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Vascular Endothelial Growth Factor Supplementation Enhance Skin Antioxidant Capacity in Hyperglycemic Rats

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Highlights

- This paper focuses on oxidative stress during wound tissue healing phases.
- Vascular Endothelial Growth Factor therapy arrange oxidative stress in hyperglycemic rats.
- Topical Vascular Endothelial Growth Factor decreased NOx level in the inflammation phase.

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Abstract

The fundamental reasons for delayed wound healing in diabetic animals include inadequate production of growth factors or their increased devastation. Vascular Growth Factor (VEGF) has a biological role in the healing process of mucosal and skin wounds, especially in the process of new vessel formation. We planned to examine the oxidant-antioxidant events that occur during healing with topical VEGF application in diabetic rats. Experiments were performed 36 adults female Wistar albino rat diabetes induced by streptozotocin. The incisional wounds were made on the dorsal region in the rats. Rats were separated to 3 groups: the untreated (negative control) group (n=12), the chitosan group (n=12), the chitosan + VEGF group (n=12). The treatments were continued for 3 and 7 days, excluding the control and negative control groups. Then, the animals were sacrificed on the 3rd and 7th days of wound healing. Antioxidant and oxidant parameters in skin tissue were measured using biochemical methods. Topical VEGF application was decreased the NOx levels on the 3rd day compared to other groups. Moreover, it increased wound tissue GSH and AA levels, subsequently contributing to the enhance tissue antioxidant capacity. In conclusion, VEGF application increases the antioxidant capacity of the tissue and simultaneously reduces the oxidative stress and thus gives a positive acceleration to the wound healing process.

1. INTRODUCTION

Wound formation is a permanent or temporary impairment of the integrity of the structures that form the skin and mucosal. Wound healing is a renovation activity starting from the time of wounding and consisting of relational stages to put the anatomic and functional features of the wounded tissue back to their regular continuity [1]. Wound healing in a mature tissue generally consists of the following three stages: the start of the inflammation process (hemostasis, inflammation), continuity of proliferation in renewed cells, and shaping of the wound (remodeling, maturation) [2-6]. In the healing process of acute wounds, a sequential, orderly and organized process usually follows each other. However, in chronic wounds, the situation does not progress exactly as desired. Diabetic wounds, which are in the category of chronic wounds, are characterized by high amputation and mortality rates, and studies on this subject are also very up-to-date [7].

Diabetes is a heterogeneous metabolic disorder based on various etiology, leading to multiple complications. In diabetic wounds, angiogenesis and re-epithelization are delayed, and collagen accumulation and endothelial cell proliferation decrease [8]. People with diabetes experience weak healing in many aspects. Invalid immunity functions bring together abnormal healing. Also, slow collagen synthesis and collection and low angiogenesis lay the ground for more downward tension between the wound and

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wound opening [5]. Diabetes is a disease associated with both metabolic effects and increased free radical formation. Oxidative stress arises when the delicate balance between antioxidants and oxidants shifts in favor of oxidants.

Long-term hyperglycemia in diabetics enhance in the formation of mitochondrial reactive oxygen species (ROS), as a result of which the cellular antioxidant system weakens and functional macromolecules are damaged and wound healing is delayed.

Increased free radicals in diabetes cause loss of membrane integrity, structural and functional changes, and genetic mutations in proteins by interacting with all cellular biopolymers. In order to deal with the effects of these harmful radicals, the organism has some defense systems that are enzymatic and non-enzymatic molecules [9-12]. Also, in diabetes, exogenous antioxidants are prescribed, and the effects of free radicals can be overcome [9].

Chitosan is a widely used cationic polymer [13]. Chitosan, formed by deacetylation of chitin shells, exhibits film-forming and gelling properties [14]. In addition to its features such as chitosan biocompatibility and biodegradability, studies have shown that chitosan induces inflammatory cells; thus, it has been shown to promote granulation and organization [15, 16]. Chitosan is frequently used because it has a regulatory contribution on the wound repair by making use of these properties. [17,18]

Under normal conditions, successful wound healing is maintained by the internal balance of growth factors, chemokines, and cytokines, the majority of which are functional polypeptides. [19]. VEGF, which has a multifunctional effect, is a member of the VEGF family and has a specific effect on endothelial cells.[20]. Wound healing is controlled by the VEGF, which is primarily a pro-angiogenic factor. The VEGF is a factor with a 40-45 kDa homodimeric glycoprotein structure, located on chromosome 6p21.3, regulating the permeability of blood vessels [6, 21, 22]. The VEGF binds two high-affinity tyrosine kinases receptors to each other and reveals its effect on vascular cells. These cells are referred to as the VEGFR-1 and the VEGFR-2. It sends signals via these receptors and regulates proliferation, migration, and new blood vessels needed by the endothelial cells [23]. Keratinocytes are the primary source of the VEGF; however, dermal fibroblasts and macrophages are produced as a to respond to wounding [24, 25]. They carry the receptors for the VEGF on the endothelial cells, which do not make the VEGF synthesis. Inflammatory cytokines, hypoxia, NO, ROS, and hormones influence its release. And the VEGF values reach their peak a few days after the injury [26, 27].

The VEGF is the most functional angiogenetic factor in wound healing [24, 28]. Some studies show that the topical VEGF application positively increases the wound healing and closure rates [29, 30]. A study on diabetic mice reported that VEGF-loaded nanoparticle treatment increases wound healing considerably [30]. Again in the diabetic mice model, topical VEGF application increases the wound closure significantly on the different days of wound healing [31], speeds up angiogenesis [32], and is a helpful factor in terms of vascular microsurgery due to its positive effects in vein structure process [33]. In addition, it removes the positive impact on wound healing in diabetic mice to which anti-VEGF antibodies were applied [29].

New vessel formation is essential especially for diabetic wounds to heal themselves and to contribute to other wound healing stages. At the same time, increasing the antioxidant capacity and reducing oxidative stress in this process will also contribute to the recovery of diabetic wounds. In this context, we planned to investigate the changes in the antioxidant capacity and oxidative events that occur in the wound tissue at different days with the application of exogenous VEGF. Consequently, the time-dependent effects of the VEGF was investigated to the dorsolateral incisional wounds formed in the diabetic rats' skin tissue, on oxidative events.

MATERIAL METHOD

2.1. Animals

Adult wistar albino rats (200-250 g, ♀) were housed at the Gazi University Laboratory Animals Raising and Experimental Research Center (GUDAM). With the 6 rats in each group, 6 different rat groups were formed (Table 1). (The rats were first divided into 3 groups, then each group was divided into two subgroups: 3 and 7 day (Table 1)). The experimental part of the study was made in GUDAM. Then they were left in an environment -with each group in a separate cage- which is enlightened in line with the daylight cycle for the duration of the experiment.

Table 1. *Experimental groups*

GROUPS	***PROCEDURE***
1. Untreated Groups (n=12)	*wound created, on day 3 (n = 6)* *wound created, on day 7 (n = 6)*
2. Chitosan Groups (n=12)	*wound created and treatment with chitosan on day 3 (n = 6)* *wound created and treatment with chitosan on day 7 (n = 6)*
3. 3. VEGF + Chitosan Groups (n=12)	*wound created and treatment with VEGF on day 3 (n=6)* *wound created and treatment with VEGF on day 7 (n = 6)*

2.2. The Formation of Diabetic Rat Model

Ethics committee approval was obtained for experimental animals for this study. (GU ET-10.118). To create diabetes, the 36 rats, after 12 h of fasting, were injected with a single dose of 55 mg/kg streptozotocin (STZ) intraperitoneally (i.p.) [34]. 3 days after the daily freshly prepared STZ injection, blood glucose levels were measured with the help of a glucometer to determine whether the rats had diabetes or not, and the rats with blood glucose above 250 mg/dl were counted as diabetes. [16]. Seven days after the creation of diabetes, incisional wound models were formed.

2.3. The Preparation of VEGF-Chitosan Gel Combination

The VEGF preparation used in the experiments (VEGF containing chitosan gel) was prepared at Gazi University, Faculty of Pharmacy, Pharmaceutical Technology Department. Firstly, we worked with the lactic acids with 1% and 1.5%. Chitosan's (Sigma C-3646) gels of 1%, 2%, and 3% were prepared in various lactic acid concentrations. Half of the distilled water was added to lactic acid, and chitosan was added in the required amounts and stirred. It was incubated overnight at 22℃ to remove the bubbles. The gel pH values were measured. As the chitosan gel pH should be close to skin pH (in other terms, pH 4-5), it was decided that the gel with the most relative pH to this one should be used. The components of the resulting formulation were determined as 3% chitosan and 1% lactic acid. The pH of the formulation was measured as 4.7. After chitosan gel was prepared, it was dispersed onto the gel to make the VEGF's (Sigma V3638) final concentration 7 ng/ml, and thus the VEGF containing chitosan gel was prepared.

2.4. The Formation of Incisional Wound Model

The experimental animals were left hungry for one day and were weighed at 10:00 in the morning on a standard scale. They were injected with IM ketamine (Ketalar 50 mg/kg) and xylazine (Rompun 5 mg/kg); thus, general anesthesia was achieved. In the dorsal region in rats, a dorsolateral incisional wound of 4 cm in length was made on both sides of the backbone. Later, the wound lips were adapted with sutures. The paracetamol in drinking water was used at 2 mg/ml to maintain the analgesia after the operation. The rats' wounds in the groups were applied VEGF (7 ng/ml) within a chitosan gel through a topical way once every day and around the same time every day throughout the study. And in the chitosan group, the same amount of empty chitosan gel was applied.

2.5. Biochemical Aanalysis

NOx Determination
The NOx levels were determined in the tissue by the Griess method [35].

TBARs Determination
The TBARs levels were determined in the wound tissue by Cassini et al. method [36].

GSH Determination
The GSH levels were determined in the tissue by Ellman [37].

Ascorbic Acid Determination
AA levels were determined in the tissue by Kuether's method [38].

SOD Determination
The SOD activity in the tissue was studied in compliance with Sun et al. (1988) [39].

2.6. Statistical Analysis

The significance of the differences between data sets was tested with one-way analysis of variance (ANOVA) and Tukey post-hoc test ($p<0.05$ significant).

3. RESULTS

All biochemical parameters are shown in Table 2.

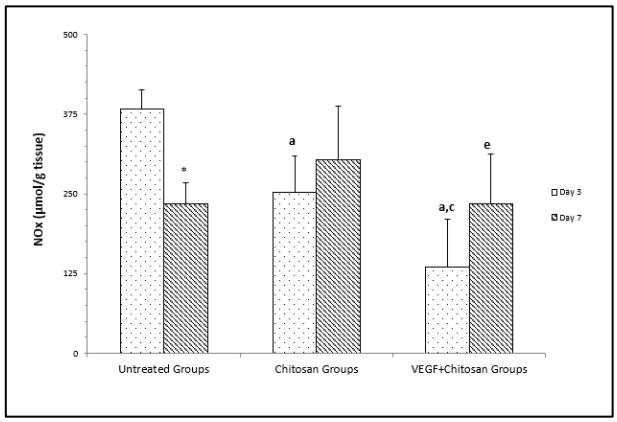
Table 2. *All biochemical results of the skin tissues*

Groups		***NOx*** ***(µmol/g tissue)***	***TBARs*** ***(nmol/g tissue)***	***GSH*** ***(µmol/g tissue)***	***AA*** ***(mg/g tissue)***	***SOD*** ***(U/g tissue)***
Untreated Groups	***Day 3 (A)***	383.61 ± 30.06	22.12 ± 5.37	0.84 ± 0.32	0.10 ± 0.01	286.75 ± 11.07
	Day 7 (B)	233.85 ± 33.09[a]	22.15 ± 6.72	1.18 ± 0.79	0.10 ± 0.01	295.46 ± 7.36
Chitosan Groups	***Day 3 (C)***	252.86 ± 55.93[a]	20.96 ± 5.63	1.33 ± 0.65	0.13 ± 0.03	293.42 ± 13.37
	Day 7 (D)	303.54 ± 83.77	26.50 ± 5.41	1.57 ± 0.96	0.16 ± 0.01[b]	285.86 ± 9.24
VEGF + Chitosan Groups	***Day 3 (E)***	134.54 ± 75.99[a,c]	33.02 ± 5.26[a,c]	2.55 ± 1.17[a,c]	0.15 ± 0.02[a]	293.69 ± 3.82
	Day 7 (F)	233.69 ± 78.17[e]	33.43 ± 8.86[b]	1.13 ± 0.39[e]	0.14 ± 0.02[b]	289.03 ± 4.28

[a] $p<0.05$ as compared to A
[b] $p<0.05$ as compared to B
[c] $p<0.05$ as compared to C
[d] $p<0.05$ as compared to D
[e] $p<0.05$ as compared to E

3.1. NOx Levels

With exogenous VEGF application, tissue NOx levels diminished on both days. The NOx levels displayed a significant decrease on day 7 compared to day 3 in the negative control group ($p<0.05$). On day 3 in the chitosan group, it was compared to the 3rd day of the untreated group, and a substantial decrease in the NOx was detected ($p<0.05$). Upon comparing the VEGF group's 3rd day to the same day of other groups, a significant decrease in the NOx was detected ($p<0.05$). A meaningful increase was seen on day 7 of the VEGF group compared to day 3 ($p<0.05$). When the chitosan group was evaluated within itself, no notable difference was found regarding NOx levels ($p>0.05$) (Figure 1).

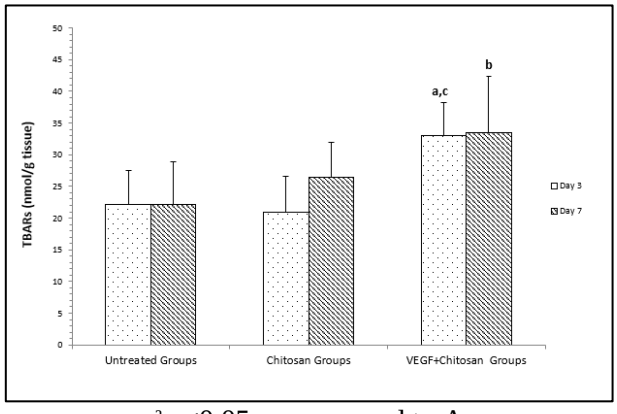


[a] $p<0.05$ as compared to A
[b] $p<0.05$ as compared to B
[c] $p<0.05$ as compared to C
[d] $p<0.05$ as compared to D
[e] $p<0.05$ as compared to E

Figure 1. *NOx levels*

3.2. TBARs Levels

With exogenous VEGF application, tissue TBARs levels increased both on days. When the chitosan group and untreated group compared each other, no remarkable difference was found regarding TBARs levels between the 3rd and 7th day ($p>0.05$). The TBARS levels of group E and F increased when compared to the other two groups ($p<0.05$) (Figure 2).

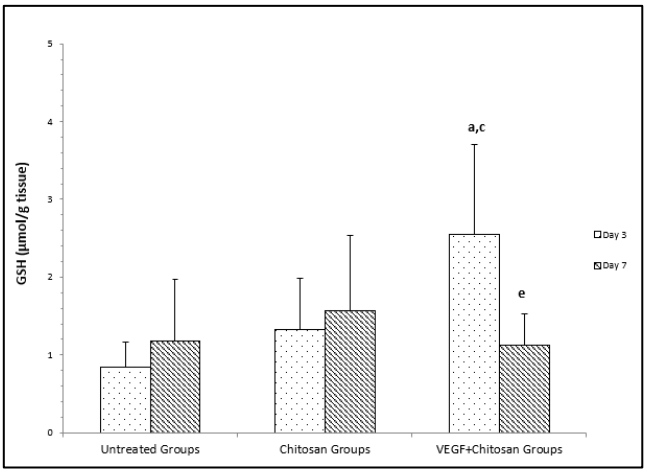


[a] $p<0.05$ as compared to A
[b] $p<0.05$ as compared to B
[c] $p<0.05$ as compared to C
[d] $p<0.05$ as compared to D
[e] $p<0.05$ as compared to E

Figure 2. *TBARs levels*

3.3. GSH Levels

With VEGF application, tissue GSH levels increased on the 3rd day when compared to all groups ($p<0.05$). When the VEGF group was evaluated within itself, it was detected that the GSH levels displayed a meaningful decrease on the 7th day ($p<0.05$). Upon the comparison of untreated and chitosan groups evaluated within themselves, no remarkable difference was detected between the 3rd and 7th days ($p>0.05$) (Figure 3).

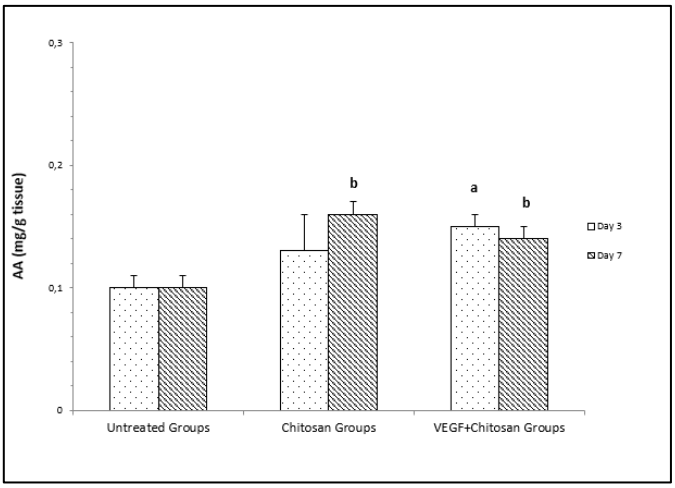


[a] $p<0.05$ as compared to A
[b] $p<0.05$ as compared to B
[c] $p<0.05$ as compared to C
[d] $p<0.05$ as compared to D
[e] $p<0.05$ as compared to E

Figure 3. GSH levels

3.4. AA Levels

Like the GSH levels, tissue AA levels with VEGF application were significantly increased on both days of wound healing compared to untreated groups. When day 7 of the chitosan group was compared to group B a meaningful increase was identified in the AA levels ($p<0.05$). When the VEGF + Chitosan 3rd day was compared to group A, a significant increase was identified in the AA levels ($p<0.05$). When the VEGF + Chitosan 7th day was compared to group B, a meaningful increase was identified in the AA levels ($p<0.05$) (Figure 4).

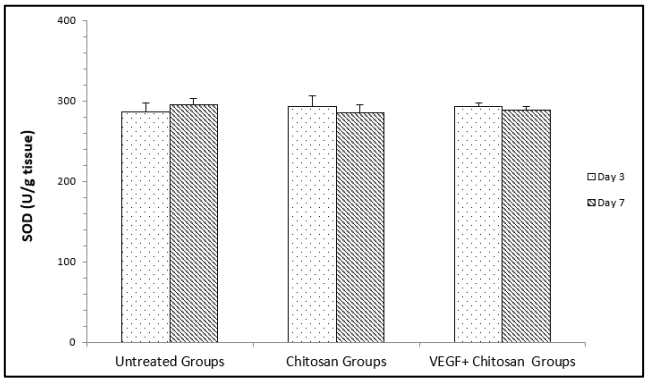


[a] p<0.05 as compared to A
[b]p<0.05 as compared to B
[c]p<0.05 as compared to C
[d]p<0.05 as compared to D
[e]p<0.05 as compared to E

Figure 4. *AA levels*

3.5. SOD Activities

In terms of the SOD activity, the values of the 3rd and 7th days were compared both among the groups and within the groups, and no statistically meaningful difference was found (p>0.05) (Figure 5).



[a] p<0.05 as compared to A
[b]p<0.05 as compared to B
[c]p<0.05 as compared to C
[d]p<0.05 as compared to D
[e]p<0.05 as compared to E

Figure 5. *SOD activities*

4. DISCUSSION

Our findings are quite remarkable in terms of revealing the role of VEGF application, which has an antioxidant effect, both in the regulation of inflammation and in increasing the antioxidant capacity of the wound in the experimental model of STZ-induced diabetes in rats.

Compared to group A, chitosan, which we used as a carrier, significantly reduced tissue NOx levels. In line with this finding of our study, Hwang et al. [40] also stated that chitin and chitin-like molecules suppress nitric oxide production in the cell culture medium and similarly, chitosan may have suppressed nitric oxide synthesis, especially inflammation phase. It can be said that exogenous application of another growth factor, Platelet-derived Growth Factor (PDGF), in two different studies using chitosan as a carrier, reduced the NOx levels of the wound tissue in both normoglycemic and hyperglycemic rats, and in fact, the presence of chitosan in the wound tissue had an effect on this decrease. [16, 34]. Although the carriers are the same, different NO levels on different days may be due to due to the difference in wound healing processes of normal and diabetic rats.

Contrary to the results of our study, Sönmez and Coşkun-Cevher found a statistically significant increase in NO levels in the groups that received both chitosan and VEGF in the serum, in contrast to the NO levels of the wound tissue [18]. This difference may be due to the fact that it represents the whole of NO levels (especially iNOS derived) that change systemically rather than locally. In another study, Di et al. [41] reported that VEGF-B supplementation has been successful in diabetic corneal nerve regeneration by reducing oxidative stress in diabetic neurons using the PI-3K/AKT-GSK3β-mTOR signaling pathway. In fact, in this study, the direction of oxidative events in the wound tissue was tried to be determined by VEGF application. We should think that with the topical application of exogenous VEGF to the healing wound, this unique growth factor itself can act as an antioxidant molecule in biochemical events and provide success with a new therapeutic approach, especially in prolonged inflammation in diabetic wounds. As a matter of fact, the finding of the study conducted by Di et al. [41] who have achieved successful results with this molecule, which we have applied here and which comes from the same superfamily, and which shows a very high homology with VEGF-B, also support us here.

Tissue TBARS levels as an indicator of lipid peroxidation is a proven parameter that has been used in the scientific community for a long time. In order to better understand oxidative events in wound healing processes, the indirect effect of oxidative events in the relevant time period in the wound tissue is shown by tissue TBARS levels. Following diabetes, on day 3 of the group which applied VEGF, the level of TBARs, the final product of lipid peroxidation, increased remarkably compared to the other groups. In our study, an unexpected sudden TBARS level was detected with VEGF application in both inflammation and proliferation phases following diabetic wound healing. It is seen that various growth factor applications have different results in terms of lipid peroxidation in wound healing studies performed with either normoglycemic or hyperglycemic rats [16, 34, 42]. In addition, MDA levels of tissue in the PDGF supplementation group with the chitosan greatly increase on day 3 but, tissue MDA levels diminish on day 7 [16]. Besides, the tissue MDA levels on day 7 decreased on the same days in all treatment groups, unlike the control [43].

In another study, the wound tissue MDA levels with the PDGF supplementation at a great rate rise on day 3 but this increase not statistically significant on day 7 compared to all groups. [34]. In another wound healing study, but in a systemic study examining serum TBARs levels, serum MDA levels decrease in the VEGF application with chitosan group when compared to all groups and exogenous administration of VEGF regulated oxidative events in serum with a reducing effect. [18]. In our study, TBARS levels of the tissue precipitately increased in the VEGF application group on all days and when compared to all groups in the diabetic rats. The unexpected results of wound tissue TBARs can be attributed to the effect of oxidative events resulting from high blood sugars of our diabetic experimental animals, and the partial insufficiency of the dose of VEGF, especially in the wound tissue or the exogenous VEGF may also be due to premature degradation by prolonged diabetes. In addition, the success of VEGF in wound healing under oxidative events may be masked by diabetes, which is too high to be eliminated, and the adverse effects and complications of diabetes. In the future, exogenous VEGF applications can be planned in different doses, durations, and easily accessible to the wound area and usable by the cells in the wound bed.

Glutathione is synthesized in almost all eukaryotic cells and approximately 85-90% is found in the cytoplasm and also it was recently reported to upregulate GPx activity. In addition, GSH provides amino acid transport across the plasma membrane and regenerates some important antioxidants. Vitamin E and vitamin C are regulated by GSH [44]. Therefore, there is a close relationship between GSH and vitamin C.

In the literature review, many studies have been found that reveal the role of GSH in wound healing. There are studies on the fact that GSH levels, which are at the forefront of the wound healing process and are the first line of defense against free radicals, are consumed in the scar tissue to reduce oxidative stress in late-healing or chronic wounds [45]. In studies with some growth factors in the presence of chitosan in both normoglycemic and hyperglycemic animals, it has been reported that the GSH capacity of the scar tissue increases [16, 34, 43, 46]. It is known that there is a tight relationship between VEGF-B and enzymes in antioxidant pathways, especially there is an accelerated upward increase in gene expression between Gpx1 and other antioxidative genes [47, 48]. Like VEGF B, VEGF A can be considered as a potent antioxidant because they are from the same VEGF family, function through the same receptor, have with approximately 47 % homology, and have sulfhydryl groups in terms of molecular structure. In the light of all this information, they share a potentiating pathway between VEGF A and GSH, Gpx1 an important intracellular antioxidant in our study. As a result, it can be said that VEGF A application contributes to wound healing by enhancing the antioxidant capacity over GSH levels. New pathways for VEGF A to regulate intracellular GSH levels through e-NOS activity were announced to the scientific world in 2007 by Langston et al. [49]. Finally, GSH positively regulates VEGF A-induced eNOS activity. Similarly, in our study, VEGF application decreases the NOx levels in the inflammation phase of the wound healing (day 3), while the GSH levels of the wound tissue increase in order to provide this aforementioned regulation. It can be seen that exogenous VEGF administration, with a system that actually controls or supervises each other in the inflammatory phase of wound healing, regulates oxidative events with a modulation between NO levels and GSH levels in the wound, while the other decreases.

Vitamin C plays a physiological role in a very broad perspective in many crucial events such as immune enhancement, immunomodulation, wound healing, neurotransmitter release, and collagen production [50]. Vitamin C itself, in a complex process such as wound healing, is especially effective in the inflammation phase as an immunomodulatory and in all phases of wound healing with its contribution to collagen synthesis. In wound healing models of growth factors, PDGF administration has been shown to increase AA levels at 3 and 7 days of wound healing [16,43]. In models with human studies, AA levels have also been reported to be elevated in both diabetic and nondiabetic patients [51]. There are also studies in the literature that contradict this situation [34, 45]. In our study, parallel findings were obtained with the findings of Gökşen et al. [34] and VEGF increased tissue AA levels.

It can be said that there is a controlled feedback between this unique growth factor VEGF and tissue AA levels that includes all phases of wound healing in terms of reducing oxidative stress and increasing antioxidants. In addition to, due to its molecular nature, VEGF A may have shown antioxidant properties just like VEGF B and regulated the levels of wound tissue AA over GSH, because it is known that tissue AA levels are also affected when GSH changes in the wound tissue. With exogenous EGF application, Güleç-Peker et al. detected a change in the GSH and vitamin C levels in the wound tissue at 1,3 and 5 days, affecting each other's turnover [52]

SOD activity in serum increased in patients with diabetes [51, 53]. It has been reported that SOD enzyme activity did not change in the experimental wound healing model [16,34], whereas wound healing increased in the proliferation phase, that is, only on the 7th day, in PDGF-treated rats [43]. In our study, tissue SOD activity were not change in the all experimental groups. The SOD enzyme activity did not change significantly because the scavenging of radicals that occur in diabetic wound healing may have occurred in the first place and directly through other antioxidant enzymes such as GSH and AA.

5. CONCLUSION

Chitosan, which we used as a carrier of VEGF in this study, effectively reduced tissue NO levels in the inflammation period of wound healing. Thus, the biocompatible chitosan itself provided this by suppressing NO synthesis. VEGF itself, on the other hand, contributed to this formation by being effective especially in the proliferation phase of wound healing, reducing tissue NO levels independently of chitosan. Both chitosan and VEGF applications showed a regulatory effect by increasing the antioxidant capacity over GSH and AA, turning the increased oxidative events into a positive direction. In order to better understand

the relationship of topical VEGF application with oxidative events in wound tissue, more comprehensive studies on the basis of days and doses are needed in the future.

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CONFLICTS OF INTEREST

No conflict of interest was declared by the authors.

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