

Research Article

The effect of 12 weeks of aerobic exercise intervention on bone mineral density, expression of lymphocyte alkaline phosphatase gene and bone turnover markers in overweight postmenopausal women: a randomized controlled trial

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Abstract

The aim of our study was to examine the effect of 12 weeks of moderate-intensity aerobic exercise on bone mineral density (BMD), lymphocyte alkaline phosphatase (ALP) mRNA expression, and biochemical markers of bone turnover in postmenopausal women (PMWs). Twenty-four healthy sedentary PMWs aged 45-70 years were randomly assigned to exercise (EX, n=12) and control (C, n=12) groups. The EX group performed walking/jogging (50-60min/day, 3days/week at 65%-70% HRmax reserve) for 12-week while the C group participated in no intervention and continued their normal lifestyle. The BMD and lymphocyte ALP mRNA were determined by DXA and qRT-PCR, respectively. After 12 weeks, the increase in the lymphocyte ALP mRNA expression and its serum (P=0.008 and P=0.001), PTH (P=0.001), Vit-D (P=0.002), and VO2max (P=0.001) were significantly higher in the EX group compared to the C group, whereas body fat was significantly decreased (P=0.028). Our study indicates that 12 weeks of moderate-intensity aerobic exercise intervention improves bone turnover by increasing the ALP mRNA expression, serum levels of PTH, ALP, and Vit-D which can lead to the prevention of aging-induced osteopenia among PMWs.


Key Words: Aerobic exercise, Bone, Alkaline phosphatase, Menopause, Bone turnover

Introduction

Menopause status is related to changes in sex hormones (Khalafi et al., 2021; Malandish et al., 2020a; Malandish et al., 2020b; Tartibian et al., 2017) as well as bone disorders (Malandish et al., 2016; Sheikhlou et al., 2016). Bone loss and osteoporosis among postmenopausal women (PMWs) have been identified as a global health problem (Eastell et al., 2019; Edwards, 2017), and fractures resulting from these defects are the main causes of mortality in older people (Cauley et al., 2012; Eastell et al., 2019). Also, sedentary-induced disorders are other factors that affect osteoporosis. Besides the genetic and environmental factors affecting bone mineral density (BMD) (Stewart & Ralston, 2000), aerobic exercise and cellular-molecular mechanisms are also involved in BMD (Tartibian, Hajizadeh Maleki, Kanaley, & Sadeghi, 2011; Yu et al., 2019). It seems that a reduction in estrogen and progesterone after menopause in women leads to disturbances in bone metabolism regulation systems such as skeletal (Azadpour, Tartibian, & Koşar, 2016; Karinkanta, 2017; Malandish et al., 2016; Tartibian, Hajizadeh Maleki, Kanaley, & Sadeghi, 2011) and cardiovascular systems (CVS) (Azadpour, Tartibian, & Koşar, 2016). The aerobic exercise is an important non-medication factor in old women to take care of their health and BMD later in life span (Karinkanta, 2017; Tartibian, Hajizadeh Maleki, Kanaley, & Sadeghi, 2011). In other words, moderate intensity aerobic exercise intervention is one of the most effective ways of reducing risk factors of bone loss and/or osteoporosis and mortality (Malandish et al., 2016; Tartibian et al., 2018a; Tartibian et al., 2018b; Tartibian, Hajizadeh Maleki, Kanaley, & Sadeghi, 2011). However, the role of gene expression and cell signaling cascades are not fully understood in BMD and bone metabolism following aerobic exercise intervention and there are currently no studies regarding the association between the human ALP gene expression and exercise in scientific databases. Interestingly, several studies have examined the effects of exercise on serum markers of bone metabolism and BMD, but their results are completely co-

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-ntradictory. Therefore, the first purpose of this study was to examine the effect of 12 weeks of moderate-intensity aerobic exercise on BMD in PMWs. Also, an attempt was made to examine the effect of 12 weeks of moderate-intensity aerobic exercise on lymphocyte ALP gene expression and BMD. The third purpose was to determine the changes in serum levels of bone markers following moderate-intensity aerobic exercise intervention. In addition, estimating the correlations between BMD and ALP gene expression with serum levels of bone markers and other variables of this study were the fourth purpose of our study.

Materials and Methods

Healthy sedentary PMWs as participants

This study was performed in a randomized control design. All stages of this study were approved by the Ethics Committee at Urmia University of medical sciences with code ir.umsu.rec.1394.453 and were conducted in January 2016 at Urmia University, Iran. During the public notice of exercise and PMWs, 100 sedentary PMWs were selected, out of 100 PMWs recruited into the study, 50 participants obtained initial conditions. Finally, after evaluating BMD by using Dual-energy X-ray Absorptiometry (DXA-HOLOGIC®, USA), 26 participants were eliminated due to osteopenia/osteoporosis and 24 human volunteers as participants met the inclusion criteria for the study (of the 76 participants excluded, 25 PMWs had hypertension, 20 PMWs had pharmacological interventions, 5 PMWs had electrocardiogram abnormalities, and 26 PMWs had BMD disorders based on T score values including osteopenia and osteoporosis). The inclusion criteria of healthy sedentary PMWs as participants included: (1) PMWs with the age range of 45 to 70 years spending at least 1 year and/or less than 5 years at menopause, as this was controlled by evaluating the normal menopause serum levels of 17β-estradiol (11-65 pg/ml) and progesterone (0.1-1 pg/ml); (2) No history of bone fractures or current diseases affecting bone metabolism as well as any clinical problem in the skeletal system and CVS; (3) No history of regular exercise, weight gain, weight loss, smoking and medications for at least 6 months before the start of the study; and (4) having normal BMD values at the whole body, lumbar spine (L1–L4), and femoral neck. Bone mass was measured by using DXA based on the world health organization (WHO) definition from normal BMD or T score values (Normal>-1SD; -2.5SD<Osteopenia≤-1SD; and Osteoporosis≤-2.5SD). The CVS was evaluated based on the normal electrocardiogram (ECG) (Esaote Spa, Firenze, Italy). Exclusion criteria of human volunteers as participants included: (1) Identification and diagnosis of chronic diseases affecting bone metabolism as well

as any medical disease during 12-week; (2) Discontinue of regular exercise protocol during 12 weeks; and (3) dietary regimen, weight gain, weight loss, and any pharmacological treatment during 12 weeks. Physical activity level of PMWs was measured by physical activity assessment (PAA) method (Strath

Table 1: Physiological characteristics, menopause status, and bone parameters of PMWs at baseline and week 12 in the EX and C groups.

Variables	EX (n=12)	C (n=12)	P ^a value	P ^c value	Eta coefficient (%)
Physiological characteristics					
Age (yr)					
Baseline	53.36±3.98	53.00±3.26	ns	ns	ns
Week 12	53.36±3.98	53.00±3.26	ns		
P ^a value	ns	ns			
Height (cm)					
Baseline	157.45±4.5	158.40±5.42	ns	ns	ns
Week 12	-	-	ns		
P ^a value	ns	ns			
Weight (kg)					
Baseline	73.18±10.30	74.72±14.10	0.706	0.329	0.050 (5%)
Week 12	72.72±10.11	77.27±15.98	0.484		
P ^a value	0.526	0.199			
Body mass index (kg/m ²)					
Baseline	30.26±4.91	29.58±5.35	0.759	0.053	0.183
Week 12	29.67±5.17	30.68±6.34	0.688		(18.3%)
P ^a value	0.185	0.157			
Total body fat (%)					
Baseline	42.03±5.10	40.54±5.50	0.914	†0.028	0.228
Week 12	41.15±4.87	41.45±5.55	0.898		(22.8%)
P ^a value	†0.023	0.188			
Systolic blood pressure (mmHg)					
Baseline	120.45±16.37	116.80±17.66	0.484	0.714	0.119
Week 12	111.63±20.08	123.20±14.30	0.841		(11.9%)
P ^a value	0.373	0.088			
Diastolic blood pressure (mmHg)					
Baseline	75.18±8.85	79.00±13.82	0.456	0.490	0.001 (0.1%)
Week 12	76.81±5.91	80.70±10.49	0.304		
P ^a value	0.603	0.355			
Resting heart rate (beats/min)					
Baseline	80.81±10.94	76.72±10.70	0.186	0.472	0.031 (3.1%)
Week 12	78.54±6.81	76.27±9.23	0.287		
P ^a value	0.307	0.657			
VO ₂ max (ml/kg/min)					
Baseline	29.00±2.15	30.03±5.74	0.713	†0.001	0.534 (53.4%)
Week 12	33.30±2.10	29.15±5.56	0.174		
P ^a value	†0.001	0.132			
Walking & Jogging time to exhaustion (min)					
Baseline	9.93±2.62	9.77±2.76	0.894	†0.001	0.551 (55.1%)
Week 12	12.16±1.97	9.62±2.61	†0.027		
P ^a value	†0.001	0.468			
Metabolic equivalent of task (METs) h/week					
Baseline	11.60±0.81	12.00±1.13	0.349		
Week 12	14.93±1.00	11.89±1.13	†0.001	†0.001	0.979 (97.9%)
P ^a value	†0.001	0.187			
Menopause status					
Menopause age (yr)					
Baseline	3.18±1.25	2.62±1.18	0.342	ns	ns
Week 12	3.18±1.25	2.62±1.18	0.342		
P ^a value	ns	ns			

Continue of table 1.

17β-estradiol (pg/ml)					
Baseline	31.53 \pm 16.28	41.06 \pm 12.65	0.202	0.123	0.117
Week 12	29.73 \pm 15.36	23.85 \pm 12.30	0.402		(11.7%)
P ^a value	0.693	*0.031			
Progesterone (pg/ml)					
Baseline	0.18 \pm 0.10	0.30 \pm 0.14	0.084	0.495	0.026 (2.6%)
Week 12	0.14 \pm 0.07	0.20 \pm 0.17	0.401		
P ^a value	0.104	*0.033			
Bone parameters					
BMD total whole body (g/cm²)					
Baseline	1.022 \pm 0.07	1.000 \pm 0.08	0.523	0.475	0.029 (2.9%)
Week 12	1.022 \pm 0.07	0.981 \pm 0.12	0.368		
P ^a value	0.952	0.390			
BMD L1 (g/cm²)					
Baseline	0.910 \pm 0.11	0.865 \pm 0.11	0.418	0.382	0.048 (4.8%)
Week 12	0.929 \pm 0.12	0.904 \pm 0.13	0.672		
P ^a value	0.208	0.113			
BMD L2 (g/cm²)					
Baseline	1.069 \pm 0.10	1.026 \pm 0.10	0.381	0.256	0.080 (8%)
Week 12	1.044 \pm 0.10	1.023 \pm 0.11	0.660		
P ^a value	0.084	0.853			
BMD L3 (g/cm²)					
Baseline	1.091 \pm 0.11	1.050 \pm 0.10	0.441	0.679	0.011 (1.1%)
Week 12	1.072 \pm 0.12	1.024 \pm 0.09	0.365		
P ^a value	0.317	0.071			
BMD L4 (g/cm²)					
Baseline	1.084 \pm 0.11	1.074 \pm 0.11	0.854	0.264	0.077 (7.7%)
Week 12	1.088 \pm 0.10	1.060 \pm 0.13	0.587		
P ^a value	0.692	0.289			
BMD Total lumbar vertebral (g/cm²)					
Baseline	1.045 \pm 0.10	1.012 \pm 0.09	0.501	0.775	0.005 (0.5%)
Week 12	1.039 \pm 0.11	1.00 \pm 0.10	0.538		
P ^a value	0.515	0.710			
BMD Femoral neck (g/cm²)					
Baseline	0.779 \pm 0.04	0.829 \pm 0.10	0.188	0.356	0.053 (5.3%)
Week 12	0.773 \pm 0.04	0.829 \pm 0.09	0.328		
P ^a value	0.422	0.999			
T score Total whole body					
Baseline	-0.918 \pm 0.80	-0.960 \pm 0.60	0.895	0.231	0.079 (7.9%)
Week 12	-0.909 \pm 0.90	-1.200 \pm 0.92	0.475		
P ^a value	0.884	0.247			
T score Total lumbar vertebral (L1-L4)					
Baseline	1.045 \pm 0.10	1.012 \pm 0.09	0.474	0.676	0.011 (1.1%)
Week 12	1.039 \pm 0.11	1.00 \pm 0.10	0.560		
P ^a value	0.515	0.710			
T score Femoral neck					
Baseline	-0.618 \pm 0.41	-0.187 \pm 0.97	0.205	0.785	0.005 (0.5%)
Week 12	-0.618 \pm 0.26	-0.175 \pm 0.88	0.321		
P ^a value	0.676	0.644			

Note. EX=Exercise group; C= Control group; VO₂max= Maximal oxygen uptake.

Values are mean \pm SD; *Pa < 0.05, significantly different from baseline values by paired samples t-test (within groups, baseline vs. week 12). †Pb < 0.05, significantly different baseline and also week 12 values by independent samples t-test (between groups, baseline EX vs. baseline C; and week12 EX vs. week 12 C). ‡Pc < 0.05, significantly different between groups during the time (interaction) analyzed by univariate analysis of variance. Eta= Partial Eta squared or estimates of effect size.

et al., 2013). All human volunteers as participants attended the exercise venue and received information about the protocols and procedures, as well as the possible risks and benefits involved in the study. Informed written consent was obtained from all the human volunteers as participants. The human volunteers as participants (PMWs) were then randomly assigned to the EX (n=12) or C (n=12) groups by true random number generation (Table 1).

Dietary assessment

Dietary data was assessed via a 3-day food record (Bailey et al., 2009) in the first and last weeks of the exercise program (over 2 weekdays and 1 weekend day). The human volunteers as participants were requested to maintain their normal diet during the period of study and were instructed to consume a diet as similar as possible in each sampling day. Information on the use of medications/supplements was also obtained through standard and self-reported questionnaires (Bailey et al., 2009). Nutrition and dietary data were analyzed by nutrition analysis software (Nutrition data proTM v1.1, StarApps Company, USA) (Table 2).

Aerobic exercise program

Prior to the beginning of the study, each human volunteer as participant underwent a graded exercise treadmill test (GXT-Turbo Fitness, LX740, Taiwan) to determine the maximal oxygen uptake (VO₂max). By using the Borg scale, rating of perceived exertion (RPE) was recorded in the last 10 s of each stage. Two of the following four criteria were required for a test to be considered maximal: (1) a plateau in VO₂ despite increasing workload; (2) respiratory exchange ratio (RER) \geq 1.10; (3) maximal heart rate within 10 beats of age predicted max [HR_{max} = (208 - (0.7 \times age in year))]; and (4) RPE \geq 17. The EX group participated in 12 weeks W-WJMIAEP-R on treadmill, 50 to 60 min/day, 3 days/week (180-200 min in week) at 65%-70% of each individual's maximal heart rate reserve (HR_{max}). In the first week of the study, the EX group performed W-WJMIAEP-R at 50% of each participant's HR_{max}. During the second and third weeks, the training intensity was increased to 60% of HR_{max} and then was progressed to 65%HR_{max} in the fourth to seventh weeks. In the last 5 weeks, human volunteers as participants were trained at 70% HR_{max}. Each training session included 10 min of prior warm-up and 5 min of cooling down (active-recovery) at the ambient temperature of 22–25°C and relative humidity of 44%. All exercise sessions were performed between 08:00 and 10:00 AM and participants were supervised by trained physical education teachers. The C group participated in no intervention and continued their normal sedentary lifestyle, current physical activity level and dietary habits during 12 weeks.

Measurements

Physiological characteristics

Physiological characteristics of participants including age, menopause age and height, weight, and body mass index (BMI) were measured by ID card, self-reported menstrual history, wall-meter (Beurer, Germany), digital scale machine (Beurer, Germany), and weight/height² formula, respectively. Total body fat was measured by Dual-energy X-ray absorptiometry (DXA) (Hologic, USA). Diastolic and systolic blood pressure and heart rate were measured by digital blood pressure monitor (WDF-BP001, Brisk, Germany). The physiological parameters were evaluated two times at baseline and week-12, except height values (Table 2).

Blood sampling and assays

Following a 12-hour overnight fasting, blood samples (10 ml) were taken in the early morning (between 07:00 and 08:00 AM) from a brachial vein of the left hand by Venoject needles in sit-down situation and relaxation. Blood samples (5 ml) were collected twenty-four hours before and after the 12-week training program in order to measure serum markers (ALP, PTH, Vit D, Ca²⁺, 17 β -estradiol, and progesterone) in the EX and C groups. To separate serum levels, blood samples were centrifuged at a speed of 3000 rpm for 15 min at 4°C by centrifuge machine after 15 min clotting at chamber temperature. Serum PTH, Vit D, 17 β -estradiol and progesterone measurements were assayed using an enzyme-linked immunosorbent assay (ELISA) kit (DiaSorin, USA), Bioactive Diagnostica (Homburg, Germany), (1561-DRG, Euroimmun, Germany), and (2633-DRG, Euroimmun, Germany), respectively. Serum levels were measured by ELISA device (Stat Fax@4200- Awareness Technology, USA). Serum ALP and Ca²⁺ levels were measured by standard automated laboratory techniques/BT-1500 machine. The other 5 ml remaining blood samples (total blood coated to an anticoagulant ethylenediaminetetra-acetic acid [EDTA]) were used to extract ribonucleic acid (RNA) in the expression of ALP and β -actin genes. The blood sample of gene expression stored at -70°C for 30 min, and then was quickly frozen in liquid nitrogen until subsequent analysis.

RNA extraction from peripheral blood lymphocytes (PBL) and complementary deoxyribonucleic acid (cDNA) synthesis

After removing blood samples from the liquid nitrogen and defreezing them in the laboratory, the rapid genomic DNA extraction (RGDE) method was performed (Saremi, Saremi, & Tavallaei, 2008), and then RNA was extracted using the RNX-Plus RNA Extraction Kit (SinaClon, Iran) according to the manufacturer's instructions. The concentration and quality or purity of total RNA

was assessed on the basis of OD260/280 ratio (ng/ μ l) measurements using a NanoDrop 1000 Spectrophotometer (ThermoScientific, Wilmington, USA) and electrophoresis on a 1.2% agarose gel in 0.5 (ng/ μ l) ethidium bromides, respectively.

The RNA samples were converted to cDNA after treating with DNase I to eliminate any genomic DNA contamination. RNA reverse transcription (RT) was performed in a final volume of 20 (μ l) reaction mixtures containing 1 (μ l) template RNA, 1 (μ l) dN6 primer, and 18 (μ l) DEPC-DNase water by AccuPower® RocketScript™ RT-PreMix cDNA kit (k-2101, Bioneer, Germany). According to the manufacturer's protocol, the reactions on the samples were performed by real time PCR (E750, ThermoScientific, Belgium). The synthesized cDNA samples were immediately stored at -70°C for using expression of ALP and β -actin genes.

Primer design

Specific primers of ALP and β -actin (as a housekeeping/reference gene) genes were designed using GenScript online primer software and were checked by primer Blast and OligoAnalyzer idt tools. Then, for the preparation of primers, their lyophilized powders were prepared by Bioneer Company, Daejeon from South Korea. The sequences of primers for genes are shown in Table 3.

Quantitative real-time reverse transcription polymerase chain reaction (qRT-PCR) assay

To measure ALP and β -actin genes from qRT-PCR device (Applied BioSystem A, Step One™, USA), the manufacturer's protocols were applied. The reactions were performed on the basis of Syber Green-I (Maxima SYBR-Green/ROX qPCR MasterMix k-0221, ThermoScientific, Germany) with melting curve for both groups in the range of 60 to 95°C to evaluate the specific sequences of β -actin gene and ALP gene along with their temperature cycles. The protocols of ALP and β -actin genes for qRT-PCR reaction were as follows, respectively: initial denaturation at 95°C for 5 min (Holding step), followed by 40 cycles of denaturation at 95°C for 10 seconds (Denaturation step), annealing at 61°C for 10 seconds (Annealing step), and extension at 72°C for 20 seconds (Extension step). Initial denaturation at 95°C for 5 min (Holding step), followed by 40 cycles of denaturation at 95°C for 10 seconds (Denaturation step), annealing at 60°C for 15 seconds (Annealing step), and extension at 72°C for 20 seconds (Extension step). The reaction was carried out in a final volume of 12 μ l based on the desired amounts. To ensure for presence of DNA in the products of the qRT-PCR device, electrophoresis on a 1.2% agarose gel in 0.5 (ng/ μ l) ethidium bromides was applied. Finally, the mean of thres-

-hold cycle (CT) data was calculated by the $2^{-\Delta\Delta Ct}$ method (Livak & Schmittgen, 2001).

BMD and T score measurements

BMD measurements (g/cm²) were evaluated at the whole body, anterior-posterior lumbar vertebral (L1-L4) and the non-dominant proximal femur, including femoral neck, using DXA. T scores for the whole body, lumbar vertebral and the femoral neck were also calculated.

Statistical analysis

All data were expressed as means±standard deviation (SD) and checked for normality using Kolmogorov-Smirnov and homogeneity of variances (Levene's test). Between-group differences were determined by independent samples t-test for baseline or week 12 values in the two groups. In addition, univariate analysis of variance (ANCOVA) test in a general linear model was applied to determine the changes of all variables between the two groups during the time (group-time interaction). The difference between baseline- and week 12 values for each group was investigated using paired samples t-test. To determine the correlations between BMD of the whole body, lumbar vertebral, femoral neck and other variables among PMWs at baseline and week 12, Pearson's correlation coefficient was applied. The statistical software program SPSS (SPSS Co, Chicago IL, version 23) for windows were used for data analysis. Statistical significance was considered at a $P < 0.05$ of two-tailed for all tests. It should be noted that the sample size was calculated using G*Power software for ANCOVA-fixed and main effects and interactions for detecting a medium effect size (Cohen $d = 0.4$) with an alpha of 0.05 and power of 95%. The total sample size was calculated as 24 participants.

Results

Baseline characteristics of participants did not show any significant differences between groups in physiological characteristics, menopause status, and bone parameters ($p > 0.05$, Table 1). At the end of 12 weeks of aerobic exercise intervention, total body fat percentage (TBF) decreased significantly while VO₂max, walking-jogging time to exhaustion (WJTE), and physical activity level or metabolic equivalent of task (METs) increased significantly in the EX group ($p < 0.05$, Table 1). Moreover, 17 β -estradiol and progesterone decreased significantly in the C group. The significant group-time interactions showed that the EX group compared with C group experienced significantly higher reduction in TBF ($p = 0.028$), higher increase in the WJTE ($p = 0.001$) and higher increase in the METs ($p = 0.001$) as well as higher increase in the VO₂max ($p = 0.001$) (Table 1). Dietary intake status in total energy intake

Table 2. Differences in dietary intake for PMWs at baseline and week 12 in the EX and C groups.

Variables	EX (n=12)	C (n=12)	P ^a value	P ^c value
Total energy intake (kcal/d)				
Baseline	2242.88±546.82	2366.12±515.60	0.602	0.341
Week 12	2303.36±335.22	2297.25±312.63	0.966	
P ^a value	0.557	0.419		
Fat (g)				
Baseline	103.11±29.13	104.22±32.18	0.935	0.646
Week 12	103.14±26.04	100.29±21.14	0.787	
P ^a value	0.997	0.566		
Carbohydrate (CHO) (g)				
Baseline	252.20±114.00	293.97±78.48	0.345	0.389
Week 12	264.74±85.26	282.18±63.76	0.605	
P ^a value	0.436	0.200		
Protein (g)				
Baseline	88.75±23.28	77.96±26.58	0.334	0.470
Week 12	90.06±20.39	79.07±21.33	0.243	
P ^a value	0.693	0.656		

Note. EX= Exercise group; C= Control group; TEI= Total energy intake; Values are mean±SD. * $P < 0.05$, significantly different from baseline values by paired samples t-test (within groups, baseline vs. week 12). † $P < 0.05$, significantly different baseline and also week 12 values by independent samples t-test (between groups, baseline EX vs. baseline C; and week12 EX vs. week 12 C). ‡ $P < 0.05$, significantly different between groups during the time (interaction) analyzed by univariate analysis of variance (between groups, baseline and week 12 EX vs. baseline and week 12 C).

(TEI), fat, carbohydrate (CHO), and protein did not show any significant within/between group differences among PMWs ($p > 0.05$, Table 2). The two groups were similar regarding food intake at the beginning and at the end of 12 weeks of the study, and no change was occurred in food intake over time.

As illustrated in Table 4, the mean changes of ALP gene expression and serum ALP increased significantly during the time in the EX group compared to the C group, respectively ($p = 0.008$ and $p = 0.001$). Serum PTH increased significantly in the EX group

Table 3. Sequences of primers of ALP & β -actin genes for qRT-PCR.

Genes and Oligonucleotide	Primer length	Sequence of primers (5' → 3')	Product size (bp)
Alkaline phosphatase (ALP)			
Forward primer	20	TGAGAGTGACGAGAAAGCCA	92
Reverse primer	20	GGGAGTGCTTGTATCTCGGT	
β-actin			
Forward primer	20	TGGACTTCGAGCAAGAGATG	137
Reverse primer	20	GAAGGAAGGCTGGAAGAGTG	

Note. qRT-PCR= Quantitative Real-Time Reverse Transcription Polymerase Chain Reaction.

($p=0.031$) while it showed a significant decrease in the C group ($p=0.023$). The PTH change during the time was significantly different between the two groups ($p=0.001$). Vit D showed significant increase in the EX group ($p=0.001$) while remained unchanged in the C group ($p=0.307$). Also, the change in Vit D during the time was significantly different between the groups ($p=0.002$). Serum Ca^{2+} did not change in the EX ($p=0.953$) or C ($p=0.809$) groups. There was no significant difference between the two groups regarding these changes during the time ($p=0.990$). The effect size (Eta) coefficient showed 22.8%, 53.4%, 55.1%, 97.9% 43.1%, 86.7%, 44.9% and 57.9% increase in TBF, VO_{2max} , WJTE, METs, ALP gene expression, serum ALP, serum PTH and serum Vit D respectively in the EX group compared to the C group, whereas the other factors did not show considerable changes.

Discussion

The present study demonstrated that increase in the lymphocyte ALP gene expression was associated with normal BMD based on the T score values. The new findings of this study are: (1) 12 weeks of aerobic exercise improved cardiorespiratory fitness (CRF) through positive adaptations in physiological indices, including increase in the VO_{2max} , WJTE, and METs and decrease

in TBF among PMWs; (2) Normal T score values were maintained in healthy sedentary PMWs after 12 weeks of moderate-intensity aerobic exercise intervention while 3 PMWs (1 PMW at the lumbar vertebral and 2 PMWs at the femoral neck; 25%) experienced aging-induced osteopenia due to physically inactive in the C group; (3) Significant decrease was observed in serum levels of 17β -estradiol and progesterone after week 12 due to a rapid decline in sex hormones during menopause in the C group; (4) 12 weeks of aerobic exercise increased lymphocyte ALP gene expression in PMWs; (5) 12 weeks of moderate-intensity aerobic exercise intervention increased circulating level of PTH in PMWs; (6) 12 weeks of moderate-intensity aerobic exercise intervention increased the circulating levels of ALP and Vit D in PMWs; and these findings clearly show that the increases of ALP mRNA and circulating levels of PTH, ALP and Vit D with the promotion of CRF provides numerous benefits for bone turnover. To our knowledge, our study is the first to concurrently investigate the impact of moderate-intensity aerobic exercise on BMD, lymphocyte ALP gene expression, and biochemical markers of bone turnover including serum levels of PTH, ALP, Vit D, 17β -estradiol and progesterone among PMWs.

Recent studies have reported that increase in CRF such as WJTE, VO_{2max} and METs is associated with reduced risk factor

Table 4. ALP Gene expression, serum levels, BMD and T scores among PMWs at baseline and week 12 in the EX and C groups.

Variables	EX (n=12)	C (n=12)	P ^a value	P ^c value	Eta coefficient (%)
ALP gene expression (fold change)					
Baseline	1.63±2.61	1.00±0.00	0.475	*0.008	0.431 (43.1%)
Week 12	2.80±2.96	0.42±0.34	*0.030		
P ^a value	0.551	*0.001			
ALP serum (dl/mg)					
Baseline	125.18±34.70	134.90±33.97	0.514	*0.001	0.867 (86.7%)
Week 12	217.27±56.83	136.55±30.96	*0.001		
P ^a value	*0.001	0.419			
PTH serum (ml/ng)					
Baseline	31.42±13.71	32.74±13.89	0.825	*0.001	0.449 (44.9%)
Week 12	42.11±14.58	19.89±15.14	*0.002		
P ^a value	*0.031	*0.023			
Vit D serum (ml/ng)					
Baseline	3.99±1.62	5.30±3.03	0.291	*0.002	0.579 (57.9%)
Week 12	11.41±3.34	3.67±0.84	*0.001		
P ^a value	*0.001	0.307			
Ca²⁺ serum (dl/mg)					
Baseline	9.43±0.35	9.28±0.28	0.105	0.990	0.001 (0.1%)
Week 12	9.42±0.45	9.38±0.91	0.988		
P ^a value	0.953	0.809			

Note. EX= Exercise group; C= Control group; Values are mean±SD. *Pa< 0.05, significantly different from baseline values by paired samples t-test (within groups, baseline vs. week 12). †Pb< 0.05, significantly different baseline and also week 12 values by independent samples t-test (between groups, baseline EX vs. baseline C; and week12 EX vs. week 12 C). ‡Pc< 0.05, significantly different group - time interaction analyzed by univariate analysis of variance (between groups, baseline and week 12 EX vs. baseline and week 12 C). Eta= Partial Eta squared or estimates of effect size.

of osteopenia and/or osteoporosis in PMWs (DeFina et al., 2016). Furthermore, increased CRF is associated with lower all-cause and cardiovascular mortality (Tartibian, Botelho Teixeira, & Baghaiee, 2015) which can promote healthy cognitive and psychosocial function among PMWs (McKinney et al., 2016). It seems that increased CRF and decreased TBF lead to a reduction in sarcopenia indices such as aging-induced osteopenia (Lee et al., 2016) and obesity sarcopenia (Fukuda et al., 2018) in the EX group compared to the C group. In other words, it is possible that increased efficiency of cardiorespiratory system (CRS) and healthy skeletal system can delay aging-induced risk factors such as osteopenia, osteoporosis, and sarcopenia in menopause although the mechanism is unclear (McKinney et al., 2016). Also, our study showed that the ground reaction force (GRF) caused by 12 weeks W-WJMIAEP-R on treadmill did not provide the necessary pressure to stimulate BMD at three areas in the EX group; although, T score values remained normal. On the other hand, it is possible that aging-induced physically inactive and rapid reduction of sex hormones such as 17β -estradiol and progesterone in the first 1-5 years of menopause (Boonen et al., 2008) will facilitate the aging process and aging-induced disease such as osteopenia or osteoporosis, as 3 osteopenic PMWs (25%) were observed after 12 weeks in the C group.

It seems that increased ALP gene expression modulates osteoblast cells after 12 weeks of aerobic exercise in PMWs. It exerted mechanical stress into the biochemical cellular signaling of osteoblasts (Tartibian, Hajizadeh Maleki, Kanaley, & Sadeghi, 2011), thus, based on the hypothesis of mechanical stress, it is likely to increase calcium phosphate/hydroxyapatite and/or maintain normal BMD values at three areas in PMWs. Since osteoblast cells can be considered as a major source of ALP (Edwards, 2017; Goseki-Sone et al., 2005; Tartibian, Hajizadeh Maleki, Kanaley, & Sadeghi, 2011), increasing the ALP gene expression as well as its serum levels is a sign of stimulation in osteoblast cells (Goseki-Sone et al., 2005; Nabipour et al., 2008), and as a result, cellular signaling will increase with increasing BMD (Goseki-Sone et al., 2005; Nabipour et al., 2008). The ALP gene-induced changes have been reported to be important determinants of age-related bone loss, and the phosphate metabolism pathway is considered as a new purpose in preventing osteoporosis (Sharma, Pal, & Prasad, 2014). Also, since Akt signaling is required in all phases of osteoblast differentiation and is essential for osteogenesis and mineralization (Mukherjee & Rotwein, 2009), it is possible that activation of Akt signaling pathway is required for ALP-induced osteoblast differentiation and mineralization process in PMWs. On the other hand, elevation of ALP gene expression was associated with elevated serum ALP in the EX group, which is related to the bone mineralization process due to the role of serum ALP as a marker for osteogenic activity in hard tissue formation and bone metaboli-

-sm (Nabipour et al., 2008). Besides, another control of the ALP gene expression is applied through the action of Vit D (Maehata et al., 2006; Paredes et al., 2004), PTH (Wang et al., 2006), and retinoic acid (mediating ALP gene expression through unique pathways) and interaction with important regulatory systems (Maehata et al., 2006; Paredes et al., 2004; ; Rey et al., 2007; Wang et al., 2006). Furthermore, in the present study, there was a significant difference for serum PTH in between-groups, as it showed an increase in the EX group and decrease in the C group. Since osteoblast cells have PTH receptors (Yavropoulou, Michopoulos, & Yovos, 2017), it is likely that increased ALP gene expression stimulates PTH receptors and also increases the circulating levels of PTH, which is probably due to activation of PTH receptors which is probably due to the surface of osteoblasts and osteocytes (Datta & Abou-Samra, 2009). Also, elevated PTH serum is associated with increased bone formation and/or the life span and number of osteoblasts by prevention of osteoblast apoptosis (Jilka et al., 1999). Recent studies demonstrated a possible role for Wnt signaling pathway on PTH actions in bone metabolism (Yavropoulou, Michopoulos, & Yovos, 2017). On the other hand, it is possible that the activation of osteoblast cells by increased ALP gene expression is associated with elevated PTH serum as well as elevated ALP serum after 12 weeks of aerobic exercise in the EX group. However, according to the findings of previous studies, the PTH duality effects on increasing and/or decreasing bone formation remain unclear (Rey et al., 2007; Yavropoulou, Michopoulos, & Yovos, 2017); therefore, increasing or decreasing this hormone by aerobic exercise depends on several factors, such as intensity, duration, and the level of exercise (Tartibian, Hajizadeh Maleki, Kanaley, & Sadeghi, 2011). Moreover, insufficient serum levels of Vit D with decreased serum PTH are associated with risk factors of bone resorption or osteoporosis and mortality (Cerdá Gabaroi et al., 2010; Tartibian, Hajizadeh Maleki, Kanaley, & Sadeghi, 2011), as observed in the C group of our study. Recent evidences indicate that Vit D plays an important role in the aging process (Berridge, 2016). The ability of Vit D synthesizes by human skin declines with aging process (Berridge, 2016). Also, dysregulation of the Ca^{2+} signaling and mitochondrial dysfunction are associated with aging process (Decuypere et al., 2011). Furthermore, Vit D acts by maintaining the totality of cell signaling pathways such as Ca^{2+} and reactive oxygen species (ROS) signaling pathways (Berridge, 2016). It is possible that decreased Vit D levels result in an increase in the activation of Ca^{2+} and ROS signaling pathways (Berridge, 2016) that not only lead to accelerate the process of aging, but also facilitate the aging process-related diseases such as osteopenia and/or osteoporosis and consequently increases a high risk of mortality in PMWs (Cerdá Gabaroi et al., 2010). In contrast, elevated serum levels of Vit D are associated with osteoblast differentiation and bone formation (VanDriel & VanLeeuwen, 2014). In other words, since osteoblast cells have Vit D receptors

(Peppel & VanLeeuwen, 2014; VanDriel & VanLeeuwen, 2014; Wang, Zhu, DeLuca, 2014), Vit D plays a direct role on osteoblasts whose magnitude is related to the presence of other hormones (such as PTH) (VanDriel & VanLeeuwen, 2014) and other signaling molecules (such as Wnt signaling) (Woeckel et al., 2013). Further, increasing Vit D has a stimulatory effect on ALP mRNA, protein synthesis, and activity of osteoblasts in humans and/or PMWs (VanDriel & VanLeeuwen, 2014). In addition, a recent study has reported that increased Vit D level is associated with improved CRF including VO₂max in the women; although, the mechanism is unclear (Marawan, Kurbanova, & Qayyum, 2018; Marawan, Kurbanova, & Qayyum, 2019).

The findings of our study showed significant positive correlation between BMD whole body and ALP gene expression ($P=0.034$) in the EX group at week 12 while the significant negative correlation was observed between BMD and Progesterone ($P=0.010$) in the C group. ALP is an important marker for determining age-related changes in bone turnover (Zhou et al., 2013). Also, the results of studies revealed that increased ALP mRNA and Vit D, as well as elevated PTH increase bone turnover or BMD by increasing the life span and number of osteoblasts (Jilka et al., 1999; Zhou et al., 2013). It seems that 12 weeks of moderate-intensity aerobic exercise intervention exerts the mechanical stress on whole body, and as a result, ALP mRNA expression and serum ALP will increase with maintaining and/or increasing BMD whole body during menopause. In addition, significant positive correlation was observed between BMD lumbar vertebral and WJTE ($P=0.001$) in the EX group, which confirms the positive correlation between CRF and BMD in PMWs. The significant negative correlation was observed between BMD femoral neck and menopause age ($P=0.037$) in the EX group, whereas the correlations of other variables were not statistically significant. The studies have reported that due to a decline in sex hormones such as estrogen and progesterone levels in the first 5 years of menopause (Boonen et al., 2008), PMWs are four times more likely to develop aging-induced diseases such as osteopenia and osteoporosis (DeFina et al., 2016), and as a result, a rapid reduction in BMD occurs in the first 1-5 years immediately following menopause (Boonen et al., 2008) which was similar in the C group.

The current study had some limitations. We only measured ALP gene expression at the mRNA level, but not at the protein level. Also, the human volunteers as participants in our study were only healthy sedentary PMWs aged 50-60 years and consequently the findings of our study can't be generalized to the other statistical populations such as young women, girls, men, and boys.

Conclusion

The findings of present study showed that 12 weeks of moderate-intensity aerobic exercise intervention at 65%-70% HR_{max} increa-

-sed lymphocyte ALP gene expression and circulating levels of ALP and Vit D in PMWs, which was associated with CRF and numerous benefits of bone turnover. In contrast, it seems that inadequate Vit D levels, decreased PTH, and decreased estrogen and progesterone due to 12-week physically inactive lead to BMD loss during menopause, as PMWs (25%) experienced aging-induced osteopenia in the C group. In addition, it is possible that increasing in ALP mRNA is associated with elevated Vit D and PTH concentrations. These findings supply additional evidence to support the viewpoint that 12 weeks of moderate-intensity aerobic exercise intervention may have numerous benefits in bone formation such as increased ALP mRNA expression, increased Vit D serum, and increased PTH serum, as well as prevention of BMD loss in healthy sedentary PMWs aged 50-60 years.

What is already known on this subject?

Studies have examined the effects of exercise on serum markers of bone metabolism and BMD, but their results are completely contradictory.

What this study adds?

Twelve weeks of moderate-intensity aerobic exercise intervention improve bone turnover by increasing the ALP mRNA expression, serum levels of PTH, ALP, and Vit-D which can lead to the prevention of aging-induced osteopenia among PMWs.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

Ethical approval The all stages of this study were approved by the Ethics Committee at Urmia University of medical sciences with code ir.umsu.rec.1394.453 and were conducted in January 2016 at Urmia University, Iran.

Informed consent Participants signed a written informed consent form that was approved by the ethical committee.

Author contributions

Conceptualization: A.M., Z.Sh.; Methodology: B.T., M.R-Y.; Software: A. M., Z. Sh.; Validation: B.T.; Formal analysis: M.R-Y.; Investigation: B.T., M.R-Y.; Resources: A.M.; Data curation: Z.Sh.; Writing - original draft: A.M., Z.Sh.; Writing - review & editing: B.T., M.R-Y.; Visualization: B.T., M.R-Y.; Supervision: A.M., Z.Sh.; Project administration: A.M.; Funding acquisition: Z.Sh.

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