Histological Alterations from Condyle Repositioning with Functional Appliances in Rats

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Objective: *This study was designed to assess the morphological and histological alterations of the condyle of rats undergoing forward mandibular repositioning via functional appliance.*

Materials and Methods: *Functional appliances were mounted onto the upper jaws of rats. Morphological analysis was conducted on micro-CT images of sacrificed animals. Histological changes in condyle were examined by immunohistochemistry using proliferating cell nuclear antigen (PCNA), matrix metalloproteases (MMPs), vascular endothelial growth factor (VEGF), tissue inhibitors of matrix metalloproteinases (TIMP-1), interleukin 1b (IL-1β), Aggrecan and Type II collagen. Osteoclast activity was identified by tartrate-resistant acid phosphatase (TRAP) staining.*

Results: *Morphological analysis confirmed the forward positioning of the condyles of rats by the appliance, but the position gradually returned to normal on days 14 after treatment. An increase in PCNA positive cells was observed in the posterior region of the condyles on days 7, whereas PCNA positive cells decreased in the anterior region. Aggrecan and Type II collagen localization increased in the posterior region throughout the entire period, but decreased in the anterior region on days 14. In both regions, IL-1β and VEGF localization was significantly increased for 14 days while MMPs localization was evident throughout the entire period. The TRAP positive cells were significantly elevated on days 3 and 7.*

Conclusions: *These results suggest that the functional appliance therapy induces significant morphological and histological changes in the anterior and posterior regions of the condyle and subsequently causes adaptive cellular functions such as chondrocyte differentiation and cartilage matrix formation.*

Keywords*: functional appliance; mandibular condyle; histological analysis; cartilage*

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INTRODUCTION

Mandibular retrusion is considered the most common characteristic of Class II malocclusion in the orthodontic patient population.[1] Orthodontists have widely utilized mandibular advancement appliances for growth modification of skeletal Class II malocclusion.[2,3] These appliances stimulate mandibular growth by forward positioning of the mandible to achieve normal jaw relationship in growing patients with recessive mandibular growth, but the effects of this therapy on the articular tissues of the temporomandibular joint (TMJ) remain unclear.[4,5] Previous studies have demonstrated skeletal mandibular changes in response to forward mandibular positioning in animal models,[6-8] while the observations in human studies are not conclusive.[9,10]

TMJ condylar cartilage is composed mainly of chondrocytes and extracellular matrix (ECM), consisting of fibrous elements and proteoglycans. Type II collagen, the major fibrous element of the condylar cartilage, forms the framework of the cartilage matrix and gives the tissue its elasticity,[11] while Aggrecan, the major large aggregated proteoglycan in cartilage, gives the tissue its resilience.[12] The expression levels of these two critical ECM components have reportedly been altered following the application of different mechanical stimuli.[13,14] Matrix metalloproteases (MMPs) are considered to be key enzymes in the degradation of the ECM as they breakdown the major components of the ECM.[15] Conversely, tissue inhibitors of matrix metalloproteases (TIMPs) are the natural endogenous inhibitors of MMPs through direct binding to MMPs.[16] Therefore, the imbalance between MMPs and TIMPs has been considered to be a major factor in the development of progressive joint destruction.[17,18]Interleukin 1b (IL-1β) reduces matrix production, diminishes chondrocyte proliferation, and stimulates the chondrocytes to release proteases responsible for cartilage degradation such as MMPs.[19] Vascular endothelial growth factor (VEGF) also regulates the production of MMP and its tissue-inhibitors.[20]

Histological and biochemical research in this field mainly aims to provide basic information about the nature of skeletal growth modification in response to functional appliance therapy. In this study, we introduce an animal model to reproduce the orthopedic effects of functional appliance therapy and define the cytological response and histological alteration of condyle such as chondrocyte proliferation, matrix formation, and immune response, following mandibular advancement in rats.

MATERIALS AND METHODS

Experimental Model

The animal protocol was approved by the Animal Ethics Committee of Kyung Hee University. Twenty-seven 5-week-old male Sprague-Dawley rats (Hanlim Inc., Seoul, Korea, body weight 350 g) were randomly divided into experimental (n=15) and control (n=12) groups. 5-week-old rats are comparable to humans in early puberty.[21] The experiment was conducted over a period from early puberty (5-weeks-old) to young adulthood (9-weeks-old), including the histological growth peak of the condyle (7-weeks-old).[12,22] All rats were kept in the same, well-controlled temperature and humidity environment. A soft diet with constant water supply was maintained throughout the experimental period.

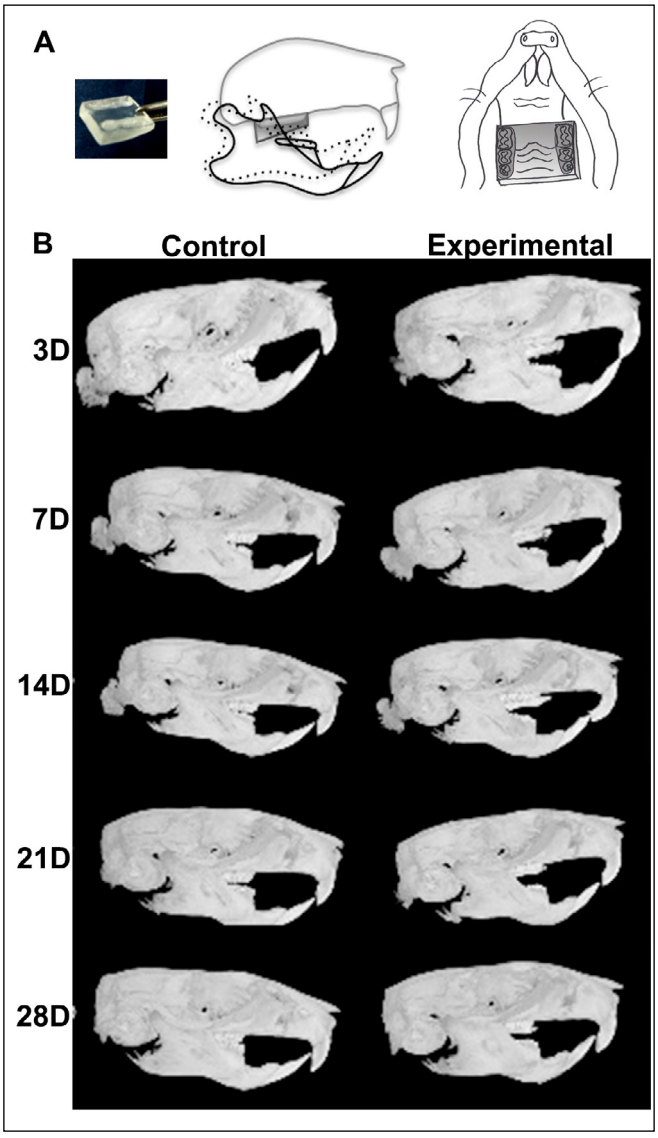
For the experimental mandibular advancement model, the dimensions of the functional appliances were based on the initial measurements (width 8 mm, length 8 mm, groove 0.8 mm). The functional appliances were designed with 2 mm maximum thickness. Grooves were made in the palatal side of the appliances for maxillary left and right molars. There was a freeway space in a tip-to-tip position of the incisors using an acrylic resin block (Figure 1A).

The appliances were placed under a 50 mg/kg intraperitoneal injection of pentobarbital sodium anesthesia. They were fixed on each rat's upper jaw using Fuji Cement (GC, Japan) in such a way that they caused mandibular forward-downward positioning while the rats were at rest and a functional bite (Figure 1A). Body weight was monitored throughout the experiment.

Micro-CT

The heads of sacrificed rats were fixed with 10% formalin and scanned using a micro-CT system (Sky-Scan 1172TM; Skyscan, Kontich, Belgium) at 60 kV and 167 μA. The image data was reconstructed using Realistic 3D-Visualization (Skyscan, Kontich, Belgium) to create three dimensional images.

Figure 1. Morphological analysis (A) Experimentally induced mandibular advancement using functional appliances. (B) Micro-CT images of the head.



Histologic Tissue Preparation

The animals were anesthetized and perfused transcardially with 10% formalin, and then the heads were immediately dissected and immersed in the same fixative overnight at 4°C. The specimens were decalcified in 10% EDTA (pH 7.4) for 6-8 weeks and then were dehydrated and embedded in paraffin. Each sample was cut sagittally into 7 μm serial sections and prepared for hematoxylin & eosin (H&E) and immunohistochemistry staining.

Immunohistochemistry and TRAP assay

For immunohistochemistry, a Vectastain ABC kit (Vector Laboratories, Inc., Burlingame, CA) was used and all procedures were performed according to the manufacturer's instructions. Each section was incubated with anti-PCNA antibody (Serotec, Kidlington, UK), anti-MMP3, anti-MMP9, anti-MMP13, anti-aggrecan (Abcam, Cambridge, UK), anti-TIMP1 (Origene, MD, USA), anti-VEGF, anti-col-II, and anti-IL-1β (Santa cruz, CA, USA) antibody for 30 minutes at room temperature. Negative controls were conducted in the absence of the primary antibody. The sections were incubated in peroxidase substrate solution (SK-4100, Vector laboratories, Inc., Burlingame, CA) until the desired stain intensity had developed. The sections were counterstained with hematoxylin and mounted. To evaluate the osteoclast activity, each slide was stained for TRAP (Sigma, MO, USA) as described in a previous study.[23] TRAP positive cells with three or more nuclei were counted as multinuclear osteoclasts. One examiner blindly performed quantitative analysis at three separate times to eliminate inter-examiner errors and bias.

RESULTS

Morphological change in the mandible

Micro-CT images showed that the condyle was displaced more anteriorly and mandible was positioned more forward and downward following functional appliance therapy. Edge-to-edge occlusion was observed on day 3, shortly after the experimental group was subjected to functional appliances. The lower incisors were progressively displaced in a backward position on days 7, 14, and 21. Morphologic response to mandibular protrusion was most evident on day 28, when normal anterior occlusion was observed (Figure 1B).

Histological changes in the condyle

The cellular structure of condyle is divided into four layers: fibrous, proliferative, hypertrophic, and bone layer.[14] Condyles in the experimental groups experienced histomorphological changes throughout the entire experiment. Cellular response to forward mandibular positioning was most evident on day 7, in which experimental rats exhibited a significantly flattened shape in the anterior and posterior regions of the condyle compared to rats undergoing natural growth. Furthermore, progressive thinning of the proliferative and hypertrophic layers in both regions of the condyle was observed on days 3 and 7, where day 7 marked the most significant decrease in size. Results from days 21 and 28 revealed condylar morphology and cartilage structure of experimental rats to be comparable to those of the control group in both anterior and posterior regions of the condyle (Figure 2A'-K' and a'-j').

Cellular events in response to mandibular protrusion, such as cell proliferation, was identified via PCNA positive cells. During natural growth, PCNA positive cells were localized mainly in the proliferation layer and bone marrow region of the condyle. During mandibular protrusion, PCNA positive cells was significantly decreased in the anterior region of the condyle from days 3 to 7, followed by a gradual increase to normal natural growth levels on days 14, 21, and 28. In contrast, the posterior region of the experimental condyle exhibited high levels of PCNA positive cells on days 3 and 7, followed by a gradual decrease to normal natural growth levels on days 14, 21, and 28 (Figure 2A-K and a-j).

Aggrecan positive cells appeared as a curved band covering the area of articular cartilage. During natural growth, aggrecan positive cells were clearly identified to reside in the transitional and hypertrophic layers of the condyle. During mandibular protrusion, aggrecan localization in the anterior region gradually diminished from days 3 to 14 in the hypertrophic layer near the proliferative layer. Days 21 and 28 revealed a marked increase in aggrecan positive cells in both transitional and hypertrophic layers. Chondrocyte enlargement was also observed in the hypertrophic region during this time. Similarly, Type II collagen exhibited an immunohistochemical staining profile similar to aggrecan. Type II collagen localization in the experimental group continually decreased from days 3 to 14 in the anterior region of the condyle, but gradually recovered to normal control levels until the end of the experiment. The highest amount of Type II collagen localization was observed on day 14 in the posterior region. Localization markedly increased in the transitional layer and hypertrophic layer adjacent to the proliferative layer (Figure 3).

VEGF and IL-1β localization was poorly indicated during natural growth in the proliferative and transitional regions of the condyle. Mandibular protrusion led to a significant increase in both markers from days 3 to 14 in the anterior and posterior regions of the condyle, followed by a gradual decrease to normal natural growth levels on days 21 and 28 (Figure 4). The strong localization was observed on day 14 in the experimental group, wherein intensification markedly increased in the proliferative and transitional layers of the condyle.

MMPs 3, 9, and 13 were weakly identified in the condyles of rats undergoing natural growth. Similarly, TIMP-1 positive cells displayed minimal localization in both the control and experimental groups (Figure 5). During mandibular protrusion, however, intensive localization of MMP 3, 9, and 13 in the anterior and posterior regions of the condyle was observed, especially in the proliferative and transitional regions throughout the entire experimental period.

TRAP positive cells, identified to measure osteoclastic activity, were observed mainly in the transitional region between the hypertrophic layer and bone during natural growth. During mandibular protrusion, TRAP positive osteoclast significantly increased on days 3 and 7, followed by a gradual decrease to the normal natural growth levels on days 14, 21, and 28 (Figure 6).

DISCUSSION

While mandibular repositioning appliances are a treatment of choice for growth modification of Class II skeletal patients with mandibular retrognathism, a clear understanding of their biologic effects on the condyles is largely lacking. Therefore, this study was designed to examine the histological changes on the condyles using a mandibular advancement animal model. Micro-CT imaging revealed active mandibular forward positioning with our fixed

Figure 2. Histological examinations (A-K and a-j) and PCNA localization (A'-K' and a'-j') in condyle after appliance attachment. (a-j and a'-j') Higher magnification views of anterior and posterior regions in G-K and G'-K'. Asterisks indicate the thinned cartilage.

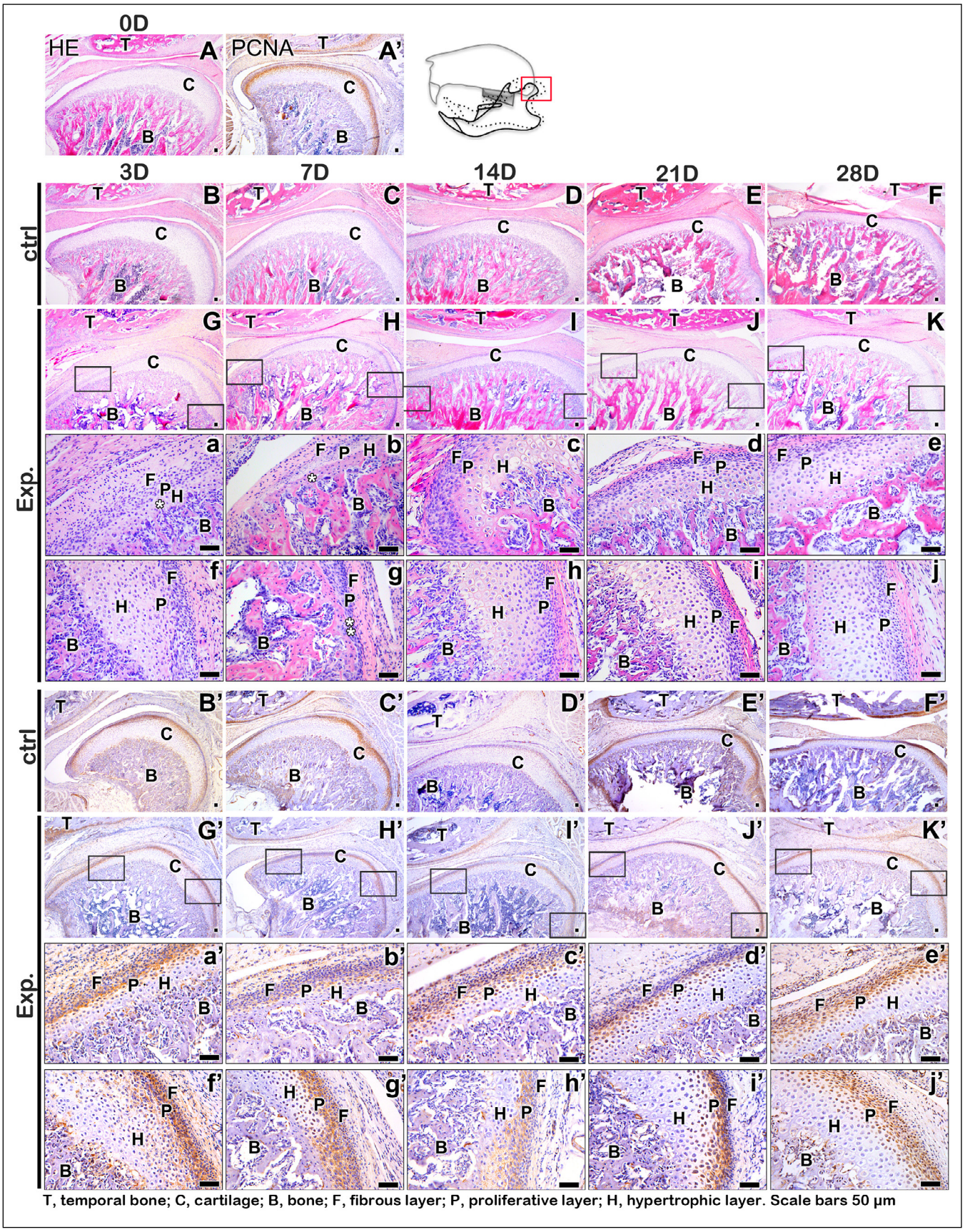


Figure 3. Localization of Aggrecan (A-K and a-j) and type II collagen (A'-K' and a'-j') after appliance attachment. (a-j and a'-j') Higher magnification views of anterior and posterior region in G-K and G'-K'.

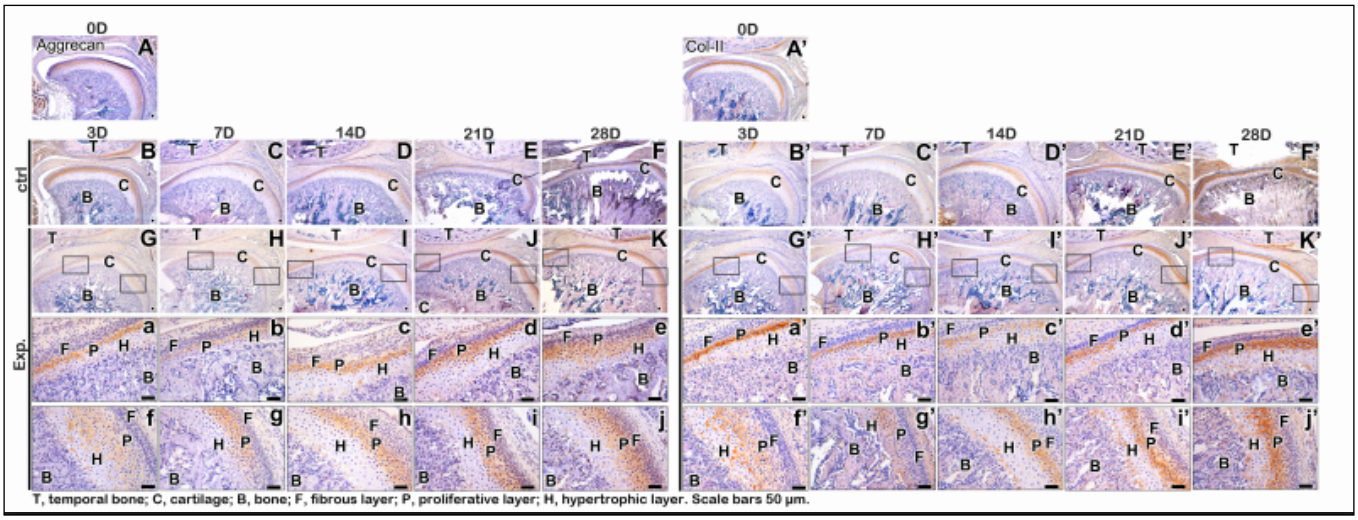
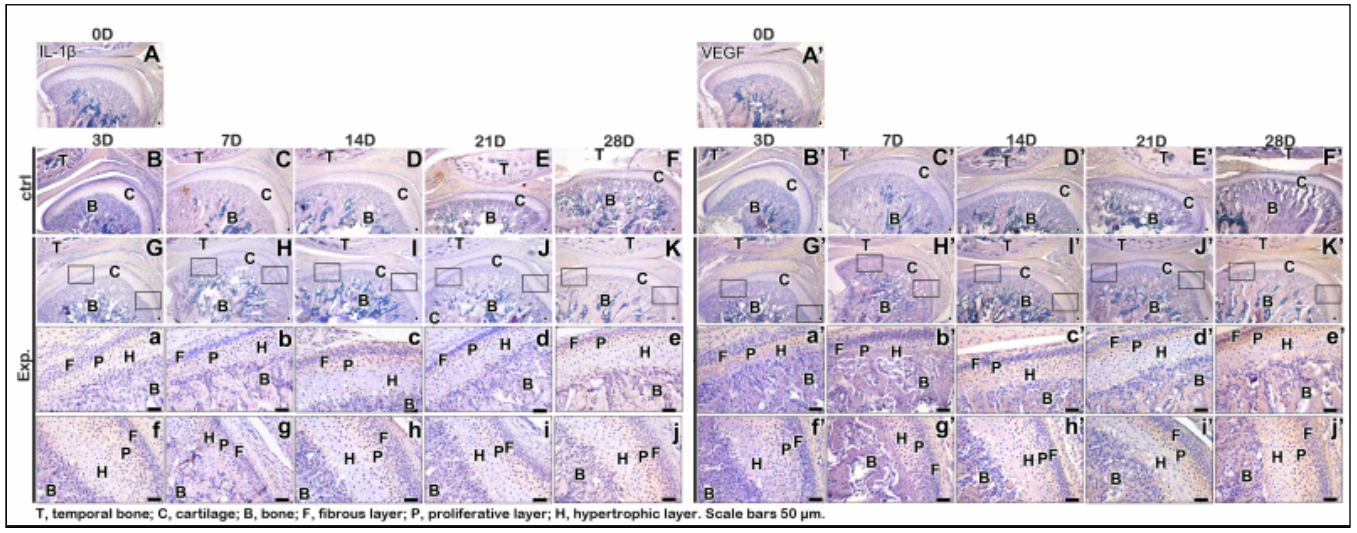


Figure 4. Localization of IL-1β (A-K and a-j) and VEGF (A'-K' and a'-j') after appliance attachment. (a-j and a'-j') Higher magnification views of anterior and posterior region in G-K and G'-K'.



functional appliance for the rats. Histological analysis showed that the appliance resulted in a compression of the anterior region and a tension of the posterior region of the condyle, leading to histomorphological and cytological changes in these regions.

The histomorphological findings demonstrate that cellular events in the posterior region of cartilage, such as proliferation, are in fact affected by biomechanical force. Normal mandibular movement was restricted at first by the appliance, but the mandibular position was considered to be adapted anteriorly and posteriorly between days 3 and 7. After adaptation, the compressive force may be reduced and may become a normal force, which increases cartilage volume. As previously reported by Rabie et al.,[8] our findings confirmed the enhanced localization of Type II collagen with mandibular advancement. Furthermore, our study examined another important component of cartilage, Aggrecan, and found that Aggrecan displayed a similar localization to Type II collagen, together accelerating and promoting chondrocyte differentiation and cartilage matrix formation in the posterior region of the condyle. Our experimental group revealed that biomechanical force resulted in destruction of hypertrophy layer and the transitional region between hypertrophy and bone layers on days 3 and 7 in both the anterior and posterior regions. After adaptation, this destruction caused an increase in Aggrecan and Type II collagen expression levels.

In this study, we considered an immune response to be a possible mechanism for the alterations of transitional regions with mandibular advancement. Cytokines such as IL-1β mediate mainly destructive and inflammatory reactions.[24] VEGF is produced by hypertrophic chondrocytes as well as osteoarthritis and mechanical overload is one of the factors responsible for VEGF induction in chondrocytes. Another study by Rabie et al.[6] identified VEGF induction as a facilitator in bone growth in the glenoid fossa during functional appliance therapy. However, in this study, we report that increased VEGF expression in our experimental group contributed to MMP induction and TIMP-2 reduction in chondrocytes.[20] Therefore, the mechanical load from the functional appliance upregulated VEGF and IL-1 β which then stimulated MMP-9 and 13 expression for cartilage breakdown.

Figure 5. Localization of MMP3 (A-K and a-j), MMP9 (A'-K' and a'-j'), MMP13 (A''-K'' and a''-j''), and TIMP-1 (A'''-K''' and a'''-j''') after appliance attachment. (a-j, a'-j', a''-j'', and a'''-j''') Higher magnification views of anterior and posterior region.

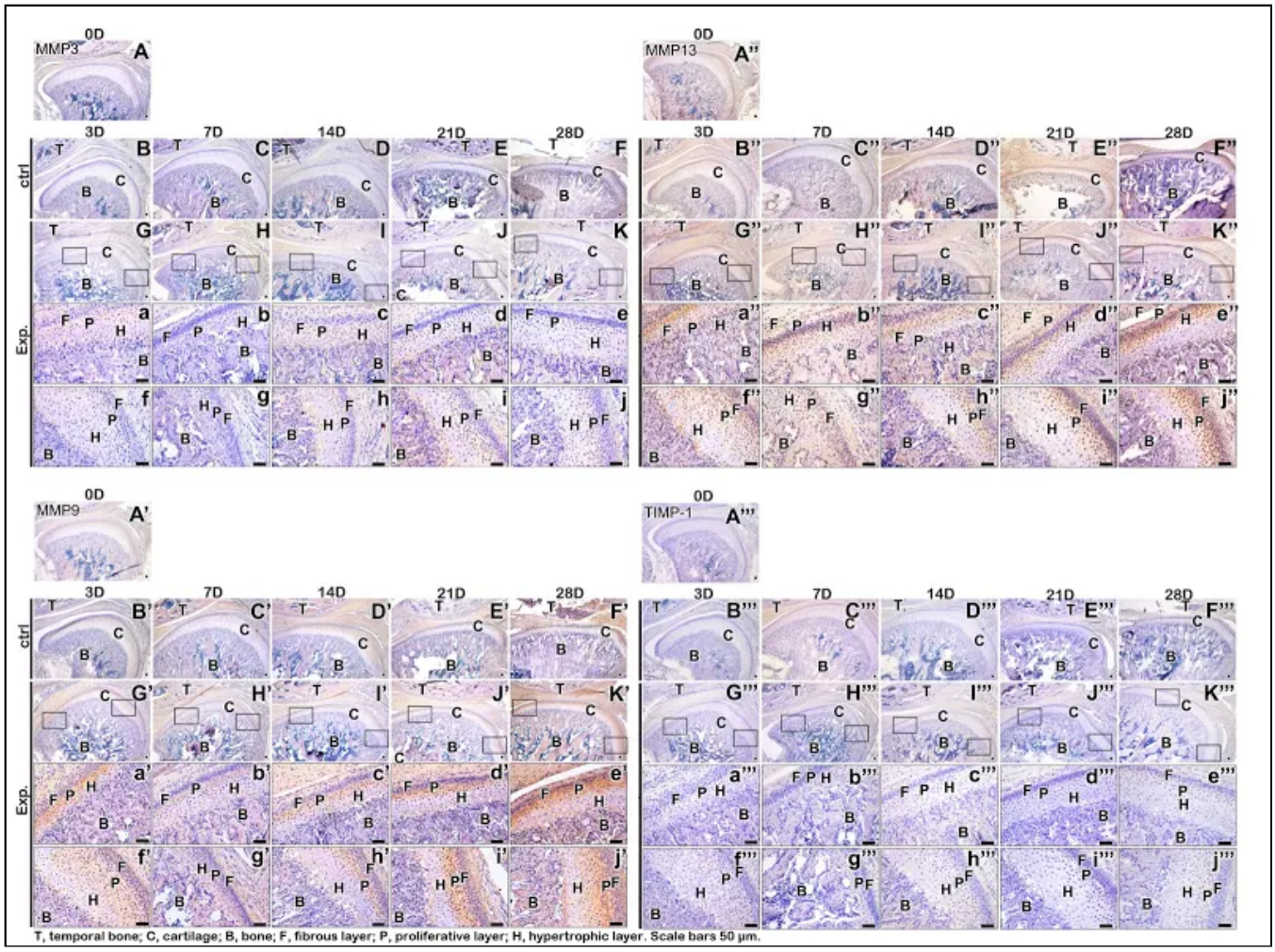
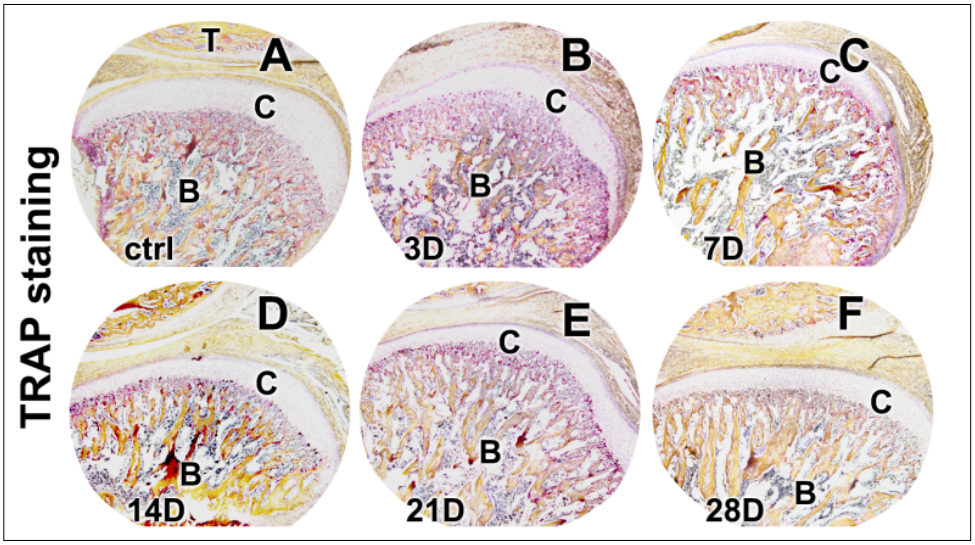


Figure 6. TRAP staining of condyle head at 0 (5 wks), 3, 7, 14, 21, and 28 days after appliance attachment.



TRAP staining further demonstrated the localization of osteoclasts in the transitional region between hypertrophic layer and bone beneath the thinned cartilage, suggesting a close relationship to the increased mechanical load. These results suggest that a retraction of the transitional region might have been caused by the osteoclastic activity.

From these results, we suggest that biomechanical stimuli from the functional appliance immediately disrupted the structural homeostasis of the transitional region and was accompanied by cellular events; however, the cellular events returned to nearly normal after 14 days of functional appliance therapy. These findings highlight the adaptive ability of the condylar head to mandibular advancement. As 14 days in rats are equivalent to approximately 2 years in humans,[21] applications in clinical orthodontics can be modified to apply step-by-step activation of functional appliance therapy.

In summary, this experimental animal model provides a reproducible method to elucidate histological and biochemical changes induced by functional appliances. In addition, this experiment indicated that mandibular forward positioning caused histological changes in the condyle of rats that leads to condylar and mandibular adaptation. Although this study provides scientific evidence supporting previous clinical case studies which only reported morphological results,[1-5] further research, including genetic and molecular studies, are recommended for a thorough understanding of biological effects of functional appliance therapy to support its clinical use.

CONCLUSIONS

After appliance attachment, compression force in anterior regions of condyle induced immune responses, showed IL-1β, VEGF, and MMPs localization, whereas tension force in posterior regions induced PCNA, Aggrecan, and Type II collagen localization by chondrocyte differentiation and cartilage matrix formation in the condyle. TRAP-positive cells were clearly present in the transitional region on days 3 and 7 and accompanied the thinning of condylar cartilage. Considering that in response to mandibular advancement the initial histochemical changes in the condyle lessen over time, it could also be reasoned that step-by-step activation of a functional appliance should cause more cellular and molecular responses and ultimately result in more significant mandibular morphological changes. However, more studies are required to evaluate this phenomenon.

REFERENCES

1. McNamara JA Jr. Components of class II malocclusion in children 8-10 years of age. Angle Orthod 51: 177-202, 1981.
2. Pancherz H, Ruf S, Kohlhas P. "Effective condylar growth" and chin position changes in Herbst treatment: a cephalometric roentgenographic long-term study. Am J Orthod Dentofacial Orthop 114: 437-446, 1998.
3. Ruf S, Pancherz H. The mechanism of Class II correction during Herbst therapy in relation to the vertical jaw base relationship: a cephalometric roentgenographic study. Angle Orthod 67: 271-276, 1997.
4. Peltola JS, Könönen M, Nyström M. A follow-up study of radiographic findings in the mandibular condyles of orthodontically treated patients and associations with TMD. J Dent Res 74: 1571-1576, 1995.
5. Peltola JS NM, Könönen M, Wolf J. Radiographic structural findings in the mandibular condyles of young individuals receiving orthodontic treatment. Acta Odontol Scand 53: 85-91, 1995.
6. Rabie AB, Shum L, Chayanupatkul A. VEGF and bone formation in the glenoid fossa during forward mandibular positioning. Am J Orthod Dentofacial Orthop 122: 202-209, 2002.
7. Sobue T, Yeh WC, Chhibber A, Utreja A, Diaz-Doran V, Adams D, Kalajzic Z, Chen J, Wadhwa S. Murine TMJ loading causes increased proliferation and chondrocyte maturation. J Dent Res, 90: 512-516, 2011.
8. Rabie AB, She TT, Hägg U. Functional appliance therapy accelerates and enhances condylar growth. Am J Orthod Dentofacial Orthop 123: 40-48, 2003.
9. Ruf S, Pancherz H. Temporomandibular joint remodeling in adolescents and young adults during Herbst treatment: A prospective longitudinal magnetic resonance imaging and cephalometric radiographic investigation. Am J Orthod Dentofacial Orthop 115: 607-618, 1999.
10. Bendeus M, Hägg U, Rabie B. Growth and treatment changes in patients treated with a headgear-activator appliance. Am J Orthod Dentofacial Orthop 121: 376-384, 2002.
11. Kuroda S, Tanimoto K, Izawa T, Fujihara S, Koolstra JH, Tanaka E. Biomechanical and biochemical characteristics of the mandibular condylar cartilage. Osteoarthritis Cartilage 17: 1408-1415, 2009.
12. Wattanachai T, Yonemitsu I, Kaneko S, Soma K. Functional lateral shift of the mandible effects on the expression of ECM in rat temporomandibular cartilage. Angle Orthod 79: 652-659, 2009.
13. Kantomaa T, Pirttiniemi P. Changes in proteoglycan and collagen content in the mandibular condylar cartilage of the rabbit caused by an altered relationship between the condyle and glenoid fossa. Eur J Orthod 20: 435-441, 1998.
14. Teramoto M, Kaneko S, Shibata S, Yanagishita M, Soma K. Effect of compressive forces on extracellular matrix in rat mandibular condylar cartilage. J Bone Miner Metab 21: 276-286, 2003.
15. Reynolds JJ. Collagenases and tissue inhibitors of metalloproteinases: a functional balance in tissue degradation. Oral Dis 2: 70-76, 1996.
16. Nagase H, Woessner JF Jr. Matrix metalloproteinases. J Biol Chem 274: 21491-21494, 1999.
17. Giannelli G, Erriquez R, Iannone F, Marinosci F, Lapadula G, Antonaci S. MMP-2, MMP-9, TIMP-1 and TIMP-2 levels in patients with rheumatoid arthritis and psoriatic arthritis. Clin Exp Rheumatol 22: 335-338, 2004.
18. Jackson C, Nguyen M, Arkell J, Sambrook P. Selective matrix metalloproteinase (MMP) inhibition in rheumatoid arthritis-targetting gelatinase A activation. Inflamm Res 50: 183-186, 2001.
19. Von den Hoff JW, Delatte M. Interplay of mechanical loading and growth factors in the mandibular condyle. Arch Oral Biol 53: 709-715, 2008.
20. Pufe T, Harde V, Petersen W, Goldring MB, Tillmann B, Mentlein R. Vascular endothelial growth factor (VEGF) induces matrix metalloproteinase expression in immortalized chondrocytes. J Pathol 202: 367-374, 2004.
21. Quinn R. Comparing rat's to human's age: how old is my rat in people years? Nutrition 21: 775–777, 2005.
22. Lee KY, Park JH, Tai K3, Chae JM. Treatment with Twin-block appliance followed by fixed appliance therapy in a growing Class II patient. Am J Orthod Dentofacial Orthop 150: 847-863, 2016.
23. Jung JK, Sohn WJ, Lee Y, Bae YC, Choi JK, Kim JY. Morphological and cellular examinations of experimentally induced malocclusion in mice mandibular condyle. Cell Tissue Res 355: 355-363, 2014.
24. Kubota E, Imamura H, Kubota T, Shibata T, Murakami K. Interleukin 1 beta and stromelysin (MMP3) activity of synovial fluid as possible markers of osteoarthritis in the temporomandibular joint. J Oral Maxillofac Surg 55: 20-27, 1997.