Journal of Photochemistry and Photobiology B: Biology 151 (2015) 180–185

Contents lists available at ScienceDirect

Journal of Photochemistry and Photobiology B: Biology

journal homepage: www.elsevier.com/locate/jphotobiol

The effects of combined low level laser therapy and mesenchymal stem cells on bone regeneration in rabbit calvarial defects ☆

R. Fekrazad [a], M. Sadeghi Ghuchani [b,*], M.B. Eslaminejad [c,*], L. Taghiyar [c], K.A.M. Kalhori [d], M.S. Pedram [e], A.M. Shayan [f], N. Aghdami [c], H. Abrahamse [g]

[a] Dental Department, AJA University of Medical Sciences, Tehran, Iran
[b] Gorgan Faculty of Dentistry, Golestan University of Medical Sciences, Golestan, Iran
[c] Department of Stem Cells and Developmental Biology, Cell Science Research Center, Royan Institute for Stem Cell Biology and Technology, ACECR, Tehran, Iran
[d] Iranian Medical Laser Association, Tehran, Iran
[e] Department of Clinical Sciences, Faculty of Veterinary Medicine, University of Tehran, Iran
[f] Tabriz Faculty of Dentistry, Tabriz University of Medical Sciences, Tabriz, Iran
[g] Laser Research Center, Faculty of Health Sciences, University of Johannesburg, South Africa

ARTICLE INFO

Article history:
Received 2 February 2015
Received in revised form 16 July 2015
Accepted 11 August 2015
Available online 11 August 2015

Keywords:
Low Level Laser Therapy (LLLT)
Bone regeneration
Stem cell
Animal study

ABSTRACT

Objective: This study evaluated the effect of Low Level Laser Therapy (LLLT) and Mesenchymal Stem Cells (MSCs) on bone regeneration.
Background data: Although several studies evaluated the effects of MSCs and LLLT, there is little information available regarding *in vivo* application of LLLT in conjunction with MSCs.
Methods: Forty-eight circular bone defects (6 mm in diameter) were prepared in the calvaria of 12 New-Zealand white rabbits. The defects of each animal were randomly assigned to 4 groups: (C) no treatment; (L) applying LLLT; (SC) filled with MSCs; (SCL) application of both MSCs and LLLT. LLL was applied on alternate days at wavelength of 810 nm, power density of 0.2 W/cm^2 and a fluency of 4 J/cm^2 using a Gallium–Aluminum–Arsenide (GaAlAs) diode laser. The animals were sacrificed after 3 weeks and then histological samples were evaluated to determine the amount of new bone formation and the remaining scaffold and inflammation.
Results: The histological evaluation showed a statistically significant increase in new bone formation of LLLT group relative to the control and the other two experimental groups ($p < 0.05$). There was no significant difference in bone formation of the control group compared to experimental groups filled with MSCs. Laser irradiation had no significant effect on resorption of the scaffold material. In addition, inflammation was significantly reduced in LLLT group compared to the control defects and the other two experimental groups.
Conclusion: Low level laser therapy could be effective in bone regeneration but there is no evidence of a synergistic effect when applied in conjunction with MSCs.

1. Introduction

Bone defects are not always straightforward to repair [1]. A variety of methods have been developed to enhance bone repair including autogenous bone grafts or synthetic materials used to fill up the defects [1,2]. Up until recently, the use of autologous grafts has been considered as the gold standard of treatment for bone regeneration [1]. However, its implementation has been restricted due to difficulties in the procedure of obtaining autogenous bone, including inadequate bone supply, donor site pain, infection, remaining scar and risk of nerve injury [1,2]. Other types of bone materials including demineralized bone matrix, Hydroxyapatite, Tricalcium phosphate, etc., are not as effective as the autogenous bone due to lower osteo-inductive capacity [3,4]. Efforts to overcome the problem led to the development of tissue engineered bone regeneration [5].

Bone tissue engineering benefits from Mesenchymal Stem Cells (MSC) along with bone material (natural or synthetic) instead of

☆ This study was funded by AJA University of Medical Sciences and Royan Institute for Stem Cell Biology and Technology.
* Corresponding authors at: P.O. Box 16635-148, Tehran, Iran.
E-mail addresses: rezafekrazad@gmail.com (R. Fekrazad), moss.sadeghi@gmail.com (M. Sadeghi Ghuchani), eslami@royaninstitute.org (M.B. Eslaminejad), leila_taghiyar@yahoo.com (L. Taghiyar), kathykalhor@yahoo.com (K.A.M. Kalhori), mpedram@ut.ac.ir (M.S. Pedram), sh_arman_ima@yahoo.com (A.M. Shayan), Nasser.Aghdami@ki.se (N. Aghdami), habrahamse@uj.ac.za (H. Abrahamse).

http://dx.doi.org/10.1016/j.jphotobiol.2015.08.002
1011-1344/

using autografts [5,6]. The bone material is actually a resorbable scaffold which carries the stem cells into the defect and helps to preserve the bone volume [4,5]. The MSCs, on the other hand, are the vital components which are responsible for new bone formation [5]. These cells are known as a group of multi-potent, self-renewing progenitor cells which are able to differentiate into all types of cells with the mesenchymal origin, including osteoblasts, chondroblasts and myoblasts [5,6]. They are easily obtainable from adult bone marrow and some other tissues such as fat tissue and periosteum [7]. Although their high proliferation and differentiation potential could be effective in regeneration of bone defects, several studies demonstrated just a moderate improvement of new bone formation by using MSCs [7,8]. In these studies, a variety of osteo-inductive techniques have been used to increase the rate, quantity and quality of bone repair, including biochemical compounds (growth factors, bone morphogenic proteins) and physical stimulus (low level laser, ultrasonic waves) [5,9].

In the past decade, several studies have evaluated the effects of Low Level Laser Therapy (LLLT) on bone regeneration [9–11]. It has been showed that *in vitro* application of LLLT could lead to higher activity of alkaline phosphatase enzyme, increased intra-cellular calcium concentration and increased activity of osteoblasts leading to a higher rate of new bone formation [12–14]. In addition, several *in vivo* studies have revealed that LLLT promotes the rate and amount of new bone formation in standardized bone defects and also improves bone healing in artificial fractures [11,15,16]. However, there is little information about the *in vivo* effects of using LLLT in conjunction with MSCs on bone healing. The aim of this study was to evaluate the effect of LLLT alone and in conjunction with autologous MSCs on bone regeneration of calvarial defects in rabbits.

2. Materials and methods

2.1. Animals

Fifteen male New Zealand white rabbits aged between 8 and 12 months and with a mean weight of 3320 ± 370 g were used in this study. The bone marrow sampling procedure commenced 1 week after adaptation. All animals were housed separately in large, well-lit standard cages in an animal laboratory controlled for temperature (21 °C) and maintained with a daily photoperiod of 12 h of light. Each animal had *ad libitum* access to food and water. All experiments followed the guidelines of the Iran Animal Care Committee and were approved by the AJA University of Medical Science animal care committee, Ethical Approval No. 88/12/12.

2.2. Bone marrow sampling

Intramuscular injection of 50 mg/kg ketamine hydrochloride (100 mg/mL, Alfasan, Woerden-Holland) and 10 mg/kg xylazine hydrochloride (20 mg/mL, Alfasan, Woerden-Holland) were used for anesthetizing the rabbits. After shaving and disinfecting the region, almost 3 mL of bone marrow were aspirated from the humerus of the animals using the Jamshidi aspiration needle and a 10 mL syringe containing 3000 U of heparin. All procedures were carefully performed under sterile conditions to avoid bacterial infection of the samples.

2.3. Mesenchymal stem cells preparation

The bone marrow aspirates were suspended in 5 mL Dulbecco's modified Eagle's medium (DMEM, Gibco, Germany) supplemented with 100 IU/mL penicillin (Sigma, USA), 100 IU/mL streptomycin (Sigma, USA) and 10% Fetal Bovine Serum (FBS). The cells were plated in 75 cm^2 culture flasks at a density of 10^5 cells/mL in an atmosphere of 5% CO_2 and 37 °C [17]. After 1 week, cells attached to the bottom of the flasks were washed by phosphate buffered saline (PBS – Gibco, USA) and culture medium was replaced with fresh DMEM [17]. The flasks were incubated and medium replacement took place twice per week until the cultures became confluent. At 60–70% confluence, second passage cultures of MSCs were washed with PBS and trypsinized with trypsin/EDTA (0.2%). MSCs labeled with vital fluorescent dye (PKH26) 24 h before the surgical procedure to track cells proliferation. The cells were then suspended in collagen type I (Koken, Japan) at a density of 10^5 cells/mL and loaded onto Bio-Oss scaffolds in preparation for surgery.

2.4. Bio-Oss resorbable scaffold

Bio-Oss was used in several studies as the scaffold for MSCs [17–19]. Bio-Oss is a resorbable natural bone substitute usually used for bone regeneration in the oral cavity [16]. It is originally deproteinized bovine bone in particulate form with high interconnecting porosity which makes it suitable for angiogenesis and also cell proliferation and migration [17–19]. It is integrated into the natural modeling and remodeling procedures and the new bone deposited directly on the Bio-Oss particles. These features make Bio-Oss a reliable scaffold in tissue engineered bone regeneration.

2.5. Surgical procedure

Animals were intramuscularly anesthetized as described above. After shaving and disinfecting the animals' head, a full thickness incision was made over the midline to expose the underlying parietal bone. Four symmetric circular full thickness bone defects were made through the calvaria using a trephine bur with an outer diameter of 6 mm as described in previous studies [20] (Fig. 1). These bone defects in each animal served as four different experimental groups: control (C), laser (L), stem cells (SC) and combination of laser and stem cells (SCL). Scaffolds and MSCs were inserted into the SC and SCL defects and the other two defects (C and L) remained empty. The L and SCL defects received one treatment of LLLT before closing the incision. Finally the periosteum and the skin were tightly sutured in two different layers with an absorbable 4-0 suture material (Vicryl, Ethicon, USA) to seal the area and keep the scaffolds in their places. All the animals received subcutaneous injection of enrofloxacin (5 mg/kg, Baytril, Bayer Corp, USA) and tramadol hydrochloride (4 mg/kg, Tehran Chemie Pharmaceutical Co, Tehran, Iran) for 5 days after the surgery. The animals were sacrificed after 3 weeks by an overdose of sodium pentobarbital.

2.6. Low level laser therapy

The laser therapy for L and SCL defects was initiated at the time of surgery and then continued every other day for 3 weeks. A continuous emission mode Gallium–Aluminum–Arsenide (GaAlAs) diode laser (THOR Photomedicine Ltd. UK) with a wavelength of 810 nm, power output of 200 mW, power density of 0.2 W/cm^2, spot size of 1 cm^2, distance of 0.5 cm, period of 20 s and fluency of 4 J/cm^2 per session was utilized as the source of LLLT.

2.7. Histologic and histomorphometric evaluation

After 3 weeks, all the animals were sacrificed and the calvariae were removed and fixed in 10% buffered formalin solution for 2 weeks. The specimens were soaked in 14% EDTA solution for decalcification while their softening or calcification was controlled frequently as they were to be cut by microtome device. After

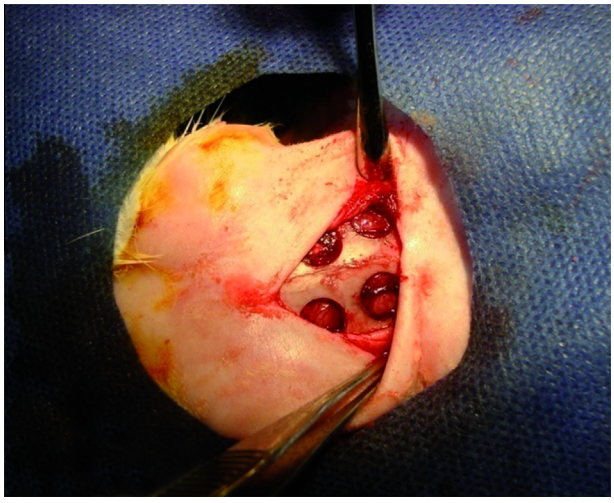

Fig. 1. Photograph of four circular bone defects created with the diameter of 6 mm.

embedding into the paraffin wax, histological sections of 5 μm thickness were prepared and then stained with hematoxilyne and eosin. The specimens' tissue was covered by a thin lamellar glass and evaluated by a pathologist blinded to the defect treatment modality using a light microscope with 40× magnification (double-head light microscope, Nikon, Eclipse E-400, Japan). Photomicrographs were taken from different areas of the defects using a digital camera (Nikon Fuji HC-300 Nikon) and the percentage of new bone formation and remaining scaffold was calculated using a computer assisted histomorphometric analysis system (Iranian histomorphometric analysis software – IHMM-version 1) [15]. The amount of inflammation was estimated for each sample, considering the frequency of inflammatory cells in the high power field (×400), ranging from grade 1 to 5: 1, less than 30%; 2, between 10% and 30%; 3, between 30% and 50%, 4, between 50% and 70% and 5, more than 70% cellular infiltration. Fluorescence illumination (Fig. 2) was used in some sections to verify the survival and proliferation of PKH26-labeled MSCs on the grafts after staining with 5 μg/mL DAPI solution (Sigma, USA).

2.8. Sample size and statistical analysis

The sample size in each group was defined considering standardized effect size (corresponding mean and standard deviations), significance level of 0.05 and 90% power, in addition to possibility of 20% drop off of the samples during the study.

The means and standard deviations of new bone region, remaining scaffold and inflammation were calculated for each group of defects. Considering normal distribution of values, one way ANOVA test and then Scheffe post hoc multiple comparison test were used to compare the data. p values less than 0.05 were considered statistically significant in each case.

3. Results

3.1. Clinical observation

Twelve rabbits recovered normally after the surgical procedure and three animals died during the recovery period after anesthesia. The surgical region repaired normally and no complications were observed among the remaining 12 rabbits. The following reported results are based on histological evaluation of 48 bone defects on 12 rabbits. The average weight of animals at the time of sacrifice was 3750 ± 530 g.

3.2. Histological analysis

Extensive proliferation of MSCs was indicated by increased fluorescence of experimental group samples (Fig. 2). The percentage of new bone formation, remaining Bio-Oss and amount of inflammation at 3 weeks after the surgery are listed in Table 1. Statistical analysis indicated a significant difference in level of bone formation among the defect groups ($p < 0.001$). Group L showed the highest level of new bone formation which was statistically significantly higher than the other three groups ($p < 0.05$). Compared to group C, groups S and SCL indicated a higher level of new bone formation but, the difference was not statistically significant ($p > 0.05$). In addition there was no statistically significant difference between groups S and SCL with respect to the level of bone formation ($p > 0.05$) (Table 1).

Bio-Oss was used as the scaffold only in groups S and SCL. After 3 weeks, there was no statistically significant difference between these two groups regarding the amount of remaining Bio-Oss ($p > 0.05$).

Histological evaluation demonstrated different levels of chronic inflammation in each group of defects. Groups SC and SCL demonstrated a statistically significant higher level of inflammation compared to the other two groups (L and C) ($p < 0.05$). However, the differences between groups SC and SCL and between groups C and L were not statistically significant ($p > 0.05$) (Fig. 3).

4. Discussion

The present study was performed to clarify the combined and individual contributions of LLLT and bone marrow derived MSCs to bone regeneration of calvarial defects in rabbits. Although several studies have reported on the isolated effects of LLLT or MSC on bone regeneration [6–9,15], there is just one other study assessing the synergistic effect *in vivo* [11].

Male adult New Zealand white rabbits were used as the animal model in this study because of their appropriate size which allowed induction of four 6 mm circular defects in each animal's calvarium. In this study, the bone defects were smaller than the Critical Size Defect (CSD) in rabbits. CSD is known as the smallest defect on a bone of specific species which will not spontaneously repair during its life time [21]. Although a range of 10–15 mm has been considered as the critical size defect of rabbits' calvaria, some studies have concluded that it is not possible to define the exact dimension of CSD [21,22]. Furthermore, it has been suggested that the term "critical size defect" should no longer be used because the repair potential of bony margin was shown to be constant despite defect size [22]. Therefore, we used four 6 mm circular defects to investigate different methods of bone regeneration in a relatively short period of time. Several studies reported significant amounts of new bone formation even after 2 weeks, so we chose 3 weeks of follow up to compare the effect of interventions on the early stages of healing process [7,23].

Histological assessment in this study demonstrated higher level of inflammation in SC and SCL groups comparing to L and C groups. Although there was no foreign body reaction or severe inflammation in neither of groups, the higher level of chronic inflammation in SC and SCL groups could be due to Bio-Oss. In spite of the fact that Bio-Oss is frequently reported as a biocompatible and osteoconductive biomaterial, it may be the reason of higher inflammation in the early stages of bone healing in this study [17,18]. In addition, slight movements of Bio-Oss particles in full-thickness bone defects may be in part responsible for more physical irritation and inflammation.

Histomorphometric assessment indicated that group L had the highest level of new bone formation ($L = 27.8\%$). Our results are

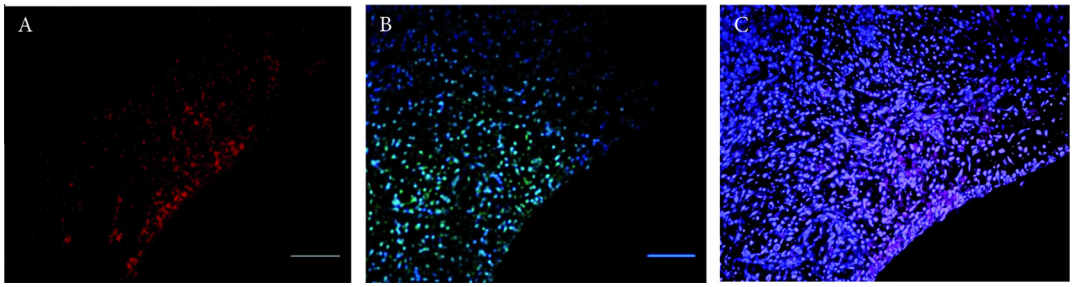

Fig. 2. Frequency and distribution of fluorescence-labeled MSCs in bone treatments of experimental group. MSCs were labeled using PKH26 in red (A) while the nuclei were stained with fluorescence-labeled DAPI indicated in blue (B). Merging A and B produced (C). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

Table 1
Mean ± SD values of the histological variables as percentages within surgically induced defects.

Defect groups	New bone formation (%)	Remaining Bio-Oss (%)	Inflammation (%)
Control (C)	10.78 ± 7.05	0.0 ± 0.0	18.2 ± 3.5
Laser (L)	27.80 ± 10.90[a]	0.0 ± 0.0	16.9 ± 4.7
Stem cells (SC)	15.23 ± 7.17	27.14 ± 5.35	59.8 ± 8.6[b]
Laser and stem cells (SCL)	16.12 ± 6.25	29.41 ± 6.46	55.3 ± 6.2[b]

[a] Statistically significant difference from all other defect groups.
[b] Statistically significant difference from the control and laser groups.

similar to those of Khadra et al., who used LLLT to enhance bone regeneration in calvarial bone defects of rabbits [9]. They employed a diode laser with a wavelength of 830 nm, power output of 75 mW and total energy density of 23 J/cm^2, to treat 2.7 mm bone defects immediately after surgery and 6 days thereafter. Their results revealed that the experimental group had significantly higher levels of bone formation, angiogenesis, and fibroblast proliferation after 2 and 4 weeks. Furthermore, they showed increased levels of calcium, phosphate and protein in the laser group. In another study by Pretel et al., a single dose of LLLT with wavelength of 780 nm and power output of 35 mW led to significantly improved bone regeneration and lower level of

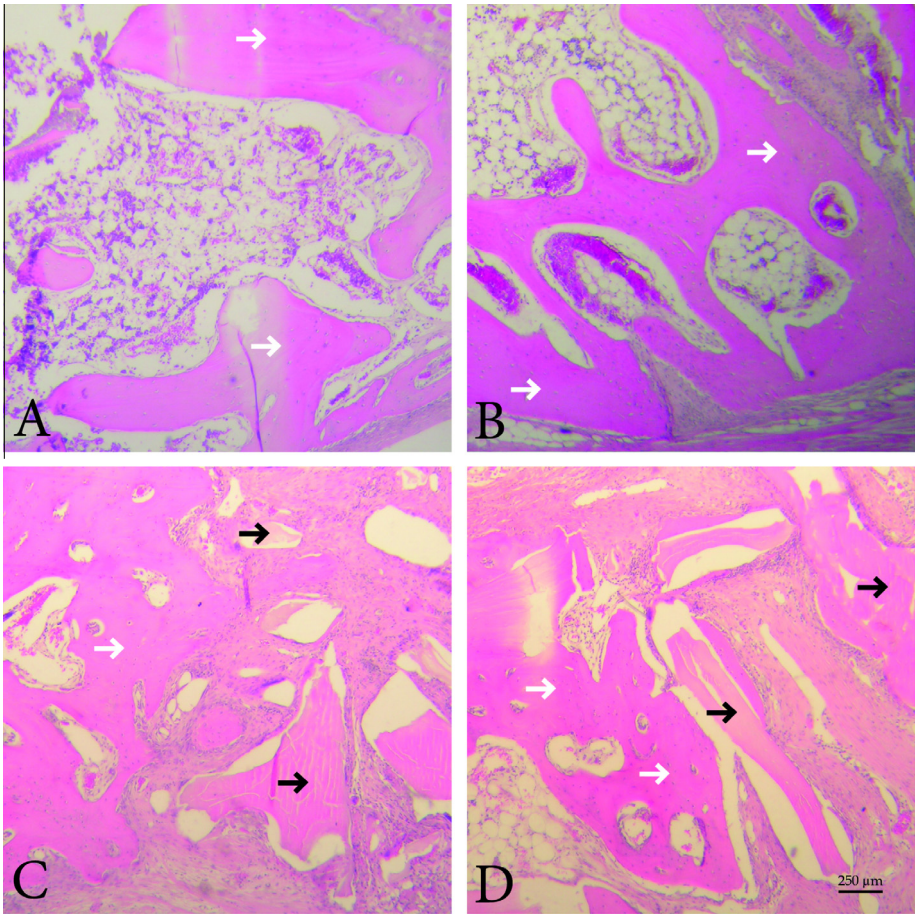

Fig. 3. Histomorphometric analysis of 4 calvarial defects after 3 weeks. New bone formation is indicated by white arrowheads. Black arrowheads indicate remaining graft material (Bio-Oss). (A) Control group, 11.9% of new bone formation. (B) Laser group, 28% of new bone formation. (C) Stem cell group, 13.6% of new bone formation and 33% of remaining Bio-Oss. (D) Stem cell and Laser group, 17% of new bone formation and 13% of remaining Bio-Oss.

inflammation in groove-shaped defects after 15 and 45 days [10]. Interestingly, they found no difference between groups after 60 days. Considering all similar studies, it seems that LLLT could be significantly effective in improving rate of bone regeneration in animals. However, this result is in contrast to findings of Choi et al., who found no statistical difference between bone regeneration of LLLT group and Acellular Dermal Matrix (ADM) group in 4 mm calvarial defects of mice [11].

Although histomorphometric evaluation of the samples after 3 weeks showed higher levels of new bone formation for groups SC and SCL (SC = 15.23%, SCL = 16.12%) compared to the control group (C = 10.78%), the difference was statistically insignificant. This indicates that using bone marrow derived MSCs and Bio-Oss could not significantly improve bone regeneration during a 3 week period of time in calvarial defects of rabbits. However, it led to relatively higher amounts of bone formation after just 21 days and if the samples were assessed for a longer period of time, it might have made a bigger difference. Similar results were obtained by Yun et al., who investigated synergistic effects of BMMSCs and Platelet-Rich Plasma (PRP) on bone regeneration [24]. They used calvarial defects of 6 mm diameter and hydroxyapatite as the scaffold for MSCs. They found no significant difference between groups after 2–4 weeks and only after 8 weeks the group with MCSs and PRP showed a higher amount of new bone formation. Another study conducted by Behnia et al., using 8 mm calvarial defects and Bio-Oss as the scaffold, also reported no significant difference between MSCs group and control group after 6 weeks [25]. However, they reported significantly higher bone formation in the experimental group after 12 weeks. Additional studies that also reported similar short term results in regards to application of MSCs in rabbits, indicate that using MSCs in regards to improving bone regeneration requires more than 3 weeks to be efficient [24]. However, in a recent experimental study Choi et al. reported significantly higher amount of bone formation just after 2 weeks by using adipose-derived mesenchymal stem cell seeded ADM on 4 mm calvarial defects of mice [11]. The probable explanation may include environmental stress (e.g. changes in temperature, available nutrition, inflammation of the region, etc.) caused by transferring MSCs from enriched media into the animal body or even the physical manipulation of cells during the surgery which affected their proliferation rate and function. Furthermore, this delayed bone formation could be due to the time that MSCs need to differentiate into functional osteoblasts. However, after a short period of time, MSCs contribute to new bone formation and accelerate the healing process [7,24,25]. High levels of inflammation in groups SC and SCL could be another contributing factor to reduced bone formation. The increased inflammation may be attributed to an immunologic response to the scaffold or even the physical irritation of the region.

According to the results of the present study, using low level laser therapy along with MSCs for a period of 3 weeks had little advantage over implementation of MSCs alone, considering the amount of new bone formation. These results are in contrast to the previously published data from Choi et al., who reported a significant positive effect of using HeNe laser in conjunction with MSCs on bone generation of mice [11]. There are also some *in vitro* studies which presented positive effects of LLL in cell cultures, including increased proliferation and differentiation of stem cells, higher amount of growth factor secretion and increased levels of calcium and alkaline phosphatase activity [26,27].

In addition to the local effects of LLLT, there are some reports on the systematic effects of LLLT as well [28]. Although these systemic effects are not as strong as the local ones, it could be considered as a limitation of the present study. So, further studies could be accomplished by selecting different defects in separate animals.

5. Conclusion

Using LLLT in the experimental conditions described for a period of 3 weeks could be significantly effective in improving bone regeneration. However, applying MSCs alone or in addition to LLLT may not make a significant difference in bone formation over a short period of time in rabbits. This study conclusively indicated no synergistic effect of MSC and LLLT in conditions described.

Conflict of interest

No competing financial interests exist.

References

[1] G.F. Rogers, A.K. Greene, Autogenous bone graft: basic science and clinical implications, J. Craniofac. Surg. 23 (1) (2012) 323–327.
[2] C.M. Misch, Autogenous bone: is it still the gold standard?, Implant Dent 19 (5) (2010) 361.
[3] P.V. Giannoudis, H. Dinopoulos, E. Tsiridis, Bone substitutes: an update, Injury 36 (3) (2005) S20–S27.
[4] R. Dimitriou, E. Jones, D. McGonagle, P.V. Giannoudis, Bone regeneration: current concepts and future directions, BMC Med. 9 (1) (2011) 66.
[5] J.C. Reichert, D.W. Hutmacher, Bone tissue engineering, in: Tissue Engineering, Springer, 2011, pp. 431–456.
[6] H. Petite, V. Viateau, W. Bensaid, A. Meunier, C. de Pollak, M. Bourguignon, et al., Tissue-engineered bone regeneration, Nat. Biotechnol. 18 (9) (2000) 959–963.
[7] P. Stockmann, J. Park, C. von Wilmowsky, E. Nkenke, E. Felszeghy, J.-F. Dehner, et al., Guided bone regeneration in pig calvarial bone defects using autologous mesenchymal stem/progenitor cells–a comparison of different tissue sources, J. Cranio-Maxill. Surg. 40 (4) (2012) 310–320.
[8] S.J. Stephan, S.S. Tholpady, B. Gross, C.E. Petrie-Aronin, E.A. Botchway, L.S. Nair, et al., Injectable tissue-engineered bone repair of a rat calvarial defect, Laryngoscope 120 (5) (2010) 895–901.
[9] M. Khadra, N. Kasem, H.R. Haanæs, J.E. Ellingsen, S.P. Lyngstadaas, Enhancement of bone formation in rat calvarial bone defects using low-level laser therapy, Oral Surg., Oral Med., Oral Pathol., Oral Radiol., Endodontol. 97 (6) (2004) 693–700.
[10] H. Pretel, R. Lizarelli, L. Ramalho, Effect of low-level laser therapy on bone repair: histological study in rats, Lasers Surg. Med. 39 (10) (2007) 788–796.
[11] K. Choi, B.J. Kang, H. Kim, S. Lee, S. Bae, O.K. Kweon, et al., Low-level laser therapy promotes the osteogenic potential of adipose-derived mesenchymal stem cells seeded on an acellular dermal matrix, J. Biomed. Mater. Res. B Appl. Biomater. 101 (6) (2013) 919–928.
[12] C. Chapple, L. Yum, M. Darendeliler, N. Hunter, The effects of low level laser irradiation on osteoblastic cells, Clin. Orthod. Res. 4 (2001) 3–14.
[13] L. Abramovitch-Gottlib, T. Gross, D. Naveh, S. Geresh, S. Rosenwaks, I. Bar, et al., Low level laser irradiation stimulates osteogenic phenotype of mesenchymal stem cells seeded on a three-dimensional biomatrix, Lasers Med. Sci. 20 (3–4) (2005) 138–146.
[14] A. Stein, D. Benayahu, L. Maltz, U. Oron, Low-level laser irradiation promotes proliferation and differentiation of human osteoblasts in vitro, Photomed. Laser Ther. 23 (2) (2005) 161–166.
[15] A.A.R. Ghahroudi, A.R. Rokn, K.A. Kalhori, A. Khorsand, A. Pournabi, A. Pinheiro, et al., Effect of low-level laser therapy irradiation and Bio-Oss graft material on the osteogenesis process in rabbit calvarium defects: a double blind experimental study, Lasers Med. Sci. 29 (3) (2014) 925–932.
[16] S.K. Shakouri, J. Soleimanpour, Y. Salekzamani, M.R. Oskuie, Effect of low-level laser therapy on the fracture healing process, Lasers Med. Sci. 25 (1) (2010) 73–77.
[17] A. Khojasteh, M.B. Eslaminejad, H. Nazarian, Mesenchymal stem cells enhance bone regeneration in rat calvarial critical size defects more than platelete-rich plasma, Oral Surg., Oral Med., Oral Pathol., Oral Radiol., Endodontol. 106 (3) (2008) 356–362.
[18] S.R. Thaller, J. Hoyt, K. Borjeson, A. Dart, H. Tesluk, Reconstruction of calvarial defects with anorganic bovine bone mineral (Bio-Oss) in a rabbit model, J. Craniofac. Surg. 4 (2) (1993) 79–84.
[19] S. Sartori, M. Silvestri, F. Forni, A. Icaro Cornaglia, P. Tesei, V. Cattaneo, Ten-year follow-up in a maxillary sinus augmentation using anorganic bovine bone (Bio-Oss). A case report with histomorphometric evaluation, Clin. Oral Implant Res. 14 (3) (2003) 369–372.
[20] P. Schmidlin, F. Nicholls, A. Kruse, R. Zwahlen, F. Weber, Evaluation of moldable, in situ hardening calcium phosphate bone graft substitutes, Clin. Oral Implant Res. 24 (2) (2013) 149–157.
[21] J.-Y. Sohn, J.-C. Park, Y.-J. Um, U.-W. Jung, C.-S. Kim, K.-S. Cho, et al., Spontaneous healing capacity of rabbit cranial defects of various sizes, J. Periodont. Implant Sci. 40 (4) (2010) 180–187.
[22] G.M. Cooper, M.P. Mooney, A.K. Gosain, P.G. Campbell, J.E. Losee, J. Huard, Testing the "critical-size" in calvarial bone defects: revisiting the concept of a critical-sized defect (CSD), Plast. Reconstr. Surg. 125 (6) (2010) 1685.

[23] J.-Y. Lee, M.-H. Choi, E.-Y. Shin, Y.-K. Kang, Autologous mesenchymal stem cells loaded in Gelfoam® for structural bone allograft healing in rabbits, Cell Tissue Banking 12 (4) (2011) 299–309.
[24] J.-H. Yun, J.-H. Yoo, S.-H. Choi, M.-H. Lee, S.-J. Lee, S.U. Song, et al., Synergistic effect of bone marrow-derived mesenchymal stem cells and platelet-rich plasma on bone regeneration of calvarial defects in rabbits, Tissue Eng. Regen. Med. 9 (1) (2012) 17–23.
[25] H. Behnia, A. Khoshzaban, M. Zarinfar, f. Mashhadi Abbas, H. bahraminasab, A. Khojasteh, Histological evaluation of regeneration in rabbit calvarial bone defects using demineralized bone matrix, mesenchymal stem cells and platelet rich in growth factors, J. Dent. School Shahid Beheshti Univ. Med. Sci. 30 (3) (2012) 143–154.
[26] H.K. Kim, J.H. Kim, A.A. Abbas, D.-O. Kim, S.-J. Park, J.Y. Chung, et al., Red light of 647 nm enhances osteogenic differentiation in mesenchymal stem cells, Lasers Med. Sci. 24 (2) (2009) 214–222.
[27] Fd.P. Eduardo, D.F. Bueno, P.M. de Freitas, M.M. Marques, M.R. Passos-Bueno, Cd.P. Eduardo, et al., Stem cell proliferation under low intensity laser irradiation: a preliminary study, Lasers Surg. Med. 40 (6) (2008) 433–438.
[28] M.S. Moreira, I.T. Velasco, L.S. Ferreira, S.K.K. Ariga, D.F. Barbeiro, D.T. Meneguzzo, et al., Effect of phototherapy with low intensity laser on local and systemic immunomodulation following focal brain damage in rat, J. Photochem. Photobiol., B: Biol. 97 (3) (2009) 145–151.