Facilitating iADMSC Differentiation into Neuronal Cells by Photobiomodulation Using Visible and Near-Infrared Wavelengths

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Abstract. The central nervous system (CNS) of mammals is limited in its repair and regeneration in the event of injury due to trauma or neurodegeneration, therefore, optimization of its regeneration capabilities is necessary. Studies have shown that this issue may be addressed through the transdifferentiation of adipose-derived stem cells (ADMSCs) into neuronal cells. This process has not been efficiently achieved with chemical and biological inducers; this study explored possible optimization through the addition of photobiomodulation (PBM). PBM uses low intensity light to stimulate intracellular processes and has been known to increase cell proliferation and aid in stem cell differentiation. This *in vitro* research aimed to differentiate ADMSCs with growth factors and chemical inducers and subsequently measure the optimization effects that PBM had on differentiation. PBM was applied as single use at a low energy density, at visible and near-infrared (NIR) wavelengths. Characterization of immortalized ADMSCs (iADMSCs) with flow cytometry was used in identifying a CD marker and early and late neuronal markers. After this, biochemical analysis was performed to observe reactive oxygen species (ROS) production, cytotoxicity, proliferation, and viability. The outcome of this study indicated that PBM is beneficial to the differentiation process, however, prior induction at a longer incubation period is needed. Findings from this research serves as contribution toward validating stem cell technology for application in *in vivo*, pre-clinical and clinical research settings. Furthermore, an optimized protocol using differentiation media and PBM should be established for *in vivo* and clinical research, that specifically targets neuronal regeneration.

1. Introduction

Neurological diseases and illnesses present a task for therapy and rehabilitation, because of the limited ability for the nervous system to repair itself. Adipose-derived mesenchymal stem cells (ADMSCs) are more flexible than any adult stem cells and can be differentiated into non-mesodermal tissues, including neurons [1]. Differentiating ADMSCs into specific neuronal lineage cells allows us to transplant the correct cell type into the nervous system. To differentiate ADMSCs into active neurons several methodologies are being studied and evaluated. Limited success has been achieved in altering ADMSC's cellular structure and operational state to become identical with neuroglial cells [2]. Photobiomodulation (PBM) has been effectively implemented not only to enhance the viability and development of ADMSCs, but also as a potential enhancer of ADMSC differentiation [3, 4]. In this in vitro study, we

examined current neuro-differentiation methodologies along with the use of PBM, for differentiation of ADMSCs into neuronal lineages.

ADMSCs display plasticity and showcase the ability to transdifferentiate into multiple phenotypes, such as osteoblasts and neurons [5]. ADMSCs are abundantly available and easily sourced through surgeries that are not as invasive when compared to the harvesting of bone-marrow stem cells [6]. This process of differentiation can be triggered by exposing ADMSCs to growth factors or chemical inducers specific for the lineage in need [6-8]. The use of these factors upregulates in vitro potential of cells to differentiate [6]. Studies have met some success in differentiating ADMSCs into neuronal cell types with inducers forskolin, 3-isobutyl-1-methylxanthine, and basic fibroblast factor (bFGF) [9, 10]. Potentially, ADMSCs may be applied clinically to aid in the repair of mechanical brain injuries or neurodegenerative diseases [11].

Applying laser light to tissue to up- or downregulate the metabolism of cells is called PBM. The increase or decrease in metabolism is dependent on the parameters of the laser light [3]. Laser parameters include the wavelength that spans the visible and near-infrared (NIR) spectrum, ranging from 400-1100 nm, and fluence which is measure in J/cm^2 [3, 4]. Fluence, or energy density, is dependent on dosage, and has been determined to be effective at 3-5 J/cm^2. Below 3 J/cm^2 little to no effects are observed, and above 5 J/cm^2 often results in a biphasic dosage response [4, 12]. PBM applied at wavelengths within the visible spectrum of light has resulted in an increase in differentiation in osteoblasts from ADMCSs [13, 14]. The aim of this study was to evaluate the effects of PBM on iADMSC transdifferentiation into neuronal-like cells.

2. Materials and Methods

Immortalized ADMSCs (iADMSCs) (ASC52Telo; ATCC) were characterized through flow cytometry by identifying protein markers including stem cell marker CD44, neuronal early marker, neuron specific enolase (NSE) and late marker, microtubule associated protein 2 (MAP2). The iADMSCs were seeded at a density of 1 x 10^5 in 35 mm treated petri dishes and incubated overnight for attachment in neuronal induction media. The cells were treated with PBM using 825 nm near-infrared (NIR), 525 nm green (G), or the combination of both wavelengths at 5 J/cm^2. Cells that were not seeded in induction media nor treated with PBM served as a standard, and cells that were seeded in induction media but not exposed to PBM served as a control group. Post PBM exposure, cells were incubated 24 h, 48 h, and 7 days. Laser parameters are indicated in Table 1. The following formula was used to determine PBM irradiation time:

$$mW/\,cm^2 = \frac{mW}{\pi r^2}$$

$$W/\,cm^2 = \frac{mW\,/\,cm^2}{1000}$$

$$Time\ (\text{seconds}) = \frac{J/cm^2}{W/cm^2}$$

Table 1. Laser Parameters

	Near infra-red (NIR)	**Green (G)**
Wavelength (nm)	825	525
Type	Diode	Diode
Emmision	CW	CW
Power (mW)	100	574
Power density (mW/cm^2)	10.394	59.66
Fluence (J/cm^2)	5	5
Time of irradiation (s)	8 min 1 sec	1 min 23 sec
Spot size (cm^2)	9.62	9.62

The CytoTox 96® non-radioactive cytotoxicity assay was performed to determine the amount of lactate dehydrogenase (LDH) released from the cells, this was measured with a spectrophotometer. Reactive oxygen species (ROS) production was determined with the Fluorometric Intracellular Ros Kit (Sigma-Aldrich, MAK143) and subsequently measure with a spectrophotometer. The percentage viability was measured using a Trypan blue assay. The CellTiter-Glo® ATP Luminescence assay was used to determine the ATP generated in each experiment; this was then measured spectrophotometrically. Significant differences between experimental groups were designated as $p<0.05$ (*), $p<0.01$ (**) and $p<0.001$ (***). All experiments were performed n=4.

3. Results

3.1. Characterization

Flow cytometry characterisation was used to detect the expression of stem cell and neuronal markers, where detection is represented by a histogram peak. An increased percentage to the right or right shift is indicative of increased expression and a left shift a decrease. Analysis (Figure 1) revealed that green and NIR-green laser light was the most effective in reducing stem-ness, as seen by the left shift in CD44 expression, indicating its effectivity in transdifferentiation. This study demonstrates the effectiveness of introducing green laser light to decrease stem-ness for transdifferentiation purposes. The right shift in early (NSE) and late (MAP2) neuronal markers 7 days post PBM treatment, albeit in very small percentages, is indicative of the iADMSCs being directed towards a neuronal fate.

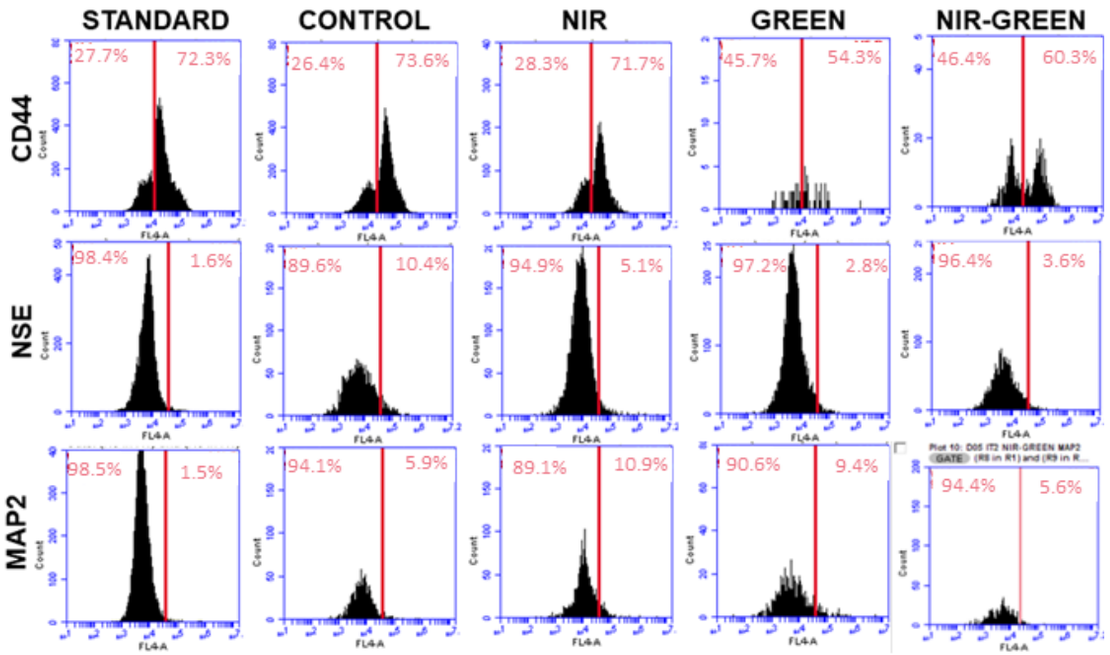

Figure 1. Flow cytometry characterization of stem cell marker CD44, early neuronal marker: Neuronal Specific Enolase (NSE), and late neuronal marker Microtubule-associated protein 2 (MAP2). A shift to the right of the red line indicated an increase in protein marker expression, whereas a shift to the left of the redline indicated a decrease in marker expression.

3.2. Cytotoxicity

Results showed an increase in LDH production in NIR and combination wavelengths 24 h following PBM treatment (Figure 2A). A significant increase was observed in all PBM treated experimental groups 48 h post PBM. NIR and green PBM significantly increased LDH concentration 7 days post irradiation. Although significant compared to the standard and control, these increases are not indicative of detrimental concentrations when compared to the positive control representing 100 percent toxicity and cell death.

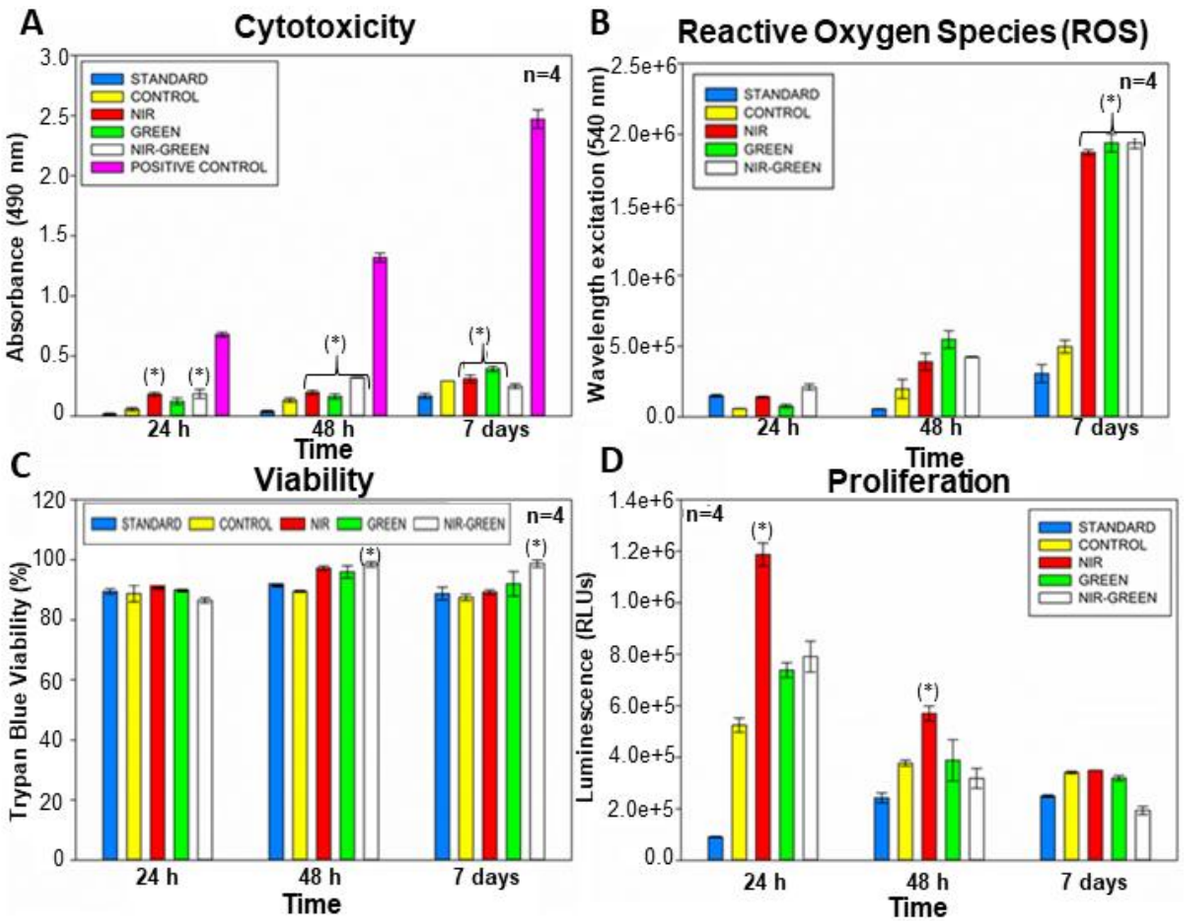

Figure 2. A. Cytotoxicity Lactate dehydrogenase (LDH) cytotoxicity assay. B. Reactive oxygen species (ROS) assay. C. Viability Trypan blue viability assay. D. Proliferation ATP luminescence assay.

3.3. Reactive oxygen species (ROS)

Increases in ROS activity is noted using NIR and concurrent PBM at 24 hours, and for all PBM groups at 48 hours albeit insignificant (Figure 2B). ROS production significantly increased in all PBM treated experimental groups 7 days post PBM exposure indicative of directing stem cell fate. As the increase in ROS was not detrimental to the cells as indicated by the viability results.

3.4. Viability

Biochemical analysis showed a consistency in percentage viability in the cells 24 h, 48 h, and 7 days post-PBM exposure (Figure 2C).

3.5. Proliferation

A significant increase in ATP was noted in the experimental group treated with NIR PBM 24 h and 48 h post PBM exposure (Figure 2D). PBM did not significantly increase proliferation 7 days post PBM exposure, however this can be due to the energy of the cells being redirected for differentiation rather than proliferation.

4. Discussion and Conclusion

Research has attempted to differentiate ADMSCs into multiple stem cell types, such as osteogenic or neuronal cells [5]. It has also been determined that these stem cells contribute to the repairing of damaged tissue cells. This study showed the ability of iADMSCs to differentiate into cells that carry neuronal cell markers and morphologically resemble premature neurons. This study also revealed that the differentiation process may be upregulated with PBM. Studies have been done to measure the effects of PBM on ADMSCs, however, the practice of consecutive wavelengths to exploit differentiation and proliferation has not been explored in great depth [3, 5], and can thus be considered a novel concept.

Analysis by flow cytometry revealed that the stem cell marker CD44, was maintained in most of the experimental groups. This suggests that large subpopulations of iADMSCs had not transdifferentiated. However, green and NIR-green PBM showed a significant decrease in CD44 marker expression, this is indicative of the cells losing their stem ness and transitioning into a neuronal fate, as previous studies using ADMSC for differentiation into various cell lineages also indicated a decrease in stem cell marker expression [22]. This study showcases that the addition of green laser, with or without consecutive use, decreases cell stemness. Furthermore, it waws seen that PBM facilitate differentiation of ADMSCs into neuronal like cells due to late neuronal marker detection seen 7 days post PBM treatment. Also, it is suggested to allow longer incubation periods for the cells to transdifferentiate which should bring about a greater increase in early and late neuronal marker expression [16].

Analysis of biochemical assays revealed that viability was maintained and remained unaffected regardless of treatment applied to the iADMSCs, therefore PBM does not have a detrimental effect on *in vitro* iADMSCs [3, 18]. The proliferation assays revealed a steady decrease in ATP production that was overall negligible. This decrease did not prove detrimental when comparing this to the cytotoxicity and viability results, perhaps indicating that cells are using their energy to differentiate rather that proliferate [19]. An assay to determine the amount of lactate dehydrogenase (LDH) released by the cell indicated significant increases by NIR and NIR-G groups 24 h post PBM, all three PBM groups 48 h post PBM, and in NIR and G PBM groups 7 days post PBM treatment. Nevertheless, when these significant increases are compared to the positive control of each period, the upregulation in LDH did not induce apoptosis. Finally, ROS studies showed that ROS concentrations increased as the population increases. Samples treated with PBM showed a significant upregulation in ROS production, however this was not detrimental to the cells when compared to the cytotoxicity studies and the viability results. A similar increase in ROS has been observed by Hu *et al* where the upregulation was tied to directing the stemness fate of the cells [20]. It should also be noted that an increase in ROS concentration is observed in neuronal developmental research and neuronal functionality studies [21].

In conclusion, the results of this study indicate that iADMSCs incubated for 7 days in media supplemented with chemical inducers and irradiated with PBM showed an upregulation in transdifferentiation. This is similar to previous research where G PBM upregulated differentiation and various wavelengths were not detrimental to cells. This study indicated that the addition of PBM prepared the cells for differentiation signified by the increase in ROS production observed in the PBM treated experimental groups. Green PBM shows significant potential for increasing the differentiation rate as seen in the decrease of CD44, indicating a loss of stem-ness at a faster rate than that of the other groups. Further studies will include incubating cells for a longer period prior to exposing cells to PBM to determine whether the goal of producing functional neuronal cells can be achieved.

References

[1] Bajek A, Gurtowska N, Olkowska J, Kazmierski L, Maj M and Drewa T 2016 *Archivum Immunologiae et Therapiae Experimentalis* **64** 443-54
[2] George S, Hamblin M R and Abrahamse H 2018 *Photomedicine and Laser Surgery* **36** 230-40
[3] Mvula B, Mathope T, Moore T and Abrahamse H 2008 *Lasers in Medical Science* **23** 277-82
[4] Wang Y, Huang Y-Y, Wang Y, Lyu P and Hamblin M R 2017 *Biochimica et Biophysica Acta- General Subjects* **1861** 441-9
[5] Van Rensburg M J, Abrahamse H and Crous A 2020 *Current Stem Cell Research & Therapy*
[6] Zuk P A, Zhu M, Mizuno H, Huang J, Futrell J W, Katz A J, Benhaim P, Lorenz H P and Hedrick M H 2001 *Tissue Engineering* **7** 211-28
[7] Ateş G B, Ak A, Garipcan B and Gülsoy M 2018 *Lasers in Medical Science* **33** 1591-9
[8] George S, Hamblin M R and Abrahamse H 2020 *Lasers in Surgery and Medicine* **52** 1032-40
[9] Jang S, Cho H H, Cho Y B, Park J S and Jeong H S 2010 *BMC Cell Biology* **11**
[10] Thompson R, Casali C and Chan C 2019 *Scientific Reports* **9** 1-10
[11] Guy R and Offen D 2020 *Biomolecules* **10** 1320
[12] Ateş G B, Ak A, Garipcan B and Gülsoy M 2020 *Cytotechnology* **72** 247-58
[13] Fekrazad R, Asefi S, Eslaminejad M B, Taghiar L, Bordbar S and Hamblin M R 2019 *Lasers in Medical Science* **34** 115-26
[14] Rosenberg N, Gendelman R and Noofi N 2020 *FEBS Open Bio* **10** 1276-87
[15] Ge Y and Fuchs E 2018 *Nature Reviews Genetics* **19** 311-25
[16] Ashjian P H, Elbarbary A S, Edmonds B, DeUgarte D, Zhu M, Zuk P A, Lorenz H P, Benhaim P and Hedrick M H 2003 *Plastic Reconstructive Surgery* **111** 1922-31
[17] Wang Y, Huang Y-Y, Wang Y, Lyu P and Hamblin M R 2016 *Scientific Reports* **6** 33719
[18] Nurković J, Zaletel I, Nurković S, Hajrović Š, Mustafić F, Isma J, Škevin A J, Grbović V, Filipović M K and Dolićanin Z 2017 *Lasers in Medical Science* **32** 151-60
[19] Birket M J, Orr A L, Gerencser A A, Madden D T, Vitelli C, Swistowski A, Brand M D and Zeng X 2011 *Journal of Cell Science* **124** 348-58
[20] Hu C, Zhao L, Peng C and Li L 2018 *Journal of Cellular Molecular Medicine* **22** 5196-207
[21] Oswald M C, Garnham N, Sweeney S T and Landgraf M 2018 *FEBS Letters* **592** 679-91
[22] Moraes, D.A., Sibov, T.T., Pavon, L.F., Alvim, P.Q., Bonadio, R.S., Da Silva, J.R., Pic-Taylor, A., Toledo, O.A., Marti, L.C., Azevedo, R.B. and Oliveira, D.M., 2016. *Stem cell research & therapy*, *7*(1), pp.1-14.