Histopathological Evaluation of Low Level Laser Therapy (LLLT) on Oral Mucosal Wound Healing in Rabbits Under Corticosteroid Therapy

AZZA H.M. HASSAN, M.D. 1, *, ALI M. SAAFAN, M.D. 2, SHERIF SAFWAT, M.D. 3 and TAREK M. IBRAHIM, M.D. 4

The Departments of Pathology 1, Faculty of Veterinary Medicine, Cairo University; Dental Laser Applications 2, 4, Department of Medical Applications of Laser, National Institute for Laser Enhancement Sciences, Cairo University and Otorhinolaryngology 3, Department of Medical Applications of Laser, National Institute for Laser Enhancement Sciences, Cairo University

Abstract

Objective: This study assessed the effect of diode laser 808nm low-level laser therapy (LLLT) on oral mucous wound healing for immunosuppressed rabbits under corticosteroid therapy.

Material and Methods: Twenty-four adult male New Zealand White rabbits were subjected to Immunosupression via dexamethasone injection, the animals were subjected to equal surgical incisions in oral mucous membrane in both sides. The left side was irradiated with Diode laser 808nm with average power 100mw for three minutes per session (18 Joule), animals were subjected to three laser sessions per week. Soft tissue specimens were collected after 0, 4, 7, 10, and 14 days and stained with Hematoxylin-Eosin, as well as Masson’s Trichrome.

Results: Fibroblast proliferation was significantly more evident in the laser irradiated groups, also there was a marked and significant increase in mitotic activity with hyperplasia of the prickle cell layer as well as formation of fibro vascular tissue in the underlying tissue in the same groups.

Conclusion: Under the circumstances of this experiment we can conclude that diode laser 808nm LLLT can accelerate wound healing in immunosuppressed experimental rabbits.

Key Words: Oral mucosa – Wound healing – Low level laser therapy – Scarless healing – Immunosupressed rabbit.

Introduction

WOUND healing is a highly ordered and well-coordinated process; involving proliferation of inflammatory cells to the wound, epithelial closure, cell differentiation, matrix deposition and remodeling; regulated by a wide variety of growth factors and cytokines [1].

Systemic corticosteroids which are frequently used as anti-inflammatory agents are well-known to inhibit wound repair via global anti-inflammatory effects and suppression of cellular wound responses, including fibroblast proliferation and collagen synthesis. Systemic steroids cause wounds to heal with incomplete granulation tissue and reduced wound contraction [2].

Corticosteroids also inhibit production of hypoxia-inducible factor-1 (HIF-1), a key transcriptional factor in healing wounds [3]. Beyond effects on repair itself, systemic corticosteroids may increase the risk of wound infection. While systemic corticosteroids inhibit wound repair, topical application produces quite different effects. Topical low-dosage corticosteroid treatment of chronic wounds has been found to accelerate wound healing, reduce pain and exudates, and suppress hyper granulation tissue formation in 79% of cases. While these positive effects are striking, careful monitoring is necessary to avoid a potential increased risk of infection with prolonged use [4].

Laser technology has continuously developed during the last 25 years partly driven by the medical demand or adapted from technical applications and transferred to the medical use. Lasers can be divided into two groups, the so-called hard lasers, which are used for surgical application as cutting, ablation, and vaporization of hard and soft tissues, they also seal blood vessels and lymphatic as well as stabilize the surgical site (e.g. CO₂, Nd: YAG, Er: YAG, Ho: YAG, excimer, and argon lasers) [5,6]. Moreover, the so-called soft lasers, which are used for biostimulation, analgesic effects and promoting wound healing (e.g. He-Ne and diode lasers) [7-9].
Low-level laser therapy (LLLT; also known as biostimulation and photo-biostimulation) is a form of phototherapy that involves the application of low-power monochromatic and coherent light to injuries and lesions in order to stimulate wound healing. LLLT has been shown to increase the speed, quality and tensile strength of tissue repair, resolve inflammation and provide pain relief (supporting literature is reviewed later in the article). Lasers are already used in a variety of medical and surgical fields, including dentistry, chiropractice, osteopathy, physiotherapy, cosmetic, pain attenuation [10-12].

While, evaluation healing process has been conducted in many researches, in our study, we evaluate healing process after LLLT (as a promoting tissue repair tool) in Immunosuppressed rabbits.

Material and Methods

A- Induction of immunosupression:

In this work, Immunosupression was induced by injection of all animals with 2mg/kg dexamethasone phosphate (DXP) three times at 6-h intervals [13].

B- Surgical procedure:

One day after administration of the last dose of dexamethasone, the surgical procedure was carried out on the immunosuppressed rabbits. After complete anaesthetization of all animals by intramuscular injection of a mixture of Xylazine (10mg/kg b.wt.) and Ketamine (87mg/kg b.wt.).

Under aseptic conditions, using a sterile Bard Parker blade no., 11 a biopsy about 4 x 4mm was excised from the upper anterior labial vestibule of both sides of oral cavity.

C- Laser treatment:

The left side of the oral mucosa were treated with a diode laser device emitting 808nm radiation with maximum output power 100mw (Luis Grees, Australia, Laser Medics) was used in the experiment while the right side didn’t receive irradiation and left as control.

The first application of the laser treatment was carried out immediately after the surgical procedure; the energy was applied over the entire area of the wound in both the horizontal direction and again in the vertical direction, to ensure complete exposure of the wounded area.

Laser beam was continuously delivered from the tip of the laser applicator and exposing the target surface for 3 minutes (average power 18 Joule) while the tip was touching the tissues. Animals were laser treated for three sessions per week.

D- Morphological studies:

On day's 0, 4, 7, 10, and 14 following surgery and laser treatment, the wounds were grossly inspected in order to register any possible disturbance on wound healing and the animals were sacrificed.

- Pathological examination:

Samples from oral mucosal wounds, at 0, 4, 7, 10, and 14 days, were fixed in 10% neutral buffered formalin. The samples were trimmed, washed and dehydrated in ascending grades of alcohol, cleaned in xylene and embedded in paraffin. Five mm-thick sections were routinely stained with Hematoxylin-Eosin, as well as Masson’s Trichrome stain for collagen [14].

Results

At zero day:

The wound of the left side of the oral mucosa (test side) received laser, meanwhile that of the right side didn’t receive irradiation (control side).

During the post-surgery period, the animals remained healthy, with excessive clotted blood on the operated site, without clinical evidence of infection.

Both the test and control groups showed the same histopathological alterations during this period (at zero day post wounding), which characterized by formation of fibrin clot that fill the wound gap with exposure of sub mucosal connective tissue (Fig. 1a). Acute inflammatory reaction, with massive infiltration of the fibrin clot with large number of polymorphonuclear cells and macrophages, was clearly demonstrated in the test side (Fig. 1b).

At 4 days post wounding:

- Control side:

In the group of animals sacrificed in 4 days, presence of clotted blood with hyperemic zone
was observed. Histologically this group, showed inflammatory phase of wound healing that is characterized by severe tissue reaction with massive infiltration of the thrombotic mass by polymorph-nuclear cells, macrophages, and fibroblasts mixed with fibrin strands (Fig. 1c). No mitotic activity was observed in this group.

- **Test side:**

The wound of the irradiated site showed yellowish granulation tissue filling the wound gap. Histologically, This group showed better regenerative activity compared with the control one. The inflammatory phase was declined and the proliferative phase was remarkably demonstrated during this period. Results of this study showed that fibroblast proliferation was significantly more evident in the laser radiated group on day 4 post wounding. Fibroplasia with presence of active plumed spindle-shaped fibroblasts infiltrating the wound site (Fig. 1d) was observed in all examined animals. Angiogenesis occurred concurrently with fibroblast proliferation with formation of newly formed blood capillaries resulting in granulation tissue formation (Fig. 1e). Tissue sections stained with Masson’s trichrome showed newly formed blood capillaries with prominent collagen fiber characteristic of granulation tissue (Fig. 1f). All examined cases showed increased mitotic activity in the prickle cell layer together with formation of fibro vascular tissue in the underlying tissue (Fig. 1 g). Decreased number of inflammatory cells with disappearance of polymorph nuclear leukocytes from the wound site was also characteristic in this group.

**At 7 days post wounding:**

- **Control group:**

The wound showed large granulation tissue formation surrounded by hyperemic border. The histopathological alterations recorded in this group were variable as in some cases, the inflammatory phase was still demonstrated and characterized by massive infiltration of the wound area by polymorph nuclear leukocytes, macrophages with exudation of edematous fluid (Fig. 1h), meanwhile other examined cases showed young granulation tissue formation which is consisted of newly formed blood capillaries, fibroblast, inflammatory cells, endothelial cells and myofibroblast (Fig. 2a). Increased mitotic activity in the prickle cell layer was clearly demonstrated in this group during this period.

- **Test group:**

This group showed marked narrowing of the wound opening. The histopathological alterations of this group were more or less similar to those demonstrated at 4 days postwounding. Increased mitotic activity in prickle cell layer was a constant feature in all examined cases. Hyperchromasia of the basal cell layer (Fig. 2b) and old granulation tissue with deposition of collagen bundles (Fig. 2c) were obvious. Re-epithelization with non keratinizing stratified squamous epithelial cells was recorded in all examined cases.

**At 10 days post wounding:**

- **Control side:**

The histopathological alterations revealed formation of fibro vascular tissue (granulation tissue) which consisted of well formed blood capillaries mixed with fibrous tissue. Increased mitotic activity in the basal and prickle cell layer with great narrowing of the wound opening and re–epithelization of the epidermal cell layer with clear intact basement membrane. In other examined sections, old granulation tissue with deposition of collagen bundles (Fig. 2d) was observed.

- **Test side:**

This group showed marked and significant increase in mitotic activity with hyperplasia of the prickle cell layer as well as formation of fibro vascular tissue in the underlying tissue (Fig. 2e). The cells of prickle cell layer showed large karyomegallic vesicular nuclei. Maturation of granulation tissue with deposition of collagen bundles (Fig. 2f) was recorded in all examined cases.

**At 14 days postwounding:**

- **Control side:**

This group showed increased mitotic activity in all cell layers of the epidermis. This group showed complete re-epithelization and remodeling of the wound. Hyperplasia of the prickle cell layer with mitotic activity of the basal cell layer was noticed. The sub mucosa showed dense fibrous tissue consisted of dense collagen fibers that appeared blue in Masson’s Trichrom stained sections (Fig. 2g).

- **Test side:**

This group showed complete re-epithelization and remodeling of the wound. Hyperplasia of the prickle cell layer with mitotic activity of the basal cell layer (Fig. 3a,b). The hyperplastic cells showed large, vesicular and karyomegallic nuclei (Fig. 3c). Increased mitotic activity in the basal and prickle epithelial cells (Fig. 3d) were a constant feature in all examined cases during this period. Sheets of hyperplastic epithelial cells filling the wound gap (Fig. 3e) and remodeling of collagen bundles (Fig. 3f) were demonstrated.
378 Histopathological Evaluation of Low Level Laser Therapy (LLLT)

Fig. (1-A): Oral mucosa at zero day post wounding showing formation of fibrin clot that fill the wound gap with exposure of sub mucosal connective tissue (H&E x200).

Fig. (1-B): Oral mucosa at zero day post wounding showing acute inflammatory reaction, with massive infiltration of the fibrin clot with large number of polymorphnuclear cells and macrophages (H&E x400).

Fig. (1-C): Oral mucosa of the Immunosuppressed group at 4 days post wounding showing severe tissue reaction with massive infiltration of the thrombotic mass by polymorphnuclear cells, macrophages, and fibroblasts mixed with fibrin strands (H&E x200).

Fig. (1-D): Oral mucosa of the test (irradiated) group at 4 days post wounding showing fibroplasia with presence of active plumed spindle-shaped fibroblasts infiltrating the wound site (H&E x400).

Fig. (1-E): Oral mucosa of the test (irradiated) group at 4 days post wounding showing granulation tissue formation which is formed of newly formed blood capillaries, fibroblast, and inflammatory cells, mostly macrophages (H&E x400).

Fig. (1-F): Oral mucosa of the test (irradiated) group at 4 days post wounding showing newly formed blood capillaries with prominent collagen fiber characteristic of granulation tissue (Masson’s trichrome x400).

Fig. (1-G): Oral mucosa of the test (irradiated) group at 4 days post wounding showing increased mitotic activity of the prickle cell layer together with formation of fibro vascular tissue in the underlying tissue (H&E x400).

Fig. (1-H): Oral mucosa of the Immunosuppressed group at 7 days post wounding showing massive infiltration of the wound area by polymorph nuclear leukocytes, macrophages with exudation of edematous fluid (H&E x400).
Fig. (2-A): Oral mucosa of the Immunosuppressed group at 7 days post wounding showing young granulation tissue formation which is consisted of newly formed blood capillaries, fibroblast, inflammatory cells, endothelial cells and myofibroblast (H&E x400).

Fig. (2-B): Oral mucosa of the Immunosuppressed group at 7 days post wounding showing Hyperchromasia of the basal cell layer (H&E x400).

Fig. (2-C): Oral mucosa of the Immunosuppressed test group at 7 days post wounding showing old granulation tissue with deposition of collagen bundles (H&E x400).

Fig. (2-D): Oral mucosa of the Immunosuppressed test group at 7 days post wounding showing old granulation tissue with deposition of collagen bundles (H&E x400).

Fig. (2-E): Oral mucosa of the test group at 10 days post wounding showing hyperplasia of the prickle cell layer as well as formation of fibro vascular tissue in the underlying tissue (H&E x400).

Fig. (2-F): Oral mucosa of the test group at 10 days post wounding showing re-epithelization and maturation of granulation tissue with deposition of collagen bundles (H&E x400).

Fig. (2-G): The sub mucosa showed dense fibrous tissue consisted of dense collagen fibers that appeared blue in Masson’s Trichrom stained sections.
Histopathological Evaluation of Low Level Laser Therapy (LLLT)

Fig. (3-A): Oral mucosa of the test group at 14 days post wounding showing Hyperplasia of the prickle cell layer with mitotic activity of the basal cell layer (H&E x200).

Fig. (3-B): Oral mucosa of the test group at 14 days post wounding showing the mitotic activity of the basal cell layer (H&E x400).

Fig. (3-C): Oral mucosa of the test group at 14 days post wounding showing The hyperplastic cells showed large, vesicular and karyomegallic nuclei (H&E x400).

Fig. (3-D): Oral mucosa of the test group at 14 days post wounding showing Increased mitotic activity in the basal and prickle epithelial cells (H&E x400).

Fig. (3-E): Oral mucosa of the test group at 14 days post wounding showing Sheet of hyperplastic epithelial cells filling the wound gap (H&E x400).

Fig. (3-F): Oral mucosa of the test group at 14 days post wounding showing remodling of collagen bundles (H&E x400).

Discussion

Intra-oral soft tissue healing has become a significant aspect of dentistry and clinicians are faced with achieving rapid healing as well as addressing biologic and functional problems [15].

Wound healing has three phases, where a substrate is laid down, then cells proliferate, and then there is remodeling of tissue. Evidence from literature suggests that laser biostimulation produces its primary effect during the cell proliferation phase of the wound healing process [16]. At cellular level,
photo-irradiation at low power causes significant biological effects such as cellular proliferation, collagen synthesis, the release of growth factors from cells [17] and macrophage and lymphocyte stimulation [18].

The goal of this study was to assess the clinical value of the use of low-level laser therapy in the oral cavity as regards rapidity and completeness of wound healing follows surgical incisions in the oral mucosa.

In the present study, the histopathological alterations observed in the test and control sides at zero day post wounding, were similar. Both groups showed formation of fibrin clot that fill the wound gap as well as acute inflammatory reaction with massive infiltration of the fibrin clot with large number of polymorph nuclear cells.

Following injury, vasoconstriction reduces hemorrhage and favors platelet aggregation. Almost concurrently, vasodilatation enables inflammatory cells to enter the site of injury and clean the wound. Polymorph nuclear cells and macrophages migrate into the wound to prevent the invasion and proliferation of micro-organisms [19].

At 4 days post wounding, the control side showed severe tissue reaction with massive infiltration of the thrombotic mass by polymorph nuclear cells, macrophages, and fibroblasts mixed with fibrin strands. No mitotic activity was observed in this group.

Meanwhile, the test (irradiated) side showed better regenerative activity compared to the control one. The inflammatory phase was declined and the proliferative phase was remarkably demonstrated during this period. Fibroplasia, angiogenesis and granulation tissue formation was significantly demonstrated, as well as, increased mitotic activity of the prickle cell layer with formation of fibro vascular tissue in the underlying tissue.

During the previous inflammatory phase, the infiltrating cells (polymorph nuclear cells and macrophages) together with resident tissue cells such as fibroblasts, release a variety of biologically active substances such as growth factors. The most important growth factors, recorded in oral mucosa, were TBF and hepatocyte growth factor (HGF). These growth factors have been shown to play important roles in mediating the differential pattern of healing between oral mucosa and the skin [20]. Oral mucosal fibroblasts, in comparison with skin fibroblasts, have been shown to produce more HGF [21,22] and keratinocyte growth factor [23].

Furthermore, LLLT can exert vaso-active effects by its actions on mast cells. There is direct evidence [24] that 660, 820, and 940nm light can trigger mast cell degranulation. Mast cells are distributed preferentially about the micro vascular endothelium in skin, oral mucosa and dental pulp. Mast cells in these locations contain the pro-inflammatory cytokine tumor necrosis factor- in their granules [25]. Release of this cytokine promotes leukocyte infiltration of tissues [26] by enhancing expression of endothelial-leukocyte adhesion molecules. In addition, mast cell proteases, such as chymase [27], alter basement membranes and facilitate entry of leukocytes into tissues.

Result of the present study showed that fibroblast proliferation was significantly more evident in the laser radiated group in 4 day post wounding, which was in agreement with previous studies in which low power laser beneficial effects were demonstrated [28]. Moreover, fibroblasts migrate into the wound, proliferate and produce the matrix proteins (fibronectin, hyaluronic acid, collagen and proteoglycans) and, in doing so, form granulation tissue [29]. They also interact with keratinocytes, releasing growth factors and cytokines that play a further role in modulating wound repair [30]. Low level laser therapy (LLLT) enhances production of fibroblast growth factor from fibroblasts and macrophages [31].

At 10 days post wounding, the test group showed marked and significant increase in mitotic activity with hyperplasia of the prickle cell layer as well as formation of fibro vascular tissue in the underlying tissue. The cells of prickle cell layer showed large karyomegallic vesicular nuclei Maturation of granulation tissue with deposition of collagen bundles was recorded in all examined cases. The hyperchromacia indicates increased ATP synthesis as confirmed by Houreld et al. [32] who demonstrated that LLLT activate the mitochondrial respiratory chain and increase ATP synthesis. The increase in ATP is a primary effect that leads to a cascade of events, including cellular proliferation and cytoprotection [33], fibroblast attachment and synthesis of collagen and procollagen, growth factor production [including keratinocyte growth factor (KGF), transforming growth factor (TGF) and platelet-derived growth factor (PDGF)], macrophage stimulation, lymphocyte stimulation [34] and induce greater rate of extracellular matrix production [35] and angiogenesis [36].

At 14 days post wounding, result of the present study showed healing of an open wound in oral
mucosa in scar less manner and this could be attributed to many factors; 1) factors related to oral mucosa itself and 2) factors related to laser.

Enoch et al. [37] attributed this type of scarless healing to the presence of ‘foetal-like’ cells or progenitor stem cells in the wounds so that the fibroblasts can be constantly replenished to expedite healing in the Oral mucosa. Enoch, Stephens [15] supposed that oral mucosal fibroblasts could have been derived from a resident, multipotent cell population within the lamina propria of the oral mucosa. This leads to the novel hypothesis that a stem cell or a ‘stem cell-like’ population may be present within the oral mucosa, which contributes to their preferential healing. Intra-oral fibroblasts generally exhibit a more fetal-like phenotype with a remodeling capacity higher than that of dermal fibroblasts [38].

Stephens et al. [38] demonstrated that oral mucosal fibroblasts have an increased ability to reorganize their surrounding extra cellular matrix (ECM). Likewise, a recent study by Shannon et al. [22] demonstrated oral mucosal fibroblasts have a better ability to reorganize three-dimensional collagen lattices than patient-matched skin fibroblasts.

Moreover the moisture environment in oral cavity play a vital role as saliva provides a unique environment favoring wound healing [39]. Saliva-induced cutaneous wounds have a reduced inflammatory reaction, faster epithelial coverage, and faster connective tissue regeneration [40]. More likely, growth factors in saliva-such as epidermal growth factor (EGF), vascular endothelial growth factor (VEGF), basic fibroblast growth factor (bFGF), and insulin-like growth factor (IGF)-enhance tissue repair [41].

Concerning factors related to laser, Laser light has been widely acclaimed to speed wound healing of ischemic, hypoxic, and infected wounds [42]. Human studies with laser light have demonstrated greater amounts of epithelialization for wound closure and stimulation of skin graft healing [43] because of the secondary effects of Laser biostimulation that include increased lymphatic flow, production of endorphins, increased microcirculation, increased collagen formation and stimulation of fibroblasts, osteoblasts, and odontoblasts.

LLLT causes the relaxation of smooth muscle associated with endothelium. This vasodilatation brings in oxygen and also allows for greater traffic of immune cells into tissue. These two effects contribute to accelerated healing [31].

LLLT effects on macrophages include increased ability to act as phagocytes, and greater secretion of basic fibroblast growth factor. Macrophages secrete fibrin as part of the demolition phase of wound healing more quickly with LLLT, because of their enhanced phagocytic activity during the initial phases of the repair response (for example, 6 hours after trauma). More rapid demolition of the wound establishes conditions necessary for the proliferative phase of the healing response to begin [31].

With LLLT, lymphocytes become activated and proliferate more quickly, while epithelial cells become more motile and are able to migrate across wound sites with accelerated closure of defects. Endothelium forms granulation tissue more quickly.

Early epithelialization, increased fibroblastic reactions, leucocytic infiltration, and neovascularization are seen in wounds irradiated using LLLT. Because of the overall impact of these influences, the time required for complete wound closure is reduced. Moreover, the mean breaking strength, as measured by the ability of the wound to resist rupture against force, is increased [44].

Furthermore, the diode laser has bactericidal activity as documented by Folwaczny et al. [45] who showed that exposure to a 308nm excimer laser reduces bacterial growth in vitro and Bayat et al. [46] who noted a decrease in the incidence of Staphylococcus epidermidis and Staphylococcus aureus, which was found in 100% of the control group, in irradiated rats (He-Ne laser, wavelength 633nm; fluence of 1.2 or 2.4 J/cm²) with second-degree burns. Enwemeka et al. [47] found that irradiation at 470nm killed methicillin-resistant S. aureus in vitro.

The forementioned reasons may be of significant clinical importance in bacterially infected wounds: Not only could LLLI increase wound closure, but it may also speed up the clearing of an infected wound a definite benefit to people with diabetes.

From the previous results and under the circumstances of this experiment we can conclude that diode laser 808nm LLLT can accelerate wound healing in immunosuppressed experimental rabbits.

References


27. MASoud PARIROKH, SHAHRIAR DABIRI, ALI REZA BAHRAmpour, MAHMoud HOMAYOn ZADEh and MOHAMMAD JATAR EGHBAL: Effect of low power laser on incisional wound healing FEJ; Volume 1, Number 4, Winter, 2006.


