Alendronate promotes osteoblast differentiation and bone formation in ovariectomy-induced osteoporosis through interferon-β/signal transducer and activator of transcription 1 pathway

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Abstract. Alendronate is commonly used for the treatment of postmenopausal osteoporosis; however, the underlying pathological molecular mechanisms of its action remain unclear. In the present study, the alendronate-treated signaling pathway in bone metabolism in rats with ovariectomy induced by osteoporosis was investigated. Rats with osteoporosis were orally administered alendronate or phosphate-buffered saline (control). In addition, the interferon-β (IFN-β)/signal transducer and activator of transcription 1 (STAT1) signaling pathway was investigated in osteoblasts following treatment with alendronate in vitro and in vivo. During the differentiation period, IFN-β (100 ng/ml) was used to treat the osteoblast cells, and the activity, viability and bone metabolism-associated gene expression levels (STAT1, p-STAT1, Fra1, TRAF6 and SOCS1) were analyzed in osteoblast cells. Histopathological changes were used to evaluate osteoblasts, osteoclasts, inflammatory phase of bone healing and osteonecrotic areas. The results demonstrated that alendronate significantly inhibited the activity of osteoporotic osteoclasts by stimulating expression of IFN-β, as well as markedly improved the viability and activity of osteoblasts compared with the control group. In addition, alendronate increased the expression and phosphorylation levels of STAT1 in osteoclasts, enhanced osteoblast differentiation, upregulated the expression levels of alkaline phosphatase and osteocalcin, and increased the expression of osteoblast differentiation-associated genes (osteocalcin, osterix and Runx2). Inhibition of IFN-β expression canceled the benefits of alendronate-mediated osteoblast differentiation. Notably, alendronate enhanced bone formation in rats with osteoporosis induced by ovariectomy. In conclusion, these findings suggest that alendronate can regulate osteoblast differentiation and bone formation in rats with osteoporosis induced by ovariectomy through upregulation of IFN-β/STAT1 signaling pathway.

Introduction

The human skeletal system comprises highly dynamic tissue that exhibits continuous bone formation by osteoblasts and bone resorption by osteoclasts to maintain homeostasis (1). Previous studies have suggested that dysfunction of remodeling and metabolism of osteoblasts and osteoclasts are associated with the progression of osteoporosis in individuals aged >50 years (2,3). Osteoporosis exhibits complex pathology and etiology, and is considered as a multifactorial polygenic disease associated with environmental, hormonal, nutritional and genetic factors. Studies have indicated that estrogen concentration is correlated with the pathogenesis of osteoporosis by regulating bone homeostasis and preventing postmenopausal bone loss (4,5). Although numerous perspective studies on postmenopausal osteoporosis have been reported, the molecular mechanisms of the pathogenesis induced by postmenopausal osteoporosis are not completely understood.

The clinical consequences of osteoporosis include fractures in the upper extremities, hip and even spine, which result in the loss of function and independence, impaired quality of life, as well as increased morbidity and mortality (6-8). Various treatments for postmenopausal osteoporosis, including farnesyl pyrophosphate synthase inhibitor, have been proposed in a large number of preclinical and clinical studies (9-11). In addition, the downregulation of levels of osteoassociative hormones have been detected in the blood plasma of postmenopausal women with and without osteoporosis in clinical trials (12,13). Furthermore, comprehensive treatments with anti-resorptive agents, such as alendronate, have been investigated for the prevention of new non-vertebral fractures in patients with postmenopausal osteoporosis (14). In the present study, the alendronate-mediated benefits and mechanism for the treatment of rats with ovariectomy-induced osteoporosis were investigated.

Previous studies have demonstrated the efficacy of long-term alendronate treatment on postmenopausal osteoporosis and
bone material properties (13,15). In addition, arterial stiffness was improved following monthly bisphosphonate treatment in women with postmenopausal osteoporosis (16). The effects of other drug treatments on endothelial function, glucose metabolism and inflammation markers have also been investigated in patients with postmenopausal osteoporosis (17-19). Furthermore, a previous study examined the effects of short-term treatment with alendronate (70 mg; once-a-week) in women with postmenopausal osteoporosis, determined by bone turnover markers, and suggested that alendronate treatment was beneficial in these patients (18).

Signal transducer and activator of transcription 1 (STAT1) signaling pathway is strongly activated by interferon-β (IFN-β) in the pathogenesis and progression of osteoporosis, and previous results contributed to further understanding the pathological signaling pathways involved in osteoporosis (20). The importance of STAT1 gene expression in monocytes has been reported, and the progression of osteoporosis was observed to be regulated by the expression and phosphorylation levels of STAT1 (21). These findings suggest that STAT1 gene expression may be associated with the initiation and development of osteoporosis.

In the present study, the therapeutic effects and molecular mechanism of alendronate were investigated in osteoblast cells and in rats with osteoporosis induced by ovariectomy. The potential involvement of the IFN-β/STAT1 signaling pathway mediated by alendronate in the progression of ovariectomy-induced osteoporosis was also examined. The findings suggested that alendronate regulated osteoblast differentiation and bone formation through upregulation of IFN-β/STAT1 pathway in the rats. These results may contribute to the clinical treatment of patients with postmenopausal osteoporosis.

Materials and methods

Ethical statement. The present study was conducted in strict accordance with the guidelines for the Care and Use of Laboratory Animals of Jining No. 1 People’s Hospital (Jining, China). All experimental protocols and animals were approved by Ethics Committee of the Animal Experiments Defense Research of Jining No. 1 People’s Hospital (Jining, China). All surgery and euthanasia procedures aimed to minimize animal suffering.

Animal study. Female Wistar albino rats (age, 6-8 weeks) were purchased from Shanghai Slack Experimental Animals Co., LTD (Shanghai, China). Rats (n=40) were fed under pathogen-free conditions and maintained in a controlled environment (temperature, 23±1°C; humidity, 50-60%), as well as an artificial simulation of 12h light/dark cycles. All rats underwent oophorectomy in order to establish an osteoporosis model and to analyze the efficacy of alendronate treatment. Experimental procedures were conducted according to a previous study (22). The induction lasted 14 days and the mandibular cortical width was used as an indicator of osteoporosis to determine whether the model was successfully established (23). Rats with osteoporosis were randomly divided into two groups (n=20 in each group) and received treatment with alendronate (2.5 mg/kg; Sigma-Aldrich; Merck KGaA; Darmstadt, Germany) or phosphate-buffered saline (PBS; 2.5 mg/kg). The treatments were performed once a day in a total of 10 times. On day 60, the therapeutic efficacies of alendronate were analyzed according to a previous study (24).

Bone mineral density. Bone tissues were isolated from experimental animals according to previous study (25). Bone cells were obtained from bone tissues as previously described (26). A total of 1x10⁶ bone cells were used to analyze bone mineral density. Bone mineral density of the tibia of experimental rats was analyzed with an automated software (N-Vivo 9.0; QSR International Ltd., Melbourne, Australia) and quantified for analysis of the efficacy of alendronate. All procedures were performed as previously described (27). Intermediate bone density was defined as mg/cm³.

Measurement of alkaline phosphatase (ALP) activity. Osteoblasts were isolated from experimental mice as described previously (28) and cultured in 6-well plates (1x10⁵ per well) with alendronate at concentrations of 1, 5, 10, 20, 50, 100, 200, 500, 1000, and 2000 μM for 48 h. The effect of alendronate treatment on osteoblast cells was analyzed by performing ALP activity assays and ALP staining (Colorimetric Alkaline Phosphatase Assay kit; ab83369; Abcam; Cambridge, MA, USA) as described in a previous study (29).

Bone resorption assays. Mature osteoclasts and osteoblasts were isolated from experimental rats with ovariectomy-induced osteoporosis and cultured in the presence of 10⁻⁶ M 1,25-dihydroxyvitamin D₃ and 10⁻⁸ M prostaglandin E₂ (Sigma-Aldrich, Merck, Darmstadt, Germany) for 7 days. Next, the cells were treated with alendronate (2 mg/ml) or PBS (2 mg/ml) for 48 h at 37°C. Subsequently, the minimum essential medium (MEM) was removed from the culture and cells were washed with PBS three times. Then, the total bone resorption was analyzed using ImagePro Plus version 4.0 (Media Cybernetics, Inc., Silver Spring, MD, USA).

Analysis of the effects of alendronate on osteoclast differentiation. Bone marrow cells (BMCs) were obtained from female Wistar albino rats as described previously (30) and cultured in MEM supplemented with 10% fetal bovine serum (FBS) for 48 h at 37°C. The medium was removed, and fresh MEM was added before cells were cultured for 72 h in 10 ng/ml macrophage colony-stimulating factor (M-CSF; Sigma-Aldrich; Merck KGaA; Darmstadt, Germany) at 37°C. Tartrateresistant acid phosphatase (TRAP) activity was measured (Sigma-Aldrich; Merck KGaA; 387A-IKT) and used to examine the osteoclast differentiation (31).

Measurement of osteoblast viability. The viability of osteoblasts was analyzed in order to examine the effects of alendronate on osteoporosis, as described in a previous study (32). All experiments were performed in triplicate.

Reverse transcription-quantitative polymerase chain reaction (RT-qPCR) assays. Total RNA from the femurs of rats was extracted using RNAeasy Mini kit (Qiagen, Gaithersburg, MD, USA). The mRNA expression levels of osteocalcin, osterix, Runx-related transcription factor 2 (Runx2), TRAP, osteoblast differentiation factor (ODF), osteoprotegerin
For histological analysis, experimental rats were anesthetized using intraperitoneal injection of sodium pentobarbital anesthesia (40 mg/kg; Invitrogen; Thermo Fisher Scientific, Inc.) and the femur was isolated from experimental rats after treatment with alendronate or PBS. Femur tissues were sectioned (4-µm thick), fixed using 10% formaldehyde for 15 min at 30°C followed by embedding in paraffin wax. Hematoxylin-eosin staining for 120 min at 30°C was used to analyze the bone quality and density using a microscope (Olympus Corporation; Tokyo, Japan) at a magnification, x400.

**Transfection with small interference RNA (siRNA).** All siRNAs were synthesized by Invitrogen (Thermo Fisher Scientific, Inc.), including the siRNA-IFN-β for endogenous inhibition of IFN-β (EI-IFN-β) and siRNA-vector. Osteoblasts (1x10^6) were transfected with 100 pmol siRNA-IFN-β using Lipofectamine 2000 (Sigma-Aldrich; Merck KGaA), according to the manufacturer's protocol. While siRNA-vector transfection was performed in the control group.

**Statistical analysis.** All data are represented as the mean ± standard error of triplicate experiments. Analysis was performing by SPSS software (version 14.0; SPSS, Inc., Chicago, IL, USA). Unpaired data were analyzed by Student's t-test, while comparisons of data between multiple groups were conducted by analysis of variance. P<0.05 was considered as an indicator of a statistically significant difference.

**Results**

Effects of alendronate treatment on the viability, ALP activity and differentiation of osteoblasts. In order to analyze the efficacy of alendronate treatment on osteoporosis, the
ALP activity and differentiation of osteoblasts were analyzed following treatment with alendronate. It was observed that alendronate significantly increased the viability of osteoblasts after 48- and 72-h incubation (Fig. 1A). In addition, ALP activity was upregulated in osteoblasts subsequent to treatment with alendronate for 48 and 72 h, as compared with the control group (Fig. 1B). It was also revealed that alendronate treatment at different concentrations (0.5-16 mg/ml) promoted early differentiation marker of osteoblasts derived from newborn rat calvaria determined by staining for ALP, with a significant effect observed at ≥4 mg/ml (Fig. 1C). Furthermore, the results indicated that alendronate (0.5-16 mg/ml) significantly enhanced ALP mRNA expression in osteoblasts (Fig. 1D). Alendronate treatment at 8 mg/ml markedly increased the mineralized area, as determined by alizarin red S staining for calcium (Fig. 1E). Notably, treatment with 8 mg/ml alendronate markedly decreased the mRNA expression levels of TRAP compared with those in the PBS-treated group (Fig. 1F). These results suggest that alendronate promotes osteoblast differentiation and bone formation.

Alendronate stimulates the expression levels of mRNAs associated with osteoblast differentiation. Next, the present study investigated the effects of alendronate on the mRNAs expression levels of osteoblast differentiation-associated factors in osteoblasts. It was observed that alendronate markedly promoted the mRNA expression levels of osteocalcin, osterix and Runx2, which are essential transcription factors for osteoblast differentiation (Fig. 2A-C). ODF, OPG and COL1A1 are essential for the osteoblast differentiation (35). As shown in Fig. 2D-F, alendronate stimulation also increased the expression levels of ODF, OPG and COL1A1 in osteoblasts. These results suggest that alendronate regulates osteoblast differentiation.
differentiation by stimulating the expression levels of various mRNAs associated with osteoblast differentiation.

Resorption ability and TRAP activity of osteoclasts following treatment with alendronate. The resorption ability and TRAP activity of osteoclasts are essential for the progression of rarefaction of bone and function of bone tissue (36). The present study results demonstrated that alendronate treatment significantly inhibited the resorption ability of osteoclasts (Fig. 3A), but promoted the proliferation of osteoblasts during the differentiation period, as determined by toluidine blue staining (Fig. 3B). In addition, the TRAP activity of osteoclasts and osteoblasts following treatment with alendronate during the differentiation period was examined, and the results indicated that TRAP-positive cells were inhibited in osteoclasts and osteoblasts following treatment (Fig. 3C and D). Furthermore, TRAP activity in osteoclasts and osteoblasts was suppressed by the treatment of alendronate during the differentiation period when compared with non-treated cells (Fig. 3E and F). Taken together, these findings suggest that alendronate treatment regulates osteoblast differentiation through upregulation of the IFN-β/STAT1 signaling pathway.

Alendronate regulates osteoblast differentiation through the IFN-β/STAT1 signaling pathway. In order to analyze the molecular mechanism of alendronate-mediated osteoblast differentiation, the IFN-β/STAT1 signaling pathway was investigated in osteoblasts. The results shown in Fig. 4A reveal that alendronate promoted IFN-β expression in osteoblasts. It was also identified that STAT1 and pSTAT1 were enhanced in osteoblasts after treatment with alendronate (Fig. 4B). Alendronate treatment also increased the mRNA expression levels of Fra1, TRAF6 and SOCS1 in osteoblasts compared with the control group (Fig. 4C). However, endogenous inhibition of IFN-β (EI-IFN-β) markedly reduced the inhibitory effects of alendronate (AEI-IFN-β) on osteoblast differentiation and differentiation-associated gene expression levels (Fig. 4D). In addition, inhibition of IFN-β expression (EI-IFN-β) downregulated of STAT1 and pSTAT1 protein levels and abolished their upregulation induced by alendronate (AEI-IFN-β) in osteoblasts (Fig. 4E), which also reduced alendronate-promoted (AEI-IFN-β) expression levels of differentiation-associated genes, including osteocalcin, osterix and Runx2 (Fig. 4F). Taken together, these results revealed that alendronate treatment regulates osteoblast differentiation through upregulation of the IFN-β/STAT1 signaling pathway.

Alendronate treatment exerts a beneficial effect in rats with ovariectomy-induced osteoporosis, determined by histological. The current study further analyzed the therapeutic effects of alendronate on rats with ovariectomy-induced osteoporosis, which was determined by histological analysis of experimental rats on day 60. The results in Fig. 5A revealed that bone structure was significantly improved in rats treated with alendronate that exhibited ovariectomy-induced osteoporosis compared with the PBS group. It was also observed that alendronate treatment prevented loss of trabeculae in rats with osteoporosis induced by ovariectomy (Fig. 5B). In addition, bone strength and bone density assays demonstrated that alendronate significantly improved osteoporosis in rats induced by ovariectomy, when compared with the PBS control group (Fig. 5C and D). Furthermore, the elastic modulus and post-yield displacement of bone were also increased in rats with osteoporosis following treatment with alendronate (Fig. 5E and F). Therefore, these data suggest that alendronate treatment was beneficial in rats with osteoporosis induced by ovariectomy, as determined by histological and index detection.
Discussion

Osteoporosis is identified as a systemic skeletal disease that affects mostly postmenopausal women characterized by reduction of bone strength and mass loss of bone mineral density (37,38). Evidence suggested that postmenopausal women have increased risk of osteoporosis, resulting in fragility fracture due to the lack of estrogen secretion (39,40). Previous studies have also demonstrated that alendronate (a bisphosphonate) is an efficient agent for the treatment of osteoporosis, functioning through inhibition of bone resorption by accumulating on the bone surface and inhibition of osteoclast apoptosis (41,42). In the present study, the therapeutic efficacy and potential underlying mechanism of alendronate treatment were investigated in a rat model of osteoporosis induced by ovariectomy. The presented results indicated that alendronate therapy promoted osteoblast differentiation in the calvarial osteoblastic cells isolated from newborn rats. In addition, the resorption ability and TRAP activity of osteoclasts were inhibited following treatment with alendronate, which may contribute to bone remodeling in rats with osteoporosis. Alendronate treatment stimulated ALP expression and activity, as well as the expression levels of mRNAs associated with osteoblast differentiation, including Fra1, TRAF6 and SOCS1. The expression levels of ODF, OPG and COL1A1 in osteoblasts were also upregulated in osteoblasts subsequent to alendronate administration. Furthermore, the findings of the current study indicated that alendronate treatment regulated the osteoblast differentiation through upregulation of IFN-β/STAT1 signaling pathway.

A previous study has demonstrated that stimulation with IFN-β leads to significant inhibition of osteoporotic
osteoclasts (20). IFN-β production is mainly secreted by fibroblasts, and subsequently binds to the IFN-α/β receptor (43,44). The activity of IFN-β depends on the transcriptional activator of the expression and phosphorylation of STAT1 and STAT2, which leads to the activation of the JAK signal pathway (45). Studies also indicated that IFN-β/STAT1 signaling pathway regulates numerous metabolism disorder-associated molecular transcriptions (46-48). Additionally, IFN-β functions as a potential drug for multiple sclerosis and exhibits various beneficial clinical outcomes (49,50). In the present study, the involvement of the IFN-β/STAT1 signaling pathway in osteoblast differentiation was analyzed. The results indicated that IFN-β/STAT1 signaling pathway was enhanced in osteoporotic osteoblasts, resulting in the increase

Figure 4. Alendronate regulates osteoblast differentiation through the IFN-β/STAT1 signaling pathway. (A) Alendronate promoted IFN-β protein expression levels in osteoblasts. (B) Protein expression levels of STAT1 and pSTAT1 in osteoblasts following treatment with alendronate. (C) Analysis of mRNA expression levels of Fra1, TRAF6 and SOCS1 in osteoblasts. Inhibition of IFN-β expression suppresses (D) ODF, OPG and COL1A1 expression levels, (E) STAT1 and pSTAT1 protein expression in osteoblasts, and (F) the differentiation-associated gene expression levels of osteocalcin, osterix and Runx2. The data are presented as the mean ± standard error. "P<0.01 alendronate vs. control (PBS) group. PBS, phosphate-buffered saline; IFN-β, interferon-β; STAT1, signal transducer and activator of transcription 1; Fra1, Fos-related antigen 1; TRAF6, TNF-receptor-associated factor 6; SOCS1, suppressor of cytokine signaling 1; ODF, osteoblast differentiation factor; OPG, osteoprotegerin; COL1A1, collagen 1A1; Runx2, Runx-related transcription factor 2; EI-IFN-β, endogenous inhibition of IFN-β; AEI, alendronate + EI-IFN-β.

Figure 5. In vivo efficacy of alendronate for the treatment of rats with ovariectomy-induced osteoporosis. (A) Bone qualities analyzed by histological assay, (B) femur bone density, (C) bone strength, (D) bone density, (E) elastic modulus and (F) post-yield displacement were evaluated in rats with ovariectomy-induced osteoporosis following treatment with alendronate or PBS. The data are presented as the mean ± standard error. "P<0.01 alendronate vs. control (PBS) group. PBS, phosphate-buffered saline.
of Fasl, TRAF6 and SOCS1 expression levels following treatment with alendronate. Furthermore, alendronate increased the differentiation-associated gene expression levels of osteocalcin, osterix and Runx2 through upregulation of IFN-β and enhanced the phosphorylated production of IFN-β. However, inhibition of IFN-β expression also suppressed STAT1 and pSTAT1 expression in osteoblasts. These findings indicated that alendronate mediated improvements in osteoporosis through regulation of IFN-β/STAT1 signaling pathway in osteoblasts. Bone strength and bone density are the most important indicators in the progression of patients with osteoporosis (51,52). Various types of treatments in ovariectomized rats have been investigated in a large number of studies (53-55). In addition, histological analysis of bone qualities is essential for examining the improvement of osteoporosis, which is regarded as an evaluation criterion for drugs used in the treatment of osteoporosis (56,57). In the present study, the therapeutic effects of alendronate-mediated improvements of osteoporosis were investigated by histological analysis, including examining the bone density and loss of trabeculae. The present study findings also indicated that bone strength, bone density, elastic modulus and post-yield displacement were significantly improved following treatment with alendronate in rats with osteoporosis induced by ovariectomy.

In conclusion, although numerous reports have provided important evidence for identifying the efficacy of alendronate treatment in osteoblast differentiation, the underlying molecular mechanism remains poorly understood (58,59). In the present study, it was demonstrated that alendronate treatment not only presents stimulatory effects on osteoblast differentiation and mineralization, but also enhances the bone formation in rats with osteoporosis induced by ovariectomy. The findings also revealed that alendronate improves bone loss of osteoporosis through upregulation of the IFN-β/STAT1 signaling pathway, which suggests that alendronate may be a potential therapeutic agent for osteoporosis.

References

1. Wong SK, Chin KY, Suhaimi FH, Ahmad F and Ima-Nirwana S: The Relationship between metabolic syndrome and osteoporosis: A Review. Nutrients 8: p.7; E347, 2016.
2. Petty SJ, Wilding H and Wark JD: Osteoporosis associated with epilepsy and the use of anti-epileptics-a review. Curr osteoporos Rep 14: 54-65, 2016.
3. Newman M, Minns Lowe C and Barker K: Spinal orthoses for epilepsy and the use of anti-epileptics-a review. Curr osteoporos Rep 14: 54-65, 2016.
4. Okada Y and Miki T: The Relationship between metabolic syndrome and osteoporosis: A Review. Nutrients 8: p.7; E347, 2016.
5. Hasler N, Gamsjaeger S, Hofstetter B, Brozek W, Klausshofer K and Paschalis EP: Effects of long-term alendronate treatment on postmenopausal osteoporosis bone material properties. Osteoporos Int 26: 339-352, 2015.
6. Massafra U, Integlia D, Broccoli S and Migliore A: Mixed treatment comparison to rank antiresorptive agents in preventing non vertebral fractures in postmenopausal osteoporosis. Value Health 18: A636, 2015.
7. Hassler N, Gamsjaeger S, Hofstetter B, Brozek W, Klausshofer K and Paschalis EP: Effects of long-term alendronate treatment on postmenopausal osteoporosis bone material properties. Osteoporos Int 26: 339-352, 2015.
8. Iba K, Sonoda T, Takada J, Dohke T and Yamashita T: Further evaluation of denosumab for the treatment of postmenopausal osteoporosis in vivo. Exp Ther Med 7: 1687-1690, 2014.
9. Zhdan VM, Kitura O, Kitura IE M, Babanina M and Tkachenko MV: Treatment of postmenopausal osteoporosis in the general medical practice (clinical case). Lik Sprava: 85-89, 2013.
10. Massafra U, Integlia D, Broccoli S and Migliore A: Mixed treatment comparison to rank antiresorptive agents in preventing non vertebral fractures in postmenopausal osteoporosis. Value Health 18: A636, 2015.
27. Leamy LJ, Kelly SA, Hua K, Farber CR and Pomp D: Quantitative trait loci for bone mineral density and femoral morphology in an advanced intercross population of mice. Bone 55: 229-235, 2015.

28. Jonason JH and O’Keefe RJ: Isolation and culture of neonatal mouse calvarial osteoblasts. Methods Mol Biol 1130: 295-305, 2014.

29. Saiyed ZM, Sharma S, Godawat R, Telang SD and Ramchand CN: Activity and stability of alkaline phosphatase (ALP) immobi-
lized onto magnetic nanoparticles (Fe3O4). J Biotechnol 131: 240-244, 2007.

30. Subramanian K, Geraerts M, Puwelykn KA, Park Y, Owens DJ, Muitjens M, Ulloa-Montoya F, Jiang Y, Verfaillie CM and Hu WS: Isolation procedure and characterization of multipotent adult progenitor cells from rat bone marrow. Methods Mol Biol 636: 55-78, 2010.

31. Idris AI, Landao-Bassonga E and Ralston SH: The TRPV1 ion channel antagonist capsazepine inhibits osteoclast and osteo-
blast differentiation in vitro and ovariectomy induced bone loss in vivo. Bone 46: 1089-1099, 2010.

32. Hiemer B, Ziebert J, Jonitz-Heincke A, Grunert PC, Su Y, Hansmann D and Bader R: Magnetically induced electrostimula-
tion of human osteoblasts results in enhanced cell viability and osteogenic differentiation. Int J Mol Med 38: 57-64, 2016.

33. Xiao S, Wang J and Xiao N: MicroRNAs as noninvasive biomarkers for the early diagnosis of bladder cancer detection: A diagnostic meta-analysis based on qRT-PCR data. Int J Biol Markers 31: e276-e285, 2016.

34. Wai-Hoe L, Wing-Seng L, Ismail Z and Lay-Harn G: SDS-PAGE-based quantitative assay for screening of kidney stone disease. Biol Proced Online 11: 145-160, 2009.

35. Johnson RW, White JD, Walker EC, Martin TJ and Sims NA: Myokines (muscle-derived cytokines and chemokines) including ciliary neurotrophic factor (CNTF) inhibit osteoblast differentia-
tion. Bone 64: 47-56, 2014.

36. Takahashi M, Kushida K, Kawana H, Hoshino H and Inoue T: Discrimination ability of pyridinoline crosslinks related markers for bone resorption in postmenopausal and osteoporosis. Endocr Res 23: 105-117, 1997.

37. Ma J, Ma Y, Liu X, Chen S, Liu C, Qin A and Fan S: Gambogenic acid inhibits osteoclast formation and ovariectomy-induced osteoporosis by suppressing the JNK, p38 and Akt signalling pathways. Biochem J 469: 399-408, 2015.

38. Song SH, Wang D, Mo YY, Ding C and Shang P: Antiosteoprotic effects of naringenin on ovariectomy-induced osteoporosis in rat. Yao Xue Xue Bao 2015: 2829-2841, 2015 (In Chinese).

39. Lee SN, Cho JY, Eun YM, Song SW and Moon KW: Associations between osteoporosis and coronary artery disease in postmeno-
pausal women. Climacteric 19: 458-462, 2016.

40. Alami S, Hervouet L, Poiraudeau S, Briot K and Roux C: Morphological analysis of bone dynamics and strength following estrogen defi-
Ciency in osteoporosis treatment model - effects of osteoporosis treatment drugs on fracture healing ability in osteoporotic mice. Histological findings in animal frac-
ture model - effects of osteoporosis treatment drugs on fracture healing ability in osteoporotic mice. J Prosthet Dent 60: 131-137, 2016.

41. Li X, Niu QT, Warnington KS, Asuncion PJ, Dwert D, Grisanti M, Han CY, Stoltenburg EB, Bergenhagen MJ, Kostenuik PJ, et al: Progressive increases in bone mass and bone strength in an ovari-
cectomy rat model of osteoporosis after 26 weeks of treatment with a sclerostin antibody. Endocrinology 155: 4785-4797, 2014.

42. Brennan O, Kuliwaba JS, Lee TC, Parkinson IH, Fazzalari NL, McNamara LM and O’Brien FJ: Temporal changes in bone composition, architecture, and strength following estrogen defi-
ciency in osteoporosis. Calcif Tissue Int 91: 440-449, 2012.

43. Ying SH, Kao HC, Chang MC, Yu WK, Wang ST and Liu CL: Fixation strength of PMMA-augmented pedicle screws after depth adjust-
ment in a synthetic bone model of osteoporosis. Orthopedics 35: e1511-e1516, 2012.

44. Simon JA, Recknor C, Moffett AH Jr, Adachi JD, Franek E, Lewiecki EM, McClung MR, Mautalen CA, Raggi-Eis S, Nicholson GC, Simon JA, Recknor C, Moffett AH Jr, Adachi JD, Franek E, Lewiecki EM, McClung MR, Mautalen CA, Raggi-Eis S, Nicholson GC, Saag KG, Agerusdei D, Hans D, Kohlmeier LA, Krohn KD, Leib ES, MacLaughlin EJ, Alam J, Simonelli C, Taylor KA and Marcus R: Trabecular bone score in patients with chronic glucocorticoid-induced osteoporosis treated with alendronate or teriparatide. Arthritis Rheumatol 68: 2122-2128, 2016.

45. Ruy TK, Kang RH, Jeong KY, Jun DR, Koh JM, Kim D, Bae SK and Choi SW: Bone-targeted delivery of nanodiamond-based drug carriers conjugated with alendronate for potential osteopa-
rosis treatment. J Control Release 232: 152-160, 2016.