Effect of atorvastatin on bone formation in ovariectomized rats

S.S. Salih and E.M. Al-Khashab

Department of Physiology, Biochemistry, and Pharmacology, Collage of Veterinary Medicine, University of Mosul, Mosul, Iraq

Abstract

The study was designed in order the effect of one class of lipophilic statins (Atorvastatin) in some biomarkers of bone formation (Alkaline phosphatase (ALP), 1,25 (OH)2D3, calcium, and phosphorous) in serum of ovariectomized female rats. Thirty adult female rats (2.5-3) months, weighing (200-220) gm were housed at conditions of controlled temperature (22-25°C), cycle (12h light - 12h dark) in the house of animals of Veterinary Medicine College of Mosul University. The animals were divided into three equal groups, sham group, ovariectomized (ovx) group, and ovx treatment orally with 20mg/kg/d of atorvastatin. After 60 days of treatment, blood from all groups was collected for ALP activity, 1,25 (OH)2D3, calcium, and phosphorous estimation, and left femur bones were excised for histological examination. The results showed that the serum ALP, calcium, and phosphorous were significantly elevated, and a significant reduction in 1,25 (OH)2D3 was noticed in the ovx group. However, treatment with atorvastatin caused a significant reduction in ALP with a non-significant elevation in 1,25 (OH)2D3. Histological results showed a low density and thin trabecular bone, a few blood vessels, high numbers of osteoclast, with low numbers of osteoblasts in the ovx group. However, the treated ovx with atorvastatin increases the thickness of trabecular bone, medium developed osteogenic tissue, and a low number of osteoblasts. In conclusion, atorvastatin has a moderate effect on bone of ovx, affecting bone formation more than bone resorption.

Introduction

Osteoporosis is a skeletal disease that affects millions of people in the world, and this disease accelerates bone loss leading to an increase in the probability of bone fracture. Menopause stimulates the loss of bone, and ovariectomy leads to bone loss because of estrogen deficiency which causes osteoporosis in humans and rats (1-3). Statins are 3-hydroxy 3-methylglutaryl-coenzyme A reductase inhibitors used to lower cholesterol levels. These drugs act by inhibiting cholesterol biosynthesis in the liver; therefore, statins reduce the risk of atherosclerosis and heart diseases (4,5). Some studies have suggested that statins may positively affect bone metabolism. Thus, taking statins to lower lipids may also impact bone metabolism; thus, statins decrease lipids and improve disorders of bone (6). Besides lowering the level of cholesterol, statins can affect bone by the decrease other molecules that produce from the mevalonate pathway, such as isoprenoid precursors, farnesyl diphosphate, and geranyl geranyl diphosphate, thus inhibiting the function of osteoclasts (4). There are two types of statins lipophilic and hydrophilic, depending on their potency of dissolving in lipid-containing media or water. Lipophilic statins (simvastatin, fluvastatin, pitavastatin, lovastatin, and atorvastatin) can move in cells quickly; however, statins dissolve in water as (rosuvastatin and pravastatin), are hepatoselectivity (7,8). Atorvastatin (Lipodar®) is an agent that inhibits cholesterol synthesis by increasing LDL receptors on the surface of the hepatocytes. It is absorbed quickly after taking it (9).
The purpose of the current work was to explore the influence of atorvastatin (dissolve in lipids) in some biomarkers of bone on ovariectomized (ovx) female rats which are considered a model of osteoporotic postmenopausal women. Ovariectomized rats have been used as a model to study the loss of bone in osteoporotic postmenopausal women. This model of rats mimics the impairment in the tissue of bone in the spine and hip.

Materials and methods

Ethical approve
Scientific ethical committee on animal experimentation at College of Veterinary Medicine, University of Mosul, UM.VET.2021.053

Laboratory animals
Thirty adult female rats 2.5-3 months, weighing 200-220 gm were obtained and housed in an environment 22-25°C, at 12-12 h dark-light in the house of animals of the College Veterinary Medicine. Water and a standard diet were provided to all animals.

Experimental design
Thirty rats were equally grouped into the following: (10 rats/ group) and divided into the following: Group 1: Sham ovaries were not removed, sham-operated, and left about 30 days after surgery) furthermore, given orally distilled water for 60 days. Group 2: ovx (ovaries were removed and left 30 days after ovariectomy) served as a model of postmenopausal osteoporosis and given orally distilled water for 60 days. Group 3: (ovaries were removed and left 30 days after ovariectomy, then treated orally with 20mg/kg (9) of atorvastatin (lipodar/dar aldawa/Jordan) for 60 days.

Ovariectomy
After anesthesia with a mixture of (5 mg/kg) of xylazine hydrochloride and 50 mg/kg of a 10% ketamine given intraperitoneal (10,11), the abdominal region with skin and musculature was incised under the last rib, then ovary was excised. The sham group did the same procedure, but the ovary was not removed.

Blood collection
After 60 days of the treatment, by using the capillary tubes, samples of blood were collected from the retro-orbital plexus (12,13) from all groups, then drawn in a gel containing tubes, and leave it to clot after that put in the centrifuge for 15 min at 3000 rpm, serum separated and kept in -18°C for biomarkers examination.

Biomarkers of bone
Biomarkers were measured included: Rat alkaline phosphatase (ALP) activity, which was estimated by using the ELISA kit (catalog No: E-EL-R1109) from Elabscience company which has been used for research only. This ELISA kit uses the sandwich-ELISA principle that the micro-ELISA plate provided in this kit has been pre-coated with an antibody specific to rat ALP, and the optical density is measured spectrophotometrically at a wavelength of 450±2nm. 1,25(OH)2D3 was estimated by using the ELISA kit (catalog No: E-EL-0016) from Elabscience company. Serum calcium was measured via spectrophotometry using diagnostic reagent kits (Biolabo, France), which include reacting calcium with the O-Cresol Phthalein to produce a complex color absorbed at 570 nm (14,15). Phosphorus in serum was determined by using Architect system operations which depend on inorganic phosphate reaction with ammonium molybdate to produce complex absorbance proportional directly to the level of inorganic phosphate in the serum.

Histological examination
In order to determine the osteoblast’s and osteoclast’s activity, left femur bones were sliced, then fixed in a solution of formalin 10% for 24h. After that, they were decalcified in formic acid 10% and embedded in paraffin blocks. Hematoxylin and eosiin were used for staining the paraffin blocks; histological sections were photographed with Omax digital camera type (16).

Statistical analysis
Mean ± SE was used to describe the results, evaluated using sigma plot version 12.5. The one-way analysis of variance (ANOVA) test was used to compare all groups with a probability of P≤0.05.

Results
A significant elevation was noticed in ALP activity in the ovx group compared to the group, as shown in table 1, but the treatment with 20mg/kg/d of atorvastatin caused a significantly decreased ALP in comparison to the ovx group but did not reach the sham group level. Ovariectomy results in a significant decrease in 1,25(OH)2D3 level compared with the sham group, while treatment with 20mg/kg/d of atorvastatin caused a non-significant increase in 1,25(OH)2D3 compared with the ovx group. Levels of calcium and phosphorus were significantly increased in the ovx group compared to the sham group, but the treatment with 20mg/kg/d of atorvastatin caused a non-significantly decrease in the calcium and phosphorus levels in comparison to the ovx group.

The histological results of bone femur rats showed intact cartilage, high density and thickness of trabecular bone, and well-developed osteogenic tissue with few osteoclasts in the sham group, as shown in figure 1. Ovariectomy caused a low density and thin trabecular bone. In addition, there were a few blood vessels and poorly developed osteogenic tissue. There are high numbers of osteoclasts with low numbers of
osteoblasts, as seen in figure 2. This means there was a change in bone caused by estrogen deficiency that leads to a condition similar to the change in the bone seen in women with postmenopausal osteoporosis. The atorvastatin-treated group showed a high-density trabecular bone (TB), few blood vessels (BV), and medium-developed osteogenic tissue (OT) (Figure 3). A significant reduction was shown in the thickness of trabecular bone/µm of ovx compared to the sham group shown in table 2, the group treated with atorvastatin 20mg/kg/d led to a significant elevation in thickness of trabecular bone in comparison to ovx group. These results mean an improvement in the bone of the ovx rat after the treatment with atorvastatin.

Table 1: Effect of atorvastatin on serum ALP, Vit D3, Ca, and P levels in ovx rats

<table>
<thead>
<tr>
<th>Biomarkers</th>
<th>Sham</th>
<th>OVX</th>
<th>OVX + A</th>
</tr>
</thead>
<tbody>
<tr>
<td>ALP (ng/ml)</td>
<td>11.64 ± 0.56 a</td>
<td>16.79 ± 0.34 a</td>
<td>14.18 ± 0.66 b</td>
</tr>
<tr>
<td>1,25(OH)₂D3 (pg/ml)</td>
<td>223.70 ± 4.52 a</td>
<td>164 ± 4.62 b</td>
<td>177 ± 5.39 b</td>
</tr>
<tr>
<td>Ca (mg/dl)</td>
<td>10.90 ± 0.08 b</td>
<td>11.51 ± 0.11 a</td>
<td>11.28 ± 0.10 a</td>
</tr>
<tr>
<td>P (mg/dl)</td>
<td>4.15 ± 0.42 b</td>
<td>5.74 ± 0.13 a</td>
<td>5.30 ± 0.27 a</td>
</tr>
</tbody>
</table>

Mean ± SE with various superscript letters in the identical row mean significant at P≤0.05. n=10 rats/group.

Discussion

Our study aimed to investigate the influence of the atorvastatin (lipophilic statin) in the bone formation of osteoporotic female rats caused by ovariectomy. Ovariectomy results in estrogen deficiency which leads to changes in bone. Ovariectomized rats were used as a model to study postmenopausal bone loss because the mimic deterioration in the rats bone is similar to estrogen reduction
seen in women with osteoporosis. So, the change in bone in rats is similar to that seen in estrogen-deficient osteoporotic women (17,18).

In our study, the level of ALP activity was elevated significantly in the ovx group compared to the sham group. This result agrees with Morgan et al. (19) and Grigoryan et al. (20) they noticed that the activity of ALP was significantly elevated in ovx rats. Also, our results agree with our prior study, which showed an increase in the level of ALP activity in the ovx group compared to the sham group (21). The decrease in estrogen production by ovaries is related to high bone turnover and high levels of markers related to bone formation and resorption. Ovariectomy increases ALP. This elevation is due to the unbalance in bone remodeling and deficiency of estrogen. This elevation in ALP refers to a high turnover of ovx rats characterized by an elevation in both formation and resorption, but the resorption process is more than the formation process. Estrogen inhibiting osteoclastogenesis, osteoclastogenesis process, and bone remodeling involve signaling pathways, like osteoprotegerin, RANK ligand, and RANK. RANK ligand stimulates activation and osteoclast differentiation and inhibits apoptosis in this cell type (22,23).

In our study, treatment of the ovx group with 20mg/kg of atorvastatin led to a significant reduction in ALP activity compared to the ovx group, meaning there is a significant improvement in the formation of bone and a decrease in bone resorption. Serum ALP is one of the biomarkers formations of bone. ALP levels, especially ALP specific for bone, are elevated in osteoporosis and in some metabolic bone diseases (24). This result indicates the utility of using atorvastatin in bone formation, and atorvastatin efficiently ameliorated ovx-induced osteoporosis. This study’s increase in ALP activity agrees with the result of Sabry et al. (25). They noticed an elevation in the serum ALP in the ovx group. In our study, the reduction in ALP in ovx rats treated with 20mg/kg atorvastatin agreed with the results of El-Nabarawi et al. (26) they showed a reduction in serum ALP after treatment with atorvastatin.

ALP plays an essential role in the formation of hard tissue. It increases and facilitates mineralization. Also, it decreases pyrophosphate levels in the fluids out of the cell. It has a crucial role in the mechanism of calcification. ALP is produced through growth and is local on the surface of the cell and matrix vesicles in all tissues (27). Our study showed a significant reduction in serum 1,25(OH)2D3 in the ovx group compared with the sham group. Ovariectomy leads to an increase in the risk of osteoporosis, and this resembles postmenopausal osteoporotic women. In addition, ovariectomized rats are an excellent standard model for examining the drugs used for the treatment and prevention of osteoporosis. Ovariectomy results in estrogen deficiency that is known to decrease 1,25(OH)2D3 (28). Our results agree with Muhammad et al. (29) they noticed a decrease in serum 25(OH)D in the ovx group. Vitamin D is considered a pro-

hormone, which is necessary for calcium homeostasis. 1,25(OH)2D3 stimulates the absorption of calcium and phosphorus from the intestine and improves the mineralization of the skeleton. In addition, 1,25(OH)2D3 with parathyroid hormone stimulates the mobilization of calcium from bone in order to prevent hypocalcemia (30,31).

Some researchers have shown a valuable role of statins on levels of 1,25(OH)2D3, but other studies have had no effect. Our results showed that rosuvastatin and atorvastatin could not reveal a significant influence on serum 25(OH)D level (32). However, in a study on patients with hyperlipidemia, they were given 10mg of rosuvastatin led to a significant increase in 25(OH)D levels (33). The exact mechanism of statins effect on 25(OH)D metabolism is not understood, but some researchers have proposed that 7-dehydrocholesterol is the precursor of vitamin D and cholesterol, and the inhibition of 3-hydroxy-3-methylglutaryl coenzyme A reductase leads to an elevation in 7-dehydrocholesterol levels, this may offer a substrate for 25-hydroxy vitamin D synthesis (34).

Our presented study indicated calcium and phosphorus concentrations were significantly elevated in the ovx group compared to the sham group. This result agrees with Yousefzadeh et al. (35) they reported elevated serum phosphorus in ovariectomized rats from the first week and continued elevation until the ninth week. Also, Ca and P elevation in our study agrees with the study of (14) that showed a significant increase in Ca and P levels after ovariectomy. Our results indicated that bone turnover because of: estrogen deficiency causes a reduction in [1,25(OH)2D3] and a decrease in calcium absorption in the intestine. Reduction in serum calcium leads to parathyroid hormone PTH secretion that causes releasing of Ca and P from the skeleton to normalize serum Ca and P levels. This process leads to excretion of Ca and P in the urine and bone loss. Calcium levels in serum depend on the level of estrogen deficiency (28).

Also, our study’s elevation in serum Ca agrees with Ohlsson et al. (36) and Naki (37). However, treatment of the ovx groups with 20mg/kg atorvastatin resulted in a non-significant reduction in Ca and P compared with ovx, which means this dose could not be returned Ca and P to the levels of the sham group. Also, the treatment with atorvastatin caused a non-significant decrease in P compared with the ovx group; this agrees with Romero et al. (24) they noticed a high level of P in all groups of their study. Calcium and phosphorus have an essential role in many functions of the body. Therefore, their regulations in plasma are made by the action of resorption/excretion in the kidney with absorption by the intestine and exchange from bone, which is considered the reservoir for Ca and P. The treatment of ovx groups with 20mg/kg atorvastatin leads to a non-significant decrease in serum Ca compared with the ovx group. Our results agree with Gokdemir et al. (9) that they reported increased calcium in the group treated with rosuvastatin. In
another study (38), they noticed a hypercalcemia condition in patients using atorvastatin, and they suggested that atorvastatin caused a high level of calcium in serum. In addition, serum calcium elevated significantly in rats treated with atorvastatin (39).

The histological results showed a reduction in trabecular bone thickness and a low number of osteoblasts, in addition to high numbers of osteoclasts, in the ovx group. These results agreed with the results of Zhang et al. (40) and Burris et al. (14) they noticed a significant 60% decrease in the volume of trabecular bone after ovariectomy. Also, there was increased osteoclastogenesis. The reduction of 50% in mass of trabecular bone, and the connectivity of trabecular bone results from osteoporosis. This means osteoporosis leads to bone loss. Atorvastatin results are a medium developed of osteogenic tissue and increase the thickness of trabecular bone (41).

Conclusions

In conclusion, the results denote that atorvastatin affects bone formation in ovariectomized rat. However, it has no negative effect on serum calcium and phosphorus level, so care must be taken into consideration when menopausal women are treated with it through measurement levels of calcium and phosphorus. The limitation of our work was that we could not estimate levels of PTH and estrogens because of the high cost of kits.

Acknowledgment

The authors appreciate the people at the University of Mosul Veterinary College and the animal home for providing facilities that enabled us to complete this study.

Conflict of interests

There are no conflicts of interest declared by the authors.

References

25. Sabry M, Mostafa S, Kamar S, Rashed L, Estaphan S. The cross-talk between matrix metalloproteinase-9, RANKL/OPG system and
Tأثير الأتورفاستاتين على بناء العظام في الجرذان المستأصلة المبايض

scratch


10. Lane NE, Yao W, Kinney H, Modin G, Balouche M, Wronska TJ. Both hPTh(1-34) and hFGF increase trabecular bone mass in osteopenic rats, but they have different effects on trabecular bone architecture. J Bone Miner Res. 2003;18(12):2105-2115. DOI: 10.1359/jbmr.2003.18.12.2105


14. Ducow EG, Ducow MW, Plum LA, DeLauca HF. Vitamin D binding protein greatly improves bioavailability but is not essential for orally administered vitamin D. Physiol Rep. 2021;9(23):15138. DOI: 10.1484/phy.2.15138


