Osteoclastic Activity in Mandibular Condyles of Rats Fed Diets of Different Physical Consistencies

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Abstract

Previous studies have shown that the morphology of the mandible and the biology at the mandibular condyle can be affected by the consistency of the diet. A recent study reported that in the mandibular condyles of rats, the bone mineral density (BMD) and bone mineral content (BMC) were significantly reduced in animals which were fed with a softer diet when compared with others fed with a hard diet (1) . Based on these results the following hypothesis was tested: Reduction in the BMD and BMC will be observed in the mandibular condyles of those animals fed with a softer diet due to an increase in osteoclastic activity in the area of endochondral ossification of the mandibular condyle. This study aims to elucidate if a difference in the physical consistency of the diet used to feed those rats involved in the previous study affected the number of osteoclasts present in the endochondral ossification area of the mandibular condyle.

In the previous study sixty-six Wistar rats were randomly assigned to three groups of twenty-two each. Each animal received either a liquid, soft or hard diet. The test subjects were sacrificed from each group at days 7, 20, and 40 (1). Fifteen (five from each group – soft, hard, and liquid diets) histological tissue slides from the mandibular condyles were obtained from the sacrificed rats. The fifteen histological slides were processed for paraffin embedded slides and then stained for TRAP in the Anatomical Sciences Department at the University of Manitoba

Digital photographs of the stained tissues were obtained by means of a Zeiss Axio Imager M2 and the number of TRAP positive osteoclasts were counted. Of the fifteen images some did not show a condyle therefore in each group the best three images were selected and used in this study. The number of TRAP positive osteoclasts were counted and the results were statistically analyzed using a one-way ANOVA followed by a *post-hoc* duncan's new multiple range test. Results showed significant differences between the control group (Hard) and the two experimental groups (Liquid and Soft). Statistical significance was found between the Hard and the Soft groups (P value: 4.956, 1% statistical significance) and between the Hard and the Liquid groups (P value: 5.021, 1% statistical significance). This work suggests that with an increase in the softness of the diet there is an

increase in the number of osteoclasts which are present, thus supporting the hypothesis that decrease in bone mineral density due to a softer diet correlated with a higher osteoclastic activity in the posterio-superior region of the mandible.

Introduction

Mastication is a process by which a group of muscles aid in the movement of the mandible within the temporomandibular joint in order to grind and break up food (2). The consistencies of the masticated food may have significant effects on the growth of the mandible, specifically the condyle. It has been previously suggested that the physical consistencies of a diet may influence the thickness of the mandibular condyle and thus the amount of bone deposition (3, 4). Low masticatory forces, as produced by a soft consistency diet, may lead to decreased growth of the condyle (3-10). The causes of these effects which are seen in the condylar cartilage are due to the weight applied via the dentition. This in turn can induce significant changes in the thickness of the cartilage (5, 11).

The process of bone formation (ossification) occurs via three different mechanisms: intramembranous ossification, sutural ossification and endochondral ossification(11). Intramembranouns ossification occurs in the mandible and flat bones of the skull. This type of bone formation occurs via direct contact with the mesenchyme. Sutural ossification is exclusively seen in the skull. This particular ossification is located in the fibrous joints of the skull. This unique location allows for the skull and face to accommodate the growth of the brain and eyes. This type of periosteal growth in turn allows for two bones to ossify independently of their shared joint.

Endochondral ossification occurs in long bones, short bones and the base of the skull. This type of bone formation is predominantly associated with the replacement of cartilage with bone. Endochondral ossification occurs when bones are pre-formed in cartilage during early embryonic development (11).

The first site of the mandibular development is formed around seven weeks via intramembranous ossification. This site forms along the lateral aspect of Meckel's cartilage; a cartilage which is not incorporated into the bone. However this serves as a scaffold for

formation of the majority of the mandible (11). By 10 weeks of development the majority of the mandible is formed via intramembranous ossification.

Following the growth of the mandible through intramembranous ossification the appearance of secondary growth cartilage becomes evident. This cartilage is deemed secondary in order to differentiate it from Meckel's cartilage. The differences between these two cartilages are seen histologically(11). There are three predominant secondary growth cartilages, which include the coronoid cartilage, symphyseal cartilage, and condylar cartilage. Condylar cartilage first appears around the 12th week of development via endochondral ossification. The cartilage is converted to bone which eventually only leaves a thin layer of cartilage surrounding the condylar head. This can be seen around 20 weeks of development. This cartilage remains past the fourth decade of life until senescence (12).

Bone growth can be influenced by both genetic and environmental factors (8, 13-17). Direct loading on the mandible can affect the morphology of the mandibular cartilage and thus the bone density. Numerous studies have found that a decrease in masticatory functional load leads to decreased growth in the mandible, specifically in the ramus region (8, 13-17). A study by Tanaka *et al.* showed that rats fed a hard diet, had a higher degree of mineralization of trabecular bone when compared to those fed a soft diet (4). Changing the consistency of the diet has been shown to alter the forces applied to the condyle during chewing (8). Conversely, there are some studies that show an increase in certain areas of the mandible with lower functional load. This can contribute to the different areas of the mandible having different types of functional loads and shearing versus tensile loads (4, 8).

Numerous cells are involved in the process of ossification. The two main types of cells are osteogenic cells and osteoclasts. Osteogenic cells are important in helping to form and maintain bone, whereas osteoclast cells function in resorption of bone(11).

The present study focuses mainly on the activity of osteoclast cells. Osteoclasts are much larger than other osteogenic cells (11). While the majority of osteogenic cells associated with bone development are mesenchymal in origin, osteoclasts originate from

hemapoietic tissue and arise from precursors of monocyte/macrophages (11, 18). They play an essential role in the process of bone remodelling. Bone remodelling is a process which occurs throughout life and modifies with age; rapid at a young age and slower in older age (11). This process consists of five phases which involve both osteoclasts and osteoblasts (11).

Osteoclasts are predominantly present in the second phase of resorption. After the activation of the osteoclasts, old bone is removed and resorption lacuna are produced. Osteoblasts are recruited to deposit new bone. The maturing of the osteoblasts causes the inhibition of osteoclastic activity. Overall, the osteocytes have been known to be able to sense the need for remodeling (11, 19).

Cytochemically the osteoclast is best detected by tartrate-resistance acid phosphatase (TRAP) staining. The TRAP technique has been used to study the osteoclast samples from rats. The objective of this study was undertaken to determine if the lower observed bone density in the posterior-superior region of the mandibular condyle in rats fed with a soft diet was correlated with the number of osteoclasts detected (1).

Materials and Methods

In a previous study, sixty-six Wistar rats were randomly assigned to three groups of twenty-two each. Each animal received a liquid, soft or hard diet and had free access to food at all times. The animals were sacrificed from each group at days 7, 20, and 40 respectively (1). Fifteen histological slides of tissues from the mandibular condyles were prepared from those animals included in the previous study. Five of the animals were fed a hard diet which consisted of a regular food pellet. Five were fed a soft diet which consisted of a pellet reduced to a powder, mixed with water in a ratio of 1:1 weight/volume. The final five animals were fed with a liquid diet of pellet powder mixed with water at a ratio of 1:4 weight/volume. Fifteen tissue samples were processed and embedded in paraffin and stained for TRAP in the Anatomical Sciences Department at the University of Manitoba (20). This study was approved by the Bannatyne Campus Protocol Management and Review Committee (Ref 10-021; July 2, 2010).

Data Collection and Evaluation

Of interest to this study was the zone of endochondral ossification which is present at the posterior-superior region of the mandible (Figure 1). As some of these slides did not appropriately show this portion of the condyle the three best slides were taken from each group. Digital images at 5x and 10x magnification were taken using a Zeiss Axio Imager M2 which is a Bright Field Microscope (Figure 2 and 3). Once the images were obtained the 10x magnification image was chosen and a 250μm^2 box was drawn over the posterior-superior region of the condyle using the program GIMP 2.8 (Figure 4). Osteoclasts were counted in each box and the number recorded. Triplicates of these numbers were obtained, by waiting one week and re-counting to account for identification and counting errors. The mean for each set of triplicates (hard, liquid and soft) was calculated. The individual averages were taken and the overall average for each of the groups was calculated (Table 1). The final overall averages were taken and a one way ANOVA analysis of variance with Duncan's Post Hoc Analysis was conducted.

Results:

Statistical analysis showed that significant differences were detected between the control group (hard fed) and the two experimental groups (liquid and soft fed). These results are presented in Table 2.

There was statistically significant differences between the hard fed group and the soft fed group (, P value: 4.956 1% statistical significance) and no statistical significance between the hard fed group and the liquid group (P value: 5.021). That observation suggest that with an increase in the softness of the diet is related to an increase in the number of osteoclasts present. These can be seen along with the respective standard deviations in Figure 5.

Discussion:

Reduction of forces which are exerted during mastication may result in reduced bone density and a decrease in bone deposition. The results from this study suggest that there is an increase in osteoclastic activity seen when less force is applied during mastication (3, 11). The decrease in forces is potentially associated with a lower functional strain produced on the mandible and thus a decrease in the bone deposition. This may result in an overall increase in bone resorption (2, 19, 21). The increase in bone resorption may result due to an increase in osteoclast activity (11, 19). Osteocytes have been shown to be highly sensitive to mechanical stimuli and are able to quickly and effectively adapt. (19).

In this study it was observed that both the Liquid and the Soft diet groups showed an increase in osteoclastic activity compared to the control group

The area of ossification on which this study focused on was the zone of endochondral ossification. We can look to other studies that examine the effects of functional loads on long bone growth in order to place this work in context.

In a study by Klein-Nulend *et* al. examining the effects mechanical loading has on bone cells it was observed that bone mass is lost when there is absence of stimuli (19). This was clarified further in a study done by You *et al.,* where osteoclastogenesis was seen to decrease with increased functional load (18). Together, these studies suggest that the more functional load that is placed on a bone, the less bone resorption is observed, suggesting an overall decrease in the number of osteoclasts present. When bones are mechanically stimulated they have an ability to send signals which inhibit the formation of osteoclasts, where as the opposite is observed when a mechanical stimulus absent, shifting the balance towards resorption (18). These known response mechanisms are consistent with the experimental observations observed in the studies above, and in the current work. Both studies by You *et al.,* and Klein-Nulend *et al.,* support our findings that the lack of mechanical loading may result in an overall decrease in bone density (1, 18, 19).

This study has a significant number of limitations, one of which being the sample size. Due to the fact that image quality was not adequate the overall sample size had to be decreased. Of the rats that were used, the days at which these animals were sacrificed differed and thus the number of osteoclasts that were counted were not an adequate representation of bone development and resorption over time. While the results observed here are consistent with the fundamental theory of bone development and supported by previous studies, an experiment with a larger sample size and longer exposure duration would increase the confidence level of these findings.

Another limitation to this study was the use of osteoclasts as a direct indicator of bone resorption. The presence of osteoclasts suggests increase resorption, however, there were some cases where at day 40 in both soft and liquid diets (L 40 4-3 and S 40 1-3) reduced osteoclast counts were detected when compared to the 20 day rats. A follow up study could be done in order to check for biomarkers, such as OPG, RANKL, or Cathepsin K, which detect osteoclastogenesis and thereby monitoring these throughout the period of the experiment (18). This could provide a more sensitive indicator of bone resorption. This would suggest that if observing an increase in osteoclastogenesis biomarkers earlier in the soft diet group along with an increase in osteoclasts via TRAP staining, would provide a second line of evidence that a softer diet does in fact cause the increase in osteoclast activity.

Conclusions:

The results of this study demonstrated that rats fed with a soft or liquid diet had a higher level of osteoclast activity in the posterior-superior region of the mandibular condyle when compared to rats fed with a hard diet. Therefore, this study supports the idea that a softer diet increases the amount of bone resorption resulting in less dense bone.

Figures:

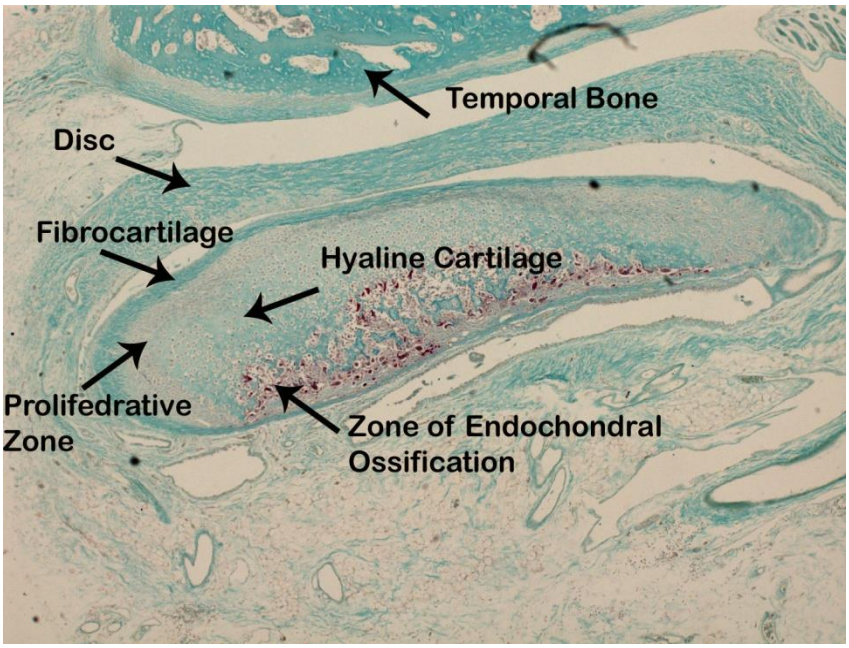

Figure 1- Condyle showing the Zone of Endochondral Ossification at 5x Magnification

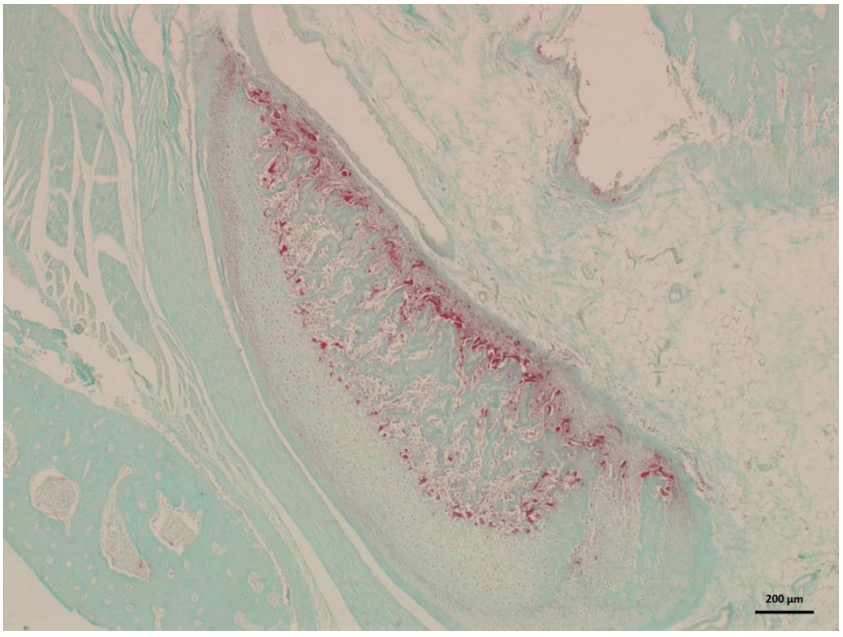

Figure 2 – Soft Diet Condyle at 5x Magnification

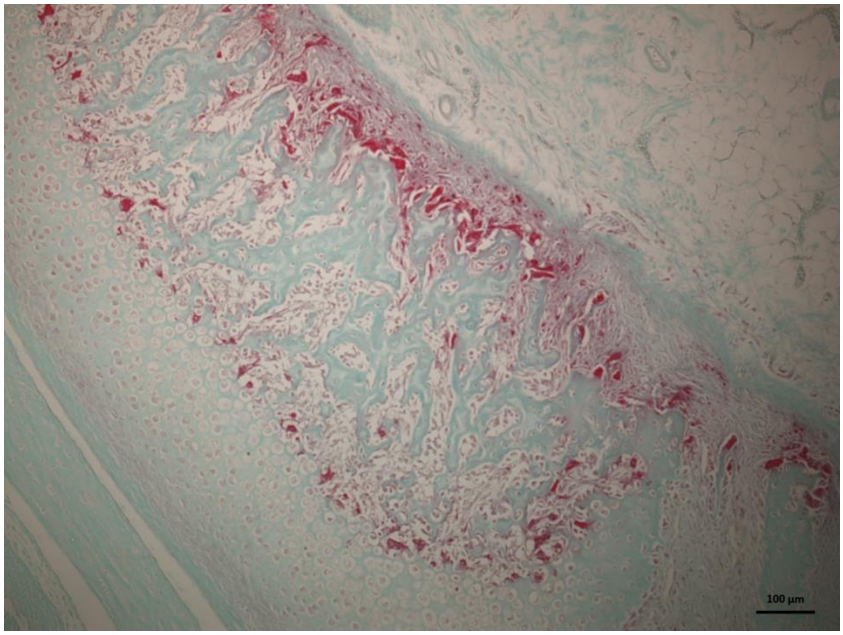

Figure 3 – Soft Diet Condyle at 10x Magnification

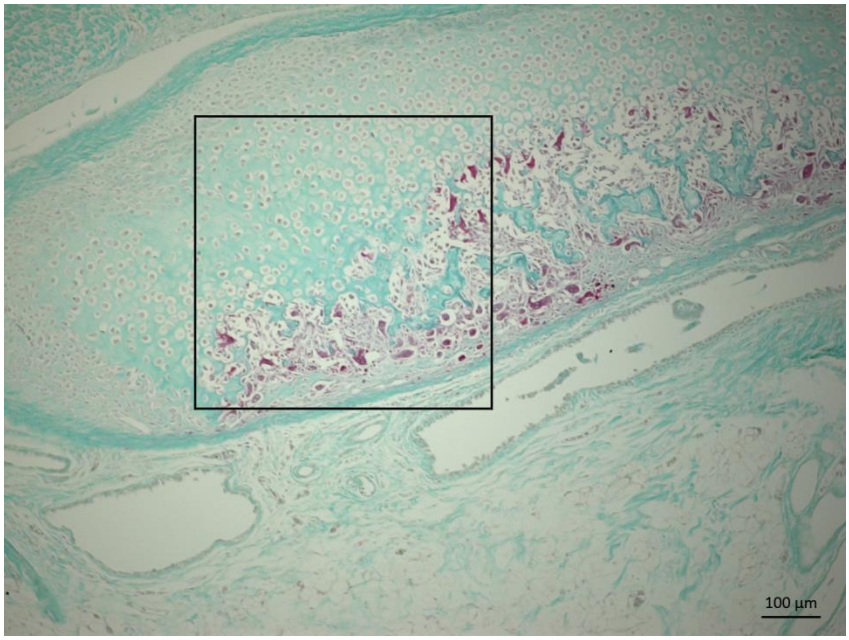

Figure 4 – 250,000µm box showing posterior-superior region of condyle

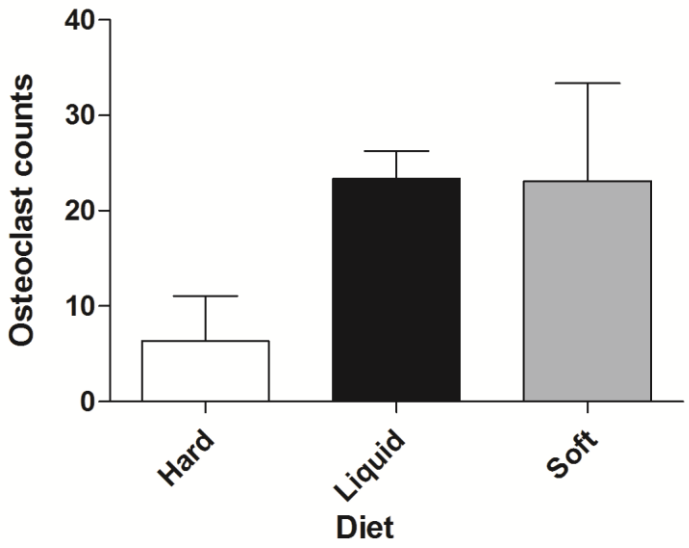

Figure 5: Osteoclast count as a function of diet. Bars represent mean ± standard error.

Tables:

Table 1 – Osteoclast counts comparing the three diet groups in this study.

Hard				**Liquid**			**Soft**		
Counts	H 40 4-3	H 20 4-3	H 40 3-3	L 20 2-3	L 20 4-3	L 40 4-3	S 40 1-3	S 20 2-3	S 20 1-3
1	17	3	1	20	28	18	2	25	37
2	15	2	1	17	29	26	6	27	41
3	15	3	0	19	29	24	4	27	39
Average	15.67	2.67	0.67	18.67	28.67	22.67	4.00	26.33	39
Overall Average	6.33			23.33			23.11		
SEM	4.7			2.9			10.2		

Table 2 – Duncan's Post Hoc Analysis comparing each group against one another for statistical significance.

Diet	**P value**	**Significant**
Hard vs. Liquid	5.021	1%
Liquid vs. Soft	0.065	NS
Soft vs. Hard	4.956	1%

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