

Effect of Bee Venom on the Structure of unwounded thick Skin in Adult Male Diabetic Rats: Histological, Immunohistochemical and Morphometric Study

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Abstract: Background: diabetes is a chronic disease that causes serious dermatologic problems. Recently bee venom (BV) has been used as a traditional medicine to treat variety of conditions. This study was designed to assess the effects of BV on the structure of unwounded thick skin in type 1 diabetes in adult male albino rats. **Materials and Methods:** Thirty adult male albino rats were divided into four groups: control group, BV control group, diabetic group and BV treated group. In the diabetic group each rat received single IP injection of 45mg/kg streptozotocin (STZ) in 100 mM citrate buffer pH 4.5. In BV treated group, each rat received STZ as in diabetic group, then after conformation of diabetes, each rat received IP injection of 0.5 mg/kg BV twice weekly for four consecutive weeks. Blood samples were taken for monitoring blood glucose levels. At the end of the experiment, thick skin was obtained from the planter surface of hind limb from all rats. Samples were processed for H&E, Mallory's trichrome stain and immunohistochemical reaction for protein gene product 9.5 (PGP-9.5). Statistical and histomorphometric studies were also done. **Results:** A significant decrease in serum glucose level was noticed in rats treated with BV, compared to untreated diabetic rats. In diabetic group, **H&E** stained skin sections showed significant decrease in epidermal thickness with a significant increase in the number of atypical cells with deeply stained nuclei and perinuclear cytoplasmic vacuolation, compared to other groups. **Mallory stained** sections of diabetic group showed disorganized and less crowded collagen bundles in the reticular dermis. Immunohistochemical reaction for **PGP-9.5** in the same group illustrated decreased immune reaction for nerve fibers in the dermis of the skin. However, BV treated group showed preservation of skin structure. The epidermis appeared almost similar to the control group with the appearance of its usual five layers. Most of collagen fibers appeared with uniform diameter and showed compact and regular arrangement. Preserved cutaneous innervation was also detected. **Conclusion:** The current study shows that bee venom is effective in preventing skin changes accompanied with diabetes mellitus type I, as it preserves the structure and innervation of unwounded thick skin.

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Key words: Bee venom, diabetes, skin, histology, PGP- 9.5, rats

1. Introduction:

Type 1 diabetes mellitus is a chronic disease that causes serious health complications, as renal failure and heart disease, in addition to dermatologic problems. It was reported that skin complications that appear during the course of the disease may be the first symptom of diabetes or may precede the diagnosis by many years. Diabetic skin also has deficient wound-healing properties and it is characterized by disturbances in collagen metabolism at the site of the unhealing wound[1]. Peripheral neuropathy is also one of the most common complications in diabetic patients[2].

Neuropathy is a common complication of both type 1 and type 2 diabetes, with predominantly small fiber involvement beginning at the distal extremities and progressively becoming more proximal with time and duration of diabetes. "Burning" or "prickly" feet are common descriptions from diabetic neuropathy patients [3].

Streptozotocin (STZ) is an antibiotic produced by *Streptomyces achromogenes*. It has been widely used for inducing experiment a diabetes mellitus in a variety of animals. It induces degeneration of pancreatic β -cells simulating the naturally occurring metabolic disorder DM [4].

Recently bee venom has been used as a traditional medicine to treat a variety of conditions as arthritis, rheumatism and skin diseases[5].

Bee venom is a natural toxin produced by the honey bee. It has an efficient and complex mixture of substances designed to protect bees against a broad diversity of predators. Bee venom therapy is a treatment modality involving the application of live bee stings to the patient's skin or, in more recent years, the injection of bee venom into the skin with a hypodermic needle. Many experiments on bee venom have proven its effectiveness in treating several pathological conditions as arthritis, pain and cancerous tumors [6].

The venom contains varieties of peptides [mellitin, apamin, secapin, tertiapin, adolapin, the mast-cell-degranulating (MCD) peptid, enzymes (phospholipase A₂, hyaluronidase, acid phosphomonoesterase, lysophospholipase), active amines (histamine, dopamine, norepinephrine, serotonin) and many other substances][7].

Most studies have investigated the remodeling of the diabetic skin in the wounded state, not at the baseline [1]. So, this study was designed to assess the effect of bee venom on the structure of unwounded thick skin in type 1 diabetes (streptozotocin-induced; STZ-induced) in adult male albino rats.

2. Materials and methods:

Animals:

Thirty adult male Wistar rats weighing 150-200 gm were used in this study. All procedures of animal care and experiments were done according to ethics committee recommendations of Ain Shams University. Animals were housed in standard conditions of illumination and ventilation. They were allowed free access to standard laboratory chow and water. The experiment was performed in the Medical Research Center (MRC), Ain Shams University hospitals.

Experimental design:

Rats were randomly divided into four groups:

Group I (control group): included five rats and served as a negative control group.

Group II (BV control group): included five rats. Each rat received IP injection of 0.5 mg/kg bee venom twice weekly (3-4 days apart) at fasting condition for four consecutive weeks [8].

Group III (the diabetic group): included ten rats. Each rat received single IP injection of 45mg/kg STZ in 100 mM citrate buffer pH 4.5 (Sigma, USA). To confirm diabetes, blood glucose level was measured three days after STZ injection. Animals were considered to be diabetic with blood glucose level > 200 mg/dl [9].

Group IV (bee venom treated group): included ten rats. Each rat received single IP injection of STZ as in group III. After conformation of diabetes, rats received IP injection of 0.5 mg/kg BV twice weekly (3-4 days apart) at fasting condition for four consecutive weeks. Bee venom was purchased from Department of allergy and clinical immunology, Faculty of Medicine, Ain Shams University.

Blood glucose level analysis:

A drop of fresh blood was collected from the animal's tail using a lancet at fasting conditions. Blood glucose levels were measured in all groups twice weekly using glucometer instrument (Accua-check, ROCHE, Germany).

Histological study:

At the end of the experiment (4 weeks after the start of BV), rats were sacrificed by ether inhalation anesthesia. Thick skin was obtained from the planter surface of right hind limb from all rats. Skin specimens were fixed in 10% buffered formalin and were prepared for paraffin blocks. Serial 5µm paraffin sections were stained with H & E and Mallory's trichrome stain [10]. Immunohistochemical reaction for nerve fibers was also done using protein gene product 9.5 (PGP-9.5). It was purchased from Biocare Medical USA.

Morphometric analysis:

Samples were analyzed by using Leica DM2500 microscope with built in camera (Wetzlar, Germany). All images were digitally acquired using an image analyzer Leica Q win V.3 program (Wetzlar, Germany) installed on a computer in the Department of Histology and cell biology, Faculty of Medicine Ain Shams University. Five different non overlapping fields from five different sections of different rats were examined in each group for measuring the mean area percentage of collagen fibers, the mean area percentage of positive immunohistochemical reaction for PGP-9.5 and the number of vacuolated cells in epidermis/HPF.

Statistical analysis:

All measurements were taken at high-power fields of magnification (×200). All data were collected, revised, and subjected to statistical analysis using one-way analysis of variance (ANOVA) performed using SPSS.21 program (IBM Inc., Chicago, Illinois, USA). The significance of data was determined by *P* values. *P* values greater than 0.05 were considered non significant (NS) and *P* values less than 0.05 were considered significant.

3. Results:

Histological Results:

Examination of *H&E* stained sections of the control group showed the layers of the epidermis. The cells of the basal layer were columnar with large oval nuclei. Cells of stratum spinosum were polyhedral with rounded nuclei. Cells of stratum granulosum appeared flattened with basophilic granules in their cytoplasm. (Figure: 1A). The epidermis of bee venom control group was formed of five layers; Stratum basal, spinosum, granulosum, lucidum and cornium (Figure: 1B). Diabetic group showed significant decrease in epidermal thickness (table2, histogram 2). Atypical cells with deeply stained nuclei and perinuclear cytoplasmic vacuolation were frequently seen in the basal and prickle cell layers with significant increase as compared to the other groups (table:2, histogram: 3). Distortion of the multilayer appearance of the epidermis was also noticed (Figure: 1C). In the bee venom treated group, the epidermis

appeared almost similar to the control group showing the normal healthy appearance of its usual five layers (Figure: 1D).

In *Mallory* stained sections, the collagen bundles in the papillary dermis of the control group and bee venom control group appeared as fine interlacing bundles. In the reticular dermis collagen was seen arranged in coarse, wavy bundles running in different directions. Most of the bundles were of uniform diameter (Figures: 2A and 2B respectively). In the diabetic group the zone of condensed collagen fibers just beneath the epidermis appeared discontinuous. While in the reticular dermis, the collagen bundles were thin, irregular, less crowded and disorganized (Figure: 2C). In bee venom treated group most of the

bundles were of uniform diameter and showed compact and regular arrangement (Figure: 2D). This was confirmed by the current histomorphometric study (table 3, histogram 4).

In *PGP-9.5* immunohistochemically treated sections, dermal nerve fibers appeared brown-stained in both control group and bee venom control group (Figures: 3A and 3B, respectively). Decreased immune reaction for nerve fibers in the dermis of the skin was seen in diabetic group (Figure: 3C). Sections of skin in diabetic rats treated with bee venom showed preserved cutaneous innervation (Figure: 3D). This was confirmed by the current histomorphometric study (table 3, histogram 5).

Histomorphometric results

Table 1: Showing the mean and SD of blood glucose level in different groups

	Group I	Group II	Group III	Group IV
Blood glucose level mg/dl	108±23.6▲	102±10.58▲	323.4±23.64*▲O	108.2±27.4▲

*Significant difference from group I. ■Significant difference from group II. ▲Significant difference from group III. O Significant difference from group IV.

Table 2: Showing the mean ± SD of number of vacuolated cells in the epidermis, and the epidermal thickness in different groups

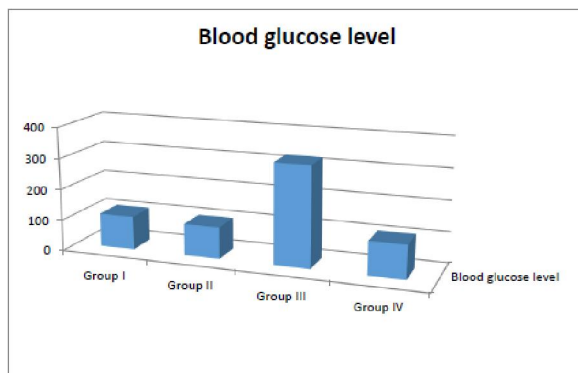
Groups	Epidermal thickness	Number of vacuolated cells in epidermis
Group I	17.4 ± 6.58	76.23 ± 14.77
Group II	15.0 ± 8.34▲	72.02 ± 16.94 O
Group III	53.6 ± 16.68*■O	53.97 ± 15.50*■O
Group IV	8.6 ± 3.36▲	62.53 ± 18.74*■▲

*Significant difference from group I. ■Significant difference from group II. ▲Significant difference from group III. O Significant difference from group IV.

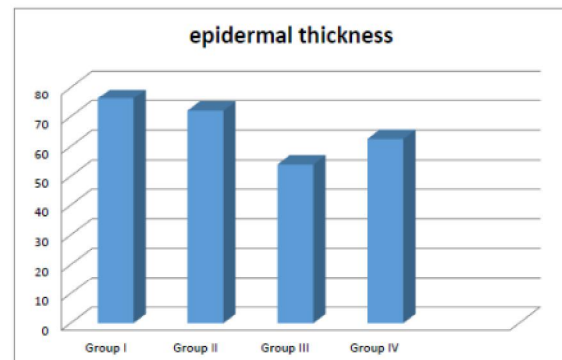
Table 3: Showing the mean and SD of area percentage of collagen fibres and positive PGP-9 expression in different groups

	Collagen fibres	PGP-9.5 expression
Group I	24.34 ± 3.26	0.499 ± 0.039
Group II	26.15 ± 4.50	0.495 ± 0.043▲
Group III	20.13 ± 4.93*■	0.201 ± 0.019*■O
Group IV	23.98 ± 3.80▲	0.455±0.045▲

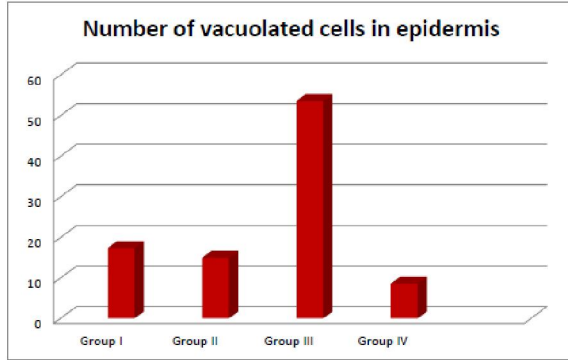
*significant difference compared to control. ■Significant difference from group II. ▲Significant difference from group III. O Significant difference from group IV.



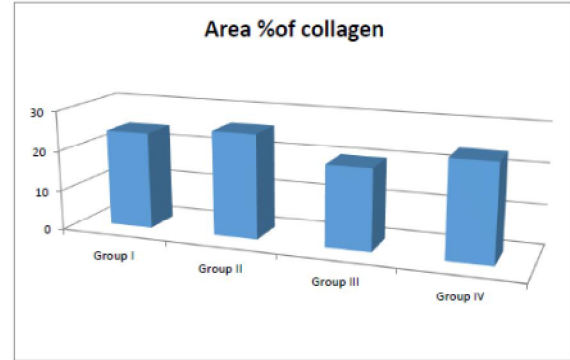
Histogram (1): Showing the mean blood glucose level in different groups



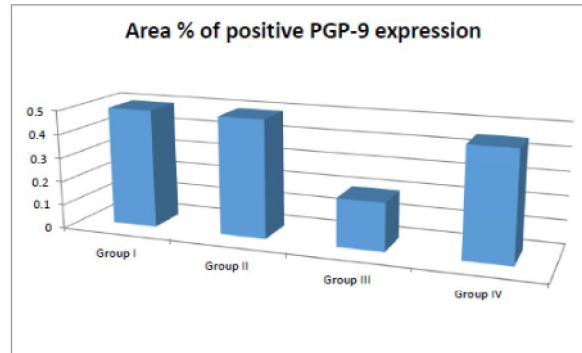
Histogram 2: Showing the mean thickness of epidermis in different groups



Histogram 3: Showing the mean number of vacuolated cells in epidermis in different groups



Histogram (4): Showing the mean of area percentage of collagen fiber in different groups



Histogram (5): Showing the mean area percentage of positive PGP-9 expression in different groups

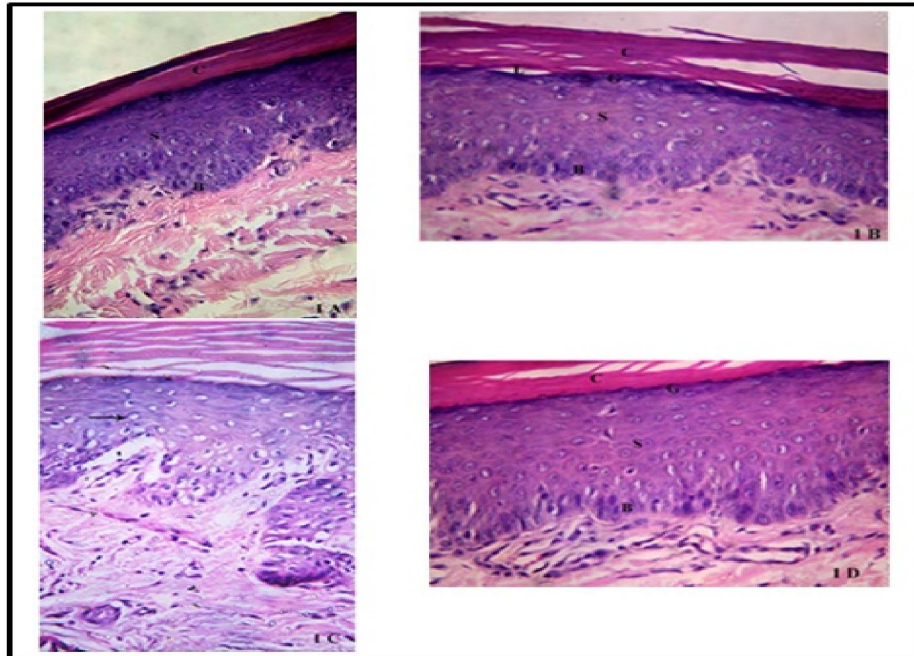


Figure 1: Photomicrographs of skin sections from: (A) **Group I** showing the layers of the epidermis in group I. The cells of the basal layer (B) are columnar with large oval nuclei. Cells of stratum spinosum (S) are polyhedral with rounded nuclei. Cells of stratum granulosum (G) appear flattened with basophilic granules in their cytoplasm. Notice the presence of stratum corneum (C). (B) **Group II** showing the epidermis of the skin formed of five layers; stratum basale (B), spinosum (S), granulosum (G), lucidum (L) and corneum (C). (C) **Group III** showing apparently decreased epidermal thickness. Atypical cells (↑) with deeply stained nuclei and perinuclear cytoplasmic vacuolation are seen in the basal and prickle cell layers. Distortion of the multilayer appearance of the epidermis is also noticed. (D) **Group IV** showing the five layers of the epidermis; stratum basale (B), spinosum (S), granulosum (G), lucidum (L) and corneum (C). (H&E×640)

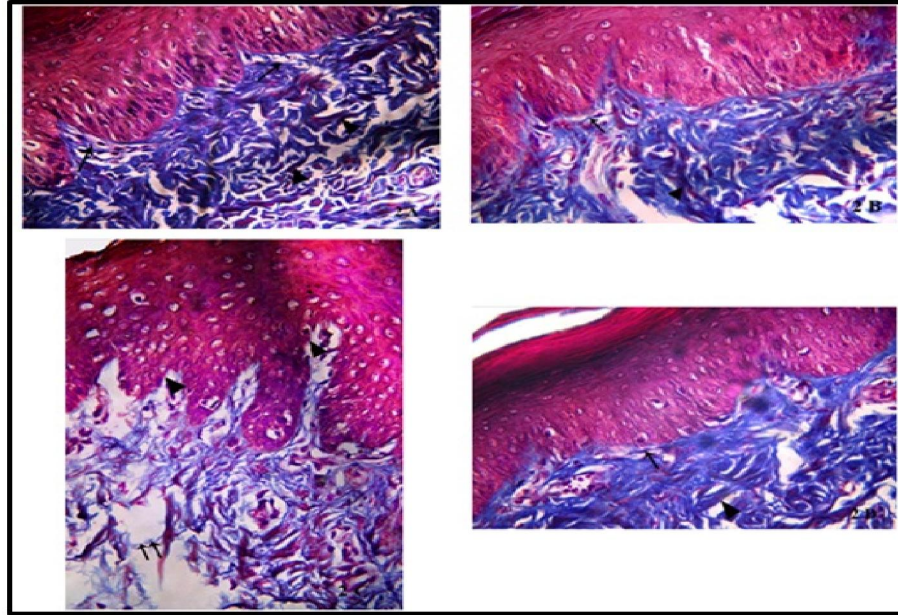


Figure 2: Photomicrographs of skin sections from (A) **Group I:** showing the collagen bundles in the dermis. In the papillary dermis they appear as fine interlacing bundles (↑). In the reticular dermis collagen is seen arranged in coarse, wavy bundles running in different directions (▲). Most of the bundles are of uniform diameter. (B) **Group II:** showing the collagen bundles. In the papillary dermis they appear as fine interlacing bundles (↑). In the reticular dermis collagen is seen arranged in coarse, wavy bundles running in different directions (▲). Most of the bundles are of uniform diameter. (C) **Group III:** The zone of condensed collagen fibers just beneath the epidermis is discontinuous (▲). In the reticular dermis, the collagen bundles are thin, irregular and disorganized (↑↑). (D) **Group IV:** showing the collagen bundles. In the papillary dermis they appear as fine interlacing bundles (↑). In the reticular dermis collagen is seen arranged in coarse, wavy bundles running in different directions (▲). Most of the bundles are of uniform diameter and show compact and regular arrangement. (Mallory's trichrome stain × 640)

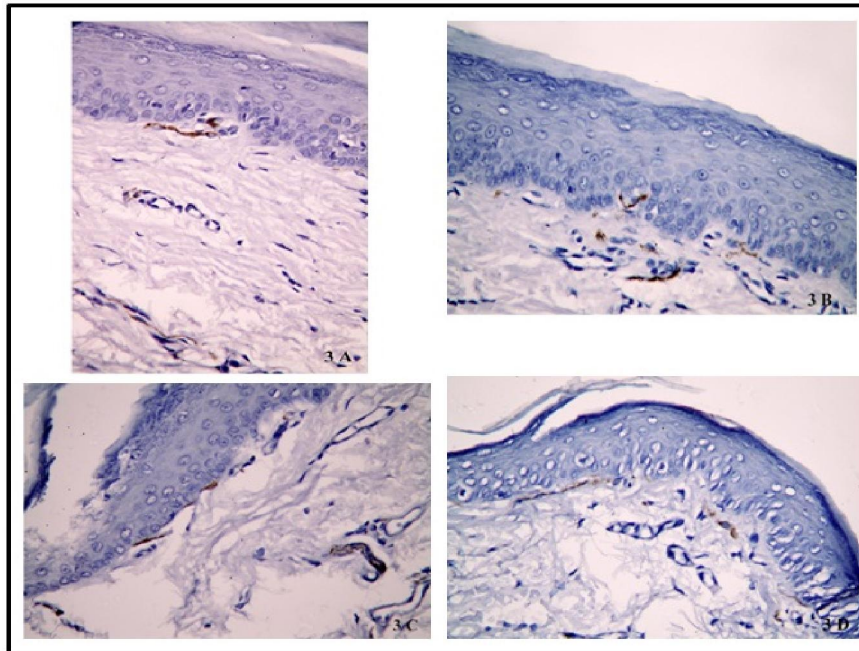


Figure 3. Photomicrographs of skin sections from (A) **Group I:** showing positive PGP-9.5 immunohistochemical reaction for nerve fibers in the dermis of the skin. (B) **Group II:** showing positive PGP-9.5 immunohistochemical reaction for nerve fibers in the dermis of the skin. (C) **Group III:** showing apparent decrease PGP-9.5 immunohistochemical reaction for nerve fibers in the dermis of the skin. (D) **Group IV:** showing positive PGP-9.5 immunohistochemical reaction for nerve fibers in the dermis of the skin. (PGP-9.5 immunohistochemical reaction × 640)

4. Discussion:

Diabetes mellitus is a chronic disease that causes serious health complications, including dermatologic problems. The diabetic skin is characterized by disturbances in collagen metabolism [1].

Chronic hyperglycaemia together with microangiopathy of vasa nervosa may lead to nerve damage, referred to as diabetic neuropathy [11].

About 56% of idiopathic neuropathy patients presenting with burning feet have impaired glucose tolerance, determined from no increase in fasting glucose but with significant elevation of 2-h postprandial glucose. This presentation may be related to an underlying small fiber neuropathy [3].

Bee venom and its components regulate pro-inflammatory cytokines. It seems to accelerate wound healing and antibacterial therapy for the treatment of inflammatory skin disease through the regulation of inflammatory signaling pathway [6].

This study was designed to study the effect of bee venom on the structure of unwounded thick skin in diabetic rats.

In the present study a significant decrease in serum glucose level in rats treated with bee venom, as compared to untreated diabetic rats was noticed. Similarly, some studies detected that glucose serum, triglyceride and total cholesterol levels in bee venom treated rats in comparison with diabetic group were significantly decreased [12]. The decrease in blood glucose may be attributed to substances like mellitin and phospholipase A2 contained in the venom which may play a role in diminishing inflammation of Islets of Langerhans and thus elevating blood insulin level. With regard to the fact that insulin regulates blood glucose level, bee venom could decrease glucose content via increasing insulin secretion [13-16].

Apparently decreased thickness of the epidermis and distortion of its multilayer appearance were noticed in diabetic rats in the current study. This could be attributed to excessive glycosylation of collagen which damaged the skin structure [1]. These results were in accordance with previous studies that detected significant epidermal thinning in mice with DM type 1 induced with STZ 2 weeks and 4 weeks after induction of diabetes, together with significant reduction of intra epidermal nerve fibers density [17]. Others also found that induction of diabetes type 1 and type 2 causes significant epidermal thinning and loss of intraepidermal nerve fibers in a rat model, and both changes were more pronounced in diabetes type 1 model [18].

Hyperglycaemia and impaired insulin signalling might participate in the pathogenesis of chronic diabetic complications related to skin, by impairing glucose utilization in skin keratinocytes, and

negatively affecting skin proliferation and differentiation [19].

A significant decrease in the mean area percentage of collagen fibers was noticed in diabetic group in the current study. This was explained by some authors who stated that in diabetes, there is an imbalance between degradation and the synthesis of extracellular matrix [1]. They added that the skin blood flow disturbances in diabetes diminish the ability to remove the degradation products of collagen, and may cause skin lesions that are characteristic of chronic diabetes. On the other hand, in the presence of hyperglycemia, glucose molecules react with the lysine and hydroxylysine – amidocyanogen of skin collagen. This leads to non-specific glycosylation of collagen with subsequent formation of advanced glycation end products, thereby disrupting the metabolism of collagen [20]. In the current study, distortion of the multilayer appearance of the epidermis was also noticed in H&E stained skin sections of the diabetic group. Also, Mallory stained sections of the diabetic group showed that the zone of condensed collagen fibers just beneath the epidermis appeared discontinuous. While in the reticular dermis, the collagen bundles were thin, irregular and disorganized. Similarly, some authors detected reduced thickness of the abdominal dermis of diabetic patients with morphological characteristics of obscured multilayer epithelium and shortened, thinned, and disorganized collagen fibrils with focal chronic inflammatory cell infiltration when compared with controls of the same age [21].

A significant decrease in the mean area percentage of PGP-9.5 expression was noticed in diabetic rats in the current study. Similar results were also reported by some authors [2,22].

Administration of bee venom resulted in a marked change of the construction of all skin layers of the tested animals. Apparent thickening of the epidermis was observed. More compact and regular arrangement of the dermal collagen fibers was observed, both in comparison to the group of diabetic rats as well as the control healthy group. Bee venom as a cosmetic ingredient may be useful as a topical agent for promoting skin regeneration or as a treatment for certain epidermal conditions, as it has shown that when wounded mice were treated topically with bee venom, increased collagen protein synthesis was demonstrated, which might be related to increased proliferation and migration of human epidermal keratinocytes [23-25].

According to the experiments performed by some authors, mellitin polypeptide present in bee venom promotes insulin secretion from Islets of Langerhans in vitro. They added that mellitin can

depolarize plasma membranes of β -cells and acts as a calcium transporter in the cell, which in turn promotes insulin granules secretion [13]. In addition others found that mellitin may promote insulin secretion through activating phospholipase A2 in Islets of Langerhans. Their results indicate that phospholipase A2 activation plays a role in compensating insulin resistance response in Islets of Langerhans[16].

Conclusions:

The current study shows that bee venom is effective in preventing skin changes accompanied with diabetes mellitus type I, as it preserves the structure and innervation of unwounded thick skin.

Recommendations:

More researches are needed to determine the most effective ingredient in BV helpful in diabetic conditions and determine its effective therapeutic dose and the effective simple and safe route of administration.

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