CA4+ Contrast Enhanced MicroCT Imaging of Fixed Articular Cartilage Tissues and Feasibility of Histology

Xueqin Gao[1,2], Amit N. Patwa[3], Zhenhan Deng[1], Hajime Utsunomiya[1], Gilberto Nakayama[1], Sarah Amra[1], Haizi Cheng[1], Walter R Lowe[1], Marc J Philippon[2], Brian D. Snyder [4], Mark W. Grinstaff [3], Johnny Huard [1, 2*]

[1]Department of Orthopaedic Surgery, McGovern Medical School, University of Texas Health Science Center at Houston, Houston, TX, USA; [2]Steadman Philippon Research Institute, Vail, CO, USA; [3]Department of Biomedical Engineering, Boston University, Boston, MA; USA [4]Department of Orthopaedic Surgery, Harvard Medical School, Boston, MA; USA. *Corresponding author Johnny.Huard@uth.tmc.edu.

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INTRODUCTION. The cationic iodinated contrast agent CA4+ is a novel positive charge contrast agent for imaging *ex vivo* bovine and human meniscus [1, 2], human cartilage [3, 4], and mouse tibia plateau cartilage[4]. The resulting contrast-enhanced attenuation directly correlates with the content and location of glycosaminoglycan (GAG) within the tissue. Furthermore, CA4+ contrast attenuation correlates with cartilage stiffness [5] and biomechanical properties [6]. As such, CA4+ is a promising new contrast agent for *in vivo* and *ex vivo* cartilage imaging. However, whether CA4+ can be used on fixed tissues and whether the tissues can be further used for histology is still unclear and; hence, prompting the current study.

METHODS. 1. **CA4+ was synthesized as previously described**[3]. 2. **CA4+ contrast labeling.** Rabbit distal femurs were dissected from 4-month-old New Zealand white rabbits (The Jackson Laboratory) (N=5/group/time point). The distal femur tissues were divided into 2 groups, the unfixed or native group and the neutral buffered formalin fixed (NBF fixed) group. For the unfixed group, all the rabbit femurs were dissected and kept at -80ºC prior to CA4+ contrast labeling and imaging. For the NBF fixed group, distal femurs were fixed in NBF for at least three days. Both groups of tissues were washed for 5 minutes with PBS, then immersed in 10 mL CA4+ (24mg/ml, PH=7.4, 400 mOSm/kg) and shaken on a horizontal shaker at 60 rpm at room temperature, and then imaged at 8, 24, and 30 hours. **3. MicroCT scanning**. After CA4+ contrast staining, the distal femurs were rinsed with deionized water for 5 minutes, and then wrapped with parafilm and scanned with a VIVA CT 40 at 38 µm resolution for the entire distal femur. After scanning, the samples were placed in cell culture sterile PBS (pH 7.4) for 5 days and then scanned via microCT using the same parameters to determine whether CA4+ binding to cartilage is stable. After the second microCT scanning, both groups of distal femur tissues were decalcified in 10% ethylenediaminetetraacetic disodium (EDTA) plus 1% NaOH (pH 7.2) for 4 months. Samples in the unfixed femur group were kept unfixed during the decalcification process. The distal femurs were then scanned a third time via microCT using the same protocol. Scanco microCT software was utilized by contouring 50 slices of femur cartilage at the level of the condyle, where both the patella groove and condyle cartilage could be visualized. We chose a threshold of 280, which is 60 higher than the threshold we used for rabbit trabecular bone. The total contoured cartilage volume and cartilage density were automatically generated by the software for 3D quantification. **4. Histology**. After the last CT scans, the distal femurs were processed for paraffin sections. H&E, Alcian blue, and Safranin O staining, as well as immunohistochemical staining of collagen type 2 (Col2) were performed. The Student t-test was used for comparing two groups; $P<0.05$ was considered statistically significant.

RESULTS. 1. MicroCT results. CA4+ effectively stained the patella groove and condyle cartilage that covered the subchondral trabecular bone, and showed higher density than subchondral bone in both unfixed and NBF fixed groups at 8, 24, and 30 hrs after labeling. The femur cartilage in the NBF fixed samples was more easily distinguished from subchondral bone than unfixed tissues (**Fig. 1A**). In the no CA4+ contrast tissues, the cartilage was not distinguishable (**Fig. 1A**). MicroCT 3D images showed the patella groove cartilage surface of unfixed femur had lower density at 8, 24, and 30 hrs post-contrast staining relative to NBF fixed tissues (**Fig. 1B**). The NBF fixed cartilage intensities were significantly higher than that of unfixed CA4+ contrast labelled tissue group (R^2=0.9993 for NBF fixed VS 0.9348 for unfixed group) (**Fig. 1C**). Cartilage volume was not statistically different for both groups at any time points (**Fig. 1D**). **2. Stability of CA4+ binding**. After soaking in PBS for 5 days, although the signals of unfixed cartilage with CA4+ contrast staining for 8 and 24 hrs decreased, the signal was still visible for the samples stained with CA4+ for 30 hrs. In contrast, the NBF fixed femur cartilage did not lose signal intensity when compared to pre-soaked cartilage, and was clearly distinguishable from the subchondral bone (**Fig. 2A**). Furthermore, after decalcification, signal in the unfixed cartilage had completely disappeared for all time points. Subchondral bone signal was also not visible due to decalcification. The NBF fixed samples clearly showed cartilage without showing subchondral bone. Some residual subchondral bone that was not fully decalcified were still visible. The control samples without CA4+ contrast (No-CA4+) in both groups showed no signal (**Fig. 2B**). **3. Histology.** H&E staining demonstrated the unfixed CA4+ labeled cartilage showed similar morphology compared with the No-CA4+ labeled tissues; however, subchondral bone tissue showed no nuclear staining. The NBF fixed tissue group exhibited enhanced red cytoplasm color that could be clearly identified from subchondral bone at all time points; a clear line between cartilage and subchondral bone were visible. Staining of the subchondral bone nuclei appeared the same as that for No-CA4+ labeled tissue (**Fig. 3A**). Alcian blue staining showed blue cartilage matrix in unfixed CA4+ labeled tissues at all time points, which was similar to that for No-CA4+ labeled tissue. NBF fixed tissues also showed blue matrix. Subchondral bone residual cartilage matrix was similar to that of the No-CA4+ group (**Fig. 3C**). Safranin O staining for unfixed CA4+ labeled tissues revealed the cartilage was stained orange red, which was similar to that of the No-CA4+ labeled tissues. However, for NBF fixed tissue, the cartilage was not stained orange red by Safranin O staining (**Fig. 3C**). This result indicates that CA4+ specifically binds to GAG, similarly to Safranin O, which also possesses a positive charge. Immunohistochemical staining of Col2 clearly showed the presence of Col2 in unfixed femur cartilage layers and No-CA4+ labeled cartilage (brown). However, cartilage of NBF fixed tissues was negative for Col2, despite the cartilage layer being clearly visible by nuclear staining (**Fig. 3D**).

DISCUSSION: In this study, we optimized the imaging protocol for *ex vivo* cartilage imaging with CA4+ and feasibility for down-stream histology. For unfixed tissues stained with CA4+, cartilage was not easily distinguished from subchondral bone. In contrast, with NBF fixed tissues, cartilage was clearly distinct and exhibited a time-dependent intensity. In conclusion, our results indicated that both unfixed and NBF fixed cartilage tissues contrasted using CA4+ can allow cartilage quantification and morphological assessment. Furthermore, it is feasible to perform H&E, Alcian blue, Safranin O, and Col2 immunohistochemical staining after decalcification of unfixed tissue labeled with CA4+. NBF fixed tissues labeled with CA4+ rendered clear microCT cartilage images, and H&E and Alcian blue staining after decalcification was amenable.

SIGNIFICANCE. The study provides an optimized CA4+ contrast-enhanced microCT imaging protocol for *ex vivo* cartilage imaging, and quantification of both unfixed tissues and NBF fixed tissues. The CA4+ contrast agent is specific for cartilage GAG and it can be successfully used for cartilage quantification.

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