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Original Article

Evaluation of in Vivo Effects of Low-Intensity Pulsed Ultrasound and Low-Level Laser Therapy on Premaxillary Suture During Rapid Maxillary Expansion

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Main Points

- During rapid maxillary expansion, low-intensity pulsed ultrasound (LIPUS) application is more effective than low-level laser therapy (LLLT) in terms of the suture width, newly formed bone areas, and vascular endothelial growth factor (VEGF) expression in the suture.
- LLLT and LIPUS do not affect the number of osteoblasts in the suture when they are applied alone, while their combined therapy significantly increases osteoblast numbers.
- Combined therapy triggers angiogenesis and osteogenesis more by increasing the expressions of bone morphogenetic protein-2, osteopontin, and VEGF in the suture compared with monotherapies.
- Combined therapy has a synergistic effect and strengthens the effects of LLLT and LIPUS on premaxillary sutural ossification.

ABSTRACT

Objective: To evaluate and compare the effects of low-intensity pulsed ultrasound (LIPUS), low-level laser therapy (LLLT), and their combined effects on sutural bone regeneration during rapid maxillary expansion (RME) of rats.

Methods: Twenty-eight Sprague-Dawley rats were randomly assigned to four groups: LLLT group, LIPUS group, combination group, and control group. RME was performed on all groups for 11 days. The Both LLLT and LIPUS groups received their respective therapies (30 J/cm^2), while the combination group received both therapies, each at 30 J/cm^2. All treated rats received their doses on days 0, 4, and 8 and were sacrificed on day 11. Numbers of osteoblasts, capillaries, and osteoclasts were counted, and suture widths and areas of newly formed bone were measured histomorphometrically. General and cellular immunoreactivity of bone morphogenetic protein-2 (BMP-2), vascular endothelial growth factor (VEGF), and osteopontin (OPN) was evaluated by immunohistochemistry.

Results: The number of osteoblasts was significantly higher in the combination group than in the control group ($p<0.05$). The combination group showed the highest general BMP-2 immunoreactivity and cellular VEGF immunoreactivity among all groups, and exhibited increased cellular OPN immunoreactivity compared with the control group ($p<0.05$). Both the area of newly formed bone ($p<0.05$) and suture width ($p<0.01$) were significantly greater in the LIPUS group than in the LLLT group.

Conclusion: LIPUS is a more effective adjuvant therapy than LLLT for increasing sutural bone formation during RME. Combined therapy with LIPUS and LLLT has a synergistic effect and accelerates sutural bone regeneration by enhancing cellular activation more than either LIPUS or LLLT alone.

Keywords: Low-level laser therapy, low-intensity pulsed ultrasound, maxillary expansion, rat

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INTRODUCTION

Rapid maxillary expansion (RME) has been used in the treatment of transverse maxillary deficiencies for over a century, since Angell first described the procedure in the 1860's.[1] The primary disadvantage of RME is the prolonged retention period required to permit new bone deposition in the expanded suture. Therefore, researchers have studied many alternative methods, such as various pharmacological agents (vitamin E, propolis, topical ozone, osthole, and simvastatin), to accelerate new bone formation, reduce relapse, and shorten the retention period by increasing the regenerative capacity of the midpalatal suture during the expansion and retention periods.[2-6] Additionally, low-intensity pulsed ultrasound (LIPUS) and low-level laser therapy (LLLT) are among the newer methods introduced in recent years to accelerate bone regeneration.[7-10]

LLLT irradiation is reported to be absorbed by cytochrome c oxidase in the inner mitochondrial membrane, stimulating the cellular energy cycle and metabolic activity.[11] Secondary mediators that arise in response to photobiomodulation activate transcription factors and signaling pathways. Many transcription factors associated with osteogenesis have been reported to be activated by laser light. In recent studies, LLLT has been used to reduce orthodontic pain and orthodontically induced root resorption, to accelerate tooth movement, and to promote bone regeneration during expansion.[8,10,12]

When low-intensity ultrasound waves are absorbed by tissue, non-thermal effects, including cavitation and acoustic streaming occur, resulting in increased cell membrane ion permeability and enhanced cellular activity.[13] Because of these properties, LIPUS is used for tissue regeneration and bone healing.[7,14]

Despite the growing literature supporting their individual effectiveness, comparative studies examining the effects of LLLT and LIPUS on bone formation remain limited. Lirani-Galvão et al.[15] performed osteotomies in rats to compare the in vivo effects of LLLT and LIPUS on bone repair. Their study concluded that LLLT promoted bone formation, while LIPUS facilitated bone resorption. Subsequently, another study investigating bone defect healing in rats reported that LLLT had positive effects on new bone formation, while LIPUS had no significant effects.[16] Babuccu et al.[14] compared the effects of LLLT, LIPUS, and their combined application on tibial osteotomies in rats. The study demonstrated that vascularization and new bone formation were higher, and inflammation was lower, in the combination group than in the other groups. Mahmoud et al.[17] reported that, in patients with dental implants, LIPUS significantly reduced post-implant marginal bone loss compared with LLLT, whereas LLLT was more effective for soft tissue healing, and combined therapy reduced pain intensity. However, the potential synergistic effect of combined LLLT and LIPUS therapy has not been investigated for RME.

The first hypothesis of this study was that LIPUS would be more effective than LLLT in stimulating cellular activation and new bone formation when applied to the premaxillary suture at equal doses during RME. The second hypothesis was that combined therapy would enhance sutural activation and increase areas of newly formed bone more effectively than monotherapies due to their distinct mechanisms of action at the cellular level.

METHODS

This animal study was carried out in year 2021. All animal study procedures were approved by the University of Health Sciences Hamidiye Local Ethics Committee for Animal Experiments (approval no.: 2020-03/05, date: 25.06.2020). Twenty-eight female Sprague-Dawley rats, aged 9-10 weeks and weighing between 100-160 g, were used in the study. Experimental animals were housed in separate plastic cages in their respective groups at 23 °C under fluorescent lighting with a 12-hour light/dark cycle. Throughout the study period, the animals were fed a standard pellet diet and provided with tap water ad libitum.

According to the power analysis (G*Power, version 3.1; effect size 0.5, α 0.05, and power 80%), seven animals were required in each experimental group, and the sample size was approved by the ethics committee. The rats were assigned to four groups (i.e., three experimental groups and a control group) each consisting of seven animals, using simple randomization performed by a technician blinded to the experimental procedure. The experimental groups were the LLLT group, the LIPUS group, and the combination (LLLT plus LIPUS) group.

Body Weights Measurements

The body weights of all animals were measured using a precision scale at the beginning and end of the study (days 0 and 11).

General Anesthesia

Placement of the RME appliances and administration of LLLT and LIPUS treatments were performed under general anesthesia. Xylazine (10 mg/kg; Xylazinbio 2%, Bioveta A.S., South Moravian, Czech Republic) and ketamine (90 mg/kg; Ketasol 10%, Richter Pharma AG, Wels, Austria) were administered via intraperitoneal injection.

Premaxillary Suture Expansion Procedure

RME appliances were fabricated from 0.014-inch stainless-steel wire and incorporated three helical springs (Figure 1A). The springs were adjusted to deliver a force of 100 g. Retention grooves were prepared on the distal surfaces of the incisors at the gingival level with a stainless-steel disc. The expansion appliances were fixed to the maxillary incisors of all animals with 0.010-inch stainless-steel ligature wires and covered with a light-curing glass-ionomer composite resin (Figures 1B and 1C). The springs were not reactivated at any point during the experimental period. The distance between the mesial edges of the maxillary incisors was defined as the 0-mm baseline at

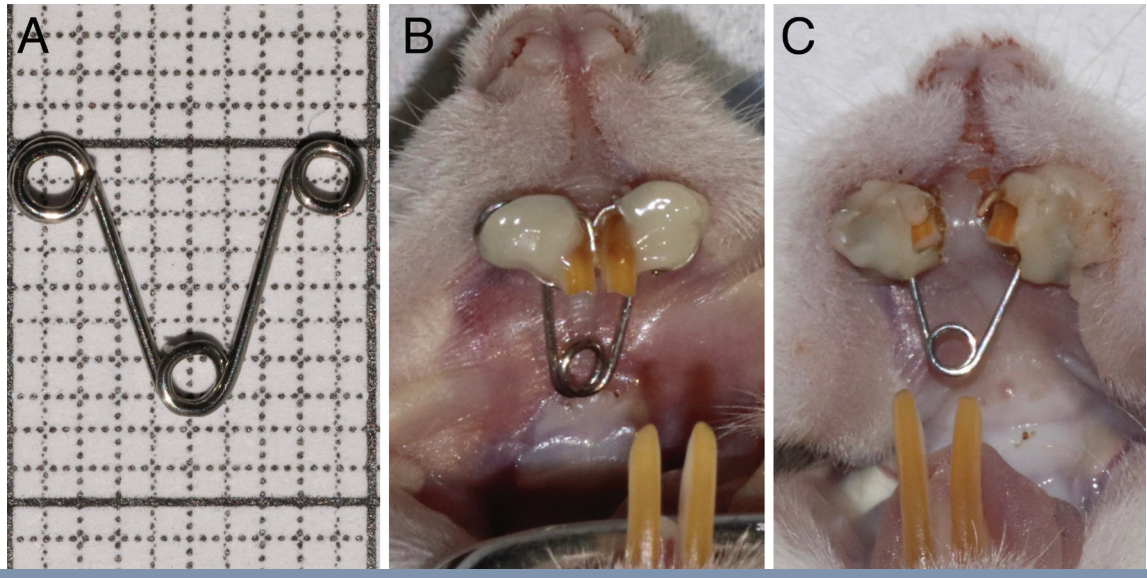

Figure 1. (A) The expansion spring on the grid; (B) The expansion appliance at the beginning; (C) The expansion appliance on 4th day.

the start of the study and remeasured at the end of the study. Occlusal radiographs were taken on days 4 and 11 to determine whether the premaxillary suture opened (Figure 2A). On day 11, an occlusal radiograph demonstrating and open premaxillary suture in a rat from the combination group is shown in Figure 2B. The RME procedure was applied to all experimental groups as well as to the control group, which did not receive any adjunctive biostimulatory treatment (e.g., LLLT or LIPUS).

LLLT Treatment

An aluminum gallium arsenide dental diode laser (Solase-976, Lazon Medical Laser Co. Ltd., Liaoning, China) was applied intraorally to the palatal mucosa immediately posterior to the maxillary incisors using a biostimulation probe (Figure 3A). Laser irradiation was performed on days 0, 4, and 8. The laser parameters used in the study are given in Table 1. Only the LLLT and combination groups received laser therapy. LLLT was applied to the combination group prior to LIPUS treatment.

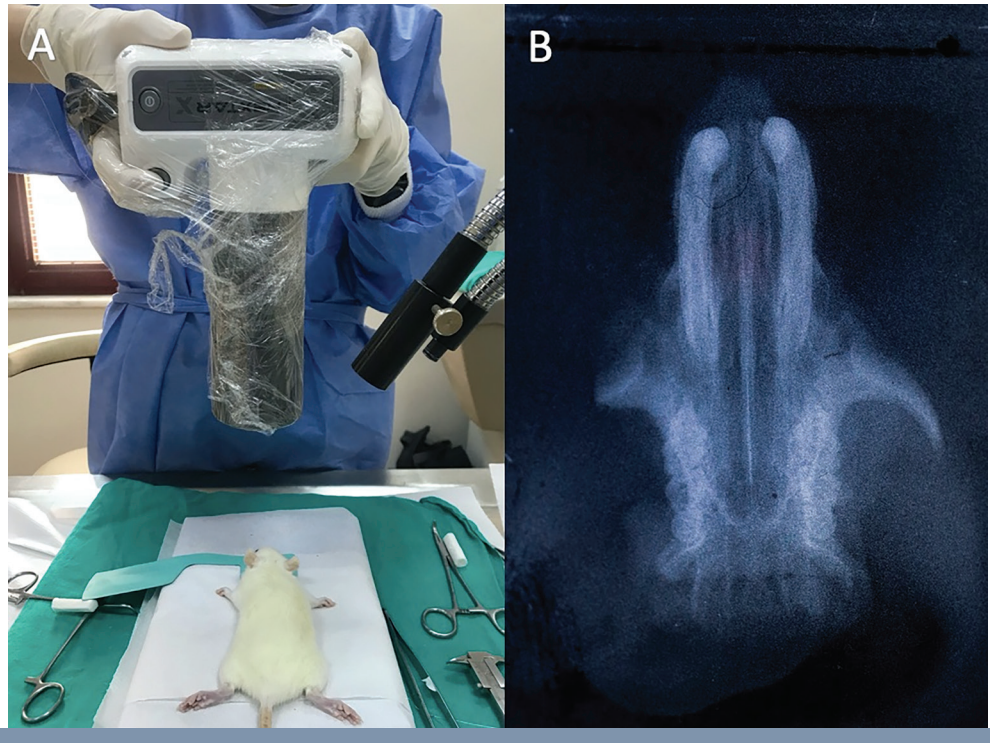

Figure 2. (A) Occlusal imaging technique with a portable X-ray device. (B) Radiographic image of an open premaxillary suture in a rat from the combination group.

LIPUS Treatment

LIPUS therapy was administered to all animals in the LIPUS and combination groups with a medical LIPUS device and a 1-cm-diameter ultrasound transducer (4710-Premium, BTL Industries Ltd., Hertfordshire, UK). After the rats' snouts were covered with coupling gel, the transducer was applied extraorally over each snout, perpendicular to the premaxillary suture (Figure 3B). Ultrasound irradiation was performed on days 0, 4, and 8. The ultrasound device parameters used in the study are given in Table 2. Only the LIPUS and combination groups received ultrasound therapy.

Specimen Preparation

On day 11, all animals were euthanized by intraperitoneal injection with an overdose of ketamine and xylazine. The premaxillae were surgically dissected, and the RME appliances were removed. After the premaxilla specimens were fixed in 10% neutral buffered formalin, they were decalcified with 10% formic acid for three weeks. Subsequently, the premaxillae were dissected perpendicular to the sagittal plane, using the incisors as primary guides. The first incision was made at the alveolar crest, and the second was made 4 mm apical to it. The tissue samples were dehydrated by passing through an ascending series of ethyl alcohol solutions, embedded in paraffin blocks and serially sectioned at 4-5 µm.

Histomorphometry

For histomorphometric evaluation, the sections were deparaffinized, rehydrated, and stained with hematoxylin and eosin. The numbers of osteoblasts, capillaries, and osteoclasts were counted in three randomly selected sections per animal. Suture width was measured between the frontal margins of the palatal bones at the most anterior region of the premaxillary suture. Newly formed bone areas were calculated by tracing the borders of the newly ossified areas of the suture using Cameram Gen III software (Argenit Ltd., İstanbul, Türkiye).

Immunohistochemistry

Sections obtained from paraffin tissue blocks were rehydrated by passing through a descending alcohol series. Following incubation in citrate buffer at high temperature, the sections were allowed to cool to room temperature. Endogenous peroxidase activity was inhibited with a 3% H_2O_2 solution. Sections were washed with phosphate-buffered saline (PBS) and then soaked in protein-blocking solution for 10 min. Anti-vascular endothelial growth factor (VEGF) (GTX22992, GeneTex

Table 1. The laser device parameters used in the study

Active medium	AlGaAs
Wavelength	976 nm
Irradiation mode	Continuous
Output power	500 mW
Irradiation time	60 sec
Energy (daily)	30 J
Dose (daily)	30 J/cm^2
Irradiation days	0th, 4th, 8th days

AlGaAs, aluminum gallium arsenide.

Table 2. The ultrasound device parameters used in the study

Intensity (I_{SATP})	200 mW/cm^2
Intensity (I_{SATA})	50 mW/cm^2
Duty cycle	25%
Irradiation time	10 min
Output power	0.1 W
Pulse repetition period	100 Hz
Frequency	3 MHz
Energy (daily)	30 J
Dose (daily)	30 J/cm^2
Irradiation days	0th, 4th, 8th days

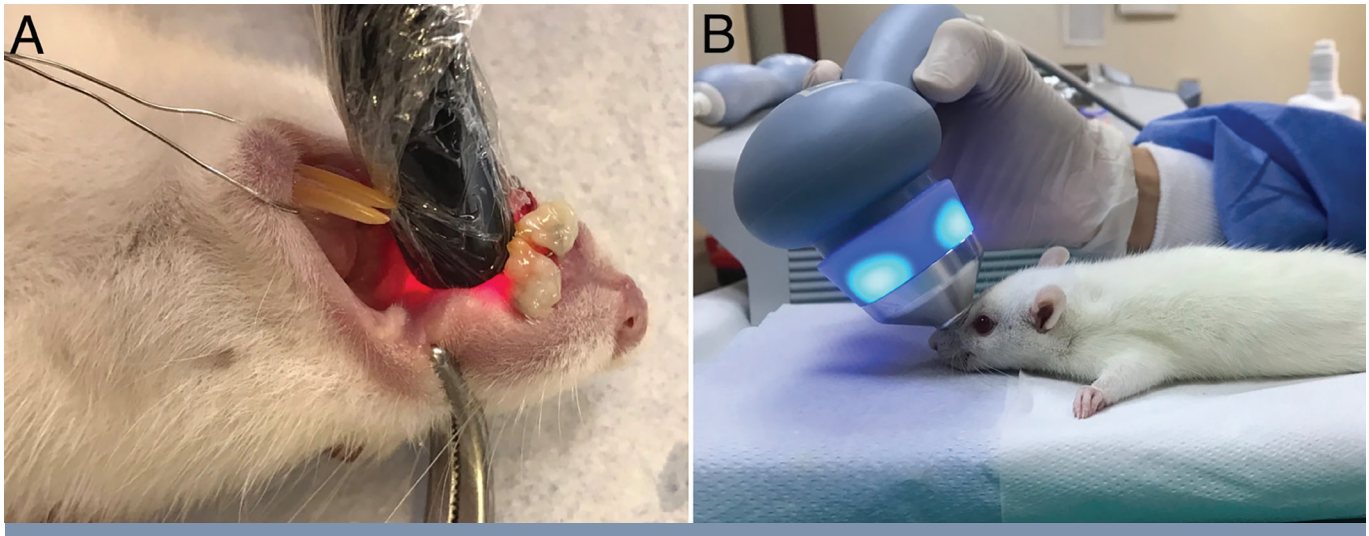

Figure 3. (A) LLLT and (B) LIPUS treatments.
LIPUS, low-intensity pulsed ultrasound; LLLT, low-level laser therap.

Inc., California, USA), anti- bone morphogenetic protein-2 (BMP-2) (GTX64355, GeneTex Inc., California, USA), or anti-osteopontin (OPN) (ab216402, Abcam plc, Cambridge, UK) primary antibodies were applied to the slides, and the sections were incubated for 24 hours at 4 °C.

After incubation, sections were washed with PBS and stained with a secondary antibody and 3,3′ diaminobenzidine. The tissue sections were counterstained with hematoxylin, passed through an ascending series of alcohols, cleared in xylene, and then mounted using Entellan mounting medium. Immunohistochemical evaluations were classified as general or cellular. For general evaluations, tissue preparations stained with anti-VEGF, anti-OPN, and anti-BMP-2 antibodies were graded as mild (+), moderate (++), or intense (+++) based on overall staining intensity. For cellular evaluations, three randomly selected areas from each section were examined, and the number of positively stained cells was graded as 1-10 (+, mild); 11-20 (++, moderate); or >20 (+++, high). All histomorphometry and immunohistochemistry assessments were performed by a single investigator who was blinded to the clinical procedures.

Statistical Analysis

Statistical analyses were performed using NCSS 2007 statistical software (NCSS LLC, Utah, USA). For data evaluation, in addition to descriptive statistics (e.g., means and standard deviations), variables were tested for normality using the Shapiro-Wilk test. One-Way ANOVA was used for comparisons of normally distributed variables; Tukey's multiple comparison test was used for pairwise comparisons among groups; the Kruskal-Wallis test was used for comparisons of variables that were not normally distributed; and the chi-squared test was used for comparisons of categorical variables. $p<0.05$ was considered statistically significant.

RESULTS

Bodyweight Change and Dental Expansion

Although the control group experienced significant weight loss, there was no significant difference between the groups in the magnitude of body weight change. Following RME, a midline diastema between the maxillary incisors occurred in all rats and measured 1.54±0.62 mm in the LLLT group, 1.83±0.32 mm in the LIPUS group, 1.69±0.34 mm in the combination group, and 2.03±0.58 mm in the control group. No statistically significant differences were detected among the groups with respect to dental expansion measurements.

Histological Observation Findings

Histological examination of hematoxylin and eosin-stained sections revealed that the width of the midpalatal suture was smaller in the LLLT group than in the other groups. Correspondingly, fewer osteoblasts were present adjacent to the new ossification areas, but they tended to be arranged in a regular pattern. In the LIPUS and combination groups, the midpalatal suture width increased compared with the LLLT group, and new ossification areas formed. The number of osteoblasts adjacent to these areas increased, and the osteoblasts were arranged in a regular pattern. In the control group, both the histological suture width and the areas of new ossification were smaller than those observed in the experimental groups. In addition, osteoblasts in the control group group had not yet achieved a regular arrangement.

Histomorphometric Findings

Histomorphometric values and intergroup comparisons are presented in Table 3.

Osteoblast, capillary, and osteoclast numbers

The combination group had a significantly higher number of osteoblasts than the control group ($p<0.05$); however, there were no significant differences among the other groups. Furthermore, no significant differences were detected among the groups regarding capillary and osteoclast numbers (Table 3).

Suture width and newly formed bone areas

Suture width was significantly greater in the LIPUS group than in the combination, LLLT, and control groups ($p<0.001$). The suture width in the control group was significantly lower than that in the combination ($p<0.01$) and LLLT ($p<0.05$) groups (Figure 4). The area of newly formed bone was significantly greater in the LIPUS group than in the LLLT group ($p<0.05$); no significant differences were observed among the other groups.

Immunohistochemical Findings

Immunohistochemical values and intergroup comparisons are presented in Table 4. Overall BMP-2 intensity in the combination group was significantly higher than in the LLLT ($p<0.05$), LIPUS ($p<0.01$), and control ($p=0.01$) groups. Also, cellular BMP-2 immunoreactivity in the combination group was higher than in the control group ($p<0.05$). There were no significant differences between the other groups in terms of BMP-2 staining ($p>0.05$).

The general intensity of VEGF staining in the LLLT group was significantly lower than that observed in the combination and LIPUS groups ($p<0.05$). Cellular VEGF immunoreactivity was significantly higher in the combination group than in the other three groups ($p<0.05$). The LIPUS group had higher scores for cellular VEGF immunoreactivity than the LLLT group ($p<0.05$).

When general OPN intensities were evaluated, no significant difference between the groups was observed ($p>0.05$). However, cellular OPN immunoreactivity in the LIPUS and combination groups was higher than in the control group ($p<0.05$) (Figure 5 and Table 4).

DISCUSSION

The aim of the study was to accelerate bone regeneration in the premaxillary suture area during RME in rats by applying

Table 3. Histomorphometric values and comparisons of experimental and control groups

Variables	LLLT	LIPUS	Combination	Control	p	Multiple comparisons**
Osteoblast*	18.06±2.23	21.08±2.44	22.33±5.83	16.21±3.24	**0.02**	LLLT-LIPUS-0.444 LLLT-Combination-0.168 LLLT-Control-0.787 LIPUS-Combination-0.922 LIPUS-Control-0.094 **Combination-Control-0.025**
Capillary*	0.841±0.734	0.667±0.476	0.988±0.533	0.234±0.274	0.071	-
Osteoclast‡	0.016±0.042	0.016±0.042	0.063±0.108	0.016±0.042	0.765	-
Suture width (µm)*	748.86±147.11	1262.71±175.31	785.71±110.27	500.14±157.5	**0.0001**	**LLLT-LIPUS-0.0001** LLLT-Combination-0.967 **LLLT-Control-0.023** **LIPUS-Combination-0.0001** **LIPUS-Control-0.0001** **Combination-Control-0.008**
Newly formed bone area (µm2)*	459152.14±197901.96	726396.57±69415.55	547663.71±103047.08	487069±276129.57	**0.046**	**LLLT-LIPUS-0.049** LLLT-Combination-0.797 LLLT-Control-0.991 LIPUS-Combination-0.276 LIPUS-Control-0.09 Combination-Control-0.922

The values marked in bold are: ($p<0.05$). *One-way ANOVA test, ‡Kruskal-Wallis test, **Tukey multiple comparison test.
LIPUS, low-intensity pulsed ultrasound; LLLT, low-level laser therapy.

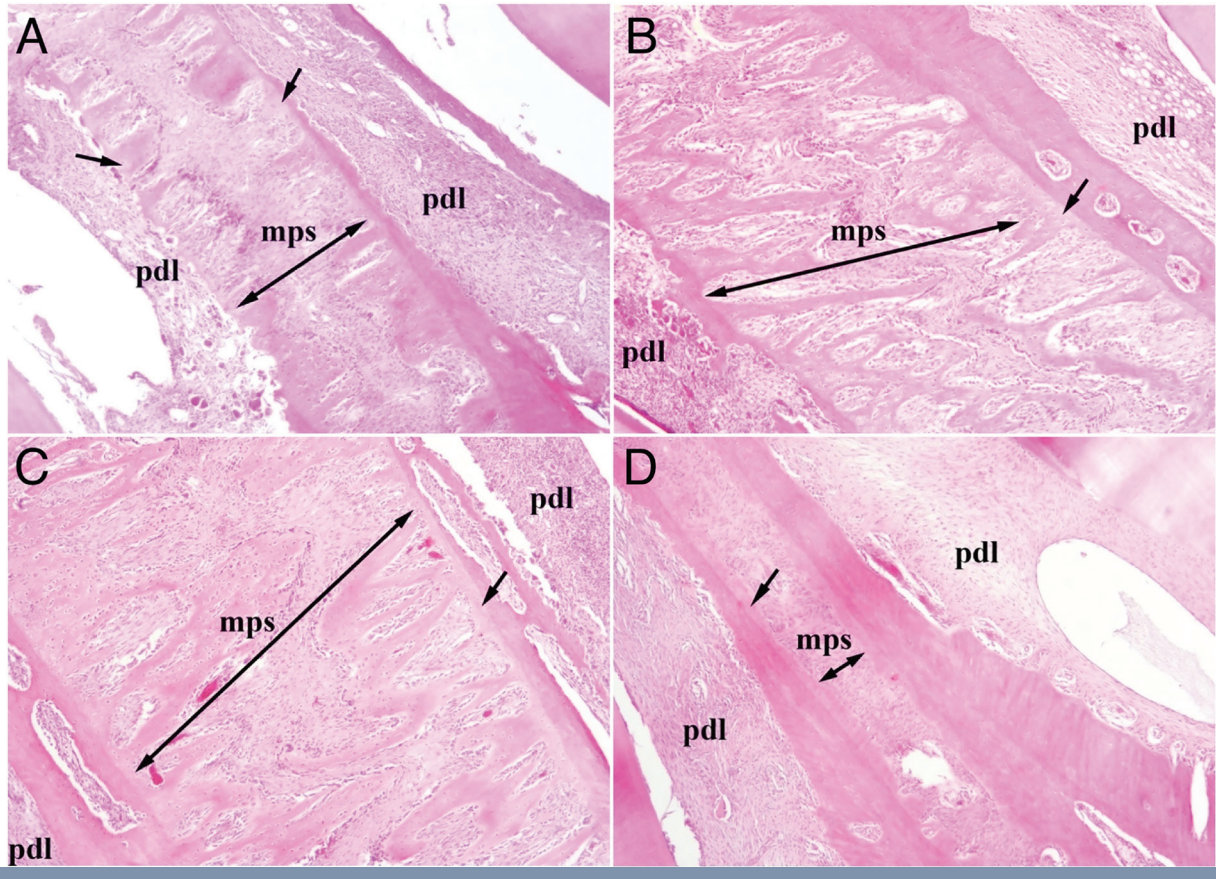

Figure 4. Morphology of midpalatal suture in the LLLT group (A), LIPUS group (B), combination group (C), control group (D) (400X magnification; mps: midpalatal suture, pdl: periodontal ligament).

LIPUS, low-intensity pulsed ultrasound; LLLT, low-level laser therapy.

Table 4. Immunohistochemical values and comparisons of experimental and control groups

Variables			LLLT	LIPUS	Combination	Control	p	Multiple comparisons**
BMP-2*	GI	(+)	57.14%	85.71%	0.00%	71.43%	**0.005**	LLLT-LIPUS-0.236 **LLLT-Combination-0.018** LLLT-Control-0.577 **LIPUS-Combination-0.004** LIPUS-Control-0.515 **Combination-Control-0.01**
		(++)	42.86%	14.29%	42.86%	28.57%		
		(+++)	0.00%	0.00%	57.14%	0.00%		
	CI	(+)	71.43%	85.71%	28.57%	100.00%	**0.03**	LLLT-LIPUS-0.515 LLLT-Combination-0.117 LLLT-Control-0.127 LIPUS-Combination-0.069 LIPUS-Control-0.299 **Combination-Control-0.021**
		(++)	28.57%	14.29%	28.57%	0.00%		
		(+++)	0.00%	0.00%	42.86%	0.00%		
VEGF*	GI	(+)	57.14%	0.00%	0.00%	42.86%	**0.041**	**LLLT-LIPUS-0.038** **LLLT-Combination-0.018** LLLT-Control-0.564 LIPUS-Combination-0.280 LIPUS-Control-0.147 Combination-Control-0.091
		(++)	42.86%	7.43%	42.86%	42.86%		
		(+++)	0.00%	28.57%	57.14%	14.29%		
	CI	(+)	71.43%	0.00%	28.57%	42.86%	**0.002**	**LLLT-LIPUS-0.018** **LLLT-Combination-0.016** LLLT-Control-0.427 **LIPUS-Combination-0.004** LIPUS-Control-0.135 **Combination-Control-0.043**
		(++)	28.57%	85.71%	0.00%	42.86%		
		(+++)	0.00%	14.29%	71.43%	14.29%		
OPN*	GI	(+)	28.57%	28.57%	0.00%	71.43%	0.061	-
		(++)	57.14%	71.43%	57.14%	14.29%		
		(+++)	14.29%	0.00%	42.86%	14.29%		
	CI	(+)	28.57%	14.29%	0.00%	71.43%	**0.006**	LLLT-LIPUS-0.515 LLLT-Combination-0.077 LLLT-Control-0.108 LIPUS-Combination-0.118 **LIPUS-Control-0.031** **Combination-Control-0.013**
		(++)	71.43%	85.71%	57.14%	28.57%		
		(+++)	0.00%	0.00%	42.86%	0.00%		

The values marked in bold are: ($p<0.05$). *Chi-square **Tukey multiple comparison test.
GI, general intensity; CI, cellular immunoreactivity; LIPUS, low-intensity pulsed ultrasound; LLLT, low-level laser therapy; VEGF, vascular endothelial growth factor; BMP-2, bone morphogenetic protein-2; OPN, osteopontin.

LIPUS and a combined LLLT-LIPUS protocol from the onset of maxillary expansion. To the best of our knowledge, this study is the first to compare the effects of LLLT, LIPUS, and their combined application (administered in equal doses during expansion) on the midpalatal suture.

In the literature, RME studies conducted in rats have applied heavy orthopedic forces to the maxillary incisors or molars. Forces ranging from 30-100 g have been applied to the maxillary incisors and are usually activated once.[2-4,7-10,18] Zahrowski and Turley[19] reported that the number of osteoprogenitor cells rose with increasing force levels up to 100 g during premaxillary expansion; however, at higher forces, cell numbers and bone formation decreased and eventually ceased. They noted that both low or high forces could result in insufficient sutural bone formation, and an expansion force of 100 g was suggested to ensure maximum sutural bone formation in the early period.[19] Therefore, although lower forces are commonly preferred in the literature, we applied 100 g of force between the maxillary incisors in our study. The expansion appliance used in our study was designed to contain three spiral springs, similar to the springs used by Aras et al.[9]

In the present study, the daily dose levels for therapeutic laser and ultrasound were determined based on Babuccu et al.,[14] who applied equal doses to compare the effects of LLLT, LIPUS, and their combination. Accordingly, LLLT and LIPUS were applied at equal daily doses of 30 J/cm^2. This approach ensured that any observed biological differences could be attributed to the biostimulation method itself, rather than to the amount of energy applied.

While no significant changes in body weight were observed in the experimental groups, the control group showed a significant decrease. LIPUS and LLLT are known to be effective in reducing orthodontic pain; therefore, the rats in the experimental groups may have experienced less pain following RME and been able to feed more comfortably, which may explain the absence of remarkable changes in these groups.[20,21]

In this study, the distance between the mesio-incisal edges of the maxillary incisors was initially set to 0 mm at baseline and measured using a caliper at the end of the study. The change in dental expansion did not differ significantly among the LLLT, LIPUS, combination, and control groups. This finding is consistent with the results of Toy et al.,[7] who reported that LIPUS did not influence the amount of dental expansion in rats during RME. However, this measurement approach assumes that the incisors of all rats were initially in full contact, which may not always be valid. Variations due to enamel wear, fractures, or positional changes resulting from applied forces might affect measurement accuracy, which is a limitation of our study. As a more reliable approach for future studies, we recommend measuring either between the mesial margins of incisors at the gingival level or between the disto-incisal edges of the incisors, both before and after activation.

Expansion of the intermaxillary suture was well tolerated by the experimental animals. No signs of inflammation or irritation were observed in the periodontal tissue, and no tooth fractures or pulpal damage occurred during appliance placement.

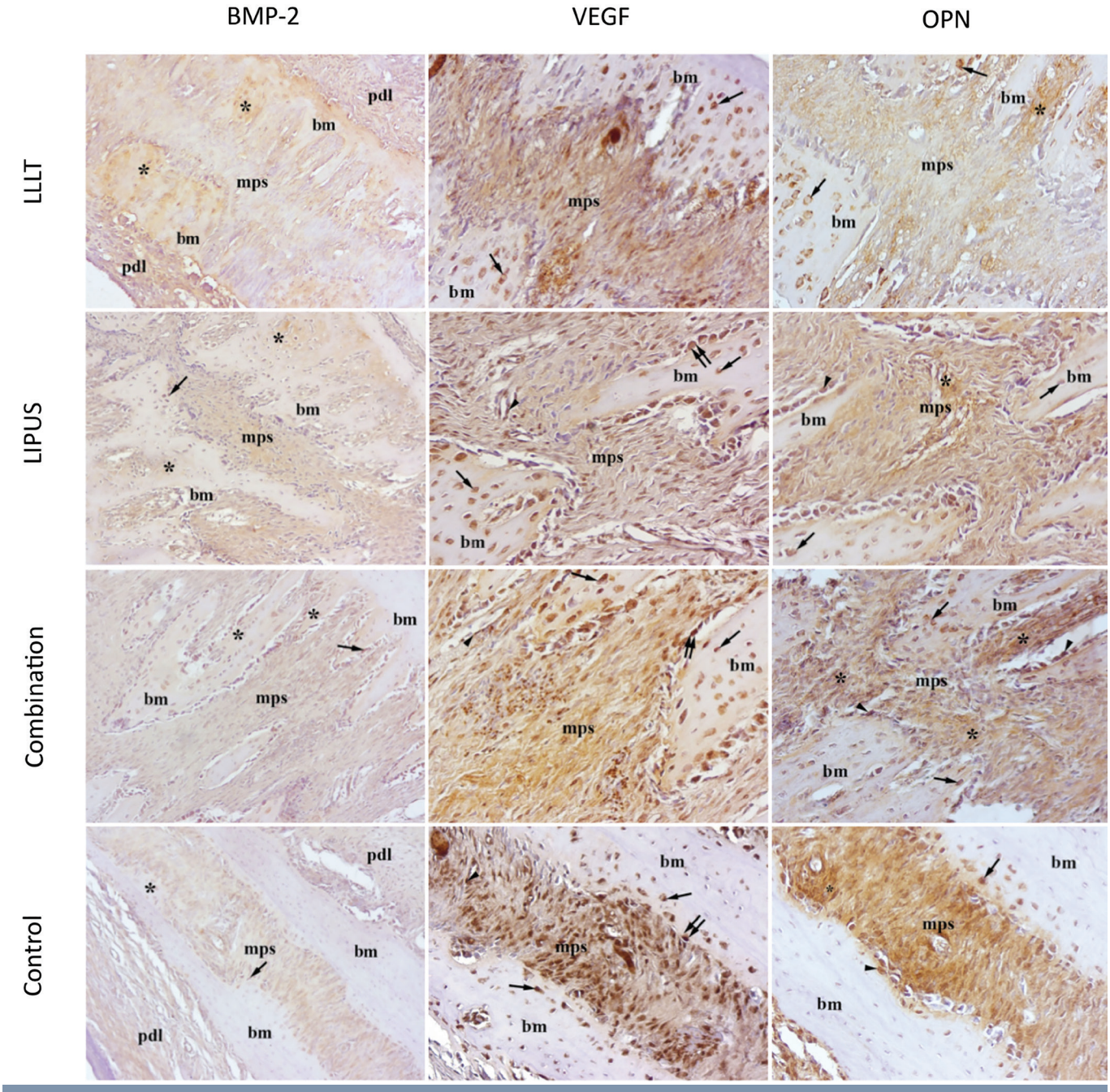

Figure 5. BMP-2, VEGF and OPN immunoreactivities of LLLT, LIPUS, combination and control groups (200X magnification; mps: midpalatal suture, pdl: periodontal ligament, bm: bone matrix).

LIPUS, low-intensity pulsed ultrasound; LLLT, low-level laser therapy; VEGF, vascular endothelial growth factor; BMP-2, bone morphogenetic protein-2; OPN, osteopontin.

However, some animals experienced appliance dislodgement and had their appliances replaced the same day.

Our results showed that the combined treatment notably increased the number of osteoblasts in the suture region compared with the control group. In contrast, LLLT and LIPUS treatments, individually, had no effect on the number of osteoblasts. Furthermore, LLLT, LIPUS, and the combination of treatments did not induce significant changes in the numbers of capillaries or osteoclasts in the premaxillary suture. Aras et al.[9] reported that LLLT did not cause any significant changes in the numbers of osteoblasts, capillaries, or osteoclasts in the premaxillary suture region on day,[17] corroborating our findings. Similarly, Toy et al.[7] reported that LIPUS treatment following RME did not significantly increase either the number of osteoblasts or the widths of capillaries, which is consistent with our findings.

Our findings indicated that LIPUS application significantly increased the sutural width and newly formed bone area compared with LLLT. Immunohistochemical analysis revealed that VEGF immunoreactivity was observed in osteocytes rather than in osteoblasts. Therefore, although the number of osteoblast, capillary, and osteoclast were similar between the two groups, the LIPUS group contained more osteocytes due to an increase in newly formed bone area; concomitantly, VEGF immunoreactivity may also have increased. No significant difference was observed between the two groups in BMP-2 and OPN expression.

Immunohistochemical evaluation demonstrated that the combination group showed the highest levels of BMP-2, VEGF, and OPN expression. In the combination group, the general BMP-2 intensity and cellular VEGF immunoreactivity were higher than those in all other groups; cellular BMP-2 and OPN immunoreactivity were higher than those in the control group; and the general VEGF intensity was significantly higher than that in the LLLT group. These results are consistent with previous studies that employed combined LLLT and LIPUS treatments.[14,22] No adverse effects were observed following combined treatment. In contrast, the combined treatment produced a synergistic effect and strengthened the outcomes of the monotherapies. This finding could be explained by different treatment methods having distinct effects at the cellular level.

BMP-2, VEGF, and OPN are key regulators of bone regeneration. Studies have shown that LLLT stimulates osteoblast differentiation and proliferation by increasing the expression of BMP-2, osteocalcin, and TGF-β1.[23] Suzuki et al.[24] demonstrated that BMP-2 expression increased when LIPUS was applied to rat osteoblasts. In the present study, BMP-2 was detected in the matrix and in some cells, especially in osteoblasts. Therefore, the increased BMP-2 expression observed in the combination group may reflect a greater number of osteoblasts.

Following RME, various tissue reactions begin in the palatal suture that are similar to those of the wound-healing process. The release of VEGF is one such tissue reaction, and angiogenesis plays a key role in healing midpalatal suture tissue and reparative bone formation under mechanical stress. LIPUS treatment during the retention period after RME has been reported to cause a significant increase in VEGF activity in mineralized and fibrous tissues.[7] However, the effects of LLLT on VEGF expression in the midpalatal suture have not been studied before. In the present study, LLLT had no effect on VEGF expression during the early stages of expansion, whereas LIPUS and combined therapy increased VEGF release by osteoblasts.

OPN plays an important role for bone remodeling under mechanical stresses. Perrien et al.[25] reported that OPN expression is biphasic, that primarily proliferating preosteoblasts express OPN, and that mature osteoblasts and osteocytes in newly ossified matrix show OPN immunoreactivity secondarily. In the present study, the LIPUS and combination groups showed a significant increase in both the number of OPN-positive cells and OPN activity, suggesting that the number of mature osteoblasts and osteocytes in the new bone matrix increased as a result of accelerated ossification and that secondary OPN expression had been initiated in the rats.

Clinical studies have shown that LLLT accelerates bone regeneration in the midpalatal suture.[26,27] However, to date, no clinical studies have evaluated the effects of LIPUS or their combined use during rapid RME. Clinical evidence supports the positive orthopedic effects of LIPUS treatment, including accelerated bone formation in fracture healing and distraction osteogenesis.[28] In addition, Maurya et al.[29] suggested that LIPUS may serve as an adjunctive therapy for treating class II malocclusion by enhancing bone remodeling of the condylar head and glenoid fossa when applied with a Forsus device. The present study can serve as a precursor to future clinical studies of LIPUS, including its combined use with LLLT during RME, particularly in young adults.

In this study, the suture width was measured only in the anterior region of the premaxilla in the transverse direction. Further histomorphological investigations and micro-computed tomography evaluations of the middle and posterior regions of the suture are required. Furthermore, the study was based on a small sample size; therefore, the findings should be validated by future in vivo studies with larger cohorts.

One advantage of LLLT is that dental laser devices are now widely used, and their costs have decreased over time. Additionally, the short application time of LLLT is advantageous for clinical use. Also, shortening treatment duration will reduce its overall cost. In contrast, LIPUS requires longer application times, and limited availability of dental-specific devices increases clinical application costs. In addition, the availability of dental laser devices equipped with small biostimulation probes suitable for application to the midpalatal suture enhances the clinical feasibility of LLLT. However, dental LIPUS devices are typically designed to accelerate orthodontic tooth movement and

have a parabolic arch (e.g., the Aevo system), making them unsuitable for use during RME. Therefore, clinical application of LIPUS during RME requires medical LIPUS devices; however, these devices often have large, bulky probes, making intraoral application in the palatal region challenging in a clinical study. Therefore, there is a need for the development of LIPUS devices specifically designed for palatal application during RME. Although these limitations currently restrict clinical applicability, increased adoption of medical and dental LIPUS technologies will lead to a wider variety of commercially available devices and reduced costs.

Study Limitations

This study has some limitations that should be considered. Firstly, in our study, the amount of dental expansion was measured between the mesioincisal edges of the maxillary incisors. The measurements obtained at the end of the study might have been affected by possible enamel wear, fractures of the incisal edges and, positional changes resulting from the expansion forces. Therefore, we recommend to measure the distance between the mesial surfaces at the gingival level for further studies to obtain more reliable results. Also, sutural width was measured only from anterior regions of the premaxilla on the transversal sections. Comprehensive histomorphological evaluations and micro-computed tomography analyses of the middle and posterior regions of the suture are necessary to provide a more complete understanding of sutural changes. Furthermore, this study was based on a small sample size; therefore, the findings should be validated by future in vivo studies with larger cohorts.

Additionally, long application time of LIPUS treatment and limited availability of dental LIPUS devices make the clinical application of the method challenging. Reducing of the device costs and devoloping of dental LIPUS devices which have smaller probes for the midpalatal suture area, are essential for the clinical application of this approach.

CONCLUSION

To accelerate sutural bone regeneration during RME, combined LLLT-LIPUS therapy was the most effective modality, followed by LIPUS therapy. The findings of this study suggest that LIPUS and combined therapy may promote more rapid cellular activation, accelerate bone regeneration, and shorten the retention period. However, further studies are needed to establish the validity of applying combined therapy in clinical practice.

Ethics

Ethics Committee Approval: All animal study procedures were approved by the University of Health Sciences Hamidiye Local Ethics Committee for Animal Experiments (approval no.: 2020-03/05, date: 25.06.2020).

Informed Consent: Not applicable.

Footnotes

Author Contributions: Surgical and Medical Practices - E.E., İ.Ö.P.; Concept - E.E., Ş.K.; Design - E.E., Ş.K., E.Ç.; Data Collection and/or Processing - E.E., İ.Ö.P.; Analysis and/or Interpretation - E.E., Ş.K., E.Ç.; Literature Search - E.E.; Writing - E.E.

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