



Effect of Co-Administration of Vitamin E Isoforms *d- α -Tocopherol* and *d- δ -Tocotrienol Rich Fraction* on the Healing of Skin Wounds in Diabetic Rats

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Abstract: Normal wound healing involves sequence of events which is believed to be altered in diabetes due to hyperglycemia, infection and oxidative stress. The latter may be reduced by antioxidants which neutralize the chain formation of free radicals. Vitamin E is a well-known antioxidant and has saturated tocopherols and unsaturated tocotrienols. The most active form being the α -tocopherol. The present study was designed to explore the combined effect of *d- α -tocopherol* and *d- δ -tocotrienol rich fraction (d- δ -TRF)* on wound healing process in both healthy and alloxan-induced diabetic rats. Diabetes was induced with alloxan (100 mg/kg S. C). 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 3 weeks. Under general anesthesia, full-thickness excisional skin wounds were created on the dorsal surface of thoracic region. Macroscopic and microscopic features of wound healing stages were recorded at weekly intervals and biochemical parameters were estimated at the end of 3 weeks. It was observed that as compared to control in the treated group there was early reappearance of epidermal and dermal components, reduced serum creatinine level, increased serum antioxidant status and total protein content and controlled glycemic status. It is concluded that oral co-administration of *d- α -tocopherol* and *d- δ -TRF* promotes skin wound healing in both healthy and diabetic rats through its antioxidant potency, therefore suggested that vitamin E isoforms hold promising future in the effective management of wounds in both otherwise healthy and diabetics.

Keywords: Antioxidant, *d- α -Tocopherol*, *d- δ -Tocotrienol Rich Fraction*, Diabetes, Skin, Wounds

1. Introduction

In diabetes the normal wound healing is altered due to hyperglycemia, infections and oxidative stress [1]. Hyperglycemia is known to cause increased production of free radicals and insufficiencies in the antioxidant system [2]. The antioxidants have the ability to reduce the diabetic complications by arresting free radical-induced damage [3]. Vitamin E has both saturated (tocopherols) and unsaturated (tocotrienols) forms and is an effective antioxidant. Tocopherol and tocotrienol have different biological activities towards free radicals [4]. α -Tocopherol is the most

abundant and active form of vitamin E in humans [4, 5] and tocotrienols are more potent antioxidants compared to tocopherols [6]. In addition tocotrienol possess antidiabetic and anticancer properties as well [7]. Interestingly, the antitumor activity of tocotrienols is not dependent on its antioxidant activity [8, 9]. The highly biopotent γ and δ -tocotrienols may play a physiological role in modulating normal mammary gland growth, function and remodeling. Nevertheless anticancer effects on mammary tumor cells by applying these compounds did not display any adverse effect on normal mammary epithelial cell growth [10 – 12].

According to Zaini et al [13], the tocotrienol-rich fraction

(TRF) treatment accelerate the wound contraction rate, enhance the reepithelialization, the regeneration process and stimulate the granulation tissue formation in deep partial-thickness burn wounds. In another study [14] it was revealed that the supplementation of TRF at 200 mg/kg was able to improve wound healing in type 1 induced diabetic rat.

TRF of palm oil consists of 25% α -tocopherol and 75% tocotrienol [13]. The concentrations of different constituents of palm oil-derived-TRF per gram are α -tocopherol at 171.1 mg, α -tocotrienol at 190.4 mg, β -tocotrienol 36.0 mg, γ -tocotrienol 211.2 mg and δ -tocotrienol 150 mg [15].

At present available studies are very limited, and that too are mainly focused on the effects of TRF on wound healing [13, 14]. TRF contains different vitamin E isoforms. Hence this study focuses on the effect of co-administration d- α -tocopherol and d- δ -TRF (90% d- δ and 10% d- γ tocotrienols) on skin wound healing in healthy and diabetic rats by using histological, histomorphological and biochemical parameters.

2. Materials and Methods

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

This present study followed the same method as described in our previous study [16] of animal care, induction of diabetes, monitoring of blood sugar level, surgical procedure, tissue sample collection and fixation.

2.1. Experimental Groups, Route and Dosage of Treatment

Animals were divided into four groups having 6 rats in each group: (1) healthy control- HC; (2) diabetic control- DC; (3) healthy d- α -tocopherol and d- δ -tocotrienol rich fraction treated- HXT and (4) diabetic d- α -tocopherol and d- δ -tocotrienol rich fraction treated- DXT (100mg/kg of d- α -tocopherol and d- δ -TRF each, orally, daily for 3 weeks. d- α -Tocopherol Myra e capsule[Vitamin E] manufactured by PT Daya- Baria laboratoria Tbk, Indonesia; Imported and packed by United laboratories, Inc, 66 United St, Philippines. Unique E Tocotrienol, tocopherol free, 90% δ and 10% γ tocotrienols, AC Grace Company, P. O Box 570, Big Sandy, TX 75755, USA). Dosage of present study treatment (200mg/kg body weight) was based on previous related studies [14, 16].

2.2. Macroscopic Examination

The macroscopic changes in the wound healing stages were observed and recorded photographically on 1st, 7th, 14th & 21st day of creation of wounds.

2.3. Histopathology and Histomorphometry

Fixed tissue samples were processed for light microscopical studies. The 5 μ m thick paraffin sections were stained with Haematoxylin & Eosin (H & E), Masson's Trichrome (MT) and Aldehyde Fuchsin with Fast Green (AF with FG).

Histomorphometry was performed on both H & E and MT stained sections. While H & E sections were used for measuring the Global Healing Index (GHI), MT stained sections were used for estimation of Global Remodeling Index (GRI). Histological features under x 4 objective lens of trinocular microscope (Olympus, BX40; Japan) were recorded by digital camera (Sony 18.2 MP, Japan) and measurements were made by using software Motic image version 2.0 [16]. Measurements related to epidermal thickness and calculation of healing indices were based on the mathematical model for healing and remodeling matrix [17].

2.4. Biochemical Estimation & Analysis

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

b. Enzymatic antioxidant

Serum catalase was assayed by colorimetry as described [18]. 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 [19]. 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 Tukeys test. All the results were expressed as mean \pm SD and $P < 0.05$ was considered as statistically significant. Student t test was used for comparing the blood sugar level in DXT group before and after treatment ($P < 0.0001$).

3. Result

3.1. Body Weight and Blood Sugar Level

Weight and blood sugar levels of all animals in each group were monitored at weekly intervals. Mean body weight in DC showed slight weight reduction whereas in all other groups (HC, HXT & DXT) remained stable at the end of study period (Table 1). Mean blood sugar levels of healthy groups (HC & HXT) remained within normal limits. In DXT the mean blood sugar level was significantly ($P < 0.0001$) reduced after 3 weeks treatment while in DC showed > 500 mg/dl throughout the experimental period (Table 2).

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 1. Body weights (g) of the animals of all groups during the period of study (Mean \pm SD).

Groups	Day 0	Day 7	Day 14	Day 21
HC	270 \pm 35.59	266.67 \pm 15.28	283.33 \pm 20.82	290 \pm 21.60
DC	277.5 \pm 25	247.5 \pm 17.08	235 \pm 23.80	227.5 \pm 22.17
HXT	270 \pm 29.44	262.5 \pm 17.09	275 \pm 20.82	288.6 \pm 19.87
DXT	272 \pm 25.88	252.6 \pm 22.33	268 \pm 23.87	279 \pm 23.02

Table 2. Blood sugar (mg/dl) level of the animals of all groups during the period of study (Mean \pm SD).

Groups	Day 0	Day 7	Day 14	Day 21
HC	146 \pm 28.21	124 \pm 19.98	160.67 \pm 18.01	167 \pm 17.06
DC	540.25 \pm 47.12	553 \pm 39.42	574.25 \pm 30.20	578 \pm 34.73
HXT	145.33 \pm 31.37	153 \pm 32.70	137.5 \pm 20.51	131.33 \pm 29.74
DXT	565.4 \pm 39.22	452.5 \pm 36.70	302 \pm 25.51	218 \pm 29.30

Note that the mean blood sugar levels of healthy groups (HC & HXT) remained within normal limits. In DXT group the mean blood sugar level was significantly ($P < 0.0001$) reduced after 3 weeks treatment while in DC showed > 500 mg/dl throughout the experimental period.

3.2. Macroscopic Observations

In treated groups progressive changes in the size of wound area were observed at the end of 14th day compared to control groups (Figure 1).

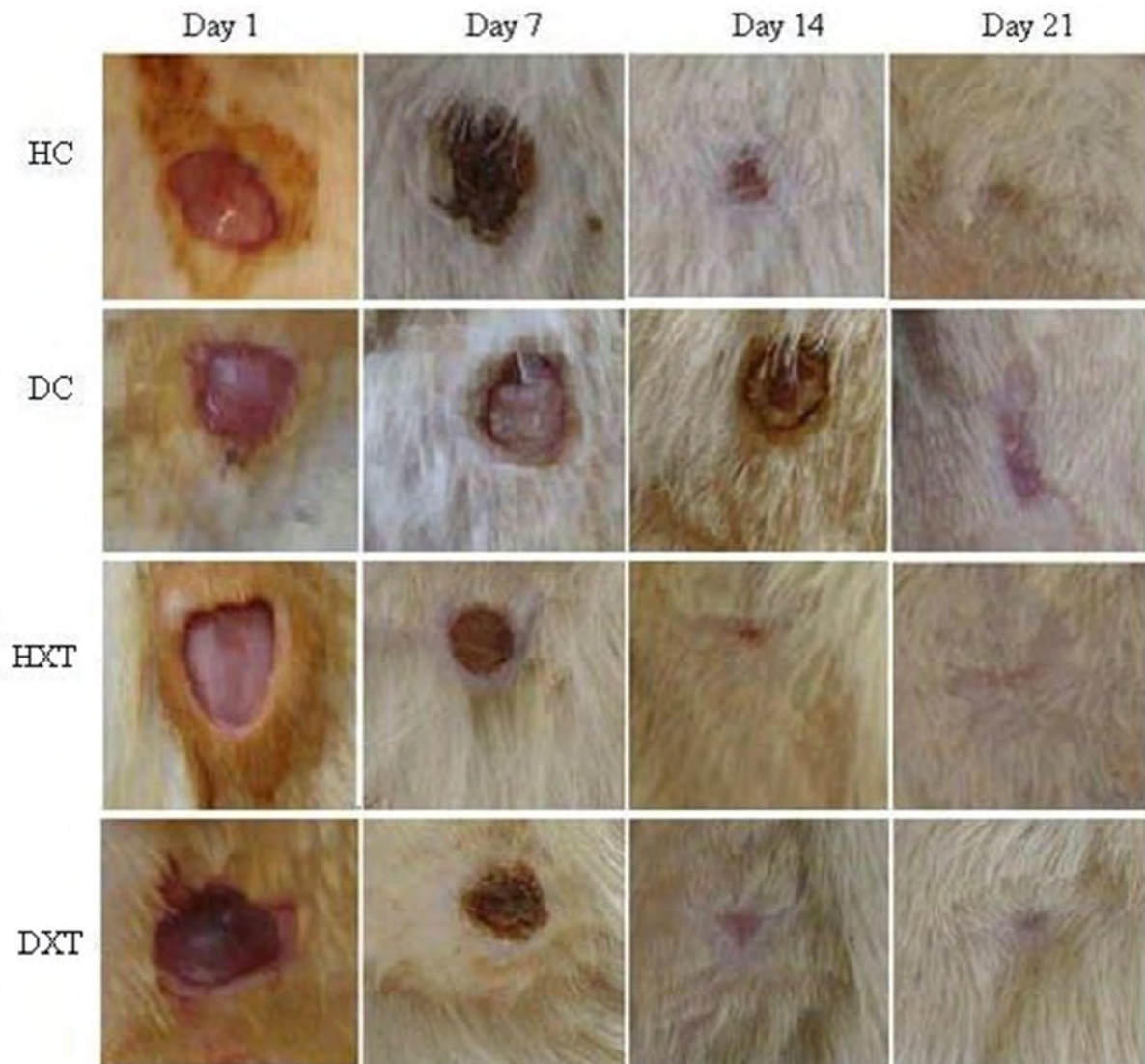


Figure 1. Photographs showing skin wounds of different groups at weekly intervals. Note that on 14th day an observable change in the size of the wound area in treated groups.

3.3. Microscopic Observations

a. Histomorphometry

In all groups the neopidermis was developed at the end of 2nd week. However, in treated groups the mean values of neopidermis were significantly thicker ($P < 0.01$) than the corresponding epidermal border thickness on 2nd and 3rd

weeks (Table 3). During the study period in treated groups the GHI and GRI were significantly higher ($P < 0.01$) compared to control groups (Figure 2 & 3).

Note that the neopidermal thickness in treated groups (HXT & DXT) much thicker than that of their respective epidermal border thickness on 2nd and 3rd weeks.

Table 3. Border & neopidermal thickness (mm; Mean \pm SD) at the end of 2nd & 3rd weeks.

Groups	2 weeks		3 weeks	
	Border epidermis	Neopidermis	Border epidermis	Neopidermis
HC	0.078 \pm 0.018	0.109 \pm 0.013	0.075 \pm 0.014	0.115 \pm 0.014
DC	0.058 \pm 0.017	0.075 \pm 0.025	0.061 \pm 0.013	0.092 \pm 0.043
HXT	0.084 \pm 0.013	0.192 \pm 0.029	0.075 \pm 0.038	0.188 \pm 0.027
DXT	0.085 \pm 0.012	0.190 \pm 0.030	0.08 \pm 0.022	0.183 \pm 0.033

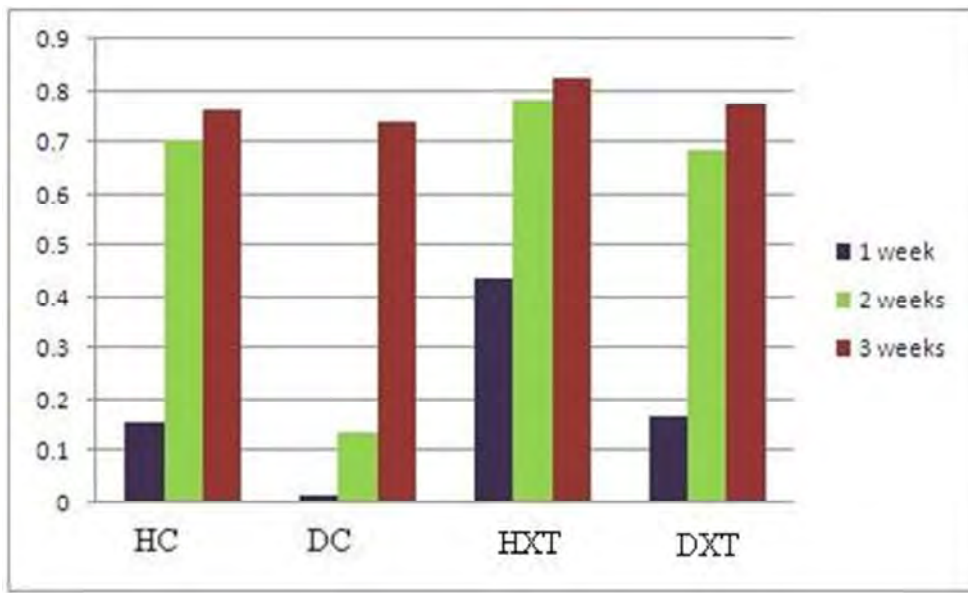


Figure 2. Weekly mean values (in mm) of Global Healing Index (GHI).

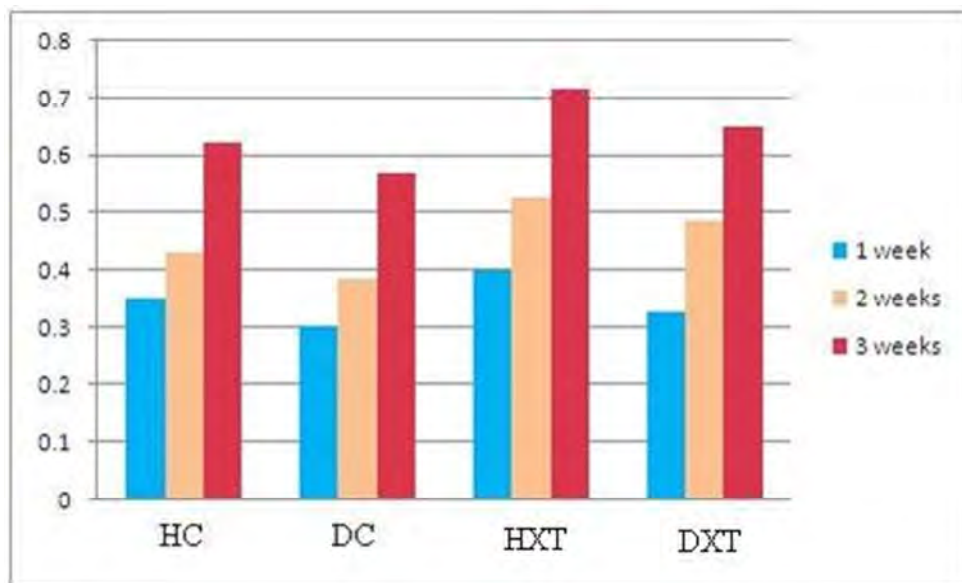


Figure 3. Weekly mean values of Global Remodeling Index (GRI).

b. Reepithelialization

Complete reepithelialization was noticed in all the groups (controls and treated). On 3rd weeks, in treated groups interdigitations appeared on the neopidermis while these features control groups were restricted at the wound margins (Figure 4 & 5).

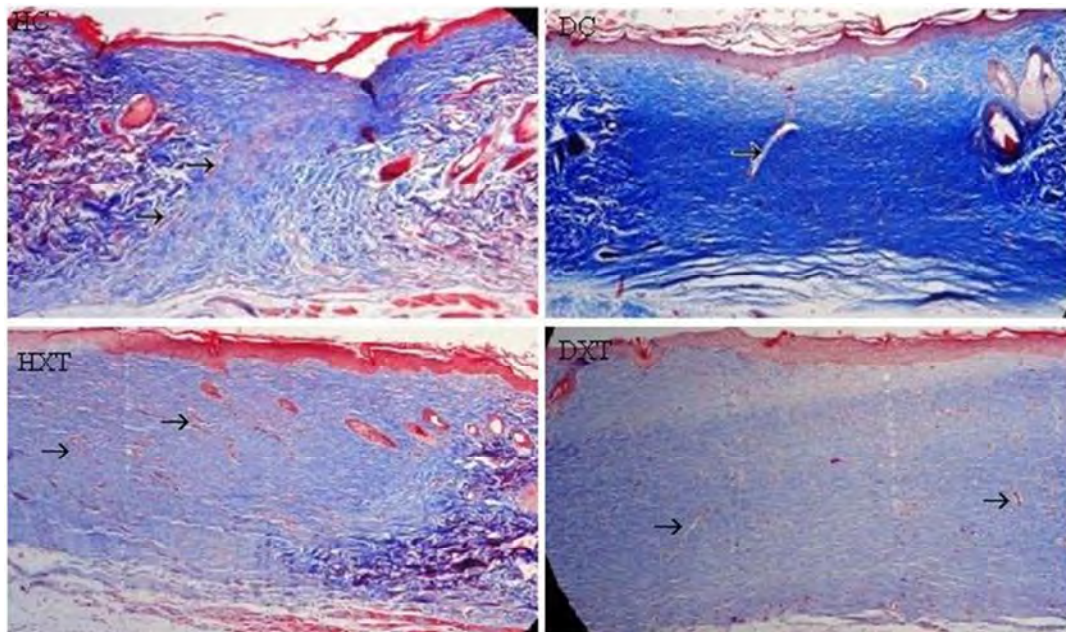


Figure 4. Representative images of MT stained sections on 3rd weeks showing presence of hair follicles and sebaceous gland in treated groups, arrangement of collagen fibres and epidermal interdigitations in all groups. Arrows (→) pointing the presence of capillary vessels at initial magnification x100.

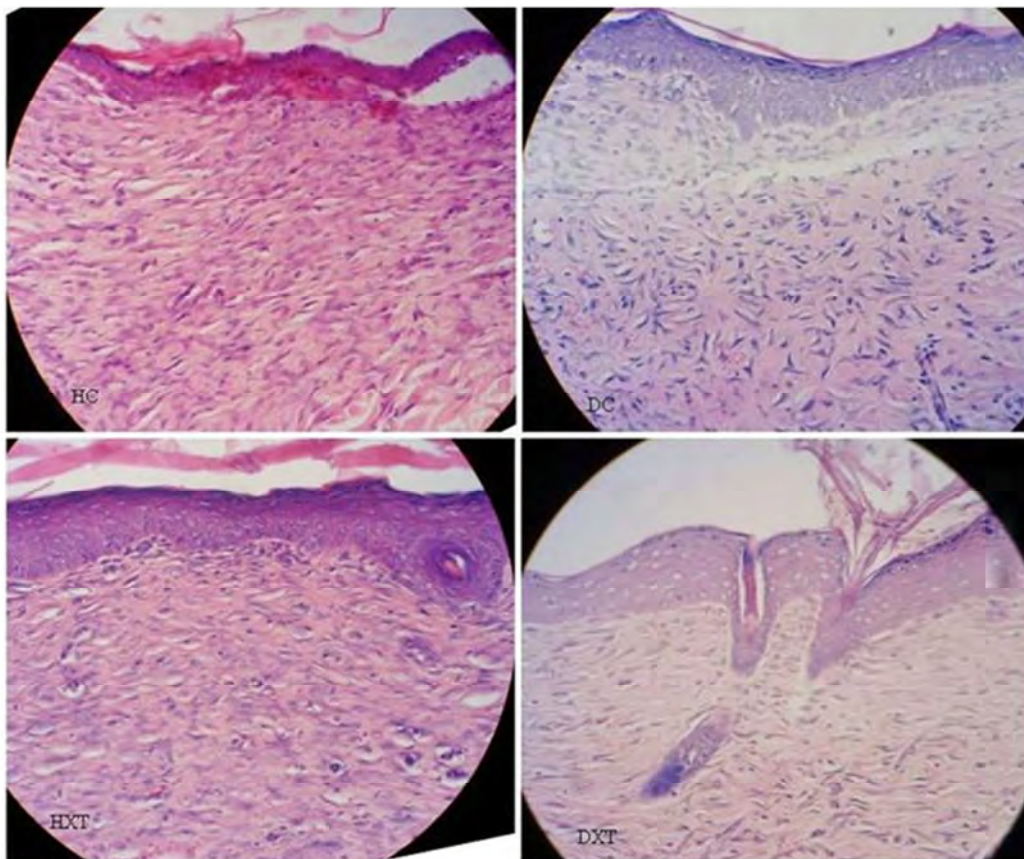


Figure 5. Representative images of H & E stained on 3rd weeks, showing arrangement of cells in all groups and presence of hairs in treated groups at initial magnification x400.

c. Cellular components

The granulation tissue consists of mainly fibroblasts on 3rd weeks and these cells were scattered, oval or spindle shaped in HC. In DC these cells were mainly stellate whereas in both treated groups spindle shaped cells lie parallel to the neoepidermis. More cellularity was observed in control groups compared to treated groups (Figure 5).

d. Neovascularization

At the end of study period, the granulation tissue contains vertically oriented blood capillaries in HC. Congested capillaries and extravasation of blood cells were seen in DC whereas in treated groups less number of such vessels was observed (Figure 4& 6).

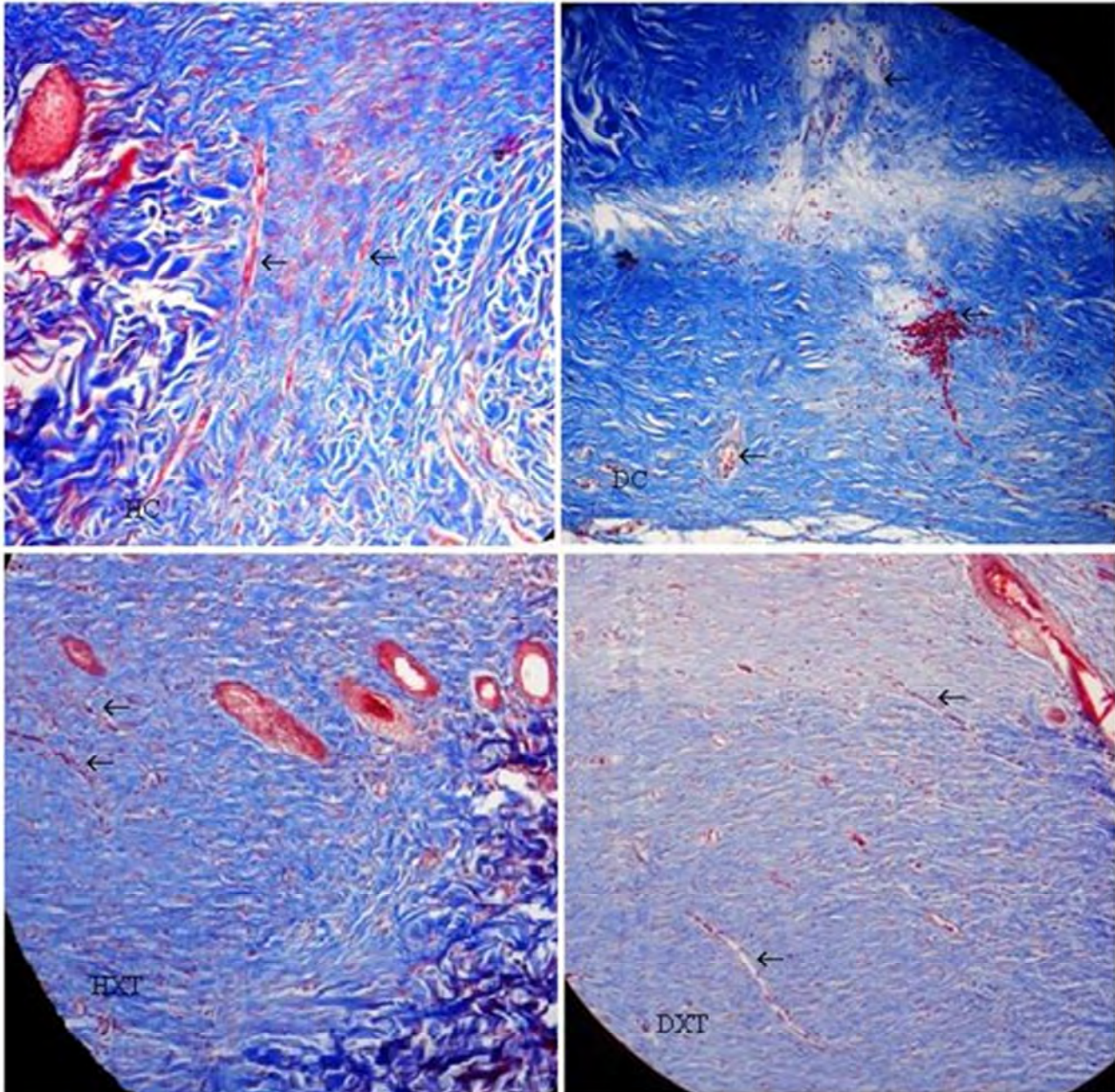


Figure 6. Representative images of MT stained sections on 3rd weeks showing the swollen capillaries and extravasation of blood cells in DC arrows (\rightarrow), all other groups' arrows (\rightarrow) pointing the presence of capillary vessels at initial magnification x400.

e. Matrix remodeling and Skin appendages

At the end of 3rd week, in treated groups the collagen fibres in regenerated dermis were mostly horizontally arranged and compactly interwoven but these fibres were more obliquely placed in HC. In DC suprahypodermal area contains poorly interlaced collagen fibres (Figure 4).

The elastin fibres in control groups were found at the wound margins while in the treated groups smaller fibrils were noticed in the regenerating dermis adjacent to the wound margin (Figure 7).

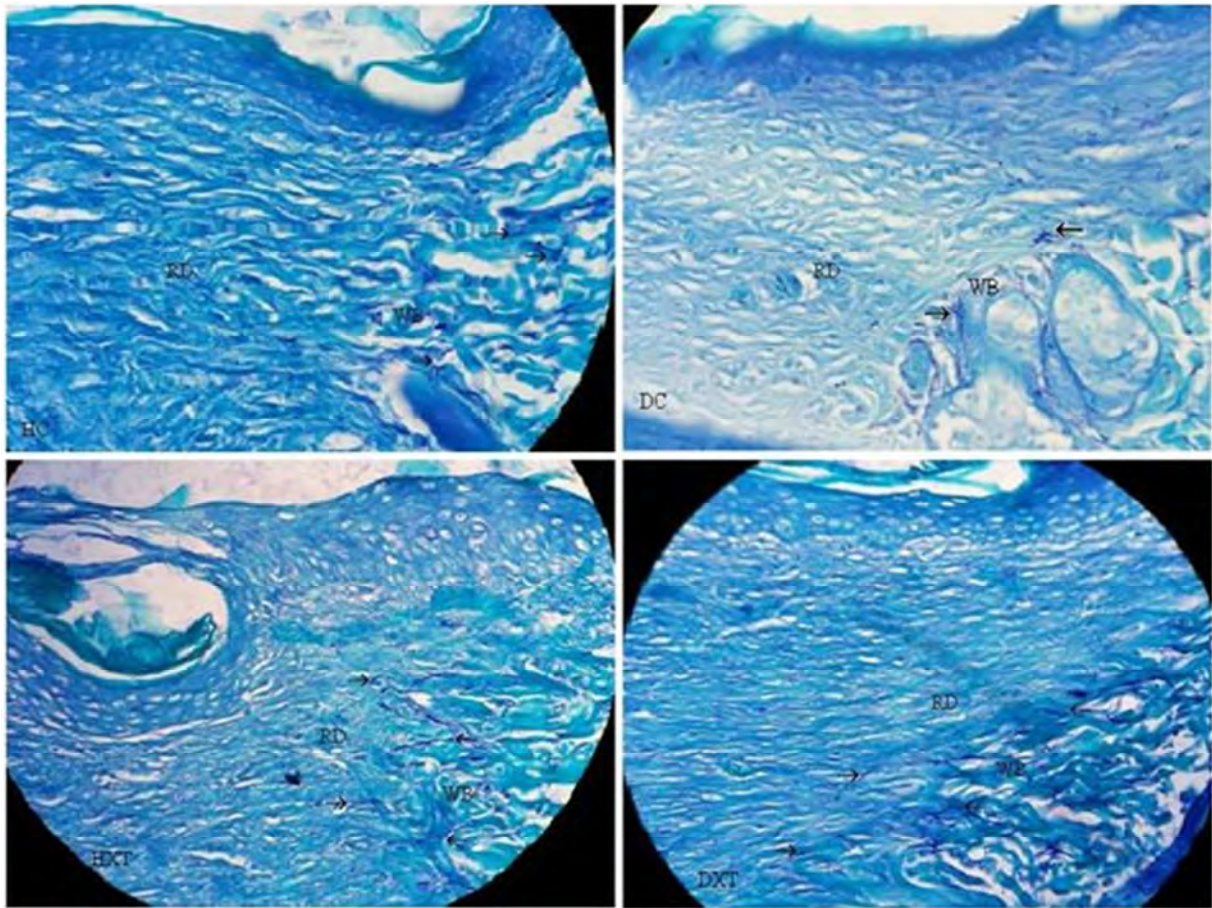


Figure 7. Representative images of AF with FG stained sections on 3rd weeks, showing elastin fibres arrangement. Arrows (→) pointing the presence of elastin fibres (violet colour) WB: wound borders; RD: regenerating dermis, initial magnification x400.

At the end of study period, in treated groups the epidermal appendages like hair follicles and sebaceous glands were in advance stage into the regenerating dermis and neoepidermal surface. In control groups hair follicles and sebaceous glands remained only at the wound margins (Figure 4 & 5).

3.4. Biochemical Analysis

a. Lipid profiles

Total cholesterol (TC), triglycerides (TG), low density lipoprotein (LDL) and very low density lipoprotein (VLDL) in DC were significantly higher ($P < 0.01$) as compared to DXT. Whereas high density lipoprotein (HDL) in DC showed significantly lower ($P < 0.01$) compared to DXT (Table 4).

Table 4. Co-administration effect of *d*- α -tocopherol and *d*- δ -TRF on Lipid profiles (Mean \pm SD).

Lipid profiles					
Groups	Total cholesterol (mg/dl)	HDL (mg/dl)	LDL (mg/dl)	VLDL (mg/dl)	Triglycerides (mg/dl)
HC	45.66 \pm 0.83	15.28 \pm 0.22	13.56 \pm 0.19	16.82 \pm 0.42	101.75 \pm 4.60
DC	54.30 \pm 1.19	11.05 \pm 0.21	18.02 \pm 0.09	25.23 \pm 0.89	171.15 \pm 11.53
HXT	45.30 \pm 0.79	16.38 \pm 0.14	13.40 \pm 0.15	16.52 \pm 0.50	103.6 \pm 2.67
DXT	48.68 \pm 1.06	16.21 \pm 0.24	14.67 \pm 0.45	17.80 \pm 0.37	119.9 \pm 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.

b. 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) showed significantly higher ($P < 0.01$) compared to control groups (HC & DC) (Table 5).

Table 5. Co-administration effect of *d- α -tocopherol* and *d- δ -TRF* on biochemical parameters (Mean \pm SD).

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

Note that all biochemical parameters reveal significantly less in DC compared to all other groups ($P < 0.05$). Catalase (u/ml)* μ - μ mol of H₂O₂ utilised/mt.

c. Enzymatic antioxidant and oxidative stress parameter

Serum catalase activity and total antioxidant capacity in treated groups (HXT & DXT) exhibited significantly higher ($P < 0.01$, $P < 0.05$) compared to control groups (HC & DC). These analyses values in DC showed significantly lower ($P < 0.05$) compared to HC group (Table 5).

4. Discussion

In diabetes the balance between oxidant and antioxidant level is disturbed which lead to accumulation of free radicals resulting into oxidative stress. These free radicals impair the normal wound healing process by being injurious to keratinocyte, endothelial cells, capillary permeability and collagen metabolism [20]. And thus oxidative stress induces cellular dysfunction and retards angiogenesis and the healing process [21]. Many studies [13, 14, 22-24] have concluded that the antioxidants help to maintain the oxidant/antioxidant balance, thereby improve the healing of wounds in diabetes. Vitamin E is a well-known antioxidant; its α -tocopherol isoform has scavenging effect on reactive oxygen species (ROS) and stabilizing effect on damaged cell membrane [25, 26]. Tocotrienols possess excellent antioxidant activity and suppress ROS production more efficiently than tocopherols [27].

In the present study, the mean body weight at the end of experimental period in DC was reduced but in DXT these were stable throughout experimental period. These findings are in agreement with related study of 4 weeks [28] having concluded that diabetic group without TRF supplementation showed significantly lower body weight than that of diabetic rat treated with TRF.

While oral co-administration of *d- α -tocopherol* and *d- δ -TRF* for 3 weeks in DXT revealed reduced mean blood sugar level, DC showed hyperglycemic state throughout the study period. These results correlate with other study [29] showing that the tocotrienol supplementation significantly increased the insulin levels and reduced the blood glucose in diabetic rats in a dose dependent manner. It supports the notion that *d- δ -TRF* is more effective in maintaining the glycemic level than *d- α -tocopherol* in diabetes [16].

Since the reepithelialization in epidermis is widely accepted to be one of the major processes in wound healing that ensures successful repair [30-32]. In the present study weekly macroscopic observation of wound area revealed that wound size reduction in treated groups even on 14th day was

suggestive of faster recovery in the treated groups than in controls.

The thickness of the epidermis is a good indicator for the superficial changes in the wound [17]. The mean values of histomorphological measurement in the present study showed that the neoepidermis is formed during the 2nd week in all groups. At the end of 2nd and 3rd week thickness of neoepidermis was much more in treated groups than their respective border epidermal thickness. The global healing and remodeling indices (GHI and GRI) are used to measure the different stages of skin wound healing and its progress and they also constitute relevant markers for direct comparison between different treatment groups. In cases of stronger wound remodeling the GRI can go up to 1 [17]. In present study the mean values of GHI and GRI were high in treated groups as compared to control groups, thereby indicating that vitamin E isoforms are beneficial for wound healing in both healthy and diabetics.

The interdigitations at dermoepidermal junction are known to provide both physical and trophic support. In treated groups interdigitations appeared on the neoepidermis whereas these were found only at the margins of neoepidermis in control groups by the end of 3 weeks. Therefore treated groups have more capacity to resist the possibility of desquamations.

Dermal regeneration has been characterized by granulation tissue rich in fibroblasts, generally oriented parallel to the epidermal layer [33]. The fibroblasts were oval or spindle shaped and scattered in HC whereas in DC these cells were mainly immature stellate shaped and less organized, indicating incomplete dermal regeneration. In treated groups spindle shaped mature thin fibroblast lay parallel to the neoepidermis, suggesting that the co-administration of *d- α -tocopherol* and *d- δ -TRF* helps the complete dermal regeneration. On 3rd week cellular components were more in control groups than treated groups. As remodeling progresses, there is a gradual reduction in the cellularity of the reparative tissue [34].

Neovascularization is characterized by well-structured capillary vessels and absence of hemorrhage [33]. By the end of 3rd week numerous vertically oriented capillary vessels that run towards the epithelial surface were seen in HC whereas in DC swollen capillaries and extravasation of blood cells in the granulation tissue, pointing towards its poor and delayed neovascularization. In advanced stage of remodeling there is a gradual reduction in the vascularity of the reparative tissue [34]. This finding is supported by the present study as it indicated that the numbers of capillary vessels were reduced in treated groups.

The collagen fibres are mainly found in the papillary and reticular layers of the dermis and they provide both mechanical and structural integrity to the dermis [35]. At the end of study period, in HC more fibres were obliquely placed while in DC the suprahypodermal area consists of poorly interlaced collagen fibres. In treated groups these fibres were horizontally placed and compactly interwoven and this horizontal alignment of collagen fibres suggests a better tissue remodeling [36].

Tough the elastin is a minor component of the dermis it has an important function in providing the elasticity of the skin [37]. On 3rd week in control groups the elastin fibres appeared at the wound margins whereas in both treated groups smaller fibrils were noticed in the regenerating dermis near to wound margins. Presence of elastin fibres in the healing wound indicates final stages of matrix remodeling [38].

At the end of the study period, hair follicles and sebaceous glands remained only at the wound margins in control groups while these structures were in advanced stage into the regenerating dermis and even newly formed hairs were found within the hair follicles and on the neopidermal surface in treated groups, which indicated a faster healing and quicker remodeling of the wound matrix [36].

The predictors of cardiovascular complications in diabetes are believed to be dyslipidemia and hyperglycemia [39-42]. The present data 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 was lower in DC, indicating significant dyslipidaemia in untreated diabetic rats [43]. The lower mean values of TC, TG, LDL and VLDL levels and high HDL level were recorded in DXT after 3 weeks combined treatment of d- α -tocopherol and d- δ -TRF. This result is in agreement with other related studies [16, 44].

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 [43]. In DXT these level were improved after 3 weeks treatment. The abnormally high level of serum creatinine was consistent with the impaired kidney function [45].

The total protein content is also known to be an indicator for the protein level and cellular proliferation of the wound tissue [46]. Result of present study shows serum total protein level was lower in DC but its level was increased in treated groups, which indicates that treatment with d- α -tocopherol and d- δ -TRF enhances protein synthesis. This is in agreement with [43] who found that the diabetic rats showed lower serum total protein level and significantly improved its level when treated with vitamin E.

Catalase is a preventive antioxidant which inhibits the initial production of free radicals. When H₂O₂ is generated in large quantities, the enzyme catalase is also used for its removal [47]. The present study showed value of serum catalase activity was lower in DC. Many other studies [48,

49] stated that the decreased catalase activity in plasma, liver and kidney of diabetic control rats. The decreased catalase activity in plasma and tissues of diabetic rats may be due to its increased utilization for scavenging the toxic products of lipid peroxidation or due to decreased availability of H₂O₂ [48]. Vitamin E treatment has been shown to normalize the catalase activity in the control group [49]. The result of present study revealed that 3 weeks supplementation of d- α -tocopherol and d- δ -TRF enhances the serum catalase activity in treated groups and this is in agreement with other related study [16].

Antioxidant capacity of plasma is the primary measure and marker to evaluate the oxidative status and potential of oxidative stress in the body [50]. Total antioxidant capacity has been shown to be significantly reduced in plasma and liver homogenate FRAP of diabetic rats compared to control animals [51-53]. The present work observed that serum total antioxidant level in diabetic control was significantly lower (P<0.05) compared to healthy control and is in agreement with the findings of above mentioned studies. The findings of present study showing significant (P<0.01) improvement in the serum antioxidant capacity after co-administration of d- α -tocopherol and d- δ -TRF in treated groups for 3 weeks is in agreement with those of previous related study [16].

5. Conclusion

Oral co-administration of d- α -tocopherol and d- δ -TRF promotes skin wound healing in both healthy and alloxan-induced diabetic rats through its potent antioxidant effect, it is therefore suggested that vitamin E isoforms hold promising future in the effective management of wounds in diabetics.

Abbreviations: AF with FG: Aldehyde Fuchsin with Fast Green; DC: Diabetic Control; DXT: Diabetic d- α -tocopherol and d- δ -tocotrienol rich fraction treated; FRAP: Ferric Reducing Antioxidant Power; GHI: Global Healing Index; GRI: Global Remodeling Index; HC: Healthy Control; H&E: Haematoxylin & Eosin; HXT: Healthy d- α -tocopherol and d- δ -tocotrienol rich fraction treated; MT: Masson's Trichrome; TAC: Total Antioxidant Capacity.

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