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# Comparative study on Photobiomodulation between 630 nm and 810 nm LED in diabetic wound healing both *in vitro* and *in vivo*

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Photobiomodulation (PBM) promoting wound healing has been demonstrated by many studies. Currently, 630 nm and 810 nm light-emitting diodes (LEDs), as light sources, are frequently used in the treatment of diabetic foot ulcers (DFUs) in clinics. However, the dose–effect relationship of LED-mediated PBM is not fully understood. Furthermore, among the 630 nm and 810 nm LEDs, which one gets a better effect on accelerating the wound healing of diabetic ulcers is not clear. The aim of this study is to evaluate and compare the effects of 630 nm and 810 nm LED-mediated PBM in wound healing both *in vitro* and *in vivo*. Our results showed that both 630 nm and 810 nm LED irradiation significantly promoted the proliferation of mouse fibroblast cells (L929) at different light irradiances (1, 5, and 10 mW/cm$^2$). The cell proliferation rate increased with the extension of irradiation time (100, 200, and 500 s), but it decreased when the irradiation time was over 500 s. Both 630 nm and 810 nm LED irradiation (5 mW/cm$^2$) significantly improved the migration capability of L929 cells. No difference between 630 nm and 810 nm LED-mediated PBM in promoting cell proliferation and migration was detected. *In vivo* results presented that both 630 nm and 810 nm LED irradiation promoted the wound healing and the expression of the vascular endothelial growth factor (VEGF) and transforming growth factor (TGF) in the

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wounded skin of type 2 diabetic mice. Overall, these results suggested that LED-mediated PBM promotes wound healing of diabetic mice through promoting fibroblast cell proliferation, migration, and the expression of growth factors in the wounded skin. LEDs (630 nm and 810 nm) have a similar outcome in promoting wound healing of type 2 diabetic mice.

*Keywords*: Photobiomodulation (PBM); light-emitting diode (LED); wound healing; diabetic ulcers.



## 1. Introduction

Type 2 diabetes mellitus (DM2) is a serious health problem with a high prevalence in the world. It is estimated that more than 360 million people will suffer the DM2 by 2030.[1] Diabetic foot ulcers (DFUs) are one of the most severe complications of patients with DM2. It is about 15% of patients will dispose to DFU during their lifetime. Long-term DFU without good care leads to infection, gangrene, amputation, and even death.[2] Various therapeutic alternatives have been used to treat DFU in clinics. However, low-efficacy or high-cost hospital care could not be applied to all patients with DFU. About 11% of DFU patients lose their lives every year.[3,4] Therefore, it is urgent to explore a harmless, feasible, long-term, and cost-effective treatment for DFU patients.

Wound healing is a complex process including four overlapping phases of blood coagulation, inflammation, proliferation, and tissue remodeling.[5] This biological process requires the coordinated integration of several cell types such as fibroblasts, keratinocytes, endothelial cells, macrophages, and mesenchymal stem cells.[6] Cell proliferation, migration, adhesion, differentiation, and extracellular matrix deposition would be activated during the process of wound healing.[7] In patients with diabetes mellitus (DM), the wound closure processes are slow because of a decrease in fibrinolysis and nutritional deficiency. There is also a decline in angiogenesis due to hyperglycemia and the migration of keratinocytes and diminished fibroblasts, causing deficient re-epithelialization, the poor production of the extracellular matrix (ECM) by fibroblasts, which lead to the problem of a deficient wound closure.[8,9]

Photobiomodulation (PBM), also known as low-level laser therapy, is a noninvasive treatment and has been widely used in clinics to accelerate the repair of tissues in human beings.[10–12] Many studies have reported that PBM mediated by laser plays a positive role in wound healing of DFU patients through promoting cell proliferation of keratinocyte and fibroblasts, neovascularization, and a reduction of inflammation.[13] In recent years, the light-emitting diodes (LEDs)-mediated PBM is going to provide DFU patients with a more feasible and lower-cost therapeutic option. Compared with laser devices, LED has several advantages, such as being smaller, lighter, lower cost, and easier for operation.[14,15] Although a few studies have demonstrated that LED irradiation is able to promote the healing of diabetic ulcers in clinics,[16,17] the dose–effect relationship and the mechanism of LED-mediated PBM are not clear. Several parameters are important for PBM, which include wavelength, light irradiance, energy, time, and frequency of the treatment. Generally, red or near-infrared (NIR) light (600–1000 nm) was considered as the optical window.[18] Laser-mediated PBM (630 nm and 810 nm) has already achieved positive results in the treatment of DFU. The light irradiance and total fluence are in the range of 1–100 $mW/cm^2$ and 1–100 $J/cm^2$, respectively.[12,19,20] However, the more accurate dose effect of PBM mediated by LED in the treatment of DFU is necessary to be explored.

In this study, the narrow bands of LED devices with central wavelengths of 630 and 810 nm were designed. The PBM effect of the LED irradiation on the mouse fibroblast cells under different light doses was evaluated. The results showed that both 630 nm and 810 nm LED irradiation significantly promoted the proliferation, migration, and secretion of cytokines of L929 cells at different light irradiances (1, 5, and 10 $mW/cm^2$). Both 630 nm and 810 nm LED significantly promoted the healing of the wounds of type 2 diabetic mice and increased the levels of vascular endothelial growth factor (VEGF)-A and transforming growth factor (TGF)-$\beta$ secreted by the tissue on the skin wounds. Overall, our results demonstrated that LED-mediated PBM is a good therapeutic option for DFU. No difference between the efficacy of 630 nm and 810 nm LED in

promoting wound healing of type 2 diabetic mice was observed.

## 2. Materials and Methods

### 2.1. *Materials and reagents*

The LED devices with a uniformity of light above 95% within 6 cm from the center of the devices were designed and made by the Institute of Semiconductors, Chinese Academy of Science. Dulbecco's modified Eagle medium (DMEM), fetal bovine serum (FBS), 0.25% trypsin, and 1% antibiotic solution (penicillin and streptomycin) were purchased from Thermo Fisher Scientific (Waltham, MA, USA). Cell Counting Kit-8 (CCK8) and ELISA kits were purchased from Beyotime Biotechnology Inc (P. R. China).

### 2.2. *Cell culture*

L929 cells (mouse fibroblast cell line) were obtained from the cell bank of the Chinese Academy of Sciences (Shanghai, China). The cells were cultured in DMEM supplemented with 10% FBS and 1% streptomycin/penicillin in a 75 $cm^2$ flask (Corning NY, USA), at 37°C in a humidified atmosphere containing 5% $CO_2$.

### 2.3. *LED irradiation*

The cell culture plate would be placed under the LED device. The distance between the LED panel and the bottom of the cell culture plate was about 5 cm, and the light irradiance could be adjusted from 0.1 mW/$cm^2$ to 20 mW/$cm^2$ through a current power supply. Before irradiation, the light irradiance was calibrated by a spectral light irradiance meter (SENSING, Zhejiang, China).

### 2.4. *Cell proliferation assay*

The L929 fibroblast cells were seeded in 96-well black plates (Corning NY, USA) at a density of 3000 cells per well. The LED light irradiation was applied 24 h after seeding. The light irradiances were 1, 5, and 10 mW/$cm^2$, and the irradiation time were 100 s, 200, 500, 1000, and 2000 s. The control group was covered by silver paper during the irradiation. The medium was replaced with a fresh culture medium immediately after irradiation. After the irradiation of 24 h, cell viability was evaluated by CCK8 following the manufacturer's protocol.

### 2.5. *Migration assay*

L929 fibroblast cells were seeded in 6-well plates with a number of $1.2 \times 10^5$ cells per well. When the cell confluence reached 80%, a cell-free area was made by scraping the cell monolayers vertically and evenly with a pipette tip. Then, L929 fibroblast cells were washed twice with PBS and incubated in DMEM with different concentrations of FBS. After 24 h of irradiation, the L929 fibroblast cells were photographed by a microscope (Olympus, Japan). The migration distances were evaluated by Image J software. The migration assay was repeated three times.

### 2.6. *Enzyme-linked immunosorbent assay (ELISA)*

L929 fibroblast cells were seeded in 6 cm plastic dishes at a density of $3 \times 10^5$ cells. The cells were incubated in DMEM without FBS before LED irradiation. After 24 h of irradiation, the cell culture medium was harvested and subjected to Enzyme-linked immunosorbent assay (ELISA) to measure target proteins, according to the manufacturer's instructions. Each assay was repeated three times.

### 2.7. *Diabetic animal model*

The protocol for animal experiments was approved by the Institutional Animal Care and Use Committee (IACUC) of PLA General Hospital, and all of the procedures were carried out in accordance with the approved guidelines. Eight-week-old female C57BL/6 mice were obtained from the Center of Experimental Animal, PLA General Hospital (Beijing, China), and maintained in a pathogen-free animal facility for at least 1 week before the experiment. The mice were used to induce diabetes following a previous method.[21] Briefly, the mice were feed food with high serum glucose and serum lipid for 3 months. Then, mice were kept fasting for 12 h before intraperitoneal injection of streptozotocin (solution 70 mg/kg, STZ) dissolved in citrate buffer solution (pH 4.0–4.2). Control animals received an equal volume of citrate buffer. Three days after administration, glucose levels were determined

in blood samples taken from the tail vein and measured on a glucose meter (San Nuo An Wen, Changsha, China). The mice were anesthetized by intraperitoneal injection of thiopental (2%). The dorsal region hire of mice was shaved by a razor and cleaned with a depilatory cream. Then, the back of the mice was washed with warm water quickly to prevent the skin injury induced by the rest of the depilatory cream. After 24 h, the wound field would be made by a press-type skin biopsy ring cutter. Each wound is a standardized circle 5 mm in diameter.

### 2.8. *Immunochemistry*

To detect the expression of VEGF-A and TGF-$\beta$ in the wound healing tissues after various treatments, the tissues were harvested after 8 d of treatments for immunohistochemistry. Briefly, the tissue samples were fixed in 10% formalin for 24 h, processed in ascending concentrations of ethanol, subsequently cleared with xylene, and embedded in paraffin. The paraffin-embedded samples of tissues were cut at a thickness of 5 $\mu$m using a microtome (Leica RM 2135, Germany). The sections were incubated with primary antibodies of VEGF-A and TGF-$\beta$ (CST, Danvers, MA) (1:100) for 1 h. Following extensive washing, sections were incubated for 30 min in the secondary biotinylated antibodies. Sections were then counterstained with Harris's hematoxylin and dehydrated in ascending grades of ethanol before clearing in xylene and mounting under a coverslip. The expression of VEGF-A and TGF-$\beta$ was first evaluated using microscopy by an independent investigator who has limited knowledge to the wound information. Images were captured by the image processing software (Kontron KS400, version 3.0, Hallbergmoos, Germany).

### 2.9. *Statistics analysis*

Statistical analysis was performed with GraphPad Prism 8. Differences were considered statistically significant at $P < 0.05$ ($n \geq 3$). Data represented the mean $\pm$ S.E.M.

## 3. Results

### 3.1. *Properties of light-emitting diode (LED) devices*

The narrow-band LED light devices with central wavelengths of 630 nm and 810 nm were designed and made. The light irradiance in the square field ($12 \times 12$ cm) was measured at a distance of 5 cm using a spectral light irradiance meter (Fig. 1(a)). The uniformity of light irradiated from the LED device was $> 95\%$. The plate seeded with cells was placed under the LED device for irradiation (Fig. 1(b)). The spectra of 630 nm LED and 810 nm LED are shown in Figs. 1(c) and 1(d). The light irradiance of both LED devices can be regulated in the range of 0–20 mW/cm$^2$.

### 3.2. *Effects of LED irradiation on cell viability*

To compare the effect of PBM between 630 nm and 810 nm LED on cell proliferation, the cell viability of L929 cells after various treatments was evaluated. As shown in Fig. 2, 100 s of LED irradiation significantly improved the cell viability at different light irradiances (1, 5, and 10 mW/cm$^2$) ($^*P < 0.05$). It continuously elevated with the extension of irradiation time from 100 s to 500 s. Over 500 s, the increase rate of the cell viability starts to decrease slowly.

### 3.3. *Effects of LED irradiation on cell migration*

To explore the effect of LED irradiation on the migration of L929 fibroblast cells, the wound healing assay was performed (Fig. 3(a)). The results showed that both 630 nm and 810 nm LED irradiation significantly accelerated the migration of L929 cells. Furthermore, the cell migration distance was positively related to irradiation time (100, 500, and 1000 s) ($^*P < 0.05$). No difference in migration distances of cells treated by 630 nm and 810 nm LED irradiation was observed (Fig. 3(b)).

### 3.4. *Influence of serum concentration on cell migration*

To determine if serum concentration has an influence on PBM mediated by LED, the cell migration assay was performed in the medium with different concentrations of serum (0, 1, 5, and 10%). The results suggested that low serum concentration decreased the migration distance of control group cells. However, compared with control groups (0, 1, 5, and 10% serum), LED irradiation significantly improved cell migration distance with rates of

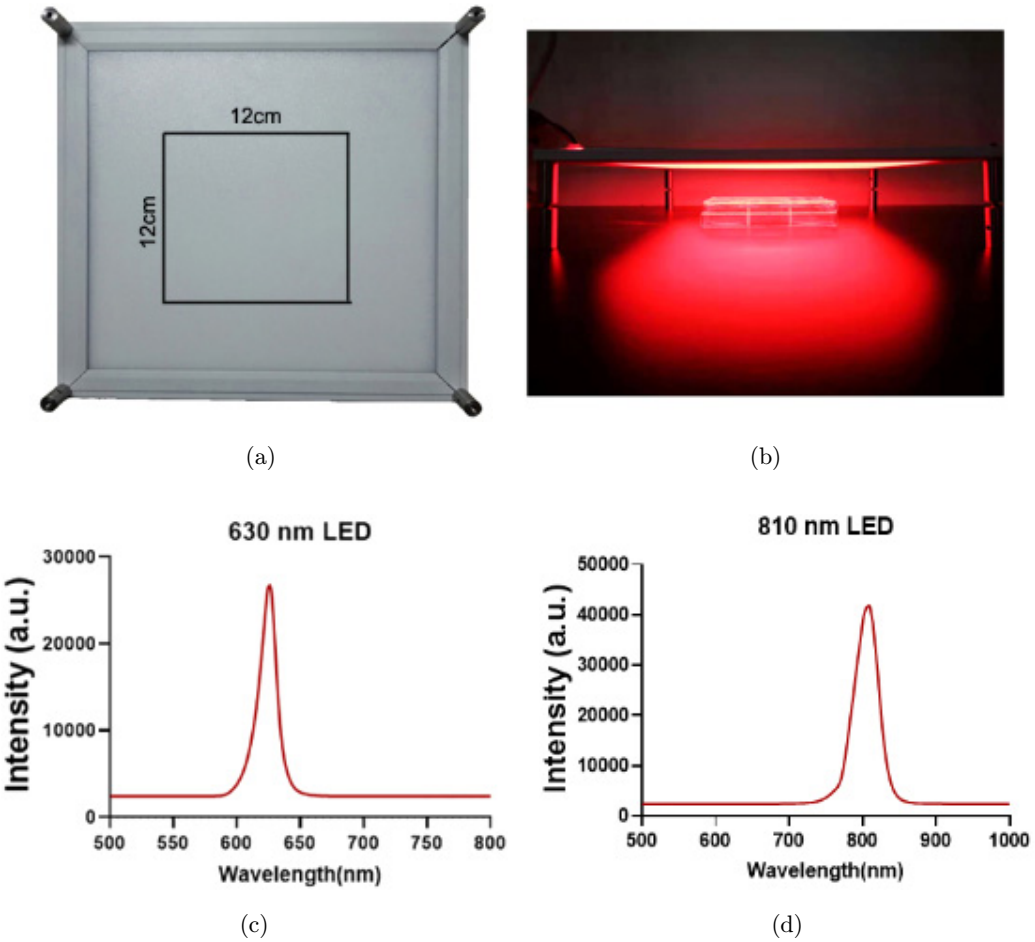



Fig. 1. Properties of LED devices. (a) Exterior of the LED device. (b) Overview of the irradiation process. (c) Spectrum of the 630 nm LED. (d) Spectrum of the 810 nm LED.

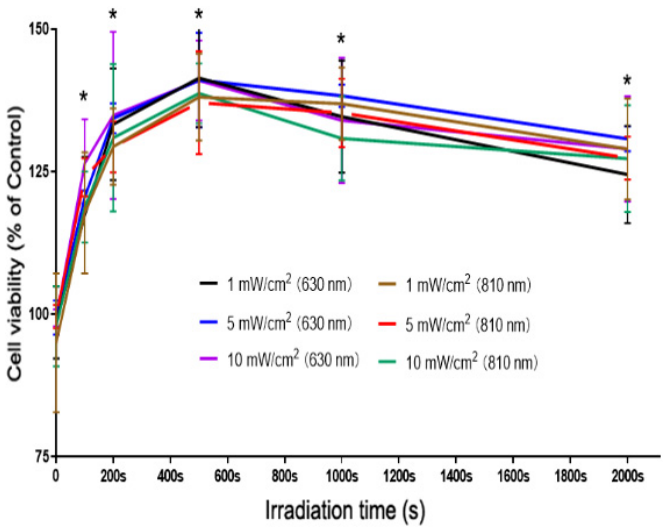
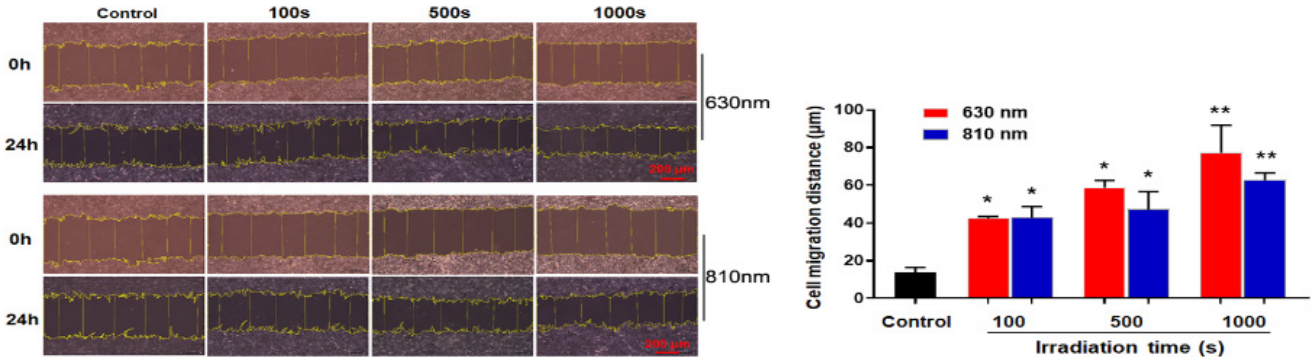



Fig. 2. Effects of LED (630/810 nm) irradiation on the cell viability of L929 fibroblast cells. LED irradiation (630/810 nm) significantly improved the cell viability with various fluence rates. 1, 5, and 10 mW/cm$^2$. ($^*P < 0.05$, compared with 0 s).

$197 \pm 12$, $188 \pm 17$, $130 \pm 11$, and $127 \pm 9\%$, respectively (Fig. 4). This result meant that LED irradiation played a better role in cells cultured in the medium with a lower serum concentration.

### 3.5. *Effects of LED irradiation on the skin wound healing of type 2 diabetic mice*

To explore the effect of LED irradiation on DFU, the wound healing model of diabetic mice was performed. The wounds on the back of diabetic mice were treated by the fibroblast growth factor (FGF) or LED irradiation. The progression of wound healing was monitored by capturing images (Fig. 5(a)). Wound contraction rates of each group were calculated and analyzed. Compared with the control group, both 630 nm and 810 nm LED irradiation significantly enhanced the wound




(a) (b)

Fig. 3. Effects of LED (630/810 nm) irradiation on cell migration of L929 fibroblast cells. (a) Photomicrographs of the wound healing assay after 630 nm LED irradiation (up) and 810 nm LED irradiation (down). (b) Quantitative analysis of the migration distance of cells in various groups (5 mW/cm$^2$) ($^*P < 0.05$).

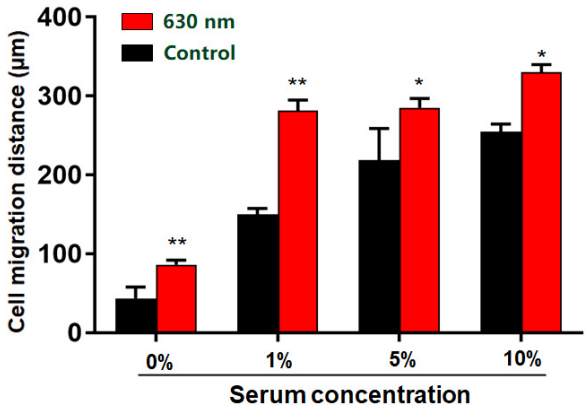



Fig. 4. Cell migration induced by 630 nm LED was influenced by serum concentration. The cell migration assay was applied in the medium with different concentrations of serum (0%, 1%, 5%, and 10%). Cell migration distances were measured and compared with the control ($^{**}P < 0.01$, $^*P < 0.05$).

healing speed. LED irradiation achieved a similar outcome on wound healing as FGF did (Fig. 5(b)).

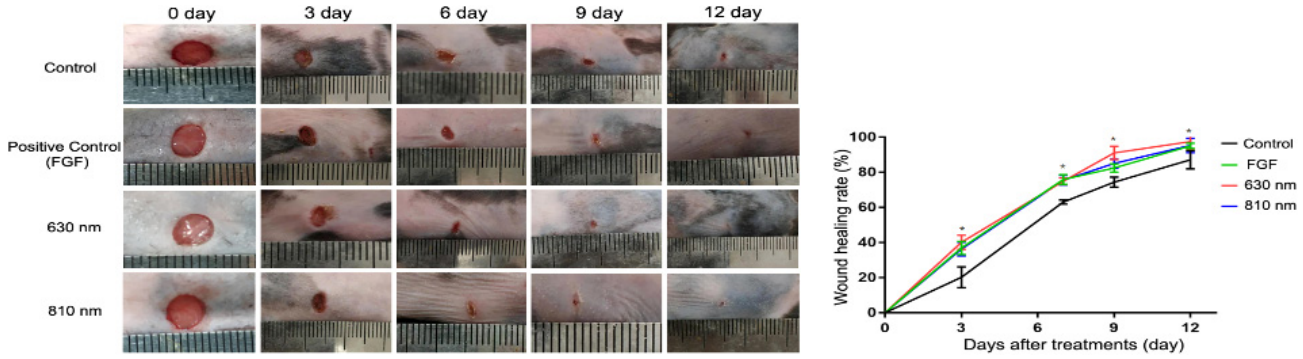
### 3.6. *Effects of LED irradiation on cytokine secretion of type 2 diabetic mice wound*

As we know that cytokines play an important role in wound healing, which enhances angiogenesis and cell proliferation. The expression of VEGF and TGF in the wounded skin of diabetic mice treated with FGF and LED irradiation was detected by immunochemistry staining (Figs. 6(a) and 6(c)). The quantitative analysis of immunochemistry results suggested that LED irradiation significantly improved the expression of VEGF and TGF in the skin of diabetic mice. There was no significant difference between the LED irradiation group and the positive group (FGF) (Figs. 6(b) and 6(d)).

## 4. Discussion

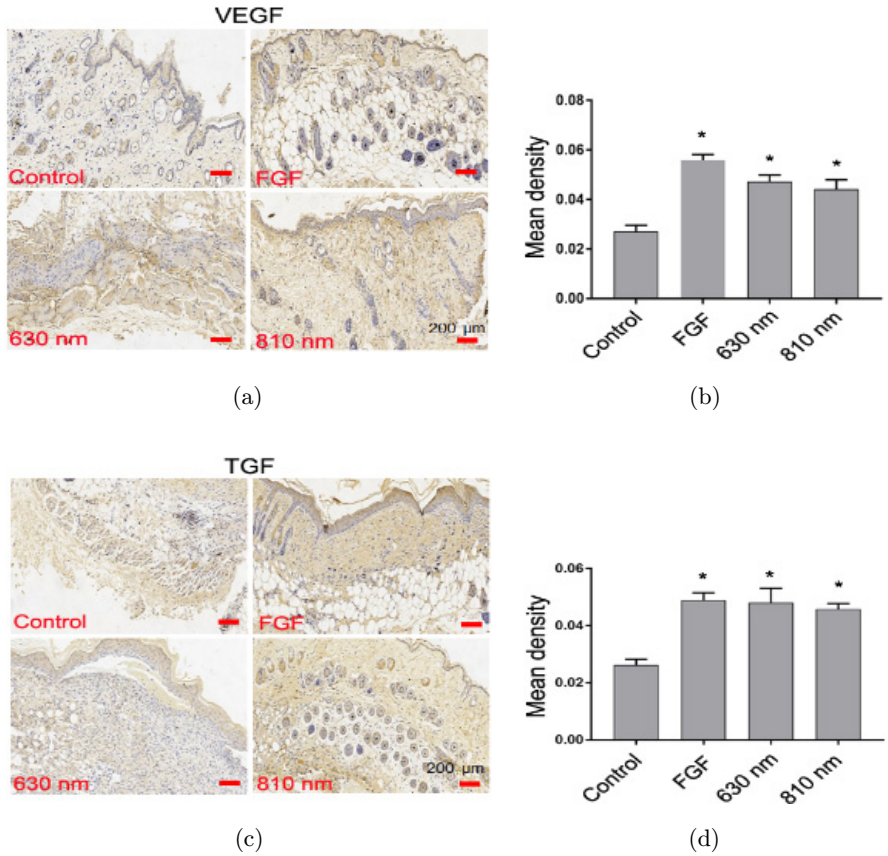
DFUs are a serious healthcare problem, which causes high morbidity, mortality, and heavy financial burden in the world. Among the current therapeutic strategies in DFU, PBM has been considered as one of the effective methods to promote wound healing and improve the life quantity of patients. The laser as the light source of the PBM has been widely used in clinics.[22] With the development of LED technology, new LED with excellent properties is going to be applied in medical devices. Compared with laser, LED has advantages of safety, covering a large area of tissues at once, feasibility, and, moreover, low cost. The cost per mW of optical power is approximately 100 times lower for LEDs compared to lasers.[23,24] In this study, the narrow bands of LED devices with central wavelengths of 630 nm and 810 nm were designed (Fig. 1(a)). For the 630 nm LED device, the half-wave width is around 20 nm (Fig. 1(c)). For the 810 nm LED device, the half-wave width is around 25 nm (Fig.1(d)). Compared with the broadband of light, the narrow band of LED has more beneficial effects in PBM.[25]

It is well known that PBM can stimulate various intracellular biological processes, such as cell proliferation, migration, and cytokine secretion. Cell proliferation is a critical physiological sign for the biostimulation effect of PBM, which involves the whole process of wound healing.[26,27] The effective wavelengths considered to accelerate cell proliferation




(a) (b)

Fig. 5. LED irradiation promoted the skin wound healing of type 2 diabetic mice. (a) Representative photomicrographs of the control, fibroblast growth factor (FGF), and LED irradiation (610/830 nm, 5 mW/cm$^2$ × 1000 s) groups showing a reduction in the wound area. (b) Comparative analysis of the wound healing rate of type 2 diabetic mice after various treatments. ($^*P < 0.05$).




(a) (b)

(c) (d)

Fig. 6. Relative expression of the VEGF and TGF of the tissue on the skin wounds after indicated treatments. (a) Immunohistochemical staining was used to evaluate the expression of VEGF on the skin wound tissue of mice in different groups. (b) Quantitative analysis of the expression of the VEGF on the skin wound tissue. (c) Immunohistochemical staining was used to evaluate the expression of TGF on the skin wound tissue of mice in different groups. (d) Quantitative analysis of the expression of the TGF on the skin wound tissue ($^*P < 0.05$, compared with the control).

during the wound healing are 630 nm and 810 nm of the laser, because cytochrome c oxidase has stronger absorption around 620 nm and 820 nm that will accelerate the synthesis of DNA and RNA in cells.[25,28] However, which one is better between these two wavelengths is not clear. So, we compared the efficacy of these two wavelengths of LED on cell proliferation at different light irradiances (1, 5, and 10 mW/cm$^2$).

No difference in the efficacy between 630 nm and 810 nm LED on the cell proliferation under the same dose of irradiation was found (Fig. 2). Interestingly, the cell proliferation of three groups reached a plateau in terms of speed during 500–1000 s. When the cells were irradiated for 2000 s with 10 mW/cm$^2$, the proliferation rate was significantly declined. This meant that the energy was overloaded to cells. So, the light irradiance of 5 mW/cm$^2$ was applied in the following experiments. Besides cell proliferation, the migration of fibroblasts also plays a very important role during wound healing. Once fibroblasts are activated, they will reduce the wound size through cell migration and the secretion of extracellular matrix proteins. During phototherapy, the tissue temperature changes in the range of 0.1–0.5°C. So, the effects of phototherapy are not from the thermal effect.[22] The most well-known mechanism of PBM is "the second signal messengers' theory". Laser/LED irradiation induces the production of ATP, nitric oxide (NO), and reactive oxygen species (ROS) in the cells. NO and ROS, as the second messengers, activate several cell signaling pathways, which transfer the signal into the cell nucleus where the transcription factors nuclear factor-$\kappa$B (NF-$\kappa$B) and activator protein 1 (AP1) will be activated. The activated NF-$\kappa$B and AP1 are able to induce the gene expression of cell proliferation and migration.[29,30] Meanwhile, ATP provides the energy for the cell biological response.[31] However, if the overdose of irradiation is delivered into cells, the production of ROS will be over the threshold of the antioxidant capacity of cells. Then, ROS will oxidize the DNA, protein, and phospholipid, which results in cell damage and even death. This is the reason why a high dose of irradiation will cause a decrease in cell proliferation. Previous studies have demonstrated that PBM could promote tissue regeneration with a light irradiance of 1 mW/cm$^2$ and the efficacy of PBM was mainly determined by irradiation time rather than light irradiance.[32–34] The obtained data (Fig. 2) is consistent with the previous study that there is no difference in cell viability among the three groups (1, 5, and 10 mW/cm$^2$). As we know that cytochrome c oxidase as the primary photoacceptor in cells plays a crucial role in the enhancement of cell proliferation.[28,35] However, the cytochrome c oxidase is inhibited by NO generated by PBM.[36] In this situation, the activation of cytochrome c oxidase will be maintained at a certain level during the irradiation. Even when the higher light irradiance was delivered, the cytochrome c oxidase would not be changed. This is the possible mechanism that light irradiance has no effect on cell viability.

Micro and macrovascular diseases are one of the pathogenesis of DFU. As we know that cell proliferation and migration need energy. Inadequate angiogenesis causing innutrition has been recognized as a major reason for prolonged healing in diabetic patients.[8] To determine whether the nutrition level will affect the PBM, we detected the cell move distance induced by LED irradiation under different concentrations of serum (0%, 1%, 5%, and 10%). The results suggested that the lower the serum concentration, the higher the efficiency of PBM. Compared with the control, LED irradiation improved cell migration distance with a rate of $197 \pm 12\%$ in the 0% serum group. Compared to the control group, the cell migration distance was improved $127 \pm 9\%$ as the serum concentration was increased to 10% (Fig. 4), which implies that the LED irradiation could achieve a better effect on DFUs than other therapeutic options.

In this study, the wound on the back of type 2 diabetic mice was used to simulate the wound of DFU. We treated the wounds with fibroblast growth factors (FGFs) or LED irradiation. Our results showed that both FGF and LED irradiation accelerated wound healing. However, LED-mediated PBM as physiotherapy is safer than FGF treatment. Because FGFs and their receptors (FGFRs) drive crucial developmental signaling pathways, which have oncogenic roles in many cancers.[37,38] DFU is a chronic wound with infection, which is hard to be cured completely. For our animal model, the wound was fresh and had a good self-healing capability. As shown in Fig. 5(a), LED irradiation did not contribute a lot to the wound healing of type 2 diabetic mice because the wound could be healed without any treatment in 12 days. However, most of the DFU wounds are in a low nutrition environment. The *in vitro* result suggested that the lower the nutrition level, the better the PBM effect of LED on cell migration. Consequently, LED irradiation is supposed to get a better effect on the wound healing of DFU.

## 5. Conclusions

The results of this study demonstrated that PBM mediated by 630 and 810 nm LED not only promoted the proliferation and migration of fibroblast

cells but also promoted the wound healing of type 2 diabetic mice. Both *in vitro* and *in vivo* results suggested that either 630 nm or 810 nm LED-mediated PBM is a good therapeutic option for DFU.

## Conflicts of Interest

The authors have no conflicts of interest relevant to this article.

## Acknowledgments

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