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Original Article

Neuroprotective effect of co-administered vitamin E isoforms in sciatic nerve crushed injury of diabetic rats

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Abstract. Diabetic peripheral neuropathy is believed to be due to vascular deficit, altered antioxidant defense mechanism and glycemic status. This study is designed to explore the effect of co-administration of some natural antioxidants e.g. vitamin E isoforms, on the regeneration of crush-injured sciatic nerve in healthy and diabetic rats. Diabetes was induced through single subcutaneous injection of alloxan at the dose of 100 mg/kg. Twenty four albino rats were divided into four groups; healthy control, diabetic control, healthy treated and diabetic treated. Treated groups received 100 mg/kg of d-α-tocopherol and d-δ-TRF each, orally and daily for three weeks. A horizontal skin incision was made on shaved right mid-thigh region and sciatic nerve was approached by splitting and retraction of surrounding muscles followed by the crushing of sciatic nerve proximal to its division with Kocher's forceps. Skin wound was closed with an absorbable suture. Sciatic functional and static indices were used to assess the functional recovery. The histopathology, histomorphology of crushed nerve and serum biochemical study were performed at the end of third week. One-way ANOVA followed by Tukey's test and Student's *t* test were used for statistical analysis of data. All results revealed that vitamin E isoforms on co-administration synergistically improve the antioxidant status, glycemic level; promote neovascularization, regeneration and remyelination of nerve fibers and matrix remodeling after crushed injuries of sciatic nerve. It is concluded that these vitamin E isoforms are potent therapeutic dietary supplements on peripheral nerve regeneration in both healthy and diabetics.

Keywords: Crush- injury, d-α-tocopherol, d-δ-tocotrienol rich fraction, diabetes, rats, sciatic nerve

Introduction

Persistent hyperglycemia, oxidative stress and vascular impairment in diabetes are known causative factors in the development of peripheral neuropathy [1]. In diabetes reactive oxygen species (ROS) have been implicated for direct damage to the peripheral neurons and altered the antioxidant defense mechanisms [2]. Hence antioxidant treatments appear to be promising therapeutics that can prevent or correct the oxidative stress, motor and sensory nerve conduction velocity in diabetic rats [3-5].

The use of antioxidant vitamin C or steroids reduces the post- injury nerve dysfunction and improves nerve regeneration of crushed sciatic nerve in healthy rats [6, 7]. Vitamin E has a central role in maintaining neurological structure and function [8]. Free-radical scavenging effects of tocopherol and tocotrienols revealed that tocotrienols appear superior because of their better distribution in the fatty layers of the cell membrane [9]. In treating of diabetes and its complications including neuropathies, a suitable treatment must contain agents that have both antioxidant and blood glucose decreasing properties [10]. As such no individual treatment has proven to have both antioxidant and blood flow enhancing effect in diabetic neuropathy [1].

Our previous studies [11, 12] also revealed that the individual supplementation of d-α-tocopherol and d-δ-TRF helped to accelerate the peripheral nerve regeneration in both healthy and diabetic rats. Hence the present study is to analyze the effect of co-administration of these isoforms in healthy and diabetic rats on peripheral nerve repair and regeneration by using functional, histopathological, histo-morphological and biochemical methods.

Materials and Methods

Twenty four albino rats of either sex each weighing 230-320g was obtained from central animal house of JN medical college, AMU, Aligarh. The study was approved by Institutional Animal Ethical Committee (No. 8937/2014).

Diabetes was induced to the diabetic groups after deprivation of food for 4 hours, followed by single subcutaneous injection (hip region) of alloxan (100 mg/kg; Alloxan monohydrate from Sigma-Aldrich). Food and water were provided after one hour of injection. Blood was

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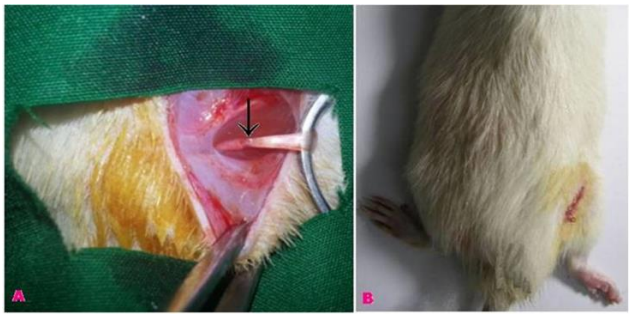

Figure 1 A. Arrow (↑) pointing to sciatic nerve crushed parts. **B**. Shows complete paralysis of right foot after sciatic nerve crushed injury。

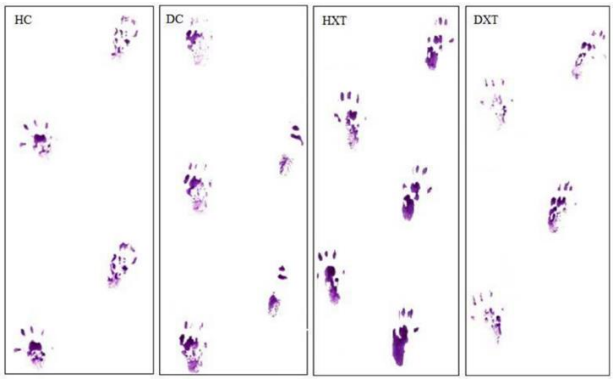

Figure 2 Photographs of the hindlimb foot prints of all groups at the end of 3rd week. Note: in diabetic control (DC) prints are not measurable. Better foot prints in co-administered (HXT and DXT) groups as compared to healthy control (HC) group.

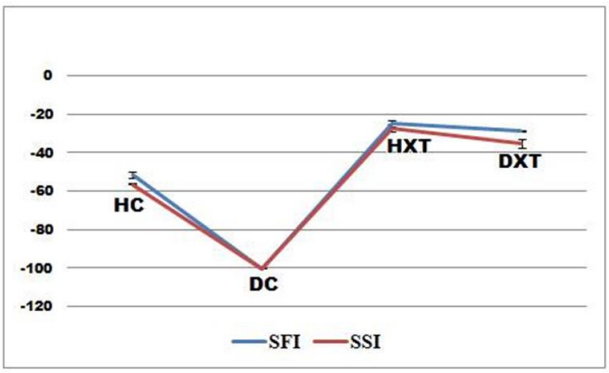

Figure 3 Showing values (Mean ± SD) of Sciatic Functional Index (SFI) and Sciatic Static Index (SSI) in all groups at the end of 3rd week. Note that in diabetic control (DC) group both SFI and SSI values indicate total impairment, in healthy control (HC) these values were significantly (P<0.01) negative compared to treated groups.

obtained via tail vein for monitoring glucose level by using Glucometer (Dr. Morepen gluco one) on the 4th day of alloxan injection. Animals with blood glucose level at 250 mg/dl and above were selected as diabetic for this study. Weight and blood glucose levels of all animals in each group were monitored at weekly intervals [13, 14].

Statistical analysis

All the data were statistically evaluated and the significance calculated using One-way 'ANOVA' followed by Tukey's test. Student's *t*-test was used for comparing the initial and final mean body weight of diabetic control (DC) and blood glucose level in diabetic administered (DXT) group before and after treatment. All results were expressed as Mean ± Standard deviation (SD) and P<0.05, in case of ANOVA and P<0.0001, in case of Student's *t*-test was considered as statistically significant.

Results

Body weight and Blood glucose level

During the experimental period, typical clinic manifestations of the diabetes such as polyphagia, polydipsia and polyuria were observed in diabetic control rats while these clinical signs were reduced in diabetic treated groups after three weeks co-administration of d-α-tocopherol and d-δ-TRF. Weight and blood glucose levels of all animals in each group were monitored at weekly intervals. Mean body weight in diabetic control (DC) group showed significant (P < 0.0001) reduction whereas in all other groups it remained stable at the end of study period (Table 1).

Mean blood glucose levels of healthy groups (HC and HXT) remained within normal limits. In diabetic co-administered (DXT) group the mean blood glucose level was significantly (P<0.0001) reduced after three weeks treatment while in DC it remained > 500 mg/dl throughout the experimental period (Table 2).

Gross observations

After sciatic nerve crushed injury, complete paralysis of the right side foot was observed in all rats (Figure 1). Since autotomy is commonly seen to begin with the nibbling of toenails, this was prevented by application of anti-nail-bite substance on the experiment side in those who showed tendency to bite. Thus none of the rats had frank autotomy or nibbling of toenails, edema, infection or ulceration on the foot [11, 12].

Functional analysis

On completion of 3rd week the better footprints were observed in treated groups compared to control groups (Figure 2).

Both SFI and SSI mean values in diabetic control (DC) showed total impairment while in healthy control (HC) values were significantly (P<0.01) negative compared to treated groups (Figure 3).

Microscopic observations at the end of 3rd week in Longitudinal Sections

1. Degenerating changes and Fibrosis

The control groups showed increased vacuolization of nerve sheath and numerous atrophic fibers with macrophages and degenerative debris whereas the treated groups had decreased vacuolization of nerve sheath and few atrophic fibers with macrophages and little degenera-

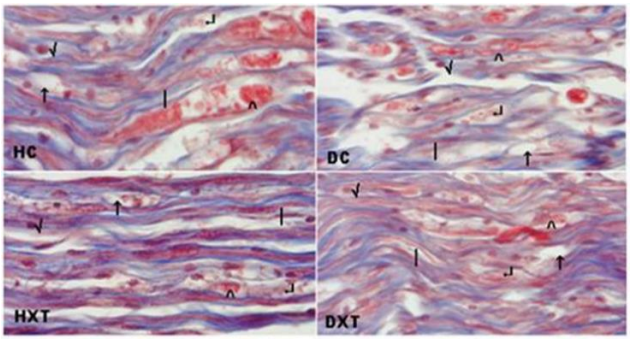

Figure 4 Representative images from all groups on completion of 3rd week showing Vacuolization (↑), Lipid droplets (↵), Debris (^), Macrophages (√) and Regenerated nerve fibres (|, red colour). Stain: Masson's Trichrome, initial magnification x1000.

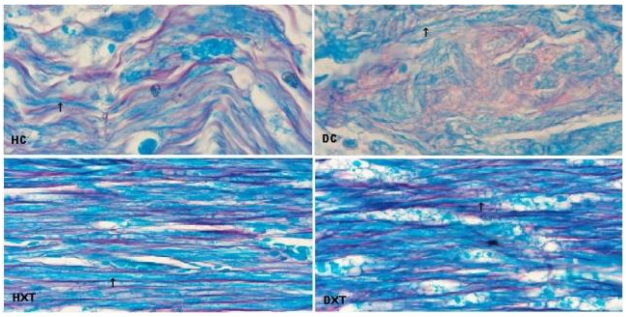

Figure 5 Representative images from all groups at the end of 3rd week showing nerve fibres (↑) and arrangements of collagen fibres (red colour). Stain: Luxol Fast Blue with PicroSirus Red, initial magnification x1000.

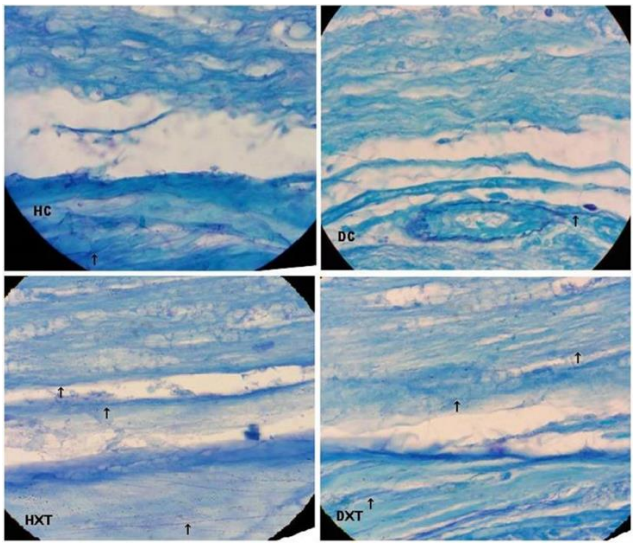

Figure 6 Representative images from all groups on completion of 3rd week showing Elastin fibres (↑, violet colour). Stain: Aldehyde Fuchsin with Fast green, initial magnification x1000.

tive debris (Figure 4).

More collagen fibers were observed in healthy control (HC) and these fibers were disorganized in diabetic control (DC) whereas in treated groups these fibers were few but more organized (Figure 5).

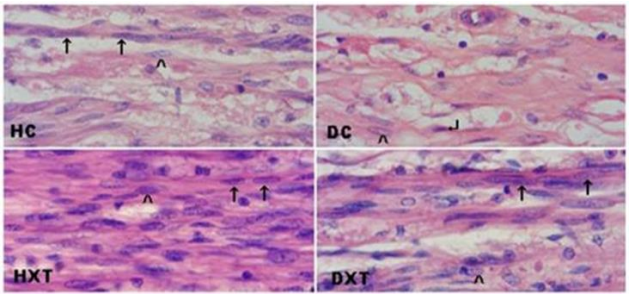

Figure 7 Representative images of all groups at the end of 3rd week showing Bands of Bungner (↑), Proliferated fibroblasts (^) and in diabetic control only Proliferated Schwann cells (↵). Stain: Haematoxylin and Eosin, initial magnification x 1000.

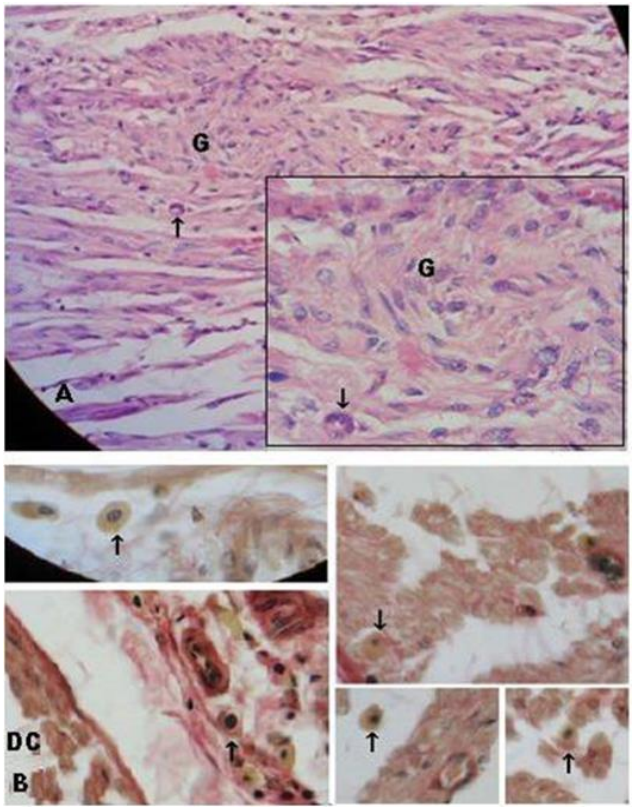

Figure 8 Representative images of Diabetic control group on completion of 3rd week showing **A**: Multinucleated giant cell (↑), G: Granuloma, **B**: Inflammatory cells (↑). Haematoxylin and Eosin stained longitudinal sections at initial magnification x400, inset image of **A** and Verhoeff van Gieson stained transverse sections at x1000 of initial magnifications.

2. Regenerating changes

a. Reappearance of Elastin fibers

The control groups revealed only few elastin fibers in the epineurium and absence of these fibers in the other connective tissue coverings whereas in treated groups these fibers were obvious in all three connective tissue coverings (Figure 6).

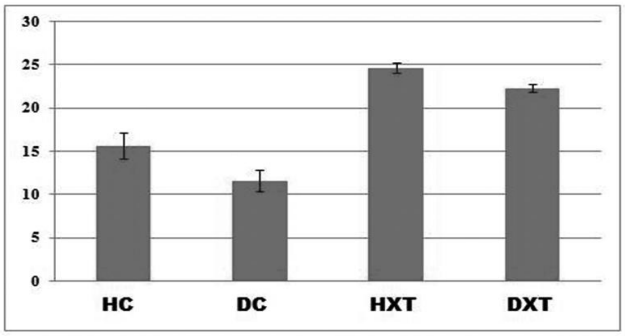

Figure 9 Number (Mean ± SD) of blood capillaries in transverse sections of all groups the end of 3rd week. Note that in control groups number of blood capillaries were significantly (P<0.01) less as compared to all treated groups.

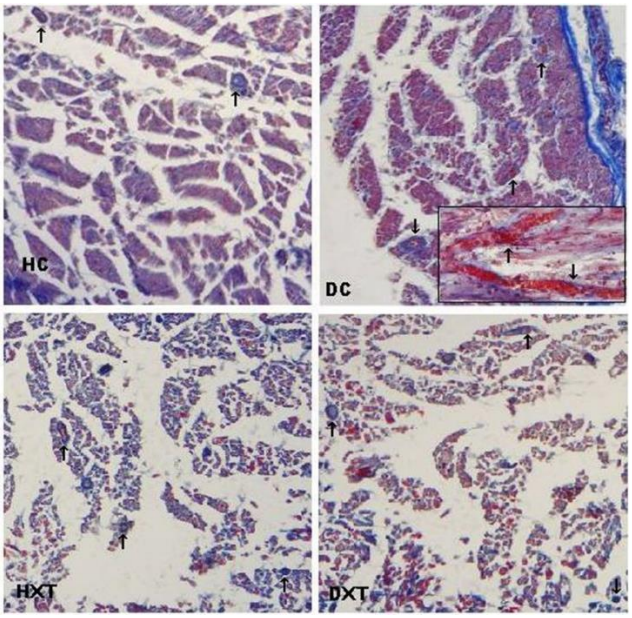

Figure 10 Representative images of all groups on completion of 3rd week showing blood capillaries (↑). Stain: Masson's Trichrome, initial magnification of transverse sections at x200 and longitudinal section at x400 (inset) in diabetic control group (DC).

b. Cellularity

The healthy control group showed moderate degree of infiltration of inflammatory cells and had only few bands of Bungner whereas the treated groups had only mild degree of inflammatory cells but more bands of Bunger (Figure 7). In diabetic control group bands of Bungner was deficient but they had few Schwann cells' proliferations (Figure 7). In addition to above features diabetic control group had also more inflammatory cells especially around capillaries and presence of multinucleated giant cells with granuloma formation (Figure 8). In general the proliferated fibroblasts were more in treated groups than control groups (Figure 7).

c. Regenerated nerve fibers

The healthy control had less number of thin non-myelinated nerves whereas the diabetic control had few short distance running neurofibrils. Presence of long distance running both myelinated and nonmyelinated nerve fibres were noticed only in treated groups (Figures 4 and 5).

Histomorphology

Neovascularization

On completion of 3rd week in control groups transverse sections the number of capillaries were significantly (P<0.01) less compared to treated groups (Figure 9). Endoneurial arteriolar walls were thicker in diabetic control group compared to all other groups (Figure 10).

Biochemical analyses at end of 3rd week

a. Enzymatic antioxidant and oxidative stress parameter

Serum catalase activity and total antioxidant capacity in treated groups were significantly higher ($P < 0.01$, $P < 0.05$) compared to control groups. These analyses values in DC were significantly lower ($P < 0.05$) compared to healthy control group (Table 3).

Discussion

In hyperglycemia enhanced generation of reactive oxygen species (ROS) is one of the known reasons of neuronal damage which leads to the development of diabetic neuropathy [1, 19 and 20]. Therefore, antioxidants administration may be potentially attractive as clinically applicable neuroprotective agents against such oxidative stress [3]. Beneficial effect of vitamin E supplementation has also been shown in diabetic neuropathy [21].

In the present study, in diabetic control group the mean body weight was significantly reduced at the end of experimental period. Reduction of body weight in diabetes is considered mainly due to the progressive muscle wasting and breakdown of tissue proteins [22]. After three weeks supplementation of vitamin E isoforms the diabetic co-administered group showed stable body weight with respect to their initial body weight. Vitamin E has also anti-hyperglycemic effect thereby it maintains the body weight in diabetic treated animals [23]. Another related four week study [24] reported that, diabetic group without tocotrienol rich fraction (TRF) supplementation showed significantly lower body weight than that of diabetic rat treated with TRF.

Mean blood glucose level was reduced in diabetic treated group after three week oral co-administration of d-α-tocopherol and d-δ-TRF but in diabetic control group showed hyperglycemic state throughout the study period. It has earlier been shown [25] that d-δ-TRF has potency to maintain the glycemic level in diabetes. This result is in agreement with other study [26] reporting that tocotrienol supplementation significantly increases the insulin levels and reduces the blood glucose in diabetic induced rats in a dose dependent manner.

Crush injury induces axonotmesis which causes severe

sensorimotor impairments and functional disabilities [27]. Nerve regeneration and functional recovery after peripheral nerve injury even today remains a clinical challenge [28]. Evaluation of functional recovery after injury is essential in assessing the nerve regeneration [29, 30], axonal reinnervation and restitution of the nerve-muscle interaction [31]. Methods used in this present study to evaluate the functional recovery of sciatic nerve after crushed injury are Sciatic Functional Index (SFI) and Sciatic Static Index (SSI). The SFI is a non-invasive method to assess the overall functional recovery of the sciatic nerve during the regeneration process because proper walking requires coordinated function involving sensory input, motor response and cortical integration [32, 33]. At the end of 3rd week better and assessable hind limb foot prints were recorded in treated groups than healthy control group. These prints were not measurable in diabetic control group. The SSI is an effective and accurate method for the assessment of the functional recovery after sciatic nerve injury in rats [16].

In the current study better indices values in treated groups support the faster functional recovery as compared to healthy control. Increased axonal repair at injured area is more likely to enhance the successful functional recovery [34]. But in diabetic control group these values indicate total impairment and the functional motor recovery is slower in the presence of persistent hyperglycemia [35].

Commonly the histological parameters are the predictors of peripheral nerve damage and regeneration [36, 37]. On 3rd week healthy control showed moderate degenerating changes whereas in diabetic control presence of numerous atrophic fibers with histiocytes and increased vacuolization of nerve sheath indicates that the Wallerian degeneration which is prerequisite for nerve regeneration is delayed in experimental diabetic rats [38-40]. Another study [41] revealed that in Wallerian degeneration of optic nerve of rabbit the degenerative debris was only partly removed even on 3rd month. The treated showed faster removal of debris thus providing appropriate environment for regeneration thereby also reducing the diabetic neuropathic complications.

The control groups showed more deposition of collagen fibers which is an indicator of more fibrosis [42] and these fibers were arranged in a disorganized manner in diabetic control. Over all reduced fibrosis and organized collagen fibers in treated groups was apparent as a result of combined effect of d-α-tocopherol and d-δ-TRF for three weeks. After three weeks supplementation of these isoforms the treated groups showed only minimal and organized collagen fibers, which maintains the structural and functional integrity of the nerves [43].

The viscoelastic properties of the peripheral nerve are due to its connective tissue supporting elements like elastin and collagen [44]. Elastin fibers are present in epineurium consisting of thick and thin fibers, perineurium with thicker band of fibers and endoneurium with thinner fibers [45]. The control groups revealed few elastin fibers only in the epineurium but in treated groups these fibers were obviously seen in all three connective tissue coverings. Presence of endoneurial elastin fibers in treated groups may provide sufficient force to impart the wavy appearance of the individual axons within the fascicle [45].

During axonal degeneration Schwann cells proliferate and dedifferentiate. Proliferations were induced by the loss of axonal contact and also stimulation by macrophages releasing growth factors [46, 47]. This dedifferentiated Schwann cells align as longitudinal columns inside the basal lamina forming bands of Bunger [48, 49]. In this present study bands of Bungner were deficient in the diabetic control and they contained only few proliferated Schwann cells and fibroblasts. But treated groups showed presence of numerous bands of Bungner and more proliferated fibroblasts than healthy control group. These bands of Bungner provide supportive environment and guide for successful axonal regeneration [47].

More infiltration of inflammatory cells was seen in control groups. In addition to above, presence of multinucleated giant cells with granuloma formation were noticed in diabetic control group. Therefore, in control groups severe immune response appears to worsen the Wallerian degeneration and consequently impair the repair and regeneration [50, 51]. Reduced inflammatory responses noticed in treated groups were possibly due to the anti-inflammatory effect of vitamin E [52].

During axonal regeneration sprouts (neurofibrils) arise from remaining part of axon that moves distally along the endoneurial tube within the basal lamina [48, 53]. In healthy control group less number of thin newly regenerated nerve fibers was noticed whereas the diabetic control showed few thin short distance running neurofibrils, indicating a partial regeneration of the nerve fibers [42]. The treated groups showed presence of thin nonmyelinated and myelinated nerve fibers. This finding is correlates with many other previous studies [21, 54 and 55] which reported the beneficial effects of vitamin E supplementation in diabetic neuropathy, sensory neuronal loss and sciatic nerve regeneration after nerve crush. The newly regenerated fibers appear to be thinner due to remodeling [56] and these fibers initially lack myelin even when the parent axon is a myelinated fiber. With time, these unmyelinated fibers will become thick and myelinated [57].

Most of the regeneration and re-establishment of normal tissue architecture during healing occurs by vessel pruning [58]. In diabetic neuropathy the thickening of arteriolar endoneurium is due to increased deposition of basement membrane material [59], these features were observed in diabetic control. Treated groups had more numbers of capillaries than control groups. Changes in capillary number and permeability and over all increased vascularization enhance successful axonal regeneration [60]. Tocotrienols are promising anticancer agent for minimizing tumor angiogenesis, tocopherol did not inhibit angiogenesis [61]. Another study [62] stated that treatment of Tocomin 50 (tocotrienol-rich oil) did not show any negative effects in preexisting vessels. In our previous studies [11, 12] number of capillaries was significantly more in d-δ-TRF administered groups than d-α-tocopherol treated groups. Some other studies [63, 64] reported that

the anticancer agents like vasostatin and endostatin may not have any inhibitory effect on new vessel formation but possibly induce vessel maturation.

Catalase is a preventive antioxidant which inhibits the initial production of free radicals and removes the excess H_2O_2 [65]. The present study showed that serum catalase activity value was lower in diabetic control group which is in agreement with other studies [66, 67]. This activity was normalizing in control group after vitamin E treatment [67].The three weeks co-administration of d-α-tocopherol and d-δ-TRF helped to increase the serum catalase activity in treated groups [25]. The antioxidant capacity of plasma is the primary measure and marker to evaluate the status and potential of oxidative stress in the body [68].The present work observed that serum total antioxidant level in diabetic control was significantly lower ($P<0.05$) compared to healthy control which is in agreement with the findings of other study [69]. Improved serum antioxidant capacity was observed in treated groups by co-administration of d-α-tocopherol and d-δ-TRF for three weeks as reported earlier [25].

Conclusion

Based on the findings of the present study it is concluded that co-administration of d-α-tocopherol and d-δ-TRF synergistically enhances the antioxidant level, maintains the glycemic status, accelerate neovascularization, regeneration, remyelination and matrix remodeling in crush-injured sciatic nerve. Hence these vitamin E isoforms appear to be quite potent nutritional option in the management of the damaged peripheral nerve of both healthy and diabetics.

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Conflict of Interest

We declared that there is no conflict of interests

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