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Research Article

Rat Bone Marrow-Derived Mononuclear Cells Outperform Folic Acid in the Treatment of Diabetic Peripheral Neuropathy in Streptozotocin-Induced Diabetic Rats

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ABSTRACT

Background: Diabetes mellitus is the most common cause of peripheral neuropathy, which itself is mediated in part via oxidative stress. Recent studies have used stem-cell-based therapies to regenerate nerve tissues following diabetic neuropathy. Folic acid supplementation promotes neuronal development and protection in some neurological diseases.

Aim: This study aims to compare the effects of bone marrow-mononuclear cells (BM-MNCs) and folic acid (FA) in the treatment of peripheral neuropathy in streptozotocin-induced diabetic rats.

Methods: Forty adult male albino rats were randomly divided into four groups: Group I (healthy control group); Group II, diabetic group (a single intraperitoneal streptozotocin injection); Group III, diabetic rats that received BM-MNCs; Group IV, diabetic rats that were treated with FA (10 mg/kg/day intraperitoneal injection) for 4 weeks. Random blood sugar was measured for all groups. The animals were euthanized, and the right sciatic nerve was carefully extracted to measure sciatic nerve conduction velocity and processed for histopathological, immunohistochemical (CD68), electron microscopic and morphometric studies.

Results: The diabetic group showed progressive histological changes characteristic of neuropathy in the sciatic nerve. Also increased number of CD68-immunopositive cells were detected. In BM-MNC transplantation group, the sciatic nerve sections showed improved histological changes characteristic of neuropathy with a decreased number of CD68-immunopositive cells. The diabetic group treated with FA showed less histological results relative to diabetic rats treated with BM-MNCs.

Conclusion: Diabetic rats treated with BM-MNC showed better improvement in diabetic neuropathy than diabetic rats treated with folic acid.

Keywords: Diabetes; Streptozotocin; Bone marrow mononuclear cells; Folic acid.

INTRODUCTION

Diabetes mellitus (DM) is a major global healthcare problem and common metabolic disorder. Impaired insulin release and/or failure to respond to insulin is the underlying cause. DM-related complications involving eyes, kidneys, blood vessels and the nervous system are a significant cause of high rates of morbidity and mortality in patients with DM [1].

Diabetic polyneuropathy (DPN) is the most common diabetic complication. Almost half of diabetic patients complain of some form of neuropathy within two decades of their disease [2]. Indeed, DPN is a leading cause of disability, as it may lead to foot ulceration, amputation, gait disturbance and fall-related injuries. Cell therapy is considered an attractive therapeutic strategy for treating diabetic neuropathy. Freshly isolated rat bone marrow-derived mononuclear cells (BM-MNCs) are used in this treatment because transplantation of BMNCs has been shown to augment neovascularization and increase a wide range of neurotrophic and

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angiogenic factors, including vascular endothelial growth factor, fibroblast growth factor-2 and angiopoietin-1 in the tissue [3,4].

Folic acid (FA) is a vitamin necessary for growth and normal cellular function. An increase in homocysteine levels or oxidative stress may be one of the possible mechanisms contribute to the development of DPN. A number of studies associated with FA deficiency and increased plasma total homocysteine levels show higher risk of vascular disease, cerebral ischemia, neuropsychiatric and neurodegenerative diseases [5]. Moreover, FA supplementation has been used to support a range of health concerns including DPN and provide neural protection in some neurological diseases [6]. In this study, we evaluated and compared the beneficial effects of transplantation of rat BM-MNCs and FA in the treatment of DPN in streptozotocin (STZ)-induced diabetic rats.

MATERIAL AND METHODS

Animals

Fifty adult male albino rats (12 weeks old) weighing 200 to 250 grams were used in this study. The animals were housed in different cages according to their groups and provided with food and water ad libitum in the animal house of the Anatomy Department, Faculty of Medicine, Suez Canal University.

Experimental design

Group I (control): This group was given an intraperitoneal injection of ice-cold 0.5 mol/l citrate buffer as a control (pH 4.5) [7].

Group II: (DA group): Diabetes was induced using an intraperitoneal injection of streptozotocin (STZ) at 40 mg/kg [8].

Group III: (DA+MNC group): Diabetes-induced plus mononuclear cell group. Two weeks after the STZ injection, the diabetic rats received BM-MNCs in the muscles of the hind limb. The MNC suspension (0.5 ml in total, 1×106 cells) was injected into 10 points in the right femoral quadriceps, right femoral biceps and right soleus muscles using a 26-gauge needle [9].

Group IV: (DA+FA): Diabetes-induced plus folic acid group; two weeks after the STZ injection, FA was injected intraperitoneally (10 mg/kg/day) for 4 weeks [5].

Group V: (FA): Folic acid-treated group. Rats in this group were treated with FA as above, without the preceding STZ injection [9].

Treatment drugs

Citrate buffer (pH 4.5) was prepared by dissolving 180 ml of sodium citrate with 100 ml of citrate acid in 10 ml of distilled water. Folic acid (FA) was purchased from Sigma Chemical Co. (Egypt) in the form of leucovorin at a concentration of 50 mg, and the FA was given (10 mg/kg/day) through intraperitoneal injection for 4 weeks [7].

Induction of diabetes

DM was induced according to the method used by Padmanbhan: a single intraperitoneal injection of 40 mg/kg STZ (Sigma Chemical Co.) in ice-cold 0.5 mol/l citrate buffer (pH 4.5). After injection with STZ, the rats were given 5 ml of 5% glucose for 3 successive days to avoid severe hypoglycemia. Blood glucose levels were measured one week after injection by the Easy Gluco instrument (ONE TOUCH Ultra Easy) as follows: the animal's tail was scratched; one drop of its blood was added to an instrument strip and the blood sugar level was measured on the monitor. Rats with blood glucose levels exceeding 200 mg/dl were considered diabetic rats [7].

Bone marrow-derived mononuclear cells

BM-MNCs were obtained from 10 6-week-old donor male rats. The isolation of BM-MNCs was performed according to the method described by Naruse et al. [3] as follows:

The donor rats were sacrificed after anesthesia by intraperitoneal injection of 60 mg/kg ketamine (Ketalar, Sigma Chemical Co., Egypt) and 5 mg/kg xylazine (Bayer, Sigma Chemical Co., Egypt) [10].

The femora and tibiae of the donor rats were excised, and all connective tissues attached to the bones were removed.

a. The BM was aspirated from the femora and tibiae.

b. After all BM was collected in 3 ml phosphate buffered saline (PBS) containing 10% fetal bovine serum (FBS), it was centrifuged for 5 minutes at 160 g.

c. The supernatant was removed, and the precipitated fraction was resuspended in 1 ml of 10% FBS.

d. The suspension was added over 0.5 ml of Ficoll-Paque.

e. Centrifugation was performed at 400 × g for 15 min and resulted in a three-layered solution in which the middle layer contained the BM-MNCs. A pipette was used to extract the middle layer.

The MNC layer was collected, washed twice with PBS, and suspended in 0.9% saline with 0.5% bovine serum albumin (BSA).

Transplantation of BM-MNCs

Two weeks after the STZ injection, the diabetic rats were deeply anesthetized with an intraperitoneal injection of 60 mg/kg ketamine (Ketalar) and 5 mg/kg xylazine [10] and were transplanted with BMMNCs via the hind limb skeletal muscles. The MNC suspension (0.5 ml in total, 1 × 10^6 cells) was injected into 10 places in the right femoral quadriceps, right femoral biceps and right soleus muscles using a 26-gauge needle [3].

Six weeks after the STZ injection, the animals from all groups were anesthetized with an intraperitoneal injection of 60 mg/kg ketamine and 5 mg/kg xylazine and then sacrificed, and the right sciatic nerves were carefully removed from each animal. The sciatic nerve was processed to measure the sciatic nerve conduction velocity. A cut section from the sciatic nerve was processed for histopathological, immunohistochemical, electron microscopic and morphometric studies [9].

Dissection of the sciatic nerve and estimation of conduction velocities

The mid-thigh posterior incision method was used to harvest the sciatic nerve from rats, according to Bala et al. in 2014, and Leal-Cardoso et al., in 2004.

a. A small (5.0 mm) vertical incision was made along the posterior region of the right thigh using scissors, and the skin was retracted laterally.

b. The muscles of the posterior thigh (including the hamstring muscles) were then split to expose the sciatic nerve, which appeared as a thick whitish cord.

c. The muscles were further split until the entire length of the sciatic nerve in the thigh region was exposed.

d. The nerve was gently lifted using forceps and removed by cutting at the proximal and distal ends

e. The test was performed 6 weeks after STZ injection, and the sciatic nerve was mounted in a nerve chamber designed for recording the action potential from the isolated nerve.

f. The nerve was dissected without, and approximately 2 cm of the nerve was positioned over the electrodes. The proximal part of the nerve was stimulated by 2 platinum stimulating electrodes, and the recording electrode was placed away from the stimulating electrode [11].

g. Electrophysiological measurements were performed using a Biopac instrument followed by computer-assisted data analysis.

h. Sciatic nerves were stimulated with square wave pulses of 200μsec duration at 1-10 volts to assess conduction velocities. Conduction velocity was measured by dividing the distance between the stimulating and recording electrodes by the latent period, which is the time elapsed between the application of stimulus and the peak of the maximum compound action potential (CAP) [12].

Light microscopic examination

Specimens from the right sciatic nerves from each group were fixed in 10% formalin for 24 hours, dehydrated in ascending grades of ethyl alcohol and cleared in xylol, impregnated in soft paraffin for 2 hours at 55°C and embedded in hard paraffin blocks. Sections were cut to 5 microns in thickness, stained with hematoxylin and eosin (H&E) and examined by light microscopy. Myelin sheaths, axons and Schwann cells were illustrated [13].

Immunohistochemistry

The presence of CD68-positive activated macrophages in the sciatic nerve was examined immunohistochemically [14]. Tissue blocks were cut into 4 mm thick sections mounted on glass slides, incubated at 4°C overnight, deparaffinized in xylene and rehydrated. Endogenous peroxidase activity was blocked with 1% hydrogen peroxide for 20 min. Slides were probed with an anti-CD68α antibody (1:200, mouse monoclonal antibody, macrophage marker, cytoplasmic staining pattern, Thermo Scientific Company, USA) [15].

Electron microscopic examination

Tissue specimens of the sciatic nerves were divided into small pieces of 1 mm^3 in size, fixed in 2.5% glutaraldehyde, dehydrated and transferred on the next day into the tip of an individually labeled capsule. Semithin sections of 0.5-1 mm thickness were obtained, stained with toluidine blue and examined with light microscopy, and ultra-thin sections of 60 nm to 100 nm in thickness were obtained, contrasted with uranyl acetate and lead citrate and examined with electron microscopy.

Morphometry

A Leica Qwin 500 LTD image analyzer and computer system were used to count the numbers of total, normal, and degenerated nerve fibers. Additionally, the numbers of CD68-stained cells were counted on slides of semi-thin sections [16].

Statistical analysis

ANOVA and post hoc Tukey's HSD comparison tests were used to statistically evaluate the results obtained from the control and experimental groups. $P<0.05$ was considered statistically significant, and $p<0.01$ was considered highly statistically significant.

RESULTS

Light microscopic results

Examination of hematoxylin-and eosin-stained sections of the sciatic nerve from animals in the control group (Group I) showed a normal histological structure, characterized by nerve fascicles containing myelinated and unmyelinated fibers. Nerve fibers were grouped as fasciculi, each surrounded by perineurium and epineurium. Axons appeared clear with a dark ring of myelin around them. Many Schwann cells surrounded nerve fibers (Figure 1A). Sections from animals in Group II (diabetes-induced group) showed swollen myelin sheaths of nerve fibers with axonal atrophy and loss (Figure 1B). Sections from animals in Group III (diabetes-induced plus mononuclear cell group) were similar to the control Group I, showing nerve fascicles containing myelinated axons, and many Schwann cells were seen surrounding the nerve (Figure 1C). Similarly, sections from animals in Group IV (diabetes-induced plus folic acid group) showed nerve fascicles containing myelinated axons. The nerve fascicle was surrounded by the perineurium connective tissue sheath. Many Schwann cells were observed enclosing the nerve (Figure 1D). Finally, Group V (folic acid-treated group) showed sciatic nerve fascicles containing myelinated axons. The nerve fascicle was surrounded by perineurium connective tissue sheaths surrounded by epineurium (Figure 1E).

Immunostaining results

CD68-immunostained sections of the control group revealed a negative immune reaction within nerve fibers (Figure 2A). In the diabetes-only group, the CD68-immunostained sections revealed abundant positive immune reactive cells (cytoplasmic) within the nerve fibers (Figure 2B). In the diabetes-induced plus mononuclear cell group, the CD68-immunostained sections were similar to those of the control group, revealing the lack of an immune reaction within the nerve fibers (Figure 2C). In the diabetes-induced plus folic acid group, the CD68-immunostained sections revealed a few positive brown immune reactions in the nerve fibers and many negative immune reactions within the nerve fibers (Figure 2D). In the folic acid-treated group, the CD68 immuno-stained sections revealed a negative immune reaction within the nerve fibers (Figure 2E).

Toluidine blue staining

Toluidine blue-stained semithin sections of the control group showed densely packed groups of different sized myelinated and unmyelinated nerve fibers that were enclosed within the endoneurium and surrounded by perineurium. The epineurium

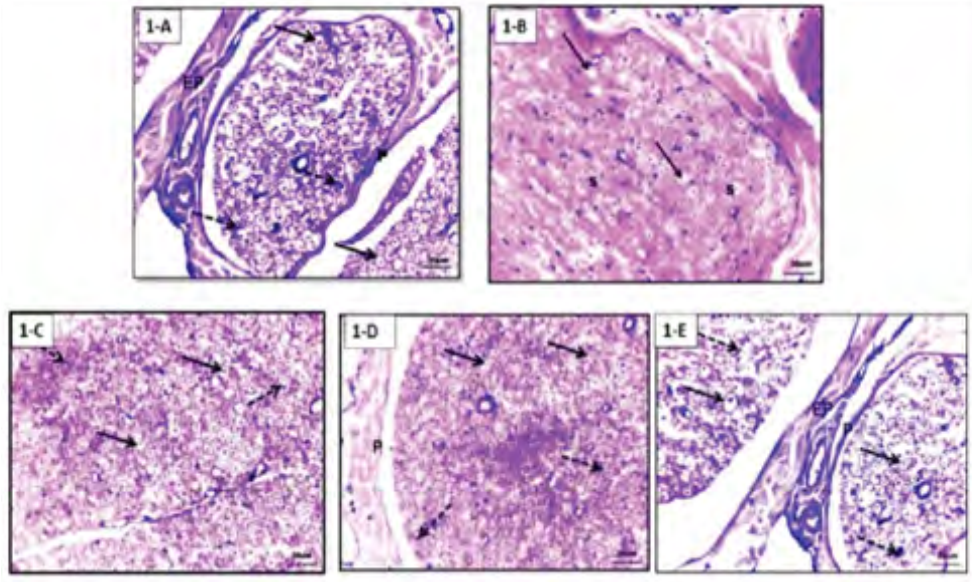

Figure 1: Images of transverse sections of rat sciatic nerves stained with H&E. (A) Control group showing nerve fascicles containing myelinated axons (arrows). The nerve fascicle is surrounded by the perineurium (P) connective tissue sheath, which is surrounded by epineurium (EP). Several Schwann cells are seen surrounding nerve fibers (dashed arrows). (B) DA group showing axonal loss (arrows) and swelling (S). (C) DA+ MNC group showing nerve fascicles containing myelinated axons (arrows) with many Schwann cells surrounding nerve fibers (dashed arrows). (D) DA+ FA group showing nerve fascicles containing myelinated axons (arrows). The nerve fascicle is surrounded by the perineurium (P) connective tissue sheath with many Schwann cells surrounding nerve fibers (dashed arrows). (E) Folic acid group showing nerve fascicles containing myelinated axons (arrows). The nerve fascicles are surrounded by perineurium (P) connective tissue sheaths that are surrounded by epineurium (EP) with many Schwann cells surrounding nerve fibers (dashed arrows) (400x).

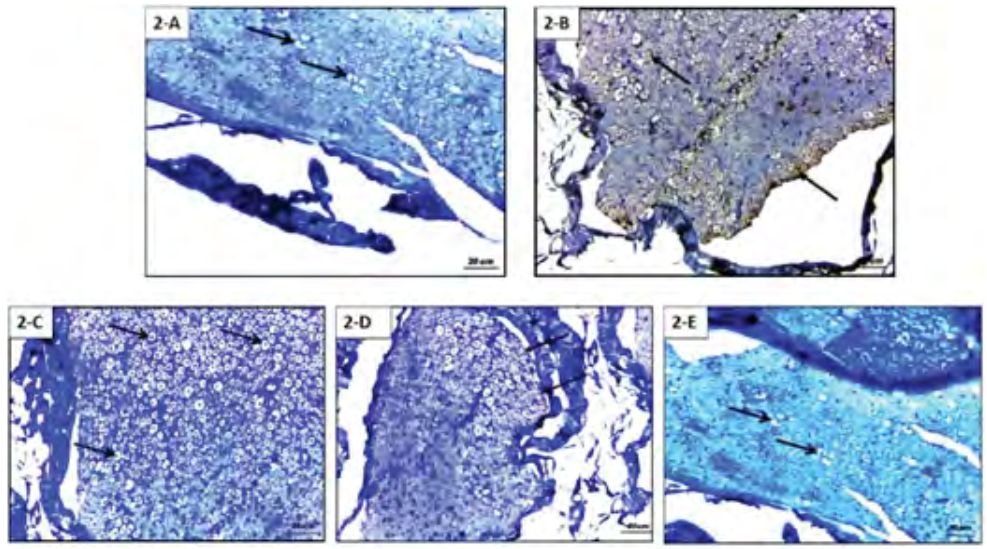

Figure 2: Images of rat sciatic stained with a CD68 antibody. **(A)** Control group showing a negative immune reaction with the nerve fibers (arrows). **(B)** DA showing abundant brown positive immune reactions within the nerve fibers (arrows). **(C)** DA+ MNC group showing a negative immune reaction within the nerve fibers (arrows). **(D)** DA+ FA group showing a positive immune reaction within the nerve fibers (arrows). **(E)** FA group showing a negative immune reaction within the nerve fibers (arrows) (immunostaining, anti-CD68, 400x).

surrounded the whole nerve trunk. The myelinated nerve fibers showed regular compact myelin with minimal infoldings (Figure 3A)**.** In the diabetic-only group, sections showed lipid droplets in the connective tissue surrounding the sciatic nerve. Marked axonal atrophy with myelin degeneration (honeycomb degeneration) was detected. We also observed changes in the myelin sheath in the form of demyelination with splitting, decompaction and degradation. Some bundles showed markedly separated nerve fibers. We also observed axonal shrinkage, axon–myelin separation and axonal retraction, and in some axons, onion-bulb formation (Figure 3B). The diabetic group treated with mononuclear cells revealed an improved histological status relative to the diabetic-only group. Most of the nerve fibers appeared normal, showing well-compacted myelin with minimal infolding. Axonal shrinkage and loss and splitting of the myelin sheath were evident only in a few fibers (Figure 3C). Meanwhile, the diabetic group treated with folic acid showed improvement in the proportion of nerve fibers with abnormalities and well-compacted myelin in most of the nerve fibers with minimal infoldings. Axonal shrinkage, loss and splitting of myelin sheath were evident only in a few fibers (Figure 3D). The

folic acid-only treated group revealed nerve fascicles containing well-compacted myelinated nerve fibers. Individual axons inside the fascicle were surrounded by connective tissue endoneurium (Figure 3E).

Electron microscopic results

Next, we examined ultrathin sections of the sciatic nerve using electron miscroscopy, in order to... In the control group, most of the nerve fibers were myelinated, with only some unmyelinated fibers observed. The myelin sheath appeared as compact electron-dense material. The axoplasm contained neurofilaments and neurotubules. The unmyelinated nerve fibers were ovoid or circular in shape and were grouped in clusters or occupied deep recesses on the surface of Schwann cells. Nerve fibers were surrounded by CT endoneurium with normal Schwann cells (Figure 4A)**.** In the diabetic-only group, ultrathin sections revealed heterogeneity of the myelin sheath. Evagination (outward bulging) and invagination (inward bulging) caused compression of the axoplasm. Demyelination was noted in most nerve fibers as evidenced by vacuolation of the myelin sheaths with formation of fermentation chambers, splitting, decompaction of myelin sheath lamellae and focal lysis of the myelin sheath. Axonal damage and total axonal destruction with accumulation of neurofilaments were observed in some fibers. Some unmyelinated axons had an irregular outer contour. Abundant collagen was noted between the fibers (Figure 4B). Ultrathin sections from animals in the diabetic group treated with mononuclear cells showed improvement relative to untreated diabetic animals – most of the nerve fibers had well-compacted myelin with minimal infoldings. The axoplasm contained neurotubules and neurofilaments. Vacuolation and lamellar separation of myelin sheath was less notable (Figure 4C).

Ultrathin sections from animals in the diabetic group treated with folic acid showed well-compacted myelin in most of the nerve fibers with minimal infolding and splitting of the myelin sheath in some fibers. The axoplasm contained neurotubules and neurofilaments (Figure 4D). Sections of the folic acid-treated group revealed that most of the nerve fibers were myelinated with only some unmyelinated fibers. The myelin sheath appeared as compact electron-dense material. The axoplasm contained neurofilaments and neurotubules. The unmyelinated nerve fibers were ovoid or circular in shape and grouped into clusters (Figure 4E).

Morphometric and statistical results

A-The number of normal and degenerated myelinated nerve fibers in toluidine blue-stained semithin sections of the sciatic nerve in all study groups: There was a significant decrease in the number of total normal nerve fibers in the DA group compared with the control group ($p<0.05$). Meanwhile, the number of degenerated fibers showed a significant increase in the DA group compared with the control group ($p<0.05$). The total number of normal nerve fibers showed a highly significant increase in both the DA+MNC group and the DA+FA group ($p<0.01$) compared with the DA group. Meanwhile, the number of degenerated fibers showed a highly significant decrease in both the DA+MNC group and the DA+FA group ($p<0.01$) compared with the DA group. The number of total normal nerve fibers showed a significant increase in the DA+MNC group ($p<0.05$) compared with the DA+FA group. Meanwhile, the number of apparently degenerated fibers

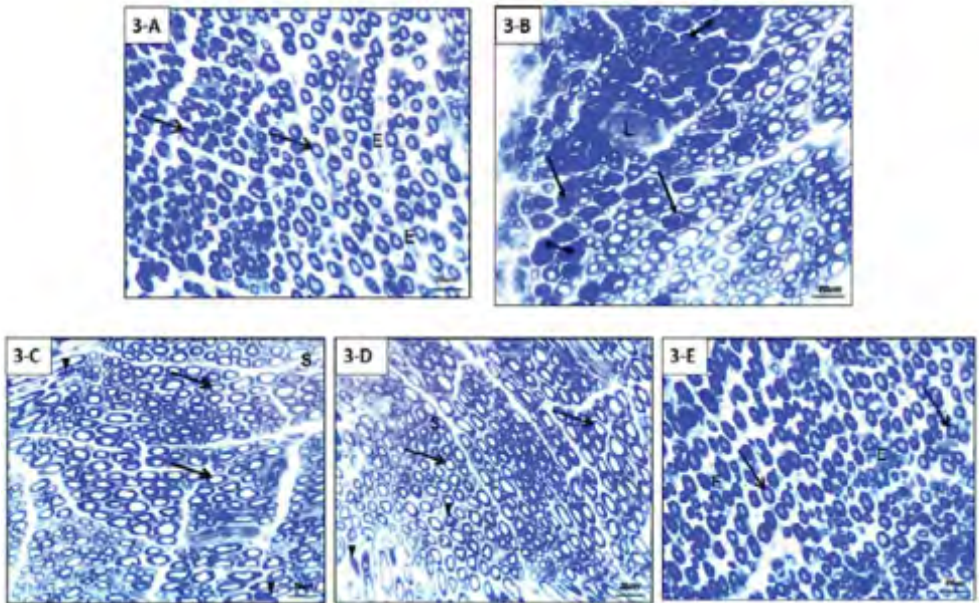

Figure 3: Images of transverse semi thin sections of rat sciatic nerves stained with toluidine blue. **(A)** Control group showing nerve fascicles containing well-compacted myelinated nerve fibers (arrows). Individual axons inside the fascicle are surrounded by connective tissue endoneurium (E). **(B)** DA group showing large lipids droplets (L) in the connective tissue surrounding the nerve marked axonal atrophy and loss (arrows) and severe myelin degeneration described as 'honeycomb' degeneration are shown (two-sided arrow). **(C)** The DA+ MNC group showing mostly normal nerve fibers, well-compacted myelin in most of the nerve fibers and minimal in folding (arrows). Axonal shrinkage and loss are evident only in a few fibers (stars). Splitting of the myelin sheath is evident only in a few fibers (S). **(D)** DA+FA group showing mostly normal nerve fibers and well-compacted myelin in most of the nerve fibers with minimal in folding (arrows). Axonal shrinkage, loss (stars) and splitting of the myelin sheath are evident only in a few fibers (S). **(E)** FA group showing nerve fascicles containing well-compacted myelinated nerve fibers (arrows). Individual axons inside the fascicle are surrounded by connective tissue endoneurium **(E).** (Toluidine blue, 400x).

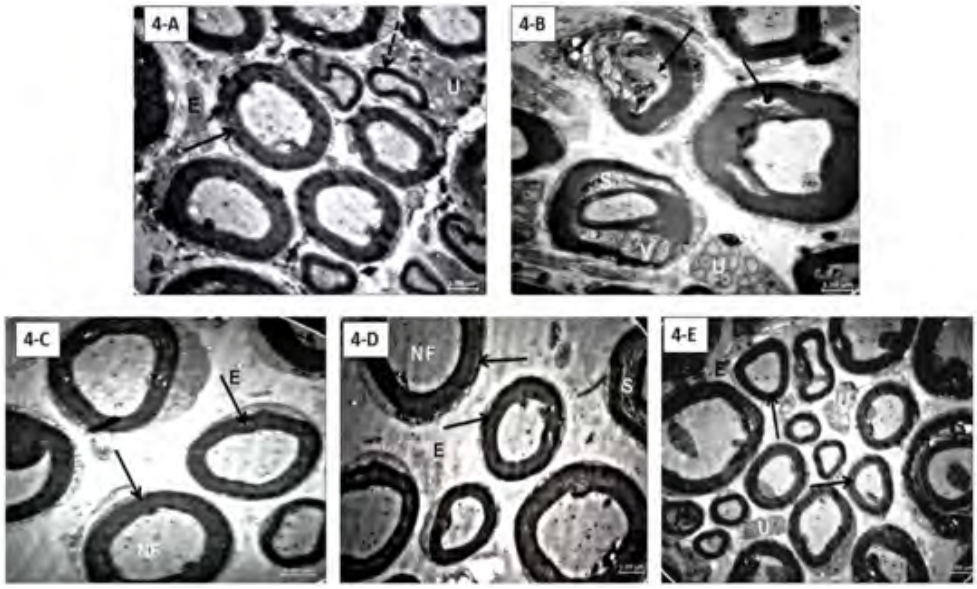

Figure 4: Electron micrograph images ratsciatic nerves. **(A)** Control group showing myelinated nerve fibers with an intact myelin sheath (arrow) and unmyelinated nerve fibers grouped in clusters (U). Nerve fibers are surrounded by endoneurium (E). Schwann cells enclose unmyelinated nerve fibers (dashed arrow). **(B)** DA group showing axonal atrophy (arrows). Vacuolation (V) of the myelin sheaths, forming fermentation chambers, focal lysis of the myelin sheath (rounded-head arrow), and splitting and decompaction of myelin sheath lamellae (S) can also be seen. Unmyelinated nerve fibers are grouped into clusters (U). **(C)** DA+ MNC group showing myelinated nerve fibers with an intact myelin sheath in most of the nerve fibers (arrows). The axoplasm contains neurotubules and neurofilaments (NF). Nerve fibers are surrounded by endoneurium (E). **(D)** The DA+ FA group showing nerve fibers with an intact myelin sheath in most of the nerve fibers (arrows). Splitting of the myelin sheath is evident only in a few fibers (S). The axoplasm contains neurotubules and neurofilaments (NF). Nerve fibers are surrounded by endoneurium (E). **(E)** FA group showing myelinated nerve fibers with an intact myelin sheath (arrows

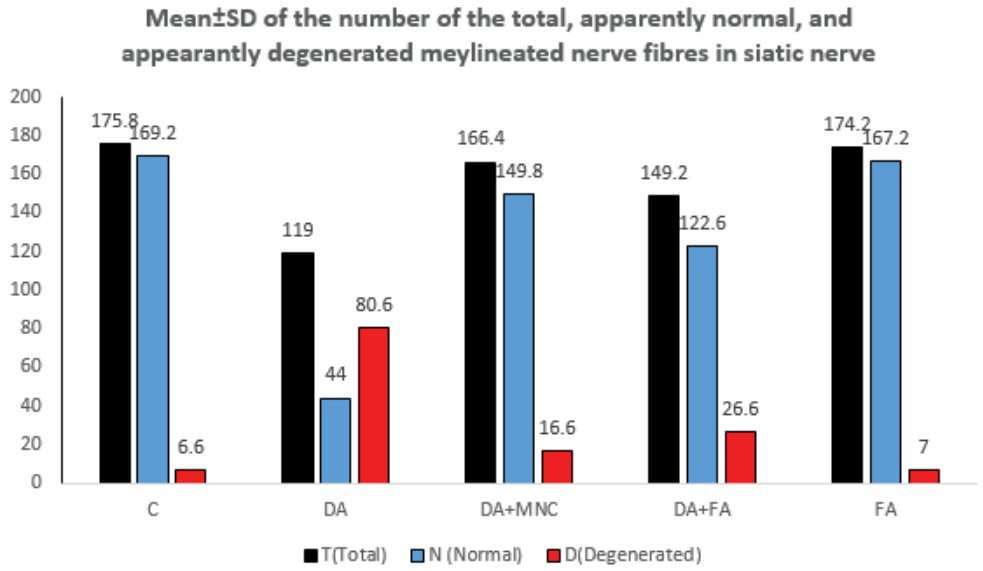

Figure 5: The number of apparently degenerated fibers significant decrease in the DA+MNC group ($p<0.05$) compared with the DA+FA group ($p<0.05$) compared with the DA+FA group.

showed a significant decrease in the DA+MNC group ($p<0.05$) compared with the DA+FA group (Figure 5).

B-The total number of CD-68immunopositive nerve fibers in all study groups: The total number of CD68-immunopositive nerve fibers showed a highly significant increase in the DA group compared with the control group ($p<0.01$). However, a highly significant decrease was observed in both the DA+MNC group and the DA+FA group compared with the DA group ($p<0.01$). Moreover, a significant decrease in the DA+MNC group compared with the DA+FA group ($p<0.05$) was observed (Table 1).

Sciatic nerve conduction velocities

The changes in sciatic nerve conduction velocities were measured from the right sciatic nerve in each animal. There was a statistically significant decrease in the sciatic nerve conduction velocities ($p<0.05$) among the DA, DA+MNC and DA+FA groups compared with the control group. In addition, a significant increase in the sciatic nerve conduction velocities of both the DA+MNC and DA+FA groups compared with the DA group was found. Moreover, a significant increase in the motor sciatic nerve conduction velocity

Table 1: The number of CD68-immunopositive macrophage in nerve fibers.

Groups	Number of positively stained macrophage in nerve fibers
Control	0
DA	281 ± 14.1[a*]
DA+MNCs	2 ± 2.8 [b,c]
DA+FA	34.2 ± 14.8[a*,b]
FA	0.2 ± 0.4

Notes: [a]DA+FA group compared to the control group. (p<0.01)*;
[b]DA+MNC group compared to the DA group. (p<0.001);
[b]DA+FA group compared to the DA group. (p<0.01);
[c] DA+MNC group compared to the DA+FA group. (p<0.05)

Table 2: Mean ± SD of sciatic nerve conduction velocities (m/s) in all study groups.

Group	MNCV (m/s)	SNCV (m/s)
Control	62.4 ± 3.6	48.26 ± 1.8
DA	35.67 ± 15.5[* a]	a*31.23 ± 13.5
DA+MNCs	51.41 ± 15.8[* a, b, c]	41.39 ± 11.9[* a, b]
DA+FA	44.85 ± 4.8[* a, b]	39.91 ± 5.87[* a, b]
FA	63.59 ± 6.4	49.14 ± 8.6

Note: (p<0.05) *ANOVA is statistically significant (p<0.05) [a] Compared to the control;
[b]Compared to the DA group;
[c]DA+MNC group compared to the DA+FA group (From a Tukey's HSD post hoc test).

(p0.05) of the DA+MNC group compared with the DA+FA group was found (Table 2).

DISCUSSION

The present study aimed to compare the effects of BM-mononuclear cells (BM-MNCs) and folic acid in the treatment of peripheral neuropathy in streptozotocin-induced diabetic rats. STZ administration led to the development of a diabetic state manifested by a significant elevation of the serum glucose level, accompanied by a significant decrease in body weight compared to the control rats. We observed a decrease in MNCV and SNCV in diabetic rats compared with the control group, as has been reported by Sharma et al. 2002, which has been attributed to impaired nerve conduction caused by demyelination and decreased nerve blood flow [18]. Our light microscopic examination of the sciatic nerve revealed that the diabetic rats showed myelin destruction indicated by vacuolation in the myelin sheaths, splitting, and decompaction of myelin sheath lamellae with focal lysis, honeycomb degeneration, axonal damage and total axonal destruction.

This myelin destruction was confirmed by morphometric analysis, which showed a decrease in the total number of normal nerve fibers and an increase in the number of degenerated fibers in the diabetic group compared to the control group. These changes are characteristic of diabetic peripheral neuropathy and have also been reported by others [1]. Onion-bulb formation was observed in the nerve sections of diabetic rats. These onion bulbs are concentric lamellar structures produced by Schwann cell processes. Axonal shrinkage and separation from the myelin sheath could explain the severe degenerative changes in the nerve fibers and the consequent decrease in nerve conduction in STZ models [19]. Moreover, we observed that neurofilaments accumulated in the axoplasm of nerve sections from diabetic rats, which may be due to stagnation of axoplasmic flow, as shown by another study [20]. In the current study, we observed an accumulation of lipid droplets in the connective tissue surrounding the nerves obtained from diabetic animals. The role of lipid droplets outside of lipid and cholesterol storage has begun to be elucidated and has been associated closely with inflammatory responses by the synthesis and metabolism of eicosanoids, signaling molecules that cause complex effects on several body systems, especially in inflammation. Lipid droplets are also involved in metabolic disorders such as DM and obesity [21].

In the current work, the number of CD68-immunopositive macrophage in nerve fibers was significantly increased in the DA group compared with the control group. This is indicative of an increased number of CD68-positive activated macrophages, as has been previously reported as a characteristic of DPN [22] and other human peripheral neuropathies, including axonal neuropathies [23]. It has been reported that the formation of advanced glycation end products may be the cause of many diabetic complications. In terms of DPN, the protein glycation cascade may result either in demyelination or in axonal atrophy. Glycation of myelin proteins would indeed cause myelin destruction and, consequently, demyelination. Moreover, glycation of collagen may lead to a reduction in nerve growth factors, resulting in axonal atrophy [24]. In the current study, sections of the sciatic nerve of rats in the DA+MNC group showed improved histology as evidenced by minimal infoldings, and by the fact that axonal shrinkage, and loss and splitting of the myelin sheath were observed only in a few fibers. A significant increase in the number of total and normal nerve fibers and a decrease in the number of degenerated fibers were also observed in this group. Additionally, there was a decrease in the number of CD68-immunopositive nerve fibers. These results were not consistent with other studies that reported that the transplantation of BM-MNCs into the unilateral hind limb skeletal muscles did not affect sciatic nerve conduction velocities [25]. Hasegawa et al.2006 has reported that in the sciatic nerve, STZ-induced diabetes caused a decrease in nerve blood flow (NBF), and this deficit was treated by BM-MNC transplantation, suggesting that BM-MNC transplantation improves blood flow in nerve vessels [26]. Han et al. noted that BM-MNCs augmented

neovascularization by elevating levels of a wide range of angiogenic factors, including FGF2, VEGF and angiopoietin 1, in the tissues [25]. Moreover, some studies have reported good therapeutic effects of BM-MNCs on experimental DN and that implantation of either peripheral blood mononuclear cells (PB-MNCs) or BM-MNCs in a rat model of DN enhanced motor nerve conduction velocity and blood flow around the sciatic nerve. These effects may be mediated via vascular endothelial growth factor (VEGF) secreted from MNCs. The researchers suggested that BM-MNCs are more effective than PB-MNCs because BM-MNCs include significantly more endothelial progenitor cells (EPCs) than PB MNCs [26].

Preventive measures, such as the early provision of adequate nutrition, can help reduce the occurrence of neuropathy. As a neuroprotective agent, FA supplementation decreases the incidence of many diseases in adults [27]. In the current study, sections of the sciatic nerves of diabetic rats treated with FA showed improvements in nerve fiber histology as compared to untreated diabetic rats as evidenced by minimal infoldings, and reduced axonal shrinkage, loss and splitting of the myelin sheath. Morphometric analysis of the DA+FA group showed increased numbers of total and normal nerve fibers and decreased numbers of degenerated fibers. These findings are in agreement with other studies that reported that some neurological disorders, such as peripheral polyneuropathy, myelopathy, retrobulbar optic neuropathy and leukoencephalopathy are associated with reduced FA [28]. Indeed, DPN may be caused by the accumulation of reactive oxygen species, particularly superoxide radicals and hydrogen peroxide, and FA supplementation ameliorates oxidative damage, as well as inflammation and insulin resistance in DM. FA is regarded as an effective antioxidant in patients, as it decreases production of free radicals [29]. In addition, FA may help prevent DPN by improving the expression of NGF and lowering malondialdehyde (MDA) levels, FA supplementation may be regarded as an adjunctive therapy in diabetic patients to improve symptoms of neuropathy [30].

Statistical analysis showed that there was significant increase in the motor sciatic nerve conduction velocity of the DA+MNC group compared to the DA+FA group, and the number of total and normal nerve fibers was significantly increased in the DA+MNC group compared to the DA+FA group. Conversely, the number of degenerated fibers was significantly decreased in the DA+MNC group compared to the DA+FA group, and the number of CD68-immunopositive nerve fibers showed a significant decrease in the DA+MNC group compared with the DA+FA group. By comparing the findings of the DA+MNC and the DA+FA group, we showed that treatment of diabetic rats with MNCs improved diabetic neuropathy more than treatment with FA.

CONCLUSION

Our results show that treatment of diabetic rats with MNCs resulted in better improvement of diabetic neuropathy than treatment with FA. This study showed the beneficial effects of folic acid in the prevention of diabetic peripheral neuropathy. Folic acid supplementation may be considered as an adjunctive preventive measure for diabetic peripheral neuropathy in diabetes. Therefore, further molecular researches are recommended to elucidate the exact mechanisms of action and examine the potential therapeutic synergistic effects of bone marrow-derived mononuclear cells together with folic acid in diabetic peripheral neuropathy particularly in humans. Moreover, the future studies need to test the use of autologous bone marrow MNCs versus allogeneic MNCs.

ETHICAL CONSIDERATIONS

All experiments were conducted in accordance with the guidelines of the Institution Animal Ethics Committee of the Suez Canal University.

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