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Research Article
Potential Effects of Oligofructose-Enriched Inulin Macromolecules (Prebiotin™) on an Ovariectomy-Induced Osteoporotic Rat Model

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Abstract

Background and Objective: Osteoporosis is the most common disease, particularly among old-age women. This study aims to examine the potential role of Prebiotin™ macromolecule on the treatment of osteoporotic female rats induced by ovariectomy and compare its effect with estradiol benzoate drug. **Materials and Methods:** A total of 50 g Prebiotin™/kg diet/day was added to an experimental ovariectomy female rat diet and estradiol benzoate drug was injected subcutaneously to another osteoporotic group at a dose of 30 g kg^{-1} b.wt. Bone mineral density, bone mineral content and bone ash mineral contents were estimated for the examined rats. Also, serum ALP, RANKL, OPG and osteocalcin levels were determined. Moreover, histopathological, body and uterus weight alterations were detected among different studied groups. **Results:** Supplementing an ovariectomized rat model with Prebiotin™ could significantly restore the reduced minerals absorption, bone mineral contents and densities, bone histological features and bone chemical composition, with a simultaneous increase in serum OPG level and decrease in serum RANKL, ALP and osteocalcin levels that resulted in regaining the balance of bone remodelling and turnover process. **Conclusion:** Prebiotin™ was a safe agent that had a therapeutic capacity against ovariectomy-induced osteoporosis in a female rat model and can be used as an adjunct for calcium supplementation to reduce bone loss in old age people.

Key words: Osteoporosis, ovariectomy, prebiotics, osteocalcin, serum ALP, postmenopausal, homeostasis

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Competing Interest: The authors have declared that no competing interest exists.

Data Availability: All relevant data are within the paper and its supporting information files.

INTRODUCTION

Osteoporosis (OP) is a persistent progressive systemic skeletal disease marked by decreased bone density and degradation of bone micro-architecture with increased bone fragility and fracture risk[1]. It is a crucial public health problem as it is estimated to affect 200 million women worldwide and results in more than 8.9 million fractures each year[2].

Hormone Replacement Therapy (HRT) using estrogen is considered the first-line strategy for the prevention and treatment of postmenopausal osteoporosis as it acts as an inhibitor of bone resorption[3]. However, many human trials and animal studies have shown that HRT as 17β-estradiol has potential risks and side effects depending on the given dose and duration of treatment period[4].

Concerning postmenopausal women, estrogen depletion changes the intestinal microbial composition and structure, leading to reduced microbial diversity, which is represented by decreased firmicutes populations, including *Clostridium* clusters IV and XIVa that possess immune regulatory effects as they boost the formation of regulatory T cells (Tregs), enhance their function and sustain immune homeostasis[5]. Hence, alteration of gut microbiota by prebiotics supplementations into a healthier microbiome has been proposed as a possible therapeutic strategy to benefit bone health and provide a promise that the intestinal microbiota may serve as a potential therapeutic tool for treating postmenopausal OP[6].

The concept of prebiotics was introduced in 1995 by Gibson and Roberfroid[7], who defined prebiotics as "a non-digestible food constituent that affects the host in a good way by selectively encouraging the growth and/or activity of one or a finite number of colonic bacteria as a result, boosts the health of host".

Firstly, for a substance to be considered a prebiotic it must be a non-digestible one so most prebiotics is found in the form of non-digestible oligosaccharides as a result, these compounds can't be absorbed in the upper gastrointestinal tract. Secondly, prebiotics must reach the colon where they undergo fermentation, act as substrates and selectively stimulate the growth or/and activity of the good endogenous bacteria like *Lactobacilli* and *Bifidobacteria* and suppress the growth of pathogenic bacteria[8]. Thirdly, a prebiotic must be able to resist the conditions of food processing such as heat and low pH so as not to be degraded or chemically changed and be available for bacterial action and metabolism in the intestine. Finally, through prebiotic fermentation, end products as Short-Chain Fatty Acids (SCFAs) (acetate, propionate and butyrate) must be produced so as for the host to be benefited from the provided energy and essential micronutrients[9].

Prebiotin™ is a prebiotic fibre supplement providing a full-spectrum prebiotic known as oligofructose-enriched-inulin (OEI) or Synergy 1 (SYN 1) extracted from chicory roots and purchased from Jackson GI Medical, USA. OEIs are considered functional food constituents because it is proven to affect the physiological and biochemical processes in many studies carried out on human and animals, leading to better health[10].

Therefore, this study aimed to find out the therapeutic effects of Prebiotin™ against induced postmenopausal osteoporosis by using an ovariectomized rat model.

MATERIALS AND METHODS

Study area: The study was carried out at the Department of Biochemistry, Alexandria University, Egypt from February, 2018 to July, 2019.

Materials: Calcium citrate (CAL-MAG®) was purchased from Hochster Pharmaceutical Industries Co., Egypt. Ketamine hydrochloride (50 mg mL^{-1}) was purchased from UMEDICA Laboratories Pvt., Ltd., India. Xylazine (Xyla-Ject®) was purchased from ADWIA Pharmaceuticals Co., Egypt. Estradiol benzoate (Folone®) was purchased from Misr Company for pharmaceutical industries Co., Egypt. Prebiotin Prebiotic Fiber (Prebiotin™) was purchased from Jackson GI Medical, USA.

Alkaline Phosphatase (ALP) kit was purchased from Biodiagnostic, Egypt. Inorganic phosphorus, calcium, Alanine Aminotransferase (ALT), Aspartate Aminotransferase (AST) and total protein kits were purchased from Spectrum Diagnostics, Egypt. Creatinine and urea kits were purchased from Diamond Diagnostics, Egypt. RANKL ELISA kit was purchased from Shanghai Sunred Biological Technology Co., China. OPG ELISA kit was purchased from Elabscience Biotechnology Inc., USA. Rat osteocalcin ELISA kit was purchased from Immutopics Inc., USA.

Methods

Experimental animals: All procedures involving laboratory animals were following the Institutional Animal Care and Use Committee, Alexandria University, Egypt (IACUC protocol No.: 04191021101), concerning the protection of animals used for scientific purposes.

Forty female albino rats weighted (250±18 g) were obtained from the Medical Research Institute, Alexandria University. The rats were allowed free access to tap water and commercially standard rodent food and kept under conventional conditions of temperature, humidity and 12 hrs light/dark cycle throughout the experimental period.

After 1 week adaptation period, the female rats were divided into four groups: A, B, C and D (10 rats/group) as follows: Sham group: Control group, OVX group: Bilaterally ovariectomized group, OVX+E group: Bilaterally ovariectomized+estradiol benzoate and OVX+P group: Bilaterally ovariectomized+Prebiotin™.

The rats in the four groups were anaesthetized with Ketamine hydrochloride (90 mg kg^{-1}, i.p.) and (10 mg kg^{-1}, i.p.). Afterwards, rats of OVX, OVX+E and OVX+P groups underwent bilateral ovariectomy using a double dorsolateral approach[11]. Sham group rats were subjected to a sham operation procedure wherein only a piece of fat around the ovaries was excised. All rats were fed with the same commercially standard rodent food and tap water for 90 days after surgery[12].

After that, for 8 weeks all groups were fed with commercially standard rodent food with limited access (15 g kg^{-1} b.wt.) added to it calcium citrate 10 g kg^{-1} diet (high calcium diet)[13] and had free access to tap water but differ in the following: Sham group: Rats received corn oil daily subcutaneously[14], OVX+E group: Rats were treated with estradiol benzoate, used as a reference drug, at a dose 30 g kg^{-1} b.wt. (it was solubilized in corn oil and administrated daily subcutaneously[14]) and OVX+P group: Rats were treated with Prebiotin™ by adding 50 g kg^{-1} diet/day to their diet[13].

Blood and tissue samples: All rats were sacrificed after 8 weeks of treatment and blood was collected from each rat in a clot activator tube to obtain serum. The collected blood was centrifuged at 3000 rpm for 20 min and the serum was kept at -80°C until further analysis.

The right femurs were rapidly removed and the muscles, ligaments and other tissues were cleaned off, washed with saline and divided randomly into two sections one was used for measuring Bone Mineral Density (BMD) and Bone Mineral Content (BMC) by Dual-Energy X-ray Absorptiometry (DEXA)[15], the other section was fixed in 10% formalin for histopathological analysis[16]. The left femurs were removed and cleaned off adhering soft tissue and used for estimation of bone ash content using the dry ashing method[17].

Assessment of body weights and uterine weights[18]: Animal body weights of each group were measured once a week throughout the treatment period. Weight changes were calculated by using the initial body weights at the beginning of the treatment period and the final body weights immediately before sacrifice. Uteri were carefully removed and cleaned of fat tissue, the adjusted uterus weight was calculated by the given formula:

$$\text{Adjusted uterus weight} = \frac{\text{Weight of uterus}}{\text{Weight of the animal}} \times \text{Average weight of the animal}$$

Determination of routine parameters: Alkaline Phosphatase (ALP) activity[19], total calcium[20], inorganic phosphorus[21], total protein[22], ALT activity[23], AST activity[23], urea level[24] and creatinine level[25] were determined in serum of all examined groups.

Determination of serum RANKL, OPG and Osteocalcin levels: Quantitative measurements of RANKL, OPG and osteocalcin using serum samples were performed using a double-antibody sandwich Enzyme-Linked Immunosorbent Assay (ELISA) technique[26].

Bone Mineral Density (BMD) and Bone Mineral Content (BMC) assay using DEXA[15]: BMD and BMC were determined by DEXA using Norland XR 46, version 3.9.6/2.3.1 instrument equipped with assigned software for small animal measurements in bone mineral density unit at Medical Service Unit, National Research Center, Dokki, Egypt. BMD was calculated from the bone mineral content (BMC) of the measured area. Such a technique provided an integrated measure of the right femur proximal, distal, mid and total areas.

Estimation of bone ash mineral content[17]: The left femurs were extracted 2 times with a 1:1 mixture of ethanol and diethyl ether for 48 hrs and one time with diethyl ether for 24 hrs. The dehydrated and defatted bones were dried for 4 hrs at 105°C in a drying oven (vacuum drying chambers model VDL 53), Germany, then ashed for 24 hrs at 600°C in a muffle furnace (OBERSAL HD-230), Spain. After that, the obtained ash weight was hydrolyzed in 4 mL of 6N HCl and the total volume was brought to 10 mL with distilled water for the determination of calcium, phosphorus and magnesium by inductively coupled plasma optical emission spectrometry (ICP-OES) using Agilent 5100 VDV, in ICP-OES Laboratory, Institute of Graduate Studies and Research, Alexandria, Egypt.

Histological study and H&E staining[16]: Previously prepared right femurs were fixed in 10% formalin for 24 hrs and then were put in nitric acid (2.5%) to soften the bone. The obtained bone samples were dehydrated in ascending series of increasing concentrations of alcohol, infiltrated and embedded in paraffin. Bone samples were then cut into 5 µm sections using an RM Leica 2135 rotary microtome (Wetzlar, Germany) followed by a hematoxylin and eosin staining.

Images were obtained using an Olympus CX31 microscope (Olympus, Japan) and images were captured with an LC-20 digital camera built into the microscope.

Statistical analysis: One-way Analysis of Variance (ANOVA) with Tukey's test for *post hoc* comparisons between individual treatment groups and control was performed using IBM SPSS software package version 20.0. (Armonk, NY: IBM Corp). The values were expressed as Means±SE and values of $p<0.05$ were considered statistically significant.

RESULTS

Effects of Prebiotin™ on serum mineral absorption markers and bone ash mineral contents in OVX rats: The OVX rats showed a significant decrease in serum levels of total calcium and inorganic phosphorus compared to the sham control group. However, a significant increase in the levels of total calcium and inorganic phosphorus was observed in both treated groups compared to the OVX group (Fig. 1).

Furthermore, the measured calcium group (Fig. 2a), phosphorus group (Fig. 2b) and magnesium group (Fig. 2c) concentrations in the femoral bone ash were significantly reduced in OVX compared to sham rats but significantly elevated in both treated groups either by estradiol or Prebiotin™ compared to the OVX.

Effects of Prebiotin™ on serum ALP, osteocalcin, OPG, RANKL levels and RANKL/OPG ratio in OVX rats: The present work showed in Fig. 3a-b that the OVX group led to a significant increase in serum osteocalcin (Fig. 3b), ALP-specific activity (Fig. 3a), In Fig. 4a-c, RANKL activity (Fig. 4a) and RANKL/OPG ratio (Fig. 4c) with a significant decrease in serum OPG levels (Fig. 4b) when compared to sham-control rats. However, OVX rats treated with either Prebiotin™ or estradiol showed a significant decrease in serum osteocalcin, ALP-specific activity, RANKL activity and RANKL/OPG ratio with a significant increase in OPG levels when compared to the OVX group.

Effects of Prebiotin™ on BMD and BMC in OVX rats: The obtained data proved that the OVX group had diminished gain in bone mass, where both BMD (Fig. 5a) and BMC (Fig. 5b) were lower than those of the sham control group with significant differences in all regions. Treating the OVX rats with either Prebiotin™ or estradiol produced remarkable enhancements in both BMC and BMD of the femoral bone. The OVX rats treated with Prebiotin™ showed a significant increase in total and mid-BMD and total, mid and proximal BMC, while estradiol resulted in a significant increase in mid-BMD and total, proximal, distal and mid-BMC values when compared to the OVX rats. Though, estradiol produced a non-significant increase in total and distal BMD values, while Prebiotin™ produced a non-significant increase in distal BMD value compared to the OVX group.

Effects of Prebiotin™ on histopathological alterations in OVX rats: Histopathological changes of different examined groups revealed normal spongy bone architecture made of thick dense bone trabeculae separated by bone marrow

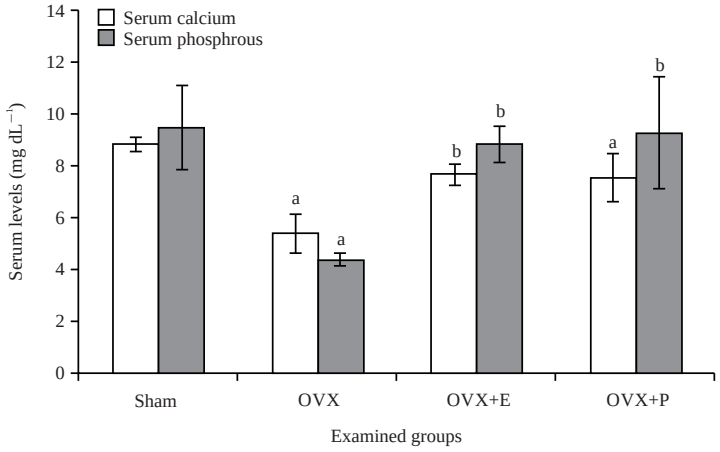

Fig. 1: Serum calcium and inorganic phosphorus levels in different examined groups

Values are expressed as Mean±Standard deviation, [a]Significant difference compared to the sham-operated control group, [b]Significant difference compared to ovariectomized group ($p<0.05$), Sham: Sham-operated control group, OVX: Ovariectomized group, OVX+E: Ovariectomized group treated with estradiol and OVX+P: Ovariectomized group treated with prebiotin

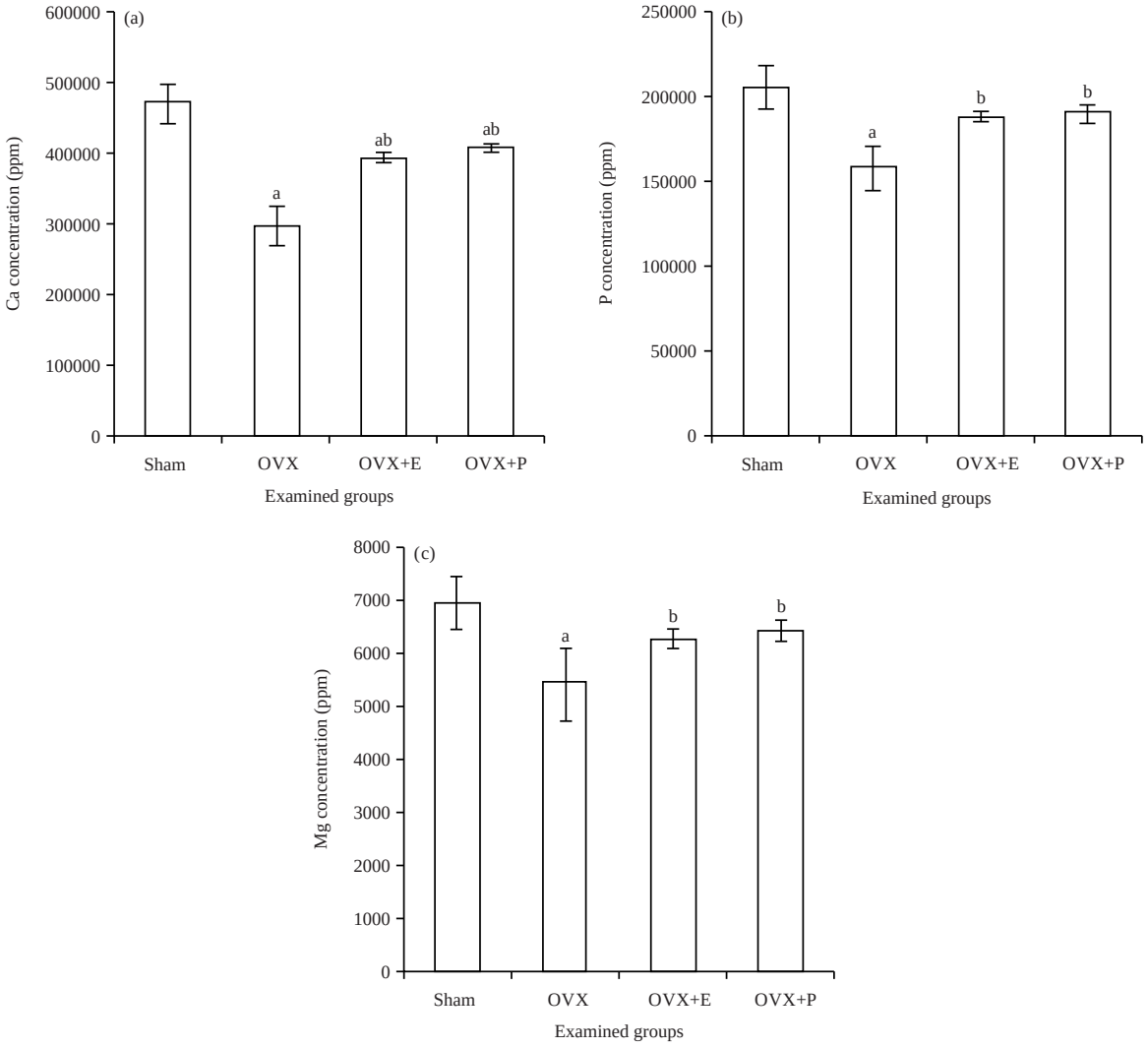

Fig. 2(a-c): Bone ash mineral contents in different examined groups

Values are expressed as Mean±Standard deviation, [a]Significant difference compared to the sham-operated control group, [b]Significant difference compared to ovariectomized group ($p<0.05$), Sham: Sham-operated control group, OVX: Ovariectomized group, OVX+E: Ovariectomized group treated with estradiol and OVX+P: Ovariectomized group treated with Prebiotin

spaces distended with hematopoietic cells and several fatty cells in the sham-operated control group (Fig. 6a). While, severe bone resorption in the form of marked thinning, sparse bone trabeculae and incomplete trabecular structure with disordered trabecular arrangement with an increased number of fatty cells were seen in the ovariectomized group (Fig. 6b). Meanwhile, the estradiol treated group showed thicker and more organized bone trabeculae with an increased number of hematopoietic cells and a reduced number of fatty cells compared with the OVX group (Fig. 6c). Although, the morphological structures of the femoral trabeculae were improved, where most of the bone trabeculae were thicker and more homogenous, some were thin and non-homogenous enclosing bone marrow having fewer fatty cells than the OVX group were seen in the ovariectomized group treated by Prebiotin™ (Fig. 6d).

Effects of Prebiotin™ on uterus weights in OVX rats: Both measured adjusted uterus weights (Fig. 7) and the live images of the bicornuate uterus in Fig. 8a-d revealed a significant increase in the uterine weights of the estradiol-treated group (Fig. 8c) if compared with the Prebiotin™-treated group (Fig. 8d), ovariectomized group (Fig. 8b) and sham-control group (Fig. 8a). On the other hand, there was a significant decrease in the uterine weights of both ovariectomized group and ovariectomized group treated with Prebiotin™ compared to the sham control group.

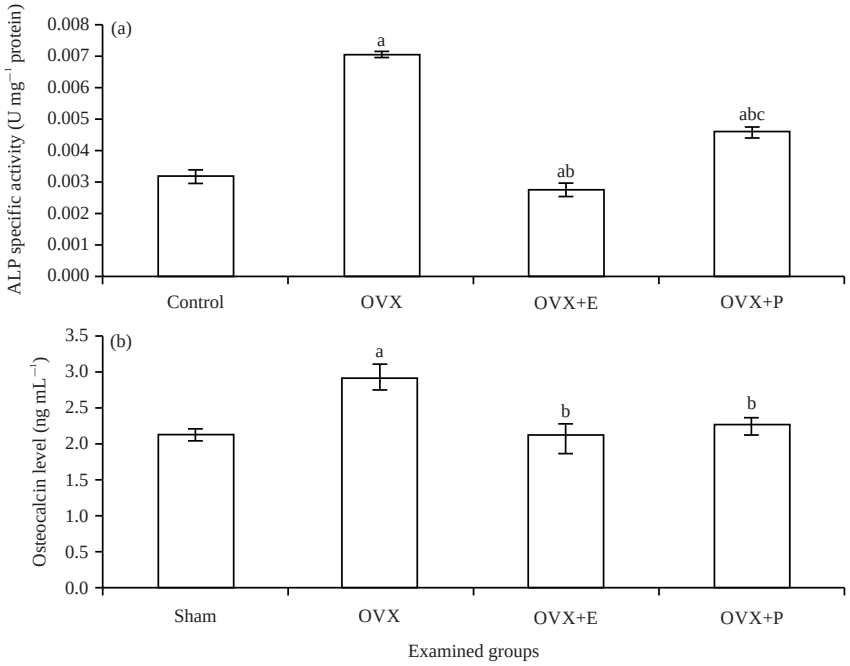

Fig. 3(a-b): (a) Serum alkaline phosphatase specific activity and (b) Serum osteocalcin levels in different examined groups

Values are expressed as Mean±Standard deviation, [a]Significant difference compared to the sham-operated control group, [b]Significant difference compared to the ovariectomized group, [c]Significant difference compared to the ovariectomized group treated with estradiol ($p<0.05$), Sham: Sham-operated control group, OVX: Ovariectomized group, OVX+E: Ovariectomized group treated with estradiol and OVX+P: Ovariectomized group treated with Prebiotin

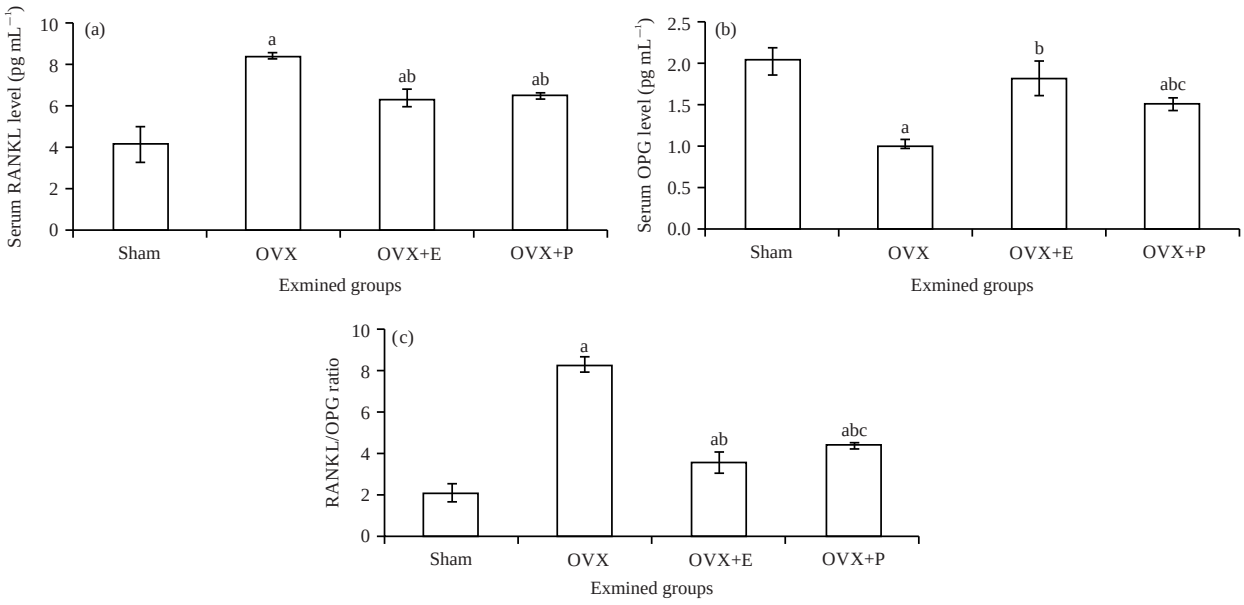

Fig. 4(a-c): Osteoclastogenesis markers in different groups, (a) Serum RANKL levels, (b) Serum OPG levels and (c) RANKL/OPG ratio in different examined groups

Values are expressed as Mean±Standard deviation, [a]Significant difference compared to the sham-operated control group, [b]Significant difference compared to the ovariectomized group, [c]Significant difference compared to the ovariectomized group treated with estradiol ($p<0.05$), Sham: Sham-operated control group, OVX: Ovariectomized group, OVX+E: Ovariectomized group treated with estradiol and OVX+P: Ovariectomized group treated with Prebiotin

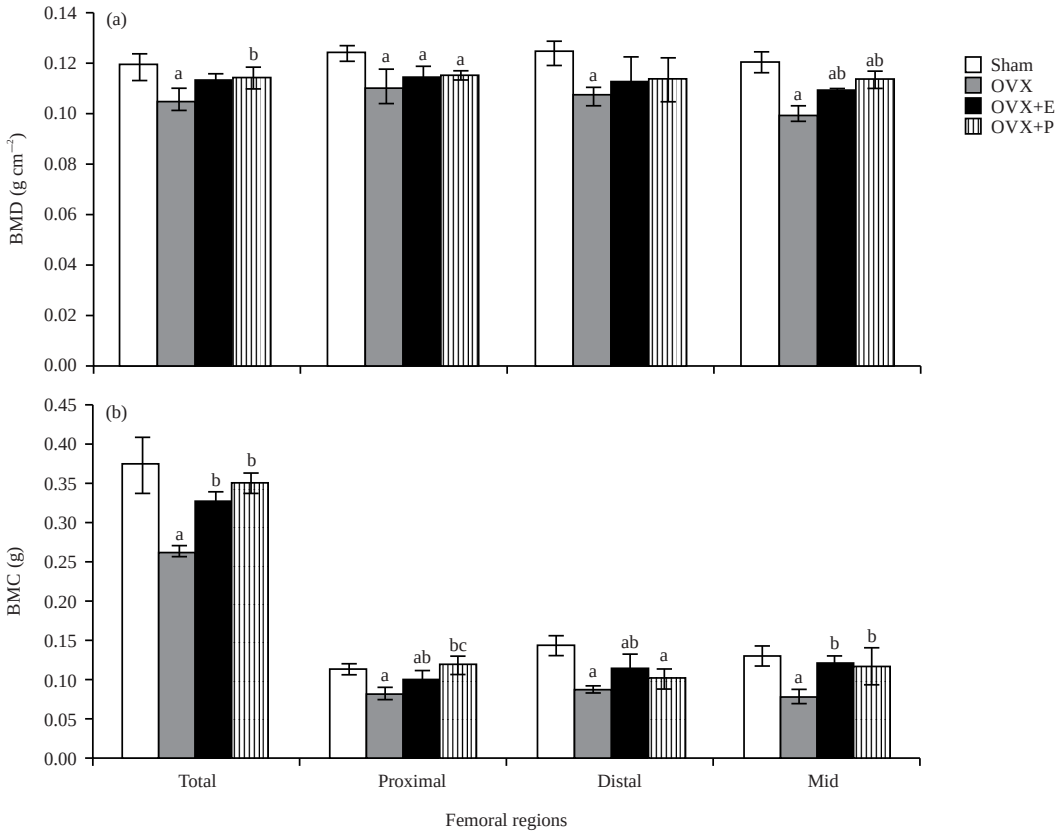

Fig. 5(a-b): (a) Bone Mineral Density (BMD) and (b) Bone Mineral Content (BMC) in total, proximal, distal and mid regions in different examined groups

Values are expressed as Mean±Standard deviation, [a]Significant difference compared to the sham-operated control group, [b]Significant difference compared to the ovariectomized group, [c]Significant difference compared to the ovariectomized group treated with estradiol ($p<0.05$), Sham: Sham-operated control group, OVX: Ovariectomized group, OVX+E: Ovariectomized group treated with estradiol and OVX+P: Ovariectomized group treated with Prebiotin

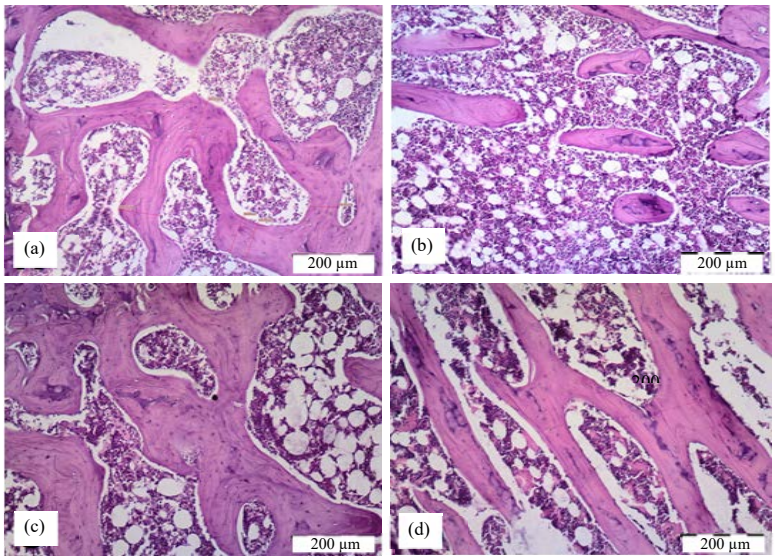

Fig. 6(a-d): Photomicrographs of rat distal femur sections of different experimental groups stained with H&E, (a) Sham-control group, (b) Ovariectomized group, (c) Ovariectomized group treated with estradiol and (d) Ovariectomized group treated with Prebiotin

Morphological changes were evaluated under optical microscopy (×200)

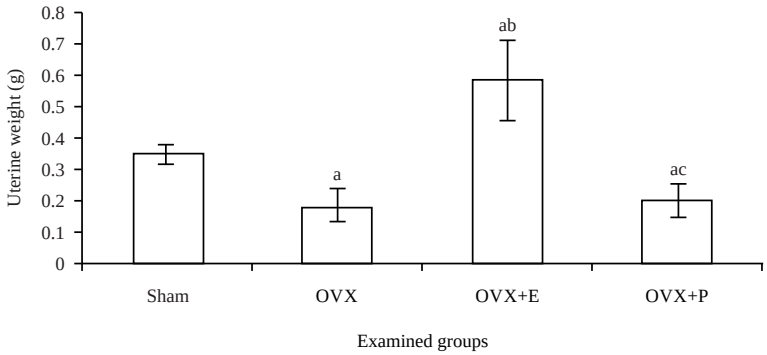

Fig. 7: Uterine weight of the different examined groups

Values are expressed as Mean±Standard deviation, [a]Significant difference compared to the sham-operated control group, [b]Significant difference compared to the ovariectomized group, [c]Significant difference compared to the ovariectomized group treated with estradiol ($p<0.05$), Sham: Sham-operated control group, OVX: Ovariectomized group, OVX+E: Ovariectomized group treated with estradiol and OVX+P: Ovariectomized group treated with Prebiotin

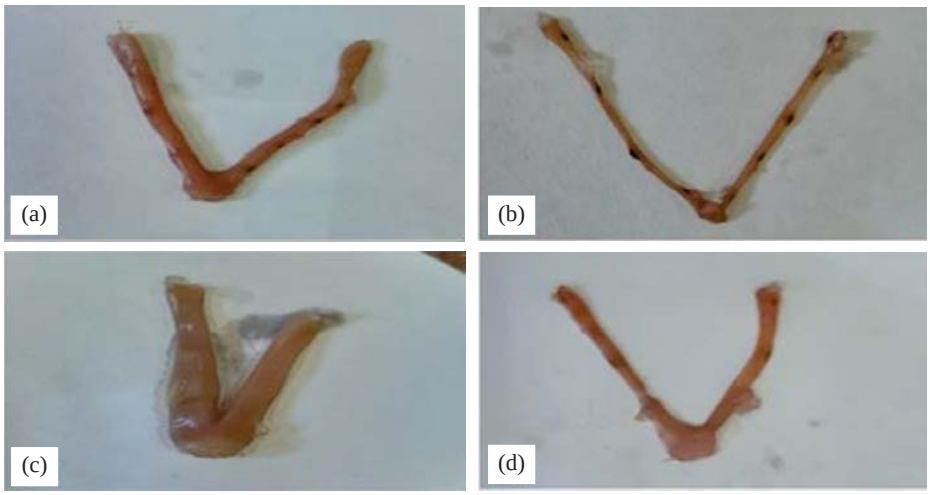

Fig. 8(a-d): Histopathological image of the bicornuate uterus of the four different groups after 8 weeks of treatment, (a) Sham-control group, (b) Ovariectomized group, (c) Ovariectomized group treated with estradiol and (d) Ovariectomized group treated with Prebiotin

Effects of Prebiotin™ on body weights in OVX rats: The body weights of the examined groups after a treatment period of 8 weeks were shown in (Fig. 9a) whereas, (Fig. 9b) showed the weekly body weight changes among the different studied groups throughout the treatment period. The obtained results showed a significant increase in the body weights of the OVX group compared to sham-control, OVX+E and OVX+P groups. Whereas, both OVX+P and OVX+E groups showed a significant decrease in their body weights compared to the OVX group, where they approached the body weights of the sham-control group.

Effects of Prebiotin™ on liver and kidney function tests in OVX rats: The obtained results (Fig. 10a-b) revealed a significant increase in serum ALT and AST-specific activities of the OVX group compared to the sham-control group, while both OVX+P and OVX+E groups showed a significant decrease in both serum ALT and AST specific activities to be less than that of OVX. Whereas, serum total protein values did not reveal any significant changes among any of the four different studied groups (Fig. 10c).

As for kidney function tests, there weren't any significant differences in BUN, serum creatinine and BUN/creatinine ratio among the different examined groups except for the BUN level of the ovariectomized group that showed significantly higher levels than sham-control, estradiol-treated and Prebiotin™-treated groups (Fig. 11a-c).

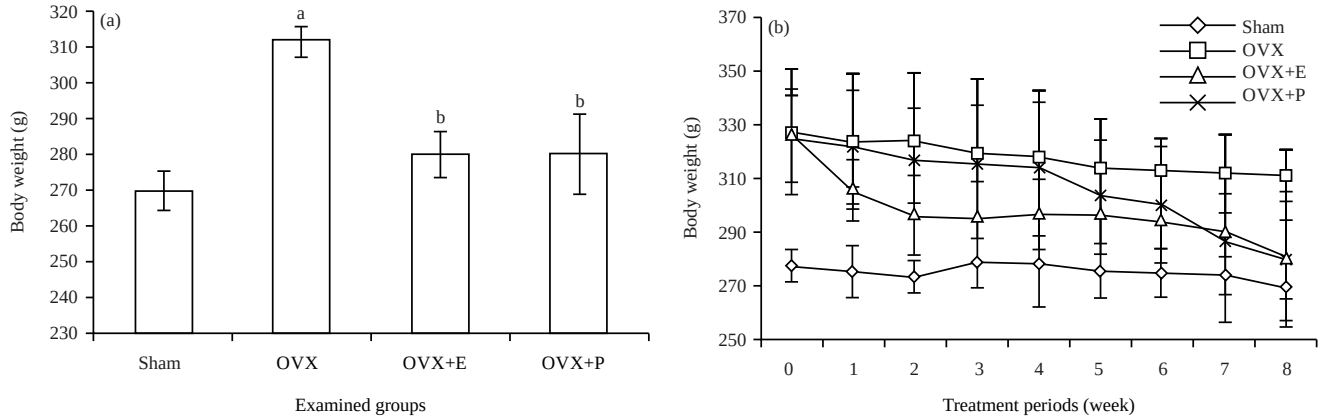

Fig. 9(a-b): Body weights and body weight changes throughout the treatment period, (a) Body weights of the different studied groups after 8 weeks of treatment and (b) Body weight changes of the different studied groups throughout 8 weeks of treatment

Values are expressed as Mean±Standard deviation, [a]Significant difference compared to the sham-operated control group, [b]Significant difference compared to ovariectomized group ($p<0.05$), Sham: Sham-operated control group, OVX: Ovariectomized group, OVX+E: Ovariectomized group treated with estradiol and OVX+P: Ovariectomized group treated with Prebiotin

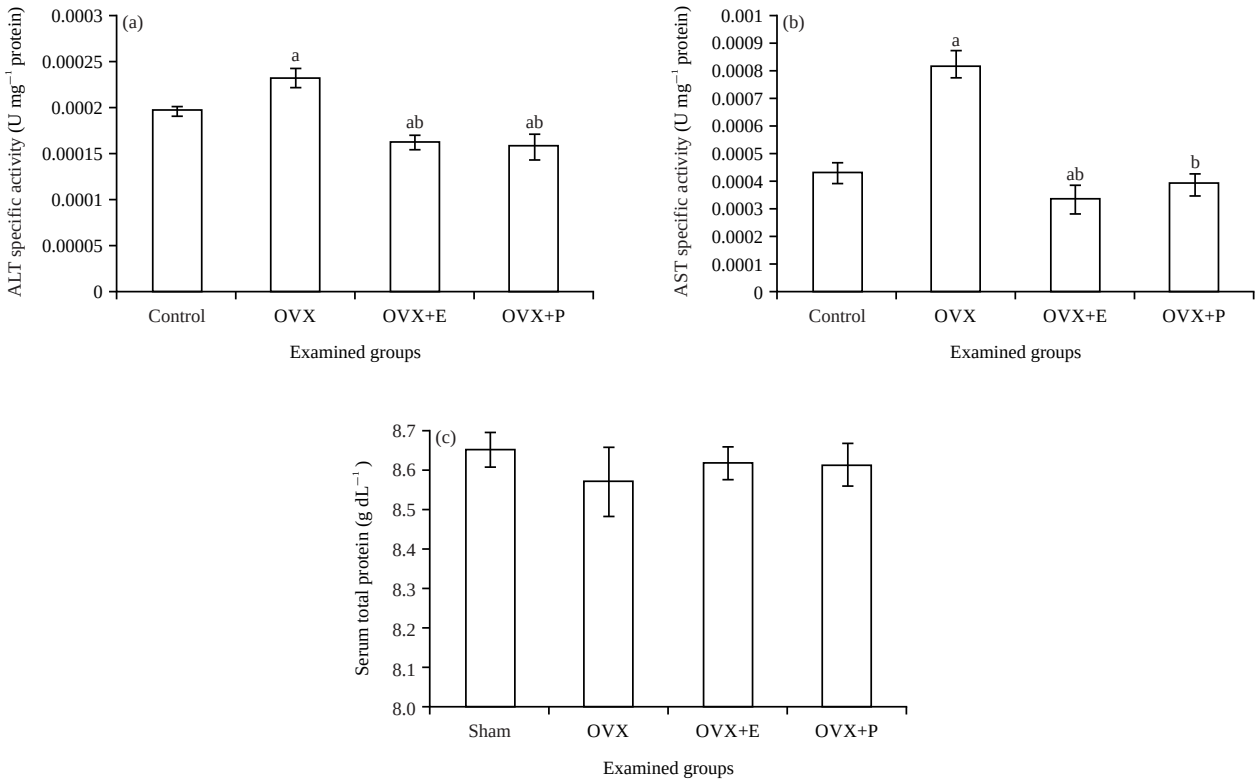

Fig. 10(a-c): Liver function tests in different groups, (a) Serum ALT specific activity, (b) Serum AST specific activity and (c) Serum total protein of the different examined groups

Values are expressed as Mean±Standard deviation, [a]Significant difference compared to the sham-operated control group, [b]Significant difference compared to ovariectomized group ($p<0.05$), Sham: Sham-operated control group, OVX: Ovariectomized group, OVX+E: Ovariectomized group treated with estradiol and OVX+P: Ovariectomized group treated with Prebiotin

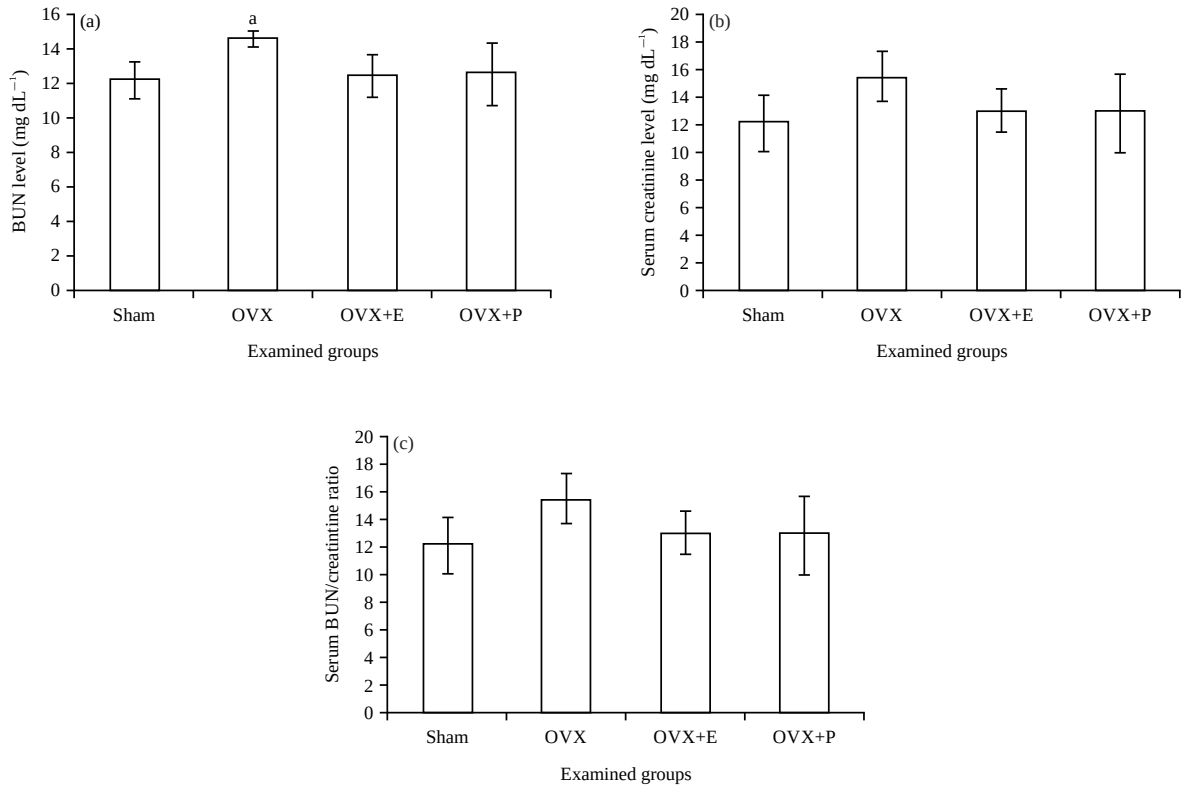

Fig. 11(a-c): Kidney function tests in different groups, (a) BUN levels, (b) Serum creatinine levels and (c) Serum BUN/creatinine of the different examined groups

Values are expressed as Mean±Standard deviation, [a]Significant difference ($p<0.05$) compared to the sham-operated control group, Sham: Sham-operated control group, OVX: Ovariectomized group, OVX+E: Ovariectomized group treated with estradiol and OVX+P: Ovariectomized group treated with Prebiotin

DISCUSSION

In this study, ovariectomized rats developed bone changes like those seen in osteoporotic women as indicated by a decrease in serum calcium and phosphorus levels, BMD and BMC values, OPG levels and an increase in both RANKL, ALP and osteocalcin levels.

The ovariectomized rat model is an approved preclinical model by the FDA for studying how reduced endogenous estrogen production by ovaries following menopause can lead to postmenopausal osteoporosis[27].

Mustafa *et al.*[28] reported that bilateral ovariectomy resulted in a dramatic reduction of bone ash contents and therefore low bone mineralization, which may be due to estrogen deficiency. Also, other studies on ovariectomized rat model reported that the bilateral ovariectomy was associated with a significant decrease in serum calcium and phosphorus levels and a marked increase in calcium and phosphorus urinary excretion as compared to the sham control group[29]. These findings agree with our results that showed diminished mineral levels in both rats' serum and bone ash of OVX compared to the sham group. These results could be attributed to estrogen depletion that occurred after bilateral ovariectomy as estrogen deficiency may affect calcium and phosphorus bioavailability[30].

Concerning BMD and BMC, they are two major factors that highlight bone rigidity and quality in terms of mineralization and resorption and are used as major determinants of OP[31]. In the present study, the obtained results revealed that both BMD and BMC of femoral bones were significantly suppressed in the OVX group. These results were in concurrence with Xu *et al.*[32], who stated that the BMD and BMC were considerably decreased in an estrogen-deficient ovariectomized animal model. These findings may be due to the previously mentioned imbalance that occurred in calcium and phosphorus absorption and excretion as a result of estrogen deficiency[33].

Furthermore, estrogen depletion especially after menopause provokes inflammatory responses represented in many aspects one of which is the increased ROS that

exclusively accumulates in bone marrow cells and results in many cascade reactions that finally produce TNF-α. TNF-α stimulates the production of RANKL and suppresses the production of OPG therefore, TNF-α indirectly induces osteoclastogenesis. Additionally, Wolski *et al*.[34], reported that despite there were multiple hormones and cytokines that regulate different aspects of osteoclastogenesis, the final effectors for the pathogenesis of osteoporosis were in the OPG/RANK/RANKL axis, which is a key element in maintaining the balance between bone resorption by osteoclasts and bone formation by osteoblasts. According to our results, OPG levels were significantly decreased in the OVX group than in the sham group while RANKL levels and RANKL/OPG ratios significantly increased in the OVX group than the sham group. These results concurred with those obtained by Eastell *et al*.[35], who found that estrogen deficiency led to imbalanced bone remodelling with increased osteoclastogenesis and subsequent bone resorption outpaced bone formation.

Additionally, ALP and osteocalcin serum levels were significantly increased in the OVX group compared to the sham group. In agreement with our results, Hassan *et al*.[29], reported increased levels of these bone markers following ovariectomy and Mukaiyama *et al*.[36], found that ALP levels tended to increase in the serum of postmenopausal women. This indicated that after menopause, osteoblasts not only remained acting and active but also their numbers increased to form bone as a compensatory mechanism to constrain the rate of bone loss due to estrogen deficiency despite the net result of bone mass loss[37].

The histopathology examinations of femoral sections of the OVX group showed a significant reduction in thickness and length of trabecular bone with the widening of inter-trabecular spaces when compared to the sham control group. These results were in agreement with Unis *et al*.[33], who reported that ovariectomy and its following consequences affected the quality of bone, consequently, the morphology and microstructure of ovariectomized group femoral bone had changed markedly resulting in the thinned disconnected trabecular bone network.

As for body and uterine weights, many previous studies have proven that complete ovary removal and therefore estrogen depletion led to increased food consumption and body weight with an associated deposition of abdominal adipose tissue[18] furthermore, is accompanied by uterus and vaginal atrophy[38]. The previous findings were consistent with our results, where the OVX group had significantly increased body weights and marked uterine atrophy changes in comparison to the sham control group.

As for liver functions, serum ALT and AST-specific activities were significantly increased in OVX rats and these findings were following previous studies that found that postmenopausal women are more prone to liver dysfunction[39,40].

On the other hand, using 17β-estradiol as a reference drug for treating the OVX rats was associated with a significant increase in serum calcium and phosphorus levels and bone ash contents when compared to the OVX group. Following such results, Liu *et al*.[41], reported that estrogen replacement therapy increased serum calcium and phosphorus in ovariectomized rats through increasing intestinal calcium absorption and renal tubular reabsorption of calcium and phosphorus.

Regarding BMC and BMD, the estradiol-treated OVX group showed recovery in both BMC and BMD compared to the OVX group and this increase was significant in mid-BMD, total, proximal, distal and mid-BMC. Our results were in concurrence with Liu *et al*.[41], who explained these positive estrogen effects on BMC and BMD may be due to the anti-osteoporotic and anti-catabolic potency of estrogen, which decreased bone resorption and regained the balance between bone resorption and bone formation by decreasing osteoclastic formation and inducing its apoptosis, decreasing cortical porosity and dwindling the resorptive area on the trabecular surface, therefore, slows the rate of bone loss as well as alleviating the symptoms of post-menopause.

In addition, estradiol administration suppressed bone formation markers, serum osteocalcin and ALP, compared to the OVX rats. Also, it led to an improved bone remodelling process and regain its balance through the significant increase in serum OPG meanwhile, there was a significant decrease in both serum RANKL levels and RANKL/OPG ratios compared to the OVX group but both were still significantly higher than the control group. Our results were consistent with the previous results reported by Alaam and Hussien[42] and Manolagas *et al*.[43], who stated that estradiol may have beneficial roles in osteoporosis treatment, by binding to its receptors on osteoblasts, OPG production was stimulated and RANKL production was suppressed, finally, osteoclastic bone resorption was reduced.

Consequently, the administration of estrogen to the OVX group was associated with significant improvement in femoral histopathological changes when compared to the OVX group as bone trabeculae showed complete, ordered and increased width structure as those of the control group. Similar results were reported by Unis *et al*.[33] and Kang *et al*.[44], who stated that estrogen recovery effect on the bone structure was related to its inhibitory activity on osteoclast cell activities.

On the other hand, estrogen was proven to have uterotrophic effects as it increased the growth, vascularity and uterine weight in rats[45] and these findings were following our results. The increase in uterine weights was mainly due to uterine water imbibition and/or a cell proliferation mediated by ERα[45]. On the other hand, the marked endometrial hyperplasia may be due to the given dose and duration of 17-β estradiol also the cessation of the menstrual period resulted in the loss of progesterone and unopposed estrogen in the ovariectomized rats[46].

As for body weight, the current results showed that the administration of 17β-estradiol to the OVX group inhibited body weight gain. Our results were consistent with the results of Russell *et al.*[45], who reported that OVX animals receiving estradiol replacement weighed significantly less than control animals. This decrease in body weight may be due to the critical role of estrogen in regulating metabolic pathways and its ability to decrease eating, at least in rats, by increasing the satiating action of Cholecystokinin (CCK)[46,47].

Concerning the liver, the administration of estradiol resulted in reducing serum ALT and AST levels to be lower than both the OVX and sham control groups. These results were following previous studies done on both ovariectomized rats[44] and postmenopausal women[48,49] which showed that estrogen therapy reduced the serum levels of ALT and AST and confirmed the hepatoprotective effects of estrogen.

It is documented from previous studies that prebiotics mainly oligofructose type not only stimulated calcium absorption but also that of magnesium in rats[50]. Therefore, supplementing the diet of the OVX rats in our study with 50 g kg^{-1} with Prebiotin™ in the presence of high dietary calcium content resulted in a significant increase in serum calcium and phosphorus levels and a significant increase in the femoral bone ash mineral contents compared to the OVX rats. These results were in agreement with Younes *et al.*[51], who demonstrated that treating adult rats with different doses of inulin and oligofructose had increased calcium and magnesium absorption and therefore reduce the risk of osteoporosis. Additionally, OEI was found to increase mineral absorption in postmenopausal women[52] and rodent animals[53]. These positive effects of OEI on mineral absorption may be mainly due to their fermentation and subsequent production of SCFAs in the lower gut that resulted in lowering the pH of the intestinal lumen and subsequently preventing calcium and other positively charged ions from complexing with negatively charged metabolites as phytates and oxalates, therefore, increasing its solubility and absorption by passive transport and subsequent bone mineralization[54]. As for phosphorus, the positive effect of OEI may be facilitated by a reduction of urinary phosphorus excretion[50].

The examination of BMD and BMC at different femoral sites by DEXA detected higher BMD and BMC in rats that were fed a diet containing OEI compared to the OVX group, which is a good indicator of increased bone mineralization. These results may indicate the protective effect of OEI against estrogen deficiency-induced loss of both trabecular bone and cortical bone in contrast with Ohta *et al.*[55], who reported that the prebiotic-treated group showed protective effects only on trabecular bone, not the mid-shaft that represented the cortical bone.

Moreover, the ingestion of a diet supplemented with OEI and high calcium levels resulted in a significant increase in serum OPG level and a significant decrease in both serum RANKL and RANKL/OPG ratio compared to the OVX group. These results were inconsistent with Bueno-Vargas *et al.*[53], who reported that serum RANKL was elevated significantly in the ovariectomized group supplemented with OEI when compared to the ovariectomized group. The mechanism that underlies this effect can be attributed to the production of butyrate from the fermentation of OEI in the lower gut. Butyrate exhibits anti-inflammatory properties where it stimulates the immune system for the production of TGF-β, IL-18 and IL-10 cytokines coupled with the stimulation and differentiation of naïve T-cells into regulatory T-cells (Tregs). Tregs inhibit osteoclast precursors to develop into mature osteoclasts by suppressing the expression of RANKL and Macrophage Colony-Stimulating Factor (M-CSF) that are often elevated after ovariectomy[56].

Furthermore, the OVX rats treated with Prebiotin™ showed a significant decrease in serum ALP and osteocalcin levels. In agreement with this finding, former animal studies[57] and human studies[52] indicated the improving effect of OEI on bone turnover markers that could be attributed to its stimulatory effect on enhancing calcium absorption and decreasing parathyroid hormone that had increased post estrogen deficiency thus reducing the osteoclastic activity and the rate of bone resorption.

Regarding the femoral histopathology, the addition of Prebiotin™ prevented bone loss, where the trabecular bone pattern was improved as trabeculae were more connected and thicker compared to the OVX group. Therefore, using a diet containing this percentage of OEI and calcium may be regarded as favourable and beneficial for restoring normal bone structure and reduced fracture risk.

In the current study, supplementing the diet with OEI didn't show any enhancing effects on estrogen deficiency-induced uterine atrophy, where the uterine weight of the Prebiotin™ group was nearly the same as the OVX group. These results were in congruence with the results reported by Ohta *et al.*[55] that showed no significant effect of different

combinations and types of prebiotics on the uterine weights. On the other hand, the body weights of the OEI group were significantly decreased compared to that of the OVX group. John *et al.*[58] reported that the OEI-induced weight loss may be attributed to the effects of prebiotics on controlling satiety and food intake that are directly linked to higher SCFA levels, which bind to the GPR 41/43 receptors on gut epithelial cells and stimulate Glucagon-Like Peptide-1 (GLP-1) production that suppress appetite by delaying gastric emptying and centrally promoting satiation.

Prebiotin™ supplementation also showed positive effects on the liver where it reduced serum AST and ALT activities compared to the OVX group. These results may be attributed to the chicory extract's beneficial properties as having hepatoprotective and antioxidant effects that resulted in reducing serum liver enzymes towards or even below the normal value[59].

Prebiotin™ could be effective in the treatment of postmenopausal osteoporosis and its related symptoms, by enhancing mineral absorption, decreasing bone turnover and modulating the bone remodelling process as it reduces the osteoclastogenesis process through increasing OPG and decreasing RANKL production and therefore, improving bone mineral density, content and microstructure. These beneficial effects of Prebiotin™ could be attributed to its bifidogenic effect or a butyrogenic effect or both, which modify the environment of the gut towards a healthier one and establish prebiotic-bone promoting interactions.

So, we recommend approving the present study by clinical trials to recommend Prebiotin™ for postmenopausal osteoporosis patients.

CONCLUSION

The present study indicates that Prebiotin™ is a promising prebiotic that can be an effective adjunct for calcium supplementation in the postmenopausal phase as it may attenuate bone loss partially through the suppression of the RANKL/OPG signalling pathway. Furthermore, it may be used as an anti-osteoporotic naturopathic agent in women who are clinically recommended not to take hormone replacement therapy.

SIGNIFICANCE STATEMENT

This study discovers the possible synergistic effect of Prebiotin™ that can be beneficial for osteoporosis-induced ovariectomized rats. This study will help the researcher to uncover the critical area of postmenopausal bone loss that many researchers were not able to explore. Thus, a new theory on this prebiotic and possibly other combinations, may be arrived at.

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