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The Effect of 8-Week Aerobic Exercise on the Expression of Regulatory Subunits of NADPH Oxidase 2 (*p47phox* and *p67phox*) in the Cardiac Tissue of Diabetic Rats

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Abstract

Introduction: Oxidative stress seems to play a major role in diabetes-induced cardiac dysfunction, known as diabetic cardiomyopathy (DCM). Nicotinamide adenine dinucleotide phosphate (NADPH) oxidase 2 is considered as one of the main enzymatic systems which primarily contributes to the production of reactive oxygen species in various organs including the heart. The present study aimed to investigate the effects of 8 weeks of aerobic exercise (AE) on the expression of *p47phox* and *p67phox*, which are regarded as the regulatory subunits of NADPH oxidase 2 in the cardiac tissue in diabetic rats.

Methods: A total of 36 male Wistar rats with a mean weight of 231±25 g were randomly divided into non-diabetic, control diabetic, and trained diabetic groups (each containing 12 rats). Nicotinamide and streptozotocin were used to induce diabetes in the rats. The cardiac muscle was removed under sterile conditions 48 hours following the last training session. Finally, the mRNA levels of *p47phox* and *p67phox* were evaluated using the real-time polymerase chain reaction.

Results: The results showed that diabetes induction significantly increased the gene expressions of *p47phox* and *p67phox* in the cardiac tissue of diabetic rats. The expression of these genes was significantly attenuated after 8 weeks of AE.

Conclusion: In general, AE was found to prevent the negative effects of diabetes by suppressing *p47phox* and *p67phox* in the cardiac tissue of diabetic rats. Therefore, this can improve cardiac function and may be a potential preventive or therapeutic modality for DCM.

Keywords: Aerobic exercise, NADPH oxidase, *p47phox*, *p67phox*, Cardiac tissue, Diabetic cardiomyopathy

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Introduction

Diabetes mellitus comprises one of the most prevalent metabolic diseases in the world with several subsequent complications.[1] In addition, diabetic cardiomyopathy (DCM), defined as myocardial dysfunction or abnormal heart structure in the absence of epicardial coronary artery disease, hypertension, or other serious valvular conditions, is considered as a major diabetic complication encompassing >50% of diabetes-related comorbidities.[2,3]

Hyperglycemia-induced oxidative stress (i.e., excessive production of reactive oxygen species, ROS) plays a critical role in the pathogenesis of DCM and the damage to cardiomyocytes in this condition.[2,3] The nicotinamide adenine dinucleotide phosphate (NADPH) oxidase is an enzymatic system contributing to the production of ROS within the cardiomyocytes[2] and has five isoforms (Nox1-5). In cardiomyocytes, both Nox2 (NADPH oxidase 2) and Nox4 (NADPH oxidase 4) isoforms are the predominant forms of NADPH oxidase.[2] Nox2 is a membrane heterodimeric protein constituting of *gp91phox* and *p22phox* subunits.[4] Further, the Nox2 is activated following the association of membrane-linked catalytic components (i.e., *gp91phox* and *p22phox*) with cytosolic subunits (i.e.,

p47phox, *p67phox*, *p40phox*, and *Rac1*).[5,6] Furthermore, NADPH oxidase activation culminates in the synthesis of superoxide radical ($O_2^{\cdot-}$). This species is highly unstable, as well as reactive and is rapidly dissociated for producing hydrogen peroxide (H_2O_2) which is a more stable and diffusible compound.[2] Several reports have recently demonstrated the primordial role of NADPH oxidase in the process of ROS synthesis within cardiomyocytes.[7,8] In particular, NADPH oxidase is noted to be the primary source of hyperglycemia-induced ROS in cultured cardiomyocytes which are derived from diabetic animal models.[7,9] Moreover, exposure to high glucose concentrations upregulates *p47phox* and *p67phox* subunits of NADPH oxidase, which, in turn, increases ROS concentration within cardiomyocytes.[9]

The role of this enzyme in DCM is well-documented.[10-12] Additionally, the inhibition of NADPH oxidase by removing its cytosolic subunits in the cardiac cells of diabetic rats reduces the production of ROS that subsequently prevents hypertrophy, cardiac fibrosis,[13] as well as myocardial remodeling,[14] and triggers myocardial contractile dysfunction.[11] Hence, prohibiting NADPH oxidase activity is suggested as a therapeutic target for diabetes-related complications including DCM.[10]

The antioxidant impacts of exercise are well-documented as well.[15,16] In fact, the exercise induces the genes which encode the subunits of antioxidant enzymes in the cardiac tissue.[17,18] However, only Sharma et al[19] investigated the impacts of AE on the NADPH oxidase activation in the cardiac tissue of diabetic rats. Therefore, the current study sought to examine the impacts of 8 weeks of AE on the expressions of *P47phox* and *p67phox*, as the regulatory subunits of NADPH oxidase 2 in the cardiac tissue of streptozotocin-induced diabetic rats.

Materials and Methods

Rats

Six to 8 weeks old male Wistar rats with a mean weight of 231 ± 25 g were obtained from the Laboratory of Bearing and Multiplying at the Baqiyatallah University of Medical Sciences, Iran. The animals were kept in standard conditions (i.e., the temperature of 22 ± 2°C, 5 humidity of 5±3%, and a reverse light-dark cycle of 12:12-h) and had free access to standard food and water. After being acclimatized to the condition (2 weeks), 36 rats were divided into non-diabetic (N-Di), control diabetic (Con-Di), and trained diabetic (T-Di) groups (n = 12 for each group).

Diabetes Induction

Briefly, 65 mg/kg streptozotocin (STZ, Sigma), dissolved in 0.1 M citrate buffer (with a pH of 4.5), was injected intraperitoneally (i.p) to induce diabetes. Nicotinamide (120 mg/kg, i.p, NA; Sigma), dissolved in normal saline, was infused 15 minutes before STZ.[20] Then, the blood sample was obtained from the tail vein, followed by measuring the fasting blood glucose (FBG) level using a glucometer (Beurer, Model: no: GL42 Germany). Animals with FBG >250 mg/dL at three days post-injection were considered as diabetic cases.

Exercise Protocol

One week after diabetes induction, rats in T-Di groups were familiarized with running on a 5-line motorized rodent treadmill (10 min/d, 10 m/min speed, grade 0%) for 5 consecutive days.[21] Exercise training was performed according to the protocol used by Kazemi et al[21] (5 days/week for 8 weeks at a 5% grade). Accordingly, 15 minutes running at 12 m/min was performed at the first week. The duration of running (5 min/wk) and its intensity (4 m/min per 2 weeks) were gradually boosted to reach 40 minutes per 20 m/min at the week 6. Eventually, the duration of exercise increased to 45 minutes at 24 m/min during the last 2 weeks.

Sample Collection

The rats were anesthetized (i.p injection of 60-80 mg/kg ketamine and 8 mg/kg xylazine) 48 hours following the last exercise session in order to avoid data misinterpretation due to residual effects of the exercise protocol. Then, chest cavities were opened and the hearts were immediately isolated. Next, the tissues were rinsed in cold normal saline 9%. Then, the left ventricle was excised and quick-frozen in liquid nitrogen. Finally, the tissues were kept in freezing (-80°C) for further analysis.

Real-Time Polymerase Chain Reaction

The total RNA extraction kit (CinnaGen, Inc, Iran) was used to extract cardiomyocytes total RNA according to the manufacturer's protocol. As instructed by the manufacturer, the extracted total RNA (5 µg) served as a template for synthesizing the cDNA (First Strand cDNA Synthesis Kit, Takara, Japan). The mRNA levels of *p47phox*, *p67phox*, and *β-actin* were then determined (LightCycler® 96 System, Roche Diagnostics). The applied primers in this study are listed in Table 1. Overall, 45 cycles were considered (94°C,15 seconds; 60°C, 25 seconds; 72°C,

Table 1. The Sequences of the Used Primers

Gene	Forward (5′ to 3′)	Reverse (5′ to 3′)
β-actin	CGTTGACATCCGTAAAGACCTC	TAGGAGCCAGGGCAGTAATCT
p47phox	TCACCGAGATCTACGAGTTC	ATCCCATGAGGCTGTTGAAGT
p67phox	GAAAGCATGAAGGATGCCTGG	ATAGCACCAAGATCACATCTCC

25 seconds) preceding by an initial denaturation phase (94°C, 4 minutes).

The melting curve analysis was performed to confirm the reaction specificity. In addition, the relative gene expression was computed using the $2^{-\Delta\Delta CT}$ method. The data were normalized to the expression of *β-actin* and expressed as fold changes relative to the N-Di group.

Statistical Analysis

The SPSS software, version 19 was applied as the statistical instrument. Further, one-way ANOVA was utilized as the univariate inferential test. Furthermore, post hoc Tukey test was applied for between-group comparisons. All the values were expressed as mean ± SD. *P* values <0.05 were considered statistically significant.

Results

Animal Characteristics

Based on the results, body weight was not significantly different between the N-Di and Con-Di groups after 8 weeks of training, though, a significant decrease was observed in T-Di group compared to the N-Di group. However, blood glucose in diabetic rats (i.e., Con-Di and T-Di groups) was significantly higher compared to N-Di. Comparing diabetic rats, blood glucose was significantly lower in T-Di group compared to Con-Di. Table 2 demonstrates the general characteristics of the rats at the time of sacrifice.

The Effects of Aerobic Exercise on the Expression of *p47phox* and *p67phox*

As shown in Figure 1, the expression of *p47phox* (2.2-fold) and *p67phox* (1.96-fold) significantly increases in the Con-Di group compared to N-Di group. Conversely, aerobic exercise (AE) leads to a significant decrease in the expression of *p47phox* (30.4% decrease) and *p67phox* (24.4% decrease) in the T-Di group compared to the Con-Di group. However, these reductions fail to reach the levels of the N-Di group.

Discussion

The present study aimed at evaluating the role of 8 weeks of AE on the expressions of *p47phox* and *p67phox* in the cardiomyocytes of diabetic rats. Both *p47phox* and *p67phox* are viewed as the regulatory components of NADPH oxidase 2. The results of the current study showed that the increased expressions of *p47phox* and *p67phox* were attenuated in the cardiac tissue of diabetic rats following AE Which are in line with those of previous studies.[19,22] For example, Sharma et al[19] have recently found that three weeks of AE attenuated the expression of *P47hox*, *p67phox*, and collagen III (i.e., an index of myocardial stiffness and DCM) in the cardiac tissue of diabetic rats. Similarly, Cunha et al[22] reported that 12 weeks of AE decreased the expressions of Nox2 and p47phox (the first at the gene and the later in protein levels). Furthermore, NADPH oxidase activity decreased in skeletal muscle tissue of the rats with a heart failure. This finding is consistent with that of Mahmoud et al[23] who demonstrated that AE reduces the expression of Nox2 at protein levels in the skeletal muscle of obese insulin-resistant adults.

AE is suggested to decrease the NADPH oxidase activity in the cardiac tissue of diabetic rats thanks to its antioxidant properties.[15,16] However, the underlying mechanisms for the reduced gene expression of *p47phox* and *p67phox* are not clearly understood. On the other

Table 2. The Characteristics of the Rats at the Time of Sacrifice

Variables	N-Di (n=7)	Con-Di (n=8)	T-Di (n=8)
Body weight (g)	279±38	250±48	220±34[a]
Blood glucose (mg/dL)	73±8	325±32[b]	179±34[cd]

Note. Values represent mean ± SD.
N-Di: Non-diabetic; Con-Di: Control diabetic; T-Di: Trained diabetic.
[a]*P*<0.012 versus N-Di; [b]*P*<0.001 versus N-Di; [c]*P*<0.001 versus Con-Di; [d]*P*<0.001 versus N-Di.

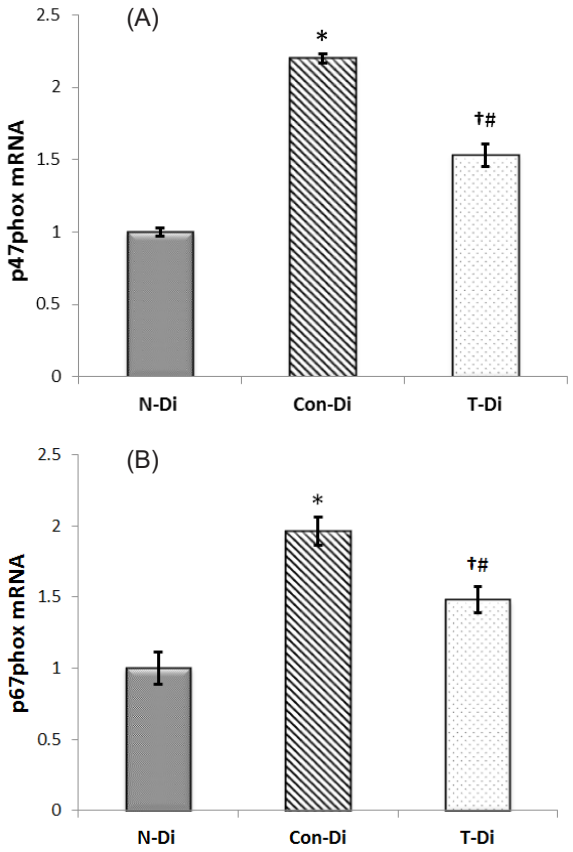

Figure 1. The mRNA Expression of (a) *p47phox* and (b) *p67phox* in Non-diabetic (N-Di), Control Diabetic (Con-Di), and Trained Diabetic (T-Di) Groups After 8 Weeks of AE. The data are presented as mean±SD. *Note*. * represents *P*<0.001 respective to the N-Di group; # denotes *P*<0.001 respective to the Con-Di group; & demonstrates *P*<0.001 respective to the N-Di group; Gene expressions are indicated as fold changes relative to the N-Di group (assigned a value of 1).

hand, the elevated blood sugar is detected to increase the circulating blood inflammatory markers including tumor necrosis factor alpha (TNF-α) which can be as mediators for the altered gene expression pattern in the cells.[24] Moreover, TNF-α participates in the NADPH oxidase-triggered superoxide production and is known as an agonist for the NADPH oxidase.[24] Based on the findings of several studies, AE reduces blood glucose[25-27] and TNF-α[28,29] up to their normal levels in patients with diabetes. Therefore, the activity of NADPH oxidase probably decreases by reducing the levels of TNF-α and returning the blood glucose to their normal levels.[19]

The results of the current study further revealed that fasting blood glucose levels significantly reduced in T-Di group compared to Con-Di group which might be responsible for the reduction in the levels of the regulatory subunits of NADPH oxidase. In addition, increased and activated protein kinase C (PKC),[30,31] angiotensin II (Ang II),[32] and advanced glycation end products (AGEs),[33] observed in the diabetic heart, are found to positively induce the NADPH oxidase activity.[8,34-36] Accordingly, the reductions of PKC, Ang II, and AGEs in the diabetic condition negatively influence the NADPH oxidase activity. In this regard, the results of Chengji and Xianjin[37] showed that an AT attenuates the gene expression of PKC in the cardiomyocytes of diabetic rats and concluded that the reduced expression of PKC can prevent or improve DCM. It was further emphasized that AE attenuates diabetes-induced elevations in AGEs[38] and Ang II[39] in the aorta and cardiac tissue of the trained rats, respectively.

Nevertheless, further investigation is required to divulge the exact mechanisms responsible for the reduced gene expression of *p47hox* and *p67phox* in the cardiomyocytes of diabetic rats. No western blot experiments or parallel tests were performed on the serum samples, which is regarded as a limitation of the current study. Therefore, similar studies are suggested to be conducted in the future considering the above-mentioned tests.

Conclusion

In general, the results indicated that 8 weeks of AE decreased the diabetes-induced elevations in *p47hox* and *p67phox* (i.e., the regulatory subunits of NADPH) oxidase 2 in the cardiomyocytes of diabetic rats. These results suggested that AE may be an effective non-pharmacological tool for preventing the oxidative stress in diabetic hearts and therefore, can prevent or improve DCM. As a result, more studies are necessary to disclose the mechanisms of beneficial effects of AE on NADPH oxidase-induced oxidative stress and DCM.

Ethical Approval

In the study, all the animal procedures were conducted in accordance with the Local Ethics Committee of the University of Isfahan under the code of IR.UI.REC.1396.061.

Competing Interests

The authors declare that they have no competing interest.

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