Effects of d-α-tocopherol on skeletal muscle regeneration in crushed injury of diabetic rats

Bijo Elsy¹, Aijaz Ahmed Khan¹, Veena Maheshwari²

¹Department of Anatomy, Jawaharlal Nehru Medical College, Aligarh Muslim University, Aligarh, India, ²Department of Pathology, Jawaharlal Nehru Medical College, Aligarh Muslim University, Aligarh, India

SUMMARY

Muscular atrophy in diabetes is believed to be due to uncontrolled hyperglycemia and oxidative stress. Vitamin E, a natural antioxidant, is considered important to maintain skeletal muscle structures and functions. The current study is designed to explore the regenerative potential of d-α-tocopherol after crushed injury of skeletal muscle in healthy and diabetic rats. Diabetes was induced through single subcutaneous injection of alloxan at the dose of 100 mg/kg at hip region. Twenty four albino rats were divided into four groups; healthy control, diabetic control, healthy treated and diabetic treated. Treated groups were administered d-α-tocopherol orally and daily at the dose of 200 mg/kg for three weeks. A horizontal skin incision was made on the shaved right mid-thigh region and after splitting of the fascia between gluteus maximus and tensor fascia lata the gluteus maximus was crushed with Kocher’s forceps. Skin wound was closed with an absorbable suture. The crushed muscle changes were studied by assessing the histopathological features, histomorphological measurements and biochemical analyses at the end of 3rd week. One way ‘ANOVA’ followed by Tukeys test and Student t test were used for statistical analysis. Results obtained through various methods indicate that the d-α-tocopherol helps in skeletal muscle regeneration by improving antioxidant status, myoblast proliferation, revascularization, reinnervation and connective tissue remodeling. Hence it is concluded that d-α-tocopherol is a useful therapeutic dietary supplement in the management of skeletal muscle crushed injuries in both healthy and diabetics.

Key words: Antioxidant – Crush-injury – D-α-tocopherol – Diabetes – Muscle – Rats

INTRODUCTION

Skeletal muscle has robust regenerative capacity and consists of two concomitant yet competitive events: myofiber regeneration and connective scar tissue production. The latter event is capable of completely inhibiting muscular regeneration when its production is excessive (Rosenblatt, 1992; Lehto et al., 1986; Jarvinen et al., 2002). Skeletal muscle regenerative capacity involves the number, activation, proliferation, and differentiation capacities of satellite cells, environment stress and oxidative stress (Lim et al., 2013; Mouly et al., 2005). Muscle-specific genes, satellite cells, stem cells, trophic factors and extracellular matrix have significant roles in the reconstruction of myofibers (Karalaki et al., 2009).

Diabetes imposes an unfavorable environment for muscle growth and functions after muscle injury (Vignaud et al., 2007). In diabetes, increased reactive oxygen species (ROS) generation leads to myopathy through failed myocyte plasma membrane repair. Plasma membrane repair is a fundamental cellular activity of the skeletal muscle myocyte. Failure to repair a plasma membrane disruption rapidly results in cell death by necrosis. Vitamin E is required for plasma membrane repair in

Corresponding author: Aijaz Ahmed Khan. Jawaharlal Nehru Medical College, Department of Anatomy, Aligarh Muslim University, Aligarh 202002 (U.P.), India. Phone: +91-9897216343. E-mail: aijazahmedkhan7@live.com

skeletal muscle myocytes (Howard et al., 2011; Labazi et al., 2015) and also protects the muscle from oxidative damage in diabetic rats (Aragno et al., 2004).

Therefore, the present study is focused to assess the regeneration promotion capacity of d-α-tocopherol in skeletal muscles after crush-injury in both healthy and diabetic rats.

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 the Institutional Animal Ethical Committee (No. 8937/2014).

This present study followed the same method as described in our previous study (Elsy et al., 2016) of animal care, induction of diabetes and monitoring of blood sugar level.

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 treated- HPT and (4) diabetic d-α-tocopherol treated-DPT (200mg/kg body weight, 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 laborato-
tories, Inc, 66 United St, Philippines).

Surgical procedure

All animals received general anesthesia via inhalation of ether. Horizontal skin incision was made on the shaved right mid-thigh region. The fascia between the gluteus maximus and tensor fascia latae was split and crush injury in the gluteus maximus was induced with Kocher’s forceps (Fig. 1). The muscles were re-approximated at proximal and distal points of crush. The overlying connective tissue and skin were closed with 3-0 Vicryl (2metric–NW2401) absorbable sterilized surgical needled suture USP (synthetic; braided coated polyglactin 910 violet; from Ethicon, manufactured in India by Johnson and Johnson Ltd, Aurangabad). 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 (Elsy et al., 2016)

Sample collection and Fixation of tissue

On completion of three weeks, animals were sacrificed under deep ether anesthesia and then the crushed parts of gluteus maximus excised with adjacent area. The excised muscle parts were immersion-fixed in 10% neutral buffered formalin. To assay the biochemical parameters, the method of serum preparation followed a previous study (Elsy et al., 2016).

Fig 1. Showing crushed parts of gluteus maximus muscle. GM- Gluteus maximus muscle; SN- Sciatic nerve; arrow (→) pointing the crushed parts.
Histopathology and Histomorphometry

Fixed tissue samples were processed for light microscopical studies. The 5 µm 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).

The longitudinal and transverse sections (LS & TS) stained with H&E were used for histomorphological measurements. The hypertrophy of myofibres was quantified by measuring the width and diameter in LS & TS with the help of Motic image version 2.0. Counting and measurement of the 100 myofibres in five LS and TS of each sample was performed. They were also used to calculate the mean diameter, width and percentage of central nuclei in regenerating myofibers at end of the study.

Biochemical Estimation & Analysis

Serum total protein content was carried out by using Avantor Benesphera™ clinical chemistry Analyzer C61.

Enzymatic antioxidant

Serum catalase was assayed by colorimetry as described (Sinha, 1972). The light absorbance of the sample was determined at 620 nm.

Non-invasive biomarker (oxidative stress parameter)

Serum total antioxidant capacity (TAC) was evaluated using ferric reducing antioxidant power (FRAP) assay (Benzie et al., 1996). 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. All the results were expressed as mean ± SD and P<0.05 and P< 0.0001 were considered as statistically significant.

RESULTS

Body weight and Blood glucose level

After induction of diabetes the typical clinic manifestations of the diabetes such as polyphagia, polydipsia and polyuria were observed in diabetic groups. 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) was reduced as compared to their initial body weight, whereas in all other groups mean body weight remained stable (Table 1). Mean blood sugar levels of healthy groups (HC & HPT) remained within normal limits while diabetic groups (DC & DPT) showed >450 mg/dl throughout the experimental period (Table 2).

Microscopic observations

Degenerating changes

At the end of 3rd week atrophic changes such as hypereosinophilic, hypertrophied myofibres and many having undulated sacrolemma were noticed. Other myopathic changes like necrotic fibres with

![Image](295.png)

**Fig. 2.** Myofibres degenerating and necrotic changes in healthy control group (HC). H&E stained sections Aстрелка up: Necrotic fibres with mineralization; D: Degenerating fibres. x100; inset стрелка down: Multinucleated giant cell, x400. Bстрелка down: Undulated sarcolemma; Cстрелка down: Fatty depositions in the epimysium; inset: Hypereosinophilic and hypertrophied myofibre, x400.
Table 1. Body weights (g) of the animals of all groups during the period of study (Mean ± SD). Note that the mean body weight in DC showed slight weight reduction while all other groups remained stable at the end of study period.

<table>
<thead>
<tr>
<th>Groups</th>
<th>Day 0</th>
<th>Day 7</th>
<th>Day 14</th>
<th>Day 21</th>
</tr>
</thead>
<tbody>
<tr>
<td>HC</td>
<td>270 ± 35.59</td>
<td>266.67 ± 15.28</td>
<td>283.33 ± 20.82</td>
<td>290 ± 21.60</td>
</tr>
<tr>
<td>DC</td>
<td>277.5 ± 25</td>
<td>247.5 ± 17.08</td>
<td>235 ± 23.80</td>
<td>227.5 ± 22.17</td>
</tr>
<tr>
<td>HPT</td>
<td>260 ± 33.17</td>
<td>250 ± 21.60</td>
<td>286.25 ± 18.87</td>
<td>293.33 ± 20.82</td>
</tr>
<tr>
<td>DPT</td>
<td>267.5 ± 29.86</td>
<td>240 ± 20.5</td>
<td>257.5 ± 17.08</td>
<td>272.5 ± 22.17</td>
</tr>
</tbody>
</table>

Table 2. Blood sugar (mg/dl) of the animals of all groups during the period of study (Mean ± SD). Note that the mean blood sugar levels of healthy groups (HC & HPT) remained within normal limits while the diabetic groups (DC & DPT) showed hyperglycemic state throughout the period of study.

<table>
<thead>
<tr>
<th>Groups</th>
<th>Day 0</th>
<th>Day 7</th>
<th>Day 14</th>
<th>Day 21</th>
</tr>
</thead>
<tbody>
<tr>
<td>HC</td>
<td>146 ± 28.21</td>
<td>124 ± 19.98</td>
<td>160.67 ± 18.01</td>
<td>167 ± 17.06</td>
</tr>
<tr>
<td>DC</td>
<td>540.25 ± 47.12</td>
<td>553 ± 39.42</td>
<td>574.25 ± 30.20</td>
<td>578 ± 34.73</td>
</tr>
<tr>
<td>HPT</td>
<td>124 ± 14.23</td>
<td>126.5 ± 17.52</td>
<td>136 ± 18.70</td>
<td>147.12 ± 18.12</td>
</tr>
<tr>
<td>DPT</td>
<td>546.5 ± 35.80</td>
<td>555.2 ± 29.95</td>
<td>507.4 ± 36.14</td>
<td>478.4 ± 36.64</td>
</tr>
</tbody>
</table>

Fig 3. Myofibres degenerating and necrotic changes in Diabetic Control group (DC). H&E stained sections. A: D: Degenerating fibres, x100; inset ↑: necrotic fibres with mineralization x400. B: PSR with FG, x100; inset ❖: undulated sarcolemma, x400. C: Fatty depositions in the epimysium, x100. D: Fatty depositions in between myofibers, x100. E: ↑: vacuolations, ↑↑: inflammatory cells, H: hyalinizations, ➜: hemorrhage in the myofibers, x100; inset, *: hemorrhage in the blood capillary x400. F: ↑: hypereosinophilic and hypertrophied myofibre, x400.
mineralization, degenerating myofibres and inflammatory cells were also noticed in control groups (Fig. 2). In addition to these features, in DC degeneration were represented by swollen, vacuolated, hyalinized and fragmented myofibers. Hemorrhage was also seen in relation to myofibers and in the blood capillaries (Fig. 3). But in treated groups atrophic, degenerative and necrotic changes were almost absent.

Regenerating changes
In DC only few activated myoblasts were observed as compared to treated groups, where numerous activated myoblast as compared to HC could be noticed (Fig. 4).

Recently regenerated myofibres were less in control groups, whereas in treated groups there were numerous regenerated myofibres (Fig. 5). Few myofibre with centrally placed nuclei were seen in control groups, but in treated groups more myofibers had central nuclei (Fig. 6).

Fibrosis and fatty connective tissue
Fatty depositions were found in epimysium of both control groups (Figs. 2 & 3), and in DC these features also found in the perimysium and interstitial connective tissues (Fig. 3). In control groups more fibrosis was marked in all connective tissue coverings as compared to treated groups (Fig. 7).

Connective tissue remodeling and Neovascularization
In HC elastin fibres were observed only in epimysium, and deficit of these fibres were noticed in DC. In treated groups these fibres were thin and seen in all connective tissue coverings (Fig. 8). The epimysium of control groups had only few proliferated blood capillaries (Fig. 9), whereas in treated possessed more proliferated capillaries in the all connective tissue coverings and these were quite marked in relation to the regenerated myofibres (Figs. 5, 9).

Functional repair by reinnervation
Epimysial nerve bundles were observed in all groups (Fig.9). In addition to this location, in treated groups regenerated nerve bundles were also obvious at the sites of regenerated myofibers as well (Figs. 5, 9).

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

Biochemical analysis on 3rd week
Serum total protein, enzymatic antioxidant and oxidative stress parameter
Serum catalase activity, total antioxidant capacity and total protein content exhibited significant (P<0.05) reduction in DC as compared to HC. All serum analyses values improved significantly after supplementation of d-α-tocopherol in HPT as compared to all other study groups (P<0.01) and in DPT as compared to DC (P<0.05, Table 3).

DISCUSSION
Marked muscle atrophy is a characteristic feature of uncontrolled diabetes (Aragno et al., 2004). Oxi-
dative stress severely impairs myocyte membrane repair, and vitamin E is to promote the myocyte plasma membrane repair and maintain the skeletal muscle homeostasis (Howard et al., 2011). Myonecrosis commonly affects the lower limb muscles (Glauser et al., 2008).

In diabetic the loss of body weight is due to the progressive muscle wasting and loss of tissue proteins (Shirwaikar et al., 2006). In the present study, the mean body weight in DC as compared to DPT revealed significant reduction at the end of experimental period (Elsy et al., 2016).

In early phases of necrosis, the fibers are enlarged with altered internal architecture, influx of calcium ions, loss of the plasmalemma and an increased number of mononucleated cells within the injured area (Musarò, 2014; Karalaki et al., 2009).

In fact, degeneration and necrosis represent a continuum of lesions and therefore are often both present within a given lesion (Crabbs, 2015). In this current study even on 3rd week control groups showed presence of necrotic fibres with mineralizations and the degenerative changes represented by hypereosinophilic, hypertrophied myofibres having undulated sacrolemma. In addition to these changes, additional myopathic changes such as swelling, vacuolation, hyalinization, fragmentation and hemorrhages were also seen in DC. After three week supplementation of d-α-tocopherol in treated groups, necrotic and degenerative changes were almost absent suggesting thereby that d-α-tocopherol is a beneficial supplement in the prevention of muscular atrophy.

Chronic inflammatory response inhibits the physi-
Fig 7. Presence and amount of fibrosis in all groups. PSR with FG stained sections. More collagen fibres (red colour) in the all connective tissue layers in control groups but in treated groups these fibres were minimal, x100.

Fig 8. Elastin fibres' reappearance during extracellular matrix remodeling. AF with FG stained sections. 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, x400.
ological activity of stem cells and could interfere with muscle regeneration (Musarò, 2014). On 3rd week, more cellular infiltrations were seen in the epimysium and between myofibres of control groups, while reduced numbers of inflammatory cells in the connective tissue of treated groups suggest that d-α-tocopherol supplementation creates a favourable microenvironment for muscle repair.

In the region of muscle regeneration some new myofibres reveal split, which may probably be due to the incomplete fusion of fibers regenerating within the same basal lamina (Blaveri et al., 1999; Bourke and Ontell, 1984). The present study also revealed that recently regenerated myofibers were less in control than treated groups support the notion that the d-α-tocopherol is beneficial for muscle regeneration.

Myofibre's diameter and the number of central nuclei are two useful markers for fusion-mediated...
muscle regeneration process (Charge and Rudnicki, 2004). Reduced myofibres' diameters were observed in DC, this is in agreement with previous study (Aughsteen et al., 2006). The recently regenerated fibres have central nuclei and increased size. These myonuclei moves to the periphery at the end of muscle regeneration (Yin et al., 2013). As compared to control groups, in the treated groups the d-α-tocopherol supplementation promotes the muscle regeneration by increasing the size of myofibres and presence of regenerated myofibers with central nuclei.

Satellite cells, once activated, can proliferate and differentiate into myoblasts which repair the damaged fibers (Karalaki et al., 2009). In the present study it was noticed that control groups had fewer myoblasts cells whereas treated groups revealed presence of large number of myoblasts suggesting thereby that d-α-tocopherol has strong potential to in accelerating the differentiation of Satellite cells.

During healing, initial fibrosis seems to be beneficial by providing support, strength and protection to the injury site. However, if the process is prolonged it leads to the overproduction of excess collagens within the injured area, which often leads to heavy scarring and the loss of muscular function (Musarò, 2014). The present study noticed that even at the end of 3rd week control groups were associated with more fibrosis, and presence of fatty depositions in the epimysium as well as perimysial connective tissue as contrast to the treated groups, where fibrosis was less evident suggesting thereby that d-α-tocopherol supplementation help to create favorable environment for the muscle functions by reducing undesirable fibrosis.

Connective tissue remodeling is a fundamental requirement for myoblast migration and fusion during development and regeneration (Lewis et al., 2000). It was observed that by the end of 3rd week the elastin fibres in HC reappear in the epimysium but remained almost absent in DC. In treated groups these fibres were thin and could be seen in all connective tissue coverings; this is in agreement with our previous studies (Elsy et al., 2016 and 2017) that the d-α-tocopherol promotes the remodeling of connective tissues.

Regeneration cannot take place until new blood vessels penetrate the area (Carlson and Faulkner, 1983). The present study revealed that in control groups the neo-vascularization were present only in the epimysium, whereas in treated groups more vessels' growth was seen in all connective tissue coverings and at the sites where regenerated myofibers were located. This result revealed that d-α-tocopherol supplementation supports the re-growth of vessels and is also in agreement with our previous study (Elsy et al., 2017).

Reinnervation is an important factor for maturation and functional recovery of newly formed myofibres. In fact 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 (Mozdziek et al., 2001; Mitchell and Pavlath, 2004; Slater and Schiaffino, 2008). In the current study epimysial reinnervations were observed in all groups. In addition to this location, in treated groups the regenerated nerve bundles were noticed at site of regenerated myofibers, suggesting thereby that the regenerated myofibers have possibly also attained the functional recovery. This result is in agreement with previous study (Elsy et al., 2017) that d-α-tocopherol supplementation accelerate the neuroregeneration after crush injuries.

The total protein content is also known to be an indicator for the protein level and cellular proliferation of the wound tissue (Lin et al., 2009). The result of the present study indicates that diabetic control rats showed marked reduction in serum total protein level and when treated with d-α-tocopherol its level improves significantly as seen in treated groups. This finding is also in agreement with other previous studies (Tavares et al., 2012; Elsy et al., 2016).

Catalase is a preventive antioxidant which inhibits the initial production of free radicals and removes the excess H₂O₂ (Vasudevan and Sreekumari, 2005). The present study showed that serum catalase activity value was lower in DC and this is in agreement with other studies (Jeyashanthi and Ashok, 2010; Shirpoor et al., 2007), and the three weeks administration of d-α-tocopherol helps to increase the serum catalase activity in treated groups which findings support from other studies (Shirpoor et al., 2007; Elsy et al., 2016). Antioxidant capacity of plasma is the primary measure and marker to evaluate the status and

### Table 3. Effects of d-α-tocopherol supplementation on biochemical parameters (Mean ± SD). Note that all biochemical parameters values were significantly less in DC compared to all other groups (P<0.05). Catalase (u/ml) u-mmol of H₂O₂ utilised/ml.

<table>
<thead>
<tr>
<th>Groups</th>
<th>Total protein (g/dl)</th>
<th>Catalase (u/ml)*</th>
<th>TAC (mmol/L)</th>
</tr>
</thead>
<tbody>
<tr>
<td>HC</td>
<td>5.05 ± 0.07</td>
<td>0.0672 ± 0.004</td>
<td>1285.5 ± 67.18</td>
</tr>
<tr>
<td>DC</td>
<td>4.5 ± 0.14</td>
<td>0.0438 ± 0.005</td>
<td>1000 ± 67.88</td>
</tr>
<tr>
<td>HPT</td>
<td>5.4 ± 0.15</td>
<td>0.088 ± 0.004</td>
<td>1481.3 ± 78.02</td>
</tr>
<tr>
<td>DPT</td>
<td>5.15 ± 0.08</td>
<td>0.066 ± 0.006</td>
<td>1309.5 ± 101.12</td>
</tr>
</tbody>
</table>
potential of oxidative stress in the body (Brahm et al., 2013). In the present work 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 (Alireza et al., 2009), which shows marked improvement after administration of d-α-tocopherol for three weeks in treated groups similar to one reported earlier (Elsy et al., 2016).

Conclusion
Based on all findings, it appears that the d-α-tocopherol supplementation helps to improve the antioxidant level, accelerate revascularization, reinnervation, myofibers’ regeneration and connective tissue remodeling. Therefore, it is concluded that d-α-tocopherol is a beneficial nutritional adjunct, and seems to be a strong future contender in the management of skeletal muscle injuries in both healthy and diabetics.

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