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The Effect of Eight Weeks of Moderate Intensity Continuous Training and High Intensity Interval Training along with Citrus Aurantium on Bone Metabolic Markers in Elderly Female Rats

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ABSTRACT

Introduction: Controlling nutrition and exercise can affect the density and metabolism of bone tissue. Therefore, the aim of the present study was to investigate the effect of eight weeks of moderate intensity continuous training (MICT) and high intensity interval training (HIIT) along with Citrus aurantium (CA) on bone metabolic markers in elderly female rats.

Methods: In this experimental study, 64 elderly female rats (14 ± 2 months old and weight of 290 ± 25 grams) were divided into 7 groups including 1) control, 2) MICT, 3) HIIT, 4) MICT+CA, 5) HIIT+CA, 6) CA and 7) sham (normal saline) groups. HIIT with an intensity of 85- 110% VO_{2max} and MICT with an intensity of 65% VO_{2max} were performed and CA was injected at the doses of 300 mg/kg/day intraperitoneally. The variables were measured using the ELISA method with the Pars Azmoun kit. The data of the present research were analyzed using one-way analysis of variance and Tukey post hoc test (P≤0.05).

Results: In MICT group the PTH and Na levels were significantly lower and Ca levels were higher than the C group (P≤0.05). PTH and Na levels in the MICT group were lower than the HIIT group (P≤0.05). Ca levels in the CA group were higher than the C group (P≤0.05). In MICT+CA and HIIT+CA groups, PTH levels were lower and Ca levels were higher than the C group (P≤0.05). Also, PTH and Na levels in HIIT+CA group were lower than the MICT+CA group (P≤0.05).

Conclusion: it seems that MICT has a better effect on bone metabolic markers than HIIT; meanwhile, HIIT with an antioxidant such as CA has more favorable effect on bone metabolic markers.

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Introduction

With the increase in life expectancy, the prevalence of chronic diseases related to aging has increased and caused muscle function disorders and decreased quality of life in this part of the population. Many of the changes that occur with aging are gradual and irreversible (1). Aging leads to many physiological changes, which changes in body composition (muscle tissue, fat tissue, and bone tissue) are more obvious (2,3). In other words, with increasing age and decreasing the synthesis of metabolic hormones, especially estrogen hormone, bone density and mass decrease, and this, along with muscle weakness, is associated with a decrease in quality of life (4). Researchers have shown that the increase in oxidative stress indicates the inability of the antioxidant system to deal with metabolic disorders. Therefore, in the bone tissue, the increase of free radicals is caused by the disruption of bone absorption and re-absorption (5). In addition to this, aging leads to disturbances in the secretion of parathormone hormone (PTH) and calcitonin, disturbances in the bone metabolism of calcium (Ca), phosphorus, sodium (Na) and potassium (K) in the bone tissue; and finally this happens with a decrease in regeneration of bone cells (6,7).

On the other hand, many studies have shown that regular and long-term exercise have favorable effects on the health and quality in life of the elderly people. Researchers have shown that exercises have a favorable effect on anabolic hormones, improve estrogen hormone secretion, improve PTH and finally improve calcium re-absorption in bone cells (7). In this context, a

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study showed that 16 weeks of aerobic training with an intensity of 65- 70% of the maximum heart rate significantly decreased PTH and increased calcitonin in middle-aged women with osteopenia (7). Also, in a study, researchers showed that eight weeks of combined training increased PTH and alkaline phosphatase (ALP) in postmenopausal women (8). In a study, the results showed that the level of physical activity was directly related to PTH; So that regular physical activity led to the improvement of calcium-phosphorus metabolism by improving PTH (9). In another study, researchers showed that regular resistance training with an intensity of 30% of the maximum repetition led to a decrease in PTH immediately after training. In addition, PTH levels were still low up to 15 minutes after training and returned to normal within 30 minutes (10). But in relation to the comparison of the type of exercise, the researchers pointed out that high intensity interval training (HIIT) and moderate intensity continuous training (MICT) both led to an increase in the mineral mass of inactive young women; But compared to MICT, only HIIT increased calcaneus stiffness index and vitamin D (11).

Studies show that although physical activities have an effect on bone metabolism, the results of studies indicate differences in the effect, type and intensity of exercise on bone metabolism. Due to the importance of nutrition along with physical activities, researchers have recently drawn attention to the use of medicinal plants along with exercise on people's health. Among the medicinal plants, Citrus aurantium (CA) includes the blossoms of the orange tree. The used parts of orange tree include the flower (blossom), fruit peel and orange juice; which all of them have nutritional and medicinal properties. CAs are white, fragrant, crisp and fragile and are placed singly or in pairs along the axis of the branches (12). CA (it grows in Iran), contains small amounts of alkaloids such as synephrine and octopamine, which act as direct and indirect sympathomimetic agonists (13). It was also reported in a study that the consumption of CA and its family, such as lemon, with its high synephrine, improved the mass of minerals, improved metabolic proteins, and improved blood supply to bones in postmenopausal women (14). In addition, hesperidin and naringenin, as two constituents of CA (15), directly inhibit RANKL and other inflammatory factors in bone tissue, leading to the improvement of the function of osteocalcin and alkaline phosphatase proteins (15). In this context, the study of *Yazdanpatast et al.,* (2018) showed that the consumption of naringenin leads to an increase in bone anabolic, and its use in ovariectomized laboratory rats led to the improvement of parathyroid hormone, the increase in the expression of estrogen receptors in bone tissue (16).

Considering the limited information regarding the type, intensity and duration of optimal exercise on bone metabolism in the old age and the lack of information regarding the simultaneous use of CA along with various types of exercises, it seems that a study should be conducted by examining the variety of exercise along with antioxidants such as CA provide more information in this field to the researchers. In addition, it seems that in old age, performing high-intensity exercises is limited and accompanied by possible risks. In addition, considering the need for the importance of diet in the results of studies, it seems important to conduct animal studies that can have the most control over disturbing interventions. Also, according to the evidence, it seems that finding the best type of exercise and using available herbal antioxidants is one of the main questions for researchers to prevent and treat metabolic diseases, especially in the elderly. Hence the present study aimed to investigate the effect of eight weeks of MICT and HIIT with CA on bone metabolic markers in elderly female rats.

Methods

Animal Caring

In this experimental study, 64 elderly female rats with 14 ± 2 months old and weight of 290 ± 25 grams were purchased and kept for one week in the Sports Physiology Laboratory of Islamic Azad University, Marvdasht Branch. During the entire research period, the standard principles of working with animals, including light, temperature, water, food, and humidity, were carefully observed. Then rats were randomly assigned to 7 groups including 1) control, 2) MICT, 3) HIIT, 4) MICT+CA, 5) HIIT+CA, 6) CA and 7) sham (normal saline) (Sh) groups. It is worth noting that all the ethical principles of working with laboratory animals in this study were observed according to the Helsinki

Convention. It is worth mentioning that the ethical principles of working with laboratory animals in this research were carried out under the supervision of the ethics committee of the Islamic Azad University, Najafabad branch, with the approved code IR.IAU.NAJAFABAD.REC.1401.014.

Training Protocols

After obtaining the average maximum speed (for evaluate the Vo_{2max}), the rats were scheduled for performing eight weeks of MICT and HIIT. In MICT and HIIT at the beginning of each session, the warm-up phase consisted of running for 3 minutes with an intensity of 10 meters per minute, then HIIT groups performed trainings with an intensity of 85- 90% VO_{2max}, which is equivalent to 7 intervals (each interval 1 minute) and a speed of 31 m/min and active rest between intervals with 6 intervals and a speed of 15 m/min was done in the first week, which was gradually increased by an average of 2 m/min per week to 10 interval (each interval 1 minute) with a speed of 55 m/min and active rest was achieved with 9 intervals (each interval 1 minute) at a speed of 25 m/min in the eighth week. MICT with a medium intensity of 65% VO_{2max} (which is equivalent to a speed of 20 m/min and a time of 15 minutes) started in the first week, which gradually reached a speed of 25 m/min and a time of 31 minutes in the eighth week. The training started with warming up for 3 minutes with an intensity of 10 m/min and 2 minutes with an intensity of 15 m and cooling down for 1 minute with an intensity of 15 m/min and ended with 2 minutes with an intensity of 10 m/min (17).

Preparation and Consumption of Citrus Aurantium

50 grams of Citrus aurantium powder which prepared from Jihad Agricultural Center of Marvdasht city was added to a balloon that contained 500 ml of distilled water, it is worth mentioning that the laboratory balloon was connected to the Cloninger device and the process of extracting the extract using it for four hours. At the end, the collected extract was dehydrated using sodium sulfate, which did not contain water, and the prepared extract remained at -20 temperature until use. 300 mg per kilogram of the prepared extract was mixed with normal saline daily and injected intra-peritoneal to rats (18).

Blood Sampling Method

Forty- eight hours after the last training session and CA consumption, the rats were anesthetized with ketamine and xylosin after 12 hours fasting, and blood samples were gathered directly from the heart of the rats. After blood collection, 5 cc of blood was poured into the falcon's nostrils. Blood samples without EDTA were kept in the laboratory environment for 40 minutes and then centrifuged at 3000 rpm for 15 minutes to prepare serum.

The Method of Measuring Variables

Phosphorus (mg/dl), PT (pg/ml), Na (mEq/L), potassium (mEq/L) and calcium (mg/dl) were measured by enzyme method and using Pars Azmoun commercial kits made by Iran.

Statistical Analysis Method

Shapiro-Wilk test was used to check the normality of data distribution. Then, one-way analysis of variance test was used to check the difference between groups and Tukey's *post- hoc* test was used to determine the location of the difference between the groups. The data of this research were analyzed in SPSS software version 19 ($P<0.05$).

Results

Mean and standard deviation levels of research variables are presented in figure 1- 5. The results of one-way analysis of variance showed that there are no significant differences in the levels of phosphorus ($P=0.70$ and $F=0.62$) and potassium ($P=0.097$ and $F=1.96$) between research groups. However, there were significant differences in the levels of PTH ($P=0.001$ and $F=52.55$), Na ($P=0.001$ and $F=7.20$) and Ca ($P=0.001$ and $F=8.53$) between research groups.

Tukey's *post- hoc* test results showed that there were no significant differences in PTH levels in the control and sham groups ($P=0.97$), but in MICT ($P=0.001$), MICT+CA ($P=0.001$), and HIIT+CA ($P=0.001$) groups were lower than control group also in MICT ($P=0.001$), MICT+CA ($P=0.001$) and HIIT+CA ($P=0.001$) groups were lower than CA group. In addition, in MICT ($P=0.001$), MICT+CA ($P=0.001$) and HIIT+CA ($P=0.001$) groups were lower than HIIT group as well as in HIIT+CA group were lower than MICT+CA group ($P=0.017$) (Figure 1).

Na levels in the control and sham groups were not significantly different ($P=0.10$). But Na levels in the MICT ($P=0.008$) and HIIT+CA ($P=0.02$)

groups were significantly lower than the control group also in MICT (P=0.002) and HIIT+CA (P=0.007) groups were lower than CA group, in MICT (P=0.004) and HIIT+CA (P=0.012) groups were lower than HIIT group, in MICT group were lower than MICT+CA group (P=0.016) as well as in HIIT+CA group were lower than MICT+CA group (P=0.04) (Figure 2).

Ca levels in the control and sham groups were not significantly different (P=0.98). But in CA (P=0.03), MICT (P=0.001), HIIT+CA (P=0.002) and MICT+CA (P=0.001) groups were higher than control group also in MICT (P=0.001), HIIT+CA (P=0.016) and MICT+CA (P=0.005) groups were higher than sham group (Figure 3).

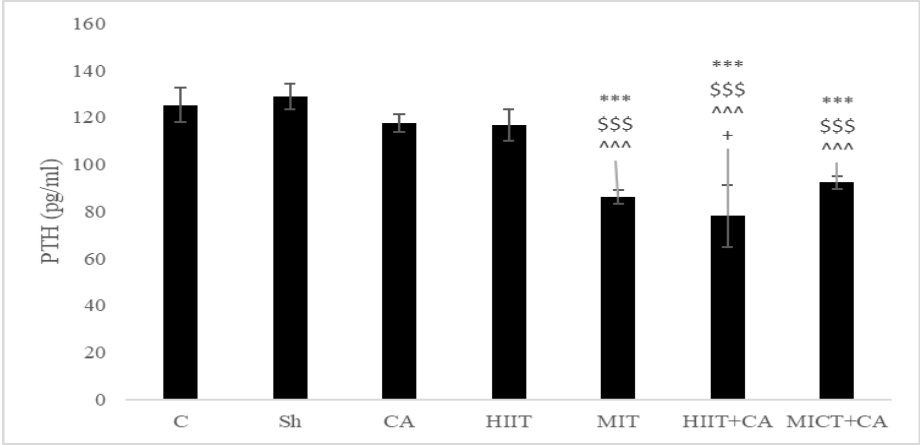

Figure 1. PTH levels in research groups
*** (P≤0.001) significant decrease compared to the control group
$$$ (P≤0.001) significant decrease compared to CA group
^^^ (P≤0.001) significant decrease compared to the HIIT group
+ (P≤0.05) significant decrease compared to the MICT+CA group

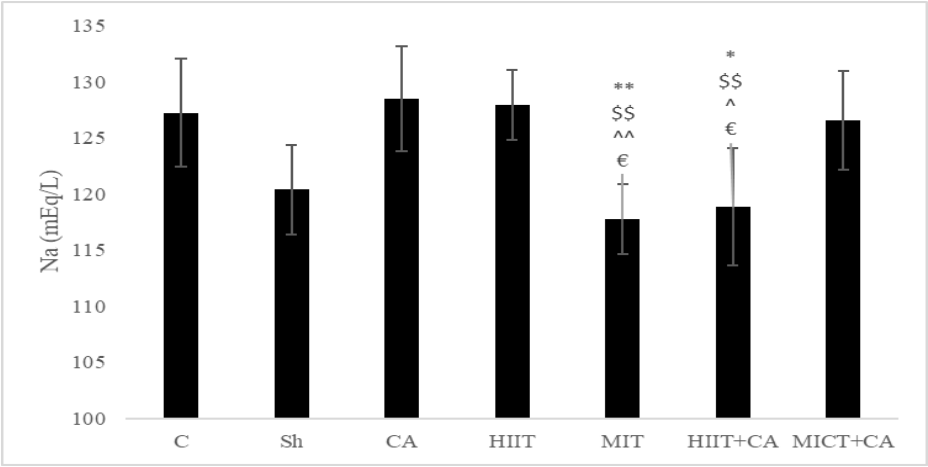

Figure 2. Na levels in research groups
* (P≤0.05) and ** (P≤0.01) significant decrease compared to the control group
$$ (P≤0.01) significant decrease compared to CA group
^ (P≤0.05) and ^^ (P≤0.01) significant reduction compared to HIIT group
€ (P≤0.05) significant decrease compared to MICT+CA group

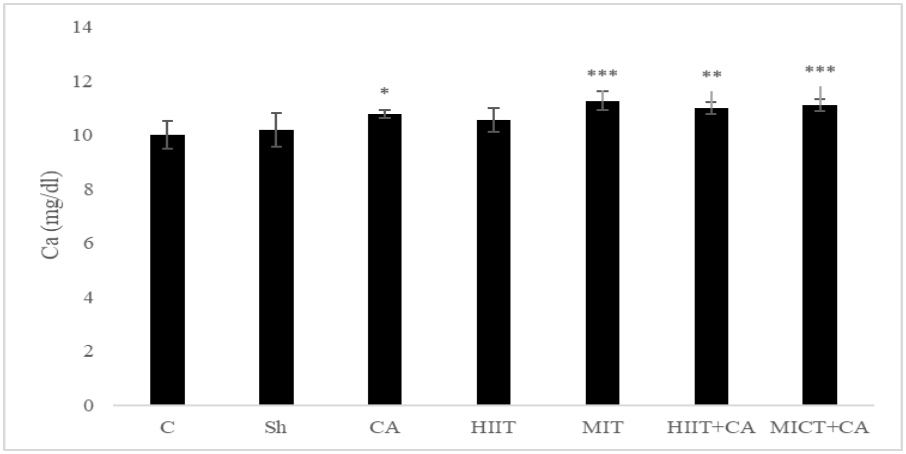

Figure 3. Ca levels in research groups
* (P≤0.05), ** (P≤0.01) and *** (P≤0.01) significant decrease compared to the control group

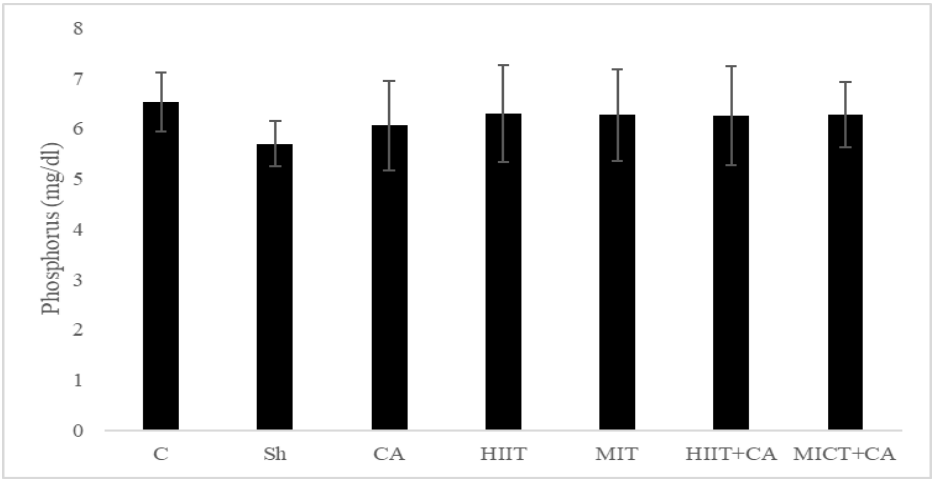

Figure 4. Phosphorus levels in research groups

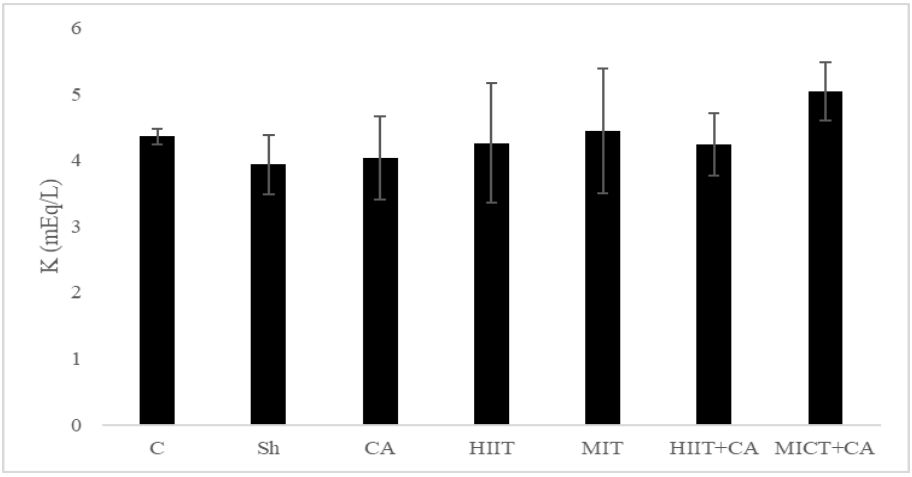

Figure 5. Potassium levels in research groups

Table 1. The protocol of HIIT and LICT exercises

Cooling down	Stage Main training	Warming up	Kind of training
Three minutes of running at a speed of 10 m/min	**First week** 7 one-minute intervals with high intensity with an intensity equal to 85 to 90% of the maximum running speed (speed 31 meters per minute) and 6 intervals with low intensity with an intensity equal to 50 to 55% of the maximum speed and a speed of 15 meters per minute **Second to eighth week** Increasing the speed by 2 meters per minute weekly and also increasing the number of repetitions from 7 repetitions with high intensity (speed 55 meters per minute) in the first week to 10 repetitions with high intensity and 9 repetitions with low intensity (25 meters per minute) in eighth week.	Three minutes of running at a speed of 10 m/min.	HIIT
Three minutes of running at a speed of 10 m/min.	**First week** Running with an intensity of 65% of the maximum running speed (equivalent to 20 meters per minute) and a duration of 15 minutes **Second to eighth week** The gradual increase in speed from the second to the eighth week to 25 meters per minute and the training time reaching 31 minutes in the eighth week.	Three minutes of running at a speed of 10 m/min.	LICT

Discussion

The results of the present study showed that in MICT group the PTH and Na levels were lower and Ca levels were higher than the control group. Also, PTH and Na levels in the MICT group were lower than the HIIT group. But HIIT had no significant effect on the changes of PTH, Na, Ca, phosphorus and potassium in elderly female rats. Studies show that physical activities with a mechanical pressure mechanism lead to the activation of pressure-sensitive receptors and thus lead to the activation of osteoblasts. In addition, regular and long-term physical activities increase anabolic hormones such as estrogen and progesterone with the mechanism of increasing muscle mass, and in this way, they are effective in bone absorption and re-absorption. One of the most important mechanisms of exercise is the parathormone hormone; which can lead to the activation of AMPK by activating the pathway of phosphatidylinositol 3-kinase (PI3), protein kinase C, phospholipase C, and protein kinase A and finally activates osteoblasts. In addition, the parathormone hormone becomes the main responsible for regulating intracellular and extracellular calcium as a main element in regulating metabolism and bone mineralization (19). It is believed that regular aerobic exercise can increase antioxidants and reduce oxidative stress in menopause (20).

Despite, studies show that the effectiveness of parathormone on exercise is depends on the intensity, type and time of training sessions. So that the researchers stated that exercises with high intensity and duration of more than 50 minutes lead to an increase in parathormone (21). While in another study, the results showed that PTH and alkaline phosphatase levels increased in the training group compared to the control group, while calcium and phosphate levels in the training group were not significantly different from the control group (19). However, in a study consistent with the results of the present study, 10 weeks of aerobic training with an intensity of 45- 60% heart rate increased the osteocalcin and decreased PTH in women with type 2 diabetes (22).

Researchers believed that parathormone and its changes are related to weight, fat percentage and vitamin D levels. In other words, the increase of parathormone in the conditions of metabolic disorder can be associated with the breakdown of vitamin D in adipose tissue and its reduction. Therefore, the increase of PTH and fat can lead to disruption of calcium re- absorption by the tubule system and reduction of ionized calcium through the binding of protein to calcium (22). It is noteworthy that in the present study, MICT increased serum calcium levels with PTH reduction. In addition, the effect of MICT on these two indicators was greater than HIIT.

The results showed that CA increased Ca levels in aged female rats, but had no significant effect on PTH, Na, phosphorus and potassium levels. Previous studies have shown that citrus constituents in flowers, fruits, peels and seeds have strong antioxidant effects. In other words, citrus ingredients with their antioxidant effects

lead to the activation of biological pathways such as AMPK, and in bone tissue, these phenols improve the functioning of the Wnt/bCatenin signaling pathway, activate sirtuins, and mitochondrial biogenesis and finally are associated with improving the function of osteoblasts (23,24). In this context, researchers showed that orange flower increased antioxidants, reduced oxidative stress, increased mineral mass and improved bone strength in a male animal model of osteoporosis (23). Also, in a study, researchers showed that 11 months of consuming lemon drink in a daily amount of 290 ml improved bone alkaline phosphatase, increased bone mineral mass, osteocalcin, and improved calcium re- absorption in postmenopausal women (13).

Studies have shown that CA and its constituents, due to the presence of synephrine and other phenols, can lead to an increase in the activity of deacetylases and are associated with the calmodulin kinase mechanism in improving calcium-related signaling in skeletal and cardiac muscle (25). Despite conducting studies in this field, the mechanism of CA on bone biology markers is still not well known, but it seems that the difference in the dosage and raw materials of this plant species are the reasons for the difference in the results. As the researchers have pointed out that long-term consumption of CA cannot have a significant effect on weight loss and body composition improvement; In addition, long-term use with doses higher than 6.37 mmHg even increases systolic and diastolic pressure (26).

In this context, the researchers showed that the amount of 4 x 10(-3)-1 x 10(-1) g/ml of CA leads to an increase in L-type calcium currents in the muscles of guinea pigs; But 2 x 10(-1)-4 x 10(-1) leads to inhibition of L-type calcium channels (25). Limited information is available regarding the effect of CA on metabolic markers, but considering the relationship between metabolic disorders and the acceleration of osteoporosis. Studies have shown that 50 and 100 µl/kg CA extract have a reducing effect on LDL, cholesterol, and TG; while the dose of 25 µl/kg had no significant effect on lipid profile of rats (27). In another study, researchers showed that 30 mg/kg of CA led to the improvement of total cholesterol, TG, and LDL in the plasma of rats with hyperglycemia (17). Therefore, it seems that although the dose of CA in the present study could not significantly improve other bone metabolic markers, this dose was suitable for improving serum calcium and increased it.

The results of the present study showed that MICT+CA and HIIT+CA decreased PTH and increased Ca in elderly rats. In addition, HIIT+CA decreased Na in aged female rats. It seems that exercises, especially aerobic trainings with moderate intensity, which are more than 50 minutes long, improve muscle mass, estrogen, progesterone, parathormone function, PI3, PKC , PLC, PKA, and AMPK as well as finally lead to the activation of osteoblasts, increasing the mineralization of calcium and sodium in the bone (19).

On the other hand, although there is limited information about CA, it seems that citrus ingredients with antioxidant effects, activation of deacetylases, AMPK, sirtuins, mitochondrial biogenesis lead to the improvement of the Wnt/ β-catenin signaling pathway and ultimately are associated with the improvement of osteoblast function (23,24). But according to the background of the studies, it seems that bone tissue changes are more affected by exercise and CA has less effect on it. In confirmation of this point, the results of the present study also showed that PTH and Na levels in MICT+CA and HIIT+CA groups decreased compared to CA group. Also, the effect of MICT on reducing PTH was more favorable than the effect of CA. PTH and Na levels in HIIT+CA group were significantly lower than the MICT+CA group.

On the other hand, based on studies, it seems that oxidative stress and adaptation to HIIT have an inflammatory and oxidative effect in some tissues such as skeletal muscle; but when combined with an antioxidant such as CA, they have more favorable effects on each other's metabolic markers. In confirmation of this point, Keikhosravi *et al.,* showed that HIIT and genistein consumption improved bone alkaline phosphatase and osteocalcin in elderly rats. Also, these researchers showed that the sensitivity of bone to exercise was more favorable than the effect of genistein supplementation. However, their interaction effect was still significant (28). Also, in another study, the results showed that HIIT and genistein both alone and interactively led to an increase in bone strength. However, these two factors did not have significant effects on calcium and phosphorus in elderly rats (29).

Despite the studies, no study was found that investigated the effects of exercise and CA on bone biology markers in old age. Therefore, one of the limitations of the present study was inability of comparing this study with similar studies. Regarding to the Wnt/ β-catenin signaling pathway and its effectivity from CA supplement and exercise, it seems that the lack of evaluation of this signaling pathway is one of the limitations of the present study. Therefore, it is suggested to evaluate specific metabolic markers in bone tissue in future studies. Also, considering the role of lipid profile and oxidative stress in the density and mass of bone minerals, the lack of evaluation of these variables seems to be one of the limitations of the present study. Therefore, it is suggested to evaluate these indicators in future studies.

Conclusion

It seems that MICT has a more favorable effect on bone metabolic markers than HIIT; meanwhile, HIIT with an antioxidant such as CA has more favorable effect on bone metabolic markers. Therefore, the use of MICT and HIIT with CA is recommended in the elderly condition to prevent osteoporosis.

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