The Investigation of the Effect of Topical Vascular Endothelial Growth Factor (VEGF) Administration On Serum Oxidative Parameters in Diabetic Rats

Vildan Sönmez ÇOBAN1-*, Şule Coşkun CEVHER1

1Gazi University, Science Faculty, Biology Department, Beşevler, Ankara, Turkey

ABSTRACT

There is a positive effect of exogen vascular endothelial growth factor (VEGF) application to diabetic wound. This study aims to research the effects of topical VEGF administration on oxidative parameters in rat serum. In this study, 36 male wistar albino rats (weight: 200-250 g) were used. All rats got diabetic with streptozotocin. Dorsalateral excisional wounds (4 cm lengthy) were made on the two sides of medulla spinalis on the rats and they were randomly divided into 3 groups: untreated groups (n=12), chitosan treated (n=12) and chitosan+ VEGF (n=12) (7 ng/ml). In chitosan treated groups, wounds treated topically with equal amount blank chitosan gel. After this administration on the 3 th and 7 th days of wound, animals were sacrificed. NOx, TBARS and RSH levels were measured spectrophotometrically in serum. Results were expressed as mean ± standard deviation. Mean differences were compared by Anova Variance Analysis (p<0,05).

It can be thought that topical VEGF administration on the excisional wound of diabetic rats may have positive effect on the recovery of oxidative damage in serum.

Key Words: VEGF, Diabetes mellitus, Wound Healing, Oxidative Stress, Antioxidant

1. INTRODUCTION

Diabetes Mellitus (DM) is a chronic metabolic disease characterized with hyperglycemia and hyperlipidemia which affects the important parts of the world population.1,2 It has been reported that the prevalence of diabetes for all age-groups worldwide was estimated to be 2.8% in 2000 and 4.4% in 2030. It is estimated that the total number of people with diabetes is projected to rise from 171 million in 2000 to 366 million in 2030.3

This chronic disease causes a number of complications at micro and macro levels, such as impaired wound healing or chronic non-healing wounds.4 The impaired wound healing may be caused by reduced levels of active growth factors5, production of extreme free oxygen radical, production of insufficient NO depending on low production of eNOS, iNOS6,7 in the wound environment.5

It has been known that VEGF is the most prevalent, efficacious and long term signal molecule. Also it stimulates angiogenesis in wound.8 ROS such as H2O2 may support wound healing inducing VEGF expression in human keratinocytes in normal physiologic conditions. VEGF binds VEGF receptor stimulating NOS to produce NO which promote simultoneus vasodilation and vascular permeability.9-11 There are some complex relations between VEGF and oxidative stress. In this stage, it can be though that VEGF may play a vital role to provide a balance between oxidant and antioxidant. Higher levels of oxidative stress in diabetic
wounds stops VEGF gene expression but, oxidative stress in micro level promotes VEGF gene expression.\textsuperscript{12}

It is stated that the production of oxidants initiates VEGF oscillation, after this the production of oxidants not only causes the production of antioxidants but also promotes the production of oxidants due to respiratory explosion.\textsuperscript{5,7,13} This study aims to investigate the effects of topical VEGF administration on oxidative parameters in rat serum as an incisional wound model.

2. MATERIALS AND METHODS

All animal experimental procedures were approved by the Gazi University Local Ethics Committee for Animal Experiments (G.E.T-10.117).

Preparation of the blank chitosan gel and preparation of the (VEGF + chitosan gel)

Chitosan (C-3646) and VEGF (Sigma V3638) were purchased from Sigma-Aldrich (St. Louis, MO). Lactic acid (1 %) was added to half of the required water. The weighed amount of chitosan (3 %) was added and stirred slowly. After swelling, the remaining amount of water was added and mixed. Gel was kept at room temperature overnight before the application to remove the air bubbles. The pH of the gel was measured as 4.7. After the preparation of the chitosan gel, the required amount of VEGF solution was added and the final concentration was 7 ng/ml.

**Animals**

Experiments were performed on 36 adult female Wistar albino rats (200–250 g). Before and during the experiments, the rats were housed in individual cages with unrestricted standard rat chow and water. They were maintained in a 12 h light/12 h dark cycle at room temperature (25 ± 3 °C).

**Excisional wound model**

The rats were anaesthetised with xylazine/ketamine intramuscularly. Their backs were shaved and cleaned with tincture of iodine. 4 cm length - incisional wound models were made on the two sides of medulla spinalis on the rats. The rats were made diabetic with streptozotocin (n=36). The rats were divided into three groups: (1) untreated, (2) chitosan control, and (3) the group on which (VEGF+chitosan) (7 ng/ml) was applied. Each group was divided into two in itself to be sacrificed on the third and the seventh days. Serum TBARS, (NOx) and (RSH) levels were measured spectrophotometrically.

6 rats in each group including 6 separate groups were formed and the groups underwent the following procedures:

<table>
<thead>
<tr>
<th>Table 1. Diabetic experimental groups</th>
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</thead>
<tbody>
<tr>
<td><strong>Groups Untreated groups</strong></td>
</tr>
<tr>
<td>Group 1 untreated, n = 6, on day 3</td>
</tr>
<tr>
<td>Group 2 untreated, n = 6, on day 7</td>
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<tr>
<td><strong>Chitosan treated groups</strong></td>
</tr>
<tr>
<td>Group 3 treated by chitosan, n = 6, on day 3</td>
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<tr>
<td>Group 4 treated by chitosan, n = 6, on day 7</td>
</tr>
<tr>
<td><strong>Chitosan +VEGF treated group</strong></td>
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<tr>
<td>Group 5 treated by chitosan + VEGF, n = 6, on day 3</td>
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<tr>
<td>Group 6 treated by chitosan + VEGF, n = 6, on day 7</td>
</tr>
</tbody>
</table>

3. BIOCHEMICAL ANALYSES

**Determination of TBARS levels**

The samples were prepared according to the method described by Kurtel et al. to determine the plasma MDA levels as a lipid peroxidation indicator. In brief, 0.5 ml aliquots was added to 1 ml of a solution containing 15% TCA, 0.375% TBA, and 0.25 N HCl. Protein precipitant was removed by centrifugation at 10,000 g for 5 min, and the supernatants were transferred to glass test tubes containing 0.02% BHT to prevent further peroxidation of lipids during subsequent steps. The samples were then heated for 15 min at 100°C in a boiling water bath, cooled, and centrifuged to remove precipitant. The absorbance of each sample was determined at 532 nm.\textsuperscript{14,16}

**Determination of RSH levels**

Plasma GSH was determined by spectrophotometric method. In brief, 0.5 ml aliquots of each samples were mixed with 1 ml of a solution containing 100 mM Tris-HCl (pH:8.2), 1% SDS, and 2 mM EDTA. The mixture was incubated for 5 min at 25°C and centrifugated at 10,000 g for 5 min. DTNB (10 mM) was then added to each reaction volume and incubated for 15 min at 37°C to allow for the formation of TNB. The absorbance of each sample was determined at 412 nm. GSH content was calculated assuming a molar extinction coefficient of 13.000 at 412 nm for TNB.\textsuperscript{15}
Determination of NOx levels

The NOx values were given by the sum of nitrite and nitrate, which are the stable end products of NO. NOx levels in plasma were determined by the Griess reaction. Briefly, 0.3 M NaOH and 10% ZnSO4 were added to 0.5 ml samples for deproteinization. This mixture was then centrifuged at 3,000 g for 20 min, then the supernatants were used for the Griess assay. Sodium nitrite and nitrate solutions (1, 10, 50, 100 µM) were used as standards.16

4. STATISTICAL ANALYSIS

Mean differences were compared by Anova Variance Analysis (ANOVA). Values of P< 0.05 were considered to be statistically significant. The results were expressed as the mean ± standard deviation.

5. RESULTS

The results of TBARS, GSH and NOx levels are shown in Table 2.

Table 2. The results of serum TBARS, RSH and NOx levels in diabetic rats

<table>
<thead>
<tr>
<th></th>
<th>TBARS (nmol/ml)</th>
<th>RSH (nmol/ml)</th>
<th>NOx (µmol/l)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Untreated group (n=12)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Day 3</td>
<td>5.19 ± 0.6</td>
<td>236.51 ± 19.21</td>
<td>5.00 ± 1.52</td>
</tr>
<tr>
<td>Day 7</td>
<td>6.40 ± 1.69</td>
<td>72.81 ± 26.34</td>
<td>5.93 ± 1.62</td>
</tr>
<tr>
<td>Chitosan treated groups (n=12)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Day 3</td>
<td>8.06 ± 2.35 a</td>
<td>100.29 ± 33.51</td>
<td>8.20 ± 3.49</td>
</tr>
<tr>
<td>Day 7</td>
<td>6.87 ± 0.50 a</td>
<td>153.17 ± 42.64</td>
<td>9.10 ± 3.02 a</td>
</tr>
<tr>
<td>Chitosan +VEGF treated group (n=12)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Day 3</td>
<td>5.57 ± 1.02 c,d</td>
<td>134.98 ± 56.3 a</td>
<td>7.60 ± 2.79</td>
</tr>
<tr>
<td>Day 7</td>
<td>4.77 ± 1.05 c,d</td>
<td>174.95 ± 46.23 a bc</td>
<td>10.50 ± 2.71 ab</td>
</tr>
</tbody>
</table>

a P< 0.05 When compared to untreated group (3 day)
b P< 0.05 When compared to diabetic group (7 day)
c P< 0.05 When compared to chitosan treated groups (3 day)
d P< 0.05 When compared to chitosan treated groups (7 day)

Results of TBARS levels

Serum TBARS levels in (VEGF + chitosan) group (4.77 ± 1.05 nmol/ml) were found significantly lower (p<0.05) than those of the other two groups (diabetic untreated (6.40± 1.69 nmol/ml) diabetic chitosan (6.87±0.5 nmol/ml).

Results of Serum RSH levels

GSH levels of the untreated group showed a decrease from the third day (236.51±19.21 nmol/ml) to the seventh day (72.81±26.34 nmol/ml), whereas GSH levels in (VEGF + chitosan) group (134.98 ± 56.3 /174.95 ± 46.23 nmol/ml) showed an increase in the same period. GSH levels of the chitosan treated groups (100.29±33.51 / 153.17±42.64 nmol/ml) increased significantly from the third day to the seventh day (p<0.05). On the seventh day, the group which had significantly the highest GSH level was (VEGF + chitosan) group (174.95 ± 46.23 nmol/ml) (p <0.05).

Results of Serum NOx levels

The values for the chitosan treated groups of serum NOx level were (8.2 ± 3.49 / 9.1 ± 3.02 µmol/l) and the (VEGF + chitosan) group were (7.64±2.79 / 10.50±2.71. µmol/l). The levels of NOx increased significantly from the third day to the seventh (p<0.05). The highest level of increase in serum NOx was (VEGF + chitosan) group on the seventh day (p<0.05).

6. DISCUSSION

Wound healing of all phases are under the control of growth factors.17 Growth factors are necessary for wound healing.17 VEGF also an important growth factor in wound healing.18,19 VEGF: vasculogenesis of angiogenesis and lenfangiogenez is a signal which stimulates protein. In particular, it is a growth factor for endothelial cells which have specific effects.20

In the current study, firstly, serum TBARS levels in VEGF + chitosan group were found significantly lower
than those of the other two groups. From this, it can be concluded that exogenous VEGF application reduces the lipid peroxidation in serum.

Secondly, the study found that GSH levels of the untreated group showed a decrease from the third day to the seventh day, whereas GSH levels in VEGF + chitosan group showed an increase in the same period. GSH levels of the chitosan treated groups increased significantly from the third day to the seventh day. On the seventh day, the group which had significantly the highest GSH level was VEGF + chitosan group. As a result, it can be understood that there was a parallelism between VEGF and serum GSH levels. Moreover, VEGF can be stated to enhance the antioxidant capacity of the wound.

Thirdly, the levels of NOx increased significantly from the third day to the seventh. The highest level of increase in serum NOx was VEGF + chitosan group on the seventh day. Therefore, NOx may be considered in the organization of endothelial function in diabetic wound healing process, for there are important physiological actions of nitric oxide: for example, relaxation of vascular smooth muscle, conduction in nerve cells, and has important physiological effects such as antimicrobial activity.

In patients with diabetes, the synthesis and secretion of VEGF deteriorates due to excessive oxidative stress. 11 Oxidative stress can induce several molecular pathways in cells. 12 There are some complex relations between VEGF and oxidative stress. It has been known that in normal physiologic conditions, antioxidants especially glutathione inhibit VEGF oscillations. 13 In this stage, it can be thought that VEGF forms redox balance by balancing among oxidants and antioxidants. It can be said that in diabetic wounds decrised Vegf mRNA. VEGF supplementation to wound environment may promote the degenerated redox balance.

In conclusion, despite the known positive effects of VEGF applications on wound healing, a more detailed examination of drugs and doses is required.

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CONFLICT OF INTEREST

No conflict of interest was declared by the authors.

REFERENCES


