Mandible protraction alters Type I collagen, osteocalcin and osteonectin gene expression in adult mice condyle

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Summary

Mandible condyle remodeling is a great challenge on craniofacial growth studies. The great majority of the reports deals with growing period. However, there is a great necessity to clarify the importance of functional stimulation on adult mandible condyle remodeling. By using an adult mouse model, we investigated the influence of mandible forwarding on condyle remodeling and gene expression by bone forming cells. Tomographic and scintigraphic evaluations showed sagittal growth and cell activity enhancement. RT-PCR showed that Type I collagen, osteocalcin and osteonectin expression level can be altered. We showed that functional stimulation is necessary to maintain the regular gene expression by condyle bone forming cells in adult mice. It opens new frame for further investigations aiming new clinical approaches to temporomandibular joint problems treatment, as well as mandible retrusion treatment.

Key words: bone biology, growth evaluation, molecular biology, CT, condyle growth, gene expression.

Introduction

Head and face growth disorders are very common in all racial populations. The prevalence of craniofacial anomalies varies among different ethnicities based on genetic background, geography, socio-economic status and environmental factors (1). Mandible retrusion prevalence runs from 52 to 56% (2), and the most common treatment is condyle traction generated by the mandible forward repositioning. Mandible protrusion enhances functional stimulation on condyle and generates sagittal growth et al. (3). However, we have a huge controversy. Some reports indicate a positive growth response to condyle traction while others indicate a negative or no response to mandibular advancement (4). Therefore, there is a need to identify the markers for each stage of condylar growth and evaluate the effect of functional stimulation on growth pattern. Condyle grows by endochondral ossification, and some Authors have been studying the early stages of condyle formation, focusing on the importance of functional stimulation for chondrogenesis. However, the osteogenic phase of condyle formation has been missed. The possible gene expression alteration on this phase, generated by different pattern of stimulation, was not studied yet (5-10). Moreover, the majority of the reports deals with growing period and do not focus on adult phase (11-16). Considering not only mandible retrusion correction but also considering that remodeling alteration is the main cause for articular temporomandibular disorders affecting many adults (17), it is important to clarify the mechanisms involved on adult condyle response to functional stimulation. So, our aim was to evaluate the alteration on osteoblast gene expression pattern, on mouse adult condyle, under different functional stimulation.

Materials and methods

Mandible protrusion protocol

This study was approved by the Federal University of Minas Gerais Animal Use and Care Committee (Protocol 169/2014). 48 female C57 mice (including 24 as controls) were used in this work. We decided to investigate the effect of mandible protrusion on condyle osteoblasts gene expression. In order to generate mandible protrusion, 24 mice (experimental group) had the inferior incisors cut around 1 mm. We used a nail plyer, holding the animal in left hand and using the plyer on right hand to cut the inferior incisors. It

generates an increase on the overjet. Because of this, they had the necessity of sending the mandible forward to be able to chew (Fig. 1).
The weight of each animal was checked. Control mice only underwent weight checking to evaluate the regular food intake and consequent weight gain. The animals were provided with free access to food pellets and tap water and were housed at the animal experimentation laboratory. Every 3 days the cutting procedure was repeated, due to continuous eruption of the teeth, as well as the weight checking.

Tomography
To evaluate the condyle sagittal shape changing, we

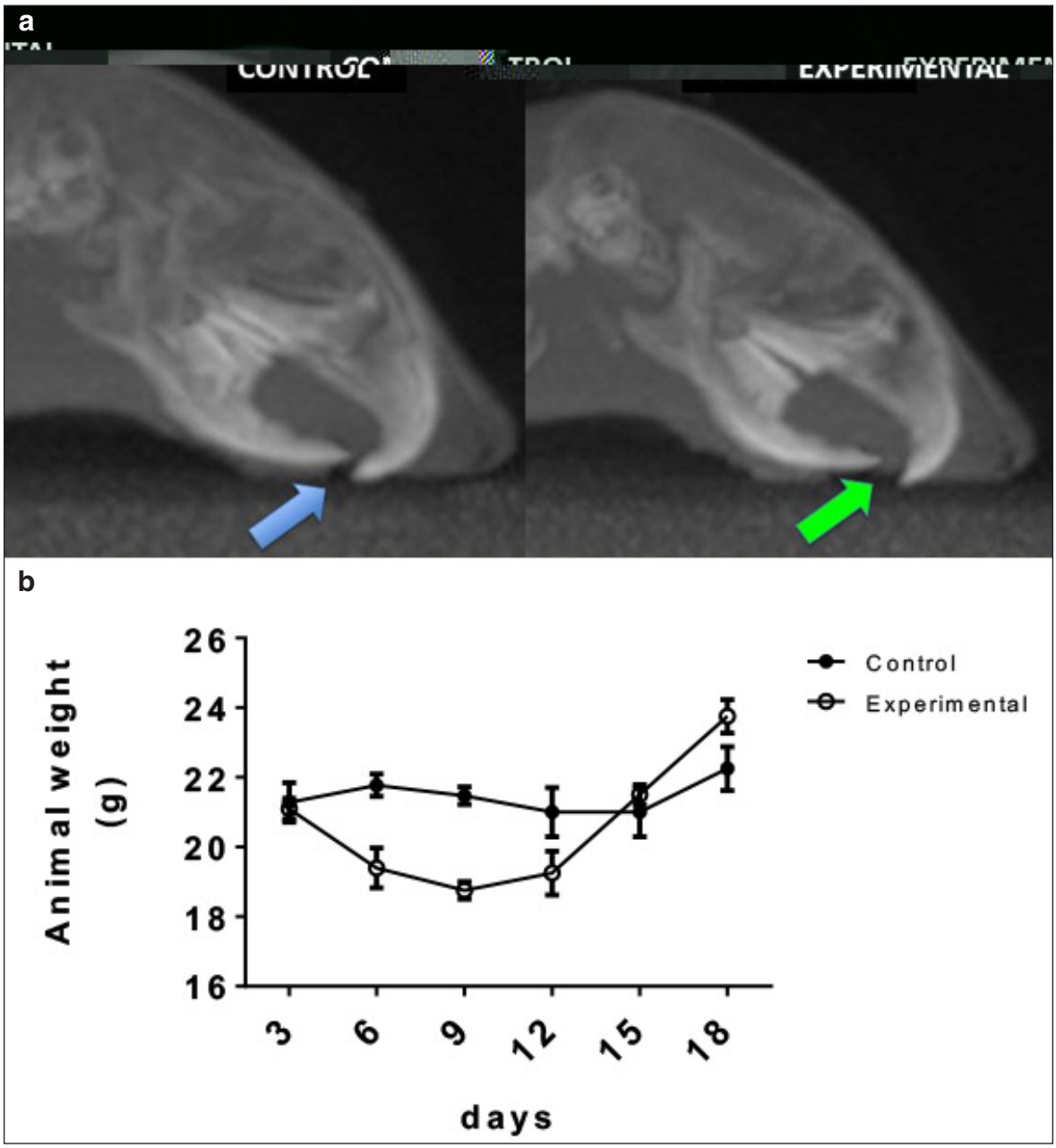

Figure 1 a, b. Mandibular advancement. (a) To generate a physiological mandibular advancement, that would lead to stimulation on condyle region, every 3 days during 21 days, the lower incisors were cut by 1 mm to induce protrusion when the animal was feeding. In left panel we can see the control animal with regular distance between inferior and superior incisor, and on right panel we can see the experimental animal immediately after cutting, showing an increase on the overjet; (b) the weight gaining of the animals indicated that on the first days the experimental group was not used to the increase of overjet and had weight loss, but after 9 days they started to eat normally and recover the weight gaining (first point of the graphic is 3 and last point of the graphic is 18, because we checked the weight on the cutting day).

used a tomographic study. The animals were anesthetized with a combination of ketamine (30 mg/kg) and xylazine HCl (2.5mg/kg) intramuscularly. Then, they were positioned on a table adapted to the equipment as showed on the Figure 2a. The condyle was localized on frontal and coronal plane and measurements were done on sagittal plane (Fig. 2b). We used a Gendex CB500 with voxel 0.125 and 4 cm high.

Scintigraphy

The ^{99m}Technetium-methylene diphosphonate (^{99m}Tc-MDP), a specific marker of the anabolic phase of remodeling, has been used in the diagnosis of a broad spectrum of conditions affecting the skeleton. The radiopharmaceutical ^{99m}Tc-MDP was obtained from UFMG Pharmacy school laboratory. To observe bone metabolic activity in the region of the condyle, bone

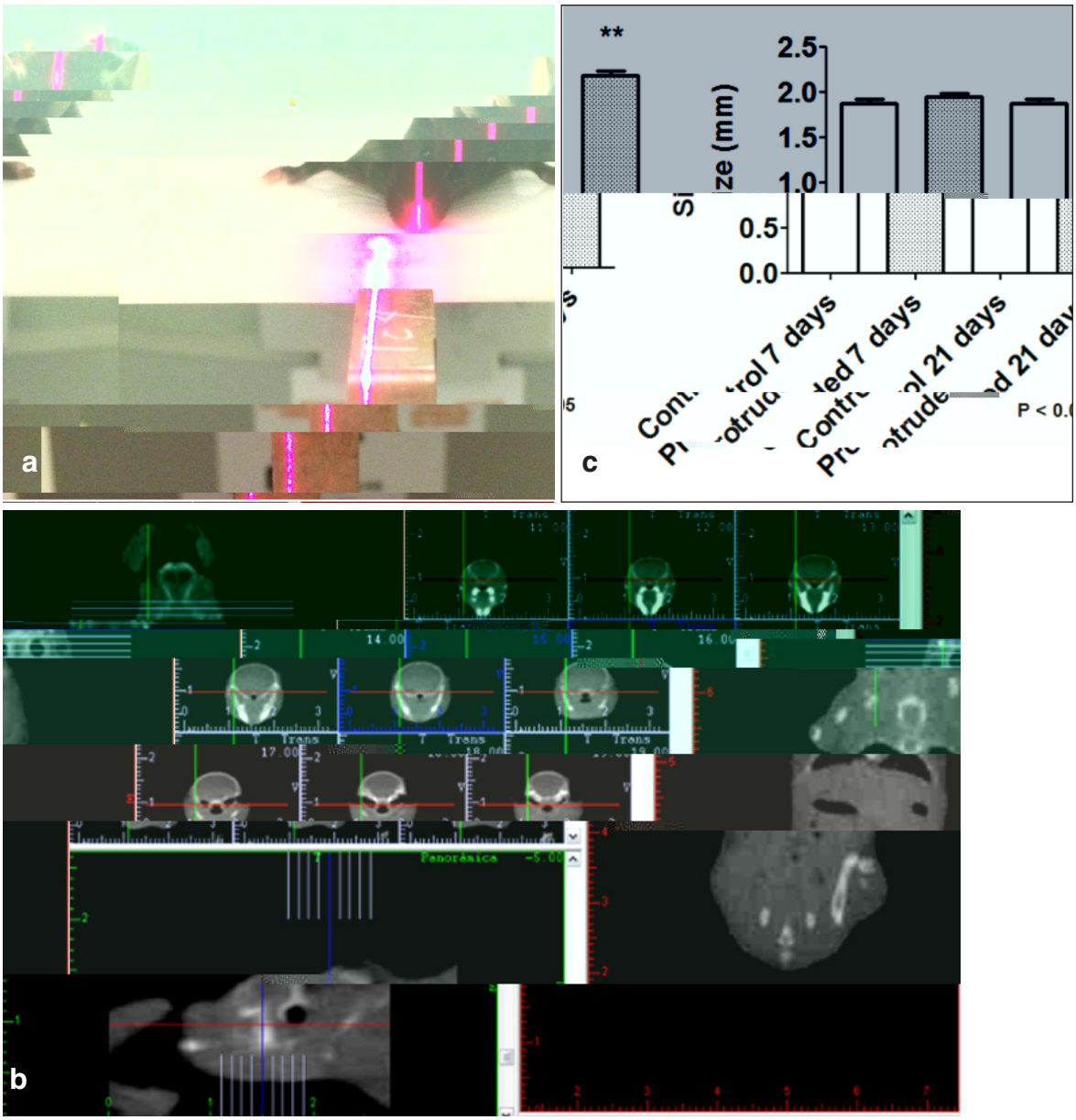

Figure 2 a-c. Tomography. (a) After anesthesia, the animals were positioned on the equipment table as showed on the picture; (b) The software used allows the user to measure linear distances in 3 dimensions. After anesthesia, the animals were analyzed in coronal (right panel) and transversal (left upper panel) plane to localize the condyle. The measurement was done on sagittal plane (left lower panel). Measurements were done separately in left and right condyle; (c) After 21 days, the experimental group showed an increase on sagittal dimension of the condyle. Results reflects means ± SD of 3 different experiments ($p< 0.05$).

scintigraphic images were taken 7 and 21 days after starting cutting, using a gamma camera (Nuclide^{TM} TH 22, Mediso, Hungary). This analysis does not require the animal to be killed, and can be repeated. The animals were anesthetized with a combination of ketamine (30 mg/kg) and xylazine HCl (2.5 mg/kg) intramuscularly. Aliquots (0.1 mL) containing 10,3 MBq of (^{99m}Tc-MDP) were injected intravenously into the tail vein of the animals. Static-planar acquisition was initiated 1 h after the injection. The animals were placed in a supine position under a gamma camera employing a low-energy high-resolution collimator. Images were acquired using a 256 × 256 × 16 matrix size with a 20% energy window set at 140 keV for a period of 10 min. The images were analyzed quantitatively, and the radioactivity was determined in the demarcation areas of the ROI (region of interest) involving the right and left condyle on experimental and control group. The uptake of ^{99m}Tc-MDP in the regions was measured. Results were analyzed using T student test unpaired. Differences were considered significant when $p<0.05$ (Fig. 3a).

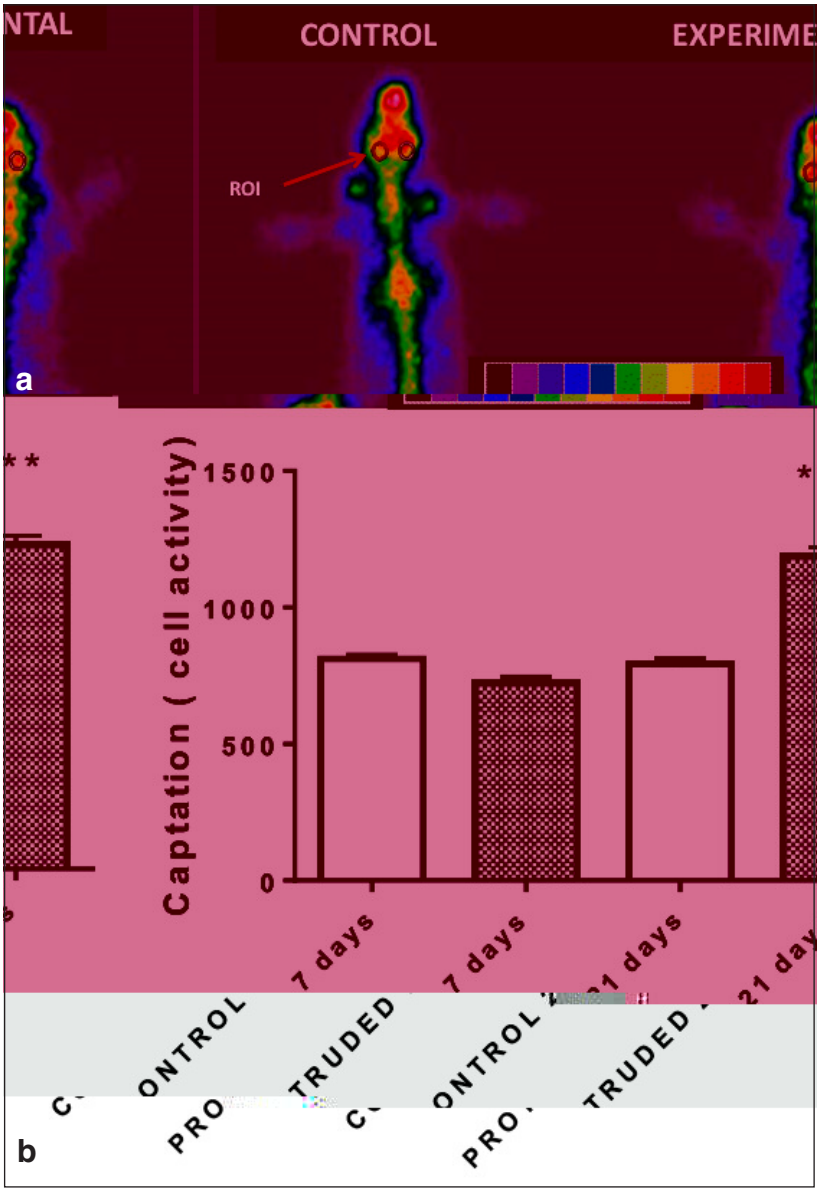

Figure 3 a, b. Bone Scintigraphy. (a) Representative scintigraphic image for the definition of the region of interest (ROI). Red arrow is reference point to show one ROI at the condyle region. The ROI (circle) was established and the radio isotope count [accumulation count of 99mtechnetium-methylene-diphosphonate (99mTc-MDP)] was measured. As inferred by the color bar scale, red shows a high accumulation of 99mTc-MDP and blue shows a low accumulation of 99mTc-MDP; (b) Uptake ratio of 99mTc-MDP at the condyle region after protrusion of the mandible. The vertical axis shows the uptake ratio. The count rates of 99mTc-MDP in the protruded mandible condyle were significantly higher than control after 21 days. Results express mean ± SD of 2 different experiments for each period ($p< 0,05$).

Condyle cell isolation and Immunofluorescence

Since our objective would be to evaluate the alteration on gene expression by osteoblasts obtained by the macerate of condyles, it was important to first check the cell population of this macerate. In order to evaluate it, we first used a sequential digestion protocol. Briefly, we dissected the condyles and freed from soft tissue, cut into small pieces and rinsed in sterile phosphate-buffered saline without calcium and magnesium (Sigma Aldrich). The condyle pieces were incubated with 1% trypsin-EDTA (Gibco) for 5 min, followed by four sequential incubations with 0.2% collagenase (Sigma Aldrich) at 37°C for 45 min each. The digestions produced a suspension of cells. After centrifugation at 1000 g for 5 min, the pellet was resuspended in 5 ml of RPMI medium (Gibco) supplemented with 10% FBS (Gibco), 1% antibiotic-antimycotic (Gibco). The cells were seeded into 25 ml tissue culture flasks (Sarstedt), and led to grow in a controlled 5% CO2 95% humidified incubator at 37°C. After confluence the cells were used for immunofluorescence using a marker for bone forming cells (osteopontine OPN). OPN is produced by cells involved in bone morphogenesis such as preosteoblasts, osteoblasts, osteoclasts, osteocytes, odontoblasts, and also hypertrophic chondrocytes. So we decided to use it to be sure that we were dealing with osteogenic cells.

For immunofluorescence, we used 6 well culture flask (Sarstedt) and platted the cells over glass cover slips (Fisherbrand). 24 hours later, the cells were fixed with 4% paraformaldehyde (Merk, Brazil) in phosphate buffered saline (PBS) for 10 min, and washed three times in PBS. The cells were incubated in blocking solution [PBS, 1% bovine serum albumin (Sigma), 5% normal goat serum (Sigma), 0.5% triton-X (Sigma Aldrich)] and then incubated with the following primary antibody: mouse antiosteopontin (R&D systems) and secondary: Alexa Fluor (Invitrogen). To evidentiate the nucleus, cells were also incubated with propidium iodide (Sigma Aldrich) (Fig. 4).

Extracting RNA from condyle in a single step

In order to evaluate differential gene expression from cells derived from experimental and control animals, condyle bones were harvested from the animals, exposing them after removing muscles. Any attached tissue was quickly removed using a scalpel before the condyle was immersed in liquid nitrogen. The samples were homogenized separately with Trizol® in porcelain mortar and pestle with liquid nitrogen. RNA was then separated and processed according to the manufacturer's protocol (Trizol® Reagent, Life Tec-

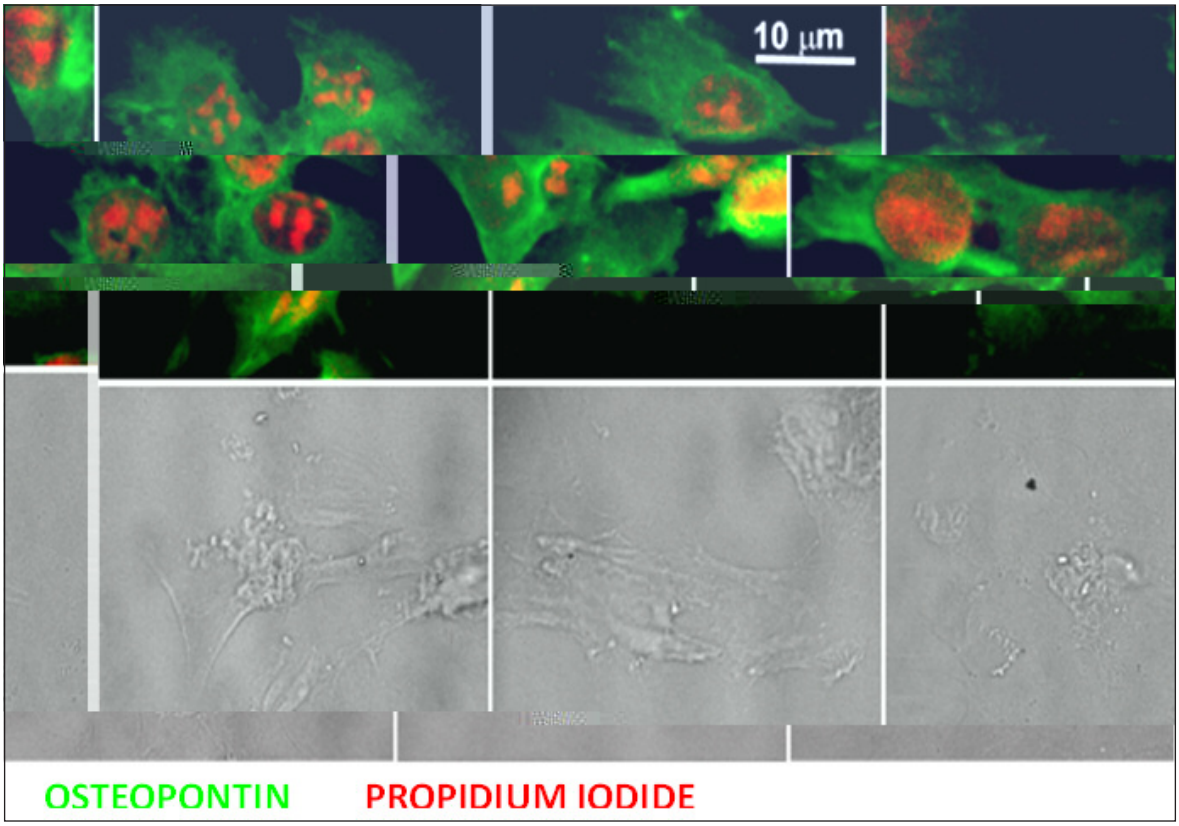

Figure 4. Immunofluorescence for osteopontin. Confocal images obtained from the condyle macerate. A great number of cells was positive for osteopontin, indicating that they were bone forming cells. Green indicates osteopontin staining and red are nucleus stained by propidium iodide. Transmission image included in merged image.

nologie, Carlsbad, California). Total RNA was quantified and then treated with DNase I® (TURBO DNA-free Kit, Ambion® Inc., Foster, California, USA).

Reverse Transcriptase Reaction

The RT for sscDNA synthesis was performed from 1 µg of total RNA in a final reaction volume of 20.4 µl per sample. Briefly, RNA was pre-incubated at 70° C for 10 minutes with 10 pmol of each reverse primer specific for the target genes, together with 10 pmol of oligo dT_{18} primer, followed by storage on ice at the bench top. Then, was added to samples mix containing RT buffer [250 mM Tris-HCl (pH 8.3), 375 mM KCl, 15 mM $MgCl_2$] and dNTP (10mM each), samples with this mix were incubated at 45° C for 2 min and then placed on ice. Finally added 1 µl of reverse transcriptase enzyme mix (40 U) into RT buffer [250 mM Tris-HCl (pH 8.3), 375 mM KCl, 15 mM $MgCl_2$] and incubated at 45°C for 1 hour along with previously described RNA and primers. The reaction was terminated at -20°C until used in the real time PCR. All reagents were from Invitrogen™ (Life Technologies, Carlsbad, CA, USA).

Real time PCR

The real time PCR was developed in 7500/ABI PRISM® Sequence Detection System equipment, using the protocol described by the reaction SYBR Green PCR Master Mix Kit (Invitrogen™ Life Technologies, Carlsbad, CA, USA). Samples in triplicate were applied to 96-well plates (ABI PRISM® Optical 96 -Well Reaction Plate with bar code Invitrogen Life Technologies, Carlsbad, CA, USA) in a final reaction volume of 20 µl each. Aliquots of 1.6 µl of sscDNA sample were pipetted into each well of the plate, subsequently adding 18.4 µl of sybrMix [10 µl of SYBR Green PCR Master Mix Kit, 1.2 µl of each primer (sense and antisense; 10 pmol / µl) and 6 µl sterile filtered water]. The plate was sealed with optical adhesive (ABI PRISM® optical adhesive Covers, Invitrogen® Life Technologies, Carlsbad, CA, USA). Real time PCR reactions occurred in the following thermal cycle: [stage 1] a cycle of 50° C/2 min; [stage 2] cycle at 95°C/10 min; [stage 3] 40 cycles of 95°C/0.15 min, followed the dissociation curve from 60˚C to analyze the specificity of the amplicons. The mRNA value for each gene was normalized relative to the housekeeping mouse S26 mRNA levels in RNA samples. Primer sequences (forward and reverse) were as follows:

S26, 5`- CGTGCTTCCCAAGCTCTATGT -3` and 5` - CGATTCCTGACAACCTTGCTATG -3`;

Bglap3, 5`-CTTGGTGCACACCTAGCAGA -3` and 5`- ACCTTATTGCCCTCCTGCT -3`;

Col1a1, 5`- GCTCCTCTTAGGGGCCACT -3` and 5`- ATTGGGGACCCTTAGGCCAT -3`;

Sparc, 5`- AAACATGGCAAGGTGTGTGA -3` and 5`- AAGTGGCAGGAAGAGTCGAA-3`.

For relative quantification was performed a comparative analysis of the expression of target transcripts genes versus endogenous control using the comparative CT method, which the endogenous control was used to normalize the expression of the target gene (target gene CT mean - mean CT endogenous control) generating ΔCT. Using the ΔCT was calculated ΔΔCT [ΔCT sample - ΔCT calibrator (reference sample)]. Then it was applied the formula $2^{-\Delta\Delta CT}$ for determining the relative levels of expression of each target gene. The results of expression levels were launched in GraphPad Prism 5 software, for statistical analysis, using t student tests unpaired. Differences were considered significant when $p<0.05$.

Results

Sequential cut of inferior incisor generates condyle sagittal remodeling

The model used to generate protrusion was adapted from Tagliaro et al. (17). In our protocol we did not use anesthesia and the cutting using a nail plyer was quick, effective and more secure than trimming using a dental motor, recommended by the Author. On Figure 1a we can see the generated increase on overjet. The weight gain of both groups was checked to evaluate the experimental group adaptation to the increased overjet. When compared to control, experimental group showed, on the first days, a decrease on weight gain, indicating that the animals were not used to the new occlusal situation leading to difficulties on chewing. We can state that the functional stimulation during this period decreased. After they get used to new occlusal scheme they regularized the food intake and achieved the same weight of the control group at 21 days as showed on Figure 1b. The tomographic images were taken in order to evaluate sagittal dimension alteration of the condyles from the animals submitted to mandible protrusion, since it is known that the condyle remodeling follows the direction of pulling (18). It was adapted a positioner for anesthetized animals as showed on Figure 2a. Images were taken after localizing the condyle on transversal and coronal plane. The measurements were made with 0,5 mm of accuracy (Fig. 2b). The results showed that mandible protrusion was able to generate sagittal condyle posterior remodeling on adult animals, when compared to no protruded controls ($p<0.05$) (Fig. 2c).

Mandible protrusion leads to increase on condyle cellular activity and enhanced expression of Type I collagen, osteocalcin and osteonectin

On the scintigraphy, the selected regions of interest (ROI) on condyle were evaluated in both sides of experimental and control animals (Fig. 3a). The plotted results indicated that on 7 days it was not possible to register a statistical difference of 99mTc-MDP capitation comparing experimental and control groups. But after 21 days it was possible to see a statistical relevant increase on the capitation by the condyles submitted to traction by protrusion ($p<0.05$) (Fig. 3b), corroborating the results obtained by the tomography. After isolation and expansion of the cell population obtained from sequential digestion of condyle macer-

ate, it was possible to show that a great number of bone forming cells were present on the population. Osteopontin antibody stained the majority of the cells as shown in Figure 4, indicating that the condyle macerate could be used for total RNA extraction to evaluate differential gene expression of bone forming markers. The expression of Type I collagen, osteocalcin and osteonectin by the bone forming cells isolated from the condyle were affected by the mechanical stimulation. During the first days, when the animals from experimental group were not able to chew properly we observed a decrease on the expression of the three genes compared to control animals. After they start to chew normally and had to protrude in order to compensate the increased overjet, there was a recovering on the expression of these genes allowing at 21 days no statistical difference on their level ($p<0.05$) (Fig. 5a-d). As soon as the animals started to increase the condyle traction, due to mandible protrusion, gene expression pattern of Type I collagen, osteocalcin and osteonectin increased.

Discussion

Little is known about the mechanisms involved on mandible remodeling under functional stimulation. In a recent integrative review conducted by our group (19) we found only 15 relevant works dealing with this subject in the last 20 years. It is well known that functional stimulation interferes with condyle remodeling during growing periods (17, 20-22). However, it remains unclear the role of functional stimulus on adult condyle remodeling. Our adapted protrusion model allowed the investigation of condyle remodeling in adult mice. On the beginning the animals diminished the food intake. The difficulty on chewing demonstrated by the animals immediately after the initial cutting is in accord to orofacial proprioception neurophysiology. It is known that body needs an adaptation period when changes in occlusal scheme occurs. It generates a delay on regular food intake (23). So, our experimental group delayed to get used to increased overjet and due to that diminished the food intake. But the curve of weight gain (Fig. 1b) indicated that the experimental group after recovering the capacity of regular chewing, even having to protrude, was able to equalize the weight gain with the control group. It indicates that the animals learned how to forward the mandible in order to chew, making our model adequated

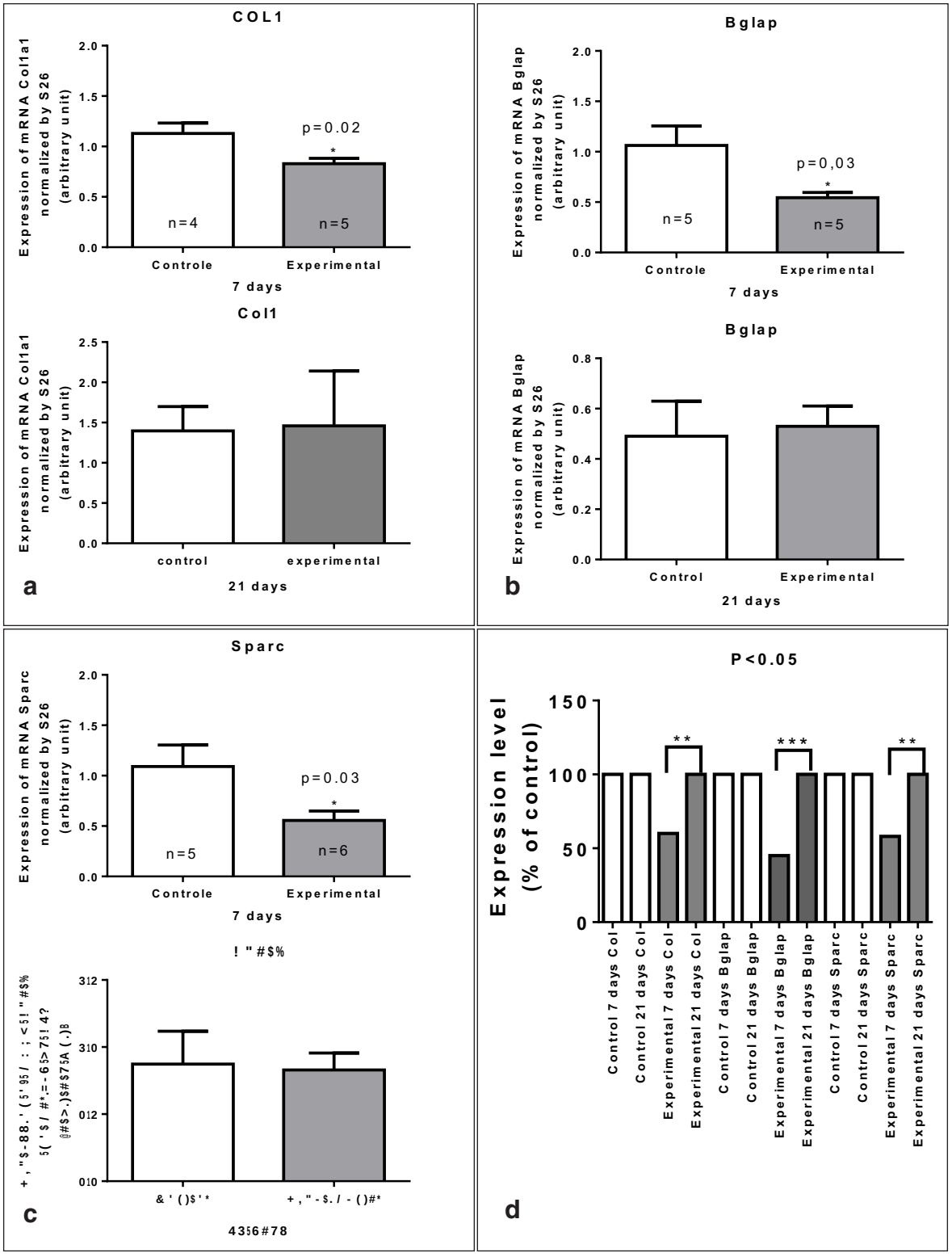

Figure 5. Real-time PCR. Diferential expression of: (a) Collagen (Col1), (b) Osteocalcin (Bglap) and (c) Osteonectin (Sparc) evaluated at 7 days and 21 days. All of them showed lower expression on experimental group at 7 days, compared to control ($p<0.05$). On 21 days no statistical difference on gene expression was observed ($p<0.05$), indicating that the experimental group showed an enhance on gene expression during the period from 7 to 21 days; (d) Differential expression of Type I Collagen, Osteocalcin and Osteonectin by experimental group analyzed in terms of control % ($p<0.05$).

References

1. Joshi N, Handam A, Fakhouri WD. Sk a ma o usion ad o m n a disord r i h a i on mor idi . J in s 1 33 .
. aum , us han H, W is in S. S a ur , h ad h i h and ro h o h r i a a . Am r hod. 13 - .
. Ar s as . h ra iona or ar or hodon i r a m n. Am r hod D n o a ia r ho . 133 1 -1 .
. a i A , A- a a A. Do s h d r o ad an in dur- in un iona a ian h ra ma 15 u r hod.Jun .
. Shi a a S, Fukuoka H, Sa o , A , Su uki . An in si u h ridi a ion s ud o h insu in- ik ro h a ors s- m in d o in ond ar ar i a o h a mous mandi . ur J His o h m. a .
. i , i son , Sun H in . D n in sia o hos ho ro- in DS a s an ss n ia ro in h os na a d - o m n and main nan o mous mandi u ar ond ar ar- i a . J His o h m o h m . 1 1 3 .
. orimo o H, a a , Han i , Doi . Dou -s rand d NA- d nd n ro in kinas r u a s insu in-s imu a d hon- dro n sis in mous ona hondro ni s, A D - . issu s. Jan 1 1 1- .
. nomo o A, Wa ahiki J, Nam o , ri , hika a , a hika- a , aki 1 . as i a ion mark d a s mandi u- ar ond ar ar i a ro h, n r ssion, and mor- ho o . Am r hod D n o a ia r ho . S 1 .
3 . S rrano J, So S, Hin on J. o s o no h si na in in mandi u ar ond ar ar i a . Ar ra io. Ju 1 3 - .
1 . m da , rao F, i a aki , oshi aki , akahashi . 1 . i ro NA u a s h D o m n o andi u ar ond ar ar i a . J D n s. Jun 1 3 - .
11. o n A , akin n , akin n , ar son DS. o- a no i and hos ha as a i i in h ra mandi a- r un iona ro rusion. Ar ra io . 13 3 .
. h n D, Wan , uo S. His o o i and his o h mi a s ud- i s on h m oromandi u ar ar i u ar dis in oun ro - in ra s a r un iona mandi u ar ad an m n s. Hua i o ian i u a hi. 133 1 .
. ou , h n , uo S, h n . ir adian rh hm han s o h s o ndo nous insu in in ro in ra ond ar ar i a a r un iona mandi u ar ro rusion. Hua i ou ian i u a hi 1 .
1 . Sh n , a i A , , hao . r ssion o o a n in ond ar ar i a durin mandi u ar ro rusion. Hua i o ian i u a hi 1 - .
1 . ion , h n , , ou S. ir adian rh hm han s o h s o ndo nous insu in in ro in ra s ood a- r un iona mandi u ar ro ru ion. Hua i o ian i u a hi 1 3 1.
1 . , h n , uo S. r ssion o m NA o os o a in in ond ar ar i a o oun SD ra s a r un iona ro rusion. hon hua o ian i u a hi - .
1 . h n J, So u , r a A, a a i , u , i s , oun . S Di r n s in hondro a ura ion in h andi u ar ond rom a D r as d. usa oadin od- . a i issu n . Au us 11 3 - 3 .
1 . Simo s WA, ro i A, S u mann J. odus o randi o anas a ian . J in dia r D n Win r. 133 1 - 3 - .
13 . a rio , a do FJ , Simo s WA. mod a i a r- a ions o J i h h us o ro ra ion a ian s an in- ra i r i r odon ia S 1 1 .
. Wa ahiki J, ama u hi , ri , Nakano H, aki , a hika a . n r ssion ro i in o mous ond ar ar i a durin mas i a ion m ans o as r mi rodiss ion and DNA arra . J D n s. a 3 .
1. Shi uka a , oun , Wu , amada S, on F, a i i- i , o ama . m oromandi u ar oin orma ion and ond ro h r uir ndian h d ho si na in . D D n. F - .
. iu D, iao F, han H , u , Jiao , han , han J, H JJ, Wu , h n D, Wan . du in di ar oad- in d r as s mous m oromandi u ar oin d rada ion indu d an rior ross i ros h sis. s oar hri is ar i a . F 1 .
. umar A, ri oriadis J, ru sson , S nsson , S nsson . s o shor - rm rainin on ha iora arnin and ski a uisi ion durin in raora in mo or ask. N uro- s i n . Ju 1 -1 .
. oko ama , A sumi , su hi a , o ama S, Sasaki . D nami han s in on m a o ism in h ra m oro- mandi u ar oin a r mo ar ra ion usin on s in i ra h . ur ra S i. Au 3 11 3 .
. oh n WA, S r ais J , our , i , u . Ar i u ar ar i- a d n ra ion in h on ra a ra non-sur i a m- oromandi u ar oin in mi i h a uni a ra ar ia dis- om . ra a ho d. F 1 -1 .
. Jahan , a sumo o A, a A , Hashimo o , nou , da a a J, S kin J ani H. F a a mo m n a s hh si na in in mandi u ar ond ar ar i a d o m n h ossi ro o hh as m hano ransdu ion m dia or. Ar ra io 1 3 1 1 -111 .
. Harris S , on a d F, Harris A, Sa a ini , Da as S, F n , hosh- houdhur N, Wo n J, und . - s o rans ormin ro h a or aon on nodu or- ma ion and r ssion o on mor ho n i ro in , os- o a in, os o on in, a ka in hos ha as , and o- a n m NA in on - rm u ur s o a ra a aria os- o as s. J on in r s. Jun 133 - .
. ro n J , D mas D, a a a , douard , ha u , uni r J. S rum on a- ro in a s i i mark r or on orma ion in os m no ausa os o orosis. an . a 13 13 1 3 1-1 .
3 . Shimoka a H. s on in, a n ro in. Nihon Shika shikai asshi. 13 - 3 1.
. arini J, Smi h S s o n sis m r a 1 A . n D roo J, k- o , hrousos , Dun an , rossman A, H rshman J , o h , a h an , N , ar , Sin r F, inik A, W i k r , di ors. ndo n rn . Sou h Dar mou h A D . om, n .
1. i iro N, Sousa S , r kk n A, on iro FJ. o o S A in on r mod in and an r-r a d on m as- asis. J io h m. Jan 1 11 1 .
. r k , o ds in AS. H drod nami sh ar s imu a s os- o a in r ssion u no ro i ra ion o on marro s ro- ma s. issu n . a -Jun 1 - .
. a J, aidi . o u ar r u a ion o m hano ransdu - ion. io h m io h s s ommun. a 1 1 .
. Wan , iu , h n J , uan S , h n . han s o os o a in and F- durin on n h nin . hin J rau- ma o . Jun 1- 1 3 1.