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The Long-term Effects of Red Light-emitting Diode Irradiation on the Proliferation and Differentiation of Osteoblast-like MC3T3-E1 Cells

TOMOKO ASAI, HIROAKI SUZUKI*, MIDORI KITAYAMA, KOUSUKE MATSUMOTO, AKIRA KIMOTO, MANABU SHIGEOKA, and TAKAHIDE KOMORI

Department of Oral and Maxillofacial Surgery, Kobe University Graduate School of Medicine
**Corresponding author*

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Low level laser therapy (LLLT) affects various biological processes, and it is said that the non-coherent light of the light-emitting diode (LED) has a similar action. The purpose of this study was to examine the effects of LED light on the proliferation and differentiation of osteoblasts-like MC3T3-E1 cells cultured in osteogenic differentiation medium (ODM) over the long term. Cells were irradiated with red LED light of 630 nm at three doses; 0.5J/cm^2, 1.5J/cm^2 or 3.0J/cm^2 for the cell proliferation activity assay, and at 0.5J/cm^2 for the osteogenic differentiation activity assay. The former activity was checked by counting the number of viable cells using Trypan blue dye. The latter activity was evaluated by alkaline phosphatase (ALP) staining and examining the mRNA expression of the osteopontin (OPN) gene using real-time quantitative PCR. The number of viable MC3T3-E1 cells showed a tendency to increase after the irradiation at all three energy densities in comparison with a non-irradiation group (control group). In particular, there was a remarkable 3.34-fold increase in the group irradiated with 3.0J/cm^2 on day 7 after starting the culture. On culture day 15, there was a tendency for the red LED irradiation group (0.5 J/cm^2) to exhibit more staining for ALP than the control group, and the expression of OPN was significantly higher in the irradiation group on culture day 16. In conclusion, low level red LED light can enhance MC3T3-E1 cell proliferation and osteogenic differentiation when the cells are cultured for a relatively long time.

INTRODUCTION

Lasers have been used extensively in a wide range of medical fields, including oral surgery, since Maiman first succeeded in exhibiting the oscillation of the ruby laser in 1962 [10, 24]. It is known that low power irradiation with a laser shows various low-level laser therapy (LLLT) effects, such as sedation and painkilling, the promotion of blood flow, the promotion of wound healing and the promotion of osteogenesis in human tissues, and various biological effects have been reported [11, 12, 18]. Various kinds of lasers are used for LLLT, and the helium-neon (He-Ne) laser, which emits red light with a wavelength of 633 nm has been used in many studies [2, 5].

In addition to the He-Ne laser, the wavelength region of the red light of light-emitting diodes (LED) has recently been demonstrated to activate cells and tissues, and it has been elucidated that this light can promote healing [15, 20, 21]. LED produce non-coherent light, whereas lasers are coherent light. However, if the actions of the red light are the same clinically, it may be possible to use an LED apparatus instead of a laser, which would be beneficial because LED devices are more cost-effective and easier to operate [14].

Kim et al. found that bone marrow mesenchymal stem cells (MSCs) cultured in osteogenic differentiation medium (ODM), which contains ascorbic acid and β-glycerophosphate dexamethasone, were promoted to undergo osteogenic differentiation by irradiation with red LED light [8]. Peng et al. reported that red LED light promotes osteogenic differentiation and reduces the proliferation of MSCs in ODM which contained osteogenic supplements, although it promoted proliferation and did not lead to the osteogenic differentiation of MSCs in normal medium without osteogenic supplements [14]. On the other hand, Pagin et al. reported that osteoblast-like MC3T3-E1 cells, which are a cell line derived from mouse calvaria, exhibit increased proliferation but no change in differentiation induced by irradiation with red LED in short-term culture in ODM [13]. However, there have been few reports that have examined the effects of the red LED light in the

Phone: +81-78-382-6213 Fax: +81-78-382-6229 E-mail:hsuzuki@med.kobe-u.ac.jp

MC3T3-E1 cells induced to undergo osteogenic differentiation, and there has so far been no study that has observed the longer-term changes induced by red LED irradiation.

Therefore, the purpose of this study was to investigate the effects of red LED light with a wavelength of 630 nm on the proliferation and differentiation of osteoblast-like MC3T3-E1 cells cultured in ODM over a relatively long period.

MATERIALS AND METHODS

Cell culture

MC3T3-E1 cells, which are a cell line derived from mouse calvaria that can differentiate into osteoblasts, were disseminated in cell culture dishes (φ 100mm, Falcon) containing alpha minimum essential medium (αMEM) with 10% fetal bovine serum (FBS) and 1% antibiotic-antimycotic solution (Penicillin G 10,000 U/ml, Streptomycin 10 mg/ml, Amphotericin B 25 μg/ml, Invitrogen). The cells were maintained in culture at 37° C in a humidified 5% CO_2 atmosphere. The culture medium was changed every three days, and the cells were passaged once. The resulting osteoblastic precursor cells were used for our experiments.

Irradiation with the red LED light

LED irradiation was carried out using a LED-based device (DAICO MFG CO., Ltd., Kyoto, Japan). Cells were exfoliated using trypsin EDTA by a conventional method right before the second-passage cultured cells reached confluency. After disseminating the MC3T3-E1 cells into ODM containing 50 μg/ml ascorbic acid (Wako), 10 mM β-glycerophosphate (Sigma-Aldrich) and 10^{-8}M dexamethasone (Wako), we allowed them to attach onto the culture dishes. Twenty-four hours from the dissemination, red LED light (wavelength of 630 nm, power density of 20 mW/cm^2) was irradiated from 20 mm above the cell layer.

Experimental groups

The MC3T3-E1 cells irradiated with LED light were cultured on φ100mm polystyrene plates (Falcon) for the cell proliferation assay and real-time quantitative PCR assays to assess the osteogenic differentiation, and on 16-well polystyrene plates (Falcon) for the assessment of ALP staining (LED (+)). Cells that were not irradiated with the LED were used as the control group (LED (-)). Furthermore, the energy density of the LED was set at three different rates: 0.5 J/cm^2, 1.5 J/cm^2 and 3.0 J/cm^2 during the cell proliferation assay, and these were defined as LED (+) 0.5J, LED (+) 1.5J and LED (+) 3.0J, respectively.

Cell proliferation assay

MC3T3-E1 cells were disseminated at 15×10^4 cells per one dish (φ 100mm) and LED light was irradiated onto the cells at an energy density of 1.5 J/cm^2 for 15 minutes. The number of viable cells with Trypan blue staining was counted using a hemocytometer (DHC-N01N) on culture days 0, 3 and 5 after the irradiation, and was compared with that in the control (non-irradiated) group (Figure 1).

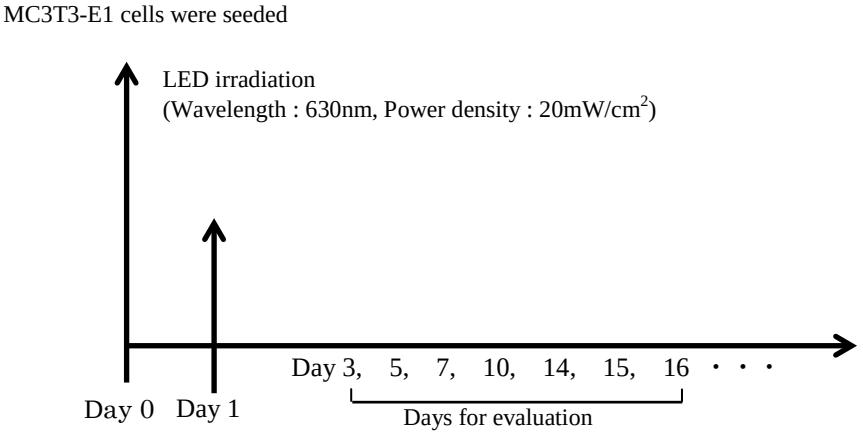

Figure 1. Experimental schedule

Then, a comparison of the effects of different energy densities was carried out. Cells were disseminated at 2.5×10^4 cells per dish (φ 100mm) and cells were exposed to LED irradiation at an energy density of 0.5 J/cm^2 (5 minutes), 1.5J/cm^2 (15 minutes) or 3.0 J/cm^2 (30 minutes), and the number of viable cells was counted on culture days 0, 3, 5 and 7 after the irradiation (Figure 1).

Osteogenic differentiation assay

Alkaline phosphatase staining and the assessment of the expression of osteopontin (OPN) mRNA, as determined by real-time quantitative PCR, were carried out.

a. Alkaline phosphatase staining

A total of 0.3×10^4 cells were disseminated per well (φ 15mm) of 16-well plates, and the cells were irradiated with LED light (0.5 J/cm^2 energy density). The culture medium was removed, and cells were rinsed in PBS once on culture days 5, 7, 10 and 15. The cells were then fixed by adding a 4% formaldehyde solution on ice for 10 minutes. After the cells were rinsed twice in PBS, ALP pre-mixture substrate liquid (Wako) was added, and was allowed to react at 37° C for 30 minutes. Subsequently, the cells were observed under a phase-contrast microscope, and the findings were compared with the control group (Figure 1).

b. Expression at osteopontin (OPN) mRNA determined by real-time quantitative PCR

A total of 1.5×10^4 cells were disseminated per dish (φ 100mm), and were irradiated with LED light (0.5 J/cm^2). Cells were exfoliated using trypsin EDTA as usual on culture days 0, 14 and 16 (Figure 1). In accordance with the method described by Shigeoka et al. [17], using a Fast SYBR®Green Cells-to-Ct™Kit (Applied Biosystems, USA), the expression level at OPN mRNA in the cells was calculated using a StepOne™ Real Time PCR System (Applied Biosystems) and was normalized to the level of glycelaldehyde-3-phosphate dehydrogenase (GAPDH) mRNA, which was used as a housekeeping gene. The primer sequences used for amplification are shown in Table I.

Table I. Primer sequences for the real-time quantitative PCR of osteopontin (OPN) and GAPDH

Gene	Forward primer (5'→3')	Reverse primer (5'→3')
OPN	GAGATTTGCTTTTGCCTGTTTG	TGAGCTGCCAGAATCAGTCACT
GAPDH	TGCCCAGAACATCATCCCTG	TCAGATCCACGACGGACACA

Statistical analysis

Student's t-tests were used to evaluate the differences between the sample of interest and its respective control. The level of significance was set at 5% (p<0.05).

RESULTS

The proliferation of MC3T3-E1 cells following irradiation with red LED light

Figure 2A shows the number of viable cells on culture days 0, 3 and 5 after red LED irradiation (□: LED (+), ×: LED (-) (control group)). The number of viable cells in the non-irradiation (control) group, which were disseminated at 15×10^4 cells/well on day 0, increased to 30.5×10^4 and 46.7×10^4 cells/well on culture days 3 and 5. On the other hand, the number of viable cells in the LED irradiation group increased to 39.0×10^4 and 55.5×10^4 cells/well on culture days 3 and 5.

Figure 2B shows the results comparing the different energy densities (◇: LED (+)0.5J, □: LED (+)1.5J, △: LED (+)3.0J, ×: LED (-) (control group)). The number of viable cells in the control group, which were disseminated at 2.5×10^4 cells/well on day 0, increased to 8.75×10^4, 24.5×10^4 and 33.7×10^4 cells/well on culture days 3, 5 and 7. On the other hand, the number of viable cells exposed to 0.5 J/cm^2 LED light increased to 11.2×10^4, 38.7×10^4 and 53.7×10^4 cells/well on days 3, 5 and 7. The 1.5J/cm^2 –treated group had values of 12.7×10^4, 31.5×10^4 and 55.7×10^4 cells/well, and those treated with 3 J/cm^2 had values of 11.2×10^4, 26.7×10^4 and 112.5×10^4 cells/well, respectively, on culture days 3, 5 and 7.

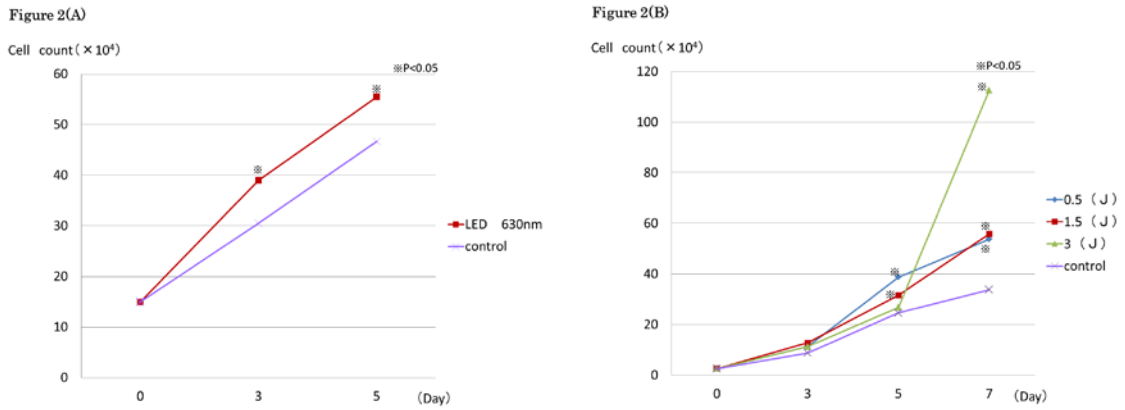

Figure 2. The proliferation of MC3T3-E1 cells in the irradiated group (LED (+)) was higher than that in the non-irradiated group (LED (-)), and differences were observed to occur in a time-dependent manner.
(A) The number of viable LED (+) cells was increased by 1.18- and 1.28-fold compared to LED (-) cells on culture days 3 and 5 (p<0.05, both time points).
(B) The number of viable LED (+) cells showed a tendency to increase compared to the LED (-) cells at all time points examined (days 3, 5 and 7), regardless of the energy density. The number of viable cells was significantly increased in the LED (+)0.5J and LED (+)1.5J cells on day 5, and in all LED (+) cells on day 7 (p<0.05). Of note, the number of viable cells increased by 3.34 times in the LED (+)3.0J cells on day 7 compared to the LED (-) cells.

Osteogenic differentiation of MC3T3-E1 cells following irradiation with red LED light

a. Alkaline phosphatase staining

Figure 3 shows representative images of the ALP staining on culture days 10 and 15 after red LED irradiation. Staining was not seen in any of the control cells, or in the groups exposed to LED irradiation group until day 10. Staining was seen in both the irradiated and non-irradiated groups on day 15, but the LED irradiation group showed a tendency to have higher staining.

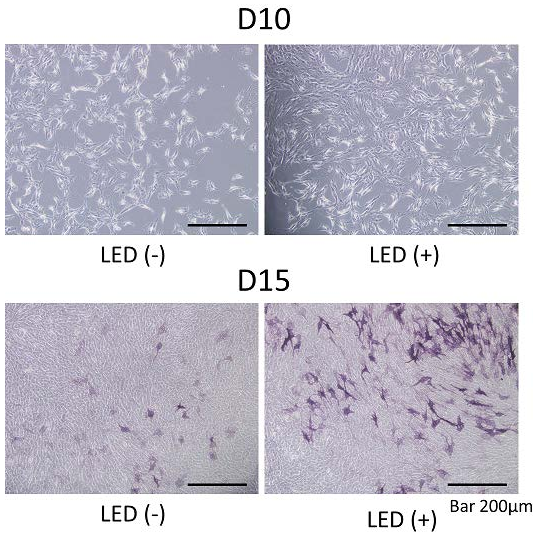

Figure 3.
The left column shows the LED non-irradiation group (control group), and the right column shows the LED irradiation group. The upper panels show the findings on culture day 10, and the lower panels show the findings on day 15. Representative images are shown. The ALP activity was measured using an ALP staining kit. The irradiation with red LED light was thought to increase the ALP activity of MC3T3-E1 cells.

b. Expression of osteopontin (OPN) mRNA

Figure 4 shows the expression of OPN mRNA, as determined by real-time quantitative PCR, on culture days 0, 14 and 16 after red LED irradiation. An increase of expression was observed on day 16 in both the LED irradiation group and non-irradiation group (control group). However, the LED irradiation group had higher expression, with a ratio of 1.7 times compared with non-LED irradiation group.

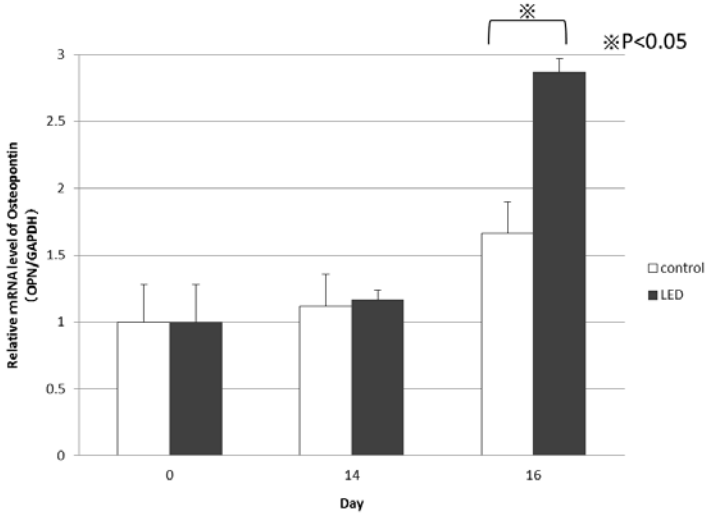

Figure 4. The results of the quantitative analysis of the OPN mRNA expression. The expression of OPN in the LED irradiation group was significantly higher on culture day 16 than that in the non-irradiation group (p<0.05). The data are shown as the fold-change in expression compared to the corresponding values on day 0 just after LED irradiation.

DISCUSSION

In this study, we evaluated the proliferation and osteogenic differentiation of MC3T3-E1 cells which were cultured for a relatively long time in ODM after irradiation with red LED light of 630 nm. In previous *in vitro* studies, many researchers have showed that the proliferation and differentiation of osteogenic cell lines, such as MC3T3-E1 cells, are promoted by low power laser irradiation [1, 13, 14, 19, 22, 23]. While lasers emit coherent light and LEDs emit non-coherent light, both bring about a similar photobiological effect when used with the same irradiation parameters [13-15, 20, 21]. In particular, we found that the red light (630-690nm) of low power does not cause negative effects such as carcinogenesis, and affected various biological processes [6, 7, 9]. According to a previous report that examined the proliferation and differentiation of osteogenic cells, the energy density of LED extends over a wide range of 0.2-10J/cm^2 and is not definded [8, 13, 14].

In this study, we first confirmed that the irradiation group showed a significant increase in the number of viable cells on culture day 5 in comparison with the non-irradiation group, which was induced by irradiation with an energy density of 1.5J/cm^2. When we reduced the number of cells initially disseminated on the plates, and found that the irradiation group had a more than three-fold increase in the number of viable cells in comparison with the non-irradiation group on day 7 at the energy density of 3J/cm^2.

The previous experiments by Pagin et al. used MT3T3-E1 cells, and they examined the effects of LED light on the cell proliferation activity using an energy density of 3 and 5J/cm^2. Unlike our results, their results showed that there was only a significant difference in the cell proliferation at 24 hours after irradiation with 5J/cm^2, and there were no significant differences in the proliferation of the irradiated and non-irradiated groups after that point [13]. Since we did not perform irradiation at the energy density of 5J/cm^2, it is hard to compare the results.

However, there are several advantages of our study compared to their study. The first point was that many cells died during the early stage of culture in their study, which may suggest that their time point for the evaluation was too early. They used 96-well plates for their cell cultures (31.2 cells/mm^2). In contrast, we cultured cells using a larger (φ 100 mm) plate and reduced the number of cells to keep longer culture periods when we performed the comparison of the different energy densities (3.1 cells/mm^2). As a result, we were able to examine the cell proliferation activity until culture day 7 under the irradiation condition (3J/cm^2) as same as that Pagin et al. performed, which was a much longer time than in the study by them.

The second point is that Pagin et al. examined the cell proliferation activity by performing the MTT assay, while we used the Trypan blue exclusion assay. In the MTT assay, the number of viable cells is measured based on the mitochondrial activity. The number of cells at 48, 72 and 96 hours after irradiation was likely relatively low in their study, because the mitochondrial activity decreases within 48 hours after plating. We excluded the dead cells based on Trypan blue staining, and compared the irradiation and non-irradiation groups by measuring the number of viable cells remaining.

To evaluate the osteogenic differentiation, we examined the ALP staining and the expression of OPN mRNA. The MC3T3-E1 cells were not stained for ALP up to culture day 10, regardless of whether they were irradiated. However, the LED irradiation group showed a tendency to have higher staining than non-irradiation group when cells became confluent on day 15. Thus, it was suggested that red LED light increased the ALP activity of MC3T3-E1 cells. Quarles et al. stated that the ALP activity reaches a peak before the cell proliferation stage is over and the bone matrix matures [16]. In our study, the cells were cultured for a relatively long time to allow them to proliferate, and it was thought that the difference in ALP activity between the irradiation group and non-irradiation group may have been due to the fact that the cells had proliferated for a longer time.

It is not possible to directly compare the results of our study with those reported by Pagin et al., because they detected the ALP activity using an absorption spectrometer after the cells were lysed. However, their results also did not show any significant difference in the cells until culture day 14 [13]. Although the details of the experiments, including the irradiation conditions and the dissemination of cells were different, Kim et al. and Peng et al. also investigated the effects of LED irradiation on osteogenic transformations using MSCs, which are undifferentiated bone marrow cells with proliferative capacity and the ability to differentiate into a variety of cells, including osteoblasts. In their study, an increase in ALP activity was observed at a relatively early stage of culture, from day 3 to 10, following LED irradiation [8, 14].

In the present study, we confirmed that the expression of OPN mRNA in the irradiation group was significantly increased compared to that in the non-irradiated group on day 16. Considering the results for ALP staining above, because both ALP and OPN are known as markers of the relatively early stage of osteoblastic differentiation [3, 4, 17], it was suggested that the irradiation of cells with LED increased the differentiation toward osteoblasts, at least at this stage. In this study, we did not investigate using marker genes for the late stage of differentiation (the appearance of mature osteoblasts), but studies using such markers would be informative.

In summary, red LED light irradiation at a wavelength of 630 nm promoted the proliferation and osteogenic differentiation of MC3T3-E1 cells cultured in ODM for a relatively long term in our experimental system. Furthermore, we consider that it is necessary to continue to elucidate the specific mechanisms that control cell proliferation and osteogenic differentiation activity in the presence and absence of LED irradiation.

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