Histomorphometric Study of the Healing of Human Oral Mucosa After Gingivoplasty and Low-Level Laser Therapy

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Background and objectives: The aim of the present study was to analyze the effects of diode laser irradiation on the healing of human oral mucosa.

Materials and Methods: After gingivoplasty, the right hemi-arch (test group) of 16 patients was irradiated with a diode laser. The left side (control group) was not irradiated. Incisional biopsies were performed on both sides at 7, 14, 21, and 60 days after surgery and morphometrically analyzed by light microscopy.

Results: Epithelium width ranged from 260.6 to 393.5 μm. Volume densities of basal (20.2%), prickle cell (55.6%), and cornified (24.2%) layers remained stable. The peak number of neutrophils were 6 cells/mm² and the mononuclear cells were 44 cells/mm². Collagen fibers (80%) and fibroblasts (14%) occupied the main volume of connective tissue. The one-way ANOVA and the paired Student’s t-test were used for statistical analysis (P<0.05).


Key words: low-level laser therapy; oral surgery

INTRODUCTION

The use of low-level laser as a therapeutic agent started with Mester in 1967, who studied its effect on the acceleration of wound healing in rats [1]. The basic principle of biomodulation of cells by laser therapy consists of the fact that irradiation at a specific wavelength is able to alter cellular behavior by acting on the mitochondrial respiratory chain [2] or on membrane calcium channels [3], promoting an increase in cell metabolism and proliferation [4]. The main effects of low-level laser therapy include the suppression of pain and the acceleration of wound healing.

The literature regarding laser therapy is controversial, either showing beneficial effects [1,5–8], or no effect at all [9–16]. It is very common to find investigations which fail to demonstrate effects because of the choice of incorrect parameters [17–19]. Therefore, the existence of various types of lasers showing different abilities of interaction with tissues should be taken into account, as well as failures in dosimetry, the mode of application, and the animal model used. The use of a laser to accelerate the wound healing process has been extensively studied; however, most investigations have been performed on animals [6,9,13–16] or in vitro [20–23]. Most human studies refer to treatment after extraction of the third molars [10,11], with only few reports in the area of periodontics being available [8,12]. Thus, to increase the application of this treatment modality in clinical dentistry, a larger number of human studies is necessary, especially histological investigations, emphasizing different aspects of the biology of tissue repair such as cell differentiation and maturation, cellular and vascular proliferation, extracellular matrix production, and the inflammatory response [15,24,25].

Based on the above considerations, the objective of the present study was to analyze histomorphometrically the effects of low-level laser therapy on the healing of human oral mucosa after gingivoplasty.

METHODS

Sixteen patients with inflammatory gingival hyperplasia in the upper or lower anterior sextants were selected. The patients signed an informed consent form according to the norms of the Ethics Committee on Human Research of the Bauru Dental School, University of São Paulo.

The patients were instructed regarding oral hygiene, scaling and root planning, and were submitted to rigorous plaque control. After 21 days, gingivoplasty took place. Following gingivoplasty, a 15-mW GaAlAs (Gallium Aluminum Arsenate) diode laser1 (670 nm wavelength) was applied in the punctual mode with mild contact to three points corresponding to the keratinized mucosa regions of teeth 11, 12, and 13 or 41, 42, and 43. The focal spot had 4 mm of diameter corresponding to the diameter of the intraoral tip. Three points of 4 mm each were enough to cover the test site. The right side represented the test side, while the left side (control) received no additional treatment. The final energy density applied was 4 J/cm² per point after compensation for a 20% loss caused by the use of an intraoral tip. The laser was calibrated by the manufacturer before the study. Laser applications were repeated every 48 hour for 1 week, for a total of four sessions. During
each session, surgical dressing (CoePack-GC America, Inc., Illinois) was removed and the wound cleaned with physiological saline.

Incisional biopsies were performed in the area of the gingival papilla between the lateral incisor and canine. The patients were randomly divided into groups of four and each group contributed with the biopsies at one of the post surgical periods which were 7, 14, 21, and 60 days after surgery. Specimens measuring approximately 2 × 3 mm were removed from the test (right) and control (left) sides of each patient. The specimens were fixed in 10% phosphate-buffered formalin for 18 hour, dehydrated in ethanol, cleared with xylene, and embedded in Histosec (paraffin + synthetic resin). Alternate 5-μm sections were cut and stained with hematoxylin and eosin.

**Morphometric Analysis of the Gingival Epithelium Tissue**

All the morphometric analysis was performed by a single calibrated blinded evaluator.

**Determination of epithelial thickness (h), volume density (Vvi) of each layer, and the luminal to basal surface ratio (Rl/b).** The area occupied by the epithelial strata (Ai), total area of the epithelium (A), and total length of the luminal (Sl) and basal (Sb) surfaces were determined in 25 histological fields randomly captured with a digitized image analysis system consisting of a Zeiss Axioscope 2 microscope equipped with a 40 × lens, a Sony CCID-IRIS RGB camera, and Kontron KS300 software (Kontron Electronic GmbH, Germany) installed on an IBM computer. Based on these data, the following variables were calculated: volume density of each epithelial layer by 

\[ V_{vi} = A_i / S_i \]

where \( A_i \) is the total area of the epithelial layer and \( S_i \) is the surface area of the tissue.

**Determination of nuclear (Vni) and cellular (Vcel) volume in germinative and spinous layer cells.** Nuclear and cytoplasmic volume density, necessary for the calculation of cellular volume, were determined by a point-counting method using a Zeiss 8 × Kpl eyepiece containing a Zeiss II quadrangular integration grid that consisted of 10 parallel lines and 100 points and a 100× immersion objective. The number of points above the nucleus (Pni) and cytoplasm (Pcyti) in each epithelial layer were determined in 25 randomly selected histological fields per patient.

Nuclear volume density (\( \rho_{ni} \)) was calculated based on the relation

\[ \rho_{ni} = \frac{P_{ni}}{S_{ni}} \]

Since the nuclear volume density thus obtained is overestimated due to the Holmes effect, this overestimation was corrected using the \( K_n \) correction factor calculated based on the following equation:

\[ K_n = \frac{(1 + 3t)}{2D} \]

where \( D = \) mean nuclear diameter and \( t = \) section thickness. The corrected nuclear (\( \rho_{ni, corr} \)) and cytoplasmic (\( \rho_{cyti} \)) densities were

\[ \rho_{ni, corr} = \frac{P_{ni}}{K_n} \quad \text{and} \quad \rho_{cyti} = 1 - \rho_{ni, corr} \]

The nuclear volume of basal and spinous layer cells was calculated based on the mean diameter (D) obtained from the orthogonal measurement of 30 intact nuclei per layer in each biopsy using an Olympus Ramsden type 10 × eyepiece micrometer and a 100× immersion objective. Nuclear volume was then calculated using the \( V_{ni} = \frac{4}{3}\pi r^3 \) formula, and cytoplasmic volume (\( V_{cyti} \)) was estimated by the

\[ V_{cyti} = V_{ni} \cdot \rho_{cyti} / \rho_{ni} \]

**Morphometric Analysis of the Gingival Connective Tissue**

**Determination of the volume density (Vvi) of collagen fibers and vessels and the number of fibroblasts and inflammatory cells (Ni) per mm² tissue.** The morphometric dimensions were obtained using a Zeiss 8 × Kpl eyepiece equipped with a Zeiss II integration grid and a 100× immersion objective. The number of points (pi) above each structure (i) of the lamina propria and above the entire lamina propria (LP) and the number of nuclear images (ni) of fibroblasts and inflammatory cells in the lamina propria were determined in 25 randomly selected histological fields per patient. Knowing the total area examined (A), \( V_{vi} \) and Ni were obtained by

\[ V_{vi} = \frac{\pi}{L} \quad \text{and} \quad Ni = \frac{ni}{A} \]

The data obtained for the test and control groups was analyzed after an arcsine transformation, by the paired Student’s t-test. Variations between postoperative periods were determined by one-way analysis of variance (ANOVA). A 95% confidence interval was applied in both tests (\( P < 0.05 \)).

**RESULTS**

**Morphological Results**

No morphological differences were observed in the maturation process of the gingival mucosa between the laser-irradiated and control groups (compare Figs. 1, 3, 5, and 7 with 2, 4, 6, and 8). In addition, the pattern of histological evolution of the epithelium and lamina propria was closely similar in the two groups.

![Fig. 1. Test side after 7 days of surgery. Epithelium (E), epithelial projections (wide arrow), basophilic granules in granular layer (thin arrow). Connective tissue (C). (H.E. original augmentation—40×). [Figure can be viewed in color online via www.interscience.wiley.com.]](image)
Thus, at 7 days after surgery, the gingival epithelium was already formed in all patients, but was still immature, i.e., it was less thick than during the subsequent periods and showed the formation and growth of epithelial projections and the presence of a basal layer with hyperstained cell nuclei, a spinous layer with ample intercellular spaces in its basal half, a granular layer containing cells with basophilic granules in the cytoplasm, and an already thin, parakeratinized stratum corneum showing moderate eosinophilia, whose cell layers tended to dissociate upon histotechnical processing. The connective tissue in the lamina propria was still disorganized (Figs. 1 and 2). At 14 days after surgery, the epithelial, basal, and spinous layers showed the same characteristics as described for the previous period, while the granular layer had disappeared, being incorporated into the parakeratinized stratum corneum. The latter layer, on the other hand, showed a significantly increased thickness and eosinophilia and the deeper cell layers close to the transition with the spinous layer were less eosinophilic than the more superficial layers. The connective tissue, which was still in the process of organization, was characterized by a large number of blood vessels (Figs. 3 and 4). At 21 days after surgery, the gingival epithelium already showed epithelial cristae and...
well-defined layers, but the basal layer still exhibited hyperstained nuclei mainly close to the projections, and ample intercellular spaces were observed in the basal half of the spinous layer. The stratum corneum showed a reduced thickness and assumed a mature pattern, i.e., a compact and highly eosinophilic aspect. The connective tissue of the papillary layer of the lamina propria was found to be more organized and contained a smaller number of vessels and inflammatory cells (Figs. 5 and 6). Sixty days after surgery, the epithelium was already completely mature, showing long and stabilized epithelial cristae, a basal layer containing less hyperchromatic cell nuclei, a well-developed spinous layer containing a larger number of cell layers and reduced intercellular spaces, and a compact and eosinophilic parakeratinized stratum corneum. The connective tissue of the lamina propria already presented characteristics of a normal oral mucosa (Figs. 7 and 8).

### Morphometric Results

Statistical analysis of the various morphometric parameters quantified did not reveal significant differences between the laser-irradiated and non-irradiated control groups for the four postoperative periods analyzed (Table 1).

Some significant differences were observed between postoperative periods within each group, confirming the morphological observations of gingival mucosal maturation between postoperative day 7 and 60 made in the two groups.

Thus, during maturation epithelial thickness increased proportionally with time from 260.5 μm at 7 days to 393.5 μm at 60 days in the control group and from 279.6 to 367.7 μm in the irradiated group. It should be emphasized that the difference observed for the test group was not statistically significant ($P > 0.05$).

With respect to the luminal-basal surface ratio, on the control side this parameter remained unchanged between days 7 and 14 ($P > 0.05$) but showed a significant decrease from 0.53 to 0.28 ($P < 0.05$) between postoperative days 14 and 60. This decline was probably due to the increase in the number, shape, and depth of the epithelial cristae during this period. On the irradiated side, despite a decrease in this ratio from 0.50 at 14 days to 0.33 at 60 days this difference was not statistically significant ($P > 0.05$).

On the other hand, no significant difference ($P > 0.05$) was observed in the volume density of the epithelial layers—basal, spinous, and stratum corneum—between periods for the control or irradiated group, i.e., the volumetric relationships between the various epithelial layers were already established at 7 days after surgery in both groups.
| Table 1: Morphometric Dimensions for the Gingival Epithelium and Connective Tissue in the Test and Control Groups in the Various Periods After Surgery |
|-----------------------------------|--------------------------|---------------------------|--------------------------|--------------------------|
|                                   | 7 days (Mean ± SEM)      | 14 days (Mean ± SEM)      | 21 days (Mean ± SEM)      | 60 days (Mean ± SEM)      |
|                                   | Test                     | Control                   | Test                     | Control                   |
| Epithelium volume density (%)     |                          |                           |                          |                           |
| Corneous                          | 24.0 ± 1.47              | 21.6 ± 2.54               | 26.6 ± 4.7               | 24.8 ± 3.26               |
| Spinous                           | 53.4 ± 1.46              | 53.9 ± 1.28               | 54.3 ± 4.78              | 58.3 ± 2.23               |
| Basal                             | 22.4 ± 1.15              | 24.4 ± 1.99               | 19.0 ± 4.21              | 16.7 ± 1.47               |
| Epithelium luminal-to-basal surface ratio | 0.4 ± 0.07           | 0.44 ± 0.09               | 0.50 ± 0.10              | 0.53 ± 0.08               |
| Epithelium width (µm)             | 279.6 ± 35.04            | 260.5 ± 11.55             | 311.9 ± 51.18            | 287.0 ± 16.75             |
| Nucleous and cell volumes (µm³)   |                          |                           |                          |                           |
| Nucleous                          | 193.2 ± 20.57            | 211.8 ± 23.59             | 197.6 ± 7.37             | 207.8 ± 22.27             |
| Basal cytoplasm                   | 415.4 ± 35.22            | 408.9 ± 79.78             | 462.8 ± 12.41            | 487.0 ± 55.62             |
| Layer cell                        | 608.7 ± 35.88            | 680.8 ± 93.63             | 660.5 ± 19.38            | 694.9 ± 76.65             |
| Nucleus                           | 318.2 ± 13.15            | 316.4 ± 18.58             | 296.1 ± 22.22            | 295.1 ± 21.04             |
| Prickle cell cytoplasm            | 2902 ± 252.01            | 3346.8 ± 439.48           | 1908.1 ± 191.11          | 2037.9 ± 335.27           |
| Layer cell                        | 3220.2 ± 258.25          | 3663.2 ± 447.70           | 2204.3 ± 207.82          | 2333 ± 343.72             |
| Fibroblasts                       | 613.8 ± 42.99            | 519.9 ± 44.23             | 858.8 ± 80.34            | 782.7 ± 119.28            |
| Mononuclear cells                 | 20.5 ± 1.06              | 12.7 ± 1.90               | 41.1 ± 13.35             | 43.8 ± 16.51              |
| Polimorphnuclear cells            | 6.1 ± 2.46               | 3.3 ± 1.43                | 1.1 ± 0.64               | 2.2 ± 1.57                |
| Collagen fibers                   | 84.5 ± 0.81              | 87.1 ± 0.83               | 77.5 ± 2.24              | 78.4 ± 2.19               |
| Vessels                           | 2.7 ± 0.74               | 2.6 ± 0.3                 | 6.6 ± 0.77               | 7.1 ± 1.61                |
| SEM, standard error of the mean; P > 0.05. |
Cell maturation evaluated in the present study by the measurement of mean cell volume in the basal and spinous layers did not reveal variations \( (P > 0.05) \) between periods for the basal layer, with cell volume ranging from 416.3 to 487.0 \( \mu m^3 \) and from 414.7 to 462.8 \( \mu m^3 \) in the control and test groups, respectively. In the spinous layer, a significant reduction \( (P < 0.05) \) in cell volume was observed for both groups, decreasing from 3663.2 \( \mu m^3 \) at 7 days to 2514.4 \( \mu m^3 \) at 21 days in the control group and from 3220.2 \( \mu m^3 \) at 7 days to 1864.0 \( \mu m^3 \) at 60 days in the irradiated group.

With respect to the maturation of the lamina propria, a significant increase was observed in the number of fibroblasts/mm\(^2\) tissue \( (P < 0.05) \) from a minimum of 519.9 cells/mm\(^2\) at 7 days to a maximum of 932.4 cells/mm\(^2\) at 60 days in the control group and from 613.8 cells/mm\(^2\) at 7 days to 946.1 cells/mm\(^2\) at 21 days in the test group. On the other hand, the volume density of collagen fibers showed a small decline during the period from 7 to 14 days, from 87.1% to 78.4% on the control side and from 84.5% to 77.5% on the experimental side, remaining unchanged thereafter in both groups.

The volume density of blood vessels increased significantly \( (P < 0.05) \) between 7 and 14 days of repair from 2.6% to 7.1% and from 2.7% to 6.8% in the control and irradiated groups, respectively, with fluctuations \( (P > 0.05) \) during the subsequent periods. Regarding inflammatory cells, the largest number of polymorphonuclear cells was found at 7 days, 6.1 and 3.3 cells/mm\(^2\) tissue in the control and test groups, respectively, with a marked decrease being observed thereafter. The number of mononuclear inflammatory cells—lymphocytes, plasma cells, and macrophages—were relatively high \( (P > 0.05) \) up to day 21, with a mean number of 26.6 and 30.1 cells/mm\(^2\) tissue in the control and irradiated groups, respectively, decreasing significantly thereafter \( (P < 0.05) \).

**DISCUSSION**

The laser irradiation parameters used in this study followed some instructions of manufacturer and were based on literature. The energy density of 4 J/cm\(^2\) was in accordance to previous studies in humans [5,11] and animals [6]. More recently, a study by Pereira et al. [26] showed that a laser dose of 3 and 4 J/cm\(^2\) increased the fibroblast numbers about threefold to sixfold comparing to control cultures. There is a variety of irradiation frequency in literature. Some authors used one single application after surgery [9–11], others twice a week [1,5], and others 3-day intervals [12]. For this study, a 48-hour interval was chosen according to previous investigations [14,15] and because it is an interval more suitable to daily practice in a dental office. Besides that, Skinner et al. [23] found that repeated irradiation increased the proliferation of fibroblasts in culture. The irradiation was performed during 1 week that was the time spent for complete reepithelization.

Morphometric analysis of the gingival epithelium and connective tissue showed that wound healing was normal in all patients. In the regeneration process of gingival epithelium, peak mitotic activity is known to occur between 24 and 48 hour after surgery, together with intense cell migration. According to Ramfjord [27], the epithelium migrates 0.5 mm/day from the borders to the center of the wound. Thus, reepithelization can last up to 14 days depending on the size of the lesion [28,29]. In the present study, reepithelization of the entire wound area was observed at 7 days after surgery in all patients, in agreement with other studies monitoring gingival healing in humans [30,31]. However, the stratified squamous epithelium formed was still immature. Over time, epithelial maturation occurred, which was characterized by an increase in thickness and in the number and size of epithelial cristae and by a reduction in the luminal to basal surface ratio. A mature, parakeratinized stratum corneum, a common characteristic of normal human oral mucosa [31], could already be observed at 21 days after surgery.

In connective tissue, the number of polymorphonuclear cells peaked at 7 days, while at 14 days a predominance of mononuclear inflammatory cells was observed, finding that agrees with literature data [27]. In addition, the number of fibroblasts increased over time, with the largest number being observed at 60 days in the control group and at 21 days in the irradiated group. In this respect, we emphasize that Ramfjord [30] and Stahl [31] found peak proliferative activity in connective tissue on the third day after surgery and fibroblast proliferation at high levels up to day 30. Ramfjord [27] reported that collagen formation is a gradual process during the first 3–4 weeks after surgery and starts with a decline in initial vascularization, with the tissue already being practically mature at 35 days. In the present study, the number of collagen fibers was high in both groups throughout the period analyzed, occupying 80% of the connective tissue volume on average, a value relatively higher than the 50%–63% reported in the literature [32,33]. This difference might be due to the thickness of the histological sections used here (5 \( \mu m \)).

In the present study, a higher percentage of vessels in connective tissue was observed on postoperative day 14, in contrast to the investigation of Novaes [29] performed on dogs, in which a larger number of vessels was noted during the first days of healing, decreasing thereafter until reaching a stable value on day 16.

The present results showed that the use of a 670-nm GaAlAs laser within the standards established did not influence gingival healing when compared to the control side. These findings agree with various studies conducted on animals [6,9,13–16], as well as humans [10–12], but differ from others [5], most of them conducted on rats [1,7] or cell cultures [20–23], which showed beneficial effects of low-level laser therapy on the acceleration of wound healing.

Several of the investigations performed on humans, which failed to demonstrate beneficial effects of low-level laser therapy, have used one of the sides of the patient as control, as done in the present study. The split mouth design has been highly criticized by some investigators who defend the theory of systemic effects of laser therapy [17–19], i.e., irradiation causes the release of substances into the bloodstream which can reach areas distant from
the irradiated site [4,9,21]. On the other hand, in studies in which better results were observed on the side where the laser was applied [5,6,8], the better response might have been due to the concomitant action of local and systemic effects.

Another point that might explain the lack of significant results in the present study is that laser therapy seems to have a greater effect on tissues affected by a pathological condition [17–19]. Tuner and Hode [18] suggested that laser irradiation directly affects the immune system and, therefore, healthy organisms do not respond as well to laser therapy because healing occurs within normal parameters. Thus, studies on diabetic rats, for example, have shown a faster closing of wounds that were irradiated with a laser [7].

Gingivoplasty is a relative simple surgical procedure, restricted only to the soft tissue and with excellent postoperative outcome in most cases. Aremband and Wade [34], comparing gingivectomies performed with a conventional or electric surgical knife, did not find significant differences in the repair process between techniques, a finding similar to the results reported here, demonstrating that healing after gingivoplasty is a very rapid and simple process which does not show significant differences even when diverse techniques are used to favor the healing process. This fact, together with the young age and absence of systemic diseases of the patients participating in the present study, might explain the lack of observation of an evident beneficial local response to laser treatment, which is probably more relevant than a possible systemic effect.

Analysis of all studies reviewed here shows that the scientific documentation of low-level laser therapy is scarce. Although the literature has demonstrated a positive influence of laser therapy on various basic biological processes, further studies are still necessary to confirm its beneficial effects on complex systems such as in vivo wound healing to establish an application protocol. Thus, there is initially the need to standardize the energy density, power, wavelength, frequency, and the specific application mode of the laser for each case, since comparison of the results of currently available studies is not possible due to extreme variations of the parameters cited above in these cases.

REFERENCES


