



Bone Regeneration of Rat Critical-Sized Calvarial Defects by the Combination of Photobiomodulation and Adipose-Derived Mesenchymal Stem Cells

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Abstract

Introduction: This study explored the synergistic effects of low-level laser therapy (LLLT) and adipose-derived stem cells (ADSCs) on cranial bone regeneration in rats, addressing the limitations of autogenous grafts and advancing bone tissue engineering with innovative photobiomodulation (PBM) applications.

Methods: Sixty Wistar rats were allocated to 5 separate groups randomly; (1) natural bovine bone mineral (NBBM); (2) NBBM+LLLT; (3) NBBM+allogenic ADSCs; (4) NBBM+allogenic ADSCs+LLLT; (5) Only defects. 8-mm calvarial defects were made in each rat in the surgical procedure. A diode laser was applied with the following parameters (continuous mode, power of 100mW, wavelength of 808nm, and 4 J/cm² energy density) immediately after the procedure and every other day. Bone samples were obtained and assessed histomorphometrically and histologically after staining with hematoxylin and eosin (H&E).

Results: Different volumes of bony material were observed in two weeks; 2.94%±1.00 in group 1, 5.1%±1.92 in group 2, 7.11%±2.82 in group 3, 7.34%±2.31 in group 4, and 2.01%±0.83 in group 5 ($P<0.05$). On the other hand, foreign body residuals were up by 23% in the groups with scaffolding by the end of 2 weeks. Four weeks of observation led to 6.74 %±1.95, 13.24%±1.98, 15.76%±1.19, 15.92%±3.4, and 3.11%±1.00 bone formation in groups 1 to 5, respectively ($P<0.05$). Generally, the difference between groups 2-4 was not statistically significant based on different types of bone and the extent of inflammation.

Conclusion: Bearing in mind the limitations of our research, it was demonstrated that ADSCs in combination with PBM have promising effects on bone tissue regeneration in sizeable bony defects. Furthermore, this study also showed that PBM usage improved the newly regenerated bone quality.

Keywords: Photobiomodulation; Bone healing; Regeneration; Adipose-derived stem cell; Mesenchymal stem cell; Laser therapy; Low-level laser.



Introduction

Recent research has demonstrated a drastic increase in bone fractures in the United States of America, and nonunion fracture sites pose a critical danger for scientists.¹ Autogenous bone tissue grafts are the golden standard treatment approach, especially in large bony defects,² because of their exact biological similarity and rapid incorporation, existing growth factors, and osteogenic potentials.³ However, there are several major problems in their clinical administration, including patient's

morbidity due to the second surgery site, restricted amount of tissue, and infection risk.⁴ Various novel therapeutic approaches have been presented to address these problems to regenerate bone, especially in critical-sized bony defects.⁵ Although bone tissue engineering in the past decades has applied a variety of biomaterials, stem cells, and bioactive molecules, until now there has not been any perfect therapeutic approach that can solve all existing shortcomings.⁶ Photobiomodulation (PBM) involves a light application designed to enhance the

healing course⁷ and tissue regeneration,⁸ reduce pain and inflammation,⁹ and change the immunologic reactions of the body.¹⁰ *In vivo*, studies have previously demonstrated that mesenchymal stem cells (MSCs) proliferation can be stimulated by PBM, possibly by altering the genetical expressions.¹¹ Cytokine release changes were demonstrated by Funk et al by PBM via the He-Ne laser.¹² Osteogenesis can be induced with PBM as it increases osteoblastic activity and decreases osteoclastic activities.¹³ Furthermore, studies have shown that bone regeneration can be enhanced by laser therapy-induced regulatory factors in the bone matrix.¹⁴ Additionally, PBM is effective in the bone healing processes of animal models by activating transcription factors such as RUNX-2 genes and escalating the bone morphogenic protein-4 (BMP-4) expression.^{15,16} Different molecular pathways have been mentioned explaining PBM effects one of which is affecting beta-catenin which its dysregulation can interfere with wound healing and chronic wound development.¹⁷ In addition, regenerative treatment modalities, specifically stem-cell-based therapies, need a dependable stem cell source¹⁸ that offers the same efficiency as the stem cells derived from bone marrow which is the most widely used source of stem cells¹⁹ besides less invasiveness and less association with donor age.¹⁸ Several alternative sources such as dental tissue,²⁰ skeletal muscles,²¹ and adipose tissues²² have been used for this purpose. Among these proposed cells, adipose-derived stem cells (ADSCs) were applied effectively in experimental and clinical research for bone reconstruction.²³⁻²⁵ ADSCs are increasingly recognized for their potential in regenerative medicine, especially for bone reconstruction, due to their advantageous properties. They are an abundant source, as they can be harvested from the body's plentiful adipose tissue. The extraction of ADSCs is less invasive and less painful compared to the process for bone marrow mesenchymal stem cells (BMMSCs). ADSCs have shown a longer capacity for proliferation and osteogenic differentiation *in vitro*, which is essential for bone tissue regeneration. Furthermore, pre-treatment with ADSCs has been associated with positive effects on bone regeneration, particularly in catabolic states where there is increased bone turnover. Additionally, ADSCs exhibit low immunogenicity, reducing the risk of rejection and making them suitable for allogeneic transplantation. Their therapeutic potential has been demonstrated in restoring wound defects and promoting bone regeneration, supporting their use in therapeutic applications.^{22,26} Studies have also shown the positive effects of pre-treatment with ADSCs and PBM in catabolic states of bone regeneration.^{1, 27}

Hence, in this research, we sought to evaluate the utilization of low-level laser therapy (LLLT) combined with ADSCs for the reconstruction of bone tissue in cranial defects in rats.

Methods

Animals

Sixty male Wistar rats were attained from Iran Pasteur Institute for this study. This trial was permitted by AJA University of Medical Sciences, and all procedures were done under their Ethics Committee guideline. The rats weighed $250 \text{ g} \pm 25$ and were sexually mature. Before starting the procedure, they were checked for systemic health and then randomly allocated into five groups ($n=12$): (1) Natural bovine bone mineral (NBBM); (2) NBBM + LLLT application; (3) NBBM + allogenic ADSCs; (4) NBBM + allogenic ADSCs + LLLT application; (5) Defects only.

Adipose-Derived Mesenchymal Stem Cell Isolation and Cultivation

Adipose tissue (3-5 mL) was excised from two separated Wistar rats (peritoneal fat tissue) and sent to the laboratory. One percentage of collagenase (I) was used to digest the obtained adipose tissue for 1 hour. Then, the solution was resuspended in Dulbecco's Modified Eagle Medium (DMEM). Finally, The ADSCs were relocated to a flask (T-25) for incubation at 37°C and $5\% \text{ CO}_2$. After attaining 80-90% confluency, 0.25% trypsin-EDTA (Life Technologies, USA) was applied to trypsinize isolated ADSCs.²⁸

Adipose-Derived Mesenchymal Stem Cells Characterization

Flow Cytometry Analysis

To characterize ADSCs, we evaluated the presence or absence of specific cell surface proteins known as cluster of differentiation (CD) markers. This was done by using flow cytometry, a technique that allows for the rapid analysis of multiple characteristics of thousands of cells in a sample.

In this context, the CD markers analyzed were:

- CD-105, CD-73, and CD-90, commonly expressed on the surface of ADSCs and indicative of their stem cell nature,
- CD-45 and CD-34, typically not expressed on ADSCs and used to distinguish them from other cell types such as hematopoietic or endothelial cells, and
- CD-44, a cell surface glycoprotein involved in cell-cell interactions, cell adhesion, and migration.²⁹

By assessing the expression of these markers, the identity of ADSCs was confirmed for further experimental procedures.

Briefly, phycoerythrin-conjugated monoclonal antibodies and fluorescent isothiocyanate-conjugated monoclonal antibodies were added to the cell culture by its explicit machine (BD Biosciences, USA). The cells that were dyed with rat immunoglobulin were used as the negative control. All samples were bathed by phosphate-buffered saline and were secured with paraformaldehyde 1%. All findings were assessed by FlowJo 7.6.1 software,

and 90%-100% expression was recognized as a positive expression of each marker.

Osteogenic Differentiation

Isolated ADSCs at passage three were induced in StemPRO (Life Technologies, Carlsbad, CA, USA) for 14 days. Afterward, the specimens were fixed with paraformaldehyde 1%. Finally, specimens were colored with Alizarin Red for 5 minutes and were portrayed under a light microscope.

Stem Cell Seeding

Natural bovine bone mineral, primarily hydroxyapatite ($\text{Ca}_{10}(\text{PO}_4)_6(\text{OH})_2$) deposited in an organic matrix of which collagen is the major constituent (NBBM) (1.0-2.0 mm crystalline size, Cerabone® granules, Botiss, Germany) which mimics bone structure, was the main material in this research. Cerabone® is composed of pure bone mineral^{1,2} with an average porosity of 40–65 vol %; 70% of the pores are 300–1200 μm and 30% around 600–900 μm . Five milligrams of the scaffold was positioned to each well of a 96-well plate and loaded by 10^6 ADSCs for two days at 37 °C and 5% CO_2 .

Surgical Procedure

Carprofen (Norbrook, New Zealand) was subcutaneously injected into all samples 2 hours before surgery (5 mg/kg). They were anesthetized with ketamine, calvarial hair was shaved, and surgical sites were prepared for the surgery. After exposure of the calvarium by a midline calvarial incision and elevating the periosteum, a trephine with an 8 mm diameter (Fine Science Tools, Inc., CA, USA) was utilized to make a critical-sized bone lesion (Figure 1). Normal saline irrigation was applied during the surgical procedure to minimize tissue necrosis. Then, the defects, according to their study group, were filled with materials or left empty. After these procedures, the periosteum was sutured, followed by skin closure with 3.0 Vicryl resorbable material (Norderstedt, Germany). The five groups of rats were divided into two subgroups ($n=6$) based on

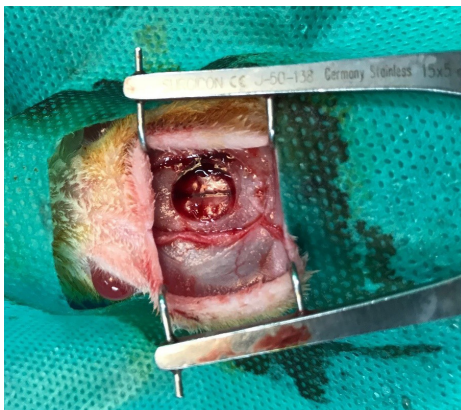


Figure 1. Critical-Sized Calvarial Bone Defect Immediately After the Elevation of the Bone

their follow-up durations and were kept for 2 or 4 weeks. Since using low-power laser therapy increases the proliferation and differentiation of MSCs, based on numerous studies, we wanted to examine early bone regeneration in this article. For this reason, 2 weeks and 4 weeks were chosen for this work.

After completion of the follow-up period, the animals were humanly euthanized with inhalation of CO_2 .

Photobiomodulation Protocol

A gallium-aluminum-arsenide (GaAlAs) diode laser (Photon Lase III, DMC Corporation, USA) was employed in this study. Its wavelength was 808 nm with an optical fiber of 0.8 cm in diameter and a spot size of 8 mm, and LLLT was irradiated over the defect site without any distance between the optical fiber and skin. Before PBM administration, its power was determined and calibrated by an experienced laser technician. The irradiation was done at 100 mW output power and a total dose of 4 J/ cm^2 for 20 seconds in groups 2 and 4 immediately after surgery (cumulative dose of 32 J/ cm^2) and then every two days until the end of the follow-up period (cumulative dose of 60 J/ cm^2). All other groups experienced sham laser administration without any irradiation at the same time points to approximate the stress level of all rats.

Histological Evaluation and Histomorphometric Analysis

The bone tissues were fixed in formalin (10%) for one week. After decalcification, the samples were periodically washed with stiller water, and then ethyl alcohol gradually dehydrated all specimens. After these processes, a paraffin block of samples was prepared. Finally, 5 μm cut along with midsagittal suture by were done a microtome device, and then all prepared sections were colored with hematoxylin and eosin.

One experienced pathologist assessed all sections with an image analytic system (Image-Pro Plus, Media Cybernetic). Light microscopy evaluations consist of the amount of bone formation and residual body. In addition, the amount and type of inflammation were determined with these measurements:

- Inflammation rate (%) counted at 40 $\times$ magnification in fields across the defect
- Type of inflammation (acute and chronic), including neutrophil, lymphocyte, plasma cell
- Foreign body reaction and the presence of xylphagous multinucleated cells around functional residual material/osteoclast cells in the margin of normal bone undergoing resorption or sequestration.

Statistical Analysis

Data were evaluated by SPSS software (SPSS 22.0, Chicago, IL). One-way analysis of variance (ANOVA) was used for comparing the means of histomorphometric quan-

titation. Furthermore, Tukey’s post hoc test was used to determine statistically significant relations between study groups, and the results were reported as mean and standard deviation ($P < 0.05$).

Results

In Vitro Findings

Flow Cytometry

Figure 2 demonstrates flow cytometry findings of isolated rat ADSCs which confirm the high expression of mesenchymal (CD-73, and -105), cell adhesion markers (CD-44, and -90), and low expression of CD-34 and -45

(hematopoietic markers).

Alizarin Red Staining

Mineralized nodule formation was observed in the cultured ADSCs (Figure 3), representing its osteogenic differentiation capability.

DAPI Staining

DAPI staining was done for survival after 48 hours in the incubator. In summary, on the second day after cell seeding, DAPI (40,6-diamidino-2-phenylindole) staining was performed, and scaffolds were rinsed with PBS three

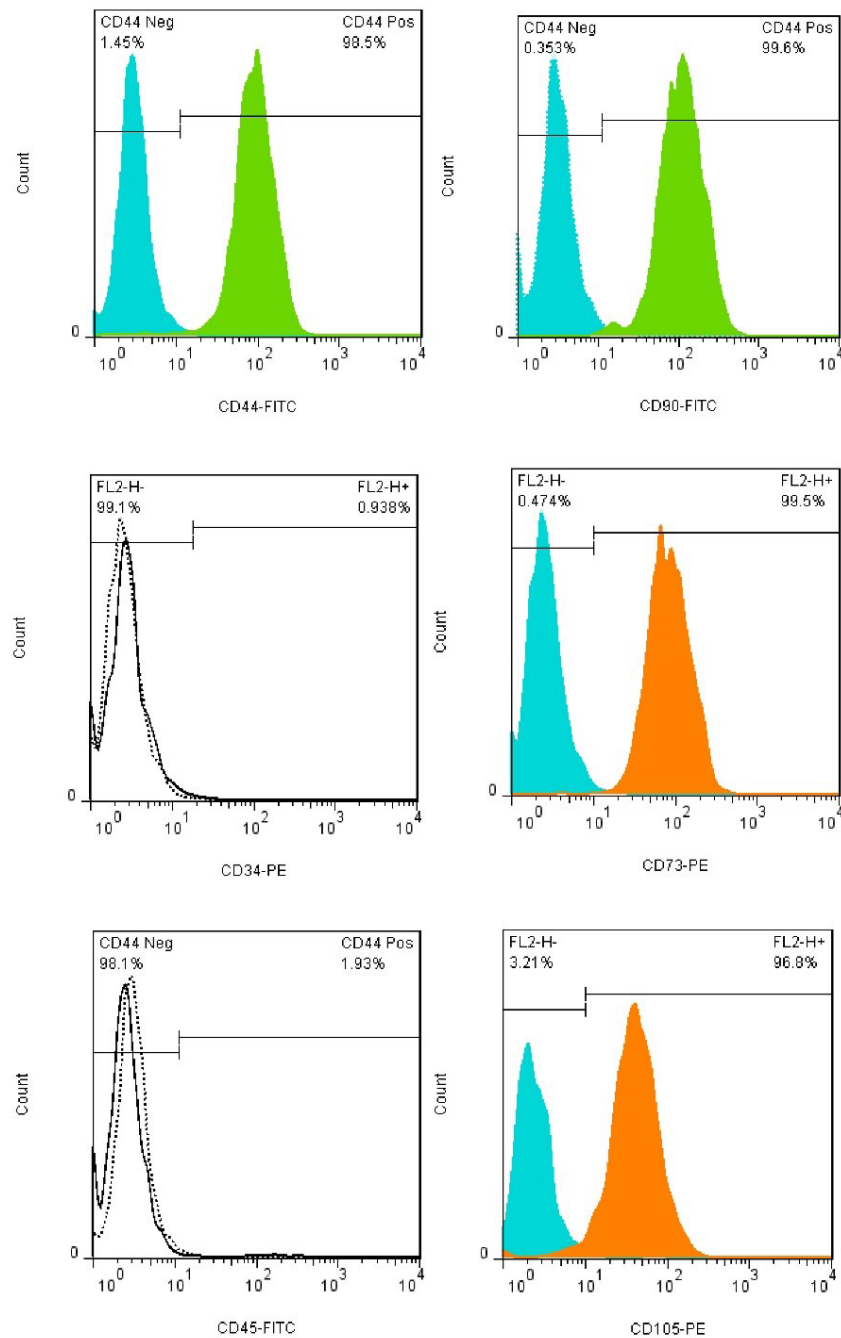


Figure 2. Flow Cytometry Findings of Adipose-Derived Stem Cells. Mesenchymal markers (CD-73 and -105) and cell adhesion markers (CD -44 and -90) were expressed higher than 90%, and CD-34 and CD-45 (hematopoietic markers) were found in a limited number of cells

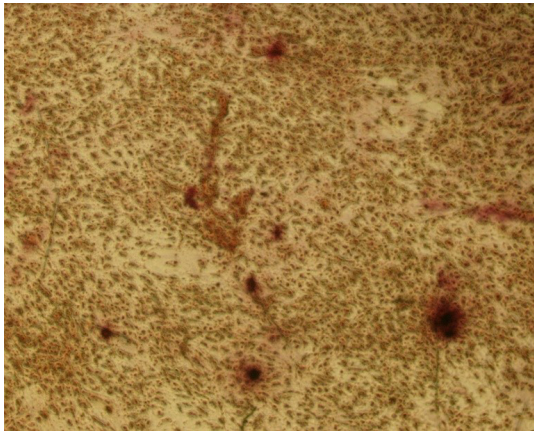


Figure 3. Mineralized Nodule Formation by Isolated Rat Adipose-Derived Stem Cells (Alizarin Red staining) ($\times 40$)

times, fixed with 4% paraformaldehyde (Sigma, USA) for 30 minutes, and rinsed again with PBS. Subsequently, the DAPI solution was added to the scaffold and incubated for 45 seconds. A fluorescence microscope was used to analyze cells (Figure 4).

In Vivo Findings

Histological evaluations (Figure 5A-G) revealed *de novo* bone formation, especially in the edges in all groups after two weeks, except group 5. None of them bridged across the defect. After two weeks, recently formed bone tissue was mostly woven in groups 2, 3, and 4. However, after four weeks, lamellar bone formation with many osteocytes in the lacunae and proliferated osteoblasts in the peripheral region were seen predominantly in groups 3 and 4. The total bone formation was $2.94\% \pm 1.00$ in group 1, $5.1\% \pm 1.92$ in group 2, $7.11\% \pm 2.82$ in group 3, $7.34\% \pm 2.31$ in group 4, and $2.01\% \pm 0.83$ in group 5 ($P < 0.05$), after 2 weeks. Newly formed bone tissues were $6.74\% \pm 1.95$, $13.24\% \pm 1.98$, $15.76\% \pm 1.19$, $15.92\% \pm 3.4$, and $3.11\% \pm 1.00$ in groups 1 to 5, respectively, after 4 weeks ($P < 0.05$) (Figure 6A). Moreover, two weeks post-operative residual foreign bodies in groups 1 to 4 were $25.97\% \pm 3.00$, $23.39\% \pm 4.74$, $23.06\% \pm 3.70$, and $23.39\% \pm 6.59$, respectively ($P > 0.05$) (Figure 6B). These amounts were $18.08\% \pm 1.05$, $17.10\% \pm 5.06$, $18.43\% \pm 4.21$, and $19.02\% \pm 2.82$ after four weeks in groups 1 to 4, respectively ($P > 0.05$) (Figure 6B).

Discussion

The reconstruction of critical-sized bone defects in cranium requires novel alternative therapeutic approaches which lead scientists to a smart solution for the drawbacks of the conventional method.³⁰ In the current study, the impact of PBM combined with ADSCs on bone healing in critical-sized calvarial defects was evaluated. Our findings indicated that the application of PBM in combination with ADSCs could synergistically improve bone healing. Although the most amount of bone formation was

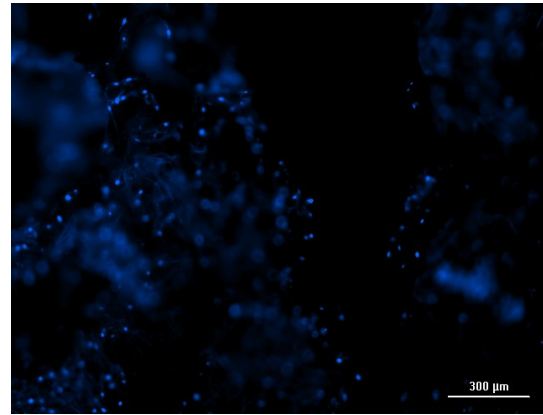


Figure 4. DAPI Staining for the Assessment of Cellular Survival

observed in group 4 (NBBM + ADSCs + LLLT), there was no statistically significant alteration found between groups 2, 3, and 5, which indicates the importance of further investigations, especially some molecular evaluations to make an accurate judgment.

Available literature accentuated the advantageous usage of ADSCs combined with biomimetic scaffolds in osteogenic differentiation and regenerating bone.^{31,32} Therefore, in this study, we used ADSCs for bone regeneration because of the evidence that showed ADSCs have excellent potential for bone healing. Although clinical investigations of the efficacy and safety of ADSCs application in patients remain scant, in agreement with our findings, ADSCs demonstrated promising results on new bone regeneration in craniofacial bony defects.^{33,27} Adipose tissue can deliver a greater quantity of stem cells, compared to bone marrow aspiration, thus suggesting the benefits of using fat as a novel source of osteoprogenitor cells.²² Yoon et al showed a 35%-72% bone development in large rat calvarial defects after 8 to 12 weeks.³⁴ Our results showed a $15.76\% \pm 1.1$ bone development in group 3 (NBBM + ADSCs) after 4 weeks. This difference can be explained by the various follow-up periods and scaffolds of the studies.

Different mechanisms take place in the process of bone formation.³⁵ In the osteoblastic differentiation pathway, OSX, RUNX -2, and other transcription factors start this activity. RUNX -2 plays a vital role in regulating osteogenic differentiation,³⁶ and OSX expression in RUNX -2 expressing precursors provokes osteoprogenitor cells to differentiate into functional osteocytes and osteoblasts at the same time as the time of bone formation.³⁷ Laser therapy increased the expression of RUNX -2, indicating its probable effect on starting and accelerating the bone-forming processes.³⁸ RT-PCR has confirmed these findings,³⁸ IHC analysis,³⁹ microarray hybridization,⁴⁰ and western blot.⁴¹ Thus, in this study, we determined a short-term (two and four weeks) follow-up. This decision was made to evaluate the initial impact of LLLT on the early stage of bone formation. In a long period (approximately

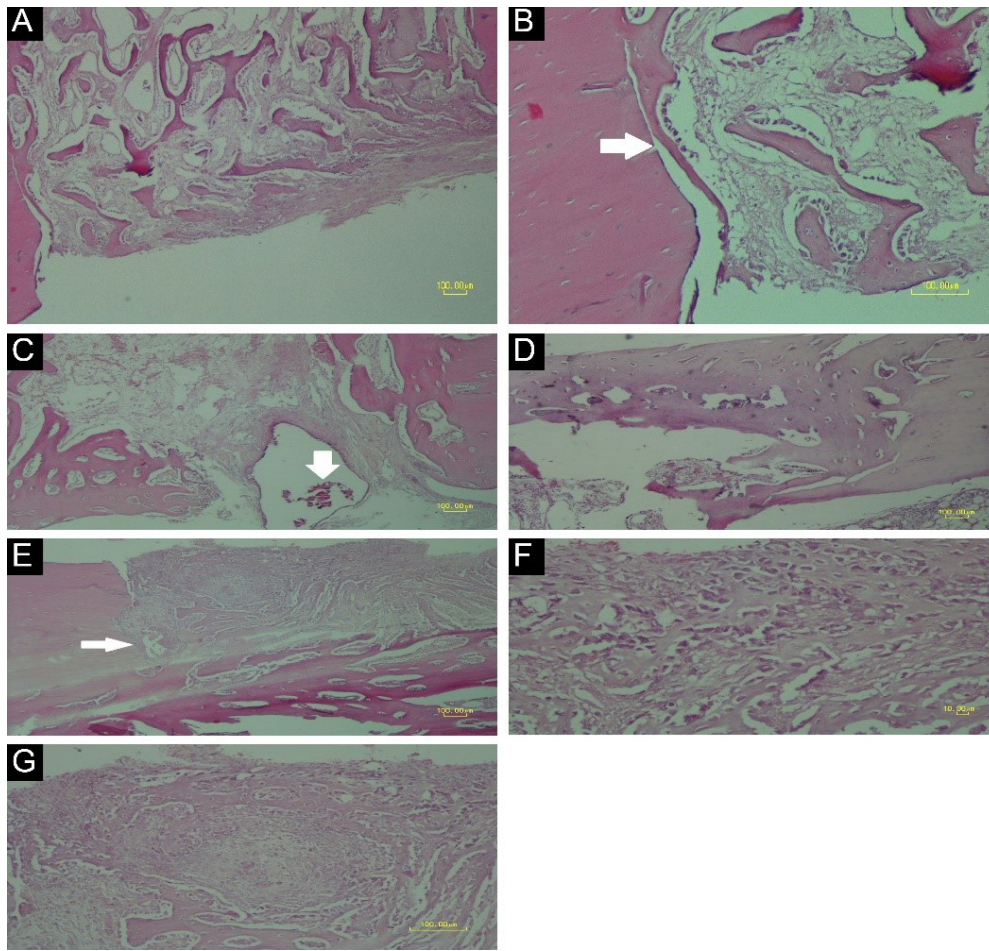


Figure 5. H&E Staining Histogram of newly formed bone in applying low-level laser therapy with adipose-derived stem cells after 4 weeks is shown in **A** and **B**. (**A**) Lamellar bone formation with surrounding fibrosis ($\times 40$). (**B**) White arrow shows the edge of the bone defect ($\times 100$). Similar time point images of adipose-derived stem cells group are demonstrated in **C** and **D**. (**C**) White arrow shows residual body ($\times 40$); (**D**) lamellar bone formation ($\times 100$). In addition, the H&E staining histogram of new bone formation in low-level laser therapy after 4 weeks is shown in **E-G**. (**E**) Lamellar and woven bone formation with surrounding fibrosis. White arrow shows the edge of the defect ($\times 10$); (**F**) Inflammatory cell infiltration ($\times 40$); (**G**) A very osteoblastic concentrated area ($\times 100$).

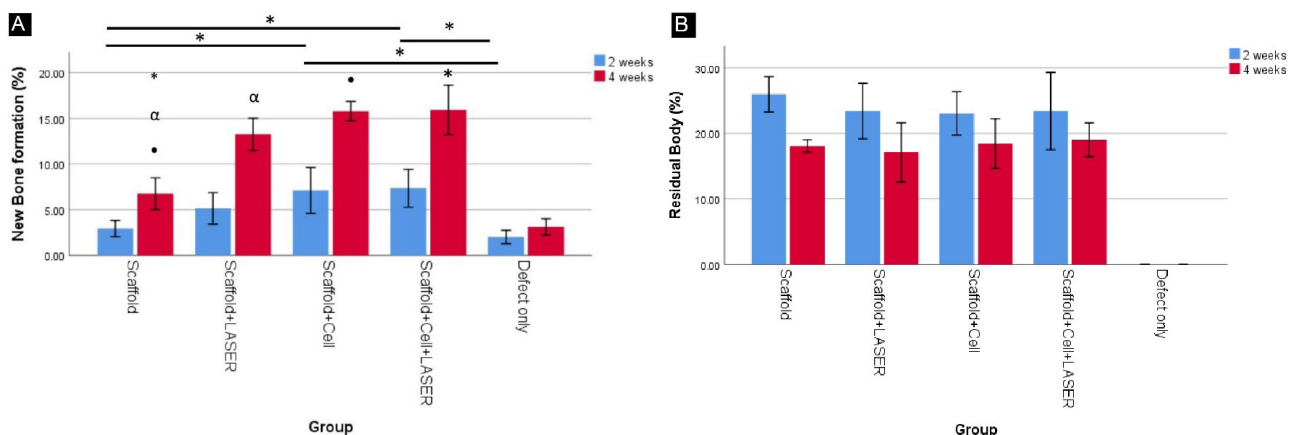


Figure 6. Quantitative Histomorphometric Analysis. **A:** Percentage of new bone formation in each group; **B:** Percentage of residual body in each group. Means $\pm$ standard deviations were compared by ANOVA and Tukey's post hoc test (*, α , and $\bullet$ indicate $P < 0.05$).

longer than 12 weeks), a rat's bone defect would be completely healed.⁴² Hence, after this time, evaluating the accelerating impact of certain kinds of treatment modalities will be undetectable.

In addition, RANKL, a tumor necrosis factor, takes

part in the activation and differentiation of osteoclasts, and ultimately bone healing.⁴³ Magri et al discovered the substantially greater expression of RANKL after Ga-Al-As laser therapy with an 808 nm wavelength for 33 seconds in rats.³⁹ Omasa et al⁴⁴ showed significantly

greater expression of BMP 2 one day post-op after the application of LLLT (830 nm continuous Ga-Al-As diode laser). Moreover, Sella et al⁴⁵ reported a significant rise in OPN and OSN in the initial stages of the bone-forming process (before day 13). They hypothesized that because of the role of these factors in the early stages of the bone generation process, laser irradiation (LLLT, 808 nm, Ga-Al-As continuous laser) can enhance bone recovery, especially in the initial stages, since they continued laser application during the follow-up period. In this regard, we applied an 810 nm Ga-Al-As continuous laser with similar energy density. These two physical properties are the most imperative factors in irradiation. Thus, our findings can be easily explained by these facts regarding the molecular impacts of LLLT (810 nm, 4 J/cm²) on bone regenerative pathways.

In 2013, Choi et al revealed bone formation from the edge of the defect 3 days after the application of LLLT in combination with ADSCs.⁴⁶ They suggested a potential impact of ADSCs on promoting bone healing after activation by LLLT at the early stages of bone regeneration. This finding was in accordance with our results. Moreover, they reported that the 8-mm calvarial bony defect was approximately completely healed after 56 days by the application of LLLT/ADSCs seeded on the acellular dermal matrix. Their stem cell carrier may cause an alteration in the volume of bone formation between their study and ours; however, both results represented a synergistic impact of PBM and ADSCs application.

On the other hand, some reports indicated a non-significant improvement in ADSCs proliferation and differentiation.⁴⁷ Despite the fact that the exact underlying mechanisms of PBM impact on cellular activity are still unclear, it seems that not only do various physical properties of these irradiations play a role in this discrepancy, but also the short duration of laser therapy can have an adverse impact on the results. Hence, we decided to perpetuate LLLT during the whole period of follow-ups. In addition, to unify the conditions of all rats, we applied sham irradiation, which meant a complete procedure of laser application without any irradiation in groups 1, 3, and 5. We used histomorphometric analysis as the gold standard bone formation assessment; however, one of our major limitations is the lack of other bone formation evaluation methods. As the next phase of our comprehensive investigation, we want to perform immunohistochemistry analysis and quantitative PCR.

Conclusion

Acknowledging the constraints of our research, we noted multiple findings. Notably, while the combination of PBM therapy and ADSCs demonstrated a cooperative effect on bone repair in significant bone deficits in animal tests, the use of PBM alone did not show a marked improvement in bone growth when compared to NBBM used with

ADSCs. Although a definitive evidence-based verdict on the specific therapeutic applications of PBM has not been reached, the outcomes of our study may pave the way for other researchers to refine their experimental designs and select more effective methods for their investigations.

Authors' Contribution

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Project administration: Reza Fekrazad.

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Supervision: Reza Fekrazad, Praveen R. Arany, Fatemeh Mashhadiabbas.

Validation: Fatemeh Mashhadiabbas, Reza Fekrazad, Praveen R. Arany.

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Writing—review editing: Sepehr Fekrazad, Reza Fekrazad, Praveen R. Arany.

Competing Interests

None.

Funding

None.

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