# *Benfotiamine reduced collagen IV contents of sciatic nerve in hyperglycemic rats*

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**Journal of Diabetes & Metabolic Disorders**

e-ISSN 2251-6581

J Diabetes Metab Disord
DOI 10.1007/s40200-020-00666-2

Springer

Journal of Diabetes & Metabolic Disorders
https://doi.org/10.1007/s40200-020-00666-2

RESEARCH ARTICLE

# Benfotiamine reduced collagen IV contents of sciatic nerve in hyperglycemic rats

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Received: 26 April 2020 / Accepted: 19 October 2020


## Abstract

**Background** Neuropathy as a common complication of hyperglycemia in diabetic patients is probably caused by metabolic and structural changes in extracellular matrix (ECM) of peripheral nerves. This study was designed to evaluate the effects of benfotiamine (BT) on the structural, biological and mechanical characteristics of rat sciatic nerve in hyperglycemic condition.

**Materials and methods** Forty eight adult male Wistar rats were assigned to 6 groups (n = 8): control (healthy rats with no treatment; C), positive control (healthy rats received BT treatment; B), negative control groups 1&2 (hyperglycemic rats kept for 4 and/or 8 weeks; 4WD and 8WD, respectively) and experimental groups 1&2 (hyperglycemic rats treated by daily oral gavage of 100 mg $kg^{-1}$ body weight BT for 4 and/or 8 weeks; 4WD + BT and 8WD + BT, respectively). Hyperglycemia was induced by a single intraperitoneal injection of of streptozotocin (55 mg $kg^{-1}$ body weight). After a period of experimental period (4 and/or 8 weeks) rats were sacrificed and from each two segments (1 cm length) of left sciatic nerve were sampled. These samples were prepared for histological examinations (light and electron microscopy), collagen IV immunohistochemistry and strength tensile test.

**Results** In comparison to control groups, in 4WD and 8WD groups the amount of type IV collagen was increased, the structure of myelin sheath and nerve fibers were extensively altered and the tensile strength was significantly decreased ($p < 0.05$) while in 4WD + BT and 8WD + BT groups these abnormalities were attenuated.

**Conclusions** It seems that BT treatment may rescue the sciatic nerve from the hyperglycemic-induced ECM structural abnormality. This beneficial advantage of BT is likely exerted through the modification of glucose metabolism pathways.

**Keywords** Benfotiamine · Sciatic nerve · Diabetes · Collagen IV · Tensile strength

## Introduction

Neuropathy is a peripheral nerve complication in diabetic patients [1] in which the structural cohesion and consequently the normal function of the peripheral nerves is impaired [2]. Because diabetes is a metabolic disorder [3] and its complications appear after a long time, metabolic or enzymatic changes and subsequent structural changes in the ECM can be one of the main causes of neuropathy [4, 5]. The clinical manifestation of neuropathy would be generally evident within ten years after the onset of diabetes and the epidemiological studies have indicated that half of the diabetic patients are affected by neuropathy [4] Several lines of evidence suggest that irregularity in multiple biochemical pathways is responsible for the cause of neuropathy [6]. It is thought that the most critical pathway involved in the development of neuropathy is the polyol

**Highlights** In clinic, benfotiamine is prescribed for the treatment of diabetic neuropathy. In the present research the preventive effect of BT on hyperglycemic-induced structural abnormalities of sciatic nerve are highlighted.

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Published online: 20 February 2021

signaling pathway in which the aldose reductase enzyme plays an axial role as a restricting enzyme [7] This enzyme is responsible for the conversion of glucose into sorbitol. Accumulation of sorbitol causes a detrimental effect on neural tissue cells such as Schwan cells through the osmotic stress and an increment in water influx into the intracellular compartment [7, 8].

Accumulation of advanced glycation end-products (AGEs) in neural tissue is another complication of diabetes [9–11]. It has been recently demonstrated that AGEs can chemically modify macromolecules, increase the rigidity of the extracellular matrix (ECM) proteins and increase their resistance to proteolysis [12].

Collagen IV is the main component of the basement membrane constituents [10] This element is primarily secreted as soluble compound and then converts into insoluble extracellular matrix. Therefore, the biosynthesis of the basement membrane is a complicated process in which different specific and non-specific enzymes are involved [13, 14] Collagen IV as a non-fibrillar collagen constitutes about 50 percent of the basement membrane [15].

Benfotiamine (BT) is a synthetic *S*-acyl derivative of vitamin B1 (thiamine) [16]. After diffusion across the plasma membrane, this compound converts into the active form of thiamine called thiamine diphosphate and then acts as a coenzyme for critical enzymes involved in glucose metabolism [17] In hyperglycemic condition three biochemical pathways, namely hexosamine, AGEs, and diacylglycerol-protein kinase C are activated [6]. It has been suggested that BT probably halts the progress of diabetic complications through the suppression of these pathways [18–21] The present study was planned to investigate firstly, the effects of experimental hyperglycemia on the structural and biomechanical characteristics of rat sciatic nerve and secondly, assess the beneficial effects of BT treatment in rescuing the sciatic nerve from hyperglycemic harms.

## Materials and methods

### Animals and grouping

In this study, 48 adult male Wistar rats (250–350 g weight) were evenly assigned to the 6 following groups (n = 8): control (healthy rats with no treatment; C), positive control (healthy rats received BT treatment; B), negative control groups 1&2 (hyperglycemic rats with no treatment kept for 4 and/or 8 weeks; 4WD and 8WD, respectively) and experimental groups 1&2 (hyperglycemic rats treated by daily oral gavage of 100 mg $kg^{-1}$ body weight BT for 4 and/or 8 weeks; 4WD + BT and 8WD + BT, respectively). Animals were kept at 22 ± 2 °C and 60 ± 5% humidity in an animal house under 12:12 hour light:dark cycle with free access to food and water *ad libitum*. All animal protocols were approved by the Ethics Committee of the Ferdowsi University of Mashhad, Iran.

### Induction of hyperglycemia and benfotiamine treatment

Hyperglycemia was induced by a single intraperitoneal (ip) injection of streptozotocin (Sigma Aldrich) in citrate buffer (pH = 4.5) at a dose of 55 mg $kg^{-1}$ body weight and confirmed by plasma glucose higher than 300 mg/dl 72 h later [22] Benfotiamine (Sigma Aldrich) treatment was performed by daily oral gavage at a dose of 100 mg $kg^{-1}$ body weight for a period of 4 and/or 8 weeks [23].

At the end of the experimental periods (4 and 8 weeks) all rats were sacrificed, the left sciatic nerves was exposed and from the middle part of it two 1 cm length segments sampled [24] The samples were then processed by different approaches as follows. (1) A number of samples were fixed in 10% formalin, paraffin embedded, sectioned (5 μm thickness) and then used for either histological examination (collagen picrofuchsin staining [25]) or collagen IV immunohistochemistry. (2) Some samples were fixed in 2.5% glutaraldehyde, resin blocked and then sectioned at 1 μm thickness. These sections were processed for transmission electron microscope (TEM) study. (3) The remaining samples were used for tensile strength test; either as whole sciatic nerve or as decellurized scaffold of sciatic nerve.

### Collagen type IV Immunohistochemistry

This procedure was carried out according to the manufacturer's protocol for collagen IV antibody (ab6586 code, Abcam company, Germany). Briefly, sections were deparaffinized and then rehydrated, using descending alcohol gradients. After that, they were incubated with a primary antibody (anti collagen IV, Abcam 6586) at a dilution of 1:400 and then kept at 4 °C overnight. In the subsequent morning, sections were firstly washed with a solution of phosphate buffered saline (PBS) + 0.25% Triton X-100. At second step, to neutralize endogenous peroxidase, sections were pretreated with a solution of 0.3% hydrogen peroxide in PBS for 15 min and finally, a few drops of the HRP-conjugated secondary antibody (Abcam 97,051) at a dilution of 1:800 were added on the sections and then they were incubated for 2 h at room temperature. To remove unbound antibodies, sections were washed in PBS solution for 10 min and then stained with hematoxylin, mounted and visualized under a light microscope [26].

## Preparation of thin and semi-thin sections

To stain the myelin sheath, the semi-thin (1 μm) sections stained with toluidine blue. Briefly, the procedure was as follows; the sciatic nerve samples were fixed in 2.5% glutaraldehyde, washed in 0.1 M sodium cacodylate buffer, dehydration by ascending gradients of ethanol and then gradually permeabilized by standard propylene oxide until the tissue samples were completely saturated with resin. This process replaces the resin solution with 100% ethanol. To obtain thin and/or semi-thin sections, the resin-embedded samples were sectioned, using an ultra-microtome (Leitz, 1512, Germany). The semi-thin sections were stained with toluidine blue [25] and examined by light microscope. The thin sections were prepared for TEM examination and then imaged [27].

## Decellularization of the sciatic nerve

Decellularization process was performed according to Sandell method [28] Briefly, after incubation of sciatic nerve segments in distilled water (7 h), they were immersed in 3% Triton X-100 in distilled water overnight and then 4% sodium dodecyl sulfate in distilled water for 24 h. The last two steps were

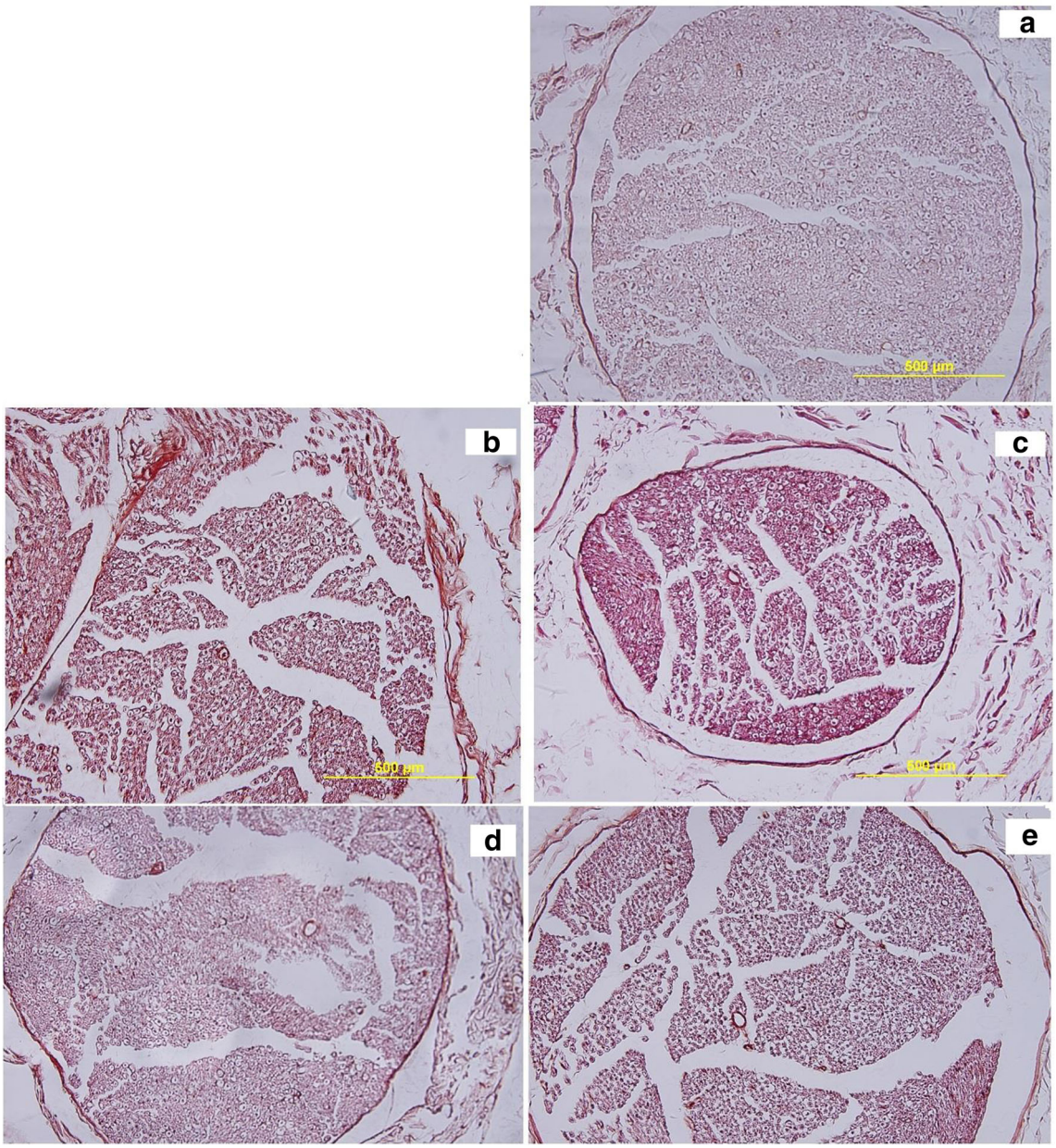


**Fig. 1** Light microscopy examinations of collagen content of sciatic nerve in different groups. A, control; B, 4WD; C, 8WD; D, 4WD + BT; E, 8WD + BT. Intensities of picrofuchsin stain are higher in 4WD (**b**) and 8WD (**c**) than control (**a**) and benfotiamine treated groups (**d** and **e**). Picrofuchsin staining, 400x

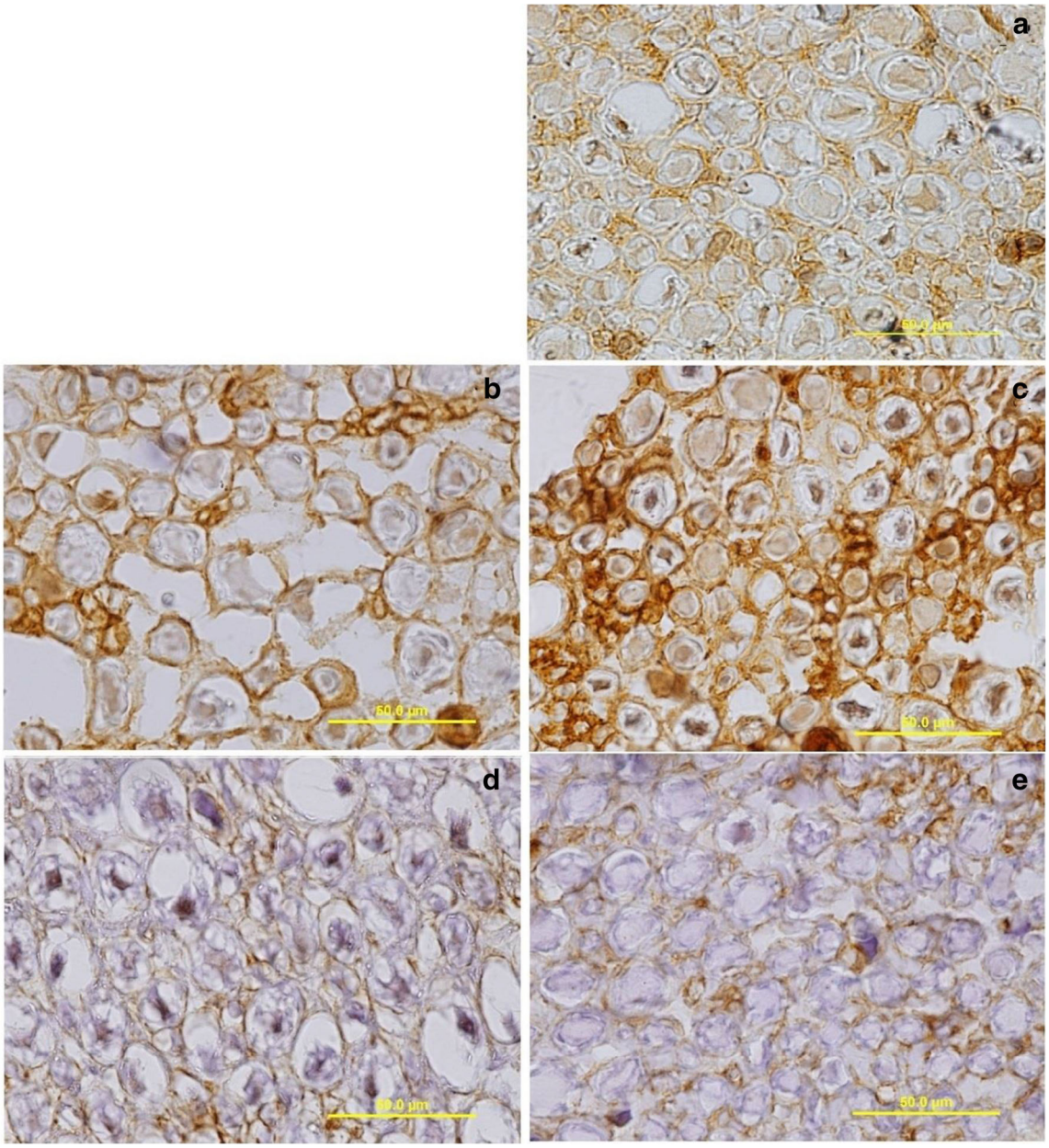


**Fig. 2** Evaluation of type IV collagen content in the sciatic nerve of different groups, using immunohistochemical technique. A, control; B, 4WD; C, 8WD; D, 4WD + BT; E, 8WD + BT. Intensities of immunostaining are higher in 4WD (**b**) and 8WD (**c**) than control (**a**) and benfotiamine treated groups (**d** and **e**). 400x

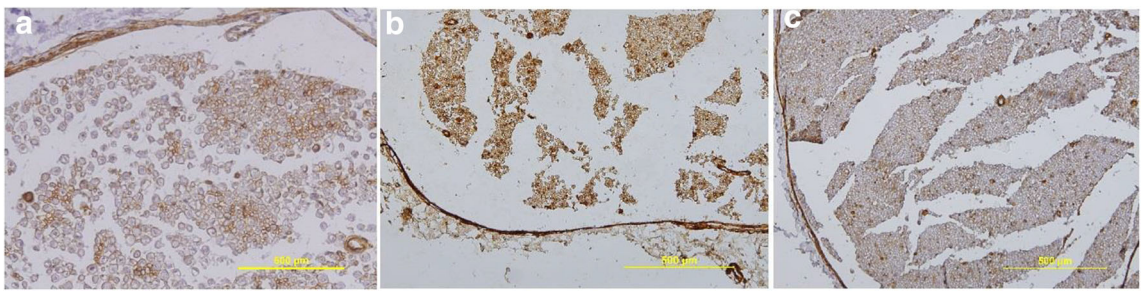


**Fig. 3** The type IV collagen contents in different compartment of sciatic nerve. The type IV collagen contents are remarkable in epineurium and endoneurium. **a** control; **b** 8WD; **c** 8WD + BT (100x)

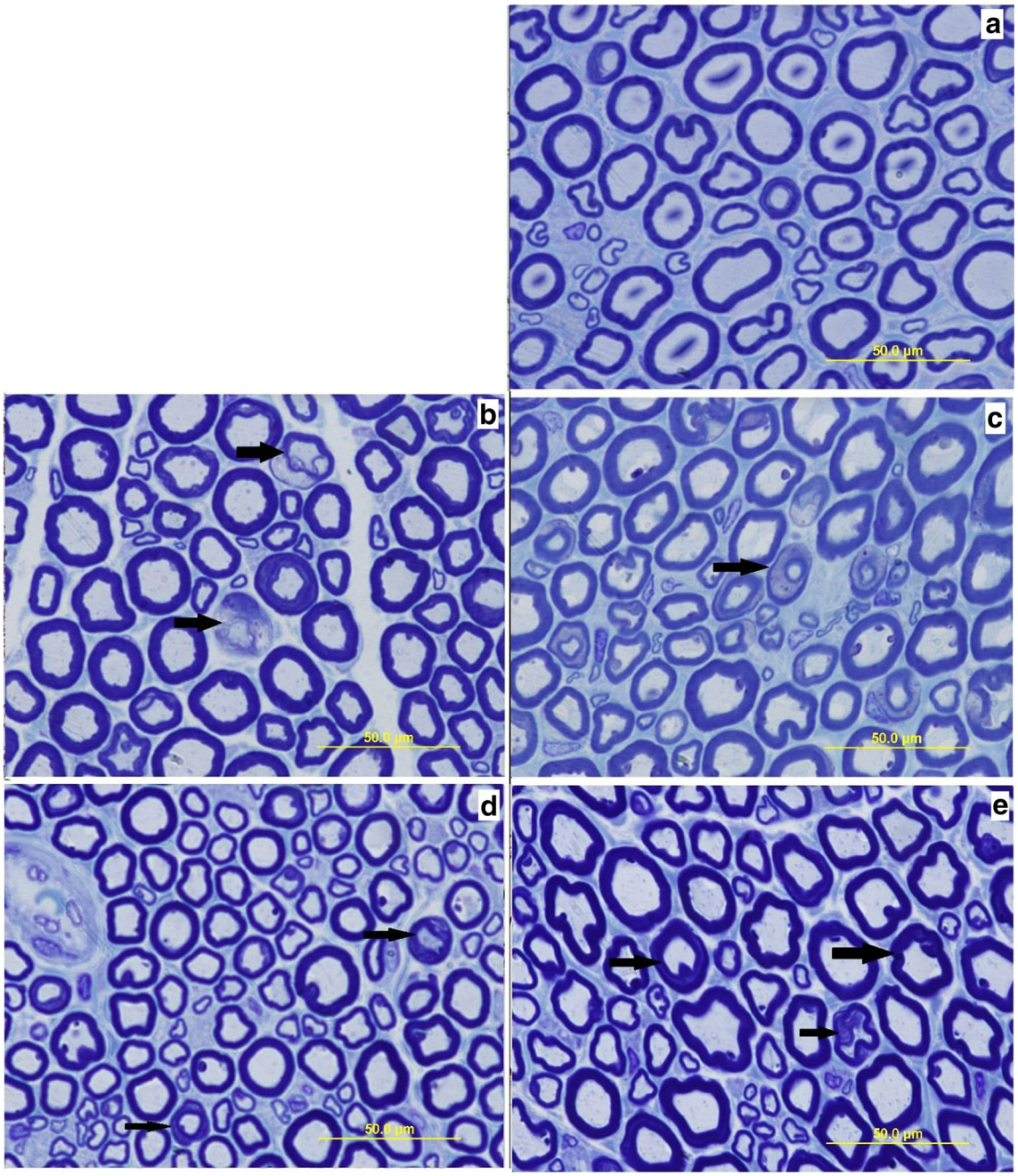


**Fig. 4** Toluidine blue stained photo micrographs of nerve fibers. **a** Control, **b** 4WD, **c** 8WD, **d** 4WD + BT, **e** 8WD + BT. The impaired nerve fibers and myelin sheath (black arrows) are more pronounced in B and C than D and E .(400x)

repeated twice. Samples were then washed with distilled water and stored in PBS (pH = 7.2) at 4 °C [28].

## Tensile strength test

For biomechanical assessments, 1 cm length segments of sciatic nerves and/or its scaffolds were fixed in the jaws of the tensile machine (SANTAM- STM20, Germany). The nerves/scaffolds were then subjected to strain tension at 1 mm/sec until rupture. The device recorded the breaking strength and maximum elongation [29].

## Statistical analysis

Data were analyzed using the SPSS software version 16.0. The differences between the experimental groups were compared by one-way analysis of variance (ANOVA), followed by Tukey´s post hoc test. The level of statistical significance was accepted if the *p*-value was less than 0.05.

## Results

### Light microscopy examinations

#### Picrofuchsin staining

Picrofuchsin as an acidic stain converts into pink-colored substances when reacting with acidophilic, such as collagen, agents. Examination of picrofuchsin stained sections show that in compared with control group the stain intensities are higher in 4WD and 8WD groups (Fig. 1). The comparison of benfotiamine untreated and treated groups (4WD and 8WD vs. 4WD + BT and 8WD + BT) show the lower stain intensities in hyperglycemic rats treated with benfotiamine (Fig. 1).

#### Collagen type IV Immunohistochemistry

As seen in Fig. 2, all samples show positive staining for type IV collagen in epineurium, perineurium, and endoneurium. In comparison to control and 4WD + BT and 8WD + BT groups, the intensities of staining are higher in 4WD and 8WD groups (Fig. 2). The staining intensities are remarkable in endoneurium and epineurium (Fig. 3).

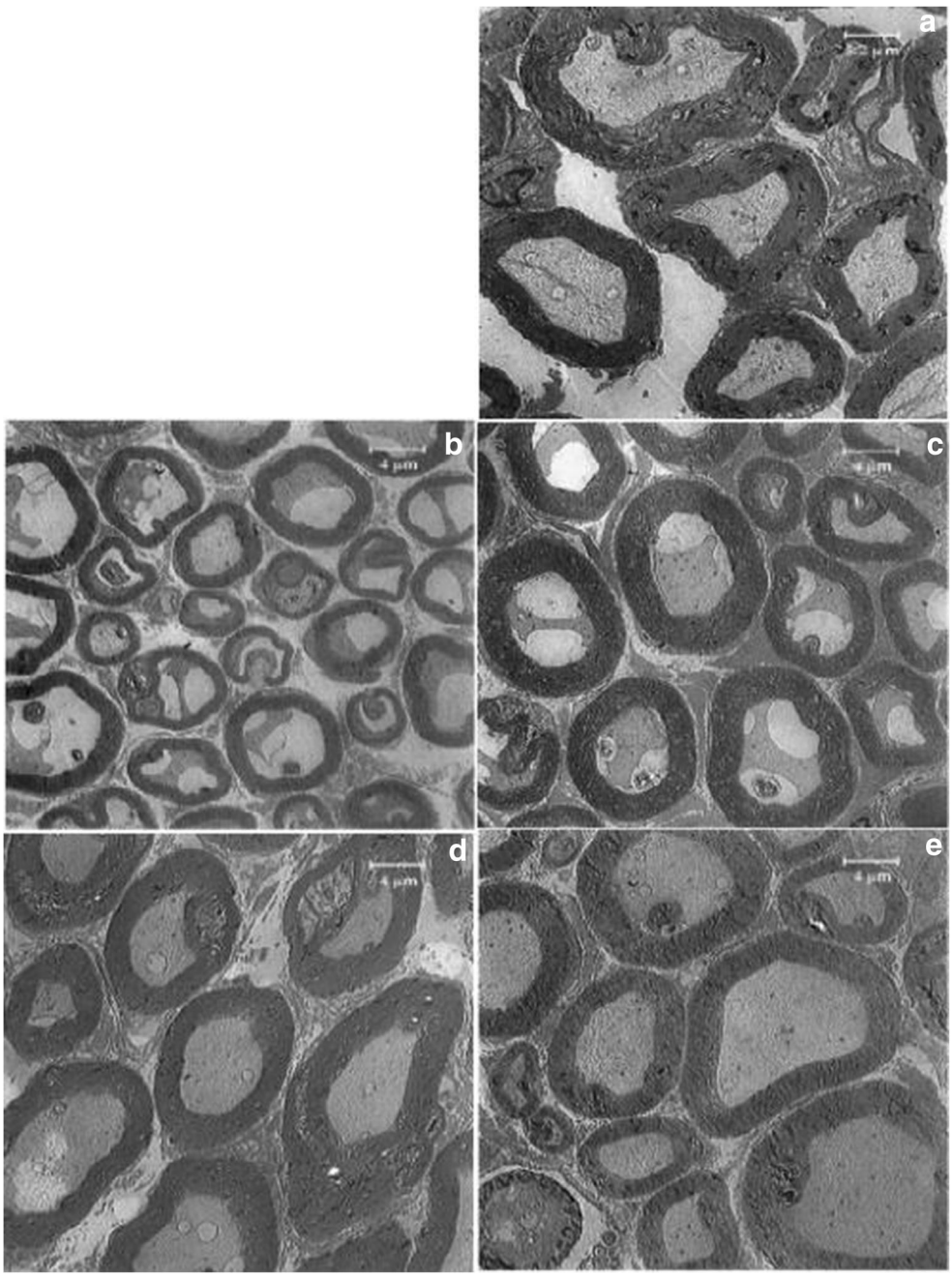


**Fig. 5** The ultra-structure of the sciatic nerves. **a** control; **b** 4WD; **c** 8WD; **d** 4WD + BT; **e** 8WD + BT. The myelin destruction and demyelination of axons are apparent in b and c. By benfotiamine treatment the degree of demyelination and separation of myelin layers are substantially declined (d and e).(500x)

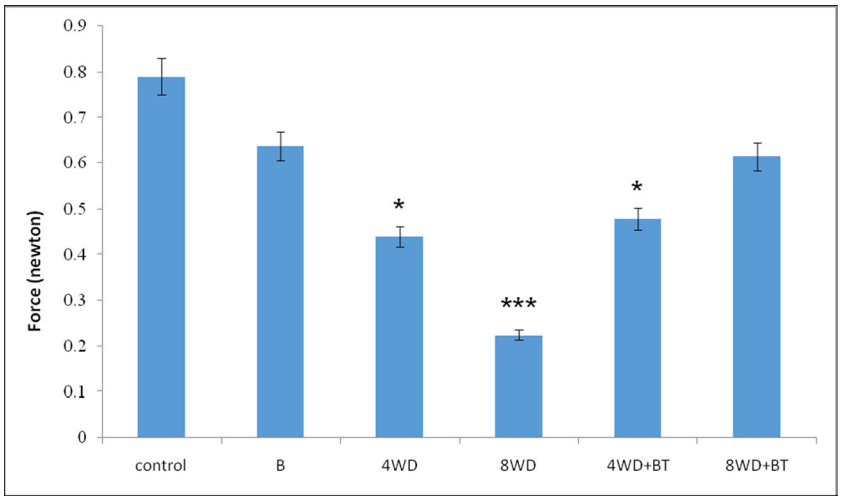


**Fig. 6** Comparison of the results of force-induced displacements (the threshold of nerve lengthening to rupture) in different groups. Data are presented as mean ± standard deviation. *$p < 0.05$ and **$p < 0.01$ compared with control (n = 8)

### Toluidine blue staining

The photo micrographs of semi-thin sections of sciatic nerves stained with toluidine blue are presented in Fig. 4. Toluidine blue stains the myelin sheath. As seen in Fig. 4, the appearance of nerve fibers and myelin sheath are normal in control (A) but in 4WD (B) and 8WD (C) sections the nerve fibers impaired so that axons lost their circular-like shape, and some protrusions and concavities are appeared in myelin sheath. These detrimental changes are more moderate in 4WD + BT (D) and 8WD + BT (E) sections

## The results of TEM examination

TEM results (Fig. 5) are in line with the findings of light microscopy. In comparison to typical morphology of sciatic nerve in control group in 4WD and 8WD samples, the sciatic nerves exhibit shrinkage and miniaturization of axons, myelin destruction, vacuolation, and separation of myelin lamellae. These abnormalities are markedly declined in 4WD + BT and 8WD + BT samples.

## The results of strain strength test

The results of strain strength test and force-induced elongation of intact sciatic nerves and its scaffolds are illustrated in Figs. 6, 7, 8 and 9. In comparison to the maximum force ($F_{max}$) required for rupture of the intact sciatic nerve of control group, the required force is significantly reduced in 4WD and 8WD groups ($p < 0.05$) (Fig. 6). Eight weeks benfotiamine treatment relatively increased $F_{max}$ force (Fig. 6). The comparison of the results of stress-strain tests of sciatic nerve scaffolds show no significant differences between control and other groups (Fig. 7).

The results obtained from the force-induced elongation test of intact sciatic nerve (Fig. 8) show that the threshold of nerve lengthening to rupture is considerably decreased in the hyperglycemic groups, when compared with the control group. However, in comparison to hyperglycemic groups, the 4WD + BT and 8WD + BT groups show a higher degree of nerve lengthening (Fig. 7). The results of this test show no significant differences between the sciatic nerve scaffolds of different groups (Fig. 9).

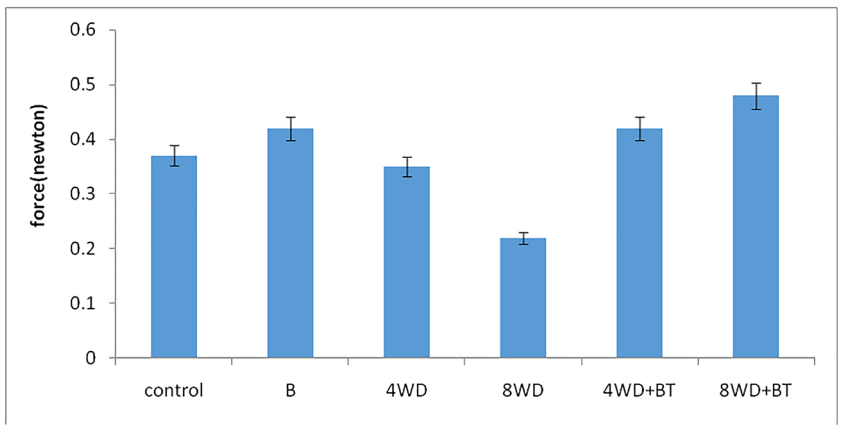


**Fig. 7** Comparison of the results of force-induced displacements (the threshold of nerve scaffold lengthening to rupture) in different groups. Data are presented as mean ± standard deviation, (n = 8)

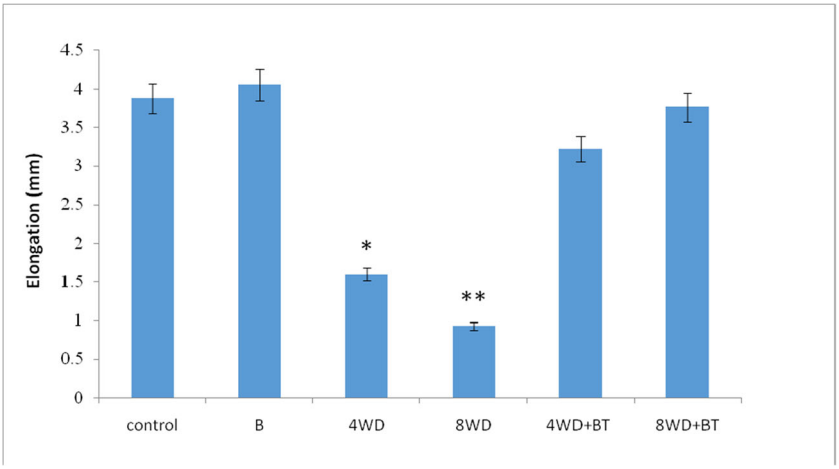


**Fig. 8** Comparison the results of strain strength test (the force required for rupture) of sciatic nerve in different groups. Data are presented as mean ± standard deviation. *$p < 0.05$ and ***$p < 0.001$ compared with control (n = 8)

## Discussion and conclusion

Collagens as extracellular proteins are the main elements of ECM responsible for the formation of the basement membranes [15] Fibrillar and microfibrillar collagens are two classes of collagen molecules produced in peripheral nerves; the former are fibril forming (collagens type I, III, and V) and the latter are the basement membrane collagens (type IV) [30] Type IV collagen is not only the primary collagen found in extracellular basement membranes but also a major component of endoneurium and epineurium. Accordingly, its location can be considered as the inner and outer connective tissue sheets of peripheral nerves [31–33].

Picrofuchsin staining and collagen IV immunohistochemistry techniques used in the present study clearly demonstrated that hyperglycemia could lead to an increase in the amounts of collagen IV in epineurium and endoneurium of sciatic nerve (Fig. 3). Previously, it has been shown that diabetic condition can increase the thickness of the basement membrane of perineurial cells in the sural nerve [34], skin nerve [35] and dorsal root ganglions [36]. Similarly, the levels of perineurium collagens that mainly consisted of type IV and V are also elevated in diabetes [37] The results obtained from the force-induced elongation test showed that the threshold of sciatic nerve lengthening to rupture considerably decreased in hyperglycemic condition (Fig. 7). In this regard, it seems that one of the reasons for vacuolization of axons and delamination of the myelin sheath seen in TEM examination (Fig. 5) is probably due to a decrease in lateral resistance of endoneurium, perineurium and epineurium of the sciatic nerve [38]. It is likely that hyperglycemia causes glycation of collagen monomers [14], and this change may prevent their polymerization and the formation of collagen fibrils [14]. Also, compared with the control group and hyperglycemic groups treated with benfotiamine (4WD + BT and 8WD + BT), the reduction of sciatic nerves elasticity (Fig. 6) and its decellularized scaffolds (Fig. 8) in hyperglycemic groups (4WD and 8WD) probably indicates the effects of hyperglycemia on the structure and content of elastic fibrils [39]. It seems that increasing the collagen type IV content of the sciatic nerve, on the one hand and decreasing the force-induced elongation test values, on the other hand, indicate that in hyperglycemic conditions, the ratio of collagen IV to collagen I, II and V may have increased. This conclusion is reinforced by the results of strain strength test of sciatic nerve scaffolds (Fig. 9). During sciatic nerve decellularization, only fibrous

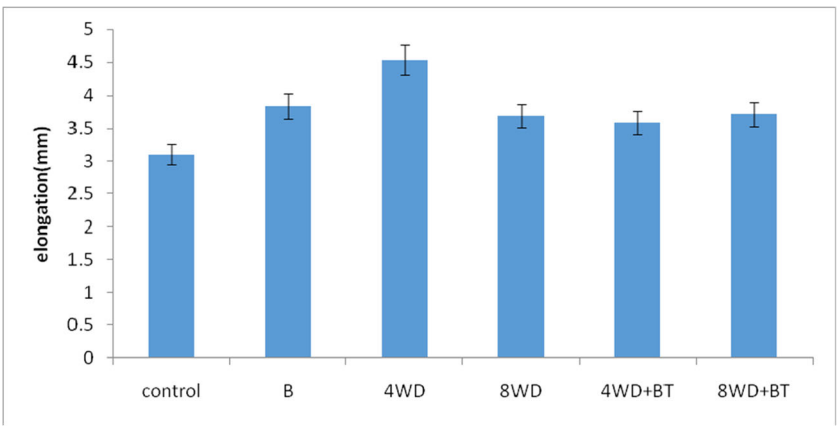


**Fig. 9** Comparison of the results of strain strength test (the force required for rupture) of decellularized scaffolds of sciatic nerve in different groups. Data are presented as mean ± standard deviation, (n = 8)

collagen is likely to remain in the scaffold so that, in addition to the cellular components, other elements that make up the ECM, including collagen type IV, are removed [39, 40].

*In vitro* assays have shown that by increasing glucose in the culture medium, the amount of collagen would be increased in the ECM of the rat sciatic nerves [37] The glycation of collagen IV influences the survival of neurons by causing significant morphological discrepancies in cultured neurons [41] Overall, although the previous studies confirm an increment of collagen IV in diabetes, they do not provide convincing explanations for the mechanisms underlying this phenomenon. In present study treatment of rats with benfotiamine may impedes an increase in the amount of collagen IV in the basement membrane probably via the suppression of non-enzymatic glycation [23, 42, 43].

Collagen can facilitate the connectivity of the Schwann cells to nerve fibers through a mechanism mediated by $\alpha 1\beta 1$ and $\alpha 2\beta 2$ integrins [44] On the other hand, it is likely that increase in the collagen contents could affect the function of Schwann cells, form of abnormal myelin [44] and change in the composition and structure of ECM integrins [11]. In hyperglycemic condition, increase in the amount of collagen in the basement membrane of the endoneurium vasculatures [45] and the thickness of the endoneurial vascular wall [46] may lead to the emergence of atrophy in nerve fibers [47, 48]. The TEM results obtained from the present study showed that, in comparison to hyperglycemic rats, in hyperglycemic animals treated with benfotiamine the axonal detachment from myelin sheath as well as axonal atrophy have declined (Fig. 5).

It has been implicated that diabetes leads to stiffness of the sciatic [49] and optic nerves [50] An increase in stiffness and decrease in flexibility of peripheral nerves might result in reduced mechanical strength of the nerves during the physical stress due to organ movements and can be associated with painful symptoms in patients with diabetic neuropathy[51]. However, despite the relationship between the severity of neuropathy and the reduction in tensile strength, it is still unclear whether a change in biomechanical properties of the nerves is responsible for the exacerbation of diabetic neuropathy or such an alteration could be considered the effects; rather than the cause. As mentioned above, the similarity of mechanical stability of scaffolds belonging to hyperglycemic and control animals (Figs. 8 and 9) suggests that some components of the ECM that have been removed during the decellularization process, contribute to the stiffness of peripheral nerves. On the other hand, the tensile strength criteria of sciatic nerve belonging to benfotiamine treated hyperglycemic rats reached to the control values (Figs. 6 and 7). Considering the fact that benfotiamine can suppress three biochemical pathways (hexosamine, AGEs, and diacylglycerol-protein kinase C pathways) [6]. which play fundamental roles in the pathogenesis of hyperglycemia-induced injuries [52] it seems that benfotiamine treatment is capable of reducing the production of AGEs [19], fine-tuning of dysregulated protein kinase C pathway [19], and decreasing the activity of aldose reductase to lower the concentration of sorbitol [19]. The potency of benfotiamine in hampering the above mentioned pathways may open up a new horizon to manage the complications of diabetic neuropathy.

Overall, our findings suggest that hyperglycemia probably causes significant changes in the composition of the ECM of sciatic nerves. Based on the results, the alteration in the collagen contents possibly influences the tensile strength of the peripheral nerves. It was also revealed that hyperglycemia led to the alterations in axonal structures and myelin sheath which may play a crucial role in the pathogenesis of diabetic neuropathy. Administration of benfotiamine considerably attenuated the deleterious effects of hyperglycemia more likely through the modulation of cellular glucose metabolism.

**Acknowledgements** This work was supported by a grant (No. 41853) from Ferdowsi University of Mashhad, which is greatly appreciated.

## Compliance with ethical standards

**Conflict of interest** The authors declare no conflict of interest.

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