

**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**

A Taş<sup>1</sup>, A Karasu<sup>1</sup>, Z Yener<sup>2</sup>, S Yıldırım<sup>2</sup>, N Atasoy<sup>1</sup>, E Düz<sup>3</sup>, E Yavuz<sup>1</sup>

**ABSTRACT**

**Objectives:** This experimental study examined 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 ( $n=100$ ).

**Materials and 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), 1–10, 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 anaesthetised 3, 7 and 15 days later. Wounded skin tissue samples were collected for histopathological and immunohistochemical analyses.

**Results:** On the seventh 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 15th day of the study, Groups 8, 9 and 10 had mature connective tissue. However, Group 1 still had exudate-containing neutrophils. Immunohistochemically, on the third 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 diabetics.

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

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From: <sup>1</sup>Department of Surgery, Faculty of Veterinary, <sup>2</sup>Department of Pathology, Faculty of Veterinary, and <sup>3</sup>Gevaş Vocational School, University of Yuzuncu Yil, 65080, Van, Turkey.

Correspondence: Dr A Taş, Department of Surgery, Faculty of Veterinary, University of Yuzuncu Yil, 65080, Van, Turkey. E-mail :abuzertas@hotmail.com

## **INTRODUCTION**

Diabetes mellitus is a chronic metabolic disorder, characterised 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 neovascularisation 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 stage, fibroplasia begins, with the formation of granulation tissue within the wound space (2).

Sildenafil is a phosphodiesterase-5 inhibitor, which is 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 University of Yuzuncu Yil, Van, Turkey.

### **Chemicals**

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

### **Animals**

One hundred adult male and female Swiss albino rats weighing approximately 200–300 g were used in this experimental study. The laboratory animals were supplied by Ataturk University Laboratory Animals Unit. 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  $22 \pm 2^\circ \text{C}$ , with 12 h of darkness and 12 h of light. The animals were not fed for 18 h 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 three 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 anaesthetised. Their backs were shaved and prepared with 10% antiseptic povidone-iodine solution (Batticon, Adeka). 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, seven and 15 days later, the rats were sacrificed after being anaesthetised.

### **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  $\mu\text{m}$ -thick sections and stained with haematoxylin-eosin stain for examination by light microscopy.

### **Immunohistochemical examination**

Immunohistochemical staining for 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, USA 1:100) was used. All the tissue sections were deparaffinised 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 min. The sections were washed with phosphate-buffered solution (PBS) and pre-treated for 5 min with a protein blocker. All the sections were then washed with PBS and incubated for 2 h at room temperature with the primary antibody. After washing with PBS, the sections were incubated for 20 min with biotinylated goat anti-rabbit antibodies at room temperature. After another PBS rinse, the sections were treated with streptavidin–horseradish-peroxidase complex for 20 min. After washing in PBS, amino-ethyl carbazole was used as a chromogen and Mayer’s haematoxylin was used for counterstaining. Non-immunised 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 third 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 third 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 seventh day of the study, epithelial regeneration was completed in Groups 8 and 9 (Fig. 1H, J). Epithelial regeneration was normal in Groups 4, 5, 6, 7 and 10 (Fig. 1D–G, K) and had just started in Groups 1, 2, 3 and 7 (Fig. 1A–C). Angiogenesis was insufficient in Group 1

(Fig. 1A). Angiogenesis was sufficient in Groups 2, 3, 5, 6 and 7 (Fig. 1B–G), but it was more marked in Groups 4, 8, 9 and 10 (Fig. 1D, H, J, K). In terms of connective tissue proliferation, partially matured connective tissue was observed in Groups 4 and 9 (Fig. 1D, J), and slack connective tissue was widespread in the other groups (Fig. 1A–H, K). 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, C, E, F, K). Only mononuclear cells were observed in Groups 4, 8 and 9 (Fig. 1D, H, J). Necrotic exudate containing a small amount of neutrophils was observed in Group 3, and necrotic exudate containing neutrophils and macrophage was observed in Group 7 (Fig. 1C, G).

On the 15th day of the study, epithelial regeneration was completed in Groups 4, 8, 9 and 10 (Fig. 2D, H, J, K), and it was at an advanced level in Groups 2, 3, 5, 6 and 7 (Fig. 2B–G). 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 seventh day. Connective tissue proliferation, in the form of slack connective tissue, was apparent in Groups 1, 2, 3 and 5 (Fig. 2A–E). Groups 6 and 7 had both slack and firm connective tissue (Fig. 2F, G). 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, H). However, the maturity of the connective tissue in Group 9 was more marked (Fig. 2H). On the 15th day of the study, Group 1 still had exudate-containing neutrophils (Fig. 2A).

### **Immunohistochemical findings**

On the third day, in Group 4, 8, 9 and 10, the iNOS reactivity of macrophages was intense, and neutrophils surrounded the wound edges (Fig. 3D, H, J, K). The level of iNOS reactivity was

moderate level in Group 6 but less distinct in Groups 1, 2, 3, 5 and 7 (Fig. 3. A–E, G). On the seventh 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 15th day, but it was insignificant in the other groups.

## DISCUSSION

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

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 seventh day of open wound healing, sildenafil citrate markedly stimulated angiogenesis and on the 15th 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 the Vit A and C and sildenafil combinations. As insufficient angiogenesis has a negative effect on wound healing in diabetic patients, treatment with the aforementioned combinations may be beneficial.

Previous study showed that collagen synthesis was slow and decreased in diabetic models compared to 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 diabetic patients (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 seventh and 15th 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 epithelialisation 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 seventh day of the study (13). The same study reported that the formation of connective tissue, in addition to epithelialisation, was complete on the 15th 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 groups.

NO is an intracellular messenger molecule with important immune functions (17). It is produced by a group of isoenzymes, which are collectively termed NOSs (18). To date, three distinct isoforms of NOS have been cloned: endothelial NOS, neuronal NOS and iNOS (17). iNOS is expressed by neutrophils, the first effector cells, which migrate into the wound and predominate during the first 24 h 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 third day of wound healing, supporting the findings of the earlier study.

With the loss of the iNOS gene, fibroblasts synthesised 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 epithelialisation, 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 epithelialisation reached the desired level.

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

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**REFERENCES**

1. Kolluru GK, Bir SC, Kevil CG. Endothelial dysfunction and diabetes: effects on angiogenesis, vascular remodelling, and wound healing. *Int J Vascular Med* 2012; 1–30.
2. Ringler, D. J. Inflammation and repair. In: Jones TC, Hunt RD, King NW, eds. *Veterinary Pathology*. 6th Edition. Williams and Wilkins, Baltimore, Maryland; 1997: 113–58.
3. Langtry HD, Markham A. Sildenafil: a review of its use in erectile dysfunction. *Drugs* 1999; **57**: 967–89.
4. Salcido RS. Viagra and wound healing: the NO connection. *Adv Skin Wound Care* 2008; **21**:106–9.
5. Jaouhari JT, Lazrek HB, Jana M. The hypoglycemic activity of *Zygophyllum gaetulum* extracts in alloxan-induced hyperglycemic rats. *Journal of Ethnopharmacology* 2000; **69**: 17–20.
6. Taylor CR, Cote RJ. *Immunomicroscopy. A Diagnostic Tool for the Surgical Pathologist*. 3rd ed. Philadelphia, Elsevier Saunders, 2005: 1–45.
7. Komesu MC, Tanga MB, Buttros KR, Nakao C. Effects of acute diabetes on rat cutaneous wound healing. *Pathophysiology* 2004; **11**: 63-7.
8. Bitar MS, Farook T, Wahid S, Francis IM. Glucocorticoid- dependent impairment of wound healing in experimental diabetes: amelioration by adrenalectomy and RU 486. *J of Surg Res* 1999; **82**: 234-43.
9. Charonis AS, Tsilibary EC, Furcht LT. Effects of nonenzymatic glycation on molecular interactions of basement membrane molecules. In: Ruderman N, Williamson J, Brownlee

- M, eds. *Hyperglycemia, Diabetes and Vascular Disease*. Oxford University Press, New York, USA; 1992: 243–50.
10. Goetzl EJ, Wasserman SI, Gigli I, Austen KF. Enhancement of random migration and chemotactic response of human leukocytes by ascorbic acid. *J of Clin Invest* 1974; **53**: 813–8.
  11. Frei B, Stocker R, Ames BN. Antioxidant defenses and lipid peroxidation in human blood plasma. *Proceedings of the National Academy of Sciences of the United States of America* 1988; **85**: 9748–52.
  12. Nicosia RF, Belser P, Bonanno E, Diven J. Regulation of angiogenesis in vitro by collagen metabolism. *In Vitro Cellular and Developmental Biology* 1991; **27A**: 961–6.
  13. Tas A, Atasoy N, Ozbek H, Aslan L, Yuksel H, Ceylan E et al. Accelerating effects of sildenafil citrate (Viagra) in the early phase of healing in open wounds induced experimentally in dogs. *ActaVet Bruno* 2003; **72**: 273–8.
  14. Gottrup F, Andreassen TT. Healing of incisional wounds in stomach and duodenum: The influence of experimental diabetes. *J of Surg Res* 1981; **31**: 61–8.
  15. Sato N, Nakamura M, Chikama T, Nishida T. Abnormal deposition of laminin and type IV collagen at corneal epithelial basement membrane during wound healing in diabetic rats. *Japan J of Ophth* 1999; **43**: 343–7.
  16. MacKay D, Miller AL. Nutritional support for wound healing. *Alternative Medicine Review* 2003; **8**: 359–77.
  17. Carew JF, Ward RF, LaBruna A, Torzilli PA, Schley WS. Effects of scalpel, electrocautery, and CO<sub>2</sub> and KTP lasers on wound healing in rat tongues. *Laryngoscope* 1998; **108**: 373–80.

18. Sinha UK, Gallagher LA. Effects of steel scalpel, ultrasonic scalpel, CO2 laser, and monopolar and bipolar electrosurgery on wound healing in guinea pig oral mucosa. *Laryngoscope* 2003; **113**: 228–36.
19. McCall T, Vallance P. Nitric oxide takes centre stage with newly defined roles. *Trends in Pharmacological Science* 1992; **13**: 1–6.
20. Ragab S, Hussein A, Abd El-Raof S, Mohammed HH. Histological and immunohistochemical study of acute incisional wound healing in male albino rat. *El-minia Med Bull* 2008; **19**: 1–15.
21. Shi HP, Most D, Efron DT, Tantry U, Fischel MH, Barbul A. The role of iNOS in wound healing. *Surgery* 2001; **130**: 225–9.

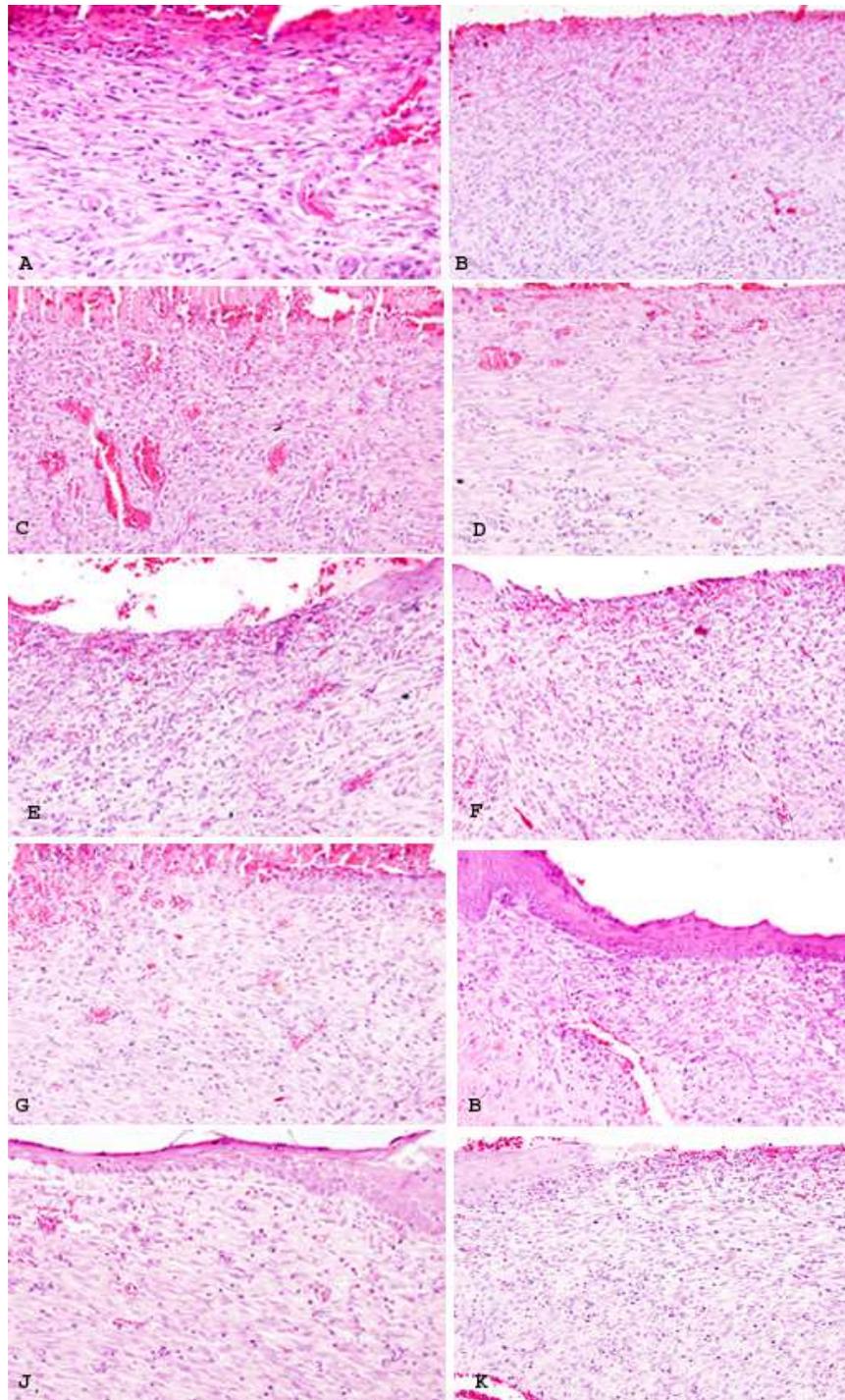


Fig.1: Histopathological findings of wound healing in dermis and epidermis in rats sacrificed on the 7<sup>th</sup> day of the study (A) 1. Group: (Control group) H.E., x 200  $\mu$ m. (B) 2. Group (Glibenclamide reference). H.E., x 200  $\mu$ m. (C) 3. Group (Insulin reference). H.E., x 200  $\mu$ m. (D) 4. Group (Sildenafil). H.E., x 200  $\mu$ m. (E) 5. Group (Vitamin A). H.E., x 200  $\mu$ m. (F) 6. Group (Vitamin C). H.E., x 200  $\mu$ m. (G) 7. Group (Vitamin E). H.E., x 200  $\mu$ m. (H) 8. Group (Vitamin A + Sildenafil). H.E., x 200  $\mu$ m. (J) 9. Group (Vitamin C + Sildenafil). H.E., x 200  $\mu$ m. (K) 10. Group. (Vitamin E + Sildenafil). H.E., x 200  $\mu$ m.

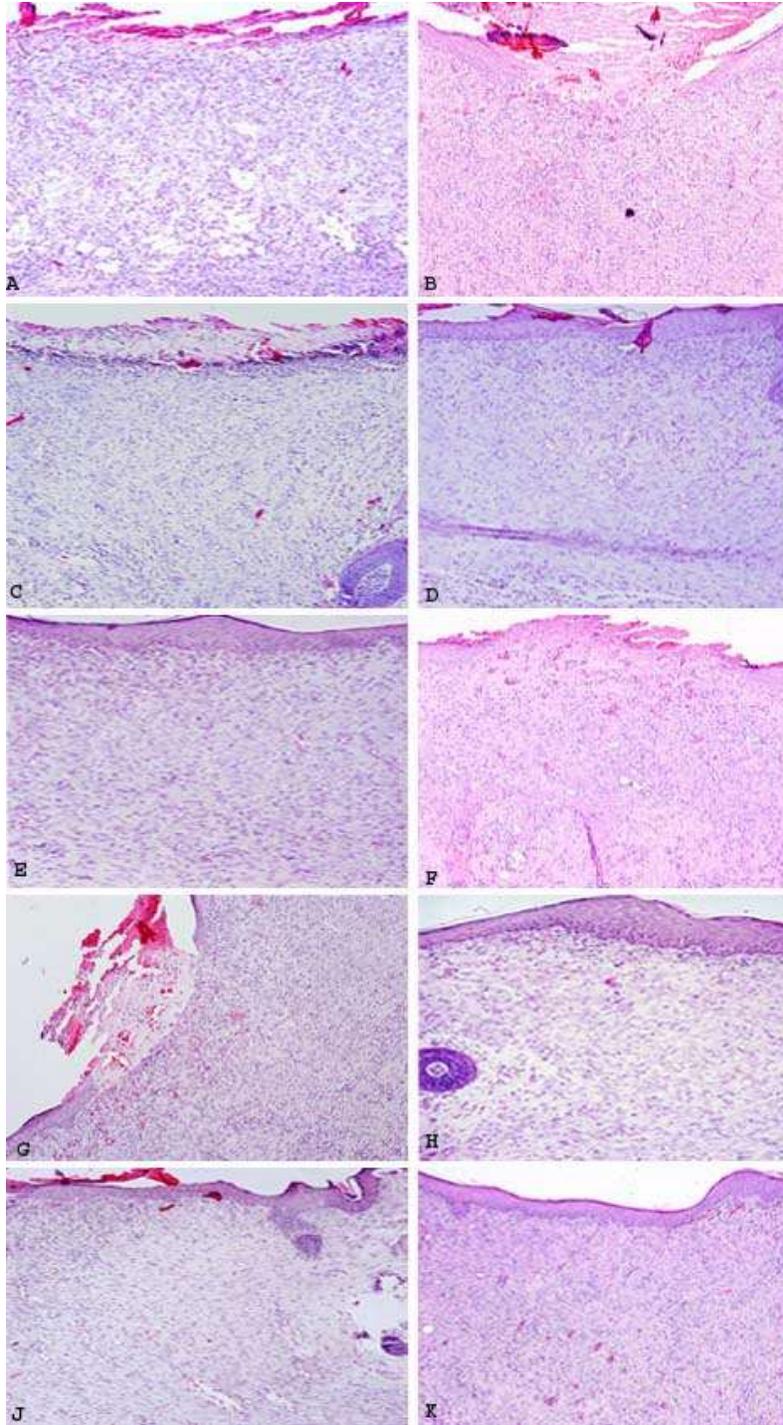


Fig. 2: Histopathological findings of wound healing in dermis and epidermis in rats sacrificed on the 15<sup>th</sup> day of the study (A) 1. Group: (Control group) H.E., x 200  $\mu$ m. (B) 2. Group (Glibenclamide reference). H.E., x 200  $\mu$ m. (C) 3. Group (Insulin reference). H.E., x 200  $\mu$ m. (D) 4. Group (Sildenafil). H.E., x 200  $\mu$ m. (E) 5. Group (Vitamin A). H.E., x 200  $\mu$ m. (F) 6. Group (Vitamin C). H.E., x 200  $\mu$ m. (G) 7. Group (Vitamin E). H.E., x 200  $\mu$ m. (H) 8. Group (Vitamin A + Sildenafil). H.E., x 200  $\mu$ m. (J) 9. Group (Vitamin C + Sildenafil). H.E., x 200  $\mu$ m. (K) 10. Group. (Vitamin E + Sildenafil). H.E., x 200  $\mu$ m.

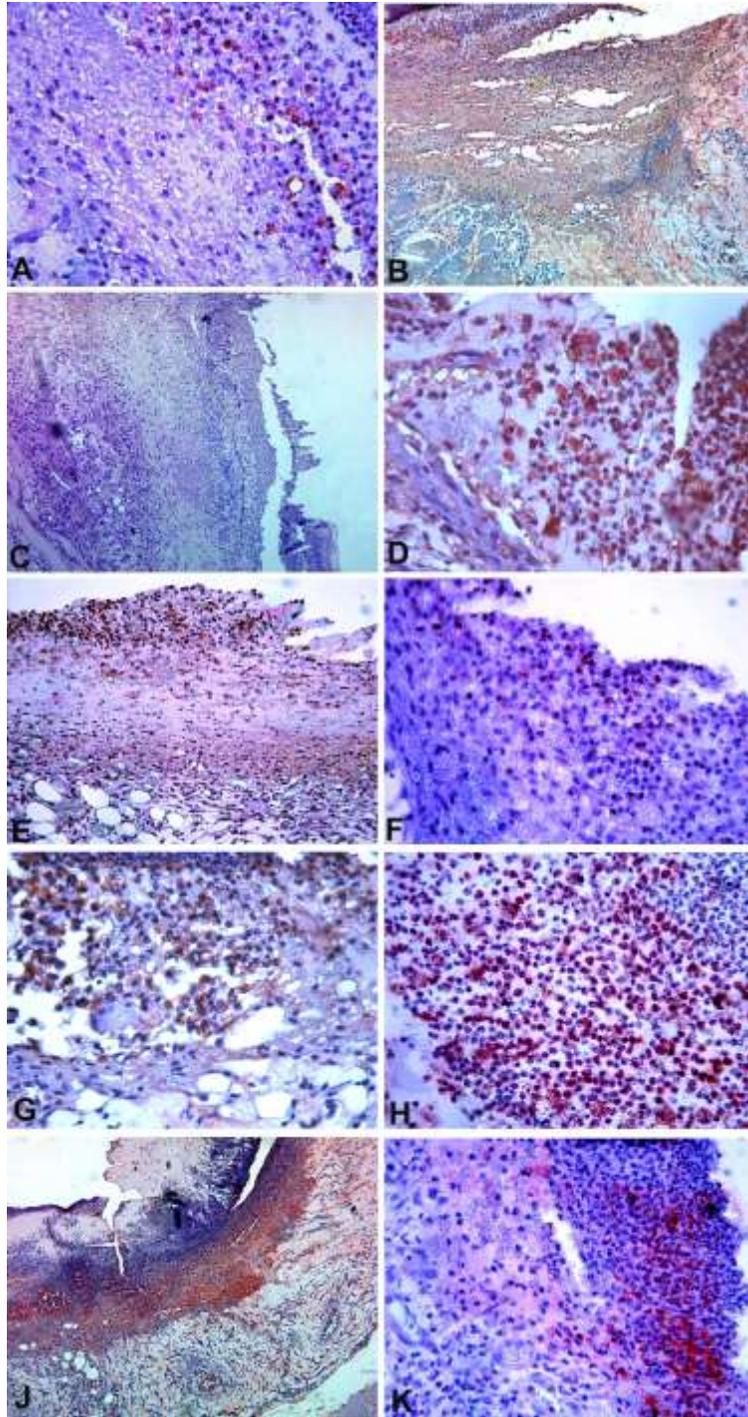


Fig. 3: Immunohistochemical findings of wound healing in dermis and epidermis in rats sacrificed on the 3<sup>rd</sup> day of the study (A) 1. Group: (Control group) H.E., x 200  $\mu$ m. (B) 2. Group (Glibenclamide reference). H.E., x 200  $\mu$ m. (C) 3. Group (Insulin reference). H.E., x 200  $\mu$ m. (D) 4. Group (Sildenafil). H.E., x 200  $\mu$ m. (E) 5. Group (Vitamin A). H.E., x 200  $\mu$ m. (F) 6. Group (Vitamin C). H.E., x 200  $\mu$ m. (G) 7. Group (Vitamin E). H.E., x 200  $\mu$ m. (H) 8. Group (Vitamin A + Sildenafil). H.E., x 200  $\mu$ m. (I) 9. Group (Vitamin C + Sildenafil). H.E., x 200  $\mu$ m. (J) 9. Group (Vitamin C + Sildenafil). H.E., x 200  $\mu$ m. (K) 10. Group (Vitamin E + Sildenafil). H.E., x 200  $\mu$ m.