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Research Report

Correlation between estrogen and alkaline phosphatase expression in osteoporotic rat model

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ABSTRACT

Background: *Osteoporosis is a systemic disease that can decrease bone density as a result of imbalance bone remodeling and bone resorption. Estrogen reduction due to menopause can increase osteoclast activity and furthermore decrease bone density. Estrogen can stimulate alkaline phosphatase (ALP) expression, collagen type I and osteocalcin in bone remodelling process. Ovariectomized rat is a common animal for studying patofisiology, diagnosis and treatment osteoporosis patient.* ***Purpose:*** *To evaluate correlation between estrogen and ALP expression in osteoporotic rat model mandible.* ***Methode:*** *18 female wistar rats, 2 months old, 200 grams were divided into 2 groups, ovariectomized group and sham surgery as control group. Surgery was done under intra muskular anesthesia using combination 2% xylazine 1cc and 10% ketamine 1cc. After 12 weeks, mandible was taken for ALP examination and blood from heart was taken to evaluate the amount of estrogen.* ***Result:*** *There was significant correlation between estrogen and ALP expression in osteoporotic rat model mandible.* ***Conclusion:*** *The amount of estrogen can influence ALP expression activity.*

Keywords: *osteoporosis; estrogen; alkaline phosphatase; ovariectomy*

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INTRODUCTION

Oral health condition of elderly patients has an important role equal to quality of life, in addition to several other life factors, such as socio-economical, physical, and psychosocial reasons. Consequently, demand to get better dental and oral cavity treatment increases. Based on global epidemiological data report, 1 out of 3 women and 1 out of every 50 men over the age of 50 years suffered from osteoporosis. Several researches even have reported that there is a correlation between systemic osteoporosis and bone resorption in oral cavity.[1]

Osteoporosis is a systemic disease that can decrease bone density as a result of imbalance between bone formation with bone resorption. Postmenopausal osteoporosis is the most common form of osteoporosis, leading to increased risk of fractures after menopause manifestations such as wrist fractures, spine fractures, and hip fractures with a mean age over 60 years.[2] Bone density is a specific factor influencing the risk of bone fractures. According to WHO, a state of osteoporosis if bone density more than 2.5 standard deviation, below the average for normal young people (T<-2.5) in dual energy x-ray absorptiometry (DEXA) measurement.[3] Moreover, an estimated 40% of women and 13% of men over at the age of 50 will experience a fracture due to osteoporosis.[4]

Osteoporosis is classified into two categories, namely primary osteoporosis and secondary osteoporosis. Primary osteoporosis is associated with menopause and aging conditions. Meanwhile, secondary osteoporosis is due to certain health conditions, along with drug therapies that influence bone density or increasing of bone density loss. The condition is generally associated with endocrine disorders (Cushing syndrome, hyperparathyroidism, insulin-dependent diabetes mellitus (IDDM), adrenal insufficiency), rheumatoid arthritis, hematological disorders, and malignancies (leukemia, lymphoma).[5] Hormonal changes that occur as the condition of

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menopause are important factors cause decrease women bone density.[6] The decline in estrogen levels associated with post-menopausal osteoporosis condition then can increase osteoclasts activity, and subsequently can lead to osteoporosis decreasing the attachment of the bone into the teeth in the oral cavity.[7]

There are several methods to measure bone density, such as inspection DEXA, which has good accuracy in diagnosis. This examination is very important to improve successful rate of dental treatment, expecially prosthodontics such as complete dentures, partial dentures, as well as dental implants. However, the use of this measuring tool has limitations in its implementation because it is conducted on a particular health facility and the cost is relative high. Therefore, simple examination is needed for early identification of osteoporosis during dental care, such as a examination of estrogen levels and ALP expression. The examination may be an option for an early indicator of osteoporosis sufferers, which is more efficient with a good degree of diagnostic accuracy.

OVX model rats are animals mostly used in a study of osteoporosis pathophysiology, focused on its diagnosis and treatment. These animals have also been validated as a clinically relevant model of post-menopausal bone density loss.[4] In this research, immunohistochemical examination (IHC) was conducted in the osteoporotic rats model mandible.

This research was conducted to evaluate estrogen levels on mandibular bone ALP expressions in both model rats suffering from osteoporosis and normal rats. Thus, the results of this research are expected to provide information about evaluation of estrogen levels and immunohistochemical examination of ALP in both normal rats and model rats suffering from osteoporosis.

MATERIALS AND METHOD

This research was a laboratory experimental research using 18 female Wistar rats, 3 months old. Those animals were not in a state of pregnancy, did not suffer from any disease, as well as did not undergo hormone therapy. This research was approved by Health Research Ethics Committee (KKEPK) from Faculty of Dental Medicine, Universitas Airlangga No. 15/ KKEPK.FKG/ I/2016.

Wistar rats then were divided into two groups, group of SHS (Sham surgery) rats as the control group and the group of OVX rats. Model rats suffering from osteoporosis were classified into the treatment group. They were weighed and had intramuscular anesthesia with a combination of 2% xylazine (1 cc) and 10% ketamine (1 cc). Ovariectomy was performed by making a ventral incision from the umbilicus to the pubis. The blood vessels of the ovary and the fallopian tubes were ligated separately. The ovarium was taken bilaterally, and then the peritoneum incision was closed using a simple interrupted suture technique. SHS was performed in the control group by making a ventral incision from the umbilicus to the pubis, and then returned to its previous position and closed the peritoneum incision by using a simple interrupted suture technique.[9] Those OVX female Wistar rats were maintained for 12 weeks in a cage with simultaneous treatment, in which they got 12 hours of light and 12 hours without light. They were also fed with Comfeed concentrate starter (calf starter) containing yellow corn, wheat bran, SBM, drops, Palm Olien essential amino acids, essential minerals, premix, and vitamin. They also got drink at the same time.

Blood samples were obtained after those rats were anaesthetized using ketamine before termination. Blood samples were taken directly from the heart through the apex as much as 3 ml using 5 ml of disposable syringe. Blood samples taken from the rat heart were then inserted into a test tube and waited for 3 hours to generate serum. The serum was centrifuged at 3000 rpm at room temperature for thirty minutes with microliter centrifuge (Micro 200, Hettich GmbH & Co.KG). Centrifugation process was performed to obtain pure serum. The serum then was added into eppendrof tubes, and stored in a refrigerator at a temperature of 4^0 C.

To determine estrogen levels in those rats, an examination with indirect ELISA method was performed. Antigens were derived from the serum of blood samples taken from the heart. The blood samples were taken from all the experimental animals treated. Indirect ELISA testing was performed using 100µL of antigens in coating buffer with a ratio of 1: 9, and then put in wells of ELISA plates and incubated overnight at a temperature of 40ºC. They were washed with PBS-Tween 3 times. They were added with 50 mL of blocking buffer (1% BSA in PBS). They were washed with PBS-Tween 3 times. They were added with 100 mL of primary antibody in 1% PBS-BSA solution with a ratio of 1: 500, and then incubated for 2 hours. They were washed with PBS-Tween 3 times, added with 100 mL of the secondary antibody in Tris buffer saline at a ratio of 1: 2500, and then incubated for 1.5 hours. They then were washed with PBS-Tween 2 times. They were added with 50 mL of pnpp substrate, incubated for 30 minutes, and added with 50 mL of 1N NaOH as the reaction stopper. The absorbance was read using ELISA reader at a wavelength of 405 nm.[10]

After blood sample was taken, rats were terminated and mandible was taken for immunohistochemistry examination. Direct method with a monoclonal antibody was used to detect a marker of cells, directly labeled with ALP enzyme. To eliminate the activity of endogenous Peroxidase, 3% H_2O_2 was used. To clean protein debris that might cover epitope with materials that would be used to detect, 0.025% trypsin in PBS was used. IHC examination then was conducted by deparaffinization, integrating successive tissue incision into xylitol, absolute ethanol, 95% ethanol, 80% ethanol, and 70% ethanol, and then watered under running water for 10-15 minutes. It was put into a solution of 3% H_2O_2 for 30 minutes, washed with PBS, put in 0.025% trypsin for 6 minutes at 37^0 C, and then washed

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Table 1. Mean and standard deviation of estrogen levels and ALP expression in the group of mandibular bone osteoporosis rats and the group of normal rats

Parameter	Group	Mean	Standard deviation	Significance of difference
Estrogen	Normal	86.5091	23.19584	0.075
	Osteoporosis	67.3417	25.65043	
ALP	Normal	1.0000	0.75366	0.001*
	Osteoporosis	4.8000	2.78959	

again using PBS. Afterwards, it was put into monoclonal antibody labeled with ALP enzyme, washed with PBS, put into chromogen substrates, washed with PBS, washed using aquadestilata, and then put into Mayer's Haematoksilin. It was washed using running water, and then dehidration-clearing-mounting was performed.[11]

Variables studied in this research included changes in both estrogen levels using ELISA and ALP expression using IHC examination. IHC examination was conducted using qualitative observation supported by quantitative data in one field of view. Quantitative data obtained were tabulated using Microsoft Office Excel, and then analyzed using SPSS 20.0 for Windows with Shapiro-Wilk analysis to determine whether the distribution of the data was normal or not. If the distribution of data was normal, the analysis would be continued using parametric analysis of independent t-test.

RESULTS

This research focused on the correlation between estrogen levels and ALP expression in all groups, used nine samples in each group, namely the group of normal rats (as the control group) and the group of OVX rats (osteoporosis). Normality test was conducted to determine the normal distribution in all the variables studied. Ekspresi ALP

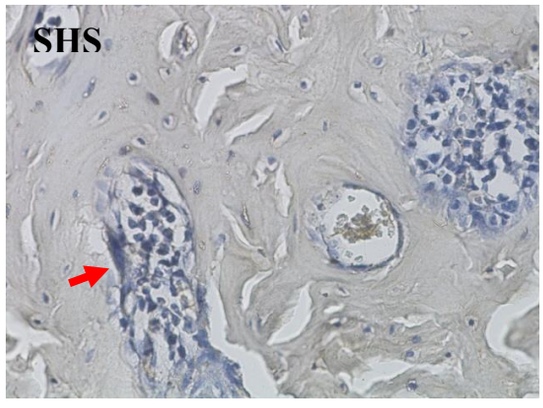

Figure 1. Expressions of ALP on immunereactive osteogenic cells (arrows) among the control groups of SHS (IHC staining, magnification 400x; H600L Nikon microscope; Fi2 300 megapixel camera DS).

Table 1 shows that the normal group had a higher mean value of estrogen than the osteoporosis group. In contrary, the osteoporosis group had a higher mean value of ALP than the normal group. Based on the results of the data distribution test, the distribution of all data was normal, so the analysis was continued using parametric analysis of Independent t-test.

Based on the results of Independent t-test, furthermore, there was no significant difference in estrogen levels between the normal group and the osteoporosis group. However, there were significant differences in ALP expression between the normal group and the osteoporosis group.

The results of Pearson correlation test showed that there was no significant correlation between estrogen levels and ALP expression.

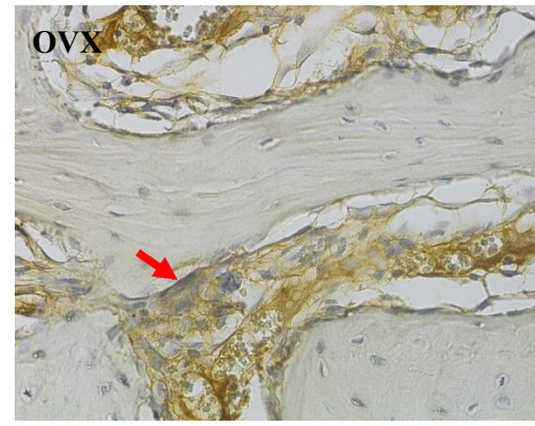

Figure 2. Expressions of ALP on immunereactive osteogenic cells (arrows) among the treatment groups of OVX (IHC staining, magnification 400x; H600L Nikon microscope; Fi2 300 megapixel camera DS).

Table 2. Correlation test on estrogen levels and ALP expression among the research groups

Group	Parameter	Coefficient	Significance of correlation
Normal	Estrogen ALP	0.162	0.635
Osteoporosis	Estrogen ALP	-0.014	0.965

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DISCUSSION

Osteoporosis in the elderly will trigger risk of fracture due to a decrease in bone density. Osteoporosis is a disease with some distinctive properties, such as low bone mass, accompanied by changes in bone microarchitecture and deterioration of bone tissue quality, increasing risk of bone fragility fractures.[12] Bone tissue continuously experiences formation and resorption (bone remodeling). At the age of 20 years old, bone formation process is highly active, far beyond bone resorption process. At the age of 20-40 years old, both processes then are nearly active, while at the age of 40 years old, bone resorption process is more active than bone formation process, leading to decrease in bone density.

Osteoporosis, furthermore, can occur in the elderly population, especially postmenopausal women because of several factors, especially estrogen levels in bone remodeling process. Estrogen plays an important role in bone metabolism contributing to an increase in the number of osteoblasts as well as their function, and also to reduce bone resorption. There are estrogen receptors in osteoblasts, osteocytes, and osteoclasts. Estrogen also has been reported to have ability in increasing levels of osteoprotegerin (OPG), a protein produced by osteoblasts, which can inhibit bone resorption by preventing RANKL from binding to RANK receptors. Therefore, estrogen plays an important role in osteoclastogenesis process. Estrogen inhibits local factors that hinder bone formation process or increase local factors that stimulate bone formation process. For this reason, a decrease in estrogen is an important pathogenic factor in bone loss associated with osteoporosis.[13]

Examination with indirect ELISA method, moreover, was conducted using blood samples to determine levels of estrogen from their heart. The results of independent t-test showed that there was a significant difference (p=0.075) between the normal group (86.5091) and the osteoporosis group (67.3417). This may happen because there are other sources of synthesis that can replace the main source of estrogen in their ovaries during osteoporosis condition. Other sources of estrogen synthesis can be found in mesenchymal cells of adipose tissue, skin, osteoblasts, osteoclasts in bone, vascular endothelial as well as some places in the brain, such as preoptic medial/anterior hypothalamus, medial basal hypothalamus, and amygdala, which are only active at the level of the local tissue with a high concentration.

At the beginning of osteoporosis, those sources of synthetic estrogen systemically controlled by the ovaries begin to decline. As a result, it will trigger the reactive properties of estrogen synthesized in the local tissue to get into the systemic circulation so that it can act as a substitute for the primary synthesis in maintaining levels of estrogen.[14] Nevertheless, the reactive properties of estrogen derived from the local tissue are only temporary and occurs only in the early stage of osteoporosis. Osteoporosis at the advanced stage then would affect the local tissue in synthesizing estrogen so that estrogen levels in the systemic circulation reached its lowest level.[15]

Similarly, a research on model animals conducted by Gao[16] shows that changes in hormones during menopause contribute to changes in body composition and fat distribution. Increased adipose tissue in a group of menopause rats triggers increased production of estrogen by the local tissue. Decreased ovarian function in the early stage of osteoporosis can also lead to an increase in adipose tissue mass not influenced by dietary adjustments and pathological metabolic conditions. Consequently, it is important to recognize clinically since in those ovariectomized rats there was an increase in fat accumulation due to estrogen deficiency and decreased physical activity.

In this research, ALP obtained showed a significant difference (p=0.001) between the normal group (1.0000) and the osteoporosis group (4.8000). Bone remodeling in osteoporosis is an ongoing process. However, bone resorption and bone formation cannot be balanced due to estrogen deficiency. This is indicated by a decline in the capacity of osteoblasts in the form of new bone matrix. In a research conducted by Lim and Kim,[17] there are significant differences in trabecular bone mass after 12 weeks of post-ovariectomy in Wistar rats. Similarly, a research on ovariectomized Wistar rats conducted by Lasota[18] also shows that after 12 weeks of post-ovariectomy there are changes in bone density and bone mineral structure. Therefore, this research used a 12-week evaluation period after ovariectomy because there has been a reduction in bone density and bone structure representing the loss of bone density after menopause.

In addition, the markers of bone remodeling are a biochemical product measured in blood or urine reflecting bone metabolism activity, but not having a function as a controller of skeletal metabolism. Markers of bone remodeling are simply categorized as markers of bone formation. Among the markers of bone formation, ALP is secreted by osteoblasts towards the extracellular fluid that can be measured in serum.[19] Besides serum activity of osteoblasts in bone formation process, type I collagen and proteoglycan can also be considered as bone matrix through a process, called ossification, which in the active condition then will produce osteoid tissue. Osteoblasts secrete a large amount of ALP in the process of bone formation. ALP plays a role in mineralization process, which is to prepare alkaline atmosphere (basic) in osteoid tissue formed so that calcium can be deposited on the tissue.[20] Increased ALP expression then indicates osteogenic differentiation. During the differentiation process from pre osteoblast to osteoblasts, ALP activity will increase. Meanwhile, at the beginning of osteoblasts formation, ALP activity decrease, but during maturation process of osteoblasts, ALP activity will increase again.[21]

Based on the results of Pearson correlation test, there was no significant correlation between estrogen and ALP in both of the normal group and the group of ovariectomized rats. This is due to estrogen levels in the systemic circulation

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affecting osteoblasts in the process of bone formation. It did not directly affect the activity of ALP expression in osteoblasts during the differentiation process from pre osteoblast to maturation. Similarly, Gao[16] mentions that the expression of ALP activity changes in accordance with the phases of bone formation in osteoblasts, but is not affected by estrogen levels in the systemic circulation. In this research, estrogen levels also were not directly related to the activity of ALP expression caused by ALP expression changes according to the phases of bone formation.

It can be concluded that estrogen levels in model rats suffering from osteoporosis are lower than normal ones. Meanwhile, mandibular bone ALP expression in model rats suffering from osteoporosis is higher than normal one. It indicates that there is no significant correlation between estrogen levels and mandibular bone ALP expression in model rats suffering from osteoporosis and normal ones.

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