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ORIGINAL ARTICLE

EFFECTS OF 970 NM DIODE LASER IRRADIATION ON MORPHOLOGY, PROLIFERATION, AND DIFFERENTIATION OF GINGIVAL MESENCHYMAL STEM CELLS

Efectos de la irradiación con láser de diodo de 970 m sobre la morfología, la proliferación y la diferenciación de las células madre mesenquimales gingivales

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ABSTRACT

Objective: The objective of this study was to investigate the morphology, proliferation, and differentiation of gingival mesenchymal stem cells (GMSCs) irradiated with a 970 nm Diode Laser (LLLT). It is essential to validate the efficacy of treatment, optimize irradiation conditions and guarantee the safety and quality of stem cells for future use in dental applications.

Materials and Methods: GMSCs were cultured in standard conditions and irradiated with a Diode laser (970 nm, 0.5W) with an energy density of 9J/cm2. Cell proliferation was assessed with the WST-1 proliferation kit. GMSCs were differentiated into chondrogenic and osteogenic lineages. Cell morphology was performed with Hematoxylin/eosin staining, and quantitative nuclear analysis was done. Cell viability was monitored with trypan blue testing.

Results: GMSCs subjected to irradiation demonstrated a significant increase in proliferation at 72 hours compared to the non-irradiated controls (p=0.027). This indicates that the 970 nm diode laser has a stimulatory effect on the proliferation of GMSCs. LLLT-stimulated GMSCs exhibited the ability to differentiate into chondrogenic and osteogenic lineages. A substantial decrease in cell viability was observed 24 hours after irradiation (p=0.024). However, after 48 hours, the cell viability recovered without any significant differences. This indicates that there might be a temporary negative impact on cell viability immediately following irradiation, but the cells were able to recover and regain their viability over time.

Conclusions: This study support that irradiation with a 970 nm diode laser could stimulate the proliferation of GMSCs, maintain their ability to differentiate into chondrogenic and osteogenic lineages, and has minimal impact on the morphological characteristics of the cells. These results support the potential use of NIR Lasers in combination with GMSCs as a promising strategy for dental treatments.

Keywords: *Photobiomodulation; semiconductor lasers; Mesenchymal stem cells; Gingiva; Cell Proliferation; Safety.*

RESUMEN

Objetivo: El objetivo de este estudio fue investigar la morfología, proliferación y diferenciación de las células madre mesenquimatosas (GMSC) irradiadas con un láser de diodo de 970 nm (LLLT). Es fundamental validar la eficacia del tratamiento, optimizar las condiciones de irradiación y garantizar la seguridad y calidad de las células madre para su uso futuro en aplicaciones dentales.

Materiales y Métodos: Las GMSC se cultivaron en condiciones estándar y se irradiaron con un láser de diodo (970 nm, 0,5 W) con una densidad de energía de 9 J/cm^2. La proliferación celular se evaluó con el kit de proliferación WST-1. Las GMSC se diferenciaron en linajes condrogénicos y osteogénicos. La morfología celular se realizó con tinción de hematoxilina/eosina y se realizó un análisis nuclear cuantitativo. La viabilidad celular se controló con prueba de azul de tripano.

Resultados: Las GMSC sometidas a irradiación demos-traron un aumento significativo en la proliferación a las 72 horas en comparación con los controles no irradiados (p=0,027). Esto indica que el láser de diodo de 970 nm tiene un efecto estimulante sobre la proliferación de GMSC. Las GMSC estimuladas con LLLT exhibieron la capacidad de diferenciarse en linajes condrogénicos y osteogénicos. Se observó una disminución sustancial de la viabilidad celular 24 horas después de la irradiación (p=0,024). Sin embargo, después de 48 horas, la viabilidad celular se recu-peró sin diferencias significativas. Esto indica que podría haber un impacto negativo temporal en la viabilidad de las células inmediatamente después de la irradiación, pero las células pudieron recuperarse y recuperar su viabilidad con el tiempo.

Conclusión: En conclusión, este estudio respalda que la irradiación con un láser de diodo de 970 nm podría estimular la proliferación de GMSC, mantener su capacidad para dife-renciarse en linajes condrogénicos y osteogénicos y tiene un impacto mínimo en las características morfológicas de las células. Estos resultados respaldan el uso potencial de láseres NIR en combinación con GMSC como una estrategia prometedora para tratamientos dentales.

Palabras Clave: *Terapia por Luz de Baja Intensidad; Láseres de Semiconductores; Células Madre Mesenquimatosas; Encía; Proliferación Celular; Seguridad.*

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INTRODUCTION

Mesenchymal stem cells (MSCs) are multipotent cells with the potential for differentiation to chondrogenic, adipogenic, and osteogenic lineages, among others.[1] Also, MSCs have immunomodulatory properties crucial in cell therapies.[1,2] Gingival Mesenchymal stem cells (GMSCs) are isolated from the gingiva, an abundant cell source, of easy access by relatively non-invasive procedures with lower morbidity rates for patients.[3] GMSCs demonstrate better proliferation rates than bone marrow and can maintain stem cell functional properties over time.[4] Thereby, its properties made them an interesting novel resource for cellular therapies.

Low-level laser therapy (LLLT) is a non-ionizing, non-thermal light treatment that, in the last years, has been widely used in managing wound healing, pain relief, and reducing inflammation.[5]

LLLT is based on Photobiomodulation (PBM), where light induces a photochemical reaction with a biological response, mainly mediated by the absorption of photons on tissues by photosensitive chromophores such as the mitochondrial cytochrome C-oxidase (CCO) among others.[6] Literature reported the "optical window" for LLLT treatment around 600-1000nm.[7,8] Although it is widely accepted that activation of CCO triggers the synthesis of adenosine triphosphate (ATP), modulating the expression of reactive oxygen species (ROS) and the induction of transcription factors.[9] The most relevant range for its activation is in the Red and Near-infrared (NIR) spectrum (600-860nm), with less absorption above 900nm.[6]

Therefore, longer wavelengths in the NIR spectrum may act on different photo-receptors.[10] One hypothesis is based on the activation of ion channels.[9,11] but recently an alternative pathway has been proposed based on the induction of water oscillation by NIR.[12]. Light effects on the mitochondria had been extensively studied; not all pathways have been completely elucidated; thus, exploring longer wavelengths for PBM on cellular models may lead us to a novel approach to regenerative medicine.

In dentistry, lasers in longer wavelengths are commonly employed in soft tissue surgery, frenectomy, mucocutaneous lesions, gingiva corrections, and implant uncover, principally due to their favorable penetration into tissues. For non-surgical purposes, longer wavelengths of Diode lasers are being used for disinfection of periodontal pockets or root canals, but with higher power outputs.[13,14]

Interestingly, they have been tested in pro-moting orthodontic movements.[15] and nerve regeneration.[16,17] Hence, there are some cli-nical recommendations for using this laser as a PBM tool, but there is still little evidence of the biological effect on cells. Thus, we aim to investigate the morphology, proliferation, and differentiation of gingival mesenchymal stem cells (GMSCs) irradiated with a 970 nm Diode Laser (LLLT). It is essential to validate the efficacy of treatment, optimize irradiation conditions and guarantee the safety and quality of stem cells for future use in dental applications.

MATERIALS AND METHODS

Characterization of GMSCs

An *in vitro* experimental study was conducted using gingival mesenchymal stem cells (GMSCs) obtained from two healthy donors (G1: GMSCs from donor one and G2: GMSCs from donor two). The study involved healthy volunteer donors of both genders, aged between 23 and 28 years. The participants' medical and dental history was meticulously reviewed, and a comprehensive dental examination was carried out by a qualified dentist. Individuals who were smokers, drug abusers, or pregnant were excluded from the study.

This study protocol (No. CEC201806) received approval from the Ethics Committee of the Universidad de Los Andes, Santiago, Chile. The protocol was designed following the principles outlined in the Declaration of Helsinki. Healthy gingival tissues were obtained from the retromolar region of patients who visited the health center (CESA, San Bernardo Hospital of the Universidad de Los Andes) and provided informed consent to participate in the study. The tissues were collected using a scalpel and immediately placed in transport media supplemented with antibiotics. Subsequently, the samples were promptly transferred to the CIBRO (Center for Investigation in Biology and Oral Regeneration) at the Dental School, Universidad de Los Andes, for further processing. GMSCs were characterized according to the criteria established by the International Society for Cellular Therapy (ISCT).[18]

Briefly, GMSCs were grown in a complete culture medium with MEM Eagle-Alpha-mo-dified medium (Invitrogen, Carlsbad, CA), 10% fetal bovine serum (HyClone, Logan, UT), and 1% antibiotic (penicillin-streptomycin, Invitrogen) at 37°C and 5% CO_2 (Sanyo, Incubator). Cells were monitored on a CKX41 inverted optical microscope (Olympus, Tokyo, Japan), and images were captured with a camera and processed with Micrometrics SE.

Laser Irradiation

Cell irradiation was carried out with a Diode laser (Siro Laser, Sirona) with the following settings: wavelength 970 nm, power output 0.5W, energy density 9J/cm^2, and single irradiation in continuous mode. The laser application was performed through the bottom of a 50mm culture plate in a perpendicular position and contact with it.

The experimental groups were G1 and G2 irradiated with 9J/cm^2 with the corresponding controls without irradiation.

Cell proliferation assay (WST-1)

Cell proliferation was assayed using WST-1TM (Roche) kit according to the supplier's instructions. Briefly, 1x10^4 cells were seeded in 96-well plates, and cell proliferation was tested at 24, 48, 72, and 96 h in triplicate. Absorbance at 570 nm was measured in a microplate reader (Rayto RT 2100C, USA).

Cytological analysis

Nuclei quantification, area measurement, and nuclei perimeter were carried out in GMSCs after irradiation at 48, 96, and 168 h. GMSCs were fixed with alcohol at 70° and stained with Hematoxylin-Eosin (H&E) with standard protocols. GMSCs

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morphology was observed in a Microscope Olympus CKX41. Four images were captured with the software Micrometrics® at 100X, and two were selected for nuclear measurements using the ImageJ® program. Results were averaged and plotted.

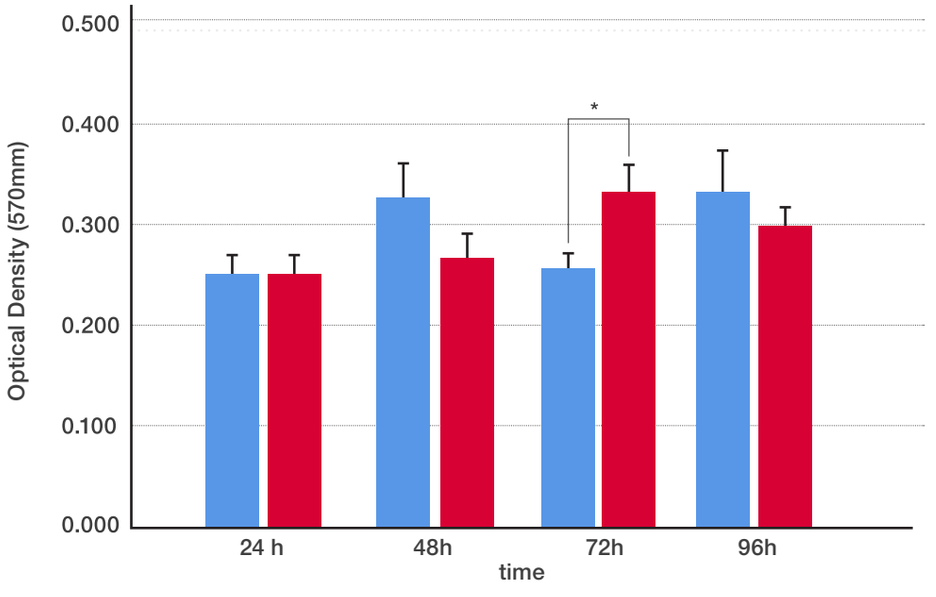
Osteogenic and Chondrogenic differentiation

For osteogenic differentiation, $3x10^4$ GMSCs were seeded with an osteogenic medium (StemPro®), which was changed every four days. On day 21st, cells were stained with Alizarin Red (A5533, Sigma(R)). In the chondrogenic differentiation, $3x10^4$ GMSCs were cultured with a chondrogenic medium (StemPro®) changed every four days. On day 14th, cells were stained with Safranin O stain (Sigma®).

Trypan Blue Exclusion Test of Cell Viability

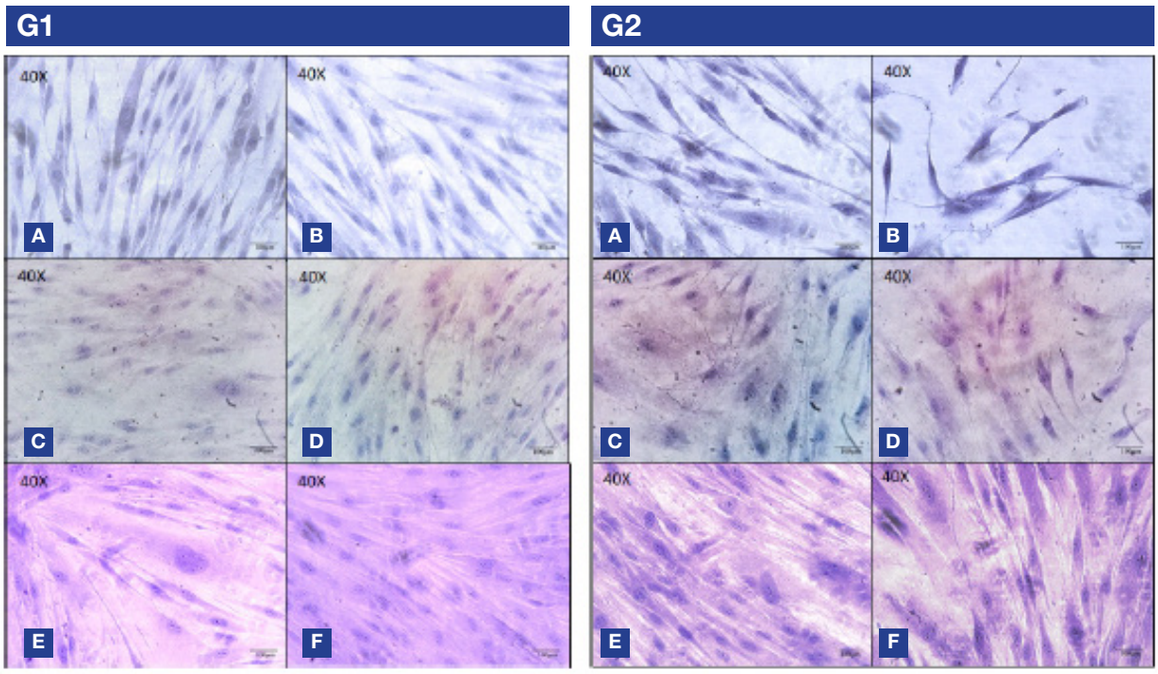
$1x10^4$ GMSCs were cultured for four days and were irradiated at $9J/cm^2$ or not. Cells were incubated for 24, 48, and 96h and were trypsinized and resuspended with 1ml of PBS 0.1M. GMSCs were mixed with 50×l PBS 0.1M and 50×uL of Trypan blue solution 0.4% (T8154, Sigma(R)), and 10×uL were counted in a Neubauer camera. Death cells were blue-stained and white cells were live cells, considered as the percentage of viability.

Figure 1 : Proliferation over time of GMSCs irradiated with LLLT. The means and standard deviation of the measurements of the arbitrary optical density units concerning time were plotted.



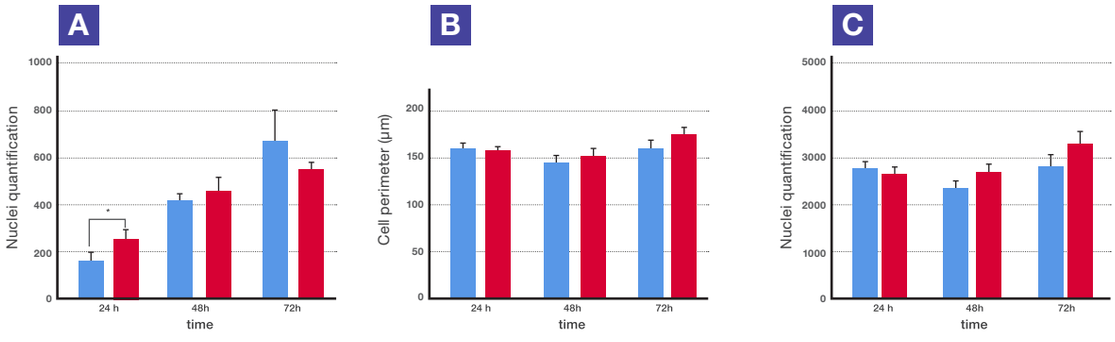
GMSC Control: Unirradiated GMSC; $9J/cm^2$: Laser. irradiated GMSC with $9J/cm^2$. *:Significant differences (p = 0.027).

Figure 2: Cell culture stained with hematoxylin-eosin. Nuclei morphology of irradiated and not irradiated GMSC from donor G1 and donor G2 with a 40X magnification.



A: GMSC at 48 hours after irradiation with LLLT 9J/cm^2 . B: Control GMSC without irradiation, at 48 hours. C: GMSC at 96 hours after irradiation with LLLT 9J/cm^2. D: Control GMSC without irradiation at 96 hours. E: GMSC at 168 hours after irradiation with LLLT 9J/cm^2. F: Control GMSC without irradiation at 168 hours.

Figure 3: Morphometry.



A: Nuclei quantification at 48, 96, and 168 hours of GMSCs irradiated with 9J/cm^2 and controls without irradiation. Significant differences were shown at 48 hours between the control and laser groups (p=0.045). The differences between groups in the other times were not significant (p>0.05).

B: Nuclei perimeter of GMSCs irradiated with 9J/cm^2 and their controls without irradiation over time in hours. The differences between the groups were not significant (p>0.05).

C: Nuclei area of GMSCs irradiated with 9J/cm^2 and their controls without irradiation over time in hours. The differences between the groups were not significant (p>0.05).

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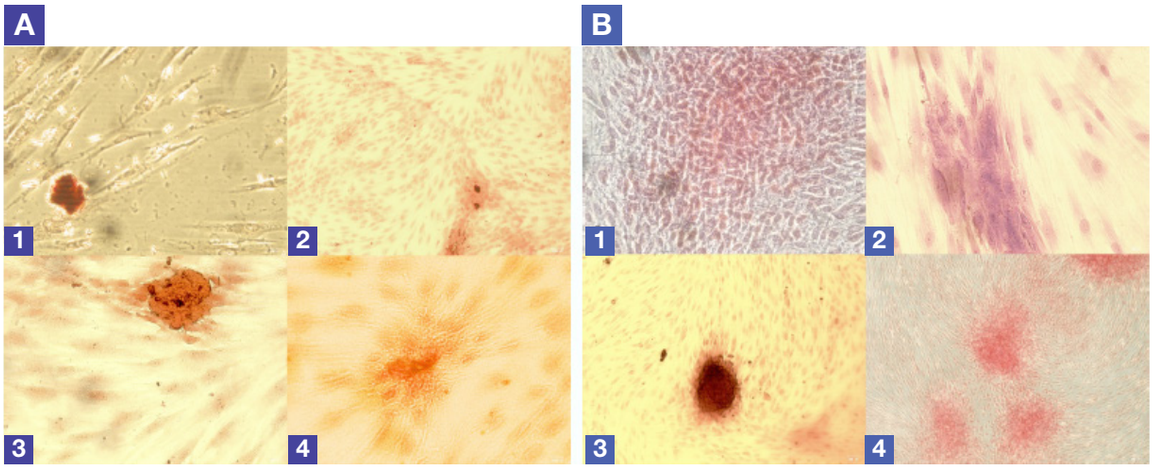
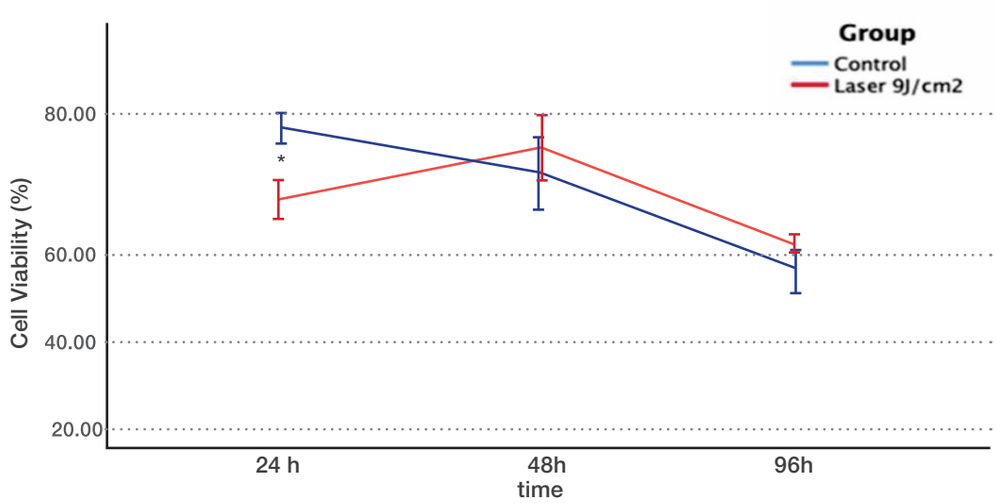
Figure 4: Differentiation.



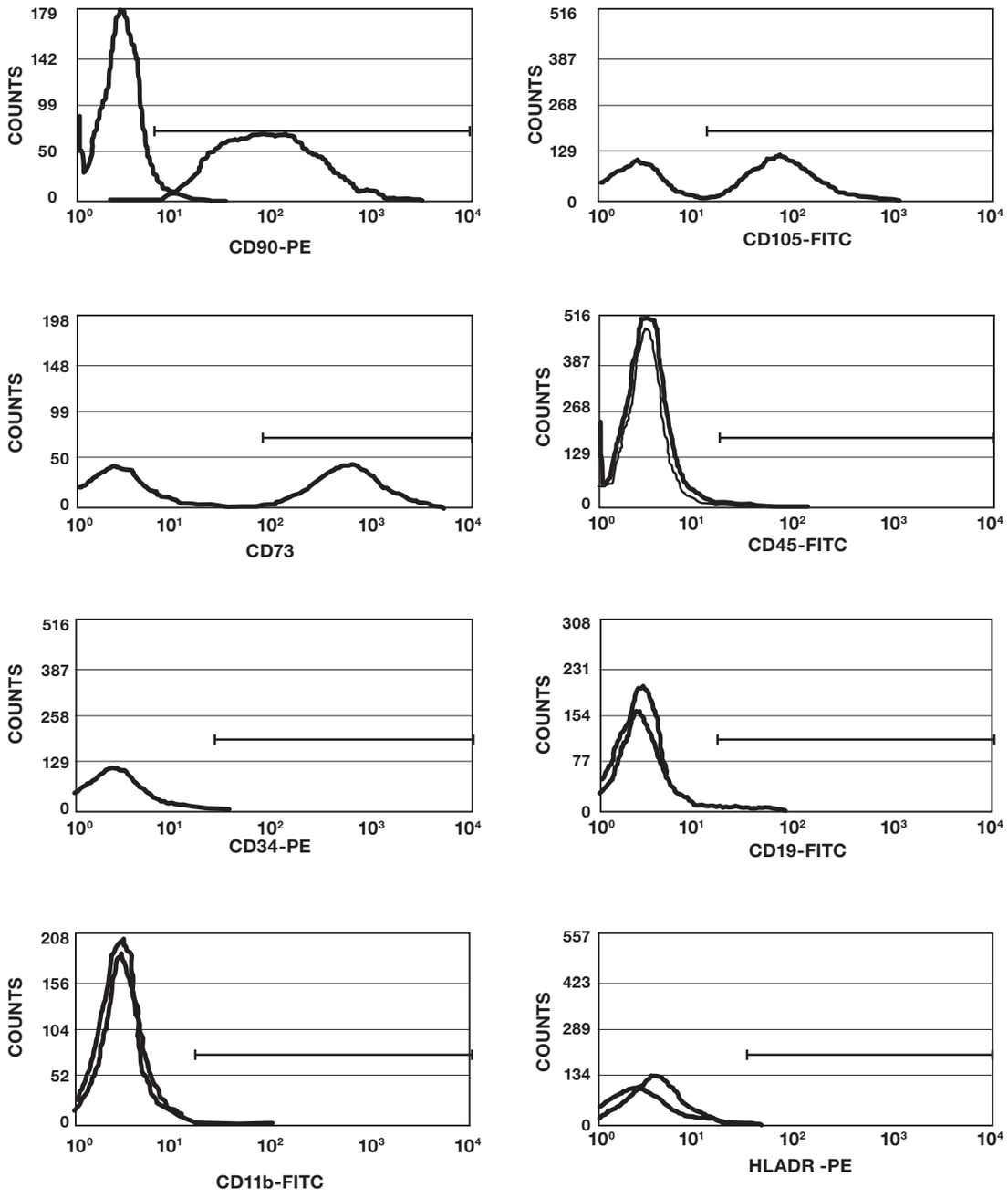
A: Osteogenic differentiation with Alizarin Red staining photographed in a 40x. 1: GMSC G1 irradiated with 9J/cm^2 laser, 2: GMSC G1 control without irradiation, 3: GMSC G2 irradiated with 9J/cm^2 laser, 4: GMSC G2 control without irradiation.
B: Chondrogenic differentiation of GMSCS stained with Safranin O. 1: GMSC G1 irradiated with 9J/cm^2 (40X), 2: GMSC G1 control without irradiation (40X), 3: GMSC G2 irradiated with 9J/cm^2 (10X), 4: GMSC G2 control without irradiation (10X).

Figure 5: Percentage of the viability of GMSCs irradiated at 9J/cm^2 compared to the unirradiated control.



Asterisk (*) represents a significant difference (p=0.024) at 24 hours. The differences between the groups at 48 and 96 hours were not significant (p=0.609 and p=0.409, respectively).

Figure 6: GMSC immunophenotype. GMSCs expressed the following markers: CD90 (73.9%), CD105 (67%), CD73 (99.6%), CD45 (0.1%), CD34 (0.1%), CD19 (0.1%), CD11b (0.1%) and HLADR (0.1%) BD Bioscience®. Marked cells were acquired in a flow cytometer (BD FACS Canto II) and the data was analyzed with the software BD FACS Diva™.



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Statistical analysis

Data were presented as means with standard deviation. The statistical comparison between groups was carried out using repeated measures analysis of variances (ANOVA-1). The student t-test was used between groups. All data were analyzed using the statistical package software IBM SPSS V.21 (ARMONK, NY, USA). A significance of 5% (p<0.05) was considered statistically significant.

RESULTS

The proliferation of irradiated GMSCs

GMSCs proliferation in both groups was observed over time with no significant differences. However, at 72h, the differences were significant between the irradiated and control group (p=0.027) (Figure 1).

LLLT effects on the morphology of irradiated GMSCs

Normal fibroblast-like morphology was observed in all cells irradiated and non-irradiated. The qualitative analysis of the irradiated samples showed the cells with larger nuclei than the cells of the control samples (Figure 2).

Nuclei quantification, area, and perimeter were evaluated in GMSCs and plotted (Figure 3). No significant changes were observed in the area or perimeter of the irradiated GMSCs (p>0.05), except in the nuclei quantification at 48 h (p=0.045).

Differentiation of irradiated GMSCs

Cells irradiated with laser 9J/cm^2 were successfully differentiated into osteogenic and chondrogenic lineages. GMSCs irradiated and stained with Alizarin Red evidenced the presence of calcium precipitates typical of osteoblasts (Figure 4A) and a chondrogenic mass stained with Safranin O staining (Figure 4B) like the non-irradiated cells.

LLLT effects on the viability of irradiated GMSCs

GMSCs irradiated with 9J/cm^2 showed a significant decrease in cell viability at 24h post-irradiation (p=0.024); however, after 48h, GMSCs irradiated recovered viability without substantial differences compared to the control. The intragroup T-test did not show significant differences (Figure 5).

DISCUSSION

Currently, strategies for cell therapies and their processing still need to be improved, and lasers could be a solution. LLLT effectiveness has been demonstrated in accelerating wound healing, enhancing bone repair, reducing pain, modulating the immune system, and others.[5,9,11] Despite this, the variability in methodology between studies hinders establishing this instrument as a standardized procedure. Evidence suggests that it could improve cellular performance. So, we aimed to evaluate the effect of 970nm Diode lasers, commonly used as an adjuvant in dental therapies on GMSCs for future regenerative procedures. Our findings indicate that longer NIR wavelengths had minimal impact on the morphological characteristics of the cells, suggesting their safety. Furthermore, this wavelength did not alter the potential for cell proliferation and differentiation.

Although, to achieve the desired effects of Photobiomodulation, it is crucial to conduct a comprehensive investigation of various dosimetry parameters.

Since the mechanism of action for wavelengths above 900nm is still not well-defined, further investigation into different dosimetry and pathways is required. A study conducted on human keratinocytes and fibroblasts compared the effects of 660nm and 980nm LED light irradiation. It was observed that the increase in ATP levels induced by NIR light returned to baseline before 24 hours, unlike red light. However, in a proliferation assay conducted at 72 hours post-irradiation, significant values were found for the 980nm wavelength. This suggests that PBM in the NIR range may not occur exclusively through ATP modulation. To investigate further, the authors tested inhibitors of COX^{-1} and ATP synthase and found that PBM still occurred to some extent even in the presence of these inhibitors. This suggests the involvement of other pathways, such as calcium (Ca^{+}) channels, nitric oxide (NO), or other mechanisms, for NIR sources.[19]

Likewise, a study[20] highlighted significant differences in the mechanisms of action between different NIR wavelengths (810nm and 980nm). Specifically, when testing calcium channel blockers and temperature settings, only the 980nm wavelength was affected, suggesting that longer wavelengths may rely on temperature-gated calcium ion channels rather than the commonly accepted cytochrome C-Oxidase (CCO) pathway. For this reason, we evaluated whether a 970 nm laser can induce PBM.

This experiment used gingival tissues from young donors, in ideal conditions for MSCs culture expansion and functional studies.[8] Our results confirmed GMSCs properties and characterization were according to the ISCT criteria[3] (Figure 6).

To evaluate proliferation, we tested laser fluence at 9J/cm^2. According to AlGhamdi *et al.*,[7] doses over 10J/cm^2 in cells could end in an inhibitory outcome. However, our findings demonstrated a significant difference between the control group and laser at 72h (p=0.027), although, at 96h, no statistical differences were observed (p>0.05).

A recent proliferation study on human gingival fibroblast irradiated with different wavelengths (635, 660, 808, 980nm) and energy densities found significant results in all groups at 24h. Still, at 72h, only 980nm remained substantial, but at 120h, all were like the control.[21] Contrary, a study on human fibroblast with differing wavelengths (1064, 980, 635, 450, and 405nm) and energy densities tended to increase proliferation in the first 24-48h but decrease at 72h[22] in longer wavelengths. Thus, PBM in longer NIR Wavelengths may have a shorter duration.

There is limited evidence indicating morphological changes in cells caused by laser irradiation, although it is not a highly researched subject. In this study, GMSCs irradiated showed a fibroblast morphology consistent with the literature.[18] The H&E staining of the irradiated cells showed

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nuclei of increased size concerning the control (Figure 2).

The quantitative analysis of the number of the nucleus (Figure 3A) showed that after 48 h, irradiated cells tend to increase nuclei numbers and there is a trend toward an increase in the nuclei perimeter and area over time (Figure 3B, 3C). Although it's not significant (p>0.05), the increase in nuclei size may indicate cell swelling and correlate with the low viability in the first 24h. Still, it could also be explained as normal development due to LLLT stimulation by increasing cell metabolism through DNA and protein synthesis. De Magalhães *et al.,*[23] study the actin filaments in cytoskeleton of 3T3 mouse fibroblast, which are essential part of cell structure, their functions are linked with cellular processes such as migration, division, structural maintenance, and wound healing. They found that regardless of wavelength employed, the total polymerized actin and number of actin filaments briefly decreased, while the nucleus area increased after irradiation. While this phenomenon was transient, its potential impact on cell functionality remains unclear.

Correspondingly, a study investigating the morpho-functional response to different laser wavelengths demonstrated that fibroblasts irradiated with NIR light ex-hibited a polygonal shape and a larger surface area. This increase was correlated with a higher level of collagen expression and deposition, suggesting a potential promotion of cell differentiation towards a (proto) myofibroblast phenotype.[24]

While these changes may be positive for this cell type, a study in adipogenic MSCs irradiated with a 1064 nm diode laser at different energies (8.8, 17.6, 26.4J/cm^2) showed significantly larger nuclei areas at higher energy fluencies, correlated with an altered adipogenic differentiation.[25]

Considering that no substantial morphological changes were observed. It is likely that the laser used does not have any detrimental effects, so differentiation was assessed.

Previous research has demonstrated remarkable differentiation capabilities of GMSCs into chondrogenic and osteogenic lineagesb.[26] And it has been suggested that NIR wavelengths have the potential to stimulate proliferation and protein expression, promoting bone formation.[27]

As a result, the use of laser therapy as an adjuvant for regeneration has been proposed, particularly in the field of periodontal treatment for infrabony defects. Recently, Thalaimalai *et al.*,[28] conducted a study investigating the effects of laser treatment combined with platelet-rich fibrin (PRF) and graft materials, which yielded positive outcomes in terms of bone regeneration. Also, the 970 nm diode laser has been proven useful in accelerating orthodontic tooth movements.[15] With this in mind, we examine whether the combination of laser therapy and GMSCs could further enhance their differentiation abilities in vitro. However, our findings indicate that the parameters used did not alter but neither did they improve the differentiation potential of GMSCs.

A significant decrease in viability was observed in the irradiated group at 24 hours (p=0.0024). This decrease may be attributed to cells adapting to the stimulus during the initial 24-hour period, where cellular stress could arise due to the high dose of energy received (0.5w, 9J/cm^2) which approached the upper limit described in literature.[29] On the other hand, this observation aligns with previous reported larger nuclei areas, which could be considered as a potential detrimental effect. However, at 48h and 96h, no differences in cell viability were found, which is consis-tent with the outcomes of proliferation and differentiation where no significant differences were observed. Thus, we believed that lower doses and different modalities should be considered. As reported by Jazaeri *et al.,*[30] in their assessment of migration and viability of human fibroblasts using a 940nm Diode laser, they found improved viability outcomes at doses of 1-4 J/cm^2 in both continuous wave (CW) and pulsed mode.

Taking into consideration the limitations of this study, we have demonstrated that irradiation with a longer wavelength diode laser, specifically at 970 nm with a dose of 9J/cm^2, did not affect the morphological characteristics of GMSCs, their proliferation, or differentiation. These findings support the safety of this approach. Therefore, further investigations should be conducted to achieve photobiomodulation for *in vitro* cell preconditioning and to facilitate the translation of LLLT and stem cell-based therapy into dental clinics.

Although current research supports the biological effects of LLLT in long Near-IR wavelengths, there are still many questions that need to be addressed. These include understanding the cell activation mechanism, determining the optimal fluences, exploring delivery modes or the use of combinations of wavelengths, investigating its impact on morphology, and exploring potential implications for cell functionality.

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CONFLICT OF INTERESTS

The authors declare no conflict of interest.

ETHICS APPROVAL

This study was approved by the Ethics Committee of the Universidad de los Andes, Santiago, Chile (No.CEC201806); Informed consent was obtained from all patients included in the study.

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AUTHORS' CONTRIBUTIONS

Ortiz J: Worked on the acquisition, analysis and complete interpretation of data, also on the experimental protocols associated with the scaffold and stem cells and approved the final version of the manuscript.

Matamala F: Contributed to writing and reviewing all the manuscripts drafts, significantly revising the draft critically for important scientific content and approval of the final version of the paper.

Andrade C: Contributed to the dental processes described in this research, especially in the taking of samples and diagnoses by specialists. Likewise, she collaborated in the writing and revision of the article and approved the final version of the manuscript.

Gutiérrez D: Contributed to the statistical analysis, reviewed the draft, revised the documents, and approved the final version.

Inostroza C: Was responsible for this research, made substantial contributions to the conception or design of this research; also, wrote the final version of this paper

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