**Effects of loading on chondrocyte hypoxia, HIF-1 α and VEGF in the mandibular
condylar cartilage of young rats**

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Objectives: To investigate HIF-1 α and VEGF expression under altered loading, and to explore the relationship between loading and hypoxia in the mandibular condylar cartilage of young rats.

Setting and Sample Population: The sample comprised of eighty Sprague-Dawley rats.

Material and Methods: The reduced loading group was fed soft food, and their incisors were cut to avoid occlusal contact. The increased loading group was fed hard food and had forced jaw-opening. Ten rats from each group (n=10) were sacrificed at 12, 24, 48, and 96 hours after initiation of the experiment. Pimonidazole hydrochloride (Hypoxyprobe-1, HP-1) was used as a hypoxia marker to confirm the hypoxic state. Hypoxic chondrocytes as indicated by HP-1, HIF-1 α and VEGF protein expressions were recognized by immunohistochemical detection. HIF-1 α and VEGF mRNA expressions were detected by semi-quantitative RT-PCR.

Results: HP-1 was confined in the upper layers of cartilage, and was most strongly expressed in the weight-bearing area of TMJ at 12 and 96 hours. Staining of HIF-1 α and VEGF was most strongly expressed in the chondrocytes of the fibrous and proliferative layer at all time points. Furthermore, expressions were also displayed in the hypertrophic and calcified layers at 48 and 96 hours. The expressions of HIF-1 α and VEGF mRNA were higher in the increased loading group than in the reduced loading group at 48 and 96 hours (p<0.05).

Conclusion: Mechanical loading seems to directly induce weight-bearing area hypoxia followed by new vessel formation, which indicates that these factors are related and important for the development of cartilage.

Key words: condylar cartilage, hypoxia, loading, HIF-1 α , VEGF

Introduction

Condylar cartilage is a primary part and an important growth site of the temporomandibular joint (TMJ).[1] Without direct blood supply, articular cartilage is avascular. [2] Thus, all nutrients, including oxygen and glucose, are diffused to the chondrocytes from surrounding sources. There are two possible routes for TMJ cartilage to obtain oxygen and other nutrients: diffusion of joint fluid from the articular surface, and transudation from the subchondral bone on the deep face. The possibility is that both co-exist, but there is controversy about the relative importance of these two routes.[3,4] Loading is an important factor for TMJ, and appropriate loading can promote the metabolism of the chondrocytes, while excessive or reduced loading leads to degenerative processes in cartilage.[5] Numerous studies have been performed to examine the oxygen pathway and the effects of loading on TMJ cartilage separately.

Hypoxia is known to be a common feature of solid tumors and has been regarded as a significant target of tumor therapy.[6] Various techniques are capable of measuring oxygen in tumor tissue, including needle electrode, imaging methods (PET, MRI), immunohistochemical method, and DNA strand break analysis.[7,8] The immunohistochemical method, which is widely used to detect hypoxia in patient biopsies, is basically based on antibody binding to bioreductive nitroimidazole compounds (e.g., pimonidazole, CCI-103F, and EF5).[9] These materials have been applied in normal tissue studies.[10,11]

The aim here was to investigate HIF-1 α and VEGF expression under altered loading, and explore the relationship between loading and hypoxia. We hypothesized that cartilage may acquire oxygen from the subchondral bone followed by angiogenesis under loading, and

that the increased loading group needs more oxygen than the reduced loading group.

Material and Methods:

Animal treatments

A total of 80 Sprague-Dawley rats (obtained from the Experimental Animal Center of Guangxi Medical University and approved by the Research Ethics Boards) were randomly divided into two groups after weaning at 21 days of age. The reduced loading group (n=40) was fed soft (powder) food, and their incisors were shortened each day to avoid occlusal contact. The increased loading group (n=40) was fed hard (pellet) homogeneous food and had forced jaw-opening (10 mm) for an hour each day under anesthesia by chloral hydrate intraperitoneally (0.3 mg/100 g).

To confirm the hypoxic state of TMJ cartilage, pimonidazole hydrochloride, known as Hypoxyprobe-1 (HP-1), was supplied (Natural Pharmacia International, Inc. Research Triangle Park, NC). Two hours before sacrifice, the rats were administered HP-1 at a dose of 60 mg/kg by intraperitoneal injection. Ten rats from each group (n=10) were sacrificed under deep anesthesia at 12, 24, 48 and 96 hours after initiation of the experiment.

Immunohistochemical staining:

The left TMJs were immediately fixed in 4% formalin for 24 hours, decalcified in 10% EDTA for 30 days, embedded in paraffin, and cut into 5 μ m sections in the sagittal direction. The three most central sections of each condyle were chosen after hematoxylin and eosin (HE) staining.

The condylar cartilage was typically divided into fibrous, proliferative, hypertrophic, and calcified layers (Figure 1c). Furthermore, the condyle was divided into nine numbered sagittal segments (Figure 1a). [12]

The sections were deparaffinized and treated with 0.2 % H₂O₂ for 30 minutes at room temperature for peroxidase omission. Thereafter, the sections were incubated with primary antibodies at 4°C overnight. The primary antibodies used were mouse monoclonal anti-pimonidazole antibody (diluted 1:50 in PBS, 0.1% PBS; NPI Inc., USA), rabbit polyclonal anti-HIF-1 α (diluted 1:100 in PBS, 0.1% PBS; Boster, China), and rabbit polyclonal anti-VEGF (diluted 1:50 in PBS, 0.1% PBS; Bioss, China). Negative controls without the primary antibody were also prepared. Applied with secondary antibody, the sections were processed with diaminobenzidine (DAB) as the chromogen. Immunostained tissue sections were visualized by using a confocal microscope (Olympus FV 1000, Japan).

Semi-quantitative RT-PCR

A two-step semi-quantitative RT-PCR method was used to measure gene expression in condylar cartilages. Total RNA of right condylar cartilages was extracted in accordance with the RNA extraction kit instructions and converted to cDNA by reverse transcriptase (TaKaRa, China). The primer sequences and optimal PCR annealing temperatures (ta) are listed in Table 1.

The PCR program for HIF-1 α and VEGF started with a 95 °C denaturation for 2 min, followed by 30 cycles of 95°C for 45 s, and 55°C for 1 min, then 72°C for 1 min, and a final extension at 72°C for 15 min. The PCR samples were analyzed by gel electrophoresis using 2% agarose. The images were captured and analyzed with the ImagJ software (NIH, Bethesda, USA). Gene expression was defined as the ratio between the band intensity of each target gene and band intensity of β -actin. The experiments were repeated three times.

Statistical Analysis

The statistical analyses were performed by using the SPSS 13.0 software. Analysis of

variance (ANOVA) was used to analyze the statistical significance. P-values less than 0.05 were considered statistically significant.

Results:

HP-1 was confined in the upper layers of cartilage at all time points in both groups. In addition, it was mainly expressed in the anterior aspect of the condyle and posterior aspect of the articular eminence at 12 and 96 hours (1-2 area of I - II). At 24 and 48 hours, it was expressed in the fibrous, proliferative, and hypertrophic layers of cartilage in both groups. Since both groups showed the same location of staining, the increased loading group was presented in Figure 2.

Staining of HIF-1 α was most strongly expressed in the chondrocytes of the fibrous and proliferative layer of cartilage at all time points. HIF-1 α staining was also expressed in the hypertrophic and calcified layers at 48 and 96 hours in both groups. Since both groups showed the same location of staining, the increased loading group was presented in Figure 3. RT-PCR analysis showed that HIF-1 α mRNA was higher in the increased loading group than in the reduced loading group at 48 and 96 hours after initiation of the experiment (Figure 5a).

Staining of VEGF was most strongly expressed in chondrocytes of the fibrous and proliferative layer of cartilage at all time points. VEGF staining was also expressed in the hypertrophic and calcified layers at 48 and 96 hours in both groups. Since both groups showed the same location of staining, the increased loading group was presented in Figure 4. The expression VEGF mRNA was lower in the increased loading group than in the reduced loading group at 24 hours. At 48 and 96 hours, VEGF mRNA was higher in the increased loading group compared to the reduced loading group (Figure 5b).

Discussion

Earlier experiments have shown that immature articular cartilage may be nourished by both synovial fluid and subchondral routes.[13] Because of the calcified barrier of subchondral bone, the view that mature cartilage gets its nutrients merely from the synovial fluid is favored by most investigators.[14,15] Oxygen tension, which has significant effects on metabolism of articular cartilage, ranges from 7% on the surface to less than 1% in the deep zone.[16,17] It is an accepted view that oxygen and other nutrients to the chondrocytes are mainly transported from the synovial fluid and assisted by cyclic loading (pumping). [18] Nevertheless, Arkill and Winlove have showed that calcified cartilage is permeable to small solutes even in mature tissue.[19] Our present results showed that the hypoxia marker HP-1 was expressed in the upper layers of the cartilage and was never seen in the calcified layer in both groups at all time points, which implies that articular cartilage is mainly nourished from direction of the deep zone by the subchondral bone in young rats under loading. The results of the present study support the possibility that hypoxia markers are useful in studies of TMJ cartilage physiology associated with oxygen tension.

The TMJ is a load bearing joint and covered by fibrocartilage which is composed of four different layers: the fibrous, proliferative, hypertrophic, and calcified cartilage.[20] Stimuli induced by loading are essential to the development of TMJ cartilage.[21] It has been speculated that the cartilage obtains oxygen by the invasion of new vasculature, which promotes cartilage replacement by bone growth and remodeling.[22] During the angiogenesis period, special factors are required to be responsible for glucose metabolism, energy generation, as well as pH regulation.[23] One important factor is HIF-1 α , which improves matrix synthesis and inhibits proliferation, but increases extracellular matrix

(ECM) production.[24] As a well-established HIF-1 α target gene, VEGF is produced by hypertrophic chondrocytes and plays a central role in angiogenesis, neovascularization and endochondral bone formation.[25,26] Our experiment showed that HIF-1 α and VEGF were most strongly expressed in the fibrous and proliferative layers at 12 and 24 hours, and also expressed in the hypertrophic and calcified layers at 48 and 96 hours. Hypertrophic chondrocytes are able to differentiate into chondrocytes, and they are required for condylar endochondral ossification. VEGF has been indicated to stimulate the proliferation and differentiation of chondrocytes, and subsequently activate new blood vessel invasion into the hypertrophic layer.[27] Our results showed that HIF-1 α and VEGF mRNA were higher in the increased loading group at 48 and 96 hours. Similarly, Jiang [22] *et al.* found out that VEGF expression increased in rat condylar cartilage more in the hard diet group than in the soft diet group. Pufe [28] *et al.* indicated that mechanical loading could induce VEGF expression in cartilage discs via HIF-1 α . These findings support our hypothesis that cartilage acquires oxygen by angiogenesis under loading. Vascular invasion is needed to supply more oxygen and other nutrients, as indicated by the increased expression of HIF-1 α and VEGF. This can explain the higher HIF-1 α and VEGF expression in the hypertrophic and calcified layers in older animal groups. It is apparent that when the loading is heavier, more new vessels are needed. It has been shown that moderate loading is significant to maintain the integrity and development of TMJ, while overloading can induce condylar cartilage degradation.[5]

Rats are very sensitive to the altered loading during the period after weaning, but before and during the establishment of occlusion.[29] In the present study, VEGF mRNA was lower in the increased loading group than in the reduced loading group at 24 hours. A

previous study has shown that absence of the fibroblast growth factor receptor 3 (FGFR-3) leads to decreased expression of VEGF.[30] There might be a feedback control mechanism to avoid excessive growth by FGFR-3 which inhibits chondrocytes proliferation and differentiation.[31] Significant differences between increased and reduced loading groups were witnessed at 48 and 96 hours compared to 12 hours, which points to the fact that the mechanical loading signal pathway is stimulated in a time-dependent manner.[32]

Although mechanical loading is a significant factor for condylar cartilage, the signal transduction mechanism remains elusive. [33] It is an interesting finding that the staining of HP-1 was primarily concentrated in the weight-bearing area of TMJ, i.e. the anterior aspect of the condyle and the posterior aspect of the articular eminence, at 12 and 96 hours. This phenomenon occurred in both groups and suggests that mechanical loading might directly induce weight-bearing area hypoxia, which may be a possible signal pathway of mechanical loading. However, it is yet unclear what is the linking factor between hypoxia and mechanical loading, and what is the specific mechanism behind the phenomenon. Apparently, further investigations are required to better understand this loading signal pathway.

Conclusion: Mechanical loading seems to directly induce weight-bearing area hypoxia followed by new vessel formation, which indicates that these factors are related and important for the development of cartilage.

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References

1. McKay GS, Yemm R, Cadden SW. The structure and function of the temporomandibular joint.

British dental journal. 1992;**173**: 127-132.

2. Jackson A, Gu W. Transport Properties of Cartilaginous Tissues. Current rheumatology reviews. 2009;**5**: 40.
3. Hewitt KM, Stringer MD. Correlation between the surface area of synovial membrane and the surface area of articular cartilage in synovial joints of the mouse and human. Surgical and radiologic anatomy : SRA. 2008;**30**: 645-651.
4. Vinel P, Anklewicz J, Bayourthe L. [Nutrition of articular cartilage]. Rhumatologie. 1972;**24**: 51-63.
5. Palla S, Gallo LM, Gossi D. Dynamic stereometry of the temporomandibular joint. Orthodontics & craniofacial research. 2003;**6 Suppl 1**: 37-47.
6. Chitneni SK, Bida GT, Zalutsky MR, Dewhirst MW. Comparison of the Hypoxia PET Tracer (18)F-EF5 to Immunohistochemical Marker EF5 in 3 Different Human Tumor Xenograft Models. Journal of nuclear medicine : official publication, Society of Nuclear Medicine. 2014;**55**: 1192-1197.
7. Koch CJ, Evans SM. Optimizing hypoxia detection and treatment strategies. Seminars in nuclear medicine. 2015;**45**: 163-176.
8. Raleigh JA, Dewhirst MW, Thrall DE. Measuring Tumor Hypoxia. Seminars in radiation oncology. 1996;**6**: 37-45.
9. Chitneni SK, Palmer GM, Zalutsky MR, Dewhirst MW. Molecular imaging of hypoxia. Journal of nuclear medicine : official publication, Society of Nuclear Medicine. 2011;**52**: 165-168.
10. Westbury CB, Pearson A, Nerurkar A, *et al*. Hypoxia can be detected in irradiated normal human tissue: a study using the hypoxic marker pimonidazole hydrochloride. The British journal of radiology. 2007;**80**: 934-938.
11. Arteel GE, Thurman RG, Yates JM, Raleigh JA. Evidence that hypoxia markers detect oxygen gradients in liver: pimonidazole and retrograde perfusion of rat liver. British journal of cancer. 1995;**72**: 889-895.
12. Pirttiniemi P, Kantomaa T, Sorsa T. Effect of decreased loading on the metabolic activity of the mandibular condylar cartilage in the rat. European journal of orthodontics. 2004;**26**: 1-5.
13. McKibbin B, Holdsworth FW. The nutrition of immature joint cartilage in the lamb. The Journal of bone and joint surgery British volume. 1966;**48**: 793-803.
14. Malinin T, Ouellette EA. Articular cartilage nutrition is mediated by subchondral bone: a long-term autograft study in baboons. Osteoarthritis and cartilage / OARS, Osteoarthritis Research Society. 2000;**8**: 483-491.
15. Ogata K, Whiteside LA. Barrier to material transfer at the bone-cartilage interface: measurement with hydrogen gas in vivo. Clinical orthopaedics and related research. 1979: 273-276.
16. Silver IA. Measurement of pH and ionic composition of pericellular sites. Philosophical transactions of the Royal Society of London Series B, Biological sciences. 1975;**271**: 261-272.
17. Ysart GE, Mason RM. Responses of articular cartilage explant cultures to different oxygen tensions. Biochimica et biophysica acta. 1994;**1221**: 15-20.
18. O'Hara BP, Urban JP, Maroudas A. Influence of cyclic loading on the nutrition of articular cartilage. Annals of the rheumatic diseases. 1990;**49**: 536-539.
19. Arkill KP, Winlove CP. Solute transport in the deep and calcified zones of articular cartilage. Osteoarthritis and cartilage / OARS, Osteoarthritis Research Society. 2008;**16**: 708-714.
20. Kaul R, O'Brien MH, Dutra E, Lima A, Utreja A, Yadav S. The Effect of Altered Loading on Mandibular Condylar Cartilage. PloS one. 2016;**11**: e0160121.
21. Betti BF, Everts V, Ket JCF, *et al*. Effect of mechanical loading on the metabolic activity of cells in the temporomandibular joint: a systematic review. Clinical oral investigations. 2017.
22. Jiang L, Xie Y, Wei L, *et al*. Identification of the vascular endothelial growth factor signalling pathway by quantitative proteomic analysis of rat condylar cartilage. FEBS open bio. 2017;**7**: 44-53.
23. Semenza GL, Roth PH, Fang HM, Wang GL. Transcriptional regulation of genes encoding glycolytic enzymes by hypoxia-inducible factor 1. The Journal of biological chemistry. 1994;**269**: 23757-23763.
24. Pfander D, Cramer T, Schipani E, Johnson RS. HIF-1 α controls extracellular matrix synthesis by epiphyseal chondrocytes. Journal of cell science. 2003;**116**: 1819-1826.
25. Blumer MJ, Longato S, Fritsch H. Structure, formation and role of cartilage canals in the developing bone. Annals of anatomy = Anatomischer Anzeiger : official organ of the Anatomische Gesellschaft. 2008;**190**: 305-315.
26. Dai J, Rabie AB. VEGF: an essential mediator of both angiogenesis and endochondral ossification. Journal of dental research. 2007;**86**: 937-950.

27. Yang YQ, Tan YY, Wong R, Wenden A, Zhang LK, Rabie AB. The role of vascular endothelial growth factor in ossification. *International journal of oral science*. 2012;**4**: 64-68.
28. Pufe T, Lemke A, Kurz B, *et al*. Mechanical overload induces VEGF in cartilage discs via hypoxia-inducible factor. *The American journal of pathology*. 2004;**164**: 185-192.
29. Kantomaa T, Tuominen M, Pirttiniemi P, Ronning O. Weaning and the histology of the mandibular condyle in the rat. *Acta anatomica*. 1992;**144**: 311-315.
30. Papadopoulou AK, Papachristou DJ, Chatzopoulos SA, Pirttiniemi P, Papavassiliou AG, Basdra EK. Load application induces changes in the expression levels of Sox-9, FGFR-3 and VEGF in condylar chondrocytes. *FEBS letters*. 2007;**581**: 2041-2046.
31. Deng C, Wynshaw-Boris A, Zhou F, Kuo A, Leder P. Fibroblast growth factor receptor 3 is a negative regulator of bone growth. *Cell*. 1996;**84**: 911-921.
32. Papachristou DJ, Papachroni KK, Papavassiliou GA, *et al*. Functional alterations in mechanical loading of condylar cartilage induces changes in the bony subcondylar region. *Archives of oral biology*. 2009;**54**: 1035-1045.
33. Papachristou DJ, Pirttiniemi P, Kantomaa T, Papavassiliou AG, Basdra EK. JNK/ERK-AP-1/Runx2 induction "paves the way" to cartilage load-ignited chondroblastic differentiation. *Histochemistry and cell biology*. 2005;**124**: 215-223.

Figure 1. The central sagittal section by HE staining.

- a:** The condylar was divided into nine sagittal segments (I -III sagittally and 1-3 vertically). I : anterior aspect; II : middle aspect; III: posterior aspect; 1: fibrous layer; 2: proliferative layer; 3: hypertrophic and calcified layer.
- b:** The central sagittal section of the condyle stained with HE. (×40) T: temporal condyle; D: articular disc; C: condyle
- c:** Four layers of the cartilage were indicated. (×100) S: fibrous layer; P: proliferative layer; H: hypertrophic layer; C: calcified layer

Figure 2. The immunostaining of HP-1: HP-1 was confined in the upper layers of condylar cartilage. At 12 and 96 hours, the staining of HP-1 was most strongly expressed in the anterior aspect of the condyle and in the posterior aspect of the articular eminence (1-2 area of I – II, arrows). At 24 and 48 hours, it was expressed in the fibrous, proliferative, and hypertrophic layers of cartilage in both groups. (×40)

Figure 3. The immunostaining of HIF-1 α : At 12 and 24 hours, HIF-1 α was most strongly expressed in the fibrous and proliferative chondrocytes of the condylar cartilage (arrows); HIF-1 α was also expressed in the hypertrophic and calcified chondrocytes at 48 and 96 hours. ($\times 100$)

Figure 4. The immunostaining of VEGF: At 12 and 24 hours, VEGF was most strongly expressed in the fibrous and proliferative chondrocytes of the condylar cartilage (arrows); VEGF was expressed in all cartilage zones at 48 and 96 hours. ($\times 100$)

Figure 5. HIF-1 α (a) and VEGF (b) mRNA expression in condylar cartilage shown as RT-PCR activity. At 48 and 96 hours, HIF-1 α and VEGF mRNA were higher in the increased loading group than in the reduced loading group. At 24 hours, VEGF mRNA dropped in the increased loading group. A-D represent 12, 24, 48 and 96 hours of the reduced loading group. E-H represent 12, 24, 48 and 96 hours of the decreased loading group. Asterisks indicates a difference between the two groups at the same time point (* P<0.05).

Table 1 PCR primer sequence, amplified product sizes and annealing temperature (ta) in condylar cartilage

Primer	Sequence (5'-3')	Size (bp)	Ta (°C)
HIF-1 α	Forward 5'-CCTGGATGGCTTTGTTATGGTG-3' Reverse 5'- CGCTGCGTGTTTTGTTCTTTC-3'	210	55
VEGF	Forward 5'- TTGAGACCCTGGTGGACATC -3'' Reverse 5'- GGATCTTGGACAAACAAATGC -3'	310	55
β -actin	Forward 5'- TATAACACCCAGCAGGCATCCA-3' Reverse 5'- GTGCTCAGTCCATTCCCGTTC-3'	150	60