Original Article

Low-Intensity Pulsed Ultrasound Stimulation of Condylar Growth in Rats

Rodrigo Oyonarte[a]; Mariana Zárate[b]; Francisco Rodriguez[c]

ABSTRACT

Objective: To test the hypothesis that low-intensity pulsed ultrasound (LIPUS) stimulation does not histologically affect the growth of mandibular condylar cartilage.

Materials and Methods: Thirty-five 20-day-old Sprague-Dawley rats were assigned to experimental and control groups. Experimental rats were stimulated with LIPUS in the temporomandibular joint (TMJ) region unilaterally, for 10 or 20 minutes for 20 days. After euthanasia, histological specimens were analyzed qualitatively and histomorphometrically at the anterior and posterior aspects of the mandibular condyle, including the condylar cartilage and the area and perimeter of subchondral bony trabeculae.

Results: LIPUS stimulation may alter the histological arrangement of the condylar bone and cartilage, showing qualitative differences on specimens treated for 10 or 20 minutes daily compared with controls. Cartilaginous layer thickness was not affected by LIPUS stimulation to a significant level, but was modified at the relative layer thickness within the cartilage at the anterior aspect of the condyle ($P < .05$). At the subchondral bone level, 20-minute stimulation significantly increases trabecular perimeter ($P = .01$).

Conclusions: LIPUS application may affect mandibular growth pattern in rats acting at the cartilage and bone level. The effect of LIPUS on the growing condyle is expressed through a variation in trabecular shape and perimeter. A greater response is achieved when stimulated for 20 minutes instead of 10 minutes daily. (*Angle Orthod.* 2009;79:964–970.)

KEY WORDS: Ultrasound stimulation; Mandibular growth

INTRODUCTION

Class II malocclusions of skeletal origin are routinely seen in the orthodontic office. These are usually due to mandibular deficiency[1,2] and highly prevalent, ranging from 18%[2] up to approximately 32%.[1]

It is known that orthopedic treatment of Class II malocclusions using functional appliances is a matter of ongoing controversy given the lack of consensus regarding the possibility of stimulating mandibular growth in a predictable manner. Despite this, it has been shown that functional orthopedic treatment of distal occlusion increases mandibular size in animal experimental models[3–5] as well as in humans.[6–8] Although these findings may be promising, these results have not been found consistently in clinical settings.[9] Apparently, the effectiveness of mandibular orthopedic treatments depends on the eventual synergy between treatment and growth, especially in individuals who are undergoing their pubertal growth spurt.[10] The development of technologies capable of accentuating the growth potential of mandibular cartilage could allow our profession to predictably intervene in the development of growing tissues.

A regenerative process that repeats several events of development is bone healing or fracture repair.[11] One stimulus capable of improving this process is the application of low-intensity pulsed ultrasound (LIPUS), which significantly accelerates bone fracture healing in humans.[12–14] LIPUS is a type of ultrasound (US) that promotes tissue healing. For such use, US is administered in pulses at lower intensity levels than in physiotherapy (0.5 to 3.0 watts per square centimeter, W/cm^2), below 0.1 W/cm^2.[15] The mechanisms involved in this process, although not well understood, include mechanotransduction of micromechanical stimuli,[11,16] increased local angiogenesis and improved blood sup-

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ply,[17] and aggrecan gene expression,[18] among other factors. LIPUS has also been used on growing cartilage. This stimulus has been effective increasing cartilaginous growth potential in primary[19] and secondary[20,21] cartilage.

El-Bialy et al[20] applied LIPUS (30 mW/cm^2, 1.5 MHz) on the temporomandibular joint (TMJ) region of growing rabbits and baboon monkeys[21] for 20 minutes daily. Their results show a significant increase in mandibular cartilaginous growth under LIPUS stimulation, especially under chronic mandibular advancement.[21] The mechanisms that may favor growth could include the same mechanisms involved when bone healing is enhanced with LIPUS.

The objective of the present study was to qualitatively and quantitatively assess the effects of LIPUS stimulation on mandibular condylar growth for 10 or 20 minutes daily.

MATERIALS AND METHODS

After review and acceptance of the research protocol by the research board of the Faculty of Odontology, Universidad de los Andes, 35 Sprague-Dawley male rats, 20 to 24 days old, were included in this study and divided in seven consecutive groups of rats. Twenty-five experimental rats were selected randomly and stimulated unilaterally with LIPUS in the TMJ region. The mandibles of experimental rats where divided into ultrasound-treated hemimandibles, the ''experimental group'' and contralateral hemimandibles, and the ''internal control group.'' The hemimandibles of the remaining rats were included as ''untreated controls.''

Ultrasound

LIPUS waves with an intensity of 0.03 W/cm^2 (1 MHz, 500-microsecond pulses) were applied using a custom-made US apparatus with a zirconate-titanate crystal transducer (Medlinne 4100, Santiago, Chile) (Figure 1A), which was calibrated on its emission parameters for this study. LIPUS stimulation was carried out under sedation with ether, after applying abundant ultrasonic gel. Twenty animals were stimulated with LIPUS in the temporomandibular region for 10 minutes and 5 were stimulated for 20 minutes a day on 20 occasions, 5 times a week over a 26-day period (Figure 1B). After the experiment, the rats were euthanized and the mandibles dissected into hemimandibles (Figure 1C).

Histological Procedure

The hemimandibles were left in buffered formalin (10%), decalcified in EDTA (20%), embedded in paraffin, and sectioned in parasagittal slides 6 μ thick.

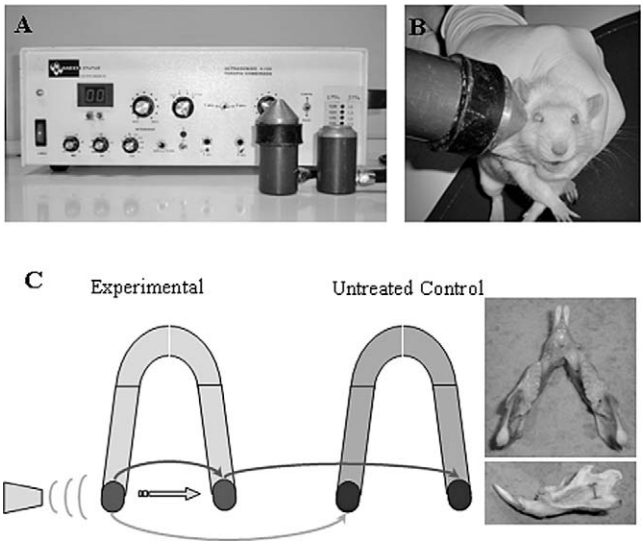


Figure 1. (A) Ultrasound device. (B) Ultrasound stimulation. (C) Experimental model.

Specimens were stained with hematoxylin-eosin (H-E), and digital images were obtained with light microscopy (Olympus CX31 microscope, Olympus Corp, Tokyo, Japan). The histological images were then analyzed qualitatively and quantitatively.

Qualitative Analysis

The qualitative assessment focused on analysis of the different condylar cartilage layers, cellular characteristics, distribution and orientation, extracellular matrix, and osseous trabeculae.

Quantitative Analysis

The quantitative histomorphometric analysis was made using the Sigmascan Pro 5.0 software (SPSS Science, Chicago, Ill) at the anterior and posterior aspects of the mandibular condyle. Measurements included the linear thickness of condylar cartilage layers and the area and perimeter of subchondral bone marrow. Each linear measurement was made in three different places along the anterior and posterior region of the condyle, and averaged, obtaining a single value for the anterior and posterior regions. The average of each measurement was used for statistical analysis. The linear measurements included absolute thickness of the mandibular cartilage, and the following layers: articular, proliferative, maturation (divided into chondroblastic and hypertrophic), and the ossification zone extending to the first bony trabecula (Figure 2A). A ratio between the proliferative and maturation zone was calculated to assess variations in the relative thickness of both layers within the cartilage structure. In the subchondral bone, medullar area and perimeter

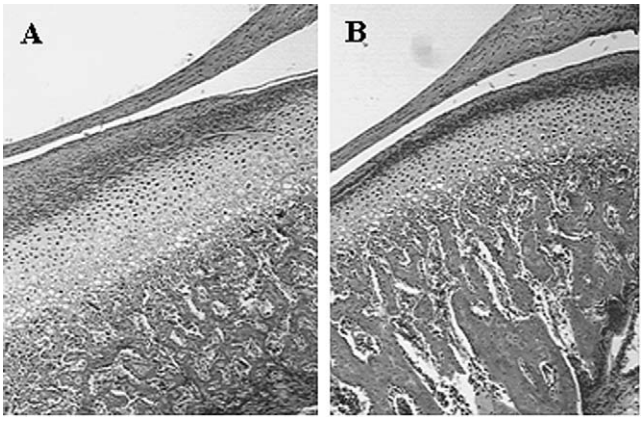


Figure 2. (A) Linear measurements. Condylar cartilage layers. I: Articular; II: Proliferative; III: Maturation (chondroblastic and hypertrophic), and IV: Erosive. (B) Bone marrow area and trabecular perimeter.

were measured in a 4 mm^2 area underlying the posterior aspect of the mandibular cartilage (Figure 2B). A calibration process was done. All linear measurements were repeated at least 4 weeks later, and measurement error was calculated using the Dahlberg formula (error between 22.4 and 55.1 μ) and Pearson correlation coefficient (r value between 0.887 and 0.994).

Statistical Analysis

The data were analyzed using Student's paired t-test for the experimental group stimulated for 10 minutes, as well as internal and external control groups. The group treated for 20 minutes was analyzed using the Wilcoxon test. Comparisons between groups were made with ANOVA and Bonferroni multiple comparison tests ($P \leq .05$). SPSS version 9 was used for statistical analysis (SPSS Science).

RESULTS

Five experimental rats died due to ether overdose, leaving a total of 30 rats. The experimental group finally included 16 rats stimulated for 10 minutes and 4 for 20 minutes. The control group remained with 10 animals.

Qualitative Histological Analysis

Experimental (stimulated) groups displayed histological changes. The extracellular matrix was more basophilic, representing increased matrix secretion at the maturation zone. Chondrocytes were more hypertrophic in the maturation zone in comparison with both control groups, showing also variations in the cellular arrangement of the same zone between experimental and control groups. More evident differences were observed in the 20-minute experimental group, including irregularity at the maturation zone, and elongated, longitudinally oriented osseous trabeculae (Figure 3).

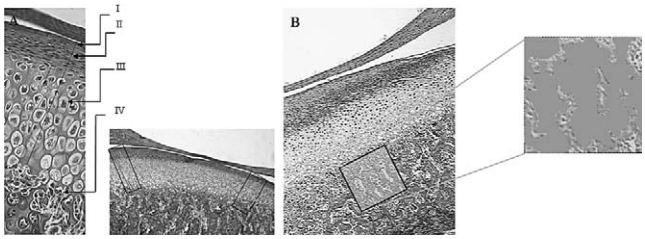


Figure 3. Temporomandibular joint condylar cartilage. (A) Untreated controls. (B) LIPUS-treated.

Quantitative Histological Analysis

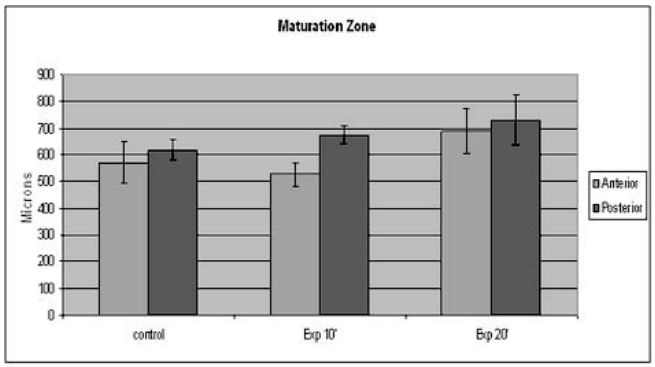
No statistically significant difference was found in the thickness of the different condylar layers between experimental and control hemimandibles at anterior and posterior zones of the condyle, either between or within groups independently from the time of application of LIPUS (ANOVA, t-test, Wilcoxon, $P > .05$). However, some tendencies were observed regarding the thickness of the proliferative, maturation and total cartilage, which differed between the anterior and posterior aspects of the condyles (Figure 4A,B).

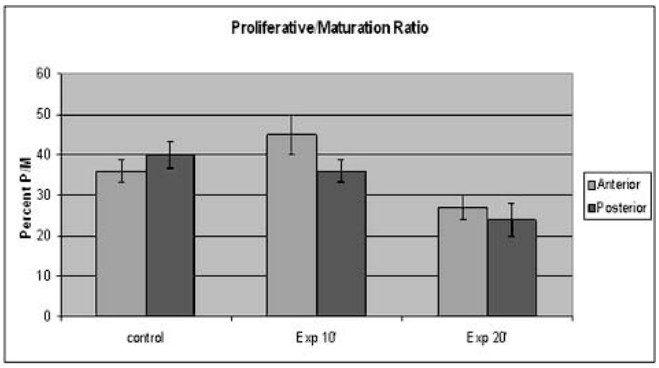
The proliferative zone/maturation zone histomorphometric ratio (P/MR) evidenced statistically significant differences at the anterior aspect of the condyles between the 10-minute experimental group, the 20-minute experimental group, and untreated controls (ANOVA, $P = .032$; Bonferroni, $P = .05$); the posterior condylar region showed no significant differences between groups (ANOVA, $P = .1$) (Figure 4C).

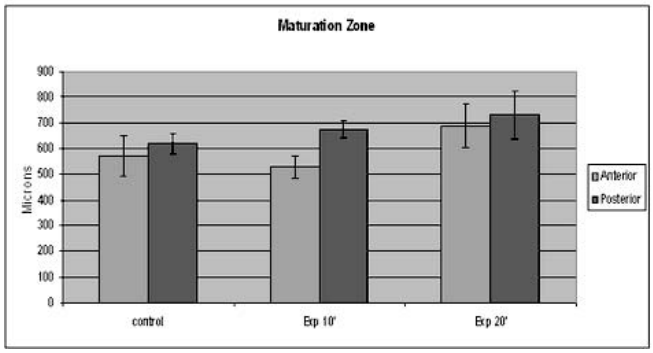
In subchondral bone, medullar area and trabecular perimeter (Figure 5) were greater in the experimental group stimulated for 10 minutes than in the control groups, without reaching significant differences (t-test, $P = .09$ and $P = .1$, respectively). Animals treated for 20 minutes displayed statistically significant differences when compared with the untreated control group for trabecular perimeter (ANOVA, $P = .01$; Bonferroni, $P = .03$). Bone marrow area measurements showed a tendency to be greater for the 20-minute experimental group, but not to a statistically significant level (ANOVA, $P < .08$).

DISCUSSION

This study was carried out using a longitudinal controlled experimental design, which was devised to analyze the effects of LIPUS stimulation on mandibular condyles of growing Sprague-Dawley rats. We used a



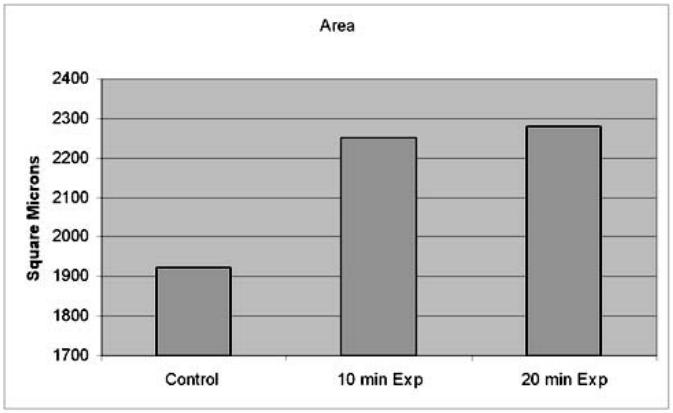


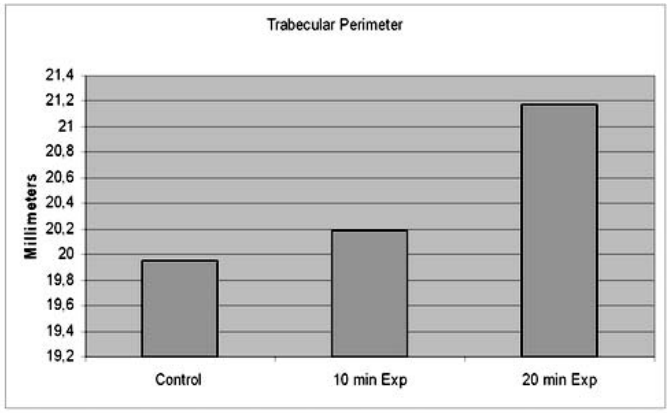


Bonferroni: p=0.05 Exp 10'-Exp 20'
Bonferroni: p= 0.11 Ctrl-20'

Proliferative / Maturation Ratio	Anterior		Posterior	
	Mean	Std. Error	Mean	Std. Error
Control	0,3552	0,0199	0,4019	0,0352
Experimental 10 minutes	0,4506	0,0501	0,3564	0,0261
Experimental 20 minutes	0,2672	0,0672	0,2368	0,0395
Total	0,3728	0,0205	0,3764	0,0247

Figure 4. (A) Proliferative zone at anterior and posterior condylar regions. (B) Maturation zone at anterior and posterior zones. (C) Proliferative/Maturation ratio at anterior and posterior zones. (D) Table showing ratio measurements.





ANOVA, p=0.01, Bonferroni, p=0,030

Figure 5. Osseous measurements: (A) Area measurements. (B) Perimeter measurements.

custom-made US apparatus especially adapted for LIPUS emission. To date, the studies that have been published in the orthodontic literature regarding the use of LIPUS and its influence on condylar growth[20,21] have been performed using the standard LIPUS device (Exogen, Caldwell, NJ), which has been extensively proven in humans,[17–19] and animals.[19,22] Despite this, other LIPUS emission settings have been reported,[23,24] varying the emission settings within the range of what is defined as LIPUS. The results presented in this animal study are particularly interesting because they point toward the biological effects of LIPUS stimulation, using a prototype LIPUS device which was able to produce US emissions of appropriate characteristics, as evaluated from the biological response secondary to its use. This is especially true if we consider that our results suggest that the biological response may vary and increases when LIPUS is applied for 20 minutes instead of 10 minutes daily.

The condylar processes of the experimental rats

were assessed qualitatively and quantitatively from a histological perspective, using LIPUS with different time application protocols. The changes observed through our qualitative analysis (Figure 3) in both experimental groups (10- and 20-minute stimulated rats) vs untreated controls show histological differences. These were seen at the maturation zone through increased matrix secretion. Chondrocytes were more hypertrophic, and the cellular arrangement differed from controls including also the subchondral bone region, expressing an elongation on the trabecular distribution and an increase in bone marrow spacing. El-Bialy et al[20,21] have reported the occurrence of evident histological effects after LIPUS stimulation both in rabbits and baboon monkeys. This was characterized by a notorious increase in thickness of the mandibular cartilage in rabbits[20] and increased bone area in monkeys.[21] Although the results hereby reported, using a different animal model, are less evident, several qualitative and quantitative differences were found between LIPUS-treated and control groups.

Our quantitative analysis consisted of measurements made at cartilage and bone tissues. LIPUS application for 10 or 20 minutes daily did not change condylar cartilage thickness to a statistically significant level. However, several tendencies were observed when experimental and untreated control groups were compared. Apparently, at the anterior and posterior condylar regions, 20 minutes of LIPUS application daily may elicit histological changes more likely to be observed than with 10-minute stimulation. No statistically significant differences were found between experimental and internal control hemimandibles. It is likely that given the intercondylar distance of approximately 18 to 20 mm of our rats, contralateral condyles could be stimulated with LIPUS to some extent (approximately 12% of the intensity at the treatment side).[20] This could explain why the greatest differences were observed between external control and experimental condyles.

One reason for not reaching statistically significant differences in several of the linear measurements may be the sample size, which was small for the 20-minute group. Given this situation, the P/MR was used to assess an eventual differential LIPUS effect within the thickness of the cartilage and between anterior and posterior aspects of the condyle. Greater than average values indicate a relative increase in the proliferative layer thickness while lower values would mean a reduction in the relative thickness of this zone. Our histomorphometric results of 0.355 at the anterior region and 0.402 at the posterior condylar region suggest a differential biological expression at a cellular level along the mandibular cartilage in response to ultrasound stimulation in the 10-minute experimental sample (Figure 4D). Those animals that were treated for 20 minutes displayed a P/MR reduction in both regions. According to Tang and Rabie,[25] condylar active growth has been associated with a decreased thickness of the cartilage. P/MR reductions could then be interpreted as an increase in the proliferative activity of the cartilage. Conversely, the 10-minute experimental group displayed different P/MR at the anterior and posterior regions. The anterior P/MR increased, while a decreased P/MR was observed in the posterior region. These results may reflect tissue's capacity to biologically react to mechanical loading elicited by LIPUS stimulation, most likely through mechanotransductive processes that confer morphogenetic competence to these micromechanical stimuli.[26] Several biological processes have been reported to play a role in the cellular response that mediates LIPUS effects on tissue healing and growth, including mechanotransductive,[11,16] microvascular,[17] metabolic,[11] and genetic[4,18,27] processes. Apparently, LIPUS stimulation can enhance their action, maximizing the growth response expressed as newly formed bone. The present results are of particular interest since they reflect that the sensitivity of condylar tissues to LIPUS stimulation differs between the anterior and posterior regions of it, and will also depend on the time stimulation protocol.

To date, most of the literature on the effects of LIPUS on growing tissues[19–21] and healing[12–14] has described the effects attained with 20-minute daily stimulation over varying periods of time. Conversely, our study included rats stimulated for 10 minutes. It was thought that bone characteristics in rats, with less secondary osteons and faster remodeling activity than the rabbit and primates,[28] would allow us to obtain in 10 minutes a growth response similar to that obtained in other species in 20 minutes. Based on the present results and those of others,[12–14,20,21] the biological effects of LIPUS apparently does not depend directly on the remodeling activity of any given species, but on the achievement of a certain threshold level of mechanical stimulation that upregulates cellular metabolism. This phenomenon is consistent with the concepts proposed by Frost[29] regarding the existence of threshold levels for skeletal adaptation.

Condylar cartilage can react differentially to LIPUS stimulation along its structure. At the anterior region, the P/MR displayed statistically significant differences between the 10- and 20-minute experimental groups (ANOVA, $P = .032$; Bonferroni, $P = .05$), while no statistically significant differences were found at the posterior region of the condyle between both groups. These results are relevant because they reflect that the sensitivity of condylar tissues to LIPUS stimulation differs between the anterior and posterior regions of the condyle, and will also depend on the duration of daily stimulation.

LIPUS application for 20 minutes daily during 4 weeks can modify the mandibular growth pattern in growing rats. Ten-minute LIPUS stimulation was not capable of significantly altering the morphologic parameters analyzed in this study. This effect can be observed in bony trabeculae underlying the endochondral ossification zone of mandibular condyles treated with LIPUS for 20 minutes, which display an increased endosteal perimeter (ANOVA, $P = .01$). Besides, there is a tendency toward an increase in bone marrow area, but not to a statistically significant level (ANOVA, $P < .08$). These findings, which are consistent with those of others, correlate with our qualitative finding that trabecular disposition appeared in a longitudinal fashion in treated animals with a greater medullar space.[21] Bone formation appears to be facilitated in the groups treated with LIPUS, especially for 20 minutes, the stimulation time that yields greater treatment results than 10-minute LIPUS stimulation.

CONCLUSIONS

- LIPUS application in mandibular condylar cartilage of growing rats produces histological changes, in a qualitative and quantitative form.
- Condylar cartilage layers of anterior and posterior regions react differentially to LIPUS application.
- LIPUS application can modify mandibular condylar growth pattern when applied for 20 minutes daily in growing rats

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