The Effect of circuit resistance training with Medicago sativa extracts on levels of osteoprotegerin and nuclear factor of Kappa-B in thin girls

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Abstract

Introduction:
Osteoporosis is the most prevalent metabolic bone disease with multiple factors involved in its pathogenesis, including low body mass index and lack of physical activity. Changes in lifestyle represents a successful strategy to prevent osteoporosis. Therefore, this study aimed to determine the interactive effects of resistance training and Medicago sativa extracts on serum level of osteoprotegerin and nuclear factor kappa B in thin girls.

Materials and Methods:
In this quasi-experimental study, 28 female students (16 to 19 years) were divided randomly into four groups of supplement, training, training-supplement and control. Resistance training program comprised 10 exercise movements at 60%-80% of one repeated maximal in 8-12 repetition (in 3 circuits), 60 min per day and 3 sessions per week for 4 weeks. Medicago sativa supplement was taken as 1 ml/kg body weight for 3 times a day. Data were analyzed with paired t and ANOVA tests.

Results:
Four weeks of resistance training, supplementation and the combined intervention were associated with a significant increase in osteoprotegerin and decrease in nuclear factor kappa B levels (P<0.05) in thin girls. Furthermore, combined intervention was associated with greater changes in these indicators in comparison with training and supplement groups (p<0.05).

Conclusion:
Regular resistance training with consumption of Medicago sativa extracts as a non-medical therapy can increase bone formation markers and also reduce the activity of the absorbency pathway in thin adolescent girls. Of course the combination of exercise and supplement are more effective than the separate affects of eachs them.

Keywords: Resistance Training, Medicago Sativa, Osteoprotegerin, Nuclear Factor Kappa B, Thinness

Introduction

Osteoporosis is a silent, asymptomatic and the most common metabolic bone disease. Today, osteoporosis is a serious health problem in most countries. According to studies, about 2.5 million women are at risk of severe osteoporosis and related...
fractures (1). Moreover, underweight and wasting are as risky as obesity for human health (2). That is, the risk of osteoporosis in people with low body mass index is higher (3). According to global statistics, young people comprise 20% of the world population (4). Given that nearly 90% of adults’ bone mass is formed before age 20, identifying variables affecting bone health in an adolescent is of great importance (5, 6). Osteoporosis starts in early adolescence and bone formation is more than bone destruction from childhood to the twenties. After the age of 30, this condition reverses for unknown reasons and bone destruction exceeds bone formation (7). Despite the prevalence of the disease among postmenopausal women, many women and young girls are now at risk due to insufficient physical activity, poor nutrition and lack of awareness of a proper lifestyle. Based on previous studies, both overweight and underweight are important risk factors for osteoporosis in young girls (8). Although the exact mechanism of bone destruction is not well known (9), a new molecular system related to the tumor necrosis factor superfamily has been identified that is involved in the regulation of bone destruction, which consists of three key proteins of receptor activator of nuclear factor kappa-B ligand (RANKL), receptor activator of nuclear factor kappa-B (RANK), and Osteoprotegerin (OPG) (10-13). OPG is a glycoprotein in the tumor necrosis factor receptor superfamily-alpha (TNFRSF). This glycoprotein along with RANKL and RANK form a molecular triad (OPG/RANK/RANKL) that regulate bone metabolism through osteoclasts control. On the other hand, RANKL binds with RANK and strengthens osteoclastogenesis and bone resorption. OPG binds with RANKL and neutralizes it, and thereby prevents bone resorption (14) and inhibits the interactions of RANKL leading to a reduction in osteoclastogenesis (15). The nuclear factor kappa-B (NF-kB) is a key signaling pathway involved in the early stages of RANKL-induced osteoclast differentiation (16), known as an evolutionary family that protects the transcription factors involved in responses to environmental changes (17).

Today, doing physical activity is recommended to prevent osteoporosis and its metabolic problems. Bone metabolism, especially bone formation markers increase with sports activities. In fact, physical activity is recommended to maintain homeostasis, bone density and also to avoid bone minerals reduction (18). During the growth, skeletal adaptation occurs with exercise and the weight-bearing resulted from resistance exercises may help with skeletal system strength, peak bone mass acquisition and thus increased bone density (19). Studies show that exercise has long-term benefits for bone markers and especially bone formation markers, such that denser bones have been reported in young athletic women who had physical activity in childhood and youth compared to sedentary women (20). However, the type of exercise affects bone density and structure. Sports with more contact and pressure have a greater influence on the osteogenic responses in comparison with aerobic exercises (21). Furthermore, regarding the impact of exercise intensity on bone formation markers compared to its duration, exercises that need weight-bearing and high-impact exercises are preferred to exercises with longer duration (22).
In addition, the results of a review study showed that combining exercises with medical supplements such as vitamins D or calcium increases bone mass density (23). Therefore, using some medicinal herbs along with exercise might be effective in sufficient calcium absorption, eliminating malnutrition, and preventing bone diseases in thin girls (24). Alfalfa, scientifically known as *Medicago sativa*, is one of the most famous traditional herbal medicine rich in vitamins A, C, E, K, and amino acids (25). It may have positive effects on the skeletal system's ability to resist fracture and to increase osteoblastic bone markers. Researchers have shown that the inclusion of 10% to 50% alfalfa powder in the diet can lead to increased absorption of calcium and positive effects on bones (26). However, no study was found on the effect of its extract on confounding markers in bone metabolism. In most studies, prevention and treatment of osteoporosis were examined in postmenopausal and middle-aged women, but today it is known that osteoporosis is not just a problem for postmenopausal women and, many young women, especially thin women are at risk for different reasons. Hence, it appears that control of the disease throughout life with the aim of having optimal bone density at a young age and reducing osteoporosis risk in later life is essential. Therefore, the present study was conducted to investigate the effects of Circular Strength Training (CST) and the consumption of alfalfa extract on NF-κB and OPG concentration in thin young girls.

**Materials and Methods**

In this quasi-experimental study, the population consisted of 16-19-year-old non-athlete thin female students with low BMI (about 18 kg/m²) in girl high schools in the urban area of Babol, Iran. Among the volunteers, 28 students were selected by cluster random sampling. They were enrolled after physical health confirmation through medical examinations, and obtaining personal and parental consents. The subjects were allowed to leave the study at any stage of the study at their will. The subjects were randomly divided into four groups of control, resistance training, alfalfa supplement, and training-supplement (combined) groups (n=7). The height and weight measurements were performed before the study.

**Resistance training program**

A week before training, participants became familiar with the environment and one repetition maximum strength (1RM) (the maximum weight that a muscle or group of muscles can lift only once) was determined for the exercises (except sit-ups) by trial and error. The exercise protocol included four weeks of CST, three times a week, 60 minutes per session, with an intensity of 60% 1RM in the first week and gradually increasing to 80% 1RM in the fourth week. Polyarticular exercises at various sets including chest press, sit-ups with bent knees, leg press, hamstring stretch, knee crunching, lateral pull, overhead press, triceps exercise with dumbbells and half squats, with 8 to 12 reps in each set in three circles with 60-second rest intervals between sets and 2-3 minutes between the circles (25).

**Alfalfa supplement**

After collecting and drying aerial parts of alfalfa, its extract was prepared using conventional methods. After filtering the extract, honey was used at a ratio of 1 to 3 to improve its taste. The amount of extract
consumption was 1 ml per kilogram of body weight, 3 times a day (10 minutes after a meal) (25). During the study, participants were asked to record any food they consumed during the day for three days in a food recall questionnaire. Accordingly, the daily calorie intake was calculated for each subject. They were also advised to follow their normal diets during the study period (especially before blood sampling).

Blood sampling and biochemical analysis
Blood sampling was performed before and after the intervention, after 12 hours of overnight fasting (with a light dinner the night before sampling), and 48 hours of no supplements or resistance training, from 8:00 am to 10:00 am, after half an hour of rest in the laboratory. For this purpose, 7 ml of blood was taken from the left brachial vein of participants who were in the luteal phase after ovulation. After separation, blood plasma was frozen at -80 °C to measure and analyze the concentrations of OPG and NF-κB. The amount of OPG was measured using Elisa technique and Cusabio a Chinese made kit, and the NF-κB was measured using Cayman commercial kit Made in the USA.

Statistical analysis
The Kolmogorov–Smirnov was used for determining the normal distribution of data, the Levene's test for determining the homogeneity of variances, the paired t-test for examining the changes within the group, and the one-way ANOVA test for comparing the mean differences of variables in the groups before and after the interventions. The Tukey's post hoc test was used to determine the differences. The significance level of the tests was considered at P<0.05. Data was analyzed in SPSS software version 16.

Results
Table 1 shows anthropometric characteristics of the subjects before the intervention. ANOVA test results showed no significant differences in anthropometric characteristics of the subjects (Table 1). In addition, OPG plasma concentration increased significantly after 4 weeks of supplementation (22.5 percent), resistance training (22.2%) and the combination of these two interventions (41.2%) (P<0.001). Meanwhile, OPG plasma concentrations were significantly higher than the control group (Table 2). The Tukey’s test showed that the effect of the combined intervention on increased OPG was significantly higher than the other two interventions (P<0.05). The four weeks of resistance training, supplementation, and the combination of the two resulted in a significant decrease in plasma concentration of NF-κB in thin girls (16.7%, 17.28%, and 33.9%, respectively, P<0.001), which were significant compared to the control group, too (P<0.05) (Table 2). Also there was a significant difference in the amount of NF-κB between the supplement and compound groups (P<0.05).
Table 1: The subjects’ BMI

<table>
<thead>
<tr>
<th>Variable</th>
<th>Supplement</th>
<th>Training</th>
<th>Training-Supplement</th>
<th>Control</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (years)</td>
<td>16.7±7.7</td>
<td>16.9±1.3</td>
<td>16.1±1.0</td>
<td>16.4±9.4</td>
<td>0.898</td>
</tr>
<tr>
<td>Weight (kg)</td>
<td>49.3±4.6</td>
<td>43.6±5.6</td>
<td>44.3±8.5</td>
<td>43.5±6.6</td>
<td>0.458</td>
</tr>
<tr>
<td>Height (cm)</td>
<td>164.4±4.4</td>
<td>161.2±6.6</td>
<td>161.6±6.4</td>
<td>161.6±2.6</td>
<td>0.659</td>
</tr>
</tbody>
</table>

Data is expressed as mean±SD.

Table 2: The mean and SD of the group variables before and 4 weeks after the interventions

<table>
<thead>
<tr>
<th>Variable</th>
<th>Groups</th>
<th>Before intervention</th>
<th>After intervention</th>
<th>P-Value</th>
<th>P-Value</th>
<th>P-Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>OPG (ng/ml)</td>
<td>Control</td>
<td>3.0±9.31</td>
<td>3.0±91.38</td>
<td>0.829</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Training</td>
<td>3.0±0.36</td>
<td>3.86±0.34</td>
<td>&lt;0.001*</td>
<td>&lt;0.001**</td>
<td>0.843</td>
</tr>
<tr>
<td></td>
<td>Supplement</td>
<td>3.0±09.32</td>
<td>3.99±0.42</td>
<td>&lt;0.001*</td>
<td>&lt;0.001**</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Training + Supplement</td>
<td>2.87±0.38</td>
<td>4.03±0.04</td>
<td>&lt;0.001*</td>
<td></td>
<td></td>
</tr>
<tr>
<td>NF-κB ng/ml</td>
<td>Control</td>
<td>8.0±0.03</td>
<td>8.11±0.34</td>
<td>0.156</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Training</td>
<td>8.03±0.37</td>
<td>6.69±0.58</td>
<td>&lt;0.001*</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Supplement</td>
<td>8.10±0.33</td>
<td>6.70±0.47</td>
<td>&lt;0.001*</td>
<td>&lt;0.001**</td>
<td>0.624</td>
</tr>
<tr>
<td></td>
<td>Training + Supplement</td>
<td>8.23±0.39</td>
<td>5.44±0.37</td>
<td>&lt;0.001*</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

* Significance of intragroup changes between before and after the intervention (using paired t-test)
** Significance of inter-group changes after the intervention (using ANOVA)
*** Significance of inter-group changes before the intervention (using ANOVA)

Discussion

Although many studies have focused on obesity, untreated weight loss and wasting provide for the development of some chronic diseases, including osteoporosis (27, 28, 8). The present study was conducted to investigate the interactive effects of resistance training and alfalfa extract on NF-κB and OPG plasma concentrations in young thin girls. According to the results, four weeks of CST, alfalfa extract supplementation, and a combination of both were associated with a significant increase in the OPG concentration in thin girls. Similarly, Bergstrom et al. reported a significant increase in OPG concentrations after a period of aerobic training for two times a week, 30 minutes of brisk walking per session in postmenopausal women (29). West et al. also suggested that OPF in sedentary girls was lower than athletic girls with and without regular menstrual cycle (13). Bone formation markers such as OPG are more sensitive than bone resorption markers. Osteoblasts and osteoclasts activities stimulation are related to sports and physical activity which are good sources for bone turnover and prevention of osteoporosis and bone metabolism problems such that changes in bone markers were more obvious after a few hours or a few days of exercise (30). However, Marcus et al. observed no changes in the OPG and RANKL levels in elderly women after eight months of resistance or aerobic training (31). This discrepancy might be due to subjects’ older age and overweight. Although the exact mechanisms of increased concentrations of OPG following exercise are not well known, studies have shown that mechanical stimuli such as...

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hemodynamic shear stress led to the inhibition of osteoclastogenesis induction through up-regulation of OPG and down-regulation of RANKL (32). In addition, mechanical stimulation through the deformation of the substrate significantly increased the amount of soluble OPG by bone-building cells (33). Although in this study the concentration of RANKL was not measured, which could be considered a limitation of this study, it is well known that RANKL stimulates its particular receptor located in dendritic cells and osteoclasts, and one of its signaling pathways is NF-κB (34).

Another finding of the research was the reduction of NF-κB following the interventions, indicating the activity of osteoclastic cells and thus reflecting the physiological adaptation resulted from resistance training in young thin girls. Results from various studies indicate that after long-term training and sports competitions, bone formation markers such as alkaline phosphatase had changes in sedentary individuals participating in a physical activity program. Professional athletes also showed changes in bone formation markers, which depend on the program intensity, while bone resorption markers remained constant (35). Also, recent studies suggested that exercise can prevent the osteoporosis process by reducing NF-κB. In this regard, Schenk et al. showed that a course of physical activity prevented NF-κB pathway activity in humans (36). These results were largely incompatible with Vella et al. findings. They stated that intense resistance training increased the amount of NF-κB phosphorylated protein for 2 hours after exercise, which returned to the basic level four hours after the exercise (37). On the other hand, Rivas et al. demonstrated that intense exercise in the elderly was associated with a 60% increase in phosphorylation of pro-inflammatory transcription factor of NF-κB, while the rate of increase was lower in young people (38). Meanwhile, some studies showed that high-intensity exercise increased the activity of NF-κB, and increasing osteoclastic activity will cause osteoporosis. Available reports suggest that an increase, decrease or no changes in the amount of NF-κB is expected depending on the type and intensity of physical activity, readiness, early state of NF-κB plasma level of the participants, and their adaptability to the exercises (16).

Although studies on the effects of alfalfa on the examined variables in human subjects are very limited, animal studies have well shown the medical effects of alfalfa (39). Walker et al. demonstrated that twice a day consumption of alfalfa powder with a proportion of 2.27 Kg of daily food intake increased the concentration of calcium, phosphorus, and alkaline phosphatase in pigs (40). Another study showed that alfalfa powder reduced osteoclastogenesis activity in bone marrow of pregnant pigs and can be used as a calcium-rich nutrient for them (27). Alfalfa might have positive effects on the skeletal system's ability to resist fracture and to increase osteoblastic bone markers. Alfalfa inhibits bone resorption by osteoclasts, so it can reduce bone resorption by preventing the proliferation, differentiation and activity of osteoclasts and increasing their apoptosis (25). In this regard, Shi et al. showed that serum levels of triglycerides, LDL-C and total cholesterol significantly reduced in mice fed with alfalfa, while the HDL-C had a significant increase (40). Al-Dosari (2012) found that administration of alfalfa extract...
for three weeks can significantly reduce the serum levels of liver markers and oxidative stress in carbon tetrachloride intoxicated rats. The histopathological examination of liver tissue of these rats showed that alfalfa extract can improve liver damages caused by exposure to oxidants (41).

In this study, the concomitant use of alfalfa supplement was associated with a further intensification of anti-osteoclastogenesis properties of resistance training. Although the mechanisms by which alfalfa supplements can affect levels of pre- and anti-osteoporotic markers are not well known yet (26), it seems that alfalfa supplement intensifies the osteoblastic effects of resistance training in thin girls due to its vitamins (25). Previous studies have shown that antioxidants can adjust the osteoclastogenesis effect and play an important role in regulating bone metabolism homeostasis by down-regulating the expression of the transcription factor of NF-κB and up-regulating OPG (39). Although the present study did not examine the markers changes related to bone homeostasis like oxidative and inflammatory agents in the studied groups, it can be suggested that oxidative stress is one of the main possible pathways of increasing OPG levels and reducing NF-κB resulted from the resistance training plus alfalfa supplementation intervention in this study.

Conclusion
In general, the results of this study indicated that although regular resistance training and alfalfa extract consumption can separately and as non-medical therapies increase bone formation markers and decrease bone resorption path activity in young thin girls, the effectiveness of the combination of exercise and supplementation is far more than the separate effects of each of these two therapeutic methods.

Acknowledgments
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Conflict of interest
The authors declare no conflicts of interest.

References:
The Effect of circuit resistance training

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