The Effect of Antioxidants on Experimentally Induced Diabetic Peripheral Neuropathy in Adult Male Albino Rats

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Abstract: Diabetes mellitus is the most common cause of peripheral neuropathy which is a major disorder that significantly affects the quality of life. There are several underlying pathophysiological mechanisms that are proposed to result in neuropathy including oxidative stress. Up to date, there is no definitive treatment for diabetic neuropathy and all the currently used medications aim to treat the symptoms rather than the underlying disease. In the current study we investigated the effect of vitamin-E intake on induced diabetic neuropathy in rats using a histological approach. We found that vitamin-E intake can partially prevent diabetic neuropathy. It can be concluded that vitamin-E is a potential safe, inexpensive antioxidant which can be used for prevention/treatment of diabetic neuropathy.

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1. Introduction

Diabetes mellitus (DM) is the commonest endocrine disorder which has a major impact on the individual health. The condition is chronic and requires continuous supervision, education and monitoring. It is associated with a decreased life expectancy and an increased morbidity resulting from the associated complications [1]**.**

DM is the most common cause of peripheral neuropathy (PN). It occurs in 50% to 90% of patients depending on the criteria used for diagnosis [2]. It has a major impact on the quality of patient's life as it may present with a variety of symptoms including pain, weakness, ataxia and impotence. Also, sensory loss may lead to ulcerations, gangrene as well as to limb amputation [2, 3].

It has been proposed that several mechanisms are involved in the pathogenesis of diabetic PN which include increased oxidative stress, advanced glycation, polyol accumulation, decreased nitric oxide, and impaired (Na+/K+)-ATPase [3].

Recent studies suggest that even minimal perturbations in blood glucose in those with impaired glucose tolerance (IGT) may lead to the development of small nerve fiber damage and neuropathy [4]. It has been shown that hyperglycemia leads to oxidative stress and glycation of proteins that produces free oxygen radicals [5-7]**.** The tissue toxicity of free oxygen radicals is based on their direct reactivity with numerous types of biological molecules and the dismutation to form hydrogen peroxide and the concomitant reduction of ferric ion to ferrous ion. The reduction of these two products yield the highly toxic hydroxyl radical that may cleave covalent bonds in proteins and carbohydrates causing lipid peroxidation and destroy the cell membrane [8-10]**.**

It has been found that experimental and clinical data suggested that the supplementation with antioxidants such as vitamin-E normalizes oxidant stress and improves both endothelium-dependent vasodilatation and insulin sensitivity [11-15].

Therefore, the present study was designed to investigate the structural changes occurring in the peripheral nerves as a result of short-term hyperglycemia in rats, and to investigate the effect of vitamin-E as an antioxidant on such changes.

2. Materials and Methods

Animals:

Fifteen healthy (6 months old) adult male albino rats weighing 250-300 gm were used in this study. All animals were housed in animal house, fed a standard balanced diet and had water ad-libitum. The animals were equally divided into three groups (5 animals each). Group A animals served as a control. Group B animals were injected intra-peritoneally with 40-mg streptozocin/kg body weight for 5 successive days in order to induce diabetes mellitus [16-18]. Group C animals were injected with streptozocin similar to the animals in group B, and then they were given a daily dose of

vitamin-E 70-mg/kg body weight orally for 6 successive weeks. All animals were sacrificed by the end of the 8th week of the experiment.

Tissue Collection and Processing:

At the time of sacrifice the animals of the all groups were anaesthetized with ether inhalation and their sciatic nerves were dissected out carefully and small pieces were taken and processed for light and electron microscope examinations.

Specimens for electron microscope were immediately fixed in 2.5% glutaraldehyde buffered with 0.1 M phosphate buffer at pH 7.4 for 2 hours at 4^0C. Speacimens were dehydrated with ascending grads of ethanol and then were put in propylene oxide for 30 minutes at room temperature impregnated in a mixture of propylene oxide and resin (1:1) for 1 hour then in a mixture of previous reagents at 48^0C for 1 hour. The specimens were embedded in Embed-812 resin in BEEM capsules at 60^0C for 24 hours. By using Leica ultra cut UCT we obtained semi-thin sections which were stained with toulidine blue for light microscope examination and ultra- thin section were obtained and stained with uranyl acetate and lead citrate and were examined with JEOL JEM 1010 electron microscope.

Biochemical Analysis:

Blood samples were obtained from treated groups two weeks after the beginning of the experiment (i.e.10 days after Streptozocin injection) to confirm the diabetic effect [16-18]. A second blood sample was also obtained just before sacrifice and immediately after Vitamin-E treatment ended. Glucose analysis (mg/dl) revealed high significant elevation in both diabetic groups when compared with the control group; this was confirmed statistically using one-way ANOVA (Table 1). Furthermore, the blood glucose concentration was significantly higher in group B than group C in the 8-weeks sample ($P<0.001$), but not in the 2-weeks sample.

Morphological Assessment:

There are several methods that have been used to quantify the morphological changes in nerve fibers in diabetic rats [19]. However, in this study we decided to use a simple method in which the nerve fibers were classified visually into either normal or abnormal (distorted) myelin sheath. For each rat, 120 nerve fibers were assessed visually from each sural nerve.

Statistical Analysis:

The results are presented as mean ± SD and the significance of differences was calculated by analysis of variance (ANOVA). An overall difference of $p < 0.05$ was considered significant.

3. Results

General Observations:

Most of the diabetic rats in the experimental groups (B and C) looked quite sick and less active, especially after the initial two days, they drank more water, lost weight, urinated more frequently, but ate well. These observations were not modified by vitamins treatment in group (C).

Biochemical Analysis:

Blood glucose analysis (mg/dl) [16-18] revealed high significant elevation in both diabetic groups when compared with the control group; this was confirmed statistically using one-way ANOVA (Table 1). Furthermore, the blood glucose concentration was significantly higher in group B than group C in the 8-weeks sample ($P<0.001$), but not in the 2-weeks sample. Interestingly, group C showed a significantly lower blood glucose level than group B, which may indicate that apart from its antioxidant effect; vitamin-E has a significant effect on blood glucose level.

Morphological Changes:

Light microscopic examination of the semi-thin sections of the sciatic nerve of group A showed many myelinated axons with few unmyelinated ones. The endoneurium between the axons contained blood vessels (Figure 1A). Ultrastructural examination of the same group revealed that some Schwann cells were enclosing myelinated axons, while others surround several unmyelinated fibers. Collagen fibrils were noticed in between nerve axons. The myelin sheaths were formed of several dark lamellae which appeared very close to each other (Figure 1B).. The axoplasm contained neurofibrils. Schwann cells cytoplasm contained rough endoplasmic reticulum. Their nuclei appeared with much euchromatin and less heterochromatin (Figure 1C). The nodes of Ranvier showed regular neurolemmal terminations as cytoplasmic swellings (Figure 1D).

Light microscopic examination of the semi-thin sections of the sciatic nerve of the diabetic group (B) showed numerous unmyelinated axons with different diameters (Figure 2A). Electron microscopic examination of the same group showed splitting in the myelin lamellae. Schwann cells nuclei appeared with much heterochromatin. Their cytoplasm contained few scattered segments of rough endoplasmic reticulum and poorly developed Golgi apparatus (Figure 2B). The nodes of Ranvier exhibited irregular neurolemmal terminations (Figure 2C). The endoneurium contained mast cells with

their cytoplasm contained numerous electron dense granules and infiltrating cells with lobulated nuclei in close relation with the distorted myelin sheath (Figs. 2D,E).

Light microscopic examination of the semi-thin sections of the sciatic nerve of the diabetic group which received vitamin-E revealed many myelinated axons. Few axons showed distorted myelination (Figure 3A). Ultrastructurally, Schwann cell appeared with much euchromatic nucleus and its cytoplasm contained several segments of rough endoplasmic reticulum. The related axon appeared with closely packed myelin lamellae (Figure 3B).

Quantitative assessment of the myelination status of the nerve fibers using light microscope photomicrographs showed a significant effect of hyperglycemia on nerve fibers myelination in group B when compared with group A ($P<0.0005$). However, this effect was reduced when comparing group C with group A ($p<0.003$), indicating a partial protective effect of vitamin-E intake (Figure 3A).

Table 1: Blood Glucose for the three groups of animals

		Mean ± SD (mg/dl)	Range	P value (versus control)
Group (A)		76.9 ±13.4	58-97	-
Group (B)	B1 (2weeks)	419.6 ±114.5	245-584	‹ 0.001
	B2 (8 weeks)	425.5 ±113.8	228-584	‹ 0.001
Group (C)	C1 (2 weeks)	330.8 ±149.5	153-547	‹ 0.001
	C2 (8 weeks)	238.3 ±104.8	150-453	‹ 0.001

This table demonstrates the level of blood glucose in all groups obtained after 2-weeks and 8-weeks from the beginning of the experiment. The results of one-way ANOVA are also shown. SD =standard deviation.

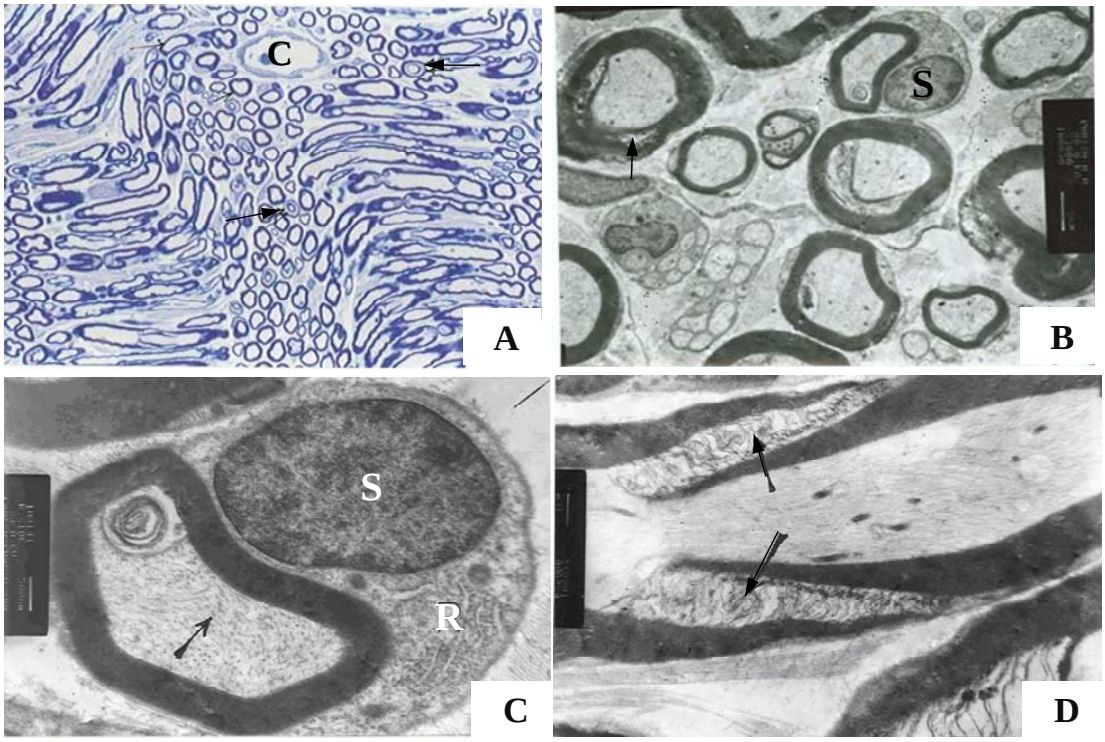

Figure 1: (A) A photomicrograph of a semi-thin section (1µm thick, Toluidine blue, X100) of the sciatic nerve of the control group (group A) showing many myelinated axons and few with distorted mylination (arrows). A blood capillary (C) is also noticed in the endoneurium**. (B)** A transmission electron micrograph (X6000) from the sciatic nerve of the control group showing many myelinated axons and few unmyelinated ones (arrows). Schwann cells (S) enclosing the axons are seen. Notice the collagen fibrils in between nerve axons. **(C)** A transmission electron micrograph (X24000) from the sciatic nerve of the control group showing a Schwann cell (S) with much euchromatic nucleus and the cytoplasm contains rough endoplasmic reticulum (R). Dark lamellae of the myelin sheath are noticed. Also, the axoplasm with its neurofibrils is seen (arrow). **(D)** A transmission electron micrograph (X10000) from the sciatic nerve of the control group showing the regular neurolemmal terminations of the nodes of Ranvier (arrows).

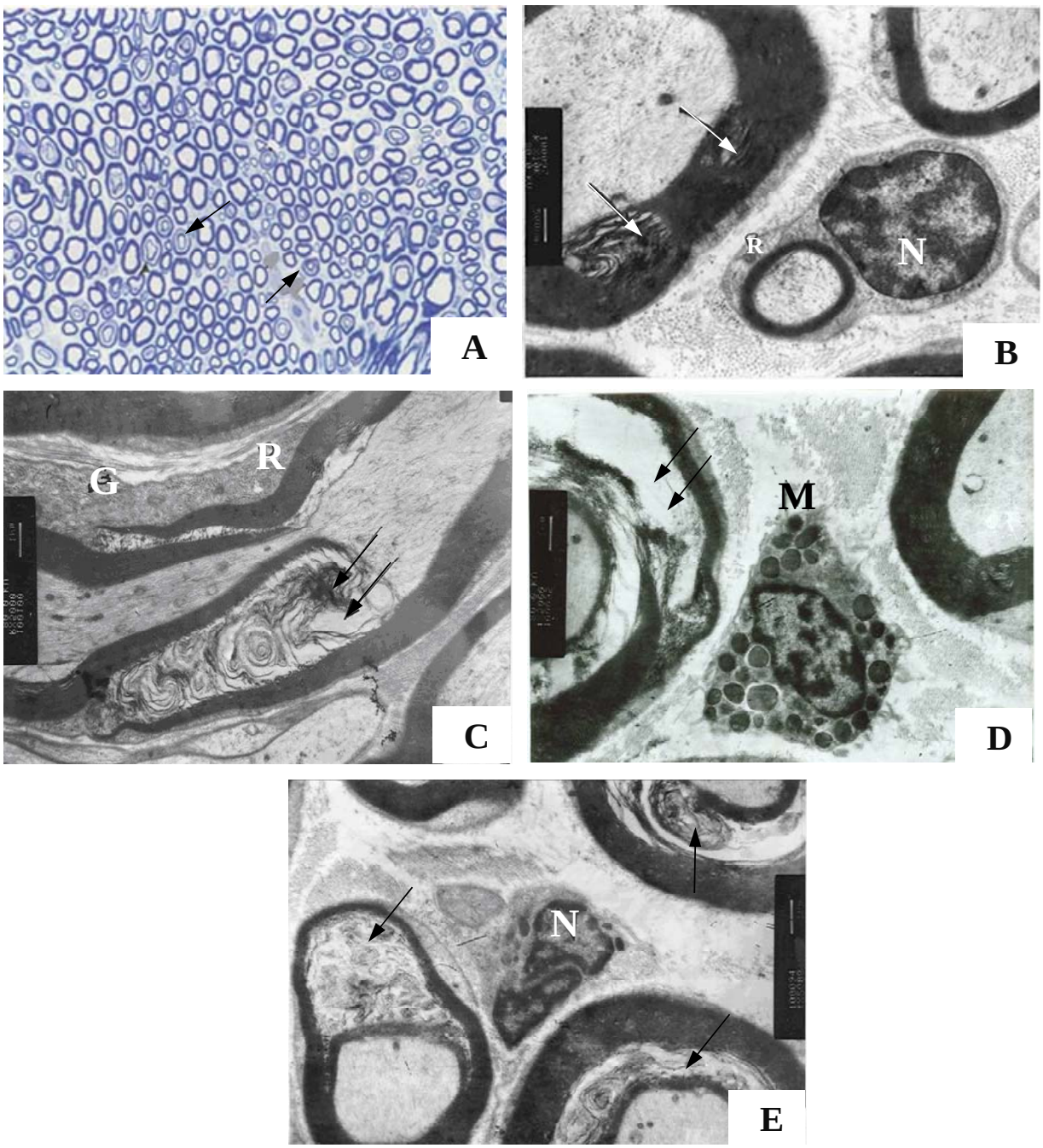

Figure 2: (A) A photomicrograph of a semi-thin section (1µm thick, Toluidine blue, X100) of the sciatic nerve of the diabetic group B showing some axons with distorted myelination (arrows). **(B)** A transmission electron micrograph (X24000) from the sciatic nerve of the diabetic group B showing a Schwann cell with heterochromatic nucleus (N) and a cytoplasm with few scattered segments of rough endoplasmic reticulum (R). Splitted myelin lamellae (arrows) are seen. **(C)** A transmission electron micrograph (X10000) from the sciatic nerve of the diabetic group B showing the irregular neurolemmal terminations of the nodes of Ranvier (arrows). A part of Schwann cell cytoplasm is seen with a poorly developed Golgi apparatus (G) and few scattered segments of rough endoplasmic reticulum (R). **(D)** A transmission electron micrograph (X10000) from the sciatic nerve of the diabetic group B showing a mast cell (M) with numerous electron dense granules in close relation with a distorted myelin lamellae (arroes). **(E)** A transmission electron micrograph (X10000) from the sciatic nerve of the diabetic group B showing an infiltrating cell with a lobulated nucleus (N) and a cytoplasm containing electron dense granules near the distorted myelin lamellae (arrows).

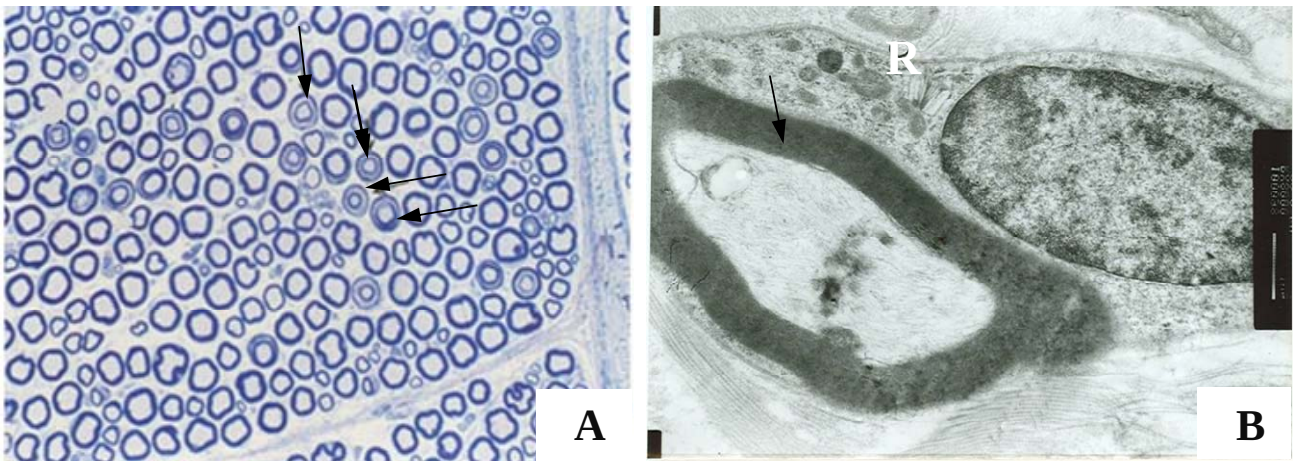

Figure 3: (A) A photomicrograph of a semi-thin section (1µm thick, Toluidine blue, X100) of the sciatic nerve of the diabetic group + vitamin-E showing many myelinated axons, some axons appear with distorted myelin sheath (arrows). **(B)** A transmission electron micrograph (X18000) from the sciatic nerve of the diabetic group + vitamin-E showing a Schwann cell with euchromatic nucleus and a cytoplasm containing multiple segments of rough endoplasmic reticulum (R). Axon appears with closely packed myelin lamellae (arrow).

4. Discussion

Peripheral nerve function is significantly affected by many diseases. Diabetic neuropathy is a common one [4]. Functional deficits may be the consequence of a loss of nerve fibers [6], myelin abnormalities, and alternations in connective tissue and vascularisation [8].

In this study we have shown, using light microscope, that short-term hyperglycemia in rats can produce significant distortion in the myelination pattern of the nerve fibers. These findings are consistent with other studies in which diabetic rats exhibited decreased myelinated fibers area, fiber density, diameter of the myelinated fibers, and axon/myeline ratio [20, 21]. Some changes in nodes of Ranveir and segmental demyelination were also reported [19]. Such structural abnormalities in patients with diabetic neuropathy have been also reported. They include nerve fiber loss and microvascular abnormalities, particularly basement membrane thickening and endothelial cell hyperplasia. Interestingly, There was a significant correlation between the extent of these changes and the severity of the neuropathy [22]. It has been shown that even impaired glucose tolerance and not only frank diabetes mellitus is associated with structural and electrophysiological changes of the nerves with clinical symptoms and signs of neuropathy [4, 23]. Many studies showed a correlation between such structural changes and the severity of the symptoms and signs of neuropathy [23-26].

We have also shown that distorted myelination of the nerve fibers can be partially reversed by short-term intake of vitamin-E. One study showed that high dose vitamin-E supplementation in rats with streptozocin-induced diabetes improved motor and sensory conduction velocity in tibial and sural nerves by 50% [27]. Low et al showed that intraperitoneal administration of alpha-lipoic acid in diabetic rats improved the biochemical (oxidative stress markers) and electrophysiological changes induced in the nerves of diabetic rats [28]. Another study reported that 3-month treatment with the antioxidant alpha-lipoic acid improved the stage of the neuropathy in patients with stage II diabetic neuropathy [29]. *In vi*tro study reported that application of the antioxidant alpha-lipoic acid prevents glucose-induced oxidative stress and cell death in cultured dorsal ganglia neurons [30, 31]. Therefore, the potential positive effect of vitamin-E on the pathological changes of the nerves may indicate that it can be used for prevention\treatment of diabetic neuropathy. However, it seems that the effect of antioxidant is partial as indicated by our results. This is supported by the findings of some studies which reported that long-term clinical trials using antioxidants did not produce therapeutic results [32, 33]. However, when antioxidants were used in combination with antihyperglycemic drugs, they showed superior protection against cellular oxidative injury (reviewed in [34]).

Interestingly, we found that vitamin-E intake reduced blood glucose level significantly. The underlying mechanisms are not clear, but it can be postulated that vitamin-E may improve endothelial function by reducing the oxidative stress associated the diabetes and, therefore, decrease insulin resistance. However, this finding may raise the argument that the beneficial effect of vitamin-E in

our study is due to its antihyperglycemic effect rather than\and its antioxidant effect. However, similar results were also reported in another study which showed that the beneficiary effect of alpha-lipoic acid on the electrophysiological changes induced in the nerves of diabetic rats was correlated with the reduction of the oxidative stress markers [28]. Another study reported that gliclazide has a beneficial effect on peripheral neuropathy in streptozocin-induced diabetic rats through its effect on oxidative stress markers irrespective of blood glucose level [20].

In conclusion, up to date there is no definitive treatment for diabetic neuropathy. All the management strategies aim to reduce the symptoms rather than the underlying pathological changes which resulted in the symptoms. Our study showed that vitamin-E can reverse the pathological changes of diabetic neuropathy, and therefore, it is a potential safe, nontoxic, and quite inexpensive supplement to improve diabetic neuropathy.

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