

The Effect of Low-Level Laser Therapy on Differentiation Ability of Human Adipocyte-Derived Stem Cells to Keratinocytes

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Abstract

Background: Low-level laser therapy (LLLT), is a clinically well-established tool for wound healing. In vitro studies have shown that LLLT has abiostimulatory effect on mesenchymal lineages and non-mesenchymal lineages of Adipose-derived stem cells (ADSCs) differentiation. The aim of this study was to evaluate the effects of LLLT at different energy intensities on differentiation of (ADSCs) to keratinocyte.

Methods: Adipose tissue were successfully obtained from 45-year-old woman by Lipoaspirates and adipose-derived mesenchymal stem cells (ADSCs) were isolated. ADSCs were divided into a control group and three low-level laser treated groups irradiated with 0.5 J/cm², 1 J/cm² and 2 J/cm² laser doses using He-Ne laser with 632nm wave length in 5 times. All groups cultured with keratinocytes media for 10-days. Cell proliferation was evaluated using 3-(4, 5-dimethylthiazol-2yl)-2, 5-diphenyltetrazolium bromide (MTT) assay. ADSCs developed a polygonal cobblestone shape characteristic of human keratinocytes. Specific keratinocyte marker 5 (*KRT5*) were analyzed by real-time PCR. Data were analyzed using one-way AVOVA and Tukey-Kramer hoc test by SPSS software version 16.

Results: Results of this study demonstrated LLLT could significantly affect proliferation of ADSCs (P<0.05) and promote ADSCs differentiation to keratinocytes by increasing expression of *KRT5* of 1 J/cm² and 2 J/cm² laser doses (P<0.01). LLLT at density of 0.5 J/cm² could not promote ADSCs differentiation significantly.

Conclusion: Considering these findings, LLLT could improve current in vitro methods of differentiating ADSCs to keratinocyte prior to transplantation. Thereby LLLT can use clinically as promoting skin wound healing.

Keywords: Adipose-derived stem cells (ADSCs), Low-level laser therapy (LLLT), Differentiation, Keratinocyte, *KRT5*.

Introduction

Laser application in medicine is specified in two fields of treatment and surgery. The low-level lasers are employed in tissue repair, wound healing, bone remodeling, skin disease treatment, and skin rejuvenation [1-3]. Indeed, low-level lasers or light-emitting diodes (LEDs) cause changes in cell function so that they improve inflammatory responses and reduce edema and pain in the tissue repair process [4]. Low-Level Laser Therapy (LLLT) is preferably utilized with red or infrared lasers with a wavelength between 600 and 1100 nm and a power of 1 to 500 mW and low energy between 0.04-50 J/cm² [5]. Multiple low-level lasers, including He-Ne, gallium-aluminum-arsenide (Ga-Al-Ar), Nd: YAG, are used in LLLT [6]. Treatment with high-power lasers in the field of dermatology and plastic surgery is carried out by converting light energy into heat. In other words, low-level lasers do not produce heat or vibration. The LLLT affects cell activity without damage and increase in cell temperature so that the photon energy is converted into chemical energy, and then biophysical changes are induced in the cell [7]. LLLT prevents cell death and apoptosis and stimulates their growth and differentiation. However, each laser has its own wavelength, power, and energy, based on which their duration and mode of radiation are also different [8]. Numerous *in vivo* and *in vitro* studies demonstrate the positive effects of LLLT [9]. Many studies have been carried out on the differentiation of Mesenchymal stem cells (MSCs) under the influence of LLLT [10, 11]. LLLT increases the survival of adipose-derived stem cells or the secretion of growth factors [12]. The definitive mechanism of the LLLT effect on cells is not fully known. In tissue repair, such as skin, the important thing

is that this complex organ to be repaired with a normal appearance and perfect function. This can be done in *in vitro* and *in vivo* environments using cells, natural or synthetic scaffolds, bioactive molecules, genetic manipulation, or a combination of all these things [10]. Despite many advances in epidermal biology and regenerative medicine, the treatment of acute and chronic large wounds has not changed much in recent decades. The conventional treatments for such wounds are often thick skin autografts [13]. The use of allograft skins also requires a long time to obtain enough cells from a small biopsy, and is often costly, and increases the risk of infection in burned skins [14]. In cell therapy, the differentiation of autologous cells, especially from adult stem cells, is very much welcome. Human adipose-derived stem cells obtained from liposuction or lipoaspirate specimens have the differentiation ability into a variety of cell lines [15]. These cells are part of a cell population isolated from lipoaspirates called stromal vascular fraction (SVF). Despite the adipocytes, the SVF is deposited in the liquid medium and adheres to the culture containers [16]. ADSc are part of mesenchymal stem cells (MSCs), which can be easily separated from subcutaneous fat by liposuction and used autologous for the individual. Therefore, the problem of immunological responses will be solved. Adipose-derived stem cells (ADSCs) have a high differentiation capacity, and these properties have transformed them into a tool for use in regenerative medicine [17]. The application of low-level lasers in the proliferation of mesenchymal stem cells (MSCs) obtained from various sources, as well as the proliferation of ADSCs is of utmost importance [18]. Nowadays, laser therapy is extensively exploited in cell proliferation, along with

growth factors [19]. The differentiation of ADSCs into various cells, including epidermal cells such as keratinocytes, has made them an efficient tool in regenerative medicine for skin protection [20]. Despite significant advances made in tissue engineering and regenerative medicine, the restoration and healing of skin wounds can yet be debatable. The use of undifferentiated cells for tissue regeneration and cell therapy-based repair strategies offers promising approaches for the treatment of chronic wounds [21]. In this study, we investigated the simultaneous effect of LLLT and keratinocyte growth factors on the ADSCs differentiation into keratinocyte cells.

Materials and Methods

Isolation of Mscs from Adipose Tissue

After obtaining written informed consent, (Ethic Committee No.: IR.IAU.PS.REC. 1398.323), adipose tissue (AT) was removed from the abdominal area of a 45-years-old woman by liposuction. After transferring to the laboratory, AT was washed three times with PBS solution containing 2% antibiotic solution and minced. Then, it was incubated with 37 mg/ml collagenase type I (Col I, Gibco Germany) for an hour at 37 °C. Next, it was centrifuged at 300 g for 5 min. The supernatant was isolated, and the cell pellet was suspended again with PBS containing antibiotics and filtered by a 70 µm cell strainer. The filtered solution was centrifuged, and the remaining cell pellet, containing ADSCs, was seeded in culture containers and incubated with Dulbecco's modified Eagle's medium (DMEM, Gibco Germany), 10% fetal bovine serum (FBS, Gibco Germany), and 1% Penicillin-Streptomycin (Pen/Strep, Gibco Germany) at 37 °C [22].

Flow Cytometry Analysis

After the third passage, Anti-human CD45, Anti-human CD44, and Anti-human CD90 antibodies were exploited by flow cytometry to detect the MSCs. 1×10^7 cells per ml were incubated with primary antibody along with 2 mM EDTA and 2% FBS for 45 minutes at 4 °C. All antibodies were prepared at a dilution of 1:200. The separation of the tubes was performed with a flow cytometry (BD FACSCa Libur, made in USA, 2009) equipped with a 15 mW argon laser. The results of flow cytometry were analyzed using Flowing software [23].

LLLT Irradiation

After the third passage, the cells were cultured in a 24-well plate until their density reached 80%. Keratinocyte growth factor (KGF, Qiagen Germany) was added to the cells at a concentration of 10 ng/ml. Then, they were divided into control group (without laser radiation) and treatment group, including group 1 (cells were irradiated with a laser at a dose of 0.5 J/cm²), group 2 (laser at a dose of 1 J/cm²), and group 3 (laser at a dose of 2 J/cm²). Simultaneously with the addition of KGF, the cells were irradiated with laser every other day from the first day of differentiation. The total differentiation period was ten days, and the cells underwent laser therapy 5 times. In this study, for low-power laser irradiation, a helium-neon (He-Ne) laser generating device (manufactured by the Atomic Energy Organization of Iran, model 2000) was used that emits a beam with a wavelength of 632.8 nm and a diameter of 2 mm with an output power of 10 mW. The diameter of the output laser is also increased to 17 mm using a Beam Expander at a distance of 15 cm to cover the entire surface of a 24-well plate. Laser characters were summarized in Table 1 [24].

Table 1 laser parameters used in ADSCs differentiation to keratinocytes

Type of laser	Helium – Neon (He-Ne)
Emission mode	632.8 nm
Time (s)	50 s, 100s ,200s
Energy	0.5J
Average power	10mW
Spot diameter at the focus	2mm
Focus spot area	17mm
Beam divergence	yes

The MTT Assay for ADMSCs Viability Assessment

An MTT assay was conducted to check the cytotoxicity of the glucose concentrations. The cultured HDFs were exposed to each glucose concentration in 96-well plates for 72 hours. Next, the supernatant was obtained by centrifugation before 200 µl of a 0.05% MTT solution (MTT, Sigma-Aldrich) was sucked in each well. Incubation was continued for 1 hour at room temperature, and then the upper solution was discarded and dimethyl sulfoxide (DMSO; Sigma-Aldrich) was used to dissolve the formazan crystal in each well. Finally, an optical density (OD) value at 570 nm was recorded with a spectrophotometer (Thermo Fisher Scientific) [25].

Admscs Differentiation into Keratinocyte

5×10⁴ cells were cultured in 24-well plates in DMEM/F12 medium and FBS

(10%) to differentiate mesenchymal stem cells into keratinocyte. After laser treatment all groups were cultured with differentiation medium containing KGF (10 ng/ml). The cells were cultured in the differentiation medium for 10 days.

Quantitative Real-Time Polymerase Chain Reaction (Qrt-PCR)

Total RNA was extracted with Favorgen Kit (Cat No.: FABRK001, Taiwan). To determine RNA quantification, Nano Drop Lite (Termo Scientific 2000) was used and light absorption in 260-280 nm indicated the RNA purification. cDNA was synthesized cDNA Synthesis Kit (Yekta Tajhiz. Iran). Real time PCR was performed using SYBR Master Kit (Sigma Aldrich) with Step One ABI Thermocycler (Thermo Fisher Scientific). Expression of keratinocyte marker KRT5 was quantified. As a control to determine relative gene expression levels, GAPDH was used (Table 2).

Table 2 Primers used in the differentiation of hADMSCs into keratinocytes

Genes	F and R Primers
<i>KRT5</i>	F: CGG CTA TGG CTT TGG AGG TGG R: TG GTCGATTTGGAGGTTGAGGGAGT
	F: GCAGGG ATGATGTTCTGG
<i>GAPDH</i>	R: CTTTGGTATCGTGGAAGGAC

Statistical Analysis

Statistical analysis was performed using one-way ANOVA software and Tukey-Kramer hoc test by SPSS software

version 16 and $P < 0.05$ is considered to be significant.

Results

Isolation, Culture, and Characterization of Adscs

Isolated ADSc from adipose tissue were evaluated by flow cytometry assay.

Cells that were positive for CD90 and CD44 antibodies and negative for CD45 antibodies were isolated and cultured in the media of DMEM/F12 and FBS 10% and antibiotics (Figure 1a). The cells had a spindle-like shape and the cell size was observed between 100-200 μm (Figure 1b).

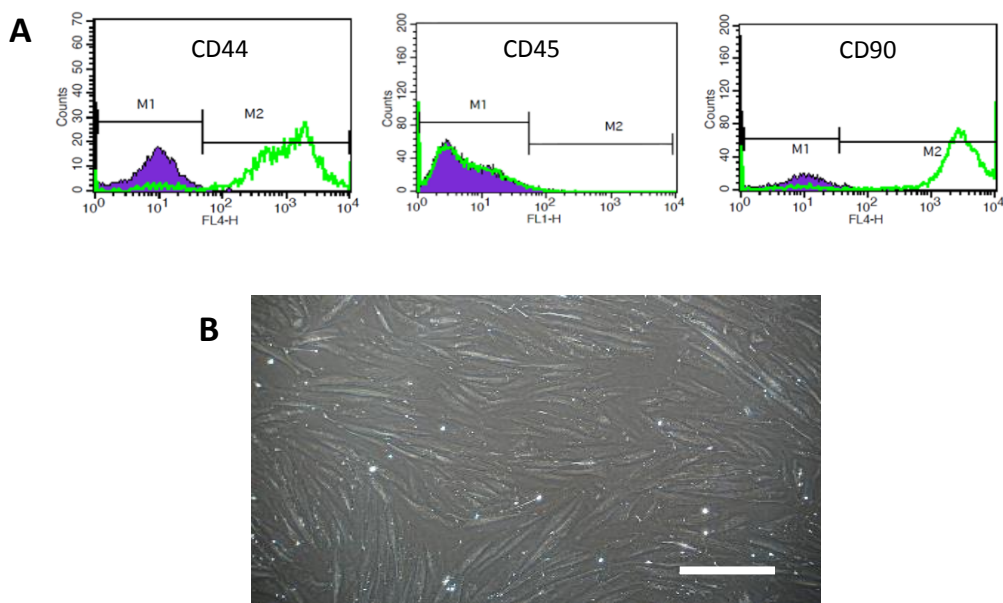


Figure 1 Isolation and characterization of ADSC. A: ASC were isolated and sorted from human fat using fluorescence-activated cell sorting. SVF expressed CD90 and CD44 and were negative for CD45 ($\text{CD90}^+\text{CD44}^+$ and CD45^-). B: Sorted ASC exhibited a spindle-like shape (80–100 μm in diameter and, 200 μm in length) Scale bars indicate 100 μm . Scale bar: 200 μm

Analysis of ADSC Cell Proliferation by LLLT

Taking into account that the rate of cell proliferation increased with increasing the rate of differentiation, the results of MTT assay were conducted on the studied groups after the end of the 10-day period of differentiation. The results demonstrated that, with increasing the laser dose, the rate of cell proliferation had a remarkable increased

compare to the control group. Thus, in group 3 (2 J/cm^2), relative to control, a significant difference in the proliferation rate was observed ($P < 0.05$). There was a difference between group 2 with group 1 and the control group that the difference was not significant between them. Thus, the highest distinction in the cell proliferation rate was observed between group 3 and other groups (Figure 2).

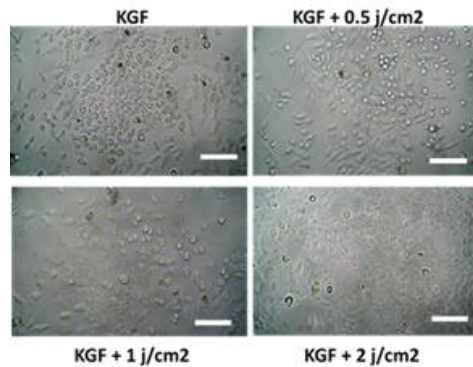


Figure 2 Effect of LLLT on keratogenic differentiation of ADSCs. Groups include a control group without laser, group 1 received 0.5 J/cm² laser, group 2 was Irradiated cells 1J/cm² laser, and group 3 was 2J/cm² laser irradiated cells. All groups received KGF at a concentration of 10ng/ml. Scale bar = 100µm.

Differentiation of ADSC Cells into Keratinocytes by LLLT

To differentiate ADSCs into keratinocytes, in addition to KGF, a three-dose low-level He-Ne laser was employed, i.e. doses of 0.5 J/cm², 1 J/cm², and 2 J/cm². The effect of laser therapy on increasing the differentiation ability of MSCs was at irradiated doses was examined. The groups receiving the irradiated laser showed more significant differentiation than the control group. Morphologically a more number of cells were changed in group 3 relative to the control group, and this difference declined by the laser dose reduction (Figure 3). In determining differentiated keratinocytes using markers expressed in these cells, it was specified by a real-

time PCR reaction and *K5 (KRT5)* gene-specific primers. The results exhibited that the *KRT5* gene expression maximizes with increasing the laser dose so that the highest *KRT5* gene expression is observed at 2 J/cm² dose and indicates a significant difference compared to the control group. Group 2, with a laser dose of 1 J/cm², shows little difference with group 3 (2 J/cm²). Groups 2 and 3 represent an increase in *K5* gene expression compared to group 1, but it is not statistically significant. Group 1 also did not show a significant difference in *K5* gene expression compared to the control group. Hence, groups 1 and 2 reflect the most significant difference with the control group in the *K5* gene expression ($P < 0.05$) (Figure 4).

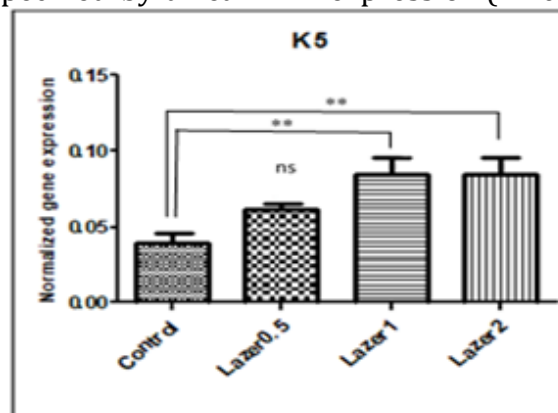


Figure 4 The expression levels of the keratinocyte genes, *KRT5 (K5)*, were measured by real-time PCR at day 10 after differentiation was induced. With increasing laser dose, *K5* gene expression shows increased expression. GAPDH was used as endogenous normalization controls for quantitation of mRNAs. Ns: non-significant.; $P < 0.01$.

Discussion

Adult adipose-derived stem cells are one of the mesenchymal stem cells (MSCs), which can be easily accessible. In addition to high plasticity and proliferative potential, these cells have the ability to modulate immune responses [26]. Adipose tissue has a heterogeneous population of cells containing adipocytes, endothelial cells, pericytes, and MSCs [27]. Since ADSCs not only have the ability to differentiate into mesenchymal stem cells, but they can also be differentiated into other cells of germ layers, and are of interest to many researchers scientifically and clinically [28]. Numerous studies have demonstrated that LLLT can increase MSCs proliferation and differentiation ability [29-30]. Although the cellular mechanism of the effect of LLLT is not yet known, some theories suggest that laser energy at a given wavelength is absorbed by members of the electron transport chain in the mitochondria and causes an increase in ROS and ATP levels. This increased expression of growth factors and cytokines eventually leads to cell proliferation [31]. Khalid *et al.* reviewed studies on the effect of LLLT on cell line culture and reported that LLLT increased MSCs proliferation in most cases, while some studies showed contradictory results [32]. Moore *et al.* reported that the maximum proliferation of fibroblasts occurred at a wavelength of 665 nm and 675 nm, while a wavelength of 810 nm inhibited their proliferation [34]. Min *et al.* (2015) studied the effect of low-power Ga-Al-As laser *in vitro* and *in vivo* on the proliferation and survival of adipose tissue-derived stem cells in mice [16]. The LLLT effect on cell proliferation depends on various factors, including laser parameters such as wavelength, energy density, power density, and (continuous or pulsed) irradiation, and of course, the type of cell being irradiated.

Hence, appropriate therapeutic parameters for specific cell lines are necessary so that the maximum biological effects on cell proliferation to be achieved [26]. Until now, there have been few studies in connection with the LLLT effects on the differentiation of ADSC into keratinocytes [33]. In this study, in addition to the impact of growth factors such as KGF on the ADSC differentiation into keratinocytes, a low-level laser was employed in three doses of 0.5, 1, and 2 J/cm², and their differences in cell differentiation were assessed. The results revealed that, with increasing dose, *KRT5* gene expression that is an evaluation index of the cell differentiation into keratinocytes increases. The cells morphology after ten days also proves this so that, at a dose of 2 J/cm², the cells clearly represent the keratinocyte phenotype compared to the control group. Likewise, the statistical analysis illustrated a significant difference between 1 J/cm² and 2 J/cm² laser groups compared to the control group and 0.5 J/cm² group in *KRT5* gene expression. In this study, the rate of cell proliferation after ten days from the beginning of differentiation was evaluated by MTT assay. The result indicated that the rate of proliferation increases with enhancing the laser dose. The findings gained from this study, in conjunction with the effects of LLLT on MSCs differentiation, are in line with studies conducted in this area. Wang *et al.*, (2019) reported the effect of Nd: YAG laser on increasing the differentiation ability of MSCs into osteocytes [34]. Several studies have revealed that LLLT is dose-dependent; that is, its biostimulatory effects are dependent on the LLLT energy density [6,35-37]. Our results revealed that increasing the dose to 2 J/cm² had more stimulatory effects on intracellular signaling relative to lower doses, due to more energy induction. However, further studies are

essential to explore the LLLT effects on the proliferation and differentiation of MSCs. We were not able to find any studies about effect of LLLT on MSCs differentiation on keratinocytes. Moreover, there were no pre-clinical and clinical studies either. To further researches in the field of effect of LLLT on MSCs differentiation to keratinocytes, it is recommended to use simultaneously the combination of two or more low-level lasers.

Conclusion

In this *in vitro* model, LLLT in combination with growth factor, accelerates the process of MSCs differentiation. LLLT in ADSCs differentiation to keratinocytes is dose dependent and in higher doses (2 J/cm²), the rate of cell differentiation increases. Thereby, it can use as promoting skin wound healing in regeneration medicine.

Declarations

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this article. The authors declare that there is no conflict of interest.

Authors' Contributions

Zeinab Piravar and Jamshid Sabaghzadeh designed this study. Zohreh Bahadorloo obtained and analyzed the data. Zeinab Piravar, Jamshid Sabaghzadeh, and Zihreh Bahadorloo proceeded to the quality control and the manuscript drafting. Zeinab Piravar revised the final version.

Conflict Declaration

The authors declare that there is no conflict in writing this manuscript.

Consent for Publications

All authors agree to have read the manuscript and authorized the publication of the final version of the manuscript.

Conflict of Interest

The authors declare that they have no conflict of interest.

Availability of Data and Material

Data are available on request from the authors.

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Not applicable.

Ethics Approval and Consent to Participate

The ethical approval was certified by Islamic Azad University Tehran Central Branch (Ethic Committee reference No.: IR.IAU.PS.REC. 1398.323).

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