Original Articles

CHANGES OF SERUM CONCENTRATIONS OF ALKALINE PHOSPHATASE AND METALLOPROTEINASE-9 IN AN OVARIECTOMIZED WISTAR RAT MODEL OF OSTEOPOROSIS

Armine V. Grigoryan, Aneliya A. Dimitrova, Krasimir G. Kostov, Adelaida L. Russeva[1], Milena A. Atanasova[2], Alexander B. Blagev[2], Tatyana M. Betova[3], Radoslav G. Trifonov[4]

Department of Pathological Physiology, Medical University – Pleven, Bulgaria
[1]Department of Clinical Laboratory, Medical University – Pleven, Bulgaria
[2]Department of Biology, Medical University – Pleven, Bulgaria
[3]Department of Patholoanatomy, Medical University – Pleven, Bulgaria
[4]Student of Medical University – Pleven, Bulgaria

Corresponding Author:
Armine V. Grigoryan
Department of Pathological Physiology,
Medical University – Pleven
1, St. Kliment Ohridski Str.
Pleven, 5800
Bulgaria
e-mail: armine14@abv.bg

Received: May 29, 2017
Revision received: July 07, 2017
Accepted: November 02, 2017

Summary

Osteoporosis is a systemic skeletal disease characterized by decreased bone mass, destruction of the microarchitectonics of bone structure and a high risk for fracture. One of the criteria for altered bone homeostasis includes the changes in serum levels of alkaline phosphatase (ALP) and the activity of matrix metalloproteinases (MMPs). The purpose of this study was to determine the serum concentrations of calcium (Ca^{2+}), phosphorus (P), magnesium (Mg^{2+}), alkaline phosphatase (ALP) and MMP-9 in ovariectomized rats. We used 35 female Wistar rats at reproductive age (2 months) divided into 2 groups: a control group (G1-SHAM) – 20 animals subjected to "false" ovariectomy and placebo-operation, and an ovariectomized group (G2-OVX) – 15 animals subjected to bilateral ovariectomy. Blood was collected from the abdominal aorta for testing levels of Ca^{2+}, P, Mg^{2+}, ALP and MMP-9. No statistically significant differences in serum concentrations of Ca^{2+}, P and Mg^{2+} were found between G2 and G1 ($p>0.05$). The values of ALP and MMP-9 in rats of G2 were statistically significantly increased, as compared to G1 ($p<0.05$). The serum activity of ALP, which is a marker for bone formation, was increased in OVX-induced osteoporosis. Elevated serum MMP-9 levels in G2 confirmed the hypothesis that it is a marker for osteoclast activity.

Key words: osteoporosis, MMP-9, ALP

Introduction

According to the WHO, osteoporosis is a progressive systemic disease of the bone tissue, characterized by decreased mass and deteriorated microartictonics of the bone, leading to increased bone fragility and risk of fractures [1].

There is a variety of causes of osteoporosis. It is estimated that 10% of the human skeleton is annually updated due to the controlled and regulated activity of osteoclasts (Oc) and osteoblasts (Ob) [2]. The pathological processes that disrupt the balance between the activity of these two cell groups lead to increased bone fragility and brittleness. During menopause or after ovariectomy the level of estrogens falls and this hormone deficiency contributes to the destruction of the bones by activating osteoclasts [3, 4] and increased expression of matrix metalloproteinases (MMPs) [5,

6].

MMPs are a family of zinc-dependent proteolytic enzymes involved in degrading the components of the extracellular matrix (ECM). MMPs, also called matrixins [7], are inactive at rest but many stimuli such as intercellular interactions, cytokines, growth factors and endocrine imbalance can rapidly trigger their expression. They can be activated by proteolytic cascade, involving several MMPs. Their activity is strictly controlled by various endogenous inhibitors in response to a number of stimuli [7, 8]. According to their substrate specificity and structural organization, MMPs are classified into five main groups: collagenases, gelatinases, stromelysins, matrilizins and membrane type I [9]. The major MMPs involved in the pathogenesis of osteoporosis are MMP-1, MMP-2, MMP-3, MMP-9 and MMP-13 [8, 10]. They are key mediators in extracellular matrix remodeling, bone growth and osteoclast bone resorption. MMPs play an important role in early bone resorption because degrade the collagen layer of the bone surface before starting demineralisation [11, 12].

Although no animal model can exactly mimic human osteoporosis, the most suitable are the Wistar line rat models. Bilateral ovariectomy (OVX) in female Wistar rats leads to bone changes similar to post-menopausal women or after ovariectomy, and are suitable for the evaluation of potential therapeutic agents for prevention or treatment of osteoporosis [13].

The purpose of this study was to determine the serum concentrations of calcium (Ca^{2+}), phosphorus (P), magnesium (Mg^{2+}), alkaline phosphatase (ALP) and MMP-9 in ovariectomized rats.

Materials and Methods

We studied 35 female Wistar rats at the age of two months at the initiation of the experiments, with initial weight 150±20 grams. All rats were grown in a standard manner, following the rules for work on laboratory animals adopted by Medical University – Pleven. The animals were prepared for the experiment by adapting them 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 acclimatised 35 rats were divided into 2 groups: control group (G1-SHAM) – 20 animals subjected to "false" ovariectomy, placebo-operation and ovariectomized group (G 2-OVX) – 15 animals subjected to bilateral ovariectomy. The osteoporosis model was performed according to the method of Kharode et al. [13]. The operative access to the ovaries was achieved by a medial incision (about 2 cm) in the ovary area at the most protruding backside. After cutting the fascia and muscles, the ovaries with the adjacent fat tissue were removed with blunt movements. The ovaries were then removed after ligation on the adipose tissue. After the double-sided OVX muscles, fascia and skin were gradually restored. In the placebo group, the same surgical procedure was performed but without ovariectomy. The procedure lasted approximately 10 minutes for each laboratory animal. Postoperatively, the field was treated with an antibacterial powder and each animal was placed in an individual cell for post-operative observation and care. Surgical sutures threads were removed 10 days after surgery [13].

Ovariectomy was performed with a combination of ketamine (90 mg/kg) and xylazine (10 mg/kg), intraperitoneally. Eight weeks after the intervention, we obtained blood by puncture of the abdominal aorta. The blood was collected in vacutainers, the serum was separated by centrifugation and then stored at -80°C for examination with ELISA.

Serum biochemical markers of bone metabolism

Serum concentrations of Ca^{2+}, P, Mg^{2+} and ALP activity were assayed in the Clinical Laboratory of the University Hospital – Pleven with Cobas Integra 400 analyzer (Roche Diagnostic kits). The calcium in the sample reacted with the 5-nitro-5'-methyl-BAPTA (NM-BAPTA) indicator in the reagent and led to a color change. During the incubation phase, a calcium-NM-BAPTA complex was formed and measured photometrically. The phosphorus concentration was determined in a reaction with ammonium phosphomolibdate. The concentration of magnesium was assayed in a color reaction with xylidilblau. The activity of the enzyme alkaline phosphatase was determined with IFCC-

approved method at 37°C. Serum levels of MMP-9 were determined using an ELISA immunological method. Antibodies were purchased from R&D Systems, with catalog number RIRMP900 rat Total-MIVP-9 Quantikine ELISA.

Statistical analysis

The data were processed with statistical software packages Statgraphics Centurion XVI and Excel for Windows. Data are presented as mean values±S.E.M. A level of $p<0.05$ was considered significant.

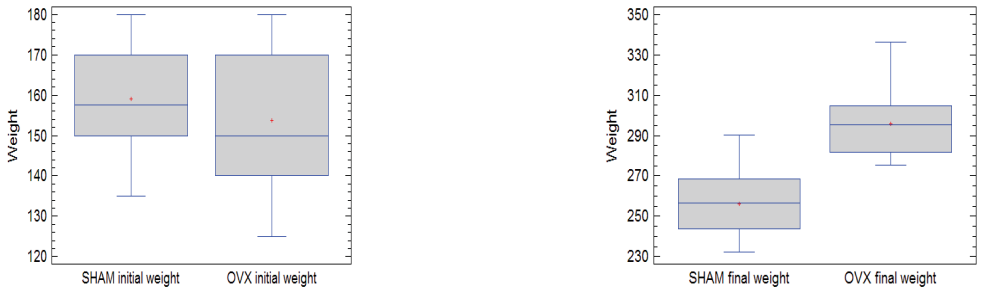
Results

All animals were weighed at the beginning and end of the experiment. Figure1 (A, B) shows changes in mean body weights in both groups at different times. At the beginning of the experiment, the average body weights were similar in both groups: G1 – 159±21 g; G2 – 153±27g ($p>0.05$). At the end of the experiment, the average body weight was significantly higher in OVX group - 295 g, as compared with the SHAM group - 256 g ($p<0.05$).

Regarding serum biochemical markers of bone turnover, we found that the serum concentration of MMP-9 was higher in G2 (5.81 ng/ml), as compared to G1 (2.85 ng/ml), ($p<0.05$; Figure 2).

The results show that there were no statistically significant differences in serum concentrations of ions: Ca^{2+} concentration in G2 (2.60 mmol/l) and G1 (2.58 mmol/l; $p>0.05$; Figure 3), P concentration in G2 (1.74 mmol/l) and G1 (1.68 mmol/l; $p>0.05$; Figure 4), and Mg^{2+} concentration in G2 (0.99 mmol/l) and G1 (0.99 mmol/l; $p>0.05$; Figure 5).

Serum ALP values in rats by G2 (74.4 U/L) were statistically significantly increased, as compared to G1 (49.9 U/L; $p<0.05$; Figure 6).



(A) (B)

Figure 1. Animal body weight at beginning (A) and end (B) of experiment in G1 and G2

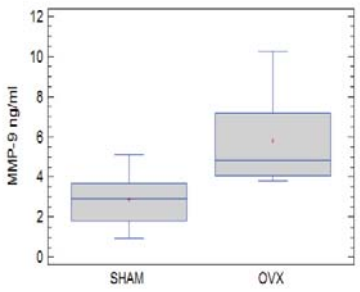


Figure 2. The serum concentration of MMP-9 in G1 and G2

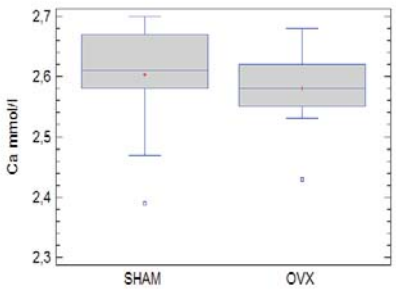


Figure 3. Serum concentrations of Ca^{2+} in G1 and G2

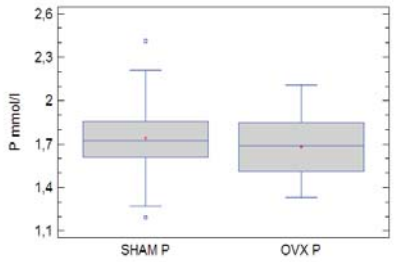


Figure 4. Serum concentrations of P in G1 and G2

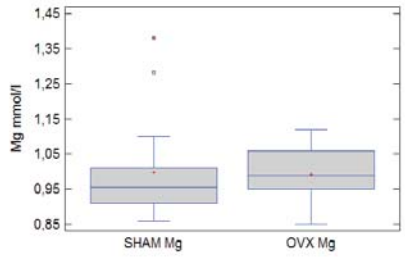


Figure 5. Serum concentrations of Mg^{2+} in G1 and G2

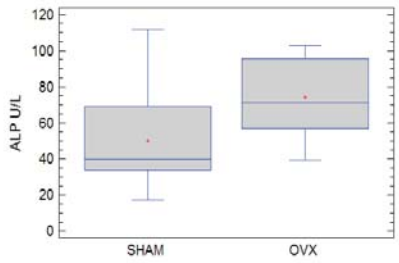


Figure 6. Serum ALP concentrations in G1 and G2

Discussion

Estrogen deficiency during menopause activates bone remodeling [6, 14]. Bone formation from Ob cannot compensate for increased bone resorption from Oc, leading to the development of osteoporosis [6]. Increased Oc activity increases the expression of MMP-9, which exerts its destructive effect on the bone structure directly and through increased production of cytokines [15, 16]. In bone tissue, MMP-9 is represented mainly in osteoclasts. It activates osteoclast resorption and degrades collagen type I [10, 15, 16].

Alkaline phosphatase is a component of the cell membrane of many tissues in the body, with the highest concentrations of this enzyme being found in bone cells (osteoblasts) and in the liver. ALP is increased in diseases of the skeletal system associated with increased osteoblast activity and bone remodeling [17].

Scientific data in relation to changes in serum concentrations of Ca^{2+}, P and Mg^{2+} are different. Some authors claim that their levels rise after ovariectomy [17], others prove the opposite data [18]. A fall in estrogen levels increases the resorptive effect of parathyroid hormone (PTH), which would lead to hypercalcemia, hyperphosphatemia [17] and hypomagnesaemia [19, 20]. However, a compensatory mechanism that reduces intestinal absorption and increases renal calcium excretion is triggered [21].

Our study has shown that the levels of Ca^{2+}, P and Mg^{2+} are not changed, which is typical of the initial phase of osteoporosis, during which no significant loss of bone mineral density due to active bone remodeling and a compensatory increased osteoblast activity is seen [22, 23]. Weight gain in animals in the G2 proves that lack of estrogens due to OVX leads to obesity [24, 25].

Conclusions

Our experiments confirmed the hypothesis that the serum activity of ALP, which is a marker of bone formation, would increase in the OVX-induced osteoporosis. Elevated levels of serum MMP-9 in G2 confirm the suggestion that matrix metalloproteinases in OVX rats play an important pathogenetic role in the development of osteoporosis and can be used as a marker for osteoclast activity.

Acknowledgements

The study was funded by Medical University – Pleven, Project №5/2016.

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