Age-related changes in the 3D hierarchical structure of rat tibia cortical bone characterized by high-resolution micro-CT

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Submitted 28 July 2011; accepted in final form 14 January 2013

Jast J, Jasiuk I. Age-related changes in the 3D hierarchical structure of rat tibia cortical bone characterized by high-resolution micro-CT. J Appl Physiol 114: 923–933, 2013. First published January 17, 2013; doi:10.1152/japplphysiol.00948.2011.—Three-dimensional hierarchical structure of female Sprague-Dawley rat tibia cortical bone was characterized as a function of age (3, 12, 32, 42, 60, and 72 wk) using a high-resolution micro-computed tomography. At the whole bone level, 3-wk samples exhibited statistically significant differences in a mean total tissue volume, mean cortical bone volume, mean cortical bone volume density, mean periosteal perimeter, and mean cortical thickness (P < 0.05) compared with all other ages. At the tissue level, there was a statistically significant increase in a mean canal number density and a decrease in a mean canal volume and diameter between 3-wk and 12-wk samples. While no significant variations were found between mean canal lengths, there was a dependence of mean canal orientation on age. At the cell level, there were no statistically significant differences in a lacuna number density and a lacuna volume density, and all lacunae element-based parameters displayed no dependence on age across age. In addition, at the microstructural level, the cannular indexes were reported separately for anterior, posterior, medial, and lateral anatomic regions. From 3 to 32 wk of age, there existed significantly fewer canals per volume of bone in the medial region of the tibia vs. other cross-sectional quadrants. Although there were changes with age, there were no statistically significant differences in the mean canal volume, mean canal diameter, and mean canal length between the four anatomic regions.

cortical bone; bone’s hierarchical structure; high-resolution micro-computed tomography; canal network; osteocyte lacunae

CORTICAL BONE is a dynamic, living tissue that has a complex and continuously evolving microstructure that changes throughout life. Cortical bone morphology has been identified as an important aspect of the overall bone quality (40) as it contributes significantly to mechanical properties of bone (34). A vital component of the cortical bone microstructure is a canal network, the interconnected system of canals that perforate cortical bone and facilitate fluid flow throughout the cortex (15, 30). The structure of this cortical canal network changes as bone develops to promote vascularization and blood perfusion and ensure the supply of adequate oxygen, minerals, and nutrients necessary for bone integrity (10, 12). Therefore, it is necessary to investigate the morphometric properties of the canal network as a function of age as they will provide insights on bone biology and better understanding of the physiological significance of canalization within cortical bone (14).

Until recently, the analysis of the cortical bone microstructure and the canal network has largely been restricted to two-dimensional histomorphometry or three-dimensional image registration of two-dimensional serial sections (2, 42, 44, 52). Although beneficial, these techniques are time-consuming and result in destruction of the specimen (11, 15). Additionally, these techniques cannot provide a complete visualization of the three-dimensional microstructure of cortical bone (15).

Micro-computed tomography (micro-CT) has come into use for the three-dimensional analysis of bone structure in vitro, as first introduced by Feldcamp et al. (17). Micro-CT offers several advantages including the ability to nondestructively provide quantitative results with little to no sample preparation. However, imaging at very small resolutions (less than a few microns) still requires careful sample preparation involving embedding of bone tissue and/or cutting it into very thin slices. Although the technique has quickly become the standard for quantification and visualization of the three-dimensional structure of trabecular bone, its application to the analysis of internal microstructures within cortical bone has been limited. Much work has been done using a desktop micro-CT to analyze the human cortical bone porosity (4, 15, 59). However, much less has been done applying this technology to the assessment of the cortical microstructure of small rodents. Due to the smaller scale of the vascular canal network of these animals, the analysis of rat and mouse cortical bone has been mainly achieved through the use of synchrotron radiation micro-CT (SRμCT) (31, 32, 46, 53). The use of SRμCT offers many advantages over conventional desktop micro-CT imaging including higher spatial resolution and the elimination of beam-hardening artifacts (8, 16), allowing for the determination of local bone mineralization (41, 50). However, access to SRμCT is often very limited, so it is desirable to obtain higher-resolution images with desktop micro-CT imaging.

In this study, the rat bone was chosen for investigation because rat animal models are frequently used to assess bone’s structural and mechanical variations due to many factors including diet (24), physical activity (25, 27, 55), and disease (21, 23). Aging is a natural process that also alters bone properties and is therefore the interest of many studies. Britz et al. (11) demonstrated the possibility of visualization and quantification of the cortical bone microstructure of the rat using a desktop micro-CT system, but an investigation of how this microstructure changes with age has not yet been conducted. Thus the primary purpose of this study was to visualize and quantify changes in the cortical bone microstructure of a rat as a function of age.

In this paper, as outlined by Schneider et al. (46), the cortical bone tissue was assessed following a hierarchical approach, beginning at the macroscopic (organ) level and proceeding down to the submicroscopic (cellular) level. In addition to the determination of various indexes at each level, the effort was made to subdivide the total intracortical porosity and identify

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the contributions of the canal network and the osteocyte lacunar system to the overall porosity. Also, the spatial orientation of osteocyte lacunae within the canal network was investigated. To our knowledge, this is the first multiscale analysis of the rat tibia structure and porosity, addressing three structural levels spanning from the organ, tissue, to cellular levels, as a function of age and anatomic position using a desktop micro-CT. Investigation of the morphology of bone at different hierarchical scales can provide valuable information on bone quality and transport in bone. Such results will be useful for subsequent studies on aging, hormone withdrawal, fracture repair, and mechanical adaptation. In addition, they can provide valuable inputs for multiscale modeling of bone’s mechanical properties and fluid flow in bone.

MATERIALS AND METHODS

Tissue Preparation

 Tibiae were excised from female Sprague-Dawley rats. Experiments were performed in compliance with an experimental protocol approved by the Institutional Animal Care and Use Committee at the University of Illinois at Urbana-Champaign. The rats ranged from infant to adult and included ages of 3 wk (n = 5), 12 wk (n = 5), 32 wk (n = 5), 42 wk (n = 5), 60 wk (n = 5), and 72 wk (n = 5). After harvesting and soft tissue removal, the bones were wrapped in gauze and soaked in a 0.1 M phosphate buffered saline solution to prevent dehydration. The samples were then stored at −20 °C in sealed freezer bags.

Bone’s Hierarchical Structure

In this study, bone is analyzed as the material with a hierarchical structure spanning across several different length scales. Within this study, the following three structural levels are considered. At the macrostructural level, cortical bone is characterized by its volume distribution and radial growth and is assumed to be compact. The canal network constitutes the microstructural level and is considered a part of the ultrastructure of the intracortical porosity. Also a part of this ultrastructure, the osteocyte lacunar system is described in the submicrostructural level due to its smaller size and variant morphology.

Micro-CT Imaging

Macrostructure: whole bone. In accordance with the convention set forth by Kuhn et al. (28), micro-CT images will herein be referred to as serial slices (three-dimensional series of two-dimensional slices), and cut bone samples will be referred to as sections. Additionally, micro-CT imaging guidelines, nomenclature, and reported parameter selection were adapted from a comprehensive review paper by Bouxsein et al. (9). For imaging at the macrostructural scale (from one to a few centimeters), a scan of the whole tibia bone was conducted. The tibiae were thawed to 4°C in a refrigerator for 24 h prior to imaging. During scanning, samples were kept wrapped in saline-soaked gauze and enclosed in plastic Eppendorf tubes. These tubes were mounted on a platform using a double-sided adhesive carbon tape to prevent sample drift over time. The samples were scanned using a SkyScan 1172 (Aartselaar, Belgium) X-ray microtomograph. The samples were rotated 360° at a rotation step of 0.7°. The X-ray settings were standardized to a source voltage of 60 kV and source current of 160 μA, and two-frame averaging was utilized to improve the signal-to-noise ratio. The object-to-source distance was set to 257 mm and camera-to-source distance to 347 mm. Total scanning time for each sample was ∼3 h, with the isotropic pixel size of 17.25 μm. Scans contained 2,000 × 1,048 pixels, which translated into the 34.50 mm × 18.08 mm field of view, with 1,048 slices taken. In these scans, three tibia were scanned simultaneously. To create a set of contiguous transverse cross-sectional slices from the acquired angular projections, the volumetric reconstruction software Ntecon 1.4 was used (SkyScan software). The reconstruction process included beam-hardening correction, alignment optimization, and ring artifact reduction.

Microstructure. Following imaging of the whole bone, the tibiae were sectioned to prepare samples suitable for imaging at the microstructural scale (20–500 μm). Tibiae were cut into transverse sections with a thickness of 400 μm using an Isomet 1000 precision diamond saw (Buehler, Lake Bluff, IL). Five such 400-μm samples were taken from each tibia from the middiaphyseal region. These sections of bone were then immediately submerged in a fixative of 2.0% paraformaldehyde and 2.5% glutaraldehyde in 0.1 M Na-cacodylate buffer for 4 h at 4°C. Following fixation, the sections were subjected to a buffer rinse of 0.1 M Na-cacodylate for 10 min. The sections were then progressively dehydrated at room temperature in 37%, 67%, and 95% ethanol, each for 10 min and finally in 100% ethanol for 30 min (solution change every 10 min). Once dehydrated, the sections were supercritically dried using a Samdri-PVT-3D Critical Point Dryer (Tousimis, Rockville, MD) to prevent drying artifacts and sample damage due to surface tension. The samples were scanned using an Xradia MicroXCT-200 (Pleasanton, CA) using a 4% lens that allowed imaging of bone sections with a 4.0-μm pixel size. Each scan involved 1,000 × 1,000 pixels, which translated into a 4 mm × 4 mm field of view, with 100 slices taken of each sample. The X-ray settings were standardized to a 40-kV source voltage and 200-μA source current. Thin sections (400 μm) were used to obtain better quality of images at this higher resolution. A total of 500 slices were obtained, corresponding to a stack height of 2 mm (corresponding to 5 × 400-μm-thick samples) in length along the middiaphysis.

Submicrostructure. Following sectioning using a slow speed saw, the remaining portions of tibiae were prepared for imaging at the submicrostructural scale (1–20 μm) and the visualization of the osteocyte lacunar system. The proximal half of each tibia was rinsed with 100% ethanol twice (10 min each) at room temperature and then left in 100% ethanol at 4°C for 24 h. After infiltration with methacrylate monomer, the samples were embedded in polymethyl methacrylate (PMMA) and sectioned into 150-μm-thick slices using a Leica RM2255 rotary microtome (Leica Microsystems, Bannockburn, IL) with a tungsten carbide blade. Then, under optical microscope a bone slice was cut using a razor blade. These cutting steps are shown in Fig. 1. Here again we cut bone to very small dimensions to ensure better quality of images at higher resolution. The bone had to be embedded in a polymer to facilitate cutting and handling. The samples were scanned using an Xradia MicroXCT-400 (Pleasanton, CA) with a 20× lens to achieve a 2.0-μm pixel size resolution. Each scan involved 1,000 × 1,000 pixels resulting in a 2 mm × 2 mm field of view. X-ray settings were standardized to a 50-kV source voltage and 200-μA source current, and exposure time per frame was 8 s.

Image Postprocessing

The micro-CT data sets were analyzed using Amira 5.3.0 (Visage Imaging, San Diego, CA). The images were translated from 16-bit grayscale images (0-65535) into 8-bit grayscale images (0-255), and void space comprising the canal network and osteocyte lacunae was identified using a standardized global threshold. The Amira Quantification+ software addition option was used to conduct quantitative analysis including automated segmentation, extraction of geometrical information, and filtering.

Quantitative Morphometry

Morphometric indexes assessed in this study were adapted from those utilized by Schneider et al. (46). Namely, at the macrostructural level, the total tissue volume (TV) comprising cortical bone and the medullary cavity, the cortical bone volume (CLBV), the cortical bone volume density (CLBV/TV), the cortical thickness (CT.Th), and the polar area moment of inertia (I) were obtained. The polar moment of
inertia \( J \) measures bone’s resistance to torsion (twisting). For a constant material property, the larger the \( J \), the more resistance the bone has to torsion.

At the microstructural level, indexes similar to the cannular parameters introduced by Cooper et al. (15) and adapted from a three-dimensional trabecular bone analysis (22) were used for quantification of the canal network. The number of canals (N.Ca), the cortical total volume (Ct.TV) (including both the cortical bone volume and void space), the number density of canals (N.Ca/Ct.TV), the total canal volume (Ca.V), and the volume density of canals (Ca.V/Ct.TV) were calculated. In addition to parameters describing the canal network as a whole, the network was broken down into single elements and analyzed using element-based morphometry, similar to that utilized previously for the analysis of trabecular bone (51). In agreement with the nomenclature introduced by Schneider et al. (46), these element-based indexes are average values calculated over the total number of elements and are designated by brackets (\( \langle \rangle \)). These average values calculated include the mean canal diameter (\( \langle \text{Ca.Dm} \rangle \)), the mean canal length (\( \langle \text{Ca.Le} \rangle \)), the mean canal orientation (\( \langle \text{Ca.\theta} \rangle \)) measured from the longitudinal axis of the tibia, and the mean canal volume (\( \langle \text{Ca.V} \rangle \)).

At the submicrostructural level, similar to the negative imprint formed by the canal network, there exists the osteocyte lacunar system. Analogous to the analysis of the canal network, this cellular network was quantified using the following indexes: the lacuna number density (N.Lc/Ct.TV) and the lacuna volume density (Lc.V/Ct.TV). Element-based indexes were also calculated and include the mean lacuna volume (\( \langle \text{Lc.V} \rangle \)), the mean lacuna length (\( \langle \text{Lc.Le} \rangle \)), and the mean lacuna orientation (\( \langle \text{Lc.\theta} \rangle \)) (measured from the longitudinal axis of the tibia). The resolution was not sufficient to view canalicular canals. We separated osteocyte lacunae from canals by using a “volume filter” option in the quantification analysis in Amira. More specifically, the porosity associated with lacunae was classified as regions 50–5,000 \( \mu^3 \) while regions greater than 5,000 \( \mu^3 \) were considered as canals. Volumes less than 50 \( \mu^3 \) were considered as noise. A similar approach was used by other studies (46, 53, 54). The morphometric indexes used in this paper are summarized in Table 1.

**Statistical Analysis**

Statistical analysis was performed using OriginPro 8.1 (OriginLab Northampton, MA). The software was used to conduct one-way analysis of variance (ANOVA) to test for differences in mean parameter values between age groups. Additionally, Tukey’s honestly sig-
significant difference (HSD) tests were performed as a post hoc analysis for pairwise comparisons among means to explicitly determine which parameters were significantly different from one another.

RESULTS

Morphometric results for the macrostructural level are summarized in Table 2. ANOVA revealed the existence of statistically significant differences between all indexes among the various age groups, with \( P < 0.001 \) for each case. Tukey tests were performed as a post hoc analysis and revealed that the 3-wk samples exhibited statistically significant differences in all indexes, except an endosteal perimeter, compared with the rest of the age groups. Among samples of 12 wk of age and older, there existed no significant differences in the mean total tissue volume, the mean cortical bone volume, the mean cortical bone volume density, the mean peristeal perimeter, the mean endosteal perimeter, or the mean cortical thickness (\( P > 0.05 \) for all cases). However, the mean polar area moment of inertia values were statistically different between all age groups except between 12-, 32-, and 42-wk groups and between 60- and 72-wk groups, which displayed no significant difference at the 0.05 level.

Table 1. Summary of abbreviations

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Definition</th>
</tr>
</thead>
<tbody>
<tr>
<td>TV</td>
<td>Total cortical tissue volume</td>
</tr>
<tr>
<td>Ct.BV</td>
<td>Cortical bone volume</td>
</tr>
<tr>
<td>Ct.BV/TV</td>
<td>Cortical bone volume density = cortical bone volume/total tissue volume</td>
</tr>
<tr>
<td>Ct.Th</td>
<td>Cortical thickness</td>
</tr>
<tr>
<td>N.Ca</td>
<td>Number of canals</td>
</tr>
<tr>
<td>Ct.TV</td>
<td>Cortical total volume = cortical tissue volume + pores</td>
</tr>
<tr>
<td>N.Ca/Ct.TV</td>
<td>Number density of canals = number of canals/cortical total volume</td>
</tr>
<tr>
<td>Ca.V</td>
<td>Total canal volume</td>
</tr>
<tr>
<td>Ca.V/Ct.TV</td>
<td>Volume density of canals = total canal volume/cortical total volume</td>
</tr>
<tr>
<td>&lt;Ca.Dia.&gt;</td>
<td>Mean canal diameter</td>
</tr>
<tr>
<td>&lt;Ca.Le&gt;</td>
<td>Mean canal length</td>
</tr>
<tr>
<td>&lt;Ca.θ&gt;</td>
<td>Mean canal orientation</td>
</tr>
<tr>
<td>&lt;Ca.V&gt;</td>
<td>Mean canal volume</td>
</tr>
<tr>
<td>N.Lc/Ct.TV</td>
<td>Lacuna number density</td>
</tr>
<tr>
<td>&lt;Lc.V&gt;</td>
<td>Lacuna volume</td>
</tr>
<tr>
<td>Lc.V/Ct.TV</td>
<td>Lacuna volume density = lacuna volume/cortical total volume</td>
</tr>
<tr>
<td>&lt;Lc.Le&gt;</td>
<td>Mean lacuna length</td>
</tr>
<tr>
<td>&lt;Lc.θ&gt;</td>
<td>Mean lacuna orientation</td>
</tr>
<tr>
<td>J</td>
<td>Polar moment of inertia</td>
</tr>
</tbody>
</table>

Abbreviations follow Refs. 9, 15, and 46.

Morphometric results for the canal network at the microstructural level are tabulated in Table 3. ANOVA revealed that all indexes except the mean canal length displayed statistically significant differences among the age groups. Specifically, there was a statistically significant (\( P < 0.05 \)) increase in the mean canal number density between 3-wk samples and 12-wk samples. After 12 wk of age, the mean canal number density displayed a gradual decrease from 163 mm\(^{-3}\) to 128 mm\(^{-3}\) at 72 wk of age. The canal volume density is a measure of the contribution of the canal microstructures to the overall bone tissue porosity. There was a statistically significant decrease (\( P < 0.05 \)) in the mean canal volume density between the ages of 3 and 12 wk followed by a gradual decrease into 72 wk of age. The mean canal volume displays a significant drop (\( P < 0.05 \)) from 742·10\(^3\) \( \mu \)m\(^3\) at 3 wk of age to 426·10\(^3\) \( \mu \)m\(^3\) at 12 wk before steadily decreasing and eventually dropping to 339·10\(^3\) \( \mu \)m\(^3\) at 72 wk. The mean canal diameter drops significantly from 29.7 \( \mu \)m at 3 wk to 21.1 \( \mu \)m at 12 wk, after which point it remains relatively constant. While no significant variations were found between the mean canal lengths for the different age groups, there was a dependence of the mean canal orientation on age (\( P < 0.05 \)). Therefore, although the average length of individual canals does not change significantly with age, the spatial arrangement of this canal network and its branches does.

Table 2. Morphometric results at the whole bone level

<table>
<thead>
<tr>
<th>Parameter</th>
<th>3 wk</th>
<th>12 wk</th>
<th>32 wk</th>
<th>42 wk</th>
<th>60 wk</th>
<th>72 wk</th>
<th>ANOVA</th>
</tr>
</thead>
<tbody>
<tr>
<td>Body mass, g</td>
<td>57.5 ± 2.8*</td>
<td>120.3 ± 10.5</td>
<td>277.0 ± 9.1</td>
<td>308.9 ± 6.3</td>
<td>331.9 ± 12.6</td>
<td>356.4 ± 9.1</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>TV, mm(^3)</td>
<td>2.49 ± 0.43*</td>
<td>7.52 ± 0.94</td>
<td>7.34 ± 0.82</td>
<td>6.48 ± 0.88</td>
<td>6.78 ± 1.11</td>
<td>6.81 ± 1.04</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Ct.BV, mm(^3)</td>
<td>1.45 ± 0.5*</td>
<td>6.32 ± 1.15</td>
<td>5.97 ± 1.09</td>
<td>5.56 ± 0.71</td>
<td>5.62 ± 0.8</td>
<td>5.39 ± 0.96</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Ct.BV/TV, %</td>
<td>58.3 ± 5.5*</td>
<td>84.1 ± 7.4</td>
<td>81.3 ± 7.2</td>
<td>85.7 ± 4.6</td>
<td>83 ± 6.2</td>
<td>79 ± 4.9</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Ct.Th, ( \mu )m</td>
<td>184 ± 36*</td>
<td>676 ± 70</td>
<td>632 ± 55</td>
<td>642 ± 71</td>
<td>640 ± 53</td>
<td>622 ± 76</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Periosteal perimeter, mm</td>
<td>3.30 ± 0.47*</td>
<td>7.10 ± 0.43</td>
<td>7.00 ± 1.02</td>
<td>6.45 ± 0.85</td>
<td>6.83 ± 0.92</td>
<td>7.22 ± 0.60</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Endosteal perimeter, mm</td>
<td>2.14 ± 0.41</td>
<td>2.85 ± 0.062</td>
<td>3.03 ± 1.0</td>
<td>2.42 ± 0.65</td>
<td>2.81 ± 0.77</td>
<td>3.31 ± 0.26</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>J, ( \mu )m</td>
<td>0.13 ± 0.03*</td>
<td>2.02 ± 0.18*</td>
<td>2.5 ± 0.20*</td>
<td>2.25 ± 0.23*</td>
<td>3.42 ± 0.41*</td>
<td>3.03 ± 0.29*</td>
<td>&lt;0.001</td>
</tr>
</tbody>
</table>

Values are mean ± SD. *Values statistically significantly different (\( P < 0.05 \)) from another sites according to Tukey’s HSD test. a,b,c Any two age groups with dissimilar superscripts for a given variable are statistically significant according to Tukey test (\( P < 0.05 \)).
Morphometric results at the submicrostructural level

Table 3. Morphometric results at the microstructural level

<table>
<thead>
<tr>
<th>Parameter</th>
<th>3 wk</th>
<th>12 wk</th>
<th>32 wk</th>
<th>42 wk</th>
<th>60 wk</th>
<th>72 wk</th>
<th>ANOVA</th>
</tr>
</thead>
<tbody>
<tr>
<td>N/Ca/Cl.TV, mm (^{-3})</td>
<td>83 ± 10.8(^a)</td>
<td>163 ± 34.4(^b)</td>
<td>159 ± 28.7(^b)</td>
<td>139 ± 25(^b)</td>
<td>132 ± 22.3</td>
<td>128 ± 27.6</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Ca/V(Cr.TV, %)</td>
<td>6.13 ± 0.7(^a)</td>
<td>2.72 ± 0.6</td>
<td>2.53 ± 0.8</td>
<td>2.10 ± 0.9</td>
<td>1.71 ± 0.4</td>
<td>1.69 ± 0.6</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Ca/V, (10^3) (\mu m)</td>
<td>742 ± 131(^a)</td>
<td>426 ± 99</td>
<td>393 ± 87</td>
<td>345 ± 123</td>
<td>360 ± 108</td>
<td>339 ± 127</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Ca/Dm, (\mu m)</td>
<td>29.7 ± 6.7(^a)</td>
<td>21.1 ± 4.1</td>
<td>18.9 ± 5.2(^b)</td>
<td>19.7 ± 4.7(^b)</td>
<td>20.3 ± 4.9</td>
<td>19.2 ± 4.4(^b)</td>
<td>0.022</td>
</tr>
<tr>
<td>Ca/Lc, (\mu m)</td>
<td>268 ± 31</td>
<td>305 ± 22</td>
<td>299 ± 15</td>
<td>287 ± 23</td>
<td>279 ± 18</td>
<td>280 ± 17</td>
<td>0.114</td>
</tr>
<tr>
<td>Ca/θ, °</td>
<td>31.2 ± 15(^a)</td>
<td>53.3 ± 13</td>
<td>57.9 ± 10</td>
<td>65.4 ± 11</td>
<td>63.1 ± 8</td>
<td>68.8 ± 8</td>
<td>&lt;0.001</td>
</tr>
</tbody>
</table>

Values are mean ± SD. \(^a\)Values statistically significantly different (P < 0.05) from another site according to Tukey’s HSD test. \(^b\)Any two age groups with dissimilar superscripts for a given variable are statistically significant according to Tukey test (P < 0.05).

Figure 4 illustrates the age-related changes in the canal volume density along with differences among various sites. At 3 wk of age, the medial region contained significantly less porosity due to canals (P < 0.05) compared with the other regions. This effect was seen throughout the 12- and 32-wk samples, although this difference was not statistically significant. After 32 wk, as age increased, the posterior region contained less porosity due to canals compared with the other sites, and this effect was significant (P < 0.05) at 42 and 72 wk of age. Similar to the trend displayed with the canal number density within the 72-wk samples, the canal volume density was also statistically significantly lower in the lateral region in the 72-wk samples.

Figures 5–8 illustrate the changes in the element-based parameters with age and differences among anatomic sites. As shown in Figure 5, although there were changes with age, there was no statistically significant difference in the mean canal volume between the anterior, posterior, medial, and lateral regions of the tibia. In fact, there was very close agreement between the mean canal volume measurements between the various sites at each age. As shown in Figure 6, the mean canal diameter was also independent of the anatomic location. Although there was less agreement between the mean canal volume measurements at each site, there were no significant differences between the mean values (P < 0.05).

Figure 7 illustrates differences in the mean canal length as a function of age and anatomic location. Although no statistically significant differences existed (P > 0.05 for all sites), there was a large decrease in the mean canal length in the posterior region of the 72-wk samples. Differences in the mean canal orientation based on anatomic location are illustrated in Fig. 8. At older ages (42, 60, and 72 wk), the posterior region of the tibia exhibited canals with mean orientations that were greater (more radially oriented) than other sites. This difference in the mean canal orientation in the posterior region was statistically significant at 60 and 72 wk of age (P < 0.05).

DISCUSSION

At the macrostructural level, the tibial mid-diaphysis of the Sprague-Dawley rat displayed a rapid increase in size and volume from 3 wk of age to 12 wk of age. This increase can be attributed to a period of rapid growth in rats taking place between 4 and 8 wk of age, as seen by the increase in rat body mass found in the present study, and as reported by Roach et al. (45) in Wistar rats and McHugh et al. (37) and Bennell et al. (7) in Sprague-Dawley rats. During this period, bone undergoes an increase in radial growth, corresponding to the increase in the total tissue volume observed between 3 and 12 wk of age. Following the period of rapid growth, the growth rate of the tibia gradually decreases until it virtually ceases after 28–30 wk of age (60). This same growth phenomenon describes the initial increase in the cortical thickness, periosteal perimeter, and cortical bone volume observed between 3 and 12 wk of age. Following the rapid growth period (samples 12 wk and older), these parameters remained relatively constant and did not exhibit statistically significant changes with age (P > 0.05). The gradual decrease in cortical thickness observed coincides with previous work done by Li et al. (29) who found that female Sprague-Dawley rats between the ages of 3 and 16 mo (~13 and 70 wk) display no changes in the tibial cortical area with age but rather a redistribution of bone mass from the endosteal region to the subperiosteal region of the tibia. This redistribution of bone mass results in gradual thinning of the cortical wall as displayed in Table 2. The gradual decrease in the cortical thickness from 12 to 72 wk of age yields the decrease in the cortical bone volume observed between that same age span. This redistribution of mass from the endosteal region to the subperiosteal region also results in a net deposition of bone mass further from the neutral axis of the bone, a compensatory mechanism designed to preserve structural strength and stiffness as the tissue level properties of bone degrade with age (13). This mass redistribution results in the gradual increase in polar area moment of inertia as seen in Table 2. Our data also show a slow increase in the endosteal and periosteal perimeters with age but these increases are not statistically significant for ages 12 to 72 wk. As mentioned above the main increase in tibia dimensions took place between 3 and 12 wk. Interestingly, the rat body mass showed a more gradual and more continuous increase at earlier ages with...
higher rate between 3 and 32 wk and a nearly linear slower increase at older ages.

It is well documented that bone formation rate and the activity of the bone-forming cells within the basic multicellular unit (BMU) are correlated with bone perfusion and the development of the vascular network (3, 26, 62). Therefore, in order for bone to sufficiently vascularize during a period of rapid growth, it is necessary that the canal network grow and develop. This growth is evidenced by the initial increase in the canal number density between 3 and 12 wk of age as seen at the microstructural level (Table 3). During the period of rapid growth between 3 and 12 wk, the vascular network grows and densifies to supply sufficient blood, nutrients, and oxygen to bone and the bone-forming cells in the BMU. Following the rapid growth period, there is a gradual decrease in the canal number density. The decrease in the canal number density indicates a suppression in the degree of the canal network vascularization and bone formation activity during the advancement into maturation and senescence (18, 33). This phenomenon is illustrated in Figure 9, which displays the densification of the canal network from 3 to 12 wk and the subsequent suppression at 60 wk. This finding coincides with the previous work done by Hagaman et al. (20), who used scanning electron microscopy (SEM) to determine that the number and density of vascular canals within Wistar rats decreases from 6 mo (~26 wk) to 24 mo (~104 wk) of age. Although the canal number density increases from 3 to 12 mo, the canal volume density decreases. This decrease in the canal volume density from 6.13% at 3 wk to 2.72% at 12 wk is statistically significant \( (P < 0.05) \) while the gradual decrease exhibited after 12 wk of age is not \( (P > 0.05) \). The significant decrease in the canal volume density from 3 to 12 wk results from a morphometric change in canal geometry during this same age span. Namely, the mean canal diameter from 3 to 12 wk significantly decreases from 29.7 to 21.1 µm, while the average canal length remains constant, causing a corresponding statistically significant decrease in the mean canal volume (Fig. 9). After 12 wk there is a small gradual decrease (not statistically significant at \( P < 0.05 \)) in the canal number density, canal volume density, mean canal volume, and mean canal diameter. These results suggest very different fluid transport characteristics in the early stage of bone growth than in a more mature bone that merit further investigation.
and mean length of canals, given in Table 3, do not obey closely an equation for the volume of cylinder. This is due to the fact that not all pores are cylindrical in shape and due to the presence of much larger canals with irregular geometries. In Schneider et al. (46) such an equation is also not satisfied precisely. Such irregular geometries make the quantitative characterization challenging.

Work done by Schneider et al. (46) on the determination of ultrastructural properties of murine cortical bone revealed a similar site dependency of the canal number density and canal volume density within femoral bone. Particularly, Schneider et al. (46) found a significant difference between the anterior and posterior regions, citing a fourfold decrease in the canal volume density and a fivefold decrease in the canal number density in the posterior region compared with the anterior region. An analogous site dependency was found by Matsumoto et al. (31) in rat tibiae harvested from 3- and 4-wk-old rats. Using monochromatic synchrotron radiation CT (SRCT), Matsumoto and colleagues found the posterior region of the tibia to be less porous than the anterior region. As mentioned above, in this study, from 3 to 32 wk of age, we found significantly fewer canals per volume of bone in the medial region of the tibia. Thus, these three findings do not report the same anatomic region with lowest canal number density. One of the challenges in assessing morphology of bone, in particular at young ages, is that bone is highly spatially heterogeneous, and void sizes may vary by an order of magnitude. Thus porosity may not necessarily scale with the number of canals. Also, studied regions are limited and may vary depending on the protocol used in a given study. These reported differences merit further investigation. The site dependency of the cortical microstructure was also investigated in turkey ulnae, where it was discovered that the cortical region containing the lowest cannular porosity was also the region subjected predominantly to compression (48, 49). Therefore, to accurately examine the causality of anatomic differences in the canal network, the information on the habitual loading of the rat tibial diaphysis in vivo is required. This subject is beyond the scope of the present study. Such further studies on bone adaptation could potentially explain the above-mentioned differences in relative po-

As shown in Table 3, the mean canal orientation increases with age, signifying that canal branches within the cannular network become more radially oriented with age. This change in the mean canal orientation is illustrated in Figure 10. Following a period of rapid growth up to about 8 wk of age (7, 37), the growth plate in the tibia of the rat decreases in width, corresponding to a decreased longitudinal growth (19, 47). After ~17 wk of age, the tibia enters a longitudinal growth plateau (6). Near 28–30 wk of age, bone essentially ceases its longitudinal growth (60); however, bone continues to remodel and redistribute its mass as it resorbs from the endosteal region and deposits on the subperiosteal region. This change in dynamic from longitudinal growth to exclusively radial redistribution of mass could explain the change in spatial arrangement in the canal network. As longitudinal growth rate decreases, the canals become more radially oriented to continue and make more efficient the transport of blood and nutrients to both the endosteal and periosteal regions (60).

Regarding the dependence of the microstructural parameters on the anatomic site, the medial region of the tibia had a lower canal number density and cannular porosity (porosity due to canals only) than the other sites. This result becomes less apparent at older ages (60+ wk) as the cannular porosity and the number density of canals increase and become comparable to other regions. At these older ages, there is also a decrease in the canal number density and cannular porosity in the posterior region of the tibia. Note that in the 3-, 12-, and 32-wk-old animals the canal number and the canal volume densities are smallest in the medial region while other parameters such as the mean canal volume do not show that trend. Similarly, at ages 42, 60, and 72 wk, the posterior region has the lowest canal number and canal volume densities but the mean canal volume does not show this trend. This may be due to the fact that limited regions were investigated at the microstructure and submicrostructure levels and that the spatial heterogeneity of the pore structure in both longitudinal and transverse directions influences morphometric parameters. Second, the canal structure shows considerable tortuosity, which makes the measurements and the interpretation of results more complex. Further studies need to be done to better understand these complex relations.

Also, it should be mentioned that the obtained morphological indexes such as the mean volume of canals, mean diameter

![Fig. 7. Age-related changes in the mean canal length at various anatomic sites.](image)

![Fig. 8. Age-related changes in the mean canal orientation at various anatomic sites.](image)
rosities at different anatomic quadrants reported by Schneider et al. (46), Matsumoto et al. (31), and the present study.

Although there existed differences in the canal number density and the canal volume density between anatomic regions, the parameters describing the single basic elements of the canal network (mean canal volume, mean canal radius, and mean canal length) were independent of the anatomic location. These findings are consistent with a similar study done by Schneider et al. (46) in which the same conclusion was drawn regarding the site independence of these element-based parameters within murine femora. Although Schneider and colleagues were comparing differences between two strains of mice, the cannular element-based parameters within each strain displayed no site dependence. While parameters describing cannular geometry were independent of site, the spatial orientation of these canals was shown to be site dependent. Specifically, in older samples (42 wk+), the posterior region of the tibia contained canals that were more radially oriented (greater mean canal orientation) than the other regions, and these differences were significant at 60 and 72 wk of age. Schneider et al. (46) also observed a site dependence of mean canal orientation within mouse femora, although those findings found the anterior and medial regions to contain canals with a greater mean orientation. These differences in the spatial orientation of canals may be due to possible differences in a nonuniform load distribution in the tibia (31, 49) in these two species.

Until recently, a vast majority of the analyses of osteocyte lacunae has been restricted to the two-dimensional realm and has been based on the microscopic analyses of histological bone sections. Although a three-dimensional analysis has been relatively restricted up until this point, the osteocyte lacunae have been analyzed and characterized since the development of the methodology for dynamic bone histomorphometry (46). As seen in Table 4, the lacuna number density was found to be independent of age. Previous studies investigating the lacunar density have reported conflicting results. Within human bone, some have determined that the lacunar density decreases with age (5, 39, 43) while others have found that it increases with age or remains constant (57). Studies of human vertebral trabecular bone and femoral cortical bone done by Vashishth et al. (56, 57) have demonstrated that the relationship between lacuna number density and age is dependent on sex and is tissue specific. This could partially account for the discrepancy between results obtained by Mullender et al. (38) and those found in this study. Specifically, Mullender et al. estimated the osteocyte number density in rats to be \( \sim 93,000 \text{ mm}^{-3} \). Although this estimation differs significantly from the average lacuna number density calculated in this study (\( \sim 42,000 \text{ mm}^{-3} \)), it is important to note that Mullender et al. analyzed femoral cancellous bone compared with the tibial cortical bone analyzed in this study. Discrepancies in values can be attributed to differences in strain as well (Wistar vs. Sprague-Dawley).

The osteocyte lacunae were ellipsoidal in shape, with one dimension being smaller than the remaining two, giving it a flattened appearance, similar to lacunae morphology determined previously (1, 36, 46) as shown in Figure 11. Figure 11
The results of this study reveal a positive relationship between the canal number density and the bone size and weight. Specifically, the canal number density scales positively with the cortical thickness and the cortical bone volume. The mean canal volume and the mean canal volume density show an inverse relation with bone size and weight. However, the lacunar morphometry does not scale with bone size. These distinct trends at different structural scales illustrate the complexity of the fluid flow in bone and its cellular activity as a function of age. Note that our analysis does not consider the canalicular network, due to the small size of these canals, which may be affected by age.

Porosity is one of the very important parameters quantifying bone structure. As summarized in Schneider et al. (46) it is one of the key parameters that contributes to bone quality. It also serves as a main input for modeling (35). One of the key contributions of this study is the quantification of the multi-scale porosity in a rat tibia as a function of age. These results are summarized in Fig. 12, which gives porosities at the tissue and cell levels, which combined give the organ level porosities.

In summary, this study provided an assessment of the cortical bone morphology within rat tibia at different hierarchical levels. Through the use of a high-resolution desktop micro-CT system and quantification software, the intracortical porosity within the rat has been decomposed into the canal network and the osteocyte lacunar system, and each of these has been quantified using new cortical morphometric indexes to describe their size, shape, and spatial orientation. The results of this study give new insights into the cortical network morphology within the rat and how this network changes with age.

Study Limitations

It is important to note that rats, unlike humans, rarely undergo osteonal remodeling. Therefore, within rat bone, regions of concentric lamellae surrounding a canal are virtually nonexistent. For these reasons, the authors caution against a direct comparison between the rat canal network and the network found within human bone. Furthermore, the analysis of this study was limited to the middiaphysis region only and assessed only the cortical bone within the rat tibia. Further work needs to be completed to determine the canal structure along the
whole bone length and to assess the differences between various bone types and species as well as the applicability of this rat model to human bone. Finally, it should be noted that the observed canal network gives only a conjecture about features of cortical vascular structures because it has not been confirmed that those canals actually contain blood vessels.

Conclusions

We characterized the hierarchical porosity of female rat tibia cortical bone as a function of age. At the organ level, 3-wk samples were statistically different in all measured parameters compared with other age groups. At the tissue level, there was a statistically significant increase in the mean canal number density and a decrease in the mean canal volume and diameter between the 3-wk samples and the remaining age groups, the mean canal lengths remained nearly constant, and there was a dependence of mean canal orientation on age. At the cell level, all lacunae parameters displayed no dependence on age. In addition, at the tissue level, the indexes were reported separately for anterior, posterior, medial, and lateral anatomic regions. From 3 to 32 wk of age, there existed significantly fewer canals per volume of bone in the medial region of the tibia (P < 0.05). There were no statistically significant differences in the mean canal volume, mean canal radius, and mean canal length between the four anatomic regions. At older ages, the posterior region of the tibia exhibited more radially oriented canals than in other sites, and this difference was statistically significant at 60 and 72 wk of age (P < 0.05).

ACKNOWLEDGMENTS

We thank Dr. L. Yin, M. Bee, and T. Ross from Visualization Laboratory at Beckman Institute and Dr. J. Li, all at the Univ. of Illinois, for help with micro-CT imaging. We thank Professor J. Gullely from the University of Illinois at Urbana-Champaign for providing rat bone tissue from his breeding colony. Additionally, some of the tissue came from retired breeders from his laboratory (purchased from Harlan Laboratories).

GRANTS

We acknowledge support of the National Science Foundation (CMMI 09-27909, Dr. K. Chong).

DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the author(s).

AUTHOR CONTRIBUTIONS

Author contributions: J.J. performed experiments; J.J. and I.J. analyzed data; J.J. and I.J. interpreted results of experiments; J.J. prepared figures; J.J. drafted manuscript; J.J. and I.J. edited and revised manuscript; J.J. and I.J. approved final version of manuscript; I.J. conception and design of research.

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