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EGCG prevents bone loss in ovariectomized mice by suppressing osteoclastogenesis via the inhibition of NF-κB, MAPK, and AKT signaling pathways

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ABSTRACT

Excessive osteoclastogenesis-mediated osteoporosis has been recognized as a global health concern. Candidate compounds derived from medicinal plants or functional foods are promising to treat osteoporosis due to their high safety and efficiency. (−)-Epigallocatechin-3-gallate (EGCG) is the most abundant and biologically active polyphenol in green tea. It can inhibit osteoclastogenesis *in vitro* by blocking receptor activator of nuclear factor (NF)-κB (RANK) signaling pathways. This study used the ovariectomized (OVX) mouse model to estimate the therapeutic effect of EGCG on osteoporosis and verified the molecular mechanism *in vivo*. The results revealed that EGCG significantly inhibited the OVX-induced body weight gain. Moreover, no adverse effects were observed on blood glucose, histomorphological features, weights, as well as indices of liver and kidney in OVX mice. EGCG could significantly ameliorate bone loss in OVX mice by inhibiting osteoclastogenesis. This effect was evidenced by the reduced number of osteoclasts and the increased trabecular bone area in the femurs. Moreover, EGCG inhibited the activities of c-telopeptide of type I collagen (CTX-I) and tartrate-resistant acid phosphatase 5b (TRACP-5b) and strengthened bone gla protein (BGP) and procollagen I N-terminal peptide (PINP) activities in OVX mice. Mechanistically, EGCG significantly downregulated the expression of osteoclastogenesis-related marker genes and proteins, including nuclear factor of activated T cells, cytoplasmic 1 (NFATc1), c-Fos, tartrate-resistant acid phosphatase (TRAP), c-Src, and cathepsin K. In addition, the phosphorylation levels of p65, c-Jun N-terminal kinase (JNK), extracellular signal-regulated kinase 1/2 (ERK1/2), p38, and protein kinase B (AKT) were significantly suppressed in OVX mice. It was found that EGCG could alleviate OVX-induced bone loss in mice by suppressing osteoclastogenesis by blocking the NF-κB, mitogen-activated protein kinase (MAPK), and AKT signaling pathways. EGCG has the potential to prevent and treat osteoclast-related diseases such as osteoporosis.

1. Introduction

Osteoporosis is a systemic and chronic bone metabolic disease characterized by reduced bone mass and density and deterioration of

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bone tissue microstructure. This condition has been identified as a severe health problem worldwide. It is often nicknamed the silent killer due to the lack of obvious symptoms in the early stage, rendering it easily overlooked[1]. The prevalence of osteoporosis increases with the growing elderly population, especially in postmenopausal women. It is estimated to affect more than 200 million individuals around the world, severely influencing the quality of life and survival of patients. The grim situation also imposes huge emotional and economic burdens on families and society[2].

The uncoordinated activities of osteoblasts and osteoclasts can disrupt bone structure and function, leading to bone diseases such as osteoporosis[3]. Current osteoporosis medications primarily include anti-resorption agents targeting osteoclasts to inhibit bone resorption and anabolic agents that target osteoblasts to promote bone formation[4]. Notably, most drugs (e.g., bisphosphonates and denosumab) have obvious side effects on patients with osteoporosis due to long-term use, such as hypercalcemia, heart attack, and breast cancer[5]. In this context, it is promising to develop candidate osteoporosis drugs derived from medicinal plants or functional foods, particularly phytochemical compounds, which exhibit high safety and efficiency[6-8].

Tea is the second most widely consumed beverage in the world. Tea consumption is reportedly conducive to human health, such as bone health[9], and its safety has been verified[10]. Various types of tea including Pu'er tea[11], black tea[12], and green tea[13], along with their main bioactive constituents such as theabrownins[14], thearubigins[15], and polyphenols[16], can effectively inhibit osteoclastogenesis *in vitro* and ameliorate ovariectomy-induced osteoporosis *in vivo*. (−)-Epigallocatechin-3-gallate (EGCG), the most abundant and biologically active polyphenol in green tea, has been shown to inhibit cancer[17], inflammation[18], and osteoporosis[16]. Many studies have reported that EGCG could suppress osteoclast differentiation and promote osteoblast differentiation through multiple signaling pathways, effectively alleviating osteoporosis[19-20]. Based on these insights, EGCG is an ideal natural candidate compound for osteoporosis prevention and treatment.

The receptor activator of nuclear factor (NF)-κB (RANK) ligand (RANKL)-induced canonical RANK signaling pathways, including NF-κB, mitogen-activated protein kinase (MAPK), and protein kinase B (AKT), play pivotal roles in osteoclastogenesis, contributing to the occurrence and development of osteoporosis[21-22]. Our previous work investigated the molecular mechanism by which EGCG inhibited osteoclastogenesis considering protein-protein interactions; we found that EGCG downregulated osteoclastogenesis *in vitro* by blocking RANKL-RANK interaction and RANK signaling pathways[23]. The present study further used the ovariectomized (OVX) mouse model to estimate the therapeutic effect of EGCG on osteoporosis and verified the molecular mechanism *in vivo*. Our results demonstrated that EGCG could prevent bone loss in OVX mice by suppressing osteoclastogenesis via the inhibition of NF-κB, MAPK, and AKT signaling pathways. This finding supports EGCG's osteoprotective effect, laying a theoretical basis for the clinical application of natural compounds derived from medicinal plants or functional foods against osteoporosis.

2. Materials and methods

2.1 Reagents

EGCG (purity > 99%) was obtained from TargetMol Chemicals Inc. (Boston, Massachusetts, USA). Raloxifene hydrochloride (RLX, 60 mg per tablet) was purchased from Jiangsu Hengrui Pharmaceuticals Co., Ltd. (Lianyungang, China). High-efficiency RIPA lysis buffer and hematoxylin and eosin (H&E) staining kit were provided by Solarbio (Beijing, China), and a tartrate-resistant acid phosphatase (TRAP) staining kit was obtained from Sigma-Aldrich (St. Louis, MO, USA). The mouse c-telopeptide of type I collagen (CTX-I), tartrate-resistant acid phosphatase 5b (TRACP-5b), bone gla protein (BGP), and procollagen I N-terminal peptide (PINP) ELISA kits were purchased from Cusabio Technology (Houston, TX, USA). The BCA protein quantification kit was provided by Beyotime Biotechnology (Shanghai, China). We obtained specific primary antibodies against NFATc1, c-Fos, TRAP, c-Src, cathepsin K, and *β*-tubulin from Santa Cruz Biotechnology (San Jose, CA, USA). In addition, antibodies against signal transduction molecules, including p-p65, p65, p-c-Jun N-terminal kinase (JNK), JNK, p-extracellular signal-regulated kinase 1/2 (ERK1/2), ERK1/2, p-p38, p38, p-AKT, and AKT, were purchased from Cell Signaling Technology (Beverly, MA, USA). Horseradish peroxidase-conjugated secondary antibodies were obtained from R&D Systems (Minneapolis, MN, USA). The rest of the biochemical agents were specified.

2.2 Mice and treatment

All animal experiments were performed in accordance with the Guidelines for Care and Use of Laboratory Animals of the Yunnan Agricultural University and approved by the Animal Ethics Committee of the Yunnan Agricultural University (No. YNAU2023 LLWYH03035). Fifty 8-week-old C57BL/6 female mice were provided by Changzhou Cawens Laboratory Animal Co., Ltd. (Changzhou, China; SCXK(Su)2021-0013). These animals were equally divided into sham, OVX, RLX (positive control, 6.5 mg/(kg·day)), OVX + low-dose EGCG (EGCG-L, 25 mg/(kg·day)), and OVX + high-dose EGCG (EGCG-H, 50 mg/(kg·day)) groups. The mice were acclimatised for 1 week before bilateral sham or ovariectomy operation. One week after the surgery, the mice received corresponding treatments via intraperitoneal injection for 8 weeks. These mice were weighed every 2 days and fasted 12 h before anesthesia. Finally, blood and other samples were collected for subsequent analysis.

2.3 Biochemisty analysis

Blood samples were allowed to clot and centrifuged at 1 200 × *g* at 4 °C for 15 min to obtain serum. The serum levels of CTX-I, TRACP-5b, BGP, and PINP were determined using the corresponding ELISA kits.

2.4 Histological examination and immunohistochemical staining

Fresh liver, kidney, and left femur tissues were fixed in formaldehyde, and the left femur tissues were decalcified. Subsequently, they were embedded in paraffin and cut into 5 μm sections, followed by H&E or TRAP staining following standard methods[24].

In addition, the expression of p-p65 in femur tissues was determined using immunohistochemical staining, as described previously[25].

2.5 Quantitative real-time polymerase chain reaction (qRT-PCR) analysis

Total RNA extraction from the right femur tissues was conducted using the TRIzol® Reagent (ThermoFisher Scientific, Carlsbad, CA, USA). qRT-PCR analysis was performed to analyze the expression of osteoclastogenesis-related marker genes, such as *NFATc1*, *c-Fos*, *TRAP*, *c-Src*, and *cathepsin K*. The detailed methods and the specific primers were described in our previous study[26].

2.6 Western blotting analysis

Proteins were extracted from the right femur tissues using RIPA lysis buffer and quantified with a BCA protein quantification kit. The proteins of equal amounts were separated by SDS-PAGE (8%) and transferred onto polyvinylidene fluoride membranes (Cytiva, Freiburg, Germany). These membranes were examined with the indicated primary antibodies at 4 °C overnight and incubated with a secondary antibody. The bands of interest were visualized using a FluorChem E chemiluminescent substrate system (ProteinSimple, San Jose, CA, USA).

2.7 Statistical analysis

All values were expressed as the mean ± standard deviation (SD). Statistical differences within groups were evaluated through one-way analysis of variance with Tukey's post hoc test using SPSS 17.0 software (SPSS, Inc., Chicago, USA). Representative images were presented, and a *P*-value lower than 0.05 was considered a significant difference.

3. Results

3.1 Effects of EGCG on body weight and blood glucose in OVX mice

The chemical structure and HPLC chromatography of EGCG are shown in Fig. 1. Fig. 2A depicts the animal experiment process. The body weights in each group consistently increased during the initial experimental stage (Fig. 2B). The mice in the OVX group showed a significant body weight gain compared to the sham mice post experiment ($P < 0.05$), and the OVX-induced body weight gain was significantly inhibited by RLX and high-dose EGCG intervention ($P < 0.05$). Additionally, no significant differences were observed in blood glucose of any animal group (Fig. 2C).

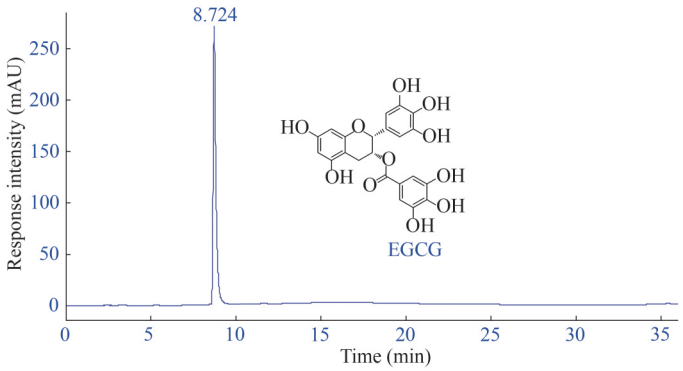

Fig. 1 Chemical structure and HPLC chromatography of EGCG. HPLC analysis method involved using Agilent 1260 HPLC instrument with an ultraviolet detector (detection wavelength: 280 nm) and a C_{18} ODS column (4.6 mm × 250 mm, 5 μm); mobile phase A of 100% acetonitrile (increased from 10% to 60% in 36 min) and phase B of 0.03% trifluoroacetic acid; a 1.0 mL/min flow rate.

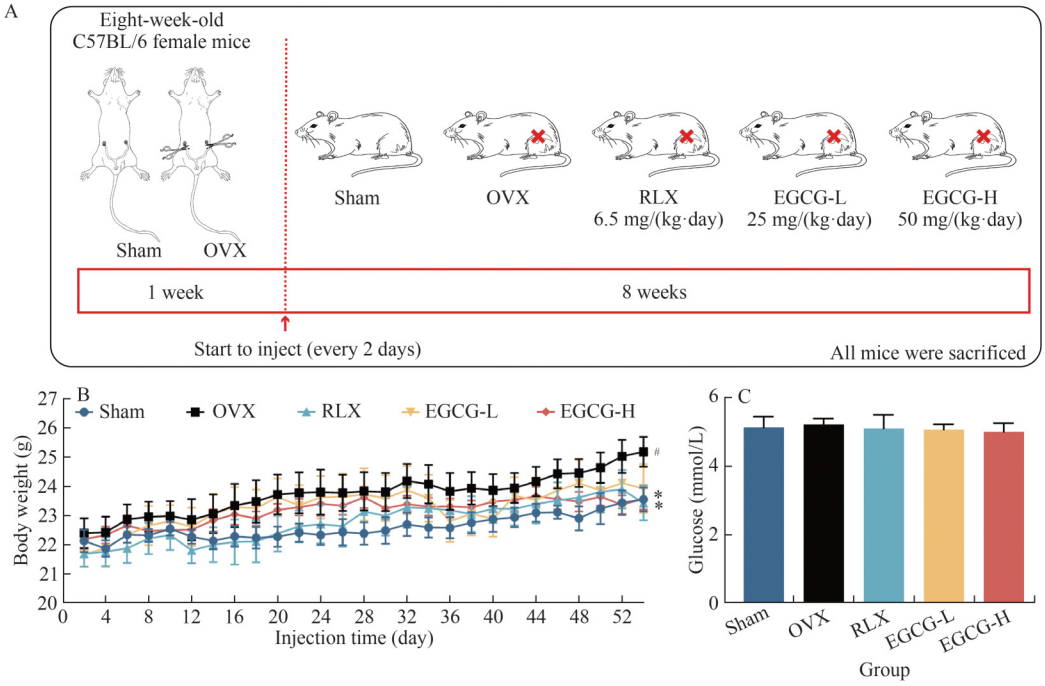

Fig. 2 Effects of EGCG on body weight and blood glucose in OVX mice. (A) The animal experiment process. (B) The body weight and (C) blood glucose in each group were monitored. Data were expressed as the mean ± SD ($n = 10$). $^{\#}P < 0.05$ indicates a significant difference compared to the sham group; $^{*}P < 0.05$ indicates a significant difference compared to the OVX group.

3.2 Effects of EGCG on the histomorphologiical features, weights, and indices of organs in OVX mice

The H&E staining for the liver and kidney in each group is illustrated in Fig. 3A. The results showed no histomorphological differences in the liver and kidney among the groups. Similarly, liver and kidney weights (Figs. 3B and C) and indices (Figs. 3D and E) were consistent. These results suggested that EGCG (25 and 50 mg/(kg·day)) intervention did not adversely affect the mice's livers and kidneys.

3.3 Effects of EGCG on bone microarchitecture and serum biochemical parameters in OVX mice

To confirm that EGCG can prevent bone loss in OVX mice by inhibiting osteoclastogenesis, TRAP and H&E staining were used separately to determine the osteoclast activity and microarchitecture changes in femur trabecular bone (Fig. 4A). As expected, RLX and EGCG treatments significantly reduced bone loss in OVX mice via the inhibition of osteoclastogenesis, as evidenced by the decreased number of osteoclasts (Fig. 4B) and the increased trabecular bone

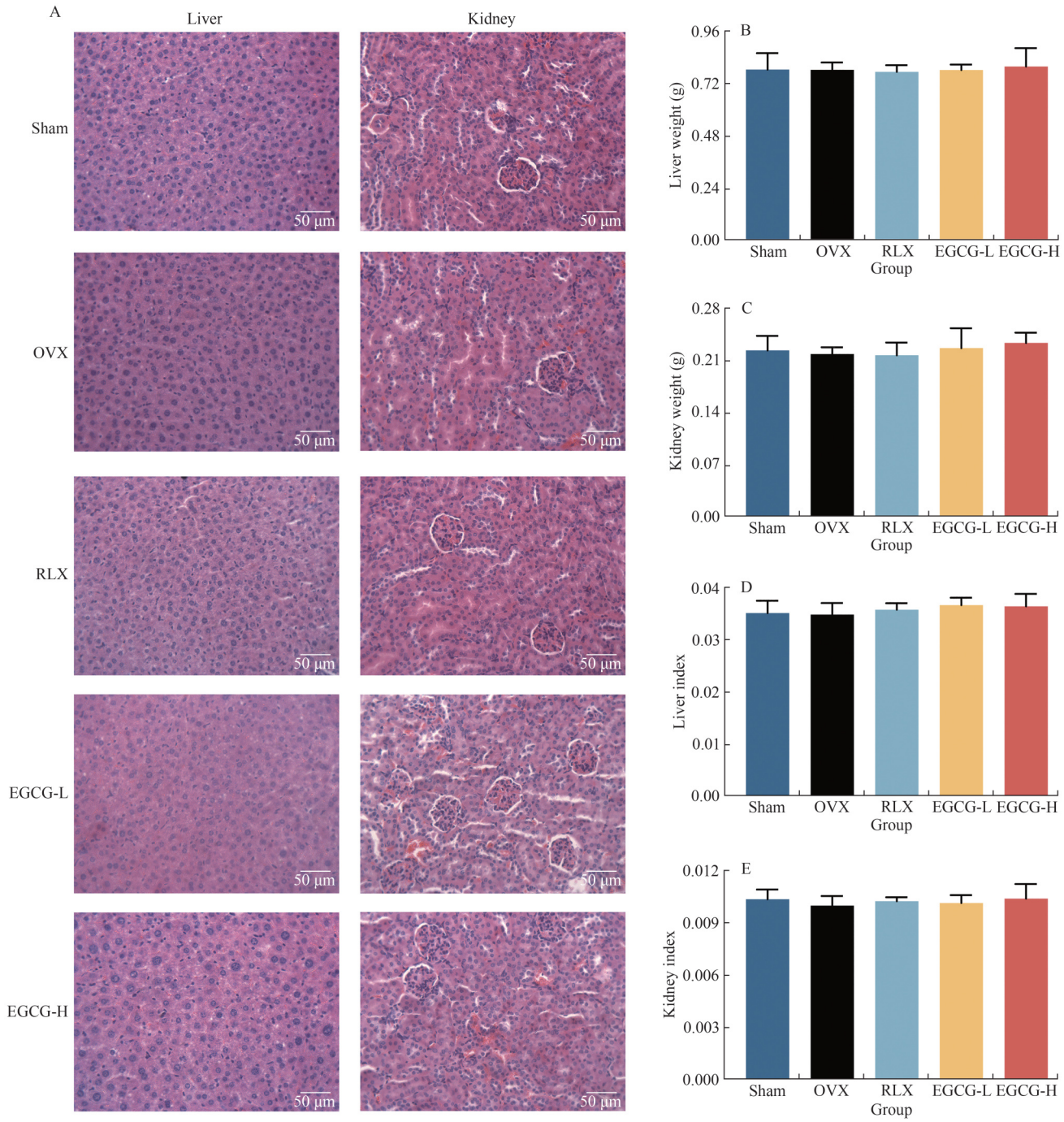

Fig. 3 Effects of EGCG on histomorphological features, weights, and indices in OVX mouse organs. (A) Liver and kidney histomorphological features, (B) liver weight, (C) kidney weight, (D) liver index, and (E) kidney index in each group. Data were expressed as the mean ± SD (n = 10).

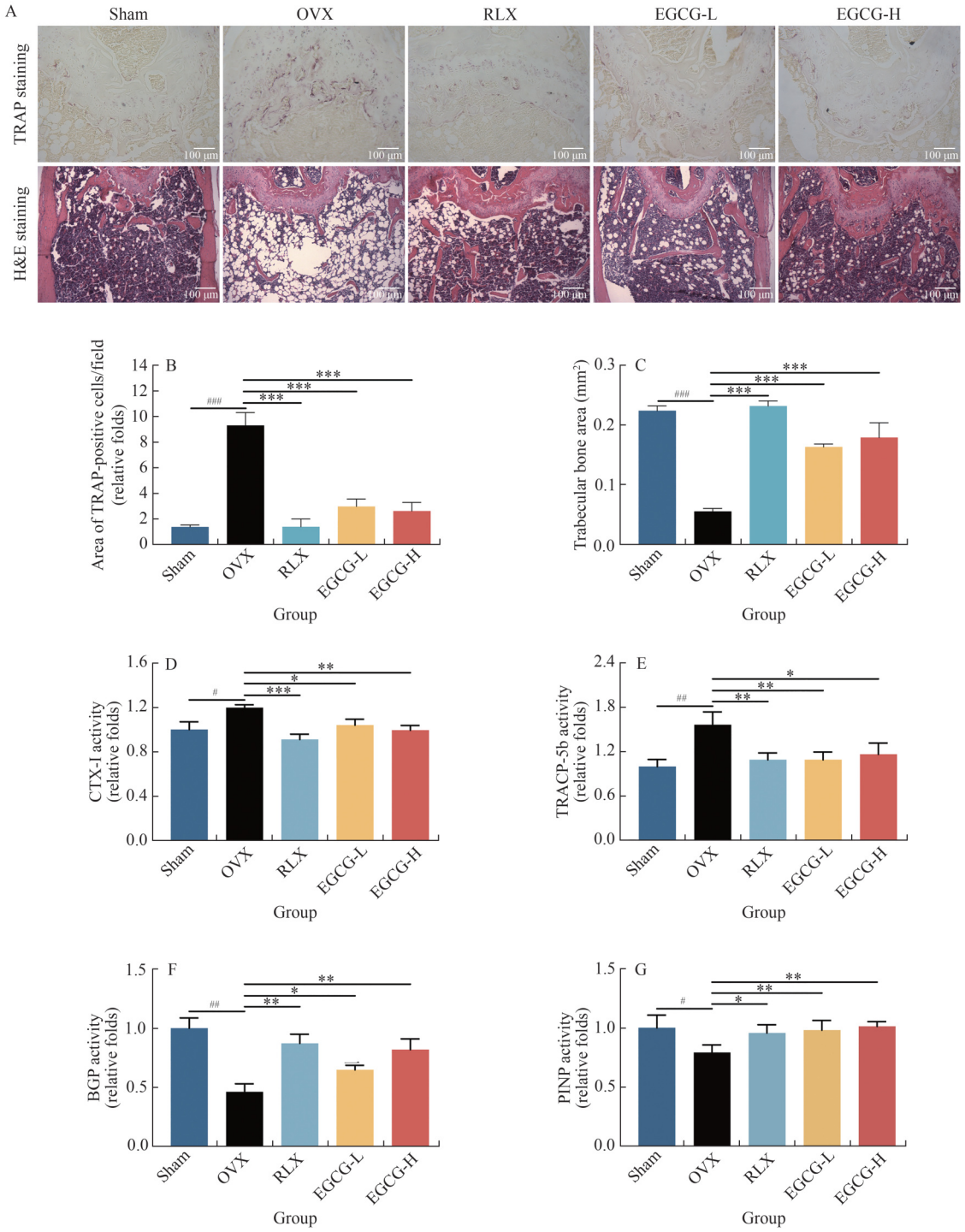

Fig. 4 Effects of EGCG on bone microarchitecture and serum biochemical parameters in OVX mice. (A) TRAP staining and H&E staining of femur sections in each group, and calculation of (B) the TRAP-positive cell counts and (C) trabecular bone area. The serum activities of (D) CTX-I, (E) TRACP-5b, (F) BGP, and (G) PINP in each group. Data were expressed as the mean ± SD (n = 10). $^{\#}P < 0.05$, $^{\#\#}P < 0.01$, and $^{\#\#\#}P < 0.001$ denote a significant difference compared to the sham group; $^{*}P < 0.05$, $^{**}P < 0.01$, and $^{***}P < 0.001$ represent a significant difference compared to the OVX group.

area (Fig. 4C) in the femur ($P < 0.001$). Consistently, the OVX mice treated with RLX and EGCG experienced a decline in CTX-I and TRACP-5b activities in the serum compared to the OVX mice ($P < 0.05$, $P < 0.01$, or $P < 0.001$; Figs. 4D and E). Moreover, BGP and PINP activities in the serum were enhanced, indicating that RLX and EGCG treatments facilitated bone formation in OVX mice ($P < 0.05$ or $P < 0.01$; Figs. 4F and G).

3.4 Effects of EGCG on osteoclastogenesis-related marker gene and protein expression in OVX mice

The effects of EGCG on the expression of osteoclastogenesis-related marker genes and proteins in OVX mice femurs are presented in Fig. 5. qRT-PCR data showed significant upregulation in the relative mRNA expression of *NFATc1*, *c-Fos*, *TRAP*, *c-Src*,

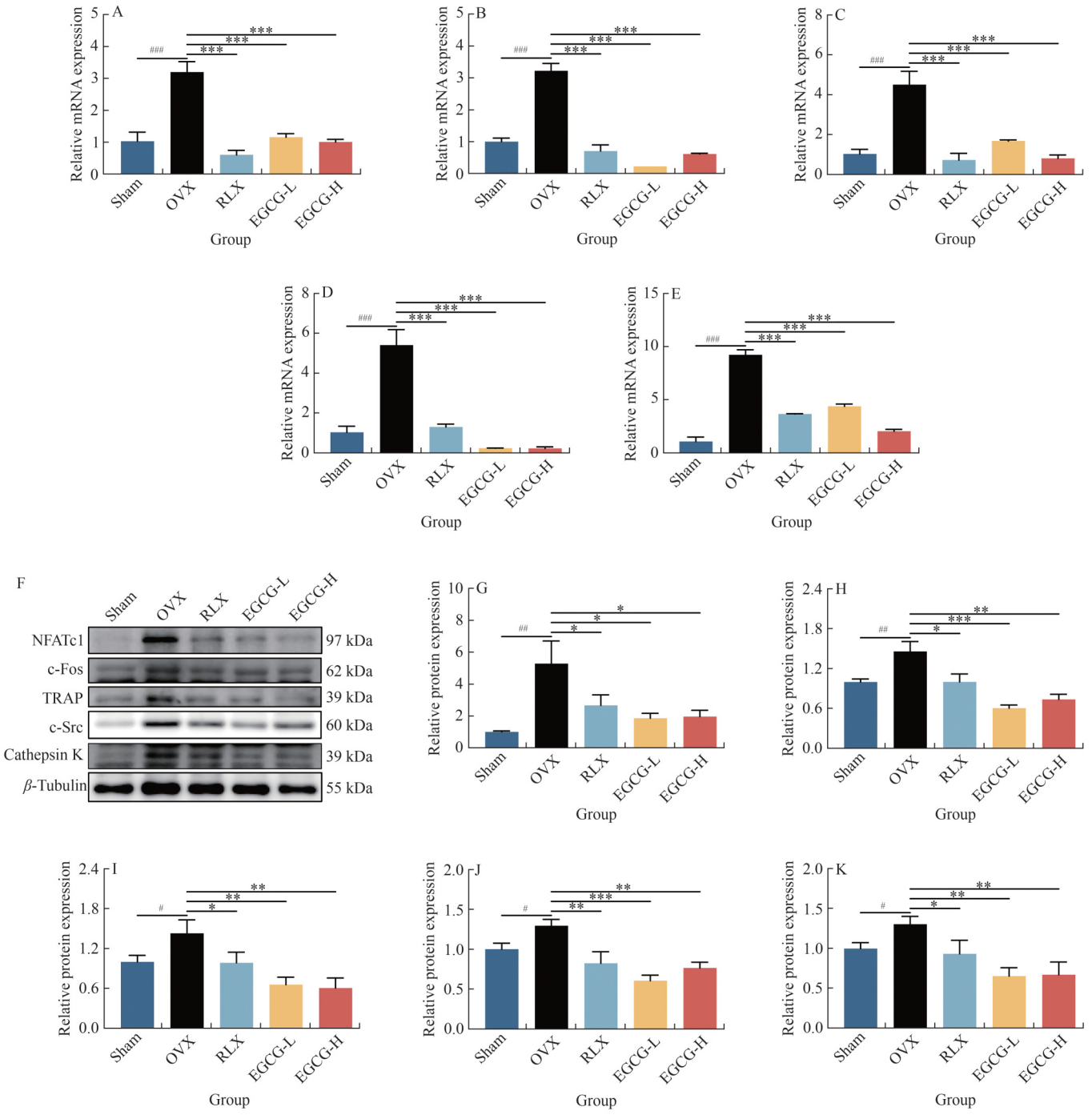

Fig. 5 Effects of EGCG on osteoclastogenesis-related marker gene and protein expression in OVX mice. The expression of osteoclastogenesis-related marker genes, including (A) *NFATc1*, (B) *c-Fos*, (C) *TRAP*, (D) *c-Src*, and (E) *cathepsin K*. (F) The expression of osteoclastogenesis-related marker proteins, such as (G) NFATc1, (H) c-Fos, (I) TRAP, (J) c-Src, and (K) cathepsin K in OVX mice, according to Western blotting analysis. Data were expressed as the mean ± SD ($n = 10$). $^{\#}P < 0.05$, $^{\#\#}P < 0.01$, and $^{\#\#\#}P < 0.001$ indicate a significant difference compared to the sham group; $^{*}P < 0.05$, $^{**}P < 0.01$, and $^{***}P < 0.001$ indicate a significant difference compared to the OVX group.

and *cathepsin K* in the OVX group compared to the sham group. RLX and EGCG treatments could significantly suppress such expression ($P < 0.001$; Figs. 5A–E). Consistently, Western blotting analysis indicated that OVX mice treated with RLX and EGCG displayed significantly downregulated expression of osteoclastogenesis-related marker proteins, including NFATc1, c-Fos, TRAP, c-Src, and cathepsin K ($P < 0.05$, $P < 0.01$, or $P < 0.001$; Figs. 5F–K).

3.5 Effects of EGCG on NF-κB, MAPK, and AKT signaling pathways in OVX mice

Immunohistochemical staining of femur sections indicated that the expression of p-p65 was significantly upregulated in the OVX group compared with the sham mice; in contrast, RLX and EGCG treatments in OVX mice led to significant inhibition ($P < 0.001$; Figs. 6A and B). According to Western blotting data, the

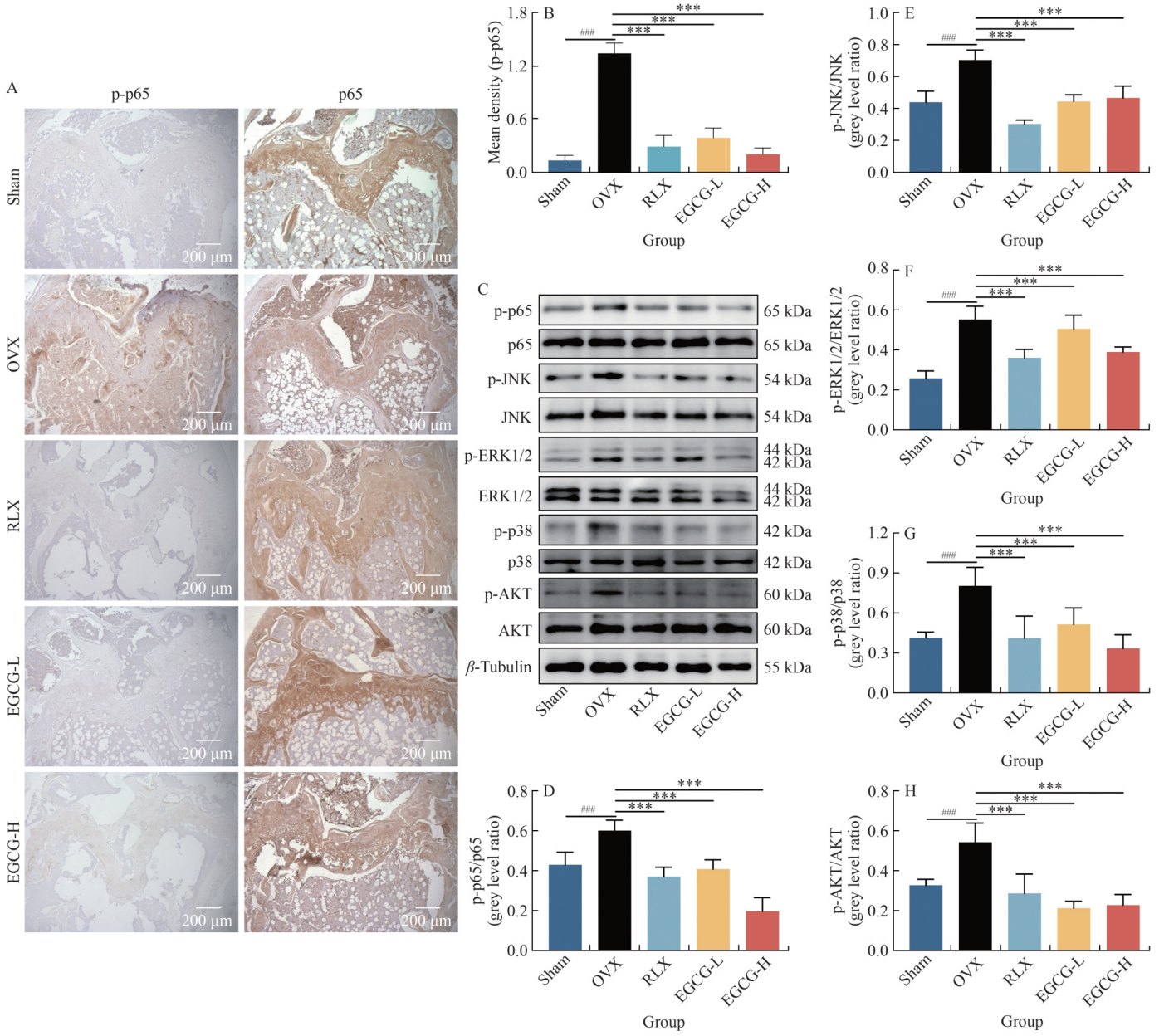

Fig. 6 Effects of EGCG on NF-κB, MAPK, and AKT signaling pathways in OVX mice. The OVX-induced expression of (A–D) p-p65, (E) p-JNK, (F) p-ERK1/2, (G) p-p38, and (H) p-AKT in femurs, based on immunohistochemical staining and Western blotting analysis. Data were expressed as the mean ± SD (n = 10). $^{###}P < 0.001$ represents a significant difference compared to the sham group; $^{***}P < 0.001$ represents a significant difference compared to the OVX group.

phosphorylation levels of p65, JNK, ERK1/2, p38, and AKT in the OVX group were significantly increased compared to the sham group ($P < 0.001$). Notably, the phosphorylation of these osteoclastogenesis-related signaling molecules was significantly inhibited after applying RLX and EGCG treatments in OVX mice ($P < 0.001$; Figs. 6C–H).

4. Discussion

Green tea holds the top position in tea production and consumption in China due to its pleasant flavor, fresh taste, and beneficial effects on human health[27]. Polyphenols in green tea are almost unoxidized due to the absence of fermentation in tea processing. As the most abundant and bioactive natural compound in tea polyphenols, EGCG contains eight phenolic hydroxyl groups and exhibits various pharmacological effects[17]. Osteoporosis induced by estrogen deficiency is prevalent in postmenopausal women. According to prior reports, EGCG can alleviate postmenopausal osteoporosis by inhibiting bone resorption and stimulating bone formation[16,28]; it is also shown to prevent dexamethasone-induced osteoporosis by promoting the production of beneficial bacteria and metabolites and enhancing immune function[29]. OVX mouse is a well-established animal model for osteoporosis investigation in postmenopausal women, as recommended by the U. S. Food and Drug Administration and the World Health Organization[11]. Considering EGCG's ability to inhibit RANKL-induced osteoclastogenesis by blocking canonical RANK signaling pathways[23], it may exhibit antiosteoporotic

activity through this mechanism. Our results confirmed the beneficial effects of EGCG on bone loss in OVX mice by suppressing osteoclastogenesis via the inhibition of NF-κB, MAPK, and AKT signaling pathways. Despite such potential, it has not been used clinically to treat osteoporosis.

Ovariectomy can increase body weight, alter serum biomarkers of bone metabolism, and deteriorate bone tissue microstructure mainly by estrogen deficiency[1]. Our data indicated that EGCG could significantly inhibit body weight gain in OVX mice, potentially related to its body weight-lowering effect[30]. Despite the proven safety of EGCG, we systematically estimated its impacts on blood glucose, liver, and kidney in OVX mice. Consistently, EGCG treatment did not adversely affect blood glucose, histomorphological features, weights, and indices of liver and kidney in OVX mice at test doses. Consistent with the results of previous studies[16,20], EGCG could effectively protect against ovariectomy-induced bone loss in mice by inhibiting osteoclastogenesis. Due to its excellent anti-inflammatory activity, EGCG can also ameliorate rheumatoid arthritis by suppressing osteoclast differentiation[31-32]. Here, we further investigated the effects of EGCG on serum biomarkers of bone resorption (CTX-I and TRACP-5b) and formation (BGP and PINP) in OVX mice. It was revealed that EGCG could inhibit the activities of CTX-I and TRACP-5b and enhance the BGP and PINP activities in OVX mice. The serum biochemistry analysis suggested that EGCG could suppress osteoclastogenesis and enhance osteogenesis. This observation is consistent with previous research[16,28]. Based on the above results, EGCG possessed antiosteoporotic property, and its mechanism of action was closely related to the inhibition of bone resorption.

Inhibition of excessive osteoclastogenesis has been an important strategy to prevent and treat osteoporosis. RANKL, a member of the tumor necrosis factor superfamily, can directly interact with its receptor RANK. Their interplay activates downstream signaling pathways, particularly NF-κB, MAPK (JNK, ERK1/2, and p38), and AKT. The activation ultimately upregulates osteoclastogenesis-related marker genes and proteins, including NFATc1, c-Fos, TRAP, c-Src, and cathepsin K[23]. We found that intraperitoneal administration of EGCG significantly downregulated the expression of NFATc1, c-Fos, TRAP, c-Src, cathepsin K, p-p65, p-JNK, p-ERK1/2, p-p38, and p-AKT in OVX mice. These results further demonstrated that EGCG could prevent bone loss by blocking NF-κB, MAPK, and AKT pathways. As a result, osteoclastogenesis was suppressed. EGCG is a widely recognized natural small molecule, and multiple interaction targets for this molecule have been reported, such as 67-kDa laminin receptor (67 LR)[33], Notch[18], p53[17], TNF-α[32], RANKL, and RANK[23]. EGCG also has limitations, such as poor bioavailability, instability, and poor water solubility[34]. As such, EGCG was administered by intraperitoneal injection in the current study. Based on the unique chemical structure of EGCG, future studies should focus on its derivatives and oxidation products, informing the development of more effective drugs against osteoporosis.

5. Conclusion

Our results demonstrated that EGCG, a widely recognized natural small molecule, could alleviate OVX-induced bone loss in mice by suppressing osteoclastogenesis via the inhibition of NF-κB, MAPK, and AKT signaling pathways. This finding suggests that regular consumption of green tea, rich in EGCG, contributes to the prevention and treatment of osteoclast-related diseases such as osteoporosis.

Conflict of interest

The authors declare that they have no conflicts of interest.

Acknowledgements

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