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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Background and Objective: The photobiological effect of laser light on cells and tissues originates from light absorption by endogenous chromophores and hence it depends on the wavelength of light source and cell type. Earlier studies regarding the biostimulation effects of green laser light investigated a wide variety of cells but not adipose tissue-derived stem cells (ADSCS). In this study we reported the *in vitro* effect of 532-nm Nd:YAG laser on proliferation, mitochondrial activity of these mesenchymal stem cells (MSCs) on the autofluorescence emission at wavelengths associated with nicotinamide adenine dinucleotide (NADH) and flavoproteins.

Materials and Methods: ADSCS were exposed to 532 nm second harmonic generation laser light at moderate power density (0.153 W/cm^2) for periods of 30, 45, 60, 180, and 300 seconds. Mitochondrial membrane potential was measured using JC1 stain and confocal laser scanning microscopy, cell proliferation rates, and cellular autofluorescence emission at 450 and 540 nm wavelengths were measured using micro plate spectrofluorometer 48 hours after irradiation.

Results: Shorter (30–60 seconds) exposure times led to significantly increased proliferation, attributed to increased mitochondrial activity ($P < 0.05$). At longer exposures we observed a significant decrease in proliferation and autofluorescence ($P < 0.05$). Strong correlation was observed between proliferation rates of cells and autofluorescence intensity.

Conclusion: Our results show that autofluorescence of the respiratory chain components and key autofluorescent metabolites offers a non-invasive method to quantify cellular response to laser irradiation. Lasers Surg. Med. 44:769–778, 2012. © 2012 Wiley Periodicals, Inc.

Key words: adipose tissue-derived stem cells; autofluorescence; confocal microscopy; low power laser; mitochondria membrane potential

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

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proliferation, secretion of growth factors, and myogenic differentiation. Kim et al. [17], reported that red light from a 647 nm laser enhances osteogenic differentiation in MSCs. In two studies by Muvla et al. [18,19], a low power laser at 635 nm laser was found to increase viability, and stimulate proliferation, gene expression, and secretion of β1-integrin. Another study [20] compared the effects of two lasers at wavelengths of 680 and 830 nm on human adipose derived stem cells. The results showed that cellular responses to laser irradiation such as cell morphology, viability, expression of stem cells markers were stronger in cells irradiated with 680 nm than 830 nm. Moreover, in a recent study by De Villiers et al. [21], irradiation of human adipose-derived stem cells using a 636 nm diode laser led to stimulation of cellular viability and proliferation but not differentiation.

Several studies regarding the biostimulation effects of green laser light have been reported in a wide variety of cells but not on stem cells. These reports showed that biological effects may be induced by green laser light across a broad range of visible spectrum. For examples, light at 514 and 532 nm has been reported to modify metabolic 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 wavelength was used to induce biostimulation of dermal fibroblasts and increased chicken fibroblast proliferation was induced 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 elucidated. 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 autofluoresence of living cells at the emission ranges of NADH and flavins as indicators of intracellular metabolic changes. We also quantified mitochondria membrane potential after irradiation by using JC-1 indicator 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-induced 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 mitochondria from rat heart was observed after low power green laser (532 nm, 30 mW) irradiation. The proliferation rate using the MTT assay as well as the mitochondrial 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 dependent 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 mitochondria where the dye forms J-aggregates whose fluorescence is red-shifted to 590 nm [29–31].

Intracellular autofluorescence is due to numerous endogenous 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]. Fluorescence detected in the region of 540 nm after excitation at 460 nm measures the cellular levels of the flavoproteins, lipomide dehydrogenase, and electron transfer flavoproteins [34–36]. Thus NADH and flavin autofluorescence provides insights into the metabolic activity and redox potential in cells.

MATERIALS AND METHODS

Cell Culture

StemPro human adipose derived stem cells (ADSCs) (Invitrogen, Sydney, Australia) were used in the experiments. 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 cm^2 tissue culture flasks and cultivated with MesenPro RS basal medium supplemented with 2% (v/v) MesenPro growth supplement, 1% (v/v) L-glutamine (2 mM), 1% (v/v) antibiotic–antimycotic mixture. Cells were incubated at 37°C, 95% humidity, and 5% CO_2. Cells were subcultured by trypsinization when they reached 80% confluence. Subculturing procedure was carried out under aseptic conditions. The MesenPro medium was aspirated from cells and the surface of cells layer was rinsed with Dulbeco Phosphate Buffer Saline (DPBS). For cells detachment, prewarmed TrypLE (0.5 ml/10 cm^2) was added and incubated at 37°C for 7–9 minutes to achieve more than 90% cell detachment. 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 210*g* 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 exclusion 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/cm^2).

Flow Cytometry Assay

In order to confirm the antigenic characteristics of ADSCS, cells from the third passage were harvested using the above subculturing procedure, a pellet of

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/cm^2 and the energy densities for each exposure time were calculated to be 5, 6.8, 9.2, 28, and 45 J/cm^2 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% CO_2 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 flourophores in the respiratory chain, NADH (excitation wavelength: 366 nm, emission wavelength 450 nm) and FAD (excitation wavelength: 460 nm, emission wavelength: 540 nm).

Proliferation Rate Experiments

A cell proliferation assay at 48 hours post-irradiation was performed using a Vybrant MTT Cell proliferation assay kit (Invitrogen). The MTT assay measures the enzymatic conversion by live cells of the water soluble MTT(3-(4,5-dimethylthiazol-2-yl)-2,5diphenyltetrazolium bromide) to an insoluble formazan, a purple dye. The formazan is then solubilized, and the concentration is determined by optical density at 570 nm. The media were removed from wells and replaced with 100 μl of fresh culture medium. Ten microliter of the 12 mM MTT stock solution were added to each well, including a negative control of 10 μl of the MTT stock solution added to 100 μl of medium alone. Microplates were incubated at 37°C for 4 hours, then 100 μl of Sodium Dodecyl Sulfate–HCL solution was added to each well and mixed thoroughly using a pipette. After 14 hours of incubation in a humidified incubator, samples were mixed using pipette. The absorbance values were measured at 570 nm using the Spectra MAX M5 Multi-Mode Microplate Reader (Molecular Devices). These values are referred to as the intensity of the proliferation index.

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 pinhole 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 Lilliefor's test of normality, which was positive at a significance 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 by using box plots [29]. The diagrams in Figure 1a represent the absorption intensity at 570 nm wavelength of 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

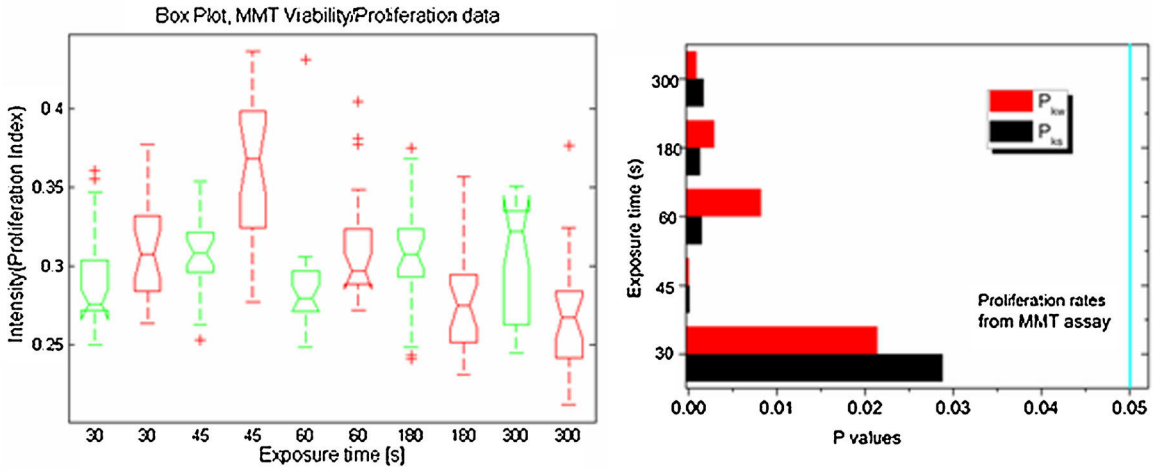

Fig. 1. Proliferation data by MMT assay, 48 hours after laser irradiation with 532 nm. **a**: Box plots of the data (24 replicates) in each of the five classes and the corresponding controls. Each diagram represents the absorption measured in the MMT assay. **b**: P-values for the statistical tests, P_{kw} (red) and P_{ks} (black). The blue line marks the value of $P = 0.05$. Laser irradiation produced statistically valid changes to viability for exposure times with P-values below 0.05. [Color figure can be seen in the online version of this article, available at http://wileyonlinelibrary.com/journal/lsm]

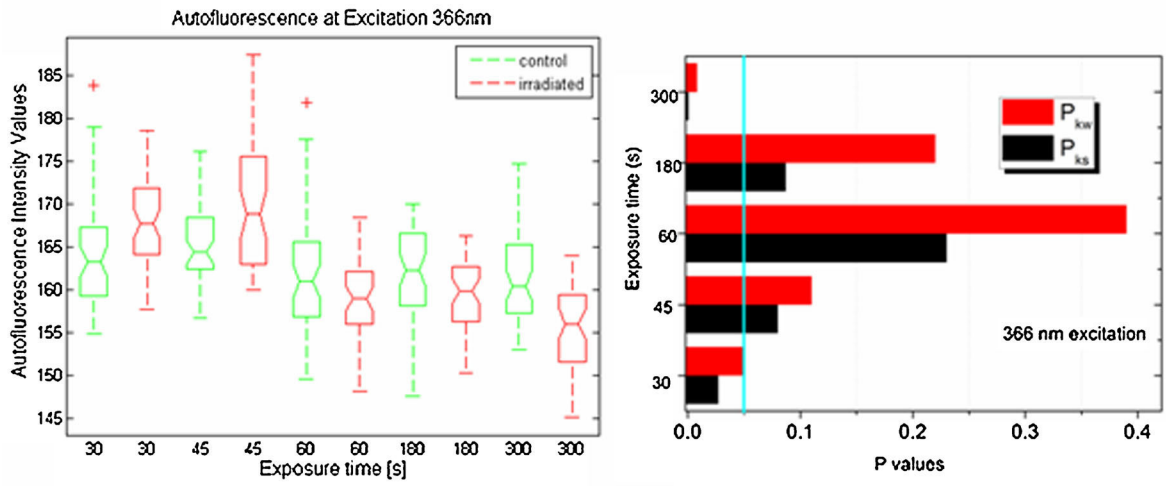

Fig. 2. Autofluorescence emission intensity of cells at 450 nm under 366 nm excitation, 48 hours after irradiation by 532 nm laser at 30, 45, 60, 180, 300 seconds exposure times. **a**: Box plots of the data (24 replicates) in each of the five classes and the corresponding controls. **b**: *P*-values 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. [Color figure can be seen in the online version of this article, available at http://wileyonlinelibrary.com/journal/lsm]

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. 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 ± 10 nm (green) and 590 ± 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

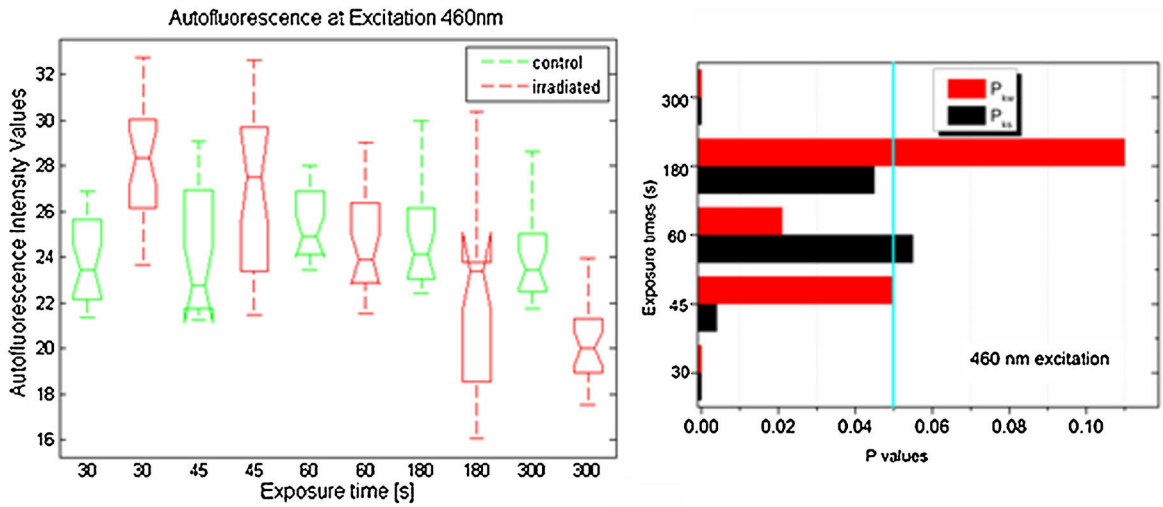

Fig. 3. Autofluorescence emission intensity of cells at 540 nm under 460 nm excitation, 48 hours after irradiation by 532 nm laser at 30, 45, 60, 180, 300 seconds exposure times. **a**: Box plots of the data (24 replicates) in each of the five classes and the corresponding controls. **b**: *P*-values 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. [Color figure can be seen in the online version of this article, available at http://wileyonlinelibrary.com/journal/lsm]

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.

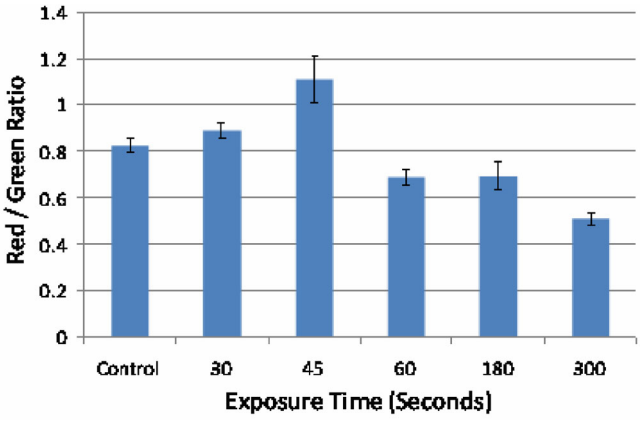

Fig. 4. Red to green fluorescence ratio of irradiated stem cells at different exposure times. [Color figure can be seen in the online version of this article, available at http://wileyonlinelibrary.com/journal/lsm]

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

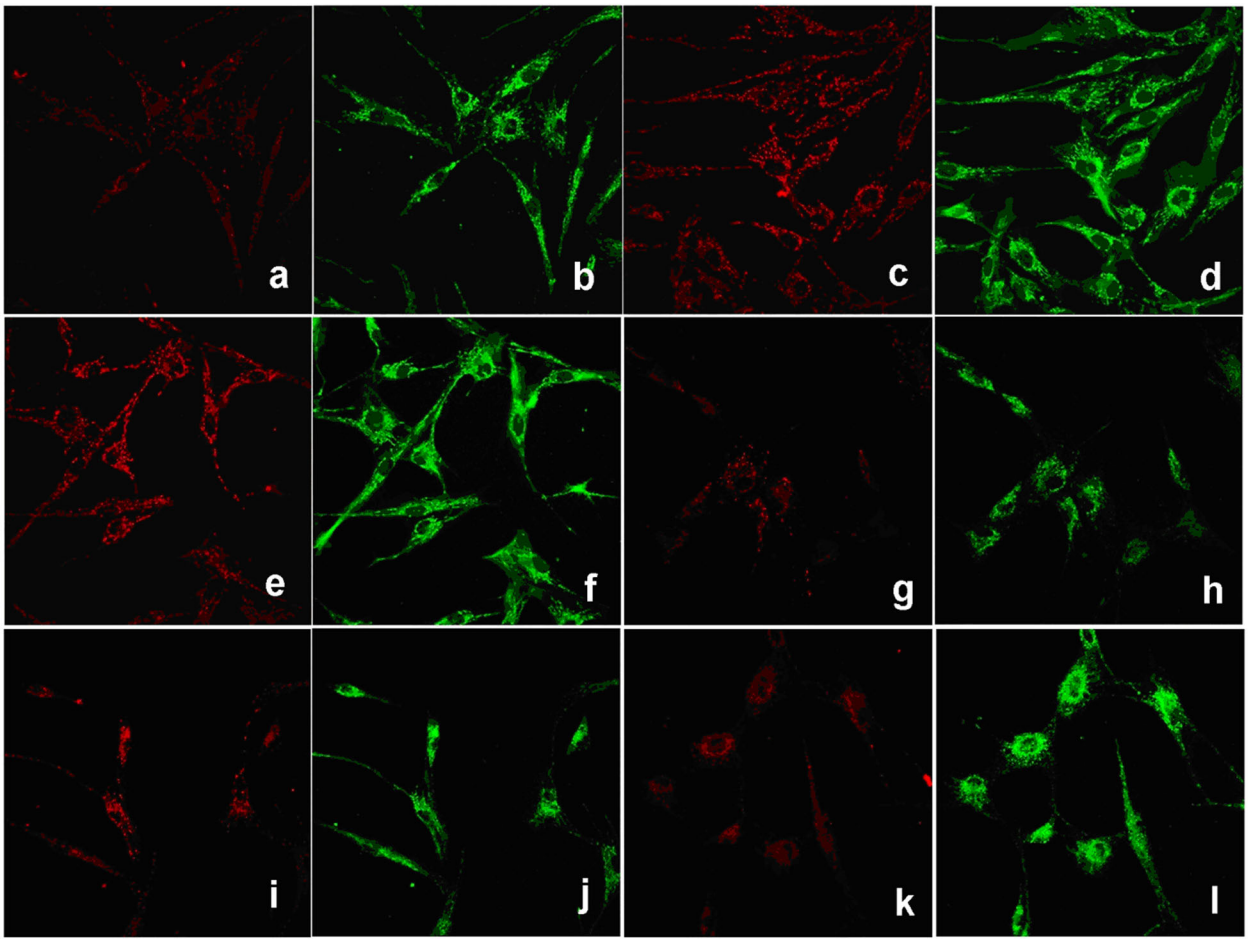

Fig. 5. Confocal laser scanning microscopy images of stem cells showing JC-1 fluorescence in two emission channels 525 nm (green images) and 590 nm(red images). **a**,**b**: control samples; **c**,**d**: 30 seconds irradiation; **e**,**f**: 45 seconds; **g**,**h**: 60 seconds; **i**,**j**: 180 seconds; **k**,**l**: 300 seconds. [Color figure can be seen in the online version of this article, available at http://wileyonlinelibrary.com/journal/lsm]

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.

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].

TABLE 1. Correlation Between Autofluorescence Emission and Proliferation Rates as Measured by the MTT Assay

Test of correlation of medians	Correlation coefficients	*P*-value
366 nm excitation	0.894	0.041
460 nm excitation	0.870	0.053

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).

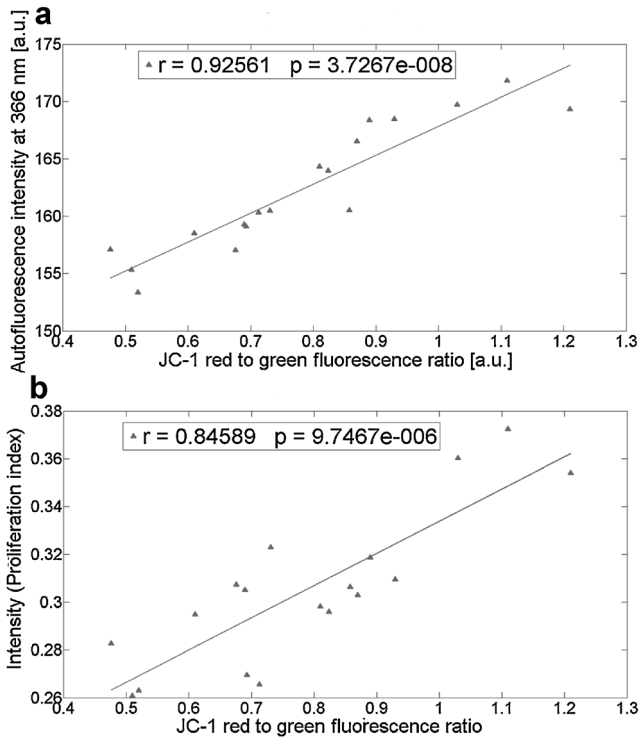

Fig. 6. **a**: Correlation between the results of the MTT assay (proliferation index) and the mitochondrial membrane potential measured by JC-1. **b**: correlation between autofluorescence at 366 nm related to NADH and the mitochondrial membrane potential. The correlation coefficients and the *P*-values are shown in the figure.

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 change, producing alterations of the fluorescence intensities [46]. Previous studies have shown that the autofluorescence of nicotinamade 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 Ca2+ flux. The Ca2+ 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 after excitation at 460 nm, most likely corresponding to flavin molecules. After 60 seconds this effect seems to cease and then to reverse direction for longer exposure times. The 30 and 45 seconds exposure times stimulate changes in the cells that lead to significantly increased proliferation, implying improved viability and mitochondrial activity. After 60 seconds there seems to be a significant decrease in proliferation/viability, perhaps due to damaging thermal effects. We observed a strong

correlation between autofluorescence intensity and proliferation rates of cells as measured by the MTT assay. A slightly stronger correlation was shown with 450 nm autofluorescence following 366 nm excitation mostly due to NADH molecules. The biostimulation effect of laser on stem cells will be beneficial in tissue engineering and regenerative medicine applications.

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