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The expression of leptin in oral wound healing in diabetes mellitus: An experimental study

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ABSTRACT

Objective: The present work evaluated histologically and immunohistochemically the expression of leptin during healing of the incisional oral mucosal wound in diabetic rats as compared to healthy rats.

Methods: Twenty-four adult male Sprague–Dawley rats weighing on average 150–200 g were allocated equally into two groups: Group I (control) and Group II (diabetic). Diabetes was induced by a single intraperitoneal injection of streptozotocin dissolved in distilled water. Each animal received experimental incision in buccal mucosa and sutured, and the specimens were collected from the buccal mucosa of each animal at intervals of 7, 14, and 21 days and routinely processed for H and E and immunohistochemical staining for leptin. All measurement data were calculated as a mean ± standard deviation.

Results: Leptin expression was observed in the epithelium and the vascular endothelial cells in both groups. In both the control and diabetic groups, the expression of leptin was significantly increased with time, and there was an extreme highly significant increase in the control group than in diabetic group after 7, 14, and 21 days ($P = 0.000$).

Conclusion: The results of the present study suggested that leptin may promote wound healing in rat's normal oral mucosa more than in diabetic. Further studies are needed to clarify the exact molecular mechanisms of leptin's effects on wound healing and to determine the usefulness of leptin as a treatment to promote wound healing in the oral mucosa in diabetic and non-diabetic patients.

Keywords: Immunohistochemical, leptin, oral mucosa, wound healing

Introduction

Diabetes mellitus is one of the most common chronic diseases in nearly all countries and continues to increase in numbers and significance, as economic development and urbanization lead to changing lifestyles characterized by reduced physical activity and increased obesity.[1] Diabetic patients often heal poorly; they have a higher incidence of wound infection and are prone to slow healing of peripheral injuries even when adequate peripheral pulses are present.[2]

Wound healing requires a well-orchestrated integration of complex biological and molecular events of cell migration, cell proliferation, and extracellular matrix deposition.[3] Over 100 known physiologic factors contribute to wound healing deficiencies in individuals with diabetes. These include decreased or impaired growth factor production,[4] angiogenic response,[5] macrophage function, collagen accumulation, epidermal barrier function, quantity of granulation tissue,[5] keratinocyte and fibroblast migration, and proliferation and also the number of epidermal nerves.[6] Injury induces tissue hypoxia leading to upregulation of growth factor, extracellular matrix degradation, and subsequently, activation of angiogenesis. This formation of new blood vessels is required to sustain the newly formed granulation tissue.[7]

Leptin is a hypoxia-inducible cytokine functionally related to the interleukin 6 cytokine family. As a well-documented angiogenic molecule, leptin may mediate wound neovascularization and has additional effects in cells involved in the healing process, including fibroblasts, macrophages, and keratinocytes. The increase observed in leptin synthesis within the wound results in a transient elevation in circulating leptin, arising directly from the wound bed.[8] The leptin is expressed in various tissues including the hypothalamus, adipose tissue, skeletal muscle, and hepatocytes. The multifunctionality of leptin and the wide distribution of its receptor suggest that leptin plays a variety of physiological roles not only as a systemic hormone but also as a local growth factor.[9]

The aim of the present study was to evaluate histologically and immunohistochemically the expression of leptin during healing of the incisional oral mucosal wound in diabetic rats as compared to healthy ones.

Materials and Methods

Animals

Twenty-four adult male Sprague–Dawley rats weighing on average 150–200 g were allocated equally into two groups: Group I (control) and Group II (diabetic). Animals were housed at the Faculty of Medicine, Alexandria University, in individual cages and received a standard diet for rodents and tap water. Room temperature and humidity were maintained at 23°C and 60%, respectively. All animal experiments were carried out in accordance with the guidelines of the National Institutes of Health for the care and use of laboratory animals.[10]

Induction of diabetes, animal euthanasia, and samples collections

Diabetes was induced by a single intraperitoneal injection of streptozotocin (STZ) (60 mg/kg, Sigma Chemical, St. Louis, MO) dissolved in distilled water. 3 days after the STZ injection, urine strips (Medi Test Combi 10; Macherey-Nagel GmbH and Co., Düren, Germany) were used to detect glycosuria in rats (a dark-green color indicated blood glucose ≥500 mg/dL).

Each animal received an experimental incision in buccal mucosa using no. 15 scalpel blade and sutured. The sutures had the same thickness (4-0) and needle (semicircular, nontraumatic with triangle section). After 7, 14, and 21 days, 4 animals from each group were sacrificed with an overdose of the anesthetics, according to the ethical guidelines, confirmed with cervical dislocation, the sutures and the subcutaneous tissue were excised from the dorsal area. The specimens were fixed in 10% buffered formalin solution, routinely processed and embedded in paraffin. Serial sections were cut at 5 μm thickness; one of each set of sections was stained with H and E for conventional histological assessment using light microscope (Leica ICC50 HD). Another set was used for immunohistochemical staining for leptin.

Immunohistochemistry technique

For the process of immunohistochemical staining, 5 μ sections were prepared from each paraffin block and were deparaffinized in xylene solution and then dehydrated in graded alcohol series. To block the internal peroxidase activity, hydrogen peroxide (3%) in phosphate buffer solution was used. Then, antigen retrieval was done in a microwave oven (Panasonic 1380W) for 10 min, under the pressure of almost 2 atmospheres in 120°C. Further incubations using pre-diluted ready to use primary mouse polyclonal antibodies against leptin (lab vision corporation, Fremont, USA) (dilution 1:10) were used as the primary antibody for 30 min and were incubated in a moist chamber in room temperature for 1 day, followed by the application of secondary antibody (for 15 min), diaminobenzidine (producing brown staining), and Meyer's hematoxylin (for background staining). The samples were placed in phosphate buffer saline (PBS) immediately after each mentioned step. The positive control was used according to the manufacturer's instructions. The negative control was prepared by the replacement of primary antibody with PBS.

Assessment of immunohistochemistry stained sections

The presence of colored reaction was considered as a positive reaction. The assessment of intensity of the immunostaining was classified as negative, mild, moderate, and intense from three fields using a blinded analysis obtained by two independent pathologists using a conventional diagnostic microscope (Eclipse 80i, Nikon, Tokyo, Japan), 0 (no staining), 1 (mild, 1–10% positive cells), 2 (moderate, 11–50% positive cells), and 3 (intense >50% positive cells), and further examination was performed by the image analyzer computer system with the software version 4.10.03, Nikon, Tokyo, Japan.[11]

Statistical analysis

Data were collected and statistically analyzed using SPSS version 20. The data were described as a mean ± standard deviation. Comparison between different means was performed using one-way analysis of variance followed by Dunnett's *post hoc* test. $P < 0.05$ was considered statistically significant difference.

Results

7 days after the incision

H and E staining

In this study, H and E staining revealed that the healing of the surface epithelium in the experimental control group was more than in diabetic group which was noticed by the increased thickness of surface epithelium [Figure 1a and b]. The total thickness of the epithelial layers in the control group was more than that in the diabetic one.

Immunohistochemical analysis of leptin expression

In oral mucosa of the experimental rats, leptin was exhibited as mild cytoplasmic staining in the surface epithelium of both experimental control and diabetic groups [Figure 1c and d]. This revealed that the healing was more in normal than in diabetic group.

14 days after incision

H and E staining

In the experimental control group, the thickness of the surface epithelium was increased with well-formed all epithelial layers,

and the rete processes were numerous and normal in shape. In experimental diabetic group, the thickness of the surface epithelium was more than that of control group after 7 days, whereas at 14 days, the epithelium thickness is markedly decreased with few, irregular, or even absent rete processes in some parts of epithelium [Figure 2a and b].

Immunohistochemical analysis of leptin expression

Leptin expression increases in the surface epithelium in both groups. The expression was intense in the control group comparing to the moderate expression in diabetic group. Leptin was intensely expressed in the endothelial cells and some stromal cells in the control group [Figure 2c], but this finding was completely absent in diabetic group with negative staining of leptin in subepithelial stroma [Figure 2d].

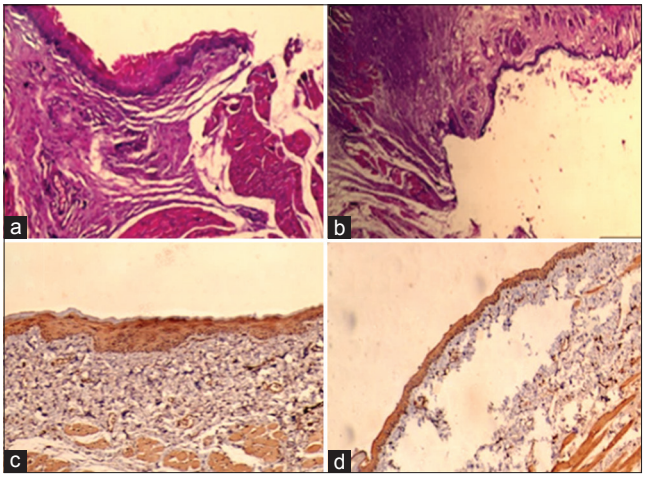

Figure 1: 7 days after the injury. (a and b) H and E staining in control and diabetic groups, respectively, showing increase thickness of surface epithelium in the control group (×100). (c and d), immunohistochemical localization of leptin in the oral mucosa of control and diabetic groups showing mild expression of leptin in the surface epithelium in both groups (×200)

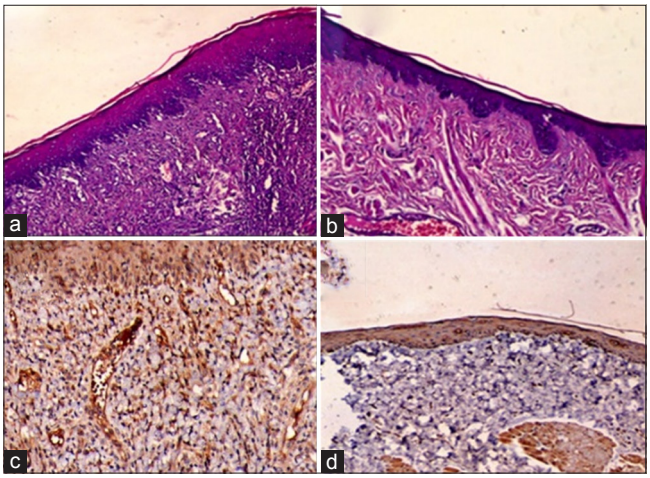

Figure 2: 14 days after the incision. (a and b) H and E staining in the control and diabetic groups, respectively, showing increased thickness of the surface epithelium in control group with well-formed epithelial layers and numerous, normal-shaped rete processes. (c) Immunohistochemical expression of leptin in the control group was intense in the surface epithelium and also in the endothelial cells and stromal cells. (d) Moderate expression of leptin in diabetic group observed only in the surface epithelium (a, b, d - ×200, c - ×400)

21 days after incision

H and E staining

Complete healing of the surface epithelium was observed in both groups. All layers and rete processes were nearly normal in both control and diabetic groups except the thickness of surface epithelium which was more increased in control group than in diabetic group [Figure 3a and b].

Immunohistochemical analysis of leptin expression

In the control group, leptin was intensely expressed in the surface epithelium and some stromal cells [Figure 3c]. In diabetic group, there was a moderate staining expression in the surface epithelium and stromal cells [Figure 3d].

Statistical results

The mean values of leptin expression in oral mucosa among control and diabetic rats at the three measuring points of time were demonstrated in Table 1. Control group showed higher means of expression of leptin after 7, 14, and 21 days than the diabetic group. The differences between the control and diabetic groups at the three measuring time periods were extremely highly significant ($P = 0.000$). Totally, the means of expression after 21 days were noticed by higher values than that after 14 days in both control and diabetic groups, while the means of expression after 7 days were the lowest one [Figure 4]. Regarding the comparison of the leptin expression among the control after 7, 14, and 21 days, increasing the means of leptin expression by increasing the time were observed with extremely higher significant difference ($P = 0.000$). Similar

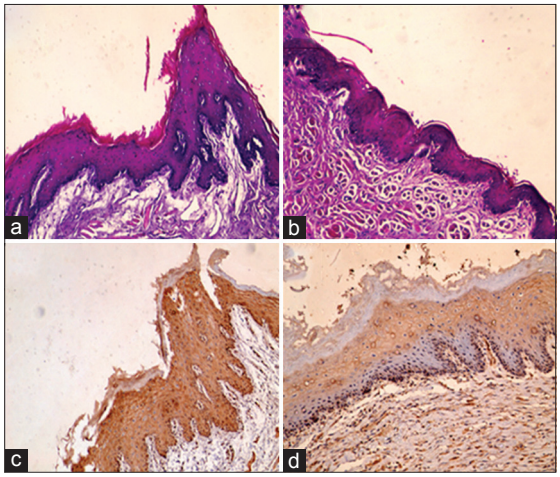

Figure 3: 21 days after the incision. (a and b) H and E staining in control and diabetic groups, respectively, all layers and rete processes were nearly normal in both control and diabetic groups. (c) Immunohistochemical expression of leptin in the control group was intense in the surface epithelium and also in some stromal cells. (d) Moderate leptin expression in diabetic group in the surface epithelium and some stromal cells (×200)

Table 1: The means and standard deviation of leptin expression among control and diabetic groups after 7, 14, and 21 days

Groups	After 7 days	After 14 days	After 21 days	*F* (*P* value)
Control				
Range	2.5–6	12–14.5	20–25	550.463 (0.000)***
Mean±SD	5.24±0.56[#]	13.01±0.85	22.78±1.78	
Diabetic				
Range	2–3.1	4–6	6–9	122.249 (0.000)***
Mean±SD	2.48±0.46	5.07±0.73	7.46±0.88	
t (*P* value)	13.192 (0.000)***	24.540 (0.000)***	26.726 (0.000)***	

***Extreme highly significant difference. SD: Standard deviation, *t*: Unpaired t-test, *F*: One-way ANOVA test

Table 2: Dunnett's *post-hoc* test pairwise comparison among the control and diabetic group after 7, 14, and 21 days

Pairwise comparison	*t* (*P* value)
Control group	
7 days versus 14 days	26.441 (0.000)***
7 days versus 21 days	31.126 (0.000)***
14 days versus 21 days	16.476 (0.000)***
Diabetic group	
7 days versus 14 days	9.241 (0.001)**
7 days versus 21 days	15.854 (0.000)***
14 days versus 21 days	7.231 (0.001)**

**Highly significant difference. ***Extreme highly significant difference

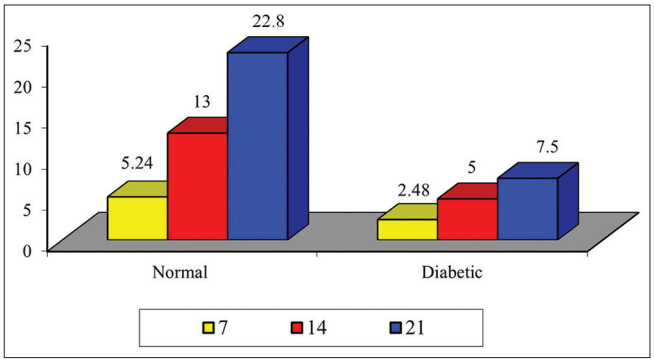

Figure 4: The expression of leptin in normal and diabetic groups was significantly increased with time ($P = 0.0001$)

result was observed among the diabetic group where the leptin expression was increasing ($P = 0.000$) [Table 1].

As regard to the pairwise comparison within the control and diabetic groups after 7, 14, and 21 days, extremely higher significant differences were found ($P = 0.000$). While, after 7 days versus 14 days and 14 days versus 21 days in diabetic group only, higher significant differences were found ($P = 0.001$) [Table 2].

Discussion

The oral mucosa serves many functions. The major one is the protection of the deeper tissues of the oral cavity; others include acting as a sensory organ and serving as the site of the glandular activity and secretion.[12] Interruption in the continuity of this lining tissue compromises these functions. The oral mucosa is easily affected by many systemic factors including diabetes. Oral wounds in diabetes often fail to heal adequately, resulting in chronic ulcer formation followed by serious systemic infections.[13,14] This in accordance with the results of the present study in which H and E staining revealed that the healing of the surface epithelium in the control group was more than that in the diabetic group which was noticed by the thickness of the surface epithelium.

It is important to establish an effective strategy to restore the structure and function of oral mucosa by promoting wound healing. Jeffcoate *et al*., 2003,[15] reported that angiogenesis is a vital component of wound repair. Despite the evidence shown by recent studies that local or systematic administration of cells and/or proangiogenic molecules could significantly improve angiogenesis and wound closure, diabetic wounds remain a significant clinical problem. However, it has been suggested that both systemic and topical leptin accelerate wound repair in diabetic mice, possibly through the direct interaction of leptin with its receptors in wounded skin, but do not appear to significantly stimulate wound angiogenesis.[16] In the present study, we evaluated the expression of leptin during healing of the incisional oral mucosal wound in both normal and diabetic rats to elucidate the role of leptin in promoting wound healing. The results of this study revealed that leptin was expressed in the epithelium and vascular endothelial cells and some stromal cells in subepithelial connective tissue. This expression is more intense in the control group than the diabetic group. These findings suggest several possibilities regarding the mechanisms by which leptin promotes wound healing. One possibility is that leptin promotes wound healing by enhancing the epithelial cell proliferation and maturation.[17] Another possibility is that leptin stimulates angiogenesis in the connective tissue beneath the wound and promotes wound healing in the oral mucosa by accelerating the supply of nutrients, oxygen, and even some bioactive substances.[18,19]

For many years, leptin-deficient mice have been used as a model system to investigate the cellular and molecular mechanisms of impaired wound healing. The severe impairment of wound healing observed in these model mice was originally explained

by the diabetic phenotype of the animals.[20] This was in agreement with our results which revealed extremely higher significant difference in the expression of leptin in oral mucosa in the control group more than in diabetic group ($P = 0.000$) after 7, 14, and 21 days.

The present results demonstrated that there was more intense expression of leptin in the epithelium and vascular endothelial cells, in the control group than in diabetic group at 14 and 21 days. Its expression increases in diabetic group by time. This is in agreement with immunohistochemical analysis for the expression of leptin protein in human and rabbit oral mucosa which performed by Umeki *et al.,* 2014,[21] they suggested that epithelial cells and vascular endothelial cells in oral mucosa are target cells for leptin, and they concluded that topically administered leptin was shown to promote wound healing in the oral mucosa by accelerating epithelial cell migration and enhancing angiogenesis around the wounded area.

Conclusion

The results of the present study suggested that leptin may promote wound healing in rat's normal oral mucosa more than in diabetic. Further studies are needed to clarify the exact molecular mechanisms of leptin's effects on wound healing and to determine the usefulness of leptin as a treatment to promote wound healing of oral mucosa in diabetic and non-diabetic patients.

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