

Phosphorylated Connexin 43 Protein Levels Attenuation in the Retina of Adult Diabetic Male Rats, by Regular Moderate Exercise

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Abstract: *Background:* Cell-to-cell interactions, via gap junctions that composed of connexin subunit, are now thought to play an important role in the molecular biology of diabetes. Increased phosphorylated connexin 43 levels are considered as an important factor involved in the development of diabetic retinopathy. Also beneficial effects of exercise in regulating glucose metabolism have been proved. Considering the effective role of exercise in diabetic patients, in this study the impact of regular moderate exercise on phosphorylated connexin43 levels were examined. *Method:* 28 male Wistar rats (250±50g) were randomly divided into four groups. Diabetes was induced by injection of streptozotocin (60 mg/Kg). One hour treadmill exercise, 5 days a week with 22 (m/min) speeds was begun, a week after induction of diabetes. Finally phosphorylated connexin 43 levels were measured by ELISA method. *Results:* This study shows, not only the meaning of blood glucose levels is significantly decreased, but also phosphorylated connexin 43 levels were reduced by increasing the days of exercise, in diabetic running group ($P<0.05$). *Conclusion:* Thus regular moderate exercise reduced the amount of phosphorylated connexin 43 levels in the retina which this application may result in partial inhibition of retinopathy observed in diabetic patient.

Key words: Blood glucose • Diabetes • Phosphorylated connexin 43 • Regular moderate exercise • Retina • Retinopathy

INTRODUCTION

Hyperglycemia is known to play a critical role in the development and progression of diabetic retinopathy [1]. Increased nonenzymatic glycation [2], activation of aldose reductase [3], oxidative stress [2], increased production of diacylglycerol and stimulation of retinal protein kinase C (PKC) [4], are the best known mechanisms that they play an important role in the pathogenesis of diabetic retinopathy.

Cell-to-cell interactions, via gap junctional communication and connexon hemichannels, are now thought to play an important role in the molecular biology of diabetes [5]. Connexins subunits in gap junctional structure are associated with the pathogenesis of both type I and type II diabetes (such as retinopathy and cardiovascular diseases) [6, 7].

Connexin 37, connexin 40 and connexin 43 are three connexin subtypes which they are expressed in the endothelium of the blood vessels. Connexin 43 can be in one non- phosphorylated state (P0) and three different phosphorylated forms (P1, P2 and P3) [8]. In the retinal endothelial cells and pericytes, connexin 43 expression is abundant [9] that suggests substantial gap junction coupling in the retinal vascular cells. Endothelial cells rely on a network of gap junctions for intracellular communication [10, 11]. Previous studies have shown that intercellular communication in endothelial cells is reduced under hyperglycemia [12] also it has been widely shown that phosphorylation of connexin 43, decreased intercellular communication through gap junctions (GJIC), probably because of the reduction in abundance at the plasma membrane [13, 14]. For example, it was shown that in smooth muscle cells, high glucose

induced the inhibition of GJIC activity through hyperphosphorylated connexin 43 [15]. In the other hand diabetes and hyperglycemia increase the P3/P0 ratio by inducing the protein kinase C activation [8] and these hyperphosphorylation elevated proteasomal degradation of connexin 43 [16].

The beneficial effects of exercise have been proven in many diabetic complications such as cardiomyopathy [17]. Furthermore exercise training has been utilized as an effective adjunct to pharmacotherapy in the management of diabetes mellitus, for a long time and physical activity influences several aspects of diabetes mellitus including blood glucose metabolism and insulin action [18, 19]. Considering previous studies, showed expression of connexin 43 was decreased and phosphorylation of it was increased in diabetic retinas and with regard to beneficial effects of exercise, including the regulation of glucose metabolism and because there are few studies that were examined the effect of exercise on diabetic retinopathy and phosphorylated connexin 43 level changes in animal models of diabetes, the effects of regular moderate exercise on phosphorylated connexin 43 level changes in retina of diabetic rat, was studied. Also pathological studies were done.

MATERIALS AND METHODS

Animals: Adult male Wistar rats (250 ± 50 g) were obtained from animal house of Tabriz University. A 12-h light/dark cycle at $22 \pm 2^\circ\text{C}$ was maintained. Animal were allowed free access to standard laboratory chow and water.

Experimental Design: Animals were randomly divided into four groups (seven each): 1- Control group: (C) [no experimental treatment], 2- Sedentary Diabetic group: (SD) [no exercise treatment], 3- Control Running group: (CR) [exercised for sixty days], 4- Diabetic Running group: (DR) [exercised for sixty days].

Experimental Protocols: Diabetes type1 in rats was induced by intraperitoneal injection of 60 mg/kg streptozotocin (STZ) (Sigma, St Louis, MO) (13). STZ was dissolved in citrate buffer (1:1 mixture of 0.1 M citric acid and 0.2 M Na_2HPO_4) just before injection. Rats in the control groups received an intraperitoneal injection of an equal volume of citrate buffer instead of STZ. 48 h after STZ injection, blood samples were obtained from the tail vein and blood glucose concentrations were measured

with an elegance glucometer, successful induction of diabetes was defined as a blood glucose level of 250mg/dl [20]. Also polyuria and polydipsia were observed in diabetic rats.

Exercise Protocols: Before beginning the formal 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) with 22 m/min speeds [21]. At the beginning of 60 minutes exercise, to warm up the rats, treadmill speed had been set at 5 m/min and progressively increased to 22 m/min. At the end of 60 minutes exercise, the speed progressively decreased to 5 m/min to cool down. Control and sedentary diabetic group animals did not perform treadmill exercise but were placed on a non moving treadmill as running groups. Exercised animals were studied 24 h after their last exercise session.

Blood Glucose and Body Weight: The blood glucose concentration was measured in blood from a tail vein in the morning in rats at the first, thirtieth and sixtieth day of the experimental period. Body weight was measured in all rats.

Sample Collection: After the experimental period, all of the rats (control and diabetic groups) were anesthetized by intraperitoneal injection ketamine 100 mg/kg and xylazine 5 mg/kg [22]. Eyes were enucleated, the retina was separated from the other parts of the eye and then they were placed in liquid nitrogen immediately and finally stored at -80°C for later homogenization and biochemical assays. Also optic nerves were separated for pathological studies. To ensure that streptozotocin induces necrosis in pancreatic tissue, pancreatic tissue was collected.

ELISA Assay: All tissues were homogenized in phosphate buffer saline (PBS) that stored overnight at -20°C . After two freeze-thaw cycles were performed to break the cell membranes, the homogenates were centrifuged for 5 minutes at 5000 xg. The supernatant was assayed and removed immediately. The assay was performed as per the manufacturer's protocol of phosphorylated connexin 43 (Cosabio CSB-E17273r).

Histological Studies: To avoid of optic nerve and pancreatic tissues autolysis after, rats were euthanized

immediately and then tissue samples collected from optic nerve and pancreas with 0.5 cm dimensions. Samples fixed in 10% formalin solution. After a stage of fixation, dehydration, clearing, embedding, blocking, sectioning and staining with H&E technique, slides were studied with light microscopy.

Data Analysis: Results are expressed in mean \pm SEM and data were analyzed by using ANOVA. The SPSS statistical software package (version 16.0) was used to perform all statistical calculations.

RESULT

Effects of Type1 Diabetes on Optic Nerve Andpancreas

Tissues: Swelling of myelinated axons was observed in the sedentary diabetic groupthat was attenuated with exercise (Fig. 1).

Streptozotocin-induced necrosis in the beta cells of the Langerhans islets (Fig. 2)

Effects of Diabetes on Metabolic Characteristic: Initial fasting blood glucose measurements were showed significant increase in blood glucose levels in the diabetic rats compared with those of the control rats. At the beginning of exercising period means \pm SEM of diabetic running and sedentary diabetic groups did not show a significant difference, but after exercising for sixty days the blood glucose levels in diabetes running group was significantly decreased compared with those of the sedentary diabetic group (Table 1). Also the fasting blood glucose of sedentary diabetic group were showed a significant increase throughout of experimental period ($P < 0.05$).

The sedentary diabetic groupwas showing significant weight loss during the test. Regular moderate exercise not only inhibit weight loss that was observed in the sedentary diabetic group but also a partial increase in weight of these groups can be seen (Table 1).

Phosphorylatedconnexin 43 Analysis:

Phosphorylatedconnexin 43 levels were significantly increased in sedentary diabetic rats compared with those of control rats ($P < 0.05$) (Fig. 3).

60-day exercise decreases the phosphorylatedconnexin 43 levels in control running group with compared of those control rats but it is not significant ($P < 0.05$) (Fig. 4).

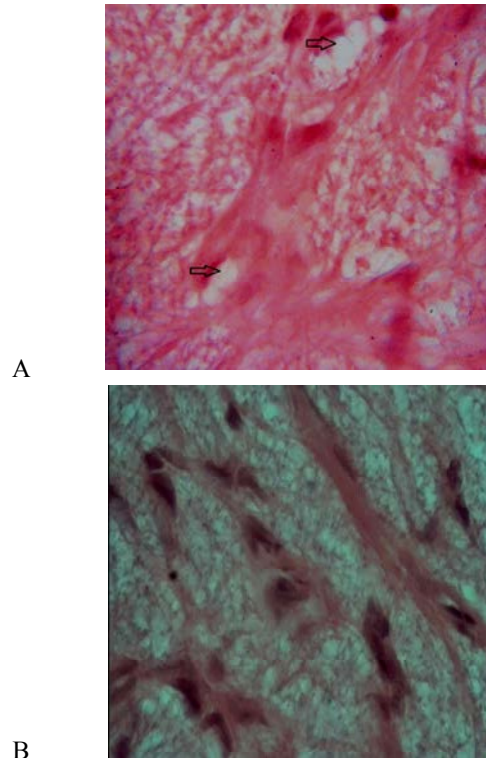


Fig. 1: Photomicrograph of optic nerve tissue. The arrow was shown swelling of myelinated axons in A: Sedentary Diabetic B: Diabetic Running ($\times 400$)



Fig. 2: Photomicrograph of pancreatic tissue. The arrow was shown necrosis part of Langerhans islet ($\times 400$)

The treatment with 60-day exercise led to a significant decrease of phosphorylatedconnexin 43 levels in the retina of diabetic running rats compared with those of sedentary diabetic rats ($P < 0.05$) (Fig. 5).

Phosphorylatedconnexin 43 levels at the end of the exercise period, in the diabetic running group is higher than control running group, but it is not significant ($P < 0.05$) (Fig. 6).

Table 1: General characteristics of STZ induced diabetic rats

Parameter	Diabetic Running	Control Running	Sedentary Diabetic	Control
Initial Blood Glucose (mg/dl)	318.5±21.1 ^{ab}	83.5±3.6 ^{cd}	406.8±43.3 ^{ab}	88.3±5.3 ^{cd}
Final Blood Glucose (mg/dl)	203.5±27.5 ^{abd}	91.3±5.2 ^{cd}	431.5±37.24 ^{abc}	81.5±3.4 ^{cd}
Initial Body Weight (g)	294.5±8.6	265.3±6.5	281.3±1.0	271.7±4.3
Final Body Weight (g)	304.0±24.5 ^d	259.1±9.7 ^d	183.0±10.1 ^{abc}	280.1±8.1 ^d

^aDifferent from Control, ^bdifferent from Control Running, ^cdifferent from Diabetic Running, ^ddifferent from Sedentary Diabetic (P<0.05)

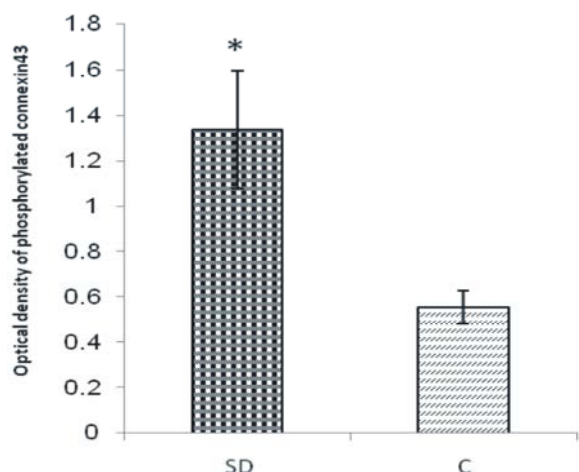


Fig. 3: Optical density of Phosphorylated connexin 43 in Control (C) and Sedentary Diabetic (SD). Data are means±SEM. Data statistically compared with (*P<0.05)

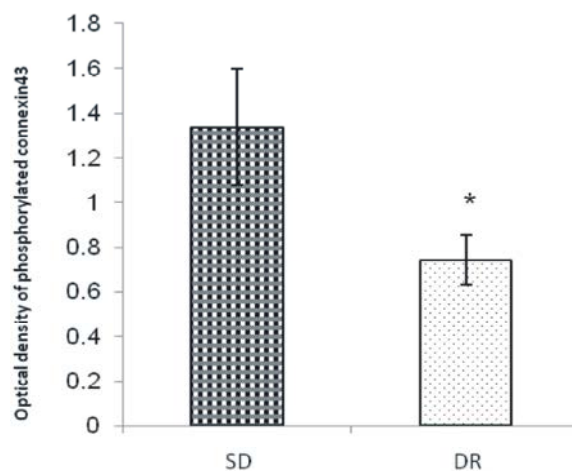


Fig. 5: Optical density of Phosphorylated connexin 43 in Sedentary Diabetic (SD) and Diabetic Running (DR). Data are means±SEM. Data statistically compared with (*P<0.05)

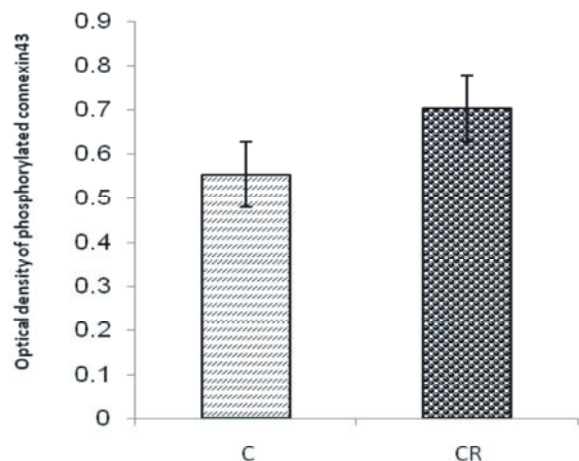


Fig. 4: Optical density of Phosphorylated connexin 43 in Control (C) and Control Running (CR). Data are means±SEM. Data statistically compared with (P<0.05)

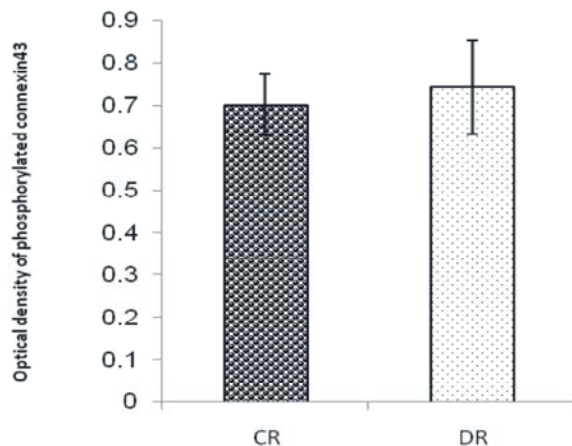


Fig. 6: Optical density of Phosphorylated connexin 43 in Control Running (CR) and Diabetic Running (DR). Data are means±SEM. Data statistically compared with (P<0.05).

DISCUSSION

The effect of type1 diabetes (STZ-induced diabetes) and exercise on phosphorylated connexin 43 protein levels in the retina have been investigated by using an ELISA assay.

The main finding of this study was that the amount of phosphorylatedconnexin 43 in the sedentary diabetic group was significantly increased (Fig. 3), which is in line with a study that was shown, high glucose-induced connexin 43 down regulation that this can occur through increased phosphorylation and elevated proteasomal

degradation of connexin 43 [16]. Blood glucose levels were increased in rats that received STZ (Table 1). STZ enters to beta cells and by induction of necrosis in the pancreatic beta cells (Fig. 2) decreased insulin levels and increased blood glucose levels [23], so these high blood glucose levels increase the protein kinase C activation that enhanced the phosphorylation of connexin 43 levels, thus the phosphorylated connexin 43 levels were increased.

Previous studies indicate that high glucose induces a reduction of gap junctional intracellular communication (GJIC) [15, 24]. The other studies were demonstrated that diabetes reduces connexin 43 expression in the retina [25, 26], that this down regulation can cause a reduction of GJIC and promote vascular cell loss in it [25]. More recently, connexin 43 down regulation and a concomitant reduction in GJIC activity was shown to be sufficient to induce apoptosis [27]. Furthermore previous in vitro studies shows that high glucose reduces connexin 43 gene expression in retinal endothelial cells and pericytes [28, 29].

60-day regular moderate exercise showed a significant decrease in blood glucose level in a diabetic exercise group than in the sedentary diabetic group (Table 1). The finding of this study was consistent with 60-day voluntary exercise in diabetic rats [30]. Exercise by increasing the sensitivity of GLUT4 in muscle and increased insulin receptor substrate plays an important role in reducing blood glucose levels [31]. So that glucose uptake in muscle sports, more than the maximum amount that can be taken with insulin stimulation [32].

Few studies have been undertaken on the impact of exercise on phosphorylated connexin 43 levels, however, finding of this research showed that phosphorylated connexin 43 levels in diabetic 60-day exercise group was decreased compared to sedentary diabetic group (Fig. 5).

Furthermore for the pathological cases a few works have been done up to now which this report was shown that swelling of myelinated axons that was observed in sedentary diabetic group attenuated with exercise (Fig. 1).

CONCLUSIONS

Taking into consideration the result of this study and previously available data, it is suggested that exercise with reducing the blood glucose can be reversed the STZ-induced elevation of blood glucose stimulates PKC-mediated phosphorylation of connexin 43, which in

turn suppresses gap junction intracellular communication, vascular cell loss and induce apoptosis.

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