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Role of 17-β Estradiol and Ramipril in OPG/RANKL Pathway in a Rat Model of Post-Menopausal Osteoporosis

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ABSTRACT

Background: Primary osteoporosis and other metabolic bone disorders have been linked to the proteins osteoprotegerin (OPG) and receptor activator of nuclear factor-kappa B ligand (RANKL).

Aim and objectives: Our study's goal was to examine the effects of co-administering estradiol (E2) and ramipril (ACEI) on bone markers in ovariectomized rats and to assess the potential interactions between these medications in order to address the function of the OPG/RANKL system as a potential mechanism of action.

Materials and methods: 40 female rats, randomly divided into 5 groups, each group included 8 rats. • Group 1: Control group (sham operated). Group 2: Ovariectomized rats (OVX). Group 3: Ovariectomized rats (OVX)+E2. Group 4: Ovariectomized rats (OVX)+ACEI. Group 5: Ovariectomized rats (OVX)+E2+ACEI.

Results: OVX rats showed a significant decrease in serum Ca^{2+} and OPG levels with significant increase in serum RANKL, osteocalcin, alkaline phosphatase activity and urinary hydroxyproline levels compared to control group. Treatment with ramipril as well as E2 led to a significant improvement in bone markers levels with a significant increase in serum OPG level with a significant reduction in serum RANKL level compared to OVX group.

Conclusion: Ramipril as ACEI had more significant effect on decreasing serum bone markers level than 17-β estradiol in ovariectomized rats. So, we can draw the conclusion that altering OPG/RANKL signalling may be a possible mechanism by which E2 and ACEI prevent osteoporosis.

Keywords: 17-β Estradiol; Ramipril; OPG/RANKL; Rat Model; Post-Menopausal Osteoporosis.

INTRODUCTION

Osteoporosis, a systemic skeletal disorder characterised by decreasing bone mass, is one of the most significant issues impacting postmenopausal women, deteriorated bone tissue, and increased fragility and fracture susceptibility **(1).**

Bone is a dynamic tissue that undergoes ongoing remodelling as a way to integrate external chemical, hormonal, and biomechanical cues as well as its own internal mechanism for self-regeneration. At the cellular level, bone remodelling occurs in a series of recurrent cycles of bone production by osteoblasts, which are descended from pluripotent mesenchymal stem cells, and bone resorption by osteoclasts, which are derived from the monocytic/macrophagic lineage **(2).**

The majority of metabolic bone illnesses are brought on by disturbances in osteoclast quantity or activity, which lead to abnormally high levels of bone resorption that are greater than osteoblasts' ability for compensation **(3)**. The most common option for simulating some of the most crucial clinical aspects of postmenopausal bone loss is the ovariectomized (OVX) rat model **(4)**.

It's well known that the estrogen exerts a direct protective effect on the bone through its receptors; it decreases the rate of production of osteoclasts, their activity and their survival and exerts an anabolic effect on osteoblasts. Additionally, oestrogen is known to control the development and activity of various pro-inflammatory cytokines, including RANKL, which has been shown to increase bone resorption and limit bone formation **(5).** One accurate measure of the differentiation status of the osteoblast and osteoclast is the OPG/RANKL ratio, and the RANKL/RANK/OPG system has been related to a range of skeletal and immune-mediated illnesses, including numerous types of osteoporosis, which are characterised by accelerated bone resorption and bone loss (postmenopausal, glucocorticoid-induced, and senile osteoporosis **(6).**

In order to assess the potential effects of ramipril (ACEI) and estradiol on bone markers in oestrogen deficiency-induced bone loss in ovariectomized rats, our study set out to compare the two drugs, to discuss the role of OPG/RANKL system as a possible mechanism of action, also to compare the effect of co-administration of estradiol and ramipril on bone markers in ovariectomized rat.

MATERIALS AND METHODS

The goal of the current investigation was to assess the potential effects of ramipril (ACEI) and estradiol on bone markers in rats with estrogen deficiency-induced bone loss as well as to discuss the potential relevance of the OPG/RANKL ratio as a mechanism of action.

Ethical consideration:

The experimental techniques and animal care were approved by Institutional Review Boards (IRB) at Faculty of Medicine's Research Ethics Committee, Cairo University. Code: MD- 252-2020. Rats were handled according to National Institutes of Health (NIH) guidelines for animal experimentation. This work

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has been carried out in accordance with the "Guide for the care and use of Laboratory Animals" for the use and welfare of experimental animals, published by the US National Institutes of Health (NIH publication No. 85–23, 1996).

Inclusion criteria: Species: Wister Rats, gender: female, age: 4–5-month-old and weight=200 -250 g.
Exclusion criteria: Used in prior studies/experiments, any signs/symptoms of illness.

Experimental animals: The 40 mature female Wistar rats used in the current investigation were between 200 and 250 g in weight. Throughout the trial, rats were housed at room temperature, fed on conventional rat cuisine, and given unlimited access to food and water. One week of acclimation time was permitted before the experimental protocol.
Experimental protocol: Prior to the trial, animals were given a week to get used to their surroundings. Water from the tap and standard rat food were accessible throughout the trial.

Rats were divided randomly into 5 groups (8 rats each): Group 1: Control group (sham operated), sham operated control rats received sesame oil orally by gavage daily for 8 weeks. **Group 2: Ovariectomized rats (OVX),** rats were subjected to ovariectomy at the beginning of the study then were administered sesame oil orally by gavage daily for 8 weeks from the day after surgery. **Group 3: Ovariectomized rats (OVX)+E2:** OVX rats received 17b-estradiol (from Sigma Chemicals, USA) 0.5 mg/kg dissolved in sesame oil daily orally by gavage for 8 weeks from the day after surgery [(7)]**. Group 4: Ovariectomized rats (OVX)+ACEI**: OVX rats received ramipril (from Sigma, USA) 5 mg/kg dissolved in water daily orally by gavage for 8 weeks from the day after surgery [(8)]**. Group 5: Ovariectomized rats (OVX)+ E2+ACEI:** OVX rats received E2 (0.5 mg/kg) and ramipril (5 mg/kg) daily orally for 8 weeks from the day after surgery.

Experimental drugs: Estradiol: We bought estradiol (E2) from Sigma Chemical Co. (St. Louis, MO, USA). Exogenous 17-estradiol (EB) is frequently used (E2). Sesame oil was used to dissolve the hormone. In group 3 and 5: OVX rats received 17b -estradiol 0.5 mg/kg dissolved in daily orally by gavage for 8 weeks. **Ramipril:** Ramipril (ACEI) was purchased from Sigma Chemical Co. (St. Louis, MO, USA). **Sesame Oil:** Sesame oil is a refined vegetable oil from sesame seeds, which are strangely high in oil, around half of seed weight [(9)]**.**

The following parameters were measured: Serum total calcium, osteocalcin in blood was examined using ELISA, urine hydroxyproline, alkaline phosphate activity[(10)], receptor activator of nuclear factor kappa B ligand (RANKL) concentrations, and osteoprotegerin (OPG) by ELISA.
Examination of histopathology utilizing a light magnifying instrument:

Femur Bone Staining: To examine the histopathological changes by light microscopy, the right femur from each rat was embedded in paraffin slices and stained with hematoxylin and eosin (H&E).

Statistical Analysis

Version 28 of the Statistical Package for the Social Sciences was used to code and input the data (IBM Corp., Armonk, NY, USA). The data were summarised using the mean and standard deviation. To compare the groups, an analysis of variance (ANOVA) with multiple comparisons of **Bonferroni** post hoc test were utilised. P-value of 0.05 or less was considered statistically significant·

RESULTS

OVX rats showed a significant decrease in serum Ca2+ with significant increase in serum osteocalcin, alkaline phosphatase activity and urinary hydroxyproline levels compared to control group. Treatment with Ramipril as well as E2 led to a significant improvement in bone markers levels compared to OVX group.

Table 1: The mean ± standard deviation of bone markers parameters measured in the five different groups

Bone markers	Control group	OVX group	17b -estradiol + OVX group	Ramipril + OVX group	17b -estradiol + ramipril + OVX group
Serum calcium (mg/dl)	9.51±0.07	7.13±0.31 *	9.16±0.04 *#	9.08±0.04 *#	9.29±0.02 *#
Serum osteocalcin (ng/ml)	4.33±0.09	6.55±0.24 *	4.33±0.03 #	4.21±0.05 #	4.33±0.03 #
Serum alkaline phosphatase activity (u/l)	164.52±0.6	245.94±1.05 *	174.44±0.44 *#	172.12±0.35 *#$	166.92±0.7 *#$@
Urinary hydroxyproline (mg/dl)	15.16±0.22	25.71±0.66 *	16.39±0.2 *#	15.76±0.12 *#$	15.24±0.14 #$@

OVX: ovariectomized, *: statistically significant compared to control group. #: statistically significant compared to OVX group. $: statistically significant compared to 17b -estradiol + OVX group. @: statistically significant compared to ramipril +OVX group.

OVX rats showed a significant decrease in OPG levels with significant increase in serum RANKL levels compared to control group. Treatment with Ramipril as well as E2 led to a significant improvement in serum OPG level with a significant reduction in serum RANKL level compared to OVX group.

Table 2: The mean ± standard deviation of serum RANKL and OPG measured in the five different groups

Biochemical measures	Control group	OVX group	17b -estradiol + OVX group	Ramipril +OVX group	17b -estradiol + ramipril + OVX group
Serum RANKL (pg/ml)	55.95±0.39	115.19±2.17 *	89.04±0.15 *#	78.8±0.35 *#$	59.9±0.29 *#$@
Serum OPG (pg/ml)	15.71±0.24	8.22±0.12 *	14.94±0.13 *#	15.09±0.12 *#	15.74±0.04 #$@

OVX: ovariectomized, OPG: osteoprotegerin, RANKL: receptor nuclear factor-kappa B ligand.
*: statistically significant compared to control group.
#: statistically significant compared to OVX group.
$: statistically significant compared to 17b -estradiol + OVX group.
@: statistically significant compared to ramipril +OVX group.

Histopathological study

Figures 1-5 show the effects of the used treatment in each group on sections from the femur bones.

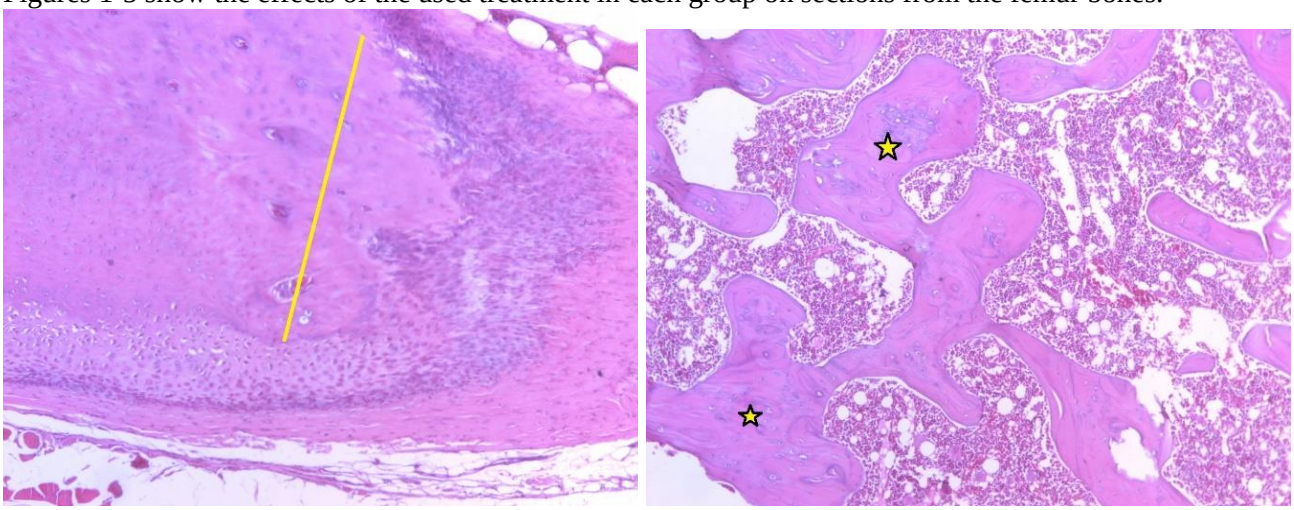

Figure 1: The photomicrograph of a section in the femur bone of control group **(Group I)** showing normal bone architecture with normal cortical bone thickness (CBT) (yellow lines) and normal trabecular bone density (TBD) with osteoblasts (yellow stars) (Hematoxylin and eosin stain, left X100; right X200).

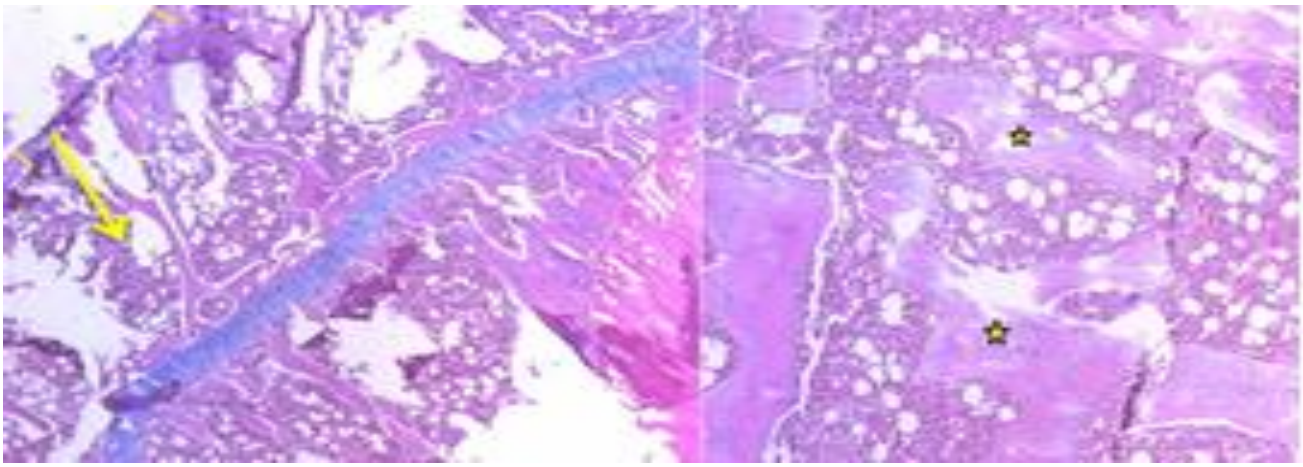

Figure 2: The photomicrograph of a section in the femur bone of OVX group **(Group II)** showing thinner cortical bone and discontinuous thin cancellous bone trabeculae having blind ends and areas of apparent faintly stained matrix together with minor fractures and multiple pores (decrease CBT) (yellow arrows) with areas of bone resolution (decreased TBD) (yellow stars) (Hematoxylin and eosin stain, left X100; right X200)

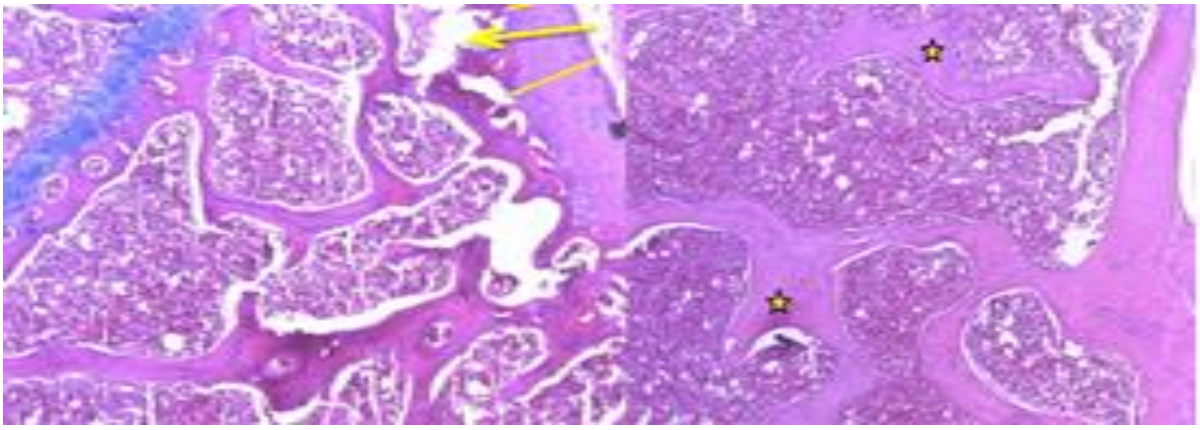

Figure 3: The photomicrograph of a section in the femur bone of OVX + E2 group.

(Group III) showing nearly normal CBT and increased osteoblastic number (yellow arrows) and mild reduction in TBD with new bone formation (yellow stars) (Hematoxylin and Eosin stain; right x100; let x200)

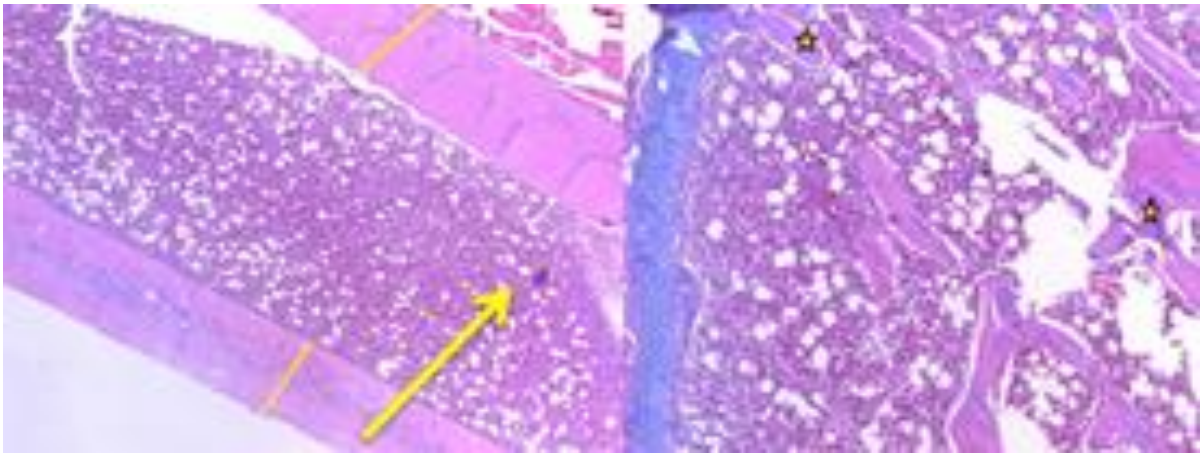

Figure 4: The photomicrograph of a section in the femur bone of OVX + ACEI group.

(Group VI) showing network of relatively thin trabeculae of cancellous bone and having faintly stained areas or small pores (yellow arrows) and apparent increased osteocytes inside their lacunae and regular continuous endosteal surface lining the bone marrow cavity (yellow stars) (Hematoxylin and Eosin stain, left X100; right X200)

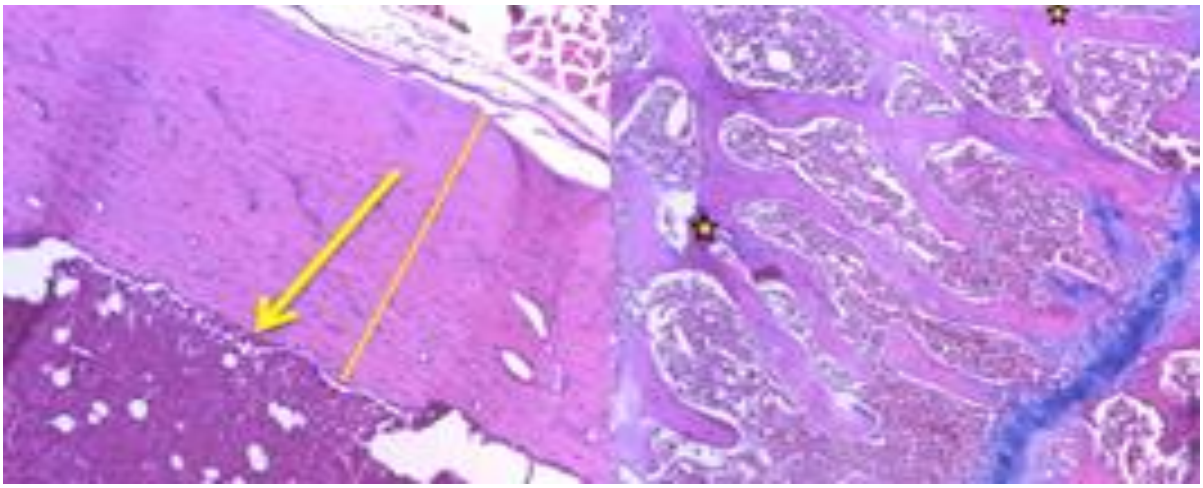

Figure 5: The photomicrograph of a section in the femur bone of OVX + E2 + ACEI group (**Group V)** showing normal CBT and configuration (yellow line) and TBD (yellow stars) (Hematoxylin and Eosin stain; X200)

DISCUSSION

The goal of the current study was to assess the potential effects of ramipril (ACEI) and estradiol on bone markers in rats with oestrogen deficiency-induced bone loss, as well as to address the potential involvement of the OPG/RANKL ratio as a mechanism of action. According to the results of the current investigation, ovariectomy caused the serum calcium levels of the OVX rats to significantly drop when compared to the control group. This was consistent with **Xiaohua *et al.*** [11], who found that plasma Ca^{2+} levels in the OVX rats considerably decreased compared to the sham operated control group 8 weeks after ovariectomy.

Because of increased mineral resorption and decreased intestinal absorption of calcium, a drop in oestrogen levels results in bone thinning [12]. Additionally, according to **Nie *et al.*** [12]**,** postmenopausal osteoporosis is linked to decreased oestrogen levels in the body as well as diminished calcium absorption in the intestines of C57BL/6 mice.

The consequences of present work showed that, bone turnover among OVX rats essentially expanded as proven by expanded bone development that was appeared by a critical expansion in plasma osteocalcin and serum alkaline phosphatase action, notwithstanding a huge expansion in bone resorption that was appeared by a critical expansion in urinary hydroxyproline level when contrasted and control bunch. These discoveries are steady with those of **Holstein *et al.*** [13], who detailed that ovariectomized rats showed an impressive expansion in bone turnover as seen by a critical ascent in both bone thickness and bone turnover markers. This is additionally reliable with the discoveries of the **Mukherjee *et al.*** [14] study, which showed that ovariectomized rats had more significant levels of the two markers. Since E2 lack expands the blend and action of various cytokines, including TNF alpha and RANKL, bone resorption and its related pointers are expanded [15].

Additionally**, Choudhary *et al.*** [16] found that the degrees of osteocalcin and alkaline phosphatase in the OVX group were significantly more prominent than those in the sham group **.** That's what our exploration uncovered, when contrasted with the sham group, ovariectomized rats had essentially higher blood RANKL levels and altogether lower serum OPG levels. This was in concurrence with the findings of **Xiaohua *et al.*** [11].

They discovered that there was a significant difference between the control group and the ovariectomized rats in the levels of serum OPG and RANKL. These findings are consistent with those from **Streicher *et al.*** [17], who found that an oestrogen deficit increases bone resorption, in part by up-regulating RANKL, which in turn causes bone loss. OPG binds to RANK and, by competitive inhibition, inhibits the binding of RANKL and RANK. A measure of osteoclast differentiation state is the OPG/RANKL ratio [18]. The findings of the current study demonstrated that, as compared to untreated ovariectomized rats, oral administration of ramipril and 17-estradiol to ovariectomized rats caused significant alterations in serum calcium levels. This is in line with **Ghosh and Majumdar** [19] findings that captopril had a protective impact on bone, and that ACEI-treated patients had higher bone mineral densities (BMD) and lower fracture risk. According to a study by **Rianon *et al.*** [20] the BMD of black men who used ACEI for a long time, including the whole hip joint, femoral neck, and total BMD, considerably increased as compared to the control group·

According to the current research, ovariectomized rats fed 17- estradiol and ramipril orally had significantly lower levels of blood alkaline phosphatase activity, osteocalcin, and urine hydroxyproline than untreated ovariectomized rats. In comparison to the OVX group, the levels of alkaline phosphatase and osteocalcin were significantly lower in the ACEI group and the oestrogen group.

Choudhary *et al.* [16] discovered, supporting the current study's finding that ACEI decreases osteoclast activity, reduces bone resorption, enhances osteoblast activity, increases bone mineral density, improves bone microstructure, and relieves osteoporosis symptoms in OVX rats. In the study of **Kaneko *et al.*** [21] as per the review, angiotensin II receptor type 1a (AT1a) is a negative controller of bone renovating as seen by the expanded bone turnover and bone mass in AT1a-lacking mice. Diminished osteoblast and osteoclast movement is brought about by AT1a motioning through non-cell independent components. Throughout directing the **Kaneko *et al.*** [21] examination, it was uncovered that both hereditarily and pharmacologically hindering the AT2 receptor prompts higher bone mass and expanded bone creation.

The aftereffects of present work showed that oral organization of 17-β estradiol in ovariectomized rats brought about critical decline in RANKL with the expansion in serum OPG. Likewise, E2 supplementation in **Garcia *et al.*** [22] study showed a significant expansion in serum OPG with the decline in RANKL. Furthermore, **Bord *et al.*** [23] showed that modulating this process may be important for oestrogen effects on osteoclast development in human cells as well, as evidenced by the fact that human osteoblasts respond to low dose oestrogen treatment by suppressing RANKL while preserving OPG expression.

The results of present work showed that oral administration of ramipril in ovariectomized rats resulted in significant decrease in RANKL with the increase in serum OPG. Moreover, ramipril had superior effect over 17-β estradiol in lowering serum RANKL and urinary hydroxyproline level. Also, co-administration of estradiol and ramipril were superior to administration of each of them alone in both increase of serum RANKL and decrease of serum OPG. Also, **Chen *et al.*** [24] reported that the administration with captopril in orchidectomized mice for six weeks enhanced the ratio of OPG/RANKL mRNA expression.

In this study we found that ramipril as ACEI has more significant effect on decreasing serum alkaline phosphatase activity and urinary hydroxyproline level than 17-β estradiol in ovariectomized rats. This can be explained by significant increase in OPG and significant decrease RANKL in rats treated with ramipril than 17-β estradiol treated rats. Also, co-administration of estradiol and ramipril were superior to administration of each of them alone. The same finding was reported by **Garcia *et al.*** [(22)] study; as they showed that ACEI has more significant effect on decreasing bone markers than E2 in OVX rats.

CONCLUSION

Ramipril as ACEI had more significant effect on decreasing serum alkaline phosphatase activity and urinary hydroxyproline level than 17-β estradiol in ovariectomized rats. So, we can draw the conclusion that altering OPG/RANKL signalling may be a possible mechanism by which E2 and ACEI prevent osteoporosis.

DECLARATIONS

- **Consent for Publication:** I attest that all authors have agreed to submit the work.
- **Availability of data and material:** Available
- **Competing interests:** None
- **Funding:** No fund
- **Conflicts of Interest:** The authors assert that there is no conflict of interest regarding the publishing of this paper.

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