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IMPACT OF IN-VIVO LOW-LEVEL LASER THERAPY COMBINED WITH RABBIT BONE MARROW MESENCHYMAL STEM CELLS TO PROMOTE BONE REGENERATION. MORPHOLOGICAL AND GENE EXPRESSION ASSESSMENTS.

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ABSTRACT

Background: Regenerative procedures are intended to restore both function and architecture of the destroyed periodontal tissue. Bone marrow mesenchymal stem cells (BMMSCs) showed high efficacy in the potentiation of bone regeneration. Additionally, Low-level laser therapy (LLLT) enhances tissue repair.

Purpose: This study aims to compare the regenerative impact via LLLT, BMMSCs in chitosan carrier gel either alone or in combination of both treatments on rabbit femoral bony defects.

Materials and Methods: 12 New Zealand albino rabbits were enrolled in the study. Two holes were drilled in each femur of all rabbits. The rabbit population was divided into 4 groups. Group A injected with chitosan carrier gel; Group B was injected with Rabbit-BMMSCs in chitosan; Group C where LLLT (diode laser, 980 nm, 0.3 W, continuous mode, and 250 mW) was applied on each hole loaded with chitosan carrier gel. Finally, Group D received both treatments in group B and C. Rabbits were sacrificed after 8 weeks. Morphological and genetic assessments were performed. All data were statistically analyzed.

Results: Best bone healing results were seen in the combined group of LLLT with BMMSCs followed by the BMMSCs group alone. LLLT group showed inferior healing to the BMMSCs group but still better than the control group. These findings were confirmed by RT-PCR which revealed the highest OC and ALP and lowest OPG in the combination group followed by BMMSCs, LLLT, and control groups in descending order.

Conclusion: The combination of LLLT application to the BMMSCs transplanted in the rabbit femur defects provided the greatest bone regeneration results.

KEYWORDS: BMMSCs, LLLT, OPG, ALP, OC

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INTRODUCTION

Periodontal treatment associated with the control of local and systemic predisposing factors is the most effective method to reduce the progression of periodontal disease. Regenerative procedures are intended to restore both function and architecture of the destroyed periodontal tissue.[1] Periodontal regeneration comprises the use of occlusive barrier membranes, hard- and soft-tissue grafts, root biomodification, laser biostimulation, tissue engineering procedures, or a combination of these procedures.[2]

The tissue engineering field is based on the integration between progenitor cells, natural or synthetic scaffolds, and specific signaling molecules to enhance the regeneration of new tissue.[3] Bone marrow mesenchymal stem cells (BMMSCs) are widely used in tissue engineering. They showed a wide variety of cellular differentiation lineage including odontoblasts, osteoblasts, and fibroblasts in response to different culture conditions. Kawaguchi *et al*.[4] verified the ability of BMMSCs to potentiate bone regeneration in class III furcation defects created in dogs. Other studies confirmed the high osteogenic capacity of BMMSCs and considered it to be one of the most suitable stems cell used for bone regeneration.[5,6] The second key element in the process of tissue engineering is the scaffold. Chitosan is a biodegradable naturally occurring carbohydrate biopolymer. It is biocompatible and has proved to be beneficial to enhance wound healing and bone formation. Thus, it has the characteristics that enable it to be utilized both as a bone substitute and as a scaffold to facilitate cells adhesion, migration, and proliferation.[7]

On the other hand, laser technology has emerged with advantageous properties that enable it to be utilized in tissue healing. Low-level laser therapy (LLLT) plays important role in tissue repair, analgesic, and anti-inflammatory action through photochemical tissue interaction (Laser Biostimulation).[8] Photochemical LLLT act within the laser wavelength from 623 nm (diode laser) to 1064 nm (Nd-YAG laser).[9] Cellular absorption of LLLT occurs through Cytochrome-C chromophores inside the mitochondrial respiratory chain increasing ATP levels, the release of growth factors, and collagen synthesis. Moreover, the anti-inflammatory and anti-edematous effects exerted by laser occur through an acceleration of microcirculation.[10] Thus, LLLT has been reported to enhance induction of specific cellular differentiation, cellular division, proliferation, and tissue organic and non-organic matrix production.[11]

In addition, LLLT was demonstrated to suppress the gene expression event of proinflammatory interleukins such as IL-1α, IL-8, IL-1β, and IL-6 as well as tumor necrosis factor-alpha.[12] LLLT was used in the different periodontal clinical applications including the field of bone regeneration.[13]

The current study was designed to compare the regenerative impact via LLLT alone, BMMSCs in chitosan carrier gel alone, and a combination of both treatments on rabbit femoral bony defects.

MATERIALS AND METHODS

The authors adopted the guidelines of the National Research Council's Guide for the Care and Use of Laboratory Animals.[14] The study was approved by the research ethics committee of the Faculty of Dentistry, Ain-Shams University, Egypt with registration no. ***FDASU-Rec R122105***.

Animals

12 New Zealand albino rabbits 6 months old (3.5–4.5 kg in weight) were enrolled in the present study. The experiment was held in the animal house of the Faculty of Medicine, Cairo University. All rabbits were kept under the same nutritional and environmental conditions in the experimental animal house with respect to the Five Freedoms of Farm Animal Welfare Council.[15] The rabbit population was divided into 4 groups, 3 rabbits each

according to the Three Rs guiding principles for the use of animals in research.[16] Animals were kept in polypropylene cages (two rabbits/cage) with ad libitum access to water and normal diet. The room temperature was about 22-24°C and the animals were exposed to 12:12 hours of light-dark cycles. The rabbit colony was health observed according to recommendations by Federation of European Laboratory Animal Science Associations.[17]

Sample size calculation

Primary outcome for sample size calculation of Osteoprotegerin (OPG) gene expression during healing of bony defect according to Koch et al.,[18] a sample of 3 cases per group is the minimum required number (Minitab software version 16). Thus 12 New Zealand white rabbits enrolled in the experiment for anticipated missing data. The rabbit population was divided as follows; 3 rabbits/group, 4 holes/ rabbit, 2 holes/ femur with total of 12 holes in each group.

Anesthetic protocol:

Rabbits were anesthetized using intramuscular injection of Xylazine* 5mg/kg body weight and Ketamine hydrochloride** 30mg/kg body weight.

Surgical protocol

A linear 20 mm incision was performed in both femurs of each rabbit in the craniocaudal direction, using a No. 24 scalpel blade. The bone was exposed after dissection of skin, muscle, and periosteum. Two holes were drilled using a rounded bur mounted on a high-speed handpiece under copious irrigation with a distance of 10 mm between the centers of bone defects. The rabbits received Cataflam*** 750 mg (10 mg/kg) and Flumox**** 500 mg vial after surgery.

Stem cells preparation technique:

Rabbit BMMSCs isolation, culture, and characterization were performed at the Unit of Biochemistry and Molecular Biology at the Medical Biochemistry Department, Faculty of Medicine, Cairo University, Egypt. Rabbit BMMSCs were prepared according to Zhang et al.[19]

Laser application:

Single dose of low level laser (LLL) was applied using Zolar Photon Plus soft tissue diode laser device (Zolar Technology & Mfg Co. Canada) with wavelength 980 nm, custom 1 mode, 0.3 W, continuous mode, and energy output 250 mW.

Different treatment groups:

The 12 New Zealand white rabbits were divided into 4 groups as follows; 3 rabbits/group, 4 holes/ rabbit, 2 holes/femur, with a total of 12 holes in each group as follows.

1- **Group A (Control):** Each hole was injected with chitosan carrier gel only.

2- **Group B (BMMSCs only)**: Each hole was injected with 250 million Rabbit-BMMSCs in chitosan carrier gel with a total of 500 million Rabbit-BMMSCs/femur.

3- **Group C (Laser only)**: LLL was applied on each hole loaded with chitosan carrier gel for 10 seconds with a total of 20 seconds/ 2 holes in each femur.

4- **Group D (Laser + BMMSCs group)**: Each hole was injected with BMMSCs in chitosan carrier gel similar to group B then LLL laser was applied for 10 seconds on it with the same laser parameters of the group C.

* Chanazine, Chanelle pharmaceutical manufacturing Ltd., Loughrea, Co. Galway, Ireland.

** Ketamine, pharmazeutische proparate, Pfaffen-Schwabenheim, Germany.

*** Dicrofenac Potassium 75MG 3 amp. Manufactured by Novartis. Egypt

**** Amoxycillin (as trihydrate) 250 mg + Flucloxacillin (as monohydrate) 250 mg. Manufactured by Egyptian Int. Pharmaceutical Industries CO. (E.I.P.I.CO.), Egypt.

Rabbit Sacrifice

Rabbits were sacrificed after 8 weeks by injecting an overdose of a Pentobarbital* ≥ 150 mg/kg body weight into an ear vein according to the American Veterinary Medical Association (AVMA) guidelines for the euthanasia of animals.[20]

Assessments

Morphological Assessments

All studied groups were examined by light microscope (Model XSZ-07 Series Biological-Microscope – LT-XSZ-107BN LED) and photographed by a microscopic camera with software S-EYE 1.3.2.297@2016 connected to HP Pavilion x 360 Convertible Laptop using windows 10.

Specimens' processing and examination

The bony defect specimens were fixed in 10 % buffered formalin for 48 hours. The specimens were then washed and decalcified in 17 % Ethylene diaminetetra-acetic acid (EDTA). The samples were then dehydrated in ascending grades of alcohol, cleared in xylol then embedded in paraffin blocks. Each specimen was cut longitudinally to show the newly formed bone in the bony defect. 5μ thick sections were prepared on regular glass slides for histological examination. Prepared sections were stained with Hematoxylin and Eosin stain according to the conventional method for routine histological examination.[21]

Genetic Assessment

Real-time Polymerase chain reaction (RT-PCR) was performed by PCR device supplied with thermal cycler and spectrometry (Applied Biolase, ABI 7300. U.S.A) and densitometry (Leica Q500, Cambridge, UK) in the Biochemistry department - Faculty of Medicine- Cairo University. The RNA Extraction was prepared using RNA Extraction RNeasy Mini Kit Catalog no. 74104 and Reverse transcription of RNA into cDNA using Omniscript Reverse Transcriptase Kit Catalog no. 205110. All kits, chemical reagents, and primers were purchased from Qiagen company. Hilden, Germany through Clinilab co. in Egypt according to manufacturer's instructions in accordance with Hassouna et al.[22]

The following genes were detected with primer sequence as in table (1):[23–25]

a) Bone turn over gene; OPG.

b) Mineralized deposited bone matrix genes; Alkaline phosphatase (ALP) and Osteocalcin (OC).

c) GAPDH (Glyceraldehyde-3-phosphate dehydrogenase) was also included to ensure proper reactions of the RT-PCR.

TABLE (1): Showing Primer gene sequence.

Primer	Gene sequence
OPG	Forward: 5-TGGCACACAGTGATGAATGCG-3 Reverse: 5-GCTGGAAAGTTTGCTCTTGCG-3
OC	Forward: 5-ATG AGA GCC CTC ACA CTC CTC-3 Reverse: 5-GCC GTA GAA GCG CCG ATA GGC-3;
ALP	Forward: 5-CCC AAA GGC TTC TTC TTG-3 Reverse: 5-CTG GTA GTT GTT GTG AGC AT-3
GAPDH	Forward: 5-GGG CTG CTT TTA ACT CTG CT-3 Reverse: 5-TGG CAG GTT TTT CTA GAC GG-3

Statistical analysis

RT-PCR gene expression Numerical data results were presented as mean and standard deviation (SD) values. Shapiro-Wilk's test was used to test for normality. Homogeneity of variances was tested using Levene's test. Data were parametric and showed variance homogeneity so a one-way ANOVA test followed by Tukey's post hoc test was used to analyze intergroup comparisons and repeated measures ANOVA followed by Bonferroni post hoc test was used to analyze intragroup comparisons. The significance level was set at $p<0.05$ within all tests. Statistical analysis was performed with R statistical analysis software version 4.1.2 for Windows.***

* Diazepam, Roche, France.

** R Core Team (2022). R: A language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, Austria. URL https://www.R-project.org/.

RESULTS

Morphological results

Control group A (chitosan gel carrier); showed an early phase of bone healing by the formation of newly formed bone in the form of an osteoid matrix at the periphery of the defect and granulation tissue at the center. There was a space separating the newly formed bone from the old bone as in fig. 1-(a). While group B (diode laser only); showed an osteoid matrix of newly formed bone filling the whole defect and the area surrounding the defect showed numerous bone trabeculae in the bone marrow space of the femur as in fig.1- (b). Regarding group C (BMMSCs group); the bony defect was filled with a great amount of bone trabeculae on the outer surface filling the defect with and wide bone marrow spaces were observed as in fig.1- (c). Finally, in group D (BMMSCs with diode laser) a fully mature bone filled the bony defect indicating complete bone healing as in fig.1- (d).

RT-PCR gene expression statistical results

Regarding bone turnover gene OPG that is found in osteoblasts and osteocytes, the highest value with statistical significance was found in the control group (A), followed by the diode laser only group (B), then BMMSCs only group (C). While the lowest value was found in the diode laser + BMMSCs group (D).

Concerning mineralized bone matrix genes ALP and OC, the highest value with statistical significance was found in diode laser + BMMSCs group (D), followed by BMMCs only group (C), then diode laser only group (B), while the lowest value was found in the control group (A) as shown in table (2) and fig. (2).

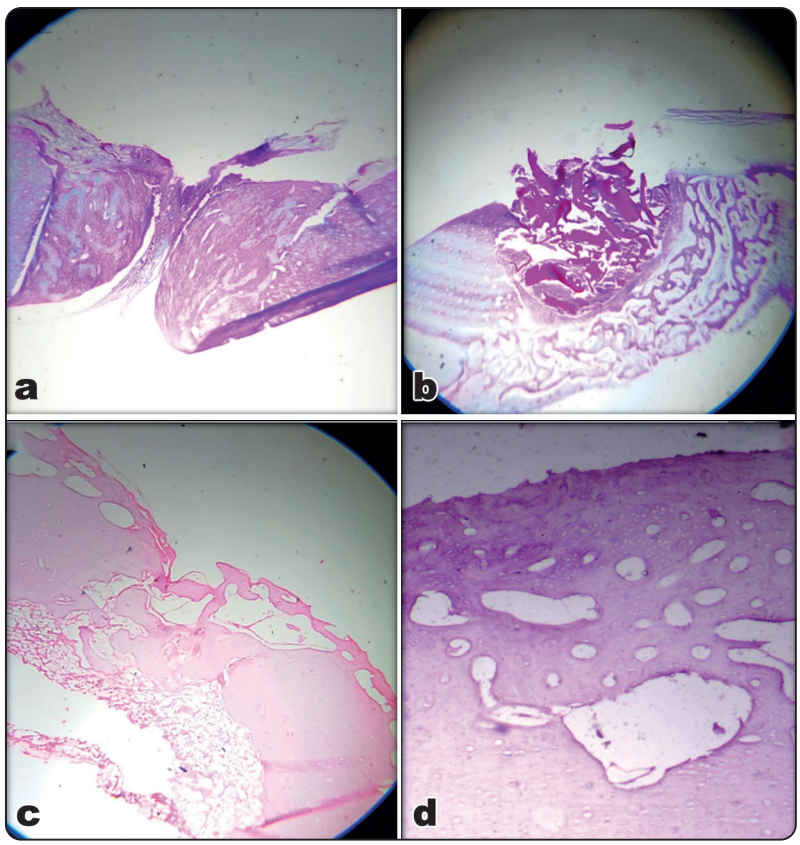

Fig. (1). Photomicrograph of longitudinal section of bone showing the healing of the bony defect after 8 weeks in control group (a); showing area of granulation tissue filling the defect. Laser only group (b) showing red areas of newly osteoid tissue filling the defect. BMMSCs only group (c) displayed bone trabeculae with bone marrow spaces filling the defect. BMMSCs with Laser group (d) revealed; complete closure of the defect with fully mineralized lamellar bone intervening non lamellar highly cellular bone trabeculae.

(H&E stain) (4 X)

TABLE (2): Comparisons of bone deposition RT-PCR gene expression markers

Genes	*(Mean±SD)*				*p-value*
	Laser only	*BMMCs only*	*Laser + BMMSCs*	*Control*	
ALP	2.14 ± 0.70^{Ca}	3.33 ± 0.82^{Bb}	4.69 ± 0.68^{Ab}	1.05 ± 0.02^{Da}	**<0.001***
OPG	0.75 ± 0.12^{Bb}	0.39 ± 0.10^{Cc}	0.22 ± 0.04^{Dc}	0.87 ± 0.03^{Ab}	**<0.001***
Osteocalcin	2.60 ± 0.48^{Ca}	4.17 ± 0.98^{Ba}	7.27 ± 0.21^{Aa}	1.07 ± 0.02^{Da}	**<0.001***

Means with different upper and lowercase superscript letters within the same row and column respectively are significantly different; *significant (p<0.05)

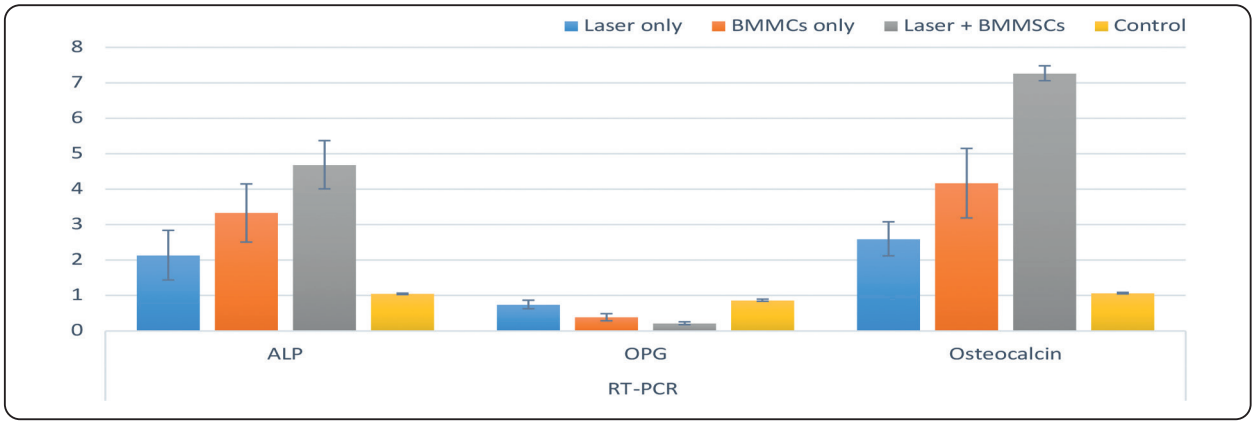

Fig. (2) Bar chart showing mean and standard deviation values for bone deposition RT-PCR gene expression markers in different groups.

DISCUSSION

This study was performed to compare the efficacy of BMMSCs, LLLT, or a combination of both treatment modalities. Chitosan, a deacetylated polysaccharide from chitin, was utilized as a scaffold with all treatment groups. It was used by the authors due to its beneficial characteristics as biodegradability, biocompatibility, mucoadhesive and antimicrobial properties. Moreover, chitosan was proved to promote osteogenesis.[26] Aguilar et al.[27] reported that using chitosan as a scaffold enhances new bone regeneration and improves neovascularization in vivo. Chitosan was also injected as a sole gel in the control group in order to exclude the effects of chitosan gel when comparing the different treatment modalities.

LLLT device used was Zolar Photon Plus soft tissue diode laser with wavelength 980 nm, custom 1 mode, 0.3 W, continuous mode and energy output 250 mW. The advantage of this type of laser is that it is available in the market and easily transported. The parameters used in the present study were adjusted manually to observe its effect on bone biostimulation which could be further suggested to the manufacturer to program the device with it.

Rabbit-BMMSCs were used in the present study to transplant BMMSCs from the same animal species (allogeneic transplantation) in order to

benefit from the advantage of the regenerative effect of BMMSCs transplantation over other stem cells lineage.[28]

Histological findings presented in (Fig. 1) showed the best bone healing results with the normal architecture of bone in the combined group of LLLT with BMMSCs followed by the BMMSCs group alone. BMMSCs group alone showed better healing than LLLT when used alone. Although in the LLLT group the newly formed bone hadn't reached full maturity yet, LLLT stimulated the undifferentiated stem cells in the bone marrow of the femur to differentiate into osteoblasts forming bone trabeculae surrounding the defect. The least healing results were observed in the control group. The combined group of BMMSCs with LLLT results of the present study were in agreement with Nagata et al.[29] who postulated that bone marrow aspirate combined with a single dose of LLLT using Induim Gallium aluminum arsenide (InGaAlAs); TheraLase®, DMC Equipamentos Ltda, São Carlos, SP, Brazil), with a wavelength of 660 nm with a power of 0.035W for 4 s/point, energy of 0.14 J/point and energy density of 4.9 J/cm^2 /point) exhibited better bone healing results when compared to the control group. Moreover, Fekrazad et al.[30] postulated better bone healing histological results upon combining LLLT with BMMSCs using multiple-dose repeated every 3 days using 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, per session when compared to control group where the defect filled with blood clot in normal healing.

Better BMMSCs findings of the current study were in agreement with Kumar and Ponnazhagan[31] who displayed better bone healing upon mobilization of BMMSCs in vivo augments bone healing in a mouse model of the segmental bone defect.

LLLT histological observations of the existing study were in accordance with Abdelaal and Saad[32] who observed better bone healing results in the lased group over the non-lased group where multiple doses of laser were applied with laser device ASA IDEA Terza laser device GaAlAs laser with wavelength at 905 nm and power 150 mW,10 J/cm^2 doses per point every 48 hours during the period of distraction osteogenesis. However, the favorable single LLLT biostimulatory parameter is still under investigation.[9]

Histological findings were further affirmed by RT-PCR assessment of OPG, OC, and ALP gene expression.

OPG is a soluble decoy receptor for the osteoclast differentiation factor RANKL. It is a member of the "Tumor Necrosis Factor Receptor" superfamily and is considered as a main regulator of osteoclastogenesis, it inhibits both the formation and development of osteoclasts. OPG is secreted in high concentrations by cells of the osteoblast lineage and it inhibits excessive bone resorption by binding to RANKL to prevent its binding to RANK.[33] Thus, OPG is used as a reliable marker for alveolar bone turnover as well as organic matrix formation.[34] Since OPG, as previously expressed; is produced by the osteoblasts which takes the upper hand during the stage of organic matrix formation, therefore; its increase indicates a stage of an early phase of bone formation.[35]

In the present study, the highest results for OPG were observed in the control group, followed by laser, BMMSCs, and LLLT with BMMSCs in descending order. These results indicate that after eight weeks maturation of osteoblasts with initial phase of bone formation was seen in the control group. This interpretation was supported by Gori et al.[36] observations which displayed the increase of OPG gene expression during osteoblast differentiation and bone formation phase during bone remodeling. Furthermore, the combined treatment group (LLLT with BMMSCs) showed the least OPG results which suggests that this group had reached final maturation of bone matrix. This suggestion confirms the results of Kon et al.[37] who proved that OPG was elevated

through early repair process in rat fracture with two peaks detected within 24 h after fracture and at day 7 while at day 14 (late phase of repair process) there was a significant decrease in OPG levels.

On the other hand, OC is an HA-binding protein that represents the major non-collagenous protein in bone.[35] It is synthesized by osteoblasts and osteocytes, secreted into the bone matrix, and aids in calcium-binding and mineral deposition.[38] Manolagas[39] confirmed the promoting role of OC in the mineralization phase of the bone matrix. OC is considered as structural molecules in the bone matrix linking the organic and inorganic matrices and contributing to the structural integrity of bone.[40] Thus, it is regarded as a bone formation indicator. Whereas, ALP is highly expressed in the cells of mineralized tissue and plays a critical function in the formation of hard tissue.[41]

In the current study, the highest results for both OC and ALP were detected in the combined group of BMMSCs with LLLT which can be attributed to the full maturation of the bone matrix. These results were in agreement with Abramovitch-Gottlib et al.[42] who demonstrated that irradiated mesenchymal stem cells (MSCs) samples demonstrated a significant increase in ALP activity which confirms LLLT biostimulatory effects on the differentiation of MSCs into osteoblasts and induction of ossification. The results of the current study showed that the degree of mineralization of the BMMSCs group was higher than the LLLT group but still less than the combination group. It was proved that the transplantation of MSCs into defect area promote regeneration by increasing the precursor cells and also by enhancing the release of growth factors and immunomodulatory cytokines.[43] Moreover, the present results also showed that the degree of mineralization of the LLLT group was higher than the control group. In agreement, Kiyosaki et al.[44] advocated that LLLT can enhance mineralization by enhancing the expression of insulin-like growth factor I and the production of bone morphogenic proteins via Runt-related transcription factor 2 expression and extracellular-signal-regulated kinase phosphorylation in the osteoblasts.

Thus, the results of the existing study suggested that BMMSCs demonstrated superior regenerative impact compared to LLLT. This could be related to the ability of BMMSCs to stimulate more than one organelle in the cell especially those included in cell signaling pathways through lysosomes over the enhancement of respiratory effect through production of ATP in mitochondria.[45] While the laser biostimulatory effect acts on mitochondria only.[46] It was confirmed that LLLT increases metabolism and cell proliferation which leads to improvement of healing.[47] Likewise, LLLT induces epithelial cells and fibroblasts mitotic activity and stimulates their production of collagen which in turn leads to biostimulation of the tissue repair process.[10]

Moreover, the present study showed that when LLLT was used in combination with BMMSCs; superior results were obtained than if either BMMSCs or LLLT were used alone which indicates that LLLT enhances BMMSCs regeneration effect. This could be related to the ability of LLLT to enhance angiogenesis and to promote the production of endogenous reactive oxygen species by altering the membrane potential of the mitochondria which in turn will increase the osteogenic differentiation of stem cells.[48] This was in accordance with Mvula et al.[49] who examined the effect of LLLT on adipose-derived stem cells and demonstrated that LLLT enhanced the migration, proliferation and differentiation of progenitor cells. Likewise, Wu et al.[50] proved that LLLT can restrain the inflammatory response to ADSCs by modulating intracellular cyclic AMP level and NF-$\varkappa$B activity and also inhibiting the expression of Lipopolysaccharide-induced proinflammatory cytokine. The author confirmed the advantageous effect of LLLT application as an anti-inflammatory therapy in presence of stem cell therapy.

CONCLUSION

Within the highlights of the present study results, the greatest bone regeneration was observed genetically and morphologically with complete healing in the combined group of BMMSCs and LLLT (diode laser with wavelength 980 nm, custom 1 mode, 0.3 W, continuous mode, and energy output 250 mW) with the highest OC and ALP levels and least OPG level. Moreover, the BMMSCs group expresses regenerative effects both morphologically and genetically over the LLLT group followed by the control group.

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