Enhancement of Osteogenic Proliferation and Differentiation in Osteoblast-Like Cells using Blue Light-Emitting Diode Radiation

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Abstract: The use of phototherapy in medicine, that is, the administration of quasi-monochromatic light for the treatment of a wide range of medical conditions, has been thoroughly investigated \textit{in vitro} and \textit{in vivo}. The purpose of this study was to investigate the effect of light-emitting diodes (LEDs) of various wavelengths on the proliferation of osteoblast-like cells (SaOS-2 cells). We studied the biologic response of SaOS-2 to stimuli of LEDs of four different wavelengths (control, red, green, and blue). Biological properties stimulated by LEDs were evaluated using \textit{in vitro} (osteoblast cell lines, SaOS-2) tests, such as cell attachment, proliferation, and differentiation of cell lines, to assess the enhancement of proliferation and differentiation using a proliferation assay to determine alkaline phosphatase (ALP) activity and von Kossa stain. Real-time polymerase chain reaction was used to analyze gene expression for osteocalcin, osteonectin, and osteoprotegerin. The cell proliferation assay ($p < 0.001$), and cell migration assay ($p=0.001$) for the blue LED group showed significant improvement compared to other groups. Molecular biologic analysis also showed significant differences for the blue LED groups of osteocalcin ($p=0.039$), and osteonectin ($p=0.010$). The irradiation of osteoblast-like cells with the blue LED promoted proliferation and differentiation. The full benefits of LED radiation must be established by further investigations using well controlled protocols.

Key words: blue light-emitting diode, osteogenic proliferation, osteogenic differentiation

1. Introduction

Phototherapy in medicine, defined as the use of quasi-monochromatic light for treating a wide variety of medical conditions, has been systematically studied \textit{in vitro} and \textit{in vivo}.\textsuperscript{1,4} Coherent light can initiate the modulation of physiological processes by the incremental production of adenosine triphosphate (ATP), stimulating microcirculation, increasing collagen fiber deposition, promoting higher fibroblast cell proliferation in the site of the lesion, and reducing the number of inflammatory cells.\textsuperscript{4,8}

Another potential source of light for phototherapy is light-emitting diodes (LEDs), which have a longer life span; they are more economically accessible and can be as efficient as laser light.\textsuperscript{5} Casalechi et al. demonstrated the effectiveness of LEDs in the regeneration of the Achilles tendon, reducing the number of fibroblasts and improving the quality of remodeling.\textsuperscript{10} Another study compared the effects of lasers and LEDs on tissue repair in diabetic rats and found similar effects; the LED was more efficient than the laser at reducing wound diameters.\textsuperscript{11}

Research on clinical applications has primarily focused on low-level laser studies, and the use of alternative light sources, such as LEDs, has received little attention. In a recent study by Vinck \textit{et al}., LED radiation was shown to enhance fibroblast growth and proliferation.\textsuperscript{7,12} However, no previous studies have reported on the effect of LED radiation on osteoblasts.

We investigated the effects of LED radiation on the biologic and morphologic responses of an osteoblast-like cell line (SaOS-2) \textit{in vitro} based on the proliferation (cell proliferation assay), differentiation [alkaline phosphatase (ALP) activity and von Kossa stain], attraction capability (cell migration assay), and messenger RNA (mRNA) expression (osteocalcin, osteoprotegerin, and osteonectin) using real-time polymerase
chain reaction (PCR).

2. Materials and Methods

Three LED light sources were used: red light (wavelength 630 nm), green light (wavelength 530 nm), and blue light (wavelength 460 nm).

2.1 Irradiation with Light-Emitting Diode

SaOS-2 cells (0.5 mL; 5×10⁴ cells/mL) were seeded on 8-cm² plate dishes in a medium containing 1% fetal bovine serum (FBS), and the plates were exposed to LED radiation. This treatment did not increase the temperature (±0.1°C) of the culture medium. The cells were irradiated once for 10, 20, and 30 minutes. The culture plates were exposed at a distance of 15 cm. Nonexposed cells (control) were maintained outside the incubator under the same conditions as the exposed cells.

2.2 Cell Proliferation Assay

The cells were incubated (37°C, 5% CO₂, 95% humidity) for 24, 48, 72, and 96 hours. The medium was exchanged with fresh medium before measuring cell proliferation using the CellTiter 96® nonradioactive cell proliferation assay (Promega, Madison, WI) according to the manufacturer’s protocol. This assay is a colorimetric method for determining the number of viable cells. The amount of formazan formed can be measured by its absorbance at 450 nm using a plate reader and is directly proportional to the number of viable cells in the culture. Using this kit, dye solution (15 µL) was placed on a stainless steel sample holder/tray, and the cells were incubated in 5% CO₂ at 37°C for 4 hours. After stop solution (100 µL) was added, cells (100 µL) were transferred by pipette to a 96-well plate (Nunc A/S, Roskilde, Denmark). Cell proliferation was measured at 450 nm using a spectrophotometer (EL 312e; BioTek Instruments, Winooski, VT).

2.3 Alkaline Phosphatase Activity and the Von Kossa Stain

ALP activity was measured, followed by incubation for 7 and 10 days. The medium was removed, and the cells were washed with phosphate-buffered saline (PBS) three times to remove as much of the serum in the culture fluid as possible. Next, 1 mL of 0.02% Triton X-100 was placed on the stainless steel sample holder/tray to lyse the cells. Cytolytic solution was transferred to a 1.5-mL tube, and the cells were sonicated. The tube was centrifuged (14,000 rpm, 4°C, 15 minutes), and the supernatant was transferred to a new 1.5-mL tube. Then, 100 µL of 1 mol/L Tris–HCl, 20 µL of 5 mmol/L MgCl₂, and 20 µL of 5 mmol/L p-nitrophenyl phosphate were added to the supernatant. The mixture was allowed to react at 37°C for 30 minutes, and 50 µL of 1 N NaOH was added to stop the reaction. Using p-nitrophenol as a standard, the absorbance was measured at 410 nm using a spectrophotometer. The measured ALP activity was expressed as p-nitrophenol production divided by the reaction time and the quantified protein synthesis, as measured by a protein assay kit (Bio-Rad, San Jose, CA). The von kossa staining cells were rinsed in water and stained with silver nitrate for 30 minutes under a UV light. Cells were then rinsed in water and allowed to dry.

2.4 Cell Migration Assay

We placed each LED lamp in a Transwell® chamber containing 24-well plates (Costar, Cambridge, MA) and put 500 µL of 1% FBS medium in the lower chamber. We prepared 0.5 mL of SaOS-2 cells (5×10⁴ cell/mL) in serum-free medium, added the cell suspension and culture fluid to the upper chamber above the polycarbonate filter (8-µm pores), and cultured the cells for 24 hours at 37°C in 5% CO₂. Then, we removed the medium from the upper chamber and removed the membrane and cells that had not migrated using a swab. To count the migrated cells, we placed the chamber in a new well, added 200 µL of extraction solution to the chamber, shook the plate on an orbital shaker for 10 minutes, transferred 100 µL of each sample to a 96-well plate, and measured the absorbance at 560 nm using a spectrophotometer.

2.5 Real-Time PCR

SaOS-2 cells (0.5 mL; 5×10⁴ cells/mL) were seeded on 8-cm² plate dishes in a medium containing 1% FBS, and the plates were exposed to LED radiation. Next, the cells were incubated for 4 days. The medium was exchanged for fresh medium before extracting RNA. The medium was removed from the incubated cells and irrigated with PBS twice; then, TRIzol reagent (Invitrogen, Carlsbad, CA) was added. Each 0.5-mL sample was placed in a 1.5-mL tube, and chloroform (Sigma, St. Louis, MO, USA) was added, followed by strong vortexing for 20 seconds and centrifugation at 13,000 rpm. After isopropanol (Sigma) was added, the mixture was shaken and allowed to react for 10 minutes at room temperature, and the supernatant was removed by centrifugation (13,000 rpm, 4°C, 15 minutes). The mixture was again centrifuged by adding 1 mL of 70% ethanol to the sediment, and after removing the ethanol, the sediment was melted by adding diethylpyrocarbonate (DEPC)-treated distilled water, and the RNA was measured using a spectrophotometer.

Total RNA (1 µg), separated to synthesize complimentary DNA (cDNA), was admixed with 50 µM of oligo (dT)₂₀ and 10 mM of