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The effects of estrogen deficiency on cortical bone microporosity and mineralization

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ABSTRACT

Recent studies have demonstrated matrix-mineral alterations in bone tissue surrounding osteocytes in estrogen-deficient animals. While cortical bone porosity has been shown to be a contributor to the mechanical properties of bone tissue, little analysis has been done to investigate the effects of estrogen deficiency on bone's microporosities, including the vascular and osteocyte lacunar porosities. In this study we examined alterations in cortical bone microporosity, mineralization, and cancellous bone architecture due to estrogen deficiency in the ovariectomized rat model of postmenopausal osteoporosis. Twenty-week-old female Sprague–Dawley rats were subjected to either ovariectomy or sham surgery. Six weeks post-surgery tibiae were analyzed using high-resolution micro-CT, backscattered electron imaging, nanoindentation, and dynamic histomorphometry. Estrogen deficiency caused an increase in cortical bone vascular porosity, with enlarged vascular pores and little change in tissue mineral density in the proximal tibial metaphysis. Measurements of cancellous architecture corresponded to previous studies reporting a decrease in bone volume fraction, an increase in trabecular separation, and a decrease in trabecular number in the proximal tibia due to estrogen deficiency. Nanoindentation results showed no differences in matrix stiffness in osteocyte-rich areas of the proximal tibia of estrogen-deficient rats, and bone labeling and backscattered electron imaging showed no significant changes in mineralization around the vascular pores. The findings demonstrate local surface alterations of vascular pores due to estrogen deficiency. An increase in cortical vascular porosity may diminish bone strength as well as alter bone mechanotransduction via interstitial fluid flow, both of which could contribute to bone fragility during postmenopausal osteoporosis.

1. Introduction

Estrogen protects the skeleton from bone loss by suppressing turnover and maintaining a balance between bone formation and resorption [1]. Estrogen deficiency after menopause can lead to osteoporosis and an increased risk of bone fracture. While there are many studies in humans and rats that report a decrease in bone volume fraction due to estrogen deficiency, especially in cancellous bone, only recently have high-resolution analyses been performed to assess changes in bone microporosity, including the vascular porosity that houses the bone vasculature and the lacunar porosity that houses osteocytes [2–4].

The vascular pores in cortical bone play an important role in load-induced interstitial fluid flow because they allow relaxation of fluid pressure in the lacunar-canalicular pores when bone is mechanically loaded [5, 6]. Measurements of strain-generated potentials in bone specimens undergoing cyclic loading demonstrate that local spatial voltage gradients, which correspond to the pressure gradients that drive fluid flow, are 10 to 30 times greater near vascular canals compared to the spatial gradients across a whole bone specimen [7]. Theoretical models that include vascular pores clearly demonstrate that the primary relaxation of the excess bone fluid pressure occurs from the lacunar-canalicular system emptying into the vascular pores [8, 9].

In addition to providing conduits for interstitial fluid flow, the cortical microporosities associated with the vascular and lacunar-canalicular systems also contribute to the overall mechanical properties of bone tissue [10–13]. In humans, the loss of cortical rather than trabecular bone has been shown to predominate in cases of radial fracture [14]. Increase

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of cortical porosity due to aging has also been linked to loss of bone strength [15]. Because of the important contributions of cortical porosity to bone strength as well as to the transport of nutrients and removal of waste products to maintain bone cell function, changes in cortical microporosity, including the vascular and lacunar porosities, should be evaluated during diseases such as osteoporosis.

The distribution of bone matrix mineral, which also contributes to the mechanical quality of bone, has been shown to be altered with estrogen deficiency [16]. Matrix mineralization has been shown to be reduced in osteoporotic patients [17, 18]. However, there are contradictory findings related to bone mineralization changes after a drop in estrogen levels in rats, with some studies reporting no changes and others reporting reduced bone stiffness and hardness [19, 20].

Our recent studies using high-resolution microscopy demonstrate that estrogen deficiency alters the submicron lacunar-canalicular porosity surrounding osteocytes in cortical and cancellous bone [21]. We found that the increase in effective canalicular size measured using a small molecular weight tracer in ovariectomized rats was due to nanostructural matrix-mineral changes at the osteocyte lacunar-canalicular surface. We have also found changes in molecular transport due to mechanical loading in the rat ovariectomy model [22].

The objective of this study was to further our investigations into how estrogen deficiency affects bone interstitial fluid pathways by assessing cortical microporosity and mineralization in the ovariectomized rat model of postmenopausal osteoporosis. Several high-resolution techniques were utilized to assess bone alterations, including 3D assessment of cortical bone vascular porosity and osteocyte lacunar porosity using micro-CT [23], assessment of mineralization density around vascular pores using backscattered electron imaging, and assessment of matrix stiffness using nanoindentation. Cancellous bone architectural changes due to estrogen deficiency were also measured, and bone turnover was quantified using fluorescent bone labels.

2. Materials and methods

2.1. Animal model

The ovariectomized rat model of postmenopausal osteoporosis [24] was used to investigate the effects of reduced estrogen levels on bone microporosity, microarchitecture, and mineralization. Skeletally mature, 20-week-old rats were used because the rate of bone turnover in the proximal tibia at that age is very low [25]. All procedures were approved by the Institutional Animal Care and Use Committee.

At 20 weeks of age, female Sprague Dawley rats (Harlan Laboratories) were either subjected to ovariectomy (OVX, n = 18) or to a sham surgery consisting of exposure of the ovaries without removal (SHAM, n = 18). The animals were fed ad libitum for one week after surgery, and then the OVX group was pair-fed to the average food intake of the SHAM group (20 g standard rat chow per day). A subset of the animals (n = 12 in the OVX group and n = 12 in the SHAM group) received two bone formation markers via the intraperitoneal (IP) cavity at 11 days and 3 days before sacrifice (15 mg/kg calcein and 90 mg/kg xylenol orange, respectively) to assess histomorphometric indices of bone formation rate, mineral apposition rate, and percent mineralizing surfaces.

Six weeks after surgery (at 26 weeks of age) all animals were sacrificed, and uterine horns were weighed to assess the effectiveness of ovariectomy. Tibiae were harvested and either put immediately in 10% neutral buffered formalin for 48 h or frozen at −20 °C. The analysis focused on the cortical and cancellous bone of the proximal tibial metaphysis because our recent studies demonstrated alterations in the lacunar-canalicular porosity surrounding osteocytes in this region [21, 22]. Tibiae from a subset of animals were used for three analyses: a micro-CT analysis to assess cortical microporosity and cancellous microarchitecture, a backscattered electron imaging and nanoindentation analysis to assess mineralization, and a histomorphometry analysis to assess bone turnover, as detailed below.

2.2. Micro-computed tomography imaging

Tibiae were scanned using a high-resolution micro-CT system (SkyScan 1172, Bruker microCT) to quantify cortical vascular and lacunar porosities, tissue mineral density (TMD), and cancellous microarchitecture (OVX: n = 6; SHAM: n = 6). Before scanning, the bones were immersed in phosphate buffered saline and brought to room temperature in a custom low X-ray attenuation plastic holder that held each bone in place during scanning. For cortical microporosity and TMD measurements, images of the proximal tibiae were acquired with a voxel size of 1 μm ("1-μm scans") and for cancellous measurements images were acquired with a voxel size of 4 μm ("4-μm scans"). A 10-MP digital detector, 10-W power energy setting (100 kV and 100 μA), and a 0.5-mm aluminum filter were used, as in our previous work [23]. The 1-μm scans had a limited field of view due to the high resolution, thus requiring separate scans for the anterior and posterior regions of the proximal tibia, whereas the 4-μm scans allowed imaging the entire tibia. For all scans, X-ray projections were generated from the sample every 0.3°, with the projections averaged 5 times to produce high-contrast, low-noise images. Hydroxyapatite rods (2-mm radius, densities of 0.25 and 0.75 g/cm^3 hydroxyapatite) were also scanned at 1-μm voxel size to calibrate for TMD.

A modified back-projection reconstruction algorithm (NRecon v.1.6.5, Skyscan, Bruker microCT) was used to generate cross-sectional images from the X-ray projections. Images were optimized using a standard post-alignment compensation algorithm, treated with a Gaussian smoothing filter (kernel = 4 pixels for 1-μm scans, and 1 pixel for 4-μm scans), and corrected for ring artifacts and beam hardening. All parameters except post-alignment compensation were set identically for all samples with the same voxel size.

2.3. Quantification of cortical vascular and lacunar porosities and TMD using micro-CT

Cortical bone microporosity was measured for the 1-μm scans utilizing a methodology previously developed in our laboratory to accurately assess cortical features, including vascular canals and osteocyte lacunae [23]. The scans were segmented using a global thresholding procedure to exclude soft tissue, with the threshold value (0.45 g/cm^3) chosen by analyzing the SHAM images with an edge detection algorithm to accurately segment cortical pore edges [23]. Cortical bone from the metaphysis of the proximal tibia was selected as the anatomical region of interest, starting 1 mm distal to the most distal point of the growth plate (Fig. 1).

Cortical vascular canal porosity (%), vascular canal diameter (μm), osteocyte lacunar porosity (%), lacunar volume (μm^3), and lacunar density were calculated in both the anterior and posterior regions of the proximal tibia using two cylindrical volumes of interest (VOIs) (diameter 250 μm, height 2 mm) for each region. All the cortical pores were identified using a "sphere fitting" algorithm (CTAn v.1.11.0, SkyScan, Bruker microCT) and separated by volume. As in our previous work, osteocyte lacunae were isolated using a 3D despeckled filter that removed objects outside the range of 100 μm^3 to 600 μm^3 in volume (Fig. 2) [23]. Some of the cortical bone analyses from the SHAM group were previously reported in an analysis of the effect of micro-CT resolution and threshold method on the 3D assessment of cortical bone porosity [23].

The tissue mineral density was calculated for the 1-μm scan VOIs using the density calibration values obtained from the hydroxyapatite rod scans.