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Low-level laser irradiation promotes the proliferation and maturation of keratinocytes during epithelial wound repair

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Abstract

Low-level laser therapy (LLLT) has been extensively employed to improve epithelial wound healing, though the exact response of epithelium maturation and stratification after LLLT is unknown. Thus, this study aimed to assess the *in vitro* growth and differentiation of keratinocytes (KCs) and *in vivo* wound healing response when treated with LLLT. Human KCs (HaCaT cells) showed an enhanced proliferation with all the employed laser energy densities (3, 6 and 12 J/cm², 660nm, 100mW), together with an increased expression of Cyclin D1. Moreover, the immunoexpression of proteins related to epithelial proliferation and maturation (p63, CK10, CK14) all indicated a faster maturation of the migrating KCs in the LLLT-treated wounds. In that way, an improved epithelial healing was promoted by LLLT with the employed parameters; this improvement was confirmed by changes in the expression of several proteins related to epithelial proliferation and maturation.

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CONFLICT OF INTEREST

The authors wish to declare no conflict of interests.

Keywords

Laser Therapy; Low-Level; Wound Healing; Keratins; p63 protein; Cyclin D1; Photobiomodulation; HaCaT Keratinocytes

1. INTRODUCTION

Low-level laser therapy (LLLT) has shown great efficacy to accelerate wound repair. There are several studies showing the benefits of LLLT in wound healing and injury recovery due to its ability of biostimulation [1–3], which occurs by the interaction of visible or near-infrared light with the cells, promoting the excitation of intracellular chromophores such as endogenous porphyrins, mitochondrial and membranal cytochromes and flavoproteins [4].

After being absorbed by mitochondrial chromophores in the skin cells the photons increase electron transport, adenosine triphosphate production, nitric oxide release, blood flow, reactive oxygen species, and activates diverse signaling pathways that are linked to beneficial effects in dermatology [5]. Among these beneficial effects are skin rejuvenation [6], reduction of acne scars [7, 8], reduction of hypertrophic scars [9], and healing of burns [10, 11]. In addition, faster healing of an aseptic injured epithelium i.e. wounds may also be achieved by LLLT [12].

LLLT is a noninvasive and safe technique that has been widely used to prevent and treat non-healing ulcers [13, 14]. Nevertheless, there is a lack of studies that focus on the maturation and differentiation of the wounded epithelium after LLLT and throughout the healing process, although a study has demonstrated that the use of green LEDs increased the production of HB-EGF and VEGF [15], which do promote the migration of keratinocytes [16]. Most of the published literature is concerned with the clinical and morphological response of wound healing [17, 18] or the response of mesenchymal cells in either soft [19, 20] or hard tissues [21, 22] in tissue repair.

The modulation of inflammatory response and the role of stem cells have also been assessed in several studies that deal with LLLT for wound healing [23–25]. Therefore, this study sought to analyze the ability of LLLT to stimulate the healing process of skin injuries by assessing the proliferation capacity and the maturation state of KCs. In order to do so, the expression of specific cytokeratins and proliferation biomarkers was evaluated in cultured KCs and also in migrating KCs at the wound edge.

2. MATERIALS AND METHODS

All experiments described were performed in compliance with the relevant laws and institutional guidelines and approved by the Ethics Committee of Animal Care of the present institution under the protocol number 11/08.

2.1 Low-level laser device

The low-intensity laser device used in this study was a semiconductor diode laser (Photon Lase III; DMC Equipment, São Paulo, Brazil), 660nm wavelength, output power of 100mW and laser beam area of 0.028cm².

2.2 Light irradiation of cells and cell viability assay

HaCaT cell line [26] was cultured in Eagle medium modified by Dulbecco (Sigma-Aldrich, St. Louis, MO, USA) supplemented with 10% fetal bovine serum and 1% penicillin/streptomycin (Sigma-Aldrich) at 37 °C under 5% CO₂. For the cell viability assay, cells were cultured in 96-well black half-area tissue-culture treated microplates (Greiner – Bio-one, Brazil) at 1×10^4 /well (n=4); incubated for 24 h and then irradiated with LLLT (100mW power; power density of 3.57 W/cm², fluences of 3; 6 or 12 J/cm² (total energy of 84, 168 and 336 mJ) were delivered after 0.84; 1.68 or 3.36 seconds, respectively). The laser tip touched the bottom of the well plates and was always perpendicular to the surface. After light stimulation, cellular viability was determined with a 3-hour MTS assay at 12, 24, 48 and 72 hours (Promega, Wisconsin, USA).

2.3 Immunofluorescence

The cellular localization of CK10 and Cyclin D1 was analyzed with immunofluorescence microscopy (anti-Cytokeratin 10 – mouse/anti-human, Clone DE K10, Dako; anti-Cyclin D1 – rabbit, #2922, Cell Signaling, Danvers, MA, USA). HaCaT cells were seeded on coverslips and then received LLLT (6 J/cm²; 168 mJ); we adjusted and spaced the number of irradiation points accordingly to deliver the same fluence to the same number of seeded cells. Cells were then fixed and permeabilized in cooled absolute methanol at -20 °C for 6 minutes. Briefly, the cells were incubated with a blocking solution (1% bovine serum albumin) for 30 min and followed incubation with each antibody described previously for 90 min at room temperature in a humidified chamber. Next, the cells were washed in PBS (Phosphate Buffered Saline) and incubated with a fluorescein isothiocyanate (FITC) conjugated secondary antibody (Vector Laboratories, Ind. Burlingame, CA, USA) for 45 min in the dark. After PBS washing, the coverslips were mounted using mounting media containing DAPI (Vectashield: DAPI, Vector Laboratories, CA, USA) and imaged with a Zeiss Axio Imager.A1 microscope (Carl Zeiss, Germany).

Images were quantified as the following: four photographs (same magnitude and resolution) of either the laser or control group were randomly selected and set in RGB mode to produce a color histogram that was based on the pixels' brightness in an arbitrary proportional scale between the experimental groups [27].

2.4 Experimental groups and surgical procedure

Forty female rats (*Rattus norvegicus albinus*, Wistar) weighing approximately 220g were subjected to surgical procedures. The animals were caged individually and had free access to water and solid food. The animals were anesthetized with an intraperitoneal injection of chloral hydrate (400 mg/kg) and sodium diethylbarbiturate (50 mg/kg), and shaving of the dorsal region was performed.

The surgical procedure consisted of one circular excision performed with a 6-mm diameter punch on the dorsum of each rat. The animals were randomly divided into two groups of 20 animals each and received the following treatments: Group 1 (control), animals had no local or systemic treatment; Group 2 (LLLT); immediately after the surgical procedure the wounds received laser irradiation. After that, the animals were subdivided into five groups according to the time of killing (1, 3, 5, 7, or 14 days) (n=4).

2.5 In vivo laser irradiation

For the *in vivo* experiment the protocol of laser irradiation followed a previous report of successful healing stimulation with LLLT [12]: contact mode with a fluence of 117.85 J/cm²; total energy of 3.3 J; power of 100mW; power density of 3.57 W/cm² and 33 seconds per point; irradiation was performed as one point on each skin wound.

2.6 Immunohistochemistry

After the surgical and irradiation procedures, full-thickness dorsal samples were obtained from each animal at each time of sacrifice; these samples were fixed in 10% buffered formaldehyde for a period of 24 h and routine laboratory procedures followed until paraffin embedding. Sections of 3 µm were obtained from the paraffin-embedded material, mounted on slides, treated with 3-aminopropyltriethoxy-silane (Sigma Chemical Co., St. Louis, MO, USA), deparaffinized, and hydrated. Endogenous peroxidase was quenched by incubation in 3% hydrogen peroxide in methanol (1:1) for 30 minutes at room temperature. Sections were then treated for antigen retrieval that consisted on 95 °C citric acid bath for 60 minutes.

Immunohistochemistry was performed on a Dako Autostainer (Dako Corp., Carpinteria, CA, USA); naked primary antibodies (Dako) were incubated for 40 minutes (anti-p63 – mouse/anti-human, Clone 4A4, Dako, Denmark; anti-Cytokeratin 10 – mouse/anti-human, Clone DE K10, Dako; and anti-Cytokeratin 14 – mouse, NeoMarkers, Fremont CA, USA). The anti-p63 antibody was elected for immunohistochemistry due to its close relation to epithelium maturation and stratification, since basal epithelial cells that express p63 serve as a source of differentiating cells from the stratified skin epithelium [28, 29].

The primary incubation was followed by peroxidase blocking with 3% H₂O₂ incubation with biotin-labeled anti-mouse secondary antibody, and peroxidase-conjugated streptavidin (Kit LSAB Peroxidase K0690; Dako). Visualization employed a 30-minute incubation with diaminobenzidine (Dako Liquid DAB plus, K3468; Dako) and subsequent counterstaining with Mayer hematoxylin. Negative control samples were treated as above, but using a solution of 1% bovine serum albumin (BSA) in Tris-HCl, pH 7.4 instead of the primary antibody.

2.7 Immunohistochemical analyses

The analysis consisted of dividing the immunohistochemically stained wound areas into 5 fields: center of the wound; immediately adjacent epithelium (left and right sides); and epithelium distant from the wound (left and right sides). The immunohistochemical staining was analyzed for each and every field; the fields were then classified according to the percentage of stained cells (p63 expression) or the stained area (CK10 and CK14

expression) [30, 31] when analyzed with the help of the ImageJ software (National Institutes of Health, Bethesda, Maryland, USA).

2.8 Statistical analysis

The immunohistochemical analyses were further evaluated through two-way ANOVA tests followed by Bonferroni tests, with a level of significance of 5%. The statistical significances concerning cell viability assays and immunofluorescence experiments were assessed through two-way ANOVA tests followed by Bonferroni tests, with a level of significance of 5%. The software used for the analyses was Graphpad Prism 6 (GraphPad Software, Inc; La Jolla, CA 92037, USA).

3. RESULTS

3.1 Keratinocyte proliferation assay

Figure 1 illustrates the response of cultured KCs irradiated with 3; 6 and 12 J/cm² of 660nm low-power laser light. There was a statistically significant difference between the 12 J/cm² and the other groups at 48 hours ($p<0.0001$; $p<0.0001$; and $p<0.0001$, between 12 J/cm² group and control group, 3 J/cm² group and 6 J/cm² group, respectively); however, a very distinctive difference in proliferation rate was observed between laser and control groups at the 72-hour time point ($p<0.0001$; $p<0.0001$; $p<0.0001$, when comparing the control group to 3 J/cm², 6 J/cm² and 12 J/cm² groups, respectively). Still, at 72 hours, the 6 J/cm² showed the highest proliferative capacity, being significantly more proliferative than even the 3 J/cm² group ($p=0.0102$) (Figure 1).

3.2 Immunofluorescence

The HaCaT KCs expressed a meaningful amount of CK10 in culture (Figure 2), though this expression did not differ between the experimental groups. However, Cyclin D1 expression was significantly augmented in the laser-irradiated cells, in agreement with the higher proliferation rates induced by LLLT (Figure 2).

3.3 Immunohistochemical analyses

The *in vivo* experiments showed that there were significant differences in CK10, CK14 and p63 immunohistochemical expression levels between the control and laser groups. Indeed, wounds of the LLLT group closed considerably faster (5 or 7 days after the surgical procedure) than the wounds of the control group (only at 14 days after surgical excision).

While a more proliferative profile was found for the laser group with increased p63 expression versus control at several time points ($p=0.0475$; $p=0.0002$; $p=0.0193$ at 3, 5 and 7 days, respectively) (Figure 3); this higher growth rate was also linked to a more rapid epithelial maturation shown by both CK14 and CK10 expression (Figures 4; 5). The reduced expression of CK14 in the superficial layers of the laser group at 14 days ($p<0.0001$) (Figure 4) as well as the increased expression of CK10 in the superficial layers ($p=0.0011$; $p=0.0098$ at 3 and 5 days, respectively) (Figure 5) of the healing epithelium implied that there was a faster maturation of the tissue produced by LLLT.

4. DISCUSSION

Epithelial integrity is crucial for maintenance of life. When this integrity is lost, the connective tissue underneath the epithelium is exposed, making the organism susceptible to bleeding and infections that could be transitory or even fatal. If the protective barrier represented by the skin is somehow disrupted, there is therefore an urgent need for re-epithelialization.

Normal human interfollicular epidermis primarily consists of KCs [32]; and upon injury the KCs adjacent to the wound must respond quickly to repair the defect. Once wound closure is achieved the KCs undergo differentiation, which consists of a complex series of morphological and biochemical changes in keratin expression and adhesion properties that take place while the cells are differentiating into suprabasal (spinous) KCs [32].

Thus, differentiated KCs such as basal and superficial KCs can be distinguished from each other by the expression of a particular set of intermediate filament proteins [33–37]. In normal epidermis, basal KCs express intermediate filament keratins 5, 14, and 15, while KCs committed to terminal differentiation begin to express keratins 1 and 10 [32]. In this study, the KCs' differentiation and migration was assessed in the repairing epithelium.

The acceleration of wound repair achieved with LLLT is well documented in many models [5, 38, 39] and involves the response of KCs and dermal fibroblasts [15]; however, although several studies have shown that the proliferation of fibroblasts and KCs can be enhanced with light irradiation of different wavelengths [40–44], the mechanisms by which low-power laser irradiation works in this particular case remain unclear.

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 [5]; 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), as utilized herein, 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 that we desired with this methodology [5]; we can also assume that a certain spread of the laser light happened by scattering [45, 46], better distributing the light through the cells either *in vivo* or *in vitro*.

Besides the activation of stem cells that allows for increased tissue repair and healing [5], the pertinent literature shows that specific low-level laser parameters can accelerate wound healing in mice [47]. The healing process starts with clot formation at the wound site and moves on to re-epithelialization characterized by epithelial tongue migration from the wound borders at 72 hours. Wounds can achieve closure at 5 or 7 days with the help of LLLT, while untreated groups only show complete epithelium development at 14 or 16 days after wounding [12, 47]. The results found herein are consistent with the literature, once the wounds of the LLLT group closed considerably faster than the control group.

Previous studies have indeed shown that cells from different origins can be stimulated to grow after LLLT [1, 48–50]. A recent study demonstrated that HaCaT KCs had their

motility enhanced by green and red LED stimulation, which was confirmed by a migration assay [15]. In addition, this same report [15] showed that KCs had the production of HB-EGF and VEGF increased after green LED light irradiation; and these mediators promoted the migration of KCs [16].

In agreement with the previous data, we found that the proliferation of KCs was significantly increased after LLLT, and this increase was detected with all laser fluences employed [48]. Interestingly, a single light irradiation was able to promote enhanced proliferation of KCs, and although the higher fluence (12 J/cm²) did impair the cell growth at 48 hours, the same irradiated cells had a higher cell cycle capacity compared to control cells at 72 hours.

The laser groups showed a higher expression of Cyclin D1, confirming the enhanced cell proliferative index already demonstrated with the cell viability assays. A previous study showed that light irradiation with lower wavelengths could modulate the expression of keratin 1, 10 and involucrin in HaCaT cells [51]. Nevertheless, as these cultured cells were not injured, a different CK10 expression between control and laser groups was not found; in addition, epithelial cells in general behave very differently in 2D culture when compared to *in vivo* [52].

Therefore, we decided to assess epithelial stratification and maturation with an *in vivo* experiment. A previous study showed that rats with a dorsal wound showed faster healing with LLLT (same parameters as utilized herein), which induced enhanced manifestation of young fibroblasts along with a faster closure of the lesions [12]. In agreement with that report, the present study showed that the epithelial tongue cells that migrated towards the wound did indeed express significantly higher amounts of p63, a reliable indicator of epithelial cell proliferation and stratification [28, 29].

The ability to differentiate found in the p63-expressing cells that were light irradiated was linked to a quicker epithelial maturation shown by both CK14 and CK10 expression. There was a faster reduction of CK14 expression in the epithelial superficial layers, as well as an increased expression of CK10 in the superficial layers of the healing epithelium; that implied that LLLT provoked a faster maturation of the proliferating tissue. It is also worth mentioning that the fluence delivered *in vitro* was lower than the used *in vivo*, once a single layer of cultured cells may be much more sensitive to light irradiation than a whole piece of living tissue. Nevertheless, both fluences worked really well in terms of biostimulation.

To the best of our knowledge, this is the first time that the quicker wound healing promoted by LLLT has been linked to the rapid maturation of KCs, which was confirmed by the accelerated expression of CK10 (terminal differentiation biomarker [32]) by these cells. The significantly higher proliferation of *in vivo* KCs (p63 expression) was also confirmed *in vitro* with the improved Cyclin D1 expression for the laser groups. In conclusion, a correlation between the *in vitro* and *in vivo* results was established and may help to elucidate the *in vivo* laser mechanisms as well as to support upcoming studies.

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Figure 1.

Cell viability assay illustrating higher proliferation rates for cells irradiated with low-level laser therapy. Statistically significant results (*) obtained with ANOVA two-way followed by a post-hoc Bonferroni test (level of significance of 5%).

Figure 2.

Immunofluorescent expression of CK10 (red channel) and Cyclin D1 (green channel). Blue color illustrates the nuclei of the cells (DAPI staining). **A** – Control group; **B** – Laser group; **C** – Quantification of immunofluorescence (arbitrary units of pixel brightness) of CK10 and Cyclin D1 for both Control and Laser groups: difference (*) obtained with ANOVA followed by Bonferroni test (level of significance of 5%).

A

Figure 3.

A – Immunohistochemical quantification of p63 expression in the migrating epithelium during the evaluation periods. Statistically significant differences (*) obtained with ANOVA followed by Bonferroni test (level of significance of 5%); and immunohistochemical expression of p63 protein in **B** – Control (scarce in the migrating epithelium at 3 days) and **C** – Laser group (high percentage of stained cells in the migrating epithelium at 3 days) – ME: migrating epithelium; C: crust; CT: connective tissue. Dashed lines indicate the

division between each quantified field: CW: center of the wound; IAE: immediately adjacent epithelium.

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Figure 4.

Immunohistochemical quantification of CK14 expression in the migrating epithelium during the evaluation periods. Statistically significant differences (*) obtained with ANOVA followed by Bonferroni test (level of significance of 5%); and immunohistochemical expression of CK14 protein in **B** – Control (all epithelial layers at 14 days) and **C** – Laser group (attenuated expression restricted to the epithelial bottom layers at 14 days). Dashed lines indicate the division between each quantified field: CW: center of the wound; IAE: immediately adjacent epithelium.

Figure 5.

Immunohistochemical quantification of CK10 expression in the migrating epithelium during the evaluation periods. Statistically significant differences (*) obtained with ANOVA followed by Bonferroni test (level of significance of 5%); and immunohistochemical expression of CK10 protein in **B** – Control (no staining at 5-day evaluation) and **C** – Laser group (advanced expression in the already healed epithelium at 5-day evaluation) – ME: migrating epithelium; C: crust; CT: connective tissue. Dashed lines indicate the division

between each quantified field: CW: center of the wound; IAE: immediately adjacent epithelium.

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