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Alterations in the Osteocyte Lacunar-Canalicular Microenvironment due to Estrogen Deficiency

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Abstract

While reduced estrogen levels have been shown to increase bone turnover and induce bone loss, there has been little analysis of the effects of diminished estrogen levels on the lacunar-canalicular porosity that houses the osteocytes. Alterations in the osteocyte lacunar-canalicular microenvironment may affect the osteocyte's ability to sense and translate mechanical signals, possibly contributing to bone degradation during osteoporosis. To investigate whether reduced estrogen levels affect the osteocyte microenvironment, this study used high-resolution microscopy techniques to assess the lacunar-canalicular microstructure in the rat ovariectomy (OVX) model of postmenopausal osteoporosis. Confocal microscopy analyses indicated that OVX rats had a larger effective lacunar-canalicular porosity surrounding osteocytes in both cortical and cancellous bone from the proximal tibial metaphysis, with little change in cortical bone from the diaphysis or cancellous bone from the epiphysis. The increase in the effective lacunar-canalicular porosity in the tibial metaphysis was not due to changes in osteocyte lacunar density, lacunar size, or the number of canaliculi per lacuna. Instead, the effective canalicular size measured using a small molecular weight tracer was larger in OVX rats compared to controls. Further analysis using scanning and transmission electron microscopy demonstrated that the larger effective canalicular size in the estrogen-deficient state was due to nanostructural matrix-mineral level differences like loose collagen surrounding osteocyte canaliculi. These matrix-mineral differences were also found in osteocyte lacunae in OVX, but the small surface changes did not significantly increase the effective lacunar size. The alterations in the lacunar-canalicular surface mineral or matrix environment appear to make OVX bone tissue more permeable to small molecules, potentially altering interstitial fluid flow around osteocytes during mechanical loading.

Keywords

osteoporosis; osteocyte; lacuna; canaliculi; periosteocytic remodeling

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1. Introduction

Bone's lacunar-canalicular porosity is formed by a three-dimensional network of lacunae and small canals that houses the osteocytes and their cytoplasmic processes and allows communication between neighboring osteocytes, osteoblasts, bone lining cells, and the vascular porosity. A pericellular fiber matrix surrounds the osteocyte processes, which have been shown to be tethered to the canalicular wall [1]. With their distribution throughout the bone matrix and their high degree of interconnectivity, osteocytes are in an ideal position to sense mechanical deformations and translate them into biochemical signals to enable bone resorption and formation [2, 3].

Mobile exchangeable interstitial fluid external to the osteocytes fills the lacunar-canalicular porosity and is thought to play an important role in bone maintenance [4]. When cyclic mechanical loading is applied to bone, interstitial fluid flows around the osteocytes in the lacunar-canalicular porosity and drains into and out of the larger pores surrounding the blood vessels and nerves in the vascular porosity. The movement of interstitial fluid in the canalicular network affects bone cells in two ways: it is believed to activate osteocytes via deformation of transmembrane links between the cell process cytoskeleton and the canalicular wall [5,6], and it is involved in mass transport favoring the passage of nutrients and signaling molecules and the removal of waste products from the osteocytes [7–11].

Reduction of estrogen levels has been shown to increase bone turnover, induce bone loss, and increase osteocyte apoptosis [12–14]. Modification of bone macroarchitecture via a decrease in bone volume fraction is frequently reported in ovariectomized rats [13,15–17], yet there is little analysis in the literature related to changes in the lacunar-canalicular porosity that houses the osteocytes. Reduced estrogen levels have been shown to increase osteocyte apoptosis, which may reduce the communication between neighboring osteocytes [14,18–20]. Alterations in bone mineral content and collagen fibrils have been documented in ovariectomized rats [21,22], but whether these changes at the nanostructural level affect the interstitial fluid pathways is not known. Alterations in the geometry or the interconnectedness of the lacunar-canalicular network may affect interstitial fluid movement and could alter the transduction of mechanical forces to the osteocyte [23,24].

Because changes in the osteocyte lacunar-canalicular microenvironment have the potential to impair mechanotransduction that is vital for bone health, the goal of this study was to determine whether the osteocyte microenvironment is altered after loss of ovarian function. Using the rat ovariectomy model of osteoporosis, this project utilized high-resolution microscopy techniques to assess several aspects of the osteocyte lacunar-canalicular network. Confocal microscopy was used to assess changes in lacunar-canalicular porosity, lacunar and canalicular size, and the number of canaliculi per lacuna; and scanning and transmission electron microscopy were used to assess canalicular diameter and structural aspects of the peri-lacunar-canalicular matrix.

2. Materials and methods

2.1. Animal model and experimental design

The ovariectomized (OVX) rat was used as a model for postmenopausal osteoporosis [12]. Twenty-week-old rats were used to avoid the early, rapid growth stage that produces significant bone turnover in the proximal tibia [25,26]. Bone changes were assessed 6 weeks after loss of ovarian function, a duration shown previously to produce significant bone loss in the proximal tibia [27,28]. All procedures were approved by the Institutional Animal Care and Use Committee at the Hospital for Special Surgery.

A total of 35 female Sprague Dawley rats (Harlan Laboratories) were used in the analyses. Eighteen rats underwent OVX at 20 weeks of age and were fed ad libitum for one week after surgery. The OVX rats were then pair-fed to the average food intake of control rats (20 g standard rat chow per day). The control groups included age-matched (CTRL, n=11) and sham-operated (SHAM, n=6). SHAM surgery was identical to OVX except the ovaries were not removed. At 26 weeks of age all rats were sacrificed, and uterine horn weights were recorded to assess the effectiveness of OVX versus SHAM.

The work was divided into three analyses that examined the osteocyte lacunar-canalicular network, with focus on cortical and cancellous bone from the proximal tibia. A subset of rats was used for each analysis: 2D confocal study: OVX, n=6, CTRL, n= 6; SEM study: OVX, n=6, CTRL, n=5; TEM and 3D confocal study: OVX, n=6, SHAM, n=6.

2.2 Sample preparation for the confocal microscopy, SEM, and TEM analyses

For the confocal microscopy analyses, harvested tibiae were placed immediately into either a fixative used routinely for electron microscopy (0.5% glutaraldehyde, 2% paraformaldehyde in 0.05M cacodylate-sodium buffer, pH 7.4) or 10% phosphate buffered formalin solution at room temperature. Transverse cortical bone sections (300–400 μm) were cut with a diamond blade saw (Buehler) from the tibial mid-diaphysis and from the metaphysis 2–3 mm distal to the growth plate and then fixed for 24 hours at room temperature. The remainder of the proximal tibia, used to analyze cancellous bone from the metaphysis and epiphysis, was fixed for 48 hours at room temperature. After fixation, the cortical sections were ground down to a final thickness of 30–50 μm using Carbimet paper discs (800 and 1200 grit; Buehler) and dehydrated in a series of ascending graded ethanol (EtOH) solutions. The proximal tibia samples containing cancellous bone were also dehydrated in a series of ascending graded EtOH solutions. To label the lacunar-canalicular porosity all the samples were stained using a protocol developed in our lab [29]. Briefly, the cortical sections and cancellous samples were stained while gently mixed in a rotator for 4 hours in a 1% filtered FITC solution (fluorescein isothiocyanate isomer I from Sigma, product #F7250). The cancellous samples were then embedded in PMMA and sagittal or frontal sections (400–500 μm) were cut from the plastic blocks with a diamond blade saw. Cancellous sections were ground down to a final thickness of 40–70 μm using Carbimet paper discs (600 and 1200 grit; Buehler), and dried in ascending graded EtOH solutions. Finally, all the cortical and cancellous sections were placed on slides and coverslipped with mounting media (Richard-Allan Scientific).

For the SEM analyses, ~5 mm thick sections were cut from the proximal end of the tibial metaphysis using a diamond blade saw. The specimens were then cut longitudinally to expose cancellous and cortical bone surfaces. After sonication for 40 minutes in a 20 ml aqueous solution (2% dimethyl sulfoxide, 2% Triton 100X, and 2% detergent) to remove cellular debris, the samples were rinsed in running water and sonicated for 30 additional minutes in fresh water. Samples were then dehydrated in 100% EtOH overnight, mounted on a sticky stub, and coated with palladium (300 Å coat thickness).

For the TEM analyses, 1–2 mm sections were cut from the proximal tibial metaphysis 2 mm distal to the growth plate and fixed in 0.5% glutaraldehyde, 2% paraformaldehyde in 0.05M cacodylate-sodium buffer, pH 7.4 for 24 hours at room temperature. After fixation undemineralized tibial samples were treated with 0.5% reduced osmium tetroxide (EM Sciences) and 1.5 % anhydrous potassium ferricyanide in distilled water, and then dehydrated in a series of ascending EtOH solution. Final dehydration was done with propylene oxide, and samples were then placed overnight in a mixture of Epon resin (Embed-812, Electron Microscopy Sciences) and propylene oxide (1:1 and then 3:1 overnight) at room temperature. Finally, the samples were embedded in a 1:1 mixture of

Epon resin and nadic methyl anhydride, using 0.5 ml of 2,4,6-Tri(dimethylaminomethyl)phenol (DMP-30) catalyst. Thin (80 nm) sections of cancellous bone were cut using an Ultracut microtome with an ultra-diamond knife and mounted on single-slot Formvar-coated grids.

2.3 Quantification of osteocyte lacunar-canalicular porosity and lacunar area using 2D confocal microscopy

The osteocyte lacunar-canalicular porosity was first quantified using 2D confocal microscopy scans (Leica TCS SP2). A 40x oil immersion lens with a 1.25 numerical aperture was used with the pinhole set at 1 Airy unit. A wavelength excitation of 488 nm was used to illuminate FITC.

2.3.1 Quantification of osteocyte lacunar-canalicular porosity of cortical bone cross-sections—To quantify the lacunar-canalicular porosity of cortical bone from the tibial diaphysis and metaphysis, confocal images spanning an entire cross-section were taken at a resolution of 2048×2048 pixels with a field of view of $375 \mu\text{m} \times 375 \mu\text{m}$ (650 gain, -5 offset). For each region a high-resolution confocal image of an entire cross-section was obtained by creating a montage of the field-of-view images using Photoshop 7.0 (Adobe Systems) (Fig. 1). Each cross-section image was then transformed to grayscale and thresholded using Otsu's method (ImageJ, National Institutes of Health). Otsu's method is a widely used global thresholding algorithm that extracts objects from their background. The algorithm assumes an image is composed of two classes of pixels, object and background, and then computes an optimal threshold value that minimizes the intra-class variance [30]. To ensure that all images were captured similarly, imaging was performed at the same z-depth from the specimen surface and the same gains were applied. This ensured that there would not be large intensity differences between images. The thresholding produced a binary image with the porous areas stained with FITC represented by white pixels, and the impermeable area of the mineralized matrix represented by black pixels. After first marking the vascular pores (including resorption canals if present), the lacunar-canalicular porosity was calculated as the difference between the pixels representing the total FITC-stained regions and the pixels occupied by the vascular pores and resorption canals, divided by the cross-sectional cortical area. The endosteal and periosteal surfaces were excluded for all measurements to avoid the accumulation of FITC at these surfaces. For the cortical metaphysis sections, an additional analysis was also performed to assess the lacunar-canalicular porosity in the regions of trabecular remnants as well as the lamellar regions found in this portion of the tibia (these regions are created during normal long bone growth when marrow space between cancellous struts is filled in with new lamellar bone [31]).

2.3.2 Quantification of osteocyte lacunar-canalicular porosity of cancellous bone—To quantify the lacunar-canalicular porosity of cancellous bone from the proximal tibia, fourteen randomly selected areas were analyzed for each rat, seven from the metaphysis and seven from the epiphysis (Fig. 2). Confocal images were taken at a resolution of 2048×2048 pixels with a field view of $375 \mu\text{m} \times 375 \mu\text{m}$ (515 gain, 1.5 offset). The images were transformed to grayscale and thresholded using Otsu's method, with the threshold values determined for each image in a region containing canaliculi. The lacunar-canalicular porosity was quantified as the percentage of white pixels (FITC-stained space) contained in the cancellous bone area examined, excluding marrow space.

2.3.3 Quantification of osteocyte lacunar area—To quantify lacunar area, lacunar pores in the 2D confocal images were marked using ImageJ, and the average area taken up by each lacuna (including the osteocyte body) was measured. Multiple bone sectors, each containing three to seven lacunae, were analyzed for cortical and cancellous bone from the

tibial metaphysis. For the cortical metaphysis, both the trabecular remnant and lamellar regions were analyzed using six bone sectors for each region. For the cancellous metaphysis region, seven bone sectors were analyzed for each rat. Thus for each anatomical region approximately 20 lacunae were analyzed per rat. The lacunar areas were averaged first for each rat and then for each group.

2.4 Qualitative assessment of bone mineral using backscattered electron microscopy

To further assess the bone microstructure in regions that appeared impermeable to FITC as viewed using confocal microscopy, a randomly chosen subset from the 2D confocal microscopy study (OVX, n=3 and CTRL, n=3) was imaged using scanning electron microscopy (SEM) in backscattered mode (FEI Quanta 600). Slide coverslips from cortical cross-sections from the tibial diaphysis and metaphysis were removed after being immersed in 100% xylene. Sections were then gently polished with 1200 grit sandpaper to eliminate mounting media and xylene residues, mounted on a microscope stud holder, and visualized with SEM in backscattered mode. Cross-sectional images of the entire cortical mid-diaphysis and metaphysis were obtained and compared to confocal microscopy images of the same sections.

2.5 Quantification of canalicular diameter using 2D confocal microscopy, SEM, and TEM

Canalicular diameter was measured for cortical and cancellous bone from the proximal tibial metaphysis using 2D confocal images, SEM images, and TEM images. For the confocal analysis of cortical and cancellous bone from the metaphysis, at least 20 canaliculi were identified and 4 diameter measurements were made over the length of each canaliculus, resulting in approximately 80 measurements per rat per region. For the SEM analysis, canalicular diameters were measured using the built-in microscope software (FEI Quanta 600) on images taken at 10^4 magnification. At least 40 canalicular diameters were measured per rat for the two regions analyzed (cortical and cancellous metaphysis). Measurements were made on the smallest dimension of the canalicular opening (Fig. 3a, b). For the TEM analysis, canalicular diameters from cancellous bone were measured using the built-in microscope software (Olympus 5.1) on TEM images taken at 19000x magnification (JOEL 100CX-II). Only circular cross-sections of canalicular diameters were measured (Fig. 3c), and at least 60 canalicular diameters were measured per rat.

2.6 Quantification of osteocyte lacunar density, number of canaliculi per lacuna, and lacunar and canalicular volume using 3D confocal microscopy

Additional 3D confocal microscopy analyses were performed on cortical bone from the proximal tibial metaphysis, a region found to undergo changes in the 2D confocal analysis. A Leica TCS SP2 microscope was used with a 63x oil immersion lens (1.4 numerical aperture). The pinhole was set at 1 Airy unit, and a wavelength excitation of 488 nm was used to illuminate FITC. A linear intensity compensation for gain was applied to compensate for loss in laser intensity at increasing depth into the sample, and each image was averaged four times to minimize noise. The counter was blinded for all analyses.

2.6.1 3D quantification of osteocyte lacunar density—To calculate volumetric osteocyte lacunar density (N.Lc/BV), confocal images were taken at a resolution of 1024×1024 pixels with a field of view of $150 \mu\text{m} \times 150 \mu\text{m}$ and a $1 \mu\text{m}$ z-step between images for a scan depth of $25 \mu\text{m}$. The 3D z-stack images were loaded in ImageJ and lacunae were point-counted directly through the z-stack using the cell counter tool. Partially sectioned lacunae at the top surface of the z-stack were included, while partially sectioned lacunae at the bottom surface or image sides were excluded. The number of lacunae (N.Lc) was divided by the analyzed bone volume (BV) and averaged for three z-stacks per rat to obtain

the volumetric osteocyte lacunar density. If the average tissue volume surrounding one osteocyte lacuna is represented by a cube, the length (L) of one side of the cube can be calculated as $\sqrt[3]{1/(N.Lc/BV)}$ (Fig. 4a). Because L was found to be approximately 25 μm for the SHAM group (Table 2), the next set of confocal scans was taken with this dimension to quantify the number of canaliculi per osteocyte lacuna.

2.6.2 3D quantification of the number of canaliculi per osteocyte lacuna—To calculate the number of canaliculi per osteocyte lacuna, confocal images of single lacunae were taken at a resolution of 1024×1024 pixels with a field of view of $25 \mu\text{m} \times 25 \mu\text{m}$ and a 244 nm z-step for a scan depth of 25 μm , with an osteocyte lacuna in the middle of the scan (Fig. 4b). All z-stacks were reconstructed using Volocity (Perkin Elmer), and the number of canaliculi penetrating each of the six faces of the cubic volume (considered secondary canaliculi), were then point-counted for three z-stacks per rat and averaged (Fig. 4c). The same confocal z-stacks were then cropped close enough to visualize each osteocyte lacuna, and primary canaliculi, considered to be all unbranched, single processes directly connecting to the lacuna, were also point-counted for three z-stacks per rat and averaged (Fig. 4d).

2.6.3 3D quantification of osteocyte lacunar and canalicular volumes—The same confocal z-stacks that were used to quantify the number of canaliculi were also used to quantify osteocyte lacunar volumes. Images were thresholded in ImageJ using Otsu's method and were imported into Mimics 3D reconstruction software (Materialise) where segmentation tools were used to isolate lacunar volumes (Fig. 4e).

To calculate canalicular volumes, confocal images consisting of only canaliculi between two osteocytes were obtained at a resolution of 2048×2048 pixels with a field of view of $10 \mu\text{m} \times 10 \mu\text{m}$ and a 122 nm z-step for a scan depth of 10 μm (Fig. 4f). Scanned images were thresholded in ImageJ using Otsu's method and imported into Mimics, which enabled creation of 3D volumes to calculate canalicular volume.

A parametric analysis was performed to assess the effects of threshold on the volume z-stack measurements. First, a parametric analysis was performed for lacunar volume z-stacks using the Otsu threshold, along with a 25% higher and 25% lower threshold. There was less than a 10% difference in lacunar volume for the three thresholds (data not shown). Second, for the canalicular volume measurements, Otsu thresholding was applied using two approaches: (1) thresholding image-by-image within the z-stack and (2) using an average threshold for all images within the z-stack. Both approaches resulted in the same outcome (data not shown), and thus the second approach, using a global Otsu threshold for each z-stack, was utilized using ImageJ.

2.7 Statistical analyses

For the 2D confocal microscopy study, lacunar-canalicular porosity measurements were analyzed using two-way repeated-measures ANOVA with Bonferroni post-hoc t-tests for the two factors: treatment (OVX and CTRL) and location (cortical bone: diaphysis and metaphysis and cancellous bone: metaphysis and epiphysis). Two-way repeated-measures ANOVA was also used to analyze the lacunar area and canalicular diameter measured using 2D confocal microscopy for the different anatomical regions: cortical metaphysis (trabecular remnant and lamellar regions) and cancellous metaphysis. For the SEM and TEM studies, unpaired, two-tailed t-tests were performed to compare canalicular diameter in OVX and control groups. Unpaired, two-tailed t-tests were also used to assess differences between the SHAM and OVX groups for the 3D confocal microscopy measurements (volumetric osteocyte lacunar density, side length (L) of cubic volume of bone surrounding an osteocyte,

the number of primary and secondary canaliculi, osteocyte lacunar volume, and canalicular volume). Finally, unpaired, two-tailed t-tests were used to compare animal and uterine horn weights between OVX and controls. All the statistical analyses were performed with the Prism 5 statistics software package (GraphPad Software Inc.). The normality of all data sets was confirmed before using parametric tests, and the significance level was set at $p < 0.05$.

3. Results

3.1 Effectiveness of ovariectomy

The effectiveness of ovariectomy was verified by the reduced weight of the uterine horns in the OVX rats ($0.19 \pm 0.03\text{g}$) compared to SHAM ($0.58 \pm 0.13\text{g}$). Despite pair-feeding, at 26 weeks of age the OVX rats weighed more than the age-matched and sham-operated control rats: OVX ($n=18$): $314 \pm 27\text{ g}$; controls ($n=17$): $279 \pm 36\text{ g}$.

3.2 Confocal microscopy measurements and observations: osteocyte lacunar-canalicular porosity, canalicular and lacunar size, and number of canaliculi per lacuna

The high-resolution 2D confocal microscopy measurements, which measured effective penetration by a small molecular tracer, showed that ovariectomized rats had an increased effective lacunar-canalicular porosity in the cortical and cancellous bone of the tibial metaphysis compared to controls, with no significant differences in the cortical bone of the diaphysis or cancellous bone of the epiphysis (Fig. 5). Cancellous bone of the metaphysis showed the largest increase in lacunar-canalicular porosity in OVX compared to CTRL (+56%), and the cortical metaphysis also had significantly higher lacunar-canalicular porosity (+16%) compared to CTRL.

Qualitative comparisons of cortical bone sections imaged with both confocal microscopy and backscattered electron imaging demonstrated that areas with an absent or disconnected canalicular network, where the FITC tracer could not penetrate, had a higher mineralization density, whereas areas with lower mineralization densities had a higher canalicular density (Fig. 6). Cortical bone in the rat tibial metaphysis of both OVX and controls demonstrated trabecular remnants surrounded by lamellar regions (Fig. 6a–c). The increase in the effective lacunar-canalicular porosity in the cortical bone of the tibial metaphysis in the OVX group was due to a 24% increase in effective lacunar-canalicular porosity in the lamellar regions; the trabecular remnants of the cortical metaphysis had no change in lacunar-canalicular porosity due to OVX.

Regions found to have an increased effective lacunar-canalicular porosity after OVX demonstrated larger effective canalicular diameter and no change in lacunar area as measured with 2D confocal microscopy (Table 1). In cancellous bone from the tibial metaphysis, the effective canalicular diameter measured using 2D confocal microscopy was larger (+48%) in OVX compared to CTRL. In the lamellar regions of cortical bone from the metaphysis, the effective canalicular diameter was also significantly larger (+26%) in OVX compared to CTRL, while there were no differences in canalicular diameter in the trabecular remnants in the cortical metaphysis. OVX did not significantly change osteocyte lacunar area in cortical or cancellous bone of the tibial metaphysis (Table 1).

The high-resolution 3D confocal microscopy measurements of cortical bone from the tibial metaphysis showed a 34% increase in effective canalicular volume in OVX compared to SHAM (Fig. 7). In addition, no significant differences between SHAM and OVX were found for osteocyte lacunar density, side length of cubic volume surrounding one lacuna, lacunar volume, and number of primary and secondary canaliculi per lacuna (Table 2).

3.3 SEM and TEM measurements and observations: changes in lacunar-canalicular interfaces

The SEM and TEM measurements did not demonstrate significant differences in canalicular diameter due to OVX; however, morphological alterations at the lacunar-canalicular interfaces were evident. While the canalicular diameter measured from SEM images of cancellous bone from the metaphysis was similar in both groups (CTRL: 335 ± 32 nm; OVX: 341 ± 32 nm), loose collagen fiber arrangements that diffusely covered the canalicular openings and masked their edges were seen in OVX compared to CTRL (Fig. 3a, b). These fibers made it difficult to find canalicular openings that could be measured in the SEM images and prevented measurement of canalicular diameter in the cortical metaphysis samples. While the canalicular diameter measured from TEM images of cancellous bone from the metaphysis was also not significantly different between groups (SHAM: 228 ± 11 nm; OVX: 242 ± 22 nm), morphological differences were observed in lacunae and canaliculi of OVX rats. The SHAM group demonstrated smooth lacunar and canalicular boundaries, while loose collagen fibers were observed invading the lacunar and canalicular spaces of the OVX group, throughout the areas analyzed (Fig. 8). Matrix debris and collagen fragments were also observed in the pericellular spaces of lacunae of OVX rats.

4. Discussion

Using high-resolution analyses, this study has demonstrated alterations in the osteocyte microenvironment in the rat OVX model of postmenopausal osteoporosis. Confocal microscopy analyses demonstrated that OVX increased the effective lacunar-canalicular porosity surrounding osteocytes in cortical and cancellous bone from the rat tibia metaphysis, with the increase in porosity largely explained by increased effective canalicular porosity, as measured by penetration of a small molecular weight dye. No significant changes were found in osteocyte lacunar density, lacunar size, or the number of canaliculi per lacuna. TEM and SEM analyses demonstrated that the larger effective canalicular size in the estrogen-deficient state was due to nanostructural matrix-mineral level differences around the osteocyte canaliculi. These matrix-mineral differences were also found in osteocyte lacunae in OVX, but the small surface changes did not significantly increase the effective lacunar size. The nanostructural changes of the osteocyte lacunar-canalicular microenvironment suggest that the lacunar-canalicular walls in the OVX rats became more permeable, which could affect interstitial fluid flow around osteocytes during mechanical loading.

Because estrogen deficiency leads to increased bone turnover, it is important to assess whether the lacunar-canalicular differences seen in OVX rats were related to the maturation of osteocytes in newly laid down bone. Osteocytes observed in the TEM analyses were slender cells with reduced cytoplasm and diminished quantity of endoplasmic reticulum, Golgi complex, and mitochondria. Such characteristics are typical of mature osteocytes rather than newly formed osteocytes [32,33]. In addition, the nanostructural matrix changes observed in the EM analyses of osteocyte lacunae and canaliculi were found uniformly throughout the cancellous bone of the OVX rats, and only rarely in osteocytes in the SHAM group. The osteocyte morphology and distribution of changes of the lacunar-canalicular surfaces in OVX lead us to believe that the osteocyte lacunar-canalicular changes documented in this study were not simply changes due to new bone laid down post-OVX. In future studies, histomorphometric analysis of fluorochrome label-based bone dynamics will facilitate the identification of newly formed bone regions that arise due to remodeling changes associated with estrogen-deficiency, and quantitative backscattered electron imaging can be used to assess mineral heterogeneity.

Contradictory results exist in the literature regarding mineral content present in osteoporotic samples at the micro and nano level. Some studies show decreased mineral content in bone tissue due to estrogen deficiency [34–36], or find no changes in mineral content [37], while others have shown an increase in mineral content [38,39]. An increase in mineral heterogeneity is often noted due to increased bone turnover [40]. Alterations in collagen composition have also been shown in osteoporotic patients [41]. TEM analysis has indicated structural changes in bone fibril architecture in OVX rats, showing irregular and disturbed collagen fibril arrangement compared to a more organized arrangement seen in controls [21]. Shrinkage of the D-spacing in collagen fibril periodicity has also been seen in OVX bone [21,42]. Modification in the concentrations of noncollagenous proteins or changes in their molecular orientation have also been shown in osteoporotic bone [43]. Defects in mineralization, changes in matrix fiber architecture or cross-linking may also be explored in future studies analyzing changes in bone architecture due to reduced estrogen levels.

The lack of response to loss of ovarian function in the tibial diaphysis and epiphysis, and the active response in the metaphysis is similar to bone macroporosity changes reported in the literature, where the proximal metaphysis of the rat tibia has demonstrated significant cancellous bone loss as measured by bone volume fraction, with little change in the diaphysis or epiphysis [15,16,27]. It is possible that there is a link between osteocyte-level changes in estrogen-deficient bone and osteoclast removal of bone, as some studies have suggested [44–46]. Ikeda et al. [44] showed osteocytes in the metaphyseal trabecular bone of OVX rats expressed osteopontin mRNA, which tended to be resorbed after estrogen withdrawal. Furthermore, osteocyte mRNA expression differed in the metaphyseal compartment compared to the epiphysis. Emerton et al. [45] showed that when osteocyte apoptosis was suppressed, it prevented OVX-induced osteoclast resorption, thus demonstrating a direct link between osteocyte apoptosis and osteoclast resorption in the estrogen-deficient state. Expression of RANKL has also been demonstrated in osteocytes, which are now believed to be the largest source of RANKL [47,48]. RANKL deletion in osteocytes has been shown to prevent osteoclast-dependent bone resorption due to unloading and has also been shown to lead to an osteopetrotic phenotype [48,49]. It is plausible that loss of estrogen may stimulate bone resorption in part by increasing osteocyte apoptosis and thereby RANKL expression [50]. Future work assessing osteocyte-related changes in this rat OVX model will include evaluation of osteocyte apoptosis.

While not directly assessed in this study, a proposed mechanism by which the lacunar-canalicular surface is altered after OVX is through periosteocytic remodeling, the process by which osteocytes may modify their immediate environment through matrix modification. The capability of osteocytes to adapt their lacunar wall under certain conditions was suggested in early studies [51,52] and then largely dismissed due to criticisms related to the methods of measurement of lacunar size and as well as the mounting evidence that osteoclasts were responsible for bone resorption [53]. However, recent studies continue to suggest that osteocytes can remodel their local environment in response to hyperparathyroidism, limb immobilization, lactation, and parathyroid hormone administration [54–57]. While none of the early studies included detailed measurements of changes in canalicular size, likely because of the resolution of the approaches used, an increased canalicular volume has recently been documented in lactating rats [57]. There is increasing evidence that osteocytes are metabolically active cells and can modify the matrix surrounding them via production of collagen-digesting proteins such as matrix metalloproteinases that have been demonstrated to be secreted during the development of osteocyte processes and are suggested to remain active in mature osteocytes [58,59].

The assessment of canalicular distribution in this study is one of the few 3D analyses of the number of canaliculi that emanate from osteocyte lacunae. Significant branching of the

primary canaliculi was found in both OVX and SHAM groups. In addition, a lower number of osteocytic processes emanating from the osteocyte cell body have been documented in other 3D confocal studies [60], which may be due to differences in species, animal age, and type of bones analyzed. Nonetheless, the number of primary canaliculi per lacuna reported here is in the range (41–115) previously estimated for different species [61].

Contradictory results have been reported concerning osteocyte lacunar density differences in osteoporotic bone. While some studies have shown significantly higher lacunar density in human osteoporotic bone [62], other reports demonstrate lower lacunar density in osteoporotic females compared to healthy females [63]. In our study, we did not find differences in lacunar densities between SHAM and OVX rats. In addition, the average distance between two osteocyte lacunae found in the present study ($\sim 25 \mu\text{m}$) is very similar to a previous 3D confocal study in chicks [60]. Similarly, lacunar volumes are in agreement with previously reported values of osteocyte volume for different species using confocal microscopy [60,64]. While a reduced lacunar area has been reported in human osteoporotic samples [62], our study showed no significant lacunar volume differences between SHAM and OVX groups.

It should be noted that the confocal microscopy analyses present a limitation related to overestimation of porosity due to resolution and distortion effects. The diameter of canaliculi is of the same order of magnitude as the resolution of confocal microscopy ($\sim 200 \text{ nm}$), which causes confocal images to overestimate the canalicular porosity due to partial volume effects. This overestimation only applies to the measurement of the canaliculi and not the lacunae because the lacunar size (order $10 \mu\text{m}$) is well-characterized at a resolution of 200 nm . In addition, a mismatch in the index of refraction between the immersion oil and mounting media can result in z-axis geometrical distortion in confocal images. We have not accounted for any elongation along the z-axis because we estimate this distortion will affect all the measurements similarly since canaliculi weave in and out of all three dimensions and the lacunae analyzed were not oriented in any preferential direction. The SEM and TEM studies were designed to quantify the canalicular diameter and to assess the amount of overestimation of canalicular size occurring in confocal images, since EM has a higher resolution than confocal microscopy. The SEM analysis gave an average canalicular diameter approximately 30% smaller than that measured with confocal microscopy, whereas the TEM analysis gave an average canalicular diameter approximately 50% smaller than that measured with confocal microscopy. The difference between measurements using the two methodologies may arise because SEM samples go through minimal processing and topographical images are obtained, while TEM samples are dehydrated and embedded in a resin prior to obtaining very thin sections. Another important step needed to calculate lacunar-canalicular microporosities was the image thresholding. Much investigation went into the choice of threshold, and the standard method chosen (Otsu's method [30]) is appropriate for this application, since we have emphasized the relative changes in microporosities between ovariectomized and control animals without focusing on the actual porosity values.

Other limitations of this study relate to the animal model used. While the ovariectomized rat model is not exactly equivalent to postmenopausal osteoporosis in humans, it has characteristics similar to that of postmenopausal bone loss, including increased bone turnover with resorption greater than formation, more trabecular than cortical bone loss, and an initially rapid loss of bone followed by a slower loss of bone [12,27,28]. Twenty-week-old rats were used to avoid the rapid growth stage that produces significant bone turnover in the proximal tibia [25,26]. While some studies show that the rat tibia continues to grow until approximately 26 weeks of age, with a slower rate in the last several weeks, other studies have demonstrated that beyond 20 weeks of age tibial growth has essentially stopped, even

though the growth plate remains open [65,66]. Another limitation of the rat ovariectomy model was that despite pair feeding, the body weight of the OVX group was greater than the controls six weeks post-ovariectomy. It has been shown that pair feeding reduces ovariectomy-induced weight gain in the rat, but it does not prevent increased weight gain compared to age-matched controls [67]. The weight gain in OVX rats has been suggested to counteract some of the effects of bone loss due to loss of ovarian function.

The physiological implications of having a more permeable lacunar-canalicular wall around the osteocyte in the estrogen-deficient state relate to the potential altering of the effects of interstitial fluid flow due to mechanical loading [24]. An increased permeability of the lacunar-canalicular wall may alter both solute transport and interstitial fluid velocities around osteocytes during mechanical loading. It is also possible that alterations in the surface of the lacunar-canalicular network could disrupt the connections of the osteocyte process to the canalicular wall, impairing the capability of the osteocyte to perceive mechanical stimuli and potentially affecting the viability of the osteocyte [68,69]. If the connections between osteocytes and the surrounding matrix are diminished due to ovariectomy, postmenopausal osteoporosis could be a remodeling response to reduced perceived loading in the presence of actual continued loading, where the bone cells cannot process strain-related information as effectively [68]. Further work to identify the mechanisms by which the osteocyte microenvironment is altered in estrogen-deficient rats as well as to investigate how the alterations in the osteocyte microenvironment affect interstitial fluid flow around osteocytes is warranted and is underway.

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Highlights

- Used high-resolution microscopy techniques to assess the osteocyte lacunar-canalicular microstructure in the rat ovariectomy (OVX) model of postmenopausal osteoporosis.
- Confocal microscopy analyses indicated that OVX rats had an increased effective lacunar-canalicular porosity in the proximal tibia metaphysis.
- OVX lacunar size remained unchanged, but the effective canalicular size measured using a small molecular weight tracer was larger.
- SEM and TEM demonstrated nanostructural matrix-mineral changes surrounding osteocytes in the estrogen-deficient state.
- Mineral or matrix changes may make the OVX lacunar-canalicular surface more permeable, possibly altering interstitial fluid flow around osteocytes.

Fig. 1.

(a) A typical tibial diaphysis section from an OVX rat created from approximately 50 montaged confocal images; scale bar = 300 μm . (b) Enlarged image of square region in (a), which demonstrates the high resolution of the images; scale bar = 50 μm . (c) Enlarged image of the square region in (b), further illustrating the details of the lacunar-canalicular network; scale bar = 10 μm . Osteocyte lacunae (short arrows), and canaliculi (long arrows) are indicated in (b) and (c).

Fig. 2.
 (a) A montage light micrograph of the proximal rat tibia showing the cancellous regions sampled with $375\ \mu\text{m} \times 375\ \mu\text{m}$ field of view images from the metaphysis (dashed line boxes) and the epiphysis (solid line boxes); scale bar = $800\ \mu\text{m}$. Typical metaphysis (b) and epiphysis (c) confocal images; osteocyte lacunae (short arrows) are indicated; scale bars = $50\ \mu\text{m}$.

Fig. 3.

SEM images of cancellous bone from the tibial metaphysis for (a) a CTRL specimen and (b) an OVX specimen showing canalicular openings (white arrows); loose collagen fibers are visible in the OVX specimen (black arrow). Canalicular diameter was measured as the smallest dimension of the opening (black lines). (c) TEM image of cancellous bone from the tibial metaphysis from SHAM showing canalicular cross-sections and measurement of canalicular diameter (black lines). TEM sections were counterstained with uranyl acetate for 40 minutes and lead citrate for 5 minutes.

Fig. 4.

(a) Schematic of cubic volume of bone (average side length L) surrounding one osteocyte lacuna. Secondary canaliculi intersect the faces of the cube. (b) Confocal scan of cubic volume of bone tissue centered on one osteocyte lacuna demonstrating the canalicular network spanning in all directions; scale bar = 5 μm . (c) Confocal scan of a $25\ \mu\text{m} \times 25\ \mu\text{m} \times 25\ \mu\text{m}$ cubic volume surrounding one osteocyte lacuna with only secondary canaliculi rendered viewable on all six faces of the cube; scale bar = 5 μm . (d) Cropped scan from (c) showing primary canaliculi emanating directly from the osteocyte lacuna; scale bar = 3 μm . (e) Isolated osteocyte lacuna from same scan constructed in Mimics software; scale bar = 2 μm . (f) Higher resolution scan of canaliculi taken between two osteocytes; scale bar = 2 μm .

Fig. 5. Relative lacunar-canalicular porosities measured using 2D confocal microscopy for (a) cortical diaphysis and cortical metaphysis cross-sections and (b) cancellous bone from the epiphysis and metaphysis of the proximal tibia. All measurements were scaled to the CTRL mean for each porosity; bars represent mean values $\pm$ standard deviations; * statistical difference between CTRL (n=6) and OVX (n=6) ($p < 0.05$).

Fig. 6.

(a) Confocal reconstructed image of a typical OVX tibial metaphysis cross-section (cancellous bone in the medullary region was not preserved in these unembedded sections); scale bar = 400 μm . (b) Confocal and (c) backscattered electron images of the rectangular region indicated in (a); the region of trabecular remnants is enclosed within the yellow lines and lamellar bone is marked with red asterisks. Scale bar in (c) = 200 μm . (d) Confocal and (e) backscattered electron images of the same portion of a CTRL tibial diaphysis section; a red line traces the same pathway in each image. Areas with an absent or disconnected canalicular network (black regions surrounding osteocyte lacunae in the confocal images) correspond to higher mineralization (whiter regions in the backscattered electron images), whereas greater canalicular density areas correspond to areas of lower mineralization. Scale bar in (e) = 200 μm .

Fig. 7.

Box plots showing 3D confocal microscopy measurements of canalicular volume (%) from cortical bone from the proximal tibial metaphysis. + indicates the mean of each group. A significant difference (34% increase) in canalicular volume was found between SHAM (n=6) and OVX (n=6) (* $p < 0.05$).

Fig. 8. TEM images of cancellous bone from the tibial metaphysis showing (a) smooth surfaces in a SHAM osteocyte lacuna (red arrows) and (b) collagen fibers in an OVX lacuna giving the border a rough appearance (yellow arrow). (c) Smooth SHAM canaliculi (red arrow); scale bar = 500 nm and (d) OVX canaliculus with collagen fibers at the surface (yellow arrow); scale bar = 500 nm.

Table 1

Osteocyte lacunar area and canalicular diameter measured using 2D confocal microscopy images of cortical and cancellous bone from the tibial metaphysis. Values expressed as mean $\pm$ standard deviation. CTRL: control (n=6); OVX: ovariectomized (n=6).

	Lacunar area (μm^2)		Canalicular diameter (nm)	
	CTRL	OVX	CTRL	OVX
Cortical bone: metaphysis				
trabecular remnants	49.4 $\pm$ 4.5	50.4 $\pm$ 5.9	520 $\pm$ 42	542 $\pm$ 33
lamellar region	49.2 $\pm$ 7.0	51.1 $\pm$ 5.2	553 $\pm$ 33	697 $\pm$ 51 ^a
Cancellous bone: metaphysis	60.9 $\pm$ 6.9	67.5 $\pm$ 13	483 $\pm$ 24	714 $\pm$ 82 ^a

^a
p < 0.05 versus CTRL

Table 2

Lacunar-canalicular network parameters measured using 3D confocal microscopy images of cortical bone from the tibial metaphysis. Values expressed as mean $\pm$ standard deviation. SHAM: sham-operated (n=6); OVX: ovariectomized (n=6).

	SHAM	OVX
Osteocyte lacunar density (#/mm ³)	6.73 $\times$ 10 ⁴ $\pm$ 1.4 $\times$ 10 ⁴	7.70 $\times$ 10 ⁴ $\pm$ 2.5 $\times$ 10 ⁴
Side length (L) of cubic volume surrounding one osteocyte lacuna (μ m)	24.8 $\pm$ 1.7	23.9 $\pm$ 2.1
Osteocyte lacunar volume (μ m ³)	352 $\pm$ 30	393 $\pm$ 92
Number of primary canaliculi per lacuna	83.9 $\pm$ 14	89.7 $\pm$ 15
Number of secondary canaliculi per lacuna	387 $\pm$ 34	365 $\pm$ 40

^a
p < 0.05 versus SHAM