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Exploring the Effects of 630 nm Wavelength of Light-Emitting Diode Irradiation on the Proliferation and Migration Ability of Human Biceps Tendon Fibroblast Cells

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Background: Light-emitting diode (LED)-based photobiomodulation is used as an inducer of cell regeneration. Although numerous *in vitro* and *in vivo* orthopedic studies have been conducted, the ideal LED wavelength range for tendon healing has not yet been determined. This study, thus, focused on the effects of LED of a 630 nm wavelength on the cell viability, proliferation, and migration of human biceps tendon fibroblast cells.

Methods: Human tendon fibroblast cell culture was performed using the biceps tendon of patients who had undergone biceps tenodesis. Human biceps tendon fibroblasts from two patients (male, aged 42 and 69 years) were isolated and cultured. The cell type was confirmed by a morphological analysis and using tendon and fibroblast specific markers. They were then split into three groups, with each receiving a different irradiation treatment: no LED treatment (control), 630 nm LED, and 630 nm + 880 nm LED for 20 minutes each. After the LED treatment, cell viability, proliferation, and migration assays were performed, and the results were compared between the groups.

Results: Twenty-four hours after LED treatment, cell viability and proliferation were significantly increased in the 630 nm LED and 630 nm + 880 nm LED treatment groups compared to that in the control group ($p < 0.05$). Under the same conditions, compared with the control group, the 630 nm LED alone treatment group showed a 3.06 ± 0.21 times higher cell migration rate ($p < 0.05$), and the 630 nm + 880 nm LED combination treatment group showed a 2.88 ± 0.20 times higher cell migration rate ($p < 0.05$) in three-dimensional migration assay.

Conclusions: In human tendon fibroblast cells, 20 minutes of LED treatment at 630 nm and 630 nm + 880 nm exhibited significant effects on cell proliferation and migration. Our findings suggest the potential of LED therapy as an adjuvant treatment for tendon healing, and hence, further research is warranted to standardize the various parameters to further develop and establish this as a reliable treatment regimen.

Keywords: *Biceps tendon, Fibroblast cells, Proliferation, Migration, Light-emitting diode irradiation*

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Tendon-related diseases and injuries constitute a significant proportion of musculoskeletal disorders. A growing geriatric population and an escalation in various sporting activities have been the main causes for the yearly increase in prevalence of such diseases, which also puts a strain on the social cost of the nation.[1] Hence, there has been a greater focus on studies related to tendon degenerative lesions and injuries, which have provided extensive information about the mechanical and biological properties of tendons.[2] Moreover, this has also led to the development of newer surgical and conservative therapies that can prevent major disabilities caused by tendon diseases.

In the treatment of degenerative tendinopathy or after tendon repair surgery, it is important to enhance healing and regeneration. For this, various methods, such as extracorporeal shockwave therapy,[3] prolotherapy,[4] platelet-rich plasma injections,[5] stem cell therapy,[6] polydeoxyribonucleotide,[7] and atelocollagen treatments,[8] have been attempted, each with its own rationale. In addition, the biologics of bioaugmentation methods, such as mesenchymal stem cells and growth factors, are being introduced for tissue healing during tendon surgery.[6] However, the treatment of tendon diseases and injuries remains precisely unestablished; hence, it is quite challenging to treat.

Photobiomodulation (PBM) treatments involve low-level light therapy (LLLT) or light-emitting diode (LED) therapy using red and near infrared light wavelengths.[9] In PBM, the photon of the LED light source is absorbed by a chromophore or photoacceptor in the cell tissue and promotes the metabolic activity of the cell. Light absorbed by photoreceptors, such as intracellular cytochrome c oxidase, increases reactive oxygen species (ROS) and adenosine triphosphate (ATP) synthesis and releases nitric oxide (NO). ROS is involved in gene transcription related to growth factor production, cell proliferation, and cell motility. It promotes photodegradation of NO, enzyme activation, mitochondrial metabolism, and ATP production. As a result, the overall metabolic activity of the cell is high, and hence, light plays a major role in cell regeneration.[10,11] Based on this evidence, PBM has been employed in various therapeutic areas, such as wound healing and nerve and bone treatments.[9,12,13] Several *in vivo* studies related to tendons have been conducted, and positive effects on tendon healing in cases of injured tendons or tendinitis have been reported.[14] However, it is not yet widely used in the field of orthopedics, in particular, clinical applications involving tendon regeneration. Thus, with the intention of developing PBM as a routine treatment procedure, the purpose of this study was to verify the effect of LED at a 630 nm wavelength on the survival, proliferation, and migration of human biceps tendon fibroblasts (HBFs).

METHODS

Culturing of HBFs

The human biceps tendon tissues were collected from two patients (men, aged 42 years and 69 years) undergoing biceps tenodesis, with consent of the patients, and approval of the Institutional Review Board of Pusan National University Yangsan Hospital was obtained (No. PNUYH-04-2021-014). Tissues were washed three times with phosphate-buffered saline (PBS; Welgene, Gyeongsan, Korea), minced with a sterile scalpel, and then placed in a 6-well tissue culture plate (SPL Life Sciences Co. Ltd., Pocheon, Korea) in Dulbecco's Modified Eagle Medium (Gibco, Grand Island, NY, USA) supplemented with 10% fetal bovine serum (FBS; Gibco). They were treated with 100 IU/mL penicillin and 100 μg/mL streptomycin and cultured in a humidified 5% CO_2 incubator at 37°C for 2 weeks until they reached 90% confluence. The cells were then trypsinized (0.02% trypsin, 0.02% ethylenediaminetetraacetic acid [EDTA] in PBS) for 5 minutes and then centrifuged for 5 minutes at 200 × *g*, and the culture expanded by a second passage. The cells were then harvested with trypsin/EDTA and cryopreserved. These cryopreserved third-passage cells were later thawed and used for all experiments in the current study.

Irradiation of Cells by LED

The following experimental setup was designed to observe the effect of PBM on HBFs. The light irradiation module was attached to the lid of a 6-well culture plate, and each well was irradiated by the LED in the top-bottom direction. Light of a wavelength of 630 nm and 880 nm was used to irradiate the HBFs. This setup irradiated the cells with an intensity between 10–100 mW/cm^2 per cell per well. Additionally, the light irradiation module was designed to have a frequency of 10–100 Hz and irradiated cells by combining the wavelength, intensity, and frequency during the experiment (Fig. 1A).

Cells were seeded onto a 6-well plate (SPL Life Sciences Co. Ltd) at density of 1 × 10^5 cells/mL along with culture medium. After 18 hours, the culture medium was replaced with fresh medium, and then the cells were exposed to LED at 630 nm (10 mW/cm^2, 100 Hz) or 630 nm (10 mW/cm^2, 100 Hz) + 880 nm (40 mW/cm^2, 100 Hz) for 20 minutes.[15] Control cells were treated in the same manner, excluding the LED irradiation.

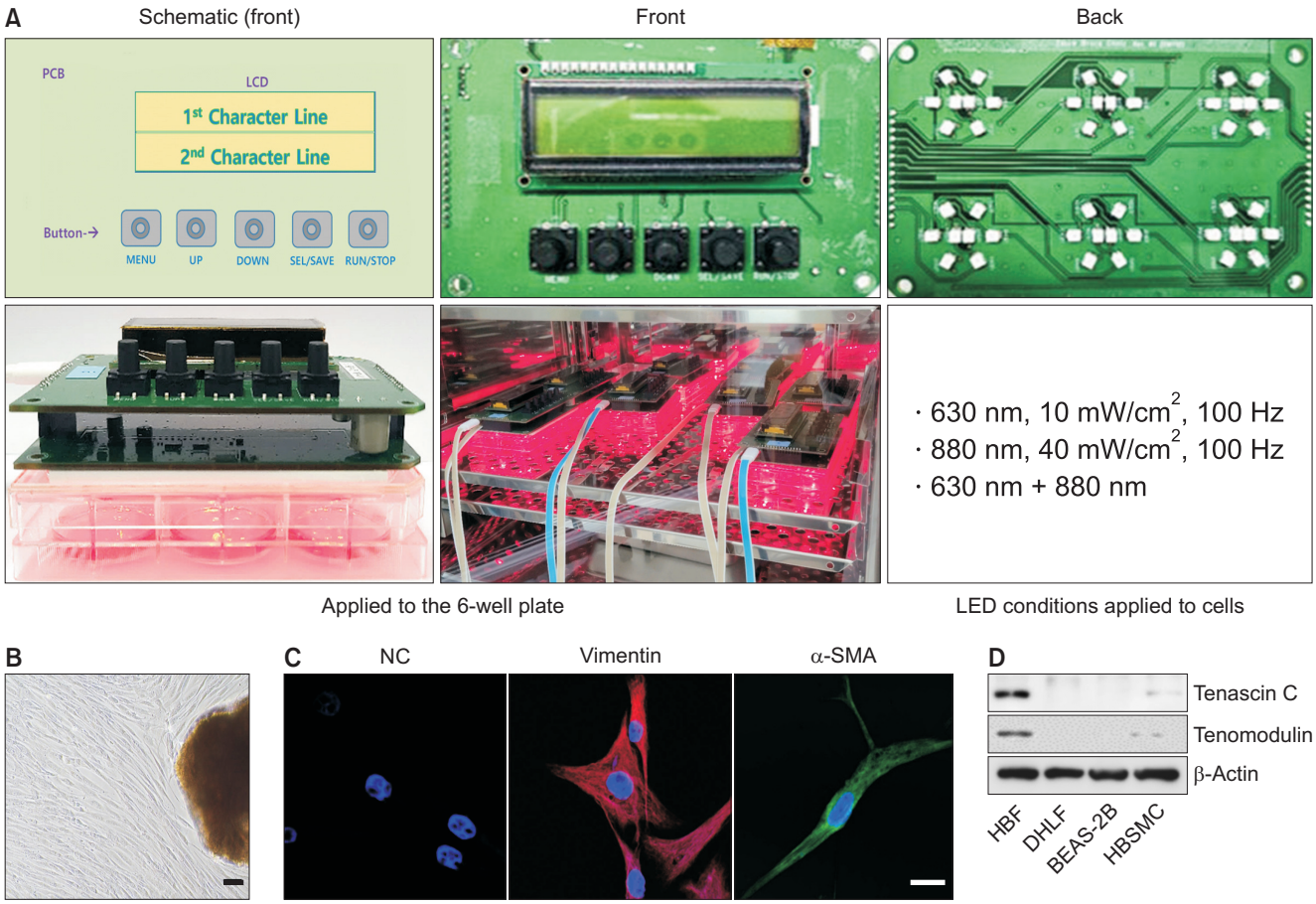

Fig. 1. Identification of fibroblast or tendon by a specific protein marker in human biceps tendon fibroblasts (HBFs) primary cells. (A) Photographs showing the lighting condition during exposure of HBFs to light-emitting diode (LED) light in an incubator. An LED irradiation was placed on the 6-well cell culture plate and maintained inside the incubator for 20 minutes. (B) Phase-contrast appearance of primary cell growth from connective tissue fragments. Elongated spindle-shaped morphology of fibroblast cells. Scale bar represents 100 μm. (C) Fibroblast positively stained for the marker vimentin (red) or alpha smooth muscle actin (α-SMA; green). Images were taken of cells at passage 2. Scale bar represents 30 μm. (D) Western blotting expression of tendon marker, tenascin C, and tenomodulin in HBFs. Diseased human lung fibroblast (DHLF), human bronchial epithelial cells (BEAS-2B), and human bronchial smooth muscle cells (HBSMCs) were used as negative controls (NCs). PCB: printed curcuit board, LCD: liquid crystal display.

Cell Viability Assay

In order to investigate the effect of LED treatment on the viability of HBFs, cell viability was determined using a 3-(4,5-dimethylthiazole2-yl)-2,5-diphenyl tetrazolium bromide (MTT) reagent (5 mg/mL in PBS, Duchefa Biochemie, Haarlem, The Netherlands). The MTT assay procedure was performed as previously mentioned.[16] In brief, HBFs were seeded at 1×10^5 cells/mL in 6-well plates and cultured for 24 hours before LED irradiation. On the following day, the culture medium was replaced with serum-free medium, and then the cells were exposed to LED at 630 nm (10 mW/cm^2, 100 Hz) or 630 nm (10 mW/cm^2, 100 Hz) + 880 nm (40 mW/cm^2, 100 Hz) for 20 minutes (Fig. 2A). Control cells were treated in the same manner, excluding the LED irradiation. After 24 hours, 5 μg/mL MTT solution was added to each well and incubated for 2 hours at 37°C in dark. The supernatant was removed, and the formazan crystals in each well were dissolved in 1 mL of dimethyl sulfoxide (Sigma-Aldrich, St. Louis, MO, USA) for 30 minutes at 20°C–25°C by shaking. Optical densities at 570 nm were then measured using a microplate reader (Tecan, Infinite M200, Grödig, Austria). Data were calculated as a percentage of viable cells in comparison with the control.

Cell Proliferation Assay

Cell proliferation was determined by 5-bromo-2'-deoxyuridine (BrdU, Sigma-Aldrich) incorporation as described previously.[17] Briefly, HBFs were grown on round cover glass coated with poly-$_L$-lysine (Sigma-Aldrich) for 24

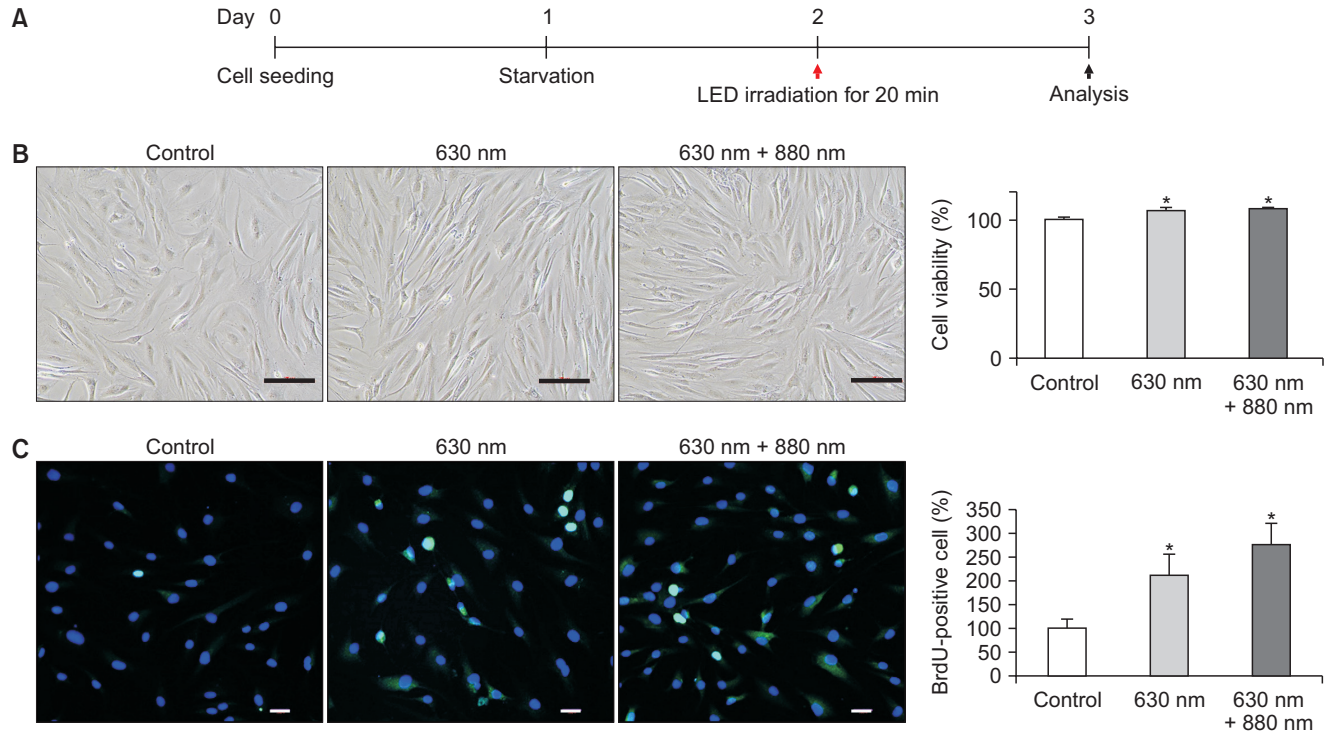

Fig. 2. Increased human biceps tendon fibroblasts (HBFs) cell viability and proliferation by light-emitting diode (LED) irradiation. (A) Experimental scheme of LED irradiation for cell viability and proliferation. (B) Effect of LED on HBFs cell viability. Cells were exposed to 630 nm LED or 630 nm + 880 nm LED irradiation for 20 minutes. Cell viability was measured by 3-(4,5-dimethylthiazole-2-yl)-2,5-diphenyl tetrazolium bromide assay. Data are shown as the mean ± standard deviation (SD) obtained from three independent experiments (n = 3). (C) 5-Bromo-2'-deoxyuridine (BrdU) immunofluorescence staining was performed in HBFs after treatment with LED irradiation for 20 minutes. Immunocytochemistry showed localization of BrdU-labeled nuclear (green) and nuclear staining (blue). Bar graphs represent the quantification of BrdU-positive cells. Data are shown as the mean ± SD of three independent experiments (n = 3). Scale bar represents 50 μm. *$p < 0.05$, compared with control.

hours at 1×10^5 cells/mL in 6-well plates. After 24 hours, the culture medium was replaced with serum-free medium, and then the cells were exposed to LED at 630 nm (10 mW/cm^2, 100 Hz) or 630 nm (10 mW/cm^2, 100 Hz) + 880 nm (40 mW/cm^2, 100 Hz) for 20 minutes. Next day, HBFs were incubated at 37°C for 3 hours with 10 μM of BrdU. Cells were fixed with 4% paraformaldehyde for 30 minutes, washed three times with PBS, and permeabilized by incubation in 0.2% Triton X-100 in PBS for 30 minutes. To measure BrdU incorporation, cells were incubated in 2 M hydrochloric acid (HCl) for 30 minutes at 37°C in order to denature the DNA. Cells were then incubated for 5 minutes in 0.1 M sodium borate (pH 8.5) to neutralize the residual acid and washed three times with PBS. Cells were incubated in a blocking solution containing 1% normal goat serum for 1 hour at 20°C–25°C and then incubated with monoclonal anti-BrdU (1 : 100 dilution; Thermo Fisher Scientific, Waltham, MA, USA) in blocking solution at 4°C for 18 hours. After washing with PBS thrice, the cells were incubated with Alexa Fluor 488 goat anti-mouse immunoglobin G (IgG) (1:500 dilution; Thermo Fisher Scientific, Rockford, IL, USA) in the dark for 1 hour. BrdU-positive cells were counted in five distinct fields per slide and expressed as a percentage of total cells counted. Total DNA was determined using 4',6-diamidino-2-phenylindole (DAPI).

Cell Migration Assays

Migration assays were performed as described previously.[18] Briefly, a culture-insert (ibid, Martinsried, Germany) was attached to the 6-well plate. The cells (7.5×10^5 cells/mL; 80 μL) were seeded into each well and incubated for 24 hours in culture media with 25 μg/mL mitomycin C (Sigma) for 30 minutes to inhibit cell division and proliferation. The culture-insert was taken out, the cells were irradiated, and migration was analyzed in 0, 12, and 24 hours. The migration of cells into the cell-free gap created by the removal of the culture-insert was monitored at indicated time points and photographed using a microscope (Nikon, Tokyo, Japan). Through quantitative assessments of the data, the velocity

of migration was determined. All data presented are from at least three independent experiments performed in triplicate. The trans-well migration assay was performed using the CytoSelect Cell Migration Assay Kit containing polycarbonate membrane inserts (8 μm-pore membrane; Cell Biolabs, San Diego, CA, USA) according to the manufacturer's instructions. The trans-well migration procedures were as described previously.[18] A cell suspension containing 1×10^5 cells/mL in serum-free medium was prepared, and 2 mL of the cell suspension was added to the upper chamber of each insert. The lower chamber containing medium with 10% FBS allowed cell migration towards the lower face of the trans-well culture inserts. Cells were irradiated with 630 nm or 630 nm + 880 nm LED for 20 minutes and then incubated for 12 hours at 37°C with 5% CO_2. Non-migrating cells on the inner side of the trans-well culture inserts were gently removed with a cotton-tipped swab. Migrated cells remaining on the bottom surface were stained with 0.1% crystal violet for 15 minutes. Photomicrographs of five individual fields per insert were taken using a microscope (Nikon) and analyzed using ImageJ software (version 1.49; National Institutes of Health, Bethesda, MD, USA) to calculate the average number of cells that had migrated.

Western Blotting

A Western blot analysis was performed as described previously.[19] Protein lysates were made from HBFs using radioimmunoprecipitation assay buffer (Thermo Fisher Scientific) containing a protease inhibitor cocktail (Xpert protease inhibitor cocktail solution, GenDEPOT) and phosphatase inhibitors (Xpert phosphatase inhibitor cocktail solution, GenDEPOT). The cell lysates were incubated for 30 minutes on ice with intermittent vortexing and were clarified by centrifugation at 16,609 × *g* (13,000 rpm; Hanil, Incheon, Korea) at 4°C for 20 minutes. After centrifugation, the supernatant was separated and stored at –70°C until use. Protein concentration in cell lysates was quantified using a Pierce bicinchoninic acid protein assay kit (Thermo Fisher Scientific). Cell lysates were boiled in 5X sample buffer and separated by 8%–15% sodium dodecyl sulfate-polyacrylamide gel electrophoresis, and the proteins were transferred onto polyvinylidene difluoride membranes (Millipore, Darmstadt, Germany). The membranes were blocked with 5% skimmed milk in TBST (50 mM Tris HCl pH 7.4, 150 mM NaCl, 0.1% Tween 20) and incubated with specific primary antibodies at 4°C for 18 hours. The primary antibodies used were as follows: Tenascin C (1 : 1000; Abcam, Cambridge, MA, USA), tenomodulin (1 : 1000; Abcam), vimentin (1 : 1000; Cell Signaling, Danvers, MA, USA), and β-actin (1 : 3000; Sigma). The membranes were then washed three times with Tris-buffered saline with Tween-20 and incubated with secondary antibodies conjugated with horseradish peroxidase (1 : 5000, Cell Signaling) for 1 hour. Immunodetection was performed using an enhanced chemiluminescence kit (Amersham Pharmacia, Piscataway, NJ, USA) with Fusion Solo X (Vilber Lourmat, Collegien, France) for detection. The relative protein level was calculated using β-actin as a loading control.

Immunocytochemistry

HBFs were cultured on round cover glass coated with poly-$_L$-lysine for 24 hours at 2.5×10^4 cells/well (500 μL) in 24-well plates. The cells were fixed with 4% paraformaldehyde in 0.1 M PBS for 30 minutes, washed three times with 1X PBS, and preincubated in a blocking buffer containing 1% normal goat serum and 0.1% Triton X-100 for 2 hours at 20°C–25°C under gentle rotation. The cells were incubated with monoclonal anti-actin, α-smooth muscle-FITC antibody (1 : 500, Sigma), and vimentin (1 : 500, Cell Signaling) in blocking solution and then incubated at 4°C for 18 hours. Samples excluding the addition of primary antibodies were used as negative controls. After washing three times in PBS, the cells were incubated in the dark for 1.5 hours with Alexa Fluor 594-conjugated anti-rabbit IgG fluorescent secondary antibody diluted 1 : 500 in PBS. The cells were washed three times and stained with 1 μg/mL of DAPI for nuclei staining. The stained cells were wet-mounted on glass slides and observed using a confocal laser scanning microscope (K1-Fluo; Nanoscope Systems, Daejeon, Korea).

Statistical Analysis

The data are expressed as mean ± standard deviation. Significant differences between groups were analyzed using an unpaired Student *t*-test (OriginPro2020; OriginLab Corp., Northampton, MA, USA). A $p < 0.05$ was considered as the criterion for statistical significance.

RESULTS

Identification of HBFs

Morphological observations of cells isolated from human biceps tendon tissue and subsequently cultured showed spindle- or star-shaped features typical of fibroblasts (Fig. 1B). Expression of fibroblast markers (vimentin and alpha smooth muscle actin) and tendon-specific markers (tenascin C and tenomodulin proteins) were confirmed by immunofluorescence assay and Western blotting, respectively

(Fig. 1C and D).

Effect of LED Treatment on the Viability and Proliferation of HBFs Cells

The LED treatment group had a significantly increased survival rate than the control group; however, there was no significant difference between the 630 nm LED alone treatment group (106.76% ± 2.30%) and the 630 nm and 880 nm combination treatment group (108.04% ± 0.90%) (Fig. 2B). As shown in Fig. 2C, The proliferation rate of HBF cells was significantly increased in the LED-treated group compared to the control group. The 630 nm LED treatment resulted in a 2.21 ± 0.76-fold increased cell proliferation rate ($p < 0.05$), and the 630 nm + 880 nm LED combination treatment group showed a cell proliferation rate of 2.87 ± 0.91 folds and higher ($p < 0.05$).

Effect of LED Treatment on the Migration of HBFs Cells

In order to replicate cell migration to wound site, the migration in 2D was observed in monolayer cells, and the effect of LED treatment was assessed. The monolayer cultured HBFs cells were treated with LED for 20 minutes, and then the distance the HBFs cells moved during the 12 hours and 24 hours period was measured (Fig. 3A). The cell migration was unaffected until 12 hours after LED treatment: however, at 24 hours, it was significantly increased in the 630 nm LED alone group ($p < 0.05$) and the 630 nm + 880 nm LED treatment group compared with the control group. ($p < 0.05$) (Fig. 3A). To further

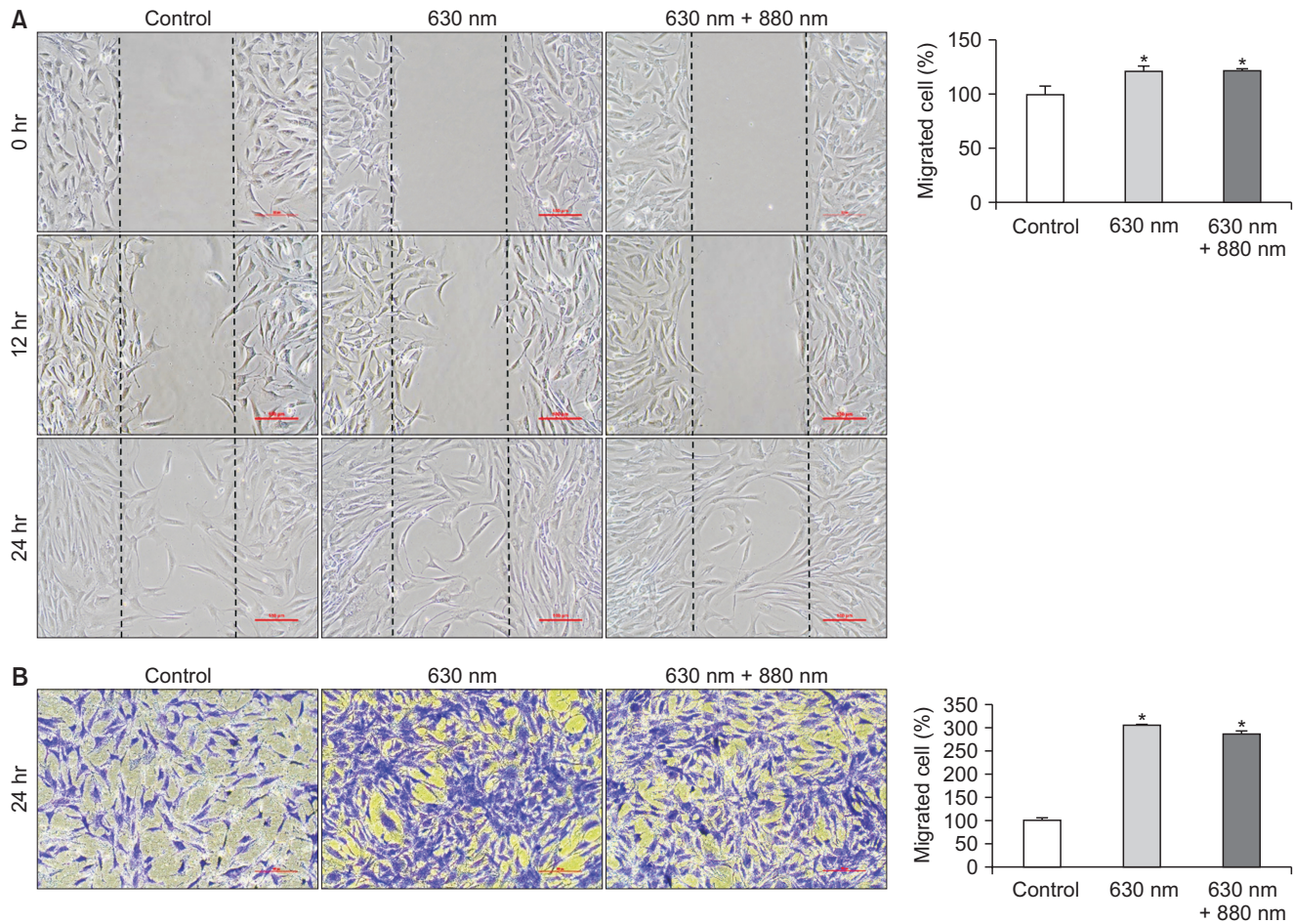

Fig. 3. Light-emitting diode (LED) irradiation-enhanced human biceps tendon fibroblasts (HBFs) migration. Cells were exposed to 630 nm LED or 630 nm + 880 nm LED irradiation for 20 minutes. (A) Representative phase-contrast images at 0, 12, and 24 hours, showing the migration of HBFs into the cell-free gap, left by the removal of the culture-insert. Bar graphs represent the quantification of migrated cells. Data are shown as the mean ± standard deviation (SD) of three independent experiments (n = 3). (B) Representative microscopic images of migrating cells by trans-well migration assays. Bar graphs represent the quantification of migrated cells. Data are shown as the mean ± SD of three independent experiments (n = 3). *$p < 0.05$, compared with control.

confirm these results, a three-dimensional migration assay was carried out using trans-wells, and we observed similar results. The 630 nm LED alone treatment group showed a 3.06 ± 0.21 times higher cell migration rate ($p < 0.05$), and the 630 nm + 880 nm LED combination treatment group showed a 2.88 ± 0.20 times higher cell migration rate than the control group ($p < 0.05$) (Fig. 3B).

DISCUSSION

The results of this study allowed us to confirm the initial effect of LED treatment on fibroblasts using HBF culture. Cell viability, proliferation, and migration all showed significant differences in the 630 nm LED and 630 + 880 nm LED treatment groups compared with the control group. PBM using LLLT or LED therapy was first introduced by Endre Mester in the early 1960s.[20] PBM accelerated healing, mediated by increased cell activity generated by stimulation of the mitochondrial and cell membrane photoreceptors synthesis of ATP.[9] Such modulations on these cells can promote fibroblast proliferation, growth factor synthesis, collagen production, and angiogenesis.[9,10,20] Based on this rationale, various studies and animal experiments have been conducted in the field of orthopedic surgery. According to Lopes Silva et al.,[13] a positive effect of LLLT/LED was reported on tendon damage. Rosso et al.[21] showed that PBM has beneficial effects on the recovery of nerve lesions. It has also been suggested by *in vitro* experiments that PBM may facilitate tissue homeostasis, thus stimulating the components of the articular tissue and promoting chondroprotective effects.[22] Our experiments used fibroblasts obtained from human tendons on the basis of previously established studies, and we observed > 2-fold cell proliferation and ≥ three-fold cell migration in the LED-treated group compared with the control group. As mentioned earlier, PBM treatment promotes reduction of inflammatory cells, increase of proliferation of fibroblasts, stimulation of angiogenesis, formation of granulation tissue, and increase of collagen synthesis. One of the strengths of our study is that it revealed the statistical significance of PBM treatment on fibroblast proliferation, which is one of the effects of PBM treatment.

Low-level lasers were used for PBM in the 1960s. Since the 1990s, LED lights have replaced lasers and have been proven to have the same medical benefits.[23] LED lights also provide the advantage of easy adjustability of light intensity, longevity, and homogeneity of light dose at an optimal intensity.[23] It also allows the use of lights with various wavelengths independently or at the same time. The wavelengths commonly used in PBM are red light and near-infrared light (600–1,100 nm). There are effective reports on blue and green light (400–500 nm) in some studies; however, more research is needed because the boundary with ultraviolet (< 400 nm), which causes DNA damage, is vague. Furthermore, at wavelengths below 600 nm, the absorption and scattering of light in tissue are higher than red light, and at wavelengths greater than 1,100 nm, water absorbs infrared light. Therefore, red and near-infrared light (600–1,100 nm) is used in PBM.[24,25] Lam[26] found that when fibroblasts were irradiated with light with a wavelength of 633 nm, procollagen synthesis was increased four times compared with the baseline. In another study, irradiation at 830 nm accelerated fibroblast-myofibroblast transformation and mast cell degranulation. Additionally, the chemotaxis and phagocytic activity of leukocytes and macrophages were enhanced by cell stimulation.[27] Hence, Russell et al.[28] suggested that there could be a synergistic effect when 633 nm and 830 nm were applied simultaneously. Hence, in our study, not only 630 nm treatment but also 630 nm and 880 nm combined irradiation was used. However, in our study, no synergistic effect was observed in the group treated with 630 nm and 880 nm wavelengths simultaneously. Moreover, since the 880 nm alone treatment did not affect cell viability, the 880 nm alone group was excluded from the study results. In some other studies, only 635 nm was considered as a potential effective option for bone regeneration in comparison of 808 nm, 635 nm, and 405 nm. However, since it shows various results depending on the light conditions and irradiation time, a more specific PBM modality should be made in the future.[28,29]

This study focused only on the effects of wavelength because it is one of the major factors affecting cell activity. However, other conditions, such as light source, energy density, light pulse structure, and LED application time can also have a significant effect and need to be investigated in future studies. Furthermore, because the assays were all performed *in vitro*, the relevance of these results in a clinical scenario needs to be assessed. PBM is presently used in the field of dermatology, where direct contact of tissues with light is possible. However, in orthopedic cases, further research is needed to determine whether light can penetrate the tissues present under the skin, such as ligaments, nerves, and bones. The present study, while elucidating the effects of PBM on cell activity, has not examined inflammatory effects among others and hence opens up several avenues for future research to establish PBM as a routine treatment procedure.

LED irradiation at 630 nm and 630 nm + 880 nm for 20 minutes significantly affected cell proliferation and

migration in human tendon fibroblast cells. LED therapy, thus, offers promising potential for use as an adjunct treatment for tendon healing, and hence, further research on various conditions is essential to establish this as a routine treatment regimen.

CONFLICT OF INTEREST

No potential conflict of interest relevant to this article was reported.

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