

■ **CARTILAGE**

The effect induced by alternated mechanical loading on Notch-1 in mandibular condylar cartilage of growing rabbits

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Aims

The aim of our study is to investigate the effect induced by alternated mechanical loading on Notch-1 in mandibular condylar cartilage (MCC) of growing rabbits.

Methods

A total of 64 ten-day-old rabbits were randomly divided into two groups according to dietary hardness: normal diet group (pellet) and soft diet group (powder). In each group, the rabbits were further divided into four subgroups by feeding time: two weeks, four weeks, six weeks, and eight weeks. Animals would be injected 5-bromo-2'-deoxyuridine (BrdU) every day for one week before sacrificing. Histo-morphometric analysis of MCC thickness was performed through haematoxylin and eosin (HE) staining. Immunochemical analysis was done to test BrdU and Notch-1. The quantitative real-time polymerase chain reaction (qRT-PCR) and western blot were used to measure expression of Notch-1, Jagged-1, and Delta-like 1 (DII-1).

Results

The thickness of MCC in the soft diet group was thinner than the one in normal diet group. Notch-1 was restricted in fibrous layer, proliferative layer, and hypertrophic layer. The expression of Notch-1 increased from two weeks to six weeks and then fell down. Notch-1 in normal diet group was higher than that in soft diet group in anterior part of MCC. The statistical differences of Notch-1 were shown at two, four, and six weeks ($p < 0.05$). The result of western blot and quantitative real-time PCR (qRT-PCR) showed the expression of DII-1 and Jagged-1 rose from two to four weeks and started to decrease at four weeks. BrdU distributed in all layers of cartilage and subchondral bone. The number of BrdU-positive cells, which were less in soft diet group, was decreasing along with the experiment period. The significant difference was found at four, six, and eight weeks in anterior and posterior parts ($p < 0.05$).

Conclusion

The structure and proliferation of MCC in rabbits were sensitive to dietary loading changes. The proper mechanical loading was essential for transduction of Notch signalling pathway and development of mandibular condylar cartilage.

Cite this article: *Bone Joint Res* 2021;10(7):437–444.

Keywords: mandibular condylar cartilage, Notch-1, BrdU

Article focus

- Find the evidence that the development of mandibular condylar cartilage (MCC) was effected by altered loading as results of previous studies have shown: the structure and proliferation of MCC decreased due to lower loading.
- Whether Notch-1 expression would change in MCC because of altered loading.
- Preliminarily explore the effect on Notch signalling pathway brought by altered loading.

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doi: 10.1302/2046-3758.107.BJR-2020-0234.R2

Bone Joint Res 2021;10(7):437–444.

Key messages

- Mastication plays an important role in the structure and proliferation of MCC during the growing period.
- Like other factors and signalling pathways which have been studied before, Notch-1 and Notch signalling pathway in MCC were sensitive to altered loading as well.
- Lower loading did not only lead to changing expression of factors and signalling pathways in MCC, but also abnormal development of MCC.

Strengths and limitations

- Unweaned growing rabbits were used, as their temporomandibular joint (TMJ) had not loaded before, and it was easier and more direct to observe the change brought by altered loading.
- There are few studies about the relationship among Notch signalling pathway and mastication.
- This study was preliminary, so the result could not display the interaction between Notch signalling pathway and altered loading and how it worked.

Introduction

Mandibular condylar cartilage (MCC) is a kind of secondary cartilage, and consists of chondrocytes and extracellular matrix (ECM). According to earlier studies, MCC is divided into the five following layers: fibre layer, proliferative layer, hypertrophic cartilage layer, calcified cartilaginous layer, and subchondral bone.^{1,2} In these layers, different factors and signalling pathways are essential players in growth and repair process at both synthesis and degradation levels.³

As the temporomandibular joint (TMJ) plays an important role in oral function, it is necessary to establish how its development, and particularly the MCC, is affected by mechanical loading (mastication or chewing pressure). Many experimental evidences have confirmed that a liquid or powdered diet has an unfavourable influence on the maxillofacial growing.^{4,5} Compared with young mice or rats fed on a normal diet, those with a long-term liquid or powdered diet might have smaller forms of mandible and a decreased expression of signal pathways, such as Indian hedgehog-parathyroid hormone-related peptide (Ihh-PThrP pathway).¹ Chen et al⁴ stated that altered functional loading would reduce the thickness of condylar cartilage and chondrocyte maturation, and the positive reactions of expression of morphogenesis regulation factors and their signalling were found to decrease. Ishida et al⁵ indicated that decreased masticatory loading could impact morphology of condylar cartilage during the early stage of growth period.

The mammalian Notch genes consist of a family of transmembrane signalling receptors, Notch-1, Notch-2, Notch-3, and Notch-4, and their corresponding ligands, Delta and Jagged.^{6,7} It had been proved that Notch-1

was closely connected with proliferation, differentiation, and maturation of chondrocytes,⁸ which had been well documented. Notch-1 is also a marker for progenitor.⁹ In human articular cartilage, it has been reported to maintain cells in an undifferentiated state and stimulate the ability of self-renewal.¹⁰ Studies on humans and animals have revealed that Notch signalling pathways were involved in different stages of cartilage physiology and pathology.^{8,11} Serrano et al¹² revealed the fact that Notch signalling plays a necessary role in the condylar cartilage.

Our study was conducted to find out the effect on the growth of MCC induced by a different diet, and the potential impact of mechanical loading on Notch signalling pathway related with condyle development.

Methods

Animals. A total of 64 ten-day-old New Zealand growing rabbits (mean weight 150 g (standard deviation (SD) 20)) were selected and allowed to acclimatize to the laboratory conditions with food and water available *ad libitum*, housed in light- and temperature-controlled rooms for five days before the experiment began. At the age of 15 days, the animals (mean weight 230 g (SD 20)) were randomly divided into two groups according to dietary hardness: normal diet (pellet) and soft diet (powder). In each group, the rabbits were further divided into four subgroups by feeding time: two weeks, four weeks, six weeks, and eight weeks. The rabbits at this age were chosen, because the MCC had never taken any stress before, and it was easier to analyze the differences induced by altered loading in growing period.

In order to ensure the animals grew healthily, breast-feeding and processed feed were provided during the first two weeks of the experiment. All the animals were weaned after two weeks. Throughout the experiment period, the rabbits from soft diet group were fed on a powdered diet of crushed pellets, while the other group was fed on a normal diet. All these animals were given unrestricted access to food and water and weighed every day.

All procedures and animal care were approved by the University Ethics Committee and performed according to institutional guidelines. We have included an ARRIVE checklist to show that we have conformed to the ARRIVE guidelines.

Condylar cartilage and joint disc harvest. Each rabbit would be injected with 5-bromo-2'-deoxyuridine (BrdU) (30 mg/kg, Sigma, USA) every day for one week before sacrificing. The rabbits were killed under anaesthesia at the exact timepoints of the experiment: two weeks, four weeks, six weeks, and eight weeks. All the efforts were made to minimize suffering.

The whole head of animal was removed, and both sides of mandibular condyle were dissected carefully. The condylar cartilage on the left side was isolated with all the soft-tissues and frozen in liquid nitrogen container in a snap, used for quantitative real-time polymerase chain

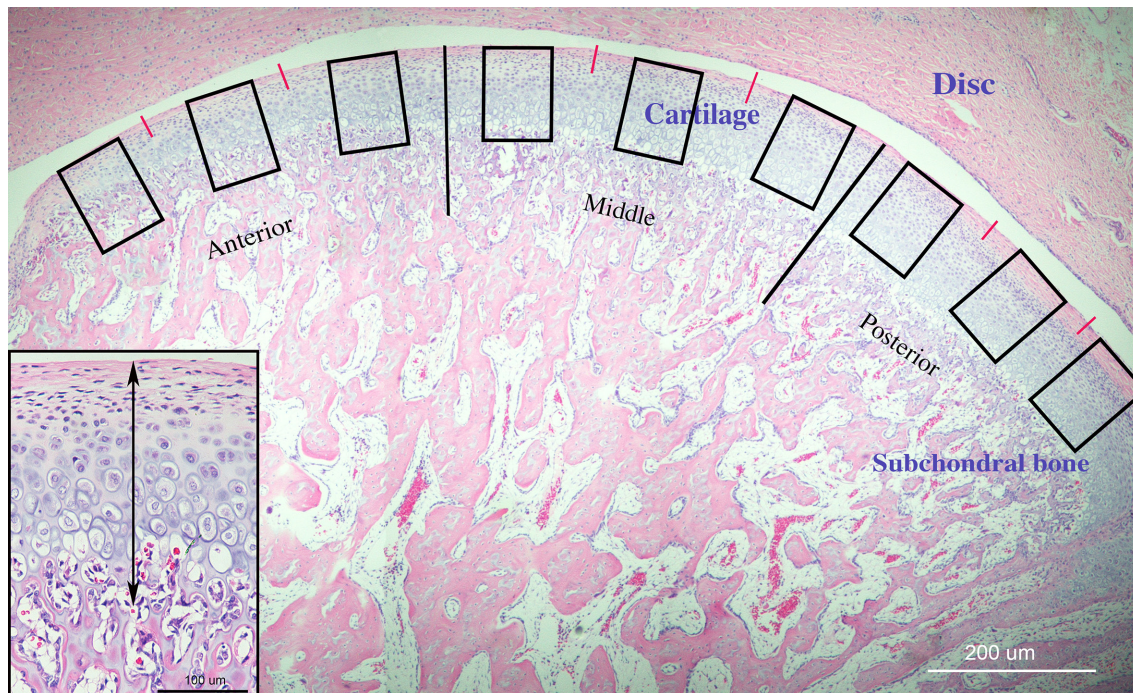


Fig. 1

The method, in which mandibular condylar cartilage was measured and divided, was shown in haematoxylin and eosin staining.

reaction (qRT-PCR) and western blot. The right side of condyle was used to perform immunohistochemistry and haematoxylin and eosin (HE) staining.

Immunohistochemistry. Immunolocalization in the samples was performed following standard protocols. The sections were rehydrated in ethanol after deparaffinization. Subsequently, the sections were pretreated with 1% ethylenediaminetetraacetic acid (EDTA) under high pressure at high temperature and with 3% H_2O_2 following endogenous peroxidase blocking. The solution was then removed by blotting without touching the sections. Staining was performed with Harris haematoxylin staining and diaminobenzidine tetrahydrochloride (DAB). The distribution of positive cells was observed at a 400 \times magnification using a DMI 3000 microscope (Leica, USA).

Quantitative analysis. According to the surface length, the cartilage was divided into three equal parts: anterior part, middle part, and posterior part. With the same method, each part was divided into three equal subparts where the samples were picked. Three equal areas ($370 \times 490 \mu m^2$) were chosen from each subpart in a 200 \times microscopic field to calculate the sum of positive cells and the total cells in the condylar cartilage (Figure 1). Then, the positive rate in the three parts was described as follows: positive rate = n/N (%); n = number of positive cells counted; N = number of all cells counted in all layers.

The same MCC dividing method was used to measure the thickness in anterior part, middle part, and posterior part. The thickness was measured from the surface of fibrous layer to the bottom of calcified cartilaginous layer. The centre of each subpart was regarded as the starting

point to calculate. Measurements in three parts of each sample were tested three times. Mean value of subparts was used as final value in each part (Figure 1)

RNA extraction. Total RNA was extracted from condylar cartilage following the standard protocol for animal tissues. For isolation of RNA from the samples, TRIzol (Qiagen, China) was added and the samples were grounded to powder. Chloroform was added (0.2 ml/ml TRIzol) and the tubes were mixed and centrifuged at 12,000 g for 15 minutes at 4 $^{\circ}C$. Culture supernatant of mtecs was collected and transferred into 1.5 ml centrifuge tubes, mixed with 70% alcohol sufficiently. The supernatant was placed in Spin Cartridge and centrifuged at 12,000 g for 15 seconds at room temperature, discarding the waste. Contaminating genomic DNA was removed from the isolated RNA using RNase-Free DNase Set (Qiagen). The quantities and qualities of RNA were checked by ultraviolet spectrophotometer and electrophoresis.

Quantitative real-time PCR. The real-time PCR analyses were performed as the standard protocol required. The primers were designed using Premier 6.0 (Premier, Canada) and Beacon designer 7.8 (Premier Biosoft, USA) software. To maximize the fluorescence response for real-time PCR, every primer was assessed in primer matrix to determine the combination of sense (S) and anti-sense (AS) primer concentration. The primer sequences were Notch-1 (CACTTGGCTGCC CGATACTCT/GCCCATGTTGTCTGGATGTT) 87 bp, DII-1 (GCTTTCGGCCACAGCACCTAT/CATTGTCATCCTCGCAG AATCCA) 79 bp, and Jagged-1 (CACGACCCCTTGTAAGTGTATTG/GTGAATTTGCCTCCCGACTGACT) 138 bp. Each sample was tested three times. Samples along with primers

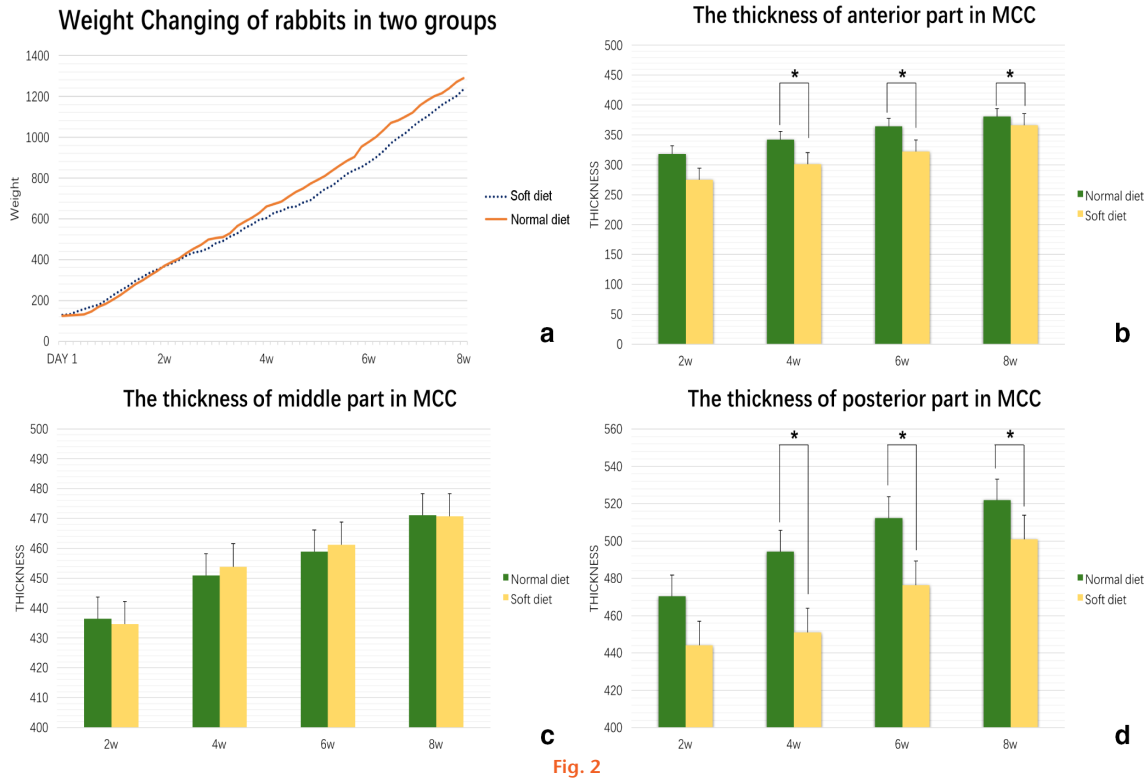


Fig. 2

Graphs demonstrating a) the animals' weight (g) changing, and b) to d) the difference between the thickness of three parts of the mandibular condylar cartilage (MCC) (μm). (* $p < 0.001$).

and SYBR Green Master Mix (Applied Biosystems, USA) were loaded in 384-well plates and the reaction was run in the CFX384 Multiplex Quantitative PCR System (Bio-Rad, USA). The standard curve method was used to calculate the real-time PCR data. Serial dilutions of cDNA (1:20) were made for the calibration curve and loaded into 384-well plates.

Western blot. The western blot analyses were performed as the standard protocol required. The total proteins were extracted from sample tissues with T-PER Tissue Protein Extraction Reagent (Thermo Pierce, 78510, USA), following the protocol strictly. The supernatants were separated by sodium dodecyl-sulfate polyacrylamide gel electrophoresis (SDS-PAGE) analysis with a 5% stacking gel and a 12% separating gel for two hours. Then it was transferred to PVDF membranes, immunoblotted with primary antibodies (Abcam, ab109536, ab109536; SC-373891, USA) at 4°C overnight, and immunoreactivity was visualized with Goat anti-Mouse IgG (Thermo Pierce, 31160, USA), using the ECL detection kit. The optical density value was evaluated by BandScan 5.0 software. Every result was tested three times.

Statistical analysis. Estimation of the differences in immunohistochemistry, western blot, and RT-PCR results of Notch-1, BrdU, Dll-1, and Jagged-1 in relation to time (two, four, six, and eight weeks) and food types (soft and normal) was performed by applying two-way analysis of variance (ANOVA), including paired t -test and non-parametric test. A value of $p < 0.05$ was considered statistically significant. Experimental data were processed with SPSS for Mac (version 22.0, IBM, USA).

Results

General condition of the rabbits. The rabbits remained in a good condition with no symptoms of any diseases, and were weighed every week during the experiment. The weight of all the animals increased all the time; there was no significant difference between the soft diet group and normal diet group (Figure 2).

Thickness of MCC. All three parts grew thicker throughout the experiment period. The anterior part was the thinnest, while the posterior part was the thickest. Compared with the thickness in the normal diet group, the cartilage was thinner in the soft diet group. We observed a statistical difference both in the anterior and posterior parts at two weeks, four weeks, and six weeks ($p < 0.001$, paired t -test, non-parametric test). In the middle part, there was no statistical difference ($p > 0.05$ paired t -test, non-parametric test) (Figures 2b to 2d)

Notch-1. Notch-1 positive cells were brown, located in the fibrous layer, proliferative layer, and hypertrophic layer of MCC (shown in Figures 3a to 3h). The labelling rate of Notch-1-positive cells was higher in the normal diet group than in the soft diet group. Notch-1-positive cells kept increasing to the top at six weeks, and then decreased. It had fallen to the minimum level by eight weeks during the experiment. The significant differences of the expression of Notch-1 were found at two weeks, four weeks, and six weeks in the anterior part of MCC ($p < 0.001$, paired t -test, non-parametric test) (Figure 4).

BrdU. The brown cells were BrdU-positive, located in all layers of cartilage and subchondral bone (Figure 5). There were

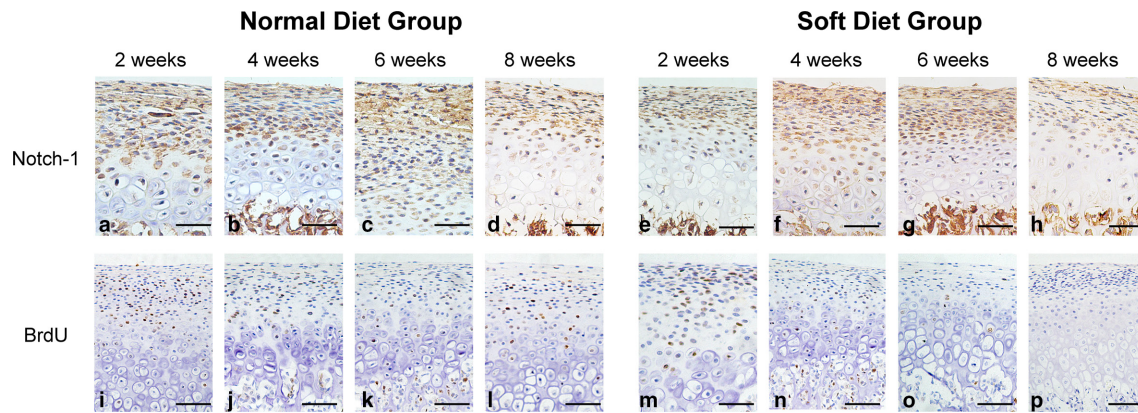


Fig. 3

With altered loading, Notch-1- and 5-bromo-2'-deoxyuridine (BrdU)-positive expression in anterior part of mandibular condylar cartilage (MCC) during the for-review-only experiment. a) to h) showed the change of Notch-1-positive cells in the anterior part of MCC between the normal diet group and the soft diet group in four periods during the experiment. i) to p) showed the change of BrdU-positive cells in the anterior part of MCC between the normal diet group and the soft diet group in four periods during the experiment (bar: 200 μm).

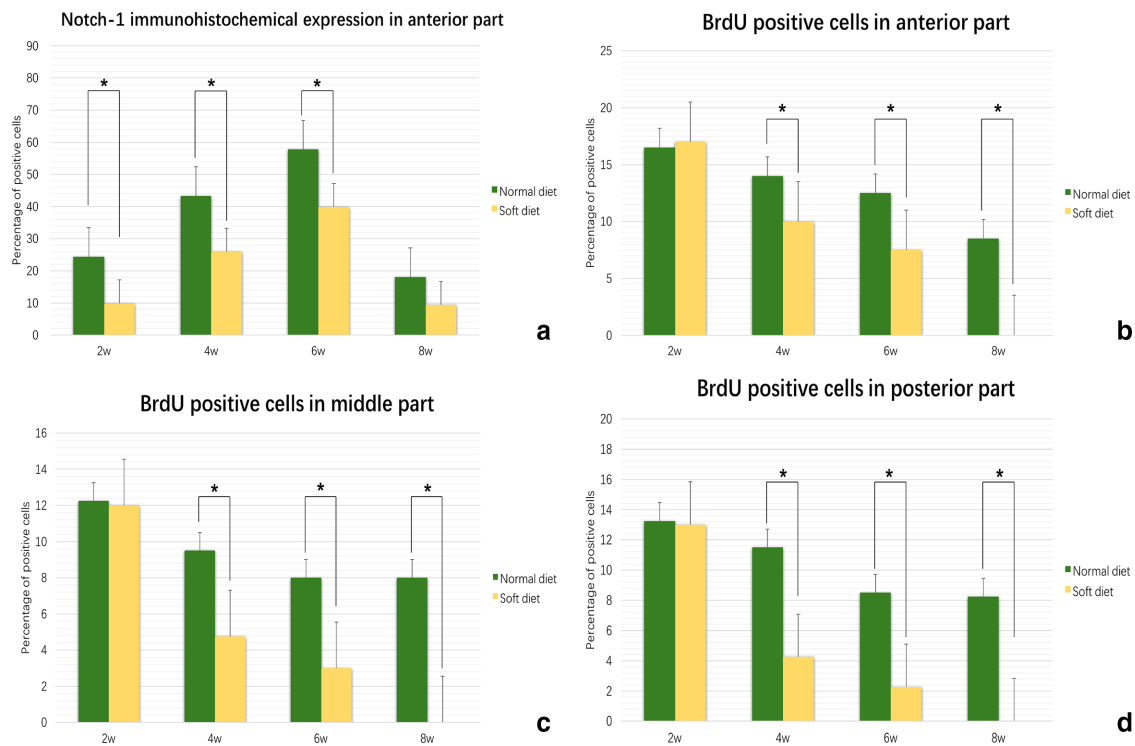


Fig. 4

The statistical differences in a) Notch-1- and b) 5-bromo-2'-deoxyuridine (BrdU)-positive cells in anterior part of mandibular condylar cartilage (MCC). Dotted lines showed the expression tendencies. b), c), and d) showed BrdU-positive cells in the three parts of MCC in four different time periods (* $p < 0.05$ paired t -test, non-parametric test).

only a few positive cells in disc, concentrated in synovium (Figure 5b). As the experiment went on and the animals grew, the number of BrdU-positive cells decreased continuously; at eight weeks, the positive cells could barely be observed in MCC in the soft diet group (Figure 3). The expression was associated with time ($p < 0.001$, paired t -test, non-parametric test). BrdU expression induced by altered loading was less in the soft diet group than that in the normal group, and the

differences were illustrated from four weeks to eight weeks ($p < 0.001$, paired t -test, non-parametric test) (Figures 4b to 4d). **Western blot and real-time PCR.** Greater Notch-1, Dll-1, and Jagged-1 expression in the normal diet group was shown in western blot and real-time PCR. In western blot tests, expression of Notch-1 rose from two weeks to six weeks. After reaching to the top, the expression decreased from six to eight weeks. Differences were observed at two weeks, four weeks,

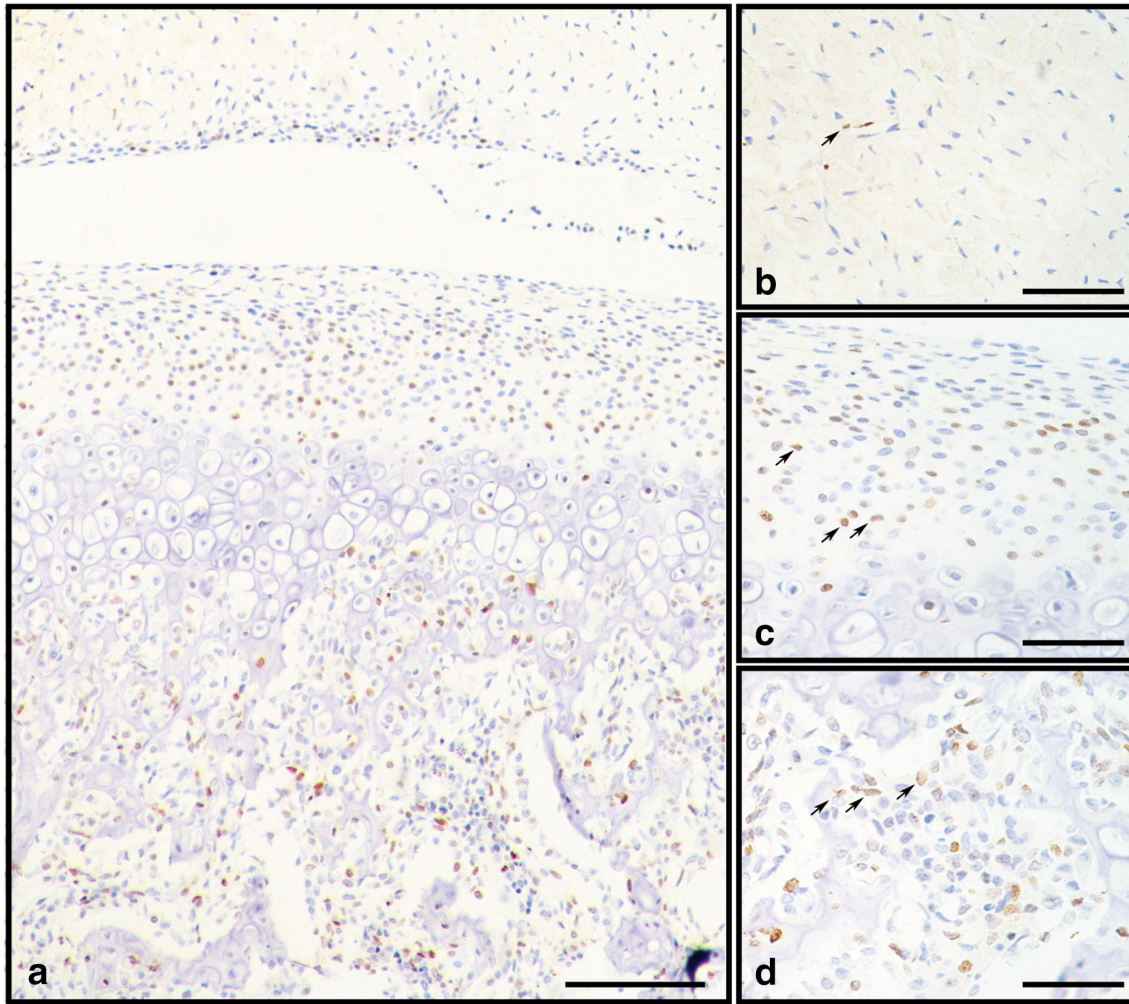


Fig. 5

5-bromo-2'-deoxyuridine (BrdU) distribution in mandibular condyle at two weeks. a) shows BrdU distribution in the anterior part of the mandibular condyle and the disc in the same side. b) to d) show BrdU expression in disc, cartilage, and subchondral bone respectively. The brown cells were BrdU-positive cells (pointed out by black arrows), which were found in large numbers in all layers of cartilage and subchondral bone. There were only a few positive cells in disc. a) bar: 200 μ m; b) to d) bar: 50 μ m).

and six weeks ($p < 0.001$, paired *t*-test, non-parametric test). Dll-1 shared a similar expression trend with Jagged-1: the amount of these factors began to increase rapidly from two weeks. The peak level was shown at four weeks; after that, the expression had decreased by eight weeks. At eight weeks, the productions of Dll-1 and Jagged-1 in the soft diet group almost surpassed the normal diet group. The significant differences were found at four weeks and six weeks ($p < 0.001$, paired *t*-test, non-parametric test). As for real-time PCR tests, the productions of Notch-1, Dll-1, and Jagged-1, respectively, started to increase at similar stages and decreased at four weeks. Significant differences were found at four weeks and six weeks ($p < 0.001$, paired *t*-test, non-parametric test). The expression was associated with time ($p < 0.001$, paired *t*-test, non-parametric test) (Figure 6).

Discussion

Several studies in animals and humans have suggested that the change of mechanical loading, such as tooth loss,

hardness of food, masticatory habits, would have a remarkable influence on the development of MCC.¹³ Like the other studies, the hardness character of fodder was changed.^{4,5} The growing rabbits were fed on powered food, which was made from pellet, in order to observe the development of TMJ and loading-related change of MCC. In this study, the body weight of the rabbits kept growing along with MCC throughout the experiment period according to the prior research.¹⁴

Like most results of studies, the present study showed that anterior part was the thinnest and posterior part was thickest in MCC. The thickness of MCC has been demonstrated to be relied on the mechanical loading, which had a great effect on proliferation.¹⁵⁻¹⁷ Compared with the normal diet group, MCC harvested from the soft diet group was thinner. The significant difference was mainly found in anterior and posterior part of MCC, which is similar to Rusu et al's¹⁸ findings. Their research found that vascular layers appeared at the anterior and posterior parts of the condyles. This implies

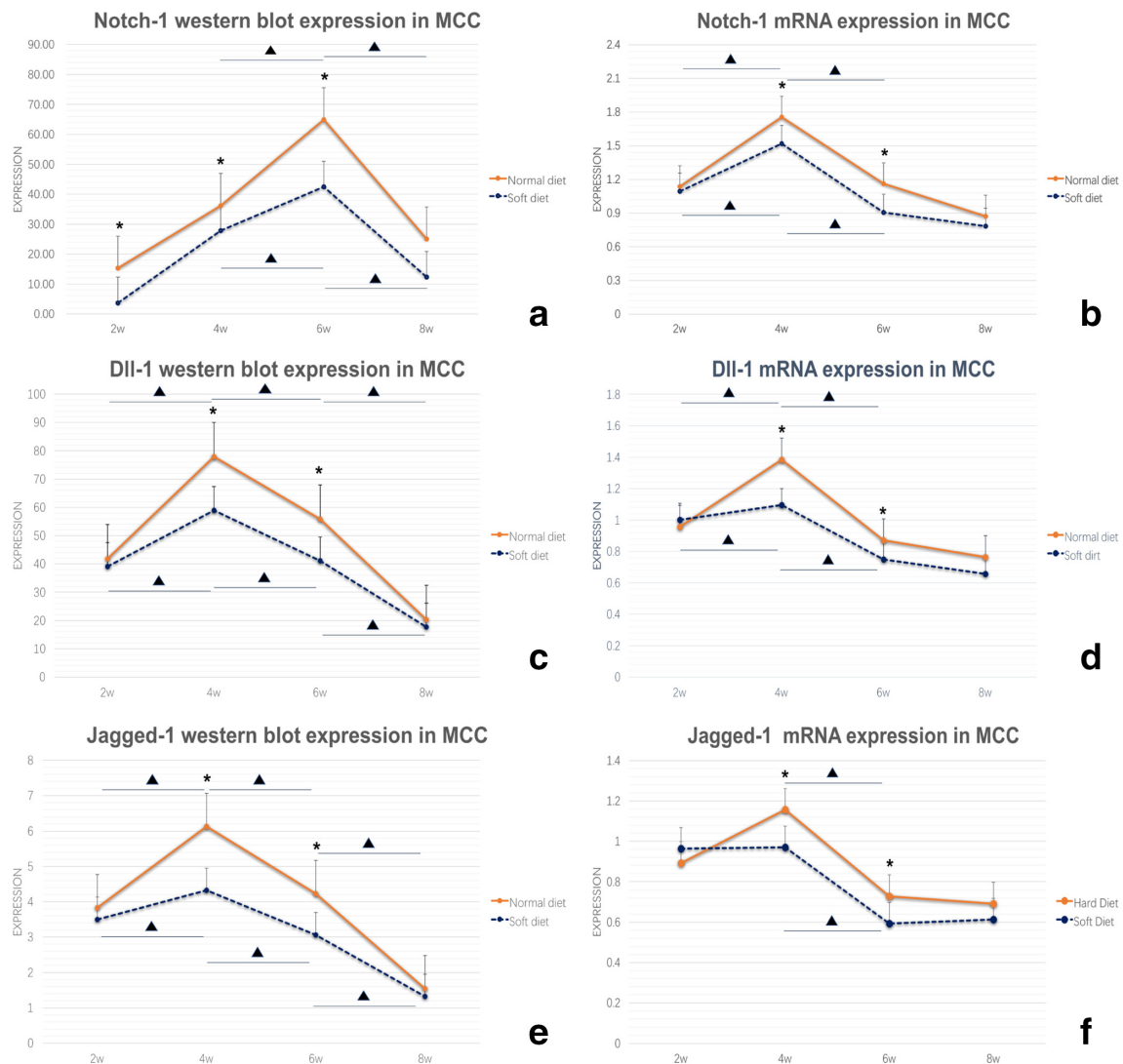


Fig. 6

With altered loading, the differences in Notch-1, Delta-like 1 (DII-1), and Jagged-1 in mandibular condylar cartilage (MCC) were shown. a), c), and e) showed the statistical differences of western blot results about Notch-1, DII-1, and Jagged-1 respectively. b), d), and f) showed the statistical differences of real-time polymerase chain reaction results for Notch-1, DII-1, and Jagged-1 respectively. *The difference between two groups at the same timepoint: $p < 0.001$; ▲ represents the difference among four timepoints in the same group: $p < 0.001$, paired t-test, non-parametric test. mRNA, messenger RNA.

that the anterior and posterior parts might play important roles in MCC development. However, more proliferative cells, which were marked by BrdU, could be counted in the anterior part in our research. A previous study by Pirttiniemi et al¹⁹ gave solid evidence that the anterior part of MCC was easier impacted by loading due to the direct connection with mastication through TMJ disc. The anterior part of MCC was suggested to be sensitive to mechanical loading.^{17,19} Even though the thickness of middle part was growing, we considered that the stress sensibility of middle part was not as strong as the other two parts, because of the smaller number of BrdU-positive cells.

Notch-1 is involved in determining the destination of cell: proliferation, differentiation, and apoptosis.²⁰ Our results showed that Notch-1 was in the fibrous layer, proliferative layer, and hypertrophic layer. It was hard to track Notch-1

in the zone close to calcified cartilaginous layer when the animals were growing. The similar phenomenon was explored in earlier research.^{21,22} In previous studies, Notch-1 was recommended as a marker for progenitor cells, and the idea that there were progenitor cells helping articular cartilage growth and repair was proposed years ago.^{9,10} Few results currently track progenitor cells in condylar cartilage. We hypothesized those Notch-1-positive cells, gathered in MCC, might have a strong capacity to proliferation and prepare for development, self-healing, and so on.

It had been proved that Notch-1 was highly associated with osteoarthritis.²³ Overexpression of Notch-1 would imply the fact that articular joint was in an unhealthy condition.²⁴ In our study, the amount of Notch-1 protein expression kept increasing from two weeks to six weeks. Via real-time PCR, the peak level was found at four weeks, earlier than western

blot and immunohistochemistry test. The production of Notch-1 was speculated to lessen when the experimental animals were in a fast-growing stage. Thus, we believed that Notch-1 was a vital player in developing MCC, as well as a sign of disease. The healthy condition of MCC was regulated by internal and external factors.^{1,2} Compared to the discrepancy of BrdU expression induced by altered loading, the expression of Notch-1 was similar: the production was less in the soft diet group. There were some tight connections between Notch-1 expression and loading: lower loading might lead to weak expression of internal factors, even developmental delay.¹⁵

According to the change of Notch-1, we speculated that the expression of Notch signalling pathway could also be influenced by mastication. Since Jagged-1 and Dll-1 were essential for second cartilage differentiation and proliferation in growing animals,^{11,12} they were tested to provide more evidence between the Notch signalling pathway and mastication. The results of our study showed a similar expression trend and discrepancy with Notch-1. At eight weeks, the productions in soft diet group nearly surpassed the normal diet group. We hypothesized that the effect of external factors, such as loading, was offset by the regulation of some internal factors of MCC in the late development period of rabbits, such as gene regulation and hormones.

In conclusion, the condylar cartilage structure and proliferation in rabbits were sensitive to dietary loading changes. The proper mechanical loading was essential for the development of mandibular condylar cartilage and Notch signalling pathway.

Supplementary material



An ARRIVE checklist is included to show that the ARRIVE guidelines were adhered to in this study.

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- J. Feng: Conceptualization, Writing - review & editing.
- L. Yang: Formal analysis.
- C. Shi: Investigation.

Funding statement:

- This study was funded by the National Natural Science Foundation of China (81200802). No benefits in any form have been received or will be received from a commercial party related directly or indirectly to the subject of this article.

Ethical review statement:

- All procedures and animal care were approved by the University Ethics Committee and performed according to institutional guidelines.

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