Sub-Critical Impact Inhibits the Lubricating Mechanisms of Articular Cartilage

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Abstract

Although post-traumatic osteoarthritis accounts for a significant proportion of all osteoarthritis, the understanding of both biological and mechanical phenomena that lead to cartilage degeneration in the years to decades after trauma is still lacking. In this study, we evaluate how cartilage lubrication is altered after a sub-critical impact (i.e., an impact to the cartilage surface that produces surface cracking but not full thickness fissuring). Through utilizing a Stribeck-like framework, the elastoviscous transition, we evaluated changes to both the innate boundary lubricating ability of cartilage after impact and also the effectiveness of high viscosity lubricants to lower friction after impact. Increases in boundary friction coincided with changes in lubricin localization after impact. However, larger increases in friction coefficient were observed in mixed-mode lubrication which can be predicted by increases in surface roughness due to cartilage fissuring. The data here reveal distinct mechanisms of cartilage lubrication that can fail after traumatic impact and may explain a key mechanical phenomenon that predisposes cartilage to development of osteoarthritis after injury.

Introduction

Although articular cartilage is regarded as one of the most remarkable bearing surfaces found in nature, the failure of this tissue in osteoarthritis (OA) is the leading cause of severe disability within the United States (Murphy et al., 2008). For most OA cases, the mechanisms of initiation and progression of this disease are not well understood; however, a significant portion of cases (~12%) are connected to an earlier traumatic injury (Brown et al.). These cases of secondary OA are considered post-traumatic OA (PTOA), and it is highly likely a traumatic injury such as an anterior cruciate ligament tear will lead to PTOA. In fact, 78% of anterior cruciate ligament tears resulted in radiographic changes consistent with OA 14 years post-injury (von Porat, 2004). Although traumatic injury is considered an initiating event in cases of PTOA, the mechanisms by which this disease manifests are not fully understood. Both mechanical and biological factors are known to play a role with significant interaction between these factors (Anderson et al., 2011; Buckwalter et al., 2013).

To study the complex etiology of PTOA, both *in vivo* and *in vitro* post-traumatic models have been developed to investigate the mechanical and biological events following trauma. Strategies for controlling cartilage injury include imposing specific loads, displacements, and impact energies on the tissue (Alexander et al., 2012; Bonnevie et al., 2015a; Borrelli et al., 2002; Burgin and Aspden, 2007; Finlay and Repo, 1978; Jeffrey et al., 1995; Morel and Quinn, 2004; Quinn et al., 1998; Sah et al., 1991). Biologically, impact stresses on the order of 15-20 MPa and local strains on the order of 0.10 are known to cause significant cell death (Bartell et al., 2015; Morel and Quinn, 2004), and impacts below these thresholds can often induce matrix damage (Quinn et al., 2001), catabolic responses (Natoli et al., 2008), and mitochondrial depolarization leading to chondrocyte apoptosis (Huser and Davies, 2007). While the acute

biological consequences of trauma have been studied extensively, there is less known about the direct mechanical consequences of cartilage trauma. Impacts on the order of 20 MPa are known to cause catastrophic matrix fissuring and can alter the water content of the tissue (Torzilli et al., 1999), which can influence the mechanics of cartilage (Mow et al., 1980). Recently, we have reported superficial cartilage cracking at impact stresses as low as 14 MPa when delivered by a spring-loaded impacting device (Bonnevie et al., 2015a). These superficial cracks, which may be on the order of tens of microns wide, would be very difficult to visualize or observe *in vivo* using conventional imaging practice (Novakofski et al., 2016). Although one may hypothesize, it is currently unknown whether cracks like these have any functional consequences on tissue.

Functional changes to the naturally low friction and shear environment of cartilage can have profound biological consequences for tissue health. Increased friction has been linked to chondrocyte apoptosis (Waller et al., 2013), mitochondrial dysfunction (E.D. Bonnevie et al., 2016), loss of superficial zone cellularity (Karamchedu et al., 2015), and matrix wear and degradation (Jay et al., 2007). However, it is currently unknown if cartilage trauma affects lubrication and what role inferior lubrication may have in the initiation and progression of PTOA.

The naturally low friction of cartilage is due to cooperation of several mechanisms leading to some of the lowest friction coefficients found in nature (McCutchen, 1962). Fluid pressurization within the cartilage matrix is a key factor that reduces friction by supporting a substantial portion of the contact load and consequently reducing the stresses on contacting solid asperities (Ateshian, 2009; Bonnevie et al., 2011; Krishnan et al., 2004). Additionally, the frictional stresses on the contacting asperities are reduced by lubricating molecules found in synovial fluid (Schmidt et al., 2007). Lubricin, a boundary lubricant, reduces frictional stresses

under high load and low motion (Gleghorn et al., 2009; Jay et al., 2001), and hyaluronic acid, a highly viscous polysaccharide, reduces friction at higher speeds due to formation of viscous films (Bonnevie et al., 2015b; Das et al., 2013). However, it is not currently known how a traumatic impact to cartilage can affect the efficacy of these lubricants to reduce cartilage friction.

In this study, we explore the effects of sub-critical impact on the lubricating behavior of cartilage. Specifically, we focused on the intrinsic boundary lubricating ability of the tissue and the role of high viscosity lubricants in reducing friction after cartilage impact. By using a Stribeck-like framework (Hersey, 1914), the elastoviscous transition (Bonnevie et al., 2015b; Dunn et al., 2014), we mapped both boundary and mixed mode friction coefficients using lubricants with viscosities that varied over 4 orders of magnitude. By analyzing specific mechanisms of cartilage lubrication and assessing cartilage structure by histology, optical profilometry, mechanical testing we identified key structural alterations that lead to compromised lubrication.

Methods

Tissue Source

Neonatal bovine stifles were obtained from a local abbatoir within 24 hours of sacrifice. Cartilage plugs (6 mm diameter by 3 mm thick) were extracted aseptically from the femoral condyles and stored at -20°C prior to testing.

Impacting Device and data acquisition

A recently described, spring-loaded impactor was used in this study (Alexander et al., 2012; Bonnevie et al., 2015a). Briefly, an 8 mm diameter cylindrical impacting missile was used

to impact articular cartilage plugs in unconfined compression at stress levels previously determined to produce surface fissures without full thickness cracks (Bonnievie et al., 2015a). Impact loads were collected using a load cell mounted in parallel to the impacting missile at 50 kHz (PCBPiezotronics, Depew, NY). A linear variable differential transducer (LVDT; RDP Electronics, Pottstown PA) was mounted in parallel with the impacting tip to measure the deformation of the cartilage plug.

India ink staining and histology

To reveal the extent of surface fissuring inflicted by the impact, cartilage was exposed to india ink before being wiped with a kimwipe to remove unbound dye and surface photographs were collected. Also, impacted and non impacted cartilage plugs were fixed in buffered 10% formalin for 48 hours, dehydrated in ethanol, embedded in paraffin, and mounted on slides. For histological staining, slides were cleared with xylene and rehydrated before staining with either Safranin-O or Picrosirius Red. Samples were imaged on a light microscope. Picrosirius Red-stained slides were viewed under crossed polarizers to view collagen organization.

Lubricin IHC

Lubricin immunohistochemical staining was conducted as previously described (Bonnievie et al., 2014). Briefly, samples were cleared and rehydrated as above. Antigen retrieval was conducted at 90°C for 20 minutes in citric acid followed by rinses in TRIS buffered saline with 0.5% TWEEN-20. Blocking was conducted using a solution containing normal serum, bovine serum albumin, Triton X-100, and TWEEN-20. Primary lubricin antibodies (Abcam: ab28484) were applied overnight at 4°C. Biotinylated Secondary antibodies (Vectastain ABC, Vector) were applied for 30 minutes followed by 30 minute incubation in avidin-biotin complex

(Vectastain ABC, Vector). Staining was conducted using a peroxidase substrate (ImmPACT DAB, Vector) for 2 minutes.

Surface Profilometry

Surface profiles of non-impacted and impacted cartilage were analyzed using a noncontact, scanning white light profilometer (ADE Phase Shift MicroXAM Optical interferometric profiler). Scans analyzing the height distribution in a 209 μm x 179 μm window were collected from the center of each sample as recently reported (Bonnevie et al., 2016). The surface roughness, S_q , was calculated for each sample. The window size was chosen to minimize effects of sample curvature on roughness values and was limited by the actuation length of the scan head as has been previously reported (Shekhawat et al., 2009).

Compression Testing

The bulk mechanical effects of sub-critical impact were tested using a confined compression set-up mounted on a Bose Enduratec testing frame. Briefly, samples were placed in a confining chamber, bathed in PBS, and compressed in four strain increments of 5% strain. The stress-relaxation curves were fit to a poroelastic model to calculate aggregate modulus and hydraulic permeability (Cross et al., 2010).

Friction Analysis

Cartilage lubricating properties were analyzed using a custom-built tribometer (Bonnevie et al., 2015b, 2014; Gleghorn et al., 2009). Cartilage plugs were 6 mm diameter by 3 mm thick cylinders and slid against a glass counterface under 25% normal strain. An *in situ* view of the contact geometry is presented in the supplemental material. To mitigate effects of interstitial fluid pressurization on friction coefficients, all friction coefficients were measured after a 60 minute equilibration period (Bonnevie et al., 2015b; Gleghorn and Bonassar, 2008) using a

stationary contact area configuration (Caligaris and Ateshian, 2008). Using a recently described framework, the boundary mode friction coefficient and the transition to mixed lubrication were mapped using an array of three different lubricants with viscosities that varied by over five orders of magnitude (Bonnevie et al., 2015b): phosphate buffered saline (PBS, 1 mPas), 10 mg/ml 630 kDa hyaluronic acid (HA, 151 mPas), and a hydrophobicized HA derivative, (HYADD4, 72,000 mPas) (Finelli et al., 2009). Friction coefficients were recorded over a range of sliding speeds for each sample ranging from 0.1 mm/s to 10 mm/s. Friction coefficients were presented as a function of the Sommerfeld number, S , which takes into account the sliding speed (V), applied normal load (F_N), lubricant viscosity (η), and the contact width ($a = 6$ mm) (Dunn et al., 2014; Unsworth, 1991):

$$S = V\eta a / F_N \quad (\text{equation 1})$$

The boundary mode friction coefficient, μ_B , the minimum friction coefficient, μ_{min} , and the Sommerfeld number at the midpoint between high and low friction, S_t , were all determined through fitting the friction data of impacted and non-impacted cartilage combined for all three viscosities by using the following equation of friction coefficient, μ , as a function of the Sommerfeld number, S (Bonnevie et al., 2015b):

$$\mu(S) = \mu_{min} + (\mu_B - \mu_{min})e^{-(S/S_t)^d} \quad (\text{equation 2})$$

Statistical Analysis

Statistical analysis for comparisons between non-impacted and impacted cartilage were conducted using a Student's t-test for significance. Uncertainties for the curve fit parameters in equation 2 were determined through 15 Monte Carlo simulations based on the point-by-point standard deviations of friction coefficient. Curve fits parameters for equation 2 were determined

through RMS error minimization, and goodness of fit for equation 2 to friction coefficients was determined through coefficient of variation of the RMS error. Significance was set at $p < 0.05$.

Results

The applied impacts to cartilage were considerably repeatable. Coefficients of variation for stress, stress rate, strain, and strain rate were all less than 7% for 4 representative samples. These impacts resulted in peak impact stresses of 17.2 ± 0.49 MPa, peak stress rates of 24.8 ± 1.0 GPa/s, peak strains of 0.29 ± 0.019 , and peak strain rates of 218 ± 8.1 s⁻¹ as determined from Gaussian model fits (Bonnevie et al., 2015a) (Figure 1).

India ink application to the articular surface after impact revealed surface fissuring that was not present in non-impacted cartilage (Figure 2A). Further, lubricin staining revealed less localized lubricin in the impacted cartilage, but not total loss of lubricin localization at the tissue surface (Figure 2B). Safranin O and Picrosirius red staining viewed under polarized light revealed fibrillation of the cartilage matrix at the articular surface without cracks extending deeper than the superficial zone (Figure 2FH, arrows). Impact did not affect either proteoglycan localization or collagen organization based on histological staining (Figure 2CD).

To better understand surface roughness changes after mechanical impact, scanning light white profiles were collected of non-impacted and impacted cartilage. For non-impacted cartilage, surfaces were relatively smooth. The height distribution was roughly Gaussian and average RMS roughness, S_q , was 2.23 ± 0.43 μm . In contrast, a representative surface scan of impacted cartilage revealed a crack along the length of the scan. Consequently, the height distribution was not Gaussian, but followed the form of a bi-modal distribution. For impacted cartilage, RMS roughnesses were 72% higher with average roughness of 3.85 ± 1.2 μm ($n = 4-5$, $p = 0.02$). Additionally, the surface profile of impacted cartilage revealed that the surface fissures

were on the order of 50 μm wide (Figure 3B). Fitting the height profile to a bimodal distribution of surface heights revealed that surface fissures were on the order of 8 μm deep, determined from the distance between the two maxima in the distribution (gray shaded regions, Figure 3D).

Mechanical compression revealed no changes in the aggregate modulus or hydraulic permeability. Moduli for non-impacted and impacted cartilage were 640 ± 130 kPa and 760 ± 150 kPa, respectively ($n = 10-11$, $p = 0.064$). Hydraulic permeability for non-impacted and impacted cartilage were 3.60 ± 1.4 $\text{m}^2/\text{Pa/s}$ and 3.40 ± 1.2 $\text{m}^2/\text{Pa/s}$, respectively ($n = 10-11$, $p = 0.73$).

Both non-impacted and impacted cartilage fit well to elastoviscous transition curves that presented friction transition from a higher boundary friction plateau to a lower mixed lubrication regime. For non-impacted and impacted cartilage the coefficients of variation of the RMS error were 0.62% and 2.6%, respectively. Overall, the friction coefficients for impacted cartilage were 41% higher than non-impacted cartilage. In the boundary regime of lubrication, impacted cartilage friction was 11% higher than non-impacted controls (Figure 4C). However, in mixed mode lubrication friction for impacted cartilage was 94% higher than non-impacted controls (Figure 4D). Additionally, the transition number, S_t , increased 63% percent after impact (Figure 4E). Collectively these data revealed that although boundary friction coefficients rose, the most drastic changes occurred in mixed lubrication and the transition towards it.

Discussion

This study revealed a connection between structural changes to articular cartilage after a sub-critical traumatic impact and changes to the effectiveness of high viscosity lubricants in reducing cartilage friction. While the intrinsic boundary lubricating ability of the cartilage was altered after impact (Figure 4C), the most pronounced changes were discovered in mixed

lubrication and the transition away from boundary to mixed mode (Figure 4DE). This altered tribological behavior not only has implications concerning cartilage homeostasis after impact injury, but also in understanding how therapeutics may function to restore proper joint lubrication.

The most drastic changes to the intrinsic lubrication of cartilage after trauma were observed in the lubrication by lubricants with high viscosities (i.e., HA: 150 mPas and HYADD4: 72,000 mPas). Both mixed mode friction coefficients and the transition to mixed lubrication were significantly altered after impact (Figure 4DE). Two key structural changes that may play a role in these friction changes were the loss of lubricin from the articular surface (Figure 2CD) and increased surface roughness (Figure 3). We recently observed that lubricin facilitates the transition towards mixed lubrication and facilitates aggregation of HA near the cartilage surface (Bonnevie et al., 2015b). Similarly, lubrication by adsorbed mucin layers revealed a similar mechanism of facilitating the transition towards mixed lubrication (Yakubov et al., 2009). However, this effect of promoting mixed lubrication was abrogated for roughened surfaces (Yakubov et al., 2009). With these previous results in mind, it is likely that both altered lubricin localization and increased surface roughness play a role in inhibiting the transition to mixed lubrication for impacted cartilage.

The factor which likely plays a major role in the increased minimum friction coefficient in mixed lubrication was the altered surface roughness as a consequence of impact. A recent study on the lubrication mechanisms of hydrogels hypothesized that increased surface roughness allows lubricating films at the interface to be drainable (Yashima et al., 2014). In this case, increased friction in mixed lubrication observed in the present study may be due to hyaluronic acid becoming drainable through surface fissures. This phenomenon can cause fluid

depressurization at the interface. Consequently higher stresses would be transferred to the contacting asperities increasing the friction coefficient. Additionally, this effect may have further implications in interstitial lubrication that were not probed in this study (Ateshian, 2009). As pressurized fluid may become less sustainable at the cartilage interface (i.e., if there is a higher effective permeability), it likely cannot support as large of a portion of the contact stress and consequently friction will be increased.

Not only was mixed lubrication of impacted cartilage altered, but boundary lubrication was affected as well. Lubricin staining revealed less lubricin localized at the tissue surface. Consistent with previous studies, the boundary friction coefficient, especially at low speed, increased on the order of ~20% (Bonnevie et al., 2015b; Gleghorn et al., 2009). While the present study focused on the intrinsic lubricating ability of cartilage after trauma, it is important to note that lubricin within synovial fluid also plays a key role in the low friction of cartilage. However, in cases of trauma such as ACL rupture, lubricin concentration can fall by ~80% as early as 32 days after injury and take a full year to rebound to baseline levels (Elsaid et al., 2008). With this in mind, the loss of intrinsic boundary lubricating ability, along with the possibility of detrimental changes to synovial fluid composition highlights that the importance of boundary lubrication should not be understated.

These changes in both the intrinsic boundary lubrication and mixed lubrication of cartilage after trauma may have functional consequences fueling the progression of damage consistent with development of PTOA. Cartilage homeostasis is dependent on a balance of frictional forces that can stimulate chondrocytes without causing detrimental effects (Nugent et al., 2006; Waller et al., 2013). Shear stimulus of synovial fluid and chondrocytes, and active deformation of the cartilage matrix are pivotal to the transport of nutrients to deeper tissue and

also to the stimulation of important genes such as proteoglycan 4 (i.e., lubricin encoding gene) (Nugent et al., 2006) and the activation of signaling molecules, such as transforming growth factors (Albro et al., 2012). However, overstimulation of cartilage in the form of elevated friction can acutely lead to chondrocyte apoptosis and necrosis (Waller et al., 2013), and mitochondrial dysfunction (Bonnevie et al., 2016). In the longer term, increased friction can lead to a loss of cellularity in the cartilage superficial zone (Karamchedu et al., 2015). The question remains as to whether the lubrication changes observed here are drastic enough to initiate any of these biological consequences. However, it is important to note that even in cases where trauma may not cause frictional changes that can lead to adverse effects such as chondrocyte apoptosis acutely, degeneration due to the elevated friction (Jay et al., 2007) may lead to adverse effects later on and may be a factor in the long, in some cases decades long, incubation period of PTOA.

From connecting structural changes to lubrication changes, it is very likely that the surface cracking plays a key role in inhibiting lubrication. In this study, we specifically explored a sub-critical impact model, that is, a model that causes surface damage to cartilage in the absence of full-thickness fissuring. These injuries would be impossible to detect through imaging modalities such as MRI or contrast-enhanced CT, and they would be difficult to detect even through arthroscopy especially compared to the detection of full-thickness cartilage lesions (Novakofski et al., 2016). However, we have seen here that these surface fissures, which can be on the order of only 50 μm wide and 10 μm deep, can have detrimental effects on the lubricating environment. They may pose a target in developing *in vivo* imaging modalities that can detect defects on their size scale, and after their detection, suitable therapeutics may be applied.

One avenue for therapeutic intervention is tribosupplementation, the process injecting a joint with lubricants (Balazs and Denlinger, 1993; Flannery et al., 2009; Teeple et al., 2011). In

the form of viscosupplementation, injecting hyaluronic acid is currently a \$1B industry world-wide, with varying results depending on many factors. While HA can reduce friction compared to PBS, and although HA still reduces friction in impacted cartilage, this effect is somewhat muted as evidenced by the increased transition number and minimum friction coefficient (Figure 4DE). Consequently, it is likely that post-traumatic cartilage is predisposed to friction-mediated degeneration. The variation between HA efficacy on fissured and non-fissured cartilage may describe one mechanism by which HA is or isn't effective on a case-by-case basis. Boundary lubrication by lubricin poses a promising path for tribosupplementation (Flannery et al., 2009; Teeple et al., 2011). Lubricin replenishment can not only restore lubricating ability in boundary mode friction, but it also enhances the lubrication of HA through lubricant synergy (Bonnievie et al., 2015b; Das et al., 2013). Likely for these reasons, lubricin has proven effective at limiting cartilage degeneration in multiple animal models of traumatic OA.

Although this study has revealed multiple paths through which the intrinsic lubricating mechanisms of cartilage are altered after traumatic impact, there are several limitations to be discussed. While the use of neonatal bovine cartilage provided a consistent source of healthy cartilage, it is likely that the surface damage seen in this study would happen at different stress and strain levels in both different aged animals and in different species. Additionally, the boundary conditions of the impact (i.e., cartilage plugs without subchondral bone, and flat ended impactor tip) may change thresholds for damage as well. Also of note was the small window sizes in the interferometer measurements which were limited by macroscale curvature and actuation range of the scanning head. Nevertheless, the effect of damage on lubrication would likely be similar. Further, due to a lack of availability of post-traumatic synovial fluid and exogenous lubricin, this study focused on the innate lubricating mechanisms of cartilage and the

role of viscous lubricants. Future studies should evaluate the role replenishing boundary lubricants on the lubrication of post-traumatic cartilage and also post-traumatic *in vivo* studies should focus on the lubricating ability of synovial fluid after trauma.

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Conflict of Interest Statement

Devis Galesso and Cynthia Secchieri are employees of Fidia Farmaceutici, SpA. Lawrence Bonassar is a paid consultant for Fidia Farmaceutici, SpA.

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Figure 1: (A) The spring-loaded impacting device utilizes both a linear variable displacement transducer and load cell to quantify the strains and stresses, respectively. (B-E) Applied impacts resulted in peak impact stresses of 17.2 ± 0.49 MPa, peak stress rates of 24.8 ± 1.0 GPa/s, peak strains of 0.29 ± 0.019 , and peak strain rates of 218 ± 8.1 s⁻¹ as determined from Gaussian model fits using a recently described model to eliminate inertial artifacts from accelerating a load cell mounted on the impacting piston. Insets reveal typical deviations of impact parameters.

Figure 2: (AB) Surface fissuring in impacted cartilage was visualized after India ink application. (CD) Less lubricin was localized to the surfaces of impacted cartilage. (EF) Safranin O staining exposed no macroscopic changes in proteoglycan localization. (GH) Picrosirius red staining visualized under polarized light revealed no macroscopic changes to collagen structure and organization. Insets in (F) and (H) highlight local areas of cartilage fissuring.

Figure 3: (AB) Representative surface scans of non-impacted and impacted cartilage revealed relatively smooth surfaces in non-impacted cartilage, but fissuring in impacted cartilage, with fissures on the order of 50 μm wide (outlined region). (C) Non-impacted cartilage had a normal Gaussian surface height distribution, but (D) impacted cartilage had a bimodal height distribution. The difference in heights between the two modes of surface heights revealed surface fissures were on the order of 8 μm deep. (E) The average surface roughness of impacted cartilage was 72% higher than non-impacted cartilage (* $p < 0.05$, $n = 4-5$).

Figure 4: (AB) Friction coefficients as a function of Sommerfeld number fit very well to elastoviscous transition curves for both non-impacted and impacted cartilage ($CV < 2\%$ for both, $n = 4$). (C-E) Fit parameters from the model curve (equation 2) revealed how mechanisms of lubrication were altered after cartilage impact. (C) Boundary friction coefficient rose 11% after impact (* $p < 0.05$). (D) Minimum friction coefficient rose 94% after impact (** $p < 0.01$). (E) Transition number rose 62% after impact (** $p < 0.01$).