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# Effect of voluntary exercise on intracellular signalling pathways of angiogenesis in the sciatic nerve of type 1 diabetic castrated male rats

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*Objectives:* Impaired angiogenesis in sciatic nerve is a major complication of diabetic neuropathy. Protein kinase B (AKT) and extracellular signal regulated kinase (ERK) signaling pathways play critical roles during capillary-like network formation in the angiogenesis process. *Methods:* Twenty-four adult male Wistar rats (weight: 250–300 g) were used in the research. The rats were randomly divided into four groups ($n = 6$): (1) diabetic (Dia), (2) diabetic + castration (Dia-Cas), (3) diabetic + exercise (Dia-E), and (4) diabetic + castration + exercise (Dia-Cas-E). Type 1 diabetes (T1D) was induced with streptozotocin (50 mg/kg). After 6 weeks, sciatic nerve was separated and used for histological evaluation and determination of phosphorylated AKT (P-AKT) and phosphorylated ERK (P-ERK) levels by ELISA method. *Results:* Glucose levels decreased in the Dia-E group compared to the Dia-Cas group ($p < 0.01$). In addition, our finding shows that exercise in the Dia-Cas group diminished blood glucose levels compared to the Dia-Cas group but this effect of exercise was not significant. Voluntary exercise in the diabetic castrated group decreased P-AKT protein and increased P-ERK 1/2 protein levels in the sciatic tissue compared to the diabetes group significantly ($p < 0.05$). Histopathological findings showed that Dia-Cas group with 6-week exercise training significantly raised the number of microvascular density in the sciatic tissue of diabetic rats compared to the diabetic group ($p < 0.05$). *Conclusions:* Voluntary exercise in diabetic rats increases angiogenesis in the sciatic nerve. The possible mechanism is the increase of P-ERK 1/2 but not P-AKT levels in the sciatic nerve of T1D rats.

**Keywords:** angiogenesis, diabetic neuropathy, sciatic nerve, testosterone, voluntary exercise

## Introduction

Diabetes mellitus (DM) is a metabolic disorder and a chronic healthcare problem (9). Nowadays, because of alterations in lifestyle, such as lack of exercise, intake of high-fat diet, subsequent obesity, and the aging population, the prevalence of DM is increasing quickly all over the world (24). Diabetes-induced hyperglycemia results in macro- and microvascular complications, such as diabetic neuropathy (DN), nephropathy, and retinopathy (1). In both types of diabetes, DN remains the most severe form of complications affecting 40%–50% of people in the world. According to the International Consensus on the Diabetic Foot (8), every 30 s, somewhere in the world, a lower limb or part of it is lost due to DM (7). Therefore,

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for improvement of DN complications, comprehensive studies considering molecular details are needed.

Angiogenesis plays a critical role in the formation of new blood vessels from preexisting ones. The diabetic condition has a paradoxical effect on the vascular beds. In specific organs such as the kidney and the retina, diabetic condition increases neovascularization, whereas it decreases angiogenesis in coronary heart disease and peripheral nervous disease (23).

Among dozens of molecular mediators related to angiogenesis, protein kinase B (AKT) and extracellular signal-regulated kinase (ERK 1/2) signaling pathways are significant as they control proliferation and survival of endothelial cells. There are evidences that AKT and ERK 1/2 signaling pathways enhance angiogenesis in normal conditions and undergo changes in diabetic conditions (2). In addition, one of our previous studies showed that the protein levels of AKT and ERK 1/2 decreased in peripheral nerves in diabetic rats (29).

Testosterone is the major gonadal androgen in men that also modulates endothelial functions and angiogenesis. Testosterone deficiency is predominant in men with diabetes and in streptozotocin (STZ)-induced diabetic male rats (26). Moreover, a high prevalence of DN has been reported among men compared to women, but sex-specific differences in DN are rarely discussed (14).

Recently, physical activity has been considered to reduce diabetic complications (19). It has been shown that exercise therapy plays a critical role in patients with diabetes by improving, for instance, glycemic control, weight management, certain cardiovascular measures, and body composition (19). In addition, the previous study has shown that low-intensity exercise decreases peripheral nerve myelin breakdown in rats with diabetes. Exercise can be classified into two types: voluntary and involuntary. Voluntary exercise can be of mild to moderate intensity, and many beneficial effects have been attributed to it (16). To our knowledge, effectiveness of voluntary exercise has not been investigated on angiogenesis in sciatic nerves of diabetic male rats. Therefore, in this study, we aimed to investigate the effects of exercise therapy on angiogenesis in the sciatic nerve and its corresponding mechanisms in castrated rats with diabetes.

## Methods

### *Animals and study design*

Twenty-four adult male Wistar rats (weight: 250–300 g) were used in the present research. These rats were obtained from the colony of Tabriz University of Medical Sciences, Tabriz, Iran. The rats were kept under a 12-h light–dark cycle at 23 ± 1 °C condition and access to food and water ad libitum. Experiments on rats were carried out under the accepted procedure on human use and care of laboratory animals for biomedical study published by National Institutes of Health (8th ed., revised 2011) and conformed to the Declaration of Helsinki. The experimental protocols were approved by the ethics committee of Tabriz University of Medical Science (no. 92198). The rats were transferred to the laboratory for adaptation to the environmental condition. They were randomly divided into four groups including ($n = 6$): (1) diabetic (Dia), (2) diabetic + castration (Dia-Cas), (3) diabetic + exercise (Dia-E), and (4) diabetic + castration + exercise (Dia-Cas-E).

### *Castration*

In the castrated group, the rats were first anesthetized by injection of ketamine hydrochloride (80 mg/kg) and xylazine hydrochloride (5 mg/kg). Afterward, they were placed in

supine position, and after a small surgical incision, testis and vasodefran were removed completely.

*Induction of diabetes*

Type 1 diabetes (T1D) was induced with STZ (50 mg/kg; Sigma, St. Louis, MO, USA). STZ was dissolved in cold normal saline and injected immediately. According to this method, 72 h after injection, the fasting blood glucose levels were determined by a glucometer device (Boehringer Mannheim, Indianapplis, IN, USA). Rats with blood glucose ≥300 mg/dl were regarded to be diabetic (15). It should be mentioned that induction of diabetes was performed 7 days after the surgery.

*Voluntary exercise*

In exercise groups, animals had free access to run in a stainless-steel vertical running wheel against a resistance of 100 g (Tajhiz Gostar, Tehran; diameter = 34.5 cm, width = 9.5 cm) 24 h a day for 6 weeks. Each wheel was equipped with a magnetic switch that was linked to a separate counter, which was located outside the rats' cage and monitored the rotation per hour. The rats that had run less than 2,000 m/day were excluded from the study (18, 25).

*Tissue sampling and protein measurement*

In the final stage of the experimental procedure, rats were anesthetized with an i.p. injection of ketamine (80 mg/kg) and xylasine (5 mg/kg). Then, sciatic nerve was separated and after quick freezing by liquid nitrogen, all samples transferred to −70 °C temperature in order to measure phosphorylated AKT (P-AKT; serine 473) and phosphorylated ERK 1/2 (P-ERK; threonine 202/tyrosine 204) proteins in the tissue. The sciatic nerve samples were weighed, and then homogenized with phosphate-buffered saline (pH 7.2–7.4) and centrifuged for 10 min at a speed of 10,000 rpm at 4 °C temperature. Afterward, supernatants were isolated and used to measure the P-AKT and P-ERK levels through sandwich rat ELISA Kits, according to the manufacturer's protocol (Rat p-AKT, N-16 Torrance, USA, Lot: 20141111. ERK 1/2, Abcam Lot: GR196140-1).

*Immunostaining for PECAM-1/CD31*

Sciatic nerves were fixed in 10% formalin and paraffin embedded for evaluating the angiogenesis. In a subsequent stage, paraffin blocks of the sciatic nerve were cut with a microtome into 3-μm-thick slices; later, the slices were floated onto charged glass slides. The slides were deparaffinized in xylene and were dehydrated in a graded series of ethanol. Tissue sections were incubated in proteinase K and treated by 0.3% hydrogen peroxide for blocking endogenous peroxidase activity. The sections were covered by primary antibody CD31 (Santa Cruz, USA) – a marker of angiogenesis – and were incubated at +4 °C overnight. At the next step, slices were washed and incubated with a standard avidin–biotin complex (Santa Cruz) according to the manufacturer's instructions. Following this, di-amino-benzidine (Santa Cruz) was added to the slides as chromogen and counterstained with Mayer's hematoxylin. Eventually, the sections were cleared in xylene, were fixed in Entellan, and were assessed by light microscope (Olympus BX 40, Japan). In this protocol, the degree of angiogenesis was evaluated by measuring the immunostaining density, which was scored according to the following pattern: 0 (<10%), 1 (10%–25%), 2 (25%–50%), 3 (50%–75%), and 4 (75%–100%) (29).

*Statistical analysis*

All values were analyzed by one-way analysis of variance (ANOVA), and Tukey's test was used to compare quantitative data. Values less than 0.05 were considered statistically significant in all cases. The results are expressed as mean ± standard error of the mean.

## Results

*Blood glucose and body weight*

According to Fig. 1, exercise significantly ($p < 0.05$) decreased blood glucose levels in the Dia group. In addition, this figure shows that castration increased blood glucose level compared to the Dia group, but this increasing effect of castration was not significant. After 6 weeks of voluntary exercise training, we found a significant decrease in glucose levels in the Dia-E group compared to the Dia-Cas group ($p < 0.01$). Furthermore, our finding shows that exercise in the Dia-Cas group diminished blood glucose levels compared to the Dia-Cas group, but this decreasing effect of exercise was not significant.

Representative profiles of body mass index for the different groups are presented in Table I. This table illustrates that body weight in all groups significantly ($p < 0.05$) decreased during the experiment. In addition, according to Table I, castration significantly decreased ($p < 0.05$) body weight compared to the Dia group. As shown in Table I, 6 weeks of treatment

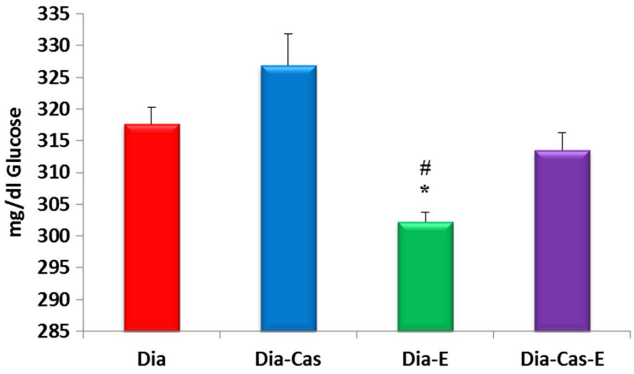


*Fig. 1.* Blood glucose levels in experimental rats (Dia: diabetes; Cas: castrated; E: exercise). Data are expressed as mean ± SEM for rats. *$p < 0.05$ vs. Dia group, #$p < 0.05$ vs. Dia-Cas group

*Table I.* Body mass index of different group of rats before and after the experiment

| Group | Body mass index | | |
|---|---|---|---|
| | *N* | Before | After |
| Dia | 6 | 236.00 ± 5.45 | 211.16 ± 9.10* |
| Dia-Cas | 6 | 251.66 ± 2.80 | 227.50 ± 4.27* |
| Dia-E | 6 | 247.33 ± 3.29 | 227.50 ± 4.33* |
| Dia-Cas-E | 6 | 247.66 ± 4.36 | 218.33 ± 4.04* |

Data are expressed as mean ± SEM for rats. Dia: diabetes; Cas: castrated; E: exercise.
*$p < 0.05$

with exercise in the Dia and the Dia-Cas groups significantly ($p < 0.05$) increased body weight compared to the Dia group.

*Effects of voluntary exercise on P-AKT levels in the sciatic tissue*

Our data indicated that after 6 weeks of voluntary exercise in the diabetic castrated group, P-AKT protein levels were significantly decreased in the sciatic tissue compared to the diabetes group. As shown in Fig. 2, P-AKT protein levels were also decreased in the Dia-Cas and Dia-E groups compared to the Dia group, although these results were not significant.

*Effects of voluntary exercise on P-ERK levels in the sciatic tissue*

Figure 3 shows that 6 weeks of exercise significantly ($p < 0.05$) increased P-ERK 1/2 protein levels in the Dia-Cas-E group compared to the Dia group. P-ERK 1/2 protein levels in the Dia-Cas-E group was increased compared to the Dia-Cas group, but not significantly. Moreover, these results indicated that levels of P-ERK were enhanced in the Dia-Cas and the Dia-E groups compared to the Dia group, but not significant.

*Histopathological findings*

*Effects of voluntary exercise on microvascular density (MVD) and in the Dia-Cas-E group.*

To investigate the function of angiogenesis in the sciatic nerve, immunohistochemistry staining was used to assess the expression pattern of CD31 marker in the endothelial cells, and was regarded as an angiogenesis indicator. One-way ANOVA illustrated that in the Dia-Cas group, the 6-week exercise training protocol significantly ($p < 0.05$) elevated the

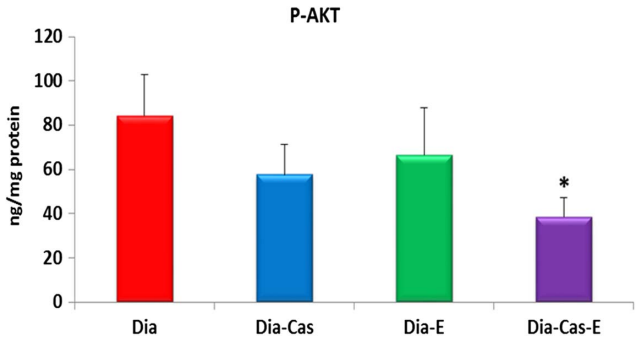


*Fig. 2.* Effect of 6 weeks of exercise training on P-AKT protein levels in the sciatic nerve tissue of diabetic and diabetic castrated groups (Dia: diabetes; Cas: castrated; E: exercise). Data are expressed as mean ± SEM for rats. *$p < 0.05$ vs. Dia group

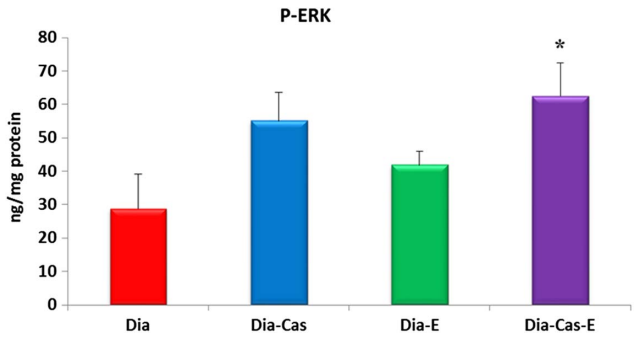


*Fig. 3.* Effect of 6 weeks of exercise training on P-ERK protein levels in the sciatic nerve tissue of diabetic and diabetic castrated groups (Dia: diabetes; Cas: castrated; E: exercise). Data are expressed as mean ± SEM for rats. *$p < 0.05$ vs. Dia group

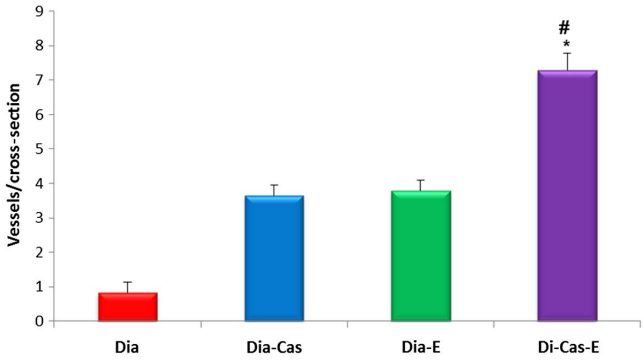


*Fig. 4.* Effect of 6 weeks of exercise training on microvascular density in the sciatic nerve tissue of diabetic and diabetic castrated groups (Dia: diabetes; Cas: castrated; E: exercise). Data are expressed as mean ± SEM for rats. $^{*}p < 0.05$ vs. Dia group, $^{\#}p < 0.05$ vs. Dia-Cas group

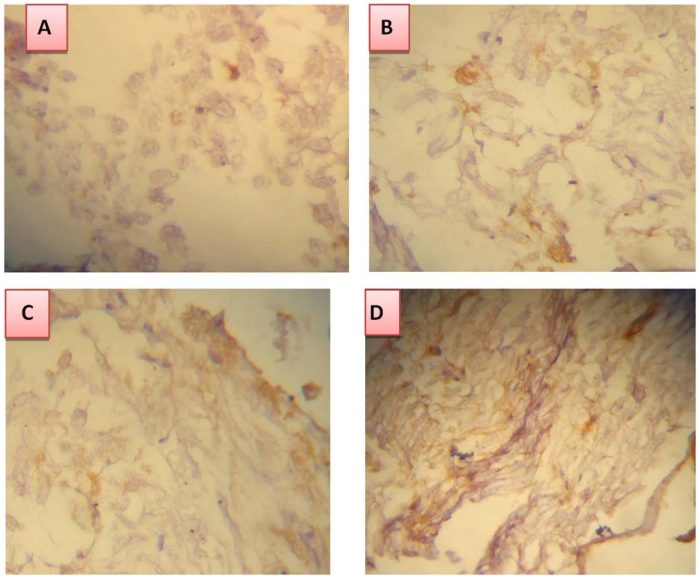


*Fig. 5.* The histological light microscopy images of the sciatic nerve tissues stained with hematoxylin and eosin (40× HE). (A) Dia, (B) Dia-Cas, (C) Dia-E, and (D) Dia-Cas-E

number of MVD in the sciatic tissue of diabetic rats compared to the diabetic group (Fig. 4). In addition, in the Dia-Cas-E group, 6-week exercise training ($p < 0.05$) significantly increased the number of MVD in the sciatic tissue of diabetic rats compared to Dia-Cas. Our data indicated that treatment of diabetic animals with exercise enhances angiogenesis compared to the Dia group; however, exercise had no significant effects (Fig. 5).

## Discussion

This study showed that glucose levels in diabetic condition were decreased by voluntary exercise. The study provided novel evidence that voluntary exercise stimulated production of P-ERK 1/2 and had no effect on expression of P-AKT in the sciatic tissue of diabetic rats. Immunohistochemistry staining in sciatic nerves showed the positive effect of exercise on angiogenesis. Our findings indicated that castration impaired the activation of AKT but enhanced activation of ERK 1/2 in the sciatic tissue of diabetic rats.

In our investigation, body weight in all groups of the diabetic rats significantly decreased during the trial and this effect was more intensive in the diabetic castrated group than in other rats. These results were in accordance with a previous study on gonadectomized rats (18). Supporting our work, Seino et al. (22) showed that, under diabetic conditions, the body produces energy from proteins and fat; therefore, the mass of muscle and fat decreases. In addition, several studies demonstrated that testosterone deficiency is common in men with diabetes and STZ-induced diabetic male rats (9, 21), and Cobo et al. (5) suggested that hypogonadism is related to physical inactivity and muscle atrophy. Regarding the level of blood sugar, a previous study revealed that total testosterone levels were inversely correlated with fasting plasma glucose in diabetic rats (4). In agreement with our results, Rao et al. (20) suggested that testosterone may improve glucose metabolism.

Huang et al. (12) reported that exercise plays an important role in the control of blood glucose in T1D patients via two general ways: insulin secretion and insulin-instigated glucose absorption in the skeletal muscles. To date, the regulatory mechanisms of exercise on blood glucose in T1D have remained unclear (12). Our data indicated that exercise decreased glucose levels in diabetic and the diabetic castrated groups. In accordance with our results, Chodari et al. (4) have shown a correlation between exercise and blood glucose in T1D rats.

The diabetic condition changes neovascularization mechanisms and impairs vascular homeostasis (2). The peripheral nerves in patients with diabetes are impaired and lose sensation. Consequently, there is reduced awareness of injury, and skin ulcers develop more commonly in the lower extremities (17). Data of this study showed that exercise improves capillary density in the Dia and Dia-Cas groups. In accordance with our work, Chodari et al. (2) showed that exercise increased capillary density in the heart tissue of diabetic rats. CD31 (also called PECAM-1) is a marker of angiogenesis that is produced in all endothelial cells and plays a key role in angiogenesis (28). Our results showed that exercise increased CD31 levels and promoted sciatic nerve angiogenesis in a diabetic model but this increase was not significant. We suggest that neural function can be associated with angiogenesis. In addition, analysis of our immunohistochemistry images demonstrated that the number of blood vessels in cross sections after being exposed physical activity for 6 weeks was increased in castrated rats. Based on our results, the stimulatory effect of exercise on angiogenesis in the Dia-Cas group is more prominent than in the Dia group. It seems that exercise in the absence of testosterone has a stronger effect. To elucidate the mechanisms of exercise that promote angiogenesis, we assessed the expression of P-AKT and P-ERK 1/2, which are proteins strongly related to angiogenesis. In this study, we provided novel information that exercise increased angiogenesis in the sciatic nerve. The possible mechanism is the increase of P-ERK 1/2 but not P-AKT levels in the sciatic nerve of T1D rats.

In this study, exercise had no effect on AKT activation and castration decreased (not significantly) the expression of P-AKT in the Dia-Cas group as compared to the Dia group. It seems that testosterone exerts an increasing effect on AKT activation, and castration eliminates this effect, and exercise in the Dia-Cas-E group could not compensate for this decreasing effect of castration. In line with our work, it is reported that castration of diabetic rats decreased P-AKT levels in the heart tissue (3). However, with regard to exercise, a study has shown that exercise has an increasing effect on P-AKT level. This contradiction can be attributed to the diabetic status, type of tissue, type of exercise, and exercise protocol. One important pathogenic mechanism of DN is decreased angiogenesis. The PI3K/AKT and RAS/RAF/MEK/ERK are two central signal pathways that control cell proliferation, survival, motility, and metabolism in response to stimuli from outside the cell (27).

The AKT pathway regulates venous specification, whereas the ERK pathway regulates arterial specification during developmental vasculogenesis (11). Zanjani et al. (29) reported that levels of P-AKT are decreased in sciatic nerves in STZ diabetic rats. The amount of P-ERK is also decreased in the heart tissue in diabetes (3). Our results showed that exercise has an effective impact on P-ERK levels in the sciatic nerve. In this study, exercise increases P-ERK levels in the Dia-E (not significantly) and in the Dia-Cas-E groups.

Furthermore, this study showed that castration increases P-ERK levels compared to the Dia group. Previous studies, which are similar to ours, have shown that testosterone increases P-AKT levels (10, 13). Therefore, the present findings support the idea that ERK pathway can be restrained by AKT through phosphorylation of RAF in this pathway (6).

## Conclusions

In conclusion, our findings demonstrated that voluntary exercise treatment in diabetic castrated rats (significantly) and in rats with diabetes (not significantly) diminished histological abnormalities and increased angiogenesis in the sciatic nerve. Moreover, voluntary exercise can compensate for the effects of testosterone deficiency and this effect may be related to the increase of P-ERK levels in the sciatic nerve.

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