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CHANGES IN THE SERUM LEVELS OF ESTRADIOL AND IN THE EXPRESSION OF ESTROGEN RECEPTOR ALPHA IN AN EXPERIMENTAL MODEL OF OSTEOPOROSIS

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Abstract

Introduction. The decrease in serum estrogens concentration at menopause disrupts the metabolic balance and leads to visceral obesity, which causes an increase in serum estradiol levels, through aromatase activity. Also, estrogen deficiency is a reason for the development of osteoporosis.
The objective of the study was to investigate the serum estradiol levels and changes in bone alpha estrogen receptor expression in an experimental model of osteoporosis.
Materials and methods. The study included 20 female Wistar rats at a reproductive age of two months, divided into two groups: group 1 (G1) – 10 ovariectomized rats, and group 2 (G2) –10 rats sham-operated. SPSS 20 software (SPSS, Inc., Chicago, IL, USA) was used for statistical analysis of the results. For the immunohistochemical study, the nonparametric test – Mann Whitney was used, by comparing the median values. Data were presented as mean ± standard deviation, with $p < 0.05$ as the limit for statistical significance.

Résumé

Modifications des niveaux sériques d'estradiol et de l'expression du récepteur alpha des œstrogènes dans un modèle expérimental d'ostéoporose

Introduction. La diminution de la concentration sérique en œstrogènes à la ménopause perturbe l'équilibre métabolique, conduisant à l'obésité viscérale, qui provoque une augmentation des taux sériques d'œstradiol, par l'activité de l'aromatase. La carence en œstrogènes est également une cause du développement de l'ostéoporose.
L'objectif de l'étude a été d'étudier les taux sériques d'œstradiol et les changements dans l'expression des récepteurs osseux des œstrogènes alpha dans un modèle expérimental d'ostéoporose.
Matériels et méthodes. Nous avons utilisé 20 rats Wistar, femelles, en âge de procréer , de deux mois, répartis en deux groupes : groupe 1 (G1) –10 animaux ovariectomisés et groupe 2 (G2) –10 animaux simulés. Toutes les analyses statistiques ont été effectuées à l'aide du logiciel SPSS 20 (SPSS, Inc., Chicago, IL, USA). Pour l'étude immunohistochimique, nous

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Results. All animals of G1 had weight gain compared to group G2. The results showed that the values of serum 17β-estradiol in rats of G1 statistically significant increased compared to G2 ($p<0.05$). The immunohistochemical analysis revealed no difference in estrogen receptor expression between the groups. The histological analysis of femur in rats from G1 showed pronounced osteoporosis.
Conclusions. Ovariectomy led to the development of obesity, which caused an increase in serum estradiol levels, through aromatase activity. However, this increase of serum estradiol did not prevent osteoporosis.

Keywords: estrogen deficiency, obesity, 17β-estradiol, osteoporosis.

List of abbreviations:
E2 – 17β-estradiol
ERs – estrogen receptors
ELISA – enzyme-linked immunosorbent assay
IHC – immunohistochemical
OVX – ovariectomized rats
SHAM – sham-operated rats
H&E – hematoxylin & eosin staining
OPG – osteoprotegerin
RANKL – receptor activator of nuclear factor kappa-ligand

Résultats: Tous les animaux de G1 ont pris du poids par rapport au groupe G2. Les résultats ont montré que les valeurs de 17β-estradiol sérique chez les rats de G1 augmentaient statistiquement par rapport à G2 ($p <0,05$). L'analyse immunohistochimique n'a révélé aucune différence dans l'expression des récepteurs aux œstrogènes entre les deux groupes. L'analyse histomorphologique du fémur des rats de G1 a montré la présence d'une ostéoporose prononcée. **Conclusions.** L'ovariectomie a entraîné le développement de l'obésité, qui a provoqué une augmentation des taux sériques d'œstradiol, par l'activité de l'aromatase, mais ce processus n'a pas empêché le tissu osseux de développer l'ostéoporose.

Mots-clés: déficit en œstrogène, obésité, 17β-estradiol, ostéoporose.

Introduction

Menopause is a physiological process that leads to changes in reproductive and non-reproductive organs. The endocrine changes that occur affect various systems and organs, such as cardiovascular, endocrine, bones and others[1]. The decrease in serum estrogens concentration disrupts the metabolic balance and changes the lipid profile, which often leads to visceral obesity[2,3]. Obesity is an abnormal or excessive fat accumulation that may impair health[4]. Obesity and metabolic syndrome are found in the menopausal period three times more often than before menopause[5]. In the postmenopausal period, with a deficiency of estrogens, androgens from adrenal glands and fat tissue produce circulating estrogens due to aromatase enzyme[6]. The enzyme catalyzes the conversion of androstenedione to estrone, with its further intracellular conversion to 17β-estradiol (E2). Aromatase is expressed in white adipose tissue in both women and men, and its production increases with age[7,8]. Estrogen production via aromatase activity occurs in the stromal cells of the adipose tissue rather than in adipocytes, as previously considered[9]. Aromatase-derived estrogens do not appear to be associated with a protective status of insulin sensitivity and cardiovascular disease, and the issue of osteoporosis prevention remains debatable[1,10]. After menopause, the bone tissue is often irreversibly altered by osteopenia and osteoporosis, sometimes without symptoms[2,11]. Osteopenia is the precursor of osteoporosis[12]. Osteoporosis is a chronic condition that reflects reduced bone strength associated with an increased risk of fracture. Menopausal obesity combined with increased E2 synthesis is a compensatory mechanism[10] against the development of osteoporosis, because E2 exerts its influence by binding to its receptors (ERα and ERβ) in bone tissue[13]. Both ERα and ERβ bind E2 with high affinity in bone[14]. The tissue sensitivity to estrogens depends on the number of estrogen receptors in the tissue[1].

The objective of the study was to investigate the serum estradiol levels and changes in the immunohistochemical expression of estrogen receptor alpha in bone tissue, in an experimental model of osteoporosis.

Materials and methods

The experimental study was approved by the Scientific Ethics Committee of Medical

University-Pleven, Bulgaria (approval no. 556/07.05.2019). The experiment was performed on 20 female Wistar rats at a reproductive age of two months, with an initial weight of 150±20 grams, who were divided into two groups: group 1 (G1) –10 animals with bilateral ovariectomy (ovx), and group 2 (G2) –10 animals sham-operated (SHAM).

All rats grew in standard rules of work with laboratory animals adopted from Medical University-Pleven, Bulgaria. The animals were prepared for the experiment by acclimating to the conditions for one week prior to the experiment. They were accommodated in an air-conditioned room (relative humidity 45-65%) over a 12-hour light/dark cycle, at 22±2°C, with free access to food and water.

The model of osteoporosis was created according to the method of Kharode et al[15]. For operational purposes, a cocktail of xylazine (10 mg/kg) and ketamine (90 mg/kg) was administered intraperitoneally. We calculated for each animal a dose of anesthesia according to their weight, which is detailed in the experimental protocol. After the period of 60-days necessary for the development of osteoporosis, the animals were again anesthetized, and blood was collected by abdominal aorta puncture. The blood was collected in vacutainers, the serum was separated by centrifugation and then stored at –80 °C for examination by the enzyme-linked immunosorbent assay (ELISA) method of enzyme activity. The serum level of 17β-estradiol was determined using the ELISA immunological method. Antibodies for 17β-estradiol were purchased from R&D Systems, with catalog number KGEO014. Following animals' euthanasia, the left femur was dissected for the preparation and execution of histomorphological and immunohistochemical (IHC) study. According to Fonseca et al., the estrogen deficiency leading to osteoporosis is best observed in the proximal femur[16]. Each sample was subjected to routine histological testing by applying hematoxylin & eosin (H&E) and Van Giesson (VG) staining. For IHC studies of the estrogen receptors alpha (ERα), we used anti-estrogen receptor alpha antibody [EPR4097] ChIP Grade ab108398, according to the instructions of the manufacturer (Abcam, UK). The IHC analysis was semi-quantified by a pathologist involved in the project. The following scale was used for the intensity of the estimation: 0 – no coloring; 1 – weak; 2 – moderate; 3 – strong[17]. All statistical analyses were performed using the SPSS 20 software (SPSS, Inc., Chicago, IL, USA). For the IHC study the nonparametric test – Mann Whitney was used, by comparing the median values. Data were presented as mean ± standard deviation, with a p-value < 0.05 as the limit for statistical significance.

Results

Results of weight determination

The results of the study showed that, at the beginning of the experiment, there was no significant difference of animals' weights between the two groups. At the end of the two-month period, the animals of the group G1 (336.32±41.72 g) had significantly increased their weight compared to the group G2 (270.3 5±18.27 g), (*p <0.05) (Figure 1).

Results of the determination of serum E2 concentrations

The concentrations of E2 in G1 (129.12±18.6 pg/mL) were significantly increased compared to G2 (77.05±28.2 pg/mL), with a significant difference between the two groups (* p<0.05) (Figure 2).

Histology results

The successful development of the osteoporosis model was confirmed by histological studies, stained with HE (Figure 3).

Results of immunohistochemistry

The results showed that IHC staining for ERα was primarily nuclear. It was not found a statistically significant difference in the IHC staining of ERα between the group with osteoporosis (G1) and the control group (G2) (Figure 4).

Discussion

The results of our study contradict that obesity is protective against fracture and suggest that obesity is a risk factor for fractures. During menopause, adipose tissue is an important extragonadal site of E2 biosynthesis[10]. However, not only the presence, but also the sensitivity of the bone tissue to the circulating E2 is important. Tissue quantification of estrogen receptors is an important factor in assessing tissue sensitivity to estrogen[1,18]. E2 exerts its influence by binding to the receptors ERα and ERβ[19,20]. Several reports describe a beneficial effect of obesity accompanied by high serum concentrations of E2 on bone tissue[21,22], because by IHC analysis ERs were found in osteoblasts, osteoclasts, and osteocytes[23]. In our experiment, all ovx rats were found to be overweight and had high estradiol concentrations compared to healthy controls, confirming the activation of aromatase activity after ovx. The results regarding the expression of ERs receptors in bone tissue in case of estrogens deficiency are also ambiguous. Some authors claim that the expression of ERs is reduced by estrogen deficiency[24], and it changes during osteoporotic fracture[25]. Other authors report that the

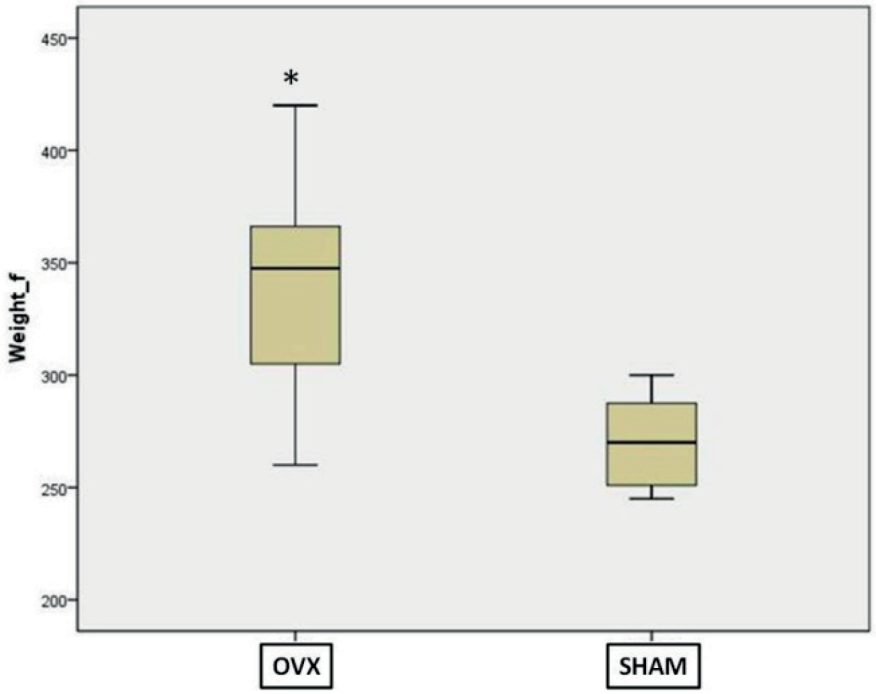

Figure 1. The animal weight at the end of the experiment, with statistically significant differences in the weights in G1 and G2. Data are presented as median, minimum and maximum value and standard deviation (*p <0.05).

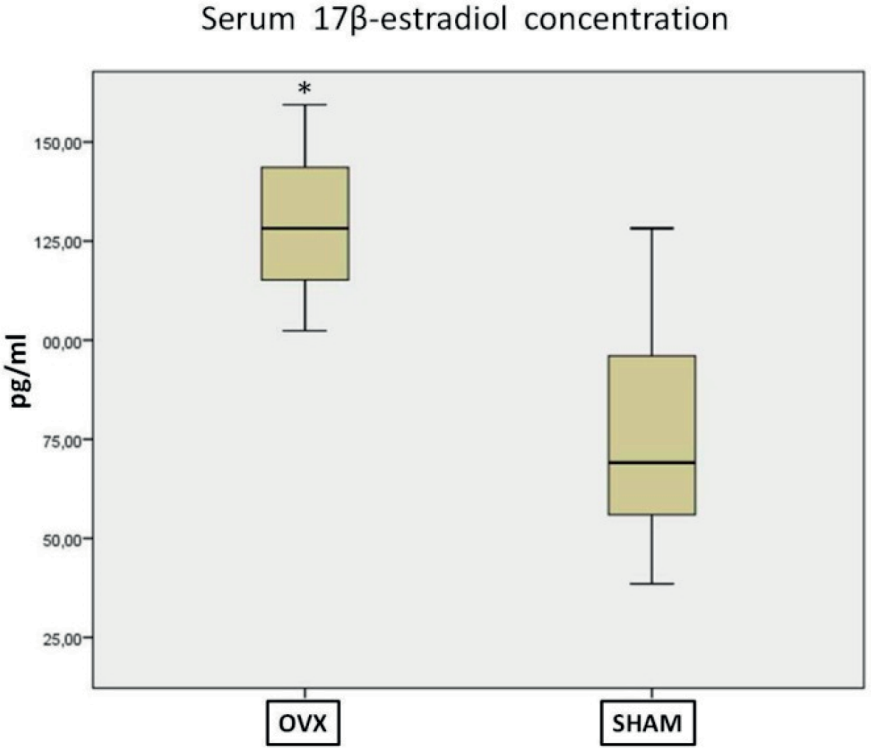

Figure 2. Serum E2 concentration. Significant difference in E2 between groups G1 and G2. Data are presented as median, minimum and maximum values and standard deviation, in pg/mL, n = 20.

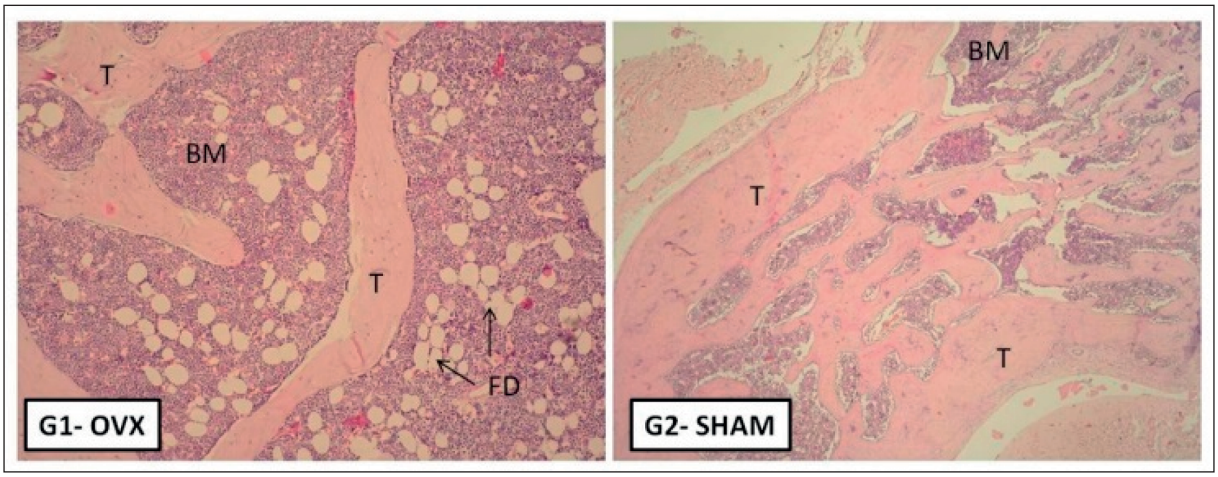

Figure 3. Preparations of femoral bone stained with HE. Magnification x 100. In animals with osteoporosis (ovx), there was thinning of the bone trabeculae (T) and disruption of the relationship between them, reduction of bone mass and increase in osteoclastic activity, reduced amount of bone marrow (BM) and the presence of fatty degeneration (FD).

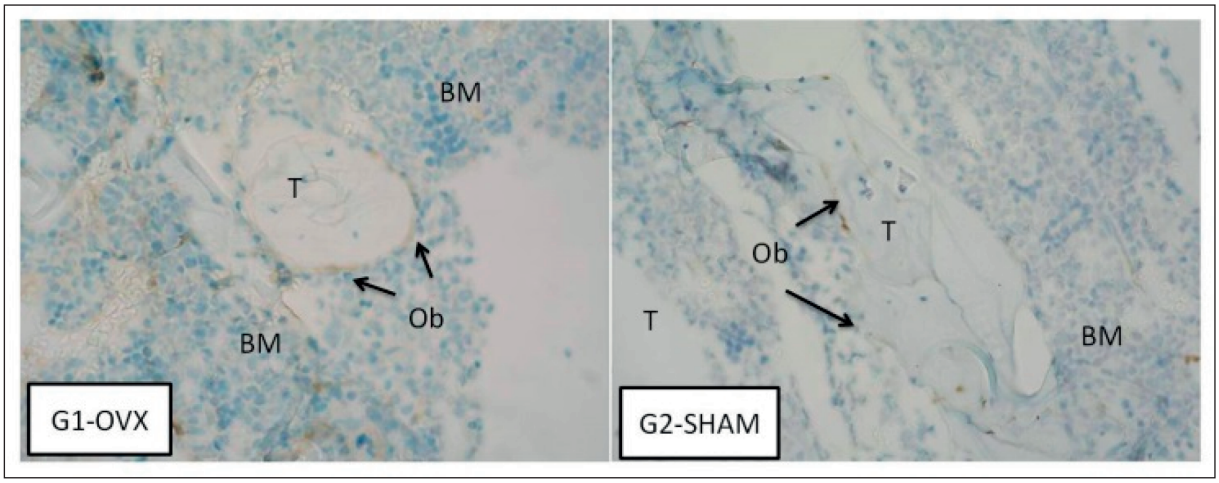

Figure 4. IHC response of ERα in femur preparations. An animal from group G1: weakly nuclear immunostaining for ERα in osteoblasts. An animal from group G2: weakly nuclear immunostaining for ERα in single osteoblasts. Light brown staining, magnification x 400. T-trabeculae, BM-bone marrow, Ob-osteoblast.

expression of receptors increases after estrogens deficiency, which is associated with changes in osteoblastic function, namely increased alkaline phosphatase activity[26]. Our studies confirmed the existence of the ERs in osteoblastic cells. We have reported high levels of alkaline phosphatase after ovx[27], but nevertheless we did not find statistically significant changes in estrogen receptor expression between ovx and SHAM group. Like the study of Ikegami et al.[28], we confirm that E2 does not alter ERs expression. Various research teams claim that estradiol prevents osteoporosis[28-31]. However, we found that overweight animals with high estradiol concentrations had significant osteoporotic changes in the femur. The explanation probably consists in the fact that the adipose tissue is a source of pro-inflammatory cytokines[32,33], which promote osteoclast activity and bone resorption through modifying the receptor activator of nuclear factor kappa-ligand (RANKL)/RANK/osteoprotegerin (OPG) system[27,34]. The unchanged expression of ERs and overproduction of inflammatory mediators induced by obesity and endocrine imbalance explain the increased bone resorption leading to osteoporosis[35-37] despite high estradiol levels. In addition, adipocytes and osteoblasts originate from a common progenitor (pluripotential mesenchymal stem cell) that has an equal tendency to differentiate into adipocytes or osteoblasts, such as alteration of the Wnt/β-catenin pathway[38]. This process is stimulated by transcription factors, proinflammatory cytokines and bioactive compounds, called adipokines, produced by adipose tissue especially in the presence

of estrogen deficiency. The link between estrogen deficiency and disorders in the catenin-dependent pathway leading to fatty degeneration of the bone marrow has already been described[39]. Accordingly, obesity leads to fatty degeneration of the bone marrow, with an increased risk of fractures[40]. Therefore, unchanged expression of ERs and overproduction of inflammatory mediators induced by obesity explain the increased bone resorption leading to osteoporosis, despite high estradiol levels.

Conclusions

Obesity has a detrimental effect on bone health, in both animals and humans. High levels of E2 in obese rats cannot be regarded as a protective factor against osteoporosis. Ovx did not alter estrogen receptor expression despite high alkaline phosphatase and E2 levels.

Author Contributions:

Conceptualization, A.V.G.; methodology, T.M.B. and A. B. B.; software, A.V.G.; validation, T.M.B., A. B.B.; formal analysis, A.A.D. and A.V.G.; investigation, T.M.B. and A.B.B.; resources, T.M.B., A.B.B. and A.V.G.; data curation, A.A.D.; writing–original draft preparation, A.V.G.; writing–review and editing, T.M.B., A.B.B and A.A.D.; visualization, A.V.G.; supervision, A.A.D.; project administration, A.V.G. All the authors have read and agreed with the final version of the article.

Compliance with Ethics Requirements:

„The authors declare no conflict of interest regarding this article."

„The authors declare that all the procedures and experiments of this study respect the ethical standards in the Helsinki Declaration of 1975, as revised in 2008(5), as well as the national law."

"All institutional and national guidelines for the care and use of laboratory animals were followed"

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