Original Article

Histological Study on the Effect of Insulin and Platelet-Rich Plasma on Skin Wounds Induced in Diabetic Adult Male Albino Rats

Salwa Metwaly Ali, Dalia El Marakby, Sarah Mosaad Amer and Hanan Abdallah

Department of Medical Histology and Cell Biology, Faculty of Medicine, Cairo University, Manial, Cairo, Egypt

ABSTRACT

Introduction: Diabetes mellitus (DM) is a metabolic disorder that has many complications like cardiovascular diseases, diabetic nephropathy and unhealed diabetic skin wounds.

Aim of the Work: To evaluate the possible curative effect of insulin and platelet rich plasma (PRP) on diabetic skin wounds induced in adult male albino rats.

Materials and Methods: 50 adult male albino rats, with an average body weight 150-200 gm, were divided into (control and experimental groups). The control groups (20 rats) that were equally subdivided into two groups (control I: normal healthy rats with intact skin and control II: diabetic rats with non-treated skin wounds managed with local intradermal saline injections 500 µL once daily at wounds margins). Experimental groups (30 rats) that were equally further subdivided into three groups; Insulin treated group I: diabetic rats with local intradermal insulin injections 0.5 U/500 µL once daily at wounds margins. PRP treated group II: diabetic rats with local intradermal PRP injections 500 µl as a single dose at wounds margins. Combined treated group III: diabetic rats with local intradermal combined PRP and insulin injections with 2 hours intervals. Each group in the control and experimental groups was further subdivided into 2 subgroups A and B according to the time of sacrification after 14 and 21 days post wounds induction respectively.

Results: The experimental groups showed significant improvement in wound closure, epidermis regeneration and increased collagen deposition as compared to non -treated control group II. The insulin and combined treated groups showed increased collagen deposition as compared to PRP treated group, however, the combined group showed the best results.

Conclusion: Local combined treatment with insulin and PRP showed a rapid rate of diabetic wounds closure.

Received: 15 July 2021, **Accepted:** 08 October 2021

Key Words: Diabetic skin wounds; e-cadherin; insulin; PRP; vimentin.

Corresponding Author: Sarah Mosaad Amer, MD, Department of Medical Histology and Cell Biology, Faculty of Medicine, Cairo University, Manial, Cairo, Egypt, **Tel.**: +20 11 1816 8158, **E-mail:** sarahm.amer@hotmail.com

ISSN: 1110-0559, Vol. 46, No.1

INTRODUCTION

Diabetes mellitus has many complications like diabetic nephropathy and diabetic foot ulcers. This is secondary to peripheral neuropathy, tissue hypoxia, microangiopathy and decreased collagen synthesis[1].

Although growth factors and stem cells have shown efficacy in promoting wound healing, these therapies are highly expensive and their safety remains to be evaluated. Therefore, low-cost and safe strategies to improve wound healing will be of great social and economic value[2].

Insulin is a peptide hormone and growth factor that can restore damaged skin[3]. The systemic insulin treatment reduces infections after surgical procedures[4]; however, this treatment has a disadvantage of inducing hypoglycemia and hypokalemia. In contrast, topical insulin improves wound healing without changing blood glucose levels in diabetic and non-diabetic patients[5].

Nowadays, the use of platelet-rich plasma (PRP) offers a new approach in wound healing[6]. Platelets act as a rich source of growth factors promoting wound-healing and cytokines such as vascular endothelial growth factor (VEGF), transforming growth factor-β (TGF-β) and platelet-derived endothelial cell growth factor (PDGF)[7].

E-cadherin is a well-known epithelial marker and its loss within a part of the cell membrane of epithelial cells can mediate cell migration. A recognized example of this occurs during the process of re-epithelialization in the wound healing in the skin[8]. The keratinocytes at the edge of the wound downregulate E-cadherin but maintain their association with their neighbors to pull them as a sheet over the gap in the tissue. The epithelial sheet cells over the wound site forms a structure known as an epithelial tongue and is a form of collective cell migration that proficiently closes the gap within the tissue[9].

Vimentin, known as fibroblast intermediate filament, is considered as a mesenchymal marker. Vimentin has a role in wound healing as it acts as a coordinator for fibroblast proliferation, keratinocyte differentiation, collagen accumulation and re-epithelialization. Loss of vimentin disrupts this coordination, leading to slow, poor, and incomplete wound healing[10].

 DOI: 10.21608/ejh.2021.86084.1527

MATERIALS AND METHODS

Drugs

1. Streptozotocin: Used for induction of D.M (STZ; Sigma, USA), 55 mg/kg, dissolved in sodium citrate buffer (0.1 ml, PH 4.5)[11].
2. Insulin: Human isophane insulin suspension (Insulatard 100 IU/ML, Novo nordisk company, Denmark)[12].
3. Platelet rich plasma (PRP): 2 ml volume of autologous blood was drawn from each rat in the PRP and combined treated groups (from a rat tail vein) into vacuum tubes containing 10% sodium citrate. The PRP were prepared according to a double centrifugation protocol[13].

Animals

This study included 50 adult male albino rats with an average body weight of 150-200 gm. Animals were provided with veterinary care by the Animal House of Kasr El Ainy, Faculty of Medicine, Cairo University, according to the guidelines for animal research approved by Animal Ethics Committee, Faculty of medicine, Cairo University. The animals were housed in stainless-steel cages under standard environmental conditions with free access to standard diet and water throughout the experimental period.

Experimental design (Table 1)

The rats were divided into the following groups:

1. Control groups (20 rats): were equally subdivided into 2 groups:

- Control (I): Healthy rats without wounds induction or D.M.
- Control (II): Diabetic rats with non-treated skin wounds. The wounds were managed with local intradermal saline injections 500 µL at wounds margins and dressings of sterile gauze[14].

2. Experimental groups (30 rats): were equally subdivided into 3 groups:

- Insulin treated group (I): Diabetic rats with insulin treated skin wounds. The wounds were managed with local intradermal insulin injections at wounds margins 0.5 U/500 µL saline[12], once daily until the end of the experiment.
- PRP treated group (II): Diabetic rats with PRP treated skin wounds. The wounds were managed with local intradermal PRP injections at wounds margins 500 µl as a single dose[15].
- Combined treated group (III): Diabetic rats with combined treated skin wounds. The wounds were managed with local intradermal combined insulin and PRP injections with 2 hours intervals between them.

Each group in the control and experimental groups was further subdivided into 2 subgroups A and B according to the time of sacrification after 14 and 21 days post wounds induction respectively.

Table 1: demonstrating the control and experimental groups with number of rats, doses of drugs, routes and durations of administration and time of sacrification

	Groups	Number of animals	Dose, route and duration of drug administration	Time of sacrification
1. Control groups	Control group I	10	Healthy rats without wounds induction or D.M	Subgroup A= at day14th. Subgroup B= at day21.
	Control group II	10	Diabetic rats with non-treated skin wounds. The wounds were managed with local intradermal saline injections 500 µL at wounds margins and dressings of sterile gauze[14].	Subgroup A= at day14th. Subgroup B= at day21.
2. Experimental groups	Insulin treated group I	10	Diabetic rats with insulin treated skin wounds. The wounds were managed with local intradermal insulin injections at wounds margins 0.5 U/500 µL saline[12], once daily until the end of the experiment.	Subgroup A= at day14th. Subgroup -B= at day21.
	PRP treated group II	10	Diabetic rats with PRP treated skin wounds. The wounds were managed with local intradermal PRP injections at wounds margins 500 µl as a single dose[15].	Subgroup -A= at day14th. Subgroup -B= at day21.
	Combined treated group III	10	Diabetic rats with combined insulin + PRP treated skin wounds. The wounds were managed with local intradermal combined insulin and PRP injections with 2 hours intervals between them.	Subgroup -A= at day14th. Subgroup -B= at day21.
	Total	50		

Induction of diabetes mellitus

The experimental rats and rats in control group II were fasted overnight before being injected intraperitoneally (I.P) with streptozotocin (STZ) at a dose of 55 mg/kg body weight[11]. The solutions were prepared freshly and injected. After 3 days from STZ injections, the body weights and the fasting blood glucose levels (FBG) were measured with a glucometer (Accu-Chek Active, Roche Diagnostics, Germany) to confirm the development of diabetes. Only those animals that showed hyperglycemia (blood glucose level > 250 mg/dl) were considered to be diabetic and were included in the study[11]. The body weights and FBG were measured again at days 14, 21 with time of sacrification as a continuous monitoring and to assess the effect of local insulin injections on the systemic blood glucose level[12].

Induction of skin wounds

Wound induction was carried out after one week of induction of diabetes to make sure of establishment of diabetes. The rats were anesthetized by I.P injections of ketamine hydrochloride (50 mg/kg/rat), and the back hair was shaved[16].

A full-thickness wounds including subcutaneous tissue were created in the interscapular region of the upper back of each rat with a punch biopsy instrument (diameter 8 mm). The wounds sites were high near the back of the neck, away from their reach[11].

The day of wounds induction was defined as day 0. Immediately after surgery, antibiotics (Ampicillin; 50mg/kg IP) and analgesic (Meloxicam 1-2mg/kg S.C) were administered for three consecutive days[17].

The treatment was started in control group II (saline) and experimental groups at the 3rd day post wounds induction.

Evaluation of wounds closure

Continuous daily monitoring of wound closure was assessed. The photographing and measurement of wounds diameters were recorded at days 14th and 21th post wounds induction (with time of sacrification). The rats were photographed in a standard prone position using an 18 mega pixels digital camera. All wounds were measured using a millimeter graded graph paper[18].

The percentage (%) of wounds closure was assessed using the Wilson's formula stated as % of wounds closure = [(Area on 0 day − Area of X days)/Area on 0 day] × 100%[19]. It was presented as mean ± standard deviations (SD) and analyzed using one-way analysis of variance (ANOVA).

Rats sacrification & samples collection

The animals were sacrificed (at day 14th and 21th post wounds induction) by I.P injections of a pentobarbital overdose[16]. The skin wounds area were dissected with 1 cm of the surrounding normal skin margin. Specimens were fixed immediately in 10% buffered formalin for 1 day. Paraffin embedded tissues were processed and serial sections at 5 µm thicknesses were cut using a microtome and mounted on glass slides. Other sections were mounted on positive charged slides for immunohistochemistry.

1. Hematoxylin and Eosin (H&E) stain to determine structural changes[20].
2. Masson's trichrome stain to demonstrate collagen fibers deposition in the dermis and wounds' beds[20].
3. Immunohistochemical staining using:
    A. Anti E-cadherin for detection of cell-cell adhesion and establishment of epithelial integrity. It is a mouse monoclonal anti-E-cadherin antibody (In*vitro*gen, Thermofisher scientific. US. Cat. No. 13-1900.). It was stored at 4°C. Species reactivity: mouse E-cadherin. Cross reactivity: This antibody reacts with human and dog E-cadherin. The cellular staining pattern is positive membranous and cytoplasmic brown color
    B. Anti Vimentin for detection of mesenchymal cells including: fibroblast, macrophages, and lymphocytes in the dermis. It is a mouse monoclonal anti-Vimentin antibody (Cell Marque Corporation, Toll-Free North America. Cat. No. 347M-18). It was stored at 2-8°C.Species reactivity: mouse, rats and human. The cellular staining pattern is positive cytoplasmic brown color.

Detection System

Ultravision large volume detection system: Anti -polyvalent kit (ready to use); [catalogue number TP -060 -HL].

Diaminobenzidine tetra hydrochloride (DAB) substrate system

[Catalogue number TA -060 -HD] and was supplied as 6 ml DAB chromogen and 6 ml DAB substrate to be stored at 4 -6˚C.

Counterstain

Mayer's hematoxylin [catalogue number TA -060 -MH] was used for counterstain. For the detection specificity of primary antibody additional sections of skin were processed in same way but omitting steps of applying the primary antibody (negative control).

Morphometric study and Statistical Analysis

Morphometric study was done using Leica "Qwin 500C" image analyzer (Leica LTD, Cambridge, UK). The image analyzer consists of a colored video camera (Olympus), colored monitor, hard disk of IBM personal computer connected to the microscope, and controlled by Leica Qwin 500C software. The following parameters were measured:

1. The fasting blood glucose levels (FBG) and the body weight were measured and presented as mean ± standard deviations (SD).
2. The mean values of the percentage of wounds closure in the groups.
3. In Masson's stained sections: the mean area percent of collagen fibers in standard low power fields (x 100)
4. In E-cadherin immunohistochemical stained sections: the mean area percent of immunopositivity in standard high power fields (x 400).
5. In Vimentin immunohistochemical stained sections: the mean area percent of immunopositivity in standard high power fields (x 400).

The previous measurements (3:5) were taken in 10 non overlapping randomly chosen fields for all specimens of each animal of all groups. All Quantitative data were collected as means and standard deviations (SD) then compared by using one-way analysis-of-variance (ANOVA). Any significant ANOVA was followed by post hoc Tukey test to detect which pairs of groups caused the significant difference. *P-values*<0.05 were considered statistically significant. Calculations were made on SPSS software version 16 (SPSS, Chicago, IL).

RESULTS

Gross evaluation of rats (Plate 1, Histogram 3)

Daily observation of rats revealed deaths only in diabetic rats. About 10 rats out of 40 died within 3-4 days after STZ injection. These rats showed anorexia with deterioration in the general body health then died. Dead rats were excluded from the total number of experimental rats. Gross morphology of wounds at days 14^{th}, 21^{th} in groups showed:

Control group II-A revealed increased wound diameter however, control group II-B showed slight decrease of wound diameter and the wound were covered by scabs. Insulin treated group I -A revealed prominent decrease of wound diameter and the insulin treated group I-B showed almost complete wound closure in some rats. PRP treated group II-A revealed significant decrease of wound diameter and the PRP treated group II-B showed complete wound closure in some rats. The combined treated group III-A revealed marked decrease in wound diameter and the combined treated group III-B showed complete wound closure in most rats.

Histological Results

Haematoxylin and Eosin Stained Sections (Plates 2, 3)

Control group I-A &B; all rats showed similar results and showed the well-known normal histological structure. Skin sections showed epidermal layer as a stratified squamous epithelium. Control group II-A revealed large wounds gaps and wide areas of separation between wounds beds and epidermis. The epidermis showed diffuse disfigurement of keratinocytes with dark pyknotic fragmented nuclei and vacuolated cytoplasm. Control group II-B showed the epidermis at wound margin with improvement of keratinocytes appearance with some cells showed shrunken nuclei. Insulin treated group I-A revealed the epidermis at the wound margin with apparently normal many cells however, some cells showed shrunken nuclei and vacuolated cytoplasm. Insulin treated group I-B showed the epidermis at the wounds margins with increased cell layers, apparently regular many keratinocytes however, some cells still showed disfigurement. PRP treated group II-A revealed the epidermis at wounds margins with some cells showed pyknotic nuclei and vacuolated cytoplasm. PRP treated group II-B showed improvement of the structure and the arrangement of the keratinocytes however, some cells showed shrunken nuclei and vacuolated cytoplasm. Combined treated group III-A revealed the epidermis with apparently normal keratinocytes. Some cells with shrunken nuclei and vacuolated cytoplasm are also detected. Combined treated group III-B showed complete wounds closure in most skin sections. The epidermis revealed marked increased of cell layers and apparently normal most keratinocytes with remnant pyknotic cells.

Masson's Trichrome stained Stained Sections (Plates 4 , 5, Histogram 4)

Control group I-A &B showed the dermis with well organized, tightly packed collagen bundles. The dermis was formed of fine collagen fibers in upper papillary layer while the lower reticular layer contained thick collagen bundles. Control group II-A&B showed the wounds beds were filled with fine, irregularly distributed collagen fibers. Insulin treated group I- A&B showed thick, irregularly arranged collagen bundles occupying all the wounds beds. PRP treated groups II- A showed the regenerated dermis with densely arranged collagen fibers aligned horizontally in one direction. PRP treated group II-B showed the papillary dermis with thin collagen bundles and the remaining dermis showed diffuse deposition of thicker and denser collagen arranged in a network manner. Combined treated group III-A showed the regenerated dermis with densely arranged collagen fibers aligned horizontally in one direction. Combined treated group III-B showed diffuse deposition of thick collagen bundles arranged in a network manner occupying most of the regenerated dermis.

Immunostaining with anti-E-cadhrin (Plate 6, Histogram 5)

Control group I-A &B revealed strong immune reaction in the form of membranous brown color in almost all epidermal cells. Control group II -A&B showed the epidermis at the wounds margins with weak to negative immune-reaction at the cell membrane of epidermal cells. Insulin treated group I- A&B showed the epidermis with mild to moderate immune-reactions. PRP treated group II- A&B showed the epidermis with moderate immune-reactions. Combined treated group III- A&B showed the

epidermis with moderate to strong immune-reactions.

Immunostaining with anti-vimentin (Plate 7, Histogram 6)

Control group I-A &B revealed immune-positive cytoplasmic reaction in cells at the dermis resembled fibroblasts. Control group II-A&B showed immune-positive inflammatory cells infiltration at the epidermis at the wounds margins and the wounds beds. Insulin treated group I- A&B showed the wound bed with numerous immune-positive fibroblasts. PRP treated group II- A revealed in the wound bed many immune-positive inflammatory cells however group PRP treated group II- B showed the regenerated dermis with decreased inflammatory cells. Combined treated group III- A showed the regenerated dermis with many fibroblasts. Combined treated group III- B showed the regenerated dermis with fibroblasts and some inflammatory cells.

Statistical results

All values were expressed as mean ± standard deviation (SD). One-way ANOVA was used to determine level of significance. The differences were statistically significant at $P < 0.05$.

1- Descriptive statistics of the fasting blood glucose level (FBG) and the body weight represented as mean ± SD (Histograms 1,2) FBG values at the beginning of the experiment for all rats were (96.2± 1.31). After 3 days from STZ, the diabetic rats (control group II and experimental groups) showed a significant increased FBG as compared to control group I ($p<0.05$). The continuous monitoring of rats along the whole experimental duration revealed significant increased FBG in control group II (the diabetic non treated rats) as compared to all groups ($p<0.05$).

The measurement of the body weight at the beginning of the experiment for all rats showed the average body weight value (164±9.9). The continuous monitoring of rats along the experimental durations revealed significant decrease in the body weight in control group II as compared to all groups ($p<0.05$)

2- Descriptive statistics of the mean values of the percentage of wound closure in the experimental groups: (Histogram 3)

The percentage of wound closure was assessed at 14^{th} and 21^{th} days with time of sacrification. It showed a significant improvement in experimental groups ($p<0.05$) as compared to control group II ($p<0.05$), with no significant differences between these experimental groups ($p>0.05$). However, control group II showed non-significant improvement ($p>0.05$).

3. The mean area percent of collagen fibers deposition in Masson's trichrome stained sections: (Histogram 4)

At day 14^{th} (subgroup A): The insulin treated group I and combined treated group III showed a significant increase in mean area % of collagen fibers deposition as compared to PRP treated group II and diabetic non-treated group (control II).However, none of the experimental groups reached the control group I as all of them showed a significant decrease in collagen fibers deposition as compared to the control I ($p<0.05$).

At day 21(subgroup B): The insulin treated group I and combined treated group III showed a significant increase in mean area % of collagen fibers deposition as compared to non-treated groups (control II) and PRP treated group II ($p<0.05$) with no significant difference to the control group I ($p>0.05$).

4- The mean area percent of E-cadherin immunohistochemical stain in studied groups (Histogram 5):

At day 14^{th} and 21^{th} (subgroup A &B): the insulin (I), PRP (II) and combined treatment (III) groups showed significant increase in mean area % of E-cadherin as compared to diabetic non- treated (control group II) ($p < 0.05$).PRP treated group II showed a significant increase in E-cadherin as compared to insulin treated group I ($p < 0.05$). As regarding the combined treated group III, it showed a significant increase in E-cadherin area % ($p < 0.05$) as compared to all experimental groups with no significant difference ($p > 0.05$) to control group I.

5- The mean area percent of vimentin immunopositive cells: (Histogram 6)

At day 14^{th} (subgroup A): the diabetic non-treated (control group II) showed a significant increase in mean area % of vimentin as compared to all groups ($p < 0.05$). However, no significant difference was seen between insulin (I), PRP (II) and combined treatment (III) groups ($p>0.05$). All groups were significantly increased in mean area % of vimentin as compared to the control group I ($p < 0.05$).

At day 21 (subgroup B): the diabetic non-treated (control group II) and insulin treated group I showed significant increased mean area % of vimentin as compared to all groups ($p < 0.05$). All groups were significantly increased in mean area % of vimentin as compared to the control group I ($p < 0.05$).

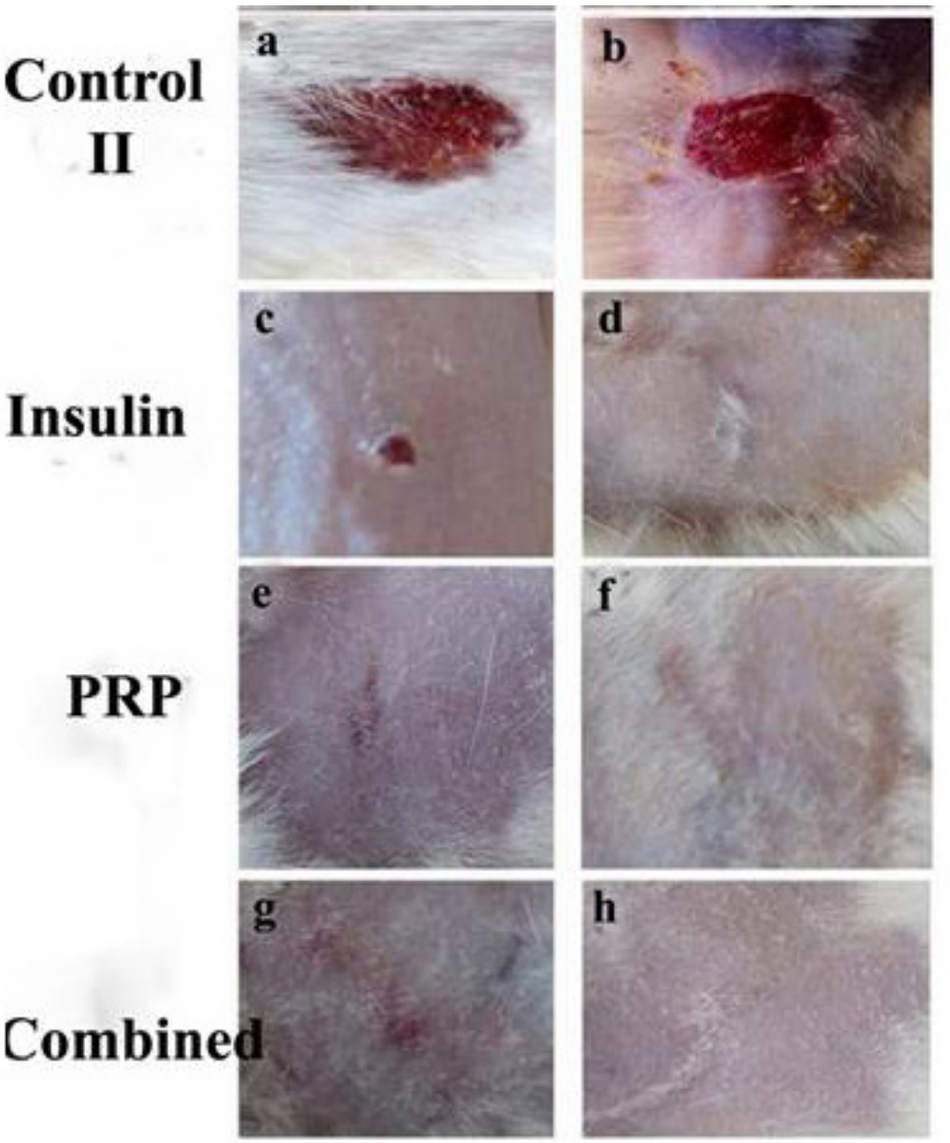


Plate. 1: Gross morphology of wounds at days 14th, 21th in groups.
(a): control group II-A reveals increased wound diameter.
(b): control group II-B shows slight decrease of wound diameter and the wound are covered by scabs.
(c): insulin treated group I-A reveals decreased wound diameter.
(d): insulin treated group I-B shows almost complete wound closure in some rats.
(e): PRP treated group II-A reveals significant decrease of wound diameter.
(f): PRP treated group II-B shows complete wound closure in some rats.
(g): combined treated group III-A reveals marked decrease in wound diameter.
(h): combined treated group III-B shows complete wound closure in most rats.

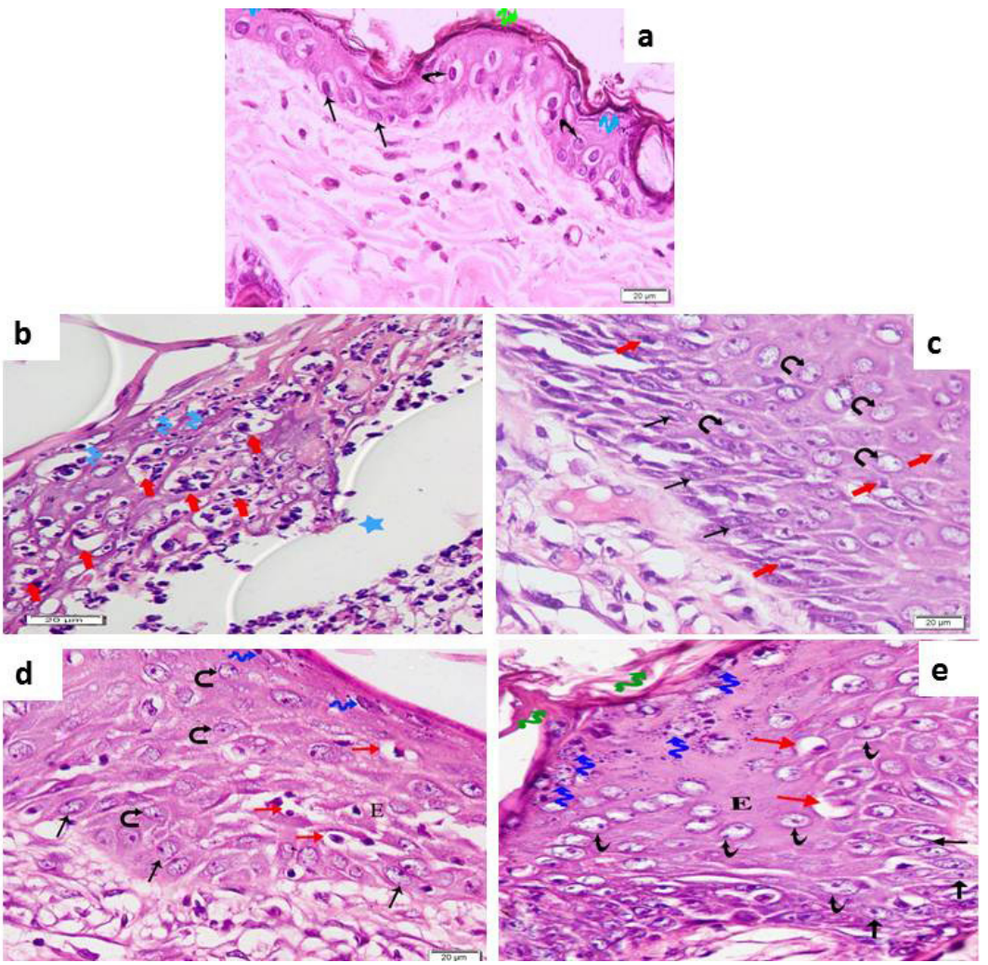


Plate. 2: Photomicrographs of H&E stained sections in rat skin X400:
(a): control group I-B showing the epidermis at the upper surface is formed of keratinized stratified squamous epithelium. It is formed of basal cell layer (black arrow), spinous cells layer (curved arrows), granular cell layers (blue zigzag arrows) and stratum corneum (green zigzag arrows).
(b): control group II-A showing diffuse disfigurement of keratinocytes with dark pyknotic fragmented nuclei and vacuolated cytoplasm at the spinous cells layer (red arrows) and the granular layer (blue zigzag arrows). There is a wide area of separation between wound bed and epidermis (blue star).
(c): control group II-B; showing the epidermis at wound margin with apparently normal cells at basal (thin black arrows) and spinous cells layers (curved arrows). Some cells with shrunken nuclei (red arrows) are also seen.
(d): Insulin treated group I-A showing the epidermis at the wound margin with apparently normal many cells at basal (thin black arrow), spinous cells layer (curved arrows), granular (blue zigzag arrow) and stratum corneum cells (green zigzag arrow) are obvious. Some cells with shrunken nuclei and vacuolated cytoplasm (red arrows) are also seen.
(e): Insulin treated group I-B showing epidermis at the wound margin with increased cell layers, apparently normal basal cell (thin black arrows), the spinous cells layer (curved arrow), granular layer (blue zigzag arrows) and stratum corneum layer (green zigzag arrows). Some cells with shrunken nuclei and vacuolated cytoplasm (red arrows) are still present.

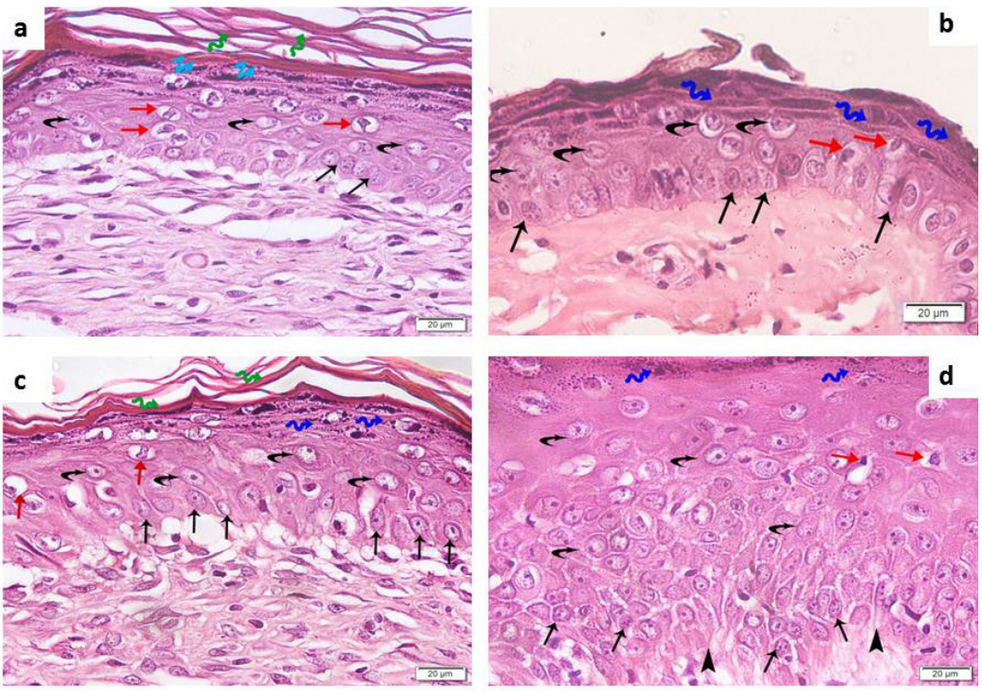


Plate. 3: Photomicrographs of H&E stained sections in rat skin x 400:
(a): PRP treated group II-A showing apparently normal basal cells (thin black arrows), the spinous cells layer (curved black arrows), granular cells (blue zigzag arrows) and stratum corneum layer (green zigzag arrow) are obvious. Some cells with shrunken nuclei and vacuolated cytoplasm (red arrows) are seen.
(b): PRP treated group II-B showing apparently normal basal cells layer (black thin arrows), the spinous cells layer (curved arrows) and granular cells (blue zigzag arrows) are observed. Some cells with shrunken nuclei and vacuolated cytoplasm (red arrows) are observed.
(c): combined treated group III-A showing apparently normal basal cells (black thin arrows), the spinous cells layer (black curved arrows), granular (blue zigzag arrows) and stratum corneum layer (green zigzag arrows) are obvious. Some cells with shrunken nuclei and vacuolated cytoplasm (red arrows) are seen.
(d) :combined treated group III-B showing the regenerated epidermis with marked increase of cell layers and apparently normal most keratinocytes. Clear basement membrane (arrow heads) is observed. Apparently normal most basal cells (thin black arrows), the spinous cells layer (curved arrows); granular cells (blue zigzag arrows) are seen. Remnant pyknotic cells (red arrows) are also observed.

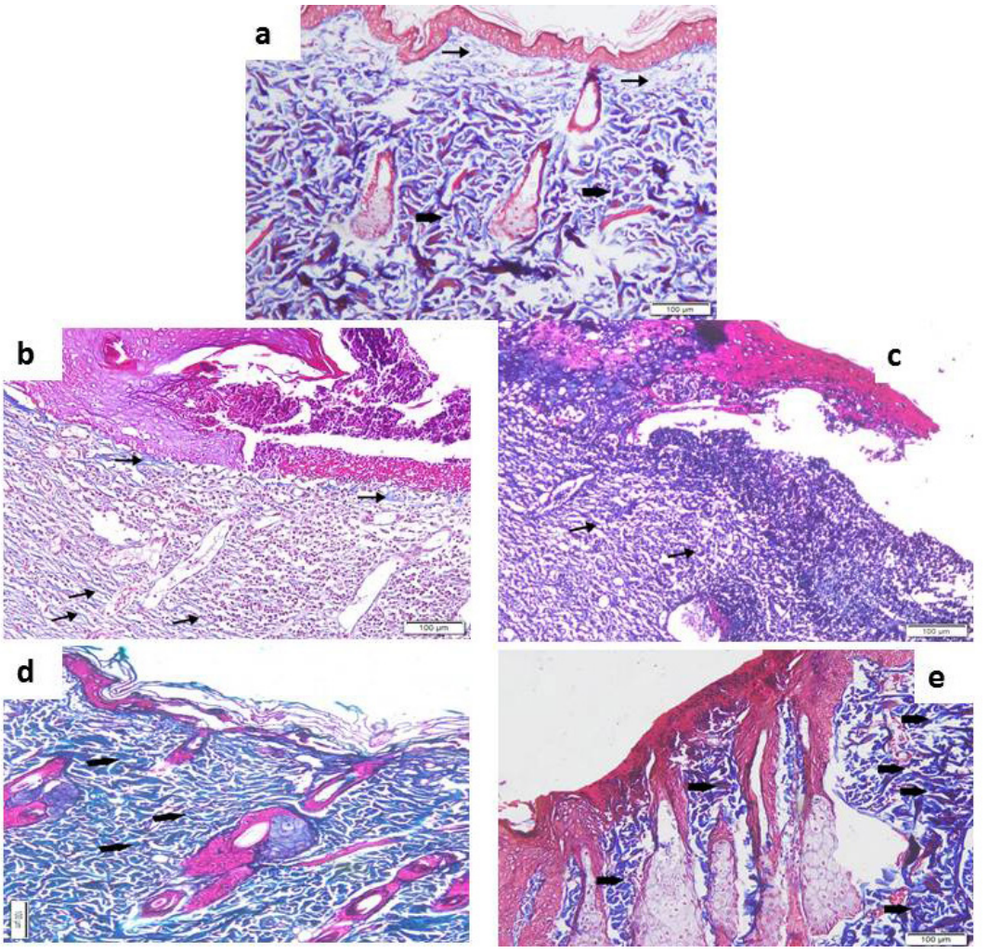


Plate. 4: Photomicrographs of Masson's stained sections in rat skin x100:
(a): control group I-B showing the dermis is formed of fine collagen fibers (thin arrows) in the upper papillary layer while the lower reticular layer contains thick collagen bundles (thick arrows).
(b): control group II-A showing the wound bed is filled with fine, irregularly distributed collagen fibers (thin arrows).
(c): control group II-B showing the wound bed is filled with fine, irregularly distributed collagen fibers (thin arrows).
(d): Insulin treated group I-A showing the wound area with diffuse deposition of thick collagen bundles (thick arrows) in the papillary and reticular dermis.
(e): Insulin treated group I-B showing the wound area with diffuse deposition of thick, irregularly arranged collagen bundles (thick arrows).

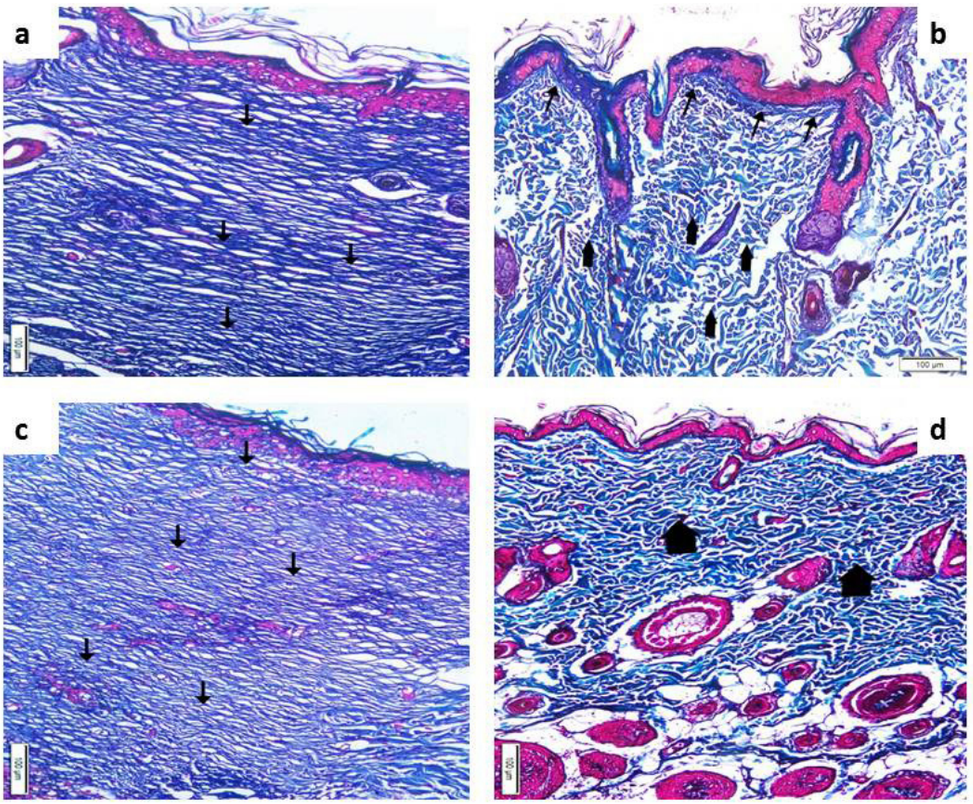


Plate. 5: Photomicrographs of Masson's stained sections in rat skin x100:
(a): PRP treated group II-A showing the regenerated dermis with densely arranged collagen fibers aligned horizontally in one direction (black arrows).
(b): PRP treated group II-B showing the papillary dermis with thin collagen bundles (thin arrows) and the remaining dermis shows diffuse deposition of thicker and denser collagen bundles (thick arrows) arranged in a network manner.
(c): combined treated group III-A showing the regenerated dermis with densely arranged collagen fibers aligned horizontally in one direction (black arrows).
(d): combined treated group III-B showing diffuse deposition of thick collagen bundles (thick arrows) are observed arranged in a network manner occupying most of the regenerated dermis.

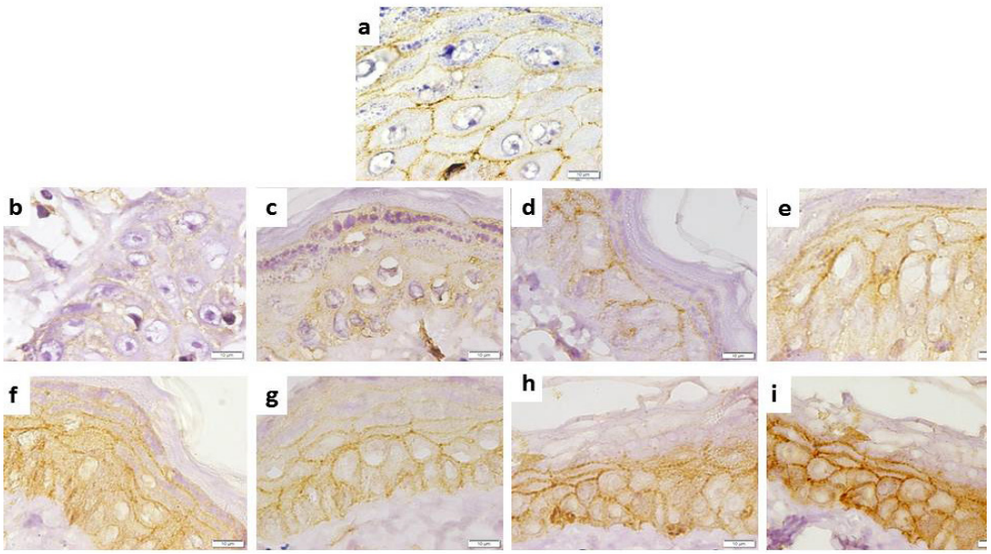


Plate. 6: Photomicrographs of E-cadherin immunostained sections in rat skin from the epidermis x1000:
(a): control group I-B showing strong membranous immune- reaction.
(b): control group II-A showing the epidermis at the wounds margins with negative immune-reaction.
(c): control group II-B showing the epidermis at the wounds margins with weak immune-reaction.
(d): Insulin treated group I-A showing mild immune-reaction.
(e): Insulin treated group I-B showing mild to moderate immune-reaction.
(f): PRP treated group II-A showing moderate immune-reaction.
(g): PRP treated group II-B showing moderate immune-reaction.
(h): combined treated group III-A showing moderate to strong immune-reaction.
(i): combined treated group III-B showing strong immune-reaction.

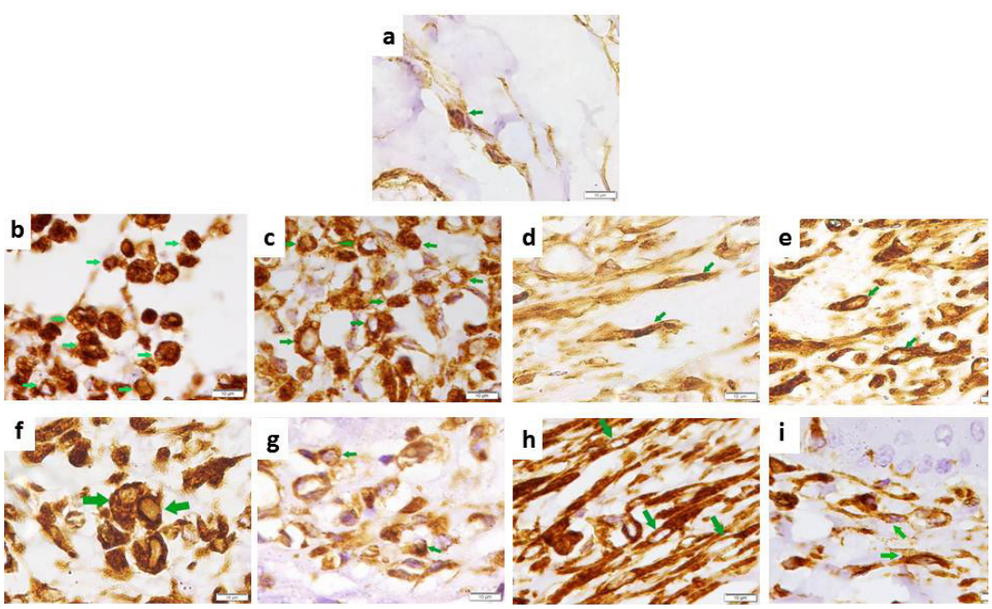
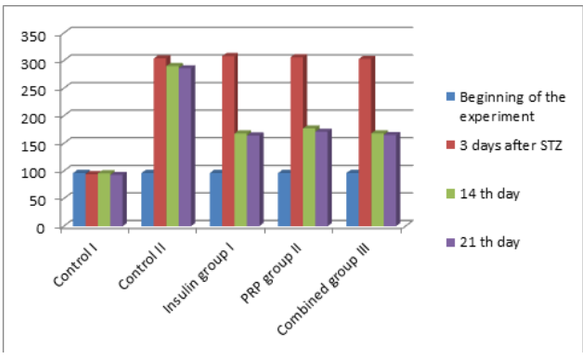
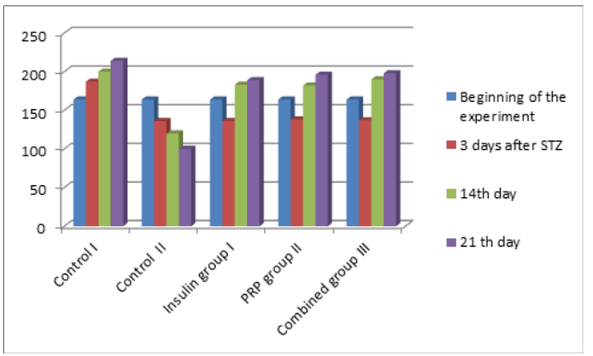


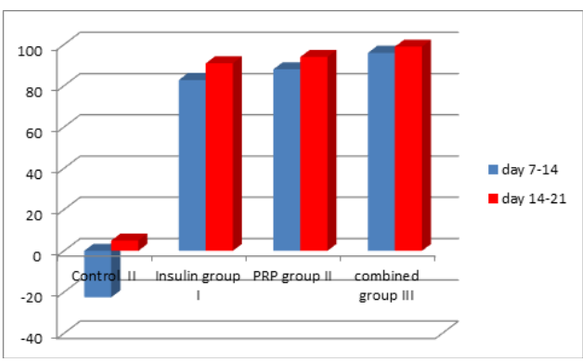
Plate. 7: Photomicrographs of Vimentin-immunostained sections in rat skin x1000:
(a): control group I-B showing the dermis with cells resemble fibroblasts (green arrows).
(b): control group II-A showing the wound bed with cells resemble inflammatory cells (green arrows).
(c): control group II-B showing the wound bed with cells resemble inflammatory cells (green arrows).
(d): Insulin treated group I-A showing the wound bed with cells resemble fibroblasts (green arrows).
(e): Insulin treated group I-B showing cells resemble fibroblasts (green arrows).
(f): PRP treated group II-A showing the wound bed with cells resemble inflammatory cells (green arrows).
(g): PRP treated group II-B showing the regenerated dermis with cells resemble inflammatory cells (green arrows).
(h): combined treated group III-A showing the regenerated dermis with many cells resemble fibroblasts (green arrows).
(i): combined treated group III-B showing the regenerated dermis with some cells resemble fibroblasts (green arrows).



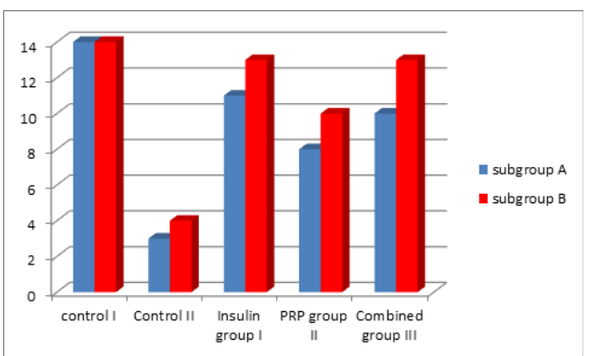
Histogram 1: The mean values of FBG in studied groups



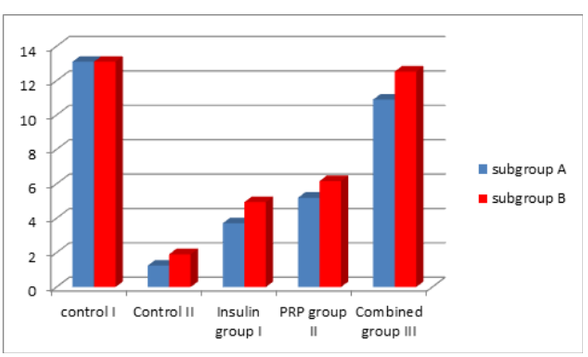
Histogram 2: The mean values of body weight in studied groups



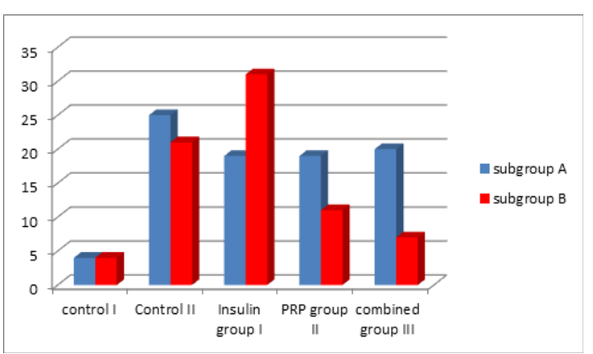
Histogram 3: The mean values of the percentage of wound closure in studied groups



Histogram 4: The mean area percent of collagen fibers deposition in the studied groups



Histogram 5: The mean area percent of E-cadherin immunohistochemical stain in studied groups



Histogram 6: The mean area percent of vimentin immunopositive cells in studied groups

DISCUSSION

Wound healing is a complicated process that takes place at an optimal rate in healthy individuals, but it is usually impaired for patients in DM[21].

During animal observation, deaths were recorded in the diabetic rats as about 25 % of rats died 3-4 days after STZ injection. This was in accordance to Wang *et al*,(2014)[22] who observed a high mortality rate in rats after a single dose of STZ injection. The deaths might be associated with infection, malnutrition and toxicity of STZ.

The present study measuring the fasting blood glucose (FBG) in diabetic non-treated rats (control group II) revealed a significant increase in FBG as compared to all groups. The insulin (I), PRP (II) and combined treated (III) groups showed a significant increase in FBG as compared to the control group I; however, no significant difference was observed between these experimental groups. This might be clarifying that local insulin treatment had no effect on the blood glucose level. This was in accordance to Bhittani *et al*, (2020)[23] who observed no significant hypoglycemia with local insulin treatment.

The diabetic non-treated rats (control group II) revealed a significant decrease in the body weight as compared to all groups. The experimental groups (The insulin I, PRP II and combined treated III groups) showed a significant increase in the body weight as compared to control group II. However, no significant difference was observed between these experimental groups. These findings were supported by Azevedo et al, (2015)[12] who mentioned that weight loss is associated with poor nutrient intake and contributes partially to the wound-repair defect observed in diabetic animals.

In the present study control group II revealed gross and histological delay of wound healing in the form of failure of wound closure, diffuse disfigurement of keratinocytes and defective collagen formation in the wound bed. Most of these findings were observed also by kamar *et al*, (2019)[16]. These findings might be due to the prolonged inflammatory response in diabetic wounds resulting in high levels of inflammatory cytokines, which became destructive and promoted apoptosis in the diabetic wounds.

The insulin treated group I revealed significant improvement of wound closure as compared to control group II. In addition, the H&E stained sections showed a more or less apparently normal keratinocytes. This was in accordance to Bhittani *et al*, (2020)[23] who reported that the rate of wound closure was found to be higher in the topical insulin treated wounds than the normal saline treated wounds.

Swaminathan, (2014)[24] mentioned that the direct application of insulin to the injured skin surface restores the decreased levels of DNA synthesis of basal epithelial cells to normal values, thereby stimulating active cell proliferation. Moreover, Yu *et al*,(2017)[25] added that the basal layers of the epidermis of healed wounds are better organized and differentiated when insulin was applied to the wounds.

The PRP treated group II revealed significant improvement in wound closure compared to control group II. This could be related to the growth factors accumulated in the platelet granules. These factors include VEG, which promotes angiogenesis, PDGF, and TGF-β[26].

This was in line with Rezende *et al*,(2020)[27] who observed reduction in wound size in the PRP treated wounds after 14 and 21 days as compared to the non-treated groups. Moreover, examination of H&E stained sections of PRP treated group II after 14 days revealed disfigurement of the keratinocytes at wounds margins. However, after 21 days most skin sections revealed improvement in the structure and arrangement of the keratinocytes with complete wound closure in many skin sections. These findings were in line with Farghali *et al*, (2017)[18] who mentioned that the healing effect of PRP needed more time to eliminate the inflammatory phase of the wound healing.

Moreover, on the level of histological evaluation, the combined treated group III showed the best results in epidermis regeneration evidenced by the apparently normal almost all keratinocytes. This could be explained by the synergistic healing effect of insulin growth factor and PRP growth factors.

Masson's trichrome stained skin sections from control group II at the wounds beds, after 14 and 21 days, revealed fine irregularly distributed collagen fibers. The observed defective collagen fibers formation could be related to defective fibroblasts proliferation in the dermis as a result of DM. In accordance, Tan *et al*. (2019)[21] postulated that many proteases were activated in diabetic wounds, which easily decompose growth factors in the wound site, leading to defect of diabetic skin ulcer healing.

The insulin treated group I revealed the diffuse deposition of thick collagen bundles. This was in accordance to Wang and Xu, (2020)[2] who reported that the topical insulin improves wound closure, reduces wound healing time and improves wound remodeling.

The PRP treated group II after 14 days showed diffuse deposition of densely arranged collagen fibers aligned horizontally in one direction however, after 21 days improvement in the collagen deposition and arrangement was observed. This can be explained that the PRP needed more time to stimulate maturation of the granulation tissue. This was in agreement with Farghali *et al*, (2017) [18]. This leads to a conclusion that insulin had more effect on collagen deposition than PRP.

The collagen deposition in combined treatment group III suggested that the combined insulin and PRP had a better effect on collagen remodeling than each treatment alone. Moreover we reached to a fact that insulin had the upper hand in collagen synthesis that might be reached to the level of fibrosis or scar tissue formation with

increased doses. This was in agreement with (Martinez-Jimenez *et al*, 2013)[28].

Immunohistochemical staining of control group I stained with anti E- cadherin antibody revealed strong reaction in cell membrane of almost all epidermal cells .This was in line with Bakry et al,(2016)[29].

The control group II showed negative to weak immune reaction. This might be explained by the diffuse disfigurement of keratinocytes with loss of cell junctions between them. In the insulin treated group I there was significant improvement of E-cadherin expression compared to control group II .This might be explained by the healing effect of insulin on the epidermis keratinocytes. Moreover, the PRP treated group II showed a significant improvement of E-cadherin as compared to insulin treated group I. This might be explained that PRP had more healing effect than insulin on the level of E-cadherin deposition between keratinocytes. On the other hand, the combined treated group III showed the best results because there was strong immune reaction and significant increased E-cadherin immune reactivity as compared to all groups with no significant difference to the control group I. This might be due to the synergistic effect of both treatments on skin wound healing.

Immunohistochemical staining of control group I stained with anti-vimentin antibody revealed the dermis with immune-positive fibroblasts and few macrophages as a normal cells present in the dermis. This was in line with Aslan *et al*,(2005)[30] who mentioned that in skin tissue, vimentin-immunoreactivity was present in the fibroblasts, endothelial cells of vessels, dentritic cells, Langerhans cells, and fibroblasts. The control group II showed numerous immune-positive cells in the epidermis and the wounds margins. These cells could be inflammatory cells infiltration as a result of defective inflammatory phase termination of the wound healing. This was declared by Devaux *et al*,(2019)[31] who stated that the reduction of the surface expression of E-cadherin on epithelia could be accompanied by alteration in the cell membrane permeability that leads to alteration of the anti-bacterial immune responses of the epidermis and infiltration of inflammatory cells between keratinocytes.

On the other hand, the insulin treated group I showed marked increased vimentin immune reactivity that could be explained by increased fibroblasts deposition and reactivity as a result of insulin treatment. Histological assessment of vimentin immune stained section of the PRP treated group II after 14 days showed immune positive cells that were most probably inflammatory cells that decreased significantly after 21 days. This might be explained that insulin had a better anti-inflammatory effect as compared to PRP.

In addition, the mean area % of vimentin in combined treated group III (after 21 days) was significantly decreased as compared to all experimental groups. However, it was significantly increased as compared to the control group I. This could be explained that the wound healing process was about to be completed.

CONCLUSION

This study demonstrated the importance of local therapeutic effects of insulin and PRP in treatment of diabetic wounds by accelerating the wound healing process . Insulin local treatment had better effects than PRP on the level of collagen deposition. PRP local treatment had better effects than insulin on rate of wound closure , epidermis regeneration and establishment of the cell junctions. Local combined treatment with insulin and PRP showed a rapid rate of wound closure, the best results in epidermis regeneration, keratinocytes morphology, establishment of cell junctions and collagen deposition in the regenerated dermis.

CONFLICT OF INTERESTS

There are no conflicts of interest.

REFERENCES

1. Cancela AM, Lana JF, Annichino-Bizzachi JM, Belangero, WD and Luzo AC: Use of Platelet-Rich Plasma (PRP) in Treating Chronic Wounds. In Platelet-Rich Plasma; Springer Berlin Heidelberg. (2014); 281-8.

2. Wang J and Xu J: Effects of Topical Insulin on Wound Healing: A Review of Animal and Human Evidences. Diabetes, Metabolic Syndrome and Obesity: Targets and Therapy (2020):13 719–727. doi.org/10.2147/DMSO.S237294.

3. Wang L, Yang B, Jiang H, et al. : The molecular mechanism study of insulin in promoting wound healing under highglucose conditions. J Cell Biochem. (2019);120(9):16244–16253.

4. Vatankhah N, Jahangiri Y, Landry GJ, Moneta GL and Azarbal AF : Effect of systemic insulin treatment on diabetic wound healing. Wound Repair Regener; (2017); 25(2):288–291. Doi:10.1111/wrr.2017.25.issue-2

5. Sridharan K and Sivaramakrishnan G: Efficacy of topical insulin in wound healing: a preliminary systematic review and meta-analysis of randomized controlled trials. Wound Repair Regener. (2017);25(2):279–287. doi:10.1111/wrr.2017.25.issue-2.

6. Badis D. and Omar B: The effectiveness of platelet-rich plasma on the skin wound healing process: A comparative experimental study in sheep. Veterinary World, (2018); 11(6): 800-808. doi: 10.14202/vetworld.2018.800-808.

7. Farzad N, Milad D, Marzieh M and Farshad N : An Innovative Approach to Platelet-Rich Plasma Application in Military Medicine; a Review Article. J Arch Mil Med. (2015); 3(2); 7 pages.

8. Schafer M and Werner S: Cancer as an overhealing wound: an old hypothesis revisited. Nat Rev Mol Cell Biol. (2008); 9; 628 –638.

9. Bhatt T, Rizvi A, Batta SPR, Kataria S, and Jamora C: REVIEW ARTICLE: Signaling and Mechanical Roles of E-cadherin. Cell Communication & Adhesion, (2013) 20: 189–199. DOI: 10.3109/15419061.2013.854778.

10. Cheng F, Shen Y, Mohanasundaram P, Lindström M, Ivaska J, Ny T and Eriksson JE : Vimentin coordinates fibroblast proliferation and keratinocyte differentiation in wound healing via TGF-β-Slug signaling. Proc. Natl. Acad. Sci. USA (2016), 113, E4320–E4327.

11. Yumun G, Kahaman C, Kahaman N, Yalcınkaya U, Akcılar A, Akgul E and Vural AH: Effects of hyperbaric oxygen therapy combined with platelet-rich plasma on diabetic wounds: an experimental rat model. Arch Med Sci (2016); 12, 6: 1370–1376.

12. Azevedo F, Pessoa A, Moreira G, Dos Santos M, Liberti E, Araujo E, Carvalho C , Saad M, and Lima M H: Effect of Topical Insulin on Second-Degree Burns in Diabetic Rats. Biological Research for Nursing. (2015); 1-12. DOI: 10.1177/1099800415592175.

13. Sonnleitner D, Huemer P and Sullivan DY: A simplified technique for producing Platelet-rich plasma and Platelet Concentrate for intraoral bone grafting techniques: A technical note. Int J Oral Maxillofac Implants. (2000); 15:879 -882.

14. Stephen S, Agnihotri M, Kaur S : A randomized, controlled trial to assess the effect of topical insulin versus normal saline in pressure ulcer healing. Ostomy Wound Manag. (2016); 62(6):16–23.

15. Karina Samudra, M, Rosadi I, Afini I, Widyastuti T, and Sobariah S et al. : Combination of the stromal vascular fraction and platelet-rich plasma accelerates the wound hea ling process: pre-clinical study in a Sprague-Dawley rat model. Stem Cell Investigation. (2019); 6, 18-18.

16. Kamar SS, Abdel-Kader DH, and Rashed LA: Beneficial effect of Curcumin Nanoparticles-Hydrogel on excisional skin wound healing in type-I diabetic rat: Histolo gical and immunohistochemical studies. Annals of Anatomy 222 (2019); 94–102. doi.org/10.1016/j.aanat.2018.11.005.

17. Abdollahi M, Yi Ng TS, Rezaeizadeh A, Aamidor S, Twigg SM, Min D, McLennan SV : Insulin treatment prevents wounding associated changes in tissue and circulating neutrophil MMP-9 and NGAL indiabeticrats. PLoS ONE (2017); 12(2):e0170951.

18. Farghali HA, AbdElKader NA, Khattab MS and AbuBakr HO: Evaluation of subcutaneous infiltration of autologous platelet rich plasma on skin-wound healing in dogs. Bioscience Reports. (2017); (37) BSR20160503.DOI: 10.1042/BSR20160503

19. Chen L, Tredget EE, Wu PY, Wu Y: Paracrine factors of mesenchymal stem cells recruit macrophages and endothelial lineage cells and enhance wound healing. PLoS One (2008); 3:e1886.

20. Bancroft JD, Layton C: The Hematoxylin and eosin. In: Suvarna SK and Layton C and Bancroft JD editors. Theory & Practice of histological techniques. (2013); 7th ed., Churchill Livingstone of El Sevier. Philadelphia; Ch.10 and 11,173-214.

21. Tan W S, Arulselvan P, Ng S F, Mat Taib CN, Sarian MN, and Fakurazi S :Improvement of diabetic wound healing by topical application of Vicenin-2 hydrocolloid film on Sprague Dawley rats. BMC Complementary and Alternative Medicine (2019) 19:20. doi.org/10.1186/s12906-018-2427-y.

22. Wang YJ, Xie XS, Feng SG, Long QX, Ai N, Wang BF:Causes of death in STZ-induced rat models of diabetes mellitus]. Sichuan Da Xue Xue Bao Yi Xue Ban. (2014) Jul;45(4):691 -5. Chinese. PMID: 25286701.

23. Bhittani MK, Rehman M, Humera N, Altaf HN, Altaf AS: Effectiveness of Topical Insulin Dressings in Management of Diabetic Foot Ulcers. World J Surg. (2020); (44) 2028–2033. doi. 10.1007/s00268-019-05321-3.

24. Swaminathan R: Effectiveness of topical insulin in management of chronic diabetic foot ulcers. Indian J Basic Appl Med Res 3. (2014); (3):455–459.

25. Yu T, Gao M, Yang P, Pei Q, Liu D, Wang D, Zhang X, Liu Y: Topical insulin accelerates cutaneous wound healing in insulin-resistant diabetic rats. Am J Transl Res (2017);9(10):4682-4693.

26. Golebiewska EM and Poole AW: Platelet secretion: From haemostasis to wound healing and beyond. Blood Rev. (2015), 29: 153-162.

27. Rezende RS de, Eurides D, Alves EGL, Venturini GC, Felipe RL de : Co-treatment of wounds in rabbit skin with equine platelet-rich plasma and a commercial ointment accelerates healing. Cienc. anim. bras. (2020); 21, e-56274. DOI: 10.1590/1809-6891v21e-56274.

28. Martinez-Jimenez M, Aguilar-García J, Valdés-Rodríguez R, Metlich-Medlich M, Dietsch L, Gaitán-Gaona F, KolosovasMachuca E, González F and Sánchez-Aguilar J : Local Use of Insulin in Wounds of Diabetic Patients. Plastic and Reconstructive Surgery. (2013), 132(6), pp.1015e-1019e.

29. Bakry OA, Hagag MM, Kandil MA, Shehata WA: Aquaporin 3 and E-Cadherin Expression in Perilesional Vitiligo Skin. Journal of Clinical and Diagnostic Research. (2016) 10(12): WC01 -WC06 DOI: 10.7860/JCDR/2016/22730.8959

30. Aslan S, Kocamifi H, Nazli M, Gulmez: Immunohistochemical Distribution of Desmin and Vimentin in the Skin of Zavot Cattle. Turk J Vet Anim Sci.29 (2005) 325-329.
31. Devaux CA, Mezouar S, Mege J-L : The E-Cadherin Cleavage Associated to Pathogenic Bacteria Infections Can Favor Bacterial Invasion and Transmigration, Dysregulation of the Immune Response and Cancer Induction in Humans. Front. Microbiol. (2019); 10:2598. doi: 10.3389/ fmicb.2019.02598.

الملخص العربى

دراسة هستولوجية على التأثير المحتمل للإنسولين والبلازما الغنية بالصفائح الدموية على جروح الجلد المستحدثة في ذكور الجرذان البيضاء البالغة المصابة بداء السكري

سلوي متولي علي، داليا المراكبي، سارة مسعد عامر، حنان عبد الله

قسم الهستولوجيا الطبية وبيولوجيا الخلايا - كلية الطب- جامعة القاهرة- المنيل -القاهرة - مصر

المقدمة: داء السكري له العديد من المضاعفات مثل أمراض القلب والأوعية الدموية ، واعتلال الكلي السكري ، و عدم التئام الجروح الجلدية الناتجة عن مرض السكري.

الهدف من العمل: تقييم التأثير العلاجي المحتمل للأنسولين والبلازما الغنية بالصفائح الدموية على جروح الجلد المستحدثة في ذكور الجرذان البيضاء البالغة المصابة بداء السكري.

المواد والطرق المستخدمة: ٥٠ من ذكور الجرذان البيضاء البالغة ، بمتوسط وزن جسم ١٥٠-٢٠٠ جم ، تم تقسيمهم إلى المجموعات التالية (مجموعات ضابطة وتجريبية).

المجموعات الضابطة (٢٠ جرذًا) تم تقسيمهم بالتساوي إلى مجموعتين (المجموعةالضابطة الأولى: الجرذان طبيعية مع جلد سليم والمجموعة الضابطة الثانية: الجرذان مصابة بداء السكري مع احداث جروح جلدية و التي تم علاجها بحقن محلول ملحي داخل الأدمة ٥٠٠ ميكرولتر مرة واحدة يوميًا عند اطراف الجروح.

المجموعات التجريبية (٣٠ جرذا) تم تقسيمهم بشكل متساوٍ إلى ثلاث مجموعات.

المجموعة الاولي المعالجة بالانسولين : الجرذان مصابة بداء السكري مع حقن الأنسولين الموضعي داخل الأدمة ٠,٥ وحدة / ٥٠٠ ميكرولتر مرة واحدة يوميًا عند اطراف الجروح.

المجموعة الثانية المعالجة بالبلازما الغنية بالصفائح الدموية: الجرذان مصابة بداء السكري مع حقن البلازما الغنية بالصفائح الدموية الموضعية داخل الأدمة ٥٠٠ ميكرولتر كجرعة وحيدة على اطراف الجروح.

المجموعة الثالثة المعالجة بالعلاج المركب: الجرذان مصابة بداء السكري مع حقن الأنسولين والبلازما الغنية بالصفائح الدموية معا داخل الأدمة بفاصل زمني ساعتين .

تم تقسيم المجموعات الضابطة والتجريبية إلى مجموعتين فرعيتين أ و ب وفقًا لوقت التشريح بعد ١٤ و ٢١ يومًا بعد احداث الجروح على التوالي.

النتائج: أظهرت المجموعات العلاجية تحسنًا ملحوظًا في التئام الجرح وتجديد البشرة وزيادة ترسب الكولاجين مقارنة بالمجموعة الضابطة الغير معالجة. أظهرت مجموعة الأنسولين والمجموعة المعالجة بعلاج مركب زيادة في نسبة ترسب الكولاجين مقارنة بالمجموعة المعالجة بالبلازما الغنية بالصفائح الدموية، ومع ذلك ، أظهرت المجموعة المعالجة بعلاج مركب أفضل النتائج.

الاستنتاج: أظهر العلاج المركب بالأنسولين والبلازما الغنية بالصفائح الدموية معدلًا سريعًا لالتئام جروح السكري.