Effect of Increasing Low-Intensity Pulsed Ultrasound and a Functional Appliance on the Mandibular Condyle in Growing Rats

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Objectives—Functional appliances are used for treatment of lower-jaw deficiencies in growing individuals; however, their effectiveness is debatable. Low-intensity pulsed ultrasound (US) is a noninvasive method, which has been shown to stimulate cartilage and bone formation with 20 minutes of application. This study was designed to test the hypothesis that increasing low-intensity pulsed US application from 20 to 40 min/d will enhance mandibular condylar growth in growing rats, especially when combined with a functional appliance.

Methods—Fifty-four Sprague Dawley rats were divided into 6 groups (n = 9): control, low-intensity pulsed US for 20 minutes, low-intensity pulsed US for 40 minutes, the functional appliance, the functional appliance plus low-intensity pulsed US for 20 minutes, and the functional appliance plus low-intensity pulsed US for 40 minutes. Low-intensity pulsed US was applied for 28 days. All rats were then euthanized, and their mandibles were dissected for morphometric, histomorphometric, and micro–computed tomographic analyses.

Results—Among all study groups, the 20-minute US group showed significant increases in most of the measured variables ($P < .05$) except for condylar process length ($P = .18$), whereas the functional appliance-plus-40-min US group showed the least favorable results. The 20-minute US group showed increases in proliferative and hypertrophic cell counts and widths and enhanced microarchitecture of trabecular bone compared with the 40-minute US group. The functional appliance-plus-20-minute US group showed better results compared with the functional appliance–alone and functional appliance-plus-40-minute US groups.

Conclusions—A daily application of low-intensity pulsed US for 20 minutes in growing rats affects mandibular growth, either alone or in combination with a functional appliance. Further study with a longer observation period is required to study the long-term effects and stability of newly formed bone.

Key Words—functional appliance; low-intensity pulsed ultrasound; mandibular condyle; musculoskeletal ultrasound

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Abbreviations
CT, computed tomographic; US, ultrasound

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The temporomandibular joint is the only movable joint in the craniofacial region and is important because of its role in critical activities, such as mastication, speaking, and clenching. This joint is covered by fibrocartilage, which serves not only as a cushion for dispersion of mechanical load but also as a main growth center for the craniofacial region.[1] Class II malocclusion, in which the

lower teeth bite in a backward position from the upper teeth, is one of the prevalent nonsyndromic deformities usually characterized by mandibular retrusion (backward position).[2] Not only does it affect jaw function and the aesthetic appearance, but it may also cause airway obstruction and functional limitations, such as speech, mastication, and swallowing.[3] Class II malocclusion affects 15% of the world population and is present in most patients attending orthodontic clinics.[4]

Treatment of a mandibular deficiency depends on the patient's age, severity of the problem, expected success rate, and impact on the facial profile.[5] For a growing patient with a retruded mandible, a functional appliance, which is also known as a forward bite-jumping appliance, can be one of the treatments of choice. Functional appliances are devices that are believed to enhance condylar growth by displacing the mandible forward and downward and eliminating abnormal muscular forces that interfere with normal dental and skeletal development of the mandible.[6] Several animals and human studies have shown improvements in mandibular forward projection with functional appliance use.[7–10] However, the effectiveness of these appliances is still debatable, especially with regard to the patient's age, treatment duration, and long-term effectiveness of mandibular growth modification. Some authors reported that the effect of functional appliances was not skeletal but only dentoalveolar (teeth and their surrounding bone),[9] whereas others reported that their best skeletal effects were observed when treatment was started just before the growth spurt.[10]

The mandibular condylar head undergoes endochondral ossification during growth, whereas bone formation in the glenoid fossa occurs by intramembranous ossification. Cellular activity in the glenoid fossa is slower than that in the condylar head. This factor could be the reason for failure or insubstantial clinical results after functional appliance treatment to modify mandibular growth.[11] In recent years, low-intensity pulsed ultrasound (US) has shown positive results in bone growth and regeneration in several studies.[12–15]

Low-intensity pulsed US was shown to be effective in increasing the intracellular calcium level in bone and cartilage cell cultures and increasing secretion of angiogenesis-related cytokines, which is important for endochondral bone formation involved in the mandibular condyle.[13] Human studies have also shown enhanced bone fracture healing,[14] reduced treatment durations, and increased bone strength in distraction osteogenesis.[16] Previous studies have shown significant increases in mandibular growth with 20-minute low-intensity pulsed US application in combination with the use of a functional appliance.[7,17,18] However, in a human study,[18] it took a year to show a clinical improvement in mandibular growth. Therefore, there is a need for an optimized technique that may reduce the treatment time to a few months in humans. Previous studies have shown that 40-minute low-intensity pulsed US application enhanced chondrogenic differentiation in vitro[19] and bone regeneration during distraction osteogenesis[20] compared with a daily application for 20 minutes.

The aim of this study was to investigate the effect of increasing the daily low-intensity pulsed US treatment duration with or without a functional appliance on mandibular condylar growth in growing rats. We hypothesized that increasing daily treatment with low-intensity pulsed US would have additive effect on mandibular condylar growth, particularly if combined with functional appliance therapy.

Materials and Methods

Animal Care and Experimental Design

This study was approved by University of Alberta Animal Care and Use Committee. Fifty-four 21-day-old male Sprague Dawley rats were divided into 6 groups ($n = 9$): (1) control (no functional appliance or low-intensity pulsed US application); (2) low-intensity pulsed US for 20 min/d; (3) 40 low-intensity pulsed US for 40 min/d; (4) the functional appliance alone; (5) the functional appliance plus low-intensity pulsed US for 20 min/d; and (6) the functional appliance plus low-intensity pulsed US for 40 min/d. The rats were acclimatized for a 1 week. The functional appliance was attached to upper and lower incisors when the rats were 28 days old (first day of the experiment), and they received low-intensity pulsed US for 20 or 40 minutes for 28 days according to the treatment group. All animals were weighed on the first day of acclimatization and after 7 days until the end of the procedures. Twenty-four hours after the final US application, the animals were euthanized by using an intraperitoneal injection of pentobarbital sodium.

Functional Appliance

The functional appliance was made from self-cured polymethyl methacrylate orthodontic resin (Dentsply,

Milford, DE) for each rat and was cemented to the upper and lower incisors when they were 28 days old with Panavia F2.0 resin cement (Kuraray Medical, Inc, Okayama, Japan). The appliance induced 3 mm of vertical displacement of the jaw[21] and had an identical inclined plane to cause anterior displacement of the mandible on mouth closing (Figure 1, panel 1). All animals with the functional appliance were fed with powdered rat chow, whereas the others were fed with normal rat chow (Lab Diet, St Louis, MO).

Ultrasound device

The low-intensity pulsed US device was custom made and was provided by SmileSonica, Inc (Edmonton, Alberta, Canada). The device generated a 200-microsecond burst of a 1.5-MHz sine wave with a repetition rate of 1 kHz and a temporal-average intensity of 30 mW/cm^2. These parameters have been used in our laboratory and have been approved by the Food and Drug Administration in the United States for clinical use in fracture healing.[7,14,17–20,22,23] The device was calibrated at the beginning and end of experiment to evaluate the consistency of the US parameters. The right mandibular condyle was used as the experimental side, which was shaved, and coupling gel was applied to ensure US wave propagation (Figure 1, panel 2).

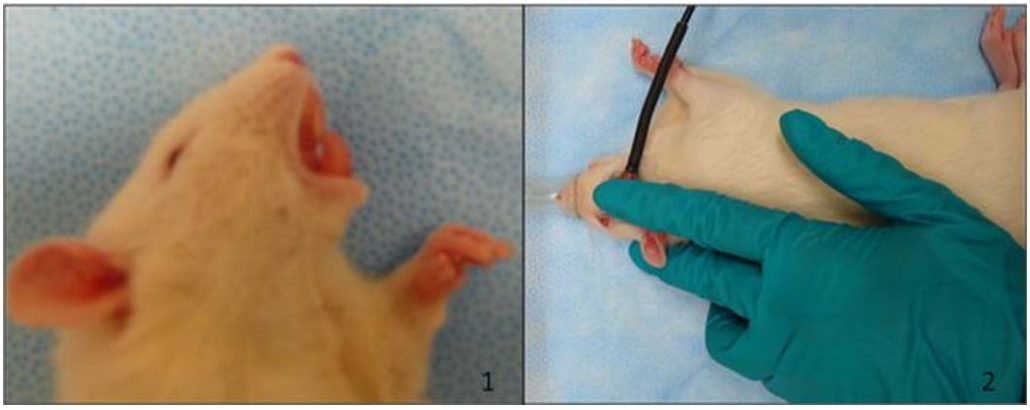
Morphometric Measurements

Mandibles were dissected and fixed in a 10% formalin solution (Sigma-Aldrich, St Louis, MO) for 24 hours at room temperature. Landmark measurements were performed with digital calipers. Figure 2 shows the landmarks and linear measurements analyzed, and a description of the variables is presented in Table 1.

Histologic Analysis and Histomorphometric Measurement

Condylar processes were separated from the mandibles and were decalcified with Cal-Ex II (Fisher Scientific, Ottawa, Ontario, Canada) for 2 weeks. Decalcified condyles were embedded in paraffin and sectioned at a 6-μm thickness. Slides were stained with the periodic acid-Schiff reagent and Alcian blue stain to identify the cartilage and new bone formation.[24,25] Alcian blue stains the cartilage blue, whereas it is stained magenta with periodic acid-Schiff, which shows calcification of the cartilage matrix. The proliferative layer is composed of undifferentiated mesenchymal cells that stain positive for Alcian blue. The extracellular matrix is stained blue in the hypertrophic layer with Alcian blue, whereas the chondrocytes in the erosive layer where calcification begins are stained magenta with periodic acid-Schiff. Photographs were taken with a Fluorescent digital microscope and a charge-coupled device digital camera (Leica, Wetzlar, Germany), processed with RS Image version 1.73 software (Photometric; Roper Scientific, Inc, Tucson, AZ), and analyzed with MetaMorph software (Molecular Devices, LLC, Sunnyvale, CA). The magnification used was ×20. The condylar cartilage was divided into 4 zones: resting, proliferative, hypertrophic, and erosive. The cell number and width of the proliferative and hypertrophic layers were measured. Readings from 3 slides of each sample were then averaged to get the final reading for every sample. For the thickness measurement, 6 lines were drawn in the proliferative and

Figure 1. 1, Functional appliance attached to the upper and lower incisors. **2**, Ultrasound transducer applied to the right side of the mandible.

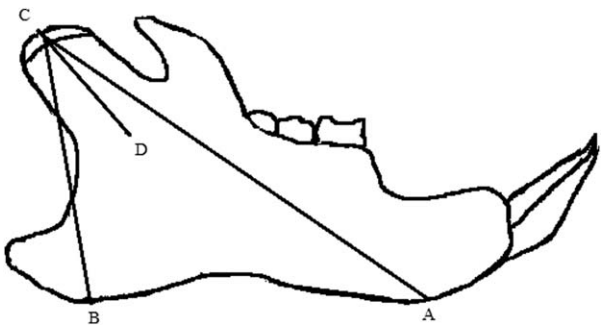


hypertrophic layers separately and then averaged to get the final value from a single slide. To measure the cell count for the proliferative and hypertrophic layers, a fixed measurement frame of 500 × 500 μm was drawn in the posterior region (Figure 3).

Micro-Computed Tomography

Dissected mandibles were scanned with a Skyscan 1176 micro–computed tomographic (CT) imager (Skyscan, Kontich, Belgium) using vendor-supplied imaging control software. The resolution selected was 18 μm. All scans were conducted at 85 kV and 293 μA with a 10-mm aluminum filter through a 180° rotation at a 0.5° step increment. The analysis was performed with CTAn version 1.12.0.0 software (Skyscan). Trabecular bones of the condylar head areas were manually selected by using the first appearance of the trabecular bone as the reference point up to the 100th slice. The bone structural parameters studied were bone volume-to-tissue volume ratio (bone volume fraction), trabecular thickness, trabecular number, trabecular separation, and bone mineral density. From each sample, 5 readings were taken, and values were averaged to get the final value.

Figure 2. Landmarks and linear measurements of the mandible: mandibular length (A–C), distance from the posterosuperior-most point on the condylar head, which is the anteroinferior point on the lower border of the mandible; ramus height (C–B), distance from the condylar point to the gonion tangent point, which is the posteroinferior point on the lower border of the mandible; and condylar process length (C–D), distance from the lower border of the mandibular foramen to the condylar point.



Statistical Analysis

Data were first analyzed for a normal distribution by the Kolmogrov-Smirnov test. For normally distributed data, a 1-way analysis of variance followed by a Bonferroni post hoc test using SPSS version 21.0 software (IBM Corporation, Armonk, NY) was used. The significance level was set at $P < .05$. Intrarater reliability for 6 randomly selected animals for each measured variable was tested by the intraclass correlation coefficient test, and measurements were performed twice at an interval of 2 weeks for morphometric analysis and 4 weeks for histologic and micro-CT analyses.

Results

All measured variables showed normally distributed data except for trabecular number; hence, the Kruskal-Wallis test was applied. The intraclass correlation coefficient test also showed absolute agreement ($r > 0.78$–0.99).

Body Weight

There was no difference in body weight among treatment groups on the first day of the experiment ($P = .18$), but there was a significant difference among the groups ($P < .001$) at the end of the experiment (Figure 4). However, there was no difference between the functional appliance and functional appliance-plus-

Table 1. Morphometric Landmarks and Linear Measurements of the Mandible

Landmark	Description
Point	
Menton	The most inferior point on the mandibular symphysis
Gonion tangent point	Assuming that the mandible is placed on a plane, the point of the mandibular gonion at its junction with that plane
Condylar point	The most posterior and superior point on the mandibular condyle
Mandibular foramen	The entry of the mandibular nerve and blood vessels in the mandibular canal
Linear measurement	
Menton-condylar point	Total mandibular length, the distance measured between the menton and the condylar point
Condylar-gonion tangent point	Ramus height, the distance measured between the condylar point and the gonion tangent point
Condylar process length	The distance measured from the mandibular foramen to the condylar point

20-minute US groups and the 20- and 40-minute US groups. Means and standard deviations of the weights on days 1 and 29 (day of euthanasia) are presented in Table 2.

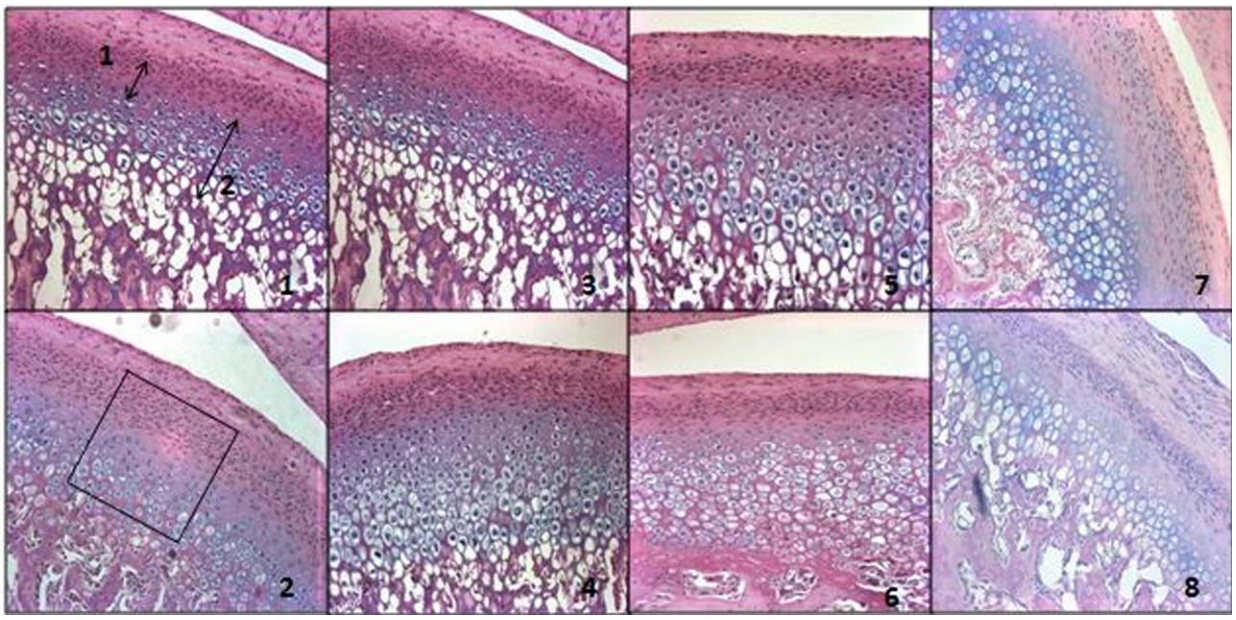
Morphometric Analysis

Linear measurements of mandibular length, ramus height, and condylar process length, as shown in Figure 2, are presented in Table 3. There were statistically significant increases in all 3 measurements in the 20-minute US group ($P < .05$) except for condylar process length between the 20- and 40-minute US groups ($P = .182$). Mandibular length showed the highest value in the 20-minute US group, followed by the 40-minute US group, compared with the control group. However, the mean difference between the control and 40-minute US groups was small (0.2 mm) and was not statistically significant ($P > .05$). There was no significant difference between the control and functional appliance groups, control and 40-minute US groups, and functional appliance and functional appliance-plus-40-minute US groups (Figure 5, panel 1). Ramus height showed an increase in the 20-minute US group compared with the control group, and the difference was statistically significant ($P < .05$; Figure 5, panel 2). Condylar process length also showed an increase in the 20-minute US group, followed by the 40-minute US group. There were also significant differences between the 20-minute US and functional appliance groups and the functional appliance-plus-20-minute US and functional appliance-plus-40-minute US groups (Figure 5, panel 3). Overall, in comparisons of the means of all treatment groups, the 20-minute US group showed statistical increases in all 3 linear measurements, whereas the functional appliance-plus-40-minute US group showed decreases in all of the measurements (Table 3).

Histomorphometric Analysis

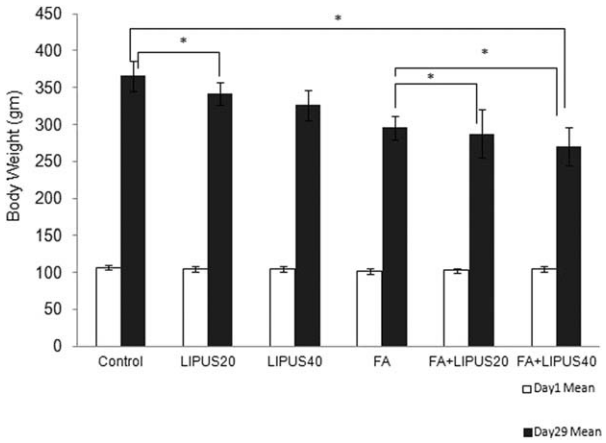
Qualitatively, the widths of the proliferative and hypertrophic cell layers are evident from the slides (Figure 3). The 1-way analysis of variance showed significant differences in all of the measured histomorphometric variables: ie, proliferative cell count and width and hypertrophic cell count and width. The 20-minute US group showed a significant difference from all other treatment groups in the proliferative cell count ($P < .05$) whereas there was no significant difference between the control and functional appliance groups and the control and functional appliance-plus-40-minute US groups (Figure 6, panel 1). For proliferative

Figure 3. 1, Line 1 shows the width of the proliferative layer, and line 2 shows the width of the hypertrophic layer. **2**, Square of 500 × 500 μm drawn in the posterior region of the condyle; the cells of the proliferative and hypertrophic layers were counted within the square. **3**, Control. **4**, Low-intensity pulsed US for 20 minutes. **5**, Low-intensity pulsed US for 40 minutes. **6**, Functional appliance. **7**, Functional appliance plus low-intensity pulsed US for 20 minutes. **8**, Functional appliance plus low-intensity pulsed US for 40 minutes.



layer width, again the 20-minute US group showed a significant increase, followed by the 40-minute US and functional appliance-plus-20-minute US groups. There was no significant difference between the control and functional appliance groups and the control and functional appliance-plus-40-minute US groups ($P > .4$ in both cases; Figure 6, panel 2). For hypertrophic cell count, there was no significant difference between the control and functional appliance groups and the functional appliance and functional appliance-plus-40-minute US groups (Figure 6, panel 3). For hypertrophic width, there was no difference between the control and functional appliance groups, 20- and 40-minute US groups, and 40-minute US and functional appliance-plus-20-minute US groups (Figure 6, panel 4). Means and standard deviations are shown in Table 3.

Figure 4. Weights measured on days 1 and 29. No significant differences in the body weights of the animals were seen in on day 1, whereas there were significant differences among the groups on day 29, especially in the functional appliance (FA) groups (*$P < .05$). LIPUS indicates low-intensity pulsed US.



Micro-CT Analysis

There was a significant increase in the blood volume fraction in the treatment groups with 20 minutes of US application compared with the control group. The 20- and 40-minute US groups showed the highest increases, whereas the functional appliance-plus-40-minute US showed the lowest value (Figure 7, panel 1). There was a significant difference between the 20-minute US group

Table 2. Body Weight of Treatment Groups on Days 1 and 29

	Body Weight, g					
Day	**Control**	**20-min LIPUS**	**40-min LIPUS**	**FA**	**FA + 20-min LIPUS**	**FA + 40-min LIPUS**
1	106.6 ± 3.5	104.5 ± 4.2	104.6 ± 4.5	102 ± 3.9	102.8 ± 3.3	105.2 ± 3.7
29	365.7 ± 20.7	342.2 ± 15.6	326.1 ± 21.1	295.6 ± 15.9	287.2 ± 32.6	270.4 ± 26.2

Data are presented as mean ± SD. FA indicates functional appliance; and LIPUS, low-intensity pulsed US.

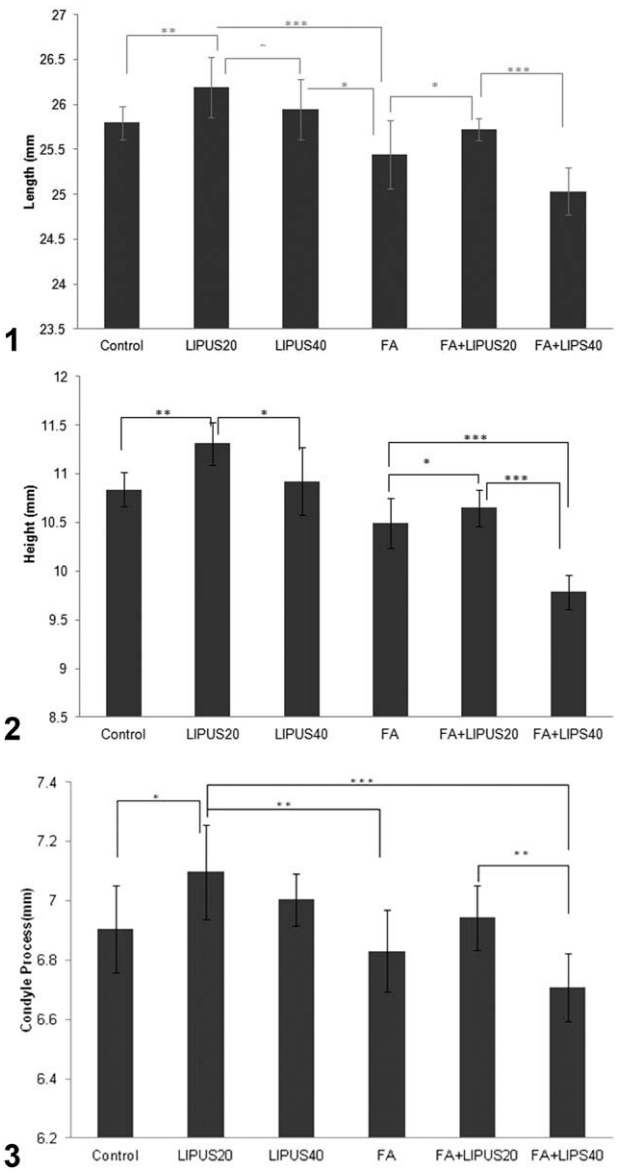
Table 3. Values of All Treatment Variables

Parameter	**Control**	**20-min LIPUS**	**40-min LIPUS**	**FA**	**FA + 20-min LIPUS**	**FA + 40-min LIPUS**
Mandibular length, mm	25.8 ± 0.2	26.2 ± 0.3	25.9 ± 0.3	25.4 ± 0.4	25.7 ± 0.1	25.0 ± 0.2
Ramus height, mm	10.8 ± 0.2	11.3 ± 0.2	10.9 ± 0.3	10.5 ± 0.2	10.6 ± 0.2	9.8 ± 0.1
Condylar process length, mm	6.9 ± 0.1	7.1 ± 0.1	7.0 ± 0.08	6.83 ± 0.13	6.94 ± 0.10	6.71 ± 0.1
Proliferative cell count, n	74.3 ± 5.5	96.9 ± 3.8	85.5 ± 6.0	72.6 ± 7.4	84.8 ± 6.1	70.1 ± 4.8
Proliferative width, μm	90.7 ± 1.8	110.9 ± 6.1	100.3 ± 8.2	84.8 ± 4.6	96.6 ± 4.7	74.5 ± 5.3
Hypertrophic cell count, n	90.6 ± 8.0	116.4 ± 6.1	100.6 ± 4.5	84.4 ± 4.0	97.0 ± 4.2	76.4 ± 6.9
Hypertrophic width, μm	346.0 ±18.2	403.7 ± 11.5	387.75 ± 15.9	339.1 ± 17.0	368.9 ± 11.13	295.0 ± 11.1
Bone volume fraction, %	60.9 ± 3.9	73.9 ± 3.7	68.9 ± 0.9	57.3 ± 4.0	63.5 ± 3.0	42.9 ± 4.3
Trabecular thickness, mm	0.09 ± 0.005	0.1 ± 0.006	0.1 ± 0.007	0.1 ± 0.008	0.1 ± 0.004	0.08 ± 0.006
Trabecular separation, mm	0.086 ± 0.006	0.089 ± 0.006	0.107 ± 0.007	0.104 ± 0.010	0.105 ± 0.010	0.13 ± 0.02
Trabecular number, 1/mm	5.2 ± 0.3	6.2 ± 0.2	5.7 ± 0.4	4.9 ± 0.3	5.4 ± 0.2	4.1 ± 0.6
Bone mineral density, g/cm^3	1.5 ± 0.2	2.1 ± 0.1	1.8 ± 0.2	1.2 ± 0.2	1.3 ± 0.07	0.92 ± 0.3
Condylar volume, mm^3	20.5 ± 1.9	26.8 ± 1.9	21.6 ± 2.4	19.5 ± 2.4	21.0 ± 1.8	16.1 ± 1.5

Data are presented as mean ± SD. FA indicates functional appliance; and LIPUS, low-intensity pulsed US.

and the other treatment groups for trabecular thickness (Figure 7, panel 2). Trabecular separation showed the highest value in the functional appliance–plus 40-minute US group, and the difference was statistically significant (Figure 7, panel 3). There was no significant difference between the control and 20-minute US groups, and these groups had the least mean values. The 20-minute US group showed a significantly ($P < .05$) higher trabecular number compared with the other groups, except for the 40-minute US group ($P > .05$; Figure 7, panel 4). Bone mineral density showed a significant increase in the 20-minute US group, and the difference was statistically significant. The functional appliance-plus-40-minute US group showed the lowest bone mineral density value. Among the groups that received the functional appliance, there was no significant difference between the functional appliance and functional appliance-plus-20-minute US groups ($P > .05$), whereas there was a significant difference between the functional appliance-plus-20-minute US and functional appliance-plus-40-minute US groups ($P < .05$; Figure 7, panel 5). Means and standard deviations are shown in Table 3.

Figure 5. Anthropometric measurements of the right mandible. **1**, Mandible length. **2**, Ramus height. **3**, Condylar process length. Low-intensity pulsed US (LIPUS) for 20 minutes showed significant increases in mandibular length, ramus height, and condylar process length compared with other treatment groups, whereas the functional appliance (FA) plus low-intensity pulsed US for 40 minutes showed the least values in all of the measured variables (*$P < .05$; **$P < .001$; and ***$P < .0001$).



Discussion

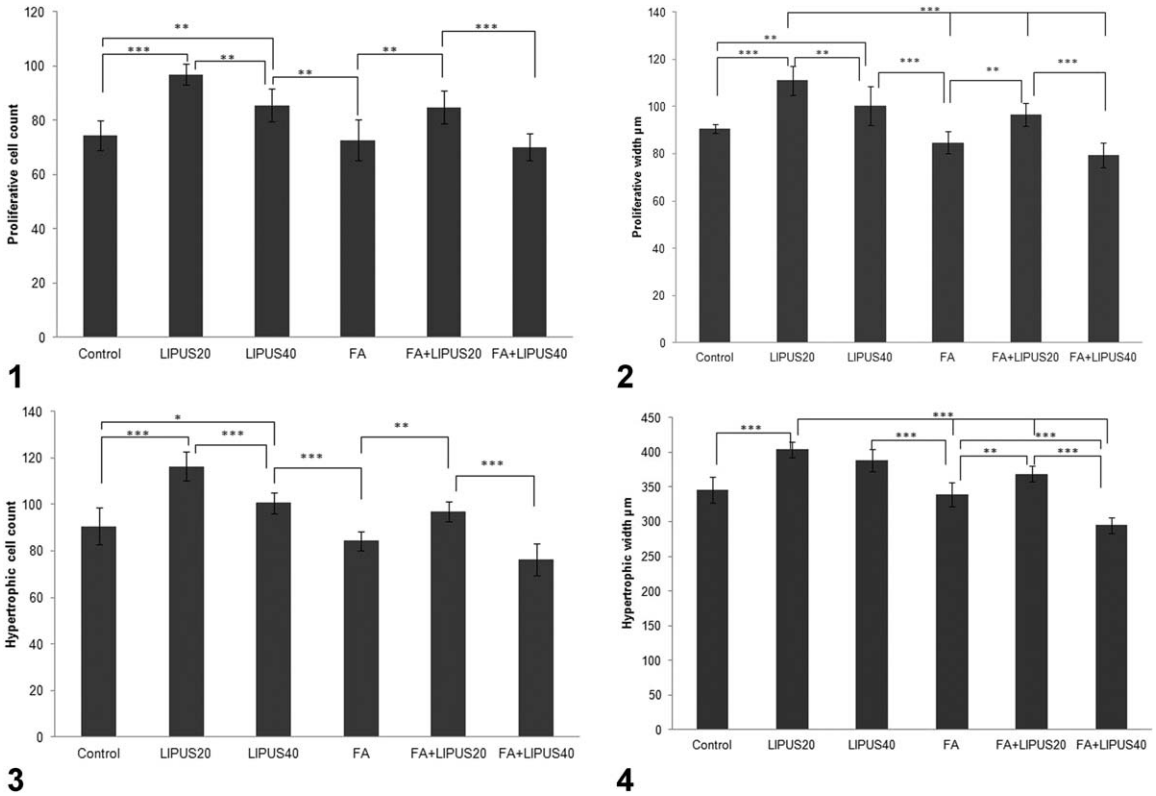
Morphometric results demonstrated that low-intensity pulsed US application for 20 min/d increased condylar and mandibular length and height in the young growing rats, whereas application for 40 min/d, either alone or in combination with the functional appliance, showed insignificant results compared with either the control group or 20-minute US group. This finding is in contrast to other dose-response studies reported.[19,20] The mandibular condyle is the primary growth center, where the growth mainly takes place by endochondral ossification. Histologically, the condyle is divided into 4 zones: (1) the fibrous or articular layer, which is the most superficial layer, composed of dense collagen matrix; (2) the proliferative layer, which contains undifferentiated mesenchymal cells; (3) the hypertrophic layer of mature chondrocytes; and (4) the erosive layer, where chondrogenesis ends and osteogenesis begins. In this study, groups treated with low-intensity pulsed US for 20 min/d showed significant increases in the proliferative and hypertrophic cell counts and layer widths, and the results are supported by earlier published articles on both in vitro and in vivo studies in which low-intensity pulsed US application had a stimulatory effect on chondrocyte proliferation and collagen synthesis.[22,23,26]

Bone is an essential part of the musculoskeletal system and is well connected to its surrounding muscles, which influence bone shape and strength via application of muscular forces. The trabecular bone constitutes only

20% of bone, but its turnover rate is higher (≈8 times) than that of the cortical bone.[27] Hence, it is the primary site for detection of changes following any therapeutic treatment. Basic parameters studied for trabecular microarchitecture are the bone volume fraction, trabecular thickness, trabecular number, and trabecular separation 3-dimensional analyses. The bone volume fraction is the ratio of the volume of bone present to the total volume of interest and is a surrogate measure of bone density.[28] Bone mineral density is the volumetric density of calcium hydroxyapatite in the bone in grams per cubic centimeter and is the best predictor of fracture risk.

The micro-CT analysis demonstrated that low-intensity pulsed US application for 20 min/d, either alone or with the functional appliance, had a higher bone volume fraction, trabecular thickness, trabecular number, and bone mineral density and lower trabecular separation than the 40-minute US and control groups. Bone volume fraction values indicate that the trabecular network is well connected, thus protecting the overlying cortical bone. The well-connected network is expected to better diffuse the load because of its structure and hence increases bone strength.[28] Trabecular separation represents the average distance between trabecular strands in the region of interest and was lower in the 20-minute US treatment group and higher in the 40-minute group. Although low-intensity pulsed US application for 20 min/d already has been shown to create better bone formation in animals and clinical studies, some in vitro studies and distraction osteogenesis cases found that application for 40 min/d showed better results by increasing chondrogenic gene marker and extracellular matrix deposition[19] and enhancing bone regeneration in the distracted callus.[20] In contrast to these studies, this study showed better microarchitecture of trabecular bone (and presumably more strength) with 20 minutes of daily low-intensity pulsed US application. This adverse effect could have been due to a thermal effect of

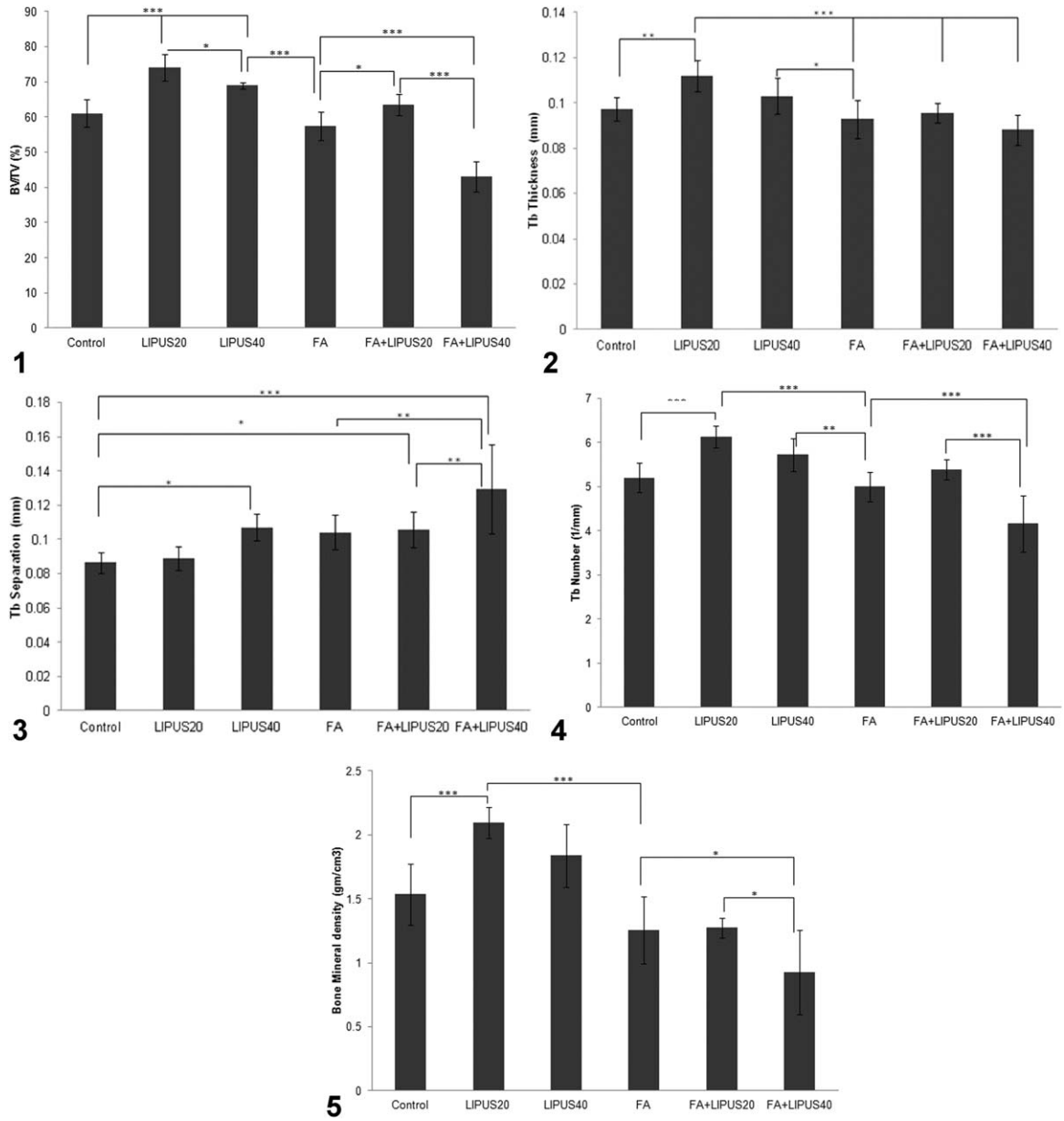
Figure 6. Histomorphometric analysis of the right mandible. **1**, Proliferative cell count. **2**, Proliferative width. **3**, Hypertrophic cell count. **4**, Hypertrophic width. After 28 days of the experimental procedure, low-intensity pulsed US (LIPUS) for 20 minutes showed increases in proliferative and hypertrophic cell counts and layer width. Similar results were seen in the functional appliance (FA) groups, in which low-intensity pulsed US for 20 minutes in combination with the functional appliance showed significant increases in all of the measured variables (*$P < .05$; **$P < .001$; ***$P < .0001$).



low-intensity pulsed US when it was applied for 40 minutes.[29,30] Also, the longer period of anesthesia for the 40-minute US group could have had a negative effect on general body growth, including mandibular growth, compared with the 20-minute US group, since a study by Kim et al[31] showed increased DNA damage caused by oxidative stress in rats exposed to isoflurane anesthesia for 30 and 60 minutes.

The exact mechanism of action of low-intensity pulsed US is still under investigation, with several possible

Figure 7. Micro-CT analysis of the trabecular bone of the condylar head of the right mandible. **1**, Bone volume (BV) fraction of total volume (TV). **2**, Trabecular (Tb) thickness. **3**, Trabecular separation. **4**, Trabecular number. **5**, Bone mineral density. The subchondral bone showed increases in bone volume fraction, trabecular thickness, trabecular number, and bone mineral density with low-intensity pulsed US (LIPUS) for 20 minutes either alone or in combination with the functional appliance (FA), whereas trabecular separation was increased with low-intensity pulsed US for 40 minutes either alone or in combination with the functional appliance (*$P < .05$; **$P < .001$; and ***$P < .0001$).



mechanisms being proposed. First, US waves cause movement of the fluid around the cells, which could have an effect on cell permeability and activation of secondary messengers.[32] Second, US waves cause mechanical pressure on the cell surface, which could activate the stretch receptors of cation channels on the cell membrane and affect intracellular gene expression.[33] Third, since bone has the ability to remodel depending on functional demands, the mechanical stresses produced by low-intensity pulsed US could help in remodeling of the bone microarchitecture.[34,35]

Mechanical stresses induced by low-intensity pulsed US can cause potential differences in osteoblasts and alter bone remodeling.[36] Chondrocytes are mechanosensitive cells, and mechanical forces are required for growth and maintenance of the cartilage. These stresses are sensed by transmembrane receptors (integrins), whose stimulation leads to intracellular signaling and hence affects chondrocyte activity. Previous studies have shown that mechanical stress produced by low-intensity pulsed US increased gene expression and protein synthesis in chondrocytes and osteoblasts via integrin activation.[37,38] Functional appliance studies have also indicated the roles of integrin, mechanical forces, and cellular proliferation in the condylar cartilage.[39,40] In these studies, an increase in integrin expression and cellular proliferation was shown in cartilage with the use of a functional appliance. Hence, it could be hypothesized that low-intensity pulsed US along with the functional appliance stimulated integrin receptors present on the chondrocyte membrane and led to an increase in cellular proliferation and microarchitectural properties of the trabecular bone, as an increase in hypertrophic chondrocytes has been closely related to the subchondral bone.

Low-intensity pulsed US application for 40 min/d showed lower condylar growth compared with the 20-minute. The detrimental effect could be due to the thermal effect of low-intensity pulsed US on the bone. The biological effects of low-intensity pulsed USi are due to a combination of thermal and nonthermal effects, which depend on energy density and are products of the US intensity applied and exposure time.[29] In in vitro[30] and in vivo[29] studies, low-intensity pulsed US at 30 mW/cm^2 showed a 3°C increase in temperature after 20 minutes. It is possible that the temperature increase could double after 40 minutes of low-intensity pulsed US application. In our experiment, it was difficult to measure temperature because of the inaccessibility of the intraoral site. This process could cause trauma in the oral cavity of anesthetized rats, as the mandibular condyle is located superiorly and posteriorly in the glenoid fossa of the skull and is covered with a thick layer of oral tissue, which requires deep dissection to reach the condyle. The thermal effect of low-intensity pulsed US application for 40 minutes might have resulted in cell destruction, decreased gene expression for bone proteins, and, hence, lower microarchitecture of the trabecular bone. Alternatively, the longer anesthesia used in the 40-minute US group might have affected general body growth, including the condyles. Future studies in larger animals that can use low-intensity pulsed US with sedation (rather than anesthesia) or an equal length of anesthesia for all groups (including control) might better reveal the role anesthesia plays in low-intensity pulsed US effects.

One confounding factor in this study was the food consistency. Masticatory forces are important, as they can stimulate bone remodeling. These forces are transmitted not only to the body and alveolar process but also to the condylar head, which affect its growth and remodeling.[41] The rats fed with the powdered diet in the functional appliance groups had a reduced stimulatory effect, as the force required to cut and chew food was minimal compared with the groups fed with the normal pellet diet. There might not have been enough of a mechanical load to stimulate the bone structure in these groups. However, the decrease in the body weight of the rats could not be due to the change in the food consistency, since both food types had similar protein and fat contents. In a study by Bozzini et al,[42] in which the rats were given soft- and hard-pellet diets, no changes in the total body weights were observed. Hence, the effect of food consistency on body weight could be excluded. In this study, US was applied to one side of the mandible only, whereas functional appliance treatment is usually bilateral. Hence, bilateral low-intensity pulsed US application is recommended in future studies to get clinically relevant results. Moreover, psychological stress caused by a functional appliance and prolonged anesthesia (ie, 40 versus 20 minutes) might also have an effect on the rat metabolism.[4] Stress leads to an increase in the metabolism and hence body weight loss.[43]

In conclusion, within the limitations of this study, a 20-minute daily application of low-intensity pulsed US showed more growth in the condyle compared with a 40-minute application. The clinical application of low-

intensity pulsed US along with functional appliance treatment will reduce the orthodontic treatment duration. Before that happens, further long-term studies are advocated using a similar diet in all groups to evaluate the combination effect of a functional appliance and low-intensity pulsed US on the mandible condyle and to study the long-term effects of combination therapy. Last, it will be important to follow the observed changes in temporal patterns for a longer time, since bone is in a continuous process of remodeling.

References

1. Delatte M, Von den Hoff JW, van Rheden REM, Kuijpers-Jagtman AM. Primary and secondary cartilages of the neonatal rat: the femoral head and the mandibular condyle. *Eur J Oral Sci* 2004; 112:156–162.
2. McNamara JA Jr. Components of class II malocclusion in children 8–10 years of age. *Angle Orthod* 1981; 51:177–202.
3. Ritto AK. Class II malocclusion: why, when and how to treat this anomaly in mixed dentition with fixed functional appliances. *J Gen Orthod* 2001; 12:9–21.
4. Proffit WR, Fields HW Jr, Moray LJ. Prevalence of malocclusion and orthodontic treatment need in the United States: estimates from the NHANES III survey. *Int J Adult Orthodon Orthognath Surg* 1998; 13: 97–106.
5. Bock NC, Ruf S. Dentoskeletal changes in adult Class II division 1 Herbst treatment: how much is left after the retention period? *Eur J Orthod* 2012; 34:747–753.
6. Graber TM, Rakosi T, Petrovic AG. *Dentofacial Orthopedics With Functional Appliances*. St Louis, MO: CV Mosby Co; 1997.
7. El-Bialy T, El-Shamy I, Graber TM. Growth modification of the rabbit mandible using therapeutic ultrasound: is it possible to enhance functional appliance results? *Angle Orthod* 2003; 73:631–639.
8. Ghislanzoni LTH, Toll DE, Defraia E, Baccetti T, Franchi L. Treatment and posttreatment outcomes induced by the mandibular advancement repositioning appliance: a controlled clinical study. *Angle Orthod* 2011; 81:684–691.
9. Pangrazio MNK, Pangrazio-Kulbersh V, Berger JL, Bayirli B, Movahhedian A. Treatment effects of the mandibular anterior repositioning appliance in patients with class II skeletal malocclusions. *Angle Orthod* 2012; 82:971–977.
10. Cozza P, Baccetti T, Franchi L, De Toffol L, McNamara JA Jr. Mandibular changes produced by functional appliances in class II malocclusion: a systematic review. *Am J Orthod Dentofacial Orthop* 2006; 129(5):599.e1–599.e12.
11. Owtad P, Park JH, Shen G, Potres Z, Darendeliler MA. The biology of TMJ growth modification: a review. *J Dent Res* 2013; 92: 315–321.
12. Ustun Y, Erdogan O, Kurkcu M, Akova T, Damlar I. Effects of low-intensity pulsed ultrasound on dental implant osseointegration: a preliminary report. *Eur J Dent* 2008; 2:254–262.
13. Doan N, Reher P, Meghji S, Harris M. In vitro effects of therapeutic ultrasound on cell proliferation, protein synthesis, and cytokine production by human fibroblasts, osteoblasts, and monocytes. *J Oral Maxillofac Surg* 1999; 57:409–420.
14. Snyder BM, Conley J, Koval KJ. Does low-intensity pulsed ultrasound reduce time to fracture healing? A meta-analysis. *Am J Orthop (Belle Mead NJ)* 2012; 41:E12–E19.
15. Baker KG, Robertson VJ, Duck FA. A Review of therapeutic ultrasound: biophysical effects. *Phys Ther* 2001; 81:1351–1358.
16. Dudda M, Hauser J, Muhr G, Esenwein SA. Low-intensity pulsed ultrasound as a useful adjuvant during distraction osteogenesis: a prospective, randomized controlled trial. *J Trauma* 2011; 71:1376–1380.
17. El-Bialy T, Hassan A, Albaghdadi T, Fouad HA, Maimani AR. Growth modification of the mandible with ultrasound in baboons: a preliminary report. *Am J Orthod Dentofacial Orthop* 2006; 130: 435.e7–435.e14.
18. El-Bialy T. Nonsurgical treatment of hemifacial microsomia by therapeutic ultrasound and hybrid functional appliance. *Open Access J Clin Trials* 2010; 2:29–36.
19. Schumann D, Kujat R, Zellner J, et al. Treatment of human mesenchymal stem cells with pulsed low intensity ultrasound enhances the chondrogenic phenotype in vitro. *Biorheology* 2006; 43:431–443.
20. Chan CW, Qin L, Lee KM, Cheung WH, Cheng JCY, Leung KS. Dose-dependent effect of low-intensity pulsed ultrasound on callus formation during rapid distraction osteogenesis. *J Orthop Res* 2006; 24:2072–2079.
21. Rabie ABM, She TT, Hägg U. Functional appliance therapy accelerates and enhances condylar growth. *Am J Orthod Dentofacial Orthop* 2003; 123:40–48.
22. Oyonarte R, Zárate M, Rodriguez F. Low-intensity pulsed ultrasound stimulation of condylar growth in rats. *Angle Orthod* 2009; 79:964–970.
23. Naruse K, Miyauchi A, Itoman M, Mikuni-Takagaki Y. Distinct anabolic response of osteoblast to low-intensity pulsed ultrasound. *J Bone Miner Res* 2003; 18:360–369.
24. Rabie AB, Zhao Z, Shen G, Hägg EU, Dr O, Robinson W. Osteogenesis in the glenoid fossa in response to mandibular advancement. *Am J Orthod Dentofacial Orthop* 2001; 119:390–400.
25. Rabie ABM, Xiong H, Hägg U. Forward mandibular positioning enhances condylar adaptation in adult rats. *Eur J Orthod* 2004; 26: 353–358.
26. Rawool NM, Goldberg BB, Forsberg F, Winder AA, Hume E. Power Doppler assessment of vascular changes during fracture treatment with low-intensity ultrasound. *J Ultrasound Med* 2003; 22:145–153.
27. Fajardo RJ, Müller R. Three-dimensional analysis of nonhuman primate trabecular architecture using micro-computed tomography. *Am J Phys Anthropol* 2001; 115:327–336.

28. Kazama JJ, Koda R, Yamamoto S, Narita I, Gejyo F, Tokumoto A. Cancellous bone volume is an indicator for trabecular bone connectivity in dialysis patients. *Clin J Am Soc Nephrol* 2010; 5:292–298.
29. Xue H, Zheng J, Cui Z, et al. Low-intensity pulsed ultrasound accelerates tooth movement via activation of the BMP-2 signaling pathway. *PloS One* 2013; 8:e68926.
30. Leskinen JJ, Hynynen K. Study of factors affecting the magnitude and nature of ultrasound exposure with in vitro set-ups. *Ultrasound Med Biol* 2012; 38:777–794.
31. Kim H, Oh E, Im H, et al. Oxidative damages in the DNA, lipids, and proteins of rats exposed to isofluranes and alcohols. *Toxicology* 2006; 220:169–178.
32. Sachs F. Mechanical transduction by membrane ion channels: a mini review. *Mol Cell Biochem* 1991; 104:57–60.
33. Bassett CA. Electrical effects in bone. *Sci Am* 1965; 213:18–25.
34. Behari J, Singh S. Ultrasound propagation in "in vivo" bone. *Ultrasonics* 1981; 19:87–90.
35. Schortinghuis J, Stegenga B, Raghoebar GM, de Bont LGM. Ultrasound stimulation of maxillofacial bone healing. *Crit Rev Oral Biol Med* 2003; 14:63–74.
36. Schuster A, Schwab T, Bischof M, et al. Cell specific ultrasound effects are dose and frequency dependent. *Ann Anat* 2013; 195:57–67.
37. Whitney NP, Lamb AC, Louw TM, Subramanian A. Integrin-mediated mechanotransduction pathway of low-intensity continuous ultrasound in human chondrocytes. *Ultrasound Med Biol* 2012; 38: 1734–1743.
38. Cheng K, Xia P, Lin Q, et al. Effects of low-intensity pulsed ultrasound on integrin-FAK-PI3K/Akt mechanochemical transduction in rabbit osteoarthritis chondrocytes. *Ultrasound Med Biol* 2014; 40:1609–1618.
39. Marques MR, Hajjar D, Oliveira Crema V, Kimura ET, Santos MF. A mandibular propulsive appliance modulates collagen-binding integrins distribution in the young rat condylar cartilage. *Biorheology* 2006; 43:293–302.
40. Marques MR, Hajjar D, Franchini KG, Moriscot AS, Santos MF. Mandibular appliance modulates condylar growth through integrins. *J Dent Res* 2008; 87:153–158.
41. Kuroda S, Tanimoto K, Izawa T, Fujihara S, Koolstra JH, Tanaka E. Biomechanical and biochemical characteristics of the mandibular condylar cartilage. *Osteoarthritis Cartilage* 2009; 17:1408–1415.
42. Bozzini C, Picasso E, Champin G, Bozzini CE, Alippi RM. Effect of physical consistency of food on the biomechanical behaviour of the mandible in the growing rat. *Eur J Oral Sci* [published online ahead of print September 4, 2015]. doi:10.1111/eos.12209.
43. Li Q, Zhang M, Chen YJ, Zhou Q, Wang YJ, Liu J. Psychological stress alters microstructure of the mandibular condyle in rats. *Physiol Behav* 2013; 110-111:129–139.