Original Article

The Effect of Low-Level Laser Therapy in Combination with Leukocyte- and Platelet- Rich Fibrin on Bone Regeneration in Rabbits' Calvarial Defects: Histologic and Histomorphometric Studies

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Abstract

Objective: Bone regeneration is a desired treatment outcome in implant dentistry. The primary goal of the current investigation was to assess the joint effect of low-level laser therapy (LLLT) and leukocyte- and platelet-rich fibrin (PRF) on new bone formation.

Materials and Methods: During this experiment study, forty bone defects (8 mm in diameter) were generated in the calvaria of ten New-Zealand white rabbits. defects were filled with autogenous bone defined as the control group, autogenous bone with leukocyte- and PRF (PRF group), autogenous bone and low-level diode laser radiation (LLLT group), and autogenous bone with leukocyte- and PRF and low-level laser radiation (LP group). Laser irradiation was done every second day for 2 weeks after surgery. Five rabbits were randomly selected to be sacrificed on postoperative weeks 4 and 8. On one and two-month post-surgery, histological and histomorphometric parameters including bone formation, fibroblast, and osteoblast were assessed.

Results: The histological panel depicted that the ratio of fresh bone formation increased at one-and two-month post-surgery in all treatment groups compared to the control group. The most favorable results were seen in the LP group, followed by the PRF group. Based on the ANOVA test, bone neoformation was statistically significant in the LP group in comparison with the control group (P<0.001). One-month post-surgery, a higher degree of fibroblast was seen in the control group, while the last place was for LP group (118.6 ± 6.9 vs. 24.0 ± 3.2). In the PRF group, the percentage of bone formation was higher than that in the control group (13.2 ± 2.8 vs. 2.0 ± 1.2), but no significant difference when compared to the LP group (13.2 ± 2.8 vs. 19.0 ±.3.8).

Conclusion: The combined L-PRF and LLLT was more likely to have a positive effect on accelerating bone regeneration and reducing fibrosis.

Keywords: Bone Regeneration, Leukocyte- and Platelet-Rich Fibrin, Low-Level Laser Therapy

Introduction

xBone regeneration is a desired treatment outcome. It is well-documented that bone regeneration could be obtained through the grafting of bone (1). The main feature that clinicians are seeking, is to provide osteoinductivity which can be achieved by combining bone grafts with bioactive growth factors or with their containing compounds (2-4).

Platelet-rich plasma (PRP) and platelet-rich fibrin (PRF) have been identified as biological sources encompassing high levels of necessary growth factors for bone regeneration (5). PRF is an autologous blood-derived platelet concentrate created using a simplified procedure that involves no biochemical processing of blood (6). PRF contains a dense fibrin network in which platelets and leukocytes are trapped (7, 8). It seems that the high fibrin content of PRF improves the growth factors and cytokines stability by conserving them from proteolytic degradation and increasing their longevity (9). Finally,

leukocyte content can play an essential role in minimizing inflammation and preventing infection (7).

Studies have reported the benefits of PRF including increased vascularization and an increase in graft stability when combining PRF with bone graft material (5, 10). In addition, some studies have suggested that the use of platelet concentrates can accelerate the bone healing process. This could be because these substances contain platelet-derived growth factors and vascular endothelial growth factors which in combination with a suitable scaffold can transfer the required molecules to the bone regeneration region (6, 11). However, limited evidence showed the effect of PRF on the bone regeneration process (5). For instance, one systematic review addressing the effect of PRF indicated that most studies have reported improvement in soft tissue regeneration and reduction in dimensional changes post-extraction (12).

Low-level laser therapy (LLLT) is regarded as a

promising treatment to accelerate bone metabolism. LLLT employs directional non-ionized electromagnetic radiation in a monochromatic and coherent manner. This can lead to stimulation of bone repair via increasing the osteoblasts' activity, vascularization, and organization of collagen fibers (13). Results from in vivo and in vitro investigations have shown that this therapy could induce bone repair by stimulating the secretion of osteogenic factors (14). Additionally, LLLT can provoke cell proliferation as well as angiogenesis which is an essential factor in bone formation in the primary stage of repairing (14, 15). In recent decades, *in vitro* studies addressing the effect of LLLT on bone regeneration have shown an increase in the activity of the alkaline phosphatase enzyme. Thus, increased intracellular calcium concentrations and osteoblastic activity lead to a higher amount of bone formation (16, 17).

The low-level laser therapy has been proved to impact the proliferation and variation of bone cells, reducing the time of osseointegration of dental implants, preimplantitis therapy, and periodontitis as well as accelerating dental orthodontic movement (15). Although positive effects of LLLT have been reported, there are still studies with contradictory results (18). Such discrepancies might be due to components such as standardized radiation protocol for the surgical procedure or the diversity of experimental models (15, 19).

In recent years, the research on the effect of low-level laser and leukocyte- and PRF on bone regeneration has received wide currency, but there is still controversy on the effects of Leukocyte- and Platelet- Rich Fibrin therapy as well as LLLT on bone regeneration. In the present study, we made an effort to address the effect of leukocyte- and PRF therapy in combination with the LLLT. This histomorphometric study aimed to evaluate the effect of low-level laser combined with PRF and could be a step toward improving bone repair treatments, especially in periodontal interventions.

Materials and Methods

Animals

Ten adult male New Zealand white rabbits, aged close to 6 months, weighing about 2.5 to 3 kg, and raised at the Pasteur Institute of Iran-Tehran, were used in this study. They were kept for 4 and 8 weeks in individual cages at the Iranian Tissue Bank and Research Center, Imam Khomeini Medical Complex (Tehran, Iran), and were provided unrestricted access to food and water. In the laboratory, they were housed in a temperature- and humidity-controlled environment with a 12-hour light/dark cycle.

Experimental design

In the present experimental study, the rabbits were randomly selected for operation. The rabbit's Calvaria bone, the matching bone to the human's Mandible (jaw bone), was selected as a model for bone defect,

which as well, allowed us to observe the repair process 3 to 4 times faster compared to humans (20). Rabbits are equally divided into two groups; five rabbits were randomly allocated to evaluate histological variables at one-month post-surgery and the rest were used two-monthmonths post-surgery. Four treatment groups were defined: i. Defects filled with autograft bone (control group), ii. Autogenous bone mixed with leukocyte- and PRF (PRF group), iii. Autogenous bone and low-level diode laser radiation (LLLT group) and iv. Autogenous bone with leukocyte- and platelet -rich fibrin and low-level diode laser radiation (LP group). On days 30 and 60 after the operation, histological parameters including the number of fibroblasts, percentage of new bone formation, and osteoblast were measured. Histological analyses were performed by two pathologists independently. The experiment was done during the 10:00-17:00 hours light phase.

Preparation of the leukocyte- and platelet-rich fibrin

The Choukroun protocol was used to prepare PRF. Concisely, the tubes containing 5 ml of blood samples, with the source of cardiac, were centrifuged at 2700 rpm for 8 minutes. Following that, L-PRF was removed from the blood cells, and then it was mixed with the autogenous bone to fill the defects (21).

Surgical procedure

The animals were anesthetized using an intramuscular injection of ketamine hydrochloride (10%, 30 mg/kg) and 2% xylazine (Alafason, Woeden, Holland, 3 mg/kg). The rabbit's heads were shaved and the scalp was prepped with povidone-iodine solution. Using a surgical blade a longitudinal anteroposterior incision (10 cm) was created along the midline of the skull from the midpoint of the base of the ears (No. 15). Before cutting the periosteum, the skin was retracted using a surgical mosquito and then using a periosteal elevator, the periosteum was separated from the bone surface cranial to caudal.

Four defects (8 mm in diameter) were generated in the parietal bone (Fig.1). Defects were on both sides of the sagittal suture without crossing the midline employing an electric 2000 rpm handpiece (Dio company, South Korea) and 8mm in diameter round surgical trephine. The obtaining bone by trephine bur was crushed by the bone mill and used as autograft bone in each defect. The first defect was filled with autograft bone. The autograft bone containing Leukocyte- and Platelet-Rich Fibrin was used in the second defect. The third, was filled with autograft bone and radiated by low-level laser. Finally, a mixture of the autograft bone containing leukocyte- and platelet-rich fibers were placed into the fourth defect before using low-level laser radiation. A clockwise counter was applied with no pressure, to fully avoid the particle's entrance to the meningeal zone while filling the defects. Then, the periosteum and the calvarium skin were sutured with

4-0 simple absorbable sutures and 3-0 silk respectively. When animals were brought to full consciousness, they were placed into cages. To prevent infection, one-day post-operation, cefazolin (20 mg/kg, IM) was injected. Tramadol (20 mg/kg, i.m) was also and administrated to relieve pain. Skin sutures were removed 10 days following the surgery.
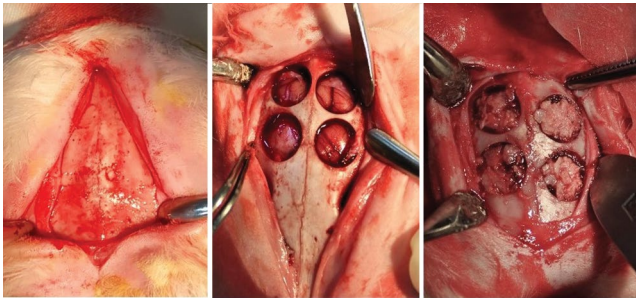

Fig.1: Photographic images of the critical-sized bone defects (8 mm in diameter) in rabbit's calvaria left to right: Flap elevation, defects preparation with trephine bur, defect filling with materials.

Laser irradiation

In the third and fourth defects of each rabbit, Aluminum Gallium Arsenide (GaAlAs) laser (Konf™-Konftec Corporation, Taiwan), wavelength 808nm, power 250 mW, density 0.4 Watt/cm², and spot size 0.5 cm² with frequency 5 J/cm² for 20 seconds were applied. Laser irradiation was done every other day for two-week post-surgery. The center of each defect was marked by a non-absorbable suture on the skin and laser irradiation was done in the center of this marking to avoid any mistakes.

Histological assessment

On 30- and 60-days post-surgery, animals were euthanized with xylazine (Alafason, Woeden, Holland), and the harvested tissue (defect area of calvarial bone) was fixed in the 10% neutral buffered formalin (NBF, pH=7.26) for 48 hours. The samples were decalcified in 10% EDTA, processed, and embedded in paraffin. Then, 5 µm thick sections were prepared and stained with hematoxylin and eosin (H & E), and Masson trichrome (MT). The histological slides were independently assessed by two pathologists using light microscopy (Olympus BX51, Olympus, Tokyo, Japan). The percentage of the new bone formation was assessed in the total area of the defect section. To differentiate the autogenous bone graft (ABG) in defects area from the new bone formation, the area with live osteocyte lacuna was identified as a new bone formation. In addition, to perform histomorphometric analysis, the number of fibroblast and osteoblast was assessed and pictured utilizing Image-Pro Plus® V.6 (Media Cybernetics, Inc., Silver Spring, USA).

Statistical analysis

The sample size was determined based on the effect size of the relevant studies, considering a significance level of 0.05 and 80% study power. Descriptive statistics were reported with mean, frequency, standard deviation (SD), and percentage of parameters in each group. Considering testing normal assumptions for all parameters, one-way ANOVA and Kruskal Walis test were used to calculate differences between groups. Hence, the Bonferroni test and Dunn Post-Hoc test were conducted to test for differences in all possible pairs. Man-Witney test was used to compare means of parameters between the samples one month and two-month post-surgery. A P<0.05 was considered a significant value. All statistical analyses were performed using statistical package for the social sciences for windows, version 25 (SPSS, Inc., Armonk, NY, IBM Corp).

Ethics statement

The protocol of the present research was reviewed and approved (IR.SHAHED.REC.1397.054) by the Shahed University of Medical Sciences Ethics Committee. All experiments followed the guidelines of the Iran Animal Care Committee.

Results

Micrographs of the normal calvarial and histological findings after one- and two months post-surgery can be seen in Figures 2 to 4. In the control group, the defect area was repleted with fibrous connective tissue (FCT) and ABG at one-month post-surgery. After two months, the new bone formation (NB) was negligible and the autogenous bone graft was also removed from the defect area via multi-nucleated giant cells, and the defect area was filled by FCT. In the treatment groups, less fibrous tissue and larger areas of NB were observed compared to the control group. Histomorphometric analysis of four calvarial defects after one-month post-surgery depicted a small area consisting of new bone formation around the ABG in the LLLT group (Fig.3).

At one-month post-surgery, the results showed that fibroblast level was significantly different among the four experimental groups (Table 1, Fig.3). Based on the result, a higher degree of fibroblast appeared in the control group, while the lowest was in the LP group. Moreover, the percentage of the new bone formation was higher in LP, followed by the PRF, but the number of osteoblasts was higher in the LP group in the first month after surgery. There was a statistically significant difference in the amount of bone neoformation among all groups (P=0.001). Results from pairwise comparisons showed that there were significant differences between LP and other groups except for the PRF group. The highest percentage of new bone formation was seen in the LP group compared to the control group (19.0 ± 3.8 vs.

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2.0 ± 1.2). Similarly, the number of osteoblasts was statistically different between LP and the other groups (P<0.05), but no significant difference was observed between LP and PRF (19.0 ± 3.8 first then 13.2 ± 2.8).

and the control group in both samples (Tables 1, 2).

Table 1: Comparison of the mean scores and standard deviations of histological variables between treatment groups after one-month post-operation

Group	Between groups	Fibroblast	Osteoblast	Bone formation
C		118.6 ± 6.9	1.1 ± 0.4	2.0 ± 1.2
	C-LLLT (p)	0.1	0.18	0.26
	C-PRF (p)	0.045*	0.041*	0.052
	C-LP (p)	0.000*	0.000*	0.001*
LLLT		87.2 ± 5.9	7.8 ± 3.3	4.2 ± 0.84
	LLLT-PRF (p)	0.18	0.17	0.8
	LLLT-LP (p)	0.045*	0.04*	0.038*
PRF		63.0 ± 6.4	18.4 ± 1.4	13.2 ± 2.8
	PRF-LP (p)	0.18	0.2	0.22
LP		24.0 ± 3.2	31.8 ± 2.6	19.0 ± 3.8

Data are presented as mean ± SD. C; Control, LLLT; Low-level laser therapy, PRF; Leukocyte-platelet rich fibrin, LP; PRF+LLLT, (p); P value, and *; Significant difference between groups (significant level=0.05).
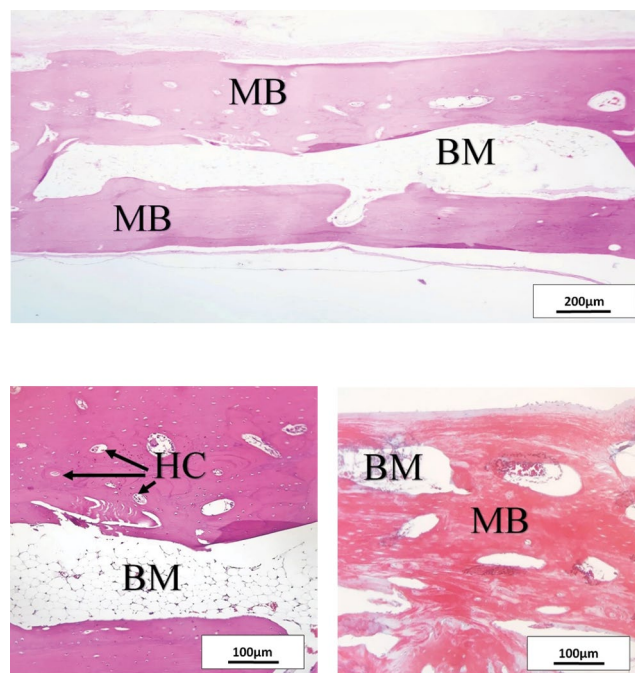

Fig.2: Histopathological evaluation of the normal calvarium. MB; Mature bone, BM; Bone marrow, HC; Haversian canal, H & E; Hematoxylin and eosin, and MT; Masson trichrome.
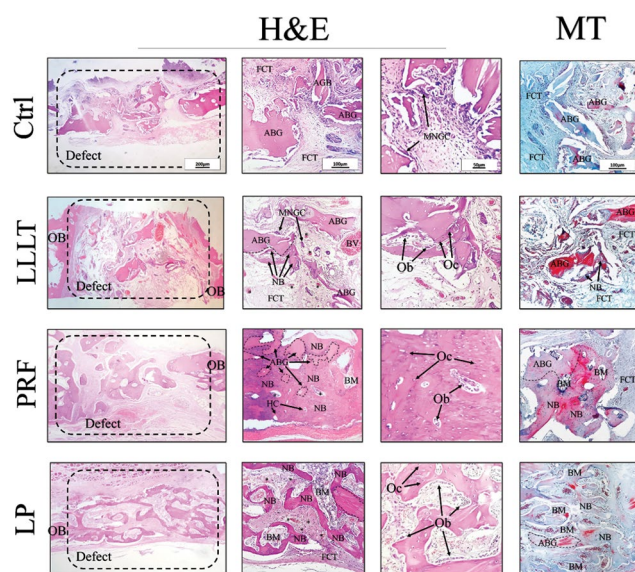
Likewise, after two months, the results showed that fibroblast level, number of osteoblasts, and percentage of bone neoformation were statistically different among all groups (Table 2, Fig.4). The LP group had a significantly higher percentage of bone formation and osteoblast compared to the other groups. In contrast, the degree of fibroblast proliferation was higher in the control than in the LP (186.1 ± 8.6 vs. 13.8 ± 16.9). The results revealed that the amount of bone neoformation was higher in the LP group compared with the control group (63.8 ± 28.1 vs. 5.8 ± 1.6). Meanwhile, there was no significant difference between PRF and LP groups, while significant differences were seen between LP and other groups.

The findings suggested significant differences in the level of fibroblast, the percentage of new bone formation, and osteoblast level between two one-and-two months after surgery (Figs. 3, 4). Additionally, the combined effect of LLLT and PRF on bone formation, osteoblast, and fibroblast was significant as there was a statistically significant difference between the LP

Fig.3: Histological findings for the effect low-level laser therapy and leukocyte and- platelet rich fibrin on fibrous tissue formation on calvarium bone regeneration in rabbit, 1-month post-surgery. OB; Old bone, ABG; Autogenous bone graft, MNGC; Multi nucleated giant cell, FCT; Fibrous connective tissue, BV; Blood vessels, Ob; Osteoblasts, Oc; Osteocytes, *; Newly formed blood vessels, NB; New bone formation, HC; Haversian canal, BM; bone marrow, H & E; Hematoxylin and eosin, MT; Masson trichrome, Ctrl; Control, LLLT; Low-level laser therapy, PRF; Leukocyte-platelet rich fibrin, and LP; PRF+LLLT.

Fig.4: Histological findings for the effect of low-level laser therapy and leukocyte and- platelet rich fibrin on fibrous tissue formation on calvarium bone regeneration in rabbit, 2-month post-surgery. FCT; Fibrous connective tissue, ABG; Autogenous bone graft, RABG; Residue of autogenous bone graft, Ob; Osteoblasts, Oc; Osteocytes, *; Newly formed blood vessels, NB; New bone formation, HC; Haversian canal, BM; Bone marrow, OS; Osteoid, MB; Mature bone, H & E; Hematoxylin and eosin, MT; Masson trichrome, Ctrl; Control, LLLT; Low-level laser therapy, PRF; Leukocyte-platelet rich fibrin, and LP; PRF+LLLT.
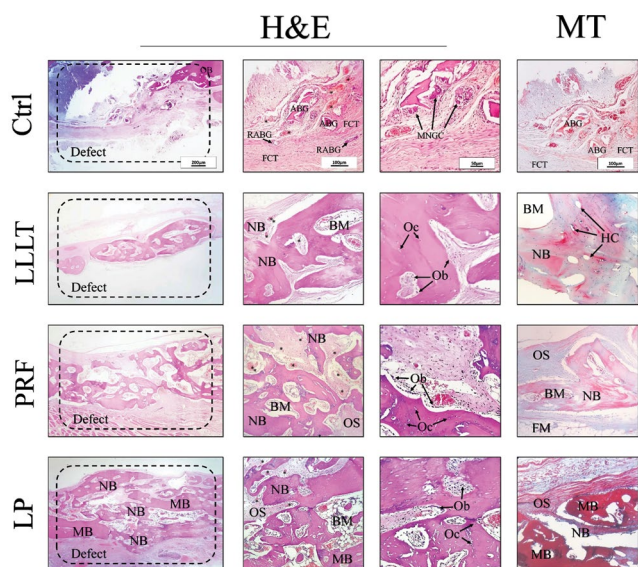
Table 2: Comparison of the mean scores and standard deviations of histological variables between paired treatment groups after two-month post-operation

Group	Between groups	Fibroblast	Osteoblast	Bone formation
C		186.1 ± 8.6	3.2 ± 0.5	5.8 ± 1.6
	C-LLLT (p)	0.65	0.44	0.65
	C-PRF (p)	0.028*	0.035*	0.02*
	C-LP (p)	0.002*	0.003*	0.003*
LLLT		40.8 ± 6.2	47.2 ± 5.6	41.8 ± 2.8
	LLLT-PRF (p)	0.22	0.47	0.18
	LLLT-LP (p)	0.28	0.55	0.36
PRF		29.8 ± 5.3	54.2 ± 3.3	52.0 ± 2.8
	PRF-LP (p)	0.45	0.47	0.59
LP		13.8 ± 16.9	74.4 ± 31.7	63.8 ± 28.1

Data are presented as mean ± SD. C; Control, LLLT; Low-level laser therapy, PRF; Leukocyte-platelet rich fibrin, LP; PRF+LLLT, (p); P value, and *; Significant difference between groups (significant level=0.05).

Discussion

Bone structure is capable of regeneration and repair itself, but this process can be hampered due to certain diseases and the size of the bone lesion (15). To date, numerous methods have been proposed to speed up the process of bone healing. While our lit review revealed that drug therapy and surgery are the most recognized methods, others including laser therapy and using bioactive material have been also suggested (19). The present study aimed to evaluate the effect of the LLLT in combination with PRF on calvarium bone regeneration in rabbits.

As suggested by Kramer et al. (22), we created four circled defects of 8mm in diameter on the parietal bones of rabbit's calvaria. Using standardized defects of 8mm allows a remarkable increment in their interaction with bone graft materials without affecting the other defects (23).

The histological assessment showed that bone neoformation and the number of osteoblasts and fibroblasts were not different between PRF and LP groups, while a pairwise comparison of PRF with the control group indicated a significant difference. As results showed, the effect of PRF was observed in creating osteoblast and new bone formation within the defect area.

Chang and Zhao (24) reported that PRF increases phosphorylated extracellular signal-regulated protein kinase, osteoprotegerin, and alkaline phosphatase activity which provide benefits for periodontal regeneration in human osteoblast cell and pulp cells and suppress osteolytic activity. Additionally, Leucocytes secrete a considerable amount of vascular endothelial growth factor (VEGF) and platelets that contain angiogenesis stimulators including VEGF and basic fibroblast growth factors (25). A study on the effect of PRF on the rabbit's cranial lesions showed that the degree of immunostaining for VEGF was higher compared to the control group. As suggested, PRF can increase the number of bone marrow cells in calvarial defects (26). Another experimental study on rabbits found that there was more new bone formed around the defect area in the PRF group than in the control group after one-month post-surgery, but no significant difference was seen between the PRF group and the other treatment groups including biphasic calcium phosphate and Bio-Oss (27). On the other hand, an *in vivo* study on the effect of PRF and leukocytes on bone regeneration including hemispheres implanted in rabbit calvaria reported no additional effect on bone regeneration at 1 week, 5 weeks and 12 weeks after surgery. As suggested by Knapen et al. (21), further investigations are required using critical size defect model.

In the low-level laser therapy group, a small area of new bone formation around the autogenous bone graft was observed one-month post-surgery, but there was no significant difference between this group and the PRF group. After two months, the percentage of bone formation increased, although osteointegration and mineralization of the bone matrix were inadequate and immature. Recently, Atasoy et al. (28) reported that GaAlAs 940 nm laser with different energy intensities (5,

10 and 20 J/cm²) have no significant impact on the course of bone healing in both stages of bone formation. The bio modulatory effects of laser are dose-dependent and highly influenced by the method of use. There is no standard energy density for the stimulation of bone healing. Some reports suggested energy densities of 1-5 J/cm2 while others referred to a total energy density of 16 J/cm2 seems to be more efficient for bone metabolism (29). One systematic review on the effect of the low-level laser therapy on the maxillofacial bone defects supported that the improvement in bone density can be obtained when using LLLT after maxillofacial bone defects surgery. It has been reported that LLLT has anti-inflammatory and analgesic potential and accelerates the healing process. However, the authors suggested that protocols for using LLLT should be standardized before drawing any concrete conclusions (15). Another review found that low-level laser treatment reduces the duration of the bone healing process, although there are no standardized protocols for the surgical procedure (19).

In this study, a significant increment in formation of new bone was seen in the LP group compared to the control group, whereas no significant difference was found between LLLT and the control group. On contrary, several studies supported that the isolated effect of LLLT was significant on bone regeneration, but the synergistic effect of combined LLLT could not improve bone regeneration significantly. For example, a study on the synergistic effect of LLLT (GaAlAs, 810 nm) and mesenchymal stem cells on bone regeneration in rabbit's calvarial defects reported that although LLLT significantly enhanced bone regeneration, there was no significant synergistic effect of combined LLLT and mesenchymal stem cells (30). In addition, a histological study revealed that the isolated effect of low-level laser therapy and low-intensity pulsed ultrasound boosted bone formation in the rabbit calvarium, but combined therapy failed to produce an additive effect on the reconstruction of defects (31). The mechanism of how LLLT enhances tissue healing is not completely understood, but it seems that absorbed laser light by tissue increases mitochondrial activity, local blood circulation, ATP synthesis, collagen synthesis, and the release of VEGF (32). Another study addressing the effect of LLLT and platelet concentration on bone repair in rats found that LLLT reduced inflammation and increased bone formation. However, platelet concentrate therapy with autogenous failed to increase bone repair alone or in combination with LLLT. The ineffectiveness of LLLT in the combined group is questionable, but in the platelet concentrate group, the result is predictable due to the use of sodium citrate as an anticoagulant in platelet concentrate. To support our result, the ineffectiveness of LLLT could be due to not having a single documented protocol for using low-level laser therapy which could be regarded as shortcoming of the present study. We suggest further investigations on this topic with more sample size as well as a longer-term evaluation.

Conclusion

In the present study, Leukocyte- and PRF improved bone regeneration by increasing the formation of new bone and reducing fibrosis. The best treatment results were in the Leukocyte- and PRF group with low-level laser therapy, but low-level laser treatment alone did not significantly improve the bone regeneration process.

Acknowledgments

Authors' Contributions

F.T., F.Sh., M.T.; Contributed to conception and design. A.Kh., M.H.T., M.T., F.Sh.; Contributed to all experimental work, data and statistical analysis, and interpretation of data. F.T., F.Sh.; Were responsible for overall supervision. F.Sh., M.H.T.; Drafted the manuscript, which was revised by A.Kh., F.T. All authors read and approved the final manuscript.

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