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RESEARCH ARTICLE

IMPACT OF EXPERIMENTAL HYPERGLYCEMIA ON THE SCIATIC NERVE OF ALBINO RATS - A HISTOLOGICAL AND HISTOMORPHOMETRIC STUDY

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ABSTRACT

The complications of chronic diabetes affect many systems including the nervous system wherein the peripheral neuropathy is the most common presenting feature and which if not controlled may necessitate limb amputation. Since the exact mechanism of hyperglycemia-induced peripheral neuropathy and its treatment are not completely known, the present study was aimed at an effort to further the information in this regard. After ethical clearance, 36 animals were divided into six groups having six rats each. The groups consisted of age-matched controls and experimental groups of two weeks, one month, two months, four months and six months duration of diabetes. Diabetes was induced by a single dose of streptozotocin (60 mg/kg, intraperitoneal). At the end of each experimental period, animals were euthanized and perfusion fixed with Karnovsky's fixative. The blood serum was subjected to biochemical analysis and the sciatic nerve tissue blocks were processed for paraffin sectioning, routine and special staining for light microscopy. Biochemical, histomorphological findings and histopathological features revealed that the prolonged duration of hyperglycemia resulted in the raised serum creatinine, lowered serum total protein, reduction of myelinated fibers and remarkable thickening of endoneurial, perineurial and epineurial collagen. It is concluded that the biochemical alterations, change in the ratio of myelinated fibers and heaping up of collagen fibers at every level of organization appear to be important contributing factors for the deterioration of peripheral nerve functions in chronic diabetes.

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INTRODUCTION

Diabetes mellitus is one of the most common metabolic disorders (Naik *et al.,* 2015). Complications of chronic diabetes include peripheral neuropathy, nephropathy, angiopathy and retinopathy (Biessels *et al*., 1994; Pek *et al.,* 2017; Swaminathan *et al*., 2017). Involvement of large fiber conduction velocity is considered as an earliest functional marker of glucose neurotoxicity. This is typically present even before the decrease in axonal diameter or structural disruption of the myelin in central or peripheral nervous systems (Zochodne *et al*., 2004; Tomlinson *et al.,* 2008; Mohseni *et al*., 2017). Length-dependent nature of the peripheral neuropathy is characterized by a disturbance in axonal transport at most distal parts of long nerves as a result of pathological alterations in the cytoskeleton as well as axonal atrophy (Sima *et al.,* 1999; Prior *et al*., 2017). Diabetic neuropathy reveals diverse clinical manifestations such as chronic sensorimotor distal symmetric poly-neuropathy and the autonomic neuropathies (Boulton *et al*., 2005). The characteristic findings in diabetic neuropathy are sensory predominant nerve fiber degeneration, axonal loss, and thickening of endoneurial arterioles (Dyck *et al.,* 1996). Thickening of endoneurial arterioles and a reduction in the luminal area correlated well with subsequent nerve fiber loss in diabetic nerves (Malik *et al*., 2005). Lately experimental diabetes mellitus has been shown to induce distal small-fiber neuropathy (Bischhoffshausen *et al*., 2017) which initiate a syndrome of distal uncomfortable tingling sensation (Rolim *et al.,* 2017) and is also associated with distal sympathetic dysfunction (Maser *et al.,* 2017) in the form of allodynia, vasomotor changes, pallor alternated with rubor, cyanosis, and mottling (Low, 2003; Ugwu, 2017). The diabetic neuropathy represents a major health problem due to its clinical manifestations such as excruciating neuropathic pain, diabetic foot ulceration, and amputations, which are associated with substantial morbidity, reduced quality of life, and increased mortality (Ziegler, 2017). Recently, marked alterations in the peripheral nerve myelin thickness have been also reported in longstanding streptozotocin (STZ)-diabetic rats (Lee *et al*., 2017). Therefore, the present study was aimed at demonstrating these and possibly other changes in the arrangement of collagen fibers, nerve fibers and myelin by using special staining for collagen and myelin along with histomorphological and biochemical analyses in experimentally induced diabetic rats after 2 weeks and after 1, 2, 4 and 6 months. In a study "The laboratory rat: relating to its

age with human's" , it is described that one month of rat's life is equivalent to three human years (Sengupta, 2013). Therefore, 6-months duration of diabetes in rat may be considered to be equivalent to18 years of diabetes in human for purpose of assessment of pathophysiological parameters associated with chronic diabetes.

MATERIAL AND METHODS

Animal preparation and experimental design: Age-matched control and non-diabetic rats of either sex (total 36 rats) weighing ~250g were obtained from the central animal house, AMU, Aligarh, after approval from Institutional Animal Ethics Committee (D. No: 9025/2014). Prior to commencement of the experiment, all animals were placed in a clean, well ventilated, properly maintained new environmental condition and monitored daily with regard to body weight, urine sugar (strip method) for a period of one week; they were supplied standard pellet diet and water ad libitum and maintained on a 12/12 h light/dark cycle.

After one week, animals were divided into six age-matched groups having six rats each: (1) non-diabetic healthy control, (2) diabetic since two weeks (2W), (3) diabetic since one month (1M), (4) diabetic since two months (2M), (5) diabetic since four months (4M) and (6) diabetic since six months (6M).

Induction of diabetes and tissue preparation: After 12 hour fasting, experimental diabetes was induced by a single dose of STZ (60 mg/kg, aqueous solution, intraperitoneal). Blood from lateral tail vein was used to monitor blood sugar level with Glucometer (GlucoOne BG03 Blood Glucose Meter, Dr. Morepen). Animals with fasting blood sugar level 250 mg/dl and above were considered as diabetic (Blood sugar analysis was done after overnight fasting). Body weight and blood glucose levels were monitored biweekly until the end of the experiment. Animals were euthanized at the end of the experimental period with an overdose of ether general anesthesia and the whole body was perfusion-fixed with Karnovsky's fixative.

Histopathology and histomorphometry: After two days of fixation; sciatic nerves from both sides were carefully dissected out and processed for paraffin embedding. Hematoxylin and eosin, Luxol fast blue, and Picrosirius red stained, 5 µm thick sections were observed and relevant findings were recorded at X400 and X1000 magnification in a trinocular microscope (BX40, Olympus, Tokyo, Japan) equipped with a digital camera (18.2 MP, Sony, Tokyo, Japan); measurements were made using software Motic (Xiamen, People's Republic of China) Image version 2.0 for histomorphometry. The histomorphometric analysis for nerves was performed in an area of 10^5 μm^2 and the nerve fibers were divided into small myelinated fibers (< 7 µm), and large myelinated fibers (> 7 µm).

Biochemical estimation and analysis: Blood glucose levels were measured from lateral tail vein blood twice a week. At the end of the assigned experimental period, blood samples were obtained from direct puncture of heart and collected into sterilized plastic vials. Samples were allowed to clot, centrifuged at 2500 rpm for 30 minutes; the serum was separated, stored and subsequently assayed for serum total protein content and serum creatinine level by using clinical chemistry Analyzer C61 (Avantor Benesphera^{TM}, Center Valley).

Statistical analysis: The data related to the total number of myelinated nerve fibers as well as the segregated quantitative data from small and large-sized fibers, serum total protein and serum creatinine levels were statistically analyzed and the significance calculated using one way ANOVA followed by Tukey's test. All numerical values were expressed as mean ± standard deviation and the value of $P<0.05$ was considered as statistically significant.

RESULTS

General observations, body weight, and blood sugar: Throughout the experimental period after induction of diabetes, all diabetic groups exhibited the classical clinical manifestations of diabetes such as polyphagia, polydipsia and polyuria. The mean values of body weight were reduced as well as blood sugar level showed hyperglycemia (> 500 mg/dl) in all diabetic groups throughout experimental periods

Histopathology: The peripheral nerve of all groups exhibited bundles of myelinated nerve fibers. All nerves fibers, nerve bundles and the entire nerve were surrounded by connective tissue of different thickness. In Luxol fast blue and Picro-Sirus red stained section, one could observe the epineurium, which covered the entire peripheral nerve. In 2W and 1M diabetic groups, the amount of dense connective tissue in epineurium looked minimal but with the progression of the duration of hyperglycemia, the amount of epineurial connective tissues increased. Compared with age-matched control the long-standing diabetic groups of 2, 4 and 6M exhibited numerous thickened epineurial collagen fibers around the entire nerve (Figure 1 and 2). Thin perineurial connective tissue was thin and uniform in 2W, 1M and 2M diabetic groups. However, in 4M and 6M diabetic groups quite thick perineurial collagen was observed as compared to age-matched control groups (Figure 1). Individual nerve fibers were surrounded by thin endoneurial connective tissues. Hematoxyline and eosin stained sections showed large nuclei of Schwann cells over the myelin sheath as well as small nuclei of fibrocytes in the endoneurium. In 2, 4 and 6M diabetic groups the collagen fibers in the endoneurium were thicker than in age-matched control groups. In 2W and 1M diabetic groups, only slight changes were observed (Figure 3). In LFB and PSR stain, 4 and 6M diabetic groups the myelin took faint staining or remained unstained but in 2W, 1 and 2M group myelin stained very lightly as compared with age-matched control groups (Figure 1). In 2W and 1M diabetic groups, the connective tissue around the perineurial blood vessel had few collagen fibers. And the same was observed in epineurium also. In 2, 4 and 6M diabetic groups similar finding were also observed but there was a slight difference in the collagen fiber thickness around blood vessels (Figure 4).

Histomorphometry: The evaluation of nerve morphology was based on the diameter of the nerve fiber. There was a reduction in the total number of myelinated fibers with loss of small diameter myelinated fibers and increase in large-sized myelinated fibers which were significant ($P<0.05$) in the sciatic nerve of 1, 2, 4 and 6M diabetic groups compared to the age-matched control groups (Table 1).

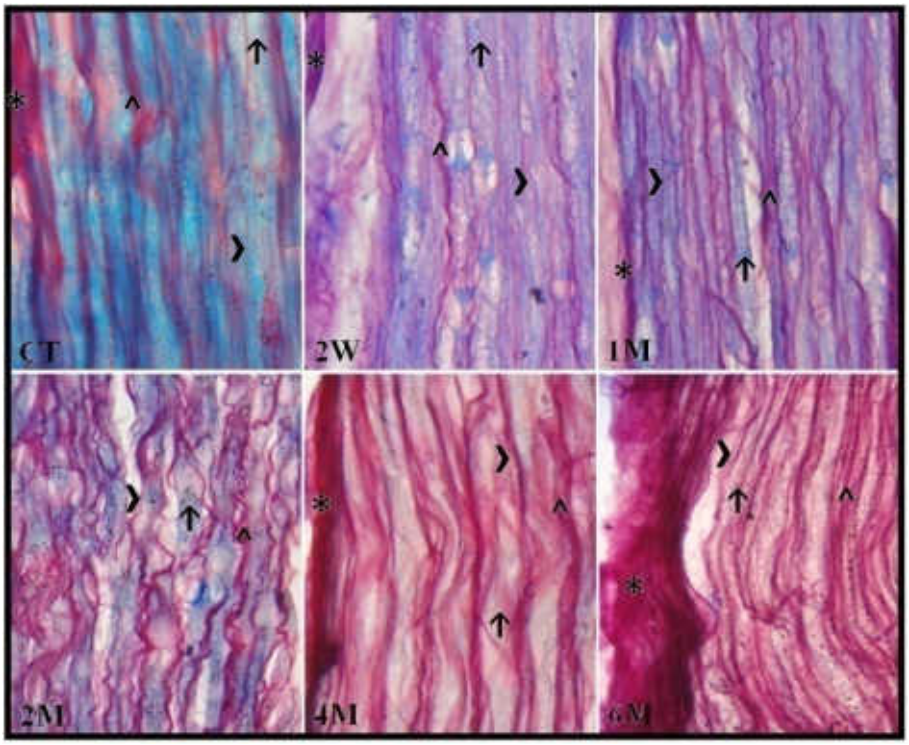

Figure 1. Longitudinal sections of the sciatic nerve from control and all diabetic groups. Note the varying sized myelinated fibers in different groups of the sciatic nerve (↑), endoneurium (>), perineurium (^), and epineurium (*). Luxol fast blue and Picrosirius red. X1000

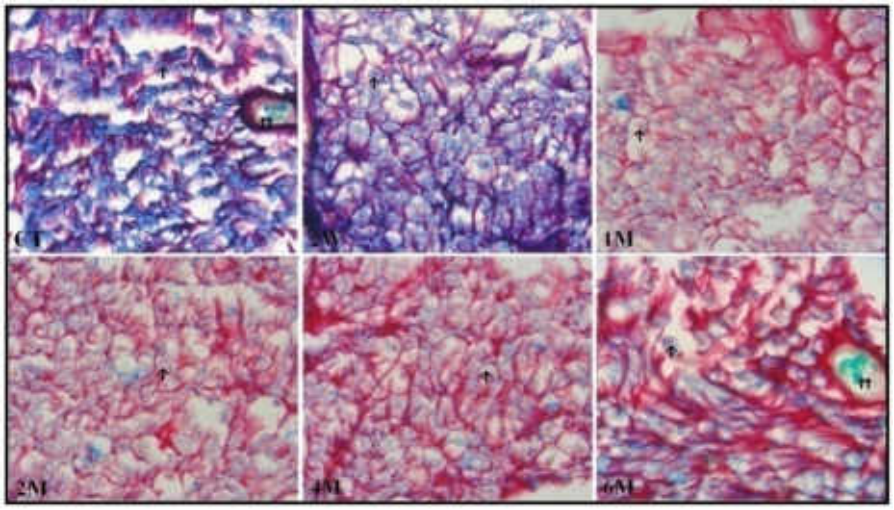

Figure 2. Transverse sections of the sciatic nerve from control and all diabetic groups. Note: myelinated nerve fibers (↑) blood capillaries, (↑↑). Collagen fibers (red) along the nerve fibers and around the blood vessels in 1, 2, 4 and 6M diabetic groups are thicker and numerous. Picrosirius red with Luxol fast blue. X1000

Table 1. Quantitative changes in the myelinated axons of the sciatic nerve in diabetic animals as compared with age-matched control groups (number ± standard deviation; N = 6 for each group).

Group	Number of myelinated fibers	Number of small myelinated fibers (< 7 µm)	Number of large myelinated fibers (> 7 µm)
2W-Control	412.13 ± 18.92	195.01 ± 09.47	217.13 ± 07.89
2W-Diabetic	402.25 ± 24.92	183.12 ± 14.43	219.13 ± 06.44
1M-Control	408.25 ± 22.52	202.38 ± 06.52	205.88 ± 09.20
1M-Diabetic	401.63 ± 20.19	174.25 ± 18.05	227.38 ± 09.41
2M-Control	418.38 ± 23.69	188.25 ± 18.14	230.13 ± 07.53
2M-Diabetic	385.13 ± 18.98	149.25 ± 17.29	235.88 ± 05.79
4M-Control	421.88 ± 29.47	182.75 ± 33.81	239.13 ± 08.16
4M-Diabetic	351.13 ± 17.13	106.88 ± 16.05	244.25 ± 10.26
6M-Control	423.75 ± 26.30	178.63 ± 30.93	245.13 ± 08.39
6M-Diabetic	305.13 ± 07.13	094.75 ± 7.38	210.37 ± 10.77

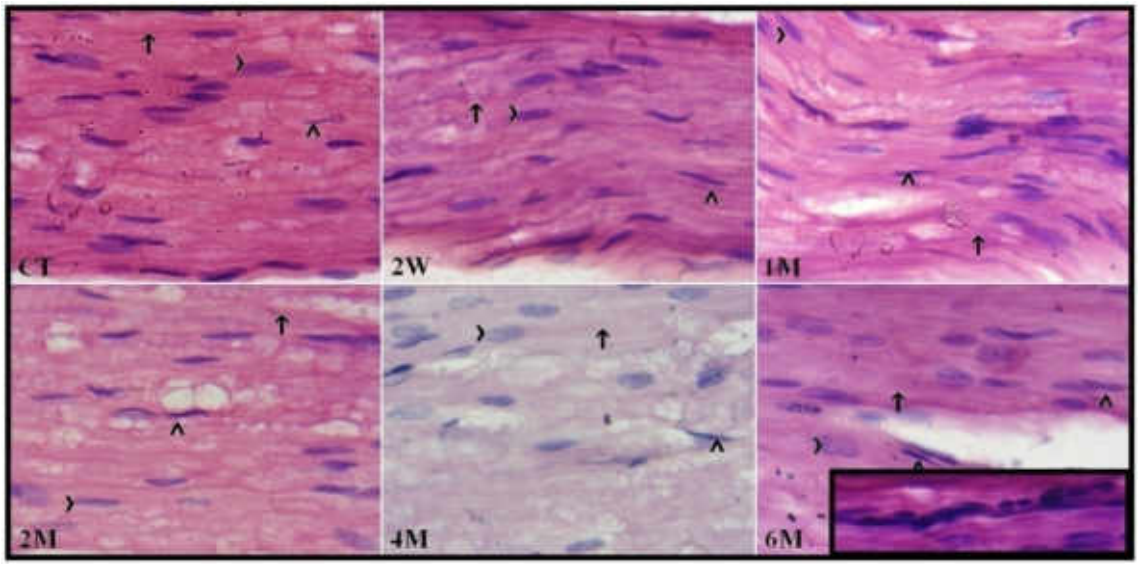

Figure 3. Longitudinal sections of the sciatic nerve. Note: myelinated nerve fibers (↑) Schwann cell nucleus, (>) and nucleus of fibrocytes (^). Inset shows aggregation of Schwann cell nuclei. H &E stain. X1000

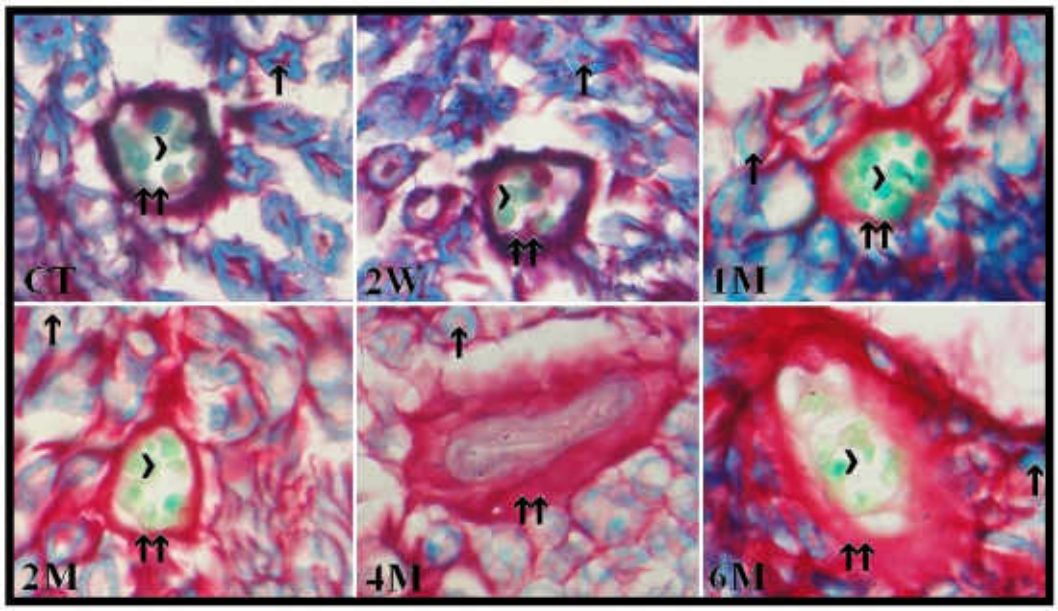

Figure 4. Transverse sections of the sciatic nerve showing blood vessels, (↑↑) RBC (>) and myelinated nerve fibers (↑) and collagen fibers (red) along the nerve fibers and in the adventitia of the blood vessels. The adventitia is of comparable thickness among control, 2 weeks, 1 and 2 months while it is thicker in 4 and 6M diabetic groups compared to the diameter of vessels. Picro-Sirius red with Luxol fast blue. X1000

Biochemical analysis: Serum creatinine levels were significantly increased ($P<0.05$) in 1, 2, 4 and 6M diabetic groups as compared to age-matched control groups. Conversely, serum total protein levels significantly decreased ($P<0.05$) in all diabetic groups as compared to age-matched control groups.

DISCUSSION

Diabetes mellitus is the most common disorder associated with impairment in the metabolism of carbohydrates, lipids and proteins (American Diabetes Association, 2017) in which tissues fail to react properly to insulin in the form of glucose resistance and glucose intolerance, resulting in hyperglycemia (American Diabetes Association, 2015). Hyperglycemia-induced oxidative stress and altered antioxidant levels may lead to potentially severe secondary complications in a number of organ systems and development of peripheral and central nervous systems abnormalities (Vincent *et al.,* 2004; Faizal *et al.,* 2017). In the present study, the general changes observed in diabetic animals were the same as previously reported (Faizal *et al.,* 2017). There was a reduction of body weight in all diabetic groups which could be attributed to muscle wasting and loss of tissue proteins as a result of lack of insulin, causing increased glycolysis and gluconeogenesis (Air *et al*., 2002; Jain, 2014). These findings were also in agreement with previous related studies (Doddigarla *et al*., 2016; Faizal *et al*., 2017). Arrangement and direction of peripheral nerve fibers in the present study appeared similar to other related studies (Malak *et al*., 2015). However, long-standing hyperglycemia-induced structural alteration in the peripheral nerve e.g., in 4M and 6M diabetic groups there was poor staining of myelin sheath due to subtle demyelination of nerve fibers. One of the earlier studies also showed similar results in STZ-induced diabetic rats (Lee *et al*., 2017) concluding that it might be due to irreversible damage to the peripheral nerve.

Connective tissues provide structural support to nerve bundles and blood vessels at all level of organization (Faizal *et al*., 2018). It has been noted that prolonged hyperglycemia initiates inflammation, associated fibrosis and excess deposition of extracellular matrix in the perivascular connective tissue, followed by a vascular occlusion, ischemia and cell atrophy (Kundalic *et al*., 2014). Some researchers have demonstrated thicker collagen fibers in the connective tissue of endoneurium, perineurium, and epineurium in diabetic groups than in controls (Kundalic *et al*., 2014; Elgayar *et al*., 2017). In the current study, the control and 2W diabetic groups showed the presence of connective tissue with thin collagen fibers in endoneurium, perineurium, and epineurium and also around the blood vessels inside the peripheral nerve. However, 2M, 4M and 6M diabetic groups showed progressively increasing the thickness of collagen fibers in the endoneurium, perineurium, and epineurium of the peripheral nerve. Earlier studies demonstrated advanced glycation end products (AGEs) are concerned with the advancement of submesothelial fibrosis and neoangiogenesis (De Vriese *et al.,* 2003). Progressive fibrosis in a diabetic heart by PKC-β and p38 mitogen-activated protein kinase expression (Olubunmi *et al*., 2016), and excess collagen accumulation in endoneurial compartment adversely affects the regeneration and growth of nerve fibers supported by Schwann cells (Bradley, 2000). In diabetic neuropathy, epineurial vessels are most prone to macrovascular changes like occlusion of blood vessels and thrombosis.

These changes also impair nerve blood flow and cause hypoperfusion, basement membrane thickening, and tunica intima cells proliferation (Llewelyn *et al.,* 1998; Ibrahim *et al*., 1999; Tesfaye *et al*., 2005), Epineurial arteriolar attenuation with venous tortuosity and distension also results in the reduction of endoneurial blood flow (Tesfaye *et al*., 1993). In the chronic hyperglycemic state, endothelial aldose reductase increases polyol pathway activity as well as vasoconstriction in peripheral nerve producing ischemic pathogenesis resulting in neuropathy (Dyck *et al*., 1985). Advanced glycation end products are formed from proteins like laminin, fibronectin and collagens, mainly I, III, IV and VI (Nukada *et al*., 1996; Goh *et al.,* 2008) and neurofilaments in the nerve axonal fibers and myelin protein in Schwann cells may also be modified by AGEs in nerve fibers influencing the repair mechanism of damaged nerves (Wada *et al.,* 2005). In the current study, fibrosis was observed around the blood vessels in prolonged hyperglycemia groups due to deposition of collagen fibers. Comparison of the current result and previous reports on perineurial and endoneurial fibrosis and thickening of collagen fibers around the adventitia of nerve capillaries (Faizal *et al.,* 2017) suggests that hyperglycemia accelerates fibrosis. The vascular concept of peripheral neuropathy implies that diabetes-induced endothelial dysfunction resultant decrease in nerve blood flow, vascular reactivity, and endoneurial hypoxia plays a key role in functional and morphological changes in the diabetic nerve. Fibrosis impairs the balance of endoneurial homeostasis and regenerating ability of the nerve fibers. Sciatic nerve, which is the thickest peripheral nerve containing the sensory, motor, and autonomic nerve fibers (Gewandter *et al.,* 2017), was the object of the current study. The difference between large-size and small-size nerve fibers concerns anatomy, transmitting pathways, neurogenesis, energy utilization, ion distribution, relationship with glial cells and sensitivity to differing neurotrophic factors (Zotova *et al.,* 2008). Small-diameter neurons degenerate in experimental diabetic neuropathy (Beiswenger *et al.,* 2008).

In the current study, the myelinated nerve fibers of the sciatic nerve at many locations appeared disorganized and degenerated with varying grades of defects in myelin sheath in addition to their focal variable thickness. Reduction of the size of myelinated nerve fiber in diabetic rats may be attributed to a loss of neurofilaments due to decreased protein synthesis in the neuronal cell bodies (Yagihashi *et al.,* 1990). The loss of axonal cytoskeletal elements may aggravate the vulnerability of diabetic nerves to environmental factors followed by fiber degeneration and eventually fiber loss. In addition prolonged hyperglycemia, oxidative stress activates polyol pathway, induces sorbitol accumulation and impairs nerve Na^{+}-K^{+}-ATPase activity leading to nerve dysfunction in terms of slowing of nerve conduction (Tarr *et al.,* 2013) and structural abnormalities of diabetic neuropathy (Sima *et al.,* 1985). In the current study long-standing hyperglycemic rats, myelinated nerve fibers in the sciatic nerve showed quantitative changes like reduced number of total myelinated fibers and loss of small-sized myelinated fibers resulting into the increased proportion of large myelinated fibers.

The serum creatinine formed from amino acid metabolism is known to be a significant marker of diabetic nephropathy (Ceriello *et al.,* 2000; Ronco *et al.,* 2010). Our results showed a high serum creatinine level in all diabetic groups parallel to the severity of hyperglycemia as compared with age-matched control. However, the serum total protein levels were reduced in all diabetic groups. Those results hint to a positive correlation of hyperglycemia and the development of diabetic nephropathy (Sjoholm *et al.,* 2006) with a low-grade inflammatory process (Sjoholm *et al.,* 2006). Similar observations have been shown in the other related study (De Almeida *et al.,* 2012). Based on the findings of the present study it is concluded that the prolonged hyperglycemic state leads to increased serum creatinine and reduced serum total protein levels and overall decrease in small-sized nerve fibers as well as heaping up of collagen around the nerve and blood vessels, which may constitute important contributing factors in development of diabetic peripheral neuropathy.

Conflict of interest

The authors declare to have no conflict of interest.

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