

Resveratrol prevents osteoporosis by upregulating FoxO1 transcriptional activity

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Abstract. Resveratrol (3,5,4-trihydroxystilbene, RES), a natural antioxidant, prevents bone loss by attenuating damage caused by oxidative stress. Our previous research revealed that the forkhead box O1 (FoxO1)/ β -catenin signaling pathway affected the proliferation and differentiation of osteoblasts through its regulation of redox balance, and RES regulated the expression of FoxO1 to control white adipose tissue and then ameliorate an overweight condition. Based on previous research, we hypothesized that RES regulates FoxO1 transcriptional activity through the phosphatidylinositol-3-kinase (PI3K)/AKT signaling pathway to achieve an antioxidative effect on osteoporosis and then we confirmed this hypothesis in the present study. An ovariectomized (OVX) rat model of osteoporosis and a H_2O_2 -induced oxidative cell injury model in RAW 264.7 cells were established to explore the underlying molecular mechanisms of how RES confers an antioxidant effect and prevents bone loss. The obtained results demonstrated that RES strongly prevented bone loss induced by oxidative stress *in vivo*. More specifically, RES effectively decreased the receptor activator of nuclear factor- κ B ligand (RANKL) together with the tartrate-resistant acid phosphatase-5b (TRAP-5b) level, but elevated the osteoprotegerin (OPG) level and attenuated bone microarchitecture damage. Notably, RES, due to its antioxidant effect, suppressed RANKL production and then inhibited osteoclastogenesis in the OVX rats. *In vitro*, RES improved the oxidative stress status of cells and thus inhibited the mRNA expression of osteoclast-specific enzymes. These data indicate that RES has a significant bone protective effect by antagonizing oxidative stress to suppress osteoclast activity, function and formation both *in vivo* and *in vitro*. Moreover, at the molecular level, we confirmed, for the first time, that RES upregulated FoxO1 transcriptional activity by inhibiting the PI3K/AKT signaling pathway, and hence promoted resistance to oxidative damage and restrained

osteoclastogenesis. Inhibition of the PI3K/AKT signaling pathway may be induced by RANKL. FoxO1 is a major action target of RES to confer anti-osteoporosis function, and whose effect stems from its power to improve redox balance.

Introduction

Osteoporosis is a metabolic disease of bone, characterized by bone mass decrease, bone microarchitecture deterioration and increasing risk of fracture, since the bone resorption by osteoclasts surpasses the bone formation by osteoblasts. A variety of local and circulating hormones, cytokines and intermediary metabolites may affect the number and activity of both bone-forming osteoblasts and bone resorption osteoclasts (1,2). Currently, there is a growing body of study demonstrating that oxidative stress plays a crucial role in the pathophysiology of osteoporosis (1,3-7). Here, oxidative stress is caused when the production of reactive oxygen species (ROS) exceeds the antioxidant defense capacity (8). Abundant evidence has demonstrated that ROS can elevate the receptor activator of nuclear factor- κ B ligand (RANKL) expression in mouse osteoblasts and in human MG63 cells, and then stimulate the differentiation of osteoclasts from its precursor and therefore result in increased bone resorption (9-11). Accordingly, in the environment of the bone, the elevated level of ROS leads to increased bone resorption and impaired osteoblast function. That is, oxidative stress indeed takes part in the regulation of bone mass and is the chief culprit of osteoporosis (12).

In addition, ROS can trigger activation of specific physiologic signaling pathways. Cells protect themselves against the adverse effect of ROS by upregulating enzymatic scavengers or expression of DNA damage repair genes. This reaction involves dephosphorylation and subsequent activation of a small family of ubiquitous transcription factors known as Forkhead box O (FoxOs) (13-15). FoxOs, as important antioxidant defense factors, promote oxidative stress resistance in mammals (16,17). Rached *et al* reported that, among the 4 FoxO proteins (FoxO1, FoxO3, FoxO4 and FoxO6), only FoxO1 is required for proliferation and redox balance in osteoblasts through cell-specific deletion and molecular analyses, and as a result FoxO1 controls bone formation (12). FoxO1 activation is normally restrained by the phosphatidylinositol-3-kinase (PI3K)/AKT signaling pathway, which prevents FoxO1 translocation into the nucleus, and it counteracts the generation of ROS by upregulating the

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expression of antioxidant enzymes [glutathione peroxidase (GSH-PX)] and superoxide dismutase (SOD) (18-20). In addition, the oxidative stress induced by homocysteine deranges insulin-sensitive FoxO1 and MAP kinase signaling cascades to decrease osteoprotegerin (OPG) and increase RANKL synthesis in osteoblast cultures, leading to the stimulation of the osteoclast formation (21). Furthermore, Bartell *et al* elucidated that macrophage colony-stimulating factor (M-CSF) and RANKL promote the accumulation of H₂O₂ in osteoclasts along with their progenitors via an AKT-mediated repression of FoxO transcription which lowers catalase protein level.

Resveratrol (3,5,4-trihydroxystilbene, RES), a natural polyphenolic component extracted from red grapes, peanuts and other plants, is known to exert many beneficial pharmacological effects such as antitumor, scavenging free radicals, anti-inflammation, cardioprotection and vasoprotection activities (22-24). Clinical and experimental studies have shown that RES prevents bone loss by attenuating the damage caused by oxidative stress. RES can induce the production of major cellular antioxidant enzymes, such as GSH-PX, heme oxygenase and SOD, resulting in marked attenuation of oxidative stress (25,26). Significantly, RES was also shown to inhibit mitochondrial production of ROS in the vasculature (27). Furthermore, a previous study confirmed that RES intake relieved alveolar bone resorption and improved systemic oxidative stress in a rat periodontitis model (28). Bhattarai *et al* also demonstrated that RES treatment exhibited multiple beneficial effects for suppressing alveolar bone loss and these effects were the result of its antioxidative, anti-inflammatory, bone formation stimulating, and anti-osteoclastogenic activity (29). It is believed that the bone protective effect of RES is primarily due to its antioxidant activity. More importantly, RES exhibited no toxic effects, and therefore RES can be safely used for the treatment and/or prevention of osteoporosis, even if used for a long time (30). However, the explicit molecular mechanisms of how RES, as a natural antioxidant, plays a major role in preventing bone loss, have not yet been well understood.

Additionally, our previous research revealed that the effects of the FoxO1/ β -catenin signaling pathway on the proliferation and differentiation of osteoblasts are achieved through its regulation of redox balance via the upregulation of antioxidant enzyme levels as well as the expression of DNA damage repair-related gene Gadd45, while inhibiting the expression of apoptosis-related gene Bim along with the osteoblast inhibition gene peroxisome proliferator-activated receptor- γ (PPAR- γ). On the other hand, RES regulates the expression of FoxO1 by inhibiting the PI3K/AKT signaling pathway to control white adipose tissue and then ameliorate an overweight condition induced by a high fat diet. Based on the above studies, we speculate that RES regulates FoxO1 transcriptional activity via the PI3K/AKT signaling pathway to protect against oxidative damage in osteoporosis. In order to further confirm this speculation, in the present study, we established an ovariectomized (OVX) rat model of osteoporosis *in vivo* and an osteoclast oxidative stress model induced by RANKL and H₂O₂ in RAW 264.7 cells *in vitro* to explore the underlying molecular mechanisms of how RES exhibits an antioxidant effect and prevents bone loss. Implementation of this project revealed the effect of RES against oxidative stress damage together with its molecular mechanisms, and also

provides a new scientific basis for the clinical application of RES in the prevention and/or treatment of osteoporosis.

Materials and methods

Ovariectomy and administration of RES. Thirty female Sprague-Dawley (SD) rats, aged 3 months, body weight 220 \pm 19.27 g, were purchased from Gansu University of Chinese Medicine and were equally divided into three groups randomly. Ten rats were administered sham operation (control rats; Sham group) and 20 rats underwent bilateral OVX under 10% chloral hydrate anesthesia (OVX group). One week postoperative, all rats were administered the medicine by subcutaneous injection: the Sham group and OVX group received equal amount of sesame oil (vehicle; Sigma-Aldrich, St. Louis, MO, USA); the third group (OVX+RES group) received RES solution (40 mg/kg body weight, once daily; Sigma-Aldrich). After treatment for 10 weeks, the rats were sacrificed, and the blood along with the bone were collected for further analyses. All experiments performed involving SD rats were approved by the Animal Experimental Ethical Inspection of Gansu University of TCM (permission code 2015-060).

Bone μ -CT scanning. We dissected the femurs, cleaned the soft tissues, fixed them in 10% neutral formalin for 48 h, and then immersed them into 70% ethanol. Bone microarchitecture in the middle and distal femur was scanned by using micro-computed tomography (μ -CT; VivaCT 40; Scanco, Brüttisellen, Switzerland) with 15 μ m resolution, tube voltage of 70 kV and tube current of 114 μ A. The reconstruction and 3D quantitative analyses were performed using software provided by a desktop μ -CT system (VivaCT 40; Scanco). In the femur, cortical bone volume (Ct.Vo) and cortical thickness (Ct.Th) were analyzed with the help of the reference point at the mid-diaphysis, with whole cortical bone, where the region of interest (ROI) was 1.0 mm. Trabecular bone region reference point was at the growth plate joining, offset 2.0 mm, ROI 2.0 mm. The following 3D parameters in the defined ROI were analyzed, including the relative bone volume over total volume (BV/TV, %), trabecular number (Tb.N), trabecular thickness (Tb.Th), trabecular separation (Tb.Sp), connectivity density (Conn.D), structure model index (SMI) and bone mineral density (BMD).

Bone histomorphometric analysis. The right isolated tibias maintained in 70% alcohol were used for the bone shape analysis. The processes of alcohol dehydration, xylene transparency undecalcified plastic embedding, sectioning (thickness of 30 μ m) followed by Van Gieson staining were included. Bone morphological measurement indices were analyzed using IPP 6.0 analysis software.

Enzyme-linked immunosorbent assay (ELISA) measurement. Rat OPG, RANKL and tartrate-resistant acid phosphatase-5b (TRAP-5b) ELISA kits as well as a ROS ELISA kit were purchased from Myhalic Biotechnology Co., Ltd. (<http://www.myhalic.com/>; Wuhan, China). MDA, SOD and GSH-PX were measured by employing specific kits (Jiancheng, Nanjing, China) following the manufacturer's instructions.

Table I. RT-PCR primers employed for amplification of specific mRNAs.

Gene	Forward (5'-3')	Reverse (5'-3')
Actb	CATCCGTAAAGACCTCTATGCCAAC	ATGGAGCCACCGATCCACA
MMP-9	CCATGCACTGGGCTTAGATCA	GGCCTTGGGTCAGGCTTAGA
TRAP	GTGCTGGCTGGAAACCATGA	GTCCAGCATAAAGATGGCCACA
Cathepsin K	CACCCAGTGGGAGCTATGGAA	GCCTCCAGGTTATGGGCAGA

TRAP, tartrate-resistant acid phosphatase; MMP-9, matrix metalloproteinase-9.

Cell culture. The mouse macrophage cell line RAW 264.7, purchased from the American Type Culture Collection (ATCC, TIB-71™; Manassas, VA, USA), was cultured in high-glucose Dulbecco's modified Eagle's medium (DMEM; Gibco, Grand Island, NY, USA) containing 10% fetal bovine serum (FBS; TAN, South America) in a 25 cm² culture flask at 37°C in a 5% CO₂ incubator. Culture media were changed every other day. Cells were incubated in an osteoclastic induction medium supplemented with 100 ng/ml RANKL (315-11; PeproTech, Rocky Hill, NJ, USA) for 7 days. This cell line has been shown to express receptor activator of NF-κB (RANK) and differentiate into TRAP-positive, functional osteoclasts when co-cultured with soluble RANKL (31,32). The RAW 264.7 cells were cultured to 80% confluence, and the medium was replaced according to the specific group. The cultured cells were randomly divided into four groups: the RANKL control group, 10⁻⁴ M H₂O₂ group, 10⁻⁵ M RES group, and 10⁻⁴ M H₂O₂ plus 10⁻⁵ M RES group. We would like to stress that in the latter three groups, the cells were also incubated in an osteoclastic induction medium supplemented with 100 ng/ml RANKL. H₂O₂ was added 1 h prior to treatment with RES. All of the experiments were repeated 3 or 5 times.

Cytotoxicity assay. The cytotoxic effects of RES or H₂O₂ were detected using Cell Counting kit-8 (CCK-8; Jiancheng), according to the manufacturer's instructions. Briefly, 8,000 cells were seeded per well on a 96-well plate. The cells were treated with 10⁻⁵ M RES or 10⁻⁴ M H₂O₂ and cultured for 24, 48 and 72 h, respectively, to measure the toxicity at 450 nm using a microplate reader.

TRAP staining. After induction of culture for 7 days, TRAP staining was performed to evaluate TRAP(+)-multinucleated osteoclast formation. RAW 264.7 cells were cultured on a 24-well plate at a density of 2x10⁴ cells/well and allowed to adhere overnight. DMEM was then replaced, and the cells were treated with new DMEM containing 100 ng/ml RANKL. After induced culture for 7 days, the cells were stained for TRAP expression using an Acid Phosphatase, Leukocyte (TRAP) kit (387-A; Sigma-Aldrich) following the manufacturer's instructions.

Assessment of the culture supernatant MDA, SOD and GSH-PX. We harvested the culture supernatant after the RAW 264.7 cells were cultured as mentioned in the different groups in the induction medium for 24 h on 96-well plates at a density of 8,000 cells/well, and we assessed the MDA,

SOD and GSH-PX by employing the relevant kits (Jiancheng) according to the manufacturer's instructions.

Intracellular ROS formation. The generation of ROS was probed with the ROS fluorescent probe-dihydroethidium (DHE). Cells were plated on 6-well plates at 6x10⁴ cells/well, and were induced-cultured in terms of the above groups for 24 h. After this treatment, the cells were washed with PBS and then incubated with 20 μM DHE for 30 min at 37°C. DHE is oxidized by ROS when it diffuses into the cells, and then the intracellular oxidized DHE was measured via flow cytometry.

Real-time PCR. Cells were plated in a 25 cm² flask at 2x10⁵ cells/flask, and induced-cultured in regards to the above groups for 7 days. Total RNA was extracted from the cultured cells applying RNAiso™ Plus (cat. no. 9108; Takara, Tokyo, Japan). The RNA concentration was determined spectrophotometrically, and only pure RNA (A260:A280 ratio in 1.8-2.1) was used for further analysis. Total RNA (5 μl) (500 ng) from each sample was reverse-transcribed using PrimeScript™ RT Master Mix (perfect real-time) (code: RR036A; Takara). Complementary DNA was mixed with SYBR Premix Ex Taq™ II (Tli RNaseH Plus) (code: RR820A; Takara) and then was subjected to PCR amplification using the LightCycler/LightCycler 480 system real-time PCR. Denaturation was carried out at 95°C for 30 sec (ramp rate 4.4°C/sec) 1 cycle; PCR (quantification) at 95°C for 5 sec (ramp rate 4.4°C/sec) and 60°C for 30 sec (ramp rate 2.2°C/sec) 40 cycles; melting (melting curves) was performed at 95°C for 5 sec (ramp rate 4.4°C/sec) followed by 60°C for 60 sec (ramp rate 2.2/sec) and 95°C (ramp rate 0.11°C/sec, acquisition mode: continuous, acquisitions: 5 per) 1 cycle; and cooling was carried out at 50°C for 30 sec [ramp rate 2.2°C/sec) 1 cycle]. The primers used for real-time PCR are listed in Table I.

Western blot analysis. The cells were plated in a 25 cm² flask with 2x10⁵ cells/flask, and induced-culture was carried out in terms of the above groups for 7 days. Cells were lysed using a lysis buffer containing 50 mM Tris (pH 7.4), 150 mM NaCl, 1% Triton X-100, 1% sodium dexycolate, 0.1% SDS, sodium orthovanadate, sodium fluoride, EDTA, and leupeptin inhibitor (code P0013B; Beyotime, Shanghai, China). The lysates (20-30 μg) were separated using 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to nitrocellulose blotting membranes. After blocking with 5% skim milk (detection of phosphorylated proteins with BSA) for 1 h at room temperature, the membranes were incubated with

Table II. Trabecular microstructural and cortical geometric properties of the right femurs evaluated *ex vivo* using μ -CT.

Parameters	Sham	OVX	OVX+RES
BV/TV (%)	47.48±6.00	20.88±7.53 ^b	36.14±8.62 ^{a,c}
Tb.N (1/mm)	4.90±0.61	2.94±0.79 ^b	4.79±0.26 ^d
Tb.Th (μ m)	81.43±16.64	51.80±3.74 ^a	78.67±14.76 ^c
Tb.Sp (mm)	0.13±0.03	0.24±0.04 ^b	0.13±0.02 ^d
Conn.D (1/mm ³)	155.59±22.56	108.78±18.59 ^b	144.86±28.12 ^c
SMI	0.42±0.13	1.87±0.27 ^b	1.23±0.20 ^{b,d}
BMD (mg HA/ccm)	732.42±15.68	693.79±11.46 ^a	729.66±4.81 ^c
Ct.Vo (mm ³)	4.86±0.64	5.18±0.50	5.52±0.87
Ct.Th (mm)	2.82±0.90	2.72±0.27	2.86±1.17

Results are presented as the mean \pm SD. ^aP<0.05 and ^bP<0.01 vs. Sham group; ^cP<0.05 and ^dP<0.01 vs. OVX group (n=5-7/group). OVX, ovariectomized; BMD, bone mineral density; SMI, structure model index; Tb.N, trabecular number; Tb.Th, trabecular thickness; Tb.Sp, trabecular separation; Conn.D, connectivity density; Ct.Vo, cortical bone volume; Ct.Th, cortical thickness.

anti- β -actin (1:5,000; ab6276), anti-AKT (1:500; ab28422), anti-p-AKT (1:500; ab8932), anti-FoxO1 (1:1,000; ab52857), and anti-p-FoxO1 (1:500; ab131339) (all from Abcam, Cambridge, UK) antibodies overnight at 4°C, and then were incubated with the secondary antibody (1:1,000; Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA) for 1 h at 37°C.

Analysis of apoptosis. Cells were plated on 6-well plates at 6×10^4 cells/well, and cultured in terms of the above groups for 3 days. After this treatment, cells were washed with phosphate-buffered saline (PBS) and then apoptosis was assessed using the Annexin V/PI double staining assay via flow cytometry.

Statistical analysis. Data are expressed as mean \pm standard deviation (SD). The values were analyzed by employing a one-way ANOVA of variance followed by a LSD test for multiple comparisons. The statistical significance was defined as P<0.05. Error bars in all figures represent SD.

Results

RES prevents the deterioration of trabecular bone micro-architecture induced by OVX. One week postoperative, we administered RES (40 mg/kg body weight, once daily) to OVX rats for 10 weeks to examine its protective effects against bone loss. The animals were all sacrificed 11 weeks after the operation. When compared with the Sham group rats, OVX significantly induced deterioration of the trabecular bone microarchitecture, and reduction in BV/TV, Tb.N, Tb.Th, Conn.D and BMD (P<0.05 and P<0.01) (Table II). In contrast, other microstructural parameters such as Tb.Sp and SMI were sharply increased in response to OVX (P<0.01). However, treatment of OVX rats with RES at 40 mg/kg body weight markedly reversed changes in these parameters induced by OVX. In μ -CT images of the distal femur, these changes in trabecular bone parameters were readily observed (Fig. 1A). Ct.Vo and Ct.Th as determined by μ -CT were also analyzed (Table II). OVX did not affect the cortical bone volume and thickness of the femur mid-diaphysis (P>0.05). Simultaneously, bone histomorphology of the right tibiae detected using Van Gieson staining were carried out and

also exhibited similar results (Fig. 1B). The trabecular number was decreased and spaces between trabeculae were broader in the OVX group compared with the Sham group. Nevertheless, treatment with RES significantly inhibited the OVX-induced deleterious effects, as demonstrated in the OVX+RES group via an increase in the trabecular number and a decrease in the trabecular space. The analysis of the properties of trabecular and cortical bone in the middle and distal femurs indicated that OVX induces damage of the trabecular bone micro-architecture in rats, yet RES was able to attenuate the damage effects.

RES suppresses osteoclast activity and formation. In order to determine the possible regulatory effects of RES on osteoclast activity and formation, we examined the production of TRAP-5b, OPG and RANKL in the serum of rats. TRAP-5b is released by osteoclasts, reflecting osteoclast activity and the bone resorption status. Upon comparison with the Sham group, the TRAP-5b content in the serum was increased following OVX. Yet, as shown in Fig. 2A, RES markedly reversed the change (P<0.01). OPG and RANKL are primarily released from osteoblasts. Osteoblast-derived RANKL binds to RANK on osteoclasts, resulting in osteoclast activation. OPG, osteoclastogenesis inhibitory factor, has also been demonstrated to compete with RANKL for binding to RANK, leading to suppression of osteoclast formation. Thus, osteoclast formation is reflected by the ratio of OPG/RANKL. Compared with the Sham group, as shown in Fig. 2B, the level of OPG was reduced, while RANKL was enhanced, and OPG/RANKL was significantly decreased in response to OVX (P<0.01). However, RES intervention recovered at least part of the changes (P<0.01) in the parameters induced by OVX. Our results revealed that, under OVX, osteoclast activity and formation were enhanced, while RES greatly weakened the OVX effects.

RES improves the antioxidant power in the rats. To further confirm the fact that bone protective effects of the RES are primarily caused by antagonizing the OVX-induced oxidative stress damage, the levels of MDA, ROS, SOD and GSHPX in the serum of rats were determined. Analysis of the oxidative

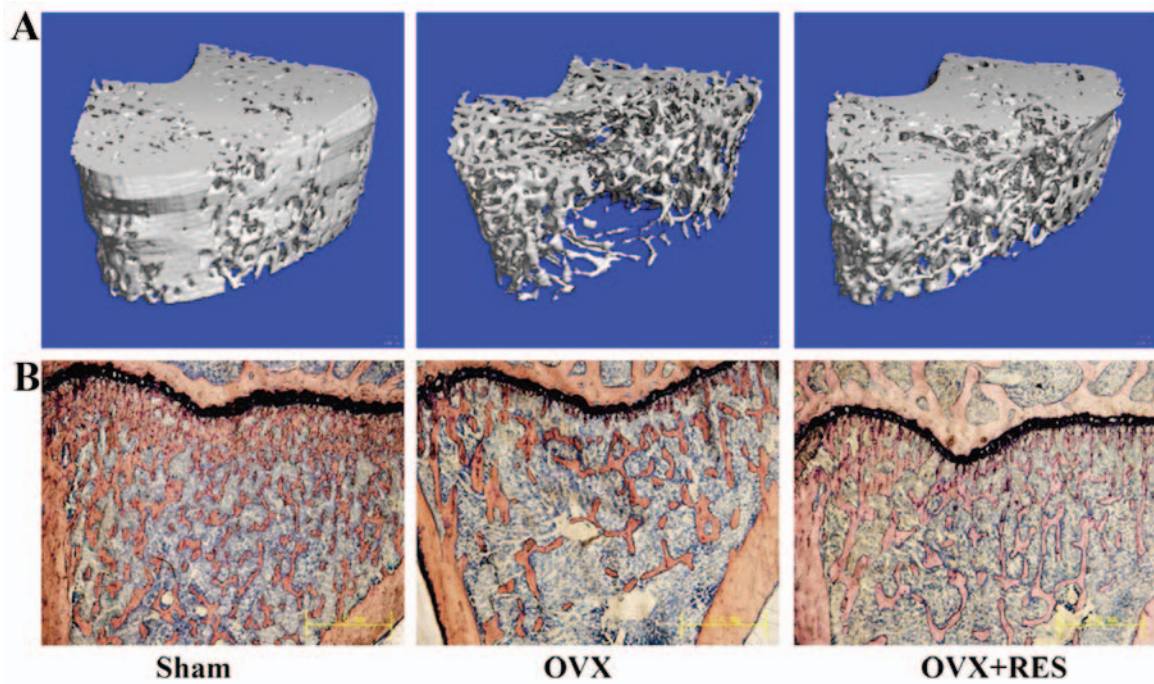


Figure 1. Resveratrol (RES) prevents bone microarchitecture damage induced in ovariectomized (OVX) rats. (A) The right femurs of rats were scanned using μ -CT in the Sham group, OVX group and OVX+RES group. (B) Bone histomorphology of the right tibias was assessed using Van Gieson staining in the Sham group, OVX group and OVX+RES group (10 x 4).

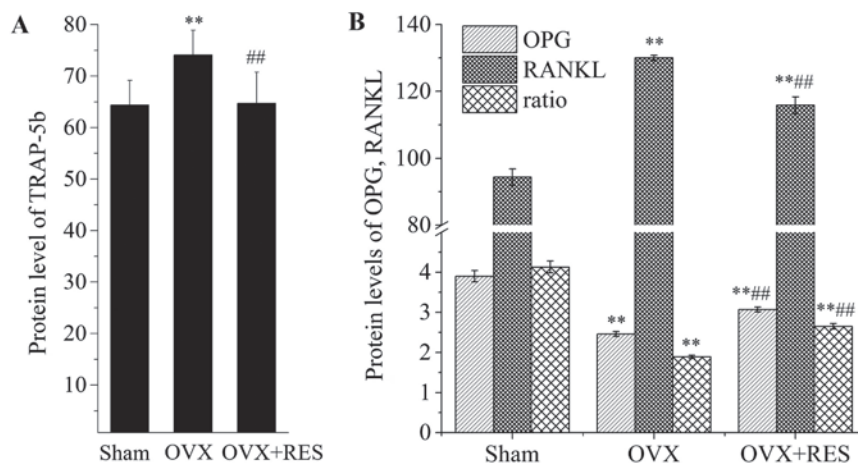


Figure 2. Osteoclast activity and formation are restrained by resveratrol (RES) *in vivo*. The protein levels of tartrate-resistant acid phosphatase-5b (TRAP-5b), osteoprotegerin (OPG) and receptor activator of nuclear factor- κ B ligand (RANKL) in the serum of rats were examined by applying enzyme-linked immunosorbent assay (ELISA) kits. (A) RES lowered the TRAP-5b level in the serum of rats in the ovariectomized (OVX)+RES group. (B) RES intervention elevated the OPG/RANKL ratio (namely, OPG enhancement vs. RANKL decrease). Values are expressed as mean \pm SD. ** P <0.01 compared with the Sham group; ## P <0.01 compared with the OVX group (n=5-7/group).

stress status of the experimental rats indicated that the Sham group also generated traces of MDA and ROS. When rats underwent the OVX operation, the MDA and ROS concentrations were increased, while the contents of SOD and GSH-PX were reduced, as compared with the Sham group. Upon treatment with RES to the OVX rats, changes were also observed when compared to the OVX group; that is, MDA and ROS levels were highly decreased while the antioxidant enzymes such as SOD and GSH-PX were markedly elevated (P <0.05 and P <0.01) (Fig. 3). On the whole, these findings indicated that OVX induced oxidative stress, resulting in a lowered OPG level but enhanced RANKL and TRAP-5b levels, and

an increase in osteoclast formation together with activity, and eventually bone microarchitecture damage. Notably, RES enhanced the OPG content, lowered the RANKL and TRAP-5b levels, and finally reduced bone resorption by improving the oxidative stress status. Subsequently to explore the hidden molecular mechanisms of the bone protective effects of RES, we stimulated the RAW 264.7 cells with RANKL to induce osteoclasts *in vitro*. Before detecting the effects of H_2O_2 and RES on osteoclast activity along with function, the cell viability (Fig. 4) was determined. The potential cytotoxicity of these agents was tested since their toxicity would influence the cell survival and consequently lower the cell number. Either

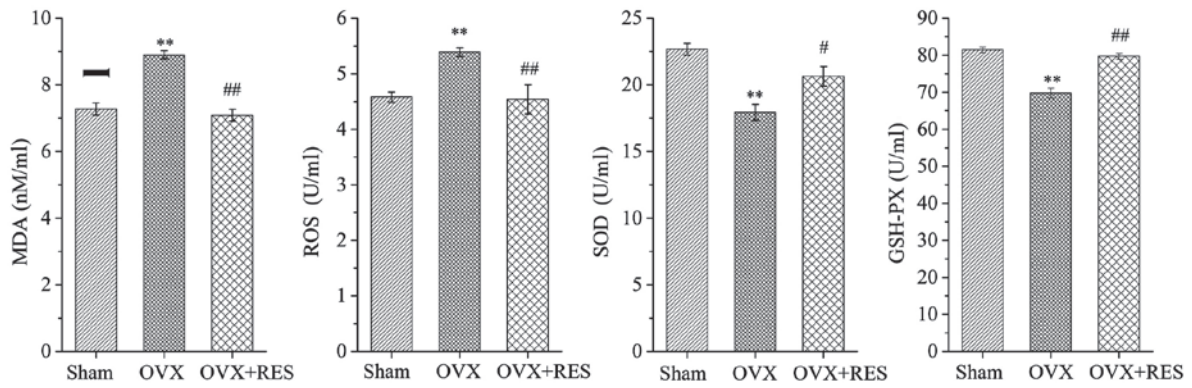


Figure 3. The ovariectomized (OVX)-induced oxidative stress damage is antagonized by resveratrol (RES) *in vivo*. The levels of MDA, reactive oxygen species (ROS), SOD and glutathione peroxidase (GSH-PX) in the serum of rats were determined. Following treatment of the OVX rats with RES, MDA and ROS levels were highly decreased and the antioxidant enzymes such as SOD and GSH-PX were markedly elevated. Values are expressed as mean \pm SD. * $P < 0.05$ and ** $P < 0.01$ compared to the Sham group; # $P < 0.05$ and ## $P < 0.01$ compared to the OVX group ($n = 5-7/\text{group}$).

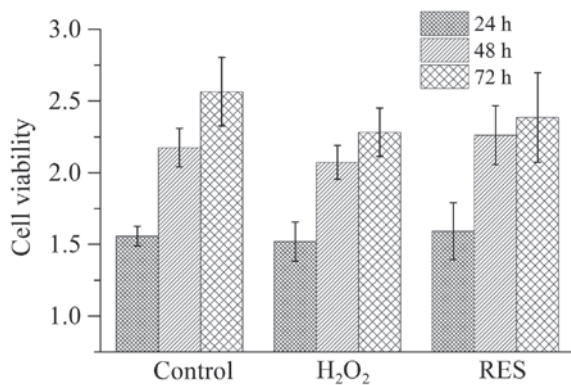


Figure 4. Cell viability as determined with Cell Counting kit-8 (CCK-8) assay. Results are expressed as OD values. Each bar represents the mean \pm SD of five independent experiments. Resveratrol (RES) at 10^{-5} M increased the RAW 264.7 cell viability and 10^{-4} M H₂O₂ decreased the cell viability, but statistical differences were not achieved compared with the control group ($P > 0.05$).

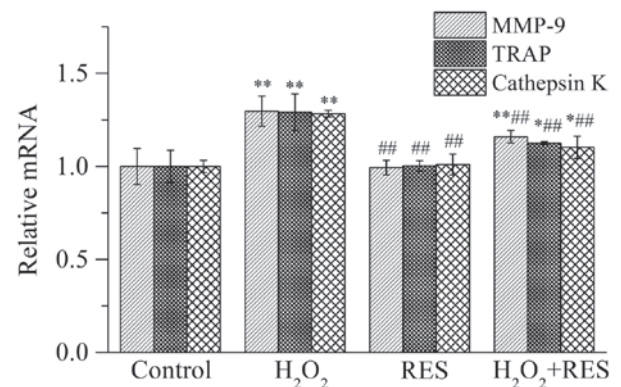


Figure 5. Expression of osteoclast marker enzymes was reduced by resveratrol (RES). To evaluate osteoclast activity, the mRNA expression of osteoclast marker enzymes (MMP-9, TRAP and cathepsin K) was assessed using RT-PCR. In the presence of RES, the effects of the stimulation of mRNA expression of osteoclast marker enzymes by H₂O₂ were greatly abolished. Results are presented as the mean \pm SD. * $P < 0.05$ and ** $P < 0.01$ vs. the control group; # $P < 0.05$, ## $P < 0.01$ vs. the H₂O₂ group.

10^{-4} M H₂O₂ or 10^{-5} M RES did not have a cytotoxic effect on RAW 264.7 cells ($P > 0.05$).

RES reduces the expression of osteoclast-related marker enzymes. In order to evaluate the osteoclast activity *in vitro*, the expression of osteoclast marker enzymes was assessed by employing RT-PCR, including matrix metalloproteinase-9 (MMP-9), TRAP, and cathepsin K. Expressed specifically by osteoclasts, these three enzymes are important indices which reflect osteoclast activity as well as bone resorption function. As shown in Fig. 5, compared with the control group, the mRNA expression levels of MMP-9, TRAP and cathepsin K were increased ($P < 0.01$) in the presence of 10^{-4} M H₂O₂, indicating that osteoclast activity along with bone resorption function was facilitated by H₂O₂. In contrast, following treatment of the cells with RES at 10^{-5} M, the expression levels of the osteoclast marker enzymes were reduced compared with the H₂O₂ group ($P < 0.01$), indicating that RES inhibited osteoclast activity and bone resorption capacity.

RES abrogates oxidative stress in cells. To elucidate whether the inhibitory effects of RES on osteoclast activity and bone

resorption power are linked to its antioxidant properties, the MDA, ROS, SOD and GSH-PX levels were detected. As shown in Fig. 6, when RAW 264.7 cells were exposed to 10^{-4} M H₂O₂, the production of MDA and ROS was strongly enhanced, while the SOD and GSH-PX levels were decreased ($P < 0.01$). The above results demonstrated that H₂O₂ stimulated oxidant generation and led to oxidative stress in RAW 264.7 cells. However, when the cells were treated with RES at a concentration of 10^{-5} M, MDA and ROS production was inhibited to some degree, while the SOD and GSH-PX levels were higher compared with the H₂O₂ group ($P < 0.05$ and $P < 0.01$). The study revealed that RES improves the oxidative stress status of the cells, and the suppressive effects of RES on the expression of osteoclast marker enzymes are linked to its antioxidant activity.

RES regulates FoxO1 transcriptional activity by inhibiting the PI3K/AKT signaling pathway. The PI3K/AKT/FoxO1 signaling pathway plays a crucial role in osteoclastogenesis. Furthermore, to confirm the RES-attenuated oxidative stress damage and RES-suppressed osteoclastogenesis by inhibiting the PI3K/AKT signaling pathway, we evaluated the effects of

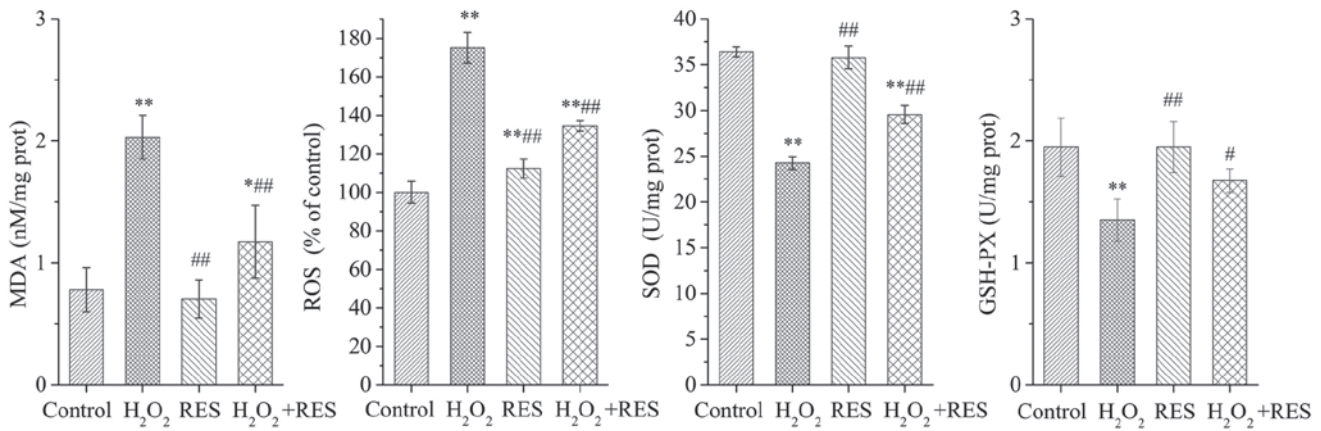


Figure 6. The oxidative stress status of the cells is improved by resveratrol (RES). RES suppressed the H₂O₂-induced elevation of MDA and reactive oxygen species (ROS) levels, yet improved the H₂O₂-induced decrease in antioxidant enzyme [SOD, glutathione peroxidase (GSH-PX)] levels. Results are presented as the mean \pm SD. *P<0.05 and **P<0.01 vs. the control group; #P<0.05 and ##P<0.01 vs. the H₂O₂ group.

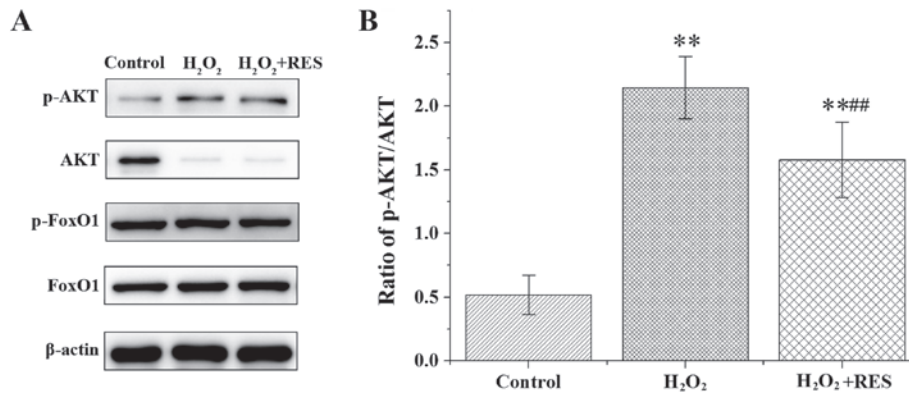


Figure 7. Forkhead box O1 (FoxO1) protein level is elevated by resveratrol (RES). (A) The protein levels of p-AKT, AKT, p-FoxO1 and FoxO1 were determined by western blot analysis. (B) RES lowered the ratio of p-AKT/AKT in osteoclasts from the H₂O₂+RES group in comparison with this ratio in osteoclasts from the H₂O₂ group. Results are presented as the mean \pm SD. *P<0.05 vs. the control group; **P<0.01 vs. the H₂O₂ group.

RES on the PI3K/AKT/FoxO1 signaling pathway following stimulation with 100 ng/ml RANKL in RAW 264.7 cells. AKT, phosphorylated AKT (p-AKT), FoxO1 and p-FoxO1 were examined by western blot analysis. Compared with the control group, H₂O₂ elevated the ratio of p-AKT/AKT and the protein content of p-FoxO1, yet the FoxO1 protein level was reduced (P<0.05 and P<0.01) (Fig. 7). According to these results, FoxO1 transcriptional activity was suppressed by H₂O₂. Following treatment with RES, the above effects of H₂O₂ were abolished, at least in part (P<0.05 and P<0.01). These data indicate the RES was able to upregulate FoxO1 transcriptional activity by inhibiting the PI3K/AKT signaling pathway. In brief, our results revealed that RES upregulated FoxO1 transcriptional activity to achieve attenuation of oxidative stress damage and inhibition of osteoclastogenesis.

RES regulates the apoptosis of RAW 264.7 cells. The inhibition of the PI3K/AKT signaling pathway leads to dephosphorylation and nuclear translocation of active FoxO1, which causes cell cycle arrest and apoptosis (33). Finally, we examined whether RES induces apoptosis by inhibiting the PI3K/AKT signaling pathway. Cells were cultured by adding H₂O₂ or H₂O₂+RES for 3 days. After this treatment, apoptosis was determined. Treatment of RAW 264.7 cells with 10⁻⁵ M RES

promoted apoptosis (P<0.01) (Fig. 8). Based on the data, it was revealed that RES induced apoptosis and as a result restrained osteoclastogenesis via suppressing the PI3K/AKT signaling pathway.

Discussion

RES, a natural polyphenolic component, is known to exert numerous beneficial pharmacological effects including antitumor, scavenging free radical and anti-inflammatory activities (22-24). Clinical and experimental investigations suggest that RES prevents bone loss by attenuating the damage of oxidative stress (25,26). Furthermore, a previous study of our group demonstrated that OPG production was boosted, whereas RANKL synthesis was decreased in RES-treated OVX rats, and hence consequently osteoclast formation and differentiation were prevented (34). More importantly, RES had no toxic effect and therefore can be safely used even for the long-term treatment and/or prevention of osteoporosis (30). However, the underlying molecular mechanisms of how RES, a natural antioxidant, plays a major role in preventing bone loss, has not yet been fully elucidated. In the present study, we explored the potential molecular mechanisms of RES against osteoporosis.

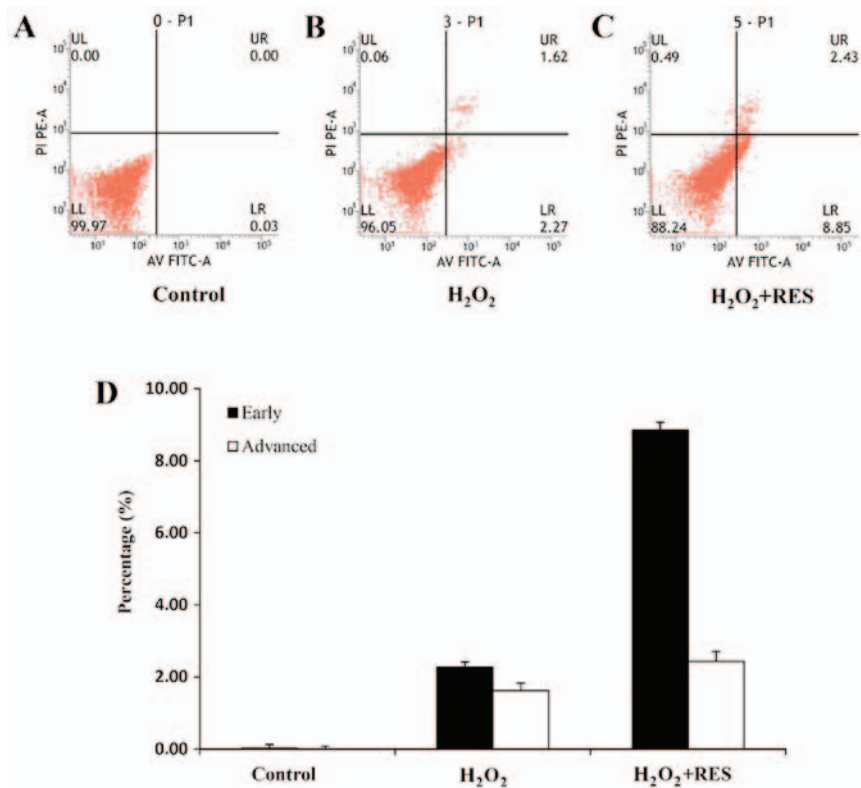


Figure 8. RAW 264.7 cell apoptosis is induced by resveratrol (RES). RAW 264.7 cells were incubated with H₂O₂ or H₂O₂+RES for 3 days. At the end of the incubation period, the cells were harvested, and apoptosis analysis was performed. Cell apoptosis (A-C) was statistically analyzed (D). $P < 0.05$.

Our results demonstrated that, by improving the oxidative stress status, RES enhanced the ratio of OPG/RANKL (namely, OPG enhancement vs. RANKL decrease), suppressed osteoclastogenesis, and thus eventually attenuated bone resorption and prevented bone loss *in vivo*. Simultaneously, *in vitro*, the activity along with the function of osteoclasts was facilitated following exposure to 10^{-4} M H₂O₂, but these were inhibited in the presence of RES owing to its effect of relieving the oxidative stress damage. Apart from the above findings, RES was able to induce apoptosis of RAW 264.7 cells and thus restrained osteoclastogenesis. Moreover, at the molecular level, we confirmed that RES upregulated the transcriptional activity of FoxO1 by inhibiting the PI3K/AKT signaling pathway, and caused protection against oxidative damage and inhibited osteoclastogenesis in osteoporosis.

Oxygen-derived free radicals are produced as by-products of aerobic metabolism. This process occurs primarily in mitochondria due to electron escape passing through the electron transport chain (35,36), and generates highly reactive and short-lived superoxide that is rapidly converted to the more stable and less reactive H₂O₂ (37-39). H₂O₂, as the most abundant form of ROS, diffuses freely through the mitochondrial membranes into the cytosol (37-39). Oxidative stress is the result of elevated ROS, which damages protein, lipids, and DNA and eventually triggers cell death. H₂O₂ additionally serves usually as both an extracellular and intercellular signal molecule (40). In the present study, 10^{-4} M H₂O₂ was used to induce oxidative stress.

RANKL and its receptor, pivotal factors required for osteoclast differentiation, are fundamental and necessary to

promote osteoclastogenesis (41). RANKL plays a dominant role in activation of the osteoclast differentiation program, including the necessary genes required for bone resorption and for fusion of monocyte progenitor cells (42). When it binds to RANK, it triggers several intracellular signaling pathways in osteoclast precursor cells, ultimately inducing the expression of osteoclast-specific genes. OPG, as an inhibitor of RANKL, also binds to RANK to antagonize the effect of RANKL and thereby regulates osteoclast activity and function in the bone. We here employed the mouse macrophage cell line, namely, RAW 264.7 cells, and stimulated the cells with 100 ng/ml RANKL to induce osteoclastogenesis (32).

There have been several previous studies providing key evidence that ROS take part in bone regulation. ROS, especially H₂O₂, may be involved in the regulation of osteoclast formation (43), and has been observed both *in vitro* and *in vivo* to be produced by osteoclasts (44-46). On the other hand, H₂O₂ production in differentiated osteoclasts can also be stimulated by RANKL (47). Bartell *et al* found that RANKL promotes the accumulation of H₂O₂ in osteoclasts and in their progenitors. In turn, H₂O₂ improves osteoclast progenitor proliferation (11). Furthermore, Kim *et al* provided evidence that ROS play an important role in osteoclast differentiation through NF- κ B regulation, while the antioxidant α -lipoic acid inhibits osteoclast differentiation by reducing NF- κ B DNA binding and has a potential therapeutic effect against bone erosive diseases (48). Additionally, ROS enhanced the expression of RANKL in mouse and human MG63 cells (9). Therefore, the increased ROS (particularly the H₂O₂) level is a critical regulatory-step of osteoclastogenesis and hence bone

resorption, which is not a mere epiphenomenon of increased mitochondria number and/or function required to meet the high-energy demands of osteoclastic bone resorption (11).

Our findings showed that OVX induced oxidative stress (ROS and MDA increased, yet SOD and GSH-PX decreased in the OVX group, compared with the Sham group), and simultaneously promoted a decrease in the OPG level, an increase in the RANKL level, increased production of TRAP-5b and damaged bone microstructure. TRAP-5b in the serum, as a bone resorption marker enzyme, released by osteoclasts, well reflects the osteoclast activity directly along with bone resorption status *in vivo*. Meanwhile, *in vitro*, 10^{-4} M H_2O_2 used to induce oxidative stress resulted in the enhanced expression levels of osteoclast-specific enzymes (MMP-9, TRAP and cathepsin K), indicating that osteoclast activity and function were elevated. In contrast, the treatment of the OVX rats with RES significantly reversed these changes *in vivo*. RES, conferring antioxidant power, effectively decreased RANKL together with the TRAP-5b level, but elevated the OPG level and attenuated bone microarchitecture damage. Notably, the results demonstrated that RES, due to its antioxidant effect, suppressed the RANKL production in the OVX rats. In other words, the increased ROS level promoted the production of RANKL and then stimulated osteoclast differentiation and bone resorption. This conclusion is consistent with previous studies (9,11,47,48). Subsequently, the *in vitro* study demonstrated that RES at the concentration of 10^{-5} M improved the oxidative stress status of cells and inhibited the expression of osteoclast-specific enzymes. These data indicate that RES has a significant bone protective effect via antagonizing oxidative stress to suppress osteoclast formation together with bone resorption activity both *in vitro* and *in vivo*. Therefore, the reduction of ROS production may be a rational approach for the treatment of diseases associated with high bone resorption, including, for example, osteoporosis and arthritis. FoxO1 activation in osteoclasts could be one valid approach to achieve this goal. Notably, an important finding of this study is that the redox regulator FoxO1 is a target of RES resisting oxidative stress and inhibiting osteoclastogenesis.

As important protein resistance to oxidative stress, FoxOs regulate the expression of antioxidant enzymes, where FoxO1 is a major member of the FoxO family. Through cell-specific deletion and molecular analyses, among the 4 FoxO proteins, FoxO1 is the only factor required for proliferation and redox balance in osteoblasts, and as a result FoxO1 controls bone formation (12). FoxO1 is regulated predominantly through the PI3K/AKT signaling pathway and the FoxO1 protein is phosphorylated by the PI3K/AKT pathway, leading to inhibition of FoxO1-dependent transcription and impaired ability of DNA binding (49). AKT has been shown to directly phosphorylate and inactivate FoxO1, which results in cytoplasmic retention, inactivation and inhibition of the expression levels of FoxO1-regulated genes that control various processes such as metabolism, cell cycle, cell death and oxidative stress (50). In contrast, inhibition of the PI3K/AKT pathway induces dephosphorylation and nuclear translocation of active FoxO1, and then these processes enhance FoxO1 transcriptional activity, causing cell cycle arrest and apoptosis (33). In hematopoietic stem cells, FoxO1 has been demonstrated to reduce ROS by upregulating the expression of antioxidant enzymes including

peroxiredoxins, GSH-PX and catalase (19,20). After the deletion of FoxO1 in cells of the hematopoietic lineage, the number of osteoclast progenitors is increased in the bone marrow (51). Particularly, Bartell *et al* showed that RANKL promotes the accumulation of H_2O_2 in osteoclasts and their progenitors via an AKT-mediated repression of FoxO1 transcription that lowers catalase protein level. They also demonstrated that as a consequence of the loss of FoxO1 function, the osteoclast number and bone resorption are prevented by the systemic administration of catalase (11). This chain of events identify that FoxO1 is a major control node of osteoclastogenesis and bone resorption, both in physiologic or pathologic conditions. Furthermore, other studies elucidated that FoxO1 also plays a crucial role in bone metabolism by influencing osteoblast physiology and function, due to its ability to maintain redox balance via ROS-dependent or -independent mechanisms (12,51,52).

In view of the facts that FoxO1 can maintain redox balance and play a central role in bone metabolism and FoxO1 protein can be phosphorylated by AKT, the inhibition of the PI3K/AKT pathway and regulation of FoxO1 transcriptional activity could be regarded as an effective and novel strategy for the prevention and/or treatment of osteoporosis. Moreover, our previous studies revealed that the effects of the FoxO1/ β -catenin signaling pathway on the proliferation and differentiation of osteoblasts is achieved through its regulation of the redox balance (upregulating antioxidant enzyme levels as well as the expression of DNA damage-repair-related gene Gadd45, while inhibiting the expression of apoptosis-related gene Bim along with the osteoblast inhibition gene PPAR- γ). In contrast, RES regulates the expression of FoxO1 by inhibiting the PI3K/AKT signaling pathway to control white adipose tissue and then to ameliorate overweight condition induced by a high fat diet. Based on the above studies, we speculated that RES regulates FoxO1 transcriptional activity by the PI3K/AKT signaling pathway to remedy oxidative damage in osteoporosis. In order to support this hypothesis, we explored the effect of RES on the PI3K/AKT pathway. In this study, we revealed that more osteoclasts were formed, and the osteoclast activity as well as function were much higher in the presence of 10^{-4} M H_2O_2 used to induce oxidative stress. This enhancement was confirmed by the higher mRNA levels of osteoclast-specific enzymes.

Moreover, the p-FoxO1 protein level and the ratio of p-AKT/AKT were increased in the osteoclasts in response to H_2O_2 . The results indicated that 10^{-4} M H_2O_2 promoted osteoclast formation via the AKT-mediating inhibition of FoxO1 transcriptional activity. Taken together based on the findings *in vivo*, oxidative stress provokes RANKL production and RANKL promotes the accumulation of H_2O_2 in osteoclasts via AKT-mediated repression of FoxO1 transcription that lowers catalase protein level (11). Thus, we concluded that an antioxidant can upregulate the FoxO1 transcriptional activity by lowering the RANKL level. In addition, FoxO1 transcription activation can elevate the antioxidant enzyme levels, then lower H_2O_2 in osteoclasts, finally restraining osteoclastogenesis. This notion is consistent with previously studies showing that FoxO1 reduces ROS by boosting the antioxidant enzyme expression (19,20). *N*-acetylcysteine, a radical scavenger, induces FoxO1 nuclear translocation and replenishes the FoxO1 level in homocysteine-treated osteoblasts (21). Here, the present study demonstrated that RES,

a natural antioxidant, at a concentration of 10^{-5} M enhanced the FoxO1 protein level together with transcriptional activity via suppressing AKT phosphorylation. In contrast, in the presence of RES, the antioxidant power of cells was enhanced, and RAW 264.7 cell apoptosis was also promoted. It was confirmed that RES enhanced the FoxO1 protein level and transcriptional activity to prevent oxidative damage, induction of apoptosis and inhibition of osteoclastogenesis. Accordingly, FoxO1 may be a novel and effective target of RES to play the role of resistance to osteoporosis.

RANKL binding to RANK on osteoclast precursors causes the recruitment of TNF receptor associated factor 6 (TRAF6). TRAF6 can activate several downstream signaling pathways, including NF- κ B, c-Jun N-terminal protein kinase (JNK), extracellular signal-regulated kinase (ERK), p38, and PI3K/AKT (53-55). In particular, RANKL-induced activation of the PI3K/AKT signaling pathway has been shown to regulate osteoclast survival and differentiation (56). As mentioned above, RANKL-induced AKT activation downregulates FoxO1 transcription (11). Leonurine hydrochloride negatively regulates osteoclastogenesis by suppressing the NF- κ B and PI3K/AKT signaling pathway. We here demonstrated that RES provoked FoxO1 dephosphorylation and upregulated FoxO1 transcriptional activity via suppressing the PI3K/AKT signaling pathway to achieve protection against oxidative damage, and inhibition of osteoclast activity, function, and formation. Moreover, our *in vivo* results demonstrated that RES inhibited RANKL production in OVX rats. Based on the above findings, we conclude that inhibition of the PI3K/AKT signaling pathway by RES was induced by RANKL, which requires further study to confirm.

In conclusion, the present study provides pivotal evidence that RES upregulates FoxO1 transcriptional activity by inhibiting the PI3K/AKT signaling pathway to obtain preventive effects against oxidative damage and inhibition of osteoclastogenesis. The study revealed the molecular mechanisms involved in the prevention of oxidative stress damage and inhibition of osteoclastogenesis by RES, and provides a new scientific basis for the clinical application of RES for the prevention and/or treatment of osteoporosis.

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