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Effects of low-intensity pulsed ultrasound on bone formation after the expansion of the inter-premaxillary suture in rats: a histologic and immunohistochemical study

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Background: Orthodontic maxillary expansion is a commonly-performed treatment approach to correct transverse maxillary deficiencies by separating the mid-palatal suture. To obtain a successful treatment result and prevent relapse, the expanded maxillary sutures require maintenance by means of qualified bone reorganisation.

Aim: To assess the effects of low-intensity pulsed ultrasound (LIPUS) on sutural bone formation after the expansion of the inter-premaxillary suture in rats.

Methods: Sixteen male Wistar rats, 6 to 8-week old, were used. The expansion appliance comprised a helical spring fabricated from 0.014 inch stainless steel wire (Dentaurum, Ispringen, Germany). The rats were divided into two equal groups and randomly assigned to the LIPUS treatment group or a sham-operation group. LIPUS was delivered via a 2.5 cm diameter ultrasound transducer (Exogen, Smith and Nephews, Inc., Memphis, TN, USA) for 20 minutes per day during 7 days of post-expansion retention. Following retention, the rats' maxillae were surgically removed and histologic and immunohistochemical specimens were prepared and examined.

Results: The number of osteoblasts and blood vessel dimensions in the ultrasound group increased but was not significant, compared with the control group. A statistically significant difference in osteocalcin, VEGF and TGF-β immunoreactivities ($p < 0.01$) was found in the area of the mineralising tissue. Only VEGF immunoreactivity was significant between two groups ($p < 0.01$) in the fibrous tissue area.

Conclusions: The area of mineralising tissue in the LIPUS-applied group expressed activity markers for osteocalcin, VEGF and TGF-β compared with a surrounding area of fibrous tissue. Cellular activation in the LIPUS group was greater than that of controls. Therefore, LIPUS may be accepted as a useful approach to enhance sutural bone formation.

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Introduction

Cranial and facial sutures have been defined as the entire complex of cellular and fibrous tissue lying between the bones of the skull.[1] It has been demonstrated that sutures are the growth sites of intramembranous bones of the craniofacial system.[2] The application of tensile forces across the sutures produces anabolic bone activity related to collagen synthesis and cell proliferation.[3,4] Orthodontists direct forces to facial sutures to manage certain malocclusions by the stimulation of bone growth. Maxillary expansion is a commonly-performed treatment

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option to correct transverse maxillary deficiencies by separating the mid-palatal suture.[5,6] As the maxillary processes move laterally, a series of biological events within the expanding suture generates new bone formation.[7]

The expanded maxillary sutures require maintenance by means of qualified bone organisation to prevent collapse, which is interpreted as relapse.[8] In a recent computerised tomographic study, complete osseous in-fill of the mid-palatal area between the anterior and the posterior nasal spines following an 8 to 9-month retention period after sutural expansion, was reported.[9] The concept of accelerating new bone formation along the mid-palatal suture was expected to improve the stability of the expansion and shorten the retention period. Several chemical and external treatment modalities, such as periostin-like-factor, boron, propolis, bisphosphonate and low-power laser irradiation, have been tried to increase the deposition rate, quality and volume of new bone.[8,10-13] Reports of the influence of transforming growth factor-β1, vitamin D analog and vitamin E injections on sutural bone formation have also been published and advocated.[14-16]

Defined as an acoustic pressure wave at frequencies greater than human hearing, ultrasound is transmitted through living tissues and serves as a tool in diagnostics, therapeutics and surgery.[17] The management of a healing fracture has been improved by using therapeutic ultrasound, particularly low-intensity pulsed ultrasound (LIPUS).[18] Numerous studies have shown that LIPUS stimulates the healing of a fresh bone fracture, assists in the resolution of non-union or delayed union, and aids other healing processes.[19] Despite the pronounced effects of LIPUS, the underlying mechanisms of LIPUS stimulation remain unclear.

Immunohistochemical staining has been used to demonstrate osteoblastic activity and, for this purpose, osteonectin, osteocalcin, vascular endothelial growth factor (VEGF) and transforming growth factor beta (TGF-β) have been primarily evaluated.[10,14,20] Osteonectin, a bone-specific protein, shows highly specific binding to hydroxyapatite and collagen.[21] Osteocalcin exists in bone and dentine as a non-collagenous protein and is referred to as bone gamma-carboxyglutamic acid-containing protein (BGLAP). Also known as a calcium binding protein, osteocalcin collects in mineralised bone and binds to hydroxyapatite crystals to stimulate bone growth.[22] This protein actively governs the processes of ossification, extra cellular matrix remodelling and maturation. VEGF, as a glycoprotein growth factor, has major effects on tissue formation, cell migration, inflammation and wound healing.[23] It plays an important role in the restoration of the oxygen supply to tissues when blood circulation is deficient. TGF-β, a secreted protein and cytokine involved in immunity, is known to regulate proliferation and cellular differentiation in most tissues. Several studies have described the biological influences of TGF-β on osteogenic cells.[14,24]

The effects of LIPUS have been examined from an orthodontic perspective in an investigation into the repair process of orthodontically-induced resorption.[25] The healing effects of LIPUS were evaluated during mandibular distraction osteogenesis[26] and also in an examination of mandibular growth as a result of anterior bite-jumping appliances.[27] However, no information has been found regarding the influence of LIPUS on new bone deposition after mid-palatal expansion. Therefore, the aim of the present study was to histologically and immunohistochemically assess the effects of LIPUS on sutural bone formation after expansion of an inter-premaxillary suture in the rat.

Materials and methods

The experimental protocol was approved by the Animal Research Ethics Committee at the İnönü University School of Medicine in Malatya, Turkey (2011/A-57). The study was conducted in compliance with the Declaration of Helsinki, Guiding Principles in the Care and Use of Animals in Research. The present study was organised as a parallel group design with one group receiving the experimental protocol and the other serving as a control group. A power analysis was performed with G*Power Ver.3.0.10 (Franz Faul, Universität Kiel, Germany) software. Based on 1:1 ratio between groups, a sample size of 16 animals was predicted to provide more than 80% confidence to detect differences at an $\alpha = 0.05$ significance level. Sixteen male, 6 to 8-week old, Wistar rats, with a mean weight of 192.63 ± 21.49 grams, were individually caged with a 12 hour light-dark cycle at a constant temperature of 23°C and fed water and nourishment, ad libitum.

Inter-premaxillary suture expansion

A helical spring appliance, fabricated from 0.014 inch

stainless steel wire (Dentaurum, Ispringen, Germany) was used for suture expansion. The animals were anaesthetised by a subcutaneous injection of ketamine hydrochloride at a dose of 90 mg/kg (Alfamine; Alfasan International BV Woerden, Netherlands) and 30 mg/kg xylazine hydrochloride (Alfazyne; Alfasan International BV, Woerden, Netherlands). The arms of the expansion spring were inserted into pre-prepared grooves close to the gingival margins of the upper incisors. The appliance was activated to deliver a force of approximately 50 gm and not re-activated during the 5-day experimental period (Figure 1) at which time the spring was deactivated. The distance between the distal edges of the upper incisors was measured, at the start and at the end of expansion, using a digital micrometer (Mitituyo, Tokyo, Japan).



Figure 1. Intra-oral view of used expansion appliance.



Figure 2. Ultrasound application to rats under anesthesia (Exogen, Smith and Nephew, Inc., Memphis, TN, USA).

Application of LIPUS

The rats were randomly divided into two groups of eight animals and designated the LIPUS treatment group and the sham-operation group. LIPUS treatment was conducted under anaesthesia with ketamine-xylazine (90 ml/kg – 30ml/kg i.m.) and delivered via a 2.5 cm diameter ultrasound transducer (Smith and Nephew Inc., Memphis, TN, USA). The transducer was placed on the rat's snout which was smeared with ultrasound covering gel (Figure 2). The ultrasound signal consisted of a 200-ms application of a 1.5 MHz wave, repeating at 1.0 kHz and delivering 30 mW/cm^2 spatial and temporal average incident intensity. The duration of ultrasound application was 20 minutes per day in the morning throughout the 7 days of a retention period following the expansion.

Specimen preparation

After the retention period, the animals were euthanased via an overdose of ketamine and xylazine and each rat's maxilla containing the expanded suture was surgically removed. The specimens were subjected to fixation in a 10% neutral buffered formalin solution at room temperature for 24 hours.

Following fixation, the retaining wire was unfastened, and the premaxilla was decalcified at 4°C using formic acid at a concentration of 15%. After decalcification, the premaxillae were segmented via cuts perpendicular to the sagittal plane using the incisor as the primary orienting guide. One section passed through the centre of the incisor crown at its gingival level. The segments were processed using an autotechnichon, following which they were embedded in paraffin and serially sectioned at 5µm intervals.

All tissue blocks were evaluated at the Laboratory of Pathology, Meram Medicine Faculty, Necmettin Erbakan University, Konya, Turkey. Orientating sections were stained with haematoxylin and eosin (Harris haematoxyline, 09-182-1, DDK Italia, Italy; Eosin, MOS, 0712012, Turkey) (Figure 3).

The immunohistochemical staining procedure required that sections be stored in xylene in an incubator at 56°C overnight and subsequently rehydrated via a descending sequence of ethanol solutions. After being incubated in citrate-buffered solution at high temperature (100°C) for 20 minutes,

sections were left to cool to room temperature. The sections were washed in distilled water and PBS, and incubated with a non-immune serum (Ultra V block, cat. No: TA-125-UD, Lab Vision) prior to incubation with primary antibodies – polyclonal rabbit anti-osteonectin (SPARC, 251503, Abbiotec, San Diego, CA, USA), monoclonal mouse anti-osteocalcin (OC4-30, Novus Biologicals, Cambridge, UK), polyclonal rabbit anti-VEGF (PU483-UP, Biogenex, San Ramon, CA, USA) and polyclonal rabbit anti-TGFβ (ab66043, Abcam plc, Cambridge, UK) – for one hour at 4°C. After washing, sections were incubated with biotinylated secondary antibody, followed by streptavidin peroxidase for 20 minutes (Lab. Vision Cat:TS-125-HR, Thermo Scientific, Fremont, CA, USA). Immunostaining was achieved using DAB substrate (Lab. Vision CAT: TS-125-HR). The incubated sections were finally washed with distilled water, counterstained with Mayer's haematoxylin (Lillie's modification, ScyTek Laboratories, UT, USA), and mounted in an aqueous mounting medium. Sections were examined with a BX43 bright-field microscope (Olympus, Tokyo, Japan). A red-brown precipitate indicated positive findings for the primary antibodies. A single examiner, blinded to the clinical procedures, performed the evaluations and the results were taken as an average of the counts. The mean values of the immunohistochemical staining intensities were graded as mild (+), moderate (++), or strong (+++). The numbers of active osteoblasts were scored as + (1-10), ++ (11-20), +++ (>20) in 40 times magnification (Figure 4). The widths of the vessels were scored as + (narrow), ++ (moderate), +++ (wide).

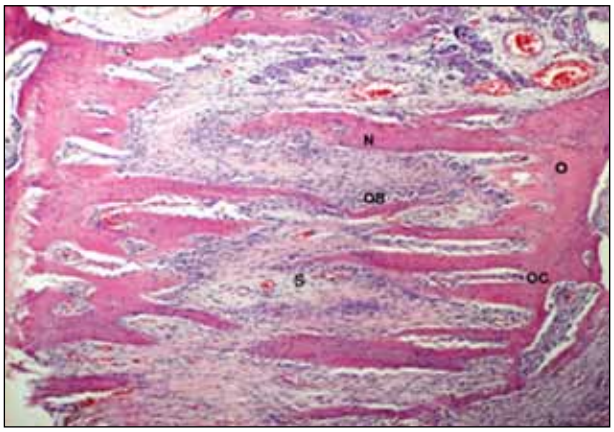


Figure 3. Haematoxylin and eosin (H&E) staining photomicrographs in ultrasound applied group. O, old bone; N, new forming bone; OB, osteoblastic activity; OC, osteoclastic activity; S, Sutural region (20X magnification).

A microscope with an attached digital camera system (Olympus BX5, Olympus Corp. Japan; Pro 150ES Digital Camera System, Pixera Corp., Los Gatos, CA, USA) and computer-assisted image analysis software (BS 200 Pro Plus, BAB Digital Imaging System, Ankara, Turkey) were used for image acquisition and histomorphometric evaluation (Figure 5). One section from each animal was randomly selected and analysed to determine the extent of the mineralised area (mm²), fibrotic area (mm²), mineralised area/fibrotic area (%) within the premaxillary suture, according to previous descriptions reported by Parfitt et al.[28]

Statistical analysis

The data were analysed with the statistical package for the social sciences 20.0 (SPSS for Windows, SPSS Inc.,

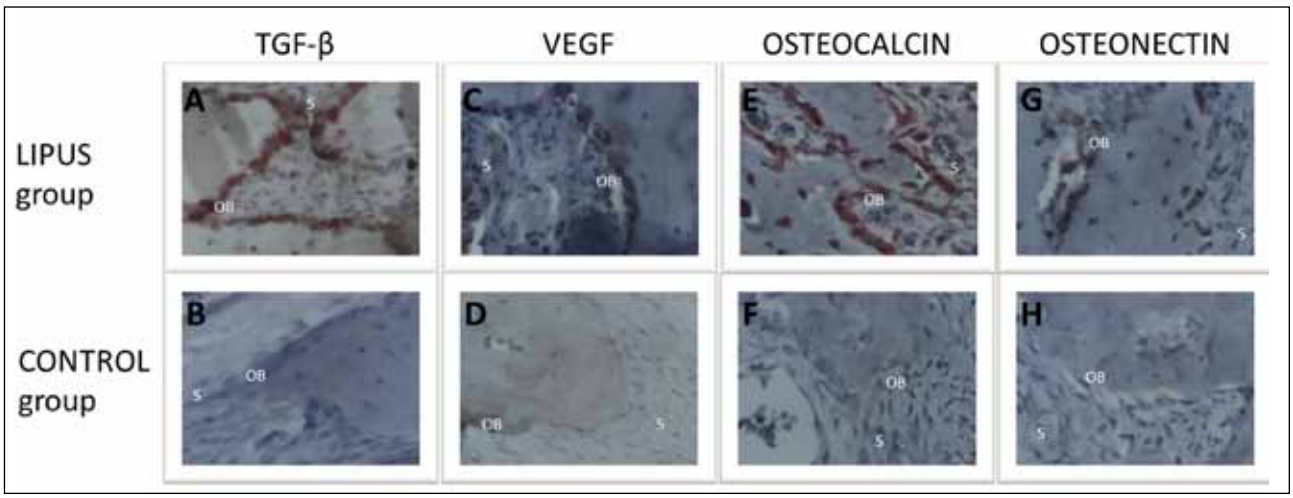


Figure 4. Photomicrographs of immunohistochemical sections from sutural areas stained with anti-TGF- β (A,B), anti-VEGF antibody (C,D), anti-osteocalcin antibody (E,F) and anti-osteonectin antibody (G,H). OB, osteoblast; S, suture connective tissue (40X magnification).

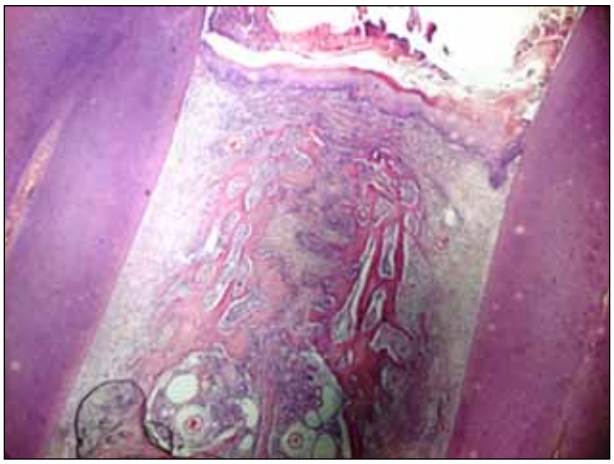


Figure 5. Histological section stained with haematoxylin-eosin for histomorphometric evaluations (4X magnification).

Chicago, IL, USA). Descriptive statistics were given as median, minima, and maxima for ordinal data, and as mean and standard deviations for continuous data. The normality of the data was evaluated by the Shapiro-Wilk test. Group differences were assessed by the Mann-Whitney U test for ordinal data and an independent samples *t*-test for continuous data. *P* values less than 0.05 were considered to be statistically significant.

Results

The rats experienced minor initial feeding difficulties after the application of the expansion appliance but no weight loss was recorded during the expansion or retention periods. Sutural opening was evident via dental inspection but no statistically significant difference was found between the two groups (Table I) and so both samples were accepted as similar.

When histomorphometric parameters were evaluated, the rats to which ultrasound was applied showed an increased area of mineralised tissue and a decrease in the area of connective tissue compared with control animals. The ratio between the areas of mineralised tissue and connective tissue correspondingly decreased. However, there were no statistically significant differences between the groups (Table II). A comparison of histologic measurements revealed that the number of osteoblasts and blood vessel dimension in the ultrasound group increased; however, this was found to be insignificant when compared with the control group (Table III).

The immunohistochemical evaluations of tested samples are presented in Table IV. In the area of mineralised tissue, there were statistically significant differences in the osteocalcin, VEGF and TGF-β immunoreactivities ($p < 0.01$). Only VEGF immunoreactivity was significant in the area of fibrous tissue between two groups ($p < 0.01$) (Figure 4).

Discussion

A method for shortening orthodontic treatment time and increasing the quality of bone and periodontal tissue would be beneficial to both patients and orthodontists. Non-invasive techniques such as low level laser therapy and LIPUS have been used for this purpose. These methods are readily tolerated and useful for stimulating the bone repair process by improving osteogenic remodelling, and angiogenesis.[29] In a recent study, Gu et al.[30] stated that ultrasound application accelerated new bone

Table I. Comparison of biometric analysis for determination of the amount of dental expansion (mm).

	Ultrasound			Control				
	N	Mean	SD	N	Mean	SD	*p* value	Significance
Amount of expansion	8	2.318	0.079	8	2.303	0.376	0.910	NS

N, sample size; *p* value, independent samples *t*test; NS, not significant

Table II. Descriptive values and comparisons of histomorphometric measurements (mm^2).

	Ultrasound			Control				
Parameters	N	Mean	SD	N	Mean	SD	*p* value	Significance
Mineralised tissue	8	0.5072	0.1142	8	0.4667	0.1207	0.502	NS
Connective tissue	8	0.4469	0.1979	8	0.4676	0.2194	0.846	NS
Mineralised tissue/ connective tissue	8	1.2511	0.4271	8	1.1601	0.4770	0.694	NS

N, sample size; *p* value, independent samples *t*test; NS, not significant

Table III. Descriptive values and comparisons of histologic measurements.

	Ultrasound				Control					
	N	Median	Min	Max	N	Median	Min	Max	*p* value	Significance
Number of osteoblasts	8	1.5	1	3	8	1	1	2	0.268	NS
Vessel width	8	2	2	3	8	2	1	3	0.199	NS

N, sample size; *p* value, Mann-Whitney U test; NS, not significant

Table IV. Immunohistochemical evaluation of tested samples.

		Ultrasound				Control					
Parameters		N	Median	Min	Max	N	Median	Min	Max	*p* value	Significance
Osteonectin	Mineralised tissue	8	1.5	0	3	8	1.5	0	2	0.663	NS
	Fibrous tissue	8	1	0	2	8	1	0	1	0.396	NS
Osteocalcin	Mineralised tissue	8	3	2	3	8	2	1	2	0.005	**
	Fibrous tissue	8	2	0	2	8	1	0	2	0.079	NS
VEGF	Mineralised tissue	8	3	2	3	8	1.5	1	2	0.002	**
	Fibrous tissue	8	2	1	3	8	1	1	1	0.010	**
TGF-β	Mineralised tissue	8	3	2	3	8	1.5	1	2	0.003	**
	Fibrous tissue	8	1	0	2	8	1	0	1	0.333	NS

N, sample size; *p* value, Mann-Whitney U test; NS, not significant; **, $p < 0.01$

formation and healing of the periodontal tissues and bone defects associated with canine teeth in Beagle dogs. In addition, it has been found that ultrasound significantly decreased orthodontically-induced inflammatory root resorption by improving the formation of a pre-cementum layer and reparative cellular cementum.[31] Therefore, the present study was planned in order to determine whether the effects of LIPUS were able to produce positive results and improve the quality of new bone formation after inter-premaxillary suture expansion in rats.

Saito and Shimizu[12] examined low-power laser irradiation and its stimulatory effects on bone regeneration in the rat sutures during expansion. Laser irradiation reportedly accelerated bone regeneration and laser therapy reduced relapse while shortening the retention period. Santiago et al.[32] studied the influence of soft laser treatment on the process of bone repair in dogs and reported that the laser affected the nature of the restoring process and contributed to suture reorganisation and palatal bone osteogenesis. Soft laser treatment is a form of extremely intensified non-ionizing radiation that results in thermal, photochemical and nonlinear actions.[32] As ultrasound generates an acoustic pressure wave, tissue overheating does not occur. Furthermore, LIPUS is a non-invasive, easily-applied and well-tolerated technique. Since the LIPUS machine is a relatively small and portable device, the application can be self-administered by a patient at home, after proper instruction.

Many clinical reports have suggested that 3 to 6 months retention is necessary for sutural reorganisation after human rapid maxillary expansion.[33,34] The retention period of the present animal study was determined according to the comparative assessment of the life span between rats and humans.[35] Therefore, a maximum of 6 month-retention in humans equals approximately 7 days in a rat's life span. Uysal et al.[36] also applied this retention period in rats.

Ultrasound acts as a mechanical disturbance on the extra-cellular matrix of the applied tissue. The energy produced by LIPUS, as a longitudinal wave with compressions and refinements, affects the biological tissue depending on its structural composition. The delivered waves pass through the transducer and specimen via a coupling gel.[37] Therapeutic ultrasound, with frequencies varying between 0.5-1.5 MHz and intensities varying between 30-200 MHz, has been proven to accelerate the bone healing process[38] and accepted by the Food and Drug Administration (USA) for improving fracture healing. Applying ultrasound to injured bone has been demonstrated to accelerate

healing time and impede the loss of bone during a repair period.[19] Therefore, in the present study, the histologic and immunohistochemical effects of LIPUS were explored on an osteoblast cell line subsequent to new bone deposition after mid-palatal expansion.

The present study indicated that LIPUS stimulated new bone formation after expansion of inter-premaxillary sutures in rats. The immunohistochemical results revealed that there was statistically significant immunoreactivity involving osteocalcin, VEGF and TGF-β in the area of mineralised tissue. Only VEGF showed significant immunoreactivity in the fibrous tissue area of the ultrasound applied group and the controls. These findings may suggest that osteoblastic activity was greater in the mineralising tissue in the LIPUS applied group compared with the control group.

Sawada and Shimizu[14] indicated that there were high levels of the active form of TGF-β in osteoblasts, fibroblasts, and endothelial cells during the first day of expansion. It was considered that this result might be a synergistic effect on ossification in response to mechanical and biological impulses. In the present study, TGF-β receptor staining was applied via immunohistochemistry to assess osteoblastic activation. The significantly increased staining of osteocalcin, VEGF and TGF-β in the mineralised tissue of the current samples suggested marked proliferative cellular immunoreactivity following the LIPUS application.

The number of osteoblasts and blood vessel dimension in the ultrasound applied group were seen to histologically increase compared with the control group in the present study. Several reports have revealed that LIPUS has various biologic outcomes but triggers a different signal pathway which is involved in bone remodelling.[19,26,39] Pilla et al.[40] conducted a placebo-controlled study of mid-shaft tibial osteotomies in rabbits and indicated that ultrasound application to bone intensified the recovery of torsional strength and stiffness which reached stability in half the time of untreated bone.

The application of several chemical and pharmacological agents to improve new bone formation in the expanded sutures has been studied. The effects of vitamin E (α-tocopherol) administration with injections on bone regeneration were assessed at the end of the expansion of the inter-premaxillary suture in rats.[15] Researchers reported that the application of α-tocopherol stimulated bone formation and shortened the retention period in the test group. In addition, the influence of bone morphogenetic proteins (BMP) on periosteal and endosteal bone deposition was examined following elevated mechanical loading using a rat tibial bending model.[41] Experimental rats were given intraperitoneal injections of BMP which acted differentially to increase endosteal but not periosteal bone formation in the rat tibia. In a recent study, Ozturk et al.[10] evaluated the effect of injected bisphosphonates on sutural new bone formation and relapse after maxillary expansion in rats. Computerised tomographic measurement of expansion and relapse revealed that these agents showed positive outcomes in the stimulation of bone regeneration and a decrease the relapse ratio. However, an association between bisphosphonate therapy and jaw osteonecrosis was found after tooth extraction.[42] In light of these facts, it can be maintained that LIPUS has an important advantage over other available invasive treatment modalities, and also poses no risk of adverse side effects.

Conclusions

Within the limitations of this study, it was concluded that:

- Cellular activation in the LIPUS-treated group was greater than that seen in the controls.
- When LIPUS was applied to rats following expansion, the area of mineralised tissue revealed increased reactivity for osteocalcin, VEGF and TGF-β, compared with the area of fibrous tissue.

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