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Original Article

MicroRNA-145, Wnt3a, and Dab2 Genes Expression Changes of the Cardiomyocytes in Hypercholesterolemic Rats Exposed to the Aerobic Training

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The current study aimed to investigate the effect of a 12-week endurance training (ET) on microRNA-145 (miR-145) changes and *Wnt3a* and *Dab2* cardiomyocytes genes expression of hypercholesterolemic Wistar male rats. Thirty-two male Wistar rats (191.2±19 g, 6-8 weeks age) were randomly assigned into the aerobic exercise-normal nutrition (ANN; n=8), hypercholesterolemic (HCL; n=8), aerobic exercise- hypercholesterolemic (ACL; n=8), and normal nutrition (NN; n=8). Hypercholesterolemia was created by adding 1% cholesterol to the food of the HCL and ACL rats. ET was done five sessions per week on nonconsecutive days for 12 weeks. Twenty-four hours after the last training session, the rats were killed, and the cardiomyocytes were removed. The expression of miR-145, *Wnt3a*, and *Dab2* genes in cardiomyocytes was assessed by real time PCR method. The expression of miR-145 significantly increased in the ANN group in comparison with other groups (P = 0.001). Also, *Dab2* gene expression significantly decreased in the ANN group in comparison with ACL (P = 0.001) and HCL (P = 0.001) groups. The results also showed that the *Wnt3a* in the ANN group was significantly different from NN (p=0.001), ACL, and HCL (p=0.001) groups. It can be concluded that aerobic training and cholesterol-rich foods play an essential regulatory role in the expression of miR-145, *Dab2*, and *Wnt3a* genes. However, cholesterol-rich foods appear to play a more significant regulatory role than aerobic exercise training.

Key words: Diet, high-fat, myocytes, cardiac, miR145, microRNA

Exercise training leads to the restoration of the physiological structure of the heart (1). Depending on the exercise training mode, structure restoration is conducted by several factors and different signaling pathways (2), and requires a modulation of the expression of many genes involved in the heart structure and function (3). Studies conducted on healthy individuals, as well as cardiovascular disease (CVD) patients, have shown that the low capacity of the aerobic system due to

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physical inactivity is more than other risk factors responsible for high mortality (4). On the other hand, lipid profile disorders such as hypercholesterolemia will increase CVD risk factors (5). Accordingly, the heart attack risk is three times higher in patients with hypercho-lesterolemia in comparison with those with normal blood lipid contents (6). It has been predicted that up to 40% of all deaths will be related to CVD by 2030 (7). However, some of the influential factors for cardiovascular disorders include microRNAs (miRNAs) (8, 9). MiRNAs are a class of short, 20- to 22-nt-long regulatory RNAs expressed in plants and animals (10). It has been reported that up to 4% of the human genome is predicted to code for differnt miRNAs, estimated to regulate at least 30% of all human genes (11).

Researches have shown that some miRNAs are specifically expressed in specific tissues (12, 13). In his regard, the downregulation of miRNAs causes many diseases, including CVD and cancer (13). Among these miRNAs, it can be referred to as microRNA-145 (miR-145), expressed only in the muscle tissue. Therefore, it is called MyomiR. MyomiR regulates various muscle processes such as proliferation, differentiation, contractility, hypertrophy, atrophy, and stress response by regulating the expression of essential genes involved in muscle control (14). Besides, it appears to be a balancing mean of growth processes, myoblast differentiation, muscle growth-regulating, and development cycle (15).

In general, previous studies have shown that miR-145 protects cardiomyocytes against oxidative stress, and improves the immediate myocardium by increasing myocytes' autophagy speed during infarction (16). It has also been reported that excessive miR-145 expression reduces the expression of Kruppel-like factor 4 (*KLF4*) and could be considered as a therapeutic target to limit the atherosclerotic platelets morphology (15).

It has been shown that Wnt/β-catenin signaling pathway regulation is essential during the process of cardiomyocytes differentiation (17). On the other hand, the disabled homolog 2 (*Dab2*) gene is known as a *Wnt3* inhibitor (18), which regulates the endothelial development (19). However, there are few studies on the effects of aerobic exercise and *Wnt3* expression. In this regard, Fujimaki et al. (2014) noted that four weeks of running voluntarily on the treadmill caused an increase in the *Wnt* expression after exercise training (19). Furthermore, Liao et al. (2018) showed that exercise training significantly modulated miR-145 expression and Akt phosphorylation (20).

Accordingly, it is essential to know more about the effects of a high-fat diet and various exercise training, especially endurance trainings, on cardiomyocytes' changes and the molecular mechanisms involved. Therefore, the current study's aim was to investigate the effect of long-term aerobic training on the expression of miR-145, *Dab2*, and *Wnt3a* genes in the heart of male desert Wistar rats.

Materials and methods

Animals

Thirty-two Wistar male rats (191.2 ±19 g, 6-8 weeks' age) were provided from Pasteur Institute of Iran, and transferred to the animal laboratory. The animals were maintained in standard laboratory conditions in groups of three animals in transparent polycarbonate cages with a length of 30 cm, width, and height of 15 cm, at an ambient temperature of 22 ± 2 °C, with a 12:12 hours light to darkness cycle and a humidity of 50%, with proper ventilation until completing the tests and the exercise training period. It should be noted that all of the ethical principles of the present study were performed conformed to the institutional guidelines and the principles of working with laboratory animals approved by Helsinki guidelines. Moreover, this research has been approved by the ethics committee of the Islamic Azad University of

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Hypercholesterolemia induction

Before starting aerobic training, hypercholesterolemia was induced in animals. Accordingly, to make cholesterol-rich foods, cholesterol powder (Merck, Germany) was mixed with water and added to the regular food to rich an amount of 1%. Using a mixer, this combination was turned into the pellet form, and then dried in the oven at the temperature of about 40-45 ºC (21). The rats in the cholesterol-rich food group were fed with this type of food for three months until reaching hypercholesterolemia (the amount of plasma cholesterol was periodically checked by taking blood from the rats' tails), and food intervention was conducted until the end of the training period. At the same time, the rats in the regular food group were fed with the standard food . The normal food (Javaneh Khorasan Company, Iran) was in the form of pellets, and its composition was such that it could meet the nutritional needs such as energy, protein, calcium, phosphorus, methionine, lysine.

Familiarizing animals with exercise training protocols

After a week of familiarity with the treadmill, the rats in the hypercholesterolemia group and regular nutrition were distributed separately and randomly in the aerobic exercise- regular nutrition (ANN; n=8), hypercholesterolemic nutrition (HCL; n=8), aerobic exercise-hypercholesterolemic (ACL; n=8), and regular nutrition (NN; n=8) groups. The rats were homogenous in terms of diet, size, weight, age.

The ANN and ACL animals practiced a 12-week aerobic exercises training (5 d/wk). Moreover, the HCL and NN groups were kept in cages for 12 weeks and did not participate in any exercise training. Throughout the study, the rats were transferred and manipulated by only one person. Before starting the primary aerobic training protocol, the animals needed to become familiar with the treadmill and running on it. For this purpose, a 5-weekly running session was imposed to the animals at a low speed.

Aerobic exercise training

The aerobic training was performed in three phases. In the first stage (introduction stage), the rats walked on the treadmill every day for 10-15 min at a speed of 10 m/min. In the second stage (overload stage), the rats ran on the treadmill for 20 min at a speed of 20 m/min. Then, gradually, during four weeks of exercise training, the duration and speed of the exercise training increased until it reached the final rate of 60 min and the speed of 25 m/min, respectively. Finally, in the third stage (load maintenance or stabilization stage), the aerobic training reached the desired duration and speed within two weeks and was continued for the remaining eight weeks with the constant speed and duration.

In addition to the mentioned time, 5 min were allocated for warming up (8 m/min) and 5 min for cooling up (8 m/min) (22, 23). It should be noted that during the study, the control group did not have any exercise training. However, in order to create the same conditions, the animals in the control group were immobilized on the treadmill during the five sessions in the week for 10 to 15 min in each session. The aerobic training protocol is presented in Table 1. The rats were weighed once every three days using a digital scale (Sartorius, Germany) with a sensitivity of 0.01 g.

Biochemical analysis

Forty-eight hours after the last training session and following a fasting night, the animals were anesthetized by intramuscular injection of ketamine (50 mg/kg) and xylazine (30 mg/kg). Cardiomyocytes of rats were immediately extracted and maintained in liquid nitrogen at -80 °C. In the laboratory, about 50 mg of cardiomyocytes were poured into a tube along with 700 µL of TRIzol, and homogenized by a tissue homogenizer. After adding 170 µL chloroform and centrifuging the

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mixture at 12000 rpm for 15 min at 40 °C, the upper liquid phase was transferred into a new tube to extract the miR-145. cDNA synthesis for miR-145 was conducted according to the kit miScript II RT Kit (Qiagen, Cat No, 218161) instructions.

Moreover, Real-time PCR reactions were conducted using specific primers and SYBR Green (Qiagen, Germany) PCR kit in the thermal cycle apparatus (ABI FAST 7500, USA). Temperature conditions of the primers used for measuring the expression of miR-145 include the initial activation step (95 °C for 15 min), 40 cycles (94 °C for 15 s, followed by 55 °C for 30 s, and 70 °C for 34 s). U87 was used as reference gene for normalization of miR-145 exxpression. DNA synthesis for *Dab-2* and *Wnt3a* genes was conducted according to the instructions of QuantiNova Reverse Transcription Kit (Qiagen, Cat No, 205411).

Furthermore, real-time PCR reactions were conducted using the specific primers and a Quanti Fast SYBR Green PCR kit (Qiagen, Cat No, 204052) in the ABI FAST 7500 thermal cycle apparatus. A two-step kit was used for real-time PCR reactions. Real-time PCR reactions were conducted according to the instructions of the manufacturer(Qiagen, Germany), which includes an initial activation cycle (95 °C for 5 min), 40 cycles (95 °C for 10 s, 55 °C for 30 s), and cooling (kept at 40 °C). Expression changes in the exercise training and nutrition treatment groups were calculated for the *Dab2* and *Wnt3a* genes compared to the control (*Hprt*) using the $2^{-\Delta\Delta CT}$ method (24).

Table 1. Endurance training protocol on the treadmill.

Training session	Speed (m/min)	min	Session/Week	Week
First stage	10	10-15	5	1
Second stage (overload stage)	20-25	20-60	5	4
Third stage	25	60	5	8

Warming up and cooling up for 5 min (8 m per min) per session.

Table 2. Primers used for gene expression analyzes.

miR-145	5′-CCTTGTCCTCACGGTCCAGT-3′	forward primer
	5′-AACCATGACCTCAAGAACAGTATTT-3′	reverse primer
Dab2	5-TAATCCAACAGAAAGCAGAG-3	forward primer
	5-GAGGTGACTCCATTTGTTAAG-3	reverse primer
Wnt3a	5-AACACAGCAGCTTAATGAC-3	forward primer
	5-ATCTCCACGTAGTTCCTG-3	reverse primer

Statistical analysis

Firstly, the normal distribution of data was done by the Kolmogorov-Smirnov test, and the homogeneity of the variances was examined using Levine's test. One-way analysis of variance (One way-ANOVA) was used to determine the variance of the studied variables. Wherever a significant data was observed, the Tukey post hoc test was applied to determine the source of significance. All of the statistical analyses were done by SPSS software version 22. The level of significance was also considered to be $P \leq 0.05$.

Results

The data for miR-145, *Dab2*, and *Wnt3a* expression in cardiomyocytes are shown in figures 1-3.

One way-ANOVA revealed a significant difference in miR-145, *Dab2*, and *Wnt3a* (P=0.001) expressions between the groups (F values were 42.34, 82.95, and 31.72 for miR-145, *Dab2*, and *Wnt3a*, respectively). Tukey post hoc test showed that the expression of miR-145 in the ANN group was significantly different in comparison with the NN (P=0.001), ACL (P = 0.001) as well as HCL (P=0.001) groups; however, no significant difference was observed between the other groups (P 0.05) (Figure 1).

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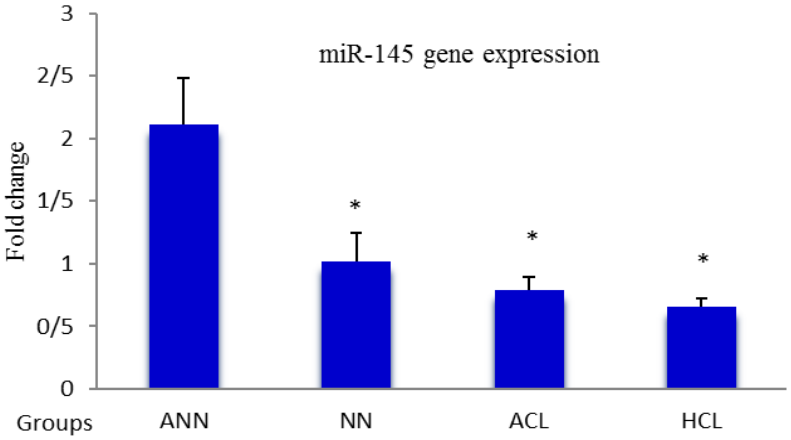

Fig. 1. Relative expression of miR-145. ANN: aerobic exercise-normal nutrition; NN: normal nutrition; ACL: aerobic exercise-hypercholesterolemic; HCL: hypercholesterolemic. * significant difference with ANN.

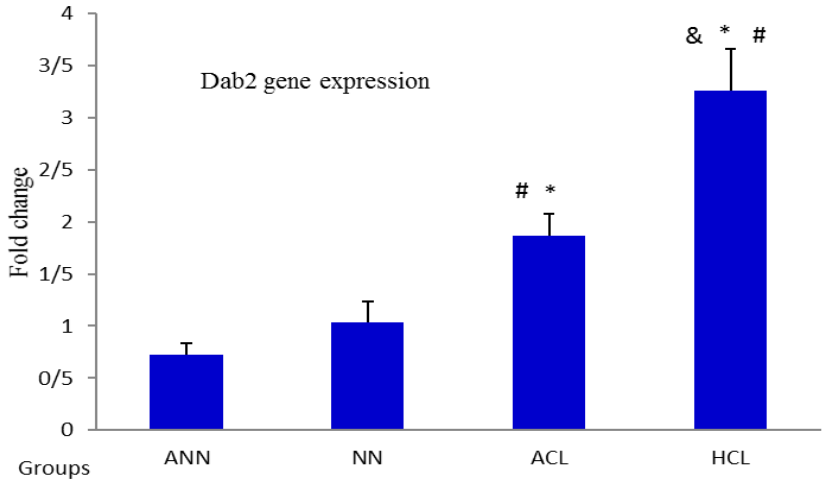

Fig. 2. Relative expression of *Dab2*. ANN: aerobic exercise-normal nutrition; NN: normal nutrition; ACL: aerobic exercise-hypercholesterolemic; HCL: hypercholesterolemic. * significant difference with the ANN; # significant difference with the NN; & significant difference with the ACL.

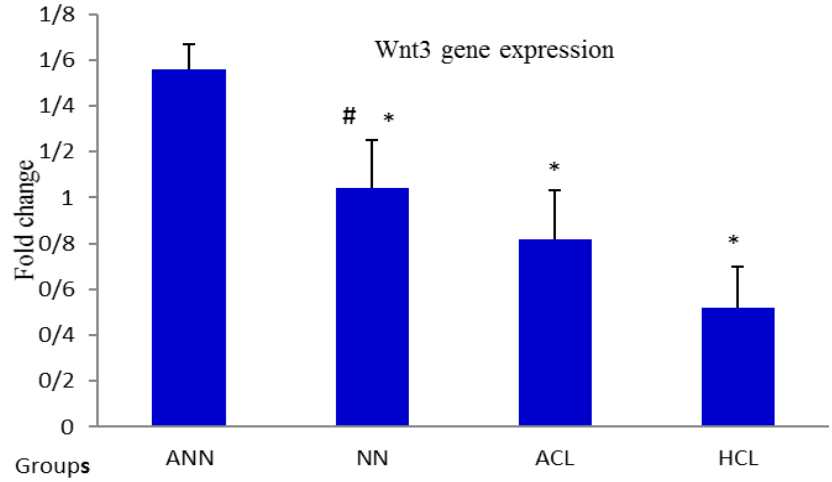

Fig. 3. Relative expression of *Wnt3a*. ANN: aerobic exercise-normal nutrition; NN: normal nutrition; ACL: aerobic exercise-hypercholesterolemic; HCL: hypercholesterolemic.* significant with the ANN; # significant difference with the HCL.

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Tukey post hoc test showed that *Dab2* presented a significant difference in the ANN group in comparison with the ACL (P = 0.001) and HCL (P = 0.001) groups. Furthermore, it was also found that changes in the NN group were also significant in comparison with the ACL (P = 0.001) and HCL (P = 0.001) groups. Finally, a significant difference in *Dab2* expression of the ACL and HCL (p=0.001) groups was observed (Figure 2).

The results also showed that the *Wnt3a* expression in the ANN group was significantly different from the NN (P = 0.001), ACL, and HCL (P = 0.001) groups.

Furthermore, a significant difference was also observed between the expression of *Wnt3a* of the rats in the NN and HCL (P = 0.002) groups (Figure 3).

Discussion

The current study aimed to investigate the relative expression of miR-145 and its target genes following a high-cholesterol diet and aerobic exercise training. For this purpose, of 32 tested rats, 16 rats were kept under a high-cholesterol diet for three months and then randomly assigned into two aerobic exercise-hypercholesterolemic and hypercholesterolemic nutrition.

The results showed that the high concentration of the stable cholesterol inhibited the expression of miR-145 in the rats' cardiomyocytes.

It was also observed that aerobic exercise training caused a relative increase in the expression of the miR-145 in the cardiomyocytes of the exercised rats. This finding is consistent with Liao et al. (2018) and Nielsen et al. (2014) (19, 25) while contradicting the results of Gonzalo-Calvo et al. (2015) (26). It seems that aerobic exercise training can cause a relative increase in the miR-145, but one single session of aerobic physical activity does not have such effects.

In a study on animals, some researchers reported that the physiological hypertrophy of the heart was associated with miR-145 modulation due to swimming exercise, which happened by targeting the components of the PI3K/AKT/mTOR signaling pathway, including phosphoinositide-3- kinase catalytic alpha polypeptide (*PIK3α*), phosphatase and tensin homolog (*PTEN*), tuberous sclerosis complex 2 (*TSC2*) (27). Silva et al. (2017) (28) also showed that miR-145 and Akt phosphorylation expression were significantly normalized due to the exercise training. In cardiac vascular smooth muscle cells where the expression of miR-145 increased, Akt phosphorylation reduced significantly (20).

Several studies have shown that miR-145 has the most frequency in the miRNAs in terms of differentiating vascular smooth muscle cells, and plays a vital molecular role in replacing vascular smooth muscle cell phenotype (29). In addition, miR-145 has been identified as a phenotypic indicator of smooth vascular muscle cells, the regulator of vascular new intima formation control, direct stimulator of transferring myocardial translation, and inhibitor of KLF4 and KLF5 receptor transcription (30). Cheng et al. (2009) also indicated that the excessive expression of miR-145 reduced *KLF4* expression and increased myocardium in rats. Thus, miR-145 can be considered a therapeutic target for limiting the morphology of the atherosclerotic platelets (11), which also inhibits the proliferation of vascular smooth muscle cells (31).

The findings also showed that the increase in the relative expression of miR-145 was higher in the aerobic exercise-normal nutrition group than in the aerobic exercise- hypercholesterolemia group, the control group without exercise training, and the hypercholesterolemia control group. *Dab2* is the target of miR-145 (15). Evidently, miR-145 can reduce the expression of *Dab2* in cardiomyocytes (29). In the present study, it was found that exposure to high concentrations of stable cholesterol increased the expression of the *Dab2*.

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Furthermore, it was also observed that normal nutrition increased the expression of the *Wnt3a*, and cholesterol-rich nutrition inhibited the expression of *Wnt3a*. In both normal nutrition and cholesterol-rich nutrition groups, aerobic exercise training reduced *Dab2* expression and increased *Wnt3a* expression. Aerobic exercise training also increased the expression of miR-145 in both nutritional groups. However, the effect of nutrition type was higher than the exercise training.

Therefore, it can be concluded that high-cholesterol concentrations affect the expression of miR-145, as well as the expression of *Dab2* and *Wnt3a* in cardiomyocytes.

By decreasing the activity rate and increasing the high-cholesterol diet, the expression of miR-145 in the cardiomyocytes of the desert rats decreased; as a result, the expression of *Dab2* gradually increased while the expression of *Wnt3a* decreased. It was found that the expression of *Dab2* increased due to hypercholesterolemic induction, while the expression of miR-145 decreased. It has been reported that overexpression of miR-145 significantly decreased *Dab2* mRNA and protein expression in the left ventricular myocardium of diabetics rats (29). It seems in the recent study that excessive expression of miR-145, resulting from aerobic exercise training, can reverse the expression of *Dab2* and *Wnt3a* due to cholesterol induction. The findings of this study are consistent with the findings of previous studies, indicating that miR-145 decreased in patients with coronary artery disease and the animal model of acute myocardial infarction (32). Therefore, miR-145 can be targeted as a new treatment strategy for managing diabetic heart disease, which is thought to be involved in repairing myocardial infarction or having protective roles against apoptosis caused by oxidative stress.

The present study indicated the molecular regulation of miR-145 in the downstream gene regarding the expression of *Dab2* cardiomyocytes under hypercholesterolemic conditions. It was also shown that by decreasing the miR-145, the expression of *Dab2* increased, and by increasing the *Dab2* expression, Wnt3a expression decreased in the cardiomyocytes. MiR-145 was considered as a therapeutic target for reducing atherosclerosis in rats (14). A better understanding of the precise mechanisms of miR-145 action under hypercholesterolemic conditions can provide a novel insight into the progression of treatment for cardiovascular diseases. Although aerobic exercise training has positive impacts on the lipid profile (33), considering the results of this study, it seems that aerobic exercise merely cannot be effective in increasing the relative expression of miR-145 while following a diet rich in cholesterol. Thus, the protective effects of aerobic exercise have little or no impact on the expression of miR-145, as well as the protective effects of the heart.

The reason behind this issue is that the aerobic exercise training failed to improve the lipid profile in this study, and could also indicate the predominant effect of diet or the type and duration of the aerobic exercise. Moreover, comparing the results of the control group with the exercise training-cholesterol-rich nutrition group confirms this finding. The lowest expression of the miR-145 gene, and thus the highest risk of heart disease, was in the unexercised group with cholesterol-rich diets, indicating a combination of physical inactivity along with a high-fat diet was likely to bring the highest risk of cardiovascular disorders.

It can be concluded that aerobic training and cholesterol-rich foods play an essential regulatory role in the expression of miR-145 and the expression of *Dab2* and *Wnt3a* genes. However, cholesterol-rich foods appear to play a more significant regulatory role than aerobic exercise in this process.

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Conflict of Interest

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The authors declare that they have no competing interests.

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