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Association Between Simvastatin Therapy and Bone Health in Cyclophosphamide-Induced Osteoporosis in Rats

Attaa S. Dawood[1], Ahsan F. Bairam[1*]
[1]Department of Pharmacology and toxicology, Faculty of Pharmacy, University of Kufa, Kufa, Iraq.
***Corresponding author Email**: ahsanf.bairam@uokufa.edu.iq
ORCID iD: 0000-0002-0832-6502
Phone: +9647709838385

Abstract

Osteoporosis, including drug-induced type, is a crucial health problem because it affects directly the quality of life and increases mortality. Simvastatin (SIM), shares the mevalonate pathway as nitrogen-containing bisphosphonate drugs which may impose a beneficial effect on bone health. This study aimed to explore the beneficial effects of SIM alone or in combination with alendronate (ALD) to prevent cyclophosphamide (CPA)-induced osteoporosis in rats. Six study groups (n = 7 rats each) were generated from 42 healthy female albino rats at the age of six months: Group 1 (control) received 1 ml/day of 0.9% NaCl orally for 6 weeks. Group 2 received CPA (4.5 mg/kg/day) orally for 15 days. Group 3 received ALD (1 mg/kg/day) orally for 6 weeks plus CPA. Group 4 received SIM (20 mg/kg/day) orally for 6 weeks plus CPA. Group 5 received a combination of the corresponding doses of ALD plus SIM in addition to CPA. Group 6 received SIM alone orally for 6 weeks. Finally, serum receptor activator of nuclear factor kappa-β ligand (RANKL) and tartrate-resistant acid phosphatase-5b (TRACP-5b) levels were evaluated in addition to the histopathological analysis of the right tibia. The osteoporosis effect of CPA was significantly reduced by SIM. Additionally, it is interesting to note that ALD plus SIM combination demonstrated an additional protective effect against osteoporosis compared to mono-therapy. Our findings suggest that SIM may be associated with bone-protective effect, the combination therapy administered group demonstrated an extra protective effect compared to mono-therapy.

Keywords: Simvastatin, osteoporosis, cyclophosphamide, alendronate, bones.

Introduction

Low bone mass and microstructural degeneration of bone tissue, which cause bone fragility and fracture susceptibility, are indicators of osteoporosis, a prevalent chronic metabolic bone disease, that is associated with significant morbidity and mortality [1].

Osteoporosis is one of the top 10 global diseases [2]. An osteoporotic fracture occurs every 3 seconds, accounting for more than 8.9 million fractures annually [3]. An osteoporotic fracture has substantial consequences for the patient and the healthcare system, making osteoporosis an expensive problem for society and the healthcare system [4,5].

Bone remodelling helps to heal microdamage in the bone matrix and prevents the buildup of old bone. It is also crucial in maintaining plasma calcium homeostasis [6,7]. The remodelling is controlled by two important signalling pathways receptor activator of nuclear factor kappa-β (RANK)/ RANK ligand (RANKL)/osteoprotegerin (OPG) and canonical Wnt signalling. The regulating function of these pathways affects the balance and timing of bone resorption and production throughout the remodelling cycle making them potentially attractive targets for pharmaceutical treatments in disease states such as osteoporosis [6,8]. RANKL, expressed from the surface of preosteoblastic cells and osteocytes, binds to RANK on the surface of osteoclastic precursor cells and is required for osteoclastic cell development, fusion into multinucleated cells, activation, and survival [9,10]. OPG is secreted by

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osteoblasts and osteocytes, suppressing osteoclastic bone resorption by inhibiting the actions of RANKL [10,11]. Consequently, the RANKL: OPG ratio plays an essential role in controlling bone resorption, bone mass, and skeletal integrity, and it is influenced by a range of systemic conditions [6]. On the other hand, the Wnt signalling pathway is vital in inducing osteogenic differentiation from Mesenchymal stem cells (MSCs). Moreover, Wnt ligands also promote osteoblast proliferation and maturation [8].

Chemotherapy contributed to osteoporosis in cancer patients has more attention in recent years [12,13]. Cyclophosphamide (CPA), an alkylating agent, is still used to treat various types of cancer. CPA or CPA regimens caused premature menopause in women [14], resulting in osteoporotic phenotype at both trabecular and cortical bone due to ovary damage in females and decreased androgen levels in males [15,16]. Estrogens inhibit bone resorption and loss via binding to osteoblast and osteoclast estrogen receptors alpha and beta (ERα and ERβ, respectively), which leads to an increase in the OPG: RANKL ratio [17,18]. Due to decreased estrogen levels, follicle-stimulating hormone (FSH) and luteinizing hormone (LH) increase, which inhibits bone production like postmenopausal osteoporosis [19,20]. CPA suppresses Wnt-signaling thus reducing osteoblastogenesis and osteoblast differentiation [16].

Bone loss is prevented with bisphosphonates, estrogen, and selective estrogen receptor modulators [21]; These medications prevent fractures but don't increase bone formation [22] and cause significant adverse effects like jaw osteonecrosis with bisphosphonates [23], increase the likelihood of cardiovascular disease and breast cancer with estrogen [24], and selective estrogen receptor modulators [21]. So, it is imperative to find a new effective, well-tolerated, and safe medication with fewer side effects.

The use of statins for the treatment of cholesterol is common among the elderly who are also at increased risk of developing osteoporosis [25]. Statins suppress the enzyme hydroxy-methylglutaryl-coenzyme A (HMG-CoA) reductase, which is the rate-limiting step in the conversion of HMG-CoA to mevalonate [26]. In 1999, researchers published the first research investigating the effect of statin on bone, simvastatin (SIM) and lovastatin, were shown to increase gene expression of the bone morphogenetic protein–2 (BMP-2) in bone cells [27]. Based on that, the current study investigates the ability of SIM either alone or in combination with ALD to prevent CPA from induced osteoporosis in rats.

Materials and methods

Ethical approval

The Ethical Committee at the College of Pharmacy, University of Kufa, Kufa, Iraq, approved the research procedures of the current study.

Laboratory animals

A total of 42 female albino rats ranging in weight from 160 to 200g and aged six months were used. Animals were kept in the animal house of the Faculty of Pharmacy, University of Kufa, inside a group caging system in an isolated chamber at 25±2 C° and ambient humidity with the 12-hour dark/light cycle. The rats were given tap water and a conventional meal.

Experimental design

All rats were randomly divided into six study groups (n = 7 each): Group 1 (control) received 1 ml 0.9% NaCl orally every day for 6 weeks. Group 2 received CPA (4.5 mg/kg daily) orally for 15 days (CPA group) [28,29]. Group 3 received alendronate (ALD) (1 mg/kg daily) orally for 6 weeks plus CPA (CPA+ALD group). Group 4 received SIM (20 mg/kg daily) orally for 6 weeks plus CPA (CPA+SIM group) [30]. Group 5 received a combination of ALD plus SIM in addition to CPA (CPA+ALD+SIM group). Group 6 received SIM alone orally for 6 weeks (SIM group).

Al-Qadisiyah Journal of Veterinary Medicine Sciences
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Collection of blood sample

After administering 100 mg/kg of ketamine and 10 mg/kg of xylazine, 5 ml of blood was collected from each rat's heart using a disposable 5 ml syringe. Blood was collected in a gel tube with a clotting activator, allowed to coagulate at 37 degrees Celsius, then centrifuged at 3000 rotations per minute for 15 minutes to separate the serum. Serum samples were collected and frozen in Eppendorf tubes at -4°C to be utilized later for evaluation of serum rat RANKL and TRACP-5b.

Determination of serum RANKL and TRACP-5b levels

Both RANKL and TRACP-5b serum concentrations were evaluated using an enzyme-linked immunosorbent assay (ELISA) kit, from Elabscience company, with catalog numbers E-EL-R0841and E-EL-R0939 respectively. The procedure was done following the directions provided by the manufacturer.

Histopathological evaluation

Following the collection of the blood sample, rats were sacrificed and the right tibia was taken and fixed in formalin 10% for 24h. Followed by decalcification in 10% formic acid and embedded in paraffin then 5 µm sections were prepared and stained with hematoxylin and eosin (H&E) for examination under a light microscope.

Statistical analysis

Statistical Program for the Social Sciences version 25 was used to analyze the data. The mean and standard error mean (SEM) were used to report the results. One-way Analysis of Variance (ANOVA) was used for all group comparisons. After that, the post-hoc with LSD testing where done. P values less than 0.05 have been determined to be statistically significant in every test.

Results

Effect of oral simvastatin and/or alendronate on serum RANKL level

As shown in Figure (1), the RANKL level is significantly increased in CPA administered group in comparison with the control group, whereas all groups treated either with SIM and/or ALD demonstrated significantly lower RANKL levels than that in the CPA-treated group. Importantly, the RANKL level in rats administered SIM plus ALD combination decreased much more significantly than that in rats received either SIM or ALD. Finally, rats administered SIM alone without CPA revealed a non-significant change in their RANKL levels as compared with control rats.

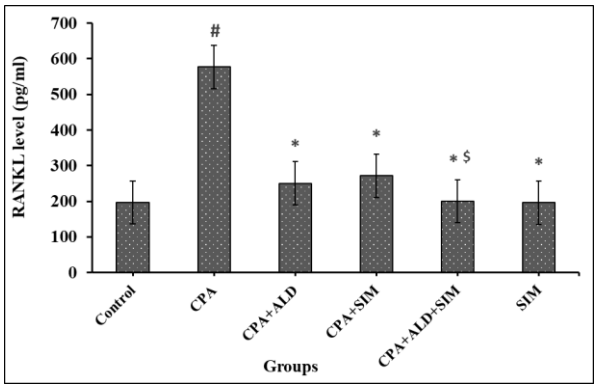

Figure 1: Receptor activator of nuclear factor kappa B ligand (RANKL) level in different study groups. Data were expressed as Mean ± SEM. CPA refers to cyclophosphamide. ALD refers to alendronate. SIM refers to simvastatin.

#Represents a significant difference compared with the control group ($p<0.05$).

*Represents a significant difference compared with the CPA-administered group ($p<0.05$). $Represents a significant difference compared with CPA+ALD administered group ($p<0.05$).

Effect of oral simvastatin and/or alendronate on serum TRACP-5b level

The TRACP-5b level was significantly elevated following CPA administration in Group 2 when compared with the control group. Other rats treated either with SIM and/or ALD demonstrated significantly lower TRACP-5b levels than that in the CPA-treated

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group. It is clearly noticed that the TRACP-5b level in rats administered SIM together with ALD combination was significantly lower than that in animal groups that received either SIM or ALD. Furthermore, rats administered SIM alone (without CPA, Group six) did not demonstrate significant variation in their corresponding TRACP-5b levels in comparison with the control group, as seen in Figure (2).

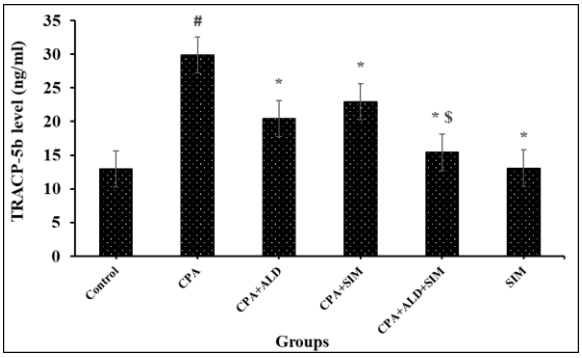

Figure 2: Rat Tartrate-Resistant Acid Phosphatase-5b (TRACP-5b) level in different study groups.
Data were expressed as Mean ± SEM.
CPA refers to cyclophosphamide. ALD refers to alendronate. SIM refers to simvastatin.

#Represents a significant difference compared with the control group ($p<0.05$).

*Represents a significant difference compared with the CPA-administered group ($p<0.05$).

$Represents a significant difference compared with CPA+ALD administered group ($p<0.05$).

Histopathological examination

The histological results of bone tibia rats showed normal bone tissue characterized by normal osteoblasts and osteoclasts on the border of thick compact bone with narrow Haversian canals and the presence of osteocytes within the lacuna. Additionally, there were normal osteoblasts and osteoclasts on the border of the thick trabecular and there is normal bone marrow in the control group and the SIM group (Figure 3 A, F) .

In the CPA-treated rat, there was an active osteoclast with decreasing cortical bone thickness and loss of lamellar bone with few numbers of osteocytes within the lacuna. Additionally, there were marked trabecular perforations which result from loss of trabecular bone, and marked reduction of the lamellar bone matrix with few numbers of osteocytes and active osteoclasts (Figure 3B) .

In the CPA+ALD treated group, there was a thickening of cortical bone and the presence of lamellar bone with moderate numbers of osteocytes within the lacuna. Additionally, there was marked narrow perforation of trabecular bone with signs of proliferation, thickening trabecular bone with the presence of lamellar bone, a high number of osteoblasts, few osteoclasts, and moderate numbers of osteocytes within the lacuna (Figure 3 C) .

In the CPA+SIM treated group, there was thick cortical bone with the presence of lamellar bone and proliferation of active osteoblast with few osteoclasts within a few wide Haversian canals with moderate numbers of osteocytes within lacuna. Additionally, there was marked thickening of trabecular bone with the presence of lamellar bone, a high number of osteoblasts, few osteoclasts, and moderate numbers of osteocytes within the lacuna (Figure 3 D) .

In the CPA+ALD+SIM treated group, there was normal compact bone with narrow Haversian canals, there are active osteoblasts within the canal with high numbers of osteocytes within the lacuna in the compact bone. Additionally, there was marked thickening of trabecular bone with signs of proliferation, the presence of lamellar bone, a high number of active osteoblasts, and high numbers of osteocytes within the lacuna (Figure 3 E).

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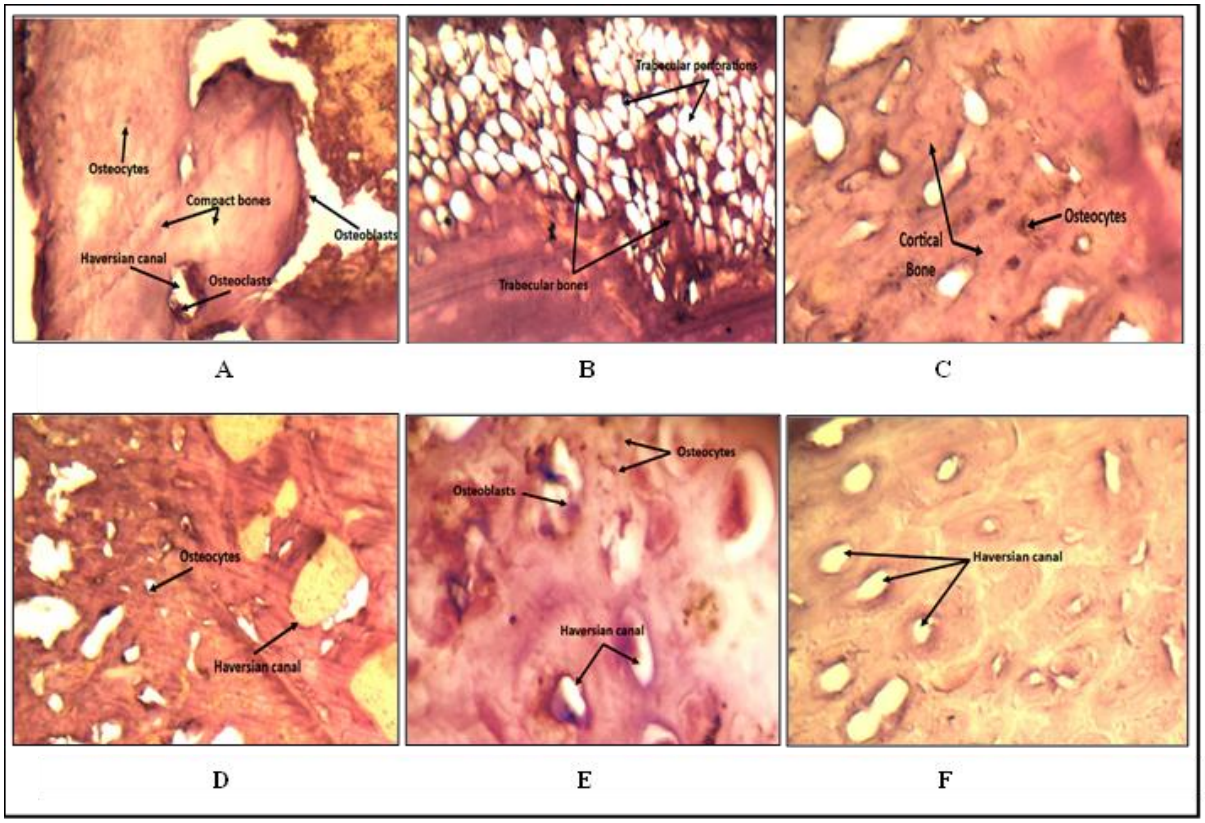

Figure 3: Histopathological examination of the rats' tibia in different study groups. A) Control group shows normal bone tissue, B) CPA-treated group shows marked trabecular perforations which result from bone loss, C) CPA + ALD-treated group shows thick cortical bone with moderate numbers of osteocytes within the lacuna, D) CPA + SIM-treated group shows thick cortical bone with few osteoclasts within few wide Haversian canals and moderate numbers of osteocytes within lacuna, E) CPA+ALD+SIM-treated group shows normal compact bone with narrow Haversian canals, there are active osteoblasts within the canal with high numbers of osteocytes within the lacuna, F) SIM-treated group shows normal bone tissue. H&E. 10x.

Discussion

A greater number of senior people, especially women, suffer from osteoporosis and related complications [31]. The bone loss that can occur as a side effect of chemotherapy treatment has gained widespread attention in recent years. This link between chemotherapy and osteoporosis in cancer patients has more attention in recent years .[12,13]

High doses or prolonged usage of CPA may cause rapid bone loss and increase the risk of osteoporotic fractures [29]. CPA has been connected to both the onset of amenorrhea and early menopause. As a result, ovarian failure is probably the primary cause of CPA-induced bone loss.[14,15]

The current investigation showed that CPA-treated rats had significantly increased serum levels of RANKL and TRACP-5b when compared to the control group. These data may suggest that CPA therapy can lead to high-turnover osteoporosis, which is supported by the findings obtained from our histological examination. CPA-induced osteoporosis was utilized as a model in rats. It was observed that rats exposed to this treatment for 15 days had conventional osteoporotic symptoms such as reduced bone trabecular density and thickness with increased trabecular separation.[29]

This investigation was done on healthy rats, i.e., rats without tumors, which were administered CPA to induce bone loss, and

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evaluate the role of SIM in preventing bone loss due to CPA treatment in a well-defined animal model; To the best of our knowledge, there was no research on the effects of SIM on CPA-induced osteoporosis. The results of the present study indicate that concurrent administration of SIM and/or ALD prevented the adverse effects of bone resorption caused by CPA as shown by decreased levels of RANKL and TRACP-5b that because the ability of statins to inhibit osteoclastogenesis by inhibiting the OPG/RANKL/RANK signaling pathway and do that by enhancing the expression of ERα [32]. When estrogen binds to ERα stimulates the synthesis of OPG while simultaneously lowering levels of RANKL. This, in turn, encourages the proliferation and activity of osteoblastic cells, slows the death of osteocytes (apoptosis), and slows the differentiation and maturation of osteoclastic precursors [33]. This finding was supported by a recent study [34], which showed that administering SIM to ovariectomy rats, used as a model for induction osteoporosis, caused a decrease in RANKL levels in those animals.

Additionally, these findings were similar to that reported by another previous study which stated that oral administration of SIM markedly lowered serum TRACP-5b, bone resorption marker, level in the ovariectomized rat model [35]. Furthermore, the combination therapy of both ALD and SIM led to a significant decrease in their corresponding RANKL and TRAP5b levels ($P<0.05$) compared to monotherapy.

Bisphosphonates prevent the disintegration of hydroxyapatite crystals, hence reducing bone resorption [36]. Nitrate-containing bisphosphonates, such as ALD, have been shown in previous research to inhibit the synthesis of isoprenoid intermediates, compounds that are essential for bone health because they are responsible for the prenylation of proteins attached to the cell membranes of osteoclasts, proteins that are essential for the function and survival of osteoclasts [37]. As a result, ALD is able to prevent bone loss by limiting prenylation, through inhibiting the enzyme FPP synthase in the mevalonate pathway.[36]

On the other hand, statins which block HMG-CoA reductase lower cholesterol synthesis in the mevalonate pathway. Thus, statins decrease substrate availability in the mevalonate pathway, decreasing prenylation and osteoclastic bone resorption like ALD [38,37]. Indicating that blocking the mevalonate pathway with two different medications at two different levels improves the deterioration of CPA on bone.

Conclusion

The findings of our research confirmed the anti-osteoporotic effects of ALD and/or SIM in rats that were given CPA, and they indicated that blocking the mevalonate pathway with two different medications at two different levels may improve the treatment of osteoporosis. Comparing the effectiveness of combination therapy versus mono-therapy in the present study, the combination therapy group showed more improvement in the tested biomarkers of bone formation and resorption over their mono-therapy group .

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Conflict of interest

The current work is declared with no conflict of interest.

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