

NEOVASCULARIZATION AND MANDIBULAR CONDYLAR BONE REMODELING IN ADULT RATS UNDER MECHANICAL STRAIN

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1. ABSTRACT

The present study was designed to explore the relationship between neovascularization, hypertrophic cartilage and the microstructural properties of cancellous bone in adult rat's condyle in response to mechanical strain produced by mandibular advancement. Seventy-eight 120-day-old female Sprague-Dawley rats were randomly allotted to six groups, nine animals in each experimental group according to different time points. Mandibular advancement appliances were used to produce mechanical strain onto the mandibular condyles of rats. Immunostaining of VEGF and type X collagen were carried out. Tartrate-Resistant Acid Phosphatase (TRAP) reaction was used to assess the activity of chondroclasts. Direct three-dimensional morphometric analysis was carried out with microcomputed tomography (Micro-CT) scanning to evaluate the properties of microstructure of cancellous bone in the mandibular condyles. Results showed that mechanical strain produced by mandibular advancement induced neovascularization in the posterior condyle marked by the increased expression of VEGF. Neovascularization coupled the remodeling of calcified cartilage as marked by the expression of type X collagen and new bone formation. The new bone formed in the adult condyle was characterized by thinner trabecular thickness, more trabecular number and increased trabecular space. In conclusion, mechanical strain produced by mandibular advancement induces neovascularization and osteogenesis leading to adaptive growth of condyle in adult rats.

2. INTRODUCTION

Most vertebrate embryonic and post-embryonic skeletal tissue formation occurs through endochondral ossification in which cartilage serves a transitory role as the anlage for bone structure. Mandibular condyle, a major growth center of the craniofacial complex, undergoes

endochondral ossification during growth. However, endochondral ossification in the condyle may stop or become inactive in adults. It was reported that endochondral bone of the rat's condyle could be replaced by chondroid bone, a unique calcified tissue which has morphological properties intermediate between cartilage and bone, along with the increase of age (1). Recently, it was also shown that chondrocytes in the cartilage in the adult rats' condyle have only two to three layers and there is no obvious hypertrophic layer in the cartilage as revealed by the immunostaining of type X collagen (2). A possible explanation to the change of the histological picture with age could be attributed to the transformation of the role of condyle from growth center to articular function (3). Interestingly however, when mechanical strain produced by active mandibular forward positioning was brought onto the adult rats' condyles, endochondral ossification was reactivated in the posterior condyle as marked by the increase of expression of type II collagen and type X collagen. This reactivated endochondral ossification ultimately results in new bone formation in the condyles of adult rats (2).

Endochondral ossification is a multistep process that is controlled by an array of factors expressed by the chondrocytes (4). The last event of endochondral ossification is that avascular cartilage template is replaced by highly vascularized bone tissue. During this process, chondrocytes first become hypertrophic and produce calcified cartilaginous matrix and angiogenic stimulators, thus becoming a target for capillary invasion and angiogenesis (5). The vasculature provides a conduit for the recruitment of the cell types involved in cartilage resorption and bone deposition and provides signals necessary for normal morphogenesis (6). It was also reported that neovascularization is a crucial event in endochondral ossification responsible

for mandibular condylar growth (4, 7).

Vascular endothelial growth factor (VEGF) is a potent angiogenic peptide with specific mitogenic and chemotactic action on endothelial cells. In addition to the effects on endothelial cells, VEGF was also reported to indirectly induce proliferation and differentiation of osteoblasts. It was shown that VEGF is an essential coordinator of chondrocyte death, chondroclast function, extracellular matrix remodeling, angiogenesis and bone formation in growth plate (8). Mechanical strain produced by forward mandibular positioning couples the expression of VEGF and subsequently results in more new bone production in the condyles of young rats (7, 9). It could be postulated that VEGF plays similar role in the reactivated endochondral ossification produced by mechanical strain in adult rats. The present study was designed to investigate the neovascularization marked by temporal expression of VEGF in mandibular condyles of adult rats in response to mechanical strain.

During the process of neovascularization, hypertrophic cartilage with its matrix comprised of mainly type X collagen, acts as a target for capillary invasion and angiogenesis with the synthesis of angiogenic activators (5). Neovascularization is intimately linked to chondrocyte hypertrophy. When hypertrophy is inhibited, neovascularization and endochondral ossification are subsequently blocked (10). Thus, it is of great importance to assess chondrocyte hypertrophy while evaluating the neovascularization.

It is known that bone remodeling in the adult skeleton is carried out by groups of several types of cells which are referred to as basic multicellular units (11). The capillary at the heart of each unit is ideally situated to coordinate the coupling of formation to resorption (11). Therefore, bone metabolism or remodeling is closely related to neovascularization. However, little is known about the relationship between bone microarchitecture and neovascularization. Previously, it was reported that condylar cancellous bone in adults is composed of dense and compact trabecular bone (12); compared with younger age, the thickness of the trabeculae in the condyle increases with age and the trabeculae is fewer in number (13). Since there is close relation with the neovascularization and bone formation in mandibular condyle (7, 9), it is necessary to relate neovascularization to the change of microarchitecture of bone in response to the mechanical strain. Recently, a high resolution micro X-ray computed tomography (Micro CT) system was developed (14). Non-invasive high precision imaging and quantitative morphometry in three dimensions became available for the evaluation of change of microarchitecture of condylar cancellous bone.

Thus, the present study was designed: 1. to identify the expression of VEGF in adult rats condyle and correlate that to its expression in response to mechanical strain produced by mandibular forward positioning; 2. to identify the change in thickness of hypertrophic layer in the cartilage of adult rats condyle and to correlate that to the expression of VEGF; 3. to identify the change of

microarchitecture of condylar cancellous bone and to correlate to the neovascularization.

3. MATERIALS AND METHODS

Animal experiment was approved by the Committee on the Use of Live Animals in Teaching and Research of The University of Hong Kong (CULATER 586-01).

3.1. Animal experiment and tissue processing

Seventy-eight 120 days old Sprague-Dawley rats were randomly allocated into six groups. In each group, there were nine rats fitted with bite-jumping devices for mandibular advancement (15). Other four rats in each group served as controls. All animals were kept under standard conditions provided with water *ad libitum*, artificial light and grounded normal rat pellets (Laboratory Rodent Chow 5010, PMI Feeds Inc, St.Louis, USA) in the Laboratory Animal Unit of The University of Hong Kong. The appliances were fitted under anesthesia (10% ketamine and 2% xylazine, 2:1, 0.1ml/100gm). Light curing Panavia F (Kuraray Medical Inc., Japan) was used as bonding material to provide enough retention for appliance.

The animals in experimental groups and their matched controls were killed on days 3, 7, 14, 21, 30, 60 by intraperitoneal injection of 20% dormal (200mg/ml pentobarbital sodium, Alfasan). Immediately after death, the heads were skinned and fixed in 4% paraformaldehyde for 48 hours. The heads were then carefully dissected along the middle sagittal plane, and the temporomandibular joints on the left halves were harvested and decalcified with 20% EDTA. The specimens were then embedded in paraffin. Serial sections of 4µm were cut through sagittal plane and then mounted on glass slides. Neighboring sections were selected for the different methods of histological assessment. Right halves of mandible from days 30 and 60 group (four experimental and four control samples in each group) were free of soft tissue and served for specimens of micro-CT scan.

3.2. Histological and immunohistochemical staining

Tartrate-Resistant Acid Phosphatase (TRAP) activity was used for the identification of osteoclasts and was performed using a staining kit (Sigma, St Louis, USA) following the manufacture instruction. Immunostaining protocol followed previously described avidin-biotin complex (ABC) method (16). Two different primary antibodies (VEGF, goat polyclone, Santa Cruz Bio. Inc., A-20, 1:50; Type X collagen, mouse monoclonal, Quartett, 211406, 1:30) were used for the immunolocalization for VEGF and type X collagen respectively.

3.3. Microcomputed tomography (Micro-CT) scanning

The specimens were scanned using a microtomographic system (µCT-20; Scanco Medical AG, Zurich, Switzerland) in high resolution mode. To standardize specimens, condylar processes were separated from the mandibular skulls. The height of each specimen was about 5mm. The specimen was then inserted into a homemade columniform abutment (Figure 1). The abutment

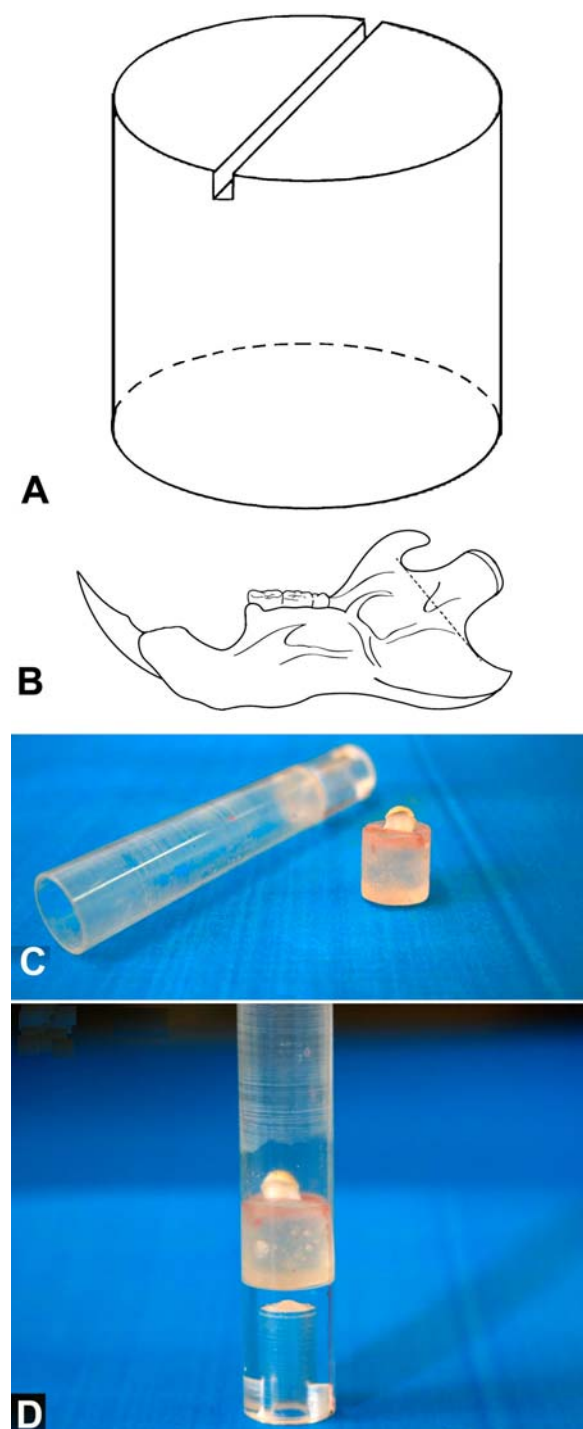


Figure 1. A homemade abutment (A) is used for fixing the specimens for micro-CT scan. Condylar process is cut from the mandible along the dotted line shown by (B) and is then inserted into the groove of the abutment in an identical direction (C). The abutment with the specimen is put into cylindrical sample holder (D) for micro-CT scan.

with specimen was then placed into an 11.3 mm cylindrical sample holder filled with paraformaldehyde for micro-CT scan (Figure 1). The groove of the abutment should be in

accordance with the marker line in the sample holder to make an identical direction for each specimen during scanning. For each specimen, the scanning began from scout view to determine the reference line. The first slice was selected at the section of the utmost point at the superior direction (Figure 2). From the reference line, 100 slices with an increment of $15\mu\text{m}$ were scanned. A cuboid ($726 \times 726 \times 451\mu\text{m}$) of volume of interest from slice 20th to 50th in the posterior condyle was selected for the model independent three-dimensional morphometric analysis. Fixed variables for the filter width (1.0), filter support (2.0) and the threshold (100) were used during the evaluation of all the specimens. Bone volume fraction (BV/TV), bone surface density (BS/BV), trabecular number (Tb.N*), trabecular thickness (Tb.Th*), trabecular separation (Tb.Sp*) and degree of anisotropy (DA) were selected for the evaluation of microstructure of the condylar cancellous bone in the present study.

3.4. Quantitative analysis and statistics

The thickness of type X collagen positive layer and area percentage of VEGF protein expression were quantified by a true-colour RGB computer-assisted image analyzing system with a digital camera (Leica DC 300 V 2.0, Wetzlar, Germany) and Leica Qwin V 2.4 software. The quantification was conducted at magnification of $\times 180$ and a fixed measurement frame of $1104 \times 811\mu\text{m}$ in the posterior condyle, where the newly formed cartilage was induced (2).

Statistical analysis was processed with SPSS for Windows (Release 11.0.0, standard version, SPSS Inc., Chicago, USA.) for one-way ANOVA with Bonferroni multiple comparisons test and Independent-Sample *t* test.

4. RESULTS

In adult rats, growth of the condyle was inactive; the expression of VEGF (Figure 3 A) and type X collagen (Figure 3 B) was weak. Bone remodeling at adult stage was not active as revealed by the TRAP activity (Figure 3 C). Mechanical strain produced by mandibular advancement led to an increased expression of VEGF (Figure 3 D, G, J) and type X collagen (Figure 3 E, H, K) in experimental adult rats. Active bone formation could be identified by the gradually increased TRAP activity in the posterior condyle (Figure 3 F, I, L).

Quantitative analysis showed that the expression of VEGF was significantly increased from day 14 to 30 compared with the expression in controls (Figure 4). The highest expression of VEGF was shown on experimental day 21 (Figure 4). The expression of type X collagen in the experimental groups showed similar pattern with the temporal expression of VEGF (Figure 5). The thickness of hypertrophic layer is significantly increased on experimental day 21 and day 30 (Figure 5).

Mechanical strain produced by mandibular advancement resulted in the remodeling of cancellous bone. During the process of remodeling, tomographic

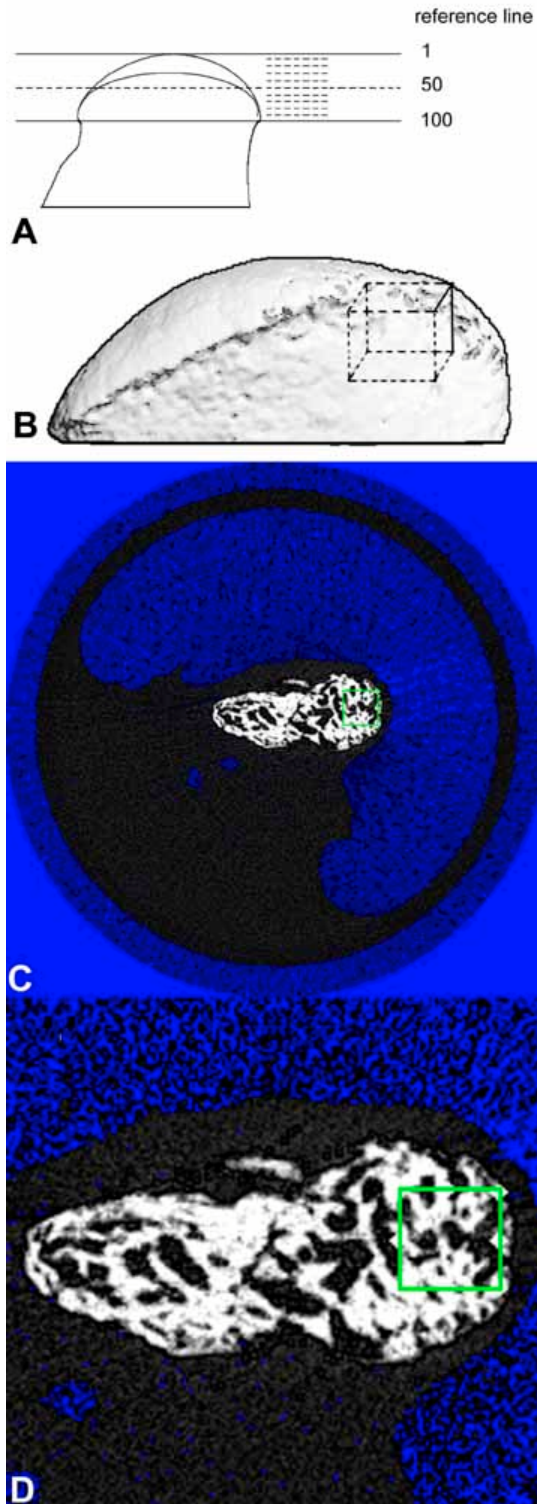


Figure 2. The reference line is selected from the section of the utmost point in the superior surface of condyle A) and a total of 100 sections are defined for micro-CT scan. A cuboid (726×726×451μm) of volume is segmented for 3D evaluation (B) by segmenting area of interest (green frame) in the posterior area of condyle in each section (C, D, D is the magnification of C)

properties of cancellous bone revealed significant changes in experimental day 30 as shown in Table 1. On experimental day 60, the variables returned to the level of controls. 3D reconstruction of the condylar head showed that there was new bone formation in the posterior condyle (Figure 6).

5. DISCUSSION

The present study was undertaken to evaluate the relationship between neovascularization, hypertrophic cartilage and the microstructural properties of newly formed cancellous bone in adult rat's condyle in response to mechanical strain produced by mandibular advancement. The results of the study showed that mechanical strain induced neovascularization in the posterior condyle marked by the increased expression of VEGF (Figure 3. D, G, J, Figure 4). Neovascularization coupled the remodeling of calcified cartilage as marked by the expression of type X collagen (Figure 3. E, H, K, Figure 5) and new bone formation (Figure 6). Such new bone was characterized by thinner trabecular thickness, more trabecular number and increased trabecular space (Table 1).

Endochondral ossification involves not only the processes of cellular differentiation but also the establishment and controlled resorption of cartilaginous matrix (17). During this process, mesenchymal cells proliferate and differentiate into chondrocytes. The chondrocytes synthesize cartilaginous matrix. The cartilaginous matrix in the cartilage is remodeled and calcified as the chondrocytes become hypertrophic. Eventually, the cartilage is resorbed during the process of neovascularization. Each differentiation step occurs in a precise temporo-spatial pattern and is controlled by the different molecular regulators (4). Neovascularization is the crucial step and marks the onset of ossification. It is known that VEGF is an essential mediator of neovascularization (18). Mechanical strain has recently been proved to up-regulate the expression of VEGF in different types of cultured cells (19-21). In chondrocyte culture model, cyclic tension was shown to activate the Cbfa/MMP13 pathway and increases the expression of terminal differentiation hypertrophy and terminal differentiation markers such as type X collagen and VEGF (22). In young rats, the expression of VEGF is up-regulated by mechanical strain and the expression is closely related with new bone formation in condyle and glenoid fossa in response to mandibular advancement (7, 23, 24). The present study showed significant increase in the expression of VEGF in experimental groups (Figure 3. D, G, J, Figure 4). This result is in accordance with the conclusion that genetic mechanisms regulating fetal skeletogenesis are also involved in regulating adult skeletal regeneration (25).

In agreement with a previous study in young rats (7), the expression of VEGF was limited to the hypertrophic layer. It has been known that the hypertrophic layer was either thinner or absent in the condylar cartilage



Figure 3. Mechanical strain induces the expression of VEGF (A, D, G, J), type X collagen (B, E, H, K) and TRAP activity (C, F, I, L). In the controls, the expression of VEGF (A) and type X collagen (B) is weak. Almost no TRAP activity can be identified in the posterior condyle. On experimental day 14, expression of VEGF increases (D), while the expression of type X collagen is still not obvious in the cartilage. At this time point, the cartilage layer becomes thicker (F). However, TRAP is not active at this stage. On experimental day 21, the thickness of cartilage layer in the posterior continually increases, and there is high expression of VEGF (G) and type X collagen (H). Underneath the hypertrophic chondrocyte, TRAP-expressing cells (dark purple, arrow showed) accumulate at site of erosive front (I). On experimental day 30, the cartilage layer is thinner than in day 21. The expression of VEGF maintains at a high level (J), as well as the expression of type X collagen. A large number of TRAP-expressing cells can be identified in the lacuna of erosive front underneath the cartilage (L).

of adult rats, thus it could explain the weak staining of VEGF and type X collagen in the controls and early phase of mandibular advancement in experimental animals. Earlier, VEGF has been considered to be an endothelial-specific factor based on the exclusive distribution of its tyrosine kinase receptors, VEGFR-1 (Flt-1) and VEGF-2

(Flk-1) in endothelial cells (26-28). While more recently, accumulating evidence showed that VEGF expression and VEGF receptors are present in non-endothelial tissues such as osteoblasts, chondrocytes, trophoblast cells and uterine smooth muscle cells. (29, 30). The role of VEGF in endochondral bone formation

Bone remodeling in adult condyles under force

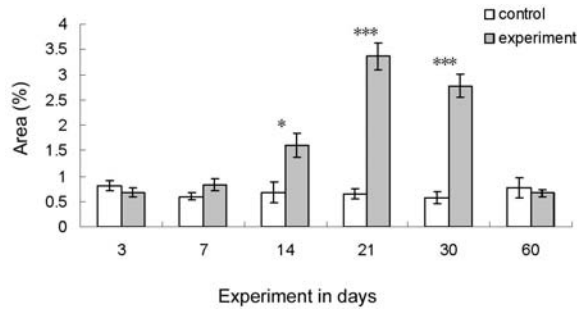


Figure 4. Graph showing the temporal expression of VEGF in the posterior condylar cartilage of control (n=4) and experimental (n=9) animals. Values are mean±SEM. Significant difference between control and experimental animals are marked with asterisks (* $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$).

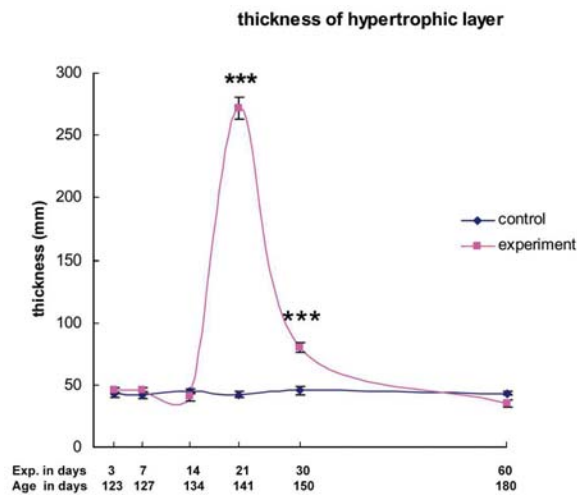


Figure 5. Graph showing the changes of the thickness of hypertrophic layer marked by expression of type X collagen in the posterior condylar cartilage of control (n=4) and experimental (n=9) animals. Values are mean±SEM. Significant difference between control and experimental animals are marked with asterisks (* $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$).

was reported to be involved in controlling chondrocyte proliferation and maturation, osteoblasts and chondroclast recruitment (8, 31). In the process of cartilage resorption, chondroclasts cooperate with the function of endothelial cells (32). Results of the current study showed that a number of TRAP positive cells (chondroclasts) were present in the erosive front of newly formed cartilage in the posterior condyle (Figure 3. I, L) on experimental days 21 and 30, when higher expression of VEGF was identified (Figure 4).

In the present study, the expression of type X collagen showed the similar pattern with expression of VEGF (Figure 5). In order to produce neovascularization, hypertrophic chondrocytes need to be in a correct extracellular matrix microenvironment. It was shown that single hypertrophic chondrocytes in a custom suspension have inhibitory effects on endothelial cell migration and

invasion. However, when the hypertrophic chondrocytes were transferred to the matrix resembling *in vivo* cartilage, the angiogenic activity resumed (33). This indicated that hypertrophic cartilage matrix could also play an important role in the process of neovascularization in response to the mechanical strain in adult rats.

Since endochondral ossification was reactivated in the posterior condyle of adult rats by mechanical strain, it is necessary to investigate the bony structure of newly formed bone. In this study, we used a direct three-dimensional morphometric analysis to explore the structure of cancellous bone in the condyles. Traditional three-dimensional morphometric indices are derived from 2D images. Such method should assume a fixed structural model such as plate models or rod models. It has been known that trabecular bone might change its structure type continually due to aging or disease (34). The advantage of direct three-dimensional morphometric analysis is the possibility of exploring the microarchitecture of cancellous bone directly without making assumptions on the structure type (34). Results of such analysis showed that values of trabecular number (Tb.N*), trabecular space (Tb.Sp*) and bone surface density were higher than controls on experimental day 30, while the values of bone volume ratio (BV/TV) and trabecular thickness were lower than that of controls at the same time point. On experimental day 30, the degree of anisotropy (DA) presented lower values in experimental samples. This implies that the trabecular bone in experimental group revealed more plate-like mode (35). All the values returned to the control levels on experimental day 60. Thus, the reactivation of new bone formation was a transient stage that led to osteogenesis on experimental day 30. Since mechanical strain induced neovascularization in the posterior condyle and resulted in new bone formation (Figure 6), it is not surprising that there were more trabecular bone and higher bone surface density. Along with the increase of neovascularization, more blood vessels invaded, thus, there was more marrow space seen in experimental groups, which was presented by the increase of trabecular space (Table 1). The character of bony structure in adult rats has been described previously. The subchondral bone is very dense and compact at 4 months of age in rat's condyle (12). There are fewer trabecular bone number and few marrow space than that of growing animal (12). Thus, the property of bony structure of newly formed bone presented on experimental day 30 reveals some characteristics of the bone presented in younger age condyles.

6. CONCLUSION

Mechanical strain produced by mandibular advancement induces neovascularization and osteogenesis leading to adaptive growth of condyle in adult rats.

7. ACKNOWLEDGMENTS

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Table 1. Values of mandibular condylar cancellous bone morphometry (Mean±SD) in experimental and control groups

	30 days			60 days		
	Experiment	control	difference	experiment	control	difference
BV/TV(ratio)	0.812±0.107	0.967±0.019	-0.155 ¹	0.826±0.119	0.914±0.047	-0.088 ns
Tb.N ¹ (mm ⁻¹)	11.212±1.887	8.341±0.494	2.871 ¹	9.451±0.349	9.382±0.303	0.069 ns
Tb.Th ¹ (mm)	0.104±0.013	0.152±0.010	-0.048 ²	0.115±0.019	0.136±0.005	-0.021 ns
Tb.Sp ¹ (mm)	0.080±0.014	0.035±0.003	0.045 ²	0.059±0.002	0.045±0.014	0.014 ns
BS/BV(mm ⁻¹)	9.797±1.271	5.139±1.754	4.658 ²	9.129±2.272	6.408±1.225	2.721 ns
DA (ratio)	1.383±0.130	3.146±0.581	-1.753 ²	2.507±0.312	3.016±0.333	-0.509 ns

¹ P < 0.05, ² P < 0.01, ³ P < 0.001 (experiment VS. control) ns, not significant

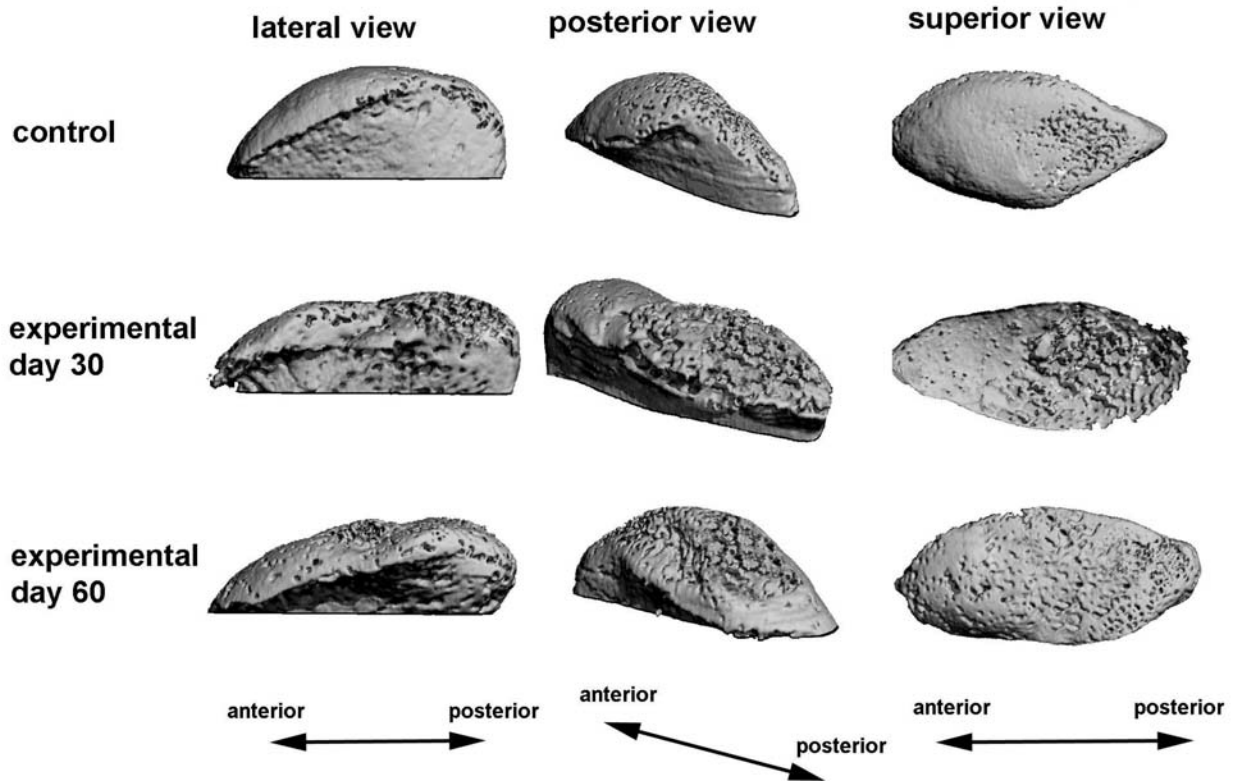


Figure 6. Three-dimensional reconstruction of mandibular condylar head of adult rats. From different views, it can be noticed that mechanical strain induces new bone formation in the posterior condyle and subsequently changes the shape and size of condylar head.

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Key Words: Mechanical strain, Condylar growth, Adult, VEGF, Micro-CT, Type X collagen

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