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Research Article

The Combination Effect of Voluntary Exercise and Crocin on Angiogenic miRNAs in High-Fat Diet/Low-Dose STZ-Induced Type2 Diabetes in Rats: miR-126 and miR-210

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Abstract

Background: As one of the major complications of diabetes, cardiovascular disease might result in early death in people with diabetes. miR-126 and 210 expressions undergo alterations in cardiac disease and cause heart failure.

Methods: Animals were divided into the 5 groups of control (Con), diabetes (Dia), diabetic-crocin (Dia-Cro), diabetic-voluntary exercise (Dia-Exe), and diabetic-crocin-voluntary exercise (Dia-Cro-Exe). Type 2 diabetes was induced by the use of a high-fat diet (4 weeks) and injection of streptozotocin (STZ) (i.p, 35 mg/kg). Animals received crocin orally (50 mg/kg), and voluntary exercise was performed alone or together for 8 weeks. QRT–PCR method was used to determine the levels of miR-210 and miR-126 in cardiac tissue.

Results: The levels of miR-210 and miR-126 in the cardiac tissue augmented in both the crocin and voluntary exercise groups in comparison with the non-treated group ($p<0.001$). The use of combination therapy with exercise and crocin magnified their effects on miR-210 and miR-126 levels ($p<0.001$). Moreover, MiR-210 levels were lower in the crocin group compared to the exercise group ($p<0.001$).

Conclusion: The results indicated that voluntary exercise combined with crocin might provide a novel therapeutic plan for cardiovascular disease through increasing miR-210 and miR-126 expression.

Introduction

Type 2 diabetes is the more prevalent form of diabetes, resulting from the combination of insulin resistance and/or a β-cell secretory defect.[1] This disease which is a major risk factor for the development of cardiovascular complications and cardiovascular disease accounts for 80% of all diabetic mortality.[2,3] It was estimated that the number of adults affected by diabetes in the world would grow from 135 million in 1995 to 300 million in 2025.[4] Hyperglycemia might cause heart injury by multiple mechanisms, one of which is the accumulation of advanced glycation end products (AGEs) that leads to development of coronary artery disease.[5,6]

Crocin, a carotenoid pigment of saffron, has the structure of crocetin di-gentiobiose ester.[7] In line with most types of carotenoids, it is widely known that the antioxidant, anti-inflammatory, and anti-cancer properties of crocin are capable of protecting us against coronary artery diseases.[8-11] Many studies have reported the cardioprotective effects of crocin that are related to the improvement of the antioxidant function and efficient markers of cardiac disease.[12-14] Recently, evidence has showed that crocin has a protective effect on the hypoxic injury of myocardial cells by raising vascular endothelial growth factor (VEGF), which is a proangiogenic factor.[15] Our previous studies indicated that crocin could increase miR-126 and miR-210 in healthy rats and was subsequently able to increase angiogenesis. However, the effect of crocin on these types of miRs in type 2 diabetes has not been reported to date.[16]

Regular physical exercise is associated with beneficial changes in blood pressure, lipid metabolism, glucose metabolism, neurohormonal factors, body weight, and shear stress.[17-19] Recent experimental studies have demonstrated that training exercise by decreasing oxidative stress and increasing VEGF expression could ameliorate cardiovascular disease in type 2 diabetes.[20,21] It has been indicated that forced exercise generates reactive oxygen

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species and increases oxidative stress that could cause injuries to the body.[22] Voluntary exercise is another form of physical activity whose severity ranges from mild to moderate levels. This type of exercise is an efficient therapeutic approach for the prevention of cardiovascular disease.[23] MicroRNAs are 18–22 nucleotides RNAs in length which play a pivotal role in the post-transcriptional regulation of gene expression at the mRNA level.[24] There is increasing evidence showing that certain miRNAs are involved in insulin production and secretion, pancreatic islet development, β-cell differentiation, and insulin resistance in type 2 diabetes. Furthermore, they are influential in various processes of vascular biology, including angiogenesis, vasculogenesis, and incidence of vascular diseases as well as inflammation.[25,26] miR-210 induced by hypoxic and its expression is altered in several diseases such as heart disease and diabetes.[27] It is considered as endothelial cell-specific miRNA, and is strongly expressed in the heart endothelium which plays a significant role in neoangiogenesis.[28] Previous studies have shown that the expression of miR-126 could be reduced in patients with cardiac disease unlike its expression in patients without cardiovascular disease.[29] Moreover, it has been demonstrated that the upregulation of miR-126 and miR-210 in cardiovascular disease could inhibit cell apoptosis and rescue the cardiac system from lethal damage that leads to the upregulation of angiogenesis and protects the cardiovascular system.[30] Hence, considering the protective effects of crocin and voluntary exercise on cardiovascular disease, the present research was designed to evaluate the effect of crocin combined with voluntary exercise on miR-210 and miR-126 in the heart tissue of type 2 diabetic rats.

Materials and Methods

Animals

Male Wistar rats used for this study were purchased by the Medical Faculty of Tabriz University of Medical Sciences (Tabriz, Iran). Animals were housed at a constant room temperature (24˚C), a relative humidity of 50%, and a 12h dark / light cycle with access to food and water ad libitum. All the rats were kept in 4-rat cages for the duration of study except the rats in the exercise groups that were singly housed.

All the groups except Dia-Cro and Dia-Cr0-Exe received NaCl 0.9 % solution orally. Twenty-eight male Wistar rats (200-250 g) were randomly divided into five groups (n=7):

1. Control (Con): rats that received normal saline (8 weeks)
2. Diabetic (Dia): rats that received a high-fat diet (4 weeks) and a low dose of STZ (35mg/kg)
3. Crocin-diabetic (Dia-Cro): diabetic rats that received crocin (50 mg/kg, 8 weeks)
4. Exercise-diabetic (Dia- Exe): diabetic rats that performed exercise (8 weeks)
5. Exercise-crocin-diabetic (Dia-Cro-Exe): diabetic rats that received crocin and performed exercise

Crocin (Sigma, Germany, CAS Number: 42553-65-1) was diluted by normal saline (0.9%) and was administrated (50 mg/kg) 6 days a week for 8 weeks. For the assessment of voluntary exercise, rats were housed individually in a cage containing a wheel (1.00 m circumference, TajhizGostar). This stainless-steel running wheel was equipped with a digital magnetic counter which is activated by wheel rotation. Every exercising rat had a separate running wheel in its cage that enabled it to run voluntarily during 8 weeks of the study. The rats whose running distance was lower than ~2000 m per day were eliminated before statistical analysis.[31]

Induction of Type 2 diabetes in rats by high-fat diet and STZ

Induction of diabetes was performed using STZ that was purchased from Sigma (Germany CAS Number: 18883-66-4). The rats were fed for 4 weeks with a high fat diet that was formulated with carbohydrate 48%, protein 20% and 22% fat mixed with standard laboratory chow containing 53% carbohydrate, 23% protein and 5% fat. At the end of dietary manipulation period, STZ (low dose, 35 mg/kg) was injected i.p (intraperitoneally). Subsequently, the rats were allowed to have free access to standard food and water.[32] After 72 hours of STZ injection, the animals with FBS (fasting blood glucose) ≥300 mg/dl were considered as type 2 diabetic rats.[33]

Preparation of heart tissue

At the end of the experiment, the rats were anesthetized by ketamine/xylazine (88/10 mg/kg, i.p). AGE and glucose were measured by blood samples that were collected from aorta. Then, the heart tissue was immediately removed from rat chest and was washed with 0.9% saline buffer. Subsequently, the heart tissue was weighted and homogenized in 4 ml of ice-cold 0.25 M sucrose buffer (10 mM 2-mercaptoethanol and 0.1 mM EDTA). The prepared homogenates were centrifuged at 30,000 g for 30 min at 4°C. The separated supernatant was kept in -80°C deep freezer for subsequent experiments.

To carry out miRNA analysis, small pieces of the heart tissue were resected, weighed and stored in liquid nitrogen.[34]

Determination of biochemical variables

The blood samples of the control and diabetic rats were taken by cardiac puncture at the end of the study. Serum glucose was measured by an enzymatic glucose oxidase assay performed by an AutoAnalyzer (Abbott, model Alcyon 300, USA). AGE measurement was conducted according to previous methods.[35] Fluorescence intensity was reported as the percentage of fluorescent emission (F %) and recorded at 440 nm following excitation at 350 nm.

miRNA extraction, cDNA synthesis and quantitative PCR

miR-210 and miR-126 expressions were measured by Q-PCR. Each sample was performed in Triplicate. miRNA was extracted from the heart tissue that was stored in liquid nitrogen by miRCURYTM RNA Isolation Kit (Exiqon, Denmark) according to the protocol proposed by the manufacturer. This kit system uses the spin column

method and separates RNA from cell components using a proprietary resin as a separation matrix for RNA. RNA purity and content were measured by a Nanodrop 1000 spectrophotometer (Thermo scientific, Wilmington DE 19810 USA) at a wavelength of 260–280 nm.

LNA universal RT miRNA PCR kit (Exiqon, Denmark) was used for cDNA synthesis. MiRNA was convert to cDNA using a poly (T) primer with a 50 universal tag and a 30 degenerate anchor. Briefly, 20 ng of the extracted RNA was reversely transcribed to cDNA y thermal cycler (Eppendorf, Germany) that was adjusted to 60 min for 42 °C and 5 min at 95 °C.

Real time PCR was performed by Syber Green qPCR Mix (Exiqon denmark) using Rotor-Gene 6000 Corbett (Corbett Life Science, Australia).

The reaction was performed with 1 µL of microRNAs LNA primers (Exiqon, Denmark), 4 µL of diluted cDNA template and 5 µL of PCR master mix (Exiqon) blended in the PCR tube. The reaction was performed at 95 °C for 10 min, followed by 40 cycles of 95 °C for 10 s, and 60 °C for 1 min as it had been recommended in the manufacturer's protocol. Relative quantitative levels of miR-210 and miR-126 were determined using the $2^{-(\Delta\Delta Ct)}$ method. The results were reported as the fold change in comparison with the control group. Mir-1 was considered as he endogenous control miRNA.

Statistical analysis

SPSS Statistics 18 was used for statistical analysis. All the data were expressed as the means±SEM. Two-way ANOVA followed by Tukey post-hoc test were used for intra-group parameters analysis. P<0.05 was considered as a significant difference.

Results

The Effects of crocin combined with voluntary exercise on serum glucose and AGE

Glucose levels in the control and diabetic animals have been presented in Figure 1. In all diabetic animals, the glucose levels were higher than the control group (p<0.001). Crocin and voluntary exercise significantly reduced the serum glucose levels in Dia-Cro and Dia-Exe groups (p<0.05). Surprisingly, crocin combined with exercise triggered a significant decrease in serum glucose compared to the Dia-Cro and Dia-Exe groups (p<0.05).

Upon the accomplishment of the study, the AGE levels in the diabetic groups were significantly higher than the control group (p<0.001 and p<0.01) (Figure 2). The level of AGE concertation was markedly decreased in the diabetic groups that were treated with crocin and voluntary exercise compared to the Dia group (p<0.001). Nevertheless, there was no significant decrease in the Dia-Cro-Exe group in comparison with Dia-Cro and Dia-Exe groups.

The Effects of crocin combined with voluntary exercise on miR-126 expression in the heart tissue

Real-time PCR measurement confirmed that miR-126 was highly upregulated in the heart tissue of crocin and exercise treated diabetic rats compared to the non-treated rats (p<0.001) (Figure 3). A clear upregulation in miR-126 expression was detected in the heart tissue 2 months after the treatment of diabetic rats using a combination of crocin and voluntary exercise compared with the Dia-Cro and Dia-Exe groups (p<0.001). There was no significant difference between the Dia-Cro and Dia-Exe groups.

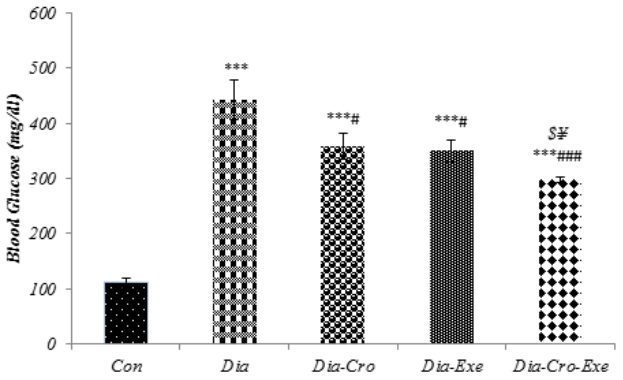

Figure 1. The effect of crocin and voluntary exercise on blood glucose levels. Data are shown as mean ± S.E.M. for 7 animals, ***p<0.001 indicated significantly change compared with Con group, #p<0.05 and ###p<0.001 indicated significantly change compared with Dia group, $p<0.05 indicated significantly change compared with Dia-Cro group and ¥p<0.05 indicated significantly change compared with Dia-Exe group.

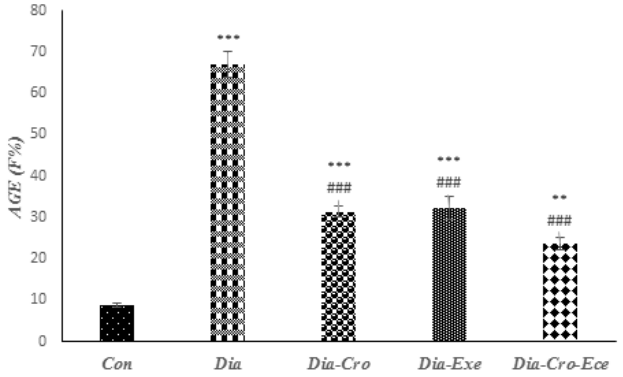

Figure 2. The effect of crocin and voluntary exercise on AGE levels. Data are shown as mean ± S.E.M. for 7 animals, ***p<0.001 and **p<0.01 indicated significantly change compared with Con group and ###p<0.001 indicated significantly change compared with Dia group.

The Effects of crocin combined with voluntary exercise on miR-210 expression in the heart tissue

Two-way ANOVA indicated that the miR-210 levels were significantly higher in the rats treated with crocin or voluntary exercise (p<0.001) than in non-treated rats. The expression of heart miR-210 significantly increased in the rats that underwent voluntary exercise and simultaneously received crocin for 8 weeks compared to the Dia-Cro, and Dia-Exe (p<0.001) groups (Figure 4). Furthermore, there was a significant difference between the Dia-Cro and Dia-Exe groups (p<0.001).

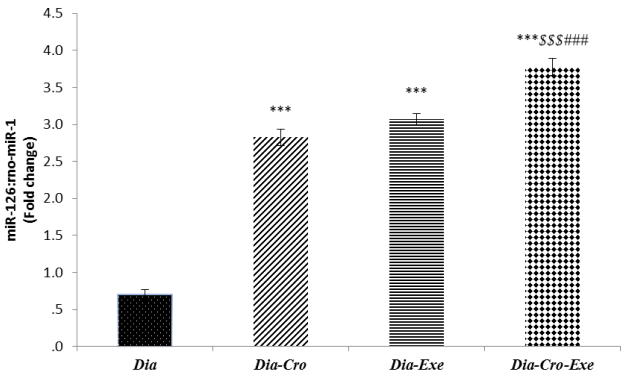

Figure 3. The effect of crocin and voluntary exercise on miR-126 expression levels. Data are shown as mean ± S.E.M. for 7 animals, ***p<0.001 and *p<0. 05 indicated significantly change compared with Con group, ###p<0.001 indicated significantly change compared with Dia group, $$$p<0.001 indicated significantly change compared with Dia-Cro group and indicated significantly change compared with Dia- Exe group.

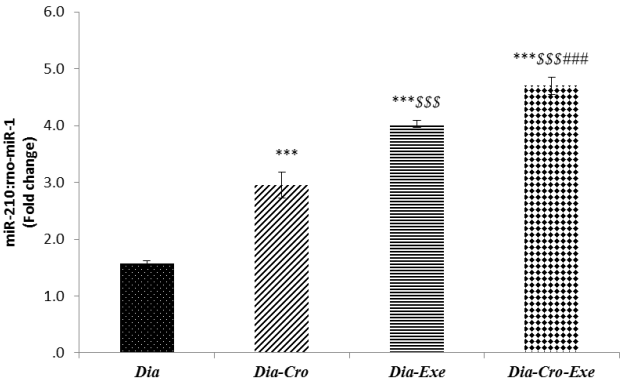

Figure 4. The effect of crocin and voluntary exercise on miR-210 expression levels. Data are shown as mean ± S.E.M. for 7 animals, ***p<0.001 and *p<0. 05 indicated significantly change compared with Con group, ###p<0.001 indicated significantly change compared with Dia group, $$$p<0.001 indicated significantly change compared with Dia-Cro group and indicated significantly change compared with Dia- Exe group.

Discussion

Our results indicated that crocin together with voluntary exercise had a significant effect on FBS. Nonetheless, no remarkable effect was observed on AGE in type 2 diabetic rats. Moreover, we found that miR-126 and miR-210 expressions increased in cardiac myocytes of type 2 diabetic rats by crocin and voluntary exercise treatment that was augmented by the combined use of crocin and exercise. As our data showed, miR-126 and miR-210 could be induced in the heart tissue of diabetic rats by crocin and voluntary exercise.

In recent years, the prevalence of diabetes has increased. Moreover, apart from the other cardiovascular risk factors, the incidence of cardiovascular disease has increased.[4] Insulin resistance is the main hallmark of type 2 diabetes that leads to high levels of blood glucose and subsequently the increasing level of AGE.[36] Glucose lowering effects of crocin and exercise have been shown in in vitro studies.[37,38] It was indicated in the present study that the simultaneous administration of crocin and voluntary exercise decreased the levels of blood glucose and AGE in type 2 diabetic rats. However, the combination of crocin and exercise had synergistic effects on blood glucose but not AGE. The effect of voluntary exercise and crocin on the reduction of blood glucose level and the inhibition of protein glycation might be related to its antioxidant potential.

In the diabetic heart tissue, signaling pathways leading to angiogenesis are disturbed and collateral vessel formation is interrupted.[39,40] Although the increased expression of VEGF (pro-angiogenic factor) promotes angiogenesis, miR-210 and miR-126 are involved and have significant regulatory functions.[41] MiR-210 targets the receptor tyrosine kinase ligand EphrinA3 (EFNA3), enhancing cell migration and capillary-like structure formation induced by VEGF.[42] Moreover, the essential developmental role of miR-126 has been confirmed due to its activation of the survival kinases ERK1/2 and Akt (VEGF signaling pathway) as well its role in increasing pro-angiogenic signaling.[43,44]

Recent evidence has indicated that under diabetic conditions and the existence of increasing risk factors for type 2 diabetes, the expression level of miR-126 could decrease.[45] Furthermore, the recent investigation of miRNA patterns revealed that the upregulation of miR-210 could rescue the cardiac function after myocardial disease by increasing angiogenesis in the heart tissue.[46] In the present study, we showed that miR-126 and miR-210 upregulated in the heart tissue of high-fat-diet-induced type 2 diabetic rats in response to crocin and voluntary exercise. However, a few studies have reported that exercise and crocin could influence miR expression in the cardiac tissue. These results are in line with the study carried out by Anja Bye *et al.*[47] They indicated that exercise activity could cause a significant up-regulation in miR-210 expression level in patients with low Vo2max.However, Baggish *et al.*[48] have reported that there was no significant difference in miR-210 expression between two responses before and after a 90-day period of rowing training that might be due to the duration of the exercise protocol and special type of training. According to the results of our study, the significant increase of the miR-210 takes approximately eight weeks. The first week of training exercise may induce more hypoxemia, and this acute hypoxemia could increase the intensity of hypoxia-inducible factor 1-alpha (HIF- 1α). Eventually, it leads to an increase in the expression of miR-210 that we observed in our research. MiR-210 upregulation in the cardiac tissue could stimulate the formation of capillary-like structures as well as VEGF-driven cell migration.[49]

Furthermore, in line with our results, Bao *et al.* (2018) reported that miR-126 levels could increase after acute aerobic exercise.[50] The major findings of the study carried out by Fernandes *et al.* (2012) confirm that exercise training could restore the levels of peripheral miR-126 associated with revascularization in hypertension.[51] On the other hand, losses of circulating miR-126 and other miRNAs were observed in subjects with type 2 diabetes.[52] Interestingly, the miR-126 reduction of the cardiac tissue

in rats with high-fat diet-induced type 2 diabetes was restored by voluntary exercise in the present study that could be indicative of the potential mechanisms involved in exercise-induced angiogenic miRNA on the cardiovascular complication of type 2 diabetes.

Moreover, in this research, we reported the increasing expression of miR-126 and 210 in type 2 diabetic rats in response to crocin administration. Crocin, known as saffron carotenoid pigment, has wide pharmacological effects on different tissues. Previous studies have revealed that the antioxidant activity of crocin and its improvement of cardiac biomarkers enable it to have cardioprotective effects.[12] The novelty of our research lies in the fact that miR-126 could be regulated by crocin in type 2 diabetes. Furthermore, the level of miR-126 could be decreased in diabetic rats leading to a reduction of cardiac angiogenesis. Likewise, it could be stated that crocin might improve the cardiovascular complication caused by diabetes by miR-126 upregulation. MiR-126, endothelium angiogenesis miR, stimulates its targets PIK3R2, a regulatory subunit of PI3K, and Spred-1 (Sprouty-related protein-1). Moreover, it promotes angiogenesis.[43] The downregulation of these targets enhances the actions of VEGF and increases the cardiac angiogenesis.[28] In addition, as we reported, crocin could enhance the cardiac miR-210 expression in type 2 diabetes, and the high expression of miR-210 in cardiomyocyte cells could increase the ability of cardiac cells to form blood vessels. Also, it has been reported that miR-210 might facilitate angiogenesis through the negative regulation of its target gene, ephrin A3, which is a significant member of the ephrin angiogenesis regulatory gene family.[42] Hence, it could be stated that crocin might improve the cardiac function in type 2 diabetic rats by increasing the miR-126 and miR-210 levels. Finally, we observed that in combination with voluntary exercise, crocin could elevate the miR-126 and miR-210 levels more significantly compared to either crocin or voluntary exercise alone.

Conclusion

In conclusion, the results of the present study reveal that the combination of crocin and voluntary exercise could increase miR-126 and miR-210 expressions (pro-angiogenic miRNAs) in the heart tissue of high-fat-diet-induced type 2 diabetic rats. Since these miRNAs could regulate the expression of multiple target genes leading to angiogenesis, the simultaneous use of crocin and exercise could influence an angiogenesis gene network through upregulated miR-210 and miR-126, and thereby modify the cardiovascular complication of diabetes.

Ethical Issues

This study was approved by the Animal Ethics Committee in accordance with the instruction for the care and use of laboratory animals prepared by Tabriz University of Medical Sciences (permit number 93/5/4).

Conflict of Interest

The authors declare they have no conflict of interest.

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