The masticatory system under varying functional load. Part 2: effect of reduced masticatory load on the degree and distribution of mineralization in the rabbit mandible

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SUMMARY A reduction in mechanical loading of the mandible brought about by mastication of soft food is assumed to decrease the remodelling rate of bone, which, in turn, might increase the degree of bone mineralization.

The effect of a reduction in masticatory functional load on the degree and distribution of mineralization of mandibular bone was investigated in male juvenile New Zealand White rabbits. The experimental animals (n = 8) had been raised on a diet of soft pellets from 8 to 20 weeks of age, while the controls (n = 8) had been fed pellets of normal hardness. The degree of mineralization of bone (DMB) was assessed at the attachment sites of various jaw muscles, the condylar head, and the alveolar process. Differences between groups and among sites were tested for statistical significance using a Student's *t*-test and one-way analysis of variance, respectively.

The DMB did not differ significantly between the experimental and control animals at any of the sites assessed. However, in the rabbits that had been fed soft pellets, both cortical bone at the attachment sites of the temporalis and digastric muscles and cortical bone in the alveolar process had a significantly higher DMB than cortical bone at the attachment site of the masseter muscle, while there were no significant differences among these sites in the control animals.

The results suggest that a moderate reduction in masticatory functional load does not significantly affect the remodelling rate and the DMB in areas of the mandible that are loaded during mastication but might induce a more heterogeneous mineral distribution.

Introduction

Bone is a dynamic tissue, which continuously undergoes adaptive remodelling, i.e. resorption and apposition, to meet the requirements of its functional environment. The remodelling rate is a major determinant of the degree of mineralization of bone (DMB; Boivin and Meunier, 2002). A higher remodelling rate decreases the time available for secondary mineralization, which results in bone with a lower DMB (Boivin *et al.*, 2009).

The remodelling rate of bone is related to the magnitude of intermittent mechanical loading and the resulting dynamic strains in the tissue (Lisková and Hert, 1971; Turner, 1998). In general, more heavily loaded bone has a higher remodelling rate and is therefore less mineralized and less stiff than lower loaded bone (Rubin and Lanyon, 1985; Cullen *et al.*, 2001). Regional differences in the DMB of cortical bone have been described in a number of species (Riggs *et al.*, 1993; Loveridge *et al.*, 2004; van Ruijven *et al.*, 2007). This regionally heterogeneous organization of bone mineral has been attributed to regional differences in the magnitude and mode of strain brought about by mechanical loading (Skedros *et al.*, 1994).

Under physiological conditions, intermittent mechanical loading of bone is caused predominantly by muscle contractions. The muscles thus provide an important mechanical stimulus for bone remodelling by inducing strains in the skeletal system (Turner, 2000). The significance of muscle-generated bone loading is illustrated by the effect on the skeleton under conditions of increased or decreased muscle activity. For example, the loss of normal physiologic loading after spinal cord injury causes rapid severe bone loss in the paralyzed extremities of affected individuals, which can be counteracted by long-term electrical stimulation of muscles (Dudley-Javoroski and Shields, 2008).

In the masticatory system, long-term alterations in the pattern of muscular strains can be enforced by changing the consistency of the available food (Yamada and Kimmel, 1991; Kiliaridis et al., 1996). For instance, the continuous intake of a soft diet during growth and development has been shown to reduce the functional capacity of jaw muscles (Kiliaridis and Shyu, 1988; Liu et al., 1998) and to influence the morphology (Abed et al., 2007; Ödman et al., 2008; Enomoto et al., 2010) and internal bone structure of the mandible (Bresin et al., 1999). The reduction in intermittent mechanical loading of the mandible during mastication of a soft diet may also decrease the rate of bone remodelling (Bouvier and Hylander, 1981), which, in turn, would increase the DMB. As mechanical loading during mastication is not evenly distributed over the mandible, this increase might be regionally different. For instance, changes in the DMB as a result of altered mechanical stimulation might be most pronounced in areas where muscle contractions load mandibular bone directly, such as the attachment sites of the jaw muscles or in areas where muscle contractions create reaction forces, such as the alveolar process and temporomandibular joint.

The aim of this study was to investigate the effect of a reduction in masticatory load on the mineralization of mandibular bone. For this purpose, the degree and distribution of mineralization was assessed in mandibles of rabbits that had been fed diets of different physical consistency during late postnatal development. Since the DMB is assumed to be related to the mechanical loading generated by muscle contractions, it was hypothesized that the DMB of mandibular bone would show region-specific increases, especially at the sites of muscle attachment, in response to reduced food hardness.

Materials and methods

Animal experiment and tissue preparation

The animal experiment has been fully described in Part 1. In brief, 16 male New Zealand White rabbits were randomly divided into two equal-sized groups at the age of 8 weeks. The experimental group was fed a diet of soft pellets requiring significantly reduced peak loadings (10 N/cm²) to break the pellet in comparison with the standard pellets (120 N/cm²) fed to the control group. At 20 weeks of age, the animals were killed, their mandibles were dissected, carefully freed from soft tissues, and split in half at the symphysis. The tooth-bearing fragments were separated from the ascending rami by vertical cuts carried out dorsal to the crowns of the molars. Care was taken not to cut the bone at the attachment sites of the masseter and medial pterygoid muscles. All bone samples were obtained within 8 hours *post mortem* and stored in methanol at 4°C before analysis.

Degree and distribution of mineralization

The right hemimandibles were scanned in a micro-computed tomography system (CT40; Scanco Medical AG, Brüttisellen,

Switzerland) at an isotropic spatial resolution of 18 m, as described in detail elsewhere (Mulder *et al.*, 2004). The computed linear attenuation coefficient of the X-ray beam for each volume element (voxel) was represented by a grey value in the reconstruction. This attenuation coefficient is proportional to the local DMB (Nuzzo *et al.*, 2002; Mulder *et al.*, 2004).

The DMB was determined in eight predefined volumes of interest (VOI) of each hemimandible as the mass of the mineralized bone tissue relative to the volume of bone. This parameter is independent from the total volume or the amount of bone present in the VOI. The VOIs were selected at the attachment sites of the superficial masseter (M1–M3, ventral to dorsal), superficial temporalis, medial pterygoid, and digastric muscles, in the alveolar process adjacent to the second molar, and within the condylar head (Figure 1). The VOIs contained only cortical bone, except for that selected at the condylar head, which contained both cortical and cancellous bone.

Three-dimensional reconstructions of the VOIs were segmented to discriminate bone from background. The optimum thresholds for the VOIs were visually determined in four scans by gradual variation and comparison of the outcome with the original scan (Renders et al., 2006). The mean values were applied as fixed thresholds to the segmentation of all VOIs to allow comparison of the samples (Ding et al., 1999). This procedure was performed separately for the VOIs containing only cortical bone and those containing both cortical and cancellous bone. In a segmented image, only voxels with a linear attenuation value above the threshold, i.e. those representing bone, kept their original grey value, while voxels with a linear attenuation value below the threshold were transparent. The two outermost voxel layers characterized as bone were disregarded as these layers were likely to be corrupted by partial volume effects. Each grey value was then converted into a DMB value, using reference measurements of a calibration phantom containing hydroxyapatite in concentrations of 0, 50, 200, 800, and 1200 mg/cm³ (QRM GmbH, Möhrendorf, Germany). The error of the method, determined as the relative difference between the measured and actual mineral density, was less than 3 per cent.

Statistical analysis

Mean values, standard deviations (SDs), coefficients of variation, and frequency distributions of the DMB were calculated for each VOI. The width of each distribution curve was calculated as twice the value of the SD. Differences between experimental and control groups were tested for statistical significance, for each VOI separately, using a Student's *t*-test, after the data had been tested for normality (Kolmogorov-Smirnov test). Differences among VOIs were tested for statistical significance, for each group of animals separately, using one-way analysis of variance with Holm-Sidak's method as the *post hoc* pairwise comparison



Figure 1 Lateral (top) and medial (bottom) views of a reconstructed right hemimandible showing the volumes of interest. C: condylar head. Selection cranial to the greatest medio-lateral extent and limited to the anterior fourth of the condylar process. M1-M3: attachment site of the superficial masseter muscle. Selections are bounded by lines perpendicularly intersecting a reference line, which connects the notches ventrocaudal and craniodorsal to the mandibular angle, at 25, 50, 75, and 100 per cent of its length. T: attachment site of the superficial temporalis muscle. A: alveolar bone. Selection medial to the second molar and cranial to a reference line parallel to the lower border of the mandible at the height of the incisor alveolar process. D: attachment site of the digastric muscle. Selection dorsal to the symphysis and between two reference lines parallel to the lower border of the mandible at 50 and 100 per cent of the distance between the lower border of the mandible and the most cranial part of the symphysis. P: attachment site of the medial pterygoid muscle. Selection is bounded by lines perpendicularly intersecting a reference line, which connects the notches ventrocaudal and craniodorsal to the mandibular angle, at 33 and 66 per cent of its length.

procedure. Statistical analyses were performed using SigmaStat 3.5 (Systat Software Inc., Point Richmond, California, USA) with *P*-values of less than 0.05 considered statistically significant.

Results

Mean values, SDs, and coefficients of variation of the DMB in the VOIs studied are shown in Table 1. Statistical testing revealed no significant differences between the experimental and control groups. However, in both the experimental and control groups, there were statistically significant differences in the DMB among the VOIs. In the experimental group, all

	Experimental*		Control*	
	DMB (mg HA/cm ³)	CV (%)	DMB (mg HA/cm ³)	CV (%)
M1**	1151.32 ± 122.44^{a}	10.63	1171.37 ± 80.53^{a}	6.87
M2**	1093.10 ± 79.86^{b}	7.30	1116.08 ± 58.38 ^b	5.23
M3**	1034.55 ± 67.88 ^{c,d,e}	6.56	1076.34 ± 46.31	4.30
[**	1175.62 ± 77.01 ^{c,f}	6.55	1154.27 ± 94.94°	8.22
)**	1150.04 ± 94.87^{g}	8.24	1183.04 ± 70.75^{d}	5.98
)**	$1236.83 \pm 108.63^{d,h}$	8.78	$1195.46 \pm 146.38^{\circ}$	12.24
\ **	1277.96 ± 103.78 ^{b,e,i}	8.12	$1212.80 \pm 70.24^{\rm f}$	5.79
]**	987 60 + 36 39 ^{a,f,g,h,i}	3.68	996 62 + 34 63 ^{a,b,c,d,e,f}	3.47

*Statistically significant differences among volumes of interest, one-way analysis of variance P < 0.05.

**Volumes of interest selected at the attachment sites of the masseter (M1–M3), temporalis (T), medial pterygoid (P), and digastric (D) muscles, in the alveolar process (A) and within the condylar head (C). For detailed explanation, see Figure 1.

Within each column, groups depicted by the same superscript letters are statistically significantly different in *post hoc* pairwise comparison, Holm-Sidak's method P < 0.05.

VOIs containing only cortical bone, except for those in the mid- (M2) and dorsal (M3) parts of the attachment site of the masseter muscle, had a higher DMB than that selected within the condylar head. In addition, the attachment sites of the temporalis and digastric muscles and the alveolar bone medial to the second molar had a higher DMB than the dorsal part (M3) of the attachment site of the masseter muscle. The alveolar bone was also more highly mineralized than the mid-part (M2) of the attachment site of the masseter muscle. In the control group, all VOIs containing only cortical bone, except for that selected in the dorsal part (M3) of the attachment site of the masseter muscle, had a higher DMB than the VOI in the condylar head. These findings were similar to those in the experimental group. In contrast to the experimental group, there were no significant differences among the VOIs containing only cortical bone in the control group.

The frequency distribution curves of the DMB (Figure 2) did not differ significantly in width between the groups for any of the VOIs, suggesting that the degree of variation in DMB within the individual VOIs was similar in the experimental and control animals. The difference in their relative positions, i.e. the wider spread of the curves, indicated a greater heterogeneity in DMB among the sites studied in the experimental animals.

Discussion

The present study investigated the effect of a masticatory functional change on the mineralization of mandibular bone. It was assumed that a reduction in intermittent



Figure 2 Mean distributions of the degree of mineralization of bone in the various volumes of interest studied in the experimental (top) and control (bottom) groups. For explanation of legend see Figure 1. HA, hydroxyapatite.

mechanical loading during mastication of a soft diet would decrease the rate of bone remodelling and increase the DMB. The results showed that reduced food hardness did not cause significant changes in the DMB at the examined sites. These findings differ from the results of other studies, which have shown that feeding diets of different consistency to growing rats might lead to a reduction in the rate of bone apposition (Yamada and Kimmel, 1991), resulting in lower bone mass (Bresin *et al.*, 1999) and alveolar bone density (Mavropoulos *et al.*, 2004, 2005) as well as in a higher degree of mineralization of mandibular bone (Tanaka *et al.*, 2007).

There are a number of possible reasons for this difference. One possibility is that the experimental period in the present research might have been too short to induce significant changes in the DMB. However, in the current study, the rabbits were fed diets of different consistency for 12 weeks, which, considering their life span, was comparable with experimental periods of 4–6 weeks typically used in similar studies on rats (Bresin *et al.*, 1999; Maki *et al.*, 2002; Mavropoulos *et al.*, 2004, 2005). Furthermore, changes in mandibular morphology resulting from decreased masticatory function have been reported in rabbits as early as 40 days after a reduction in dietary consistency (Tuominen *et al.*, 1993). Taking these considerations together, the duration of diet

change in the present study was considered sufficiently long to induce changes in mandibular bone properties.

Another possible reason for the above difference in the findings might be a greater interindividual difference, which would mask the changes produced by the alteration in masticatory load, particularly with regard to the very small differences in the DMB between the experimental and control groups reported elsewhere (Tanaka *et al.*, 2007). This, however, is probably not the case because interindividual variation in the DMB (Table 1) was low, as shown by the low coefficients of variation, and was comparable with the results of the study of Tanaka *et al.* (2007). This finding supports the hypothesis proposed by Reid and Boyde (1987) that under physiological conditions, the rate of bone remodelling at a particular site can be considered a constant biological parameter.

Similar to earlier studies, the present investigation was carried out on juvenile animals as functional alterations influence bone tissue more effectively during adolescence (Parfitt, 1994). However, it has to be noted that changes in the properties of growing bones cannot, other than in a mature organism, solely be attributed to adaptive remodelling, i.e. resorption and apposition, but may be influenced by modelling, i.e. bone deposition during growth.

Most likely, the above disparity in results is based on the difference in food hardness used in various studies. Significant changes in mandibular bone properties in response to reduced food consistency have been reported in animals fed powdered (Maki et al., 2002; Tanaka et al., 2007) or liquefied (Yamada and Kimmel, 1991; Bresin et al., 1999; Mavropoulos et al., 2004, 2005) food. Although this experimental approach imposes greater differences in masticatory functional loads on experimental and control animals, it also alters more than just the dietary consistency. Powdered or liquefied food eliminates the need for mastication (Mavropoulos et al., 2004) and changes the pattern of food uptake from incising and chewing into licking and sucking (Kitagawa et al., 2004). In contrast to this experimental approach, the present study used purpose-made soft pellets, which did not change the feeding behaviour of the experimental animals. With regard to the difference in the consistency of the standard pellets fed to the control animals, these pellets mimic a 10-fold difference in the compressive strength between hard and soft foods normally eaten by humans (Yanagisawa et al., 1985). The continuous intake of these pellets did not induce a significant alteration in the DMB in the present study. This result is in accordance with the finding of Maki et al. (2002) who, comparing powdered and kneaded diets with pellets of normal hardness, found a significantly different mandibular DMB in the animals fed a powdered diet, but not in those fed a kneaded diet with a consistency similar to the soft pellets used in the present study. Considering these findings, it appears that the DMB tends to increase only as a result of a significant reduction in masticatory activity induced by an unusually soft diet, such as a powdered diet.

The importance of bone remodelling in determining the average level of bone mineralization is generally accepted. In adult bone, the rate of remodelling is the major biological determinant of the DMB (Boivin et al., 2009). The DMB increases when bone formation is suppressed and decreases when new bone formation is increased (Boivin and Meunier, 2002). The significantly higher DMB in the mandibles of rats raised on a powdered diet has been attributed to a reduction in the strain stimulus for new bone formation (Tanaka et al., 2007). This reduction may have led to a preponderance of bone resorption as a result of disuse atrophy (Ferretti et al., 2003), which is always observed in periods of physical inactivity (Forsén et al., 1994). The findings of the present study suggest that the gentle loading during mastication of the softer pellets might have been sufficient exercise to prevent disuse atrophy. These considerations are in accordance with the finding that low-level mechanical signals can inhibit osteoclastic activity in the growing skeleton (Xie et al., 2006).

Regional differences in the DMB have been reported for various bones and species (Riggs et al., 1993; Loveridge et al., 2004; van Ruijven et al., 2007). The present investigation revealed significant differences in the DMB among the mandibular sites studied. In both the experimental and control groups, the DMB in the condylar head was lower than that at the cortical sites of the mandibular body, most probably because of the presence of cancellous bone in the condylar head. Differences in the DMB between cancellous and cortical mandibular bone are well documented (Mulder et al., 2006; van Ruijven et al., 2007; Willems et al., 2007) and have been attributed to a higher remodelling rate in cancellous bone compared with cortical bone (Renders et al., 2006). The DMB also differed significantly among cortical sites of the mandible but only in the experimental group. The attachment site of the masseter muscle was less highly mineralized than those of the temporalis and digastric muscles and the alveolar bone site. These findings suggest a greater heterogeneity in the DMB in the mandibles of the experimental animals.

Regional adaptations in material organization of bone reflect regional variations in strain magnitude (Skedros *et al.*, 1994). It is plausible that the attachment site of the masseter muscle, which is the main generator of force during mastication (Weijs *et al.*, 1989), is more heavily loaded than the attachment sites of the digastric and temporalis muscles or the bone of the alveolar process. In the present study, a lower DMB was found at the attachment site of the masseter muscle, which most likely resulted from a higher remodelling rate. It has been suggested that any adaptive remodelling influences the material properties of bone so as to achieve some mechanical advantage or to minimize material while maintaining a constant safety factor between peak functional stress and appropriate yield stress (Lanyon *et al.*, 1979). By rendering the bone more elastic, the lower DMB at the attachment site of the masseter muscle might constitute an advantage as it allows more bending of the bone during muscle contractions. The higher DMB at the attachment sites of the digastric and temporalis muscles and in the alveolar process might have been caused by suppression of bone formation relative to bone resorption. As it is advantageous to maintain bone weight as low as possible, this might reflect the body's endeavour to optimize energy use by minimizing the amount of material needed to maintain structural integrity under altered loading conditions.

Bone mineralization is influenced by the strain distribution in cortical and cancellous bone (van Ruijven *et al.*, 2007). The greater heterogeneity in the DMB in the experimental group might also have resulted from a relative strain distribution in the mandible, which was different from that in the control group. It is reasonable to assume that the intake of soft pellets led to less deformation of the mandible during mastication, and the local strains at the attachment sites of jaw muscles had, therefore, more influence on the DMB.

Adaptive responses depend on timing, duration, and intensity of a given stimulus. In the present investigation, the experimental stimulus, i.e. the reduction in masticatory load, induced significant changes in the phenotypic properties of the less recruited jaw muscles (see Part 1) but did not cause significant changes in the DMB of the less loaded mandibular bone. Studies using similar experimental stimuli have shown that reducing the mechanical loading of the mandible during growth and development can be effective in increasing the DMB in the mandible (Tanaka et al., 2007). It seems, therefore, unlikely that the timing or the duration of the stimulus was ineligible to induce an adaptive change in the DMB of mandibular bone. However, it is possible that the stimulus was not sufficiently intense to cause significant changes in the mandibular DMB. The remodelling rate of mandibular bone, which is the main determinant of its DMB, might be under stronger genetic control and less easily influenced by environmental factors than the phenotypic properties of the jaw muscles. For this reason, a more intensive stimulus might be required to induce changes in the DMB of the mandible. Taken together, these considerations lend support to the idea that different intensities of a given stimulus might be necessary to modify the properties of muscular and skeletal craniofacial tissues.

Conclusions

The results of the present study suggest that a reduction in masticatory load within the range of physical consistency of foods eaten under normal life conditions does not strongly affect the DMB of mandibular bone in areas in which muscle contractions load mandibular bone directly or indirectly but might induce a more heterogeneous mineral distribution.

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