

The effect of low level laser irradiation on adult human adipose derived stem cells

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Abstract This study investigated the effect of low level laser irradiation on primary cultures of adult human adipose derived stem cells (ADSC) using a 635-nm diode laser, at 5 J/cm² with a power output of 50.2 mW and a power density of 5.5 mW/cm². Cellular morphology did not appear to change after irradiation. Using the trypan blue exclusion test, the cellular viability of irradiated cells increased by 1% at 24 h and 1.6% at 48 h but was not statistically significant. However, the increase of cellular viability as measured by ATP luminescence was statistically significant at 48 h ($p < 0.05$). Proliferation of irradiated cells, measured by optical density, resulted in statistically significant increases in values compared to nonirradiated cells ($p < 0.05$) at both time points. Western blot analysis and immunocytochemical labeling indicated an increase in the expression of stem cell marker $\beta 1$ -integrin after irradiation. These results indicate that 5 J/cm² of laser irradiation can positively affect human adipose stem cells by increasing cellular viability, proliferation, and expression of $\beta 1$ -integrin.

Keywords Primary human adipocyte stem cells · 635 nm diode laser · Trypan blue test viability · ATP luminescence · $\beta 1$ -integrin differentiation expression

Introduction

Stem cells have the ability to self-renew, an extensive proliferative potential and an ability to give rise to one or more differentiated cell types [1, 2]. Due to their broad differentiation potential, stem cells are of particular interest from a therapeutic point of view. One may envisage a scheme in which a patient's own somatic stem cells from a particular tissue might be used in autologous cell therapy to replace tissue(s) of the same or different tissue [3]. However, before such therapies are able to be applied, further research is necessary to determine the differentiation potential of different stem cell types, the means by which to stimulate controlled differentiation and the stability and safety of the differentiated tissue(s).

Adipose tissue, like bone marrow, is derived from the mesenchyme and contains a supportive stroma that is relatively easily isolated. ADSCs have shown a rather impressive differentiation potential in vitro, from adipocytes, to osteogenic, myogenic, chondrogenic, and neurogenic lineages when treated with established lineage-specific factors [4]. Signals through cell–cell contact, extracellular matrix (ECM) proteins and other factors secreted from surrounding tissue are instrumental in triggering specific pathways that subsequently lead to differentiation. Many of these inducing factors have yet to be elucidated; however, known growth factors and signals are currently being researched in great detail to further define and understand their roles in tissue growth and differentiation. Stem cells can be identified by their expression of certain genes and proteins. One such protein expressed on surface membranes of stem cells is $\beta 1$ -integrin. $\beta 1$ -integrin is a known stem cell marker and has previously been shown to be expressed in adipose-derived stem cells [5, 6].

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Laser irradiation at different intensities has been shown to inhibit and stimulate cellular processes. Recent findings suggest that at the cellular level, laser energy of a particular wavelength can initiate signaling cascades, such as those that promote cellular proliferation [7]. Studies on low level laser therapy (LLLT) and stem cells have shown that low level laser irradiation increased migration of stem cells and suggests that LLLT could affect the metabolism of stem cells [8], which in turn, could also be indicative of increased cell proliferation.

Materials and methods

Isolation of adipose tissue

Adipose tissue from consenting donors undergoing abdominoplasty was used for the isolation of the adipose stem cells. Ethical approval in accordance with the Human Tissue Act 65, 1983, was obtained from the Academic Ethics Committee of the Faculty of Health Sciences, University of Johannesburg.

Adipose tissue was separated from the dermal layer using a scalpel and placed in a sterile beaker with Hanks Balanced Salt Solution (HBSS; Adcock-Scientific SA., P04-34500) containing 10,000 U/ml penicillin/streptomycin (Pan Biotec-GmbH, SA, PO6-07100) and 250 µg/ml fungizone (GIBCO, SA, 15290-026). The covered beaker containing the tissue was then stored at room temperature overnight protected from light. The adipose tissue was removed from the beaker and cut into 3- to 5-mm pieces using two scalpels. Equal volumes (12.5 ml) of the minced tissue and a collagenase solution containing 600 U/ml collagenase type-1 (240 U/mg, Pan Biotec-GmbH, SA, LS0004196), HBSS, and 5 M CaCl were then placed in 50-ml Falcon tubes, sealed with parafilm and incubated in a shaking incubator (labcon, Instrulab, SA) at 20×g for 80 min at 37°C. After incubation, equal volumes of complete medium, consisting of Dulbecco's Modified Eagle Medium (DMEM- (GIBCO, SA, 21331-020) supplemented with 10% fetal calf serum (FCS; deltabioproducts, SA, 14-501BI), 0.1% penicillin/streptomycin, and 1 µg/ml fungizone were added to the tubes, and inverted to mix. After centrifugation of the suspension at 300×g for 5 min at 20°C, the oil layer on the surface of the suspension was removed and discarded using a plastic Pasteur pipette. The infranatant was then removed, resuspended, and filtered through a 40-µm filter (BD Biosciences, SA, 352340). The resultant suspension was spun at 650×g for 5 min, and the supernatant was removed. Cell pellets from the same sample were pooled and resuspended in complete medium, and spun at 650×g for 5 min.

The supernatant was removed, and the pellets were resuspended in Erythrocyte Lysis Buffer [(ELB; NH₄Cl, KHCO₃, ethylenediaminetetraacetic acid (EDTA)] and incubated for 10 min at room temperature. The solution was spun at 650×g for 5 min and the pellet resuspended in complete medium. Using 75 cm² tissue culture flasks, in 75 cm² 2.5 ml of the cell suspension was added to 17.5 ml of complete culture medium. The cells were incubated at 37°C in a humidified atmosphere of 5% CO₂. ADSCs were observed in the flasks 24 h after incubation. The cells were grown to semi-confluence before passage, and the medium was changed once a week. Semi-confluent cells were subcultured in 3.3 cm diameter culture plates for laser irradiation.

Laser irradiation

Semi-confluent mono-layers of ADSC were irradiated in the dark at room temperature with 5 J/cm² at 635 nm using a diode laser (Oriol, USA). Laser irradiation was delivered to the culture plate of 3.3 cm diameter via an optical fiber with a spot size of 3.3 cm diameter covering the entire surface of the culture dish uniformly. On average, 50 mW of power output was measured, and this was calculated to take 15 min to deliver 5 J/cm² irradiation at a power density of 5.5 mW/cm². Nonirradiated control cells were kept under the same conditions. Both irradiated and nonirradiated samples were re-incubated at 37°C in a humidified atmosphere of 5% CO₂.

Cell morphology

Morphological observations using an inverted light microscope (Olympus CKX41) were performed at 24 and 48 h postirradiation on both irradiated and nonirradiated cells. A digital camera (Olympus C5060-ADUS) coupled to the microscope was used to document digital micrographs.

Cell viability

Trypan blue

Cellular viability was measured by using trypan blue [9]. Trypsin/EDTA (0.02–0.05 µg) cell suspensions of the culture ADSCs were used to measure cell viability through trypan blue and adenosine triphosphate (ATP) luminescence. A mixture of cell suspension (500 cells/µl) in complete medium (20 µl) and (20 µl) trypan blue reagent (Sigma, SA, 200-786-7) was incubated at room temperature for 5 min. Viable and nonviable cells were counted using a hemocytometer with Neubauer rulings using a light microscope (Olympus CKX41), and the percentage viability was determined. The percentage viability was calculated

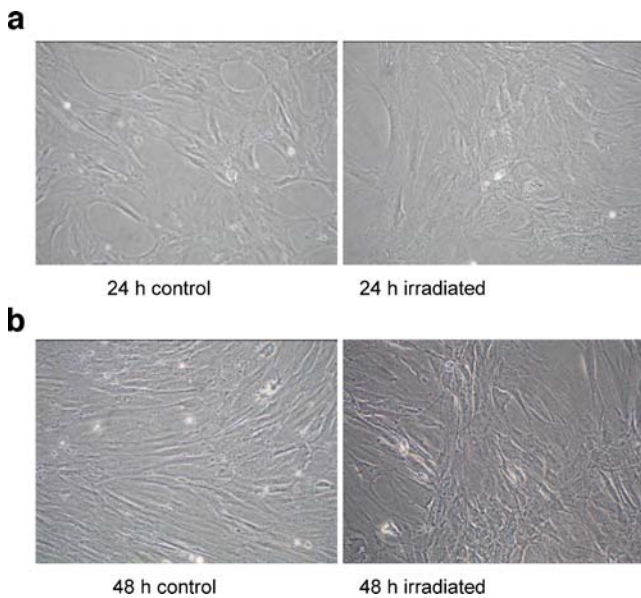


Fig. 1 Morphology of ADSCs. Monolayer of ADSCs 24 h (**a**) and 48 h (**b**) post irradiation showing typical smooth elongated cell shape. No discernable change was observed although there was an increase in cell number

by dividing the number of viable cells (translucent) by the total number of cells and multiplied by 100.

Metabolically active cell detection: adenosine triphosphate (ATP) luminescence

The cell Titer-Glo luminescent cell viability assay (Promega, SA, G7571) provides a homogeneous method for determining the number of viable cells in culture based on quantitation of ATP, which indicates the presence of metabolically active cells (Product information, Fact sheet # G757rev02). According to the manufacturer's protocol, a mixture of cell suspension (500 cells/ μ l) in complete medium (50 μ l) was mixed with equal volume of Glo reagent (1 ml buffer and 0.007 g substrate) and mixed on a vortex for 2 min to induce lysis. The mixture was incubated for 10 min at room temperature and read on a luminometer (Hygiena International, Pi-102, Germany).

Table 1 Cell viability

Time postirradiation (h)	Control (nonirradiated; %)	Irradiated (%)
24	96	97
48	97	98.6

Percentage cell viability using trypan blue ($n=6$) showed an increase in irradiated cells evaluated at 24 and 48 h compared to the nonirradiated. The increase was however not statistically significant.

Cell proliferation

Optical density (OD) was used to measure cell proliferation. A hundred microliters of cell suspension in complete medium (DMEM) was read at $A_{540 \text{ nm}}$ in a microplate reader (BioRad, Benchmark Plus Microplate spectrophotometer).

Protein expression

Expression of $\beta 1$ -integrin

Cells were seeded onto coverslips, by slowly pipetting 200 μ l of cell suspension (500 cells/ μ l) into 1.8 ml of complete medium in 3.3 cm diameter culture plates containing heat sterilized glass coverslips (22 \times 22 mm) (Deckglaser, Lasec, SA). The cells were allowed to attach to the coverslips and grow for 4 days to semi-confluence. The cultured cells were then irradiated. Cells cultured on the sterile glass coverslips were rinsed twice with ice-cold phosphate-buffered saline (PBS) bovine serum albumin (BSA)/azide buffer (PBS, 0.1% w/v BSA, Sigma Aldrich, SA, 9048-46-8; 0.01% w/v azide, BDH Lab supplies, UK, 103692K) and then incubated with $\beta 1$ -integrin (0.8 μ g/ml, 1:250; Whitehead Scientific Group, SA, Sc-9970) in PBS/azide for 30 min on ice. Cells were then rinsed three times with PBS BSA/azide buffer and incubated with the secondary fluorescent antibody (0.4 μ g/ml, 1:1,000, Goat anti-mouse IgG-Rhodamine; Whitehead Scientific Group, SA, Sc-2092) in PBS/azide for 30 min on ice, protected from light. Cells were rinsed three times as before and fixed in 3.7% formalin for 10 min. After fixation, cells were rinsed once briefly with PBS, and then once, with tap water before being stained with 4'-6-diamidino-2-phenylindole (DAPI, Sigma Aldrich, SA, D9564) and mounted on glass

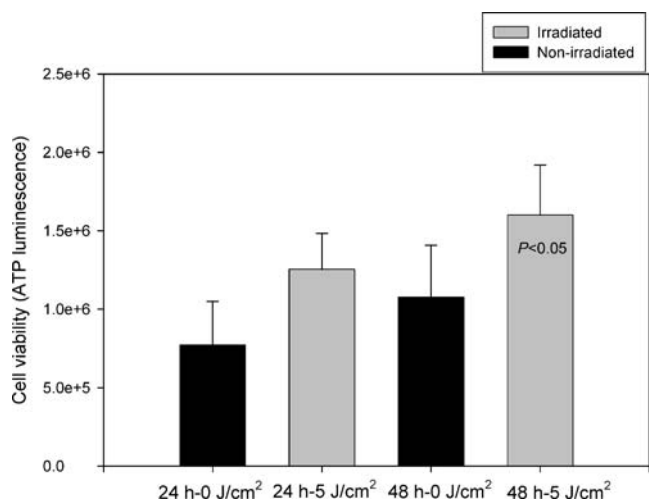


Fig. 2 Cell viability. ATP luminescence showed an increase in cell viability in irradiated cells compared to nonirradiated cells both at 24 and 48 h. The increase was statistically significant at 48 h ($P<0.05$)

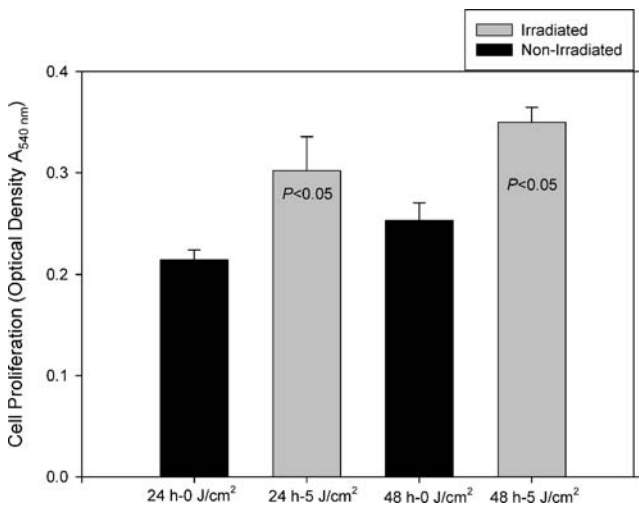


Fig. 3 Cell proliferation. Optical density assay ($n=6$) showed a statistically significant increase in cell proliferation in irradiated cells at 5 J/cm² compared to the nonirradiated cells ($P<0.05$) at 24 and 48 h postirradiation

slides. The slides were viewed through a fluorescent microscope (Olympus BX41).

Western blotting

Cultured cells were lysed in lysis buffer (equal volumes of HBSS and sample buffer-2 M Tris (hydroxymethyl) amino-methane pH 6.8, BioRad, SA, 161-0719; 2% sodium dodecyl sulfate (SDS), BioRad, SA, 161-0302; 100% glycerol, Separation Scientific, SA, 56-40-6 and H₂O) on ice. Cell extracts were sonicated, protein was determined using Bicinchoninic Acid Protein Kit Assay (BCA™, Pierce, USA, 23228) [10].

Ten microgram of protein was loaded in each lane. After SDS-polyacrylamide gel electrophoresis, proteins were transferred to polyvinylidene difluoride membranes, 0.2 μm, (Immunoblot PVDF membrane, BioRad, SA, 162-0177). Membranes were blocked overnight in blocking buffer containing Tris buffer saline (TTBS—50 mM Tris; 150 mM NaCl, Separation Scientific, SA, 7647-14-5) containing 0.1% Tween 20 and 5% nonfat milk. This

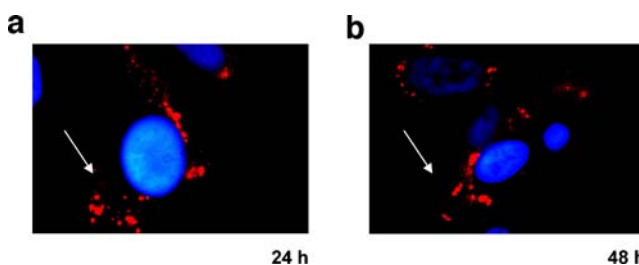


Fig. 4 β1-integrin expression. Postirradiation surface expression of β1-integrin (red) in ADSCs. Localization of β1-integrin on the cell surface is shown by the arrows on both diagrams (a) and (b). Nuclei are counter stained with DAPI (blue)

reduced background and prevented binding of the primary antibody to the membrane [11]. The membranes were then incubated in primary antibody (2 μg/ml, 1:100, β1-integrin, Whitehead Scientific Group, SA, Sc-9970) diluted in blocking buffer (as above) at room temperature for 1 h. The membranes were washed in TTBS and then incubated in secondary antibody (0.2 μg/ml, 1:1,000, goat anti-mouse horseradish peroxidase, Whitehead Scientific, SA, sc-2005) diluted in blocking buffer (as above) at room temperature for 2 h.

The membranes were washed as before and incubated in chemiluminescent substrate (SuperSignal West Pico, Pierce, USA, 34080) for 10 min protected from light. The blots were then exposed to X-ray film (Kodak MXG, Rockester, USA, 326052) for 2 and 4 min. The films were developed and then viewed.

Statistical analysis

All laser irradiation experiments were performed at least six times ($n=6$). Statistical analysis was performed using Sigma plot 8.0 software. Differences between groups were determined using the Student *T* test for each independent variable (viability, proliferation, protein expression).

Results

Cell morphology

A single layer of smooth elongated fibroblast-like cells were observed as shown in Fig. 1. The observation revealed no difference between the irradiated and the control cells at both 24 and 48 h.

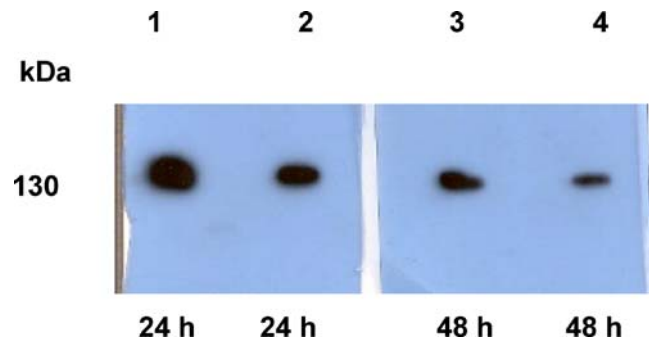


Fig. 5 Western blot analysis of the expression of β1-integrin in irradiated and nonirradiated cells at 24 and 48 h postirradiation. Expression of β1-integrin appears to be higher in irradiated cells compared to nonirradiated cells. Lanes 1 irradiated 24 h; 2 non-irradiated 24 h, 3 irradiated 48 h, and 4 nonirradiated 48 h

Cell viability

Trypan blue indicated an increase in percentage viability in cells that were irradiated compared to nonirradiated cells at 24 and 48 h; however, the difference did not prove to be statistically significant (Table 1). The increase in viability may indicate a stimulatory effect of laser irradiation. ATP luminescence showed that there was a statistically significant increase in ATP concentration at 48 h ($p < 0.05$) in irradiated cells when compared to nonirradiated cells at 24 and 48 h as shown in Fig. 2.

Cell proliferation

Irradiated cells both at 24 and 48 h showed a statistically significant increase ($p < 0.05$) in OD compared to their respective controls (Fig. 3)

Protein expression

Immunocytochemical live cell labeling showed the expression of $\beta 1$ -integrin in irradiated cells (Fig. 4). Western blot analysis showed an increase in $\beta 1$ -integrin expression in cells irradiated at 24 and 48 h (Fig. 5).

Discussion

To our knowledge, this is the first study investigating the effect of low level laser irradiation on human ADSCs in vitro. Adipose tissue contains an abundant, accessible source of adult stem cells, and the stem cells present from this tissue are termed adult adipose derived stem cells [12, 13]. ADSCs have been shown to be able to differentiate into bone, fat, cartilage, neuron, and smooth muscle when treated with specialized induction media in vitro [14, 15]. Once differentiated, these cells could potentially be used in stem cell therapy to replace or repair damaged tissues and organs. A number of disorders that are amenable to this approach include neurological, cardiovascular diseases, and bone defects and diabetes [15]. In this study, ADSCs were derived from adipose tissue, and the effect of low level laser irradiation was evaluated in vitro at 24 and 48 h postirradiation.

LLLT has been shown to have a variety of biostimulatory effects such as wound healing [16, 17], fibroblast proliferation [18–20], nerve regeneration [21], and collagen synthesis [22]. Studies on LLLT and stem cells have shown that LLLT increases migration of stem cells [8], but as yet, there have been no studies on the effect of LLLT on ADSCs.

In this study, we found that LLLT increased cell viability, cell proliferation, and the expression of $\beta 1$ -

integrin in ADSCs. Cell viability increased at both 24 and 48 h in irradiated cells compared to nonirradiated cells. Although the trypan blue exclusion assay did not show a statistically significant increase in viability, the more sensitive and reliable ATP luminescence assay showed a significant increase 48 h after irradiation.

Optical density measurements at both 24 and 48 h indicated an increase in cell proliferation in the irradiated cells. Proliferation was greater after 48 than at 24 h. This is in agreement with the results of Hawkins and Abrahamse [23], who found that there was an increase in cellular proliferation of human skin fibroblast cells (WS1) when treated with a Helium Neon laser at the same dosage used in this study and wavelength of 632.8 nm.

In addition, this study found that $\beta 1$ -integrin showed an increased expression in irradiated cells, and the expression was greater after 24 h. $\beta 1$ -integrin is a cell surface marker for ADSCs [24]. Immunocytochemical live cell surface labeling also confirmed the expression of $\beta 1$ -integrin at 24 h postirradiation.

We conclude that low level laser irradiation at 5 J/cm², a power density of 5.5 mW/cm² and a wavelength of 635 nm can positively affect ADSCs in vitro by increasing cell viability, cell proliferation, and the expression of $\beta 1$ -integrin. We therefore suggest that low level laser irradiation could enhance the viability, proliferation, and the maintenance of the stem cell properties of ADSCs in culture, therefore, not only expanding the number of the stem cells, but also avert premature differentiation of these cells into other tissue types. By preventing spontaneous differentiation in vitro, the ADSCs could possibly be cultured for longer periods without the loss of their stem cell characteristics. This, in turn, would also allow for further studies to be conducted into the stem cell dynamics of ADSCs, as well as their differentiation potentials, further aiding the development of future stem cell treatments and therapies.

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