Lasers Med Sci (2018) 33:445–449
DOI 10.1007/s10103-017-2176-z

BRIEF REPORT

# Epithelial cell-enhanced metabolism by low-level laser therapy and epidermal growth factor

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Received: 23 August 2016 / Accepted: 20 February 2017 / Published online: 11 March 2017


**Abstract** Reepithelialization and wound closure are the desired outcome for several ulcerative conditions. Such resolution reduces the possibility of wound contamination and maintenance of the injury and improves the reestablishment of tissue morphology and functions. Investigators are seeking adjuvant therapies that can accelerate wound healing and are developing new strategies for clinical applications. This study compared the effects of epidermal growth factor (EGF) application and low-level laser therapy (LLLT) on cultured epithelial cells. Cells were seeded in 24-well plates. After a 24-h incubation, the epithelial cells were either treated with EGF (100 μM in serum-free DMEM for 72 h) or subjected to LLLT (780 nm, 25 mW, 0.5, 1.5, and 3 J/cm$^2$) by three applications every 24 h. Seventy-two hours after cells were treated with EGF or LLLT, cell migration, viability, proliferation, and collagen synthesis were assessed. Cells treated with EGF showed increased cell viability, proliferation, and collagen synthesis compared with those cells that received no treatment. LLLT enhanced cell migration; however, no significant effects of laser irradiation on other cell functions were observed. Comparison of both therapies demonstrated that EGF and LLLT enhanced specific epithelial cell activities related to wound healing.

**Keywords** Epithelial cells · Low-level laser therapy · Epidermal growth factor · Cell proliferation · Cell migration

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## Introduction

Tissue regeneration is the goal of the healing process, to reestablish local morphology and function [1, 2]. However, wound healing is dependent on several cellular and molecular mechanisms that involve reepithelialization and wound closure [2, 3].

It is known that epithelial cells play a fundamental role in wound healing [4]. Cell proliferation and migration are of particular importance, since these events are responsible for initial wound closure [3, 5]. Therefore, different therapies have been tested to stimulate the repair process, including local application of growth factors and low-level laser therapy [2, 6].

Growth factors are strong mitogenic agents capable of increasing cell proliferation and migration [1]. Epidermal growth factor (EGF) is highly expressed in the epithelium, and several studies have demonstrated that this molecule enhances epithelial cell proliferation [7, 8]. However, the use of EGF for improving skin and mucosal healing has not yet been fully assessed, and the use of growth factors can promote hyperplastic tissue formation [9].

Low-level laser therapy has also shown promising results when applied for cell and tissue biostimulation, mainly in wound-healing models. This therapy, at specific parameters, can increase cell proliferation and migration, in addition to the synthesis of growth factors and other molecules related to tissue regeneration [6, 10–13].

In addition to previous efforts to demonstrate the beneficial effects of different adjuvant therapies, the comparative analysis of available strategies is crucial for clinicians attempting to select from different options. Therefore, this study aimed to evaluate the effects of both therapies, i.e., EGF application or LLLT at selected parameters, on the proliferation and migration of epithelial cells and other cellular functions related to the wound-healing process.

**Table 1** Experimental and control groups

| Groups | EGF | LLLT |
|---|---|---|
| G1 (control) | – | – |
| G2 | 100 μM | – |
| G3 | – | 0.5 J/cm$^2$ |
| G4 | – | 1.5 J/cm$^2$ |
| G5 | – | 3 J/cm$^2$ |

## Materials and methods

### Cell culture

According to the laboratory protocol followed in this investigation, epithelial cells (HaCaT) were seeded at different densities in sterilized 24-well plates with Dulbecco's Modified Eagle's Medium (DMEM, Gibco, Carlsbad, CA) containing penicillin, streptomycin, and glutamine (Gibco) and supplemented with 10% fetal bovine serum (Gibco) for 24 h. The cells were then exposed to EGF or subjected to laser therapy (Table 1).

### EGF application

Cells were treated with EGF (Sigma-Aldrich, St. Louis, MO) at 100 μM in FBS-free DMEM and incubated for 72 h.

### LLLT

In vitro LLLT was performed by means of a specific diode device (LaserTABLE, 780 nm, 25 mW; spot area of 2 cm$^2$; distance of 2.5 cm) at 0.5, 1.5, and 3 J/cm$^2$ [12, 14–16]. These parameters were selected based on the results from previous studies that demonstrated cell biostimulation and acceleration of cell functions related to tissue repair, such as proliferation, migration, and growth factor expression [10, 11, 14]. Cells were subjected to three irradiations every 24 h. All parameters of LLLT used in this investigation are shown in Table 2.

**Table 2** LLLT parameters applied for the study

| | |
|---|---|
| Device information | InGaAsP laser diodes (12 laser diodes) |
| Irradiation parameters | 780 nm ± 5 nm |
| | Continuous wave Gaussian beam profile |
| Treatment parameters | |
| Beam spot size at target | 2 cm$^2$ |
| Distance | 2.5 cm |
| Irradiance at target | 12.5 mW/cm$^2$ |
| Exposure duration | 40, 120, and 240 s |
| Energy denstity | 0.5, 1.5, and 3 J/cm$^2$ |
| Area irradiated | 2 cm$^2$ |
| Number and frequency of sessions | 3 irradiations, each 24 h |

### Cell viability

Cell viability was assessed at the fourth day, 24 h after the final irradiation and 72 h following exposure to EGF. The MTT assay used in this in vitro study was carried out as previously described [11]. Briefly, 1 mL of serum-free DMEM containing 10% MTT solution (methyltetrazolium salt, 5 mg/mL in PBS; Sigma-Aldrich) was applied to cells exposed to laser or EGF and incubated for 4 h at 37 °C. After this period, formazan crystals were dissolved in acidified isopropanol, and absorbance of each sample was analyzed by spectrophotometry (Synergy H1, Biotek, Winooski, VT).

### Cell proliferation

Cell proliferation was detected by crystal violet staining. After being exposed to the laser or EGF, cells were fixed in 70% ethanol for 1 h at 4 °C, stained with 0.1% crystal violet for 15 min at room temperature, and then rinsed with deionized water. Photomicrographs of nine fields (×20 magnification) were obtained by means of an optical microscope (Olympus BX51) equipped with a digital camera (Olympus C5060), from cells treated or not with the laser or EGF. The number of cells for each sample was determined by ImageJ Software (Wayne Rasband, National Institutes of Health, Bethesda, MD).

### Cell migration–wound healing

For the in vitro wound-healing method, cells were seeded in wells of 24-well plates ($8 \times 10^4$ cells/well). After 24 h of incubation at 37 °C, an in vitro wound was created with the tip of a 5-mL micropipette. The cells were then exposed to EGF or LLLT. After treatment, cells were fixed in 70% ethanol at 4 °C, washed with deionized water, and stained for 15 min with 0.1% crystal violet solution (Sigma-Aldrich). Wound areas were evaluated by light microscopy, and wound area was measured by ImageJ Software. Cell migration was detected by evaluation of the reduced wound area, where cell repopulation was noted.

### Total collagen synthesis

Collagen synthesis was determined by the Sirius Red method, with aliquots of culture medium from each sample. After treatment, the culture medium was stored at −20 °C until analysis. Then, a 400-μL quantity of direct red dye (Sigma-Aldrich), diluted at 0.1% in saturated picric acid, was added to 400 μL

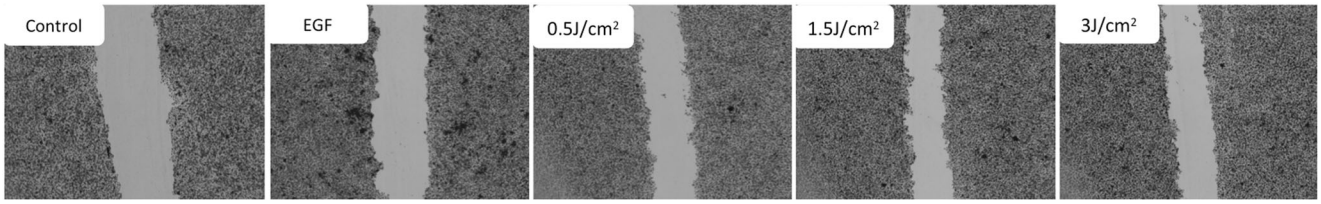


**Fig. 1** Photomicrographs of cell migration (wound areas) of groups subjected to EGF treatment or LLLT (original magnification ×20)

of culture medium and incubated for 1 h at room temperature under shaking (400 rpm). After this, samples were centrifuged at 12,000 rcf for 10 min, and supernatant was gently discarded. Pellets were washed in sodium chloride (0.1 M), and samples were subjected to new centrifugation. Finally, supernatant was discarded again, and pellets were diluted in sodium hydroxide (0.5 M). Two aliquots of each sample (100 μL) were transferred to a 96-well plate, and total collagen was determined by absorbance evaluation at 555 nm in a spectrophotometer (Synergy H1).

### Statistical analysis

Since normal distribution was observed, data were statistically evaluated by ANOVA, complemented by Tukey's test, at a 5% significance level.

## Results

Resulted data demonstrated that the application of EGF on epithelial cells promoted a significant increase on cell proliferation (40%) and viability (20%) as well as collagen synthesis (116%) when compared to control group; however, migration of epithelial cells was not stimulated by EGF treatment (Figs. 1, 2, 3). On the other hand, laser irradiation at 1.5 and 3 J/cm$^2$ enhanced epithelial cell migration in about 32 and 29%, respectively, while this therapy did not stimulate other cell functions evaluated in the present study (Fig. 2).

## Discussion

Significant research effort has been exerted for the development of adjuvant therapies for accelerating wound healing [2]. Previous studies have demonstrated the biostimulatory effects of EGF on fibroblasts and epithelial cells, which presented increased proliferation and metabolism, accelerating tissue repair [7, 8].

This study aimed to supply a comparative evaluation of the effects of EGF and LLLT at specific parameters on the biostimulation of epithelial cells, mainly for cell functions related to the acceleration wound healing, i.e., viability, proliferation, migration, and collagen synthesis [4, 5].

In this in vitro study, epithelial cells exposed to EGF showed enhanced viability, proliferation, and collagen

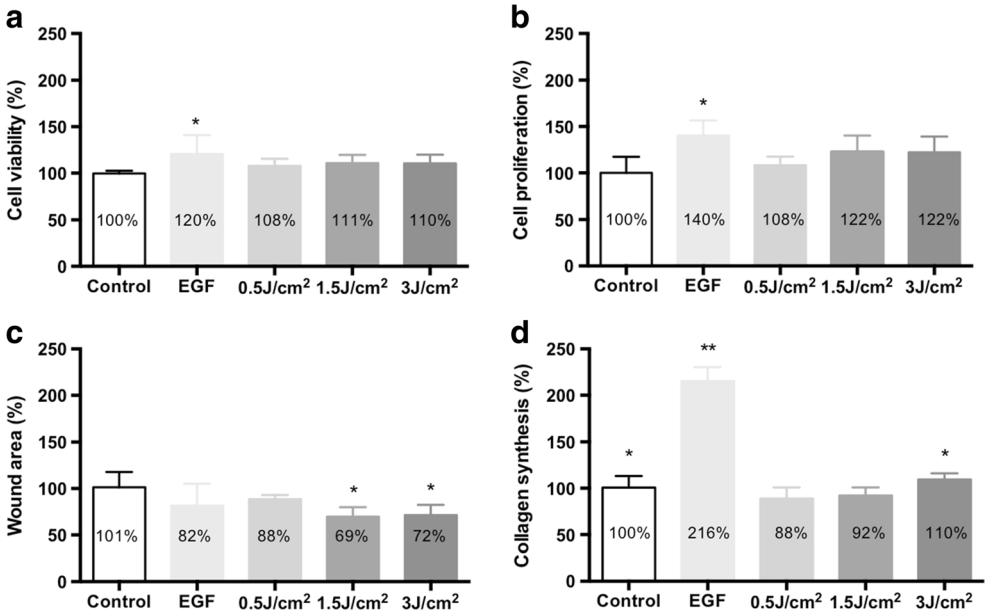


**Fig. 2** Results of cell viability, proliferation, migration, and collagen synthesis for epithelial cells treated with EGF or LLLT. *Bars* indicate mean and standard deviation, $n = 6$. *Asterisks* and *double asterisks* indicate statistically different groups (Tukey, $p < 0.05$)

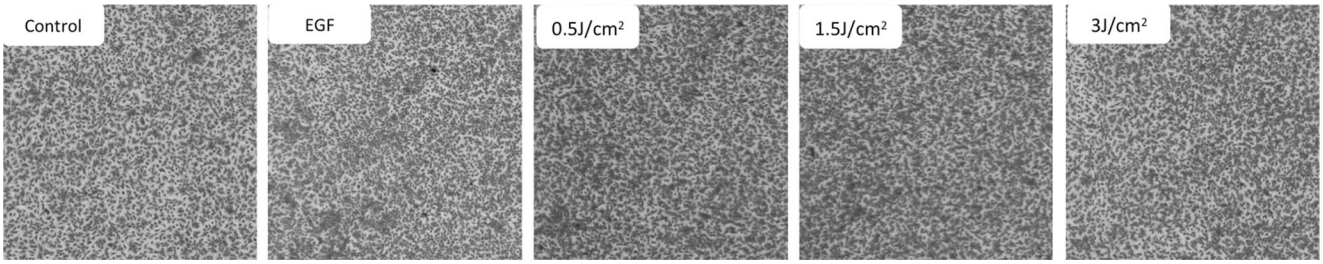


**Fig. 3** Photomicrograps of cells stained in crystal violet for groups treated with EGF or LLLT. Number of cells was evaluated using ImageJ Software (original magnification ×20)

synthesis. The specific parameters of LLLT used in this investigation also increased cell proliferation and migration as well as the synthesis of growth factors and other extracellular molecules that play fundamental roles in wound closure [6, 10–13, 16, 17]. Additionally, Basso et al. (2015) [15] reported that LLLT biomodulates inflammation by reducing the expression and synthesis of inflammatory cytokines, accelerating tissue repair, and avoiding delayed wound healing.

Cell migration is crucial for wound closure, and therefore, the stimulation of this cell function leads to the acceleration of tissue repair [1, 4, 5]. It was previously shown that cell migration may be enhanced by a chemotactic effect, such as low concentrations of inflammatory mediators or increased concentrations of growth factors capable of causing cell attraction and motility [7, 8]. In the present study, EGF exerted a slight tendency toward a chemotactic effect on epithelial cells, decreasing the in vitro wound area. Conversely, LLLT enhanced cell migration, since the mechanically created wound area was decreased by about 30% after laser application at 1.5 and 3 J/cm$^2$. Similar data have been reported by other authors when epithelial cells [13–18] and gingival fibroblasts [11] were subjected to low-power laser irradiation. This positive effect may be caused, at least in part, by increased synthesis of some growth factors, such as vascular endothelial growth factor (VEGF), basic fibroblastic growth factor (bFGF), and even EGF by cells subjected to laser irradiation [11].

While a significant increase of total collagen synthesis occurred when the epithelial cells were exposed to EGF treatment, LLLT did not affect this cellular function. Collagen is one of the major extracellular proteins involved in the wound healing process [5]. For epithelial tissue, synthesis of collagen type IV is related to the maintenance of tissue organization [5]. However, exacerbated synthesis of collagen may also result in hyperplastic formation [9]. In the present investigation, EGF promoted a massive increase in total collagen synthesis. In spite of the fact that the data obtained from our in vitro study cannot be directly extrapolated to clinical situations, one may suggest that the local application of growth factors, such as EGF, may be considered with caution. Therefore, further in vivo studies are needed.

In general, the concentration of EGF tested in this study stimulated more cell functions than the specific parameters of LLLT used. However, the laser irradiation of epithelial cells enhanced migration of epithelial cells, which is a major event that takes place at the beginning of wound healing. Based on the methodology used in the present in vitro study, it can be concluded that both therapies, EGF and laser irradiation, can biostimulate cultured epithelial cells, improving their functions relative to oral mucosal healing.

**Compliance with ethical standards**

**Funding** This study was funded by São Paulo State Research Support Foundation – FAPESP (Grant: 2013/05879-0) and the National Council for Scientific and Technological Development – CNPq (Grants: grants: 303599/2014, 307696/2014 and 157779/2015-7).

**Conflict of interest** The authors declare that they have no conflicts of interest.

**Ethical approval and informed consent** Not applicable.

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