Effect of Topical Resveratrol Formulation on Healing of Experimental Full Thickness Wound in Diabetic Male Albino Rats: (Histological and Immunohistochemical study)

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Abstract: Impaired healing of wounds is one of the most serious diabetes mellitus microvascular complications. Resveratrol (RSV), a natural polyphenol compound, has antioxidant, anti-inflammatory and antidiabetic activities. Our study aimed to evaluate the potential of topical resveratrol formulation on histological, immunohistochemical and anti-oxidative findings in experimentally induced full-thickness wound model in induced diabetic rats. Seventy - two albino rats were divided into two main groups: normal control group (18 rats) and diabetic group (54 rats) which divided equally into three sub groups: sub group (I) received plain ointment base and considered as control diabetic positive; sub group (II) received topical standard marketed product for wound healing containing β-Sitosterol (MEBO) (0.25% w/w) and considered as reference group, while sub group (III) received topical Resveratrol 0.5% ointment, and considered as test group. Wound surface area (W.S.A) and oxidative enzymes, Histological and Immunohistochemical studies were carried out for each group throughout treatment period (1st, 2nd, and 3rd weeks). The obtained results showed that topical resveratrol ointment showed more potential healing effect on diabetic wound than the reference product. Resveratrol has percent wound contraction rate high than the wound closure rate than MEBO. Wound treated with resveratrol (group II) showed re-epithelialization and increased epidermal thickness compared with rats in control diabetic. So, topical application of resveratrol ointment has potential effect on enhancing wound healing process in diabetic conditions, and this activity may be attributed – mainly – to its free-radical scavenging activity.

[Wardah Abdullah Alasmari Naser A. ElSawy, Mohammed A.S. Abourehab, Eman Mohamed Faruk, Ashwaq Abdullah Alasmari. **Effect of Topical Resveratrol Formulation on Healing of Experimental Full Thickness Wound in Diabetic Male Albino Rats: (Histological and Immunohistochemical study).** *Life Sci J* 2018;15(10):6-20]. ISSN: 1097-8135 (Print) / ISSN: 2372-613X (Online). http://www.lifesciencesite.com. 2. doi:10.7537/marslsj151018.02.

Keywords: Resveratrol - Wound Healing - Diabetic Rats - topical formulation

1. Introduction

The prevalence of diabetes is increasing globally, where, it had been estimated that world prevalence of diabetes in 2013 was 8.3% (1). And chronic non healing foot ulcers was developed in 15% of diabetic patients (2).

Skin-wound healing process is sequential overlapping phases: hemostasis, inflammation, new tissue formation (proliferation) and remodeling (3). An aberrant course of inflammation has been demonstrated in wounds of diabetic patients (4). Prolonged hyperglycemia has been estimated to decrease the healing rate of foot ulcers (5). Therefore, delayed healing rate of wound is one of the long-term complications of diabetes (6 & 7). Which is mainly mediated by methylglyoxal (8). In a study on diabetic animal models of impaired wound healing, it was found that the wound healing process was accelerated by suppressing reactive oxygen stress (9). One of the pathogenic factor in delayed diabetic wound complications is Oxidative stress (10). Delayed wound healing in diabetes was demonstrated to be mainly, associated with hyperglycemia, over-expression of inflammatory mediators (cytokines), oxidative stress, decreased synthesis of collagen, and reduced angiogenesis in addition to the infections of the wounds (11), where, different studies proved that ROS may promote the secretion of different factors as epidermal growth factor (EGF), fibroblast basic growth factors, and G protein-coupled receptors (12). But, at higher concentrations of ROS, severe tissue damage may be induced and in some cases it may lead to malignant transformation (13 & 14). Cells develop cellular defensive mechanisms which maintain balanced redox state, these mechanisms include the antioxidant enzymes superoxide dismutase (SOD),

catalase (CAT), glutathione peroxidase, glutathione reductase, in addition to the other endogenous free radical scavengers as total reduced glutathione (GSH) (15 & 17 & 18).

Resveratrol (3,5,4′-trihydroxystilbene), a natural polyphenolic flavonoid compound found in many plants, is widely distributed in edible fruits and vegetables, including red grapes. Resveratrol showed antioxidant, anti-inflammatory, anti-hyperlipidemic, and antitumor activities (18). Resveratrol has been demonstrated to prevent cell damage caused by free radicals by virtue of its strong antioxidant properties, as well as by inhibiting cell death (19 & 20). Moreover, resveratrol appears to protect against diabetes (21). As it has been reported that resveratrol reduces hyperglycemia in humans through the mechanism of increasing insulin sensitivity. Resveratrol was demonstrated to have the ability to enhance stimulation of glucose uptake in the absence of insulin (22).

Recent study has demonstrated that oral resveratrol supplementation enhances the healing of the foot ulcer and decreases plasma fibrinogen level in type 2 diabetic patients (23).

Due to its hydrophobicity, resveratrol is poorly absorbed following oral administration, Because of its low bioavailability and extensive rapid first-pass metabolism (24), resveratrol is a suitable candidate for topical applications. No previous studies had been carried out to evaluate the potential effects of topical formulations of resveratrol on wound in streptozotocin-induced diabetic rats, therefore, so the present work designed to evaluate the wound healing and antioxidant potential of topical resveratrol formulation in diabetic rats compared with a standard marketed reference formulation.

2. Material and Methods

2.1. Materials

Resveratrol was purchased from Xian Lukee Bio-Tech Co., Ltd., (Xi’ an, Shaanxi Sheng, China), Streptozotocin (STZ) was purchased from Sigma-Aldrich Chemical Co. (St. Louis, MO, USA). White soft paraffin, hard paraffin, liquid paraffin, Cetostearyl alcohol and bees wax were purchased from (BDF, UK), MEBO (Batch No. 0202, Gulf Pharmaceutical Industries, Ras Al Khaimah, U.A.E.), other solvents and materials were of analytical grades and used without further purification.

2.2. Animals

Ninety-four adult male Albino rats (150-180 gm). Animals were obtained from the animal house of Faculty of Medicine - Umm Al Qura University. Our experiment was done according to the International Principles of Laboratory Animal Research of the Faculty of Medicine, Umm Al Qura University, KSA. Animals were individually housed in well-ventilated stainless steel cages in a controlled temperature (23-25°C) and relative humidity of 45-50 % with a light dark cycles of 10 and 14 h, respectively. Animals were kept in this standard conditions as an acclimatization period to the laboratory environment for one week prior to the study. During the entire period of the study, animals were provided with standard rodent pellet diet and tap water *ad libitum*.

Rats were divided into two groups: normal control group (18 rats) and diabetic group (54 rats) which divided equally (18 rats each) into three sub groups sub group (I) received simple ointment base and considered as control positive; sub group (II) received topical standard marketed product for wound healing containing β-Sitosterol (**MEBO**) 0.25% and considered as reference group, while sub group (III) received topical Resveratrol 0.5% ointment, and considered as test group.

2.3. Experimental

2.3.1. Formulation of Resveratrol Ointment:

Resveratrol was prepared with simple ointment base B.P. in a concentration of (0.5 % w/w) by fusion method. The prepared ointment formulation was kept in a tightly closed glass container used for topical application and stored at 4 ºC.

High reputation marketed ointment formulation for wound healing, 0.25% w/w β-Sitosterol Ointment USP was selected as a reference for comparison.

2.3.2. Dermal Irritation Study:

A primary skin irritation test was done on animals to show any skin allergy that happen from topical application of ointment. Animals that showed preexisting skin irritation or abnormalities was excluded from the study. A suitable amount of the formula was applied to 6 cm^2 intact area on each animal which was then caged separately. After 4 h of ointment application, the amount of ointment was removed and the sites of ointment application (25).

2.3.3. Induction of Diabetes:

Experimental diabetes was induced in animals by a single intraperitoneal injection of streptozotocin (80 mg/kg body weight) freshly prepared in Citrate Buffer (0.1 M, pH 4.5) after overnight fasting. All the animals were given 5% glucose solution to avoid sudden post-injection hypoglycemia- first phase hypoglycemic condition (26). Blood samples were obtained from the rat tail, 72 h after the injection for estimation of blood glucose level using Glucometer. The animals showing stable blood glucose level >250 mg/dL were considered diabetic then full-thickness excision wound model was persistent. Blood glucose level was estimate two hours after creation of the wounds (28). and after treatment every 1,2, 3 weeks (27).

2.3.4. Wound Creation:

The upper back of rats was shaved by electrical small animal clipper, and the animals were examined for any skin abnormalities. The shaved areas were disinfected with 70% alcohol, then, rats were anesthetized by the open mask method with anaesthetic ether, then 2x2.5 cm wound area was created (a full-thickness excisional wound of circular area was performed, as (500 mm^2) wound area was excised from the back of all rats by surgical blade The same researcher performed all surgical procedures. Animals were strictly observed for any signs of infection, and those arise any signs of infection were excluded from the study protocol.

2.3.5. Experiment Design (Protocol):

After creation of wound, rats of diabetic group were randomly divided into three groups (eighteen rats each) [29]:

Group (IIa) received plain ointment base and considered as control positive;

Group (IIb) received topical standard marketed product for wound healing containing β-Sitosterol (MEBO) and considered as reference group.

Group (IIb) received topical Resveratrol 0.5% ointment and considered as test group.

2.3.5.1. Treatment:

As wound dimensions were approximately (20 mm x 25 mm x 2 mm) so amount of different ointment treatments about (1.5 cm^3) used to cover wound cavity and boundaries of wound through using a sterile syringe; this amount was fixed for all treatments throughout all time of experiment. The treatment started in each group just 2 hours after wound creation. For all animals, the treatments were applied once daily with topical application for all treatments. Periodically, we follow the wound healing by the biochemical and histological parameters in which the tissue was obtained at 7^{th}, 14^{th}, and 21^{st} day of wound creation.

2.3.5.2. Wound Surface area, percent wound contraction and epithelialization time measurements:

By placing a transparent tracing paper over the wound we measure the wound area then counted the squares and the area was recorded [30].

The percentage of wound contraction was calculated using the following formula:

Wound Area Contraction (%) = $[(A_0 – A_t)/A_0] * 100$

Where: A_0: the initial wound surface area

A_t: the wound surface area at time t

For each rat, measurement of wound area was repeated three times at the end of (1^{st}, 2^{nd}, and 3^{rd} weeks) time intervals of treatment. Additionally, wounds were photographed by digital camera at these intervals. At the end of the specified intervals (1^{st}, 2^{nd}, and 3^{rd} weeks), 8 rats from each group were randomly selected and sacrificed then the skins of wound areas were dissected and isolated for histological and immunohistochemical examination.

2.3.5.3. Enzymatic and non-enzymatic antioxidant assay

From the wound area, the small part of granuloma tissue was used for antioxidant assay. The granuloma tissues were homogenized in phosphate buffer (pH 7.0) and centrifuged under cold condition. The clear supernatant was taken to assay of antioxidants level. Catalase activity was estimated according to the method described by [31]. Superoxide dismutase (SOD) activity was measured, according to the method of [32]. Total reduced glutathione (GSH) level was determined in tissues homogenates, according to the method described by [33]. For Lipid peroxidation analysis, we used the method described by [34].

2.3.5.4. Histological study:

The animals were anaesthetized by inhalation using diethyl ether and sacrificed, skin specimens were taken from the wound area with the surrounding normal skin. Skin samples were collected and cleaned properly from connective tissue and stored in 10% neutral buffered formaldehyde. Then tissues were dehydrated, cleared in xylol, and then embedded in paraffin blocks and sectioned (4□5 µm thickness) were prepared using microtome. Finally, skin sections were stained with haematoxylin and eosin (H & E) stain and Masson's trichrome. Then examined under light microscope. Sections were assessed for, collagen maturation, angiogenesis and epithelialization. [35].

2.3.5.5. Immunohistochemically study

For detection of the cytokeratin intermediate filaments on keratinocytes. The deparaffinized and rehydrated Sections were treated with 0.01m citrate buffer (pH 6.0) for 10min and heated twice in a microwave oven to unmask antigens. To abolish endogenous peroxidase activity sections were incubated in 0.3% hydrogen peroxide for 30min Slides were incubated with the primary antibody (1: l500 monoclonal mouse anticytokeratin) (Dako Biotechnology, Denmark) for 2h, then washing, then incubated with biotinylated secondary antibodies (ABC kit, 1: l200) and then with the avidin–biotin complex. Slides were counterstained for 1 min with Mayer's hematoxylin, and dehydration, clearing, and mounting were carried out to be examined under light microscope. Keratinocytes containing cytokeratin appeared brown, whereas nuclei appeared blue in color. [36].

2.3.5.6. Morphometric study

a. Epidermal total thickness and the number of fibroblasts were estimated in H & E-stained sections. b. The area percentage of the collagen fibers in the dermis was estimated in Masson's trichrome-stained sections. c. cytokeratin intermediate filaments

expression. All above were quantified in in five non overlapping fields from ten different sections for each group at × 400. by using Image-Pro Plus program version 6.0 (Media Cybernetics Inc., Bethesda, Maryland, USA) in the Histology Department, Faculty of Medicine, Benha University which consisting of a microscope equipped with a high resolution video camera.

2.3.5.7 Statistical Analysis

Data were collected and analyzed as means ± SD for eight rats in each group. Statistical comparison was performed using one-way analysis of variance (ANOVA) followed by Tukey's Multiple Comparison test using SPSS statistical version 16.0 software package (SPSS® Inc., USA). Values were considered statistically significant when $p<0.05$.

3. Results

Dermal Irritation Study:

Showed negative signs of dermal reaction towards the applied formulation, as, very slight erythema was observed only after 24 hrs. of application, and this erythema was disappeared after 48 hrs. No edema was observed in any treated rats

Wound Surface Area (WSA):

The representative photographs of the wound for treated animals of each group taken at different time intervals are given in Figure (1).

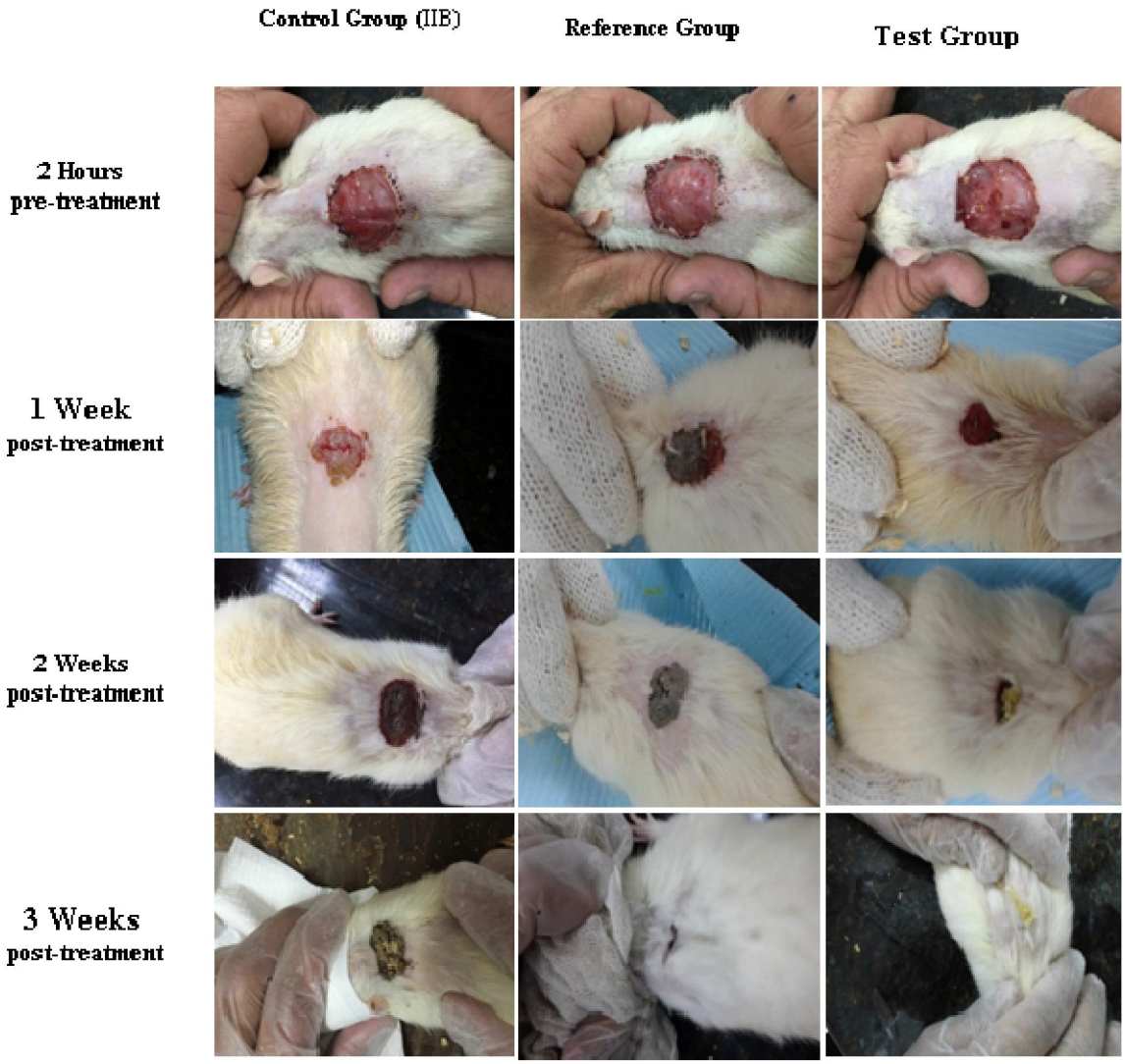


Figure (1): Examples of captured photographs by digital camera providing comparative images for wounds in different groups pretreatment and after

Table 1: Effect of different treatments on Wound Surface Area

Post-wounding (days)	Wound Surface Area (mm^2) (mean±SD)		
	Control positive (diabetic group)	Reference Mebo ointment (0.25%, w/w)	Resveratrol ointment (0.5%, w/w)
0	506.86 ± 4.82	507.36 ± 9.59	511.06 ± 9.28
7	374.81 ± 4.13	259.21 ± 5.78*	244.18 ± 4.67*
14	264.06 ± 7.76	50.82 ± 2.88*	37.60 ± 3.08*
21	152.60 ± 7.11	05.39 ± 1.61*	00.00 ± 0.00*

Results are expressed as mean±SD.
*significantly different compared to control group ($p<0.05$)
ns not significantly different compared to reference group ($p>0.05$).

The effect of different treatments on excision wound model in diabetic rats are given in Table (1). The obtained results revealed a significant reduction in wound surface area during treatment period (21 days) within all three groups. The topical application of resveratrol ointment significantly ($p<0.05$) decreased the wound surface area compared to the control diabetic group. After the first week of treatment, the mean value of WSA in test group was about (374.81 ± 4.13) and it was (259.21 ± 5.78) for reference group while it was (244.18 ± 4.67) for control group. While, at the end of the experiment, after 21 days of treatment, the mean value of WSA in test group was about (00.00 ± 0.00) and it was (05.39 ± 1.61) for reference group while it was (152.60 ± 7.11) for control group. From the results, it is clear that only rats treated with topical resveratrol formulation showed complete healing of the wound (WSA = 00.00) at the end of experiment.

Rate of healing studies:

The results of wound healing are shown in Table (2). The results demonstrate that, the percentage of healing in diabetic group was significantly lower than those of treated groups.

After the first week of treatment, the mean value of healing rate in test group was about (52.22±0.91), and it was (52.52±2.07) for reference group while it was (26.05±0.81) for control group. Analysis of results by one-way ANOVA revealed that there was statistically significant difference between group the test and reference groups compared to the control group as (P values < 0.05, but the difference was not significant between the test and the reference group ($p>0.05$).

At the end of the experiment, after 21 days, rats treated either with topical resveratrol or reference topical formulation showed nearly complete healing of the wound with healing rate about (100.0% ±0.00) and (98.94±0.32) respectively, while the healing rate mean for animals in the control group was (69.89±1.40). These results showed that wound closure and healing were accelerated in diabetic rats treated topically with resveratrol and the reference formulation compared with control diabetic rats.

Table (2): Comparison of wound contraction and epithelialization period.

Post-wounding (days)	Percent wound contraction % (Healing Rate %) (mean±S.E.)		
	control positive (Diabetic G)	Reference Mebo ointment (0.25%, w/w)	Resveratrol ointment (0.5%, w/w)
0	0	0	0
6	26.05±0.81	52.52±2.07*	52.22±0.91*
14	47.90±1.53	89.98±0.57*	92.64±0.60*,ns
21	69.89±1.40	98.94±0.32*	100.00±0.00*,ns
Epithelialization period (days)	Incomplete	19.53 ± 0.61	18.63 ± 0.54

Results are expressed as mean±SD (n=8).
*significantly different compared to control group ($p<0.05$)
ns not significantly different compared to reference group ($p>0.05$)

The mean period of epithelialization in control diabetic rats cannot be calculated as the healing was not complete, where it was (19.53±0.61) and (18.63±0.54) days for animals in reference and test groups respectively. As results showed in Table (2), the difference was not significant between the reference and the test group ($p>0.05$).

Biochemical analysis

Blood Glucose Level:

Blood glucose level was determined at the time of creation of the wounds and after treatment every 1,2 and 3 weeks. The obtained results for blood glucose level throughout the experiment period are shown in Table (3). The results revealed that the diabetic rats in the three groups exhibited significant elevation ($p<0.001$) in the glucose levels compared to the normal non-diabetic rats. Results also revealed that, blood glucose levels were not significantly ($p>0.05$) decreased upon treatment with either topical resveratrol or the reference drug compared to control group.

Table (3): Comparison of fasting serum glucose (mg/dl) mean values after (1st, 2nd and 3rd weeks) between groups.

Mean ± SD	1st week	2nd week	3rd week
Normal Control group (NC)	96.64±2.72	95.68±5.40	91.13±2.96
Control Diabetic group (group IIA)	395.35±5.21***	388.46±5.05ns,***	367.99±5.93ns,***
Mebo ointment (0.25%, w/w) (Reference group IIB)	392.11±3.24***	381.20±6.88ns,***	360.13±7.31ns,***
Resveratrol ointment (0.5%, w/w) (Test group IIC)	394.07±4.21***	377.85±6.79ns,***	356.83±7.45ns,***
P value	< 0.001	< 0.001	< 0.001

Results are expressed as mean±SD. *** Significant difference compared to normal control group ($p<0.001$). ns not significantly different compared to diabetic control group ($p>0.05$)

-Anti-oxidant activity:

The enzymatic assays for antioxidants during wound healing processes in skin tissues are shown in table (4).

The wound tissue from diabetic rats showed decreased extracellular SOD activity as compared to non-diabetic rats. Treatment of diabetic rats with resveratrol or the reference drug showed significant ($p<0.05$) increase in the SOD levels. Similar results were obtained for both CAT and GSH parameters. On the other hand, diabetic non-treated rats, showed elevated levels of LPO, which is a marker for lipid peroxidation, compared to normal control non-diabetic rats. Treatment of diabetic rats with resveratrol or the reference drug showed significant ($p<0.05$) decrease in the LPO levels. The results for antioxidant activity of resveratrol indicate potent antioxidant activity through decreasing lipid peroxidation, and increasing the levels of reduced glutathione (GSH), SOD and CAT activities. This validates the potent wound healing activity of resveratrol

Table (4): Effect of different treatments on different biochemical parameters of tissues from diabetic wound.

Parameter	LPO (nmoles/mg protein)	GSH (µmol/50mg tissue)	Catalase (µmol/50mg tissue)	SOD (µg/50 mg) tissue
Normal Control group (NC)	0.53±0.02	26.65±0.55	37.51±1.82	34.95±0.78
Control Diabetic group (IIA)	6.66 ± 0.25	10.72±0.42	16.83±0.31	10.89±0.38
Mebo ointment (0.25%, w/w) (Reference group IIB)	1.75 ± 0.13*	20.40±0.63*	24.79±0.90*	22.28±0.76*
Resveratrol ointment (0.5%, w/w) (Test group IIC)	0.78 ± 0.05**, ***	26.48±0.51**, ***	34.35±0.65**, ***	28.47±0.91**, ***

Results are expressed as mean±SD (n=8).

*** significantly different compared to control group ($p<0.001$) * significantly different compared to reference group ($p<0.05$) ns not significantly different compared to reference group ($p>0.05$)

Histological results:

Hematoxylin and eosin stain:

Group I: Sections of control un-wounded skin (-ve control G) revealed a normal histological structure of the skin of two basic layers: the epidermis and the dermis. The epidermis appeared stratified squamous keratinized epithelium resting on a wavy basement membrane with four distinct cell layers: stratum basale (columnar cells), stratum spinosum (polyhedral cells), stratum granulosum, plus and on the top there is superficial noncellular acidophilic horny layer (stratum corneum). The epidermal-dermal junction showed many dermal papillae. The underlying dermal layer contained thin superficial papillary layer with connective tissue and thick deep reticular layer with dense connective tissues and collagen fibers, the epidermal appendages (hair follicles & sebaceous gland) were seen in dermis (Fig.2 A.B). While in diabetic group without any treatment (control positive), skin specimens showed loss of covering epidermal layer (wound area after 7 days) (Fig.3A). There is granulation tissue entangling mononuclear inflammatory cells and congested blood vessels filling the wound gap in the untreated diabetic group after 2weeks of wound (Fig.3B). While in three weeks' post wounding the wound showing loss of the

epidermal bridges and presence of inflammatory cells and keratocytes migration (Fig.3 C). In the test group (treated with resveratrol) there is an epidermal tongue seen growing from the wound with scab and decrease of granulation tissue in 1st and 2nd week and was appeared more vascular with deposition of collagen fibers and presence of hair follicles (Fig. 4 A, B) the wound totally covered with epidermis and apparent normal after 3 weeks of wound (F.4 C). While in reference drug treated group the wound totally uncovered with epidermis in 1st week (Fig.5A) and after 2nd and 3rd weeks the wound covered with very thin epidermis that showed vacuolated and degenerated keratocytes cells with little deposition of collagen fibers in dermal layer in contrast to the control normal skin (Fig.5B, C).

-Masson's trichrome stain:

In the specimen of the normal skin of control group revealed in the papillary layer of the dermis thin interlacing bundles of collagen fibers, while coarse collagen fibers in the reticular layer (Fig. 6A). In diabetic untreated group, the collagen fibers in the dermis were disorganized at the wound edge. (Fig. 6B). In the test resveratrol group an apparent increase in collagen content in the papillary dermis was seen (Fig. 6C), while in the reference group, there are less collagen fiber in the dermis compared with the control skin (Fig. 6D). There was a significant increase in the collagen content of the papillary dermis in the reference group wound after 7 days compared with the untreated diabetic wound at the same periods (Table 5).

-Immunohistochemically staining:

There were normal apparent of immunohistochemically staining of cytokeratin intermediate filaments in the keratocytes cells of the epidermis (Fig.7A**)**. In diabetic control group without any treatment, epidermal cells showed loss of immunohistochemically staining of cytokeratin intermediate filaments (Fig.7B**)**, whereas in the test group the immunohistochemically staining of cytokeratin intermediate filaments appeared increase as in normal skin (Fig. 7C). There is less the immunohistochemically staining of cytokeratin intermediate filaments in the epidermal cells compared with the control skin (Fig. 7D).

-Morphometric results:

a. The thickness of the epidermis showed a significant decrease in the un treated diabetic group ($P < 0.01$) whereas that of the test group showed a significant increase in the untreated diabetic group ($P < 0.01$) with a nonsignificant change ($P > 0.05$) in reference group (Table 5).

b. The area percentage of collagen fibers was significantly decreased ($P < 0.01$) in the untreated diabetic group (group IIA) and not significantly changed ($P > 0.05$) in the test group (group IIB) as compared with that of the control group (Table 4). The mean area percentage of collagen fibers was 43.32 ± 1.12% in the control group compared with 40.23 ± 1.15% in the test group, which was statistically significant (P=0.02). (Table 6).

c. The area percentages of cytokeratin intermediate filaments expression was 18.72 ± 0.3% in the resveratrol -treated group compared with 20.77 ± 0. 33% in the control-ve group. The difference in area percentages of cytokeratin intermediate filaments expression between the two groups was statistically significant ($P<0.001$). The area percentage of cytokeratin intermediate filaments expression was 16. 13 ± 0.56% in the Mebo-treated group compared with 20.77 ± 0. 33% control –ve group. Expression of cytokeratin intermediate filaments was statistically significant in the control +ve group compared with the control- ve group ($P<0.001$) (Table 7).

Table (5): showing the mean epidermal thickness (mean ± SD) in the different groups compared with control-ve group.

Groups	Control (-ve) group	Diabetic group	Resveratrol ointment (0.5%, w/w)	Mebo ointment (0.25%, w/w)
1st week post wound	39.79± 9.11	11.21 ± 1.21	24.23±0.12	19.96 ± 0.18
2nd week post wound		15.94± 0.21	31.12±0.82	28.25 ± 1.51
3rd week post week		20.89 ± 0.34	38.19±0.15	32.73 ± 1.82
Significance		S	S	NS

SD = standard deviation S = Significant NS = Significant

Table (6): The mean area percentage of collagen fibers in the different groups studied.

Groups	Control (-ve) group	Diabetic group	Resveratrol ointment (0.5%, w/w)	Mebo ointment (0.25%, w/w)
1st week post wound	43.32 ± 1.12	10.11 ± 0.21	37.23 ± 2.05	29.34 ± 0.19
2nd week post wound		18.14±0.41	39.19 ± 0.45	34.28 ± 0.13
3rd week post week		26.79 ± 0.48	40.23 ± 1.15	37.31 ± 0.62
Significance		S	S	NS

SD = standard deviation S = Significant NS = Significant

Table (7): Showing the area percentage of cytokeratin intermediate filaments expression in positive cells compared with control-ve group.

Groups	Control (-ve) group	Diabetic group	Resveratrol ointment (0.5%, w/w)	Mebo ointment (0.25%, w/w)
1st week post wound	20.77 ± 0. 33	9.12 ± 1.09	15.03 ± 0.15	14.39 ± 2.84
2nd week post wound		11.04±2.91	16.18 ± 0.98	15.26 ± 1.19
3rd week post week		12.39 ± 2.08	18.72 ± 0.30	16. 13 ± 0.56
Significance		S	s	NS

SD = standard deviation S = Significant NS = Significant

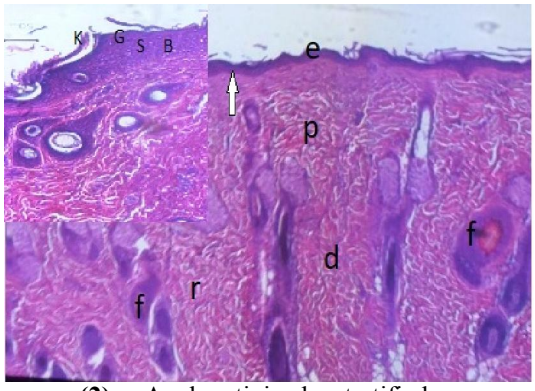
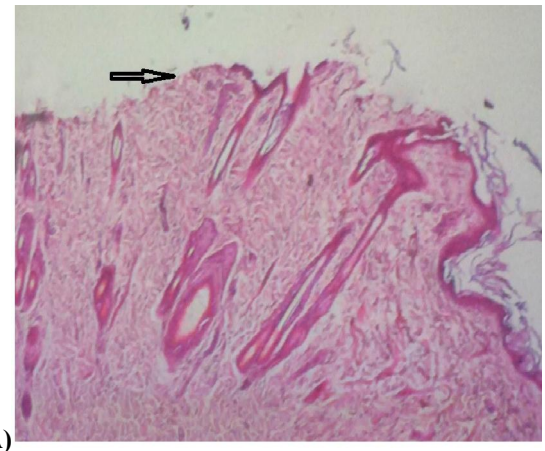
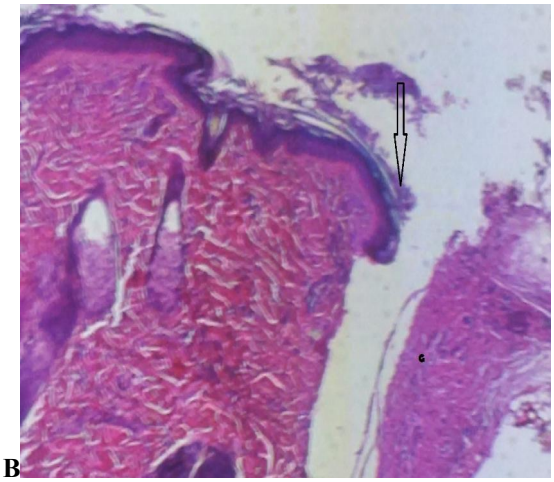


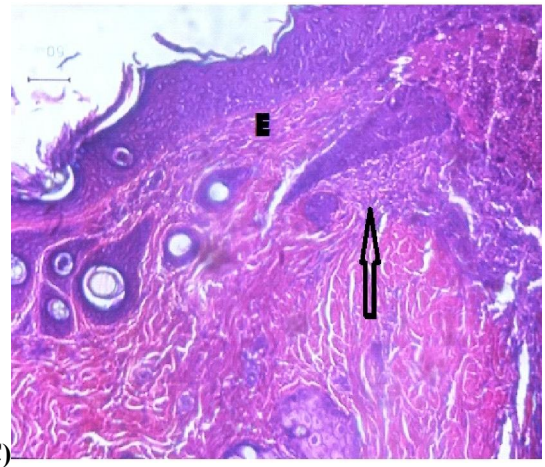
Figure (2). A keratinized stratified squamous epithelium of skin epidermis (e) in skin from a control rat (group I). The dermis (d) with upper thin papillary layer (p) and basal thick reticular layer (r). Apparent epidermal–dermal junction (arrow) with hair follicles (f). Above magnify photo showing the layers of the epidermis: stratum basale (B), stratum spinosum (S), stratum granulosum (G), and stratum corneum with keratin (K). H & E × 200. (B) H & E × 400.



A)



B



C)

Figure (3). (A) Photomicrographs of skin wounds from a control +ve rats (IIA) showing loss of the epidermis at the site of the wound defect (arrow). (B) Two weeks' post wounding the wound showing granulation tissue filled the wound gap and creeping epithelium filling the defect (arrow). Note that granulation tissue is formed of fine fibrous material entangling mononuclear inflammatory cells and containing congested blood vessels (G). three weeks' post wounding the wound showing loss of the epidermal bridges (E) and presence of keratocytes migration with inflammatory cells (arrow). H & E × 200.

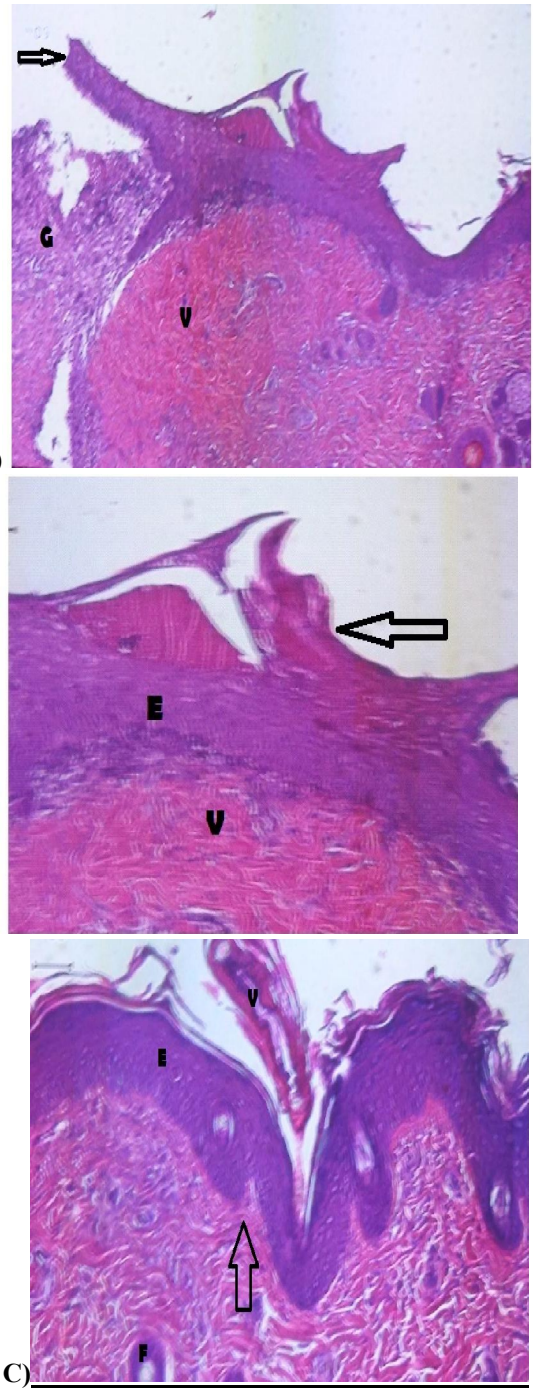


Figure (4). (A) Photomicrographs of skin wounds from group IIB (topical resveratrol formulation treated rats) showing Discontinuity in the epidermis and it partially covering the wound with presence of skin tongue (arrow) with underlying granulation tissue (G) (one-week post wound). Note that the granulation tissue appears more vascular and more fibrous (V). (B) two -weeks post wound apparent increase in the epidermal epithelium (E) with presence of wound scab (arrow). Note increase vascularization in dermis (V) (C) three -weeks post wound the epidermis is totally covers most of the wound and an apparent increase in epidermal thickness (E) with presence of epidermal ridges (arrow). Note the hair follicle (F). (H & E × 200).

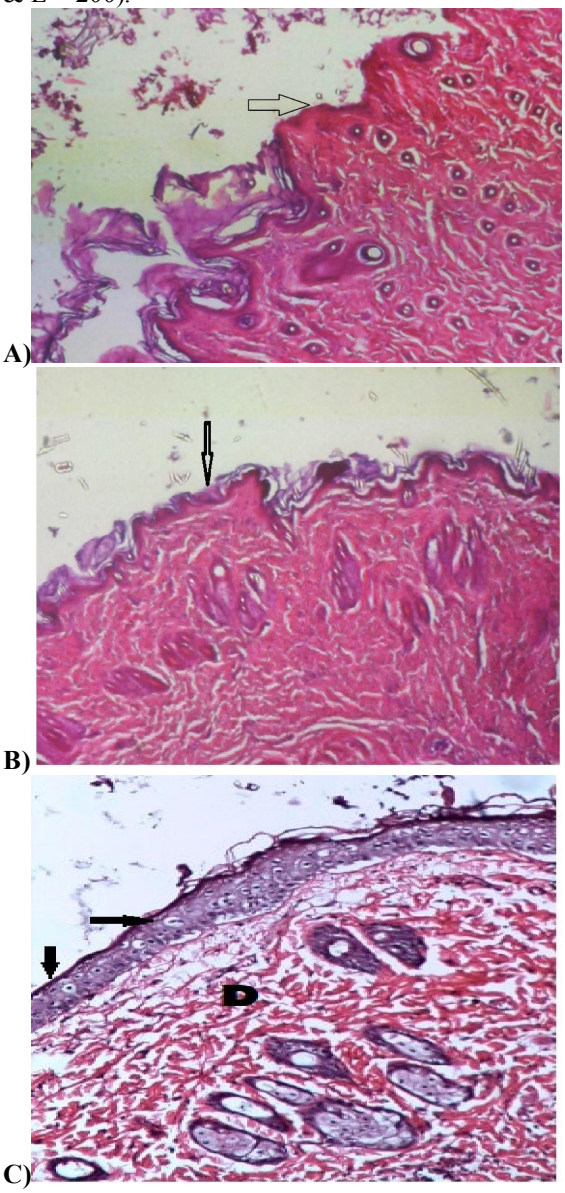
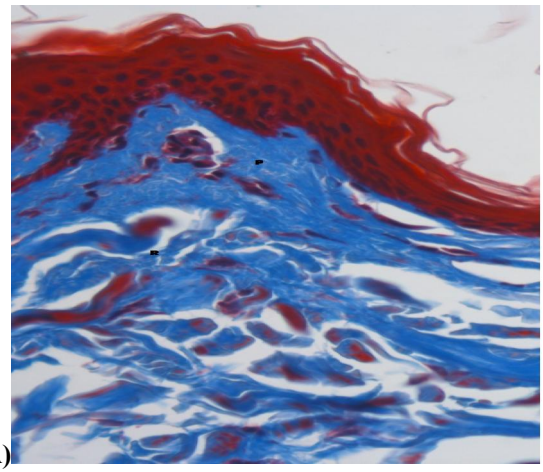


Figure (5). (A) Photomicrographs of skin wounds from group IIC (topical mebo treated rats) showing loss in the epidermis with scab (arrow) with no underlying granulation tissue (one-week post wound). (B) two weeks' post wound an apparent thinning out of the epidermis (arrow), loss of epidermal ridges, and dermal papillae as compared with that of the control rats. The dermis shows less dense connective tissue fibers. (C) three weeks' post wound the epidermis completely cover the wound with loss of its ridges and vaculation of keratocytes (arrow). Note increase connective tissue in dermal layer (D) (H & E × 200.



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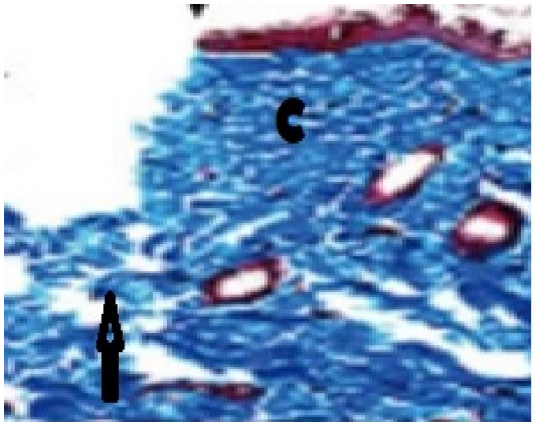

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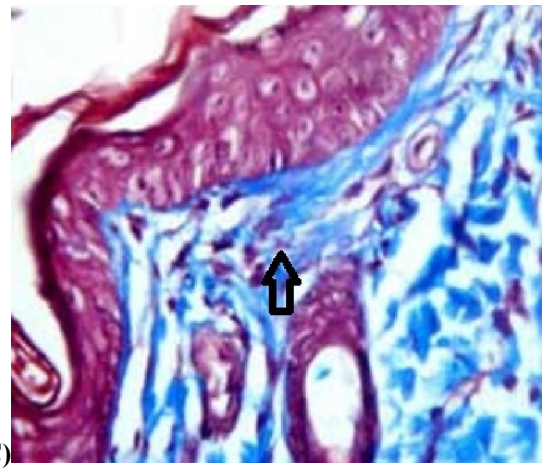

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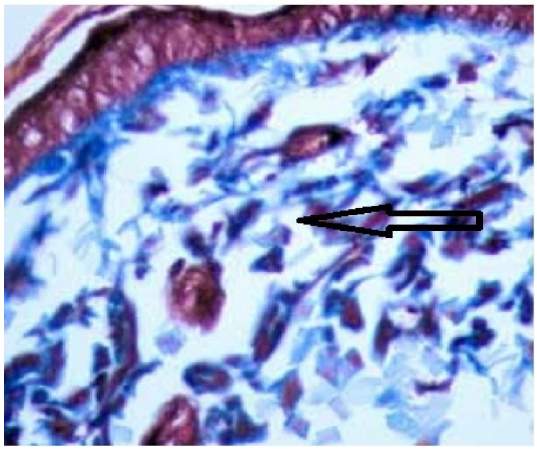
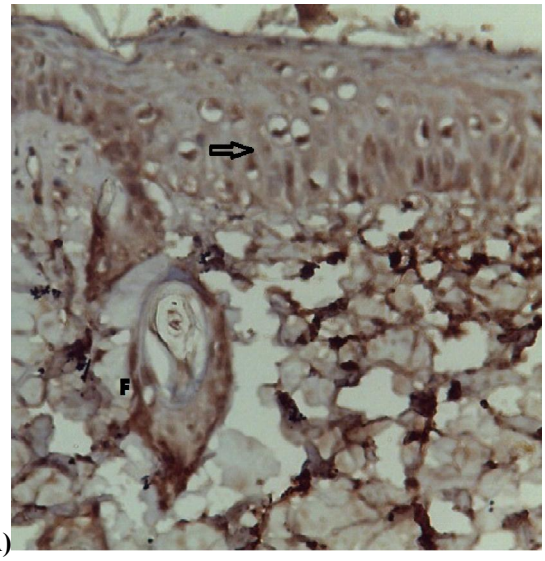


(D)

Figure (6). A) A photomicrograph of a section of rat skin of the diabetic control group (group I) showing the collagen fiber content in dermis. Thin collagen bundles are present in the papillary layer of the dermis appear (P), Notice that collagen fibers in the reticular dermis appear as coarse, wavy bundles (R). (B) A photomicrograph of a section of rat skin of the diabetic group (group IIA) showing more collagen fiber content (c) and irregular arrangement of collagen fibers at the wound area (arrow). (C) A photomicrograph at the skin of group IIB showing regular arrangement of collagen fibers in dermis (arrow). (D) A photomicrograph at the skin of subgroup IIC showing less collagen fiber content with irregular arrangment of them in dermis compared with control skin. (arrow). Masson's trichrome stain, × 400.



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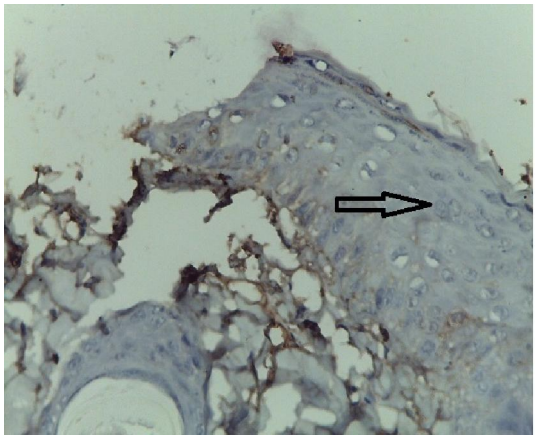

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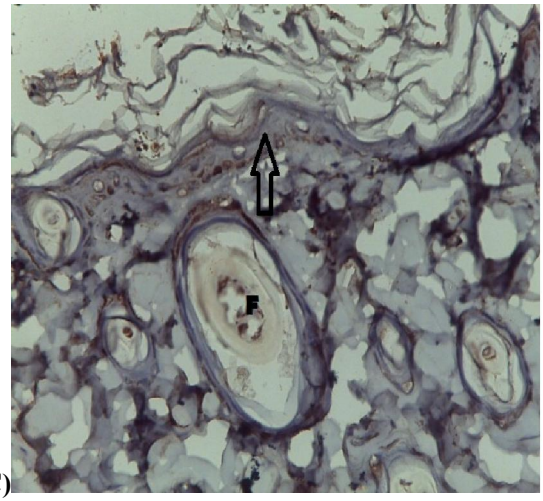

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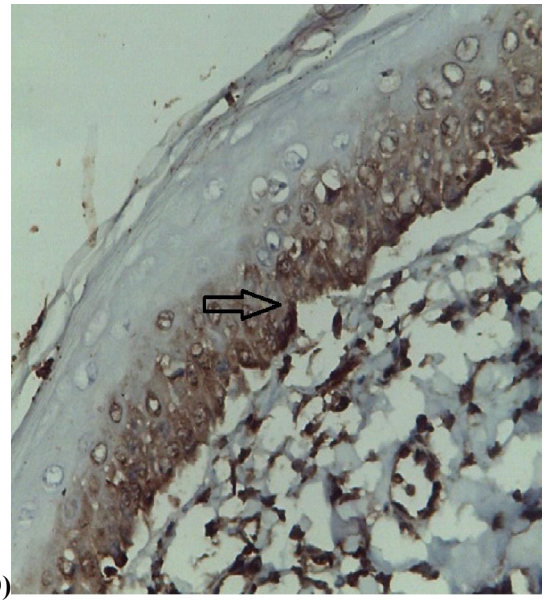

(D)

Figure (7). (A) A photomicrograph of a section of rat skin of the –ve control group showing the positive cytokeratin intermediate filaments (brown color) in the epidermal cells (arrow) and around hair follicles (F). (B) A photomicrograph of a section of rat skin of the diabetic group (group IIA) showing an apparent decrease in the cytokeratin intermediate filaments in the epidermis (arrow) as compared with that of the control group. (C) A photomicrograph at the skin of group IIB showing apparent increase in the cytokeratin intermediate filaments in the epidermis (arrow) as compared with that of the diabetic un treated group. Note positive staining in hair follicle (F). (D). A photomicrograph at the skin of group IIC showing apparent moderate increase in the cytokeratin intermediate filaments in the epidermis (arrow) as compared with that of the diabetic untreated group. Cytokeratin Immunohistochemical staining × 400

4. Discussions

Wound healing is the process in which an affected tissue is restored its normal structure and it depends mainly on both; the repairing ability and the general health state of the tissue [37]. In diabetic wound conditions, impairment of healing occurs as a result of ischemia, excessive production of reactive oxygen species (ROS), and inflammatory mediators [38]. High blood glucose levels may lead to occlusion of capillary vessels as a consequence for endothelial damage as well as hyperglycemia-induced leukocyte dysfunction and phagocytosis [39].

In experimental diabetic wound, healing process is characterized by delayed cellular infiltration, impaired granulation tissue formation, decreased collagen deposition, and increased epithelialization time [40].

In our study an excision a full-thickness wound model was done for the assessment of wound healing and the effect of topical formulation of resveratrol. The healing of skin wounds in our study was significantly delayed in diabetic rats, and this may be attributed to the high blood glucose levels, where, it delayed cellular infiltration through abnormal physiological response. While, in untreated diabetic rats there is delayed wound healing when compared with treated animals as naked eye examination of the wound surface area after 7 and 14 days revealed that incomplete closure of the wound. The epithelization time is an important factor that can be used to assess the wound healing process. In the diabetic control animals, epithelial reorganization process was very slow compared to that of the treated animals. The obtained results show that topical application of resveratrol clearly enhanced wound healing from the first stage, and this effect can be attributed to its known angiogenic and mutagenic potential.

Also, histological examination of tissue samples showed loss of the epidermis and dermis which suggested a delayed wound healing stated by some authors [41] and the wounded areas of the untreated

group (group I) did not show complete closure till the end of experiment with presence of wound scab and keratocytes migration. These above data were observed by several studies that explained the presence of scab filled with inflammatory cells and keratinocyte migration beneath the scab from thickened epidermis on the second day, but the whole wound was covered only after 14 days [42].

The percentage of collagen fibers of the same group increased progressively in an irregular manner and this results are in agreement with results obtained by Gal et al. (43), who noticed an increase in proteoglycans, glycoproteins, and collagen synthesized by fibroblasts one week after wounding [43]. In diabetic patient the delayed healing occurs as result of poor neovascularization, decreased growth factors, with failure of migration of keratocytes and so failure of reepithelialization and wound closure [44]. While other study revealed decreased cellular infiltration and delayed collagen fiber formation and reorganization [45].

Our study, showed that the wound of resveratrol treated rats had highly improvement effects in wound healing as increase granulation tissue formation, angiogenesis, epithelialization, keratinization, restoration of hair follicles, and collagen fibers deposition with prominent increase in cytokeratin immunohistochemical staining. This results are in agreement with results obtained in a previous study which demonstrated that the thickness of the newly formed epidermis was not similar to intact epidermis [46 & 47]. In our study there are regularly appearing of collagen fibers in the dermis and this as the results of other experimental studies [48]. Our study revealed that there is mild positive reaction for cytokeratin immunohistochemical staining in the skin of the untreated diabetic group and this is due decrease kertaocytes formation as they produce cytokeratins for protection and providing mechanical support to the cells [49]). The mechanical support of keratins is the ability to flexible easily to resist as well as softness, strength, self-repair [50], but the wound treated with MEBO did not completely heal as the wound of resveratrol treated rats and this as shown in [51].

Flavonoids have astringent and antimicrobial property which lead to accelerate the wound healing process and so wound closure [52]. Oxidative stress, originated from over production of reactive oxygen species (ROS), may lead to cytotoxicity and delayed wound healing, therefore, elimination of ROS may be a key strategy for chronic wounds healing [53]. The antioxidants have been reported to hasten wound healing by decreasing the free radicals [54]. Results in this study on the antioxidants revealed that topical resveratrol had significant antioxidant activity which is directly expressed as decrease in inflammation and oxidative damage and enhancing the healing process.

Flavonoids have been estimated to exert several biological effects on collagen synthesis process, as enhancing collagen synthesis, promoting the collagen fibers cross-linking, decreasing soluble collagen degradation in addition to acceleration of the conversion of soluble collagen into insoluble collagen [55]. Collagen- major protein of the extracellular matrix- imparts strength to wound. Breakdown of collagen results in free hydroxyproline and its peptides, therefore, hydroxyproline content has been used as an index for collagen turnover. High content of collagen in the excision wound is a clear indicator for faster collagen turnover which accelerates wound healing process [39 & 40].

Resveratrol has been demonstrated to have a significant enhancing effect on skin fibroblast proliferation in addition to its anti-collagenase activity. It also promotes maturation of mesenchymal stem cells in adipose tissue in a dose-dependent scheme [56]. Furthermore, flavonoids are known to decrease lipid peroxidation through improving vascularity and decreasing the onset of cell necrosis. Therefore, drugs which reduce lipid peroxidation are thought to enhance the collagen fibrils viability by increasing the collagen fibers strength, increasing the circulation and preventing the cell damage [57].

The main valuable pharmacological effect of resveratrol which may be directly affect wound healing is attributed to its inhibitory activity on matrix metalloproteinases (MMPs) which play the key role in diabetic wound healing, where, these enzymes promote collagen and other extracellular matrix of the skin degradation [58]. High level of MMP-8 and MMP-9 in diabetic wounds revealed a negative sign of wound healing process [59].

The obtained data from biochemical, histopathological and immunohistochemical studies in the present study are in line with the regular findings of normal wound healing phases, and this results are similar to that obtained from other studies using different natural constituents for treatment of excision wound in diabetic rats [58].

In conclusion, the observation and results obtained from the present study indicated that topical resveratrol possesses a powerful modulating effects on wound healing, as it promotes wound healing by reepithelization and regular collagen fibers synthesis. This study confirms the promising wound healing activity of topical resveratrol in diabetic animals, and deserve for more investigations on the scale of cellular level in future and further studies may be required to determine the underlying mechanisms through which resveratrol affects wound healing and other body

system to establish resveratrol as a potential candidate for clinical settings.

Disclosure and Conflicts Statement

The author declares no conflicts of interest.

Acknowledgements:

The authors would like to thank Institute of Scientific Research and Revival of Islamic Heritage at Umm Al-Qura University, Makkah, KSA (Project No: 43509022) for the financial support.

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10/4/2018