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Abstract. Keratinocytes play a central role in wound healing by responding to tissue injury through the activation of cellular proliferation and migration. Current clinical evidence suggests that the laser phototherapy (LPT) accelerates wound healing in a variety of oral diseases; however, the molecular mechanisms involved in response to LPT are not fully understood. Oral keratinocytes (NOK-SI) maintained under nutritional-deficit culture medium (2% fetal bovine serum) were irradiated with InGaAlP laser (660 nm; 40 mW; 0.04 cm² spot size) in punctual and contact modes. The energy densities used were 4 and 20 J/cm² corresponding to 4 and 20 s of exposure times and 0.16 and 0.8 J of energy per point, respectively. Three sessions of irradiations were applied with 6-h intervals. Further, the impact of LPT over cellular migration, proliferation, and activation of the mammalian target of rapamycin (mTOR) pathway, known to play a major role in epithelial migration and wound healing, was analyzed. Compared with control cells, the LPT-treated cells showed accelerated cellular migration without any changes in proliferation. Furthermore, LPT resulted in an increase in the phospho-S6 ribosomal protein, indicating activation of the mTOR signaling pathway. Collectively, these findings suggest that the LPT activates mTOR signaling pathway, promotes epithelial cell migration, and accelerates healing of oral mucosa. © 2014 Society of Photo-Optical Instrumentation Engineers (SPIE) [DOI: 10.1117/1.JBO.19.2.028002]

Keywords: keratinocytes; laser phototherapy; epithelial cell migration; PI3K; rapamycin; low-level laser therapy.

Paper 130793R received Nov. 5, 2013; revised manuscript received Jan. 14, 2014; accepted for publication Jan. 17, 2014; published online Feb. 14, 2014.

1 Introduction

Laser phototherapy (LPT) has been clinically used to accelerate the wound healing in several diseases including those from the oral cavity.1 The LPT has proven to be efficient in treating slow healing skin ulcers,2 chronic diabetic leg and foot ulcers,3,4 and several oral diseases, such as chemotherapy- and radiotherapy-induced oral mucositis,5,6 herpes simplex infections,9 and bone osteonecrosis.10,11 Diverse cell types, like fibroblasts,12–14 osteoblasts,15 endothelial,16,17 and keratinocytes,18–20 when growing in culture in adverse conditions respond positively to LPT. This treatment improves cell proliferation, migration, and transcription of genes involved in wound healing.21

Epithelial cells play an important role in wound healing by restoring the epithelial barrier, followed by re-establishment of tissue homeostasis.22 Rapid migration and proliferation of epithelial cells located in the basal layer of the epithelium adjacent to the injured area are important events of wound healing. Several molecular pathways are activated during wound healing including the AKT/mTOR pathway.23–26 In fact, augmented epithelial migration and accelerated wound healing have been observed as a result of in vivo overexpression of the AKT/mTOR pathway in genetically defined animal models.22 Additionally, mammalian target of rapamycin (mTOR) signaling pathway was proven to be a key player in the accelerated migration phenotype of epithelial and muscle cells.23,27

Only three studies analyzed the effects of LPT on epithelial cells, looking for understanding the mechanisms underlying the biostimulation in these cells.5,12–20 It is known that the mechanisms involved in the LPT are those related to the improvement in the respiratory metabolism8,20 and changes in mitochondrial membrane potential,30 leading to an increase in the mitochondrial respiration and ATP synthesis.31 However, it remains unclear how LPT influences epithelial cell proliferation and migration. Thus, this study searched the molecular mechanisms underlying the effects of LPT on oral keratinocytes proliferation and migration, and further the molecular circuitry involved in this process was dissected.

2 Materials and Methods

The study received approval from the Research Committee of School of Dentistry, Universidade Federal do Rio Grande do Sul under the process number 24114.

2.1 Cell Lineage and Cell Culture

Normal oral keratinocyte spontaneously immortalized (NOK-SI) cell line isolated from the retromolar area of the oral cavity was...
previously established and kindly provided by Dr. Gutkind from the National Institute of Dental and Craniofacial Research (NIDCR/NIH). The cells were maintained in Dulbecco's-modified Eagle's medium (DMEM, Hyclone, Thermo Fisher Scientific, Waltham, Massachusetts) supplemented with 10% fetal bovine serum (FBS) (Hyclone, Thermo Fisher Scientific), 100-U/ml penicillin, 100-μg/ml streptomycin, and 250-ng/ml anfotericyn B (Hyclone, Thermo Fisher Scientific) at 37°C in a humidified atmosphere with 5% CO2.

Cells plated on 6- or 96-well tissue-culture plates were grown to 70% of confluency. Cell stress was induced by using culture medium with low concentration of FBS (DMEM with 2% FBS, nutritional deficit). All cell culture experiments were performed under laminar flow (biosafety class II), and cells were monitored daily using a phase contrast microscope (AO Biostar, American Optical, Reichert, Depew, New York).

2.2 LPT

The irradiations were performed using a continuous-wave indium-gallium-aluminum-arsenide (InGaAsP) diode laser (MM Optics Ltd., São Carlos, SP, Brazil) with a spot size of 0.04 cm2, operating at a wavelength of 660 nm, and an output power of 40 mW. The energy densities used were 4 and 20 J/cm2 corresponding to 4 and 20 s of exposure times, respectively. Laser was applied perpendicularly and in contact with the tissue-culture plates. When using 96-well culture plates (MTS (3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium) assay), the laser was applied in a single point; whereas, using 6-well culture plates (“scratch assay”), the irradiations were applied in five points. Three sessions of irradiations were applied with 6-h intervals. The output power of the equipment was checked using a power meter (Laser Check; MM Optics LTDA, Sao Paulo, Brazil). The total energy per point and the total energy used in each culture plate after all the irradiations are described in Table 1.

Because the distance between the laser source and the surface of application is critical, the LPT was performed through the bottom of the optically clear plates. The irradiation was carried out in partial dark conditions without the influence of other light. Control groups were treated under identical conditions without activating the laser source (sham irradiation).

2.3 In Vitro ‘Scratch Assay’

In vitro scratch-induced wound model was chosen to assess the migratory ability of the cells upon irradiation with diode laser. NOK-SI cells were seeded on 6-well culture dishes maintained at 37°C and grown to confluence in normal growth media. Three groups were established: control (0 J/cm2), 4 J/cm2 laser group, and 20 J/cm2 laser group. Two hours before the scratch, culture medium was substituted with nutritional-deficit media in all groups (DMEM with 2% FBS). A wound was made in the monolayer of the cells by completely scratching the cells in a line with a 200-μl pipette tip. The time of scratching the wound was designated as time zero. Three irradiations were applied in five points with 6-h intervals between each application. The first irradiation was performed immediately after the scratching (T0). Cells were allowed to proliferate and migrate into the scratch wound for 48 h since the time zero. Migration of the cells into the wound was photographed at 0, 12, 24, 36, and 48 h under a phase contrast microscope. Relative wound closure (%) was measured by using a computerized image analyzer system (AxioVision 4.8.1, Carl Zeiss, Thornwood, New York). “Scratch assay” was performed in triplicate.

2.4 Cell Mitochondrial Activity Analysis (MTS Assay)

To determine the cell proliferation rate, the Cell Titer 96TM Aqueous nonradioactive cell proliferation kit (Promega, Fitchburg, Wisconsin) was used. Cell proliferation was determined by the reduction of MTS (inner salt) following the manufacturer’s protocol. In brief, 2.5 × 104 cells were seeded onto the 96-well plates 24 h prior to irradiation. Culture media from all the samples were replaced with the nutritional-deficient media (2% FBS) after cellular attachment (6 h after seeding). Three groups were established: control (0 J/cm2), 4 J/cm2 laser group, and 20 J/cm2 laser group. Cells received either sham (control) or laser irradiation in eight replicates for each treatment. Twenty-four hours after the first irradiation, the cells were incubated with MTS for 4 h at 37°C. The absorbance at 595 nm was measured using a microplate reader (Promega).

2.5 Western Blotting

NOK-SI cells were seeded into the 6-well culture dishes and maintained at 37°C in a humidified atmosphere with 5% CO2 in DMEM supplemented with 10% FBS. Three groups were established: control (0 J/cm2), 4 J/cm2 laser group, and 20 J/cm2 laser group. Six hours before irradiation, the medium was substituted with nutritional-deficient medium in all groups (DMEM with 2% FBS). Epithelial growth factor (EGF) was used as a positive control. The laser groups received three irradiations applied in five points with 6-h intervals. Eighteen hours after the first irradiation, the cells were washed with ice-cold PBS and lysed with radio immunoprecipitation assay (RIPA) buffer. Protein lysates were cleared of cellular debris by centrifugation at 4°C for 10 min, resolved in 10% SDS-PAGE gels, and transferred to polyvinyl difluoride membrane (Millipore, Billerica, Massachusetts). After the transfer of proteins, the membranes were blocked at room temperature for 1 h with 5% milk solution, washed three times with Tris-buffered saline (TBS)-Tween, and incubated overnight with anti-phospho S6 (Cell Signaling, Danvers, Massachusetts), anti-phospho AKT (Cell Signaling), and GAPDH (Calbiochem, Gibbstown, New Jersey), which was used as a loading control. The membranes were washed three times for 5 min each with TBS-Tween and then incubated for 1 h at room temperature with appropriate secondary antibodies conjugated with horseradish peroxidase. The reactions were visualized using the ECL.
Super Signal West Pico Substrate (Pierce Biotechnology, Rockford, Illinois).

2.6 F-Actin Polarization Assay
Culture medium containing 10% FBS was substituted by DMEM supplemented with 2% FBS 6 h before the first irradiation. Two groups were established: control group receiving 0 J/cm² (sham irradiation) and LPT group receiving 4 J/cm². The LPT group received two irradiations with 6-h interval. F-actin staining was performed in two different time points: 10 min and 12 h after the first irradiation. In brief, cells were washed with cold PBS, fixed with fresh 4% paraformaldehyde, and permeabilized using 0.1% Triton for 5 min. Cells were incubated with 100-nM rhodamine/phalloidin for 30 min (Cytoskeleton, Denver, Colorado), followed by DNA staining using Hoechst 33342. Slides were mounted using aqueous mounting media, and images were acquired using a Nikon Eclipse 80i Microscope and QCapturePro software (Nikon, Melville, New York).

2.7 Statistical Analysis
Statistical analyses were carried out using GraphPad Prism 5 (GraphPad Software, San Diego, California). Cellular migration was assessed by two-way analysis of variance (ANOVA), followed by the Bonferroni multiple comparison test. Statistical analysis of cellular proliferation was performed by one-way ANOVA, followed by Tukey’s multiple comparison test. Asterisks denote statistical significance [*p < 0.05; **p < 0.01; ***p < 0.001; and not significant (NS) p > 0.05].

3 Results

3.1 LPT Accelerates Oral Keratinocyte Migration
In vitro scratch-induced wound model was chosen to assess the migratory ability of the cells upon irradiation with diode laser [Fig. 1(a)]. Oral keratinocytes responded to diode laser biostimulation by accelerating the epithelial cell migration. Compared with sham-irradiated control group, LPT-treated cells showed significant acceleration of migration in 12 h after scratch (4 J/cm² ***p < 0.001 and 20 J/cm² ***p < 0.01), followed by wound closure within the first 24 h (*p < 0.05 for 4 and 20 J/cm²) [Fig. 1(b)]. Epithelial cells did not show significant differences in migration when irradiated by either 4 or 20 J/cm² energy density (NS p > 0.05).

3.2 LPT Did Not Influence the Proliferation Rate of Oral Keratinocytes
Cell viabilities of control and irradiated groups 24 h after irradiation are represented in Fig. 2. There were no differences in the amount of viable cells among the groups (p > 0.05).

3.3 Diode Laser Irradiation Activates the AKT/mTOR Signaling Pathway in Oral Keratinocytes
To further explore the mechanistic details of accelerated epithelial cell migration, the involvement of the AKT/mTOR signaling pathway especially the phosphorylation levels of S6 protein, a downstream marker of mTOR signaling, was tested. LPT was able to induce the activation of the mTOR signaling in both irradiated groups (4 and 20 J/cm²). Further, treatment of cells with rapamycin, a specific pharmacological inhibitor of

![Fig. 1](https://www.spiedigitallibrary.org/journals/Journal-of-Biomedical-Optics.028002-3(Fig.1).jpg)

Fig. 1 Oral keratinocyte migration upon laser irradiation. (a) Scratch wound assays in NOK-SI oral keratinocytes cell line monolayers from control and LPT groups (4 and 20 J/cm²) during whole experimental time (T0 to T48). Wounds were generated after cell confluence (T0). LPT irradiations were applied three times with 6-h interval. In vitro cell migration was assessed every 12 h. Scale bars represent 50 μm. (b) Graph shows epithelial cell migration, represented as the percentage of open wound of all groups in each experimental time (h). Compared with control group, LPT-treated cells show significant accelerated migration by 12 h after scratch (***p < 0.001—4 J/cm² and **p < 0.01—20 J/cm²), followed by accelerated wound closure within the first 24 h (*p < 0.05). There were no significant differences in migration when comparing both irradiated groups (4 and 20 J/cm²) (NS p > 0.05).
mTOR, completely ablated the LPT-induced phosphorylation of S6 protein (Fig. 3). AKT phosphorylation at serine 473 (AKT

3.4 Low-Power Diode Laser Promotes F-Actin Polymerization

Cell migration requires directional reorganization of the actin cytoskeleton and can be mediated by a variety of extracellular factors including growth factors and extracellular matrix. To test if LPT interferes with the cytoskeletal reorganization during epithelial cell migration, changes in F-actin organization were observed at two time points: 10 min and 12 h after the first irradiation using the optimal energy density of 4 J/cm² as previously established (Fig. 1). It was observed that LPT induced a robust accumulation of F-actin in NOK-SI cells compared with sham irradiation (Fig. 4). It was also observed that LPT induced the polarization of F-actin as early as 10 min after the initial irradiation, which was intensified after 12 h (Fig. 4, arrows). This was also accompanied by changes in cellular morphology (Fig. 4) to one that is typical of highly motile cells.

4 Discussion

Wound healing is a key process essential for tissue repair and restoration of cellular homeostasis. It plays important roles in several physiological and pathological conditions involving ulcerated lesions that constitute significant clinical challenges such as burn ulcers, mucositis, diabetes with slow or nonhealing ulcers, pressure, and chronic venous ulcers. In an epithelio-centric view, wound healing is characterized by a multistep process comprising epithelial proliferation and migration. Notably, epithelial proliferation is often observed at the original wound edge, characterized by an increase in epithelial thickness. However, the initial stages in epithelial cell migration are mainly observed in the epithelial tongue, characterized by the layers of epithelial cells that migrate into the wound bed, thereby promoting healing of the wound. The epithelial tongue is primarily composed of migratory cells and a very few proliferating cells. It is interesting to note that the cells from the epithelial tongue do not undergo a terminal differentiation program compared with the cells localized at proliferating wound edge, suggesting that these two compartments are driven by distinct sets of active molecular processes.

Clinically, LPT has been shown to be effective in wound healing by accelerating the closure of lesions in addition to reducing pain and discomfort. These observations suggest that the LPT may influence mucosal wound healing by accelerating epithelial cell migration over the wounded site. However, only three studies analyzed the effects of LPT on epithelial cells and the molecular mechanisms by which LPT can promote biostimulation in these cells.

In the present study, we sought to explore the specific effects of diode laser irradiation on oral keratinocytes during wound healing using a keratinocyte cell line originally isolated from the retromolar area of the oral cavity. Our results suggest that the LPT accelerates the healing of oral mucosa by activation of oral keratinocytes migration and mTOR signaling pathway independent from cellular proliferative mechanisms.

Recent studies including those from our laboratory have identified key molecules responsible for enhancing and impairing epithelial cell migration. We had shown that the PI3K/mTOR signaling pathway plays a crucial role in driving the re-epithelialization process. In fact, depletion of phosphatase and tensin homolog (PTEN), a negative modulator of the PI3K/mTOR signaling pathway, resulted in accelerated epithelial cell migration and overall wound healing. The identification of new therapeutic strategies to enhance the epithelial wound healing is aimed at the discovery of clinically viable solutions for slow healing ulcers. Recent advances in the field of photo-biomodulation have shown a great deal of promise in this regard. However, molecular mechanisms have not been completely clear. The use of low-power diode laser or
LPT has been shown to modulate several biological processes including wound healing; however, only a few reports have showed the effect of LPT on epithelial cells. In addition, the impact of LPT over keratinocytes from the oral mucosa and the associated molecular mechanisms involved in accelerated migration remain unknown. In the present study, we investigated the effect of LPT on wound-healing process of oral keratinocytes and explored the molecular circuitry involved in epithelial cell migration. To get in-depth analysis on cellular response to laser stimulation, we used two different LPT parameters: 660-nm diode laser/40 mW, 4 and 20 J/cm² with same frequency of irradiation and irradiation time intervals. This choice was made based on the literature which showed that the small energy densities of irradiation lead to better effects compared with higher energy densities in wound healing. These studies were in accordance to the Arndt–Schultz law (e.g., a small stimulus can excite physiological activities, whereas a higher stimulus can inhibit them). Then, 4 and 20 J/cm² were used as lower and higher energy densities, respectively. By maintaining the same power in both groups, the cells were irradiated with energy per point of 0.16 J in respect. By maintaining the same power in both groups, the small energy densities of irradiation lead to better effects compared with higher energy densities in wound healing.43–48 The current mechanism by which LPT induces cellular response involves the activation of mitochondrial respiratory chain. LPT irradiation activates cellular cytochromes that promote a cascade of events leading to the generation of reactive oxygen species (ROS), changes in Ca²⁺ flux, no binding to cytochrome c oxidases that affect the levels of cyclic nucleotides, and DNA and RNA synthesis, thereby modulating a variety of cellular functions.51 We also found that LPT activates the mTOR signaling pathway in epithelial cells. Apart from its role in cell growth, shape, polarity, and size,52-58 mTOR also acts as a metabolic sensor and has been shown to regulate both the resting oxygen consumption and oxidative capacity. Therefore, mTOR activity correlates with the overall mitochondrial function, and pharmacological inhibition using rapamycin significantly reduces mitochondrial membrane potential.59 In all our experimental conditions, we noticed that LPT increased the phosphorylation of S6 protein and rapamycin treatment impaired this phosphorylation, indicating that LPT triggers mTOR signaling pathway. AKT phosphorylation was not influenced by LPT, suggesting that LPT activation of mTOR may be through mTORC1 and independent of mTORC2, which is known to phosphorylate AKT at serine 473. The physiological implications of this mechanism are yet to be elucidated in detail.

**Fig. 4** Low-power diode laser promotes F-actin polymerization. Immunofluorescence images of F-actin staining in NOK-SI oral keratinocytes cell line. Oral keratinocytes cells were seeded on 6-well culture dishes supplemented with 10% FBS. Culture media were substituted by DMEM supplemented with 2% FBS 6 h before the initial irradiation. Two groups were established: control group (0 J/cm²) and 4 J/cm² laser group. The laser groups received three irradiations with 6-h intervals. F-actin staining was performed 24 h after administration of nutritional-deficit medium. Phalloidin (F-actin staining—red), DAPI (DNA staining—blue). LPT induced a robust accumulation of F-actin in treated cells (arrows), associated with the changes in the cellular morphology. Scale bars represent 50 μm.
Our findings have advanced the current understanding on the molecular circuitry involved in the use of a low-power diode laser in oral epithelial cells. Our data strongly indicate that LPT is a promising therapeutic strategy for diseases of the oral cavity such as oral mucositis, resulting from chemotherapy and radiotherapy, herpes simplex infections, and postsurgical management. Nonetheless, new studies are under way to better understand the effect of LPT over oral keratinocyte physiology and to further dissect the molecular mechanisms involved in photobiomodulation.

5 Conclusion
Based on the limits of this in vitro study, we concluded that the improvement of keratinocyte migration after LPT can occur through activation of mTOR signaling pathway.

Acknowledgments
This work was funded in part by the University of Michigan, School of Dentistry startup, by the Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES), and by the Universidade Federal do Rio Grande do Sul (UFRGS). The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript. There was no industrial funding for the study.

References

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