sciendo

DOI: 10.2478/aoj-2013-0002

Morphological effects of mesenchymal stem cells and pulsed ultrasound on condylar growth in rats: a pilot study

Rodrigo Oyonarte,[*] Daniela Becerra,[*] Jaime Díaz-Zúñiga,[*] Victor Rojas[*] and Flavio Carrion[†]

Faculty of Odontology[*] and Faculty of Medicine,[†] Universidad de los Andes, Las Condes, Santiago, Chile

Aim: The aim of this study was to assess and describe the morphological effects of an intra-articular injection of Mesenchymal Stem Cells (MSCs) and/or Low Intensity Pulsed Ultrasound (LIPUS) stimulation on the mandibular condyles of growing rats, using cone beam computed tomography (CBCT) and histology.

Methods: Twenty-six young (23-day-old) rats were divided into 5 groups identified as LIPUS-stimulated (20 minutes daily using 50 mW/cm^2, 1MHz, 0.2 millisecond pulses), MSCs injected (1 x 10^5 cells/kg), LIPUS + MSCs, medium injected, and untreated controls. All treatments were performed in the left temporomandibular joint of each rat (TMJs). At day 21, CBCTs were obtained for cephalometric analysis and 3D reconstructions. After animal sacrifice, left and right TMJ sections were histologically prepared and examined. The Wilcoxon sign rank test and the Kruskal-Wallis 2 test were applied for statistical comparison.

Results: Imaging results showed that left condyles were wider in all LIPUS-treated groups ($p < 0.05$), while the LIPUS-only group had a greater left sagittal condylar length. LIPUS-treated groups displayed a lower midline shift to the right ($p < 0.02$). No significant differences were observed in the MSC group. Bone marrow morphology and vascularity differed between the groups as LIPUS-treated groups exhibited increased vascularity in the erosive cartilage zone.

Conclusion: It was established that LIPUS and MSC application to the TMJ region of growing rats favoured transverse condylar growth, while LIPUS application alone may enhance sagittal condylar development. The MSC injection model had little effect on sagittal condylar growth.

(Aust Orthod J 2013; 29: 3-12)

Received for publication: May 2012
Accepted: March 2013

Rodrigo Oyonarte W: royonarte@miuandes.cl; Daniela Becerra G: dani_becerra@hotmail.com; Jaime Diaz Z: zaidemiaj@gmail.com; Victor Rojas O: vhrojas@miuandes.cl; Flavio Carrion A: fcarrion@uandes.cl

Introduction

Several treatment alternatives are available for the management of mandibular retrognathism. However, each option has generated controversy, relating to treatment efficiency and effectiveness, the achievement of functional and aesthetic goals, and long-term stability. Although mandibular functional orthopaedic treatment for Class II malocclusion offers an alternative, randomised control trials have failed to prove appliance capacity to increase mandibular length in the long term. Functional orthopaedics elicits changes that are mainly dento-alveolar in nature,[1,2] which makes the search for new therapies for the treatment of mandibular deficiency, clinically relevant.

The regulation of condylar growth may be achieved by local, hormonal or genetic factors. Among the latter, Indian hedgehog,[3-7] IGF,[8] Sox 9[9,10] and VEGF[11,12] genes play vital roles in the development of the mandibular condylar process. Gene expression prompts an increase in the availability of undifferentiated mesenchymal cells at the endochondral ossification zone, which

are required for condylar growth. Alternatively, local factors such as low-intensity pulsed ultrasound (LIPUS)[13,14] and mandibular forward posturing[12,15] are reported to enhance angiogenesis, extracellular matrix production, as well as chondroblastic proliferation and differentiation, with a resultant stimulation of endogenous MSCs. The number of MSCs has been directly correlated with the growth potential of the mandibular condyle.[5,15]

LIPUS is a form of mechanical energy transmitted through tissue at energy levels lower than 0.1 W/cm.[4,16] Its use has been studied extensively in medicine for accelerating bone fracture healing.[17,18] Its proposed mechanism of action in a healing callus involves stimulation of proteoglycan formation and chondrocyte differentiation, within a local angiogenic environment through the expression of Vascular Endothelial Growth Factor (VEGF), and other growth factors.[19] Several studies have shown a positive effect of LIPUS on endochondral growth, in vivo and in vitro[13,20-23] including changes in the endochondral growth pattern of the mandibular condylar process in animals.[13,14,20] LIPUS also has been shown, in cell culture, to stimulate chondrocyte differentiation[19,24-26] and cell culture expansion.[24] It may stimulate osteoblast differentiation, extracellular matrix formation and maturation,[27-29] and therefore, enhance expression of bone morphogenetic protein.[30]

Recently, regenerative medicine has attracted attention due to the use of stem cells, particularly mesenchymal stem cells (MSCs), for therapeutic purposes. These multipotent cells are capable of differentiating into several types of cells within the mesenchymal lineages[31] which offers new possibilities for tissue repair and regeneration. At an orofacial level, MSCs have been used experimentally for craniofacial tissue regeneration of injuries to bone and cartilage[32] and for periodontal and endodontic regeneration within biocompatible and biodegradable scaffold materials.[33-36] There is no evidence, however, supporting the use of MSCs in orofacial growth therapies.

In an in vivo pilot study, El-Bialy et al.[24] reported the use of LIPUS to enhance the performance of tissue-engineered mandibular condyles in rabbits in an attempt to expand the scope and role of regenerative medicine of the temporomandibular joint (TMJ). Tissue engineered mandibular condyles combining cartilage and bone tissues cultured from MSCs within a scaffold, were used for condylar replacement with promising results in the treatment of severely damaged joints. To date, no minimally invasive alternatives exist which might promote condylar growth using MSCs. An approach utilising a scaffold-free local exogenous supply of MSCs in conjunction with LIPUS stimulation could favour condylar growth in growing subjects.

The aim of the present study was to assess and describe the morphological effects of intra-articular mesenchymal stem cell injection into healthy mandibular condyles of growing Sprague-Dawley rats with and without LIPUS stimulation using CBCT scans and histology.

Materials and methods

Twenty-six, 23-day-old Sprague-Dawley rats, with an average weight of 65 grams were used. All were housed under controlled conditions in the animal house of Universidad de los Andes (Santiago, Chile). The sample was divided into five groups incorporating an experimental group A (LIPUS), consisting of six rats stimulated daily with LIPUS in the left TMJ region for 20 minutes for 20 consecutive days; an experimental group B (MSCs), which included six rats treated surgically with an intra-articular injection of MSCs in the left mandibular condylar region on two occasions (days 1 and 5) of the 21 day experimental period. Experimental group C (LIPUS and MSCs) comprised six rats that received stimulation with LIPUS in the left TMJ as described for group A and MSCs in the left mandibular condyles as described for group B. Control rats were assigned to groups D and E. Group D included four rats that received culture medium without MSCs according to the above injection protocol (injected control), into the left TMJ side. Four untreated rats made up Group E (control). All left-sided treatments were performed under general anaesthesia delivered by Ketamine (Ketamine hydrochloride 10% Troy Laboratories, Smithfield NSW, Australia) and Xylazine (Xylazine base 20% Centrovet Laboratories, Santiago, Chile), in a proportion of 8:1 with a dose of 1µL/mg of body weight. The research project was approved by the research committee, Faculty of Odontology, Universidad de los Andes, and all protocols were performed according to accepted criteria for the care and experimental use of laboratory animals, by the Institutional Animal Care and Use Committees of the Universidad de los Andes School of Medicine, Chile.

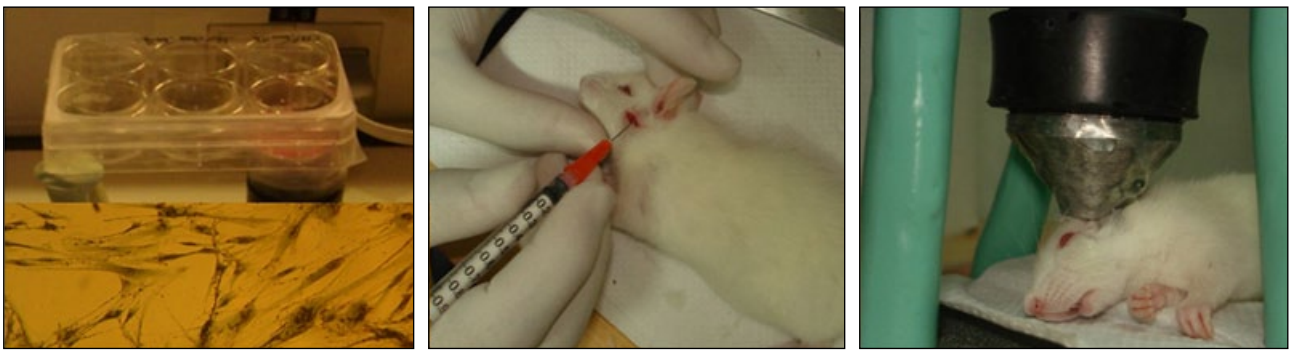


Figure 1. Cell culture at 7 days after passage 4 and its LIPUS stimulation (left), injection of MSCs in the TMJ region (centre), and LIPUS stimulation in the TMJ region under sedation (right).

Topical injection of allogenic cultured MSCs, obtained from femoral bone marrow of Sprague-Dawley rats (isolated by flushing) was performed using a cell suspension inoculation protocol. Briefly, MSCs isolated from rat bone marrow cultures, were grown and expanded in T75 flasks (Nunc TM) in culture medium composed of α – MEM (Minimum Essential Medium, GIBCO, Invitrogen, Carlsbad, CA, USA) enriched with fetal bovine serum 20% (MSC Qualified Fetal Bovine Serum, 100 mL, GIBCO, Invitrogen, Carlsbad, CA, USA) and 1% antibiotic (Penicillin and streptomycin, PenStrep, GIBCO, Invitrogen, Carlsbad, CA, USA). These cells were serially passaged with Triple select (Invitrogen, Carlsbad, CA, USA) upon nearly reaching confluency. Passage number was defined by cell growth with the medium used in this study. Cultures possessed the characteristics of fibroblastoid morphology, plastic adherence and proliferative states using a volume of 1 µL/mg of body weight, in estimated quantities of 1 x 10^5 cells/kg of MSCs in passage 4. For this purpose, a minimally invasive standardised protocol of injection of MSCs including the incision of the facial skin on the left TMJ region, was developed. Non-suspended cells were kept cultured and were monitored until differentiation into mesenchymal lineage cells (chondrocytes, osteocytes and fat) occurred.

All the injections of MSCs or culture medium were performed by the same calibrated operator (JD). The MSCs from the bone marrow of Sprague-Dawley rats used in this study were cultured in the laboratory of immunology and cell therapy of the Faculty of Medicine of the Universidad de los Andes. Cell culture was performed according to an established international protocol (Stemcell Technologies, Technical Manual 2008) modified through the stimulation of the cell culture with LIPUS (50mW/cm2) for 20 minutes, twice a day, to facilitate cell culture expansion[24] (Figure 1) and chondrocyte differentiation.[19,26]

LIPUS application

LIPUS was applied using a Medlinne-4100-Combination Therapy device (Santiago, Chile) equipped with a ceramic zirconate titanate transducer and customised to emit LIPUS waves at a frequency of 1 Mhz. LIPUS was applied for 20 minutes daily for 20 consecutive days, at an intensity of 50 mW/cm^2 (1 MHz) in pulses of 0.2 milliseconds.

Cone beam computed tomography imaging (CBCT)

Cone beam computed tomographic images of the heads of all rats were obtained using an Accuitomo 3D scanner (XYZ Slice View Tomograph, J. Morita, Kyoto, Japan). Exposure parameters were set at 60 kV, 5.0 mAmp (17 seconds exposure) in a window of 40 x 40 mm, with an isotropic voxel size of 0.08 millimetres (80 microns) displayed in a 13-bit grayscale. All imaging procedures were performed under sedation using a Ketamine-Xylazine mixture. The captured images were processed and interpreted by a single operator (JD) using the i-Dixel software (J Morita MFG Corp. Kyoto, Japan) (Figure 2).

Three-dimensional reconstructions were generated for each rat. One reconstruction oriented the head with the intercondylar axis parallel to the horizontal axis and midsagittal plane parallel to the vertical axis. The other reconstructions were oriented along each hemi-mandibular long axis (Figure 2). Cephalometric points were identified (Table I) and a cephalometric analysis was conducted which included 6 linear measurements quantifying mandibular characteristics

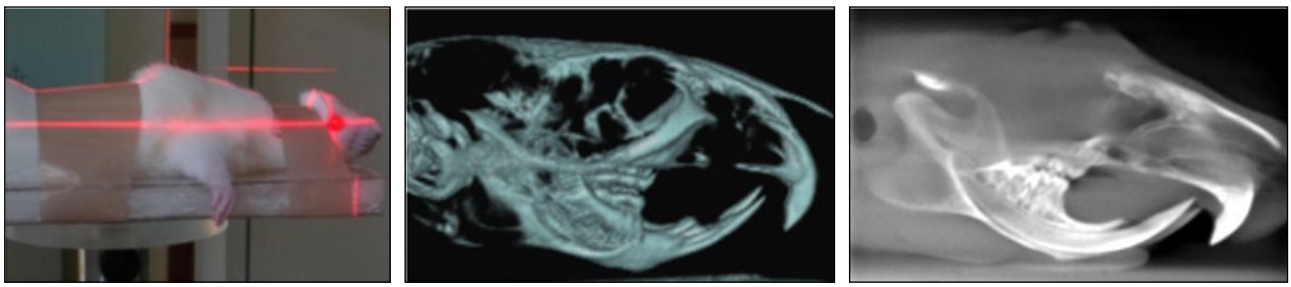


Figure 2. Imaging procedure, from the positioning of the rat in the CBCT scanner, to the acquisition of images.

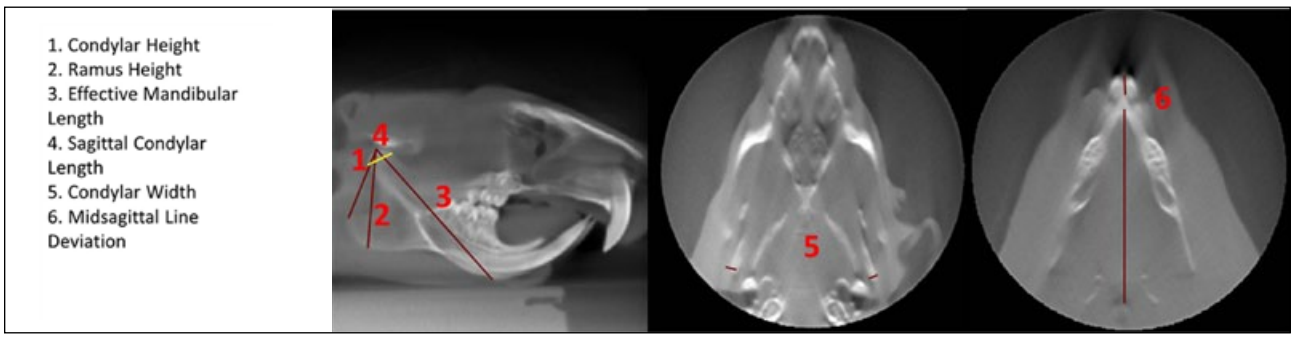


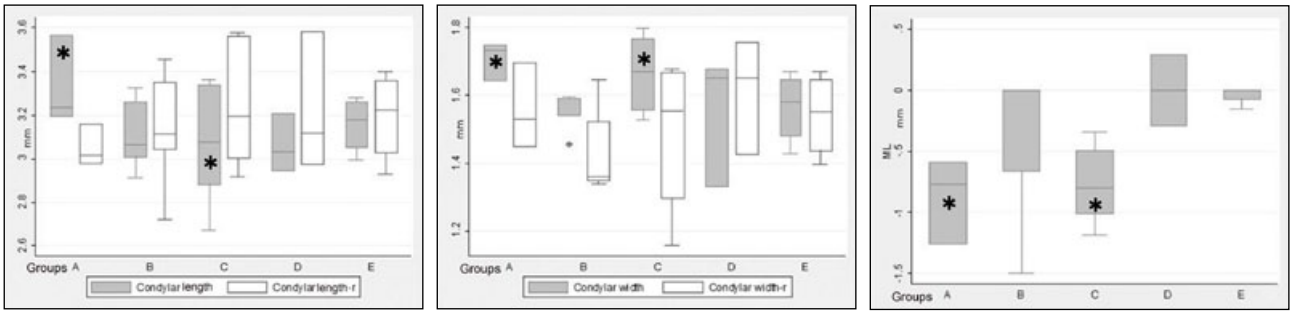
Figure 3. Cephalometric variables.

Table I. Cephalometric points.

Cephalometric point	Description
Condylar (Cd)	Most posterior and superior point of the mandibular condyle in the lateral view
Angular process (Ap)	Most posterior and superior point of the angular process in the lateral view
Gonion (Go)	Most distal and inferior point of the mandibular body
Menton (Me)	Most inferior and anterior point of mandible
Posterior Pole (PP)	Most posterior point of the mandibular condyle in the lateral view
Anterior Pole (AP)	Most anterior point of the mandibular condyle in the lateral view
Medial Pole (MP)	Innermost point of the medial pole of the mandibular condyle in the axial view
Lateral Pole (LP)	Most lateral point of the lateral pole of the mandibular condyle in the axial view
Dental Midlines	Midsagittal line of maxillary and mandibular dental arches, between the central incisors

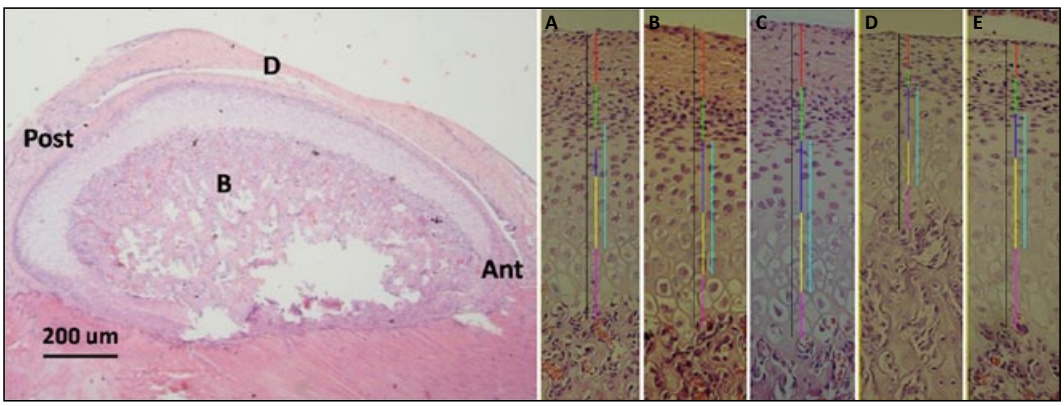
Table II. Cephalometric measurements.

Cephalometric measurements	
Condylar Height (CH)	Distance between Ap and Cd
Ramus Height (RH)	Distance between Go and Cd
Effective Mandibular Length (EML)	Distance between Cd and Me
Sagittal Condylar Length (SCL)	Distance between PP and AP
Condylar Width (CW)	Distance between LP and MP
Midsagittal Line Coincidence (ML)	Distance between upper and lower dental midlines



(a) (b) (c)

Figure 4. (a) Condylar length was increased at the LIPUS only treated condyles ($p = 0.046$) and decreased in combined LIPUS-MSC treated condyles ($p = 0.027$).
(b) Condylar width was increased at the left side for the LIPUS ($p = 0,046$) MSCs ($p = 0.079$) and Combination groups ($p = 0.027$).
(c) Midline (ML) deviation was recorded towards the untreated side in LIPUS-treated animals (Groups A and C).



(a) (b) (c) (d) (e)

Figure 5. Left side: A panoramic view of a condyle (40 X). The anterior cartilage zone (Ant) relating to the anterior insertion of the temporomandibular disc (D), and the posterior zone (Post) relating to the posterior insertion of the disc. The subchondral bone (B) is also identified.
Right side: Representative sections of each of the five experimental condyles and their cartilage layers measurements: articular layer (red), proliferative layer (green), maturation layer (cyan), precondroblastic layer (blue), hypertrophic layer (yellow), erosive layer (pink) and total length (black).

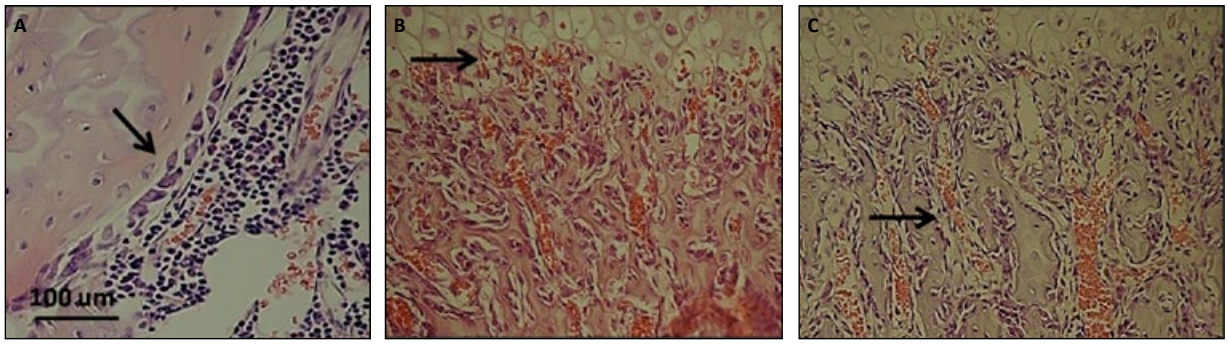
and midline coincidence between upper and lower arches (Table II, Figure 3). Each radiographic measurement was bilaterally performed to the nearest tenth of a millimetre and taken at three consecutive time points, seven days apart. The average was used for analytical purposes. All rats were sacrificed at day 21 (44 days of age) by an ether overdose (ethyl ether 99.9%).

Preparation of histological samples

Each left hemi-mandible was dissected and fixed in 10% buffered paraformaldehyde for at least 24 hours. The mandibles were decalcified in 20% EDTA at 37°C and dehydrated with alcohol of increasing concentrations and xylol. Specimens were embedded in paraffin wax with the anterior condylar pole orientated upside down. Parasagittal 5 micron sections were cut and stained with haematoxylin and eosin and PAS. Digital optical microscopic images of areas of interest were acquired at 40 X, 100 X and 400 X magnification (Nikon Labophot Optical Microscope).

Histological analysis

The histological characteristics of the cartilage and underlying bone tissue were recorded. A qualitative assessment analysed osteoblastic morphology, the characteristics of the condylar bone marrow spaces and vascularity. Sigmascan 5.0 software (SPSS Science, Chicago, II) was utilised for a quantitative assessment. Digital images (100 X magnification) of the anterior and posterior condylar regions were measured in three representative zones of each



(a) (b) (c)

Figure 6. (a) Increased osteoblastic activity (arrow) was observed in groups A, B and C.
(b) Blood vessels (arrow) were more abundant in the erosive zone in LIPUS-treated groups.
(c) Increased vascularity as observed in the underlying bone area (arrow) in injected condyles (MSCs and other injected groups).

microscopic field, established perpendicular to a line tangent to the articular surface. The zones were 50 microns apart. The average of the three obtained values was used for analytical purposes. The anterior condylar zone was located immediately distal to the anterior disc insertion, while the posterior condylar zone was located immediately anterior to the posterior disc insertion on the condylar head (Figure 4). The measurements included total cartilage thickness, articular, proliferative, maturation, erosive layer and the proliferative/maturation layer ratio (Figure 5). The perimeter and area of bone marrow spaces were measured within the first 100 microns of subchondral bone layer.

Statistical analysis

A Wilcoxon sign rank test and the Kruskal-Wallis 2 non-parametric statistical analyses ($p < 0.05$) were applied to determine within and between group imaging comparisons. The Kruskal-Wallis 2 analysis was used to assess between group histological comparisons. The statistical analysis was performed using Stata 11 statistical software.

Results

One rat died in group D and another from group B was isolated due to infection. Therefore, the final sample consisted of 24 rats; 6 in the LIPUS and MSC+LIPUS groups, 5 in the MSC group, 3 injected controls and 4 untreated controls. All animals were included in the imaging analyses. After the samples were processed for histology, the final composition was 4 sections for the LIPUS group, and 3 sections for each of the remaining groups, for a total of 16 samples.

Cone Beam CT imaging

Within group paired comparisons, left versus right side (Wilcoxon Sign Rank Test)

Compared with the contralateral condyle, the LIPUS Group A displayed statistically significant differences in sagittal condylar length (SCL, $p = 0.0277$; Figure 4a) and condylar width (CW, $p = 0.0464$, Figure 4b), as the left side was wider and longer. Group C (LIPUS + MSCs) showed statistically greater sagittal condylar length (SCL, $p = 0.0277$; Figure 4a) on the right side, and significant differences were found in condylar width (CW, $p = 0.0277$; Figure 4b) on the left side.

No statistically significant differences were found within the MSCs, injected controls and untreated control groups (B, D and E), and no significant differences were found for intra-group comparisons in relation to mandibular length, ramus height or condylar height (Wilcoxon $p > 0.05$).

Between group left side comparisons (Kruskal-Wallis Test)

The only variable that displayed statistical significance was the 'mid-sagittal line' due to differences between the LIPUS-treated groups A and C and the controls of group D and E (Figure 4c).

Histology

Qualitative analysis

Experimental groups receiving LIPUS and/or MSCs (A, B and C) showed signs of increased cellular activity and increased numbers of cuboidal shaped osteoblasts (Figure 6a).

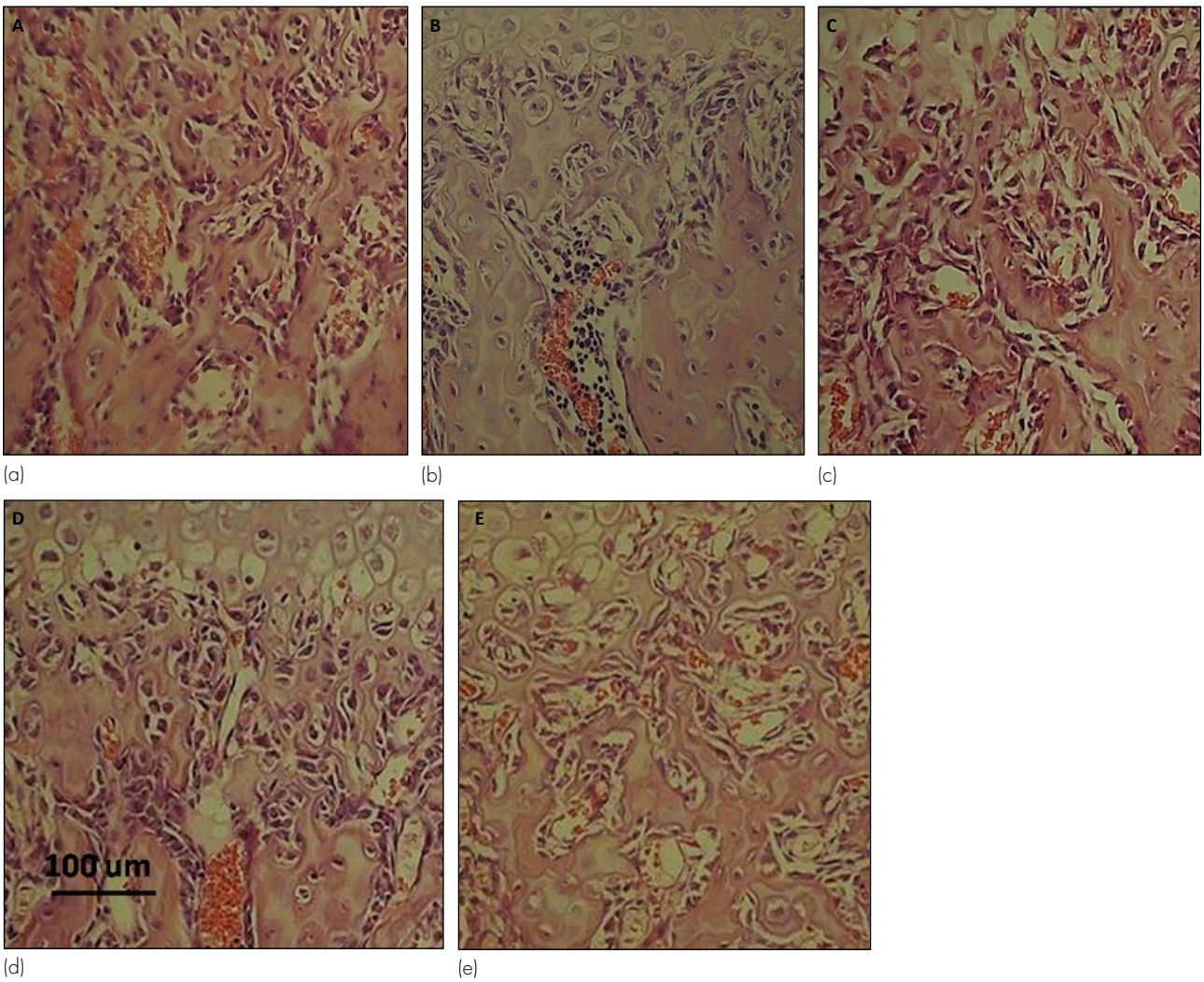


Figure 7. Bone marrow morphology- representative histological samples of groups A, B, C, D and E are shown. Groups A and C display elongated bone marrow spaces.

In comparison with controls and non-LIPUS treated groups, LIPUS treated groups (A and C) showed abundant small blood vessels in the erosive layer and profuse blood invasion in the empty chondrocyte spaces (Figure 5b). Conversely, the injected groups (B, C and D) displayed wide thin-walled blood vessels in the bone region next to the erosive layer (Figure 5c).

Group C (LIPUS and MSCs) demonstrated numerous small vessels in the erosive layer, as well as an increased vascularity in the subchondral bone (Figures 6 b, c). An evaluation of marrow spaces showed differences in the quantity and disposition of marrow spaces in LIPUS-treated groups. These presented elongated bone marrow spaces, unlike the other groups which displayed a discontinuous relationship of their medullar spaces (Figure 7).

Quantitative analysis

No statistically significant differences (Kruskal Wallis 2, $p > 0.05$) in thickness were observed between the different layers of condylar cartilage at either the anterior or posterior zones. Although the proliferative zone was thicker posteriorly in the LIPUS treated groups A and C (Figure 7a), this was not significant ($p = 0.053$) in comparison with other treatment groups. For groups A and C, the proliferative/maturation zone index appeared to trend towards higher values at the posterior condylar zone (Figure 7b). However, this finding was not statistically significant ($p = 0.071$). No significant differences were found for bone marrow area or perimeter.

Discussion

The present in vivo pilot study was conducted to assess the morphological effects of LIPUS application, MSC injection and their combination, into the condyles of growing Sprague-Dawley rats. A further aim was to explore the effects of physical stimulation and cellular therapy using a scaffold-free approach on active mandibular condylar growth. The results were assessed using CBCT and histology which were intended to show tendencies and direct future research in the field.

In vitro and in vivo studies have reported that LIPUS may promote proliferation[37] and differentiation of MSCs to chondrocytes,[21,22,26] and its application may increase the relative thickness of the proliferative condylar layer which is known to contain endogenous MSCs in Sprague-Dawley rats.[24] The present study's repeated injections of MSCs were used to ensure persistence of the transplanted cells. Given the known effects of LIPUS on MSCs,[22,24,38,39] the likelihood of retaining and differentiating MSCs in mandibular cartilage could increase, and therefore promote enhanced condylar growth beyond that described for LIPUS stimulation alone.[13,14,20]

For the formation and ossification of new bone, the presence of undifferentiated mesenchymal cells is essential.[5,12] These cells are present in the proliferative zone of mandibular condylar cartilage, bone marrow and surrounding blood vessels,[12] which may eventually supply more cells to the local population of MSCs as bone neo-vascularisation proceeds. LIPUS promotes VEGF expression[19] as well as chondrogenic differentiation,[19,24-26] and improves aggrecan gene expression.[40-42] The differences observed in condylar width and length seen in the present study may be related to the LIPUS stimulation process.

According to El-Bialy et al.,[24] LIPUS stimulation facilitated chondrogenic and osteogenic differentiation of bone marrow stem cells which was beneficial for the tissue engineering of a mandibular condyle in vivo (rabbit model) using a scaffold matrix. The development of treatment techniques allowing the use of MSCs without scaffolds appeared to be an attractive alternative for the cartilage engineering process.[43,44] In the presence of LIPUS stimulation, collagen II and aggrecan gene expression were enhanced, thereby improving matrix formation,[44] which is relevant if growth enhancement is intended through the use of scaffold-free techniques with MSCs.

In the present study, imaging was performed using high definition clinical CBCT 3D reconstructions, with an 80 μm voxel size. Although a higher resolution can be obtained using micro CT scans, a recent report comparing the performance of similar CBCT volume images (76 μm voxel size) with 41 μm voxel micro CT images showed that their performance was comparable.[45] CBCT also had the advantage of generating in vivo intermaxillary measurements.

Six cephalometric measurements were chosen to assess morphological changes in the mandible and also their effect on dental midline coincidence. Among them, condylar width, sagittal condylar length and midline coincidence displayed significant differences. Accordingly, LIPUS and MSCs may lead to increased condylar width, but not sagittal condylar length. Condylar length was increased only in the LIPUS group, and significantly decreased in the combined LIPUS and MSCs group. It is possible that this difference could be related to the trauma associated with the injection procedure. The mandibular midline deviation towards the untreated side observed in the LIPUS group suggested that its effect on condylar length may result in a mandibular asymmetry, which is consistent with previous research.[13,20]

Histologically, all groups displayed increased vascularity relative to untreated controls. The fact that increased vascularity was found in the erosive zone in the LIPUS group instead of the subchondral bone layer may be related to endochondral bone formation through varied pathways.[5,12] This has been supported by previously reported data.[13,14,20]

The present study reported differences in the effects of LIPUS on condylar growth compared with earlier studies in rabbits and baboons.[13,20] An increase in mandibular cartilage thickness was observed in LIPUS-treated rabbits,[20] and increased condylar bone area in baboon monkeys was accompanied by reduced cartilage thickness.[13]

However, consistent with the results of the present investigation, both studies[13,20] found a positive LIPUS effect on mandibular growth, with a marked effect on osseous vascularity, along with ossification during endochondral growth. The present study revealed that LIPUS did not significantly alter the thickness of the cartilage, nor the proliferative/maturation layers in the posterior zone of the condyle in LIPUS-treated groups and control groups D and E. Nevertheless, a thicker proliferative zone was observed in the posterior region

in groups A and C, which approached significance (p = 0.053), despite the current small sample size. These results are consistent with a previous report.[14] Histological observations raise the possibility that with this experimental model, the effect of LIPUS on developing mandibular condyles of rats may act specifically at the proliferative layer and at a vascular level, thereby facilitating growth to a moderate extent. This phenomenon likely relates to the qualitative expression of different bone marrow space morphology and vascularity between groups.

The present results showed no effect from an intra-articular injection of MSCs on the sagittal and vertical condylar growth processes. It is possible that the properties of the cartilaginous matrix do not allow the diffusion of MSCs. Matrix density and the relatively large size of the cells (20 – 30 μm) make diffusion unlikely. Only low weight molecules such as matrix liquid components can permeate,[46] which supports the use of scaffolds in regenerative TMJ procedures.[24] Given the high complexity of its cellular and extracellular matrix arrangement, a MSC scaffold-free approach is inappropriate in eliciting orthopaedic condylar growth changes. These may be eventually achieved in rodents with mechanical stimuli such as forward mandibular posturing[5,15] or LIPUS stimulation.[14,20]

The MSC condyle injection protocol used in the present study is novel in its attempt to generate a favourable scaffold-free environment for growth enhancement. It is apparent that the use of MSCs, either with or without a scaffold, would be useful in healing and joint reconstruction, but not for sagittal mandibular growth enhancement, at least under the current experimental conditions. Conversely, LIPUS was effective in eliciting vascular and osseous morphological changes, which accompanied a favourable sagittal condylar response.

Conclusion

The application of LIPUS, with and without the application of MSCs to the TMJ region of growing rats, favours transverse condylar growth, while LIPUS alone may favour sagittal condylar development. In addition, LIPUS to the TMJ of growing rats increases vascularisation of the erosive cartilage layer.

The exogenous supply of MSCs into the TMJ of rats did not stimulate mandibular sagittal condylar growth.

Given the high complexity of its cellular and extracellular matrix arrangement, the injection of mesenchymal stem cells in the TMJ region using a scaffold-free approach is inappropriate in eliciting orthopaedic condylar growth changes.

Acknowledgments

Dr Andrés Briner, for the enormous help provided with the acquisition and processing of imaging data.

Dr Valeria Ramirez, for her valuable help with the statistical analysis of the data.

Dr Natividad Sabag, for her continuous support and advice regarding the morphological data of this experiment.

Fabiola Diaz for her invaluable and expert help during the experimental phase of the study.

The work was supported by a FIC-ODO research grant, Faculty of Odontology, Universidad de los Andes, Santiago, Chile.

Corresponding author

Professor Rodrigo Oyonarte Weldt
Discipline of Orthodontics
Facultad de Odontología
Universidad de los Andes
San Carlos de Apoquindo 2200
Las Condes
Santiago
Chile

Email: royonarte@miuandes.cl

References

1. Tulloch JF, Phillips C, Proffit WR. Benefit of early Class II treatment: progress report of a two-phase randomized clinical trial. Am J Orthod Dentofacial Orthop 1998;113:62-72.
2. O'Brien K, Wright J, Conboy F, Sanjie Y, Mandall N, Chadwick S et al. Effectiveness of early orthodontic treatment with the Twin-block appliance: a multicenter, randomized, controlled trial. Part 1: Dental and skeletal effects. Am J Orthod Dentofacial Orthop 2003;124:234-43.
3. Tang T, Rabie AB, Hagg U. Indian Hedgehog: a mechanotransduction mediator in condylar cartilage. J Dent Res 2004;83:434-8.
4. Ng TC, Chiu KW, Rabie AB, Hagg U. Repeated mechanical loading enhances the expression of Indian hedgehog in condylar cartilage. Front Biosc 2006;11:943-8.
5. Rabie AB, Wong L, Tsai M. Replicating mesenchymal cells in the condyle and the glenoid fossa during mandibular forward positioning. Am J Orthod Dentofacial Orthop 2003;123:49-57.
6. Teixeira VC, Teixeira AC, Luz JG. Skeletal changes alter experimentally displaced condylar process fracture in growing rats. J Craniomaxillofac Surg 2006;34:220-5.

7. Sugito H, Shibukawa Y, Kinumatsu T, Yasuda T, Nagayama M, Yamada S et al. Ihh signaling regulates mandibular symphysis development and growth. J Dent Res 2011;90:625-31.
8. Ramirez-Yañez G, Smid J, Young W, Waters M. Influence of growth hormone on the craniofacial complex of transgenic mice. Eur J Orthod 2005;27:494-500.
9. Bi W, Deng J, Zhang Z, Behringer R, De Crombrugghe B. Sox-9 is required for cartilage formation. Nat Genet 1999;22:85-9.
10. Hattori T, Müller C, Gebhard S, Bauer E, Pausch F, Schlund B et al. SOX9 is a major negative regulator of cartilage vascularization, bone marrow formation and endochondral ossification. Development 2010;137:901-11.
11. Carvelaro, MF, Cermelli S, Cancedda R, Descalzi Cancedda F. Vascular endotelial growth factor (VEGF) in cartilage neovascularization and chondrocyte differentiation: auto-paracrine role during endochondral bone formation. J Cell Sci 2000;113:59-69.
12. Rabie AB, Shum L, Chayanupaktul A. VEGF and bone formation in the glenoid fossa during forward mandibular positioning. Am J Orthod Dentofacial Orthop 2002;122:202-9.
13. 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.e7-14.
14. Oyonarte R, Zarate M, Rodriguez F. Low intensity pulsed ultrasound stimulation of condylar growth in rats. Angle Orthod 2009;79:964-70.
15. Rabie AB, Leung FY, Chayanupaktul A, Hägg U. The correlation between neovascularization and bone formation in the condyle during forward mandibular positioning. Angle Orthod 2002;72:431-8.
16. Schortinghuis J, Stegenga B, Raghoebar GM, de Bont LG. Ultrasound stimulation of maxillofacial bone healing. Crit Rev Oral Biol Med 2003;14:63-74.
17. Kristiansen TK, Ryaby JP, McCabe J, Frey JJ, Roe LR. Accelerated healing of distal radial fractures with the use of specific, low-intensity ultrasound. A multicenter, prospective, randomized, double-blind, placebo-controlled study. J Bone Joint Surg Am 1997;79:961-73.
18. Busse JW, Kaur J, Mollon B, Bhandari M, Tornetta P, Schünemann HJ et al. Low intensity pulsed ultrasonography for fractures: systematic review of randomized controlled trials. BMJ (Clinical research ed) 2009;27:338-51.
19. Kobayashi Y, Sakai D, Iwashina T, Iwabuchi S, Mochida J. Low-intensity pulsed ultrasound stimulates cell proliferation, proteoglycan synthesis and expression of growth factor-related genes in human nucleus pulposus cell line. Eur Cell Mater 2009;17:15-22.
20. El-Bialy T, El-Shamy I, Graber TM. Growth modification of rabbit mandible using therapeutic ultrasound: Is it possible to enhance functional appliance results? Angle Orthod 2003;73:631-9.
21. Lee HJ, Choi BH, Min BH, Son YS, Park SR. Low-intensity ultrasound stimulation enhances chondrogenic differentiation in alginate culture of mesenchymal stem cells. Artif Organs 2006;30:707-15.
22. Cui JH, Park SR, Park K, Choi BH, Min BH. Preconditioning of mesenchymal stem cells with low-intensity ultrasound for cartilage formation in vivo. Tissue Eng 2007;13: 351-60.
23. Choi BH, Choi MH, Kwak MG, Min BH, Woo ZH, Park SR. Mechanotransduction pathways of low-intensity ultrasound in C-28/I2 human chondrocyte cell line. Proc Inst Mech Eng H 2007;221:527-35.
24. El-Bialy T, Uludag H, Jomha N, Badylak SF. In vivo ultrasound-assisted tissue-engineered mandibular condyle: A pilot study in rabbits. Tissue Eng Part C Methods 2010;16:1315-23.
25. Ebisawa K, Hata K, Okada K, Kimata K, Ueda M, Torii S et al. Ultrasound enhances transforming growth factor beta-mediated chondrocyte differentiation of human mesenchymal stem cells. Tissue Eng 2004;10:921-9.
26. Cui JH, Park K, Park SR, Min BH. Effects of low-intensity ultrasound on chondrogenic differentiation of mesenchymal stem cells embedded in polyglycolic acid: An in vivo study. Tissue Eng 2006;12:75-82.
27. Saito M, Soshi S, Tanaka T, Fujii K. Intensity-related differences in collagen post-translational modification in MC3T3-E1 osteoblasts after exposure to low- and high-intensity pulsed ultrasound. Bone 2004;35:644-55.
28. Sena K, Leven RM, Mazhar K, Sumner DR, Virdi AS. Early gene response to low-intensity pulsed ultrasound in rat osteoblastic cells. Ultrasound Med Biol 2005;31:703-8.
29. Tam KF, Cheung WH, Lee KM, Qin L, Leung KS. Osteogenic effects of low intensity pulsed ultrasound, extracorporeal shockwaves and their combination – an in vitro comparative study on human periosteal cells. Ultrasound Med Biol 2008;34:1957-65.
30. Suzuki A, Takayama T, Suzuki N, Kojima T, Ota N, Asano S et al. Daily low-intensity pulsed ultrasound stimulates production of bone morphogenetic protein in ROS 17/2.8 cells. J Oral Sci 2009;51:29-36.
31. Fortier L. Stem cells: classifications, controversies, and clinical applications. Vet Surg 2005;34:415-23.
32. Salinas CN, Anseth KS. Mesenchymal stem cells for craniofacial tissue regeneration: designing hydrogel delivery vehicles. J Dent Res 2009;88:681-92.
33. Donzelli E, Salvadè A, Mimo P, Viganò M, Morrone M, Papagna R et al. Mesenchymal stem cells cultured on a collagen scaffold: in vitro osteogenic differentiation. Arch Oral Biol 2007;52:64-73.
34. Moioli EK, Clark PA, Chen M, Dennis JE, Erickson HP, Gerson SL et al. Synergistic actions of hematopoietic and mesenchymal stem/progenitor cells in vascularizing bioengineered tissues. PLoS One 2008;3:3922.
35. Lin NH, Gronthos S, Bartold PM. Stem cells and future periodontal regeneration. Periodontol 2000 2009;51: 239-51.
36. Ji YM, Jeon SH, Park JY, Chung JH, Choung YH, Choung PH. Dental stem cell therapy with calcium hydroxide in dental pulp capping. Tissue Eng Part A 2010;16:1823-33.
37. Ruan JL, Wang YN, Crum L, Mitchell S. Effect of low intensity pulsed ultrasound on mesenchymal stem cells. J Acoust Soc Am 2011;129:2576.
38. Caplan AI. Review: mesenchymal stem cells: cell-based reconstructive therapy in orthopedics. Tissue Eng 2005;11:1198-211.
39. Sorrell JM, Caplan AI. Topical delivery of mesenchymal stem cells and their function in wounds. Stem Cell Res Ther 2010;1:30.
40. Yang KH, Parvizi J, Wang SJ, Lewallen DG, Kinnick RR, Greenleaf JF et al. Exposure to low-intensity ultrasound increases aggrecan gene expression in a rat femur fracture model. J Orthop Res 1996;14:802-9.
41. Parvizi J, Wu CC, Lewallen DG, Greenleaf JF, Bolander ME. Low-intensity ultrasound stimulates proteoglycan synthesis in rat chondrocytes by increasing aggrecan gene expression. J Orthop Res 1999;17:488-94.
42. Zhang Zi, Huckle J, Francomano CA, Spencer RG. The influence of pulsed low-intensity ultrasound on matrix production of chondrocytes at different stages of differentiation: an explant study. Ultrasound Med Biol 2002;28:1547-53.
43. Irie Y, Mizumoto H, Fujino S, Kajiwara T. Reconstruction of cartilage tissue using scaffold-free organoid culture technique. J Biosci Bioeng 2008;105:450-3.
44. Uenaka K, Imai S, Ando K, Matsusue Y. Relation of low-intensity pulsed ultrasound to the cell density of scaffold-free cartilage in a high-density static semi-open culture system. J Orthop Sci 2010;15:816-24.
45. Maret D, Molinier F, Braga J, Peters OA, Telmon N, Treil J et al. Accuracy of 3D reconstructions based on cone beam computed tomography. J Dent Res 2010;89:1465-9.
46. Spaeth E, Klopp A, Dembinski J, Andreeff M, Marini F. Inflammation and tumor microenvironments: defining the migratory itinerary of mesenchymal stem cells. Gene Ther 2008;15:730-8.