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Original Article

Effect of mesenchymal stem cells injection and low-level laser therapy on bone formation after rapid maxillary expansion: an animal study

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Abstract: Introduction: One of the most common orthodontic problems is maxillary constriction, which is mostly treated by rapid palatal expansion (RPE). However, its high rate of relapse and prolonged retention period have led to some challenges for orthodontists. To encounter these issues, accelerating bone regeneration can provide long-term stability of expanded maxilla. The present study aimed to evaluate the effect of low-level laser therapy (LLLT), bone marrow-derived mesenchymal stem cells (BMSCs) and their combination on promoting bone regeneration of the inter-maxillary suture after RPE in rats. Materials and method: Total of 60 rats went under RPE treatment. After 7 days, retention period started and interventions (group A, Control (saline); group B, LLLT; group C, BMSCs; group D, LLLT + BMSCs) were performed in the sutural area. After 21 days, radiographic and histological analyses were done. Histological analyses were conducted to evaluate the following criteria of the newly formed bone: the number of osteoblasts, new bone formation, vascularization, connective tissue. Moreover, sutural width was assessed in histologic images. To evaluate bone density at suture area, gray scale and Hounsfield Unit values were measured based on the occlusal radiographic and Micro-Computed topography images respectively. Results: Only in group C and D, osteoblasts and new bone formation were observed in all of the samples. There were no significant differences among the study groups regarding the post-treatment sutural width ($P > 0.05$). In the radiographic analysis, only group D showed more bone density compared to the control group ($P = 0.022$). Similarly, in micro-CT analysis, the most bone density was observed in group D which was significantly more than the control group ($P = 0.013$). Conclusion: Our findings suggest that the application of LLLT and BMSCs is the most beneficial approach in accelerating bone regeneration in the inter-maxillary suture.

Keywords: Mesenchymal stem cells, low-level light therapy, palatal expansion technique, orthodontic treatment

Introduction

Maxillary constriction, defined as a dental or skeletal transverse discrepancy of the maxillary bone, is one of the most common orthodontic problems among patients [1]. Maxillary constriction may lead to breathing, esthetic, and functional problems [2-4]. Among various treatment approaches, rapid palatal expansion (RPE) and surgically assisted expansion are common options in growing and non-growing patients respectively [5-7]. However, the most challenging problem regarding RPE is its high possibility of relapse following the treatment. To decrease the possibility of post-treatment relapse, which occurs as a result of insufficient

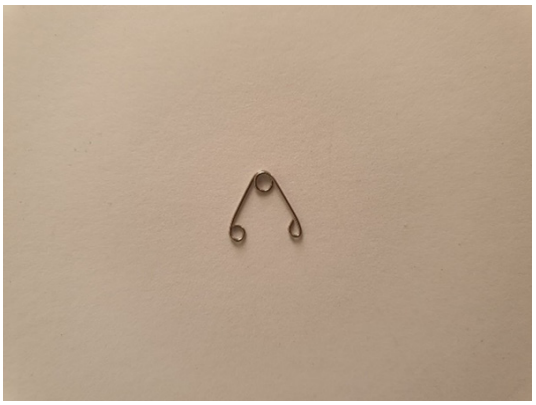

Figure 1. Spring fabricated from 0.14-inch stainless steel orthodontic wire.

sutural new bone formation, the three to four months of the post-treatment retention period is inevitable [8-10]. This prolonged treatment can cause enamel decalcification, poor oral hygiene, and oral mucosa disease [11].

Moreover, enhancing bone regeneration in the inter-maxillary space made by RPE, will reduce the probability of treatment relapse [12]. The positive impact of transforming growth factor-β1 (TGF-β) [13], Vitamin D [14], ozone administration [15] and intermittent compression [16] on sutural bone formation have been reported previously in animal studies. Applying nonionized radiation, which is called laser therapy (LLLT), may reduce the duration of retention period through its anti-inflammatory effects and its ability to increase blood circulation and wound healing [4]. The positive effect of LLLT has been indicated in animal studies while human studies did not show clinically significant effect for LLLT [17-23].

Application of mesenchymal stem cells (MSCs) is another way to increase the rate of bone formation. These cells can differentiate into several cell lines including osteoblasts [24, 25]. MSCs can be isolated from several oral tissues such as periodontal ligament and dental pulp [26]. Considering the availability of these cells, they can be recruited to increase the rate of bone formation in sutural area after RPE treatment. Ekizer et al. analyzed the effect on BMSCs and mentioned its positive effect on the rate of mid-palatal bone formation after expansion [27].

This study is done in order to analyze the effect of BMSCs, LLLT and their combination on mid-palatal suture following expansion in Wister rats.

Material and method

Animals

Ethical permission was obtained from the Experimental Animal Use Protocol of Ethical Committee of Shahid Beheshti Medical University and IRI Health Ministry (Code: IR.SBMU.DRC.REC.1397.070). Sixty 84-day-old male Wister rats with a mean weight of 200 ± 10 g were used in this study. Animals were housed in cages with constant temperature and day/night light cycles.

Rapid palatal expansion

Rats were anesthetized by injecting 50 mg/kg ketamine (100 mg/mL, Alfasan, 85 Woerden, Holland) and 10 mg/kg xylazine (20 mg/mL, Alfasan, Woerden, Holland). Helical spring fabricated from 0.14-inch stainless steel orthodontic wire was used as the expansion device (**Figure 1**). Springs were able to apply a force of 50cN. Adhesion system and bonding resins were used to increase the retention of the springs. Springs were activated only at the beginning of the study and were not reactivated during the expansion or retention period.

Retention period

After 7-days of expansion, 3-week retention period initiated. Animals without palatal expansion or with spring displacement were excluded. The gap between maxillary incisors were filled using a light cure composite as retainer and the spring was removed. Rats were randomly allocated to the five study groups (**Table 1**). In order to measure the length of the sides of the region of interest (ROI), after 7 days of expansion, group E samples went for specimen preparation phase without facing interventions or retention.

Interventions: local delivery of BMSCs

BMSCs Isolation and characterization: Bone mesenchymal stem cells were isolated from two green fluorescent protein (GFP) transgenic rats. The intended area of the animals was disinfected using betadine 10%. A specific part of the iliac bone was punched and the mar-

Table 1. Semi-quantitative histology analyses

Criteria	Mild	Moderate	Strong
Osteoblast count	Less than 5 cells	6-10 cells	More than 10 cells
New bone formation	Irregular bone formation	Regular bone formation	Mature-looking bone
Vascularization	Large disorganized vessels	Small vessels, irregularly distributed	Small vessels, evenly distributed
Quality of connective tissue	Loose	Dense, Disorganized	Dense, Organized

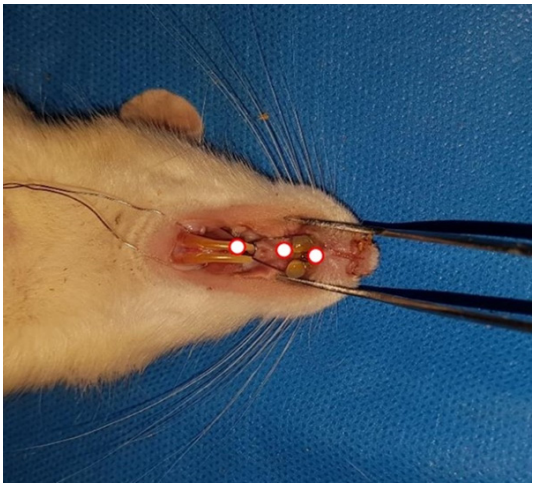

Figure 2. Defined points for laser therapy.

row cavity rinsed with PBS. A total of 8-10 ml bone marrow suspension was aspirated through a heparin-coated syringe and centrifuged and resuspended in Dulbecco's Modified Eagle Medium (DMEM) (Gibco, Paisley, UK) growth containing 10% fetal bovine serum (Gibco, Paisley, UK) and 1% penicillin. Cultures were maintained at 37°C in a humidified 5% carbon dioxide environment. When the cultures reached 90% of confluence, cells were detached using 0.25% trypsin-ethylenediaminetetraacetic acid (EDTA) (Life Technologies) and passaged three times.

In order to evaluate cell surface markers, flow-cytometry analysis performed on third passage stem cells. Cells were detached from the flask by incubation with 0.15% EDTA solution for 5 min at room temperature. Cells were centrifuged and suspended in PBS. Then antibodies of intended markers (CD29, CD90 and CD45; Abcam) were added and the solution incubated at 4°C for 30 minutes. After that, cells were rinsed with PBS and analyzed.

In order to evaluate differentiation capacity, the third passage cells were cultured in osteogenic medium (DMEM plus 0.2 mol/L ascorbic acid 2-phosphate (Sigma, USA), 10^{-8} mol/L dexamethasone (Sigma), and 10 mmol/L β glycerol phosphate) and adipogenic medium (DMEM plus 0.2 mmol ascorbic acid 2-phosphate (Sigma), 10 mmol beta-glycerophosphate (Sigma), and 50 mg/ml indomethacin (Sigma)) and incubated for 21 days. In order to analyze osteogenic and adipogenic differentiation, cells were rinsed and died with alizarin red and oil red solutions, respectively.

Cell delivery: A total of 0.1 ml stem cell suspension with the concentration of 2×10^6 BMSCs/mL was injected at the anterior portion of the intermaxillary suture in groups C and D. The same amount of normal saline was injected into the control group. The injection performed at the time of installing the retainers.

Interventions: low-level laser therapy

LLLT was applied in three points of the mid-palatal suture using an 810-830 nm allium-aluminum-arsenide (GaAlAs) diode laser (whitening lase II, DMC©, Sao Paulo, Brazil) in continuous wave mode with 250-300 mW output power (**Figure 2**). The Fiber tip was in contact with the mucosa. The approximate dose during exposure was 4-6 J/cm^2. LLLT was performed in groups B and D twice at the time of retainer placement and 7 days after that (14 days after expansion).

Specimen preparation

On day 28, rats were sacrificed through chloroform vapor in a desiccator. Rats with infection or weight loss were excluded. The maxilla was dissected out using an orthodontic cutter (Fattah Teb Pouya, Tehran, Iran) and fixed in 10% formalin for two days.

Radiographic analysis

Occlusal radiographs were taken from the species through the intra-oral digital device

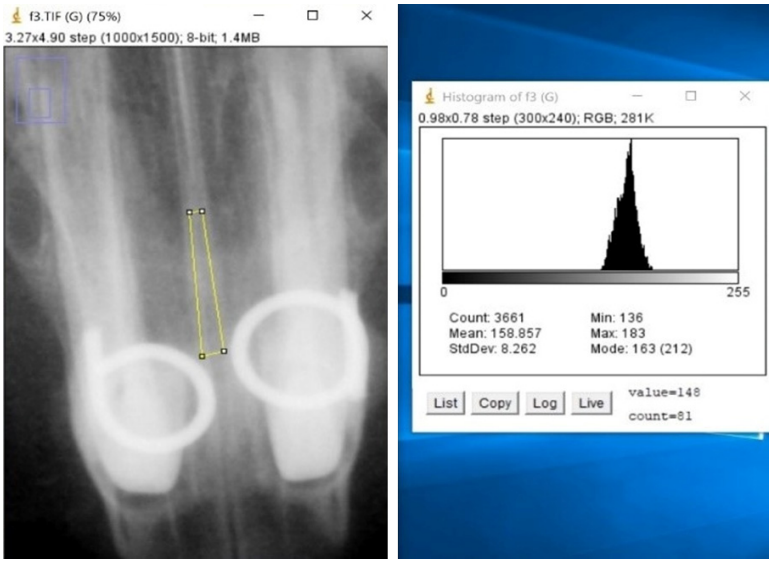

Figure 3. Trapezoid shaped ROI and Grayscale measurement using Image J software.

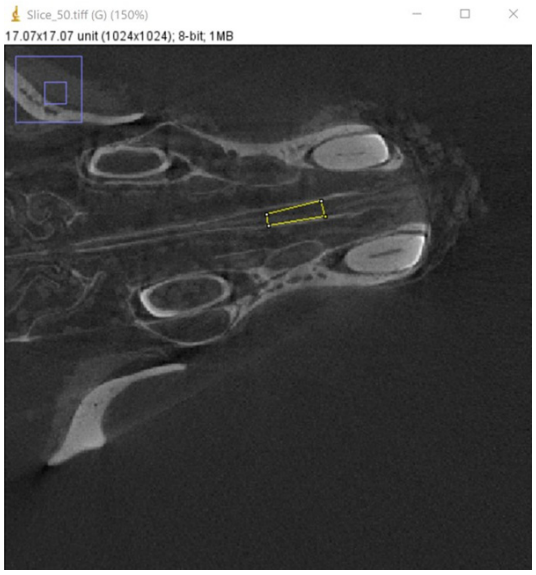

Figure 4. Region of interest for Hounsfield unit measurement.

(Planmeca 142 Prostyle™, Helsinki, Finland). Exposure parameters were 64 kw and 0.06 s. Pictures were processed using Owandy XIO Stand Alone (Owandy Radiology, Croissy-144 Beaubourg, France). The grayscale of the trapezoid-shaped region of interest (ROI) was measured using Image J software version 1.8 (U. S. National Institutes of Health, Bethesda, Maryland, USA). The length of the bases of the trapezoid was defined based on the sutural widths of the samples of group E. The mean sutural width of group E samples, adjacent to incisors and 3.5 mm away from incisors, were considered as lengths of the bases of the trapezoid-shaped ROI (**Figure 3**). The aforementioned ROI was used in all of the samples to measure sutural bone density. Finally, the equivalent aluminum thickness of ROI was calculated using a calibrated step wedge.

Micro computed topography

Tissue species were scanned through the high-resolution scanner (LOTUS- in Vivo, Behin Negareh Co., Tehran, Iran) set at 80 kV, 100 μA, 8 w. The slice thickness was 25 μm. After the scanning procedure, 3D images were constructed based on Feldkamp, Davis, Kress algorithm [28]. The mineral density of the mid-palatal suture measured using the Hounsfield Unit (**Figure 4**) [29]. Picture analyses performed with Image J software version 1.8 (U. S. National Institutes of Health, Bethesda, Maryland, USA). The mean sutural width of group 5 samples, adjacent to incisors and at 45 pixels away from incisors, were considered as lengths of bases of the trapezoid-shaped ROI. The aforementioned ROI was used in all of the samples to measure sutural bone density.

Histological examination

Maxilla was decalcified with 7% formic acid for seven days. Samples were dissected into three sections with two coronal cuts. Specimen were rinsed, trimmed and embedded in paraffin and cut into 5 μm sections by microtome (SCILAB Co Ltd, Barnsley, UK).

Histological sections were stained with hematoxylin and eosin and analyzed under a light microscope (E400, Nikon, 114 Japan) by a blinded examiner. Semi-quantitative analysis was performed based on previous studies [5]. The grading system and assessed criteria are mentioned in **Table 2**. In addition, the mean sutural width of the samples was measured based on the histology images using Image J

Table 2. Study groups

Study Group	Sample Size	Expansion (day 7)	Retention (day 21)	Intervention
A	12	+	+	Control (Saline) (day 7)
B	12	+	+	LLLT (days 7,14)
C	12	+	+	BMSCs (day 7)
D	12	+	+	LLLT (day 7, 14) + BMSCs (day 7)
E	12	+	-	-

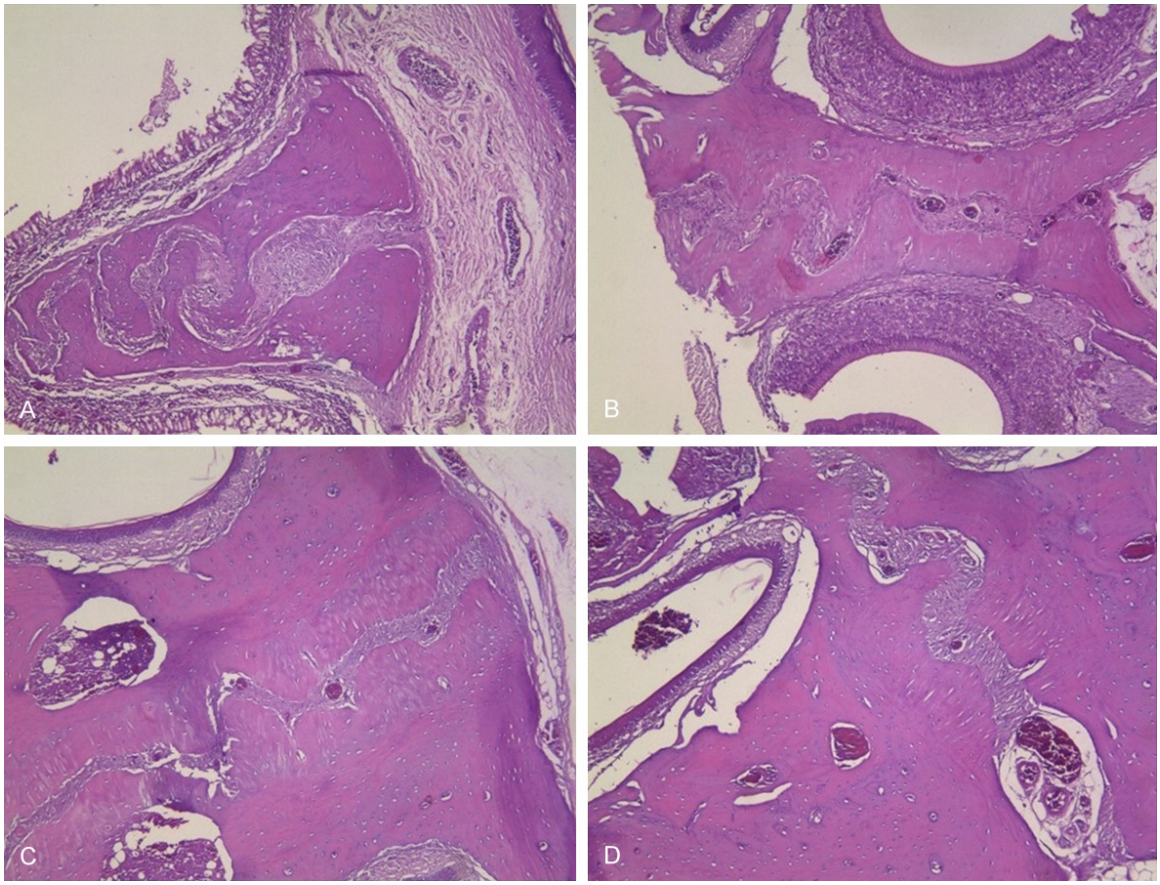

Figure 5. Hematoxylin and eosin staining of the palatal suture in the (A) control group, (B) LLLT group, (C) BMSC group, (D) BMSC + LLLT group (100 ×).

software version 1.8 (U. S. National Institutes of Health, Bethesda, Maryland, USA).

Statistical analyses

In order to compare the means of groups, the Man-Whitney U test was performed for histology results. For radiographic and micro-CT results, two-way ANOVA followed by Tukey's post-hoc test was performed. Data were analyzed using SPSS v.18 computer software (SPSS, Chicago, IL, USA) at the significance level of 0.05.

Results

DPSC characterization

Flow cytometry tests revealed that cells were positive for CD29 and CD90 markers and negative for CD45. Alizarin red and Oil red staining confirmed the osteogenic and adipogenic differentiation ability of stem cells respectively.

Histology results

In order to evaluate the effect of BMSCs injection and laser therapy on bone formation, four

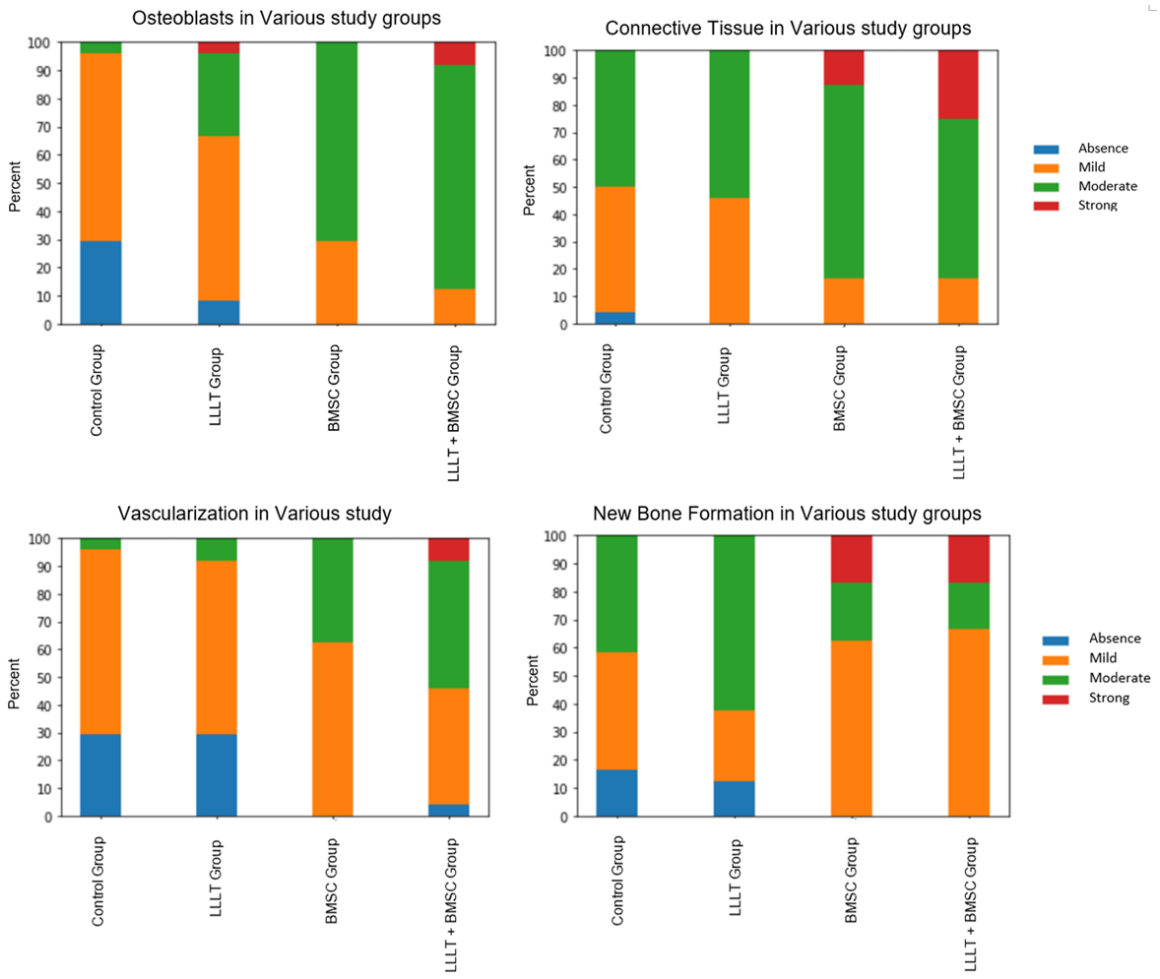

Figure 6. Semiquantitative Histology analyses.

criteria were analyzed from histology images (**Figure 5**).

Number of osteoblasts

Experimental groups showed better results compared to the control group. Osteoblasts could be seen in all of the BMSCs and BMSCs + LLLT treated samples. However, some of the LLLT samples did not have osteoblasts. Among all of the interventions, merely laser therapy caused significantly better results in the case of osteoblast counts compared to the control group ($P = 0.004$) (**Figure 6**).

New bone formation

Control and LLLT groups had samples without new bone formation. Interventions caused no significant effect on the quality of newly formed bone compared to the control group ($P > 0.05$) (**Figure 6**).

Vascularization

All of the groups had samples with no capillary formation except group C. In the case of vascularization, group B and group D had no significant difference with the control group ($P > 0.05$). However, samples treated with BMSCs showed significantly better vascularization than the control group ($P < 0.001$) (**Figure 6**).

Quality of connective tissue

Connective tissue formation could be seen in all of the experimental groups. No samples with dense, organized connective tissue could be seen in group B. Besides, there were no significant differences in the quality of connective between group B and control. In comparison with the control group, the quality of connective tissue was significantly better in group C ($P = 0.005$) and group D ($P = 0.002$). However, the

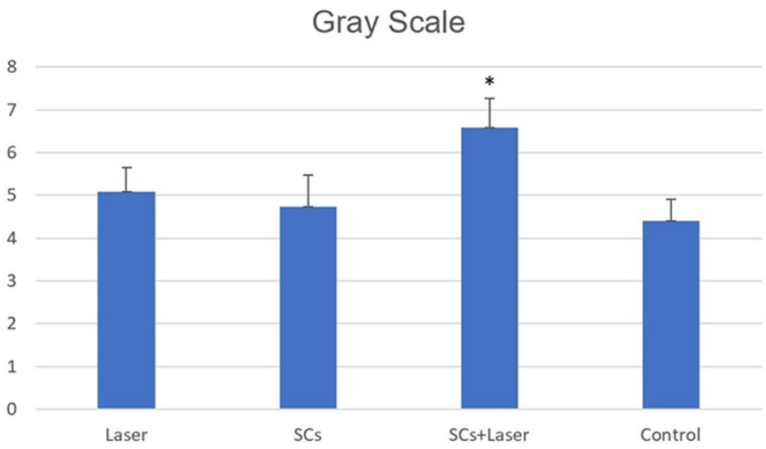

Figure 7. Measurement of bone density at the retention period (Grayscale). *P < 0.05 vs control.

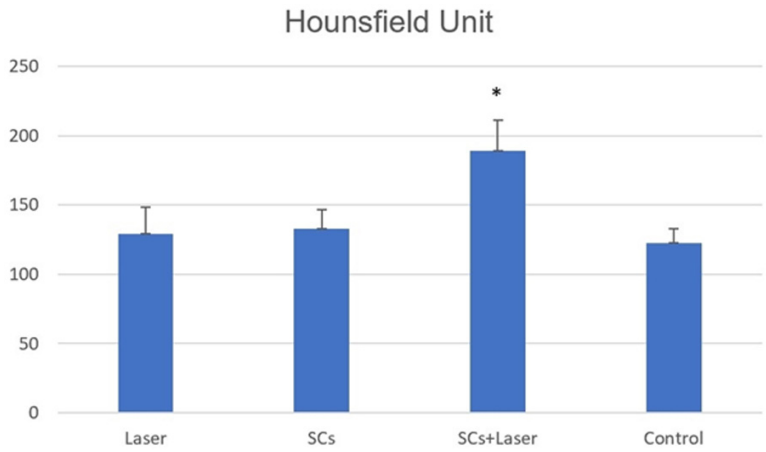

Figure 8. Measurement of bone density at the retention period (Hounsfield Unit). *P < 0.05 vs control.

difference between groups C and D was not significant (P = 0.46) (**Figure 6**).

In addition, in comparison with control group (0.31 ± 0.11 mm), it has been shown that LLLT (0.28 ± 0.10 mm), BMSCs (0.23 ± 0.8 mm) and BMSCs + LLLT (0.18 ± 0.05 mm) had no significant effect on the post-treatment sutural width (P > 0.05).

Radiographic results

Based on the mean sutural width of group E samples, the smaller base and larger base of the trapezoid-shaped ROI were 0.31 mm and 0.49 mm, respectively. In order to analyze the effect of LLLT and BMSCs on mid palatal bone density, the equivalent aluminum thickness of groups was analyzed. In comparison with the control group (4.40 ± 1.00 mm-Al), LLLT + BMSCs (6.58 ± 1.37 mm-Al) increased the grayscale of expanded suture significantly (P = 0.022). However, LLLT (5.09 ± 1.11 mm-Al) and cell delivery (4.73 ± 1.48 mm-Al) did not increase bone density significantly (P > 0.05) (**Figure 7**).

Micro-CT results

The smaller base and larger base of the trapezoid-shaped ROI were 7.93 pixels and 10.81 pixels, respectively. Hounsfield unit (HU) values of groups were compared to assess the effect of interventions on suture mineral density. LLLT (129.17 ± 38.82 HU) and BMSCs (132.67 ± 27.43 HU) did not cause significantly higher bone density compared to the control group (122.67 ± 20.85 HU). However, in comparison with control group, LLLT + BMSCs (189.23 ± 44.48 HU) increased the mid-palatal bone density significantly (P = 0.013) (**Figure 8**).

Discussion

Nowadays, RPE has been widely used in orthodontic procedures to treat crossbites [30], dental crowding [31], pseudo-class III malocclusion [32], *etc*. However, its long retention period leads to frequent relapse and, consequently a relatively high rate of failure [33]. To improve RPE's long-term stability and reduce its retention period, bone formation in intermaxillary suture should be enhanced [33]. Saito and Shimizu [34] reported that inadequate bone regeneration in the intermaxillary region is the main reason for RPE relapse. Therefore, various approaches have been suggested in the literature to increase the stability of maxillary expansion by enhancing bone regeneration, including low-level laser therapy [35], LED phototherapy [36], local delivery of bisphosphonates [37], lithium [38], matrine [33] and vitamin D analog [39] as well as injection of BMSC [27]. In the present study, we applied the low-level laser

and BMSC on the intermaxillary region of rats. Moreover, since it has been reported that the simultaneous use of LLLT and mesenchymal stem cells may have synergic effects regarding bone regeneration [40], we assessed their concurrent application on the suture. Our study results showed that combine application of LLLT and BMSCs had the highest impact on accelerating bone formation.

It has been reported that the local delivery of mesenchymal stem cells would enhance cartilage formation [41] and vascularization [42]. Badiavas et al. [43] showed that local delivery of BMSCs would be an effective treatment approach in patients with chronic wounds by enhancing regenerative processes. The results of the current study showed that local injection of BMSCs alone into inter-maxillary suture could not enhance bone regeneration at a significant level. In contrast to our study, Ekizer et al. [27] suggested that applying BMSCs into mid-palatal suture could be an effective treatment strategy to improve RPE's long-term stability. They sacrificed their rats on day 10, however, we sacrifice them on day 21 after injection of BMSCs. So, it can be concluded that BMSCs can enhance bone regeneration in the earlier days following its application, but over time, that is unlikely to observe any differences.

Regarding the application of LLLT on mid-palatal suture, various animal studies [19, 36] and clinical studies [18, 44] have been conducted. Despite the limited evidence, most of the studies showed the success of LLLT [45]. However, our study results regarding the application of LLLT alone showed that it has no beneficial effect on increasing bone regeneration in the region of interest. Unlike our study, Altan et al. [17] showed that LLLT lead to an increase in the number of osteoblasts and new blood vessels in inter-maxillary suture in inter-maxillary suture. All the differences in the results obtained regarding the success of LLLT application in various studies are mostly due to its application protocol, including the number of sessions, radiation dose, and radiation site [35]. Other than LLLT stimulatory effects, it has some other advantages like reducing pain following RPE [35], convenient clinical application [45], and lack of side effects [19].

Among all the interventions assessed in the study, the combined application of LLLT and BMSC showed the most promising results. To the extent of our knowledge, no studies found that used this treatment approach regarding bone regeneration in the inter-maxillary suture. However, Kouhkheil et al. [46] demonstrated that LLLT and BMSCs simultaneous applications would be beneficial and have synergistic effects regarding wound healing. Ginani et al. [40] reported that LLLT increases the proliferation rate of mesenchymal stem cells. Although the exact molecular mechanism of this phenomenon is unknown, it seems that the cause could be the effect of the laser on the increased synthesis of growth factors, nitric oxide, reactive oxygen species, ATP, RNA, and DNA [47]. On the other hand, LLLT increases the expression of various growth factors [48]. Furthermore, LLLT has a dose-dependent effect on the differentiation of BMSCs into osteoblasts [49].

In this study, we used rat models to evaluate bone regeneration in the intermaxillary suture, which was previously used in similar studies. It was mainly because of its easy maintenance requirements and the possibility of easy and fast RPE [33]. However, further clinical evaluations on bigger animals or humans are required before their widespread use in practice. In addition, the effect of interventions on the rate of post-treatment relapse was not analyzed in this study. Moreover, studies which investigated the effect of the length of the retention period on the post-treatment relapse are required.

Conclusion

Considering the limitations of our study, the results implied that using LLLT and BMSCs simultaneously could be an effective treatment approach for enhancing the long-term stability of RPE through accelerating bone regeneration. Further studies will be needed to assess the combined effect of LLLT and BMSC on bone regeneration.

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Disclosure of conflict of interest

None.

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