Estrogen Deficiency Increases the Variability of Mineralization of Bone Surrounding Teeth

Thesis

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ABSTRACT

Estrogen deficiency increases bone remodeling. Increased remodeling activity yields bone tissue with a higher percentage of immature/newly formed bone, ultimately leading to increased variability of mineralization. Due to the functional demands of mastication, alveolar bone is inherently a highly remodeled region of bone tissue. Does estrogen deficiency affect alveolar bone’s remodeling and subsequent mineralization?

Objectives: Using a rat model of ED, the objectives of this study were 1) to assess regional variation of mineralization in the mandible, and 2) to assess the effects of estrogen deficiency on the magnitude of regional variation. Methods: Ovariectomized (OVX, n=10) and sham surgery (Sham, n=10) rat mandible sections were scanned by micro-computed tomography (micro-CT) with a voxel resolution of 20 micrometers. The images of each section were manipulated using imaging software to isolate a portion of alveolar bone (AB) within 200 micrometers of root surface and a control bone (CB) region 200 micrometers removed from all internal and external mandibular bone borders. CT attenuation values were maintained and gray level voxel values were reported in Hounsfield Units (HU). Histograms were generated to analyze gray level frequency distribution. HU parameters analyzed were Mean, Standard Deviation (SD), Coefficient of Variation (COV) (to normalize SD) and percentile values (5th = Low5, and 95th = High5). Paired t-tests were used to assess regional variation of mineralization between AB and CB for all specimens. The magnitude of regional variation (how different AB
and CB were from each other) was compared for all parameters between OVX and Sham groups by expressing the difference of AB from CB, as a percentage of CB. The treatment groups were compared using ANOVA.

**Results:** AB and CB were significantly different (p<0.001) in all parameters measured within all subjects confirming significant regional variation, independent of estrogen deficiency. Comparisons between Sham and OVX groups for the percentage difference of AB resulted in percentage differences moderately different for SD (p<0.073) and significantly different for COV (p<0.039) and Low$_5$ (p<0.019). The percentage difference of Mean and High$_5$ were not significantly different (p>0.093) between Sham and OVX groups. **Conclusions:** An increase in variability (COV and Low$_5$) of mineralization represents a relative increase in bone remodeling. Higher variability of mineralization observed in AB of OVX group indicates that estrogen deficiency amplifies the active bone remodeling of AB already present due to mastication.
Dedicated to Laura, Xavier and Grady.
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With sincerity, I would like to acknowledge the animal subjects that we too often take for granted. Modern medicine has progressed as it has due to our ability to utilize animals in research endeavors such as in this present study.

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CHAPTER 1

INTRODUCTION

Post-menopausal Osteoporosis

Osteoporosis, the most common metabolic bone disorder, effects every third post-menopausal female (Bauer and Link 2009). Post-menopause estrogen deficiency leads to increased and imbalanced bone turnover. Infinitely complex and not completely understood, estrogen deficiency leads to increased formation, recruitment and prolonged lifespan of osteoclasts. Osteoblast activity and lifespan is also increased by relatively less than that of osteoclasts. The resultant altered bone turnover activity, or disproportionate remodeling, results in the net bone loss and reduction of bone quality that characterizes post-menopausal osteoporosis (Dervis 2005; McDonnell McHugh and O’Mahoney 2007; Seeman and Delmas 2006; Weitzmann and Pacifici 2006). Osteoporotic bone changes increase the risk of fracture up to 40%, most commonly affecting the femoral neck, vertebrae and the distal radius (McDonnell McHugh and O’Mahoney 2007). The dramatic impact of osteoporosis is obvious as the associated fractures compromise life quality and shorten life expectancy (Cockerill et al 2004) (Center et al 1999). While the long bone and vertebral implications are well established, the effects of osteoporosis on the jaw bones are less understood.
The Jaw Bones, Remodeling and Mineralization in a Disease-Free State

As tooth-bearing bones, the maxilla and mandible are arguably more complex than most bones. The functional demands on the jaw bones increase the complexity of both the structural makeup and the subsequent mechanical properties. The forces of mastication are transmitted directly to alveolar bone and these forces produce stresses, strains and ultimately micro-damage that stimulates remodeling (Seeman and Delmas 2006). A gradient of this remodeling activity, decreasing from the alveolar process, exists in the mandible (Tricker Dixon and Garetto 2002).

Remodeling is the biological determinant of mineralization (Grynpas 1993). During remodeling the bone formation that follows resorption is a multi-step process. Organic osteoid is laid down and shortly thereafter begins primary mineralization to form the basic structural unit (BSUs), either the cortical osteon or the cancellous bone packet. About 50% of the mineralization potential is achieved, relatively rapidly, with primary mineralization. Secondary mineralization follows as a slow and gradual maturation of the mineral component (Boivin and Meunier 2003). The individual BSUs are all produced at different points in time, resulting in a heterogeneously mineralized material that reflects the turnover, mineralization kinetics and average bone matrix age (Roschger et al 2008; Ruffoni et al 2007). High remodeling activity decreases tissue mineralization as mature, more mineralized bone is replaced with immature, less mineralized bone. Conversely, low remodeling activity leads to increased mineralization as the bone continues to be subject to secondary mineralization (Boivin and Meunier 2003; Grynpas 1993; van der Linden et al 2001a).
As remodeling determines mineralization, the gradient of remodeling found in the mandible should yield a gradient of mineralization, and bone quality should follow. In fact, significant regional variation of bone quality within the mandible has been demonstrated. The alveolar processes have higher rates of remodeling, decreased mineralization and subsequent decreased mechanical stability compared to bone more distant from teeth (Chun and Lim 2009; Lettry et al 2003; Park et al 2008; Peterson Wang and Dechow 2006; Rapoff et al 2008; van der Linden et al 2001b). Even within the alveolar process, alveolar bone proper has been found to be more compliant than adjacent cortical bone (Huja et al 2007). Together, these studies support the idea of a global gradient of structure and property within the mandible, which manifests as regional variation.

*Estrogen Deficiency and the Jaw Bones*

There is debate as to the magnitude of osteoporosis effects on the bone quality of the jaws. Not all skeletal sites are equally susceptible to estrogen deficiency induced bone loss just as not all post-menopausal women suffer with osteoporosis. Many studies have shown that mandibular bone is affected in a state of estrogen deficiency (Binte Anwar et al 2007; Ejiri et al 2008; Kuroda et al 2003; Mavropoulos Rizzoli and Ammann 2007; Rawlinson et al 2009; Tanaka et al 2002). There is debate among these studies as to the relative effects compared to other bones. And still, some studies refute any effects of estrogen deficiency on mandibular bone relative to long bones or vertebrae (Moriya Ito and Murai 1998; Orrico et al 2005; von Wowern 2001). Although the effects of osteoporosis on the jaw bones have not been clearly elucidated, significant implications for alveolar bone have been demonstrated.
Investigators have found osteoporotic effects on the onset and progression of periodontal disease and subsequent tooth loss. Osteoporosis is a risk factor for periodontal disease and epidemiologic studies have demonstrated a trend that post-menopausal women have fewer remaining teeth with advancing age (Gur et al 2003; Jeffcoat 2005; Krall Garcia and Dawson-Hughes 1996a; Payne et al 1999; Taguchi et al 2004; Wactawski-Wende et al 1996). Estrogen deficiency suppresses bone formation in alveolar wound healing following tooth extraction (Ejiri et al 2008; Pereira et al 2007). Osteoporosis can also be superimposed on degenerative temporomandibular joint changes (Gruber and Gregg 2003; Kirk 1998). Even orthodontic tooth movement has been found to be more rapid, and perhaps subsequently more unstable, in the absence of estrogen (Arslan et al 2007; Yamashiro and Takano-Yamamoto 2001). Histomorphometry in these studies revealed significantly increased osteoclast counts (Arslan et al 2007; Yamashiro and Takano-Yamamoto 2001) and relatively decreased osteoblast counts (Arslan et al 2007). Pharmacologically amplified bone turnover, similar to that induced with estrogen deficiency, has led to increased tooth movement as well (Ashcraft Southard and Tolley 1992; Verna Dalstra and Melsen 2000).

Although the mechanisms aren’t clearly elucidated and there remains some debate, it seems reasonable to conclude that there are osteoporosis implications with the jaw bones. Oral health status has a dramatic impact on quality of life (McGrath and Bedi 2004) and if post-menopausal osteoporosis has significant oral consequences, then it is possible that quality of life can be compromised beyond the commonly associated skeletal fractures.

Mineralization Assessment
Mineralization of bone is a significant parameter of bone quality that gives insight into bone remodeling and can be assessed with three-dimensional (3D), non-invasive imaging techniques. The local variation in mineral content (tissue mineral density (TMD) distribution), assessed with radiographic techniques, can be described and quantified as a histogram of gray level frequency distribution (Roschger et al. 2003; Roschger et al. 2008; Ruffoni et al. 2007; Ruffoni et al. 2008). Bone mineral distribution has been shown to change significantly in the presence of disease states or clinical treatments that effect bone metabolism. TMD distribution parameters have been shown to be able to distinguish between bone conditions and treatments (Boivin and Meunier 2003). With untreated disease-free bone as a reference, bone with high turnover activity shows a higher proportion of less mineralized tissue and subsequent increased variability (Boivin and Meunier 2003; Grynpas 1993; Roschger et al. 2003; Roschger et al. 2008; Ruffoni et al. 2008), thus demonstrating that remodeling plays an important role in TMD distribution.

**Overall Objective**

The overall goal of this study is to develop a novel method of region of interest isolation and subsequent TMD distribution analysis that can be applied to the clinical cone beam computed tomography scans we obtain of our dental patients. With continued development of this method and the technology it utilizes, we can obtain more detailed, objective information about our patient’s bone. This will ultimately allow us to better diagnose and treatment plan for the needs of our patients with respect to their individual bone quality conditions.

**Specific Aim**
In this study we hypothesized that estrogen deficiency osteoporosis, induced in a rat model, would amplify the regional variation of TMD distribution in mandibular bone, as assessed by 3D micro-computed tomography (micro-CT). The objectives of this study were; 1) to verify regional variation of TMD distribution within mandibular bone and, 2) to compare the regional variation between estrogen deficient and control jaw bone.
CHAPTER 2

MATERIALS AND METHODS

Twenty 6-month old Sprague-Dawley female rats were utilized following experimental protocol approved by the Institutional Animal Care and Use Committee of The Ohio State University. Ten rats were ovariectomized (OVX) and ten rats were subject to a sham surgery (Sham) at Harlan Laboratories (Harlan Laboratories, Inc., Indianapolis, IN). The rats received an intraperitoneal calcein (25mg/kg) injection 3 days prior to euthanization. At 59 days post-surgery, the rats were euthanized using an overdose of pentobarbital sodium (100 mg/kg). One side of the mandible was randomly chosen and tooth-bearing 5 mm mandibular bone sections were obtained using a low speed saw with two parallel diamond blades cutting under water irrigation (A.1). Cuts were made perpendicular to the occlusal plane of the molars in the bucco-lingual direction. One cutting surface of each bone section was polished and calcein labels on the surface of bone were observed under epifluorescence to verify turnover activity (A.2).

The mandible specimens were scanned and reconstructed using 3D micro-CT with 20 micrometer voxel resolution. The data file that contained the raw CT image was imported into ImageJ (NIH). CT attenuation values were maintained and the voxel gray level/mineral density values were reported in Hounsfield Units (HU) (A.3). The image
was cropped and negative gray level values were converted to zero. The image was then loaded into ModelPrep (FEM processing program) and the data volume was written as a standard image stack. The stack was then run through a threshold program using a heuristic algorithm at a minimum contrast level of 20 percent (of the maximum voxel value in the central spherical sample) to detect edges between bone (including dental hard-tissue) and non-bone. The heuristic algorithm consisted of five successive detecting procedures: normalization, edge detection, continuity crawling, final thresholding, and connectivity testing as previously published (Kim et al 2004; Yeni et al 2005). This process resulted in a thresholded image stack. The thresholded image stack was then imported and processed to yield a binary data image (bone voxels = 1, non-bone voxels = 0). The binary image was multiplied by the converted density image to result in an image with non-bone data thresholded out and bone data density values maintained.

The density image, originally in 16-bit format, was converted to an 8-bit, binary image. Both ModelPrep and ImageJ were utilized to systematically segment the molar and incisor teeth from the mandible sections. Continuous voxel connections between bone and teeth were present. In order to segment the teeth from the bone, these connections needed to be eliminated. "Pencil" and "fill" tools were used, while scrolling through the image stacks (in ImageJ), to eliminate major connections and erase teeth. This represented the only portion of the method that introduced manual selection and potential subjectivity and error and as such is the reason for testing reliability of the method as will be described later. A connectivity test was then performed in Modelprep. This test identified and removed any segments (teeth) that were not directly connected to the bone segment. These processes were alternated until segmentation was complete,
thus isolating the mandibular bone segment. This segment was multiplied by the density image to result in a mandibular bone segment that maintained density values with teeth removed.

To isolate the alveolar bone (AB) segment region of interest (ROI), the mandibular bone image stack was subtracted from an 8-bit, binary formatted, thresholded image. This resulted in a teeth-only image from which the incisor was removed. The teeth were then dilated 10 voxels in the X, Y, and Z planes using both ImageJ and a volume rotation algorithm in ModelPrep. The dilation command in ImageJ only dilates in the two planes (X and Y) of the individual 2-dimensional slices of the stack. To expose the third plane for dilation, the teeth image was rotated in ModelPrep. This rotated image was dilated (in the Y and Z plane) and then rotated back to the original orientation. The two dilated images were added and resulted in a 3-dimensionally dilated, 8-bit, binary teeth image stack. The dilated teeth image was then multiplied by the density bone segment to result in the AB ROI consisting of a uniform layer of bone within 200 micrometers of the root surfaces.

A control bone (CB) ROI, removed from significant modeling and remodeling effects, was isolated for comparison. This was accomplished by eroding the bone 10 voxels from all internal and external bone borders in the X, Y, and Z planes. The 3-dimensional erosion method was similar to that used for the dilation. The eroded image was multiplied by the density bone segment to result in the CB ROI 200 micrometers removed from all internal and external bone borders. As the rat has quite limited naturally occurring cortical bone remodeling inside mandibular bone (Meta et al 2008; Turner
2001), the CB of this study represented mature bone removed from significant remodeling or modeling influences.

Image manipulations and ROI isolations are described and depicted in figures A.4 and A.5.

**Statistical Analysis**

Histograms of gray level frequency were generated to analyze the TMD distribution parameters (A.6). Gray level mean (Mean) was computed by dividing the sum of gray levels by the total voxel count. The standard deviation (SD) of the Mean was obtained and the coefficient of variation (COV) was calculated (COV=SD/Mean). The gray level value representing the fifth and 95th percentiles (Low₅ and High₅, respectively) were reported. Paired t-tests were used for intra-specimen comparison between AB and CB regions for the mean values of gray level parameters. The magnitude of regional variation (how different AB was from CB) was compared between Sham and OVX groups by analyzing the difference of AB as a percentage of CB (|AB-CB|/CB×100). This statistical method allowed CB to serve as a reference for AB differences. Analysis of variance was performed for the inter-group comparisons. To demonstrate reliability of the method, three images were manipulated a second time to isolate the ROI. Shrout and Fliess intra-class correlation coefficients (ICCs) were generated for each parameter. Statistical analysis was performed with SAS (Cary, NC).
CHAPTER 3

MANUSCRIPT

Abstract:

Estrogen deficiency increases bone remodeling. Increased remodeling activity yields bone tissue with a higher percentage of immature/newly formed bone, ultimately leading to increased variability of mineralization. Due to the functional demands of mastication, alveolar bone is inherently a highly remodeled region of bone tissue. Does estrogen deficiency affect alveolar bone’s remodeling and subsequent mineralization?

Objectives: Using a rat model of estrogen deficiency, the objectives of this study were 1) to assess regional variation of mineralization in the mandible, and 2) to assess the effects of estrogen deficiency on the magnitude of regional variation. Methods: Ovariectomized (OVX, n=10) and sham surgery (Sham, n=10) rat mandible sections were scanned by micro-computed tomography (micro-CT) with a voxel resolution of 20 micrometers. The images of each section were manipulated using imaging software to isolate a portion of alveolar bone (AB) within 200 micrometers of root surface and a control bone (CB) region 200 micrometers removed from all internal and external mandibular bone borders. CT attenuation values were maintained and gray level voxel values were reported in Hounsfield Units (HU). Histograms were generated to analyze gray level frequency
distribution. HU parameters analyzed were Mean, Standard Deviation (SD), Coefficient of Variation (COV) (to normalize SD) and percentile values (5th = Low_5, and 95th = High_5). Paired t-tests were used to assess regional variation of mineralization between AB and CB for all specimens. The magnitude of regional variation (how different AB and CB were from each other) was compared for all parameters between OVX and Sham groups by expressing the difference of AB from CB, as a percentage of CB. The treatment groups were compared using ANOVA.

**Results:** AB and CB were significantly different (p<0.001) in all parameters measured within all subjects confirming significant regional variation, independent of estrogen deficiency. Comparisons between Sham and OVX groups for the percentage difference of AB resulted in percentage differences moderately different for SD (p<0.073) and significantly different for COV (p<0.039) and Low_5 (p<0.019). The percentage difference of Mean and High_5 were not significantly different (p>0.093) between Sham and OVX groups. **Conclusions:** An increase in variability (COV and Low_5) of mineralization represents a relative increase in bone remodeling. Higher variability of mineralization observed in AB of OVX group indicates that estrogen deficiency amplifies the active bone remodeling of AB already present due to mastication.
Introduction:

Estrogen deficiency leads to disproportionate bone remodeling by increasing osteoclast resorption activity more than osteoblast formation activity. This altered bone turnover activity results in the net bone loss and reduction of bone quality that characterizes post-menopausal osteoporosis (Dervis 2005; McDonnell McHugh and O'Mahoney 2007; Seeman and Delmas 2006). While these effects on the long bones and vertebra are well established, the effects on the jaw bones are less understood. As tooth-bearing bones, the maxilla and mandible are arguably more complex than most bones. The functional demands on these bones increase the complexity of both the structural makeup and the mechanical properties. Within the maxilla and mandible there is significant regional variation in bone quality as the alveolar processes demonstrate higher rates of remodeling and subsequent decreased mineralization density and mechanical stability compared to bone more distant from teeth (Huja et al 2007; Rapoff et al 2008).

Although the specific effects of osteoporosis on regional variation of bone quality have not been clearly elucidated, significant implications for alveolar bone have been demonstrated. Investigators have found osteoporotic effects on the onset and progression of periodontal disease and subsequent tooth loss (Gur et al 2003; Jeffcoat 2005; Krall Garcia and Dawson-Hughes 1996b; Payne et al 1999; Taguchi et al 2004; Wactawski-Wende et al 1996) and in alveolar wound healing (Ejiri et al 2008; Pereira et al 2007). Even orthodontic tooth movement has been found to be more rapid, and subsequently more unstable, in the absence of estrogen (Arslan et al 2007; Yamashiro and Takano-Yamamoto 2001). Oral health status has a dramatic impact on quality of life (McGrath and Bedi 2004) and if post-menopausal osteoporosis has significant oral consequences,
then it is possible that quality of life can be compromised beyond the commonly associated skeletal fractures.

Mineralization of bone is a significant feature of bone quality that gives insight into bone remodeling and can be assessed with three-dimensional (3D), non-invasive imaging techniques. Bone tissue is composed of individual cortical osteons and trabecular bone packets that are produced at different points in time. This results in a heterogeneously mineralized material that reflects bone turnover activity, mineralization kinetics and average bone matrix age (Roschger et al 2008; Ruffoni et al 2007). The local variation in mineral content (tissue mineral density (TMD) distribution), assessed with radiographic techniques, can be described and quantified as a histogram of gray level frequency distribution (Roschger et al 2003; Roschger et al 2008; Ruffoni et al 2007; Ruffoni et al 2008).Bone mineral distribution has been shown to change significantly in the presence of disease states or clinical treatments that effect bone metabolism. With untreated disease-free bone as a reference, bone with high turnover activity shows a higher proportion of less mineralized tissue and subsequent increased variability (Roschger et al 2003), thus demonstrating that remodeling plays an important role in TMD distribution.

In this study we hypothesized that estrogen deficiency osteoporosis, induced in a rat model, would amplify the regional variation of TMD distribution in mandibular bone, as assessed by 3D micro-computed tomography (micro-CT). The objectives of this study were; 1) to verify regional variation of TMD distribution within mandibular bone and, 2) to compare the regional variation between estrogen deficient and control jaw bone.
Materials and Methods:

Twenty 6-month old Sprague-Dawley female rats were utilized following experimental protocol approved by the Institutional Animal Care and Use Committee. Ten rats were ovariectomized (OVX) and ten rats were subject to a sham surgery (Sham) at Harlan Laboratories (Harlan Laboratories, Inc., Indianapolis, IN). The rats received an intraperitoneal calcein (25mg/kg) injection 3 days prior to euthanization. At 59 days post-surgery, the rats were euthanized using an overdose of pentobarbital sodium (100 mg/kg). One side of mandible was randomly chosen and tooth-bearing 5 mm mandibular bone sections were obtained using a low speed saw with two parallel diamond blades cutting under water irrigation (A.1). Cuts were made perpendicular to the occlusal plane of the molars in the bucco-lingual direction. One cutting surface of each bone section was polished and calcein labels on the surface of bone were observed under epifluorescence to verify turnover activity (A.2).

The mandible specimens were scanned and reconstructed using 3D micro-CT with 20 micrometer voxel resolution. Bone voxels were segmented from non-bone voxels using a heuristic algorithm consisting of five successive detecting procedures: normalization, edge detection, continuity crawling, final thresholding, and connectivity testing as previously published (Kim et al 2004; Yeni et al 2005). A CT attenuation value (gray level) for each voxel was maintained and reported in Hounsfield units (HU) (A.3). The 3D images of each specimen were manipulated using imaging software (ImageJ, National Institutes of Health) to isolate the regions of interest (ROI) for this study (A.5). Multiple isolating techniques were utilized to separate the teeth from the mandible. The isolated teeth image of each specimen was three-dimensionally dilated by
10 voxels, binarized and multiplied by the separated mandible segment. This process provided a 3D alveolar bone (AB) ROI within 200 micrometers of the tooth roots (Fig. 1b). A control bone (CB) ROI was isolated by three-dimensionally eroding the separated mandible segment 10 voxels (200 micrometers) from all internal and external bone borders. As the rat has quite limited naturally occurring cortical bone remodeling inside mandibular bone (Meta et al 2008; Turner 2001), the CB of this study represented mature bone removed from significant remodeling or modeling influences.

Histograms were generated to analyze the gray level distribution parameters (A.6). Gray level mean (Mean) was computed by dividing the sum of gray levels by the total voxel count. The standard deviation (SD) of the Mean was obtained and the coefficient of variation (COV) was calculated (COV=SD/Mean). The gray level value representing the fifth and 95th percentiles (Low5 and High5, respectively) reported. Paired t-tests were used for intra-specimen comparison between AB and CB regions for the mean values of gray level parameters. The magnitude of regional variation (how different AB and CB were from each other) was compared between Sham and OVX groups by analyzing the difference as a percentage of the control bone ((|AB-CB|)/CB×100). Analysis of variance was performed for the inter-group comparisons. To demonstrate reliability of the method, three images were manipulated a second time. Shrout and Fliess intra-class correlation coefficients (ICCs) were generated for each parameter. The total voxel count of the segmented bone image and the corresponding histograms of the ROI were compared. Statistical analysis was performed with SAS (Cary, NC)
Results:

OVX rats had significantly heavier weight than Sham rats (336±20.645 g vs. 288±24.138 g, p<0.001). However, the total volume of specimens after digitally removing teeth was significantly smaller for the OVX group (47.312±3.109 mm$^3$) than for the Sham group (51.235±3.829 mm$^3$) (p<0.022). These results were consistent with other studies and help demonstrate that the rats did realize estrogen deficiency effects (Elovic Hipp and Hayes 1995a; Elovic Hipp and Hayes 1995b; Jiang Matsumoto and Fujii 2003; Wronski Cintron and Dann 1988).

Despite the minimal subjectivity present with the occasional manual deletion of voxels connecting tooth and bone, reliability was demonstrated. Shrout and Fliess ICCs confirmed reliability of the nearly fully automated method (A.7). Calcein labels confirmed the presence of newly formed bone tissue and demonstrated remodeling in AB (A.2).

The ROIs were successfully isolated using the 3D micro-CT image of each specimen (A.5). The heuristic segmentation method utilized in this study provided the normal distribution of gray level histogram (A.6). This shape of histogram resembles the calcium content frequency distribution as introduced in the previous studies to compute the Low$_5$ and High$_5$ following the mineralization law (Roschger et al 2003; Roschger et al 2008; Ruffoni et al 2007).

The mean values of Mean, Low$_5$ and High$_5$ of AB were significantly lower than those of CB for both Sham and OVX groups (p<0.001) (A.8, A.9). In contrast, the mean values of standard deviation (SD) and coefficient of variation (COV) of AB were significantly higher than those of CB for both groups (p<0.001). The magnitude of
regional variation (how different AB and CB were from each other) compared between groups was moderately (p<0.073) higher for SD and significantly higher for COV and Low5 in the OVX group compared to the Sham group (p<0.04) (A.10). Mean and High5 regional differences were not significantly different between Sham and OVX groups (p>0.093).

Discussion:

Alveolar bone plays an important role in sustaining mechanical stability of teeth. Masticatory forces applied to teeth directly transfer to the alveolar bone. Bone remodeling, activated by the masticatory stimulus, alters tissue properties of the alveolar bone compared to mandibular bone more distant from the teeth. It is well known that estrogen deficiency increases bone remodeling. As such, postmenopausal alveolar bone may have more altered tissue properties than disease-free alveolar bone. Change of tissue mineral density (TMD) distribution is a detectable indicator for the alteration of tissue properties resulting from bone remodeling. Thus, the objective of this study was to investigate the effect of estrogen deficiency on TMD distribution of alveolar bone. We found that the alveolar bone region had significantly less TMD (Mean, Low5 and High5) but more variability (SD and COV) of TMD than a control region of mandibular bone, independent of estrogen deficiency. A state of estrogen deficiency amplified these regional differences for the variability and the TMD of less mineralized portion (Low5) while maintaining the TMD of mean and high mineralized portion at the normal level. These findings indicated that bone remodeling increased by estrogen deficiency would
yield a higher percentage of immature bone resulting in increased variability of mineralization.

Regional variation of mandibular bone properties has previously been analyzed using microscopic observation based on histological section of specimens. This traditional histological method inherently involved a destructive sectioning process to obtain a two-dimensional slice. In contrast, micro-CT is a non-destructive 3D imaging technique that has been widely used for bone research. Resolution of micro-CT images is more than 100 times higher than that of conventional clinical CT. As such, the detailed 3D image obtained by micro-CT technique could be used to investigate a limited small region including alveolar bone. Using the micro-CT images, we successfully isolated a uniform portion of alveolar bone for analysis in this study.

The micro-CT image of bone also provides the CT attenuation value (gray level) that is determined depending on the amount of mineral within a bone tissue region corresponding to a 3D voxel of image. This tissue mineral density (TMD) is different from traditional bone mineral density (BMD) measured using two-dimensional dual X-ray absorptiometry (DXA). The rough resolution of DXA image provides BMD that includes not only bone tissue mineral density but also pores of the scanned bone area. A more accurate measurement method was needed for assessing mineral density at tissue level. The reproducibility of micro-CT based morphology and tissue mineral density analyses using rat alveolar bone model were recently evaluated (Park et al 2008). This evaluation demonstrated that micro-CT images can be utilized for 3D measurement of TMD of the small alveolar bone region as used in this study. To our best knowledge, our
study is the first application of the 3D micro-CT image to directly compare regional variation of TMD in the mandibular bone.

Distribution of mineral density at the tissue level reflects bone mineralization following remodeling. During remodeling the bone formation that follows resorption is a multi-step process. Organic osteoid is laid down and shortly thereafter begins primary mineralization until the basic structural unit (either cortical osteon or cancellous bone packet) is formed. About 50% of the mineralization potential is achieved, relatively rapidly, with primary mineralization. Secondary mineralization follows as a slow and gradual maturation of the mineral component (Boivin and Meunier 2003). As bone consists of tissue packets (i.e. osteons and trabecular packets) under these different ages of mineralization, distribution of TMD varies. High turn-over activity due to amplified bone remodeling increases the amount of less mineralized new packets of bone, resulting in increased variability of TMD (Roschger et al 2008). Exposure of alveolar bone to the direct stresses and strains from masticatory forces ultimately leads to high bone remodeling. The calcein labels observed at the region surrounding teeth in this study indicated that new bone was forming in the alveolar region (A.2). Taken together, it is likely that increased bone remodeling is responsible for decreased degree of mineralization and higher variability of TMD at the alveolar region (AB) compared with the mature control bone region (CB).

It is well known that postmenopausal estrogen deficiency increases bone remodeling (Dervis 2005; McDonnell McHugh and O'Mahoney 2007; Seeman and Delmas 2006). For alveolar bone, the combination of the estrogen deficiency effects and the mechanical masticatory load may give rise to more severe alteration of the TMD
distribution. We found that the difference of variability parameters (SD and COV) of TMD between AB region and CB region increased more for the OVX group than for the Sham group. These finding indicate that estrogen deficiency has more effects on the alveolar bone region than the control bone region of the OVX group. Increase in the magnitude of regional variation of the less mineralized portion of bone (Low5) for the OVX group but no difference of the TMD of higher mineralized portion (High5) between the two groups together give insight that the primary mineralization of newly formed bone tissue of OVX group plays more of a role in controlling the TMD distribution than the secondary mineralization of the pre-existing mature bone tissue.

The limitation of this study was the use of an animal as a model of estrogen deficiency. In this study, fully matured 6 month old rats were used comparable with the human age of postmenopause. The OVX rat is an acceptable animal model to investigate the effects of postmenopausal estrogen deficiency on bone properties (Turner 2001). However, it is still controversial whether the OVX rat model can appropriately mimic the postmenopausal condition of human bone.

The use of micro-CT is currently limited to animal studies because of its high radiation dose. Thus, it remains to be verified whether the micro-CT based results of this study can be used to explain clinical observation of postmenopausal patients. The finest resolution of clinical 3D cone beam CT (CBCT) is about 200 micrometers which is 10 times coarser than the 20 micrometer of resolution used in this study. It is possible that the micro-CT image based analysis of TMD distribution used in this study is applicable to CBCT images commonly used in clinical practice. Further evaluation is required to confirm this speculation.
Increasing heterogeneity of TMD has been observed for bone with a high turnover rate (Kneissel Boyde and Gasser 2001; Roschger et al 2003). In bone tissue models based on micro-CT, it has been shown that apparent modulus decreases up to 21% with an increase in mineral heterogeneity (Renders et al 2008). Thus, it is likely that loading on the top of two adjacent bone tissues with different variability of TMD produces more deformation of the bone tissue that has higher TMD variability. If the difference of variability between the two tissues increases, more deformation would develop in the bone tissue having higher TMD variability. We found that the alveolar bone tissue adjacent to teeth had higher TMD variability than the control bone tissue and this regional variation was increased in the OVX group. This result suggests that the alveolar tissue of the OVX group would be more deformed under loading compared to that alveolar tissue of the Sham group. The more deformation of alveolar tissue of OVX group may be partly responsible for the increased orthodontic tooth movement observed in OVX rats (Arslan et al 2007; Yamashiro and Takano-Yamamoto 2001). Decrease in the mechanical stability of alveolar tissue may play a significant role in the onset and progression of periodontal disease, especially as observed in postmenopausal patients (Buencamino Palomo and Thacker 2009). The results of this study may help to understand the underlying mechanism of these clinical issues.

In conclusion, we found that the estrogen deficiency amplified the regional variation of TMD variability in the rat mandible. This increased regional variation is attributed to the greater presence of immature, less mineralized bone tissue. These findings suggest that regional variation of TMD distribution is altered by estrogen
deficiency, and the subsequent increased bone remodeling, and this variation may compromise the mechanical stability of the tooth-bearing alveolar bone.

References


Alveolar bone plays an important role in sustaining mechanical stability of teeth. Masticatory forces applied to teeth directly transfer to the alveolar bone. Bone remodeling, activated by the masticatory stimulus, alters tissue properties of the alveolar bone compared to mandibular bone more distant from the teeth. It is well known that estrogen deficiency increases bone remodeling. As such, postmenopausal alveolar bone may have more altered tissue properties than disease-free alveolar bone. Change of tissue mineral density (TMD) distribution is a detectable indicator for the alteration of tissue properties resulting from bone remodeling. Thus, the objective of this study was to investigate the effect of estrogen deficiency on TMD distribution of alveolar bone. We found that the alveolar bone region had significantly less TMD (Mean, Low$_5$ and High$_5$) but more variability (SD and COV) of TMD than a control region of mandibular bone, independent of estrogen deficiency. A state of estrogen deficiency amplified these regional differences for the variability and the TMD of less mineralized portion (Low$_5$) while maintaining the TMD of mean and high mineralized portion at the normal level. These findings indicated that bone remodeling increased by estrogen deficiency would
yield a higher percentage of immature bone resulting in increased variability of mineralization.

Regional variation of mandibular bone properties has previously been analyzed using microscopic observation based on histological section of specimens. This traditional histological method inherently involved a destructive sectioning process to obtain a two-dimensional slice. In contrast, micro-CT is a non-destructive 3D imaging technique that has been widely used for bone research. Resolution of micro-CT images is more than 100 times higher than that of conventional clinical CT. As such, the detailed 3D image obtained by micro-CT technique could be used to investigate a limited small region including alveolar bone. Using the micro-CT images, we successfully isolated the alveolar bone region at the micro distance from teeth in this study.

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CHAPTER 5

COMPREHENSIVE REFERENCES


APPENDIX A:

FIGURES AND TABLES
A.1. 5mm tooth-bearing mandible sections were obtained from each rat. The sections were scanned with micro-CT with a 20 micrometer resolution to result in the blown-up 3D reconstruction.
A.2.  Calcein labels of newly formed alveolar bone tissue surrounding a tooth.
A.3. Distribution of voxel gray levels (HU) based on 3D micro-CT image.
A.4. Segmentation techniques were applied to thresholded micro-CT volumes (A.) to yield a teeth-only volume (B.) and a bone-only volume (C.). The teeth were binarized, dilated 10 voxels in 3-D and then superimposed on the bone-only volume to determine the region of interest (represented in the overlay of the 2-D slices (D.)). The result was a volume of alveolar bone (AB) isolated within 0.2mm of the root surfaces (E.). To isolate the control bone (CB), the bone-only volume was binarized and eroded 10 voxels from all internal and external mandibular bone borders. This eroded volume was superimposed on the bone-only volume (E.) to result in the control bone volume (G.).
A.5. Isolation of the AB and CB regions of a rat mandibular bone using its 3D micro-CT image. a) 2D slice of the bucco-lingual view and b) reconstructed 3D view of the whole specimen.
A.6. A typical histogram of gray level with parameters examined in this study.
A.7. Shout and Fliess ICCs confirmed reliability of the method.

<table>
<thead>
<tr>
<th>Parameter</th>
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</tr>
<tr>
<td>COV</td>
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</tr>
<tr>
<td>Low&lt;sub&gt;5&lt;/sub&gt;</td>
<td>0.99877</td>
</tr>
<tr>
<td>High&lt;sub&gt;5&lt;/sub&gt;</td>
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A.8. Comparison of regional variation (AB vs CB) of gray levels (HU) within individual rats.

<table>
<thead>
<tr>
<th></th>
<th>Sham</th>
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<th>OVX</th>
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<tr>
<td></td>
<td></td>
<td>Paired t-test</td>
<td></td>
<td>Paired t-test</td>
</tr>
<tr>
<td>Mean</td>
<td></td>
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<tr>
<td>AB</td>
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<td>3610.046</td>
<td>±84.623</td>
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<tr>
<td></td>
<td>4889.503</td>
<td>±146.777</td>
<td>5042.313</td>
<td>±117.907</td>
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<tr>
<td>SD</td>
<td>786.240</td>
<td>±48.726</td>
<td>816.911</td>
<td>±57.257</td>
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<tr>
<td></td>
<td>536.143</td>
<td>±67.489</td>
<td>505.508</td>
<td>±53.148</td>
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<tr>
<td>COV</td>
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<td>0.226</td>
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A.9. An example of a histogram comparing the regions within one mandible section.

The distributions are different in all parameters analyzed.
A.10. Comparisons between Sham and OVX groups for the % differences ((AB-CB)/CB×100). The differences were moderately higher for SD (*; p<0.073) and significantly higher for COV (**; p<0.04) and Low5 (**; p<0.02) between the two groups. Mean and High5 were not significantly different (p>0.093). Negative % accounts for that the value of AB is lower than that of CB.