Effect of Diet Hardness on Mandibular Condylar Cartilage Metabolism

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Aims: *To study the effect of diet hardness on condylar cartilage thickness, extracellular matrix composition, and expression of matrix metalloproteinase (MMP) -3, -8 and tissue inhibitor of metalloproteinase-1 (TIMP-1), by using immunohistochemical and morphometric methods.* ***Methods:*** *Seventy-two female Sprague Dawley rats were exposed to different dietary hardness, from soft to hard. MMP -3, -8, and TIMP-1 expression, cartilage thickness, cell count, and expression of type II collagen were studied. Analysis of variance among treatments was carried out followed by Bonferroni's comparisons test.* ***Results:*** *The ratio of MMP-3 and TIMP-1 immunopositive cartilage cells were similar in all age groups, whereas the number of MMP-8 positive cells decreased with age. A change of diet from soft to hard caused a significant decrease in the number of MMP-3 and MMP-8 and an increase in TIMP-1 positive cells. Cartilage thickness and area of type II collagen-positive staining were significantly affected by diet hardness.* ***Conclusion:*** *The results show that a soft diet during growth increases collagenolytic activity and may increase the vulnerability of condylar cartilage.* J OROFAC PAIN 2011;25:68–74

Key words: condylar cartilage, diet hardness, mandibular condyle, MMP, TIMP

Mechanical loading of articular cartilage is considered essential in regulating the metabolic activity of chondrocytes and in maintaining normal extracellular matrix (ECM) properties. Studies using different diet hardnesses and occlusal alterations have shown changes in temporomandibular joint (TMJ) structure. Animals fed a soft diet are characterized by a significantly thinner condylar cartilage and a reduction in trabecular bone density deep to the cartilage.[1–3] Loading is considered important for condylar cartilage growth to maintain both ideal proliferation and matrix chondrocyte production.[4]

In rats, continuous soft diet and suppressed incisal mastication have been shown to cause a marked reduction in proliferative activity and proteoglycan synthesis in condylar cartilage. These changes have been associated with an increase in matrix metalloproteinase-3 (MMP-3) expression and activation.[4] MMP-8 has been shown to be involved in the cleavage and denaturation of type II collagen in articular cartilage, and increased expression has been shown in osteoarthritic (OA) human cartilage specimens.[5,6] Together with other metalloproteases, including MMP-8, MMP-3 can synergistically degrade the major components of the extracellular matrix, including proteoglycan and type II collagen.[5,7] Tissue inhibitor of metalloproteinase-1 (TIMP-1) is considered the most common tissue inhibitor of metalloproteinases.

The aim of this study was to determine the effect of diet hardness on condylar cartilage thickness, extracellular matrix composition and expression of MMP-3, -8 and TIMP-1 by using immunohistochemical and morphometric methods. The hypothesis was that a change in diet hardness would cause a significant change in proteolytic enzyme expression and cartilage composition. The levels of MMP-3 and -8 were expected to be higher in soft diet groups, while a hard diet was assumed to cause a decrease in the expression of MMPs and an increase in TIMP expression. Different cartilage areas were expected to respond differently to dietary changes since the central area of the condylar cartilage is assumed to be under the most loading.

Table 1 Animal Groups With Different Durations of Diet Manipulations: 30 to 33 days, 50 to 53 days, and 200 days after weaning (n = 72)

Group	
1	30 days soft diet
2	30 days soft diet, 3 days hard diet
3	33 days normal diet
4	50 days soft diet
5	50 days soft diet, 3 days hard diet
6	53 days normal diet
7	30 days soft diet, 170 days hard diet
8	50 days soft diet, 150 days hard diet
9	200 days normal diet

Materials and Methods

Animals and Tissue Preparation

After weaning at 21 days, 72 female Sprague-Dawley rats were divided into nine groups, each consisting of eight animals (Table 1). Two groups comprising eight animals each were fed a soft (powder) diet for 30 or 50 days (groups 1 and 4), two groups were on a soft diet for 30 or 50 days after which the diet was switched into hard (pressed pellet) diet for 3 days before sacrifice (groups 2 and 5). Groups 7 and 8 had soft diet for 30 or 50 days, after which the diet was switched into hard for 170 or 150 days, respectively. Groups 3, 6, and 9 were fed a normal diet (porous pellet) for 33, 53, or 200 days. Pressed pellets were cylindrically shaped, with a diameter of approximately 10 mm and 25 mm in length (SDS diets). Soft diet consisted of fine powder of a slightly grainy consistency (Lantmännen animal feeds division). Animals were exposed to CO_2 inhalation for 10 minutes prior to killing by decapitation. The protocols were approved by the Animal Experiment Committee of the University of Oulu. The right TMJs were decalcified with 5% formic acid and heated in a microwave oven (Micromed T/T MEGA) for 5h × 6 times at 37°C, followed by embedding in paraffin. Serial sections of 6 µm were cut parallel to the sagittal plane of the condyle. Deparaffinized sections were stained by toluidine blue, and the most central sections were selected for histological observation.

Immunohistochemical Analysis

Immunohistochemistry was carried out with previously described methods.[4] The most central sagittal sections were chosen for immunohistochemistry. Deparaffinized sections were pre-treated with 0.4% pepsin for MMP-3, -8, and TIMP-1 staining for 60 minutes at 37°C. Endogenous peroxidase activity was quenched by treatment with 0.2% H_2O_2 for 3 hours. Nonspecific binding of antibodies was blocked by normal goat (MMP-8) and normal horse (type II collagen, TIMP-1, MMP-3) serum treatments. The sections were incubated with diluted monoclonal mouse antichicken type II collagen (0.004 µg/µL), polyclonal antigoat MMP-3 (0.2µg/µL) antibody (Santa Cruz Biotechnology), and polyclonal antirabbit MMP-8 (1µg/µl) antibody (Chemicon International) overnight at +4°C. For negative controls, polyclonal nonimmuno goat serum was used. As suggested by the antibody manufacturer, human and rat odontoblasts for TIMP-1 and MMP-8[8] and breast cancer tissue for MMP-3 were used as positive controls. The immunostaining was visualized with a Vectastain Elite kit (Vector Laboratories) using a peroxidase and diaminobenzine substrate. The sections were counterstained with Mayer's hematoxylin (Histolab Products) and subsequently examined with an image analyser in a light microscope (Leica Leitz DMRB/E) at a magnification of 150. The number of cell layers was counted in the anterior, the most superior central, and the posterior areas of the condylar cartilage. Each of the three areas was estimated to form one third of the total length of the condylar surface in the anteroposterior direction (Fig 1). The most superior central area was defined as the highest point of the condylar surface opposite to the mandibular fossa. A vertically positioned line starting from the cartilage surface and ending at the erosion front was positioned in the middle of each of three cartilage areas. The total number of cells and positively immunostained cells intersected by the line in each group was calculated twice.

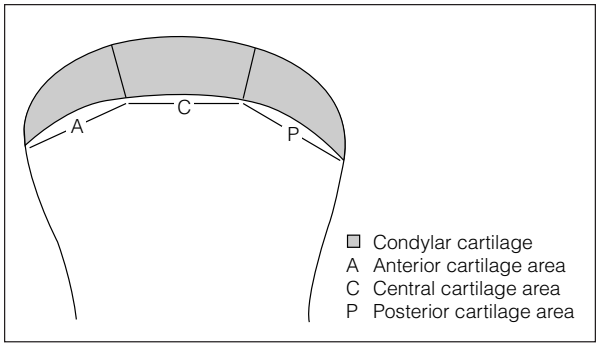

Fig 1 Schematic presentation of the areas for histomorphometric analysis of the mandibular condylar surface.

Histomorphometric Analysis

For histomorphometric analysis, the total thickness of condylar cartilage was measured in the most superior central area of the condylar surface. The number of cells was counted at the most central point of each area (Fig 1).

Statistical Analysis

The intraclass correlation coefficient for the number of MMP-3, -8, and TIMP-1 immunopositive cartilage cell double measurements was calculated, and three specimens were selected from each group for secondary analysis. The intraclass correlation coefficient was found to be 0.725. Positively immunostained cartilage cells were calculated, and comparisons were made in subgroups of 30 to 33, 50 to 53, and 200 days. Analysis of variance among treatments was carried out, followed by Bonferroni's comparisons test. *P* values less than .05 were considered significant. SPSS statistical software (version 14.0, IBM) was used for data analysis.

Results

Immunostaining of MMP-3, -8, and TIMP-1

Immunohistochemical staining revealed a positive reaction for MMP-3, -8, and TIMP-1 in cartilage cells in all three areas of the mandibular condylar cartilage in all three age groups. Both MMP-3, -8 and TIMP-1 positive cells were found in all cartilage zones.

MMP-3 Immunostaining

In rats fed the soft diet for 50 days, a change to the hard diet for 3 days caused a statistically significant decrease in MMP-3 positive cells in the anterior ($P < .01$) and posterior ($P < .001$) cartilage areas. (Figs 2 and 3). A significant decrease in the number of MMP-3 positive cells was also found in rats fed the normal diet for 53 days in the anterior ($P < .01$) and posterior ($P < .001$) cartilage areas compared to the rats fed the soft diet for 50 days followed by hard diet for 3 days. In rats fed the soft diet for 50 days and hard diet for 150 days, the number of MMP-3 positive cells was greater in the anterior area of the condyle compared to rats fed the normal diet for 200 days ($P < .01$) (Fig 3).

MMP-8 Immunostaining

In the normal diet group of 53 days, there was a significantly lower number of MMP-8 positive cells in the most superior central ($P < .01$) and posterior ($P < .001$) cartilage areas when compared to the group of 50 days on the soft diet. A significant decrease in MMP-8 positive cells in the posterior cartilage area was also seen when the diet was switched to hard for 3 days after 50 days of soft diet ($P < .001$). Rats fed the normal diet for 53 days also showed a decrease in the number ($P < .01$) of MMP-8 positive cells in the most superior central cartilage area when compared to rats fed the soft diet for 50 days followed by a switch to hard diet for 3 days (Figs 2 and 3).

TIMP-1 Immunostaining

Rats fed the normal diet for 33 days had a significantly larger number of TIMP-1 positive cells in the anterior and posterior cartilage areas ($P < .01$), whereas in the central area, the number of TIMP-1 positive cells remained similar to the two other 30- to 33-day groups (Fig 3). A switch of diet to the hard diet for 3 days after 30 days of soft diet caused a statistically significant increase in the number of TIMP-1 positive cells in the anterior and posterior ($P < .01$) and in the most superior central ($P < .05$) cartilage areas. In rats with 50 to 53 days of hard diet, the number of TIMP-1 immunopositive carti-

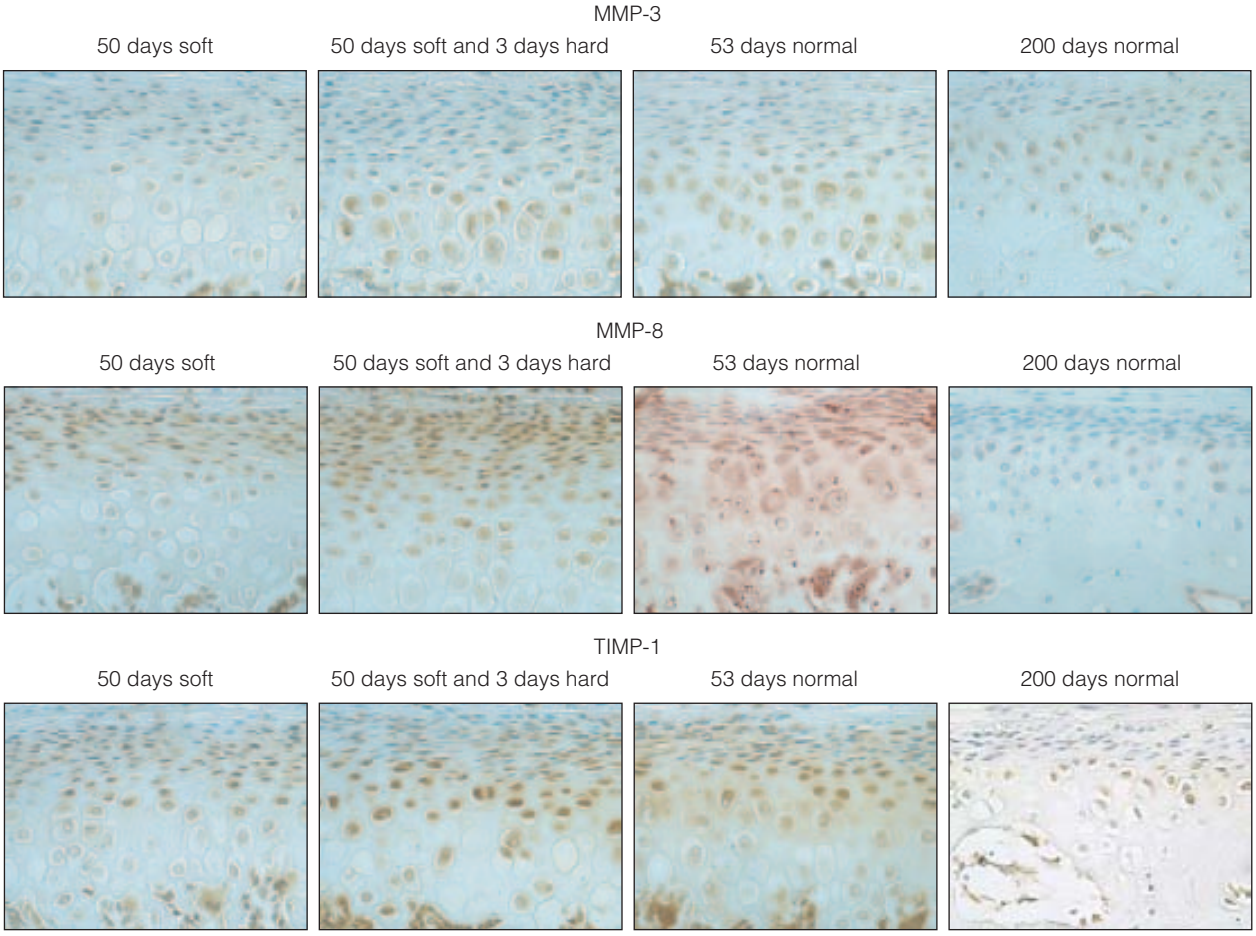

Fig 2 Immunostaining for MMP-3, MMP-8, and TIMP-1 in the central area of the mandibular condylar cartilage. Four different types of diet hardness are presented: Animals fed a soft diet for 50 days, 50 days of soft diet switched to 3 days of hard diet, 53 days of normal diet, and 200 days of normal diet.

lage cells in the most superior central area decreased compared to the anterior and posterior cartilage areas (Fig 2). In rats fed for 30 days with the soft diet followed by 170 days of the hard diet, the number of TIMP-1 positive cells was significantly ($P < .01$) larger compared to 200 days of normal diet in the anterior cartilage area. In rats fed for 50 days with the soft diet followed by 150 days of the hard diet, the number of TIMP-1 positive cells in the anterior ($P < .01$), the most superior central ($P < .05$), and posterior ($P < .001$) cartilage areas (Fig 3) was significantly larger compared to that in rats fed for 200 days of normal diet.

Histomorphometric Analysis

Animals fed for 30 or 50 days with the soft diet after weaning showed the thinnest cartilage thickness compared to animals fed with 33 or 53 days of normal diet ($P < .05$) (Fig 4). A change from 30 days of soft diet to 3 days of hard diet increased the cartilage thickness, but it was still significantly thinner ($P < .05$) compared to rats fed the normal diet for 33 days (Fig 4). The number of cartilage cells was largest in the 30- to 33-day groups of normal dietary loading. No statistically significant differences were seen in the three different age groups.

Immunostaining of Type II Collagen

The positively stained area of type II collagen was measured and compared to total cartilage area of the condylar cartilage (Fig 4). When the diet was switched to hard for 3 days after 30 or 50 days of soft diet, the area of type II collagen positive immunostaining was significantly smaller when compared to rats fed with soft diet for 30 ($P < .001$) or 50 ($P < .05$) days and with normal diet for 33 days

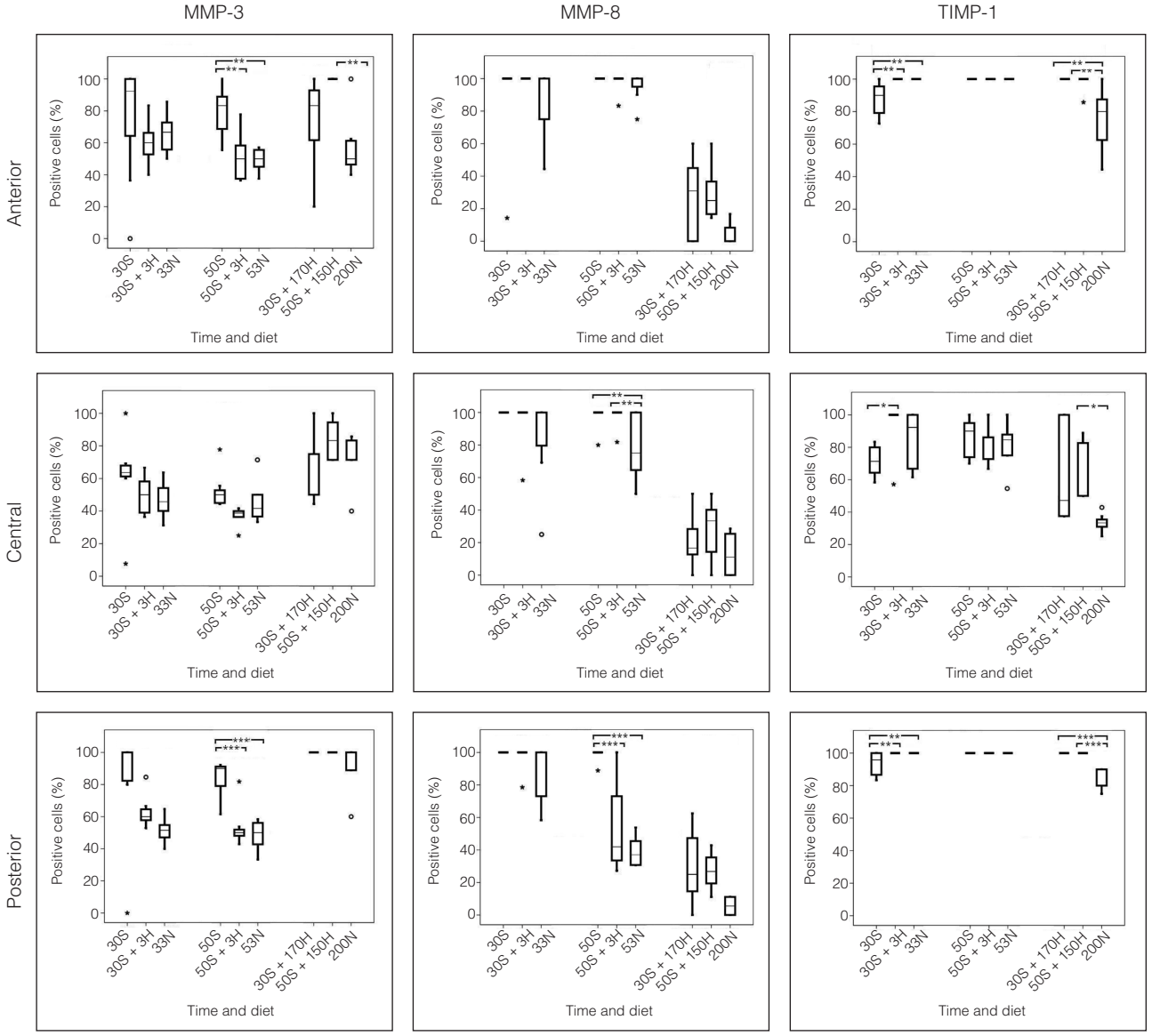

Fig 3 Relative number (%) of MMP-3, MMP-8, and TIMP-1 immunopositive cartilage cells in the anterior *(top row)*, central *(middle row)*, and posterior *(bottom row)* areas of the mandibular condylar cartilage. Nine groups are presented as three different age and dietary (S: soft diet; H: hard diet; N: normal diet) groups with 30 to 33 days, 50 to 53 days, or 200 days of dietary loading. Statistically significant differences marked as $*P < .05$, $**P < .01$, and $***P < .001$.

($P < .01$). In rats fed the soft diet for 30 or 50 days, the hard diet for 170 or 150 days, and the normal diet for 200 days, there were no statistically significant differences between the three subgroups.

Discussion

The results of the present study show that mandibular condylar cartilage is sensitive to changes in diet hardness. The presence of proteolytic enzymes and one of their tissue inhibitors was seen at all time points, and the expression was affected by age of the animal and hardness of the diet. It has earlier been found that articulatory function is able to retard the calcification process in the mandibular condyle under organ culture conditions and that the simulation of functional appliances will delay the onset of synthesis of type II collagen in the posterior region of the condyle in the rabbit.[9,10] Maturation of the condylar cartilage cells has been observed to be retarded in areas regarded as stress-bearing.[11] It has been shown that the rate of differentiation and maturation of mesenchymal cells into chondrocytes seems

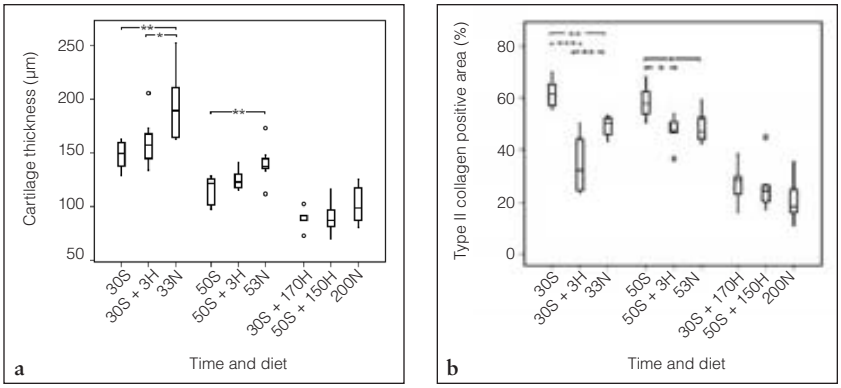

Fig 4 *(a)* Total cartilage thicknesses (µm) in the most superior central area of the mandibular condylar cartilage of nine different age and dietary (S: soft diet; H: hard diet; N: normal diet) groups; 30 to 33 days, 50 to 53 days, and 200 days of dietary loading (means for groups in homogeneous subsets are displayed). A decrease in total thickness of the mandibular condylar cartilage can be seen with increasing age of the animal groups. $*P < .05$, $**P < .01$, and $***P < .001$. *(b)* Percentages of type II collagen-positive area (μm^2) in the mandibular condylar cartilage of the nine groups. A decrease in positive immunostaining for type II collagen can be seen with increasing age of the animal groups. $*P < .05$, $**P < .01$, and $***P < .001$.

to be controlled by mechanical factors.[11] Thickness of the cartilage cell layers, the number of chondrocytes, and the deposition of type I and II collagens are sensitive to alterations in loading.[3] Maturation of cartilage cells has been shown to be slower under increased mechanical loading, and mature cartilage cells have been found together with type II collagen deposition in cartilage. Generally, type II collagen is considered a marker of mature collagen. This might explain the present finding that the area of type II collagen-positive immunostaining was largest in animals fed with soft diet without changes in diet hardness. In this study, a change of diet from soft to hard caused a decrease in both MMP-3 positive cells and the type II collagen-positive area of immunostaining in animals fed a soft diet for 50 days and a hard diet for 3 days. The slowed maturation of chondrocytes caused by the hard diet may explain this simultaneous decrease of MMP-3 and type II collagen. It could be assumed that the prolonged soft diet may cause a more rapid maturation process with a smaller number of cartilage cells and a catabolism of the cartilage tissue. The most intense immunostaining for MMPs and TIMPs (including MMP-3, -8, and TIMP-1) in condylar cartilage has been found in early stages of growth, followed by a decreased expression during maturation and aging.[12] Movements of the mandible are affected by dietary consistency. Soft diet is assumed to decrease the amplitude of loading, and it also affects masticatory function, as the food is not chewed but licked or sucked.

There have been no studies of the effect of diet hardness on MMP-8 and TIMP-1 expression in the condylar cartilage. In the authors' previous study, a short-term hard diet caused a significant decrease in the expression of TIMP-3 compared to a soft diet after 6 hours of dietary loading, after which the expression increased.[13] Cartilage cells under increased hydrostatic pressure have been shown to express increased levels of TIMP-1.[14]

In this study, the expression of MMP-3, -8, and TIMP-1 was significantly affected by diet hardness. When diet was switched to hard for 3 days after 50 days of the soft diet, the number of MMP-3 and -8 positive cells was significantly reduced, which is in line with the previous finding of elevated levels of MMP-3 expression cartilage under soft dietary loading.[4] Simultaneously, a significant increase in TIMP-1 expression was seen in rats fed the soft diet for 30 days, followed by a switch to the hard diet for 3 days, which supports the earlier *in vitro* findings.[14] In rats fed the soft diet for 30 or 50 days followed by normal diet for 170 or 150 days, the number of MMP-3 and -8 positive cells was significantly larger compared to rats fed the normal diet for 200 days, respectively. Bae et al[15] studied age-related changes in gene expression patterns of matrix metalloproteinases, including MMP-8, in the condylar cartilage of the rat TMJ. The enzyme

was expressed in the cells in all cartilaginous cell layers at ages 4 and 8 weeks and was later localized only in mature cells. In the present study, decreased MMP-8 expression was also seen in older animals of 200 days under dietary loading, which supports the view of a minor role of MMP-8 after the growth period of the TMJ condylar cartilage. The number of TIMP-1 positive cells was also significantly lower in rats fed the normal diet for 200 days compared to rats fed the combination of soft and hard diets.

The differences found in MMP and TIMP immunostainings in different cartilage areas were likely to be linked to unequal loading, since the central cartilage area is considered to be under the most loading.[16,17] When the diet was switched from soft to hard for 3 days, the number of TIMP-1 positive cells increased in all examined areas, whereas the number of MMP-3 positive cells decreased only in the anterior and posterior areas and the number of MMP-8 positive cells was decreased in the posterior part. Thus, the expression of TIMP-1 seems to be most sensitive to sudden changes of loading, most likely due to its being an inhibitor of MMPs. The finding that the anterior and posterior cartilage areas responded differently from the central area may reflect the differences in masticatory movements during different diets. A hard diet is supposed to require larger masticatory movements, where larger areas of the condyle become loaded and respond quickly with biochemical changes.

It can be concluded that the mandibular condylar cartilage maintains an adaptive response from the period of growth and development into older age. These findings suggest that early exposure to a soft diet might maintain higher collagenolytic activity in condylar cartilage for a relatively long time despite a later change to a hard diet. The production of ECM and the expression of metalloproteinases and their tissue inhibitor in condylar cartilage cells seem to be sensitive to diet hardness. However, the diminished area of type II collagen in the condylar cartilage as a result of less masticatory function can be seen even after a longer period of hard diet. This finding may indicate a diminished adaptive response in older age.

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