

## Histopathological and Immunohistochemical Study of the Effect of Sildenafil Citrate, Vitamin A, Vitamin C and Vitamin E on Wound Healing in Alloxan-induced Diabetic Rats

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### ABSTRACT

**Objective:** To examine the impact of sildenafil citrate, vitamin A (Vit A), vitamin C (Vit C) and vitamin E (Vit E) on wound healing in a rat model of diabetes (n = 100).

**Methods:** Diabetes was induced in the rats by intraperitoneal (i.p.) administration of alloxan. After anaesthesia, a standard wound was created on the back of each rat using a 10-mm sterile punch. The rats were randomly divided into 10 groups (n = 10 in each), as follows: normal saline, glibenclamide, insulin, sildenafil, Vit A, Vit C, Vit E, Vit A + sildenafil, Vit C + sildenafil, Vit E + sildenafil daily for 15 days. The rats were sacrificed after being anaesthetized 3, 7 and 15 days later. Wounded skin tissue samples were collected for histopathological and immunohistochemical analyses.

**Results:** On the 7<sup>th</sup> day, epithelial regeneration was completed in groups 8 and 9. Angiogenesis was insufficient in group 2. In terms of connective tissue proliferation, partially matured connective tissue was observed in group 4. On the 15<sup>th</sup> day of the study, groups 8, 9 and 10 had mature connective tissue. However, group 1 still had exudate-containing neutrophils. Immunohistochemically, on the 3<sup>rd</sup> day, the level of inducible nitric oxide synthase (iNOS) reactivity was intense in macrophages and neutrophils surrounding the wound edges in groups 4, 8, 9 and 10. The level of iNOS reactivity was moderate in group 6 and less distinct in groups 1, 2, 3, 5 and 7.

**Conclusion:** Sildenafil citrate, together with Vit A and Vit C, is beneficial in wound healing of diabetic rats.

**Keywords:** Diabetes, sildenafil, vitamin A, C, E, wound healing.

### INTRODUCTION

Diabetes mellitus is a chronic metabolic disorder characterized by hyperglycaemia due to a lack of or resistance to insulin. Patients with diabetes mellitus frequently have ischaemic vascular disease or wound-healing defects. It is well known that type 2 diabetes mellitus causes amplification of the atherosclerotic process, endothelial cell dysfunction, glycosylation of extracellular matrix proteins, and vascular denervation. These complications

ultimately lead to impairment of neovascularization and diabetic wound healing (1).

Wound healing involves a complex chain of cellular and biochemical events designed to restore tissue integrity and function. There are two major components of wound healing in the early phase: an inflammatory stage and a new tissue formation stage. In the inflammatory stage, various inflammatory cells, such as polymorph nuclear neutrophil cells and macrophages, infiltrate the injured area. In the new tissue formation

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stage, fibroplasia begins with the formation of granulation tissue within the wound space (2).

Sildenafil is a phosphodiesterase-5 inhibitor usually used in the treatment of erectile dysfunction, as it enhances vasodilatation and increases tissue blood flow by relaxing the smooth muscle in the vessels; it also inhibits platelet aggregation and improves microcirculation (3). Sildenafil stimulates the release of nitric oxide (NO) at cellular and endovascular levels, and this relaxes the arterial walls and increases blood flow. The upregulation of NO has a positive influence on multiple aspects of wound healing, including angiogenesis, inflammation, endothelial and epithelial cell proliferation, matrix deposition and remodelling (4).

The purpose of this study was to histopathologically and immunohistochemically investigate the effect of sildenafil citrate, vitamin A (Vit A), vitamin C (Vit C) and vitamin E (Vit E) on wound healing in alloxan-induced diabetic rats.

## MATERIALS AND METHODS

All the experiments in this study were performed in accordance with the guidelines for animal research of the National Institutes of Health and were approved by the Committee on Animal Research at Yuzuncu Yil University, Van, Turkey.

### Chemicals

Alloxan monohydrate (Sigma Aldrich, St. Louis, MO, USA; stored at 4°C), glibenclamide (Nobel, İstanbul, Turkey), normal saline (Adeka, İstanbul, Turkey), insulin (Novo Nordisk, Denmark, France), sildenafil (Pfizer, İstanbul, Turkey), Vit A (AksuFarma, İstanbul, Turkey), Vit C (Bayer, İstanbul, Turkey), and Vit E (AksuFarma, İstanbul, Turkey) were used in this study.

### Animals

A total of 100 adult male and female Swiss albino rats, weighing approximately 200–300 g, were used in this experimental study. The laboratory animals were supplied by Atatürk University Laboratory Animals Unit, Erzurum, Turkey. They were accommodated in standard cages, and food and water were provided *ad libitum*. The animals were housed in a room at a temperature of 22 ± 2°C, with 12 hours of darkness and 12 hours of light. The animals were not fed for 18 hours before each treatment. Before starting the study, the approval of the Ethics Council of Yuzuncu Yil University Medical Faculty was obtained.

### Alloxan-induced diabetes

Hyperglycaemia was induced by an intraperitoneal (i p) injection on three consecutive days of alloxan monohydrate (120 mg/kg), which was dissolved in distilled water (5%). Diabetes was confirmed 3 days after the last alloxan dose by determining the blood glucose concentration (day 6). Only animals with blood glucose levels over 250 mg/dL were used (5).

### Surgical procedure

The rats were anaesthetized. Their backs were shaved and prepared with 10% antiseptic povidone-iodine solution (Batticon, Adeka, İstanbul, Turkey). A circular, full thickness, standard wound was created on the back of each rat using a 10-mm sterile punch.

### Animal experiments

The rats were randomly divided into 10 equal groups (n = 10 in each): group 1: control group, normal saline (1 ml/day i.p.); group 2: glibenclamide (0.5 mg/kg/day orally); group 3: insulin (0.5 IU/kg/day i.p.); group 4: sildenafil (0.5 mg/kg/day i.p.); group 5: Vit A (4000 IU/kg/day orally); group 6: Vit C (100 mg/kg/day i.p.); group 7: Vit E (100 mg/kg/day i.p.); group 8: Vit A (4000 IU/kg/day orally) + sildenafil (0.5 mg/kg/day i.p.); group 9: vitamin C (100 mg/kg/day i.p.) + sildenafil (0.5 mg/kg/day i.p.); group 10: Vit E (100 mg/kg/day i.p.) + sildenafil (0.5 mg/kg/day i.p.).

The wounds were clinically observed in all groups every day. Three, 7 and 15 days later, the rats were sacrificed after being anaesthetized.

### Histopathological examination

Wounded skin tissue samples were collected after sacrificing the rats for histopathological examination purposes. The tissue samples were fixed in 10% neutral-buffered formalin solution, embedded in paraffin wax, cut into 5-µm-thick sections, and stained with haematoxylin and eosin stain for examination by light microscopy.

### Immunohistochemical examination

Immunohistochemical staining for inducible nitric oxide synthase (iNOS) was performed using the avidin-biotin immunoperoxidase complex method (Universal LSAB 2 kit, DAKO, CA, USA). As primary antibody, anti-human monoclonal mouse anti-serum (Thermo Scientific, Waltham, MA, USA; 1:100) was used. All the tissue sections were deparaffinized in xylene and rehydrated in graded alcohol. Endogenous peroxidase was blocked by immersing the sections in 0.3% hydrogen peroxide

in absolute methanol for 30 minutes. The sections were washed with phosphate-buffered solution (PBS) and pre-treated for 5 minutes with a protein blocker. All the sections were then washed with PBS and incubated for 2 hours at room temperature with the primary antibody. After washing with PBS, the sections were incubated for 20 minutes with biotinylated goat anti-rabbit antibodies at room temperature. After another PBS rinse, the sections were treated with streptavidin–horseradish peroxidase complex for 20 minutes. After washing in PBS, aminoethyl carbazole was used as a chromogen and Mayer's haematoxylin was used for counterstaining. Non-immunized mouse or rabbit serum was used as a negative control (6).

## RESULTS

### Histopathological findings

Similar wound healing was observed among the groups of rats that were sacrificed on the 3<sup>rd</sup> day of the study. Almost all the groups had a mass on top of wound sites that consisted of various amounts of necrotic exudate. Underneath necrotic exudate, a mass consisting of macrophages, microphages, erythrocytes and plasmatic exudate was observed. In the lower sections, active connective tissue proliferation and new capillary proliferation were observed. On the 3<sup>rd</sup> day of the study, angiogenesis and inflammatory cells were more distinct in groups 4, 8, 9 and 10 than in the other groups (1, 2, 3, 5, 6 and 7).

On the 7<sup>th</sup> day of the study, epithelial regeneration was completed in groups 8 and 9 (Fig. 1H, 1J). Epithelial regeneration was normal in groups 4, 5, 6, 7 and 10 (Fig. 1D–1G, 1K) and had just started in groups 1, 2, 3 and 7 (Fig. 1A–1C). Angiogenesis was insufficient in group 1 (Fig. 1A). Angiogenesis was sufficient in groups 2, 3, 5, 6 and 7 (Fig. 1B–1G), but it was more marked in groups 4, 8, 9 and 10 (Fig. 1D, 1H, 1J, 1K). In terms of connective tissue proliferation, partially matured connective tissue was observed in groups 4 and 9 (Fig. 1D, 1J), and slack connective tissue was widespread in the other groups (Fig. 1A–H, 1K). Connective collagen-rich tissue was observed in group 9 (Fig. 1J). Inflamed cells were observed in group 1 (Fig. 1A). A small amount of neutrophils was observed in group 7 (Fig. 1G), and lymphoplasmocytic and isolated neutrophils were observed in groups 2, 3, 5, 6 and 10 (Fig. 1B, 1C, 1E, 1F, 1K). Only mononuclear cells were observed in groups 4, 8 and 9 (Fig. 1D, 1H, 1J). Necrotic exudate containing a small amount of neutrophils was observed in group 3,

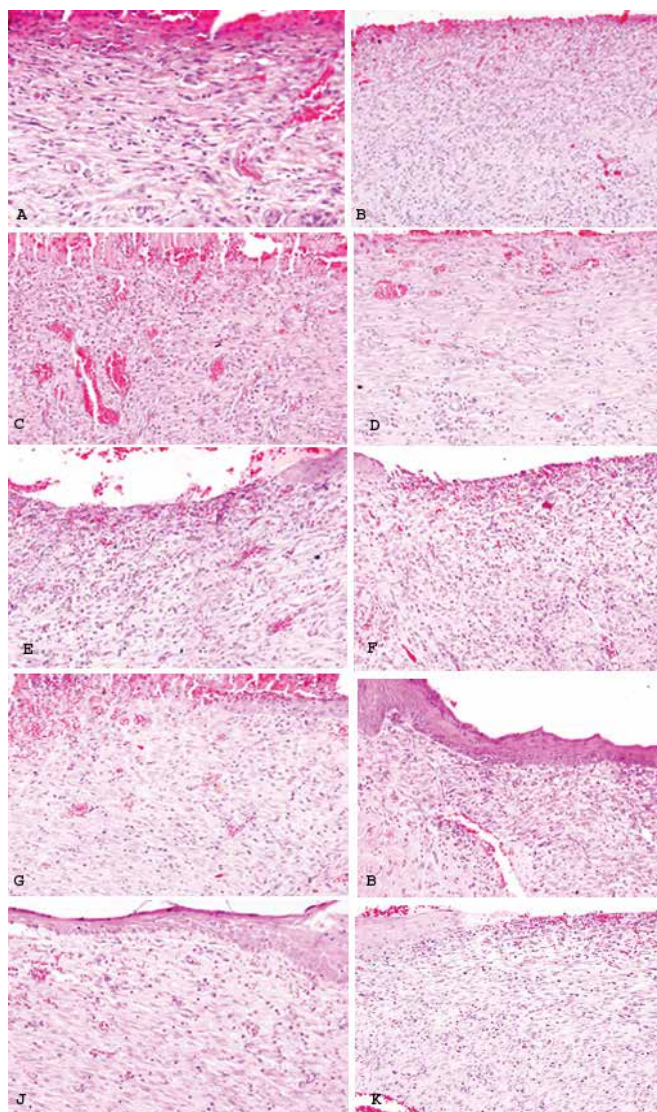


Fig. 1: Histopathological findings of wound healing in dermis and epidermis in rats sacrificed on the 7<sup>th</sup> day of the study (A) Group 1 (control group) H&E,  $\times 200 \mu\text{m}$ . (B) Group 2 (glibenclamide) H&E,  $\times 200 \mu\text{m}$ . (C) Group 3 (insulin) H&E,  $\times 200 \mu\text{m}$ . (D) Group 4 (sildenafil) H&E,  $\times 200 \mu\text{m}$ . (E) Group 5 (vitamin A) H&E,  $\times 200 \mu\text{m}$ . (F) Group 6 (vitamin C) H&E,  $\times 200 \mu\text{m}$ . (G) Group 7 (vitamin E) H&E,  $\times 200 \mu\text{m}$ . (H) Group 8 (vitamin A + sildenafil) H&E,  $\times 200 \mu\text{m}$ . (J) Group 9 (vitamin C + sildenafil) H&E,  $\times 200 \mu\text{m}$ . (K) Group 10 (vitamin E + sildenafil) H&E,  $\times 200 \mu\text{m}$ .

and necrotic exudate containing neutrophils and macrophage was observed in group 7 (Fig. 1C, 1G).

On the 15<sup>th</sup> day of the study, epithelial regeneration was completed in groups 4, 8, 9 and 10 (Fig. 2D, 2H, 2J, 2K), and it was at an advanced level in groups 2, 3, 5, 6 and 7 (Fig. 2B–2G). On the other hand, epithelial regeneration was not observed at all in group 1 (Fig. 2A). The level of angiogenesis and inflammatory cells were similar to those observed on the 7<sup>th</sup> day. Connective tissue proliferation, in the form of slack connective tissue, was

apparent in groups 1, 2, 3 and 5 (Fig. 2A–2E). Groups 6 and 7 had both slack and firm connective tissue (Fig. 2F, 2G). Partially mature connective tissue was present in group 4 (Fig. 2D), and mature connective tissue was present in groups 8, 9 and 10 (Fig. 2G, 2H). However, the maturity of the connective tissue in group 9 was more marked (Fig. 2H). On the 15<sup>th</sup> day of the study, group 1 still had exudate-containing neutrophils (Fig. 2A).

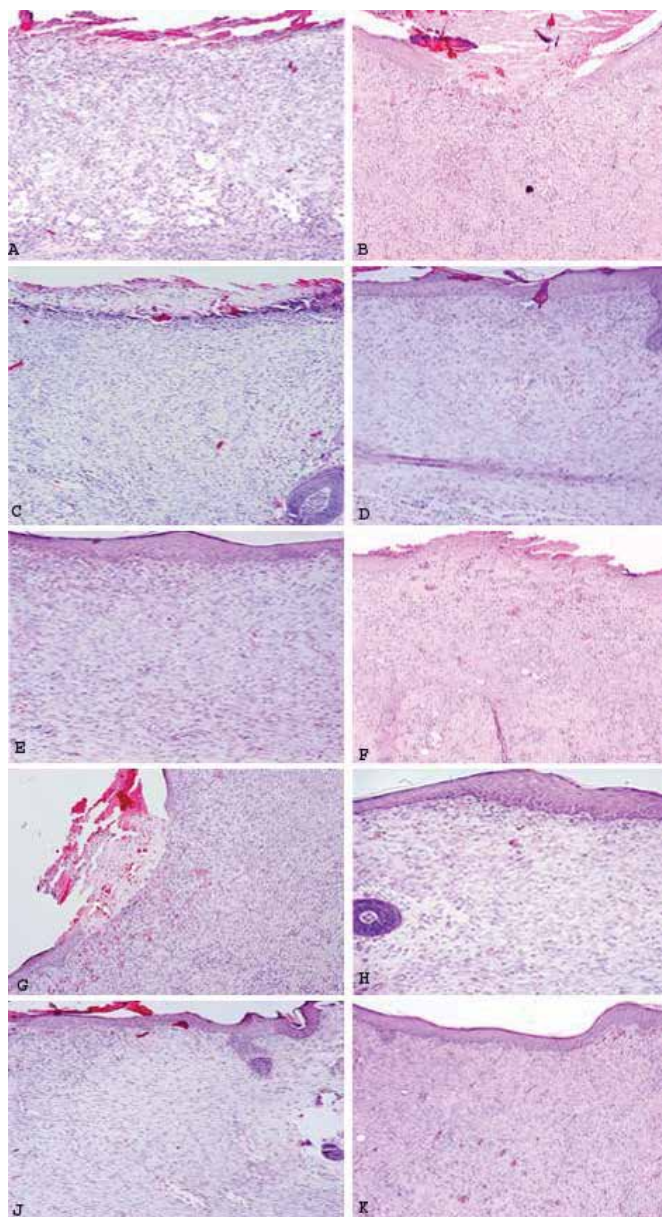


Fig. 2: Histopathological findings of wound healing in dermis and epidermis in rats sacrificed on the 15<sup>th</sup> day of the study (A) Group 1 (control group) H&E,  $\times 200$   $\mu\text{m}$ . (B) Group 2 (glibenclamide) H&E,  $\times 200$   $\mu\text{m}$ . (C) Group 3 (insulin). H&E,  $\times 200$   $\mu\text{m}$ . (D) Group 4 (sildenafil) H&E,  $\times 200$   $\mu\text{m}$ . (E) Group 5 (vitamin A) H&E,  $\times 200$   $\mu\text{m}$ . (F) Group 6 (vitamin C) H&E,  $\times 200$   $\mu\text{m}$ . (G) Group 7 (vitamin E) H&E,  $\times 200$   $\mu\text{m}$ . (H) Group 8 (vitamin A + sildenafil) H&E,  $\times 200$   $\mu\text{m}$ . (J) Group 9 (vitamin C + sildenafil) H&E,  $\times 200$   $\mu\text{m}$ . (K) Group 10 (vitamin E + sildenafil) H&E,  $\times 200$   $\mu\text{m}$ .

### Immunohistochemical findings

On the 3<sup>rd</sup> day, in groups 4, 8, 9 and 10, the iNOS reactivity of macrophages was intense, and neutrophils surrounded the wound edges (Fig. 3D, 3H, 3J, 3K). The level of iNOS reactivity was moderate in group 6, but less distinct in groups 1, 2, 3, 5 and 7 (Fig. 3A–E, 3G). On the 7<sup>th</sup> day, iNOS reactivity was intensive in groups 1, 2 and 3 and moderate in groups 5, 6 and 7. It was slight in groups 4 and 10 and barely detectable in groups 8 and 9. The level of iNOS reactivity was significant in group 1, which showed complete epithelial regeneration on the 15<sup>th</sup> day, but it was insignificant in the other groups.

### DISCUSSION

In the present study, on the 3<sup>rd</sup> day of wound healing, inflammatory cells were evident in the wound areas of all the groups (Fig. 1A). On the 7<sup>th</sup> and 15<sup>th</sup> days, the infiltration of inflammatory cells continued in groups 2, 3, 6, 7 and 10; see Fig. 1A, 1C, 1F, 1G, 1K and Fig. 2A, 2C, 2E, 2F, 2G, 2K. On the 7<sup>th</sup> day of wound healing, inflammatory cell infiltration was not observed in groups 4, 5, 8 and 9 (Fig. 1D, 1E, 1H, 1J).

In a rat model of diabetes, the infiltration of inflammatory phase cells to the wound area was delayed compared to that of a control group, and the migration of these cells from the wound area was also delayed (7). Another experimental study revealed that the number of inflammatory cells in a diabetic wound-healing model was less than in a control group (8).

Angiogenesis plays an important part in feeding newly formed cells during wound healing and in cleaning debris during tissue formation conglomeration (9). Vit C (ascorbic acid) enhances the functions of neutrophils, in addition to collagen production (10–12). It also stimulates angiogenesis and has a strong antioxidant effect (10–12). In a study by Taş *et al* (13), on the 7<sup>th</sup> day of open wound healing, sildenafil citrate markedly stimulated angiogenesis and on the 15<sup>th</sup> day, widespread capillary veins filled with blood were present within granulation tissue in a dog model of wound healing. In the present study, angiogenesis was insufficient in group 1. It was more pronounced in the groups dosed with sildenafil citrate. The rate of angiogenesis was best in the groups treated with Vit A and Vit C and sildenafil combinations. As insufficient angiogenesis has a negative effect on wound healing in patients with diabetes, treatment with the aforementioned combinations may be beneficial.

Previous study showed that collagen synthesis was slow and decreased in diabetic models compared to

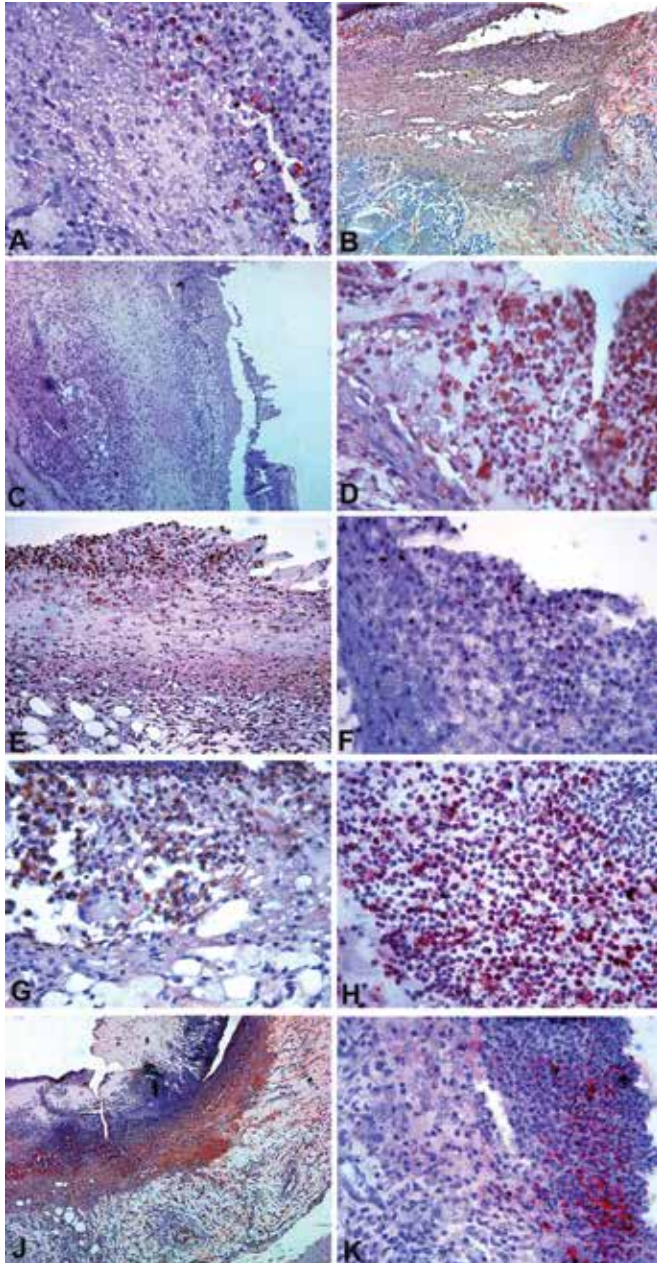


Fig. 3: Immunohistochemical findings of wound healing in dermis and epidermis in rats sacrificed on the 3<sup>rd</sup> day of the study (A) Group 1 (control group) H&E,  $\times 200 \mu\text{m}$ . (B) Group 2 (glibenclamide) H&E,  $\times 200 \mu\text{m}$ . (C) Group 3 (insulin) H&E,  $\times 200 \mu\text{m}$ . (D) Group 4 (sildenafil) H&E,  $\times 200 \mu\text{m}$ . (E) Group 5 (vitamin A) H&E,  $\times 200 \mu\text{m}$ . (F) Group 6 (vitamin C) H&E,  $\times 200 \mu\text{m}$ . (G) Group 7 (vitamin E) H&E,  $\times 200 \mu\text{m}$ . (H) Group 8 (vitamin A + sildenafil) H&E,  $\times 200 \mu\text{m}$ . (I) Group 9 (vitamin C + sildenafil) H&E,  $\times 200 \mu\text{m}$ . (K) Group 10 (vitamin E + sildenafil) H&E,  $\times 200 \mu\text{m}$ .

non-diabetic ones. Another study reported that collagen fibres were sparse in diabetic rats (8). One study concluded that healing in the duodenum and abdominal area of rats with experimental diabetes was low due to increased mechanical resistance and low collagen content (14). Collagen formation was delayed and irregular

in corneal wound healing in patients with diabetes (15). In the present study, the development of connective tissue in the control group was insufficient compared to that reported in the literature on both the 7<sup>th</sup> and 15<sup>th</sup> days of the study (8, 14, 15).

One study indicated that Vit E inhibited collagen synthesis when it was administered locally but that it had no effects other than enhancing the resistance of wounds against infections when it was used in parenteral form. On the other hand, Vit E reduced both the formation of connective tissue and epithelialization healing (16). With regard to the present study, the findings clearly revealed marked delays and decreases in both connective tissue proliferation and epithelial regeneration in the groups treated with Vit E.

A previous study of the impact of sildenafil citrate on wound healing in dogs reported that the formation of collagen was significantly greater than that of a control group on the 7<sup>th</sup> day of the study (13). The same study reported that the formation of connective tissue, in addition to epithelialization, was complete on the 15<sup>th</sup> day of the study. In the present study, the comparison of the histopathological findings of the 10 groups suggested that connective tissue proliferation and granulation tissue formation seemed to be best in the Vit C group.

Nitric oxide is an intracellular messenger molecule with important immune functions (17). It is produced by a group of isoenzymes collectively termed NOSs (18). To date, three distinct isoforms of NOS have been cloned: endothelial NOS, neuronal NOS and iNOS (17). Inducible NOS is expressed by neutrophils, the first effector cells, which migrate into the wound and predominate during the first 24 hours after injury (19). In a previous study, the expression of iNOS was strongest in the first 2 days post-wounding (20). In the present study, the level of iNOS was greatest in macrophages and neutrophils on the 3<sup>rd</sup> day of wound healing, supporting the findings of the earlier study.

With the loss of the iNOS gene, fibroblasts synthesized much less collagen in response to 10% foetal bovine serum and NO plays a significant role in fibroblast collagen synthesis (21). In the present study, collagen synthesis was greatest in the groups where iNOS was intensively detected (groups 4, 8, 9 and 10), and it was highest in group 9.

The results of the present study revealed that neither glibenclamide nor insulin was able to eliminate the delay in the wound healing of diabetic animals. In addition, in this rat model of diabetes, the application of Vit E did not have a positive impact on wound healing, Vit A had

a positive influence on epithelialization, and Vit C had a positive effect on the proliferation of connective tissue. Sildenafil citrate had a positive impact on improving wound healing, as well as ensuring that the early phase of wound healing was concluded successfully and that the proliferation of both connective tissue and epithelialization reached the desired level.

In conclusion, sildenafil citrate, together with Vit A and Vit C, may be recommended to improve wound healing in diabetic rats.

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