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ORIGINAL STUDY

SERUM CONCENTRATIONS OF MATRIX METALLOPROTEINASE-9, -13 AND TIMP-1 IN AN OVARIECTOMIZED WISTAR RAT MODEL OF OSTEOPOROSIS

Armine V. Grigoryan[1], Anelia A. Dimitrova[1], Krasimir G. Kostov[1], Adelaida L. Ruseva[2], Milena A. Atanasova[3], Alexander B. Blazhev[3], Tatyana M. Betova[4]

[1] Department of Physiology and Pathophysiology, Medical University – Pleven, Bulgaria
[2] Department of Clinical Laboratory, Medical University – Pleven, Bulgaria
[3] Department of Biology, Medical University – Pleven, Bulgaria
[4] Department of Pathoanatomy, Medical University – Pleven, Bulgaria

Abstract

Introduction. Osteoporosis is a disease characterized by decreased bone density and destruction of the microarchitectonics of the bone structure. This leads to increased bone fragility and risk of fracture particularly of the hip, spine, wrist and shoulder. Osteoporosis is known as „The Silent Epidemic of the Century" because bone loss occurs without symptoms. Altered ovarian function is one of the most common causes of osteoporosis. Indicators for altered bone homeostasis are the changes in serum levels of matrix metalloproteinases (MMPs) and their tissue inhibitors (TIMPs).
Objective. The aim of current study was to determine the activity of alkaline phosphatase (ALP) and serum concentrations of MMP-9, MMP-13 and TIMP-1 in the ovariectomized rats.
Materials and Methods. An experiment was performed on 35 female Wistar rats at reproductive age – 2 months divided into 2 groups: group 1 (G1)-20 animals were sham-operated (sham) and group 2 (G2)-15 were ovariectomized (ovx).

Résumé

Concentrations du sérum des métallo-protéinases matricielles-9, –13 et TIMP-1 dans un modèle d'ostéoporose à déficit ostrogénique d'un rat Wistar femelle

Introduction. L'ostéoporose est une maladie caractérisée par une diminution de la densité de la masse osseuse et la destruction de la micro-architecture de la structure osseuse. Cela conduit à une fragilité osseuse accrue et à un risque de fracture, en particulier de la hanche, de la colonne vertébrale, du poignet et de l'épaule. L'ostéoporose est connue comme «l'épidémie silencieuse du siècle» parce que la perte osseuse se produit sans symptômes.L'altération de la fonction ovarienne est l'une des causes les plus fréquentes de l'ostéoporose. Les indicateurs de l'altération de l'homéostasie osseuse sont les changements dans les taux sériques des métallo-protéinases matricielles (MMPs) et de leurs inhibiteurs tissulaires (TIMPs).
Objectifs. Le but de cette étude était de déterminer l'activité de la phosphatase alcaline (ALP) et

Corresponding author: Armine Grigoryan
Department of „Physiology and Pathophysiology", Medical University-Pleven
„Kliment Ohridski" Str., № 1, 5800 Pleven, Bulgaria
e-mail: armine14@abv.bg; phone: 0886-31-99-33

Results. The concentrations of ALP, MMP-9, MMP-13 and TIMP-1 in G2 were significantly increased compared to G1 ($p<0.05$).
Conclusion. Our study confirmed that the serum activity of ALP, which is a marker of bone formation, was elevated in rats with OVX-induced osteoporosis. Although the level of TIMP-1 is increased, the level of MMP-9 in G2 is also increased, that confirms the thesis that MMP-9 may be a marker for osteoclast activity.

Key words: osteoporosis, MMP-9, MMP-13, TIMP-1, ALP.

Abbreviations:
MMPs- matrix metalloproteinases
TIMPs-tissue inhibitors of matrix metalloproteinases
ALP- alkaline phosphatase
ECM –extracellular matrix
RANKL- nuclear factor kappa B ligand
OPG-osteoprotegerin

Matériel et méthodes. Une expérience a été réalisée sur 35 rats Wistar femelles en âge de procréer – 2 mois répartis en 2 groupes: groupe 1 (G1) –20 animaux ont été opérés de façon factice (factice) et groupe 2 (G2) –15 ovariectomisés (ovule).
Résultats. Les résultats ont montré que les valeurs de ALP, MMP-9, MMP-13 et TIMP-1 chez les rats de G2 étaient statistiquement significativement augmentées par rapport à G1 ($p<0,05$).
Conclusion. L'activité sérique de l'ALP, marqueur de la formation osseuse, est augmentée dans l'ostéoporose induite par l'OVX. Des niveaux significatifs d'augmentation de MMP-9 dans G2 malgré les niveaux élevés de TIMP-1 confirment la thèse selon laquelle il s'agit d'un marqueur de l'activité des ostéoclastes.

Mots-clés: ostéoporose, MMP-9, MMP-13, TIMP-1, ALP.

Abréviations:
MMPs – matrix de métallo-protéinases
TIMP – inhibiteurs tissulaires des métallo-protéinases matricielles
ALP – phosphatase alcaline
ECM – matrice extracellulaire
RANKL facteur nucléaire kappa B ligand
OPG – ostéoprotégérine

Introduction

Osteoporosis is a metabolic disorder of the bones characterized by decreased bone mass and microarchitectural deterioration of bone tissue. This consequently leads to bone fragility and susceptibility to fractures. According to the World Health Organization, osteoporosis is known as „The Silent Epidemic of the Century", because bone loss occurs without symptoms[1]. Altered ovarian function is one of the most common causes of primary osteoporosis. Estrogen deficiency contributes to the development of osteoporosis, by an imbalance between bone formation and bone resorption. An important role in the pathogenesis of this process plays the matrix metalloproteinases (MMPs)[2].

MMPs are a family of zinc-dependent endopeptidases, which can degrade various components of extracellular matrix (ECM). Different stimuli, such as intercellular interactions, cytokines, growth factors and endocrine imbalance, are able to induce their expression. They can be activated by proteolytic cascade involving several MMPs. Although the group of metalloproteinases continues to increase, nowadays, the following classification is established. MMP family can be divided into six groups: collagenases, gelatinases, stromelysins, matrilysins, membrane-type MMPs, and other non-classified MMPs[3]. Their activity is strictly controlled by various endogenous inhibitors in response to a number of stimuli[3,4]. There are four members of the family of tissue inhibitors of metalloproteinases (TIMPs) classified as TIMP-1, TIMP-2, TIMP-3 and TIMP-4[5]. Changes of TIMPs levels are very important, because they directly affect the level of MMPs activity. Studies show that TIMP-1 inhibits all of the examined MMPs, except for MT1-MMP[6]. Changes in serum levels of MMPs and TIMPs[6,7] are the indicators for altered bone homeostasis.

The major MMPs involved in the pathogenesis of osteoporosis are MMP-1, MMP-2, MMP-3, MMP-9 and MMP-13[4,8]. They are key mediators in the ECM remodelling, bone growth and osteoclast (Oc) bone resorption. MMPs are essential for early bone resorption, because they degrade collagen layer of the bone surface before demineralisation begins[9].

The aim of the study was to determine the concentrations of calcium (Ca^{2+}), phosphorus (P), magnesium (Mg^{2+}), alkaline phosphatase (ALP), MMP-9, MMP-13 and TIMP-1 in serum of ovariectomized rats.

Materials and Methods

The experiment was performed on 35 female Wistar rats at reproductive age – 2 months divided into 2 groups: group 1 (G1)-20 animals were sham-operated (sham) and group 2 (G2)-15 of which were ovariectomized (ovx). Animals are selected with initial weight 150 ± 20 g.

A bilateral ovariectomy female Wistar rat leads to bone changes similar to post-menopausal women[10]. All rats grew in a standard manner Rules of work with laboratory animals adopted from MU-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 YP et al[10]. At surgery, rats were initially anesthetized intraperitoneally with a mixture of xylazine (10 mg/kg) and ketamine (90 mg/kg). We calculated for each animal a dose of anesthesia, according to their weight, which is detailed in the experimental protocol. The operative access to the ovaries was made after a medial incision (about 2 cm) in the ovary area (at the most protruding back). After incising the fascia and the muscles, the adipose tissue was pulled away until the uterine tube and the ovary surrounded by a variable amount of fat were identified. The ovaries were 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 field was treated with an antibacterial powder and each animal was placed in an individual cell for post-operative observation and care. High degree of aseptic procedure was maintained throughout the operation. Surgical sutures threads were removed 10 days after surgery[10]. Eight weeks after the intervention from anaesthetized animals we obtained blood by puncture of the abdominal aorta. The blood was collected in vacutainers, the serum is separated by centrifugation and then stored at –80°C for examination by a method ELISA of enzyme activity.

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 xylidil blue. The activity of the enzyme alkaline phosphatase was determined with IFCC- approved method at 37°C. Serum levels of MMP-9, MMP-13 and TIMP-1 were determined using an ELISA immunological method. Antibodies for MMP-9 were purchased from R&D Systems, with catalogue number RIRMP900 rat Total-MIVP-9 Quantikine ELISA. Antibodies for MMP-13 were purchased from CUSABIO, with catalogue number is a CSB-E07412r, Rat MMP-13 ELISA Kit. Antibodies for TIMP-1 were purchased from R&D Systems, with catalogue number is a RTM100 Rat TIMP-1 Immunoassay, Quantikine ELISA.

Statistical analysis

Statgraphics Centurion XVI software (Statpoint Technologies, Inc., Warrenton, VA, USA) was used for statistical analyses. The significance of the differences between groups was assessed by Fisher's F-test (ANOVA). The data are represented as means ± SD and $p < 0.05$ was considered statistically significant.

Results

I. Body weights

All animals were weighed at the beginning and end of the experiment. At the beginning of the experiment, the body weights were similar in both groups G1-159±13,8g and G2-153±19,2g. At the end of the experiment, the body weight was significantly higher in OVX group 295±17,7g compared with the SHAM group 256±16,0g, ($p<0.05$). Fig. 1 shows changes in mean body weights in both groups at the end of the experiment.

II. Concentration of MMP-9, MMP-13 and TIMP-1 in the serum:

Fig. 2 shows the serum concentration of MMP-9. We found that the serum concentration of MMP-9 is higher in G2 (5.81 ng/ml), compared to G1 (2.85ng/ml), ($p<0.001$).

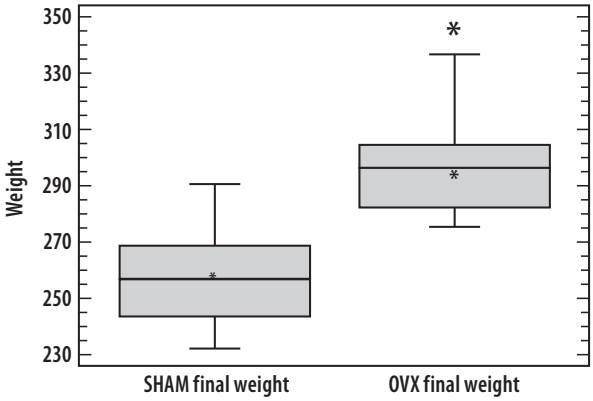

Figure 1. Animal weight at the end of experiment in G1and G2 groups,* p<0.05.

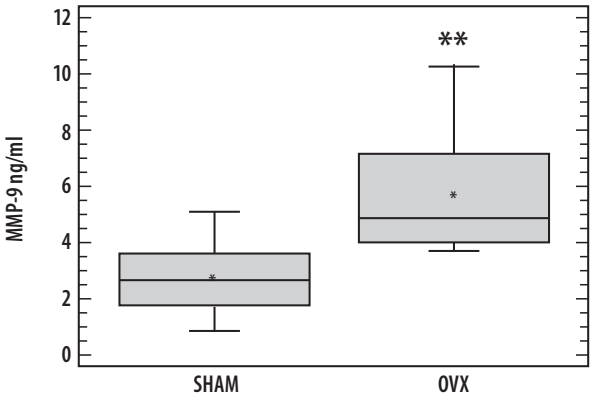

Figure 2. The serum concentration of MMP-9 in G1 and G2 groups,* * p<0.001.

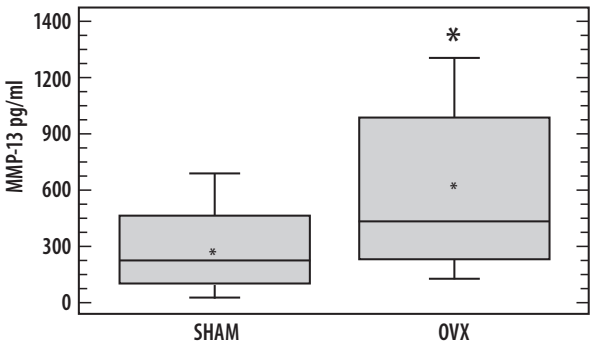

Figure 3. The serum concentration of MMP-13 in G1 and G2 groups, * p<0.05.

Fig. 3 shows the serum concentration of MMP-13. We found that the serum concentration of MMP-13 is higher in G2 (625.3 pg/ml), compared to G1 (311.6 pg/ml), (p<0.05).

Fig. 4 shows the serum concentration of TIMP-1. We found that the serum concentration of TIMP-1 is higher in G2 (435.0 pg/ml), compared to G1 (287.4 pg/ml), (p<0.05).

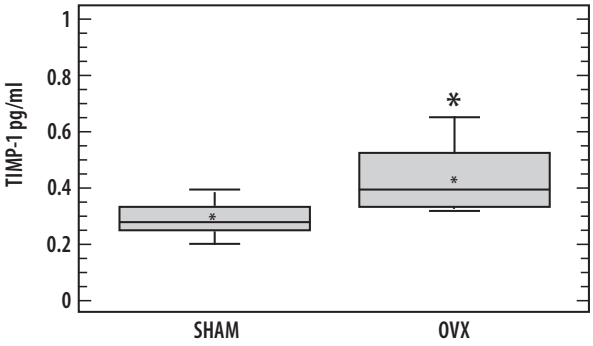

Figure 4. The serum concentration of TIMP-1 in G1 and G2 groups, * p<0.05.

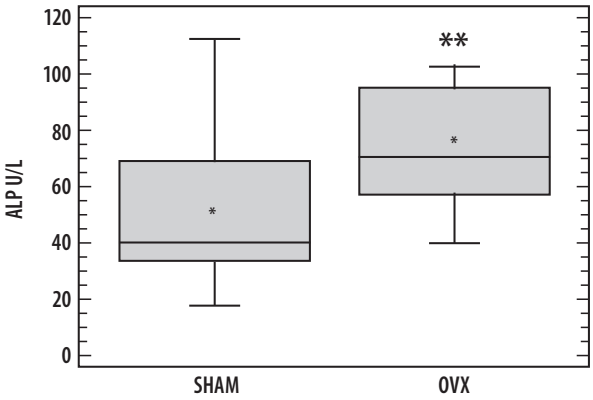

Figure 5. Serum ALP concentrations in G1 and G2 groups, ** p<0.001

III. Serum calcium (Ca^{2+}), magnesium (Mg^{2+}) and phosphate (P) concentrations:

The results show that there were no statistically significant differences in serum concentrations of ions between G1 and G2: Ca^{2+} concentration were (2.60 mmol/L) in G2 group and (2.58 mmol/L) in G1 group; P concentration were (1.74 mmol/l) in G2 group and (1.68 mmol/L) in G1 group; Mg^{2+} concentration were (0.99 mmol/L) in G2 group and (0.99 mmol/L) in G1 group.

Serum ALP levels: Serum ALP levels were (74.4 U/L) in rats by G2 group were statistically significantly increased, as compared to G1 group (49.9 U/L), (p<0.001) – Fig. 5.

Discussion

Estrogen deficiency during menopause activates bone remodelling[8]. Bone formation from osteoblasts (Ob) cannot compensate for increased bone resorption from Oc, leading to the development of osteoporosis[2]. Estrogen insufficiency increases osteoclastogenesis through the production of the

pro-osteoclastogenic cytokines: tumor-necrosis factor (TNF)-α and receptor activator of nuclear factor kappa B ligand (RANKL). It also reduces the expression of its soluble receptor osteoprotegerin (OPG), which has protective effects, because it interferes with RANKL and blocks its action, thus preserving bone[2]. Estrogen deficiency alters the regulatory interaction between T-cells and stromal cells, which also resulting in greater production of a number of cytokines[2]. T-cells activate osteoclastogenesis by the production of RANKL and secretion of various proinflammatory cytokines such as interleukin (IL) –1, IL-6, interferon (IFN) –γ or IL-4 [2], which stimulate the production of matrix metalloproteinases[11].

Osteoblast cells, like targeting cells of estrogen, can release various matrix metalloproteinases (MMPs) involved in bone remodelling. Bone loss caused by estrogen deficiency is closely related to abnormal expression of multiple MMPs which activates osteoclasts[9,11]. 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. In bone tissues MMP-9 are represented mainly in osteoclasts. It activates osteoclast resorption and degrades collagen type I[8,12].

MMP-13 is produced by osteoblasts and osteocytes. Matrix metalloproteinases 13 degrade collagens type II and I in bone matrix[3,7,8,13] and is mainly associated with mineralized bone matrix[8]. By interacting with cathepsin-K and MMP-9 produced by osteoclasts, MMP-13 produced by cells originating from osteoblasts may also be involved in the degradation of organic components in the bone resorption process[13]. Researches show that 17-b-estradiol can inhibit the expression of MMP-13 in osteoblastic cells[11] and our study confirmed this thesis.

TIMP-1 is expressed from osteoblasts and osteocytes[11,14], and the activity of MMP-9 and MMP–13 is inhibited by TIMP-1[6,9].

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 the liver. ALP increases in diseases of the skeletal system associated with increased osteoblast activity and bone remodelling[9,14].

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[16], others prove the opposite[17]. Our study showed that the serum levels of Ca^{2+}, P and Mg^{2+} do not change, which is typical for the initial phase of osteoporosis, where despite reduced bone mineral density, serum ion balance is preserved, due to active bone remodelling and a compensatory increased Ob activity[18,19].

Weight gain in animals in the G2 proves that lack of estrogens due to OVX leads to obesity[20].

Conclusion

Our study confirmed that the serum activity of ALP, which is a marker of bone formation, was elevated in rats with OVX-induced osteoporosis. The concentrations of MMP-9, MMP-13 and TIMP-1 in G2 were significantly increased compared to G1 ($p<0.05$). Although the level of TIMP-1 is increased, the level of MMP-9 in G2 is also increased, confirming the thesis that MMP-9 may be a marker for osteoclast activity and play an important role in the pathogenesis of osteoporosis. Degradation of collagens type-I and type-II from MMP-13 is an important factor for bone demineralisation, which slowly leads to the development of osteoporosis. Therefore, MMP-13 may also be considered as an important marker for development of osteoporosis.

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References

1. Lorentzon M, Cummings SR. Osteoporosis: the evolution of a diagnosis. *Journal of Internal Medicine*. 2015;277(6):650-661.
2. Faienza MF, Ventura A, Marzano F, Cavallo L. Postmenopausal osteoporosis: the role of immune system cells. *Clinical and Developmental Immunology*. 2013; Article ID 575936.
3. Nagase H, Visse R, Murphy G. Structure and function of matrix metalloproteinases and TIMPs. *Cardiovascular Research*. 2006;69(3):562-573.
4. Galliera E, Banfi G, Corsi MM. Human bone disorders: pathological role and diagnostic potential of matrix metalloproteinases. *The International Journal of Biochemistry & Cell Biology*. 2010;42(10):1590–1593.
5. Gomez D, Alonso D, Yoshiji H, Thorgeirsson U. Tissue inhibitors of metalloproteinases:structure, regulation and biological functions. *European Journal of Cell Biology*. 1997;74(2):111-122.
6. Visse R., Nagase, H. Matrix metalloproteinases and tissue inhibitors of metalloproteinases. *Circulation Research*. 2003; 92(8): 827-839.
7. Kevorkian L, Young D, Darrah C, et al. Expression profiling of metalloproteinases and their inhibitors in cartilage. *Arthritis& Rheumatology*. 2004;50(1):131-141.
8. Sun B, Sun J, Han X, et al. Immunolocalization of MMP 2, 9 and 13 in prednisolone induced osteoporosis in mice. *Histology and Histopathology*. 2016;31(6): 647-656.
9. Bord S, Horner A, Hembry RM, Reynolds JJ, Compston JE. Distribution of matrix metalloproteinases and their inhibitor, TIMP-1, in developing human osteophytic bone. *Journal of Anatomy*.1997;191(1):39–48.
10. Kharode YP, Sharp MC, Bodine PV. Utility of the ovariectomized rat as a model for human osteoporosis in drug discovery. *Osteoporosis: Methods and Protocols*. 2008:111-124.

11. Li J, Liao E, Dai R, Wei Q, Luo X. Effects of 17 β-estradiol on the expression of interstitial collagenases-8 and-13 (MMP-8 and MMP-13) and tissue inhibitor of metalloproteinase-1 (TIMP-1) in ovariectomized rat osteoblastic cells. *Journal of Molecular Histology.* 2004; 35(8-9): 723-731.
12. Zhao H, Cai G, Du J, Xia Z, Wang L, Zhu T. Expression of matrix metalloproteinase-9 mRNA in osteoporotic bone tissues. *Journal of Tongji Medical University.* 1977;17(1):28-31.
13. Johansson N, Saarialho-Kere U, Airola K, et al. Collagenase-3 (MMP-13) is expressed by hypertrophic chondrocytes, periosteal cells, and osteoblasts during human fetal bone development. *Developmental Dynamics* 1997; 208(3):387-397.
14. Dobnig H, Sipos A, Jiang Y, et al. Early changes in biochemical markers of bone formation correlate with improvements in bone structure during teriparatide therapy. *The Journal of Clinical Endocrinology & Metabolism.* 2005; 90(7), 3970-3977.
15. Hatori K, Sasano Y, Takahashi I, Kamakura S, Kagayama M, Sasaki K. Osteoblasts and osteocytes express MMP2 and –8 and TIMP1, –2, and –3 along with extracellular matrix molecules during appositional bone formation. The anatomical record. Part A, *Discoveries in molecular, cellular, and evolutionary biology.* 2004; 277(2):262-271.
16. Kim SK, Lee MH, Rhee MH. Studies on the effects of biomedicinal agents on serum concentrations of Ca2+, P and AlP activity in osteoporosis–induced rats. *Journal of Veterinary Science*. 2003;4(2): 151–154.
17. Parikh P, Suresh B, Rangrez A. Osteoprotective effect of Litsea glutinosa in ovariectomized wistar rats. *Electronic Journal of Pharmacology and Therapy.* 2009;(2):81-6.
18. Andrusishina IN. Diagnostic values of calcium and magnesium forms determined in human serum and saliva. *Journal of Elementology.* 2010;15(3):425–433.
19. Alam MR, Kim SM, Lee JI. Effects of Safflower seed oil in osteoporosis induced-ovariectomized rats. *The American Journal of Chinese Medicine*. 2006;34(04):601-612.
20. Lizcano F, Guillermo G. Estrogen deficiency and the origin of obesity during menopause. *BioMed Research International.* 2014; Article ID 757461.