

The Effect of Endurance Training on Fibronectin Gene Expression of the Sciatic Nerve in Diabetic Rats

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Abstract- Diabetic neuropathy can cause disorders in axon transmission, changes in the extracellular matrix, and peripheral nerve damages. However, its mechanism, along with the beneficial effects of exercise on these disorders is not entirely clear. The aim of the current study was to assess changes in fibronectin mRNA gene expression level of the sciatic nerve in rats with streptozotocin-induced diabetes after endurance training. Eighteen male Wistar rats (10 weeks old with 250 ± 20 gr weight) were randomly assigned to three groups, including healthy, induced diabetes and induced diabetes plus endurance training. Induction of diabetes was conducted using an intraperitoneal injection of a single dose of streptozotocin (STZ). Neuropathy was confirmed using the behavioral tests. Rats in induced diabetes plus training group had 8 weeks of moderate and increasing intensity endurance training on the treadmill. The Fibronectin mRNA gene expression level of the sciatic nerve was assessed using Real-time-PCR. Changes in fibronectin protein and myelin thickness were measured by immunohistochemistry and luxol fast blue staining. The mean and standard deviation was used to report descriptive data. Data were entered into SPSS 22. Fibronectin mRNA gene expression level (1.90) of sciatic nerve fibronectin protein and myelin thickness reduced significantly due to diabetes ($P < 0.05$). Eight weeks of endurance training increased fibronectin gene expression of sciatic nerve fibronectin protein and prevented further destruction of myelin, which was statistically significant. The results showed that diabetes leads to changes in the extracellular matrix and the reduction of the sciatic nerve myelin thickness. Endurance training as a non-drug strategy is effective in preventing these damages.

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Introduction

Diabetic peripheral neuropathy (DPN) is a common complication of diabetes. It is related to axonal atrophy, destruction of myelin, action potential speed reduction, and loss of peripheral nerve fibers (1). Factors such as hyperglycemia, insulin deficiency, and dyslipidemia impact on the neuropathy. Diabetes also increases oxidative stress and the mitochondrial dysfunction that causes the destruction of neurons and reduces the capacity of the recovery and nervous remodeling (2). Therefore, in diabetes, nerve tissue, especially peripheral nerves are vulnerable (3). Despite progress in understanding the causes of DPN, there is no approved drug therapy for the treatment of painful DPN. Therefore, the identification of new treatment strategies will be considered again. DPN do not develop uniformly

in terms of time or biochemical, so medical management requires a systematic and multipurpose approach. The purposes are inhibition of pathogenic mechanisms, control of inflammation, and increase of cytoprotection support. It was found that long-term exercise, as part of the treatment of diabetes, can delay or prevent the development of painful DPN (4). Yet its impact mechanisms are not well identified. Several studies have shown that exercise on a regular basis can change gene expression in various tissues and strengthen the body's protective mechanisms (5). Exercise is able to positively regulate glycoprotein (6).

Fibronectin is a multidimensional glycoprotein that consists of a series of repetitive structures. It consists of 12 repeats of fibronectin type I, 2 repeats of fibronectin type II, 15 repeats of fibronectin type III, a non-homologous variable (V), or linking segment area (7).

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Fibronectin exists in two forms: soluble and insoluble. The non-soluble form is detected as the extracellular matrix. In the extracellular matrix, fibronectin provides the development of cell migration routes. Various studies have shown that fibronectin plays an important role in the direction and migration of nerve tissue cells, such as fibroblasts, neurons, macrophages, and Schwann cells (8). Therefore at the time of injury, fibronectin plays an important role in healing (9). Fibronectin protein aggregation creates cable form strings that facilitate cellular bonding and migration, including fibroblasts, neurons, macrophages, and Schwann cells. Fibronectin substrates provide a pathway for transmission of nerve growth factor (NGF), as well as a suitable matrix for the regeneration of axons. Studies have shown that the fibronectin platform can act as a substrate for the cells, direct cells in the correct orientation in vivo, and in vitro conditions. The use of fibronectin as a substrate in the in-vivo model resulted in nerve regeneration and growth of neurites (axons and dendrites that grow from a cell body) and Schwann cell migration (10). This is a process that is highly regarded in peripheral nerve and spinal cord injury recovery (11).

Exercise has been considered as an effective strategy for the prevention and treatment of the peripheral nervous system damages (12). In this regard, Wilhelm *et al.*, (2012) studied the expression of Brain-derived neurotrophic factor (BDNF) in neurons and Schwann cells and showed that exercise increases this factor, which can increase the rate of healing of peripheral nerves (13). The exercise through the upregulation of connective tissue cells surface factors such as fibronectin or collagen is able to repair connective tissue structures (14). However, few studies have examined changes due to exercise in peripheral nervous tissue fibronectin levels of diabetic samples. Therefore, in this study, fibronectin changes of the sciatic nerve in Wistar rats with diabetic neuropathy due to endurance training were assessed.

Materials and Methods

Eighteen male Wistar rats (10 weeks old with 250 ± 20 gr weight) were purchased from the Pasture Institute of Iran. After transportation to the new environment, they were kept in controlled conditions, 12 hours of light and 12 hours of darkness (Lighting started from 6 AM, and darkness started from 6 PM), Temperature ($22 \pm 3^\circ$ C) and humidity (about 45 percent). Three to five rats were kept in plexiglass cages with lace door and dimensions $25 \times 27 \times 43$ cm so they could freely

have access to standard water and food. All the process of maintenance and killing of rats was performed based on animal ethics committee protocol, after a week of familiarization with the laboratory animals randomly assigned to three groups. Three groups were healthy ($n=6$), induced diabetes ($n=6$), and induced diabetes plus endurance training ($n=6$). Induction of diabetes was conducted using an intraperitoneal injection of a single dose of streptozotocin (Sigma, USA) dissolved in sodium citrate buffer, pH 4.5. The STZ dose was 45 mg/kg. The injection was conducted after 12 hours of food deprivation. The equivalent volume of citrate buffer was injected to non-diabetic rats. To confirm diabetes, 72 hours after administration of STZ, a small wound was created in the rats' tail with the Lancet, and a drop of blood was placed on the strip glucometer. Blood sugar levels were determined with a glucometer and recorded. Rats with glucose greater than 250 mg/dl were considered diabetic. Before the beginning of endurance training protocol and two weeks after induction of diabetes, behavioral tests of neuropathic pain (including mechanical allodynia and hyper heat analgesia) were conducted as an indicator of the creation of pathological conditions of diabetic neuropathy which was followed by training for 8 weeks.

Training protocol

Diabetic rats assigned to the exercise group did the training protocol 5 times a week for 8 weeks. The pace and duration of exercise were increased gradually and based on the schedule in Table 1. Other groups were kept in laboratory conditions during the implementation of the protocol. It should be mentioned that during training programs, no shock was used, and if it was necessary, by using hands or create audio stimuli on the rails of the treadmill, the animals were forced to continue practicing.

Table 1. Training protocol

Week	Speed (Metter/minute)	Duration (Minute)
First	8	5
Second	10	10
Third	10	14
Forth	14	18
Fifth	14	22
Sixth	17	26
Seventh	20	30
Eighth	20	30

Tissue evaluation

After 8 weeks, all rats were anesthetized by injection of ketamine (70 mg/kg) and Zaylaryn (10 mg/kg). The

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left sciatic nerve of rats (via a longitudinal section of the great trochanter to the mid-thigh area) was dissected to a length of two centimeters. Dissected nerves were placed Paraformaldehyde (Macrogen, Korea) (4%) overnight at 4° C. After 24 hours of the initial consolidation phase, 5 micrometers cut of the sciatic nerve was prepared and stained with Luxol Fast Blue method, and the myelin of sciatic nerve was investigated.

Gene expression

To study the molecular level of gene expression, the RNA extraction was carried out from tissue in all groups, according to the manufacturer's protocol (Qiagen, Germany). To do this, the 200 µL of extract solution was added to the samples, and they were incubated for 24 hours at -80° C. Plaque in cryotube was crushed in a semi-frozen state and in order to sample lysis, 100 µL chloroform was added for 1 minute. The resulting solution was centrifuged at 12,000 rpm for 10 minutes. The supernatant, containing the RNA, was gently removed and placed in a diethylpyrocarbonate (DEPC) microtube. One ml of isopropyl alcohol was poured onto a transparent RNA and was stirred by hand for 1 minute. Samples were centrifuged at 12,000 rpm

for 10 minutes. The liquid was removed, and 1 ml of 70% ethanol was added to the remaining sediment. After vortexing, the mixture was centrifuged at a speed of 750 rpm for 10 minutes. The liquid was removed, and the plaque in cryotube was dried. Twenty µL of distilled water at a temperature of 60° C was poured onto the plate and it was placed for 5 minutes at 60° C. After extracting the RNA with high purity and concentration from all samples, cDNA synthesis process was performed according to the manufacturer's protocol (Fermentas, USA). Then synthesized cDNA was used for reverse transcription reaction. Primers were designed based on fibronectin and GAPDH genes in the NCBI GenBank using the online Primer3 version 0.4.0 software (<http://primer3.ut.ee>) and were synthesized by Macrogen, Inc, Korea. The sequence of used primers is presented in Table 2. Gene glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was used as a control gene. Measuring the expression levels of fibronectin was performed using a quantitative method of Real time-PCR according to the following steps: activation at 95° C for 15 minutes, followed by amplification at 95° C for 35 seconds and 55° C for 40 seconds for 35 cycles.

Table 2. Primer sequences were used in this study

Gene name	Oligo sequence 5'-3'	Accession Number
Fibronectin	F 5' CGACTCTCCTCCCATCCACTCA 3'	XM_006245159.3
	R 5' AGCCTGCCTCTTGCTCTTCCC 3'	
GAPDH	F 5' CAT ACT CAG CAC CAG CAT CAC C 3'	XM_017593963.1
	R 5' AAG TTC AAC GGC ACA GTC AAG G 3'	

Immunohistochemistry

Neuropathy was confirmed using a behavioral test in rats. After 8 weeks of training, (in diabetic group+exercise) as well as other groups within that time, rats were anesthetized using an intraperitoneal injection of ketamine and Zaylaryn and then a part of the sciatic nerve immediately extracted and placed in 10% formalin. After fixing the samples, molded paraffin-embedded tissues, 5 mm slices were cut by a microtome. In order to recover antigen, obtained slices were incubated in buffer TBS1X (pH: 9.2) for 20 min at 70° C. Triton 3.0 percent was used for 30 minutes to make cell membrane permeable. After washing with PBS, 10% goat serum for 30 minutes was added to block the reaction of the secondary antibody as additional color fields. Anti-fibronectin diluted primary antibody (1:100) was added to the sample overnight with PBS and incubated at a temperature of 2 to 8 degrees. After washing with PBS, secondary antibody bound with

FITC color was added and incubated at 37° C for 2 hours in the dark. Finally, in order to stain the nucleus, DAPI was added to the samples. In the final step for marker confirmation, the samples were observed by fluorescent microscope (Olympus, 400x).

Statistical tests

The mean and standard deviation was used to report descriptive data. Data were entered into SPSS 22. After confirmation of normal distribution of data with the Shapiro-Wilk test, one-way analysis of variance (ANOVA) and Tukey post hoc test were used to analyze the data. The level of significance was considered $P < 0.05$ in all tests.

Results

Changes in mRNA gene expression levels of fibronectin are shown in Figure 1. Fibronectin mRNA

gene expression level of the sciatic nerve in the diabetic group showed a significant decrease (1.90) in comparison to the control group ($P<0.05$) while the implementation of 8 weeks of aerobic training increased the fibronectin gene expression levels in diabetic group with exercise (Figure 1).

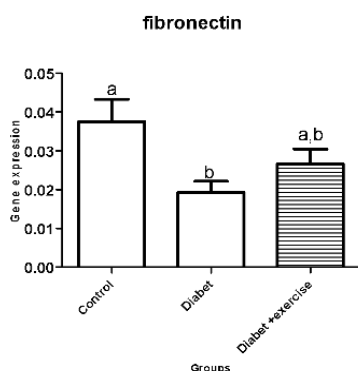


Figure 1. Fibronectin expression at mRNA level in the different study groups compared to the control group ($P<0.05$)

In line with the changes in gene-based in Figure 1, in the diabetic group, fibronectin protein in Figure2 was significantly reduced (26%) compared to the control group ($P=0.023$). The exercise in diabetes plus exercise group, fibronectin protein levels were raised significantly (41%) compared to the diabetic group ($P=0.002$).

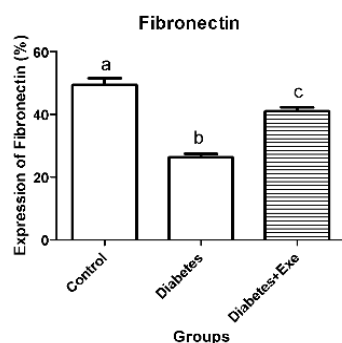


Figure 2. Average expression changes of fibronectin protein

Fibronectin protein level changes were also evaluated with the immunohistochemistry method (Figure 3). To study the protein expression, tissue imaging was performed from 5 sections with microscope 40X. Images were analyzed with Image J software. The percentage of protein expression was calculated by determining the ratio of green area to the entire surface, and it was reported as Mean and Standard Deviation. Based on Figure 3, there were significant differences in the expression of this protein between study groups.

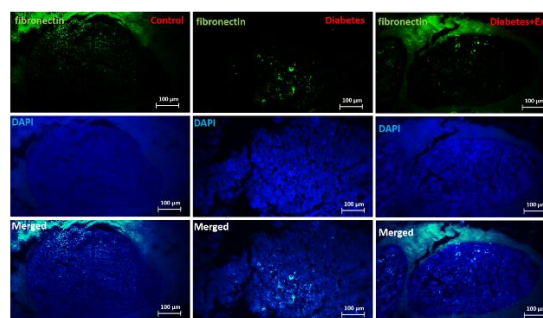


Figure 3. Fibronectin protein levels. The above images show that the fibronectin protein levels in the diabetic group were lower than the control group. It was higher in diabetes+exercise group compared to the diabetes group

To assess nerve damage in the study groups, the sciatic nerve myelin thickness changes were assessed and presented in Figure 4.

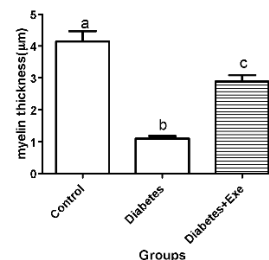


Figure 4. Changes in the thickness of myelin of sciatic nerve

Based on the above Figure, the thickness of myelin of sciatic nerve in diabetic rats Based on the above graph thick myelinated sciatic nerve in diabetic rats compared to the control group was significantly different compared to the control group ($P<0.05$) while 8 weeks of endurance training protocol prevents further damage to nerve myelin in Figure 5.

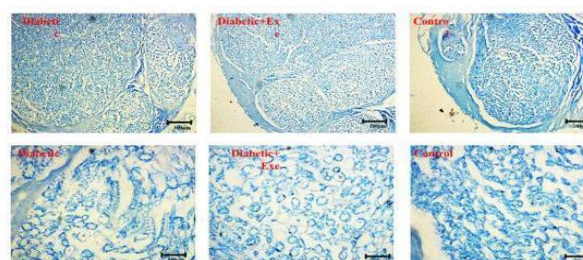


Figure 5. Myelin of sciatic nerve using Luxol Fast Blue method

Discussion

Neuropathy is the most common complication of diabetes that is created following metabolic disorders and pathological changes of the extracellular matrix. Complications of neuropathy and peripheral nerve damage with irreparable environmental injuries are the

most common problems in patients with diabetes. Therefore, the development of therapies, especially nervous tissue regeneration methods, is crucial to improve the quality of life in diabetic patients with neuropathy (1).

Results of this study showed that diabetic rat's sciatic nerve tissue levels of gene expression and protein fibronectin fell sharply, while the exercise for 8 weeks was able to increase fibronectin (increased levels of protein) in the sciatic nerves of diabetic rats. The results indicated that endurance training led to the prevention of demyelination of the sciatic nerve. In order to better understand the cellular and molecular changes and the effect of exercise in the current study, the related literature first was reviewed, and then our results will be examined.

Based on cellular studies, neurons are specific cell types, which are dependent on the spread movement for transfer of the protein, mRNA, and other nervous components from the cell body to the tips of axon and dendrite (16). Kinesins and Dyneins direct respectively forward and backward transfer in axons, respectively. Studies have shown that forward axonal transfer and BDNF decrease in diabetic rats.

Alrashdan *et al.*, (2010) showed that increased activity in the form of 30-minute electrical stimulation after nerve damage caused an increase in the number of sensory neurons, the axons, the myelin thickness, and increase BDNF gene expression in sensory neurons (18). Also, Rahmati *et al.*, (19) also reported a beneficial effect of endurance training on improved performance of sensory neurons in rats with diabetic neuropathy.

Chen *et al.*, (2012) related the effects of exercise on improving the performance of sensory neurons to the reduction of expression of beta-1 Interleukin and Tumor Necrosis Factor Alpha TNF- α in the sciatic nerve (20). These studies show that exercise can improve sensory neurons' condition by reduction of the inflammatory cytokines and pro-inflammatory and neurotrophic factors. However, the role of exercise and its results to changes in extracellular matrix proteins has not been investigated. The study was carried out on fibronectin gene changes caused by endurance training.

Fibronectin, as a multifunctional protein, is the most abundant component of the extracellular matrix of nervous tissue that is secreted by Schwann cells (20). Fibronectin is a glycoprotein which works as a ligand, with at least 11 different integrin heterodimer. It supports adhesion of different cell types. It also promotes the progress of neuritis with the growth of nerve cells in the PNS and CNS (22,23). Other studies

have shown that integrin $\alpha 5\beta 1$ is one of the most important fibronectins, which is expressed to axonal restore (24,25). In the Schwann cells, fibronectin, along with type IV collagen, and laminin create a network that causes adhesion, proliferation, differentiation, and migration. In addition, fibronectin plays an important role in the development, homeostasis, and regeneration of nervous tissue. Studies have shown that extracellular matrix proteins such as fibronectin have direct interaction with neural growth factors (27,28). Therefore, any factor that increases the expression of them can play an important role in peripheral nerve repair and restoration.

The results of the current study showed that diabetes caused a negative impact on the regulation of fibronectin through impacting on extracellular matrix. However, it is suggested that future studies simultaneously assess neurotrophic factors with fibronectin.

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