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Original Article

Du-Zhong (*Eucommia ulmoides Oliv.*) cortex extract prevents bone loss in ovariectomized diabetic rats by suppressing bone turnover and upregulating OPG/RANKL ratios

Shanshan Qi[1], Jia He[1], Chen Chen[2], Hongxing Zheng[2], Zhijian Zhang[2]

[1]*Vitamin D Research Institute, College of Biological Science and Engineering, Shaanxi University of Technology, Hanzhong 723000, Shaanxi, China;* [2]*Chinese-German Joint Laboratory for Natural Product Research, College of Biological Science and Engineering, Shaanxi University of Technology, Hanzhong 723000, Shaanxi, China*

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Abstract: Incidence rates of osteoporosis in postmenopausal diabetic women are very high due to severe bone loss. The current study established a rat osteoporosis model, *in vivo,* to observe the protective effects and mechanisms of Du-Zhong (*Eucommia ulmoides Oliv.*) cortex extract (DZCE) on osteoporosis induced by diabetes, combined with estrogen deficiencies. Thirty 8-week-old female Sprague-Dawley rats were divided into the control group (vehicle treatment), OVX/T1DM group, and OVX/T1DM-DZCE group (OVX/TIDM treated with DZCE), with 10 rats in each group. Bone histomorphometry parameters, bone mineral density (BMD), serum bone turnover markers, and bone marrow adipogenesis were analyzed after 60 days of DZCE administration. Results showed that consumption of DZCE at doses of 200 mg kg^{-1} increased the BMD of ovariectomized diabetic rats. DZCE decreased serum bone turnover marker levels. At the same time, the bone structure and number of osteoclasts, as well as bone marrow adipogenesis, were normal. Runt-related transcription factor 2 (RUNX2), as well as the OPG/RANKL ratios, were increased by DZCE treatment in OVX/T1DM rats. Results of this study indicate that oral administration of DZCE can prevent osteoporosis due to estrogen deficiencies and diabetes. These effects are mainly related to inhibition of bone turnover, inhibition of bone marrow adipogenesis, and upregulation of bone RUNX-2 and OPG/RANKL expression ratios.

Keywords: Du-Zhong cortex extract, osteoporosis, diabetes, bone turnover, bone histomorphometry

Introduction

Postmenopausal women are prone to osteoporosis due to estrogen deficiencies. This type of osteoporosis is called postmenopausal osteoporosis (PMOP), characterized by bone loss and destruction of bone structure [1, 2]. Decreased bone mineral density (BMD) and increased incidence of fractures are the main clinical symptoms of PMOP [3]. Type 1 diabetes mellitus (T1DM) is a group of metabolic disorders characterized by islet beta cell destruction and hyperglycemia [4]. Hyperglycemia has been associated with systemic abnormal bone remodeling and bone loss [5]. Evidence has shown a high rate of bone destruction in individuals with chronic hyperglycemia [6]. Patients with T1DM have been reported to have high rates of osteoporosis and fractures [7-10]. Women with postmenopausal diabetes, experiencing a high incidence of fractures, have been given special attention regarding management of their bone health and prevention of fractures [11].

Estrogen and insulin deficiencies play important roles in the pathogenesis of osteoporosis [12]. A study by Raehtz et al. showed that bone loss can be aggravated when combined with estrogen deficiencies and hyperglycemia [13, 14]. Although estrogen and insulin can inhibit bone turnover, hormone replacement therapy increases the risk of endometrial, breast, and ovarian cancers [15, 16]. Therefore, it is neces-

sary to find other safe treatments for postmenopausal diabetic osteoporosis.

Du-Zhong (*Eucommia ulmoides Oliv.*) is a kind of kidney-tonifying herbal Chinese Medicine. It has a long history of treating fractures in China. No side effects have been reported thus far. It contains rich phenolic compounds, such as flavonoids, phenolic acids, and lignans [17]. It has been reported that Du-Zhong cortex extract (DZCE) not only prevents postmenopausal osteoporosis, but also provides hypoglycemic effects on diabetic rats [18-20]. However, it is still unknown whether it provides protective effects for estrogen deficiencies combined with diabetes-induced bone loss. It was hypothesized that DZCE maybe have protective effects on bone lose in ovariectomized diabetic rats.

In this study, an osteoporosis model of rats was established to study the protective effects of DZCE on estrogen deficiencies combined with diabetes-induced osteoporosis, examining the underlying mechanisms. This study systematically investigated bone turnover markers, bone mineral density (BMD), bone histomorphometry parameters, and bone marrow adipogenesis. Since the OPG/RANKL axis plays a vital role in bone neosynthesis [21] and RUNX2 controls skeletal development via regulating osteoblasts differentiation [22], OPG/RANKL axis and RUNX2 expression levels were detected, aiming to study the underlying mechanisms. This study aimed to provide a theoretical basis for the potential therapeutic use of DZCE supplements for prevention of bone loss in postmenopausal diabetic women.

Materials and methods

Du-Zhong cortex extract preparation

Dried Du-Zhong cortex was purchased from an herbal drug store in the city of Hanzhong, China. It was homogenized to make a fine powder. Extraction methods were as follows: Powdered Du-Zhong cortex (100 g) was boiled in 70% alcohol for 4.5 hours, then filtered with a filter paper. It was concentrated and freeze dried. The final extraction rate was 10% (w/w). The extract contained 35% of total isoflavones (the ratio of genistein: daidzein: glycitein was 1.3:1:0.3), 7-12% protein, 6% ash, and 5% moisture. The remaining 43% consisted of other natural phytocomponents.

Animals and treatments

Eight-week-old SD rats (female), weighing 212 ± 16 g, were purchased from the Experimental Animal Center of Xi'an Jiaotong University (Xi'an, China). Care and operation of the rats was conducted in strict accordance with the plan approved by the Animal Ethics Committee of Shaanxi University of Technology. Throughout the experiment, the rats were housed in individual cages in a room with constant humidity (50 ± 18%) and temperatures (24°C). The rats were given free access to standard ingredient chow (solid) and distilled water.

After 7 days of adapted feeding, the rats were assigned to the control group (vehicle treatment), OVX/T1DM group (ovariectomized diabetic rat model), and OVX/T1DM-DZCE group (OVX/TIDM treated with DZCE). Three groups of rats were intraperitoneally injected with sodium pentobarbital 30 mg kg^{-1} for anesthesia. OVX/T1DM and OVX/T1DM-DZCE rats were underwent ovariectomy operations. The control rats underwent sham operations. Fifteen days after the ovariectomy operation, in OVX/T1DM and OVX/T1DM-DZCE groups, the rats were injected with 60 mg kg^{-1} streptozotocin (STZ) intraperitoneally. The dose of STZ was selected based on methods described by Carbonel et al. [23, 24]. Control group rats were intraperitoneally injected with an equal amount of vehicle. After 7 days, blood glucose was detected using a glucometer (Sinocare Inc., Changsha, China). Rats were considered to be diabetic when blood glucose levels were above 250 mg dl^{-1}. Rats in the OVX/T1DM-DZCE group were intragastrically administered DZCE (200 mg kg^{-1} body weight) for 60 days. DZCE supplementation was started after STZ injections. A supplemental dose of DZCE was selected according to the research of Zhang and Liu et al. [18]. OVX/T1DM rats were given intragastric deionized water instead of DZCE.

Serum bone turnover markers, Ca, P, OPG, RANKL, and RUNX2 detection

After 60 days of DZCE treatment, the rats were fasted overnight and sacrificed in excess of isoflurane. Abdominal aorta blood was taken and centrifuged at 4°C for 15 minutes to extract serum. An atomic absorption spectrometer was used to analyze serum calcium (Ca) and phosphorus (P) levels of the rats. Protocols of the

Table 1. qPCR primer sequences

Primer name	Primer sequence (5-3')
β-actin-F	CGT TGA CAT CCG TAA AGA C
β-actin-R	TAG GAG CCA GGG CAG TA
RUNX2-F	CGA AAT GCC TCT GCT GTT AT
RUNX2-R	TTC TGT CTG TGC CTT CTT GG
OPG-F	TGA GTG TGA GGA AGG GCG TTA C
OPG-R	TTC CTC GTT CTC TCA ATC TC
RANKL-F	ATC AGA AGACAG CAC TCA CT
RANKL-R	ATC TAG GAC ATC CAT GCT AAT GTT C

enzyme-linked immunosorbent assay kits (Beijing kits Sinogene Bio-Technology Company, China) were used to detect serum bone turnover markers (ALP, osteocalcin, CTX-1, PINP, and TRACP 5b), as well as serum OPG, RANKL, and RUNX2.

Bone mineral density measurement

Bone mineral density (BMD) levels of the left femurs and lumbar vertebrae (L1-L4) of the rats were measured using a dual energy X-ray absorptiometry (DEXA) scanning system (Lunar, Wisconsin, USA).

Bone histomorphometric analysis

Histomorphological analysis was performed using methods previously reported [25, 26]. The right femurs and tibial bone tissues were fixed with 4% paraformaldehyde (PFA) solution for 24 hours. Next, the bone tissues were decalcified in 10% EDTA for 4 weeks at 4°C in a refrigerator [27]. The bone tissue was dehydrated with ethanol and transparent with xylene, then embedded with paraffin. Bone tissues were cut into 5 microns and stained with hematoxylin and eosin. Histological examinations were performed under the Leica DM 3000 microscope (Leica Microsystems, Wetzlar, Germany). Cortical or trabecular thickness (Ct.T, μm; Tb.Th, μm), trabecular separation (Tb.Sp, μm), and bone volume per tissue volume (BV/TV, %) were measured using Image Pro Plus 5.0 analytic software. An acid phosphatase kit (Jiancheng Bio-Technology Company, China) was used to stain the femur slides with tartaric acid phosphatase (TRAP). The number of osteoclasts was quantified using Image Pro Plus 5.0 analytic software [28].

Bone marrow adipocyte parameters analysis

Hematoxylin and eosin stained tibial slices were observed under Leica DM 3000 (Leica Microsystems, Wetzlar, Germany). According to published methods [29, 30], the mean adipocyte diameter (m) and adipocyte numbers (number/mm^2) in the tibial bone marrow were analyzed using Image Pro Plus 5.0 analysis software.

Immunohistochemistry

Femoral slides (5 m thick) were incubated with 1% Triton x-100 solution at room temperature for 30 minutes. They were then incubated in citric acid buffer solution in a microwave oven for 12 minutes. The sections were washed three times with PBS-T, then blocked with 3% bovine serum albumin. Primary antibodies of RUNX2, OPG, and RANKL (Invitrogen, USA) were added to the femoral slides and incubated at 37°C for 1.5 hours. The negative control slides were added with rabbit immunoglobulin G (IgG). Next, 1.5 hours later, the slides were washed with PBS-T. Horseradish peroxidase (HRP) secondary antibodies were added and incubated at 37°C for 2 hours, then washed with PBS-T three times. The nuclei were stained with hematoxylin after adding DAB solution to the slides. Images were observed using the Leica DM 3000 microscope (Leica Microsystems, Wetzlar, Germany). Finally, percentages of RUNX2, OPG, and RANKL positive regions were quantitatively analyzed using Image Pro Plus 5.0.

Quantitative real-time PCR

RUNX2, OPG, and RANKL gene expression levels were detected using real-time quantitative PCR. Total RNA from bone tissues was extracted using RNA TRIzol Reagent (Sigma-Aldrich, Steinheim am Albuch, Germany). Next, cDNA was obtained with PrimeScript™ RT Master Mix (TaKaRa, Japan). Real-time quantitative PCR analysis of gene expression levels of RUNX2, OPG, and RANKL was conducted using the primer sequences listed in **Table 1**. Gene relative variation expression was analyzed with the $2^{-\Delta\Delta CT}$ method.

Statistical analysis

Data was recorded using an excel database. Statistical analyses were performed using SPSS18.0 analytic software (IBM Corporation, Armonk, NY, USA). Ten different regions per slice were selected from 5 slices per group under a 400× light microscope. Trabecular thickness, trabecular separation, and optical

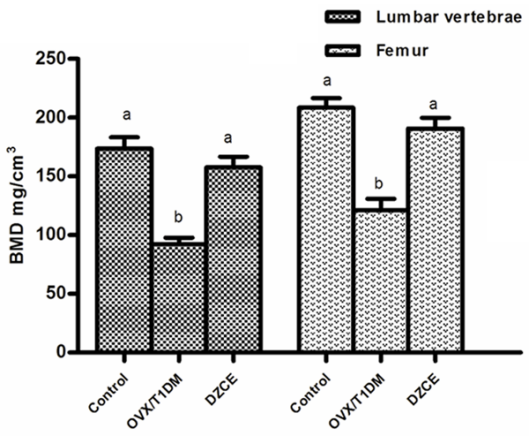

Figure 1. Lumbar vertebrae and femur BMDs of rats in each group. Values are presented as means ± SD. [a,b]Data with the same letters are not statistically different.

density levels of RunX2, OPG, and RANKL, as well as the mean adipocyte diameter, were measured by Image Pro Plus 5.0. Differences between the two groups were analyzed using SPSS version 18.0 one-way ANOVA and Duncan's test. Differences are considered significant when $P < 0.05$.

Results

DZCE increased bone mineral density of ovariectomized diabetic rats

DEXA analysis showed that bone mineral density (BMD) levels in the lumbar spine (L1-L4) and femurs of OVX/T1DM rats were restored by DZCE treatment (OVX/T1DM-DZCE). There were no significant differences in BMD between the OVX/T1DM-DZCE group and control group ($P > 0.05$) (**Figure 1**), suggesting that DZCE can improve BMD in ovariectomized diabetic rats.

DZCE decreased serum glucose and bone turnover markers, but increased serum OPG and RUNX2

As shown in **Table 2**, blood glucose levels, serum bone turnover markers (ALP, CTX-1, osteocalcin, TRACP 5b, PIPN), and serum RANKL in the OVX/T1DM group were significantly higher than those in the control group ($P < 0.01$), which were recovered to normal by DZCE treatment (OVX/T1DM-DZCE). Levels of serum Ca, P, OPG, RUNX2, and OPG/RANKL ratios in the OVX/T1DM group were significantly lower than those in the OVX/T1DM-DZCE and control groups ($P < 0.01$). After 60 days of DZCE treatment, levels of serum glucose, bone turnover markers, calcium (Ca), phosphorus (P), OPG, RANKL, and RUNX2 were returned to normal. There were no significant differences in the above indexes between OVX/T1DM-DZCE and control groups.

DZCE repaired bone morphology and bone histomorphometry parameters

Histological observation showed morphologic changes in femoral trabecular and tibial cortical thickness in ovariectomized diabetic rats. As shown in **Figure 2A1-C1**, the femoral trabecular spacing of the OVX/T1DM group was increased, while the femoral trabeculae were broken (**Figure 2B1**). The femoral bone structure in the OVX/T1DM-DZCE group was returned to normal (**Figure 2C1**). Tibia cortical thickness (Ct.T) was decreased in the OVX/T1DM group (**Figure 2B2**) and it was restored in DZCE treated group (**Figure 2C2**).

After 60 days of DZCE administration, bone histomorphometric parameters, including bone volume per tissue volume (BV/TV, **Figure 3A**), trabecular thickness (Tb.Th, **Figure 3B**), trabecular separation (Tb.Sp, **Figure 3C**), and cortical thickness (Ct.T, **Figure 3D**,) were all returned to normal in the OVX/T1DM group. Results indicate that DZCE could restore estrogen deficiencies and diabetes-induced bone structure disorders in rats.

As shown in **Figure 4A**, osteoclasts are giant cells with multiple nuclei. Compared with the control group, the number of osteoclasts per bone perimeter was increased in OVX/T1DM rats ($P < 0.05$). The number was returned to normal by DZCE treatment for 60 days (**Figure 4B**).

DZCE inhibited bone marrow adipogenesis

Histological observation showed that the number of adipocytes in the bone marrow of OVX/T1DM rats (**Figure 5B**) was higher than that of the control group (**Figure 5A**), while it was significantly decreased by DZCE treatment in the OVX/T1DM-DZCE group (**Figure 5C**). As **Figure 5D, 5E** indicate, the bone marrow adipocyte density and mean adipocyte diameters (μm) of the OVX/T1DM group were significantly incre-

Table 2. Serum glucose, bone turnover markers, Ca, P, OPG, RANKL, and RUNX2 in each experimental group

Parameter	Control	OVX/T1DM	OVX/T1DM-DZCE
Glucose (mg/dl)	87.90 ± 8.23[a]	421.78 ± 31.24[b]	97.87 ± 10.17[a]
ALP (U/dL)	107.45 ± 11.31[a]	193.49 ± 19.54[b]	119.89 ± 13.43[a]
CTX-1 (ng/mL)	26.67 ± 4.21[a]	110.89 ± 15.73[b]	31.34 ± 3.97[a]
Osteocalcin (ng/mL)	18.89 ± 3.01[a]	40.56 ± 6.08[b]	28.67 ± 4.89[c]
TRACP 5b (U/L)	1.90 ± 0.34[a]	3.78 ± 0.83[b]	2.04 ± 0.47[a]
PINP (μg/L)	41.89 ± 5.77[a]	63.09 ± 7.16[b]	45.56 ± 6.42[a]
Ca (mg/dL)	9.85 ± 0.88[a]	4.68 ± 0.65[b]	9.02 ± 0.76[a]
P (mg/dL)	7.97 ± 0.66[a]	3.46 ± 0.58[b]	5.51 ± 0.83[c]
RUNX2 (ng/mL)	10.78 ± 2.01[a]	3.21 ± 0.89[b]	9.73 ± 2.90[a]
OPG (ng/mL)	8.90 ± 2.56[a]	2.34 ± 0.77[b]	8.78 ± 1.97[a]
RANKL (ng/mL)	2.01 ± 0.68[a]	7.33 ± 1.49[b]	2.54 ± 0.49[a]
OPG/RANKL ratio	4.04 ± 0.59[a]	0.43 ± 0.12[b]	3.69 ± 0.53[a]

Values are presented by mean ± SD. Different letters (a, b, c) within rows were used to indicate statistically significance differences ($P < 0.05$).

ased ($P < 0.01$). They returned to normal in the DZCE treatment group (OVX/T1DM-DZCE), indicating that DZCE can inhibit bone marrow adipogenesis.

DZCE increased RUNX2 mRNA expression and OPG/RANKL mRNA ratios in bone tissues of ovariectomized diabetic rats

As shown in **Figure 6**, RUNX2 and OPG mRNA expression was decreased, RANKL mRNA expression was increased, and OPG/RANKL mRNA ratios were decreased in ovariectomized diabetic rats (OVX/T1DM group), compared with the control group ($P < 0.01$). All levels were recovered to normal levels after 60 days of DZCE treatment.

DZCE increased OPG and RUNX2 protein expression in the bone tissues of ovariectomized diabetic rats

Bone immunohistochemistry results showed that the bone protein of OPG and RUNX2 was decreased and RANKL was increased in the OVX/T1DM group, compared with the control group. DZCE supplementation effectively increased OPG and RUNX2 in bone tissues of ovariectomized diabetic rats, decreasing expression levels of RANKL (**Figures 7** and **8**). As shown in **Figure 8**, there were no differences in the positive staining area of OPG, RANKL, and RUNX2 between the OVX/T1DM-DZCE group and control group ($P > 0.05$).

Discussion

Many studies have shown that estrogen deficiencies and T1DM affect bone turnover and bone integrity [31-33]. When menopausal women have T1DM, bone loss increases and bone turnover is accelerated [34]. DZCE has been reported to prevent osteoporosis caused by estrogen deficiencies. It also provides hypoglycemic effects on diabetic rats [18-20]. However, there is currently no evidence that supplementation with DZCE is beneficial for bone loss caused by estrogen deficiencies combined with hyperglycemia. In this study, results indicated that 60 days of DZCE treatment can improve severe bone loss in ovariectomized diabetic rats.

There were decreased BMD levels, increased serum glucose, increased bone turnover markers, increased osteoclasts numbers, and increased bone marrow adipocyte density levels, as well as destroyed bone structure, in ovariectomized diabetic rats in this study. Results indicate that the osteoporosis animal model was successful. After treatment with DZCE for 60 days, these indicators were significantly improved. This suggests that DZCE provides protective effects on bone loss caused by estrogen deficiencies and diabetes in rats.

In the development and research of anti-osteoporosis drugs, bone turnover biomarkers (BTMs) are important indicators in the evaluation of anti-osteoporosis drugs. They reflect bone formation and absorption [34, 35]. BTMs include bone resorption and formation markers [36]. Osteoblasts synthesize markers of bone formation, including osteocalcin, ALP, and PINP, which reflect the body's osteogenic function [37]. Bone formation markers in postmenopausal osteoporosis patients are increased. Thus, it has become a possible predictor of postmenopausal osteoporosis [38, 39]. CTX-I and TRACP 5b are bone resorption markers [40, 41]. TRACP 5b and CTX-I levels have been negatively correlated with female bone mineral

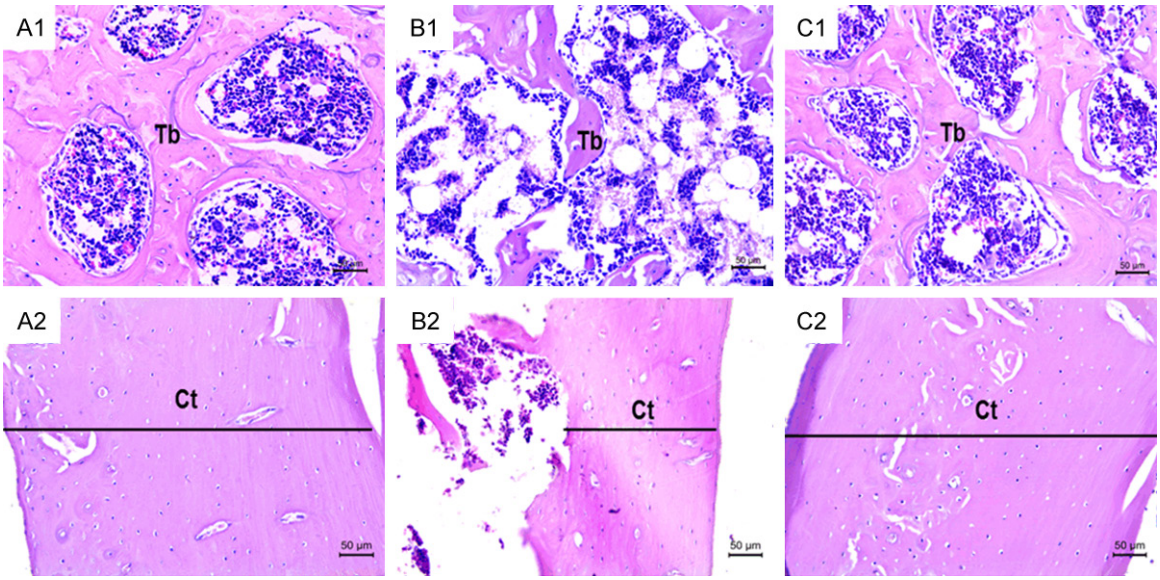

Figure 2. Femoral and tibia morphology of rats in each group. A1. Femur metaphysis in a rat of control group; B1. Femur metaphysis in a rat of OVX/T1DM group; C1. Femur metaphysis in a rat of the OVX/T1DM-DZCE group; A2. Tibia in a rat of control group; B2. Tibia in a rat of OVX/T1DM group; C2. Tibia in a rat of the OVX/T1DM-DZCE group. Hematoxylin and eosin staining, magnification: 200×. Tb. Trabecular bone. Ct: Cortical bone.

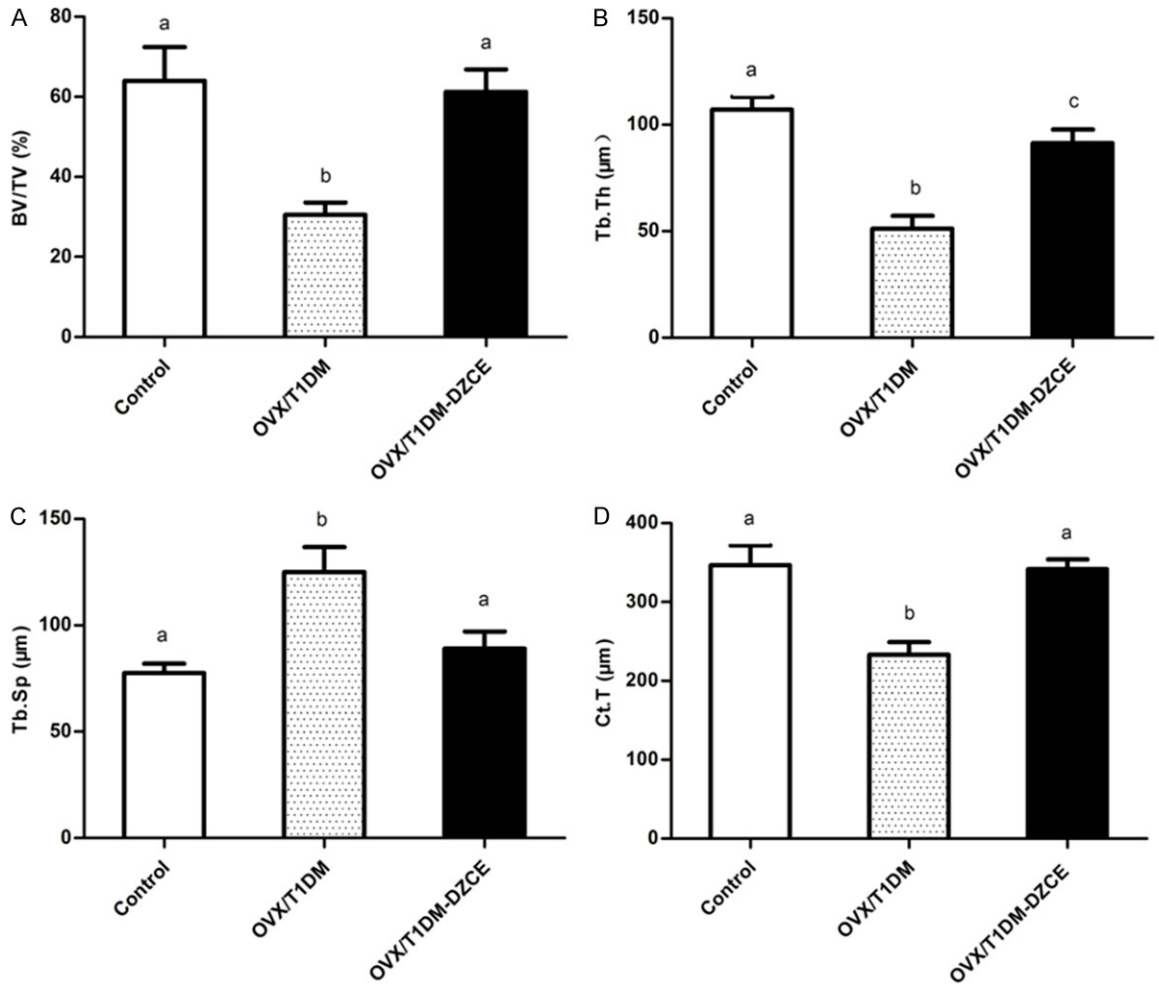

Figure 3. Bone histomorphometric parameters in all experimental groups. A. Bone volume per tissue volume (BV/TV, %); B. Trabecular thickness (Tb.Th, μm); C. Trabecular separation (Tb.Sp, μm); D. Cortical thickness (Ct.T, μm). Values are presented as means ± SD. Different letters indicate statistically significant differences ($P < 0.05$).

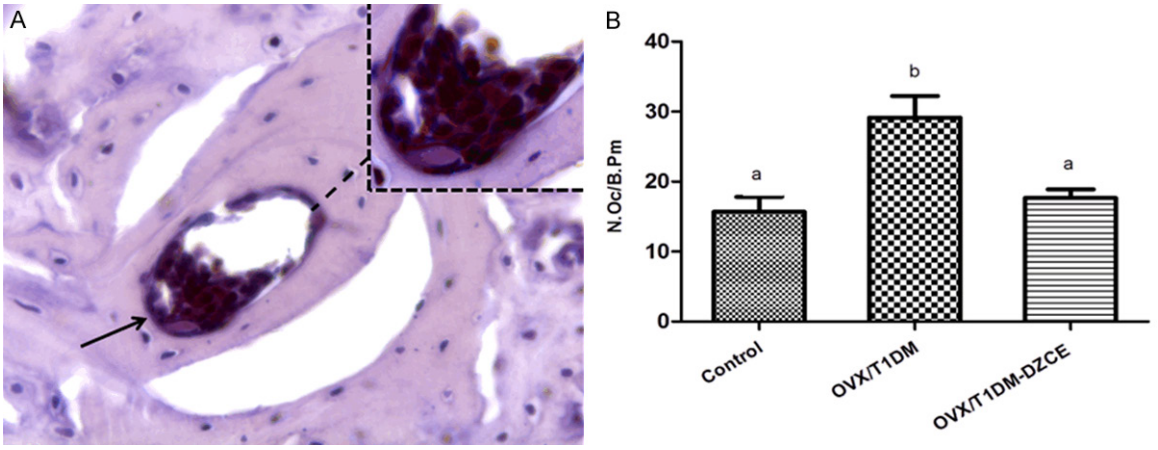

Figure 4. The number of osteoclasts per bone perimeter (N.Oc/B.Pm) in all experimental groups. A. Osteoclast in bone tissue (stained by TRAP), the arrow points to the osteoclast; B. The number of osteoclasts per bone perimeter (N.Oc/B.Pm); Values are presented as means ± SD. Different letters indicate statistically significant differences ($P < 0.05$).

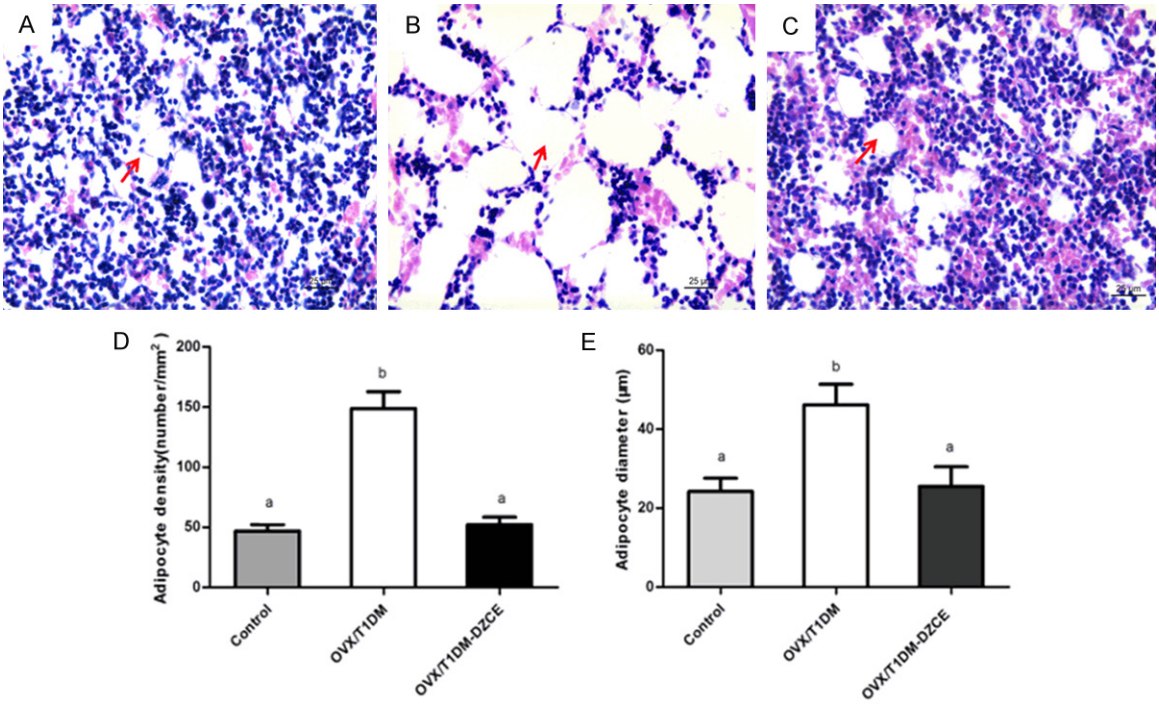

Figure 5. Bone marrow adipogenesis in all experimental groups. A. Tibia bone marrow in the rats of control group; B. Tibia bone marrow in the rats of OVX/T1DM group; C. Tibia bone marrow in the rats of OVX/T1DM-DZCE group; Hematoxylin and eosin staining, magnification: 400×; D. Adipocyte density of tibia bone marrow in each group (n = 10 in each group); E. Mean adipocyte diameter of tibia bone marrow in each group. Values are presented as means ± SD. Different letters indicate statistically significant differences ($P < 0.05$). Red arrows point to adipocytes.

density [42, 43]. In this research, markers of bone formation and bone resorption were significantly increased in ovariectomized diabetic rats, indicating increased bone turnover. The

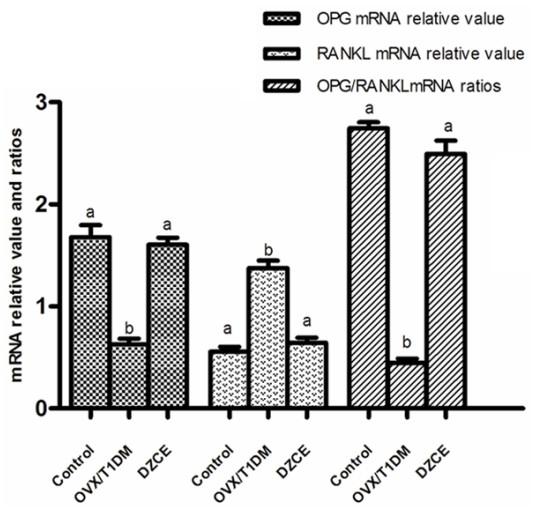

Figure 6. Expression of RUNX2, OPG, and RANKL mRNA, as well as OPG/RANKL mRNA ratios, in bone tissues of all experimental groups. Values are presented as means ± SD. Different letters (a, b) indicate statistically significant differences ($P < 0.05$).

main reason for increased serum bone formation markers in OVX/T1DM rats may be that osteoblasts attempt to compensate for bone loss induced by type 1 diabetes and ovariectomy procedures.

Bone structure parameters are the main evidence of bone degeneration [44]. In this study, the bone structure of the OVX/T1DM group was destroyed and BMD levels were decreased. However, 60 days of treatment with DZCE increased BMD levels, trabecula thickness, and bone volume. It also decreased the number of osteoclasts, adipocyte density levels in bone marrow, and improved the bone tissue structure. Results suggest that DZCE supplementation provides protective effects against bone lose in ovariectomized diabetic rats.

Many studies have reported that bone marrow adipocytes are increased in osteoporosis patients, suggesting them as alternative indicators of osteoporosis [45-47]. In this experiment, the number of bone marrow adipocytes and the average diameter of adipocytes (μm) of ovariectomized diabetic rats were increased. They were restored by DZCE treatment (OVX/T1DM-DZCE). Osteoblasts and adipocytes share common precursor cells [48]. Decreased bone density in OVX/T1DM rats may be responsible for the easier differentiation of precursors into adipocytes rather than osteoblasts. DZCE treatment can effectively reduce bone marrow lipogenesis in OVX/T1DM rats, suggesting this treatment as an important mechanism in prevention bone loss.

Runt-related transcription factor 2 (RUNX2) is a multifunctional transcription factor that regulates osteoblast differentiation by regulating gene expression of extracellular matrix proteins [49]. In this study, expression of RUNX2 in serum and bone tissue of OVX/T1DM rats was decreased, suggesting osteogenic dysfunction. Levels were recovered after 60 days of DZCE treatment, indicating that DZCE can promote osteogenesis by upregulating RUNX2. Osteoprotegerin (OPG) and receptor activator of nuclear factor-κB ligand (RANKL) are key factors mediating osteoclast differentiation. OPG/RANKL axis plays an important role in bone neo-synthesis and remodeling [50-52]. A decreased OPG/RANKL ratio is indicative of increased osteolysis [53, 54]. In this study, oral DZCE administration for 60 days increased OPG and OPG/RANKL ratios and decreased RANKL levels in serum and bone tissues of OVX/T1DM-DZCE rats. These findings suggest that DZCE prevents type 1 diabetes and ovariectomy-induced bone loss by upregulation of RUNX2 expression and OPG/RANKL ratios.

Conclusion

Present results suggest that oral DZCE could prevent osteoporosis caused by estrogen deficiencies and diabetes. The protective mechanisms of DZCE against ovariectomy- and diabetes-induced bone loss are that DZCE inhibited bone turnover, inhibited bone marrow adipogenesis, and upregulated OPG/RANKL expression ratios. Therefore, Du-Zhong cortex extract may be a potential drug or functional food for treatment of osteoporosis in postmenopausal diabetic women.

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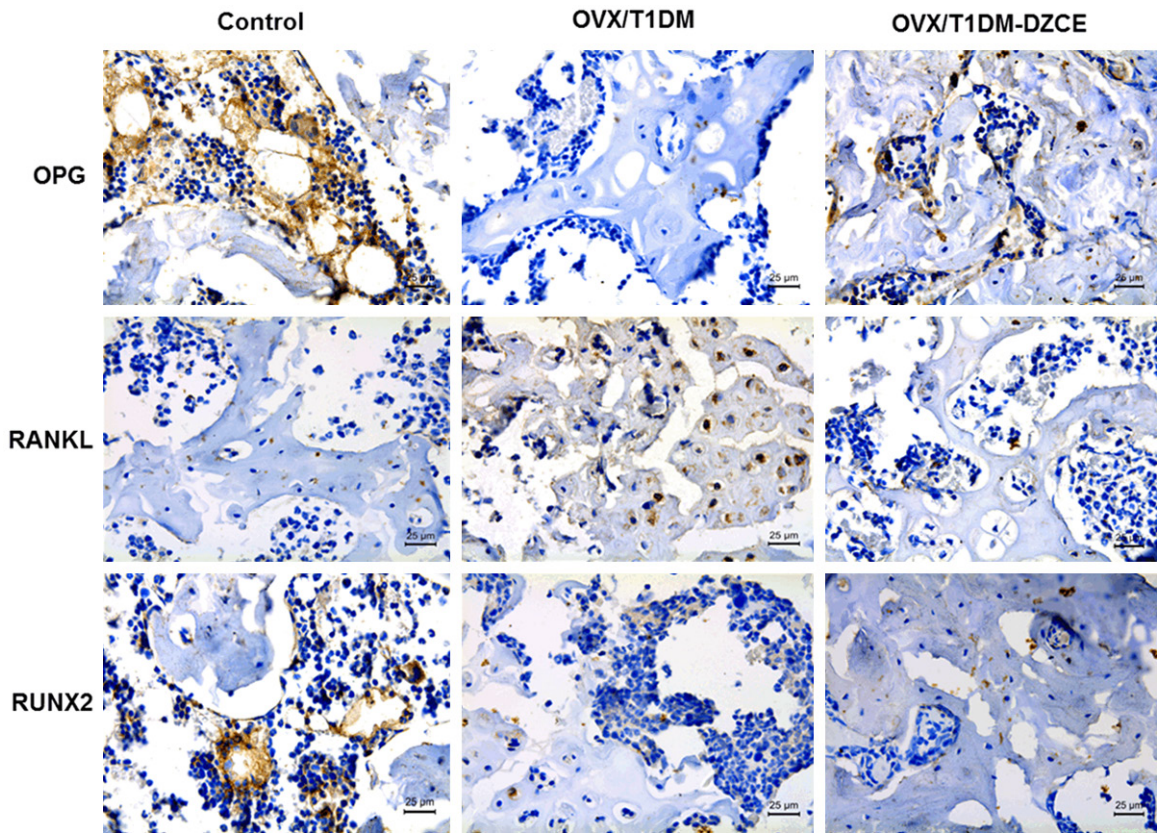

Figure 7. Expression of OPG, RANKL, and RUNX2 protein in the femoral bone tissues of each group. Immunohistochemical staining, the cells with positive expression of OPG, RANKL, and RUNX2 are shown in brown. Magnification: 400×.

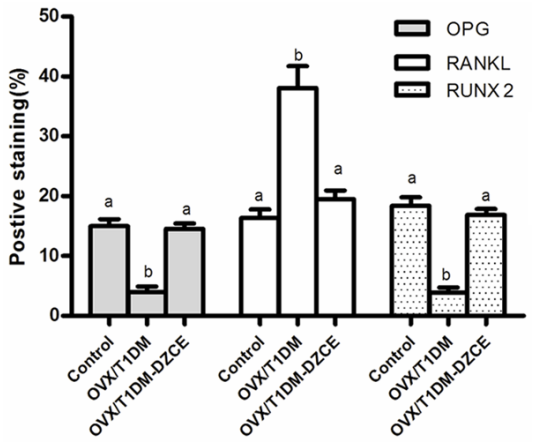

Figure 8. Percentage (%) of the positive staining area of OPG, RANKL, and RUNX2 in the femoral bone tissues of each group. Values are presented as means ± SD. Different letters (a, b) indicate statistically significant differences ($P < 0.05$).

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Disclosure of conflict of interest

None.

Address correspondence to: Chen Chen, Hongxing Zheng and Zhijian Zhang, College of Biological Science and Engineering, Shaanxi University of Technology, Chaoyang Road, Hantai District, Hanzhong 723000, Shaanxi, China. E-mail: cchen2008@yahoo.com (CC); zhenghongxing100@126.com (HXZ); zhangzhijian@snut.edu.cn (ZJZ)

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