Photobiomodulation with 655-nm Laser Light to Induce the Differentiation of PC12 Cells

Emel Bakay[1],
[1]Department of Biomedical Technologies
Izmir Katip Çelebi University, Izmir, Turkey
emelbakay@hotmail.com[1]

Nermin TOPALOĞLU[2]
[2]Department of Biomedical Engineering,
Izmir Katip Çelebi University, Izmir, Turkey
nermin.topaloglu@ikcu.edu.tr[2]

***Abstract*— The healing effect of light at low power and energy density can be used as a treatment or alternative supportive method in various diseases. The photobiostimulation effect created on neural cells is also a very promising approach in the treatment of important neurodegenerative diseases such as Alzheimer's disease. In this study, the response of PC12 cells to photobiomodulation was investigated as a result of the low level laser therapy with 655 nm diode laser after triple treatment. The red light at an energy density of 1, 3 and 5 J/cm^2 was applied to PC12 cells three times with 24h intervals. The differentiation capacity of the cells and the elongation rates of neurites were assessed. The neurite lengths were calculated by analyzing the microscopic images of the cells. Neurite-forming capacity and differentiation rate of PC12 cells was at the maximum level after the application with 1 J/cm^2 energy, nearly 2 times of the control group. 5 J/cm^2 of energy density strongly inhibited the cell proliferation and the elongation of the neurites. The cell viability percentages of the cells showed that 5 J/cm^2 energy density inhibited cell viability with a rate of nearly 30%. The outcomes of this study emphasized that the adjustment of light parameters in photobiomodulation applications may result in biostimulation or bioinhibition depending on the intensity and the irradiance levels applied on the cells.**

Keywords—photobiostimulation; 655-nm, differentiation; PC12 cells; neural diseases.

I. INTRODUCTION

The interaction mechanisms between light and the matter has been investigated and used for different purposes from past to present. In particular, the use of light in therapeutic and diagnostic approaches has led to the emergence of different research areas, such as photomedicine. It is known that light used at appropriate wavelength and power has a healing effect on the human body. In addition, many disease factors are eliminated and treated when these application doses are optimized [1]. Laser beams are also widely used in medicine today due to their different advantageous properties such as being monochromatic, coherent, and collimated [2]. Photons from laser light can interact with different tissue chromophores, creating changes in cell metabolism and functioning [3].

Photobiomodulation (PBM) is a novel description for Low Level Light Therapy that is used to increase cell proliferation, reduce inflammation and/or trigger tissue repair. Due to the use of low power laser light it does not cause any damage on the target tissue and generally there is no need to use a chemical agent. PBM is used for different treatment purposes such as osteoarthritis, wound healing, nerve injuries, and pain relief [4].

PBM has a promising therapeutic effect depending on the different parameters, such as wavelength, irradiance, intensity, application time, and cycle. The most important part is that the laser light should have a specific wavelength to be absorbed by the chromophores of the target tissue. In PBM, red to infrared region of the electromagnetic spectrum (600-1000 nm) is commonly used. It is also noted that the level of the light used in this region should be of low power and energy [5]. It is very important to transfer the appropriate power and energy density depending on the targeted tissue. In a study using 635 nm of wavelength, the effect of different power and energy densities on fibroblast cells was investigated. In the experimental groups formed with the use of different energy intensities and powers, 1 J/cm^2 generally showed stimulatory effect at 30, 50 and 125 mW/cm^2 power densities. However, the same energy density with 200 mW/cm^2 of irradiance and 3 J/cm^2 energy density with 50 mW/cm^2 of irradiance showed lower cell viability [6].

The PBM applications on brain is an innovative approach for the treatment of brain disorders, neurologic and physiological conditions. The application of red or near infrared light to the brain tissue can change the cellular and systemic activity of the brain [7]. Although the cellular mechanism of action is not fully known, the target of light is known to be the mitochondria inside the cell. Cytochrome c oxidase (complex IV), which is an element of the electron transport chain in mitochondria, is stimulated and finally responsible for the increased ATP production [7, 8]. Besides, both the cellular and the functional connections between the neurons are increased, too [9]. Due to the use of low power of light at the red and near-infrared spectrum, it is known that there is no critical increase in temperature after the light applications. Thus, it appears to be very suitable for the use on sensitive cells such as neurons [10]. In a study of adult mice, PBM has been shown to support proliferation and differentiation on neuroprogenitor cells with 25 mW/cm^2 of irradiance at 808-nm of wavelength [11]. In this study, we aimed to assess the effect of 655 nm laser light on PC12 cells. In order to increase the photo-biostimulative effect clearly, light treatment was applied in triplicate with 24h intervals. The triple administration of 655-nm laser light at specific energy

978-1-7281-8073-1/20/$31.00 ©2020 IEEE

densities (1, 3, and 5 J/cm^2) was investigated to induce PBM for the differentiation capability of PC12 cells.

II. MATERIALS AND METHODS

A. Materials

Collagen (type IV, 0.5–2 mg/ml), RPMI-1640, nerve growth factor (NGF, Vipera lebetina venom), trypsin, L-glutamine, 3-(4, 5-dimethythiazol- 2-yl) - 2, 5-diphenyl tetrazolium bromide (MTT) assay, and fetal bovine serum (FBS) were supplied from Sigma-Aldrich (St. Louis, MO, USA). Gentamicin were purchased from Gibco (Dublin, Ireland). Donor horse serum (DHS) was purchased from Capricorn (Ebsdorfergrund, Germany).

B. Cell Culture

Rat pheochromocytoma cell line 12 (PC12) were purchased from DSMZ (Braunschweig, Germany) and cultured in RPMI-1640 containing 10% DHS, 1% FBS, 1% L-glutamine and 0.1% gentamicin at 37 °C and 5% CO_2 humidified atmosphere. Cells were cultured in collagen-coated flasks to facilitate adherent cell growth.
Three wells that receiving light with equal intensity were determined on 96-well plate. These wells were coated with collagen for the attachment of the cells before any application. 5000 PC12 cells were seeded in each collagen-coated well and incubated in cell culture medium for 24 h at 37 °C.

C. Light Source and Optical Setup

Diode pumped laser device (PSU III.LED; Changchen New Industries optoelectronics Tech Ltd.) that emits a wavelength of 655 nm with a maximum output power of 1 Watt was the light source of this study. The optical fiber of the laser device was fixed vertically to an optical table where culture plate was placed on. Beam area was adjusted to illuminate specific wells that contained cells and the output power of light was checked with a power meter (Thorlabs, USA) for homogenous light application. The output power of the laser device was kept constant and the application times and energy densities were changed to illuminate the cells with specific parameters. Table 1 shows the light parameters used in the experiments.

Table 1. Laser parameters for experimental groups

Wavelength (nm)	Output Power (mW)	Energy Density (J/cm^2)	Irradiation Time (s)
655 nm	50 mW	1 J/cm^2	251 s
655 nm	50 mW	3 J/cm^2	754 s
655 nm	50 mW	5 J/cm^2	1256 s

D. Experimental Setup

For PBM applications, four groups are formed. (1) Control: without any light application, (2) Group 1: with 1 J/cm^2 laser light application, (3) Group 2: with 3 J/cm^2 laser light applications, and (4) Group 4: 5 J/cm^2 laser light applications. In all groups, half of the cell culture medium was removed from the cells before the application and the remaining was completed with the fresh cell culture medium containing 50 ng/ml NGF. Then, the cells in all groups were incubated for 30 minutes before light applications.

After incubation, laser light was applied at day 0 to all experimental groups, except the control group. Then, the laser application was repeated two more times at day 1 and day 2 with 24h intervals. The medium of PC12 cells was changed every 3 days with the fresh cell culture medium containing 50 ng/ml NGF.

E. Differentiation of PC12 cells and Neurites Elongation Analysis after Light Application

PC12 cells were analyzed throughout the experimental procedure at day 0, day 1, day 2, day 3, and day 6. The microscopic images of the living cells were captured with an inverted microscope (Olympus, CKX41). 3 different wells were analyzed and 10 pictures (40X magnification) were captured from each well. The length of the neurites and the number of differentiated cells were calculated with the help of ImageJ software in these wells, then the mean values were used for the comparison with the control group.

F. Cell Viability Assay

MTT (3-(4, 5-dimethythiazol- 2-yl) - 2, 5-diphenyl tetrazolium bromide) assay was used to calculate the viability of PC12 cells. First, the stock 5 mg/ml MTT solution prepared then added to the wells to be 10%. The initial yellow color of MTT turns dark purple color by interacting with the enzyme succinate dehydrogenase that is found in the mitochondria of the living cells. All groups prepared as 100 µl of 10% MTT solution in each well were left for 2h incubation. After incubation, MTT solution was removed and DMSO was added with the same volume. After 30 minutes of incubation with DMSO, absorbance values were measured at 570 nm by microplate reader (Multimode Microplate Reader Biotek Synergy HTX, Biotec). All the steps of MTT assay were carried out in the dark. The measured absorbance values were used in calculations of the cell viability. Because these values were directly proportional to the cellular metabolism of the living cells.

G. Statistical Analysis

Student t-test analysis was used to determine the statistically significant cell viability, neurite length, and differentiated cell number results of the laser groups. All experimental groups were compared with the control group and *p*-value was set to <0.05.

III. RESULTS

A. Differentiation of PC12 cells and Neurites Elongation Analysis

In order to observe the differentiation capacity of PC12 cells after PBM applications at different energy densities, the microscopic images were taken at day 0, day 1, day 2, day 3 and day 6. Fig. 1 shows PC12 cells before the first light application (day 0) and then day 1, day 2, day 3 and day 6. It was observed that there was no difference between the groups at day 0. At day 1, PC12 cells started to differentiate in all experimental groups. At day 2, the differentiation of the cells was seen in all groups, but it was observed that the

differentiation rate was higher in the 1 J/cm^2 and 3 J/cm^2 groups. The microscopic image of the cells at day 3 shows that the differentiation rate was significantly increased in the laser group of 1 J/cm^2. On the 6th day after the first laser application, the rate of neural interaction between the cells and differentiation capacity was more intense in the laser group of 1 J/cm^2 than other groups.

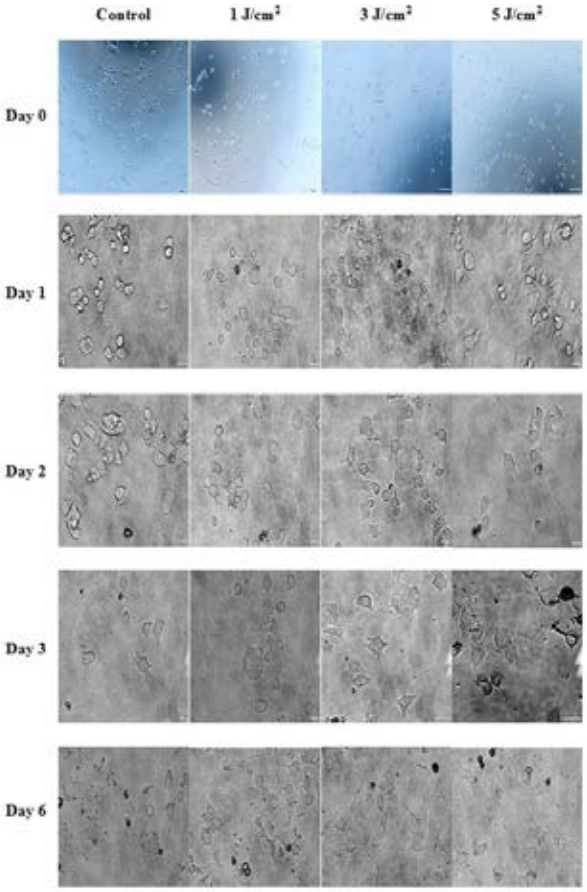

Fig. 1. Microscopic images of PC12 cells in control, 1 J/cm^2, 3 J/cm^2 and 5 J/cm^2 groups at Day 0, 1, 2, 3, and 6.

Neurite lengths were calculated according to energy densities on the determined days. 1 J/cm^2 showed the highest neurite length average in all days. Although the 3 J/cm^2 group and the control group had similar neurite lengths, 5 J/cm^2 showed a lower neurite length when compared with other groups (Fig. 2).

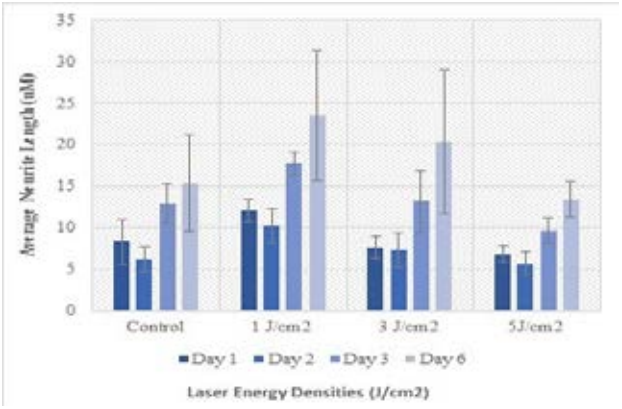

Fig. 2. Average neurite length for different energy densities at Day 0, 1, 2, 3, and 6.

Differentiated cell numbers were calculated depending on the total number of cells in laser groups with different energy intensity and in the control group, and expressed in percentage values. All laser groups showed an increasing number of differentiated cells throughout 6 days compared to control, and the 1 J/cm^2 group had the highest differentiated cell count (Fig. 3).

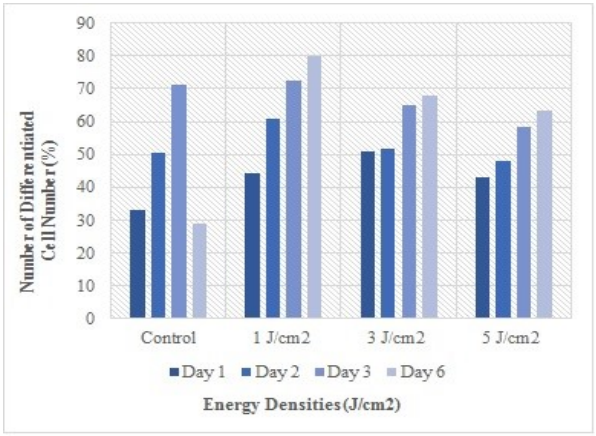

Fig. 3. Differentiated cell number for different energy densities at Day 0, 1, 2, 3, and 6.

B. Cell Viability Analysis

After the 6th day of light applications, cell viability test was performed using MTT assay. As a result of MTT analysis, cell viability was determined as 99.27% in 1 J/cm^2 group, 76.00% in 3 J/cm^2 group and 69.59% in 5 J/cm^2 group. It was understood that the 1 J/cm^2 group gave similar results with the control group, while the 5 J/cm^2 of application caused a statistically significant decrease in the cell viability (Fig. 4).

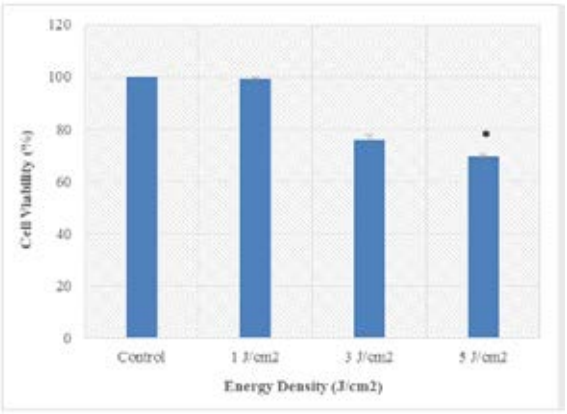

Fig. 4. Cell viability percentages of the control, 1 J/cm^2, 3 J/cm^2 and 5 J/cm^2 groups after triple laser treatment with 655 nm laser device.

IV. Discussion

In this study, PC12 cells were followed up for 6 days after the PBM applications with 655-nm laser light at different light intensities. Neurite forming capacity and differentiation rates of cells were analyzed with microscopic images taken at determined intervals. In the laser group applied with a constant 50 mW output power and 1 J/cm^2 energy density, the differentiation rates were significantly higher than the control and other laser groups on the 1st, 2nd, 3rd and 6th days, except for the first day. The application with this energy intensity revealed the strong photobiomodulation effect on PC12 cells with the microscopic images and the neurite length outcomes of the differentiated cells. The cell viability analysis showed that the light application induced differentiation mechanism, instead of proliferation and did not negatively affect the cell viability.

It was also found that the light application at 3 J/cm^2 induced differentiation, too. The differentiation capacity was higher than that of the control group, but still lower than that of the laser group of 1 J/cm^2. Besides, the cell viability of the cells in this group decreased with a rate of nearly 25%. Nevertheless, this decrease was not statistically significant compared to the control group. This outcome also supported that the influence of the light application at determined energy density was to induce differentiation, instead of cell proliferation.

The maximum energy density was 5 J/cm^2 in this study and this light intensity resulted in similar differentiation capacity and neurite length with the control group. The cell viability analysis of this laser group showed that the triple laser light application at 5 J/cm^2 had a negative effect on PC12 cells and induced a statistically significant decrease in cell viability with rate of 35%. It has been understood that 5 J/cm^2 energy density resulted in bioinhibition on PC12 cells.

As a conclusion, it is known that low level power and energy density supports photobiomodulation. In this study, photobiomodulation or bioinhibition can be induced by changing the light intensities of 655 nm laser light at constant 50 mW output power. For this reason, it is very important to optimize the light parameters specific to the target tissue for a desired outcome. It has been proved that the PBM effect manifests itself for a long period of time and the energy density which is absorbed by the target cells should be adjusted properly to induce a specific mechanism such as differentiation, biostimulation or bioinhibition. Finally, it can be said that PBM application with 655-nm laser light can be a promising therapy for the biostimulation of neural cells in the treatment of neurodegenerative diseases such as Alzheimer and Parkinson diseases.

Acknowledgment

The authors thank Assoc. Prof. Mustafa Şen, Tansu Gölcez, Ziyşan Buse Yaralı Çevik ve Fikri Seven for their support and valuable suggestions throughout this study.

References

[1] Whinfield, A. L., & Aitkenhead, I. (2009). The light revival: does phototherapy promote wound healing? A review. *The foot*, *19*(2), 117-124.

[2] Basford, J. R. (1995). Low intensity laser therapy: still not an established clinical tool. *Lasers in surgery and medicine*, *16*(4), 331-342.

[3] Lubart, R., Eichler, M., Lavi, R., Friedman, H., & Shainberg, A. (2005). Low-energy laser irradiation promotes cellular redox activity. *Photomedicine and Laser Therapy*, *23*(1), 3-9.

[4] Hawkins, D., & Abrahamse, H. (2006). Effect of multiple exposures of low-level laser therapy on the cellular responses of wounded human skin fibroblasts. *Photomedicine and Laser Therapy*, *24*(6), 705-714.

[5] de Freitas, L. F., & Hamblin, M. R. (2016). Proposed mechanisms of photobiomodulation or low-level light therapy. *IEEE Journal of selected topics in quantum electronics*, *22*(3), 348-364.

[6] Düzgören, İ., Ruhi, M. K., & Gülsoy, M. (2017, June). Effect of different laser power densities on photobiomodulation of L929 cell line. In *European Conference on Biomedical Optics* (p. 1041702). Optical Society of America.

[7] Salehpour, F., Mahmoudi, J., Kamari, F., Sadigh-Eteghad, S., Rasta, S. H., & Hamblin, M. R. (2018). Brain photobiomodulation therapy: a narrative review. *Molecular neurobiology*, *55*(8), 6601-6636.

[8] Poyton, R. O., & Ball, K. A. (2011). Therapeutic photobiomodulation: nitric oxide and a novel function of mitochondrial cytochrome c oxidase. *Discovery medicine*, *11*(57), 154-159.

[9] Mitrofanis, J., & Henderson, L. A. (2020). How and why does photobiomodulation change brain activity?. *Neural Regeneration Research*, *15*(12), 2243.

[10] Caruso-Davis, M. K., Guillot, T. S., Podichetty, V. K., Mashtalir, N., Dhurandhar, N. V., Dubuisson, O., ... & Greenway, F. L. (2011). Efficacy of low-level laser therapy for body contouring and spot fat reduction. *Obesity surgery*, *21*(6), 722-729.

[11] Yang, L., Tucker, D., Dong, Y., Wu, C., Lu, Y., Li, Y., ... & Zhang, Q. (2018). Photobiomodulation therapy promotes neurogenesis by improving post-stroke local microenvironment and stimulating neuroprogenitor cells. *Experimental neurology*, *299*, 86-96.