Determining Collagen Distribution in Articular Cartilage by X-ray Micro-computed Tomography

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Disclosures:

Introduction: Current histology techniques are based on staining thin tissue sections. This allows qualitative investigation of collagen distribution within articular cartilage (AC) using collagen-specific staining or antibodies. However, section-based characterization of collagen distribution in AC has following limitations: 1) the spatial distribution of collagen across the entire joint surface cannot be characterized from just a few tissue sections; 2) the sample preparation process is destructive and may modify the sample. Proteoglycan (PG) distribution in AC can be investigated with contrast-enhanced micro-computed tomography (CEµCT) [1]. In contrast to conventional histology, this approach allows one to characterize the sample composition in a volume, or even an entire joint in a small animal. However, there are no studies demonstrating the capacity of the CEµCT approach to detect collagen distribution in AC. Therefore, the aim of this study is to demonstrate the feasibility of using CEµCT to evaluate collagen distribution in AC. To accomplish this, we apply phosphotungstic acid (PTA) as a contrast agent that binds to collagen molecules [2] and contains a heavy element (Tungsten) to attenuate X-rays.

Methods: Limbs from three horses were collected from the local slaughterhouse (Veljekset Rönkä Oy, Kemi, Finland). Fetlock joints were prepared and the proximal phalanx was carefully dissected. Osteochondral samples (\(n = 12\), dia. = 6mm) were prepared from the anterior medial (AM, \(n = 3\)), posterior medial (PM, \(n = 3\)), anterior lateral (AL, \(n = 3\)) and posterior lateral (PL, \(n = 3\)) of the proximal phalanx. Each sample was cut in two; first half CEµCT, whereas the other was prepared for Fourier transform infrared imaging (FTIRI). Samples for CEµCT were formalin fixed for five days and stored in 70% EtOH. Before CEµCT the samples were stained with 1% w/v PTA using 70% EtOH as base solution (\(pH = 5.5\)). Images were collected at eight time points during the PTA staining: 0h, 18h, 36h, 54h, 72h, 90h, 180h and 270h. Samples were scanned (80kV, 300mA, 658 projections, exposure 1050ms/frame, 0.5 mm Al filter + 0.038mm Cu filter) with an in-vivo µCT device at isotropic 8.71µm voxel size length (Skyscan 1176, Bruker microCT). Drying was prevented by keeping the samples in sealed containers with moistened cotton balls during the imaging. The acquired X-ray images were reconstructed using manufacturer software with beam hardening correction and ring artefact reduction. Coronal images were used for the analysis.

As a reference, FTIRI measurements were conducted for adjacent tissue section using a Hyperion 3000 FTIRI Microscope (Bruker Inc.) with a 5.4 x 5.4 µm pixel size (2 x 2 binning, spectral resolution 4 cm-1, average of 32 acquisitions per pixel). Chemical maps from Amide I distribution were used to represent collagen distribution in cartilage and bone [3]. FTIRI and CEµCT profiles were normalized to max. AC thickness. Following this, image intensity profiles were achieved by calculating the mean and SD row by row from the surface of AC to the tidemark. Subsequently, attenuation profiles of AC from PTA-stained CEµCT images were compared against the collagen distribution profiles of the FTIRI images and correlation analysis was applied.

Results: CEµCT images showed increasing X-ray attenuation between the superficial AC and the tidemark from the 36h time point onwards (Figs. 1-2). PTA reached the tidemark in all samples with the used concentration and pH by 36h. PTA staining prolonged for more than 36h appeared to increase the X-ray attenuation at the tidemark and superficial AC. The highest match between the X-ray attenuation profile and FTIRI collagen profile (highest Pearson correlation coefficient, i.e. \(r = 0.974 \pm 0.012, n = 11, p < 0.001\) for all samples) was observed at 36h.

Discussion: Both CEµCT and FTIRI detected the higher collagen concentration at the AC surface as well as the increasing collagen concentration as a function of increasing depth into the AC. The attenuation profile in PTA-stained AC samples correlated with the collagen profile in the FTIRI measurement. The highest correlation coefficient was observed at 36h. There were slight differences in the collagen distribution between these two techniques; the thickness of the superficial collagen layer is apparently greater as recorded by µCT as compared to that recorded by FTIRI. This might be partially due to the weaker spatial resolution of the µCT. Another potential reason may be the accumulation of PTA in superficial AC. However, the underlying mechanism of temporal accumulation of PTA at the AC surface and tidemark is unclear. This needs further attention in order to harness the full potential of this approach to detect collagen distribution. Furthermore, PTA diffusion appears slow in the calcified cartilage and compact bone. Therefore, the collagen quantification may take place only where PTA penetrates, i.e., between superficial AC and the tidemark.

We conclude that PTA distribution in AC is related to collagen distribution and may allow 3D assessment of collagen distribution in non-calciﬁed AC. With the used species, sample size, and staining protocol, full staining is achieved in 36 hrs.

Significance: The proposed method is the first CEµCT based technique to determine collagen distribution in AC. This is a first step towards 3D quantiﬁcation of collagen distribution in in vitro AC samples and in small animal joints.
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Figure 1: Normalized μCT image intensity (X-ray attenuation) and FTIRI image intensity (Amide I intensity) as a function of articular cartilage (AC) depth (analysis limited from AC surface to tidemark). The profiles was calculated as an average of all samples (n = 11) at each time point. One sample was excluded from analysis due to failure in sample preparation. Best match of FTIRI and μCT average profiles were observed at 18 or 36 h suggesting that the different methods provide similar information on collagen distribution in AC.
Figure 2: A. Representative contrast-enhanced μCT image (X-ray attenuation) of articular cartilage (AC) sample hypothesized to visualize collagen. B. FTIRI image (Amide 1 intensity) of adjacent AC tissue demonstrating the reference collagen distribution within the sample. The red lines demonstrate segmented contours of AC surface (top), tidemark (middle) and AC-bone interface (bottom). The intensity distributions obtained with different methods were similar between AC surface and tidemark, whereas contrast agent penetrated poorly into calcified cartilage. The result suggests that μCT can be used to visualize collagen and detect collagen distribution in AC using phosphotungstic acid staining. FTIRI image shows a section-related artefact (lighter contrast) not visible in μCT.

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