Photobiomodulated Differentiation of Adipose-derived Stem Cells into Osteoblasts.

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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 due to the characteristics of SCs including self-renewal and trans-differentiation into various cell types [4]. Adipose-derived Mesenchymal Stem Cell (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 trans-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 photobiomodulation (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]. 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], whereas wavelengths of 495–570 nm had been seen to affect differentiation [14]. The aim 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 the proliferation and differentiation effectivity of ADMSCs into osteoblast cell lineages.

2. Materials and Methodology

Immortalized ADMSCs (ASC52telo hTERT, ATCC®SCRC-4000™) were cultured for one week in osteogenic differentiation media comprised of Dulbecco's Modified Eagle Media (DMEM) media, 10% Fetal Bovine Serum, 0.5% Penicillin-Streptomycin, 0.5% Amphotericin B solution, 50 nM Dexamethasone, 10 nM Beta-glycerol phosphate disodium and 0.2 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 treated culture dishes with a radius of 1,75 cm with 2 mL of osteogenic differentiation media. Prior to irradiation, the osteogenic differentiation media was refreshed. Cells were irradiated from above in the dark at room temperature with the petri dish lid taken off prior to irradiation to avoid negligible factors. The time of irradiation was calculated as follows:

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

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

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

This study included an experimental standard whereby cells did not receive osteogenic growth factors nor PBM treatment and an experimental control of which cells received osteogenic trans-differentiation growth factors but weren't exposed to PBM treatment. 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 as a qualitative assay, identified SC protein markers CD44 and CD166 presence on the immortalized ADMSCs. Morphology was identified by inverted light microscopy and biochemical assays such as Adenosine triphosphate (ATP) proliferation (Promega, G9241) [15], Trypan blue viability (Invitrogen™, T10282) and Lactate dehydrogenase (LDH) cytotoxicity (Promega, G1780) were performed. For statistical analysis, biochemical assays included n=3 biological repeats with technical duplicates. Statistical analysis was performed using SigmaPlot version 12 and the data was evaluated by a student t-test and one-way ANOVA. The statistical variances amongst the experimental groups were 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 (sec)	93	86

3. Results

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. Flow cytometry analysis (Figure 1) demonstrated a left shift, CD44 and CD166 SC protein marker expression by all experimental groups at 7 days post-PBM treatment. This implies the effective use of PBM to reduce stem-ness and be an efficient tool for trans-differentiation of immortalized ADMSCs into osteoblasts.

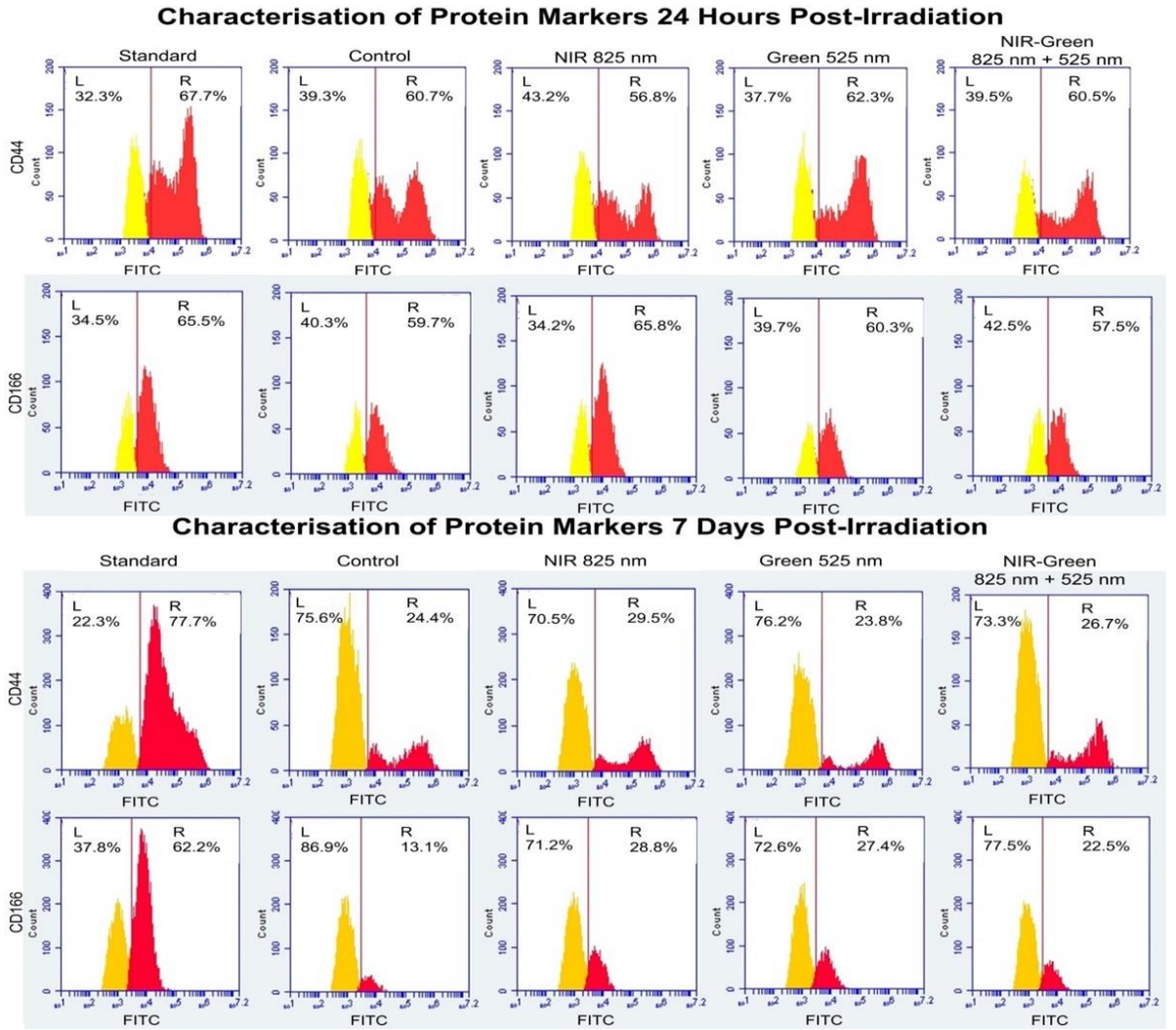

Figure 1. Flow cytometry characterization of immortalized ADMSC CD44 marker and CD166 markers at 24 hours and 7 days post-irradiation treatment. The red histogram is indicative of a right shift and increase in protein marker expression and the yellow histogram is indicative of a left shift, a decrease in protein marker expression.

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.

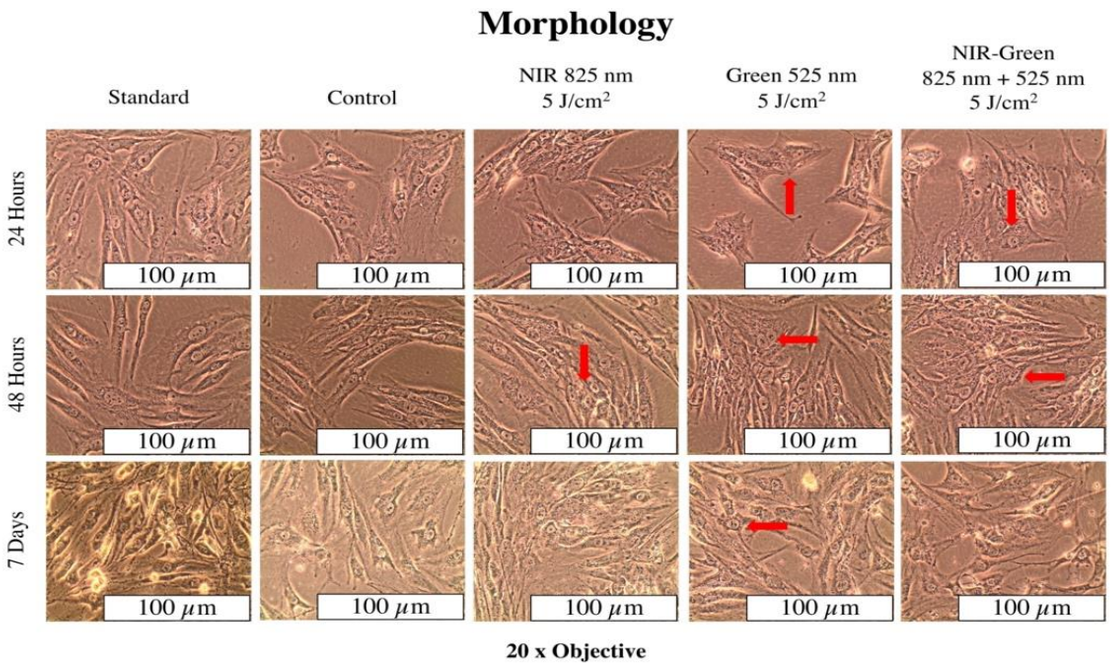

Figure 2. Morphology of immortalized ADMSC differentiation post-PBM treatment using Inverted Light Microscopy.

A statistically significant decrease in ATP (Figure 3A) was identified for all experimental groups compared to the standard, at 24 hours and in the Green and NIR-Green experimental groups at 48 hours post-PBM treatment. Proliferation was decreased for all experimental groups compared to the standard at 7 days, albeit insignificant. The overall decrease in proliferation seen may suggest that ATP is being redirected for cell differentiation instead of cell proliferation [16]. The viability assay (Figure 3B) suggested a consistent cell percentage viability over time implying that PBM does not negatively impact cell health instead maintains the overall health of cells. LDH results (Figure 3C) 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. As the cell viability was seen to been maintained over time. The slight LDH leakage identified may be explained by changes in cell membrane permeability due to trans-differentiation [17], or due to contact inhibition and nutrient depletion within the cell culture medium after an extended cell culture period [18].

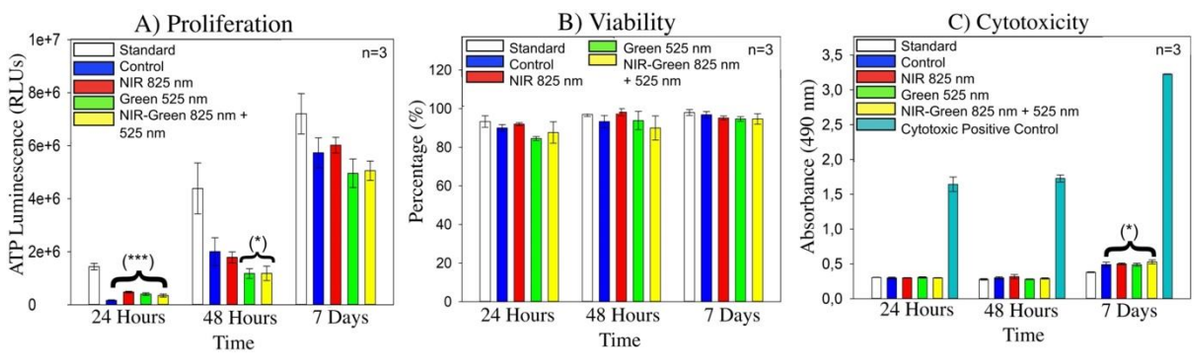

Figure 3. Biochemical Analysis of immortalized ADMSC differentiation at 24 hours, 48 hours, and 7 days post-PBM treatment.

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4. Discussion and Conclusion

Osteoporosis is the consequence of a decline in bone-forming mature osteoblast populations [19] 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 [20]. 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 [21]. 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 [22–24], however, limited studies have explored the outcome of combining PBM wavelengths to aid multiple cellular functions [25].

Flow cytometry analysis indicated a reduction in CD44 and CD166 immortalized ADMSC stem-ness protein markers at 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 trans-differentiation of immortalized ADMSCs. Wang et al. concluded their findings on the detection of trans-differentiated ADMSCs into osteogenic lineages with the aid of Green PBM after 7 days post-PBM treatment [14].

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 [26]. This change in cell morphology suggests cell differentiation of immortalized ADMSCs into osteoblasts.

Analysis of biochemical assays identified a statistically significant decrease in cell proliferation at 24 hours post-PBM treatment amongst all experimental groups. At 48 hours post-PBM treatment, a decreased cell proliferation occurred amongst the Green PBM and NIR-Green PBM experimental groups. However, the decrease in proliferation 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 [27]. 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 [24]. 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 [28]. 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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