The effect of Tumor Necrosis Factor alpha inhibitor on wound healing of oral mucosa in induced diabetic rats

Received: 7/1/2015                                      Accepted: 20/5/2015

Rafah Al-Marooof *   Farhad Fareed **

Abstract

Background and objective: Impaired wound healing is a major complication of diabetes mellitus. This study was carried out to determine the healing process of oral mucosa in diabetic rats and the role of systemic tumor necrosis factor alpha inhibitor (infliximab).

Methods: Thirty eight male rats were divided into two groups, the normoglycemic group (11 rats), and diabetic group (27 rats) that were rendered diabetic by alloxan injection. Two months later, wound was created in the lateral side of the tongue for both groups. The diabetic group was then subdivided into two subgroups, 14 rats received 5mg/kg infliximab subcutaneous injection at the day of wound creation while the other 13 rats received saline injection. After 7 days, biopsies of the tongue were collected and subjected to histological and histochemical procedure.

Results: Histological examinations showed delayed healing in the diabetic group with persistence of epithelial discontinuity, large amount of granulation tissue and destruction of the underlying muscle fibers. In the subgroup injected with infliximab, reepithelialization of the wound was demonstrated with well arranged underlying collagen fibers. Using PAS stain, diabetic group revealed a dramatically high amount of PAS positive precipitants in the lamina properia, especially in the wall of the blood vessels, while with infliximab injection, the PAS+ve precipitants were more prominent than normoglycemic group but less than diabetic group without infliximab.

Conclusion: These findings suggest that infliximab accelerated mucosal wound healing in the diabetic rats with the formation of well organized connective tissue.

Keywords: Diabetes, infliximab, wound healing.

Introduction

Type 1 diabetes mellitus is a common chronic autoimmune disease, induced by permanent destruction of β pancreatic cells, and characterized by defects in insulin secretion and hyperglycemia. Long-term manifestations of diabetes include retinopathy, neuropathy, nephropathy, defect in blood vessels, periodontitis, and other complications, such as impaired wound-healing. The delayed wound healing in diabetes is caused by complex factors such as reduced keratinocyte proliferation, diminished fibroblast migration and differentiation, and higher rate of apoptosis. Several of these defects have been linked to greater inflammation and proinflammatory cytokine production. In normal inflammation, necrotic tissues are removed to create a healthy environment for the growth of new tissue. In a pathological circumstances, such as that of diabetics, sluggish removal of necrotic tissue delays the onset of healing and results in chronic inflammation. A large number of growth factors are important in stimulating and organizing cellular events that occur during normal wound healing. Among them, cytokines and chemokines are especially noted because of their roles in promoting inflammation, budding new blood vessels, leukocyte migration, and reepithelialization. Proinflammatory cytokines that are

*Department of oral diagnosis, college of dentistry, Hawler Medical University, Erbil, Iraq.
**Ministry of health, Erbil, Iraq.
http://dx.doi.org/10.15218/zjms.2016.0008

Elevated presently after wounding both in human and animal models include IL-1, IL-6, and TNF-alpha. In diabetic models, increased levels of the proinflammatory cytokines such as TNF-alpha and IL-6 and decreased levels of anti-inflammatory IL-10 are observed in diabetic wound tissue compared to nondiabetic healing wound. TNF-alpha is found threefold higher in diabetic mouse wounds than wounds in normal mice. Application of TNF-alpha causes a decrease in wound strength that may be due to decreased collagen type I and type III expression. In contrast, inhibition or deletion of TNF-alpha generally enhances repair processes. Genetic ablation of TNF receptor-1 improves wound healing by enhancing angiogenesis, collagen production, and reepithelialization. Similarly, administration of anti-TNF-alpha antibody in mice significantly promotes collagen deposition.

Infliximab is a monoclonal antibody that targets and inactivates TNF-alpha, and approved by the Food and Drug Administration (FDA) for the treatment of many autoimmune diseases, such as rheumatoid arthritis, Crohn’s disease, ulcerative colitis and some skin autoimmune diseases like psoriasis. Infliximab binds to the soluble form of TNF-alpha and inhibits binding of TNF-alpha to its cognate receptors. Delayed or incomplete healing of wounds has been well documented in diabetic humans and in animal models of diabetes. Therefore, this study was conducted to investigate the relation between wound healing in diabetes mellitus and the role of anti tumor necrosis factor alpha.

**Methods**

Thirty-eight albino male rats, weighting (250-350 gm) were used in this study. They were housed in the animal house facilities of the Department of Anatomy, College of Medicine of Hawler Medical University. The rats were left to acclimatize for one week to the laboratory conditions prior to the experimental manipulation and all the rats were checked for the general health by a specialist veterinarian. They had free access to the food and water at a room temperature of (19-25) C. The rats then were divided into 2 groups, the normoglycemic group (11 rats) and experimental diabetic group (27 rats). The experimental group was rendered diabetic by depriving the rats from the food for 12 hours (overnight fasting) giving them just access to the water, and for the next day all the rats of the experimental groups were injected with 90mg/kg/body weight alloxan mixed with normal saline in the peritoneal area under the thigh using insulin syringes. Three days after injection of the alloxan, the rats of the experimental groups were checked for both random blood glucose and weight, the rats were considered to be diabetic if blood glucose level was 200mg/dl or more, and this was done by collecting the blood from the tip of the tail and checking it with glucometer (ACCU-CHECK active). Routine checking of the blood glucose and weight were done for the diabetic group every 4 days and continued for 2 months. After two months, wound was created in the lateral border of the tongue of both diabetic and normoglycemic groups by the use of 0.5 cm square biopsy punch in the left side. After wound creation, the experimental diabetic group was subdivided into 2 subgroups. Fourteen rats from the diabetic group were injected with 5mg/kg/body weight infliximab subcutaneously. This was done by mixing the infliximab (100mg) with 10ml normal saline, then left for 15 minutes for dissolving without shaking. The remaining subgroup (included 13 rats) received saline subcutaneous injection and...
considered as control diabetic group. Seven days following the wound creation, the rats were executed in gas chamber and sample collections from the tongue included the wounded area was performed for all diabetic and normoglycemic groups. The biopsies were placed in 10% formalin to be preserved and prepared for slide preparation, histological analysis using Hematoxylin & Eosin stain, and histochemical analysis using Periodic Acid Schiff (PAS) stain to localize the presence of carbohydrates in wounded area. The evaluation of the PAS stained slides and determination of the stain intensity was done by two experts blindly. The H&E slides were used to count the number of inflammatory cells using digital motic microscope and its software program under high power field (400X). The cell's counting was performed using grid containing 30 square measures (993 * 994 micrometer). Using SPSS version 17.0 software, statistical analysis including descriptive statistic, ANOVA, and LSD tests were done for the collected data (P value ≤0.05 was considered statistically significant). The experiments were carried out with the approval of the ethic committee of Hawler Medical university/college of Dentistry.

Results

The clinical examination of the rat tongue seven days after wound creation revealed a great difference between the three groups. In both, the normoglycemic and diabetic experimental group injected with infliximab, although the wound area was demonstrated as a depression at the side of the tongue, it demonstrated clinical healing with well recognized covering of the underlying tissue (Figure 1a & 1c) respectively, while in the diabetic group with saline injection, the healing was not completed and the open wound was easily detected clinically, where epithelial breakdown was still obvious exposing the underlying deeper structures (Figure 1b).

Figure -1: Clinical picture of the rat tongue 7 days after wound creation. a-normoglycemic group. b- diabetic saline group. C- diabetic group injected with infliximab.
Histological Results

I- Normoglycemic Group: The microscopical examination showed complete reepitheliazation of the mucosa with considerably thick epithelial layer that approaching the normal appearance. In spite of that, the junction between the epithelia with underlying lamina properia appeared to be varies from the normal features with lack of ordinary interdigitations. The underlying lamina properia demonstrated an area of chronic inflammation and accumulation of granulation tissues at site of the wound. The underlying skeletal muscle appeared to be destructed with obvious spacing between their fibers with infiltration of chronic inflammatory cells (Figure 2). The mean of the inflammatory cells was 146.54±9.15 (Figure 3).

Figure 2 (H & E): Microscopical picture of normoglycemic rat tongue 7 days after wound creation. a: reepitheliazation (arrow) of mucosa and large amount of cellular granulation tissues (arrow head) (40X). b: ill defined basement membrane (thick arrow) with infiltration of the basal cell layer (B) and the lamina properia (LP) with chronic inflammatory cells (arrows) (400X). C: chronic inflammatory cells (arrows) invading destructed skeletal muscle fibers (M). (400X).

Figure 3: The mean of the number of chronic inflammatory cells
NS: normoglycemic, D: diabetic with saline injection, DF: diabetic with infliximab injection
II-Diabetic group with saline injection: the microscopic examination revealed discontinuity of the epithelia at the wound area which was denuded from the epithelial bridge necessary for healing and the process of reepithelization appeared to take a vertical direction instead of horizontal direction. The lamina properia was extensively infiltrated with chronic inflammatory cells (241.53± 17.134) (Figure 3) that filled the granulation tissues and the interdigitations between the epithelia and lamina properia were obscure. (Fig 4a). On the edge of the wound, the epithelial layer with high magnification power, showed complete destruction of the epithelium, inflammatory cell infiltration and signs of cell death represented as cell hypertrophy with pyknotic nucleus (Figure 4b). The lamina properia contained very limited number of fibroblasts and collagen fibers (Fig 4c). One of the most prominent features in this group was the presence of thickened wall inflamed blood vessels, when compared with normoglycemic group and their walls were infiltrated with chronic inflammatory cells and the lumen appeared to be constricted (Figure 4d).

Figure 4 (H & E): Microscopical picture of diabetic with saline group 7 days after wound creation. a: discontinuity of epithelia(arrow) and extensive chronic inflammatory cells in the lamina properia (LP). (100X). b: epithelial cells (E) at the edge of the wound. Basal layer cells (arrows) are hypertrophied with pyknotic nuclei. (400X). C: granulation tissues highly cellular filled with chronic inflammatory cells, limited fibroblast and lytic collagen fibers (arrows). (400X). d: thickening of blood vessel’s wall (arrow) infiltrated with chronic inflammatory cells. (400X).
Ill-Diabetic Experimental group injected with infliximab: the microscopical exami-
nation revealed complete reepitheliazaion of the mucosa in a feature close to that of
normoglycemic group but with thinner epithelia. The interdigitations between the
lamina properia and the epithelial layer were arranged well and more prominent
than the non injected diabetic group. The underlying lamina properia demonstrated
area of chronic inflammatory cells but with less cellularity than diabetic non injected
group (192.71±12.51) (Figure 3), and the superficial skeletal muscles demonstrated
histological changes with destruction and spacing (Figure 5a). The high power
magnification showed layers of the epithelia covered with well formed keratin
layer over the epithelial bridge, the basal cells showed mitotic activity and the
interdigitation between the epithelium and underlying lamina properia was uniform
and easily identified. The inflammatory cells concentrated near the basement
membrane more than the deeper area of the granulation tissues with a considerable
amount of newly formed collagen fibers accompanied by active fibroblasts and
lymphocytes with small number of macrophages. Many newly blood
vessels formed in the lamina properia especially near the basement membrane
(neovascularization) (Figure 5a & b). The deep skeletal muscle fibers in both cross
and longitudinal sections retained their normal histological architectures with the
cross striation of the muscle fibers and peripherally located nuclei, the superficial
layer were infiltrated with chronic inflammatory cells mainly lymphocytes and
some of their nuclei were present in the center of muscle fibers (Fig 5c).
The number of inflammatory cells was significantly higher than normoglycemic
group, at the same time it was significantly lower than diabetic group injected with
saline (f= 147.72) (P <0.001). LSD test revealed highly significant difference
(p<0.000) between each two groups, where the mean difference between the
diabetic groups with saline injection and infliximab injection were (-94.993 ±5.539)
and (-46.169 ±5.448 ) respectively. While for normoglycemic group and diabetic
group with saline, the mean differences were (46.169 ±5.448 ) and (-48.824
±5.208 ) respectively.

Figure 5 (H & E): Microscopical picture of diabetic rat tongue injected with infliximab.
a: complete re-epitheliazation with keratin layer (circle), cellular granulation tissue in the
lamina properia (arrows)(100X). b: basal cell (B) have deeply stained large nuclei. The
lamina properia filled with new blood vessels ( arrow) & newly formed collagen fiber(400X).
d: muscle fibers (M) superficially infiltrated with chronic inflammatory cells (arrow) while
deep fibers appeared normal with obvious cross striation. (400X).
PAS stain results
I- Normoglycemic group: In the epithelium, only the prickle cell layer demonstrated PAS +ve reaction. In the lamina properia, positive PAS reaction was demonstrated in the collagen fibers in different locations and the inflammatory cells throughout the newly formed granulation tissue (Figure 6a). The skeletal muscle demonstrated limited sites of PAS +ve reaction within the muscle fibers, only in the basement membrane around each muscle fiber (Figure 6b).

II- Diabetic group: In order to determine the baseline data about the PAS stain in diabetic rats, biopsies were taken from the tongue of diabetic rats without creation of wound. In these rats, the epithelium was covered with a keratinized layer and the reaction to PAS stain was strong in the basement membrane together with the underlying lamina properia where highly PAS +ve reaction was concentrated in the collagen fibers located just beneath the basement membrane (Figure 7a). The muscular tissues showed multiple positive precipitations where the PAS +ve reaction was well demonstrated throughout the muscle fibers in longitudinal section and high concentration of PAS +ve in the basement membrane around muscle fibers with obviously higher intensity when compared with wounded normoglycemic group (Figure 7b).

Figure 5 (PAS): Microscopical picture of normoglycemic group rat tongue 7 days after wound creation. a: PAS +ve reaction in prickle cell, discrete reaction in collagen fiber (arrows) of the underlying lamina properia (LP) (400X). b: muscle fibers with positive reaction in basement membrane (arrows).(400X).

Figure 6 (PAS): Microscopical picture of diabetic rat tongue (without wound) a: lamina properia with collagen fiber reactive to PAS (thick arrow) that is concentrated beneath the epithelia (E), with PAS +ve blood vessels (arrow).(400X). b: muscle fibers heavily precipitated with reactive dark granules & highly PAS +ve basement membrane (arrow) of the muscle cells. (400X).
About the histological features of the diabetic rat tongue 7 days after the wound creation, epithelia was more reactive to PAS stain than in the normoglycemic group but in the same manner of distribution where the prickle cell layer was highly reactive to PAS stain (Figure 8a). The underlying lamina properia which was highly cellular in this group demonstrated highly PAS +ve collagen fibers which were intermingled with large number of blood vessels. The cells of the lining wall of blood vessels (endothelia) revealed more intensive positive reaction to PAS stain and the entire wall of the vessel was thickened and showed positive reaction for the stain in many locations. All blood vessels were surrounded by large number of chronic inflammatory cells and PAS +ve collagen fibers (Figure 8b&c). The skeletal muscle fibers in both cross and longitudinal sections showed many histological variations from that of normal rat tongue. They were heavily deposited with the dark PAS +ve granules accumulated in the muscle tissues extensively; muscle striations became obscured by highly PAS +ve reaction along the thickness of muscle fibers (Figure 8d).

**Figure 8 (PAS)** a: Microscopical picture of the diabetic rat tongue 7 days after wound creation. a: the prickle layer is highly reactive (arrow). (400X). b: the lamina properia is heavily infiltrated with reactive inflammatory cells (arrow) within reactive collagen fiber and many congested PAS+ve blood vessels (stars) (400X) C: blood vessel (star) with PAS +ve thick wall especially the endothelium (400X). d: muscle fibers are heavily precipitated with darkly PAS stained granules (arrows)(400X).
III-Diabetic group injected with infliximab

Using PAS stain, positive prickle cell layer was demonstrated as in the previous normoglycemic and diabetic group, and the epithelia rested on PAS +ve basement membrane. The underlying lamina properia demonstrated discrete weak PAS +ve collagen fibers which were obviously less than diabetic group (both wounded and unwounded) and even with less darkly stained deposits granules (Figure 9a & b). Many thickened wall blood vessels were present in the lamina properia showed PAS +ve reaction distributed throughout their walls. The muscle fibers demonstrated PAS +ve reactive basement membrane. Generally the reactivity to PAS stain was greatly less intense than that in the diabetic group (Figure 9c).

Figure 9 (PAS): Microscopical examination of the diabetic rat tongue injected with infliximab 7 days after wound creation. a: moderate reactive PAS stain in the prickle cell layer and moderate reactivity in the collagen fibers (arrow). (400X) b: lamina properia contain blood vessels with +ve reaction to PAS stain (arrow) and mild deposition of darkly stained granules on the muscle fibers (M). (100X). c: moderate PAS +ve reaction throughout the blood vessel especially in endothelium (arrow) (400X).
Diabetes, a complex metabolic disorder, is becoming a major health concern. Among diabetic complications, impaired wound healing that is often results in infection, and chronic ulceration. This study was conducted to determine the histological events of healing process after excisional wounding of oral mucosa in experimentally induced diabetic rats and to detect the influence of using systemic TNF-alpha antagonist single injection on the healing process. In the present study, the normoglycemic group of the rats 7 days after wound creation showed evidence of complete healing with reepithelization and deposition of newly formed collagen fibers which come in agreement with previous studies that reported healing process of excisional skin wounds in animal model without systemic diseases can be detected within 7-8 days after wound creation.

Inside oral cavity, Suragimath et al and Lee et al reported epithelial proliferation 4 days following excisional palatal and dorsal tongue wound respectively with completely covered epithelium and well formed connective tissue without any residual inflammatory cell in the underlying muscular tissue. Migration and proliferation of keratinocytes and the close relationship between the migratory cells and the collagen fibers of the newly formed connective tissue determine the rate of the reepithelization of the wound. The collagen fibers from normoglycemic specimens appeared more packed and presumably better aligned, which suggests the beginning of macro molecular organization of granulation tissue. The presence of PAS +ve reaction in the epithelial tissue was localized in the prickle layer of the normoglycemic group in agreement with Shinsuke et al who collected specimens from buccal and gingival region of patients undergo oral surgery and found that PAS+ve cells present among the upper prickle layer which were digested by diastase, thus suggesting the presence of glycogen in these cells. Regarding the presence of the glycogen in the muscle fibers, it was found that the muscle fibers were reactive to the PAS stain with high concentration in their basement membrane. This result come in accordance with Candeke et al who analyzed the presence of glycogen in skeletal muscle fibers using of PAS stain computerized image technique in the skeletal muscle of pigs leg and found that the amount of glycogen was related to the location of the muscle, oxygen availability and the muscle activity related to rest or in movement situation. In the diabetic saline group, the wound remain uncovered exposing the underlying structures which was consisted of highly cellular granulation tissue covering destructed underlying skeletal muscle. This result agreed with Haihong et al and Siqueira et al who studied wounds healing in the trunk and scalp of diabetic rats respectively and recorded impaired healing with incomplete contraction and absence of epithelial coverage. Valander et al also reported delayed healing with the damage of the epithelial cells at both end of the wound in the diabetic Yorkshire pig’s skin. The massive infiltration of chronic inflammatory cells into the granulation tissues was an obvious finding in the present study especially lymphocytes and macrophage which appeared as a hallmark in the inflammatory phase where they increased in number more than the normoglycemic group. These findings agreed with Gottrup et al who investigated the presence of chronic inflammatory cells in the granulation tissue in buccal mucosa ulcer of diabetic mice. The amount of collagen fibers in the diabetic group in this study was lowered dramatically and less than that of the control group. This result supported the finding of Park et al who studied the effect of hominis placenta on cutaneous wound healing in normal and diabetic mice. They reported thinner collagen fiber layer in diabetic mice than that of normal mice throughout the entire experiment (14 days) with higher number of macrophages.
The reason behind that could be due to the destruction of the fibroblasts by the action of TNF alpha which cause apoptosis for these cells and the action of IL_1 that influenced by the excess level of TNF alpha which are mitogenic for the fibroblast.34 The high glucose level was greatly associated with decreased migration, proliferation, and increased apoptosis of fibroblasts.13,16,35,36 The blood vessels of the diabetic group had been changed in their histological appearances and expressed with thickening in their wall, constriction of the lumen, and infiltration of the wall with chronic inflammatory cells. These changes may be due to many factors, one of these factors is the action of excessive TNF alpha production, which causes clotting of the blood over the internal wall of the vessel through inactivation of the heparin like material presented in the endothelial cells that enclose the wall of the vessel, atheroma like structure laying down, and destruction of the smooth muscle fibers of the vessel.37 The blood vessel wall of the diabetic rats 7 days after wound creation presented with strong PAS +ve reaction especially in the endothelial layer lining the lumen. There are evidences from other previous studies concluded the same results particularly striking the PAS positive diastase resistant thickening of the vessel walls and the swelling and proliferation of endothelial cells which in many cases produced luminal obliteration in the gingival and periodontal tissues of diabetic patients.38 The intensity of PAS +ve prickle cell layer in the diabetic group was obviously more prominent than both normoglycemic and diabetic group injected with infliximab. This come in accordance with Kuroki et al study, where PAS staining and diastase-digested PAS staining showed a positive correlation between the distribution of cells containing glycogen with the differentiation gradient and the presence of cells containing glycogen in the upper 2/3 area of the prickle cell layer. In this study, when TNF-α was specifically inhibited by the injection of infliximab, healing of the wounds was improved and figured clinically. The underlying connective tissues was covered with newly formed epithelial bridge which come in accordance with the study done by Goren et al.28 who found that in ob/ob mice, the punch wound that created in the back skin was clinically healed after application of anti TNF alpha. The anti-TNF- α treatment of ob/ob mice depleted monocytes from the circulation and caused a marked reduction of macrophage numbers in wound site. More importantly, reduction of circulating and wound monocyctic cells in ob/ob mice resulted in a rapid transformation of chronic wounds into healing tissue. Microscopically, in this study, the collagen fibers orientation and their amount, as well as presence or absence of inflammatory cells and fibroblasts were observed. TNF-α inhibition significantly enhanced fibroblast density, together with a deposition of a remarkable amount of collagen fibers. It was obvious that the effect of diabetes on fibroblast proliferation and apoptosis was reversed by blockade of TNF-α. This result supported the study done in vivo, where nude mice with the implant of TNF-alpha producing cells exhibited a decrease in collagen synthesis and collagen I gene mRNA in the skin and mucosa and displayed impaired healing of skin wounds.26 Lu¨gering et al reported a marked induction of apoptosis in circulating monocytes by infliximab in patients with Crohn’s disease. These findings are strongly supported by the potency of the anti-TNFa antibodies infliximab to selectively mediate caspase-dependent apoptosis in cultured human monocytes, a process that is accompanied by a significaate decrease in cytokine release from the cells.41 Angiogenesis is another indispensable event for granulation tissue formation. TNF-, the chief inflammatory mediator of microangiopathy, elicits a sequence of events that end in apoptosis of vascular endothelial cells.22 Moreover, infliximab is able to inhibit inflammation
induced angiogenesis,\textsuperscript{42} and selectively deplete immature blood vessels.\textsuperscript{43} In regard to the PAS stain, up to our knowledge, there is no previous study concerning the effect of infliximab on glycogen picture but the reduction in the intensity of PAS +ve reaction in the diabetic rats received infliximab in this study may be due to the reduction in the inflammatory reaction caused by infliximab that in turn reduce the amount of carbohydrates present in the tissue. Since the systemic application of an anti-TNFα antibody markedly increased insulin receptor expression in wound tissue.\textsuperscript{44}

**Conclusion**

Anti TNF-alpha (Inflixiamb), single systemic injection improve healing process of oral mucosa in type I uncontrolled diabetic rats. Anti TNF-alpha (infliximab), single systemic injection reduces the inflammatory reaction and reduces the glycogen precipitation in the oral mucosa and the underlying muscle in type I uncontrolled diabetic rats.

**Conflicts of interest**

The authors report no conflicts of interest.

**References**

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http://dx.doi.org/10.15218/zjms.2016.0008


