Drug-Herb Interaction Between Selective Estrogen Receptor Modulators and Estrogenic Herbal Medicine Er-Xian Decoction in Bone in Vivo and in Vitro

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Abstract

Background: Er-Xian decoction (EXD), a traditional Chinese Medicine for managing menopausal syndrome and osteoporosis in China, could exert osteoprotective action via activation of estrogen receptor (ERs) and regulation of serum estradiol without causing severe side effects. However, no fundamental studies have explored its potential interaction in the combined use of prescription drugs, Selective Estrogen Receptor Modulators (SERMs), regarding the osteogenic and uterotrophic effects. The present study evaluated the estrogenic effects of EXD and its potential interactions with tamoxifen and raloxifene in bone and uterus using a mature ovariectomized (OVX) Sprague-Dawley (SD) rat model and human osteoblastic MG-63 cells.

Methods: Six-month-old female SD rats were randomly assigned to Sham-operated group or seven OVX groups: vehicle, 17ß-estradiol (E2, 1.0 mg/kg.day), Tamoxifen (Tamo, 1.0 mg/kg.day), Raloxifene (Ralo, 3.0 mg/kg.day), EXD (EXD, 1.6 g/kg.day), EXD+Tamoxifen (EXD+Tamo) and EXD+Raloxifene (EXD+Ralo). The effect of EXD on bodyweight, bone mineral density (BMD), bone microarchitecture, biochemical analysis of serum and urine, and uterus were evaluated. In addition, Alkaline phosphatase assay and activation of estrogen-responsive element mediated by EXD and in its combination with SERMs were investigated in MG-63 cells.

Results: *In vivo,* EXD could interact with SERMs to modulate the serum estradiol, follicle-stimulating hormone, osteocalcin level as well as BMD and bone properties in OVX rats. Moreover, EXD could relieve the uterotrophic effect of SERMs. *In vitro*, EXD crude extract and EXD-treated serum could promote ALP activity. In particular, EXD-treated serum could interact with SERMs on regulating ALP activity in MG-63 cells.

Conclusion: Our study demonstrated that EXD *in vivo* and EXD-treated serum *in vitro* did not weaken the osteogenic effect of SERMs. Interestingly, EXD seems to ameliorate the uterotrophic effects of SERMs. Therefore, the combined use of EXD and SERMs may be considered safe and effective in managing postmenopausal osteoporosis.

Introduction

Osteoporosis is a metabolic bone disease characterized by decreased bone mineral density (BMD) and compromised bone microarchitecture that result in an increased risk of fracture. Over 200 million postmenopausal women worldwide are currently estimated to suffer from this disease, causing more than 8.9 million fractures annually [1]. Dramatic decrease in circulating estrogen level due to ovarian dysfunction is believed to be the major cause of bone loss in postmenopausal women [2].

Selective estrogen receptor modulators (SERMs) are estrogen receptor (ERs) ligand and behave as agonists or antagonists depending on tissue type [3]. They are commonly prescribed to postmenopausal women to manage estrogen-related diseases, tamoxifen for ER-positive breast cancer treatment and raloxifene for osteoporosis treatment [3, 4]. Indeed, both tamoxifen and raloxifene act as ER agonist and exert extensive bone protective effects [3]. However, administration of tamoxifen is associated with the occurrence of endometrial polyps (8-36%) and endometrial hyperplasia (1-20%) in women [5]. Therefore, the modification of SERMs usage should be considered.

Traditional Chinese medicine (TCM) have been clinically prescribed as an alternative approach for treating bone diseases with a long history of safe use. The development of postmenopausal osteoporosis is due to kidney deficiency according to the principles of TCM. Er Xian decoction (EXD), one of the most popular kidney-tonifying Chinese formulas, has been clinically used to relieve postmenopausal osteoporosis for more than 60 years with high effectiveness, fewer side effects on reproductive organs, and at relatively low cost [6].

EXD contains six herbs, including *Herba epimedii* (HEP, 仙灵脾) and *Curculigo orchioides* Gaertn (XM, 仙茅) as the principal drugs, while the other four herbs as the adjuvant drugs, *Morinda officinalis* (BJT, 巴戟天), *Rhizoma Anemarrhenae* Bunge (ZM, 知母), *Phellodendron amurense* Rupr (HB, 黄柏), and *Radix Angelicae Sinensis* (DG, 当归) [7]. The main active components of EXD were flavonol phytoestrogens, ER ligands exhibiting estrogenic activities which account for its efficacy in treating osteoporosis [8]. A systemic review and meta-analysis involving 677 patients in 5 clinical trials indicated that EXD was clinically effective in relieving menopausal syndrome via increasing circulating estradiol (E2) [9]. A study clearly revealed the modulatory impact of EXD on the hypothalamic-pituitary axis [10], which might partially account for its bone protective effects through hormonal regulation. Our *in vivo* study demonstrated the inhibitory effects of EXD on bone turnover in mature ovariectomized (OVX) rats [11] while our *in vitro* study found that EXD dramatically exerted ER-dependent cell proliferation and differentiation and activated estrogen response element-dependent transcriptional activity in rat osteoblastic UMR-106 cells [12], indicating the estrogen-like actions of EXD. These findings suggest that phytoestrogen-containing EXD could safely exert bone protective effects via ERs.

With the increasing popularity of TCM in treating menopausal symptoms, it is anticipated that EXD might be a good candidate to use in combination with SERMs exacerbating the side effect of SERMs without weakening SERMs' osteoblastic effects and. However, there is no fundamental study on the interaction between SERMs and EXD in combination regarding the estrogenic effects of SERMs at bone as well as reproductive tissues, especially uterus. The present study aimed to systematically investigate the estrogenic activity of EXD in bone and uterus as well as the interactions between EXD and SERMs (tamoxifen and raloxifene) using a mature OVX rat model and human osteosarcoma MG-63 cells.

Methods

Authentication, extraction and quality control of EXD

Six herbal medicines used in EXD, including HEP, XM, BJT, ZM, HB, and DG at a ratio of (9:9:9:6:6:9) were purchased from mainland China and authenticated by High-performance liquid chromatography (HPLC) assays. HPLC was conducted to ensure that these herbs' quality fulfills the requirement of the China Pharmacopoeia and/or the Hong Kong Chinese Materia Medica Standard. Upon authentication, herbs were delivered to Xi'an Pincredit Bio-tech Co., Ltd for water extract preparation as described previously [11]. The quantities of chemical markers in EXD extract was determined by Liquid chromatography–mass spectrometry (LC-MS).

Experimental design and animal treatment

The animal experiment protocol was approved by the Hong Kong Polytechnic University Animal Subjects Ethics Sub-committee (ASESC Case: 15-16/31-ABCT-HMRF). Eighty six-month-old female Sprague Dawley (SD) rats

were given OVX or sham operation. Upon two-week recovery, the OVX rats were orally administrated with vehicle, 17ß-estradiol (E2, 1.0 mg/kg.day), tamoxifen (1.0 mg/kg.day), raloxifene (3.0 mg/kg.day), EXD (1.6 g/kg.day) or its combinations with tamoxifen (EXD+Tamo) and raloxifene (EXD+Ralo) for 12 weeks (n=10/group). Sham-operated rats treated with vehicle were used as control. Dosages of E2, tamoxifen, raloxifene, and EXD were chosen based on equivalent human dosages and/or previous preclinical studies [11, 13]. During the whole treatment, the animals were pair-fed with phytoestrogen-free diet (AIN-93M, Research diet, New Jersey, USA) to remove the influence of phytoestrogens in the diet. Bodyweight was measured every two weeks. Urine, serum and uterus were collected and stored at -80°C for further detection. The left leg and lumbar spine were collected for micro-CT analysis. Sample size (n = 10/group) was determined at alpha of 5 % and power of 90 % based on our previous data of serum osteocalcin in rats (1 % suppression) [14].

Biochemical analysis of serum and urine

Calcium (Ca) and phosphorus (P) level in serum and urine as well as urinary level of creatinine were measured by Arsenazo III UV (Shanghai Kehua Bio-Engineering Co. LTD, Shanghai, China) with Hitachi 7100 automatic biochemical analyzer. Urinary deoxypyridinoline (DPD) was determined by enzyme-linked immunosorbent assay (ELISA) kit (QUIDEL, San Diego, USA) and normalized by urinary creatinine. Serum level of osteocalcin (OCN, Alfa Aesar, Massachusetts, USA), estradiol (Alfa Aesar, Massachusetts, USA), luteinising hormone (LH, CayMan Chemical, Ann Arbor, USA), and follicle-stimulating hormone (FSH, Cloud-Clone Corp., Texas, USA) were measured by ELISA kit.

BMD and Micro-CT analysis

Bone properties of trabecular bone at proximal tibia, distal femur and lumbar vertebra (L4) were determined by Micro-computed tomography (Micro-CT, µCT40, Scanco Medical, Switzerland). The source energy selected was 70 KVp and 114 µA, with a resolution of 21 µm. Approximately 200 slices were done for each scan. The distal/proximal were defined as 4.2 mm and 2.2 mm away from the femur/tibia head. Scanning was done at the metaphyseal area located 0.63 mm below the lowest point of the epiphyseal growth plate and extending 2.0 mm in the proximal direction. Bone mineral density (BMD, mg HA/ccm^2) and bone morphometric properties, including bone volume over total volume (BV/TV), connectivity density (Conn.D, 1/mm^3), structure model index (SMI), trabecular bone number (Tb.N, mm^{-1}), trabecular bone thickness (Tb.Th, mm) and trabecular bone separation (Tb.Sp, mm), were evaluated by contoured volume of interest (VOI) images.

Hematoxylin-eosin staining

The morphological change of uterus was examined by Hematoxylin-eosin staining (H&E). Uterus samples were collected and fixed in 4% paraformaldehyde for 6 hours. After dehydration (Leica TP1020, Leica biosystem, Buffalo Grove, USA), tissues were embedded in paraffin. Sections for histology were cut at 5 microns from the paraffin blocks using a standard microtome (Leica biosystem, Buffalo Grove, USA) before mounting and heat-fixed on glass slides. Standard H&E staining was performed. A minimum of 5 sections from each sample were observed using 400× magnification and photographed using a photoscope (Olympus BX51, Olympus corporation, Tokyo, Japan)

Real-time PCR

The mRNA expression histone H3, a marker of proliferation for endometrium, was measured in rat uterus. Total RNA was isolated from the uterus of rats in Trizol reagent (Thermofisher, MA, USA) by using Precellys 24 homogenizer (Bertin Technologies SAS, France). RNA was reverse-transcribed into cDNA by using High-Capacity cDNA Reverse Transcription Kits (Applied Biosystems, MA, USA) in Veriti™ 96-Well Thermal Cycler (Applied Biosystems, MA, USA). The specific primer for histone H3 and GAPDH (histone H3 forward 5' CTACCAGAAGTCGACCGAGC 3' , reverse 5' TCCTTGGGCATGATGGTGAC 3' ; GAPDH forward 5' CAAGTTCAACGGCACA

GTCAAGG 3'; reverse 5' ACATACTCAGCACCAGCATCACC 3') were used to perform RT-PCR with TB Green detection (TaKaRa Bio, Kyoto, Japan) using QuantStudio 7 Flex Real-Time PCR System (Applied Biosystems, MA, USA). For each gene, a standard curve was established to determine the relative quantity of mRNA, and the melting curve was used to assess the specificity of the amplification.

Preparation of EXD-treated serum

The metabolites of TCM usually considered as the functional component, which require biological activation. To observe the direct estrogenic actions of EXD *in vitro*, EXD-treated serum (EXD-Ts), a biologically activated form of EXD, were prepared in OVX rats. OVX rats were given vehicle or EXD at 16.0 g/kg.day (n=10/group) for three consecutive days and pair-fed with phytoestrogen-free diet. Upon the last oral administration on day 3, the rats were fasted overnight and given drugs one more time in the following morning. Rats were then sacrificed after an hour, and serum was prepared and stored at -80°C. LC-MS analysis of the serum was performed to confirm the presence of major chemical markers from each herb. Methanol extract of serum was prepared, and extract of 1ml serum was dissolved in 1ml of ethanol, and the concentration of this solution was defined as "1". Microsep™ Advance Centrifugal Device (3K, Pall Corporation, NY, USA) was used to remove small molecules, including steroids in serum extract, and the solution was sterilized with 0.22 μm membrane. The final dilution (10^{-5}, 10^{-4}, 10^{-3}, and 10^{-2}) of the serum extract (EXD-Ts) was used for cell studies.

Cell culture and ALP assay

Human osteosarcoma MG-63 cell (ATCC® CRL-1427™) was cultured in dulbecco's modified eagle's medium (DMEM, Gibco, MA, USA) supplemented with 100 U/ml penicillin, 100ug/ml streptomycin (Invitrogen, MA, USA), and 10% Fetal Bovine Serum (FBS, Gibco, MA, USA). The cultures were maintained in an incubator at 37°C in a 97% humidified atmosphere of 5% CO_2. Cells were cultured with phenol red-free (PRF) medium containing 5% charcoal-stripped fetal bovine serum (cs-FBS) 24 hours before subjecting to treatment with vehicle, E2 (10^{-8} M), crude EXD extract (1-200 μg/ml), EXD-Ts (10^{-4}-10^{-2} dilution), tamoxifen (10^{-12} to 10^{-6} M), raloxifene (10^{-12} to 10^{-6} M) or their combinations with EXD-Ts at the optimal concentration in PRF medium for 48 h. Upon treatment, 100 μl of Passive Lysis Buffer was added to each well to lyse cell. The ALP activity of cell lysate was measured by a LabAssay™ ALP kit (Wako, Osaka, Japan) following the manufacturer's instruction.

Statistical analysis

Data were reported as mean ± SEM. Inter-group differences of *in vivo* experiment were analyzed by one-way ANOVA with Tukey's as post hoc test. Inter-group differences in *in vitro* experiment were analyzed by t-test. Interactions between drugs were analyzed by two-way ANOVA with Bonferroni as a post hoc test. A value of *p*

0.05 was considered statistically significant. The correlation between serum hormone level and BMD at tibia in rats were analyzed using Pearson's correlation.

Results

EXD extract preparation and authentication

Raw herbs were authenticated according to the presence and quantities of specific chemical markers (see Additional file 1 and 2). They are icariin for HEP, curculigoside for XM, ferulic acid for DG, berberine hydrochloride for HB, timosaponin BII for ZM, *and* nystose for BJT. HPLC profile confirmed that the quality of all raw herbs had fulfilled their respective requirements. After water extraction, the amount of each chemical marker in EXD extract was quantified by LC-MS (Table 1).

EXD could interact with SERMs to alter bodyweight, uterus weight, or biochemical parameters in OVX rats

In table 2, the change of bodyweight increased in OVX rats (18.72% ± 5.01%,), and the increase was dramatically reduced by treatment with E2 (-0.93% ± 0.25%), tamoxifen (-0.88% ± 0.23%), raloxifene (6.57% ± 1.76%), and their combinations with EXD (2.38% ± 0.64% for Tamo, 3.80% ± 1.02% for Ralo) , but not EXD alone (20.34% ± 5.09%). Two-way ANOVA analysis indicated that EXD interacted with tamoxifen and raloxifene to suppress bodyweight gain in OVX rats ($p<0.0001$). Besides, the uterus index was significantly decreased in rats upon ovariectomy (0.33 ± 0.02 mg/g), suggesting that the surgery was successful. E2 (1.55 ± 0.05 mg/g) and tamoxifen (0.70± 0.02 mg/g) significantly increased uterus index in OVX rats. In contrast, EXD alone (0.37± 0.01 mg/g) and in combination with SERMs (0.57 ± 0.03 mg/g for Tamo, 0.60 ± 0.02 mg/g for Ralo) did not alter uterus index in OVX rats. Two-way ANOVA indicated interaction between EXD and tamoxifen ($p=0.0098$), but not raloxifene in altering the uterus index. Serum Ca and P as well as urinary P excretion in rats, were not significantly altered by ovariectomy nor treatments with EXD, SERMs, and their combinations, while urinary Ca excretion was significantly increased in EXD+Tamo treatment group (0.137 ± 0.011 mg/L). Two-way ANOVA indicated no interactions between EXD and SERMs on modulating these biochemical parameters, except urinary P (Tamoxifen x EXD: $p=0.0021$; Raloxifene x EXD: $p=0.0011$).

EXD could interact with SERMs to increase BMD and improve bone properties in OVX rats

Figure 1A clearly indicated that ovariectomy significantly reduced BMD at distal femur (111.88 ± 11.89 mgHA/ccm^2), proximal tibia (114.49 ± 12.40 mgHA/ccm^2), and lumbar vertebra (199.78± 21.05 mgHA/ccm^2) in rats when compared to Sham group. Treatment with E2 , EXD, SERMs alone and in combination with EXD significantly increased BMD in OVX rats at all three sites ($p<0.01$ vs. OVX). EXD, SERMs, and their combinations dramatically improved bone microstructure at the distal femur (Figure 1B) and trabecular bone properties at the distal femur (Table 3), proximal tibia, and lumbar spine (see Additional file 3) in OVX rats. As expected, OVX in rats significantly decreased BV/TV, Conn.D, Tb.N, and Tb.Th while significantly increased SMI and Tb.Sp at all the three bone sites ($p<0.05$ vs. Sham). Treatment of OVX rats with E2, tamoxifen, raloxifene, and EXD alone significantly increased BV/TV, Conn.D, Tb.N, and Tb.Th as well as decreased SMI and Tb.Sp of bone at all three sites ($p<0.05$ vs. OVX). Treatment of OVX rats with EXD combined with tamoxifen or raloxifene also significantly improved bone properties at three sites ($p<0.05$ vs. OVX). BMD and bone properties at all three sites in OVX rats treated with SERMs alone were not statistically different from those in OVX rats treated with

respective SERMs combined with EXD. Two-way ANOVA analysis indicated that EXD interacted with tamoxifen and with raloxifene to alter BMD at all three sites in OVX rats (Figure 1A, Tamoxifen Î EXD, distal femur: p=0.0003, proximal tibia: p=0.0022, lumbar vertebra: p=0.0005; Raloxifene Î EXD, distal femur: p=0.0199, proximal tibia: p=0.0208, lumbar vertebra: p=0.0014). And also, EXD interacted with both SERMs on improving trabecular bone properties at all three sites in OVX rats (Table 3, p<0.05).

EXD could interact with SERMs to restore serum E2 or FSH in OVX rats

In figure 2, serum E2 level was decreased in OVX rats (9.72 ± 0.86 pg/ml), accompanied with the significant increase in FSH (12.54 ± 1.43 ng/ml) and LH (5.75 ± 0.94 ng/ml) when compared to Sham group. As expected, E2 treatment in OVX rats significantly increased serum E2 (173 ± 34 pg/ml) and suppressed the increase in FSH (8.27 ± 0.48 ng/ml) and LH (2.35 ± 0.52 ng/ml) levels. Serum E2 increased in OVX rats upon the treatment with tamoxifen (29.01 ± 2.19 pg/ml), raloxifene (33.99 ± 13.78 pg/ml), EXD alone (88.88 ± 23.35 pg/ml) as well as their combinations (50.66 ± 8.62 pg/ml for Tamo, 23.01 ±2.06 pg/ml) without reaching statistical significance. Serum FSH levels were reduced in OVX rats in response to treatment with tamoxifen (9.41 ± 0.59 ng/ml), raloxifene (8.38± 0.3 ng/ml), EXD alone (9.2 ± 0.44 ng/ml) and EXD+Ralo (10.73 ± 0.42 ng/ml), but not EXD+Tamo. Serum LH levels were significantly decreased in all treatment groups in OVX rats (p<0.001 vs. OVX). Interestingly, the levels of serum reproductive hormones in OVX rats treated with SERMs alone were not statistically different from those co-treated with SERMs and EXD. Two-way ANOVA analysis indicated EXD and SERMs interacted to alter reproductive hormones (Tamoxifen Î EXD, FSH: p=0.0023, LH: p=0.0204; Raloxifene Î EXD, E2: p=0.0107, FSH: p=0.0012, LH: p=0.0141) in OVX rats. Correlation analysis found that there is a positive correlation (r =0.4304, p<0.01) between the serum E2 level and the BMD at tibia in rats while the serum FSH (r =-0.4520, p<0.01) and LH (r =-0.4698, p<0.001) level were negatively correlated with the BMD at tibia in OVX rats.

EXD could interact with SERMs to serum OCN, but not urinary DPD

As shown in figure 3, serum OCN (28.93± 0.77 ng/ml) and urinary DPD (142.12 ± 5.92 nmol/mmol) were significantly increased in OVX rats while the treatment of OVX rats with E2 significantly restored changes in OCN (14.31 ± 1.09 ng/ml) and DPD (84.92 ± 4.78 nmol/mmol). Treatment with both tamoxifen and raloxifene also significantly suppressed the increase in OCN and DPD levels in OVX rats (p<0.001 vs. OVX). However, the treatment with EXD alone significantly restored E2 deficiency-induced change in the OCN level (21.13 ± 2.45 ng/ml), but not urinary DPD (109.12 ± 5.08 nmol/mmol) in OVX rats. Co-treatment of EXD and SERMs in OVX rats also markedly reduced both serum OCN and urinary DPD (p<0.01 vs. OVX). Two-way ANOVA analysis suggested no interaction between EXD and tamoxifen while EXD interacted with raloxifene on suppressing serum OCN (raloxifene Î EXD, p=0.0334).

EXD relieved the uterotrophic effect of SERMs

In figure 4A, the mRNA expression of histone H3 was significantly increased by E2 (1.36 ± 0.16) and raloxifene (1.39 ± 0.18). The combination of EXD and raloxifene (0.8 ± 0.19) could further suppress raloxifene-induced mRNA expression of H3 in rat uterus. Two-way ANOVA analysis suggested the interaction between EXD and SERMs on suppressing H3 mRNA expression (tamoxifen Î EXD: p=0.0005, raloxifene Î EXD: p=0.0493). In figure 4B, OVX in rats obviously reduced the uterus epithelial cell layer's thickness (red line) compared to the sham

group. Treatment of E2 and tamoxifen alone could apparently reverse OVX-induced change of it. Raloxifene alone, EXD alone, and EXD combined with SERMs did not to increase the thickness of the epithelial cell layer in the uterus when compared to the OVX group. Indeed, there was an observable difference in layer's thickness between the group treated with tamoxifen alone and EXD combined with tamoxifen.

EXD interacted with SERMs on ALP activity in human osteosarcoma MG-63 cells

In figure 5A, both crude EXD extract and EXD-Ts significantly increased the ALP activity in MG-63 cells in 48 hours (p<0.05 vs. control). Crude EXD extract at the concentrations of 10 µg/ml or above exerted impressive efficacy in increasing ALP activity in MG-63 cells (1.37 ± 0.04), which was more potent than E2 (1.26 ± 0.03). EXD-Ts at 10^{-3} dilution (1.21 ± 0.03) exerted the most potent stimulatory effect on ALP activity, which was slightly weaker than E2. Both tamoxifen and raloxifene dramatically stimulated ALP activity of MG-63 cells from 10^{-12} to 10^{-6} M (Figure 5B and C, p<0.05 vs. control). Upon co-treatment for 48 h, ALP activities were decreased in co-treatment of EXD-Ts and tamoxifen at 10^{-12} and 10^{-10} M (Figure 5C, p<0.05 vs. tamoxifen alone) while ALP activity was increased in co-treatment of EXD-Ts and raloxifene at 10^{-12} M (Figure 5D, p<0.05 vs. raloxifene alone). Two-way ANOVA analysis indicated that EXD interacted with raloxifene (raloxifene Î EXD-Ts: p=0.0031 at 10^{-12} M; p=0.0006 at 10^{-10} M; p=0.0433 at 10^{-8} M; p=0.0023 at 10^{-6} M) and tamoxifen at 10^{-8} M (tamoxifen Î EXD-Ts: p=0.0010) to alter ALP activities in MG-63 cells.

Moreover, crude EXD extract at 100 and 200 µg/ml significantly promoted ERE luciferase activity (Figure 5D, p<0.01 vs. control) in MG-63 cells. However, EXD-Ts did not stimulate the ERE luciferase activity in MG-63 cells.

Discussion

Er Xian decoction (EXD) has been prescribed for the treatment of osteoporosis for decades, possibly via modulating the hypothalamus-pituitary-gonadal (HPG) axis and ER-mediated estrogen signaling. The present study confirmed the anti-osteoporotic activity in OVX rats and human osteoblastic MG-63 cells of EXD. Moreover, we are the first to address EXD at its clinical dose could interact with two clinically prescribed SERMs (tamoxifen and raloxifene) to attenuate estrogen deficiency-induced bone loss in mature ovariectomized rats and relieving the uterotrophic effect of SERMs. Our study also suggested that the inhibition on FSH and LH were correlated with the osteoprotection of EXD and *in vitro* study confirmed the direct estrogenic activity of both crude EXD extract and biologically activated EXD and further reported the existence of interaction between biologically activated EXD and SERMs at certain concentrations in human osteosarcoma MG-63 cells.

The dose of EXD, tamoxifen and raloxifene used in the present was selected based on our previous study and converted from its clinical dosages [11], while dosages of tamoxifen and raloxifene used in the present study were chosen based on our previous study in OVX rats [13], which are equivalent to 11 mg/kg.day and 33.3 mg/kg.day in human, respectively. These dosages are close to the clinical dosages of them (10-40 mg/kg.day of tamoxifen for breast cancer treatment, 60mg/kg.day of raloxifene for treatment and prevention of osteoporosis as well as prevention for breast cancer). As expected, EXD and SERMs was effective in attenuating bone loss at three bone sites regarding BMD and bone microarchitecture in mature ovariectomized rats. EXD and SERMs could restore the serum OCN level, a specific osteoblast product, and is considered a biomarker for bone formation [15] while only SERMs could restore urinary DPD level, a breakdown product of

collagen during bone resorption and is a biomarker for bone resorption in OVX rats [13]. Afterward, we demonstrated that EXD could interact with SERMs to exert bone protection. This interaction could be explained by the similar underlying mechanisms of EXD and SERMs mediated by ERs. Estrogen receptors α (ER-α) have been detected in osteoblasts, osteocytes, and osteoclasts that mediate the direct effects of estrogen, resulting in decreased bone resorption and formation activity [16]. Previously, our group [17] and others [18] address that EXD, tamoxifen, and raloxifene could activate estrogenic signaling, exciting osteoblastic activity of osteoblasts. Therefore, it is possible that they could interact with each other in bone by sharing the similar mechanisms. Further study is needed to investigate the estrogenic pathway which EXD interact with SERMs in bone remodeling.

Our study was the first to compare the bone protective activities between EXD and SERMs and found that EXD at its clinical dose exerted a similar but weaker bone protective effect than SERMs in OVX rats. Although EXD and SERMs could activate ER-α, their binding affinity to ER-α might illustrate their bone protection effectiveness. EXD is a mixture of phytoestrogen or phytoestrogen-like natural compounds. For example, HEP, as one of the principal herbs in EXD, has been reported to possess phytoestrogens (e.g., icariin, icaritin, and baohuoside) and act via ERs [19]. Phytoestrogens, as alternatives to estrogen, also exert estrogen-like activities, possibly via their direct but weak affinity for ERs [20] when compared to SERMs. The weaker estrogenic bone protection of EXD might be a possible reason that EXD did not alter SERMs' action in bone.

During menopause, the decrease in E2 level and increase in FSH level in serum accounts for bone loss [21, 22]. Thus, we studied the hormonal regulation by EXD and SERMs in OVX rats, mimicking the endocrinological condition in menopause. We demonstrated EXD, SERMs, and their combination could dramatically reverse OVX-induced suppression of E2 level and upregulation of the FSH level in rats. These results are consistent with previous studies EXD was found to promote the E2 synthesis through stimulating aromatase activity in the ovary, liver, and fats as well as decrease FHS production by hypothalamic-pituitary axis. It is anticipated that the increase of E2 level by EXD is resulted from the promoted synthesis in liver and fats [7]. Also, raloxifene and E2 was proved to decrease the FSH level in postmenopausal women, while tamoxifen [23] was reported to increase the E2 level in women. These might explain the interactive regulation of hormone level in OVX rats after treatment of EXD with SERMs in the present study. Most importantly, these treatments' hormonal changes likely contributed to the bone protection of EXD and SERMs. A recent study reports that FSH could directly stimulate osteoclastogenesis and bone resorption, and its circulating level is associated with changes in bone turnover biomarkers in postmenopausal women [24]. Besides, E2 could govern female bone remodeling through ERs [21, 22]. In agreement with the report, a dramatic elevation was also observed in the present study in OVX rats in which the changes in BMD and bone turnover markers appeared to be associated with the changes in circulating FSH and E2 in OVX rats. We also showed that the BMD in rats was positively correlated with serum E2 and negatively correlated with serum FSH and LH level. In sum, our results might suggest that the modulation of HPG axis, including stimulating E2 and suppressing FSH, might be involved in mediating the *in vivo* bone protective actions of EXD and SERMs. Further study is needed to systemically review the regulation of brain-bone axis by EXD and SERMs.

As EXD could increase the circulating E2 in OVX rats, we studied the interaction between EXD and SERMs in the uterus, one of the sensitive target tissues to estrogen and the tissue that the side effects of HRT and SERMs are frequently reported [3, 12]. Indeed, our study confirmed the stimulatory effects of E2 and tamoxifen on the

uterus indicated by the significantly increased uterus weight and uterine epithelial cell growth in OVX rats while E2 and raloxifene increase a uterine proliferation marker histone H3 mRNA expression in rat uterus. In contrast, EXD did not stimulate the growth of the uterus and histone H3 expression suggesting a tissue-selective action of EXD. Interestingly, EXD was shown to relieve the side effects of tamoxifen in the uterus which might be resorted to its direct interaction with ER or indirect modulation of the E2 level. Our results agree with the previous studies that tamoxifen causes endometrium cancer via modulating endogenous E2 level and being an ER-α agonist promoting the uterus's growth [25]. EXD and its abundant isoflavone, icariin [26] were found to maintain a healthy epithelial layer of uterus in OVX rats, probably via suppressing the protein expression of cancer-promoting ER-α and increasing the protein expression of cancer-inhibiting ER-beta (ER-β) in uterus. Also, a phenolic glycoside (curculigoside) [27], and an alkaloids (berberine) [28] in EXD, were found to improve histopathological lesion of uterus in mice and suppress the growth of endometrium cancer, respectively. Despite tamoxifen could stimulate the growth of the uterus via ER-α, EXD and its phytochemical seems to counteract it through targeting the same responder and regulating uterine growth. Even though EXD increased the E2 level, phytochemicals in EXD (icariin, ferulic acid and berberine) might exert estrogen-like activities as phytoestrogens, which have been reported to facilitate the clearance of estrogens from local tissues, such as the uterus and breast tissue, and catabolize the estrogens to more benign 2-hydroxylated metabolites, regulating the E2 level in local tissue [29]. Therefore, it is possible that EXD might enhance estrogens' clearance from reproductive tissues and (or) the catalysis of E2, thereby reducing the potential side effect of tamoxifen in the uterus. Taken together, EXD probably reduce the uterotrophic effect of tamoxifen via modulation of ERs and local E2 level.

In vitro study confirmed the estrogen-like activities of crude EXD extract in promoting ALP activity and ERE-dependent transcriptional activities in human osteosarcoma MG-63 cells. As mentioned above, EXD is a complex mixture of chemicals that might not be completely bioavailable, and its estrogenic actions might require metabolic activation *in vivo*. To observe the direct estrogenic activities of EXD and possible interactions between SERMs, biologically activated EXD in the form of serum derived from EXD-treated OVX rats were also applied to MG-63 cells. Additional file 4 shows the presence of major polyphenol and alkaloid groups in EXD-treated serum extract and the EXD crude extract, suggesting that TCMs extract and their metabolites were being absorbed and transported in rat circulation. Our results showed that EXD-Ts dose-dependently induced ALP activity while the stimulatory effect seemed less potent than that of crude EXD extract. Moreover, unlike crude EXD extract, EXD-Ts at 10^{-3} dilution did not stimulate ERE -dependent transcriptional activities. These results suggest that the estrogenic activity of biologically activated EXD was different from that of crude EXD extract. Two-way ANOVA analysis indicated that EXD-Ts interacted with SERMs (tamoxifen at certain concentrations and raloxifene at all concentrations applied) and altered the effects of SERMs at lower concentrations. Our previous study showed that serums obtained from these TCM-treated animals were too dilute, and the major markers and metabolites of TCM were undetectable. Therefore, EXD at high dosage, which was ten times to its clinical dose, was used for preparing biologically activated EXD for use in the *in vitro* studies. Our observation that biologically activated EXD could alter the effects of SERMs in MG-63 cell, but not in OVX rats, are likely due to the high dosages of EXD being used for harvesting EXD-treated serum. Thus, it is possible that upon metabolic activation and clearance, the level of phytoestrogens and their metabolites in EXD will not be high enough to compete with SERMs for binding towards ERs. Future studies will be needed to characterize major phytoestrogens' tissue distribution in OVX rats upon long-term treatment with EXD.

As summarized in figure 6, bone protective TCMs formula EXD at its clinical equivalent dose selectively exert estrogenic actions in bone without causing undesirable effects in the uterus. EXD interacts with SERMs while co-treatment with EXD does not significantly suppress the responses of bone tissue to SERMs in OVX rats and tends to relieve the side effect of tamoxifen in the uterus. Crude EXD extract, but not EXD-treated serum, directly increases ERE activities in human osteoblastic MG-63 cells, indicating actions of crude EXD extract might be different from metabolically activated EXD. The present study provided evident that the combined use of EXD and SERMs could be a modified therapeutic approach to treat postmenopausal osteoporosis while relieving the side effect of SERMs.

Conclusions

To our knowledge, this is the first study to examine the drug-herb interaction between EXD and SERMs using an OVX model and biologically activated EXD. The results suggest that proper use of bone protective TCMs formula EXD alone and, in combination with SERMs, might be safe for managing menopausal symptoms. The results of interaction studies can provide insights for understanding the systemic actions of TCMs.

Abbreviation

BMD, Bone mineral density; BV/TV, Bone volume/Total volume; Conn.D, Connective Density; DPD, Deoxypyridinoline; EXD, Er-Xian decoction; ER, Estrogen Receptors; ERE, Estrogen Response Element; FSH, Follicle Stimulating Hormone; HEP, *Herba Epimedii*; HPG, Hypothalamus-pituitary-gonadal; HRT, Hormone Replacement Therapy; LH, Luteinizing hormone; OCN, Osteocalcin; OVX, Ovariectomy; SERMs, Selective Estrogen Receptor Modulators; SMI, Structure Model Index; Tb.N, Trabecular bone number; Tb.Th, Trabecular bone thickness; Tb.Sp, Trabecular bone separation; TCMs, Traditional Chinese medicine; XM, *Curculigo orchioides* Gaertn

Declarations

Ethics approval and consent to participate

The animal experiment protocol of the present study was approved by the Hong Kong Polytechnic University Animal Subjects Ethics Sub-committee (ASESC Case: 15-16/31-ABCT-HMRF).

Consent for publication

Not applicable.

Availability of data and materials

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

Competing interests

The authors declare that they have no competing interests.

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Tables

Table 1 Chemical constituents of EXD extract by LC-MS

Herbs	Chemical marker	Contents (mg/g)
Herba Epimedii (Yinyanghuo, HEP)	Icariin	1.605
Rhizoma Curculiginis (Xianmao, XM)	Curculigoside	0.002
Morindae Officinalis (Bajitian BJT)	Nystose	4.344
Phellodendron amurense Rupr (Huangbo, HB)	Berberine hydrochloride	1.814
Radix Angelicae Sinensis (Danggui, DG)	Ferulic acid	0.007
Rhizoma Anemarrhenae Bunge (Zhimu, ZM)	Timosaponin BII	-

Note: "-" Trace amount

Table 2 Bodyweight, uterus weight and biochemical parameters in OVX rats

Group (n=10)	Bodyweight (% to baseline)	Uterus index (mg/g)	Serum biochemistry			
			Serum Ca (mg/L)	Serum P (mg/L)	Urinary Ca/Cr (mg/mg)	Urinary P/Cr (mg/mg)
Sham	12.69 ± 3.52	1.89 ± 0.15	100.77 ± 2.27	47.77 ± 2.09	0.076 ± 0.015	0.29 ± 0.06
OVX	18.72 ± 5.01	0.33 ± 0.02***	95.21 ± 1.79	45.05 ± 1.62	0.077 ± 0.008	0.22 ± 0.03
E2	-0.93 ± 0.25^^^	1.55 ± 0.05^^^	99.44 ± 2.92	52.24 ± 2.40	0.088 ± 0.016	0.26 ± 0.01
Tamo	-0.88 ± 0.23^^^	0.70 ± 0.02^	89.96 ± 2.37	46.33 ± 1.89	0.071 ± 0.011	0.31 ± 0.03
Ralo	6.57 ± 1.76^^^	0.62 ± 0.03	94.35 ± 1.95	46.62 ± 1.65	0.085 ± 0.012	0.38 ± 0.03
EXD	20.34 ± 5.09	0.37 ± 0.01	95.25 ± 1.83	46.81 ± 1.52	0.118 ± 0.005	0.28 ± 0.03
EXD+Tamo	2.38 ± 0.64^^^	0.57 ± 0.03	92.57 ± 0.81	50.13 ± 1.51	0.137 ± 0.011^^	0.14 ± 0.04
EXD+Ralo	3.80 ± 1.02^^^	0.60 ± 0.02	92.17 ± 2.45	40.79 ± 2.31	0.095 ± 0.007	0.19 ± 0.04
p value of SERMs X EXD interaction						
Tamo X EXD	<0.0001	0.0098	0.4904	0.5435	0.2864	0.0021
Ralo X EXD	<0.0001	0.4097	0.5824	0.6948	0.1372	0.0011

Ratio of uterus weight (mg) to bodyweight was defined as uterus index. Calcium (Ca) and phosphorus (P) level in serum and urine as well as urinary level of creatinine were measured by Arsenazo III UV method with an automatic analyzer HITACHI7100. Data was expressed as mean±SEM. n=7-9 rats per group. Differences between groups were determined by one-way ANOVA followed by Tukey's test for post hoc comparison. Interactions between EXD and SERMs were determined by Two-way ANOVA followed by Bonferroni test as post test. ***$p<0.001$ vs sham; ^$p<0.05$, ^^$p<0.01$, ^^^$p<0.001$ vs OVX. Ca: calcium; Cr: creatinine

Table 3 Trabecular bone properties at distal femur of OVX rats

Group (n=10)	Trabecular bone properties (distal femur)					
	BV/TV	Conn.D 1/mm^3	SMI	Tb.N mm^{-1}	Tb.Th mm	Tb.Sp mm
Sham	0.51±0.02	44.61±2.29	-0.82±0.25	4.13±0.10	0.16±0.005	0.21±0.01
OVX	0.08±0.01***	11.69±1.49***	2.07±0.14***	1.53±0.05***	0.11±0.004***	0.65±0.02***
E2	0.39±0.04^^^	35.75±1.85^^^	0.12±0.42^^^	3.20±0.24^^^	0.15±0.010^^	0.29±0.03^^^
Tamo	0.32±0.02^^^	36.98±2.16^^^	0.69±0.21^^	2.91±0.19^^^	0.13±0.004	0.33±0.03^^^
Ralo	0.31±0.03^^^	30.12±1.42^^^	0.77±0.24^^	2.59±0.19^^^	0.13±0.005^	0.41±0.04^^^
EXD	0.22±0.01^^^	21.60±0.77^	1.35±0.13	2.00±0.05	0.13±0.003^	0.54±0.02
Tamo+EXD	0.29±0.02^^^	33.38±0.87^^^	1.05±.0.21	2.80±0.07^^^	0.13±0.003^	0.39±0.02^^^
Ralo+EXD	0.30±0.03^^^	27.51±1.61^^^	0.57±0.33^^^	2.49±0.21^^^	0.14±0.007^^	0.42±0.04^^^
p value of SERMs X EXD interaction						
Tamo X EXD	<0.0001	<0.0001	0.0054	0.0124	0.0093	0.0011
Ralo X EXD	0.0033	0.0001	0.2808	0.0732	0.3435	0.0764

Bone properties of trabecular bone at proximal tibia were determined by Micro-CT (μCT40, Scanco Medical, Switzerland). Data was expressed as mean±SEM. n=7-9 rats per group. Differences between groups were determined by one-way ANOVA followed by Tukey's test for post hoc comparison. Interactions between EXD and SERMs were determined by Two-way ANOVA followed by Bonferroni test as post test. ***$p<0.001$ vs sham; ^$p<0.05$, ^^$p<0.01$, ^^^$p<0.001$ vs OVX.

Additional Material

Additional file 1. Authentication of raw herbs contained in EXD using HPLC HPLC chromatograms of *Herba Epimedii* (**A**), *Rhizoma Curculiginis* (Xianmao, **B**), *Radix Angelicae* (Danggui, **C**), *Phellodendron amurense Rupr* (Huangbo, **D**), *Rhizoma Anemarrhenae Bunge* (Zhimu, **E**) and *Sinensis Morindae Officinalis* (Bajitian, **F**) were presented.

Additional file 2. Quantification of standard in raw herbs of EXD by HPLC

Quantities of standards in different herbs have been examined by HPLC and presented in percentage. All herbs fulfilled the requirement of China pharmacopeia about the minimal percentage of standards presented in herbs.

Additional file 3. Effects of estradiol, tamoxifen, raloxifene, EXD and their combinations on trabecular bone properties at proximal tibia and lumbar spine of OVX rats

Bone properties of trabecular bone at the distal femur and lumbar vertebra were determined by Micro-CT (μCT40, Scanco Medical, Switzerland). Data were expressed as mean±SEM. n=7-9 rats per group. Differences between groups were determined by one-way ANOVA followed by Tukey's test for post hoc comparison. Interactions between EXD and SERMs were determined by Two-way ANOVA followed by Bonferroni test as a post-test. ***$p<0.001$ vs sham; ^$p<0.05$, ^^$p<0.01$, ^^^$p<0.001$ vs OVX.

Additional file 4. Major polyphenol and alkaloids detected in EXD extracts and EXD-treated serum by LC-MS in ESI (+) and ESI (-) mode
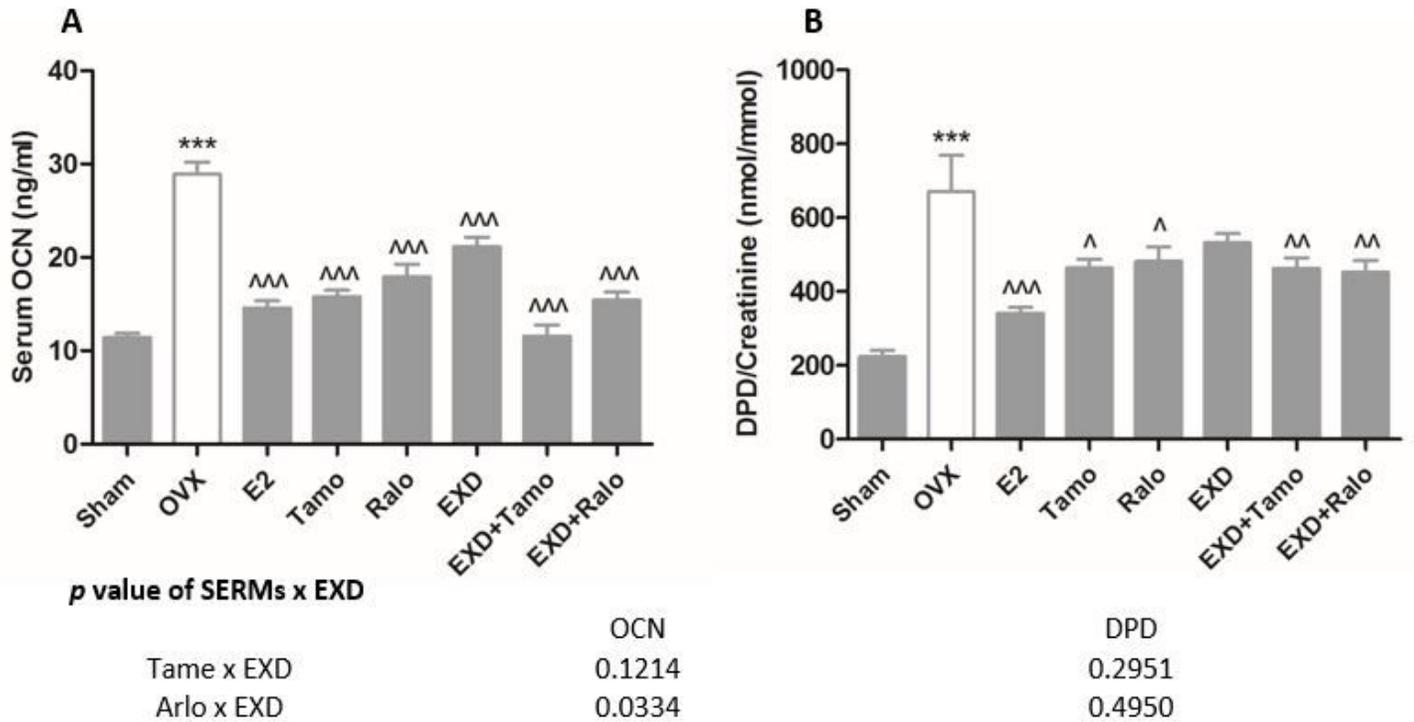
Figures
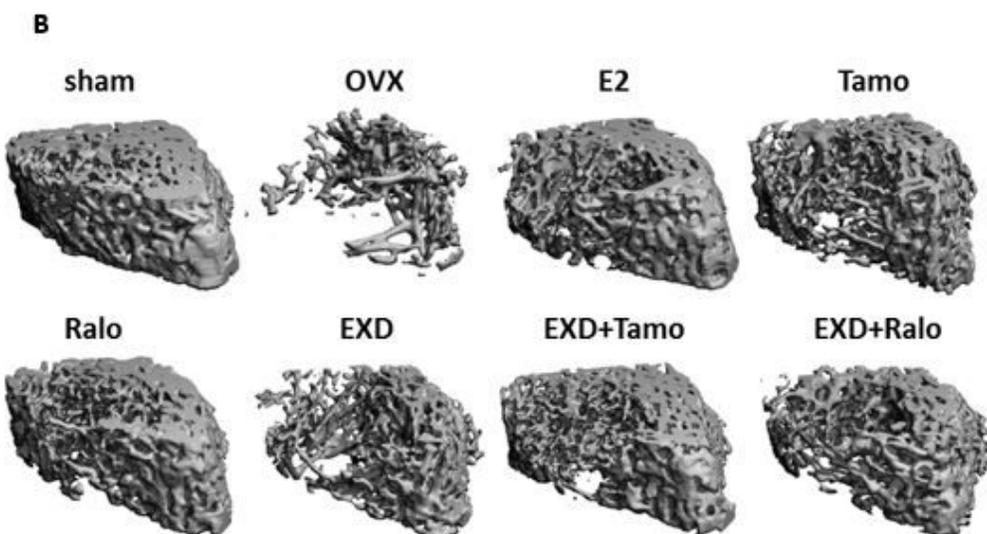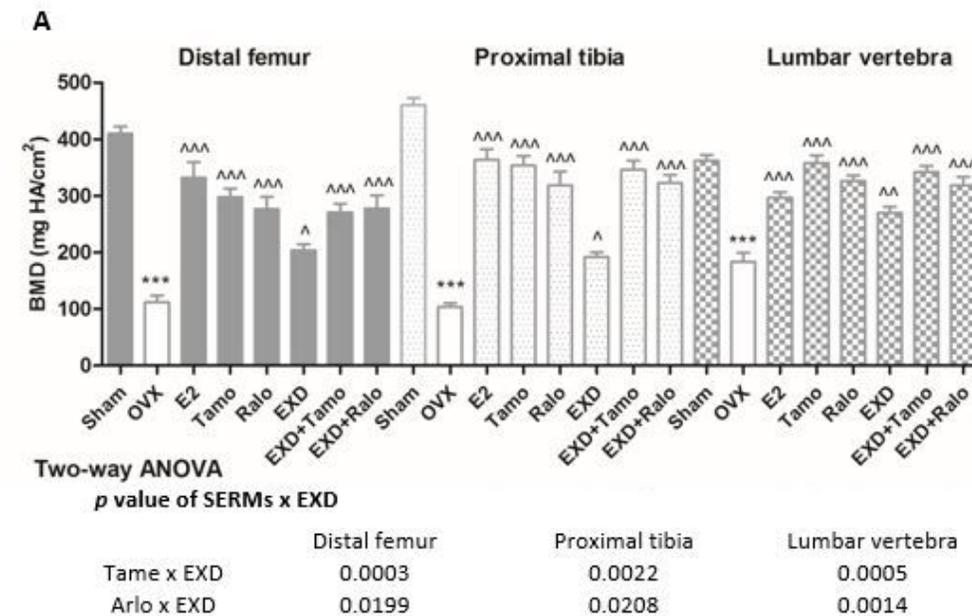
Figure 1

Estrogenic effects of EXD, SERMs and their combinations on bone properties in ovariectomized rats 6-month-old mature Sprague Dawley sham-operated (Sham) or ovariectomized (OVX) rats were treated with either vehicle, E2 (1.0 mg/kg.day), tamoxifen (Tamo, 1.0 mg/kg.day), raloxifene (Ralo, 3.0 mg/kg.day), EXD (1.6 g/kg.day) or its combinations with respective SERMs for 12 weeks. Upon treatment, the whole left leg and lumbar spine were collected. Bone mineral density at the distal femur, proximal tibia, and lumbar vertebra (A), as well as bone microstructure at distal femur (B), were analyzed by Micro-CT. Data was expressed as mean ± SEM. n=5 to 12. Differences between groups were determined by one-way ANOVA followed by Tukey's test for post hoc comparison. Interactions between EXD and SERMs were determined by Two-way ANOVA followed by Bonferroni test as a post-test. ***p<0.001 vs sham; ^p<0.05, ^^p<0.01, ^^^p<0.001 vs OVX.
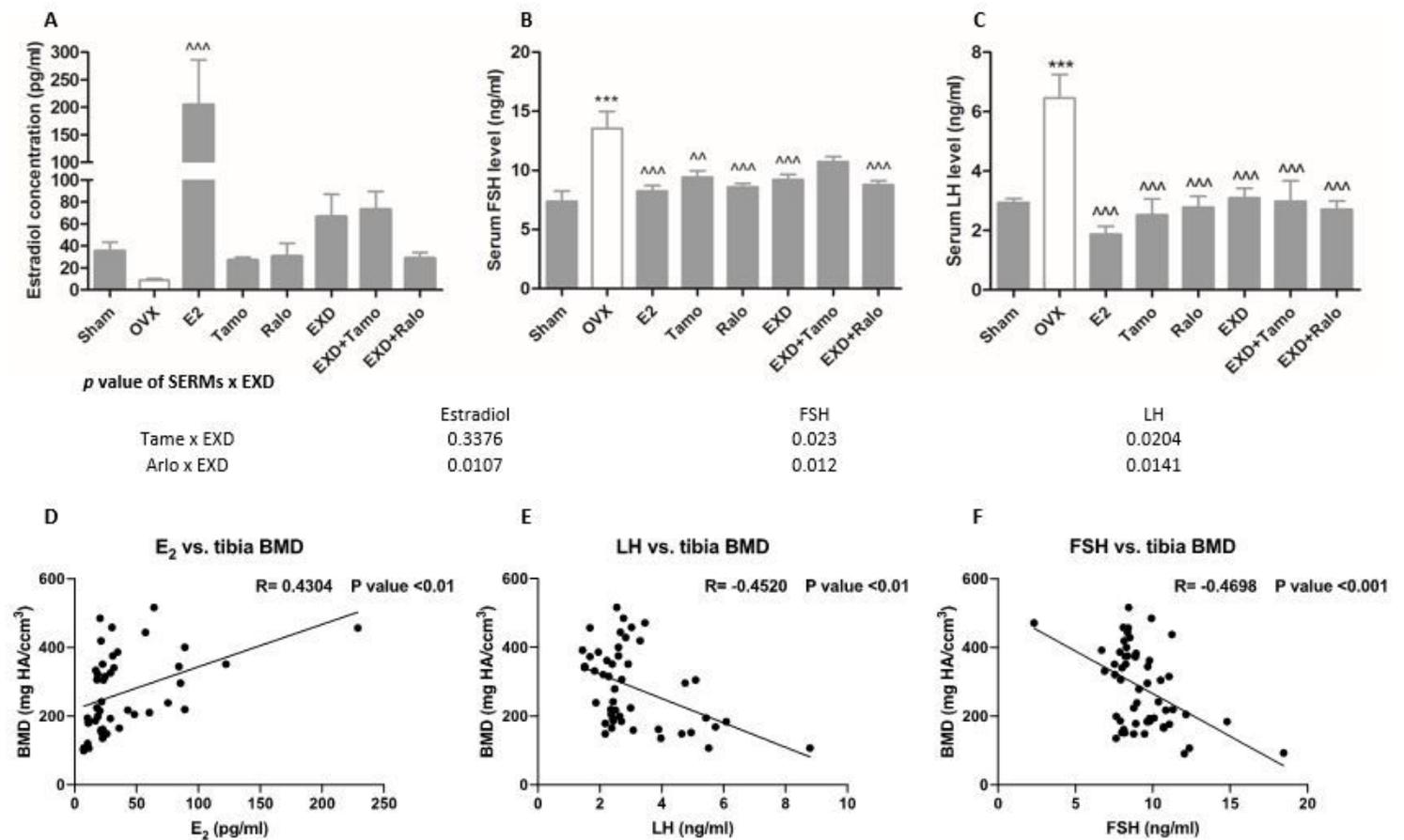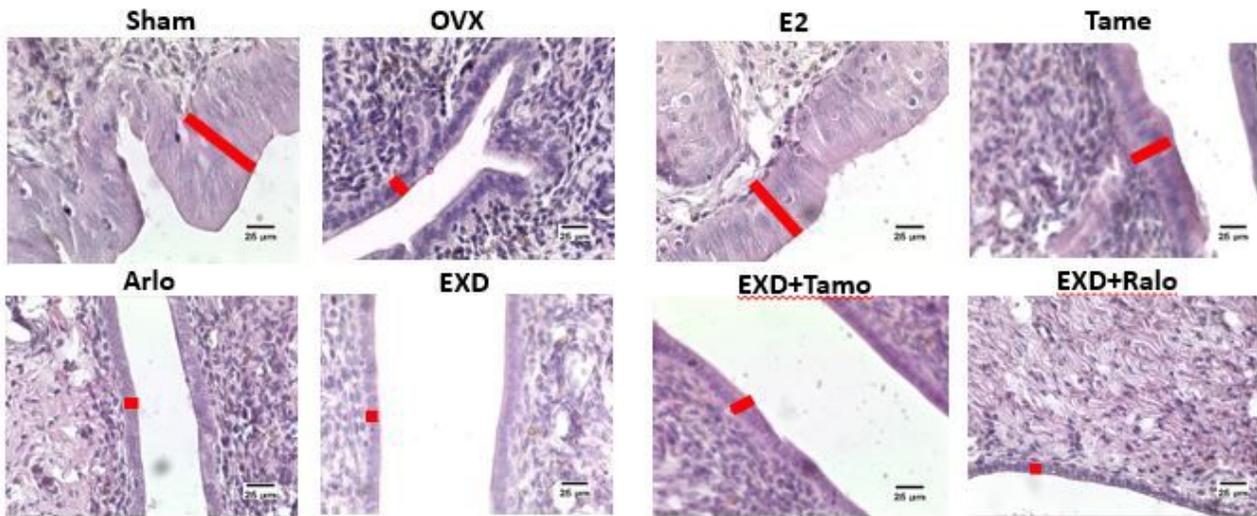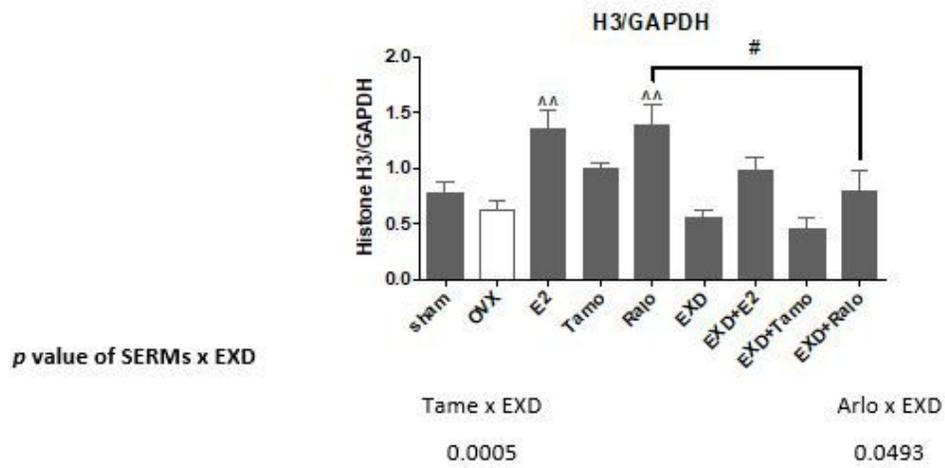

Figure 2

Estrogenic effects of EXD, SERMs and their combinations on serum reproductive hormone in ovariectomized rats Six-month-old mature Sprague Dawley sham-operated (Sham) or ovariectomized (OVX) rats were treated with either vehicle, E2 (1.0 mg/kg.day), tamoxifen (Tamo, 1.0 mg/kg.day), raloxifene (Ralo, 3.0 mg/kg.day), EXD (1.6 g/kg.day) or its combinations with respective SERMs for 12 weeks. Upon treatment, serum was collected, serum level of estradiol, follicle-stimulating hormone (FSH), and luteinizing hormone (LH) were measured by ELISA kits. A. Circulating estradiol level; B. Circulating FSH level; C. Circulating LH level. Data was expressed as mean ± SEM. n=6 to 12. Differences between groups were determined by one-way ANOVA

followed by Tukey's test for post hoc comparison. Interactions between EXD and SERMs were determined by Two-way ANOVA followed by Bonferroni test as a post-test. ***p<0.001 vs sham; ^^p<0.01 vs OVX, ^^^p<0.001 vs OVX. Correlation analyses were performed to study the correlation between E2 (D), FSH (E) or LH (F) with BMD at tibia of rats. Pearson's correlation coefficients (r) and p values were shown.

p value of SERMs x EXD

|	OCN	DPD
Tame x EXD	0.1214	0.2951
Arlo x EXD	0.0334	0.4950

Figure 3

Estrogenic effects of EXD, SERMs, and their combinations on bone turnover biomarkers in ovariectomized rats Upon treatment, serum and urine samples were collected. Serum level of osteocalcin (OCN) and urinary deoxypyridinoline (DPD) were measured by ELISA kits by following manufacturers' instruction. A. Serum level of OCN; B. Urinary level of DPD. Data was expressed as mean ± SEM. n=5 to 12. Differences between groups were determined by one-way ANOVA followed by Tukey's test for post hoc comparison. Interactions between EXD and SERMs were determined by Two-way ANOVA followed by Bonferroni test as a post-test. ***p<0.001 vs sham; ^p<0.05, ^^p<0.01, ^^^p<0.001 vs OVX.
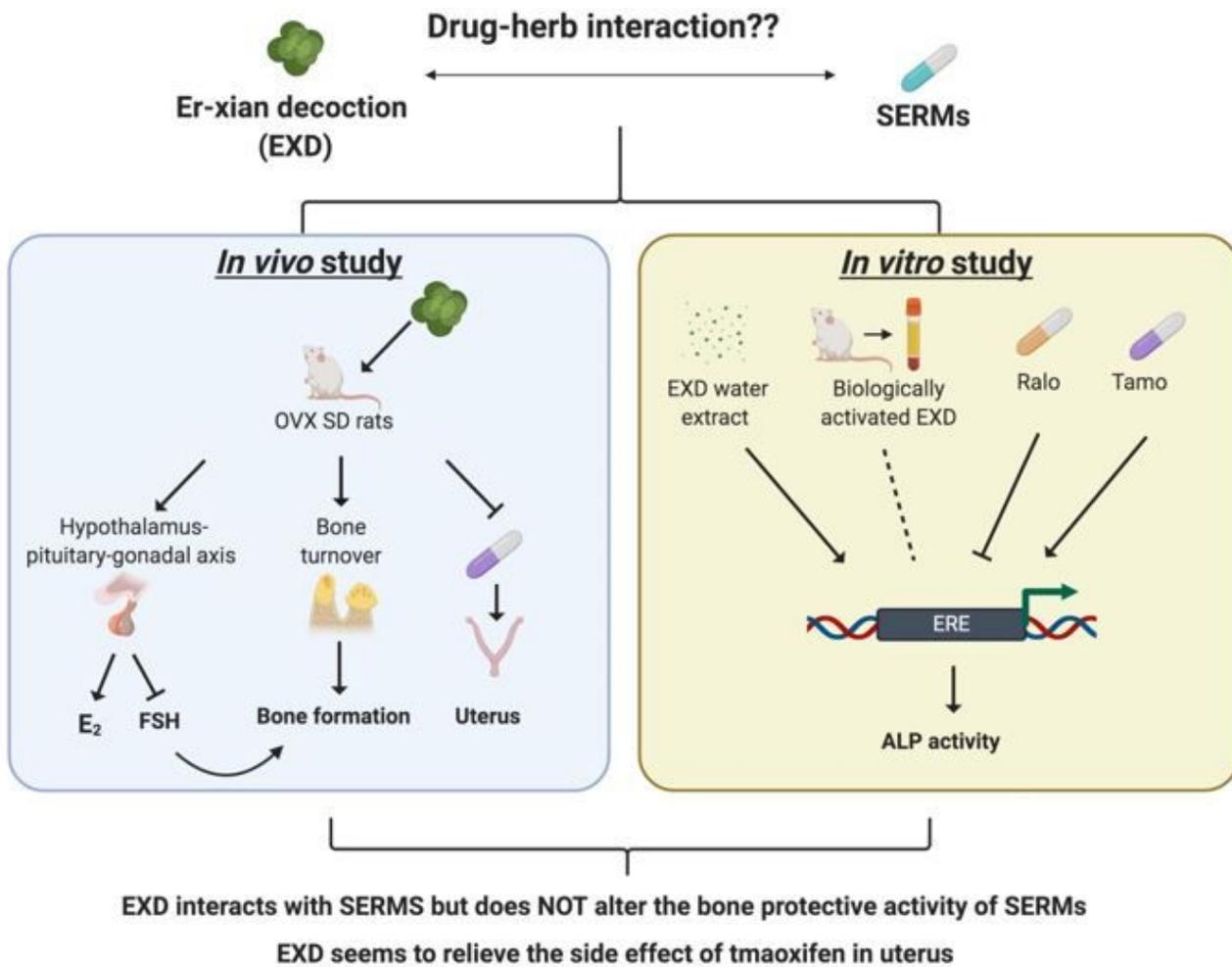
Figure 4

Uterotrophic effect of SERMs and EXD in ovariectomized rats. Uterus of OVX rats were isolated from OVX rats. (A) mRNA expression of H3 in uterus was measured by RT-RCR. (B) The thicknesses of epithelial cell layers of the rats' uterus after treatment were studied by H&E staining and indicated with red lines on the histology image (400× magnification).
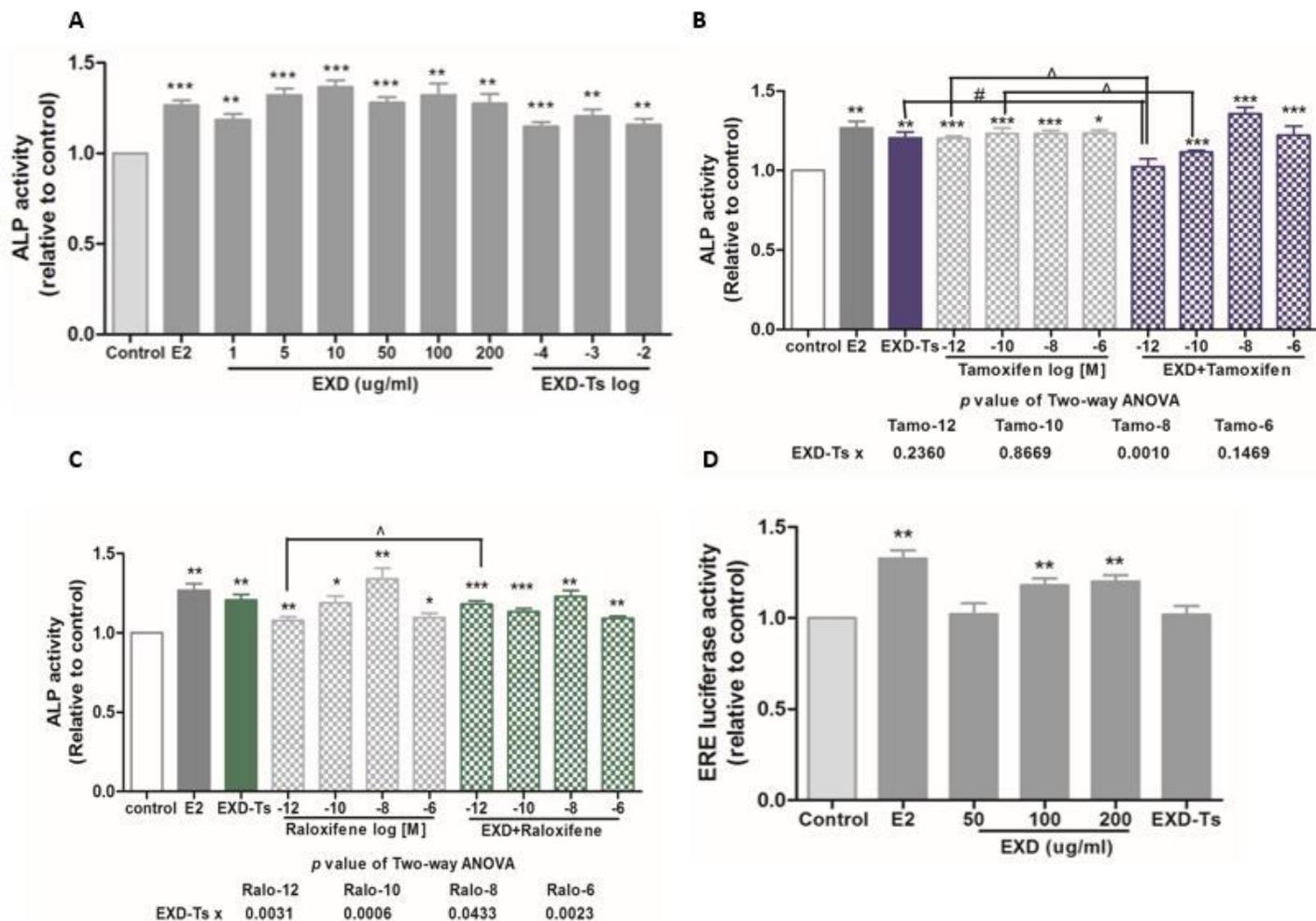
Figure 5

Estrogenic effects of EXD, SERMs and their combinations on ALP and ERE-luciferase activity in vitro Human osteosarcoma MG-63 cells were routinely cultured and treated with EXD (crude EXD extract and EXD-treated serum), tamoxifen, raloxifene and their combinations for 48 hr. Upon treatment, ALP activity and estrogen response element [12] luciferase activity were measured by ALP assay and Dual Luciferase® Reporter Assay System, respectively. Results were expressed as a ratio to control. n=3 or more. Differences between groups were determined by independent t-test. Interactions between EXD and SERMs were determined by Two-way ANOVA followed by Bonferroni test as a post-test. *p<0.05, **p<0.01, ***p<0.001 vs control; ^p<0.05 vs SERM alone; #p<0.05 vs EXD-Ts alone.

Figure 6

Bone protective activity of EXD alone and in combination with SERMs In vivo study suggests that EXD promotes bone formation by regulating the release of E2 and FSH and suppressing bone resorption. In vitro study indicates that crude EXD extract or SERMs could increase ALP activity in osteoblasts via modulating ERE while biologically activated EXD promotes ALP activity without ERE activation. Both in vivo and in vitro studies demonstrate that EXD might interact with SERMs but do not alter the bone protective activity of SERMs. (Created with BioRender.com)

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