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The Effect of High- and Moderate-intensity Endurance Training on Some Anabolic/Catabolic Osteokines in Old Male Wistar Rats

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ABSTRACT

Background: Bone-related osteokines are crucial for bone function and metabolic response to physical activity. The present study aimed to shed light on the effect of different intensities of continuous and interval endurance training on the serum levels of some osteokines that are associated with wingless-related integration site (WNT) signaling pathway and Receptor Activator of Nuclear Factor (NF)-κB Ligand (RANKL) in old male Wistar rats.

Materials and Methods: A total of 24 old male Wistar rats (mean age: 23 months, mean weight: 437.93 g) were randomly assigned to three groups: interval endurance-training group (n=8), continuous endurance-training group (n=8), and control group (n=8). The continuous and interval training interventions comprised 8 weeks of treadmill exercise, 5 days a week. The continuous endurance-training group started to exercise at 60% of velocity at maximal oxygen uptake (vVO_2max) for 16 min during the first week. It continued with 70% of vVO_2max for 45 min from the fourth week onward. In contrast, the interval endurance-training group switched on exercise with 40%-80% of vVO_2max from the first week and persisted with 30%-110% of vVO_2max from the fourth week onward. As the exercise bout was completed, the enzyme-linked immunosorbent assay was applied to measure the study dependent variables. Statistical analysis was further performed using 1-way analysis of variance, considering the significance level of $P\leq0.05$.

Results: The study results demonstrated a significant difference in the levels of Oteoprotegerin (OPG) (P=0.036) and RANKL (P=0.001) in the experimental (namely, interval and continuous training) groups compared with the controls following the exercise bout. However, the level of sclerostin was not significantly changed (P=0.549).

Conclusion: High-intensity endurance training in the RANKL/OPG and WNT pathways decreased sclerostin and RANKL levels, but this decrease was significant at the RANKL level. It was effective with regard to the intensities of different types of endurance exercise.

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Introduction

Among the changes that come with aging is osteoporosis, in which the bone gets thinner and more fragile and makes people more susceptible to bone fracture [1, 2]. Bone regeneration can thus significantly contribute to maintaining bone mass and skeletal structure in adulthood and old age. In this respect, osteoclasts are in charge of decreased bone resorption, and osteoblasts are responsible for bone formation. Osteocytes (as the most abundant bone cell types) are also crucial for regulating the activities of bone formation with osteoblasts and osteoclasts [3]. With increasing age, osteoclasts become more active, and osteoblast activity is diminished, reducing bone mass [4]. So, the relationship between these types of cells in controlling bone resorption and remodeling is being investigated. Numerous biochemical and biomechanical factors can also contribute to regulating these processes [5].

Sclerostin is among the proteins secreted by osteocytes with antianabolic properties during bone formation [5]. It can be reduced by inhibiting the wingless-related integration site (WNT) signaling pathway [6]. Other Bone Marrow Stromal Cells (BMSCs) play a definite role in bone regeneration by binding to various cytokines and calcium hormones. They can be further detected among the members of the large Tumor Necrosis Factor (TNF) receptor family, including the Receptor Activator of Nuclear Factor (NF)-κB Ligand (RANKL) and Osteoprotegerin (OPG) [7]. Of note, OPG is considered a trap for RANK, secreted from osteoblasts, and can thus prevent the differentiation and secretions of osteoclasts and consequently increase their apoptosis [8]. In this sense, higher OPG levels are associated with a drop in the number of osteoclasts and a rising trend in bone strength and density in animal models. The WNT pathway also stimulates OPG production and secretion; therefore, it is a RANK antagonist.

Also, the OPG/RANK ratio is a sensitive regulator of osteoclast structure and bone resorption. Besides, RANK is a bone resorption marker, but its presence alone is insufficient to stimulate resorption and demands an OPG-related reduction [9]. Overexertion can thus stimulate osteoblasts to induce bone resorption and calcification [10]. Optimal stimulation for skeletal development is further achieved with weight-bearing exercises, affecting bone formation [11, 12].

The mechanisms by which exercise shapes bone metabolism are not yet fully understood. It seems that exercise and not getting enough physical activity can augment RANKL levels in osteocytes [13, 14]. It has been additionally confirmed that osteogenic responses induced by exercise are strong [15, 16]. Therefore, exercise in varying intensities results in different cellular responses and consequently bone adaptations [17].

Scientific research evidence shows that not all exercise regimes are equally efficient in increasing bone tissue. For instance, some practices can significantly remodel bone quality [18]. Also, exercise intensity can influence bone density, and some studies have explained the effectiveness of exercise using high-intensity training [19]. In this regard, the effect of eight weeks of continuous endurance training and interval endurance training has been investigated on the biomechanical properties of rats (aged seven weeks) bone. It was found that both types of endurance exercise had enhanced bone characteristics [20]. It has also been well confirmed that exercise-induced osteogenic responses are strongly related to intensity [21, 22]. Therefore, it is hypothesized that training in different intensities results in various cellular responses and consequently many bone adaptations [23]. However, research shows that not all training regimes are equally effective in increasing bone mass. High-intensity interval training has been more effective in physical fitness than low- to moderate-intensity exercises [24]. According to the American College of Sports Medicine, people should get involved in moderate- to vigorous-intensity aerobic exercise (viz. weight-bearing) activities to keep and boost bone quality [25]. In addition, the intensity of exercise training can be a useful factor in increasing bone density.

For instance, some studies report that only high-intensity training can influence metabolism and bone density [26] because lifting more weight and adding to bone pressure by enhancing muscle contraction can both affect bone density [27]. High-intensity exercise can further stimulate bone resorption but does not lead to bone formation. In one study, bone resorption had augmented, but bone formation had remained unresponsive after intense running in active and endurance-trained men [28]. Long-distance running has been further reported to have a positive effect on bone mass [29]. In this regard, Ziegler et al. determined the plasma concentrations of OPG and RANKL before and immediately after running 15 and 42.195 km, respectively. Accordingly, a significant decrease in circulating RANKL was observed during running in both groups of endurance runners, which could be attributed to the running distance. The rising

trend in the OPG levels had been further observed only in marathon runners. The authors had hypothesized that the positive effect of long-distance running on skeletal mass might be due to the RANKL/OPG system [30].

Given the prominent role of the OPG pathway in osteogenesis and RANKL and sclerostin and DKK1(Dickkopf WNT signaling pathway inhibitor 1) in osteoporosis, it is of utmost importance to understand how mechanical pressure can control osteoporosis to reflect better on age-related changes in the serum levels of these proteins. Therefore, the present study aimed to determine the effect of endurance training on RANKL and sclerostin levels as negative regulators and OPG as a positive one in bone formation. We hypothesized that stimulation provided by training in old age might serve as a valuable way to prevent intracellular signaling, giving rise to osteoporosis.

Materials and Methods

A total of 24 old male Wistar rats with a mean weight of 438.27 g and a mean age of 23 months were obtained from the Pasteur Institute of Iran. They were kept at a temperature of 22±3°C at 12:12 h light: dark cycle and fed with rat diet and water. In addition, all study processes followed the guidelines of the Ethics Committee for the Care and Use of Laboratory Animals at Borujerd Branch, Islamic Azad University, Borujerd, Iran. After one week of familiarity with the laboratory environment, the animals were weighed with a 2000-g digital scale (the SF-400A model) with an accuracy of 0.1 g. Based on initial weight matching, these animals were randomly assigned to three homogenous groups of eight: Control group (C), Moderate-Intensity Continuous Endurance Training (MICET), and High-Intensity Interval Endurance Training (HIIET). Of note, the control group did not receive particular interventions, and the animals were only run on a treadmill for 15 min at a speed of 2 m/min for simulation purposes, three times a week.

Determining velocity at maximal oxygen uptake (vVO_2max)

To determine the velocity at maximum oxygen uptake (vVO_2max), a running test of 10 three-minute steps was run on a rodent treadmill. According to Leandro et al. [25], the animals started with a basic running speed equal to 0.3 km/h (the slope was 0%). Then, the speed was enhanced every 3 min by 0.3 km/h. At each step of the vVO_2max test, when the animal could not complete running at that velocity, it was considered vVO_2max instantly before the animal failed to achieve the given velocity [31].

Exercise protocol

The rats were trained 5 sessions per week, for 8 weeks, at a rate similar to the VO_2max, which was presented as m/min. The animals were retested, and the exercise rate was determined based on a new test at the end of the fourth week. High-intensity and moderate-intensity endurance training regimes had three sections: warm-up, training, including high-intensity interval activities linked to high-intensity endurance-training group, and continuous training related to moderate-intensity endurance-training group and cooling (Table 1).

Blood sampling

The rats were anesthetized 72 hours after the last training session with the intraperitoneal injection of ketamine (30-50 mg/kg) and xylazine (10 mg/kg). Then, the blood samples, approximately 8 mL from each rat, were directly collected from the heart and poured into standard tubes for serum isolation. Serum separation was further performed through centrifugation at 3000 rpm for 15 min, and the isolated serums were kept at -80°C.

Statistical analysis

Levene's s test and the Shapiro-Wilk test were used to check the normal distribution of data and the equality of variances, respectively. The 1-way Analysis of Variance (ANOVA) was also used to evaluate the efficacy of the interventions concerned. These statistical tests were completed using the SPSS (ver. 21). The significance of the data differences was also considered at the level of $P \geq 0.05$.

Results

Table 2 presents the weight changes of the study Wistar rats in the study groups. The results of 1-way ANOVA indicated that the levels of OPG (P=0.036) and RANKL (P=0.001) changes were significant compared with the control group (Table 3). The post hoc test results also revealed that OPG in the continuous endurance training group significantly reduced compared with the controls (P=0.035), and RANKL in both continuous and interval endurance training groups significantly dropped compared with the controls (P=0.006, P=0.04) (Table 4).

Table 1. Continuous and interval endurance training protocol

		vVO_2max		
Activity	**Warm-Up**	**Moderate-Intensity Activity**	**High-Intensity Activity**	**Cool-Down**
	5 min	vVO_2max	Each repetition/2 min	5 min
		vVO_2max test Average 34.4 m/min	vVO_2max test Average 33.8 m/min	
First week 16 min	5 min with 40%-50% of vVO_2max	6 min with 60% of vVO_2max	Two repetitions with 80% and one repetition with 40% of vVO_2max	40%-50% of vVO_2max
Second week 24 min	5 min with 40%-50% of vVO_2max	14 min with 65%of vVO_2max	Four repetitions with 90% and three repetitions with 40% of vVO_2max	40%-50% of vVO_2max
Third week 35 min	5 min with 40%-50% of vVO_2max	22 min with 70% of vVO_2max	Six repetitions with 100% and five repetitions with 40% of vVO_2max	40%-50% of vVO_2max
Fourth week 40 min	5 min with 40%-50% of vVO_2max	30 min with 70% of vVO_2max	Eight repetitions with 110% and seven repetitions with 30% of vVO_2max	40%-50% of vVO_2max
		vVO_2max test Average 40.5 m/min	vVO_2max test Average 39.4 m/min	
Fifth to eighth week	5 min with 40%-50% of vVO_2max	22 min with 70% of vVO_2max	Eight repetitions with 100%and 7 repetitions with 40%of vVO_2max	40%-50% of vVO_2max

Discussion

Understanding how the bone senses and responds to exercise training in varying intensities has become a significant part of recent research. Changes in the duration, intensity, and repetition of exercise can thus have different effects on bone remodeling. In the present study, the mechanical load was evaluated based on 8 weeks of endurance exercise with different intensities on the levels of OPG and RANKL in old male Wistar rats to generalize the effect of mechanical load on osteogenesis. Since exercise intensity plays a leading role in the differentiation, development, and function of osteoblasts and osteoclasts [31], various intensities of endurance exercise were examined. Following 8 weeks of endurance exercise as the intervention with varying intensities, a significant difference was observed in the serum levels of sclerostin, OPG, and RANKL in the study groups.

Sclerostin and endurance training

In this study, sclerostin levels in the control group were 16.56 pmol/L, which in both training groups, sclerostin levels decreased. Sclerostin levels also increase with age, which might help increase age-related bone loss [32]. Some situations associated with bone resorption are also related to higher sclerostin levels.

Ardawi et al. further found that even a slight rise in mechanical loading could induce a significant decline in serum sclerostin levels and a growth in bone remodeling markers [33]. Spatz et al. also reiterated that bone mineral levels could significantly dwindle after 90 days of home rest, and such changes were associated with an increase in sclerostin levels [34]. Similarly, in inactive postmenopausal women due to stroke, sclerostin levels were higher than those in the controls, which coincided with a decrease in the hardness level of bone obtained by ultrasound [35]. Reflecting on athletes, Lombardi et al. reported elevated sclerostin levels in women than in men [36]. In a study of postmenopausal women, Sheng et al. similarly found higher sclerostin levels in these cases without osteoporosis than in the same group suffering from this condition [37]. Besides, Xu et al. demonstrated a significant relationship between sclerostin levels and

Table 2. Rat body weight (g)

Groups	**Mean±SD**		
	MICET	**HIIET**	**Control**
Weight before intervention (g)	447.4±24.09	432.75±48.46	445.50±24.07
Weight after four weeks (g)	438.2±21.62	422.87±49.56	452.00±30.92
Weight after eight weeks (g)	434.88±15.68	416.12±42.25	441.87±27.95

MICET: Moderate-Intensity Endurance Training; HIIET: P High-Intensity Interval Endurance Training; C: Control.

Table 3. Comparing sclerostin, OPG, and RANKL levels in the experimental and control groups after 8 weeks of intervention

Variables	Groups	Mean±SD	F	P
Sclerostin (ng/mL)	MICET	12.765±3.058	0.774	0.549
	HIIET	12.591±3.253		
	C	16.565±2.895		
DKK1 (ng/mL)	MICET	6.552±0.704	1.093	0.375
	HIIET	6.162±0.343		
	C	6.498±0.436		
OPG (ng/mL)	MICET	2.395±0.263	2.885	0.036
	HIIET	2.563±0.883		
	C	3.322±0.470		
RANKL(pg/mL)	MICET	65.954±11.925	5.883	0.001
	HIIET	72.575±17.214		
	C	101.042±27.011		
OPG/RANKL	MICET	36.31±8.23	4.26	0.43
	HIIET	35.31±6.45		
	C	32.87±9.58		

OPG: Osteoprotegerin; RANKL: Receptor Activator of Nuclear Factor (NF)-κB Ligand; MICET: Moderate-Intensity Tndurance Training; HIIET: high-Intensity Interval Tndurance Training; C: Control.

lumbar mineral density [38]. As sclerostin increases, for example, with increasing age, the mechanism of action is likely to be as follows. Sclerostin secreted by osteocytes travels to bone surfaces and then binds to the low-density lipoprotein receptor-related protein 5 and 6 corticosteroids. Next, it inhibits the WNT pathway, and osteoblastogenesis (osteoblast growth and differentiation) and bone formation are reduced [39].

However, in the present study, it seems that endurance training with moderate (viz. continuous) and high (i.e., interval) intensities diminished sclerostin levels, so such a reduction may lead to sclerostin inhibition of the WNT pathway and augment bone formation. An antibody aimed at sclerostin also lowers sclerostin levels while enhancing bone density, which is currently being investigated in a clinical trial [40]. Therefore, a sclerostin-

Table 4. Tukey's Post Hoc Test results for sclerostin, OPG, and RANKL

Variables	Groups/Comparison	Mean Difference	Standard Error	P
OPG (ng/ml)	MICET/HIIET	-0.168	0.296	0.42
	HIIET/C	0.759	0.315	0.13
	MICET/C	-0.927	0.307	0.035*
RANKL (pg/ml)	MICET/HIIET	-6.620	9.128	0.949
	HIIET/C	-28.465	9.723	0.044*
	MICET/C	-35.085	9.46	0.006*

OPG: Osteoprotegerin; RANKL: Receptor Activator of Nuclear Factor (NF)-κB Ligand; MICET: Moderate-Intensity Tndurance Training; HIIET: High-Intensity Interval Tndurance Training; C: Control.

inhibitory therapy can be exploited as a treatment to reduce bone density, which will be significantly effective if applied following exercise.

In the present study, sclerostin levels decreased by approximately 20% in the high-intensity endurance-training group. These results were consistent with the findings reported by Ardawi et al. [41]. However, in the investigation by Gombos [42] on 50 people undergoing a walking endurance program with a 50% maximum of three sessions per week, an elevation in sclerostin levels was observed. Such discrepancy might be attributed to differences in intensities and types of endurance training. At present, in moderate-intensity (viz. continuous) endurance training, 30 min of exercise with 70% maximum was done on the treadmill, with 100% maximum in the high-intensity endurance training. This difference in intensity and duration of exercise could cause an inconsistency in the present study, in line with the results reported by Gombos et al. [42]. High-intensity endurance training accordingly seems to be effective in lowering sclerostin as an inhibitor of the WNT anabolic pathway.

The study results also showed that OPG levels in high- and moderate-intensity endurance training did not alter significantly. These results were in agreement with the outcomes of the research by Karaarslan et al. [43], in which 40 older women had received a high- and low-intensity resistance-training program for 12 weeks with an increase in Bone Mineral Density (BMD) while no change in OPG levels had been observed. In addition, in another study on older women, three sessions of moderate-intensity aerobic exercise had been fulfilled every week for 8 months, with no significant changes in OPG levels, which was in line with the present study results [44].

Most studies did not demonstrate any significant changes in OPG circulation. An elevation in OPG after a long aerobic exercise program for one year in postmenopausal women [41] and after a long-distance during a marathon [45] further indicated that the effect of OPG on the protection of bone mass and its role as a mediator in mechanical loading in humans was possible. High-intensity exercise accordingly seems only to increase bone resorption and have no effect on bone formation. This rising trend in OPG emerges to be only a compensatory mechanism against increased bone resorption [46].

DKK1 and endurance training

Dickkopf WNT signaling pathway inhibitor 1 (DKK1) is an antagonist of the Wnt/β-catenin signalling pathway that acts by isolating the LRP6 co-receptor so that it cannot aid in activating the WNT signaling pathway. The result in our study showed no significant difference between the mean serum levels of DKK1 in the experimental groups and the control group. In general, a few studies have examined the effect of exercise on DKK1 levels. In particular, sports exercise with different intensities has not been considered. In the study of Jennifer Decker et al., the effect of plyometric exercise on anabolic and catabolic osteokines in girls and adolescents concluded that plyometric exercise in adolescents reduces DKK1 after 24 hours [45]. On the other hand, Kim et al. showed that a 12-week workout reduced DKK1 levels in a breast cancer patient [46]. Rahimi et al. examined the effect of 12 weeks of high-intensity interval training in 57 patients with diabetes, and the results showed that exercise reduces DKK1 levels [47]. The results of the above research are inconsistent with our study results. Perhaps the reason for this discrepancy is the type of training and intensity of training and, secondly, the age of rats in the research results. In fact, with increasing age, the estrogenic effect of mechanical load force is less effective, indicating a loss of bone sensitivity to chemical and physical signals [48-50]. This is probably one of the factors in the lack of effect of recent endurance training on the estrogenic response [51]. In addition, the relatively short duration of the training period in the present study may have been another possible reason for the lack of changes in DKK1 serum levels in rats. Most previous studies showing the effectiveness of endurance programs in the bone had a longer training period [52, 53].

Endurance training and serum levels of OPG and RANKL

The OPG/RANKL ratio has been recently shown to be crucial for bone circulation and regeneration. Numerous studies have thus far attempted to elucidate the link between bone markers and Bone Mineral Density (BMD). Previous research had further suggested that BMD could or could not be positively [54] or negatively [55] correlated with serum OPG levels [56]. Patients with low BMD have been accordingly found to have higher OPG and RANKL levels [58, 59], or a negative association between OPG/RANKL ratio and BMD has been observed [60]. This negative correlation has drawn researchers toward hypothesizing that RANKL is related to BMD and it is a reflection of osteoclastogenesis. Moreover, OPG may increase or act as a compensatory mechanism contrary to the RANKL mechanism and decrease bone resorption. In recent years, researchers have also paid particular attention to the effects of physical activity on bone-related osteokines. In this sense, Hinton et al. found no changes in OPG and RANKL levels

among skiers and athletes in the control group [11]. No significant changes had been additionally reported in postmenopausal women doing six weeks of aerobic exercise in terms of RANKL levels (walking and jogging with 60% VO_2max) [61].

In 2006, Hinton et al. performed aerobic exercises on obese men and women for six weeks at 60% of VO_2max. In this study, no changes in RANKL levels [62] had been reported, inconsistent with the changes observed in the present study. However, the study findings were in line with the research results by Mezil et al. using high-intensity exercise on bone-related cytokines in young men [56] and Kish et al. recruiting high-intensity plyometric training on the OPG/RANKL pathway in boys and young men [60]. One of the reasons for such uniformity is that the bone responds to exercise. On the other hand, proper mechanical stimulation can enhance the proliferation of osteoblasts [63]. The researchers had further concluded that weight-bearing aerobic exercise might shape the balance between bone resorption and formation during weight loss [64]. In postmenopausal women, after 8 weeks of aerobic exercise, a decline in insulin resistance and osteocalcin levels had been observed. However, no significant changes were seen in OPG levels [65]. In the present study, a decrease in OPG was reported in both high- and moderate-intensity endurance-training groups.

The OPG/RANKL ratio is a vital regulator of bone formation and resorption performance [66]. As well, RANKL indicates bone resorption, although increased RANKL levels are associated with elevated bone resorption [66, 67]. The presence of RANKL alone is sufficient to stimulate bone resorption independently [66]. For RANKL to be reabsorbed, OPG levels should be reduced [67]. Therefore, it seems that OPG changes are considered for the effectiveness of RANKL ones in bone density. Because of the significant reduction in OPG in moderate-intensity endurance training, the highest bone resorption was observed in this group. In the present study, it seems that OPG changes were significant only in the moderate-intensity endurance-training group, along with the reduction of RANKL in high- and moderate-intensity endurance-training ones. In this study, a decrease of approximately 30% in serum RANKL levels in high- and moderate-intensity endurance-training groups could confirm that such a training program inhibited RANKL-RANK as a significant biological marker in bone resorption. On the other hand, significant reductions in RANKL during endurance training increased the OPG/RANKL ratio, which can, in turn, reduce osteoclastosis (making osteoclast) and shift to the bone formation by suppressing catabolic osteokines.

Conclusion

The present study reflected on the direct effect of endurance training on bone-related osteokines. It showed that this exercise with appropriate repetitions and intensities could positively affect bone formation signaling pathways. These results were consistent with previous studies, suggesting that muscle contractions could significantly stimulate bone formation or resorption. Such changes were evident in decreased serum levels of sclerostin and RANKL following endurance training. As a whole, endurance training seems to be very effective in planning and preventing or treating osteoporosis. However, it is suggested to measure gene expression to reach accurate conclusions.

Ethical Considerations

Compliance with ethical guidelines

The Ethics Committee of Borujerd Azad University of Medical Sciences, Borujerd approved the study (Code: IR.JAU.B.REC.1396.5).

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Authors contribution's

All authors equally contributed to preparing this article.

Conflict of interest

The authors declared no conflict of interest.

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