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Impact of bilateral ovariectomy on bone biomarkers, the RANK-RANKL pathway, and hormones, in rabbits

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***Abstract*— Bone homeostasis and skeletal growth are mostly influenced by estrogen. Osteoclastic bone resorption in women is often accelerated by estrogen insufficiency following menopause. Examining the relationship between estrogen and the RANK/RANKL/OPG pathway as well as other biomarkers and their correlation with bone loss was the goal of the current investigation. Twenty female rabbits with ovariectomies were split into two groups at random: the ovariectomized group (OVX group) and the control group. Six weeks following ovariectomy, blood samples were obtained for biochemical analysis in order to determine the blood's calcium percentage and to evaluate the serum hormones estrogen, progesterone, LH, and FSH (OPG, RANKL, and RANk). Additionally, femur samples were obtained for histological sectioning analysis. According to the data, rabbits that had ovariectomy had significantly higher levels of RANk and RANkL, however OPG analysis showed that these rabbits' levels were significantly lower than those of the control group. According to the findings, rabbits with osteoporosis had lower serum levels of progesterone and estrogen than the control group. In contrast, the osteoporosis-affected rabbits' serum levels of FSH and LH were significantly higher than those of the control group.However, the calcium levels were higher than in the control group, according to the data. The rabbits' femur underwent histological examination, which showed osteoporosis, a high concentration of osteoclasts, and bone tissue reabsorption with thinner bone laminae. The results of the light microscope analysis of the bones showed that there were variations between the control and ovariectomized groups.**

***Keywords* — Osteoporosis, Ovariectomy, Estrogen Receptors and Rabbits.**

INTRODUCTION

The Humans can have osteoporosis for many years without realizing it. It is a lack of bone mass that makes fractures more likely. particularly in the hip and spine (**1 ,2)**. About 25 million people in the US suffer from osteoporosis, a condition that is common throughout the world. An imbalance between osteoblasts' and bone-building cells' functions is the primary cause of osteoporosis. Oestrogen hormone, a key medication in the deposition of minerals in the tissues (bone mineralization), is reduced as a result of numerous circumstances, including natural and surgical ones (**3**). However, it also affects several key elements for bone growth and development, including insulin-like growth and osteoprogestins, and it triggers the apoptotic process of osteoblasts or bone marrow cells (4). Additionally, it limits calcium absorption and initiates the adsorption process.Together, the three crucial proteins RANK, RANK-L, and OPG control osteoclast growth and activation (5). PGE2 also increased the production of Interleukin 6 (IL-6), increased the production of RANK by osteoclasts, and inhibited the secretion of OPG (6).

Various Techniques for Producing Animal Models of Premature Ovarian Failure Chemotherapy drugs, radiation therapy, genetic induction, D-galactose, natural ovarian aging, oophorectomy, 4-vinylcyclohexene diepoxide, autoimmunity, psychological stress, and POF are currently the most common animal models of premature ovarian failure (7). Along with further methods of using methionine to cause osteoporosis (6). In women, bone loss begins prior to menopause and oestrogen shortage. According to research on cells, animals, and humans, the rise in follicle-stimulating hormone (FSH) occurs before the fall in estrogen and may be the reason for bone loss prior to menopause. According to cellular research, osteoclasts, osteoclast precursors, and mesenchymal stem cells all possessed the FSH receptor (FSHR), whereas osteoblasts did not. FSH had minimal impact on osteoblasts but increased osteoclast differentiation, activity, and survival (8). Because oestrogen insufficiency decreases calcium absorption and increases excretion, menopause causes women to lose bone rapidly; hence, bone loss greatly exceeds bone deposition. Although people with osteoporosis typically have acceptable serum calcium levels, their total body calcium reserves are significantly reduced (9). The management of phosphorus and magnesium is intimately related to calcium. The production of follicles from oocytes in the rat ovary marks the beginning of the ovarian estrus cycle's follicular phase, which is triggered by low levels of follicle stimulation hormone (FSH), which is

generated by the pituitary.Additionally, a steady rise in estradiol levels is noted during this time. Diestrus 1 or metestrus is the first day of this phase, which lasts around two days. Diestrus 2 or simply diestrus is the second day. While diestrus cells are primarily composed of leukocytes, metestrus is also marked by the activity of the corpus luteum, which generates progesterone and is cytologically characterized by nucleated and cornified cells (10, 11,12). Estradiol levels sharply rise during proestrus, which is the pre-ovulatory phase. This causes the pituitary to release more luteinizing hormone (LH), which in turn causes ovulation, and initiates the release of gonadotrophin-releasing hormone (GnRH). A few hours prior to ovulation, progesterone rises and aids in this process. Ovulation takes place 10–12 hours after the release of progesterone and LH into the bloodstream. Vaginal cytology reveals a large number of non-cornified nucleated epithelial cells during the proestrus stage. Estrus, which coincides with the actual day of ovulation, is the period of time when the female is sexually receptive. Estradiol and progesterone return to normal levels during this time, which follows the LH surge and ovulation. 75% of cells are nucleated and 25% are cornified during the estrus period, which typically lasts 25–27 hours (10,11).

The study aims to determine the extent of the impact of low levels of estrogen on the body, especially on the occurrence of osteoporosis, on the inhibition of the RANK (Receptor Activator of Nuclear Factor Kappa-B), RANK-L (RANK Ligand), OPG (Osteoprotegerin), pathway, measure RANKL, RANK, OPG and and other Biomarkers levels, given the importance of this condition in society, as a high percentage of women suffer from osteoporosis after menopause.

MATERIALS AND METHODS

Experimental Animals

Twenty sexually and physically mature female rabbits were used in the study. They were kept in wooden cages designed specifically for rabbits and had the best possible habitat, food, and water. Second-stage broiler feed (crude protein 19, crude fat 4%, fiber not to exceed 1.9%, energy 3200 kcal kg (feed) was given to them. They were split into two groups.

1. There are ten clinically female members in the first group.

2. Ten clinically female patients who have both of their ovaries surgically removed and receive antibiotic treatment for five days following the procedure make up the second group.

Surgical removal

The abdominal region was completely shaved, sterilized with a sterile solution (povidone liquid 10%), and anesthetized by intramuscular injection of 40 mg/kg of ketamine and 2 mg/kg of xylazine. A 5–10 cm incision was then made on the midline of the abdominal surface in the area confined between the first and second nipples, penetrating the abdominal layers to reach the abdominal cavity. A surgical hook was used to remove the ovaries, which were then tied with a Vicryl surgical thread (0–2) after being secured to the uterine body with arterial clamps for removal with a surgical blade. Vicryl thread (0–2) was then used to suture the abdominal muscles with simple interrupted stitches, and a silk thread (0–2) was used to suture the skin with simple interrupted stitches (13).

Collect of the blood samples

Direct heart punctures are used to obtain blood, and ketamine and xylazine are used to anesthetize the animals in order to relax and control them prior to the 6-week blood draw. Blood samples were allowed to clot at room temperature for 30 minutes in non-heparin tubes. After 15 minutes at 3,000 rpm in the centrifuge, the tubes were taken out and put in a fresh tube, which was then stored in a freezer until it was needed. Furthermore, assess levels of progesterone and estrogen, FSH and LH, and calcium using Biomarker ELISA (RANK, RANKL, OPG).

Serum biomarker

A unique Elisa kit from ELK Biotechnology China was used to measure the levels of serum calcium, estrogen and progesterone, FSH, and LH.

Examination of bone mineral density by X-ray

A combination of I/M ketamine (100 nmg/kg) and xylazine (10 mg/kg) was used to anesthetize the rabbits (14). On the scan, the sedated rabbit was placed dorsally recumbent. DXA (Hologic QDR-1000 System, Hologic Inc., Waltham, USA) was used for all scans. The rabbits' femur's bone mineral density (BMD) was assessed using a high-resolution scan.

Histological

Histological examination of femur bone with E and H dye. Femur bone was collected and processed using the following methods: fixation, decalcification, dehydration, clearing, infiltration, embedding, tissue sectioning with a rotary microtome, tissue attachment, de-wax and hydration, staining with hematoxylin and eosin, and finally mounting (15). Sections of tissue were examined under a microscope using descriptive histology. A light microscope, also referred to as a motile microscope, was used for the microscopic analyses in this investigation.

Ethical approve

Under the reference number UOK.VET. PH.2024.095 ،the study was conducted at the Kerbala University, College of Veterinary Medicines' anatomical facility in Iraq.

Statistical analysis:

Statistical analysis of data for experiments in the present study was performed by prism V8.0 on the basis of one way and two way analysis of variance (ANOVA) using significant level of ($P<0.05$) **(16).**

RESULT

1- Effect of ovarictomy on femoral bone X-Ray:

In the current study the control group show a normal bone density at 6 weeks of the experiment while the ovarictomy group show a significant decrease in the bone density osteoporosis femoral and pelvic bones especially at the middle and epiphysis of bones, as shown in the figure (1)

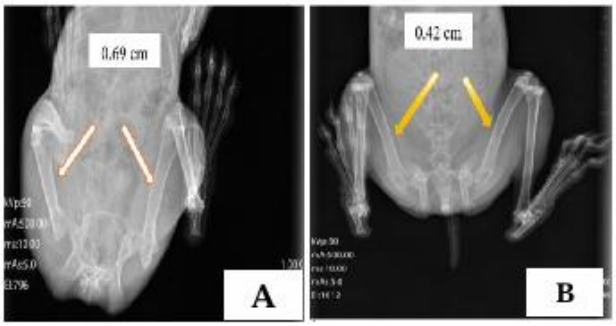

Figure 1. Radiographic image of the bones with white arrow of control group showing normal bone density (A). While Radiographic image of the bones with ovarictomy group (B) show a lees bone density as camper with control group (after six month).

2- Hormonal and Biomarker effected by ovariectomized:

A- Estrogen level:

In the current study there was a significant ($p \leq 0.5$) decrease in the level of estrogen in the serum in the OVX group compared to the control group as a result of ovariectomized (Figure 2) **.**Shows the estrogen level in the control group 34.37+-0. , 28 while the estrogen level in the OVX group 13.44+-0.49 . We note a decrease in the estrogen level in the animals that underwent OVX, as the removal of the ovaries is a factor affecting the estrogen level.

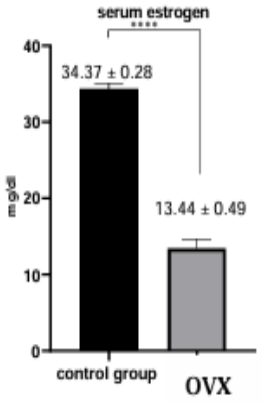

Figure 2. Estrogen serum measurement in the control group and the OVX group. Values expressed as mean±, standard deviation, control group, and ovariectomized

B- Progesterone level:

Figure 3 shows the progesterone level in the control group 32.418+-0.40 , while the progesterone level in the OVX group 7.4+-0.84 . We notice a decrease in the progesterone level in the animals that were subjected to OVX, a s the removal of the ovaries is a factor affecting the progesterone level, with a significant ($p \leq 0.5$) decrease in the level.

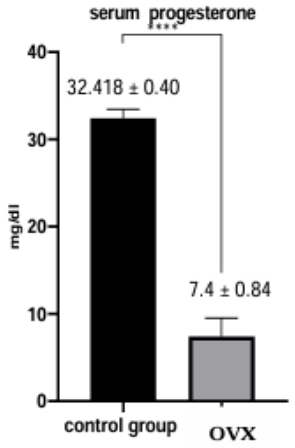

Figure 3. progesterone serum measurement in the control group and the OVX group. Values expressed as mean±, standard deviation, control group, and ovariectomized

C- Calcium level:

The significant increase in level of calcium in OVX group. the percentage of calcium in the control group9.22 +-0.80 , while the percentage of calcium in the OVX group 32.37 +-0.41. We note an increase in the calcium level in the animals that underwent OVX, as the removal of the ovaries is a factor affecting the calcium level, as show in Figure 4 .

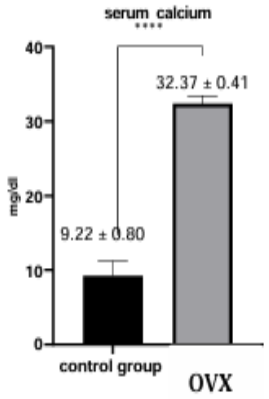

Figure 4. calcium serum measurement in the control group and the OVX group. Values expressed as mean±, standard deviation, control group, and ovariectomized .

D- FSH level:

Figure 5 shows the FSH ratio in the control group 16.21+-0.42 , while the FSH ratio in the OVX group 22.56 +-0.424. We note an increase in the FSH level in the animals that underwent OVX, which is considered an effective factor in removing the ovaries on the FSH level, with the significant ($p \leq 0.5$) effect.

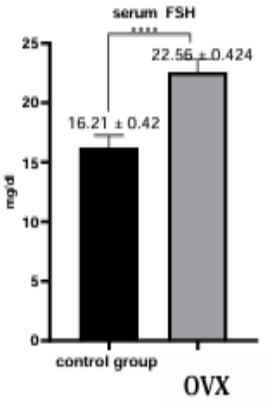

Figure 5. FSH serum measurement in the control group and the OVX group. Values expressed as mean±, standard deviation, control group, and ovariectomized

E- LH level:

Figure 6 shows the LH ratio in the control group 13.38 +-0.35 , while the LH ratio in the OVX group 23.12 +-0.88 . We notice an increase in the LH level in the animals that underwent OVX, which is considered an effective factor in removing the ovaries on the LH level.

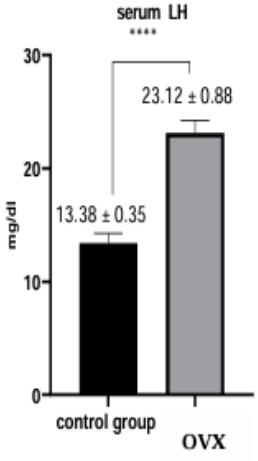

Figure 6. LH serum measurement in the control group and the OVX group. Values expressed as mean±, standard deviation, control group , and ovariectomized .

F- RANK level:

Figure 7 shows the RANK ratio in the control group 1.7416 +-0.17 , while the RANK ratio in the OVX group 2.85 +-0.27 . We notice an increase in the RANK level in the animals that underwent OVX, as the removal of the ovaries is a factor affecting the RANK level, with The significant ($p \leq 0.5$) increase.

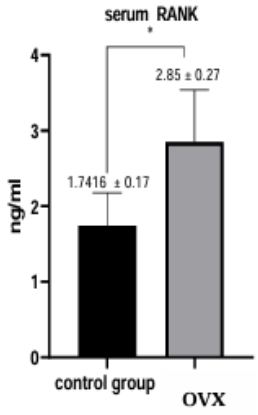

Figure 7. RANK serum measurement in the control group and the OVX group. Values expressed as mean±, standard deviation, control group, and ovariectomized

G- s RANKL level:

The percentage of s RANKL in the control group 135.05 +-22.07, while the percentage of s RANKL in the OVX group 264.006 +-26.56. We note an increase in the level of s RANKL in the animals that were subjected to OVX, as the removal of the ovaries is a factor affecting the level of s RANKL. Figure 8 shows The significant ($p \leq 0.5$) increase.

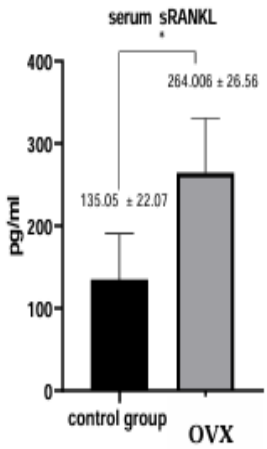

Figure 8. s RANKL serum measurement in the control group and the OVX group. Values expressed as mean±, standard deviation, control group, and ovariectomized

H- OPG level:

Figure 9 shows the percentage of OPG in the control group 48.06 +-5.40 , while the percentage of OPG in the OVX group 31.15 +-1.87 . We note a decrease in the level of OPG in the animals that were subjected to OVX, as the removal of the ovaries is a factor affecting the level of OPG, with The significant decrease ($p \leq 0.5$) in OVX.

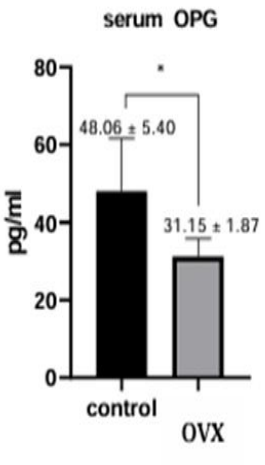

Figure 9. OPG serum measurement in the control group and the OVX group. Values expressed as mean±, standard deviation, control group , and ovariectomized

3- Histological result

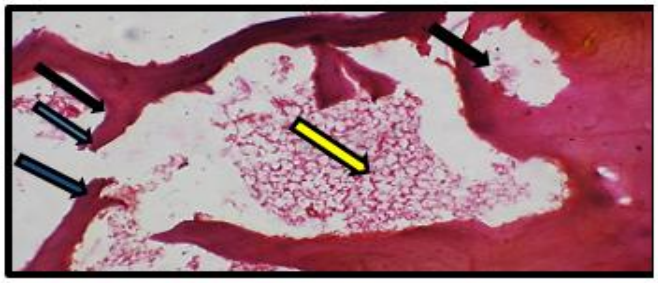

Figure 10. Histopathological section of rabbits femur longitudinal of control positive group 6 week post Ovariectomy showed illustrated the trabecula bone formation characterized by thin wall with longitudinal specula's (black arrow) with adipocytes predominance in bone marrow tissues (yellow arrow) ,some of these bone specula's not anastomosis with others and have bland end (blue arrow) (H&E x10)

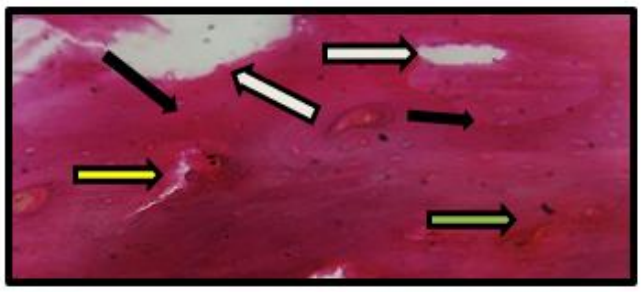

Figure 11. Histopathological section of rabbits femur longitudinal of control positive group 6 week post Ovariectomy showed lacunae with osteocytes (green arrow) & without osteocytes (black arrow), with osteoclasts infiltration (yellow arrow), and widening in the Haversian canal (white arrow) (H&E x40)

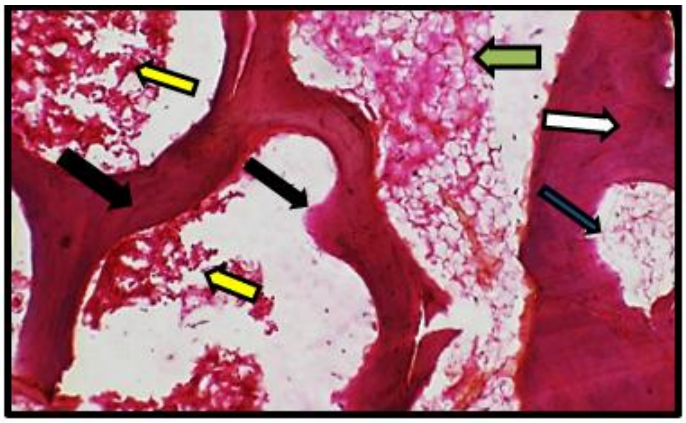

Figure 12. Histopathological section shows longitudinal view of rabbits femur bone of control negative group illustrated normal architecture bone that contain many mature long thick trabecula bone (black arrow) that consist of osteocytes cells inside the lacuna with large empty cavity between the mature trabecular bone formation (white arrow) with large size haversian canal (blue arrow) & hematopoietic cells (yellow arrow) with adipocytes predominance (green arrow) in bone marrow cavity (H&E x10)

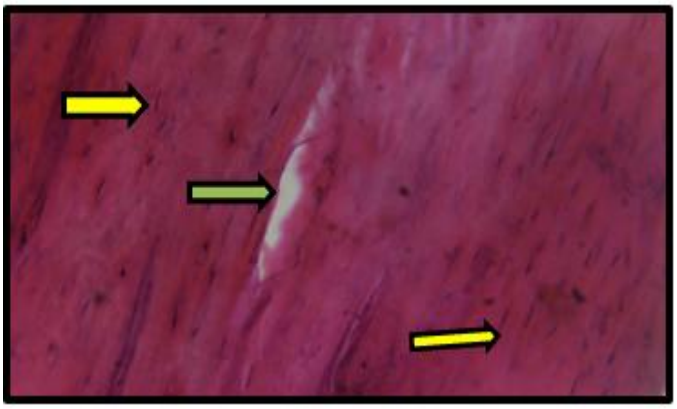

Figure 13. Histopathological section of rabbits femur longitudinal of control negative group showed the cortical bone has a normal shape with osteocytes inside their lacunae (yellow arrow) & Haversian canals run longitudinally through compact bone (green arrow) (H&E x40)

DISCUSSION

Important information regarding the impact of decreased estrogen on osteoporosis can be obtained from X-rays. The OVX group's bones are less dense than those of the control group, which has normal bones with the right density. The probability of osteoporosis is shown via X-rays. Osteoporosis causes loss of bone mass and increases the risk of fractures and falls. This severe health problem affects millions of people worldwide This is consistent with our results (17,18). The current study showed a decrease in serum estrogen in the ovariectomized rabbit group compared to the control group, where the OVX group was 13.44+-0.49 compared to the control group 34.37+-0. 28. Overall bone resorption results

from an estrogen shortage, which raises the number of osteoclasts and lowers the number of osteoblasts. In order to reduce the function of nuclear factor-KB ligand RANKL and promote osteoproteogerin (OPG), estrogen connects with the estrogen receptor, preventing the development of osteoclasts (19). Estrogen receptors are expressed by osteoblasts, osteocytes, and osteoclasts. Furthermore, estrogen directly affects bone through local growth factors and cytokines. Because IL-6 helps attract osteoclasts and suppresses IL-6 release, estrogen plays a role in osteoporosis. (20) nuclear factor-kappa B ligand receptor activator The last common mechanism for bone resorption is the RANKL/receptor activator of nuclear factor-kappa B (RANK) osteoprotegerin (OPG) system. To encourage osteoclast differentiation, RANKL binds to RANK, which is expressed by osteoclasts and osteoclast precursors. By binding and sequestering RANKL, OPG, a soluble decoy receptor, suppresses RANK-RANKL (21,22).

The current study showed a decrease in serum progesterone in the ovariectomized rabbit group compared to the control group, as the OVX group was7.4+-0.84 compared to the control group 32.418+-0.40 . Progesterone is a steroid sex hormone produced in the ovaries and involved in pregnancy, development, and the menstrual cycle. It has been demonstrated to have bone protective properties (22), and it is directly controlled by osteoblast progesterone receptors (23). Both osteoblasts and osteoclasts contain them (24,25). and are elevated by estrogen levels, which may indicate that progestogens are partially controlling the effects of estrogens on bone (26, 27 24). According to reports, progesterone inhibits bone resorption indirectly through metalloproteinases and glucocorticoid receptors in addition to directly affecting osteoblasts (22). The current study showed an increase in serum calcium levels in the ovariectomized rabbit group compared to the control group, where the OVX group was 32.37 +-0.41 higher than the control group. 9.22 +-0.80 . The amount of calcium, phosphorus, and magnesium in bone ash decreases as a result of ovariectomies. Both calcium and phosphorus are used as indicators of bone development, play a crucial part in bone 62 calcification, and are linked to osteoporosis and a loss in bone mass (28). Over 99 percent of calcium is contained in teeth and bone, making it a crucial element for bone production (29). The results of this study were consistent with (30), who noted a significant decrease in femur ash weight and a decrease in calcium and phosphorus in bone ash of ovx rabbits. Additionally, the findings of (31), who reported a reduction in bone ash weight, percentage ash, calcium, phosphorus, and magnesium in the ovx rabbits, agreed with the findings of this study. Since vitamin D improves the absorption of calcium and phosphorus, which are necessary for bone formation, the drop in calcium and phosphorus in bone ash may be caused by the ovx rabbits' low vitamin D levels. The current study showed an increase in serum FSH hormone in the ovariectomized rabbit group compared to the control group, where the OVX group was 22.56 +-0.424compared to the control group16.21+-0.42 . FSH's direct impact on bone cells. FSH causes osteoclast precursors to produce more TNFα and express more RANK. Additionally, it improved the processes that lead to the differentiation of osteoclasts (8). The current study showed an increase in serum LH hormone in the ovariectomized rabbit group compared to the control group, where the OVX group was23.12 +-0.88 compared to the control group 13.38 +-0.35 .

In this investigation, we used OVX as a model for estrogen insufficiency; however, OVX also causes high levels of gonadotrophins (FSH and LH) in addition to low levels of estradiol. It's likely that either an increase in FSH or a decrease in estradiol is the reason for the rise in BMAT after OVX (32). Additionally, elevated FSH levels may also influence adipocytes' production of RANKL (33). Nevertheless, the existing evidence indicates that secreted RANKL has little effect on bone turnover or volume (33, 34). The current study showed an increase in RANk in the ovariectomized rabbit group compared to the control group, where the OVX group 2.85 +-0.27 was higher than the control group 1.7416 +-0.17 RANk is expressed as a tumor necrosis factor that is mostly expressed on the surface of osteoclasts, which are involved in the process of bone resorption, because ovariectomy results in a decrease in estrogen, which is a major factor in osteoporosis and the activation of osteoclasts. This study supports the findings of (35). According to this study, the OVX group's RANKL increased significantly in the OVX group 264.006 +-26.56 compared to the control group's RANKL of 135.05 +-22.07. It is commonly acknowledged that the RANK/RANKL/OPG system is a crucial signal transduction route for preserving the balance of bone metabolism and OC differentiation (36, 37) .Estrogen inhibits osteoclastogenesis, which involves numerous signaling pathways, including RANA ligand (RANKL), receptor activator of nuclear factor-KB (RANK), and osteoprotegerin (OPG). RANKL promotes the activation and differentiation of osteoclasts (20). The present study showed that the serum concentration of OPG in the OVX group 31.15 +-1.87 was significantly lower than that of the control group 48.06 +-5.40 (38) To preserve bone density and stop loss, the ratio of OPG to RANKL must be balanced. Increased bone resorption results from RANKL's promotion of osteoclast production and activation by its binding to its receptor on the surface of osteoclast precursors. However, OPG suppresses the production and activation of osteoclasts and competitively binds to RANKL, blocking its interaction with RANK (39) Histological sections of the bones in the present study (control group) showed normal bone structure with a compact osteophyte and prominent osteolytic canals surrounding normal medullary cavities, as shown in Figures 10 and 11 Animals treated with bilateral ovariectomies OVX Figure 12 also revealed significant histological changes manifested by marked thinning of the osteophytes, characterized by thin walls with longitudinal views and irregular eroded borders, intense resorption and perforation of the compact bone with irregular atrophic osteocytes, and marked cavity formation with numerous typical multinucleated osteocytes emanating from the surface of the osteophytes and typical large multinucleated osteocytes in the osteophyte with irregular lamellar margins. Also in Figure 13, a histological section of the longitudinal femur of rabbits with bilateral ovariectomies OVX shows lacunae with osteocytes and a osteophyte plate without

osteocytes. Irregular margins with osteoclast infiltration and Haversian canal dilatation. These findings are scientifically consistent with previous studies by(40,41, 42)

CONCLUSION

Through the current study conclude that as low estrogen has a direct effect on osteoporosis and the deterioration of bone-forming tissues and cells.

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