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Determining collagen distribution in articular cartilage using contrast-enhanced micro-computed tomography

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SUMMARY

Objective: Collagen distribution within articular cartilage (AC) is typically evaluated from histological sections, e.g., using collagen staining and light microscopy (LM). Unfortunately, all techniques based on histological sections are time-consuming, destructive, and without extraordinary effort, limited to two dimensions. This study investigates whether phosphotungstic acid (PTA) and phosphomolybdic acid (PMA), two collagen-specific markers and X-ray absorbers, could (1) produce contrast for AC X-ray imaging or (2) be used to detect collagen distribution within AC.
Method: We labeled equine AC samples with PTA or PMA and imaged them with micro-computed tomography (micro-CT) at pre-defined time points 0, 18, 36, 54, 72, 90, 180, 270 h during staining. The micro-CT image intensity was compared with collagen distributions obtained with a reference technique, i.e., Fourier-transform infrared imaging (FTIRI). The labeling time and contrast agent producing highest association (Pearson correlation, Bland–Altman analysis) between FTIRI collagen distribution and micro-CT -determined PTA distribution was selected for human AC.
Results: Both, PTA and PMA labeling permitted visualization of AC features using micro-CT in non-calcified cartilage. After labeling the samples for 36 h in PTA, the spatial distribution of X-ray attenuation correlated highly with the collagen distribution determined by FTIRI in both equine (mean ± S.D. of the Pearson correlation coefficients, $r = 0.96 \pm 0.03$, $n = 12$) and human AC ($r = 0.82 \pm 0.15$, $n = 4$).
Conclusions: PTA-induced X-ray attenuation is a potential marker for non-destructive detection of AC collagen distributions in 3D. This approach opens new possibilities in development of non-destructive 3D histopathological techniques for characterization of OA.

Introduction

To better understand osteoarthritis (OA) greater knowledge about the structural and compositional changes in articular cartilage (AC) with progression of OA is required. Histopathological evaluation is a standard *in vitro* approach to detect changes in osteochondral tissue morphology and related OA stage. Progressing OA alters water, proteoglycan (PG), and collagen content and distribution in AC, as well as size, distribution, orientation, and density

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of chondrocytes[1–3]. The collagen network, which is important for the biomechanics of AC[4,5], is disrupted in OA. This is evident as superficial fibrillation, clefts, and collagen condensation around chondrons[6]. The degeneration can lead to tissue failure and cell death[7] particularly at the transitional zone. It is unclear whether this kind of degeneration induces apoptosis[8,9].

The standard technique to determine collagen distribution in AC is histological sectioning and subsequent staining of the collagen distribution in thin (ca. 5 μm thick) section. Commonly, sections are stained by using collagen labels such as phosphotungstic acid (PTA) or phosphomolybdic acid (PMA)[10]. Another option is to image the collagen within the slice using a label-free approach such as Polarized LM[11], Autofluorescence[12] or FTIRI[13,14]. All section-based techniques are time-consuming and destructive since they involve sample preparation such as, fixation, de-calcification, and cutting of histological sections. 3D reconstruction from serial sections is impractical. Currently, one can study 3D distribution of collagen using contrast-enhanced MRI[15], but MRI lacks the resolution to visualize tissue constructs, e.g., cells, in histologically relevant detail.

There are non-destructive techniques to detect PG distribution in AC using micro-CT and contrast agents such as Hexabrix[16], CA4+/CA1+[17], sodium iodide[18], tantalum oxide nanoparticles[19] and gadopentetate[20]. However, no corresponding methods to detect collagen distribution in the AC volume exist in the literature. In this study, we investigated: (1) whether PTA and PMA can penetrate the AC matrix and label collagen for micro-CT imaging, and (2) if these contrast agents can serve as markers to reveal the spatial distribution of collagen in AC. After first verifying the suitability of the technique with equine AC, we aimed to demonstrate the approach in human AC.

Method

Horse samples

Fresh joints from three horses were obtained from a local slaughterhouse (Veljekset Rönkä Oy, Kemi, Finland). Osteochondral cylinders (n = 12, diameter = 6 mm) 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 proximal phalanxes. Each sample was cut into three pieces [Fig. 1(A)] and subjected to the following procedures: first two pieces (two quarters) were fixed in 4% formalin 5 days (maintenance of tissue structure) and subjected to PTA or PMA staining as described later; the third piece [one half, Fig. 1(A)] was formalin-fixed for 2 days (maintenance of tissue structure), de-calcified with EDTA for 30–60 days (fixes the sample and enhances microtome sectioning), embedded with paraffin (holds the sample during sectioning), and finally sectioned with a microtome (produces 5 μm thick tissue slices). The sections were subjected to FTIRI[7], our reference, to determine the collagen distribution in unstained tissue sections (5 μm thick slices) [Fig. 1(A)].

Human samples

Human osteochondral cylinders (n = 4, diameter = 4.6 mm) were prepared from two patients undergoing total knee arthroplasty; one *tibial* and one *femoral* plug from each of the donors (donor 1: male, age 77 yrs; donor 2: male, age 58 yrs) [Fig. 1(B)]. Institutional ethics approval (PSSHP 78/2013) and patient consents were obtained. Each sample was cut into one half and two quarters [Fig. 1(B)]. Each part (one half and one quarter were used) was fixed, decalcified, and sectioned like the horse samples. The collagen distribution in these unstained sections was assessed by FTIRI and LM using Masson's trichrome staining [Fig. 1(B)][10]. One quarter of the sample was immersed in X-ray contrast agent using the protocol chosen based on the equine tests.

Contrast agent labeling and micro-CT

The horse samples were imaged multiple times with micro-CT: The sample was first imaged at 0 h without markers. Next the sample was immersed in 70% EtOH containing 1% w/v PTA (pH = 2.71) or 1% w/v PMA (pH = 2.35) for 270 h [Fig. 2(A)]. At predefined time points 18, 36, 54, 72, 90, 180 and 270 h the sample was removed from the contrast agent solution, rinsed in 70% ethanol and imaged with micro-CT. The samples were scanned with an *in vivo* micro-CT device (Skyscan 1176, Bruker microCT; settings: 80 kV, 300 mA, 658 projections, exposure 1050 ms/frame, average of 2 frames per projection, 32 min imaging time, 0.5 mm Al filter & 0.038 mm Cu filter, isotropic 8.7 μm voxel side length), while kept in sealed containers with cotton balls moistened with 70% ethanol to prevent drying. The acquired X-ray projections were reconstructed using Skyscan NRecon software (v. 1.6.9, Bruker microCT, Kontich, Belgium) in conjunction with beam hardening and ring artifact corrections. In the resulting images greater attenuation is indicated with brighter contrast similar to conventional X-ray images in which bone appears as brighter contrast and soft tissues appear as darker contrast or with no contrast.

The staining method that most prominently revealed collagen distribution in horse AC (i.e., greatest association between FTIRI collagen distribution and micro-CT -determined PTA distribution as determined by Pearson correlation and Bland–Altman analyses), was selected for the human samples. Following staining, the samples were rinsed in 70% ethanol and scanned with a micro-CT device (Nanotom 180NF, Phoenix X-ray Systems/GE; settings: 80 kV, 100 μA, 1200 projections, exposure 750 ms/frame, average of 7 frames per projection, 2 h 15 min imaging time, no filters, isotropic 3.2 μm voxel side length), while kept in sealed containers with cotton balls moistened with 70% ethanol to prevent drying. The acquired X-ray projections were reconstructed using datos|x reconstruction software (version 1.3.2.11, GE Measurement & Control Solutions/Phoenix X-ray, Fairfield, CT, USA).

Reference methods

The reference collagen distribution was detected from unstained histological sections using FTIRI (Hyperion 3000 FTIRI Microscope, Bruker Inc., Billerica, MA, USA; imaging settings: 2 x 2 binning, 5.4 × 5.4 μm^2 pixel size, 4 cm^{-1} spectral resolution, on average 32 acquisitions per pixel). Collagen content and distribution were directly evaluated from the area under the Amide I peak (1590–1720 cm^{-1}) of the spectrum. This approach has been validated for collagen distribution within normal and osteoarthritic AC histological slices[7,21,22].

Image analysis

The micro-CT image stacks for the horse samples were aligned and re-sliced with the Automated Image Registration software (version 5.3.0, University of California, Berkeley, CA)[23] using a linear rigid body model (6 degrees of freedom) [Fig. 2(B)]. One micro-CT image slice (8.7 μm thickness) per sample was analyzed. The image slice was chosen close to the location from which the histologic section was prepared. The same image location was used for the image sets obtained at all time points. The AC surface, tidemark, and AC-bone interface were manually segmented in the FTIRI and micro-CT images (Fig. 3). The interfaces were then straightened, i.e., each column of the image matrix was normalized (followed by