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Protective effect of vitamin B complex in diabetic peripheral neuropathy - Histopathological study

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SUMMARY

Vitamin B complex has been used for peripheral neuropathy for a long time and continues to be part of current practice despite lack of strong evidence for its use and its non-inclusion in treatment guidelines. So this study was carried out to verify the neuroprotective effect of vitamin B complex from morphological changes of the diabetic rat sciatic nerve. A total number of 30 adult male albino rats were used and divided into three groups. Group I: normal vehicle control (N=10). Group II: streptozotocin-induced diabetic rats (N=20), which is equally divided into two subgroups; IIa (diabetic vehicle control) and IIb (diabetic vitamin B complex –treated, at a dose of 1mg/kg/day for 6 weeks).

Diabetes was induced by a single intraperitoneal injection of streptozotocin (STZ) at a dose of 80mg/kg. Specimens from sciatic nerve were processed for light, electron microscopy and immunohistochemistry investigations. Morphological indices including the average myelin sheath thickness, the average myelinated nerve fiber area, endoneurial capillary density and perineurial index were measured and statistically analysed. Vitamin B complex treatment for six weeks markedly protected the sciatic nerve from the deleterious effect of hyperglycemia and preserved normal structural features of the perineurium, Schwann cells and their myelin sheath, nerve fibers, blood capillaries and the interstitium. The results were verified by immunohistochemistry (using CD 31, CD 68 and anti caspase-3 antibodies) and the morphological indices including the myelin sheath thickness, perineurial index and endoneurial capillary density.

In conclusion, vitamin B complex supplementation might provide a long-term, drug approach for protection from diabetic peripheral neuropathy.

Key words: Vitamin B complex – Diabetic neuropathy

INTRODUCTION

Diabetes is a globally prevalent condition and peripheral neuropathy is one of its major chronic complications. Effective therapies for diabetic complications need the potential therapeutic interventions that might prevent diabetic complications, in order to inhibit mechanisms induced by hyperglycemia's toxic effects and also enhance the endogenous protective factors.

The therapeutic use of vitamins meets two main objectives: preventing and treating either deficiencies or vitamin-dependent metabolic diseases (Naurath et al., 2001). Water-soluble vitamins B1 (thiamine), B2 (riboflavin), B6 (pyridoxine), B9 (folic acid), and B12 (cyanocobalamin) are cofactors in numerous enzymatic activities and proton and electron transfers (Krautler, 2005). Pyridoxine use is a double-edged sword, as high doses are neurotoxic (Scott et al., 2008).

Vitamin B complex has been used for peripheral neuropathy for a long time and continues to be part of current practice despite the lack of strong

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evidence for its use and its non-inclusion in treatment guidelines. Vitamin B complex consists of eight water soluble vitamins that are necessary for health but cannot be manufactured by the body.

Following 4 months of treatment of type 2 diabetic neuropathy patients, diabetic neuropathy symptoms were significantly reduced in the B complex-vitamin supplemented group as compared to placebo (Farvid et al., 2011). A meta-analysis of 7 published studies reported that supplementation with vitamin B complex mixtures containing B12 significantly improved pain and paresthesia in diabetic neuropathy patients (Sun et al., 2005).

In experimental studies, B vitamins are reported to alleviate neurophysiological properties of the peripheral nerves and neuropathic pain in diabetic rats (Iwata et al., 1979; Jolivalt et al., 2009, respectively). In addition, some studies evaluated some structural changes of peripheral nerve after diabetic neuropathy (Kirschner and Eichberg, 1994; Koura, 2003). However, to the best of our knowledge, protection of the structure of the peripheral nerves by B complex vitamins supplementation has never been investigated histologically in diabetic peripheral neuropathy. So this work was carried out to assess the histo-morphological indices of vitamin B complex protection of DPN. Such study is needed to extend its recommendation in this pathology.

MATERIALS AND METHODS

Animals

A total number of 30 adult (5 month old) male *Wistar* rats (200 g body weight) were used in this study. They were purchased from Central Animal House, Faculty of Medicine, Assiut University. All animal procedures were in accordance with the standards set forth in guidelines for the care and use of experimental animals by the Committee for Purpose of Supervision of Experiments on Animals (CPCSEA) and according to National Institute of Health (NIH) protocol, and approved by the Institutional Ethics Committee of Assiut University. The animals were housed in clean capacious cages (up to 3 per cage) under normal day-and-night cycles and appropriate temperature (25 ± 5°C), fed rat chow (standard rat pellets) and water *ad libitum*.

Animal groups

Animals were divided into two main groups. Group I (normal vehicle control) consisted of 10 rats that were injected subcutaneously once daily with 0.5 mL saline for 6 weeks. Group II: Streptozotocin (STZ)-induced diabetic group, consisted of 20 rats that were fasted overnight 12 hours before STZ injection, then each one received a single intraperitoneal injection of STZ (purchased from Sigma Chemical Co, St, Louis, MO, USA) at a dose of 80 mg/kg freshly dissolved in 0.5 mL of 0.9% sterile saline (Jolivalt et al., 2009). Some failure of diabetes induction has been experienced with lower STZ doses, possibly due to reasons related to animals, environment or drug stability in a country with a very hot climate despite considering all necessary measures. After 3 days of diabetes induction, fasting blood glucose level was measured by Dextrostix glucometer (Ames, Elkhart, Indianapolis, IN, USA). Rats with fasting blood glucose above 300 mg/dL were considered diabetic (Han et al., 2004) and were equally divided into two subgroups; group IIA (diabetic vehicle control) was injected subcutaneously once daily with 0.5 mL saline for 6 weeks. Group IIB (diabetic vitamin B complex-treated) was injected subcutaneously once daily with vitamin B complex (purchased from Alpha Chemical Co, India), the doses of the vitamins in the complex mixture per 1gm of the mixture were (B1, 40mg), (B2, 122mg), (B3, 66mg), (B5, 50mg), (B6,13mg), (B7, 55mg), (B9, 33mg), (B12, 6mg). Vitamin B complex dissolved in 0.5 mL saline (0.9% NaCl, pH 7.35) at a dose of 1 mg/kg (Jolivalt et al., 2009) for 6 weeks.

At the end of the experiment, the animals were anaesthetized with ether, their hearts were exposed, and then perfusion was done.

Histological study

For light microscope, five rats from each animal group were used and perfused intra-cardiac with 10% formaldehyde solution. After perfusion, specimens from the sciatic nerve were cut and immersed into the 10% formaldehyde solution to continue fixation for two more days. Then the specimens were processed for preparation of paraffin blocks. Paraffin sections (5 µm) of sciatic nerve were cut using a microtome (Leica RM 2125RT, Germany), mounted on glass slides, and every 10^{th} section was stained with hematoxylin-eosin (H&E) (Drury and Wallington, 1980). 10 sections per animal were studied. Other sections were processed for Masson's trichrome stain for demonstration of collagen fibers (Drury and Wallington, 1980).

For electron microscope, five rats from each group were perfused intra-cardiac with 4% glutaraldehyde in cacodylate buffer (pH7.4). Sciatic nerve specimens were cut and immersed in 4% glutaraldehyde in cacodylate buffer (pH 7.4) for 24 hours and post fixed in 1% osmium tetroxide in phosphate buffer for two hours. Tissues were rinsed in the same buffer, dehydrated with alcohol, cleared with propylene oxide and embedded in Epon-812 substitute (SPI- Pon Araldite Kit, Cat. no. 02635- AB., SPI- chem. USA). For Polymerization, the embedded samples were kept in the incubator at 35 ºC, 45 ºC and 60 ºC for one day each (Gupta, 1983). Semi-thin sections (0.5-1µm) were cut with glass knives on the ultramicrotome (LKB Bromma 8800 Ultratome^{R} III, 3518, Sweden) and stained with 1% toluidine blue (TB) (pH 7.3) for

examination on a light microscope (Olympus, Bx50. Model Bx50F-3, SC09160, Tokyo, Japan). Ultrathin sections (50-80 nm) were cut from selected areas of the blocks on a Reichert ultramicrotome (Leica WILDM3Z, 89386, Austria) placed on copper grids (G 300, 3.05 mm, Polaron Equipment Ltd. Watford. England) and contrasted with uranyl acetate and lead citrate. These sections were examined using the transmission electron microscope (Jeol E.M.-100 CX11; Japanese electron optic laboratory, Tokyo, Japan) and photographed at 80 kV.

Immunohistochemical study

Expression of CD31 (endothelial cell marker), CD68 (macrophage marker) and Caspase 3 (apoptotic marker) was detected in formalin- fixed paraffin- embedded sections. Sections (5 µm) were deparaffinized in xylene and rehydrated in alcohol. Sections were boiled in 10mM citrate buffer, pH 6.0 for 10 minutes followed by cooling at room temperature for 20 minutes. CD31 monoclonal mouse antibody (Clone TLD-3A12, Novus Biologicals) was used at 1:100 dilution for 30 minutes at room temperature. CD68 monoclonal mouse antibody Ab-3 (Clone KP1 Lab Vision Corp, Neo Markers Inc/ Lab Vision, Fremont, CA, USA) was used at 1:100, dilution, for 30 minutes at room temperature. Caspase-3 (CPP32) Ab-4, Rabbit Polyclonal Antibody (Thermo Fisher Scientific, Fremont, CA 94538-7310, USA) was used at 1:100, dilution, for 30 minutes at room temperature. Sections were processed according to the manufacture instructions using the universal kit (Ecno Tek HRP Anti-Polyvalent, DAB) (ScyTek Laboratories, Inc. 205 South 600 West Logan, UT 84321 USA). After completion of the reaction, counterstaining was done using Mayer's haematoxylin, dehydrated and cover-slipped using DPX (Oxford laboratory reagents, Bombay, India).

Morphometric study

Using computer-assisted image analysis (Soft Imaging System, AnalySIS-2004, Olympus Company, Tokyo, Japan), the following parameters were measured per mm^2 in the studied groups; thickness of myelin sheath, myelin sheath area, endoneurial capillary density (the ratio of capillaries/nerve area) and perineurial index. The endoneurial capillary density was determined by counting all endoneurial capillaries directly in all fascicles of the whole nerve and relating it to the fascicular area to calculate a density (Soley et al., 2003). The perineurial index (P index) was calculated according to the formula: P index = $[(DO-DI)/DO]$. [D0 is the distance between two spots on the opposite sides of outer perineurial surface which pass through the center of fascicule, D1; distance between opposite two spots on the inner perineurial surface which pass through the center of the fascicule] (Tesfaye et al., 2005). We measured the outer "distance between two spots on the opposite sides of outer perineurial surface which pass through the center of fasciculae—DO" and the inner "distance between opposite two spots on the inner perineurial surface which passes through the center of fasciculae—DI" diameter of the measured fasciculae in order to calculate the perineurial index (P index) according to formula P index = [(DO - DI/ DO] X100 (Tohgi et al., 1977; Ugrenović et al., 2011; Kundalić et al., 2014).

Measurements were done in semithin sections using x100 oil immersion lens in five non-overlapping fields in ten randomly chosen sections from three different animals for each group.

Statistical analysis

The morphometric data of each animal group were statistically analyzed using Statistical Package for the Social Sciences (SPSS). One-way analysis of variance (ANOVA) was employed to compare the studied animal group. The results were expressed as mean ± standard deviation (SD). *P*-value < 0.05 was considered significant.

The data were tested for normality using Anderson-Darling test and for homogeneity variances prior to further statistical analysis. Continuous variables were described by mean, SD and median.

ANOVA was used to compare between continuous normally distributed variables, followed by LSD post hoc test to compare between continuous abnormality distributed variables followed by Mann Whiteny U test to compare between each two groups.

A two tailed $p < 0.05$ was considered statistically significant. All analyses were performed with the IBM SPSS 20.0 software.

RESULTS

I - Light Microscopy

The sciatic nerve of the control rats was constructed of bundles or fascicles of nerve fibers, each of which was surrounded by a collagenous connective tissue layer; the perineurium. The fascicles were enveloped by a yet connective tissue layer; epineurium. The endoneurium, which was the innermost connective tissue, enclosed the nerve fibers with their axons and covering Schwann cells (myelinating or non-myelinating), blood capillaries; formed of endothelial cells surrounded by pericytes; and connective tissue cells (Fig. 1A). Collagen fibers were detected in the endoneurium, perineurium and epineurium (Fig. 1B).

The sciatic nerve of the diabetic group exhibited an increase in thickness of the perineurium and the wall of the endoneurial blood capillaries (Fig. 2A), and an increase in collagen deposition in the perineurium and the endoneurium (Fig. 2B).

In the vitamin-treated group the histological structure of the sciatic nerve was close to that of the control group with regard to the wall of the eno-

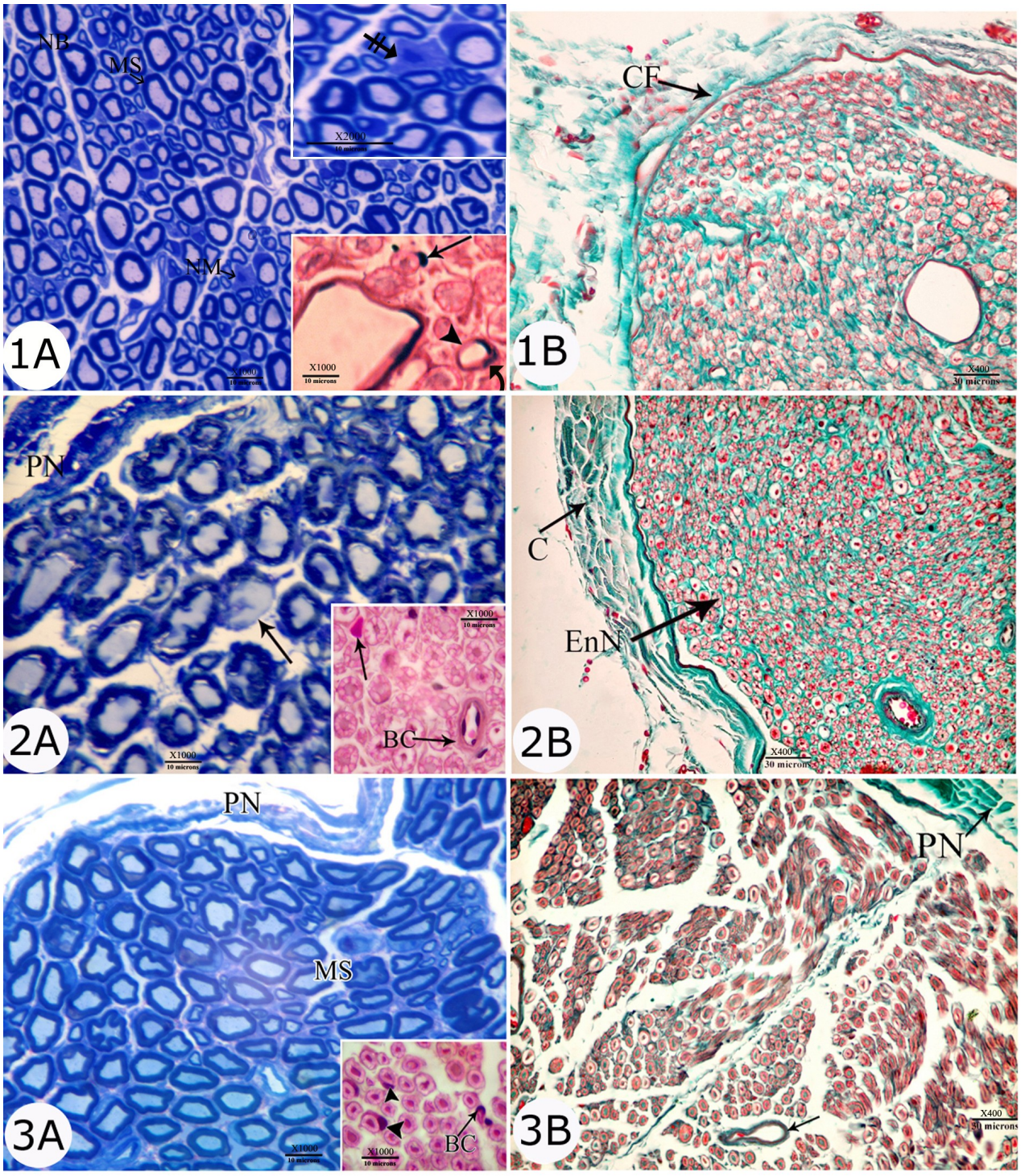


Fig 1A. Photomicrograph showing part of a nerve bundle (NB). It is formed of nerve fibers ensheathed by myelin sheath (MS). They are separated by endoneurial connective tissue. (Control, TB). Upper inset: shows non-myelinating Schwann cell enclosing non-mielinated nerve fibers (hatched arrow). Lower inset: Each nerve fiber is formed of an axon surrounded by Schwann cell (↑). Note the endoneurial blood capillary lined by an endothelium (▲) and surrounded by a pericyte (▲). (Control, H&E). **Fig 1B**. Collagen fibers (CF) in the perineurium and the endoneurium. (Control, Masson's trichrome). **Fig 2A.** Semithin section showing increased thickness of the perineurium (PN), deformation, damage and/or loss of the myelin sheath (↑). (Diabetes, TB). Inset: shows cellular infiltration (↑) of the endoneurium and thickening in the wall of blood capillaries (BC). (Diabetes, H&E). **Fig 2B.** Increased collagen deposition in the perineurium (C), endoneurium (En N) and around the endoneurial blood capillaries. (Diabetes, Masson's trichrome). **Fig 3A.** Part of a nerve fascicle showing minor changes in the nerve fibers. PN (perineurium). MS (myelin sheath). (Vitamin, TB). Inset: The endoneurium reveals no noticeable thickening of the blood capillary wall or cellular infiltration. Myelinating Schwann cells (▲). (Vitamin, H&E). **Fig 3B.** Part of a nerve fascicle. The endoneurium reveals no noticeable collagen fibers infiltration or accumulation around the endoneurial blood vessels (↑). PN (perineurium). (Vitamin, Masson's trichrome).

neurial blood capillaries (Fig. 3A, inset) and the endoneurial collagen fibers (Fig. 3B).

II - Ultrastructure

In ultrastructure, the perineurium of the control rats was composed of concentric layers of flat perineurial cells with flattened nuclei, about four laminae were usually present (Fig. 4). Each perineurial cell layer is surrounded by basal lamina. Pockets containing extracellular matrix are present between the perineurial cell layers. Immediately internal to the innermost layer of the perineurium, there were flattened perineurial fibroblasts similar to those seen within the endoneurium. The diabetic rats revealed thickened basement membrane of perineurial cells (Fig. 5A) and apoptotic changes in their nuclei (Fig. 5B). In vitamin B complex treated rats, there was no thickening of the perineurial cells basal lamina (Fig. 6).

Control sections showed Schwann cells wrapping myelinated and unmyelinated axons. The Schwann cells were surrounded by distinct basal laminae. Their cytoplasm showed normal content of cell organelles including mitochondria, Golgi body and free and attached ribosomes (Fig.7). In the diabetic group some myelinating Schwann cells revealed signs of increased activity as thickened basal lamina (Fig. 8) and numerous well-developed Golgi bodies (Figs. 9), while others exhibited degenerative changes as dense nucleus and cytoplasm (Fig. 10). Laminated myelin-like cytoplasmic inclusions have been observed inside

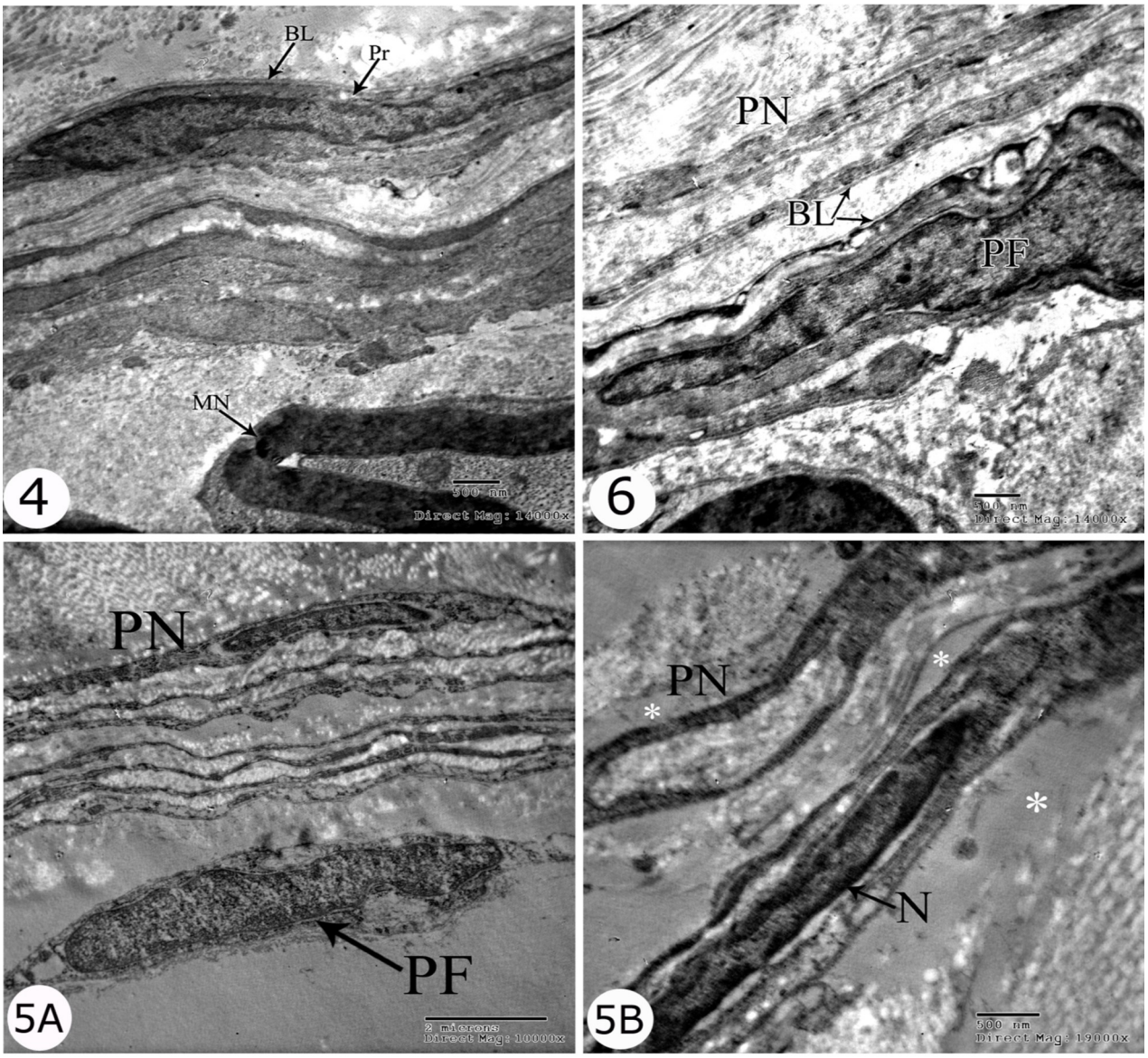


Fig 4. An electron micrograph showing a part of the perineurium and a myelinated nerve fiber (MN). Note the basal lamina (BL) around the perineural cell (Pr). (Control). **Fig 5A.** Image showing thickened perineurium (PN). (Diabetes). **Fig 5B.** Perineurium (PN), a perineurial cell with marginated nuclear chromatin (N) and thickened basal lamina (*). (Diabetes). **Fig 6.** The perineurium (PN) showing no remarkable thickening of the perineurial basal lamina (BL). (Vitamin).

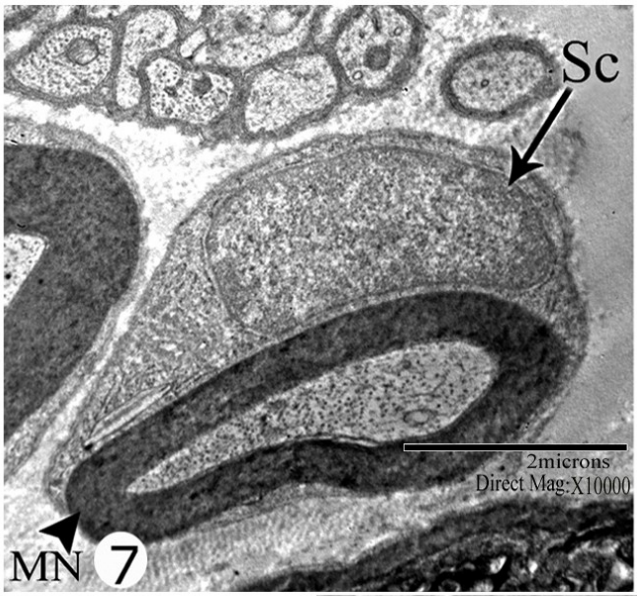
the cytoplasm of some myelinated Schwann cells (Fig. 11). In the vitamin-treated group, some myelinating Schwann cells revealed cytoplasmic laminated inclusions (Fig. 12), but the majority demonstrated vesicular nucleus and well developed organelles (Fig. 13).

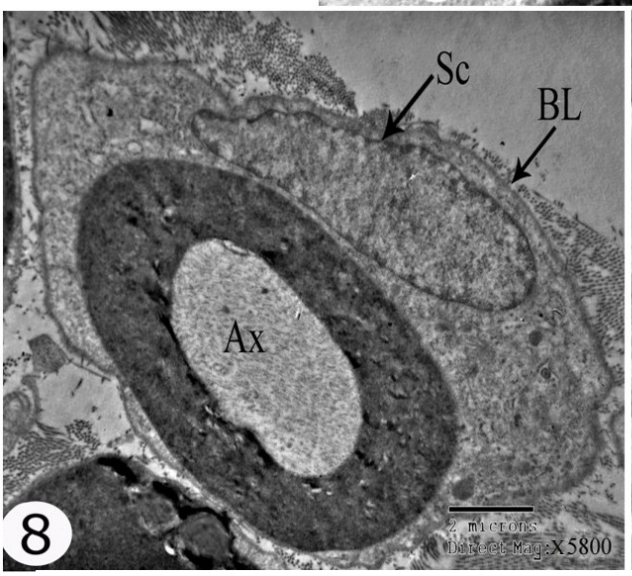
In cross section, myelinating Schwann cells of the control group formed a thick compact band of myelin sheath surrounding the axon (Fig. 7). In the diabetic group, the myelin sheath exhibited abnormal figures in the form of infolding (Fig. 14A), splitting (Fig. 14A, B) or focal thickening (Fig. 14C). The innermost layers of the myelin sheath may become reflected to extend in-between the nerve fibers dividing them to several compartments (Fig. 14D).

The nerve fibers of the diabetic group might reveal degeneration with reduction of the axon diameter (Fig. 14C). In the vitamin-treated group, the structure of the nerve fibers and their surrounding myelin sheath were similar to those of the control group (Fig. 13).

The endoneurial blood capillaries of the control group were lined by continuous endothelial cells surrounded by pericytes (Fig. 15). The endoneurial blood capillaries demonstrated collapse (Fig. 16) and attenuated endothelium (Fig. 16, inset). In the vitamin-treated group, the endoneurial blood capillaries revealed patent lumen (Fig. 17) and endothelial cells exhibited no thickening of the basal lamina.

The endoneurial fibroblasts of the control group appeared as flattened cells with lateral processes and prominent rough endoplasmic reticu-





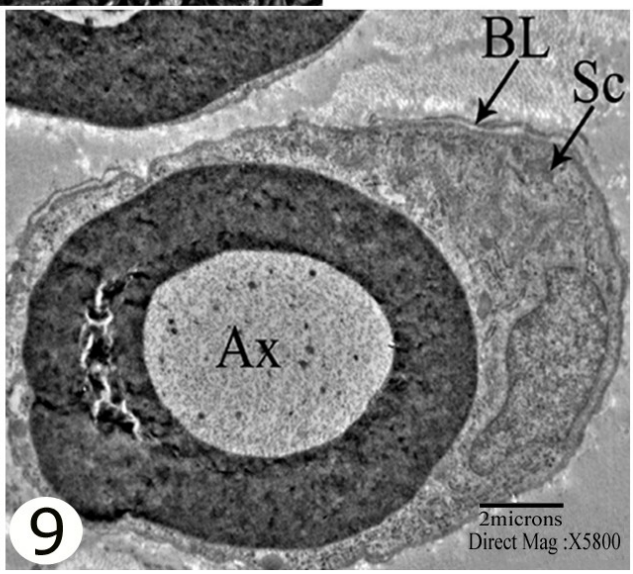


Fig 7. Electron micrograph showing cross section in a Schwann cell (Sc) forming a thick compact band around an axon of myelinated nerves (MN). (Control). **Fig 8.** Cross section of a Schwann cell (SC) enclosing a myelinated axon (Ax). Note the thickened basal lamina (BL). (Diabetes). **Fig 9.** Cross section of a Schwann cell with numerous cytoplasmic organelles. (Diabetes).

lum (Fig. 18). In the diabetic group, some fibroblasts revealed myelin debris and numerous lysosomes in their cytoplasm (Fig. 19). The vitamin treated group demonstrated endoneurial cells with numerous cytoplasmic phagosomes of variable electron density and some of these cells were in close vicinity of collagen fibrils (Fig. 20).

III - Immunohistochemistry

Immunohistochemistry of the control group revealed negative expression for anticaspase-3 (Fig. 21A), positive expression of CD 31 antibodies in endothelial cells lining the endoneurial blood capillaries of the sciatic nerve (Fig. 21B) and negative expression for CD 68 (Fig. 21C). The diabetic group revealed positive expression for anticaspase -3 antibodies in perineurial cells (Fig. 22A) and myelinating and non-myelnating Schwann cells (Fig. 22A, inset). Expression of CD 31 antibodies in endothelial cells lining the endoneurial blood capillaries was negative, while both Schwann cells and perivascular endoneurial cells were positive. CD 68 + immune reaction was detected in several endoneurial cells (Fig. 22C). In the vitamin-treated group the immune reaction for anticaspase antibodies was negative (Fig. 23A), whereas that for CD31 antibodies was + in the endothelium (Fig. 23B) and numerous endoneurial cells revealed +

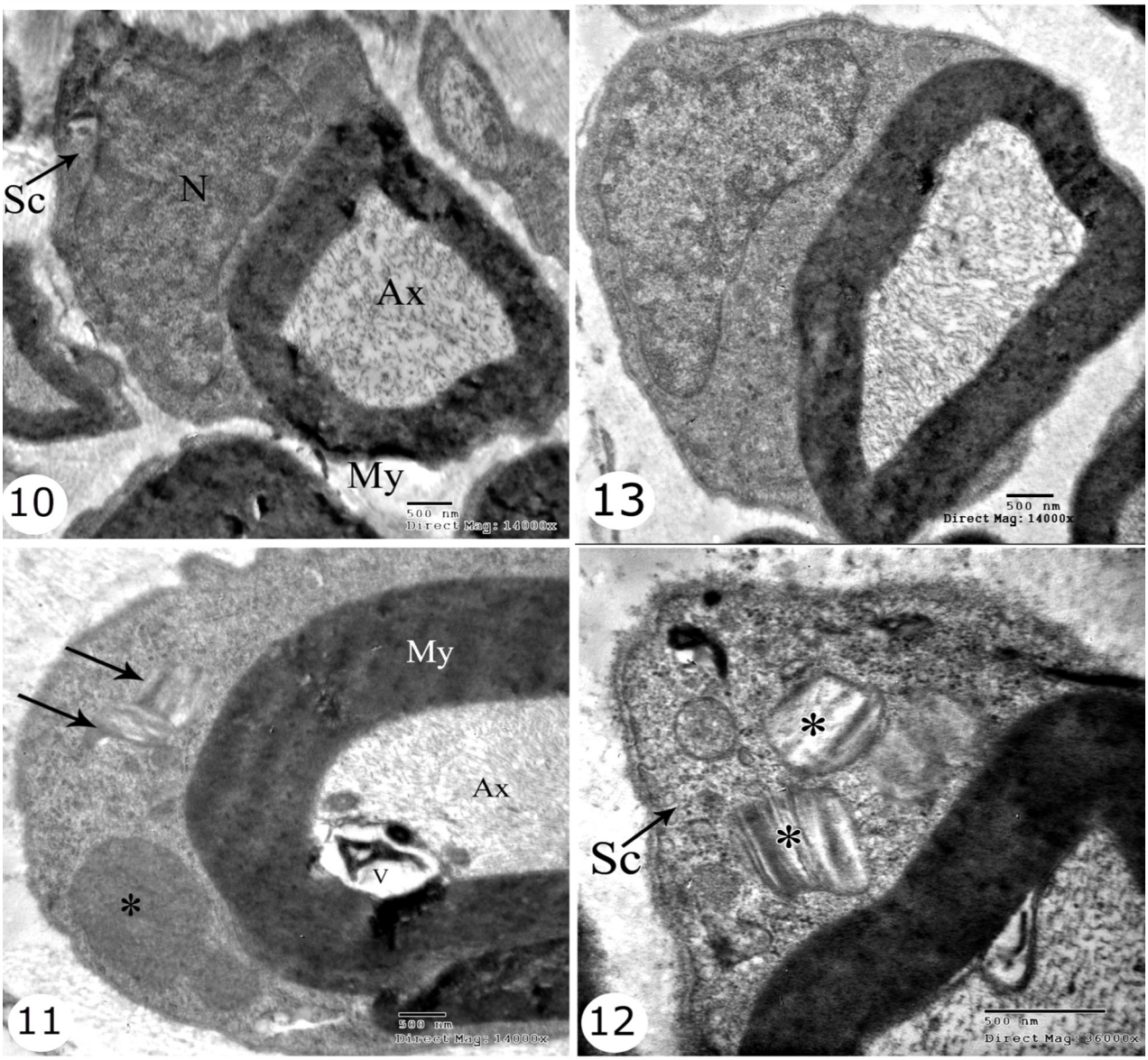


Fig 10. Another cross section of a Schwann cell (Sc) which wraps around a single myelinated (My) axon (Ax).The nucleus (N) is dense and the cytoplasm is poor in organelles. (Diabetes). **Fig 11.** Myelinated nerve fiber (Ax) with vacuolated (V) myelin sheath (My). Both dense (*) and laminated inclusions (↑) are present inside Schwann cell cytoplasm. (Diabetes). **Fig 12.** Myelinated nerve fiber enclosed by a Schwann cell soma (Sc) containing laminated inclusions (*). (Vitamin). **Fig 13.** An electron micrograph of a Schwann cell with an ovoid nucleus, enclosing a myelinated nerve fiber. (Vitamin).

reaction for CD68 antibodies (Fig. 23C).

IV - Morphometry

Thickness of myelin sheath

A significant decrease in myelin sheath thickness was observed in both the diabetic and the treated groups compared to the control group (P < 0.001) (Table 1, histogram 1). However, the treated group showed greater thickness compared to the diabetic group and this difference was significant (P < 0.001).

Myelinated nerve fiber area

There was significant decrease in myelinated nerve fiber area in both the diabetic and treated groups compared to the control group (P < 0.001, P = 0.001 respectively) (Table 2, histogram 2).The treated group showed a non-significant difference compared to the diabetic group (P >0.05).

Endoneurial capillary density

There was a significant decrease in the endoneurial capillary density in the diabetic group compared to the control group (P = 0.003) (Table 3, histogram 3). The treated group showed significant increase compared to the control and the diabetic group (P = 0.026, P = 0.001 respectively).

Perineurial index

There was a significant increase in the perineurial index in both the diabetic and the treated group

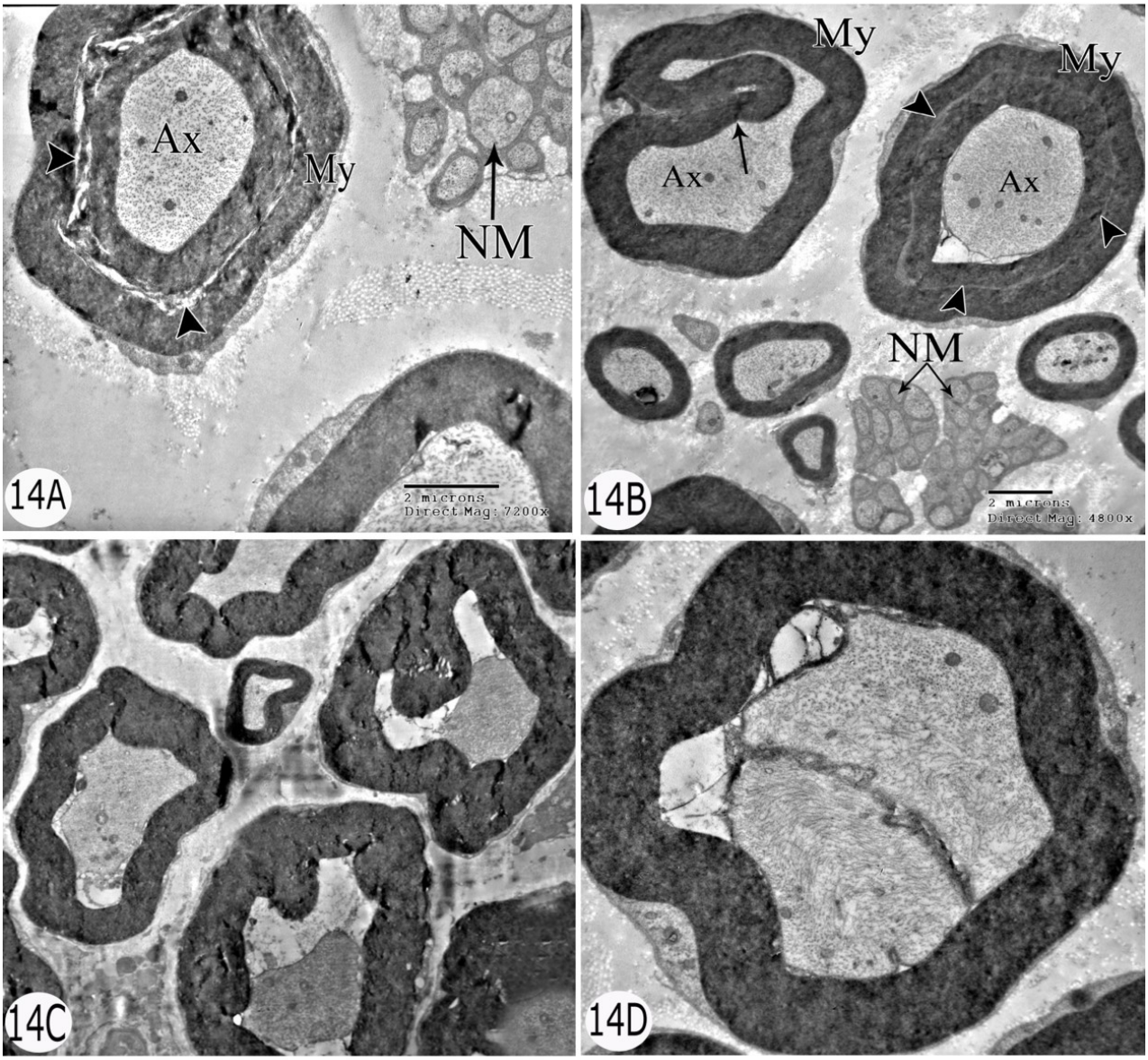


Fig 14A. Electron micrograph showing splitting (▲) of the myelin sheath (My) surrounding a nerve fiber (Ax). NM (non-myelinated). (Diabetes). **Fig 14B.** Folding (↑) of the myelin (My) ensheathing a nerve fiber and splitting (Ax) in another sheath (▲). NM (non-myelinated fibers). (Myelin). **Fig 14C.** Cross section in myelinated nerve fibers. Note the atrophied nerve fibers (Ax) leaving a large vacuolar space (*) where the myelin sheath became thickened (↑). (Diabetes). **Fig 14D.** Cross section in a myelinated nerve fiber showing retroflection of the myelin sheath at several points (↑) extending through the axon. (Diabetes).

compared to the control group (P < 0.001, P = 0.015 respectively) (Table 4, histogram4). However, the treated group had a significant lower perineurial index compared to the diabetic group (P = 0.008)

DISCUSSION

This work demonstrates the structural neuroprotection of a combination of B-vitamins for neuropathy in a rat model of diabetic PN, as many of the pathological changes detected in sciatic nerve have been attenuated. The changes included decreased thickness of the perineurial cell layers, perineurial and Schwann cells apoptosis, axonal atrophy, myelin abnormal figures and endoneurial microangiopathy, in addition to the frequent detection of phagocytic cells in vitamin B-treated group.

The increase in the thickness of the perineurium cell layers and perineurial cells basement membrane has previously been reported by some investigators (Hill and Williams, 2002). However, the apoptotic changes we observed in the perineurial cells by ultrastructure and immune reaction for anticaspase-3 antibodies have not been previously reported.

A few Schwann cells revealed activated secretory activity in the form of hyperplasia and hypertrophy of Golgi bodies and increased basal lamina thickness in the diabetic untreated group, but numerous cells revealed variable stages of apoptotic death. Impaired neurotrophin production as conse-

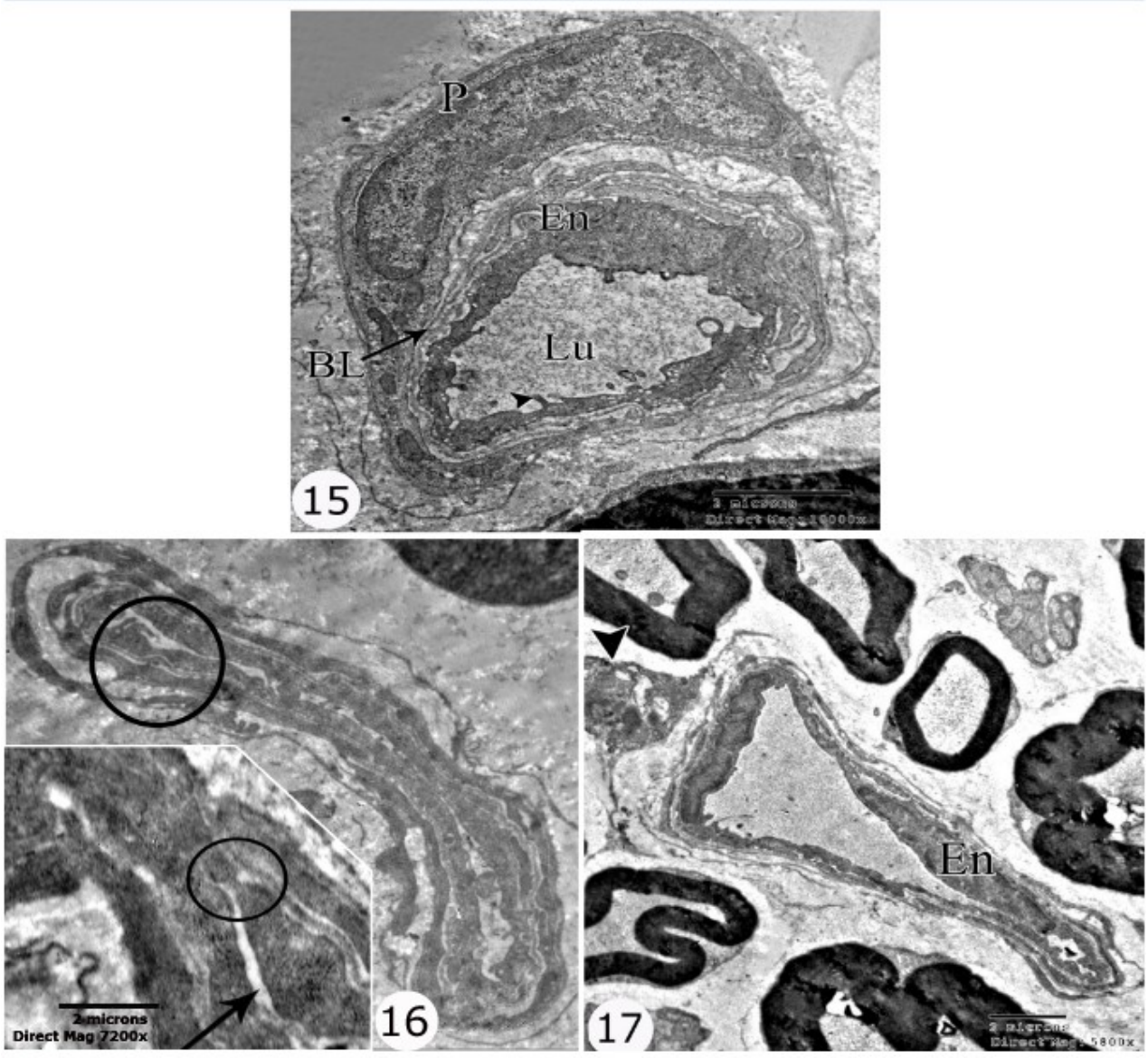


Fig 15. Cross section of an endoneurial blood capillary lined by continuous endothelial cells (En), with folds (▲) extending to a patent lumen (Lu). It is surrounded by a basal lamina (BL) which splits to enclose a pericyte (P). (Control). **Fig 16.** Collapsed endoneurial blood capillary with an attenuated lining endothelium (o). (Diabetes). Inset: Shows the attenuation of the lining endothelium (o) at higher magnification of Lu (lumen). Note the collapsed lumen (↑). (Diabetes). **Fig 17.** Endoneurial blood capillary and a nearby phagocytic cell (▲). En (endothelium). (Vitamin).

quences of hyperglycemia in Schwann cells are supposed to influence their viability (Kazunori et al., 2008). Moreover, the oxidative damage induced by mitochondrial dysfunction under hyperglycemic conditions can activate the apoptotic cascade (Nishikawa and Araki, 2007). In addition, methyl glyoxal (MG), which is a reactive dicarbonyl precursor of advanced glycation end products (AGEs), has been shown to induce apoptotic cell death of primary cultured rat Schwann cells (Fukunaga et al., 2004) and aldose reductase, which is the first enzyme in the polyol pathway, is localized to Schwann cells in the peripheral nerves (Kern and Engerman, 1982).

Treatment of the diabetic group with vitamin B complex attenuated apoptosis in both the perineurial cells and Schwann cells. Thus use of vitamin B complex preserved the integrity of these cells, and consequently their normal functions. Perineurium forms metabolically active diffusion barrier in the peripheral nerve (Allt and Lawrenson, 2000), whereas Schwann cells activate the process of clearance of degenerated myelin, which is a crucial step for successful nerve regeneration and production of cytokines and neurotrophic factors necessary for axon regeneration. The anti-apoptotic ability of vitamin B1 (thiamine) and benfotiamine in human umbilical vein endothelial cells and bovine retinal pericytes cultured in high glucose by measuring DNA fragmentation and caspase-3 activity (Beltramo et al., 2004) lend support to our findings. Vitamin B3 (niacinamide) has also

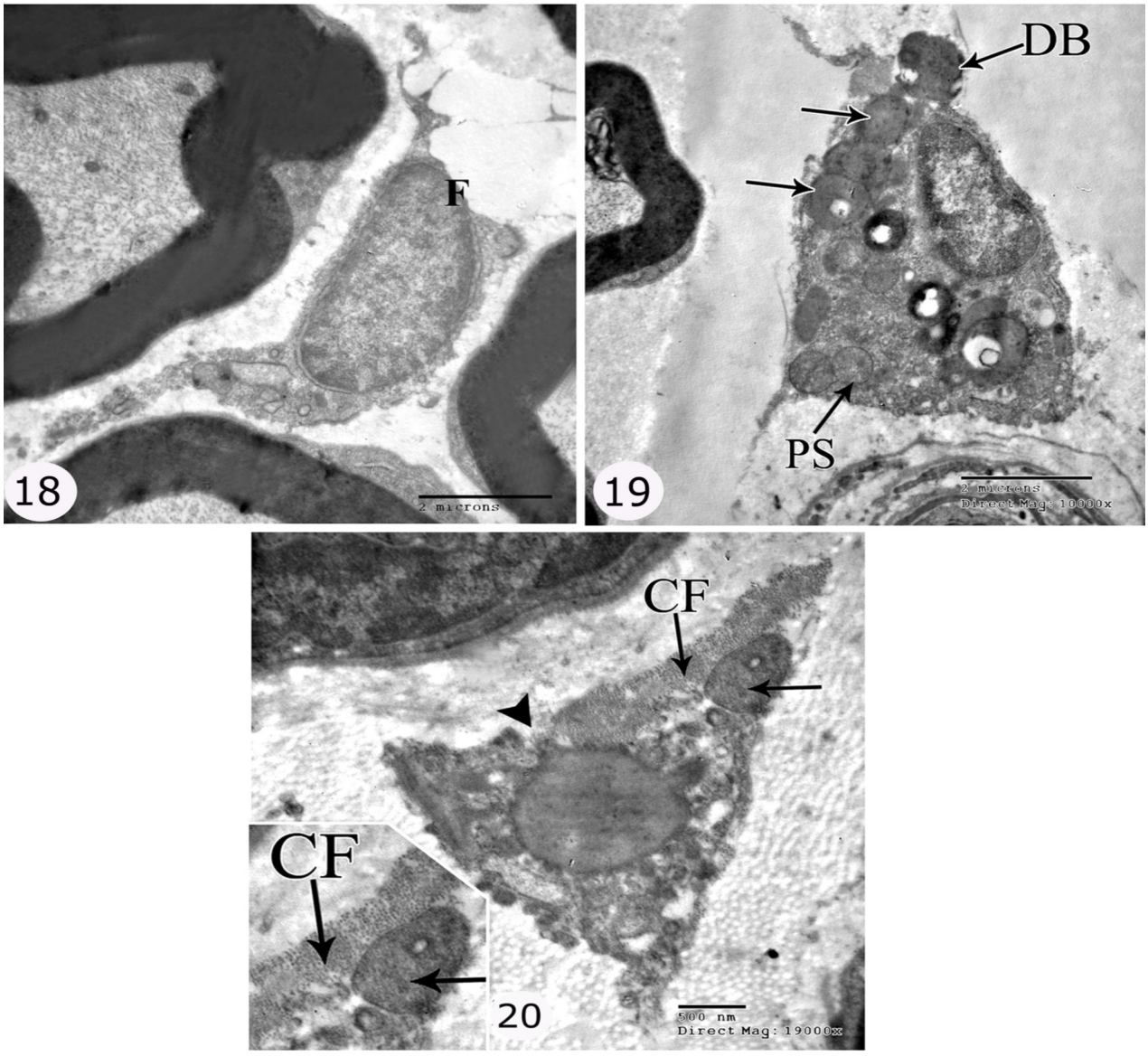


Fig 18. An endoneurial fibroblast (F) with cytoplasmic processes and an ovoid nucleus. The scanty cytoplasm contains well developed RER. (Control). **Fig 19.** Phagocytic cell with thin cytoplasmic processes and a peripheral nucleus. The cytoplasm contains lysosomes (↑) and peroxisomes (PS). Note the extracellular dense body (DB). (Diabetes). **Fig 20.** Soma of a phagocytic cell with a large phagosome (▲). Note the extracellular dense body (↑) and the collagen fibrils (CF) which are shown at higher magnification in the inset. (Vitamin).

been shown to inhibit apoptosis (Maiese and Chong, 2003; Shen et al., 2004), exhibit antioxidant properties (Melo et al., 2000; Shen et al., 2004), regulate neuronal calcium fluxes (Shen et al., 2004), reduce superoxide and hydrogen peroxide production and dose-dependently counteracted glucose mediated cell death in dorsal root ganglion neurons, exposed to high (45 mM) glucose (Vincent et al., 2005). In addition, Chong, et al.

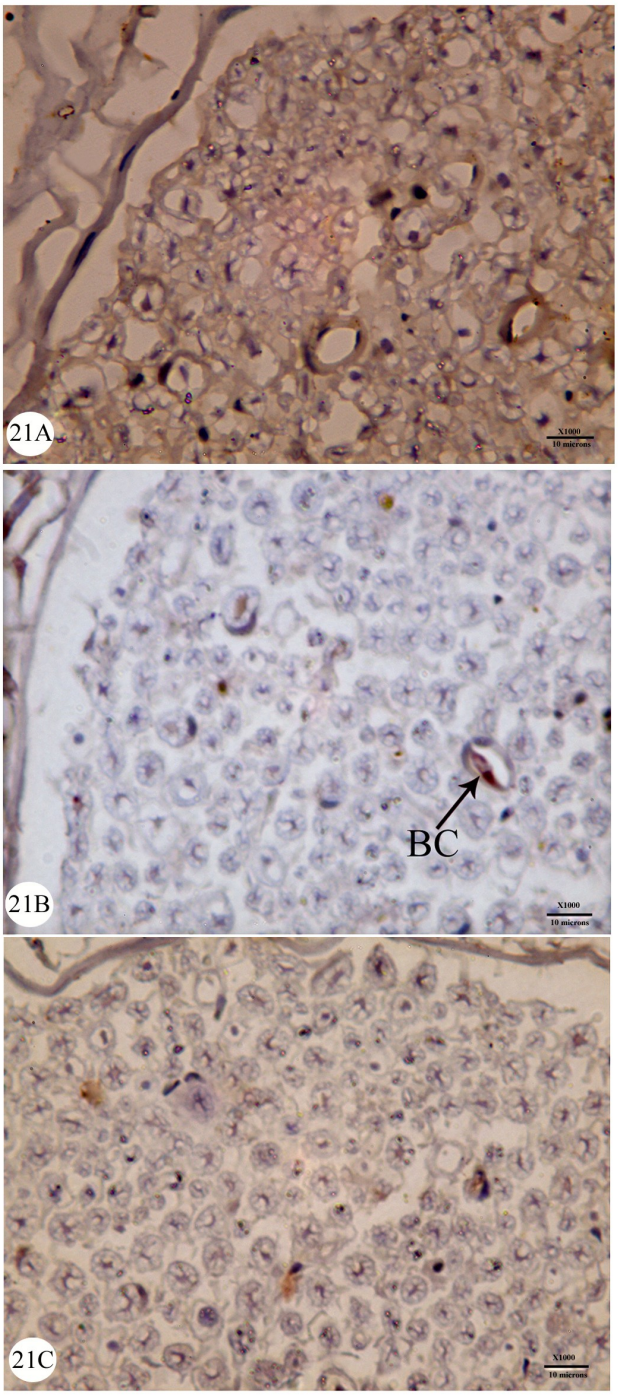


Fig 21A. Negative reaction for anticaspase-3 antibodies. (Control). **Fig 21B.** Positive immune reaction for CD31 in the endothelium lining a blood capillary (BC). (Control). **Fig 21C.** Negative reaction for CD68. (Control).

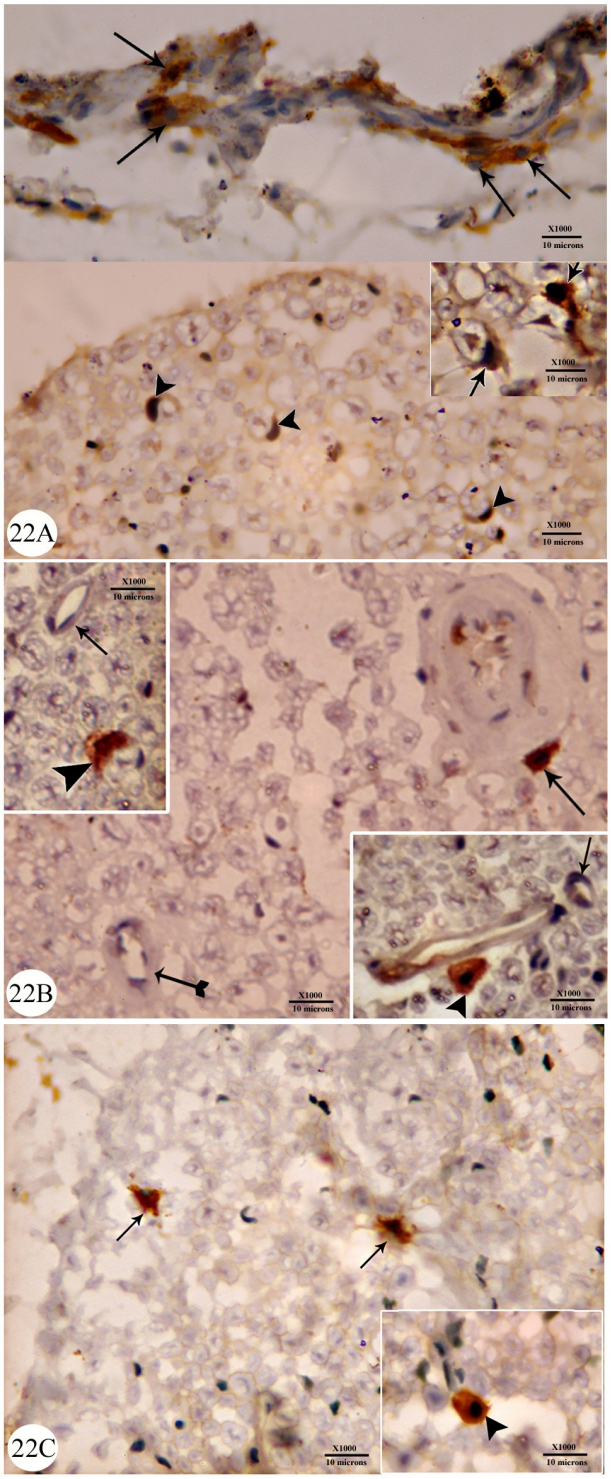


Fig 22A. Positive immune reaction for anticaspase-3 antibodies in the perineurial cells (↑) and in myelinating Schwann cells (▲). (Diabetes). Inset: shows + immune reaction for anticaspase-3 in a myelinating (↑) and a non-myelinating Schwann cells (▲). (Diabetes). **Fig 22B.** Positive immune reaction for CD31 in an endoneurial perivascular cell (↑), negative immune reaction in the endothelial lining of a blood capillary (hatched arrow). (Diabetes). Upper left inset: shows + immune reaction for a myelinating Schwann cell (▲), negative immune reaction in the endothelial lining of a blood capillary (↑). Lower right inset: shows + immune reaction for an endoneurial perivascular macrophage (▲), negative immune reaction in the endothelial lining of a

blood capillary (↑). **Fig 22C.** Positive immune reaction for CD68 in endoneurial cell (↑). (Diabetes). Inset: showing + immune reaction for an endoneurial macrophage (▲).

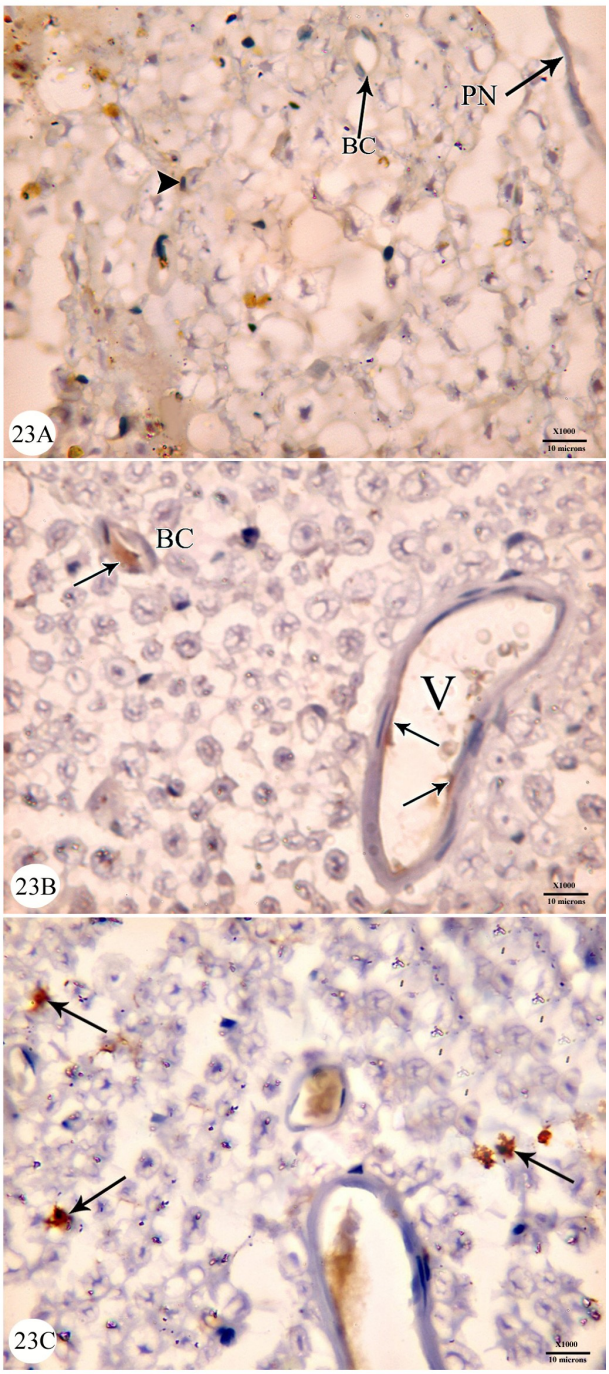


Fig 23A. Negative immune reaction for anticaspase-3 antibodies in the perineurial cells (PN) and Schwann cells (▲). BC: blood capillary. (Vitamin). **Fig 23B.** Positive immune reaction for CD31 in endothelial cells (↑) lining a blood capillary (BC) and a venule (V). (Vitamin). **Fig 23C.** A photomicrograph showing + immune reaction for CD68 in endoneurial phagocytic cells (↑). (Vitamin).

(2004) showed that niacinamide provides broad, but concentration-specific, protection against apoptotic genomic DNA fragmentation and membrane phosphatidylserine (PS) exposure in hippocampal neurons during oxidative stress through activation of the protein kinase B (Akt1) pathway. Niacinamide inhibits several pro-inflammatory cytokines, such as interleukin-1b, interleukin-6, interleukin-8, tissue factor, and tumor necrosis factor-a (TNF-a) in pancreatic islets (Reddy et al., 2001). Niacinamide could exert a direct neuroprotective effect in both neurons and glial cells (Stevens et al., 2007). Niacinamide (25 mM) prevented high (30 mM) glucose induced overexpression of poly (ADP-ribosylated) proteins in human Schwann cells. In adult animals it prevents depolarization of the mitochondrial membrane, prevents pore formation in the mitochondrial membrane and reduces release of cytochrome *c* into the cytoplasm and reduces activation of caspase-9, which reduces the activation of caspase-3, all of which may be secondary to niacinamides effect on oxidants and free radicals (Maiese et al., 2009).

Besides, vitamin B12 and dexamethasone upregulated brain derived neurotrophic factors (BDNF) expression, in the injured sciatic nerves (Hongzhi et al., 2012). BDNF is one of the neurotrophic factors essential in supporting axon regeneration, during peripheral nerve repair. In galactose-fed rats, BDNF inhibits the in motor neuron conduction velocity. However, it was not capable of neuropathic attenuating deficit in sensory nerve conduction velocity (Mizisin et al., 1997).

Treatment with vitamin B complex increased the phagocytic endoneurial cells and Schwann cells detected in this work in the diabetic group using immunohistochemical reactivity against CD31 and CD68 antibodies and verified by ultrastructure. According to Mc Kenney et al. (2001), CD31, which is generally regarded to be the most sensitive and specific endothelial marker in paraffin sections, may also be useful as a non-lysosomal marker of macrophages. Elevated numbers and densities of endoneurial macrophages have been reported in diabetic tibial nerves at late stages of diabetes (12 and 14 weeks) (Kennedy and Zochdne, 2000) and correlated with increased number and density of degenerating axon profiles. Ge Ârard et al. (2003) also detected mononuclear cell infiltrates in patients with multifocal diabetic neuropathy.

The endoneurial phagocytic cells detected in our study resembled the structure of fibroblasts. They possessed cytoplasmic thin and long processes, in addition to their being surrounded by numerous collagen fibrils. The phagocytic capacities of endoneurial fibroblasts have been previously reported by Schubert and Friede (1981). They demonstrated cellular transformations of the endoneurial fibroblast population upon intraneurial injections of india ink or of a purified preparation of rat sciatic myelin were studied for intervals of from 6 hours to 3 days after injection which suggested their capacity for myelin degradation. A subsequent massive

increase in phagocytic endoneurial cells appeared to develop from, and at the expense of, the normal endoneurial fibroblast population has also been suggested (Schubert and Friede, 1981).

Fibroblasts are known to demonstrate phagocytosis. Apoptotic neutrophils are phagocytosed by fibroblasts (Hall et al., 1996). Fibroblasts also ingest beads coated with collagen or fibronectin in culture (Lee et al., 1996; Mc Culloch et al., 1993; McKeown et al., 1990). Gingival fibroblasts have been shown to engulf type I, III, and IV collagen-coated beads (Knowles et al., 1991).

Coinciding with our findings**,** Richard et al. (2014) reported that some endoneurial fibroblast-like cells share some immunophenotypic similarities with pericytes, which have progenitor cell potentials. When activated by experimental nerve lesion, these endoneurial fibroblast-like cells could have a different proliferative and/or regenerative potential than others, and may play a role in the initial phase of nerve repair (Richard et al., 2014). In addition, Goodrum et al. (1994) confirmed that some lipid catabolism takes place in Schwann cells and endoneurial fibroblasts prior to infiltration of the nerve by macrophages.

The pathological changes detected in sciatic nerve fibers as axonal atrophy and abnormal figures of myelin sheath coincide with the work of several groups of investigators, i.e., segmental demyelination and axonal degeneration in nerves of rats with chronic experimental diabetes (Sahgal et al., 1972; Powell et al., 1977; Yagihashi et al., 1979; Zangiabadi et al., 2011). However, our morphometric data which revealed a significant decrease in myelinated nerve fiber area as well as myelin sheath thickness compared to the control group contradict with those of Kennedy and Zochodne (2000) who reported an unchanged fiber and axonal diameter and area along with myelin sheath thickness in diabetic tibial fascicles. In accordance with our work Britland et al. (1985) and Bhoyrul et al. (1988) showed a reduction of the myelinated axonal area in the sciatic and tibial nerves of STZ diabetic rat and Sharma et al. (1985) and Mc Callum et al. (1986) showed a reduction of the axonal caliber in the tibial and sural nerves of STZ-diabetic rats. In further support to our findings, Sun et al. (2012) found that dexamethasone and vitamin B12 promoted the proliferation of Schwann cells and regeneration of myelinated nerve fibers promoted recovery of sciatic functional index and sensory nerve conduction velocity**.** Vitamin B12 reduced GSH depletion in peripheral nerve of diabetic rats (Mizukami et al., 2011).

Since vascular and neural integrity are closely related, our study focused on the endoneurial blood capillaries. Micro-angiopathy of the endoneurium included basement membrane thickening, reduced luminal area of endoneurial blood capillaries and endothelial cell hyperplasia and a significant decrease in endoneurial capillary density compared to the control group.

In support with our results, endoneurial microangiopathy has been demonstrated in diabetic patients with established neuropathy and related to neuropathic severity (Bradley et al., 1990; Britland et al., 1990; Malik et al., 1992). Stephen et al. (1990) reported that there was increase in capillary wall thickness, BM thickness and capillary diameter, the latter was expected to indicate a long-term morphological adaptation toward reducing the resistance to nerve blood flow.

Reduction in capillary luminal area (Soley et al., 2003) and significant increase in capillary BM area and endothelial cell profile number (Malik et al., 2005) have been detected in diabetic patients. However, no significant differences in endoneurial capillary density have been reported between diabetic patients and control subjects.

Vitamin B complex provided marked improvement in the structure of the endoneurial capillaries of the diabetic group, a finding which correlates well with the neuroprotective effects of vitamin B12 have been explored against superoxide-induced cell injury in human aortic endothelial cells (Edward et al., 2011).

The endoneurial vascular endothelium together with the ensheathing perineurium provide a specialized endoneurial microenvironment within which, axons, Schwann cells and other resident cells of peripheral nerves function. They restrict as well as regulate the exchange of materials between the endoneurial microenvironment and the surrounding extracellular space, and are more appropriately described as blood nerve interface rather than blood nerve barrier. Besides, according to what is known as vascular hypothesis of diabetic neuropathy, which is based on structural alteration contributing to nerve perfusion, the endoneurial microangiopathy could be a potential cause of reduced nerve perfusion, hypoxia and neuropathy.

The results of this work demonstrated attenuation of the detrimental pathological hyperglycemic effect on the sciatic nerve which has been assessed using morphological, morphometric and immunohistochemical techniques. Consequently, it might enhance the repair process by reducing apoptosis and thus maintaining the cells structure and function, influencing the clearance by influencing the phagocytic cells and improving the integrity of the endoneurial micro-vessels making it an attractive potential agent for the protection of diabetic peripheral neuropathy. Therefore, treatment with the vitamin B complex is recommended to reduce peripheral neuropathy in patients with diabetes.

REFERENCES

Allt G, Lawrenson JG (2000) The blood-nerve barrier: enzymes, transporters and receptors - a comparison with the blood-brain barrier. *Brain Res Bull*, 52: 1-12.

Bancroft JD, Gamble M (2008) Theory and practice of histological techniques. Sixth edition. Ch. 9-10, pp 126, 150.

Bell DS (2010) Metformin-induced vitamin B12 deficiency presenting as a peripheral neuropathy. *South Med J*, 103(3): 265-267.

Bhoyrul S, Sharma AK, Stribling D, Mirrlees DD, Peterson RG, Farber MO, Thomas PK (1988) Ultrastructural observations on myelinated fibres in experimental diabetes: effect of the aldose reductase inhibitor ponalrestat given alone or in conjunction with insulin therapy. *J Neurol Sci*, 85(2): 131-147.

Bradley J, Thomas PK, King RHM, Llewelyn JG, Muddle JR, Watkins PJ (1990) Morphometry of endoneurial capillaries in diabetic sensory and autonomic neuropathy. *Diabetol*, 33: 611-618.

Britland ST, Sharma AK, Duguid IG and Thomas PK (1985) Ultrastructural observations on myelinated fibres in the tibial nerve of streptozotocin-diabetic rats: effect of insulin treatment. *Life Support Syst*, 3 Suppl 1: 524-529.

Britland ST, Young RJ, Sharma AK, Clarke BF (1990) Relationship of endoneurial capillary abnormalities to type and severity of diabetic polyneuropathy. *Diabetes*, 39: 909-913.

Chong ZZ, Lin S H, Maiese K (2004) The NAD+ precursor nicotinamide governs neuronal survival during oxidative stress through protein kinase B coupled to FOXO3a and mitochondrial membrane potential. *J Cereb Bl Flow Met*, 24: 728-743.

Edward SM, Nicola EB, June Y (2011) Vitamin B12 protects against superoxide-induced cell injury in human aortic endothelial cells. *Free Rad Biol Med*, 51(4): 876-883.

Farvid MS, Homayouni F, Amiri Z, Adelmanesh F (2011) Improving neuropathy scores in type 2 diabetic patients using micronutrients supplementation. *Diabet Res Clin Prac,* 93: 86-94.

Fukunaga M, Miyata S, Liu BF, Miyazaki H, Hirota Y, Higo S, Hamada Y, Ueyama S, Kasuga M (2004) Methylglyoxal induces apoptosis through activation of p38 MAPK in rat Schwann cells. *Biochem Biophys Res Commun*, 320(3): 689-695.

Geârard S, Catherine L, Pierre L, Angeále R, Violaine P, David A (2003) Inflammatory vasculopathy in multifocal diabetic neuropathy. *Brain*, 126: 376-385.

Gomez Sanchez JA, Carty L, Iruarrizaga-Lejarreta M, Palomo-Irigoyen M, Rey M, Griffith M, Hantke J, Macias-Camara N, Azkargorta M, Aurrekoetxea I, De Juan VG, Jefferies HB, Aspichueta P, Elortza F, Aransay AM, Martínez-Chantar ML, Baas F, Mato JM, Mirsky R, Woodhoo A, Jessen KR (2015) Schwann cell autophagy, myelinophagy, initiates myelin clearance from injured nerves. *J Cell Biol*, 210(1): 153-168.

Goodrum JF, Earnhardt T, Goines N, Bouldin TW (1994) Fate of myelin lipids during degeneration and regeneration of peripheral nerve: an autoradiographic study. *J Neurosci*, 14(1): 357-367.

Hill RE, Williams PEA (2002) Quantitative analysis of perineurial cell basement membrane collagen IV, laminin and fibronectin in diabetic and non-diabetic human sural nerve. *J Anat*, 201: 185-192.

Kazunori S, Hiroko Y, Koichi K, Noriaki K, Hiroko H, Kazuhiko W (2008) Differential effects of high glucose and methylglyoxal on viability and polyol metabolism in immortalized adult mouse Schwann cells. *Open Diab J*, 1: 1-11.

Kennedy JM, Zochodne DW (2000) The regenerative deficit of peripheral nerves in experimental diabetes: its extent, timing and possible mechanisms. *Brain*, 123: 2118-2129.

Kern TS, Engerman RL (1982) Immunohistochemical distribution of aldose reductase. *Histochem J*, 14: 507-515.

Knowles GC, McKeown M, Sodek J, McCulloch CAG (1991) Mechanism of collagen phagocytosis by human gingival fibroblasts: importance of collagen structure in cell recognition and internalization. *J Cell Sci*, 98: 551-558.

Kräutler B (2012) Biochemistry of B12-cofactors in human metabolism. *Subcell Biochem,* 56: 323-346.

Kundalić B, Ugrenović S, Jovanović I, Stefanović N, Petrović V, Kundalić J, Pavlović M, Antić V (2014) Analysis of fascicular structure and connective tissue sheaths in sural nerve during aging. *Sci J Fac Med Niš*, 31(2): 113-120.

Lee W, Sodek J, McCulloch CA (1996) Role of integrins in regulaton of collagen phagocytosis by human fibroblasts. *J Cell Physiol*, 168: 695-704.

Maiese K, Chong ZZ (2003) Nicotinamide: necessary nutrient emerges as a novel cytoprotectant for the brain. *Trends Pharmacol Sci*, 24: 228-232.

Maiese M, Chong ZZ, Jinling Hou J, Shang YC (2009) The vitamin nicotinamide: translating nutrition into clinical care. *Molecules*, 14(9): 3446-3485.

Malik RA, Tesfaye S, Newrick PJ, Walker D, Rajbhandari SM, Siddique I, Sharma AK, Boulton AJM, King RHM, Thomas PK, Ward J (2005) Sural nerve pathology in diabetic patients with minimal but progressive neuropathy. *Diabetol*, 48: 578-585.

Malik RA, Veves A, Masson EA, Sharma AK, Ah-See AK, Schady W, Lye RH, Boulton AJ (1992) Endoneurial capillary abnormalities in mild human diabetic neuropathy. *J Neurol Neurosurg Psychiatry,* 55: 557-561.

Mc Callum KN, Sharma AK, Blanchard DS, Stribling D, Mirrlees DJ, Duguid IG, Thomas PK (1986) The effect of continuous subcutaneous insulin infusion therapy on morphological and biochemical abnormalities of peripheral nerves in experimental diabetes. *J Neurol Sci*, 74(1): 55-67.

Mc Culloch CAG, Knowles GC (1993) Deficiencies in collagen phagocytosis by human fibroblasts in vitro: a mechanism for fibrosis? *J Cell Physiol*, 155: 461-471.

Mc Keown M, Knowles GC, Mc Culloch CAG (1990) Role of the cellular domain of fibronectin in the phagocytosis of beads by human gingival fibroblasts in vitro. *Cell Tissue Res*, 262: 523-530.

Melo SS, Arantes MR, Meirelles MS, Jordão AA Jr, Vannucchi H (2000) Lipid peroxidation in nicotinamide-deficient and nicotinamide-supplemented rats with streptozotocin-induced diabetes. *Acta Diabetol*, 37(1):

33-39.

Mizisin AP, Bache M, DiStefano PS, Acheson A, Lindsay RM, Calcutt NA (1997) BDNF attenuates functional and structural disorders in nerves of galactose-fed rats. *J Neuropathol Exp Neurol*, 56: 1290-1301.

Mizukami H, Ogasawara S, Yamagishi S, Takahashi K, Yagihashi S (2011) Methylcobalamin effects on diabetic neuropathy and nerve protein kinase C in rats. *Eur J Clin Invest,* 41(4): 442-450.

Naurath HJ, Riezler R, Putter S, Ubbink JB (2001) Does a single vitamin B-supplementation induce functional vitamin B deficiency? *Clin Chem Lab Med*, 39: 768-771.

Nishikawa T, Araki E (2007) Impact of mitochondrial ROS production in the pathogenesis of diabetes mellitus and its complications. *Antioxid Redox Signal*, 9: 343-353.

Powell H, Knox D, Lee S, Charters A C, Orloff M, Garrett R, Lampert P (1977) Alloxan diabetic neuropathy: electron microscopic studies. *Neurology*, 27: 60-66.

Reddy S, Young M, Ginn S (2001) Immunoexpression of interleukin-1beta in pancreatic islets of NOD mice during cyclophosphamide-accelerated diabetes: co-localization in macrophages and endocrine cells and its attenuation with oral nicotinamide. *Histochem J,* 33 (6): 317-327.

Richard L, Védrenne N, Vallat J, Funalot B (2014) Characterization of endoneurial fibroblast-like cells from human and rat peripheral nerves. *J Histochem Cytochem*, 62(6): 424-435.

Sahgal VK, Roy S, Ahuja MM, Singh N (1972) Diabetic neuropathy. An experimental study in alloxanised rats, with special reference to insulin therapy. *Acta Diabetol Lat*, 9: 983-1006.

Schubert T, Friede RL (1981) The role of endoneurial fibroblasts in myelin degradation. *J Neuropathol Exp Neurol,* 40(2): 134-154.

Scott K, Zeris S, Kothari MJ (2008) Elevated B6 levels and peripheral neuropathies. *Electromyogr Clin Neurophysiol*, 48(5): 219-223.

Sharma AK, Duguid IG, Blanchard DS, Thomas PK (1985) The effect of insulin treatment on myelinated nerve fibre maturation and integrity and on body growth in streptozotocin diabetic rats. *J Neurol Sci*, 67 (3): 285-297.

Shen CC, Huang HM, Ou HC, Chen HL, Chen WC, Jeng KC (2004) Protective effect of nicotinamide on neuronal cells under oxygen and glucose deprivation and hypoxia/reoxygenation. *Biomed Sci,* 11: 472-481.

Soley T, Rayez AM, Lars BD, Peter W, Karl FE, Ingmar R, Jesper P, Douglas AG, Göran S (2003) Endoneurial capillary abnormalities presage deterioration of glucose tolerance and accompany peripheral neuropathy in man. *Diabetes*, 52: 2615-2622.

Sorrell JM, Caplan AI (2009) Fibroblasts-a diverse population at the center of it all. *Int Rev Cell Mol Biol*, 276: 161-214.

Stephen TB, Robert JY, Ashutosh KS, Basil FC (1990) Relationship of endoneurial capillary abnormalities to type and severity of diabetic polyneuropathy. *Diabetes*, 39: 909-913.

Stevens MJ, Li F, Drel VR, Abatan OI, Kim H, Burnett D, Larkin D, Obrosova IG (2007) Nicotinamide reverses neurological and neurovascular deficits in streptozotocin diabetic rats. *J Pharmacol Exp Ther*, 320(1): 458-464.

Sun H, Yang T, Li Q, Zhu Z, Wang L, Bai G, Li D, Li Q, Wang W (2012) Dexamethasone and vitamin B12 synergistically promote peripheral nerve regeneration in rats by upregulating the expression of brain-derived neurotrophic factor. *Arch Med Sci,* 8(5): 924-930.

Sun Y, Lai MS, Lu CJ (2005) Effectiveness of vitamin B12 on diabetic neuropathy: systemic review of clinical controlled trials. *Acta Neurol Taiwan*, 14(2): 48-54.

Tesfaye S, Chaturvedi S, Eaton SEM (2005) Vascular risk factors and diabetic neuropathy. *New Engl J Med*, 352: 341-350.

Tohgi H, Tsukagoshi H, Toyokura Y (1977) Quantitative changes with age in normal sural nerves. *Acta Neuropathol*, 38(3): 213-220.

Ugrenović S, Jovanović I, Vasović L (2011) Morphometric analysis of human sciatic nerve perineurial collagen type IV content. *Microsc Res Tech,* 74(12): 1127-1133.

Vincent AM, Stevens MJ, Backus C, McLean LL, Feldman EL (2005) Cell culture modeling to test therapies against hyperglycemia-mediated oxidative stress and injury. *Antioxid Redox Signal*, 7(11-12): 1494-**1**506.

Yagihashi S, Nishihira M, Baba M (1979) Morphometrical analysis of the peripheral nerve lesions in experimental diabetes rats. *Tohoku J Exp Med*, 129: 139-149.

Zangiabadi N, Asadi-Shekaari M, Sheibani V, Jafari M, Shabani M, Asadi AR, Tajadini H, Jarahi M (2011) Date fruit extract is a neuroprotective agent in diabetic peripheral neuropathy in streptozotocin-induced diabetic rats: A multimodal analysis. *Oxid Med Cell Longev*, 2011: 976948. doi: 10.1155/2011/976948.