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ORIGINAL ARTICLE

# Wound-healing effects of 635-nm low-level laser therapy on primary human vocal fold epithelial cells: an in vitro study

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**Abstract**

Low-level laser therapy (LLLT) has been promoted for its beneficial effects on tissue healing and pain relief for skin and oral applications. However, there is no corresponding literature reporting on vocal fold wound healing. Our purpose was to assess the potential wound-healing effects of LLLT on primary human vocal fold epithelial cells (VFECs). In this study, normal vocal fold tissue was obtained from a 58-year-old male patient who was diagnosed with postcricoid carcinoma without involvement of the vocal folds and underwent total laryngectomy. Primary VFECs were then cultured. Cells were irradiated at a wavelength of 635 nm with fluences of 1, 4, 8, 12, 16, and 20 J/cm$^2$ (50 mW/cm$^2$), which correspond to irradiation times of 20, 80, 160, 240, 320, and 400 s, respectively. Cell viability of VFECs in response to varying doses of LLLT was investigated by the Cell Counting Kit-8 (CCK-8) method. The most effective irradiation dose was selected to evaluate the cell migration capacity by using the scratch wound-healing assay. Real-time polymerase chain reaction (RT-PCR) was used to detect the gene expression of TGF-β1, TGF-β3, EGF, IL-6, and IL-10. Irradiation with doses of 8 J/cm$^2$ resulted in 4% increases in cell proliferation differing significantly from the control group ($p < 0.05$). With subsequent doses at 48 and 72 h after irradiation, the differences between the experimental and the control groups became greater, up to 9.8% ($p < 0.001$) and 19.5% ($p < 0.001$), respectively. It also increased cell migration and the expression of some genes, such as EGF, TGF-β1, TGF-β3, and IL-10, involved in the tissue healing process. This study concludes that LLLT at the preset parameters was capable of stimulating the proliferation and migration of human vocal fold epithelial cells in culture as well as increase the expression of some genes involved in tissue healing process. Additionally, successive laser treatments at 24 h intervals have an additive beneficial effect on the healing of injured tissues.

**Keywords** Low-level laser therapy (LLLT) · Vocal fold epithelial cells (VFECs) · Wound healing · Anti-inflammation

## Introduction

Vocal folds are vulnerable to injury from trauma, penetrating neck wounds, surgical intubation, laryngeal infections, inflammation, and other mechanical, biological, and chemical challenges. Aberrant healing from injury or scarring can result in the loss of the vibratory function of the vocal folds, negatively affecting quality and efficiency of voice production. Currently, treatment options for vocal scars produce inconsistent and often unsatisfactory results [1]. Thus, a more efficacious management method is needed to promote vocal fold wound healing while reducing scarring.

Low-level laser therapy (LLLT) has been used in the treatment of several oral diseases and wounds to the skin with the main goal of stimulating tissue healing capacity, such as oral mucositis [2], surgical incisions, and perhaps the most difficult to treat ulcerations [3, 4]. Tissue healing involves an intense activity of diverse cell types such as epithelial cells and fibroblasts which play a key role in this process [5]. Previous studies have evaluated the effect of LLLT on the proliferation and migration of human gingival fibroblasts as well as other cellular effects and responses such as protein production and growth factor expression [6]. Nevertheless, there is no literature on vocal fold epithelial cells (VFECs) after LLLT. The reason for this may be due to the lack of vocal fold epithelial cell line.

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Vocal folds are a well-defined layered structure consisting of numerous tissue types including epithelium, lamina propria, and muscle. Among them, the multilayered epithelium protects the underlying tissues from environmental and mechanical insults. Once injured, vocal fold epithelial cells likely play an important role in tissue healing by the expression of growth factors [7]. In addition, the epithelium is the first tissue to be irradiated during the treatment of vocal fold lesions with LLLT. Therefore, therapies that can biostimulate and increase the proliferation of epithelial cells could have a beneficial effect on the healing of injured tissues. Considering the beneficial effects of LLLT in dermatology and dentistry, we hypothesized that it may have the potential wound-healing effects on primary human vocal fold epithelial cells. Thus, the aim of this study was to investigate the effect of LLLT on vocal fold epithelial cells by evaluating their proliferation, migration, and gene expression involved in the tissue healing process after irradiation with a diode laser.

## Materials and methods

### Epithelial cell culturing

Normal vocal fold tissue was obtained from a 58-year-old male at the Department of Otolaryngology—Head and Neck Surgery at the Eye, Ear, Nose, and Throat Hospital of Fudan University in Shanghai, China. The patient was diagnosed with postcricoid carcinoma without involvement of the vocal folds and underwent total laryngectomy. This study was approved by the local ethics committee, and informed consent was obtained from the patient. A 3 × 3 mm$^2$ area of normal vocal fold mucosa was identified by an experienced surgeon (Chief of Surgery, 20 years of clinical experience) and carefully excised under the supervision of a pathologist. The surrounding mucosa was histologically examined to confirm absence of tumor invasion. Primary VFECs were cultured according to the methods published by Leydon [8] and Mizuta [9]. Briefly, vocal folds were washed with sterile phosphate-buffered saline (1 × PBS; Hyclone, Logan, UT, USA) and soaked overnight at 4 °C in dispase II (2 U/ml; Roche, Indianapolis, IN, USA) to separate the epithelium from the underlying lamina propria. Sheets of epithelium were placed in 0.25% trypsin EDTA (Life Technologies, Paisley, UK) for 10 min to isolate cells, then vortexed by Vortex Mixers (Kylin-Bell, Jiangsu, China), and submerged in soybean trypsin inhibitor (STI; Sigma, St. Louis, MO, USA). The cell suspension was filtered through a cell strainer (40 μM; Falcon, BD Biosciences, San Diego, CA, USA) and centrifuged in the Thermo Scientific refrigerated centrifuge at 188 g for 5 min at 4 °C. Supernatant was removed, and isolated cells were cultured in culture medium. The medium was composed of F12/DMEM (3:1 ratio; Sigma, St. Louis, USA), fetal bovine serum (2.5% FBS; Gibco, USA), hydrocortisone (0.4 μg/ml; Sigma, St. Louis, MO, USA), cholera toxin (8.4 ng/ml; Sigma, St. Louis, MO, USA), insulin (5 μg/ml; Sigma, St. Louis, MO, USA), adenine (24 μg/ml; Sigma, St. Louis, MO, USA), epidermal growth factor (10 ng/ml EGF; Sigma, St. Louis, MO, USA), penicillin (100 U/ml; Sigma, St. Louis, MO, USA), and streptomycin (0.01 mg/ml; Sigma, St. Louis, MO, USA). Cells were plated in a 25-cm$^2$ tissue culture flask, placed in a 37 °C incubator (Thermo Fisher Scientific, MA, USA) with 5% $CO_2$. Epithelial cells were used between the third and ninth passages.

### Identification of epithelial cells

VFECs were identified by immunocytochemistry staining of Cytokeratin13 (CK13; Abcam, Cambrigde, UK) [9]. VFE cells were fixed in 4% paraformaldehyde (Sigma, St. Louis, MO, USA) overnight at 4 °C. Then, cells were washed with phosphate-buffered saline (1 × PBS; Hyclone, Logan, UT, USA) and incubated in serum-blocking solution (3% bovine serum albumin; Sigma, St. Louis, MO, USA). Cells were then incubated with anti-cytokeratin13 antibody (1:100; Abcam, Cambrigde, UK) at a dilution of 1:100 overnight at 4 °C. After rinsing with PBS, cells were incubated with fluorescein isothiocyanate-labeled goat anti-rabbit secondary antibody (1:1000; Jackson ImmunoResearch, West Grove, PA, USA) and Hoechst (Sigma, St. Louis, MO, USA) for 60 min. Then, the cells were visualized with a fluorescence microscope (Nikon, Tokyo, Japan).

### Low-level laser irradiation

The laser device used in this study was a diode laser (Hi-Tech Optoelectronics, Beijing, China) with a wavelength of 635 ± 5 nm (central wavelength, 635 nm). The power output was measured by a Liconix laser power meter (Molecular Devices, Sunnyvale, CA, USA). Power measurements were made at the center point of the incident beam spot and at each quadrant 1 cm from the center of the incident beam. The power density was maintained at an average irradiation of 50 ± 0.36 mW/cm$^2$ within the 35-mm-diameter spot (well diameter of 6-well plates). For the evaluation of cell proliferation, the radiation was delivered on the base of each 96-well plate (Thermo Fisher Scientific, MA, USA) with energy doses of 1, 4, 8, 12, 16, and 20 J/cm$^2$, and irradiation times of 20, 80, 160, 240, 320, and 400 s, respectively. The 96-well plates containing the control cells were maintained at the same irradiation times used in the respective irradiated groups, though without activating the laser source (sham irradiation). After 24-h irradiation (active or sham), the proliferation of the cells was evaluated using the CCK-8 assay (described below). In order to investigate a possible effect of successive laser treatments, irradiation was carried out twice and thrice with a 24-h interval prior to analyzing the cultured cells. Based on cell proliferation assay results, the most effective irradiation dose was selected to evaluate the cell migration capacity by using the scratch wound-healing assay and to detect the gene expression of transforming growth factor beta1 (TGF-β1), epidermal growth factor (EGF), interleukin-6 (IL-6), interleukin-10 (IL-10), and transforming growth factor beta3 (TGF-β3) by using the real-time polymerase chain reaction (RT-PCR), as described below.

### Cell proliferation assay

VFECs were seeded in 96-well plates at an initial density of $5 \times 10^3$ cells/well and incubated for 12 h at 37 °C under 5% $CO_2$ atmosphere prior to exposure to laser irradiation. For the cell proliferation assay, 100 μL F12/DMEM (3:1 ratio) with 10% Cell Counting Kit-8 (CCK-8, Yeasen, Shanghai, China) solution was added to each well. After 3 h, the absorbance was measured at 450 nm using a Synergy H1 multimode reader (BioTek, Winooski, VT, USA).

### Scratch wound-healing assay

VFECs were plated into 6-well plates at $3 \times 10^5$ cells/well. LLLT was performed, and a linear wound was immediately created using a sterile 100 μL tip (Sigma, St. Louis, MO, USA). Cells were then cultured in culture medium. Photos were taken immediately 6, 12, and 36 h after treatment via an inversion fluorescence microscope (Olympus, Tokyo, Japan) and analyzed using the software ImageJ (National Institutes of Health, Bethesda, MD, USA). Wound closure rates were defined as the mean rates of blank area reduction at five selected points of each well.

### Gene expression analysis

Cells were collected, and real-time polymerase chain reaction (RT-PCR) was performed at 12, 24, 48, and 72 h after LLLT. Total RNA was extracted using Ultrapure RNA Kit (CWBio, Shanghai, China). Quantification of total mRNA was performed by spectrophotometry (NanoDrop Technologies, Inc., Wilmington, NC, USA). Reverse transcription was performed using PrimeScript RT Master Mix (RR036; Takara, Kusatsu, Japan). The cDNA was then subjected to real-time RT-PCR reactions for quantification of mRNA levels of GAPDH, TGF-β1, EGF, IL-6, IL-10, and TGF-β3 using the SYBR Premix EX Tap (Takara, Kusatsu, Japan). Primer sequences used to amplify various cDNA are shown in Table 1. A typical real-time RT-PCR protocol was performed under the following conditions: 30 s at 95 °C, followed by 40 cycles (95 °C denaturing for 5 s; 60 °C annealing for 30 s), melting at 60 °C until 95 °C for 90 s, and finally cooling to 35 °C. The specificity of the SYBR Green assays was confirmed by melting point analysis. Gene expression of the housekeeping gene glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used for normalization of the results. The delta-delta cycle threshold (ΔΔCt) was used to calculate the relative fold changes.

### Statistical methods

The cell metabolism data had a normal distribution and were analyzed statistically by one-way ANOVA and Tukey's test for multiple comparisons of groups. Data for the gene expressions had a non-normal distribution and were analyzed statistically by the Kruskal-Wallis and Mann-Whitney nonparametric tests. According to the sample size, we estimated the power value (1-β) was greater than 0.8. A significance threshold of $\alpha = 0.05$ was used. Power analysis was performed with the use of the NCSS-PASS computer program (NCSS, Kaysville, UT, USA). All data were analyzed using SPSS 22 software (IBM, Armonk, NY, USA).

## Results

### Analysis of cell proliferation (CCK-8 assay)

VFECs were identified with anti-cytokeratin13 antibody (Fig. 1). Results of proliferation assay in both experimental and control groups are illustrated in Fig. 2. The cells irradiated with 1 J/cm$^2$, 4 J/cm$^2$, 12 J/cm$^2$, 16 J/cm$^2$, and 20 J/cm$^2$ presented no statistically significant difference between the irradiated group and non-irradiated control group ($p > 0.05$). Conversely, irradiation with doses of 8 J/cm$^2$ resulted in a 4% increase in cell proliferation differing significantly from the control group ($p < 0.05$, power = 0.947) (Fig. 2a). Then, we irradiated the cells with a dose of 8 J/cm$^2$ for three successive times at 24 h intervals (Fig. 2b). On day 1, results showed a 3.6% increase in cell proliferation compared to the control group ($p < 0.05$, power = 0.893). At 48 and 72 h after irradiation, the differences between the case and the control groups became greater, up to 9.8% ($p < 0.001$, power = 0.983) and 19.5% ($p < 0.001$, power = 0.991), respectively. The results of this in vitro study revealed that increased levels of cell proliferation could be achieved if successive treatments were given to the cells to show the effects of laser irradiation on the cell proliferation rate.

### Cell migration assay

A scratch wound-healing assay was used to evaluate the effect of LLLT on cell migration (Fig. 3). Twelve and 36 h after LLLT, the migration of epithelial cells was significantly increased ($p < 0.01$, power = 0.993), especially 36 h ($p < 0.001$, power = 1.000). Therefore, treatment with LLLT was associated with significant changes to VFECs migration.

**Table 1** Sequences of primers used for real-time polymerase chain reaction

| Target | Oligo | Sequence | GenBank ACC |
|---|---|---|---|
| GAPDH | Forward primer | 5′-TGAAGGTCGGAGTCAACGGATT-3′ | NM_001289746.1 |
| | Reverse primer | 5′-TTGACGGTGCCATGGAATTTGC-3′ | |
| TGF-β1 | Forward primer | 5′-CGACTCGCCAGAGTGGTTAT-3′ | NM_000660.6 |
| | Reverse primer | 5′-TAGTGAACCCGTTGATGTCCA-3′ | |
| EGF | Forward primer | 5′-GCCATGCTCCAGCAAAATCAA-3′ | NM_001963.5 |
| | Reverse primer | 5′-GTGCAGGACCCACACAAGTAG-3′ | |
| IL-6 | Forward primer | 5′-CATCCTCGACGGCATCTCAG-3′ | NM_000600.4 |
| | Reverse primer | 5′-AGCTCTGGCTTGTTCCTCAC-3′ | |
| IL-10 | Forward primer | 5′-TACGGCGCTGTCATCGATTT-3′ | NM_000572.3 |
| | Reverse primer | 5′-TAGAGTCGCCACCCTGATGT-3′ | |
| TGF-β3 | Forward primer | 5′-ATGACCCACGTCCCCTATCA-3′ | NM_003239.4 |
| | Reverse primer | 5′-CAGACAGCCAGTTCGTTGTG-3′ | |

### Expression profiling of LLLT-treated VFECs

We explored the time-dependent effects of LLLT on the mRNA levels of genes associated with wound healing. Level of IL-6 was significantly reduced, and levels of EGF, TGF-β1, TGF-β3, and IL-10 were elevated (Fig. 4). The expression of some genes returned to normal levels 72 h after LLLT, including EGF, TGF-β3, and IL-6. Other genes retained differences for more than 72 h.

## Discussion

Low-level laser therapy is typically of narrow spectral width in the red or near-infrared spectrum (600–1000 nm), with a power density between 1 mW and 5 W/cm$^2$. Because of its low-power range (output power below 500 mW), the effects of the low-level laser on treated are commonly attributed to non-thermal photobiostimulation [10]. LLLT was first used by Mester et al. for treatment of chronic ulcers that were not responsive to routine treatments [11]. Recent studies have demonstrated that LLLT is capable of promoting proliferation and increasing gene expression and protein production of cells in addition to attenuating inflammatory processes and accelerating tissue healing when used in the treatment of oral alterations [2, 12]. However, there is no study on LLLT in the treatment of vocal fold wound healing. In this study, we cultured primary human vocal fold epithelial cells to study vocal fold healing post-injury. Vocal fold epithelial cells play an important role in tissue healing for its expression of some growth factors, and it is the first tissue to be irradiated during the treatment of vocal fold lesions with LLLT. Many studies have demonstrated that the photobiomodulation effect of LLLT depends on the wavelength and energy density applied to the target cells [13]. In the present study, we used a diode laser with a wavelength of 635 nm to irradiate the epithelial cells. This is because the wavelength between 600 and 700 nm is regarded as the most effective [14, 15]. We found that cells irradiated with doses of 8 J/cm$^2$ have a significant beneficial effect on proliferation while cells irradiated with 1 J/cm$^2$, 4 J/cm$^2$, 12 J/cm$^2$, 16 J/cm$^2$, and 20 J/cm$^2$ presented no statistically significant differences. We hypothesized that lower energy density with relatively short exposure time did not produce enough cytokines to promote cell proliferation, and when the energy density was more than 10 J/cm$^2$, according to the research of the Hawkins et al. [16], it decreased the cell viability and mitochondrial activity with an increase in percentage cytotoxicity and DNA damage. Thus, the energy density of 8 J/cm$^2$ is the most effective irradiation dose to promote cell proliferation, and the results of downward trend of cell viability under doses of 12 J/cm$^2$, 16 J/cm$^2$, and 20 J/cm$^2$ are consistent with those of Hawkins et al.

Vocal fold healing post-injury is likely dictated by the interactions between various cells types, growth factors and components of the extracellular matrix. Here, we focused on two endogenous growth factors that are known to play key

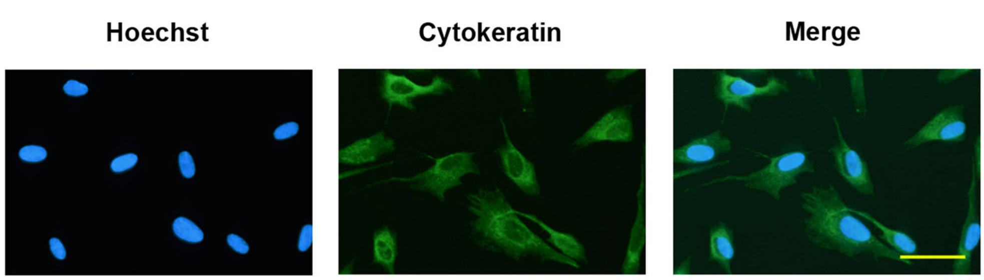


**Fig. 1** Identification of VFECs with anti-cytokeratin13 antibody. Hoechst in blue, cytokeratin in green, and merged pictures are shown. VFECs vocal fold epithelial cells. Scale bar represents 50 μm

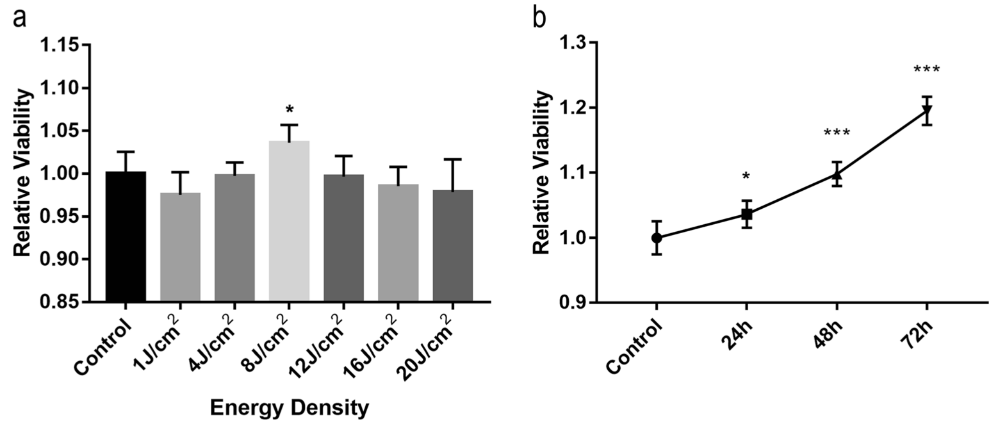


**Fig. 2** Proliferation of human vocal fold epithelial cells was analyzed by CCK-8 assay. **a** VFECs were incubated for 12 h and irradiated with energy doses of 1, 4, 8, 12, 16, and 20 J/cm$^2$. Relative viability was analyzed by CCK-8 assay 24 h after irradiation. **b** Irradiation was carried out twice and thrice with at 24 h intervals to investigate a possible effect of successive laser treatments. *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$ versus control. CCK-8, cell counting kit-8; VFECs, vocal fold epithelial cells

roles in wound healing, epidermal growth factor (EGF), and transforming growth factor beta (TGF-β). EGF is secreted by various cell types involved in wound healing including epithelial cells [17], fibroblasts, macrophages, and platelets [18]. Endogenous EGF has been shown to play a critical role in guiding acute [19] and chronic [20] epithelial response to damage through paracrine and autocrine signaling in airway epithelia. Further, it promotes epithelial regeneration by regulating intercellular junction disassembly and increasing cell proliferation after injury [21]. The role of TGF-β has been better explored in vocal fold wound healing; however, its effects are not fully understood. TGF-β has three isoforms, TGF-β1, TGF-β2, and TGF-β3. TGF-β1 and TGF-β2 are associated with fibrotic healing in adult wounds, whereas TGF-β3 is associated with regenerative healing in fetal wounds [22, 23]. It has been shown that LLLT can trigger natural intracellular photobiochemical reactions involved in wound healing, including TGF-β modulation [24–26]. In this study, we found the LLLT could stimulate the epithelial cells to release EGF, TGF-β1, and TGF-β3. As we know, TGF-β1 induces collagen secretion and fibroblast differentiation to promote tissue repair. However, if this tissue repair phase does not properly terminate, persistent myofibroblast activation can lead to excessive wound contraction, extracellular matrix (ECM) deposition, and eventually scar formation. TGF-β3 could stimulate sufficient fibroblast activation and differentiation to achieve wound closure and maintain appropriate ECM synthesis while protecting against the excessive activation that is associated with fibrosis [27]. Although TGF-β1 and TGF-β3 have different effects on wound healing, we hypothesize that concurrent secretion can produce synergistic effects that promote wound healing while reducing scar formation.

The effective control of inflammation is of great importance in accelerating wound healing. The interleukins (IL-6 and IL-10), as inflammation cytokines, have a major role in the modulation of inflammation [28]. IL-6 is a cytokine with a central role in inflammation after injurious processes. In acute inflammation, we often see an increase in IL-6 [29]. In contrast, IL-10 is known for its anti-inflammatory activity. IL-10 inhibits both the expression of the interleukins (IL-6) and the migration of inflammatory cells at the lesion site including macrophages and monocytes [30, 31]. It has recently been proposed that poor regulation of interleukin (IL)-6 signaling

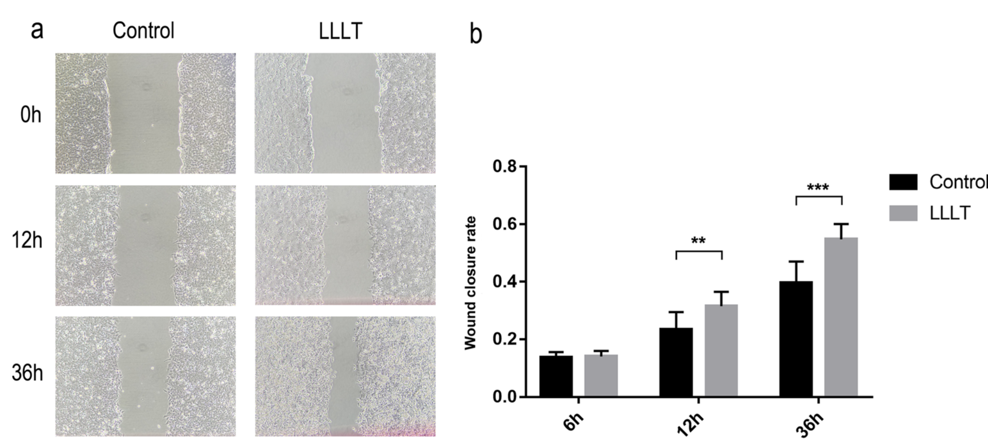


**Fig. 3** Cell migration ability was increased by LLLT using scratch wound-healing assay. **a** Scratches were made immediately after treatment, and representative images were obtained immediately, 12, and 36 h after scratch. **b** LLLT increased the wound closure rate of VFECs. *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$ versus control. LLLT, low-level laser therapy; VFECs, vocal fold epithelial cells

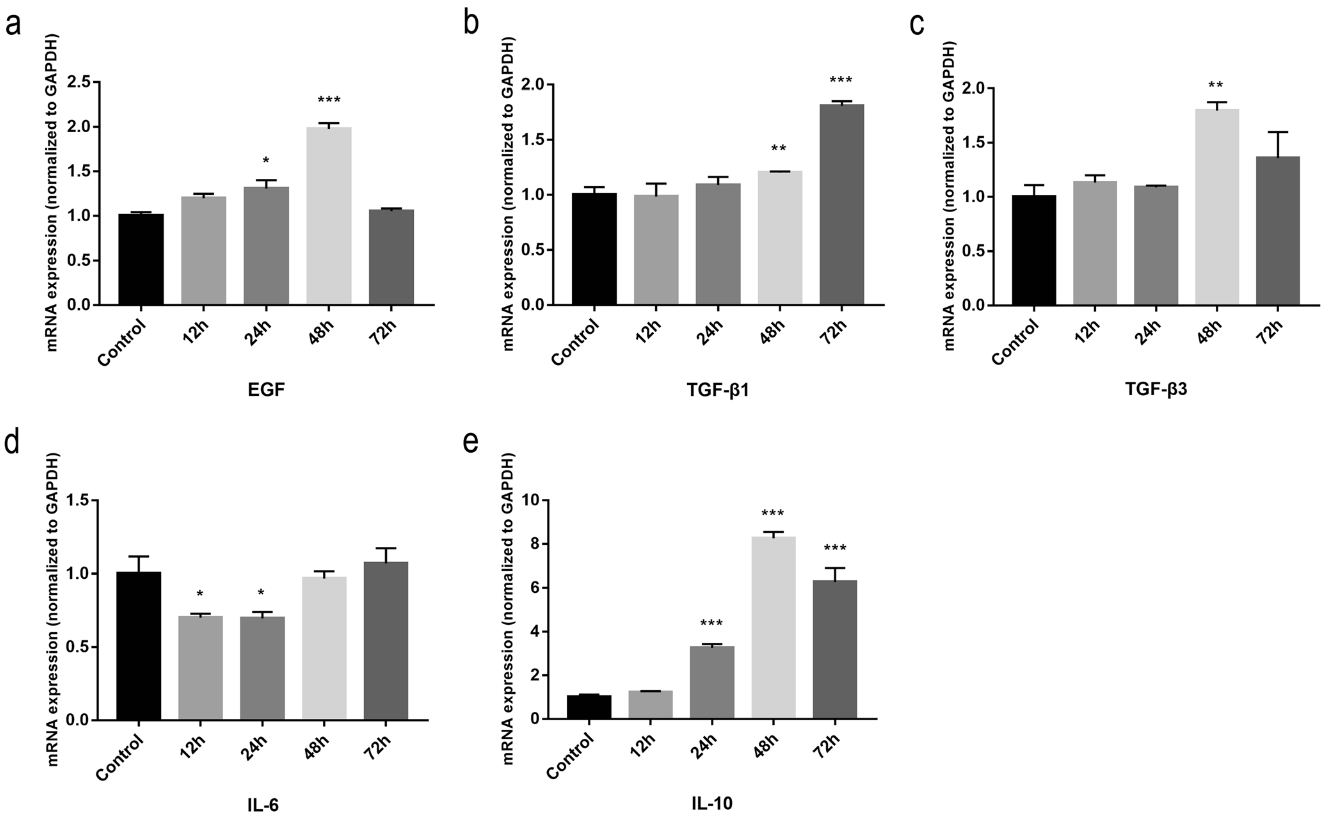


**Fig. 4** mRNA levels of genes related to wound healing after LLLT. VFECs were treated with LLLT, and real-time polymerase chain reaction was performed 12, 24, 48, and 72 h after LLLT. The values are represented by the mean values and error bars are SDs. $*p < 0.05$; $**p < 0.01$; $***p < 0.001$ versus control. EGF, epidermal growth factor; TGF-β1, transforming growth factor beta1; TGF-β3, transforming growth factor beta3; IL-6, interleukin-6; IL-10, interleukin-10; VFECs, vocal fold epithelial cells

pathways has a significant role in abnormal wound healing, and many previous studies have demonstrated the effects of LLLT on decreasing IL-6 [32] and increasing IL-10 [33] mRNA levels. In this study, we found that the LLLT can promote the epithelial cells to release the IL-10 and inhibit the production of IL-6, which provides a theoretical basis for accelerating the wound healing of injured vocal cord tissue.

Although much of the mechanism of action of lasers on the tissue is mediated via photothermal effects, LLLT typically causes low or imperceptible temperature changes [34]. In the study of Schindl A et al. [35], the investigators used a 632-nm HeNe laser to irradiate the skin on the foot and found an inconspicuous temperature increase of 1.06 °C at the end of 30 min of treatment, which would not cause thermal damage to the tissue. Considering no significant temperature rise is found, biologic change may be produced by LLLT. However, the mechanism associated with the cellular photobiostimulation by LLLT is not yet fully understood. Currently, most researchers believe that the basic biological mechanism behind the effects of LLLT is thought to be through absorption of red and NIR light by mitochondrial chromophores, in particular cytochrome c oxidase (CCO) which is contained in the respiratory chain located within the mitochondria [36]. Then, the absorption of light energy may cause photodissociation of inhibitory nitric oxide from CCO leading to enhancement of enzyme activity, electron transport, mitochondrial respiration, and adenosine triphosphate (ATP) production [37]. In general, LLLT alters the cellular redox state which induces the activation of numerous intracellular signaling pathways and alters the affinity of transcription factors concerned with cell proliferation, survival, tissue repair, and regeneration. Further experiments are required to fully understand this phenomenon.

Low-level laser therapy has been used in the treatment of several oral diseases and wounds to the skin with the main goal of stimulating tissue-healing capacity, such as oral mucositis, surgical incisions, and ulcerations. In the field of laryngology, there is no related research about the impact of LLLT on the superficial lamina propria and vocal fold mucosal wave at present. In the future, we hope this LLLT can treat Reinke's edema and reflux laryngitis and promote the repair of vocal folds after operation. We plan to operate under topical anesthesia with fiberoptic laryngoscope in the office, which is feasible in theory, because we often use the potassium-titanyl-phosphate (KTP) laser to treat laryngeal lesions such as papillomas, polyps, and even early glottis cancer under topical anesthesia through the channel of a flexible laryngoscope in the office. Of course, this study has many limitations. For instance, we obtain the normal vocal fold tissue from one patient, which may cause selective bias to the results of this experiment. However, it is indeed difficult to derive many samples for this type of study. In addition, we only studied the effect of low-level laser on vocal fold epithelial cells. The vocal folds as a whole have many cells involved in wound healing, in which the role of fibroblasts cannot be negligible. In a follow-up study, we will co-culture epithelial cells with fibroblasts to study the effects of low-level lasers on both cells and the interaction between cells.

## Conclusions

Based on the finding of the present study, it could be demonstrated that LLLT at the preset parameters was capable of stimulating the proliferation and migration of human vocal fold epithelial cells in culture as well as the expression of some genes involved in the tissue healing process. Additionally, considering the irradiation protocols used in this in vitro study, LLLT at an energy dose of 8 J/cm$^2$ was the most effective in promoting biostimulation of the cultured cells. Successive laser treatments at 24 h intervals have an additive beneficial effect on the healing of injured tissues.

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### Compliance with ethical standards

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

**Ethical approval** Ethical approval was obtained from the ethics committee of the Eye, Ear, Nose, and Throat Hospital of Fudan University (2017061).

**Informed consent** Informed consent was obtained from all individual participants included in the study.

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