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Evaluating Sex Related Differences in the Osteocyte Lacunar Canalicular Network Across the Lifespan: A Confocal Laser Scanning Microscopy Approach

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Evaluating Sex-Related Differences in the Osteocyte Lacunar-Canalicular Network Across the Lifespan: A Confocal Laser Scanning Microscopy Approach

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I. Introduction

The osteocyte lacunar-canalicular network (LCN) is an extensive cellular network within bone tissue. Osteocytes, the most abundant cells in bone, account for 90 to 95 percent of all bone cells in a mature human skeleton (Lanyon 1993). They reside in cell spaces called lacunae. Lacunae are connected via dendritic projections, the canaliculi. This network serves multiple functions required for maintaining bone homeostasis and sensing mechanical stimuli. Osteocytes act as mechanical sensors and perceive external force on bone and facilitate bone remodeling in response to mechanical strain (Bonewald and Johnson 2008). Strain induced on bone changes fluid-flow within the osteocyte LCN. There is strong evidence that osteocytes transmit strain induced changes in fluid-flow into biochemical signals that initiate bone remodeling (Klein-Nulend et al. 2013). Solutes that trigger bone remodeling travel between osteocyte lacunae via canaliculi. Transport rate has been shown to increase linearly with increasing numbers of canaliculi (Zhou et al. 2009). In pathological states, such as osteoporosis, this bone maintenance machinery is impaired and mechanosensing is altered.

Osteoporosis affects more than 200 million people worldwide (Sözen et al. 2017). This disease is characterized by accelerated bone resorption coupled with decreased bone formation (Bala et al. 2014). Osteoporosis preferentially affects women. Women aged 50 years and older are four times more likely than males of the same demographic to develop osteoporosis in their lifetime (Alswat 2017). Furthermore, the International Osteoporosis Foundation recently reported that 1 in 3 women will experience an osteoporotic fracture in their lifetime, whereas 1 in 5 men will experience an

osteoporotic fracture in their lifetime (Sözen et al. 2017). It is hypothesized that osteoporotic incidence is more prevalent in females due to physiological changes associated with menopause. Post-menopausal, estrogen deficient rat models have revealed changes in osteocyte LCN microarchitecture. Estrogen deficiency has been shown to increase vascular porosity in bone, leading to an increase in fluid-flow, thus triggering osteocytes to signal for bone remodeling (Gatti et al. 2018). In addition, osteoporosis and overall bone deterioration preferentially affects the elderly population. Overall osteocyte LCN volume decreases with increasing age (Carter et al. 2013).

Previous studies have failed to fully describe changes in bone microarchitecture as it relates to osteoporosis. When imaging bone microarchitecture, the primary limitation is resolution. The average diameter of a single canaliculus in human bone is 259 ± 129 nm (Nango et al. 2016). Given this small value, many imaging modalities that are traditionally employed (i.e. micro-CT and synchrotron-radiation micro-CT) fail to capture canaliculi. Confocal laser scanning microscopy (CLSM) has been utilized as an effective method for imaging the entire osteocyte LCN, including the canaliculi (Ciani et al. 2009). Previously, CLSM has been used to analyze the osteocyte LCN in two dimensions; one study revealed a decline in osteocyte LCN volume in females with increasing age, primarily due to the loss of canaliculi (Ashique et al. 2017). The osteocyte LCN has not been explored three-dimensionally (3D) using CLSM. Therefore, the objective of the present study is to answer a number of questions revolving around age-related bone deterioration utilizing CLSM in 3D including: 1) do males and females exhibit differences in osteocyte LCN volume?, and 2) whether perimenopausal females exhibit a pronounced decline in osteocyte LCN volume. Given the preferential bias of

osteoporosis and fracture incidence in women, it is hypothesized that female individuals will exhibit a lower osteocyte LCN volume when compared to males. Furthermore, middle-aged females (35 to 54 years old) will display a lower osteocyte LCN volume when compared to younger females (15 to 34 years old).

II. Materials and Methods

Sample Preparation

Cadaveric left femur samples were sourced from local medical schools, universities, and Medical Examiner's offices including the University of Toledo, Kent State University, Cleveland State University, Northeast Ohio Medical University, and the New York City Office of Chief Medical Examiner. The collection included samples from modern males and females ranging from 19 to 101 years at death. Individuals with known bone-affecting conditions were excluded from the study. Sample processing began with maceration. This involved soaking the samples in a protease solution in water for 3 to 4 hours at 45°C. Soft tissue was scraped from the femora using dental tools. Once clean of soft tissues, the femora were placed in 70% ethanol. After soaking in ethanol for at least 24 hours, samples were removed and allowed to air dry for 24 hours. One-millimeter transverse thick sections were cut using a Buehler Isomet 1000 precision saw (Buehler, Lake Bluff, IL) equipped with a diamond blade. Thick sections were hand-ground to an ideal thickness of 75 μm utilizing a Buehler EcoMet 30 grinder/polisher (Buehler). In the event of damage to the bone (i.e. rips or tears in the thin section), grinding ended prior to the 75 μm thickness. Once the appropriate thickness was achieved, the specimens were polished using a Buehler EcoMet 30

grinder/polisher instrument. MasterPrep polishing suspension fluid (Buehler) was applied to a polishing disc to remove unwanted striations produced during grinding. Samples were washed in distilled water using an ultrasonicator to remove any leftover polishing fluid.

Processed and dried samples were stained using a 1% fluorescein isothiocyanate (FITC) fluorescent dye (Sigma-Aldrich, St. Louis, MO). FITC is an effective fluorescent dye for osteocyte LCN imaging using confocal laser scanning microscopy (Ciani et al. 2009). All staining procedures were performed in a dark room to avoid excitation of the stain. Once stained, excess dye was removed via three 20-minute washes in 100% ethanol. Samples were air dried and mounted on glass slides using ProLong Glass (Fisher Scientific, Hampton, NH) for CLSM imaging. Prepared slides were stored in a light-concealing container and frozen until imaging.

Confocal Laser Scanning Microscopy Imaging

The anterior portion of each prepared slide was imaged using an inverted Leica TCS SPE confocal laser scanning microscope, equipped with a motorized Z-Galvo stage (Leica Microsystems, Wetzlar, Germany). Immersion oil (Immersol 518 F oil, Zeiss, Jena Germany) was applied to a 63x objective lens and the sample was placed on the stage. A laser of wavelength 488 nm was set to 32.5% intensity and the spectral window was set to 485 nm to 585 nm. Once in focus, a z-stack of 29.99 μm was established to produce a 3D image stack. Individual slices were taken at increments of 0.3 μm for a total of 101 slices per sample. The scan speed for individual images was programmed to 400 hz. Images from three regions of interest within the anterior aspect of the femur (endosteal, intracortical, and periosteal) were obtained for a total of nine

images per sample. Individual images were taken at a resolution of 1024 by 1024 pixels. For each region, the horizontal stage was not manipulated to ensure the three images were in line vertically. Three-dimensional renderings were optimized using Leica Application Suite X software. Gain and smart-offset were adjusted accordingly to provide an image with minimal noise. The pinhole was set at 1 AU and the zoom was 1.5. Individual, three-dimensional stacks were 116.4 μm by 116.4 μm by 29.99 μm deep for a total sample volume of 4.06×10^5 μm^3.

Image Processing and Data Extraction

The 3D volume of the osteocyte LCN network was quantified using Amira 6.0 (Thermo Fisher Scientific, Waltham, MA). Image stacks were segmented to isolate the osteocyte LCN. Once isolated, the osteocyte LCN was designated as a new material to ensure its separation from the extracellular environment. The material statistics feature provided a total volume of the osteocyte LCN for the given image stack. For each sample, two image stacks per region were segmented and osteocyte LCN volume was extracted. These two volumes were then averaged to provide a regional average (V_{region}). Three regional averages (periosteal, endosteal, and intracortical) were added to create a representative total osteocyte LCN volume (V_{total}) for each individual sample.

Statistical Analyses

V_{total} was compared between sexes using a student's *t*-test. Five age categories (15-34, 35-54, 55-74, 75-99, and 99+) were compared across sexes. A Shapiro-Wilk test was conducted in JMP Pro 14 (SAS Institute, Cary, NC) to ensure normality, a necessary condition when performing an Analysis of Variance (ANOVA). V_{total} was not normally distributed. V_{total} was log transformed to ensure the data followed a normal

distribution. Once normalized, age category was compared using a one-way ANOVA and age category and sex were compared using a two-way ANOVA. A Tukey's post-hoc analysis revealed significant differences between the groups compared in each ANOVA. A *p*-value of less than 0.05 indicated a significant difference in V_{total} for all performed statistical analyses.

III. Results

The results of this project are part of a larger, ongoing study. Therefore, the author does not wish to report results at this time.

IV. Discussion

The results contain unpublished data that is part of a larger, ongoing publication. Therefore, a discussion of the results is not warranted at this time.

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