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The Effect of Diode Laser on Palatal Mucosal Wound Healing: An Experimental Study in Rabbits

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ABSTRACT

To evaluate the effect of diode laser therapy on healing of palatal mucosal wound in rabbits. Twenty male local breed rabbits (average weight 2Kg) were included in this study. One cm full thickness incisions of the anterior part of the palatal mucoperiosteum were created on either side of the midline. Immediately after wound incision, the right sided palatal wound was irradiated with single application of 0.5W diode laser for 5 minutes (1 minute for every 2mm of the incision). The left sided incisions were kept as a control. The animals were sacrificed after 1, 3, 7 and 14 days and the mucosal wounds were subjected to histological and histomorphometric under light microscope. Clinical observation revealed immediate arrest of bleeding of the lasered wound. Histological examination showed massive infiltration of inflammatory cells with edema in the laser and non-laser sides in the first postoperative day. After 3 days, there was significant reduction in the inflammatory cells (neutrophil and macrophages) in the lasered side, as compared to non- lasered side (p<0.01). At 7 and 14 days, the density of collagen fibers in the lasered side was significantly denser than non- laser side. The thickness of epithelial layer was significantly thicker in the lasered side than non-lasered side (P<0.01) at 7 and 14 days. Single application of diode laser application was found to improve the healing and regeneration process of surgically created palatal wounds in rabbit model.

Key words: Diode Laser, Healing, Palatal Mucosa, Rabbit

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INTRODUCTION

Surgical wounds heal through a series of complex coordinated sequence of overlapping events, broadly classified as inflammatory, proliferative and remodeling phases. The inflammatory phase begins with haemostasis and subsequent inflammation and usually lasts for 1-2 days. The proliferative phase, which usually dominates for 4 weeks, is characterized by granulation tissue formation and its subsequent replacement by fibrous tissue. In the remodeling stage, there is maturation of the wound that can be extended from 6 months to 2 years [1].

Various disorders create conditions that retard the normal sequence of wound healing, causing many patients to eventually develop chronic wounds. A variety of treatment modalities have been attempted to improve healing of soft tissue wounds, including ultrasound, electrical stimulation, growth factors, platelet rich plasma, hyperbaric oxygen and laser.

The word laser is an acronym for Light Amplification by Stimulated Emission of Radiation, although common usage today is to use the word as a noun (laser) rather than as an

acronym (LASER). High intensity lasers, or also called "surgical lasers", generate heat and is used in surgery for cutting and ablating tissues. In a completely different manner, the low-intensity lasers or called" soft lasers" have the ability of altering cell behaviour and function and usually the temperature changes associated with treatment are negligible. If the density of laser is less than 760mW/cm^2, the laser is called low-power one [2].

Therapeutic application of low-level laser was first used in late 60s by Endré Mester and colleagues, in an attempt to improve wound healing in rats [3]. Diode lasers have been used in a variety of soft tissue surgical procedures and have many advantages such as less pain, bleeding, scar formation and infection. Numerous research studies have demonstrated that low level laser therapy (LLLT) is effective for some specific applications in dentistry including: oral mucositis [4], recurrent aphthous ulcers [5], and post-surgical wound healing [6].

It is well known that LLLT stimulate lymphocytes, deactivate mast cells, and increase mitochondrial ATP synthesis, which boost mitotic activity of various cell types [7]. In addition, these lasers stimulate microcirculation which leads to edema absorption and elimination of intermediary metabolites [7]. LLLT leads to increase in ascorbic acid in the fibroblasts, which augments hydroxyproline production and consequently, collagen synthesis. Furthermore, these lasers lead to increase in mitotic activity of epithelial cells and fibroblasts [7]. On the vascular level, lasers improve proliferation of the endothelial cells, which leads to increased number of blood vessels, as well as, increased production of granulation tissue [7]. Moreover, LLLT promotes the release of endorphins, which are natural pain killers [8].

The objective of the present study was to analyze the effects of low-level laser therapy on the healing of palatal mucosal incisional wounds in rabbits.

MATERIALS AND METHODS

The study was conducted using 810-nm GaAlAs diode (A.R.C. laser GmbH, Germany). The laser treatment parameters are: power (0.5W), spot size (2.18 mm), and dose (5 J/cm^2).
Twenty healthy male local breed rabbits (lepus cuniculus demostica) have been included in the present study; their age was 8 - 12 months and the mean weight was 2.0 Kg.

The animals were kept in metallic cages in large room which had a constant temperature of 25±1°C with a 12-hour-light and 12-hour-darkness cycle and 50% relative humidity under veterinary supervision. They were a fed standard laboratory diet and water *ad libitum.* Their cages were cleaned every day to keep the animals healthy. European Commission Directive 86/609/EEC for animal experiments was strictly followed. The animals were weighted to calculate the dose of anesthetics. Each rabbit was anesthetized with an intramuscular injection of a combination of 5 mg/kg xylazine (Sanofi; Sante Nutrition, Laballasrere-3301, Libonne Codex, France) and 35 mg/kg Ketamine (Rotex-medicr GMBH, Germany).

By using blade number 15, two full thickness incisions, of 1cm in length, were made in the anterior part of the hard palate mucosa on either side of the midline. Immediately after incision, the right sided palatal incision was irradiated with a single application of 0.5 W for a total time of 5 minutes (1-minute application for every 2mm of the incision). The left sided incision was not irradiated and was kept as a control. Animals were killed by injection of 100 mg/kg of Zoletil 50 (Vibrac Laborato -ries, Carros, France) after 1, 3, 7, and 14 days post application, with 5 animals in each time interval.

Biopsy samples from the irradiated and control palatal wounds were fixed immediately in 10% neutral buffered formalin for 48 hours, dehydrated in a graded ethanol series, and embedded in paraffin wax. Sections of 5µm were prepared using a microtome (Leitz 1512, Germany). After mounting onto glass slides, the sections were stained with haematoxylin and eosin (H&E), as well as Masson's trichome stains for detection of collagen fibers. The sections were examined using light microscope (Olympus, Japan) with a digital colour camera attachment (Sony, Japan).

The histomorphometric analysis was done by image J software program (NIH, Betheseda, USA), after image digitization. The microscope was coupled to an image-capturing digital camera, and this was connected to a microcomputer. All the images were digitized before the quantification and cell counting, thus standardization of the microscope light intensity and condenser height is

ensured. The number of neutrophils and macrophages per mm^2 were calculated in 25 randomly selected fields at 40x.The mean epithelial thickness (mm) and also the density of collagen fibers per mm^2 were analyzed at the 7^{th} and 14^{th} postoperative irradiation days.

Statistical Analysis

Statistical analyses were performed using the Student's *t*-test for comparison of data. Differences were considered significant when the *p* level was < 0.05.

RESULTS

Lasered wounds showed a faster hemostasis, in comparison to control ones. The mean time for arresting bleeding for lasered side was 10 (±3) seconds and 150 (±23) seconds for the control sides. The difference was significant (P<0.05). Macroscopically, three days postoperatively the lasered wounds showed more improvement of wound healing with the incision line appeared more welded as compared to nonlasered wounds (Figure 1). After seven days and also14 days, the control wounds still showed more marked incision line than the non-laser ones.

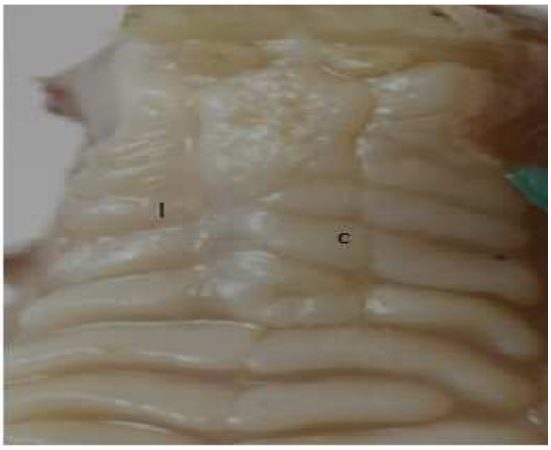

Figure 1: Photograph of palatal wounds three days postoperatively. L: lasered side; C: control side

Histological observation at day one shows infiltration of inflammatory cells and edema in the laser irradiated wound, in addition to fibrin clot formation with minimal haemorrhage. The control wounds showed severe inflammatory infiltrate and massive haemorrhage. At day three, the laser irradiated wound showed resolution of edema and marked reduction in inflammatory reaction. Moreover, there was beginning of fibroblasts infiltration and angiogenesis, which are more marked in lasered wounds

At day seven, the laser irradiated wound demonstrated more collagen formation and greater number of active of fibroblasts than control ones (Figure 2). Furthermore, the lasered wound showed obvious re-epithelization, robust new blood vessels formation and narrowing of wound edge, while the control side showed less collagen formation and less angiogenesis (Figure 3).

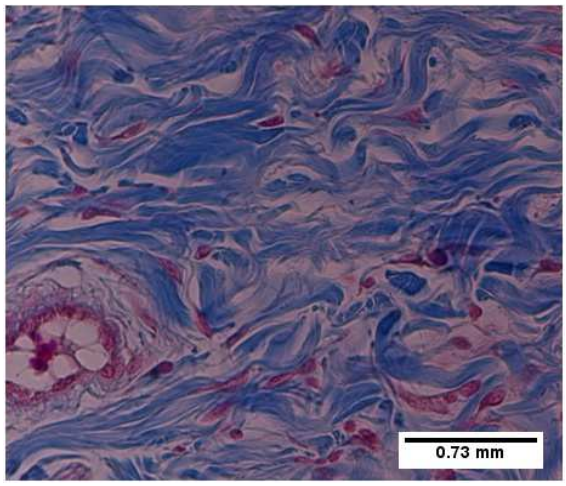

Figure 2: Microscopic picture of palatal mucosa with laser application after 7 days showing abundant collagen fibers deposition with active fibroblasts. Masson`s Trichrome (40X)

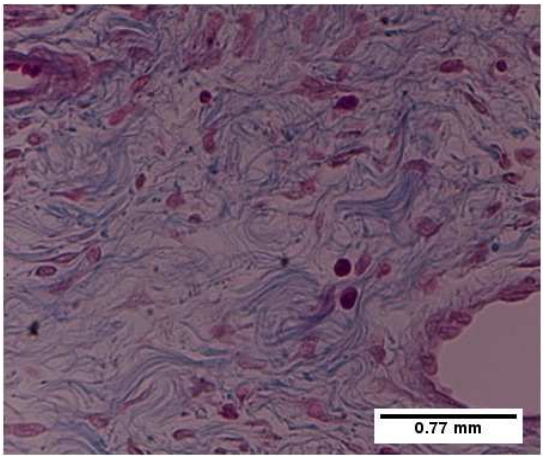

Figure 3: Microscopic picture of palatal mucosa without laser after 7 days showing scanty collagen fibers Masson`s Trichrome (40X)

At day14, for laser sides there was collagen bundles remodeling and complete re-

epithelialization with hyperplasia of epithelial layers. The control wounds sides demonstrate less collagens fibers and less fibroblasts proliferation in the sub mucosal with incomplete re-epithilization of the mucosal, appearing as very thin layer of para-keratinized squamous epithelium and many empty spaces along the incision line.

Table 1 shows the number of neutrophils and macrophages in the lasered and control wounds at days 1, 3, 7 and 14. There was no significant difference in neutrophil number between the two groups at days 1, but the lasered wounds showed a significantly less neutrophil than control wounds at days 3, 7. There was also no significant difference in macrophage number between the two groups at day 1, but the lasered wounds had significantly higher number of macrophages than control wounds at days 3 and 7.

Table 1: Neutrophil and macrophage number of lasered and control palatal wound

Days post-treatment	Palatal Wounds	Neutrophils/ mm² Mean±SD	P value	Macrophages/ mm² Mean±SD	P-value
Day 1	Lasered	8 ±5.7	>0.05	2.5±0.6	>0.05
	Control	8.4 ±5.3		2.9±1.0	
Day 3	Lasered	6.3 ±2.6	<0.05	6.5±2.4	<0.01
	Control	10.4±3.8		3.7±1.1	
Day 7	Lasered	1.7±0.4	<0.05	1.3±1.0	<0.05
	Control	2.8±0.8		0.7±0.8	
Day 14	Lasered	1.8 ±0.9	>0.05	0.7±0.6	>0.05
	Control	2.1±0.6		0.5±0.5	

Table 2: Thickness of epithelium (mm) of palatal wounds of lasered and control sides

Days post-treatment	Palatal Wounds	Thickness of the epithelium (mm) Mean±SD	P-value
7	Lasered	1.2±0.5	<0.01
	control	0.6±0.2	
14	Lasered	1.5 ±0.4	<0.01
	control	0.5±0.4	

Table 3: Collagen fiber density / mm² of lasered and control palatal wounds

Days post-treatment	Palatal wounds	Collagen fibers density /mm² Mean±SD	P-value
7	Lasered	1.7±0.2	<0.01
	Control	1.6±0.3	
14	Lasered	2.0±0.3	<0.01
	control	1.6±0.3	

Table 2 shows the thickness of epithelium of the healed wound, in millimeters, at 7th and 14th postoperative days. The lasered wounds showed a significantly thicker epithelium than control wounds at the two-time intervals. Table (3) shows the collagen fiber density, per mm², at 7th and 14th postoperative day. The lasered wounds had significantly denser collagen fibers than control wounds at the two-time intervals.

DISCUSSION

The present study has evaluated the effects of LLLT on in vitro responses in a model of wound healing using rabbit palatal wound model. Low-level laser therapy was delivered as a single exposure from 810-nm GaAlAs diode. Several studies have been conducted to evaluate the effects of LLLT on the healing of different types of wounds, including oral wounds, with contradictory results. The use of LLLT in the present study had positive effects on healing of incisional palatal wound.

The histological findings showed an attenuated inflammation, faster re-epithelialization, mature granulation tissue, and an abundant collagen deposition of the laser-irradiated wound, as compared to the control wound. The results indicate that LLLT promote wound healing by reducing inflammation without compromising the proliferation of fibroblasts and keratinocytes. The mean number of neutrophils in the laser irradiated wounds was significantly reduced as compared to the control wounds.

Our result is in accordance with studies in which increase in collagen fibers and faster elimination of inflammation was reported after laser exposure. Fahimipour *et al.,* [9] showed that, on the 3rd and 7th day after the surgery, the number of polymorphonuclear cells (PMN) in laser exposed palatal wounds groups was significantly lower than that of the control group and collagen density fibers in laser treated groups were also significantly higher than that of the control groups. Firat *et al*., [10] using a GaAlAs laser at a wavelength of 940 nm and a dose of 10 J/cm², elicited a positive healing effect on palatal mucoperiosteal wounds likely via the induction of fibroblasts. Lacjaková *et al*., [11] reported less inflammation and faster re-epithelization following laser administration at different doses. Busnardo and Biondo-Simões [12] reported less PMNs after laser treatment. Suzuki and Takakuda [13] found that LLLT with a 660-nm diode laser with energy density of 1 and 5 J/cm2 enhanced wound healing in a rat incisional wound model

and that higher radiation energy density yielded no significant enhancement.

In contrary to our study, other studies were unable to show wound healing acceleration after laser exposure [14-16]. Many factors may influence the results of low level laser exposure and generate conflicting results. These include wound type and aetiology of wounding, laser type, application time and technique, energy density, output power and wavelength. Variation in physical parameters in different studies may hinder comparison of their results and there should be a consensus on the physical variables for using LLLT in intraoral wounds. The use of 810-nm GaAlAs diode, applied with different densities can lead to different cellular responses, and this may preclude comparisons. Hawkins and Abrahamse [17] applied doses of 0.5, 2.5, 5, 10, and 16 J/cm^2 to human skin fibroblasts on two consecutive days and found that 5 J/cm^2 stimulated mitochondrial activity, cell proliferation, and fibroblast migration. However, higher doses decreased cell viability and proliferation and damaged the cell membrane and DNA.

Fibroblast cells are essential in the healing process of injured tissues as they synthesize collagen fibers and ground substance. In the present study, the density of collagen fibers at first and second week of incision was significantly higher in the laser irradiated wounds, as compared to control wounds. This observation was also noted by previous studies [18-20]. The stimulatory effect of LLLT on fibroblast activity has been attributed to the increase in the expression of collagen genes type I alpha 1 (COL1α1) and vascular endothelial growth factor (VEGF) in the fibroblast [21] and to the release of interleukin-6 and basic fibroblast growth factor Esmaeelinejad and Bayat [22]. In the present study, we use Masson's trichome staining to measure the quantity of collagen fibers which is the main component of healing wound. Masson's trichome staining has three colours and is beneficial in differentiating connective tissue from cells. The collagen is stained blue, keratin and myofibers appear red, and the cytoplasm stain pink. The control wounds at the 14th day of incision showed granulation tissue formation with less collagen and proliferating fibroblast in the submucosal layer, in addition, this collagen appeared resistant to Masson's trichome stain. This is because of immature fibers, and as density of protein network directly related to the staining reaction and penetration of the stain. Suvarna [23].

In the present study, the histopathological examination revealed that the proliferation of the epithelial cells was faster than the control group. These results are in agreement with a study that reported that LLLT induced faster epithelialization that culminated in better formation of the epidermis with formation of normal epidermis and disappearance of the scar [24]. Basso *et al*., [25] found that LLLT at energy doses ranging from 0.5 to 3 J/cm2 promoted the most significant biostimulatory effects on cultured keratinocytes. It is thought that photons are absorbed by mitochondrial chromophores in skin cells and increase reactive oxygen species, adenosine triphosphate, nitric oxide release, blood flow and activate diverse signaling pathways [26]; that may correlate to the acceleration of epithelium maturation as seen in this study. In addition, the wavelength range in between 600 and 650nm (red light) is able to penetrate through the epidermis and dermis, reaching approximately from 1.0 and up to 2.0mm depth, which certainly fits the purpose of superficial skin healing [26].

CONCLUSION

Single application of low level laser improves the healing process of incisional wound of the palate. It diminishes the inflammatory process, increases collagen deposition and hastens epithelialization.

Conflict of interest

The authors declare no any conflict of interest

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