Effect of Transforming Growth Factor Beta 1 on Wound Healing in Induced Diabetic Rats

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Abstract:

Objective: Delayed wound healing is one of the complications of diabetes mellitus, exhibited by profound inflammation and decreased granulation tissues. The current study was carried out to evaluate wound healing in both normal and diabetic rats. In addition, it evaluated the potential protective effect of transforming growth factor β1 (TGF β1), that has the broadest spectrum of actions, affecting all cell types that are involved in all stages of wound healing to accelerate wound healing in normal & diabetic rats.

Methods: The present study was performed on 40 male albino rats. Each 10 rats were designed as a group. Group I saved as control. They received incisional wound in their tongues 1 cm length and 1/2 cm depth. Group II received 500 ng/kg of TGF β1 5 minutes before wounding. Group III diabetes was induced then rats were treated as second group. At the 14th day post wounding, sections of tongues were taken for hematoxylin and eosin and Masson's trichome staining to examine the histological changes. The intracellular actions of TGF β1 were studied by TEM.

Results: A higher cell proliferation rate and a denser and more organized new extracellular matrix and complete wound closure was detected at the 14th days in the TGF β1 treated wound in comparison with the 14th days for the untreated, control groups. There were delayed wound healing in diabetic rats, decreased re-epithelialization, granulation tissue thickness, matrix density, number of infiltrated cells, and number of capillaries. In TGF β1 treated diabetic rats, showed significant healing improvement was obvious as compared with diabetic rats.

Conclusions: A single intravenous injection of TGF β1 was sufficient to enhance wound healing in rat's tongue. This approach represents a new strategy that may be applied to the treatment of incisional wounds in human diabetic patients.

Key words: Transforming growth factor β1, wound healing, diabetes mellitus, tongue mucosa.

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Introduction:
Wound healing process can be defined as a complex cascade that relies on several mechanisms for tissue repair that optimally leads to restoration of tissue integrity and function including coagulation, inflammation, ground substance and matrix synthesis, angiogenesis, wound contraction and remodeling. (1)

Jorge de la Torre reported that wound healing process is best organized into 3 phases. The inflammatory phase is clinically characterized by cardinal sign of redness, hotness, swelling, pain and loss of function begin immediately upon tissue injury which is initiated and maintained by the coagulation cascade, the arachidonic acid pathway and creation of growth factors and cytokines. The proliferative phase begins approximately 2-3 days after wounding and is signed by arrival of fibroblast which proliferates and syntheses glycosaminoglycan and proteoglycan, the building blocks of the new extracellular matrix of granulation tissue and collagen. During fibroblast proliferation keratinocytes and endothelial cell population are also stimulated to increase their number. The maturation phase is characterized by new collagen production from the first week until the sixth week, remodeling of collagen into more organized structure occur during wound maturation to increase wound tensile strength. (2)

Terranova reported that diabetes mellitus delays wound healing as hyperglycemia leads to decrease oxygenation, perfusion and limits PMN function and produces malnutrition by increasing hormones that cause catabolism. (3)
Unfortunately, this study has found data to support evidence of dysfunction in poly morph nuclear leukocyte, macrophage and fibroblasts with prolonged inflammatory phase. In addition, diabetes decreases biosynthesis of collagen and glycosaminoglycans (GAGs) which result in significant delay in formation of granulation tissue. (4)

In addition number of growth factors essential for wound healing including TGF-beta, PDGF and FGF have been found to be reduced in experimental diabetic wound. (5)

Matsuda et al. investigated several growth factors in the wound space and border, such as platelet-derived growth factor (PDGF), transforming growth factor (TGF), epidermal growth factor (EGF), and fibroblast growth factor (FGF), which have biological activities to stimulate infiltration of inflammatory cells into the wound space. (6) These growth factors induced proliferation of keratinocytes and fibroblasts, led to new formation of capillaries in the granulation tissue and modulated extra-cellular matrix deposition and reconstitution of the injured area. Also they claimed that topical application of growth factors was successful to accelerate healing of full thickness wound in normal mice and normalize a delayed healing response of diabetic rats.

Miyzono and Heldin reported that TGF-beta is a family of multifunctional 25KDa protein (TGF-beta 1, 2, 3) which stimulates collagen and fibronectin formation in variety of fibroblast cell lines. (7)

Also, TGF-beta is known to regulate the differentiation of cells, induce chemotaxis of inflammatory cells and induce the accumulation of extra cellular matrix protein in vivo.TGF-beta stimulates the repair of soft as well as hard tissue and it acts as a potent immunosuppressant. (8)

Marston proved that TGF-beta initiates and terminates tissue repair. As TGF-beta enhances the deposition of extracellular matrix component "EMC" which continually degraded by proteases. (9) In addition, TGF-beta acts simultaneously as cellular stimulator to increase synthesis of most matrix protein by several folds, a cellular suppressor to decrease the production of inhibitors of certain collagen protease and modulator of integrin expression in a manner that increases cellular adhesion to the matrix. These effect on EMC reflects the diverse biologic properties of TGF-beta and may also be part of a negative feedback loop that normally regulates its own expression.

Spom et al. stated that TGF-beta 1 is a human DNA-derived polypeptide growth factor that induces normal soft tissue repair mechanism and reverses deficient repair rates. This growth factor is released by platelets, monocytes/macrophages, endothelial cells and fibroblasts, cells that are essential to the repair process. (10)

Roberts et al. found that TGF-beta 1 played a central role in wound healing. It influenced the inflammatory response, angiogenesis, granulation tissue formation, re-epithelization, extra-cellular matrix deposition and remodeling
so promoting healing and contributing to scar formation. \(^{(11)}\)

As few researches concerned with the effect of TGF on wound healing so this current study will concern on the effect of TGF beta 1 on tongue wound healing in normal and diabetic rats.

The aim of the present study is to examine the effect of transforming growth factor-bet1 on tongue wound healing in normal and alloxan-induced diabetic rats using light and transmission electron microscopes.

**Materials and Methods:**

**Animals**

Forty adult male rats will be used in this study with average body weight 225:250 grams. All rats will be fed standard laboratory diet and tap water for at least one week prior to experiment.

Then rats will be divided into 4 equal groups:

**Group I (control group)**

It consists of 10 rats that will be injected intravenously (IV) by 0.05 mol/L citrate buffer then oral mucosal wound will be made on the dorsal surface of the tongue lateral to midline 1 cm length and 1/2 cm depth then will take 1 to 2 sutures over the wound. \(^{(12)}\)

**Group II**

It consists of 10 rats that will receive the same treatment as the previous group. In addition they will receive single intravenous injection of TGF-beta1 500 ng/kg 5 minutes before wounding. \(^{(13)}\)

**Group III**

It consists of 10 rats, they will be injected with alloxan monohydrate to induce diabetes mellitus then a wound will be made in the dorsal surface of the tongue.

**Group IV**

It consists of 10 rats, they were subjected to diabetes mellitus, then rats were received single intravenous injection of TGF-\(\beta\)1 500 ng/kg 5 minutes before wounding. Then a wound was made in the tongue.

**Tissue preparation**

All rats will be then euthanized at 14 day post wounding and their tongues will be dissected and prepared for:

**Light microscopic examination**

Anterior half of the tongue including the wound area specimens will be fixed in 10% formol calcium for 48 hours routinely prepared and embedded in paraffin sections of 4-6 micron thickness will be cut and stained with hematoxylin and eosin (H&E) and Masson’s trichrome stain (MTC).

**Electron microscopic examination**

The other half of the wound will be trimmed into cubic 1 mm and fixed in mixture of 4% paraformaldehyde and 1%glutaraldehyde for 2 hours at 4C. Then, they will be rinsed and post fixed in 1% osmium tetroxide, following fixation, samples will be dehydrated in a graded series of ethanol, and embedded in epon. Ultrathin sections will be cut, stained with uranyl acetate and lead citrate and examined with Jeol Transmission Electron Microscope (100CX), in Faculty of Science Alexandria University.

**Light Microscopic Results**

1- Hematoxylin and eosin

**Control group (Group I)**

After wounding LM examination showed obvious improvement in epithelial thickness & structure. The CT showed decreased number of fibroblasts and endothelial cells simultaneously the amount of collagen increased (Fig. 1).

**Group II (TGF-\(\beta\)1 group)**

Fig. 1: Light micrograph of group I showing obvious improvement ep. Thickness & structure(E). The CT showing decreased number of fibroblasts and endothelial cells and increased the amount of organized collagen fibers (arrow). (H&E. X100)
LM examination showed the epithelial regeneration and remodeling of the connective tissue were almost completed. Normal CT papillae and slight invagination than the surface (Fig. 2).

Fig. 2: Light micrograph of group II showing almost normal ep. Thickness & structure (E) with almost normal CT papillae (arrow) and slight invagination than the surface. (H&E, X100)

Deeply stained epithelial basal cell layer and the CT showed increased endothelial cell proliferation and congested blood vessels with RBCs (Fig. 3).

Fig. 3: Higher magnification of the previous figure showing deeply stained ep. Basal cell layer (arrow). The CT showed increased endothelial cell proliferation, congested blood vessels with RBCs (arrow head). (H&E X400)

**Group III (diabetic group)**

All rats received 150mg/kg alloxan monohydrate. Diabetes was confirmed 3 days later. Rats wounded in the middle of their tongues and showed healing as follow:-

LM examination showed slight improvement in epithelial & CT structures with ill-defined filiform papillae and thin keratin layer. The CT showed few collagen fibers. There was obvious separation between muscles from underlying CT (Fig. 4). Epithelial basal cell layers were less stained and CT thickness was decreased (Fig. 5).

Fig. 4: Light micrograph of group III showing slight improvement in ep. (E) & CT structures with ill-defined filiform papillae (F) and thin keratin layer. There is obvious separation between muscles from underlying CT (arrow). (H&E X100)

Fig. 5: Higher magnification of the previous figure showing ep. Basal cell layer less stained (arrow) and decreased keratin layer with decreased thickness of CT (arrow head). (H&E)

**Group IV (diabetic + TGF-β1 group)**

LM examination showed almost completed epithelial regeneration with normal keratinization and spreading over the wound surface (Fig. 6). The granulation tissue apparently well-organized that rich in fibroblasts. There were moderate collagen fibrils, increased collagen bundles between muscles and increased endothelial cell proliferation (Fig. 7).
II- Masson’s Trichrome

**Group I (Control group)**
After wounding MTC showed moderate staining reaction of collagen fibers with organized proliferating fibroblasts and endothelial cells. Also there were numerous capillaries & few inflammatory cells (Fig. 8).

**Group II (TGF-β1 group)**
After wounding MTC showed intense staining reaction of collagen fibers with increased CT thickness, and more organized granulation tissue (Fig. 9).

**Group III (diabetic group)**
After wounding MTC showed mild staining reaction of collagen fibers, decreased CT thickness, and separation of muscles from underlying CT (Fig. 10).
**Group IV (diabetic + TGF-β1 group)**

After wounding MTC showed moderate to intense staining reaction of collagen fibers (Fig. 11).

![Fig. 11: Light micrograph of group IV showing moderate to intense reaction of collagen fibers and apparently organized CT (G). (Masson’s Trichrome X100)](image)

**Electron Microscopic Result**

**Group I (Control)**

After wounding the inflammatory cells decreased. Fibroblasts were large and contained dilated RER (Fig. 12). Increased segments of collagen fibers, which in some places were arranged in small-unorganized bundles (Fig. 13).

![Fig. 12: TEM micrograph of group I showing fibroblast with dilated RER cisternae (X27000)](image)

**Group II (TGF-β1 group)**

After wounding most of the fibroblasts showed a mature appearance with almost normal RER. Many of the collagen fibers were concentrated close to the fibroblastic surface; fibers often located parallel to the main axis of the cell, (Fig. 14). Irregular fibroblast with slightly dilated RER, surrounded by numerous L.S & T.S bundles of collagen fibers (Fig. 15).

![Fig. 13: TEM micrograph of group I showing fibroblast with dilated RER (*) surrounded by collagen fibers (arrow) (X14000)](image)

![Fig. 14: TEM micrograph of group II showing fibroblasts surrounding normal bundles of the collagen fibers (arrow). (X10000)](image)
Group III (diabetic group)
After wounding, TEM showed abnormal granulation tissue with increased numbers of lymphatics & blood vessels (Fig. 16). Fibroblasts were inactive with notched nucleus, dilated RER and moderate collagen fibrils (Fig. 17).

After wounding wounds were almost similar to control group. Fibroblasts had abundant RER and were surrounded by an expressive amount of thin and well-organized collagen fibrils (Fig. 18 & 19). Some superficial epithelial cells showed double nuclei (cell division). (Fig. 20).
Discussion:

As wound repair is a dynamic, complex process that, in short, marshals the disparate elements of inflammatory cells and especially macrophages, platelets, and coagulation factors, fibroblasts, endothelial cells, and keratinocytes. (14) So, identification of methods to enhance wound healing is a goal of significant potential benefit. Wounds in high-risk settings as diabetes mellitus would benefit from enhancing early wound healing. Other, more typical wounds would also benefit from accelerating the wound response in terms of minimizing patient discomfort and disability.

This study was carried out on rat’s mucosa because healing of oral mucosal is clinically distinguished of both its rapidity and lack of scar formation. (15) As well as many previous studies had suggested that saliva containing abundant amounts of cytokines, growth factors, and protease inhibitors is the primary factor that accounts for rapid oral wound healing. (16)

Thus, we have chosen the tongue as a good site of intra-oral injury, since it gives the best reproducibility and adequate space for an incisional wound. Additionally, the dorsal surface of the tongue is covered with keratinized epithelium, similar to skin so we can compare the results with dermal researches. (17)

One to two sutures were used. This provided a high reproducibility of healing conditions than that can be achieved by using a standardized open healing model. Moreover, higher risk of infection by using an open wound healing model removed by using suturing techniques. (18)

Diabetes mellitus was selected because this disease decreases the patient's general metabolism, (19) and changes the cellular and humoral immunity, increasing the risk of infections especially when the patient is submitted to surgical procedures. (20) Moreover, the delay in cell proliferation, the decrease of collagen metabolism and all other granulation tissue components, such as glycoproteins and mucopolysaccharides, are direct consequences of the disease that severely affects the tissue repair process. (21)

In addition, decreased healing capacity in diabetes is the result of multiple factors, including elevated blood glucose levels, suppressed cell-mediated immunity, local ischemia, and free radical generation. Inadequate oxygenation, such as that seen in local ischemia, causes production of extremely reactive metabolites (called free oxygen radicals) that impair normal wound healing by damaging keratinocyte endothelial cells, capillary permeability, and collagen metabolism. (22)

In the current study alloxan-induced diabetes is a well-documented model of experimental diabetes. This compound causes severe necrosis of pancreatic β-cells. This effect was previously explained on the basis of alloxan’s ability to produce hydrogen peroxide and other free radicals, including O2 and OH that damage β-cells hence leading to their death. β cells dysfunction eventually culminates in reduction of insulin release leading to hyperglycemia. (23)

The use of TGF β1 as an improved therapy for poorly vascularized or non-healing wounds in the lower equine limb and in immunocompromised, diabetic, or elderly individuals could provide good healing of acute wounds leading to limb salvage that would be of significant personal, economic, and social advantage. (24)

In the current study, we injected a single systemic dose of TGF β1 to adult rats in which healing was impaired with diabetes to increase the rate of healing of incisional wound to a level similar to that of normal rats. (25)

It is possible that TGF β1 becomes available to the extravascular environment and thus may be capable of priming cells for increased responsiveness to normal regulatory factors related at sites of injury. (26)

In the current work, histological study using hematoxylin and eosin stain, of all groups revealed variable wound healing consisting of new stroma, often called granulation tissue, begins to invade the wound space approximately four days after injury. Numerous new capillaries endow the new stroma with its granular appearance. Macrophages, fibroblasts, and blood vessels move into the wound space at the same time. Epithelium at the edge of the wound was several cell layers thick. These finding were in agreement with that found by Skalli and Gabbiani. (27)

The rate of healing at the 14th day after wounding in control rats (group I) showed obvious improvement in epithelial thickness &
At the 14th day after wounding of the TGF-β1 treated rats (group II) showed almost completed epithelial regeneration and remodeling of the connective tissue. Connective tissue was characterized by abundant collagen bundles proliferation. These were coincided with results of Desmoulière and Gabbiani. In addition TGF-β1 depicted increased endothelial cells, congested blood vessels with RBCs, extensive collagen fibers surrounding fibroblastic cells. Leucocytic infiltration was mild. These results were in agreement with the results of Beck et al. and Dallas et al. (30, 31).

On the other hand wound healing of diabetic rats (group III) at the 14th day there were slight improvement in epithelial & connective tissue structures with ill-defined filiform papillae and thin keratin layer. The connective tissue showed few collagen fibers. There was obvious separation between muscles from underlying connective tissue this result were in agreement with that of Kucine et al. (1997). (32)

These previous observation means delayed diabetic wound healing that account for some of the reduction in granulation tissue. The reduced collagen content of the granulation tissue may also be a manifestation of this delay. Conceivably, the delayed healing in the untreated diabetic rats may be due to less collagen in the wound tissue and possibly due to fewer myofibroblasts, which play a crucial role in wound contraction. (34)

The wound of group IV (TGF-β1+ diabetic group) at the 14th day wounds showed almost completed epithelial regeneration with normal keratinization and spreading over the wound surface as compared with group III. The granulation tissue apparently well-organized and rich in fibroblasts generally oriented parallel to the epithelium layer, moderate collagen fibrils, collagen bundles between muscles and increased endothelial cell proliferation these results was confirmed by Benn et al. (35).

Moreover, Jude et al. found that TGF-β1-deficient mice display delayed wound healing, and decreased TGF-β1 expression and lack of TGF-β1 up-regulation have been documented as major defects in diabetes mellitus. Furthermore, in alloxan induced diabetic rats, the transfer of TGF-β1 DNA to wounds resulted in a marked improvement in wound healing, suggesting that TGF-β1 may be an effective clinical therapy for diabetic-impaired wound healing. In addition to TGF-β1, the migration and proliferation of fibroblasts are thought to be key features of successful healing, as these fibroblasts are responsible for the synthesis of new extracellular matrix proteins, primarily types I and III collagen, which are the initial components of granulation tissue that leads to the formation of scar tissue. (37)

In most histological studies, collagen, when viewed in sections stained with hematoxylin and eosin, could not be identified. So with the application of Masson's trichrome stain, the concentration and the blue stain of collagen fibers became more identifiable.

In the current study, the wound healing of control rats at 14th day revealed intense staining reaction of collagen fibers, with increased connective tissue thickness, and more organized granulation tissue. Proliferating fibroblasts, endothelial cells, few inflammatory cells and abundant mature collagen bundles were obvious. These results were confirmed by Moyer et al. (38).

This improvement in wound healing in TGF-β1 groups could be explained by that TGF-β1 has three prolonged effects on extracellular matrix deposition. At the same time TGF-β1 decreases the secretion of proteases responsible for the breakdown of the matrix and it also stimulates the protease inhibitor, tissue inhibitor of metaloprotease. In skin incisions in rabbits, TGF-β1 triggered synthesis and rapid maturation of collagen in early wounds. (42)

Wound healing of diabetic group (group III) MTC 14th day revealed decreased connective tissue thickness, and separation of muscles from underlying connective tissue. Disorganized granulation tissue mass with decreased number of fibroblasts, inflammatory cells and short irregular collagen fibers with mild staining reaction, these results were in agreement with results of Grotendorst et al. (43).

Compared to diabetic group (diabetic+ TGF-β1 or group IV) MTC 14th day revealed collagen deposition in wounds increased to the level of control, non diabetic animals. This coincided with Grotendorst et al. (44) MTC showed moderate to intense staining reaction of
collagen fibers, apparently organized granulation tissue, proliferated fibroblasts and formed capillaries. These results had similarities with that found by Greenhalgh.\(^{(45)}\)

This could be explained by that during the proliferative phase of healing TGF-β1 has been implicated by its demonstrated ability to stimulate angiogenesis and collagen deposition in normal and compromised tissue.\(^{(46)}\)

**Ultrastructure examination** of the current study of control group (group I) at 14\(^{th}\) day the inflammatory response was decreased. Fibroblasts were large and containing dilated RER. Short segments of collagen fibrils were arranged in small-unorganized bundles. Epithelial cells showed some divisions, increments of tonofilaments and prominent mitochondria. These results were confirmed by Ross and Benditt.\(^{(47)}\)

Healing process of group II at the 14\(^{th}\) day showed mature fibroblastic appearance with almost normal RER. Many of the thin collagen fibers were concentrated close to the fibroblastic surface, fibers often located parallel to the main axis of the cell. These results were coincided with Darby et al.\(^{(48)}\) In addition, there were increased blood vessels containing RBCs, and surrounded by well-developed endothelial cells. Epithelial cells regeneration had been indicated by parabasal cell divisions these results confirmed by Rappolee et al. and Cordeiro et al.\(^{(49, 50)}\)

Another observation was found at the 14\(^{th}\) day of group II was irregular fibroblasts with slightly dilated RER that may suggest their involvement in an active synthetic activity surrounded by numerous L.S & T.S bundles of collagen fibers. This observation was in agreement with Grinnell, Clark and Badid et al.\(^{(51, 52, 53)}\) They found that fibroblasts displayed a considerable degree of inter- and intra-site heterogeneity in phenotype and activity. Also fibroblasts showed differences in migration, integrin expression, cell proliferation, and collagen synthesis obtained from different phases of wound healing.\(^{(53, 54)}\)

Healing of group IV at the 14\(^{th}\) day revealed fibroblasts had abundant RER and was surrounded by an excessive amount of thin and well-organized collagen fibrils. Some superficial epithelial cells showed double nuclei (cell division). These results were in agreement with Pierce et al.\(^{(42)}\) Abnormal shaped fibroblasts surrounded by collagen fibers were detected, these cells assumed to be myofibroblasts characterized by the abundance of cytoplasmic microfilaments and indented nucleus These cells also showed dilated RER suggesting their involvement in an active synthetic activity these observations were coincided with Ramadori et al. and Schiifer et al.\(^{(55, 56)}\) This revealed that the wound was completely re-epithelized. The predominant cell type was large fibroblasts that were rich in rough endoplasmic reticulum (RER) and usually surrounded by an organized ECM containing collagen fibrils.\(^{(57)}\)

Diabetes mellitus impair healing primarily as it affects fibroblasts. TGF-β1 has been shown to recruit blood-borne cells stimulatory to fibroblasts, as well as fibroblasts themselves, in addition to its direct stimulatory effect on fibroblasts. Wounded rats receiving systemic dose of 500 ng/Kg of TGF-β1 showed improvement in healing by increased collagen deposition.\(^{(58, 59)}\)

Although TGF-β1 has repeatedly been shown to enhance healing in unimpaired wound-healing models in vivo, other reports of enhanced healing in impaired models have been reported. Glucocorticoid-induced wound-healing impairment in rats and pigs was reversed by administration of TGF-β1. Rats treated with doxorubicin showed decreased collagen and collagen gene expression in wounds, both were increased by the addition of TGF-β1.\(^{(60)}\) Few of these studies investigating the effect of TGF-β1 on diabetic impaired wound-healing models used ultrastructure study to evaluate TGF-β1 effect. Linear incisions closely mimic surgical incisions clinically and TEM may better reflect wound dehiscence potential than other models of wound healing.

The observations reported here indicated that single systemic administration of TGF β1 can profoundly alter cellular function that influences the wound healing cascade. TGF β1 reversed the healing impairment associated with diabetes mellitus, a finding that may suggest that healing impairment associated with diabetes mellitus share common cellular events that is respond to growth factor manipulation.
References:
46. Hosgood: Wound Healing: The Role of Platelet Derived Growth Factor and


