Original Article

Attenuation of Phosphorylated Connexin-43 Protein Levels in Diabetic Rat Heart by Regular Moderate Exercise

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Abstract

Background: High blood glucose levels increase the ratio of phosphorylated to non-phosphorylated connexin-43 amounts, which leads to the decomposition of the hyperphosphorylated connexin-43. This can cause heart arrhythmia in diabetic patients. Considering the effective role of exercise in diabetic patients, and because there are few studies regarding the effect of exercise on phosphorylated connexin-43 protein levels, in the present study the impact of different periods of moderate regular exercise on phosphorylated Connexion-43 levels were examined.

Methods: Sixty (60) male Wistar rats (300 ± 50 g) were randomly divided into six groups (n = 10). A week after induction of diabetes by injection of streptozotocin, one hour treadmill exercise, 5 days a week with 22 (m/min) speeds was undertaken. Left ventricles of hearts were isolated and immediately frozen. Finally, phosphorylated connexin-43 protein levels were measured by ELISA method.

Results: The means of blood glucose levels were significantly decreased (P < 0/05) by increasing days of exercise. The means of blood glucose levels were significantly decreased (P < 0/05) by increasing days of exercise. Regular moderate exercise reduced the connexin-43 levels by increasing days of exercise (P < 0.01).

Conclusion: It is concluded that regular moderate exercise reduces the amount of phosphorylated connexin-43 protein levels in the ventricular myocardium, by reducing blood glucose levels. This can result in partial inhibition of cardiac arrhythmia observed in diabetic patients.

This research was done in Faculty of Natural Sciences, University of Tabriz, Tabriz, Iran.

Keywords: Arrhythmia, blood glucose, exercise, phosphorylated connexin-43

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Introduction

Diabetes mellitus type I is a risk factor for diabetic cardiomyopathy.[1] Ventricular fibrillation and sudden death are a higher incidence of cardiac arrhythmias in diabetic patients.[2] In heart, intercalated discs which contain gap junctions connect the adjacent cardiomyocytes to each other.[3] Structurally, in gap junctions that conduct electrical signals,[4] there are intramembranous proteins called connexin.[5] The dominant connexin of the left ventricle is connexin-43.[6] Connexin-43 is a kind of phosphor-protein. Potential mechanisms controlling the level of intracellular communications in the heart include a regulation of connexin-43 dynamics and its phosphorylation.[7] Protein kinase C plays an important role in regulating the permeability of gap junctions with phosphorylation of connexin-43. The activity of protein kinase C remarkably increases in diabetic patients.[8] High blood glucose levels increase the ratio of phosphorylated to non-phosphorylated connexin-43, which leads to degradation of the hyperphosphorylation connexin-43. Degradation of connexin-43 would reduce the total number of channels in gap junctions and electrical conductivity, and consequently may lead to arrhythmias of ventricular myocardium.[2,4]

Although insulin and other glucose-lowering drugs are the most common treatments of diabetes, the beneficial effects of exercise in regulating glucose metabolism and insulin activity have also been proved (Loganathan et al., 2007), (Chipkin et al., 2001).[9,10] Previous studies have demonstrated the beneficial effects of exercise on cardiac performance in diabetes mellitus; for example, exercise training has been shown to reverse bradycardia and hypotension in diabetic rats.[11] Exercise may also delay the progression of diabetic complications such as cardiomyopathy.[12] End-systolic volume, end-diastolic volume and ejection fraction reduction can be noted as indicators of recovery of left ventricular function that are other benefits of exercise.[13,14] Considering the beneficial effects of exercise, including regulation of glucose metabolism, and the fact that the amount of phosphorylated connexin-43 increases by hyperglycemia and diabetes, as well as the fact that there are few studies on the effects of exercise on phosphorylated connexin-43 protein levels in animal model of diabetes, in this investigation the effects of regular moderate exercise on phosphorylated connexin-43 protein levels in left ventricles of hearts of diabetic rats were studied. Also, some pathological studies were done.

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Materials and Methods

Animals

Adult male Wistar rats (300 ± 50 g) were obtained from the Animal House of the University of Tabriz. The rats were maintained in a 12-hr light/dark cycles at 22 ± 2°C and were allowed free access to standard laboratory chow and water. Additionally, care was taken to use the minimum number of animals possible in each experiment.

Experimental designs

Animals were randomly divided into six groups (10 each): 1) Sedentary Control group (SC), 2) Sedentary Diabetic group (SD), 3) Healthy Control with 15-day Exercise group (H15E), 4) Healthy control with 60-day Exercise group (H60E), 5) Diabetes with 15-day Exercise group (D15E), 6) Diabetes with 60-day Exercise group (D60E). Diabetic animals were housed triad in cages.

Experimental protocols

Diabetes type I was induced in rats by intraperitoneal injection of 60 mg/Kg Streptozotocin (STZ) (Sigma, St Louis, MO).[15] STZ was dissolved in citrate buffer (1:1 mixture of 0.1 M citric acid and 0.2 M Na2HPO4) just before injection. Rats in the control groups received an intraperitoneal injection of an equal volume of citrate buffer instead of STZ. Forty-eight h after STZ injection, blood samples were obtained from the tail vein and blood glucose concentrations were measured with a Surestep glucometer. Successful induction of diabetes was defined as blood glucose level equal to 250mg/dL [16] Also, polyuria and polydipsia were observed in diabetic rats.

Exercise protocols

Before beginning the formal 15 and 60 day exercise protocol, animals were habituated to treadmill running (5–20 min/day) for 5 consecutive days. After this period of habituation, the exercised animals performed 5 days of consecutive treadmill exercise (60 min/day) at 22 m/min speeds.[17] At the beginning of the 60-minute exercise, to warm up the rats, the treadmill speed was set to 5 m/min and progressively increased to 22 m/min. At the end of the 60-minute exercise, the speed progressively decreased to 5 m/min to cool down. Mild electrical shocks were used sparingly to motivate animals to run. Control animals did not perform treadmill exercise but were placed on a non-moving treadmill for 60 min/day for 5 days a week.[18] Exercised animals were studied 24 h after their last exercise session.

Measurement of blood glucose levels and body weight

The blood glucose concentrations of diabetic rats were measured in blood collected from the tail vein in the morning, at the beginning and end of each experimental period. Furthermore, the blood glucose levels of diabetic running groups were measured on the seventh (fifteenth day of exercise) and thirtieth days (sixtieth day of exercise) of the running period. The body weight of all rats was measured.

Sample collection

After the experimental periods, all rats (control and diabetic groups) were anesthetized by intraperitoneal injection of 100 mg/Kg ketamine and 5 mg/Kg xylazine.[19] The hearts were immediately removed and washed with cold 9% normal saline; seven left ventricles were excised from hearts and placed in liquid nitrogen and then stored at -80°C for later homogenization and biochemical assays. Also, three left ventricles of the sedentary control, sedentary diabetic and diabetes with 60-day exercise groups were separated for pathological studies. Pancreatic tissues were studied histopathologically to confirm necrosis induced by STZ.

ELISA assay

All tissues were homogenized in phosphate buffer saline (PBS) and stored overnight at -20°C. After two freeze-thaw cycles performed to break the cell membranes, the homogenates were centrifuged for 5 minutes at 5000×g. The supernatants were removed immediately. The assays were performed according to the manufacturer's protocol (Cosabio CSB-E17273r).

Histopathological studies

To avoid autolysis, immediately after euthanasia, tissue samples collected from left ventricles of hearts and pancreases were fixed in 10% formalin solution. After fixation, dehydration, clearing, embedding, blocking, sectioning and staining with hematoxilin-eosin, slides were studied with light microscopy.

Data analysis and statistics

Data were analyzed using analysis of variance (ANOVA). The post hoc tukey was performed to determine which condition differed significantly from another.

Results

Effects of streptozotocin on left ventricle and pancreatic tissue

Numerous lipid vesicles were observed in the sedentary diabetic group but were decreased by exercise in diabetic rats with 60-day exercise. Also, the lipid vesicles diameter in 60-day exercise group was reduced by exercise (Figure 1).

Streptozotocin-induced necrosis was observed in the beta cells of the Langerhans islets (Figure 2).

Effects of diabetes on metabolic characteristics

At the beginning of the experiment, initial fasting blood glucose measurements showed significant increases in blood glucose levels in the diabetic rats compared with the healthy rats ($P < 0.05$) (Table 1). At the beginning of the exercise period, means ± SEM of blood glucose levels in diabetic running groups and sedentary diabetic group did not show any significant difference, but there was a significant difference in blood glucose levels between diabetes with 15-day exercise group and sedentary diabetic group at the end of the experiment ($P < 0.05$). Also, after exercise for sixty days, in diabetics with 60-day exercise group the blood glucose levels were significantly decreased compared with those of the sedentary diabetic group ($P < 0.05$) (Table 1). The fasting blood glucose of the sedentary diabetic group showed a significant increase throughout the experimental period ($P < 0.05$). Furthermore, diabetics with 60-day exercise showed a significant decrease in fasting blood glucose levels compared with diabetic with 15-day exercise rats ($P < 0.05$). The sedentary diabetic group showed significant weight loss during the test ($P < 0.05$). It was clear that regular moderate exercise inhibits weight loss that was observed in the sedentary diabetic group. Also, a partial increase in weight was seen in these groups (Table 1).

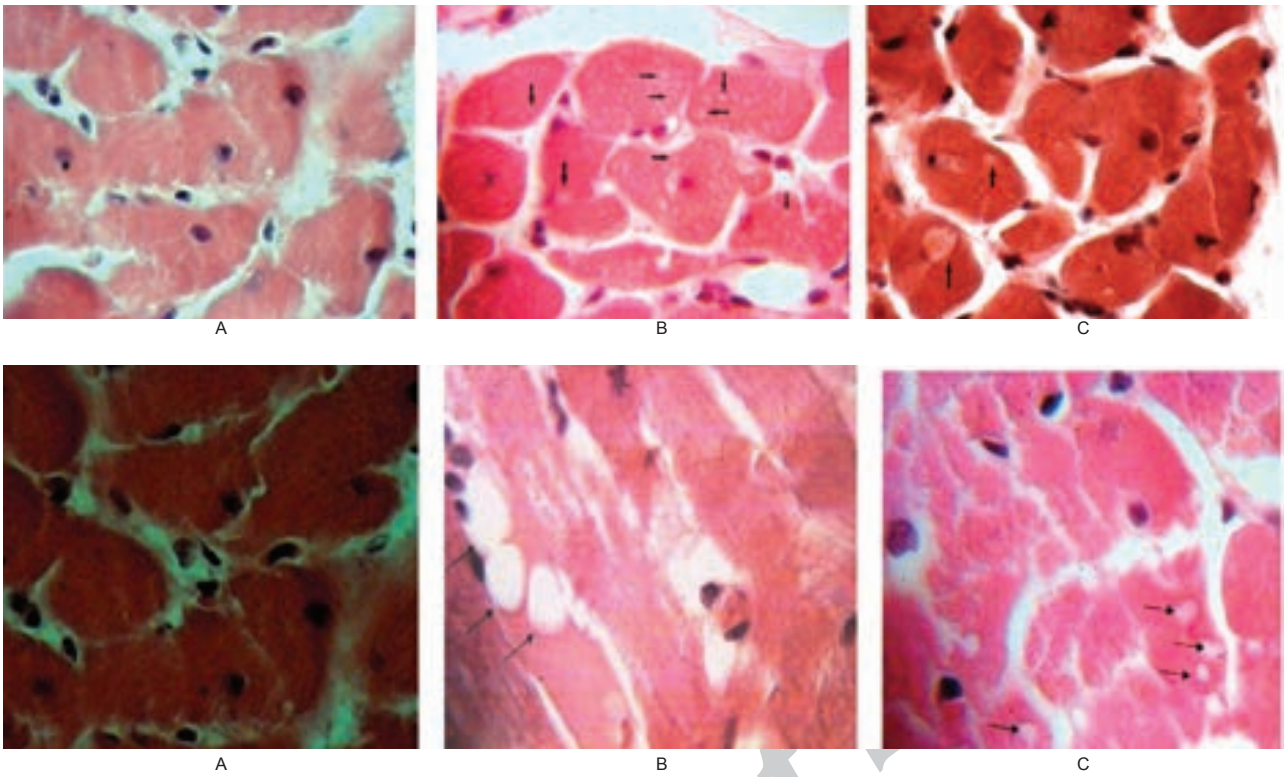

Figure 1. Photomicrograph of left ventricle tissue. Lipid vesicles are seen in myocytes (arrows). **A)** Sedentary Control, **B)** Sedentary Diabetic, **C)** Diabetes with 60-day Exercise (×400).

Table 1. Blood glucose levels and body weight of diabetics and healthy rats. Sedentary Control group (SC), Sedentary Diabetic group (SD), Healthy Control with 15-day Exercise group (H15E), Healthy Control with 60-day Exercise group (H60E), Diabetes with 15-day Exercise group (D15E), and Diabetes with 60-day Exercise group (D60E).

Parameter means ± SEM	SC	SD	H15E	D15E	H60E	D60E
Initial Blood Glucose (mg/dL)	85.3 ± 4.3*	378.8 ± 39.1	89.0 ± 4.4*	390.3 ± 41.1	91.3 ± 5.2*	324.8 ± 17.7
Final Blood Glucose (mg/dL)	88.3 ± 5.2*#	455.5 ± 33.8#	88.5 ± 3.8*#	317.3 ± 26.9*#	81.5 ± 3.4*#	175.8 ± 34.8*
Initial Body Weight(g)	271.6 ± 4.2	281.3 ± 10.2	268.1 ± 6.0	275.7 ± 7.9	265.3 ± 6.5	285.8 ± 5.9
Final Body Weight(g)	280.1 ± 8.1*	183.0 ± 10.9	279.8 ± 8.6*	261.8 ± 14.0*	259.1 ± 9.7*	288.8 ± 23.5*
*Significantly different from SD and #significantly different from D60E ($P < 0.05$).						

Phosphorylated Connexin-43 analysis

Phosphorylated connexin-43 protein levels were significantly increased in sedentary diabetic rats compared with sedentary control rats ($P < 0.01$) (Figure 3). Phosphorylated connexin-43 protein levels in diabetics with 15-day exercise did not show a significant decrease compared to the sedentary diabetic group but the treatment with 60-day exercise led to a significant decrease in phosphorylated connexin-43 protein levels in left ventricles of the diabetics with 60-day exercise compared with the sedentary diabetic rats ($P < 0.01$) (Figure 3). Means ± SEM values of phosphorylated connexin-43 protein levels in diabetics with 60-day exercise were significantly lower than diabetics with 15-day exercise ($P < 0.01$) (Figure 3). 60-day exercise decreased the phosphorylated connexin-43 protein levels in healthy 60-day exercise group in comparison with those of sedentary control rats but it was not significant (Figure 3).

Discussion

The results showed that type I diabetes caused a significant increase in the levels of phosphorylated connexin-43 in the left ventricles of sedentary diabetic rats compared to sedentary control rats (Figure 3) which is similar to the results of other studies.[2,4,20,21] Connexin-43 can be in one nonphosphorylated state (P0) and three different phosphorylated forms (P1, P2, and P3). In previous studies, it was found that diabetes and high blood glucose levels can cause an increase in protein kinase C activation and thus enhance the phosphorylated connexin-43 protein levels.[8] In other words, high blood glucose level significantly increases the P3/P0 ratio. STZ enters beta cells and by induction of necrosis in them, decreases insulin levels and increases blood glucose levels.[22] These high blood glucose levels increase protein kinase C activation and this enhances the phosphorylation of connexin-43 protein and elevating its level. Also, according to a study in 2008 by Howarth et al., diabetes does not alter expression of connexin-43

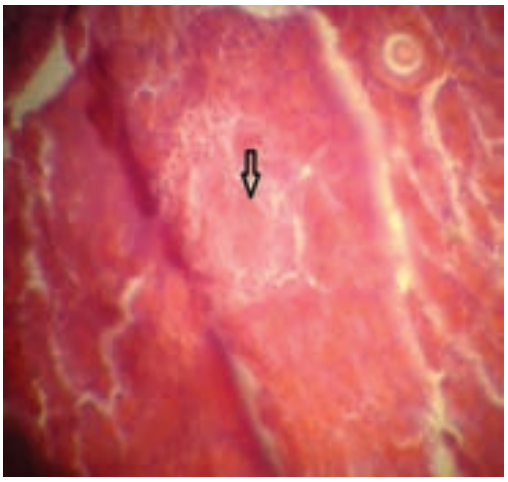

Figure 2. Photomicrograph of pancreatic tissue. Necrosis is obvious (arrow) (×400).

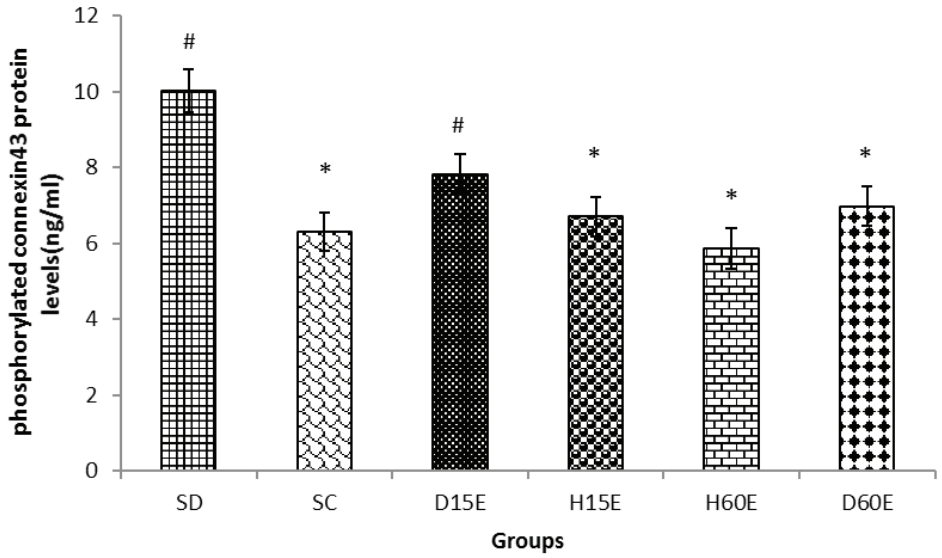

Figure 3. Phosphorylated connexin-43 protein levels in Sedentary Control group (SC), Sedentary Diabetic group (SD), Diabetes with 15-day Exercise group (D15E), and Diabetes with 60-day Exercise group (D60E), Healthy with 15-day Exercise group (H15E) and Healthy with 60-day Exercise group (H60E).
*Significantly different from SD and # significantly different from D60E ($P < 0.01$).

mRNA and nonphosphorylated connexin-43 amounts.[2] For this reason, the effect of different periods of regular moderate exercise on phosphorylated connexin-43 protein levels were examined by ELISA method.

The results of the present study show that 15-day regular moderate exercise training decreases the blood glucose levels in diabetic running group and by continuing the exercise, blood glucose levels will be closer to normal levels (Table 1). These results are consistent with findings in 60-day voluntary exercise treatment[23] but it is in contrast with some other studies.[24,25] The reason for this difference could be due to the type, intensity and duration of exercise. Exercise plays an important role in reducing blood glucose levels by increasing the sensitivity of GLUT4 in muscle as well as the insulin receptor substrate.[26]

Few studies have addressed the impact of exercise on phosphorylated connexin-43 protein levels; however, the results of this investigation showed that phosphorylated connexin-43 protein levels in diabetic 15-day exercise group was decreased insignificantly while in diabetic 60-day exercise group the amount of phosphorylated connexin-43 was significantly decreased compared to the sedentary diabetic group (Figure 3). In other words, by following this type of exercise, the phosphorylated connexin-43 protein levels will be closer to the sedentary control group.

STZ with increase in blood glucose levels, increases the activity of protein kinase C that promotes phosphorylation of connexin-43, which in turn suppresses gap junction intracellular communication and electrical conduction, and consequenty may lead to arrhythmias of ventricular myocardium.[2] The findings of the present study showed that 60-day exercise reduces the blood glucose levels more than 15-day exercise; this result also applies in the case of phosphorylated connexin-43 protein levels, so it can be concluded that there is a direct relationship between phosphorylated connexin-43 and blood glucose levels.

Pathological studies indicate that the diameter of lipid vesicles

increased in the sedentary diabetic myocytes compared to sedentary control rats. This may be caused by cell metabolic dysfunction. Loganathan et al., in 2012 demonstrated that exercise has an important role in improving cellular function by reducing type I diabetes complications[27] as our study showed that 60-day exercise can reduce cell complications in diabetes.

Thus, the major finding of this research points to another effective role of regular moderate exercise on improvement of the microcellular (hyperphosphorylation of phosphorylated connexin-43 protein levels) and macrocellular (Increase the lipid vesicles diameter) complications of diabetes.

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