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The Effect of Eight Weeks Aerobic Training with Citrus Aurantium on miR-1 and miR-22 in Left Ventricle of Elderly Female Rats; An Experimental Study

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Abstract

Background: *Heart attack is the main cause of mortality in the elderly. Although the beneficial role of exercise and medicinal plants on heart health has been reported, the simultaneous effect of these factors on micromolecular pathways in heart tissue is still unknown. This study aimed to investigate the effect of eight weeks aerobic training (AT) with Citrus Aurantium (CA) on miR-1 and miR-22 in left ventricle of elderly female rats.*
Methods: *In this experimental study, 35 female Sprague-Dawley rats (age 14-18 months and weight of 270-320 g) were randomly divided into (1) control (C), (2) sham (normal saline/Sh) (3) CA, (4) AT and (5) AT+CA groups. The AT groups performed aerobic training for eight weeks, three sessions per week each lasting 25-41 minutes at a speed of 20-27 meters per minute. The CA groups received 300 mg/kg CA intra-peritoneally. One-way analysis of variance with Tukey's post-hoc tests was used for statistical data analysis (Pvalue≤0.05).*
Results: *miR-1 and miR-22 levels in AT and AT+CA groups were significantly higher than in C and CA groups (P-value=0.001). miR-1 levels in AT+CA group were significantly lower than AT group (P-value=0.005) and miR-22 levels in AT group were significantly lower than AT+CA group (P-value=0.001).*
Conclusions: *Aerobic training seems to be an effective factor on the improvement of miRs involved in left ventricular hypertrophy; nevertheless, Citrus Aurantium has no significant effect, or even modulates the effect of training.*
Keywords: Training, Citrus Aurantium, miR-1, miR-22, Left ventricle, Elderly.
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Introduction

Heart attack is the main cause of mortality in the elderly; So that this chronic and progressive disease occurs with complex signs and symptoms following the structural change of the heart. It is also predicted that the number of deaths due to heart diseases in the elderly will almost double from 2016 to 2030[1]. It seems that heart diseases in elderly people are multifaceted, but differences in race, genetic characteristics, and gender are important factors in heart structure changes and the occurrence of diseases. Hormonal changes and the decrease in the function of sex hormones have caused elderly women to be more prone to pathological hypertrophy of the heart, heart attack, and cardiovascular fibrosis than men[2]. It is believed that the pathological hypertrophy of the heart tissue in response to the increase in the workload of the heart tissue, disruption in the supply of cardiac energy for repeated contractions, and disruption of mitochondrial function is related to the occurrence of heart diseases[3]. New findings show that disruption of non-coding RNAs (ncRNAs), as important regulators of the signaling pathway in heart tissue, has been the target of many studies[4]. In other words, micro RNAs (miRNAs) affect the cardiac tissue during aging via transcription pathways of physiological and pathological hypertrophy genes, and among these miRs, miR-22 is known as a regulator whose overexpression following aging in cardiac tissue leads to an increase in the number of collagen and fibrinogen as well as is associated with hypertrophy of the elderly heart[5]. Also, miR-1 is one of the best microRNAs that intervenes in the calcineurin- NFAT signaling pathway and reduces the expression of calmodulin, leading to cardiac tissue regeneration and modulating cardiac hypertrophy[4].
On the other hand, considering the need of human societies to prevent diseases related to the elderly, exercises have been suggested as a non-invasive and effective solution for physical and psychological health in old age conditions[6]. In other words, regular exercises in elderly people improve body metabolism and lipid profile and reduce cardiovascular risk markers[7]. Researchers believe that exercises with their biological effects lead to changes in the expression of miRs and in this way lead to changes in the structure of the heart[8]. The researchers showed that high-intensity interval and moderate-intensity continuous trainings decreased miR-260 levels, increased heat shock protein 60 (HSP60), improved cardiac function, and decreased apoptotic markers in the heart tissue of diabetic rats[9]. In another study, researchers showed that one session of intense exercise decreased levels of miR-20a and increased levels of miR-21, but no significant change was observed in the plasma levels of miR-1 in healthy men[10]. Studies show that a wide range of miRs have been identified, however, there is little information regarding the effect of exercise on them.
At the same time, the favorable effects of exercise on improving cardiac function have been reported in many studies. The researchers believe that in addition to exercise, a proper diet along with antioxidants and medicinal plants is a good solution for heart health. Citrus Aurantium (CA) has been used for a long time due to the presence of flavonoids, isoflavones, and glycosides, which have anti-inflammatory effects, and improve lipid profile, glucose, and insulin metabolism[11]. CA seems to play a role in mitochondrial biogenesis and

transcription of metabolic genes by the mechanism of improving biological pathways such as activating the AMP-activated protein kinase (AMPK) pathway[12]. A study showed that CA consumption led to the improvement of sleep quality in the elderly with heart disease[11] and menopausal women[13]. It has been reported that CA led to the inhibition of the inflammatory pathway related to tumor necrosis factor-alpha (TNFα) in cells derived from adipose tissue by reducing the expression of miR-155 [14]. In addition, continued and interval training along with CA consumption improved balance and pain tolerance threshold[15], mitochondrial biogenesis markers[12], and autophagy in skeletal muscle tissue[16] in aging conditions.

However, considering the importance of miR in the regulation of cellular-molecular pathways related to heart tissue hypertrophy and the effectiveness of heart tissue structural changes to exercise and antioxidants, conducting fundamental studies in this field can provide more information to health researchers. Therefore, the present study aimed to investigate the effect of aerobic training (AT) along with CA on miR-1 and miR-22 in the left ventricle of elderly female rats.

Materials and Methods

In this experimental study with a post-test design along with a control group, 35 female rats with a weight range of 270- 320 grams and age range of 14- 18 months (they were able to do exercises and were free of any disease and infection) were selected as a statistical sample and transferred to the Animal Sports Physiology Laboratory of Islamic Azad University (Marvdasht Branch). It should be noted that the rats were kept in the laboratory for one week to adapt to the environment according to the Helsinki Convention and the ethical principles of working with laboratory animals. During the entire period, the rats were kept under standard conditions in terms of light (12 hours of darkness and 12 hours of light), temperature (22-24 degrees Celsius), and relative humidity (55- 60%) in polycarbonate cages (which could autoclave) made from Razi Rad Company. Food and water were freely available to the animals during the entire period. In addition, sterile wood grates were used to absorb urine and moisture from the bottom of the cages, and depending on the conditions of the cages, they were washed and replaced every three days.

After the adaptation period, to homogenize the menstrual cycle of rats, 550 micrograms of estradiol valerate and 3 milligrams of progesterone per kilogram of body weight of rats along with sesame oil were injected into the thigh muscle for two consecutive days. Then vaginal smears of rats were prepared and the homogeneity of the sex cells was confirmed with a light microscope, and rats were randomly divided into (1) control (C), (2) Sham (CA/Normal Saline) (Sh), (3) CA, (4) AT and (5) AT+CA groups.

Before performing AT, rats were first introduced to the treadmill for one week, five sessions per week at a speed of 8 m/min. Then maximum running speed of rats was measured based on the protocol mentioned in the research of Li *et al.,*[17]. Next, rats in the AT and AT+CA groups, trained for 15 minutes at a speed of 65% of the maximum speed (equivalent to 20 m/min) in the first week. After the second week, 1 m/min was added to the speed of the treadmill for each week until it reached 27 m/min in the last week. In addition, the duration of training gradually increased from 15 minutes in the first week to 31 minutes in the eighth week. It is worth noting that the duration of 5 minutes at the beginning of the exercises and 5 minutes at the end of the exercises at a speed of 10 m/min for warming up and cooling down was added to the total time of the exercise in each session. Also, exercises were done for eight weeks and three sessions per week[18].

To prepare CA extract, 100 grams of CA powder, which was previously approved by Marvdasht Agricultural Jihad, was added to 500 ml of distilled water; then, the solution was kept in the laboratory environment for 2 days, and was extracted. It is worth mentioning that this process took four hours to prepare the extract at a low temperature. Also, the extract was dehydrated using sodium sulfate and remained at a temperature of -20 until use. In addition, according to the required dose, 1500 mg of the extract was dissolved in 2.4 cc of normal saline and then 30 International Units (IU) of the solution was injected intra-peritoneally to each rat. It is worth noting that Sh group received only normal saline[19].

Forty-eight hours after the last training session, rats were anesthetized by intra-peritoneal injection of 50 mg/kg of ketamine and 15 mg/kg of xylazine after 12 hours of fasting. To diagnose unconsciousness, tail pinch, and foot touch tests were used, and after confirming unconsciousness, the chest cavity was opened using a 15-size surgical blade, and the heart tissue was carefully extracted. Next, after weighing and washing, the aorta part was separated, and using a fine surgical blade, the right part, which was the atrium and the right ventricle, was removed, and the remaining part, which formed the left ventricle, was immediately immersed in a nitrogen tank and was kept at -70 temperature until the molecular cell tests were performed.

For this purpose, the left ventricle was first placed in a special oven, and after lysing and homogenizing the tissue and removing damaged cells and connective tissues, RNA extraction was performed according to the protocol of the FavorPrep™ Tissue Total RNA Mini Kit (Taiwan). After ensuring the purity of RNA by spectrophotometric device and observing the purity with light absorption property at 260 nm wavelength, the samples were prepared for cDNA synthesis. For cDNA synthesis, the protocol recommended in the commercial kit of Thermo Fisher Scientific, Inc., Waltham, MA, USA was used. Then, the primers of the current research, which were prepared using the NCBI site guide, were determined by using the online software of this site, and after ensuring the optimal efficiency of the primers, cDNA with the primers for the reverse transcription reaction in the presence of phosphorus element was added to the well of Step One Real-Time PCR System (Italy) were added. It is also worth mentioning that the internal control gene U6 was used for quantification (Table 1). Further, after finishing the system and reaching the threshold cycle (CT), data quantification was done using the formula $2^{-\Delta\Delta CT}$

The Shapiro-Wilk test was used to check the data distribution. Also, due to the normality of data distribution, a one-way analysis of variance was used to analyze the differences between groups, and Tukey's *post-hoc* test was used to determine the differences between groups. The data of the present research were analyzed using Graph Pad Prism 8.3.0 software at a significance level of less than 0.05.

Table 1. Primers designed for the present research

miR-1	Forward: CGTCCTTCAAGTAATCCAGGA
	Reverse: GCAGGGTCCGAGGTATTC
miR-22	Forward: TGCGCAGTTCTTCAGTGGCAAG
	Reverse: CCAGTGCAGGGTCCGAGGTATT
U6	Forward: CTCGCTTCGGCAGCACA
	Reverse: AACGCTTCACGAATTTGCGT

Results

According to the results of the one-way analysis of variance test, there were significant differences in miR-1 and miR-22 levels between research groups (P-value=0.001).
Tukey's *post-hoc* test showed that there was no significant difference in miR-1 levels between C and Sh groups (P-value=0.11); Also, there was no significant difference between CA and C groups (P-value=0.41); But in AT and AT+CA (P-value=0.001) groups was significantly higher than C, Sh and CA groups. It was also significantly lower in the AT+CA group than in the AT group (P-value=0.005) (Figure 1).

miR-22 levels in Sh (P-value=0.056) and CA (P-value=0.09) groups were not significantly different from C group; But in the AT (P-value=0.001) and AT+CA (P-value=0.019) groups were significantly lower than the C group. Also, in AT and AT+CA groups were significantly lower than Sh group (P-value=0.001). The levels of miR-22 in the AT and AT+CA groups were significantly lower than in the CA group (P-value=0.001). It was also significantly lower in the AT group than the AT+CA group (P-value=0.001) (Figure 2).

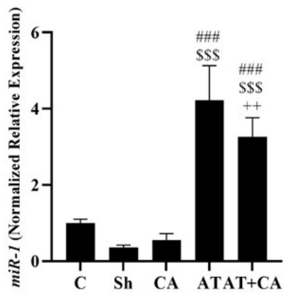

Figure 1. Expression levels of miR-1 in heart tissue of rats in research groups

(P-value=0.001) significant increase compared to C and Sh groups. $$$ (P-value=0.001) significant increase compared to CA group.

++ (P-value=0.01) significant increase compared to AT group

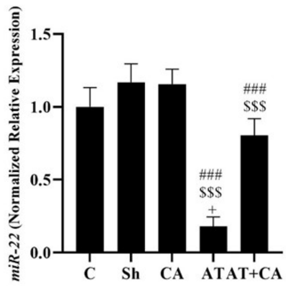

Figure 2. Expression levels of miR-22 in heart tissue of rats in research groups

(P-value=0.001) significant decrease compared to C and Sh groups. $$$ (P-value=0.001) significant decrease compared to CA group.

+ (P-value=0.05) significant decrease compared to AT+CA group

Discussion

The results of the present study showed that AT increased the expression of miR-1 and decreased miR-22 in the left ventricle of elderly rats. Based on the conducted studies, it seems that miR-22 levels increase in response to oxidative stress in the heart tissue, and by participating in reactants such as calcineurin and myocyte enhancer factor-2C, activation of cell differentiation as well as tumorigenesis leads to hypertrophy of heart tissue[20]. It also seems that some mechanisms of exercise such as the angiogenesis pathway, change in hypoxia-inducible factor 1-alpha (HIF-1α), and vascular endothelial growth factor

(VEGF) with the participation of miR-1 lead to angiogenesis in cardiac tissue and is effective in physiological hypertrophy of heart tissue[10]. In this context, Zhou et al.'s study showed that one session of exercise increased plasma levels of miR-21 and decreased miR-20a in healthy men; While significant changes in miR-1, miR-126, miR-133a, miR-133b, miR-146, miR155, miR-208a, miR-208b, miR-210, miR-221, miR-222, miR- 328, miR-378, miR-499 were not observed[10]. Also, in the study of Delfan et al., the results showed that both continuous and high-interval training decreased miR-206 as an inhibitory factor of HSP60 in the heart tissue, and inhibited apoptosis in the heart tissue of diabetic rats via this pathway[9]. However, there is limited information regarding the effect of exercise on miR-1 and miR-22 in the heart; For example, in a study, researchers showed that cardiac ischemia leads to a decrease in miR-1 and an increase in miR-214; However, exercise prevented the decrease of miR-1 and the increase of miR-214 following myocardial infarction, and these two lead to the improvement of cardiac function through the calcium-dependent pathway[21]. On the other hand, it seems that exercise activates physiological hypertrophy pathways and inhibits pathological hypertrophy pathways. It can also be concluded from the conducted studies that the increase or decrease of miRs expression, especially miR-1 and miR-22, are expressed differently in different disease conditions, and its disruption is associated with disruption in cellular pathways.

The results showed that CA consumption had no significant effect on miR-1 and miR-22 levels in the left ventricle of elderly rats. Researches have shown that the CA consumption or the ethanol extract of 100, 200, and 400 mg/kg citrus peel initially activates the lipolysis pathways by improving the metabolism of lipids; which leads to an increase in the fatty acid's transporter GLUT4 in skeletal and cardiac muscle cells. Also, by activating transcription proteins, the ethanolic extract of CA leads to the activation of Transforming growth factor-1 (TGF-1), inhibition of nuclear transcription factor kappa-B (NFKB), activation of cell and nuclear differentiation proteins as well as PGC-1α and PGC-1β. In addition, the ethanolic extract of citrus peel with high doses leads to a decrease in the size of myocardial infarction, necrosis, and granular degeneration of heart cells[22]. However, these mechanisms depend on the dose and duration of use, and it seems that higher doses up to 400 mg/kg have more favorable effects than lower doses. Also, in this context, researchers showed that taking a single dose of 13 mg of p-synephrine extracted from citrus leaves had no significant effect on systolic blood pressure, diastolic blood pressure, and heart rate of women[23]. In a study, researchers showed that CA led to a decrease in the expression of miR-155 and TNFα in cells derived from adipose tissue and improved lipolysis via this way[14].

The results showed that the combination of AT and the consumption of CA increased the expression of miR-1 and decreased miR-22; In addition, the factor affecting miR-1 and miR-22 was more influenced by training. Consumption of CA, due to the presence of P-synephrine, it acts like catecholamines and activates some biological mechanisms such as AMPK, Peroxisome proliferator-activated receptor gamma coactivator 1-alpha (PGC-1α), nuclear respiratory protein (NRF) 1 and 2 by interacting with β3-adrenergic receptors and ultimately leads to the improvement of blood pressure, regulation of vague nerve tone, and heart rate during exercise[24]. In addition, CA with its antioxidant properties leads to inhibition of inflammatory and apoptotic pathways caused by free radicals in the heart[24]. Also, in a study, continuous and interval training with CA consumption led to an increase in aerobic power and heart weight as well as a decrease in fat weight in elderly rats (Hosseini, 2020). In the study of Shykholeslami et al., the favorable effects of CA and exercise training on PGC-1α and AMPK pathways in liver tissue were reported[12]. However, in another study, the researchers reported the toxicity effects of CA in trained rats, so that 28 days of 10 and 50 mg/kg of synephrine led to an increase in systolic and diastolic blood pressure; But it is worth mentioning that the combination of synephrine with caffeine in this study was one of the possible causes of adverse effects on the heart system. Because caffeine itself increases blood pressure[25].

From this study and other studies, it appears that exercise and CA have almost the same biological effects on heart health. Despite the favorable effects of both interventions on heart function, no study was found that investigated the simultaneous effect of AT and CA on this pathway (microRNAs) in the path of cardiac hypertrophy. Therefore, the limitation of the present research is comparing the results of this study with other studies, and it is suggested to conduct more studies in this field. Also, considering the contradictory role of microRNAs in the heart tissue depending on the type of damage, it seems that the lack of evaluation of proteins that are affected by these two factors such as TGF-1, PGC-1α and AMPK pathways, pathological hypertrophy pathways such as Akt and mTOR is another limitation of the present study. Therefore, it is suggested to evaluate more paths in future studies.

It seems that AT is an effective factor in the improvement of miRs involved in left ventricular tissue hypertrophy, and CA has no significant effect or even moderates the effect of AT.

Ethical Considerations

It should be noted that the rats were kept in the laboratory for one week to adapt to the environment according to the Helsinki Convention and the ethical principles of working with laboratory animals. Researchers received introduction letters from Marvdasht Branch of Islamic Azad University with the ethics code IR.IAU.M.REC.1399.032.

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Conflict of Interest

The authors declared no conflict of interest.

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