In-Vivo Raman Spectroscopy Can Serially Monitor Focal Cartilage Defect Progression in a Porcine Impact Model

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INTRODUCTION: Post-traumatic osteoarthritis (PTOA) is an incapacitating, chronic condition among individuals who sustain traumatic joint injuries. Up to 87% of patients experiencing chondral contusion advance to PTOA within 15 years of injury. The pathophysiology of joint contusion injury involves a combination of mechanical stress disrupting the structural integrity of the articular cartilage, to which the extent depends on the impact energy, which solicits a robust cellular response involving synoviocytes, chondrocytes, and osteocytes adjacent the injured tissue provoking the production of inflammatory mediators and matrix-degrading enzymes that promulgate cell death and tissue dysfunction. Diagnostics that rely on visual arthroscopic grading schemes (Outerbridge [OB] scoring) reflect macroscopic changes in tissue morphology, but do not provide insight into changes in tissue composition governing the material properties of the tissues fundamental to cartilage performance. Quantitative MRI (T1ρ, T2*) metrics are only moderately correlated with cartilage tissue composition and functional material properties[1]. Raman spectroscopy is a label-free optical technique that quantifies the vibrational modes of the biochemical building blocks (amides, sulfates, hydroxyls) of key cartilage ECM-specific biomarkers (GAG, COL, H_2O) that contribute to the material properties intrinsic to tissue function. We developed a novel Raman spectroscopy needle probe and real-time spectral analysis platform capable of performing both *ex-vivo* and *in-vivo* measurement of ECM-specific compositional biomarkers for cartilage with a high degree of accuracy[2]. The aim of this pilot study is to demonstrate that *in-vivo*, real-time Raman spectral analysis of cartilage can map instantaneous changes in tissue composition immediately after a contusion injury *in-situ*, providing insights not possible in the artificial setting of isolated chondral plugs growing in bioreactors.

METHODS: ***Raman Probe***: A custom, Raman probe (In Photonics) comprised of a threaded needle tip (∅2.75mm) with a distal ∅1mm sapphire ball lens was fiber-coupled to a 785nm laser (100mW output; IPS) and spectrometer (Eagle; Ibsen) ***Raman Spectra***: Spectra in the fingerprint (800-1800cm^{-1}) and high wavenumber (800-3800cm^{-1}) ranges were acquired. Spectra were preprocessed by background subtraction and area-under-curve normalization. The cartilage fingerprint spectra was fit to a multivariate linear regression model: $\mathbf{Cartilage_{spectra}}=GAG_{score}*(\mathbf{GAG_{REF}})+COL_{score}*(\mathbf{COL_{REF}})+H_2O_{score}*(\mathbf{H_2O_{REF}})+Bone_{score}*(\mathbf{Bone_{REF}})$, where: $\mathbf{GAG_{REF}}$, $\mathbf{COL_{REF}}$, $\mathbf{H2O_{REF}}$, and $\mathbf{Bone_{REF}}$ are reference spectra of purified reference chemicals for each ECM constituent; "scores" are the "fit" regression coefficients reflecting the relative contribution of each component to the acquired composite spectra (**FIG1A,B**) ***Ex-Vivo Validation***: Porcine, human, equine, and bovine cartilage surfaces were mapped using the hand held Raman probe to generate spatial composition maps. Plugs corresponding to the Raman "optical biopsy" sites were cut from the articular surfaces and tested for biochemical content and compressive modulus. ***Injury Model*:** With IACUC approval, 5 contusions were created on the distal femur of a 6-year-old male castrated Yucatan mini-pig through a parapatellar arthrotomy using a hemispherical spring-powered impactor[3]. Three impacts were delivered to the medial facet of the trochlea, the lateral facet providing an uninjured control, and two impacts were delivered to the medial condyle. An in-line high-speed load cell allowed the derivation of the impact energy delivered at each site. Fiducial marks were created along the margins of the trochlea , allowing for locating the contusion sites during future imaging. ***In-Vivo Raman Measures*:** The needle probe was autoclaved, and optics were draped in sterile plastic. Spectra were acquired immediately before and ~10-30 minutes after impacts, and at 1 month follow-up via the same surgical approach. Holding the probe normal to the surface with minimal contact force, spectra were acquired for 15 seconds at 5 points per site for the trochlea (**FIG2A,B,C**). ***MRI***: Quantitative MRI (T1ρ, T2*) was conducted using high-field MRI at 1-month follow-up. ***Statistics***: The GAG score at each defect site before, immediately after contusion, and 1 month after contusion was compared using 2-way ANOVA with Tukey-Kramer multiple comparison; significance threshold α=0.01.

RESULTS: ***Ex-Vivo*:** The hand-held Raman needle probe generated detailed cartilage composition maps detailing gradients in GAG content over the joint surfaces (**FIG1C**) that were highly correlated to standard DMMB assays of GAG content (**FIG1D**) and tissue compressive modulus (**FIG1E**). ***In-Vivo*:** Before impact, the spatial gradient of GAG composition over the trochlea was like the *ex-vivo* mapping (**FIG2A**). Immediately after impact the GAG score *increased* at all 3 sites along the medial trochlea; at 1-month follow-up the GAG scores were below pre-injury values at all injury and control sites (**FIG2D**). After aggregating all data points at the impact sites, the increase in GAG score from pre-injury to post-injury was significant. The decrease in GAG score immediately after impact to 1-month follow-up was also significant at all impact sites. MRI revealed a single defect with increased water signal, which did not extend into the subchondral bone or marrow space (**FIG3**). Outerbridge scoring by visual inspection identified only 1 defect with some cartilage fissuring (OB 1).

DISCUSSION: While the literature suggests that there is a decrease in GAG after impact [4], this fails to reflect the immediate tissue response to injury, where real-time Raman spectroscopy revealed a temporary increase. We have two hypotheses to explain the transient increase: 1) our probe is most sensitive to the composition of the superficial and middle zones, so mobile GAG liberated by tissue fracture at impact facilitates diffusion from the GAG-rich deep zone to the relatively GAG-poor superficial zone, where it is detected by the probe; 2) the probe is pressed against the tissue with a small tare force to ensure lens contact, which squeezes fluid out of the tissue, increasing the relative concentration of non-water constituents GAG and COL. Because of structural failure, the cartilage is more compliant and compresses more under the same load, enhancing the relative concentration of GAG and COL. In addition to the injury sites, the control sites also demonstrated a decrease in Raman GAG score at 1 month, indicating a generalized catabolic response to inflammatory cytokines released into the synovial fluid after the injury. These results highlight the limitations of conventional arthroscopic grading schemes, such as Outerbridge, which only reflect gross surface morphology but fail to characterize cartilage composition critical to joint function.

SIGNIFICANCE: This pilot study proves that real-time Raman spectroscopy obtained using our needle probe platform can detect *in-vivo* compositional changes in tissue composition on very short time scales enabling new techniques for basic science research of cartilage injury. *In-situ* compositional mapping of cartilage after injury over time and space *in-vivo* can provide insights as to boundary conditions for transport kinetics and facilitate repeated measures for *in-vivo* assessments of the efficacy of chondroprotective therapies. Compatible with surgical workflows, real-time Raman spectroscopy can transform clinical practice by enabling objective diagnosis of cartilage injury, monitoring disease progression, and evaluating the efficacy of therapeutic interventions.

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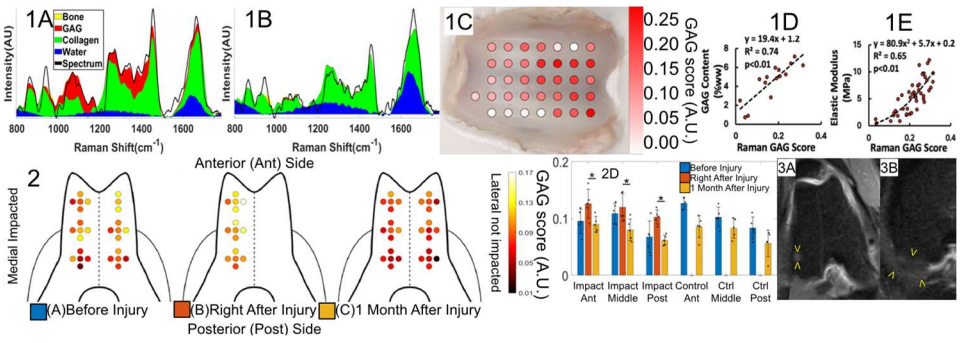

FIG1: *Ex-vivo* validation. Representative high(**A**) and low(**B**) GAG cartilage spectra, where scores represented as colored stacked area. **C**: map porcine trochlea showing gradient in GAG content. Grid points colored by GAG score spaced 1/6 inch. Validation DMMB GAG assay (**D**) and indentation modulus(**E**). **FIG2**: *In-Vivo* Raman Data. Schematic of trochlear with GAG scores before (B), immediately after (**B**) and 1 month after (**C**) injury. **D**: Comparison GAG score between sites over time, *=P<0.01. **FIG3** MRI Images from the coronal(**A**) and transverse planes(**B**).