Protective effect of *Rhizoma Dioscoreae* extract against alveolar bone loss in ovariectomized rats via regulation of IL-6/STAT3 signaling

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Abstract. The aim of the present study was to assess the effectiveness of *Rhizoma Dioscoreae* extract (RDE) on preventing rat alveolar bone loss induced by ovariectomy (OVX), and to determine the role of interleukin-6 (IL-6)/signal transducer and activator of transcription 3 (STAT3) signaling pathway in this effect. Female Wistar rats were subjected to OVX or sham surgery. The rats that had undergone OVX were treated with RDE (RDE group), vehicle (OVX group) or 17β-estradiol subcutaneous injection (E2 group). Subsequently, bone metabolic activity was assessed by analyzing 3-D alveolar bone construction, bone mineral density, as well as the plasma biomarkers of bone turnover. The gene expression of alveolar bone in the OVX and RDE groups was evaluated by IL-6/STAT3 signaling pathway polymerase chain reaction (PCR) arrays, and differentially expressed genes were determined through reverse transcription-quantitative PCR. The inhibitory effect of RDE on alveolar bone loss in the OVX group was demonstrated in the study. In comparison with the OVX group, the RDE group exhibited 19 downregulated genes and 1 upregulated gene associated with the IL-6/STAT3 signaling pathway in alveolar bone. Thus, RDE was shown to relieve OVX-induced alveolar bone loss in rats, an effect which was likely associated with decreased abnormal bone remodeling via regulation of the IL-6/STAT3 signaling pathway.

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Key words: alveolar bone loss, interleukin-6/signal transducer and activator of transcription 3 signaling pathway, herbal medicine, ovariectomized rats, *Rhizoma Dioscoreae*

Introduction

Osteoporosis is a common postmenopausal disease that markedly affects the quality of life of the patients (1). There is a known association between alveolar bone loss and osteoporosis in women following pausimenia (2). Furthermore, alveolar bone provides essential tooth support through desmodontal fiber anchoring. It was previously reported that alveolar bone mass maybe reduced and alveolar bone structure may be altered in patients suffering from osteoporosis (3). During the early postmenopausal period, alveolar bone loss occurs rapidly, but this process is leveled out in the ~6th postmenopausal year, which likely results from postmenopausal estrogen reduction (4). Alveolar bone loss causes tooth loss or mobile teeth, severely compromising postmenopausal quality of life (5).

Estrogen (6), parathyroid hormone (PTH) (7) and bisphosphonates (8) are currently used for preventing postmenopausal alveolar bone loss. However, it was indicated that long-term use of these agents may be associated with side effects, including higher risk of endometrial and ovarian cancer (9,10), nervous system disorders (11), osteonecrosis of the jaws (12) and venous thromboembolism (13). A substitutive method or drug with verified safety and efficiency is urgently required for treating alveolar bone loss. In recent decades, certain herbal medicines or botanical drugs have been widely recognized as effective remedies for relieving or treating alveolar bone loss (14-16).

Rhizoma Dioscoreae (RD), a Chinese medicinal herb/medicinal food, has long been used to promote bone and tooth strength in China (17). Our previous study demonstrated that RD extract (RDE) exerts a protective effect on maintaining alveolar bone among rats subjected to ovariectomy (OVX) through the regulation of p38 mitogen-activated protein kinase (MAPK) and Wnt signaling pathway (18). However, it has not been fully elucidated whether this effect is associated with other signaling pathways.

Previous studies have demonstrated that the interleukin-6 (IL-6)/signal transducer and activator of transcription 3 (STAT3) signaling pathway in osteoclasts and osteoblasts plays a central role in osseous metabolism and remodeling (19,20). Moreover, using bioinformatics methods in our previous

study, we predicted that RDE protection against alveolar bone loss may be associated with the IL-6/STAT3 signaling pathway (21). The aim of the present study was to analyze the inhibitory effect of RDE on alveolar bone loss among OVX rats, and investigate the association between this effect and the IL-6/STAT3 signaling pathway.

Materials and methods

Preparation of RDE. RDE was prepared as previously reported (22), and that same batch of extract was used in the present study.

Grouping and treating animals. A total of 48 Wistar female virgin rats (aged 6 months and weighing ~310±20.0 g) were acquired from the Experimental Animal Center in the Academy of Military Medical Sciences [SCXK-(Military) 2014-005, Beijing, China]. The protocol involving animals in the present study was authorized by the Institutional Ethics Committee of China Academy of Chinese Medical Sciences (approval no. 2015-009). Sham surgery (n=12) or bilateral OVX (n=36) using a dorsal incision was conducted on the rats following acclimatization. The rats undergoing OVX were divided in three groups based on the treating agent, namely control (OVX), RDE and 17β-estradiol (E2) groups. Each group contained 12 rats. Subsequently, 17β-estradiol (Sigma-Aldrich, St. Louis, MO, USA) was dissolved in ethanol and diluted with olive oil. The preparation was used for daily subcutaneous injection in the E2 rats at a dosage of 30 μg/kg body weight. RDE was dissolved with distilled water and force-fed to RDE rats at a dosage of 1.3 g/kg body weight/day, which was calculated using the human recommended dosage (30 g/day) proposed by Chinese Pharmacopeia and the weight ratio of rat and human. OVX and sham rats were force-fed equal quantities of distilled water, and standardized rat food was provided to all subjects throughout the study (Animal Center of the Fourth Military Medical University, Xi'an, China). The rats were treated with agents or distilled water for 7 days postoperatively and the treatment was maintained until the 13th week, with body weight monitoring of each subject once weekly.

Preparation of specimens. On the day after the final treatment, xylazine (12 mg/kg) and ketamine (80 mg/kg) were intraperitoneally injected to anesthetize the animals, which were subsequently sacrificed by exsanguination. The uteri were excised and immediately weighed (23). The abdominal aorta was punctured prior to death to collect blood specimens into heparinized tubes. Subsequently, the blood specimens were separated by centrifugation at 3,000 x g at a temperature of 4˚C for 10 min, and then aliquoted and preserved at -80˚C until use. The left mandibles were excised and preserved at -80˚C for reverse transcription-quantitative polymerase chain reaction (RT-qPCR) and microarrays. The right mandibles were excised, immersed into normal saline solution and preserved at -20˚C, with the aim of measuring bone mineral density (BMD) and studying the microscopic structure using microscopic computed tomography (micro-CT).

Biomarkers of bone turnover. Enzyme-linked immunosorbent assays (ELISA; Sunbio, Inc., Beijing, China) were conducted to assess plasma concentrations of bone formation and bone absorption biomarkers, such as alkaline phosphatase (ALP) and tartrate-resistant acid phosphatase (TRAP), in control, standardized and duplicated experiments. An ELISA reader (Bio-Tek, Colmar, France) was used to read 450 nm absorption values.

Micro-CT analyses. Untreated right mandibles were scanned with high-resolution micro-CT (SkyScan 1172 micro-CT system; SkyScan, Antwerp, Belgium) which applied cone beam reconstruction for the determination of the cone geometric construction of the X-ray source. The desktop SkyScan micro-CT system was operated as previously described (24). The desirable resolution value of 6.8 μm was obtained by placing a specimen on the rotating stage and translating it along the persistently varying magnifying stage. The rotation angle of a specimen was 185˚, and an image was generated with the specimen rotating for 0.9˚. The repeatability of this protocol was verified by performing repetitive scanning from the start of this experiment. The resulting gray level images were denoised with a low-pass filter, and a constant threshold value was used to determine the trabeculae.

Following image capture (100 keV, 100 μA), a quadrate region of interest (ROI) (1x1 mm) was constructed using CT analyser, which was affiliated to SkyScan on the sagittal planes of the first molar teeth. None of the selected ROIs overlapped with any tooth roots. As a 3-D rebuilding software affiliated to SkyScan, NRecon was applied for establishing a volume of interest (VOI) with a cubical mass (1x1x1 mm) underneath the bottom of the first molar crown, with a vertical distance of 1.5 mm, and the trabeculae in VOI were measured morphologically by the standardized SkyScan software package. Subsequently, degree of anisotropy (DA), structure model index (SMI), trabecular number (Tb.N), trabecular separation (Tb.Sp), trabecular thickness (Tb.Th), bone volume fraction (BV/TV) and BMD were assessed for a certain VOI using 3-D analysis (25).

RT-qPCR array assay. Alveolar bone from 6 OVX and 6 RDE rats was used for RT-qPCR. The differential expression profiles of IL-6/STAT3 signaling pathway-related genes were analyzed by the rat IL-6/STAT3 signaling pathway PCR array (PARN-160Z; Qiagen, Valencia, CA, USA) obtained from Kangchen Biotech (Shanghai, China). A total of 84 essential genes that may be involved in the activation of the IL-6/STAT3 signaling pathway and downstream reactions were profiled by this PCR array. RNA was extracted and utilized to synthesize First-Strand cDNA by RT2 First Strand kit (Qiagen, Manchester, UK) on the basis of standardized protocol and the cDNA template was mixed with RT2 SYBR-Green qPCR Master mix (Qiagen, Germantown, MD, USA), which was ready to be used in the specific kit. Subsequently, mixed reagent and template was injected into wells on PCR array plates, which contained cytokines and IL-6/STAT3 signaling-associated genes, at a dosage of 25 μl for 96-well plates, to perform RT-qPCR. An instrument-specific software was utilized to calculate quantification cycles (Cq) of all genes throughout each PCR experiment and the $2^{-\Delta\Delta Cq}$ approach was applied for calculating fold-change in the gene-expressing profiles for comparing between any two means.

Table I. Primers used for RT-qPCR analysis.

Transcript		Sequence (5'-3')
Gapdh	F:	GGAAAGCTGTGGCGTGAT
	R:	AAGGTGGAAGAATGGGAGTT
Akt1	F:	CACGACCGCCTCTGCTTT
	R:	CACAGCCCGAAGTCCGTTA
Ccl4	F:	TGCTGCTTCTCTTACACCTCC
	R:	TCATTCACATACTCATTGACCCA
Cxcl3	F:	CAGTGCCTGAAGACCCTACCA
	R:	GATCGACTCGGACGTTATTTGA
Stat3	F:	TTAACATTCTGGGCACGAACA
	R:	TCAGTGACAATCAAGGAGGCA
Tnfsf11	F:	TACCTGGATAACCCTTGATGACC
	R:	TCTCCAGAAATCCCTACAACGG
Cd4	F:	TCAGCCCGACAGCAACACTT
	R:	AGCACGACAGCCAGGAACAT
Csf3r	F:	GGTTCCATTCAAGACCCCAG
	R:	TGTTTCCCTCAGGACCAGTAGA
Hgf	F:	TATTGCCCTATTTCCCGTTGT
	R:	CCATCCACCCTACTGTTGTTTG
Il12a	F:	CAGCACTTCAGAGCCACAATC
	R:	GCCGCTGTGATTCAGAGACC
Il13	F:	AGTCCTGGCTCTCGCTTGC
	R:	TGTGTGATGTTGCTCAGCTCCT
Il1r1	F:	AAGTGGAATGGGTCGGAAAT
	R:	AAGCAGATGAACGGATAGCG
Il2	F:	CACTTGGAAGACGCTGGAAAT
	R:	CACAGTTGCTGGCTCATCATC
Il6st	F:	CGTGGCAGAAGTCCTCCTACA
	R:	GGATCGCTTGAGCCTACATAAC
Jak2	F:	AGAAGGGTGCCCAGACGA
	R:	GGTTGACATTGTTGTTCCAGC
Lifr	F:	CCGCCCTCTTATCCATCTTT
	R:	ACCAGTCCCGTTATCCTTCC
Mapk14	F:	CTGTATTGTCAGGATTCTCGGA
	R:	GCAGTGATGGGCTCTGGTTAG
Mapk1	F:	CAGGAAAGCATTACCTTGACCAG
	R:	CAGAGCCTGTTCAACTTCAATCC
Met	F:	GAAAATACCTCAACAGCGGCA
	R:	AAAGATTTGGTCGGGTGGATT
Mtor	F:	CCAACTACCTTCGGAACCTC
	R:	CTTCACTTCAAACTCCACATACTC
Nfkb1	F:	ACTCAAGAACAGCAAGGCAGC
	R:	GGTGTCGTCCCATCGTAGGT
Osm	F:	CAATGTTTACTGCATGGCTCG
	R:	GGTCTGATTCTGTGGTCTCCCT
Osmr	F:	ACTGTCCCAACCTTTAGTCATCA
	R:	GCGTCATCTACCATAGCCCTTA
Pias3	F:	CTCCTTCCCAATACTCAGCG
	R:	CAACCTTTATTGTAGGCGAGAA
Src	F:	TGCTTCATACTGGGTGACGAG
	R:	TGGGTAGAGTGGGTTGAGGTT

RT-qPCR, reverse transcription-quantitative polymerase chain reaction; F, forward; R, reverse.

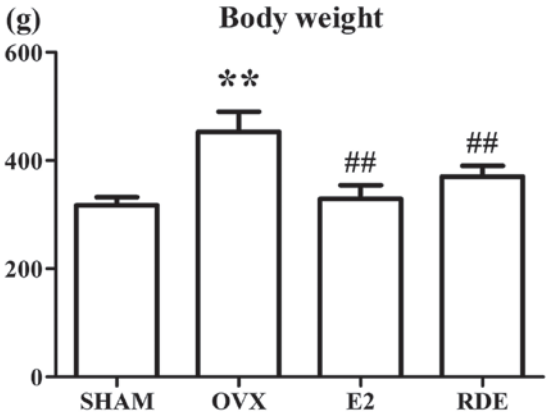

Figure 1. Effect of RDE on body weight after 12-week treatment; **P<0.01 vs. sham group; $^{\#\#}$P<0.01 vs. OVX group. RDE, *Rhizoma Dioscoreae* extract; OVX, ovariectomy; E2, 17β-estradiol.

Confirmation by RT-qPCR. Alveolar bone from another 6 OVX and 6 RDE subject rats was used for RT-qPCR. The RNeasy mini kit (Qiagen, Valencia, CA, USA) was utilized to purify total extracted RNA and SuperScript First Strand Synthesis system (Invitrogen; Thermo Fisher Scientific, Carlsbad, CA, USA) was used to reversely transcribe 4 μg RNA into cDNA. In an ABI-7500 Sequence Detection system (Applied Biosystems, Foster City, CA, USA), cDNA synthesized in the process of RT-qPCR was detected by SYBR-Green based on the manufacturer's instructions. The primers of the RT-qPCR analyses are listed in Table I. The RT-qPCR conditions in the present study were as follows: 10 sec of initiation at 95˚C, 5 sec of thermal denaturation at 95˚C, and 34 sec of annealing at 60˚C for 40 cycles. The expressing quantities of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) were used to normalize PCR results and $2^{-\Delta\Delta Cq}$ was applied for data analysis. The purity of the amplified products was assessed by melting curves of each RT-qPCR assay containing negative control without templates.

Statistical analysis. Data are presented as mean ± standardized difference and were statistically analyzed by SPSS 13.0 software (SPSS, Inc., Chicago, IL, USA). Analysis of variance and the least significant difference (LSD) test were used to determine inter-group differences in the parameters under evaluation. The normality of all data was proven by Kolmogorov-Smirnov tests, and a P-value of <0.05 was set as the threshold of statistical significance.

Results

Effect of RDE on body and uterine weight. The weight of sham rats was significantly lower compared with that of OVX rats (Fig. 1). Administration of E2 or RDE markedly inhibited weight gain induced by OVX during the 12-week treatment.

Compared with the sham group, OVX was associated with marked atrophy of the uterus, which indicated a successful surgery. E2 injection markedly reduced the atrophy of uterine tissue compared with OVX rats, whereas administration of RDE exerted a mild uterotrophic effect (Fig. 2).

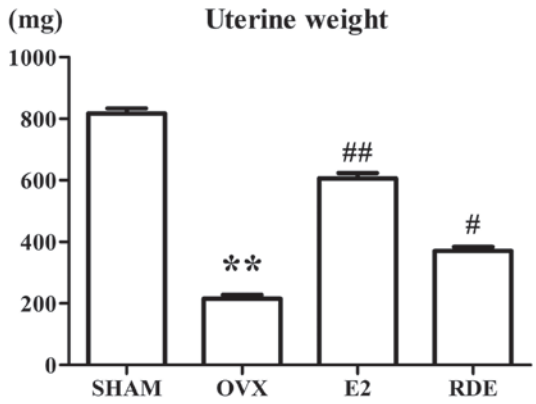

Figure 2. Effect of RDE on uterine weight after a 12-week treatment; **P<0.01 vs. sham group; #P<0.05 vs. OVX group; ##P<0.01 vs. OVX group. RDE, *Rhizoma Dioscoreae* extract; OVX, ovariectomy; E2, 17β-estradiol.

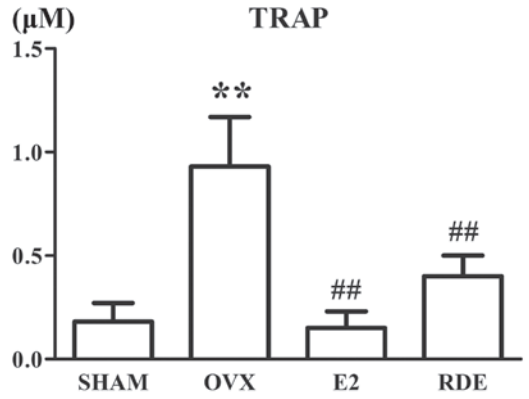

Figure 4. Effect of RDE on TRAP in plasma after 12-week treatment; **P<0.01 vs. sham group; ##P<0.05 vs. OVX group. RDE, *Rhizoma Dioscoreae* extract; OVX, ovariectomy; E2, 17β-estradiol; TRAP, tartrate-resistant acid phosphatase.

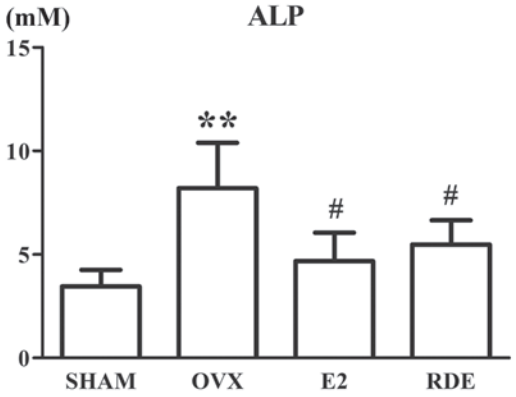

Figure 3. Effect of RDE on ALP in plasma after 12-week treatment; **P<0.01 vs. sham group; #P<0.05 vs. OVX group. RDE, *Rhizoma Dioscoreae* extract; OVX, ovariectomy; E2, 17β-estradiol; ALP, alkaline phosphatase.

Effect of RDE on bone turnover biomarkers. The plasma concentrations of ALP and TRAP in different subject rats after a 12-week treatment are shown in Figs. 3 and 4. After 12 weeks, the ALP and TRAP levels in OVX rats were significantly higher compared with those in sham rats (P<0.01). Moreover, the plasma ALP and TRAP levels in RDE and E2 rats were significantly lower compared with those in OVX rats (P<0.01).

Effect of RDE on BMD and trabecular bone microarchitecture. It was revealed by analyzing morphological measurements that Tb.N, trabecular BV/TV and BMD were significantly decreased (P<0.01), while DA, SMI and Tb.Sp were significantly increased (P<0.01) in OVX rats compared with sham rats. Treatment with RDE or E2 relieved OVX-induced bone loss and limited the OVX-induced damage to alveolar bone trabeculae (Fig. 5 and Table II).

Effect of RDE on gene expression profile. It was revealed by IL-6/STAT3 signaling pathway PCR arrays that the expression of 24 genes from alveolar bone exhibited differences of >3-fold between OVX and RDE rats (Table III). Specifically, 2 genes were upregulated, while 22 were downregulated.

Confirmation of differential levels of gene expression by RT-qPCR. With the aim of confirming the differential gene expression determined using IL-6/STAT3 signaling pathway PCR arrays, all 24 genes listed in Table III were verified using RT-qPCR and the results are presented in Fig. 6. Alveolar bone from 6 OVX and 6 RDE rats was used for the RT-qPCR assay. In the majority of the cases, the variations of genes in microarray analyses conformed to the RT-qPCR results, apart from 4 genes (*Cxcl3*, *Hgf*, *Il2* and *Il12a*). The role of the IL-6/STAT3 signaling pathway in the inhibitory effect of RDE on osteoporosis is schematically represented in Fig. 7.

Discussion

In our previous study, using bioinformatics and PCR or western blotting to confirm the expression of *Stat3*, it was demonstrated that RDE inhibited osteoporosis in OVX rats, an effect which was associated with the IL-6/oncostatin M (OSM)/STAT3 pathway via miRNA regulation (21). The putative miRNAs targets' gene prediction and pathway analysis are both bioinformatics methods, and the results occasionally do not fully reflect the true effects of RDE; however, the results raise the question whether RDE exerts an anti-osteopenic effect via another IL-6 family cytokine pathway. The IL-6 cytokine family includes 10 members: IL-6, IL-31, IL-27, IL-11, neuropoietin, cardiotrophin-like cytokine, cardiotrophin-1 (CT-1), ciliary neurotrophic factor (CNTF), OSM and leukemia inhibitory factor (LIF). All IL-6 family cytokines employ the transducing receptor β-subunit gp130 as part of a multimeric receptor complex (26). The present study aimed to determine the association between the IL-6 family of cytokines pathway and the anti-osteopenic effect of RDE.

Our results demonstrated that treatment with RDE for 12 weeks prevented loss of uterine wet weight and body weight gain resulting from lack of estrogen in rats of the OVX group (Figs. 1 and 2). It was deduced that RDE exerts a mild estrogen-like effect, which may slow down OVX-induced uterine atrophy and weight gain.

Following ovary removal, OVX rats exhibited markedly reduced BMD, resulting from increasing alveolar bone metabolism, compared with the sham rats. By contrast, treatment with RDE or E2 increased the BMD of alveolar bone.

The significant and coincident increases in plasma ALP and TRAP verified mature female OVX rat as an appropriate

Table II. Effect of RDE on BMD and trabecular bone microarchitecture.

	SHAM	OVX	E2	RDE
BMD (g/cm^3)	0.861±0.105	0.344±0.017[a]	0.601±0.004[c]	0.499±0.089[b]
BV/TV (%)	27.708±4.371	7.839±1.364[a]	17.631±1.765[c]	13.886±2.888[b]
Tb.Th (μm)	24.989±0.165	24.473±0.173	24.568±0.518	24.491±0.595
Tb.Sp (μm)	48.646±12.107	93.224±7.745[a]	60.646±12.360[c]	66.763±12.060[b]
Tb.N (1/mm)	0.011±0.002	0.003±0.000[a]	0.007±0.001[c]	0.006±0.001[b]
SMI	1.206±0.118	2.090±0.122[a]	1.523±0.115[c]	1.680±0.147[c]
DA	1.330±0.150	1.834±0.095[a]	1.455±0.267[b]	1.584±0.111

Values are presented as means ± standardized difference (n=12 in each group). [a]P<0.01 vs. SHAM group; [b]P<0.05 vs. OVX group; [c]P<0.01 vs. OVX group. RDE, *Rhizoma Dioscoreae* extract; BMD, bone mineral density; OVX, ovariectomy; E2, 17β-estradiol; BV/TV, bone volume fraction; Tb.Th, trabecular thickness; Tb.Sp, trabecular separation; Tb.N, trabecular number; SMI, structure model index; DA, degree of anisotropy.

Table III. Differential expression of genes (≥2-fold) in alveolar bone from 6 RDE and 6 OVX rats.

Symbol	P-value	Fold-change
Akt1	0.000264	-3.09
Ccl4	0.001408	-3.26
Cd4	0.000581	-2.34
Csf3r	0.001814	-4.45
Cxcl3	0.023671	-11.72
Hgf	0.004032	-3.30
Il12a	0.027687	-2.87
Il13	0.019136	2.06
Il1r1	0.000045	-3.85
Il2	0.027486	5.58
Il6st	0.005906	-2.88
Jak2	0.000009	-2.31
Lifr	0.009917	-3.25
Mapk14	0.000994	-2.81
Mapk1	0.040108	-1.64
Met	0.000938	-5.16
Mtor	0.010758	-2.03
Nfkb1	0.000017	-3.05
Osm	0.040752	-4.52
Osmr	0.016804	-2.01
Pias3	0.007814	-2.75
Src	0.030115	-2.34
Stat3	0.004448	-2.57
Tnfsf11	0.007872	-6.58

RDE, *Rhizoma Dioscoreae* extract; OVX, ovariectomy.

and reliable animal model for studies on early postmenopausal osteoporosis characterized by high-turnover bone loss. Treating rats with RDE or E2 for 12 weeks inhibited the excessive bone metabolism, as shown by significantly decreased ALP and TRAP levels (Figs. 3 and 4).

From analyses of 3-D bone microarchitecture by micro-CT, it was indicated that the loss of alveolar bone among rats treated with E2 or RDE was less severe compared with that in OVX rats, which was inferred through significant variations in SMI, Tb.Sp, Tb.N and BV/TV. The inhibition of alveolar bone loss with E2 was superior to that of RDE (Table II and Fig. 5).

The results of bone metabolic biomarkers, micro-CT and BMD demonstrated that RDE significantly inhibited alveolar bone loss.

To investigate the association between the IL-6/STAT3 signaling pathway and the anti-osteopenic effect of RDE, the differential expression of genes by was screened using IL-6/STAT3 signaling pathway array. A total of 20 genes (19 downregulated and 1 upregulated) from alveolar bone were successfully identified and validated; their expression profiles differed significantly among RDE rats compared with the OVX group. The IL-6/STAT3 signaling pathway was downregulated following RDE treatment (Fig. 7).

In the IL-6 family cytokine signaling cascade, oligomerization of receptor subunits induced by a specific ligand may activate janus protein-tyrosine kinases (JAKs), which further activate the MAPKs or the STATs (mainly STAT1 and STAT3). Another signaling cascade activated by cytokines of the IL-6 family is the phosphoinositide-3-kinase/AKT (27,28). Receptor complexes that are capable of signaling after being activated by cytokines of the IL-6 family may provide specificity in a given signal transduction pathway. For example, OSM is considered to be a unique gp130-binding cytokine, as it binds first to gp130, and then develops a signaling complex with either the OSM receptor (OSMR) or the LIF receptor (LIFR), which are both capable of intracellular signaling (29,30).

Cytokines of the IL-6 family exert a major effect on osseous remodeling through osteoclasts or osteoblasts, despite seemingly functioning as a double-edged sword. These cytokines maintain bone generation while driving bone absorption induced by a variety of osteolytic factors. This dual effect is apparent in two main signaling pathways (STAT and MAPK signaling) bidirectionally affecting osteocytes (19).

Certain members of the IL-6 family (OSM, CNTF, LIF, IL-11 and IL-6) may promote bone formation. This effect was mainly reflected in three aspects: First, these cytokines promote the differentiation of osteoblast precursors or maturing osteoblasts isolated from the bone marrow or calvaria. During this process, STAT3 must be activated (31,32). Thus, the expression of osteoblastic biomarkers, such as bone sialoprotein, osteocalcin or ALP, was increased, while extracellular matrix

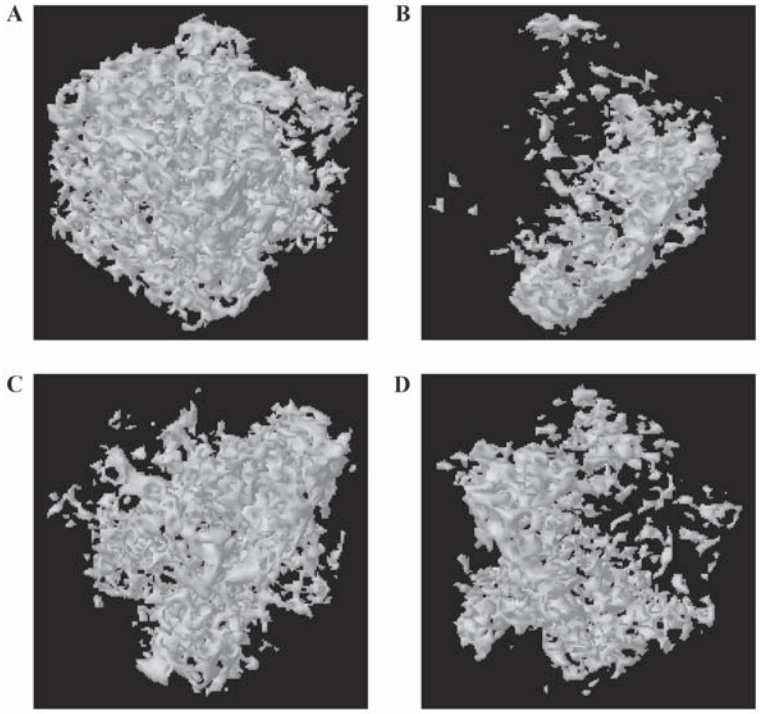

Figure 5. Representation of 3-D architecture of alveolar bone beneath the lowest point of the first molar crown in the (A) sham, (B) OVX, (C) E2 and (D) RDE groups. RDE, *Rhizoma Dioscoreae* extract; OVX, ovariectomy; E2, 17β-estradiol.

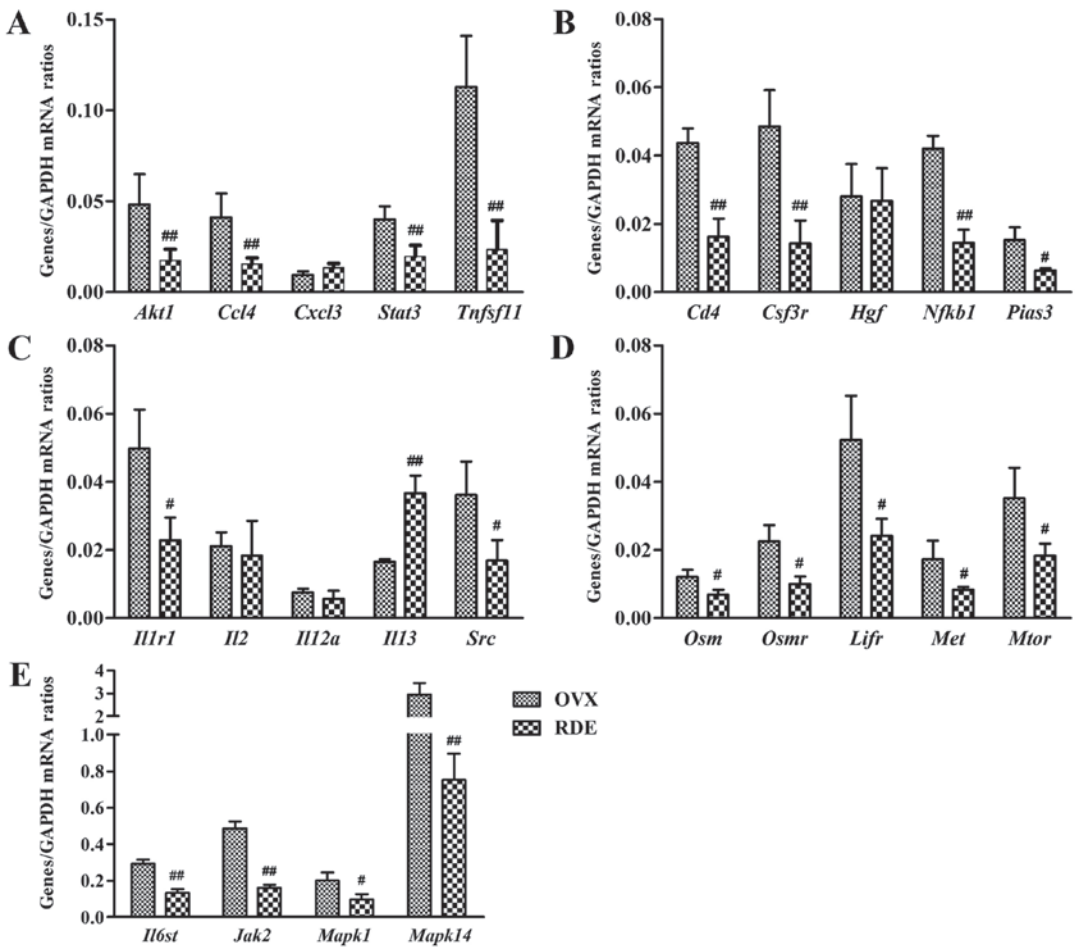

Figure 6. Validation of 24 differential expression genes identified by microarray and IPA in a replicated experiment by RT-qPCR. (A-E) Effect of RDE on the expressions of *Akt1*, *Ccl4*, *Cd4*, *Csf3r*, *Cxcl3*, *Hgf*, *Il12a*, *Il13*, *Il1r1*, *Il2*, *Il6st*, *Jak2*, *Lifr*, *Mapk14*, *Mapk1*, *Met*, *Mtor*, *Nfkb1*, *Osm*, *Osmr*, *Pias3*, *Src*, *Stat3* and *Tnfsf11*. $^{\#}$P<0.05 and $^{\#\#}$P<0.01 compared with the OVX group. Alveolar bone prepared from 6 RDE and 6 OVX rats was used. IPA, Ingenuity Pathway Analysis; RDE, *Rhizoma Dioscoreae* extract; OVX, ovariectomy; RT-qPCR, reverse transcription-quantitative polymerase chain reaction.

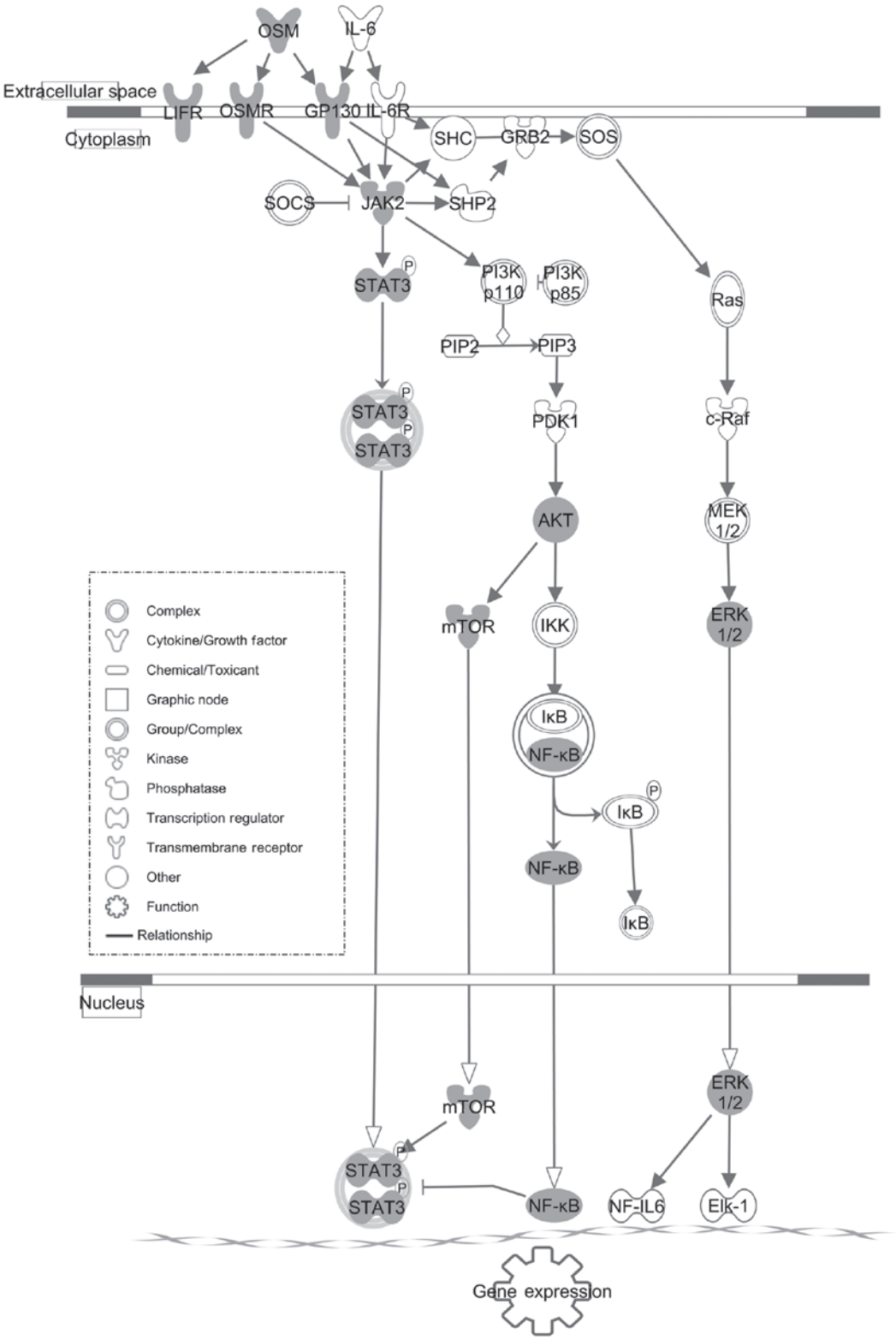

Figure 7. Schematic diagram illustrating the role of IL-6/STAT3 signaling pathway in an anti-osteopenic effect of RDE. The downregulated genes appear in gray. The genes that are not specified but are incorporated into the network through association appear in white. RDE, *Rhizoma Dioscoreae* extract; IL-6, interleukin-6; STAT3, signal transducer and activator of transcription 3.

mineralization and bone nodule formation were enhanced. Second, the biomarkers inhibit certain osteoblasts from proliferating via activating gp130/STAT3 and increasing the expression of $p21^{WAF1}$, which is a cell cycle inhibitor and is also required for cytokines of the IL-6 family to induce ALP (33). Finally, these cytokines may protect osteoblasts from apoptosis caused by tumor necrosis factor-α (TNF-α) or serum depletion (34).

As regards bone absorption, cytokines of the IL-6 family promote osteoclast differentiation and bone absorption by accelerating the interactions between osteoclasts and osteoblasts. In co-cultures of osteoclast precursors and stromal cells

or osteoblasts, certain IL-6-type cytokines (OSM, CNTF, LIF, IL-11 and IL-6) are able to promote osteoclast differentiation and bone absorption. In fact, these cytokines induce production of various osteoblastic downstream effectors that, in turn, promote osteoclast differentiation or activity, such as IL-1, receptor activator of nuclear factor-κB (NF-κB) ligand (RANKL), prostaglandin E2 and PTH-related protein. Recent studies implicated activation of IL-6-type cytokines/STAT3 signaling as the pivotal event in the induction of RANKL in osteoblasts, which leads to pro-resorption action of osteoclasts (35,36).

It was observed that the gene expression of certain signaling molecules (*Osm*, *Osmr*, *Lifr*, *Il6st*, *Jak2*, *Stat3*, *Mtor*, *Nfkb1* and *Mapk1*) in the IL-6/STAT3 pathway were downregulated following RDE treatment (Fig. 7), which subsequently inhibited IL-6/STAT3 signaling. The RT-qPCR analysis of *Osm*, *Osmr*, *Lifr*, *Il6st*, *Jak2*, *Stat3*, *Mtor*, *Nfkb1* and *Mapk1* expression (Fig. 6) proved that RDE effectively reduced excessive alveolar bone formation and bone absorption synchronously caused by attenuated canonical IL-6/STAT3 signaling following OVX.

Further studies are required for RDE to be developed into a novel promising drug for the prevention or treatment of alveolar bone loss in postmenopausal women.

In conclusion, RDE was effective in inhibiting rat alveolar bone loss caused by OVX, by simultaneously inhibiting bone formation as well as bone resorption through regulation of the IL-6/STAT3 signaling pathway. The present study verified that RDE may be used as an oral agent for the treatment of alveolar osteopenia in postmenopausal women.

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