Visible 532 nm Laser Irradiation of Human Adipose Tissue-Derived Stem Cells: Effect on Proliferation Rates, Mitochondria Membrane Potential and Autofluorescence

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Introduction

Adipose tissue-derived stem cells (ADSCS) show significant promise in a variety of regenerative medicine applications because of their multipotent differentiation potential [1] as well as their secretion of bioactive factors that promote healing. Recent work [2,3] discussed the therapeutic potential of adult stem cells to regenerate injured or diseased tissues via migration to injury sites and secretion of soluble biofactors that enhance tissue regeneration, and stimulate proliferation and migration. These cells exhibit extensive self-renewal capacity, due to the ability to undergo asymmetric cell divisions. They are also able to exist in a mitotically quiescent form [4,5]. In vitro studies indicate that mesenchymal stem cells (MSCs) can be expanded and terminally differentiated into osteoblasts, chondrocytes, and adipocytes in response to suitable stimuli and/or microenvironments [6].

Low-level laser therapy has become a clinically accepted tool in regenerative medicine. This is because low energy laser irradiation with output powers in the milliwatt range modulates biological processes in living cells both in vitro and in vivo with a variety of beneficial effects attributed to photostimulation of cellular processes [7–11]. However, there are conflicting reports about detailed effects of low energy laser on cell proliferation, especially visible laser light on cells in culture. A range of studies have shown that low power laser produces significant and often desirable effects on irradiated cells. These include altered gene expression and anti-inflammatory effects, enhanced proliferation rates and motility, protein secretion, stimulation of calcium influx and mitosis rate, and activation of ATPase enzyme [12–15].

The literature concerned with investigating the effects of laser light on stem cells is quite limited. Liat et al. [16], found that 632.8 nm He–Ne laser at 10 mW power irradiation can stimulate the osteogenic phenotype of MSCs seeded in a three-dimensional biomatrix. Hou et al. [14] found that irradiation of bone marrow stem cells using a 635 nm diode laser at 60 mW power activated cellular...
laser light on human adipose derived stem cells
biostimulating effects of low power 532 nm Nd:YAG
dated. In order to fill this gap, our study focuses on the
induced using an LED at the wavelength 570 nm [23].
blasts and increased chicken fibroblast proliferation was
length was used to induce biostimulation of dermal fibro-
activity in rat myocardial cells, while absorption of green
band light by fibroblasts lead to DNA synthesis activation
[22]. Frequency-doubled Nd:YAG laser at 532 nm wave-
length was used to induce biostimulation of dermal fibro-
and increased chicken fibroblast proliferation was
using an LED at the wavelength 570 nm [23].

Despite these earlier studies, the effects of 532 nm
green laser light on human stem cells have yet to be eluci-
dated. In order to fill this gap, our study focuses on the
biostimulating effects of low power 532 nm Nd:YAG laser light on human adipose derived stem cells in vitro.
This was achieved by quantifying proliferation rates of stem cells after irradiation using the MTT assay. We have
also quantified autofluorescence of living cells at the emis-
sion ranges of NADH and flavins as indicators of intracel-
lar metabolic changes. We also quantified mitochondria
membrane potential after irradiation by using JC-1 indi-
cator dye.

Mitochondria under physiological conditions play a key
role in the maintenance of normal cellular metabolism
and it is generally accepted that the mitochondria are the
initial sites for interaction of light in cells. The light-in-
duced biostimulation effects in cells occur mainly through
the activation of the mitochondrial respiratory chain and
the initiation of cellular signaling [24–26]. Kssak et al.,
reported that mitochondrial alteration in isolated mito-
chondria from rat heart was observed after low power
green laser (532 nm, 30 mW) irradiation. The prolifera-
tion rate using the MTT assay as well as the mitochondri-
al membrane potential were significantly higher in
irradiated cells [27].

Mitochondrial membrane potential has been used as a
marker for mitochondrial activity in different cells [28].
We employed the most common probe used to measure
changes in mitochondria membrane potential, the cationic
dye JC-1. The accumulation of JC-1 in mitochondria is de-
pendent on the membrane potential. At low membrane
potential the dye remains in a monomeric form where it
exhibits green fluorescence at 525 nm. High membrane
potential leads to increased accumulation in the mito-
chondria where the dye forms J-aggregates whose fluores-
cence is red-shifted to 590 nm [29–31].

Intracellular autofluorescence is due to numerous en-
dogenous fluorophores present in cells. In this study, we
focus on reduced nicotinamide adenine dinucleotide
(NADH, NADPH) in the cytoplasm and mitochondria,
which serves as a coenzyme and a principal electron
donor within the cell for both oxidative phosphorylation
and glycolysis. Fluorescence emission of NADH is
detected in the region of 450 nm with excitation at
366 nm. Another important fluorophore is flavin adenine
dinucleotide (FAD), which exists mostly as cofactors of
enzymes involved in the redox reactions [32,33]. Fluores-
cence detected in the region of 540 nm after excitation at
460 nm measures the cellular levels of the flavoproteins,
lipomide dehydrogenase, and electron transfer flavopro-
teins [34–36]. Thus NADH and flavin autofluorescence
provides insights into the metabolic activity and redox poten-
tial in cells.

MATERIALS AND METHODS

Cell Culture

StemPro human adipose derived stem cells (ADSCs)
(Invitrogen, Sydney, Australia) were used in the experi-
ments. These cells are isolated from human adipose
tissue through mechanical and enzymatic digestion.
Before cryopreservation, the ADSCs are expanded for one
passage in MesenPRO RS medium (Invitrogen). After
thawing, cells were plated into 75 cm² tissue culture
flasks and cultivated with MesenPro RS basal medium
supplemented with 2% (v/v) MesenPro growth supple-
ment, 1% (v/v) L-glutamine (2 mM), 1% (v/v) antibiotic–
antimycotic mixture. Cells were incubated at 37 C, 95%
humidity, and 5% CO₂. Cells were subcultured by trypsi-
nization when they reached 80% confluence. Subculturing
procedure was carried out under aseptic conditions.
The MesenPro medium was aspirated from cells and the sur-
face of cells layer was rinsed with Dulbeco Phosphate
Buffer Saline (DPBS). For cells detachment, prewarmed
TrypLE (0.5 ml/10 cm²) was added and incubated at 37 C
for 7–9 minutes to achieve more than 90% cell detach-
ment. Equivalent of twice the volume used for TrypLE
of prewarmed MesenPro medium was added and the cells
were transferred to a 15 ml conical tube, centrifuged at
210g for 5 minutes at room temperature, followed by
removal of supernatant and re-suspension of the cell
pellet in a minimal volume of prewarmed medium. The
viability of cells was determined using trypan blue exclu-
sion test. The viability of cells was observed to be at least
90% for each subculturing. The seeding density of cells
was approximately 5,000 cells/cm².

Flow Cytometry Assay

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centrifuged cells was suspended in a minimal volume of DPBS. Cells were incubated with appropriate volumes of anti-human monoclonal antibodies (CD14, CD29, CD44, CD45, CD90, and CD105) conjugated with fluorescence dyes (PE, FITC, PerCP-Cy 5.5; eBioscience.com), then fixed at 3.7% paraformaldehyde for 20 minutes at room temperature. Cells were washed with PBS to remove fixing solution and resuspended with PBS for flow cytometry.

**Laser Irradiation Procedure**

A diode pumped solid state (DPSS) second harmonic generation Nd:YAG Laser (Viasho, China) was used in this study. This system is operated at the continuous wavelength of 532 nm and an adjustable power output from 0 to 150 mW. Power output was kept constant at 30 mW in all irradiation experiments. All irradiation experiments were done inside a class II biosafety hood under aseptic conditions. Cells from the third passage were harvested by trypsinisation as above, washed twice and suspended in the medium. Cells were transferred by pipetting into five groups of 0.5 ml Eppendorf tubes. Each cell group was exposed to laser light using exposure time of 30, 45, 60, 180, and 300 seconds. Each cell group irradiated for a fixed exposure time was subdivided into 24 replicates. During irradiation the laser was placed vertically on top of the tubes. The distance between the laser aperture and the target surface was 10 cm producing a laser spot of 5 mm diameter, matching the Eppendorf tube. The power density was calculated to be 0.153 W/cm² and the energy densities for each exposure time were calculated to be 5, 6.8, 9.2, 28, and 45 J/cm² for 30, 45, 60,180, and 300 seconds, respectively. Prior to each irradiation, the cells were shaken well to break up agglomerates. The control groups received no laser light but they were exposed to the same environment for the same duration as the laser irradiated groups. The non-irradiated cells were also divided into the same number of replicates, as a control. All groups and replicates were cultured in wells of a 96 well cell culture plate and incubated at 37°C in a humidified atmosphere of 5% CO₂ for 48 hours.

**Autofluorescence Experiments**

After 48 hours of incubation, the autofluorescence properties of irradiated and non-irradiated cells were measured using a Spectra MAX M5 Multi-Mode Microplate Reader (Molecular Devices). Prior to autofluorescence measurements the media was replaced with 1X phosphate buffer saline to avoid the fluorescence from medium. The software of the Microplate reader system was set on fluorescence mode to measure the emission intensity at 450 nm after excitation with 366 nm, and emission intensity at 540 nm after excitation with 460 nm. We have chosen these excitation and emission parameters corresponding to the spectral properties of two important fluorophores in the respiratory chain, NADH (excitation wavelength: 366 nm, emission wavelength 450 nm) and FAD (excitation wavelength: 460 nm, emission wavelength: 540 nm).

**Mitochondria Membrane Potential Experiments**

The previously irradiated cells were first incubated for 48 hours after irradiation. Subsequently, the JC1 stain from (Invitrogen) was used for labeling stem cells at 2 μM concentration followed by incubation for 20 minutes at 37°C. Labeled cells were imaged using a Leica SP2 confocal microscope. Each group of control and irradiated cells were imaged using 488 nm excitation wavelength and the emission signals were obtained through 525 and 590 emission channels at the same parameters of pin-hole aperture and detector voltage. The ratio between red to green fluorescence was measured using ImageJ program.

**Data Analysis and Statistics**

The purpose of our statistical analysis is to conclude, at the significance level of 5% (95% confidence) that there are differences in the optical absorption measured in the MTT assay or autofluorescence intensity between irradiated and non-irradiated stem cells. In our study, we have five classes of data corresponding to 30, 45, 60, 180, and 300 seconds exposure time, each having a corresponding non-irradiated control group. Each measurement of autofluorescence and/or viability was carried out in independent 24 replicates, so the sample size in each class was 24. Each sample was checked by using a statistical Lilliefors’s test of normality, which was positive at a significance level of 0.01, thus distributions were not normal. This could be related to the existence of subpopulations within the examined cells. Similarly, the Bartlett’s test of equal variances produced a negative result, which means that there was only a very small probability of the same variance. For the data analysis we have therefore applied the Kruskal–Wallis test, which does not assume normal distributions. This test compares the medians of the groups, and returns the P-value for the null hypothesis that all
samples are drawn from the same population. For additional verification, we used a non-parametric Kolmogorov–Smirnov test of different source distributions. This test is based on a minimum distance estimation used to test the equality of one dimensional probability distributions and can be used to compare two samples by the quantification of distance between sample empirical distribution functions. When testing two samples the null hypothesis states that the samples are drawn from the same distribution [37]. The Kruskal–Wallis and Kolmogorov–Smirnov tests were applied to the 24 replicates in each class comparing the (empirical) fluorescence or absorption intensity distributions for irradiated cells to the same quantities in the control cells that were not exposed to laser light.

RESULTS

Effect of Laser Radiation on Proliferation Rate of Cells

The MTT assay was employed to determine if the exposure times affected proliferation of cells incubated for further 48 hours. Cell viability measured in this assay was analysed following irradiation by the 532 nm laser for varying exposure times (30, 45, 60, 180, and 300 seconds). These and all other data have been plotted using box plots [29]. The diagrams in Figure 1a represent the absorption intensity at 570 nm wavelength for all five exposure times for both irradiated and control groups.

The results in Figure 1a indicate a very significant effect of laser irradiation on proliferation rate, and hence viability. For 30, 45, and 60 seconds exposures the proliferation rates increase while the cells irradiated for 180 and 300 seconds showed considerable and very significant decreases in proliferation rate.

These data were analysed by applying the Kruskal–Wallis test for the difference in the medians to each pair of 24 replicates for each class. The results are shown in Figure 1b where we have plotted the obtained P-values ($P_{KW}$). Applying the Kolmogorov–Smirnov test (testing if the underlying distributions are different) produced the values $P_{KS}$ also shown in this figure.

Our statistical analysis (Fig. 1b) showed that our results are statistically very robust and that all classes of cells have significantly modified proliferation rates over the control cells (at 5% significance level).

Effect of Laser Radiation on Autofluorescence

Figure 2 shows the autofluorescence intensity of cells following irradiation by the 532 nm laser for varying exposure times (30, 45, 60, 180, and 300 seconds). Each diagram in this box plot represents the emission intensity at 450 nm after excitation with 366 nm wavelengths for each exposure time. In this spectral region autofluorescence is related to NADH.

A significant increase in autofluorescence emission can be observed for cells irradiated for 30 and 45 seconds, compared to non-irradiated cells. On the other hand a significant decrease in autofluorescence is observed for the 300 seconds exposure.

The box plot in Figure 3 shows the autofluorescence properties of cells 48 hours after irradiation by 532 nm laser at 30, 45, 60, 180, and 300 seconds exposure times in the spectral area where flavins are detected. Each diagram represents emission intensity at 540 nm under 460 nm excitation, where FAD makes a significant contribution.

As previously (Fig. 1b), these data were analysed by applying the Kruskal–Wallis and Kolmogorov–Smirnov tests.
tests. The results are shown in Figures 2b and 3b. The data shown in Figure 2b indicates that autofluorescence at 366 nm excitation increases upon laser irradiation after 30 seconds exposure and it decreases after 300 seconds exposure. Figure 3b for 450 nm excitation confirms the same trend. There is clear evidence of autofluorescence increase at 30 and 45 seconds, and a clear decrease at 300 seconds exposure.

Effect of Laser Radiation on Mitochondria Membrane Potential

The data in Figure 4 illustrate the effect of laser irradiation at different exposure times on mitochondrial membrane potential of stem cells reflected in red/green fluorescence ratios of JC-1 dye. Significant increase ($P < 0.05$) in the red/green fluorescence ratio can be observed at 30 and 45 seconds exposure times compared with the control group. This indicates an increased membrane potential at short exposure time laser irradiation while a significant decrease ($P < 0.05$) in red/green ratio and membrane potential can be observed at long exposure times at 60, 180, and 300 seconds.

Confocal laser scanning microscopy images of control stem cells and irradiated cells are illustrated in Figure 5. The fluorescence emission of stem cells stained with JC-1 was recorded through two emission channels in confocal microscopy: 525 $\pm$ 10 nm (green) and 590 $\pm$ 10 nm (red). As also shown in Figure 4a clear increase in red to green emission intensity can be observed at 30 and 45 seconds laser exposure times comparing with control group, while the intensity ratio between red to green decreased at longer times of irradiation.

Correlation Between Autofluorescence, Proliferation Rate, and Mitochondrial Membrane Potential

In order to establish the correlation between proliferation rates and autofluorescence characteristics, we subtracted the median value of each of the control groups from the irradiated data (control baseline removal) for the 366/450 nm emission, and separately for 460/540 nm autofluorescence data. The same procedure was applied to the MMT data. We then calculated the correlation between auto fluorescence and MMT proliferation/viability. The correlation coefficients and associated $P$-values that give significance to the test for correlation are shown in Table 1. These results show a very significant correlation between cell proliferation rates and cellular autofluorescence.

We also present the correlation plots between the autofluorescence at 366 nm and the mitochondrial potential as measured by JC-1 red to green fluorescence ratio (Fig. 6a) and between the MMT proliferation index and the mitochondrial potential (Fig. 6b). These parameters are highly correlated as indicated by the correlation coefficients which are close to unity (see Fig. 6) and very low $P$-values.

DISCUSSION

The photobiological interaction between laser light and living cells depends on parameters such as wavelength, fluence, power density, and exposure time. The influence of these parameters has been shown in many types of cells, including the few studies concerning the
biostimulation effects of green lasers on living cells [38]. The wavelength of laser irradiation is a crucial parameter in biostimulation, since the photobiological effects of laser light is based on the light absorption by primary endogenous chromophores (mitochondrial enzymes, porphyrins, flavins, and cytochromes) [39].

In our study, we investigated the stimulating effect of green Nd:YAG laser at 532 nm wavelength on the proliferation and mitochondrial activity of adipose derived stem cells in correlation with intracellular autofluorescence. We found no other reports in the literature concerning the biostimulation effect of 532 nm laser on adipose derived stem cell as well as detection autofluorescence after irradiation.

We selected the MTT assay to confirm our hypothesis that green laser light has an effect on the mitochondrial activity and cellular proliferation. The reduction reaction in the MTT assay is attributed mainly to mitochondrial enzymes and electron carriers and it is regarded as an indicator of cell redox activity [28]. When the concentration of MTT in the cytoplasm is increased, more formazan products are formed and higher absorption spectrum by cells is detected, indicating greater cell viability [40]. In experiments where cell numbers vary significantly the MTT metabolic assay mainly reflects the changes in cell number while variations of mitochondrial biochemistry make a limited contribution. Cell proliferation is a very important physiological effect for low power laser irradiation. Increased proliferation following laser irradiation in many cell types in vitro including, endothelial cells, fibroblasts, osteoblasts, and stem cells [25,41–44]. The results and statistical analysis in Figure 1 show that laser light stimulates the cells’ proliferation significantly at short exposure times, 30 and 45 seconds. This may be due to increasing the activity of respiratory chain components that absorb laser light at 532 nm wavelength. This increased activity could be followed by increased ATP levels resulting in increased proliferation. Absorption of light by respiratory chain components can increase mitochondrial membrane potential leading to increased energy availability and signal transduction. These cellular changes lead to macroscopic effects such as increased cell proliferation. The reverse effect was observed at longer exposure times (180–300 seconds), which may be due to damaging thermal effects.

![Fig. 3. Autofluorescence emission intensity of cells at 540 nm under 460 nm excitation, 48 hours after irradiation by 532 nm laser at 30, 45, 60, 180, 300 seconds exposure times. a: Box plots of the data (24 replicates) in each of the five classes and the corresponding controls. b: P-values for the statistical tests, $P_{kw}$ (red) and $P_{ks}$ (black). The blue line marks the value of $P = 0.05$. Laser irradiation produced statistically valid changes to autofluorescence for exposure times with $P$-values below 0.05.](image1)

![Fig. 4. Red to green fluorescence ratio of irradiated stem cells at different exposure times.](image2)
The results of measuring mitochondrial membrane potential using JC-1 gave an indication that the cellular mitochondria were affected directly by laser irradiation leading to an increase or decrease in the cellular metabolic activity depending on laser irradiation dose. As with proliferation rate results we found that the mitochondrial activity increased at shorter laser irradiation exposures (30 and 45 seconds). The mitochondrial membrane potential is a sensitive indicator of the condition of the mitochondria and therefore the whole cell [45]. In our results, the data in Figure 4 showed a significant increase in the mitochondrial membrane potential at short exposure times (30, 45 seconds). This correlates with the results of cells proliferation in Figure 1. High mitochondrial membrane potential means increased efficiency of proton pumps transporting charge through mitochondrial membranes leading to a greater electrochemical gradient that is used for ATP synthesis [28].

The results of our study indicated that 532 nm laser light can induce changes in the intracellular autofluorescence properties in the irradiated cells. We verified this by measuring the intracellular autofluorescence of two important components of the respiratory chain in mitochondria, NADH and flavins. We demonstrated a significant increase in the intracellular autofluorescence of cells at the emission range of NADH (Fig. 3) and flavin (Fig. 4). In interpreting the results one needs to take into account that NADH and flavin molecules are both involved in the redox reactions of the inner mitochondrial membrane and consequently, the oxidation state of NADH and FAD may

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change, producing alterations of the fluorescence intensities [46]. Previous studies have shown that the autofluorescence of nicotinamide adenine dinucleotide (NADH) can be used to monitor the metabolic state of living tissues in various species [47]. Highly significant correlation between proliferation and autofluorescence emission of NADH as well as FAD can be seen in our results (Table 1) and it seems that the correlation coefficient for NADH is higher than for FAD. This agrees with earlier reports that the effect of low power laser on cells is related to an increase in mitochondrial oxidation metabolism caused by excitation of respiratory chain components [48].

Various mechanisms for the effect of low intensity laser irradiation have been proposed, including absorption of light by mitochondrial enzymes with localized heating, photon absorption by flavins and cytochromes in the mitochondrial respiratory chain effecting electron transfer [25]. A mechanism by which low intensity lasers may induce biostimulation of cell activity has been well described by Karu [49,50]. Laser irradiation is postulated to intensify the formation of a transmembrane electromechanical proton gradient in mitochondria [50]. Karu proposed a chain of molecular events starting with the absorption of light by photoacceptor (chromophore), which leads to signal transduction and amplification, and finally results in photoresponse [51]. In this way light is absorbed by components of the respiratory chain, which causes an activation of the respiratory chain and oxidation of the NAD pool, which leads to changes in the redox status of both the mitochondria and the cytoplasm. This in turn has an effect on membrane permeability/transport, which changes in the Na\(^+\)/H\(^+\) ratio and increases ATPase activity, which in turn has an effect on the Ca\(^{2+}\) flux. The Ca\(^{2+}\) flux affects the levels of cyclic nucleotides, which modulates DNA and RNA synthesis and these, in turn, modulate cell proliferation and protein synthesis [52]. Our results are in agreement with this model.

Enhanced proliferation and mitochondrial activity of cells observed in this work might be a consequence of minute amount of reactive oxygen species (ROS) generation upon irradiation with low laser doses at short exposure times. As the cellular redox state has a key role in maintaining the viability of the cell, the redox changes tend to be reflected in cell activity and proliferation. It is known that low power laser irradiation produces a shift in the overall cell redox potential and the cytosolic response to this change involves several transcription factors including the nuclear factor B(NF-B) which is significant for cell growth [53,54]. Several studies regarding generation of ROS within the cells following low power laser irradiation found that laser light between 400 and 500 nm may produce ROS via a photochemical process involving flavins, and ROS may be also produced in the mitochondria at longer wavelengths [55].

Our results showed that biostimulation effect of laser light occurred mainly using 30 and 45 seconds exposure times corresponding to 5 and 6.8 J/cm\(^2\) energy densities. These values are in agreement with Hawkins and Abrahams [56] results who found that helium–neon laser irradiation of 5 J/cm\(^2\) resulted in an increase in cellular proliferation of human skin fibroblast cells. Muvla et al. [18], found that low power laser light increased cell viability and proliferation at 24 and 48 hours at 5 J/cm\(^2\) energy density.

CONCLUSIONS

Significant differences were observed in the autofluorescence properties of stem cells following irradiation by 532 nm laser using different exposure times at 0.15 mW/cm\(^2\). This is predominantly seen in autofluorescence emission at 540 nm related to NADH and the mitochondrial membrane potential. The correlation coefficients and the P-values are shown in the figure.
REFERENCES


