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Histopathological and Immunological Evaluation of Topical Treatment with Leptin on the Healing of Oral Soft Tissue and Cutaneous Wounds

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ABSTRACT

Aims: Histopathological and immunological studies were conducted to elucidate the effect of topical application of leptin on the healing of cutaneous and oral mucosal wounds.

Materials and Methods: Fifty apparently healthy adult male rabbits of mixed breed were used.A standard round 0.8 cm diameter full-thickness skin wound and a second identical oral mucosal wound were aseptically induced in each rabbit using a locally made device. The animals were then divided into two equal groups: control (no treatment), leptin (treatment with leptin and orobase). The treatment protocol for material was once daily for 3 continuous days. Biopsies were collected from the oral and skin wounds for 5 rabbits from each group at 1, 3, 7, 15 and 30 days, and processed histopathologically. Blood samples were collected from all rabbits and were used for measurements of the serum level of Il-6 and TNF-α using ELISA Kits.

Results: Histopathological and statistical findings of this study indicated that the inflammation was more extensive and re-epithelialization was higher in both oral and skin wounds treated with leptin than control group. Significant differences ($P < 0.05$) were found in concentrations of IL-6 and TNF-α in sera of control and leptin treated groups.

Conclusions: Inflammation was found to be more pronounced in oral mucosal and skin wounds in leptin treated group than in control group. Reepithelialization was faster in mucosal and skin wounds for the leptin treated group than in control group. There were significant fluctuations in serum concentrations of Il-6 and TNF-α in both control and the leptin treated rabbits for the 1, 3, 7, 15 and 30-day periods.

Key words: Leptin, Oral Soft TissueWound Healing, Cutaneous Wound Healing.

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INTRODUCTION

Leptin, the "Satiety hormone" is a 16 KD protein that is produced by adipocytes and induces weight loss in both normally and generally obese ob/ob (mutated obesity gene) mice. ob/ob mice are obese, have multiple metabolic abnormalities, and exhibit impaired wound healing. Exogenous administration of leptin to these animals induces weight loss and correct their metabolic defects. Leptin levels are rapidly increased by many active phase cytokines, such as tumor necrosis factor alpha (TNF-α), interleukin-1 (IL-1), interleukin-6 (IL-6) and other factors[1,2]. Frank et al. (2000)[3] demonstrated that systemically and topically supplemented leptin improved re-epithelialization of wounds in ob/ob mice. Leptin completely reversed the atrophied morphology of the migrating epithelial tongue observed in the wound margin of leptin-deficient animals into a well-organized hyperproliferative epithelium, moreover, topically administered leptin accelerated normal wound-healing condition in wild type mice. Circulating leptin level after incisional wounds were created in obese and in non-obese mice, increased rapidly in the first 24h [4]. A high serum

level of leptin appears to promote cutaneous wound healing in obese mice [5]. It was not possible to establish a positive link between chronic periodontitis in terms of differences in the levels of adiponectin, leptin and TNF-α between obese and normal weight subjects with and without periodontitis[6]. Leptin in human milk may suppress intestinal microbial pathways associated with intestinal inflammation. Increased acetate production, reduced host pyruvate metabolism and suppress bacterial protease activity may suppress intestinal inflammation in 2 weeks life of infant [7] .

Tadokoro et al. (2015) [8]studied the role of topically administered leptin in wound healing of the skin. They found that Ob-R (leptin receptor) was expressed in epidermal cells of human and mouse skin. Leptin has been found to possess a potent direct angiogenic factor that stimulates endothelial cell migration and activate in vitro, as well as angiogenesis in vivo [9,10,11]. In addition, leptin seems to participate in clinical angiogenesis as it promotes the formation of new blood vessels [12,13].

MATERIALS AND METHODS

Fifty apparently healthy adult male rabbits (of mixed breed) were used in this study. The animals were bought from a local vendor and their average weight was 1.5±0.5 kg. The rabbits were kept in an animal house with constant temperature of 24^0C, good ventilation, 12 hours light-dark cycle, and were given food (vegetable, grain and corn) and water ad libitum (as desired). Leptin used in this study was imported from GenScript (860 Centennial Ave., Piscataway, NJ 08854,USA). Concentration of leptin of 0.00015g/100ml[14] of orabase paste according to the procedure described by Al-Nema (2000) [15] was used in this study. The rabbits were randomly divided into 2 equal groups of 25 rabbits, each rabbit was anesthetized using 40mg/kg. Ketamin injection [16] intramuscularly in the thigh muscle, mixed with xylazine 4mg/kg [17]. Following induction of anesthesia, a standard round 0.8 cm full thickness skin defect was induced by using punch biopsy (locally made device). Identical defect is induced in the oral mucosa of the right cheek. The treatment protocol for the treatment group was done once daily for three continuous days. Biopsies were collected from the oral and skin defects of five rabbits from each of the groups at 1,3,7,15 and 30 days. Both skin and oral biopsies were preserved in freshly prepared 10% formalin for 48 hours. Blood sample for measurements of IL-6 and TNF-α were collected from the rabbits at the same intervals as indicated above. Following fixation of tissue specimens they were stained with hematoxylin and eosin (H&E) stain and examined under light microscope to evaluate the inflammatory response and re-epithelialization depending on the score described by Camacho-Alonso et al., 2005; Camacho-Alonso et al.,2008; Lopez-Jornet et al., 2009 and Albannaa, 2010[18,19,20,21].

The concentration of TNF-α in serum was measured using TNF-α ELISA kit provided by MyBiosource.com. and IL-6 serum concentration was determined using the IL-6 ELISA kit provided by MyBiosource.com.

Statistical analyses

Analysis of the results was done using SPSS 19 computer software program. Mann–Whitney NPar Test was used to compare between means of various groups. Statistical significance was accepted for P–value ≤ 0.05. Furthermore, results of estimations of serum concentration of IL–6 and TNF–α in various groups and during the various post–wounding days were analyzed using the one–way analysis of variance. Values of $P \leq 0.05$ were considered statistically significant.

The Results

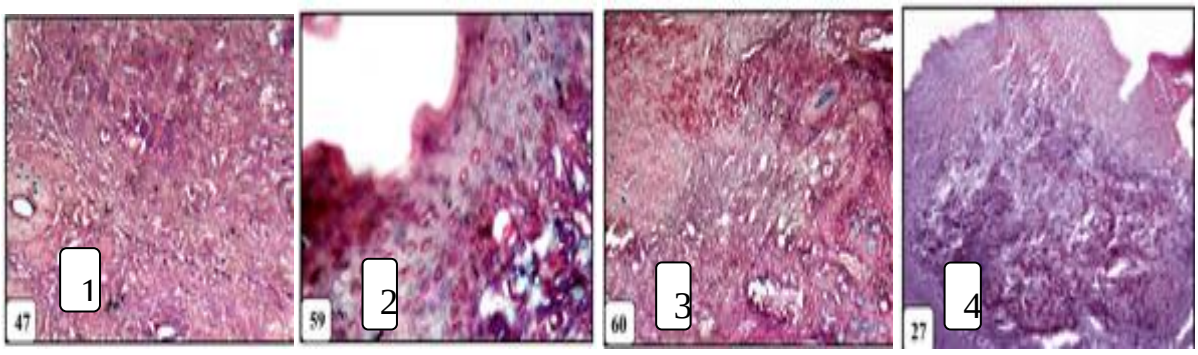

Fig. (1): A 15-days skin wound treated with leptin. Mature fibrous tissue is present in the dermal wound. H&E, X100.

Fig. (2): A high power view of the mucosa of a 7-days oral mucosal wound treated with leptin. The mucosa consist of well – differentiated epithelial cells. H&E, X400.

Fig. (3): A view of the submucosal part of a 7-days oral mucosa wound treated with leptin. Only minimal amount of fibrous tissue could be seen in the submucosal wound. H&E, X100.

Fig. (4): A 7-days skin wound in a control rabbit. A thickened epidermis could be seen over the wound and granulation tissue is evident in the dermal wound. H &E, X100.

The mean scores of inflammation and re-epithelialization for the whole rabbits of control and leptin groups and for all time periods 1,3,7,15 and 30 days all are shown in (Table 1 and 2).

Table(1) :Mean values of scores of inflammation and re-epithelialization of skin and oral mucosal wounds in control group

	Skin		Mucosa	
Days	Inflammation	Re-epitheli-alization	Inflammation	Re-epitheli-alization
1	0.52	0.0	0.34	0.36
3	1.8	0.53	1.8	2.95
7	3.25	2.15	3.85	3.65
15	3.30	2.70	3.65	4.00
30	3.75	3.75	3.85	3.90

Table(2): Mean values of scores of inflammation and re-epithelialization of skin and oral mucosal wounds in leptin treated group

	Skin		Mucosa	
Days	Inflammation	Re-epithe-lialization	Inflammation	Re-epithe-lialization
1	0.56	0.93	0.65	0.8
3	2.4	1.2	2.5	3.1
7	3.05	2.15	4.1	3.8
15	3.9	3.65	4.25	4.2
30	4.2	4.15	4.4	4.25

Mann-Whitney statistical test showed significant differences between inflammation and re-epithelialization of the healing skin wounds in control and leptin groups (Table 3). Inflammation was more extensive in skin wounds treated with leptin than control skin wounds. However, significant differences were found only between inflammation in control and leptin treated wounds during the 3-day and 15-day periods (Table 3 and Fig.5). Re-epithelialization was significantly higher ($P<0.05$) in leptin treated skin wounds than in control skin wounds for the 1,3 and 15 day periods (Table3 and Fig.6). Inflammation was significantly higher ($P<0.05$) in leptin treated mucosal wounds than in control mucosal wounds for the periods 1,15 and 30 day periods (Table4and Fig.7). Re-epithelialization was significantly higher ($P<0.05$) in leptin treated mucosal wounds than control mucosal wounds for the periods 1,15 and 30 day (Table4 and Fig.8).

Table (3): Mann-Whitney test comparing between inflammation and re-epithelialization in control and leptin treated skin wounds.

Duration	Inflammation (P-value)	Re-epithelialization
Day 1	0.585	0.008 (*)
Day 3	0.023 (*)	0.015 (*)
Day 7	0.390	0.955
Day 15	0.014 (*)	0.021 (*)
Day 30	0.161	0.055

Significance (*): P value ≤0.05

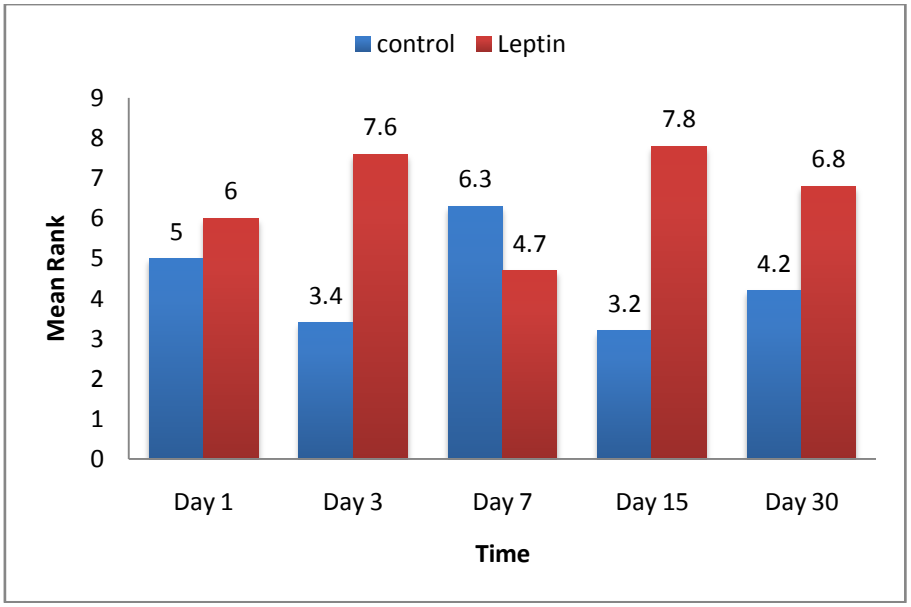

Fig. (5): Mann-Whitney mean ranks for inflammation in control and leptin treated skin wounds.

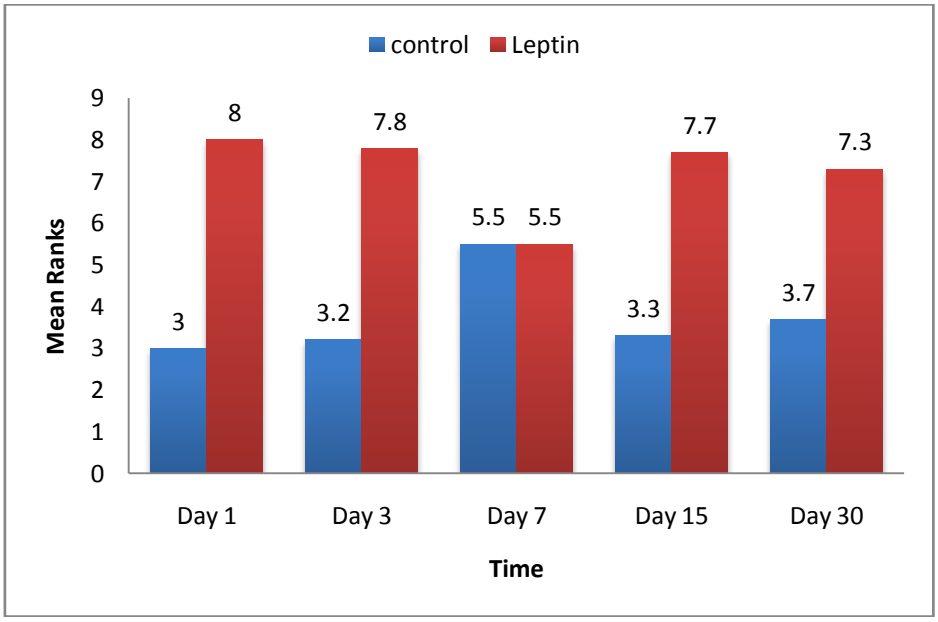

Fig. (6): Mann-Whitney mean ranks for re-epithelialization in control and leptin treated skin wounds

Table (4): Mann-Whitney test comparing between inflammation and re-epithelialization in control and leptin treated mucosal wounds.

Duration	Inflammation (P-value)	Re-epithelialization
Day 1	**0.091 (*)**	**0.011 (*)**
Day 3	**0.014 (*)**	**0.445**
Day 7	**0.329**	**0.584**
Day 15	**0.010 (*)**	**0.034 (*)**
Day 30	**0.051**	**0.033 (*)**

Significance (*) P value ≤0.05

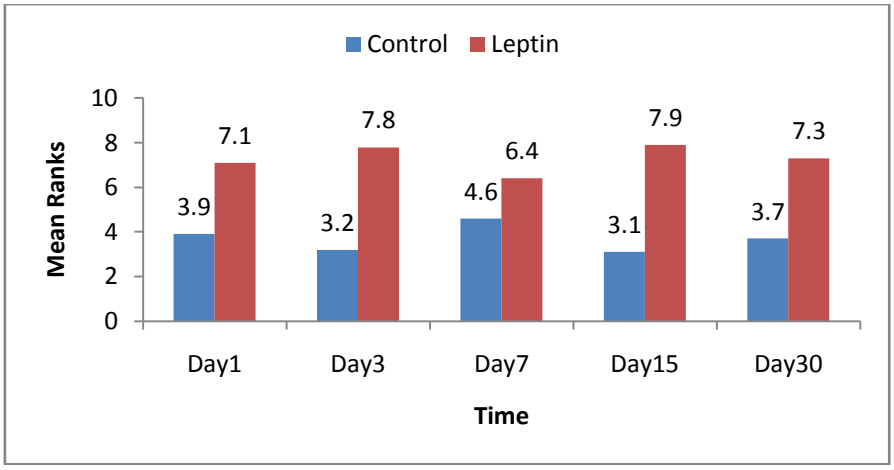

Fig. (7): Mann-Whitney mean ranks for inflammation of control and leptin treated mucosal wounds.

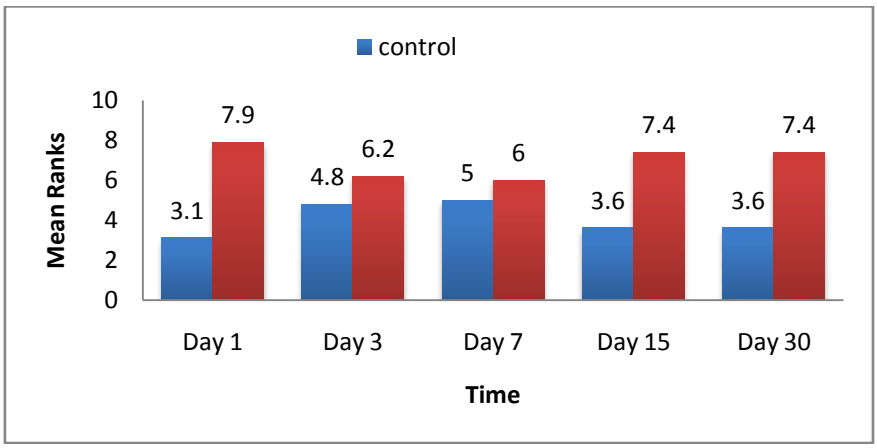

Fig. (8): Mann-Whitney mean ranks for re-epithelialization of control and leptin treated mucosal wounds.

Serum concentration of IL-6 and TNF-α in control and leptin treated groups showed fluctuation during various time periods 1,3,7,15 and 30 post-wounding days (Table 5 and 6).

The ANOVA statistical test comparing between serum concentration of IL-6 in control and leptin treated groups showed significantly lower (P<0.05) concentration in leptin treated group in the 1 and 15 post-wounding days and significantly higher (P<0.05) concentration in leptin treated group in the 7 post-wounding days (Table 5), while ANOVA statistical test comparing between serum concentration of TNF-α in control and leptin treated groups showed significantly higher (P<0.05) concentration in 3 and 30 post- wounding day (Table 6).

Table (5): The mean values of serum concentration of IL-6 of control and leptin treated groups at various post-wounding days.

Groups	Day 1	Day 3	Day 7	Day 15	Day 30
Control group	50.00(*)	12.50	0.00(*)	12.50(*)	1.50
Leptin group	16.25	5.00	13.60	2.60	8.67

Significance (*), P value < 0.05.

Table (6): The mean values of serum concentration of TNF-α of control and leptin treated groups at various post-wounding days.

Groups	Day 1	Day 3	Day 7	Day 15	Day 30
Control group	25.00	20.50(*)	35.00	37.50	37.50(*)
Leptin group	34.25	88.00	38.00	35.00	85.00

Significance (*), P value < 0.05.

DISCUSSION

There are several possibilities regarding the mechanisms by which leptin promotes wound healing. The first possibility is that leptin promotes wound healing by enhancing the epithelial cell proliferation [22,23]. Another possibility is that leptin enhances wound healing by accelerating epithelial cell migration [24]. A third possibility is that leptin promotes wound healing by stimulating angiogenesis [10,13,24]. In view of the fact that wound healing is a dynamic and complex process, it is likely that these mechanisms contribute toward the wound healing concurrently.

Results of the present study indicated that topical application of the recombinant leptin increased the rate of re-epithelialization and enhanced angiogenesis of the skin wounds. In support of this finding are the results of other workers [8,25]. Thus, Tadokoro et al. (2015) [8]found that leptin enhanced wound healing in mouse skin by accelerating proliferation, differentiation /function and migration of epidermal keratinocytes and enhancing angiogenesis around the wounded area. Furthermore, Liapakis et al. (2008)[25] found that exogenous administration of recombinant leptin increased early tissue angiogenesis in the burn wound level of an experimental animal model (rats). Manjunathan and Ragunathan (2015) [26]found that human recombinant leptin (HRL) has the ability to induce new vessel formation at the treated area of the chicken chorio–allantoic membrane and growth of the newly formed vessels and cellular morphological changes occur in a dose dependent manner. The finding of elevated mRNA and protein level expression of VEGF165 and MMP2 along with the activation of ECs as demonstrated by the presence of CD34 expression supported the neovascularization potential of HRL. Murad et al. (2003) [23]demonstrated that leptin expression is rapidly induced in experimental wounds in mice, that neutralizing anti–leptin antibodies impair healing progression in experimental wounds, and that wound–derived leptin leads to a transient increase in circulating leptin levels after wounding.

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