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Dose analysis of photobiomodulation therapy on osteoblast, osteoclast, and osteocyte

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Abstract. The objective of this study was to evaluate the effects of varying light doses on the viability and cellular activity of osteoblasts, osteocytes, and osteoclasts. A light application device was developed to apply 940-nm wavelength light from light-emitting diodes on three cultured cells, MC3T3-E1, MLO-A5, and RANKL-treated RAW264.7 cells. The doses (energy density) on cells were 0, 1, 5, and 7.5 J/cm^2. The corresponding light power densities at the cell site were 0, 1.67, 8.33, and 12.5 mW/cm^2, respectively, and the duration was 10 min. The results showed that the three cell types respond differently to light and their responses were dose dependent. Low-dose treatment (1 J/cm^2) enhanced osteoblast proliferation, osteoclast differentiation, and osteoclastic bone resorption activity. Osteocyte proliferation was not affected by both low- and high-dose (5 J/cm^2) treatments. While 1 J/cm^2 did not affect viability of all three cell types, 5 J/cm^2 significantly decreased viability of osteocytes and osteoclasts. Osteoblast viability was negatively impacted by the higher dose (7.5 J/cm^2). The findings suggest that optimal doses exist for osteoblast and osteoclast, which can stimulate cell activities, and there is a safe dose range for each type of cell tested. [DOI: 10.1117/1.JBO.23.7.075008]

Keywords: photobiomodulation; osteoblast; osteocyte; osteoclast; cell viability; proliferation; differentiation; light dose.

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1 Introduction

Photobiomodulation (PBM) therapy has been reported to have major effects on stimulating bone modeling and remodeling (BMR), resulting in clinical benefits such as tissue repair or accelerated tooth movement. The therapy involves applying light from laser or light-emitting diode (LED) with certain intensity (mW) to the tissue for a period of time to achieve clinical effects. There have been applications in the fields of orthopedics, orthodontics, as well as pain management.[1–16] Conversely, there have been studies that do not show definitive effects.[9,10,12,17]

Applications of PBM have been reported. The light sources were characterized by the following parameters: wavelength (nm), energy or energy density (J or J/cm^2), power or power density (mW or mW/cm^2), the application time, and application frequency (no. of application per day or week). The dose is defined as the amount of energy density delivered per application. The unit is J/cm^2, which is calculated by the product of input power density (mW/cm^2) and application duration (s). Energy density (J/cm^2) is typically used as the dose, a parameter that best represents the energy the cell receives. The light was typically applied to the tissue surface of the region of interest. The primary purpose of this research was to investigate whether the PBM can increase BMR activities[1,2,7,18–21] and stimulate cell proliferation.[18,22–25] Research on cell response to PBM demonstrates positive effects, supporting the conclusions of the animal studies that PBM can accelerate cell proliferation and increase BMR.[22,24,25]

BMR is the key for achieving various clinical outcomes and is accomplished by the three primary bone cell types: osteoblasts, osteoclasts, and osteocytes. Studies have shown that the cells receive the light energy, which result in cell proliferation;[26] impact signaling pathways,[27] which promote cell proliferation by activating protein kinase C[28] and Akt signaling pathways,[27] and affect signaling pathways regulated by the tyrosine protein kinase receptor[26] and Hedgehog signaling pathways involved in osteoblast proliferation.[29] Low-power irradiation is also shown to upregulate expression of receptor activator of nuclear factor kappa-B ligand (RANKL) in osteoclasts, thereby stimulating osteoclast formation.[30]

In general, the level of stimulation on cell dictates its response. A cell may not respond if the stimulation is too weak or may be damaged if the stimulation is too strong. Thus, there must be an optimal stimulation within a range that maximizes cell activity. However, the optimal stimulation is still unknown. In previous studies, PBM intensity and experimental designs varied significantly. The light was applied to the tissue surface, such as skin or soft tissues, resulting in uncertain doses on the cells. This uncertainty certainly causes significant variation in the experimental or clinical outcomes. The pressing question is what energy density level (dose) triggers the cell response.

It is imperative to identify the range of the dose that is not harmful to the cells and the optimal doses that stimulate their activities in order to maximize the therapeutic effects. The goal is to find the ranges. The objective of this study was to prove that these ranges exist and to determine the effects of varying

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doses on the cellular activity of osteoblasts, osteocytes, and osteoclasts.

2 Materials and Methods

Osteoblasts, osteocytes, and osteoclasts were tested at four doses: 0, 1, 5, and 7.5 J/cm^2. The light (940-nm wavelength) was delivered through a custom-made device. After each stimulation, cell activities (cell proliferation, osteoclastic activity, and bone resorption activity) were evaluated at 12, 24, and 48 h, while cell viability was evaluated at 12 and 24 h.

2.1 *Cells*

Three types of cells were used for this study: MC3T3-E1 osteoblasts (ATCC, Manassas, Virginia), MLO-A5 osteocytes (a gift from L. Bonewald), and RAW264.7 macrophages (ATCC, Manassas, Virginia). MC3T3-E1 cells were cultured following the manufacturer's protocol. They were cultured in minimum essential alpha medium (αMEM; Invitrogen, Grand Island, New York) containing 10% fetal bovine serum (FBS) (Invitrogen) and antibiotics (50 units/mL penicillin and 50 μg/mL streptomycin; Lonza, Basel, Switzerland). MLO-A5 cells were cultured in αMEM containing 5% calf serum (Hyclone, Logan, Utah) and 5% FBS and penicillin/streptomycin.[31] RAW264.7 cells were cultured in Dulbecco's modified Eagle medium (Invitrogen) supplemented with 10% FBS and antibiotics.[32] To induce osteoclast formation, RAW264.7 cells were treated with 35 ng/mL murine RANKL for 5 or 6 days until many multinucleated cells have formed.

2.2 *Application of Photobiomodulation*

2.2.1 *Equipment*

A device was created to deliver controlled light to the cells with a specified dose [Fig. 1(a)]. Twenty-four well plates (six columns; four wells per each column) were used to grow the cells. A lid holding LEDs with 940-nm wavelength was designed to irradiate the cells in each well. For each well, three LEDs arranged in a triangular pattern were installed in the lid to provide evenly distributed light stimulation [Fig. 1(b)]. To prevent light coupling among the wells, only three columns were used for light treatment and an empty column was placed between the treated columns. The LEDs were also isolated by the walls in the lid to further prevent light interference. The light power was controlled by adjusting the input voltage of each group of LEDs with a custom-made controller. The dose at the cell level was validated by a wavelength/power meter (Optical Power and Wavelength Meter OMM-6810B, Silicon Power/Wavehead OMH-6722B, ILX Lightwave Corporation, Bozeman, Montana).

2.2.2 *Dose*

The cells were cultured in three columns of the 24-well plates. Each column of cells was stimulated using the same dose. The light intensity was measured at the cell level, which was used to calculate the dose on cells. There were three testing groups based on the dose: the control, low-dose, and high-dose groups. The control group received no light, while the low- and high-dose groups received 10-min light stimulation with two different lighting power density, 1.67 and 8.33 mW/cm^2, which delivered 1- and 5-J/cm^2 doses (energy density), respectively (Table 1). A 7.5-J/cm^2 dose was applied to osteoblast after the 5-J/cm^2 dose did not affect the cell viability. Selection of the doses was based on the doses used in the previous studies.[22,25] The values in Table 1 are the average light intensity at the cell site. The variation among different locations was 18%.

2.3 *Cell Proliferation Assay*

Proliferation of MC3T3-E1 and MLO-A5 cells was quantified using the cell proliferation assay, Click-iT Plus EdU Alexa Fluor 594 Imaging Kit (Life Technologies, Grand Island, New York). The kit employs a nucleoside analog EdU (5-ethynyl-2′-deoxyuridine) to detect active DNA synthesis. Briefly, 12, 24, and 48 h after light stimulation, cells were incubated with EdU solution for 2 h and then fixed with 3.7% formaldehyde in PBS for 15 min and permeabilized with 0.5% Triton-X in PBS for 20 min at room temperature. Cells were then incubated with Click-iT reaction cocktail for 30 min at room temperature. The red fluorescent, proliferating cells and all cells stained with DAPI, a fluorescent stain, were counted using a fluorescence microscope (Nikon, Melville, New York). For data presentation

Table 1 The light intensity (power density) at the cell site, stimulation duration, and dose (energy density) in each column.

Dose	Control (0 J/cm^2)	1 J/cm^2	5 J/cm^2	7.5 J/cm^2
Light intensity (mW/cm^2)	0	1.67	8.33	12.5
Duration (min)	10	10	10	10

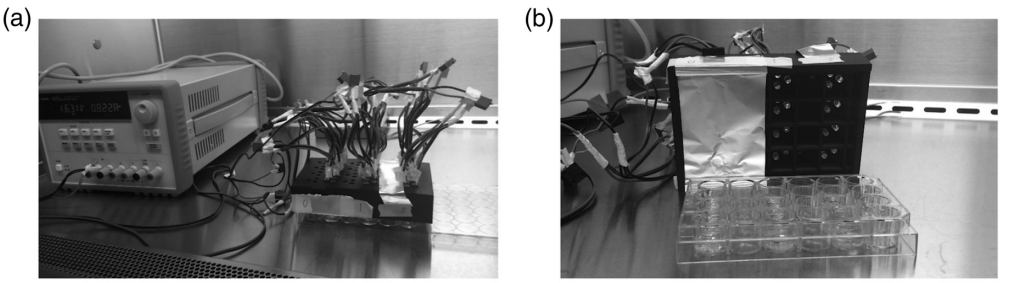

Fig. 1 (a) The PBM station consists of a controller that can adjust individual light output power of the 12 channels in our experiment, a 24-well cell plate, and controllable light sources for each of the 12 wells used for this study. (b) A 24-well cell plate and a custom-made lid with three LEDs applying light stimulation to cells in each well. Three columns of cells with different doses were used for our experiments.

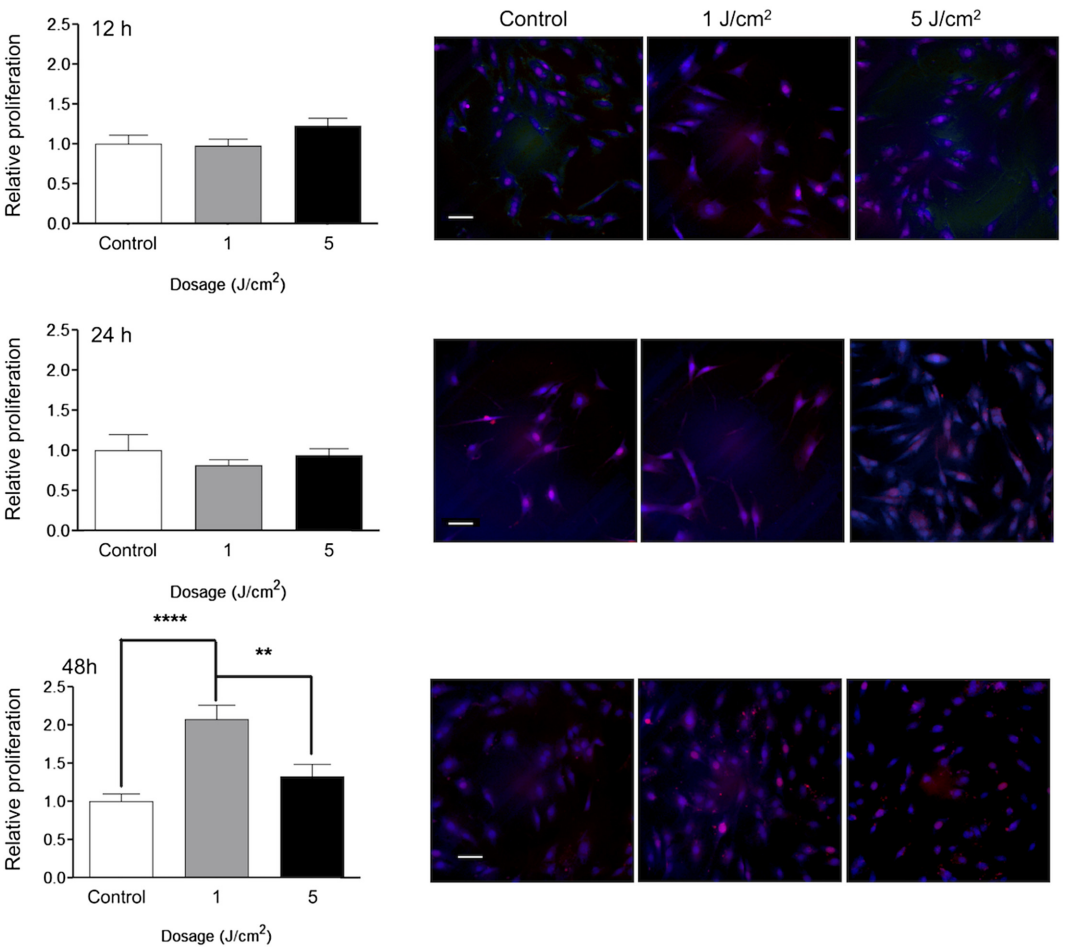

Fig. 2 Proliferation of MC3T3-E1 cells at 12, 24, and 48 h after PBM stimulation. The ratios of the number of proliferating (EdU+) and all cells were normalized to the untreated control group. Significant higher proliferation occurred at 48 h under 1 J/cm^2. ** $p < 0.01$, **** $p < 0.0001$. Scale bars: 100 μm.

in Figs. 2 and 3, the ratios of the number of proliferating (EdU+) and all cells were normalized to the untreated control group.

2.4 *Osteoclast Differentiation Assay*

To quantify osteoclast differentiation, tartrate-resistant acid phosphatase (TRAP) staining was used.[33] After 12, 24, or 48 h of light application, osteoclasts were treated with 10% formalin neutral buffer for 5 min and then tartrate-containing buffer for 30 min at room temperature. The area of the bone-resorbing osteoclasts, shown as red, was quantified. For data presentation in Fig. 4, the ratios of the area of the red-stained cells to the total area within the region of interest were normalized to the untreated control group.

2.5 *Bone Resorption Activity Assay*

To quantify osteoclastic bone resorption activity, we used Bone Resorption Assay Kits (Cosmo Bio). The kit consists of fluoresceinated calcium phosphate-coated plate. Fluoresceinamine-labeled chondroitin sulfate, bound to the plate, is released by osteoclastic resorption activity. After 24 h of light stimulation, fluorescence intensity was measured via the fluorescence microscope, and the data were normalized to the control group. To measure the pit area, cells were removed in the well by treating the plate with 5% sodium hypochlorite for 5 min. The plates were washed and dried. Using a microscope, five different regions in each well were randomly selected to measure the pit area. The area was measured by ImageJ (NIH) and normalized to the untreated control group.

2.6 *Cell Viability Assay*

The LIVE/DEAD Cell Imaging Kits (Life Technologies) were used to quantify cell viability. After 12 or 24 h of light stimulation, cells were treated with the kit according to the manufacturer's protocol. Briefly, cells were incubated with a mixture of Live Green and Dead Red Reagents for 15 min at room temperature. Then, green fluorescent and red fluorescent cells were counted as live and dead cells, respectively, under a fluorescent microscope (Nikon). For data presentation in Fig. 5, the ratios of the number of live to total (live + dead) cells were normalized to the untreated control group. A higher dose (7.5 J/cm^2) was also applied to MC3T3-E1 cells to validate the viable range of the light dose.

2.7 *Statistical Analysis*

Statistical analysis was performed using Prism 7 software (GraphPad, La Jolla, California). The unpaired student's *t*-test was used to compare differences between two experimental groups. One-way analysis of variance with Dunnett's *post hoc* test was used for multiple comparisons. A *p*-value <0.05 was

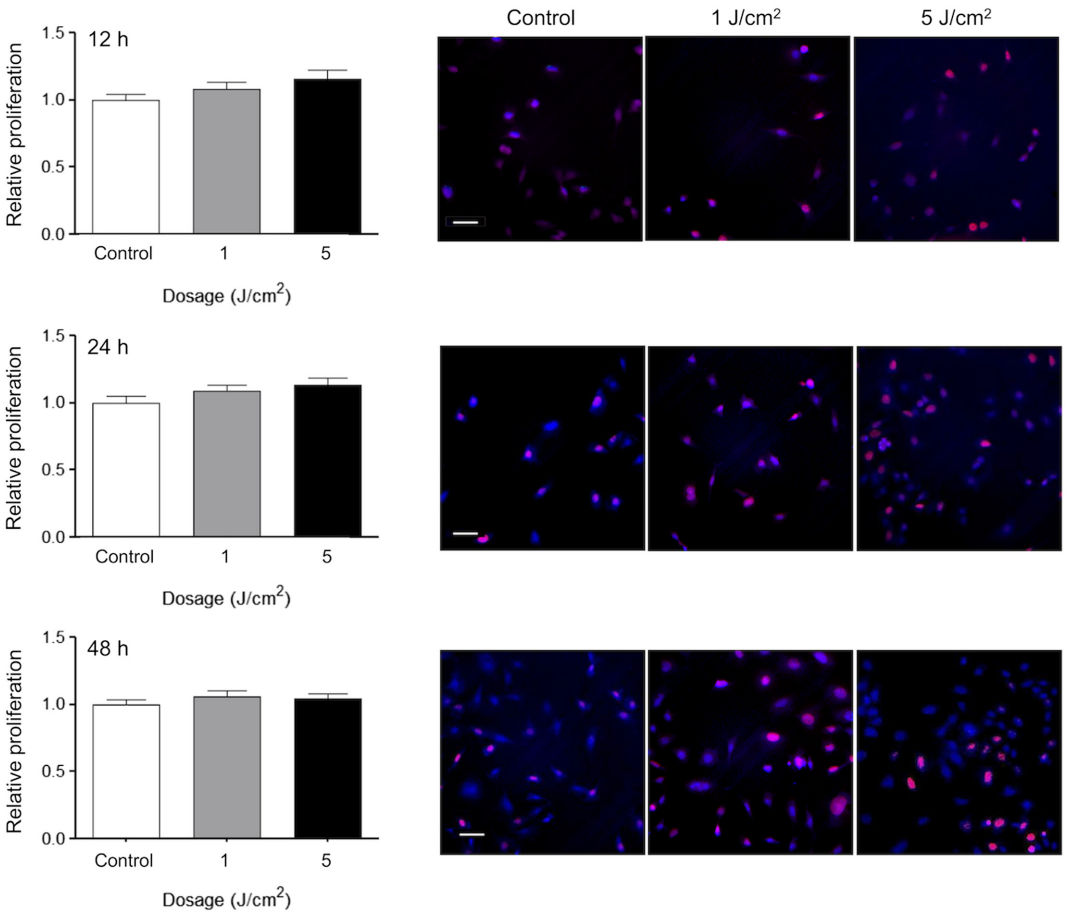

Fig. 3 Proliferation of MLO-A5 cells at 12, 24, and 48 h after PBM stimulation. The ratios of the number of proliferating (EdU+) and all cells were normalized to the untreated control group. There were no significant differences in proliferation among the three groups. Scale bars: 100 μm.

considered significant. At least, three independent experiments were performed for each condition.

3 Results

3.1 *Effect of Photobiomodulation on Cell Proliferation*

We first tested whether different intensities of light by PBM affect cell proliferation. The results are shown in Figs. 2 and 3. Two different intensities (1 and 5 J/cm^2) of light were applied to cells, and cell proliferation was measured at 12, 24, and 48 h. Both light intensities did not affect MC3T3-E1 proliferation until 24 h (Fig. 2). However, at 48 h, 1 J/cm^2 substantially increased proliferation ($p < 0.0001$), whereas 5 J/cm^2 did not. The difference between the two treatment groups was also significant ($p < 0.01$). We also tested MLO-A5 proliferation in response to light (Fig. 3). There were no significant changes in proliferation for 48 h regardless of the light intensity. These data suggest that low-dose treatment stimulates osteoblast proliferation, which may positively affect bone formation.

3.2 *Effect of Photobiomodulation on Osteoclast Differentiation*

Next, we evaluated the effects of light intensity on osteoclast differentiation. RANKL-treated RAW264.7 cells were used as a model of osteoclasts. Twelve to 18 h prior to light application, cell culture medium, including RANKL, was replaced with RANKL-free medium. Osteoclast differentiation was quantified by measuring the surface area occupied by TRAP-stained osteoclasts. The data revealed that the increase in osteoclast differentiation occurs primarily in the low-dose (1 J/cm^2) treatment group [Fig. 4(a)]. The increase peaked at 12 h ($p < 0.01$), then gradually reduced, which was still significant ($p < 0.05$) at 24 h. At 48 h, 1-J/cm^2 treatment group was not significantly different from the control. The results indicate that the effect of the low dose appeared to decrease over time. While the high-dose (5 J/cm^2) treatment group did not show a significant change during the entire 48 h as compared to the control group, it showed a significant difference as compared to the low-dose treatment group. The results suggest that low-dose treatment stimulates osteoclastic activity as early as 12 h after light application, which may lead to increased bone resorption.

3.3 *Effect of Photobiomodulation on Bone Resorption Activity*

The osteoclastic bone resorption activity was further evaluated by two different methods based on fluorescence intensity [Fig. 4(b)] and pit formation area [Fig. 4(c)]. The activity was measured 24 h after light application. The results showed a similar trend as those of the osteoclast differentiation as shown

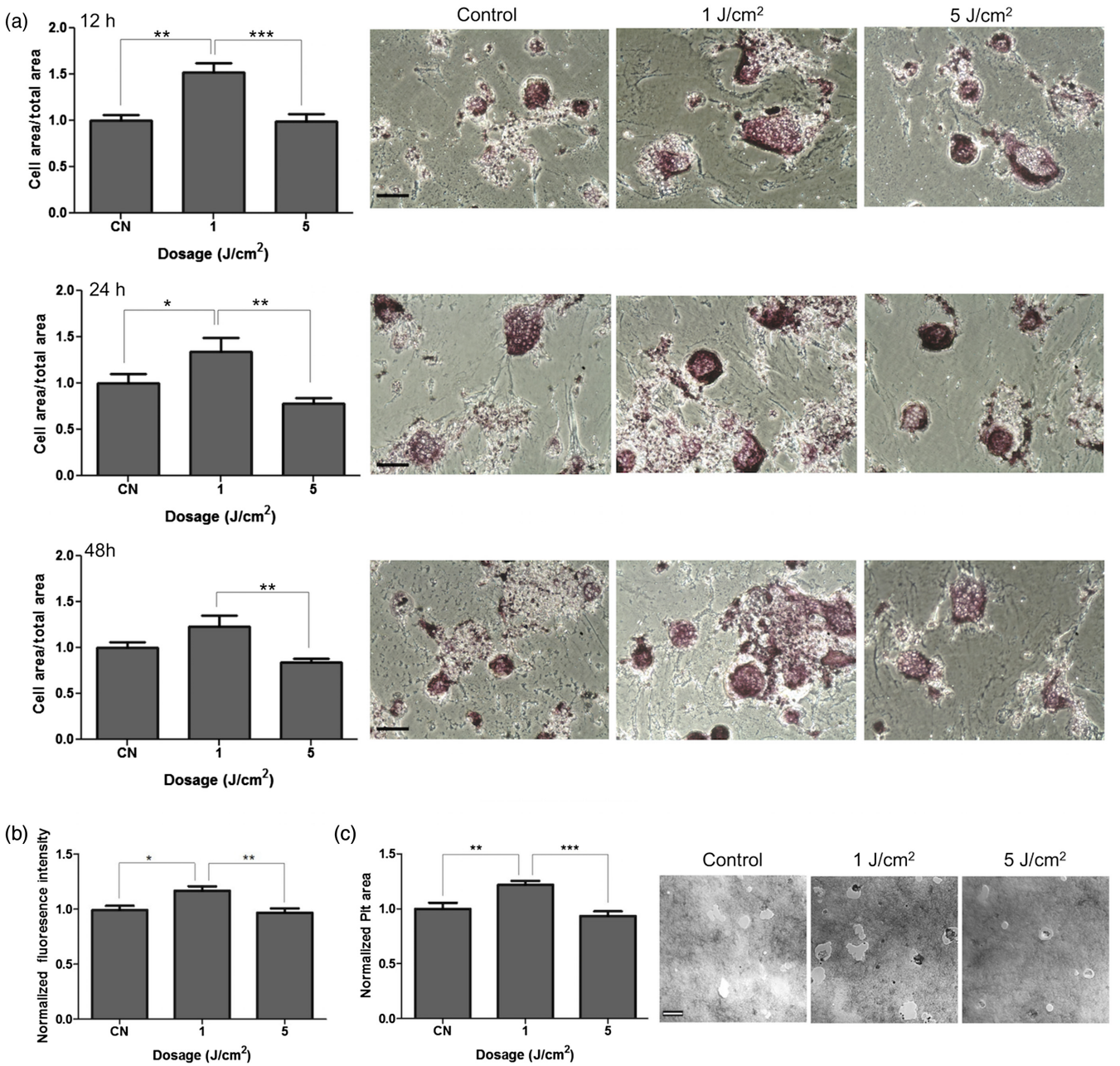

Fig. 4 Osteoclast response. Data were normalized to the untreated control group. (a) Osteoclast differentiation in response to PBM. Significant higher activities occurred at 12, 24, and 48 h under 1 J/cm^2. 5 J/cm^2 did not significantly affect osteoclast differentiation. The ratios of the area of the osteoclasts ("cell area") to the total area in the region of interest were normalized to the untreated control group. (b) and (c) Osteoclastic bone resorption activity measured by fluorescence intensity (b) and pit area (c). *, **, and *** $p < 0.05$, 0.01, and 0.001, respectively. Scale bars: 100 μm.

in Fig. 4(a). The low-dose treatment group showed significant increased activities compared to both the control and high-dose treatment group. However, high-dose treatment group did not show any significant changes. These data suggest that 1 J/cm^2 stimulates osteoclastic bone resorption activity.

3.4 *Effect of Photobiomodulation on Cell Viability*

The effect of light application on the viability of MC3T3-E1, MLO-A5, and RANKL-treated RAW264.7 cells was quantitatively evaluated. The data showed that both low- and high-dose treatment did not affect viability of MC3T3-E1 cells [Fig. 5(a)]. MLO-A5 cells showed a significant reduction in cell viability under 5 J/cm^2 as early as 12 h after light application and showed significant cell death at 24 h [Fig. 5(b)]. However, 1 J/cm^2 did not significantly affect cell viability of MLO-A5. RANKL-treated RAW264.7 cells did not show a significant difference between the experimental and control groups within the first 12-h period. However, the high-dose group showed significant cell death at 24 h [Fig. 5(c)].

To confirm the negative effects of high-dose treatment on the MC3T3-E1 cell viability, we tested the cell with a higher dose

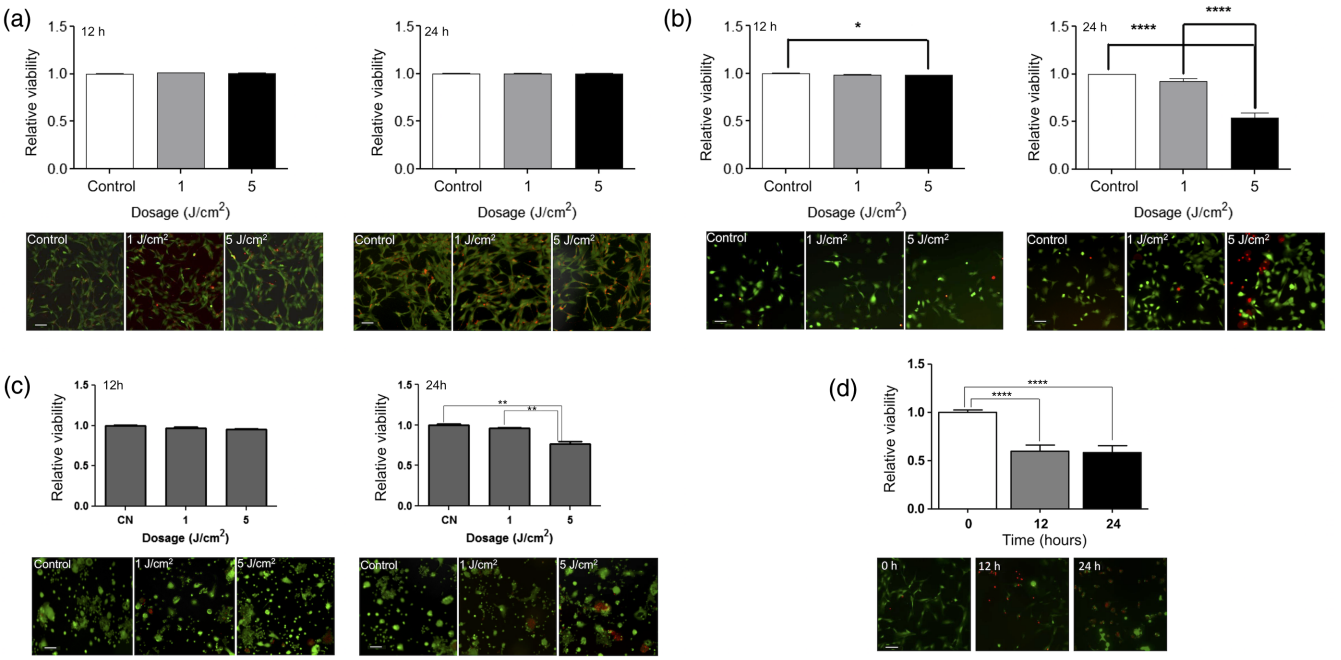

Fig. 5 Cell viability response to PBM. The ratios of the number of live to total (live + dead) cells were normalized to the untreated control group. (a) Viability of MC3T3-E1 cells at 12 and 24 h after PBM stimulation. There was no significant difference in viability among the three groups. (b) Viability of MLO-A5 cells at 12 and 24 h after PBM stimulation. (c) Osteoclast viability at 12 and 24 h after PBM stimulation. (d) Viability of MC3T3-E1 cells in response to 7.5-J/cm^2 dose. *, **, and **** $p < 0.05$, 0.01, and 0.0001, respectively. Scale bars: 100 μm.

(7.5 J/cm^2). The results revealed a substantial reduction (50%) of the treatment group in cell viability [Fig. 5(d)].

4 Discussion

This study focused on the effects of the light dose on cell responses. Methods were developed to reliably deliver the desired dose to the three types of cells: osteoblasts, osteocytes, and osteoclasts, respectively. Light dose-dependent cell viability, proliferation, and bone resorption activity were quantitatively evaluated. While other cellular activities, such as osteoblastic differentiation, might also affect BMR, we have focused, in the present study, on proliferation of osteoblasts and osteocytes as well as osteoclastic differentiation because these would play a major role in BMR.

The results showed that osteoblast proliferation was affected by PBM (Fig. 2). The increase of proliferation was not shown initially, but occurred at 48 h after the stimulation, suggesting the stimulation initiated a process that took more than 24 h to show the increase. Importantly, the effect is dose dependent. The lower dose (1 J/cm^2) had 100% increase while the higher dose (5 J/cm^2) had about 25% at 48 h. Our results are consistent with the previous reports demonstrating that PBM increases osteoblast proliferation.[22,25] Bloise et al.[22] demonstrated that osteoblast proliferation was promoted under 1 and 3 J/cm^2. The major increase occurred at 48 h after irradiation. Migliario et al.[25] defined the dose as J rather than J/cm^2; thus, their results cannot be directly compared with ours.

Little is known about the effect of light on osteocyte proliferation. Our results suggest that PBM does not stimulate osteocyte proliferation at both low and high doses (Fig. 3). Osteocytes reside in bone and serve as the sensor to regulate activities of osteoblasts and osteoclasts. They are not expected to proliferate under external stimulation.

We also observed that osteoclast activities were affected by PBM. In response to 1 J/cm^2, osteoclastic differentiation was significantly increased (50%) within 12 h and gradually reduced as the time elapsed [Fig. 4(a)]. This temporal profile was different from that of proliferation of osteoblasts (Fig. 2) and osteocytes (Fig. 3). In this study, all samples were exposed to a single of light with different energy density levels (i.e., 0, 1, 5, and 7.5 J/cm^2). These results suggest that multiple light stimulations may be required to sustain or enhance osteoblast proliferation and osteoclast differentiation. The observed osteoclastic differentiation was also confirmed through our bone resorption activity data [Fig. 4(b)]. Taken together, the results suggest that osteoclasts respond to light stimulation quicker than osteoblasts. The response was also dose dependent. The increase did not occur in the high-dose group as the time elapsed. There has not been any cell study on the effects of PBM on osteoclasts yet.

PBM has been reported to be able to regulate BMR through its application to the cells. Our results have confirmed that the PBM can stimulate both osteoblastic and osteoclastic cellular activities and can, therefore, be used to regulate BMR. However, it is important to understand that the effects are dose and time dependent. The timeline associated with the cell responses should be considered when PBM is used to regulate BMR. To further evaluate the range of light dose that does not harm the cell, we conducted cell viability tests. The results showed that three cell types differently responded to the light stimulation. Osteoblast viability was not affected by the light at both low and high doses [Fig. 5(a)], which is agreeable with the result reported previously.[22] Osteocyte viability was also not affected by the low dose [Fig. 5(b)]. Interestingly, osteocytes were

significantly damaged by the high dose as early as 12 h even though their proliferation rate was unchanged (Fig. 3). These data suggest that the high dose (5 J/cm^2) might differently regulate cell-signaling pathways responsible for cell proliferation and viability. Osteoclasts showed significant damage (~25%) at 24 h in the high-dose treatment group [Fig. 5(c)]. These data suggest that major reduction of live osteocytes and osteoclasts under the high-dose treatment could reduce the cells' ability to regulate BMR, and thus should be avoided. While osteoblasts were not affected by 5 J/cm^2, they were significantly damaged by 7.5 J/cm^2 [Fig. 5(d)], which again indicates existence of a limit the cell can withstand depending on the cell type.

While it is not clear how the cells respond to the light energy, previous studies suggest that light stimulation affects signaling pathways that regulate various cell activities, including cell viability, proliferation, and differentiation. For example, light stimulation influences cell proliferation via the extracellular signal-regulated protein kinase pathway,[34] PI3K/Akt pathway,[27] and Src pathway.[35] Low-power irradiation is also shown to promote the proliferation and osteogenic differentiation via cyclic adenosine monophosphate.[36] Similarly, to our observation on the dose-dependent cell activities, another study observed that low-power irradiation enhanced cell proliferation via protein kinase C while high-power irradiation induced cell apoptosis.[28] Recently, hedgehog signaling has been reported to promote the proliferation of MC3T3-E1 cells in response to light stimulation.[29] Differentiation and activation of osteoclasts are also enhanced by light stimulation via RANK expression.[30] These reports together with our data on dose-dependent cell activities indicate that PBM affects various signaling pathways that lead to cell proliferation and viability as well as osteoclastic differentiation and activity.

Our results demonstrate that light application affects various cell responses and that there is a limit that cells can tolerate without negative outcomes. The light dose is what the cells sense, which may not be used as the criterion when choosing light intensity for human or animal tests. In these cases, the light is applied to the tissue surfaces. The light intensity attenuates when it penetrates the tissues and reduces due to reflections, thus will reduce when it reaches the cell. The attenuation rate of the tissues should be quantified.

5 Conclusions

a. The osteoblast proliferation, osteoclast differentiation, and bone resorption activity are dose dependent. The cells responding to different dose showed different reactions.

b. There are optimal doses for osteoblast and osteoclast.

c. The major PMB effect on osteoblast occurs later than osteoclast.

d. PBM does not stimulate osteocyte's proliferation at both low and high doses.

e. There are dose limits for these three types of cells.

Disclosures

The authors declare that they have no conflicts of interest.

Acknowledgments

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