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NEUROPROTECTIVE EFFECTS OF d-δ-TOCOTRIENOL RICH FRACTION ON CRUSHED SCIATIC NERVE IN DIABETIC RATS

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ABSTRACT

Background: Peripheral neuropathy in diabetes is believed to be due to the reduced blood flow, altered antioxidant defense mechanism and glycemic status. **Aims:** Since vitamin E is an antioxidant and its potent neuroprotective form is tocotrienols, the current study was designed to explore the neuroprotective effects of d-δ-tocotrienol rich fraction (d-δ-TRF) on sciatic nerve regeneration not only in healthy but also in diabetic rats. **Methods and Material:** Diabetes was induced through single subcutaneous injection of alloxan (100 mg/kg). Twenty four albino rats included in the study were divided into four groups; healthy control, diabetic control, healthy treated and diabetic treated. Treated groups were administered d-δ-TRF orally and daily at the dose of 200 mg/kg for three weeks. Right sciatic nerve was approached through mid-thigh skin incision and was crushed with Kocher's forceps. Functional, histopathological, histomorphological and biochemical parameters were analyzed on 3rd week. **Statistical analysis:** One way 'ANOVA' followed by Tukeys test and Student t test were used for statistical significance. **Results:** It was noticed that d-δ-TRF significantly improves the antioxidant level, glycemic status and revascularization. It also accelerates regeneration and remyelination of nerve and reorganization of extracellular matrix in both healthy and diabetic rats. **Conclusions:** It is concluded that the d-δ-TRF is a strong antioxidant and antidiabetic dietary supplement in the management of crushed nerve injury in diabetics.

KEYWORDS: Antioxidant, d-δ-tocotrienol rich fraction, Diabetes, Rats, Sciatic nerve.

INTRODUCTION

In hyperglycemia enhanced generation of reactive oxygen species (ROS) is believed to cause neuronal damage which leads to the development of diabetic neuropathy.[1-3] Antioxidants administration may be potentially attractive as clinically applicable neuroprotective agents against such oxidative stress.[4] Beneficial effect of vitamin E supplementation has also been shown in diabetic neuropathy.[5] More lately rats[6, 7] it has also been shown that the use of antioxidant vitamin C or steroids is either reduces the post- injury nerve dysfunction or improves nerve regeneration in healthy sciatic nerve crushed. Therefore, it appears logical a suitable treatment protocol for treating of diabetes and its complications including neuropathies must include certain agents which have both antioxidant and antihyperglycemic properties.[8] In addition, presently there is no individual treatment that has proven to have both antioxidant and blood flow enhancing effect in diabetic neuropathy.[1]

At present informations regarding the effectiveness specifically that of tocotrienol isoforms' on the healthy and diabetic peripheral nerve regeneration are scarce and also there is dearth of data related to any single agent having multiple properties to counteract diabetic complications. Hence the present study was attempted to explore the properties of d-δ-TRF on peripheral nerve repair in both healthy and diabetic rats by using functional, histopathological, histomorphological and biochemical parameters.

MATERIALS AND METHODS

After approval of the project from the Institutional Animal Ethical Committee (No. 8937/2014), twenty four albino rats of either sex each weighing 230-320g were obtained from central animal house of JN medical college, AMU, Aligarh. This present study followed the same method as described in previously regarding animal care, induction of diabetes and monitoring of blood sugar level.[9]

Experimental groups and route of treatment

Animals were divided into four groups having six rats in each group: (1) healthy control- HC; (2) diabetic control- DC; (3) healthy d-δ-TRF treated- HTT and (4) diabetic d-δ-TRF treated- DTT. The treatment group of animals received d-δ-TRF (200mg/kg body weight, orally, daily) for three weeks. The d-δ-TRF (Unique E Tocotrienol, tocopherol free) obtained from AC Grace Company, P.O

Box 570, Big Sandy, TX 75755, USA) consisted of, 90% δ and 10% γ tocotrienols. In order to ensure the ingestion of desired oral dose of tocotrienol, the oil-based d-δ-TRF capsule ready for human consumption was puncture and squeezed gently to get a drop formed (one drop ~30mg weight) which was directly touch on the rat's tongue which followed by standard rat diet (Laboratory animal feeds; Ashirwad Industries, 1544-Sector 38B, Chandigarh-10036, India).

Surgical procedure

All experimental animals received ether general anesthesia. Horizontal skin incision was made on the shaved right mid-thigh area. Muscles were retracted to gain access to the sciatic nerve. Kocher's forceps was used to induce crushed injuries on the sciatic nerve proximal to its division. The nerve was replaced, muscles were re-approximated and skin incision were closed with 3-0 Vicryl (2metric-NW2401)-absorbable suture USP (synthetic; braided coated polyglactin 910 violet; from Ethicon, manufactured in India by Johnson and Johnson Ltd, Aurangabad).[10] Povidone-iodine solution (antisepsis) was applied on the wound and 0.5 ml Voveran (analgesic) and 2 mg single shot of Gentamycin (antibiotic) were also injected simultaneously.[9]

Functional Evaluation of Sciatic Nerve

Foot print analysis

Footprint analysis was performed once a week, all experimental animals' hind feet were dipped in an ink solution and they were permitted to walk down the track upon a strip of white paper. The prints by the ink were left to dry. These foot prints were used to calculate the Sciatic Function Index (SFI) and the Sciatic Static Index (SSI).[10]

Sciatic functional index (SFI) - Calculation is based on the method described elsewhere.[11] The lengths of the third toe to its heel (PL), the first to the fifth toe (TS) and the second toe to the fourth toe (IT) were measured on the experimental side (E) and the opposite normal side (N) in each rat. SFI of each animal was calculated by the following formula:

SFI = [(-38.3× PLF) + (109.5× TSF) + (13.3× ITSF)] - 8.8

Wherein different factors were calculated as under:

PLF = (EPL-NPL)/NPL

TSF = (ETS-NTS)/NTS

ITSF = (EIT-NIT)/NIT

Sciatic static index (SSI) - Calculation is based on method described[12] by using the static factors, not considering the print length factor (PL), according to the equation:

SSI = [(108.44 × TSF) + (31.85 × ITSF)] - 5.49

In both SFI and SSI, an index score of zero was considered normal and an index of −100 indicated total impairment. When no footprints were measurable, the index score of −100 was given.

Sample collection and Fixation of tissue

On completion of three weeks animals were sacrificed under deep ether anesthesia and then the sciatic nerve was excised and immersion-fixed in 10% neutral buffered formalin. To assay the biochemical parameters, the method of serum preparation were followed as described in our previous study.[9]

Gross examination

Daily observation was made to check for any autotomy or nibbling of toenails, edema, infections and ulcerations on the nerve crushed hind limb.

Histopathology and Histomorphometry

Fixed nerves samples were processed for light microscopic studies. The 5µm thick paraffin sections were stained with Haematoxylin & Eosin (H & E), Masson's Trichrome (MT), Aldehyde Fuchsin with Fast Green (AF with FG), Luxol Fast Blue with PicroSirus Red (LFB with PSR), Periodic Acid Schiff with Haematoxylin (PASH) and Verhoeff Van Gieson (VVG).

In histomorphometry single blind experiment was performed for counting the number of capillaries on transverse sections of nerves stained with H & E, MT and VVG.

Biochemical Estimation & Analysis

a. All lipid profiles and serum creatinine levels were carried out by using Avantor Benesphera™ clinical chemistry Analyzer C61.

b. Enzymatic antioxidant

Serum catalase was assayed by colorimetery as described earlier.[13] The light absorbance of the sample was determined at 620 nm.

c. Non-invasive biomarker (oxidative stress parameter)

Serum total antioxidant capacity (TAC) was evaluated using ferric reducing antioxidant power (FRAP) assay.[14] The absorbance of sample was measured at 620 nm using photo colorimeter.

Statistical Analysis

All the data were statistically evaluated and the significance calculated using one way 'ANOVA' followed by Tukeys test. Student t test were used for comparing the initial and final mean body weight of DC and blood sugar level in DTT group before and after supplementation of d-δ-TRF. All the results were expressed as mean ± SD and $P<0.05$ and $P< 0.0001$ were considered as statistically significant.

RESULTS

Gross observations

After sciatic nerve crushed injury, complete paralysis of the right side foot was observed in all rats (Figure 1). Since autotomy starts 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 autotomy or nibbling of toenails, edema, infection or ulceration on the foot.[10]

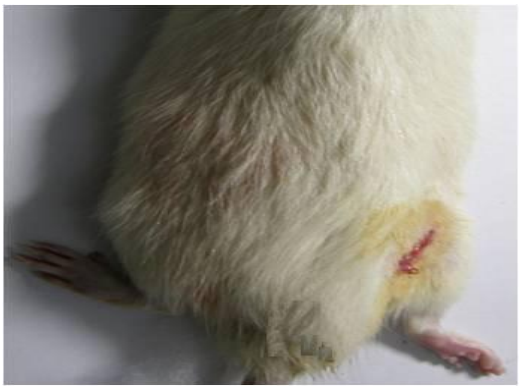

Figure 1: Showing complete paralysis of right foot after sciatic nerve crushed injury

Body weight and Blood sugar 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 markedly reduced after three weeks supplementation of d-δ-TRF in diabetic treated groups.

Body weight and blood sugar levels of all animals in each group were monitored at weekly intervals. At the end of study period mean body weight in DC significantly (P<0.0001) reduced as compared to their initial body weight whereas in all other groups mean body weight remained stable (Table 1).

Table 1: Body weights (g) of the animals of all groups during the period of study (Mean ±SD)

Groups	Day 0	Day 7	Day 14	Day 21
HC	270 ± 35.59	266.67 ± 15.28	283.33 ± 20.82	290 ± 21.60
DC	277.5 ± 25	247.5 ± 17.08	235 ± 23.80	227.5 ± 22.17
HTT	270 ± 26.46	266 ± 14.26	272.5 ± 17.07	285 ± 20.82
DTT	271.25 ±20.97	251.25 ± 20.5	265 ± 23.80	275 ± 20.82

Note the mean body weight significantly (P<0.0001) reduced in DC at the end of study period while body weight of all other groups remained stable.

Mean blood sugar levels of healthy groups (HC & HTT) remained within normal limits. In DTT the mean blood sugar level was significantly (P<0.0001) reduced after three weeks supplementation of d-δ-TRF while in DC showed > 500 mg/dl throughout the experimental period (Table 2).

Table 2: Blood sugar (mg/dl) of the animals of all groups during the period of study (Mean ±SD)

Groups	Day 0	Day 7	Day 14	Day 21
HC	146 ± 28.21	124 ± 19.98	160.67 ± 18.01	167 ± 17.06
DC	540.25 ± 47.12	553 ± 39.42	574.25 ± 30.20	578 ± 34.73
HTT	150 ± 35.36	170 ± 30.53	117.5 ± 21.92	134.67 ± 29.69
DTT	574.71 ± 33.74	406.25 ± 1.31	286.75 ± 19.96	195 ± 31.10

Note that the mean blood sugar levels of healthy groups (HC & HTT) remained within normal limits. In DTT group the mean blood glucose level was significantly (P<0.0001) reduced after three weeks treatment while in DC showed > 500 mg/dl throughout the experimental period.

Functional analysis

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

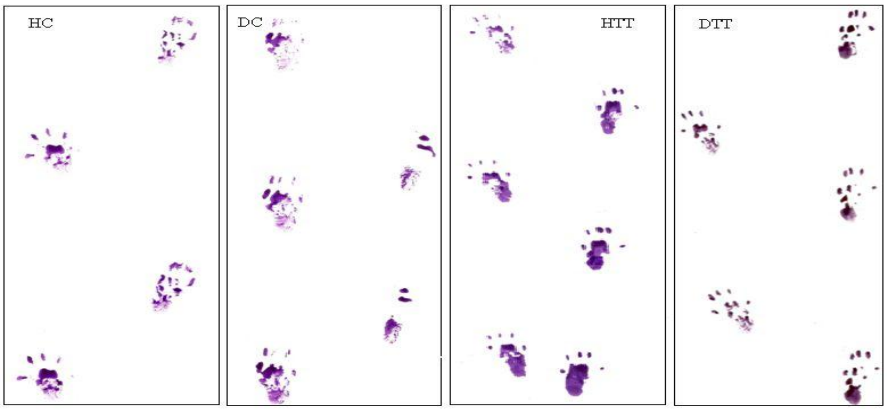

Figure 2: showing foot prints of all groups on 3rd week

Both SFI and SSI mean values in DC showed total impairment, in HC these values were significantly ($P<0.01$) negative compared to treated groups (Figure 3).

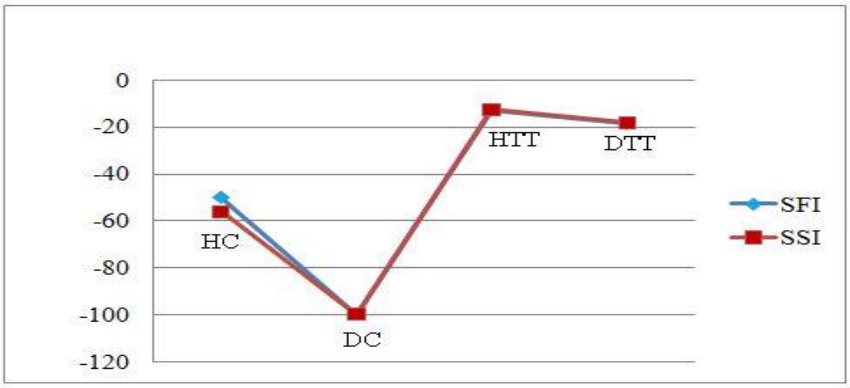

Figure 3: showing both SFI and SSI mean values of all groups on 3rd week

Microscopic observations on 3rd week

1. Degenerating changes and Fibrosis

The control groups revealed marked vacuolization of nerve sheath and numerous atrophic fibres with histiocytes and degenerative debris whereas the nerves in treated groups were associated with lesser vacuolization of nerve sheath and fewer atrophic fibres with histiocytes and little degenerative debris (Figure 4).

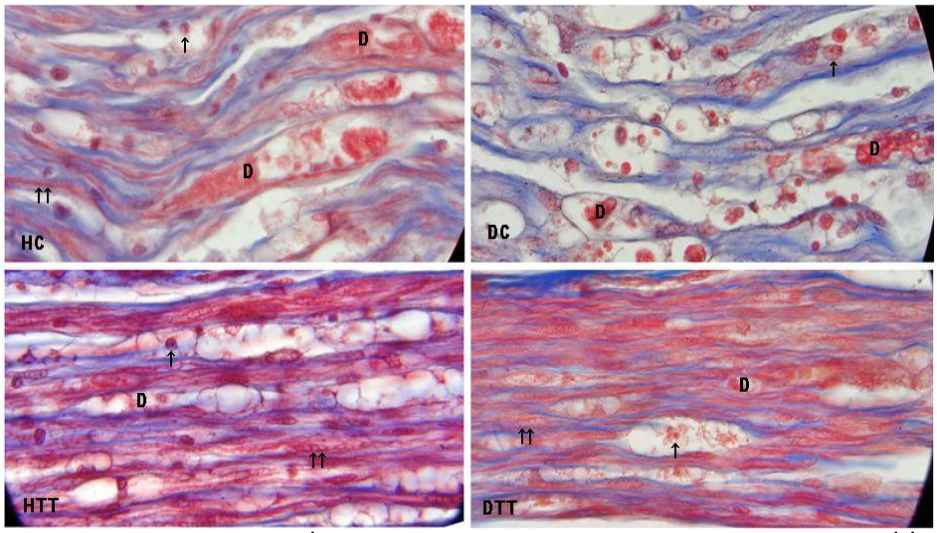

Figure 4: MT stained sections showing ↑- vacuolization of nerve sheath with histiocytes,↑↑- nonmyelinated nerve fibres in HC and myelinated nerve fibres in treated groups. D- Degenerative debris, at initial magnification x1000.

More collagen fibres were observed in HC, these fibres were disorganized in DC whereas they were well organized and fewer in the treated groups (Figure 5).

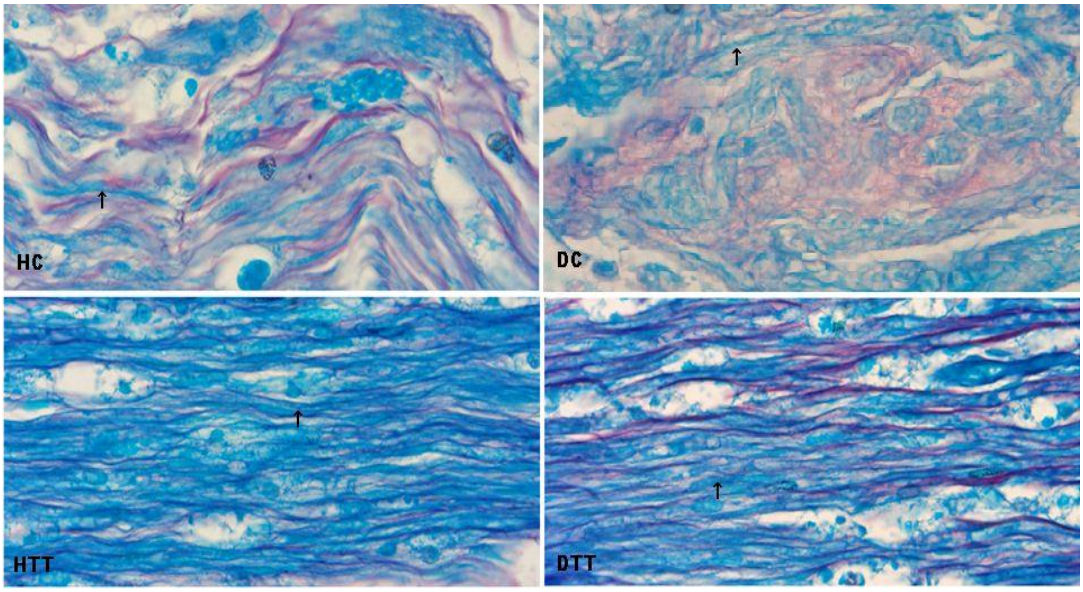

Figure 5: LFB with PSR stained sections showing arrangements of Collagen fibres (Red colour) and arrow (↑) pointing the nerve fibres at initial magnification x1000.

2. Regenerating changes

a. Reappearance of Elastin fibres

It was noticed that the control groups had only few elastin fibres in the epineurium and absence of these fibres in the other connective tissue coverings whereas in treated groups these fibres were well developed and noticed in all three connective tissue coverings (Figure 6).

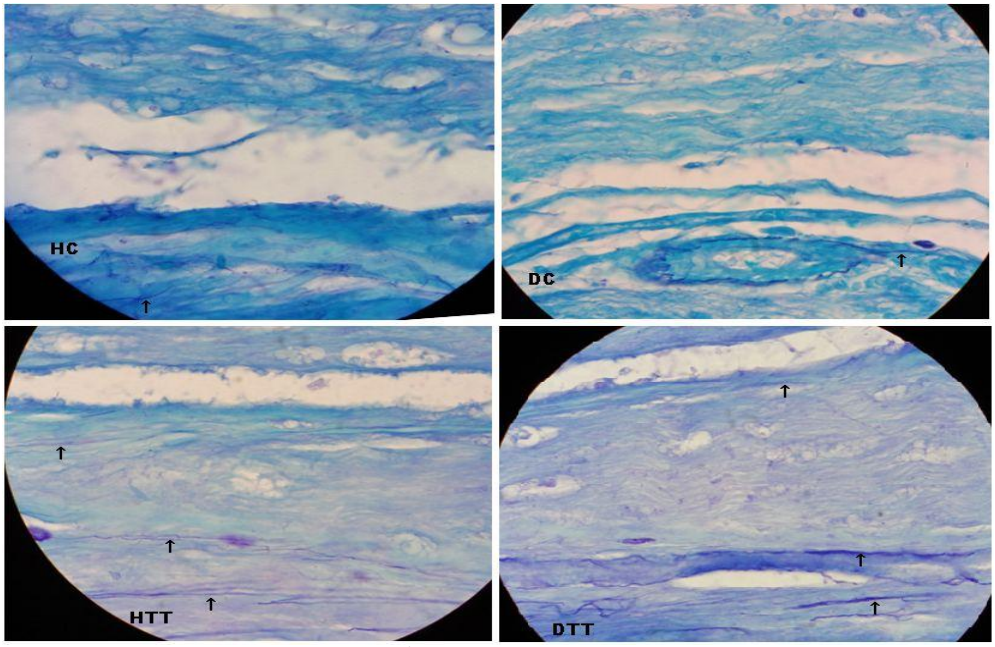

Figure 6: AF with FG stained sections, arrow (↑) pointing Elastin fibres (Violet colour) at initial magnification x1000.

b. Cellularity

The HC were associated with moderate degree of infiltration of inflammatory cells and had only fewer bands of Bungner (Figure 7). In DC bands of Bungner were deficient (Figure 7) and there was marked infiltration of inflammatory cells especially around capillaries and even multinucleated giant cells with granuloma also made their presence (Figure 8 A & B). Mild degree of inflammatory cells, marked proliferation of fibroblasts cells and numerous bands of Bunger were observed in treated groups (Figure 7).

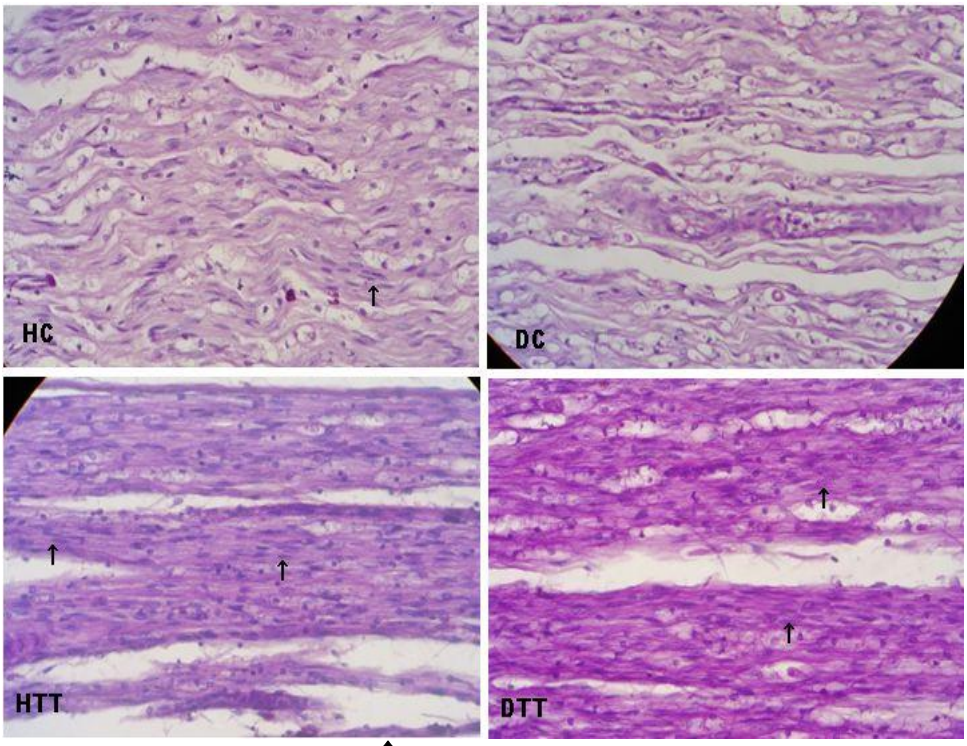

Figure 7: PASH stained sections, arrow (↑) pointing Bands of Bungner at initial magnification x400.

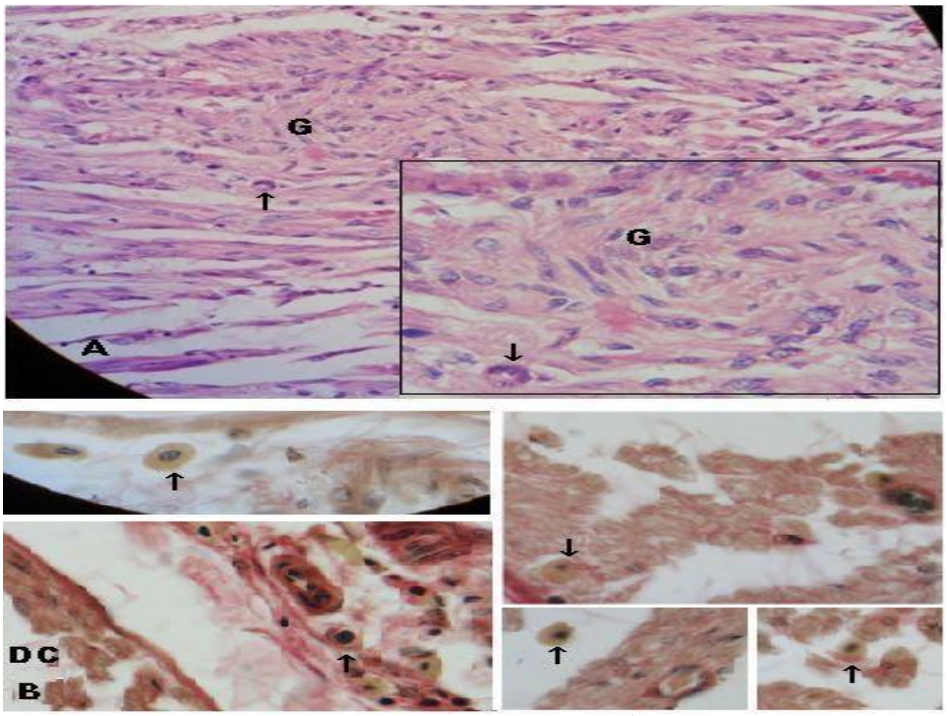

Figure 8: A: H&E stained Longitudinal Sections of DC, arrow (↑) pointing multinucleated giant cell, G: Granuloma at initial magnification x400 and x1000 (inset). B: VVG stained Transverse Sections (↑) pointing inflammatory cells at initial magnification x1000.

c. Regenerated nerve fibres

The HC had less number of thin nonmyelinated nerves whereas in DC had fewer nerve fibres that too running for very short distance. Numerous myelinated and nonmyelinated nerve fibres running for longer distance were noticed in treated groups (Figure 4 & 5).

Histomorphometry

Neovascularization

The counting performed in the transverse section of the nerves on 3rd week revealed that in control groups the mean values of number of capillaries were significantly ($P<0.01$) less compared to treated groups (Figure 9).

Endoneurial arterioles' walls were thicker in DC compared to all other groups (Figure 10).

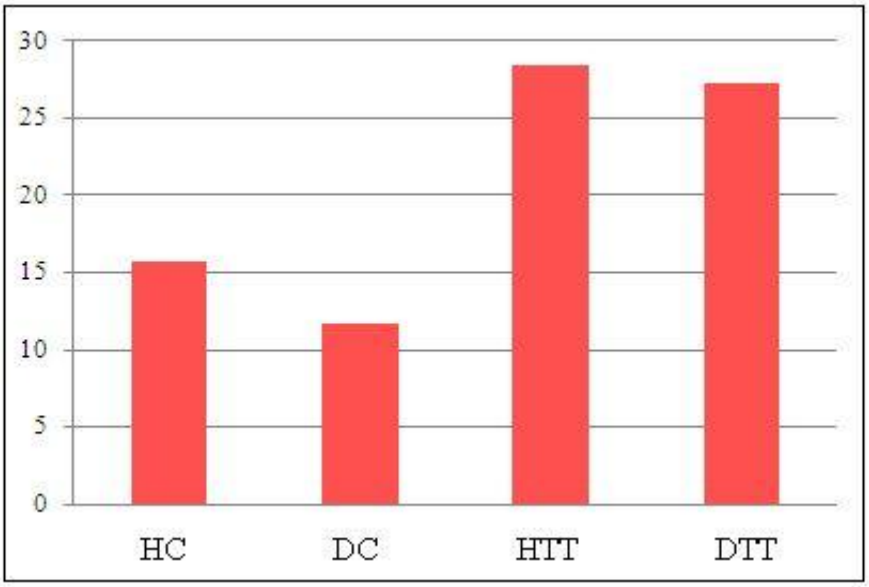

Figure 9: Mean values of number of blood capillaries on 3rd week transverse sections

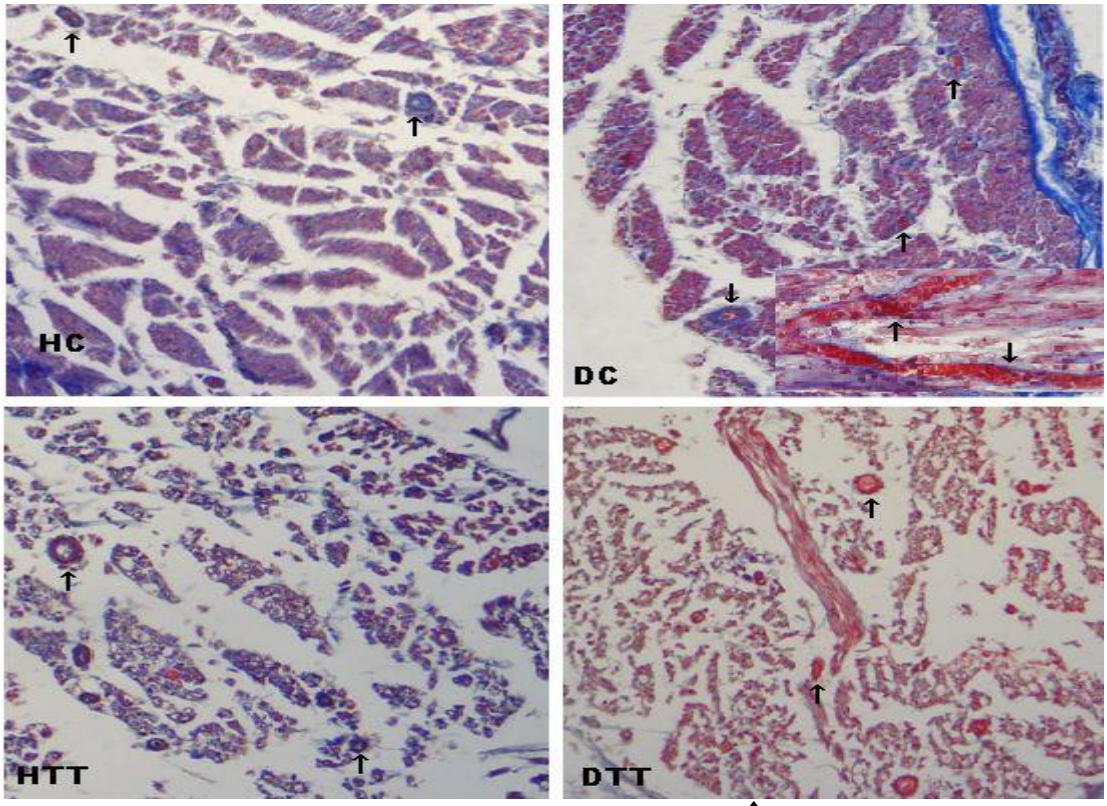

Figure 10: MT stained Transverse and Longitudinal sections, arrow (↑) pointing blood capillaries at initial magnification x200 and x400 in DC Longitudinal section (inset).

Biochemical analysis on 3rd week

a. Lipid profiles

Total cholesterol (TC), triglycerides (TG), low density lipoprotein (LDL) and very low density lipoprotein (VLDL) in DC were significantly higher ((P<0.01) compared to other groups. The high density lipoprotein (HDL) in DC showed significantly lower (P<0.01) values as compared to all other groups (Table 3).

Table 3: Effects of d-δ-TRF supplementation on Lipid profiles (Mean ± SD)

Lipid profiles					
Groups	**Total cholesterol (mg/dl)**	**HDL (mg/dl)**	**LDL (mg/dl)**	**VLDL (mg/dl)**	**Triglycerides (mg/dl)**
HC	45.66 ± 0.83	15.28 ± 0.22	13.56 ± 0.19	16.82 ± 0.42	101.75 ± 4.60
DC	54.30 ± 1.19	11.05 ± 0.21	18.02 ± 0.09	25.23 ± 0.89	171.15 ± 11.53
HTT	45.82 ± 0.42	17.08 ± 0.10	12.84 ± 0.11	15.90 ± 0.21	101.25 ± 1.48
DTT	46.46 ± 0.29	16.98 ± 0.16	13.15 ± 0.05	16.33 ± 0.08	110.6 ± 0.42

Note that in DC mean values of TC, TG, LDL, VLDL were significantly higher (P<0.01) and HDL significantly lower (P<0.01) compared to all groups.

b. Serum creatinine level

Serum creatinine level in DC were significantly higher (P<0.01) compared to all other groups (Table 4).

c. Enzymatic antioxidant and oxidative stress parameter

The serum catalase activity and total antioxidant capacity (TAC) in treated groups exhibited significantly higher values (P<0.01, P<0.05) compared to control groups. The aforesaid activity and capacity showed significantly lower values (P<0.05) in DC as compared to HC group (Table 4).

Table 4: Effects of d-δ-TRF supplementation on biochemical parameters (Mean ± SD)

Serum Analyses				
Groups	**Creatinine (mg/dl)**	**Total protein (g/dl)**	**Catalase (u/ml)***	**TAC (μmol/L)**
HC	0.425 ± 0.010	5.05 ± 0.07	0.0672 ± 0.004	1285.5 ± 67.18
DC	0.790 ± 0.022	4.5 ± 0.14	0.0438 ± 0.005	1000 ± 67.88
HTT	0.430 ± 0.013	5.85 ± 0.08	0.156 ± 0.006	2063.3 ± 72.89
DTT	0.441 ± 0.020	5.5 ± 0.01	0.105 ± 0.007	1888.3 ± 72.78

Note that all biochemical parameters reveal significantly less in DC compared to all other groups (P<0.05). Catalase (u/ml)*u-μmols of H_2O_2 utilised/mt.

DISCUSSION

Long standing hyperglycemia, oxidative stress and vascular impairment in diabetes are believed to be causative factors in the development of peripheral neuropathy.[1] The antioxidant treatments are promising therapeutics adjuvant that can prevent or correct reduced motor and sensory nerve conduction velocity in diabetic rats.[15, 16] Vitamin E has a central role in maintaining neurological structure and function. Tocotrienols are naturally occurring potent neuroprotective form of vitamin E and are routinely consumed by humans with no documented adverse effects.[17] The absorption of tocotrienols has been shown to be negligible when administered via intraperitoneal and intramuscular route and incomplete when administrated via the oral route in rats[18, 19] nevertheless its potent antioxidant and neuroprotective effects have also been shown after oral administration by others. Bioavailability of all tocotrienol analogues after oral administration has been shown to markedly increase when taken with food.[20]

The tocotrienol supplementation significantly increases the insulin levels and reduces the blood glucose in diabetic induced rats in a dose dependent manner.[21] The present study also revealed that DC had hyperglycemic state throughout the study period whereas DTT had reduced mean blood sugar level which is in agreement with the previous report[21] thereby reducing the complications of diabetic neuropathy.

Axonotmesis, commonly seen in crush injury, causes severe sensorimotor impairments and functional disabilities.[22] Nerve regeneration and functional recovery after peripheral nerve injury remains a clinical challenge.[23] Methods used in the present study to evaluate the functional recovery of sciatic nerve after crushed injury are Sciatic Functional Index (SFI) and Sciatic Static Index (SSI). The sciatic functional index (SFI) provides a non-invasive method and foundation 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.[24, 25] Sciatic static index (SSI) is an effective and accurate method for the assessment of the functional recovery after sciatic nerve injury in rats.[12] In the present study the mean values of these indices in treated groups were significantly improved complimenting the faster functional recovery whereas HC had more negative values. In DC these values showed total impairment,

these findings indicate that the functional motor recovery is slower in the presence of persistent hyperglycemia.[26]

In general, the histological parameters are good predictors of peripheral nerve damage and regeneration.[27, 28] On 3rd week in HC showed moderate degenerating changes whereas in DC presence of numerous atrophic fibres with histiocytes and increased vacuolization of nerve sheath indicated that the Wallerian degeneration is prerequisite for nerve regeneration, which is impaired in experimental diabetic rats.[29-31] Another study[32] revealed that in Wallerian degeneration of optic nerve the degenerative debris was only partly removed even on 3rd month. But the treated groups of the present study most of the debris was removed on 3rd week indicating that the supplementation of d-δ-TRF hastens debris removal and creates a suitable environment for nerve regeneration.

The control groups showed more deposition of collagen fibres which is an indicator of more fibrosis.[33] and these fibres were more haphazardly laid down in DC. The treated group had lesser amount of collagen but arrangement of such fibres was in a well organized form. The viscoelastic properties of the peripheral nerve are due to its connective tissue supporting elements, elastin and collagen.[34] Elastin fibres are present in epineurium with thick and thin fibres, perineurium with thicker band of fibres and endoneurium with thinner fibres.[35] In the present study the control groups had fewer elastin fibres primarily in the epineurium but in treated groups these fibres were observed in all three connective tissue coverings. The endoneurial elastin fibres may provide sufficient force to produce the wave-like or unstretched position of the individual axons within the fascicle.[35]

The HC group showed moderate degree of inflammatory cells and only few proliferated Schwann cells organized as bands of Bungner.[36] In DC found severe infiltration of inflammatory cells especially around capillaries, presence of multinucleated giant cells with granuloma formation and absence of Schwann cells bands of Bungner. Mild degree of inflammatory cells, marked proliferation of Schwann cells and presence of numerous bands of Bungner were seen in treated groups, which give supportive environment for successful axonal regeneration.[37]

The HC had less number of thin nonmyelinated nerves whereas in DC had fewer nerve fibres that too running for very short distance, indicating a partial regeneration of the nerve fibers.[33] In treated groups presence of numerous nonmyelinated and myelinated nerve fibres running for longer distance were observed and these fibres appear to be thinner due to remodeling.[38] The regenerating units will initially lack myelin even when the parent axon is a myelinated fiber. With time, these unmyelinated fibers will become myelinated.[39]

A prominent finding in diabetic neuropathy is thickening of endoneurial arterioles due to increased deposition of basement membrane material[40], these features were observed in DC. Changes in capillary number and permeability and increased vascularization enhances successful axonal regeneration.[41]

Most of the regeneration and re-establishment of normal tissue architecture during healing occurs by vessel pruning.[42] Tocotrienols have been shown to be a promising anticancer agent for minimizing tumor angiogenesis[43] without having negative effects in preexisting vessels.[44] Thus, sensitivity of malignant tumor growth and physiologic wound healing to inhibition of angiogenesis are different. Vasostatin and endostatin are angiogenic inhibitors but may not have an inhibitory effect on new vessel formation in healing wounds and thus therapeutic inhibition of tumor angiogenesis may be achieved without impairment of tissue repair. Vasostatin induced a higher degree of vessel maturation.[45, 46] The present study has shown significantly less number of capillaries in control groups compared to treated groups which suggesting that the d-δ-TRF may create a suitable environment for re-establishment of vascularization.

The predictors of cardiovascular complications in diabetes are believed to be both dyslipidemia and hyperglycemia.[47-50] The data of the present study indicated that mean values of total cholesterol (TC), triglycerides (TG), low density lipoprotein (LDL) and very low density lipoprotein (VLDL) levels were higher and high density lipoprotein (HDL) level lower in DC, which in conformity with the finding of previous study[51] indicating significant dyslipidemia in untreated diabetic rats. The lower mean values of TC, TG, LDL and VLDL levels and high HDL level were recorded in DTT after three weeks treatments. This result is in agreement with other related study.[52]

The serum creatinine level is known to be a significant marker of diabetic nephropathy. Our result showed the serum creatinine level was higher in DC than all other groups and almost similar observation has been shown in the STZ-induced diabetic rat earlier.[51] In DTT these level were improved after treatment very similar to the level of healthy groups (HC & HTT). The abnormally high level of serum creatinine is usually consistent with the impaired kidney function.[53]

Catalase is a preventive antioxidant which inhibits the initial production of free radicals and removes the excess H_2O_2.[54] The present study showed serum catalase activity value was lower in DC this is in agreement with other studies.[55,56] This activity has been shown to normalize in control group after vitamin E treatment[56] and this is in agreement with three weeks administration of d-δ-TRF in treated groups of the present study. Antioxidant capacity of plasma is the primary measure and marker to evaluate the status and potential of

oxidative stress in the body.[57] The present study it was 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.[58] Improved serum antioxidant capacity was observed in treated groups by supplementation of d-δ-TRF for three weeks.

CONCLUSION

Based on the findings of present study it is concluded that d-δ-TRF enhances the antioxidant level, controls the glycemic status and creates an appropriate environment for early revascularization to accelerate the regeneration, remyelination and extracellular matrix reorganization of crushed sciatic nerve. Hence the d-δ-TRF appears to be a strong contender as a nutritional adjuvant in the management of peripheral nerve crush injuries in both healthy and diabetics.

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Conflict of Interest

We declared that there is no conflict of interests.

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