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Ptychographic X-ray CT characterization of the osteocyte lacuno-canalicular network in a male rat's glucocorticoid induced osteoporosis model

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ABSTRACT

Ptychographic X-ray computed tomography (PXCT) is a quantitative imaging modality that non-destructively maps the 3D electron density inside an object with tens of nanometers spatial resolution. This method provides unique access to the morphology and structure of the osteocyte lacuno-canalicular network (LCN) and nanoscale density of the tissue in the vicinity of an osteocyte lacuna. Herein, we applied PXCT to characterize the lacunae and LCN in a male Wistar rat model of glucocorticoid-induced osteoporosis (GIO). The ptychographic images revealed significant ($p < 0.05$) differences in the number of canaliculi originating from the lacuna per ellipsoidal surface unit, Ca.Nb ($p = 0.0106$), and the 3D morphology of the lacuna ($p = 0.0064$), between GIO and SHAM groups. Moreover, the mean canalicular diameter, Ca.Dm, was slightly statistically un-significantly smaller in GIO (152 ± 6.5) nm than in SHAM group (165 ± 8) nm ($p = 0.053$). Our findings indicate that PXCT can non-destructively provide detailed, nanoscale information on the 3D organization of the LCN in correlative studies of pathologies, such as osteoporosis, leading to improved diagnosis and therapy.

1. Introduction

Bone is a dynamic "organic-inorganic" tissue able to adapt to environmental and temporal changes through remodeling processes performed by osteoclasts, osteoblasts and osteocytes (Henkel et al., 2013). Osteocytes are the last differentiated state of the "osteoblast-osteocyte" lineage. They are the most long-lived cells in the skeleton, embedded within an extensive and intricate network of cavities and channels in the bone matrix, called *lacunae* and *canaliculi*, respectively (Bonewald, 2011). Thanks to their interconnectivity, osteocytes can communicate and exchange nutrients and waste products (Fritton and Weinbaum, 2009). The movements of extracellular fluids within the *lacuno-canalicular* network (LCN) mediate external stimuli to the osteocytes, implicating the architecture of this network in mechano-transduction pathways and bone remodeling. The morphology of the lacunae housing the osteocytes has been shown to depend on the osteoarticular physiopathological context, varying, *e.g.*, in osteoarthritis, osteoporotic bone, and osteopetrotic osteocytes (van Hove et al., 2009; Schneider et al., 2010; Schneider et al., 2011). The lifecycle and health of individual osteocytes affects the state of the LCN within bone tissue (Dallas and Veno, 2012). Hence, the functional cellular syncytium and the common fluid space defined by the LCN are interrelated, and depend on the viability of the osteocytes and the health of the bone tissue (You et al., 2004). Osteocytes change in size and shape as they lose viability, and the bone remodeling cycle may be initiated to remove non-viable cells. Lacunae previously occupied by healthy cells may remain empty, or become mineralized and occluded (Frost, 1960; Currey, 1964).

Consequently, a three-dimensional (3D) characterization of the LCN is necessary to improve our understanding of bone functionality and

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bone diseases. The key outcome of this study was a comprehensive, non-destructive and quantitative characterization of the LCN in high 3D resolution *via* ptychographic X-ray computed tomography (PXCT), allowing us to examine in detail the physiological impact of glucocorticoid-induced osteoporosis (GIO) at the sub-cellular level in cortical bone. We characterized the LCN in the presence of GIO in male rats and made a comparison with healthy paired-age rats.

Glucocorticoid (GC) therapy is used extensively, especially in chronic inflammatory and autoimmune diseases. Patients receiving long-term GC treatment present fractures in 30–50% of cases (Briot and Roux, 2015). Osteoporosis is one of the most widespread diseases in the world, resulting in bone fragility and enhancing the risk of fracture, and GIO is the most frequent cause of secondary osteoporosis (van Staa, 2006). In GIO, bone fragility occurs before changes in bone mineral density (BMD) are detectable, and the increases in fracture risk are larger than those expected on the basis of BMD changes (Van Staa et al., 2003; Weinstein, 2010; Van Staa et al., 2002). Fractures induced by GC therapy could, therefore, result from other factors than a decrease in BMD. The pathophysiology of GIO has not yet been completely explained (Frenkel et al., 2015). Among other mechanisms GC interferes with the adipocytic pathway but also with Wnt signalling pathway notably the upregulation of its inhibitor Dickkopf-1 (Ohnaka et al., 2004). At the cellular level, GC excess has adverse effects on bone cells (Weinstein, 2000; Weinstein, 2011; Weinstein et al., 2002), causing osteoblast (Weinstein, 2000) and osteocyte apoptosis (Weinstein et al., 1998; Jia et al., 2006; O'Brien et al., 2004; Sambrook et al., 2003; Achiou et al., 2015). Experiments strongly suggest that GC excess adversely affects bone vasculature due to direct effects on osteocytes (Weinstein et al., 2010).

Glucocorticoid-induced osteocyte apoptosis (Achiou et al., 2015) and decreasing bone vasculature could be implicated in a loss of bone strength before changes in BMD occur (Weinstein et al., 2010; Canalis et al., 2007). This could account for the observed mismatch between BMD and fracture risk in patients with GIO (Van Staa et al., 2003; Van Staa et al., 2005; Kanis et al., 2004).

The motivation for this study is to build upon these previous results, investigating GC induced modifications of the LCN structure at the nanoscale, to give new insights into how this network is modified in conditions of high rates of osteocyte apoptosis, and the correlation between mineral matrix and organic tissue. A thorough understanding of the nanoscale structure of cortical bone could shed light on the effects of GC treatment at the sub-cellular level and if so, provide the means to design effective co-administered therapies.

X-ray imaging exploits short wavelength radiation to produce images with high density contrast and high spatial resolution, simultaneously separating bone from soft tissue, and revealing the nanostructure of each component. Ultramicroscopy using X-ray optics has been achieved fairly recently (Langer and Peyrin, 2016; Schroer et al., 2002). Coherent 3D X-ray imaging has been demonstrated for the particular case of ultra-microscopy on bone using ptychographic X-ray computed tomography (PXCT) (Dierolf et al., 2010) and holography (Langer et al., 2012). Other options for performing ultramicroscopic imaging of bone tissue in 3D are transmission electron microscopy (Rubin et al., 2003; Everts et al., 2012), serial sectioning focused-ion beam scanning electron microscopy (Schneider et al., 2011), and confocal laser scanning microscopy (Jones et al., 2005). Compared to X-ray microscopy these methods obtain information from very thin specimens, or regions of a sample that are close to the surface, and both the sample preparation and the imaging are destructive in nature.

PXCT is a scanning coherent X-ray diffraction microscopy technique in which high-resolution images of a sample are obtained from diffraction data with iterative phase retrieval algorithms. Information from scanning a sample in a coherent beam such that illuminated areas overlap is used to aid the recovery of the unmeasurable phases that are associated to the far field diffraction intensities (Faulkner and Rodenburg, 2004; Thibault et al., 2008). Fig. 1 shows a schematic illustration of the experimental geometry for PXCT image acquisition.

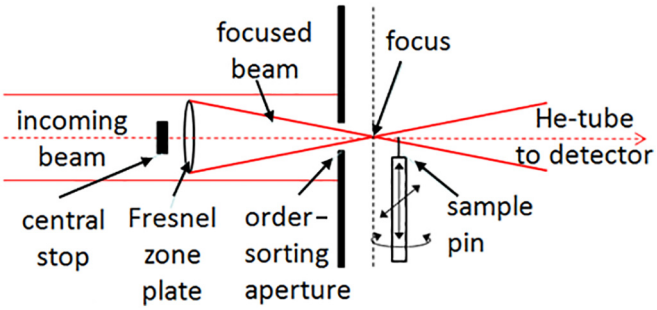

Fig. 1. A schematic illustration of the experimental geometry for ptychographic data acquisition. The beam is focused by a Fresnel zone plate, increasing the flux density on the sample, which is supported on a scanning and rotation stage. The diffraction signal is measured by a detector in the far field (not shown).

Complex-valued maps of the transmission function retrieved in this way represent the projection of the X-ray absorption and refraction properties of the sample. Different projections are achieved by rotating the sample around an axis perpendicular to the beam. Quantitative 3D images of the electron density within objects are obtained using CT algorithms, such as filtered backprojection (FBP) to invert the phase component of the projection images (Dierolf et al., 2010).

Cortical bone samples from the tibial diaphysis of rat bones were analyzed through a quantitative method we have developed, previously reported by Ciani et al. (2016) which provides a microarchitectural characterization of the LCN in terms of a morphological description of the osteocyte lacunae and a geometrical description of the canaliculi. Fig. 2 shows a sketched cross-section of an osteocyte lacuna and canaliculi as a guide to the following discussion.

Lacuna morphology was analyzed in terms of shape, volume, and degree of anisotropy. At the canalicular level, the porosity, spatial density of the network connections to the lacuna surface, and the mean canalicular radius was computed. The LCN structure of GIO samples were compared with the microarchitecture in healthy (SHAM) samples. To our knowledge, this is the first time that the effects of GC treatment on the LCN in a GIO rat experimental model have been studied at this scale of spatial resolution in 3D.

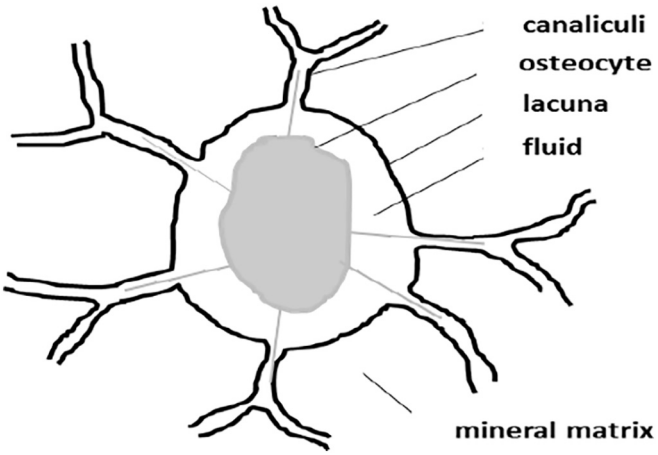

Fig. 2. A sketch of an osteocyte section with its lacuna and canaliculi and surrounding matrix. The osteocyte (cell body + cytoplasmic processes) is represented in gray. The lacuna, or the cavity in which the cells are located, and the canaliculi are outlined in black.