

Betulinic Acid Mitigates Estrogen Deficiency–Related Femoral Bone Loss: Evidence from an Ovariectomized Rat Model

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ABSTRACT

Objective: Postmenopausal osteoporosis (OP) is characterized by estrogen deficiency–induced bone loss and deterioration of trabecular microarchitecture. Betulinic acid (BA), a natural pentacyclic triterpenoid, has demonstrated antioxidant and osteoprotective properties. This study aimed to evaluate the histopathological effects of BA on femoral bone tissue in ovariectomized rats.

Methods: Thirty-two female Wistar albino rats were randomly divided into four groups (n=8): Sham, OVX, BA+OVX (BA administered before OVX), and OVX+BA (BA administered after OVX). BA was given orally at 3 mg/kg/day. Pelvic bone mineral density (BMD) was assessed using dual-energy X-ray absorptiometry (DEXA). Serum calcium levels were measured, and femoral bone tissues were evaluated histopathologically for trabecular thinning and loss of connectivity.

Results: OVX resulted in a significant reduction in pelvic BMD and marked deterioration of trabecular bone architecture compared with the Sham group. BA administration prior to OVX significantly attenuated OVX-induced bone loss, as evidenced by higher pelvic BMD and preserved trabecular structure compared with the OVX group ($p<.05$). In contrast, BA treatment initiated after OVX did not significantly reverse bone loss or histopathological damage. Serum calcium levels did not differ significantly among the groups.

Conclusion: BA exerts a protective effect against OVX-induced bone loss when administered prior to estrogen deficiency, preserving both bone mineral density and trabecular microarchitecture. These findings suggest that BA may have a preventive, rather than therapeutic, role in estrogen deficiency–related (OP).

Keywords: Betulinic acid, bone microarchitecture, ovariectomy, osteoporosis, rat model

Introduction

Osteoporosis (OP) is a progressive skeletal disorder characterized by decreased bone mass, deterioration of bone microarchitecture, and increased fracture risk, particularly in postmenopausal women (Eastell et al., 2016; Lorentzon et al., 2021). Estrogen deficiency plays a central role in the pathogenesis of postmenopausal OP by disrupting the balance between bone formation and resorption, favoring osteoclast-mediated bone loss (De Villiers, 2023). Experimental ovariectomy (OVX) in rodents is a well-established and widely used model that closely mimics postmenopausal bone loss and associated structural changes in bone tissue (Yousefzadeh et al., 2020).

Oxidative stress (OS) has emerged as a critical contributor to estrogen deficiency–induced bone loss (Lean et al., 2005). Increased production of reactive oxygen species (ROS) and impaired antioxidant defense mechanisms promote osteoblast apoptosis, enhance osteoclast differentiation, and impair bone remodeling (Marques-Carvalho et al., 2023; Zhang et al., 2023). These processes lead to thinning of trabecular bone, loss of trabecular connectivity, and reduced bone mineral density (BMD) (Giulia et al., 2019). Therefore, agents with antioxidant and anti-inflammatory properties have gained increasing attention as potential therapeutic strategies for the prevention or attenuation of OP-related bone damage (Marcucci et al., 2023).

Betulinic acid (BA) is a naturally occurring pentacyclic triterpenoid found in various plant species, particularly in the bark of birch trees (Ríos & Máñez, 2017). BA has been reported to exhibit a wide range of pharmacological properties, including antioxidant, anti-inflammatory, anti-apoptotic, and osteoprotective effects (Lao et al., 2025; Wei et al., 2020). Experimental studies have demonstrated that BA can modulate OS pathways, suppress inflammatory signaling, and influence bone metabolism by regulating osteoblast and osteoclast activity (Choi et al., 2016; Huang et al., 2021). However, data regarding the effects of BA on bone microarchitecture and histopathological changes in estrogen-deficient conditions remain limited.

In this context, the present study aimed to investigate the histopathological effects of BA on femoral bone tissue in ovariectomized rats. BMD was assessed using dual-energy X-ray absorptiometry (DEXA) together with histological evaluation to provide a comprehensive assessment of BA's protective effects against OVX-induced bone loss. Serum calcium levels were evaluated as a general indicator of systemic mineral homeostasis in estrogen deficiency–related bone loss (Emmanuelle et al., 2021). Although circulating calcium is not a specific marker of bone turnover, alterations in bone remodeling may interact with systemic calcium regulation through endocrine mechanisms involving parathyroid hormone (PTH) and vitamin D (Mashadiyeva-Bayramova et al., 2025).

The present study was designed to evaluate whether BA administration may attenuate estrogen deficiency–related bone deterioration in an OVX model. Although the osteoprotective effects of BA have been reported in OVX-induced bone loss models, the influence of treatment timing remains insufficiently clarified (Jeong et al., 2020; Lo et al., 2010; Park et al., 2014; Sardar et al., 2022; Wei et al., 2020). In particular, it is unclear whether BA is more effective when administered prior to estrogen deprivation or after the establishment of OVX-related skeletal alterations. Therefore, the present study compared

preventive BA administration before OVX (BA+OVX) with therapeutic administration initiated after OVX (OVX+BA). Bone changes were assessed using densitometric and histopathological analyses in order to evaluate structural outcomes such as BMD and trabecular architecture. The study was primarily focused on structural skeletal effects rather than on direct investigation of OS–related molecular mechanisms.

Methods

Animals

This study included 32 female Wistar albino rats (3 months old, 200–250 g) obtained from the Inonu University Laboratory Animals Research Center. Animals were housed under controlled conditions (21 ± 2 °C, $60 \pm 5\%$ humidity) with a 12:12 h light–dark cycle and had free access to standard pellet chow and tap water. Rats were randomly assigned to experimental groups, and all data collection and analyses were performed by investigators blinded to group allocation to minimize bias. All procedures complied with the National Institutes of Health guidelines for animal research and the ARRIVE guidelines (Percie du Sert et al., 2020). The experimental protocol was approved by the Inonu University Faculty of Medicine Ethics Committee on Animal Research (approval no: 2016/A-47) on March 29, 2016.

Chemicals

BA (CAS Number: 472-15-1, Sigma-Aldrich, St. Louis, MO, USA), ketamine (Ketasol 10% w/v injectable solution; Richter Pharma Ag, Wels, Austria), xylazine (XylazinBio 2% injectable solution, Bioveta PLC, Ivanovice na Hané, Czech Republic) and povidone-iodine 10% w/v (Batticon antiseptic solution, ADEKA Pharmaceuticals, İstanbul, Türkiye) were purchased.

Experimental Design

Wistar albino female rats were divided into 4 groups (n=8).

Sham Group: OVX was not performed on the rats in this group; instead, a physiological saline containing 5% dimethyl sulfoxide (DMSO), used as a vehicle solution, was administered orally once daily at a volume of 1 mL for 28 days. Rats in this group underwent a sham surgical procedure under anesthesia. A midline abdominal incision was performed, and the ovaries were exposed but not removed. The incision was then sutured.

OVX Group: Rats that underwent bilateral OVX were administered orally a single daily dose of 1 mL of the vehicle solution for 28 days.

BA+OVX Group: After administering a single daily oral

dose of 3 mg/kg BA to rats for 28 days, bilateral OVX was performed and the rats were observed for 28 days.

OVX+BA Group: After bilateral OVX, rats were administered 3 mg/kg of BA orally once daily for 28 days.

The post-OVX observation period was limited to 28 days to focus on early skeletal alterations associated with estrogen deficiency, as rapid trabecular deterioration in OVX rat models is known to occur within the first 1–4 weeks after surgery (Laib et al., 2001). The experimental design was intended to distinguish between preventive and therapeutic BA administration strategies. In the BA+OVX group, BA treatment was initiated prior to OVX and continued throughout the experimental period to evaluate its potential prophylactic effect against estrogen-deficiency-induced bone loss. In contrast, in the OVX+BA group, BA administration was initiated after OVX to assess its therapeutic potential once estrogen deficiency had been established.

The dosage and route of administration of BA were referenced from a previous study (Bilgiç et al., 2022). The body weights of the rats were measured at the beginning and end of the experiment. To create the OVX model, anesthesia was induced by administering 75 mg/kg ketamine and 8 mg/kg xylazine intraperitoneally to the rats. The abdominal region of the rats was disinfected with a 10% povidone-iodine solution prior to bilateral OVX. The incision was closed with 3.0 silk sutures after OVX.

On the 29th day of the OVX procedure, bone density was measured in the pelvic region of rats under ketamine/xylazine anesthesia using a DEXA device. BMD was measured using DEXA device (Osteosys Primus®, OsteoSys Co., Ltd., Seoul, Republic of Korea). The measurements were performed using the manufacturer-provided software in animal mode to ensure accurate densitometric evaluation in small laboratory animals. Representative DEXA images illustrating pelvic densitometric evaluation in rats are shown in Figure 1. Intracardiac blood was collected from anesthetized rats, after which they were euthanized by surgical exsanguination. Femur tissues were collected and analyzed histopathologically. BMD was measured using DEXA because the pelvic region contains a substantial trabecular bone component and provides a reliable indicator of systemic bone mineral changes in OVX models (Anish et al., 2023). For histopathological evaluation, femoral tissue was selected since the distal femur represents a standard site for assessing trabecular bone microarchitecture in experimental OP studies (Shaheen et al., 2021). This combined approach allowed both quantitative assessment of BMD and detailed evaluation of trabecular microstructural changes. At the end of the experimental period, intracardiac blood samples were collected under

ketamine/xylazine anesthesia. Blood samples were transferred into plain tubes and centrifuged at 2500 rpm for 7 min to separate the serum. The separated serum samples were stored at -80°C until analysis. Serum calcium levels were measured at the Central Laboratory of Inonu University Turgut Ozal Medical Center using an automated biochemical analyzer in accordance with the manufacturer's instructions.

Histopathological Analyses

The femoral head obtained at the end of the experiment was fixed in 10% formaldehyde. After undergoing decalcification and tissue tracking procedures, sections 4–5 μm thick were taken from the prepared paraffin blocks. Hematoxylin-eosin (H-E) staining was applied to the sections to determine the general morphological structure.

Histological evaluations were performed to determine thinning of the bone trabeculae in the metaphysis and reduction in the connections between the trabeculae. Histological changes were scored according to their prevalence: 0: none, 1: less than 25%, 2: 26–50%, 3: more than 50% change. Evaluations were performed using a Leica DFC-280 research microscope and the Leica Q Win Image Analysis System (Leica Micros Imaging Solutions Ltd., Cambridge, UK).

Statistical Analyses

The bioinformatics software developed by the Department of Biostatistics and Medical Informatics at Inonu University was used in the analyses (Arslan et al., 2018). The normality of the data distribution was assessed using the Shapiro–Wilk test. Variables showing normal distribution, such as pelvic BMD, were analyzed using one-way analysis of variance (ANOVA) followed by Tukey's post-hoc test for pairwise comparisons. Variables that did not follow a normal distribution, including body weight, serum calcium levels, and histopathological scores, were analyzed using the Kruskal–Wallis test, followed by Conover pairwise comparisons. These post-hoc procedures inherently account for multiple comparisons. A p -value $< .05$ was considered statistically significant. Data were presented as mean $\pm$ standard deviation for normally distributed variables and as median (minimum–maximum) for non-normally distributed variables.

Results

Rat Body Weights

As shown in Table 1, the initial body weights of the rats were comparable among the Sham, OVX, BA+OVX, and OVX+BA groups, and no statistically significant difference was observed at baseline ($p=.295$). Similarly, although a numerical increase in final body weight was noted in the

Table 1.*Changes in the weight of rats*

Parameters*	Groups				<i>p</i>
	Sham	OVX	BA+OVX (Preventive treatment group)	OVX+BA (Therapeutic treatment group)	
Initial body weight of rats (g)	212 (200-245)	218 (203-242)	221 (189-258)	218 (191-257)	.295
Final body weight of rats (g)	231 (213-247)	247 (210-288)	250 (225-263)	227 (166-288)	.096

No statistically significant difference was observed between the groups.

*: Variables are summarized as 'median (min-max)'.

Table 2.*Pelvic dual energy x-ray absorptiometry results*

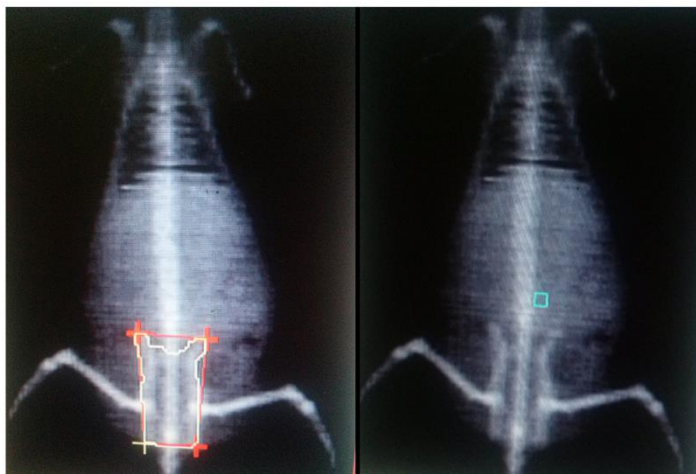
Parameters*	Groups			
	Sham	OVX	BA+OVX (Preventive treatment group)	OVX+BA (Therapeutic treatment group)
Pelvic bone area density (g/cm ²)	0.348 ± 0.059	0.29 ± 0.047	0.363 ^a ± 0.037	0.336 ± 0.048

^a: There is a statistically significant difference compared to the OVX group ($p < .05$).

*: Variables are summarized as 'arithmetic mean ± standard deviation'.

Figure 1.

Image showing the examination of pelvic densitometry in rats using a DEXA device



OVX and BA-treated OVX groups compared with the Sham group, these differences did not reach statistical significance ($p = .096$). Overall, neither OVX nor BA administration produced a significant effect on body weight changes during the experimental period.

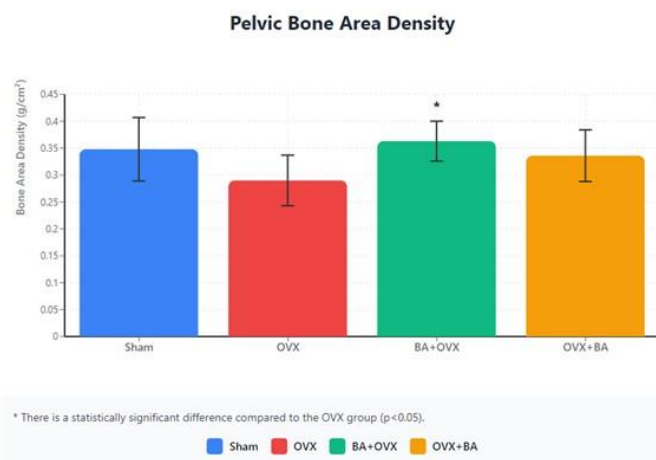
Pelvic Dual Energy X-Ray Absorptiometry Results

Pelvic DEXA analysis findings presented in Figure 2. Specifically, pelvic BMD values were as follows: Sham group (0.348 ± 0.059 g/cm²), OVX group (0.290 ± 0.047 g/cm²),

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Figure 2.

Pelvic bone area density of experimental groups.



* There is a statistically significant difference compared to the OVX group ($p < 0.05$).

BA+OVX group (0.363 ± 0.037 g/cm²), and OVX+BA group (0.336 ± 0.048 g/cm²). Pelvic DEXA analysis demonstrated a pronounced decrease in pelvic BMD in the OVX group compared with the Sham group (Table 2). In contrast, BA treatment significantly mitigated OVX-induced bone loss, as indicated by a significantly higher pelvic BMD in the BA+OVX group compared with the OVX group ($p < .05$; Table 2). Although the OVX+BA group exhibited numerically increased pelvic bone density values relative to the OVX group, this difference did not reach statistical significance.

Table 3.*Changes in serum calcium levels*

Parameters*	Groups			
	Sham	OVX	BA+OVX (Preventive treatment group)	OVX+BA (Therapeutic treatment group)
Calcium levels (mg/dL)	9.50 (8.50-10.20)	9.20 (8.50-10.30)	8.95 (8.70-9.50)	9.15 (8.40-10.40)

No statistically significant difference was observed between the groups.

*: Variables are summarized as 'median (min-max)'.

Table 4.*Histopathological score results for each group.*

Parameters	Sham Group	OVX Group	BA+OVX Group (Preventive treatment group)	OVX+BA Group (Therapeutic treatment group)
Histopathological score	0.0 (0.0 – 1.0)	1.0 (1.0 – 2.0) ^a	1.0 (0.0 – 1.0) ^{b,c}	1.0 (1.0 – 2.0)

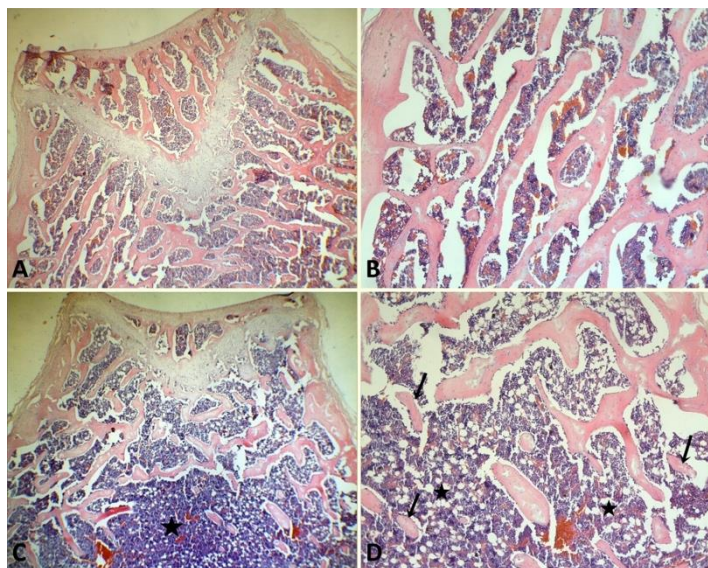
^a Statistically significant increase compared to the Sham group ($p=0.003$).

^b Statistically significant decrease compared to the OVX group ($p=0.031$).

^c Statistically significant decrease compared to the OVX+BA group ($p=0.010$).

*: Variables are summarized as 'median (min-max)'.

Figure 3. In the sham group (A and B), trabecular bone with a normal histological appearance is observed in the metaphysis region. In the OVX group (C and D), a decrease in bone trabeculae (stars) and disruption of trabecular connections (arrows) are observed. A and C; 4x, B and D; 10x.

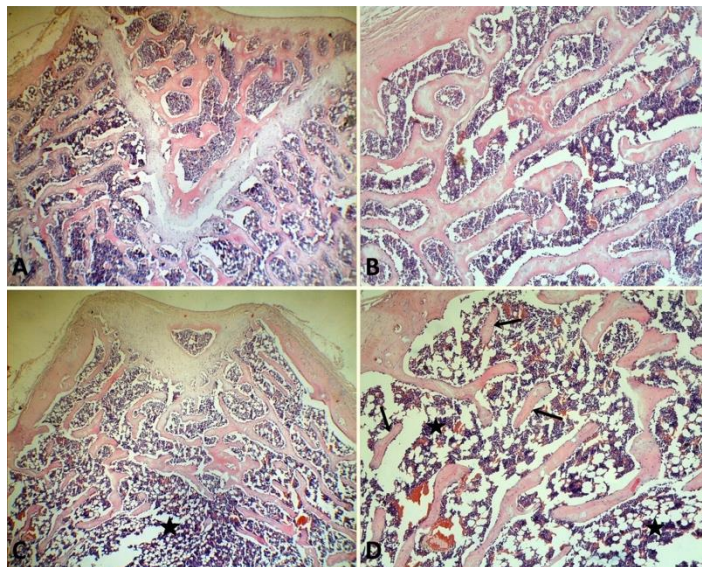


Serum Calcium Levels

Serum calcium levels were measured to provide a systemic indicator of mineral metabolism in the context of OVX-induced bone loss (Anish et al., 2023). Although serum calcium is not a specific marker of bone turnover, it can reflect alterations in calcium homeostasis associated with increased bone resorption and was included to complement the densitometric and histopathological

Figure 4.

In the BA+OVX (preventive) group (A and B), the trabecular bone structure exhibits a normal histological appearance similar to that of the sham group. In the OVX+BA (therapeutic) group (C and D), the histological changes observed in the OVX group continue. Stars indicate areas without bone trabeculae, and arrows indicate thinned bone trabeculae. A and C; 4x, B and D; 10x.



evaluation of bone changes. Therefore, in OVX models, serum calcium can be reported as a complementary indicator of systemic calcium homeostasis, but densitometry, histopathology, and dedicated bone turnover markers are required to characterize bone changes (Guo et al., 2022; Yousefzadeh et al., 2020). As

presented in Table 3, serum calcium levels were comparable among the Sham, OVX, BA+OVX, and OVX+BA groups, with no statistically significant differences observed between the groups. Although a slight numerical decrease in calcium levels was noted in the OVX and BA-treated OVX groups compared with the Sham group, these variations did not reach statistical significance. Overall, neither OVX nor BA administration significantly affected systemic serum calcium levels during the experimental period.

Histopathological Findings

In the Sham group, the bone tissue in the trabecular structure was observed to have a normal histological appearance, with continuous connections between the trabeculae (Figure 3A and B). In the OVX group, compared to the Sham group, there was a marked thinning and reduction in bone trabeculae, along with disruption of trabecular connections ($p=.003$) (Figure 3C and D). On the other hand, in the BA+OVX group, histological changes in trabecular bone associated with OVX were found to be significantly reduced (Figure 4A and B) ($p=.031$). However, in the OVX+BA group, similar to the OVX group, a decrease in trabeculae and disruption of trabecular connections were observed (Figure 4C and D). The difference between the BA+OVX and OVX+BA groups in terms of histological changes was found to be statistically significant ($p=.010$). Specifically, histopathological scores were as follows: Sham group [0.0 (0.0–1.0)], OVX group [1.0 (1.0–2.0)], BA+OVX group [1.0 (0.0–1.0)], and OVX+BA group [1.0 (1.0–2.0)]. The histopathological score results for the groups are presented in Table 4.

Discussion

A key aspect of the present study is the direct comparison between preventive and therapeutic administration of BA in an estrogen-deficient model. While previous studies have demonstrated the osteoprotective properties of BA in OVX models, most of these investigations primarily focused on its ability to attenuate ongoing bone loss (Sardar et al., 2022; Wei et al., 2020). In contrast, our experimental design allowed us to differentiate between preventive administration before estrogen deprivation and therapeutic administration after the establishment of estrogen deficiency. The findings indicate that BA is significantly more effective when administered prior to OVX, suggesting that its primary benefit lies in preventing early osteoporotic changes rather than reversing established bone loss.

The present findings indicate that OVX is associated with deterioration in bone microarchitecture and a reduction in pelvic BMD, suggesting the development of an estrogen-deficient OP-like condition in this experimental model.

Consistent with previous reports, OVX was accompanied by thinning of trabecular bone, loss of trabecular connectivity, and decreased bone density. Similar structural alterations have been widely reported in OVX animal models, including thinner trabeculae, disrupted connectivity, widened trabecular spaces, and reduced BMD. In rat long bones and vertebrae, OVX has been associated with decreased bone volume fraction (BV/TV), trabecular number (Tb.N), and connectivity density (Conn.D), together with increased trabecular separation (Tb.Sp) and structure model index (SMI), reflecting a shift from plate-like to rod-like trabecular architecture (Guo et al., 2022; Li et al., 2021; Liu et al., 2015; Rocabado et al., 2018). Early after OVX, micro-computed tomography (μ CT) and histological analyses have been reported to show sparse loss of trabecular interconnectivity and widening of intertrabecular spaces, accompanied by progressive thinning and occasional disruption of trabeculae (Guo et al., 2022; Lei et al., 2025). In a systemic rat study, BMD reductions of approximately 70–75% at the tibia and femur and about 37% in the spine were reported by 36 weeks, accompanied by pronounced trabecular deterioration in long bones, the spine, and the ilium (Liu et al., 2015). Rapid trabecular loss has been reported during the first 4–10 weeks following OVX, after which the rate of bone loss appears to slow. In later stages, some secondary trabecular thickening and mineralization may occur, which could partially compensate the remaining trabecular structures, although the original trabecular architecture is generally not fully restored (Li et al., 2021; O'Sullivan et al., 2019; Roberts et al., 2019). Across different species and skeletal sites, OVX-induced estrogen deficiency has been reported to be associated with thinning of trabecular bone, loss of trabecular connectivity, and reductions in trabecular bone density, reproducing several structural features observed in postmenopausal OP.

The absence of significant differences in BMD between OVX and Sham groups may be related to the relatively early post-OVX observation period. In 3-month-old rats, femur BMD at 4 weeks post-OVX is not significantly decreased vs. Sham, but a clear deficit appears by 8 and 12 weeks (Lo et al., 2010). Another rat study found femur BMD differences emerging at 6 weeks and becoming stronger at 8 weeks post-OVX vs. Sham (Park et al., 2010). μ CT work shows that some trabecular parameters differ as early as 4 weeks, but others only from 12 weeks onward (Guo et al., 2022). In a systematic age–site–time analysis, the proximal tibia showed the earliest and largest OVX–Sham differences; effects depended on both skeletal site and weeks post-OVX (Francisco et al., 2011). In experimental OVX models, trabecular microarchitectural alterations may occur before pronounced densitometric changes become detectable (Boyd et al., 2006; Guo et al., 2022). Furthermore,

circulating calcium levels are tightly regulated by endocrine mechanisms and may therefore remain within normal ranges despite structural bone alterations. Reviews highlight that when body calcium is low, regulatory hormones pull calcium from bone to protect other organs, underscoring bone's role as a "buffer" for plasma calcium (Hurwitz, 1996; Spiardi & Geara, 2022; Wawrzyniak & Balawender, 2022).

One of the principal observations of this study is that BA administration prior to OVX is associated with protective effects on bone tissue. The BA+OVX group showed relatively preserved pelvic BMD and improved histopathological architecture compared with the OVX group. These findings suggest that BA contributes to increased resistance of bone tissue to estrogen deficiency-related alterations. Although the present study did not directly evaluate molecular pathways, previous OVX studies indicate that estrogen deficiency is associated with early increases in OS, inflammatory cytokines, and disrupted bone remodeling that may contribute to skeletal fragility (Hsu et al., 2024). Previous studies suggest that BA may influence bone metabolism through multiple pathways (Park et al., 2014; Sardar et al., 2022). Although OS and inflammatory signaling pathways have been implicated in OVX-induced bone loss and have been reported to be influenced by BA in previous studies, these parameters were not directly evaluated in the present study. Therefore, the discussion of OS- and inflammation-related pathways should be considered as providing biological context rather than direct mechanistic evidence derived from the current experimental findings. Previous studies have reported that oral BA may attenuate bone loss in OVX mice, with improvements in trabecular morphometry and normalization of serum bone turnover markers, suggesting reduced excessive remodeling under conditions of estrogen deficiency. In experimental models combining cancer and estrogen deficiency, BA has also been reported to reduce osteolytic signaling, inhibit RANKL-induced osteoclastogenesis and resorption, and attenuate bone loss in both tumor-inoculated and OVX mice. (Park et al., 2014). Previous studies have reported that BA formulated as peroxisome proliferator-activated receptor gamma (PPAR- γ)-inhibiting nanoparticles may promote osteoblastogenesis and suppress adipogenic differentiation of bone marrow stromal cells through activation of wingless-related integration site (Wnt)/ β -catenin signaling pathway. These effects have been associated with reduced bone marrow adiposity and attenuation of OVX-induced BMD loss in experimental models (Sardar et al., 2022).

In contrast, BA administration initiated after OVX (OVX+BA group) does not significantly reverse bone loss or histological deterioration under the conditions tested,

suggesting that BA may be more effective when administered prior to OVX rather than after the establishment of estrogen deficiency. Consistent with this observation, many experimental studies have investigated BA primarily in preventive designs targeting OVX- or inflammation-induced bone loss rather than late-stage reversal (Wei et al., 2020). Previous studies have also reported that BA may attenuate inflammatory bone loss induced by lipopolysaccharide (LPS), primarily through suppression of receptor activator of nuclear factor- κ B ligand (RANKL)-driven osteoclast formation and function rather than through direct stimulation of bone regeneration. (Jeong et al., 2020). Similar preventive anti-resorptive effects have also been reported when BA is used to attenuate bone loss associated with breast cancer bone metastasis and estrogen deficiency in mouse models (Park et al., 2014). Although the present study did not directly evaluate molecular signaling pathways, previous experimental studies suggest that BA may influence bone metabolism through mechanisms involving RANKL-mediated osteoclastogenesis, Wnt/ β -catenin signaling, ROS regulation. Therefore, these pathways should be considered potential mechanisms that may contribute to the osteoprotective effects of BA rather than mechanisms directly demonstrated in the present study. Future studies incorporating molecular analyses will be necessary to confirm whether these signaling pathways are involved in BA-mediated bone protection.

The DEXA findings were generally consistent with the histopathological observations. In the BA+OVX group, preservation of pelvic BMD was accompanied by relatively maintained trabecular thickness and connectivity, whereas the OVX and OVX+BA groups showed comparable reductions in both densitometric and microscopic bone parameters. This concordance may support the combined use of densitometric and histological assessments in experimental OP studies. Consistent with these observations, previous studies in ovariectomized mice have reported that BA may attenuate bone loss and help preserve trabecular architecture, with μ CT analyses demonstrating improvements in BV/TV, Tb.Th, and Tb.N compared with untreated OVX controls (Wei et al., 2020). Previous studies have reported that BA nanoparticles in OVX rats may attenuate reductions in BMD and reduce bone marrow adiposity. μ CT and histological analyses in these models have also suggested improvements in trabecular structure following treatment (Sardar et al., 2022). Experimental evidence indicates that BA may reduce osteoclast formation and activity in several models, which has been associated with preservation of bone mass and structure in OVX mice and inflammatory bone loss models (Jeong et al., 2020; Park et al., 2014; Wei et al., 2020).

Serum calcium levels were evaluated as a general indicator of systemic mineral metabolism rather than a specific marker of bone turnover. Interestingly, serum calcium levels did not differ significantly among the experimental groups. This observation may indicate that OVX-related bone alterations and the effects observed with BA were not reflected in measurable changes in circulating calcium under the conditions of the present study. In many OVX animal models, bone loss has been associated with alterations in systemic calcium balance and calciotropic hormones, suggesting an interaction between skeletal remodeling and systemic calcium regulation. However, circulating calcium levels are tightly regulated physiologically, and therefore may remain within a relatively narrow range despite structural bone changes. Consistent with this, OVX has also been reported to influence serum calcium levels and urinary calcium excretion, reflecting systemic calcium regulation rather than a purely local bone effects (Aeimlapa et al., 2019; Franco et al., 2024; Mao et al., 2021). Dietary calcium availability has been reported to influence OVX-induced bone loss. In experimental models, low-calcium diets may exacerbate OVX-related skeletal deterioration, whereas calcium supplementation has been associated with partial attenuation of OVX-related BMD decline and improvements in trabecular architecture, suggesting the involvement of systemic calcium supply and regulation. (Dereka et al., 2025; Mao et al., 2021).

Similar observations have been described in OVX models, where marked bone microarchitectural changes may occur without detectable alterations in circulating calcium levels. Across OVX rodent and large-animal models, early trabecular deterioration has been widely reported, whereas circulating calcium often remains within normal ranges due to compensatory endocrine regulation. In many of these studies, bone turnover markers rather than serum calcium levels are emphasized as indicators of OVX-related skeletal alterations (Campbell et al., 2008; Guo et al., 2022; Yoon et al., 2012). Methodological reviews of OVX models indicate that OP in OVX rats is commonly verified by reductions in BMD and trabecular indices within approximately 14–60 days after OVX. In contrast, circulating calcium levels are often reported to remain within normal ranges, likely due to compensatory hormonal regulation involving PTH and vitamin D, despite the presence of skeletal bone loss (Yousefzadeh et al., 2020). In a femoral-head OVX rat study, reductions in local bone calcium content together with microstructural damage were reported. In the same model, serum calcium levels in OVX groups were observed to be higher than those in Sham controls, illustrating that structural bone alterations may occur without parallel hypocalcemia (Atwa et al., 2021).

The osteoprotective effects observed in the BA+OVX group may be related to the reported antioxidant and anti-inflammatory properties of BA. Estrogen deficiency has been associated with increased ROS production, enhanced osteoblast apoptosis, and stimulation of osteoclastogenesis (Kim et al., 2020; Manolagas, 2010; Marques-Carvalho et al., 2023). Elevated ROS levels have been reported to activate mitochondrial apoptotic pathways and contribute to osteoblast and osteocyte apoptosis. In contrast, estrogen signaling, including estrogen receptor-dependent pathways, has been associated with suppression of ROS production, enhancement of antioxidant enzyme activity, and reduced apoptosis in osteoblasts and osteocytes (Manolagas, 2010; Yang et al., 2022). Estrogen deficiency has been associated with increased levels of pro-inflammatory cytokines, enhanced T-cell activation, and upregulation of RANKL expression in stromal cells, T cells, B cells, and osteocytes. These changes may contribute to amplified osteoclastogenesis, potentially involving ROS-dependent signaling mechanisms (Cheng et al., 2022; Fischer & Haffner-Luntzer, 2021; Marcucci et al., 2023; Weitzmann & Pacifici, 2006; Yang et al., 2022). Although molecular markers were not assessed in the present study, the histological preservation observed supports a role for BA in mitigating oxidative and inflammatory bone damage.

Several limitations of this study should be acknowledged. First, molecular analyses evaluating OS markers, inflammatory cytokines, and osteoclast/osteoblast signaling pathways were not performed. Second, only a single BA dose (3 mg/kg) and treatment duration were evaluated, which limits conclusions regarding dose dependency and long-term effects. In addition, classical indicators confirming OVX-induced estrogen depletion, such as uterine weight, circulating estrogen levels, or biochemical bone turnover markers, were not assessed. Although pelvic BMD reduction and trabecular microarchitectural deterioration indicated OVX-related bone loss, the inclusion of endocrine and biochemical markers would have strengthened model validation.

Furthermore, the post-OVX observation period was limited to 28 days, which likely represents an early stage of estrogen-deficiency-induced bone loss (Laib et al., 2001). Longer experimental durations may lead to more pronounced trabecular deterioration and provide additional insight into the long-term effects of BA treatment. The absence of significant differences in systemic parameters such as serum calcium may reflect the tight physiological regulation of calcium homeostasis rather than the absence of skeletal alterations. Accordingly, the interpretation of BA's protective effects in this study is primarily based on densitometric and histopathological findings rather than systemic calcium levels.

It should also be considered that initiating BA administration prior to OVX in the BA+OVX group may introduce potential confounding effects by influencing bone metabolism before estrogen depletion occurs. However, this design was intentionally employed to distinguish between preventive and therapeutic administration strategies. Future studies incorporating molecular endpoints, longer observation periods, multiple dosing regimens, and additional OVX validation parameters would help clarify the mechanisms underlying BA's osteoprotective effects and further define its preventive versus therapeutic potential in estrogen deficiency-related bone loss.

Conclusion and Recommendations

The present findings suggest that BA administration prior to OVX attenuates OVX-induced bone loss and help preserve both BMD and trabecular microarchitecture. In contrast, BA administration initiated after OVX did not produce comparable effects under the conditions of the present study, which may indicate a greater benefit in preventive settings. These findings suggest that BA could represent a potential natural compound of interest for the prevention of postmenopausal OP; however, further studies are required to clarify its underlying mechanisms and possible clinical relevance.

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Conflict of Interest: The author of this article, Hakan Parlakpınar, was a member of the journal's Editorial Board at the time the manuscript was submitted. This situation constitutes a potential conflict of interest. A double-blind peer-review process was implemented, and his role was not disclosed to the reviewers. Members of the Editorial Board do not have decision-making authority regarding manuscripts. All stages of the evaluation process were conducted in accordance with the journal's ethical policies and international ethical guidelines, including those of COPE and ICMJE.

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