Changes in Cardiac Levels of Caspase-8, Bcl-2 and NT-proBNP Following 4 Weeks of Aerobic Exercise in Diabetic Rats

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Abstract

Introduction: Cardiac apoptosis is one of the most important cardiovascular complications of diabetes. We aimed to investigate the changes of caspase-8, Bcl-2, and N-terminal pro B-type natriuretic peptide (NT-proBNP) in cardiac tissue after 4 weeks of aerobic exercise in male rats with diabetes.

Methods: Forty adult male rats were randomly allocated to healthy control, diabetes, control + exercise and exercise + diabetes groups. Diabetes was induced by intraperitoneal injection of streptozotocin (STZ) solution (55 mg/kg). Two weeks after injection, fasting blood glucose levels were measured. After the induction of diabetes, the exercise program was performed for 4 weeks (5 sessions per week) at a speed of 15 to 18 m/min for 25 to 44 minutes. Forty-eight hours after the last training session, the subjects were anesthetized and the heart muscle was removed. Caspase-8, Bcl-2 and NT-proBNP levels were measured by ELISA method.

Results: The induction of diabetes in the control group resulted in a significant increase in caspase-8, and NT-proBNP levels while an insignificant increase was observed for Bcl-2 levels ($P < 0.05$). In non-diabetic groups, exercise caused no changes in caspase-8, NT-proBNP and Bcl-2 ($P < 0.05$). Exercise in diabetic groups significantly decreased NT-proBNP while no changes were observed in caspase-8 and Bcl-2 ($P < 0.05$).

Conclusion: Our findings showed that diabetes increases the pro-apoptotic and anti-apoptotic agent. In addition, 4 weeks of regular aerobic exercises can be used as a non-pharmacological strategy to reduce the complications of apoptosis in diabetic cardiomyocytes.

Keywords: Apoptosis, NT-proBNP, Streptozotocin, Aerobic exercise

Introduction

Cardiovascular disorders are the main cause of morbidities and mortalities in diabetic patients, not only due to coronary artery disease and related high blood pressure but also due to direct adverse effects of diabetes on the heart and independent of other pathological factors. Research has shown that apoptosis plays a major role in the pathogenesis of diabetes-induced heart disease.¹ Collagen is precipitated when heart cells are reduced by apoptosis. Ultimately, this reduction in cardiac

compliance increases the tension of the heart muscle cell wall, causing ventricular dysfunction. The occurrence of apoptosis in the pathway of diabetes-induced myocardial damage has been proven by activating pathway components of apoptosis and caspase activity. In addition, the death of myocardial cells is known as a main contributor to the development of diabetes-induced cardiovascular damage.¹⁻⁵ In fact, various studies have shown that diabetes significantly increases apoptosis in cardiac cells.⁵ In the early stages of heart

damage, the number of cells that are lost is greater, which indicates the activation of the incremental regulation of anti-apoptotic pathways after the reduction of the cells.⁴ The process of apoptosis and planned cell death is regulated by some mitochondrial proteins including B-cell lymphoma-2 (Bcl-2) proteins, which are divided into 2 parts of the anti-apoptotic proteins (Bcl-2, Bcl-XL, Bcl-W, Bfl-1 and Mcl-1) and pro-apoptotic proteins (Bax, Bak, Bad, Bcl-Xs, Bid, Bik, Bim and Hrk) which play a leading role in accelerating the onset of an attack.⁶ Whereas apoptosis is regulated by anti-apoptotic proteins by preventing the release of cytochrome c from the mitochondria, pro-apoptotic proteins accelerate its release.⁶

As a proto-oncogene antagonist of apoptosis at mitochondrial levels, with a weight of 28 kD, Bcl-2 prevents the oxidative damage to the cell and is known as one of the most prominent inhibitors of apoptosis proteins, which, in addition to enhancing the release of cytochrome c from mitochondria, the integrity of the mitochondrial membrane by attaching H⁺ ions to the apoptotic protease activating factor (apaf-1), prevents the activation of caspase-9.^{6,7} A mitochondrion is an inseparable component of the internal pathway of apoptosis and the site of deposition of many of the proteins interfering in the early stages of this process including the members of the Bcl-2 family.⁸ Mitochondrial functions are impaired as a result of DNA damage leading to irreversible cholesterol; therefore, Mitochondria participate in both the internal and external pathways of programmed cell death.⁹

In general, the pathways involved in stimulating the apoptosis process are divided into 2 categories as follows: internal pathway (or mitochondrial pathway), which is regulated by Bcl-2 family proteins and by activating Bak/Bax, leads to the permeability of the mitochondrial membrane and the external pathway (or the pathway for death receptors), which is started following the inclusion of the TNF receptor ligand coating and is resulted in the activation of caspase-8 and, consequently, caspase-3.^{5,9} The process of apoptosis is carried out by a family of cysteine proteases called caspase.¹⁰ The external pathway of apoptosis begins through death receptors and activates caspase-8. After activation, caspase-8 can directly activate the active caspases or act through Bid protein.¹¹ In the internal pathway, the release of cytochrome c from mitochondrion activates caspase-9 and ultimately activates caspases.¹⁰ There is a close relationship between these two pathways so that Bid protein as caspase-8 substrate releases cytochrome c after transfer to mitochondrion.¹³ Previous reports have noted that diabetes increases the levels and activity of caspase-8.¹⁴ Studies also showed that the level of prohormone brain natriuretic peptide (NT-proBNP) is significantly higher in diabetic subjects than in healthy subjects.¹⁵⁻¹⁷ BNP levels have a significant positive correlation with cardiac failure and severity of hypertrophy and poor ventricular diastolic function.¹⁸⁻²¹ Researchers

have argued that NT-proBNP level can be measured as a predictor of left ventricular malfunction and increased risk of death due to damage to the heart muscle.²²⁻²⁵ Research has shown that regular exercises modify many metabolic disorders in the diabetic population in addition to their beneficial effects on systemic changes associated with obesity and type 2 diabetes.^{4,15} Such fluctuations are due to indirect effects of systemic alternations due to exercise and their direct effects on cardiac contractile activity during exercise.¹⁵ Exercise plays a protective role in the heart against complications of diabetes through the reduction of oxidative stress and apoptosis in heart cells.¹⁶ Due to a dearth of research on the effects of exercise on the adverse effects of diabetes on heart muscle damage and apoptosis, we examined the effects of a 4-week course of increasing aerobic exercise on cardiac markers of caspase-8, Bcl-2 and NT-proBNP in diabetic male rats.

Methods

Animals

Based on the model used in the study done by Chow et al in the selection of clinical trial samples,²⁶ 40 male Wistar rats weighing 200.63 ± 17.47 were obtained from the Animal Breeding Center of Pasteur Institute of Iran-Tehran and transferred to the Animal Laboratory of Faculty of Physical Education and Sports Sciences of Mazandaran University. All subjects were kept under controlled environmental conditions with an average temperature of $22 \pm 3^\circ\text{C}$, a 12-hour light cycle, a 12-hour cycle of darkness and free access to water and food. Mouse food was purchased from Behparvar Company, Iran.

Allocation and Training Protocol

The animals were randomly divided into 4 groups of 10 rats,²⁶ including control, diabetes, control + exercise, and exercise + diabetes. Before performing the exercise protocol, the subjects were introduced to the treadmill for 1 week. The familiarization program consisted of 5 walking and running sessions at a speed of 5 to 8 m/s without slope for 8 to 10 minutes. The training program included running on a non-slip treadmill with a progressive overload principle between 25 minutes in the first week and 44 minutes in the fourth week (one minute increase in training time per session compared to the previous session) and between 15 m/min in the first week, up to 18 m/min in the fourth week (1 m/min/wk), and 5 sessions per week for 4 weeks.^{27,28} To warm up, the subjects ran at 7 m/min for 3 minutes at the beginning of each training session, and then to reach the desired speed, the speed of 2 m/min for each minute was added to the treadmill. In order to cool the body in every training session, the speed of the treadmill decreased steadily until it reached the initial speed.

Diabetes Induction

Diabetes induction was done by an intraperitoneal

injection of streptozotocin (STZ) solution from Sigma Aldrich Germany (CAS 18883-66-4-Calbiochem), soluble in citrate buffer (pH=4.5 and 0.1 mol concentration) and 55 mg/kg body weight.²⁹ Fourteen days after STZ injection, blood glucose concentration was measured using blood samples collected from animals with a glucometer. The criterion to be diabetic was the blood glucose level greater than 250 mg/dL. For the control group, in order to equalize the effect of injection of 0.1 µm citrate buffer, the same volume was injected intraperitoneally.¹⁷

Caspase-8, Bcl-2, and pro-BNP Measurement

Forty-eight hours after the last training session, all groups were anesthetized under completely similar conditions and fasting with intravenous injection of peritoneal ketamine (50 mg/kg body weight) and xylazine (3 mg/kg body weight) and the chest was split and the heart tissue was collected. In order to measure the indices, the nitrogen fluid was applied for powdering the heart tissue, and then 0.1 g (100 mg) of the powder was homogenized with 1 mL of PBS buffer, and then the extracted solution was centrifuged for 15 minutes at a speed of 5000 rpm, and its serum was used to measure the indices.¹⁸ To detect the cardiac index, Caspase-8 ELISA kit made by American MyBioSource Company (MBS2022115 96 tests) was used applying a quantitative sandwich method (sensitivity of 0.023 ng/mL).¹⁸ Cardiac levels of Bcl-2 were also measured by ELISA kits by MyBioSource Inc (MBS704330) made in the United States using a quantitative sandwich method (sensitivity of 0.65 pg/ml).¹⁹ Cardiac levels of NT-proBNP were also measured by ELISA kits by MyBioSource Inc (MBS2509359) made in the United States using a quantitative sandwich method (sensitivity of 2.49 ng/L). All of the above steps were carried out in the Biochemistry Laboratory of the Faculty of Physical Education and Sports Science in Mazandaran University.

Statistical Analysis

Shapiro-Wilk test was run to measure the normal distribution of data. Regarding the nature of the distribution of data, for comparing the groups in the variables studied, two-way analysis of variance (ANOVA) was used. In addition, a Tukey test was conducted as the post hoc test. The level of significance was $P < 0.05$. Statistical procedures were done using SPSS software package version 22.0.

Results

Figure 1 shows the mean and standard deviation of caspase-8 levels in heart tissues in the present study. As it can be seen, caspase-8 levels in diabetic rats were significantly higher compared to control group ($P = 0.001$). On the other hand, the results indicated that caspase-8 levels in the exercise group were significantly lower compared to the diabetes group ($P = 0.001$).

Moreover, the results showed a significant increase of caspase-8 levels in exercise + diabetes group compared to exercise group ($P = 0.001$).

Figure 2 shows the levels of Bcl-2 in various study groups in terms of mean and standard deviation. As it can be seen, there is no significant difference between the groups.

Figure 3 shows the mean and standard deviation of NT-proBNP in heart tissue of different groups in the current study. Our results indicated that NT-proBNP in the diabetes groups was significantly higher compared to the control group ($P = 0.001$). However, NT-proBNP significantly decreased in the exercise group compared to control group ($P = 0.001$). It was also observed that NT-proBNP significantly reduced in the exercise and exercise + diabetes compared to the diabetes group ($P = 0.001$ and $P = 0.014$).

Discussion

This study aimed to determine the changes in cardiac levels of caspase-8, Bcl-2 and NT-proBNP, as markers for apoptosis and inhibition of heart apoptosis, in STZ-induced diabetic rats after 4 weeks of aerobic exercise

Figure 1. The mean and standard deviation of caspase-8 in picograms per milliliter. * $P = 0.001$, ** $P = 0.001$, and *** $P = 0.014$ represent a significant difference between the groups. $P < 0.05$ was considered as the level of significance.

Figure 2. The mean and standard deviation of Bcl-2 in picograms per milliliter. * $P = 0.001$, ** $P = 0.001$ and *** $P = 0.014$ represent a significant difference between the groups. $P < 0.05$ was considered as the level of significance.

Figure 3. The mean and standard deviation of NT-proBNP in terms of nanograms per milliliter. * and ** $P = 0.001$ indicates a significant difference between the groups. $P < 0.05$ was considered as the level of significance.

(running). The results revealed that intraperitoneal injection of STZ (55 mg/kg body weight) resulted in a significant increase in caspase-8, and NT-proBNP levels and no significant changes in Bcl-2 levels. Diabetes is the most prevalent metabolic disorder in the developing world. Extensive studies are underway to find suitable treatments for diabetes.²⁰

Similarly, Kanter et al reported histological disorders including loss of muscle fibers and irregularities in striated muscles after induction of diabetes.¹⁶ Moreover, Shamsaei et al observed that after induction of diabetes in rats, necrosis and apoptosis occurred in the neurons of the hippocampus.³⁰ Kim et al,³¹ in contrast, showed that caspase-3 increased in the eyes of diabetic rats. In addition, the expression of pro-apoptotic protein Bax and Bcl-2 increased in the eyes of diabetic rats. The reason for this discrepancy in the change of Bcl-2 protein can be due to differences in the tissue levels and the expression of the protein gene since changes in the level of gene expression vary with the changes in the tissue levels, and these changes are not necessarily associated.^{22,32} Dousar et al also revealed the incidence of apoptosis in myopathy of diabetic rats.³³ Joussen et al demonstrated the activation of caspase pathways, damage to retinal cells, apoptosis, and endothelial cell loss in diabetic rats, confirming the results of the present study.³⁴ Kang et al stated that the phosphorylation of the pro-apoptosis protein Bad decreases, emphasizing the progression of the apoptosis in the mesenchymal cells in high concentrations of glucose. These disorders in the expression and phosphorylation of the Bcl-2 family are associated with releasing of cytochrome c and the activation of caspase. It was reported that oxidative stress in mesenchymal cells exposed to high concentration of glucose is an important incidence in the activation of the cell death program that results in mitochondrial dysfunctions and activation of caspase-3.³⁵

In line with the current study, Shiroo et al observed severe

apoptosis in cardiac cells of untreated diabetic rats. Furthermore, diabetes in the rats significantly increased the lipid peroxidation rate, the levels of carbonyl protein as an index of protein oxidation, and the superoxide dismutase.³⁶ Diabetes increases the level of oxidative stress, which leads to elevated levels of reactive oxygen species and reduces the antioxidant defense capacity, resulting in the programmed death of heart cells and apoptosis.²¹ However, precise molecular mechanisms of apoptosis have not yet been determined by high glucose concentrations. Scholars have argued that the mechanism of apoptosis, where glucose induces cell death, varies depending on the cell and tissue studied.¹⁶ Research has reported that the cause of diabetes-induced cardiac apoptosis, in addition to increased stress or oxidative stress, is the occurrence of inflammatory processes and the presence of cytokines such as TNF- α , IL-1 β , and IFN- γ . In addition, their effect on nitric oxide causes the appearance of Fas ligand by inflammatory and cardiac cells, which ultimately leads to the activation of caspase signaling and ultimately cell death by apoptosis in cardiac cells.³⁷

It was also observed that in non-diabetic groups, exercise performance did not cause any changes in caspase-8, NT-proBNP and Bcl-2. On the other hand, the exercise in diabetic groups led to a significant decrease in NT-proBNP and no changes in caspase-8 and Bcl-2 were observed. Previous studies showed that increased activity of antioxidant enzymes and decreased lipid peroxidation levels which are followed by exercise have important effects on the prevention of complications of apoptosis caused by diabetes and tissue damage caused by oxidative stress following the disease.²⁴ Regular exercise activity has been shown to increase the activity of antioxidant enzymes, increase the resistance to oxidative stress, and thus reduce oxidative damage.²⁵ Previous evidence has shown that regular exercise is effective in preventing and delaying diabetes, increasing insulin sensitivity and improving glucose metabolism.³⁸ It has also been shown that exercise before ischemia results in a decrease in the ratio of pro-apoptotic proteins and anti-apoptotic proteins, such as Bcl-2, and decreased signaling of caspase pathway activation, especially caspase-3 (final caspase of apoptosis pathway).³⁹ The inhibitory capability of free radicals is probably one of the most important mechanisms in the field of cell defense against cardiac damage. Active oxygen species in the mitochondrial electron transfer chain are produced as a natural product; however, when their level exceeds the antioxidant capacity of the cell, they can lead to cell death. Oxidative stress induced by active oxygen species is highly associated with diabetes and its complications and can cause cell death through various pathways.³⁹

NT-proBNP is a precursor of the BNP hormone with 108 amino acids that is broken down by a protease series into 2 CT-BNP molecules with 77 to 108 amino acids and NT-proBNP with 1 to 76 amino acids after

production and release from the left ventricle. Research has shown that BNP and NT-proBNP levels increase in ventricular hypertrophy and left ventricular dysfunction, atrial fibrillation, severe hypertension, congestive heart failure, myocardial infarction, pulmonary hypertension, pregnancy and chronic renal failure hypertension, and metabolic abnormalities such as diabetes. It has been determined that NT-proBNP enjoys a very high diagnostic value of cell wall stress due to higher half-life. This is consistent with the results obtained in this research. In previous studies, it has also been observed that running exercise reduces the levels of NT-proBNP. These results are consistent with the current research. Cellular and molecular factors are associated with each other through cascade signaling. Following external stimulus and stress, this intercalating cascade of signaling occurs. Protein kinase B is the main agent in the signaling pathway of phosphatidylinositol-3 kinase, which plays a role in many cellular processes, including cellular survival, metabolism, cell growth and proliferation. Increasing the expression and enhancement of protein kinase B activity inhibit apoptosis pathways by phosphorylation of the anti-apoptotic proteins of the Bcl-2 family and inactivating the apoptotic precursor protein such as Bax or by directly controlling caspase activity.³¹ Studies have reported that levels of protein kinase B decrease in animal samples with diabetes mellitus.³¹ Furthermore, probably another mechanism of cellular protection from sporadic exercises against apoptosis is associated with the important effects of exercise in enhancing the expression of protein kinase B since it has been shown that protein kinase B encounters an increase in aerobic exercise.⁴⁰

Conclusion

Finally, it can be concluded that 4 weeks of aerobic exercise probably reduces the severity of apoptosis in diabetic rats.

Competing Interests

Authors declare that they have no competing interests.

Ethical Approval

All the ethics of work with animals were examined by the Ethics Committee of Razi University of Kermanshah and approved with code 024-2-396.

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