Therapeutic potential of d-δ-tocotrienol rich fraction on excisional skin wounds in diabetic rats

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ABSTRACT

Introduction: Long-standing hyperglycemia in addition to many of its associated complications also hampers normal wound healing which may be further aggravated in the presence of infection and oxidative stress. Therefore, antioxidant supplementation appears to be strategically relevant for wound healing. This study is designed to explore the therapeutic potential of d-δ-tocotrienol rich fraction (d-δ-TRF) on skin wound healing in both healthy and diabetic rats. Materials and Methods: Diabetes was induced through single subcutaneous injection of alloxan at the dose of 100 mg/kg at hip region. 24 albino rats were divided into four groups; healthy control, diabetic control, healthy treated and diabetic treated. d-δ-TRF was administered to treated groups (200 mg/kg), orally, daily for 3 weeks. Full thickness excisional skin wounds were. Wound area was studied by assessing the morphological, histomorphological and histological features at weekly intervals and biochemical analyses were performed at the end of 3rd week. Results: The findings of present study revealed that d-δ-TRF accelerated the skin wound healing by means of early regeneration of both epidermal and dermal components; enhancement of serum protein synthesis, improvement of antioxidant status, maintenance of glycemic condition and controlling serum creatinine levels in diabetic rats. Conclusion: It is concluded that d-δ-TRF has significant therapeutic potency on the healing of skin wounds in both healthy and diabetics.

Key words: Antioxidant; d-δ-Tocotrienol; Diabetes; Rat; Skin; Wounds
The tocotrienol-rich fraction (TRF) of palm oil consists of 25% α-tocopherol and 75% tocotrienol [14]. 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]. Therefore, TRF being an excellent antioxidant has been effectively used as a nutritional supplement due to its potential therapeutic benefits [16].

In deep partial-thickness burn wounds, the TRF treatment has been shown to accelerate the wound contraction rate, enhance the reepithelialization, the regeneration process and stimulate the granulation tissue formation [14]. According to Musalmah et al [17], supplementation of TRF at 200 mg/kg was able to improved wound healing in type 1 induced diabetic rat.

Data related to the effects of specific tocotrienol isoforms treatment on skin wound healing are scarce. The available very limited studies mainly focused on wound healing effects of TRF [14,17]. Since TRF contain different vitamin E isoforms, it was not possible to determine which isoforms was specifically responsible to promotes skin wound healing. Hence the present study is focused to assess the therapeutic antioxidant potency of d-δ-TRF on full thickness excisional skin wound healing in healthy and diabetic rats by using histological, histomorphological and biochemical parameters.

**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 was approved by Institutional Animal Ethical Committee (No. 8937/2014). Prior to commencement of the experiments, animals were acclimatized to the new environmental condition for a period of one week. They were kept in a well ventilated room and maintained on a standard pellet diet and water [18].

**Induction of Diabetes**

Diabetes was induced to the diabetic group after deprivation of food for 4 hours, followed by single subcutaneous injection (hip region) of alloxan (100 mg/kg; Alloxan monohydrate from Sigma-Aldrich). Food and water were provided after one hour of injection. Blood was obtained via tail vein for monitoring sugar level by using Glucometer (Dr Morepen gluco one) on the 4th day of alloxan injection. Animals with blood sugar level at 250 mg/dl and above were selected as diabetic for this study. Weight and blood sugar levels of all animals in each group were monitored at weekly intervals [18].

**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-δ-TRF treated - HTT and (4) diabetic d-δ-TRF treated - DTT (200 mg/kg body weight, orally, daily for 3 weeks. Unique E Tocotrienol, tocopherol free, 90% δ and 10% γ tocotrienols, AC Grace Company, PO Box 570, Big Sandy, TX 75755, USA). Orally supplemented tocotrienol was rapidly taken up by the skin [19]. Dosage of d-δ- tocotrienol rich fraction (200 mg/kg body weight) was based on previous studies of TRF [15,17,20].

**Surgical Procedure**

All animals received general anesthesia via inhalation of ether and after that, the dorsal surface of thoracic region was shaved and antisepsis was performed over the shaved area. Full thickness excisional skin wounds of 8.5 ± 0.48 mm diameter (an area equivalent to 46.74 ± 0.32 mm²) was made on pinched skin fold of shaved area. Type and size of wound model were very akin to the murine excisional wound model described earlier [21]. 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 [18].

**Sample Collection and Fixation of Tissue**

On completion of 3 weeks animals were sacrificed under deep ether anesthesia and then excised the healed parts of skin with adjacent area. The excised tissues were immersion-fixed in 10% neutral buffered formalin. Blood samples were collected into sterilized vials by direct puncture of heart at the time of sacrifice. Samples were allowed to clot, centrifuged at 2500 rpm for 30 min, the serum was separated and stored in vials and used to assay all biochemical parameters [18].

**Macroscopic Examination**

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

Fixed tissue samples were processed for light microscopical studies. The 5 μm thick sections were stained with Haematoxylin & Eosin (H & E), Masson’s Trichrome (MT), Aldehyde Fuchsin with Fast Green (AF with FG) and PicroSirus Red with Fast Green (PSR 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. Measurements related to epidermal thickness and calculation of healing indices were based on the mathematical model for healing and remodeling matrix [22].

Biochemical Estimation & Analysis

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

b. Enzymatic antioxidant

Serum catalase was assayed by colorimetry as described [23]. 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 [24]. 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. All the results were expressed as mean ± SD and P < 0.05 was considered as statistically significant. Student t test was used for comparing the blood sugar level in DTT group before and after supplementation of d-δ-TRF (P < 0.0001).

RESULT

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, HTT& DTT) remained stable at the end of study period (Table 1). 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 3 weeks supplementation of d-δ-TRF while in DC showed > 500 mg/dl throughout the experimental period (Table 2).

Macroscopic Observations

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

Microscopic Observations

Histomorphometry

In all groups the neoepidermis was developed at the end of 2nd week. However, in treated groups the mean values of neoepidermis 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 (Figs 2 and 3).

Reepithelialization

Complete reepithelialization was noticed in all the groups (controls and treated). On 2nd and 3rd weeks, in treated groups well defined interdigitations at dermoeidermal junction appeared on the entire length of neoepidermis (Figs 4, 5a and 7). On 2nd weeks in HC poorly defined interdigitations at wound margins while in DC absence of interdigitations (Fig. 4). On 3rd weeks

<table>
<thead>
<tr>
<th>Table 1: Body weights (g) of the animals of all groups during the period of study</th>
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<tbody>
<tr>
<td>Groups</td>
</tr>
<tr>
<td>HC</td>
</tr>
<tr>
<td>DC</td>
</tr>
<tr>
<td>HTT</td>
</tr>
<tr>
<td>DTT</td>
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</table>

Note 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>Table 2: Blood sugar (mg/dl) level of the animals of all groups during the period of study</th>
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<tbody>
<tr>
<td>Groups</td>
</tr>
<tr>
<td>HC</td>
</tr>
<tr>
<td>DC</td>
</tr>
<tr>
<td>HTT</td>
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<tr>
<td>DTT</td>
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</table>

Note that the mean blood sugar levels of healthy groups (HC & HTT) remained within normal limits. In DTT 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
in control groups these features were restricted to only at the wound margins (Fig. 5a and 7).

**Cellular components**

At the end of study period, the granulation tissue consists of mainly fibroblasts in all groups. The fibroblasts appeared oval or spindle shaped and scattered in HC. In DC these cells were mainly stellate whereas in treated groups spindle shaped cells lie parallel to the neoeipidermis. More cellularity was observed in control groups as compared to treated groups (Fig. 6).

**Neovascularization**

In treated groups, well formed vertically oriented blood capillaries appeared by the end of 2nd week while they appeared late in HC by the end 3rd week. Swollen capillaries and extravasation of blood cells were seen in DC granulation tissue on 3rd weeks whereas in treated groups less number of vessels was observed on 3rd weeks (Figs 5a and 5b).

**Matrix remodeling and Skin appendages**

On 2nd and 3rd weeks, in treated groups the collagen fibres in the regenerated dermis were mostly horizontally arranged and compactly interwoven but these fibres were more obliquely placed in HC on 3rd weeks. In DC on 2nd weeks collagen fibres were arranged as wavy pattern and on 3rd weeks these fibres were poorly interlaced in the suprahypodermal area (Figs 4, 5a and 7).

The elastin fibres in control groups were found in the wound margins while in the treated groups these fibres

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**Table 3: Border & neoeipidermal thickness (mm; Mean ± SD) at the end of 2nd & 3rd week**

<table>
<thead>
<tr>
<th>Groups</th>
<th>2 weeks</th>
<th>3 weeks</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Border epidermis</td>
<td>Neoeipidermis</td>
</tr>
<tr>
<td>HC</td>
<td>0.078±0.018</td>
<td>0.109±0.013</td>
</tr>
<tr>
<td>DC</td>
<td>0.058±0.017</td>
<td>0.075±0.025</td>
</tr>
<tr>
<td>HTT</td>
<td>0.093±0.017</td>
<td>0.248±0.035</td>
</tr>
<tr>
<td>DTT</td>
<td>0.088±0.014</td>
<td>0.237±0.038</td>
</tr>
</tbody>
</table>

Note that the neoeipidermal thickness in treated groups (HTT & DTT) is thicker than that of their respective epidermal border thickness on 2nd and 3rd weeks

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**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.

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

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

**Figure 4:** Representative images of MT stained sections on 2nd weeks, showing presence of interdigitations and hair follicles at initial magnification x100.
were noticed in one step advanced stage and newly formed smaller fibrils were diffusely arranged in the regenerating dermis on 3rd weeks (Fig. 8).

On 2nd weeks, in control groups the hair follicles were confined to wound margins while in treated groups hair follicles were notice almost in the central part of the wound (Fig. 4). At the end of study period, in treated groups hair follicles and sebaceous glands were in advance stage into the regenerating dermis and newly formed hairs found within the hair follicles and neocidermal surface. In control groups hair follicles and sebaceous glands remained only at the wound margins (Figs 6 and 7).

Biochemical Analysis

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 DTT. Whereas high density lipoprotein (HDL) in DC showed significantly lower (P<0.01) compared to DTT (Table 4).

Serum creatinine level and serum total protein content
Serum creatinine level in DC were significantly higher ((P<0.01) compared to all other groups. Serum total protein content in treated groups (HTT & DTT) showed significantly higher (P<0.01) compared to control groups (HC & DC) (Table 5).

Enzymatic antioxidant and oxidative stress parameter
Serum catalase activity and total antioxidant capacity in treated groups (HTT & DTT) 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).

![Figure 5a: Representative images of MT stained sections on 3rd weeks, showing collagen fibres arrangements and interdigitations. Arrows (→) pointing the presence of capillary vessels at initial magnification x100.](image)

![Figure 5b: Representative images of MT stained sections on 3rd weeks DC showing the presence of swollen capillaries and extravasation of blood cells in the regenerating dermis at initial magnification x400.](image)

![Figure 6: Representative images of H & E stained sections on 3rd weeks, showing arrangement of cells and presence of hairs at initial magnification x400.](image)

![Figure 7: Representative images of PSR with FG stained sections on 3rd weeks, showing collagen fibres arrangement, interdigitations and hairs within the hair follicles at initial magnification x100.](image)
Impaired wound healing is a well-documented phenomenon in both experimental and clinical diabetes [25]. Free radicals impair the normal wound healing by damaging keratinocyte, endothelial cells, capillary permeability and collagen metabolism [26]. Oxidative stress induces cellular dysfunction and retards angiogenesis and the healing process [27]. Thus, elimination of ROS is an important strategy to improve the healing of wounds in diabetes mellitus patients [28]. The unsaturated isoforms of vitamin E e.g., tocotrienols possess excellent antioxidant activity and suppress ROS production more efficiently than saturated forms e.g., tocopherols [29].

In the present study, in DC reduced mean body weight at the end of experimental period but in DTT these were stable throughout experimental period. These findings are in agreement with related study [20] whose had demonstrated that, diabetic group without TRF supplementation showed significantly lower body weight than that of diabetic rat treated with TRF for 4 weeks.

While oral administration of δ-TRF for 3 weeks in DTT revealed reduced mean blood sugar level and in DC showed hyperglycemic state throughout the study period. These results correlate with other study [30] showing that the tocotrienol supplementation significantly increased the insulin levels and reduced the blood glucose in diabetic induced rats in dose dependent manner.

Macroscopic observation of healing wounds revealed remarkable changes in the wound size in treated groups even on14th day, suggestive of faster recovery in the treated groups. The reepithelialization in epidermis is widely accepted to be one of the major processes in wound healing that ensures successful repair [31- 33]. Basal keratinocytes from both the wound edge and epidermal appendages such as hair follicles, sweat glands and sebaceous glands constitute the main sources for cells responsible for the reepithelialization [34].

Thickness of the epidermis is a good indicator for the superficial changes in the wound [22]. The mean values of histomorphological measurement in the present study showed that although the neoepidermis regenerated during the 2nd weeks in all groups, in treated groups the neoepidermal thickness was remarkably higher than the border epidermal thickness at the end of 2nd and 3rd weeks.
The global healing and remodeling indices (GHI and GRI) are used to measure the different stages of skin wound healing and its progress Lemo et al [22]. In cases of stronger wound remodeling the GRI can go up to 1. The mean values of GHI and GRI in the present study were high compared to control groups, indicating the positive therapeutic effects of d-δ-TRF in both healthy and diabetics.

Progression of wound healing revealed that while complete reepithelialization took 3 weeks in control group it took only two weeks in the treated group suggesting that d-δ-TRF promotes wound healing.

The interdigitations at dermoepidermal junction are known to provide both physical and trophic support. In treated groups well developed interdigitations appeared on entire length of neoepidermis on 2nd weeks and were well defined than those in the control groups on 3rd weeks. Therefore, the neoepidermis in treated groups has 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 [25]. On 3rd weeks cellular components were more in control groups than treated groups. The fibroblasts were oval or spindle shaped and scattered in HC whereas in DC these cells were mainly stellate shaped, indicating incomplete dermal regeneration. In treated groups spindle shaped fibroblast lie parallel to the neoepidermis, suggesting that the d-δ-TRF supplementation helps the complete dermal regeneration.

Neovascularization is characterized by well-structured capillary vessels and absence of hemorrhage [25]. Numerous, well formed vertically oriented capillary vessels that run towards the epithelial surface were seen by the end of 3rd week in HC on whereas in treated groups these were observed by the end of 2nd week thus indicating an early and good neovascularization in the treated group. Swollen capillaries and extravasation of blood cells were seen in DC granulation tissue even on 3rd week, pointing towards its poor and delayed neovascularization. As remodeling progresses, there is a gradual reduction in thecellularity and vascularity of the reparative tissue [35]. This finding is supported by the present study as it indicated that the numbers of capillary vessels were reduced in treated groups on 3rd weeks.

At the end of study period, in control groups hair follicles and sebaceous glands remained only at the wound margins. In treated groups hair follicles and sebaceous glands were in advance stage and had their made their presence into the regenerating dermis and even newly formed hairs were found within the hair follicles and on the neoepidermal surface, which indicated a faster healing and quicker remodeling of the wound matrix [37].

The predictors of cardiovascular complications in diabetes are believed to be dyslipidemia and hyperglycemia [40-43]. 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 lower in DC, indicating significant dyslipidemia in untreated diabetic rats [44]. The lower mean values of TC, TG, LDL and VLDL levels and high HDL level were recorded in DTT after 3 weeks treatments. This result is in agreement with related study [45].

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 [44]. In DTT these level were improved after 3 weeks treatment and similar to the level of healthy groups (HC & HTT). The abnormally high level of serum creatinine was consistent with the impaired kidney function [46].
The total protein content is also known to be an indicator for the protein level and cellular proliferation of the wound tissue [47]. The result of present study also indicates that the δ-δ-TRF treatment enhances protein synthesis in treated groups and its level lower in DC, which is in agreement with [44] who found that diabetic rats showed lower serum total protein level and when treated with vitamin E its level improves significantly.

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 [48]. The present study showed that the serum catalase activity was lower in DC. Many other studies [49,50] stated that the catalase activity had decreased in plasma, liver and kidney of diabetic control rats. The decreased catalase activity in plasma and tissues of STZ-diabetic rats may be due to its increased utilization for scavenging the toxic products of lipid peroxidation or due to decreased availability of H₂O₂ [49]. Vitamin E treatment has been shown to normalize the catalase activity in the control group [50]. The result of present study revealed that δ-δ-TRF supplementation enhances the serum catalase activity in treated groups.

Antioxidant capacity of plasma is the primary measure and marker to evaluate the status and potential of oxidative stress in the body [51]. Total antioxidant capacity has been shown to be significantly reduced in plasma and liver homogenate FRAP of diabetic rats compared to control animals [52-54]. The observation of present work with significantly lower (P<0.05) serum FRAP level in diabetic control compared to healthy control is in agreement with the findings of above mentioned workers. In treated groups, after 3 weeks control is in agreement with the findings of present work with significantly lower (P<0.05) serum antioxidant capacity.

CONCLUSION

Based on findings of the present study it is concluded that δ-δ-TRF promotes skin wound healing in both healthy and diabetic rats and thus indicative of its strong therapeutic potential in future in the management of skin wounds.

ACKNOWLEDGEMENTS

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Abbreviations

AF with FG: Aldehyde Fuchsin with Fast Green; DC: Diabetic Control; DTT: Diabetic 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; HTT: Healthy δ-tocotrienol rich fraction treated; ME Masson’s Trichrome; TAC: Total Antioxidant Capacity; PSR with FG: Picro Sirus Red with Fast Green.

REFERENCES


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