Original Article

Bone-Protective Effect of Simvastatin against Cyclophosphamide-Induced Osteoporosis in Rats

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Abstract

Background: Osteoporosis, as well as osteoporosis caused by chemotherapy, is regarded as a serious medical condition that lowers the quality of life, has a high prevalence of mortality and morbidity, and is expensive to treat. Simvastatin (SIM) is an HMG-CoA reductase inhibitor that works through the same mevalonate route as bisphosphonate medications containing nitrogen to promote bone growth. **Objectives:** This study aimed to explore the protective effects of SIM on cyclophosphamide (CPA)-induced osteoporosis in rats. **Materials and Methods:** In total, 42 healthy female albino rats aged 6 months old were randomly divided into six study groups (n = 7 rats per group): Group 1 (control) received 1 mL 0.9% NaCl orally for 6 weeks, Group 2 administered CPA (4.5 mg/kg daily) orally for 15 days, Group 3 administered alendronate (1 mg/kg daily) orally for 6 weeks plus CPA, Group 4 administered SIM (20 mg/kg daily) orally for 6 weeks plus CPA, Group 5 administered a combination of alendronate plus SIM in addition to CPA, and Group 6 administered SIM alone orally for 6 weeks. Finally, serum alkaline phosphatase and type I collagen cross-linked c-telopeptide levels were evaluated in addition to histological analysis of the right tibia. **Results:** SIM significantly improved the bone deleterious effects of CPA when compared to the CPA group. Interestingly, the alendronate plus SIM combination showed an additional improvement in the tested clinical biomarkers versus SIM monotherapy. **Conclusion:** Our study findings may ensure the bone-protective effect of SIM as monotherapy or in combination with alendronate.

Keywords: Alendronate, cyclophosphamide, osteoporosis, simvastatin

Introduction

The word osteoporosis has Greek origin, which dates back to the 1820s and comes from the word osteon (means bone) and poros (means a small hole or pore).[1] Osteoporosis is a serious medical condition that not only lowers the quality of life but is also linked to increased risk of morbidity, death, and financial burden. Osteoporosis has been defined by the World Health Organization as a "systemic skeletal disease characterized by low bone mass and microarchitectural deterioration of bone tissue which leads to bone fragility and susceptibility to fracture."[2]

Osteoporosis considers one of the 10 disorders that are most prevalent around the world.[3] Globally, 200 million women are thought to be affected by osteoporosis.[4] After age 50, one of three women can develop osteoporotic fracture.[5] When an osteoporotic fracture occurs, it seriously impacts the patient and the healthcare system, making it possible to consider osteoporosis a costly problem for the healthcare system and society.[6,7] Osteoporosis induced by chemotherapy treatment has emerged as a major problem for cancer patients. In recent years, chemotherapy has been recognized as a potential cause of osteoporosis in cancer survivors.[8,9]

Cyclophosphamide (CPA) is an alkylating agent and is still extensively utilized as a chemotherapeutic drug for the treatment of many different forms of cancer.[10] CPA or CPA regimens promoted the premature onset of menopause in women,[11] leading to the development of osteoporotic phenotype at both trabecular bone and cortical bone because of the ability of CPA to cause

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ovary damage in females and the decrease in androgen levels in males,[12,13] that increases bone loss by reducing estrogens production. Estrogens reduce bone resorption and loss through binding to the estrogen receptors alpha and beta (ERα and ERβ, respectively) in osteoblasts and osteoclasts, and that leads to an increase in the production of osteoprotegerin (OPG) and decreases the receptor activator of nuclear factor-B ligand (RANKL) that is responsible for osteoclast differentiation and proliferation.[14,15] Furthermore, follicle-stimulating hormone and luteinizing hormone are increased due to reducing in estrogen levels; both of them are bone formation inhibitors, which is similar to what happens in postmenopausal osteoporosis.[16,17] CPA also decreases bone formation by inhibiting osteoblastogenesis and osteoblast differentiation by suppressing Wnt/-catenin signaling.[13,18] Bisphosphonates, estrogen, and selective estrogen receptor modulators (SERMs) are used to prevent bone loss[19]; these drugs prevent fractures, but they don't enhance bone production[20] and may have substantial adverse effects like osteonecrosis of the jaws with bisphosphonates,[21] estrogen increases the risk of coronary artery disease and breast cancer,[22] and SERMs increase risk of venous thromboembolism.[19] So, it is imperative to find a new efficient, safe, and well-tolerated agent with fewer adverse effects.

Statins are often used to treat cholesterol in older people, who are also more likely to have osteoporosis.[23] Statins inhibit HMG-CoA reductase (named 3-hydroxy-3-methylglutaryl-coenzyme A reductase), which converts HMG-CoA to mevalonate, an early rate-limiting step in the mevalonate pathway.[24]

Mundy *et al*.[25] published the first research investigating the effect of statin on bone (simvastatin [SIM] and lovastatin) were shown to have osteogenic effects when given orally and subcutaneously in a rodent study. Based on that, the current study aims to explore the bone protective outcome of SIM on CPA-induced bone loss in rats.

Materials and Methods

Laboratory animals

A total of 42 healthy 6-month-old female albino rats weighed 160–200 g were included in the study. These animals were kept in the animal house of the Faculty of Pharmacy, University of Kufa, in a group caging system in an isolated chamber at 25 ± 2°C and ambient humidity with the 12-h dark/light cycle. Animal enclosures have free water and feed access.

Experimental design

All rats were randomly divided into six study groups ($n = 7$ each): Group 1 (control group) rats administered orally a single daily dose of 1 mL 0.9% NaCl for 6 weeks; Group 2 (CPA group) animals administered orally a single daily dose of CPA (4.5 mg/kg) for 15 days[26]; Group 3 (CPA + ALD group) administered alendronate orally (1 mg/kg daily) for 6 weeks plus CPA (4.5 mg/kg) for 15 days; Group 4 (CPA + SIM group) administered SIM orally (20 mg/kg daily) for 6 weeks, plus CPA (4.5 mg/kg) for 15 days[27]; Group 5 (CPA + ALD + SIM group) received a combination of oral alendronate (1 mg/kg daily) and SIM (20 mg/kg daily) for 6 weeks in addition to CPA (4.5 mg/kg) for 15 days; In Group 6 (SIM group), the rats administered oral SIM (20 mg/kg daily) alone for 6 weeks.

Collection of blood sample

At the end of the treatment, the rats were given 100 mg/kg ketamine and 10 mg/kg xylazine, and a disposable 5 mL syringe was used to puncture their hearts and draw about 5 mL of blood. To obtain serum, blood was placed in a gel tube with a clotting activator, allowed to coagulate at 37°C, then centrifuged at 3000 rpm for 15 min. The serum samples were stored in Eppendorf tubes at -4°C to be utilized later for evaluation of the study's parameters.

Measurement of alkaline phosphatase (ALP) and type I collagen cross-linked c-telopeptide (CTX-1) serum levels

Both ALP and CTX-1 serum concentrations were evaluated using an enzyme-linked immunosorbent assay (ELISA) kit from Elabscience company; the Sandwich ELISA concept is employed by this ELISA kit. The procedure was done following the directions provided by the manufacturer.

Histopathological evaluation

After blood sampling, rats were sacrificed, and the right tibia was removed and fixed in formalin 10% for 24 h. Later, the bones were then decalcified in 10% formic acid and embedded in paraffin for light microscope investigation. Sections with 5 μm thickness were prepared and stained with hematoxylin and eosin (H&E). The trabecular thickness and trabecular perforations in the tibia were examined by using Oculometer.[28]

Statistical analysis

The statistical analysis was carried out using version 25 of the SPSS (statistical program for the social sciences, IBM, Chicago, IL, USA). The results were reported using the mean together with the standard error mean (SEM). The various comparisons between groups were carried out using analysis of variance, followed by post-hoc testing with the LSD technique. *P* values less than 0.05 have been determined to be statistically significant in every test.

Ethical approval

This experiment followed the national criteria for laboratory animal care and use. All protocols, followed by the Institutional Animal Care and Use Committee

(IACUC) in the University of Kufa, Kufa, Iraq, according to document number 2236, on March 1, 2023 to get this approval.

Results

Effect of oral SIM and/or alendronate on serum ALP level

As seen in Figure 1, it shows that the ALP 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 ALP levels than that in the CPA-treated group. Importantly, ALP levels in rats administered SIM plus ALD combination decreased much more significantly than that in rats received either SIM or ALD.

Effect of oral SIM and/or alendronate on serum CTX-1 level

As demonstrated in Figure 2, the CTX-1 level in CPA administered group was significantly greater than that in the control group. However, groups treated with SIM and/or ALD showed a significant decrease in CTX-1 level. In addition, rats administered a combination of SIM plus ALD in addition to CPA showed significantly more

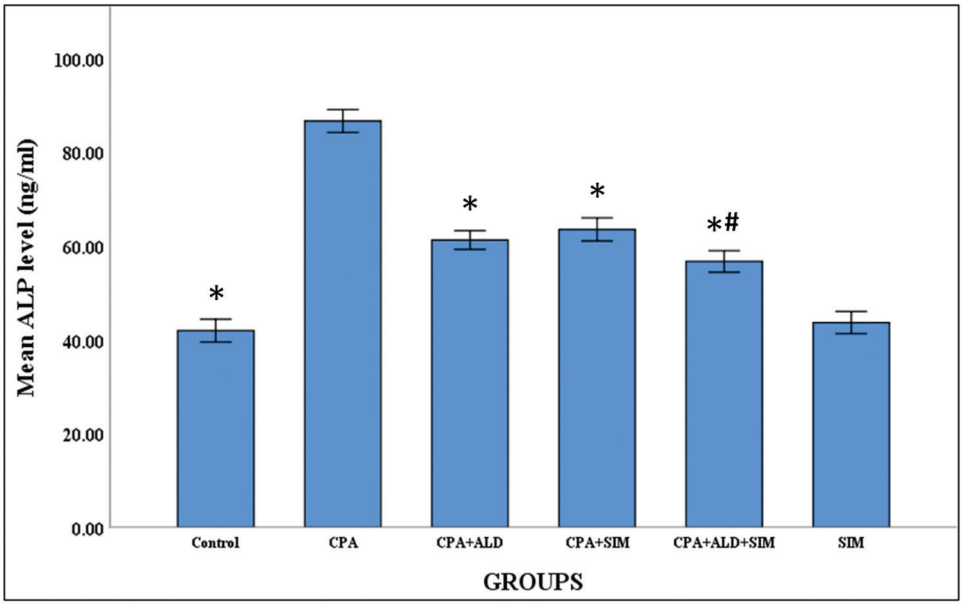

Figure 1: Alkaline phosphatase level in different study groups as mean ± SEM. * Represents a significant difference with the CPA group ($P < 0.05$). # Represents significant difference with CPA + ALD group ($P < 0.05$)

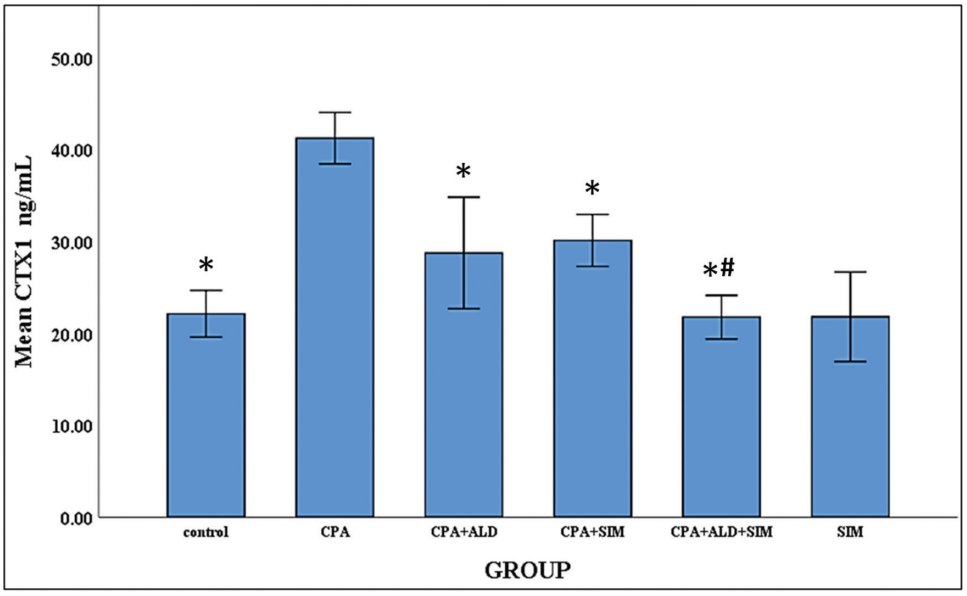

Figure 2: Type-I collagen cross-linked C-telopeptide level in different study groups as mean ± SEM. * Represents a significant difference with the CPA group ($P < 0.05$). # Represents significant difference with CPA + ALD group ($P < 0.05$).

reduction in their CTX-1 level than that detected in rats received ALD monotherapy with CPA (Group 3). Finally, rats administered SIM alone without CPA (Group 6) revealed non-significant changes in their CTX-1 levels as compared with control rats.

Histopathological examination

As shown in Figures 3 and 5, in the CPA-treated group, there was a clear significant decrement in the trabecular bone thickness versus the control. In rats treated with SIM, ALD, or SIM plus ALD combination, the trabecular bone thickness levels were significantly higher than those in the CPA-treated group. Importantly, comparing the histopathological changes in bones of rates received a combination of SIM plus ALD to that in rats administered ALD monotherapy (Group 3) showed a significant elevation in trabecular bone thickness. However, there was no significant difference in the corresponding bone thickness between the control group and the SIM group (Group 6).

Regarding trabecular separation, histopathological examination demonstrated a significant elevation in trabecular bone separation compared with that in the control group, as seen in Figures 4 and 5.

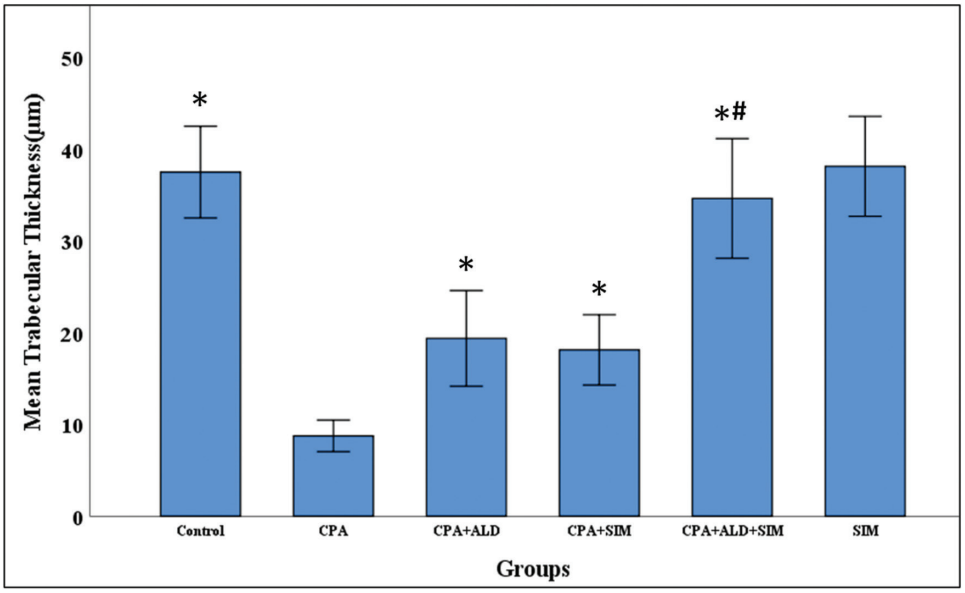

Figure 3: The trabecular bone thickness of rats in different study groups as mean ± SEM. * Represents a significant difference with the CPA-treated group ($P < 0.05$). # Represents significant difference with CPA + ALD-treated group ($P < 0.05$)

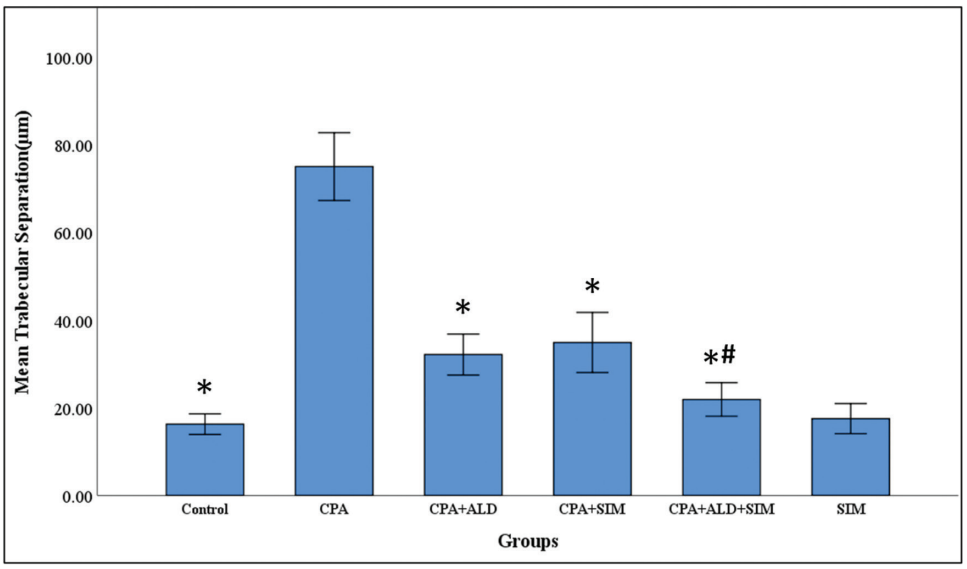

Figure 4: The trabecular bone separation of rats in different study groups as mean ± SEM. * Represents a significant difference with the CPA-treated group ($P < 0.05$). # Represents significant difference with CPA + ALD-treated group ($P < 0.05$)

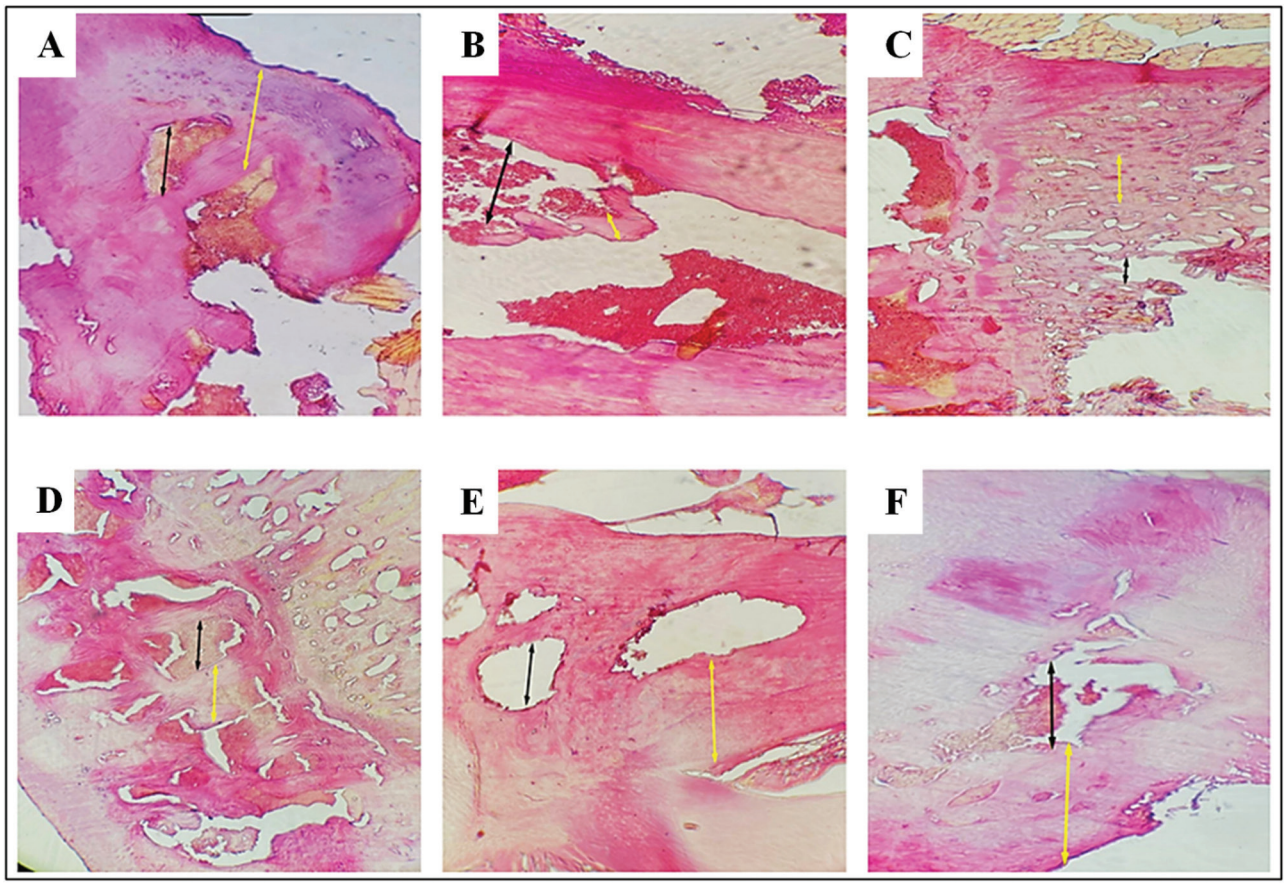

Figure 5: Histopathological examination of the rats' tibia in different study groups. Yellow arrows refer to photomicrographs of the rat's trabecular bone thickness. Black arrows refer to photomicrographs of rat's trabecular bone separation

In rats administered SIM, ALD, or SIM plus ALD combination therapy, the examined trabecular bone separation was significantly lower than that observed in the CPA-treated group. Furthermore, histopathological changes in trabecular bones of rates received a combination of SIM plus ALD displayed a significant reduction in their bone separation results than that obtained in rats treated with ALD (Group 3). Finally, no significant differences were shown in the trabecular bone separation between the control group and the SIM group (Group 6).

Rat tibia sections were stained by H&E stain in different animal groups:

A. Control group,
B. CPA-treated group,
C. CPA + ALD-treated group,
D. CPA + SIM-treated group,
E. CPA + ALD + SIM-treated group,
F. SIM-treated group.

Discussion

Osteoporosis and its complications affect a significantly greater number of the elderly population, especially women, due to a lack of estrogen.[29] Chemotherapy-induced osteoporosis is now recognized as a significant issue for cancer patients. Recent years have seen an increased awareness that chemotherapy may contribute to osteoporosis in patients with cancer.[8,9] CPA may cause fast bone loss and raise the risk of osteoporotic fractures if it is used in high doses or for an extended period of time.[30] Amenorrhea and early menopause have been linked to CPA. Therefore, ovarian failure is likely to be the main reason for CPA-induced bone loss.[11,12] The current study found that compared to the control group, the CPA-treated rats exhibited higher serum levels of ALP and CTX-1 and also had a significant decrease in their trabecular bone thickness as well as a significant increase in trabecular separation.

These findings may indicate that CPA therapy may cause high-turnover osteoporosis, which is confirmed by our histopathological findings. CPA-induced osteoporosis is already used as a model in rats; 15 days of exposure to this chemotherapy was reported to exhibit classic osteoporotic features such as reduced bone trabecular density and thickness with increased trabecular separation.[30] The bone formation biomarker (ALP) is released by osteoblasts to provide a high concentration of phosphate during bone mineralization.[31] According to Vasikaran

et al.[32], ALP has been observed in both people and animals who have osteoporosis and other bone problems. The results of our study showed that giving alendronate and/or SIM can reverse the bad effects of CPA on bone. Alendronate and/or SIM significantly decreased the ALP and CTX-1 levels compared to the CPA-treated group, associated with a clear elevation in trabecular thickness as well as a reduction of trabecular separation. A close study used ovariectomy (OVX) for induction of osteoporosis found that ovariectomized female rats, treated for 4 weeks with either alendronate or SIM, showed a significant decrease in their serum ALP levels compared to control rats. Furthermore, the combination therapy of both alendronate and SIM led to a significant decrease in their corresponding ALP levels ($P < 0.05$) compared to monotherapy.[33]

Another study showed that oral administration of SIM by rats for 12 weeks significantly decreased serum CTX-1 and P1NP concentrations and trabecular separation, as well as an increase in trabecular thickness compared with the control group.[34] Different induction method of osteoporosis in rats was applied by other research groups which utilized glucocorticoids for that purpose; after induction, it was reported that SIM plus risedronate combination significantly decreased rat's serum level of CTX-1 with increasing trabecular thickness in comparison to osteoporotic non-treated rats.[35] CTX-1 is released when osteoclasts break down collagen; CTX-1 represents an accurate and selective measure of bone resorption that can quickly show how well bisphosphonate treatment is working in osteoporosis after menopause.[36]

The impact of statins medications on bone growth is owing to the capacity of statins to stimulate the expression of the bone morphogenetic protein-2 gene, which in turn increases runt-related transcription factor 2, a major transcription factor linked with osteoblast differentiation from mesenchymal stem cells.[37,38] In addition, statins suppress glucocorticoid receptor function, which accelerates bone resorption and prevents the proliferation of osteoblastic cells; this occurs by reducing the cellular farnesyl pyrophosphate (FPP), which are key intermediates in the production of sterols and related metabolites via the mevalonate pathway.[39]

Statins inhibit osteoclastogenesis by inhibiting the OPG/RANKL/RANK signaling pathway and by enhancing the expression of Erα.[40] 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.[41]

Bone resorption is minimized by nitrogen-containing bisphosphonates because they inhibit hydroxyapatite crystals dissolution.[42] Previous studies have shown that nitrate-containing bisphosphonate, such as alendronate, blocks the formation of isoprenoid intermediates, which are crucial for bones because these compounds are responsible for the prenylation of proteins connected to the cell membranes of osteoclasts; these proteins are needed for osteoclasts to function and survive.[43] As a result, alendronate is able to prevent bone loss by limiting prenylation by inhibiting the enzyme FPP synthase in the mevalonate pathway.[42]

On the other hand, statins, which are inhibitors of the enzyme HMG-CoA reductase, also work to reduce blood cholesterol levels by inhibiting the same route. Consequently, statins reduce substrate availability in the mevalonate pathway, which leads to decreased prenylation and osteoclastic bone resorption like alendronate.[43,44]

Additionally, we propose that SIM supplementation might provide a quick and low-cost technique that can be utilized in conjunction with CPA medication to lessen its negative effects. Additional functional investigations may offer critical insights and pertinent data on the functions of SIM in protecting bone health. To conclude, our study validated the anti-osteoporotic benefits of alendronate and/or SIM in CPA-treated rats and demonstrated that inhibiting the mevalonate pathway by two medicines at two levels may improve the treatment of osteoporosis. Comparing the effectiveness of combination therapy versus monotherapy in the present study, the combination therapy group showed more improvement in the tested biomarkers of bone formation and resorption over their monotherapy group.

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Nil.

Conflicts of interest
There are no conflicts of interest.

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