Laser-induced differentiation of Adipose Stem Cells to neuron-like cells

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Abstract. Adipose Stem Cells (ASCs) possess better survivability and are capable of differentiation, which makes them an ideal choice for replacement therapies in clinics. Particularly, differentiating ASCs into neuron-like cells allow us finding solutions to maladies of Central Nervous System (CNS) for cell therapies based on patient's own genetic background. This study was performed using cells isolated from healthy human subjects undertaking abdominoplasty in clinics. Harvested cells were maintained in culture medium supplemented with 10% fetal calf serum and passaged repeatedly, while been characterized based on their surface protein markers CD44/90/133/166. Cultured cells were proliferated and induced to differentiate into neuron-like cells using Fibroblast Growth Factor, basic (bFGF) and forskolin in the presence of Near Infra-Red (NIR) lasers. Proportion of ASCs growing in the culture and capable of differentiation by induction was estimated as 6.3% based on the expression of CD90, a key stem cell marker. Exposure to 5, 10 and 15 J/cm^2 of NIR and growth factors for 14 days resulted in the initiation of differentiation of these ASCs into neuron-like cells (as revealed by the expression of nestin, an *early* neuronal marker). However, these neuron-like cells failed to express βIII-tubulin, a late neuronal marker under this regimen of differentiation. This indicates that the light energy can initiate differentiation of human primary ASCs to neuron-like cells. More such initiatives are essential for our understanding and standardization of light or lasers in altering cellular phenotype towards addressing the demand of neurons for autologous therapy.

1. Introduction

Lasers are produced when electrons within an active medium are stimulated from their ground stages and they emit radiation as return to their normal energy state. These emitted photons collide with other excited photons causing additional emission of energy. Cells absorb photons mainly by mitochondria, which are the cellular battery, where energy for biological activities are generated by the transport of electrons [1, 2]. The net result of this electron transfer is manifested as an increase in the mitochondrial consumption of oxygen for the synthesis of cellular energy, nucleic acids and proteins [3, 4]. Thus, the effect of lasers (light) on living cells and organs, referred as photobiomodulation (PBM), is capable of bringing morphological and functional changes to stem cells. PBM is a therapeutic modality were photons could stimulate and/or inhibit biological functions by altering cellular activities

[5, 6]. PBM involves exposure of intact or diseased cells/tissues to laser or light emitting diodes that can alter survivability, proliferative and migratory capabilities [7, 8].

Adipose-derived stem cells (ASCs) are located in the subcutaneous fat deposits and has the lowest amount of apoptosis [9]. Additionally, fat tissues from abdominal region has significantly more ASCs as compared to the hip and thigh regions with no variations in the growth rate and phenotype [10]. They are capable of differentiation in laboratory conditions, which make them the best choice for stem cell replacement therapies in clinics. In many occasions light along with biological and chemical agents can induce, lineage-specific differentiation of ASCs often harvested from the body fats of adult humans. However, our understanding of differentiating them to any specific-lineages under the influence of photons (light) is very limited. This study gives us indications on how the fate of primary human ASCs can be manipulated by light in favour of neuro-differentiation. Similar initiatives are vital for elucidating the role of physical forces like light on biological systems.

2. Methodology

Adult stem cells with the major role of repair and regeneration are the resident of many body organs including fat tissues. These adult stem cells were harvested from consented patients undergoing cosmetic surgery in hospitals and was obtained as a kind gift by Prof. Michael Pepper, University of Pretoria, South Africa. Briefly, the harvested lipoaspirate was washed thrice in 1x PBS (Phosphate Buffered Saline) and centrifuged to separate fat layer, blood cells and residues for the isolation of adult stem cells. Subsequently, the lipoaspirate was decanted onto sterile culture plates and subjected to enzymatic digestion using 0.1% collagenase Type I (Sigma, C9407-25mg). The sample was incubated for 45 minutes with agitation for the mechanical breakdown of adipose tissue at 37ºC in a 5% CO_2 incubator [11]. The digested adipose tissue was suspended in *complete medium* consisting of Dulbecco's Modified Eagle's Medium (DMEM) and 10% Foetal Bovine Serum (FBS). Finally, this solution was centrifuged to obtain the Stromal Vascular Fraction (SVF), which was seeded on to tissue culture flasks for propagation under standard conditions in the laboratory.

Table 1: Laser parameters applied on adipose stem cells for neuronal differentiation

Diode light source	**Near-infrared laser**
Wavelength (nm)	825
Power output (mW)	104
Power density (mW/cm^2)	11.45
Spot size (cm^2)	9.1
Emission	Continuous wave
Energy density(J/cm^2) = Irradiation time	05 J/cm^2 = 07 min 15 sec 10 J/cm^2 = 14 min 30 sec 15 J/cm^2 = 21 min 45 sec

Following harvest, $5*10^5$ cells were seeded onto 35 mm diameter culture dishes (Corning, 430165) in *complete medium* and incubated at 37°C with 5% CO_2. After 48 hours, cells were shifted to *neuronal differentiation media* consisting of DMEM supplemented with 1% FBS and 100 ng/ml Fibroblast Growth Factor, basic (bFGF) for a week and with 10 µM forskolin for another week [12]. Further, these cells were subjected to Low-Level Laser Irradiation (LILI) using a Near Infrared (NIR) diode laser of wavelength, λ=825 nm and

fluences at 5, 10 and 15 J/cm^2. Culture dishes (Corning, 430165) with the lids off were irradiated from above in the dark using laser parameters as mentioned in **Table 1**. LILI was repeated on every third day with media changes and the cells were analysed of neuronal differentiation on day 14th as well as day 21st. Cells treated under similar regimen of growth inducers with media changes and without laser irradiation (0 J/cm^2) served as baseline control.

Immunostaining combined with flow cytometry analysis offer a powerful tool to detect and quantify proteins specific to a cell type (markers). For immunostaining, cells were blocked by 5% goat serum to minimise non-specific binding and false positive signals. These cells were incubated with monoclonal antibodies specific for human neuronal markers at a dilution of 1: 200 for one hour. This was followed by the addition of a fluorochrome-conjugated secondary antibody at 1: 500 and incubation for 30 minutes in the dark. Flow cytometry and analysis with Accuri™ C6 (BD biosciences) quantifies both cell surface as well as intracellular markers. The immunostained cell suspension was drawn through the flow chamber into a stream created by surrounding sheath of isotonic fluid. A monochromatic beam of light intersects with these labelled cells and the photomultiplier tubes detected these signals corresponding to each flourochrome and recorded as events for analysis.

3. Results

The SVF obtained by enzymatic digestion of adipose tissue is an admixture of resident cells, which necessitates its identification by immunostaining. These cells were adherent to plastic surface of tissue culture flask and expressed ≥ 95% reactivity towards surface markers CD73/90/105 (standards set by the international society of cellular therapy). Upon seeding harvested cells attached to the tissue culture coated plastic surface for logarithmic expansion with a mean doubling time of 4-6 days [13]. This was followed by a stationary phase or a decline in growth due to senescence or death. After repeated subcultures, these primary cells were analysed by flow cytometry for the markers CD44/90/133/166 (**Table 2**). Based on the expression of CD90, a key stem cell marker, the percent of ASCs within these primary cells capable of differentiation by induction was estimated as 6.3%. Thus, the proportion of ASCs declined rapidly during the subculture of human primary cells in laboratory [14].

Table 2: Changes in adipose stem cells markers during propagation in *complete medium*

Stem cell markers	**Percent positive cells**
Anti-human antibody	Passage #8 after isolation
CD44	12.6%
CD90	6.3%
CD133	19.4%
CD166	56.9%

The biological effect induced by a light source or laser is explained by the changes in the rate transfer of electrons in mitochondria with production of oxide free radicals and reactive nitrite ions [2, 5]. Thus, light induces an increase in mitochondrial activity of cells, often manifested as an increase in cellular respiration and production of bioenergy [15, 16]. Red light and NIR specifically stimulates mitochondrial respiratory chain components resulting in changes to cellular Reactive Oxygen Species (ROS) and Adenosine Triphosphate (ATP). This marks the beginning of increased protein synthesis and changes to fatty acid metabolism, often manifested as proliferation and/or differentiation [17, 18].

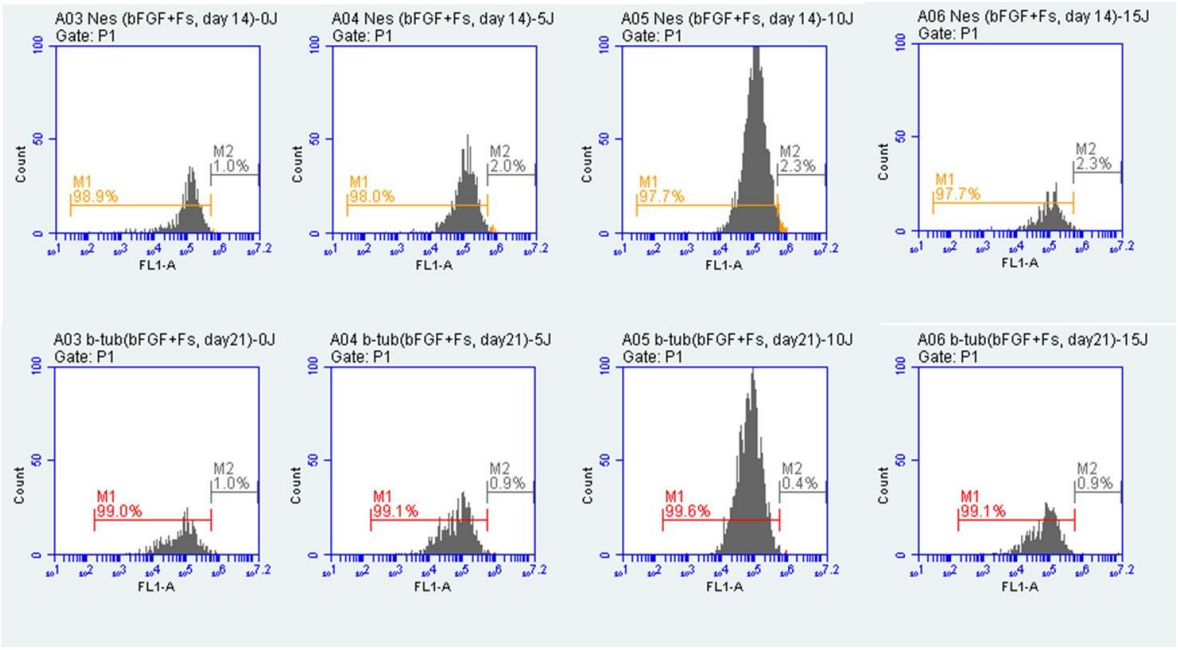

Figure 1. Expression of markers during neuronal differentiation: Harvested cells were maintained in *neuro-differentiation medium* with inducers [12]. Further, these cells were irradiated with 0, 5, 10 and 15 J/cm^2 of near infrared laser. Analysis of *early* and *late* neuronal markers in differentiating ASCs was performed by immunostaining and flow cytometry.

Analysis show that LILI induces neuronal differentiation of human primary ASCs in the presence of specific biological and chemical inducers. Neuronal differentiation was initiated by all fluences of NIR laser in the presence of growth factors (**Figure 1**). Nestin, an *early* neuro-marker is an intermediate filament protein expressed by the neuronal precursor cells, which localizes in growth cone and play a role in the axon elongation [19]. Irradiation with NIR fairly induced expression of nestin indicating the initiation of neuro-differentiation (**Figure 2**). However, these neuron-like cells failed to progress into mature or functional neurons expressing βIII-tubulin, a *late* marker. Tubulin is a major component of the cytoskeleton and exists as a heterodimer of two polypeptides, α and β in microtubules. The β-tubulin class III is expressed exclusively at the later stages of neuronal differentiation [20].

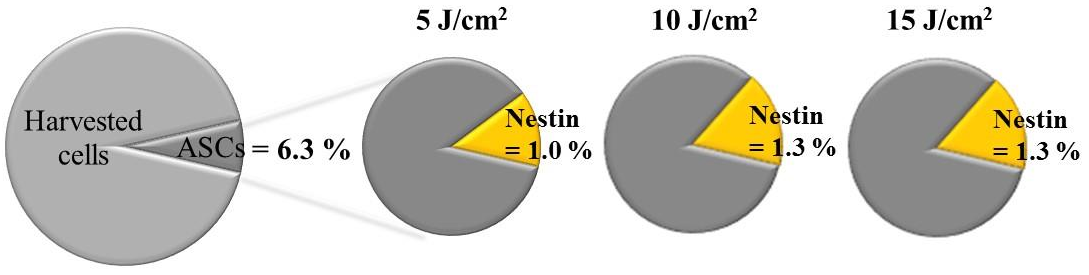

Figure 2. Laser-induced differentiation of ASCs to neuron-like cells: Treatment with NIR laser and growth inducers resulted in the differentiation of ASCs to neuron-like cells as revealed by the expression of nestin, an *early* neuronal marker. Percent of differentiating ASCs expressing CD90 (a key stem cell marker) within the harvested cells using 5, 10 and 15 J/cm^2 of NIR were normalized to untreated control (0 J/cm^2). *ASCs, Adipose Stem Cells.*

4. Conclusion

Lasers are polarized light with coherence that have additional biological benefits as compared to monochromatic light from a conventional light source or LED. Red light and NIR are absorbed by cytochrome C located in the inner mitochondrial membrane leading to the oxidation of NADH and $FADH_2$ in the respiratory chain. This is followed by changes in the rate of electron transfer for the production of ATP and formation of free radicals [21]. Altogether, light can lead to the increased expression of genes related to anti-inflammatory signalling, cell migration, proliferation and differentiation [22]. This study shows that light enhances differentiation of human primary ASCs to neurons-like cells. Therefore, lasers could be used as an adjuvant to biological and chemical inducers of neuro-differentiation.

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