Effects of d α-Tocopherol on Progression of Reepithelialization, Matrix Remodeling and Appearance of Epidermal Appendages in Secondary Skin Wounds of Diabetic Rats

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Abstract

In diabetes the delayed wound healing is believed to be due to many reasons such as hyperglycemia, infection, suppressed immunity and oxidative stress. Since vitamin E is an effective antioxidant and its active form is tocopherol, this study was designed to explore its role in wound healing process in both healthy and alloxan-induced diabetic rats. Twenty four albino rats were divided into four groups; healthy control, diabetic control, healthy treated and diabetic treated. Treated groups received d α-tocopherol (200 mg/kg body weight) orally daily for 3 weeks. Under general anesthesia, full-thickness excisional skin wounds were created on the dorsal surface of thoracic region. Progression of wound healing was assessed by macroscopic and microscopic features of wounds recorded at weekly intervals. Serum biochemical parameters were also estimated for each animal at the end of 3 weeks. It was observed that re-epithelialization, matrix remodeling and reappearance of epidermal appendages were earlier in treated groups as compared to control groups and this was also associated with significantly increased serum antioxidant status and total protein content. It is concluded that oral administration of d α-tocopherol promotes skin wound healing in both healthy and alloxan-induced diabetic rats, suggesting that its antioxidant potency is reasonably effective in the management of skin wounds.

ABBREVIATIONS

AF with FG: Aldehyde Fuchsin with Fast Green; DC: Diabetic Control; DPT: Diabetic d α-tocopherol treated; FRAP: Ferric Reducing Antioxidant Power; GHI: Global Healing Index; GR: Global Remodeling Index; HC: Healthy Control; H&E: Haematoxylin & Eosin; HPT: Healthy d α-tocopherol treated; MT: Masson’s Trichrome; TAC: Total Antioxidant Capacity; VVG: Verhoeff Van Gieson

INTRODUCTION

In many ways oxygen plays a pivotal role in the wound healing process such as by oxidative bacterial killing, collagen synthesis and epithelialization. That why it is believed to be one of the reasons that the wound healing process is impaired under hypoxic conditions [1,2]. In the inflammatory phase of wound healing, neutrophils and macrophages arrive at the wound site and secrete large amount of reactive oxygen species (ROS) along with pro-inflammatory cytokines [3]. Since, ROS being cytotoxic, redox balance must be strictly controlled in order to achieve normal wound healing. Interestingly, an optimal ROS level is distinctive for each step of wound healing. Hence, each antioxidative enzyme needs to be fine tuned as per requirement to maintain ROS levels suitable for each process of wound healing [4]. Both non-enzymatic antioxidants (e.g., glutathione, vitamin C, vitamin E) and enzymatic antioxidants (e.g., SOD, GPX, PRDX, and catalase) are involved in the fine tuning of ROS [5]. ROS are also involved in reepithelialization [4]. Superoxide a member of ROS has been shown to be linked with the activation of receptors for epidermal growth factor (EGF) and the keratinocyte growth factor (KGF) [6,7] which also supports the migration and proliferation of epidermal cells.

In a 10 days study [8] it has been shown that topical tocopherol
treatment enhances the rate of wound closure in streptozotocin-induced diabetic rat. Oral administration of both palm vitamin E and α-tocopherol (200 mg/kg) for 10 days significantly reduced oxidative stress markers and improved rates of wound closure in streptozotocin-induced diabetic rats when compared with control group [9,10].

Thus, there are only few short-term (10 days) studies [8-10] conducted on streptozotocin-induced diabetic rats by the time when wound is not fully closed. And therefore, the present study has been undertaken to evaluate the antioxidant effects of d-α-tocopherol on skin wound healing for an extended period of 21 days by using gross, histological, histomorphological and biochemical parameters.

MATERIALS AND METHODS

After clearance from Institutional Animal Ethical Committee (No.8937/2014), 24 albino rats of either sex each weighing 230-320g were obtained from central animal house of JN medical college, AMU, Aligarh. 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 were supplied standard pellet diet.

Induction of Diabetes

After deprivation of food for 4 hours, single dose of alloxan (100 mg/kg of body weight; Alloxan monohydrate from Sigma-Aldrich) was injected subcutaneously at hip region. Food and water were provided after one hour of injection. Blood sugar level was monitored 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 glucose levels of all animals in each group were monitored at weekly intervals.

Experimental Groups

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 treated- HPT and (4) Diabetic d-α-Tocopherol treated- DPT (200mg/kg body weight, orally, daily for 3 weeks. d-α-Tocopherol Myra e capsule [Vitamin E] manufactured by PT Daya- Baria laboratorla Thk, Indonesia; Imported and packed by United laboratories, Inc, 66 United St, Philippines). Dosage of d-α-tocopherol (200mg/kg body weight) was based on previous studies carried out [9,10].

Creation of Skin Wound; Collection and Fixation of Tissue and Blood Samples

Under ether general anesthesia, dorsal surface of thoracic region was shaved and from the pinched thickness of 8.5 ± 0.48 mm diameter (an area equivalent to 46.74 ± 0.32 mm²) excisional wounds were made. Type and size of wound model were very akin to the murine excisional wound model described earlier [11]. Povidone-iodine solution was applied on the wound and 0.5 ml Voveran (analogesic) and 2 mg single shot of Gentamycin (antibiotic) were also injected simultaneously. On completion of 3 weeks animals were sacrificed under deep ether anesthesia and skin bearing healing wound were excised in a manner to include some adjacent normal skin also. 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 subsequently assayed for serum catalase activity, total antioxidant capacity and total protein content.

Macroscopic Examination

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

Histopathology and Histomorphometry

Fixed tissue samples were processed for light microscopical studies. The 5µm thick sections were stained with Haematoxylin & Eosin (H & E), Masson’s Trichrome (MT), Verhoef Van Gieson (VVG) and Aldehyde Fuchsin with Fast Green (AF with FG).

Biochemical Estimation and Analysis

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

b. Enzymatic antioxidant: Serum catalase was assayed by colorimetry as described [13]. The light absorbance of the sample was determined at 620 nm.

c. Non-invasive biomarker (oxidative stress parameter): Serum total antioxidant capacity (TAC) was evaluated using ferric reducing antioxidant power (FRAP) assay [14]. The absorbance of sample was measured at 620 nm using photocolorimeter.

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.

RESULTS

Weight and blood glucose levels of all animals in each group were monitored at weekly intervals. Mean body weight in healthy control (HC) and treated groups (HPT & DPT) remained stable while diabetic control group (DC) showed slight decrement at the end of study period. Mean blood glucose levels of healthy groups (HC & HPT) remained within normal limits while diabetic groups (DC & DPT) showed > 450 mg/dl throughout the experimental period (Table 1,2).
Table 1: Body weights (g) of the animals of all groups during 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>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>266.25 ± 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>

Note the mean body weight in healthy control (HC) and treated groups (HPT & DPT) remained stable while diabetic control group (DC) showed slight decrement at the end of study period.

Table 2: Blood sugar (mg/dl) of the animals of all groups during 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>

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.

Macroscopic Observations

Progressive wound healing was observed in both control and treated groups which lead to gradual decrease in the actual wound areas. However, by the end of 14th day those in treated groups were remarkably smaller than diabetic control (Figure 1).

Microscopic Observations

Histomorphometry: Neoeipidermis in general develops in all groups by the end of 2nd week. However, on completion of 3 weeks, in both the treated groups (HPT & DPT) the neoeipidermis was found to be significantly thicker (P < 0.01) than the thickness of the epidermis on their wound borders (Table 3). During the study period GHI and GRI in HPT were significantly higher (P < 0.01) as compared to all other groups. And even the said values in DPT remained significantly high (P < 0.01) as compared to DC (Figure 2,3) suggestive of beneficial effect of treatment in both healthy and diabetics.

Reepithelialization: At the end of study period, complete reepithelialization were noticed in all the groups and interdigitations at dermoepidermal junction appeared in major portion of the wound in treated groups whereas in control groups these features were restricted at the margins of wounds (Figure 4,5).

Matrix remodeling and Skin appendages: The collagen fibres in the regenerated dermis were mostly horizontally arranged and interwoven compactly in treated groups (HPT & DPT) but they were found to be obliquely placed in HC. Poorly interlaced collagen fibres in the suprahypodermal area were observed in DC (Figure 5,6). Elastin fibres in control groups were found in the wound margins while in the treated groups they were noticed one step beyond the border towards the central part of the wound (Figure 7). In treated groups hair follicles and sebaceous glands were in advance stage into the regenerating dermis whereas in control groups they remained at wound margins (Figure 4,5).

Biochemical Analysis

Effects of d α-tocopherol supplementation on 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
**DISCUSSION**

There are many studies which suggest that hyperglycemia promotes generation of highly reactive free radical and leads to the development of oxidative stress [15] which in turn accelerates the development of diabetes and its associated complications [16]. α-Tocopherol is known to be the most abundant and active form of vitamin E in humans [17,18] and it is also known to have scavenging effect on reactive oxygen species and also has an stabilizing effect on damaged cell membrane [19,20].

Since simple murine excisional wounds provide a valid and reproducible wound model that heals by both contraction and reepithelialization [11], in the present study full thickness
Articular cartilage in the knees was obtained from patients undergoing surgery for knee arthroplasty. The cartilage tissue was harvested using a specialized tool designed to minimize damage to the underlying bone. The tissue was then processed and prepared for analysis.

In the present study, articular cartilage samples were subjected to biochemical analysis to determine the presence of specific markers. The results showed that the cartilage tissue contained high levels of glycosaminoglycans (GAGs) and proteoglycans, which are characteristic of healthy cartilage. The analysis also revealed decreases in the expression of matrix metalloproteinases (MMPs), which are enzymes that degrade cartilage matrix. These findings suggest that the prepared cartilage tissue is suitable for further biomechanical and molecular characterization studies.

Table 4: Effects of d α-tocopherol supplementation on biochemical parameters (Mean ± SD).

<table>
<thead>
<tr>
<th>Serum Analyses</th>
<th>HC</th>
<th>DC</th>
<th>HPT</th>
<th>DPT</th>
</tr>
</thead>
<tbody>
<tr>
<td>Catalase (u/ml)*</td>
<td>0.0672 ± 0.004</td>
<td>0.0438 ± 0.005</td>
<td>0.088 ± 0.004</td>
<td>0.066 ± 0.006</td>
</tr>
<tr>
<td>TAC (µmol/L)</td>
<td>1285.5 ± 67.18</td>
<td>1000 ± 67.88</td>
<td>1481.3 ± 78.02</td>
<td>1309.5 ± 101.12</td>
</tr>
<tr>
<td>Total protein (g/dl)</td>
<td>5.05 ± 0.07</td>
<td>4.5 ± 0.14</td>
<td>5.4 ± 0.15</td>
<td>5.15 ± 0.08</td>
</tr>
</tbody>
</table>

Note that all biochemical parameters reveal significant reduction in diabetic control group (DC) as compared to all other groups (P <0.05). Catalase (u/ml)* indicates the utilization of H₂O₂.

Antioxidant activity of plasma is the primary measure and marker to evaluate the status and potential of oxidative stress in the body [27]. Total antioxidant capacity showed significant reduction (P <0.001) in plasma and liver homogenate FRAP of diabetic rats when compared with control animals [28-30]. The observation of present work with significant reduction (P <0.05) in serum FRAP of diabetic control when compared with healthy control is in agreement with the findings of above mentioned workers. Antioxidant power in diabetic treated group has been found significantly (P <0.05) improved with d α-tocopherol treatment as compared to diabetic control group similar to one observed earlier [29].

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 [31]. It has been shown that the catalase activity in plasma, liver and kidney of diabetic rats are significantly decreased when compared with those of control rats [32,33]. The results of the present study also demonstrated that in diabetic control group serum catalase activity decreased significantly (P <0.05) as compared to healthy control which is in agreement with the observations of above studies [32,33]. It has been suggested that decreased catalase activity in plasma and tissues of streptozotocin diabetic rats may be due to its increased utilization for scavenging the toxic products of lipid peroxidation or due to decreased availability of H₂O₂ [32]. Vitamin E treatment has been shown to normalize the catalase activity in the control group [33]. The findings of present study showing significant (P <0.05) improvement in the catalase activity by supplementation of d α-tocopherol in diabetic treated rats for 3 weeks as compared to diabetic control is in agreement with those of previous study [33].

The total protein content is also known to be an indicator for the protein level and cellular proliferation of the wound tissue [34]. Diabetic rats commonly show marked reduction in serum total protein level and when treated with vitamin E its level improves significantly [35]. The result of present study indicates that the tocopherol treatment enhances protein synthesis in treated groups (HPT & DPT) as compared to control groups (P <0.05, P <0.01). This finding is also in agreement with other previous studies [34,8]. Thus the enhancement of wound healing in the present study is supported by the increased protein level-dependent collagen synthesis. From afore-
mentioned observations it appears that supplementation of the d α-tocopherol leads to increased serum catalase activity, total antioxidant capacity, total protein content, regeneration of epithelium, matrix remodeling and reappearance of epidermal appendages in both treated groups as contrast to control groups.

CONCLUSION

From the present experimental study it is concluded that oral administration of d α-tocopherol promotes skin wound healing in both healthy and alloxan-induced diabetic rats. Therefore, d α-tocopherol seems to hold strong therapeutic potential in the management of skin wounds in future.

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REFERENCES
