Densitometric Evaluation Jaws and Skulls of Ovariectomized Rats Following Atorvastatin Administration: The Role of Nitric Oxide

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Abstract- Statins affect the bone metabolism. Considering the role of nitric oxide (NO) in many physiological processes, this study assessed the effects of atorvastatin (ATOR) and NO on the mandible and skull bone density (BD) in ovariectomized rats. This study evaluated 48 female Sprague-Dawley rats in 6 groups (n=8). Groups 1 and 2 underwent sham surgery. Group 1 (sham) did not receive any medication, but group 2 (sham/ATOR) received atorvastatin. Groups 3 to 6 underwent ovariectomy. Group 3 (OVX) did not receive any medication, group 4 (OVX/ATOR) received atorvastatin, group 5 (OVX/L-NAME) received L-NG-nitro arginine methyl ester (L-NAME), and group 6 (OVX/ATOR/L-NAME) received both atorvastatin and L-NAME. Atorvastatin (40 mg/kg) was gavaged and L-NAME (3 mg/kg) was administered intraperitoneally for 4 weeks. All rats underwent lateral cephalometry before and after the interventions, and BD was measured at 2 points in the mandible and skull before and after the intervention by a digital densitometer. Data were analyzed by t-test, ANOVA, and Sidak test (alpha=0.05). The change in BD was 26.5±10.17 in the mandible and 22.17±9.45 in the skull in OVX group. These values were 25.63±5.55 and 28±8.59 in OVX/ATR, 1.5±7.78 and -1.88±4.39 in OVX/L-NAME, and 6.63±7.37 and 4.33±6.35 in OVX/ATOR/L-NAME, respectively. OVX/ATOR showed no significant difference (P=1), but OVX/L-NAME (P<0.001) and OVX/ATOR/L-NAME (P<0.001) groups showed significant differences with OVX group. The present findings indicated that atorvastatin had no significant effect on BD, but administration of L-NAME prevented osteoporosis in ovariectomized rats.

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Keywords: Bone density; Atorvastatin; Ovariectomy; Nitric oxide; Rats

Introduction

According to the National Institute of Health consensus conference in 1993, osteoporosis is a systemic skeletal disease characterized by a reduction in bone mass and microscopic changes in bone tissue, which increases the susceptibility to fracture (1). Osteoporosis is an age-related disease. According to the World Health Organization, osteoporosis, cancer, cerebrovascular accident, and myocardial infarction are the four main enemies of humans, and according to recent statistics, the morbidity and mortality of osteoporosis exceed those of cancer (2).

Hormone replacement therapy has long been the main treatment option to prevent osteoporosis. However, this treatment is not well accepted due to its long-term adverse effects on the reproductive system and risk of malignancy (3). Therefore, the search is ongoing to find an efficient alternative treatment.

Atorvastatin is a cholesterol lowering medication that inhibits the β-Hydroxy β-methylglutaryl-CoA (HMG-CoA) reductase and subsequently blocks its transformation to mevalonate in the cholesterol synthesis cascade (4,5). Like bisphosphonates, statins inhibit the

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activation of osteoclasts by preventing the production of mevalonate. They prevent prenylation of small GTPases and, consequently, disrupt the downstream intracellular signaling pathways in osteoclasts (6). Accordingly, statins inhibit bone resorption by osteoclasts (7-9). Evidence shows that statins increase bone formation in rodents and enhance the bone volume when orally administered to rats (10). Statins also increase the synthesis of bone anabolic factors such as bone morphogenetic protein-2 (11), which increases osteoblast differentiation. Cardiovascular diseases and osteoporosis, which are both highly prevalent, often develop in almost the same age range in patients. Many patients at risk of osteoporosis use atorvastatin. It has been hypothesized that atorvastatin may be prescribed for patients with high serum cholesterol levels not only to decrease their serum cholesterol level, but also to prevent osteoporosis in them.

Nitric oxide (NO) is a biological molecule that plays an important role in the regulation of bone metabolism. It is synthesized by osteoblasts and is believed to regulate the anabolic effects of estrogen on bone by inhibition of osteoclasts and stimulation of osteoblasts. NO metabolites decrease after menopause, although estrogen therapy can increase their level (12-15). NO has a direct inhibitory effect on bone resorption by osteoclasts (16-18).

Several factors affect the development of osteoporosis such as several medications and biological molecules. Considering the role of NO in many physiological processes, this study aimed to assess the effects of atorvastatin and NO on bone density (BD) of the mandible and skull in ovariectomized rats.

Materials and Methods

This animal study was conducted on 48 female Sprague-Dawley rats with a mean weight of 250±25 g that were obtained from the Pasteur Institute of Iran. The study was conducted in accordance with the guidelines for the care and use of laboratory animals.

The rats were randomly assigned to six groups (n=8). All rats were kept under standard conditions in terms of temperature and moisture, and 12-hour light/12-hour dark cycles. The rats in each group were kept in separate cages, and their weight and group were written on a card attached to each cage. The rats were weighed at baseline, and on a weekly basis. The rats were allowed 4 weeks for the purpose of acclimation, and also to reach the desired weight.

Initial lateral cephalometry

All rats then underwent lateral cephalometry (Intra; Planmeca, Helsinki, Finland) with the exposure settings of 66 kVp, 6 mA, and 0.25 s using #2 E-speed 31x41 mm periapical films (Kodak, Japan) (Figure 1).

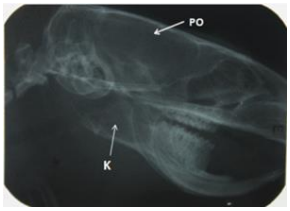

Figure 1. Lateral cephalogram of a rat. Selected points marked by arrows were used for bone densitometry

For this purpose, each rat's head was perfectly adapted to the film, and the source-to-image receptor distance was adjusted to be 50 cm. The rats were positioned on their side in a specific chamber, the X-ray tube was positioned perpendicular to the chamber and in contact with it, and a plastic cephalostat held the head in a fixed position.

Surgery

After the induction of general anesthesia, the rats were fixed to the operating table. The rats in groups 1 and 2 underwent sham surgery while those in groups 3 to 6 underwent bilateral ovariectomy with a dorsal approach. Both ovaries were resected. In groups 1 and 2, all surgical procedures were similar to those in groups 3 to 6 except that the ovaries were not removed. The rats were allowed one week for recovery and then received the following interventions:

Group 1 (sham): The rats in this group did not receive any medication after the sham surgery.

Group 2 (sham/ATOR): The rats in this group received 40 mg/kg atorvastatin (Chemi Daru. Iran, Tehran) (19) after the sham surgery.

Group 3 (OVX): The rats in this group did not receive any medication after ovariectomy.

Group 4 (OVX/ATOR): The rats in this group received 40 mg/kg atorvastatin (19) after ovariectomy.

Group 5 (OVX/L-NAME): The rats in this group received 3 mg/kg L-NG-nitro arginine methyl ester (L-NAME; a non-selective NO synthase inhibitor) (Sigma Aldrich. US) (19) after ovariectomy.

Group 6 (OVX/L-NAME/ATOR): The rats in this group received 3 mg/kg L-NAME (19) and 40 mg/kg ATR (19) after ovariectomy.

The medications were administered daily according to the weight of rats. Atorvastatin was administered through

gavage, while L-NAME was administered intraperitoneally. Carboxymethyl cellulose (Chemi Daru, Tehran. Iran) was used as a carrier for atorvastatin. To standardize the conditions and eliminate the possible confounding effects of carboxymethyl cellulose and the stress related to gavage, all other groups also received carboxymethyl cellulose gavage on a daily basis.

Four weeks after the onset of administration of medications (20,21), all rats underwent final lateral cephalometry with the same conditions and parameters as explained for baseline cephalometry. At the end of week 4 and after final lateral cephalometry, blood samples were collected from all rats through intra-cardiac puncture, their blood serum was separated by centrifugation, and the serum level of stable NO metabolites was measured using the Grace kit.

The rats were generally anesthetized prior to initial and final lateral cephalometry, sham surgery/ovariectomy, and blood collection by intraperitoneal injection of 10% xylazine (0.1 mL; Rooyandarou, Tehran, Iran) and 50% ketamine (0.1 mL; Sterop Brussels, Belgium). Also, all radiographs were processed at the same time using the same automatic film processing machine (Velopex).

Assessment of BD

After film processing, all lateral cephalograms were evaluated by a digital densitometer (Macbeth PD 504), and the radiographic BD was measured at two points on each cephalogram. The first point was at the inferior border of the skull cortex (PO) and the second point was in the mandible anterior to the mandibular foramen (K). The values were reported as optical density (OD)×0.01.

D1: Initial radiographic BD of the mandible

D1F: Final radiographic BD of the mandible

D2: Initial radiographic BD of the skull

D2F: Final radiographic BD of the skull

Statistical analysis

The initial and final radiographic BD of the mandible and skull were reported as mean and standard deviation, and the change in BD after the intervention was calculated and reported. The t-test was applied to compare the values pairwise while ANOVA was applied for multiple comparisons, followed by the Sidak post-hoc test for pairwise comparisons. The level of significance was set at 0.05.

Results

Effect of ovariectomy on BD

To ensure the effect of ovariectomy on BD, first the sham and OVX control groups were compared regarding change in their BD. The results showed that the mean change in BD of the mandible (5.9±1 vs. 26.5±10.1, $P<0.001$) and skull (4.50±5.23 vs. 22.17±9.45, $P<0.001$) was significantly lower in the sham group.

Comparison of mandibular BD change of the ovariectomized groups

A significant difference was found in mandibular BD changes of the four ovariectomized groups ($P<0.001$). Pairwise comparisons (Table 1) revealed that the change in mandibular BD in OVX/L-NAME group was significantly lower than that in OVX ($P<0.001$) and OVX/ATOR ($P<0.001$) groups. Also, the change in mandibular BD in OVX/ATOR/L-NAME group was significantly lower than that in OVX ($P<0.001$) and OVX/ATOR ($P<0.001$) groups.

Table 1. Pairwise comparisons of the four ovariectomized groups regarding mandibular BD changes (OD×100)

Group 1	Group 2	Mean density changes difference (group 2- group 1)	*P*
OVX *(26.50 ± 10.17)	OVX/ATOR (25.63±5.55)	0.88(SE=4.15)	1/00
	OVX/L-NAME (1.50 ±7.78)	25(SE=4.15)	< 0.001
	OVX/ATOR / L-NAME (6.63± 7.37)	19.88(SE=4.15)	< 0.001
OVX/ATOR (25.63±5.55)	OVX/L-NAME (1.50 ±7.78)	24.13(SE=3.84)	< 0.001
	OVX/ATOR / L-NAME (6.63± 7.37)	19(SE=3.84)	< 0.001
OVX/L-NAME (1.50 ±7.78)	OVX/ATOR / L-NAME (6.63± 7.37)	- 5.13(SE=3.84)	0.73

*Mean±SD
SE=Standard Error

Comparison of skull BD change of the ovariectomized groups

A significant difference was found in skull BD changes of the four ovariectomized groups (P<0.001). Pairwise comparisons (Table 2) revealed that the change in skull BD in OVX/L-NAME group was significantly lower than that in OVX (P<0.001) and OVX/ATOR (P<0.001) groups. Also, the change in mandibular BD in OVX/ATOR/L-NAME group was significantly lower than that in OVX (P<0.001) and OVX/ATOR (P<0.001) groups.

Table 2. Pairwise comparisons of the four ovariectomized groups regarding skull BD changes (OD×100)

Group 1	Group 2	Mean density changes difference (group 2-group 1)	*P*
OVX *(22.17±9.45)	OVX/ATOR (28±8.59)	- 5.83(SE=3.71)	1.00
	OVX/L-NAME (-1.88±4.39)	24.04(SE=3.71)	< 0.001
	OVX/ATOR / L-NAME (4.33±6.25)	15.92(SE=3.71)	0.001
OVX/ATOR (28±8.59)	OVX/L-NAME (-1.88±4.39)	29.88(SE=3.71)	<0.001
	OVX/ATOR / L-NAME (4.33±6.25)	21.75(SE=3.44)	< 0.001
OVX/L-NAME (-1.88±4.39)	OVX/ATOR / L-NAME (4.33±6.25)	- 8.12(SE=3.44)	0.15

*Mean ±SD
SE=Standard Error

Effect of atorvastatin on BD

The mandibular BD change was 1±5.93 in the sham group and 5.71±3.55 in the sham/Ator group, which were not significantly different (P=0.09). The skull BD change was 4.5±5.23 in the sham and 7.71±1.98 in the sham/ATOR group, which were not significantly different either (P=0.15).

BD change of the mandible versus the skull

The mean change in BD of the mandible and skull was first compared within each group (Table 3); however, the difference was not significant in any group (P>0.05). The mean percentage of change in BD (compared with baseline value) was also calculated for both the mandible and skull in each group. The mean percentage of change in the mandible was slightly, but not significantly, higher than the skull in OVX group (P=0.06). This difference was not significant in any other group either (P>0.05).

Table 3. Comparison of the mean change in BD of the mandible and skull (OD×100) within each group

group	D_m	D_S	D_m- D_S	Standard deviation	Standard error	*P*
OVX	26.5	22.17	4.33	5.47	2.23	0.11
OVX/ATOR	25.63	28	-2.38	10.43	3.69	0.54
OVX/L-NAME	1.5	-1.88	3.38	5.88	2.08	0.15
OVX/ATOR/L-NAME	6.63	6.25	0.38	9.88	3.49	0.92

Dm: Changes of radiographic BD in the mandible
Ds: Changes of radiographic BD in the skull
Dm-Ds= Difference between radiographic BD changes of the mandible and skull

Serum level of NO metabolites

A significant difference was noted in the mean serum level of NO metabolites among the study groups (P<0.001). Thus, pairwise comparisons were carried out (Table 4), which showed that the mean serum level of NO metabolites in the OVX /L-NAME group was significantly lower than that in the OVX (P<0.001) and OVX /ATOR (P<0.001) groups. Also, the mean serum level of NO metabolites in the OVX /ATOR /L-NAME group was significantly lower than that in the OVX (P<0.001) and OVX /ATOR (P<0.001) groups.

Table 4. Pairwise comparisons of the groups regarding the serum level of NO metabolites

Group 1	Group 2	Mean NO serum level difference (group 2- group 1)	Standard Error	*P*
OVX *(10.50±1.82)	OVX/ATOR (9.33±1.22)	1.16	0.75	0.58
	OVX/L-NAME (4.37±1.41)	6.13	0.77	< 0.001
	OVX/ATOR /L-NAME (5.28±0.94)	5.22	0.75	< 0.001
OVX/ATOR (9.33±1.22)	OVX/L-NAME (4.37±1.41)	4.97	0.68	< 0.001
	OVX/ATOR /L-NAME (5.28±0.94)	4.07	0.66	< 0.001
OVX/L-NAME (4.37±1.41)	OVX/ATOR / L-NAME (5.28±0.94)	- 0.91	0.68	0.73

*Mean±SD

Discussion

This study assessed the effects of atorvastatin and NO on BD of the mandible and skull in ovariectomized rats. Use of ovariectomized female rats in the present study was because this model has the highest resemblance to human osteoporosis according to Kalu (22). Comparison of the radiographic BD change in the sham and OVX groups revealed a significant reduction in BD in the OVX group. A 4-week period was allocated for the changes to take place after ovariectomy since Wronski *et al*., (20) reported a significant reduction in trabecular bone volume 2 weeks after ovariectomy compared with the sham control group. Also, Irie (21) reported a significant reduction in trabecular bone volume at the femoral neck 4 weeks after ovariectomy. In the present study, the final lateral cephalograms taken 4 weeks after ovariectomy revealed a significant reduction in BD, confirming the role of ovariectomy in reduction of BD and development of osteoporosis. Comparison of the OVX/ATOR and OVX groups did not reveal any significant difference in BD, indicating that 40 mg/kg atorvastatin did not affect BD in ovariectomized rats. Moreover, BD in the sham/ATOR group had no significant difference with that in the sham group, indicating that atorvastatin did not affect BD in non-ovariectomized rats either.

The effect of different statin drugs on bone metabolism has been widely investigated. However, studies regarding atorvastatin are limited. Fisher *et al*. (9) revealed that lovastatin increased BD by inhibiting bone resorption. Maeda *et al*, (11), Ayukava *et al*, (23), Du *et al*, (24), and Chuengsamarn *et al*., (25) evaluated the effects of simvastatin on BD and concluded that it increased BD and had therapeutic effects on osteoporosis. However, their results contrasted with the findings of the present study. Nevertheless, different results were reported regarding atrovastatin in the recent years. Gradosova *et al*, (26) in two separate studies reported that atorvastatin increased BD. Also, Goes *et al*., (27) showed that administration of atorvastatin decreased bone resorption in rats. Such results are different from the present findings.

McCann *et al*., (28) did not detect any bone regeneration after administration of atorvastatin. Chang *et al*., (29) assessed the effects of atorvastatin and reported that despite the reduction in serum level of bone resorption markers, no change was detected in BD. Handal *et al*., (30) found that atorvastatin did not have any significant effect on BD and strength. Braatvedt *et al*., (31) and Bone *et al*., (32) assessed the effects of atorvastatin on BD and metabolism in humans and did not observe any alteration in serum level of bone biomarkers or BD. Their results agreed with the present findings.

To evaluate the effect of NO on BD, L-NAME which is a nitric oxide synthesis inhibitor was used in the present study. To confirm the effects of L-NAME, serum levels

of nitric oxide metabolites were measured. Evaluation of serum levels of these metabolites in the study groups revealed that L-NAME significantly decreased the serum levels of NO metabolites in the OVX/L-NAME group in comparison with the OVX and OVX/ATOR groups, and also in the OVX/ATOR/L-NAME group in comparison with the OVX and OVX/ATOR groups.

Data analysis revealed that radiographic BD change in the OVX/L-NAME group was significantly smaller than that in OVX group, indicating that reduction of serum NO inhibits the reduction of BD in both the mandible and skull.

The scientific literature is controversial regarding the effects of NO on BD. Lowik *et al.*, (16) and Kasten *et al*., (17) showed that NO inhibited osteoblastic bone resorption. This result was in contrast to the present findings. Moreover, unlike the present study, Wang *et al*., (33) reported that administration of a drug releasing NO increased BD in the femur and tibia. However, the results of studies about the effects of L-NAME on orthodontic tooth movement somehow confirm the present results. Shirazi *et al*., (34) Hayashi *et al*., (35) and Akin *et al*., (36) evaluated orthodontic tooth movement following L-NAME administration and concluded that it decreased orthodontic tooth movement in rats. Histological analysis of the samples in the study by Shirazi *et al*., (34) revealed a significant reduction in number of osteoclasts in L-NAME administrated group, which confirms the present findings.

The combined effects of atorvastatin and L-NAME were also evaluated in the present study in the OVX/ATOR/L-NAME group. The results revealed that the change in radiographic BD in this group was not significant in comparison with the OVX/L-NAME group. This finding reveals that atorvastatin and NO did not have synergistic effects on BD.

To assess the difference in BD change in the mandible and skull, the mean value of radiographic BD change was compared between the mandible and skull in the present study. The results revealed no significant difference in any group; although in the ovariectomized control group, osteoporotic changes in the mandible were slightly greater than the skull. Similar results were reported by Talaeipour *et al*., (37).

Optical densitometry which was adopted in the present study to measure the change in BD of rats is accurate enough to reveal any change in BD after 4 weeks. This method of BD measurement was also used by Barbosa *et al*., (38) and Horner *et al*., (39) and its accuracy for this purpose has been well documented. Dual energy X-ray absorptiometry is also used for measurement of BD. However, due to popularity of optical densitometric method and its application in numerous studies, it was also used in the present study.

This study only assessed the effect of one dosage of atorvastatin, which was a limitation. Future studies on different doses of atorvastatin are recommended. The effects of other statin drugs should also be evaluated in future studies. Moreover, NO donor and specific NO synthase inhibitors can be used to further assess the effects of NO on BD.

The present results revealed that administration of 40 mg/kg atorvastatin for 4 weeks did not affect BD of the skull and mandible in ovariectomized rats. However, administration of 3 mg/kg L-NAME caused a reduction in serum levels of NO metabolites and inhibited BD reduction in the mandible and skull. Atorvastatin and L-NAME did not have synergistic effects on BD.

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