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Protective effect of aqueous extracts from *Taxillus chinensis* (DC.) Danser on ovariectomy-induced osteoporosis in rats

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Abstract

As one of classic Chinese kidney-tonifying herbal medicines, *Taxillus chinensis* (DC.) Danser is widely applied in Chinese folk medicine as herbal tea for nutraceutical purposes including nourishing kidney, strengthening muscle and bone to against weakness, *etc*. In the present study, we investigated the protective effect of aqueous extracts from *Taxillus chinensis* (AETCs) on the ovariectomy (OVX)-induced osteoporosis in rats. Results showed that AETC treatment at the dose of 12.0 g raw materials/kg for 12 weeks significantly enhanced the bone mineral densities of femur and tibia, improved the trabecular microarchitecture compared with OVX controls. The serum levels of osteogenic markers alkaline phosphatase and osteocalcin were increased, and the levels of bone resorption biomarkers tartrate-resistant acid phosphatase 5b and telopeptides of collagen type I were suppressed by AETCs. Moreover, 12.0 g raw materials/kg AETC treatment significantly reduced the OVX-induced up-regulation of cyclooxygenase (COX)-2 mRNA and protein expression in bone tissues. Data suggested that AETCs showed anti-osteoporotic effect in OVX rats, possibly *via* promoting bone formation and inhibiting bone absorption. The down-regulation of inflammatory COX-2 might be involved in the protection of AETCs against OVX –induced osteoporosis.

Keywords: *Taxillus chinensis* (DC.) Danser; aqueous extracts; osteoporosis; bone mineral density; COX-2.

Practical Application: This study provided *in vivo* experimental evidence supporting the application of *Taxillus chinensis* (DC.) Danser in Chinese folk as herbal tea for nutraceutical purpose of strengthening muscle and bone.

1 Introduction

As a common orthopedic disorder being highly prevalent among the elderly, in particular, postmenopausal women, osteoporosis (OP) is defined as a systemic skeletal disease resulting from a steady-state metabolic imbalance manifested as excessive bone resorption and/or decreased bone formation during remodelling, which leads to reduction in bone strength, degradation of bone microstructure and increased susceptibility to fracture (Li et al., 2021a; Zhu et al., 2021). Several pharmacological agents such as bisphosphonates and hormone replacement therapy are currently the treatment of choice for OP. However, the concerns remain about their efficacy, cost-effectiveness, the potential serious adverse effects on endocrine and cardiovascular systems, as well as the tendency of high-risk groups for noncompliance, which severely limit the clinical use of these therapies (Li et al., 2021a; Zhu et al., 2021). Considering annually increasing incidence of OP with the aging of global population, as well as loss of health, societal social burdens and economic consequences caused by OP, it is necessary to explore new alternative anti- osteoporosis therapies possessing therapeutic efficacy, safety and economy (Zhu et al., 2021).

Traditional Chinese Medicine (TCM) approaches have been demonstrated to elicit curative effects toward OP (Zhu et al., 2021; Zhao et al., 2021; Lin et al., 2017; Yang et al., 2018). According to the TCM principle that the healthy bone metabolism is supported by a kidney "essence", the condition congruent with OP mainly results from the deficiency of this essence. Accordingly, the kidney-tonifying prescription is essential in TCM therapy for OP (Yang et al., 2018; Deng et al., 2012). As a semi-parasitic plant that grows on host tree and shrubs, *Taxillus chinensis* (DC.) Danser is one of classic Chinese kidney-tonifying herbal medicines. The leaves and branches of *Taxillus chinensis* (DC.) Danser are called as *Taxilli* Herba or loranthus (Sangjisheng in Chinese and Sang-gi-saeng in Korean) (Noh et al., 2021; Wei et al., 2020). Up to date, *Taxillus chinensis* (DC.) Danser is still officially recorded in the current edition of the Chinese Pharmacopoeia, and is widely used in TCM clinics in China. In this study, we attempted to determine whether the aqueous extracts from *Taxillus chinensis* (AETCs) have the protective effect on ovariectomy (OVX)-induced OP in rats.

2 Materials and methods

2.1 Materials

Commercial plant materials of *Taxillus chinensis* (DC.) Danser were supplied by the Beijing Tongrentang Co., Ltd. China, and were authenticated by Dr. Song Liu who is an expert in

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pharmacognosy. The dried materials (1000 g) were crashed then extracted with boiling water (w/v = 1:10) for 2-3 h. The extracted liquid was filtrated then concentrated to 100 mL volume using a rotary evaporator. The concentration of obtained AETCs was equivalent to 10 g original weight per ml.

2.2 Animal model and treatment protocol

Sprague-Dawley rats (female, 3 months old, 200-220 g) were purchased from Hubei Experimental Animal Research Center (Wuhan, China). After a 1-week acclimation period, the animals underwent either bilateral laparotomy (Sham group, n = 10) or bilateral ovariectomy (OVX group, n = 30) as previously described (Xiao et al., 2021). Then the OVX animals were randomly divided into 3 groups of 10 rats in each group: OVX control, OVX with low dose AETC (6.0 g raw materials/kg)–treated group and high dose AETC (12.0 g raw materials/kg)–treated group. Rats in the Sham and OVX control groups were administered with the same volume of normal saline. Vehicle and AETCs were administered orally through an oral gavage once per day. The treatments started at 3 days after surgery and lasted for 12 weeks. At 1 h after the last administration of AETCs, the rats were sacrificed after collection of whole blood by cardiac puncture. The animal experimental protocol was performed strictly in accordance with the procedures approved by the Animal Ethic Committee of the Wuhan University of Science and Technology.

2.3 Analysis of Bone Mineral Density (BMD)

The BMDs of the left femurs and tibia isolated from adjacent tissues were determined by a dual-energy X-ray absorptiometer (DEXA; XR-36; Norland, Fort Atkinson, WI) using a mode for small subjects as described previously (Li et al., 2012).

2.4 Histopathological analysis

After being fixed in formaldehyde sodium phosphate for 24 to 48 h, the right proximal femurs were cut longitudinally into 6 μm-thick sections using a microtome (Polycut E, Leica, Nussloch, Germany) then stained with hematoxylin and eosin (H&E).

2.5 Assessment of bone turnover markers in serum

Blood samples collected by cardiac puncture were allowed to clot for 30 min, then centrifugated at 1500 g for 10 min to separate serum. The levels of biomarkers for bone formation, including alkaline phosphatase (ALP) and osteocalcin (OC), in serum were respectively estimated using ALP Activity Assay Kits (Nanjing Jiancheng Bioengineering Institute, Nanjing, China) and Rat Osteocalcin EIA Kit (Biomedical Technologies, Stoughton, MA) according to the manufacturers' instructions. Serum levels of biomarkers for bone resorption, including tartrate-resistant acid phosphatase 5b (TRACP5b) and telopeptides of collagen type I (CTx), were evaluated using commercial ELISA kits (TRACP5b: Westang, Shanghai, China; CTx: Immunodiagnostic Systems Ltd., Boldon, UK).

2.6 Real-time PCR

The expression levels of cyclooxygenase (COX)-2 mRNA in the lumbar (L1-L3) tissues were measured by real-time PCR. Total RNA isolated from lumbar bone tissues was extracted using the TRIzol reagent (Invitrogen, Carlsbad, CA, USA). The primer set sequences of COX-2 and β-actin were 5'-ACCGTGGTGAAT GTATGAGCATAGGA-3' (forward) and 5'-TCAGGTGTTGCACGTAGTC TTCGAT-3'(reverse), and 5'- TGTTTGAGACCTTCAACACCCC-3'(forward) and 5'-ACGTCACACTTCATGATGGAA -3' (reverse), respectively. The relative amount of COX-2 mRNA was normalized to the amount of β-actin as a reference in the same sample.

2.7 Western blotting analysis

The protein was extracted from lumbar bone tissues using ProteoJET™ Mammalian Cell Lysis Reagent (MBI fermentas, Canada), separated by 10% SDS-PAGE, and then transferred onto a nitrocellulose membrane by electroblotting. The membrane was incubated with goat polyclonal COX-2 or β-actin antibody (Santa Cruz, USA). The relative protein expression levels of COX-2 were corrected by the amount of β-actin as a reference in the same sample.

2.8 Statistical analysis

All data were expressed as the mean ± SD, and analyzed using one-way analysis of variance (ANOVA) with post hoc Tukey test by SPSS 22.0 software. P value of less than 0.05 was considered statistically significant.

3 Results and discussion

3.1 AETCs prevented bone loss of OVX rats

BMD is believed as the gold standard for the evaluation of individuals at risk for OP (Shevroja et al., 2021; Akkawi & Zmerly, 2018). As shown in Figure 1A, rats treated with AETCs at the dose of 12.0 g raw materials/kg for 12 weeks exhibited significantly higher femur BMD (increased by 23.6%) and tibia BMD (increased by 26.0%) compared with OVX controls ($P < 0.01$), suggesting a protective effect against the BMD loss of OVX-induced OP rats. The improvement of BMD in AETC-treated OVX animals was further supported by H&E staining of femur sections (Figure 1B). Alteration of trabecular architecture is considered to be responsible for decreased bone strength in postmenopausal OP, and is a good predictor of OVX-induced bone loss and bone quality deterioration (Li et al., 2021b). Histopathological analysis of femur specimens showed the trabecular bone density and microarchitecture were markedly improved in AETC-treated rats compared with the vehicle-treated OVX rats.

In Chinese folk, *Taxillus chinensis* (DC.) Danser as a bitter, sweet and non-toxic traditional medicine (Shen et al., 2021) is widely applied as herbal tea for nutraceutical purposes including nourishing kidney, strengthening muscle and bone to against weakness, *etc*. In this study, we provided *in vivo* experimental evidence supporting the clinical application of *Taxillus chinensis* (DC.) Danser for OP prevention and control.

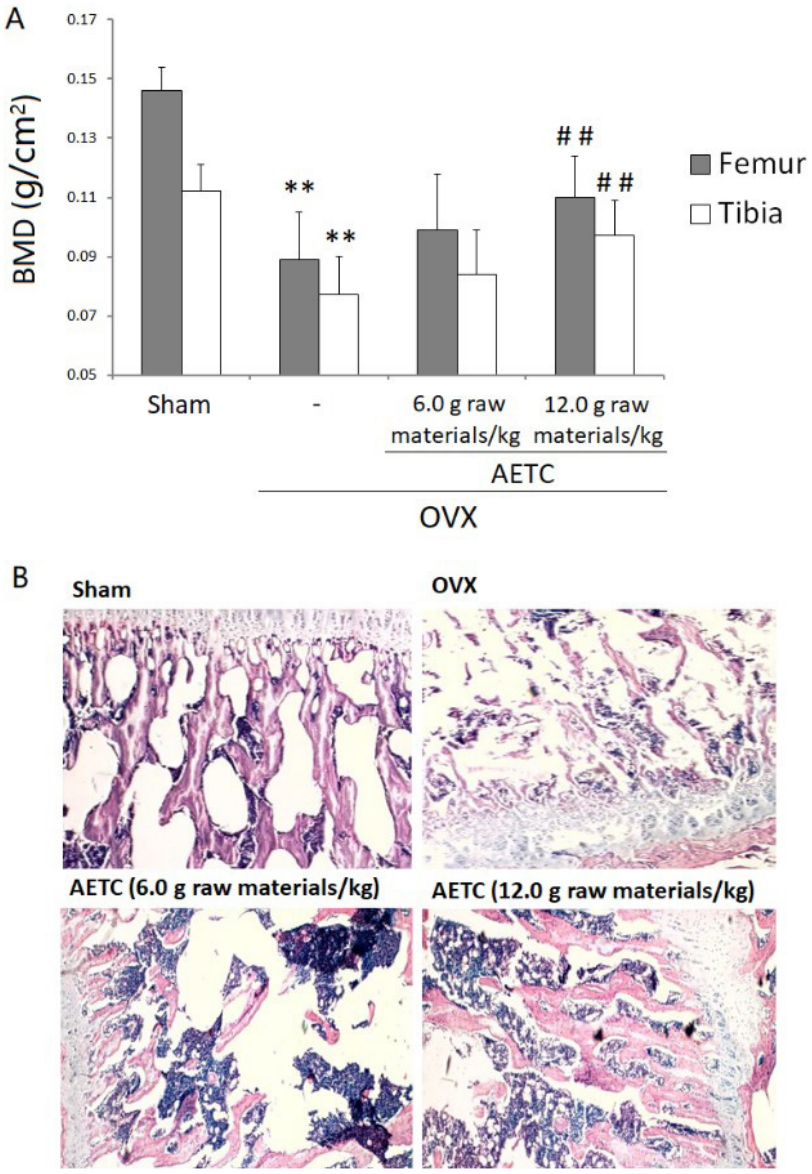

Figure 1. Effect of AETCs on OVX-induced bone loss in rats after 12 weeks treatment. A: BMDs of the left femurs and tibias measured by dual-energy X-ray absorptiometry. Data are the mean ± SD (n = 10). ** $P < 0.01$ *vs.* Sham group; ## $P < 0.01$ *vs.* OVX group. B: Bone architecture of the proximal femurs measured by H&E staining.

3.2 AETCs promoted bone formation and inhibited bone resorption

As a metabolic bone disease, OP has been widely accepted to result from the imbalance of bone build-up (formation) and break down (resorption) (Shi et al., 2022; Bai et al., 2019; Hardy & Fernandez-Patron, 2020). To determine whether AETCs exerted an anti-osteoporotic activity by influencing the bone metabolism, serum levels of bone turn over markers including ALP, OC, TRACP-5b, and CTX were measured in OVX rats.

These bone turnover biomarkers are produced from the bone remodeling process (Akkawi & Zmerly, 2018). Among them, ALP is associated with bone metabolism and differentiation of osteoblasts; OC acts as one of the very few molecules exclusively produced by osteoblasts. The activities of these two biomarkers are the most common indicators of osteoblast differentiation and osteogenic properties (Akkawi & Zmerly, 2018; Noh et al., 2020). On the other hand, TRACP 5b and CTx are well known major markers for bone resorption. CTx is a bone related degradation product from type I collagen, the latter of which accounts for more than 90% of the organic matrix of bone and is degraded and released into circulation during renewal of the skeleton (Bai et al., 2019). Serum TRACP 5b is secreted mainly by osteoclasts, therefore, its activity is widely used as an reliable indicator of osteoclast activity and number with high clinical sensitivity (Mori et al., 2018).

As shown in Figure 2, in line with the characteristic of high bone metabolism in OP of OVX rats and postmenopausal women

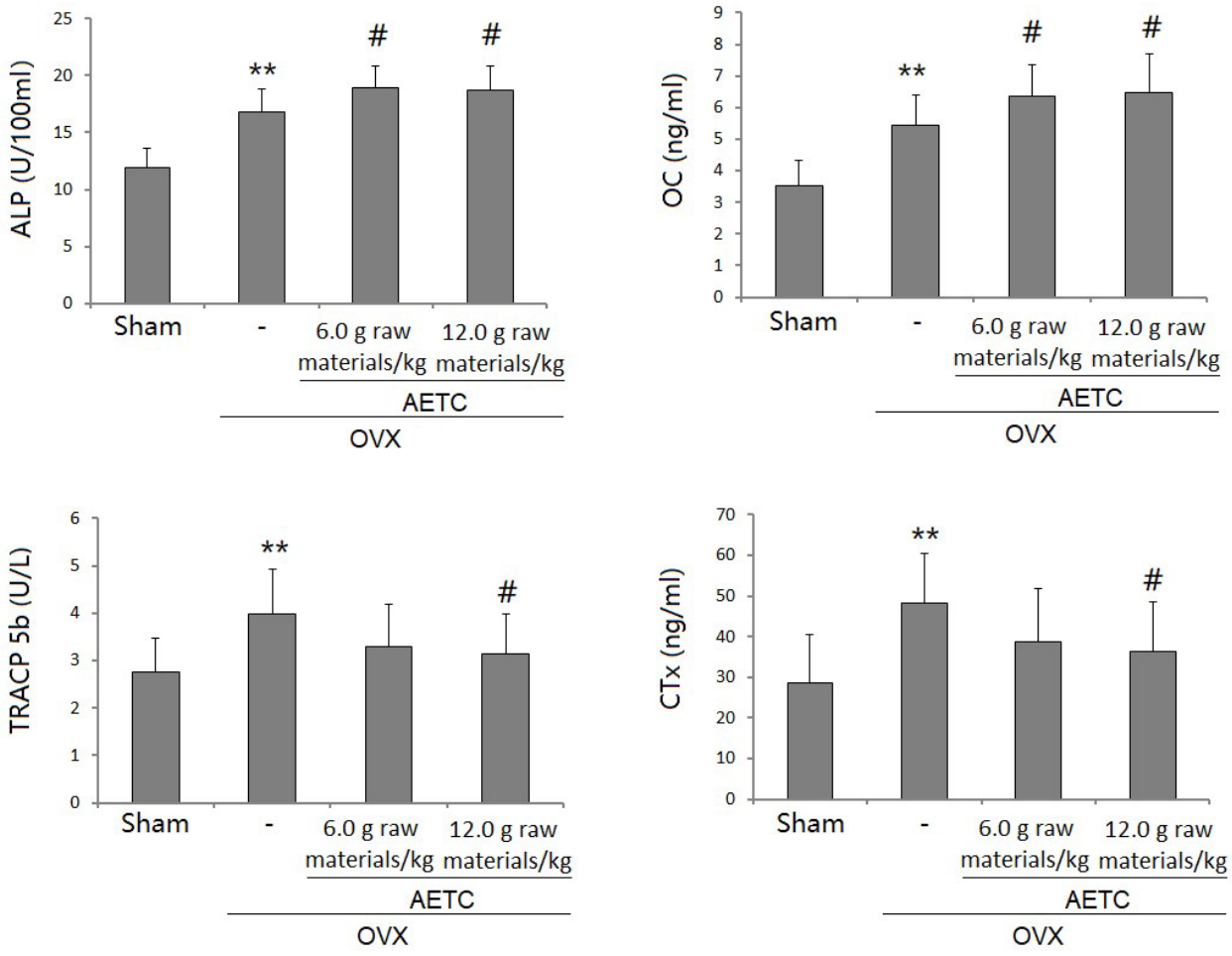

Figure 2. Effect of AETCs on the serum levels of bone turnover markers including ALP, OC, TRACP 5b, and CTx in OVX rats. Data are the mean ± SD (n = 10). ** $P < 0.01$ *vs.* Sham group; # $P < 0.05$ *vs.* OVX group.

(Akkawi & Zmerly, 2018), serum levels of bone turnover markers including ALP, OC, TRACP 5b, and CTx were increased in the OVX group compared to those in the Sham group ($P < 0.01$). However, low- and high-dose AETC treatment for 12 weeks further enhanced the levels of osteogenic markers ALP and OC compared to the OVX group ($P < 0.05$); while the high dose of AETCs significantly suppressed the OVX-induced increases of markers for bone resorption (TRACP 5b and CTx) in serum ($P < 0.05$). These date suggested that AETCs might exert their anti-osteoporosis activity *via* promoting bone formation and inhibiting bone absorption, thus contributing to the bone recovery.

3.3 AETC inhibited the OVX-induced COX-2 up-regulation in bone tissues

Then, we sought to explore the possible anti-osteoporotic mechanism of AETCs in OVX-induced OP rats. Up to date, accumulative evidences (Fang et al., 2020; Al-Daghri et al., 2017) have shown a close link between inflammation and OP. Especially for postmenopausal OP, systematic inflammation activation due to estrogen deficiency has been considered as a driving force for OP development. Accordingly, anti-inflammatory agents have been believed as a valuable tool for prevention and control of postmenopausal OP (Fang et al., 2020; Al-Daghri et al., 2017). Interestingly, in TCM, *Taxillus chinensis* (DC.) Danser has been commonly applied to treat arthritis and other inflammatory disorders (Shen et al., 2021). The anti-inflammatory activity of the *Taxillus chinensis* (DC.) Danser extracts has been demonstrated in lipopolysaccharide and interferon -γ activated RAW 264.7 macrophages (Zhang et al., 2011).

As a key factor in inflammation, COX-2 is an inducible enzyme expressed during inflammation that is responsible for the production of pro-inflammatory mediators. It has been demonstrated that COX-2-related inflammation is responsible to the osteoclastic bone resorption (Xie et al., 2021; Nadia et al., 2012). The efficacy of selective COX-2 inhibition for bone loss prevention has been found in estrogen deficiency animals (Kasukawa et al., 2007). Clinical studies have also showed that regular use of relative COX-2 selective nonsteroidal anti-inflammatory drugs was associated with higher BMD at multiple skeletal sites in men and women (Ahmad et al., 2020; Carbone et al., 2003). Especially, inhibition of the COX-2 enzyme

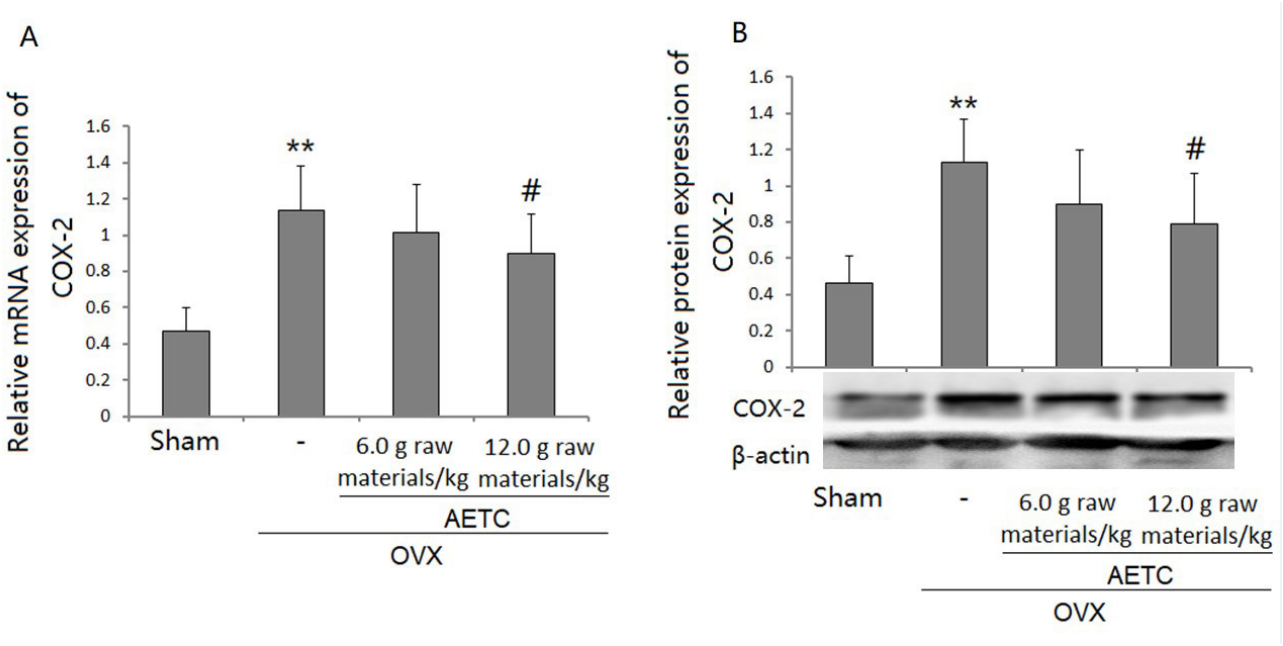

Figure 3. Effect of AETCs on COX-2 expression in bone tissues of OVX-induced OP rats. A: The mRNA expression of COX-2 detected using Real-time PCR; B: The protein expression of COX-2 using Western blotting. Data are the mean ± SD (n = 10). ** $P < 0.01$ *vs.* Sham group; # $P < 0.05$ *vs.* OVX group.

in postmenopausal women may prevent menopausal bone loss (Richards et al., 2006).

Considering the potential anti-inflammatory activity of AETCs and the role of COX-2 in OP development, we focused on the effect of AETCs on the COX-2 expression in bone tissues of OVX-induced OP rats. As shown in Figure 3, AETC treatment at dose of 12.0 g raw materials/kg significantly reduced the OVX-induced COX-2 up-regulation in bone tissues ($P < 0.05$). Compared to the OVX control group, the mRNA and protein expression of COX-2 in the high-dose AETC group was declined by 21.2 and 30.2%, respectively. This finding suggested the possible involvement of COX-2 down-regulation in the anti-osteoporotic effect of AETCs in OVX rats. However, further researches are needed to confirm whether and how such a mechanism underlies the potential contribution of AETCs to OP control.

4 Conclusions

In the present study, we reported the anti-osteoporotic effect of AETCs in OVX rats for the first time. The mechanism of this effect might in part be attributed to the bone turnover-modulating activity of AETCs through promoting bone formation and suppressing bone resorption. Importantly, the down-regulation of inflammatory COX-2 might be involved in the protection of AETCs against OVX –induced OP.

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