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Resveratrol Administration Reverses The Osteoporotic Bone In Independent-Manner Of Ahr-Esr1 Axis In Rats

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ABSTRACT

The objective of this work was to analyze the involvement of AhR in bone metabolism using a rat model of experimental osteoporosis and to analyze the mechanisms behind its activity. Rats were assigned randomly to the subsequent groups; Control, received no treatment; ovariectomized (OVX) rats; Sham; Sham+RES received resveratrol; OVX+RES and OVX+CH received AhR's antagonist, CH223191 (CH); and finally OVX+CHR group received both AhR antagonist along with resveratrol. Resveratrol and AhR antagonist treatment started 7 days after surgery and continued to 45 days. The serum of osteocalcin (OC) and Ca^{+2} was measured by ELISA and spectrophotometer, respectively. X-ray was used to estimate bone density of rats. In molecular levels, *Ahr*, *Esr1* and *Esr2* gene expression were quantified in the Control, OVX, OVX+RES, OVX+CH and OVX+CHR groups. Supplementation of RES and CH223191 significant increased ($P<0.05$) in serum Ca^{+2} and bone density in treated groups compared OVX groups while serum OC decreased. The *Ahr* gene expression was significantly higher in OVX+RES than in OVX+CHR, OVX and Control groups. *Esr1* gene was actively expressed than *Esr2* gene in the bone metabolism in this study. In conclusion, the administration of phosphoric acid with/without combination with ovariectomy procedure had enhanced the mediation of osteoporosis while administration of resveratrol led to resumption of nearly normal bone metabolism via the overexpression of AhR signaling and reduction of osteocalcin production in estrogen-independent manner.

Keywords: *AhR, ESR1, ESR2, osteocalcin, experimental osteoporosis, resveratrol.*

1. INTRODUCTION

The aryl hydrocarbon receptor (AhR) is a ligand-dependent transcription factor, recognized chiefly for its function in regulating the toxicity of environmental pollutants, including 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) and benzo[a]pyrene (B[a]P). Between the cytoplasm and nucleus, AhR protein is constantly translocated. Its nuclear import is enhanced by the binding of either endogenous or exogenous ligands **(Haidar et al., 2023).** AhR integrates signals from the environment, nutrients, and microbes; it is expressed in barrier organs such the skin, colon, and lungs, and in related immune cells **(Fernández-Gallego et al., 2021).** Once AhR and its ligand make contact, the receptor moves from the cytoplasm to the nucleus and attaches to the AhR nuclear translocator (ARNT). The resulting compound subsequently associates with the xenobiotic responsive element (XRE) **(Bonati et al., 2023)**. Some members of the cytochrome P450 family, including CYP1A1, CYP1A2, and CYP1B1, have their gene expression controlled by the AhR/ARNT complex **(Fujii-Kuriyama and Mimura, 2005).** The nuclear transcription site is revealed when the AhR binds with two types of ligands: exogenous ligands like TCDD and endogenous ligands like indoxyl 3-sulfate (I3S). This modification enables the insertion of AhR into the nucleus, where it heterodimerizes with the ARNT **(Liu et al., 2020).** To activate the promoter of an XRE or a dioxin response

element (DRE), the AhR/ARNT heterodimer needs to be present in the nucleus. One member of the cytochrome P450 family, CYP1A1, and another member of the cytochrome P450 family, CYP1B1, are involved in this process. These enzymes promote gene transcription, which in turn leads to various biological outcomes such as toxicity, immune response, biological development, and bone remodeling **(Park et al., 2020).**

Resveratrol (RES) is a naturally occurring polyphenolic molecule, chemically analogous to the estrogen diethylstilbestrol, mostly located in grapes, peanuts, and several other sources. RES can competitively interact with estrogen receptors in vitro and demonstrate estrogenic actions; thus, RES is regarded as a phytoestrogen **(Khera et al., 2019).** RES has several notable bioactivities, such as estrogenic modulation of lipid function, anti-platelet aggregation, antioxidative effects (**Dawood and Alghetaa, 2023; Alghetaa et al., 2023a**), and anti-tumor capabilities **(Jia et al., 2019; Lei and Chen, 2018 , Khayoon and Al-Rekabi, 2020; Abdulla and Al-Okaily, 2022)** and anti-inflammatory agent **(Alghetaa et al., 2023b, Al-Khaqani and Mohammed, 2024).** RES could significantly hinder bone erosion, as demonstrated by in vitro experiments on rats experiencing bone mass reduction due to insufficient physical activity and estrogen deficiency **(Feng et al., 2018).** Bone metabolism encompasses two opposing processes: bone resorption and bone synthesis, interconnected inside the bone remodeling unit. Bone production occurs prior to bone resorption during the remodeling process. Bone resorption involves the breakdown of bone minerals, the catabolism of matrix components by osteoclasts, and the release of matrix materials, leading to the formation of a resorptive cavity and the efficient removal of organic and mineral components. The production of transforming growth factor β (TGFβ) and other matrix-associated chemicals, as well as calcium and matrix components, by osteoclast activity might impact other cells in the surrounding microenvironment **(Delaisse et al., 2020).**

When a new bone is formed, osteoblasts fill the empty space left by resorption with a matrix of growing bone. Then, the process of primary mineralization begins quickly, while secondary mineralization is more gradual. The N-terminal and C-terminal pro-peptides of type I procollagen, osteocalcin (OC), and bone-specific alkaline phosphatase (bone-ALP) are all tools for evaluating bone development **(Lau et al., 2022).**There are numerous hormonal and local variables that continuously control bone metabolism. A number of hormones, including insulin, growth hormone, gonadal hormones, cytokines, and growth factors, have a major effect on bone metabolism, including calciotropic hormones, particularly parathyroid hormone, vitamin D, and calcitonin **(Kini and Nandeesh, 2012).** In metabolic bone disorders such osteoporosis, inadequate bone metabolism leads to bone loss, occasionally accompanied by alterations in microarchitecture that increase fragility and result in fractures. The creation of serum and urinary assays for biochemical markers indicative of osteoblast and osteoclast enzymatic activity, as well as bone tissue breakdown products, has been essential to exploring the intricate pathways of bone metabolism and their modifications in bone diseases **(Saeki et al., 2024).** This study seeks to investigate the regulation of AhR on bone metabolism through upregulation and downregulation utilizing resveratrol, an AhR agonist, and CH223191, an AhR antagonist, respectively.

2. MATERIAL AND METHODS

Ethical statement:

The study was conducted in compliance with the University of Baghdad's Animal Welfare regulation with the authorization of the local Institutional Animal Care and Use Committee at the College of Veterinary Medicine, University of Baghdad (AUP#: 1181/P.G).

Animals of study

Seventy female adult albino rats were utilized in the current study. All animals are contained in plastic containers under a regulated environment (22-25°C) at the animal care facility of the College of Veterinary Medicine, University of Baghdad. They kept for a minimum of two weeks for acclimatization to the housing surroundings. The food and water were freely accessible all the time of the study period.

Ten Wistar adult female naïve rats will serve as control (C). Forty adult female rats will be ovariectomized according to **Lemini et al. (2015) and Alghetaa et al. (2023c)**. While, another twenty rats, sham rats and exposed to the same surgical conditions of ovariectomized rats except that the ovaries were not excised. All surgically operated rats (n=60) then followed up for three days to ensure there are no complications or wound infections. One week later, all surgical sutures were removed, and rats were inspected for any defects. Osteoporosis induction begin by giving all ovariectomized and sham rats a 10% phosphoric acid in drinking water for 30 consecutive days **(Mohammed, 2018).**

Preparation of treatments

Resveratrol (exogenous ligands of ESR and AhR)

Resveratrol solution was prepared at a concentration of 100 mg/kg body weight, following the methodology from a previous study **(Bordbar et al., 2022**; **Alghetaa et al., 2023a,b**). This entailed diluting 400 mg of Resveratrol in 4 cc of 1% carboxymethyl cellulose (CMC) buffer. The solution was thoroughly blended for uniformity with a vortex mixer, and each rat was administered a dose proportional to its weight (1µL/1g B.W.) of the resultant solution CH-223191 **(AhR antagonist)**

At a concentration of 10 mg/kg body weight, a CH-223191 solution was made using the procedures described in earlier research **(Cao et al., 2022; Bustani et al., 2024).** The procedure involved dissolving 40 mg of CH-223191, sufficient for 20 participants, in a 2 ml DMSO solution. The solution was meticulously blended for uniformity with a vortex mixer, and each rat administered a dosage proportional to its weight (0.5µL for 1 g of body weight) of the prepared solution.

3. EXPERIMENTAL DESIGN

Treatment of osteoporotic female rats with AhR antagonist (CH223191) and resveratrol and explore of *Ahr*, *Esr1* and *Esr2* genes in bone metabolism in femoral bone of osteoporotic female rats .

Animals grouping

Seventy mature female rats were assigned to different experimental groups according to the treatment protocol. Ten non-osteoporotic rats serve as control subjects. The other sixty osteoporotic rats were divided into the following groups at random:

1-**Sham group:** rats of these groups exposed to laparotomy operation without removing ovaries and divided into two groups :

first, ten rats serve as Sham group ,rats of this group no receive treatment .

second, Ten rats will receive resveratrol orally daily at a dose of 100mg/kg (**Alghetaa et al., 2018)** for 45 days serve as Sham group +RES.

2- Ovariectomized groups split into four groups :

Firstly, ovariectomized (OVX) group , Ten OVX-rats act as positive control. **Secondly,** OVX+RES, Ten OVX-rats was administrated resveratrol orally daily at a dose of 100mg/kg for 45 days.

Tertiary OVX+CH, these group Ten OVX-rats given CH223191 Antagonist i.p. every 3 days for 45 days (**Zhao et al., 2010 ; Bustani et al., 2024).)**.

And lastly, Ten OVX-rats given CH223191 antagonist i.p every 3 days + resveratrol daily at a dose of 10mg/kg for 45 days.

Confirmation of osteoporosis:

All ovariectomized rats and sham rats treated with 10% phosphoric acid were confirmed with osteoporosis status by X-Ray imaging.

Blood collection

At the end of the experiments, 45 days, blood collection from rats performed by heart puncture (**Subhi and Al-Okaily, 2024)** under anesthesia by using a mixture of xylazine and ketamine at a dose 10mg/kg and 80mg/kg respectively (**Dodelet-Devillers., 2016**). then obtained serum and transferred into lab to analysis

Assessment of serum Calcium concentration (mg/dl):

Rat serum calcium concentration was measured by automation analyzers (spectrophotometer).

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

Rat serum Osteocalcin concentration was measured by using **Osteocalcin** kit.

Radiographing of experimental animals:

X-ray images were taken for experimental rats at the end of the experiment (45 day) . The radiographed animals were control, sham, OVX, OVX+RES, OVX+CH, OVX+CHR. Briefly, all targeted animals in imaging were generally anesthetized . Once the animals were fully unconscious, they were put on imaging stage of X- RAY apparatus (Hindland – India , on their left side to be radiographed in whole-body capture with X-ray set on 100mlamber ,mass 50 and kup50. All these steps are performed by senior radiologists under standard settings. Then all taken images were printed out on specific X-ray films(CR =CR10X –AGFA,CRMD 10 –AGFA)for further investigations and analysis.

Bone density measurement:

A free-available software called ImageJ (**Schneider et al., 2012**), a JAVA programming software was utilized to analyze the X-ray scanned films (**Schmid et al., 2010**). Briefly, all films were scanned with Canon Scanner at a default setting with a resolution of 600 dpi, then they were uploaded separately to ImageJ software. Before performing any image analysis, the background noise was removed by built-in tool then fifteen different areas were determined by oval shape from the right side of each animal distributed as follows: 3 areas from femur, 5 areas from vertebrae column (3 lumbar and 2 thoracic), 3 areas from radius bone, 2 areas from mandible bone and 2 areas from maxillary bone. The measurements were set as gray mean value through choosing it from analyzing tab. The mean the bone density of each mentioned point by calculating the mean of gray contrast per inch of surrounded area through calculating pixels divided by respective area. Then all analyzed images were saved as TIFF format in inverted background while raw and mean integrated density values were exported as CSV files

(**Geiger et al., 2016; Roessler et al., 2023**).

RNA extraction from bone tissues:

All experimental rats were euthanized under an overdose of xylazine and ketamine combination .the right femoral bone which immediately kept in liquid nitrogen for few minutes before being crushed down to small pieces and transferred to triazole reagent to total RNA extraction and DNA synthesis according to (**Chomczynski, 1993).**

Amplification of *Ahr, Esr1, Esr2* and *Gapdh* .

PCR was used to amplify the *Ahr, Esr1, Esr2 and Gapdh* genes by using the following primers.

No	Gene ID	forward (5'→3′)	reverse (5'→3′)	NCBI accession number
1	*Ahr*	GTC AGC CAT GGT CAG TCC TCA G	GCT CGG ACT CTG AAA CTT GCT T	NM 013149.3
2	*Esr1*	CTG CCT TGA TCA CAC ACC GC	GCC ACC CTG GTT CAA AA	NM 012689.1
3	*Esr2*	5-CTG GAC AGG GAT GAG GGG AA-3	5-GTG TCA GCT TCC GGC TAC TT-3	NM 012754.3
4	*Gapdh*	AGA GAC AGC CGC ATC TTC TT	ATG AAG GGG TCG TTG ATG GC	NM 017008.4

Normalized gene expression was calculated according to $2^{-\Delta\Delta Ct}$ and fold change was expressed for *Ahr, Esr1 and Esr2* in normalization to *Gapdh* .

Statistical analysis

The experimental data was analyzed by using GraphPad Prism 8.0. The descriptive statistics are expressed in the form of mean ± standard deviation. The data were compared by Tukey's multiple comparisons test- one-way analysis of variance (one-way ANOVA) and p value <0.05 was considered statically significant.

4. RESULTS

Assessment of serum Calcium concentration (mg/dl):

Figure 1 exhibits the average serum calcium concentrations in the Control, Sham, Sham+RES, OVX, OVX+RES, OVX+CH, and OVX+CHR groups during the experimental periods. Following a 45-day investigation, the results indicated no significant difference in serum calcium concentration among the Control, Sham, Sham+RES, and OVX+RES groups. Compared to the Sham+RES, OVX, OVX+RES, and OVX+CH groups, the Sham group of rats (treated with phosphoric acid and a surgical wound) exhibited minimal changes in serum calcium levels. However, rats that received 10% phosphoric acid along with surgery (OVX) showed a significant (P<0.05) drop in serum Ca+2 concentration at 45 days compared to the other groups (Control, Sham, and Sham+RES), while there were no significant differences between the OVX+RES, OVX+CH, and OVX+CHR groups at the end of the experiment.

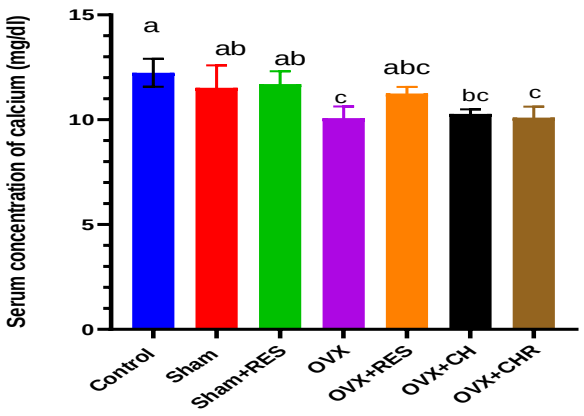

Figure 1: Concentration of calcium after 45 days of experiment . rat administration of 10% of phosphoric acid after surgical wound (Sham).administration of resveratrol (100 mg /kg to Sham group(Sham +RES). Induction group(phosphor 10% +surgical operation)without treatment (OVX). administration of resveratrol (100 mg /kg) to induction rat by (phosphor 10% +OVX (OVX +RES). administration of CH223191 (10 mg /kg) to induction rat by (phosphor 10% +OVX (OVX +CH). administration of resveratrol (100 mg /kg) + CH223191 (10 mg /kg) to induction rat by (phosphor 10% +OVX (OVX +CHR). Results presented as means with ± SD.. Small letters indicate significant differences between groups (P<0.05)

Assessment of serum Osteocalcin concentration (ng/ml)

Figure 2 displays the average blood osteocalcin hormone levels in the Control

group and six treatment groups throughout the trial periods. Following 45 days of treatment, the results indicated a significant elevation (P<0.05) in serum OC concentration in the OVX group administered 10% phosphoric acid post-surgery, in comparison to the control and other groups. Conversely, at the conclusion of the trial, rats subjected to phosphoric acid treatment with surgical wounds (Sham group) demonstrated a substantial increase (P<0.05) in comparison to the control group. Sham+RES and OVX+RES cohorts. There is no significant difference between the OVX + CH and OVX + CHR groups.

Oral administration of resveratrol to female ovariectomized rat in Sham +RES group (and) exhibited not a significant compared control , OVX+RES, OVX+CH and OVX+CHR groups after 45days of the experiment .Within the time, the results showed a significant (P<0.05) increase in OC concentration in OVX+CH and OVX+CHR groups at end of the experiment as compared to Control, Sham+RES and OVX+RES groups. As well as, on significant differences between OVX+CH and OVX+CHR groups and Sham +RES group in OC concentration after 45 days of experiment. Whereas treatment of ovariectomized rat with resveratrol in OVX +RES group showed non-significant differences in OC concentration after 45 days of the experiment as compared to Control and Sham+RES groups.

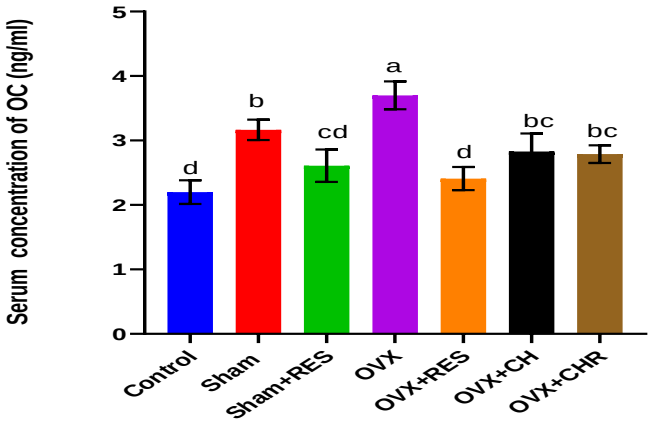

Figure 2: Concentration of osteocalcin (OC) after 45 days of experiment . rat administration of 10% of phosphoric acid after surgical wound (sham).administration of resveratrol (100 mg /kg to sham group(sham +RES). Induction group(phosphor 10% +surgical operation)without treatment (OVX). administration of resveratrol (100 mg /kg) to induction rat by (phosphor 10% +OVX (OVX +RES). administration of CH223191 (10 mg /kg) to induction rat by (phosphor 10% +OVX (OVX +CH). administration of resveratrol (100 mg /kg) + CH223191 (10 mg /kg) to induction rat by (phosphor 10% +OVX (OVX +CHR). Results presented as means with ± SD.. Small letters indicate significant differences between groups (P<0.05)

Measurement of bone density

To determine if resveratrol and the AhR antagonist CH-223191 alleviated bone loss resulting from estrogen deprivation, OVX rats were administered resveratrol (100 mg) and CH-223191 (10 mg) for a duration of 45 days. Figure 4 illustrates that

the bone density of the Control group was statistically insignificant when compared to the Sham+RES, OVX+RES, OVX+CH, and OVX+CHR groups. The bone calcium concentration in femoral rats was considerably decreased in the OVX group ($p<0.05$) relative to the Control and treatment groups.

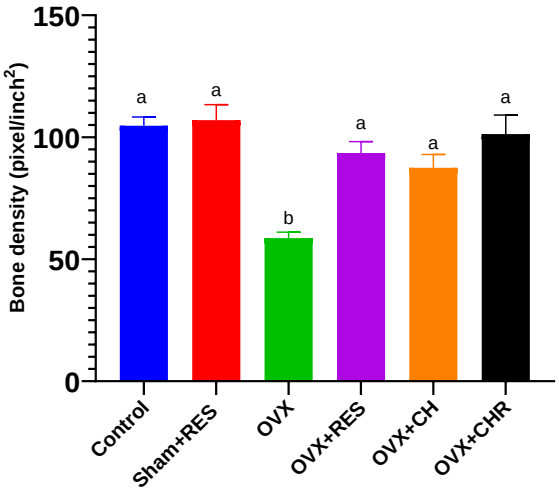

Figure 3. statical analysis of X-RAY image and shows Effect of resveratrol and AhR antagonist on osteoporotic rats induced by ovariectomy procedure agonized by administration of 10% of phosphoric acid after 45 days of treatment on bone density. Bone density was calculated by using ImageJ software. Results in bar represent as mean ± SD. One-way ANOVA test employed to investigate the significant differences at P <0.05. OVX, ovariectomized rats; RES, resveratrol; CH, AhR antagonist; CHR, treated with RES and CH.

***Esr* genes expression**

The fold change values represent the relative change in Esr1 gene expression between (OVX, OVX+RES, OVX+CH, OVX+CHR) groups compared to the Control group. The Control group served as the guideline for the other groups. Since in the OVX group, the gene expression shows a significant increase compared with control and treated groups, indicating an absence of ligand (estradiol) in these groups and leading to overexpression of estrogen receptor in bone (*Esr1*). While there are insignificant differences between control groups and treated groups (OVX+RES, OVX+CH, OVX+CHR; Figure 4A). Estrogen receptor 2, *Esr2* gene expression showed no significant changes among the study groups (Figure 4B).

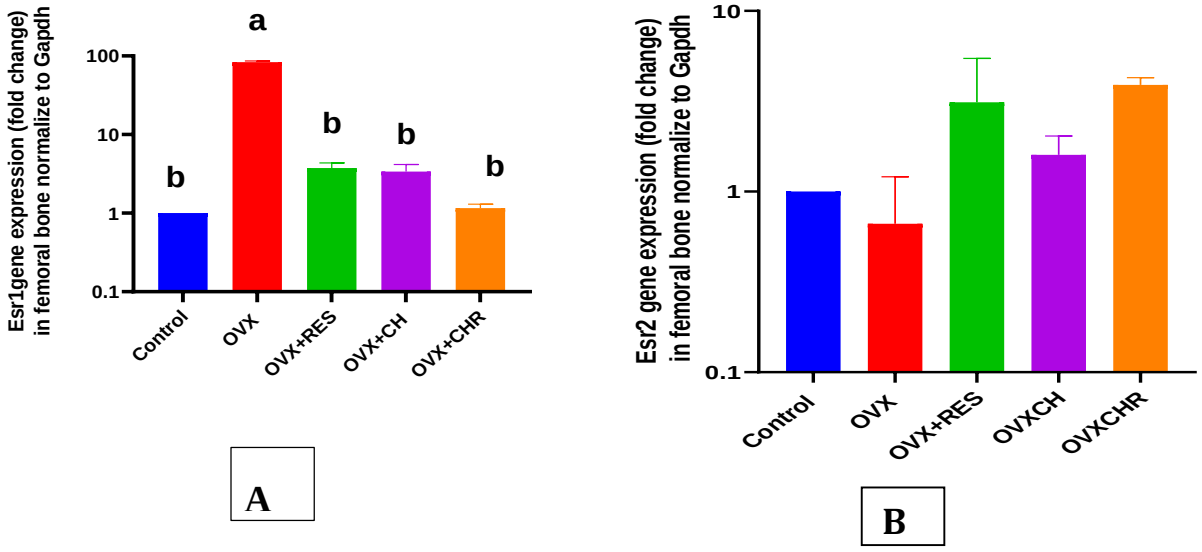

Figure 4. Effect of resveratrol and AhR antagonist on osteoporotic rats induced by ovariectomy procedure agonized by administration of 10% of phosphoric acid after 45 days of treatment on A) *Esr1* fold chain in femoral bone of rats B) *Esr2* fold chain in femoral bone of rats . Results in bar represent as mean ± SD. One-way ANOVA test employed to investigate the significant differences at P <0.05..OVX; ovariectomized rats; RES, resveratrol; CH, AhR antagonist .

2-*Esr2* gene expression

***Ahr* gene expression**

Figure-5 illustrated the fold change values of Ahr gene expression in control group and treated groups (OVX, OVX+RES, OVX+CH, OVX+CHR). The control group, serving as the baseline with a fold change of 1, establishes a reference point for comparison, since in the OVX+RES group, the gene expression of *Ahr* shows an increase significantly compared with other groups. Whereas the *Ahr* gene expression in OVX+CHR exhibited decrease significantly comparison with OVX+R group ,while the expression higher than (Control, OVX, OVX+CH). In OVX group , the gene expression of *Ahr* showed significant decrease simile with OVX+RES and OVX+CHR groups, in contrast the expression of *Ahr* gene in this group showed significant increase compared with control and OVX+CH groups. When comparing the OVX+CH group to other treatment groups, the fold change shows a substantial drop in gene expression; however, when compared to the control group, there is no significant difference in the fold-change. Here, after continuous exposure to an AhR antagonist, we examined gene expression in rat bone along the AhR pathway.

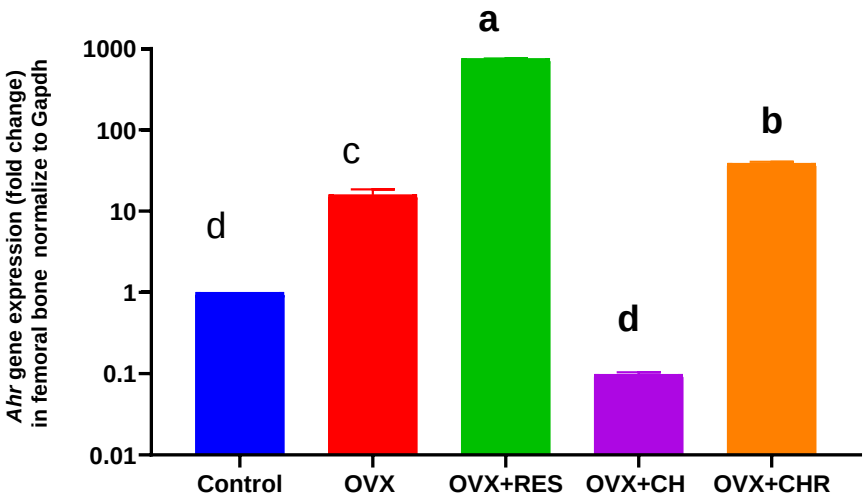

Figure 5. Effect of resveratrol and AhR antagonist on osteoporotic rats induced by ovariectomy procedure agonized by administration of 10% of phosphoric acid after 45 days of treatment on fold chain of *Ahr* gene of femoral bone of rats . Results in bar represent as mean ± SD. One-way ANOVA test employed to investigate the significant differences at P<0.05. OVX; ovariectomized rats; RES; resveratrol; CH ; AhR antagonist .

5. DISCUSSION

Postmenopausal osteoporosis is a metabolic disorder characterized by increased bone resorption by osteoclasts exceeding bone synthesis by osteoblasts, attributable to estrogen shortage **(Cheng et al., 2022)**. In order to learn how AhR contributes to healthy bones and how to reverse osteoporosis, this study used resveratrol in conjunction with an AhR antagonist, CH223191.

Ovariectomy-induced osteoporosis resulted in hypocalcaemia in the OVX group compared to the Control groups (Figure 1), these findings in agreement with many studies **(Saleh and Saleh, 2011; Saleh et al., 2020; Kadhem et al., 2023).** Ovariectomy induces osteoporosis by causing changes in bone development and structure, resulting in decreased estrogen and calcium levels in the blood, along with an increase in alkaline phosphates (**Saleh et al., 2020).** Post-menopausal women may experience less osteoblastic activity in the bones and bone matrix, along with a reduction in calcium and phosphate buildup, contingent upon the severity of estrogen shortage **(Baloğlu and Özkorkmaz, 2019).** Similarly, post-ovariectomy, there will be an alteration in calcium and phosphorus metabolism, leading to diminished blood and bone calcium concentrations, heightened urine calcium excretion by the kidneys, and ensuing calcium deficiency. Elevated blood and urinary phosphorus levels impair bone formation and calcification, resulting in decreased mineral deposition, inadequate bone material, reduced bone density, diminished bone mass, increased bone fragility, and leading to osteoporosis and potential fractures **(Zhang et al., 2020; Hassan and Farhan, 2024**)

The RES action improves postmenopausal osteoporosis through the activation of the estrogen receptor, activation of osteoblast differentiation, and suppression of osteoclast differentiation. Additionally, RES displays chemical similarities to both natural and synthetic estrogens, thus classifying it as a phytoestrogen. Furthermore, RES in several studies interacted with membrane-bound estrogen receptors and regulated non-genomic estrogenic activity. In additional to disrupted

steroidogenesis and estrogen production at several stages of the pathway **(Wang et al., 2020 and Qasem, 2020)**. The results of the present study showed reduction in serum concentration of calcium and increase in serum concentration of osteocalcin (OC) in OVX group (Figures 1&2). Whereas, when ovariectomized rats treated with RES, the Ca^{+2} and OC concentrations returned to normal state of bone metabolism. Previous study indicated that resveratrol reduces ALP and OC levels in the blood of ovariectomized rats, thereby influencing the metabolism of collagen and non-collagen in bone, decreasing the increased bone turnover rate associated with ovariectomy, promoting bone formation, and inhibiting bone resorption **(Zhang et al., 2020)**.

Moreover, we utilized CH223191, an AhR antagonist, to address the effects of estrogen deficiency in status of AhR's pathway inhibition, in OVX+CH and OVX+CHR groups. Consequently, CH223191 effectively inhibited the action of AhR in bone (Figures 1&2), from one side, and reduced bone resorption and increased bone density (Figure 3) from the other side. *Ahr* gene deficient rats showed an increase in trabecular bone volume, trabecular number, trabecular bone connection density, and bone mineral density compared to rats with the wild type gene **(Yu et al., 2014).**

The AhR and RANKL signaling pathways share NF-kB as a transcription factor. Thus, the activation of AhR creates competition for NF-kB, thereby limiting RANKL-induced osteoclast formation (**Voronov et al., 2008**). According to **Herlin et al. (2013)**, AhR activation also reduced matrix mineralization and increased bone porosity in vivo.

In a molecular study, the exploration of *Esr1*, *Esr2*, and *Ahr* genes in the regulation of bone cells and bone metabolism revealed that estrogen receptor type 1 plays a predominant role in safeguarding bone against osteoporosis, whereas *Esr2* is ineffective in treating osteoporotic rats (Figure 4A and B), these findings are aligned with results of **Almedia et al. (2017)**. Furthermore, there was a markedly high expression of *Esr1* in the OVX group, while the fold change in *Esr2* expression showed no significant differences compared to the control group (Figure 4A and B). The influence of estrogen on the bone is primarily governed by its engagement with two estrogen receptors ER1 and ER2. Both are expressed by chondrocytes and osteogenic cells (**Almeida et al., 2017),** it regulates the expression of several target genes. Numerous studies indicate that mechanical stimulation increases *Esr1* expression in the fracture callus, especially in OVX rodents **(Wehrle et al., 2015; Chow et al., 2014; Haffner-Luntzer et al., 2018**). In another work, *Esr1* expression is diminished in the fracture callus of OVX mice compared to non-OVX counterparts **(He et al., 2011)**. Furthermore, ER1 is crucial for the protective effects of estradiol in the mandible at alveolar, cortical, and trabecular sites, but ER2 is nonessential (**Vinel et al., 2018),** which comes in alignment with our results (Figure 4). Moreover, RES is a natural phytoestrogen, and its estrogen-mimetic capability demonstrates a greater affinity for ER-1, hence facilitating bone production through the activation of the ER1 isoform **(Shah et al., 2022)**. Bone mass is governed by various elements, including internal and environmental factors, such as nutrition and pollutants. The aryl hydrocarbon receptor acts as a dioxin receptor and is involved in various clinical and physiological processes (**Rejano-Gordillo, 2022).** According to earlier studies, AhR signaling changes bone remodeling via regulating the NF-κB, Wnt, and MAP kinase pathways, which in turn affect the activity and differentiation of osteoblasts and osteoclasts **(Ye et al., 2022; Øvrevik et al., 2014; Wang et al., 2016).**

In current study, the expression of *Ahr* gene highly expressed in OVX, OVX+RES and OVX+CHR groups compared with Control group (Figure-5) due to low activity of osteoblast, these results in agreement with **Yu et al (2014).** Interestingly, in this study using of CH223191 led to inhibition of *Ahr* gene expression that may drive of reversing the osteoporotic bones to be healthier (Figure -5). These findings are streamed with prior studies **(Choi et al., 2012; Tong et al., 2017)**.

6. CONCLUSIONS

This study highlights the crucial role of AhR in bone metabolism, particularly in osteoporosis. Ovariectomy-induced osteoporosis led to decreased bone density and altered calcium metabolism, while resveratrol and AhR antagonist CH223191 improved bone health. RES as a phytoestrogen compound enhanced calcium levels and reduced osteocalcin in AhR-independent manner, while CH223191 effectively inhibited AhR activity, decreasing bone resorption. This could suggest that AhR inhibition and phytoestrogen supplementation could be a promising potential therapeutic strategies for osteoporosis management.

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Novelty Statement.

This study is the first to demonstrate that resveratrol reverses ovariectomy-induced osteoporosis through the upregulation of the Ahr-Esr1 axis. By employing a combination of resveratrol and the AhR antagonist CH-223191 in a rat model, we reveal a novel molecular mechanism in which resveratrol not only restores calcium balance and bone density but also modulates key regulatory genes—specifically enhancing the expression of AhR and estrogen receptor alpha (*Esr1*) that is critical for bone metabolism. These findings provide fresh insights into the interplay between environmental and hormonal signaling

pathways in osteoporosis and suggest new therapeutic targets for the management of postmenopausal bone loss.

Conflict Of Interests

The author declared that there is no conflict of interest.

Author Contribution

HA: designed, mentored, analyzed collected data, sorted them and reviewed the draft and final versions of this manuscript. SA: performed all experiments, collected data and wrote the initial draft version of this manuscript. Both HA and SA approved the current form of the manuscript.

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