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REPEATED MEASUREMENT OF MECHANICAL PROPERTIES IN VIABLE OSTEOCHONDRAL EXPLANTS FOLLOWING A SINGLE BLUNT IMPACT INJURY

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Abstract

Objective—To develop a method for repeated same-site measurement of mechanical properties suitable for the detection of degenerative changes in a biologically-active explant model after a single blunt impact injury.

Background—Focal blunt impact injuries to articular surfaces lead to local cartilage degeneration and loss of mechanical properties. We employed a repeated measurement methodology to determine variations in mechanical same-site properties before and after injury in living cartilage with the hypothesis that normalization with initial mechanical properties may provide a clearer evaluation of impact effects and improve our understanding of the biologic responses to impact injury.

Materials and Methods—Bovine osteochondral explants were cultured for up to 14 days after impact injury. Indentation tests were performed before and after impact injury to assess relative changes in mechanical properties.

Results—Creep strain increased significantly in impacted explants after 7 days and in both impacted and control explants after 14 days. Further analysis at 14 days revealed decreases in stretch factor β , creep time constant and local compressive modulus.

Conclusion—A repeated measures methodology reliably detected changes in mechanical behavior viable osteochondral explants after a single impact injury.

Introduction

Post-traumatic Osteoarthritis (PTOA) accounts for approximately 12% of the overall prevalence of symptomatic osteoarthritis (OA), affecting 6 million people annually[1]. Current understanding suggests that structural disruption and chondrocyte death at the site of injury result in a cascade of biochemical events that lead to degradation of the extracellular matrix (ECM) in and around impact sites. To this end, studies have provided significant insights on injury-induced cell viability patterns in articular cartilage [2–5]. At the molecular level, pro-inflammatory and catabolic activities are also apparent upon injury induction with a simultaneous suppression of anabolic precursors, indicating the onset of a degradative

cascade. Acute responses to mechanical injury vary in a magnitude-dependent manner [6], suggesting that rate of progressive degeneration may be partly dependent on the severity of the initial damage inflicted on the tissue. In concert with abnormal loading, soluble biochemical factors from injured cartilage may spread to the surrounding tissue [7], causing the spatial progression of cell death and matrix destruction. With this evidence in mind, the onset and development of PTOA is believed to be multifactorial and current paradigms are focused on understanding the spread of degeneration in articular cartilage with the objective of formulating strategies of forestalling the progression of this disease. To study the phenomenon of a spatial progression, an *in vitro* model that allows temporal and spatial progression of injury-induced cartilage degeneration initiated by a focal trauma is warranted. Previous studies have shown that cartilage damage can be best simulated *in vitro* in an osteochondral model, since mechanical characteristics of the underlying bone influences the injury dynamics significantly. The presence of the subchondral bone significantly decreases the degree of matrix damage and distribution of cell death [8–10]. Mechanically, the effective moduli of an osteochondral structure when compared to a chondral explant would vary considerably, affecting its physical response to high strain rate conditions such as impact loads [11, 12].

In this study, we developed a methodology to monitor material property trends of the cartilage after injury in a biologically active osteochondral explant model. Flat-platen cartilage indentation was selected for mechanical characterization so that *in situ* measurements could be performed. Current post-processing methodologies in extracting intrinsic cartilage properties involve complex modeling [13] and computation intensive procedures [14] that may not be suitable for routine screening. Hence, we present a linear regression and curve fitting approach in describing the mechanical trends observed in our study. Also the extent of mechanical variation in normal cartilage leads to obscurity in distinguishing subtle degenerative changes [15]. Our methodology enabled repeated assessment of the same site at pre- and post-injury times, circumventing the potential confounding effects of spatial variations in mechanical properties. This approach was used to test the hypothesis that the cartilage mechanical integrity can be followed over time with same-site testing using a repeated measures methodology.

Materials and Methods

Bovine knees with intact joint capsules were obtained from a local abattoir. Osteochondral explants (25mm × 25mm) were carefully harvested from the lateral tibial plateaus of the joints since the curvature of the chondral surface was minimal and well-suited for the study purpose. Medial-laterally, this region was identified from the base of the intercondylar eminence towards the meniscus. In the anterior posterior direction, explant edges fell roughly equidistant from the intercondylar eminence (Fig. 1). Subchondral bone thickness was maintained > 25mm. Subchondral bone depth that is 10-fold greater than average cartilage thickness (~ 2.5mm), would obviate the effects of variations in the effective moduli of osteochondral explants [11]. The subchondral bone was cut parallel to the cartilage surface. The explants were harvested in an aseptic environment and transported in a sterile solution of HANKS buffered salt solution (HBSS-supplemented with Penicillin/Streptomycin and Amphotericin B) maintaining *in vivo* joint osmolarity. Explants were incubated and tested in culture medium (10% Fetal Bovine Serum, Dulbecco Modified Eagle Medium) under standard conditions (37°C, 5% CO₂) during the whole study to maintain tissue viability.

On the day of testing, a polyethylene hollow cylindrical adapter (Halo) was used to hold the specimen in the mechanical testing jig (Fig. 2B). The sterile halo was interfaced with the osteochondral explant using stainless steel screws and the mounted specimens were left

undisturbed for 7 or 14 days. This ensured no change in test location and orientation throughout the testing period. A 4mm diameter location on the explant that superimposed with the center of radius of the polyethylene halo was selected as the area of interest for mechanical characterization and subsequent impact. The fixture materials were sterilized with 70% ethanol overnight and washed with sterile HBSS prior to use.

Mechanical Testing

The protocol was designed to determine elastic mechanical behavior as well as visco-elastic response (both in compression). A stepper motor driven actuator (Ultramotion®, NY; resolution:~ 1µm) along with a tension compression load cell ($\pm \sim 100\text{N}$, Model 34 Sensotec®, OH) along with a high performance signal conditioner (UV-10 Sensotec®, OH) were used in a custom set-up for mechanical characterization. Data acquisition and motion control were programmed using Labview® 7.0. A 4mm diameter flat-cylindrical non-permeable indenter initially contacted the surface of the cartilage with a tare load of 0.5N. The rest period of 15 minutes was sufficient for contact stresses to reach close to 0 MPa. Following the wait period, the testing protocol (Fig. 3A) consisted of compression cycles (10 cycles between 0MPa and 0.5MPa) and then a slow compressive ramp (0.5 MPa at 0.05MPa/s). Ten cycles of equilibration was the minimum required to decrease transients and stabilize the hysteresis area in load-displacement curves. After a 300 second rest, a compression creep testing (0.5 MPa in 10s and held for 15 min) was performed. Prior to testing protocol, a reference value for cartilage thickness at the testing/impact site was determined using a sharp indenter and a digital caliper at 4 locations around the test site (Fig. 2A). The operator used a sterilized kirschner (K) wire/polymer stopper pushing the sharp end in to the cartilage manually until resistance was felt, indicating the encounter with the subchondral bone. The polymer stopper was placed flush to the cartilage surface and the distance between the depressed wire tip and the stopper was measured as the cartilage thickness using a digital caliper (Fig. 2C). The thickness process was performed under aseptic conditions and validated using an ultrasound thickness measurement device (Sonopen®, Olympus NDT). The mean of the four thickness measurements was assumed to be the cartilage thickness at the testing site. This measurement was used as an input parameter for finer load control during the creep testing.

The mechanical force-displacement data were adjusted to a reference zero at the start of the testing after the tare load was applied. Cartilage thickness data was used to convert displacement values to corresponding strain equivalents. Local compressive modulus was determined from the stress-strain curves generated from the slow compression ramp by using linear regression fit for the region of the curve between 0.25MPa and 0.5MPa. This region was selected using a linear regression criterion of $R^2 = 0.99$ (Fig. 3B).

The time-dependant displacement from the viscoelastic creep test was curve-fit to a stretch exponential polynomial function:

$$\varepsilon = (\varepsilon_F - \varepsilon_I) e^{-(t/\tau)^\beta} + \varepsilon_I \quad (1)$$

Where ε_F and ε_I are final and initial strains respectively, t is time, τ is the creep time constant and β is the stretch of the exponential ($0 < \beta < 1$). This function has been applied previously to describe creep behavior and was found to be more representative of creep response than an exponential function [16]. Programming and curve fitting utilities of MATLAB 7.0 were utilized for this purpose. Non-linear least squares (robust fitting method) with the Trust-region algorithm was employed with a lower limit of $R^2 = 0.98$ (Fig. 3C).

Testing methodology

On the day of testing, the test site on each explant was subjected to a single blunt impact with a flat-ended non-porous platen of 5mm diameter using a custom built device (Fig. 2A). The drop tower based system was used to inflict a single impact injury on cartilage explants. This device consists of a drop mass guided by roller bearings riding along two cylindrical columns. The drop mass dropped from a desired height using a solenoid release mechanism. An indenter resting on the cartilage initiated the injury after being struck by the drop mass. Mechanical assessments were performed with a 4mm diameter indenter inside the 5mm impact sites. A load of 2 Kg was released from a height of 12 cm (nominal impact energy ~ 2.35 J). The injury simulated here was comparable to 2.5 J/cm² energy density applied to a rigidly fixed specimen. Explants were randomly distributed into groups of 7 and 14 day time points and tested before impact, at 1 hour after impact, and at 7 days or 14 days to monitor the progress of mechanical changes. To assess the mechanical stability of cartilage explants in culture, a group of non-impacted control explants underwent mechanical characterization at Day 0 and either Day 7 or Day 14 (Fig. 4). The 14 day endpoint was selected based on prior experiments (data not shown) showing cartilage viability and biosynthetic activity maintained to near day 0 states in an osteochondral bovine model.

Explants were subsequently fixed in 10% formalin, decalcified (5% formic acid) and processed for paraffin histology. Serial 5µm sections were stained with Safranin-O/Fastgreen Weigert's haematoxylin to analyze proteoglycan distribution and general morphological condition using standard histological protocols. Sections were scanned using an Olympus BX-40 Optical Microscope (10X magnification) with an automated stepper motor driven stage to image an entire section of cartilage including subchondral bone. Cartilage thickness measurements were determined from histological sections with calibrated scales and correlated with initial in-situ measurements done prior to the testing. The average cartilage thickness measured from all explants involved in this study was 2.40mm ± 0.24 mm.

Statistical analysis

Repeated Measures One –Way ANOVA was employed to determine the effect of impact on measured elastic and visco-elastic parameters. Post hoc tukey test was used for pair wise comparison to determine relative changes within a treatment group. Significance level was set at p<0.05. Direct comparison of injured and non-injured groups was performed using student t-test.

Results

A total of 46 explants were utilized for this study with a minimum of 10 samples for analysis in each group. Preliminary studies measuring regional local compressive moduli at 5 predetermined sites showed a mean variance of 28% among sites [17]. The data show measurements (mean ± sd) along with normalized equivalents (mean ± sd in parentheses) of local compressive moduli, creep strain, stretch factor 'β' and relaxation constant 'τ' (Table 1). These data were consistent with previously measured values of normal cartilage mechanics under comparable conditions [18, 19]. Direct comparison of impact and control data did not reveal significant difference between the groups at all time points measured. Non-impacted controls showed no appreciable variation in their moduli values through 14 days in culture. Day 0 values (6.62 ± 1.82 MPa) did not vary significantly at Day 7 (6.7 ± 1.45 MPa) or Day 14 (6.50 ± 1.33 MPa). In the impact group, local compressive modulus showed a modest 4% decrease at Day 7 and a significant 10% decrease (p=0.04) at Day 14. Local compressive modulus measured 1 hour after impact did not change when compared to pre-impact values.

Under compressive creep, cumulative strain and viscoelastic parameters β and τ were calculated. After impact, a significant increase in creep strain was observed at Day 7 (9% $\uparrow$ $p=0.023$) whereas there was no such change in our control samples. At 14 days, a significant increase in both impacted (14% $\uparrow$; $p<0.001$) and control (11% $\uparrow$; $p<0.001$) samples was observed. Similar trends were observed in values of β (Control: 4% $\downarrow$; $p=0.013$; Impact: 6% $\downarrow$ $p<0.001$) and τ (Control: 7% $\downarrow$; $p=0.006$; Impact: 12% $\downarrow$; $p=0.001$). Also, creep strain, β and τ measured at 1 hour after impact varied significantly against 14 day data but did not when compared to day 0 pre-injury values. Based on these results, impacted explants lost the majority of their mechanical integrity in the first week of culture.

Histological analyses showed that impacted explants suffered superficial disruption and clefts extending into the transitional zone. Figure 5 shows a compilation of typical test site changes at various time points. Mild delamination of the superficial zone was also observed in some of the impacted sections. Variable decreases in Safranin-O staining intensity (lightest intensity at the superficial zone) were observed in impacted osteochondral explants on day 7 (Fig. 5D) and day 14 (Fig. 5E.) particularly in areas adjacent to cracks. Control explants showed a decrease in Safranin-O staining after 14 days in culture. At Day 14, several empty lacunae (remnants of cells) were observed immediately below the impact site suggesting widespread cell death whereas this event was not observed in our control explants (Fig. 5B, 5C).

Discussion

Our data demonstrate that mechanical characteristics can be monitored over time in viable osteochondral explants using a sequential same-site testing methodology. The findings support the hypothesis that mechanical properties continue to decline in the absence of mechanical stimulation. The immediate structural damage revealed by our histological data did not appear to affect mechanical properties at impact sites. Rather, mechanical integrity losses may be partly associated with proteoglycan depletion 7 days after injury. Our study resonates with findings of Natoli and his associates [20], who observed similar trends in their cartilage osteochondral impact model.

Our data also show that repeated measures greatly facilitate detection of injury-induced cartilage degeneration. Previous studies have shown that biological and mechanical composition varies across joints and joint surfaces [21, 22]. In account of this inherent variability, mechanical testing has been suggested to be valuable in monitoring progressive changes via sequential testing but may not be as reliable in distinguishing a degenerated cartilage from a healthy counterpart [15]. To our knowledge this is the first study that takes advantage of repeated measures to determine the temporal progression of impact induced mechanical damage in a fully viable osteochondral explant.

The drop tower system used in this study (Fig. 2A.) has been previously employed to show that injury induced oxidative stress may be responsible for chondrocyte death [3, 23] and progression of cartilage injury induced degeneration may be biochemically mediated by mitogen activated protein kinases (MAPK) [24]. The injury on osteochondral explants have been characterized at different energy densities varying from 7J/cm² to 14 J/cm². The energy density used in this study (12J/cm²) simulated a visible gross injury without severe delamination, rendering the articular cartilage suitable for mechanical characterization.

Mechanical trauma has been shown to cause cellular necrosis [25] and induction of pro-inflammatory cytokines such as IL-1 β and TNF- α [26] and matrix degrading enzymes. Decreases in biosynthetic activity and apoptosis of chondrocytes ensue [27, 28], leading to depletion of proteoglycans [29, 30] and eventually secondary OA. Levin and associates [7]

showed in their cartilage explant culture model that soluble intracellular signaling may be responsible for the spread of cell death after impact to the surrounding tissues, providing evidence that spatial progression of impact induced changes are observable *in vitro*. The bovine osteochondral model developed in this study may provide the much needed spatial and temporal resolution to study biological changes and connect them to impending mechanical changes. Although our study did not measure the spread of degeneration, it has clearly paved the way for future studies focused on post-traumatic loading effects on articular cartilage, providing basis for clinically significant questions such as in rehabilitation after ruptured ACL blunt trauma.

Mechanical properties are believed to be closely related to ECM composition since cartilage response to physical stimuli is predominantly governed by the presence of the water attracting proteoglycans and collagen fibers among other constituents [31, 32]. Our histological analyses (Safranin-O) revealed a modest but detectable decline in superficial to transitional zone PG at impact sites that might underlie the observed changes in mechanical properties. Control explants also showed a loss of Safranin-O staining after 14 days in culture, consistent with our mechanical results. Recent work from Bian and coworkers [33, 34] indicated that functional properties of immature cartilage are more responsive to endogenous factors conditions than mature cartilage. In our study we used osteochondral explants from mature bovine joints and did not observe any of the effects previously observed [35]. Cartilage culture is commonly performed in the absence of mechanical loading for a matter of simplicity. *In vitro*, several studies have shown the effect of mechanical load in regulating tissue metabolism [36–38]. Our study describes the effect of tissue mechanical properties in the absence of any mechanical loads. We predict that the presence of mechanical stimulation may be more physiologically relevant in studying disease pathology in cartilage.

The physical dimensions of our explant model were selected to minimize artifacts commonly encountered in other systems. For mechanical properties assessments, indentation stiffness in bovine cartilage stay relatively reproducible when measurements are performed at locations that are away from the edges (>1.5mm) [39]. The 25×25 mm specimens used in our experiments have low surface to volume ratios (<20), which contribute to their stability in culture [40–42]. In addition, most investigators perform impacts in an unconfined configuration. The lack the circumferential support from surrounding cartilage leads to unrealistic stresses and strains during impacts, exaggerating the severity of the resulting structural damage. In our model, more realistic boundary conditions are provided by impacting an area of the explant surface that is surrounded by a centimeter or more of undisrupted cartilage creating a physiological semi-confined structure. Thus, problems associated with non-physiologic boundary conditions were obviated by limiting the impact site to a small area surrounded by intact cartilage.

In this study, we did not attempt to determine intrinsic material properties of the articular cartilage. Although aspect ratios of the cartilage and indenter diameter were available, the methodology of estimating material parameters requires use of rigorous computational procedures that require sophisticated algorithms and finite element methods [43–45]. Also, to accurately determine equilibrium parameters, experimental compression of cartilage must be performed for durations significantly long which we considered to be harmful to the viability of the tissue *in-vitro*. More accessible methods that do not require any elaborate software requirements are available [46] that may be employed in the future if repeated tests of significant duration are found to be “friendly” to the biologic status of the cartilage in long-term culture.

In our model, more realistic boundary conditions are provided by impacting an area of the explant surface that is surrounded by a centimeter or more of undisrupted cartilage creating a physiological semi-confined boundary condition. Investigating mechanical and biological outcomes of injured cartilage before and after application of treatment modalities can be achieved in the current model, taking in to account inherent spatial variations that may facilitate a better analysis of future interventions. Our results indicate that it is possible to detect time-dependent mechanical property changes after a blunt impact injury using a site-specific indentation method that does not itself damage cartilage. Because chondrocyte viability and function can be preserved throughout multiple testing sessions, biologic responses can be studied in parallel with mechanical property changes to better understand the progression of injury induced degeneration in cartilage.

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Figure 1.

Anatomical location of the osteochondral explant is depicted in this cartoon representation of an exposed bovine tibial plateau. Tibial plateaus were inspected for gross damage and signs of visible degeneration before allocation for explant harvest.

Figure 2.

The drop tower assembly (A.) was used to induce the impact injury on the osteochondral explant. The explant was secured in the impactor using steel pins interfaced with the subchondral bone to ensure rigidity. The polyethylene halo (B.) facilitated convenient handling of the tissue during mechanical testing. Prior to mechanical testing, cartilage thickness at the testing site was estimated manually (by the operator) using a K-wire/polymer stopper instrument and a digital caliper. The estimation was performed as an average of four measurement around the test/injury site (as shown in A.)

Figure 3.

Mechanical Testing protocol (A) employed in this study to determine mechanical property of test sites on osteochondral explants consisted of cyclic compression and creep. Local compressive modulus was determined using linear regression on the stress-strain curve generated (B). Creep data (C) was utilized to determine visco-elastic parameters β and τ using stretch exponential function curve fitting along with accumulated creep strain.

Figure 4. Chronological testing flowchart depicting explant allocation for various groups and time points.

Figure 5.

5 μ m paraffin sections through the central region of the test site of osteochondral explants (A) were used for histological analysis. Superficial zone indicated decrease in Safranin-O staining in control explants between Day 7 (B) and Day 14 (C). Impacted samples showed similar decline in staining at day 7 (D) with radiating cracks and de-lamination. Empty lacunae suggesting prior cell presence was observed 14 days after impact along with impact induced structural damage (E). Scale bar represents A ~ 200 μ m and B, C, D, E ~ 45 μ m.

Table 1

Mechanical properties of non-injured and impacted osteochondral explants (mean $\pm$ standard deviation) at various time points are tabulated.

		Time points	Local Compressive Modulus MPa	Creep Strain NA	Stretch Factor β NA	Creep Relaxation Constant s
Non Injured control		0	6.59 $\pm$ 2.13 (1.00)	0.19 $\pm$ 0.01 (1.00)	0.86 $\pm$ 0.04 (1.00)	278.36 $\pm$ 16.57 (1.00)
		7 Day	6.60 $\pm$ 1.49 (1.05 $\pm$ 0.17)	0.19 $\pm$ 0.02 (1.03 $\pm$ 0.12)	0.85 $\pm$ 0.02 (0.99 $\pm$ 0.27)	274.53 $\pm$ 12.20 (0.98 $\pm$ 0.04)
		14 Day	6.50 $\pm$ 1.33 (1.03 $\pm$ 0.09)	0.21 $\pm$ 0.01 (1.11 $\pm$ 0.08) *	0.83 $\pm$ 0.05 (0.96 $\pm$ 0.03) *	258.96 $\pm$ 29.44 (0.93 $\pm$ 0.05) *
Injured		0	6.09 $\pm$ 2.04 (1.00)	0.18 $\pm$ 0.02 (1.00)	0.84 $\pm$ 0.05 (1.00)	271.32 $\pm$ 24.45 (1.00)
		1Hr	5.83 $\pm$ 1.58 (0.99 $\pm$ 0.21)	0.19 $\pm$ 0.03 (1.04 $\pm$ 0.11)	0.83 $\pm$ 0.03 (0.98 $\pm$ 0.06)	262.11 $\pm$ 29.53 (0.99 $\pm$ 0.10)
		7 Day	4.74 $\pm$ 1.21 (0.96 $\pm$ 0.21)	0.21 $\pm$ 0.03 (1.09 $\pm$ 0.11) *	0.81 $\pm$ 0.04 (0.98 $\pm$ 0.05)	249.46 $\pm$ 28.45 (0.96 $\pm$ 0.11)
		14 Day	5.66 $\pm$ 1.66 (0.90 $\pm$ 0.20) *	0.21 $\pm$ 0.02 (1.14 $\pm$ 0.13) *, α	0.80 $\pm$ 0.03 (0.94 $\pm$ 0.05) *, α	238.81 $\pm$ 26.15 (0.88 $\pm$ 0.06) *, α

Values normalized to corresponding Day 0 mechanical measurements are shown in parentheses.

* represents $p < 0.05$ Vs Day 0 baseline;

α represents $p < 0.05$ Vs $t = 1$ hr.