Effect of Photobiomodulation on Wound Healing of the Corneal Epithelium through Rho-GTPase

Yun-Hee Rhee1,2, Kyong Jin Cho2,3, Jin-Chul Ahn1,4, Phil-Sang Chung1,2,5

1Beckman Laser Institute Korea, Dankook University, Cheonan, Korea
2Laser Translational Clinical Trial Center, Dankook University Hospital, Cheonan, Korea
3Department of Ophthalmology, College of Medicine, Dankook University, Cheonan, Korea
4Department of Biomedical Science, College of Medicine, Dankook University, Cheonan, Korea
5Department of Otolaryngology-Head and Neck Surgery, College of Medicine, Dankook University, Cheonan, Korea

Background and Objectives
In recent years, photobiomodulation (PBM) using low-level light has been applied to diverse clinical approaches because of its potential to elevate the cell metabolism or regulate various signaling pathways. This study evaluated the possibility of a short term effect of PBM on the wound healing of corneal epithelial cells. Rapid healing of the corneal epithelium and the return of an intact basement membrane can restore the eye’s normal mechanical barriers. The migration, proliferation, attachment, and cytoskeletal rearrangement play a critical role in the wound healing process of the corneal epithelium.

Materials and Methods
To determine which wavelength was most effective on corneal epithelium wound healing, light emitting diode (LED) arrays with wavelengths of 470, 530, 660, 740, and 850 nm were used. The proliferative effect was assessed using a MTT assay, cell cycle assay, and BrdU immunofluorescence (IF) staining, and the motility effect was examined using a wound healing assay after PBM. The cytoskeletal rearrangement effect of PBM was also evaluated by Western blot analysis and IF staining.

Results
PBM had no effect on cell proliferation; the cell cycle portion and BrdU were not changed after the PBM treatment, whereas cell survival was decreased at 470 nm. On the other hand, PBM at wavelengths greater than 660 nm affected migration. In particular, 740 nm was the most effective. The expression of Rho A and Rho C increased after PBM at wavelengths greater than 660 nm. The levels of cdc42 and mTORC2 expression were similar.

Conclusion
This study showed that PBM could increase the corneal epithelial cell migration capacity without cell proliferation in a short time via the activation of a part of Rho-GTPase pathways without the effect of the upstream signals. These findings may be used for the future development of PBM-based therapy for acute ocular surface diseases.

Key words
Photobiomodulation; Corneal epithelium; Wound healing; Rho-GTPase
INTRODUCTION

The corneal epithelium is the outermost mechanical barrier of an eye in mammalian. Various corneal injuries result in corneal epithelial cell damage and break down. Although corneal epithelial cell had full vitalities, damages of corneal epithelium by fatal injuries such as alkali burn, ulcer, and surgical operation are difficult to repair and require transplantation in extreme cases. A repair system of injured corneal epithelium is delicate cross-talk of various signaling pathways for wound healing events such as proliferation, migration, adhesion, and differentiation of corneal epithelial cells. Rapid healing of the corneal epithelium and the return of an intact basement membrane can restore the eye’s normal mechanical barriers and prevent various epithelium-derived growth factors from leaking into the stroma.

Meanwhile, the use of low levels of visible or near-infrared (NIR) light for reducing pain, inflammation, and edema, promoting healing of wounds, deeper tissues and nerves, and preventing tissue damage has been known. There are many studies on the use of light aiming to positively stimulate the healing process, but no report about corneal wound healing process. In this study, we hypothesized that photobiomodulation (PBM) using low-level light could restore the corneal epithelial damage faster than the typical corneal recovery time. Based on the corneal epithelial wound healing process, we investigated the effect of PBM on human corneal epithelial cells (HCE-T) using different wavelengths of light emitting diode (LED) array by evaluating of cell proliferation, migration, attachment and cytoskeletal rearrangement pathways.

MATERIALS AND METHODS

Chemicals

3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl-tetrazolium bromide (MTT), dimethyl sulfoxide (DMSO), crystal violet, propidium iodide (PI), RIPA buffer, protease and phosphatase inhibitors were purchased from Sigma (Saint Louis, MO, USA). Protein DC kit was supplied by Bio-Rad (Hercules, CA, USA). BrdU, RhoA, RhoB, RhoC, phospho-Rac, Rac-123, cdc42, and mTOR were purchased from cell signaling technology (Beverley, MA, USA). β-actin was purchased from Sigma.

Cell

The human corneal epithelial cell line HCE-T was purchased from ATCC (Manassas, VA, USA) supplemented with 0.05 mg/ml bovine pituitary extract (BPE), 5 ng/ml epidermal growth factor (EGF), 500 ng/ml hydrocortisone in keratinocyte-serum free medium (Life Technologies, Grand Island, NY, USA). The cells were maintained at 37°C in a 5% CO₂ humidified environment.

Photobiomodulation condition for low-level light therapy

The light sources were 470, 530, 660, 730, and 850 nm light emitting diode (WON Technology Co., Ltd, Daejeon, Korea). The elliptical fiber shape of the diode laser output had a diameter of 1.7 mm. The light device information is described in Table 1. The irradiance at the surface of the cell monolayer was measured by a power meter [FieldMAXII, Coherent Inc. Santa Clara, CA]. We attached a supplementary data file of LED and the photobiomodulation parameter evaluation is provided in Table 1 and Fig. 1. The irradiance or power density was measured as 100 mW/cm² and the operating mode was a continuous wave. The light dose measure for energy density or fluency was 30 J/cm² with the duration of each treatment set.

Proliferation assay

Cells were inoculated into a 96-well, flat-bottomed microplate at a volume of 100 μl (2,000 cells) for a stationary culture and incubated overnight in growth medium to allow the cells to adhere the bottom of wells. Cells underwent by dual PBM treatment at 30 J/cm² once and 10 J/cm² three times then incubated for 24, 48 and 72 h in 5% CO₂ at 37°C. After incubation 50 μl MTT solution (2 mg/ml) was added to each well. Four hours h after incubation in 5% CO₂ at 37°C, media of each well was removed and 100 μl DMSO was added to dissolve violet blue crystals. The growth of cells was determined by measuring the absorbance at 570 nm using ELISA reader (TECAN, Männedorf, Switzerland).

Cell cycle analysis

To analyze cell cycle distribution at different stages in corneal epithelial cells with and without PBM, cell cycle analysis was performed by flow cytometry. Briefly, Cells

| Table 1. Photobiomodulation (PBM) by low level light condition |
|---------------------------------------------------------------|
| Irradiation parameters (nm) | 470, 530, 660, 740, 850 |
| Treated surface diameter (mm) | 35 |
| Treated area (cm²) | 9.61 |
| Power input (mW/cm²) | 5.2 |
| Irradiation time (sec) | 600 |
| Total energy (J) | 30 |
| Total energy density (J/cm²) | 3.12 |
were treated with PBM at 30 J/cm² and incubated at 37°C and 5% CO₂ for 24 h. Then the cells were collected by trypsinization and washed with PBS. Next, the cells were incubated with propidium iodide (50 μg/ml) (Sigma) for 15 minutes. The cell cycle distribution and sub-G1 DNA content were determined and analyzed by flow cytometry (Accuri C6, BD, CA, USA). The percentages of viable and dead were determined as 10,000 events per sample using an FL-2 filter and compared with control to study the efficacy of SFE. The histogram was prepared for showing a change in the percentage of cell numbers at different subpopulation.

**Migration assay**

Cells were seeded into 12-well plate and grown to confluence. The Wound was created by scraping confluent cell monolayers with a pipette tip. The cells were allowed to migrate for 8 h after 30 J/cm² of PBM treatment. At 0 h and 8 h after scratching, the plate was stained with 0.5% crystal violet and wound images were taken under the inverted microscope to assess the ability of the cells to migrate into the wound area.

**Western blot analysis**

The expressions of Rho-GTPase signaling proteins were analyzed by Western blot technique. Cells were treated with same procedures of cell cycle assay described above, and the proteins were extracted in RIPA buffer (50 mM Tris-HCl, pH 8.0, 1% NP-40, 0.5% sodium deoxycholate, 150 mM NaCl, and 0.1% sodium dodecyl sulfate with protease and phosphatase inhibitor cocktail (Sigma) and centrifuged at 15,000 rpm for 30 minutes at 4°C. The protein concentration was determined using the Bradford Protein Assay Reagent. Equivalent amounts of protein from each sample were loaded onto polyacrylamide gels and separated by electrophoresis. Then the proteins were transferred to PVDF membranes (Immu-no-Blot PVDF; BioRad Laboratories, Hercules, CA, USA). Both electrophoresis and blotting were performed by using a PowerPac200 electrophoresis system (BioRad Laboratories). The membranes were blocked for 2 hours at room temperature in Tris-buffered saline containing 0.1%
Tween-20 (TBST) and 3% BSA, and incubated overnight at 4°C with the primary antibody diluted with 3% BSA in TBST. The membranes were probed with horseradish peroxidase-conjugated anti-mouse IgG, anti-goat IgG or anti-rabbit IgG antibody for 2 hours. The protein band was developed by ECL Western Blotting detection reagents (GE Healthcare, Buckinghamshire, UK) and the pictures were taken and quantified by the image analyzer (Bio-Rad Laboratories).

**Immunofluorescence staining**

Cells were seeded onto 11 mmØ round coverslip in 24-well plate and grown to confluence. After 30 J/cm² of PBM treatment, cells were incubated for 24 h, then fixed with chilled methanol. Fixed cells were blocked with 3% BSA and then incubated with primary antibodies (mTOR or BrdU) for overnight at 4°C in a humidified chamber. Cells were then incubated in a 1:100 dilution of Alexa488 or Alexa650 probed anti-rabbit IgG and observed using confocal laser scanning microscope (FV3000, Olympus, Tokyo, Japan). The green or red positive cells were counted under ×40 magnification.

**Statistical analysis**

The results are expressed as mean ± SD. Unpaired Student’s t-tests were used for comparisons between two means. The comparisons were performed by analysis of one way ANOVA, Tukey test (Graph Pad, Prism®, La Jolla, CA, USA). *p<0.1, **p<0.05 and ***p<0.0001 were considered statistically significant.

**RESULTS**

**The PBM induced the migration of HCE-T corneal epithelial cells**

To investigate whether PBM has an effect on the migration of corneal epithelial, a scratch wound healing assay was performed. As shown in Fig. 2, PBM treatment groups exhibited the increase of cell migration except for 470 nm irradiated group. Interestingly, both 740 nm and 850 nm showed the significant cell migration.

**The PBM effect on proliferation of HCE-T corneal epithelial cells**

To determine whether PBM-induced cell migration was due to cell proliferation, we performed the BrdU staining and cell proliferation assay. Since energy dose could affect the proliferation of cells, we performed the separated cell proliferation assay using 30 J/cm² once and 10 J/cm² three times. As shown in Fig. 3A and B, cell proliferative ratio was no significance between a control group and PBM group. The cell proliferative ratio with or without PBM was similar to that of control. In addition, there was no difference in the results of the cell proliferation between irradiated 30 J/cm² at one time or 10 J/cm² three times. The IF staining also showed the same results of cell proliferation (Fig. 3C). The BrdU positive cell number of a control group was no different from PBM treated group.

**The PBM effect on cell cycle progression of HCE-T corneal epithelial cells**

The cell cycle progression under PBM was evaluated...
Fig. 3. The effect of PBM on cell proliferation of HCE-T cells. (A, B) Cells were seeded onto 96-well at a density of 2,000 cells/well and incubated overnight in a growth medium. Cells underwent by dual PBM treatment at 30 J/cm² (A) once and 10 J/cm² three times (B) then incubated for 24, 48 and 72 h in 5% CO₂ at 37°C. After incubation, 2 mg/ml of MTT solution was added to each well then was replaced by 100 μl DMSO after 4 h. The growth of cells was determined by measuring the absorbance at 570 nm. (C) Cells were seeded onto 11 mmΦ round coverslip in 24-well plate and were underwent PBM treatment at 30 J/cm². After 24 h, cells were fixed by chilled methanol. Fixed cells were blocked with 3% BSA and then incubated with BrdU (1:100) for overnight at 4°C in a humidified chamber. Cells were then incubated in a 1:100 dilution of Alexa650 probed anti-rabbit IgG and observed using confocal laser scanning microscope. The red positive cells were counted under × 40 magnification. Every assay was performed 3 times and the results are expressed as mean ± SD. One-way ANOVA (Tukey test) were used for comparisons between two means.
via flow cytometry. A representative example depicting the effect of PBM on cell cycle phase distribution in the HCE-T cell line is shown in Fig. 4. At 24 h after PBM treatment, the percentage of cells in each phase were analyzed. There were no differences between control group and PBM treated group. Taken together, PBM had no effect on cell proliferation within 24 h whereas had an effect on migration.

The PBM effect on Rho-GTPase expression of HCE-T corneal epithelial cells
If so, we wondered what would have been possible for

Fig. 4. The effect of PBM on cell cycle progression of HCE-T cells. Cells were treated with PBM at 30 J/cm² and incubated at 37°C and 5% CO₂ for 24 h. Cells were collected by trypsinization and were incubated with propidium iodide for 15 minutes. The cell cycle distribution and sub-G1 DNA content were determined and analyzed by flow cytometry. The percentages of viable and dead were determined as 10,000 events per sample using an FL-2 filter and compared with control. Every assay was performed 3 times and the results are expressed as mean ± SD. One-way ANOVA (Tukey test) were used for comparisons between two means.
Fig. 5. The effect of PBM on Rho-GTPase activity of HCE-T cells. Cells were treated with same procedures of cell cycle assay described above, and the proteins were extracted in RIPA buffer. Equivalent amounts of protein were analyzed by Western blotting. The expression of Rho-GTPase was taken and quantified by the image analyzer. Every assay was performed 3 times and the results are expressed as mean ± SD. One-way ANOVA (Tukey test) were used for comparisons between two means. *p<0.1, **p<0.05, ***p<0.001.
cell migration without proliferation. For cell movement, intracellular adherent and cellular structure should be remodeled. We investigated the expression marker of cytoskeletal rearrangements such as Rho family of small GTPase including Rho, Rac, and cdc42 acts as molecular switches to regulate processes such as cell migration, adhesion, proliferation and differentiation. As shown in Fig. 5, the expression of Rho A and Rho C was increased after PBM treatment at over 660 nm whereas the expression of RhoB and phospho-Rac was elevated only at 660 nm. The expression of cdc42 showed no difference.

The PBM effect on mTOR expression of HCE-T corneal epithelial cells

Next, we investigated the expression of mTORC2 after PBM for concerning the upstream of Rho/Rac activity. As shown in Fig. 6, the expression of mTOR was seemed to increase in PBM treatment group over 660 nm, but not significantly. However, the expression of mTORC2 has significantly decreased after PBM treatment at 470 and 530 nm.

![Image](image-url)
DISCUSSION

There is evidence that multiple mammalian cell types can respond to low-level light irradiation. For wound healing type studies, these cells are likely to be endothelial cells, fibroblasts, keratinocytes, and possibly some classes of leukocytes.

Corneal epithelial repair involves a multifaceted series of events with specific physiologic functions. At this phase, metabolic activity was increased and the cellular structure was reorganized for allowing the epithelial cells migration over the wound surface. Additionally, intercellular adherens and gap junctions are also lost, desmosomes are remodeled, and structural proteins and actin filaments are assembled in preparation for cellular migration. The epithelial healing process is referred to as the migration phase, in which cells begin to move and cover the epithelial defect. This begins with the flattening of cells at the wound edge into a monolayer, and the formation of lamellipodia and filopodia that aid in cellular movement. Focal contacts of migrating cells to the provisional extracellular matrix, and subsequent contraction of the cytoskeletal actin filaments, allow the layer of cells to slide together as a sheet, eventually fully covering the wound bed. It is important to note that throughout the latent and migration phases, there is no mitotic activity of the cells in or around the area of the epithelial defect; these initial processes are completely independent of cellular proliferation. Based on this initial wound healing process of corneal epithelial cells, we hypothesized that PBM could induce cell motility in short time. Wound closure is usually accomplished within 2 to 4 days following a corneal injury, the entire epithelial healing process typically requires weeks after to be fully restored. In this study, we observed that PBM regulate cell migration but not proliferation in corneal epithelial cells. We evaluate the cell proliferation using BrdU assay, MTT assay (Fig. 3), and cell cycle progression assay (Fig. 4), however, PBM could not elevate the corneal epithelial cell proliferation. Thus, we focused the Rho-GTPase activity after PBM treatment. The Rho family of small GTPases, including Rho, Rac, and Cdc42, are small monomeric G proteins that cycle between an inactive GDP-bound form and an active GTP-bound form and regulate actin cytoskeleton, cell migration, and proliferation. Rho regulates actin polymerization, resulting in the formation of stress fibers and the assembly of focal adhesion complex. Rho has been implicated in cell migration, actin organization, focal adhesion formation, as well as adherents and gap junction assembly in the corneal epithelium. Rho kinases (ROCKs) were initially characterized for their roles in mediating the formation of RhoA-induced stress fibers and focal adhesions through their effects on the phosphorylation of myosin light chain. In the cornea, ROCKs have been suggested to be involved in epithelial differentiation, cell cycle progression, cell-cell adhesion, endothelial barrier integrity, stromal cell phenotype conversion, cytoskeleton reorganization, contractility, and cell-matrix interaction. Thus, we investigated the Rho-GTPase activity by PBM and found that the expression of RhoA and RhoC was significantly increased after PBM treatment. However, the expression Cdc 42 after PBM was not changed (Fig. 5). The precise mechanism of Rho-GTPase during corneal epithelial cells should be more investigated, however, we suggested that the Rho-GTPase had a critical role during corneal epithelial wound healing but not all Rho-GTPase factors are involved. Next, we investigated whether Rho-GTPase activation by PBM treatment was downstream of mechanistic target of rapamycin catalytic domain 2 (mTORC2). Although both mTORC1 and mTORC2 are central mediators of growth factor responses and cellular metabolism, mTORC1 is uniquely activated by environmental cues such as adequate nutrients (amino acids), energy (ATP/AMP), and oxygen availability, which results in activation of pathways leading to cellular growth (protein, DNA, and lipid synthesis) and inhibition of autophagy. Conversely, mTORC1-mediated cellular growth is inhibited by cellular stresses such as DNA damage, low energy states, and hypoxia. mTORC2, by comparison, is more specifically activated by growth factor signaling, and facilitates cell survival and cytoskeletal reorganization to promote cell migration and adhesion. In addition, the Rho-GTPase family has been known to the target of mTORC2 kinases. Unlike what we expected, the expression of mTORC2 was significantly decreased after PBM treatment at 470 and 530 nm, and not increased by PBM treatment over 660 nm (Fig. 6). This results suggested that under 530 nm of wavelength inhibited the cell migration via mTORC2 regulation, nevertheless over NIR wavelength did not increase or affect the expression of mTORC2.

In summary, the current study revealed that PBM could increase corneal epithelial cell migration capacity in short time. The activation of Rho-GTPase pathways was involved in this process. In addition, mTORC2, the upstream of Rho-GTPase was not changed by PBM treatment. These results may be used for future development of PBM-based therapy for acute ocular surface diseases.
ACKNOWLEDGEMENTS

This research was supported by a grant of the Korea Health Technology R&D Project through the Korea Health Industry Development Institute (KHIDII), funded by the Ministry of Health & Welfare, Republic of Korea (grant number: HI15C1524) and was a part of the project titled “Development of marine material based near infrared fluorophore complex and diagnostic imaging instruments”, funded by the Ministry of Oceans and Fisheries, Korea.

REFERENCES

1. Torricelli AA, Santhanam A, Wu J, Singh V, Wilson SE. The corneal fibrosis response to epithelial-stromal injury. Exp Eye Res 2016;142:110-8.
2. Ljubimov AV, Saghizadeh M. Progress in corneal wound healing. Prog Retin Eye Res 2015;49:17-45.
3. Santhanam A, Torricelli AA, Wu J, Marino GK, Wilson SE. Differential expression of epithelial basement membrane components nidogens and perlecan in corneal stromal cells in vitro. Mol Vis 2015;21:1318-27.
4. Torricelli AA, Marino GK, Santhanam A, Wu J, Singh A, Wilson SE. Epithelial basement membrane proteins perlecan and nidogen-2 are up-regulated in stromal cells after epithelial injury in human corneas. Exp Eye Res 2015;134:33-8.
5. Kwon T, Kwon DY, Chun J, Kim JH, Kang SS. Akt protein kinase inhibits Rac1-GTP binding through phosphorylation at serine 71 of Rac1. J Biol Chem 2000;275:423-8.
6. Iwata TN, Ramirez-Komo JA, Park H, Iritani BM. Control of B lymphocyte development and functions by the mTOR signaling pathways. Cytokine Growth Factor Rev 2017;36:47-62.
7. Moore P, Ridgway TD, Higbee RG, Howard EW, Lucroy MD. Effect of wavelength on low-intensity laser irradiation-stimulated cell proliferation in vitro. Lasers Surg Med 2005;36:8-12.
8. Hawkins D, Abrahamse H. Biological effects of helium-neon laser irradiation on normal and wounded human skin fibroblasts. Photomed Laser Surg 2005;23:251-9.
9. Yu HS, Wu CS, Yu CL, Kao YH, Chiou MH. Helium-neon laser irradiation stimulates migration and proliferation in melanocytes and induces repigmentation in segmental-type vitiligo. J Invest Dermatol 2003;120:56-64.
10. Fujimaki Y, Shimoyama T, Liu Q, Umeda T, Nakaji S, Sugawara K. Low-level laser irradiation attenuates production of reactive oxygen species by human neutrophils. J Clin Laser Med Surg 2003;21:165-70.
11. Yin J, Yu FS. Rho kinases regulate corneal epithelial wound healing. Am J Physiol Cell Physiol 2008;295:C378-87.
12. Hall A. Rho GTPases and the actin cytoskeleton. Science 1998;279:509-14.
13. Kaibuchi K, Kuroda S, Amano M. Regulation of the cytoskeleton and cell adhesion by the Rho family GTPases in mammalian cells. Annu Rev Biochem 1999;68:459-86.
14. Kozma R, Ahmed S, Best A, Lim L. The Ras-related protein Cdc42Hs and bradykinin promote formation of peripheral actin microspikes and filopodia in Swiss 3T3 fibroblasts. Mol Cell Biol 1995;15:1942-52.
15. Anderson SC, Stone C, Tkach L, SundarRaj N. Rho and Rho-kinase (ROCK) signaling in adherens and gap junction assembly in corneal epithelium. Invest Ophthal Vis Sci 2002;43:978-86.
16. Nakamura M, Nagano T, Chikama T, Nishida T. Role of the small GTP-binding protein rho in epithelial cell migration in the rabbit cornea. Invest Ophthal Vis Sci 2001;42:941-7.
17. Leung T, Chen XD, Manser E, Lim L. The p160 RhoA-binding kinase ROK alpha is a member of a kinase family and is involved in the reorganization of the cytoskeleton. Mol Cell Biol 1996;16:5313-27.
18. SundarRaj N, Kinchington PR, Wessel H, Goldblatt B, Hassell J, Vergnes JP, et al. A Rho-associated protein kinase: differentially distributed in limbal and corneal epithelia. Invest Ophthal Vis Sci 1998;39:1266-72.
19. Freedson PS, Brentley K, Ainsworth BE, Kohl HW 3rd, Leslie E, Owen N. New techniques and issues in assessing walking behavior and its contexts. Med Sci Sports Exerc 2008;40(7 Suppl):S574-83.
20. Harvey SA, Anderson SC, SundarRaj N. Downstream effects of ROCK signaling in cultured human corneal stromal cells: microarray analysis of gene expression. Invest Ophthal Vis Sci 2004;45:2168-76.
21. Kim A, Matthew Petroll W. Microtubule regulation of corneal fibroblast morphology and mechanical activity in 3-D culture. Exp Eye Res 2007;85:546-56.
22. Petroll WM, Vishwanath M, Ma L. Corneal fibroblasts respond rapidly to changes in local mechanical stress. Invest Ophthal Vis Sci 2004;45:3466-74.
23. Laplante M, Sabatini DM. mTOR signaling in growth control and disease. Cell 2012;149:274-93.