Research Article

Ghrelin and Leptin Changes in Serum of Postmenopausal Osteoporotic Iraqi Women after Three Months Oral Estrogen Therapy

Elaf Mahmood Shihab*[1], Mustafa G. Al-Abbassi[1], Khudair Z. AL-Bedri[2], Inas Taha Ahmad[3]
[1]Department of Pharmacology and Toxicology, College of Pharmacy, Al-Mustansirya University, Baghdad, Iraq, [2]Department of Rheumatology, College of Medicine, Baghdad University, Baghdad, Iraq, [3]Al-Elwyia Maternity Teaching Hospital, Baghdad, Iraq.
***Corresponding author's E-mail:** Phelafmahmood86@yahoo.com

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ABSTRACT

Randomized single blind clinical trial was carried out in Rheumatology outpatient Department of Baghdad Teaching Hospital / Medical city complex, from January 10th till June 12th, 2016. The sample size was (50) postmenopausal osteoporotic patients; who were initially involved in the study; (10) cases were excluded according to exclusion criteria, (10) cases couldn't be followed and (6) cases were again excluded because didn't ingest the drug daily. The remaining (24) women were enrolled in the study. They had the following inclusion criteria; they were 47 to 65 years, with amenorrhea for more than 12 consecutive months, a high follicle stimulating hormone, (30.6-106.3 mIU/mL), diagnosed to be osteoporotic; with a bone mineral density, measured by Dual Energy X-Ray Absorptiometry, of 2.5 standard deviation or more below the young female adult mean (T-score less than or equal to –2.5 Standard Deviation) and a body mass index >19.0 kg/m^2 and < 30 kg/m^2. Ghrelin level was significantly elevated in estrogen group users; from (1068.17 ± 537.98) at baseline, reaching 1171.08 ± 87.26) after end of estrogen treatment; (P=0.011). Leptin; on the other hand, showed a significant increase in estrogen group users as well compared to its baseline level before estrogen treatment, (P<0.001), 49.13 ng/ml.

Keywords: Ghrelin, Leptin, Oral Estrogen, Postmenopausal Women.

INTRODUCTION

Osteoporosis is a pored and weak bone that is incapable of maintaining normal bone functions[1], characterized by low bone mass and structural deterioration of bone tissue, leading to an increased risk of fractures[2]. It is diagnosed clinically or radio graphically. It affects approximately one-tenth of women aged 60 worldwide[3]. This disease causes more than 8.9 million fractures annually, resulting in an osteoporotic fracture every 3 seconds. According to the World Health Organization (WHO) criteria; osteoporosis is defined as a Bone Mineral Density) BMD) that lies 2.5 SD or more below the average value of a young healthy women (a T-score of less than -2.5 SD). The most widely validated technique to measure the body mass index (BMD) is Dual Energy X-ray Absorptiometry (DXA) and the diagnostic criteria based on the T-score for BMD are the recommended entry criterion for the development of pharmaceutical interventions in osteoporosis[4]. The word 'menopause' is derived from men and pauses[5]. Any woman who has experienced 12 consecutive months without menstruation is postmenopausal. An estimated 75% of women ages 50 to 55 are considered to be postmenopausal. Postmenopausal osteoporosis is a progressive loss of bone density which results in pathological fracture within 10-20 years of the onset of menopause. Estrogen normally prevents bone loss by way of multiple effects on bone marrow and bone cells which cause decreased osteoclast formation, increased osteoclast apoptosis, and decreased capacity of mature osteoclasts to resorb bone[6]. Estrogen is the most important sex steroid in preventing osteoporosis in women. It has two forms of receptors, detected in osteoblasts and osteoclasts. It is an inhibitor of bone resorption that decreases both osteoclast numbers and activity. It promotes apoptosis and has anabolic effects on osteoblasts. Its action on osteoclasts is superior in comparison with that on osteoblasts[7]. Ghrelin the "hunger hormone"; a 28 amino acid, produced by ghrelinergic cells in the gastrointestinal tract, which functions as a neuropeptide in the central nervous system and significant role in regulating distribution and rate of use of energy[8]. Ghrelin, was discovered to be the natural ligand of the type 1a growth hormone (GH) secretagogue receptor (GHS-R1a). Thus, it was considered as a natural GHS additional to growth hormone-releasing hormone (GHRH), although later on ghrelin has mostly been considered a major orexigenic factor[9], and this considered as the most powerful orexigenic peptide hormone that leads to increase food intake and decrease energy expenditure. Ghrelin has been shown to modulate osteoblast differentiation and function, both directly and perhaps also through regulation of the GH–insulin-like growth factor axis. However, recently it has also been shown that ghrelin interacts with leptin in modulating bone structure, constituting a new mechanism that couples bone metabolism with energy homeostasis[9]. Leptin is a cytokine produced by the differentiated white adipose tissue, with various functions on the immune and endocrine systems, including reproduction, glucose homeostasis, hematopoiesis, angiogenesis, osteogenesis, wound healing, and inflammation[10]. This hormone

regulates caloric expenditure and intake, playing a central action in energy balance. It is directly correlated with fat mass and is elevated in obese patients, who are also leptin resistant. This hormone is known to regulate food intake but has also emerged as a significant factor in the regulation of bone mass. In humans, states of energy deprivation with low serum leptin have been associated with low bone mass. Leptin regulates bone metabolism indirectly in the hypothalamus thereby activating the sympathetic nervous system (SNS). In addition to the SNS, leptin also interacts with various hypothalamic neuropeptides, such as cocaine- and amphetamine regulated transcript, neuropeptide Y and/or neuromedin U, which might modulate the effects of leptin on bone. In osteoblasts sympathetic signaling is further gated by the transcriptional factors called molecular clock. As a result, bone loss is accelerated showing that the central effect of leptin seems to be anti-estrogenic. Additionally, leptin has a direct anabolic effect within the bone driving the differentiation of bone marrow stem cells into the osteoplastic cell lineage. Besides, the interaction between the central and peripheral pathways, the overall effect of leptin on bone might be bimodal depending on leptin serum concentrations[11]. Conjugated Estrogens is an active pharmaceutical ingredient (API) obtained from a natural source. It contains a mixture of many steroidal and non-steroidal components derived from pregnant mares' urine. The term (conjugated estrogen) refers to mixtures of at least 8 compounds, including sodium equiline sulfate, derived from wholly or in part from equine urine or synthetically from estrone and equilin. Conjugated estrogens contain as concomitant components the sodium sulfate conjugates of 17 alpha dihydroequilin, 17 beta dihydroequiline and 17 alpha estradiol[12, 13].

Aim of the study

To evaluate the effect of estrogen therapy on levels of Ghrelin and Leptin in serum of postmenopausal osteoporotic women**.**

Methodology

Randomized, single blind clinical trial was carried out in the Rheumatology Outpatient Department of Baghdad Teaching Hospital / Medical city complex; from the 10th of Jan to the 12th of June 2016. The protocol was approved by the local Committee from College of Pharmacy, Al-Mustansirya University and Medical Ethics Committee in Baghdad College of Medicine, University of Baghdad, Iraq. The patients who attended the unit were screened for eligibility according to the inclusion and exclusion criteria. Each participant gave a written consent of agreement to participate in.

Women excluded from the study were; those with history of Diabetes Mellitus, osteoporotic fracture, thyroid disease, smokers, steroid therapy, severe liver disease. cerebrovascular, cardiovascular and thromboembolic diseases, patients with undiagnosed vaginal bleeding, endometriosis, genital neoplasia, breast neoplasia and family history of breast carcinoma, severe hyper triglyceridemia, history of migraine, renal impairment and patients using supplements containing antioxidant as vitamin A, C and selenium, using hormone replacement therapy and antihypertensive drugs and patients with malabsorption disease (irritable bowel syndrome, ulcerative colitis), non-compliance with treatment & patients suffering from side effects of estrogen treatment. The sample size was (50) postmenopausal osteoporotic patients; who were initially involved in the study; (10) cases were excluded according to exclusion criteria, (10) cases couldn't be followed and (6) cases were again excluded because didn't ingest the drug daily. The remaining (24) women were enrolled in the study, had the following inclusion criteria; they were 47 to 65 years old , amenorrheic for more than 12 consecutive months, a high follicle stimulating hormone, (30.6-106.3 mIU/mL), osteoporotic; with a bone mineral density measured by Dual Energy X-Ray Absorptiometry , of 2.5 SD or more below the young female adult mean (T-score less than or equal to –2.5 Standard Deviation) and a body mass index >19.0 kg/m2 and <30 kg/m^2**.** A written concept for agreement to participate was obtained from each case. They were divided into two groups. The two groups of patients were equally divided; 12 patients for each group as follows: Group A; 12 postmenopausal osteoporotic women, treated by conjugated equine estrogen 0.625 mg single daily tablet (Premarin) for 90 days. Group B: 12 cases of postmenopausal osteoporotic women, given a placebo capsule (filled with starch), daily for the same duration. Five cc of blood was aspirated from each patient, put in a sterile syringe and transferred into plastic test tube without anticoagulant, centrifuged for 15-20 minutes at 2000 rpm.The serum sample was separated in to two pin tubes and stored in deep freeze (- 20°C- 40°C)**.** This sample from participants at day zero of treatment was considered as a base line, then repeated after 90 days of treatment, to obtain serum for measurement of Ghrelin & Leptin levels pre and post treatment. Specific kits, chemicals and reagents used in this study were of the highest available purity and needed no more purification; they are listed in table 1.

Table 1: Drugs and chemicals with their manufactures

Drugs and Kits	Manufacturers
(PREMARIN ® conjugated equine estrogen tablets) 0.625mg	Pfizer company
GGhrelin Elisa kit	Human, USA
Leptin Elisa kit	Human, Germany

The Equipments used were; Bioelisa reader, Centrifuge Universal 16A, Vortex mixture while Instruments Micropipette multichannel 50-300 µl, Tips yellow and blue, pin tubes and Micropipette 100 µl -1000 µl.

The RayBio Human Ghrelin ELISA kit is an in vitro enzyme-linked immunosorbent assay for the quantitative measurement of human GHRL/Ghrelin in serum, plasma

and cell culture supernatants. This assay employs an antibody specific for human GHRL/Ghrelin coated on a 96-well plate. Standards and samples are pipetted into the wells and GHRL/Ghrelin present in a sample is bound to the wells by the immobilized antibody. The wells are washed and biotinylated anti-human GHRL/Ghrelin antibody is added. After washing away unbound biotinylated antibody, HRP-conjugated streptavidin is pipetted to the wells. The wells are again washed, a TMB substrate solution is added to the wells and color develops in proportion to the amount of GHRL/Ghrelin bound. The Stop Solution changes the color from blue to yellow, and the intensity of the color is measured at 450 nm. The results were measured by pg/ml.

Human leptin Elisa kit Assay principle

The Demeditec ELISA for Leptin is a so-called Sandwich-Assay using two specific and high affinity antibodies. The Leptin in the samples binds to the first antibody coated on the microtiter plate. In the following step the second specific anti-Leptin-Antibody binds in turn to the immobilised Leptin. The second antibody is biotinylated and will be applied in a mixture with a Streptavidin-Peroxidase-Enzyme Conjugate. In the closing substrate reaction the turn of the colour will be catalysed quantitatively depending on the Leptin-level of the samples. The result measured by ng/ml.

Statistical Analysis Data of the participated women were entered and analyzed using the Statistical Package for Social Sciences (SPSS), version 24, IBM, US, 2013.

Descriptive statistics were presented as mean, standard deviation (SD), Difference rate as (%). Analysis of Variances (ANOVA) was used to compare the means of each studied parameters across the two studied groups; Estrogen and Placebo, multiple comparison in between groups was performed using the post hoc test (LSD). Paired t test was used to assess the differences in the mean levels of each studied parameter within each group for Pre-treatment vs. Post treatment levels. Level of significance (P-Value) of ≤ 0.05 considered as significant. Finally, the results and findings presented in tables and figures with an explanatory paragraph for each, using the MS Word 2010software for windows.

RESULTS

The mean age of patients was 56.3 ± 6.14 (47 – 65 years). The mean Body Mass Index (BMI) of the participants was 26.86 ± 2.1 (22.3 – 29.4) kg/m^2. Comparison of mean age and BMI across the two studied groups revealed no statistical significant differences in these two variables (P>0.05), as presented in table 2.

Table 2: Age and BMI mean values of the studied groups

Variable	Statistic	Group		P. value
		Estrogen (A)	Placebo(B)	
Age (year)	Ma	57.1 ± 6.1	56.1 ± 6.4	0.91*
BMI (kg/m^2)	Mean	26.9 ± 2.1	26.5 ± 2.4	0.78*

Group A: Estrogen users, Group B: Placebo users * Not significant

Ghrelin level was significantly elevated in estrogen user's group from (1068.17 ± 537.98) at baseline to reach 1171.08 ± 87.26) after treatment (with a P value = 0.011), with a mean difference of (102.92 ± 34.31) and an increment rate of 7.2%, compared to (3.73 ± 1.24) mean difference in placebo group, with an increment rate of 0.4%. However; the changes in the two groups were statistically insignificant, (P>0.05), as shown in table 3.

Table 3: Comparison of changes in Ghrelin levels among the studied groups

Ghrelin mean ± SD (pg/ml	Groups		P.value
	Estrogen group(A)	Placebo group(B)	
Pre treatment	1068.17 ±537.98	1027.25 ± 390.87	0.973*
Post treatment	1171.08 ± 87.26	1030.98 ± 95.24	0.012**
mean difference (pre - post)	102.92 ± 34.31	3.73 ± 1.24	< 0.001***
Difference rate (%)	7.2%	0.4%	
P value (pre vs. post)	0.011**	0.476*	

*= Not significant, **= Significant, ***= Highly significant

Leptin level showed a significant increase in estrogen group users compared to its baseline level, (P<0.001). On the other hand; the mean difference in Leptin was significantly much higher than placebo group; (P<0.05) as revealed in table 4.

Table 4: Comparison of changes in Leptin levels among the studied groups

Leptin (ng/ml)	P. value (between groups)		
	Estrogen (A)	Placebo(B)	
Pre treatment	46.40 ± 24.23	46.88 ± 20.08	0.993*
Post treatment	55.76 ± 26.31	45.71 ± 19.44	0.57*
mean difference (pre – post)	9.36 ± 3.12	1.17 ± 0.39	0.001**
Difference rate (%)	20.2%	2.5%	
P. value (pre vs. post)	< 0.001**	0.866*	

*= Not significant, **= Highly significant

DISCUSSION

Estrogen increases the availability of ghrelin receptors by promoting the gene encoding for growth hormone secretagogue receptors. Alternatively; administration of estrogen reduces the somatostatin inhibitory drive on growth hormone secretion, as reported by relevant pilot studies, in accordance with finding that central effect of ghrelin is at least partially mediated through the counteraction of somatostatin potency. Finally; estrogen exposure promotes growth hormone synthesis and enhances growth hormone releasing hormone stimulating secretion, with a greater availability of growth hormone in the pituitary somatotrophic cells, as demonstrated by in vitro and in vivo investigations in primates and rodents[14]. The current study revealed that level of Ghrelin was increased with significant differences after treatment with per oral estrogen. This was found by Kellokoski Eija, *et al* as well; a study that showed a significant increase in ghrelin levels after a 6-month treatment with oral estrogen, while trans dermal estrogen did not change ghrelin levels significantly[15]. Moreover; it is in agreement to that found by Soni, Amy C, *et al*., study in 2011[16]. Kellokoski *et al*. on the other hand; recently reported that estrogen therapy in hysterectomized women increases plasma active ghrelin levels by 14% especially following oral administration as compared to transdermal[16.] In addition; treatment of post-menopausal women with trans-dermal 17β-estradiol at a dose of 50 µg/day in a continuous regimen for at least 24 months and nomegestrol at a dose of 5 mg/day for 12 days/month in a sequential regimen resulted in higher ghrelin levels as compared to untreated post-menopausal women[17]. On the contrary; the current results were not in agreement with that found by Purnell *et al*., who reported no changes in total ghrelin levels between users and non-users of hormone therapy[18]. Furthermore; Veldhuis *et al*.,[19] reported no alterations in total ghrelin levels after transdermally administering estrogen 2 (E2) in escalating doses to mimic late follicular-phase estrogen 2 (E2) concentrations in 10 post-menopausal women compared to placebo group. In contrast; Chu *et al*.,[20] found a decrease in total ghrelin levels following oral or transdermal estrogen therapy in obese postmenopausal women with metabolic syndrome. Dafopoulos, K., *et al*.,[21] in his study revealed that plasma ghrelin levels were significantly decreased in post-menopausal than in pre-menopausal women. All these variations between the studies may be attributed to; the difference in sample size collection, the way, the time and for how long the patients had the estrogen.

There are matches in mechanism of action between estrogen and leptin in manipulating the process of bone restoration. It may be considered that the role of estrogen can be replaced by leptin[22]. Leptin acts on bone through two indirect regulation mechanism discovered by Ducy et al. on mutant mice. When they were injected with leptin in intra cerebroventricular region , it caused decrease in bone mass, because leptin stimulated the brain to release Hypothalamic Osteoblast Inhibitory Factor (HOBIF). Activation ObRb (long isoform of leptin receptor) in the hypothalamus stimulates HOBIF, which, if secreted, it will reduce ability of bone matrix formation by osteoblasts[22]. Another indirect regulation mechanism by which leptin acts on bone is by; the neuropeptide Y (NPY) and its receptor (Y2) which motivates the secretion of HOBIF. Loss of leptin or Y2 receptor will decrease productivity and rise osteoblast HOBIF. In the current study; didn't have any obese participant, as all of them were either overweight or normal (BMI<30), because leptin level normally increases in obese patients. That's why with treatment estrogen in those participants, it was found that the level of leptin was found to be significantly increased. This is concordant with Syed, Farhan A., *et al*[23] study who revealed that serum leptin levels increased significantly after estrogen treatment, whereas marrow adipocyte parameters showed a decrease. However; changes in serum leptin levels in either group did not correlate with changes in bone or adipocyte parameters. Consistent with the current findings, estrogen treatment of mice has been shown to increase leptin mRNA levels and estrogen also stimulates leptin secretion from human subcutaneous adipose tissue fractions in vitro. However, leptin has complex effects on bone metabolism, with evidence for both central (hypothalamic) negative effects on bone mass as well as peripheral effects enhancing osteoblast differentiation and inhibiting adipocyte differentiation of bone marrow stromal cells[23]. Moreover it is in agreement with Dedeoğlu EN[24] study, who concluded that hormone therapy administration increase the serum leptin levels while maintaining body weight

and body fat distribution. On the other hand; some studies have showed an increase in serum leptin, after one month of therapy with transdermal estradiol alone or in combination with transvaginal progesterone, after 2 months of treatment with 2 mg of oral estradiol, as well as after 6 months of oral estrogen therapy[25]. On the contrary; Di Carlo *et al*. showed a decrease of serum leptin to premenopausal levels after transdermal hormonal replacement therapy; a finding confirmed by the same group in a subsequent study[26]. Further to differences in study design and regimens used, this discrepancy may be explained by the indirect lowering effect of hormone therapy on leptin through the reduction of adipose tissue content, as opposed to a direct estrogen-mediated stimulatory effect on leptin secretion [25].

CONCLUSIONS

Ghrelin significantly increases in post-menopausal women when treated with estrogen therapy , whereas the increment of leptin is highly significant in those women .That mean postmenopausal osteoporotic patients might be beneficial if they were prescribed Estrogen tablet 0.625 mg daily for 3 months.

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