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A Study of Endurance Exercise Type, Citrus Aurantium (CA) Supplementation and Their Interactive Effects on the Antioxidant Status, CRP, and AngII of Cardiac Tissue in Elderly Rats

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ABSTRACT

Introduction: The current study was aimed at determining the interactive effects of two training types of HIIT and MICT along with citrus aurantium supplementation on the antioxidant status, CRP and AngII of cardiac tissue in elderly rats.

Methods: Thirty-five elderly female Sprague-Dawley rats with the mean age of 14 months and mean weight of 270-320 g were divided into 7 groups of 5 animals, including High-Intensity Interval Training (HIIT), Moderate-Intensity Continuous Training (MICT), High-Intensity Interval Training and Citrus Aurantium (HIIT+CA), Moderate-Intensity Continuous Training and Citrus Aurantium (MICT+CA), Citrus Aurantium (CA), Sham (Normal Saline), and Control. HIIT was conducted at 85-110%Vo2max and MICT was performed at 65-75%Vo2max. 300 mg/kg/day CA was injected peritoneally into each rat. HIIT, MICT, and CA supplementation protocols were performed before the statistical test. SOD, CAT, GPX activity levels (by ELISA), and CRP and AngII gene expression levels (by Real-time PCR) were measured in cardiac tissue.

Results: Training, CA, and training+CA significantly increased SOD ($P \leq 0.05$), and CAT ($P \leq 0.05$) activity levels and reduced GPX ($P \leq 0.05$) activity levels. Also, training and CA significantly increased AngII gene expression levels ($P \leq 0.05$).

Conclusion: The results showed that training and CA supplementation instigated a significant change in SOD, CAT, and GPX activity levels. Thus, it is suggested that HIIT, MICT, and CA should be used in order to improve the antioxidant status in elderly conditions. Also, the interactive combination of Training and CA seems to have a better effect on the antioxidant status in the elderly.

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Introduction

The process of aging comprises a decline in a set of functions. In this process, a set of cellular and molecular mechanisms (including excessive oxidative stress and low-grade chronic inflammation), take place that follow the aging process of cardiovascular function (1). the The strongest risk factors concerning coronary artery disease (CAD) include age (2) and gender (3). Older women are more subject to certain problems related to heart disease. According to the American Heart Association (AHA) (2019), the incidence of cardiovascular diseases (CVD) was 77.2% in men and 78.2% in women from ages 60 to 79 years. On the other hand, in adults above 80 years of age, the incidence of CVD was 89.3% in men and 91.8% in women (2). Nonethless, the CVD risks increase with age in both men and women; these risks correspond to a general decrease in sex hormones, mainly estrogen and testosterone (2).

Estrogen and testosterone are often recognized for their cardioprotective role. In a study, low levels of testosterone were found to be associated with CAD in postmenopausal women (2). In another study, it was reported that men may develop heart disease 10–15 years earlier than women due to the gradual decline in estrogen levels after puberty. On the contrary, men of 70 years of age have lower total cardiovascular risk as compared with women at

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age 50. The average age of menopause in women, which is a strong indicator of estrogen decline, has a greater effect on CVD risks in women than in men. Moreover, the risk for CVD rises vividly by as much as 2–4 times in women at the onset of menopause (2).

Since the Renin Angiotensin System (RAS) is an important regulator of cardiovascular, alterations in this system (RAS) are tightly linked to CVD (4). In many CVDs, the role of the renin-angiotensin-aldosterone system (RAAS) has been recognized. The octapeptide angiotensin (AngII) is the major mediator of the RAAS system. Excessive AngII levels produce heart failure. Studies have revealed that AngII causes an inflammatory phenotype in the heart, resulting in cellular hypertrophy and enhanced deposition of matrix proteins. Besides, it has been shown that AngII inhibition prevents cardiac hypertrophy and fibrosis. Cardiac side effects appear to be facilitated by the AngII receptor type1 (AT1) (5). Activation of the AT1 receptor stimulates vascular contraction, vascular cell hypertrophy and hyperplasia, sodium retention, ROSs production, and induction of inflammatory, thrombotic, and fibrotic processes (4). Exercise interacts with Angiotensin Converting Enzyme (ACE), AngII, AT1, and AngII receptor type2 (AT2) receptors in a varying manner (4). Accordingly, Silva et al., maintained that AngII levels in the renal artery of healthy rats with intrinsic hypertension decreased after 3 months of low-intensity exercise training (6). Also, in the research of Ren et al., it has been reported that sports training reduces the production of AngII through ACE downregulation and ACE2 upregulation (7).

Another marker of vascular inflammation is C-reactive protein (CRP) which is a mediator of atherosclerosis and is linked with an enhanced risk of coronary heart disease (8, 9). CRP levels have been associated with myocarditis, congestive heart failure, atherosclerotic disease, atrial fibrillation, aortic valve disease, and heart transplantation, revealing that it has an active role in the pathophysiology of CVD. Many factors including age, and gender can alter baseline CRP levels. Also, studies have confirmed that the oral sex steroid hormone estrogen may influence CRP levels in older men and women and postmenopausal women (8). Furthermore, in a meta-analysis and systematic review, Fedewa et al. reviewed the effect of exercise training on C reactive protein. It was concluded that engaging in exercise was associated with decreased levels of CRP irrespective of the individuals' age or sex (10). Also, Ihalainen et al., studied the effects of 24 weeks of combined aerobic and resistance training on inflammation markers which were performed on the same day or different days. It was revealed that training significantly rdeclined circulating CRP in the training groups (11).

Increased inflammation and oxidative stress hurt the elderly, so they should be minimized (12). On the other hand, chronic exposure to oxidative stress may result in cardiovascular disorders (13). Glutathione peroxidase (GXP), superoxide dismutase (SOD), and catalase (CAT) are antioxidant enzymes that have been implicated in the prevention of CVD, vascular diseases, atherosclerosis, hypertension, and inflammatory diseases. Their deficiency openly triggers an intensification in vascular oxidative stress along with attendant endothelial dysfunction (14). Exercise affects the production of free radicals and antioxidant capacity, which can upset the balance between the two. A lot of studies on exercise and aerobic metabolism, such as swimming or running, show an increase in the production of free radicals as well as escalated activity of antioxidant enzymes such as GPX, SOD, and CAT (15). Souissi et al., reviewed the effect of different modalities of running exercise (i.e., continuous running exercise (CR); intermittent running exercise (15/15); intermittent running exercise (30/30)) on post-exercise oxidative stress markers in trained athletes. The researchers reported that SOD increased after CR and also remained unchanged after 15/15, and decreased after 30/30. In all experimental sessions, GPX did not change after exercise (15). Also, Accattato et al., reviewed the effects of acute physical exercise on oxidative stress and inflammatory status in young, sedentary obese subjects (normal weight; overweight to moderate obesity; severe obesity). The researchers reported that SOD and GPX levels increased in the normal weight and severe obesity groups, and decreased in the overweight to moderate obesity group, but these changes were not statistically significant (16). In another study, Bouzid et al., conducted a study on four groups (young sedentary (YS), young active (YA), old sedentary (OS), and old active (OA)). Resting SOD activities were higher in YA in comparison with OA. After exercise, a significant increase in

SOD and GPX activities was observed in YS, YA and OA (17). However, Bessa et al., conducted a study on nineteen male athletes who carried out a combination of high-intensity aerobic and anaerobic training exercises. The researchers reported that SOD levels remained fixed across all-time points. In response to exercise, CAT activity increased at 3 and 24 hours after exercise (18). In addition, González-Bartholin et al., in a study of eight male and two female healthy older adults reported that GPX activity increased after all exercises. The subjects of the study performed 30 min of moderate-intensity concentric (CONC-M: 50% maximum power output; POmax) and eccentric cycling (ECC-M: 50% POmax) and high-intensity eccentric cycling (ECC-H: 100% POmax) (12). Chaki et al. reviewed the extent of high-intensity exercise-induced oxidative stress in sedentary pre- and post-pubertal boys. Participants were sixty-four sedentary pre-pubertal (n=32) and post-pubertal (n=32) boys who performed incremental treadmill running exercise at 80 percent of the age-predicted maximum heart rate till volitional exhaustion. SOD and CAT activities increased in both groups following exercise (13). Pal et al., reviewed the high-intensity exercise-induced oxidative stress of 44 sedentary postpubertal boys and girls. The results showed that CAT activity increased significantly in both groups after exercise (19).

Acute exercises increase the level of oxidative stress (12). Such oxidative stress induced by exercise is associated with a parallel increase in the activity of enzymatic antioxidants in the body (13). Dietary supplements with antioxidants may be helpful in this situation. Exogenous and endogenous antioxidants reduce oxidative stress (20). Citrus aurantium Linné (CA), is also known as Seville orange, marmalade orange, or sour orange (21). It is rich in vitamin C, flavonoids, and volatile essential oils (22). Fruits, flowers, essential oils, and plant ingredients of this plant have several biological potentials, such as antimicrobial, antioxidant, anti-cytotoxic, antianxiety, anti-diabetic, anti-obesity, and anti-inflammatory effects (23).

Aerobic exercise training has become an important part of cardiac rehabilitation. Two common exercise strategies are HIIT and MICT training. HIIT versus MICT is more effective in improving the cardiovascular risk profile and requires lower exercise duration. Also, through a single exercise session, a higher amount of work is done at a higher intensity, which is accomplished by alternating HIIT with low-intensity exercise or rest intervals (24-26). Considering the previous research studies conducted by the researchers, in most of the studies, CA and Training have been investigated separately on the variables of the current research. In this vein, the present study attempted to examine the interactive effects of CA and Training. In case either factor (CA or Training) can strengthen another one, either protocol may be used alone provided that there is no CA sensitivity and Training restriction. Accordingly, one purpose of the present study is to fill the gap between the studies that have been conducted so far. In addition, the present study can verify and support the findings of the previously conducted studies. Therefore, the present study aimed to determine the interactive effect of HIIT, MICT, and CA consumption on the antioxidant status, CRP, and AngII of cardiac tissue in elderly rats.

Materials & Methods

In this experimental study, Thirty-five elderly female Sprague-Dawley rats with the mean age of 14 months and mean weight of 270-320 g were divided into 7 groups of 5 animals, including High-Intensity Interval Training (HIIT), Moderate-Intensity Continuous Training (MICT), High-Intensity Interval Training and Citrus Aurantium (HIIT+CA), Moderate-Intensity Continuous Training and Citrus Aurantium (MICT+CA), Citrus Aurantium (CA), Sham (Normal Saline), and Control. HIIT+CA, MICT+CA, and CA groups received 300 mg/kg/day of the hydroalcoholic extract of CA (6 days a week except Fridays/ 10 am) peritoneally one hour after finishing the training (27, 28).

Citrus Aurantium Supplement

For the preparation of the hydroalcoholic extract of Citrus aurantium, first, the Citrus Aurantium plant was prepared from Jahade Daneshgahi Center and was approved by a botanist. Then the plants were milled, and their extracts were prepared by the percolation method. 90% ethanol solvent was used to prepare the extract (29).

Extract Preparation Method

To use CA extract, daily, 2.4 grams of CA extract was dissolved in 7.2 ml of normal saline, and 300 mg/kg/day/weight of CA extract was injected into rats peritoneally. The reason for peritoneal

administration was adaptation of this method to previous studies (27, 28).

Endurance Exercise Protocol

First, the maximum velocity was evaluated to determine the maximum oxygen consumption in all rats (to plan for eight weeks of HIIT and MICT training). To determine the maximum oxygen consumption, the standard increasing test of Bedford1 et al., was used (30), which was standardized by Leandro et al., for rats (31). This test consists of 10 stages of three minutes. The speed in the first stage is 0.3 km/h and in the next stages, 0.3 km/h should be added to the speed of the turntable, while the slope is zero in all stages. In each stage of the test when the animal can no longer continue the work, the speed at that stage is considered equivalent to the speed of the animal at the maximum oxygen consumption.

HIIT and MICT training (8 weeks of HIIT and MICT training/ 3 sessions per week) were performed in such a way that at the onset of each session, the warm-up phase was performed, which included running for 3 minutes at an intensity of 10 m/min. Following that, the HIIT groups trained with an intensity of 85 to 90%VO2 max, equal to 7 attempts in 1 minute and a speed of 31 m/min, and active rest between intervals of 6 attempts and a speed of 15 m/min in the first week. With an average increase of 2 m/min per week, the attempts gradually reached 10 1-min attempts at a speed of 55 m/min, intensity 110%VO2max, and finally, active rest reached with 9 1-min attempts (between intervals) at a speed of 25 m/min in the eighth week. In fact, the HIIT protocol consisted of three parts: warm-up (3 min), exercise including 1-min interval repetitions (1x1) (with a 1-min active rest period between each interval), and cool-down (5 min) (Figure1). Meanwhile, the MICT group trained with an intensity of 65%VO2max, which started with a speed of 20 m/min and duration of 15 minutes in the first week, which gradually reached a speed of 25 m/min, at the intensity of 75% VO2max, and the duration of 31 minutes in the eighth week. The training protocol began with warming up for 3 minutes (with an intensity of 10 meters per minute) and 2 minutes (with an intensity of 15 meters) and then cooling up for 1 minute (with an intensity of 15 m/min) and 2 minutes (with an intensity of 10 m/min) (Figure2) (32).

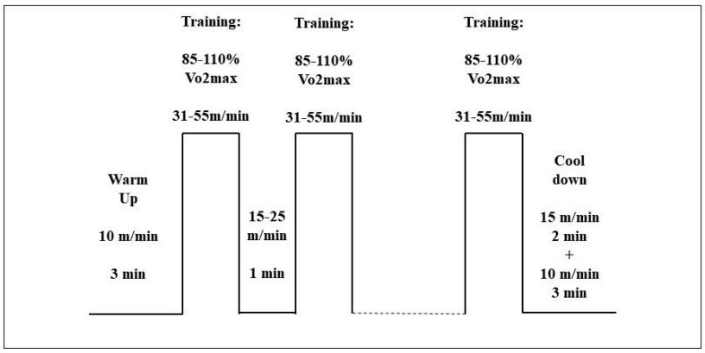

Figure1. Schematic view of the HIIT protocol

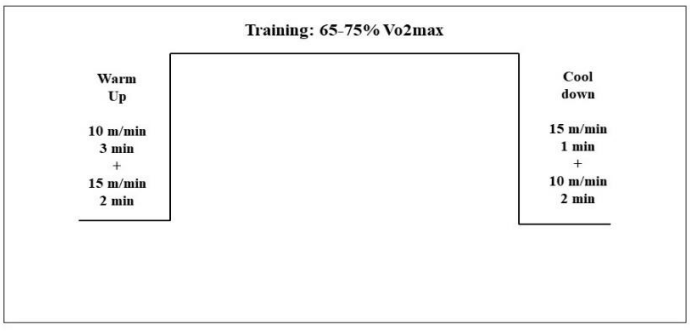

Figure2. Schematic view of the MICT protocol

Dissection and Histology

48 hours after the last training session and after 10 hours of fasting, the rats were anesthetized with ketamine and xylazine (32) (ketamine and xylazine were mixed at a ratio of 3:1 and injected intraperitoneally into the rats). After ensuring the complete anesthesia rats through the pain test with pressure on the soles of the rats' feet and in a state of complete analgesia, the chest of the rats was carefully split open, and the cardiac tissue was extracted by laboratory specialists, and immediately after weighing and washing with normal saline, it was placed in special tissue storage microtubes, and in the shortest possible time after immersion in a nitrogen tank, it was transferred to a temperature of -80 and for further measurements to measure SOD, GPX, CAT, CRP, and AngII were sent to molecular cell laboratory.

The Activity of Antioxidant Enzymes

SOD, GPX, and CAT tissue activity levels were assayed using commercial standard kits (Nasdox™, Nagpix™, and Nactaz™ Navand Salamat Company, Urmia, Iran). The absorbance rates were recorded by an FLUO star OMEGA microplate reader and software (BMG Labtech Ltd., Aylesbury, UK) at 405, 340, and 550 nm for SOD, GPX, and CAT, respectively. The results of SOD, GPX, and CAT are reported in U/mg protein, nmol/mg, and nmol/mg protein, respectively (33). For SOD activity, the reduction of color development at 405 nm was used to determine SOD activity, which is considered an inhibitory activity. An absorbance rate of 550 nm was considered to determine CAT after incubation for 10 min at room temperature. The protein content was assayed based on the Bradford method (34) by a commercial standard kit (Nadford™, Navand Salamat, Iran).

RNA Isolation and Real-Time PCR Analysis

Total RNA was isolated from the cardiac tissue using an RNA extraction kit (FavorPrep™ Tissue Total RNA Mini Kit, Taiwan). The purity, integrity, and concentration of RNA were determined by measuring the optical density 260/280 and agarose gel (1%) electrophoresis. Complementary DNA (cDNA) was synthesized from 1 µg of RNA using RevertAid™ first strand cDNA synthesis kit (Thermo Fisher Scientific, Inc., Waltham, MA, USA). Real-time PCR was performed according to the protocol of RealQ Plus 2x Master Mix Green (Ampliqon Inc.) in applied Biosystems StepOne™ Instrument (ABI, Step One, USA). Real-time PCR for expression analysis of the primer pairs for AngII, Crp, and β2m was designed, as shown in Table 1. The β2m housekeeping gene was also used as the internal control of real-time PCR reactions. The real-time PCR conditions were set for 10 minutes at 94°C followed by 40 cycles of 15 seconds at 94°C, 60 seconds at 60°C, and extension steps. After each real-time PCR run, melting curve analysis was carried out to confirm the specific amplification of targets. The amplification signals of different samples were normalized to β2m Ct (cycle threshold), and then the delta-delta CT (2-ΔΔCT) method (Livak and Schmittgen, 2001) was applied for comparing mRNA levels of test versus control, which represented a fold change in data analysis (35).

Table 1. Sequence of primers used in the present study

Genes	Primer Sequences	Sizes (bp)
B2m	Forward:5- CGTGCTTGCCATTCAGAAA -3 Reverse:5-ATATACATCGGTCTCGGTGG -3	244
AngII	Forward:5- GCTGGAGCTAAAGGACACACA-3 Reverse:5- GATGTATACGCGGTCCCCAG-3	130
Crp	Forward:5- TCAGGCTTTTGGTCATGAAGACAT-3 Reverse:5-GACTCTGCTTCCAGGGACAC-3	90

H&E Staining Analysis

Pathological evaluation was performed to evaluate the heart tissue. The heart tissues were excised, washed with ice-cold phosphate buffer saline (PBS), and fixed in 10 % formol saline for 24 hours, which was followed by dehydration at increasing concentrations of ethanol, clearing with xylene, and embedding with paraffin.

Specimens for 4 µm sections were prepared from paraffin blocks. The sections were stained with a hematoxylin and eosin stain and were examined using Olympus light microscopy at ×400 magnification.

Ethical Considerations

The training protocol was performed by international guidelines and agreements for the care and use of laboratory animals. All the ethical and legal aspects of this research were done with the license number IR.IAU.M.REC.1400.003 of Islamic Azad University, Marvdasht Branch. These animals were maintained in the laboratory for one week to adapt to the environment. The standard conditions, including a standard temperature of 22-24°C, a humidity of 55-60%, a light-dark cycle of 12/12 hours, and free access to water and special food for rats were observed. In compatibility with the Declaration of Helsinki to preserve the life and comfort of laboratory animals, the minimum sample of the study for the implementation and obtaining a reliable conclusion was selected. Also, during the study period, appropriate living conditions and feeding were provided. The conditions of transportation, maintenance, and appropriate feeding for research were met. Besides, to comply with the ethical principles, the minimal appropriate doses of ketamine and xylazine solutions were injected, and the surgery was conducted by an expert in the field.

Statistical Analysis

To determine the normal distribution of the data, the Shapiro-Wilk test was used. Due to the fact that there were two independent variables in the present study and the distribution of data was normal, two-way ANOVA test was used to investigate the effect of training and CA as well as their interactive effect. The independent t-test, two-way analysis of variance (ANOVA), and Bonferroni post-hoc test were used to make between-group comparison. The results were analyzed using SPSS software version 22 at a significant level of $P<0.05$.

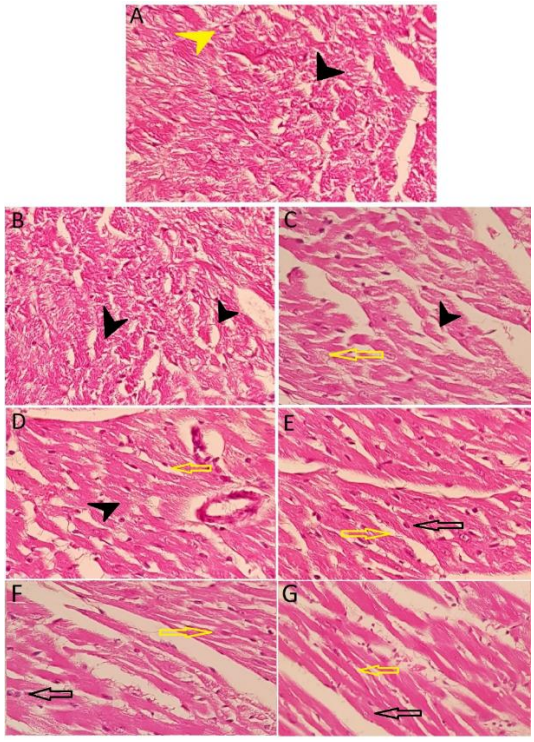

Figure 3. Hematoxylin and eosin (H&E)-stained heart sections from rat. Normal architecture of cardiac myocytes (Yellow arrow) with centrally placed nuclei (Black arrow). Myocyte necrosis with loss of cross striations and loss of nuclei (Black Arrowhead) and the nuclei of cardiac myocytes showed changes in the form of pyknosis (Yellow Arrowhead); (In total, normal cells were indicated with arrows and abnormal cells with arrows) (HF&E, ×400). The results showed that the abnormal cardiac myocytes decreased in training CA groups. This improvement is much greater in the MICT+CA and HIIT+CA groups. (A) Control group. (B) Sham group. (C) CA group. (D) MICT group. (E) HIIT group. (F) MICT+CA group. (G) HIIT+CA group.

Results

H&E staining Analysis

Histological changes were assessed by a pathologist unaware of the type of practice and treatment (Figure 3).

On hematoxylin and eosin staining, the area of myocardial infarction can be easily recognized by viewing the typical areas of necrosis when compared to the surrounding border zone and the healthy myocardium. In detail, classical myocardial morphology loss, necrotic cell death with the loss of the nuclei, as well as the complete loss of entire muscle fibers can be observed. The results showed that the myocardial infarction index decreased in training CA groups. This improvement is much greater in the MICT+CA and HIIT+CA groups.

SOD, GPX, and CAT activity levels as well as CRP and AngII gene expression are shown in Figures 4-8, respectively. The results of two-way ANOVA showed that training (P=0.001), CA (P=0.001), and the interactive effects of training+CA (P=0.001) had a significant effect on SOD activity levels in the elderly female rats.

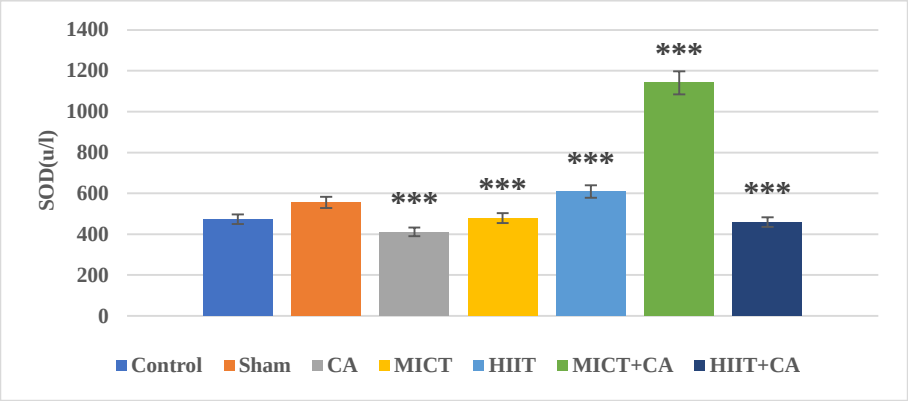

Figure 4. Levels of activity of SOD change in different study groups.
***Significant effect at P≤0.001.

The results also showed that training+CA (Eta=0.896) had more effects than training (Eta=0.853) and CA (Eta=0.547) (Figure 4). The results of Benferroni's post-hoc test showed that there was a significant difference between HIIT and MICT groups on SOD activity levels (P=0.001), and both HIIT (P=0.045) and MICT (P=0.001) triggered a significant increase in SOD activity levels. Also, the results showed that MICT training had more effects on increasing the levels of activity of SOD than HIIT.

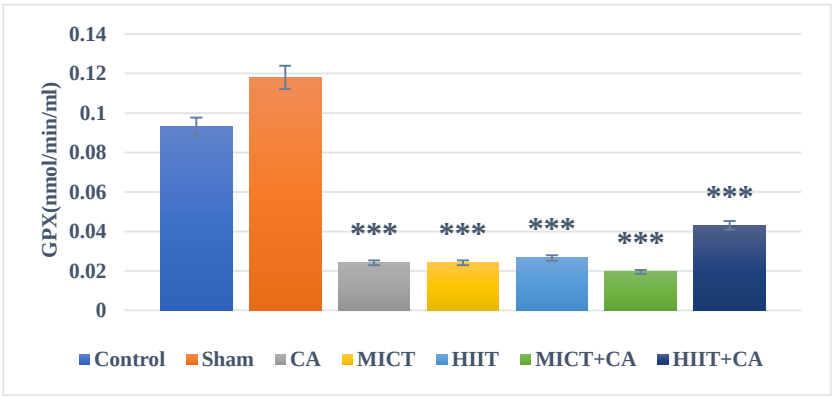

Figure 5. Levels of activity of GPX change in different study groups.
***Significant effect at P≤0.001.

The results of two-way ANOVA showed that training (P=0.001), CA (P=0.001), and also the interactive effects of training+CA (P=0.001) had a significant effect on GPX activity levels in the elderly female rats. Also, it was revealed that training+CA (Eta=0.610) had more effects than

training (Eta=0.532) and CA (Eta=0.304) (Figure 5). The results of Benferroni's post-hoc test indicated no significant difference between HIIT and MICT groups on GPX activity levels (P=0.390). Both HIIT (P=0.002) and MICT (P=0.001) brought about a significant decline in GPX activity levels. Also, the results showed that HIIT had more effects on levels of activity of GPX than MICT.

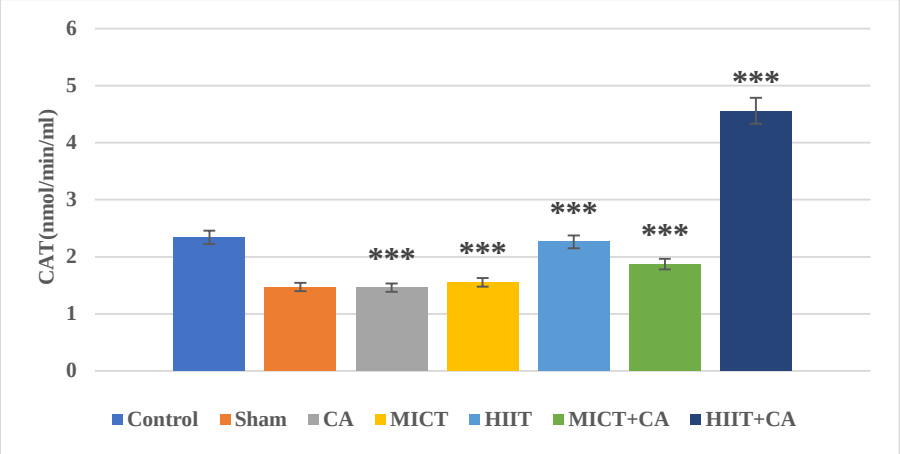

Figure 6. Levels of activity of CAT change in different study groups.
***Significant effect at P≤0.001.

The results of two-way ANOVA showed that training (P=0.001), CA (P=0.001) as well as the interactive effects of training+CA (P=0.001), had a significant effect on CAT activity levels in the elderly female rats. The results also showed that training (Eta=0.668) had more effects than Training+CA (Eta=0.519) and CA (Eta=0.299) (Figure 6). The results of Benferroni's post-hoc test revealed a significant difference between the HIIT and MICT groups on CAT activity levels (P=0.001). While HIIT (P=0.001) caused a significant increase in CAT activity levels, MICT (P=0.001) caused an increase in CAT activity levels which was not statistically significant. The results also showed that HIIT has more effects on increasing CAT CAT activity levels than MIC.

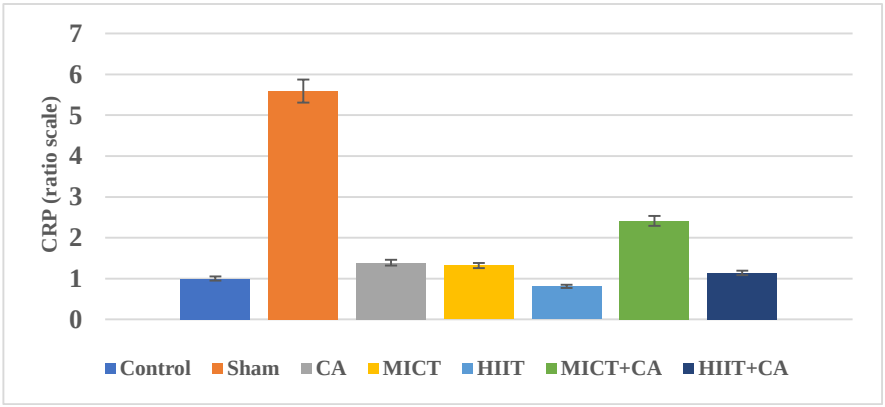

Figure 7. Levels of CRP gene expression change in different study groups.

The results of two-way ANOVA showed that training (P=0.125), CA (P=0.768), as well as the interactive effects of Training+CA (P=0.067) had no significant effect on CRP gene expression in the elderly female rats (Figure 7).
The results of two-way ANOVA showed that training (P=0.001) and CA (P=0.007) had a significant effect on AngII gene expression in the elderly female rats. However, the interactive effects of training+CA (P=0.100) had no effect on AngII gene expression in the elderly rats. In addition, based on the resulrs, training (Eta=0.663) had more effects than CA

(Eta=0.227) and training+CA (Eta=0.147) (Figure 8).

According to Benferroni's post hoc test, there was a significant difference between the HIIT and MICT groups on AngII gene expression levels (P=0.001). While HIIT (P=0.001) triggered a significant increase in AngII gene expression, MICT (P=0.762) triggered an increase in AngII gene expression, though this increase was not statistically significant. The results also showed that MICT had more effects on AngII gene expression than HIIT.

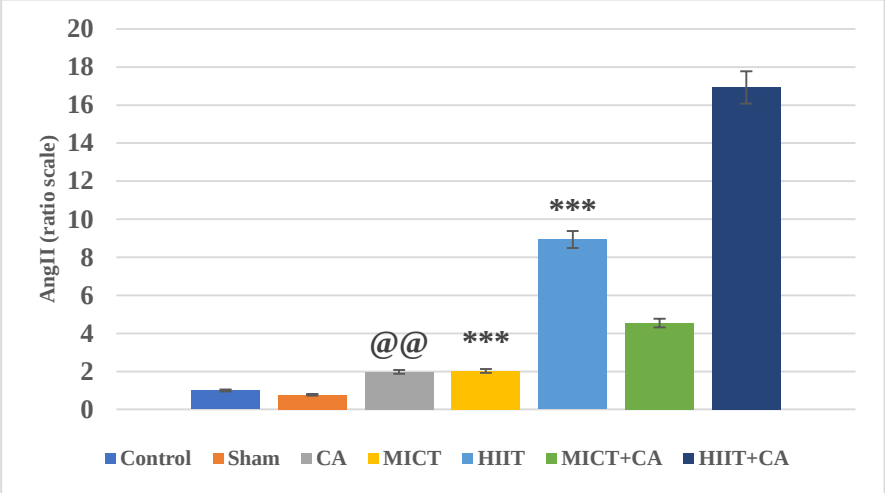

Figure 8. Levels of AngII gene expression change in different study groups.
***Significant effect at P≤0.001.
@@Significant effect at P≤0.001.

Discussion

Based on the findings of this research, Training, CA, and Training+CA significantly increased SOD activity levels. The results showed that Training+CA compared to Training and CA, as well as MICT compared to HIIT had more increasing effects on SOD activity levels. This finding is in line with the results of the research (15-17) that revealed enhanced SOD antioxidant enzyme activity after exercise, nevertheless, it is inconsistent with the results of the studies (16, 18) that reported unchanged or decreased SOD activity levels. Previous studies reported that oxidative stress parameters were associated with various running conditions (aerobic, anaerobic, intermittent, and continuous) and a rise in the activity of antioxidant enzymes such as SOD, GPX, and CAT (12, 15-18). A major finding of this study was that following HIIT, MICT training, and CA supplementation, the oxidative stress parameters (SOD, GPX, CAT) presented different responses depending on the training and supplementation conditions. Continuous running appears to be a prominent inducer of superoxide (-O2) radical production. Consequently, SOD activity levels, as the forefront of antioxidant defense, will be augmented to guard against the damaging effects of O2 radicals. After intermittent exercise, the balance between free radicals and antioxidants appears to be established, and most of the O2 radicals created are unceasingly neutralized by existing antioxidant defenses (15).

Training leads to an increase in AMP, intracellular calcium concentration, and an increase in ROS. These changes trigger the activation of some intracellular signaling pathways, including calmodulin calcium-dependent protein, AMPK, and p38-AMPK, which play an essential role in the upregulation of PGC-1a activity followed by mitochondrial biogenesis; AMPK with a change in NAD^+ to NADH ratio accelerates the process of mitochondrial biogenesis (36, 37). With increasing age, AMPK levels decrease significantly and can play an important role in reducing PGC-1a (38). Increasing access to nitric oxide (NO) caused by shear stress is another important mechanism for explaining the protective role of exercise against endothelial dysfunction (39, 40). With NO production and vasodilation, blood circulation improves during exercise, and this can cause a decrease in FR production and, as a result, a decrease in the oxidative stress marker. NO

bioavailability occurs through the balance between the amount of enzymatic and non-enzymatic NO production and on the other hand the elimination of NO, in reaction to ROS. In addition, it depends on the extent of its formation through mitochondria or enzymes such as NAD(P)H oxidase (NOX) and Xanthioxidase (XO) and the extent of ROS elimination through the antioxidant defense system or other reactions (41). However, the effect of exercise depends on the type, intensity, and duration of training, and the mechanism of the type of training on this molecular cellular mechanism is still not well known (42).

Recently, researchers have attracted their attention to the use of natural antioxidants and medicinal plants in addition to physical activities (43). Among the antioxidant plants that have nutritional and medicinal properties, we can mention CA (44). Synephrine-p, ephedrine, salicin, and caffeine are among the main effective substances in CA, which have the most biological effects in this plant. Also, this medicinal plant can have favorable effects on the cardiovascular system by improving the function of prostaglandin F2a. This natural antioxidant can reduce ROS, increase NO enzyme, activate guanylyl cyclase, inhibit extracellular dependent $Ca2^{+}$, adjust K^{+} and its adaptations in calcium-potassium channels, and also improve the function of the ryanodine receptor in the heart (45). It also appears that CA plays a role in the phosphorylation of PGC-1a through the mechanism of AMPK activation, but this mechanism depends on the dose and duration of consumption (46). In the previous research, the beneficial and positive effects of HIIT, MICT and CA supplements on the elderly and cardiovascular patients have been well demonstrated. However, it seems that the combination of training and CA has more synergistic effects than training and CA alone on increasing SOD. In addition, MICT has greater effects on increasing SOD than HIIT.

The present study showed that Training, CA, and Training+CA reduced GPX activity levels significantly. It was also revealed that Training+CA in comparison with Training and CA, as well as HIIT in comparison with MICT had more effects on GPX activity levels. This finding is consistent with Accattato et al.'s, study (16), yet it is inconsistent with Wajedi Souissi et al., and some other researchers, who showed that GPX activity did not respond to exercise stress, remained constant or increased (12, 15-17, 47). In their research, Accattato et al., examined the effect of exercise training on three groups of people with normal weight, moderate obesity, and severe obesity. The results showed that GPX levels decreased after exercise in the group of people with moderate obesity, which is in line with the results of the current research. Also, researchers in their study reported that after exercise, GPX levels increased in the groups of people with normal weight as well as severe obesity, but this increase was not statistically significant, which is inconsistent with our results (16). Wajedi Souissi et al., investigated the effects of different running posture modes on post-exercise oxidative stress markers in skilled athletes. The researchers reported that GPX did not change in all experimental periods (15).

CA leads to the neutralization of ROSs. Ephedrines present in CA mostly affect the AMPK/PGC-1a axis (48), therefore the effect of ephedrine interacts with the molecular cellular adaptations of catecholamines and can yield to augmented capacity of total antioxidant, expression of the electron transport chain family proteins and rate of oxidative phosphorylation in the heart tissue (49). In addition, in line with exercise, flavonoids are able to remove free radicals and have a protective effect against lipid peroxidation; they can also reduce endothelial NO metabolism, which leads to the production of NO radicals, and modulate the activity of NADPH oxidase (50). Thus, antioxidant mechanisms of flavonoids comprise 1) suppressing the formation of ROSs by inhibiting the enzymes involved in their production; 2) eliminating ROSs, and 3) regulating and protecting the antioxidant defense systems (50). Considering the levels of SOD after exercise in the present study, it seems that most of the O2 radicals created after exercise are neutralized by SOD in the forefront of antioxidant defense. The result is that just a small amount of H2O2 radical is created, which explains the decline in GPX activity (15) following HIIT, MICT training, and CA supplementation.

Training, CA, and Training+CA significantly increased CAT activity levels in this study. The results showed that Training compared to Training+CA and CA, as well as HIIT compared to MICT had greater effects on the increase in levels of activity of CAT. This suggests that exercise

stress can regulate the activity levels of this antioxidant gene, which copes with high-intensity hydrogen peroxide produced during exercise. These findings could be consistent with previous studies indicating that CAT activity levels respond to exercise stress (13, 19). However, in terms of the percentage of difference in CAT activity levels after exercise, a significant difference was seen between the study groups, so that CAT enzyme activity levels significantly changed in all groups of rats.

CA causes PGC-1a phosphorylation by the mechanisms of improving prostaglandins, increasing antioxidants, regulating ion channels (45), and activating AMPK (46). HIIT is likely to have a more favorable effect than MICT training due to its adaptability, which challenges the oxidative stress system and leads to the activation of the FOXO3a pathway (51). Therefore, it seems that following HIIT along with CA consumption, the FOXO3a cellular redox pathway is simultaneously activated, but MICT with CA consumption contributes to mitochondrial biogenesis by the mechanism of increasing AMPK phosphorylation (45, 46, 52). Considering the positive and antioxidant effects of HIIT, MICT, and CA supplements, some of which were mentioned above, the increase in CAT in HIIT, MICT, and CA supplementation protocols is justified and not far from expected.

Training, CA, and Training+CA significantly reduced CRP gene expression levels, but this reduction was not statistically significant. Our finding was in line with previous studies that showed that CRP gene expression levels respond to exercise in a decreasing manner (10, 11). Many factors, including age, sex, weight, and fat levels, can alter baseline CRP levels (8). CRP is an acute-phase homopentamer inflammatory protein that increases its expression in inflammatory conditions such as some cardiovascular diseases and infections. Based on evidence, CRP is not merely an inflammation or infection marker; rather, it is an important regulator in the process of inflammation. Complement pathway, phagocytosis, apoptosis, NO release, and cytokine production are major areas of inflammation and host response to infection that are modulated by CRP.

Exercise training can improve CRP levels probably through several mechanisms. Performing regular exercise directly by enhancing the secretion of NO from the endothelial tissue leads to the improvement of endothelial function and the increase of antioxidant factors, which results in the reduction of general inflammation and as a result the reduction of inflammatory cytokines of the endothelial system smooth muscles (53). The reduction of the pro-inflammatory CRP marker following training in the current study can lead to a reduction in the release of chemical mediators and a decline in pro-inflammatory transcription factors, such as NF-kß, and so play a role in modulating vascular inflammation (54). In addition, various studies have demonstrated that the increase of ROS activates the NF-kB pathway. Evidence has shown that phenolic extract of CA (TPE-CA) can inhibit MAPK and NF-κB signaling pathways, thereby reducing inflammatory symptoms and acting as an anti-inflammatory (55). Therefore, any intervention that leads to the reduction of this marker can be effective in preventing cardiovascular problems or contributing to the treatment of cardiovascular diseases. Considering the findings of the present research, the implementation of HIIT, MICT, and CA supplementation can reduce CRP levels in elderly rats.

Regarding the results of the present research, training and CA elevated AngII gene expression levels significantly; also, Training+CA increased AngII gene expression levels, but this increase was not statistically significant. In addition, training compared to CA and training+CA, as well as MICT compared to HIIT had more effects on AngII gene expression. These findings contradict previous studies showing that levels of AngII gene expression respond to exercise in a reduced manner (6, 7). Silva et al., reported that AngII levels in the renal artery of healthy rats with intrinsic hypertension decreased after 3 months of low-intensity exercise training (6). In the research of Ren et al., it has been reported that sports training reduces the production of AngII through ACE downregulation and ACE2 upregulation (7). RAS proteins are involved in many types of tissue mechanisms, cellular aging, and longevity. AngII is a determining factor in this process, which increases oxidative stress and inflammation through the AT1 receptor. One of the reasons for this inconsistency is the difference in the type, intensity, and duration of training. Considering the type, intensity, and duration of training, a wide range of changes are created in the body. High and moderate intensity

training can increase free radicals production and inflammation, which in turn elevate AngII levels .

Training can have beneficial effects on AngII levels in several ways. The first effect is a decrease in the levels of AT1 and AT2 receptors (56). Inhibition of AngII/AT1 prevents the production of ROS. As stated earlier, ROS contributes to the production of inflammation and deformation of vascular structure through activating transcription factors NF-KB, MCP-1, and IL-1 (57). Also, the inhibition of the AT1 receptor allows for inhibition of Profilin-1 and PKC, therefore controlling and inhibiting the downstream pathways of Profilin-1, namely ERK/MAPK, and PKC, namely, JAK/STAT, respectively, which directly affect the vessel's smooth muscle cells (57).

By performing exercise training in one session, free radicals in the body increase, but gradually by creating adaptation, the body's antioxidant/antioxidant defense is strengthened as well. Therefore, after participating in a rehabilitation training session, the amount of free radicals scavenged by the body's antioxidant enzymes increases. This ability of the body is able to reduce the effects of ROSs in the production of AngII, so the plasma levels of AngII decrease (58). Another adaptation of regular exercise training is the increase in plasma and blood hematocrit levels, and because the secretion of AngII is somewhat influenced by changes in plasma levels, therefore, in a trained body, less fluctuations in plasma levels can lead to less secretion of AngII (59). The possible cellular mechanisms that develop in adaptation to aerobic training may affect the sympatho-stimulatory process by reducing oxidative stress and increasing NO (60). Participation in HIIT or MICT, through triggering shear stress and nitric oxide production (eNOS), oxidative defense enzymes including SOD, improving vascular endothelium functional disorder as well as reducing O2- levels and pro-inflammatory cytokines such as TNF-a and IL-6 can result in declined levels of AngII and reduced levels of vasoconstrictor response induced by this vasoconstrictor (56, 61).

A major limitation of this study is not having access to the western blot tool. Considering that the measurement of the variables of the present study by the western blot method can confirm the results of this research, it is recommended that in the analogous studies, changes in the variables of this study should be investigated by the western blot method. The lack of access to human subjects is another limitation of this study. Still again, another limitation is the lack of measurement of CRP and AngII activity, which can be attributed to the absence of research funding. Moreover, the present study lacks administering a pre-test between groups at the onset of the study to confirm the absence of differences before the training intervention due to financial shortages; hence in citing the absence of differences before the intervention, the researchers sufficed to deal only with the control group. Still, another restriction of this study is the lack of investigation of the effect of long-term activities and follow-ups. So it is suggested that in the following studies, researchers consider the impact of several months of training and follow-up on the indicators discussed in this study. It is also suggested that the comparison of the mentioned training model with resistance training be examined more widely.

Conclusion

Exercise training can bring about beneficial changes in the antioxidant defense system and inflammation of the heart tissue. These outcomes were observed in HIIT and MICT protocols and the combination of both training methods with CA consumption. Also, it seems that the interactive combination of Training+CA in the elderly condition has a better effect on the antioxidant status, even though further studies are required to make generalization of the results to human beings and to understand the mechanism of action of their combination.

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