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Effect of Fibroblast Growth Factor 2 and Low-Level Laser Therapy on the Adhesion and Proliferation of Periodontal Ligament Stem Cells

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Abstract

Introduction: The adhesion ability of mesenchymal stem cells can significantly affect their viability and is considered a prerequisite for cell therapy. The current study sought to evaluate the effect of fibroblast growth factor 2 (FGF2) and low-level laser therapy (LLLT), either individually or in conjunction, on the adhesion and proliferation of periodontal ligament stem cells (PDLSCs) when applied on the first day of cell seeding.

Methods: The experimental groups of this study comprised a control group and different combinations of adjunctive FGF2 (50 ng/mL) and LLLT with an 808 nm diode laser in one (LLLT-1) or two sessions (LLLT-2) of irradiation. The proliferation and adhesion of cells were evaluated by using the methylthiazolyl tetrazolium (MTT) assay and 4′,6-diamidino-2-phenylindole (DAPI) staining. All experiments were done in triplicates on the first, third, and fifth days after cell seeding. Two-way ANOVA and post hoc Tukey tests were used to analyze the data of the MTT assay. $P<0.05$ was considered statistically significant.

Results: One-day post-culture, only significant differences were found between the control group and the FGF2 ($P=0.04$) and FGF2 + LLLT-2 application ($P=0.04$) groups. After three days post-cell culture, only a significantly higher proliferation rate was found in the control group than in the FGF2 group ($P=0.01$). After five days, the control group and LLLT-2 groups showed significantly higher amounts of proliferation compared to the other groups ($P<0.05$). DAPI staining qualitatively confirmed the results of the MTT assay.

Conclusion: The LLLT can be applied to PDLSCs on the day of seeding without causing a notable decrease in their viability and adhesion. Conversely, the administration of FGF2 should be restricted on the seeding day and postponed to subsequent days as it may have adverse effects on their adhesion and proliferation.

Keywords: Cell adhesion; Cell proliferation; Fibroblast growth factor 2; Lasers, Semiconductor; Mesenchymal stem cells

Introduction

The importance of cell adhesion in intercellular regulation and communication, as well as in cellular functionality and development, is inevitable.[1] Based on the "cell adhesion model," a cell's level of adhesion is directly correlated with the quantity of chemical bonds present on its surface.[2,3] Mesenchymal stem cells showed high amounts of expression of surface adhesion molecules, like ICAM-1 and VCAM-1.[4]

Biomaterials and biomolecules specifically designed for tissue engineering applications are essential for promoting increased cell adhesion capabilities, thereby facilitating subsequent biosynthesis and proliferation processes.[5] These features may be particularly notable in the context of mesenchymal stem cell therapy, as their capacity for adhesion can have a substantial impact on their viability and is considered an essential requirement for cell-based therapeutic interventions.[6] Among the mesenchymal stem cells, the periodontal ligament stem cells (PDLSCs) showed a significant enhancement of the regeneration of periodontium regarding both bone fill and attachment gain when employed in cell therapy.[7]

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Growth factors are considered as one of the adjunctive treatments showing the potential for enhancing true regeneration.[8] Fibroblast growth factor 2 (FGF2) is one of the growth factors investigated in many previous research and has shown promising outcomes in the regeneration of periodontium.[9] Low-level laser therapy (LLLT) shows potential as a treatment modality in the field of regenerative medicine. Studies have demonstrated that LLLT with diode lasers emitting wavelengths of 660 nm, 808 nm, and 940 nm shows promise in promoting the proliferation of PDLSCs.[10,11]

The potential of these treatments may be integrated with cell therapy to pave the way for future advancements in regenerative medicine, particularly in the context of periodontal regeneration. The current study aimed to evaluate the effect of FGF2 and LLLT, both separately and in combination, on the adhesion and proliferation of PDLSCs when introduced on the first day of seeding to observe if they interfere with the adhesion of these cells. To the authors' knowledge, no study has elaborated on the effect of these treatments on the seeding day. The findings of this study would be beneficial for future research focusing on utilizing cell therapy in regenerative medicine since the viability and adhesion of the cells to the scaffolds or tissues are crucial factors for achieving a desirable result and might be affected by the protocol of applying the adjunctive treatments like growth factors or LLLT.

Materials and Methods

The protocol of the current study has received approval from the Ethics Committee of the Research Institute of Dental Sciences, Dental School of Shahid Beheshti University of Medical Sciences (IR.SBMU.DRC.REC.1402.072).

Materials

Cells

The authors obtained the PDLSCs from the Iranian National Center of Genetic and Biologic Resources (IBRC C11326). The surface characterization of stem cells was CD34- (1.27%), CD45- (1.08%), CD90 + (99.6%), and CD105 + (95.6%).

Culture and Test-Related Materials

Dulbecco's modified Eagle's medium (DMEM)-high glucose, trypsin/ethylenediaminetetraacetic acid (trypsin/EDTA), fetal bovine serum (FBS), and penicillin/streptomycin (pen/strep) were purchased from Biosera, France. FGF2 was purchased from Royan Institute, Iran. Methyl thiazolyl tetrazolium (MTT) solution, 4′,6-diamidino-2-phenylindole (DAPI) Solution, dimethyl sulfoxide (DMSO), paraformaldehyde, and phosphate-buffered saline (PBS) were bought from Sigma Aldrich, United States, and ethanol was purchased from Merck, Kenilworth. A diode laser with an 808 nm wavelength (DX82, Komftec, Taiwan) was used in the experiments.

Assessment Tools

ELISA Reader Device: ELX800, BioTek, United States.

Fluorescent Microscope: Cytation3, BioTek, United States.

Preparation of Solutions

FGF2: 10000 ng of the FGF2 powder was mixed with sterile water to form the study stock. 2.5 µL of this solution containing 0.5 mL of medium culture was added to each well. The concentration of FGF2 was adopted from the study by Hyun et al,[12] who explored the effect of FGF2 on teno/ligamentogenic differentiation.

Preparation of Standard Medium

The standard medium contains high-glucose DMEM supplemented with 15% FBS and 1% pen strep.

Study Design

Experimental Groups

Table 1 describes the details of treatments in each experimental group. The experimental groups comprised six different groups: the control group containing only PDLSCs; the FGF2 group containing cultured PDLSCs in a standard medium culture supplemented with 50 ng/mL FGF2; the LLLT1 and LLLT2 groups containing cultured PDLSCs radiated with an 808 nm diode laser (250 mW power; 0.5 W/cm^2 power density; 4 J/cm^2 energy density, 8-second radiation; 8 mm tip and 0.5 cm^2 area; with 1 mm distance from the culture surface) with irradiation in single (LLLT1: day 0) or two sessions (LLLT2: day 0 and 2 post culture); the FGF2 + LLLT1 and FGF2 + LLLT2 groups containing cultured PDLSCs in a medium culture supplemented with 50 ng/mL FGF2 radiated with an 808 nm diode laser (250 mW power; 0.5 W/cm^2 Power density; 4 J/cm^2 energy density, 8-second radiation; 8 mm tip and 0.5 cm^2 area; with 1 mm distance from the culture surface) with radiations on days 0 and both 0 and 2, respectively. Figure 1 depicts the characteristics and timeline of the study.

Cell Culture

Initially, 4000 PDLSCs were counted by using a hemocytometer and added to 48-well plates with 0.5 mL of the standard culture at 37 °C, 98% humidity, and 5% CO_2. The treatment of cells started from the day of seeding. The standard culture medium was changed every two days. All experiments were done in triplicates.

MTT Assay

A 0.5 mg/mL concentration of the MTT solution was added to each well after 1, 3, and 5 days of culture;

Table 1. Description of the treatment details in study groups.

	Groups	Description
1	Control	Cultured PDLSCs
2	FGF2	Cultured PDLSCs + FGF2 (50 ng/mL)
3	LLLT1	Cultured PDLSCs + LLLT with 808 nm diode (on the day of seeding)
4	LLLT2	Cultured PDLSCs + LLLT with 808 nm diode (on the day of seeding and two days after)
5	FGF2 + LLLT1	Cultured PDLSCs + FGF2 (50 ng/mL) + LLLT with 808 nm diode (on the day of seeding)
6	FGF2 + LLLT2	Cultured PDLSCs + FGF2 (50 ng/mL) + LLLT with 808 nm diode (on the day of seeding and two days after)

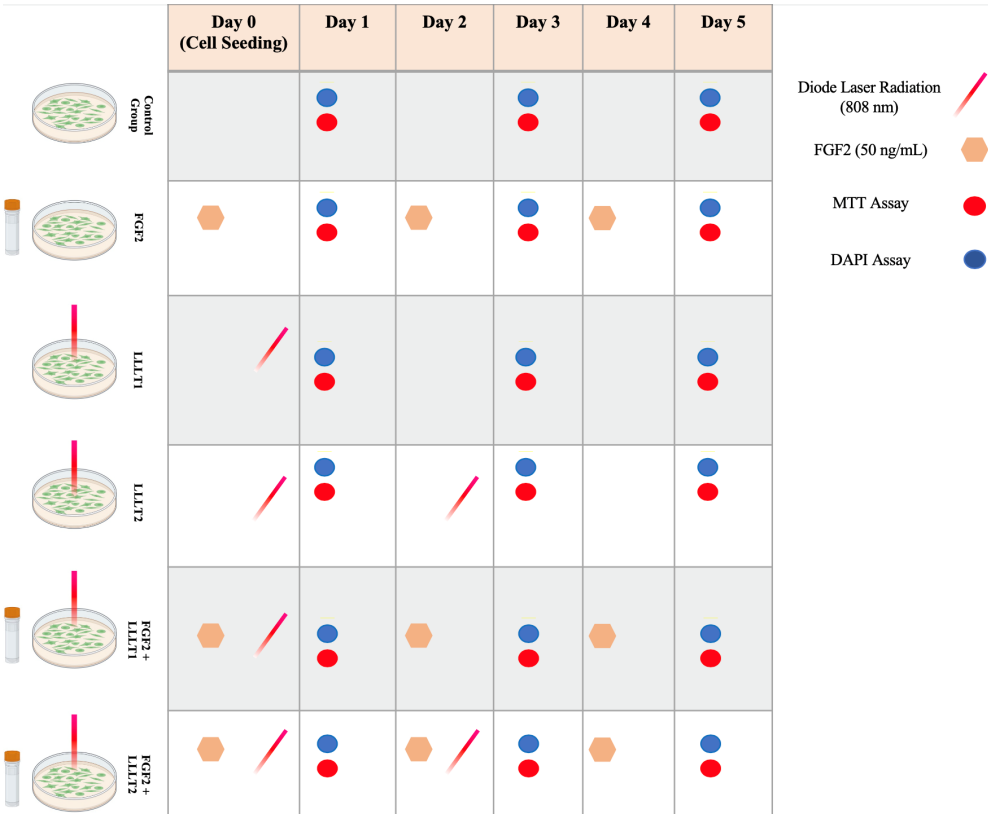

Figure 1. Characteristics and Timeline of the Study Groups

then, the plate was incubated for 3 hours at 37 °C, 98% humidity, and 5% CO_2. The 0.1 mL of DMSO was added to each well, and the culture mediums were transferred to a 96-well plate to be read by an ELISA reader device at a 570 nm wavelength.

DAPI Staining

The seeded cells were gently washed with PBS 2-3 times and then fixed by using 4% paraformaldehyde for 45 min. Following the fixation of the cells, they underwent an additional 2-3 washes with a PBS buffer. The stock of the study was formed by the 1 mg/mL concentration of DAPI solution in sterile water. The solution was introduced into individual wells, which were allowed to incubate for a duration of 45 seconds. Subsequently, the cells were washed with PBS and imaged by using a fluorescent microscope to visualize the cell nuclei.

Statistical Analysis

The statistical analysis was conducted by using GraphPad Prism v9 software. The MTT assay results were analyzed using a two-way ANOVA with the Tukey post hoc test. $P<0.05$ was deemed statistically significant.

Results

MTT Assay

The results of the two-way ANOVA showed significant differences between the study groups regarding cell proliferation ($P<0.0001$). Also, the interaction between time and study group variables was significant ($P<0.0001$). Figure 2 illustrates the outcomes of the analysis conducted considering the groups and time.

Only the control ($P=0.03$) and FGF2 + LLLT2 ($P=0.01$) groups showed a significant increase in the cell proliferation rate from day 1 to 5.

On the first day of the assay, the control group showed

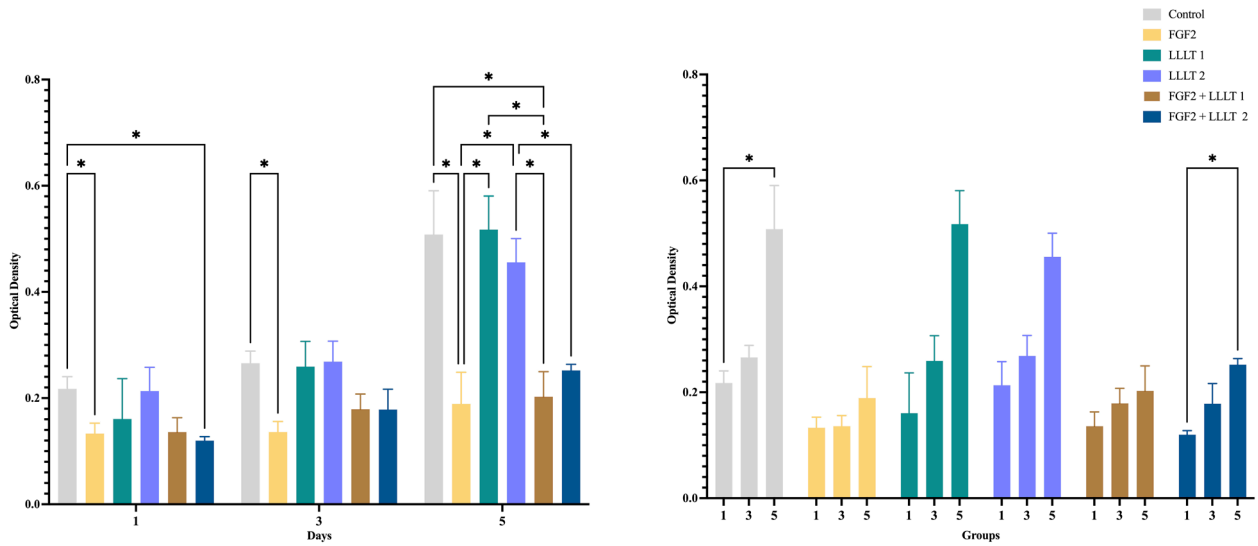

Figure 2. Results of the Cell Proliferation Assay Based on the Groups and Time. * Signs indicate a *P* value lower than 0.05

significantly higher cell proliferation than the FGF2 ($P=0.04$) and FGF2 + LLLT2 ($P=0.04$) groups. On the third day of the assay, only a significantly higher cell proliferation was seen in the control group in comparison with the FGF2 group ($P=0.01$). On the fifth day of the assay, the control group showed significantly higher cell proliferation compared to the FGF2 ($P=0.03$) and FGF2 + LLLT1 ($P=0.04$) groups. The LLLT1 group demonstrated significantly higher amounts of cell proliferation than the FGF2 ($P=0.02$) and FGF2 + LLLT1 ($P=0.01$) groups. Furthermore, it was found that the LLLT2 group had significantly higher cell proliferation than the FGF2 ($P=0.02$), FGF2 + LLLT1 ($P=0.01$), and FGF2 + LLLT2 ($P=0.04$) groups.

Table 2 shows all *P* values resulting from the post hoc comparison between the amounts of cell proliferation between different groups.

DAPI Staining

Figure 3 presents the DAPI staining results for different days. The results show that the control, LLLT1, and LLLT2 had higher amounts of cell viability and adhesion than other experimental groups, which qualitatively confirms the results of the MTT assay.

Discussion

The current study aimed to evaluate the effect of FGF2 and LLLT on the adhesion and proliferation of PDLSCs when they were used on the seeding day. Many factors like surface energy, surface roughness, pH, temperature, fluid flow, and different cell treatments can affect the cell adhesion ability to the substrate.[1,13] In this study, the majority of these variables were controlled to be consistent across various experimental groups, with the exception of the cell treatments, which were the primary focus of investigation in this study. The rationale for these outcomes may be attributed to a "differentiative shock" caused by FGF2 when the cells have not yet adhered to the plate or are functional. The inverse relationship between cell differentiation and cell proliferation[14] suggests that a differentiative shock can lead to down-regulation in the proliferation capacity of the cells.

LLLT has demonstrated stimulatory effects such as enhancing the proliferation and differentiation of various cell types[11,15] as well as the stimulation of growth factors secretion.[15] Moreover, its positive effect on both viability and differentiation of PDLSCs has been shown.[11] FGF2, as a signaling molecule, can also promote cell proliferation, particularly in PDLSCs.[16,17] Additionally, FGF2 has demonstrated positive effects on the differentiation of these cells[12]; nevertheless, this effect has been explored on the adhered cells. On the other hand, LLLT also demonstrated its positive effect on both viability and differentiation of PDLSCs.[11] In the present study, LLLT did not exhibit any negative impact on the viability and proliferation of PDLSCs, even in cases where the cells were freshly seeded and had not yet adhered to the culture plates. Nevertheless, the viability and proliferation of PDLSCs that were freshly seeded were notably diminished by the presence of FGF2. The variation in the impact of FGF2 and LLLT on the viability and adhesion of PDLSCs, despite both demonstrating beneficial effects on the viability and adhesion of PDLSCs, may be attributed to the dose-dependent nature of FGF2, as previously observed. Although the dosage utilized may be deemed safe for cells that are already adhered to and functional, it could potentially hinder the growth and functionality of cells that have not yet been adhered to the surface.

This research was constrained by certain limitations. The adhesion ability of the stem cells could be further investigated through a more comprehensive approach involving the analysis of surface adhesion protein

Table 2. League Table Containing *P* Values Associated With Comparing Each Pair of Experimental Groups' Cell Proliferation Resulting From Two-Way ANOVA and Tukey Post Hoc

0.21 ± 0.02	Day 1						
0.26 ± 0.02	Day 3	**Control**					
0.50 ± 0.08	Day 5						
0.13 ± 0.02	Day 1	0.04 *					
0.13 ± 0.02	Day 3	0.01 *	**FGF2**				
0.18 ± 0.06	Day 5	0.03 *					
0.16 ± 0.07	Day 1	0.80	0.98				
0.25 ± 0.04	Day 3	0.99	0.12	**LLLT1**			
0.51 ± 0.06	Day 5	1.00	0.01 *				
0.21 ± 0.04	Day 1	1.00	0.27	0.88			
0.26 ± 0.03	Day 3	1.00	0.06	0.99	**LLLT2**		
0.45 ± 0.04	Day 5	0.90	0.02 *	0.74			
0.13 ± 0.02	Day 1	0.09	1.00	0.98	0.31		
0.17 ± 0.02	Day 3	0.08	0.42	0.32	0.17	**FGF2 + LLLT1**	
0.20 ± 0.04	Day 5	0.04 *	0.99	0.01 *	0.01 *		
0.12 ± 0.01	Day 1	0.04 *	0.87	0.91	0.22	0.89	
0.17 ± 0.03	Day 3	0.16	0.60	0.36	0.21	1.00	**FGF2 + LLLT2**
0.25 ± 0.01	Day 5	0.10	0.59	0.05	0.04 *	0.59	

P values lower than 0.05 indicate a significant difference and are marked with a * sign. The first column at the left demonstrates the amount of optical density (mean ± standard deviation) related to each group on each day.

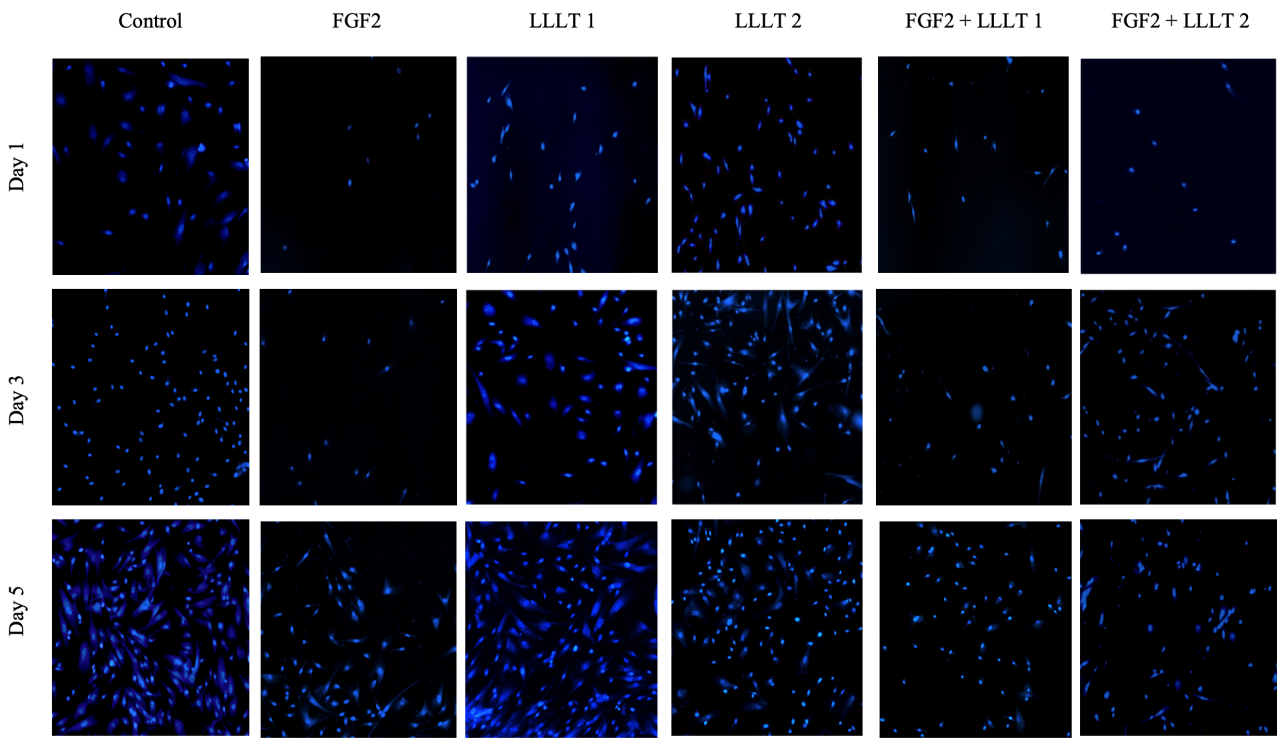

Figure 3. Results of the 4′,6-Diamidino-2-Phenylindole Staining

expression at the protein level. Additionally, the impact of alternative adjunctive therapies beyond FGF2 or LLLT on cell adhesion and viability warrants further exploration.

Conclusion

Considering the limitations of the study, it can be concluded that LLLT is viable for application on the day of seeding on PDLSCs without causing a notable detrimental impact on their viability and adhesion; nevertheless, the use of FGF2 should be restricted on the seeding day and postponed to the subsequent day, as its immediate application may have adverse effects on the

adhesion and viability of PDLSCs.

Authors' Contribution

Conceptualization: Amirhosein Mahmoudian, Hanieh Nokhbatolfoghahaie, Neda Hakimiha, Fazele Atarbashi-Moghadam, Ali Azadi.
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Software: Ali Azadi.
Supervision: Fazele Atarbashi-Moghadam.
Validation: Hanieh Nokhbatolfoghahaie, Neda Hakimiha.
Visualization: Hanieh Nokhbatolfoghahaie, Fazele Atarbashi-Moghadam, Ali Azadi
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Writing–review & editing: Amirhosein Mahmoudian, Hanieh Nokhbatolfoghahaie, Neda Hakimiha, Fazele Atarbashi-Moghadam, Ali Azadi.

Competing Interests

The authors have no conflict to declare regarding this manuscript.

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