Non-Invasive Diagnostic Methods to Monitor the GAG Content and Biomechanical Properties of Articular Cartilage

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BACKGROUND

Hyaline cartilage is a soft hydrated tissue providing an almost frictionless surface that transmits and distributes forces across articular joints. It is primarily made up of proteoglycans (5-10% by wet mass), type II collagen and water. Osteoarthritis (OA) is a disease characterized by the degeneration of hyaline cartilage in articular joints due to wear and tear. It is estimated that 10% of adults over the age of 50 and 70% of adults over the age of 65 show symptoms of OA[1]. This disease affects more than 20 million people in the United States alone and its treatment accounts for $125 billion in health care costs every year. The NIH estimates that the number of people suffering from OA will increase to 40 million over the next 23 years. Cartilage has a very limited capacity to heal itself, and efforts to stimulate cartilage regeneration have met with mixed success. While there is no cure for OA, early diagnosis may lead to treatments that can significantly slow the progression of the disease or reverse it altogether.

Most clinicians rely on patient-reported symptoms and plane radiographs to make the diagnosis of osteoarthritis. However radiographs are diagnostic only when patients have reached a relatively advanced stage of OA[2]. Traditionally, plain radiographic images have been used to diagnose OA based on joint space narrowing and osteophyte formation. Joint space narrowing is not a reliable marker for early OA, since many individuals over the age of 60 exhibit joint space narrowing but do not exhibit symptoms of OA. Osteophyte formation is more specific for OA but osteophytes are apparent at a stage when OA progression is often irreversible.

The loss of glycosaminoglycans (GAGs) from the extracellular matrix of hyaline cartilage ECM is the hallmark of early OA and is observed before any radiographic changes are evident[4]. Diagnosing and observing the progression of OA can be achieved by monitoring the GAG content of articular cartilage. Proteoglycan degradation results in deterioration of the static and dynamic mechanical properties of articular cartilage in the earliest stages of OA. Techniques that use advanced bioimaging to monitor GAG content in articular cartilage are at the cutting edge of OA diagnostics. Non-invasive methods capable of measuring changes in the proteoglycan content of hyaline cartilage in vivo are emerging as the most effective approach to diagnose OA early and to monitor the progression or regression of OA in response to treatment.

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DELAYED GADOLINIUM ENHANCED MAGNETIC RESONANCE IMAGING OF CARTILAGE

Delayed Gadolinium-Enhanced Magnetic Resonance Imaging of Cartilage (dGEMRIC) has been validated as a feasible clinical method to monitor GAG concentrations in-vivo[5]. The technique depends on the distribution of a charged contrast agent (Gd-DTPA2-) through the ECM of hyaline cartilage. Due to the fixed negative charge density of proteoglycans, the anionic contrast agent Gd-DTPA2- diffuses in inverse proportion to the GAG content of the cartilage and diffuses preferentially into areas where GAGs have been depleted by OA (Fig. 1).

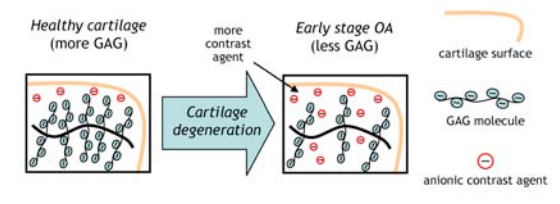

Figure 1: GAG-dependent diffusion of an anionic contrast agent into cartilage tissue. In the earliest stages of OA, GAG molecules begin to leave the cartilage ECM, leading to a loss of compressive stiffness. With an anionic contrast agent, the diffusion is inversely proportional to GAG concentration.

The diffusion of Gd-DTPA2- in inverse proportion to the GAG content of cartilage results in a proportionate change in the T1 relaxation times measured by MRI. Studies have confirmed that dGEMRIC can differentiate between healthy and arthritic cartilage, both in vitro and *in-vivo* in human clinical trials[6,7].

While dGEMRIC can measure the earliest biochemical changes in cartilage induced by OA non-invasively, its widespread acceptance has been hindered by the high costs and long acquisition times inherent to MRI, as well as the necessity of specialized pulse sequences and software programs required for the analysis. In comparison, CT imaging is cheaper, more

widely accessible and able to achieve higher resolution at faster acquisition times. Recent advances in CT such as multi-row detector instruments will allow the simultaneous 3D reconstruction of cartilage and the underlying bone without any specialized sequences.

QUANTITATIVE CT ARTHROGRAPHY USING ANIONIC, IODINATED CONTRAST AGENTS

Recently, iodinated contrast agents along with CT have been used to study changes in the ECM of cartilage [8,9]. In principle, these techniques are similar to the dGEMRIC technique in that they provide a quantitative assessment of the spatial distribution of GAG in the cartilage matrix based on changes in the diffusion of a charged contrast agent. The high density of negatively charged proteoglycans in cartilage normally limits the extent to which an anionic contrast agent is able to permeate the cartilage matrix. In tissues where the GAG content has been depleted due to cartilage degradation, the contrast agent is able to diffuse into the ECM to a greater extent. Both dGEMRIC and CT imaging techniques exploit this inverse relationship between GAG content and the extent that a charged contrast agent can diffuse into the cartilage matrix. It has been shown that contrast enhanced CT imaging can be used to monitor GAG concentrations in cartilage tissue explants using a procedure analogous to dGEMRIC [8,9]. However, to the best of our knowledge there have not been any studies that have demonstrated that the diffusion of an anionic, iodinated contrast agent into cartilage measured by CT can be used to measure the GAG content and biomechanical properties of articular cartilage. In this study, we demonstrate that quantitative CT arthrography (qCTA) may be used as a diagnostic tool to assess the biomechanical and biochemical properties of articular cartilage *ex-vivo* using mated bovine osteochondral plugs to model articular cartilage degeneration observed in human diarthrodial joints.

MATERIALS AND METHODS

Specimen preparation:

Twelve pairs of mated cartilage on cartilage osteochondral plugs (7 mm diameter) were harvested from the patella-femoral joint of four mature cows using a water-cooled, diamond tipped cylindrical cutter (Fig. 2). The samples were immersed in chilled saline (4° C) during the coring operation. The mated osteochondral plug pairs were divided into a normal cartilage group (n=4) and a trypsin degraded cartilage group (n=4). The cartilage was degraded by immersing the osteochondral plugs in trypsin (0.1 mg/mL in 50 mM Tris, 20 mM $CaCl_2$, at pH 7.8) at room temperature for 30 min. Prior to imaging, each mated pair of osteochondral plugs were immersed overnight in an anionic, triiodinated contrast agent (Cysto Conray II) at 4°C to allow sufficient time for the contrast agent to diffuse into the cartilage. The contrast agent was mixed with an antibiotic cocktail to prevent bacterial contamination of the cartilage.

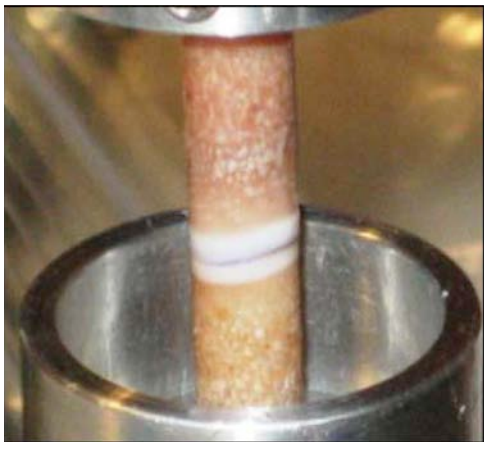

Fig. 2
Setup showing the mated cartilage on cartilage osteochondral plug harvested from patella-femoral joint.

qCTA:

Excess contrast agent was removed from the samples and loaded onto custom holders for CT. All the imaging was performed in air using a pQCT machine (XCT Research SA+ Stratec). Sequential, 100μ thick, transaxial pQCT images were obtained at 70μ in plane resolution and 100μ inter-slice distance. The CT data sets were imported into Analyze™ (BIR, Mayo Clinic) for image-processing and to create 3D reconstructed images of the osteochondral plugs. The mean x-ray attenuation for cartilage was obtained by averaging over the transaxial CT images that contained only cartilage for each osteochondral plug.

Mechanical testing:

Each pair of osteochondral plugs was immersed in PBS overnight to desorb the contrast agent. Using a custom built fixture, each pair of plugs were mounted collinearly in a mechanical testing machine (Instron 8511). Load was measured using a 10 lb load cell (Honeywell sensotec, Columbus, Ohio, USA) and displacement was measured using a linear variable displacement transducer (Instron 8511). Incremental, stepwise compressive stress relaxation tests were performed. An initial tare load of 0.25N was applied and allowed to creep to zero to ensure contact between the opposing cartilage surfaces. The samples were then loaded in four 5% incremental strain steps to 20% of the total undeformed thickness of the mated cartilage surfaces. (Average thickness for the femoral and patellar samples was 1.355 ± 0.185 mm and 1.66 ± 0.09 mm, respectively.) Between each incremental strain step the samples were allowed to relax until the change in force was less than 0.05 N/min. The equilibrium compressive modulus (E) was obtained from the slope of the linear regression fit to the equilibrium stress and strain data. All parameters were computed using custom software written in MATLAB (Mathworks, Natick MA).

Biochemical Assessment of GAG Content:

The samples were prepared for biochemical analysis using the 1,9-dimethylmethylene blue (DMMB) colorimetric assay [10]. The articular cartilage was separated from the subchondral bone using a razor blade and the wet mass of the cartilage was obtained. After weighing the hydrated cartilage, the cartilage was lyophilized for 24 hours to obtain the dry weight. The tissue was digested in Papain at 65°C for 24 hours and then diluted 10 to 100 times for the assay. A linear calibration curve was generated using chondroitin-4-sulfate (Sigma 27042) dissolved in PBS at concentrations ranging from 10-100 μg/mL. 10 μL of each chondroitin-4-sulfate calibration solution was combined

with 100 μL of 1,9-dimethyl-methylene blue (DMMB) solution in a 96-well plate. The absorbance of each solution at 520 nm was monitored using a plate reader (Beckman Coulter AD340). The total GAG weight per mg wet weight and dry weight of the cartilage was calculated accounting for the dilution.

Statistics:

Unless otherwise noted, all data is presented as mean±SEM. Differences in CT attenuation, GAG content and E were compared between groups using student's t-test (α=0.05). The relationships among CT attenuation, GAG content and E were evaluated using linear regression analysis.

RESULTS

qCTA:

Consistent with the results of Palmer *et al*, the diffusion of the anionic triiodinated contrast agent was inversely proportional to the GAG content of the articular cartilage ECM. In degraded osteochondral plugs, the increase in CT attenuation was most evident in the superficial zone progressing towards the middle zone of the articular cartilage (Fig. 3). The mean CT attenuation for the control group was lower than the trypsin degraded group (Fig. 4a).

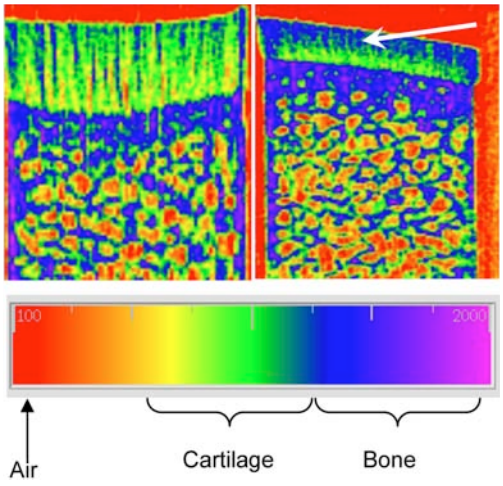

Fig. 3 (Left) shows the pQCT color map image of the control sample. The image on the right is for the degraded sample. It can be seen the degraded sample shows more contrast agent diffusing from the top (blue color). The white arrow shows the articular cartilage surface.

Unconfined compressive stress relaxation test:

The equilibrium compressive modulus (E) decreased with progressive loss of GAG from the articular cartilage ECM indicating a loss of bound water and altered mechanical integrity of the tissue. The average value of E for the degraded osteochondral plugs was lower than the control group ($p<0.05$, Figure 4b).

Biochemical assessment using DMMB assay:

The GAG content of the control group was higher than that of the osteochondral plugs treated with trypsin for 30 min ($p<0.05$, Figure 4c).

DISCUSSION:

Non invasive diagnostic methods capable of measuring the earliest biochemical and biomechanical changes in articular cartilage associated with arthrosis are important to optimize treatment protocols and monitor the response of articular cartilage to treatment. Our work suggests that qCTA may be a sensitive and specific technique to monitor non-invasively the earliest changes in articular cartilage associated with arthrosis. Using a commercially available, tri-iodinated, anionic, contrast agent suitable for arthrography, changes in CT attenuation were inversely proportional to changes in the GAG content and equilibrium modulus of articular cartilage. Ongoing work in our lab has shown that we can also measure these changes in articular cartilage in small animal joints, *ex vivo* and *in vivo*. Multi-detector, helical, CT machines are more accessible, have better resolution with faster acquisition times compared to MRI. Furthermore post-processing of sequential transaxial CT images through the joint allows 3D reconstruction of the cartilage surface and subchondral bone. It has been shown that the subchondral bone architecture is also affected during OA. Hence, the excellent ability of qCTA to visualize bone

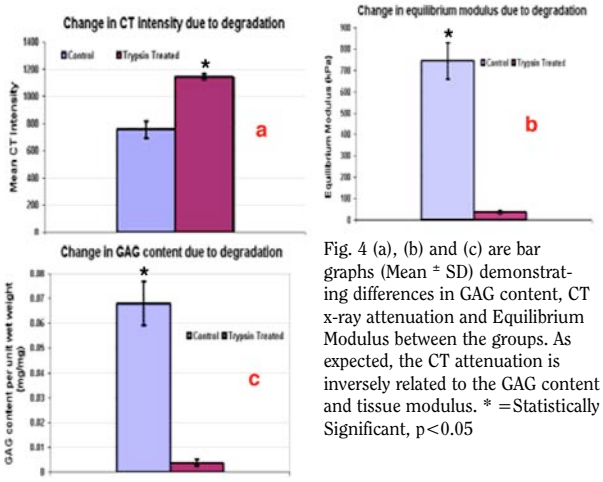

Fig. 4 (a), (b) and (c) are bar graphs (Mean ± SD) demonstrating differences in GAG content, CT x-ray attenuation and Equilibrium Modulus between the groups. As expected, the CT attenuation is inversely related to the GAG content and tissue modulus. * =Statistically Significant, $p<0.05$

simultaneously with articular cartilage without any specialized sequences adds to the attractiveness of this technique. Further, we evaluated the biomechanical properties using mated osteochondral plugs obtained from conformal surfaces of bovine patella-femoral joints. This model is physiologically relevant as opposed to the ubiquitous cartilage against metal artificial surface model. We believe that this *ex-vivo* model better mimics *in vivo* conditions, albeit partially. The values of E obtained using this model are 24-41% higher for normal cartilage than reported using unconfined compression tests. This may be attributed to differences in the *ex-vivo* models used. However, further validation is required

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