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Original Article

Effect of atorvastatin, 3-hydroxy-3-methylglutaryl coenzyme a reductase inhibitor (HMG-CoA) on the prevention of osteoporosis in ovariectomized rabbits

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Abstract: Osteoporosis is a systemic and metabolic skeleton disease characterized as low bone mineral density and micro-architectural deterioration, resulting in increased bone fragility and fracture risk. Although current drugs such as bisphosphonates, estrogen replacement treatment, and selective estrogen receptor modulators, had been used to treat osteoporosis in clinical, they are not the ideal ones due to their insufficient curative effects and adverse side effects. Recently, atorvastatin are used to treat osteoporosis more frequently. However, its clinical effect and treatment mechanism is still unknown. In present study, the bilateral ovariectomy of rabbits was duplicated to construct osteoporosis model. The effect of atorvastatin *in vivo* was determined, and its treatment mechanism was studied *in vitro* after the curative effect was explored. The results show that atorvastatin significantly increased the mechanical parameters such as maximum load, stiffness, and energy absorbing capacity, and improved the microarchitecture. They indicated that the antiosteoporosis activity of atorvastatin may be due to the promotion of differentiation of osteoblasts by inducing synthesis of vascular endothelial growth factor, bone morphogenetic protein 2 (BMP2), and core-binding factor alpha 1 (CBFα1), and inhibition osteoclasts formation through osteoprotegerin(OPG)-receptor activator for nuclear factor κB ligand (RANKL) system. Atorvastatin was effective in treating experimental osteoporosis. This study provides necessary experimental evidence for the clinical application of atorvastatin in osteoporosis treatment.

Keywords: Osteoporosis, ovariectomy, atorvastatin, osteoblasts, osteoclasts

Introduction

Osteoporosis is a chronic, metabolic, and systemic skeletal disorder characterized by diminished bone mass, microarchitectural deterioration, predisposing to decrease bone mineral density (BMD) and increase the risk of a broken bone [1, 2]. It is believed to currently affect more than 200 million people worldwide [3]. Population studies have consistently shown osteoporosis to be about two to four fold as common in women as in men because a sharp decrease in ovarian estrogen production causes rapid bone loss during the first decade after menopause [4]. Osteoporosis itself has no symptoms; its main consequence is the increased risk of bone fractures. The most common osteoporotic fractures occur in the vertebral column, rib, hip and wrist. Debilitating acute and chronic pain in the elderly is often attributed to fractures from osteoporosis and can lead to further disability and early mortality. By 2025, the annual rate of fractures is projected to increase by 50% from 2005, and annual costs are expected to reach $25 billion [5]. Therefore, prevention, detection, and treatment of osteoporosis are essential in reducing the personal and socioeconomic burden of osteoporotic fractures.

Current treatments for osteoporosis target either the osteoclast, by inhibiting bone resorption (antiresorptive agents), or the osteoblast, by stimulating bone formation (osteoanabolic agents) [6]. However, the decrease in bone resorption induced by antiresorptives is soon

accompanied by a decrease in bone formation, due to the coupling effect, thereby limiting their efficacy [7]. Likewise, increased bone formation by osteoanabolics is followed by an increase in bone resorption. Thus, there is a pressing medical need to develop new drugs that achieve uncoupling of bone formation from resorption.

Recently, statins, as a class of cholesterol-lowering drugs, have been proposed to prevent fractures and treat osteoporosis [8]. Apart from the inhibitory effect on osteoclasts differentiation through osteoprotegerin (OPG)-receptor activator for nuclear factor κB ligand (RANKL) system (also known as OPG/RANKL/RANK), statins also can enhance bone formation by inducing synthesis of vascular endothelial growth factor, bone morphogenetic protein 2 (BMP2), and core-binding factor alpha 1 (CBFα1) [9-11]. Similar results were also reported in clinical studies showing beneficial effects on blood lipids disorders, osteoporosis, and fracture prevention. Atorvastatin is being used to treat osteoporosis more frequently, and clinical studies have given controversial results. However, its clinical effect and molecular mechanism underlying atorvastatin acting on osteoporosis are still elusive. This study aimed to investigate the effects and the underlying mechanisms of atorvastatin on the prevention of osteoporosis in ovariectomized rabbits. Body weight observation, biochemistry, histomorphometry, mechanical test, micro-computed tomography (micro-CT), histology, and component analysis were performed. The effect of atorvastatin in vivo was determined, and its functional mechanism was studied in vitro.

Materials and methods

Animals

Seventy-five 6-month-old female New Zealand White rabbits (weight ranged from 2.5 to 3.0 kg) were purchased from the Animal Center of Bengbu Medical College for this study. They were housed in individual cages (320 cm×180 cm×160 cm) and allowed for 2 weeks to habituate housing conditions before the beginning of the experimentation. Food and water were available ad libitum for the duration of the experiments unless otherwise specified. The rabbits were maintained in a constant temperature (21±2°C) and humidity-controlled room (55±2%) with a 12 h light-dark cycle (lights on at 08:00 h). This study was carried out in strict accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of AnHui Laboratory Animal Monitoring Institute, the National Laboratory Animal Monitoring Institute of China. All experimental procedures performed in this studies involving animals were approved by the Ethical Committee of University of Bengbu Medical College. All efforts were made to minimize pain and suffering of the rabbits.

Experimental design

After a 2-week acclimatization period, the rabbits were randomly allocated to an ovariectomy model group (OVX) (n=55) and a sham operation group (n=22). Rabbits in the ovariectomy group underwent bilateral ovariectomy as previously described under general anesthesia with intravenous injection of pentobarbital sodium (40 mg/kg), and the sham operation was to expose the ovaries without excision. Postoperatively, all rabbits were deprived food and water for 12 h, and then intramuscularly injected benzylpenicillin (0.3×10^6 IU/kg) for 1 week to prevent inflammation. Even so, two rabbit from the sham group and five rabbits from the ovariectomy group died within 180 days after the surgery. To verify experimental animal model of osteoporosis, ten rabbits from each group were randomly picked out after ovariectomy to measure BMD of vertebrae, serum biochemical parameters, mechanical properties, and microarchitecture of the lumbar vertebrae. Once animal models had been successfully set up, the administration of atorvastatin was immediately performed following 180 days treatment. The treatment dosage was 40 mg/kg every day, and atorvastatin was administered orally. After medical treatment with atorvastatin for 90 and 180 days, respectively, the serum containing atorvastatin was separated. Finally, the micro computed tomography (microCT) analysis, the bone biomechanical test, and histopathological evaluation were carried out.

Histological examination

The sections of the third lumbar vertebra which was harvested 90 or 180 days after treatment were prepared as described previously. Samples were settled in 10% neutral formol-

saline for 5 days, dehydrated in graded ethanol, and embedded in paraffin after being decalcified in 10% ethylenediaminetetraacetic acid for 30 days. Then, the blocks were cut into 5-μm slices perpendicular to the longitudinal axis at the middle of the lumbar vertebra. The sections were examined for morphology under a light microscope after being stained with hematoxylin and eosin.

BMD analysis

Rabbits were anesthetized by intravenous injection of pentobarbital sodium (40 mg/kg), and the BMD of the vertebrae was measured by dual-energy X-ray absorptiometry (Lunar Prodigy Advance, GE Lunar, Madison, WI, USA) as previously described, and specific software for small animals (GE Medical Systems, enCORE 2004, version 8.80.001) was used. BMD measurements were conducted 0 days (180 days after bilateral ovariectomy), 90 days, and 180 days after treatment.

Biochemical assays of serum

Blood samples were collected from the central ear artery 0, 90, and 180 days after treatment. The serum was promptly separated and stored at -80°C. The serum calcium was determined with a Hitachi 7080 biochemical automatic analyzer (Hitachi, Tokyo, Japan), and the serum concentrations of bone alkaline phosphatase (BALP), tartrate resistant acid phosphatase 5b (TRAP5b), and N-terminal telopeptide of type I collagen (NTX) were measured using rabbit enzyme-linked immunoassay kits. All the samples were run in the same assay unless an individual value needed to be obtained again.

Micro-computed tomography (micro-CT) scanning

The LV4s were scanned using a desktop preclinical specimen micro-CT (μCT-40, Scanco Medical, Bassersdorf, Switzerland). Briefly, the vertebral bodies were aligned perpendicularly to the scanning axis for a total scanning length of 6.0 mm at custom isotropic resolution of 8-lm isometric voxel size with a voltage of 70 kV p and a current of 114 μA [12]. Three-dimensional (3D) reconstructions of mineralized tissues were performed by an application of a global threshold (211 mg hydroxyapatite/cm^3), and a Gaussian filter (sigma =0.8, support =2) was used to suppress noise. A volume of interest (VOI) containing only trabecular bone within the vertebral body extracted from the cortical bone with 1.80-mm thick (150 slices) was acquired 1.0-1.2 mm from both cranial and caudal growth plate-metaphyseal junctions [13]. The three-dimensional reconstructed images were used directly to quantify microarchitecture, and the morphometric parameters including bone volume fraction (BV/TV), trabecular number (Tb.N, 1/mm), trabecular thickness (Tb.Th, mm), trabecular separation (Tb.Sp, mm), structure model index (SMI, 1), and connective density (Conn.D, 1/mm^3) were calculated with the image analysis program of the microCT workstation (Image Processing Language v4.29d, Scanco Medical, Switzerland) [14].

Mechanical testing

The second lumbar vertebrae harvested 0, 90, and 180 days after treatment were frozen at -20°C, and the mechanical properties was measured as described previously. Bones were thawed at room temperature before the mechanical tests and were kept fully moist using gauze soaked in 0.9% NaCl solution during the entire test period. The vertebra was prepared by cutting off the end plates from the vertebral body to create parallel planar surfaces using a diamond wafer saw. The vertebral samples were then placed centrally between two steel parallel plates attached to the materials-testing machine (Instron 5565, Intstron, Norwood, MA, USA) and were tested along the longitudinal axis at a constant compressive speed of 1 mm/min [15]. The specimens were loaded to failure, and the mechanical parameters (maximum load, displacement, stiffness, and energy-absorption capacity) were calculated from the load-displacement curves. Briefly, the maximum load (N) was taken as the maximum force on the curve, displacement (mm) was taken as the ultimate deformity before failure, stiffness (N/mm) was determined from the slope of the linear portion, and the area under the load-displacement curve was defined as the energy-absorption capacity (mJ) [16].

Preosteoblast cell line culture with serum containing atorvastatin

Blood samples were collected from the central ear artery 1 h, 90 days, and 180 days after

Table 1. Summary of BMD, serum biochemical parameters and Micro-architecture parameters after ovariectomy for successive180 days

Characteristics	Sham group (n=10)	OVX group (n=20)
BMD	0.25±0.01	0.23±0.022)
Ca 2+	3.42±0.01	3.295±0.111)
BALP (mU/mL)	0.62±0.10	1.128±0.232)
TRAP5b (mU/mL)	5.92±0.81	8.61±1.122)
NTX (pmol/mL)	7.61±0.92	10.61±1.012)
Tb.N (1/mm)	1.12±0.11	0.72±0.132)
Tb.Th (mm)	0.33±0.03	0.22±0.012)
Tb.Sp (mm)	0.82±0.03	1.24±0.042)
BV/TV (%)	0.35±0.12	0.14±0.082)
BS/BV (1/mm)	6.17±0.38	7.43±0.242)

Note: BMD, bone mineral density, BALP bone alkaline phosphatase, NTX N-terminal telopeptide of type I collagen, OVX ovariectomy, TRAP5b tartrate-resistant acid phosphatase 5b, Tb.N trabecular number, Tb.Th trabecular thickness, Tb.Sp trabecular separation, BV/TV bone volume/total volume, BS/BV bone surface/bone volume. 1)$P < 0.05$, 2)$P < 0.01$.

treatment. Serum was promptly separated and stored at -80°C. The MC3T3-E1 cell line, derived from newborn murine calvariae and exhibiting a developmental sequence similar to that of primary osteoblasts, has been used extensively. The detailed culture protocols for osteoblasts were performed according to [17]. After resuscitation of MC3T3-E1 cells, 1×10^5 cells were added to the culture tubes on 96-well culture plates, with Dulbecco's modified Eagle's medium/F-12 (containing 10% fetal bovine serum) as the medium (100 μL per well). The cells were cultured under a humidified atmosphere of 5 % CO_2 at 37°C for 12 h to allow the osteoblasts to adhere to the 96-well plates, and then serum containing atorvastatin was added. Serum was diluted with Dulbecco's modified Eagle's medium to final concentrations of 20, 10, 5, and 2.5%. The medium was changed 2 days later, and cells were collected after another 2 days. Serum containing atorvastatin from 90 and 180 days after treatment was used to intervene in the preosteoblast cell line culture. The MC3T3-E1 cell proliferation was observed and measured.

RNA extraction

Isolation of total RNA was carried out according to manufacturer's instructions (NucleoSpin RNA II kit, Macherey-Nagel). Total RNA was extracted and prepared separately from each individual tissue. The frozen femur tissue samples were lysed and dounze-homogenized in the presence of highly denaturing ß-mercaptoethanol-containing buffer, which immediately inactivates RNases. Ethanol was added to provide appropriate binding conditions, and the sample was then applied to an RNeasy Mini spin column, where the total RNA binded to the membrane and contaminants were washed away. RNA was then eluted in 100-200 μL RNase-free water. The eluates were stored at -80°C.

Quantitative real-time PCR assay

The expression of four genes (BMP2, CBFα1, OPG, and RANKL) was measured with an Opticon fluorescence (SYBR Green II) quantitative real-time PCR system (MJ Research, USA). Briefly, total RNA of the femur tissue from each sample were reverse-transcribed using an Omniscript reverse transcription kit (Qiagen) following the manufacturer's instructions. And then the RT product (cDNA) subjected to 40 cycles of amplification with corresponding PCR primers, in which SYBR Green II was used as the signal fluorescence and ROX as the control fluorescence. The mRNA reverse transcription took place on an Applied Biosystems' GeneAmp PCR system 9700 and real-time PCR was carried out using the ABI7500 Real-Time PCR machine (Applied Biosystems). All samples were processed at the same time to avoid inter-experiment variance. Each real-time experiment was performed three times to confirm the data, and the mean value was used in the analysis. Six rabbits replicates per group were included in qRT-PCR in mRNA measured experiment. Samples were analyzed by the double delta CT (ΔΔCT) method. Delta CT (ΔCT) values represent normalized target genes levels with respect the internal control. Normalization was based on a single reference gene (β-Actin). Delta CT (ΔΔCT) values were calculated as the ΔCT of each test sample minus the mean ΔCT of the calibrator samples for each target gene. The fold change was calculated using the equation $2^{(-\Delta\Delta CT)}$.

Table 2. Effects of atorvastatin BMD, serum biochemical parameters and Micro-architecture parameters in osteoporotic rabbits

Characteristics	90 days			180 days		
	Shame (n=10)	OVX (20)		Shame (n=10)	OVX (n=20)	
		Control	Atorvastatin		Control	Atorvastatin
BMD	0.33 ± 0.03	0.27 ± 0.01	0.29 ± 0.02	0.31 ± 0.03	0.28 ± 0.02	0.32± 0.03[4)]
Ca^{2+} (mmol/L)	3.51±0.11	3.21±0.14	3.41±0.14	3.51±0.11	3.23±0.18	3.47±0.21
BALP (mU/mL)	0.65±0.13	1.13±0.25[2)]	0.85±0.21	0.65±0.13	1.12±0.15[2)]	0.78±0.12[3)]
TRAP5b (mU/mL)	6.11±1.39	8.49±0.42[2)]	7.14±0.79	6.11±1.39	8.35±1.71[2)]	6.36±0.69[4)]
NTX (pmol/mL)	7.59±0.19	10.54±1.21[2)]	8.161±0.29[3)]	7.59±0.19	10.62±1.15[2]	7.76±1.93[4)]
Tb.N (1/mm)	1.23±0.03	0.75±0.19[2)]	0.98±0.12[2),3)]	1.23±0.03	0.77±1.12[2)]	1.02±0.14[4)]
Tb.Th (mm)	0.33±0.05	0.21±0.02[2)]	0.23±0.07[1)]	0.34±0.04	0.26±0.03[2)]	0.32±0.16[4)]
Tb.Sp (mm)	0.86±0.08	1.24±0.09[2)]	1.05±0.12[1),4)]	0.86±0.08	1.24±0.08[2)]	0.88±0.18[4)]
BV/TV (%)	0.36±0.07	0.29±0.04[2)]	0.29±0.05[2),3)]	0.37±0.09	0.20±0.05[2)]	0.34±0.03[4)]
BS/BV (1/mm)	6.27±0.31	7.44±0.36[2)]	6.86±0.45[1),3)]	6.27±0.31	7.57±0.39[2)]	6.47±0.31[4)]

Note: [1)]$P < 0.01$ (analysis of variance, compared with the sham group), [2)]$P < 0.05$ (analysis of variance compared with the OVX control group), [3)]$P < 0.01$ (analysis of variance compared with the OVX control group), [4)]$P < 0.05$ (analysis of variance, compared with the sham group).

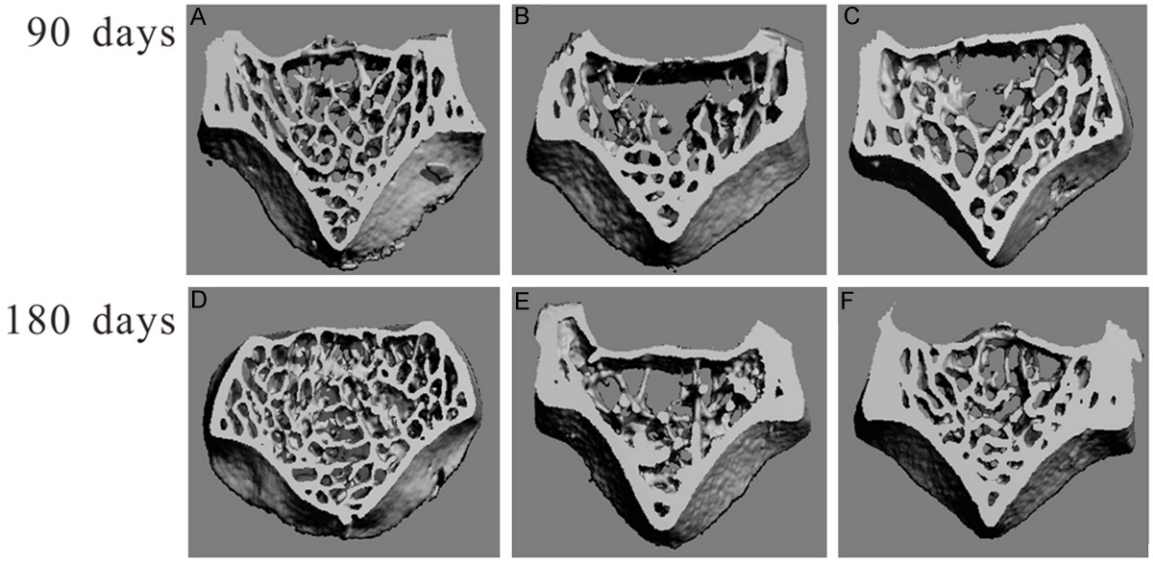

Figure 1. Three-dimensional reconstructions with micro computed tomography of the first lumbar vertebrae after 90 days (A-C) and 180 days (D-F) of treatment with atorvastatin: (A, D). Representation sham group; (B, E). Representation ovariectomy control group; (C, F). Representation ovariectomy atorvastatin group.

Statistical analysis

All experimental data were processed with the statistical system SPSS 18.0 and are presented as the mean ± SD. Differences in the mean values of BMD, serum biochemical parameters, mechanical parameters, and structural parameters between two groups 180 days after ovariectomy were compared with the independent-samples t test, and those between five groups at the same time point after treatment were performed using one-way analysis of variance with the Bonferroni post hoc test. $P < 0.05$ was considered statistically significant.

Results

Female New Zealand White rabbits underwent bilateral ovariectomy, and the animal model was measured 180 days after the operation (**Table 1**). We found that the BMD of the whole body of the variectomy group was significantly

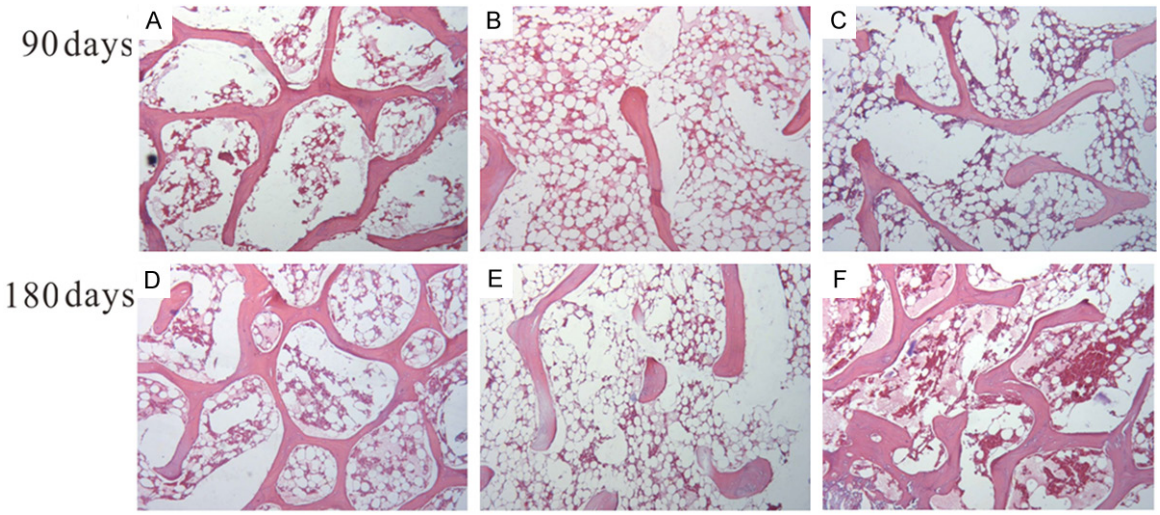

Figure 2. Morphology of the third lumbar vertebrae after 90 days (A-C) and 180 days (D-F) of treatment with atorvastatin (hematoxylin and eosin, × 40); (A, D) Sham group; (B, E) Ovariectomy control group; (C, F) Ovariectomy atorvastatin group.

lower than that of the sham group after ovariectomy. Compared with the sham group, for the ovariectomy group, the levels of bone turnover biomarkers such as Ca^{2+}, BALP, TRAP5b, and NTX were significantly higher, the biomechanical parameters such as the maximum load, displacement, stiffness, and energy absorbing capacity were significantly lower. The microarchitecture parameters TB.N, Tb.Th, and BV/TV were significantly lower, and the microarchitecture parameters Tb.Sp and BS/BV were significantly higher. All data confirmed that the osteoporotic rabbit models were set up successfully 180 days after ovariectomy.

Atorvastatin was injected orally to the osteoporotic rabbit model at a dosage of 40 mg/kg per day. After treatment for 90 and 180 days, respectively, the serum comprising atorvastatin was separated. The serum biochemical parameters test, micro-CT analysis, the bone biomechanical test, and histopathological evaluation were performed. The results showed that atorvastatin significantly decreased the level of bone turnover biomarkers such as BALP, and NTX (**Table 2**), increased the mechanical parameters such as the maximum load, stiffness, and energy-absorbing capacity, increased the microarchitecture parameters TB.N, Tb.Th, and BV/TV, decreased the microarchitecture parameters Tb.Sp and BS/BV and improved the microarchitecture (**Figures 1**, **2**) and BMD, which indicated that atorvastatin was effective in treating experimental osteoporosis.

The messenger RNA analysis showed that atorvastatin significantly increased the expression of BMP2, CBFα1 and OPG, especially CBFα1, but decreased the expression of and RANKL (**Figure 3**). This suggests that atorvastatin is effective in treating experimental osteoporosis, and the antiosteoporosis activity of atorvastatin might be due to the promotion of osteoblasts and the inhibition of osteoclasts.

Serum including atorvastatin can improve preosteoblast cell line proliferation. The results demonstrated that serum containing atorvastatin activated cell proliferation significantly: the best effect was obtained at a final concentration of 10%, and the effect was better at 90 days than at 180 days (**Figure 4**). These results suggest that the antiosteoporosis activity of atorvastatin may be due to the promotion of proliferation and differentiation of osteoblasts. Serum containing atorvastatin increased the expression of BMP2, CBFα1 and OPG, and decreased RANKL expression. The effect was greater at 90 days than at 180 days. Altogether, these results suggest that the antiosteoporosis activity of atorvastatin could be attributed to the promotion of differentiation of osteoblasts and, suppression of differentiation of osteoclasts (**Figure 5**).

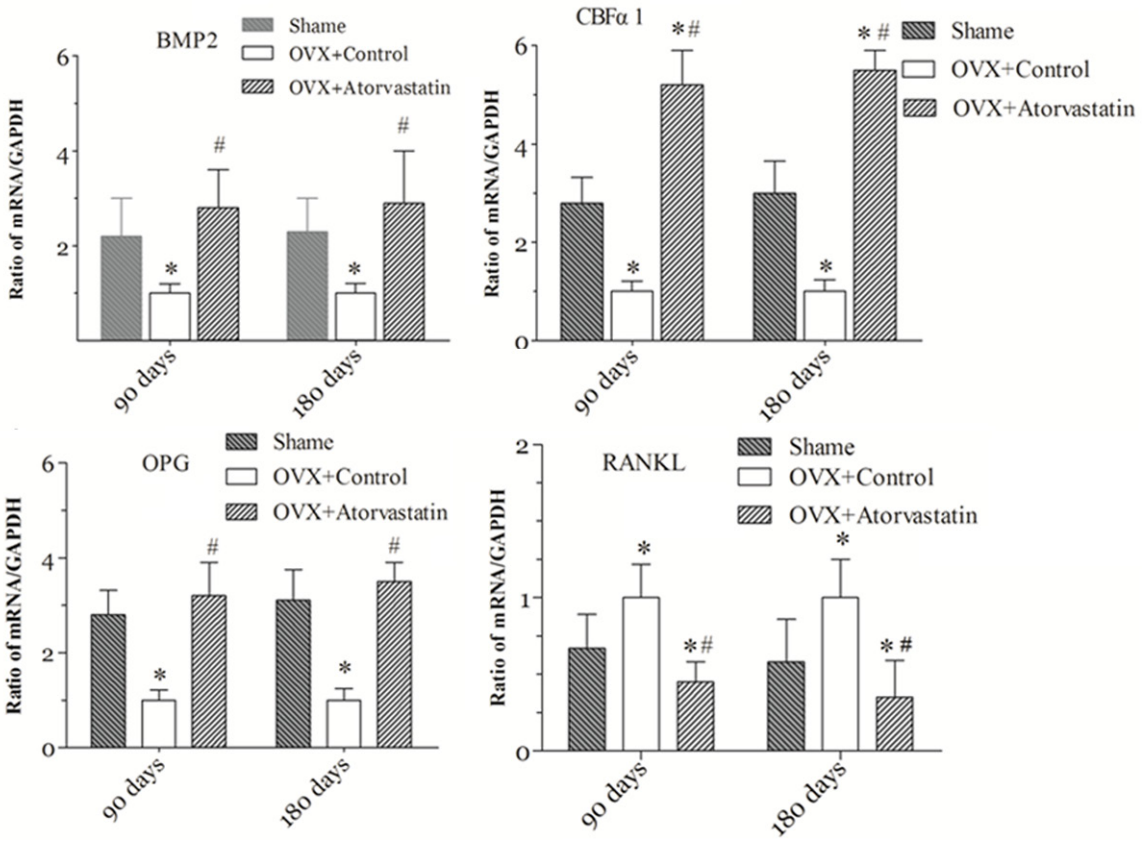

Figure 3. The femur tissue expression of four genes: bone morphogenetic protein 2 (BMP2), core-binding factor alpha 1 (Cbfa1), osteoprotegerin (OPG), and receptor activator for nuclear factor κB ligand (RANKL). *Sign $P < 0.05$ (compared with the sham group), #Sign $P < 0.05$ (compared with the ovariectomy control group).

Discussion

Osteoporosis, osteoporosis related fractures and large bone defects are the current problems in orthopedic research. In a normal bone, there is a balance between new bone formation and osteoclastic reaction which is responsible for bone health and mechanical properties [18-20]. In osteoporotic patients, such balance is altered; thus the biomechanical and morphological features of such bone are significantly altered which result in bone weakening and fracture injuries. After the occurrence of vehicle traumas, high energy traumas, comminuted-, compound-, multiple- and complicated fractures, bone tumors, burns, osteonecrosis, osteomyelitis and osteoporosis related fractures, it is often necessary to remove the damaged bony segment/s and stabilize the remaining bone. Prevention, management and treatment of such diseases and injuries are challenging [21]. Although many surgical and pharmaceutical options are available, because healing of the injured bone is a multifactorial process, treatment of such diseases is a state of art.

Statins, 3-hydroxy 3-methyl glutaril coenzyme A (HMG-CoA) reductase inhibitors, were first developed to control and treat patients with hyperlipidemia and hypercholesterolemia [22]. In 1999, statins were found to have osteogenic effects which may be beneficial for osteoporotic patients [23, 24]. To date, we know much more about statins than before. Since the discovery of statins, it has been shown that statins not only are beneficial for managing osteoporosis but also they are strong modulators of bone healing responses. Based on the recent investigations, statins have the ability to modulate inflammation, enhance osteoinduction, osteogenesis and angiogenesis, and inhibit osteoblast apoptosis and osteoclastogenesis [25, 26]. However, its clinical treatment mechanism

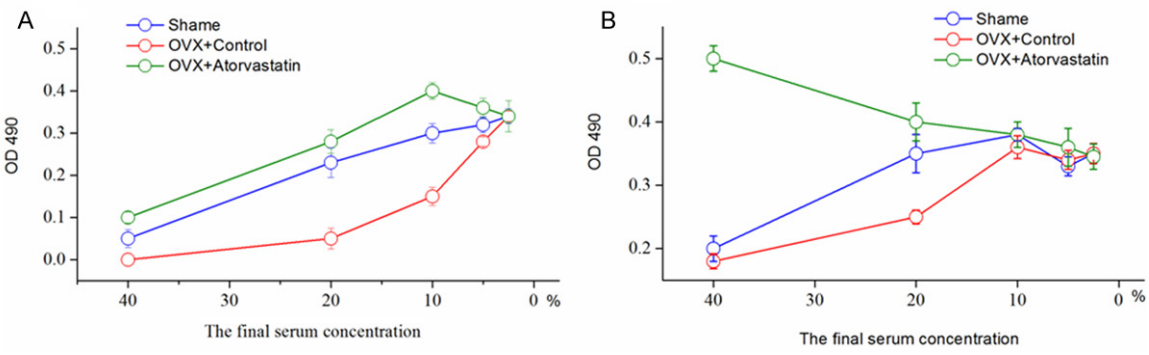

Figure 4. Effects of atorvastatin containing serum at (A) 90 days and (B) 180 days on MC3T3-E1 cell proliferation.

is still unknown. With the use of animal models, the treatment effectiveness of atorvastatin on experimental osteoporosis was investigated and the functional mechanism was preliminarily explored in this study.

On the basis of the animal models, our results demonstrated that atorvastatin significantly decreased the level of bone turnover biomarkers such as BALP, TRAP5b, and NTX, increased the mechanical parameters such as the maximum load, stiffness, and energy-absorbing capacity, increased the microarchitecture parameters TB.N, Tb.Th, and BV/TV, decreased the microarchitecture parameters Tb.Sp and BS/BV, and improved the microarchitecture and BMD. Atorvastatin was significant effective in treating experimental osteoporosis, and the findings support those of previous studies [27-29].

Then, we further explore the molecular mechanisms underlying the atorvastatin treatment of osteoporosis in vivo and vitro respectively. The data in vivo showed that atorvastatin significantly increased the expression of BMP2, CBFα1 and OPG, especially CBFα1, but decreased the expression of and RANKL. The BMP2 and CBFα1 genes are important signals in osteoblast proliferation and differentiation [30, 31], whereas the OPG and RANKL genes are critical factors in osteoclast proliferation and differentiation [32]. BMP-2 and Cbfα1 act as two important regulators of osteoblast differentiation and maturation process, they not only promote osteoblast differentiation, but also affects osteoblasts after differentiation and maturation of function. On the one hand, BMP-2 can bind to and activate the mesenchymal cell surface-specific receptors, causing the signal transduction and thereby promoting osteogenesis; on the other hand, it can promote osteocalcin, alkaline phosphatase enzymes osteoblast phenotype-related gene expression in myoblasts [33]. Furthermore, BMP-2 also promotes and inhibits differentiation of preosteoblast cells and fat precursor cell, receptively [34]. Cbfα1 overexpressed in non osteoblast or preosteoblast cells can upregulate specific markers of osteoblast such as collagen α1 and osteocalcin [35]. In addition, Cbfα1$^{-/-}$ mice is unable to survive due to the complete lack of ossification in the skeletal system [36]. BMP-2 can promote osteogenesis by upregulation Cbfa1, in turn, Cbfa1 increase the expression of BMP-2 through positive feedback. Thus, BMP-2 and Cbfα1 collaborative facilitate ossification differentiation and adjust the balance of bone metabolism [31, 37, 38]. OPG/RANKL/RANK system is recently found a crucial signal transduction pathway about osteoclast action on osteoclasts and differentiation. RANKL upon binding to its cell surface receptors RANK, immediately activates nuclear factor-κB (NF-κB), c-Jun N-terminal kinase (c-Jun N-terminal kinase, JNK), p38 and extracellular signal regulated kinase by RANKL/RANK/TRAF6 axis, and thereby regulating osteoclast differentiation, activation, maturation and bone resorption activity [39, 40]. OPG is mainly secreted by osteoblasts as a tumor necrosis factor receptor-associated protein. It can be used as a decoy receptor which can block the binding of RANKL and RANK, resulting in repress maturation and differentiation of osteoclast cells. Therefore, osteoblasts can modulate maturation and differentiation in osteoclastic cells by regulating the expressed ratio of OPG and RANKL. Our results suggested

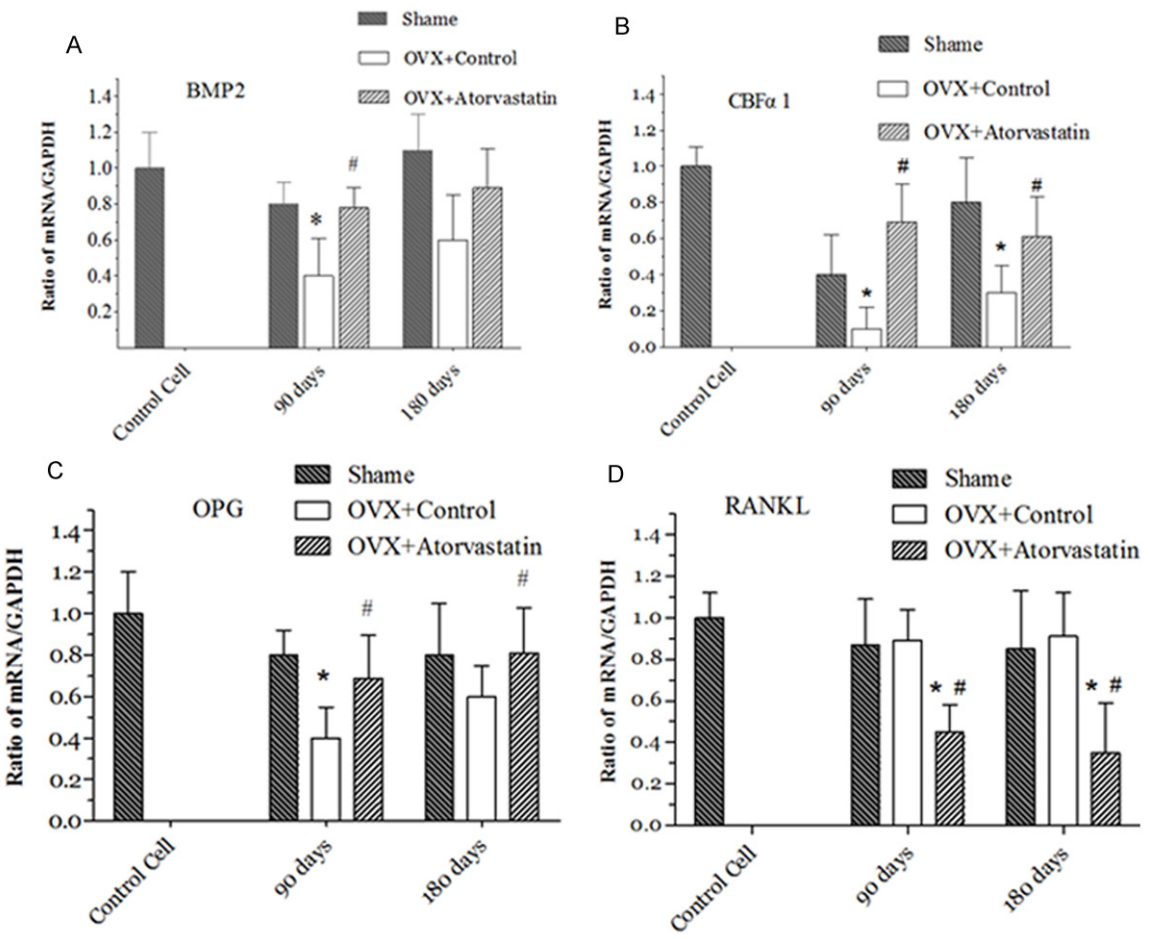

Figure 5. Effects of atorvastatin containing serum at 90 and 180 days on MC3T3-E1 cell expression of four genes: bone morphogenetic protein 2 (BMP2), core-binding factor alpha 1 (Cbfa1), osteoprotegerin (OPG), and receptor activator for nuclear factor κB ligand (RANKL). *Signs $P < 0.01$ (compared with the sham group), #Signs $P < 0.01$ (compared with the ovariectomy control group).

that atorvastatin was effective in treating experimental osteoporosis, and the antiosteoporosis activity of atorvastatin might be due to the promotion of osteoblasts and the inhibition of osteoclasts.

In view of atorvastatin as an inactive lactone prodrug, it can not suppress mevalonate synthesis in vivo, unless converted to an open-loop β-hydroxy acid form (i.e. simvastatin acid) within the body by the action of esterase [41]. Direct intervention by the original drug atorvastatin cultured cells may not play its efficacy. Containing serum refers to blood serum collected in human or animal administration of drug for a period of time. The method of serum pharmacology is an analog and trying to alternative drugs itself for the experimental study. Recently, this method caused a vertical multi researchers' attention, and did extensive research, showing good prospects [42-44]. Therefore, in this study we used orvastatin-containing serum to intervene in cultured cells, in order to better simulate the pharmacodynamics of simvastatin. Date from vitro showed that serum containing atorvastatin can improve preosteoblast cell line proliferation. Serum containing atorvastatin can elevate the expression of BMP2, CBFα1, OPG and reduce RANKL expression in the MC3T3-E1 cell line. This suggests that the antiosteoporosis activity of atorvastatin may be due to the promotion of proliferation and differentiation of osteoblasts and the inhibition of osteoclast proliferation and differentiation. The in vitro results were compatible with the in vivo data.

Our results support previous findings that atorvastatin has advanced effects on bone formation in vivo and in vitro [11, 45-47]. To our knowledge, our study for the first time confirmed atorvastatin have effect on Atorvastatin has a stimulant effect on MC3T3-E1 cells in vitro, and enhances the expression of osteogenesis genes, such as OPG. But serum containing atorvastatin can improve pre-osteoblast MC3T3-E1 cell line proliferation, with different effects on the stage at 90 and 180 days. The different effects indicate that other components could participate in the functional stimulation. Metabolic analysis by ultraper formance liquid chromatography-tandem mass spectrometry proved that the biomarkers related to osteoporosis were different between the control group and the model group [48]. Which factors participate in the functional stimulation remains unclear. This needs to be investigated further.

In summary, the in vivo and in vitro data indicate that atorvastatin was effective in treating experimental osteoporosis, and it has potential clinical application for osteoporosis treatment.

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Disclosure of conflict of interest

None.

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