International Journal of Pharmacology

ISSN 1811-7775

science alert

ansinet
Asian Network for Scientific Information

OPEN ACCESS International Journal of Pharmacology

ISSN 1811-7775
DOI: 10.3923/ijp.2021.217.228

Research Article
Synergistic Protective Effects of Resveratrol and Estradiol on Estrogen Deficiency-Induced Osteoporosis Through Attenuating RANK Pathway

[1]Basem H. Elesawy, [2,3]Hussein F. Sakr and [3,4]Amr M. Abbas

[1]Department of Clinical Laboratory Sciences, College of Applied Medical Sciences, Taif University, P.O. Box 11099, Taif 21944, Saudi Arabia
[2]Department of Physiology, College of Medicine and Health Sciences, Sultan Qaboos University, Muscat, Oman
[3]Department of Medical Physiology, Faculty of Medicine, Mansoura, University, Mansoura, Egypt
[4]Department of Medical Physiology, Faculty of Medicine, King Khalid University, Abha, Aseer, Saudia Arabia

Abstract

Background and Objective: Loss of ovarian function after menopause affects bone metabolism and structure. The present study examined the synergistic effects of Estradiol (E2) and Resveratrol (RES) on Bone Mineral Contents (BMC) and Bone Mineral Density (BMD) and biomarkers of bone remodelling in ovariectomized rats. **Materials and Methods:** For this purpose, this study was conducted on five (n = 10) groups of female Sprague Dawley rats which were classified into: (1) Sham group, (2) Ovariectomized rats (OVX), (3) Ovariectomized then treated with resveratrol (OVX+RES), (4) Ovariectomized treated with estradiol (E2) (OVX+E2) and (5) Ovariectomized treated with (OVX+RES+E2). After 16 weeks, we measured Bone Mineral Density (BMD), Bone Mineral Content (BMC) were assayed in the tibia. Also, we assayed the serum levels of the bone turnover markers as deoxypyridinoline (Dpd), N-telopeptide of type I collagen (NTxI), Alkaline Phosphatase (ALP) and osteocalcin (OC) as well as Receptor Activator of Nuclear Factor Kappa B (RANk) and Osteoprotegerin (OPG). **Results:** OVX reduced both BMD, BMC, OPG, ALP, OC and increased RANK, DPD and NTXI significantly ($p<0.05$) as compared to sham rats. RES or E2 corrected the hypogonadism induced osteoporosis by inhibiting osteoclastogenesis as compared to OVX rats. RES and E2 produced comparable effects as compared to RES and E2 treated rats. **Conclusion:** As RES and E2 increased BMD and BMC after ovariectomy a combination of RES and E2 might be used as a potential therapeutic for osteoporosis in the female after menopause.

Key words: Osteoporosis, resveratrol, female hypogonadism, osteocalcin, bone mineral content, bone mineral density, collagen

Citation: Elesawy, B.H., H.F. Sakr and A.M. Abbas, 2021. Synergistic protective effects of resveratrol and estradiol on estrogen deficiency-induced osteoporosis through attenuating RANK pathway. Int. J. Pharmacol., 17: 217-228.

Corresponding Author: Hussein F. Sakr, Department of Physiology, College of Medicine and Health Sciences, Sultan Qaboos University, Muscat, Oman

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

Menopause is known as the lasting termination of menstruation resulting from loss and atresia of the ovarian follicles[1,2]. The loss of ovarian activity produces a change in the hormonal pattern, with a decrease in the levels of circulating estrogen affecting many tissues of the body and producing a variety of signs and symptoms[3]. Bilateral ovariectomy either surgically or by drugs in rats was used for studying the effects of ovarian hormone absence in human menopause[4]. This procedure results in a rapid dramatic loss of ovarian function rather than a gradual decline that occurs in perimenopause[5]. The ovariectomized rat with female hypogonadism model remains the most popular choice as it has been proven to represent some of the most important clinical features of human menopause as bone loss[6], cardiovascular dysfunction[7], metabolic changes[8] and Oxidative Stress (OS)[9]. Treatment of the hot flushes and nervousness with estrogen is markedly utilized, however, its use revealed multiple drawbacks including increased risks of breast, endometrial and ovarian cancers[10]. Given the former risks, diverse authors suggest alternative ways of treatment for menopausal disorders including non-hormonal drugs, herbal remedies, minerals, antioxidants and vitamins[11].

Osteoporosis is known as a systemic skeletal disease characterized by loss of bone mass and density with deterioration of the micro architectural as well as a consequent upstroke in bone fragility and susceptibility to fracture[12]. According to data released by the World Health Organization (WHO), osteoporosis affects approximately 75 million people throughout Europe, the USA and Japan[13]. The incidence of osteoporosis increases dramatically with life expectancy. Accordingly, the risk of osteoporotic fractures and their associated costs is rising rapidly due to population ageing[12]. In the elderly, hip fractures are closely associated with mortality[14]. Accumulating data have indicated that the loss of estrogen at menopause is a major contributor to the pathogenesis of osteoporosis because this hormone is a principal negative regulator of osteoclast activity and osteoclasts are the chief effector's cells responsible for bone resorption in osteoporosis. Osteoporosis is twice as common in women as in men and approximately one in three women over 50 years old experiences an osteoporotic fracture in their lifetime[12].

Recently, it is reported that the route of action of estrogens on bone remodelling signalling involves osteoprotegerin (OPG), receptor activator of NF-κB (RANK) and RANK ligand (RANKL), identified as the major factors involved in osteoclastogenesis. RANK is located on the surface of mature osteoclasts and their precursors, while RANKL is a protein that belongs to the Tumour Necrosis Factor (TNF) family[15]. The main role of RANKL is to inhibit apoptosis of osteoclasts and stimulate the differentiation and activation of these cells. OPG directly inhibit the binding RANKL with RANK; thus its effects are antagonistic to the effects of RANK[15]. Estrogens affect osteoclastogenesis by regulating the production of RANKL/OPG by stromal cells and osteoblasts[16]. After menopause, bone loss arises because the RANKL action is favoured above the OPG. The decreased estrogen level reduces the OPG activity and improves the RANKL activity, resulting in increased resorption and bone loss[16,17].

In recent years, the potential benefits to bone health of the natural compound resveratrol (RES) have attracted attention. Resveratrol is a polyphenolic phytoestrogen that naturally occurs in the skin of red grapes, various other fruits, peanuts and root extracts of the weed *Polygonum cuspidatum*[18,19]. *In vitro* evidence has shown the ability of Res to promote the survival and activity of osteoblasts and suppress the differentiation and action of the osteoclasts (bone-resorbing cells)[20,21]. Also, *in vitro* RES increased the activity of Alkaline Phosphatase (ALP) (a biomarker for osteoblast differentiation) in a dose-dependent manner, thus indicating its ability to stimulate differentiation of osteoblasts[22]. However, the *in vivo* effect of Res is still not well-demonstrated.

The involvement of oxidative stress in osteoporosis is of growing interest[23]. Some studies demonstrated that oxidative stress is an important mediator of bone loss in postmenopausal osteoporosis by generating a more oxidized bone microenvironment[24]. *In vivo* support of this hypothesis is found from experiments in which OVX induces oxidative stress and impairs bone antioxidant expression in adult rats[25]. Moreover, it was reported that oxidative stress levels were negatively associated with bone mineral density and antioxidant levels were lower in osteoporosis patients[26]. In addition, vitamin C intake showed beneficial effects in increasing bone mineral density in women and, more recently, NAC and vitamin C inhibited ovariectomized-induced bone loss in a rodent osteoporosis model[27].

Therefore, this work aimed to compare the effects of the estrogen replacement therapy (17 β estradiol) and antioxidant resveratrol supplements on osteoporosis in ovariectomized rats.

MATERIALS AND METHODS

Study area

Animals housing and diet: Fifty female Sprague-Dawley rats, 12-14-weeks-old, weighing 365±10 g were purchased from the Vaccine and Immunization Authority (Helwan, Cairo, Egypt) and housed (Animal House, Department of Medical Physiology, Faculty of Medicine, Mansoura University, Egypt) under same conditions (temperature of 23±1°C and a 12 hrs light/dark cycle). The animals were allowed free access to food and tap water. Experiments were performed according to the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH publication No. 85-23, revised 1996). All experimental procedures in this study were approved by the Medical Research Ethics Committee of Mansoura University, Egypt. The study was carried out in the Department of Medical Physiology, College Of Medicine, Mansoura University between May, 2018-December, 2019.

Experimental design: After 1 week of acclimatization to the laboratory environment, the animals were randomly divided into five groups of ten rats each. Group 1 (sham) included sham-operated rats that underwent sham surgical procedure received the vehicle (dimethyl sulfoxide) (Sigma, St. Louis, MO, USA) daily by oral gavage for 16 weeks. Group 2 (OVX) included OVX rats that were subjected to ovariectomy at the beginning of the experiment through a midline incision under ether anaesthesia and allowed to recover while being treated with the vehicle (dimethyl sulfoxide) (Sigma, St. Louis, MO, USA) daily by oral gavage for 16 weeks. Group 3 (OVX+E2) included OVX rats that received 17b-estradiol (E2) (25 µg/kg/day, orally by gavage) for 16 weeks[28]. Group IV (OVX+RES) included OVX rats that received RES (45 µg/kg/day, orally by gavage)[29] 16 weeks. Group V (OVX+E2+RES) included OVX rats that received E2 and RES in the same route and doses[28,29], as previously mentioned, for 16 weeks. All the drugs were dissolved in the vehicle (dimethyl sulfoxide) (Sigma, St. Louis, MO, USA). Treatments were initiated 4 weeks after OVX and lasted for 16 weeks.

Sampling protocol

Blood samples: At the end of the experimental period, blood samples were collected by cardiac puncture under diethyl ether anaesthesia. These blood samples were collected without anticoagulant, left for 10 min and then centrifuged for 10 min at 4,000 r min^{-1} to obtain serum, which was stored at -20°C until further biochemical analysis for determination of serum calcium, Alkaline Phosphatase (ALP), osteocalcin, acid phosphatase, osteoprotegerin and RANKL.

Tissue samples: Tibias from rats of all groups were harvested. The right tibia from each rat was stored in formalin buffer 10% for dual-energy X-ray absorptiometry (DEXA). Bone mineral density (BMD) and bone mineral content (BMC) of each tibia were measured by DEXA using Norland XR-46, version 3.9.6/2.3.1 instrument (Norland X-R-46 version 3.9.6, Peachtree City, GA, USA) equipped with dedicated software for small animal measurements. The other tibia from each rat was stored in liquid nitrogen for gene expression of RANKL and osteoprotegerin.

Image analysis procedure: Tibia from each rat was embedded in paraffin sections and was stained with hematoxylin and eosin for image analysis technique as following, Slides were photographed using an Olympus® digital camera installed on the Olympus® microscope with 1/2 X photo adaptor, using a 40X objective. It is made in Vietnam. The resulting images were saved as TIFF files to be analyzed with Intel® Core I3® based computer using Video Test Morphology® software (Russia) with a specific built-in routine for length and area fraction measurement.

Cortical bone thickness was measured by a freehand line tool which is calibrated against a micrometre slide photographed under the same conditions.

The trabecular bone area was manually extracted with the aid of a genius® G-Pen F509® tablet. The total area of the extracted trabecular bone was measured. Then the empty areas were subtracted according to colour variation and the area was measured again. Area fraction was calculated in percentage. Mean Cortical Bone Thickness (CBT) (µm) and mean trabecular bone density (TBD) (%) were obtained by measuring 5 fields/slide from 5 slides for each rat. The reading of each animal was considered as one variable.

Serum measurements: Serum total calcium (25) and alkaline phosphatase (ALP) activity (26) were measured by colorimetric method (Chemroy, Biochemical Trade, Inc. USA) in Beckman Coulter DU-70 spectrophotometer (Beckman Coulter Inc., CA, USA). and osteoprotegerin (OPG), Tartrate-resistant acid Phosphatase 5b, Cross-linked N-Telopeptide of type 1 collagen (NTXI), osteocalcin (OC), Deoxypyridinoline (DPD) and Receptor Activator Of Nuclear Factor Kappa B (RANk) were determined using rat specific ELISA kits (Cat. No. MBS2700368, Cat. No. MBS27101236, Cat. No. MBS2702692; Cat. No. MBS2700254, Cat. No. MBS2701838, Cat. No. MBS2506789 and Cat. No. MBS2704130, respectively) (MyBioSource, CA, USA). All samples were analyzed for n = 10 rats per group and as per the manufacturer's instructions.

Estimation of tibial bone oxidative and anti-oxidative parameters: The tibia was cleaned from surrounding soft tissue and specimens were removed, weighed, crushed and homogenized in cool distilled water and then centrifuged at 3000 r.p.m. the supernatant was kept at -80°C until used for the analysis of MDA, GSH, CAT and SOD using a colorimetric kit (Bio-Diagnostics, Dokki, Giza, Egypt) according to the manufacturer's instructions.

Statistical analysis: Statistical analysis for all measured parameters was done using GraphPad Prism statistical software package (Version 8/Australia). Normality was tested using the Kolmogorov-Smirnov test. Differences among the experimental groups were assessed by the Kruskal-Wallis non-parametric analysis. Data were presented as Mean±SD. Values will be considered significantly different when $p = 0.05$.

RESULTS

Resveratrol and estradiol increased BMD and BMC in ovariectomized rats: As depicted in Fig. 1a-b, female hypogonadism by ovariectomy decreased both BMD and BMC significantly ($p<0.01$) as compared to the sham group. In response the oral intake of RES, E2 and both of them significantly ($p<0.01$) increased the BMD and BMC as compared to the ovariectomized rats. RES increased both BMD and BMC respectively. While E2 increased both BMC and BMD. Administration of both them increased BMC and BMD to a value non-comparable from the sham rats.

Resveratrol and estradiol increased both cortical and trabecular bone thickness in ovariectomized rats: As depicted in Fig. 2a-b, surgical ovariectomy decreased the CBT and TBD significantly ($p<0.01$) by 36 and 43% as compared to the sham group. Oral intake of RES increased CBT and TBD significantly ($p<0.05$) by 17 and 15%, respectively. However, there was a significant ($p<0.05$) increase in both CBT and TBD as compared to OVX and OVX+RES. Both RES and E2 increased both CBT and TBD significantly as compared to OVX, OVX+RES and OVX+E2 and insignificant to sham rats.

Resveratrol and estradiol increased OPG and decreased RANK: As depicted from Fig. 3a-b, the ovariectomized rats showed a significant increase in RANK ($p<0.001$) with decreased OPG of 59% ($p<0.05$). However, RES and E2 either separated or in combination increased the inhibitor of bone resorption OPG significantly ($p<0.01$) and decreased significantly ($p<0.01$) the bone resorption marker (RANK) compared to OVX rats. Interestingly, RES and E2 produced incomparable changes ($p>0.05$) to the sham group.

RES and E2 decreases circulatory levels of DPD, TRAP 5b and NTXI: As depicted from Fig. 4a-c, the bone resorption markers (DPD, TRAP 5b and NTXI) increased significantly ($p<0.01$) in OVX rats as compared to sham rats. E2 or RES decreased DPD, TRAP 5b and NTXI significantly ($p<0.05$) as compared to OVX rats. In addition, treatment of female hypogonadism with combined E2 and RES significantly decreased ($p<0.05$) and even normalized the bone resorption markers as compared to the other studied groups.

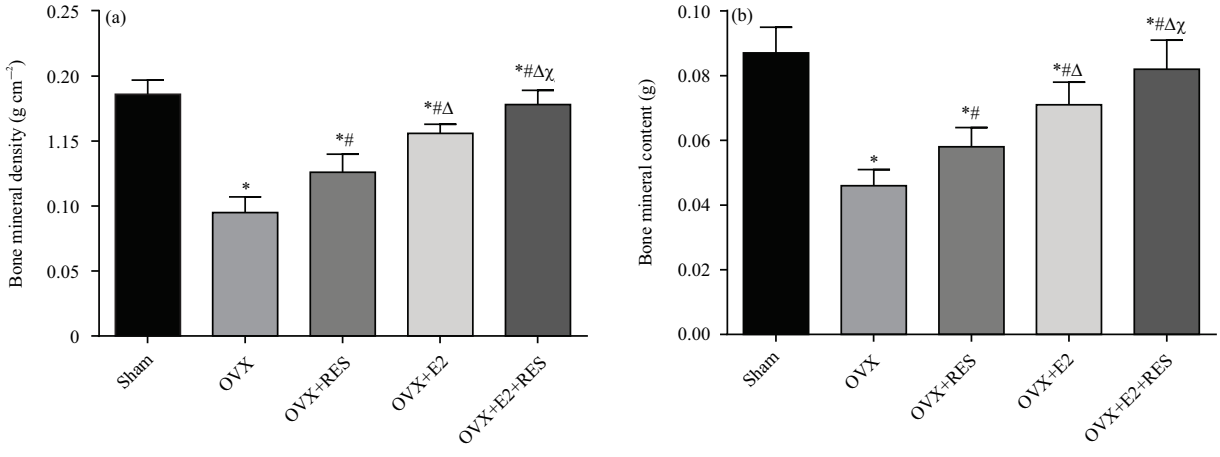

Fig. 1(a-b): Effect of ovariectomy, resveratrol and estradiol treatment on bone mineral density (BMD) and bone mineral content (BMC)

Data were expressed as mean±SD of 10 rats. *$p<0.05$ versus sham, #$p<0.05$ versus OVX, Δ: $p<0.05$ versus OVX+RES, χ: $p<0.05$ versus OVX+E2. OVX: Ovariectomized, RES: Resveratrol and E2: Estradiol

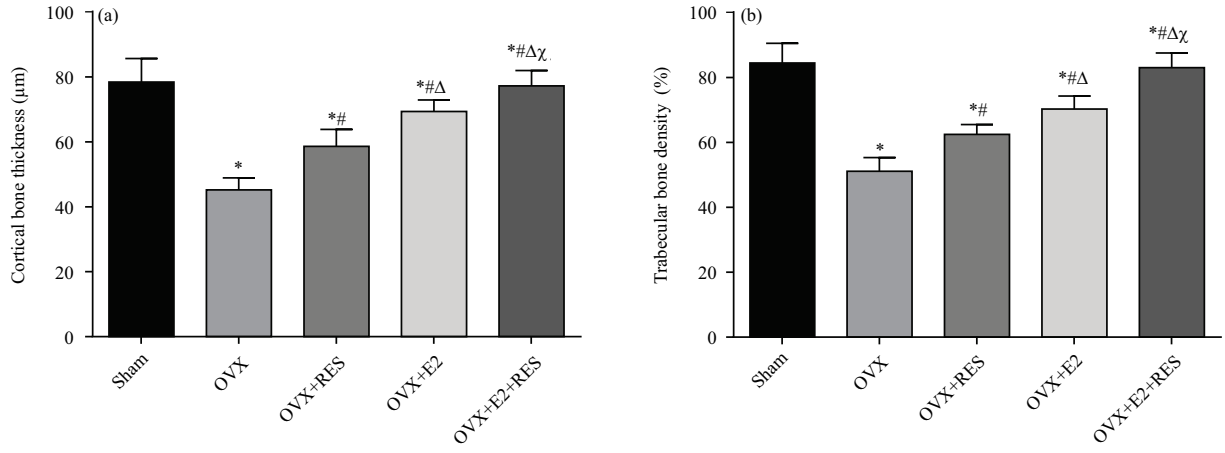

Fig. 2(a-b): Effect of ovariectomy, resveratrol and estradiol treatment on cortical bone thickness (CBT) and trabecular bone density (TBD)

Data were expressed as Mean±SD of 10 rats. *p<0.05 versus sham, #p<0.05 versus OVX, Δ: p<0.05 versus OVX+RES, χ: p<0.05 versus OVX+E2. OVX: Ovariectomized, RES: Resveratrol and E2: Estradiol

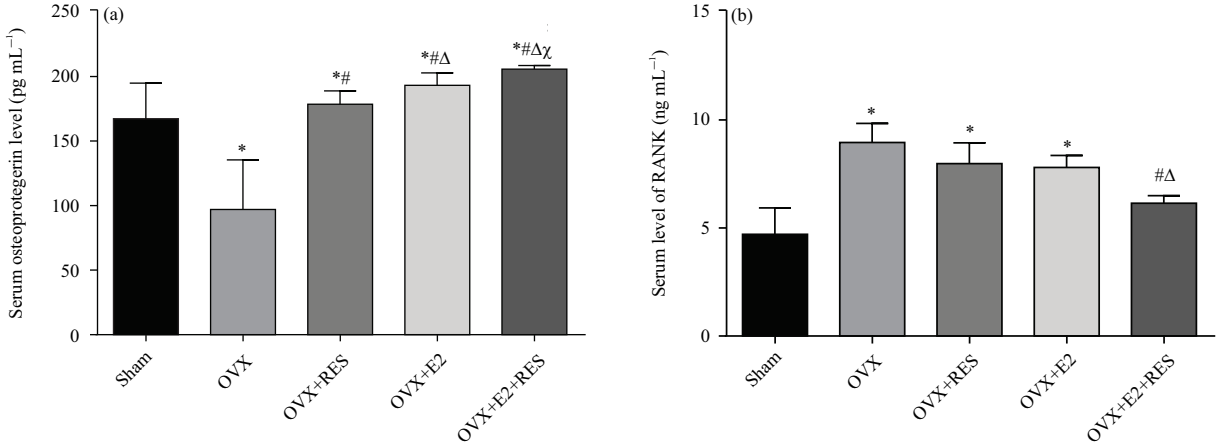

Fig. 3(a-b): Effect of ovariectomy, resveratrol and estradiol treatment on serum level of OPG and RANK

Data were expressed as Mean±SD of 10 rats. *p<0.05 versus sham, #p<0.05 versus OVX, Δ: p<0.05 versus OVX+RES, χ: p<0.05 versus OVX+E2. OVX: Ovariectomized, RES: Resveratrol and E2: Estradiol, OPG: Osteoprotegerin, RANK: Receptor activator of nuclear factor kappa B

Resveratrol and estradiol increases OC and ALP: As depicted from Fig. 5a-b, the bone formation markers were reduced significantly (p<0.05) in OVX rats as compared to sham rats. In response to E2 or RES, the serum level of OC and ALP increased significantly (p<0.05) versus the ORX rats. E2 and RES normalized the levels of OC and ALP and increased significantly (p<0.05) as compared to OVX, OVX+E2 and OVX+RES.

Resveratrol and estradiol ameliorated the oxidative stress parameters: As depicted from Table 1, OVX decreased the levels of the antioxidant enzymes [SOD from 54.14±3.14-27.14±2.51 U g^{-1} tissue, CAT from 0.52±0.02-0.27±0.0 U g^{-1} tissue and GSH from 6.42±0.12-5.75±0.19 mg g^{-1}] tissue significantly, (p<0.05) and increased MDA [from 8.21±0.14-11.34±0.16 nmol g^{-1} tissue] significantly (p<0.05) as compared to sham rats. Administration of Res or E2 increased the antioxidant enzymes significantly (p<0.05) and decreased MDA significantly (p<0.05) as compared to OVX rats. In addition, combined treatment of the ovariectomized rats with Res and E2 normalized the level of the antioxidant enzymes and decreased MDA as compared to sham rats.

Correlations between different studied parameters: As depicted from Fig. 6a-b there was a negative correlation between serum OPG level and MDA (r = -0.9177; p<0.001), while there was a positive correlation between serum RANKL and MDA (r = 0.9580; p<0.001).

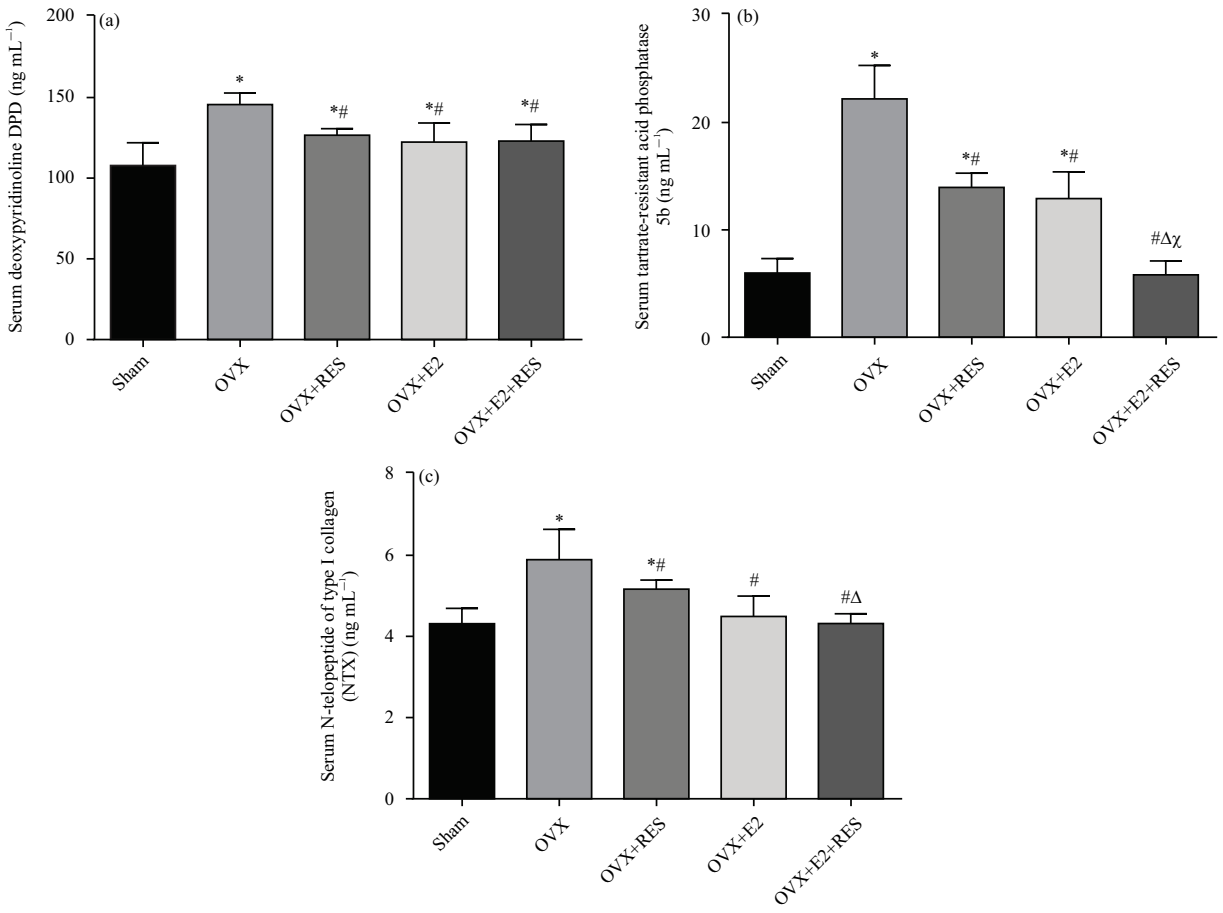

Fig. 4(a-c): Effect of ovariectomy, resveratrol and estradiol treatment on serum level of bone resorption markers (DPD, TRAP-5b and NTXI)

Data were expressed as Mean±SD of 10 rats. *p<0.05 versus sham, #p<0.05 versus OVX, Δ: p<0.05 versus OVX+RES, χ: p<0.05 versus OVX+E2. OVX: Ovariectomized, RES: Resveratrol and E2: Estradiol, Dpd: Deoxypyridinoline, NTXI: N-telopeptide of type I collagen, TRAP-5b: Tartrate-resistant acid phosphatase 5b

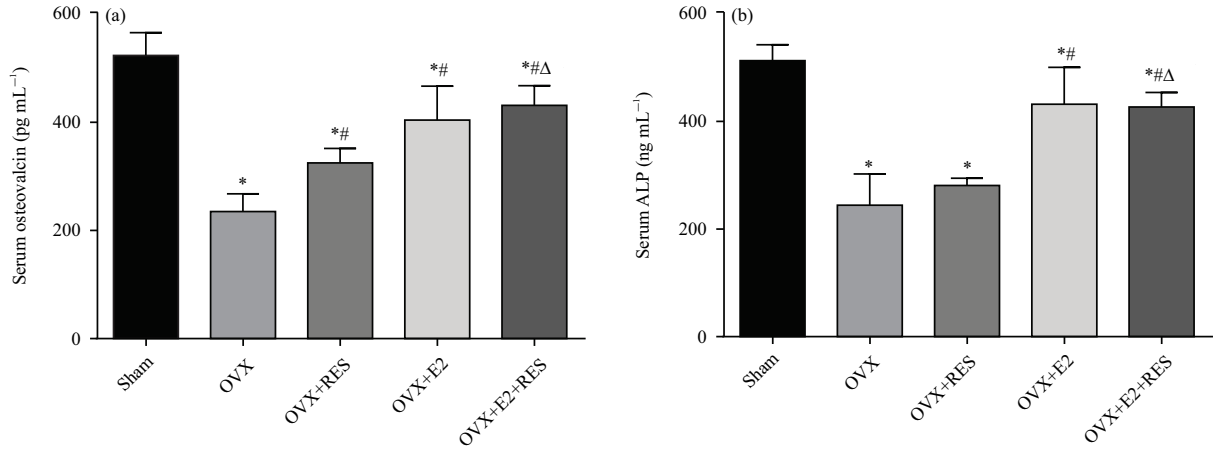

Fig. 5(a-b): Effect of ovariectomy, resveratrol and estradiol treatment on serum level of bone formation markers (OC and ALP)

Data were expressed as Mean±SD of 10 rats. *p<0.05 versus sham, #p<0.05 versus OVX, Δ: p<0.05 versus OVX+RES, χ: p<0.05 versus OVX+E2. OVX: Ovariectomized, RES: Resveratrol and E2: Estradiol, OC: Osteocalcin, ALP: Alkaline phosphatase

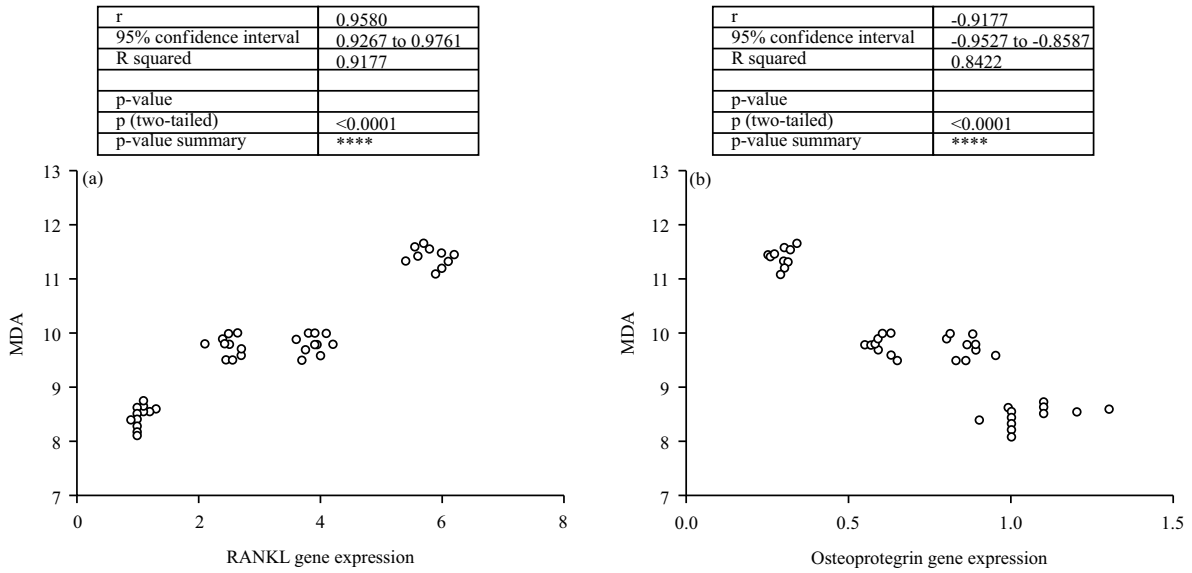

Fig. 6(a-b): Pearson correlation serum (a) OPG level and MDA and (b) RANK level and MDA
OPG: Osteoprotegerin, RANK: Receptor activator of nuclear factor kappa B

Table 1: Effect of 17 β estradiol and resveratrol on MDA, GSH, SOD and CAT in the tibia of OVX rats

Parameter	Sham	OVX	OVX+RES	OVX+E2	OVX+E2+RES
MDA (nmol g^{-1} tissue)	8.21±0.14	11.34±0.16*	9.61±0.12*#	9.87±0.13*#	8.55±0.24#Δ
GSH (mg g^{-1} tissue)	6.42±0.12	4.15±0.11*	5.75±0.19*#	5.62±0.18*#	6.22±0.17#Δ
SOD (U g^{-1} tissue)	54.14±3.14	27.14±2.51*	38.14±2.61*#	36.14±2.43*#	49.21±2.91#Δ
CAT (U g^{-1} tissue)	0.52±0.02	0.27±0.02*	0.39±0.02*#	0.36±0.02*#	0.47±0.02#Δ

Effect of ovariectomy, resveratrol and estradiol treatment on, MDA: Malondialdehyde, GSH: Glutathione, SOD: Superoxide dismutase and CAT: Catalase. Data were expressed as Mean±SD of 10 rats. *p<0.05 versus sham, #p<0.05 versus OVX, Δ: p<0.05 versus OVX+RES, χ: p<0.05 versus OVX+E2. OVX: Ovariectomized, RES: Resveratrol and E2: Estradiol

DISCUSSION

The salient findings of our study showed that female hypogonadism induced in rats by the bilateral OVX protocol decreased the estrogen levels, reduced BMD and BMC the tibia of rats and increased serum levels of major markers osteoporosis, namely, RANKL, osteocalcin, acid phosphatase. These findings were also associated with a concomitant reduction in serum levels of OPG. Additionally, the antioxidant enzymes decreased significantly with the significant increase in the MDA.

However, RES and E2 co-treatment significantly attenuated the loss in BMD and BMC and alleviated all other biochemical alterations induced by OVX.

Bone remodelling is continuing through adult life and is regulated by the balance between osteoclasts and osteoblasts activity. As a result of low estrogen level, it is markedly prevalent after menopause, thus making such patients susceptible to bone fracture. The major sits for bone fractures are the vertebrae, hips and wrist[30]. For those with hip fracture, there is overall mortality of up to 33%[31]. Many are unable to walk independently at one year[32]. Menopause and reduced circulatory sex hormone levels are major treatable causes of osteoporosis in women. Indeed, it is currently well-established that the incidence of osteoporosis in women is indirectly correlated to the reduction in circulating estrogens[33,34]. Nevertheless, locally produced and circulating sex steroid hormones can module bone formation due to the presence of aromatase, estrogen and androgen receptors[35].

The possible negative impact of estrogen deficiency on BMD and BMC was also confirmed in this rat's study. Herein, we are showing a significant reduction in the BMD and BMC in the tibia of OVX-induced rats. These findings are in agreement with the studies of Scopacasa *et al.*[36] who have shown a positive correlation with serum levels of estrogen and BMD. Supporting our data is also the finding that hip fracture incidence is increased in hypogonadal aged women.

On the other hand, the bone is continuously remodelled by bone resorption and formation mediated by both the osteoclasts and osteoblasts respectively[37]. The delicate balance between the activity of osteoblasts and osteoclasts determines skeletal integrity[38]. Osteoblasts normally secrets

RANK which acts through special receptors to suppress osteoclast apoptotic and stimulates the differentiation of the hematopoietic stem cells into mature osteoclasts[37]. OPG is the natural inhibitor of RNAK that inhibits osteoclastogenesis[38]. Nonetheless, Osteocalcin is released from mature osteoblasts and hypertrophic chondrocytes to stimulate bone mineralization BMD[39]. However, ALP is considered a valid marker for the activation of osteoblasts[40]. Also, TRAP 5b, secreted from the active osteoclasts, as we all NTXI and DPD which are released due to collagen degradation, are novel markers of bone resorption and low BMD[41,42].

To explain the decrease in BMD and BMC after OVX, we have evaluated the activities of the osteoblasts and osteoclasts by measuring the above-mentioned markers. In this study, the induction of menopause by ORX significantly increased serum levels of TRAP 5b, NIXI, DPD and RANK levels and concomitantly suppressed those of OPG and ALP, thus confirming a state of osteoclastogenesis and bone loss, in absence of estrogen. Based on these observations, we can strongly argue that estrogen deficiency is associated with increased osteoclasts activity and bone loss. These results are supported the available *in vitro* evidence which has shown that estrogen inhibits the activity of isolated osteoclasts[43]. Also, it supports the findings of others who have demonstrated that the late-onset of female hypogonadism is associated with enhanced RANK activity[44,45]. In line with these findings, previous studies reported a significant increase in serum level of RANKL in the femur of ovariectomized rats[46]. Furthermore, several other studies have shown that the urinary execration rates of DPD and NTXI are increased in patients who have low levels of testosterone and estrogen without any replacement therapy[47].

Moreover, in the present study, we found that E2 alone or in combination with RES caused a significant rise in the serum level of OPG with a significant decrease in RANK suggesting that E2 could inhibit the bone resorption and osteoclastogenesis via targeting the RANK/OPG signalling pathway. Studies performed on animal models of estrogen deficiency showed that knockout of both the α-estrogen receptor and the aromatase gene in mice leads to lower BMD than in wild-type mice. This is similar to what happens when aromatase inhibitors are used in rodents[48]. In agreement with these findings, Wu *et al.*[49] found that inhibition of RANK ameliorates bone resorption in OVX rats.

On the other hand, the novelist finding reported here is the protective effect of Res combined with E2 on bone resorption and stimulation of bone formation in OVX rats.

The markers of bone remodelling are secreted from the bone matrix or bone cells are grouped into 2 categories; bone resorption markers such as C- or N-terminal telopeptide of type I collagen (CTX or NTX) and pyridinolines that promote the bone breakdown and bone-forming markers such as procollagen type I N-terminal propeptide (PINP), alkaline phosphatase (ALP), bone-specific alkaline phosphatase, or osteocalcin that help in bone formation. So, in the current research, we measured the effect of E2 and RES on the bone resorption biomarkers including DPD, NTXI and TRAP-5b and bone-forming biomarkers including OC and ALP in ovariectomized rats. So, in the current study, we found a significant rise in the bone resorption biomarkers with a significant increase in bone resorption biomarkers. Following these findings, Sims *et al.*[50] found a systemic increase in bone resorption markers at 6 days post ovariectomy. Furthermore, this was confirmed by other studies by Yoon *et al.*[51].

Tartrate-resistant Acid Phosphatase (TRACP) is a bone-resorbing biomarker that is expressed in activated macrophages, dendritic cells and osteoclasts[52]. Serum TRACP5b is produced from bone-resorbing osteoclasts and its assay reflects the occurrence of bone resorption in multiple systemic diseases that influence bone turnover[53]. Also, it has been confirmed that serum N-telopeptide of type I collagen (NTXI) is a subtle indicator for bone resorption[54]. Also, NTXI is excreted in urine after bone resorption and considered as urinary markers of bone resorption[55]. Moreover, Deoxypyridinoline (DPD) is released into the blood as a result of collagen decomposition during bone turnover and considered as a specific marker of bone resorption[56].

Accordingly, we are showing that chronic administration of RES significantly improved values of BMD and BMC in the tibia with the attenuation of all osteoporosis markers (NIXI, TRAP 5b and DPD). However, the mechanism of protection involves increasing circulatory levels of osteocalcin, OPG and ALP. Similar to our data, RES inhibited osteoclastogenesis in an ovariectomized (OVX) rat model of osteoporosis by decreasing RANKL and TRAP 5b and increasing OPG[57]. This effect could be explained by the phytoestrogen nature of Res and its biological activity including anti-apoptotic, antioxidant and anti-inflammatory properties as discussed below.

Additionally, In both men and women, Estrogen is the best-known hormone that regulates bone metabolism in women and men during growth by inhibition of bone remodelling and resorption, increasing the thickening of bone cortices (i.e., maintain BMD) and preventing bone loss[58]. The molecular mechanisms by which estrogen stimulates bone formation are described in excellent reviews and involve, at

least, suppression of osteoblast oxidative stress, inflammation and apoptosis, as well as activating Src/Shc/ERK signalling pathway[58]. E2 replacement to the ovariectomized rats increased the BMC and BMD, with the significant increase in OPG as well bone formation markers ALP and OC. The effects of E2 were significantly non-comparable to RES. On the other hand, it reduced the circulating level of RANK, NIXI, TRAP 5b and DPD. In agreement with our results, previous studies concluded that E2 directly, but only partially, curtails human osteoclast formation[59]. Moreover, García Palacios *et al.*[60], demonstrated that estrogen inhibits osteoclast precursor differentiation and may protect bone by reducing osteoclast production.

Interestingly, the Addition of RES to E2 produced significant comparable effects versus the groups treated either by RES and E2. So, we can consider that both RES and E2 produced synergistic effects on bone. To the best of our knowledge, we are the first to investigate the combination of E2 and RES on bone health. This might help to add RES and reduce the E2 dose as well as the side effects arising from it like ovarian cancer or adenocarcinoma of the breast in female complaining from osteoporosis.

In general, bone oxidative stress and inflammation are highly associated with osteoporosis. high levels of reactive oxygen species negatively affect bone formation and remodelling by suppressing the proliferation and differentiation of mesenchymal stem cells into osteoblasts and increase the proliferation and activity of osteoblasts[61-64]. An increase in systemic and bone reactive oxygen species is increased with ageing and testosterone administrations attenuated this in aged mice[65].

On the other hand, RES is a potent estrogen agonist (phytoestrogens)[66]. Low circulating testosterone in orchiectomies male animals is associated with a reduction in locally produced estradiol which negatively affects the BMD and BMC[33]. Therefore, at this stage and in addition to its potent antioxidant and anti-inflammatory potentials, we could assume that Res stimulated bone formation and suppressed bone resorption by antagonizing estrogen which has a direct effect on bone and exerting antioxidants and anti-inflammatory effects. However, one limitation still exists here is that we couldn't measure levels of circulatory estrogen and levels of ROS, antioxidants and inflammatory markers in the different cell populations including osteoclast, osteoclast and mesenchymal stem cells to confirm these hypotheses. This requires further attention to ion future studies.

In the present, the bone antioxidant enzymes decreased significantly with increased MDA in ORX rats. Following our data, previous studies showed that ORX and estrogen deficiency is associated with bone oxidative stress, inflammation and apoptosis in the bony tissues mediated by an increase in the generation of reactive oxygen species (ROS), suppression of endogenous antioxidants and activation of nuclear factor kappa-beta (NF-κB), which are associated with bone loss, activation of osteoclasts and inhibition of osteoblasts ENREF64[64]. Interestingly, the administration of the antioxidant, Pyrroloquinoline Quinone (PQQ) significantly attenuated oxidative stress and inflammation in ORCD-induced rats and significantly increased bone osteoblastic bone formation and inhibited osteoclastic bone resorption[64].

The antioxidant and anti-inflammatory effect of RES is well reported in different organs and under various conditions[18,19]. Indeed, RES has been suggested as an effective therapy to suppress osteoporosis by inhibiting the production of ROS production in bone mesenchymal stem cells through activation of AMPK[67]. In addition, RES suppressed osteoporosis in ovariectomized (OVX) rat model and H_2O_2-induced oxidative cell injury model in RAW 264.7 cells by attenuating RES through upregulation of FOXO-1. In the same line, Res prevented bone loss during mechanical unloading by improving total antioxidant potentials.

CONCLUSION

In conclusion, our data suggest that chronic administration of RES to OVX rats increased BMD and BMC by increasing osteoblastic bone formation and inhibiting osteoclastic bone resorption. This will through a beam of light on the use of resveratrol with estradiol to reverse the osteoporosis induced after menopause.

SIGNIFICANCE STATEMENT

This studies that resveratrol alone or in combination with estradiol could be beneficial in the maintenance of the bone mass and architecture after menopause. This study will help the researcher to reveal the several mechanisms played by resveratrol to modulate the balance between osteoblasts and osteoclasts as well as the role of the circulating cytokines.

ACKNOWLEDGMENT

This research is supported by Taif University, Taif, Saudia Arabia with a grant number (TURSP-2020/127).

REFERENCES

1. Lujan-Barroso, L., K. Gibert, M. Obón-Santacana, M.D. Chirlaque and M.J. Sánchez *et al*., 2018. The influence of lifestyle, diet and reproductive history on age at natural menopause in Spain: Analysis from the EPIC-Spain sub-cohort. Am. J. Hum. Biol., Vol. 30. 10.1002/ajhb.23181.
2. Sherman, S., 2005. Defining the menopausal transition. Am. J. Med., 118: 3-7.
3. Rosenfield, R.L. and D.A. Ehrmann, 2016. The pathogenesis of polycystic ovary syndrome (PCOS): The hypothesis of PCOS as functional ovarian hyperandrogenism revisited. Endocr. Rev., 37: 467-520.
4. Koebele, S.V. and H.A. Bimonte-Nelson, 2016. Modeling menopause: The utility of rodents in translational behavioral endocrinology research. Maturitas, 87: 5-17.
5. Brooks, H.L., D.P. Pollow and P.B. Hoyer, 2016. The VCD mouse model of menopause and perimenopause for the study of sex differences in cardiovascular disease and the metabolic syndrome. Physiology, 31: 250-257.
6. Satpathy, S., A. Patra and B. Ahirwar, 2015. Experimental techniques for screening of antiosteoporotic activity in postmenopausal osteoporosis. J. Compl. Integr. Med., 12: 251-266.
7. Johnston, B.D. and W.E. Ward, 2015. The ovariectomized rat as a model for studying alveolar bone loss in postmenopausal women. Biomed Res. Int., Vol. 2015. 10.1155/2015/635023.
8. Medina-Contreras, J., R. Villalobos-Molina, A. Zarain-Herzberg and J. Balderas-Villalobos, 2020. Ovariectomized rodents as a menopausal metabolic syndrome model. A minireview. Mol. Cell. Biochem., 475: 261-276.
9. Yazğan, Y. and M. Nazıroğlu, 2017. Ovariectomy-induced mitochondrial oxidative stress, apoptosis and calcium ion influx through TRPA1, TRPM2 and TRPV1 are prevented by 17β-estradiol, tamoxifen and raloxifene in the hippocampus and dorsal root ganglion of rats. Mol. Neurobiol., 54: 7620-7638.
10. Bansal, R. and N. Aggarwal, 2019. Menopausal hot flashes: A concise review. J. Midlife Health, 10: 6-13.
11. Johnson, A., L. Roberts and G. Elkins, 2019. Complementary and alternative medicine for menopause. J. Evidence-Based Integr. Med., Vol. 24. 10.1177/2515690x19829380.
12. Sozen, T., L. Ozisik and N.C. Basaran, 2017. An overview and management of osteoporosis. Eur. J. Rheumatol., 4: 46-56.
13. Carter, M.I. and P.S. Hinton, 2014. Physical activity and bone health. Mo Med., 111: 59-64.
14. Klestil, T., C. Röder, C. Stotter, B. Winkler and S. Nehrer *et al*., 2018. Impact of timing of surgery in elderly hip fracture patients: A systematic review and meta-analysis. Sci. Rep., Vol. 8. 10.1038/s41598-018-32098-7.
15. Sakr, H.F., A.M. Hussein, E.A. Eid, A. Boudaka and L.S. Lashin, 2020. Impact of dehydroepiandrosterone (DHEA) on bone mineral density and bone mineral content in a rat model of male hypogonadism. Vet. Sci., Vol. 7. 10.3390/vetsci7040185.
16. Streicher, C., A. Heyny, O. Andrukhova, B. Haigl and S. Slavic *et al*., 2017. Estrogen regulates bone turnover by targeting RANKL expression in bone lining cells. Sci. Rep., Vol. 7. 10.1038/s41598-017-06614-0.
17. Riggs, B.L., 2000. The mechanisms of estrogen regulation of bone resorption. J. Clin. Invest., 106: 1203-1204.
18. Xia, N., A. Daiber, U. Förstermann and H. Li, 2017. Antioxidant effects of resveratrol in the cardiovascular system. Br. J. Pharmacol., 174: 1633-1646.
19. Silva, P., A. Sureda, J.A. Tur, P. Andreoletti, M. Cherkaoui-Malki and N. Latruffe, 2019. How efficient is resveratrol as an antioxidant of the mediterranean diet, towards alterations during the aging process? Free Radical Res., 53: 1101-1112.
20. Lee, A.M.C., T. Shandala, L. Nguyen, B. Muhlhausler, K.M. Chen, P. Howe and C. Xian, 2014. Effects of resveratrol supplementation on bone growth in young rats and microarchitecture and remodeling in ageing rats. Nutrients, 6: 5871-5887.
21. Boissy, P., T.L. Andersen, B.M. Abdallah, M. Kassem, T. Plesner and J.M. Delaissé, 2005. Resveratrol inhibits myeloma cell growth, prevents osteoclast formation and promotes osteoblast differentiation. Cancer Res., 65: 9943-9952.
22. Picard, F., M. Kurtev, N. Chung, A. Topark-Ngarm and T. Senawong *et al*., 2004. Sirt1 promotes fat mobilization in white adipocytes by repressing PPAR-γ. Nature, 429: 771-776.
23. Domazetovic, V., G. Marcucci, T. Iantomasi, M.L. Brandi and M.T. Vincenzini, 2017. Oxidative stress in bone remodeling: Role of antioxidants. Clin. Cases Mineral Bone Metab., 14: 209-216.
24. Tu, C., D.Z. Wu, Y.S. Huang, J.S. Zhuang and J.H. Zeng *et al*., 2020. Oxidative stress contributes to hyperalgesia in osteoporotic mice. J. Pain Res., 13: 131-142.
25. Cervellati, C., G. Bonaccorsi, I. Piva and P. Greco, 2018. Oxidative stress as a possible pathogenic cofactor of post-menopausal osteoporosis: Existing evidence in support of the axis oestrogen deficiency-redox imbalance-bone loss. Indian J. Med. Res., 147: 341-351.
26. Lee, Y.J., J.Y. Hong, S.C. Kim, J.K. Joo, Y.J. Na and K.S. Lee, 2015. The association between oxidative stress and bone mineral density according to menopausal status of Korean women. Obstet. Gynecol. Sci., 58: 46-52.
27. Sahni, S., M.T. Hannan, D. Gagnon, J. Blumberg, L.A. Cupples, D.P. Kiel and K.L. Tucker, 2008. High vitamin C intake is associated with lower 4-year bone loss in elderly men. J. Nutr., 138: 1931-1938.

28. Ström, J.O., A. Theodorsson, E. Ingberg, I.M. Isaksson and E. Theodorsson, 2012. Ovariectomy and 17β-estradiol replacement in rats and mice: A visual demonstration. J. Visualized Exp., Vol. 7. 10.3791/4013.
29. Park, E.J. and J.M. Pezzuto, 2015. The pharmacology of resveratrol in animals and humans. Biochim. Biophys. Acta (BBA) Mol. Basis Dis., 1852: 1071-1113.
30. O'Donnell, S., 2018. Screening, prevention and management of osteoporosis among Canadian adults. Health Promotion Chronic Dis. Prev. Canada, 38: 445-454.
31. Haentjens, P., J.S. Magaziner, C.S. Colon-Emeric, D. Vanderschueren, K. Milisen, B. Velkeniers and S. Boonen, 2010. Meta-analysis: Excess mortality after hip fracture among older women and men. Ann. Internal Med., 152: 380-390.
32. Haentjens, P., P. Autier, M. Barette and S. Boonen, 2005. Predictors of functional outcome following intracapsular hip fracture in elderly women. Injury, 36: 842-850.
33. Golds, G., D. Houdek and T. Arnason, 2017. Male hypogonadism and osteoporosis: The effects, clinical consequences and treatment of testosterone deficiency in bone health. Int. J. Endocrinol., Vol. 2017. 10.1155/2017/4602129.
34. Manson, J.E., J. Hsia, K.C. Johnson, J.E. Rossouw and A.R. Assaf *et al*., 2003. Estrogen plus progestin and the risk of coronary heart disease. N. Engl. J. Med., 349: 523-534.
35. Taxel, P., D.G. Kennedy, P.M. Fall, A.K. Willard, J.M. Clive and L.G. Raisz, 2001. The effect of aromatase inhibition on sex steroids, gonadotropins and markers of bone turnover in older men. J. Clin. Endocrinol. Metab., 86: 2869-2874.
36. Scopacasa, F., M. Horowitz, J.M. Wishart, H.A. Morris, B.E. Chatterton and A.G. Need, 2000. The relation between bone density, free androgen index and estradiol in men 60 to 70 years old. Bone, 27: 145-149.
37. Titorencu, I., V. Pruna, V.V. Jinga and M. Simionescu, 2014. Osteoblast ontogeny and implications for bone pathology: An overview. Cell Tissue Res., 355: 23-33.
38. Neve, A., A. Corrado and F.P. Cantatore, 2011. Osteoblast physiology in normal and pathological conditions. Cell Tissue Res., 343: 289-302.
39. Oury, J. and F. Oury, 2018. Osteocalcin, a key molecule for bone endocrine functions. Med. Sci., 34: 54-62.
40. Lumachi, F., M. Ermani, V. Camozzi, V. Tombolan and G. Luisetto, 2009. Changes of bone formation markers osteocalcin and bone-specific alkaline phosphatase in postmenopausal women with osteoporosis. Ann. N. Y. Acad. Sci., 1173: E60-E63.
41. Christgau, S., O. Bitsch-Jensen, N.H. Bjarnason, E.G. Henriksen, P. Qvist, P. Alexandersen and D.B. Henriksen, 2000. Serum crosslaps for monitoring the response in individuals undergoing antiresorptive therapy. Bone, 26: 505-511.
42. Yin, Y., L. Ding, Y. Hou, H. Jiang, J. Zhang, Z. Dai and G. Zhang, 2019. Upregulating microRNA-410 or downregulating Wnt-11 increases osteoblasts and reduces osteoclasts to alleviate osteonecrosis of the femoral head. Nanoscale Res. Lett., Vol. 14. 10.1186/s11671-019-3221-6.
43. Kameda, T., H. Mano, T. Yuasa, Y. Mori and K. Miyazawa *et al*., 1997. Estrogen inhibits bone resorption by directly inducing apoptosis of the bone-resorbing osteoclasts. J. Exp. Med., 186: 489-495.
44. Pepene, C.E., N. Crişan and I. Coman, 2011. Elevated serum receptor activator of nuclear factor kappa B ligand and osteoprotegerin levels in late-onset male hypogonadism. Clin. Invest. Med., Vol. 34. 10.25011/cim.v34i4.15365.
45. Özbek, M.N., H. Demirbilek, R.T. Baran and A. Baran, 2016. Bone mineral density in adolescent girls with hypogonadotropic and hypergonadotropic hypogonadism. J. Clin. Res. Pediatr. Endocrinol., 8: 163-169.
46. Ominsky, M.S., X. Li, F.J. Asuncion, M. Barrero and K.S. Warmington *et al*., 2008. RANKL inhibition with osteoprotegerin increases bone strength by improving cortical and trabecular bone architecture in ovariectomized rats. J. Bone Miner. Res., 23: 672-682.
47. Osterberg, E.C., A.M. Bernie and R. Ramasamy, 2014. Risks of testosterone replacement therapy in men. Indian J. Urol., 30: 2-7.
48. Couse, J.F. and K.S. Korach, 1999. Estrogen receptor null mice: What have we learned and where will they lead us? Endocrine Rev., 20: 358-417.
49. Wu, K., T.H. Lin, H.C. Liou, D.H. Lu, Y.R. Chen, W.M. Fu and R.S. Yang, 2013. Dextromethorphan inhibits osteoclast differentiation by suppressing RANKL-induced nuclear factor-κB activation. Osteoporosis Int., 24: 2201-2214.
50. Sims, N.A., H.A. Morris, R.J. Moore and T.C. Durbridge, 1996. Increased bone resorption precedes increased bone formation in the ovariectomized rat. Calcified Tissue Int., 59: 121-127.
51. Yoon, K.H., D.C. Cho, S.H. Yu, K.T. Kim, Y. Jeon and J.K. Sung, 2012. The change of bone metabolism in ovariectomized rats: Analyses of microCT scan and biochemical markers of bone turnover. J. Korean Neurosurg. Soc., 51: 323-327.
52. Janckila, A.J. and L.T. Yam, 2009. Biology and clinical significance of tartrate-resistant acid phosphatases: New perspectives on an old enzyme. Calcified Tissue Int., 85: 465-483.
53. Chao, T.Y., J.C. Yu, C.H. Ku, M.M. Chen, S.H. Lee, A.J. Janckila and L.T. Yam, 2005. Tartrate-resistant acid phosphatase 5b is a useful serum marker for extensive bone metastasis in breast cancer patients. Clin. Cancer Res., 11: 544-550.

54. García-Pérez, M.A., J. Moreno-Mercer, J.J. Tarín and A. Cano, 2006. Similar efficacy of low and standard doses of transdermal estradiol in controlling bone turnover in postmenopausal women. Gynecological Endocrinol., 22: 179-184.
55. Hanson, D.A., M.A.E. Weis, A.M. Bollen, S.L. Maslan, F.R. Singer and D.R. Eyre, 1992. A specific immunoassay for monitoring human bone resorption: Quantitation of type I collagen cross-linked N-telopeptides in urine. J. Bone Mineral Res., 7: 1251-1258.
56. DeLaurier, A., B. Jackson, K. Ingham, D. Pfeiffer, M.A. Horton and J.S. Price, 2002. Biochemical markers of bone turnover in the domestic cat: Relationships with age and feline osteoclastic resorptive lesions. J. Nutr., 132: 1742S-1744S.
57. Feng, Y.L., X.T. Jiang, F.F. Ma, J. Han and X.L. Tang, 2018. Resveratrol prevents osteoporosis by upregulating FoxO1 transcriptional activity. Int. J. Mol. Med., 41: 202-212.
58. Khosla, S., M.J. Oursler and D.G. Monroe, 2012. Estrogen and the skeleton. Trends Endocrinol. Metab., 23: 576-581.
59. Robinson, L.J., B.B. Yaroslavskiy, R.D. Griswold, E.V. Zadorozny, L. Guo, I.L. Tourkova and H.C. Blair, 2009. Estrogen inhibits RANKL-stimulated osteoclastic differentiation of human monocytes through estrogen and RANKL-regulated interaction of estrogen receptor-α with BCAR1 and Traf6. Exp. Cell Res., 315: 1287-1301.
60. Palacios, V.G., L.J. Robinson, C.W. Borysenko, T. Lehmann, S.E. Kalla and H.C. Blair, 2005. Negative regulation of RANKL-induced osteoclastic differentiation in RAW264.7 cells by estrogen and phytoestrogens. J. Biol. Chem., 280: 13720-13727.
61. Kondo, H., C. Limoli, N.D. Searby, E.A. C Almeida, D.J. Loftus and W. Vercoutere, 2007. Shared oxidative pathways in response to gravity-dependent loading and gamma-irradiation of bone marrow-derived skeletal cell progenitors. Radiats Biol. Radioecol., 47: 281-285.
62. Stolzing, A., E. Jones, D. McGonagle and A. Scutt, 2008. Age-related changes in human bone marrow-derived mesenchymal stem cells: Consequences for cell therapies. Mech. Ageing Dev., 129: 163-173.
63. Feng, Y.F., L. Wang, Y. Zhang, X. Li and Z.S. Ma *et al*., 2013. Effect of reactive oxygen species overproduction on osteogenesis of porous titanium implant in the present of diabetes mellitus. Biomaterials, 34: 2234-2243.
64. Wu, X., J. Li, H. Zhang, H. Wang, G. Yin and D. Miao, 2017. Pyrroloquinoline quinone prevents testosterone deficiency-induced osteoporosis by stimulating osteoblastic bone formation and inhibiting osteoclastic bone resorption. Am. J. Transl. Res., 9: 1230-1242.
65. Drake, M.T. and S. Khosla, 2012. Male osteoporosis. Endocrinol. Metab. Clin. North Am., 41: 629-641.
66. Gehm, B.D., J.M. McAndrews, P.Y. Chien and J.L. Jameson, 1997. Resveratrol, a polyphenolic compound found in grapes and wine, is an agonist for the estrogen receptor. Proc. Nat. Acad. Sci. USA, 94: 14138-14143.
67. Zhou, T., Y. Yan, C. Zhao, Y. Xu, Q. Wang and N. Xu, 2019. Resveratrol improves osteogenic differentiation of senescent bone mesenchymal stem cells through inhibiting endogenous reactive oxygen species production via AMPK activation. Redox Rep., 24: 62-69.