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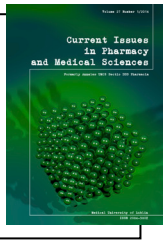

The effect of hormone replacement therapy on the expression of the alkaline phosphatase gene (*ALPL*) within the mucosal epithelium of the cheek and in peripheral blood lymphocytes

Mansur Rahnama[1], Michal Lobacz[1], Anna Szyszkowska[1], Grzegorz Trybek[2], Maryla Kozicka-Czupkallo[1]

[1] Chair and Department of Oral Surgery, Medical University of Lublin, Karmelicka 7, 20-081 Lublin, Poland
[2] Department of Oral Surgery, Pomeranian Medical University, al. Powstańców Wlkp. 72, 70-111 Szczecin, Poland

ARTICLE INFO

Received 01 July 2014
Accepted 11 August 204

Keywords:
alkaline phosphatase,
bone turnover markers,
menopausal period,
ovarectomy hormone
replacement therapy.

ABSTRACT

In adult life, proper bone metabolism requires efficient regulation of bone formation and resorption processes. Bone turnover markers allow for assessing the rate of bone formation and resorption processes. In menopausal period, female patients experience gradual reduction in blood estradiol levels. The deficit of estrogens leads to enhanced osteoclastogenesis and bone resorption. Alkaline phosphatase (ALP) is a membrane-bound enzyme that stimulates the osteoblast activity and bone mineralization. It is synthesized by osteoblasts and incorporated into the newly formed bone tissue. The produced enzyme stimulates the osteoblast activity and bone mineralization. The goal of this study is to determine the effect of hormone replacement therapy in post-menopausal women on the expression of alkaline phosphatase gene (*ALPL*) within the mucosal epithelium of the cheek and in peripheral blood lymphocytes. The studies show that hormone replacement therapy has no significant effect on the increase in *ALPL* expression within the mucosal epithelium of the cheek. Only in women having undergone ovarectomy (OV), the epithelial *ALPL* expression level was higher than in the remaining groups.

INTRODUCTION

In adult life, proper bone metabolism requires efficient regulation of bone formation and resorption processes. Imbalanced function of osteoclasts and ostaoblasts leads to adverse changes within the skeletal system, usually manifested by osteoporosis or osteopetrosis. The osteoclastic activity is a result of differentiation and combination of osteoclast progenitor cells as well as of the loss of mature cells undergoing apoptosis. Numerous humoral factors and drugs may affect osteoclastogenesis and bone resorption (12,13,22). Inhibition of the resorptive activity of osteoclasts plays an important role in the maintenance of bone tissue mass and stabilization of bone turnover at the cellular level (23). Bone turnover markers allow for assessing the rate of bone formation and resorption processes. Depending on the prevalence of either of these processes, bone tissue mass is either increased or reduced. In healthy humans, both compact and trabecular bone are constantly remodeled throughout individual's life.

In adults, about 10% of total bone mass is remodeled during a year, with compact bone turnover being about 4%, and trabecular bone turnover as much as about 25% (9,10). The values differ significantly in pathological conditions of bones and joints. Increase in marker levels reflects the intensity of bone formation or resorption processes.

In menopausal period, female patients experience gradual reduction in blood estradiol levels. After ovarian failure, the only estrogen being formed is estrone, synthesized within the adipose tissue. The deficit of estrogens leads to enhanced osteoclastogenesis and bone resorption (2).

The levels of resorption markers rise after menopause and the results of the studies suggest that bone loss correlates with the marker levels. It is a potential indication for using the biochemical markers of bone metabolism e.g. for assessing the risk of fractures. Prospective studies revealed correlation between the incidence of osteoporotic fractures and bone turnover markers regardless of the bone

Corresponding author
e-mail: lobosos@o2.pl
tel.: + 48 81 528-79-50, +48 608 399 856

 DOI: 10.2478/cipms-2014-0021

mineral density in menopausal and older women (4,5,7). The results show that in older females, whose resorption markers significantly exceed the reference ranges, the risk of fractures relative to the BMD value is twice higher compared to pre-menopausal women. The studies demonstrate that combination of BMD values and bone turnover markers may improve predicting the risk of fractures in post-menopausal women (11,15).

Hormone replacement therapy (HRT) is an established method of management of adverse symptoms caused by the cessation of the endocrine function of the ovaries. Most authors believe that the beneficial effect of HRT on the skeletal system is important for reducing the risk of post-menopausal osteoporosis, and estrogens should be one of the key elements of the management of osteoporosis in post-menopausal women. Estrogen replacement therapy significantly or completely prevents the post-menopausal loss of bone tissue mass. Used early after menopause or directly after ovarectomy, hormone replacement therapy allows for maintaining the bone tissue mass, while in women who start their treatment several years or later after last menses estrogens exert antiresorptive activity. The role of estrogens in the process consists in inhibition of the resorptive activity of osteoclasts and stimulation of the bone formative activity of osteoblasts. The effect of estrogens on osteoblasts consists in inhibition of selected cytokines that stimulate maturation and metabolic activity of osteoclasts. Even when used long after menopause, HRT reduces bone pains and improves the quality of life. Used at appropriate doses, estrogens facilitate maintenance or gain of the bone tissue mass. A 2-8% increase in BMD within spinal bones and femoral shaft has been observed. Studies show that women receiving estrogens experience fractures less frequently (16,20,21).

Hormone replacement therapy is administered via different routes: orally, in the form of patches, intravaginally, as subcutaneous implants, injections, gels for transdermal administration. The route of estrogen administration to women has principally no effect on the reactions taking place within the skeletal system. When administered subcutaneously or intramuscularly, 17-β estradiol affects bone metabolism in the same manner as following oral administration (20).

Biochemical bone turnover markers are sensitive indicators of early disturbances in cartilage and bone metabolism. Their serum, urine or articular fluid levels reflect both the synthesis and resorption of articular cartilage and bone tissue. Structurally, these markers are fragments of collagen comprising the structure of cartilage and bone as well as enzymes and proteins released into circulation upon metabolic activity of chondro- and osteoblasts as well as chondro- and osteoclasts. A group of molecules of high specificity to bone metabolic processes has been identified and methods for specific determination of these molecules have been developed. The molecules are referred to as bone turnover markers. They include:

1. Bone formation markers:
- bone alkaline phosphatase (b-ALP),
- osteocalcin (oc) (bone GLA protein);
- procollagen I N-terminal propeptide (pINp).

2. Bone resorption markers:
- pyridinoline and deoxypyridinoline (pYd and dpd),
- collagen type I cross-linked N-telopeptide (NTX);
- collagen type I cross-linked C-telopeptide (CTX) (6,8).

Alkaline phosphatase (ALP) is a membrane-bound enzyme that stimulates the osteoblast activity and bone mineralization. It is synthesized by osteoblasts and incorporated into the newly formed bone tissue. About 10-25% of the peptide is released into the circulating blood. Alkaline phosphatase, being a membrane-bound enzyme, is an established bone formation marker. The produced enzyme stimulates the osteoblast activity and bone mineralization.

Classification of alkaline phosphatase (ALP) has been established after long studies, consisting of four fractions of intestinal, placental, placental cell-like and and tissue non-specific isoforms. The osseous isoenzyme has been classified as tissue non-specific. The enzyme is bound to osteoblast membrane and increases the potential for differentiation into osteogenesis by decreasing the quantity of pyrophosphates. Pyrophosphates inhibit crystallization at calcification sites and degrade organic phosphates in order to increase the concentrations of inorganic phosphates.

The goal of this study is to determine the effect of hormone replacement therapy in post-menopausal women on the expression of *ALPL* within the mucosal epithelium of the cheek and in peripheral blood lymphocytes.

MATERIAL AND METHODS

A clinical stomatological study was carried out in a group of 60 menopausal women. The mean age of subjects was 53.0 years. The study population was divided into 4 groups of 15 subjects each:
- group M – 15 post-menopausal women not receiving HRT: a control group,
- group OV – 15 women after ovarectomy procedures not receiving HRT,
- group OV+HRT – 15 women after ovarectomy procedures and receiving HRT,
- group M+HRT – 15 menopausal women receiving HRT.

All patients provided consent to participate in the study and the remaining study procedures were approved by the Bioethics Committee of the Medical University of Lublin, decision no. KE-0254/140/99 and KE-0254/246/2001.

Blood samples of 9 ml and fragments of superficial mucosal epithelium of the cheek were collected from all patients.

The epithelium samples were suspended in 5 ml of 0.9% physiological saline and centrifuged for 15 minutes at 3000 rpm to isolate epithelial cells. The isolated epithelial cells as well as freshly collected, non-centrifuged blood samples were transferred to separate EDTA collection tubes. Thus prepared material was used to assess the expression of the reference *GAPDH* gene and the *ALPL*.

Total cellular RNA isolation was performed using Sigma TriReagent using the protocol provided by the manufacturer (modified Chomczyński and Sacchi method) (1).

After dissociation of the nucleoprotein complex (5 minutes at room temperature), 0.2 ml of chloroform was added, the mixture was shaken vigorously, incubated for 15 minutes

at room temperature and centrifuged at 12,000 rpm for 20 minutest at 4°C. The lysate separated into three phases:

- the protein-containing organic phase;
- the DNA-containing interphase;
- the upper, colorless, aqueous, RNA-containing layer

After transferring the aqueous phase into Eppendorf tubes, 0.5 ml of isopropanol was added into samples. The samples were left to stay for 10 minutes at room temperature, and than centrifuged at 12,000 rpm for 10 minutes at 4°C. The RNA precipitate was washed with 75% ethanol, centrifuged (7500 rpm) for 5 minutes, dried and finally dissolved in H_2O. Thus prepared RNA was used to prepare the cDNA.

Isolation of RNA from whole blood (1 mL) was preceded by the lysis of erythrocytes in a buffer consisting of NH_4Cl: 0.8M, $KHCO_3$: 0.05M, EDTA: 0.01M.

After 30 minutes of incubation at 4°C, blood samples were centrifuged (12,000 rpm) for 20 minutes at 4°C. The supernatant was discarded and RNA was isolated out of the precipitate according to the procedure described above. The cDNA synthesis was carried out using the High-Capacity cDNA Reverse Transcription Kit (AppliedBiosystems) according to the manufacturer-recommended protocol

Next, cDNA was used in real-time PCR to determined the expression of the reference *GAPDH* gene and the *ALPL*.

The results were subjected to statistical analysis. Arithmetic mean (M) and standard deviation (SD) values were calculated. The significance of differences between individual groups was determined on the basis of confidence intervals as determined in the analysis of variance (ANOVA). The results of statistical calculations are presented in tables while the mean values are illustrated by graphs in the figures. The differences between the control group M and the remaining groups are marked by letters in table columns (P) as well as below graphs in the figures. Means are significantly different if not labeled by the same alphabetic character.

RESULTS

The results of determinations of the expression of the *ALPL* within the mucosal epithelium of the cheek are listed in Table 1 and illustrated in Figure 1.

The average level of the expression of the *ALPL* within the mucosal epithelium of the cheek in the female patients of the control group M was 0.37, compared to a much higher value of 3.96 in the ovarectomy (OV) group). The differences between the groups was statistically significant ($P > 0.05$). In the group of women receiving HRT in the perimenopausal period (M+HRT), gene expression was reduced to 0.07; the difference compared to Group M was statistically insignificant ($P > 0.05$). In the group of patients undergoing hormone replacement therapy after ovarectomy (OV+HRT), gene expression was reduced to 0.08; the difference compared to Group M was statistically insignificant ($P > 0.05$).

The results of determinations of the expression of the *ALPL* in peripheral blood lymphocytes are listed in Table 2 and illustrated in Figure 2.

Table 1. Alkaline phosphatase gene expression within the mucosal epithelium of the cheek compared to the GADPH reference gene

Group	n	*ALPL* expression within the mucosal epithelium of the cheek (M ± SD)	Significance of difference (P*)
M	15	0.37 ± 0.54	a
OV	15	3.96 ± 0.78	b
M+HRT	15	0.07 ± 0.05	a
OV+HRT	15	0.08 ± 0.04	a

*Means are significantly different if not labeled by the same alphabetic character
n – number of samples
Group OV was significantly different than M, M+HRT and OV+HRT groups

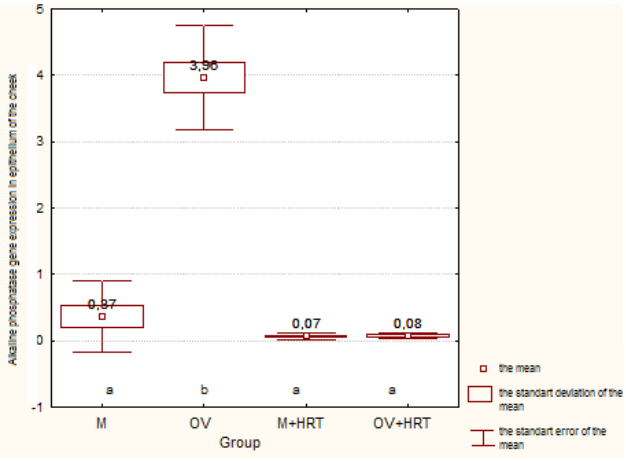

Figure 1. Alkaline phosphatase gene expression within the mucosal epithelium of the cheek compared to the GADPH reference gene

Table 2. Alkaline phosphatase gene expression in peripheral blood lymphocytes (PBLs) in tested female patient groups compared to the GADPH reference gene

Group	n	*ALPL* expression in peripheral blood lymphocytes (PBLs) (M ± SD)	Significance of difference (P*)
M	15	0.69 ± 0.87	b
OV	15	0.28 ± 0.19	a, b
M+HRT	15	0.42 ± 0.50	a, b
OV+HRT	15	0.12 ± 0.10	a

*Means are significantly different if not labeled by the same alphabetic character
n – number of samples
Group M was significantly different than group OV+HRT

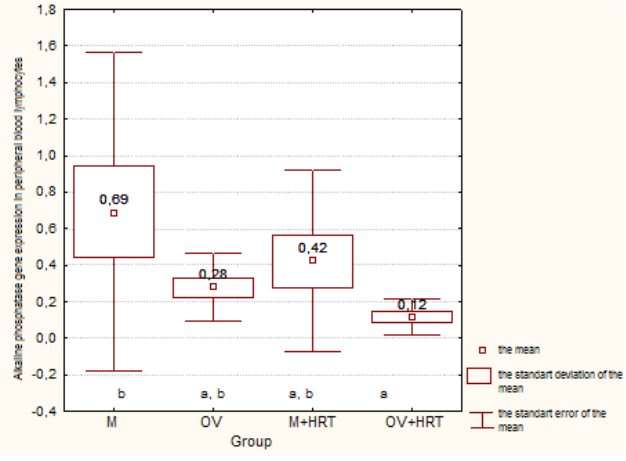

Figure 2. Alkaline phosphatase gene expression in peripheral blood lymphocytes (PBLs) in tested female patient groups compared to the GADPH reference gene

The average level of the expression of the *ALPL* in peripheral blood lymphocytes (PBL) in the female patients of the control group M was 0.69, compared to 0.28 in the ovarectomy (OV) group. The difference between these groups was statistically insignificant ($P > 0.05$).

Reduced expression of the phosphatase gene was observed in the study groups of patients receiving hormone replacement therapy (M+HRT) and undergoing hormone replacement therapy after ovarectomy (OV+HTZ) as compared to groups M and OV. The differences between the control group M and the remaining groups: M+HRT, OV, and OV+HRT were statistically significant ($p < 0.05$).

In order to study the correlation (linear relationship) between the expression of the *ALPL* within the mucosal epithelium of the cheek and the expression of the *ALPL* in peripheral blood lymphocytes, Pearson's r-coefficients were calculated for individual groups. The results are presented in Table 3.

Table 3. Pearson's r coefficients of correlation between the expression of the alkaline phosphatase gene within the mucosal epithelium of the cheek and the expression of the alkaline phosphatase gene in peripheral blood lymphocytes (PBL)

Group	n	Pearson's r coefficient
Total	60	-0.0893
M	15	-0.2783
OV	15	0.6787
M+HRT	15	0.1228
OV+HRT	15	-0.0560

In the OV group, positive correlation ($r = 0.6787$) was observed between the expression of the *ALPL* within the mucosal epithelium of the cheek and the expression of the *ALPL* in peripheral blood lymphocytes. The results show that in the group of patients having undergone ovarectomy (OV), a 1 unit increase in the of the *ALPL* within the mucosal epithelium of the cheek led to a 0.1615 unit increase in the expression of the *ALPL* in peripheral blood lymphocytes. Figure 3 presents distribution graphs with regression lines.

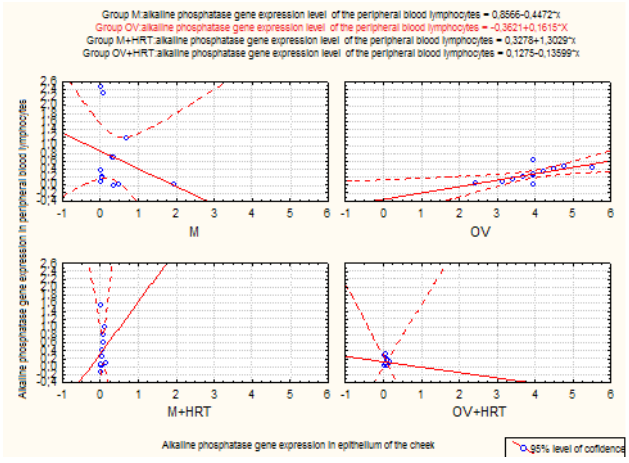

Figure 3. Distribution of the within the mucosal epithelium of the cheek and peripheral blood lymphocytes (PBL)

DISCUSSION

The studies show that hormone replacement therapy has no significant effect on the increase in *ALPL* expression within the mucosal epithelium of the cheek. Only in women having undergone ovarectomy (OV), the epithelial *ALPL* expression level was higher than in the remaining groups.

The highest expression of the *ALPL* within peripheral blood lymphocytes was demonstrated in menopausal women (M); in the remaining groups, the expression of the gene was lower, although the differences were not statistically significant.

Positive correlation was demonstrated between the levels of expression of the *ALPL* within the mucosal epithelium of the cheek and in peripheral blood lymphocytes.

Takahashi et al. concluded that the overall activity of alkaline phosphatase was poorly correlated with the rate of bone formation while the bone fraction of the enzyme was well correlated with the bone remodeling activity (19).

Further studies are recommended to assess the bone alkaline phosphatase levels as a measure of the effect of HRT on bone space. Bone alkaline phosphatase (BALP) is synthesized within the osteoblasts and released into circulation during the middle stage of bone formation, i.e. during bone matrix maturation. The enzyme is believed to impact the early stages of bone mineralization while having an additional beneficial property of long half life in blood (1-2 days), resulting in low diurnal variability of this parameter (3).

The problem of the assessment of the effect of hormone replacement therapy on the expression of the *ALPL* within the mucosal epithelium of the cheek and peripheral blood lymphocytes is due to the fact that alkaline phosphatase is not a uniform enzyme.

To date, 4 ALP-encoding genes have been identified, expressing four isoenzymes present within the plasma. The gene encoding for tissue non-specific ALP is expressed within the kidneys, osteoblasts, hepatocytes, young placenta and encodes three enzymatic isoforms: renal, bone and hepatic alkaline phosphatase. Isoforms develop as a result of differences in post-translational modifications (glycosylation)

The placental, intestinal and gonadal reproductive epithelial isoenzymes are tissue-specific. Te bone-hepatic-renal isoenzyme is detected in different tissues. All three isoforms – hepatic and bone phosphatase (in equivalent amounts) and intestinal phosphatase (absent in some individuals) are detected in healthy subjects. Since the bone isoform is subject to most dynamic changes, it largely affects changes in total ALP levels. Therefore, determination of total ALP activity is justified in bone diseases associated with enhanced bone metabolism and/or disturbed mineralization, such as rickets, osteomalacia Paget's disease, renal osteodystrophy, as well as in bone fracture healing processes, whereas in other diseases characterized by moderate increase in bone turnover, the percentage contribution of other ALP sources may be too high and significant changes in total alkaline phosphatase levels should not be expected e.g. in post-menopausal osteoporosis. Therefore, measurements of the bone fraction levels are most reliable.

The activity of bone alkaline phosphatase is affected by a number of factors. Among others, increased activity of this enzyme has been observed in children with rapid growth rates, in pregnant women and in diseases characterized by

increased bone turnover, such as Paget's disease, hyperparathyroidism, hyperthyroidism, osteomalacia, renal osteodystrophy and tumor bone metastases (6,8,9,10,22).

According to some authors, only the assessment of bone alkaline phosphatase allows for accurate estimation of the rate of bone metabolism (18). Determinations of the total levels of this enzyme have been imprecise (17).

According to Nawaw et al., hormone replacement therapy (HRT) reduces bone metabolism in menopausal patients.

Monitoring of bone markers, including total alkaline phosphatase and particularly bone alkaline phosphatase may be used to evaluate the efficacy of HRT (14).

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