Co-Administered Vitamin E Isoforms d-α-tocopherol and d-δ-tocotrienol Rich Fraction Promote Regeneration of Skeletal Muscle in Diabetics

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Abstract: In diabetes the structural and functional recovery of skeletal muscle is impaired due to persistent hyperglycemia-induced oxidative stress. Vitamin E is known to be essential antioxidant for maintains the skeletal muscle homeostasis thus preventing oxidative damages. This study is designed to explore the effect of d-α-tocopherol and d-δ-tocotrienol rich fraction (d-δ-TRF) on crushed muscle regeneration in both healthy and diabetic rats. Diabetes was induced through single subcutaneous injection of aqueous 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, daily for three weeks. Through a horizontal mid-thigh skin incision and splitting of the fascia gluteus maximus was approached and crushed with Kocher's forceps. Skin wound was closed with an absorbable suture. The crush-induced degenerative and regenerative changes in the muscle were studied by assessing the histological features, histomorphological measurements and biochemical analyses at the end of 3rd weeks. One- way 'ANOVA' and Student's *t*-test were used for statistical analysis. All results revealed that the vitamin E isoforms have potency to maintain glycemic level, improve the antioxidant capacity and hasten the process of regeneration, revascularization, reinnervation and connective tissue remodeling in skeletal muscle after crush injury. It is therefore, concluded that the vitamin E isoforms are useful nutritional supplements for skeletal muscle functional and structural recovery in both healthy and diabetics.

Keywords: Antioxidant, Crush-Injury, d-α-tocopherol, d-δ-tocotrienol Rich Fraction, Diabetes, Rats, Skeletal Muscle

1. Introduction

Skeletal muscle regenerative capacity involves the number, activation, proliferation and differentiation capacities of satellite cells as well as environment and oxidative stress [1, 2].

Marked muscle atrophy is a characteristic feature of uncontrolled diabetes [3]. Oxidative stress severely impairs plasma membrane repair which is a fundamental cellular activity of the skeletal myocyte and failure to repair of the membrane may result into cell death by necrosis. Vitamin E is required for plasma membrane repair in skeletal myocytes [4, 5] and also it protects the muscle from diabetes-induced oxidative damage in rats [3]. All above-mentioned studies are mainly based on the α-tocopherol isoform.

Another study [1] revealed that only low doses of tocotrienol rich fraction (TRF) promotes the proliferation capacity of the myoblasts but its high doses (> 200 μ g/ml) has negative effect on proliferation capacity and is cytotoxic to the myoblasts. Since TRF contain different vitamin E isoforms among which the γ -tocotrienol is the major constituent [6].

Our previous studies [7, 8] revealed that the individual supplementation of d-α-tocopherol and d-δ-TRF helped to accelerate the skeletal muscle regeneration after crushed injuries. Therefore this present study is designed to assess the effect of co-administration of these compounds on muscle repair in both

healthy and diabetic rats by using functional, histopathological, histomorphological and biochemical parameters.

2. Materials and Methods

After the approval by 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 of animal care, induction of diabetes and monitoring of blood glucose level as described in our previous study [9].

2.1. Experimental Groups, Route and Dosage 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-α-tocopherol and d-δ-TRF treated- HXT and (4) diabetic d-α-tocopherol and d-δ-TRF treated- DXT (100mg/kg of d-α-tocopherol and d-δ-TRF each, orally, daily for three weeks). d-α-Tocopherol Myra e capsule [Vitamin E] manufactured by PT Daya- Baria laboratoria Tbk, Indonesia; Imported and packed by United laboratories, Philippines. Unique E Tocotrienol, Tocopherol free [90% δ and 10% γ tocotrienols] AC Grace Company, TX 75755, USA).

2.2. Surgical Procedure

Surgical procedure (Figure 1), sample collection and fixation of tissues were followed the methods as described in our previous studies [7-9].

Figure 1. *Photograph showing crushed parts of the Gluteus Maximus muscle.*

Note: GM- Gluteus Maximus muscle, SN- Sciatic Nerve and Arrow (→) pointing the crushed parts of GM.

2.3. Histopathology and Histomorphology

Fixed tissue samples were processed for light microscopic studies. The 5µm thick paraffin sections were stained with Haematoxylin & Eosin (H & E), Aldehyde Fuchsin with Fast Green (AF with FG) and PicroSirus Red with Fast Green (PSR with FG).

Methods used in Histomorphological measurements and its calculations were same as described in our previous studies [7, 8]

2.4. Biochemical Estimation & Analysis

2.4.1. Lipid profile, Serum Creatinine and Total Protein

All lipid profiles, serum creatinine and serum total protein content were carried out by using Avantor Benesphera™ clinical chemistry Analyzer C61.

2.4.2. Enzymatic Antioxidant

Serum catalase was assayed by colorimetery as described [10]. The light absorbance of the sample was determined at 620 nm.

2.4.3. Non-invasive Biomarker (Oxidative Stress Parameter)

Serum total antioxidant capacity (TAC) was evaluated using ferric reducing antioxidant power (FRAP) assay [11]. The absorbance of sample was measured at 620 nm using photo colorimeter.

2.5. 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 were used for comparing the initial and final mean body weight of DC and blood glucose level in DXT group before and after treatment. All results were expressed as mean ± SD and P<0.05 and P< 0.0001 were considered as statistically significant.

3. Results

3.1. 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 DC was significantly (P<0.0001) reduced whereas in all other groups it remained stable at the end of study period (Table 1). Mean blood glucose levels of healthy groups (HC & HXT) remained within normal limits. In DXT 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 (Table 2).

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
HXT	270 ± 29.44	262.5 ±17.09	275 ± 20.82	288.6 ± 19.87
DXT	272 ± 25.88	252.6 ± 22.33	268 ± 23.87	279 ± 23.02

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.

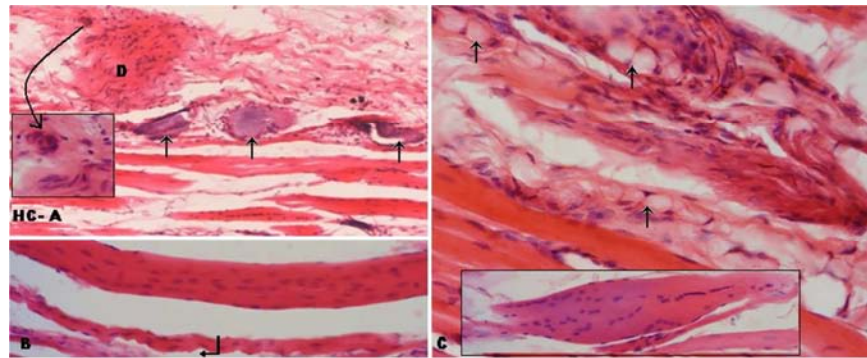**Table 2.** *Blood glucose (mg/dl) level of the animals of all groups during the period of study (Mean ± SD).*

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
HXT	145.33 ± 31.37	153 ± 32.70	137.5 ± 20.51	131.33 ± 29.74
DXT	565.4 ± 39.22	452.5 ± 36.70	302 ± 25.51	218 ± 29.30

Note that in DXT 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.

3.2. Microscopic Observations
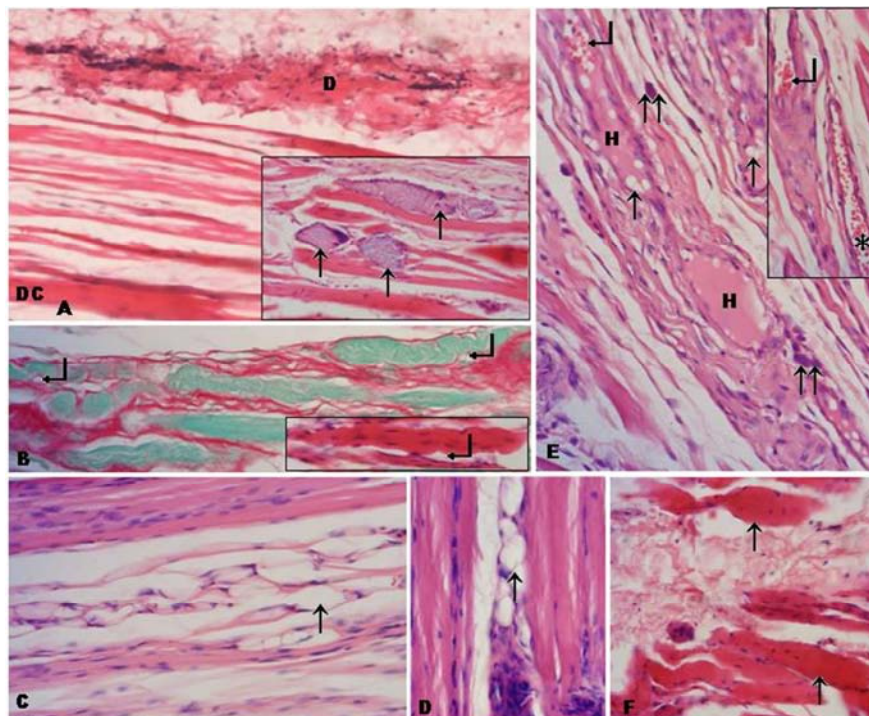
3.2.1. Changes Suggesting Degeneration

On 3rd week the control groups showed more inflammatory cells, atrophic fibres with hypereosinophilic, hypertrophied and undulated sacrolemma and necrotic fibres with mineralization (Figure 2 & 3). Other myopathic changes such as swollen, vacuolated, hyalinized, fragmented myofibers and hemorrhages in the myofibers and blood capillaries were noticed in DC (Figure 3). Whereas in treated groups these degenerative and necrotic changes were hardly seen.

Figure 2. *Showing degenerating and necrotic changes in the myofibres of HC group.*

Note: A-↑: Necrotic fibres with mineralization, D: Degenerating fibres at initial magnification x100, inset ∫ : Multinucleated giant cell at initial magnification x400.
B-↵: Undulated sacrolemma, C-↑: Fatty depositions in the epimysium, inset: Hypereosinophilic and hypertrophied myofibre at initial magnification of x400. H & E stain

Figure 3. *Showing degenerating and necrotic changes in the myofibres in DC group.*

Note: A. D: Degenerating fibres, inset↑: Necrotic fibres with mineralization at initial magnification at x100. H & E stained sections. B.↵: Undulated sacrolemma at initial magnification x400. PSR with FG and inset H & E stain. C-Fatty depositions in the epimysium, D- Fatty depositions in between myofibers, E-↑: Vacuolations, ↑↑: Inflammatory cells, H: Hyalinizations, ↵: Hemorrhage in the myofibers and inset, ∗: Hemorrhage in the blood capillary (inset), F- ↑: Hypereosinophilic and hypertrophied myofibre at initial magnification x400. H & E stain.
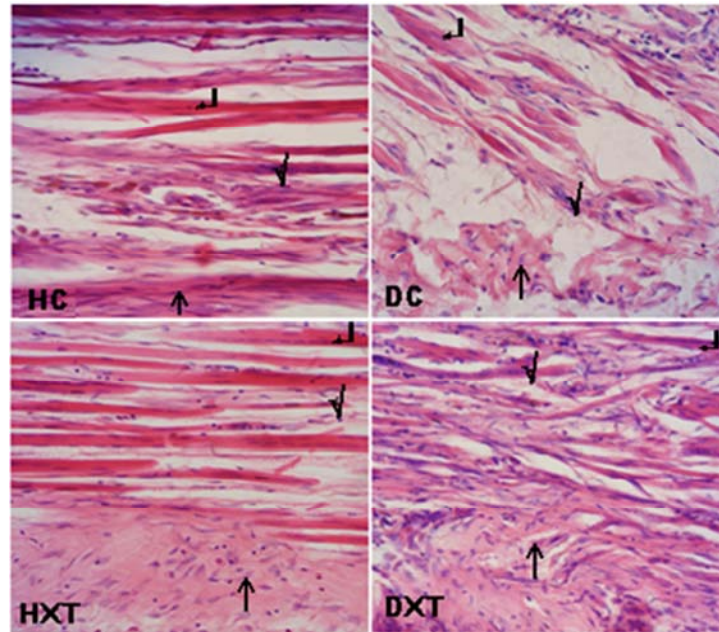
3.2.2. Changes Suggesting Regeneration

Only few activated myoblasts were noticed in control groups especially in DC as compared to treated groups (Figure 4). More newly regenerated myofibers (Figure 5), myofibers with central nuclei and split fibres were often noticed in treated groups as compared to control groups (Figure 6).
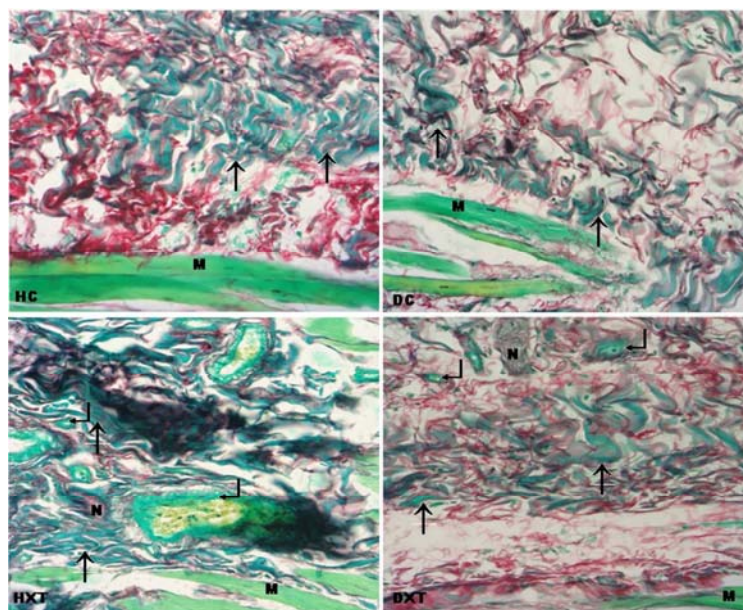
3.2.3. Fibrosis and Fatty Connective Tissue

Quite frequent fatty depositions were found in the epimysium of control groups (Figure 2, 3 & 9) as compared to treated groups (Figure 9) and in DC these features were also found in the in perimysium and interstitial connective tissues (Figure 3). In control groups fibrosis was marked in all connective tissue coverings as compared to treated groups (Figure 7).

Figure 4. *Showing Activated myoblasts in all groups.*

Note: ↑: Activated satellite cells (↑), Myoblasts with row of vesiculated nuclei (√) and centrally placed myonuclei (↵), at initial magnification x400. H & E stain.
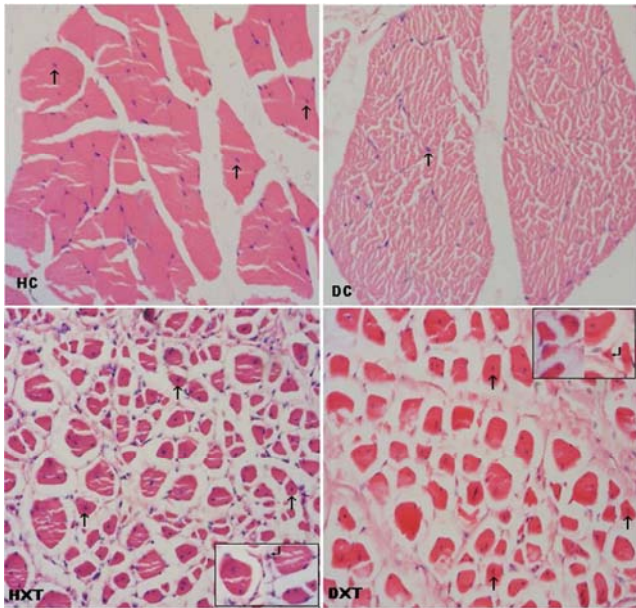

Figure 5. *Showing regenerated myofibers in all groups and vascularization and innervations of the regenerated fibres in treated groups.*

Note: ↑: Regenerated myofibers, M: Normal myofibers and presence of collagen fibres (Red colour) in all groups. N: Nerve bundle, ↵: blood capillaries. In treated groups (HXT & DXT) nerve bundles and blood capillaries were seen in relation to regenerated myofibers and, at initial magnification x400. PSR with FG stain.
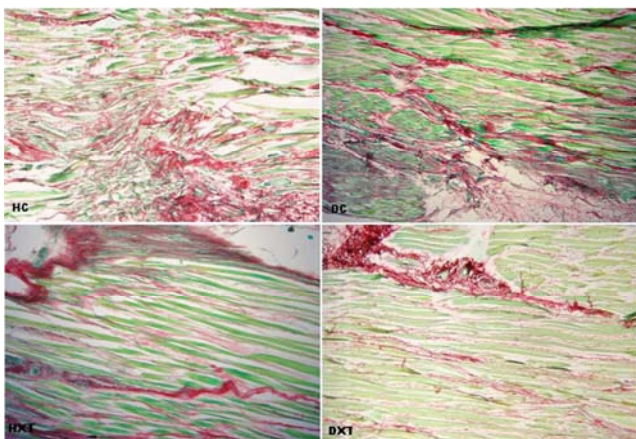
3.2.4. Connective Tissue Remodeling and Neovascularization

Only few thin epimyseal elastin fibres were noticed in HC while these fibres were almost absent in the connective tissue coverings of DC. In treated groups these fibres were prominent were distributed in all connective tissue coverings (Figure 8). In treated groups more proliferated blood capillaries have shown in the epimysium and at the site of newly regenerated myofibers (Figure 5 & 9). But these features were less and observed only in the epimysium of control groups (Figure 9).
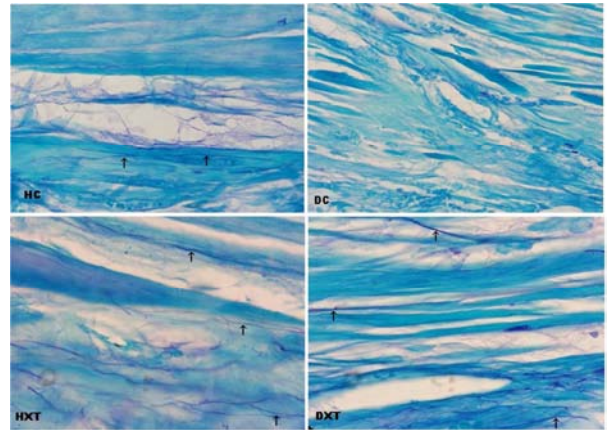

Figure 6. Showing central nuclei in all groups and split fibres in treated groups.

Note: ↑: myofibers with central nuclei, in HXT & DXT inset-↵: split fibres, at initial magnification x400. H & E stain.
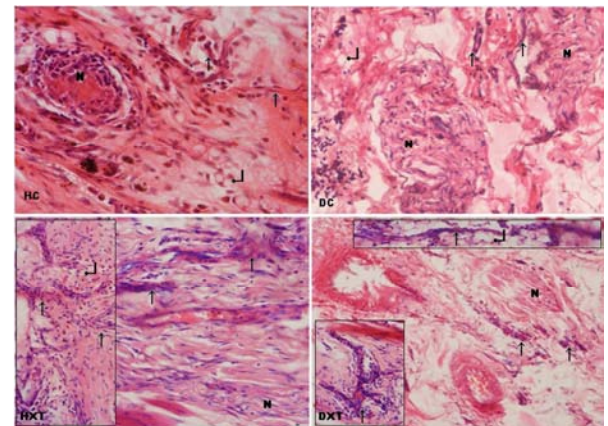

Figure 7. Showing presence and amount of fibrosis in all groups.

Note: Note- more collagen fibres (Red colour) in the all connective tissue layers of control groups but in treated groups these fibres were minimal, at initial magnification x100. PSR with FG stain.
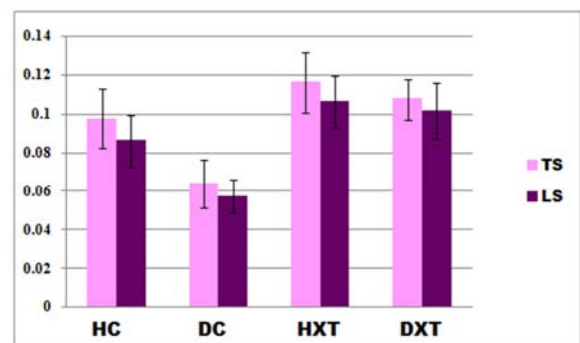

Figure 8. Showing reappearance of Elastin fibres during extracellular matrix remodeling.

Note: ↑: Elastin fibres. In HC elastin fibres only in the epimysium, DC these fibres were deficient in all connective tissue coverings but in treated groups have shown reappearance of thin elastin fibres in all connective tissue coverings, at initial magnification x400. AF with FG stain.

Figure 9. Showing neovascularization of blood capillaries and reinnervations.

Note:↑: Blood capillaries, in treated groups and its inset views have shown numerous proliferated blood capillaries N: Nerve bundle, ↵: Fat depositions, at initial magnification x400. H & E stain.

Figure 10. Showing myofibres' width in LS and diameter in TS ((in mm, Mean ± SD).

Note: In treated groups myofibers' diameter/width were significantly (P<0.01) increased as compared to control groups.

Figure 11. *Showing percentage of central nuclei in all groups in muscle TS (Mean ± SD).*

Note: The percentages of central myonuclei in treated group were significantly (P<0.01) increased as compared to control groups.

3.2.5. Functional Repair by Reinnervation

In all groups epimyseal nerve bundles were observed (Figure 9) and these features were also seen in relation to the recently regenerated myofibers of treated groups (Figure 5 & 9).

3.3. Histomorphology

In control groups myofibers width and diameter were significantly (P<0.01) lesser as compared to treated groups (Figure 10). Percentages of central myonuclei were significantly (P<0.01) high in treated groups as compared to control groups (Figure 11).

3.4. Biochemical Analysis on 3rd Week

3.4.1. 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) as compared to DXT. Whereas high density lipoprotein (HDL) in DC showed significantly lower values (P<0.01) compared to DXT (Table 3) [12].

Table 3. *Lipid profiles (Mean ± SD) after co-administration of d-α-tocopherol and d-δ-TRF.*

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
HXT	45.30 ± 0.79	16.38 ± 0.14	13.40 ± 0.15	16.52 ± 0.50	103.6 ± 2.67
DXT	48.68 ± 1.06	16.21 ± 0.24	14.67 ± 0.45	17.80 ± 0.37	119.9 ± 1.27

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 DXT group.

3.4.2. Serum Creatinine Level and Serum Total Protein Content

Serum creatinine level in DC were significantly higher ((P<0.01) as compared to all other groups. Serum total protein content in treated groups (HXT & DXT) was significantly higher (P<0.01) compared to control groups (HC & DC) (Table 4) [12].

Table 4. *Biochemical parameters (Mean ± SD) after co-administration of d-α-tocopherol and d-δ-TRF.*

Serum Analyses				
Groups	Catalase (u/ml)∗	TAC (µmol/L)	Total protein (g/dl)	Creatinine (mg/dl)
HC	0.0672 ± 0.004	1285.5 ± 67.18	5.05 ± 0.07	0.425 ± 0.010
DC	0.0438 ± 0.005	1000 ± 67.88	4.5 ± 0.14	0.790 ± 0.022
HXT	0.128 ± 0.002	2619 ± 48	5.47 ± 0.15	0.425 ± 0.016
DXT	0.081 ± 0.001	2413 ± 27.71	5.2 ± 0.14	0.545 ± 0.018

Note that serum creatinine level was significantly high ((P<0.01) and all other values were significantly low in DC compared to healthy control and treated groups (P<0.05). Catalase (u/ml)•u-µmols of H_2O_2 utilised/mt.

3.4.3. Enzymatic Antioxidant and Oxidative Stress Parameter

Serum catalase activity and total antioxidant capacity in treated groups (HXT & DXT) exhibited significantly higher (P<0.01, P<0.05) values compared to control groups (HC & DC). These analyses values in DC were significantly lower (P<0.05) compared to HC group (Table 4) [12].

4. Discussion

In diabetes increased reactive oxygen species (ROS) generation leads to myopathy due to failure of myocyte plasma membrane repair. Vitamin E promotes the myocyte plasma membrane repair and thus maintains the skeletal muscle homeostasis [4]. Myonecrosis is a complication of longstanding diabetes which most commonly affects the lower limb muscles [13].

In muscle injury the necrotic fibers are enlarged with altered internal architecture, influx of calcium ions, loss of the plasmalemma and an increased number of mononucleated cells [14, 15]. In this current study the control groups showed more inflammatory cells, atrophic fibres with hypereosinophilic, hypertrophied and undulated sacrolemma and necrotic fibres with mineralization. Other myopathic changes such as swollen, vacuolated, hyalinized, fragmented and hemorrhagic myofibers and plethora of blood capillaries

were noticed in diabetic control group. Three weeks co-administration of d-α-tocopherol and d-δ-TRF seems to hasten the replacement of degenerated myofibers in treated groups.

Muscle regeneration and physiological activity of stem cells are inhibited by chronic inflammatory response [14]. On 3rd week, more cellular infiltrations were seen in the epimysium and between myofibres of control groups. In treated groups the decreased inflammations points out towards the helpful effect of d-α-tocopherol and d-δ-TRF in creating the favorable environment for muscle regrowth after injury.

Presence of split myofibers during muscle regeneration suggests incomplete fusion of fibers regenerating within the same basal lamina [16, 17]. On 3rd week in control groups the recently regenerated myofibers were minimal. In treated groups presence of more newly regenerated myofibers along with the split fibres revealed the efficiency of vitamin E isoforms in structural recovery of injured skeletal muscle.

Diameter of myofibers and the number of central myonuclei are two useful markers for fusion-based muscle regeneration process [18]. Reduction in the diameter of hind limb myofibres were observed in STZ-diabetic rats [19] and in the present study similar histomorphological results were observed in diabetic control group. The recently regenerated fibres are larger in diameter and have central nuclei. These myonuclei moves to the periphery at the end of muscle regeneration [20]. More number of central nuclei and increased size of muscle fibres were found in treated groups than control groups. These explain the combined effect of d-α-tocopherol and d-δ-TRF on fast repair of myofibers after crushed injury.

The repair of damaged myofibers occurred by the activation, proliferation and differentiation of satellite cells into myoblasts [15]. In treated groups more myoblasts were seen as compared to control groups, suggesting thereby that both d-α-tocopherol and d-δ-TRF have potency to promote the proliferation and differentiation process of satellite cells into myoblasts.

The initial fibrosis which occurs during healing process has important role in support, strength and protection of the injury site. However, the overproduction of collagens within the injured area often leads to heavy scarring and the loss of muscular function [14]. In the present study, even on 3rd week more fibrosis and fatty depositions in the epimyseal and perimyseal connective tissue layers were observed in control groups. Treated groups had lesser fibrosis and thus providing suitable environment for muscles regeneration evidenced as superior effects of co-administration of d-α-tocopherol and d-δ-TRF.

Extra cellular remodeling is a one of the fundamental requirements for myoblast migration and fusion during development and regeneration [21]. On 3rd week in HC the reappearance of thin elastin fibres were observed only in epimysium while even these were absent in DC connective tissue coverings. Treated groups showed marked elastin fibres in all connective tissue coverings which is in

agreement with our previous study [12] that the d-α-tocopherol and d-δ-TRF have beneficial role in connective tissue remodeling. Regeneration process is completed only after the penetration of new blood vessels into the injured area [22]. In the present study control groups showed lesser proliferated blood capillaries in the epimysium as compared to treated groups. In addition to this treated groups had neovascularization at the site of regenerated myofibers as well. This result correlates with our many previous studies [23, 24] that both d-α-tocopherol and d-δ-TRF supplementation supports revascularization after crush-injuries.

Reinnervation after injury is helpful for the maturation and functional recovery of regenerating myofibres. The nerve activities can directly influence protein turnover and gene expression in multinucleated regenerating myotubes and indirectly influence the proliferation and differentiation of satellite cells [25- 27]. At the end of 3rd week the reinnervation were observed in epimysium of all groups. In treated groups the newly formed myofibers were more mature and attained functional recovery by presence of regenerated nerve bundles in relation to repaired myofibers. This result is in agreement with previous studies [23, 24] that both d-α-tocopherol and d-δ-TRF have potency in nerve regeneration after crush injuries.

Increased levels of cholesterol, triglycerides and phospholipids are the common features in hyperlipidemia [28]. Maintaining the optimal level of lipids is a necessary requirement in diabetes to prevent the diabetic microangiopathy, macroangiopathy, cerebral vascular disease and arteriosclerosis [29]. The data of the present study indicates that the 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 was lower in diabetic control group, indicating a state of significant dyslipidemia in untreated diabetic rats [30]. Lower mean values of TC, TG, LDL and VLDL levels and high HDL level were recorded in diabetic co-administered group (DXT) after three weeks treatment, this result is in agreement with other related study [31].

Catalase is a preventive antioxidant which inhibits the initial production of free radicals and removes the excess H_2O_2 [32]. The present study showed serum catalase activity value was lower in DC this is in agreement with other studies [33, 34]. This activity was normalized in control group after vitamin E treatment [34]. Three weeks co-administration of d-α-tocopherol and d-δ-TRF also helped to increase the serum catalase activity in treated groups [12].

Antioxidant capacity of plasma is the primary measure and marker to evaluate the status and potential of oxidative stress in the body [35]. 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 [36]. Improved serum antioxidant capacity was observed in treated groups after co-administration of d-α-tocopherol and d-δ-TRF for three weeks [12].

5. Conclusion

Based on all findings it is concluded that co-administration of d-α-tocopherol and d-δ-TRF have potency to maintain glycemic level, improve the antioxidant capacity and promote functional and structural recovery of myofibers after crush injuries by regeneration, revascularization, reinnervation and connective tissue remodeling. Therefore it is suggested that these vitamin E isoforms are hopeful option for the treatment of skeletal muscle crush-injuries in both healthy and diabetics.

Acknowledgements

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