Photobiomodulated Differentiation of Adipose-derived Stem Cells into Osteoblasts.

D Da Silva[1], A Crous[1] and H Abrahamse[2]

[1/2]Laser Research Centre, Faculty of Health Sciences, University of Johannesburg, P.O. Box 17011, Doornfontein, Johannesburg, South Africa, 2028

[2]**~~Corresponding author~~** ~~Prof Heidi Abrahamse;~~ Email: habrahamse@uj.ac.za

Abstract. Osteoporosis is a progressive, metabolic bone disease affecting millions across the globe. Stem cell (SC) regenerative therapy has demonstrated potential in treating osteoporosis, particularly when using Adipose-derived Mesenchymal Stem Cells (ADMSCs). Photobiomodulation (PBM) has gained international momentum due to its ability to aid in the proliferation and differentiation of stem cells. Additionally, PBM when combined with differentiation growth factors has revealed enhanced proliferation and ADMSC differentiation into osteoblasts. This *in vitro* study combined the use of osteogenic differentiation inducers and PBM at a near-infrared (NIR) wavelength of 825 nm, a green wavelength of 525 nm and their combination wavelengths (825 nm and 525 nm) using a single fluence of 5 J/cm^2 to determine the proliferation and differentiation effectivity of ADMSCs into osteoblasts. The cells were characterised via the use of flow cytometry. Morphology was investigated and the biochemical assays performed include proliferation, viability, and cytotoxicity. The successful outcome of this study provides relevant scientific knowledge and a standardization for osteogenic differentiation *in vitro* using PBM.

1. Introduction

Osteoporosis is known as the most progressive mitochondrial bone disease in humans [1]. Osteoporosis is characterized by the increase in bone fragility leading to the increase in fracture occurrence [2]. Regenerative Medicine (RM) is currently the most promising branch of medical science used to repair or heal tissues and organs damaged by severe injuries, chronic disease or age [3]. At the frontline of RM stands SC therapy because of its' ability to indefinitely self-renew and transdifferentiate into various cell types [4]. ADMSC transplantation can facilitate the development and strength of new bones, increase bone consistency and decrease the risk of fractures [5]. ADMSCs are easily isolated and harvested from adipose tissues via minimally invasive surgery, providing low risk of morbidity, a high abundancy and a high yield in cell numbers [6]. The successful differentiation of ADMSCs into osteogenic lineages *in vitro*, requires the addition of biological and chemical growth factors (GFs) within the cell culture medium [7]. However, ADMSCs have a tendency of favouring adipogenic lineages despite GF presence [8]. Therefore, lineage specific differentiation control is required via a combinational use of GF presence and a mechanical stimulant such as PBM [9]. The use of visible and NIR light by coherent or incoherent light sources on cells and tissues is known as PBM [10]. Once endogenous chromophore absorption occurs, the light within various ranges brings about photophysical and photochemical responses. PBM is understood to aid in cell function, proliferation, migration and

tissue regeneration due to the increased mitochondrial oxidative metabolism [11]. The potential stimulatory and inhibitory outcomes of PBM on ADMSCs are wavelength and fluence dependant [12]. However, cell proliferation has been suggested when stimulated by PBM using a wavelength of/between 660-850 nm and a fluence of/between 5 – 10 J/cm^2 [13].

2. Materials and Methodology

Immortalized ADMSCs were cultured for one week in osteogenic differentiation media containing complete Dulbecco's Modified Eagle Media (DMEM) media, 50 nM Dexamethasone, 10 nM Beta-glycerol phosphate disodium and 50 mM Ascorbic acid. All cultured cells were kept in Corning® cell culture flasks and incubated at 37°C in 5% CO^2 and 85% humidity. The cultured immortalized ADMSCs were seeded at 1 x 10^5 cells into 35 mm diameter treated culture dishes in 2 mL of osteogenic differentiation media. Prior to irradiation, the osteogenic differentiation media was refreshed. Each experimental cell plate was irradiated with a NIR 825 nm Diode Laser, Green (G) 525 nm Diode Laser and/or a combination (825 nm Diode Laser and 525 nm Diode Laser) of wavelengths all at a single fluence of 5 J/cm^2. The time of irradiation was calculated as follows:

$$mW/cm^2 = \frac{mW}{\pi \times (r^2)}$$

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

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

The PBM studies were performed in the dark at room temperature with the petri dish lid taken off prior to irradiation to avoid negligible factors. The laser parameters are shown in Table 1. The cell samples were collected at 24 hours, 48 hours and 7 days post-irradiation. Cell characterization, via the use of flow cytometry, identified CD44 and CD166 protein markers. Morphology was identified by inverted light microscopy and biochemical assays such as ATP proliferation, trypan blue viability and LDH cytotoxicity were performed. Statistical variances amongst the experimental groups are denoted on the figures as $P < 0.05$ (*), $P < 0.01$ (**) and $P < 0.001$ (***).

Table 1. Laser Parameters

Laser Parameters	Near infra-red (NIR)	Green (G)
Light Source	Diode Laser	Diode Laser
Wavelength (nm)	825	525
Power Output (mW)	515	553
Power Density (mW/cm^2)	53.53	57.47
Area (cm^2)	9.62	9.62
Emission	Continuous Wave	Continuous Wave
Fluence (J/cm^2)	5	5
Irradiation Time (s)	1 min 33 sec	1 min 26 sec

3. Results

Flow cytometry analysis (Figure 1) indicated a decline in CD44 and CD166 protein marker presence by all experimental groups at 7 days post-PBM treatment implying the effective use of PBM to reduce stem-ness and be an efficient tool for immortalized ADMSC differentiation. Noticeably, each experimental group reduced a particular protein marker more than another suggesting the individual influence a laser group may have on the function of a cell.

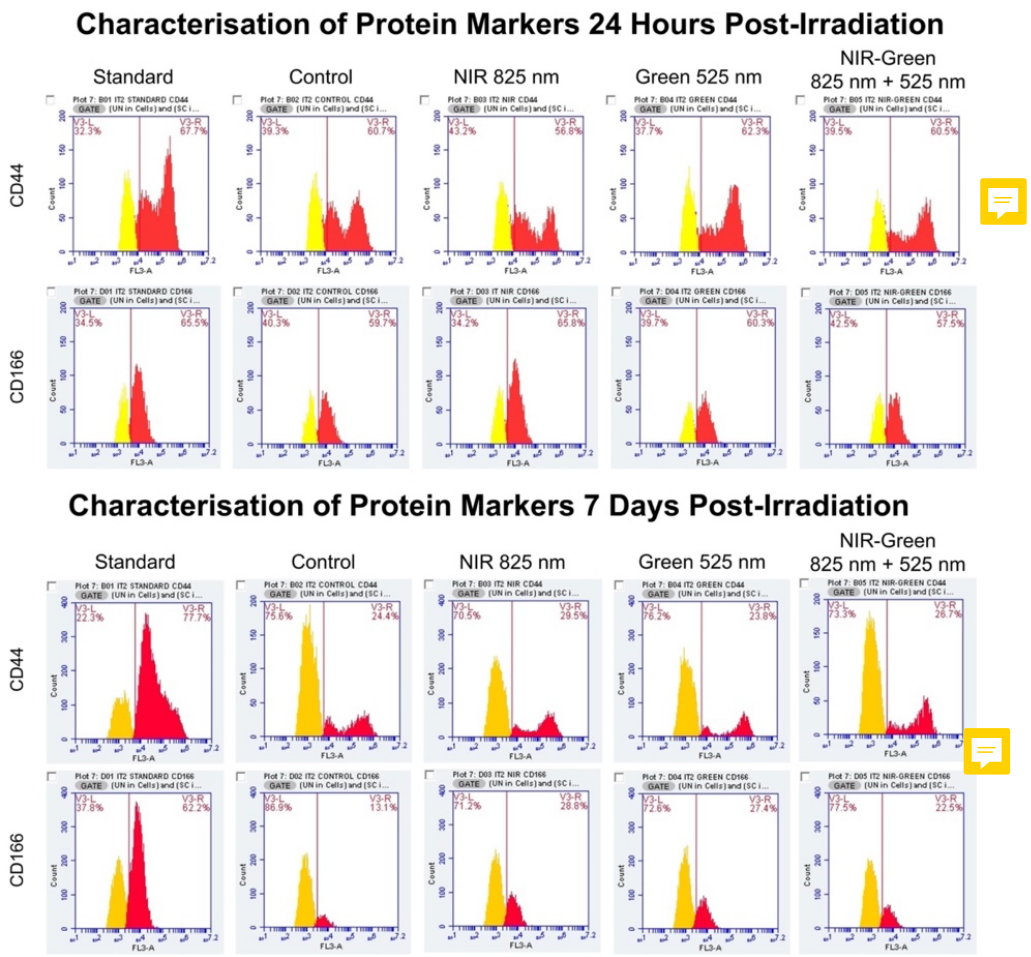


Figure 1. Flow cytometry characterization of immortalized ADMSC CD44 marker and CD166 marker at 24 hours and 7 days post-irradiation treatment.

Immortalized ADMSCs are characteristically thin and spindle in shape. A noticeable change in cell morphology (Figure 2) occurred amongst the Green treated cell groups at 24 hours, the Green and NIR-Green treated cell groups at 48 hours and amongst the NIR, Green and NIR-Green treated cell groups at 7 days post-PBM treatment. The cell shape had become rounded in appearance similarly to that of osteoblasts and a loss in the visibly thin and longitudinal initial ADMSC cell shape.

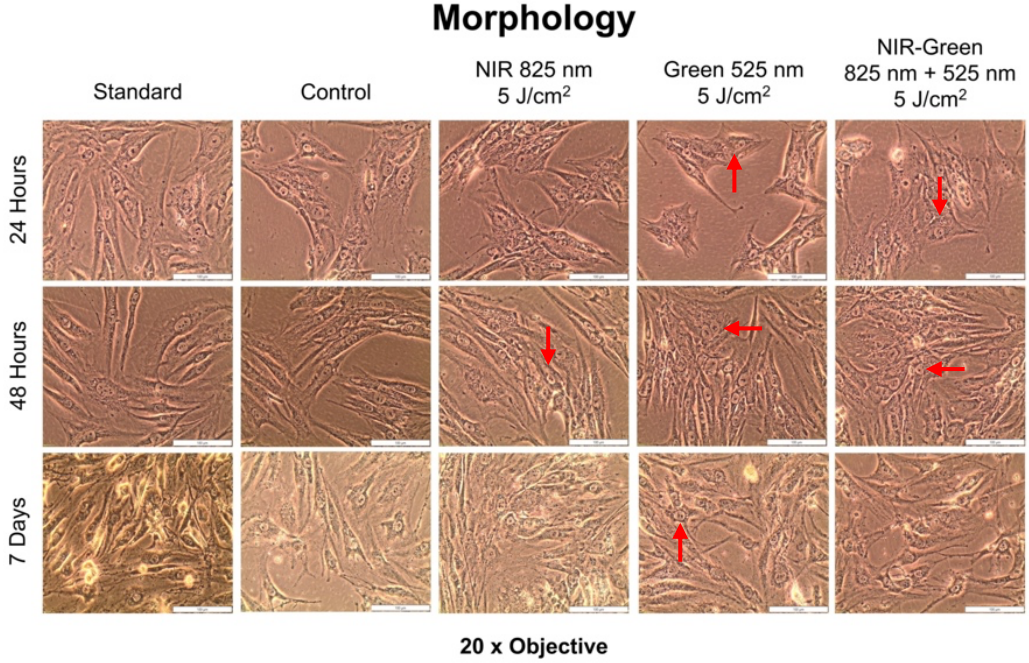
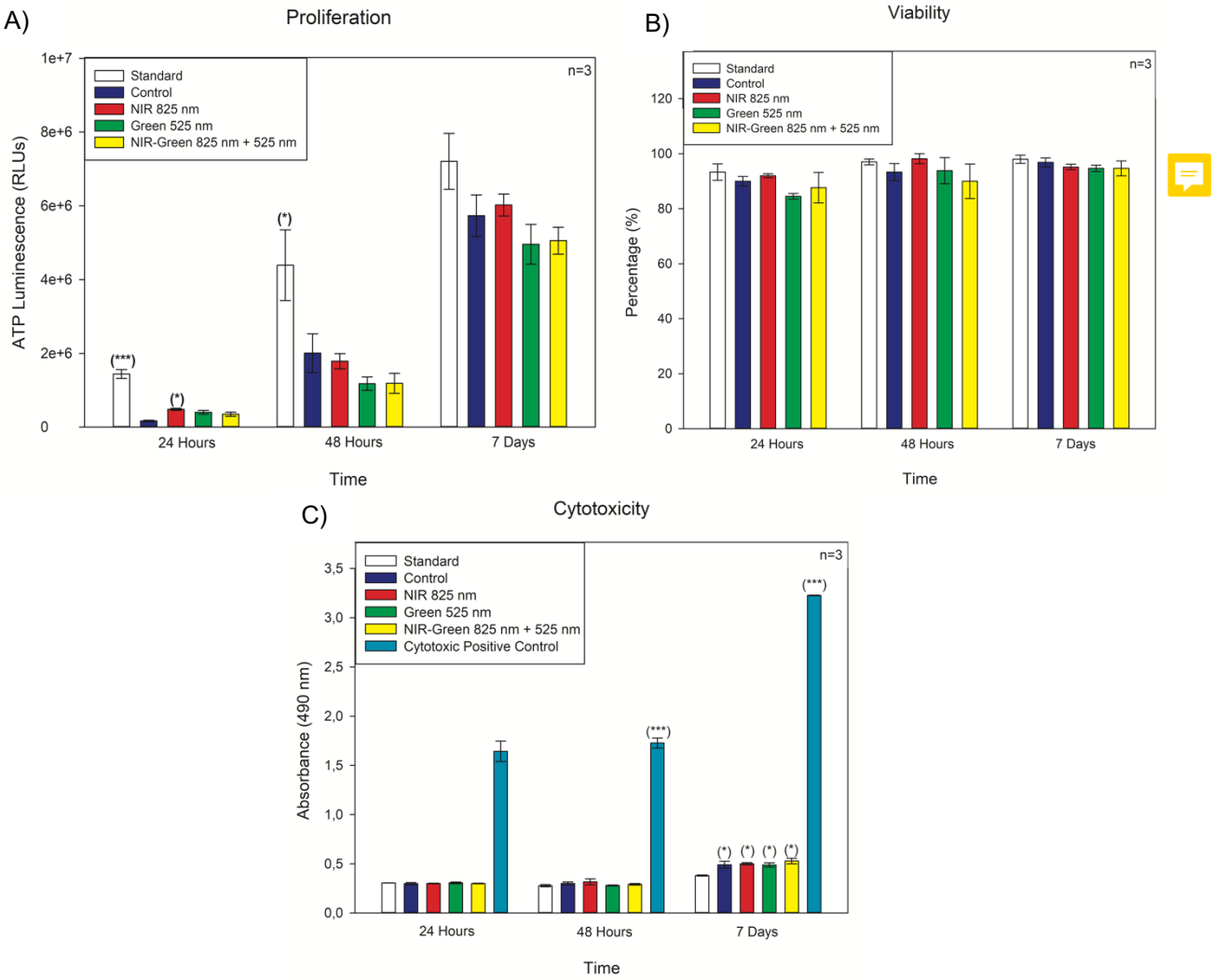


Figure 2. Morphology of immortalized ADMSC differentiation post-PBM treatment using Inverted Light Microscopy.

A significant increase in ATP (Figure 3 A) was identified in the standard for 24 hours and 48 hours. PBM did not significantly increase cell proliferation, however, this may suggest that ATP is being redirected for cell differentiation instead of for cell proliferation. The viability assay (Figure 3 B) suggested a consistent cell percentage viability of cells at 24 hours, 48 hours and 7 days post-PBM treatment implying that PBM does not negatively impact cell health instead maintains the overall health of cells. LDH results (Figure 3 C) showed no significant increase in LDH production at 24 hours and 48



hours post-PBM treatment, however, a significant increase in LDH production did occur amongst the control and all the experimental groups at 7 days post-PBM treatment. Despite the significant increase when compared to the standard, these increases are not of toxic concentrations in comparison to the cytotoxic positive control which represents a hundred percent cell toxicity and cell death. This indicates that PBM treatment has not induced plasma membrane damage to the cell populations.

Figure 3. Biochemical Analysis of immortalized ADMSC differentiation at 24 hours, 48 hours, and 7 days post-PBM treatment.

4. Discussion and Conclusion

Osteoporosis is the consequence of a decline in bone-forming mature osteoblast populations [14] instigated by changes in the biology of Mesenchymal Stem Cells (MSCs), insufficient osteoblast progenitor proliferation, a rise in apoptosis and an upsurge in the accumulation of adipocytes within the marrow [15]. The development, strength, and consistency of new bones can be aided by the transplantation of ADMSCs [5] because ADMSCs are an ample multipotent cell source capable of differentiation into osteoblast, adipocyte, and chondrocyte cell lineages [16]. PBM has been recognized to assist in cell function, proliferation, migration, and the regeneration of tissue [11]. The effects of PBM on ADMSCs have shown to be dependent based on the wavelength and fluency applied [12]. Previous studies have identified the effect of PBM at a single wavelength on ADMSCs [17–19], however, limited studies have explored the outcome of combining PBM wavelengths to aid multiple cellular functions [20]. The intention of this *in vitro* study was to combine the use of osteogenic differentiation inducers and PBM at a near-infrared (NIR) wavelength of 825 nm, a green wavelength of 525 nm and their combination wavelengths (825 nm and 525 nm) using a single fluence of 5 J/cm^2 to determine proliferation and differentiation effectivity of ADMSCs into osteoblast cell lineages.

Flow cytometry analysis indicated a reduction in CD44 and CD166 immortalized ADMSC stem-ness protein markers 7 days post-PBM treatment, implying the transition of stem cell into cell lineage. The data identified Green PBM to have a greater outcome in reducing CD44 and NIR-Green PBM to have a greater outcome in reducing CD166. This suggests Green PBM and NIR-Green PBM influence different cellular pathways, yet both are an effective aid in the differentiation of immortalized ADMSCs. Wang et al. concluded their findings on the identification of differentiated ADMSCs into osteogenic lineages with the aid of Green PBM [21].

Immortalized ADMSCs are typically identified as thin and spindle in cell shape, however, cellular morphology depicted noticeable cell shape rounding as early as 24 hours post-PBM treatment amongst the Green PBM experimental group. At 48 hours post-PBM treatment, both Green and NIR-Green PBM experimental groups presented with rounded cell and/or shorter spindle shaped cell morphology. An osteoblast is characteristically rounded in cell morphology as identified by previous research [22]. This change in cell morphology suggests cell differentiation of immortalized ADMSCs into osteoblasts.

Analysis of biochemical assays identified a statistical significance in cell proliferation at 24 hours post-PBM treatment amongst the NIR PBM experimental group. At 7 days post-PBM treatment, a decreased cell proliferation occurred amongst the Green PBM and NIR-Green PBM experimental groups. However, this decrease is suggestive that ATP had been redirected for the use of cellular differentiation instead of cellular proliferation as suggested by the findings of a study which intended for the differentiation of human embryonic stem cells into neural cells [23]. The viability assay presented with a consistent cell percentage viability of cells amongst all experimental groups at 24 hours, 48 hours and 7 days post-PBM treatment. This indicates that PBM treatment assists in the maintenance of cell health without a negative outcome [19]. Lastly, a significant increase in LDH production had been identified amongst the control group and all three experimental groups 7 days post-PBM treatment, however, these concentrations were not toxic to the cell population. In comparison to a hundred percent cell toxicity and death, the cytotoxic positive control, the increases of LDH were not a result of plasma membrane damage induced by PBM and thus, not harmful to the cells [24]. This is further supported by the proliferative and viability assays performed.

In conclusion, this study has indicated promising early osteogenic differentiation of immortalized ADMSCs using a combination of growth trans-differentiation inducers and PBM treatment. The results of this study suggest that both Green PBM and NIR-Green PBM possess the greatest potential for the differentiation of immortalized ADMSCs into osteoblasts as identified by the significant decrease of CD44 and CD166 protein markers, suggesting a loss of immortalized ADMSC stem-ness into osteogenic cell lineage and the identifiable changes in cell morphology into rounded cells characteristic of osteoblast cells. These findings further imply that Green PBM has the ability to prime immortalized ADMSCs for cellular differentiation into osteogenic cell lineages. However, further analysis such as early and late osteogenic protein marker characterisation, additional biochemical assays and genetic

expression using Real Time-PCR and ELISA will offer confirmation of efficient immortalized ADMSC differentiation into osteoblasts via the aid of PBM treatment.

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