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Du-Zhong (*Eucommia ulmoides* Oliv.) cortex extract prevent OVX-induced osteoporosis in rats

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ABSTRACT

Du-Zhong, rich in polyphenolic compounds such as lignans, phenolic acid, and flavonoids, is a kidney-tonifying herbal medicine with a long history of safe use for treatment of bone fractures and joint diseases in China. In the present study, we examined whether Du-Zhong cortex extract (DZCE) with graded doses exerted its preventive effects on estrogen deficiency-induced osteoporosis. Eighty 3-month-old female Sprague–Dawley rats were used and randomly assigned into sham-operated group (Sham) and five ovariectomy (OVX) subgroups, i.e. OVX with vehicle (OVX); OVX with 17α-ethinylestradiol (E_2, 25 μg/kg/day); OVX with DZCE of graded doses (100, 300, or 500 mg/kg/day). Daily oral administration of DZCE or E_2 started on week 4 after OVX for 16 weeks. Treatment with DZCE at higher doses (300 or 500 mg/kg/day) was found to be able to significantly prevent OVX-induced decrease in biomechanical quality of femur such as maximum stress and Young's modulus. The mechanical changes were associated with the prevention of a further bone mineral density (BMD) decrease or even with some improvements in microarchitecture. DZCE 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 phosphatese (ALP), deoxypyridinoline (DPD), and urinary Ca and P excretions. μCT 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. We conclude that 16 weeks of DZCE treatment improves bone biomechanical quality through modifications of BMD, and trabecular microarchitecture without hyperplastic effect on uterus, and it might be a potential alternative medicine for treatment of postmenopausal osteoporosis.

Introduction

The incidence of osteoporosis increases dramatically with life expectancy. This disease is characterized by a reduction in bone mass and microarchitectural deterioration of bone tissue, resulting in skeletal fragility and susceptibility to fractures [1,2]. Because hypoestrogenemia after menopause is an important cause of osteoporosis, hormone replacement therapy (HRT) was used to be a popular regime for prevention and treatment of postmenopausal osteoporosis [3,4]. Ironically, data from the Women's Health Initiative (WHI) Trial indicated that long-term acceptance and/or compliance of HRT is low due to potentially malignant effects on reproductive tissue [5,6]. Although traditional therapic agents that stimulated bone formation (e.g., sodium fluoride, growth hormone, and anabolic steroides) and antiresorptive agents (e.g., calcitonin and bisphosphonates) may prevent further bone loss in established osteoporosis, their costs are too high to benefit a large population in the developing or even the developed countries for prevention and treatment of osteoporosis. Consequently, it is necessary to develop "natural" products or synthetic substance with less undesirable side effects that can substitute or reduce the need for drugs used currently [6,7].

Through thousands of years of human experimentation, belief in the safety of "natural" products has contributed to the fairly widespread use of complementary therapies among women to relieve postmenopausal symptoms [8]. Indeed, many of commonly consumed foods, herbs and spices contain a complex array of naturally occurring bioactive molecules called phytochemicals, which may confer health benefits [9,10]. Soy food and isoflavones have received considerable attention for their potential role in preventing osteopenia induced by ovariectomy (OVX) in rats [11–14] or by menopause in women [15]. They have been characterized as naturally occurring selective estrogen receptor modulators (SERMs) with similar beneficial effects to raloxifene on bone [10,16,17]. Very recently, attention also has focused on the possible role of other polyphenols. Lignans, secoisolariciresinol diglycoside from flaxseed and isotaxiresinol from *Taxux yunnanensis* prevented bone loss

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in postmenopausal women or ovariectomized (OVX) model, respectively [18,19]. Arylnaphthalene lignans isolated from *Machilus thunbergii* increased mouse osteoblast differentiation by increasing ALP activity, collagen content and mineralization [20]. Flavonoids, rutin have been shown to inhibit estrogen deficiency-induced bone loss in OVX rats, both by slowing resorption and by increasing osteoblastic activity, resulting in increased femoral strength [21].

In the search for new naturally occurring antiosteoporosis agents in plants, we found that *Eucommia ulmoides* Oliv., also called Du-Zhong or Tu-Chung, is one of the earliest and most important edible crude herbs used for various medicinal purposes in China, Japan, and Korea. This plant, prepared from the leaf or bark, are rich in polyphenolic compounds such as lignans, phenolic acid, and flavonoids [22,23]. According to the ancient records, Du-Zhong is commonly used to reinforce the muscles and lungs, lower blood pressure, prevent miscarriages, improve the tone of the liver and kidneys, and increase longevity [24]. Based on theories of traditional Chinese medicine (TCM), the kidney is responsible for the nourishment of bone and supports gonadal functions, herein Du-Zhong shown to have kidney-tonifying activities is one of the most frequently used herbs in formulas that are prescribed for the treatment of osteoporosis in China. By far, the beneficial effect of Du-Zhong on bone and mineral metabolism has not yet been well investigated and reported as a preventive medicine for osteoporosis. In the present study, we have targeted Du-Zhong as a potential treatment for osteoporosis because its 'kidney-tonifying' and 'be rich in bioactive phytochemicals'.

Consequently, the aim of the present study was to systematically evaluate the ability of Du-Zhong cortex extract (DZCE) consumption to prevent osteoporosis induced by OVX in rats (an established model for postmenopausal osteoporosis) [25,26]. Orally dosed 17α-ethinylestradiol (E_2) was used as a reference compound for estrogenic activity on bone.

Materials and methods

Preparation of DZCE

Dried Du-Zhong (*E. ulmoides* Oliv.) cortex were purchased from a local herbal drug store in Xi'an, China, homogenized to a fine powder, and stored at room temperature (20 ± 2 °C) until use. The extraction was performed as follows: 100 g of powdered material was boiled in 60% alcohol (1:10 (w/v)) for 4 h. The extracts were filtered through Whatman No. 2 filter paper, then concentrated and spray-dried, giving a yield of extraction of 10%.

Animals and treatments

Eighty 3-month-old virgin Sprague–Dawley specific-pathogen-free (SPF) female rats (SIPPR-BK Experimental Animal Ltd., China) (body weight 250 ± 12.0 g) were housed in animal house at 22 °C and with a 12-h light and 12-h dark cycle. During the experimental period, all the rats were pair-fed [27] and allowed free access to distilled water and fed with standard rat chow (SIPPR-BK Experimental Animal Ltd., China) (Table 1).

The acclimatized rats underwent either bilateral laparotomy (Sham, $n = 10$) or bilateral OVX (OVX, $n = 70$). Four weeks after recovering from surgery, the OVX rats were randomly divided into five groups: OVX with vehicle (OVX, $n = 20$); OVX with E_2 (E_2, $n = 20$, 25 μg/kg body weight/day); OVX with DZCE of graded doses (DZCE100, $n = 10$, 100 mg/kg body weight/day), (DZCE300, $n = 10$, 300 mg/kg body weight/day) and (DZCE500, $n = 10$, 500 mg/kg body weight/day). According to the Human Rat Equivalent Dose Conversion Principle [28,29], the experimental dose for DZCE and E_2 in the present study was equivalent to the corresponding clinical prescription dose for a 60 kg human subject. Vehicle, DZCE, and E_2 were all administrated orally through a custom-made stomach tube, which started on the week 4 after OVX for 16 weeks.

The body weight of the animals was recorded weekly during the experimental period. Urine sample was collected from the rat that was housed individually for 24 h in metabolic cages without providing food 1 day before euthanizing the animals and acidified with 2 ml 1 mol/L HCL. After laparotomy using anesthetized with diethyl ether, blood sample was collected via abdominal aorta puncture, serum was then prepared by centrifugation of the collected blood (2000 rpm for 20 min). Urine and serum samples were then stored at −80 °C for biochemical determinations. Uterine, heart, liver, spleen, lung, kidney, brain and thymus were removed from each rat and immediately weighed. Femurs were dissected and filled in physiological saline and stored at −20 °C for measurement of bone mineral content (BMC) and bone mineral density (BMD) by Dual-energy X-ray absorptiometry (DXA), trabecular microarchitecture by Microcomputed tomography (μCT), and bone biomechanical quality by a three-point bending test. All studies were conducted according to the principles and procedures contained in the most recent publication of the NIH Guide for the Care and Use of Laboratory Animals National Research Council [30].

Assay for serum and urine chemistry

Serum calcium (S-Ca), phosphorus (S-P) and alkaline phosphatase (ALP) concentrations were measured by standard colorimetric methods using commercial kits (ZhongSheng BeiKong Bio-technology and Science, PRC) and analyzed by a Cobas Integra 400 Plus automatic biochemical analyzer (Roche Diagnostics, Switzerland). Urine calcium (U-Ca), phosphorus (U-P) and creatinine (Cr) concentrations were analyzed by the same method used for the serum samples. Serum osteocalcin (OC) concentration was determined using a rat OC ELISA kit (Biomedical Technologies, Stoughton, MA, USA), with a 4% intra- and 7% interassay variabilities according to the manufacturer. Urinary deoxypyridinoline (DPD) concentration was assayed using a rat DPD ELISA kit (Quidel, San Diego, USA). The intra- and interassay variabilities was 5.5% and 3.1%, respectively. Urinary excretion of Ca and DPD were both expressed as the ratio to Cr concentration (Ca/Cr; DPD/Cr).

DXA analysis

Two-dimensional the total bone mineral content (t-BMC) and the total bone mineral density (t-BMD) of the right femur were measured using Lunar Prodigy Advance by DXA (GE Healthcare, USA) equipped with appropriate software for bone density assessment in small laboratory animals as reported elsewhere [31]. BMD was calculated by BMC of the measured area.

Table 1
Composition of the experimental diets consumed by female Sprague–Dawley rats.

Ingredient	Diet (g/kg)
Protein	180
Fat	40
Crude fiber	50
Carbohydrate	180
Ca	9
P	7
Amino acids[a]	66.6
Mineral mixture[b]	9.2
Vitamin mixture[c]	1.4
Vitamin A	7000 IU/kg
Vitamin D	800 IU/kg
Vitamin E	60 IU/kg

[a] With L-Lysine, 8.2; methionine + L-cystine, 5.3; L-arginine, 9.9; L-histidine, 4.0; L-tryptophane, 1.9; L-phenylalanine + L-tyrosine, 11.0; L-threonine, 6.5; L-leucine, 4.4; L-isoleucine, 7.0; L-valine, 8.4 (g/kg).

[b] With Mg, 2000; K, 5000; Na, 2000; Fe, 100; Mn, 75; Cu, 10; Zn, 30; iodine, 0.5; Se, 0.1 (mg/kg).

[c] With vitamin K, 3; vitamin B_1, 8; vitamin B_2, 10; vitamin B_6, 6; nicotinic acid, 45; pantothenic acid, 17; folic acid, 4; biotin, 0.1; vitamin B_{12}, 0.020; choline, 1250 (mg/kg).

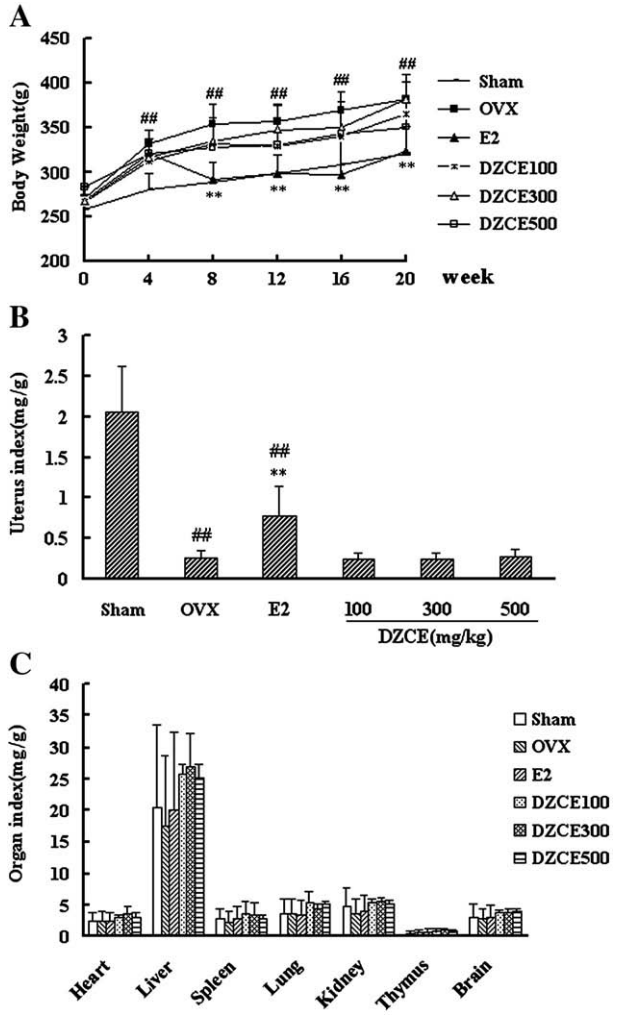

Fig. 1. Effects of 16-week treatment with DZCE or E_2 on body and organ weight of ovariectomized (OVX) rats. 3-month-old female SD rats were randomly assigned into sham-operated group (Sham) and five ovariectomy (OVX) subgroups: OVX with vehicle (OVX); OVX with 17α-ethinylestradiol (E_2, 25 μg/kg body weight/day); OVX with DZCE of graded doses (DZCE100, 100 mg/kg body weight/day), (DZCE300, 300 mg/kg body weight/day) and (DZCE500, 500 mg/kg body weight/day). Daily oral administration of DZCE and E_2 started on 4 week after OVX for 16 weeks. (A) The body weight of the animals was recorded weekly during the experimental period. (B) Uterus was isolated and weighed after euthanized, the animals and uterus index was represented as uterus weight divide by body weight. (C) Heart, liver, spleen, lung, kidney, brain and thymus were isolated and weighed after sacrificing the animals and the organ index was represented as organ weight divide by body weight. Values are mean ± S.E.M. $^{*}P<0.05$, $^{**}P<0.01$ vs. OVX; $^{\#}P<0.05$, $^{\#\#}P<0.01$ vs. Sham as evaluated by ANOVA.

Three-point bending test

Prior to mechanical testing, the left femurs were slowly thawed and held at room temperature on the day of test, the length of the femurs (distance from intermalleolar to intercondylar region) were measured with a micrometer and the middle of the diaphysis was determined. The intact femur then was placed in the material testing machine on two supports separated by a distance of 20 mm and load was applied to the middle of the diaphysis, thus creating a three-point bending test. The biomechanical quality of the left femoral diaphysis were determined using 858 Mini Bionix material testing machine (MTS, Eden Prairie, Minnesota, USA) at a speed of 2 mm/minute. The central loading point was displaced, and the load and displacement were recorded until the specimen was broken. From the load–deformation curve, maximum load (ultimate strength, F_{max}), stiffness (slope of the linear part of the curve representing elastic deformation), and energy absorption (area under the curve, W_{abs}), maximum stress (F_{max}/cross-sectional area, σ_{max}) and Young's modulus (maximum slope of the stress-strain curve, E) were obtained.

μCT analysis

After DXA measurement, three representative right femur from each group were selected for evaluating trabecular microarchitecture of the femoral metaphysis using eXplore Locus SP Pre-Clinical Specimen μCT (GE Healthcare, USA). The selection of representative sample was based on the median value of t-BMD of respective group [32,33]. The distal femur is rich in trabecular bone, compared with the proximal and middle regions. Therefore, the femur was scanned from the proximal growth plate in the distal direction (16 μm/slice). This region included 350 images obtained from each femur using 1024 × 1024 matrix resulting in an isotropic voxel resolution of 22 μm^3 [29]. The volume of interest (VOI) was selected as a region twenty five slices away from the growth plate at the proximal end of the femur to 125 slices. The 3D images were obtained for visualization and display. Bone morphometric parameters including bone volume over total volume (BV/TV), trabecula number (Tb.N), trabecula separation (Tb.Sp), trabecula thickness (Tb.Th), connectivity density (Conn.D), and structure model index (SMI) were obtained by analyzing VOI. The operator conducting the scan analysis was blinded to the treatments associated with the specimen.

Statistical analysis

Data are expressed as mean values ± S.E.M. One-way ANOVA and analysis of covariance (ANCOVA) were used to test for differences among groups. Tukey's post hoc test was made for multiple comparisons among all six groups if one-way ANOVA and ANCOVA test was found to be statistically significant. All physical measurements were analyzed using an analysis of covariance (ANCOVA) with body weight as a covariant variable for eliminating its influence on the measurement results. Dose-dependent responses of DZCE on weight by multiple linear regression; dose-dependent responses of DZCE on other variables were determined by linear regression analysis (not shown). Possible interactions between BMD and bone biomechanical quality were evaluated by

Table 2
Effects of 16-week treatment with DZCE or E_2 on biochemical parameters in serum and urine of ovariectomized (OVX) rats.

Parameters	Sham	OVX	E_2	DZCE100	DZCE300	DZCE500
S-Ca(mmol/L)	2.56 ± 0.18	2.58 ± 0.11	2.46 ± 0.27	2.45 ± 0.05	2.55 ± 0.14	2.50 ± 0.07
S-P(mmol/L)	1.50 ± 0.19	1.61 ± 0.62	1.60 ± 0.30	1.83 ± 0.28	1.82 ± 0.56	1.82 ± 0.45
U-Ca/Cr(mmol/mmol)	0.18 ± 0.03	0.37 ± 0.03$^{\#\#}$	0.24 ± 0.01**	0.33 ± 0.04**	0.31 ± 0.02**	0.27 ± 0.02**
U-P/Cr(mmol/mmol)	3.72 ± 0.27	4.88 ± 0.64$^{\#\#}$	4.27 ± 0.68*	4.37 ± 1.24	4.55 ± 0.68	3.98 ± 0.64**
ALP(U/L)	115.00 ± 17.27	251.51 ± 27.77$^{\#\#}$	161.28 ± 53.46**	226.66 ± 16.85**	204.11 ± 19.13**	184.51 ± 11.66**
OC(nmol/L)	8.15 ± 0.87	11.32 ± 0.22$^{\#\#}$	8.89 ± 0.31**	11.19 ± 0.62	10.17 ± 1.01**	10.12 ± 0.77**
DPD/Cr(nmol/mmol)	51.98 ± 3.23	85.69 ± 8.52$^{\#\#}$	66.58 ± 4.27**	84.19 ± 5.91	80.58 ± 5.69*	72.36 ± 5.58**

Note. Values are mean ± S.E.M. $^{*}P<0.05$, $^{**}P<0.01$ vs. OVX; $^{\#}P<0.05$, $^{\#\#}P<0.01$ vs. Sham as evaluated by one-way ANOVA.

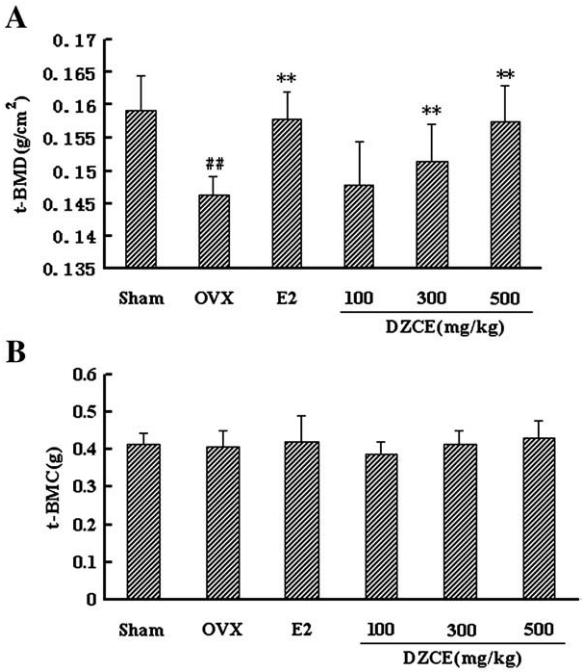

Fig. 2. Effects of 16-week treatment with DZCE or E_2 on (A) the total bone mineral density (t-BMD) and (B) the total bone mineral content (t-BMC) in right femur of ovariectomized (OVX) rats by DXA. Values are mean ± S.E.M. *P<0.05, **P<0.01 vs. OVX; #P<0.05, ##P<0.01 vs. Sham as evaluated by ANCOVA.

linear regression analysis. Statistical significance was set at P<0.05. All statistical analysis was performed using SPSS 11.0 (SPSS Inc, Chicago, IL, USA).

Results

Body and organ weights

Six groups of rats had a similar initial mean body weight. In spite of pair feeding the animals, the body weight of OVX group were significantly higher than Sham group (P<0.01) on week 4 after operation. The body weight of OVX group continued to be significantly higher than Sham group throughout the study (P<0.01 for all). E_2 completely prevented the increase in body weight associated with E_2 deficiency and returned body weight to the level maintained by Sham group 4 weeks after treatment (P<0.05). Although multiple linear regression revealed DZCE dose-dependently altered body weight, all three doses of DZCE had no significantly effect in preventing the body weight increase (Fig. 1A).

OVX caused significant atrophy of uterine tissue compared to Sham group (P<0.01), indicating the success of the surgical procedure, and administering E_2 significantly increased the uterine weight compared to OVX group (P<0.01). While DZCE at all dose levels did not elicit any uterotrophic effect (Fig. 1B). The tissue weight of heart, liver, spleen, lung, kidney, brain and thymus was not significantly different in each group (Fig. 1C).

Serum and urine chemistry

The measurement values of S-Ca and S-P did not show significant differences among all groups, while U-Ca and U-P levels in OVX group were significantly higher compared to Sham group (P<0.01 for both). All three doses of DZCE could significantly prevent the increase in U-Ca level in OVX group (P<0.01 for all), which appeared to be dose dependent; whereas, DZCE at the highest dose (500 mg/kg/day) could only significantly reduce U-P level in OVX group (P<0.01). Again, E_2 treatment had a similar effect as the highest dose of DZCE in reducing U-Ca and U-P levels in OVX group (P<0.01 and 0.05, respectively) (Table 2).

Sixteen weeks after OVX, urinary DPD/Cr ratio [34], a bone resorption marker, and plasma OC [35] and ALP [36] activity, both bone formation markers, were significantly increased in OVX group compared with Sham group (P<0.01 for all). All three doses of DZCE could significantly suppressed the increase in serum ALP level (P<0.01 for all); DZCE at higher doses (300 or 500 mg/kg/day) significantly reduced serum OC level (P<0.01 for both). In analysis of urinary DPD/Cr ratio, treatment with 300 or 500 mg/kg/day DZCE showed significantly decrease compared to OVX group (P<0.05 and 0.01, respectively). These effects appeared to be dose dependent. E_2 treatment had a similar effect as the highest dose of DZCE in reducing bone turnover (P<0.01 for all) (Table 2).

Total bone mineral content and density of the femur

There were no differences in the right femur t-BMC among any of the treatment groups. However, OVX significantly lowered the right femur t-BMD compared to the Sham group (P<0.01). The 16-week treatment with DZCE at higher doses (300 or 500 mg/kg/day) significantly increased the right femur t-BMD compared to the OVX group (P<0.01 for both), which appeared to be dose dependent. E_2 also increased the right femur t-BMD, which was significantly higher than OVX group (P<0.01) (Fig. 2).

Biomechanical quality of the femur

Twenty weeks of estrogen deficiency, maximum load, energy and stiffness showed a tendency to decrease, but any one of three biomechanical parameters did not reach statistical significance compared to Sham group. Although DZCE treatment dose-dependently altered maximum load, neither E_2 nor DZCE treatment had significant effects on values of maximum load and energy compared to OVX group. Treatment with DZCE at the highest dose (500 mg/kg/day) for 16 weeks significantly increased stiffness compared to OVX group (P<0.01), yielding values that were higher than in Sham group, which appeared to be dose dependent (Table 3).

The actual effect of treatment on biomechanical quality can only be fully evaluated if the structural biomechanical parameters (i.e., maximum load and stiffness) are corrected for changes in geometric properties of the femur midshaft, yielding material biomechanical

Table 3
Effects of 16-week treatment with DZCE or E_2 on bone biomechanical parameters in the femoral diaphysis of ovariectomized (OVX) rats.

Parameters	Sham	OVX	E_2	DZCE100	DZCE300	DZCE500
Maximum load (N)	105.26 ± 6.07	100.21 ± 9.29	105.49 ± 10.00	93.66 ± 5.77	100.74 ± 8.71	108.67 ± 4.15
Stiffness (N/mm)	158.79 ± 21.89	150.61 ± 17.27	159.93 ± 15.51	151.67 ± 9.72	174.93 ± 27.24	177.45 ± 21.21**
Energy (N×mm)	35.17 ± 3.04	33.58 ± 4.89	35.27 ± 6.75	29.00 ± 2.79	29.61 ± 6.06	33.55 ± 3.15
Maximum stress (MPa)	186.02 ± 19.57	141.53 ± 13.21##	172.79 ± 24.27**	149.34 ± 13.99	162.27 ± 17.31*	164.39 ± 2.91*
Young·modulus (MPa)	6623.26 ± 1724.51	4493.31 ± 716.55##	5924.41 ± 955.94**	5392.25 ± 1047.81	6370.89 ± 1472.49**	5812.63 ± 610.42*

Note. Values are mean ± S.E.M. *P<0.05, **P<0.01 vs. OVX; #P<0.05, ##P<0.01 vs. Sham as evaluated by ANCOVA.

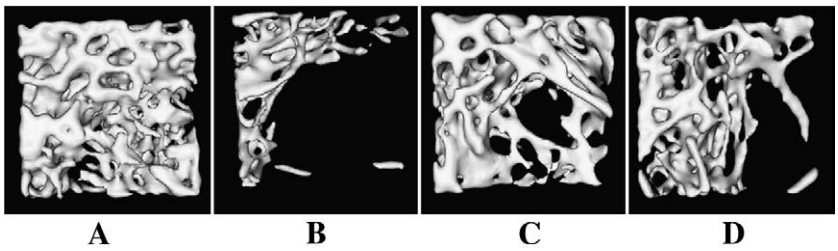

Fig. 3. Representative sample from each group: 3-D architecture of trabecular bone within the distal metaphyseal femur region. (A) Sham, (B) OVX, (C) E_2, (D) DZCE500.

parameters, that is, maximum stress and Young's modulus [37]. OVX resulted in a significantly decrease in maximum stress and Young's modulus in femur, as compared with Sham group ($P<0.01$ for both). Compared with OVX group, DZCE at 300 or 500 mg/kg/day could significantly increase Young's modulus ($P<0.05$ for both), and DZCE at higher doses (300 or 500 mg/kg/day) could significantly increase maximum stress ($P<0.05$, 0.01, respectively). The effect of DZCE on maximum load in OVX rats appeared to be dose dependent. E_2 significantly prevented the decreases in both maximum stress and Young's modulus ($P<0.01$ for both). Linear regression analysis revealed a significant positive correlation between t-BMD and maximum stress in the femur ($r=0.609$; $P\leq0.0001$) (Table 3).

μCT evaluation

Three-dimensional images of femoral metaphysis showed differences in trabecular microarchitecture among the various treatment groups as represented in Figs. 3A–D. Analysis of the representative samples data indicated that OVX significantly decreased trabecular BV/TV, Conn.D, Tb.N, and Tb.Th ($P<0.01$ for all), compared to Sham group. In contrast, SMI and Tb.Sp ($P<0.01$ for both) in the proximal femur were significantly increased in response to OVX compared to Sham group. Compared to OVX group, treatment with 500 mg/kg/day DZCE or E_2 treatment reversed the above mentioned findings at the same degree, all were statistically significant (Table 4).

Discussion

Osteoporosis is a disorder in which loss of bone and strength leads to fragility fractures. The remodeling activity in healthy bone is essential to retain bone quality and produce bone that can adapt appropriately to mechanical stimuli. Because the resorption phases of bone remodeling are short and the period required for osteoblastic replacement of the bone is long, any increase in the rate of bone remodeling will result in a loss of bone mass [38]. Menopause results in accelerated bone remodeling (an increase in bone resorption and bone formation), an uncoupling between resorption and formation, and thus more bone is resorbed by the osteoclasts on the trabecular bone surface than is replaced by the osteoblasts, and net bone loss [39,40]. The present study was designed to systematically evaluate the effect of DZCE on the protection against OVX-induced bone loss in mature rats. E_2 was included in the study as a reference compound for the effect of estrogenic activity on bone modeling and remodeling.

Table 4
μCT 3-D parameters of trabecula bone in the distal femur region.

Parameters	Sham	OVX	E_2	DZCE500
BV/TV(1)	0.4436 ± 0.037	$0.2163\pm0.013^{\#\#}$	$0.3122\pm0.054^{**}$	$0.2588\pm0.062^{*}$
Conn.D ($1/mm^3$)	55.333 ± 13.05	$32.756\pm4.149^{\#\#}$	$46.826\pm9.064^{**}$	$39.824\pm6.415^{*}$
SMI(1)	0.5314 ± 0.071	$1.1479\pm0.036^{\#\#}$	$0.4422\pm0.027^{**}$	$0.7861\pm0.041^{**}$
TB.N(1/mm)	4.4287 ± 0.370	$2.0215\pm0.177^{\#\#}$	$3.3303\pm0.400^{**}$	$3.2072\pm0.283^{**}$
Tb.Th(mm)	0.1070 ± 0.010	$0.0846\pm0.008^{\#\#}$	$0.0938\pm0.010^{*}$	$0.0957\pm0.010^{**}$
Tb.Sp(mm)	0.1243 ± 0.019	$0.3832\pm0.048^{\#\#}$	$0.2070\pm0.012^{**}$	$0.2055\pm0.023^{**}$

Note. Values are mean ± S.E.M. $^{*}P<0.05$, $^{**}P<0.01$ vs. OVX; $^{\#}P<0.05$; $^{\#\#}P<0.01$ vs. Sham as evaluated by ANCOVA.

As expected, OVX resulted in significant decrease in the femur t-BMD after 20 weeks. This loss of bone mass was accompanied by a significant increase in bone remodeling, as was evidenced by the enhanced levels of the bone turnover markers OC, ALP, and DPD. Treatment with DZCE dose-dependently prevented the decreases in t-BMD, which were reflected by the decreases in plasma OC and ALP levels, and the urinary DPD/Cr ratio. DZCE dose-dependently decreased all markers, indicating a reduction in bone turnover. In addition, an increase in fecal and urinary calcium excretions, as well as a decrease in calcium absorption efficiency, might contribute to the reduction of BMD [41,42]. DZCE dose-dependently prevented the OVX-induced increase in urinary Ca excretion, and DZCE at the highest dose (500 mg/kg/day) significantly decreased urinary P excretion. These effects of DZCE mirrored that observed with E_2 and are consistent with the maintenance of bone mass by inhibiting bone remodeling in both groups.

Although such inhibition generally would be considered beneficial, biomechanical competence of bone may be decreased if bone remodeling is inhibited excessively. A 25-day treatment with pamidronate (14 mg/kg/day), for instance, has been shown to decrease intrinsic diaphyseal bone strength in rats [43]. Performing three-point bending tests on femoral diaphysis, we found that DZCE at higher doses (300 or 500 mg/kg/day) significantly prevented maximum stress and Young's modulus decreases in OVX rats. Although BMD has been described as only a surrogate measure of bone strength [44], microarchitecture determinants are necessary to evaluate the true impact of a treatment on quality of trabecular bone because trabecular bone is more readily lost due to OVX in this animal model [45].

The preservation of trabecular microarchitecture significantly contributes to bone strength and may reduce fracture risk beyond BMD and BMC [46,47]. The effects of DZCE at the highest dose on femoral microarchitecture were only investigated by scanning with μCT. The selection of dose was based on DZCE improving most of the variables in a dose-dependent manner, especially t-BMD and maximum stress. The results indicate increased trabecular BV/TV, Tb.N, Tb.Th and Conn.D, and decreased Tb.Sp in rats treated with 500 mg/kg/day DZCE compared with OVX group. SMI distinguishes between rods and plates of bony trabeculae. SMI 0 and 3 represents bone that consists purely of plate- or rod-like structures, respectively [48]. Our results suggest a moderate but significant evolution of trabecular structures back from rods to a mixed plates and rods form in rats treated with 500 mg/kg/day DZCE in OVX group. Although DZCE in the present study had positive effects on trabecular microarchitectural properties, none of the treatments, including E_2, was able to restore trabecular bone completely. These findings are in agreement to those of other investigators [49] who were unable to observe restoration of trabecular structure after its deterioration has occurred, emphasizing the need for prevention of trabecular bone loss.

In agreement with the previous observations [33], ovarian hormone deficiency significantly increased body weight, and, as

expected, this excess body weight gain was completely prevented by E_2 administration. Although the exact mechanisms by which OVX induces increase in body weight are not clear, recent study by Dang et al. [50] suggests that estrogen plays an important role in stimulating the differentiation of progenitor cells through the osteoblast lineage and not adipocyte lineage. Estrogen may be involved directly in energy metabolism by binding to estrogen receptor (ER) within the abdominal, subcutaneous, and brown fat pads [51,52]. DZCE at all dose levels tested in the current study did not prevent the increase in body weight induced by OVX in rats. It also was devoid of any uterotrophic activity because uterine weight was not different in OVX and DZCE group. The results suggest that DZCE at these dosages did not behave like E_2 in the regulation of body weight and uterine tissue growth in the OVX rats.

There have been numerous studies on the biological activities of some plant-derived phenolic compounds, which are potent antioxidants and free radical scavengers [53,54]. It has been demonstrated recently that oxidation-derived free radicals intervene in bone resorption, promoting osteoclastic differentiation in such a manner that bone resorption is increased with oxidative stress [55–57]. The mechanism of the effects of Du-Zhong on bone appeared to be related to high contents of the polyphenolic compounds such as lignans, phenolic acid, and flavonoids, which would influence oxidative stress [58–60]. Furthermore, the previous studies confirmed that Du-Zhong extracts exhibited inhibitory effects on lipid peroxidation and oxidative damage in biomolecules [60–63]. Good correlation between the contents of polyphenolics in Du-Zhong extracts and their antioxidant activity was observed [60]. Another possibility would be that the polyphenolic compounds such as lignans and flavonoids in Du-Zhong affect bone through ER as phytoestrogens do [64], at least in part. The rat, mouse, and human ER exists as two subtypes ERα and ERβ, and ERβ is more abundant than ERα in bone tissue while ERα is mainly distributed in reproductive cells, especially those of the breast and uterus. Thus, DZCE might show higher affinity for ERβ than for ERα that produces optimal action in preventing bone loss without stimulating an unwanted proliferation of the uterine tissues. Moreover, the earlier study showed the presence of ERβ-like immunoreactivity in the nuclei of human and murine osteoblast and osteocytes and in the cytoplasm of osteoclasts and chondrocytes [65]. ERβ messenger RNA (mRNA) is present in rat osteoblasts, predominantly in those covering metaphyseal bone trabecular surface [66]. Our study showed that total lignans from Du-Zhong (*E. ulmoides* Oliv.) cortex has a direct stimulatory effect on the proliferation and differentiation of cultured rat osteoblast in vitro (not published). We proposed that DZCE stimulate osteoblastic activity and inhibit osteoclastic resorption through ERβ.

In conclusion, the present study clearly demonstrates that daily oral administration of DZCE over a 16-week period in the adult OVX rat can prevents the estrogen deficiency-induced bone loss and deterioration of trabecular microarchitecture, thereby maintaining biomechanical competence of bone. Moreover, DZCE did not act the same as E_2 on the uterus and other organs. These effects seem to be related to the high content of the polyphenolic compounds such as lignans, phenolic acid, and flavonoids. Consequently, Du-Zhong, used in traditional Chinese medicine for 'strengthening the kidney', might be a potential alternative medicine for treatment of postmenopausal osteoporosis.

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