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Quantitative assessment of articular cartilage morphology *via* EPIC-μCT

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Summary

Objective: The objective of the present study was to validate the ability of Equilibrium Partitioning of an Ionic Contrast agent *via* microcomputed tomography (EPIC-μCT) to nondestructively assess cartilage morphology in the rat model.

Design: An appropriate contrast agent (Hexabrix) concentration and incubation time for equilibration were determined for reproducible segmentation of femoral articular cartilage from contrast-enhanced μCT scans. Reproducibility was evaluated by triplicate scans of six femora, and the measured articular cartilage thickness was independently compared to thickness determined from needle probe testing and histology. The validated technique was then applied to quantify age-related differences in articular cartilage morphology between 4, 8, and 16-week-old ($n = 5$ each) male Wistar rats.

Results: A 40% Hexabrix/60% phosphate buffered saline (PBS) solution with 30 min incubation was optimal for segmenting cartilage from the underlying bone tissue and other soft tissues in the rat model. High reproducibility was indicated by the low coefficient of variation (1.7–2.5%) in cartilage volume, thickness and surface area. EPIC-μCT evaluation of thickness showed a strong linear relationship and good agreement with both needle probing ($r^2 = 0.95$, slope $= 0.81$, $P < 0.01$, mean difference 11 ± 22 μm, $n = 43$) and histology ($r^2 = 0.99$, slope $= 0.97$, $P < 0.01$, mean difference 12 ± 10 μm, $n = 30$). Cartilage volume and thickness significantly decreased with age while surface area significantly increased.

Conclusion: EPIC-μCT imaging has the ability to nondestructively evaluate three-dimensional articular cartilage morphology with high precision and accuracy in a small animal model.

Key words: EPIC-μCT, Cartilage morphology, Cartilage development, Cartilage imaging, Microcomputed tomography.

Introduction

Osteoarthritis (OA) is the most common joint disorder and is characterized by a gradual but progressive loss of articular cartilage[1]. Interest in understanding disease pathogenesis in more detail and in developing efficient models for testing disease modifying therapies has motivated the development of small animal models for joint degeneration[2,3]. Accurately quantifying morphologic changes in small animals is a critical factor in the development and evaluation of new therapies for OA. However, existing technologies for assessing articular cartilage morphology are typically destructive and time-consuming or limited by inadequate spatial resolution.

Morphologic changes in articular joint cartilage can be destructively evaluated *via* histology, needle probing[4,5], and stereophotographic techniques[6], but these methods do not allow repetitive analysis in the same sample. Nondestructive articular cartilage assessment techniques, including magnetic resonance imaging (MRI)[7,8], and ultrasonography[4], are capable of monitoring cartilage changes in humans, but do not usually provide adequate resolution for the thinner articular cartilage in small animal joints. Optical coherence tomography (OCT) is capable of imaging rat cartilage at high resolution[9–11], but only provides two-dimensional (2-D) images. A nondestructive, high resolution technique that provides accurate and precise (i.e., reproducible) quantification of cartilage morphologic parameters such as volume, surface area, and thickness would be tremendously valuable for small animal studies of joint degeneration[12,13].

Microcomputed tomography (μCT) provides three-dimensional (3-D), quantitative morphologic analysis of hard tissues at micron-level voxel resolutions and has become the "gold standard" for bone microstructural analysis. However, soft tissues such as cartilage are generally undetectable by μCT due to their low X-ray attenuation, and segmentation of cartilage from other soft tissues in such images is not possible. μCT arthrography has been used previously for *ex vivo* visualization of the rat patellar cartilage thickness[14]. Recently, Equilibrium Partitioning of an Ionic Contrast agent *via* μCT (EPIC-μCT), a nondestructive imaging technique combining μCT with a charged X-ray-absorbing contrast agent, has been shown to provide direct *in situ* visualization of articular cartilage morphology in a rabbit femur[15]. The objective of the present study was to evaluate the ability of EPIC-μCT to nondestructively assess cartilage thickness and morphology in the much thinner cartilage of the rat. Specifically, this study addressed the following research issues:

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(1) the appropriate incubation conditions for the contrast agent (Hexabrix) to optimize identification of cartilage in μCT images, (2) a suitable segmentation method to reliably distinguish cartilage from bone and non-cartilage soft tissues, and (3) the precision (reproducibility) of this novel technique to assess cartilage morphology and agreement with two independent thickness measurements. Finally, EPIC-μCT was applied to quantify age-related morphologic differences in rat articular cartilage during postnatal growth from 4 to 16 weeks of age.

Methods

CONTRAST AGENT CONCENTRATION AND INCUBATION TIME

Twelve femora were harvested from six 8-week-old male Wistar rats (Charles River Laboratories, Sparks, NV) and kept in phosphate buffered saline (PBS) at 4°C. To prevent cartilage degeneration, the PBS used throughout this study contained protease inhibitors (1% Protease Inhibitor Cocktail Set I, CalBiochem, San Diego, CA). After dissection of surrounding tissue, each femur was pre-scanned prior to incubation with the contrast medium. The distal femur was then immersed in 2 ml of a specified dilution in PBS of the ionic CT contrast agent Hexabrix 320 (Mallinckrodt, Hazelwood, MO) for 5 min at 37°C, then patted dry and immediately transferred to the μCT system for scanning. The femur was then removed, immersed in the contrast agent solution for another 5 min, and then rescanned. For each sample, this process was repeated for cumulative immersion times of 0, 5, 10, 15, 30, and 60 min to identify the time required to reach equilibrium. This procedure was repeated for femora ($n = 3$) incubated in one of four contrast agent dilutions: 20% Hexabrix/80% PBS, 30% Hexabrix/70% PBS, 40% Hexabrix/60% PBS or 50% Hexabrix/50% PBS.

All femora were consistently secured such that the longitudinal axis was aligned with the vertical axis of the μCT scanning tube. The scanning tube, containing PBS at the bottom, was sealed with parafilm to prevent dehydration during scanning. All scanning was performed in air using a μCT 40 (Scanco Medical, Bassersdorf, Switzerland) at 45 kVp, 177 μA, 200 ms integration time, and a voxel size of 12 μm with a 12 mm scanning tube. Approximately 360 slices (4.3 mm) of each distal femur were scanned. For the convenience of histogram analysis, a volume of interest (VOI) was defined for each condyle extending from the top surface of the cartilage down 31 slices, or 372 μm, which included cartilage, subchondral bone, and some trabecular bone. By using Scanco Medical software, a histogram of the X-ray attenuation values was produced, revealing two partially overlapping peaks corresponding to contrast-enhanced articular cartilage and calcified bone. Separation of the midpoint attenuation values from bone and cartilage peaks in the histograms was directly compared at equilibrium for the four contrast agent concentrations. Cartilage attenuation for each femur was determined by averaging values from both condyles.

SCANNING AND SEGMENTATION PROCEDURES

Based on results of the parametric studies (see below), incubation for 30 min in 40% Hexabrix/60% PBS at 37°C was selected as a standard incubation protocol. Subsequent analyses indicated that this protocol was also adequate to reach equilibrium in femoral articular cartilage from 4-week and 16-week-old rats. A 16 mm scanning tube was required to fit the largest samples in the study from 16-week-old animals. All femora in the remaining studies were therefore evaluated in a 16 mm scanning tube, providing a voxel size of 16 μm, and scanned at 45 kVp, 177 μA, 200 ms integration time, and 25–40 min acquisition time.

The femora were placed in a vertical position during scanning, and the μCT scanner therefore produced transverse cross-sectional slices. Cartilage, subchondral bone, and bone marrow were adjacent in the regions of analysis. Because Hexabrix diffuses into marrow, giving it similar attenuation values to cartilage, a global threshold for segmentation in the whole distal femur was not suitable. In order to accurately segment the femoral articular cartilage from bone marrow, contour lines for articular cartilage were manually drawn. Semi-automatic contouring was applied every 3–10 slices. However, semi-automatic contouring was not feasible for all the cross-sectional slices due to the curved morphology of articular cartilage and disconnected sections in these images [Fig. 1(A)]. In addition, a pilot study showed that partial volume effects in the cross-sectional images produced inaccurate cartilage boundaries in the central regions of both femoral condyles. To overcome this challenge, a new segmentation method was developed. By using Scanco Medical software, the femoral bulk image (grayscale image file) was 3-D rotated to ensure that the lateral–medial axis was aligned with the vertical axis. The femoral bulk image was re-sliced horizontally *via* manual reconstruction, to generate a series of sagittal sections [Fig. 1(B)]. After drawing contour lines to eliminate marrow space [Fig. 1(C)], an appropriate threshold was selected to segment cartilage from bone tissue according to the histogram analysis of the tissues [Fig. 1(D)]. The lower threshold was 70, and the upper threshold was 220 (Gauss filter parameters: sigma = 1.2, support = 2). The 3-D morphology of the entire articular cartilage layer was then visualized [Fig. 1(E)] and quantified in terms of average cartilage thickness, volume, and surface area using direct distance transformation algorithms[16,17]. In addition, a thickness map of the soft tissue volume was generated and presented as a pseudo color-scaled image.

PRECISION EVALUATION

To evaluate the reproducibility of measuring cartilage morphology *via* EPIC-μCT, four femora from 4-week-old rats and two femora from 8-week-old rats were scanned three times in 2 days. The distal femora were incubated in 40% Hexabrix/60% PBS for 30 min at 37°C and scanned as described above. After desorption of the contrast agent for 1 h in PBS, the femora were re-incubated with contrast agent and re-scanned. The specimens were stored in PBS at 4°C overnight until the third scan, which was performed on the second day. The cartilage volume, thickness and surface area of the three measurements were compared, and the coefficient of variation (CV) (100 times the standard deviation [SD] divided by the mean of the three measurements) was calculated for each sample. The root mean square coefficient of variation (RMS-CV) and root mean square standard deviation (RMS-SD)[18] were averaged for the six samples. All scans and analyses were performed by a single experienced operator.

THICKNESS VALIDATION *VIA* NEEDLE PROBING

Cartilage thicknesses for five specimens (two femora, three tibiae) from three additional rats were measured by slow (0.006 mm/s) insertion of a blunt needle probe (~0.1 mm in diameter) attached to a 5 N load cell (sensitivity: 0.001 N, Interface, Scottsdale, AZ) on an ELF3100 mechanical test frame (EnduraTec, Minnetonka, MN). The load and displacement outputs were recorded at 3 Hz as the needle penetrated into the cartilage tissue. A change from zero to non-zero slope on the load–displacement chart indicated contact with the cartilage surface and a sudden rise in force indicated contact with bone. The articular surface was kept hydrated with PBS solution. After probing, each sample was examined *via* EPIC-μCT and the thickness at each probe location (based on manual marking) was measured in the 3-D viewing window of the Scanco Medical software interface.

MORPHOLOGY OF 4, 8, AND 16-WEEK-OLD RAT FEMORA AND HISTOLOGY COMPARISON

Three groups of male Wistar rats of different ages (4, 8, and 16-week old, $n = 5$ each) were sacrificed and both femora were harvested. The distal femora were incubated and scanned according to the standard protocol described above. Following μCT scanning, femora were fixed fresh in 10% neutral buffered formalin overnight and decalcified in 2.5% formic acid (pH 4.2) for 10 days. Upon dehydration, samples were embedded in glycol methacrylate (GMA) according to the manual of the JB-4 embedding kit (Polysciences, Warrington, PA). For comparison with the 3-D spatial images generated by EPIC-μCT, sagittal sections were cut at 8 μm thickness, and the central sections of each condyle were examined. Sections were stained for sulfated glycosaminoglycans (sGAGs) by using a 0.5% safranin-O solution with a 0.2% aqueous solution of fast green used as a counterstain. For each femur, one central, sagittal section in each condyle was used for the thickness analysis. Digital images of each section were captured at a resolution of 0.5 μm. The cartilage thickness of each section was defined as the average value of 10 manual thickness measurements at regular intervals perpendicular to the cartilage surface (ImageJ, NIH). Femoral articular cartilage thickness was determined by averaging values from both condyles.

STATISTICAL ANALYSIS

All data were expressed as mean ± SD. Average cartilage morphology parameters during growth were evaluated using a one factor (age) repeated (left vs right) general linear model with Tukey's test for *post hoc* analysis. The histology measurements of cartilage thickness during growth were evaluated *via* one-way analysis of variance (ANOVA) with Tukey's test for *post hoc* analysis. Thickness measurements *via* EPIC-μCT and needle probing/histology were compared *via* paired *t*-tests, and the relationships and agreements between these two methods were examined *via* linear regression analysis and Bland–Altman analysis[19], separately. Statistical significance was set at $P = 0.05$ (SPSS 11, SPSS Inc., Chicago, IL).