Wnt/β-Catenin and ERK Pathway Activation: A Possible Mechanism of Photobiomodulation Therapy With Light-Emitting Diodes that Regulate the Proliferation of Human Outer Root Sheath Cells

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Background: Outer root sheath cells (ORSCs) play important roles in maintaining hair follicle structure and provide support for the bulge area. The hair growth promoting effects of photobiomodulation therapy (PBMT) have been reported, but the mechanisms for this in human ORCs (hORSCs) have rarely been studied.

Objective: The aim of this study was to investigate the effect of various wavelengths of light-emitting diode (LED) irradiation on human ORSCs (hORSCs).

Methods: LED irradiation effects on hORSC proliferation and migration were examined with MTT assay, BrdU incorporation assay and migration assays. hORSCs were irradiated using four LED wavelengths (415, 525, 660, and 830 nm) with different low energy levels. LED irradiation effects on the expression of molecules associated with the Wnt/β-catenin signaling and ERK pathway, hair stem cell markers, and various growth factors and cytokines in hORSCs were examined with real-time PCR and Western blot assay. The effect of the LED-irradiated hORSCs on cell proliferation of human dermal papilla cells (hDPCs) was examined with co-culture and MTT assay.

Results: PBMT with LED light variably promoted hORSC proliferation and suppressed cell apoptosis depending on energy level. LED irradiation induced Wnt5a, Axin2, and Lef1 mRNA expression and β-catenin protein expression in hORSCs. Phosphorylation of ERK, c-Jun, and p38 in hORSCs was observed after LED light irradiation, and ERK inhibitor treatment before irradiation reduced ERK and c-Jun phosphorylation. Red light-treated hORSCs showed substantial increase in IL-6, IL-8, TNF-α, IGF-1, TGF-β1, and VEGF mRNA. Light irradiation at 660 and 830 nm projected onto hORSCs accelerated in vitro migration. LED-irradiated hORSCs increased hDPCs proliferation when they were co-cultured. The conditioned medium from LED-irradiated hORSCs was sufficient to stimulate hDPCs proliferation.

Conclusion: These results demonstrate that LED light irradiation induced hORSC proliferation and migration and inhibited apoptosis in vitro. The growth-promoting effects of LEDs on hORSCs appear to be associated with direct stimulation of the Wnt5a/β-catenin and ERK signaling pathway. Lasers Surg. Med. © 2017 Wiley Periodicals, Inc.

Key words: ERK signaling pathway; hair growth promoting effect; light-emitting diode irradiation; photobiomodulation therapy; outer root sheath cell; Wnt5a/β-catenin pathway

INTRODUCTION

The effects of photobiomodulation therapy (PBMT) on hair growth have been demonstrated in clinical and animal studies [1–3]. Although a recent meta-analysis revealed that PBMT could be a promising tool to induce hair growth, it was difficult to assess its clinical efficacy for hair loss disorders [1,4]. This was due to the heterogeneous therapeutic conditions including light source, wavelength, energy level, disease severity, and concomitant use of other medicines. In addition, the mechanisms underlying PBMT effects on improved hair growth are not known. Light-emitting diode (LED) wavelengths are known to be absorbed by intracellular chromophores and mitochondria, which produce increased adenosine triphosphate (ATP), leading to increased cellular signaling related to proliferation [5]. PBMT could also affect microcirculation, inflammation improvement, and growth factor production [1].

Outer root sheath cells (ORSCs) surround most hair follicle components and play important roles in supporting dermal papilla cells (DPCs) and hair matrix cells. The bulge is part of the ORSC and is a reservoir for stem cells. The bulge activation theory suggests the means by which...
human DPCs (hDPCs) activate stem cells in the bulge area and cause the telogen to anagen transition. However, it is unknown whether certain hair cycle disorders result from a defect in interactive signaling between hDPCs and the bulge area. If there is a signaling defect in hDPCs in certain diseases, bulge area stimulation with PBMT might be a therapeutic option for inducing anagen re-entry or prolonging the anagen period. Growth promoting effects of PBMT on human hair cells have been mostly seen in DPCs, but its effect on human ORSCs (hORSCs) or on the interaction between hDPCs and hORSCs is not well known. PBMT effects on ORSCs have been reported in a few studies using red light. While 660 nm light efficiently promoted ORSC proliferation, 700–770 nm light was associated with less robust biochemical reactions.

The extracellular signal–regulated kinases (ERK) and Wnt/β-catenin signaling pathway upregulate genes responsible for cell proliferation and differentiation in hair cells and induction of anagen re-entry. However, the role of the ERK and Wnt/β-catenin signaling pathway in hair cells in response to PBMT has not been well studied. [6].

We investigated the effects of various light wavelengths on ORSC proliferation and migration using 415, 525, 660, and 830 nm light with different energy levels. We focused on the activation of the ERK and Wnt/β-catenin signaling pathway in hair cells in response to PBMT and the associated changes in hair stem cell marker and several cytokines and growth factors. We also investigated whether LED irradiation on hORSCs could stimulate hDPC proliferation when co-cultured.

MATERIALS AND METHODS

Light Source and Irradiation

A LED device produced for laboratory experiments was provided by Korea Electronics Technology Institute (KETI, Seongnam, Korea). Four wavelengths were manufactured as LED light sources: 415 nm (4.23 mW/cm²); 525 nm (3.85 mW/cm²); 660 nm (2.42 mW/cm²); and 830 nm (2.72 mW/cm²) and irradiated with 1, 3, 5, or 10 J/cm² of energy. The wavelength and parameter conditions are listed in Table S1. The spot size of the device was 10 × 17 cm and was made to cover the whole size of plates. Each well of a plate was exposed to LED with the same setting.

Cell Culture

hORSCs were purchased from ScienCell (San Diego, CA) and cultured as previously described [1]. hORSCs (2 × 10^5 cells per well) were seeded into 6-well culture plates in mesenchymal stem cell medium containing 1% penicillin-streptomycin, 5% fetal bovine serum and 1% mesenchymal stem cell growth factor (ScienCell) for 24 hours. hORSCs at passage 3 or 4 were used for the experiments. For LED irradiation, hORSCs were seeded in culture dish plates with defined keratinocyte serum free medium (dKSFM); then, the plates were irradiated with LED light from a distance of 2 cm. All experiments were performed at ambient temperature and pressure during LED irradiation.

Cell Viability, Proliferation, and Apoptosis Assay

Cell viability was assessed with an 3-(4, 5-dimethylthiazol-2-yl) 2, 5-diphenyl tetrazolium bromide (MTT) assay performed in the modified manner. MTT assay measures cell viability in terms of reductive activity as enzymatic conversion of the tetrazolium compound to water insoluble formazan crystals by dehydrogenases occurring in the mitochondria of living cells.

We adopted the 5-Bromo-2-deoxyuridine (BrdU) incorporation assay to examine the effect of PBMT on cell proliferation. BrdU incorporation was quantified using the BrdU Cell Proliferation Assay kit (Cell Signaling Technology) according to the manufacturer’s instructions.

The Bcl-2/bax ratio was assessed to examine the effect on apoptosis.

Migration Assay

hORSC migration was measured in Transwell plates (Cell Culture Insert Companion Plate, BD Falcon, Franklin Lakes, NJ). The Transwell system was separated from the lower compartment by a permeable membrane coated with polyethylene terephthalate (pore size: 8 mm, 24-well format, Cell Culture Inserts, BD Falcon). Cells seeded in the upper transwell plate compartment were exposed to 660 nm at 10 J/cm² or 830 nm at 10 J/cm² LED wavelengths. dKSFM media was added to the lower chamber. Migrated cells were stained with crystal violet. Cells in five randomly selected viewing fields beneath each membrane were counted.

Real Time PCR

Total RNA from the DPCs using the Trizol reagent (Invitrogen, Carlsbad, CA) and cDNA synthesis with QuantiTect Rev. Real time PCR was performed with transcription kit (Qiagen, Hilden, Germany) according to the manufacturer’s instructions. The cDNA used for real time-PCR (RT-PCR), which was carried out with SYBR Green (Bio-Rad, Hercules, CA). The primers sequences and PCR conditions are listed in Table S2.

Western Blot Analysis

Cells were harvested and lysed with RIPA lysis buffer (Pierce, Rockford, IL). Protein levels were measured using Bradford reagent (Bio-Rad, Hercules, CA) with bovine serum albumin as a standard. Cell lysates containing equal amounts of total protein were separated by electrophoresis on SDS–polyacrylamide gel and then transferred to a polyvinylidene fluoride membrane. The membranes were blocked with 5% bovine serum albumin in TBS-T and subsequently incubated with primary antibodies against phospho-ERK, total-ERK, phospho-c-Jun NH2-terminal kinases (JNK), total-JNK, phospho-Akt, total-Akt, phospho-c-Jun, β-catenin, SOX-9, CD34, CD200, and β-actin, purchased from Cell Signaling Technology, Inc. (Beverly, MA) and Santa Cruz Biotechnology (Santa Cruz, CA). Specific reactive bands were detected with a
LEDs REGULATE THE PROLIFERATION OF hORSCs

Co-Culture

Co-culture of hDPCs and hORSCs was performed as previously described [7]. hORSCs were seeded in the lower Transwell culture plate chamber (Cell Culture Insert Companion Plate, BD Falcon, Franklin Lakes, NJ) at a density of $5 \times 10^4$ cells per well. Twenty-four hours later, hORSCs were irradiated with 10 J/cm$^2$ 660 nm LED light. After that, hDPCs ($5 \times 10^4$ cells per well) were added to the upper chamber of the Transwell system, which was separated from the lower compartment by a permeable membrane coated with polyethylene terephthalate (pore size: 0.4 mm, 24-well format, Cell Culture Inserts, BD Falcon). The two cell types were then co-cultured and separately irradiated with 660 nm light. Viable DPC cell count was assessed with MTT assays after 2 days.

To obtain hORSC-conditioned medium (CM), hORSCs from passage three were seeded at a density of $1 \times 10^6$ cells in 10 cm culture dishes containing dKSFM. The cells were then exposed to LED 660 nm light at 0 or 10 J/cm$^2$, after which CM was collected. The CM contained several growth factors and cytokines secreted by hORSCs and was stored in sterile plastic tubes at $-20^\circ$ C and used within 1 month. hDPCs were seeded at a density of $5 \times 10^4$ cells into 24-well plates containing Dulbecco’s modified Eagle’s medium (DMEM) in the absence of fetal bovine serum. To observe CM effects, hDPCs were incubated with 50% DMEM plus 50% CM from the hORSC-culture. All hDPC culture plates were also irradiated with 660 nm light at 0 or 10 J/cm$^2$, and the MTT assay was performed after 48 hours.

Statistical Analysis

All data are expressed as mean ± SD. For statistical analysis, an ANOVA test was performed. All tests were one-sided, and a P-value < 0.05 was considered statistically significant.

RESULTS

Physiobiomodulative Effects of LED Irradiation on hORSC Viability

To determine the best wavelength range for LED, hORSCs were exposed to 415, 525, 660, and 830 nm LEDs at 1, 3, 5, and 10 J/cm$^2$. Cell viability was assessed by MTT assays (Fig. S1). LED irradiation with 525, 660, and 830 nm wavelengths at 5 J/cm$^2$ and all wavelengths at 10 J/cm$^2$ significantly enhanced cell proliferation (Fig. S1a). The results showed that 660 nm LED light at 10 J/cm$^2$ best stimulated ORSC proliferation. The Bcl2/bax mRNA ratio with 525 nm light and 10 J/cm$^2$ irradiation significantly increased cell survival by affecting anti-apoptosis and apoptosis, while the other wavelengths and energy levels did not (Fig. S1b–d).

Physiobiomodulative Effects of LED Irradiation on hORSC Migration Ability

Next, PBMT effects on hORSC migration were examined using a crystal violet assay. Irradiation at 660 and 830 nm at 10 J/cm$^2$ significantly enhanced hORSC migration. Irradiation with an LED wavelength of 660 nm at 10 J/cm$^2$ best induced hORSC migration compared to 830 nm light (Fig. S2).

Physiobiomodulative Effects of LED Irradiation on the ERK/Mitogen-Activated Protein Kinase (MAPK) Signaling Pathway in hORSCs

First, western blotting was used to investigate the effects of LED 660 nm light at a 10 J/cm$^2$ energy level on the ERK/MAPK signaling pathway (Fig. 1). ERK phosphorylation increased until 30 minutes after LED irradiation and persisted for 48 hours. This effect was inhibited by pretreatment with an ERK inhibitor (PD98059) at 20 µM before irradiation. A total of 660 nm LED irradiation time-dependently reduced Akt and c-Jun phosphorylation for 48 hours. Protein production of ERK, Akt, and c-Jun was reversed by treatment with an ERK inhibitor before irradiation. After irradiation with wavelengths other than 660 nm, Akt expression was totally blocked by pretreatment with an ERK inhibitor; thus, comparisons for relative fold changes were unavailable. Compared to 660 nm light, JNK expression was significantly decreased after pretreatment with an ERK inhibitor before irradiation with 415, 525, and 830 nm wavelengths, whereas c-Jun was not changed.

Second, western blotting was performed 24 hours after irradiation with all LED wavelengths. This revealed LED irradiation-induced ERK phosphorylation at all energy levels, especially 415 and 525 nm at 10 J/cm$^2$. JNK phosphorylation level was not significantly changed by LED irradiation. MAPK phosphorylation level was increased by 660 and 830 nm LED irradiation at 1 J/cm$^2$. hORSC proliferation was significantly increased by irradiation with 415 and 830 nm LED at 5 J/cm$^2$ and decreased by pretreatment with an ERK inhibitor (Fig. 2).

Physiobiomodulative Effects of LED Irradiation on the hORSC β-Catenin/Wnt Signaling Pathway

Next, we investigated PBMT effects on signaling cascades in the β-catenin/Wnt signaling pathway. The mRNA of genes responsible for β-catenin/Wnt signaling including β-catenin, Wnt5a, Axin2, and Lymphoid-enhancer factor-1 (Lef-1) was investigated by performing RT-PCR. Wnt5a, Axin2, and Lef-1 gene expression levels were increased 24 hours after various LED irradiation protocols. Although β-catenin expression did not show a significant increase in mRNA level, β-catenin protein level was increased after LED irradiation with 830 nm at 1 J/cm$^2$ and 415 and 525 nm at 5 J/cm$^2$ (Fig. 3).

Physiobiomodulative Effects of LED Irradiation on hORSC Cytokines and Growth Factors

LED irradiation at 830 nm significantly stimulated mRNA levels for various growth factors. We found...
significant increases in IGF-1 mRNA 24 after irradiation with 830 nm light at 1 J/cm². KGF mRNA was increased after irradiation with 830 nm at 10 J/cm². IL-6 and IL-8 mRNA was significantly increased after irradiation with 830 nm light at 10 and 5 J/cm², respectively. However, no significant difference was seen between FGF and VEGF mRNA levels. TGF-\(\beta_1\) mRNA was significantly enhanced 24 hours after irradiation with 830 nm light at 1 J/cm²; however, TGF-\(\beta_2\) mRNA expression change was not significant (Fig. S3).

Photobiomodulative Effects of LED Irradiation on hORSC Hair Stem Cell Markers

SOX-9 mRNA showed significantly increased expression after irradiation with 830 nm light at 10 J/cm². SOX-9 protein expression and the human follicular stem cell markers CD34 and CD200 were increased by various LED irradiation wavelengths, but the changes were not significant (Fig. S4).

LED-Irradiated hORSC Effects on hDPC Proliferation

We investigated whether LED irradiation on hORSC could increase hDPC proliferation when they were co-cultured. Co-cultured irradiated hORSCs and hDPCs showed an up to 150% increase in cell proliferation compared to controls. The growth-promoting effects of co-cultured in irradiated hORSCs were not higher than the effect of LED irradiation directly onto hDPCs. To evaluate whether the growth-promoting effects were mediated by cell-cell direct contact or paracrine effects, conditioned medium (CM) from hORSC cultures was used. CM from irradiated or non-irradiated hORSCs and hORSCs themselves was used for co-culture, mixed as 50% CM and 50% DMEM. hDPC culture under 100% DMEM with or without LED irradiation was used as a control.

A total of 50% CM from non-irradiated hORSCs mixed with 50% DMEM significantly increased hDPC proliferation compared to 100% DMEM. CM from irradiated hORSCs significantly enhanced hDPC proliferation compared to CM from non-irradiated hORSCs (Fig. 4).

DISCUSSION

PBMT with LED enhanced hORSC proliferation and migration, especially at 660 and 830 nm. The 660 nm light showed a maximum proliferative and migratory effect at 10 J/cm². Anti-apoptotic activity of PBMT was prominently observed with 525 nm light at 10 J/cm². Regulation of cell survival of hORSCs by PBMT may play a role in anagen prolongation. The Wnt/\(\beta\)-catenin and ERK signaling pathway seem to be the major pathway for hORSC proliferation or migration.

The \(\beta\)-catenin/Wnt signaling pathway is the major signaling pathway for hair morphogenesis and the hair cycle. \(\beta\)-catenin is required by bulge stem cells to promote their proliferation [8]. In this study, 830 nm PBMT at 1 J/cm² significantly induced \(\beta\)-catenin expression in hORSCs. Wnt5a is a downstream molecule in the Sonic HedgeHog signaling cascade and is specifically upregulated during early the morphogenesis stage [9,10], and
Axin2 is a direct target of Wnt. Lef-1 binds the keratin gene and differentiates hair shaft cells [11]. All mRNA of examined molecules related to the β-catenin/Wnt signaling pathway were upregulated by various PBMT conditions. Thus, one may speculate that, if PBMT could stimulate hORSCs in the bulge area to differentiate hair follicle stem cells, this would facilitate hair cycling and anagen induction in vivo.

SOX-9 is known to be specifically expressed in CD34-positive hORSCs in the bulge area and to involve in bulge formation and regulation of stem cell transcription [12–14]. SOX-9 mRNA showed increased expression...
after PBMT with 830 nm light at 10 J/cm². Sox9 is known to be a β-catenin-regulated gene; thus, elevated SOX-9 expression after PBMT might occur through activation of the Wnt/β-catenin signaling pathway [15].

The ERK pathway was the main pathway through which PBMT activated in hORSC proliferation, and these effects were reversed by pretreatment with an ERK inhibitor. p38 MAPK phosphorylation was significantly induced by 660 and 830 nm wavelengths at 1 J/cm², but not by higher energy levels. c-Jun activity can be induced by ERK or JNK pathway activation. Activated c-Jun contributes to cell cycle activation through targets such as cyclin D1. Phosphorylation of ERK, c-Jun, and Akt in response to PBMT seemed to stimulate cell proliferation and anti-apoptosis in hORSCs.

The CM from irradiated hORSCs showed greater effects on hDPC proliferation than did the CM from non-irradiated hORSCs. It seemed the CM from irradiated hORSCs contained more effective growth factors and cytokines than did the CM from non-irradiated hORSCs. Production of cytokines and growth factors was differentially affected by PBMT with different wavelengths. The 830 nm light significantly stimulated several growth factors in vitro. Since ERK phosphorylation was activated by 830 nm LED light, the increases in growth factors and cytokines seemed to be mediated by the ERK signaling pathway. Kim et al. [2] consistently compared the differential effects of PBMT on hair growth using 632, 670, 785, and 830 nm wavelengths and reported that the 830 nm wavelength showed the greatest hair growth promotion in a rat model. In this study, possible growth
factors responsible for cell proliferation were IGF-1, KGF, IL-6, and IL-8. IGF-1 is known to restore the immune activities of hair follicles and show an anagen-prolonging effect [16]. KGF is a well-known ORSC growth factor. IL-6 and IL-8 have been reported to increase hyper-proliferative skin disorders and wound regeneration [17]. TNF-α might downregulate the expression of adhesion molecules and promote ORSC migration. TNF-α is also a potent immunosuppressant that might exert its anti-inflammatory effects during irradiation. TGF-β is a well-known catagen inducer, but it has also been shown to contribute to anagen re-entry [18]. VEGF is known to accelerate hair growth and increase follicle size through follicular angiogenesis and to stimulate ORSC migration [19–20].

Theoretically, PBMT on the follicular units taken from donor sites before follicular unit transplantation could promote transplanted hair follicle survival in the recipient area and wound healing through production of various cytokines and growth factors. Our data support the presented hypothesis. A recent study investigated whether PBMT on follicular units before transplantation would lead to better outcomes [21]. Although they failed to show a positive effect of PBMT during hair transplantation, this finding needs to be verified through further studies.

We previously reported photobiomodulative effects of LED irradiation on hDPC proliferation and ex vivo hair growth (data not shown). Comparing the photobiomodulative effects on hORSCs and on the hDPCs in our previous data, both hORSCs and hDPCs showed maximal cellular proliferation after LED irradiation at 660 nm and 10 J/cm². Both cell types increased phosphorylated ERK, but little change was seen in JNK phosphorylation after LED irradiation. However, p38 MAPK phosphorylation, which is a stress-related signaling pathway, was not activated in hDPCs, but increased in hORSCs in response to LED irradiation, especially with 660 nm light at 1 J/cm². The growth-promoting effect of PBMT was decreased after ERK inhibitor pretreatment in both hORSCs and hDPCs.

Recently, Sheen et al. [6] reported that red light enhanced ORSC proliferation via activating ERK signaling in a rat model. They observed that ERK phosphorylation was involved in LED-induced hORSC proliferation, but, not p38 phosphorylation, which differs from the results of our study. The photobiomodulative effects related to cell survival and stress response seem to be different depending on the cell type and irradiation settings.
REFERENCES


SUPPORTING INFORMATION

Additional supporting information may be found in the online version of this article.