KUFA JOURNAL FOR VETERINARY MEDICAL SCIENCES

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A novel practice for Experimental Osteoporosis Model in Rats Mediated by Combining Phosphoric Acid Administration with Ovariectomy

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ABSTRACT

Osteoporosis, a metabolic condition impacts bone health by affecting calcium metabolism. Aging and decreased estrogen levels lead to imbalanced endocrine function, increasing bone resorption and decreasing bone creation. Increased release of parathyroid hormone due to high phosphate intake results in hyperphosphatemia and the removal of calcium from the bone loading. The goal of this work was to develop a new method for quickly inducing osteoporosis in contrast to existing conventional techniques. Wistar female rats were exposed to ovariectomy and subsequently 10% phosphoric acid orally for 30 days to induce osteoporosis. Thirty adult Wistar female rats were equally divided into three groups: Naïve rats served as the control group (C), Bilateral ovariectomized rats (OVX group), and Sham group were exposed to the same surgical operation steps of OVX group without removing the ovaries. One week of recovery post-surgical operation, the OVX and Sham groups were given 10% phosphoric acid in drinking water for 30 days, following the removal of all surgical sutures and an inspection for any flaws. The outcomes demonstrated that the OVX and Sham groups had significantly lower bone density than the control group, despite that they had significantly higher levels of alkaline phosphates, osteocalcin, and parathyroid hormone. Interestingly, the OVX group's serum calcium levels were significantly lower than those of the control group. In conclusion, ovariectomy and 10% phosphoric acid administration enforced osteoporosis status via hyperphosphatemia, hyperparathyroidism, and disruption in the bone metabolism in female rats in relatively short-term induction, this practice is highly useful in investigations of bone metabolism and health.

ARTICLE INFO
Article history:
Received: 7 January 2025
Accepted: 10 February 2025
Published: 30 June 2025
DOI:
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P- ISSN: 2077-9798
E- ISSN: 2959-8478

KEYWORD: Phosphoric acid, Osteoporosis, Ovariectomy, Parathyroid hormone, Osteocalcin.

INTRODUCTION

Bone is a complex tissue mineralized with calcium and phosphorus, which are essential for sustaining and safeguarding the body's structures. Bones safeguard key organs, offer attachment points for muscles, facilitate blood production in the bone

marrow, act as levers, and store essential minerals for various tasks [1]. Osteocytes function as endocrine cells and regulators of various metabolic processes by secreting certain hormones and signaling molecules [2]. For instance, osteoblasts and osteocytes create and release fibroblast growth factor 23 (FGF-23). By reducing the expression of sodium-phosphate cotransporters in the kidney's proximal tubule and preventing phosphate reabsorption, FGF-23 plays a part in bone remodeling, phosphate metabolism, and vitamin D regulation [3]. Additionally, sclerostin and osteocalcin, two additional hormones, are secreted by bone cells. According to some research, osteocalcin which is mostly made and secreted by osteoblasts is important for controlling energy metabolism, glucose tolerance, testosterone synthesis, and bone resorption [2].

Estrogen considerably regulates bone mass, Postmenopausal osteoporosis is predominantly attributable to estrogen deficiency, underscoring the critical role of estrogen in maintaining bone homeostasis in humans. Estrogen functions through two receptors: estrogen receptor-beta (ERβ, ER2) and estrogen receptor-alpha (ERα or ER1), with ERα playing a more significant role in regulating bone metabolism [4]. Estrogen safeguards bone integrity by modulating osteoclast survival, primarily through the enhanced secretion of transforming growth factor-beta (TGF-beta), which is essential for bone remodeling. TGF-beta promotes the synthesis of matrix proteins, significantly influences the activity of bone cells involved in both bone formation and resorption, and is present in high concentrations within bone tissue. [5]. Estrogen interacts with estrogen receptors to enhance the expression of osteoprotegerin (OPG) and to decrease the activity of nuclear factor-κβ ligand (RANKL), hence preventing osteoclast development and bone resorption. It can additionally stimulate Wnt/β-catenin signaling to enhance osteogenesis [6].

Postmenopausal women are susceptible to osteoporosis due to an imbalance in bone turnover, mostly resulting from estrogen insufficiency, with RANKL significantly contributing to the promotion of osteoclastogenesis. Conversely, osteoprotegerin (OPG) is a RANKL antagonist produced by osteoblast lineage cells. Estrogen induces osteoclast death and inhibits osteoclastogenesis by enhancing OPG synthesis and diminishing osteoclast differentiation through the inhibition of IL-1β and TNFα, thereby affecting M-CSF, RANKL, and IL-6 release. It can also activate the Wnt signaling pathway to enhance osteogenesis, facilitating the differentiation of mesenchymal stem cells from pre-osteoblasts to osteoblasts instead of adipocytes [6]. Estrogen deficiency leads to the uncoupling of bone resorption and formation; therefore, resulting in greater bone loss [7]. Alkaline phosphatase, a phosphomonoesterase located on the cell membrane, largely facilitates the hydrolysis of phosphate esters in alkaline environments, resulting in the release of inorganic phosphate [8] (“The correlation between serum levels of alkaline phosphatase and bone mineral density in adults aged 20 to 59 years”) It is highly expressed in tissues such as bones and liver. Additionally, alkaline phosphatase (ALP) may serve as a crucial biomarker of bone metabolism and bone mineral density (BMD), since fluctuations in its levels might signify the growth, healing, and remodeling of bones [9]. Parathyroid hormone (PTH) is an 84-amino acid peptide secreted by the parathyroid glands, which signals via the PTH and PTH-related protein receptor (also referred to as parathyroid hormone receptor 1, PTHR1), expressed in osteoblasts, osteocytes, chondrocytes, and proximal tubular cells [10].

About 80–90% of bone's mineral composition consists of calcium and phosphorus, with 85% of the body's phosphorus located in the skeleton. Proper phosphorus consumption is crucial for numerous biological functions, including bone mineralization; nevertheless, it is believed that excessive intake may adversely affect bone health. Phosphorus can directly induce apoptosis in cultured osteoblasts and elevate PTH concentrations [11].

MATERIALS AND METHOD

Ethical statement:

The study was conducted in compliance of University of Baghdad's Animal Welfare regulation with the authorization of the local Institutional Animal Care and Use Committee at the College of Veterinary Medicine, University of Baghdad (AUP no.1181/P. G).

Animals

A total of thirty mature female Wistar rats were utilized in the current study. All animals are housed in plastic cages within a specifically conditioned pathogen-free environment (22-25°C) at the Animal House of the College of Veterinary Medicine, University of Baghdad. They were retained for a minimum of two weeks for acclimation to the housing conditions. The food and water were freely accessible.

Ovariectomy procedure:

Adult female rats have been bilaterally ovariectomized (OVX) under xylazine and ketamine anesthesia. Sham animals underwent anesthesia and a dorsal incision, involving skin sectioning and suturing akin to the OVX animals, without the removal of the ovaries. [12,13].

Before appling any surgical procedure, all targeted animals were anesthetized with combination of xylazine and ketamine. A few minutes later, a toe pinch was applied to ensure that the animal was fully unconsciousness. Each animal was clipped and shaved on the lower dorsal area, just above the flank area. Then surgical incision was made for all animals to open skin, subcutaneous and peritoneal cavity. For Sham rats, the incision was sutured with silk without any further cutting. For OVX rats, the right and left ovaries plus part of fallopian duct were removed. Briefly, a little incision (1 cm) was executed through the skin and musculature of the posterior wall, aligned parallel to the body axis. The peritoneum was carefully incised to expose the ovaries. The ovaries were then located, carefully isolated from surrounding tissue and a silk thread was tightly ligated around the oviduct and ovarian blood vessels before the ovary was being removed, taking good care in leaving the knot intact. The dermis and the muscular wall were subsequently sutured using surgical silk thread. Postoperative care included close observation along with pain management for the next 48 hours [13,14].

Induction of osteoporosis by OVX and phosphoric acid

Ten Wistar adult female naïve rats served as control (C). Ten adult female rats will be ovariectomized according to [12]. While, another ten rats, Sham rats, were exposed to the same surgical conditions of ovariectomized rats except that the ovaries were not excised. All surgically operated rats (n=20), then followed up for three days to ensure there were no complications or wound infections. One week after the ovariectomy procedure, all surgical sutures were removed, and rats have been inspected for any defects. Osteoporosis induction started by giving all ovariectomized and Sham rats a 10% phosphoric acid in drinking water for 30 consecutive days [15].

Radiographing of experimental animals:

X-ray images were taken for

experimental rats in one time points; 30 days post starting of induction of osteoporosis by ovariectomy agonized by administrating phosphoric acids to the experimental animals in drinking water at concentration of 10% [15]. The radiographed animals were divided into control, Sham and ovariectomized rats. Briefly, all targeted animals in imaging were generally anesthetized by using a mixture of xylazine and ketamine at a dose of ketamine (80 mg/kg) and xylazine (10 mg/kg) [14], respectively. Once the animals were unconscious, they were put on imaging stage of X- RAY apparatus (Hindland – India, on their left side to be radiographed in whole-body capture with X-ray set on 100mlamber, mas 50 and kup50. All these steps are performed by senior radiologist under standard settings. Then all taken images were printed out on specific X-ray films (CR =CR10X –AGFA, CRMD 10 –AGFA) for further investigations and analysis.

Bone density measurement:

A free-available software called ImageJ [16], a JAVA programming software was utilized to analyze the X-ray scanned films [17]. Briefly, all films were scanned with Canon Scanner at a default setting with a resolution of 600 dpi, then they were uploaded separately to ImageJ software. Before performing any image analysis, the background noise was removed by built-in tool then fifteen different areas were determined by oval shape from right side of each animal distributed as follows: 3 areas from femur, 5 areas from vertebrae column (3 lumbar and 2 thoracic), 3 areas from radius bone, 2 areas from mandible bone and 2 areas from maxillary bone. The measurements were set as gray mean value through choosing it from analyze tab. The mean the bone density of each mentioned point by calculating the mean of gray contrast per inch of surrounded area through calculating pixels divided by respective area. Then all analyzed images were saved as TIFF format in inverted background while raw and mean integrated density values were exported as CSV file [18, 19].

Assessment of Serum ALP (IU\L) and Calcium (mg/dl) concentration.

Rat serum ALP and calcium concentration was measured by automation analyzers (spectrophotometer.) after blood sample collected by heart puncture [20,21], according to the recommendations of the manufacturers (reference number of ALP kit MI 41233, SPINREACT and manufactures in ESPANA, reference number of calcium kit MI 1001065, SPINREACT and manufactures in ESPAN.

Assessment of serum parathormone hormone (PTH) concentration (pg/ml) .

Rat serum PTH concentration was measured by using PTH kit, Catalogue Number:SL1342Hu, Sunlong Biotech Co. Chine. This ELISA kit uses Sandwich-ELISA as the method.

Assessment of serum Osteocalcin (OC) concentration (ng/ml).

Rat serum Osteocalcin concentration was measured by using Osteocalcin kit, Catalogue Number:SL3489Hu, Sunlong Biotech Co. Chine. This ELISA kit uses Sandwich-ELISA kit as the method.

Statistical analysis

The experimental data was analyzed by One-way ANOVA followed by Dunnett's multiple comparison test using GraphPad Prism 8.0 for Windows, GraphPad Software, Boston, Massachusetts USA, www.graphpad.com. The descriptive statistics are expressed in the form of mean ± standard deviation. $P<0.05$ was considered a statistically difference threshold. Significant

differences depicted as *P<0.05, **P<0.01, ***P<0.001 and ****P<0.0001 and ns = non-significant, unless stated differently.

RESULTS

Assessment of Serum ALP concentration (IU\L) and Calcium concentration (mg/dl).

Fig. 1A illustrates the mean values of serum alkaline phosphates in the control, Sham and ovariectomy (OVX) groups along the experimental periods. After 30 days of experiment, the result revealed a significant increase ($p<0.05$) in serum ALP concentration in Sham group treated with phosphoric acid 10%, in comparison with control group. On the other hand, at the end of the experiment, ovariectomized (OVX) rats administrated phosphoric acid 10% exhibited a significant ($p<0.05$) increase in serum ALP concentration at 30 days as compared to control group. Insignificant difference in serum ALP concentration between the Sham and OVX groups.

Fig.1B illustrates the mean values of serum calcium concentration in the control. Sham and ovariectomy (OVX) groups along the experimental periods. After 30 days of experiment, the results revealed insignificant differences in serum calcium concentration in Sham group treated with phosphoric acid 10%with surgical wound and control group. On the other hand, at the end of the experiment rat's administration with phosphoric acid 10% with surgical operations OVX group exhibited a significant ($p<0.05$) decreased in serum Ca+2 concentration at 30 days as compared to control group. While the serum concentration of Ca+2 in Sham group revealed insignificant differences with OVX group.

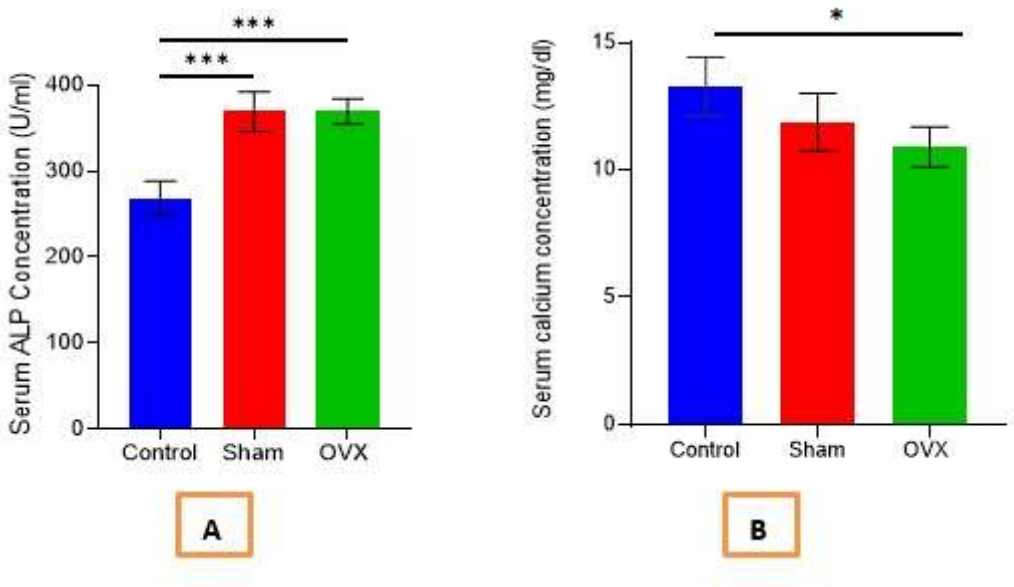

Fig. 1. Effect of ovariectomy procedure agonized by administration of 10% of phosphoric acid for 30 consecutive days on serum Concentration of A) alkaline phosphates enzyme (ALP), and B) calcium (Ca^{++}). Results in bar represent as mean ± SD. One-way ANOVA test employed to investigate the significant differences at P <0.05. * P <0.05, ***P <0.001.OVX; ovariectomized rats.

Assessment of serum PTH concentration (pg/ml).

Fig. 2A illustrates the mean values of serum parathyroid hormone in the control. Sham and ovariectomy (OVX) groups along the experimental periods. After 30 days of experiment, the results revealed a significant increase ($p<0.05$) in serum PTH concentration in Sham group treated with phosphoric acid 10% and surgical wound without surgical

operations (OVX) compared to control group. On the other hand, at the end of the experiment rat's administration with phosphoric acid 10% with surgical operations (OVX group) exhibited a significant ($p<0.05$) increase in serum PTH concentration at 30 days as compared to control group the concentration of PTH in OVX group a significant increased ($p<0.05$) compared with control and Sham groups.

Assessment of serum Osteocalcin (OC) concentration (ng/ml)

Fig. 2B illustrates the mean values of osteocalcin (OC) hormone in the control. Sham and ovariectomy (OVX) groups along the experimental periods. After 30 days of experiment, the results revealed a significant increase ($p<0.05$) in serum PTH concentration in Sham group treated with phosphoric acid 10% with surgical wound compared to control group. Since the concentration of serum PTH in Sham group revealed a significant decreased ($p<0.05$) compared with OVX group. While, at the end of the experiment rat's administration with phosphoric acid 10% with surgical operations OVX group) exhibited a significant ($p<0.05$) increase in serum OC concentration at 30 days as compared to control group, the concentration of PTH in OVX group a significant increased ($p<0. 05$) compared with control and Sham groups.

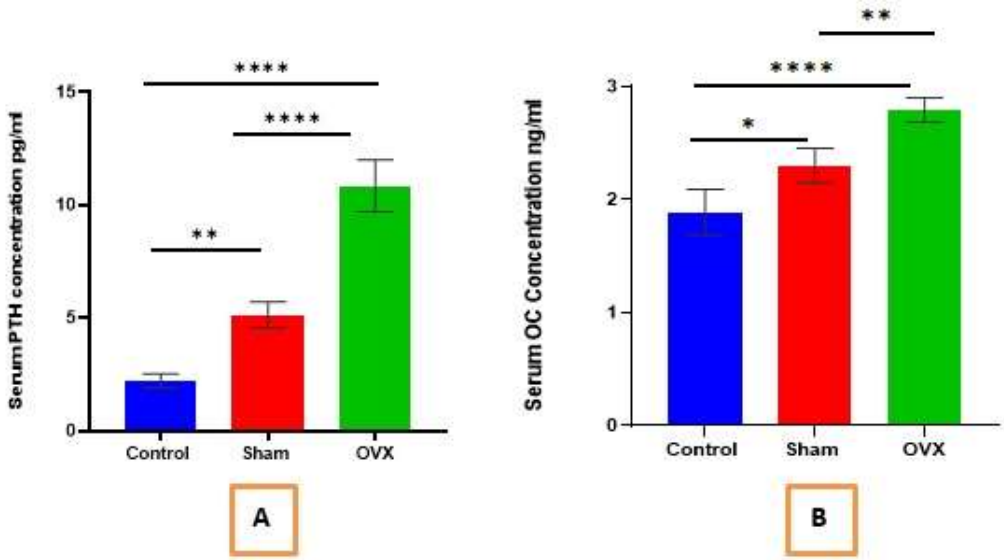

Fig. 2. Effect of ovariectomy procedure agonized by administration of 10% of phosphoric acid for 30 consecutive days on serum Concentration of A) Parathormone hormone (PTH), and B) Osteocalcin (OC). Results in bar represent as mean ±SD. One-way ANOVA test employed to investigate the significant differences at $P<0.05$. * $P<0.05$, ***$P<0.001$.OVX; ovariectomized rats

Bone mineral density

Fig. 3. illustrate the mean values of bone mineral density in the control. Sham and ovariectomy (OVX) groups along the experimental periods. After 30 days of experiment, the result revealed decrease significant diff. among means (P<0.05) in bone density in Sham group treated with phosphoric acid 10%without surgical operations (OVX) but only surgical wound and OVX group as compared to control group. On the other hand, the bone density exhibited non a significant difference (P< 0.05) between Sham and control at 30 days of experiment

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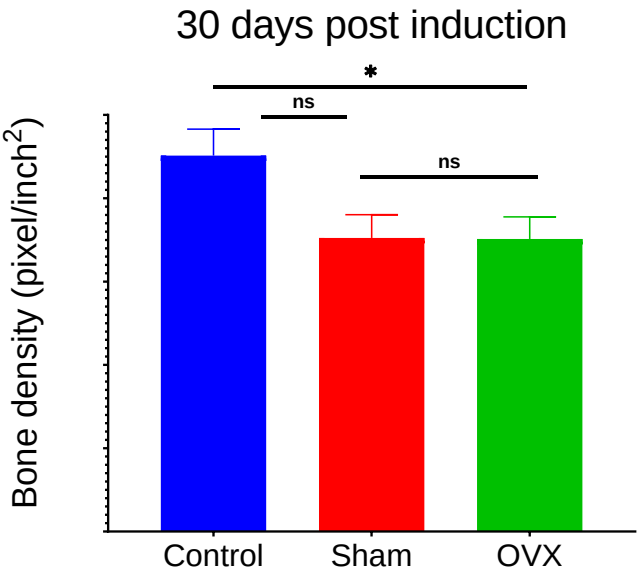

Fig. 3. Effect of ovariectomy procedure agonized by administration of 10% of phosphoric acid for 30 consecutive days on bone density. Results in bar represent as mean ±SD. One-way ANOVA test employed to investigate the significant differences at P <0.05. * P <0.05.OVX; ovariectomized rats.

DISCUSSION

The present study has aimed to induce blitz osteoporosis by bilateral ovariectomy in female rats and oral administration of phosphoric acid 10% for 30 days. The results of the present study showed a change in calcium and ALP concentrations in the serum of adult female rats treated with phosphoric acid overload after that ovariectomy. The serum calcium concentration in the Sham and OVX groups significantly decreased compared to the control group, while the serum concentration of ALP increased significantly in the Sham and OVX groups compared with control group (Figure-1 A and B). The calcium and phosphate are biomarkers in postmenopausal osteoporosis in females, Increased serum ALP levels may assist in assessing the reduction of bone mineral density (BMD) in postmenopausal women [22]. Biver et al. demonstrated elevated levels of bone alkaline phosphatase in osteoporotic patients relative to normal individuals, and these heightened levels may correlate with the occurrence of vertebral fractures [23]. Another study also demonstrated a robust negative correlation between alkaline phosphatase concentration and bone density by stepwise regression analysis [24].

Calcium metabolism is regulated by the parathyroid hormone (PTH, parathormone), which is released by the

parathyroid glands [25]. Phosphorus-rich and calcium-deficient diets result in decreased intestinal calcium absorption, lowering serum calcium levels and prompting PTH release, which subsequently induces bone resorption to restore serum calcium to homeostatic levels. Intermittent injection of human parathyroid hormone enhances bone mass in humans and rats [26]. In secondary hyperparathyroidism (SHPT) is recognized by high PTH secretion and due to decrease serum calcium or increased serum phosphate concentrations [27]. Studies show ovariectomized rats with decreased serum calcium may have impaired calcium balance, contributing to osteoporosis. Hypocalcemia may stimulate PTH, resulting in hypophosphatemia and hyperparathyroidism [28-30].

Osteoporosis is a condition marked by the deterioration of bone tissue, leading to heightened bone fragility and an elevated risk of fractures. Parathyroid hormone is crucial in the pathogenesis of osteoporosis [31]. Biochemical analysis of data in the current study showed that a significant increase in PTH in Sham and OVX groups compared with control group which might effect on bone metabolism. Elevated levels of PTH in circulation are called hyperparathyroidism, which may be due to hyperphosphatemia. These results aligned with previous study, Mice subjected to a high inorganic phosphate diet for 12 months had a significant decrease in bone mass, primarily attributable to bone resorption, maybe linked to elevated PTH levels [31, 32]. Another study demonstrated High levels of PTH for prolonged periods lead to a decrease in bone density [25]. Vitamin D is essential for calcium absorption and bone mineralization, while estrogen has a protective effect on bone density in women [33] and rat [34].

Numerous studies indicate that Pi enhances PTH secretion in the parathyroids, which subsequently promotes calcitriol synthesis in the proximal tubules, so indirectly enhancing intestinal Pi absorption [35]. Furthermore, PTH promotes bone turnover, leading to the liberation of Pi from the skeletal system [36]. Nonetheless, the overall impact of PTH is to decrease blood levels of Pi, as PTH diminishes the stability of type II Na+-Pi co-transporters (NPT2a and NPT2c) at the renal brush border membrane, hence decreasing Pi reabsorption from urine [37]. Hyperphosphatemia, a condition characterized by high serum phosphate levels due to increased dietary phosphate intake, is linked to chronic kidney disease (CKD) and secondary hyperparathyroidism and metabolic bone disease [38].

Current results revealed that the osteocalcin in OVX and Sham groups were significantly increased which is a protein secreted by osteoblasts, plays a crucial role in maintaining calcium ion homeostasis, bone mineralization, and bone remodelling, particularly in postmenopausal women with osteoporosis [39]. A prior study indicated that osteoporosis is characterized by a shortage in calcium and phosphorus levels, and as osteocalcin is a calcium-dependent biomarker with a significant affinity for the bone matrix (hydroxyapatite), it plays a crucial role in bone mineralization. Osteoporosis causes a reduction in hydroxyapatite crystal production, resulting in elevated serum osteocalcin levels [40,41]. Serum osteocalcin level measurement can be used for screening purposes in post-menopausal patients [39,42]. Moreover, the present study showed significant reduction in bone density in OVX and Sham groups, which measured by X- ray image [43] compared with control group which come parallel to the findings of [44] who observed that the mean The ALP level was markedly elevated in postmenopausal women with decreased bone mineral density. Plasma alkaline phosphatase levels were dramatically elevated in ovariectomized rats, accompanied by a notable drop in osteoblasts

and a considerable increase in osteoclast numbers, resulting in bone resorption and osteoporosis [30]. The fall of estrogen is crucial in the bone remodeling process. This method is frequently employed to create an animal model of post-menopausal osteoporosis [45]. Estrogen diminishes bone cell apoptosis, decelerates bone remodeling, and inhibits oxidative stress, NF-κB activity, and osteoblast apoptosis, so promoting ongoing bone production. RANKL reacts with osteoclast receptors, facilitating their development and resulting in an elevated rate of bone resorption. Estrogen maintains bone integrity via these mechanisms [46].

Normal bone metabolism is sustained by a balance between osteoclastic resorption and osteoblastic creation, sustaining steady bone mass. Low BMD triggers the activation of quiescent osteoblasts, leading to the formation of unmineralized bone-like tissue and undifferentiated osteoblasts. The osteoblasts grow in a feedback way and generate substantial quantities of bone alkaline phosphatase (B-ALP), markedly elevating serum ALP levels [47].

A separate study indicated that acid retention promotes bone resorption by activating osteoclasts and suppressing osteoblasts [48]. Furthermore, acid loading elevates urine calcium excretion as a result of modified tubular calcium processing. These alterations are autonomous from PTH, 1,25(OH)2D3, and tubular sodium management [49]. Consequently, dietary acid loading was correlated with a negative calcium balance and reduced bone mineral density (BMD) [48, 50, 51].

CONCLUSION

Collectively, this study can conclude that exposure of rats to long-term administration of phosphoric acid is able to induce osteoporosis via lowering the serum calcium and elevate the parathormone hormone which is responsible to enhance the bone resorption. However, lacking steroid hormones due to ovariectomy procedure has exacerbated the deleterious effects of hyperphosphatemia and enhanced the induction of osteoporosis in rats through elevation of PTH and OC levels. In current study we found that combine of ovariectomy with administration of phosphoric is a novel practical method to induce experimental osteoporosis in rats which opens wide-window for better understanding of bone–related calcium metabolism disorders.

CONFLICT INTEREST

The authors declare that there is no conflict of interest.

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