although traditionally regarded as a disease of women, especially after menopause, osteoporosis also occurs frequently in men. Men steadily lose bone mineral density with aging, and one in five men over 50 will suffer an osteoporotic fracture [35]. Almost 30% of all hip fractures are in men, and the mortality following a hip fracture is substantially higher in men than in women [36]. Men represent between 20% and 40% of all patients with each types of fracture that frequently affect the hip, vertebrae, forearm (wrist), and humerus. These fragility fractures may occur following minimal trauma such as a fall from standing height [37].

Bone loss in men has many causes, and often the same patient is affected by several of these. As in women, primary osteoporosis includes age-dependent and idiopathic osteoporosis. Age-related bone loss is dependent on estrogen production in both sexes, not only in women [38, 39]. While estrogen is considered the female sex hormone, men also produce some estrogen. And as they age, men may experience a decrease in their ability to convert male sex hormones called androgens into estrogen [40, 41]. Recent research suggests that estrogen deficiency and reduced levels of other sex hormones may be a cause of osteoporosis in men. The main causes of secondary osteoporosis in men are excessive alcohol use, treatment with glucocorticoids, and hypogonadism, including that experienced by men receiving androgen deprivation therapy (ADT) for prostate cancer [42].

It is impossible to reverse the osteoporosis to its original form; it is just possible to prevent deterioration. Analgesic drug, heat, massage, and rest can be used to relief the osteoporotic pain [43]. The use of calcium, estrogen, calcitonin and vitamin D has been recommended too. Recently, few studies concerning the role of antioxidants in osteoporosis have been published, and the results show that there is a correlation between antioxidants and osteoporosis. Free radicals play an important role in many diseases such as diabetes, degenerative disorders, and cancer [44]. In normal conditions, there is a balance between free radicals and antioxidants defensive system. Sometimes, this balance is lost, which is called oxidative stress. Oxidative stress has been the centre of attention in recent studies of osteoporosis pathogenesis [45].

4. Importance of Natural Product

Today, it is estimated that about 80% of individuals in the developing countries still rely on traditional medicine-based largely on plants and animals for their primary health care. Herbal medicines are currently in demand, and their popularity is increasing day by day [46]. Herbal drugs are fairly preferred due to their effectiveness, fewer side effects, and relatively low cost [47]. It also has a brighter prospect in the global market. The market for ayurvedic medicines is estimated to be expanding at 20% annually [48].

Due to some adverse effects or lack of efficacy of synthetic drugs, the potential efficacy of traditional medicines has stimulated the interest of scientists and doctors to turn on to traditional medicines for treatment of some chronic and difficult diseases, including the treatment for osteoporosis [49]. In traditional Chinese medicine (TCM), osteoporosis is classified as "rheumatism involving the bone" and "atrophic debility of bones" [50]. Based on the theory of "Kidney dominates bone" in TCM, many medicinal herbs have been prescribed to treat bone metabolic disorders for long time [51].

The *Huang Di Nei Jing* (*The Yellow Emperor's Classic of internal Medicine*) says, "The Meridians move the qi and blood. As a result, tendons and bones get nourished whereas joints get facilitated." *Astragalus membranaceus* Bge is one of the most popular herbs for restoring Qi and also can be applied for the osteoporosis treatment. The water extract of the root of *A. membranaceus* prevented osteoporosis induced by dexamethasone or by ovariectomy in rats by inhibiting osteoclast, decreasing bone absorption and promoting bone formation [23]. In TCM, *Curculigo orchioides* Gaertn., *Epimedium grandiflorum* Morr., *Morinda officinalis* How., *Cistanche salsa* G., *Eucommia ulmoides* Oliv., *Psoralea corylifolia* L., *Cuscuta chinensis* Lam., and *Dipsacus japonicus* Mip. possess the efficacy of tonifying kidney, strengthening Yang, and strengthening tendons and bones [24]. On

Table 1: Table revealing earlier studies on traditional medicinal plants and their usage part.

Plants	Part used	System of medicine	References
Astragalus membranaceus Bge	Roots	Chinese	[23]
Curculigo orchioides Gaertn	Rhizome	Chinese	[24]
Epimedium grandiflorum Morr.	Roots and leaves	Chinese	[24]
Morinda officinalis How.	Root	Chinese	[25]
Cistanche salsa G. Beck	Stem	Chinese	[25]
Eucommia ulmoides Oliv.	Bark	Chinese	[26]
Psoralea corylifolia L.	Fruit	Chinese	[25]
Cuscuta chinensis Lam.	Seed	Chinese	[25]
Dipsacus japonicus Mip.	Root	Chinese	[24]
Fructus Ligustri Lucidi	Fruit	Chinese	[24]
Ficus religiosa	Young bark and stem bark	Ayurveda	[27]
Cissus quadrangularis	Stems	Ayurveda	[28]
Alangium salvifolium	Root bark	Ayurveda	[29]
Christiella subpubescens	Rhizome and tuberous	Ayurveda	[29]
Diospyros chloroxylon	Stem bark	Ayurveda	[29]
Erythrina fusca	Seed	Ayurveda	[29]
Mimosa intsia	Root	Ayurveda	[29]
Phoenix loureiroi	Tender stem	Ayurveda	[29]
Rhaphidophora pertusa	Aerial roots and leaves	Ayurveda	[29]
Symplocos loba	Bark	Ayurveda	[30]
Oryza sativa Linn	Fruits and roots	Ayurveda	[31]
Eurycoma longifolia	Root	Malay	[32]
Labisia pumila	Leaves and roots	Malay	[33]
Piper sarmentosum	Leaves	Malay	[34]

the other hand, Fructus Ligustri Lucidi (FLL, Chinese name, Nvzhenzi) is one of the examples of herbs for nourishing *Yin* due to estrogen deficiency [52]. FLL can act on pituitary gland and further modulate endocrine function [53] and also useful in the prevention of bone marrow loss in cancer patients receiving chemotherapy [54].

The term "ayurveda" can be defined as knowledge *(veda)* of the lifespan *(ayu)*. This knowledge is recorded in the ancient literature of India, referred to collectively as the Veda [55]. Ayurvedic medicine is based on breathing techniques, meditation, and yoga [56]. In Ayurvedic culture, the young bark of *Ficus religiosa* (family-Moraceae) also known as Ashwatha or Ashvattha, has been widely used in the treatment of bone fracture [27]. The stem bark cleaned with urine of a boy (below 7 years old) is taken and ground. Two spoonfuls of paste were administered twice daily for 21 days. Paste is also applied on the affected part and bandaged [29]. *Cissus quadrangularis* (family-Vitaceae), commonly known as the Asthishunkala, is an indigenous medicinal plant of India [57]. Methanolic extract of Cissus quadrangularis has been reported to promote the healing process of experimentally fractured radius-ulna of dogs, proven by radiological and histopathological examinations [28].

The native of Eastern Ghats (India's eastern coast area) use different types of plants to treat bone fractures. According to Eastern Ghats folklore, *Alangium salvifolium*, *Christiella subpubescens*, *Diospyros chloroxylon*, *Erythrina fusca*, *Mimosa intsia*, *Phoenix loureiroi*, and *Rhaphidophora pertusa* may be used for the treating bone fracture [29]. The bark of Symplocos loba is used externally for poulticing fractures, as it promotes the healing of bones [30]. *Oryza sativa* Linn (family-Poaceae), commonly known as Asian rice, is used in fracture healing and as a poultice to reduce inflammation at the affected areas [31]. Table 1 reveals the earlier studies on traditional medicinal plants by different cultures and their usage part.

5. Malay Traditional Medicine

The principles of Malay traditional medicine are generally based on the Arabic *Unani* medicine and Galenic philosophy in addition with other practices of Indonesian, Chinese, Indian, and *orang asli* (indigeneous people) traditional medicines [58]. It consists of chants (*jampi*), prayers (*doa*), massage, abstinence (*pantang*), and other practices, plus various natural resources from plants, animals, microorganisms and minerals for the purpose of treating and preventing illnesses, and for rehabilitation and health promotion. Medications containing single or compound medicinal plants may be dispensed in many forms, such as powders, capsules, pills, *makjun*, medicated oils, simple distillates, decoctions, infusions, paste, and poultices. Documentation of Malay TM practices is rather scarce. Most practices rely on old references, such as *Mujarabat Melayu*, *Tajul Muluk*, *Tajus as Salatin,* and *Surat Tib Ubat* [33]. The earliest scripts on

the ethno-botanical uses of Malaysian plants dated back to the time of British colonialism. Some of these were publications [59, 60] providing monumental references for researchers on the utilization of medicinal plants in Malay traditional medicine.

6. Tongkat Ali

Eurycoma longifolia (EL) is a traditional medical plant known as *Tongkat Ali* in Malaysia, *Tung Saw* in Thailand, and *Pasak Bumi* in Indonesia. The plant is under the family of *Simaroubaceaea*. The water decoction of its root is a well-known folklore medicine to enhance sexuality, fertility, and antiaging [61]. The herb contains Quassinoid alkaloid with properties curing Malaria, allergies, alleviating fevers and reduced tumors. The water-soluble extract contains among other things, tannins, high molecular weight polysaccharides, glycoproteins, and mucopolysaccharides [62].

Studies have revealed that *EL* contain *eurycomanone, eurycomanol, eurycomalactone,* and alkaloid that may help to increase the free testosterone level in the blood and also inhibit sex hormone binding globulin [63]. Testosterone has been approved to preserve bone mass development. Therefore, testosterone can help to prevent osteoporosis. Testosterone replacement could increase the mass and density of the bone and become efficient treatment for osteoporosis [64]. The main treatment to prevent diseases related to testosterone deficiency is hormone replacement therapy whereby testosterone is injected intramuscularly [65]. Some patients may refuse this treatment because of the painful administration of testosterone and its associated adverse effect, especially prostate cancer. Tongkat Ali can be used as an alternative to increase the testosterone level since *EL* is believed to have proandrogenic effect [66].

A study has shown that EL has a great potential as an alternative agent to testosterone replacement in treating androgen-deficient osteoporosis in men. It has good safety profile and convenient oral route of administration [32]. The active compound in *EL* can increase the level of testosterone in blood. The increase of testosterone can induce the androgen receptor, which is located in osteoblast and osteoclast cell. Testosterone and 5-α-dihydrotestosterone can inhibit *receptor activator of nuclear factor kappa-β ligand (*RANKL) and colony-forming unit-macrophages and further reducing osteoclast numbers [67]. As a result, the bone resorption process will be decreased and thus bone mass density will be maintained. These properties are essential in bone fracture healing process.

7. Kacip Fatimah

Kacip Fatimah, also known as *Labisia pumila* (LP), is a member of small genus of slightly woody plants of the family Myrsinaceae [68, 69]. The locals know it by the name as *Selusuh Fatimah, Rumput Siti Fatimah, Akar Fatimah, Pokok Pinggang,* and *Belangkas Hutan* [60, 70]. Its water extract is traditionally consumed especially by the Malay women to treat menstrual irregularities and painful menstruation, help contracting birth passage after delivery, and to promote sexual health function [71]. It has also been used to treat dysentery, gonorrhoea, rheumatism, and sickness in bones [72, 73].

Postmenopausal women are prone to osteoporosis due to the reduction in estrogen level. Estrogen can induce osteoclasts apoptosis and inhibit osteoblasts apoptosis, which will indirectly reduce bone resorption and increase bone-formation activity [74]. Proinflammatory cytokines such as, IL-6 and IL-1 may influence osteoclastogenesis by stimulating self-renewal and inhibiting the apoptosis of osteoclasts progenitors [75, 76]. They promote osteoclasts differentiation which is an important stimulator of bone resorption that has been linked to accelerated bone loss seen in postmenopausal women [74]. Estrogen is able to suppress the production of these proinflammatory cytokines. LP, which has been opposed to exert phytoestrogen property, can be used as an alternative to estrogen replacement therapy (ERT) in postmenopausal inflammation-induced osteoporosis. In contrast to ERT, which can cause many harmful side effects, LP, which originated from natural resources, will not cause any side effect, if taken within its safe therapeutic dose [77].

Based on previous studies, LP has been shown to exhibit antioxidative properties due to the presence of flavanoids, ascorbic acid, beta-carotene, anthocyanin, and phenolic compounds [78, 79]. Flavonoid has been shown to be highly effective scavenger of free radicals that are involved in diseases such as osteoporosis and rheumatism, which is associated with aging due to oxidative stress [80]. Anthocyanin and phenolic, on the other hand, not only play a role as antioxidant agents, but also as anti-inflammatory agents [52, 81, 82]. These antioxidative and anti-inflammatory properties of LP extract explained the effectiveness of this medicinal plant against various diseases such as osteoporosis, rheumatism, and women sexual function.

8. Kaduk

Piper sarmentosum (PS) (*Piperaceae,* Malay name: *Daun Kaduk*) is a creeping shrub with erect branchlets that can grow up to 20 cm. In Malay culture, the water decoction of its leaves is being used for treating diabetes, hypertension, cough, and joint aches [83]. The extract of the different parts of PS plant is known to possess benefits. It also possesses antioxidant, antiplasmodial, antituberculosis, anti-inflammatory, anticarcinogenic, and hypoglycemic properties [84, 85]. The methanolic extract of PS consists of a high natural antioxidant scavenger, naringenin, a flavonoid group with high superoxide scavenging activity [83]. Past experimental studies showed that flavonoids prevented ovariectomy-induced osteopenia and strengthened bone in ovariectomised animals [86]. Therefore, fracture healing properties of PS may be attributed to the action of flavonoids present in the PS extract.

A previous study on experimental fractured animal models revealed better fracture healing following PS administration during the late phase of fracture healing [34, 87]. In addition, Ima-Nirwana et al. [26] had observed

an antiosteoporotic effect of PS aqueous extract in the adrenalectomised rats. The beneficial effect of PS on osteoporosis and fracture healing is most probably attributed to the antioxidative actions of the PS flavonoids, which may prevent oxidative stress [26]. Previous experimental studies on animals confirmed that osteopenia following oestrogen loss can be prevented by the supplementation of antioxidants [88]. It has also been found that glutathione peroxidase, an antioxidant enzyme secreted by osteoclasts, has a major role in reducing H_2O_2 [89]. Hence, supplementation of antioxidants can strengthen the bone and promote fracture healing in osteoporotic patients [90]. PS is rich in a natural antioxidant superoxide scavenger (Naringenin), which may have beneficial effect in promoting fracture healing most probably by reducing ROS through its free radical-scavenging activity. Hence, PS may have the potential to be added as antioxidant supplements to the current treatment modalities.

9. Conclusion

As a conclusion, a serious concern should be taken on osteoporosis. Because of the dormant properties of the disease, it is hard to recognize the symptoms until fracture occurs. Several studies have shown that androgen deficiency can lead to osteoporotic fractures. Therefore, various treatments should be considered to promote the healing period of the fracture. Natural products could be considered as a natural heritage from Mother Nature as a source of medicine. Thus, more extensive studies should be conducted to explore the healing properties of different types of medicinal plants to produce an alternative and effective treatment for the osteoporotic patient. A few of Malay's famous medicinal plants like EL, LP, and PS, can be used to enhance fracture healing. The plants possess the androgen-like and antioxidative properties, which are important in the promotion of bone fracture healing.

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Review Article

The Effects of Tualang Honey on Bone Metabolism of Postmenopausal Women

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Osteoporosis which is characterized by low bone mass and microarchitectural deterioration with a consequent increase in bone fragility can be associated with various stimuli such as oxidative stress and inflammation. Postmenopausal women are more prone to osteoporosis due to reduction in estrogen which may further lead to elevation of oxidative stress and lipid accumulation which will promote osteoblasts apoptosis. Proinflammatory cytokines are elevated following estrogen deficiency. These cytokines are important determinants of osteoclasts differentiation and its bone resorption activity. The main treatment for postmenopausal osteoporosis is estrogen replacement therapy (ERT). Despite its effectiveness, ERT, however, can cause many adverse effects. Therefore, alternative treatment that is rich in antioxidant and can exert an anti-inflammatory effect can be given to replace the conventional ERT. Tualang honey is one of the best options available as it contains antioxidant as well as exerting anti-inflammatory effect which can act as a free radical scavenger, reducing the oxidative stress level as well as inhibiting proinflammatory cytokine. This will result in survival of osteoblasts, reduced osteoclastogenic activity, and consequently, reduce bone loss. Hence, Tualang honey can be used as an alternative treatment of postmenopausal osteoporosis with minimal side effects.

1. Introduction

Natural product has been used for medicinal purposes long before recorded history. Wide arrays of natural products used to treat a variety of ailments are also known as traditional medicine which is defined by the World Health Organization (WHO) as the sum total of the knowledge, skills, and practices based upon the theories, beliefs, and experiences indigenous to different cultures, whether explicable or not, used in the maintenance of health as well as in the prevention, diagnosis, improvement, or treatment of physical and mental illness [1]. Recently, it has been estimated that 80% of worldwide population rely on traditional medicines in order to meet their health care needs as an alternative to the conventional medicine [2]. Alternative medicine was first discovered as early as 5000 B.C in the Middle East, followed by the development of Ayurvedic, Chinese and Western medicine. Acceleration of the use of alternative treatment nowadays is due to rise in prevalence of chronic diseases, increasing costs of medical care and public concern on the rising adverse effects of conventional medicine.

Prevalence of chronic diseases is now increasing and contributed the most in medical care spending [3]. One of the serious diseases that is now becoming an important socioeconomic burden in many countries is osteoporosis. Osteoporosis is a systemic skeletal disease characterized by low bone mass and microarchitectural deterioration with a consequent increase in bone fragility with susceptibility to fracture [4]. According to National Osteoporosis Foundation, osteoporosis has become a major public health threat. The amount of medical care expenditure is rising tremendously due to increased elderly population as a result of increased longevity [5, 6]. Main factor causing osteoporosis are age-related decline in level of sex hormones

(e.g., postmenopausal women), and secondary osteoporosis that can be due to diseases such as hypogonadism, hyperthyroidism, and gastrointestinal malabsorption syndromes [7]. Prolonged used of medications such as steroid prednisone, barbiturates, thyroxine and diabetic medications, excessive alcohol consumption, and smoking can contribute to bone loss as well [8–10]. Women are prone to tremendous estrogen reduction after menopause which will result in bone loss. This explains the fact that about 80% of the economic burden of osteoporosis has been attributed to its occurrence in women, especially in postmenopausal women [11]. Men, on the other hand, have a greater total bone mass than women, hence they are less likely to develop osteoporosis compared to women [12, 13].

To date, the main treatment of osteoporosis is hormone replacement therapy (HRT) and bisphosphonate. HRT, which contains estrogen has been shown to reduce hip fractures [14], wrist, vertebral and all nonvertebral fractures [15, 16]. Although HRT is known to be very effective in increasing sex hormone level and improving bone mass, it can also produce many adverse effects, particularly breast cancer [17]. Estrogen can enhance the rate of cell proliferation in glandular tissue of breast and could potentially act both in initiation and promotion of breast cancer [18]. A study by Collaborative Group for Hormonal Factors in Breast Cancer has found out that women who had used HRT for longer than 5 years, the relative risk of breast cancer was 1.35 [19]. Other than that, prolonged use of HRT can also result in cardiac infarction, stroke, and pulmonary emboli [20]. Route of administration of HRT can be via oral, transdermal, patches, subcutaneous, and intramuscular injection. The most widely used method of administration is via intramuscular injection due to its rapid absorption. However, this method can be very painful. Other than HRT, bisphosphonates such as alendronate and risedronate are now widely used and have been approved to be used not only for treatment but also as prevention of osteoporosis. Alendronate and risedronate are able to inhibit osteoclast-mediated bone resorption and reduce risk of fracture effectively. However, these treatments may contribute to adverse effects such as abdominal pain, joint and muscle pain, constipation [21], hypocalcemia, and osteonecrosis of the jaw bones [22].

Regardless the effectiveness of current available treatments of osteoporosis, alternative natural medicines are needed to treat this disease with minimal side effects. Honey is documented as one of the most ancient traditional remedies in history [23]. The first written reference to honey was a Sumerian tablet writing dating back to 2100–2000 B.C which mentions honey's use as a drug and ointment [24]. Since then it has become useful for both nutritional and medical purposes [25]. Honey has been of proven value in accelerating wound healing, as well as treating ulcers and skin infection [26, 27]. It has also shown to be an effective antioxidant and anti-inflammatory agent [28, 29]. Honey consists of primarily sugars such as monosaccharides, disaccharides, oligosaccharides, and polysaccharides [30] as well as enzymes such as glucose oxidase, diastase, invertase, catalase, and peroxidase [31]. Other chemical contents of honey are organic acids, ascorbic acids, vitamins, amino acids, proteins, flavonoids and phenolic acids [32, 33]. These chemical constituents of honey made it beneficial in human health.

To date, there has been a resurgence in the use of honey in treating a wide array of diseases which are not only limited to wound healing. Malaysia is one of the countries in Asia that is well known for its varieties of honeys such as Tualang, Gelam, and Belimbing honey. Tualang honey is found on Asia's largest tree, Tualang tree or *Koompassia excelsa*. It has been used widely by the local community in treating wound, as beauty products, antiageing products, and health supplements [34]. Since the last few years, Tualang honey has been used widely by the researchers in order to discover its hidden potential values. The major components of Tualang honey are furfural derivatives such as 5-(hydroxymethyl)-furfural, furfural 2-furyl methyl ketone, 5-methyl furfural, and fatty acids such as palmitic acid, ethyl linoleate, and ethyl oleate [35]. Tualang honey is known for its antimicrobial, antiparasitic, antioxidant, and anti-inflammatory effects which could be due to its chemical content that is high in antioxidative properties such as flavonoids and phenolic acids [36]. Recently, Tualang honey has been studied for its effect on bone density and a positive result was exhibited. This could make it a good potential as an alternative treatment to osteoporosis, replacing the conventional treatment. This paper focuses on the mechanisms of Tualang honey on bone density and its antiosteoporotic values particularly in postmenopausal osteoporosis women.

2. Oxidative Stress in Postmenopausal Osteoporosis and Antioxidative Role of Tualang Honey

Bone is continually remodelled throughout life where bone resorption activity by osteoclasts is always followed by bone formation by osteoblasts [37]. Osteoporosis occurs when this bone remodelling cycle is impaired. Hence, any factors that can impair the rate of bone remodelling will contribute to bone loss such as in postmenopausal women where estrogen reduction is the main factor. Estrogen deficiency following menopause can lead to bone loss via direct effects of estrogen on osteoclasts [38] and upregulation of osteoclastogenesis. Upregulation of osteoclastogenesis occurs via activation of receptor activator of nuclear factor kappa-B ligand (RANKL) [39] and diminishes production of osteoprotegerin (OPG), which function as osteoclastogenesis inhibitory factor [40]. Postmenopausal osteoporosis can be associated with oxidative stress which results from a disturbance in the balance between free radicals production and antioxidant protective activity. Estrogen can be considered as an antioxidant as it was found to exhibit antioxidant protection of lipoproteins in the aqueous system and was shown to increase the expression of glutathione peroxidase (GPx) in osteoclasts [41]. When body is subjected to high level of oxidative stress following estrogen reduction, lipid accumulation will occur. This will promote osteoblast apoptosis and simultaneously upregulating reactive oxygen

species (ROS) production, particularly hydrogen peroxide (H_2O_2) and superoxide anion [42–44]. ROS may increase bone resorption through activation of NF-κB which plays an important role in osteoclastogenesis. ROS can also promote osteoclast resorption activity directly or by mimicking receptor activator of NF-κB (RANK) signalling which results in osteoclast differentiation [45]. Some *in vitro* and animal studies have reported that oxidative stress decreases the level of bone formation by modulating the differentiation and survival of osteoblasts [46, 47]. Postmenopausal women are not only subjected to high level of free radicals and oxidative stress, but in previous studies it was also found that they exhibited higher erythrocyte nitric oxide (NO) levels compared to nonporotic women [48]. NO is able to enhance the ability of cytokines to stimulate osteoclast activity [49] and potentiates the inhibitory effects of those cytokines on osteoblast growth [50, 51].

Ovariectomized rat is often used in postmenopausal women research as a model for osteoporosis which exhibits a progressive loss of bone matrix through a process that is similar to what occurs during postmenopausal osteoporosis [52]. This include increased rate of bone turnover with resorption exceeding formation, greater loss of cancellous than cortical bone, and decreased intestinal absorption of calcium [53]. The bone loss following ovariectomy can be due to oxidative stress. In a study by Muthusami et al. (2005), there was a significant decrease in the levels of antioxidant enzymes; superoxide dismutase (SOD), glutathione-s-transferases (GST) and GPx in the femur of ovariectomized rats. Hydrogen peroxide and lipid peroxidation were found to increase. These results have shown that oxidative stress was induced in ovariectomized rats which probably is the major factor behind the bone loss in these animals [54]. The ability of cells to scavenge the harmful ROS is mainly dependent upon the efficacy of the antioxidant defense system which comprises of enzymatic antioxidants such as SOD, GPx and catalase (CAT) and nonenzymatic antioxidants such as glutathione (GSH), vitamin C and E [55, 56]. Hence, supplementation with antioxidant is required to combat the oxidative stress and subsequently preventing bone loss. Based on recent published studies, amongst the natural products that are able to prevent osteoporosis are vitamin E tocotrienol and *Labisia pumila*. Both of these natural products possess antioxidative effects [57, 58]. According to Nazrun et al. (2011) tocotrienol exert a potent antioxidative property which is able to suppress bone resorbing cytokines and thus preventing osteoporosis.

Over the past few years, a resurgence of interest in the ability of honey as an antioxidant followed by its protective effects on bone has occurred. Tualang honey has been shown to exhibit good antioxidant and antiradical activities [59, 60]. Study performed by Zaid et al. (2010) has shown that daily consumption of Tualang honey for two weeks in female ovariectomized rats was able to promote an increase in bone density [61]. This positive effect on bone of ovariectomized rats is probably due to the antioxidants found in honey such as flavonoids and phenolic acids [62, 63]. The main phenolic and flavonoid compounds in Tualang honey include kaempferol, quercetin, ellagic acid, gallic acid, hesperetin, and catechin [64].

A study on the antioxidative compounds of Tualang honey has found that Tualang honey had the highest total phenolic and protein content compared to other types of honey; Gelam, Indian forest and Pineapple honey. Among this group of honeys, Tualang honey also had the highest ascorbic acid content which may be responsible for the elevated scavenging of the ROS. In contrast to those types of honey mentioned, Tualang honey had also shown the highest DPPH radical scavenging activity, suggesting that it may contain the most effective free-radical scavenging compounds [60]. These findings have shown that Tualang honey is a good source of antioxidant that is able to scavenge free radicals, resulting in reduced bone resorption activity by osteoclasts which subsequently maintaining the bone health.

Previous studies reported that flavonoids mainly quercetin and kaempferol exert a potent inhibitory effect on osteoclastic bone resorption and apoptosis in a rabbit long bone osteoclast model [65]. They are also involved in inhibition of NF-κB and activator protein (AP-1), a transcription factor highly related to osteoclastic differentiation [66]. There were some investigations suggesting that quercetin plays an important role in bone loss inhibition by affecting osteoclastogenesis and accelerating TNF-α-induced osteoblast growth inhibition and apoptosis [67]. Flavonoids may also inhibit RANKL-induced formation of multinucleated osteoclasts and expression of osteoclastic differentiation markers; RANK and osteocalcin receptor [68]. Flavonoids have been shown to inhibit production of nitric oxide and expression of inducible nitric oxide synthase (iNOS) [69] which will result in inhibition of osteoclast activity. These protective mechanisms of flavonoids on bone strongly indicate that it can be considered as protective agent against bone loss.

3. Anti-Inflammatory Effects of Tualang Honey

Osteoporosis is more prevalent in inflammatory conditions such as rheumatoid arthritis, systemic lupus erythematosus (SLE), haematological diseases, inflammatory bowel disease and other inflammatory diseases when compared to the healthy population [70]. Proinflammatory cytokines such as tumor necrosis factor (TNF)-α, interleukin (IL)-1, IL-6, IL-7, IL-11, IL-15 and IL-17 are elevated in these conditions [71]. Elevation of these cytokines will then result in increase production of prostaglandin E_2 (PGE_2), an inflammatory mediator which consequently stimulates osteoclastic activity [72]. Therefore, osteoporosis can be strongly associated with inflammation. Activated osteoclasts are usually found in the presence of accessory cells including stromal cells, cells in osteoclast lineage and cells involved in the inflammatory responses. These cells posses the ability to express proinflammatory cytokines. Thse proinflammatory cytokines has been shown capable of stimulating osteoclastic bone resorption [73].

There is evidence from previous studies to suggest that postmenopausal bone loss may be linked to activation of osteoclasts by proinflammatory cytokines [74–76]. Estrogen

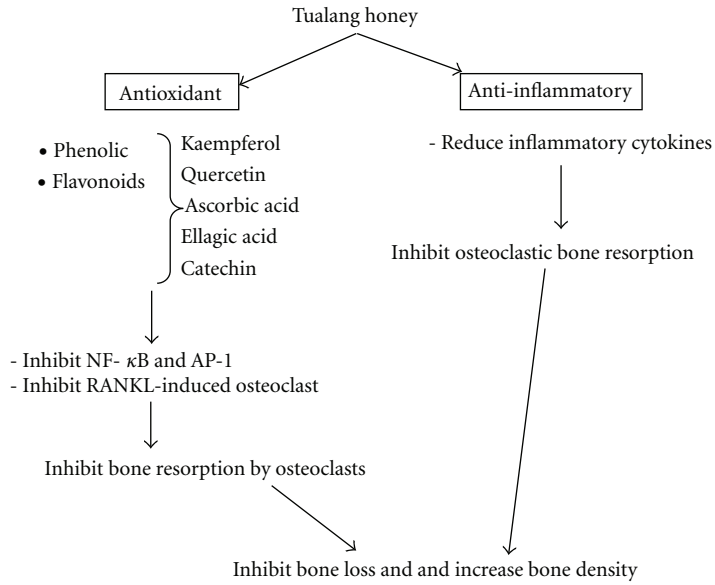

Figure 1: Schematic diagram of antioxidative and anti-inflammatory actions of Tualang honey on bone.

is able to suppress production of these cytokines and stimulate production of OPG [77, 78]. Thus, estrogen withdrawal following menopause will result in an increase in these cytokines and simultaneously lead to downregulation of OPG, resulting in local inflammation in the bone. Ovariectomy in rats was accompanied by an increase in production of TNF-α and IL-1 [79]. This may influence osteoclastogenesis by stimulating self-renewal and inhibiting the apoptosis of osteoclast progenitors [80]. They also support osteoclast formation and activation mediated by RANKL and macrophage colony-stimulating factor (M-CSF). These studies have shown that postmenopausal osteoporosis can be associated with inflammation.

Honey does not only exhibit antioxidative effects but it also possesses anti-inflammatory effects. Previous studies have shown that the level of PGE_2 was reduced after the ingestion of honey [81]. Recent study has also shown that honey was able to exert anti-inflammatory effect via inhibition of nitric oxide [82]. Consumption of Tualang honey for two weeks in female ovariectomized rats was able to increase bone density. Tibial bones of the ovariectomized rats treated with Tualang honey were comparable to those of control nonovariectomized rats [61]. This result can strongly suggests that Tualang honey was able to restore osteoporotic bone similar to the nonosteoporotic bone.

Another study performed on the effects of Tualang honey on postmenopausal women showed that daily intake of Tualang honey at 20 mg/day for four months was found to be safe and exerted the same effect on bone densitometry when compared to hormone replacement therapy [83]. Based on this result, it was shown that Tualang honey was able to produce an effective result as HRT and can be used as anti-osteoporotic agent. These positive effects of Tualang honey on bone are probably due to its anti-inflammatory property, apart from the antioxidative property mentioned. Other than that, it was found out that its anti-osteoporotic property could probably due to the presence of calcium [84] and gluconic acid. Gluconic acid could enhance calcium absorption in the bone which consequently maintaining bone mass and prevent osteoporosis [85]. These results proved that Tualang honey has the potential to be used as an alternative treatment in treating postmenopausal osteoporosis. The antioxidative and anti-inflammatory actions of Tualang honey are summarized in Figure 1.

From nutrition point of view, Tualang honey is a sugar. For sweeteners, a maximum of 40 to 50 g per day is generally accepted. However, the recommended dose is about 20 g daily [86]. This is the reason why majority of the researches were done with the dose of 20 g daily such seen in study of the effects of Tualang honey on postmenopausal women [83]. A previous study on the hepatoprotective effect of Tualang honey supplementation in streptozotocin-induced diabetic rat has shown that diabetic rats supplemented with Tualang honey had a reduced blood glucose concentration compared to the control diabetic rats group [87]. This result suggests that Tualang honey is safe to be consumed by postmenopausal diabetic women. Since Tualang honey is a food supplement, not many studies have been done on its drug interaction. Previous study has provided evidence for the in vitro inhibition of cytochrome P450 2C8 (CYP2C8) activity by Tualang honey. It revealed that this honey, through this inhibition, may have the potential to cause *in vivo* drug-food interaction with drugs metabolized by CYP2C8 [35] such as thiazolidinediones, a class of antidiabetic drug and amodiaquine, an antimalarial agent [88]. More clinical studies should be performed in order to study the safety of Tualang honey on diabetic patients and its interaction with other drug hence, providing a more accurate proof of its safety profile.

Based on the positive effects on bone density, Tualang honey has the potential to be used as an alternative treatment for postmenopausal osteoporosis due to its anti-oxidative

and anti-inflammatory properties against bone loss. As Tualang honey was able to produce results as effective as HRT, it can be used as an alternative in order to prevent bone loss with minimal side effects. However, there are few studies performed to observe the effects of Tualang honey on bone metabolism. More studies and clinical trials are required to explore the mechanism of Tualang honey on overall bone metabolism and its side effects.

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Research Article

Effects of Palm Vitamin E on Bone-Formation-Related Gene Expression in Nicotine-Treated Rats

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The study determines the effects of palm vitamin E on the gene expression of bone-formation-related genes in nicotine-treated rats. Male rats were divided into three groups: normal saline olive oil (NSO), nicotine olive oil (NO), and nicotine palm vitamin E (NE). The treatment was carried out in 2 phases. During the first 2 months, the NSO group received normal saline while the NO and NE groups received nicotine 7 mg/kg, 6 days a week, intraperitoneally. The following 2 months, normal saline and nicotine administration was stopped and was replaced with oral supplementation of olive oil for the NSO and NO groups and oral supplementation of palm vitamin E (60 mg/kg) for the NE group. Both femurs were harvested to determine the gene expression of bone morphogenetic protein-2 (BMP-2), Osterix (OSX), and Runt-related transcription factor 2 (RUNX2). Nicotine significantly downregulated the gene expression. This effect was reversed by palm vitamin E treatment. In conclusion, palm vitamin E may play a role in osteoblast differentiation and can be considered as an anabolic agent to treat nicotine-induced osteoporosis.

1. Introduction

Osteoporosis is a complex disorder with a large number of environmental risk factors that often interact in combinations. The bone deficit results from an imbalance in the normal relationship between bone formation and bone resorption, causing too little bone to be formed, too much removed, or both [1].

Most drugs available for the treatment of osteoporosis are inhibitors of bone resorption. They act either via reducing osteoclast number (such as bisphosphonates and estrogen) or osteoclast activity (cathepsin K inhibitors). However, in osteoporosis, bone loss may exceed the amount that can be restored by the inhibitors of resorption. Therefore, drugs that would act via promoting bone formation would be a tool for a highly desirable therapy [2].

Several studies have identified smoking as a risk factor for osteoporosis and bone fracture. Tobacco smoking inhibits the activity of osteoblasts and is an independent risk factor for osteoporosis. Smoking also results in increased breakdown of exogenous estrogens, lower body weight, and earlier menopause, all of which contribute to lower bone mineral density [3].

The study done by Gao et al. [4] has shown that smoke exposure can inhibit bone formation and increase bone resorption in female rat. Another study showed that nicotine has effects at the cellular level in human osteoblast cells [5]. It has also been shown to cause upregulation or downregulation of osteoblast regulatory genes and thus suppresses osteogenesis, promotes bone resorption, and delays osteoblast differentiation [6].

Vitamin E is an important fat-soluble vitamin with antioxidant properties. Of the two types of vitamin E, tocopherol is found in vegetable oils such as soy oil whereas tocotrienol is abundant in palm oil [7]. Previous studies have confirmed

the beneficial effects of palm-oil-derived tocotrienol in several experimental osteoporosis; ovariectomized rats [8], steroid-induced rats [9], ferric-nitrilotriacetate-induced rats [10], and nicotine-induced rats [11, 12]. Furthermore, recent study has shown that supplementation of palm vitamin E, especially gamma isomer, can improve bone structural and biomechanical properties of normal male rats. Therefore, palm vitamin E has the potential to be used as an anabolic agent [13].

In this study, we focused on bone morphogenetic protein-2 (BMP-2), which is one of the growth factors known to induce bone and cartilage formation, RUNX2 and Osterix (OSX) which play a key role in osteoblast differentiation. This study aimed to provide a better understanding of the negative impact of smoking on bone health and offer evidence for the therapeutic potential of palm vitamin E in osteoporotic patients following cessation of smoking to improve bone health.

2. Materials and Methods

2.1. Animals and Treatment. Thirty-two young adult (3 months old) male *Sprague-Dawley* rats weighing 250–300 g were obtained from the Laboratory Animal Resource Unit, Faculty of Medicine, Universiti Kebangsaan Malaysia (UKM). Rats were randomly assigned to three groups with eight rats in each group; that is, normal saline olive oil (NSO), nicotine olive oil (NO), and nicotine palm vitamin E (NE). All rats received normal rat chow from Gold Coin (Port Klang, Malaysia). Rats were kept two per cage under 12-hour natural light-dark cycles and were given tap water. The treatment was carried out for 4 months and was divided into 2 phases. During the first 2-month duration, the NSO group received normal saline while the NO and NE groups received nicotine 7 mg/kg, 6 days a week, intraperitoneally. The following 2 months, normal saline and nicotine were discontinued and oral supplementation of olive oil (NSO and NO groups) and palm vitamin E 60 mg/kg (NE group) was then given 6 days a week.

After 4 months of treatment, rats were sacrificed. Femur bones were extracted, attached soft tissues were removed, and the bones were rinsed with cold RNAase-free phosphate-buffered saline (Sigma, USA) before being flash-frozen with liquid nitrogen (Malaysian Oxygen, Malaysia). The bones were then stored at −70°C until RNA extraction.

2.2. Diet, Nicotine, and Palm Vitamin E. The nicotine used was nicotine hydrogen tartrate salt, which was purchased from Sigma. Nicotine (7 mg/kg) was prepared by mixing 0.07 g of nicotine in 10 mL normal saline. A total of 0.1 mL/100 g body weight of the preparation was given intraperitoneally 6 days a week according to the respective treatment duration. Palm vitamin E (3 g) was dissolved in 50 mL olive oil (Bertolli, Lucca, Italy) to obtain the 60 mg/kg rat weight dose. Palm vitamin E was obtained from Sime Darby Bioganic, Malaysia.

2.3. RNA Extraction and Quality Test. Purification of total RNA was done using the RNeasy Lipid Tissue Mini Kit according to the instructions of the manufacturer (Qiagen, Germany). RNA samples obtained were then stored at −70°C until polymerase chain reaction (PCR) analysis was carried out. All RNA samples were quantified using the NanoDrop ND-1000 for quality control. The RNA concentration which was obtained was within acceptable range.

2.4. Primer Synthesis. Both forward and reverse primers for the genes of interest in this study were synthesized using primer 3 input software (version 0.4.0). The primer sequences and PCR product for our genes of interest are shown in Table 1.

2.5. Quantitative Real-Time PCR. Quantitative real-time PCR (qRT-PCR) was conducted using the iScript One-Step RT-PCR Kit with SYBR Green (Bio-Rad, USA). The reaction cocktail was assembled with all required components except sample template (total RNA), and equal aliquots were dispensed into each reaction tube. Target sample was then added to each reaction as the final step. Replicate samples were assembled as a master mix with a single addition of sample template. The reaction mixture and protocol were tabulated in Table 2. The PCR products were resolved on 2% agarose gel. Agarose gels were photographed for densitometry analysis using gel doc UV chamber with Quality One software (Bio-Rad, USA).

2.6. Relative Quantification Method. Relative quantification mathematical model was used to determine the relative quantification of a target gene in comparison to a reference gene. Relative quantification is based on internal reference genes to determine fold-differences in expression of the target gene. The relative amount of target gene is the difference between the cycle threshold (Ct) of the gene and the Ct for the GAPDH. The crossing points, which are essential for calculation of relative expression ratios, were obtained, and relative expression ratios were calculated according to equation $\text{REV} = 2^{(\text{ct reference gene-ct target gene})}$.

2.7. Statistical Analysis. Data was expressed as mean ± standard error of the mean (SEM) and analyzed using "Statistical Package for Social Sciences" (SPSS version 15.0, Chicago, IL, USA). Parametric one-way analysis of variance (ANOVA) followed by post hoc (Tukey) tests was carried out for normally distributed data. Differences were considered significant at the levels $P < 0.05$ for all statistical analysis.

The study was approved by the Universiti Kebangsaan Malaysia Animal Ethics Committee (UKMAEC) with the UKMAEC approval number: PP/FAR/2010/NORAZLINA/20-January/285-January-2010-December-2011.

3. Results

3.1. Confirmation of Primer Specificity. Specificity of RT-PCR product was documented with high resolution gel

Table 1: RT-PCR oligonucleotide primer.

Gene description	Accession number	Primer sequence	PCR product size (bp)
GAPDH	NM 017008	F: 5′-GTG GAC CTC ATG GCC TAC AT-3′ R: 5′-TGT GAG GGA GAT GCT CAG TG-3′	129
BMP-2	NM 017178	F: 5′-TGA ACA CAG CTG GTC TCA GG-3′ R: 5′-TTA AGA CGC TTC CGC TGT TT-3′	120
RUNX2	NM 053470	F: 5′-GCC GGG AAT GAT GAG AAC TA-3′ R: 5′-GGA CCG TCC ACT GTC ACT TT-3′	200
OSX	NM 181374	F: 5′-GCT GCC TAC TTA CCC GTC TG-3′ R: 5′-GTT GCC CAC TAT TGC CAA CT-3′	137

Table 2: SYBR Green real-time PCR reaction mixture and protocol.

Reaction mixture	
Master mix	Volume per one reaction
2X SYBR Green RT-PCR reaction mix	12.5 μL
Forward primer (10 μM)	1 μL
Reverse primer (10 μM)	1 μL
Nuclease-free H_2O	9 μL
RNA template (1 pg to 100 ng total RNA)	1 μL
iScript reverse transcriptase for one-step RT-PCR	0.5 μL
Final reactions volume	25 μL
Protocol	
Process	Duration/temperature
cDNA synthesis	20 min at 50°C
iScript reverse transcriptase inactivation	4 min at 95°C
PCR cycling and detection (standard PCR cycle)	
Denaturation	10 sec at 95°C
Annealing/extension	30 sec at 61°C (data collection step)
Repeated for (30 to 45 cycles)	
	1 min at 95°C
Melt curve analysis (optional)	1 min at 55°C
	10 sec at 55°C (80 cycles, increasing each by 0.5°C each cycle)

electrophoresis and resulted in a single product with the desired length (GAPDH, 129 bp; BMP-2, 120 bp; OSX, 137 bp; RUNX, 200 bp) as shown in Figure 1.

3.2. BMP-2 Gene Expression. BMP-2 gene expression was decreased in the NO group compared to the NSO group ($P < 0.05$) (Figure 2). However, BMP-2 expression was significantly increased in the NE group, when compared to the NO group ($P < 0.05$). The gene expression in the NE group was not significantly different from the NSO group.

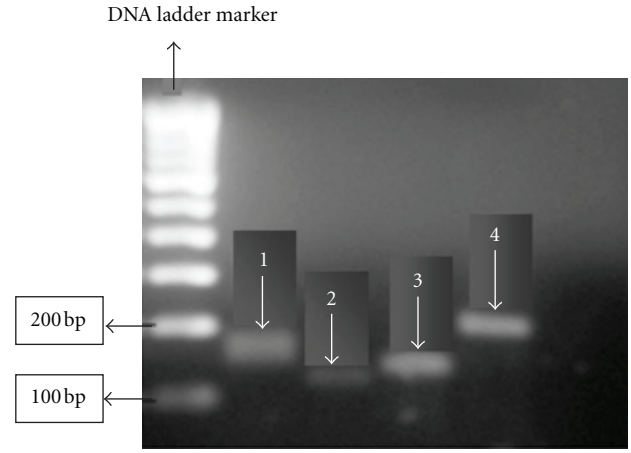

Figure 1: Agarose gel electrophoresis of PCR products. Lane1: OSX (129 bp). Lane 2: BMP-2 (120 bp). Lane 3: GAPDH (137 bp). Lane 4: RUNX2 (200 bp).

3.3. OSX Gene Expression. The expression level of OSX mRNA was markedly decreased in the NO group compared to the NSO group ($P < 0.05$). However, OSX expression level was significantly increased in NE group compared to the NO group ($P < 0.05$). The value for the NE group is still lower than the NSO group (Figure 3).

3.4. RUNX2 Gene Expression. The NO group had a lower RUNX2 gene expression as compared to the NSO ($P < 0.05$) group (Figure 4). However, the expression increased significantly in the NE group, when compared to the NO group ($P < 0.05$). The RUNX2 gene expression of the NE group was not significantly different from the NSO group ($P < 0.05$).

4. Discussion

Previous studies have shown that palm vitamin E (60 mg/kg) supplementation produced significant positive effects on bone metabolism in rats [14–17]. While a number of studies explored molecular responses associated with palm vitamin E treatment, there is paucity of studies on gene expression related to bone formation after a palm vitamin E treatment.

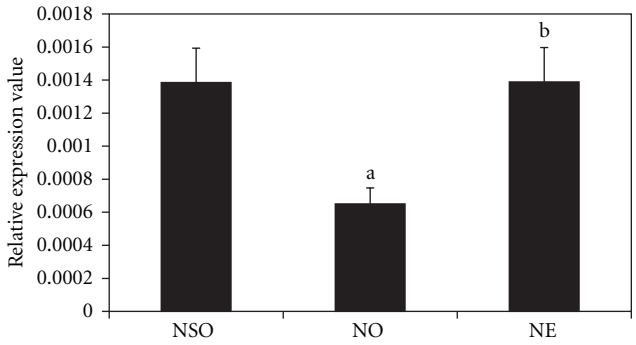

Figure 2: Effects of palm vitamin E on BMP-2 mRNA expression in nicotine-treated rats. Data is presented as mean ± SEM. NSO: normal saline olive oil group. NO: nicotine olive oil group. NE: nicotine palm vitamin E group. [a]Significantly different compared to NSO ($P < 0.05$). [b]Significantly different compared to NO ($P < 0.05$).

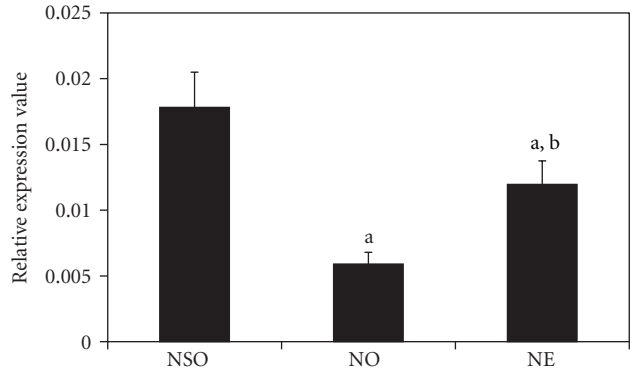

Figure 3: Effects of palm vitamin E on OSX mRNA expression in nicotine-treated rats. Data is presented as mean ± SEM. NSO: normal saline olive oil group. NO: nicotine olive oil group. NE: nicotine palm vitamin E group. [a]Significantly different compared to NSO ($P < 0.05$). [b]Significantly different compared to NO ($P < 0.05$).

In this study, we documented a significant decrease in BMP-2, OSX, and RUNX2 gene expressions in the nicotine olive oil group compared to the normal saline olive oil group. Our findings are in line with other researchers. A study done by Yanagita et al. [18] found that gene expressions of extracellular matrices and osteoblastic transcription factor (OSX) were reduced in nicotine-treated murine periodontal ligament cells. In another study, nicotine was shown to suppress osteoblast proliferation and inhibits the expression of some key osteogenic and angiogenic mediators in the *in vitro* experimental model [19]. In a more recent study, nicotine decreased RUNX2 in human alveolar bone marrow-derived mesenchymal stem cells [20].

On the other hand, palm vitamin E treatment significantly increased the expression of the above genes after two months of nicotine exposure. These results suggested that palm vitamin E treatment may enhance osteoblast cell differentiation via upregulation of these bone-formation-related genes. This mechanism could account for the increase in bone formation activity observed after palm vitamin E treatment.

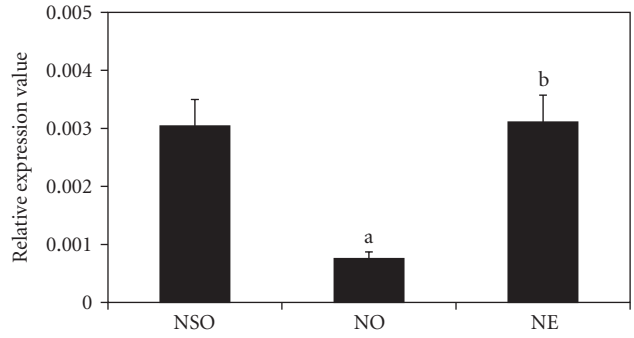

Figure 4: Effects of palm vitamin E on RUNX2 mRNA expression in nicotine-treated rats. Data is presented as mean ± SEM. NSO: normal saline olive oil group. NO: nicotine olive oil group. NE: nicotine palm vitamin E group. [a]Significantly different compared to NSO ($P < 0.05$). [b]Significantly different compared to NO ($P < 0.05$).

Nicotine may induce an oxidative stress condition. Oxidative stress has been shown to downregulate the expression of bone formation genes such as RUNX2 [21]. Therefore, palm vitamin E which possesses antioxidant properties may able to reverse the effects of nicotine on the gene expression.

Reports pertaining to vitamin E and bone-formation-related genes are limited. However, other nutrients and vitamins have been shown to influence gene expression. One study showed that vitamin D strongly accelerates expression of genes involved in differentiation of human mesenchymal stem cells, moreover, identifies as a novel regulator of osteogenesis [22]. Xing et al. [23] found that ascorbic acid upregulated OSX expression via a novel mechanism involving nuclear translocation to activate genes critical for osteoblast cell differentiation. Other researchers have demonstrated that vitamin D exerts stimulatory effect on RUNX2 mRNA expression after 48 hours of treatment [24].

Future studies on the effects of palm vitamin E on other bone-formation-related genes such as the bone morphogenetic proteins (BMP-4, -5, -7), markers of bone formation such as alkaline phosphatase and osteocalcin, and growth factors such as fibroblast growth factor receptors (1, 2, 3) may be important to support our results. In addition, bone-resorption-related genes such as RANKL and OPG are also important for future studies to further validate these results.

5. Conclusion

In summary, palm vitamin E was able to upregulate the bone-formation-related gene expressions in nicotine-treated rats. This effect may be useful in developing new anabolic agent in the treatment of metabolic bone disease such as osteoporosis.

Conflict of Interests

All authors have no conflict of interests to declare.

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Research Article

Effects of *Eurycoma longifolia* on Testosterone Level and Bone Structure in an Aged Orchidectomised Rat Model

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Testosterone replacement is the choice of treatment in androgen-deficient osteoporosis. However, long-term use of testosterone is potentially carcinogenic. *Eurycoma longifolia* (EL) has been reported to enhance testosterone level and prevent bone calcium loss but there is a paucity of research regarding its effect on the bone structural parameters. This study was conducted to explore the bone structural changes following EL treatment in normal and androgen-deficient osteoporosis rat model. Thirty-six male Sprague-Dawley rats aged 12 months were divided into normal control, normal rat supplemented with EL, sham-operated, orchidectomised-control, orchidectomised with testosterone replacement, and orchidectomised with EL supplementation groups. Testosterone serum was measured both before and after the completion of the treatment. After 6 weeks of the treatment, the femora were processed for bone histomorphometry. Testosterone replacement was able to raise the testosterone level and restore the bone volume of orchidectomised rats. EL supplementation failed to emulate both these testosterone actions. The inability of EL to do so may be related to the absence of testes in the androgen deficient osteoporosis model for EL to stimulate testosterone production.

1. Introduction

Hormonal replacement therapy with testosterone is the choice of treatment for hypogonadism in men [1–3]. It is well documented that testosterone therapy improves bone mineral density (BMD) and promote patient compliance in such cases [1, 4]. However, long-term use of testosterone is potentially carcinogenic [1, 5] and commonly causes secondary osteoporosis [6]. A suitable substitute for testosterone is needed, but there is paucity in studies discovering new alternative for the treatment of osteoporosis secondary to hypogonadism.

Several studies have shown androgen-like or testosterone-enhancing effects of *Eurycoma longifolia* (EL). These were evident in animal studies where EL has increased the copulatory activity, levator ani muscle activity, initiation of sexual performance and spermatogenesis [7–13]. Research studies on human subjects suffering from late-onset hypogonadism have shown that EL improved the patient's symptoms as well as the concentration of the testosterone serum [14]. These experimental outcomes revealed the potential of EL as a natural substitute to testosterone.

EL (Simaroubaceae family) is a traditional medical plant, known locally in Malaysia as tongkat Ali, tung saw in Thailand, pasak bumi in Indonesia, and cay ba bihn in Vietnam [15]. The root has been proven to have medicinal values, which is rich in various bioactive compounds (eurycomaoside, eurycolactone, eurycomalactone, eurycomanone, and pasak bumi-B), among which the alkaloids

and quassinoids form a major portion [15, 16]. The standardized water soluble extract of EL has also exhibited antimalarial properties and anticancer effects [17–19]. A recent study showed that EL can prevent bone calcium loss in orchidectomised rats (osteoporotic animal model) and has the potential as an alternative treatment for androgen-deficient osteoporosis [20]. This beneficial effects on bone may have been contributed by its testosterone-enhancing effects. To date, there has been no published report regarding the effect of EL on the bone structural parameters. With the current scenario that EL is taken by normal individual as a health supplement, the first part of the study was conducted to determine the effects of EL on the testosterone level and bone structure of normal male rat. The second part of the study was carried out to determine the effects of EL on the bone structural changes and testosterone level of androgen-deficient osteoporosis rat model.

2. Material and Methods

Thirty-six male Sprague-Dawley rats aged 12 months and weighing 370–500 g were used for the studies. For the first part of the study, twelve rats were divided into two groups; normal control (NC) and normal and supplemented with EL (EL) groups. For the second part of the study, the rest of the rats were divided into sham-operated (Sham), orchidectomised-control (OrxC), orchidectomised and given testosterone replacement (Orx + T) and orchidectomised and supplemented with EL (Orx + EL) groups. Before the start of the study, the rats were allowed to adjust to the new environment at least for a week. Two rats were placed in each plastic cage at 29 ± 3°C under natural day/night cycle and were fed with commercial rat chow and tap water *ad libitum*. The study was approved by the UKM Animal Ethics Committee (PP/FAR/2008/NAZRUN/13-FEB/217-FEB-2008-FEB-2010).

The body weights of the rats were measured and recorded weekly. The rats in the first part of the study were not subjected to any surgical procedures. In the second part of the study, aged orchidectomised rats were used as the model for androgen-deficient osteoporosis [21]. The rats were anesthetised with Ketapex : Xylazil (1 : 1) and scrotal approach was used to access the testis for orchidectomy [22]. A small incision 2 mm was made at the tip of the scrotum. The tunic was opened and the testis, cauda epididymis, vas deferens, and the spermatic blood vessels were exteriorized. The blood vessels and vas deferens were then ligated with 4-0 absorbable suture. The testis and epididymis were then removed. The remaining tissue was returned into the sac. The procedure was repeated for the other testis. The skin incision was closed with a nonabsorbable suture. The rats were monitored closely to avoid postsurgical complications especially chewing of the sutures and infections [22].

EL aqueous extract was supplied by Phytes Biotek Sdn. Bhd. (Malaysia). It was extracted from the root of the plant using a patented high-pressure water extraction (US Patent no: US7, 132, 117 B2). The extract was in brownish powder form and contained bioactive 22.0% eurypeptide, 41.1% glycosaponin, and 1.6% eurycomanone. EL aqueous extract powder was dissolved in normal saline. It was given to the rats through oral gavages at the dose of 15 mg/kg rat weight daily at 9 am for 6 weeks [11]. Testosterone was purchased from TCI UK Ltd (UK). It was diluted in olive oil (Bertolli, Italy), and 8 mg/kg was injected intramuscularly once daily at 9 am for 6 weeks [23]. Blood samples were collected before and after 6 weeks of treatment for measurements of testosterone serum level. Blood samples were obtained from the retroorbital vein after anesthetising the rats with ether. After 3 hours, the blood was centrifuged at 3000 rpm for 10 minutes and the serum was stored at a temperature of −70°C. After the completion of the treatment, the rats were euthanized and the femora were dissected out.

2.1. Bone Histomorphometry. Bone histomorphometry is a quantitative examination of an undecalcified bone. The bone structural changes were measured using bone histomorphometric analysis according to the American Society of Bone Mineral Research Histomorphometry Nomenclature Committee 1987 [24–26]. The undecalcified left femurs were embedded in methyl methacrylate (Osteo-Bed Bone Embedding Kit; Polysciences, USA). The femora were sectioned at 9 mm thickness using a microtome (Leica RM2155, Wetzlar, Germany). The slides were stained using a modified Von Kossa method (Von Kossa, 1974) and analyzed under Nikon Eclipse 80i microscope (Nikon Instrument Inc., USA) with an image analyzer software Pro-Plus v. 5.0 (Media Cybernetics, Silver Spring, MD, USA).

Measurements at the metaphyseal region were performed for the structural bone histomorphometry. It is located at 3–7 mm from the lowest point of the growth plate and 1 mm from the lateral cortex, excluding the endocortical region [27]. The structural parameters measured were trabecular volume (BV/TV), thickness (TbTh), number (TbN), and separation (TbSp).

2.2. Statistical Analysis. The results were expressed as mean ± standard error of mean (SEM). Data analysis was performed using Statistical Package for Social Sciences software (IBM SPSS Statistics v20). Data was tested for normality using the Test of Homogeneity of Variances. One-way analysis of variance (ANOVA) followed by *post hoc* Tukey test was used to test the data for significance.

3. Results

At the end of the study, there was no significant difference in the mean body weights of the different groups throughout the study (Figures 1 and 2).

Before the treatment, there was no significant difference in the testosterone serum levels for all the groups. At the end of the 6 weeks of treatment, in the first part of the study, the testosterone level of the EL group was not significantly different from the NC group (Figure 3). In the second part of the study, the testosterone levels of the OrxC and Orx + EL groups were significantly lower than the Sham group (Figure 4). Testosterone replacement was found to elevate the testosterone level of the Orx + T group significantly higher than the OrxC and Orx + EL groups (Figure 4).

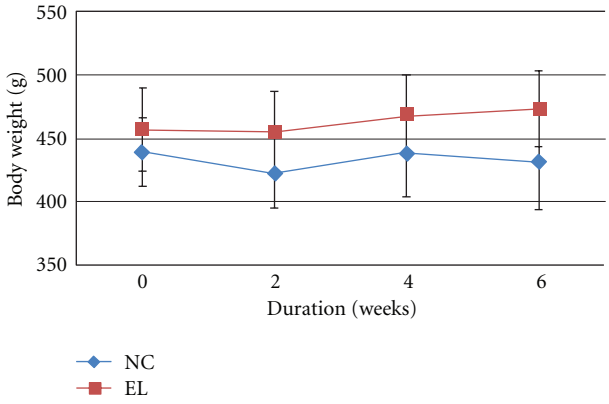

Figure 1: Mean body weight throughout the study. Data presented as mean ± SEM. There was no significant difference in the body weights between the groups. NC: normal control, EL: supplemented with EL.

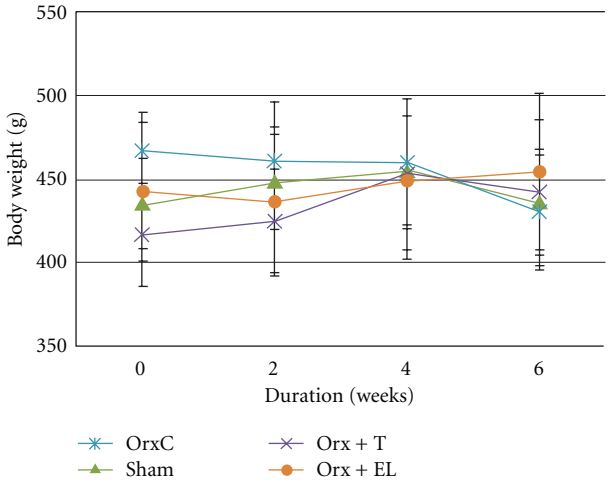

Figure 2: Mean body weight throughout the study. Data presented as mean ± SEM. All the groups have gained weight at the end of the study except OrxC group. There was no significant difference in the body weights between the groups. Sham: sham-operated, OrxC: orchidectomised-control, Orx + T: orchidectomised and given testosterone replacement, Orx + EL: orchidectomised and supplemented with EL.

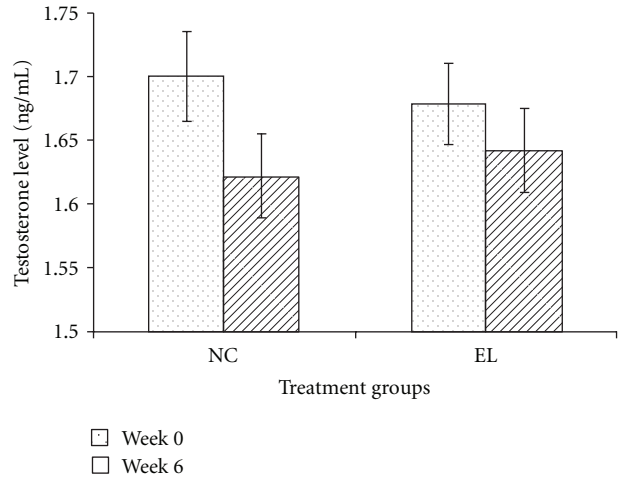

Figure 3: Mean testosterone level (ng/mL) at week 0 and week 6. Data presented as mean ± SEM. There was no significant difference in the serum testosterone levels between the groups. NC: normal control, EL: supplemented with EL.

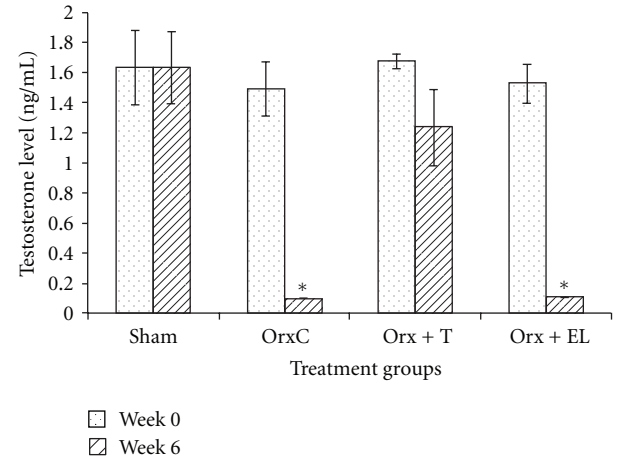

Figure 4: Mean testosterone level (ng/mL) at week 0 and week 6. Data presented as mean ± SEM ($P < 0.05$). *Significant difference of testosterone level at week 6 compared to Sham and Orx + T groups. Sham: sham-operated, OrxC: orchidectomised-control, Orx + T: orchidectomised and given testosterone replacement, Orx + EL: orchidectomised and supplemented with EL.

The bone structural parameters (BV/TV, TbTh, TbN and TbSp) were evaluated using bone histomorphometry technique. In the first part of the study, there were no significant differences in the BV/TV, TbTh, TbN, and TbSp between the NC and EL group (Figures 5, 6, 7, and 8). While for the second part of the study, orchidectomy has resulted in significantly lower BV/TV of the OrxC group compared to the Sham group. When the orchidectomised rats were given testosterone (Orx + T group), the BV/TV was restored equal to that of the Sham group. However, when the orchidectomised rats were supplemented with EL (Orx + EL), the BV/TV remained unchanged and was significantly lower than the Orx + T group (Figure 9).

There were no significant differences in the TbTh, TbN, and TbSp parameters between the different treatment groups (Figures 10, 11, and 12).

4. Discussion

In the past, neither alternative medicinal therapy received recognition in general nor herbal medicines accepted by pharmacological societies due to various reasons. Until recently, the effects of herbal products have not been standardised experimentally, and active compounds have not been isolated meticulously leading to gross dilution

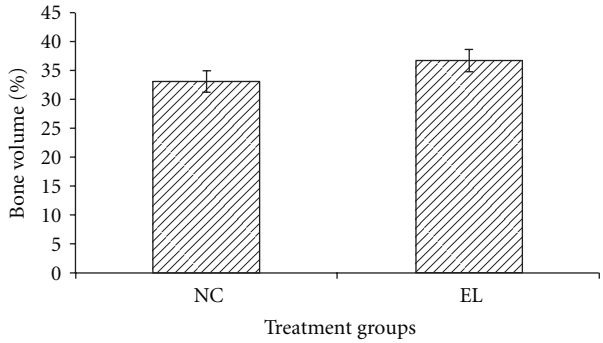

Figure 5: Mean trabecular bone volume for both groups. Data presented as mean ± SEM. There was no significant findings between the groups. NC: normal control, EL: supplemented with EL.

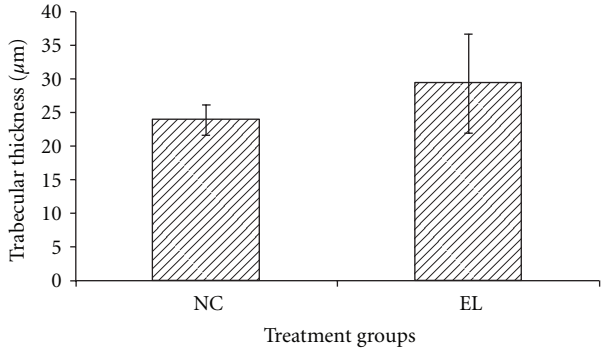

Figure 6: Mean trabecular thickness for both groups. Data presented as mean ± SEM. There was no significant difference in trabecular thickness between the groups. NC: normal control, EL: supplemented with EL.

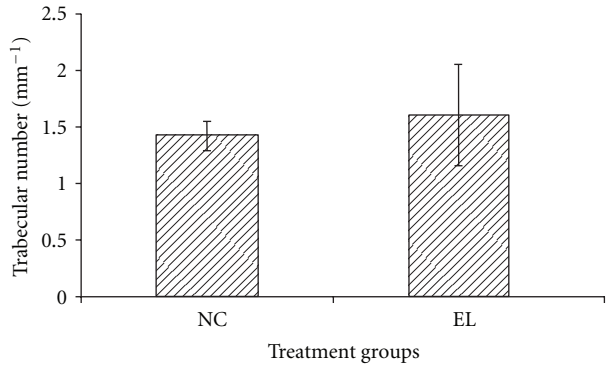

Figure 7: Mean trabecular number for both groups. Data presented as mean ± SEM. There was no significant difference in trabecular number between the groups. NC: normal control, EL: supplemented with EL.

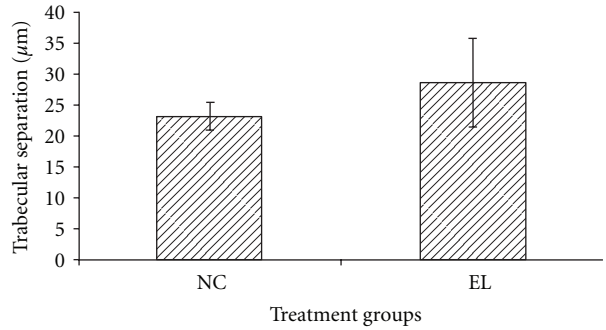

Figure 8: Mean trabecular separation for both groups. Data presented as mean ± SEM. There was no significant difference in trabecular separation between the groups. NC: normal control, EL: supplemented with EL.

of the importance of alternative medicine. Several herbal-based products have shown therapeutic potentials in certain diseases such as malaria, cancer, and many others [17–19]. Recently, it has been reported that EL may prevent bone calcium loss in orchidectomised rats and has been shown as potential alternative or complimentary therapy for androgen-deficient osteoporosis [20]. EL has also shown the ability to activate 17 α-hydroxylase/17, 20 lyase (CYP17) enzyme that will increase testosterone level [28]. CYP17 is a P450 enzyme that catalyzes the last step of androgen biosynthesis in both the testis and the adrenals.

In androgen-deficient patient (hypogonadism), there is a failure of the testis to produce physiological levels of androgen especially testosterone due to disruption of one or more levels of the hypothalamic-pituitary-testicular axis [1]. The choice of treatment for androgen deficiency syndrome is testosterone replacement therapy [1–4, 6]. Androgens play vital role in bone formation through androgen receptor (AR) which are present in most bone cells. Testosterone acts both direct and indirectly via the AR and following aromatization, via the estrogen receptor (ER), respectively [29–31]. Androgens may protect men against osteoporosis by maintaining the cancellous bone mass and expansion of the cortical bone [31].

However, the adverse effects of testosterone can be troublesome to the patients such as sleep apnea, polycythemia, liver toxicity, and more importantly, the risk of developing cancer [1, 5, 32, 33]. Hormone-dependent cancers such as metastatic prostate cancer and breast cancer may be stimulated during testosterone treatment. Testosterone is not recommended for those with underlying or high-risk factors for cancer [1, 5]. Therefore, an alternative to testosterone should be recommended to these patients. Based upon earlier studies, EL may increase the testosterone levels and preserve the bone calcium content [14, 20].

It was found that EL supplementation to normal male rats failed to elevate the testosterone levels compared to non-treatment rats. While for orchidectomised rat, the model for androgen-deficient osteoporosis [21], there was a significant reduction in the testosterone levels. Similar findings were found in several other studies which reported that the testosterone level falls immediately after orchidectomy [34, 35]. Testosterone replacement was shown to reverse the effect of orchidectomy. The testosterone levels of the orchidectomised

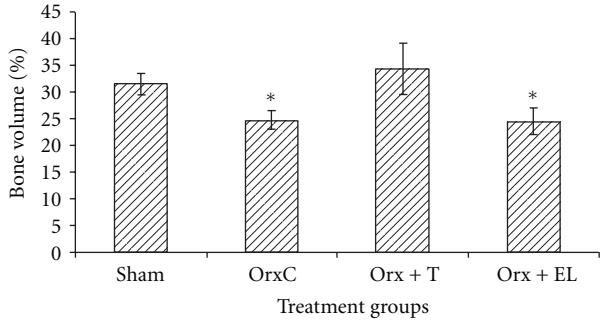

Figure 9: Mean trabecular bone volume for all the groups. Data presented as mean ± SEM. *Significant difference of BV/TV compared to Sham and Orx + T groups. Sham: sham-operated, OrxC: orchidectomised-control, Orx + T: orchidectomised and given testosterone replacement, Orx + EL: orchidectomised and supplemented with EL.

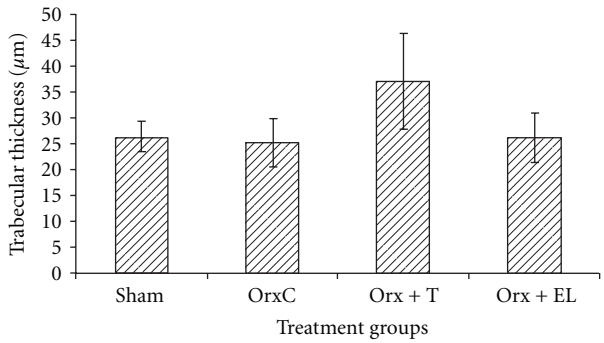

Figure 10: Mean trabecular thickness for all the groups. Data presented as mean ± SEM. There was no significant difference in trabecular thickness between the groups. Sham: sham-operated, OrxC: orchidectomised-control, Orx + T: orchidectomised and given testosterone replacement, Orx + EL: orchidectomised and supplemented with EL.

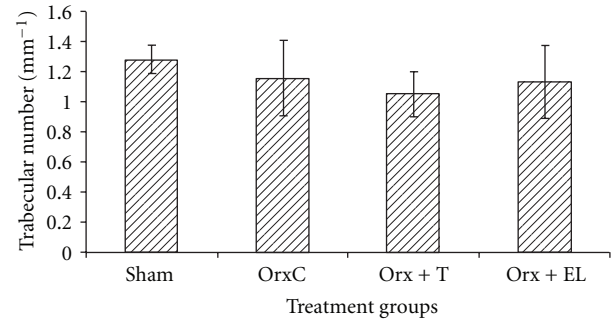

Figure 11: Mean trabecular number for all the groups. Data presented as mean ± SEM. There was no significant difference in trabecular number between the groups. Sham: sham-operated, OrxC: orchidectomised-control, Orx + T: orchidectomised and given testosterone replacement, Orx + EL: orchidectomised and supplemented with EL.

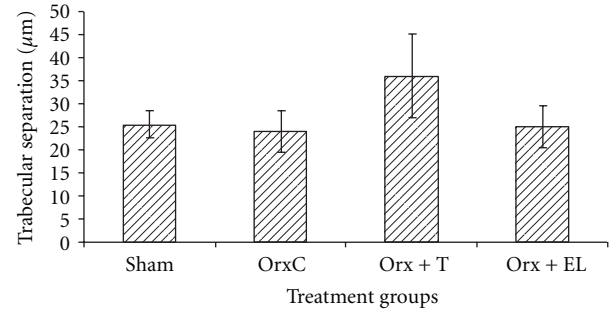

Figure 12: Mean trabecular separation. Data presented as mean ± SEM. There was no significant difference in trabecular separation between the groups. Sham: sham-operated, OrxC: orchidectomised-control, Orx + T: orchidectomised and given testosterone replacement, Orx + EL: orchidectomised and supplemented with EL.

rats remained low with EL supplementation (Figure 4). These findings showed that EL supplementation did not alter the testosterone levels of both normal and testosterone-depleted rats.

Based on the histomorphometric analysis of the first part of the study, there was no significant change in the BV/TV of normal rat with EL supplementation. In the second part of the study, the drop in the testosterone level in the OrxC group was associated with the reduction in BV/TV. When the testosterone level was raised with testosterone replacement in the Orx + T group, BV/TV was restored to that of the Sham group. Testosterone was able to improve the trabecular bone volume of orchidectomised rats; however, EL failed to emulate the testosterone action. This may be contributed by the failure of EL to raise the testosterone level of orchidectomised rats as that demonstrated by testosterone replacement. Only the BV/TV parameter produced significant findings. Although there were differences in the rest of the structural parameters, they failed to reach statistical significance among the groups.

Clinical study in human has found that the dose up to 600 mg/kg did not cause any adverse effects [36]. EL is normally recommended to be administered by men at the dose of 200–400 mg daily. The dose of EL used in the present study is equivalent to the dose of about 100 mg/day in human, which is considered safe. Acute toxicity study revealed that the LD50 for EL extract was more than 5000 mg/kg [37]. It is unlikely to cause fatality in human as the equivalent dose of EL is 35 g in a 70 kg man. In subacute toxicity study, EL extract at doses of more than 1200 mg/kg was shown to cause pathological changes in the rat liver. This dose was equivalent to approximately 8200 mg taken by a 70 kg man [37]. In the first part of the study, EL supplementation (EL group) did not seem to elevate the testosterone levels in rats with intact testes. This contradicted the finding that EL raised testosterone level in rats [8] and late onset hypogonadism patients which used different dosage [14]. However, there is a possibility that EL may only work when there is testosterone deficiency and that the testes are still intact. EL may only work to

rectify the androgen deficiency state by stimulating the testes to produce testosterone. In androgen-deficient osteoporosis model, the rats were orchidectomised; therefore, we cannot determine if that was the case. However, it was proven that EL was unable to raise the testosterone level in rats with the absence of testes.

5. Conclusion

EL failed to emulate the testosterone replacement's ability to raise the testosterone level and restore the bone structure of orchidectomised rats. It also failed to produce any effect when supplemented to normal male rats. Further studies are required to determine if EL may be useful in androgen-deficient state if the testes are still intact.

Conflict of Interests

All authors have no conflicts of interest to declare.

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Research Article

Effects of Low-Dose versus High-Dose γ-Tocotrienol on the Bone Cells Exposed to the Hydrogen Peroxide-Induced Oxidative Stress and Apoptosis

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Oxidative stress and apoptosis can disrupt the bone formation activity of osteoblasts which can lead to osteoporosis. This study was conducted to investigate the effects of γ-tocotrienol on lipid peroxidation, antioxidant enzymes activities, and apoptosis of osteoblast exposed to hydrogen peroxide (H_2O_2). Osteoblasts were treated with 1, 10, and 100 μM of γ-tocotrienol for 24 hours before being exposed to 490 μM (IC_{50}) H_2O_2 for 2 hours. Results showed that γ-tocotrienol prevented the malondialdehyde (MDA) elevation induced by H_2O_2 in a dose-dependent manner. As for the antioxidant enzymes assays, all doses of γ-tocotrienol were able to prevent the reduction in SOD and CAT activities, but only the dose of 1 μM of GTT was able to prevent the reduction in GPx. As for the apoptosis assays, γ-tocotrienol was able to reduce apoptosis at the dose of 1 and 10 μM. However, the dose of 100 μM of γ-tocotrienol induced an even higher apoptosis than H_2O_2. In conclusion, low doses of γ-tocotrienol offered protection for osteoblasts against H_2O_2 toxicity, but itself caused toxicity at the high doses.

1. Introduction

Bone is a dynamic organ that carries out major functions of the body, which include maintenance of the mechanical integrity, body support, and regulation of mineral homeostasis. Bone is continually being resorped by osteoclasts and formed by osteoblasts to maintain bone volume and calcium and phosphorus homeostasis. The balance between bone formation and resorption is known as bone remodeling. If the balance is disturbed, the volume and quality of bone will be adversely affected, as in the case of osteoporosis.

Many studies and lines of evidence have linked oxidative stress to the pathogenesis of osteoporosis. Basu et al. [1] reported that there was a biochemical link between increased oxidative stress and decreased bone mineral density (BMD) in aged men and women. Maggio et al. [2] found that there was a significant decrease of plasma antioxidant levels for elderly women who have osteoporosis. Lean et al. [3] found that the thiol antioxidants in osteoclasts were lowered during estrogen deficiency. Oxidative stress may lead to bone loss by promoting lipid peroxidation [4, 5], lowering antioxidant enzymes [5], and promoting apoptosis of osteoblasts [6]. Several osteoporosis risk factors, such as smoking [7], hypertension [8], and diabetes mellitus [9], were related to oxidative stress.

Osteoblasts are important cells that are responsible for bone formation. Any reduction in the number or function of these cells to synthesize new bone matrix may result in osteoporosis [10, 11]. Several studies have shown that free radicals and reactive oxygen species (ROS) can affect the growth and function of these cells. Mody et al. [12] and Mogi et al. [13] showed that osteoblasts can produce ROS such as nitrogen oxide (NO) and hydrogen peroxide (H_2O_2) in response to inflammatory cytokines. These ROS may

initiate lipid peroxidation [14], reduce antioxidant enzymes [15], and induce osteoblast apoptosis [16, 17]. These may adversely affect osteoblast numbers at bone formation site [18] and may contribute to bone loss [18, 19].

There is now a tendency towards the application of antioxidants in the protection and treatment of oxidative stress-related diseases. Vitamin E is a powerful biological antioxidant [20] with the ability to protect bone cells from the damages caused by lipid peroxidation [21]. Tocotrienols, the minor isomers of vitamin E, have gained scientific interest with the recent reports that they have better therapeutic potential than tocopherols [22]. Tocotrienols are the main constituent of vitamin E in palm oil *Elaeis guineensis,* and palm oil is the best source of tocotrienols, with 800 mg of tocotrienols for every kilogram of the crude oil [23]. γ-tocotrienol is the most abundant isomer in palm oil, making up 49% of vitamin E [24].

In bone studies, when the two types of vitamin E were compared in animal osteoporosis models, tocotrienol isomers were found to have better bone-protective effects than α-tocopherol. Norazlina et al. [25] have shown that tocotrienols were able to reverse bone loss induced by nicotine in rats. Palm oil-derived tocotrienols have also shown potential as prophylactic agents in prevention of glucocorticoid-induced osteoporosis in adrenalectomized rat [26]. The bone-protective mechanism of vitamin E was thought to be contributed by its antioxidant property. This was confirmed by a study which found that vitamin E especially tocotrienols protected rat bones against damage caused by free radicals released by an oxidizing agent [27]. Hermizi et al. [28] showed that γ-tocotrienol not only reversed nicotine-induced osteoporosis better than tocopherol, but also improved the bone structure until it was better than the normal control rats. This has led to a study which confirmed that vitamin E, especially tocotrienols, has bone anabolic effects on normal male rats [29]. Tocotrienols were also found to be better than tocopherol in improving the static and dynamic bone histomorphometric parameters [30]. The most recent study found that α-tocotrienol, but not α-tocopherol, prevented osteoclastic bone resorption by inhibiting RANKL expression and blocking RANKL action on osteoclast precursors [31].

Although *in vivo* studies showed that tocotrienols exhibit bone-protective activity, there is paucity of *in vitro* studies to determine the effect of tocotrienols on bone cells. Low doses of γ-tocotrienol were found to be better than α-tocopherol in protecting rat osteoblasts against H_2O_2 toxicity. However, higher doses of γ-tocotrienol were found to be toxic to rat osteoblasts [28]. This paradoxical effect of γ-tocotrienol needs further investigation on how the protective effects were not only lost at high dose of γ-tocotrienol, but it became toxic to osteoblasts.

It was suggested that at high dose, tocotrienol may become pro-oxidant or proapoptotic, which may be responsible for its toxic effects on osteoblasts. In order to confirm this, the study was focused on determining the effects of low and high doses of γ-tocotrienol on the index of lipid peroxidation and apoptosis of osteoblasts.

2. Materials and Methods

2.1. Culture of Osteoblasts. Osteoblasts were isolated using the explant culture method [32]. Briefly, Sprague-Dawley male rats (after weaning, 4–6 weeks old, weight 40–60 g) were sterilely dissected, and the long bones (femur, tibia, fibula, radius, and ulna) were collected and scraped until cleaned from the remaining muscle and connective tissues. The bones were cut into small pieces (1-2 mm) and sterilized in 50 μg/mL gentamycin (Sigma) in PBS. The bone pieces were then digested with collagenase solution (type IA, Sigma) (2 mg/mL in DMEM) for 2 hours in shaking water bath (37°C, 150 rpm) to remove the remaining soft tissues. The bone pieces were then rinsed with PBS before plated into 25 mm^2 flask containing 5 mL DMEM (10% FCS, 50 μg/mL gentamycin) and incubated in CO_2 incubator (37°C, 5% CO_2) until confluence. This study was approved by the Universiti Kebangsaan Malaysia Animal Ethic Committee (UKMAEC) with the approval number FAR/2006/NAZRUN/24-JULY/171-JANUARY-2007.

2.2. Treatment of Osteoblasts. Osteoblast number was prepared at 1×10^7 cells for measurement of MDA levels, 2×10^6 cells for measurements of glutathione peroxidase, superoxide dismutase, catalase, and caspase-3 enzymes activities, and 2×10^5 cells for single-stranded DNA analysis. Osteoblasts were incubated in CO_2 incubator (37°C, 5% CO_2) with 1, 10, and 100 μM of γ-tocotrienol extracted from palm oil (Carotech, Malaysia) for 24 hours before incubated with H_2O_2. The incubation period with H_2O_2 was 2 hours at the concentration of 490 μM, which was the IC_{50} of H_2O_2 [32].

The doses of γ-tocotrienol used were based on previous study. These doses were able to cover both spectrums of γ-tocotrienol activities at low and high doses [28]. Every concentration was repeated triplicate, using 3 different osteoblast cultures.

2.3. MDA Levels. The MDA level was measured using Biotech LPO-586 (OxisResearch, US) based on the reaction of a chromogenic reagent, N-methyl-2-phenylindole, with MDA at 45°C which yields a stable chromophore that can be measured at the absorbance of 586 nm. Briefly, 1 mL of cell supernatants that were obtained by scraping, sonicating, and centrifugation (3000 ×g, 10 min) of cells monolayer (1×10^7 cells) in cold environment (4°C) was mixed properly with 650 μL R1 reagent (N-methyl-2-phenylindole in acetonitrile diluted 3 times with ferum ion solution in methanol) and 150 μL concentrated HCl (12N, 37%). The samples were then heated in water bath (45°C, 60 min) before centrifuged (15 000 ×g, 10 min) to obtain the supernatants that were measured spectrometrically (586 nm).

2.4. Glutathione Peroxidase Activity. Glutathione peroxidase (GPx) activity was measured using the Glutathione Peroxidase Assay Kit (Cayman Chemical, US). The kit measures GPx activity indirectly by a coupled reaction with glutathione reductase (GR). Oxidized glutathione (GSSG), produced upon reduction of hydroperoxide by GPx, was

recycled to its reduced state by GR and NADPH. The oxidation of NADPH to $NADP^+$ is accompanied by a decrease in absorbance at 340 nm. The rate of decrease in the absorbance is directly proportional to the GPx activity in the sample. Briefly, 20 μl of cell supernatants that were obtained by scraping, sonicating, and centrifugation (10 000 ×g, 15 min) of cells monolayer (2×10^6 cells) in cold environment (4°C) was added with 100 μL assay buffer (50 mM Tris-HCl, pH 7.6 contains 5 mM EDTA) and 50 μL cosubstrate mixture (NADPH, glutathione, and glutathione reductase) in a 96-well plate. The reaction was started by adding 20 μL cumene hydroperoxide and the absorbance (340 nm) measured kinetically every minute for 5 minutes by using ELISA reader (Versamax, US). GPx activity was calculated by using the formula

$$\text{GPx activity} = \left[\left(\frac{\Delta_{\text{A340 min}}}{0.00373\,\mu\text{M}^{-1}}\right) \times \left(\frac{0.19\,\text{mL}}{0.02\,\text{mL}}\right) \times \text{sample dilution factor}\right], \quad (1)$$

where $\Delta_{\text{A340 min}}$ was the difference of absorbance calculated by using the formula

$$\Delta_{\text{A340 min}} = \frac{(\text{Absorbance at time A} - \text{Absorbance at time B})}{(\text{time A} - \text{time B})}. \quad (2)$$

GPx activity was stated in nmol/min/mL by assuming that 1 unit of enzyme oxidizes 1 nmol of NADPH to $NADP^+$ at 25°C.

2.5. Superoxide Dismutase Activity. The superoxide dismutase (SOD) activity was measured using the Superoxide Dismutase Assay Kit (Cayman Chemical, US). The kit utilizes a tetrazolium salt for detection of superoxide radicals generated by xanthine oxidase and hypoxanthine. One unit of SOD is defined as the amount of enzyme needed to exhibit 50% dismutation of the superoxide radical. Briefly, 10 μl of cell supernatants that were obtained by scraping, sonicating, and centrifugation (15 000 ×g, 5 min) of cells monolayer (2×10^6 cells) in cold environment (4°C) was added to 200 μL radical detector (50 μL tetrazolium mixed with 19.95 mL assay buffer, i.e., 50 mM Tris-HCl, pH 8.0 contained 0.1 mM DTPA and 0.1 mM hypoxanthine). The reaction was started by adding 20 μL of xanthine oxide in a 96-well plate. The plate was gently shaken and incubated (20 min, room temperature) before the absorbance (450 nm) measured by using ELISA reader (Versamax, US). Standard curve of linearize rate (LR) of absorbance versus SOD activities was plotted, and the SOD activities in the samples were calculated by using the formula

$$\text{SOD}\left(\frac{\text{U}}{\text{mL}}\right) = \left\{\left(\frac{[\text{LR sample} - y\text{-intercept}]}{\text{slope}}\right) \times \left(\frac{0.23\,\text{mL}}{0.01\,\text{mL}}\right)\right\} \times \text{sample dilution factor}. \quad (3)$$

The linearized rate (LR) was calculated by dividing all the absorbance values with standard absorbance value (SOD 0.0 U/mL).

2.6. Catalase Activity. The catalase (CAT) activity was measured using the Catalase Assay Kit (Cayman Chemical, US). The method was based on the reaction of CAT with methanol in the presence of H_2O_2. The formaldehyde produced was then measured chromatically (450 nm) with 4-amino-3-hydrazino-5-mercapto-l,2,4-triazole as the chromogen. Briefly, 20 μl of cell supernatants that were obtained by scraping, sonicating, and centrifugation (10 000 ×g, 15 min) of cells monolayer (2×10^6 cells) in cold environment (4°C) was added with 100 μL assay buffer (100 mM potassium phosphate, pH 7.0) and 30 μL methanol in a 96-well plate. The standard was prepared by mixing 100 μL assay buffer with 30 μL methanol and 20 μL formaldehyde (0, 5, 15, 30, 45, 60, and 75 μM). The reaction was started by adding 20 μL diluted H_2O_2 (40 μL H_2O_2 with 9.96 mL HPLC-grade water) into all wells. The plate was then incubated for 20 minutes at room temperature on a shaker. The reaction was stopped by adding 30 μL KOH 0.5 M and 30 μL chromogen, and the plate was measured spectrometrically (540 nm) using ELISA reader (Versamax, US). The standard curve of absorbance versus formaldehyde concentrations was plotted, and formaldehyde concentration in the samples was calculated by using the formula

$$\text{Formaldehyde concentration } (\mu\text{M}) = \left(\frac{[\text{sample absorbance} - y\text{-intercept}]}{\text{slope}}\right) \times \left(\frac{0.19\,\text{mL}}{0.02\,\text{mL}}\right). \quad (4)$$

CAT activity was expressed in nmol/min/mL by assuming that 1 unit of enzyme produces 1 nmol of formaldehyde at 25°C.

2.7. Caspase-3 Activity. The caspase-3 activity was measured using CaspASE Assay System Colorimetric Kit (Promega, US). Briefly, 20 μL of cell supernatants was obtained by scraping, lysing with lysis buffer and free-thaw cycles, and centrifugation (15 000 ×g, 20 min, 4°C) of the monolayer of the cells (2×10^6 cells). 32 μL caspase buffer, 2 μL DMSO, 10 μL DTT, and 78 μL deionized water were added into a 96-well plate. The reaction was started by adding 2 μL DEVD-pNA substrate and incubated (37°C, 4 h). The absorbances of the samples were measured spectrometrically (405 nm).

2.8. Single-Stranded DNA Analysis. The single-stranded DNA (ssDNA) was analyzed using ssDNA Apoptosis ELISA Kit (Chemicon, US). This procedure was based on selective DNA denaturation in apoptotic cells by formamide and detection of the denatured DNA by monoclonal antibody to single-stranded DNA. Briefly, the cells in a 96-well plate were fixed with 80% methanol in PBS before treated by formamide and denaturized by heating (75°C, 10 min) and cooling (4°C, 5 min). Negative control was prepared by adding 100 unit/mL SI nuclease and incubated (37°C, 1 hour), while positive control was prepared by adding 100 μL ssDNA solution. All wells were dried overnight before washed 3 times with PBS. After blocking the nonspecific sites with 200 μL nonfat milk 3% for 1 h, all wells were added with antibody mixture and incubated for 30 minutes before washed 3 times with PBS. ABTS solution was added, and the absorbance was read at 405 nm (Versamax, US) after incubation of 60 min.

2.9. Protein Content Determination. Protein content determination used in the analysis of MDA levels and GPx activity was measured by the method of Bradford [33].

2.10. Statistical Analysis. Every concentration was repeated triplicate and using 3 different osteoblast cultures with comparable results. All data were analyzed by one-way ANOVA by using SPSS version 13 software and expressed in mean ± standard deviation. $P < 0.05$ was considered significant.

3. Results

3.1. MDA Levels. Exposure of osteoblasts to 490 μM H_2O_2 for 2 hours significantly increased the MDA levels compared to the control group. Pretreatments with γ-tocotrienol prevented MDA elevation induced by H_2O_2 in a dose-dependent manner (Figure 1).

3.2. GPx Activity. Exposure of osteoblasts to 490 μM H_2O_2 for 2 hours significantly reduced the GPx activity compared to the control group. The group pretreated with 1 μM γ-tocotrienol had the highest GPx activity compared to other groups, while the pretreatment with 10 and 100 μM γ-tocotrienol did not prevent the reduction in GPx activity induced by H_2O_2. The group pretreated with 100 μM γ-tocotrienol also had the lowest GPx activity compared to other groups (Figure 2).

3.3. SOD Activity. Exposure of osteoblasts to 490 μM H_2O_2 for 2 hours significantly reduced the SOD activity compared to the control group. Pretreatment with γ-tocotrienol at doses 1, 10, and 100 μM for 24 hours had prevented the reduction of SOD activity induced by H_2O_2 (Figure 3).

3.4. CAT Activity. Exposure of osteoblasts to 490 μM H_2O_2 for 2 hours significantly reduced the CAT activity compared to the control group. Pretreatment with γ-tocotrienol at doses 1, 10, and 100 μM for 24 hours had prevented the reduction of CAT activity induced by H_2O_2 (Figure 4).

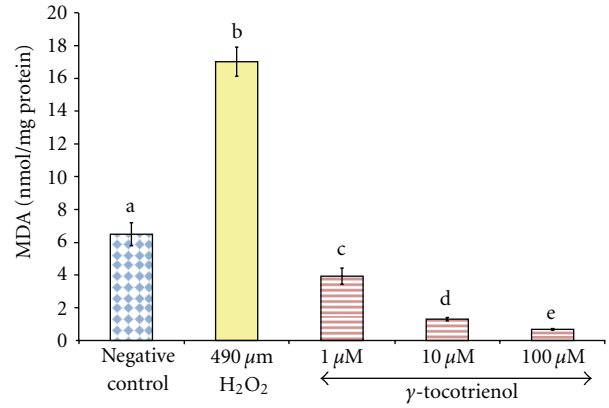

Figure 1: The effects of H_2O_2 and γ-tocotrienol on the MDA levels in osteoblasts. Osteoblasts were pretreated with 1, 10, and 100 μM γ-tocotrienol for 24 hours before treated with 490 μM H_2O_2 for 2 hours. The groups that have the same alphabet symbols (a, b, c, d, e) are not significantly different from each other ($P < 0.05$). Data are presented as mean ± SEM, $n = 3$.

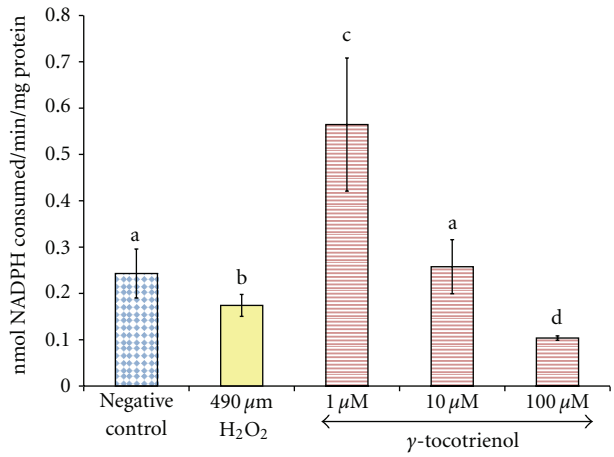

Figure 2: The effects of H_2O_2 and γ-tocotrienol on the GPx activity in osteoblasts. Osteoblasts were pretreated with 1, 10, and 100 μM γ-tocotrienol for 24 hours before treated with 490 μM H_2O_2 for 2 hours. The groups that have the same alphabet symbols (a, b, c, d) are not significantly different from each other ($P < 0.05$). Data are presented as mean ± SEM, $n = 3$.

3.5. Caspase-3 Activity. Exposure of osteoblasts to 490 μM H_2O_2 for 2 hours significantly increased the caspase-3 activity in the cells compared to the control group. Pretreatment with 1 and 10 μM of γ-tocotrienol prevented the increase in caspase-3 activity induced by H_2O_2, but pretreatment with 100 μM resulted in the highest caspase-3 activity compared to other groups (Figure 5).

3.6. ssDNA Analysis. Exposure of osteoblasts to 490 μM H_2O_2 for 2 hours significantly increased the ssDNA levels in the cells compared to the control group. Pretreatment

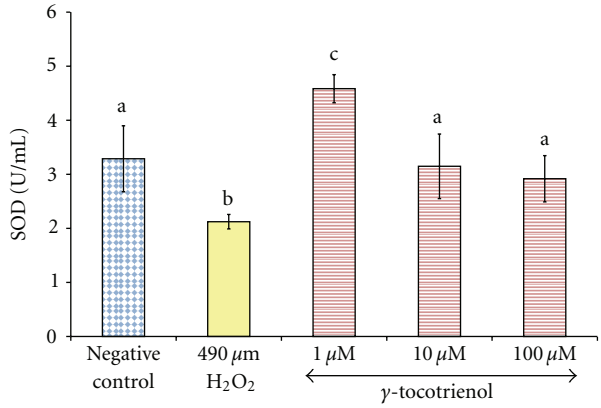

Figure 3: The effects of H_2O_2 and γ-tocotrienol on the SOD activity in osteoblasts. Osteoblasts were pretreated with 1, 10, and 100 μM γ-tocotrienol for 24 hours before treated with 490 μM H_2O_2 for 2 hours. The groups that have the same alphabet symbols (a, b, c) are not significantly different from each other ($P < 0.05$). Data are presented as mean ± SEM, $n = 3$.

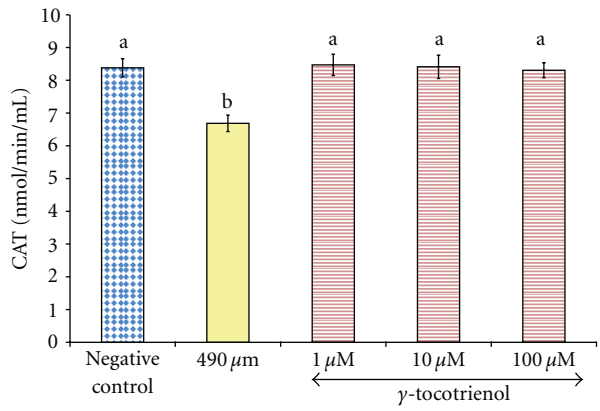

Figure 4: The effects of H_2O_2 and γ-tocotrienol on the CAT activity in osteoblasts. Osteoblasts were pretreated with 1, 10, and 100 μM γ-tocotrienol for 24 hours before treated with 490 μM H_2O_2 for 2 hours. The groups that have the same alphabet symbols (a, b) are not significantly different from each other ($P < 0.05$). Data are presented as mean ± SEM, $n = 3$.

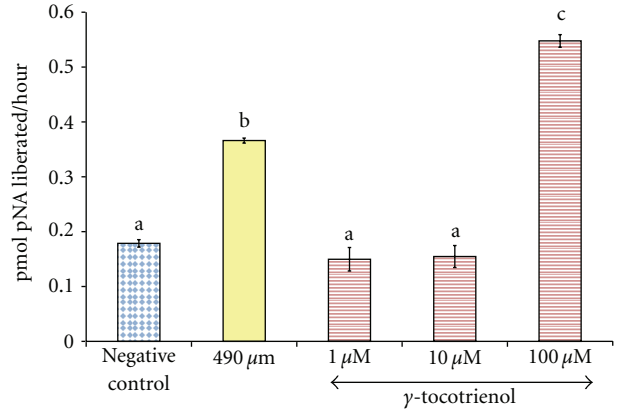

Figure 5: The effects of H_2O_2 and γ-tocotrienol on the caspase-3 activity in osteoblasts. Osteoblasts were pretreated with 1, 10, and 100 μM γ-tocotrienol for 24 hours before treated with 490 μM H_2O_2 for 2 hours. The groups that have the same alphabet symbols (a, b, c) are not significantly different from each other ($P < 0.05$). Data are presented as mean ± SEM, $n = 3$.

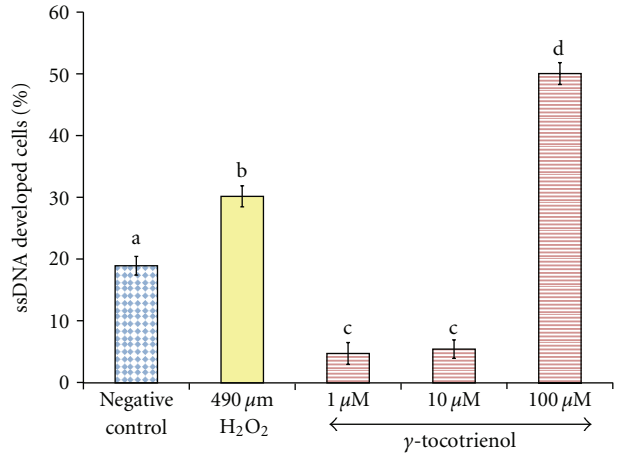

Figure 6: The effects of H_2O_2 and γ-tocotrienol on the percentage of ssDNA developed in osteoblasts. Osteoblasts were pretreated with 1, 10, and 100 μM γ-tocotrienol for 24 hours before treated with 490 μM H_2O_2 for 2 hours. The groups that have the same alphabet symbols (a, b, c, d) are not significantly different between each other ($P < 0.05$). Data are presented as mean ± SEM, $n = 3$.

with 1 and 10 μM of γ-tocotrienol for 24 hours significantly reduced ssDNA levels when compared to the control group and H_2O_2 groups. However, pretreatment of 100 μM γ-tocotrienol resulted in the highest ssDNA level compared to other groups (Figure 6).

4. Discussion

Lipid peroxidation is closely associated with osteoporosis. Parhami et al. [4] showed that the lipids that accumulated in human osteoporotic bones were oxidized and become hazardous to the bone cells. The lipids accumulation and oxidation may reverse the normal control of the local biomineralization process, by encouraging calcification in soft tissue and osteolysis [34]. Oxidized lipids promoted bone resorption [35] by promoting recruitment and differentiation of osteoclast precursor and inhibition of osteoblasts differentiation [36]. The present study showed that the MDA level of osteoblasts exposed to H_2O_2 was elevated. Similar increase in the MDA levels was reported in MC3T3-E1 preosteoblast cell line [14] and bone marrow stromal cells [37] when exposed to H_2O_2. Both the H_2O_2 and lipid peroxidation levels were reported to be elevated in the femoral tissue homogenate of ovariectomized rats [38].

In the present study, pretreatment with γ-tocotrienol prevented the MDA elevation of osteoblasts exposed to H_2O_2 in dose-dependent manner. The antilipid peroxidation property of vitamin E is contributed by the phenolic hydroxyl group of vitamin E which easily donates hydrogen atoms to

the peroxyl radical, thus creating a more stable lipid species. The efficiency of this protective mechanism is dependent on the mobility of vitamin E in the membrane and its ability to contribute electrons, which is determined by the aliphatic side chains and the number of methyl groups on the chromanoxyl ring, respectively [39].

Tocotrienols were reported to exhibit antilipid peroxidation activity in liver cells [40], brain cells [41], red blood cells [42], and low-density lipoprotein (LDL) [43]. Tocotrienol was better than tocopherol in protecting HUVEC cells exposed to arachidonic acid [43] and RAT-1 fibroblasts exposed to H_2O_2 [44]. In an *in vivo* study, Maniam et al. [45] found that the femoral bone TBARS levels decreased dose dependently with palm tocotrienol supplementation.

Serbinova et al. [46] suggested that tocotrienol has superior antioxidant activity than tocopherol due to the more uniform distribution in the membrane bilayers and higher displacement of the membrane lipids. Palozza et al. [44] hypothesized that the unsaturated double bonds of tocotrienol enable trapping of radicals in both hydrophilic and lipophilic compartment, facilitating its absorption [47] and mobility [44] in the cell membrane.

The present study found that H_2O_2 significantly reduced the GPx, SOD, and CAT activities, indicating disruptions of the endogenous antioxidant enzymes. Similar findings were demonstrated with pheochromocytoma cell lines (PC12) [48], HUVEC cells [49], rat hepatocytes [50], bone marrow stromal cells [37], and MC3T3-E1 cells [51]. The antioxidant enzyme was used up to eliminate the lipid peroxidation products or deactivated and glycated by the radicals [52, 53]. Therefore, exogenous antioxidants such as vitamin E are required to assist endogenous antioxidants in eliminating free radicals and reactive species. Vitamin E is also involved in the glutathione redox cycle which allows glutathione to be regenerated [54, 55].

In the present study, all doses of γ-tocotrienol prevented the reductions in osteoblastic SOD and CAT activities, but only the dose of 1 μM of γ-tocotrienol prevented the reduction in GPx activity. The restoration of the antioxidant enzymes activities may be contributed by the ability of vitamin E to elevate the mRNA expression of these enzymes under stressful condition [56]. Another possibility is that vitamin E may have stabilized the mRNA of antioxidant enzymes after transcription process and enhanced the translation of the derived enzyme proteins [57]. The antioxidant enzymes expression may have been modulated by vitamin E via peroxisome proliferator-activated receptors γ (PPARγ) and nuclear factor-kappaB (NF-κB) [58].

Unexpectedly, there was a paradoxical reduction in the GPx activity with the high dose of γ-tocotrienol. Antioxidants can become pro-oxidants at certain concentrations or with the presence of oxygen or metal ions [59]. Mazlan et al. [60] suggested that at high concentrations, γ-tocotrienol turns to pro-oxidant and causes toxicity to astrocytes. Low concentration (0.3 mM) of vitamin C induced the differentiation of preosteoblasts (MC3T3-E1) [61], but at high concentration (1 mM), it increased oxidative stress, reduced viability, and caused morphological changes in lung endothelial cells [62]. Other antioxidants were also reported to become pro-oxidant at high concentration such as β-carotene [63], amyloid β-peptide [64], and vitamin A [65, 66]. The presence of transition metal ions may cause an antioxidant to become pro-oxidant as in the case of α-tocopherol [67], amyloid β-peptide [68], and vitamin C [58]. However, this was unlikely without the presence of transition metal ions in the present study.

The present study found that the activation of the caspase-3 activity may have caused apoptosis of the osteoblasts exposed to H_2O_2. This was consistent with a study which found elevation of caspase-3 activity in preosteoblast cell lines (MC3T3-E1) exposed to H_2O_2 [69]. The apoptosis and caspase-3 activities were found to be elevated in human vascular endothelial cells (ECU-304), bone marrow stromal cells, and HUVEC exposed to H_2O_2 [37, 49, 70]. Caspase-3 is the main executor of the caspase group that led to the pathway of apoptosis [71]. Caspase-3 induces apoptosis by cleaving DNA repair molecules, degrading antiapoptotic protein, and cleaving the extracellular matrix protein, skeleton proteins, and related molecules [72]. In the present study, the osteoblast apoptosis by H_2O_2 was associated with the denaturation of osteoblast DNA. H_2O_2 was found to adversely affect the DNA of MC3T3-E1 cells through the inhibition of DNA synthesis [73], DNA fragmentation [69], and nuclei condensation [51], which are characteristics of apoptosis. It was also reported to inhibit osteogenic differentiation, increase the ROS levels, activate the caspase activity, and eventually induce apoptosis [74–76].

Agents that inhibit the production of reactive oxygen species or increase the antioxidant defense may prevent apoptosis and protect cells from oxygen radicals damage [77–79]. In the present study, low concentration of γ-tocotrienol was able to protect osteoblasts from H_2O_2 induced apoptosis, but α-tocopherol was not able to do so. This was consistent with studies which found that low concentrations of γ-tocotrienol (1 and 10 μM) were able to protect rat primary astrocytes [60], rat primary cerebellar cells [80], rat primary cortical neuronal cells, and SH-SY5Y cells [81] from H_2O_2-induced apoptosis. Paradoxically, higher concentration of γ-tocotrienol (100 μM) was found to promote osteoblast apoptosis. This was reflected with the excessively high caspace-3 activity of osteoblast treated with 100 μM of γ-tocotrienol. According to Then et al. [80], H_2O_2 activated both the intrinsic pathway through caspase-9 and extrinsic pathway through caspase-8 before they activate caspase-3. The present study confirmed that γ-tocotrienol had caused apoptosis via activation of caspace-3, but the actual pathway is not well understood [82].

There is still a question regarding the cause of tocotrienol to become proapoptotic at high concentration. Birringer et al. [83] found that HepG2 cells metabolized tocopherols and tocotrienols to short- and long-chain metabolites, with greater tocotrienol metabolites being produced. Recently, a study has found that the tocopherol metabolite of long chain (13′-carboxychromanol) was a strong inducer of apoptosis [84]. Although there was no studies done to confirm

this, tocotrienol metabolites may have contributed to the proapoptotic effect of tocotrienol. At low concentrations, γ-tocotrienol prevented apoptosis by increasing the endogenous antioxidant capacity, reducing lipid peroxidation, inhibiting the apoptosis pathway, and reducing the DNA fragmentation. Nanomolar concentrations of tocotrienol have been found to inhibit apoptosis pathway signals including src kinase [85, 86] and 12-lipoxygenase [87].

Based on the result of the present and previous studies, the toxic effects of γ-tocotrienol may have been contributed by its proapoptotic effects at higher doses. Although high dose of γ-tocotrienol reduced the glutathione peroxidase activity, the lipid peroxidation level was still suppressed. At low doses, γ-tocotrienol has potential to be used for the treatment and prevention of diseases related to oxidative stress including osteoporosis. However, at high doses, γ-tocotrienol may be toxic to cells by promoting apoptosis. This paradoxical effect of γ-tocotrienol at high doses may be useful for killing cancer cells.

In conclusion, low doses of γ-tocotrienol (1 and 10 μM) offered osteoblasts protection against H_2O_2-induced oxidative stress and apoptosis. Paradoxically, high dose of γ-tocotrienol (100 μM) reduced glutathione peroxidase activity and promoted apoptosis. Further studies are required to determine the exact apoptosis pathway involved and possible involvement of the tocotrienol metabolites.

Conflict of Interests

All authors have no conflict of interests.

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Research Article

Bone Micro-CT Assessments in an Orchidectomised Rat Model Supplemented with *Eurycoma longifolia*

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Recent studies suggested that *Eurycoma longifolia*, a herbal plant, may have the potential to treat osteoporosis in elderly male. This study aimed to determine the effects of *Eurycoma longifolia* supplementation on the trabecular bone microarchitecture of orchidectomised rats (androgen-deficient osteoporosis model). Forty-eight-aged (10–12 months old) *Sprague Dawley* rats were divided into six groups of sham-operated (SHAM), orchidectomised control (ORX), orchidectomised + 7 mg/rat testosterone enanthate (TEN) and orchidectomised + *Eurycoma longifolia* 30 mg/kg (EL30), orchidectomised + *Eurycoma longifolia* 60 mg/kg (EL60), orchidectomised + *Eurycoma longifolia* 90 mg/kg (EL90). Rats were euthanized following six weeks of treatment. The left femora were used to measure the trabecular bone microarchitecture using micro-CT. Orchidectomy significantly decreased connectivity density, trabecular bone volume, and trabecular number compared to the SHAM group. Testosterone replacement reversed all the orchidectomy-induced changes in the micro-CT parameters. EL at 30 and 60 mg/kg rat worsened the trabecular bone connectivity density and trabecular separation parameters of orchidectomised rats. EL at 90 mg/kg rat preserved the bone volume. High dose of EL (90 mg/kg) may have potential in preserving the bone microarchitecture of orchidectomised rats, but lower doses may further worsen the osteoporotic changes.

1. Introduction

Eurycoma longifolia, known locally as "*Tongkat Ali*" in Malaysia is a native herb in the Southeast Asia region, especially in Malaysia, Indonesia, Cambodia, Laos, and Vietnam [1]. This small plant from the *Simaroubaceae* family can grow up to more than 15 meters and starts to bear fruits after 2-3 years of cultivation. Since decades ago, almost all parts of this evergreen plant including the fruits, root, and leaves have been sought after for its medicinal uses. However, the most valuable component is the root which has been more commonly used as an aphrodisiac and also to treat a wide range of diseases, including aches, fever, malaria, sexual insufficiency and glandular swelling [2–4]. The root extract of *Eurycoma longifolia* has been used as herbal ingredients to enhance blood flow and restore vitality and energy following childbirth [5]. Due to these therapeutic values, *Eurycoma longifolia* has been commercialized in various forms of health supplement [6], and added to beverages such as coffee and tea. Since decades ago, various researches have been conducted to study and explore the active components which are responsible for these claimed benefits. These components are the quassinoids for example eurycomanone [3, 4, 7], canthine-6-one alkaloids [4, 8–12], squalene derivatives [13], and byphenylneolignans [14]. Considering the fact that this herbal extract increased the level of testosterone in sexually insufficient individuals, there was an effort to explore the role of *Eurycoma longifolia* in treatment of androgen-deficiency-related diseases. An earlier study observed the potential of *Eurycoma longifolia* for the treatment of androgen-deficient osteoporosis [15].

Osteoporosis is a systemic skeletal disease characterized by low bone mass and microarchitectural deterioration of bone tissue with a consequent increase in bone fragility and increased fracture risk [16]. Male osteoporosis which was not recognized earlier has now become an important

public health issue. Although hip fractures involve less than one-third of males, the mortality rate has been reported to be 31% and 17% in males and females, respectively [17]. Osteoporotic fracture in males is associated with significant morbidity and mortality, which have huge emotional and financial impacts on their families and the society [18].

The treatment of choice for men with androgen-deficient osteoporosis is testosterone-replacement therapy. This therapy is recommended based on the increasing understanding on the role of androgens in bone physiology and remodeling, which is made evident by numerous animal and human studies [19]. However, testosterone replacement therapy is contraindicated in patients with prostate or breast cancer [20]. Some physicians even perceived that testosterone replacement therapy is associated with increased risk of prostate cancer. A study reported that almost 35% of hypogonadal men did not receive treatment due to fear of developing prostate cancer [21]. Another contraindication which is worth mentioning is sleep apnoea as hypogonadal patients may have worsening of sleep apnoea with testosterone replacement [20]. Some may also relate testosterone replacement therapy with cardiovascular disease, liver damage, and erythrocytosis [22]. Thus, it is necessary and essential to find an alternative treatment which not only gives protection to the bone but at the same time possesses fewer side effects compared to the testosterone therapy.

Over the past few decades, *Eurycoma longifolia* has been recognized internationally especially as an aphrodisiac and is being used widely to enhance virility and sexual performance in male [23, 24]. Many studies reported that this ability of *Eurycoma longifolia* is associated with the increased level of testosterone in subjects supplemented with *Eurycoma longifolia*. Recent human trials showed that *Eurycoma longifolia* supplementation increased the level of testosterone, together with the increase in the superoxide dismutase (SOD), an antioxidant which plays an important role in slowing down the process of aging [25–28]. Despite the documented history of *Eurycoma longifolia* as a testosterone-raising herb, the underlying mechanisms for its androgenic effects remain unclear. Shawn and Kraemer (2007) in their study suggested that *Eurycoma longifolia* increased testosterone levels by way of promoting the dissociation of testosterone from sex-hormone-binding globulin (SHBG) [29]. This SHBG is known to increase in aging men and causes a decrease in the bioavailability of the active fractions of testosterone. One of the bioactive components of *Eurycoma longifolia*, eurypeptide may enhance the biosynthesis of different androgens, by activating the CYP17 (17 α-hydroxylase) enzyme, thereby boosting further the metabolism of pregninolone and 17-OH-progesterone to dehydroepiandrosterone (DHEA) [30]. It also enhances the metabolism of progesterone and 17-OH-progesterone to 4-androstenedione and testosterone [30].

The World Health Organization (WHO) defines osteoporosis as bone mineral density (BMD) value of 2.5 standard deviations or more below the young adult women mean value [31]. BMD measurement by dual energy X-ray absorptiometry (DEXA) is currently the most common technique for assessing the risk of osteoporosis. However, this BMD measurement may not reflect the actual bone strength and the risk of fragility fracture. In a prospective study involving 699 subjects, it was observed that there was an overlapping of BMD measurements between subjects with and without fractures [32]. In the same study, they found that the relationship between bone mass and fracture risk was not linear. The trabecular bone, which is predominantly affected in osteoporotic changes, is a continuous three-dimensional network of bars and plate, with varying densities and orientations. Several studies have suggested that connectivity density of the trabecular bone, rather than bone density, may be the parameter mostly affected by osteoporotic changes [33–35]. Connectivity density, with regard to bones microarchitecture, is defined as the maximal number of branches that can be broken before the microarchitecture is separated into parts [36]. Apart from trabecular bone density, histomorphometry analysis of the bone microarchitecture also contributes to the prediction of fracture risk [33, 37]. However, this conventional and static two-dimensional histomorphometry gives only limited information on bone microarchitecture [38]. A three-dimensional, nondestructive method of bone microarchitecture measurement pioneered by Feldkamp et al. in 1989, has become feasible only in recent years [39]. This microcomputed tomography (micro-CT) imaging system gives a detailed picture of three-dimensional bone architecture together with bone connectivity density. This high-resolution imaging technique has become more popular and largely applied in the field of bone research, both in basic as well as preclinical. Thus in this study, we aimed to observe the effects of *Eurycoma longifolia* supplementation on the trabecular bone microarchitecture in orchidectomised rats using micro-CT.

2. Materials and Methods

2.1. Experimental Animals and Treatment. Forty-eight *Sprague Dawley* rats aged 10–12 months old (300–400 mg) were divided into six groups of sham-operated (SHAM), orchidectomised control (ORX), orchidectomised + testosterone enanthate 7 mg/rat (TEN), orchidectomised + *Eurycoma longifolia* 30 mg/kg (EL30), orchidectomised + *Eurycoma longifolia* 60 mg/kg (EL60) and orchidectomised + *Eurycoma longifolia* 90 mg/kg (EL90). *Eurycoma longifolia* standardized aqueous extract (EL) was given 6 days a week via oral gavages, while testosterone was injected intramuscularly weekly throughout the six weeks duration of the study. The rats were housed singly in plastic cages at room temperature with a 12-hour light-dark cycle. They were fed with commercial rat chow (Gold Coin, Selangor, Malaysia) and tap water *ad libitum*. Ethical approval was obtained from the Universiti Kebangsaan Malaysia Animal Ethics Committee (UKMAEC) (PP/FAR/2011/NAZRUN/22-MARCH/362-JUNE-2011-MAY-2012).

2.2. Materials and Bone Preparation. Eurycoma longifolia standardized aqueous extract (EL) was obtained from Phytes Biotek Sdn, Bhd. (Shah Alam, Malaysia). The extract was in brownish powder form and the bioactive components were eurypeptide (22.0%), glycosaponin (41.1%) and eurycomanone (1.6%). EL aqueous powder was dissolved in

deionized water and given via oral gavages at doses of 30, 60, and 90 mg/kg rat weight at 9 am six days a week for six weeks [40]. Testosterone enanthate (Jesalis Pharma, Germany) was diluted in peanut oil and 7 mg/rat was administered via intramuscular injection once a week throughout the study period [41]. At the end of the treatment, the rats were euthanized by overdose of diethyl ether. Femora were dissected and cleaned from all soft tissues. They were then stored in 10% formalin solution until analyzed.

2.3. Micro-CT Analysis. The effect of EL supplementation on trabecular bone was assessed using micro-CT (μCT80 scanner, Scanco Medical, Switzerland). Before scanning, measurement protocols to define parameters such as source energy and image resolution were created. The left femur was placed in a sample holder in a vertical direction with the epiphyseal head facing downward. The source energy selected for this study were 70 KVp and 114 μA with image resolution set as "high" to obtain the best contrast between bone and soft tissues. The trabecular bone parameters were obtained from the distal end of the left femur. Scanning was done at the metaphyseal area located 1.5 mm below the lowest point of the epiphyseal growth plate and extending 2.0 mm in the proximal direction. This is the secondary spongiosa area, which is rich in high-turnover trabecular bone. Trabecular bone was chosen because its remodelling process is more dynamic than the cortical bone [42].

2.4. Statistical Analysis. The results were expressed as mean ± standard error of the mean (SEM). The data analysis was performed using the Statistical Package for Social Sciences software (SPSS 19.0; SPSS, Chicago, IL, USA). The data were tested for normality using the Kolmogorov-Smirnov test. For normally distributed data, the statistical tests used were the analysis of variance (ANOVA), followed by Tukey's Honestly Significant Difference (HSD) test. For data that were not normally distributed, Kruskal-Wallis and Mann-Whitney tests were used.

3. Results

There were no significant differences in body weight between the different groups at the beginning of the study. The body weight increased steadily throughout the period of the study. At the end of the study, there was a significant decrease in the body weight of the ORX group compared to the SHAM group ($P < 0.01$). The body weights of all the other groups showed no significant difference compared to each other (Figure 1).

In the present study, orchidectomy significantly decreased (−37.56%, $P < 0.05$) the trabecular bone connectivity density compared to the SHAM group (Figure 2). In contrast, the group supplemented with testosterone enanthate (TEN) showed an increase (+57.30%, $P < 0.05$) in trabecular bone connectivity density compared to the ORX group. Generally, the groups supplemented with EL showed a dose-dependent increase in the trabecular bone connectivity density. However, the bone connectivity density of these groups were significantly lower ($P < 0.05$) than the SHAM, ORX, and TEN groups. Only the group with the highest dose of EL (EL90) showed similar trabecular bone connectivity density compared to that of the ORX group.

Figure 3 depicts the three-dimensional image of the distal femur metaphysis. The bone tissue chosen as the region of interest (ROI) shown in white has been reconstructed in three dimension to see the structural context of trabecular bone. The loss of trabecular bone connectivity density is apparent in the groups treated with EL (EL30, EL60 and EL90) compared to the SHAM and TEN groups, with associated changes in other indices of trabecular bone microarchitecture.

Bone volume was significantly decreased in the ORX, EL30 and EL60 groups (−24.97, −40.72, and −33.11%, resp., $P < 0.05$ for all) compared to the SHAM group (Figure 4). EL supplementation at 90 mg/kg was effective in preserving bone volume as it showed no significant difference compared to the SHAM group.

There was a significant decrease in the trabecular number in the ORX and all EL treated groups (−29.77, −47.23, −47.79, and −39.91%, resp., $P < 0.05$ for all) compared to the SHAM group (Figure 5). Similarly, all EL treated groups showed significantly higher trabecular separation (+103.36, +92.96 and +87.36%, resp., $P < 0.05$ for all) compared to the SHAM group (Figure 6).

As for the trabecular thickness parameter, there were no significant differences between the groups (Figure 7).

4. Discussion

In males, androgen deficiency caused severe loss of bone [43], muscle, and fat mass [44, 45]. These may have contributed to the low body weight of rats in the orchidectomised control group. The body weights of rats supplemented with EL or receiving testosterone were preserved from the weight loss effects of orchidectomy. This may be due to the anabolic effects of testosterone and EL on the bone and body compositions. Androgen replacement therapy on both orchidectomised rats and hypogonadal men were found to reverse the effects of androgen deficiency on muscle mass [45–47]. The androgenic effect of EL used in this study was supported by a five-week human study, which showed that supplementation of water soluble extract of *Eurycoma longifolia* increased muscle mass and strength [48].

In the present study, the trabecular bone microarchitecture was assessed using the state of the art micro-CT. It is capable of three-dimensional bone analysis which produces high quality images, thus well accepted by the scientific communities. A comparison study revealed that all radiographic parameters (bone thickness and length) were significantly correlated to the corresponding micro-CT measurements [49]. Another study comparing three-dimensional micro-CT and two-dimensional histomorphometry, found good correlations between these two methods [50]. The same study also showed an excellent correlation in the connectivity density estimation using ConnEuler principle and micro-CT.

As expected, the present study showed that androgen withdrawal induced by orchidectomy had caused significant

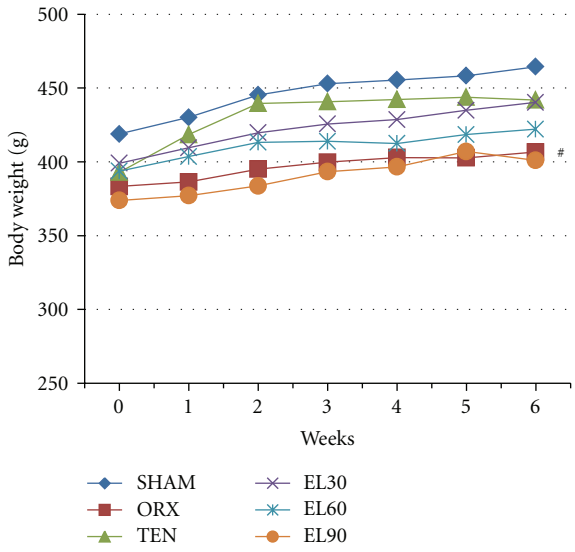


Figure 1: Mean body weight throughout the study. SHAM, sham-operated; ORX, orchidectomised-control; TEN, orchidectomised + 7 mg/rat testosterone enanthate; EL30, orchidectomised + *Eurycoma longifolia* 30 mg/kg; EL60, orchidectomised + *Eurycoma longifolia* 60 mg/kg; EL90, orchidectomised + *Eurycoma longifolia* 90 mg/kg.

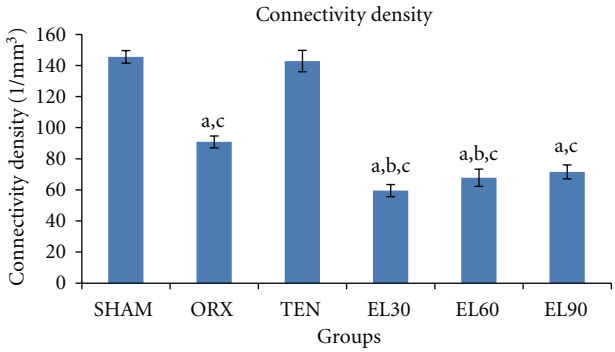


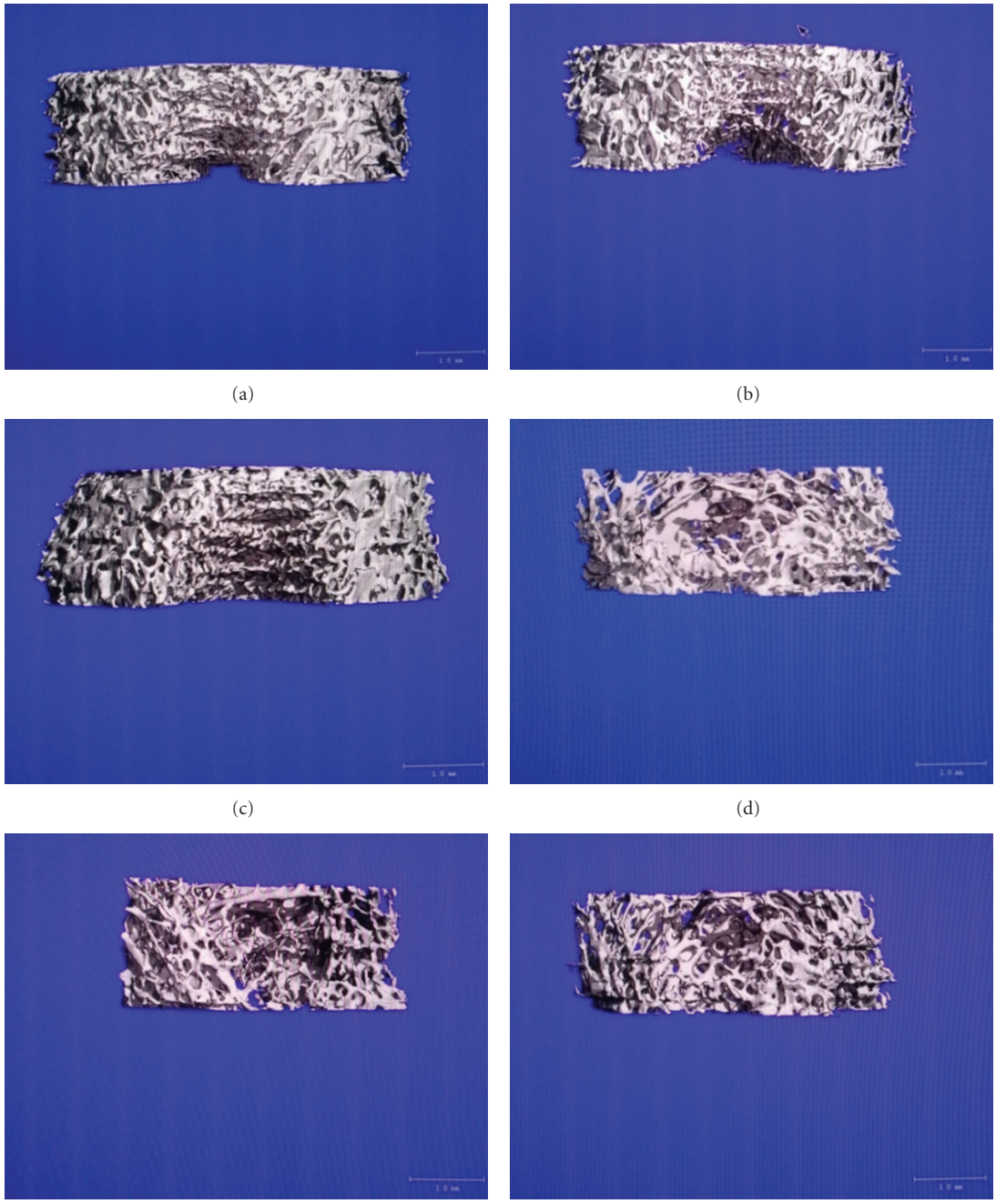
Figure 2: Trabecular bone connectivity density for all the groups after 6 weeks of treatment. SHAM, sham-operated; ORX, orchidectomised-control; TEN, orchidectomised + 7 mg/rat testosterone enanthate; EL30, orchidectomised + *Eurycoma longifolia* 30 mg/kg; EL60, orchidectomised + *Eurycoma longifolia* 60 mg/kg; EL90, orchidectomised + *Eurycoma longifolia* 90 mg/kg. $^{a}P < 0.05$ versus SHAM, $^{b}P < 0.05$ versus ORX, $^{c}P < 0.05$ versus TEN.

deteriorations of the bone microarchitecture of the rats at six weeks post-orchidectomy. These findings were supported by Yao et al. (2005) who reported that orchidectomy caused reduction in trabecular bone connectivity density but caused no change in trabecular thickness [51]. Meanwhile, studies by Yarrow et al. (2008) and Libouban et al. (2008) showed that orchidectomy caused deterioration in all trabecular bone microarchitectures which include trabecular bone number, thickness and separation [41, 52]. Testosterone replacement was able to reverse all the orchidectomy-induced changes. This was in agreement with Yarrow et al. (2008) which showed that testosterone administration to orchidectomised rats significantly improved the trabecular number, width and separation [41].

In a previous study, EL supplementation at 15 mg/kg was able to prevent bone calcium loss in orchidectomised rats, although not as effective as testosterone treatment [15]. In the present study, higher EL doses of 30, 60, and 90 mg/kg were used to determine whether EL supplementations would affect bone microarchitecture in a dose-dependent manner.

Surprisingly, data from this study showed that EL at doses of 30 and 60 mg/kg failed to protect bone from orchidectomy-induced changes. Supplementation of 90 mg/kg EL showed some protection on the bone volume. Meanwhile, EL doses of 30 and 60 mg/kg worsened all the bone microarchitecture indices such as the bone connectivity density, volume, number, and separation. There is paucity of literature on the effects of *Eurycoma longifolia* on osteoporosis. However, it can be postulated that EL may directly damage bone cells or they may be involved in the regulation of osteoblasts and osteoclasts activities, resulting in increased bone resorption. The standardized water extract of EL used in this study contains eurycomanone, which was found to be toxic to human cells [10]. However, *in vitro* toxicity studies often used methanolic root extracts of *Eurycoma longifolia* [10, 53], which is more potent, and thus may explain its toxic effects on human cells. Apart from eurycomanone, there are other *Eurycoma longifolia* constituents, which were shown to possess cytotoxic activities such as β carboline alkaloid, which was active against human lung and breast-cancer cells and canthine-6-1 alkaloid, which was active against multiple human cancerous cells [1, 10, 54]. The toxic effects of these EL constituents may have caused the deterioration of the bone microarchitecture found in this study.

Bone is composed of supporting cells (osteoblast and osteocytes), remodeling cells (osteoclasts) osteoid and inorganic mineral salts (hydroxyapatite). Osteoblasts synthesize new collagenous matrix and regulate its mineralization by concentrating calcium and phosphate, and destroying mineralization inhibitors such as pyrophosphate and proteoglycan [55, 56]. Approximately 30 to 50% of osteoblasts become osteocytes or bone-lining cells, while the majority of them undergo apoptosis [57]. The bone-lining cells differentiate into osteoblasts upon exposure to parathyroid hormone or mechanical forces [58]. The formation, activation, and resorption of osteoclasts, the bone-resorbing cells, are regulated by the ratio of receptor activator of NF-$\kappa\beta$ ligand (RANKL) to osteoprotegerin (OPG), Interleukin-1 (IL-1) and Interleukin-6 (IL-6), macrophage colony stimulating factor (M-CSF), parathyroid hormone, 1,25-dihydroxyvitamin D, and calcitonin [59, 60]. Both RANKL and M-SCF are produced by osteoblasts and marrow stromal cells, and the presence of these two cells is very important in osteoclastogenesis [61, 62]. Bone remodeling is the combination of bone resorption and formation by osteoclasts and osteoblasts, respectively, to maintain bone mass and bone strength, which can be assessed by measuring the bone microarchitecture indices. Thus, any interruption to these cytokines or bone cells regulating the activities of bone resorption and bone formation, will affect these measurements. The present study showed that EL30 and



(a) (b)

(c) (d)

(e) (f)

Figure 3: Three dimensional micro-CT images of the trabecular microstructure of distal femur metaphysis. (a) sham-operated; (b) orchidectomised-control; (c) orchidectomised + 7 mg/rat testosterone enanthate; (d) orchidectomised + *Eurycoma longifolia* 30 mg/kg; (e) orchidectomised + *Eurycoma longifolia* 60 mg/kg; (f) orchidectomised + *Eurycoma longifolia* 90 mg/kg.

EL60 groups have suffered deterioration in the bone microarchitectures. Studies have shown that estrogen withdrawal following ovariectomy caused deterioration of trabecular bone microarchitecture which was related to perforation of trabecular plates and loss of connectivity density [63–65]. In the present study, this loss of connectivity density was reversible only with testosterone replacement but not with EL30 and EL60 supplementations. A study by Yao et al. (2005) on the long bone (tibia) reported that ovariectomy caused reduction in total trabecular bone volume, connectivity, and trabecular number while trabecular thickness did not change significantly [51]. In the current study, testosterone depletion following orchidectomy showed similar results. Yao et al. (2005) also reported that connectivity density of the tibia showed contradictory findings compared to the vertebra, partly due to the slower bone loss rate in the vertebra than in the tibia: there was only 20% loss of trabecular bone in the vertebra four months postovariectomy versus

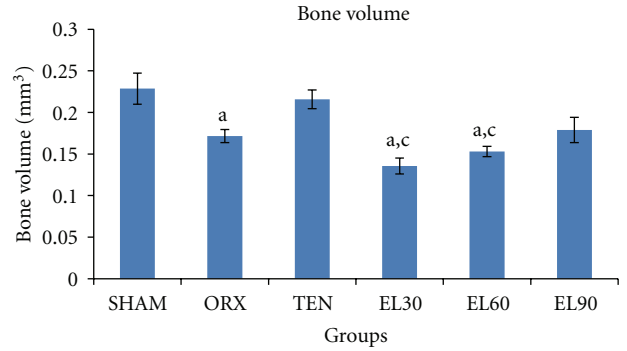


Figure 4: Bone volume for all the groups after 6 weeks of treatment. SHAM, sham-operated; ORX, orchidectomised control; TEN, orchidectomised + 7 mg/rat testosterone enanthate; EL30, orchidectomised + *Eurycoma longifolia* 30 mg/kg; EL60, orchidectomised + *Eurycoma longifolia* 60 mg/kg; EL90, orchidectomised + *Eurycoma longifolia* 90 mg/kg. $^{a}P < 0.05$ versus SHAM, $^{b}P < 0.05$ versus ORX, and $^{c}P < 0.05$ versus TEN.

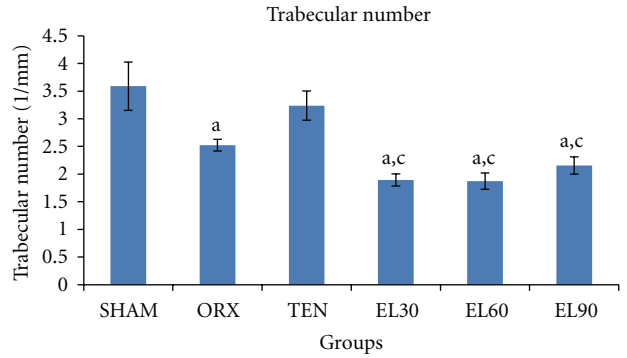


Figure 5: Trabecular number for all the groups after 6 weeks of treatment. SHAM, sham-operated; ORX, orchidectomisedcontrol; TEN, orchidectomised + 7 mg/rat testosterone enanthate; EL30, orchidectomised + *Eurycoma longifolia* 30 mg/kg; EL60, orchidectomised + *Eurycoma longifolia* 60 mg/kg; EL90, orchidectomised + *Eurycoma longifolia* 90 mg/kg. $^{a}P < 0.05$ versus SHAM, $^{b}P < 0.05$ versus ORX, and $^{c}P < 0.05$ versus TEN.

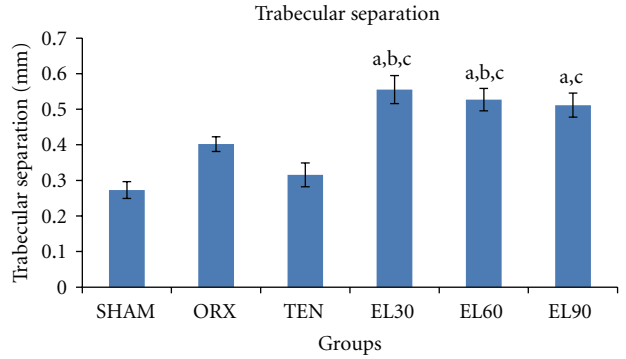


Figure 6: Trabecular separation for all the groups after 6 weeks of treatment. SHAM, sham-operated; ORX, orchidectomised control; TEN, orchidectomised + 7 mg/rat testosterone enanthate; EL30, orchidectomised + *Eurycoma longifolia* 30 mg/kg; EL60, orchidectomised + *Eurycoma longifolia* 60 mg/kg; EL90, orchidectomised + *Eurycoma longifolia* 90 mg/kg. $^{a}P < 0.05$ versus SHAM, $^{b}P < 0.05$ versus ORX, $^{c}P < 0.05$ versus TEN.

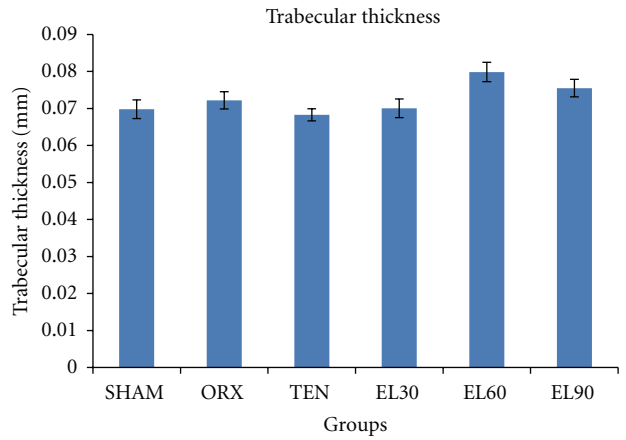


Figure 7: Trabecular thickness for all the groups after 6 weeks of treatment. SHAM, sham-operated; ORX, orchidectomised control; TEN, orchidectomised + 7 mg/rat testosterone enanthate; EL30, orchidectomised + *Eurycoma longifolia* 30 mg/kg; EL60, orchidectomised + *Eurycoma longifolia* 60 mg/kg; EL90, orchidectomised + *Eurycoma longifolia* 90 mg/kg. $^{a}P < 0.05$ versus SHAM, $^{b}P < 0.05$ versus ORX, and $^{c}P < 0.05$ versus TEN.

more than 80% in the tibia [51]. In the vertebra, estrogen depletion-induced fenestration of plates, which increased the connectivity density, and treatments with anabolic or antiresorptive agents filled in the fenestration, resulting in a lower connectivity density of the bone [51].

There were no significant changes in the trabecular thickness for all the groups. Physiologically, with the loss of bone connectivity density, reduction in trabecular number and widening of the trabecular separation, there would be a compensatory trabecular thickening. This finding is in agreement with Gasser et al. (2005) who found that zoledronic acid (antiresorptive agent) increased trabecular thickness without improving the bone connectivity density [66]. Other studies have also reported compensatory increase in the remaining trabecular following the loss of bone mass during adulthood [67–70]. This compensatory mechanism may account for the nonsignificant changes in the trabecular thickness for the orchidectomised rats seen in the present study.

Supplementation of EL at 90 mg/kg showed preservation in the trabecular volume although it was less effective than testosterone. As mentioned earlier, EL may have indirectly improved bone volume by increasing the testosterone level. Although there is paucity in literature on the effects of EL on bone microarchitecture, there are many studies on the relationship between testosterone and bone metabolism and structure. A study on hypogonadal men given testosterone treatment showed significant increases in trabecular bone volume fraction and thickness after 24 months of treatment [71]. Meanwhile, another study on supraphysiological administration of testosterone enanthate also showed

improvements on the trabecular number and width, and reduction in trabecular separation [41].

Although the exact mechanisms are still unclear, it was suggested that *Eurycoma longifolia* increased free testosterone level by enhancing the dissociation of testosterone from SHBG [29]. *Eurycoma longifolia* has also been shown to enhance the metabolism of different types of androgens, thus increasing the levels of more biologically active androgens, testosterone, and DHEA [30]. The latter mechanism may be supported by recent human trials on *Eurycoma longifolia* supplementation, which showed that water extract of *Eurycoma longifolia* increased the total testosterone and DHEA levels [25–28, 72]. In animal studies involving rats and mice, both the water and ethanolic extracts of *Eurycoma longifolia* were found to increase the sexual motivation, performance, and frequency of sexual activity [2, 23]. In one of the studies, it was shown that at 800 mg/kg, *Eurycoma longifolia* promoted the growth of the reproductive organs (seminal vesicles and ventral prostate) in male rats [23]. These effects resembled those induced by testosterone administration [73]. Testosterone may exert its effects directly via activation of the androgen receptor (AR) found on bone skeletal surfaces or indirectly via aromatization of androgens (testosterone) to estrogen [19]. Thus, the increase in androgens may play an important role in maintaining the bone volume of the rats supplemented with 90 mg/kg dose of EL.

5. Conclusion

In conclusion, EL supplementations at the dose of 30 and 60 mg/kg deteriorated the bone microarchitecture of orchidectomised rats. At higher dose of 90 mg/kg, EL supplementation was only able to preserve bone volume. Further studies are required to determine if low dose of EL is toxic to bone cells, and if higher dose than 90 mg/kg would provide better protection to bone microarchitecture.

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Research Article

The Effects of Virgin Coconut Oil on Bone Oxidative Status in Ovariectomised Rat

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Virgin coconut oil (VCO) was found to have antioxidant property due to its high polyphenol content. The aim of this study was to investigate the effect of the virgin coconut oil on lipid peroxidation in the bone of an osteoporotic rat model. Normal female Sprague-Dawley rats aged 3 months old were randomly divided into 4 groups, with 8 rats in each group: baseline, sham, ovariectomised (OVX) control group, and OVX given 8% VCO in the diet for six weeks. The oxidative status of the bone was assessed by measuring the index of lipid peroxidation, which is malondialdehyde (MDA) concentration, as well as the endogenous antioxidant enzymes glutathione peroxidase (GPX) and superoxide dismutase (SOD) in the tibia at the end of the study. The results showed that there was a significant decrease in MDA levels in the OVX-VCO group compared to control group. Ovariectomised rats treated with VCO also had significantly higher GPX concentration. The SOD level seemed to be increased in the OVX-VCO group compared to OVX-control group. In conclusion, VCO prevented lipid peroxidation and increased the antioxidant enzymes in the osteoporotic rat model.

1. Introduction

Osteoporosis is a chronic systemic skeletal disease characterized by a low bone mass and loss of bone tissue and microarchitecture. The bone becomes weak and fragile with a consequent increase in the fracture incidence [1]. According to World Health Organization (WHO), the osteoporosis is defined as having a bone mineral density (BMD) of 2.5 standard deviations below the mean for young healthy adults of the same gender or below the peak adult bone mass (T-score). Postmenopausal women who have T-scores of less than 1 SD below the mean are considered as having a low bone density and this places them at an increased risk of osteoporosis [2].

Many studies have shown that oxidative stress plays a role in the pathogenesis of osteoporosis while several risk factors for osteoporosis such as smoking, hypertension, and diabetes are associated with high levels of oxidative stress [3, 4]. The fall in estrogen levels during the menopausal period leads to a loss of protective effect of estrogen against oxidative stress and reactive oxygen species [5, 6], followed by depletion in antioxidant enzymes in bones [7]. Increased activity of reactive oxygen species (ROS) leads to overexpressions of TNF-α, RANKL, and M-CSF which enhance osteoclasts function and induce bone loss [7, 8]. Oxidative stress also suppresses bone formation by inhibiting osteoblast differentiation and decreasing the survival of these cells [9, 10]. The absence of estrogen reduces osteoblastic activity and stimulates osteoclastic activity finally leading to the development of osteoporosis [9].

Virgin coconut oil (VCO) has captured a lot of interest because of its possible role in enhancing body defense against oxidative stress. VCO is different from the ordinary coconut oil as the former contains a lot more biologically active components such as polyphenols, tocopherols,

sterols, and squalene [11]. It has been established that the antioxidant activity in VCO is higher than refined coconut oil [12, 13]. VCO has been shown to enhance antioxidant enzymes activity and inhibit the lipid peroxidation in rats [14].

The beneficial effects of VCO have been investigated in various experimental models. Other than having anti-inflammatory, analgesic, and antipyretic effects [15], coconut oil has an antiviral effect whereby it decreases the viral load and increases CD4, CD8 count in HIV patients [16]. The superior moisturizing property of VCO renders it to be more effective in the treatment of atopic dermatitis compared to virgin olive oil [17]. However, to the best of our knowledge, there has not been any work investigating the effect of VCO on bone. In the present study we used ovariectomized rats to simulate postmenopausal osteoporosis, a condition associated with oxidative stress. The aim of the study is to determine the effects of VCO on bone oxidative status in osteoporotic rats by assessing the index of lipid peroxidation and endogenous antioxidant enzymes in the bones.

2. Materials and Method

2.1. Experimental Animals and Treatment. Thirty-two Sprague-Dawley female rats aged three months old weighing 250–300 g were obtained from the Laboratory Animal Resource Unit, UKM. After being acclimatized for two weeks, they were randomly divided into four groups with eight rats in each group. Two groups of rats were ovariectomised with a group being a negative control and the other one treated with 8% VCO mixed with rat chow. The sham group was sham-operated without removing the ovaries. The baseline group was killed at the beginning of the study. All the rats except the VCO group were fed with normal rat chow diet. The rats were housed two per cage at room temperature with adequate ventilation and normal 12-hour light-dark cycle. They were allowed free access to water and food. The treatment started two weeks postoperatively and lasted for six weeks. This study was approved by the animal ethics committee of UKM (UKM AEC: PP/FAR/2009/NORLIZA/24-FEBRUARY/250-MARCH-2009-JULY-2010.

2.2. Preparation of Virgin Coconut Oil Diet. Coconut palm (*Cocos nucifera*) was used to prepare the virgin coconut oil. The virgin coconut oil was prepared based on the method by Nevin and Rajamohan with slight modification [18]. The grated coconut and its neutral water were mixed together to soften the coconut. Then, the coconut mixture was squeezed into viscous slurry until all creamy milk was expelled from the coconut mixture. After that, the creamy coconut milk was kept at room temperature for 48 hours until the fermentation process took place. Three layers were produced as follows: creamy mixture in the upper layer, the virgin coconut oil in the middle, and the water in the lower layer. The oil was gently scooped out and filtered into a container. The 8% VCO diet was prepared by mixing 8 g of VCO with 100 g of rat chow.

2.3. Ovariectomy. The rats were anaesthetized, and bilateral ovariectomy was performed for the OVX-groups through ventral approach. The fallopian tubes were tied up before the ovaries were removed. The sham-operated rats underwent the sham procedure whereby the ovaries were exposed and carefully manipulated, but they were left intact [19].

2.4. Preparation of Bone Samples. Following six weeks of treatment, the rats were sacrificed using high dose of diethyl ether. The left tibias were cleaned from the adhering muscles and kept at −80°C until they were ready to be tested for malondialdehyde (MDA), superoxide dismutase (SOD), and glutathione peroxidase (GPX).

2.5. Measurement of Lipid Peroxidation. The malondialdehyde (MDA) levels in the bones were estimated by using TBARS Assay Kit (Cayman Chemical Company, USA) [20]. The MDA represents the end product of lipid peroxidation. For homogenization of bone sample, the left tibia was ground in a porcelain mortar; 25 mg of bone tissue was weighed and put into 1.5 mL centrifuge tube. It was mixed with 250 mL of RIPA buffer solution with protease inhibitors (EDTA). Then it was sonicated at 40 V for 15 minutes, at 4°C to obtain a homogenate. After that, the homogenate was centrifuged at 1,600 ×g for 10 min (Sigma Laborzentrifugen-3k30, Osterode, Germany). The supernatant was taken and stored at −80°C. The concentration of MDA was measured spectrophotometrically at 540 nm by using Microplate Reader (MBC VERSA max, USA), and the test was performed according to the TBARS Assay Kit instructions.

2.6. Measurement of Superoxide Dismutase. First the tibia was homogenized, using this procedure: the bones were perfused with phosphate-buffered saline at PH 7.4 to remove any blood cells or clots. Then 0.25 g of bone was weighed and crushed by using mortar and pestle on ice. The bone tissue was put in 10 mL tube containing 2 mL of 20 mM HEPES buffer (20 mM HEPES buffer pH 7.2, containing 1 mM EGTA, 210 mM Mannitol, and 70 mM sucrose per gram tissue). After that, the tissue was put on ice and homogenized using tissue homogenizer. Next, the homogenized mixture was centrifuged at 1500 ×g for 5 min, 4°C. The supernatant was put in a tube for assaying.

Superoxide dismutase was measured by using Superoxide Dismutase Assay Kit, from Cayman chemical company, USA. SOD was measured spectrophotometrically at 540 nm by using Microplate Reader (MBC VERSA max. USA). The assay was performed according to the Superoxide Dismutase Assay Kit instruction [20].

2.7. Measurement of Glutathione Peroxidase. GPX was measured by using Glutathione Peroxidase Activity Assay Kit, from BIOVISION Company, USA [21]. For homogenization of bone sample: 0.1 gram was weighed and put in 10 mL tub on ice. Then 0.2 mL of cold assay buffer was added on ice. After that, the sample was homogenized by using OMNI BEAD RUPTOR 24, for 50 seconds. Then it was put on ice till the temperature decreases. The mixture was centrifuged at

10,000 xg for 15 minutes at 4°C by using Microcentrifuge 22 R (Beckman Coulter bench top refrigerated micro centrifuge, Germany). Finally, the supernatant was collected in eppendorf tube for assay. The GPX was estimated by measuring the optical density (OD) of the samples at 340 nm by using Micro Plate Reader (MBC VERSA max, USA). The assay was performed according to the Glutathione Peroxidase Activity Assay Kit instruction.

3. Results

3.1. Bone Lipid Peroxidation (TBARS). There was a significant decrease in the concentration of MDA ($P < 0.05$) in the bone of OVX-VCO group compared to OVX-control group. In addition, MDA level was significantly increased in the bone of OVX-control group compared to baseline and sham groups (Figure 1).

3.2. Glutathione Peroxidase. There was a marked improvement in the antioxidant status of the bone in OVX-VCO group which was reflected by a significant increase in the concentration of GPX ($P < 0.05$) compared to OVX-control group. In addition, the GPX level was significantly increased in OVX-control group compared to baseline group ($P < 0.05$) (Figure 2).

3.3. Superoxide Dismutase. The SOD concentration was increased in the bone of OVX-VCO group compared to OVX-control group, but the change was not statistically significant. The SOD level was significantly increased in OVX-control group compared to baseline group ($P < 0.05$) (Figure 3).

3.4. Statistical Analysis. SPSS version 19 was used for analysis of data. Data was tested for normality by using Kolmogorov-Smirnov normality test. Normally distributed data was analyzed using one-way ANOVA. The results were presented as means ± SEM.

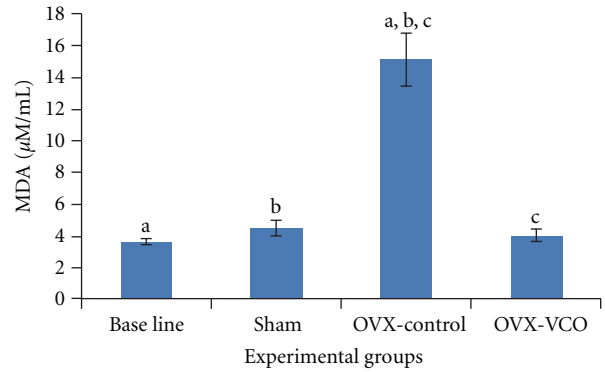

Figure 1: This figure shows MDA levels in different groups of rats. Same letters indicate significant difference-between groups at $P < 0.05$. OVX-VCO group (ovariectomised-received virgin coconut oil group). OVX-control group (ovariectomised control group). Sham group (sham-operated group).

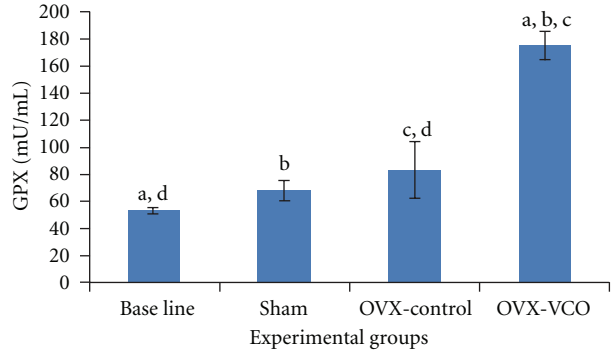

Figure 2: This figure shows GPX levels in different groups of rats. Same letters indicate a significant difference between groups at $P < 0.05$. OVX-VCO group (ovariectomised-received virgin coconut oil group). OVX-control group (ovariectomised-control group). Sham group (sham-operated group).

4. Discussion

Reduction in estrogen level is the major cause of bone loss in postmenopausal osteoporosis [22]. The ovariectomised rats are the recommended animal model for investigating preclinical therapies for postmenopausal osteoporosis [23], since the bone changes in ovariectomy and postmenopausal state are similar. The reduction in endogenous estrogen levels in both situations causes an increase in the bone turnover which leads to enhanced bone loss and a decrease in the bone mineral density [24, 25].

Postmenopausal osteoporosis is associated with oxidative stress and inhibition of the antioxidant defense system [22], resulting in the imbalance between osteoblast and osteoclast activities. Previously, we demonstrated that virgin coconut oil significantly improved the bone histomorphometric parameters, including the trabecular number, trabecular thickness, and trabecular separation in ovariectomised rats (unpublished data). The positive findings in the histomorphometric study in the bone directed us to further investigate the effect of VCO on oxidative status in the bone of osteoporotic rat model as an attempt to understand the role of VCO in enhancement of the body defense system against oxidative stress and free radicals.

The results of the present study showed significant improvement in the bone antioxidant status after VCO supplementation by a significant increase in the levels of glutathione peroxidase in OVX-VCO group compared to OVX-control group, with an increased trend of SOD levels. The positive effect on the antioxidant enzymes was supported by a low level of MDA in OVX-VCO group. In the same way, the significant increase in the levels of GPX and SOD in the ovariectomised-control rats represented the endogenous release of antioxidant enzymes, in response to oxidative stress and the high free radical activity in bone. Nonetheless, this elevation in the antioxidant enzymes was unable to suppress the lipid peroxidation which explained the significantly high level of MDA in OVX-control group.

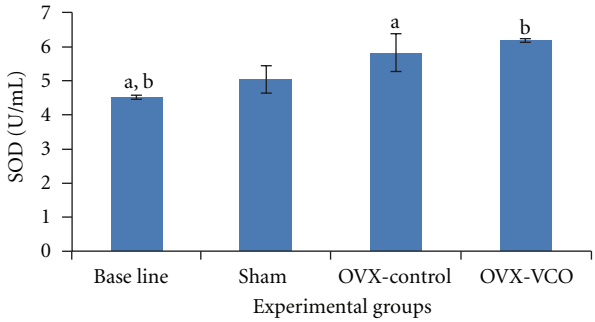

Figure 3: This figure shows SOD levels in different groups of rats. Same letters indicate a significant difference between groups at $P < 0.05$. OVX-VCO group (Ovariectomised-received virgin coconut oil group). OVX-control group (ovariectomised-control group). Sham group (sham-operated group).

Our results showed some similarities with the studies involving postmenopausal osteoporotic women, whereby the antioxidant parameters such as the total antioxidant capacity (TAC), plasma activity of SOD, catalase, and glutathione reductase were significantly increased compared to normal women, but this physiological elevation in the antioxidants was not enough to prevent the development of osteoporosis [26, 27]. In contrast, several studies indicated that the activity of GPX in the plasma was significantly reduced in postmenopausal women compared to healthy women [28, 29]. Glutathione peroxidase has an important role in reducing lipid hydroperoxide, which breaks down the oxidation chain and suppresses the free radicals release [30]. Dreher et al. [31] reported that the reduction in GPX expression could interfere with osteoblast functions and enhance the bone loss leading to osteoporosis. In addition, GPX expression by osteoblast was increased in response to oxidative stress [32].

The role of VCO in preventing oxidative stress was also manifested in other organs as well. VCO was shown to have superior suppressive effect on microsomal lipid peroxidation compared to copra oil and groundnut oil [14]. VCO stimulated the antioxidant enzymes activity and decreased the MDA and glutathione levels in healing wounds. This inhibition in lipid peroxidation promoted fibroblast proliferation, neovascularization, and healing process [33]. In addition, blending of VCO with groundnut oil or olive oil was proven to be effective in inhibiting LDL oxidation, and stimulating the activity of hepatic antioxidant enzymes [34].

The antioxidant activity of VCO is due to the high composition of polyphenol compounds in the oil [11, 14, 35]. Marina et al. estimated the total phenolic content of VCO to be in the range of 7.78–29.18 mg GAE/100 g oil, which is significantly higher than the refined, bleached, and deodorized coconut oil [35]. The major polyphenols in VCO are ferulic acid and p-coumaric acid [35]. Seneviratne and Dissanayake (2008) also detected the presence of ferulic acid, p-coumaricacid, and caffeic acid in the commercial and traditional VCO [36]. Polyphenols are stronger as antioxidants than vitamins C and E *in vitro* on the molar basis [37].

The antioxidant properties of ferulic acid have been established. Ferulic acid belongs to phenoxy carboxylic acid family [38]. Toda et al. [39] have proven that ferulic acid has the ability to scavenge the superoxide radical and suppress the lipid peroxidation induced by superoxide anion. Superoxide radicals can enhance bone resorption by degrading matrix proteins, making the bones weak and easily digested by enzymes [5]. Ries et al. [40] have reported that a superoxide radical scavenger such as Desferal-manganese complex can reduce superoxide production and decrease bone resorption by osteoclast.

The effects of ferulic acid and superoxide dismutase as antioxidants were equal in magnitude, and this characteristic made it superior to caffeic acid and p-coumaricacid as an antioxidant [39]. In addition, the effect of ferulic acid as inhibitor of lipid peroxidation was similar to the effect of α-tocopherol [39]. Castelluccio et al. [41] reported that ferulic acid was more potent as an antioxidant against LDL oxidation than ascorbic acid. It seems that VCO derives most of its effects from the free-radical scavenging and antioxidant properties of ferulic acid.

The antioxidant power of ferulic acid is due to its ability to effectively end the terminal radical chain reactions, since any free radical colliding with ferulic acid molecule can easily extract a hydrogen atom from the phenolic hydroxyl group to form a phenoxy radical which is considered a highly stable compound [38]. This phenoxy radical is unable to initiate or propagate the reactive chain reaction. This stability belongs to easy formation and lack of reactivity of phenoxy radical. Moreover, there is extended conjugation in the unsaturated side chain of phenoxy radical, and the unpaired electron may not be attached to oxygen atom, but it can move throughout the entire molecule [38].

There are few studies that investigate the effects of phenolic acids on bone loss. Sassa et al. [42] reported that ferulic acid enhanced bone remodeling process by stimulating osteoblasts to compensate for the bone loss by osteoclasts, and ferulic acid raised serum level of estrogen, progesterone in postmenopausal osteoporotic rat model. Zych et al. [43] showed that caffeic acid and p-coumaricacid increase serum estrogen levels in estrogen deficiency rat model. The authors suggested that phenolic acids such as caffeic acid may affect the metabolic pathway which regulates the extraovarian estrogen release [43]. Folwarczna et al. [44] investigated the effects of phenolic acids on bone loss in postmenopausal osteoporotic rat model, and they reported that p-coumaricacid had a positive effect on the bone mass/body-mass ratio and bone mineral mass/body-mass in bone. On the other hand, phenolic acids have no effect on bone mineral mass/bone mass in the bone of ovariectomised rats.

5. Conclusion

The imbalance between oxidative stress and antioxidant agents leads to enhancement of osteoclast activity and inhibition of osteoblast activity. Diet rich with antioxidants

is considered as a novel therapeutic agent in prevention and treatment of postmenopausal osteoporosis. VCO can prevent lipid peroxidation and increase the antioxidant enzymes in the osteoporotic rat model. Therefore, supplementation of antioxidant-enriched diet as virgin coconut oil may shed light on the development of new alternative therapy for postmenopausal osteoporosis and prevention of fractures. However, further studies are necessary in order to obtain a more complete evaluation of the therapeutic potential and safety profile of the oil.

Acknowledgment

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Research Article

Effects of Tocotrienol and Lovastatin Combination on Osteoblast and Osteoclast Activity in Estrogen-Deficient Osteoporosis

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Statins are HMGCoA reductase inhibitors and had been demonstrated to stimulate bone formation in rodents after high oral doses. Observational studies on patients treated with oral statins were varied. Delta-tocotrienol had been found to stimulate the cleavage of HMGCoA reductase and inhibit its activity. Tocotrienols were found to have both catabolic and anabolic effects on bone in different animal models of osteoporosis. The current study aimed to ascertain the effects of delta–tocotrienol and lovastatin combination on biochemical and static bone histomorphometric parameters in a postmenopausal rat model at clinically tolerable doses. 48 Sprague Dawley female rats were randomly divided into 6 groups: (1) baseline control group; (2) sham-operated control group; (3) ovariectomised control group; (4) ovariectomised and 11 mg/kg lovastatin; (5) ovariectomised and 60 mg/kg delta-tocotrienol; (6) ovariectomised and 60 mg/kg delta-tocotrienol + 11 mg/kg lovastatin. These treatments were given daily via oral gavage for 8 weeks. Delta-tocotrienol plus lovastatin treatment significantly increased bone formation and reduced bone resorption compared to the other groups. Therefore, the combined treatment may have synergistic or additive effects and have the potential to be used as an antiosteoporotic agent in patients who are at risk of both osteoporosis and hypercholesterolemia, especially in postmenopausal women.

1. Introduction

Osteoporosis is known as a silent age-related disorder, and it is considered as a major public health problem. Patients with osteoporosis have decreased bone density and microarchitectural disruption of bone tissue, leading to skeletal fragility and fractures. Postmenopausal osteoporosis is the most common type associated with high bone turnover and is due to estrogen deficiency [1]. Current available therapies are effective in the prevention of bone loss by stabilizing the bone mass through inhibition of osteoclast activity, but they are not favored to treat established osteoporosis where there is a need to increase bone volume. The United States Food and Drug Administration approved parathyroid hormone (Teriparatide) in 2002 as the first bone anabolic agent that can reduce the risk of osteoporotic fractures and increase bone mineral density [2]. However, the use of parathyroid hormone is associated with some drawbacks such as daily injection, and the possibility of tumorigenesis [3]. The identification of a well-tolerated anabolic agent that can increase bone formation and restore bone strength would represent a major therapeutic breakthrough in the treatment of any form of bone loss.

3-hydroxy-3-methylglutaryl coenzyme A (HMGCoA) reductase catalyzes the conversion of HMGCoA to mevalonic acid. Statins are competitive and reversible inhibitors of HMGCoA reductase. They are safely used as cholesterol-lowering agents and have pleiotropic actions in various systems such as the cardiovascular system, immune system, and nervous system [4]. Lovastatin is a prodrug and is converted to the active open-ring acid from its lactone by esterases. Lovastatin was the first compound identified as

a promising bone anabolic agent after examining about 30,000 compounds [5]. Statins act as an anabolic agent by promoting bone formation *in vitro* and also *in vivo* in rodents after high oral doses [5–11]. Several observational clinical studies on patients treated with oral statins showed varying results. Some had suggested that oral statins minimize the risk of fractures and increase bone mineral density [12–17], while others reported that they had no effects on bone [18–23]. Several clinical studies that compared bone biochemical markers between statin-treated patients and control populations have had varying outcomes [24–26]. However, these findings as a whole suggested that the oral statins do not have sufficient anabolic effects *in vivo* when given in cholesterol lowering doses. Therefore, high doses of statins are needed to protect the bone and induce bone formation *in vivo*. However, high doses of statins had been associated with myotoxicity and hepatotoxicity [27–29].

Tocotrienols and tocopherols are members of the vitamin E family. They are further subdivided into alpha, beta, gamma, and delta isomers. All the vitamin E isomers have antioxidant properties. In addition, tocotrienols have anticancer, neuroprotective, antiplatelet, and cholesterol-lowering activities [30]. Studies have shown that vitamin E, specifically the tocotrienols was able to maintain bone density and prevent further bone loss in different animal models of osteoporosis [31]. Recent studies offered evidence for tocotrienols as a bone anabolic agent in normal male, ovariectomised female and nicotine-treated male rats [32–35]. Tocotrienols, similar to statins, suppress the activity of HMGCoA reductase (Figure 1), although through different mechanisms [36, 37]. Statins inhibit the enzyme activity through competitive inhibition, while tocotrienols modulate the intracellular mechanism of controlled degradation of the reductase protein [38, 39]. A prior study revealed that only gamma and delta tocotrienols stimulate the degradation of HMGCoA reductase, and only the delta isomer was able to block the cleavage of sterol regulatory element-binding proteins (SREBP) [39]. Therefore, administration of statins and delta-tocotrienol together may have synergistic or additive effects. Additionally, with coadministration of delta-tocotrienol, we may be able to avoid the occurrence of the adverse effects of high doses of lovastatin in humans.

The annatto bean is one of the major sources of tocotrienols, containing 90% delta and 10% gamma tocotrienols. The annatto tree is a tropical South American tree (*Bixa Orellana*), having spinose capsules with seeds and cordate leaves that yield annatto beans. A previous study reported that coadministration of a pure extract of annatto tocotrienols lowered the effective dose of lovastatin and offered a novel approach to cancer prevention and therapy [40]. Small daily doses of delta and gamma tocotrienols isolated from annatto bean reduced serum levels of cholesterol, triglycerides, and LDL by 15–20% [41]. Annatto-derived tocotrienol was chosen for this study due to the reported efficacy above, as well as the total absence of any tocopherol isomers in the extract. Tocopherol may interfere with tocotrienol absorption and distribution and may attenuate the inhibitory effect of delta-tocotrienol on liver HMGCoA reductase [42–44]. Previous studies have found that the tocopherol isomers do not prevent bone loss in orchidectomised rats [45, 46]. Thus, it is important to use a tocopherol free extract in this study.

Ovariectomised rats are a widely accepted model of postmenopausal osteoporosis due to their appropriateness, convenience, and relevance. Furthermore, the ovariectomised rats exhibit skeletal response similar to postmenopausal women [47].

Biochemical markers of bone resorption and formation are sensitive markers that reflect the different processes involved in bone metabolism by detecting the activity of osteoclasts and osteoblasts. However, they do not show the changes in bone mass and structure [48, 49]. Osteocalcin is an osteoblast-specific noncollagenous protein. It forms about 10% of noncollagenous proteins of the bone matrix and generally serves as a specific marker for osteoblast activity and bone formation [50]. Cross-linked C-terminal (CTX) telopeptides are proteolytic fragments of type 1 collagen formed during bone resorption. CTX is known as a specific marker for osteoclast activity and bone resorption [51]. Static bone histomorphometric indices are used to examine bone histology and quantitatively evaluate the activity of the bone cells at a specific time. Therefore, a strong tool to study bone metabolism and bone morphology is through a combination of bone biochemical analysis and static histomorphometric indices.

The current study was designed to evaluate the combined effects of delta-tocotrienol and lovastatin and to compare it with delta-tocotrienol and lovastatin given individually on bone biomarkers and static bone histomorphometric parameters in the ovariectomised estrogen-deficient female rat. The findings from this study may provide an alternative medication to treat postmenopausal osteoporosis.

2. Method and Materials

2.1. Animals. Forty eight female Sprague-Dawley rats that were approximately 3 months old and weighed 200–250 g, were purchased from the Laboratory Animal Research Unit, Universiti Kebangsaan Malaysia. The rats were kept two per cage under 12 hour light-dark cycles. The rats were fed commercial rat chow (Gold Coin, Selangor, Malaysia) and tap water *ad libitum*. After one week of acclimatization, the rats were randomly divided into 6 groups with 8 rats in each group. The first group, served as a baseline control (BC), was not ovariectomised and was sacrificed upon receipt. The second group was not ovariectomised but was sham-operated (SHAM) for simulation of surgical stress. The third group was the ovariectomised control group (OVXC). The fourth group was ovariectomised and treated with 11 mg/kg of lovastatin (OVX + LOV). The fifth was ovariectomised and treated with 60 mg/kg of delta-tocotrienol (OVX + TT). And the sixth was ovariectomised and treated with 11 mg/kg of lovastatin and 60 mg/kg of delta-tocotrienol (OVX + TT + LOV). The treatment had been administrated to the rats daily via oral gavage for 8 weeks.

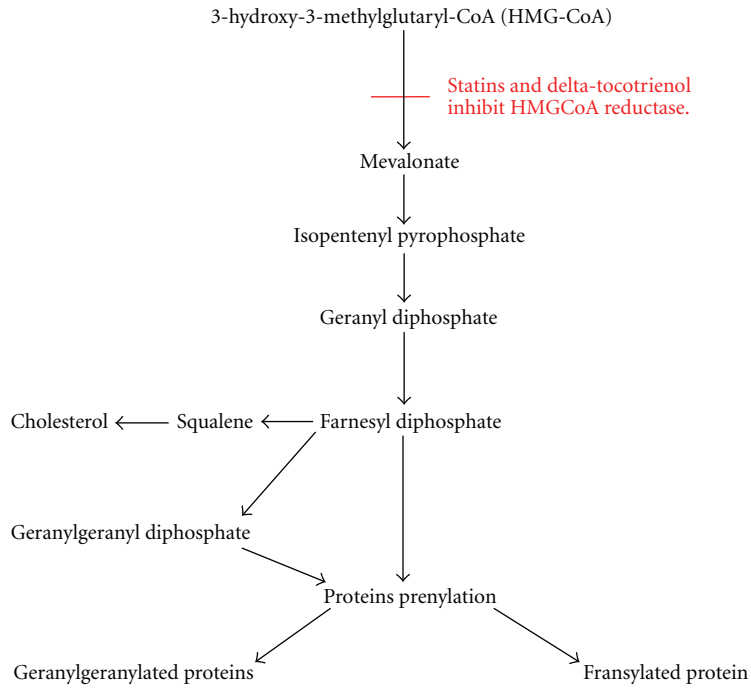

Figure 1: Mechanism of action of lovastatin and delta-tocotrienol on mevalonate pathway.

Prior approval for the study protocol had been obtained from the UKM Animal Ethics Committee, (PP/FAR/2011/IMA/27-JANUARY/352-JANUARY-2011–DECEMBER-2012).

2.2. Preparation of Treatment. The Delta Gold 70 viscous oil (American River Nutrition, Hadely, USA) is a rich delta-tocotrienol extract from the annatto bean consisting of 90% delta-tocotrienol and 10% gamma-tocotrienol. The orange-red oil was diluted in olive oil (Bertolli Classico, Italy) and administrated daily via oral gavage at a dose of 60 mg/kg delta-tocotrienol for 8 weeks. This dose was roughly equivalent to 420 mg/day for an adult human.

Mevacor tablet, containing 40 mg of lovastatin, was crushed and suspended in 0.5% carboxymethylcellulose (Sigma-Aldrich, St. Louis, USA) solution and given daily to rats via oral gavage at a dose of 11 mg/kg for 8 weeks. This dose was equivalent to 80 mg/day for an adult human. Oral gavages of the vehicles were given to SHAM and OVX groups for a similar duration of treatment. The duration of the study was based on a previous study, in which 8 weeks was shown to be adequate for significant changes in bone parameters to be observed [52].

2.3. Sample Collection. For the biochemical study, blood samples were collected at the start (pretreatment) and after 8 weeks of treatment (posttreatment) from all the groups except BC because they were sacrificed upon receipt. Blood samples were obtained from the retroorbital vessel after the rat was anesthetized with diethyl ether. After 3 hours, blood was centrifuged for 10 min at 3000 rpm, and the serum stored at −70°C for further use.

For bone histomorphometric analysis, the rats were sacrificed by high dose diethyl ether after completing the treatment period. The left femurs were removed and the distal portion kept in 70% alcohol.

2.4. Biochemical Analysis. Levels of bone biochemical markers, osteocalcin and CTX in serum were measured using an ELISA microplate reader (VERSA max, Sunnyvale, USA). The kits used were Rat-Mid Osteocalcin ELISA kit (IDS, UK) and RatLaps CTX-1 ELISA kit (IDS, UK).

2.5. Bone Histomorphometry. The left femur was decalcified with EDTA (Sigma Aldrich, St. Louis, USA) for 2 months and then embedded in histological paraffin wax. The decalcified paraffin blocks were sectioned at 6 μm with a microtome (Leica, Wetzlar, Germany) and stained with Hematoxylin and Eosin.

The static parameters, namely, osteoblast surface/bone surface (ObS/BS), osteoclast surface/bone surface (OcS/BS), eroded surface/bone surface (ES/BS), osteoid surface/bone surface (OS/BS), and osteoid volume/bone volume (OV/BV) were analysed using a quantitative stereological method for histology known as the Weibel technique.

The static histomorphometric indices were performed at the secondary spongiosa area, which is rich in trabecular bone. The selected metaphyseal region was located 1 mm from the lateral cortex and 3–7 mm from the lowest point of the growth plate.

Bone cellular average changes were analyzed and expressed using bone histomorphometric measurements as

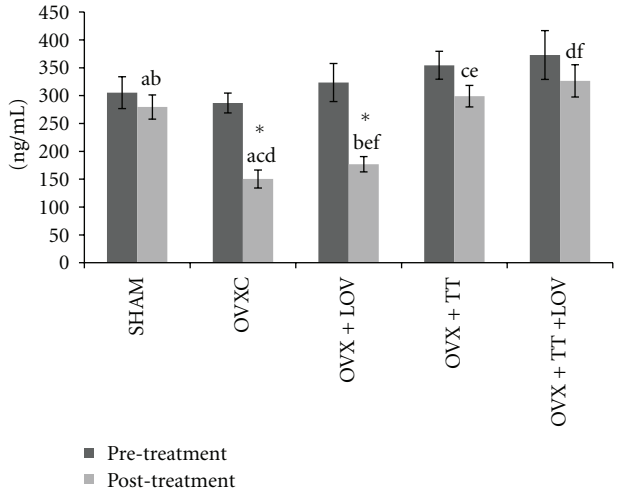

Figure 2: Serum osteocalcin levels in treatment groups. Data labeled with the same letter indicates significant difference between treatment groups. *Indicates significant difference between pre-treatment and posttreatment values for the same group. Data was presented as mean ± SEM. Significant level was taken at $P < 0.05$.

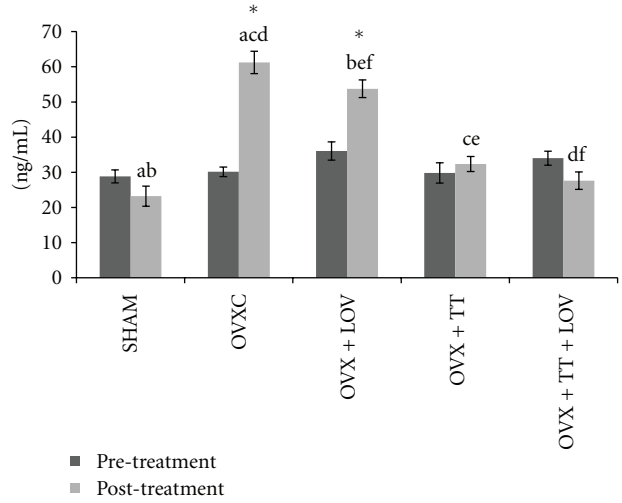

Figure 3: Serum CTX levels in treatment groups. Data labeled with the same letter indicates significant difference between treatment groups. *Indicates significant difference between pretreatment and posttreatment values for the same group. Data was presented as mean ± SEM. Significant level was taken at $P < 0.05$.

recommended by The American Society of Bone Mineral Research Histomorphometry Nomenclature Committee [53].

2.6. Statistical Analysis. Data analysis was performed using the Statistical Package for Social Sciences software (19, SPSS, Chicago, IL, USA). The Kolmgorov-Smirnov test was used as a normality test. The paired-sample t test was utilized to compare the same group before and after treatment. The ANOVA followed by post hoc Tukey's tests were used to determine the statistical significance between groups. The results were expressed as mean values ± standard error of the mean (SEM). The statistical differences were considered significant at $P < 0.05$.

3. Results

Serum osteocalcin level was significantly lower post-treatment compared to pretreatment for the OVXC and OVX + LOV groups. The posttreatment level of serum osteocalcin did not differ significantly from the pre-treatment level for the remaining groups. No significant differences were seen between the groups before treatment. After treatment, the serum osteoclacin level in the OVXC group was significantly lower than the SHAM group. The OVX + TT and OVX + TT + LOV groups had significantly higher serum osteocalcin levels compared to the OVXC and OVX + LOV groups, but they did not differ from the SHAM group. While the OVX + LOV group did not differ significantly from the OVXC group but was significantly lower than the SHAM group (Figure 2).

Serum CTX level was significantly higher posttreatment compared to pretreatment for the OVXC group. The post-treatment level of serum CTX did not differ significantly from the pretreatment level for the remaining groups No significant differences were observed between the groups before treatment. After treatment the serum CTX level for the OVXC group was significantly higher than the SHAM group. The OVX + TT and OVX + TT + LOV groups had significantly lower serum CTX levels compared to the OVXC and OVX + LOV groups, but they did not differ from the SHAM group. While the OVX + LOV group did not differ significantly from the OVXC group but was significantly higher than the SHAM group (Figure 3).

The OVXC group had significantly lower ObS/BS, OS/BS and OV/BV values than the BC and SHAM groups (Figures 4, 5, 6, 7, and 8). There were no significant changes in all static bone parameters between the BC and SHAM groups. The OVX + TT + LOV group had significantly higher ObS/BS and OV/BV values compared to the OVX + TT group; significantly higher ObS/BS, OS/BS, and OV/BV values compared to OVX + LOV and OVXC groups; and significantly higher ObS/BS, OS/BS, and OV/BV values than the BC and SHAM groups. The OVX + TT group had significantly higher ObS/BS, OS/BS, and OV/BV values compared to the OVX + LOV and OVXC groups, and significantly higher ObS/BS, OS/BS, and OV/BV values than the BC and SHAM groups. The OVX + LOV did not differ from the OVXC in all static bone parameters but had significantly lower ObS/BS, OS/BS, and OV/BV values than the BC and SHAM groups (Figures 4, 5, 6, 7, and 8).

The OVXC group had significantly higher OcS/BS and ES/BS values than the BC and SHAM groups. The OVX + TT + LOV group had significantly lower OcS/BS value compared to the OVX + TT group; significantly lower OcS/BS and ES/BS values compared to the OVX + LOV and OVXC groups; significantly lower OcS/BS value than the BC and SHAM groups; significantly lower ES/BS value than

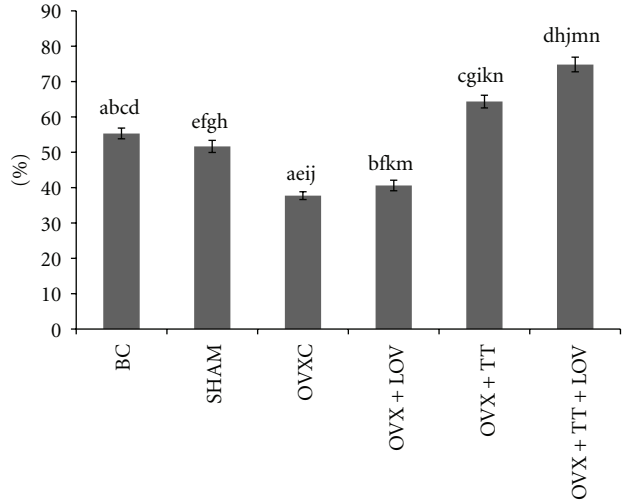

Figure 4: Osteoblast Surface/Bone Surface% (ObS/BS%) in treatment groups. Data labeled with the same letter indicates significant difference between treatment groups. Data was presented as mean ± SEM. Significant level was taken at $P < 0.05$.

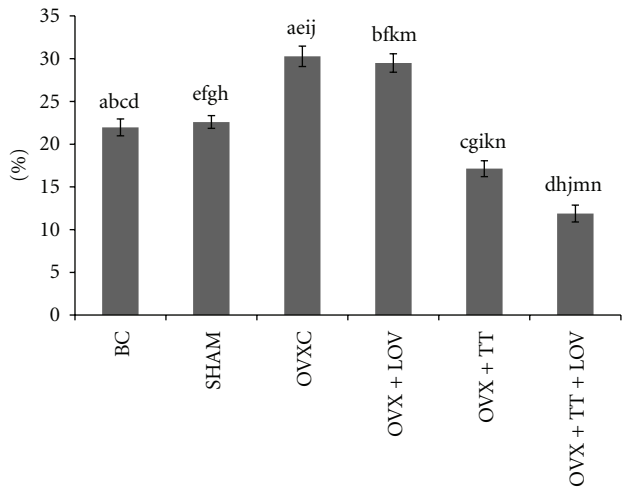

Figure 5: Osteoclast Surface/Bone Surface% (OcS/BS%) in treatment groups. Data labeled with the same letter indicates significant difference between treatment groups. Data was presented as mean ± SEM. Significant level was taken at $P < 0.05$.

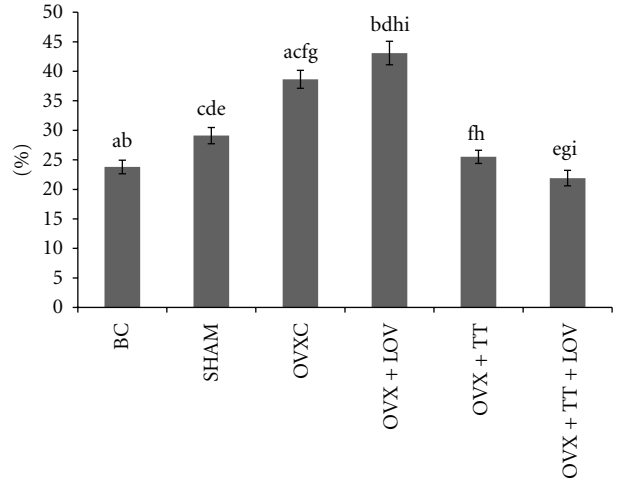

Figure 6: Eroded Surface/Bone Surface% (ES/BS%) in treatment groups. Data labeled with the same letter indicates significant difference between treatment groups. Data was presented as mean ± SEM. Significant level was taken at $P < 0.05$.

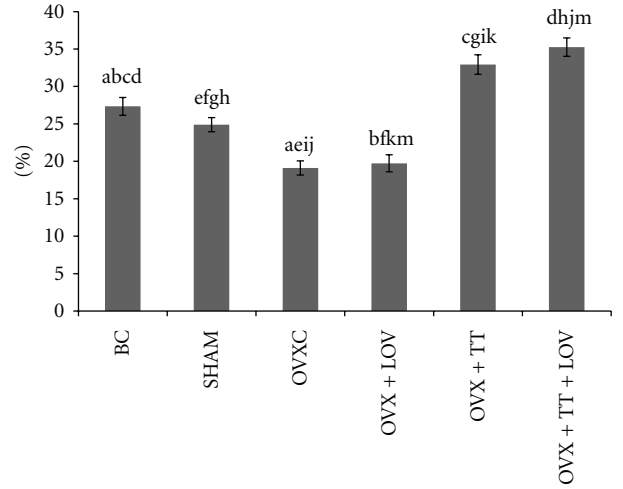

Figure 7: Osteoid Surface/Bone Surface% (OS/BS%) in treatment groups. Data labeled with the same letter indicates significant difference between treatment groups. Data was presented as mean ± SEM. Significant level was taken at $P < 0.05$.

the SHAM group. The OVX + TT group had significantly lower OcS/BS and ES/BS values compared to the OVX + LOV and OVXC groups; and significantly lower OcS/BS value than the BC and SHAM groups. The OVX + LOV group had significantly higher OcS/BS and ES/BS values than the BC and SHAM groups (Figures 4, 5, 6, 7, and 8).

4. Discussion

Both osteoblast and osteoclast cells are required for continuous bone remodeling. During bone formation, the osteoblast cells start to secrete osteoid and synthesize osteocalcin, while during bone resorption, the activated osteoclast cells dissolve the bone matrix resulting in the formation of the eroded surfaces and the release of CTX [54].

The results of the current study showed that daily supplementation of delta-tocotrienol in combination with lovastatin increased the osteoblastic bone formation and decreased osteoclastic bone resorption in ovariectomised rats as indicated by the OVX + TT + LOV group which had significantly higher serum osteocalcin, ObS/BS, OS/BS, and OV/BV values and significantly lower serum CTX, OcS/BS, and ES/BS values compared to the OVXC group. The role of the mevalonate pathway in the pathophysiology of osteoporosis suggests that critical regulatory mechanisms are needed to maintain osteoblast and osteoclast function. Inhibition of the mevalonate pathway by statins and tocotrienols

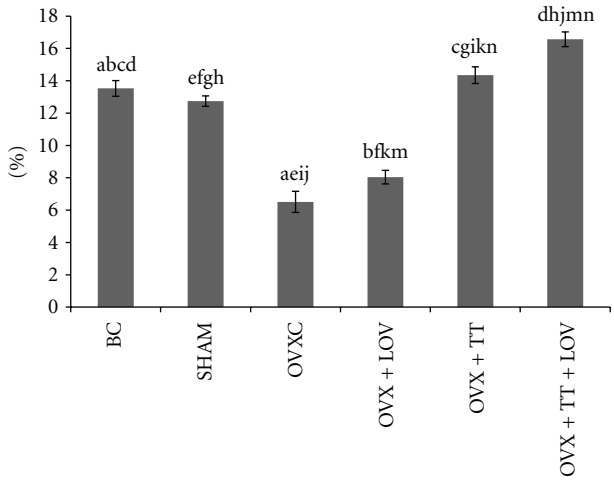

Figure 8: Osteoid Volume/Bone Volume% (OV/BV%) in treatment groups. Data labeled with the same letter indicates significant difference between treatment groups. Data was presented as mean ± SEM. Significant level was taken at $P < 0.05$.

(Figure 1) suppresses the prenylation of GTPase binding proteins and disrupts their function. Therefore, inhibition of GTPase function reduces the activity of osteoclasts and induces their apoptosis [5, 55–57]. Inhibition of GTPase function also increases osteoblast activity through enhancement of BMP-2 expression [5, 6, 57–60]. Ultimately, this will lead to stimulation of bone formation and decrease in bone resorption.

Competitive inhibition of HMGCoA reductase by statins reduces the cholesterol level. This reduction subsequently stimulates SREBP cleavage and inhibits HMGCoA reductase degradation, resulting in an increase in mRNA and HMG-CoA reductase protein expression [61]. In contrast, delta-tocotrienol inhibits the cleavage of SREBP and induces the degradation of HMGCoA reductase, thereby inducing the reduction in mRNA and protein HMGCoA reductase levels [61]. Therefore, the combination of lovastatin and delta-tocotrienol may have synergistic or additive effects on bone metabolism, while at the same time avoiding the unwanted effects of high doses and low bioavailability of lovastatin.

The current study found that delta-tocotrienol combined with lovastatin provided better bone formation and bone protection against ovariectomy-induced bone loss compared to delta-tocotrienol alone as indicated by the OVX + TT + LOV group which had significantly higher ObS/BS, and OV/BV values and significantly lower OcS/BS value compared to the OVX + TT group. The improvement in bone metabolism by the combined treatment may be due to synergistic or additive inhibition of the mevalonate pathway. Moreover, the OVX + TT group had significantly higher serum osteocalcin, ObS/BS, OS/BS, and OV/BV values and significantly lower serum CTX, OcS/BS and ES/BS values compared to the OVXC group. These results were consistent with those who found that 60 mg/kg of tocotrienols had antiosteoporotic effects in thyroidectomised, orchidectomised, oxidative stressed, adrenalectomized, nicotine treated, and ovariectomised rat models [62–69]. The dose of 60 mg/kg/day for rats is roughly equivalent to 420 mg/day for humans, taking into the account the metabolic rate of rodents is around ten times faster than that of humans. This dose is relatively low has no toxic effects. It had been reported that daily supplementation of 200 mg/kg palm vitamin E extract containing 18.43% alpha-tocopherol, 14.62% alpha-tocotrienol, 32.45% gamma-tocotrienol, and 23.93% delta-tocotrienol has no toxic effects in female mice [70].

The current study showed that the combination of delta-tocotrienol plus lovastatin increased bone formation and reduced bone loss compared to lovastatin alone as indicated by the OVX + TT + LOV group which had significantly higher serum osteocalcin level, ObS/BS, OS/BS, and OV/BV values and significantly lower serum CTX, OcS/BS, and ES/BS values compared to the OVX + LOV group. Moreover, there were no significant changes in all biochemical markers and static bone histomorphometric indices between the OVXC and OVX + LOV groups. Therefore, lovastatin alone failed to enhance bone formation and to prevent bone resorption in ovariectomised rats at clinically tolerable hypocholesterolemic doses. Statins have limited distribution to the peripheral tissues after oral administration [71]. Therefore, they yield uncertain results as bone anabolic agents when used *in vivo* at cholesterol lowering doses. Bjarnason et al. [26] reported that fluvastatin did not affect serum osteocalcin and serum and urinary CTX levels in postmenoposal women with osteoporosis and mild hypercholesterolemia when given in clinically relevant doses. A cross over clinical study showed that 40 mg/day of atorvastatin had no effect on serum osteocalcin and CTX in type 2 diabetic men with baseline hypercholesterolemia compared to placebo [72]. Similar results were seen, when a randomized clinical trial measured the serum CTX concentration in hypercholesterolemic patients treated with 20–80 mg/day of simvastatin [24]. Twenty mg/day of pravastatin did not affect the serum CTX level in hypercholesterolemic postmenopausal women [25]. Meta analysis of both observational studies and clinical trials of around 300,000 patients found that there was clinical benefit from the use of oral statins but there was no significant reduction in fracture incidence in older women [73]. Yao and his coworkers ascertained that the 0.3, 0.6, 3, 6, and 10 mg/kg of simvastatin for 60 days could not prevent or restore ovariectomy-induced osteoporosis [74]. On the other hand, previous studies showed that lovastatin and other statins enhanced bone formation and reduced bone resorption after high oral doses in rodents [5, 7–10]. This indicates that clinically nontolerable doses of oral statins are required to achieve successful prevention and treatment of osteoporosis. Myotoxicity and hepatotoxicity were associated with the high doses of oral statins [27–29]. In this study, 11 mg/kg of lovastatin was chosen, which if extrapolated to human is roughly equivalent to 80 mg/day, the highest dose of lovastatin used as an antihyperlipidemic agent.

The results of the current study found that the OVX + TT + LOV group had significantly higher ObS/BS,

OV/BV and OS/BS values and significantly lower OcS/BS and ES/BS values than the SHAM group. These current findings indicate that delta-tocotrienol in combination with lovastatin promoted better cellular bone histomorphometric parameters than the SHAM group, thus exhibiting bone anabolic effects. Therefore, the combined treatment has the potential to increase bone strength. Recently, tocotrienols were shown to have bone anabolic activity in ovariectomised female, intact male and nicotine-treated male rats [32–35], and these findings had been confirmed by the results of the current study (Figures 4, 5, 7, and 8). Therefore, combination of delta-tocotrienol plus lovastatin may have the ability to further improve the bone density in normal bone.

5. Conclusion

Supplementation of delta-tocotrienol in combination with oral statins at clinically acceptable doses has both bone antiosteoporotic and anabolic activity and was more effective than delta-tocotrienol and lovastatin given individually. Therefore, the combination of delta-tocotrienol plus lovastatin has the potential to be used as an anti-osteoporotic agent especially in patients who are at risk of both conditions, that is, osteoporosis and hypercholesterolemia. This is especially true for postmeanopausal women, and also for men of the older age group.

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Research Article

Combined Effects of *Eurycoma longifolia* and Testosterone on Androgen-Deficient Osteoporosis in a Male Rat Model

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Androgen-deficient osteoporosis in men is treated with testosterone therapy, which is associated with side effects. *Eurycoma longifolia* (EL) is known to possess androgenic properties and has been reported to protect bone from androgen-deficient osteoporosis in experimental animal models. The present study aimed to determine the effectiveness of combination therapy of EL and testosterone (T) in treating androgen-deficient osteoporosis. Forty male Sprague-Dawley rats were divided into: sham-operated (SHAM), orchidectomized-control (ORX), orchidectomized with testosterone (ORX + T), orchidectomized with EL (ORX + EL), and orchidectomized with combined T and EL therapy (ORX + T + EL). EL was administered via oral gavages daily at the dose of 15 mg/kg. T was injected intramuscularly at 8 mg/kg and 4 mg/kg for the ORX + T and ORX + T + EL groups, respectively. Following 6 weeks of treatment, the osteocalcin levels of ORX + T and ORX + T + EL groups were significantly lower than the SHAM group ($P < 0.05$). The posttreatment CTX levels of ORX + T and ORX + T + EL groups were significantly lower than their pretreatment levels ($P < 0.05$). Biomechanically, the strain parameter of the ORX + T + EL group was significantly higher than the ORX group ($P < 0.05$). Thus, the combination therapy of EL and low-dose T has potential for treatment of androgen-deficient osteoporosis. The lower T dose is beneficial in reducing the sideeffects of testosterone therapy.

1. Introduction

Partial androgen deficiency, which is common in aged men, may lead to bone loss and osteoporosis [1]. Androgen deficiency due to natural aging is the main cause of osteoporosis in men [2]. This bone-thinning disease was given more attention lately after becoming one of the main causes of morbidity and mortality in older men. In the United States, 1.5 million men over 65 years old were reported to suffer from osteoporosis [3]. One of the complications of osteoporosis is pathological fracture when the osteoporotic bone breaks with minimal force. Men are more likely than women to die following a hip fracture [4], and it is estimated that 31% of men with hip fracture died within a year after fracture [5].

Among the accepted treatment for androgen-deficient osteoporosis in men is testosterone replacement therapy (TRT). Studies have shown that TRT increased bone mass [6] and improved muscle strength and mass in testosterone-deficient male [7, 8]. It can be given via various routes, but intramuscular injection is widely used as it provides immediate testosterone surge. This form of administration is painful and associated with prostate cancer and increased haematocrit [9]. Other routes of testosterone have their disadvantages such as liver tumors with the oral form or transference to women and children by skin contact with the transdermal form.

Eurycoma longifolia (EL), classified under the *Simaroubaceae* family, is a traditional medicinal plant, known as "*Pasak Bumi*" in Indonesia, "*Cay Ba Binh*" in Vietnam, "Langir Siam" in Bahrain, and "Tung Sawa" in Thailand [10]. EL is a herbaceous plant that grows slowly. It can reach a maximum height of 15 to18 meters and bear fruit after 2 to 3 years.

Initially, the fruits are green in colour but turned dark red when ripe. The leaves are oval in shape with 10 to 30 leaflets and arranged spirally. EL was officially announced as a protected plant in many growing areas, including Malaysia [11].

It was reported that EL has aphrodisiac effects and the ability to increase the testosterone levels [12]. Supplementation of EL to male with idiopathic infertility improved their sperm concentration, motility, and morphology [13]. Due to its androgenic effects, EL may be useful in the treatment of diseases related to androgen deficiency. Recently, a study demonstrated that EL has potential in the treatment of osteoporosis due to androgen deficiency in a rat model [14]. Both EL and testosterone were found to be capable of preventing bone calcium loss in orchidectomized rats [14]. The dose of EL used in the present study was 15 mg/kg rat weight, which was very much lower than its lethal dose 50 (LD_{50}) of more than 5000 mg/kg [15]. Therefore, EL can be combined with testosterone to lower the testosterone dose, thus minimizing side effects.

The aim of the study was to determine the effects of the combination therapy of EL and low-dose testosterone on bone markers and bone strength of orchidectomized rats. Their effects on bone were compared to a group of orchidectomised rats receiving the full dose of testosterone alone. Aged orchidectomized rat is an accepted model for androgen-deficient osteoporosis [16]. The 6-week duration of treatment in the present study was similar to an earlier study [14] and should be sufficient for orchidectomy to produce significant bone changes.

2. Material and Methods

2.1. Animal Model. Forty aged male Sprague-Dawley rats (aged 10 to 12 months) were obtained from the UKM Animal House. The rats were housed at two per cage with the surrounding temperature of 29 ± 3°C and under natural day/night cycle. They were fed commercial food pellets and tap water *ad libitum*. They were allowed to adjust to the new environment for a week before the study was started. The study was approved by the UKM Animals Ethics Committee (PP/FAR/2010/NAZRUN/14-JULY/310-JULY-2010-JUNE-2011). The rats were divided into groups of sham-operated (SHAM), orchidectomized-control (ORX), orchidectomized and given EL (ORX + EL), orchidectomized and given testosterone (ORX + T), and orchidectomised and given combined testosterone and low-dose EL (ORX + T + EL). Body weight was measured before treatment and then weekly until the end of the study.

2.2. Orchidectomy Procedure. Before performing orchidectomy, the rats were anesthetized with Ketamil : Xylazil (1 : 1). A 2 cm ventral midline incision was made in the scrotum, and the skin was retracted to expose the tunica. The tunica was pierced, and the testis was pushed out and raised to expose the underlying blood vessels and tubules. The spermatic cord was clamped and tied with absorbable catgut suture at the confluence of the blood vessels and epididymis. The testis was removed, and all deferential vessels and ducts were replaced back into the tunica. Similar procedures were repeated on the other testis.

2.3. Eurycoma longifolia and Testosterone. *Eurycoma longifolia* was supplied by Phytes Biotek Sdn. Bhd. (Selangor, Malaysia), a licensed GMP manufacturer of herbal products, in the form of a freeze-dried standardized extract (batch No: TA 071210). It was extracted from the roots using a patented high-pressure water extraction process (US 7,132,117 B2), filtered at 1–4 microns and freeze-dried without maltodextrins or lactose. Physically, it was a light brown fine powder with 4–6% moisture content. Its major chemical components were proteins (31.75%), glycosaponins (41.08%), and eurycomanone (1.60%). This extract was the same form used for human consumption as health supplements.

EL aqueous extract powder was dissolved in deionized water, and 15.0 mg/kg was given to rats in the ORX + EL and ORX + T + EL groups via daily oral gavages at 9 am for 6 weeks [17]. Testosterone was purchased from TCI UK Ltd (UK). It was diluted in olive oil (Bertolli, Italy) and 8.0 mg/kg was injected intramuscularly once daily at 9 am for 6 weeks for the ORX + T group [18]. As for the low-dose T in the ORX + T + EL group, half of the dose (4.0 mg/kg) was injected once daily at 9 am for 6 weeks. The SHAM, ORX, ORX + T, and ORX + T + EL groups were also given oral gavages of vehicle (deionised water). In addition, the SHAM, ORX, and ORX + EL groups received intramuscular injections of vehicle (olive oil). Blood samples were collected before the start of treatment and after 6 weeks of treatment from retroorbital sinus after anesthetizing the rats with ether. After 3 to 4 hours, the blood was centrifuged at 3000 rpm for 10 minutes, and the serum stored at temperature of −70°C.

2.4. Parameters. At the end of the treatment, the rats were sacrificed with overdoses of ether, and the femora were dissected out and cleansed of all soft tissues. The femora were wrapped with gauze soaked with phosphate buffered solution, wrapped again with aluminium foil and placed in −70°C. The study groups were numbered to blind the operators. Each femur was placed on the Instron machine (Instron Microtester 5848, Instron Corp., USA) in a three-point bending configuration. The load was applied at the mid-diaphysis in an anteroposterior direction with a loading speed of 5 mm/min until the femur fractured. The load, stress, and strain-deflection curves were automatically calculated by the computer using the Bluehill software. The femora were kept moist at all times during the testing. The parameters measured were load, stress, strain, and Young's Modulus.

Bone formation marker (serum osteocalcin) and bone resorption marker (C-terminal telopeptide of type I collagen, CTX) were measured before and after treatment using ELISA technique with ELISA reader (VERSA MAX, Sunnyvale,USA). The kits used were the Rat Osteocalcin ELISA (Biomedical Technologies, Herlev, Denmark) and RatlapsTM ELISA CTX-1 kits (Nordic Biosciences, IDS, UK).

2.5. Statistical Analysis. For normally distributed data, the statistical test used was ANOVA followed by Tukey's HSD.

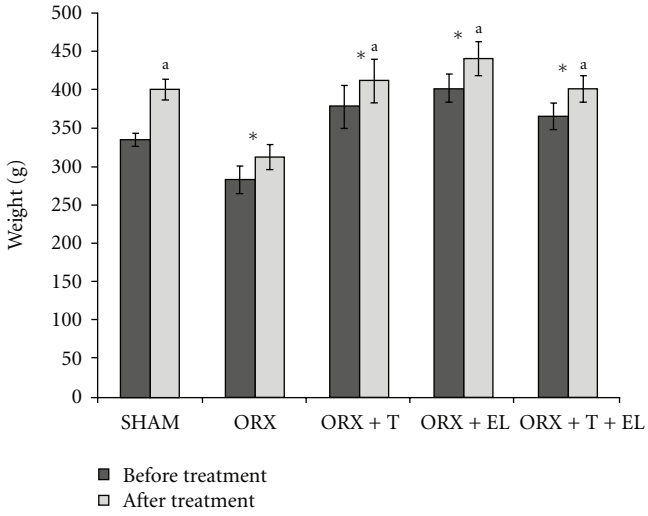

Figure 1: Mean body weight before and after treatment. Data presented as mean ± SEM. SHAM: sham-operated control. ORX: orchidectomized control. ORX + T: orchidectomized supplemented with testosterone. ORX + EL: orchidectomized supplemented with EL. ORX + T + EL: Orchidectomized and combination treatment of testosterone with EL. *Significant difference before and after treatment within the group ($P < 0.05$). [a]Significant difference after treatment compared to ORX group ($P < 0.05$).

Data that was not normally distributed was analyzed using Mann-Whitney followed by Kruskal-Wallis test if more than two groups were compared. The level of significance was taken as $P < 0.05$.

3. Results

There were no significant differences in the mean body weight for all the groups of rats at the start of the study. At the end of the study, all groups except the SHAM group showed significantly higher body weight compared to their pretreatment weights. The posttreatment weight of the ORX group was significantly lower than the rest of the groups (Figure 1).

There was no significant difference in the osteocalcin levels between the SHAM and ORX groups. The posttreatment osteocalcin levels of ORX + EL and ORX + T + EL groups were significantly lower compared to their pretreatment levels. The posttreatment osteocalcin levels of ORX + T and ORX + T + EL groups were also significantly lower compared to the posttreatment level of the SHAM group. There was also no significant difference in the pre- or posttreatment osteocalcin levels of the ORX + T + EL group compared to the ORX + T or ORX + EL group (Figure 2(a)).

There was no significant difference in the CTX levels between the SHAM and ORX groups. The posttreatment CTX levels of ORX + T and ORX + T + EL groups were significantly lower than their pretreatment levels. The posttreatment CTX level of ORX + EL group also appeared to be lower than its pre-treatment level, but the difference was not significant. There was no significant difference in the pre- or posttreatment CTX levels of the ORX + T + EL group compared to the ORX + T or ORX + EL group (Figure 2(b)).

As for the biomechanical parameters, there was no significant difference between the SHAM and ORX groups, although these parameters appeared to be lowered in the ORX group. There was no other significant difference between the groups except for the significantly higher strain parameter for the ORX + T + EL group compared to the ORX group. The ORX + T + EL group also had the highest value for the rest of the biomechanical parameters but were not statistically significant compared to the other treatment groups (Figures 3(a), 3(b), 3(c), and 3(d)).

4. Discussion

All the rats in the study had gained weight, but the orchidectomised rats failed to gain weight normally, resulting in their body weight to be significantly lower than the sham-operated rats at the end of the study. Testosterone reduction due to orchidectomy may have caused the weight loss due to loss of muscle and bone mass [19]. Testosterone, EL, or combination of both treatments could maintain the weight gain of orchidectomised rats. Testosterone has been shown to stimulate the release of growth hormone [20] which then stimulates the expression of insulin-like growth factor-1 (IGF-1). IGF-1 has anabolic effects on skeletal muscle mass, stimulates protein synthesis, decreases protein degradation, and increases the lean body mass [21–23]. EL supplementation has been found to increase the body mass index [24] and has ergogenic effects [25] in adult male subjects. Based on the findings of these studies, it is not surprising to find that the combination of EL and low-dose testosterone was able to maintain the weight gain in orchidectomised rats.

In normal aged rats, the growth plate should have stabilized resulting in balanced bone formation and resorption [26] as seen in the sham-operated rats used in this study. The bone formation (osteocalcin) and resorption (CTX) markers of the sham-operated rats were not significantly different before and after treatment. Theoretically, there should be increased bone markers with the high bone turnover due to gonadectomy in aged male rats [27]. However, this was not observed in the present study. In comparison with the findings of a previous study [14], there was also no significant change in the osteocalcin level of orchidectomised rats, but the CTX level was not similarly raised although the trend was there.

Treatment with testosterone or EL significantly lowered the posttreatment levels of osteocalcin and CTX. Studies have shown that testosterone treatment prevented cancellous bone loss in orchidectomised rats, similar to the action of estrogen on bone resorption [28, 29]. The findings of the present study were consistent with an earlier study by Shuid et al. [14] that EL was capable of preventing bone loss in orchidectomised rats. Combination of testosterone and EL was able to significantly lower both the formation and resorption markers. This meant that the combination treatment could reduce bone turnover in orchidectomised rats, much better than treatment with EL or testosterone alone. Combination of EL and testosterone seemed to produce

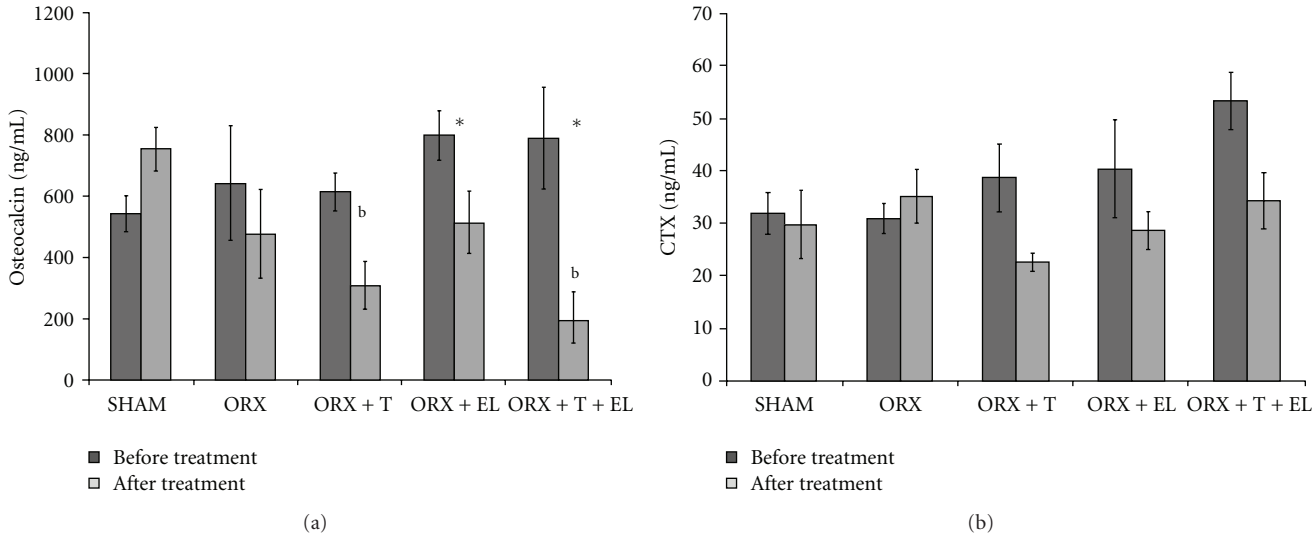

Figure 2: (a) Mean serum osteocalcin levels for all the groups before and after treatment. (b) Mean serum C-terminal telopeptide of type I collagen for all the groups before and after treatment. Data presented as mean ± SEM. SHAM: sham-operated control. ORX: orchidectomized control. ORX + T: orchidectomized supplemented with testosterone. ORX + EL: orchidectomized supplemented with EL. ORX + T + EL: orchidectomized and combination treatment of testosterone with EL. [b]the posttreatment level is significant different compared to the posttreatment level of SHAM group ($P < 0.05$). *Significant difference before and after treatment in each group ($P < 0.05$).

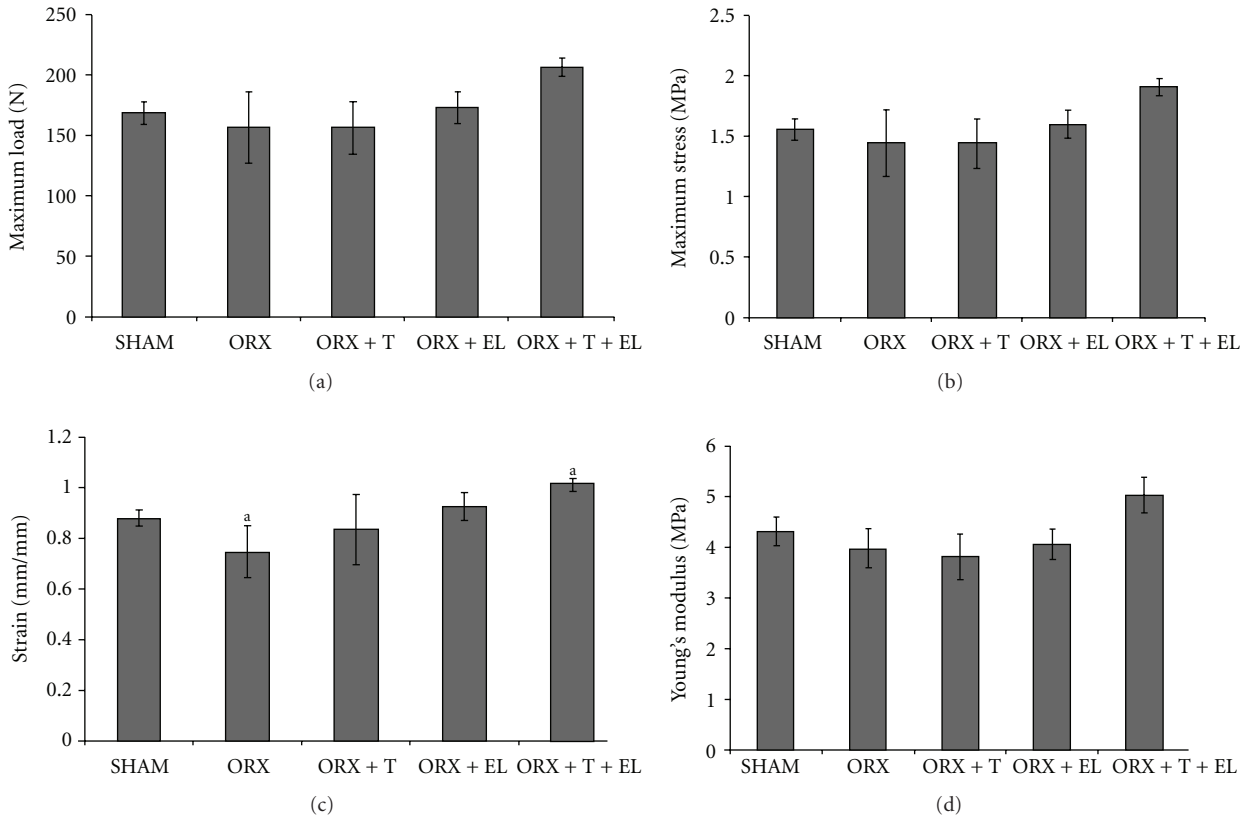

Figure 3: (a) Maximum load values for all the groups. (b) Maximum stress values for all the groups. (c) Strain values for all the groups. [a]Significant difference between the groups ($P < 0.05$). (d) Young's modulus for all the groups. Data presented as mean ± SEM. SHAM: sham-operated control. ORX: orchidectomized control. ORX + T: orchidectomized supplemented with testosterone. ORX + EL: orchidectomized supplemented with EL. ORX + T + EL: Orchidectomized and combination treatment of testosterone with EL.

synergistic effects in maintaining the bone turnover in orchidectomised rats.

The osteocalcin or CTX levels of the combination of EL and testosterone group were not significantly different compared to the testosterone or EL group. When compared to the pretreatment levels, combination of EL and testosterone has resulted in significantly lower osteocalcin or CTX levels. Treatment with testosterone or EL was only able to lower either one of the bone markers. This indicated that combination treatment could reduce bone turnover in orchidectomised rats, much better than treatment with EL or testosterone alone. This is beneficial as high bone turnover can lead to thinning of the bone, resulting in abnormal bone microarchitecture and reduced bone mineralization [30–32]. Combination of EL and testosterone seemed to produce synergistic effects in reducing the bone turnover of orchidectomised rats.

Shuid et al. [14] reported that reduction in bone calcium can be recovered with EL or testosterone treatment. To the best of our knowledge, the effects of EL on bone biomechanical testing were never reported. In the present study, there was no significant difference in the bone biomechanical strength of the orchidectomised rat compared to the sham-operated rats. Other studies have also found no significant change in the bone biomechanical strength of orchidectomised rats [33, 34]. The bone strength is determined by the bone mass and the intrinsic properties of the bone material [35, 36]. This indicated that the bone changes induced by orchidectomy may not be sufficient to affect bone strength. The bone strength of rats that have received the combination of EL and testosterone was not different from rats receiving testosterone or EL alone. However, these rats have recorded a significantly higher strain parameter than the orchidectomised rats. This meant that the femora of rats receiving the combination of EL and testosterone could resist deformation with the force applied, much better than the other groups. Treatment with EL also seemed to produce higher biomechanical parameters although they did not reach statistical significance. The combination of EL and testosterone has resulted in the highest values for all the biomechanical parameters but only the strain parameter reached statistical significance. This meant that the combination of EL and testosterone was synergistic in maintaining the bone strength against the deleterious effects of orchidectomy.

The combination of EL and testosterone was able to reduce bone turnover and improve the bone strength of orchidectomised rats more effectively than treatment with EL or testosterone alone. It is important to note that the dose of testosterone used in combination with EL was only half of that used in the ORX + T group. Thus, combination of EL and low dose testosterone was synergistic in maintaining the bone turnover and strength of orchidectomised rats.

5. Conclusion

Combination of EL and testosterone has shown potential for the treatment of androgen-deficient osteoporosis. With this combination, lower dose of testosterone can be used, therefore minimizing its side effects. This opens the door for future studies to explore the possible role of combination therapy in the treatment of androgen-deficient osteoporosis in men.

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Research Article

The Effects of *Cosmos caudatus* on Structural Bone Histomorphometry in Ovariectomized Rats

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Osteoporosis is considered a serious debilitating disease. *Cosmos caudatus* (*ulam raja*), a plant containing antioxidant compounds and minerals, may be used to treat and prevent osteoporosis. This study determines the effectiveness of *C. caudatus* as bone protective agent in postmenopausal osteoporosis rat model. Thirty-two female rats, aged 3 months old, were divided into 4 groups. Group one was sham operated (sham) while group two was ovariectomized. These two groups were given ionized water by forced feeding. Groups three and four were ovariectomized and given calcium 1% ad libitum and force-fed with *C. caudatus* at the dose of 500 mg/kg, respectively. Treatments were given six days per week for a period of eight weeks. Body weight was monitored every week and structural bone histomorphometry analyses of the femur bones were performed. Ovariectomy decreased trabecular bone volume (BV/TV), decreased trabecular number (Tb.N), and increased trabecular separation (Tb.Sp). Both calcium 1% and 500 mg/kg *C. caudatus* reversed the above structural bone histomorphometric parameters to normal level. *C. caudatus* shows better effect compared to calcium 1% on trabecular number (Tb.N) and trabecular separation (Tb.Sp). Therefore, *Cosmos caudatus* 500 mg/kg has the potential to act as the therapeutic agent to restore bone damage in postmenopausal women.

1. Introduction

Estrogen deficiency increases the risk of developing osteoporosis. Estrogen was found to have antioxidant properties [1] and was also shown to increase the expression of glutathione peroxidase in osteoclasts [2], an enzyme which is responsible for the degradation of hydrogen peroxide. Estrogen deficiency will reduce the expression of the enzyme and renders the bone susceptible to hydrogen peroxide attacks.

In osteoporosis, lipid peroxidation is increased due to the reduction in antioxidants [3], and reactive oxygen species are found to play a role in bone metabolism [4]. Free radicals have also been shown to be cytotoxic to osteoblastic cells [5]. Loss of estrogens accelerates the effects of aging on bone by decreasing defence against oxidative stress which leads to bone loss [6].

Since free radicals and lipid peroxidation are involved in bone metabolism and may be the culprit in causing bone loss, substances having antioxidative activities can overcome the detrimental effects. Our previous studies have shown the beneficial effects of palm-oil derived tocotrienols in several experimental osteoporosis, ovariectomized [7], steroid

induced [8], and nicotine induced [9]. The effects of palm-oil derived tocotrienols may be attributed to its antioxidative activities.

Thus, in finding alternatives in the treatment of osteoporosis, a local plant, *Cosmos caudatus* or locally known as "ulam raja" (King's salad), is of consideration. Previous study has shown that this plant has antioxidative activities [10]. It contains phenolic compounds that contribute to the color, antioxidant, and anticarcinogenic properties of the plants. For every 100 g of *Cosmos caudatus*, the total phenolic compound is 21.41 mg. It is also found that *Cosmos caudatus* had extremely high antioxidant capacity of about 2,400 mg l ascorbic acid equivalent antioxidant capacity (AEAC) per 100 g of fresh sample [11]. It is also believed that *Cosmos caudatus* promote the formation of healthy bones [12]. Thus, we hypothesized that *Cosmos caudatus* may exert protective effects on bone of ovariectomized rats which is a suitable animal model for studying postmenopausal osteoporosis. In this study, the effects of *Cosmos caudatus* on structural bone histomorphometry were determined.

2. Materials and Methods

2.1. Animals and Treatment. Thirty-two young adult (3 months) female Wistar rats, weighing 190 g–260 g, were obtained from the Laboratory Animal Resource Unit, Faculty of Medicine, Universiti Kebangsaan Malaysia. Rats were randomly assigned to four groups with eight rats in each group. Group 1 was sham operated (sham) while the second was ovariectomized-control group (OVX). The third and fourth groups were ovariectomized and treated with calcium 1% (Ca) ad libitum and force-fed with 500 mg/kg *C. caudatus* extract (CC), respectively. Treatment was given six days a week for eight weeks and body weight was recorded weekly. The study was approved by the Universiti Kebangsaan Malaysia Animal Ethics Committee with the approval code of PP/FAR/2008/NORAZLINA/12-AUGUST/225-SEPT-2008-AUG-2009.

2.1.1. Diet, Cosmos caudatus, and Calcium 1%. All rats received normal rat chow obtained from Gold Coin, Malaysia. The composition of rat chow is shown in Table 1. The aqueous extract of *C. caudatus* with the concentration of 500 g/300 mL was prepared by School of Chemical Sciences & Food Technology, Faculty of Science and Technology, Universiti Kebangsaan Malaysia using water extraction method which was previously described [10]. The 500 mg/kg dose was prepared by mixing *C. caudatus* with deionized water in ratio 3 : 7. Calcium 1% solution was prepared by mixing 1 g of hemicalcium lactic acid (Sigma Chemical CO., USA) with 100 mL deionized water.

2.2. Ovariectomy. Before the surgery, the rats were anesthetized with Ketamil and Ilium Xylazil-20 (Troy Laboratories PTY, Australia), given intraperitoneally, at 1 : 1 ratio. A vertical incision was made approximately 15 cm in the abdomen using a sterilized sharp knife. The right and left ovaries were cut and removed. Before the ovaries were cut, the fallopian tubes were tied to prevent bleeding. The muscle layer under the skin was stitched up by sterile and soluble suture (Serafit, Germany). Then, the outer layer of skin was sewn with nonwater soluble suture (Seralon, Serag Wiessner, Germany). The procedure for sham operated rats was just the same with ovariectomized rats, but both of the ovaries were not removed. Rats were left recuperating for 1 week before commencing the treatment.

Table 1: Composition of rat chow (Golden Coin, Malaysia).

Composition	Amount/percentage
Crude protein	21–23%
Crude fibre (max)	5.0%
Crude fat (min)	3.0%
Moisture (max)	3.0%
Calcium	0.8–1.2%
Phosphorus	0.6–1.0%
Nitrogen free extract	49.0%
Vitamin A	10 M.I.U.
Vitamin D_3	2.5 M.I.U
Vitamin E	15 g
Vitamin K	trace
Vitamin B_{12}	trace
Thiamine	trace
Riboflavin	trace
Pantothenic acid	trace
Niacin	trace
Pyridoxine	trace
Choline	trace
Santoquin	trace
Microminerals	trace

2.3. Bone Histomorphometry. Upon sacrifice, the distal part of the femur was fixed with 70% ethanol and undergoes undecalcified bone preparations. The bone samples were embedded in polymer methyl methacrylate according to Difford [13], sectioned at 9 μm thickness using a microtome, stained using von Kossa method [14], and analyzed using an image analyzer with the Video Test-Master software. The parameters measured were trabecular bone volume (BV/TV), trabecular thickness (Tb.Th), trabecular number (Tb.N), and trabecular separation (Tb.Sp). All measurements were performed randomly at the metaphyseal region, which was located 3–7 mm from the lowest point of the growth plate and 1 mm from the lateral cortex [15]. The selected area is the secondary spongiosa area, which is rich in trabecular bone. All parameters were measured according to the American Society of Bone Mineral Research Histomorphometry Nomenclature Committee [16].

2.4. Statistical Analysis. Results were presented as mean ± standard error of the mean (SEM). All data were analysed using the Statistical Package for Social Sciences software. The Kolmogorov-Smirnov test was used for normality. ANOVA followed by Tukey's HSD tests were used for normally

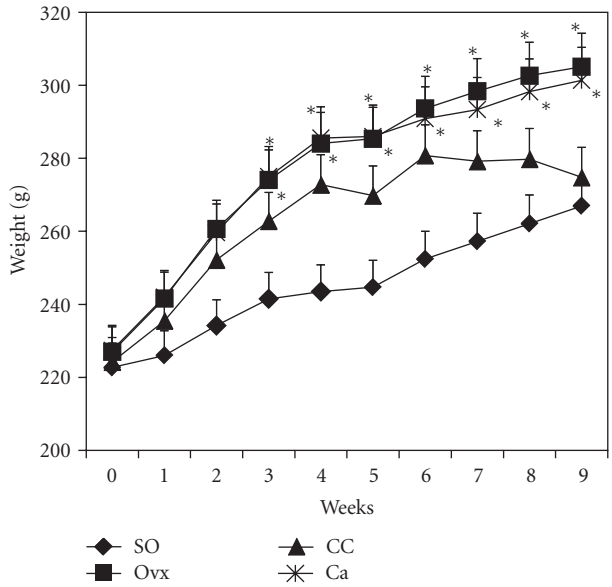


FIGURE 1: Body weight of rats for all treatment groups. *Indicates significant difference ($P < 0.05$) compared to SO group. SO, sham operated; Ovx, ovariectomized; CC, ovariectomized and supplemented with 500 mg/kg *Cosmos caudatus*; Ca, ovariectomized and supplemented with 1% calcium.

distributed data while Kruskal-Wallis and Mann-Whitney tests were used for not normally distributed data.

3. Results

Figure 1 shows the body weight of all groups throughout the study period. Ovariectomized group gained weight and was significantly different compared to sham-operated group beginning from week 3. Similar findings were observed in the calcium supplemented group. Group supplemented with *C. caudatus* also showed weight gain but did not differ compared to the sham-operated group.

Figure 2 shows the photomicrographs of trabecular bone with von Kossa staining for the different treatment groups. Ovariectomy caused a reduction in bone volume (BV/TV) and an increase in trabecular separation (Tb.Sp) compared to the control group (Figures 3 and 4, resp.). Administration of *C. caudatus* was able to improve bone volume and trabecular separation in ovariectomized rats (Figures 3 and 4, resp.) while calcium administration was only able to improve bone volume (Figure 3). In addition, the group supplemented with *C. caudatus* showed a higher trabecular number compared to ovariectomized group (Figure 5). No significant differences were seen in trabecular thickness (Figure 6).

4. Discussion

Ovariectomized rats have been used by researchers as the model for postmenopausal osteoporosis. Even though limitations exist, certain characteristics in the rat model mimic the bone changes in postmenopausal women and made the study of the human disease possible [17, 18]. Reduction in bone mineral density occurs two months after ovariectomy with greater loss seen in regions rich in trabecular bone [19].

Ovariectomized group showed increase in body weight which was statistically different compared to sham-operated group beginning from week 3 (Figure 1). This observation is common in estrogen-deficient animals since the deficient state induces hyperphagia in rats [20]. Group supplemented with calcium showed the same findings. However, rats given *C. caudatus* did not show a significant increase in body weight. *Cosmos caudatus* seemed able to prevent weight gain induced by ovariectomy. However, the exact mechanism of this occurrence is unknown.

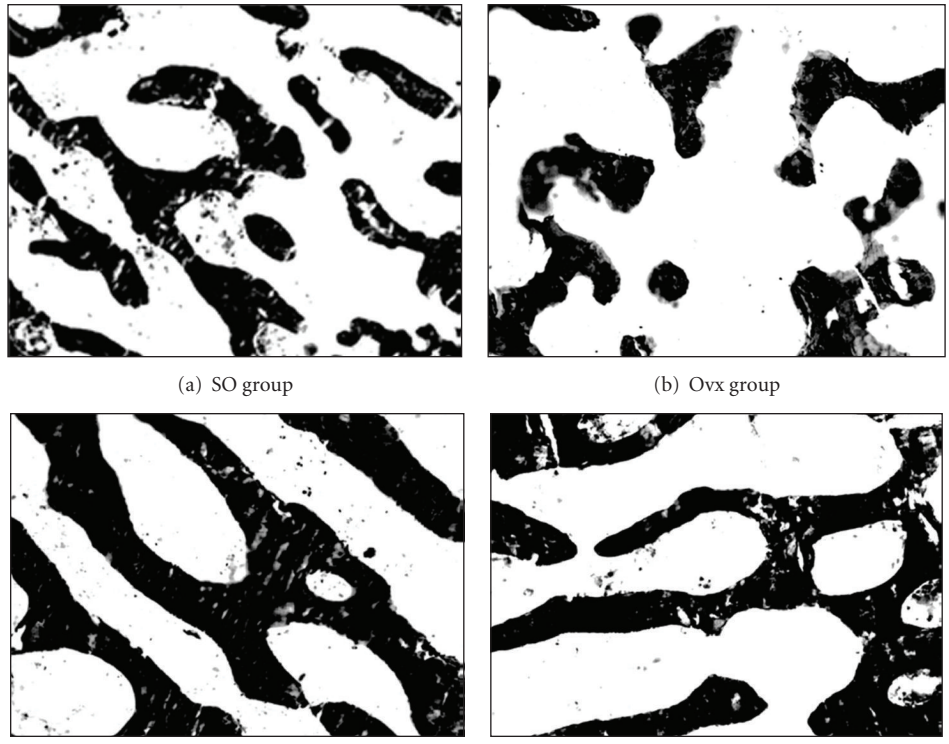
In this study, ovariectomy caused loss of bone volume, increase in trabecular separation, and decrease in trabecular number (Figures 3, 4, and 5, resp.). The bone loss is reflected in the photomicrograph of the trabecular bone in which the ovariectomized group showed perforated and discontinued trabecular bone compared to the sham-operated group (Figures 2(a) and 2(b)). Similar findings were observed in other studies [21, 22]. However, trabecular thickness did not show any significant differences between the different groups. This is in contrast with other studies [23, 24] which used micro-CT in their studies as opposed to conventional histomorphometry in our study. In another study which used older rats, ovariectomy was also shown to cause a decrease in trabecular thickness [25]. Different method and different age range of the animals may contribute to the discrepancy seen in this study.

Estrogen deficiency is the major factor which affects bone in ovariectomized rats [26]. The deficiency state induces osteocytes apoptosis which further leads to increase in osteoclastic resorption [27]. Ovariectomy has been associated with increase in oxidative stress as evident by high malondialdehyde levels [28]. The condition of oxidative stress would eventually lead to bone loss [29].

Ovariectomized rats supplemented with *C. caudatus* showed improvements in bone volume, trabecular separation and trabecular number (Figures 3, 4, and 5, resp.). Photomicrograph of the trabecular bone of rats given *C. caudatus* appears similar to the sham-operated rats (Figures 2(a) and 2(c)). *Cosmos caudatus* is shown to contain flavonoids [30] and ascorbic acid [31]. It has also been shown to exert antioxidant activity [32]. This may contribute to the bone protective effects of *C. caudatus* observed in the present study.

The dose of *C. caudatus* in this study was chosen based on previous study which used *C. caudatus* at the dose of 100, 200, and 300 mg/kg. In the previous study, the effects of *C. caudatus* on bone biochemical markers and bone histomorphometry in ovariectomized rats were determined. It was found that *C. caudatus* at all doses was able to prevent the increase in interleukin-1 and pyridinoline as seen in the ovariectomized group. However, no significant changes were seen in the bone histomorphometry parameters (unpublished data). Thus, in the present study, a higher dose, 500 mg/kg, was used.

In an acute toxicity study done previously, a single dose of *C. caudatus* was given to male rats at the dose of 50, 500, and 2000 mg/kg. The rats were kept for 7 days before sacrifice.



(a) SO group (b) Ovx group

(c) CC group (d) Ca group

Figure 2: Photomicrographs of trabecular bone. Undecalcified section (100 x magnification) shows trabecular bone (stained black) using von Kossa method. SO, sham operated; Ovx, ovariectomized; CC, ovariectomized and supplemented with 500 mg/kg *Cosmos caudatus*; Ca, ovariectomized and supplemented with 1% calcium.

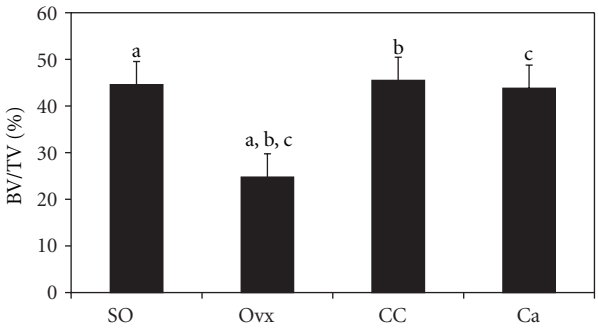


Figure 3: Effects of *Cosmos caudatus* supplementation on bone volume in ovariectomized rats. Groups which share the same alphabet indicate significant difference ($P < 0.05$). SO, sham operated; Ovx, ovariectomized; CC, ovariectomized and supplemented with 500 mg/kg *Cosmos caudatus*; Ca, ovariectomized and supplemented with 1% calcium.

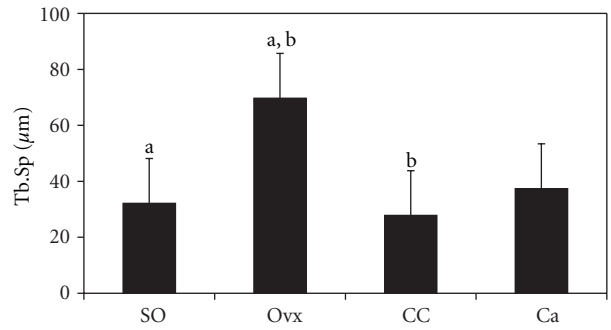


Figure 4: Effects of *Cosmos caudatus* supplementation on trabecular separation in ovariectomized rats. Groups which share the same alphabet indicate significant difference ($P < 0.05$). SO, sham operated; Ovx, ovariectomized; CC, ovariectomized and supplemented with 500 mg/kg *Cosmos caudatus*; Ca, ovariectomized and supplemented with 1% calcium.

The higher dose of *C. caudatus*, that is, 2000 mg/kg, was found to increase liver enzymes but did not cause any changes on the haematological parameters such as clotting time, bleeding time, platelet levels, and white cell count (unpublished data). The observations above imply that *C. caudatus* at the dose of 500 mg/kg is not associated with side effects.

Calcium supplement in combination with vitamin D is recommended in the treatment regime for patients with osteoporosis [33] to reduce risk of nonvertebral fractures [34]. This combination therapy is considered essential but not sufficient for the treatment of osteoporosis and additional benefit may be obtained with the addition of antiresorptive or anabolic agent [35]. In the present study, calcium

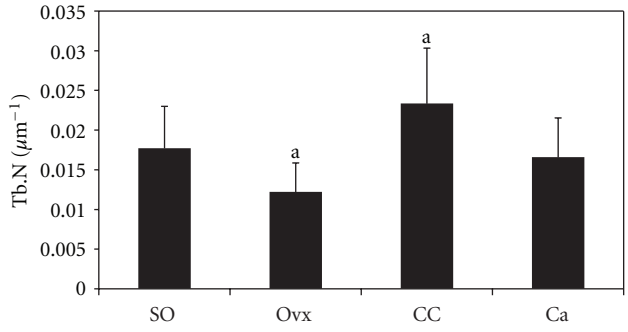


Figure 5: Effects of *Cosmos caudatus* supplementation on trabecular number in ovariectomized rats. Groups which share the same alphabet indicate significant difference ($P < 0.05$). SO, sham operated; Ovx, ovariectomized; CC, ovariectomized and supplemented with 500 mg/kg *Cosmos caudatus*; Ca, ovariectomized and supplemented with 1% calcium.

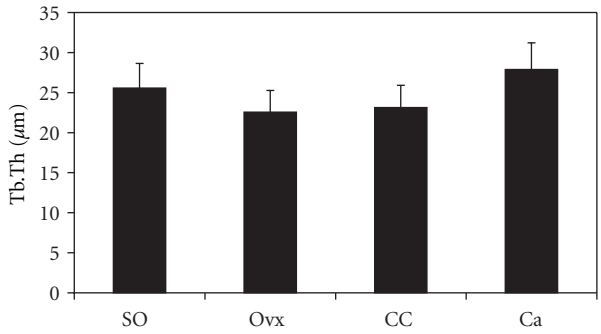


Figure 6: Effects of *Cosmos caudatus* supplementation on trabecular thickness in ovariectomized rats. SO, sham operated; Ovx, ovariectomized; CC, ovariectomized and supplemented with 500 mg/kg *Cosmos caudatus*; Ca, ovariectomized and supplemented with 1% calcium.

supplementation also reversed the effects of ovariectomy on bone volume (Figure 2) but failed to show significant changes in the other parameters. Similar findings were observed in previous study in which ovariectomized rats given calcium supplementation still had lower bone volume and trabecular number as compared to sham-operated rats [36]. In another study using ovariectomized rats, calcium supplementation is shown to improve bone fracture healing but failed to improve strength [37]. Another study showed that calcium supplementation suppresses bone formation in magnesium-deficient rats [38]. The findings above suggested that calcium may need to be present with other elements to exert beneficial effects on bone.

All rats received rat chow which contained 0.8–1.2% calcium. The Ca group received additional 1% calcium in drinking water. However, since the calcium was administered via drinking water, the amount of calcium ingested by the rats may vary between individual rats thus causing inconsistent results in the histomorphometry parameters mentioned above.

In the present study, we observed that *C. caudatus* improved bone structural histomorphometric parameters. In fact, *C. caudatus* seemed to yield better effects on bone in terms of trabecular separation and trabecular number (Figures 4 and 5). According to Nutriweb Malaysia [39], *C. caudatus* contains 270 mg calcium per 100 g of the plant. This composition, in addition to its antioxidative property, enables *C. caudatus* to have a greater effect in reversing bone changes induced by ovariectomy as opposed to calcium supplementation.

Further studies are required to establish the effects of *Cosmos caudatus* on bone such as the effects on dynamic histomorphometry, bone biomarkers, bone density, and bone calcium content. In addition, further studies are also required to ascertain the active compound and calcium content of the plant as well as its exact mechanism on bone.

5. Conclusion

Cosmos caudatus at the dose of 500 mg/kg reversed bone changes induced by ovariectomy. Thus, *C. caudatus* has the potential to be used as an alternative for the treatment of postmenopausal osteoporosis.

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Review Article

Vitamin E as an Antiosteoporotic Agent via Receptor Activator of Nuclear Factor Kappa-B Ligand Signaling Disruption: Current Evidence and Other Potential Research Areas

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Osteoporosis is a growing healthcare burden that affects the quality of life in the aging population. Vitamin E is a potential prophylactic agent that can impede the progression of osteoporosis. Various *in vivo* studies demonstrated the antiosteoporotic potential of vitamin E, but evidence on its molecular mechanism of action is limited. A few *in vitro* studies showed that various forms of vitamin E can affect the receptor activator of nuclear factor kappa-B ligand (RANKL) signaling and their molecular targets, thus preventing the formation of osteoclasts in the early stage of osteoclastogenesis. Various studies have also shown that the effects of the different isoforms of vitamin E differ. The effects of single isoforms and combinations of isoforms on bone metabolism are also different. Vitamin E may affect bone metabolism by disruption of free radical-mediated RANKL signaling, by its oestrogen-like effects, by its effects on the molecular mechanism of bone formation, by the anti-inflammatory effects of its long-chain metabolites on bone cells, and by the inhibition of 3-hydroxyl-3-methyglutaryl coenzyme A (HMG-CoA). In conclusion, the vitamin E isoforms have enormous potential to be used as prophylactic and therapeutic agents in preventing osteoporosis, but further studies should be conducted to elucidate their mechanisms of action.

1. Introduction

Osteoporosis is a metabolic disease of the skeletal system, characterized by low bone mass and deterioration of the microarchitecture of bone, leading to fragility and subsequent fractures [1]. According to an estimate done in the year 2000, nine million osteoporotic fractures occurred globally among men and women aged 50 years and above, in which 1.6 million were at the hip, 1.7 million at the forearm, and 1.4 million at the vertebrae. Most of the osteoporotic fractures occurred in Europe (34.8%), followed by the Western Pacific region, inclusive of East Asia, Australia and New Zealand (28.6%) and Southeast Asia (17.4%) [2]. With the global shift of distribution of death from younger to older ages and from communicable to noncommunicable causes [3], osteoporosis presents a growing disease burden especially in developing countries [4].

Both men and women suffer from osteoporosis, but the prevalence of osteoporosis, as indicated by osteoporotic fractures, is lower in males than in their female counterpart. The ratio of female to male osteoporotic fractures is 1.6 (61% of fractures occur in women) [2]. The major cause of osteoporosis in women is due to the presence of a phase of accelerated bone loss during menopause, which accounts for 20–30% loss of cancellous bone and for 5–10% loss of cortical bone. Despite the absence of a similar rapid bone loss in women, aging men do experience a gradual phase of bone loss which contributes to 20–25% in both cortical and cancellous bone [5]. This decline has also been demonstrated in our previous study using the calcaneal quantitative ultrasound technique in Malaysian men [6]. While bone loss in women is caused by the decline of sex steroids during menopause, bone loss in men is contributed by the decrease in bioavailability of testosterone due to the increase

in sex hormone-binding globulin level with aging [5]. The assessment of age-related changes of microarchitecture in humans is made possible due to the advancement of high-resolution peripheral quantitative computed tomography (HR-pQCT). A study by Khosla et al. reported that a cross-sectional reduction in bone volume (BV/TV) at the radius was observed in men and women. The decrease in trabecular number (Tb.N) and increase in trabecular separation (Tb.S) were more prominent in women while the reduction in trabecular thickness (Tb.Th) was more evident in men [7].

Apart from age-related hypogonadism and menopause, osteoporosis also develops as a result of the following causes (Table 1).

According to an estimate by Burge et al., the economic cost of osteoporosis (inclusive of long-term care cost) in the United States was more than 16.9 billion dollars. This cost was projected to increase to 25.3 billion dollars (an increment of 48% compared to the year 2005) by the year of 2025 due to the increase in the age of the population and bone fracture incidence [8]. In the European Union, the cost of osteoporotic fractures was € 30.7 billion in 2010 and was expected to rise to € 38.5 billion in 2025. Apart from the direct hospitalization cost and long-term care cost, the burden of osteoporosis can be measured in years of life lost due to a fracture and the disability (disability-adjusted life years/DALYs). The total DALYs lost due to osteoporotic fractures globally were estimated to be 5.8 million, which were around 0.83% of the global burden of noncommunicable diseases [9]. Unfortunately, data for health economic burden of osteoporosis in Malaysia is currently not available.

Table 1: Secondary factors of osteoporosis.

Drug
Aluminium
Anticonvulsants (phenobarbital, phenytoin)
Antidepressants
Aromatase Inhibitors
Cytotoxic drugs
Glitazones (antidiabetic agent)
Glucocorticosteroids and adrenocorticotropin
Gonadotropin-releasing hormone agonists
Heparin
Immunosuppressants
Lithium
Loop diuretics
Proton-pump inhibitor
Thyroxine
Endocrine Disorders
Hyperparathyroidism
Hyperthyroidism
Immobilization
Inflammation
Rheumatoid arthritis
Inflammatory bowel disease
Ankylosing spondylitis
Other factors
Alcoholism
Coeliac disease
Gastrectomy
Prostate cancer therapy

(Source: National Osteoporosis Foundation 2003 [16], Kok and Sambrook 2009 [17]).

2. RANKL Signaling and Osteoclastogenesis

Bone is constantly under deconstruction (resorption) and reconstruction (formation) in a process called bone remodeling. Osteoporosis occurs when the rate of bone resorption exceeds the rate of bone formation [10]. Macrophage colony-stimulating factor (M-CSF) and receptor activator of NF-κB ligand (RANKL) are cytokines secreted by osteoblast and stromal cells which are important for proliferation, differentiation, and maturation of osteoclasts [11]. M-CSF binds to colony-stimulating factor-1 receptor (c-Fms) on the osteoclast precursors and primes them to differentiate in the presence of RANKL by inducing RANK expression [12]. Humans with mutation in the gene encoding RANKL will suffer from osteopetrosis, and their bone specimens lack osteoclasts [13]. Binding of RANKL to RANK induces several signaling pathways eventually leading to the activation of genes required for osteoclast differentiation and activation. Several transcription factors, such as nuclear factor kappa-B (NF-κB) (p50 and p52), nuclear factor of activated T cells, cytoplasmic, calcineurin-dependent 1 (NFATc1), and FBJ osteosarcoma oncogene (c-Fos) function downstream of RANKL signaling and the pathways are mediated by protein kinases, such as sarcoma oncogene (Src), c-Jun N-terminus kinases (JNK), p38 mitogen-activated protein kinase, and phosphoinositide 3-kinase (PI3K). NF-κB has been shown to control RANKL-induced osteoclast differentiation, leading to activation of c-Fos prior to NFATc1 [14]. NFATc1 is also called the master switch of terminal osteoclast differentiation because without NFATc1 stem cells cannot differentiate into osteoclasts even in the presence of RANKL, and this can be reversed by exogenous NFATc1 [15].

Osteoprotegerin (OPG) is a protein that exerts protective effects on bone by binding to RANKL, thus interrupting osteoblast-osteoclast progenitor signaling and subsequently preventing osteoclast activation and differentiation [18]. Proinflammatory cytokines, such as tumor necrosis factor alpha (TNFα), interleukin 1β (IL1β), and interleukin 6 (IL6), are known to increase both RANKL and OPG expression, but the dominant outcome is a net increase in RANKL activity. They also act synergistically with RANKL in regulating osteoclastogenesis [19]. Maximal expression of RANKL under the influence of these cytokines may require p38 mitogen-activating protein kinase (MAPK) pathway [20]. Prostaglandin E_2 (PGE_2) has also been implicated in the upregulation of RANKL, as shown in increased RANKL mRNA and cell-surface RANKL protein in mouse pre-B cell line after treatment, which is probably caused by TNFα/IL1 induction of cyclooxygenase (COX)

activity [21]. PGE_2 downregulation of OPG is also implicated in lipopolysaccharide-induced osteoclastogenesis but in the same experiment, upregulation of RANKL is independent of PGE_2 [22]. Other factors that can influence RANKL/OPG ratio include parathyroid hormone (PTH), vitamin D3, transforming growth factor β (TGFβ), bone morphogenic protein (BMP) 2, glucocorticoids, 17β-estradiol, and insulin-like growth factor-1 (IGF-1) [11].

The reactive oxygen species (ROS) play a critical role in RANKL signaling. Hydrogen peroxide (H_2O_2), a ROS originating from the dismutation of superoxide, has been shown to induce differentiation of osteoclast precursor cells [23]. Thioredoxin-1 (Trx-1) also increases the capability of precursor cells to differentiate into osteoclasts and transfection with glutathione peroxidase-1 (Gpx-1), and peroxiredoxin (Prx-1) abolishes osteoclast formation. Trx-1 also augments RANKL-induced activator protein 1 (AP-1), NFκB, and NFAT gene expression in preosteoclasts [24]. Pretreatment with N-acetyl-L-cysteine (NAC) and GSH have also been shown to reduce RANKL-induced Akt activation, and pretreatment with NAC alone inhibits degradation of IκBα (which inhibits NF-κb) and RANKL-induced MAPKs (extracellular signal-regulated kinase/ERK, JNK, and p38) activation. Actin ring formation and bone-resorbing activity of osteoclasts are also inhibited by NAC [25]. Further study revealed that blocking of nicotinamide adenine dinucleotide phosphate (NADPH) oxidase (Nos) will impair RANKL signaling in bone morphogenic macrophage (BMM) cells by blocking the intracellular ROS production, activation of ERK, JNK, and p38, and ultimately preventing osteoclast differentiation [26].

From the information above, it is clear that an ideal agent to prevent osteoporosis should be able to interrupt RANKL and its downstream signaling pathway, by means of suppressing proinflammatory cytokine-induced RANKL expression or downregulation of OPG, or by disrupting ROS-mediated RANKL signaling.

3. Therapy for Osteoporosis

The current therapy for osteoporosis can be classified into antiresorptive therapy (example: bisphosphonates and estrogen) and anabolic therapy (e.g., teriparatide). Antiresorptive drugs like nitrogen-containing bisphosphonates inhibit farnesyl diphosphate synthase, thus reducing osteoclast activity. They also promote osteoclast apoptosis by activation of proapoptotic caspases [28]. There are some concerns that bisphosphonates may increase the risk of osteonecrosis of the jaw [27]. Estrogen exhibits both antiresorptive and anabolic properties because it acts on the osteoclastic and osteoblastic compartments. Estrogen reduces the responsiveness of osteoclast progenitor cells to RANKL and subsequently hinders their differentiation. It also improves osteoblast proliferation, differentiation, function, and lifespan [28]. However, the side effects of long-term estrogen use such as increased risk of breast cancer and cardiovascular disease may out weight its benefits [29]. Teriparatide functions as an anabolic agent by acting directly on osteoblastic lineage cells and indirectly through regulation of skeletal growth factors like insulin growth factor 1. However, teriparatide is very expensive, so it may not be affordable for every patient. It is also contraindicated in patients with hypercalcemia, Paget's disease, skeletal metastases, or skeletal malignant conditions and young patients [30].

Although newer pharmacological agents in the treatment of osteoporosis with fewer side effects have been introduced, the cost and accessibility of the treatment may present a barrier to the patients, especially in developing countries. The quest to find new agents which can concurrently prevent and reverse the progression of osteoporosis, reduce the disease burden of the healthcare system and the patients, and improve the quality of life in the aging population is imperative. The recent discovery of the anti-osteoporotic properties of plant oil-derived vitamin E may offer a solution to the problem. This paper is aimed at discussing the molecular mechanisms leading to osteoclastogenesis and subsequently osteoporosis, and how vitamin E could affect these pathways. All the current evidence pertaining to the antiosteoporotic properties of vitamin E and some suggestions for future studies are also presented.

4. Vitamin E and Its Bone Protective Properties

Vitamin E is a fat-soluble vitamin first isolated in green leafy vegetables. It consists of 2 major isoforms: tocopherols and tocotrienols, each with four distinct analogues (alpha, beta, gamma, and delta) due to differences in the location of methyl groups on the chromanol ring. Tocopherols are saturated forms of vitamin E, and tocotrienols are the unsaturated forms, distinguishable by the three double bonds in the tails of tocotrienols. Tocopherols and tocotrienols are commonly found in edible plant oils, such as rice bran, coconut, palm, and annatto oil in varying proportions [31]. The structures of the various vitamin E isomers are depicted in Figure 1.

The importance of vitamin E on bone metabolism was depicted when rats fed with vitamin E-deficient diet had lower calcium content in the left femur and L5 vertebra compared to control rats, and this was prevented by supplementing the rats with palm vitamin E containing alpha-tocopherol (ATF), alpha-tocotrienol (ATT), gamma-tocotrienol (GTT), and delta-tocotrienol (DTT) for eight months. Supplementation with ATF alone failed to demonstrate similar effects [32]. Further study indicated that vitamin E-deficient diet caused hypocalcaemia and subsequently hyperparathyroidism and decreased calcium content in the fourth lumbar vertebra in rats [33]. Normal male rats supplemented with various vitamin E isomers also displayed better structural, static, and dynamic bone histomorphometry, as well as better biomechanical characteristics compared to control rats, with GTT showing the best effect compared to ATT and DTT [34, 35]. Studies in male mice showed that high-dose ATF supplementation (500 mg/kg) could increase mRNA transcripts of osteocalcin and insulin-like growth factor in young and old mice. In addition to that, improvement in biomechanical strength was

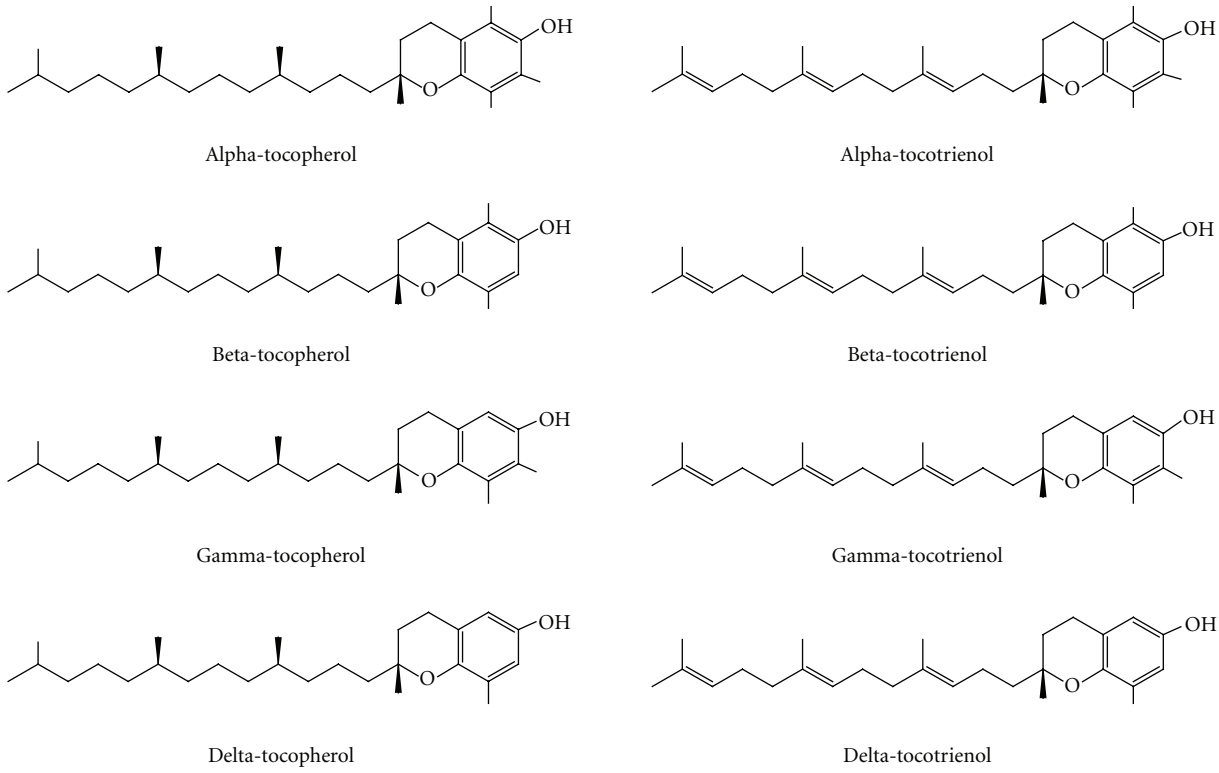

Figure 1: Chemical structures of tocopherols and tocotrienols.

best demonstrated in old mice rather than in young mice. However, there was no improvement in the bone mineral density of the mice in that study [36]. Supplementation of palm-tocotrienol containing ATT, GTT, DTT, and ATF at 100 mg/kg for four months was shown to improve redox status in bone by decreasing thiobarbituric acid-reactive substance (TBARS) and increasing glutathione peroxidase activity in the femur of rats. This effect was not seen in rats supplemented with ATF alone [37].

Vitamin E also showed beneficial effects on various animal models of osteoporosis. Studies showed that tocotrienol-enriched fraction (TEF, containing ATT, GTT, and DTT), GTT alone and ATF alone exerted beneficial effects on nicotine-induced osteoporosis in male rats by improving bone structural, cellular, and dynamic histomorphometric measurements, and the effects of GTT alone were more superior than TEF and ATF alone [38]. This effect might be contributed by the fact that nicotine-induced increase in bone-resorbing cytokines (IL1 and IL6) was abrogated by TEF and GTT supplementation, and this was indicated by lower urinary pyridinoline (bone resorption marker) and higher serum osteocalcin (bone formation marker) levels in the supplemented group as compared to the control group. ATF was also found to suppress the elevation of IL1 but not IL6, and it could not prevent the rise in pyridinoline. This might be the reason why GTT exerted better anti-osteoporotic effects than ATF [39]. Tocotrienol mixtures containing ATT, GTT, and DTT protected rodent bone from ferric-nitrilotriacetate- (FeNTA-) induced osteoporosis as indicated by the lower level of IL6 and deoxypyridinoline cross-links (DPD) and better histomorphometric parameters in the tocotrienol-treated groups compared to the control group. In the same experiment, it was also found that the tocotrienol mixture was better than ATF alone in preventing free radical-induced osteoporosis [40]. A study by Ima-Nirwana and Suhaniza showed that side effects of long-term glucocorticoid usage were prevented by GTT. When adrenalectomized rats given excessive glucocorticoids were supplemented with GTT, their calcium content in the fourth lumbar vertebrae was higher than the unsupplemented group. This effect was not shown in rats supplemented with ATF [41]. In another study, supplementation of palm vitamin E mixture containing ATF, ATT, GTT, and DTT to rats also prevented dexamethasone-induced osteoporosis by preventing BMD (whole body) and bone calcium loss at femur, but no comparison between mixtures and single isomers was conducted [42]. High-dose of ATF (500 IU/kg diet) could also prevent osteoporosis induced by hindlimb unloading in male rats, shown by improved bone histomorphometry compared to the control group. These effects could be contributed by suppression of COX-2 enzyme expression and a modest increase in bone formation by high-dose ATF [43]. In an orchidectomized rat model, supplementation with palm olein and palm vitamin E containing ATF, ATT, GTT and DTT prevented BMD loss at various sites and bone calcium loss at L5 [44]. In a recent study, tocotrienol-rich fraction containing ATF, ATT, GTT, and DTT was shown to be more effective than oestrogen-replacement therapy in

preventing osteoporosis in ovariectomized rats, indicated by improvement in bone histomorphometric parameters in the supplemented female rats [45]. A study by Norazlina et al. showed that supplementation with palm tocotrienols containing ATF, ATT, GTT, and DTT could improve femoral bone calcium levels in both intact and ovariectomized female rats while supplementation with ATF at the same dose could only increase bone calcium content in intact female rats [46]. Nazrun et al. later showed that palm tocotrienol mixtures (containing ATT, GTT, and DTT) could improve bone mechanical strength of ovariectomized female rats, and this could be contributed by reduced lipid peroxidation and induction of superoxide dismutase and glutathione peroxidase in the bone of the supplemented group. The researchers also found that the effects of supplementation with tocotrienol mixture were better than with ATF alone [47].

Overall, animal studies clearly indicate that vitamin E has anabolic effects on the skeletal system and may preserve bone health. This may be brought about by vitamin E-mediated suppression of bone-resorptive cytokines and elevation of antioxidant enzyme activity in bone, which ultimately disrupt RANKL signalling. However, the exact mechanism in which vitamin E, especially tocotrienols, prevents osteoporosis is still not completely understood and needs further studies. The differences in the anti-osteoporotic effects among different vitamin E isomers, and among different vitamin E mixtures are obvious, but the reason was not fully explored. There is also some disagreement regarding the anti-osteoporotic potential of ATF, in which some studies showed beneficial effects [43], while the others found none [41] or lesser effects [38, 40] compared to tocotrienols.

5. The Effects of Vitamin E on RANKL Signalling

In a bone marrow cell-osteoblast coculture, Trolox, a hydrophilic derivative of ATF with a carboxylic group instead of a phytyl chain, was found to suppress IL1, COX-2 enzymatic activity and subsequently PGE_2 production without affecting the expression of COX-2, phospholipase A2, and membrane-associated PGE synthase-1. IL1-induced RANKL expression was also suppressed by Trolox. It prevented osteoclast formation in the early stage (first two days in the coculture) but had no effects on mature osteoclasts. In addition, Trolox could abolish RANKL-induced c-Fos and NFAT2 protein expression without marked change in c-Fos mRNA expression. However, it did not have any effects on IL1-activated signalling pathways involving ERK, PI3K/AKT, and p38 [48].

The antiresorptive potential of ATT was demonstrated in a bone marrow macrophage- (BMM-) osteoblast coculture, in which osteoclast formation was inhibited by suppressing RANKL expression without affecting OPG expression. ATF was found to have no effects on the expression of both proteins. Treatment of BMM cells with ATT inhibited osteoclast formation, but it was only effective when added in the early stage of osteoclastogenesis (first two days in culture). ATT was discovered to suppress RANKL-induced mRNA and protein expression of c-Fos and NFATc1, acute activation of ERK (but not on JNK and p38), NF-κB activation, and pit formation (indicative of osteoclast resorptive activity) on dentin-coated plate by mature osteoclasts [49].

In contrast to the studies previously mentioned, a recent study indicated that megadose of ATF may promote osteoclastogenesis and affect bone health adversely. When normal mice and rats were supplemented with 600 mg/kg ATF (ten times higher than the effective dose used by other researchers) for eight weeks, a 20% decrease in bone mass, with concomitant increase in bone resorption and osteoclast size compared to mice/rats fed with normal diet was observed. This effect was not seen with mice supplemented with delta-tocopherol. Mice deficient in alpha-tocopherol transfer protein (TTPA), which selectively transfer alpha-tocopherol into lipoproteins, were also found to have higher bone mass compared to wild type mice and this could be reversed by alpha-tocopherol supplementation. Further *in vitro* investigation revealed that p38 pathway might be involved in osteoclast fusion and only ATF promoted osteoclast fusion, but other vitamin E isomers including ATT did not exhibit such effect. Other antioxidants, including ascorbic acid, did not also stimulate osteoclast fusion, indicating this effect of ATF was not due to its antioxidant properties [50].

Overall, the *in vitro* experiments describing the mechanism of action of vitamin E on regulating osteoclastogenesis are very limited. The studies presented indicated that Trolox and ATT can inhibit RANKL signaling by suppressing RANKL expression although the mechanism of action downstream may vary slightly. Not all the vitamin E isomers were tested in the same experiments, hence, their effects cannot be directly compared. The adverse effects of high-dose ATF are alarming. This may be contributed by the fact that increasing ATF levels, in the presence of oxidative stress and without a balance network of antioxidants, may result in an increase of ATF free radicals which cannot be reduced by other coantioxidants [51]. In addition, an *in vitro* study by Nizar et al. found that GTT at low dose (1 μM) was protective against free-radical damage on osteoblasts, but concentration beyond 50 μM was toxic to osteoblasts. ATF was not protective to osteoblast for free-radical damage at all doses used [52]. The overall mechanism of action of vitamin E is summarized in Figure 2.

6. Potential Areas of Interest in Vitamin E-Mediated Bone Remodeling

The research on the molecular actions of the vitamin E isoforms has just begun, and there are plenty of uncharted territories left for future exploration (as highlighted in Figure 3). Vitamin E, especially the tocotrienol fraction, is known to induce expression of antioxidant enzymes and quench ROS in tissue. Rats supplemented with vitamin E showed elevated concentrations of liver superoxide dismutase, glutathione peroxidase, and catalase compared to the control

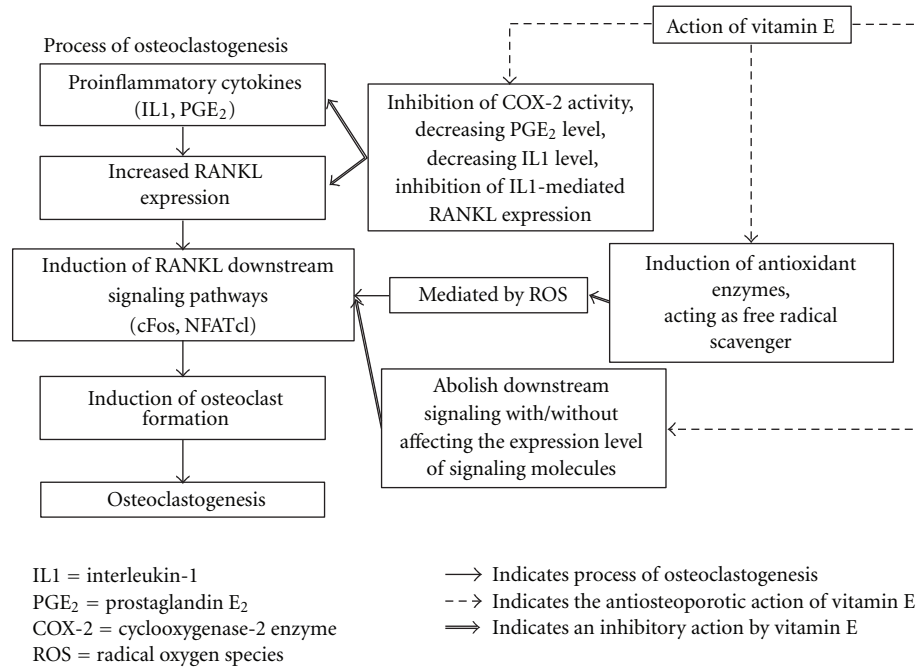

Figure 2: Mechanism of action of vitamin E in preventing osteoclastogenesis. Vitamin E affects osteoclastogenesis via three distinct mechanisms. Firstly, it inhibits COX-2 activity and subsequently PGE^2 level. It also decreases IL-1 level and thus preventing IL-1-mediated RANKL expression. Secondly, vitamin E induces upregulation in antioxidant enzymes in bone and acts as a free radical scavenger itself, thus abolishing ROS-mediated RANKL signaling. Lastly, vitamin E also abrogates downstream signaling pathways leading to osteoclastogenesis even with or without affecting the expression level of signaling molecules.

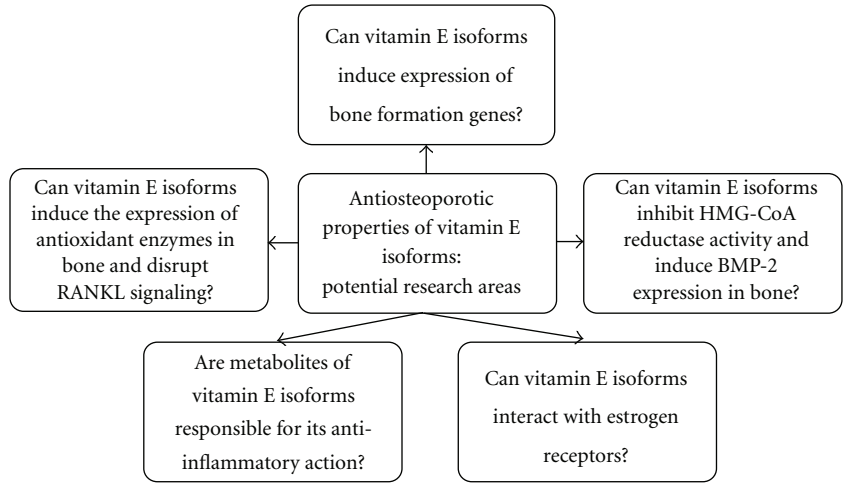

Figure 3: Potential research areas for the anti-osteoporotic properties of vitamin E isoforms.

group [53]. We have also shown that palm-tocotrienol mixture containing ATT, GTT, and DTT increased glutathione peroxidase level in femoral bones of supplemented rats [37]. Besides that, a study by Norazlina et al. indicated that ATF and tocotrienol mixtures (content not specified) can prevent bone calcium loss due to nicotine without affecting serum RANKL to OPG ratio significantly [54]. Whether this induction of antioxidant enzymes transpires in bone in the presence of vitamin E, and whether it is responsible for the disruption in RANKL signaling independent of the expression of RANKL need validation via *in vitro* studies.

A recent study also revealed that tocotrienols (GTT, DTT, and tocotrienol-rich fraction) display high affinity for estrogen receptor beta (ERβ) and interact with it by

increasing its translocation into the nucleus and activating estrogen-responsive genes in breast cancer cells [55]. Since previous studies found that gamma-tocotrienol showed anabolic effect on the skeleton of normal male and female rats [34, 35], it is reasonable to speculate whether it has similar estrogenic effects on bone. Apart from that, although some effects of vitamin E on osteoclastogenesis have been studied, information pertaining to its effects on the molecular regulation of bone formation, such as on bone formation genes in osteoblast is lacking and should be studied.

There are plenty of evidence that vitamin E can suppress the expression of proinflammatory cytokines which promote osteoclastogenesis, as reviewed by Nazrun et al. [56]. A recent finding suggested an alternative to the mechanism of the anti-inflammatory action of vitamin E. A study on inhibition of the cyclooxygenases (COX) by vitamin E revealed that long-chain carboxychromanols (metabolites of vitamin E), rather than vitamin E itself was the potent inhibitors of prostaglandin E_2 synthesis in human lung epithelial A549 cells. The inhibitory effect of vitamin E was lost when its metabolism in cells is inactivated and the metabolites are not formed. Computer simulation done on enzyme kinetics data showed that metabolites of vitamin E competed with arachidonic acid to bind with COX, and the 13′-COOH fraction of carboxychromanol was more potent than 9′-COOH fraction in enzyme inhibition [57]. Whether these vitamin E metabolites were beneficial in suppressing bone damage due to inflammation and RANKL signaling would be an interesting field of investigation.

It is known that statins, a class of lipid lowering drugs which act as 3-hydroxyl-3-methyglutaryl coenzyme A (HMG-CoA) reductase inhibitor can reduce fracture risk in the elderly population [58–60]. It was shown that statins enhanced BMP-2 mRNA expression in bone cells and induced bone formation [61], and the addition of mevalonate (a metabolite downstream of HMG-CoA reductase) could abolish the activation of BMP-2 promoter [62]. These effects were not seen in hydrophilic statins [62]. Tocotrienols such as GTT was found to cause posttranscriptional HMG-CoA expression in hepatocyte culture [63]. *In vivo* studies also found that tocotrienol mixtures (containing ATT, GTT and DTT) derived from palm oil fatty acid distillate (PFAD) can inhibit HMG-CoA reductase activity [64]. However, Ng and Khor. showed that the inhibitory activity of tocotrienols could be decreased in the presence of ATF [65]. Thus, part of the anti-osteoporotic effects of tocotrienols could be attributed by its inhibitory action on HMG-CoA reductase enzyme similar to the statins, but direct evidence is needed to elucidate the relationship between them.

7. Conclusion

Vitamin E, either together or separated into its various isomers, has enormous potential to be developed as an anti-osteoporotic agent. Animal studies have provided positive outcomes which include improvements in the majority of the histomorphometric parameters as well as biomechanical strength. However, its mechanisms of action at the molecular level need to be elucidated. The current evidence indicates that Trolox and ATT can disrupt RANKL and its downstream signaling thus preventing the formation of osteoclasts in early osteoclastogenesis. Other indirect evidence provided by application of various isoforms of vitamin E in cell lines other than bone cells also suggests vast uncharted research areas for the study of the anti-osteoporotic properties of vitamin E. Apart from that, current studies showed that different isoforms of vitamin E have different effects in bone metabolism. Thus, it is important to study each isoform on its own, as well as in combination. The properties of these isoforms may differ when given individually or in combination since there may be a previously unrecognized interaction among them. Furthermore, due to their chemical structure, the properties of the tocotrienols may differ vastly from the tocopherols and even be opposing in certain situations. Thus, there is a real need to look at the tocopherols and tocotrienols as different chemical entities and not as a single entity under the general name of vitamin E.

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Review Article

Eurycoma longifolia: Medicinal Plant in the Prevention and Treatment of Male Osteoporosis due to Androgen Deficiency

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Osteoporosis in elderly men is now becoming an alarming health issue due to its relation with a higher mortality rate compared to osteoporosis in women. Androgen deficiency (hypogonadism) is one of the major factors of male osteoporosis and it can be treated with testosterone replacement therapy (TRT). However, one medicinal plant, *Eurycoma longifolia* Jack (*EL*), can be used as an alternative treatment to prevent and treat male osteoporosis without causing the side effects associated with TRT. *EL* exerts proandrogenic effects that enhance testosterone level, as well as stimulate osteoblast proliferation and osteoclast apoptosis. This will maintain bone remodelling activity and reduce bone loss. Phytochemical components of *EL* may also prevent osteoporosis via its antioxidative property. Hence, *EL* has the potential as a complementary treatment for male osteoporosis.

1. Introduction

Traditional medicine is defined by the World Health Organization as the sum total of knowledge, skills, and practices based on the theories, beliefs, and experience indigenous to different cultures that are used to maintain health, as well as to prevent, diagnose, improve, or treat physical and mental illnesses [1]. It covers numerous alternatives that vary from country to country and is referred to as "alternative" or "complementary" medicine. Traditional medicine comprises of biologically based therapies (e.g., herbs and vitamins supplement), manipulative and body-based therapies (e.g., massage and reflexology), mind-body medicine (e.g., meditation), and holistic medical systems, such as acupuncture and ayurveda [2].

For centuries, medicinal plants (i.e., herbal medicine) have been a part of cultural heritage. More than 35,000 plant species have been reported to be used in various human cultures around the world for medicinal purposes [3]. Burkill, in his extensive compilation of economic products of the Malay peninsula, recorded not less than 1,300 plants, which were used in traditional medicine [4]. In spite of the great advances observed in modern medicine in recent decades, plants still make an important contribution to health care. Nowadays, medicinal plants play a significant role as an alternative medicine due to the damaging effects of food processing, environment, and hazardous side effects of prolonged medications [5, 6].

The earliest report on medicinal plant research in Malaysia was on the phytochemical screening of 205 plants in Sabah [7, 8], followed a few years later by the screening of 200 plant species in Peninsular Malaysia for the presence of alkaloids [9]. Subsequently, more plants were screened chemically for alkaloids, saponins, triterpenes, and steroids [10] to the extent of becoming the focus of major worldwide attention currently. Malaysia is rated as one of the 12 countries in the world with mega diversity of plants [11, 12].

According to a WHO global survey report, Malaysia was one of the nine countries that contributed a large amount of sales in herbal medicine worldwide between the year of 1999 to 2001, as seen in Figure 1 [13]. In 2008, the Malaysian market for herbal and natural products was

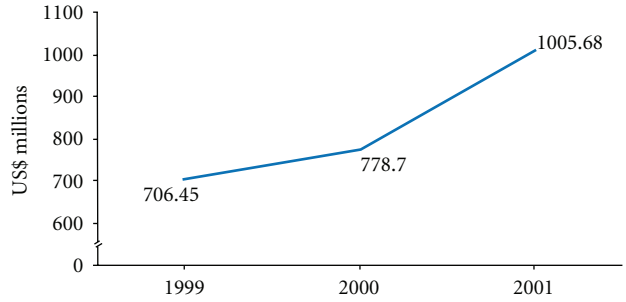

Figure 1: Sales of herbal medicine of nine representative countries. It is shown that between 1999 and 2001 alone, the sales value of herbal medicines in this group of countries increased by more than 40%. (Source: [13]). Growth in the sales of herbal medicines in a group of nine representative countries, 1999–2001 (Bhutan, Canada, the Czech Republic, Iran, Madagascar, Malaysia, Pakistan, Sudan, and Sweden).

estimated to worth approximately RM10 billion, increasing at the rate of 8% per year. Hence, it was projected that by 2050, the global market for herbal products would be around 5 US trillion dollars [14]. Examples of famous Malaysian medicinal plants are *Eurycoma longifolia* Jack (Tongkat Ali), *Labisia pumila* (Kacip Fatimah), *Piper sarmentosum* (Daun kaduk), *Ficus deltoidea* (Mas cotek), and *Centella Asiatica* (Pegaga). These herbs are known to exert antibacterial, antipyretic, antioxidant, anti-inflammatory, and potential antitumor activities [15–17].

2. Eurycoma longifolia Jack

Eurycoma longifolia Jack (*EL*) or commercially known as Tongkat Ali in Malaysia, Pasak bumi in Indonesian, Piak and Tung saw in Thailand, and Cay ba binh in Vietnam [18] is a famous medicinal plant in the family of Simaroubaceae. Besides *EL*, there are three other plant species also known locally as Tongkat Ali which literally means "Stick of a man" and " Ali" of which referring to its aphrodisiac property. The three plant species are *Entomophthora apiculata*, *Polyathia bullata*, and *Goniothalamus* sp. [19, 20]. However, *EL* is the most widely used species for its therapeutic activities. In Malaysia, *EL* is well known among various ethnic groups for treating disease and enhancing health, particularly sexual health among men. *EL* is a shrub tree that grows up to 10 metres in height, with long leaves that are green in colour. The leaves are pinnate in shape (i.e., the leaflets are arranged in pairs) [21]. The flowers of this tree are dioecious, whereas its ovoid-shaped fruits will turn to dark brown colour when they are ripe [22]. Due to the high demand of *EL* for its tremendous health benefits, *EL* preparations are now widely available in the health-food market in the form of raw crude powder where the root is dried and grinded without involving any other chemical processing steps. *EL* is also available in the form of capsules which may either contain raw crude powder or standardised *EL* extract. *EL* extract is prepared by extracting the active ingredients, adjusting the preparation to a defined content of a constituent, and followed by concentrating it to a standard level. Other than that, *EL* is available as an additive brewed with coffee and even canned processed drinks [23, 24]. It has been recommended that *EL* should be administered orally, as other means such as intraperitoneal could enhance its toxicity by approximately 100-fold [25].

A wide range of chemical compounds have been isolated, especially from the root of *EL*, which include eurycomanone, eurycomanol, eurycomalactone, canthine-6-one alkaloid, 9-hydroxycanthin-6-one, 14,15β-dihydroxyklaineanone, phenolic components, tannins, quanissoids, and triterpenes, as depicted in Figure 2 [26, 27]. Due to the presence of these chemical compounds, the root has been reported to have effective medicinal values in terms of sexual enhancement property for males, as well as antipyretic, antimalarial, antibacterial, and antitumor properties [28, 29]. *EL* has been well documented to exert antioxidative properties due to its high concentrations of superoxide dismutase (SOD) [30, 31]. *EL* is famously known for its aphrodisiac effect, which is due to its ability to stimulate the production or action of androgen hormones, especially testosterone. Hence, it can be used as an alternative for testosterone replacement therapy [21] in a variety of related conditions, for example, in the treatment of male osteoporosis due to androgen deficiency [32].

To the best of our knowledge, till date, there is no reliable review on the effects of *Eurycoma longifolia* Jack on bone remodeling and its antiosteoporotic value. Hence, this review focused on the effects of *Eurycoma longifolia* Jack on bone and its postulated antiosteoporotic mechanisms in treating osteoporosis due to androgen deficiency. This information will be useful and applicable for future researches on osteoporosis and the development of a more comprehensive natural medicine approach to bone diseases.

3. Osteoporosis

The incidences of osteoporosis and osteoporosis-related fractures are increasing and have now become a major public health issue. According to the World Health Organization (WHO), osteoporosis occurs if the bone mineral density is more than 2.5 standard deviation below the peak bone mass reference standard for young women. If a similar criterion is used for men in United States (by referring to the peak bone mass reference standard for young men, instead for young women), approximately 1-2 million men may have osteoporosis and another 8–13 million may have osteopenia [33].

Osteoporosis is categorized as one of the serious chronic diseases that has become a significant socioeconomic burden in many countries. Chronic diseases are a major factor in the continuous growth of medical care spending [34]. According to the WHO, chronic diseases are by far the leading cause of mortality in the world [35]. Osteoporosis is a silent, slowly progressive systemic skeletal disease that is characterized by low bone mass and microarchitectural deterioration of bone tissue. It does not usually present with a significant symptom until a bone fracture occurs,

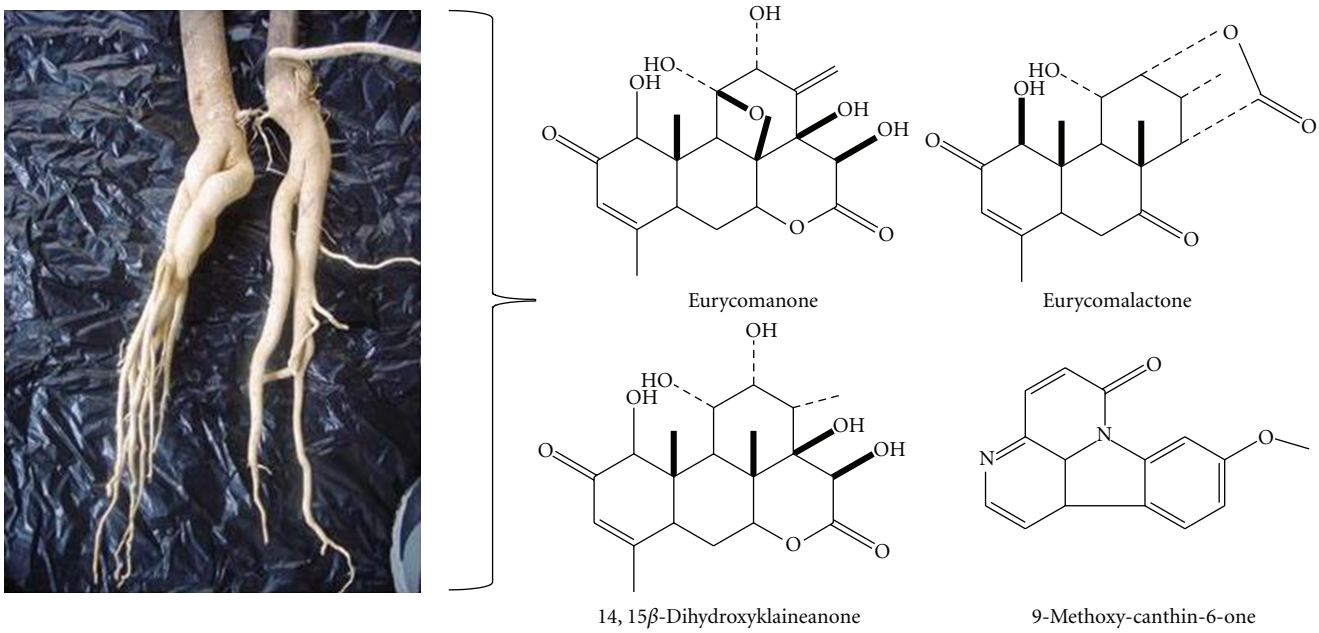

Figure 2: Some of the chemical constituents isolated from the root of *Eurycoma longifolia* Jack (Source: [27]).

hence, the so-called "silent" disease [36]. Therefore, an acute condition due to bone fracture may occur, although osteoporosis is classified as a chronic disease due to its slow and destructive progression. There are two types of this disease: primary and secondary osteoporosis. Primary osteoporosis can be caused by aging and hormonal disturbance. It is also known as senile osteoporosis or postmenopausal osteoporosis, which mainly affects women [37]. On the other hand, secondary osteoporosis is caused by exogenous factors. These include diseases affecting bone metabolism, such as Cushing's disease, hyperparathyroidism, and liver disease. Medications, such as steroid (prednisone), barbiturates, thyroxine, heparin, and diabetic medications, may also impair bone metabolism, leading to bone loss [38].

Bone is continuously remodelled throughout life, whereby bone resorption activity by osteoclasts is always followed by bone formation by osteoblasts in a perpetual cycle [39]. Osteoporosis occurs when the bone formation and bone resorption cycle is impaired. The resorption and reversal phases of bone remodelling are short, while the period required for osteoblastic replacement of the bone is long. Therefore, any factors that increase the rate of bone remodelling, such as hormonal deficiency, will result in loss of bone mass [40].

The sex hormones, androgen and estrogen, play an important role in regulating bone health. Androgens are the most abundant circulating sex steroids in both men and women that modulate bone remodelling cycle through direct androgenic activity via androgen receptor (AR) or indirect action through aromatization to estrogens [41]. Testosterone is the main androgen and predominant sex steroid in men. About 60–70% of testosterone is bound to sex-hormone binding globulin (SHBG), while the remaining proportion is free and biologically active [42]. Free testosterone can be converted to a more potent AR activator, dihydrotestosterone (DHT) via 5α-reductase enzymes [43]. DHT act by stimulating osteoblast proliferation and enhancing osteoblast differentiation, which will subsequently increase bone formation rate [44]. It can also exert pro-apoptotic effects on osteoclasts that will increase the rate of osteoclast death and indirectly reduce bone resorption activity [45, 46]. Free testosterone can be converted to estrogen via aromatase enzyme found on several tissues including adipose tissue [47] and osteoblastic cells [48, 49].

Estradiol will then activate estrogen receptors (ERs), mainly ERα receptors, which are located on osteoblasts and osteoclasts. Aromatization of androgen is important as estrogen plays a significant role not only in female, but also in male skeletal homeostasis [50, 51]. Osteoporosis is associated with various inflammatory conditions such as rheumatoid arthritis, haematological diseases, and inflammatory bowel disease. Proinflammatory cytokines such as interleukin (IL)-1, IL-6, IL-7, and tumor necrosis factor (TNF)-α are elevated in these conditions [52]. They mediate osteoclastogenesis by stimulating osteoclast differentiation and inhibiting apoptosis of osteoclast progenitors [53, 54]. Estrogen promotes the downregulation of these proinflammatory cytokines which stimulate the apoptosis of osteoclasts [55]. Receptor activator of NF-$\kappa\beta$ ligand (RANKL) plays a crucial role in osteoclasts formation. Estrogen is able to suppress RANKL production by osteoblast-lineage cells and T and B cells [56], which consequently causes the inhibition of bone resorption by osteoclasts. Other than that, estrogen also stimulates the production of osteoprotegerin (OPG), a potent antiosteoclastogenic factor. This factor blocks the binding of RANK (which is expressed on the osteoblast progenitors) to its

ligand (RANKL), thus, making it a potent antagonist of osteoclastogenesis [57].

4. Androgen-Deficient Male Osteoporosis

Males have bigger bones and a higher amount of cortical bones than females. This might be contributed by the stimulatory effects of androgen on periosteal modelling drifts and longitudinal bone growth. In contrast, estrogen, the female predominant hormone, suppresses periosteal bone expansion and longitudinal bone growth [58, 59]. Women have a higher risk of osteoporosis due to their lower bone mass and the exposure to tremendous decline of estrogen after menopause, which will lead to aggressive bone loss. Due to their greater bone mass, men usually present with osteoporotic fractures 10 years later than women. Although, once hip fracture has occurred, men have a higher mortality and morbidity rate than women [60]. Hence, osteoporosis in men is now recognized as a significant and important public health issue [61]. The factors involved in the cause of osteoporosis in men include hypogonadism, prolonged-use of glucocorticoid, alcohol consumption, inflammatory arthritis, and family history of osteoporosis. Hypogonadism, which is a reduction in circulating androgen levels or better known as androgen deficiency, is one of the major causes of osteoporosis in most men worldwide [62, 63].

According to the Endocrine Society, approximately four million men of the worldwide population have hypogonadism but less than 200,000 received treatment [64]. Hypogonadism can be classified into primary and secondary hypogonadism. Primary hypogonadism is caused by testicular impairment due to aging and diseases such as Klinefelter's syndrome and orchitis, while secondary hypogonadism is caused by pituitary and hypothalamic dysfunction. Adult men produce 3 to 10 mg of testosterone daily that is mostly secreted by the testes. Only about 0.05 mg of testosterone and other androgens, such as dihydroepiandosterone (DHEA), DHEA-sulfate, and androstenedione, are secreted by the adrenal glands [65]. As men age, the testosterone levels decrease and SHBG levels increase. Testosterone declines gradually after the age of 40 by 0.4 to 2.6% [66]. The serum free testosterone level of an 80 year-old man is approximately 50% of that in a 20 year-old.

The development and interpretation of animal models are essential for the study of the role of androgen hormone in male skeletal growth. The orchidectomized adult male rat has been widely used as a model for bone studies of androgen-deficient male. According to Gill et al., orchidectomy causes a fall in serum testosterone level by approximately 80% in male rats [67]. This may explain the reduction of lumbar vertebral and tibial bone volume, as well as the reduction of femoral bone mineral density, due to the acceleration of bone remodelling rate as demonstrated in previous studies [68]. Orchidectomy also results in reduced bone strength, body weight, and lean body mass [69, 70]. Recently, Nazrun et al. have shown that orchidectomy causes significant bone calcium loss which is consistent with osteoporosis due to androgen deficiency. In the same study, a bone resorption marker, C-terminal telopeptide of type 1 collagen (CTx), was found to be elevated in the orchidectomized rats [71]. This indicates that androgen plays a major role in male skeletal regulation, whereby orchidectomy may cause bone loss by increasing the bone resorption activity.

The loss of gonadal function also causes an upregulation of osteoclastogenesis by increasing the production of IL-1, IL-6, IL-7, and TNF-α [72], which will result in bone resorption. As the testosterone synthesized by the gonads is the major source of circulating estradiol in males, orchidectomy will not only reduce the testosterone level, but also the estradiol levels [73]. Estrogen withdrawal following androgen deficiency will result in the upregulation of RANKL and downregulation of OPG, resulting in increased osteoclastic activity.

Orchidectomy can also promote the upregulation of reactive oxygen species (ROS), which contribute to oxidative stress [74]. This leads to osteoblast apoptosis and promotion of osteoclast differentiation [75]. Estrogen (mainly estradiol) plays an important role as an antioxidative agent that will upregulate the glutathione reductase activity to combat the deleterious ROS activity. This results in stimulation of osteoclast apoptosis and inhibition of osteoblast apoptosis.

Based on the facts above, we can see that androgen deficiency not only impairs the function of testosterone on bone, but also indirectly impairs estrogen activities. Androgen-deficient osteoporosis has long been treated using testosterone replacement therapy (TRT), which is usually given via intramuscular injection to produce a stable testosterone level as well as improving calcium absorption to reduce bone resorption. TRT is not only painful and prone to cause infection, but its prolonged use may produce harmful side effects, such as prostate cancer, liver damage, cardiovascular diseases, and painful erections [76]. On the other hand, the oral form of testosterone is mostly deactivated by the liver and is associated with liver tumors. Transdermal testosterone in the form of cream and gel may cause transference to women and children by skin contact. This is also the most expensive form as it requires a high concentration of testosterone.

The other treatment options for osteoporosis in men are biphosphonates, which include alendronate and risedronate. These drugs have been shown to significantly reduce the risk of vertebral fracture. However, they can cause many adverse effects, such as abdominal pain, esophageal cancer, jawbone necrosis, muscle pain, and nausea [77]. The Food and Drug Administration (FDA) has also approved recombinant human parathyroid hormone (PTH) for the treatment of androgen-deficient osteoporosis. Several studies have shown that PTH has positive effects on bone turnover rate and significantly increases bone mineral density [78]. However, due to its high cost of treatment and adverse effects, such as marrow fibrosis, headache, and osteosarcoma, PTH should only be considered for men with severe osteoporosis. Another effective medication that is still under an ongoing multicenter international trial is strontium ranelate. Strontium ranelate has been proven to be significantly effective in reducing fractures especially in postmenopausal women by stimulating bone formation activity and simultaneously

reducing bone resorption by osteoclasts [79]. Recent studies have found that strontium ranelate is also able to increase bone mineral density in osteoporotic men [80]. Regardless the efficacy of strontium ranelate on improving bone health, it is, however, still has not been approved by FDA and may produce side effects such as nausea, diarrhea, and thrombosis [81]. Among the mentioned available treatments, TRT is widely used in male osteoporosis due to androgen deficiency. Despite the available treatments of osteoporosis, the development of an alternative treatment agent to protect against osteoporosis in men is highly desirable. This alternative agent should ideally be without any side effects and can be easily taken as a supplement.

5. Mechanism of Action of *Eurycoma longifolia* Jack in the Prevention and Treatment of Osteoporosis

The pharmacological properties of *EL* have been widely studied. Over the years, pharmacological evaluations of this plant showed that it has antimalarial [82], antibacterial [83, 84], antitumor, and antioxidant [85, 86] properties. Recently, it was established that *EL* may be used in the prevention and treatment of osteoporosis, or more specifically, male osteoporosis. In the study, it was shown that orchidectomized male rats supplemented with *EL* did not experience bone calcium loss [32]. The root of *EL* contains a wide variety of chemical compounds including alkaloids, quassinoids, quassinoid diterpenoids, eurycoma no side, eurycolactone, eurycomalactone, phenolic component, and tannins [87]. Apart from these compounds, a bioactive peptide of 4.3 kDa with aphrodisiac properties has been identified [88]. These phytochemicals may be the reasons behind the effectiveness of *EL* on various diseases. The underlying mechanism of *EL* against osteoporosis is still unclear, but studies have demonstrated that it is mainly due to its aphrodisiac property. With regard to the said aphrodisiac property, several animal model studies using rats revealed an increase of sexual motivation and performance along with the increase of serum testosterone concentrations in treated rats [89]. While animal studies do not always guarantee similar results in human, it has been shown that *EL* supplementation has increased the level of serum testosterone in most subjects in a human study [86].

The bioactive complex polypeptides from the *EL* root extract, labelled as eurypeptides, can exert and enhance their effects on the biosynthesis of various androgens [90]. Eurypeptides work by stimulating dihydroepiandosterone (DHEA). DHEA will act on androgen receptors to initiate the conversion of androstenedione and androstenediol to testosterone and estrogen, respectively [91]. These eurypeptides may also alleviate SHBG and subsequently increase the free testosterone level [92]. Due to these proandrogen properties of *EL*, it is able to stimulate osteoblast proliferation and differentiation, resulting in increased bone formation rate. High levels of testosterone and estrogen may also exert proapoptotic effects on osteoclasts, reducing the bone resorptive activity. As testosterone level decreases with age, it has been suggested that men can consume *EL* (at suitable dosage) as a supplement, to replace the famous proandrogenic drug, sildenafil (or better known as Viagra), which can cause harmful adverse effects [93].

Other than its proandrogenic properties, *EL* contains high level of nitric oxide (NO) [94] that have effects on bone. NO derived from the endothelial isoform of nitric oxide synthase (eNOS) is widely expressed in bone on a constitutive basis. It acts as a mediator, together with prostaglandin, to promote bone formation and suppress bone resorption [95]. NO in bone cells is stimulated by IL-1 and TNF. At low concentration, NO exerts a destructive effect to potentiate IL-1-induced bone resorption [96]. This effect appears to be biphasic as at high concentrations, NO has been shown to inhibit IL-1- and TNF-α-induced bone resorption [97, 98]. NO is only needed in low concentration to reduce bone resorption and enhance bone formation activity, that is why its short-acting property still makes an impact on bone. Apart from that, NO activity can be upregulated by estradiol [99]. As consumption of *EL* may increase testosterone level which later will be converted to estradiol, this will indirectly enhance the effect of NO on bone. Van't Hof and Ralston confirmed that NO is a potent inhibitor of bone resorption via two main mechanisms: by inducing osteoclast apoptosis and by inhibiting mature osteoclast activity [100]. By taking into account the NO effects on bone, the high level of NO in *EL* can be postulated to be effective in reducing bone resorption activity.

Male osteoporosis can also be explained in terms of oxidative stress mechanism. Free radicals, mainly reactive oxygen species (ROS), are efficiently scavenged in the body. However, oxidative stress will occur when there is an imbalance between increased ROS and inadequate antioxidant activity [101]. Orchidectomy, as shown in the model of androgen-deficient osteoporosis, can promote upregulation of ROS which leads to oxidative stress. Oxidative stress plays a role in osteoblast apoptosis and osteoclast differentiation [102]. Antioxidants such as tocotrienol are known to offer protection against oxidative damage. Tocotrienol can prevent lipid peroxidation by enhancing its glutathione peroxidase (GPx) enzyme activity. Due to its antioxidative property, tocotrienol can protect bone cells from damages caused by lipid peroxidation to maintain bone remodelling [103]. It has been shown that palm vitamin E prevents bone mineral density loss due to orchidectomy [104]. According to Tambi and Kamarul, *EL* contains high concentrations of superoxide dismutase (SOD), another antioxidant that plays an important role in counteracting oxidative stress [105]. Other components of *EL*, such as alkaloids and triterpenes, can also act as antioxidants that may reduce bone loss and maintain the bone formation rate.

6. Safety and Toxicity of *EL*

Although *EL* has been used in traditional medicine for generations in Malaysia, it was only in the late 1990s that researchers started to pay more attention on its safe dosage and toxicity profile. An acute toxicity study done by

Satayavivad et al. has found that the oral Lethal Dose 50 (LD_{50}) of the alcoholic extract of *EL* in mice is between 1500–2000 mg/kg, while the oral LD_{50} of the aqueous extract form is more than 3000 mg/kg [106]. Toxicity of *EL* was further tested by Shuid et al. in their acute toxicity study, where a dose of 5000 mg/kg of *EL* extract was given orally to rats within 24 hours. It was found that the LD_{50} for aqueous extract of *EL* was more than 5000 mg/kg [107]. To extrapolate this animal dosage to human, normalization of body surface area (BSA) method should be used, as shown below [108].

Formula for Dose Translation Based on BSA. Formula for dose translation from animal to human using BSA method (Source: [108])

$$\text{HED (mg/kg)} = \text{Animal dose (mg/kg)} \times \frac{\text{Animal Km}}{\text{Human Km}}. \quad (1)$$

Hence, *EL* intake is unlikely to cause fatality in human as the equivalent LD_{50} for *EL* is extrapolated to be 810 mg/kg in an adult man. In another subacute toxicity study, *EL* extract at the doses of 600, 1200, and 2400 mg/kg were given to rats, respectively, for 28 days. Pathological changes in the liver were seen in two rats, each from the group treated with 1200 and 2400 mg/kg. Clinical study in human done by Tambi has found that a dose up to 600 mg/kg did not cause any adverse effects [109]. *EL* is considered safe as long as it is not taken in high dose. Based on the results of previous toxicity studies, *EL* is normally recommended to be administered to men at the dose of 200–400 mg daily and should be used with caution, especially in the elderly. Currently, *EL* is commercially sold worldwide following this established dosage in the form of tablets for easier daily consumption.

7. Conclusion

Based on established literatures on health benefits of *EL*, it is important to conserve this valuable medicinal plant for the benefit of future generation. There are several mechanisms proposed for its antiosteoporotic effects. The main mechanism is via its testosterone enhancing effects for the prevention and treatment of androgen-deficient osteoporosis. Other mechanisms involved are through its nitric oxide contents and antioxidative properties. Due to its safety profile and potential as an alternative antiosteoporotic agent, further studies are warranted to document a better and conclusive mechanism for its therapeutic action.

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Research Article

Tocotrienol Supplementation Improves Late-Phase Fracture Healing Compared to Alpha-Tocopherol in a Rat Model of Postmenopausal Osteoporosis: A Biomechanical Evaluation

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This study investigated the effects of α-tocopherol and palm oil tocotrienol supplementations on bone fracture healing in postmenopausal osteoporosis rats. 32 female Sprague-Dawley rats were divided into four groups. The first group was sham operated (SO), while the others were ovariectomised. After 2 months, the right femora were fractured under anesthesia and fixed with K-wire. The SO and ovariectomised-control rats (OVXC) were given olive oil (vehicle), while both the alpha-tocopherol (ATF) and tocotrienol-enriched fraction (TEF) groups were given alpha-tocopherol and tocotrienol-enriched fraction, respectively, at the dose of 60 mg/kg via oral gavages 6 days per week for 8 weeks. The rats were then euthanized and the femora dissected out for bone biomechanical testing to assess their strength. The callous of the TEF group had significantly higher stress parameter than the SO and OVXC groups. Only the SO group showed significantly higher strain parameter compared to the other treatment groups. The load parameter of the OVXC and ATF groups was significantly lower than the SO group. There was no significant difference in the Young's modulus between the groups. In conclusion, tocotrienol is better than α-tocopherol in improving the biomechanical properties of the fracture callous in postmenopausal osteoporosis rat model.

1. Introduction

Osteoporosis is a disease characterized by low bone mass and microarchitectural deterioration of bony tissue, which leads to bone fragility and an increase in fracture risk [1]. Osteoporosis has affected more than 75% of the population in Europe, US, and Japan [2] and is estimated to cause more than 50% of hip fractures in Asia by the year 2050 [3, 4]. Osteoporosis causes bone loss, leading to fragile bone which fractures under slight trauma. Apart from increasing the incidence of atraumatic or osteoporotic fractures, it may also add severity to a traumatic fracture and delayed fracture healing. Several animal studies have confirmed that osteoporotic bones undergo prolonged and impaired healing process [5, 6]. The incidence of osteoporotic fractures is expected to be on the rise, with the growing number of elderly people worldwide [7, 8].

Fracture healing is a complex process with three distinct phases, that is, the reactive phase, reparative phase, and the remodeling phase [9]. In the reactive phase, inflammation and formation of granulation tissue occur immediately after fracture. This is followed by the reparative phase, where

callus is formed and lamellar bone is deposited. In the final or remodeling phase, the bone will be remodeled back to its original bone contour. Studies have shown that osteoporosis can delay and impair fracture healing [10]. Antiosteoporotic drugs such as estrogens, vitamin D, and bisphosphonates may also influence fracture healing.

Due to these complexities and ethical issues, animal osteoporotic models are more appropriate to study the effects of osteoporosis on the fracture repair process [11]. These animal models include small animals such as rat and mouse to larger animals such as dogs and sheep. Ovariectomised rat has been widely used and accepted as osteopenia model to simulate bone loss in postmenopausal women [12, 13]. There are many similar bone changes in postovariectomised rats and postmenopausal women, making the ovariectomised rat a suitable model for postmenopausal bone loss [14]. The same model can be used to study fracture healing in postmenopausal osteoporosis.

The National Institutes of Health consensus panel defined that bone quality is an important parameter for the evaluation of osteoporosis, fracture risk, and osteoporosis treatment. The panel also suggested that the bone biomechanical strength is compromised with low bone density and poor bone quality [15]. The mechanical properties of bone can be assessed at different levels from the macroscopic to the ultramacroscopic levels and under different mechanical basic assumptions, such as heterogenous or homogenous and isotropic or anisotropic assumptions [16]. Bone mineral density (BMD) measurement is used widely for the detection of osteoporosis and is the major determinant of the biomechanical functioning of an osteoporotic bone. Dual energy X-ray absorptiometry (DEXA) is a good noninvasive method for BMD assessment [17].

Bone structure is made up of cortical bone (outer layer) and trabecular bone (inner layer). The inner layer has a spongy and honeycomb-like structure which is essential for the bone strength. The strength of a bone is normally estimated indirectly from its density, whereby a high density bone is assumed to be strong. This assumption may not always be accurate as discovered with the fluoride treatment of osteoporosis. The bone density appeared to be improved with fluoride treatment but in actual fact there was no improvement in strength as the new bones formed were fragile [18–21].

The bone strength can be measured directly with biomechanical testing technique, where the strength (stress), elasticity of the bone (strain), energy needed before the bone deform (load), and elastic modulus (Young's modulus) were evaluated. Biomechanical testing provides an accurate measurement of bone strength but can only be carried out in an animal model as the bone samples need to be subjected to force until they break. This is another reason why animal model is appropriate for fracture healing studies.

The strength of bone is determined by its material composition and structure [22]. Biomechanical testing of healing fractures is a useful tool in evaluating fracture healing. Bone stiffness is important to ensure that the bones will not bend when loaded, but at the same time they must also be flexible to absorb the energy imposed by the loading via elastic and plastic deformation [23].

Bending tests was proven to be sensitive in measurements of the mechanical properties of healing callus in rat [24]. In the case of fracture healing, the biomechanical testing can be carried out on the callous, the hard bony tissue that develops around the ends of a fractured bone during healing.

Vitamin E is a lipid soluble vitamin which has a chain breaking ability to stop lipid peroxidation chain reaction. Oxidative stress has been linked to the pathogenesis of bone loss leading to osteoporosis [25]. Therefore, an antioxidant such as vitamin E is able to protect bone against osteoporosis via its antioxidant properties. There are 2 types of vitamin E, tocopherol and tocotrienol, which are further divided into 4 isomers, α, β, γ, and δ. Tocotrienol is a unique vitamin E which is abundant in palm oil from *E. guinesses* species. It has better antioxidant capacity than tocopherol [26, 27]. Recently, tocotrienol has become a research interest not only for its potent antioxidant activity but also for its anticholesterol [28], antiplatelet [29], and anticancer properties [30, 31]. A study by Norazlina et al. found that both palm vitamin E and α-tocopherol maintained bone mineral density in ovariectomised rats [32]. However, there are still limited numbers of studies focusing on the effects of vitamin E on bone, especially tocotrienol. Durak et al. reported that α-tocopherol had positive effect on the fracture healing of a rabbit model [33]. Similar findings on α-tocopherol were seen in another study by Keskin et al. [34].

The present study was designed to investigate the effects of tocotrienol supplementation on bone fracture healing in postmenopausal osteoporosis rats in comparison to α-tocopherol supplementation.

2. Materials and Methods

2.1. Animals and Treatment. 32 female Sprague-Dawley rats weighing between 250 to 300 grams were used in this study. They were obtained from the Laboratory Animal Resources Unit, Faculty of Medicine, Universiti Kebangsaan Malaysia. The rats were divided into four groups, where the first group was sham operated (SO), while the other three groups were ovariectomised. They were left untreated for two months to allow for osteoporosis to develop in the ovariectomised rats. The right femora of all the rats were then fractured using a blunt guillotine method according to Vialle et al. [35] under anesthesia, and the procedures were done aseptically. Ketamine and Xylazil (1 : 1 ratio) were given intramuscularly at the dose of 0.1 mL/100 g weight as anesthetic. Iodine was then applied and a small incision was made at the right knee. The right patella was then dislocated and a Kirshner wire (K-wire) (1.0 mm in diameter) was inserted with a drill into the intramedullary canal of the femur as the internal fixation to the bone. The patella was then relocated back and the incision was sutured using a nonabsorbable suture (nylon 4). A fracturer device was used to fracture the right femur of all the rats. This device uses the same principle as the guillotine principle, where a 500 g steel blunt bar was dropped on the mid diaphysis of the femur supported by two

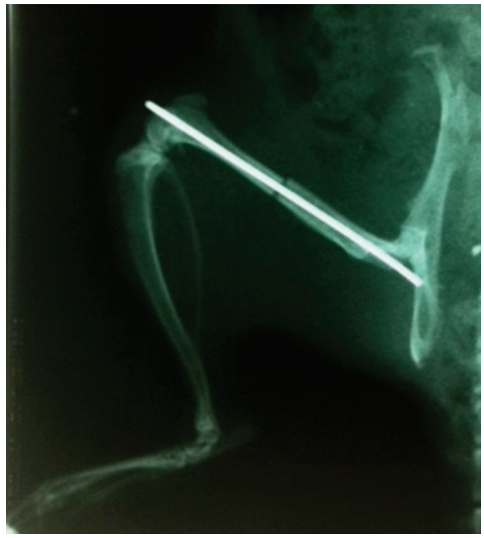

Figure 1: X-ray image taken for confirmation of K-wire insertion and the fracture is at the mid-diaphyseal region.

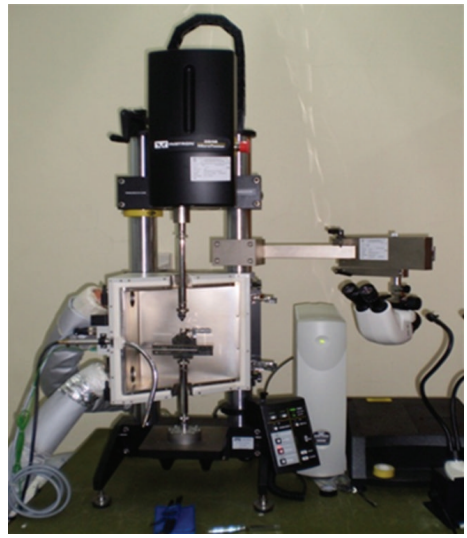

Figure 2: Instron machine with Bluehill software that we used to assess the bone strength.

metal bases. This forms a three-point system, which could produce a closed and standardized fracture. This device measuring 12.5 cm by 45 cm has three platforms, with the middle platform is 30 cm height from the lowest platform and 10 cm height to the upper platform. Triggering bar is placed at the upper platform to release the bar. The upper platform also functions to stabilize the whole structure of the device. Baytril was given intramuscularly for perioperative antibiotic prophylaxis. Buprenorphine (analgesics) was injected subcutaneously to the rats every 12 hours at the dose of 0.3 mg/kg rat weight. For confirmation purpose, X-rays were taken immediately after fracture using X-ray machine (Proteus XR/a, GE UK). This is to confirm that fractures had occurred at mid diaphysis of the right femur and the K-wires were inserted correctly (Figure 1). The rats were allowed unrestricted weight bearing after recovery from anesthesia.

The ovariectomised rats were then randomly divided into three groups, which are ovariectomised control (OVXC), α-tocopherol (ATF), and tocotrienol-enriched fraction (TEF) groups. All the rats were housed individually in separate cages at room temperature under natural day/night cycle (12 hours light/dark cycle). All the rats were given rat chow (Gold Coin, Malaysia), and deionized water was given *ad libitum*. The ATF-treated group was given α-tocopherol acetate (Sigma, USA), whereas the TEF group was given Gold-Tri E (Golden Hope Bioganic Sdn. Bhd., Malaysia), both at the dose of 60 mg/kg rat weight. The Gold-Tri E (Batch no. GHB071113196298R) was composed of 73.9 mg/g α-tocopherol, 167.1 mg/g α-tocotrienol, 41.1 mg/g β-tocotrienol, 165.2 mg/g γ-tocotrienol, and 98.5 mg/g δ-tocotrienol. On the other hand, SO and OVXC groups were given olive oil (Bertolli, Italy) which acts as vehicle. Olive oil was used as vehicle because it only contains a very small amount of α-tocopherols (51 p.p.m) and no tocotrienols. All the treatments were given via oral gavages for 2 months [36], 6 days per week. After 2 months, all the rats were euthanized and their femora were dissected out. This study has been approved by the UKM Animal Ethics Committee (FP/FAR/2008/NAZRUN/13-FEB/217-FEB2008-FEB2010).

2.2. Bone Biomechanical Test. The right femora samples were prepared for bone biomechanical test. They were wrapped individually in gauze dipped in phosphate buffer saline (PBS) to make sure that the bones did not dry out and were kept in 4°C temperature to avoid bone degradation. The biomechanical strength of the healed bones was assessed using Instron machine (Instron Microtester 5848, Instron Corp, USA) with Bluehill software (Figure 2). This machine employs the 3-point bending test, where the load was applied to the callous at the mid-point of the femoral diaphysis at the speed of 5 mm/min until it refractured. The stress, strain, and load parameters were recorded by the software. From the stress versus strain graph plotted, Young's modulus was derived from the curve gradient.

2.3. Statistical Analysis. Results were expressed as mean ± standard error mean (SEM). Analysis was done using Statistical Package for Social Sciences software (SPSS version 19.0, USA). Normality of the data was tested using Kolmogorov-Smirnov test. ANOVA was performed for normally distributed data, followed by Tukey's HSD. The level of significance was taken as $P < 0.05$.

3. Results

The strength of the healed bone (callous) was assessed using the biomechanical testing. Stress is the force implied to a unit area. Higher stress parameter indicated higher callous strength, as more force is needed to flex the callous. The callous of the TEF group had significantly higher stress parameter than the SO and OVXC groups. This indicates that the fractured femora of the TEF group had better healing

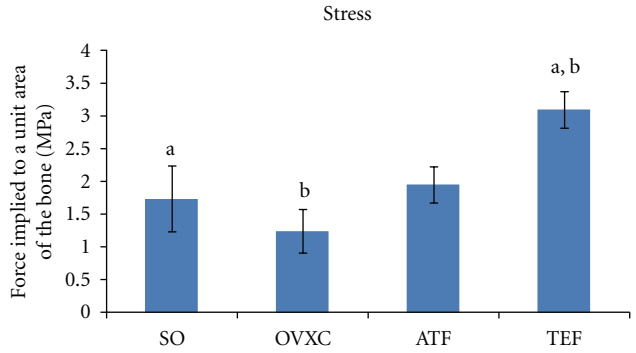

Figure 3: Bar chart shows stress parameter derived from the bone biomechanical testing. SO: sham-operated group. OVXC: ovariectomised control group. ATF: ovariectomised + ATF supplemented group. TEF: ovariectomised + TEF supplemented group. Values marked with the same alphabet are significantly different at $P < 0.05$.

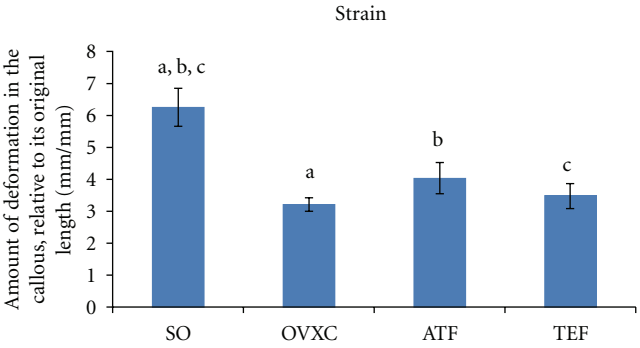

Figure 4: Bar chart shows strain parameter derived from the bone biomechanical testing. SO: sham-operated group. OVXC: ovariectomised control group. ATF: ovariectomised + ATF supplemented group. TEF: ovariectomised + TEF supplemented group. Values marked with the same alphabet are significantly different at $P < 0.05$.

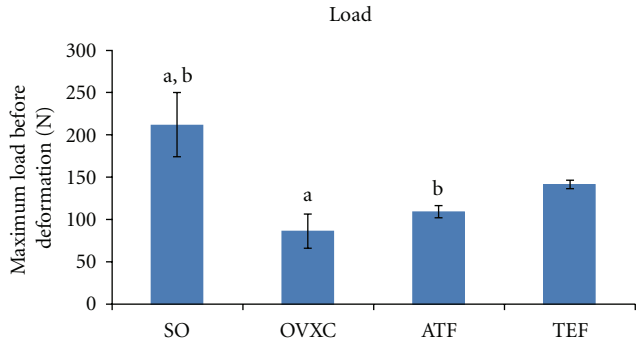

Figure 5: Bar chart shows load parameter derived from the bone biomechanical testing. SO: sham-operated group. OVXC: ovariectomised control group. ATF: ovariectomised + ATF supplemented group. TEF: ovariectomised + TEF supplemented group. Values marked with the same alphabet are significantly different at $P < 0.05$.

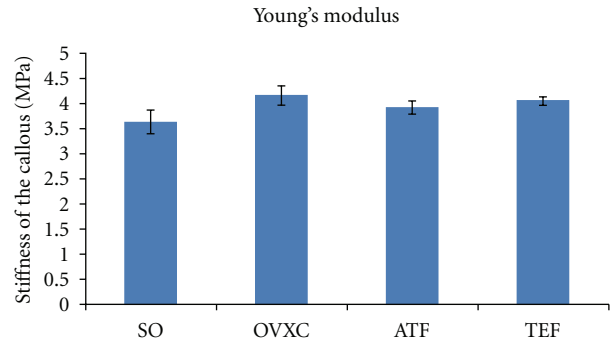

Figure 6: Bar chart shows Young's modulus derived from the bone biomechanical testing. SO: sham-operated group. OVXC: ovariectomised control group. ATF: ovariectomised + ATF supplemented group. TEF: ovariectomised + TEF supplemented group. Values marked with the same alphabet are significantly different at $P < 0.05$.

than both these groups. There was no significant difference in the stress parameter between the TEF and ATF groups (Figure 3).

Strain represents the amount of deformation in the callous, relative to its original length. Only the SO group showed significantly higher strain parameter compared to the other treatment groups. There was no other significant finding in the strain parameter between the other groups (Figure 4).

Load represents the maximum load that the callous is able to receive before it undergoes deformation. The Load parameter of the OVXC and ATF groups was significantly lower than the SO group. There was no significant difference in the Load parameter between the SO and TEF groups (Figure 5).

Young's modulus represents the stiffness of the callous. It is derived from the slope of the elastic region of the stress-strain curve. There was no significant difference in the Young's modulus between the groups (Figure 6).

4. Discussions

Osteoporotic or pathological fracture is the most common complication of osteoporosis which occurs with minimal trauma due to bone fragility. In the present study, comparison was made between the effects of ATF and TEF supplementations on the fracture healing of postmenopausal osteoporosis rat model. The healing was assessed by measuring the strength of the callous at the fracture site with biomechanical testing. The callous biomechanical properties of the treatment groups were compared to the SO and OVXC groups. The latter two groups acted as the control groups and represented the models for traumatic fracture and osteoporotic fracture, respectively.

In terms of safety, the 60 mg/kg dosage of tocotrienol-enriched fraction and α-tocopherol used in the present study were nontoxic to the rats. Toxicity studies in rat models showed NOAEL (no observable adverse effect level) with the tocotrienol dose of 130 mg/kg [37] to 2500 mg/kg weight

[38]. While for α-tocopherol, the NOAEL level was found to be at 643 mg/kg [39].

Osteoporosis has been shown to impair bone fracture healing. It influences both the early and late period of fracture healing in rat osteoporotic model [22, 23, 36]. The callous strength is an important indicator to show that the fracture healing is complete with full restoration of its structure and function. Calcium supplementation was reported to promote fracture healing in ovariectomised rats based on radiological assessments. However, biomechanical testing confirmed that the fracture healing was not accompanied by improvement in the callous strength [40]. Therefore, despite of the reports that vitamin E was able to reverse osteoporosis, it is important to determine whether vitamin E would improve the callous strength during fracture healing of osteoporotic bone.

Most bone studies have found that tocotrienols were better than tocopherols in preventing osteoporosis (Nazrun et al., 2010) [41], while Norazlina et al. found that palm vitamin E (rich in tocotrienol) was comparable to α-tocopherol in maintaining the bone mineral density of ovariectomised rats [32].

Our findings showed that the bone fractures of the ovariectomised rats healed poorly as demonstrated by the lower strain and load parameters of their calluses. Supplementation with α-tocopherol failed to improve any of the biomechanical parameters of the callous and therefore did not seem to promote the fracture healing of ovariectomised rats. Supplementation with TEF was able to significantly increase the stress and load parameters compared to OVXC group. It is also interesting to discover that the stress parameter was also significantly higher than the sham-operated group. This indicated that tocotrienol was not only able to promote fracture healing but may further strengthen the fracture callous. This is consistent with the findings by Shuid et al., which showed that normal male rats supplemented with γ-tocotrienol had better bone biomechanical strength than the normal control rats [41].

Turk et al. reported that α-tocopherol has a positive effect on fracture healing [42]. The radiological evaluation showed that α-tocopherol supplementation had improved the fracture healing of normal rats. In another study, based on the radiological scoring of fracture healing, ovariectomised rats supplemented with α-tocopherol were found to have similar fracture healing to that of the sham-operated rats (Nazrun et al., 2011) [43]. In the present study, α-tocopherol supplementation failed to improve the callous biomechanical parameters of ovariectomised rats. This meant that even though α-tocopherol seems to promote fracture healing on radiological assessments, there was no improvement in the fracture callous strength. This is in line with Arjmandi et al. [44] who found that α-tocopherol has no effects on the bone biomechanical properties of rats.

The superiority of tocotrienol may be accounted by its better antioxidant capacity than α-tocopherol. Free radicals have been shown to be involved in the process of fracture healing and their excessive levels may impair fracture healing [45, 46]. Osteoporosis itself may worsen oxidative stress as seen in postmenopausal osteoporotic subjects, who were found to be under oxidative stress [47, 48]. It is believed that tocotrienol was able to overcome oxidative stress at the fracture site to create an ideal environment for fracture healing to take place [26].

In conclusion, supplementation with tocotrienol has improved the biomechanical properties of the fracture callous in postmenopausal osteoporosis rat model. Supplementation with α-tocopherol failed to produce similar effects. Further studies are required to explore the potential of tocotrienol in promoting fracture healing of osteoporotic bone.

Conflict of Interests

The authors declare that they have no conflict of interests.

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