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ORIGINAL ARTICLE

Comparative impact of systemic delivery of atorvastatin, simvastatin, and lovastatin on bone mineral density of the ovariectomized rats

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Abstract

Purpose In addition to lipid-lowering properties, statins have been suggested to affect bone turnover by increasing the osteoblastic bone formation and blocking the osteoclastogenesis. However, there are many controversial reports regarding the beneficial effect of statins on osteoporosis. In this study, we investigated the therapeutic effects of the most important lipophilic statins administered orally for 60 days to the ovariectomized (OVX) female Sprague–Dawley rats and compared the effects on different harvested trabecular and compact bones.

Methods Thirty female rats were divided into five equal groups including the normal rats, untreated OVX rats (negative control), and the OVX rats treated with atorvastatin (20 mg/kg/day), simvastatin (25 mg/kg/day), and lovastatin (20 mg/kg/day). The osteoporotic animals were treated daily for 60 days and euthanized at the end of experiments. The effectiveness of these treatments was evaluated by biomechanical testing, histopathologic, histomorphometric, micro-CT scan, real-time PCR, and serum biochemical analysis. Moreover, the hepatotoxicity and rhabdomyolysis related with these treatments were assessed by biochemistry analysis and histopathological evaluation.

Results The results and statistical analysis showed that systemic delivery of simvastatin and lovastatin significantly increased serum calcium level, expression of osteogenic genes, bone mineral density (BMD), and biomechanical properties in comparison to the untreated OVX rats, especially in trabecular bones ($P < 0.05$). The results of different analysis also indicated that there was no statistical difference between the atorvastatin-treated animals and the negative control. Among all treatments, only atorvastatin showed an evident hepatotoxicity and myopathy.

Conclusions It was concluded that the lovastatin and simvastatin efficiently ameliorated the OVX-induced osteoporosis. Moreover, the simvastatin-treated animals showed more resemblance to the normal group in terms of BMD, expression of osteogenic genes, serum biochemical parameters, histomorphometric findings, and biomechanical performance with no significant side-effects.

Keywords Osteoporosis · Simvastatin · Lovastatin · Atorvastatin · Systemic drug delivery · OVX rat

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Introduction

Osteoporosis is a major health issue characterized by low bone mass and debilitating of bone structure leading to increased bone fragility and susceptibility to fracture [1]. Osteoporosis more frequently occurs in females than males. Post-menopausal women more commonly suffer from this systemic skeletal disease with more fractures compared to premenopausal women [2]. Patients with definite diagnosed osteoporosis may lose up to half of their bone mass at imperative sites of their skeletons [3, 4]. Nowadays, several drugs such as RANK ligand antibodies (denosumab), selective estrogen receptor modulators (SERMs),

teriparatide, and bisphosphonates, are administered to treat this disease [5]. Most of these drugs reduce bone remodeling because they result in reduced osteoclast activity and recruitment of new osteoclasts. Although these drugs have a specific function in treatment and control of this disease, there are several limitations in long-term use of these treatments. For instance, atrial fibrillation, osteonecrosis of the jaw and severe suppression of bone turnover have been associated with long-term bisphosphonates therapy [6]. Moreover, venous thromboembolism and fatal strokes have been reported due to long-term use of SERMs medication (raloxifene) [7]. Thus, an influential drug is required to not only enhance the BMD but also results in the formation of a new bone without serious side effects related to long-term medication.

Statins as lipid-lowering drugs have frequently been utilized in cardiovascular diseases [8, 9]. In addition, it has been shown that they can be helpful in bone regeneration [10]. Statins represent a dual mode of action by ameliorating osteogenesis and inhibiting osteoclast activity [11]. In fact, statins enhance osteoblastic differentiation of osteoprogenitor stem cells, down-regulate osteoblasts apoptosis, and upregulate expression of bone morphogenetic protein-2 (BMP-2) [10, 12]. Based on the experimental and clinical studies, the lipophilic statins particularly atorvastatin, simvastatin, and lovastatin seem to be the most effective statin types in the treatment of osteoporosis [13, 14]. Previous in vivo studies indicated that atorvastatin promoted the expression of BMP-2 [15] and Insulin-like growth factor 1 (IGF-1) [16] and improved osteoporotic alterations stimulated by ovariectomy [13]. Simvastatin is an inhibitor of 3-hydroxy-3-methylglutarylcoenzyme A (HMG-CoA) reductase. It has been suggested that simvastatin can increase BMD, decrease the rate of osteoporotic fractures and enhance fracture healing by its beneficial effects on bone metabolism [17]. In this regard, an interesting study showed that high-dose of simvastatin (20 mg/kg/day) significantly increase bone fracture healing, especially in OVX rats [18]. In addition, this lipophilic statin has been successfully applied to improve osteogenesis around the titanium implants [19]. Lovastatin is a bone anabolic agent which also promotes bone formation [20]. However, there is less evidence regarding its bone regenerative impact compared with the aforementioned statins. Several clinical investigations have shown controversial findings when the osteogenic markers have been compared between the statin-treated patients and the normal populations [21, 22]. On the other hand, several studies have reported no significant effect by these statins on bone mineral density [23, 24].

Quite a few studies have compared the impact of different lipophilic statins on osteoporotic animal models [25, 26]. Moreover, the majority of studies have assessed the beneficial effects of statins on BMD of trabecular bone and their effect on compact bone has not well been investigated yet [25, 27]. Another issue related to administration of statin drugs is their serious adverse effects. High doses of statins are required in vivo to preserve the bone and stimulate bone formation and such doses may result in serious side effects such as hepatotoxicity and myopathy [28, 29]. Therefore, in the current study, we aimed to 1) comprehensively and comparatively evaluate the effects of lipophilic statins including atorvastatin, simvastatin, and lovastatin on trabecular and compact bone tissues (vertebra, pelvis, and femur) in OVX-induced osteoporotic rats, 2) assess the potential skeletal muscle and liver-related side effects of these drugs via histopathology and serum biochemistry analysis.

Materials and methods

Animals and surgical procedures

A total of thirty adult female Sprague–Dawley rats (200–250 g, 10–11 weeks) were purchased from the Razi Institute, Karaj, Iran. The animals received ad libitum access to standard chow pellets and water throughout the experimental period. The animals were anesthetized by intramuscular (IM) injection of 50 mg/kg Ketamine hydrochloride (10% Ketamine, Alfasan Co., Woerden, Netherland), 2 mg/kg Xylazine (2% Xylazine, Alfasan Co., Woerden, Netherland), and 1 mg/kg Acepromazine maleate (Alfasan Co., Woerden, Netherland), for OVX surgery. After shaving off the hair on the bilateral dorsal regions, the ovaries were exposed by a 2 mm incision with the aid of straight surgical tweezers. After vascular ligation, the ovaries were resected with surgical scissors and the other exposed tissues were repositioned. The incision including muscles and skin was sutured in a routine fashion with 3.0 silk threads. Postoperative pain relief and antibiotic therapy were then provided by subcutaneous (SC) administration of 1 mg/kg Meloxicam (2% Meloxivet, Razak Co., Tehran, Iran) and IM administration of enrofloxacin (5% Enron, Irfan, Tehran, Iran), respectively, for 5 days. The animals received humane care in compliance with the Guide for Care and Use of Laboratory Animals published by the National Institutes of Health (NIH Publication No. 85-23, revised 1985). This Experiment was approved by the local Ethics Committee of "Regulations for using animals in scientific procedures" in our Medical Science University.

All treatments were started 3 months after surgery, a time which has been well established in the previous literature for general osteopenia [30]. Three animals from normal rats and OVX group at 3 months post-OVX surgery were euthanized and their bones were histologically evaluated, as

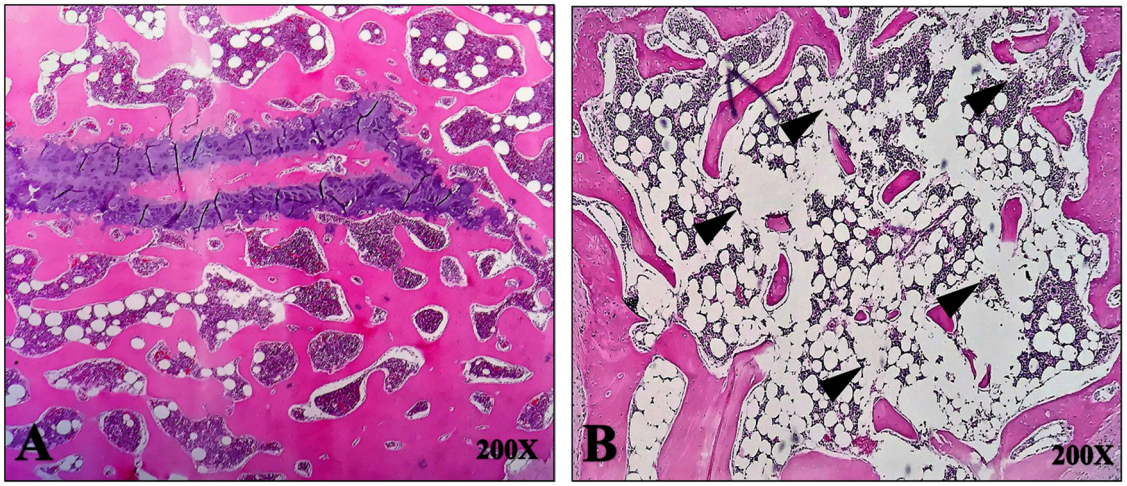

Fig. 1 Evaluation of osteopenia induced OVX before initiation of treatments. **a**. Normal rats, **b**. OVX rats. The micrographs of OVX rats showed a typical osteopenia with loss of trabecular bone volume and interconnectivity. Arrow heads: loss of interconnectivity. H&E staining

a check to be certain of the establishment of an osteopenic state at the time treatment began (Fig. 1). The animals received Phosphate buffered saline (PBS) (negativecontrol), atorvastatin (20 mg/kg/day), simvastatin (25 mg/kg/day), and lovastatin (20 mg/kg/day) by oral gavage for 2 months and the animals in the last group were also operated but were not ovariectomized and received PBS daily (Normal group) ($n = 6$ animals in each group). The oral doses of each treatment were selected according to the beneficial protective in vivo results of these drugs on bone and mineralized tissues from the previous literature [31, 32]. Because of lack of data about the specific lovastatin bone protective dosage (oral), the therapeutic dose was adjusted based on the other treatments (20 mg/kg/day). The rats were euthanized two-month post-treatment by IM injection of 50 mg/kg Ketamine hydrochloride and 2 mg/kg Xylazine hydrochloride. Then, 1 mg/kg Gallaminetriethiodide (Specia, Paris, France) was injected intracardially to stop breathing of the anesthetized animals.

Micro-computed tomography (CT) testing

Micro-computed tomograms (micro-CTs) of the harvested femoral bones were acquired at 70 kVp, 114 µA for 800 ms, using Scanco µCT35 scanner (Scanco, Wangen-Brüttisellen, Switzerland). Mid-shaft and head of femur were analyzed as compact and trabecular bones, respectively. Two hundred slices of the region of interest were evaluated by micro-CT scan (thickness of each slice = 6 µm, voxel size = 10 µm). The bone volume/total volume (BV/TV), bone mineral density (BMD), cortical bone thickness (Ct. Th) and trabecular bone thickness (Tb.Th) were evaluated based on the micro-CT scan results.

Serum and blood biochemical analysis

Five milliliter whole blood was collected from the heart in terminal anesthesia (each animal) through cardiac puncture and divided into two parts. 1.5 ml of the blood sample was transferred into a tube containing EDTA as an anticoagulant and 3.5 ml of the blood sample was transferred to a plane tube and centrifuged for 10 min at 3000 r.p.m. for serum separation. The serum samples were kept and stored at −20 °C till measurements. The percentage of hematocrit, levels of serum total protein, calcium, phosphorous, aspartate aminotransferase (AST), alanine aminotransferase (ALT), CK (creatine kinase), and alkaline phosphatase (ALP) were determined by commercially available kits (Pars Azmoon kit; Pars Azmoon Co., Tehran, Iran), using an autoanalyzer (Mindray, BS-200, China).

Histopathologic and histomorphometric evaluations

The harvested bones including lumbar vertebra (L4), right-femur, and ileum bones were dissected, to get free from soft tissues, fixed in 10% neutral buffered formalin solution for 48 h, and decalcified with 10% EDTA (pH 7.4) for 30 days. The decalcified bones were then dehydrated in graded ethanol (70–100%), cleared in xylene, embedded in paraffin, and finally, 5 µm thick sections were prepared and stained with hematoxylin and eosin (H&E). The histological sections were examined, using an ordinary light microscope (Olympus BX51; Olympus, Tokyo, Japan) and blindly scored by two independent pathologists. For histomorphometric analysis, the percentage of trabecular bones (pelvis, vertebra, femoral head) and compact bone thickness (mid shaft of femoral bone) were calculated and analyzed, using

Table 1 Primers used in qRT-PCR

Gene	Primer sequence	Size (bp)	Genebank code	Annealing temperature (°C)
Ocn	F: GAGGGCAGTAAGGTGGTGAA	135	NM_013414.1	60
	R: GTCCGCTAGCTCGTCACAAT			
Alp	F: GCACAACATCAAGGACATCG	195	NM_013059.1	60
	R: TCAGTGCGGTTCCAGACATA			
Col1a1	F:GAATATGTATCACCAGACGCAG	186	NM_053304.1	60
	R: AGCAAAGTTTCCTCCAAGAC			
GAPDH	F: GACTTCAACAGCAACTCCCAC	1306	NM_017008.4	60
	R: TCCACCACCCTGTTGCTGTA			

Ocn osteocalcin, *Alp* alkaline phosphatase, *Col1a1* collagen type 1 a1, *GAPDH* host keeping gene

computer software Image-Pro Plus®V.6 (Media Cybernetics, Inc., Silver Spring, USA). Moreover, the adverse effects of different treatments on the liver and skeletal muscle were evaluated histopathologically.

Quantitative real time-PCR (qRT-PCR) analysis

A fresh-frozen lumbar vertebra (L5) from each group was stored at −70 °C till measurements. The frozen vertebras were air dried at room temperature and immediately dipped in liquid nitrogen until reached a temperature of −195 °C, then transferred into an RNAs free crucible in order to make bone powder. Total RNA from the powdered samples were extracted, using RNeasy Micro Kit (Qiagen-74004). The RNA quality and quantity were assessed by ND-1000 spectrophotometer (NanoDrop, Wilmington, DE, USA), and its integrity was determined by the agarose gel electrophoresis. cDNA was synthesized using the Revert Aid First Strand cDNA Synthesis Kit (Fermentas, Sankt Leon-Rot, Germany, k1632) according to the manufacturer's instructions. The qRT-PCRreaction was performed with SYBR® Green PCR Master Mix (Applied BiosystemsLifeTechnologies, Inc, REF 4367659) with a real-time PCR system (Applied Biosystems Life Technologies, Inc., ABiStepOnePlus) and analyzed with StepOne software (Applied Biosystems, version 2.1). Relative quantification was performed, using comparative CT method (also known as the $2-^{\Delta\Delta Ct}$ method), where a number oftarget genes (Collagen type 1-Col1, ALP, osteocalcin-OCN) normalized against glyceraldehyde 3-phosphate dehydrogenase (GAPDH, housekeeping gene) mRNA and relative to calibrator group (normal bone samples). All reactions were performed in duplicates and all samples were collected from three biological replicates. Table 1 lists the primers.

Biomechanical evaluation

The bone samples (left-femur, and lumbar vertebra (L6), $n = 6$ for each group) were firstly removed from muscles and tendons, wrapped in saline-soaked cotton in order to prevent dehydration and were frozen at −20 °C until biomechanical evaluation. The biomechanical analysis was performed on the compact and trabecular bones as have previously been described [33]. The bone specimens were subjected to bending (compact bone) and compression testing (trabecular bone), using a universal tensile testing machine (Instron, London, UK) and Enduratec ELF 3200 testing machine (Bose Corporation, Eden Prairie, MN). For compact bones, the samples were placed horizontally on two rounded supporting bars separated at a distance of 16 mm. The third bar was carefully positioned at the midpoint of the loaded bone. The rate of loading on the bone specimen was 2 mm/min until fracturing. For trabecular bone, the compression test was performed until the bone fractured. The maximum load (N) and Stiffness (N/mm) were then calculated from the load-deformation curve and analyzed for each specimen. The biomechanical results calculated from the load-deformation curve were presented as the Mean ± standard deviation (SD).

Statistical analysis

One-way ANOVA with subsequent Tukey post-hoc test was used to compare the quantitative data between the groups. The scored values (qualitative data) were statically analyzed by Kruskal–Wallis H and non-parametric ANOVA, and if the differences were significant ($P < 0.05$), then analyzed by Mann–Whitney U test. All statistical analyses were performed by GraphPad Prism software, Version 6.00 (GraphPad Prism, Inc., San Diego, CA).

Results

Micro-CT findings

Micro-CT scan was utilized to analyze the BV/TV, BMD, Ct.Th, and Tb.Th at 60 days post-treatment (Fig. 2). Compared to the normal group, a significant reduction in BMD of cortical bone was seen in the negative control group ($P = 0.003$). The BMD of cortical bone in the simvastatin-treated group was significantly higher when compared to the negative control ($P = 0.02$). There was no significant difference between the simvastatin-treated group and the normal rats. The images of micro CT showed a significant difference between the simvastatin group and other treatment groups in terms of the trabecular BMD. In addition, the BMD of trabecular bone in the lovastatin treated group was significantly higher than the untreated group (negative control). The rats in the simvastatin group had more BV/TV of compact and trabecular bone in comparison to the atorvastatin ($P = 0.007$) and untreated animals ($P = 0.004$). The Ct.Th of the femoral bone in the simvastatin ($P = 0.04$) and normal ($P = 0.02$) rats were significantly higher than the negative control and the atorvastatin-treated groups. Analysis of the normal ($P = 0.005$) Simvastatin ($P = 0.03$) and Lovastatin ($P = 0.04$) groups showed significantly higher Tb.Th than the untreated animals. Moreover, the Tb.Th index was significantly higher in the normal ($P = 0.02$) and simvastatin groups ($P = 0.03$) in comparison to the atorvastatin-treated rats.

Serum biochemical and blood parameters

The effects of different treatments on serum biochemical and blood parameters are shown in Table 2. The serum calcium (Ca) level was significantly lower in the negative control and atorvastatin treated-group than the normal group ($P = 0.02$). The final serum Ca concentration in the simvastatin group was significantly higher than that of the negative control group ($P = 0.03$). The serum level of ALP was significantly higher in the negative control ($P = 0.005$) and atorvastatin treated-group ($P = 0.006$) than in the normal and simvastatin-treated animals. The serum level of TG in the statin-treated animals was significantly lower than the untreated and normal animals (Atr: $P = 0.008$, Sim and Lev: ($P = 0.006$). The ALT ($P = 0.007$) and CK ($P = 0.001$) serum levels significantly increased in the atorvastatin-treated animals in comparison to other treatments. However, there was no significant difference between the atorvastatin, simvastatin, and lovastatin-treated groups in terms of serum TG levels ($P > 0.05$). The serum phosphorus (P) concentration, AST, total protein, and hematocrit were not significantly different between the groups.

Histopathological and histomorphometrical findings

All H&E-stained sections from different experimental groups were comparatively evaluated histologically (Fig. 3). Normal compactness of the vertebral body and competent trabeculae were found in the normal animals. The lumbar vertebral micrographs (L4) of the negative control group showed sparse loss of the trabecular interconnectivity and thinning of the trabeculae, resulting in widened intertrabecular spaces. There was a significant increase in trabecular interconnectivity in the lovastatin treated group in comparison to the negative control; however, the thickness of trabeculae did not access to the normal statues. Although the connection of trabecula was enhanced in the atorvastatin group when compared to the untreated group, this finding was not significant via histomorphometric analysis. The simvastatin treated animals showed more resemblance to the normal group in terms of trabecular interconnectivity, trabecular bone percentage, and thickness of the trabecular bone. No significant histopathological changes were found in the tissue sections of the femoral diaphysis samples in all treated groups. The sections of the pelvis in the untreated animals showed a significant reduction in trabecular bone density (%) in comparison to the normal, simvastatin, and lovastatin-treated animals. However, the histomorphometric analysis did not show any significant differences between the negative control and atorvastatin group in terms of trabecular percentage in the pelvis samples. Thinning or even disappearance of trabeculae was also seen in the femoral epiphysis of the negative control group. The trabeculae in the negative control group were significantly fewer in comparison to the normal and simvastatin-treated animals. In overall, bone histopathology revealed a marked recovery effect of simvastatin and showed a restored architecture with this treatment regime in the ovariectomy-induced osteoporosis model in rats. The histomorphometric analysis of all groups is shown in Fig. 4a.

The liver and skeletal muscles were histopathologically evaluated to find out any hepatic toxicity and myopathy related to long-term statins therapy (Fig. 5). The histopathological findings revealed infiltration of mononuclear inflammatory cells and a severe necrotizing myopathy in the atorvastatin-treated animals. The micrographs of liver also showed severe degeneration (hydropic degeneration) and mild inflammation of hepatic cells in this group. There were no significant pathological changes in liver and skeletal muscle of other groups.

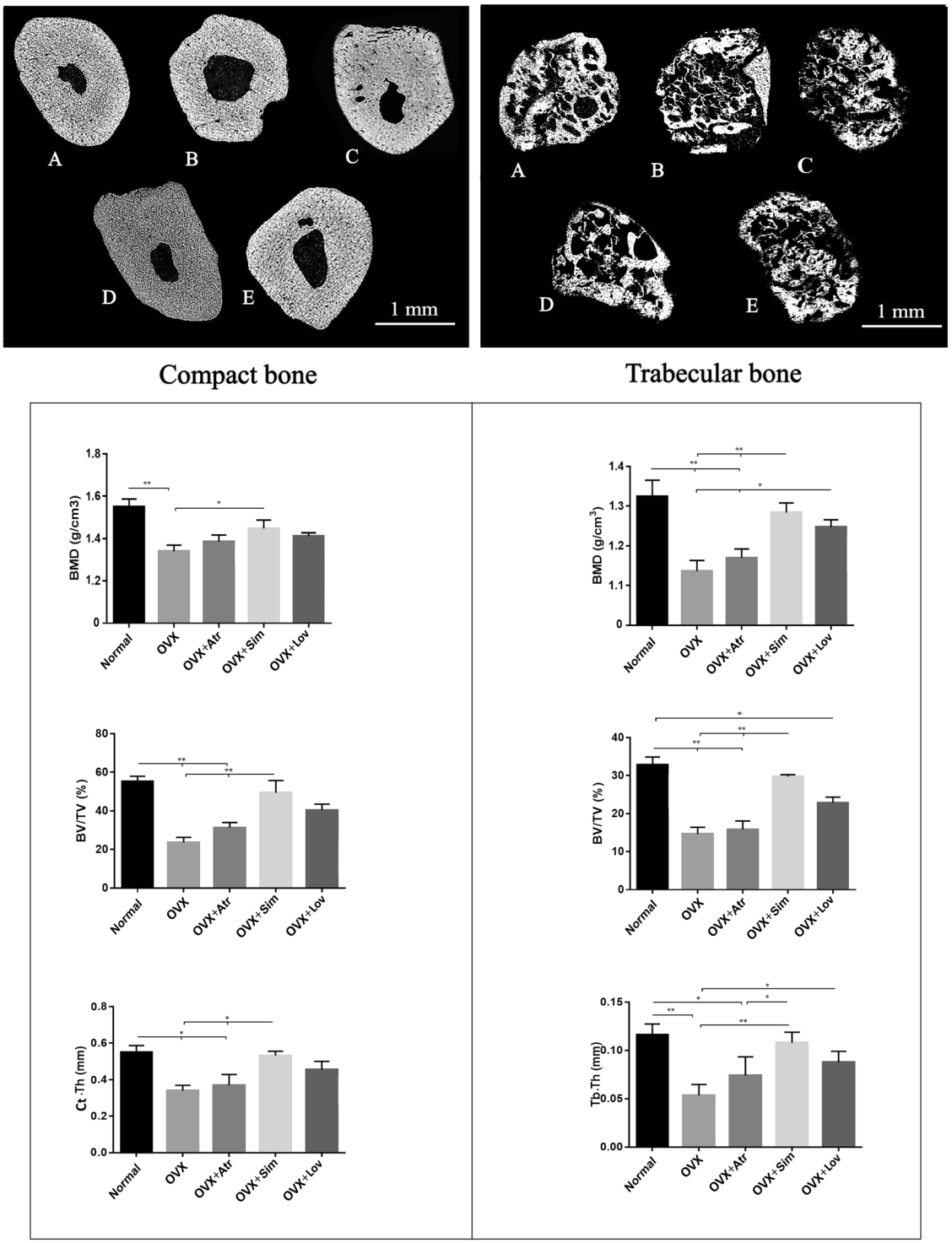

Fig. 2 Micro CT analysis of different experimental groups 60 days after treatment, A: normal, B: negative control, C: simvastatin, D: lovastatin, E: atorvastatin, BMD bone mineral density, BV/TV bone volume/total volume, Ct.Th cortical bone thickness, Tb.Th trabecular bone thickness, 6 samples were evaluated in each group. $^{**}P < 0.01$, $^{*}P < 0.05$

Osteogenic markers in bone tissue

The mRNA levels of osteoblastic marker genes in vertebral bone tissue were determined by qRT-PCR (Fig. 4b). Expressions of the Col 1 ($P = 0.005$), ALP ($P = 0.03$), and OCN ($P = 0.03$) were remarkably decreased in the negative control group compared to those in the normal group. These reductions were prevented upon treatment with simvastatin.

Table 2 Effects of statin drugs on hemaotocrit (HCT), serum total protein (TP), triglyceride (TG) serum calcium (Ca), phosphorous (P), Aspartate amino transferase (AST), Alanine aminotransferase (ALT), and alkaline phosphatase (ALP) levels in OVX rats

Factors	Normal	OVX	OVX+Atr	OVX+Sim	OVX+Lov
HCT (%)	46 ± 2	39 ± 3	43 ± 2	42 ± 1	45 ± 3
TP(g/dL)	6.9 ± 0.2	5.8 ± 0.1	6.7 ± 0.2	6.5 ± 0.3	5.7 ± 0.2
Ca(mg/dL)	10.2 ± 1.1	7.2 ± 1.2*	7.8 ± 1.6*	9.1 ± 2.8	8.9 ± 1.3
P(mg/dL)	7.1 ± 0.4	6.2 ± 0.2	6.8 ± 0.15	6.6 ± 0.52	6.9 ± 0.42
TG (mg/dl)	84.2 ± 4.5	103.6 ± 9.8	52. 6 ± 8.7**	44. 3 ± 4.1**	47. 6 ± 8.4**
AST(IU/L)	59.2 ± 7.2	55.1 ± 4.6	84.7 ± 8.1	66.1 ± 5.2	58.2 ± 3.9
ALT(IU/L)	53.5 ± 4.3	46.1 ± 6.3	113.7 ± 15.5**	68.4 ± 5.7	61.0 ± 8.4
ALP (IU/L)	74.8 ± 3.8	135.5 ± 14.4**	128.4 ± 9.6**	80.0 ± 10.1	104.2 ± 5.4
CK(IU/L)	358.4 ± 11.6	371.7 ± 8.3	1641.2 ± 23.7**	329.6 ± 14.8	363.2 ± 9.5

Data are shown as the mean ± SD ($n = 6$), evaluated by Tukey's multiple comparison test

HCT hematocrit, *TP*total protein, *Ca* calcium, *P* phosphor, *TG* triglyceride, *AST* aspartate amino transferase, *ALT* alanine aminotransferase, *ALP* alkaline phosphatase, *CK* creatine kinase, *OVX* PBS treated ovariectomized rat, *OVX+Atr* atorvastatin treated ovariectomized rat, *OVX+Sim* simvastatin treated ovariectomized rat, *OVX+Lov* lovastatin treated ovariectomized rat

*$p < 0.05$, **$p < 0.01$: Treatments vs. normal group

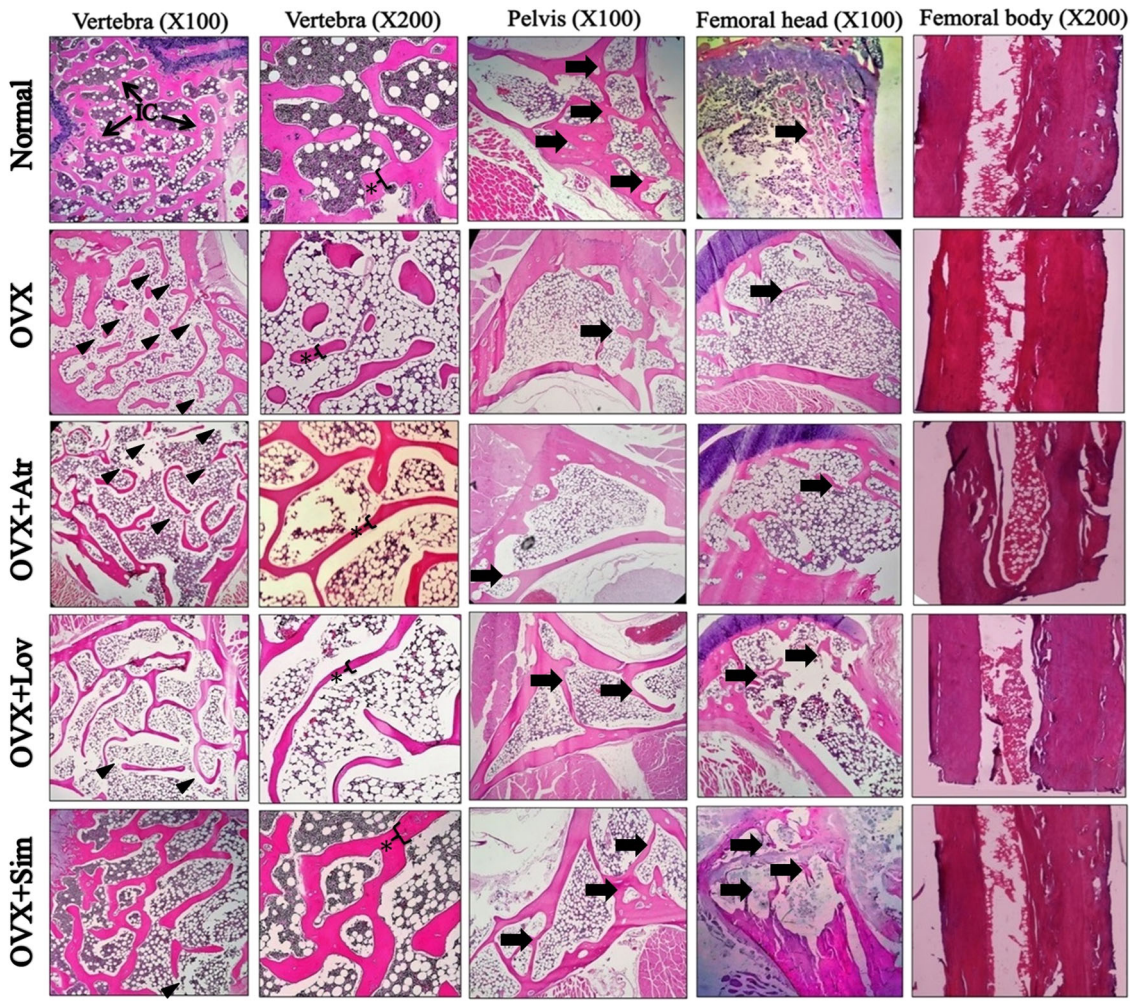

Fig. 3 Histopathology of vertebra, femur, and pelvis from the six groups on 60th day post-treatment. Among all treatments, the simvastatin-treated animals showed more resemblance to normal rats in terms of trabecular bone volume and thickness. IC: interconnectivity, arrowheads: loss of interconnectivity, asteroids: trabecular thickness, Thick arrows: trabecular bones. H&E staining

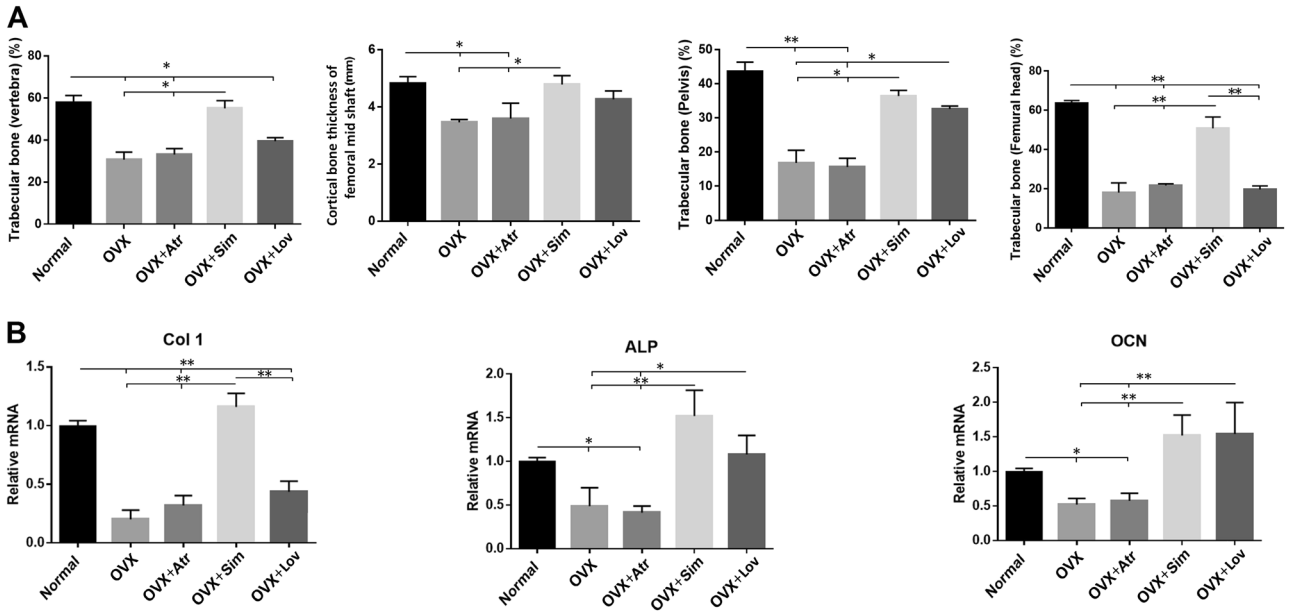

Fig. 4 a. Histomorphometric analysis of all groups including trabecular bone (%) of the pelvis, femoral head, and vertebra on 60th day post-treatment **b**. Effects of different treatment regimens on mRNA expression of osteocalcin (OCN), alkaline phosphatase (ALP), and collagen type 1 (Col 1) on the 60th day post-treatment (vertebral bone). $^{**}P < 0.01$, $^{*}P < 0.05$ by ANOVA test

Moreover, the OCN ($P = 0.003$) and ALP ($P = 0.02$) expression levels in the lovastatin-treated groups were higher than those in the untreated (negative control) and atorvastatin treated-animals.

Biomechanical performance

The data achieved from the biomechanical testing are available in Table 3. In terms of biomechanical analysis of femoral bone, the normal and simvastatin treated-rats demonstrated significantly higher maximum load (N) (Normal: $P = 0.03$, Sim: $P = 0.04$) and stiffness (N/mm) (Normal and Sim: $P = 0.02$) when compared with the negative control. Moreover, the lovastatin treated group showed higher stiffness compared to the negative control ($P = 0.03$). Biomechanical analysis of the lumbar vertebral bone showed that the normal and simvastatin groups had a significantly greater ultimate load (Normal and Sim: $P = 0.03$), and stiffness (Normal: $P = 0.01$, Sim: $P = 0.02$) when compared to those of the negative control group. In addition, the stiffness of normal and simvastatin treated-animals was significantly higher than the atorvastatin group ($P = 0.03$).

Discussion

Osteoporosis and the consequents are considered one of the most common diseases in the aging population, thus a cost-effective therapeutic approach to this disorder should be contemplated. Cardiovascular diseases are also considered as the other age-related diseases which may have common biological pathways with osteoporosis [34]. Statins, which have been widely used in the prevention of cardiovascular diseases, have other therapeutic effects including positive biological effects on bone [35]. Ovariectomized rats have been proven as one of the best models which mimic the clinical situation of bone tissue in postmenopausal human [36]. In our study, the OVX-induced osteoporosis models were confirmed by histopathological analysis 3 months post-ovariectomy. Our results in agreement with the previous investigations showed a reduced level of calcium and phosphorous in the OVX rat models compared with the normal ones [37, 38].

Many challenges regarding the application of statins in bone healing such as the most effective statin, beneficial concentrations, and potential side effects remained unanswered [39]. In the current study, we comparatively assessed the osteopromotive effect of the three most frequently applied types of statins including atorvastatin, simvastatin, and lovastatin and evaluated the related potential side effects of these drugs. Our results showed that simvastatin can promote bone mineral density of cortical and trabecular bones and also improve the biomechanical performance of the harvested bones more than the two other statins. In addition, simvastatin significantly enhanced the expression of osteogenic markers more than atorvastatin and lovastatin. Application of simvastatin and lovastatin resulted in a reduction of serum ALP level which indicates the efficiency

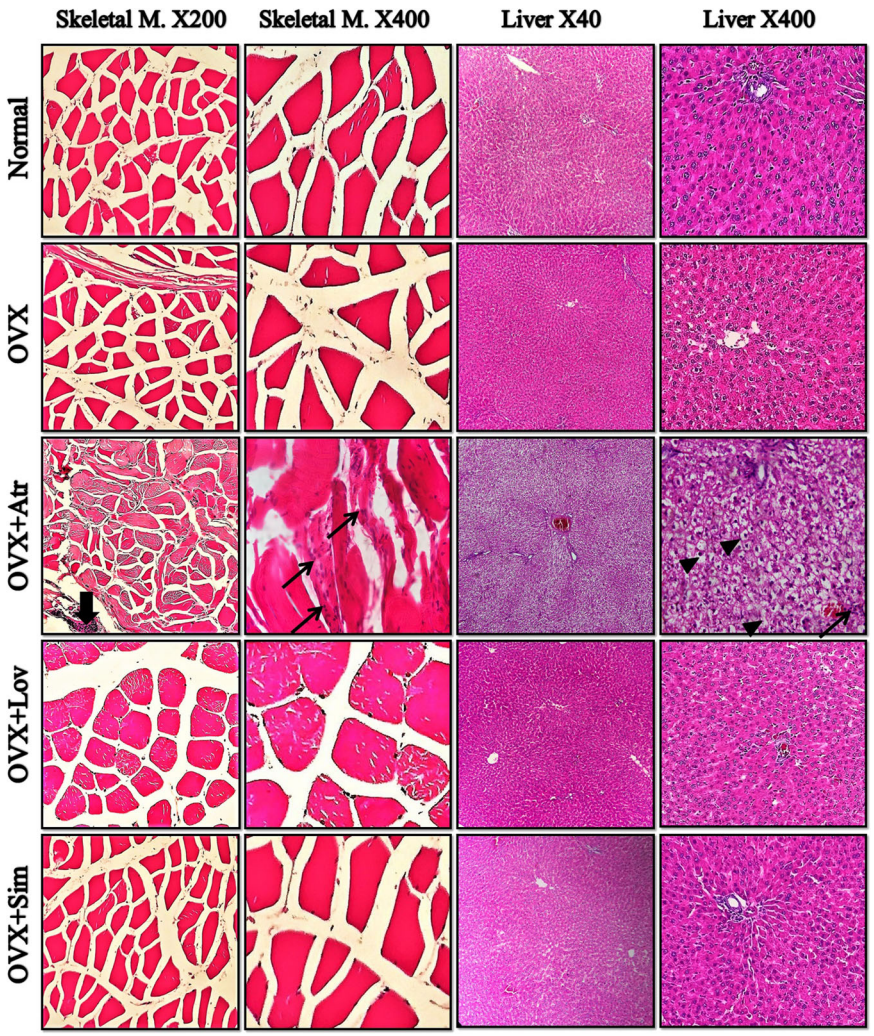

Fig. 5 Histopathology of liver and skeletal muscle samples in different groups on 60th day post-treatment. Micrographs of atorvastatin OVX treated animals showed a severe necrotizing myopathy (thin arrows) infiltrated by mononuclear inflammatory cells (thick arrow). Moreover, histopathological findings revealed atorvastatin-induced hepatotoxicity demonstrated by hepatic cell swelling and degeneration (arrowheads) and mild inflammation (thin arrows). H&E staining

of these treatment regimes in the improvement of osteoporosis.

A very interesting investigation in 2001 suggests that statins can affect bone turnover in a dose-dependent way [25]. They introduced a novel idea regarding the efficacy of statin drugs on bone turnover which indicates that low dose and dosage of statins could lead the bone turnover into resorption by increasing the activity of osteoclasts. On the other hand, high dose and dosage of these drugs can equally increase bone formation and resorption with little net change in BMD [25]. Hence, in this study, we used the high dose of lipophilic statins to prevent osteoporosis in the OVX animals. Regarding pharmacokinetics, the previous literature considered a 10 mg/kg/day dose of statins to rats about equivalent to 70 mg/day for humans, taking into account that metabolic process in rodents are 8–10 times faster than in humans [40]. 20–25 mg/kg/day of statins (140 mg/day for human) was selected as the therapeutic doses for rats according to the beneficial effects reported in previous in vivo studies which is higher than the routine dose in clinical applications (20–50 mg/day), and should be adjusted for human patients in future clinical trials.

Both histomorphometric and micro-CT scan analysis indicated no significant improvement in microstructure and volume of bone in atorvastatin group in comparison with the normal, simvastatin, and lovastatin groups. These findings were in agreement with Maritz et al. [25] and Kawane et al. [41] studies. Kawane et al. represented that lumbar and femoral BMD did not improve even in a high dose of atorvastatin. Furthermore, Bone et al. [42] in a randomized double-blind clinical trial reported no significant alteration in BMD after application of various dosages of atorvastatin. On the other hand, some studies indicated that administration of atorvastatin ameliorated osteoporotic bone tissues [43, 44]. These controversies might occur due to various application dosage and follow-up periods.

The previous literature showed that atorvastatin leads to a reduction of bone remodeling that can result in a significant reduction of various markers such as osteoprotegerin (OPN), BMP-2, and collagen type 1 [45]. Gradosova

Table 3 Biomechanical analysis at 60 days post-treatment

Value	Normal (1) Median ± SD	OVX(2) Median ± SD	OVX+Atr (3) Median ± SD	OVX+Sim(4) Median ± SD	OVX+Lov (5) Median ± SD
Femur					
Ultimate load (N)[a]	46.15 ± 8.21	40.27 ± 6.65	42.82 ± 4.28	45.73 ± 5.75	43.68 ± 3.58
Stiffness (N/mm)[b]	92.23 ± 6.43	81.54 ± 3.72	84.01 ± 6.13	88.69 ± 3.12	86.82 ± 3.27
Vertebra					
Ultimate load (N)[c]	121.23 ± 3.67	103.17 ± 4.50	105.82 ± 2.24	119.74 ± 2.27	112.18 ± 4.61
Stiffness (N/mm)[d]	1572.47 ± 28.55	1402.51 ± 23.72	1428.01 ± 32.13	1535.46 ± 44.69	1431.82 ± 53.27

OVX PBS treated ovariectomized rat, *OVX+Atr* atorvastatin treated ovariectomized rat, *OVX+Sim* simvastatin treated ovariectomized rat, *OVX +Lov* lovastatin treated ovariectomized rat

Femur:

[a]$P < 0.05$ (2 vs. 1, 4)

[b]$P < 0.05$ (2 vs. 1,4, 5)

Vertebra:

[c]$P < 0.05$ (2 vs. 1, 4)

[d]$P < 0.05$ (2 vs. 1, 4), (3 vs. 1, 4)

et al. [45] represented a lower level of collagen type 1 expression in the atorvastatin-treated bone in comparison to the normal bone tissue. To the contrary, atorvastatin resulted in higher expression of BMP-2 in the proximal part of tibia among healthy [15] or dyslipidemic [44] rats. Such controversies may emerge due to the differences between the condition of the bone tissue (baseline) and it seems that atorvastatin might be helpful in bone regeneration but not in osteoporosis prevention.

Although our volumetric and morphometric findings showed a significant improvement in osteoporosis after application of simvastatin and lovastatin in comparison with the negative control, this amelioration was more in the simvastatin-treated animals when compared to the lovastatin treated ones which can be resulted from their potency differences. The previous studies represented that combination of lovastatin with other bioactive molecules can significantly improve BMD through a synergistic beneficial effect [20, 46]. In this regard, Ibrahim et al. in their study on bone healing in fracture sites in OVX rats showed that a single dose of lovastatin resulted in improved callus strength; whereas addition of tocotrienol to lovastatin not only improved the biomechanical performance but also enhanced callus mineralization [20].

It has been shown that simvastatin can improve expression of osteogenic markers, however, its impact is dose-dependent. New investigations have shown that application of statins has no effect on these markers in osteopenic women at doses which simvastatin sufficiently prohibited the activity of HMG-CoA activity [47]. Moreover, Due et al. [14] revealed that administration of simvastatin is correlated with dental implants' osseointegration in osteoporotic rats. Our results showed that simvastatin increased expression of collagen type 1, ALP, and OCN levels in osteoporotic rats in comparison to other treatments which are in agreement with the previous findings that indicated stimulation of OCN and BMP-2 expression by simvastatin [48, 49].

It has been found that systemic delivery of statins, using clinical doses required for lipid-lowering treatment, possibly results in negative results [42]. Most studies suggested that statins had more therapeutic impact on the bone tissue when used in higher doses than the clinical doses via the same route of administration [8]. Moreover, it has been shown that higher doses of simvastatin increase bone formation and resorption while at lower doses it decreases bone formation and increases bone resorption [25]. Although higher doses of statin drugs may be more beneficial in osteoporosis treatment, with increasing the dose and dosage, the risk of statin-associated adverse effects such as hepatotoxicity, myopathy etc. greatly increases [50]. In order to evaluate the potential of atorvastatin, simvastatin, and lovastatin in inducing hepatotoxicity we assessed the serum level of AST and ALT. Although the AST level in all groups revealed no significant difference compared to the normal rats, the ALT level was significantly higher in the atorvastatin-treated animals in comparison to other treatments and normal group. These results were confirmed by our histopathologic evaluation of liver samples and with the results by Clarke et al., who also reported more hepatotoxicity in high doses of atorvastatin compared with simvastatin [51]. Statins-induced rhabdomyolysis is one of the most reported adverse effects which was also evaluated in the present study. The biochemistry analysis showed that the atorvastatin-treated animals had significantly higher levels of CK (~5 times) in comparison to other groups that

may indicate serious skeletal muscle damages at 60 days post-treatment. Histopathological analysis of skeletal muscle has also confirmed the data which obtained by serum biochemistry analysis in terms of CK levels. These findings are in accordance with the El-Ganainy et.al. study [52].

The serum biochemical ALP level was also used to identify the osteoporotic patients. ALP is derived from bones and liver and increases in blood serum as a result of high bone turnover [53]. A recent investigation revealed that the activity of serum total ALP > 129 U/L can be used as an indicator of screening for men with osteoporosis [54]. In addition, reduction in the serum ALP level has been demonstrated after treatment by anti-osteoporotic drugs [55]. In our study, the level of serum ALP was significantly increased in the untreated osteoporotic models when compared to the normal rats. The simvastatin and lovastatin-treated animals showed a considerable reduction in the serum ALP in comparison to the untreated models which indicates the therapeutic effect of these drugs. However, there was no significant difference between the atorvastatin-treated and untreated osteoporotic animals in terms of ALP levels.

In overall, micro-CT and histopathology and histomorphometry analysis of the harvested bones in this study showed that simvastatin can significantly promote BMD, BV/TV, bone thickness, and percentage of bone in both cortical and trabecular bone tissues compared to the negative control. Moreover analyzing of the serum biochemistry parameters revealed that not only simvastatin support bone tissues by increasing the Ca and P levels, but it also did not result to any liver toxicity which was confirmed via analysis of ALT and AST levels. However, the FDA's review of additional data from large clinical trials (12,000 patients) of high doses of simvastatin (from 20 to 80 mg/day) resulted in 10% increases in the incidence of myopathy, which indicates the importance of future clinical investigations to achive an effective and safe dose and dosage [56]. Finally, the biomechanical properties of the bone tissues were significantly improved in the simvastatin-treated group when compared to the negative control and other statin-treated animals. These findings are in accordance with the previous studies [10, 48].

Conclusion

Our radiographical, histopathological, biomechanical, serum biochemical, and molecular findings indicated that administration of simvastatin and lovastatin is more beneficial for bone healing in OVX rats in comparison to treatment by atorvastatin. Moreover, based on the evaluation of osteogenic markers, simvastatin can actively promote bone formation. In overall, simvastatin showed the best therapeutic effects on improving osteoporosis in OVX rats in comparison to other treatment regimes. However, further in vivo and clinical trials are required to lead us to an evidence-based decision making about these statins.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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