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Calycosin attenuates osteoporosis and regulates the expression of OPG/RANKL in ovariectomized rats *via* MAPK signaling

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We aimed at exploring the effect of calycosin (CA) on osteoporosis in ovariectomized (OVX) rats. Sprague-Dawley (SD) rats were divided into five groups: Sham group, OVX group, OVX group treated with estradiol valerate (EV), CAL group treated with 15 mg/kg/d of CA and CAH group treated with 30 mg/kg/d of CA for 12 weeks. Bone mineral density (BMD), histopathology, body weight, parameters in serum and urine were observed. Gene expression and protein level of OPG/RANKL were also studied by real-time PCR and western blot, respectively. We further identified the effect of CA on mitogen-activated protein kinase (MAPK) signaling. In comparison with OVX rats, CAL and CAH significantly increased the BMD by 8.3% and 19.0%. Treatment with CA notably inhibited the excretion of Ca, P and Cr. CAH also significantly increased the level of alkaline phosphatase (ALP) and decreased the level of tartrate-resistant acid phosphatase (TRAP) in serum of OVX rats. CA could improve the trabecular bone area, and increased the trabecular number and the trabecular connection after 12-week. CA also increased the expression of osteoprotegerin (OPG) and decreased the Receptor Activator for Nuclear Factor-κB Ligand (RANKL) mRNA expression compared with the OVX rats. In addition, CA could effectively decrease the phosphorylation of MAPKs induced by ovariectomy. In conclusion, CA had remarkable antiosteoporotic activity and therefore can be a promising candidate for the treatment of postmenopausal osteoporosis.

1. Introduction

Osteoporosis is a chronic, progressive disease of the skeleton characterized by bone fragility due to a reduction in bone mass and possibly alteration in bone architecture which leads to a propensity to fracture with minimum trauma (Aguilar et al. 2015; Gunawardene et al. 2015; Horikawa et al. 2015). As the osteoporosis typically reflects an imbalance between bone resorption and bone formation, the current therapies and agents for osteoporosis thus either inhibit bone resorption or stimulate bone formation, with efficient recovery of bone mass and bone metabolism in osteoporosis. It is reported that estrogen regulates the differentiation of mesenchymal stromal cells (MSCs) via mitogen-activated protein kinase (MAPK) signaling. The MAPK system is composed of extracellular signal regulated kinases (ERK), c-Jun N-terminal kinase (JNK) and p38 MAP kinase. Loss of estrogen results in stagnant period of MSCs differentiating into osteoblasts, accelerated differentiation of MSCs to osteoclast and enhanced bone absorption. Hormone replacement therapy is one of the most effective treatments for the prevention of bone resorption (Krantz et al. 2015; Mak 2015; Weaver et al. 2015). Receptor activator of nuclear factor-κB ligand (RANKL) and its receptor RANK are components in a signaling pathway that is essential for osteoclast differentiation, activation, and survival. Osteoprotegerin (OPG) is a decoy receptor for RANKL with a high affinity and competes with RANK for RANKL binding and thus functions as an inhibitor of RANK-RANKL interaction and inhibits osteoclast maturation and activation. At present, the chemical drugs, which are clinically used as effective medications, are associated with long treatment time and numerous side effects (Sadat-Ali et al. 2015). Therefore, the extracts, effective fractions and compounds of some natural plants with few side effects attracted our attention. These plants, containing flavonoids, steroidal compounds, phenylpropanoids and alkaloids, belong to the family Leguminosae or had the effect of strengthening the sexual behavior, and have been proved effective in anti-osteoporosis.

Calycosin is the main compound isolated from Radix astragali (Huangqi in Chinese), one of the most widely used traditional Chinese medicines in China. Huangqi has been widely used to tonify Qi (Di Cesare et al. 2015; Wu et al. 2015), and it is reported that Huangqi exhibited beneficial effects on postmenopausal women who suffer from osteoporosis (Yang et al. 2007; Cao et al. 2014; Guo et al. 2015). Total flavones of astragalus extract increased bone mineral density (BMD) of ovariectomized rats (Chen et al. 2005). The effect of CA, which is one of the flavones in Radix Astragali, on osteoporosis still remains unclear.

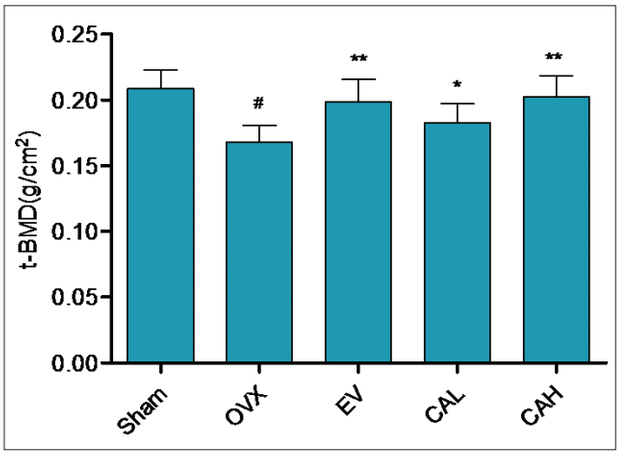

Fig. 1: Effects of CA and EV on the total BMD of ovariectomized rat (n = 8). All values are presented as mean ± SD, n = 8, $^{\#}P < 0.05$ compared to that of sham group rats; $^{*}P < 0.05$, $^{**}P < 0.01$ compared to that of OVX rats.

In this study, we attempt to identify the effect of CA on ovariectomized rats and the possible mechanisms involved. We established a rat model of osteoporosis to explore the effect of CA on the body weight, BMD, biochemical parameters, histopathological analysis, OPG/RANKL expression and MAPK signaling.

Table 1: Effects of CA on body weight of rats (n = 8)

Groups	Initial	Finally	Difference
Sham	289.2 ± 8.5	329.3 ± 14.5	40.1 ± 12.9
OVX	294.6 ± 10.3	365.4 ± 19.5$^{\#}$	70.8 ± 16.5$^{\#\#}$
EV	301 .2± 14.9	335.7 ± 21.6**	34.5 ± 18.4**
CAL	291.3 ± 9.5	359.7 ± 28.3	68.4 ± 15.1
CAH	302.3 ± 11.3	354.6 ± 15.6	52.3 ± 12.7

All values are expressed as mean ± SD, $^{\#\#}P< 0.01$ compared with Sham, $^{*}P < 0.05$ $^{**}P < 0.01$ compared with OVX.

2. Investigations and results

2.1. Body weight

The body weight of rats in each group was recorded weekly. As shown in Table 1, a gradual increase in body weight of the rats of each group throughout the experiment was observed. At the end of the experiment, the body weight gain of each group was as follows: 40.1±12.9 g for sham group, 70.8±16.5 g for OVX group, 34.5±18.4 g for EV group, 68.4±15.1 g for CAL group and 52.3±12.7 g for CAH group. Body weight gain in the OVX group was significantly higher than in the sham group ($P < 0.01$). However, the body weight gain decreased in the EV ($P < 0.01$) group with statistical difference from that in the OVX group.

2.2. Effects of CA on the bone mineral density (BMD)

As BMD is an important marker of bone quality reflecting the degree of osteoporosis, we further examined the BMD of rats in the five groups by dual-energy X-ray absorptiometry. The total BMD of OVX rats was 0.168±0.015 g/cm^2 and was significantly lower than that of sham group rats (0.208±0.014 g/cm^2) ($P < 0.05$), indicating that ovariectomy decreased the BMD of rats by 19.8% after 12 weeks. CAL and CAH increased the BMD of rats by 8.3% ($P < 0.05$) and 19.0% ($P < 0.05$) (Fig. 1), respectively, compared to that of OVX rats. In addition, administration of EV also enhanced the BMD by 18.8% compared to that of OVX rats.

2.3. Effects of CA on serum biochemical parameters

Ca, P and Cr in urine reveals the degree of calcium loss in bone. The effects of CA on the levels of Ca, P and Cr in serum of rats are shown in Table 2. 12 weeks after the operation, OVX rats exhibited significantly ($P < 0.05$) increased urinary excretion levels of Ca, P and Cr by 115.6%, 38.5% and 85.7% respectively, compared to sham group rats. In addition, treatment with CA and EV inhibited the urinary excretion of Ca, P and Cr. In addition, no obvious changes were observed among all the groups in the levels of serum Ca, Cr and P levels.

2.4. Effects of CA on the bone formation and resorption markers

Serum alkaline phosphatase (ALP) is recognized as one of bone formation markers, which was measured on an automatic analyzer. Although an increasing trend of ALP activity was indicated in CAH group compared to the sham group rats, no statistically significant changes of ALP activity were observed in all treated groups (Fig. 2A). As shown in Fig. 2B, ovariectomy led to a significant increase of TRAP (a bone resorption marker) in the OVX rats. However, CAH and EV could significantly suppress the increase of TRAP in OVX rats ($P < 0.05$).

2.5. Histopathological examination

As shown in Fig. 3, there was apparent reduction of trabecular bone area, trabecular number and trabecular connection in OVX rats, and OVX resulted in the expansion of marrow cavity compared to that of sham group rats. However, CAL, CAH and EV treatment could partially reverse this effect of OVX.

2.6. Effects of CA on OPG and RANKL expression

OPG and RANKL play an important role in osteoclast cell differentiation and bone resorption. Therefore, we further examined the mRNA and protein expression of OPG and RANKL expression in rats by RT-PCR. As shown in Figs. 4A and 4B, the mRNA expression of OPG in OVX rats was significantly decreased, while RANKL mRNA expression in OVX rats was increased remarkably in comparison with that of sham group rats. CAL, CAH and EV treatment could effectively increase the OPG mRNA expression and decreased the RANKL mRNA expression compared to that of OVX rats. In Figs. 4C and 4D, protein expression of OPG was decreased and the RANKL expression was increased in the OVX rats. CA and EV could effectively increased the OPG expression and decreased the RANKL expression with statistical significance.

Table 2: Effects of CA on urine and serum P, Ca and Cr content of rats (n = 8)

Group	Urine			Serum		
	Ca (mmol/ml)	P (mmol/ml)	Cr (mmol/ml)	Ca (mmol/ml)	P (mmol/ml)	Cr (mmol/ml)
Sham	0.032 ± 0.006	0.366 ± 0.018	35.11 ± 4.82	2.52 ± 0.55	2.55 ± 0.34	2.21 ± 0.34
OVX	0.079 ± 0.008$^{\#\#}$	0.507 ± 0.024$^{\#\#}$	65.42 ± 7.31$^{\#\#}$	2.35 ± 0.69	2.72 ± 0.60	2.08 ± 0.60
EV	0.416 ± 0.006**	0.395 ± 0.018**	40.75 ± 5.22**	2.44 ± 0.81	2.41 ± 0.47	2.17 ± 0.35
CAL	0.063 ± 0.008**	0.458 ± 0.023*	54.41 ± 7.85*	2.31 ± 0.74	2.67 ± 0.90	2.24 ± 0.90
CAH	0.052 ± 0.007**	0.426 ± 0.023**	47.84 ± 7.60**	2.30 ± 0.45	2.71 ± 0.65	2.37 ± 0.68

All values are expressed as mean ± SD, $^{\#\#}P< 0.01$ compared with Sham, $^{*}P < 0.05$ $^{**}P < 0.01$ compared with OVX.

2.7. Effects of CA on the phosphorylation of JNK, ERK and p38 MAPKs

To identify the effect of CA on the MAPK signaling, we detect the phosphorylation of p38, ERK and JNK by western blots. As shown in Fig. 6, the phosphorylation of p38, ERK and JNK was increased significantly in the OVX rats compared with the control rats. The phosphorylation of p38, ERK and JNK in OVX rats treated with CAL, CAH and EV was obviously decreased compared with that of the OVX rats.

3. Discussion

Huangqi characterized by the property of tonifying Qi is widely used as a medicinal plant in nourishing and strengthening life in

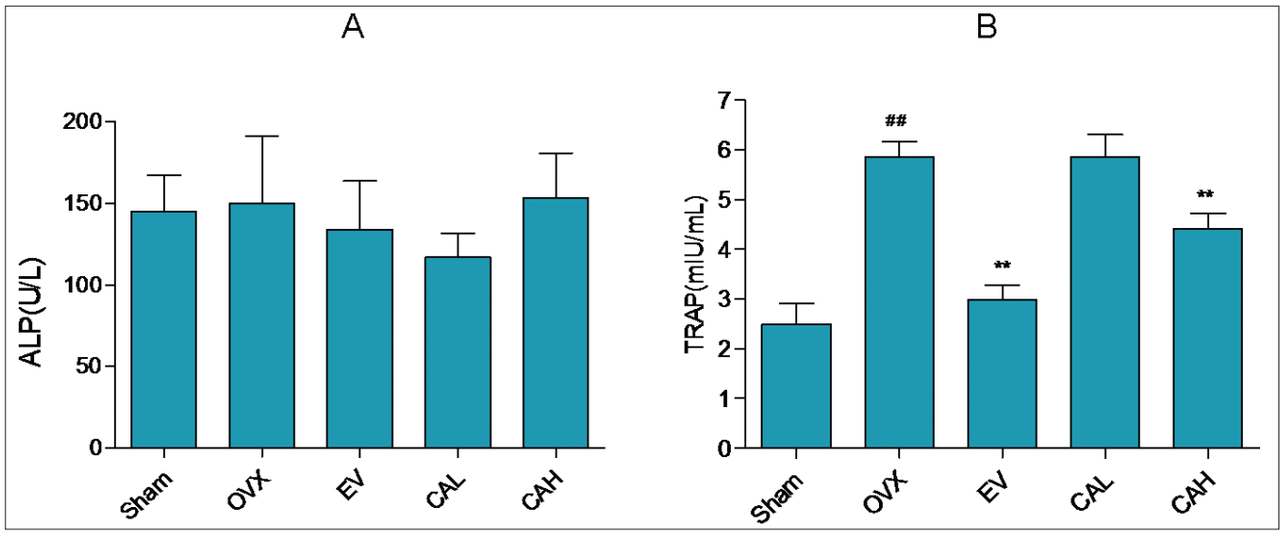

Fig. 2: Effects of CA and EV on ALP and TRAP in serum of ovariectomized rat (n = 8). All values are presented as mean ± SD, n = 8, $^{\#\#}P < 0.01$ compared to that of sham group rats; $^{**}P < 0.01$ compared to that of OVX rats.

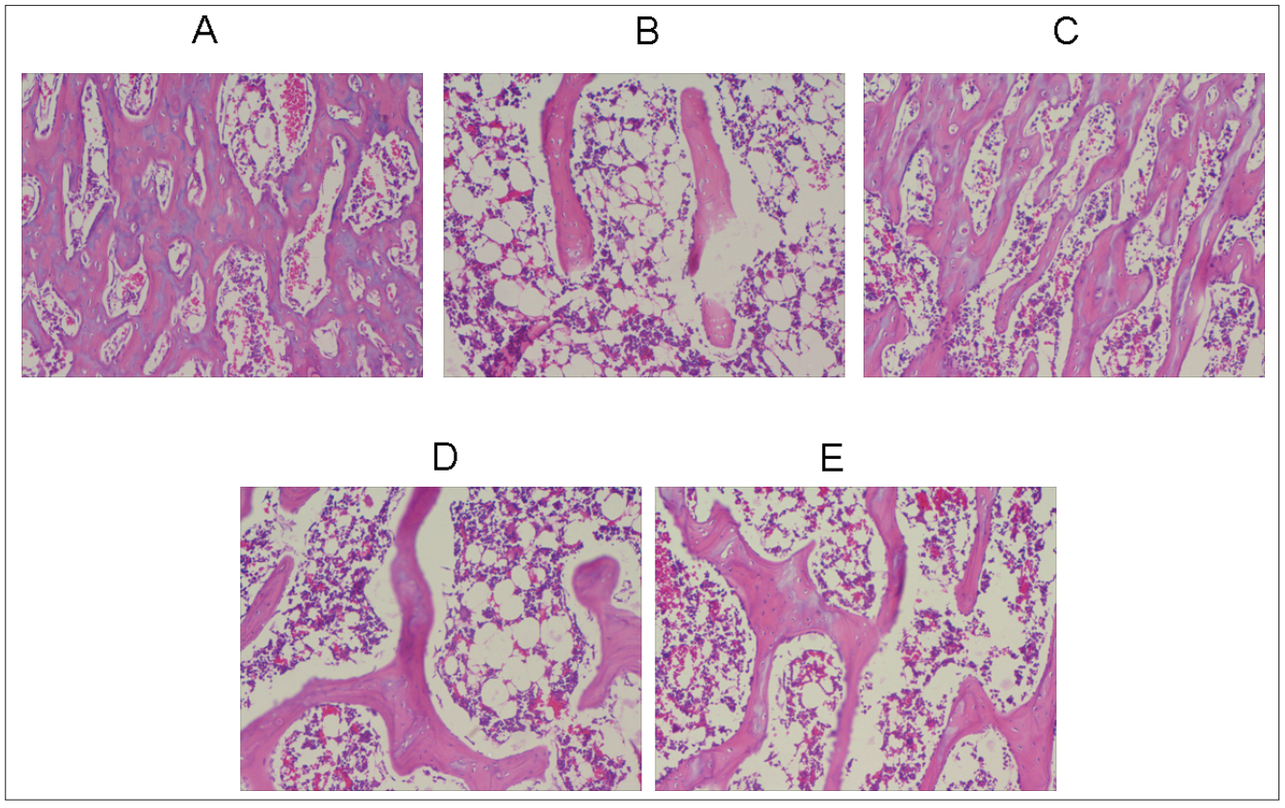

Fig. 3: Histopathological analysis of L4 vertebral body. The L4 vertebral body was stained with hematoxylin and eosin (H&E). (A) sham group rats. (B) OVX rats. (C) EV rats. (D) CAL group. (E) CAH rats.

China (Wang et al. 2015; Zhang et al. 2015). It is reported that the water extract from Huangqi notably inhibited the osteoporosis of postmenopausal women after 3-month treatment (Yang et al. 2007). Liu et al. (2005) reported that total flavones of Huangqi effectively improve bone mineral density in rats with osteoporosis induced by retinoic acid. In addition, Chen et al. (2005) reported that total flavones of Huangqi effectively improved bone health, increased bone density and decreased organ damage in OVX rats, but the possible mechanism involved and the pharmacodyamic material basis still remains unclear. In the present study, we established an animal model of ovariectomized rats to simulate osteoporosis in postmenopausal women, and investigated the antiosteoporotic activity of CA, one of total flavones in Huangqi.

Low bone mass is a major risk factor for fractures. Ovariectomy significantly decreased trabecular structural parameters. It is well known that ovariectomy can cause osteoporosis with an increase in bone resorption and body weight which are partially due to the estrogen deficiency (Chen et al. 2014; Tao and Liang 2014).

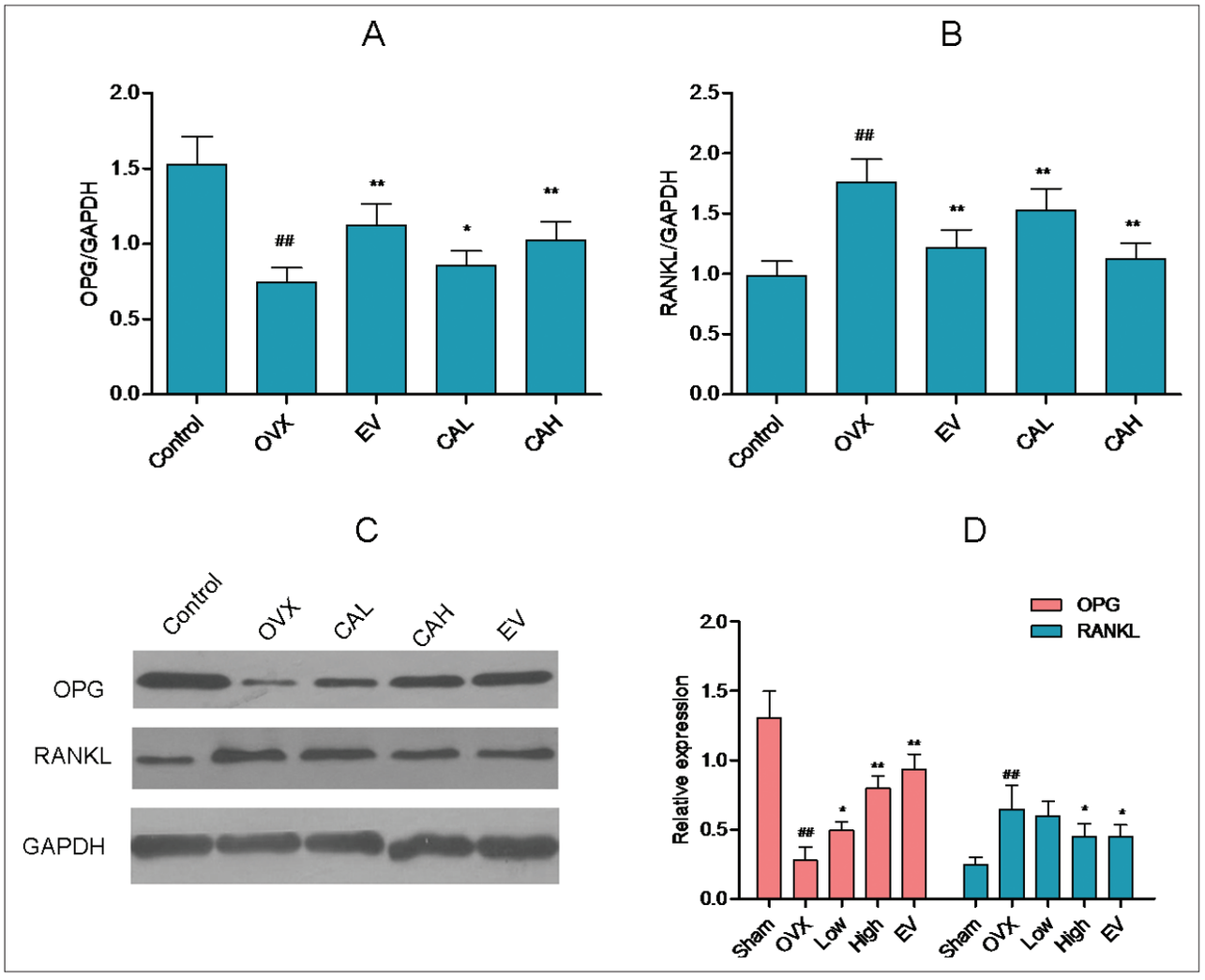

Fig. 4: Effects of CA on the expression OPG and RANKL. The expression OPG (A) and RANKL mRNA (B) were identified by RT-PCR analysis. (C, D) Protein expression of OPG and RANKL was identified by western blot analysis. All values are presented as mean ± SD, n = 8, $^{\#\#}P < 0.01$ compared to that sham group rats; $^{*}P < 0.05$ and $^{**}P < 0.01$ compared to that of OVX rats.

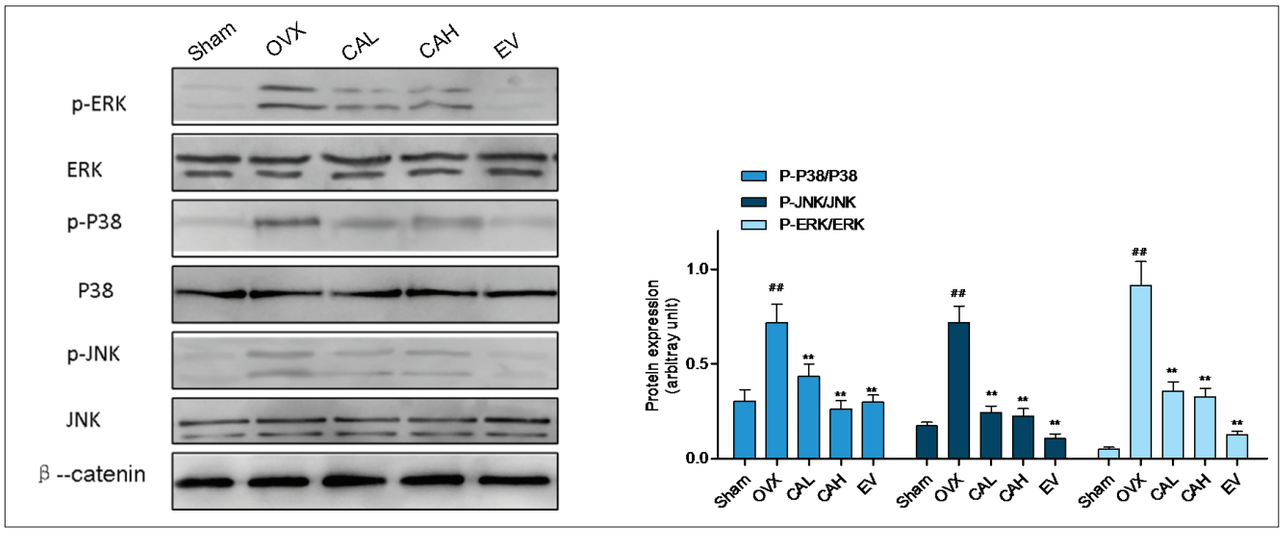

Fig. 5: Effects of CA on the phosphorylation of JNK, ERK and p38 MAPKs. The phosphorylation of JNK, ERK and p38 was measured by western blot analysis. GAPDH was also detected as the control of sample loading. All values are presented as mean ± SD, n = 8, $^{\#\#}P < 0.01$ compared to that Sham rats; $^{*}P < 0.05$ and $^{**}P < 0.01$ compared to that of OVX rats.

Reduction of bone density is a major risk factor for fractures and BMD measurement is widely used for the diagnosis of osteoporosis (Schmid et al. 2015; Tanaka et al. 2015). From our results (Table 1), CAH effectively decreased the weight increase compared to that of OVX rats. CA also increased the t-BMD of OVX rats (Fig. 2). This phenomenon suggests that CA prevented the bone loss induced by ovariectomy without stimulating the unwanted weight-gain, indicating that CA might prevent bone loss and avoid unwanted estrogen-like effects.

With regard to bone metabolic markers, both bone formation and bone resorption index were used to estimate the mechanisms of action of CA. The ALP activity has been extensively used as markers of bone formation. In our study, both of the OVX and CA treatment did not affect the activity of ALP. However, the ALP activity in OVX rats still showed a non-significant increasing trend indicating an increased rate of bone turnover. TRAP, DPD and cathepsin K were classic markers of bone resorption. In Fig. 2, CAH could effectively decrease the bone resorption marker (TRAP). All these data implied that CA possessed both bone resorption-inhibiting effects. CA also decreased urinary Ca, P and Cr excretion caused by OVX, further supporting the fact that CA could inhibit bone resorption. OVX resulted in the apparent reduction of trabecular bone area, trabecular number and trabecular connection, and the expanded marrow cavity compared to sham group rats (Fig. 4). However, CA treatment could partially reverse this effect of OVX after 12-week interventions.

RANKL plays an important role in osteoclast cell differentiation and bone resorption. OPG is a decoy receptor for RANKL with a high affinity and competes with RANK for RANKL binding. It thus functions as an inhibitor of RANK-RANKL interaction and inhibits osteoclast maturation and activation. In the present study, we found that CA could effectively regulate the OPG/RANKL expression, which suggested that CA could be used to prevent bone loss via inhibition of RANKL and promotion of OPG, and thus should be a novel therapeutic agent for the treatment of osteoporosis. It is reported that osteoclast precursor proliferation is achieved by M-CSF–induced activation of MAPKs and that osteoclast differentiation is regulated by a more persistent biphasic activation of MAPKs by RANKL, with that of p38 and JNK playing a prominent role (Choe et al. 2015; Lee et al. 2016). In the present study, CA could effectively surpress the phosphorylation of JNK, ERK and p38, which indicated that CA inhibited the osteoclast differentiation by regulating OPG/RANKL/MAPK signaling.

The above in vivo experiments proved that CA possesses a remarkable antiosteoporotic activity in OVX rats and can therefore have a promising role in the treatment of osteoporosis caused by estrogen deficiency, and the mechanisms involved in need be further studied.

4. Experimental

4.1. Animal experiments

All animal experiments were strictly performed according to the guide for the care and use of laboratory animals approved by the Bioethics Committee of the Tongji Medical College, Huazhong University of Science and Technology. Forty female Sprague-Dawley (SD) rats, aged 3 months with the body weight of 295.0±18.0g, were purchased from Experimental Animal Center of Tongji Medical College, Huazhong University of Science and Technology, Wuhan Hubei China and acclimated to laboratory conditions for 1 week before the experiment. Every four rats were kept in one cage with a standard laboratory diet and tap water under an air-controlled condition (24.0±0.5 °C, 45-50% humidity and 12 h/12 h light-dark illumination cycles). After one week of acclimation, all the rats were anesthetized with chloral hydrate (300 mg/kg, i.p.) (Sinopharm, China) under aseptic conditions and underwent either bilateral laparotomy without removing the ovaries (sham, n=8) or bilateral ovariectomy (OVX, n=32). After another one week recovery period, the 32 OVX rats were equally and randomly divided into four groups: ovariectomized orally treated with vehicle (0.5% CMC-Na) as model group (OVX); with estradiol valerate (1 mg/kg body weight/day) as positive control (EV); with 15 and 30 mg/kg body weight/day of calycosin (CA, Jiancheng Biotech, Nanjing) as low (CAL) and high dose group (CAH), respectively. Animals in each group were orally treated with an equal volume of 10 ml/kg body weight vehicle, EV or CA which started on the first week after operation and lasted for 12 weeks. Body weight was measured weekly, with the dose of vehicle, EV and CA adjusted accordingly. After the last administration, urine was collected from overnight fasted rats by metabolic cages; blood samples were withdrawn from the femoral artery of anesthetized rats (300 mg/kg chloral hydrate i.p.), allowed to clot and centrifuged at 3000 × g for 10 min to afford serum; the femora and tibia were dissected. All the samples were stored at -20 °C until further analysis.

4.2. Biochemical parameters

The content of calcium (Ca), inorganic phosphorus (P) and creatinine (Cr) in urine and serum as well as serum alkaline phosphatase (ALP) activity was measured on an automatic analyzer (Ciba-Corning 550, USA). The levels of serum cathepsin K and bone gla-protein (BGP) as well as urine deoxypyridinoline crosslinks (DPD) were determined by the corresponding reagent kits, whereas the serum tartrate-resistant acid phosphatase (TRAP) was measured according to the related literature (Jiao et al. 2009).

4.3. Bone mineral density

The bone mineral density (BMD, g/cm^2) of the right total femur was measured by dual-energy X-ray absorptiometry (Lunar, USA) using the small animal scan mode.

4.4. Histopathological analysis

Samples were obtained from the fourth lumbar (L4) vertebral body, fixed by immersion in buffered formalin for 72 h, then decalcified in 10% ethylenediaminetetraacetic acid (EDTA) for 4 weeks, dehydrated in a desiccator with graded ethanol, defatted in xylene, and embedded in paraffin. Five mm-thick longitudinally oriented sections were cut, stained with hematoxylineeosin (HE) for histopathological analysis.

4.5. Quantitative real-time PCR (RT-PCR) measurements of gene expression

Total RNA was extracted from the bone marrow cells obtained from the right femur and tibia using Trizol-Reagent (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions. RT-PCR was performed by applying a FTC-2000 RT-PCR machine (Funglyn, Toronto, Canada). The mRNA expressions of target genes in the bone marrow cells included osteoprotegerin (OPG) and Receptor Activator for Nuclear Factor-κB Ligand (RANKL). GAPDH was selected as an endogenous housekeeping gene. The primers for each gene were listed as following: 5′-CTT CGT GCC TTG ATG GA-3′ and 5′-TTG GGA AAG TGG GAT GT-3′ for OPG (product: 113 bps); 5′ -ACC AAG ATG GCT TCT ATT ACC-3′ and 5′-TCC CTC CTT TCA TCA GGT TAT-3′ for RANKL (product: 132 bps); 5′-ATC ACT GCC ACC CAG AAG-3′ and 5′-TCC ACG ACG GAC ACA TTG-3′ for GAPDH (product: 191 bps). The reaction conditions were as follows: 95 °C for 10 min; 40 cycles of 95 °C for 10 sec, 60 °C for 20 sec, and 72 °C for 30 sec; and 4 °C for 5 min. GAPDH was used as an internal control.

4.6. Western blot

The synovial tissues of mice in each group were homogenized on ice in a lysis buffer. Proteins were resolved using SDS-PAGE, and transferred onto polyvinylidene fluoride membranes by electroblotting. Membranes were incubated with primary antibodies against RANKL (Abcam), OPG (Abcam), phosphorylation (p) -ERK, ERK, p-P38, P38, p-JNK, JNK (Abcam) and GAPDH (Abcam) at 4 °C overnight. Membranes were incubated with secondary antibodies (horseradish peroxidase-conjugated rabbit antigoat IgG, dilution, 1:2000) at room temperature for 2 h. A chemiluminescence detection system was used to detect protein bands. Band intensities were analyzed using Quantity one software.

4.7. Statistical analysis

The data were analyzed using SPSS computer software Version 18.0. The data for multiple comparisons were performed by one-way ANOVA followed by LSD t-test. A value of $P < 0.05$ was considered statistically significant and all results per presented as the means ± SD.

Conflict of interest: None declared.

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