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635nm Diode Laser Biostimulation on Cutaneous Wounds

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ABSTRACT

Biostimulation is still a controversial subject in wound healing studies. The effect of laser depends of not only laser parameters applied but also the physiological state of the target tissue. The aim of this project is to investigate the biostimulation effects of 635nm laser irradiation on the healing processes of cutaneous wounds by means of morphological and histological examinations.

3-4 months old male Wistar Albino rats weighing 330 to 350 gr were used throughout this study. Low-level laser therapy was applied through local irradiation of red light on open skin excision wounds of 5mm in diameter prepared via punch biopsy. Each animal had three identical wounds on their right dorsal part, at which two of them were irradiated with continuous diode laser of 635nm in wavelength, 30mW of power output and two different energy densities of 1 J/cm^2 and 3 J/cm^2. The third wound was kept as control group and had no irradiation. In order to find out the biostimulation consequences during each step of wound healing, which are inflammation, proliferation and remodeling, wound tissues removed at days 3, 7, 10 and 14 following the laser irradiation are morphologically examined and than prepared for histological examination. Fragments of skin including the margin and neighboring healthy tissue were embedded in paraffin and 6 to 9 um thick sections cut are stained with hematoxylin and eosin.

Histological examinations show that 635nm laser irradiation accelerated the healing process of cutaneous wounds while considering the changes of tissue morphology, inflammatory reaction, proliferation of newly formed fibroblasts and formation and deposition of collagen fibers. The data obtained gives rise to examine the effects of two distinct power densities of low-level laser irradiation and compare both with the non-treatment groups at different stages of healing process.

Keywords: Laser biostimulation, wound healing, low-level laser therapy (LLLT), therapeutic

1. INTRODUCTION

A wound is the condition of biological tissue at which its normal anatomical structure and function completely or partially is defected. Accordingly, wound healing can be defined as a complex process that biological tissues endeavor to repair the defected area which will result in the restoration of anatomical and function continuity [9]. The chemical and physical changes occurring during a normal healing process of acute wounds can be examined under four distinct, but overlapping stages: *hemostasis*, *inflammation*, *proliferation* and *remodeling*.

Nowadays, researchers have been studying on investigation of methods to accelerate the healing process by means of many distinct types of stimulation, i.e., heat, light and electrical current stimulation. Therefore, these modalities are thought to provide a potential treatment opportunity for chronic non-healing wounds and enhance the patients' quality of life.

Light has been used in medicine over decades not only for surgical procedures but also for treatment purposes. Laser light particularly has been thought to be a good source of stimulation because of its physical properties. Especially low-level laser illumination is used as a therapeutic stimulator that has been estimated to have cellular, molecular and vascular effects on biological tissues. The most important consequences that are suggested to occur due laser irradiation are biostimulative, analgesic and anti-inflammatory effects. Most frequently 632.8nm HeNe and 904nm GaAs lasers have been used for therapeutic purposes with low energy densities and this method is named Low-level Laser Therapy (LLLT) [1-8].

When the cellular metabolic changes are examined following a LLLT administration, it has been observed that there is an increase in the amount of newly formed connective tissue and granulation tissue along the epithelization around the wounded area. Besides, amount of fibroblast formation and distribution along the area under investigation has also been examined in many researches since that has been an enlightening parameter about the progress of healing [3-6]. Some of the studies in literature noted that 904, 670, 692, 780 and 786nm laser therapy applications significantly increased fibroblast formation, whereas they had no positive or negative effect on collagen synthesis. However, they also claim that healing process all in all has found to be accelerated due to irradiation using those specific wavelengths, but with certain energy densities [10-12].

In addition to cell culture studies on stimulating effect of laser irradiation, the necessity of animal and even human experiments has been emphasized by many scientists, in order to understand the complicated physiology of wound healing in depth. The general idea is to find the optimum power and energy densities to reach a maximum healing effect. So far, it is widely accepted that lower energy densities compared to those of which have been used in surgical procedures have a much more therapeutic effect on normal wound healing. Studies suggest that 632.8 nm HeNe and 514.5 Argon laser irradiation on cutaneous wounds had more healing effect when applied at low energy densities [13, 14]. However, there are also many other researches claiming that similar wavelength laser applications did not have any positive effect on the healing process. Schlager et al. claimed that neither 635 nm nor 690 nm low-power laser lights produced any beneficial effects on the healing processes of burns in rats, which is not consistent with the results of similar researches obtained by Mester et al. (694.3 nm) and Rochkind et al. (632.8 nm) [15-18]. In addition to those cell culture and animal studies, there are also human subject experiments mentioned in the literature aiming to investigate the effects of LLLT on chronic wounds [19, 20].

2. MATERIALS AND METHODS

2.1 Animal Model

Our research is based on animal experiment model, which was approved by the Ethics Committee of the Boğaziçi University Istanbul-Turkey. 3-4 months old male Wistar Albino rats (n=32) weighing 330 to 350 gr were used throughout this study and randomly divided into four groups of 8 animals. The rats were subjected to general anesthesia (Ketamine 40 mg/kg, Xylazine 15 mg/kg), and three, round, full-thickness, skin wounds, 5 mm in diameter, were made on the back of each rat by means of punch biopsy. The control wounds were placed at the dorsal skin 5 cm posterior of the head and 1 cm at the lateral side of the spinal cord. Laser irradiated wounds were placed 1 cm apart from each other as illustrated below.

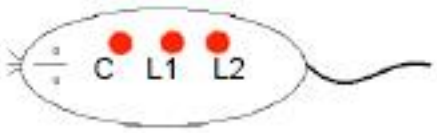

Figure 1. Wound model

2.2 Low-Level Laser Therapy (LLLT)

The biostimulative effect of light has been claimed to occur in case of low-power laser irradiation applications that has been found to accelerate normal wound healing process most probably by stimulating oxidative phosphorylation and reducing inflammatory responses in the cellular metabolism of biological tissues [21, 22].

Many studies in literature suggest that laser biostimulation with therapeutic effects on normal healing process has been observed frequently during the proliferation phase. Metabolic processes in the cellular level are claimed to enhance due to mitochondrial photoreception of monochromatic visible red light. Thus, increases in rate of respiratory metabolism of certain cells may affect their electrophysiological properties [23].

In this study, three wounds excised at the back part of the rats were classified as Control (C), Laser 1 (L1) and Laser 2 (L2) groups. L1 and L2 groups were irradiated by visible red light of 635nm wavelength and two different energy densities of 1 J/cm^2 and 3 J/cm^2. The laser output beam of 1cm in diameter reaching to the target tissue was set to 30mW. In order to reach these energy densities, laser groups were exposed for 26 seconds and 78 seconds respectively. Control group had no irradiation and has been used as reference for investigating the effects of LLLT in terms of morphological changes and histological findings during healing process.

Table 1. Laser Irradiation Specifications

Group	Wavelength (nm)	Power Output (mW)	Energy density (J/cm^2)	Time (s)
Control (C)	635	30	0	0
Laser 1 (L1)	635	30	1	26
Laser 2 (L2)	635	30	3	78

The experimental apparatus was arranged and stabilized as shown in Figure 2 in order to obtain the stated irradiation parameters that are the energy density transferred to the tissues and beam spot size covering the entire wound area.

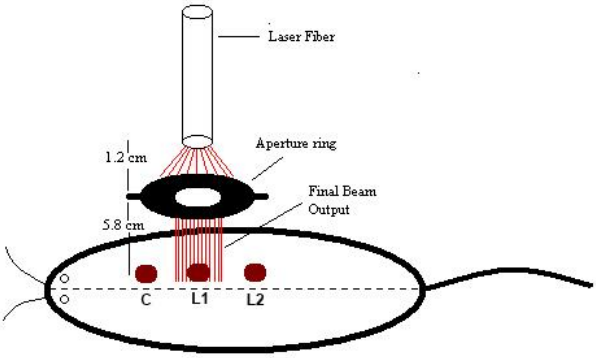

Figure 2. Experimental setup

Laser applications are performed for once on each wound immediately after wound formation. Tissue samples are removed on days 3, 7, 10 and 14 post-wounding for histological examinations. In order to be able to make statistical analysis, eight animals were used for each day. Removed skin tissue samples, covering the entire wound site and subcutaneous tissue, were kept in 10% phosphate buffered formalin for 12 hours for fixation. Paraffin embedded tissue slices of varying thicknesses between six and twelve micrometer from each sample were stained with hematoxylin and eosin.

2.3 Histological Examinations

The histological sections were examined by means of Leica DM200 light microscope. Fragments of wound site including part of the neighboring healthy tissue and subcutaneous tissue are fixed in 10% phosphate buffered formalin and embedded in paraffin (Figure 3). Six to ten micrometer thick sections were cut and stained with hematoxylin and eosin (H&E).

Figure 3. Fragment of a wound including part of the neighboring healthy tissue and subcutaneous tissue

2.4 Histological Analysis

During each phase of healing, the degree of edema, collagen and elastic fibers deposition, newly formed epithelial tissue concentration and fibroblast and granulation tissue formation were examined. Those parameters between control and laser groups for each tissue removal day and between each group for varying days following irradiation (days 3, 7, 10 and 14) were compared and investigated.

3. RESULTS

The tissue samples removed on specified days were morphologically examined in terms measuring wound diameters and calculating wound areas. The average values of wound surface areas for 3rd, 7th and 10th days following the treatment are given in Table 2. At day 14 post-wounding all the wounds were almost completely healed and closed. The differences between the control group and the experimental groups were analyzed by t-test with a significance level of $P < 0.05$ (Significant results are indicated by *). At day 3 post-wounding, although laser application caused a reduction in wound areas for both 1 and 3 J/cm^2 energy densities, there are no significant differences between any of the groups. On the other hand, there is significant difference by means of wound closure between control group and both laser groups at day 7 following laser irradiation. Similar results are obtained between control group and 3 J/cm^2 laser treatment at the 10th day of examinations. However, laser treatment groups are not significantly different from each other on 10th day examinations.

Table 2. Average of skin wound areas (mm^2) for control group and two laser treatment groups of 1 J/cm^2 and 3 J/cm^2. ("*" indicates that there is significant difference with respect to control group)

Day	Group	Mean Area ± SD (mm^2)
3rd Day	Control	57.83±11.05
	1 J/cm^2	51.33±8.15
	3 J/cm^2	50.65±4.73
7th Day	Control	21.02±1.47
	1 J/cm^2	15.24±1.78*
	3 J/cm^2	13.89±1.73*
10th Day	Control	0.90±0.35
	1 J/cm^2	1.07±0.34
	3 J/cm^2	1.33±0.19*
14th Day	Control	0±0
	1 J/cm^2	0±0
	3 J/cm^2	0±0

3.1 Day 3

At the first few days of inflammation, blot clot formation with incomplete re-epithelization is observed in all groups. The epidermis layer of control groups along the wound edge is observed to be thicker than both laser groups. Stratum corneum, which is the outermost layer of the skin, is not continuously spread over the epidermis for all groups with slight keratinization. Reticular formation of the dermis and fibroblast migration is more regularly distributed in control groups. Keratinocyte cells along the lower stratified epithelia of the epidermis have both structural and chemical importance throughout healing. These cells structurally serve as a barrier between the tissue and its environment in order to prevent the loss of moisture, heat, and other important constituents of the body. In addition, keratinocytes are responsible for secreting inhibitory cytokines in the absence of injury, and stimulating inflammation and activating Langerhans cells, in response to injury. Those changes may indicate that degree of edema is more distinctive for control wounds at the end of

inflammation phase and signs of the beginning of proliferation are not yet completed in control samples when compared to those of laser treated tissues.

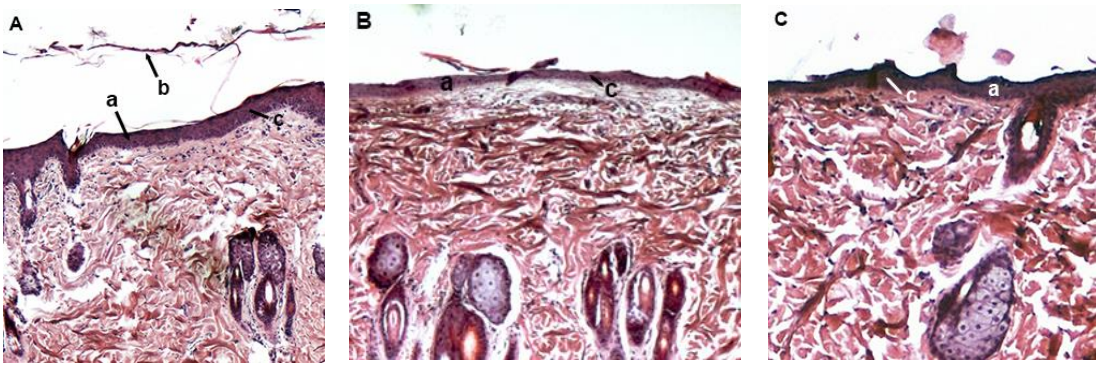

Figure 4. 3rd day hematoxylin and eosin (H&E) stained tissue samples. **A.** Control group **B.** 1 J/cm^2 laser group **C.** 3 J/cm^2 laser group (a Thicker epidermis layer of control group along the wound edge. b Keratinization of epidermis in control wounds. c Keratinocyte migration along the epidermis)

3.2 Day 7

Morphologically, scab formation with moderate keratinization of epidermis is examined in both control and laser wounds. However, epidermis thickness of control samples is observed to be fairly greater than laser wounds. This leads to the consequence of higher quantity of epidermal contents on control wounds with higher amount of keratinocytes along the layer.

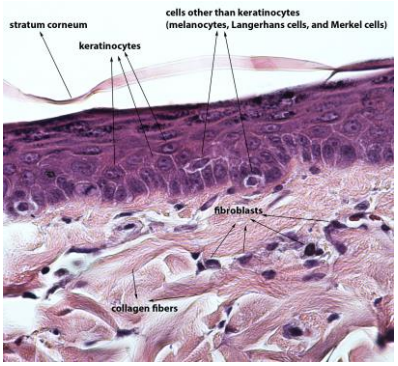

Figure 5. Structures throughout the epidermis and dermis interface of H&E stained skin samples that are visible under 40X light microscopy

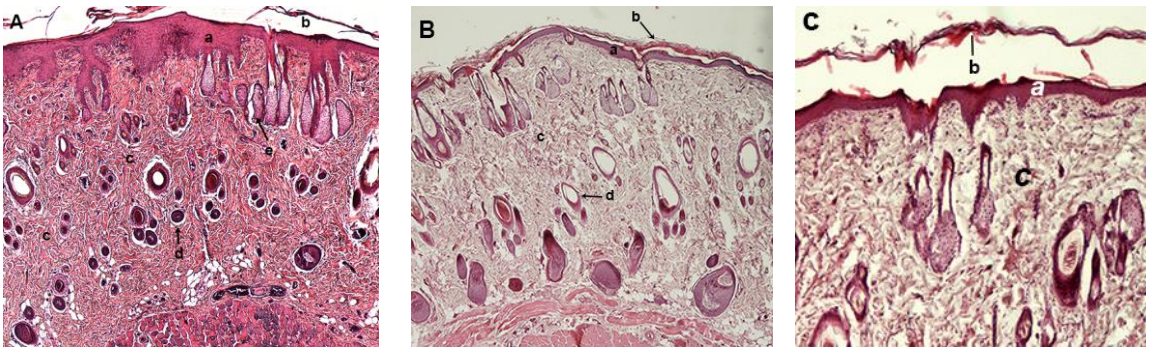

Figure 6. 7th day stained tissue samples. **A.** Control wound (a Thicker epidermis in control wounds similar to 3rd day examinations. b Discontinuous keratinized epidermis. c Dermis layer rich in collagen tissue, blood vessels and glands) **B.** 1 J/cm^2 laser treated wound (a – b Thinner epidermis with continuous keratinization. c – d Dermis layer rich in collagen tissue, blood vessels and glands) **C.** 3 J/cm^2 laser treated wound (Epidermal and dermal structures are observed to be similar to 1 J/cm^2 laser applied wounds)

3.3 Days 10 - 14

After day 10, all three groups had signs of improvement in phases of healing process. The amount of newly formed collagen is significantly higher in quantity outside the wound site than it is observed inside the wound edges (Figure 7). Epidermis thickness is reduced in all groups after day 10 with detachment of scab above the epidermal layers.

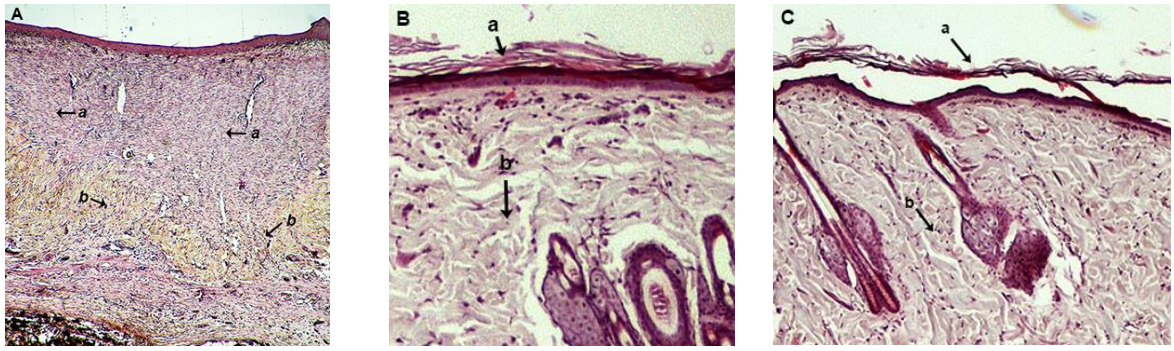

Figure 7. 10th day stained tissue samples **A.** Control wound. Cross section of the complete tissue is used in order to demonstrate the direction of healing. Healing starting from dermis layer of the wound edges spreads through the epidermal structures by time. (a Granulation tissue with small quantity of collagen, at which healing is still in progress. b Granulation tissue with significant amount of newly formed collagen) **B.** 1 J/cm^2 laser treated wound showing epidermal and upper dermal structures (a Continuous formation of keratinized stratum corneum. b Collagen formation along the dermis) **C.** 3 J/cm^2 laser treated wound mostly similar to 1 J/cm^2 laser group.

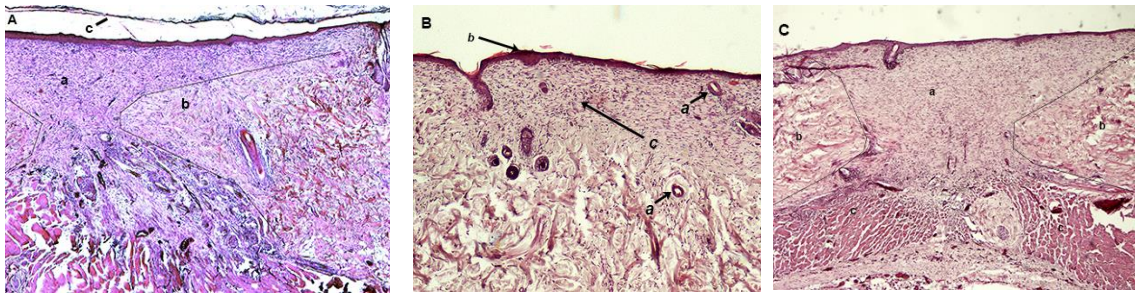

Figure 8. 14th day stained tissue samples **A.** Control group (Wound area is visibly reduced compared to day 10 control samples. a Granulation tissue with small quantity of collagen b Granulation tissue with significant amount of newly formed collagen c Keratinization above epidermis) **B.** 1 J/cm^2 laser group (a New blood vessels, b Slight presence of crust, c Complete epithelization). **C.** 3 J/cm^2 laser group (Rate of healing was observed to be fairly similar to control group and 1 J/cm^2 laser group)

4. DISCUSSION

This study aimed to investigate the biostimulative effects of low-level laser therapy on cutaneous skin wounds. Visible red light of 635nm wavelength with two different energy densities (1 J/cm^2 and 3 J/cm^2) is used as the source of irradiation. Each tissue sample removed on pre-determined days for histological examinations were morphologically observed for the assessment of wound closure rates. The results of these examinations suggest that low-level laser application with specified parameters have signs of therapeutic effects on phases of healing. The morphologic

measurements show that wound closure is accelerated by low-laser irradiation of both 1 J/cm^2 and 3 J/cm^2 energy densities approximately during the proliferation phase of healing process. Significantly different measurement results in wound areas between non-treatment group and laser treated tissues were obtained at 7th day of healing. However, there were no significant consequences of wound closure rate between the two laser irradiation groups. It is claimed that, metabolic activities that occur during phases of wound healing is accelerated by stimulation of low energy density laser application particularly during the proliferative phase of healing. Consequently, our study results seem to be consistent with the results of many studies previously published in literature. However, there is exceeding necessity of further cell culture and animal experiments on biostimulation effects of light irradiation on biological samples. This is crucially important in order to improve the understanding of cellular and metabolic changes occurring during the complicated healing process.

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