

Role of rosuvastatin in bone metabolism of ovariectomized adult rats

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Abstract

The study was planned to explore the influence of one type of hydrophilic statins (rosuvastatin) on the level of serum osteocalcin, N-telopeptide of type 1 collagen (NTx), calcium, and phosphorus. In addition to calcium, phosphorus, and magnesium in femur bone ash of female ovariectomized rats. Thirty female rats aged 2.5-3 months were divided as follows, sham group, ovariectomized (ovx) group as a model of osteoporosis, and (ovx) group treated with 20mg /kg of rosuvastatin for 60 days. Blood samples were collected after 30 and 60 days of the experiment for biochemical analysis. Besides, after 60 days of the treatment, right femur bones were excised and ashed to estimate calcium, phosphorus, and magnesium. The results showed a significant elevation in serum osteocalcin, (NTx), calcium, and phosphorus, in addition to elevation in osteoclasts number and deceased osteoblasts and thickness of trabecular bone in the (ovx) group compared to the sham, while treatment with rosuvastatin caused a significant reduction in osteocalcin, (NTx), calcium and phosphorus after 60 days. Also, the results revealed a significant reduction in the percentage of (Ca, P and Mg) content in bone ash of the (ovx) group compared to the sham group. However, rosuvastatin treatment led to a significant elevation in the percentage of calcium and inorganic phosphate in bone ash and increased the thickness of trabecular bone and development in osteocytes compared with ovariectomized rats. The conclusion of the present study, rosuvastatin has a positive effect on the bone of ovariectomized rats.

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Introduction

Osteoporosis is a metabolic disease; it is characterized by a low mass of bone and damage to the architecture in bone tissue. Osteoporosis that results from the deficiency of estrogen is the standard type. The reduction in estrogen leads to an elevation in bone turnover because of the bone's imbalance between formation and resorption that affects cortical and trabecular bones since there is a loss of connectivity, cortical thinning, and porosity (1,2). Statins have been widely used to lower cholesterol levels in the blood and prevent cardiovascular diseases (3). Statins play a critical role in the pathway of mevalonate. Moreover, they enhance bone morphogenetic protein-2 (BMP-2) expression, and this protein is necessary for osteoblast cell

differentiation (4,5). Statins share the exact mechanism of their action but differ in their chemical, structure, lipid-modifying efficacy, and pharmacokinetic profile (6). Statins can be classified into lipophilic and hydrophilic types following their solubility (7). Lipophilic statins can diffuse passively through the membrane of the cells. This diffusing reduces the hepatoselectivity because they were also able to diffuse into cells of other tissues. Hydrophilic statins such as rosuvastatin are more hepatoselectivity (8,9). Rosuvastatin is one synthetic statin type with pharmacologic characteristics, including hepatic selectivity and HMG-CoA reductase inhibition (4). Rosuvastatin has an enhancing effect on bone formation. Also, it activates osteoblast cells and stimulates the self-regeneration of mesenchymal stem cells (10). Because of the statin's ability to inhibit HMG-CoA

reductase, considered a key enzyme in the cholesterol biosynthesis pathway and the osteoclast activation process, studies have hypothesized that statins have another role in lowering cholesterol and stimulation bone formation (11,12).

Some studies have suggested that statins may positively affect bone tissue by increasing bone formation and reducing bone resorption in vivo and in vitro (13).

Materials and methods

Ethical approve

Ethical approve for this study was obtained from the laboratory animals house in College of Veterinary Medicine of Mosul University with number of approvals UM.VET.2021.054.

Animals and housing conditions

This study was conducted in the animal house in the Veterinary Medicine College/University of Mosul. Thirty adult female rats 2.5-3 months and weighing 200-220g were used. All rats were housed and maintained under 22-25°C, 40-80% humidity, and a 12/12h light /dark cycle. Water and a standard diet were provided. The animals were acclimated for 1 week before the surgical operation.

Ovariectomy

All rats were anesthetized with a mixture of 5-10 mg /kg of xylazine and 40-90 mg/kg of a 10% ketamine hydrochloride given intraperitoneal (14,15). The failure of the wink reflex and lack of reaction to pinching of the foot was used to test the success of anesthesia. Ovaries were removed from 20 rats after skin and musculature were incised under the last rib. The abdominal muscle incision was closed. These ovariectomized (ovx) groups are considered a model of postmenopausal osteoporosis. The same procedure was done for 10 rats without removing the ovaries. These groups are considered sham groups. Rats were returned to their cages and stayed 30 days after surgery.

Experimental design

After 30 days of ovariectomy, total female rats were divided, and treatment was as follows; Group 1: 10 rats sham-operated group (non-ovariectomized) given orally only D.W. Group 2: 10 rats ovariectomized (ovx) given orally only D.W. Group 3: 10 rats ovariectomized (ovx) gavage with 20mg /kg /days (7) of rosuvastatin (AstraZeneca/UK) for 60 days.

Collection of blood samples

Blood samples were withdrawn from retro-orbital plexus (16,17) after 30 days and at the end of the experimental (60 days), then placed into anticoagulant-free tubes, left for clotting, and put in centrifuged for 15 min. Serum separated, then stored at -18°C for biomarker analysis.

Biochemical markers assessment

ELISA kits were used for the estimation of serum rat osteocalcin (Elabscience Rat OC / BGP, catalog No: E-EL-R0243) (USA) and rat NTx 1 (crosslinked N-Telopeptide of Type 1 collagen) (Elabscience Rat NTx, catalog No: E-EL-R0276) (USA). The spectrophotometric method was used to estimate serum calcium (Biolabo, France) by reagent kit (18). Serum phosphorus was estimated by using Architect system operations, while the determination of calcium, phosphorus, and magnesium in bone ash as the following: After blood collection, three rats from each group were sacrificed, the right femur bones were cleaned, and soft tissues around them were removed. Ash was obtained by drying and ashing bones in muffle apparatus for 18h at 600°C. The ash samples were extracted and solubilized with 0.1MHCl, then diluted to 100 ml by deionized water. This solution was kept to determine calcium, phosphorus, and magnesium in the bone ash sample. The Ca-EDTA method detects calcium content in bone ash (19). The spectrophotometric method was used to determine serum phosphorus in ash, which depends on reacting inorganic phosphate with molybdic acid to form phosphomolybdic, which is reduced to form blue color (20). Magnesium is determined according to the method (21). To determine the osteoblasts and osteoclasts' activity, left femur bones were sliced, and 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 eosin were used for staining the paraffin blocks (22). The thickness of trabecular bone was measured using the software of a microscope camera in a micrometer as a mean of 5 measurements /field (100X) for 5 fields for each animal in the group.

Statistical analyses

The results were presented as mean \pm S.E. All groups were compared using one-way analysis of variance (ANOVA) tests with a $P \leq 0.05$.

Results

Table 1 shows a significant elevation ($P \leq 0.05$) of osteocalcin and NTx in serum of the ovx group after 30 and 60 days compared to the sham group. Treatment with 20mg/kg rosuvastatin leads to a non-significant decrease in osteocalcin and NTx after 30 days of treatment, but treatment with 20mg /kg rosuvastatin leads to a significant decrease in osteocalcin and NTx after 60 days of the treatment and returns to the level of the sham group compared with ovx group.

As shown in table 2, there was no difference in calcium and phosphorus levels among groups of the study after 30 days of treatment. Nevertheless, there was a significant increase in calcium and phosphorus in serum of the ovx group compared with the sham group after 60 days of

treatment. So, the treatment with 20mg /kg rosuvastatin led to a significant decrease in serum calcium and phosphorus compared with the ovx group, although serum calcium did not approach its level in the sham group.

Ashing content results are shown in table 3. The ovariectomy leads to a significant decrease in the ash's

calcium, phosphorus, and magnesium content compared with the sham group. Treatment with 20 mg /kg of rosuvastatin significantly elevated ash's calcium and phosphorus content compared with the ovx group.

Table 1: Effect of rosuvastatin on serum osteocalcin and NTx

Groups	30 days		60 days	
	OC	NTx	OC	NTx
sham	10.92 ± 0.52 ^b	4.76 ± 0.38 ^b	13.20 ± 0.72 ^b	5.11 ± 0.39 ^b
Ovx	14.43 ± 0.94 ^a	7.05 ± 0.41 ^a	17.64 ± 0.87 ^a	6.71 ± 0.23 ^a
Ovx + R	13.44 ± 1.20 ^{ab}	6.35 ± 0.19 ^a	13.57 ± 1.09 ^b	5.12 ± 0.35 ^b

Values expressed as mean ± S.E. with different superscript letters in the same column significant at P≤0.05. 10 animals /group.

Table 2: Effect of rosuvastatin on calcium and phosphorus in serum after 30 and 60 days

Groups	30 days		60 days	
	Ca	P	Ca	P
sham	10.60 ± 0.10 ^a	5.70 ± 0.20 ^a	10.90 ± 0.08 ^c	4.15 ± 0.42 ^b
Ovx	10.70 ± 0.10 ^a	6.10 ± 0.17 ^a	11.51 ± 0.11 ^a	5.74 ± 0.13 ^a
Ovx + R	10.60 ± 0.20 ^a	6.00 ± 0.20 ^a	11.19 ± 0.09 ^b	4.75 ± 0.24 ^b

Values expressed as mean ± S.E. with different superscript letters in the same column significant at P≤0.05. 10 animals /group.

Table 3: Effect of rosuvastatin on bone ash content Ca, P and Mg

Groups	Ca %	P %	Mg %
Sham	42.07 ± 1.48 ^a	22.10 ± 0.97 ^a	1.40 ± 0.11 ^a
Ovx	34.20 ± 2.10 ^b	16.87 ± 0.98 ^b	0.93 ± 0.08 ^b
Ovx + R	40.67 ± 0.57 ^a	21.87 ± 0.22 ^a	1.20 ± 0.05 ^{ab}

Values expressed as mean ± S.E. with different superscript letters in the same column significant at P≤0.05. 10 animals /group.

Table 4 shows the thickness of trabecular bone in all groups in this study. The thickness of trabecular bone was decreased significantly in the ovx group in comparison with the sham group, but the treatment of the ovx group with 20 mg/kg rosuvastatin caused a significant elevation in trabecular bone thickness in comparison with the ovx group. Results of the histology examination in this study revealed a high density of bone, high thickness of trabecular bone, high blood vessels, and well-developed osteogenic tissue in the bone of the sham group (Figure 1). However, the histological tests of bone rats in the ovx group showed low density, thin trabecular bone, few blood vessels, and poorly developed osteogenic tissue. Also, we observed the presence of low osteoblasts and high osteoclasts (Figure 2). The effect of rosuvastatin treatment on the bone of ovariectomized rats (Figure 3). We observed medium-density trabecular bone, blood vessels, and well-developed osteogenic tissue. Treatment with 20 mg /kg showed an excellent developed osteogenic tissue.

Table 4: Effect of the rosuvastatin on the thickness of trabecular bone

Groups	The thickness of trabecular bone/μm
Sham	86.6 ± 12.4 ^a
Ovx	50 ± 4.2 ^b
Ovx + R	73.8 ± 3.7 ^a

Values expressed as mean ± S.E. with different superscript letters in the same column significant at P≤0.05. 10 animals /group.

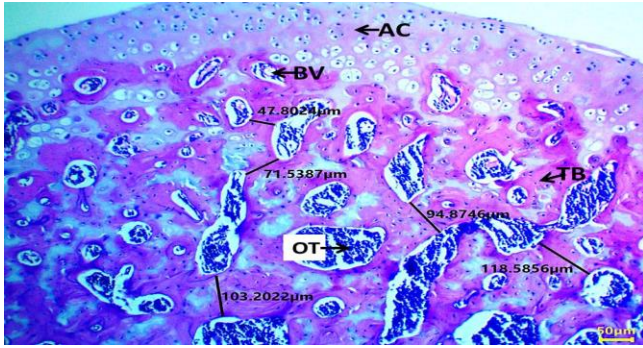


Figure 1: The photomicrograph of rat femur bone of the (sham) group shows intact articular cartilage (AC), high density and thickness trabecular bone (TB), blood vessels (BV), and well-developed osteogenic tissue (OT). H&E stain, Scale bar=50µm, 100X.

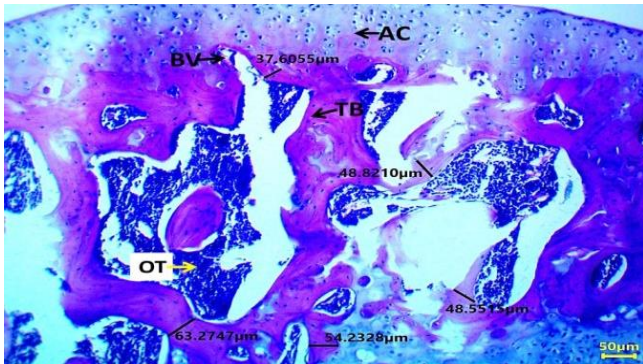


Figure 2: The photomicrograph of rat femur bone of the ovx group shows articular cartilage (AC), low density and thin trabecular bone (TB), few blood vessels (BV), and poor developed osteogenic tissue (OT). H&E stain, Scale bar=50µm, 100X.

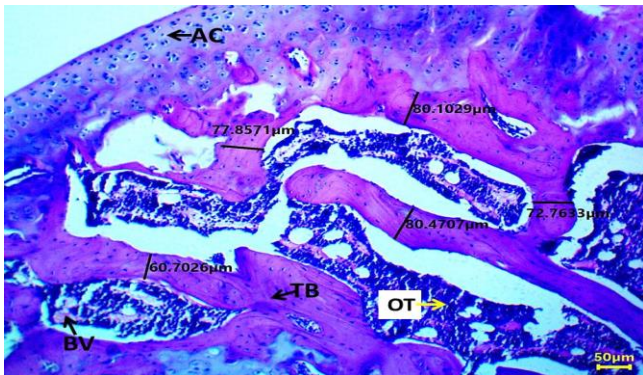


Figure 3: The photomicrograph of rat femur bone of rosuvastatin treated group shows articular cartilage (AC), medium density trabecular bone (TB), blood vessels (BV), and well-developed osteogenic tissue (OT). H&E stain, Scale bar=50µm, 100X.

Discussion

This study aimed to clarify if rosuvastatin (hydrophilic statin) treatment alleviated bone loss caused by ovariectomy. The results of our study point out that the ovariectomy process for rats developed bone turnover and bone structure changes that are similar to changes seen in osteoporotic women with estrogen deficiency. An ovariectomized rat was used as a model of menopause induced by surgery (23,24). Estrogen is considered a suitable inhibitor of bone resorption. Therefore, it prevents osteoporosis. Estrogen or androgen deficiency causes an elevation in bone remodeling. Both process formation and resorption of bone are increased, but uncoupling between these processes increases resorption more than formation (25). Also, estrogen can induce apoptosis in osteoclasts (26). Ovariectomy results in an elevation in serum osteocalcin and NTx. The elevation in serum osteocalcin after ovariectomy in our study agrees with previous studies of Srikanta *et al.* (27), El-Nabarawi *et al.* (28), and Morgan *et al.* (29), which observed an increase in osteocalcin levels after ovariectomy due to the deficiency of estrogen. Levels of NTx in serum of ovx rats were elevated significantly compared with the sham group. This elevation in NTx of the ovx group refers to a significant elevation in bone resorption because ovariectomy increases osteoclast activity. NTx is one of the biomarkers that refer to turnover of bone and is considered the best marker with CTx for bone resorption (30).

NTx mobilized from bone by osteoclasts and elevated serum levels indicate to increased bone resorption. The elevation in NTx in this study was in agreement with the study of Alrowaili *et al.* (31), they reported an elevation in NTx in DEX-induced osteoporosis compared with the control group, and they suggested that osteoclasts activity increased in estrogen deficiency after ovariectomy that leads to reduce BMD which related to increasing in NTx. During bone remodeling, some compounds are secreted into the bloodstream during bone formation, and some are released during bone resorption, so NTx is released during bone resorption. Serum NTx and CTx levels have been suggested to be increased in postmenopausal women (32).

Our results showed a significant elevation in serum calcium and phosphorus of the ovx group after more than 60 days of ovariectomy. This observation agrees with another study by Hamdoon *et al.* (33) and Morgan *et al.* (29), which pointed to a significant rise in serum calcium and phosphorus of the ovx rats.

One of the markers of bone formation is calcium with phosphorus since it plays an essential role in the calcification of bone (34). Calcium is associated with decreased bone mass and causes osteoporosis. Calcium is an essential mineral in bone. More than 99% calcium is found in bone and teeth (35). Calcium and phosphorus have essential functions in the body, so their concentration in plasma is controlled by the actions of the kidney (reabsorption

/excretion), intestine (absorption), and bone (exchange), the major reservoir of calcium and phosphorus in the body (36,37).

Our results indicated a significant reduction in calcium, phosphorus, and magnesium percentage in bone ash of the ovx group compared to the sham group. These results agree with the findings of Nagareddy and Lakshmana (38). They referred to a reduction in bone ash weight and percentage of ash (Ca, P and Mg) levels in ovariectomized group. Also, our results were in agreement with Mustafa *et al.* (39). They noticed a significant decrease in ash weight of femur bone and decreased levels of calcium and phosphorus in the ash of the ovx group compared to the sham group. Treating the ovx group with 20mg/kg rosuvastatin in our study leads to a significant decrease in osteocalcin serum and NTx after 60 days of treatment. This reduction in these biomarkers means there was a significant improvement in bone and a decrease in bone resorption by ovariectomy. A reduction in osteocalcin levels after treatment indicates an increase in bone formation, although histological tests did not show these improvements since some studies reported no changes in histological tests after treatment with statins. Rosuvastatin could reduce bone turnover because it lowered the elevation of serum osteocalcin and NTx seen in the ovx rats. Osteocalcin is a non-collagenous protein present in the matrix of bone, is synthesized by osteoblast cells, and is considered a bone marker because it is produced from newly synthesized osteocalcin released through bone formation and from osteocalcin released from the matrix during resorption of bone. Thus, osteocalcin is considered a marker of bone turnover.

NTx is released when osteoclasts degrade type 1 collagen, so NTx and CTx are released into the bloodstream. These proteins related to type 1 collagen are very bone specific in collagen turnover since osteoclasts are ineffective in degrading another type 1 collagen-containing tissue (40). Several studies have observed a significant 40% and 70% reduction in bone resorption markers like CTx and NTx when using bisphosphonates (anti-resorptive agents).

Treating the ovx rats with 20mg /kg of rosuvastatin led to a significant reduction in serum phosphorus compared with the ovx group after 60 days of treatment and returned to its levels in the sham group. This result agrees with the results of Gokdemir *et al.* (13), who found that phosphorus level was significantly reduced in rats treated with pravastatin. Furthermore, this study observed a significant reduction in the serum calcium of the ovx group treated with rosuvastatin but not returned to the sham group. This elevation in serum calcium in our study's group treated with rosuvastatin agrees with the previous study Gokdemir *et al.* (13). They found that serum calcium increased significantly in the group treated with rosuvastatin (41). This means statins may affect calcium levels in the blood; therefore, care must be taken into consideration when people are treated with statins because we have another study not published yet.

We observed calcium levels increased after treatment with atorvastatin (lipophilic statin).

Conclusions

Based on the results, we concluded that ovariectomy decreases bone content and leads to bone turnover, so the treatment with rosuvastatin positively affects this condition.

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Conflict of interests

No conflict

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دور الـروزفاستاتين في أيض عظام الجرذان البالغة المستأصلة المبايض

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الخلاصة

صممت هذه الدراسة للتحري عن تأثير الـروزفاستاتين أحد أنواع الـستاتينات المحبة للماء في مستوى الأوستيوكالسين، الكالسسيوم، الفسفور في مصل الدم بالإضافة الى تقدير الكالسسيوم، الفسفور والمغنسيوم في رماد عظم الفخذ لإناث الجرذان المستأصلة المبايض. تم استخدام ثلاثون من إناث الجرذان التي تراوحت أعمارها ما بين ٢,٥ - ٣ أشهر والتي تم تقسيمها كالاتي: مجموعة شاهدة، مجموعة مستأصلة المبايض والتي استخدمت كنموذج لهشاشة العظام، ومجموعة مستأصلة المبايض والتي عولجت ب ٢٠ ملغم / كغم من الـروزفاستاتين ولمدة ٦٠ يوماً. جمعت نماذج الدم بعد ٣٠ و ٦٠ يوم من بداية المعاملة لغرض إجراء الفحوصات الكيموحيوية، كما تم نزع عظام الفخذ الأيمن بعد ٦٠ يوم وتحويلها الى رماد لغرض تقدير الكالسسيوم، الفسفور والمغنسيوم. أشارت النتائج الى وجود ارتفاع معنوي في الأوستيوكالسين، الكالسسيوم والفسفور في مصل

الكالسيوم والفسفور في مصل الدم مع وجود زيادة في النسبة المئوية للكالسيوم والفسفور في رماد العظم وزيادة سمك العظام التريبيقية وتطور في الخلايا العظمية مقارنة مع الجرذان المستأصلة المبايض. نستنتج من الدراسة الحالية أن للروفاستاتين تأثير إيجابي في عظام الجرذان المستأصلة المبايض.

الدم، بالإضافة الى زيادة عدد الخلايا الهادمة للعظم وقلة الخلايا البانية للعظم وسمك العظام التريبيقية، فضلاً عن وجود انخفاض في النسبة المئوية للكالسيوم، الفسفور والمغنسيوم في رماد العظم للمجموعة المستأصلة المبايض مقارنة بالمجموعة الشاهدة. في حين أدت المعاملة بالروفاستاتين لمدة ٦٠ يوم الى انخفاض معنوي في الأوستيوكالسين،