Original Article

The Effect of Aerobic Exercise on Collagen Type I and IV Gene Expression and Collagen Type I Protein Changes in the Sciatic Nerve of Diabetic Rats

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Abstract

Background: Neuropathy is one of the complications of diabetes, probably due to the destruction of the extracellular matrix and the thickening of the peripheral nerve basement membrane. However, its mechanisms and the impact of exercise on these disorders has not fully understood. The purpose of the present study was to investigate the effect of aerobic exercise on *collagen* levels of *type I* and *IV* and collagen type I protein changes in the sciatic nerve of diabetic rats.

Materials and Methods: Eighteen 10-week-old Wistar male rats weighing 250 ± 20 g were randomly divided into three groups of healthy control (n=6), diabetic (n=6) and diabetic + aerobic exercise (n=6). For this purpose, after introduction and adaptation of rats to new environment, diabetes was induced by single dose injection of dissolved streptozotocin in sodium citrate buffer at pH=4.5 at 45 mg/kg intraperitoneal. After confirming neuropathic conditions (with behavioral tests), diabetic+exercise rats underwent moderate-intensity aerobic exercise on the treadmill for 8 week. At the beginning and at the end of the period, blood glucose of all rats was measured by glucometer and the mean of each group was measured separately. Changes in *collagen type I* and *IV gene* expression, and collagen type I protein levels in sciatic nerve of rats were evaluated by real-time PCR technique and immunohistochemistry, respectively.

Results: Diabetes increased *collagen type I* and *IV* gene expression and collagen type I protein levels in the sciatic nerve samples of rats. However, exercise reduced blood sugar levels and expression of *collagen type I* and *IV genes* (p=0.05) and collagen type I protein significantly reduced in sciatic nerve (p=0.001).

Conclusion: The results of the present study showed that aerobic exercise as a non-pharmacological strategy by negative regulating type I and IV collagen factors at the gene and protein level, was able to control and inhibit the effects of diabetes on extracellular matrix components in the sciatic nerve.

Keywords: Diabetes, *Collagen I*, *Collagen IV*, Aerobic exercise

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Introduction

Diabetes has become a pandemic in the 21th century and is projected to affect more than 4% of the world's population by 2030. Neuropathy is one of the most common complications of diabetes that affects the

sensory, motor, and autonomic nerves. Diabetes affects pathological changes including axonal atrophy, demyelination, damage to nerve fibers and impaired nerve repair by affecting peripheral nerve structure, especially extracellular matrix and connective tissue[1]. Although extracellular matrix contains glycoproteins, glycosaminoglycans and proteoglycans[2], however, in the present study, collagen changes as extracellular matrix glycoproteins were investigated.

So far, 26 different types of *collagen* have been identified that are involved in peripheral nerve structure, *type I, III, V, XI fibrillation collagen*, and in endomysium and perimysium, *type IV* and *VI* network *collagen*[3]. In addition to structural and mechanical roles, *collagen* also plays a role in adhesion of cells to the extracellular matrix[4].

According to studies in diabetic neuropathy, different pathways of cellular damage due to hyperglycemia, metabolic imbalance, oxidative stress, and peripheral nerve cellular mechanisms such as the extracellular matrix can affect axonal growth and neural repair[5-7].

Although the mechanism behind these changes has not been fully elucidated, it has been suggested that altering the expression of extracellular matrix genes and non-enzymatic glycation may be involved[8]. Crosslinking of extracellular matrix proteins protects against protease digestion, which results in accumulation of proteins including collagen and increased basement membrane thickness[9].

Increased basement membrane induces ischemia in peripheral nerve, damages distal nerve fibers, damages axons and numbness in limbs of diabetic patients[10]. According to the recent studies, about 45-50 % of diabetic patients develop numbness, pain and weakness in the lower and upper limbs[11].

Hill and colleagues have shown that elevated blood glucose levels increase collagen deposition in the endometrium, therefore increased collagen with diabetes is a sign of injury[12].

On the other hand, Majumder et al, who investigated the effect of exercise on diabetic neuropathy and found that exercise training increased the amount of irizine and mitochondrial biogenesis to alleviate and control the damage caused by diabetic neuropathy[13], have also reported the effects of exercise on diabetes.

Zanjani and colleagues also examined the effect of exercise on cellular signaling pathways. The results of the study showed that exercise in diabetic rats increases angiogenesis in the sciatic nerve; they attributed the possible mechanism to increased P-ERK 1/2 levels[14]. However, no studies have been performed on the effect of exercise training on extracellular matrix changes, especially *collagen* in the diabetic sciatic nerve. Therefore, in the present study, the effect of 8 week aerobic training on *collagen* levels of *type I* and *IV* and collagen type I protein in the sciatic nerve of diabetic rats was investigated.

Methods

Eighteen 10-week-old Wistar male rats weighing 250 ± 20 g purchased from the Pasteur Institute of Iran. After the transfer of rats to the new environment, the animals kept under controlled conditions with 12 hours of daylight to 12 hours of darkness (start lighting at 6 am and start off at 6 pm), temperature (22±3°C) and humidity (~45%).

Three to five rats were housed in plexiglass cages (25 x 27 x 43 cm) in such a way that they could freely access standard food and water. All rats kept and killed according to the rules of the Animal Ethics Committee (IR.IAU.PS.REC.1398.326). Animals randomly divided into three groups of healthy control (n=6), diabetic (n=6) and diabetic + aerobic exercise (n=6) after one week of familiarization with the laboratory environment.

In order to induce diabetes, streptozotocin (STZ) injection was used, so that rats were injected intraperitoneally with 45 mg/kg of STZ dissolved in sodium citrate buffer after 12h of starvation[15]. Other non-diabetic rats were injected with the same volume of sodium citrate buffer. To confirm diabetes, 72 hours after STZ injection, a small lesion was injected into the animal's tail and a blood drop was placed on the glucometer strip. Blood glucose level determined and recorded by glucometer. Rats with blood glucose levels above 250 mg / dl were considered as diabetic rats.

Prior to endurance training protocol and two weeks after induction of diabetes, neuropathic pain behavioral tests (mechanical allodynia and thermal hyperalgesia) were performed as an indicator of pathological conditions of diabetes neuropathy, and after assurance, the exercise protocol was performed for 8 week. Also throughout the training period, blood glucose was

Table 1: Training schedule.

	Speed (Meter/Min)	Duration (Minute)
1th week	8	5
2th week	10	10
3th week	10	14
4th week	14	18
5th week	14	22
6th week	17	26
7th week	20	30
8th week	20	30

measured eight times (weekly) in all rats by glucometer.

Training protocol

The rats in the diabetic+exercise group performed of exercise protocol for 8 week (5 sessions a week), and were gradually added according to the timing and duration of exercise as described below.

It is noteworthy that no training shocks were used during the training program and that animals were forced to continue training if necessary using hand or sound stimulus on the treadmill rails.

Gene expression

For molecular analysis at the level of gene expression, RNA was extracted from tissues in all studied groups according to the manufacturer's protocol (of QIAGEN, Germany). To do this, 200 µl Kiazol was added to the samples and incubated at -80°C for 24 h. The plaque in the cryotubes was crushed in semi-freezing state and 100 µl of chloroform was added for 1 min to lyse the samples. The resulting solution was centrifuged at 12000g for 10 minutes. Transparent liquid in the top of the tube containing RNA was gently removed and placed in a DEPC microtube. 1^{cc} isopropanol was poured onto transparent RNA and shaken by hand for 1 min. The samples were centrifuged at 12000g for 10 minutes. The supernatant was discarded and 1^{cc} of 70% alcohol was added to the precipitate. After vortexing, the mixture was centrifuged at 7500g for 10 minutes. The supernatant was drained and the plaque was dried in the microtube. 20 µl Distilled water (60°C) was poured onto the plaque and placed on a 60°C plate for 5 minutes. After extracting high purity and high concentration RNA from all studied samples, cDNA synthesis steps were performed according to the manufacturer's protocol (Fermentas, USA) and then synthesized cDNA was used for reverse transcription reaction. Quantitative real time-PCR method was used to measure expression levels of *collagen I* and *IV*. Design of primers based on *collagen I, IV* and GAPDH gene data was performed at NCBI gene bank by Macrogen Corporation. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) gene was used as the control gene and the expression level of the target gene was calculated. The sequences of the primers used are reported in the following table (Table 2).

Immunohistochemistry

Diabetic rats were anesthetized by intra-peritoneal injection of ketamine and xylarine after behavioral tests after 8 weeks of exercise in the diabetic + exercise group and other groups after the confirmation of neuropathy. A portion of their sciatic nerve was immediately extracted and placed in 10% formalin.

Table 2: Sequences of primers.

Gene name	Oligo sequence 5'-3'	Accession Number
Col I	F 5' TCTACCCCCCTCTTCACTCTTC 3' R 5' CTCTTACTTCCTCCCACCCCA 3'	NM_053304.1
Col IV	F 5' CTCCTCCCTCCTTGTCCTCC 3' R 5' TACCTGTCTGTCCTTCTCCTTC 3'	NM_001135009.1
GAPDH	F 5' CAT ACT CAG CAC CAG CAT CAC C 3' R 5' AAG TTC AAC GGC ACA GTC AAG G 3'	**XM_017593963.1**

Using the techniques mentioned above, after fixation of the specimens, the paraffin-embedded tissues were sectioned by microtome apparatus and cut into 5-µm thick sections[16]. Sections obtained for antigen retrieval were incubated in TBS1X buffer (pH 9.2) for 20 min at 70°C. Triton 0.3% was then used for 30 min to permeate the cell membrane. After washing with PBS, serum (10%) was added for 30 min to block the secondary antibody reaction with additional background color. Diluted anti-collagen I antibody with PBS (1:100) was added to the sample overnight and incubated at 2 to 8°C. After washing with PBS, secondary antibody conjugated with FITC dye was added and incubated in the dark at 37°C for 2h. DAPI was added to the samples for staining the nucleus. Finally, the specimens were observed by Olympus fluorescent microscope with 400 lens to confirm the markers.

Statistical test

Mean and standard deviation were used for descriptive data reporting. After confirming the normality of the data by Shapiro-Wilk test, one-way ANOVA and Tukey post hoc tests were used to determine the significance of the mean difference between the groups. The required data were analyzed by SPSS software version 22 at the significant level ($p \leq 0.05$).

Results

The results showed that diabetes increased *collagen type I* and *IV gene* expression in sciatic nerve sample of diabetic rats. However, exercise training significantly reduced the expression levels of *type I* and *IV collagen genes* (p=0.05) (Chart 1, 2).

Immunohistochemical analysis of collagen type I protein expression was performed in three groups and results are shown figure1and chart 3.

In addition, the mean changes in blood glucose of the three groups at the beginning and end of the period are shown in Table 3.

According to Table 3, the mean blood glucose at the end of the period in the diabetic + exercise group declines relative to the diabetic group.

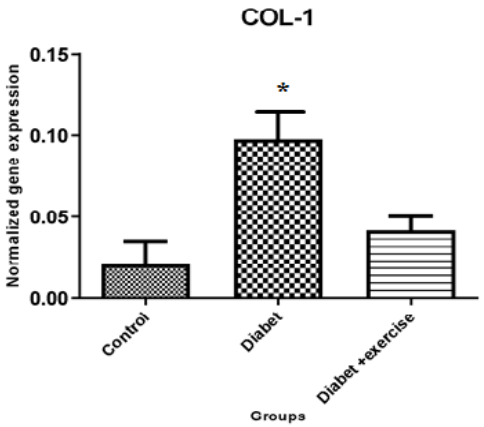

Chart 1. Collagen type I gene expression changes, collagen type I gene expression was 0.02 in the healthy group, which increased by 0.09 in the diabetic group and decreased to 0.04 by the exercise protocol, which was significant in the diabetic group (p=0.05).

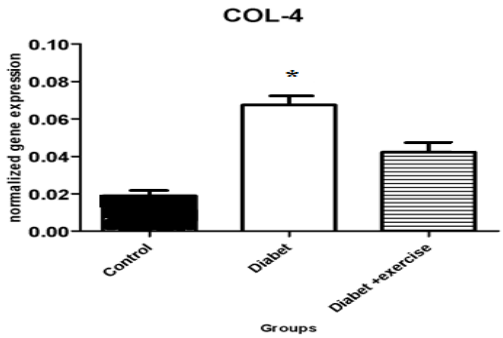

Chart 2. Changes of type IV collagen gene expression, the expression level of type IV collagen gene in healthy group was 0.02, which was increased by 0.07 in the diabetic group and decreased to 0.04 in the exercise protocol, which was significant compared to the diabetic group (p=0.05).

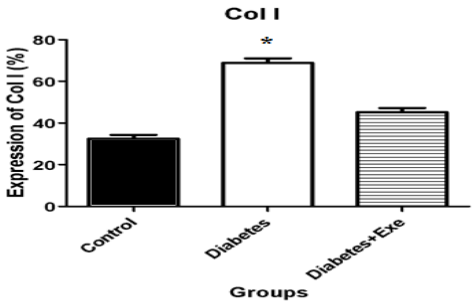

Chart 3. Type I collagen protein change, percentage of type I collagen protein change was 30.23% in the control group, which increased to 65.19% in the diabetic group and decreased to 48.78% in the diabetes + exercise group. The decrease in the proportion of the diabetic group was significant (p=0.001).

Discussion

The extracellular matrix plays a critical role in the normal function of nerve fibers and in the repair of nerve damage responses. Damage to nerve fibers is a

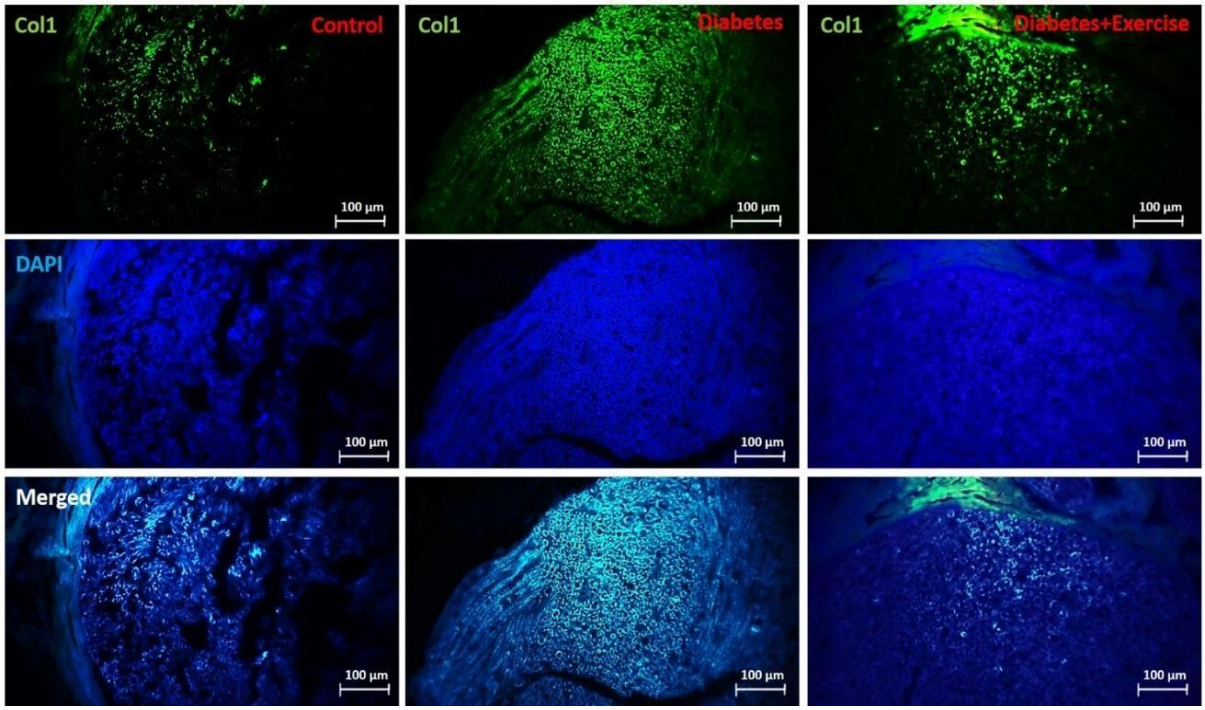

Figure 1. Collagen type I protein expression changes, according to the protein content in the control group was 30.23% which increased to 65.19% in the diabetic group and decreased to 48.78% in the diabetic + exercise group. This decrease was significant in relation to the diabetic group (p=0.001), which was consistent with changes in collagen type I mRNA levels (Graph 1).

Table 3: Blood glucose control at the beginning and end of the period.

Group	Average blood sugar at the beginning of the period	Average blood sugar at the end of the period
Healthy control	100	99
Diabetic	356	511
Diabetic + Exercise	345	457

major feature of diabetic neuropathy; however, peripheral nerve damage is associated with changes in study aimed to investigate the effect of 8 weeks aerobic exercise on the levels of *collagen type I* and *IV* (extracellular matrix components) and the amount of type I collagen protein in the sciatic nerve of diabetic rats. Studies show that diabetes by different mechanisms affects the extracellular matrix and increases collagen so that endothelial cells begin to synthesize collagen, fibronectin and laminin in hyperglycemia[7]. The results of the present study also show that induction of diabetes with STZ in Wistar rats induced a multiple-fold increase in the expression of *collagen type I* and *IV* in diabetic rats. In the healthy control group, the expression level of *type I* and *IV collagen* was 0.02, whereas with induction of diabetes the expression level of type *I collagen gene* was 0.09 and type *IV collagen* was 0.07. In the present study, the amount of collagen type I protein changes was evaluated by immunohistochemical method and the results showed that induction of diabetes in Wistar rats induced an increase in collagen type I protein level from 30.32 to 65.19 in the diabetic group.

Studies have shown that hyperglycemia can resist cross-linking of extracellular matrix proteins to protease digestion and cause protein aggregation including collagen and thickening of the basement membrane[17-18]. This increased thickness appears to inhibit the release and delivery of nutrients and damage to the perinurium and disruption of neural tissue microcirculation. In addition, collagen deposition in endoneurium prevents the growth and repair of nerve

fibers[19-21]

A study by Siironen et al. Also showed that increased collagen mRNA expression in fibroblasts, epineurium, and increased collagen I protein levels in epineurium may cause fibrous tissue in epineurium and impede axonal repair[22]. Hyperglycemia with impaired function and structure of sensory neurons reduces intercellular messaging capability, axonal transmission, nerve conduction and finally sensory nerve atrophy, which causes upper and lower limbs numbness in diabetic patients[22-24].

However, it is reasonable to argue that lowering blood sugar can reduce the effects of hyperglycemia and limit damage to the peripheral nerve. Therefore, in the present study, exercise training was used as a factor for reducing blood sugar in diabetic rats. The results of this study showed that the mean blood glucose in diabetic rats + exercise (457mg/dl) was lower than the diabetic rats (511mg/dl). The results also showed that aerobic exercise in diabetic rats for 8 week decreased collagen content. *Collagen type I* and *type IV gene* expression decreased from 0.09 and 0.07 in diabetic group to 0.04 in diabetic + exercise group, respectively. Collagen type I protein decreased from 65.19% in the diabetic group to 48.78% in the diabetic + exercise group, which was significantly lower than the diabetic group (p=0.001).

This means that aerobic exercise prevented increased *collagen type I* and *IV gene* expression and type I collagen protein and finally peripheral nerve injury. Although aerobic exercise also activates other mechanisms, studies have shown that exercise training positively regulates neurotrophic factors, in particular BDNF, which induce positive neuronal function and remodeling[25].

Studies by Chen et al. (2012) also showed that aerobic exercise significantly reduced *TNF-α, IL-1β* levels and increased *HSP72* expression in the sciatic nerve. They concluded that elevated *HSP72* levels could decrease pro-inflammatory cytokine levels and improve neuropathic pain[26]. Molteni et al. (2004) also showed that voluntary wheel-running exercise increases nerve endurance in sensory neurons after sciatic nerve injury. The researchers attributed the increased renascence of sensory neurons to increased expression of *GAP-43*, *synapsin I*, *NT-3*, and *BDNF genes*[27]. Goulart et al. (2014) showed that aerobic exercise was able to enhance the repair of sciatic nerve Schwann cells in rats[28].

However, the present study showed that hyperglycemia was reduced by aerobic exercise and the effects of hyperglycemia were reduced so that aerobic exercise had an effect on extracellular matrix and decreased expression of *type I* and *IV collagen genes* and type I collagen protein in the sciatic nerve structure, respectively. It can be considered as a protective agent and a non-pharmacological approach for diabetic patients.

Conclusion

Diabetes can increase basement membrane thickness and peripheral nerve damage by affecting extracellular matrix and increasing levels of *collagen type I* and *IV genes* and proteins. Aerobic exercise seems to be able to reduce collagen due to its positive effects on the extracellular matrix to prevent the increase in basement membrane thickness and to provide normal blood supply and nerve growth. Therefore, it can be stated that aerobic exercise prevented abnormal changes in the expression of extracellular matrix factors induced by diabetes. Since the effect of exercise on changes in other extracellular proteins in diabetes has not been investigated, it may be a subject for future research.

Acknowledgment

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