The Microarchitecture and Atomic Mineral Composition of the Rats' Mandibular Condyle Varying Masticatory Functional Loads

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Abstract

This study was performed to determine the microarchitecture of bone and the atoms' mineral composition in the rats' mandibular condyle with varying masticatory functional loads by scanning electron microscope (SEM) and SEM energy-dispersive X-ray spectroscopy (SEM-EDX) examination. A total of 24 three-week-old Wistar male rats were divided into three groups, hard diet (HD), soft diet (SD) and liquid diet (LD). The end of eight weeks, the rats was sacrificed and the right mandibular condyles examined to study the effects of masticatory functional load on bone microarchitecture and atomic mineral composition. Qualitative analysis of SEM images showed that the HD group had smaller spaces between trabeculae and interconnected thick-walled arches compared to the SD and LD groups. SEM-EDX analysis showed the composition of the most dominant atoms in the rat mandibular condylar bone were P, Ca, Cr, Fe, Ni, and Cu. The Ca/P ratio in the HD group was significantly higher than the other groups.

The results of this study support the idea that an increase in muscular activity in the masticatory muscles caused by harder consistency of the diet leads to better bone architecture quality and correlates with the properties and quantity of mineral atoms in the mandibular condyle.

Experimental article (J Int Dent Med Res 2020; 13(2): 463-468)

Keywords: Atomic mineral, mandibular condyle, microarchitecture, SEM, SEM-EDX.

Received date: 17 December 2019 **Accept date:** 22 January 2020

Introduction

According to the World Health Organization (WHO), osteoporosis is defined as a systemic bone disease that characterized by low bone mineral density, and micro-architectural changes followed by fragility as well as fractures of bone tissue.[38] Bone tissue changes continuously throughout life due to many hormonal and physical factors, especially during the period of bone formation and growth.[1,2] Under physiological conditions, bone intermittent mechanical loading is caused predominantly by the contraction of muscles. The muscles thus provide a mechanical stimulus is important for bone remodeling by inducing strains in the skeletal system.[3] In the masticatory system, long-term changes in the pattern of muscular loading on bone can be enforced by changing the consistency of diet.[4,5] Many animal studies have been confirmed that a liquid or soft diet has an unfavorable effect on mandibular morphology and alveolar bone quality.[5] There is a decrease in bone mineral density, trabecular volume and low mineral apposition on the mandibular bone.[5,6] Tanaka et al. showed that a hard diet produced a much higher level of mineralization in the trabecular bone in the condyle, compared to animals that are given a soft diet.[7]

Bone mineralization is influenced by properties of atoms that are able to make substitutions to form a composite. Each mineral atom has specific properties. Several studies have reported the composition of atoms in normal bone. Brodziak-Dopierala et al. looked at the composition of nickel (Ni), manganese (Mn), chromium (Cr), calcium (Ca), lead (Pb), copper (Cu), iron (Fe), zinc (Zn), magnesium (Mg), potassium (K), sodium (Na), and cadmium (Cd) in different slices of the femur head, showing that

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the content of various minerals in various slices can be indicative of the amount of mechanical stress.[8] The composition of bone minerals then forms a composite that determines bone microstructure.[9]

Mastication is the process of chewing food to form a bolus that is readily swallowed and digested. The masticatory performance and digestive system play an essential role in maintaining general health and normal body functions.[39] Masticatory ability is an important aspect of stomatognathic function that affects oral health-related quality of life. Low masticatory performance has been found to relate to underweight conditions in children.[40] Mastifactory function is crucial for numerous oral tasks. It is a complex process characterized by the comminution of food into small particles to increase the food's surface area, stimulating an enzymatic function and improving the food's digestibility.[41] Mechanical stress caused by chewing food affects the masticatory muscles around the mandible, the condylar width, and changes the growth and development patterns of the mandible. The mandibular condyle is the center of growth in the mandible, the site of deposition of bone through endochondral ossification.[10,11]

Therefore, most of researches are uses it to assess the effects of the consistency of food on bone development. However, few studies have studied the effects on bone microarchitecture and atomic mineral composition of the mandibular condyle in rats of varying masticatory functional load, through examination of bone microarchitecture with a scanning electron microscope and (SEM) and SEM energy-dispersive X-ray spectroscopy (SEM-EDX).

Materials and methods

Experimental design

A total of 24 three-week-old male Wistar rats (approximately 60g body weight) were randomly divided into three equal groups (eight rats in each group). They were maintained under the following conditions. Group 1, the control group, was fed with regular rat food pellets and composed the hard diet (HD group). Group 2 were administered a soft diet (SD group), fed with a slurry of pellets softened in water with a ratio of 1:1. Group 3 was administered a liquid diet (LD group) and fed a blended mixture of pellets and water at a ratio of 1:4. All groups were given water and fed ad libitum. The rats were caged individually in suspended metal cages. Apart from the diet there were no other materials or objects that could be a masticatory stimulus inside the cages. Body weight and physical condition were checked weekly to monitor the growth and health of the animals. After eight weeks of growth under the above conditions, all rats were sacrificed by exsanguination under anesthesia, with pentobarbital sodium at a fetal overdose of 50 mg/kg.

Immediately after sacrifice, the right mandibular condyles were carefully removed. Specimens were washed in physiological saline to remove synovial fluid and blood and frozen at -20ºC until testing. The samples were thawed at room temperature and air dried before assessment. Prior to electron microscopy, they were covered with gold palladium by a sputtering process (EMITECH SC7620 Sputter Coater). The mandibular condyles were taken for determination of bone microstructure by means of scanning electron microscopy (SEM) using 500x and 1000x magnification and the mineral atomic composition of the bone determined using SEM-EDX (FEI Inspect S50).

Statistical analysis

Statistical analysis was performed using SPSS 16.0 for Windows (IBM, USA). Normality of the data was tested with the Shapiro–Wilk test. Results were compared using ANOVA with Tukey's post hoc test. The differences are reported as percentages. $P<0.05$ was considered to be statistically significant.

Ethical Clearance

All experimental procedures were approved by the Ethical Committee of the Faculty of Dentistry, Universitas Indonesia (No. 27/Ethical Approval/FKGUI/VII/2016). Informed consent was obtained from all subjects after they received an explanation about the study.[39] This experimental protocol was approved by the Ethics Committee for Animal Research of Universitas Brawijaya, Malang, Indonesia, Registration No. 269/EC/KEPK/07/2017.

Results

The results showed that the three experimental groups did not show any change in their masticatory pattern in response to the varying masticatory functional load. There was no significant difference in the Lee Index between LD, SD and HD groups during 8 weeks entire experimental period (Table 1).

	Group	n	Mean	Median	Minimum	Maximum	Standard Deviation
Lee Index	HD	8	275.38	270.44	264.65	299.24	11.34
	SD	8	277.76	278.12	264.63	292.89	9.78
	LD	8	276.46	276.72	266.91	288.24	7.44

Table 1. Lee index difference in three experimental groups. HD: hard diet group; SD: soft diet group; LD: liquid diet group, for 8 weeks of experiment.

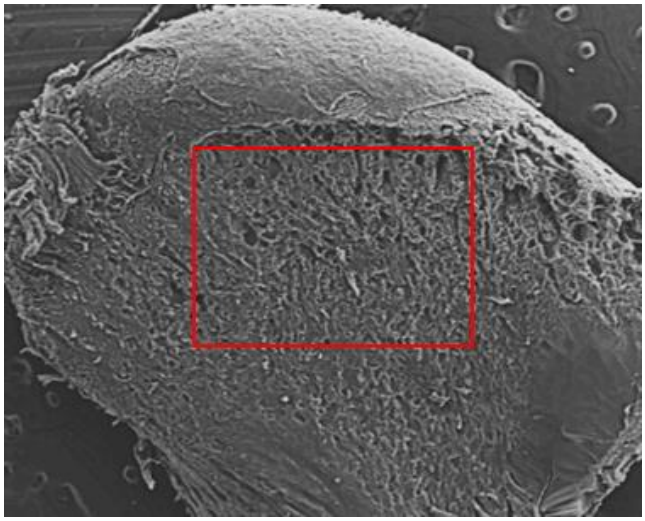

Figure 1. Scanning electron microscopy image of condyle mandibular at 100x magnification. The red box indicates the area where bone microarchitecture and atomic mineral composition assessment was performed.

On the basis of SEM analysis (Figure 1), this study found differences in microarchitecture bones between the three groups. At 500x magnification, SEM images of condyle mandibular HD group showed that the trabecular structures were still massive with interconnected thick-walled arches, without apparent granular structure. In the SD group, it began to appear the crack trabeculae, although these were still rare. The LD group showed trabeculae with cavities due to excessive resorption of the trabecular arches (Figure 2).

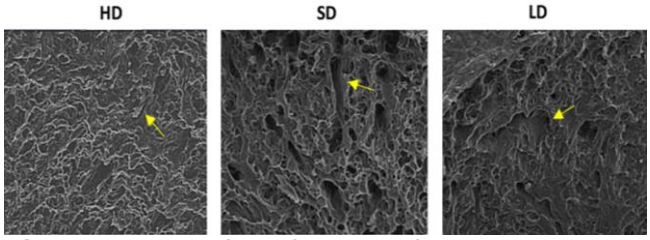

Figure 2. Scanning electron microscopy at 500x magnification showing comparative bone micro-architecture. Arrows indicate trabecular space. HD: hard diet group; SD: soft diet group; LD: liquid diet group.

At 1000x magnification, in the HD group there was no apparent granular structure and the dominant feature was the presence of interconnected fibrillar strands. The trabecular walls were still thick with a knobby surface without any cracks. The SD group showed resorption cavities but no large perforations; the trabecular walls were reduced compared to the HD group. The LD group showed the presence of perforations in several locations, surrounded by resorption cavities and trabecular stumps. The surface of the remaining trabecular structure appeared thin and flattened (Figure 3).

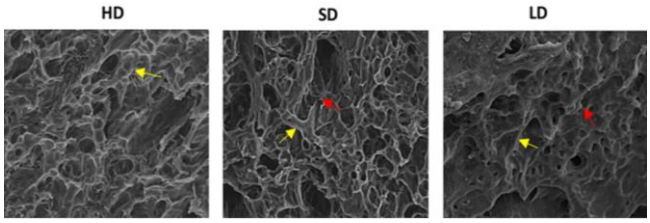

Figure 3. Scanning electron microscopy at 1000x magnification showing comparative bone micro-architecture. Yellow arrows indicate trabecular walls. Red arrows indicate trabecular stumps. HD: hard diet group; SD: soft diet group; LD: liquid diet group.

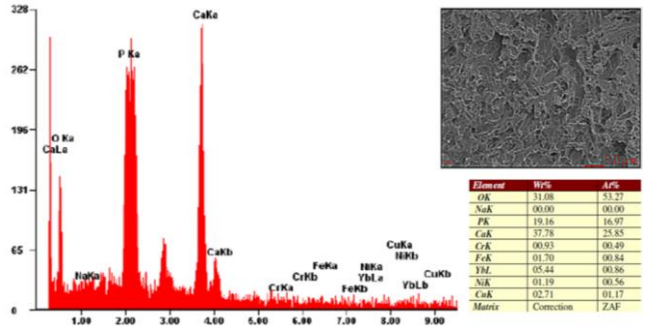

Figure 4. Representative SEM micrographs (Scale bars =50 µm) and EDX spectra with atomic concentrations (At%).

In the SEM-EDX analysis, the dominant atomic elements contained in the rat mandibular condylar bone were P, Ca, Cr, Fe, Ni, and Cu (Figure4). There were quantitative differences in atomic composition between the HD, SD and LD groups. Between the three groups, the highest atomic components in the HD group were Ca and Fe. Meanwhile, the highest in the LD group was P, Cr, Ni, and Cu. As shown by the ANOVA test, the amount of Ca atoms increased along with increasing food consistency, but there was no significant difference when compared to the SD and LD groups. Another atom with a higher concentration in the HD group is Fe. The mean P atomic levels increased significantly in the LD group compared to the HD and the SD group (both $p<0.05$). The ratio of Ca/P is more crucial for the assessment of bone health than single Ca and P concentrations. In the HD group the average Ca/P ratio was 1.28 (std. dev. 0.09), the levels were decreasing in SD group 1.22 (0.17) and the LD group was 1.05 (0.11). In the LD group, the atoms Cr, Ni, Cu, had a higher value, although not significantly different (Table 2).

	HD	SD	LD
P (%)	15.89 ± 1.68	18.97 ± 1.09[a]	19.09 ±1.99[a,b]
Ca (%)	23.11 ± 3.02	20.25 ± 1.89	20.14 ± 3.19
Cr (%)	0.77± 0.23	0.55 ± 0.34	0.89 ± 0.39
Fe (%)	1.47 ± 0.61	1.29 ± 0.43	1.05 ± 0.96
Ni (%)	1.67 ± 0.56	1.47 ± 1.14	1.79 ± 1.72
Ca/P ratio	1.28 ± 0.09	1.22 ± 0.18	1.05 ± 0.11[a]

Table 2. Average of atomic mineral concentrations. Data are reported as means ± standard deviation. HD: hard diet group; SD: soft diet group; LD: liquid diet group. [a]$P<0.05$ vs control (HD group); [b]$P<0.05$ vs SD group. ANOVA followed by Tukey's post-hoc test was used for statistical analysis.

Discussion

The results of this study demonstrate that dietary consistency does not interfere with the physical growth of animals. The Lee Index also showed no statistically significant difference among the three groups of rats (Table 1). A Lee Index value below 300 in rats is considered normal (see Lee 1928). Bone is a living material that is able to renew itself and that adapts its structure and density to changes in the mechanical environment. Bones maintain an optimal architecture; in this case, the mandibular condyle undergoes various mechanical loads, which compels the condyle to be very adaptive.[12,13] Trabecular bone is more sensitive than cortical bone to hormones and many factors such as age, diet, and health status that are involved in modulating bone metabolism.[14]

Qualitative analysis of trabecular bone of the rat mandibular condyle through examination of SEM images showed that the HD group had smaller spaces between trabeculae and interconnected thick-walled arches, showing a higher quality of bone microarchitecture compared to the SD and LD groups (Figures 2 and 3). The SD group began to see a decrease in bone microstructure that was characterized by trabecular cracks, and the structure of the whole trabecula was degenerate and thinned. However, the bone structure of the SD group was more organized compared to the LD group. The bone trabeculae of the LD group were more irregular and thinner, with a larger space between trabeculae with thin and flat walls, many cracks and granular structures. Dimensions of variable granular structures that are surround the resorption cavities and trabecular stumps. Granule formation results in increased bone porosity. It can be seen that the LD group had the lowest bone quality among the three groups with a degraded microarchitectural character.

Most studies suggest that the consistency of a harder diet led to better bone quality due to the increased muscular activity in the muscles of mastication. Some studies use bone mineral density (BMD) to assess the effects of masticatory function on the quality of animal mandibular bone.[7,15] Gurreiro et al. estimated bone density in the ramus area of the mandible by digital radiography[16], while Kufley et al. analyzed bone quality by looking at micro CT scan values of BMD.[6,16] However, other studies in humans suggest that examining bone strength obtained by measuring BMD may not be enough because it has been reported that up to 80% of low trauma fractures occur in individuals without osteoporosis through DXA examination.[17] Orkun Gül et al. states that BMD measurements are not the only criterion in the diagnosis of osteoporosis; several factors such as genetic predisposition, bone turnover, and bone microarchitecture are also important.[18]

In term of the nanomaterial scale, differences in bone microstructure are strongly

influenced by the properties of atoms that are capable of substitution to form composites.[19] SEM-EDX is capable of analyzing the atomic composition of materials by detecting atomic structures in the bone. Vora et al. states that SEM-EDX can be used for verification of the Ca/P hydroxyapatite mass ratio.[20] Many elements have a significant impact on bone metabolism.[9] In the HD group, there were high Ca and low P levels (Table 2). It has been stated previously that changes in the Ca/P ratio are very important for assessment of bone quality and is a better predictor than single Ca and P concentrations to assess bone abnormalities.[21] Calcium (Ca) and phosphorus (P) are some of the elements composing hydroxyapatite, which is a main and important component in bones. The Ca/P ratio in the HD group was significantly higher than the LD group. When correlated with SEM images, the trabecular structures of the HD group appear to describe the best bone quality among the three groups. In the LD group, the low Ca and high P values cause the Ca/P ratio in this group to also decrease. It appears that the LD group has the lowest bone quality among the three groups, with a degraded microarchitecture character.

Another element with higher concentrations in the HD group was Fe. Iron plays an important role in bone formation as an enzyme cofactor of collagen synthesis. Bone breaking strength is lower in iron-deficient animals indicating that iron deficiency plays a role in bone fragility.[22,23] In the LD group, besides P, other elemental concentrations that were found to be higher than in the HD and SD groups were Cr, Cu, and Ni. The role of Cr in bone metabolism is not yet fully understood.[24] But it is known that chromium is a trace element which stimulates the pancreas and insulin action, facilitates the diffusion of glucose into cells, and is also involved in the metabolism of carbohydrates and proteins .[25–27] Copper is a cofactor for lysyl oxidase, the enzyme responsible for the cross-linking of collagen fibers. Disorders in the formation of crosslinks lead to a weakening of bone.[28] Copper also reduces the suppression of bone turnover by osteoblasts and osteoclasts.[29–31] In humans, Ni deficiency causes a decrease in oxygen consumption and an increase in the accumulation of liver fats. This occurs mainly in soft tissues, although its presence and influence in bone metabolism have also been confirmed.[32,33]

Although increasing mineral content makes bones harder and stronger, when the optimum mineralization point or limit for mineralization is exceeded, the material loses its strength; however, within physiological limits, mineral content and ultimate strength are positively correlated.[34,35] Certain mineral atoms in the bones will provide the consequences of substitution or incorporation activities with other mineral atoms; this mechanism will determine bone microstructure, implicated in imbalances between formations and bone resorption and changes in bonding andorganic bone structure.[19,36,37]

Conclusions

In this study, consistency of the diet had an impact on bone microarchitecture thought to be based on the properties of mineral atoms in the formation of composites. The Ca/P ratio, which can be a good predictor for assessing bone quality, had its the highest value in the HD group and was significantly different compared to the SD and LD groups. Qualitative analysis of SEM images showed that the HD group had a smaller space between trabeculae, showing good quality microarchitecture of bone. This shows that besides these properties, the quantity of mineral atoms also had a correlation with bone architecture quality.

Acknowledgements

The authors gratefully thank you for the financial assistance received from the Directorate General of Strengthening for Research and Development, the Ministry of Research, Technology and Higher Education through a Doctoral Research grant No: 338.178 / UN10.C10 / PN / 2018.

Declaration of Interest

The authors report no conflict of interest.

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