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Synovial Fluid Lubricin and Hyaluronan are Altered in Equine Osteochondral Fragmentation, Cartilage Impact Injury and Full-Thickness Cartilage Defect Models

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Abstract

The objectives of this study were to evaluate temporal changes in lubricin, hyaluronan (HA), and HA molecular weight (MW) distributions in three distinct models of equine joint injury affecting the carpal (wrist), tarsal (ankle), and femoropatellar (knee) joints. To establish ranges for lubricin, HA, and HA MW distributions across multiple joints, we first evaluated clinically healthy, high-motion equine joints. Synovial fluid was collected from high-motion joints in horses without clinical signs of joint disease (n=11 horses, 102 joints) and from research horses undergoing carpal osteochondral fragmentation (n=8), talar cartilage impact injury (n=7), and femoral trochlear ridge full-thickness cartilage injury (n=22) prior to and following arthroscopically-induced joint injury. Lubricin and HA concentrations were measured via enzyme-linked immunosorbent assays, and gel electrophoresis was performed to evaluate HA MW distributions. Synovial fluid parameters were analyzed via linear regression models, revealing that lubricin and HA concentrations were conserved across healthy, high-motion joints. Lubricin concentrations increased post-injury in all osteoarthritis models (carpal fragmentation $p=0.001$; talar impact $p<0.001$; femoral trochlear ridge cartilage defect $p=0.03$). Sustained loss of HA was noted post-arthroscopy following carpal osteochondral fragmentation ($p<0.0001$) and talar impact injury ($p<0.001$). Lubricin may be elevated to compensate for loss of HA and to protect cartilage post-injury. Further investigation into the mechanisms regulating lubricin and HA following joint injury and their effects on joint homeostasis is warranted, including whether lubricin has value as a biomarker for post-traumatic osteoarthritis.

Keywords

osteoarthritis; proteoglycan 4; hyaluronic acid; horse; PTOA

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Author contributions

BTP acquired, analyzed, and interpreted data, and drafted the manuscript. RG and JS acquired and analyzed data. AJN, LAF, and MLD each contributed samples for data analyses and critically revised the manuscript for intellectual content. HLR contributed to the conception and design of the project, contributed to the statistical analysis and interpretation of data, and critically revised the manuscript for intellectual content. HLR and BTP take full responsibility for the integrity of the work as a whole, from inception to finished article. All authors read and approved the final manuscript.

Introduction

Acute joint injury, including soft tissue destabilization and articular surface injury, increases the risk of developing osteoarthritis by 10 to 20-fold, respectively[1]. In early post-traumatic osteoarthritis (PTOA), varying degrees of articular cartilage damage can be apparent as a result of the initial trauma, with progression of cartilage degradation over time secondary to altered biomechanics, impaired joint lubrication, and up-regulation of inflammatory mediators[2,3]. The principal lubricating molecules in synovial fluid, lubricin and hyaluronan, are essential for mitigation of the effects of inflammatory cytokines, maintaining low-friction joint lubrication, and protecting articular cartilage from degeneration[4]. Lubricin functions as the primary boundary lubricant, while hyaluronic acid confers viscoelastic properties to synovial fluid[5].

The autosomal recessive disease camptodactyly-arthropathy-coxa vara-pericarditis (CACP) in humans is characterized by a genetic mutation within the PRG4 gene leading to a deficiency in lubricin[6]. Studies utilizing lubricin-deficient synovial fluid from CACP patients and lubricin knock-out mice have shown inferior boundary lubricating abilities and a propensity to develop osteoarthritic changes at an early age, respectively[7,8]. Additionally, a subset of people with naturally-occurring osteoarthritis (OA) of the knee have been identified as having lubricin-deficient synovial fluid and similarly have decreased cartilage lubricating ability[9]. Further, losses of HA, particularly high molecular weight (MW) HA, have been associated with increased friction coefficients in mixed or viscoelastic lubrication regimes[10,11], and elevated friction due to loss of synovial fluid lubricants is thought to contribute to increased cartilage wear and joint degeneration[12].

Loss of synovial fluid lubricin has been detected in human knee OA associated with anterior cruciate ligament injury[13], knee destabilization models in the rat[14,15], and in a subset of people with chronic knee OA[9]. However, recent work has identified increased lubricin concentrations secondary to osteochondral fragmentation in the equine carpus[16,17], naturally-occurring acute and chronic joint injury in the equine carpal and metacarpophalangeal joints[10], and following repair of experimentally-induced cartilage defects in the femoropatellar joint of the horse[18]. Lubricin synovial fluid concentrations are also elevated in humans following intra-articular tibial fracture[19] and in advanced knee OA[20]. To date, it remains unclear whether these variations in lubricin activity are unique to specific joints or are predominantly a function of the underlying injury mechanism.

A recent study evaluating a variety of equine joints identified variations in gene expression, cell density, and response to joint trauma in articular cartilage, suggesting that inherent differences between joints may be significant factors in the development and progression of OA[21]. In humans, it is well documented that the rate of occurrence and type of OA varies significantly between joints, with a higher incidence of OA in the knee, hand, and hip compared to the ankle, wrist, elbow, and shoulder[22]. Biomechanical differences exist between the various joint types, and complex joints, such as the human knee or equine stifle, may be significantly influenced by peri- and intra-articular soft tissue structures. These biomechanical variations significantly affect the loading and shear forces exerted on the

articular structures and likely play a role in the development of OA and specific pathology commonly noted in each joint type[23].

Given this knowledge, it would be of value to investigate both healthy and OA synovial fluid obtained from biomechanically distinct human joints; however, human studies are often restricted to data from end-stage OA joints, thus minimizing the amount of data about disease progression and intervention that can be obtained[24]. Obtaining healthy tissues and fluids from age-matched controls presents an additional difficulty in human studies. Thus, a multitude of translational animal OA models have been developed in both small and large animal species. Given the similarities in joint biomechanics and cellular and biochemical composition, equine models are suggested to most closely represent the disease process in humans[24,25]. Horses are also similar to humans in that the prevalence and joint specificity of OA varies based on athletic use. As OA significantly affects quality of life and is a source of wastage in the equine industry[26], utilization of equine models offers a dual benefit for both species.

The overarching goal of this study was to determine if elevations in lubricin are specific to intra-articular fracture or fragmentation-type joint injuries. In addition, we investigated whether lubricin, HA, and HA MW distributions varied between equine joints and type of joint trauma. Synovial fluid was evaluated in three distinct equine joint injury models: carpal osteochondral fragmentation, talar cartilage impact injury, and full-thickness femoral lateral trochlear ridge cartilage defects. In order to draw meaningful conclusions from data obtained from different joints and injury models, we also aimed to establish normal ranges for and identify associations between lubricin, HA, and HA MW distributions in healthy, high-motion equine joints (tarsocrural, antebrachiocarpal, middle carpal, metacarpo/metatarso-phalangeal and femorotibial).

Methods

Samples

Synovial fluid samples were obtained from four different equine cohorts: (Cohort 1) horses donated for research purposes unrelated to clinical signs of joint disease (n=11), and research animals with joint-injuries induced in the (Cohort 2) carpus (n=8), (Cohort 3) tarsus (n=7), or (Cohort 4) stifle (n=22). The Cornell University Institutional Animal Care and Use Committee approved all experimental protocols, briefly described below, prior to the onset of each original study.

Multi-joint synovial fluid harvest (Cohort 1)

Synovial fluid was obtained via needle arthrocentesis immediately post-euthanasia from 11 horses, ranging in age from 1–15 years old (mean 8.7 years) that were euthanized for reasons unrelated to this study. The cohort consisted of castrated males (n=8) and intact females (n=3), and was predominantly composed of Thoroughbreds (n=9 Thoroughbreds; n=1 Standardbred; n=1 Quarter Horse). The horses were sound at the walk with no clinical evidence of osteoarthritis. High-motion joints, including the tarsocrural (n=22), antebrachiocarpal (n=17), middle carpal (n=18), metacarpophalangeal (n=18),

metatarsophalangeal (n=18), and femorotibial (n=9) joints were sampled via direct aspiration of synovial fluid immediately after euthanasia, resulting in a total of 102 joint samples. Centesis of all joints of interest was attempted in all horses. Maximal synovial fluid aspiration was performed in each joint and, when possible, joints were sampled bilaterally. After collection, synovial fluid was centrifuged at 4,000 × g for 60 minutes to remove cellular debris. The supernatant was retained, and the synovial fluid was frozen in aliquots of 1.5 mL at −80°C until sufficient samples were obtained for evaluation. Synovial fluid total protein (TP) was measured using a refractometer.

Osteochondral fragmentation (Cohort 2)

Synovial fluid samples from the middle carpal joints of eight young adult (2–6 years old) Thoroughbred horses (n=3 castrated males; n=5 intact females) were obtained. Horses were sound, based on AAEP subjective lameness assessment by an equine veterinarian, and were confirmed to have no radiographic evidence of carpal OA prior to study enrollment in the osteochondral fragment-treadmill exercise model of PTOA[16]. Briefly, each horse's forelimbs were randomly assigned to either sham operation (arthroscopic evaluation only) or osteochondral fragmentation in which an 8mm osteochondral fragment was arthroscopically created on the distal dorsal margin of the radial carpal bone. Horses were exercised on a treadmill (30 minutes/day; 5 days/week) starting 2 weeks after osteochondral fragmentation until cessation of the study. Serial synovial fluid samples were obtained throughout the study (days 0, 7, 14, 21, 28, 35, 42, 49, 56, 63, and 70). Post-mortem examination of the injured joints was performed for OA induction assessment.

Cartilage impact injury (Cohort 3)

A cohort of 7 young adult horses (2–5 years old; n=5 castrated males; n=2 intact females) of various breeds (n=4 Thoroughbreds, n=1 Quarter Horse, n=1 Standardbred, and n=1 Mixed breed) underwent cartilage impact injury to the medial trochlear ridge of the talus by use of a spring-loaded impacting device[27]. Horses were sound, based on AAEP subjective assessment by an equine veterinarian, and had no radiographic evidence of tarsal OA prior to inclusion in the study. Synovial fluid was sampled from the impacted tarsocrural joint on days 0, 4, 7, 14, 43, 71, and 168 post-arthroscopy. Contralateral limb sham-operation was not performed in this group of horses. Synovial fluid total protein was reported as < 2.5 g/dl or as a discrete number if the value was greater than 2.5 g/dl. Post-mortem examination of the injured joints was performed for OA induction assessment.

Full thickness cartilage defect (Cohort 4)

Synovial fluid samples were obtained from a cohort of 22 young adult (2–6 years old; n=5 castrated males; n=17 intact females) research horses of various breeds (n=14 Quarter Horses; n=3 Paint Horses; n=2 Appaloosas; n=1 Tennessee Walking Horse; n=1 Saddlebred; and n=1 Paso Fino) enrolled in a cartilage repair study[28]. Horses were sound, based on AAEP subjective assessment by an equine veterinarian, and had no radiographic evidence of stifle OA prior to inclusion in the study. Briefly, two 15mm diameter full-thickness femoral lateral trochlear ridge cartilage defects were created in one randomly assigned femoropatellar joint in each horse. Synovial fluid samples were obtained on day 0, 84 days after creation of the cartilage defect via a second-look arthroscopic surgery, and 397 days

post-injury at necropsy. Synovial fluid total protein was reported as < 2.5 g/dl or as a discrete number if the value was greater than 2.5 g/dl. Contralateral limb sham-operation was not performed in this group of horses. Post-mortem examination of the injured joints was performed for OA induction assessment.

Biochemistry

Lubricin characterization—Lubricin was quantified as previously described using a sandwich ELISA with peanut agglutinin (PNA) as the capture reagent (L0881; Sigma-Aldrich, St. Louis, MO) and monoclonal antibody 9G3 (MABT401; EMD Millipore, Darmstadt, Germany) as the detection antibody, with purified equine synovial fluid lubricin as the standard[16]. Equine synovial fluid samples from all horses at all time points were diluted 1:1,000 in phosphate buffered saline (PBS), and samples were measured in duplicate on high binding 96-well plates (Costar #3590; Corning Inc., Corning, NY). Absorbance was measured at 450nm with wavelength correction set at 540nm. To evaluate ELISA specificity for high molecular weight lubricin rather than degradation products, select samples were assessed for size relative to full-length, purified equine synovial fluid lubricin. Following electrophoresis on 3–8% NuPAGE tris-acetate gels (Life Technologies, Carlsbad, CA) and transfer to nitrocellulose, Western and lectin blotting with mAb 9G3, polyclonal antibody PA3–118 (Thermo Fisher Scientific, Waltham, MA), and biotinylated PNA (BA-0074; Vector Laboratories, Burlingame, CA) was performed (Supplemental Figure 1).

Hyaluronan characterization—A commercially available sandwich ELISA (Hyaluronan DuoSet ELISA, R&D Systems, Minneapolis, USA) was used to quantify HA concentration in synovial fluid. This assay utilizes recombinant human aggrecan as the HA capture reagent and biotinylated recombinant human aggrecan to detect bound HA using streptavidin conjugated to horseradish peroxidase. Synovial fluid samples from all horses at all time points were diluted 1:80,000 in 5% Tween 20 in PBS and measured in duplicate. Absorbance was measured at 450nm with wavelength correction set at 540nm. HA MW distribution was determined by gel electrophoresis as previously described[29]. Briefly, synovial fluid samples were diluted (1:20 in PBS) and treated overnight with proteinase K (Roche Applied Science, Mannheim, Germany) prior to electrophoresis (50V for 8 hours) on a 1% agarose gel. HiLadder (0.5–1.5 MDa) and Mega-HA Ladder (1.5–6.1 MDa) (Amsbio LLC, Cambridge, MA) were used for MW reference. Gels were stained with 0.005% Stains-All (Sigma-Aldrich, St. Louis, MO) in 50% ethanol, then de-stained with a 10% ethanol solution prior to image acquisition (Bio-Rad VersaDoc Imaging System, Hercules, CA). Where necessary, gel images were combined using the Stitching plugin[30] prior to quantification of band intensity with ImageJ software[31].

Statistical analysis

Multi-joint samples—Student's t-tests were used to confirm the absence of significant differences between left and right limbs. Samples were then grouped by joint for the remaining analyses. Shapiro-Wilk goodness-of-fit tests were performed to assess the data for normality. As HA and lubricin were not normally distributed, log transformations were performed to satisfy regression assumptions. Linear, mixed effect models were used to evaluate differences in lubricin, HA, and HA MW distribution between joints utilizing joint,

age, sex, and total protein as fixed effects. Random effects included the horse and each individual joint nested within the respective horse to account for the lack of independence between these factors. Post hoc Tukey's HSD comparisons were performed to further evaluate significant differences between joints.

Experimental OA samples—Shapiro-Wilk goodness-of-fit tests were performed to confirm normal distribution of the data. To evaluate differences in lubricin, HA, and HA MW distribution between joints and to assess the influence of joint and post-injury sampling time on synovial fluid parameters while controlling for the hierarchical nature of the data, linear, mixed effect models were used. Post hoc Dunnett's tests were performed to further evaluate significant differences between sampling time-points, with the baseline (time 0) sample used as the control comparison. All statistical analyses were performed using JMP Pro 14 (SAS Institute Inc., Cary, NC), and significance was set at $p<0.05$.

Results

Multi-joint comparison

A total of 102 joints from 11 horses (Table S-1) were analyzed for between-joint comparisons (Table 1). Lubricin, HA, HA MW proportions, and total protein were similar between left and right limbs for each respective joint (Figure 1). Lubricin concentrations did not vary significantly across the sampled joints ($p=0.13$; Table 2). However, HA concentrations did vary between joints ($p=0.002$), with the highest HA concentration in the metatarsophalangeal joint and the lowest HA concentration in the tarsocrural joint (Figure 2). The relative proportion of high molecular weight HA MW > 61 MDa was increased in the medial femorotibial joint compared to the antebrachiocarpal joint ($p=0.3$), middle carpal joint ($p=0.001$), and the metacarpophalangeal joint ($p=0.01$). For the lowest HA MW category 1.5 – 3.1 MDa, the relative proportion was lower in the medial femorotibial joint compared to the middle carpal joint ($p=0.01$) and the metacarpophalangeal joint ($p=0.02$). Relative proportions of HA MW did not vary by joint for the MW categories 3.1 – 6.1 MDa or 0.1 – 1.5 MDa. Both total protein ($p=0.01$) and age ($p<0.0001$) were significant predictors for HA concentration but not for lubricin concentration (Table 2).

Experimental OA Samples

Lubricin characterization—The intra-assay CV was 9.6%. Lubricin concentrations increased post-injury in synovial fluid samples from all joint injury models (Table 3). In the osteochondral fragment model, middle carpal joint injury, middle carpal joint synovial fluid lubricin concentrations were increased from pre-injury levels on day 7 ($p=0.005$), day 14 ($p=0.04$), and day 21 ($p=0.03$) (Figure 3). In the cartilage impact model, tarsocrural joint synovial fluid lubricin concentrations varied by day ($p<0.0001$) and were elevated from pre-injury levels at day 4 ($p=0.0006$), 7 ($p<0.0001$), and 14 ($p<0.0001$) post-injury. Horses with full-thickness femoral cartilage defects had elevated lubricin concentrations at the second time point (day 84, $p=0.04$) as compared to the pre-injury sample obtained prior to creation of the full-thickness cartilage defect (day 0). Day 84 samples also showed a trend for elevated lubricin compared to samples obtained at day 397 ($p=0.06$). Synovial fluid lubricin immunoblots revealed increased signal intensity for both mucin-domain reagents (mAb 9G3

and PNA) following joint injury, paralleling the sandwich ELISA results (Figure S-1A,D; B,E). In addition to the high MW lubricin band, the C-terminal antibody immunoblot revealed a strong signal at ~ 220 kDa which was also increased post-injury (Figure S-1C,F). These results suggest that there is a lower, ~ 220 kDa band, which may represent a non- or under-glycosylated variant of lubricin or a non-O-glycosylated lubricin cleavage product.

Hyaluronan characterization—The intra-assay CV was 7.1%. HA concentrations decreased post-injury in both the carpal fragmentation ($p<0.0001$) and talar impact ($p<0.0001$) models (Table 3). HA concentrations in middle carpal joint synovial fluid were significantly decreased following intra-articular fracture on days 7 ($p=0.002$), 14 ($p=0.02$), and 21 ($p=0.04$) post-injury (Figure 3). By the final sampling time point (day 70), HA concentrations had rebounded and were higher than pre-injury values ($p=0.02$). HA concentrations remained significantly decreased from day 4 to 168 days post-injury in the talar impact group. Altered HA concentrations were not detected in full-thickness cartilage defect synovial fluid samples on days 84 ($p=0.89$) and 397 ($p=0.88$) post-injury.

Alterations in relative proportions of HA MW were not detected at any day across HA MW categories in the talar cartilage impact model (> 6.1 MDa, $p=0.19$; 3.1 – 6.1 MDa, $p=0.67$; 1.5 – 3.1 MDa, $p=0.96$; 0.5 – 1.5 MDa, $p=0.7$) or the femoral full-thickness cartilage defect model (> 6.1 MDa, $p=0.34$; 3.1 – 6.1 MDa, $p=0.25$; 1.5 – 3.1 MDa, $p=0.66$; 0.5 – 1.5 MDa, $p=0.19$). HA MW data was not available for samples from the carpal osteochondral fragment model due to limited remaining synovial fluid volumes.

Discussion

Synovial fluid lubricin increased in all equine injury models, confirming that elevations in lubricin are not specific to intra-articular fracture. Rather, increased synovial fluid lubricin concentration appears to be a conserved response to various types of injury, including osteochondral fragmentation and two distinct models of cartilage injury across three different high-motion joints. Conversely, hyaluronan was decreased post-arthroscopy in both osteochondral fragmentation and cartilage impact injury models. The disparate responses between lubricin and HA are supported by the lack of correlation between the two lubricating molecules in this study and suggest that the mechanisms that regulate lubricin and HA synovial fluid homeostasis are distinct from one another. While the precise relationship between HA and lubricin has not been fully elucidated, it is well accepted that HA and lubricin primarily function under different loading circumstances in the joint where HA is essential for lubrication under high motion and low-load circumstances, and lubricin primarily enables low-friction lubrication while joints are under high load. Additionally, a synergistic relationship between lubricin and HA has been suggested[4], and it is possible that lubricin may be elevated secondarily to HA losses to restore synovial fluid lubricating ability in joint injury.

The mechanisms responsible for increased synovial fluid lubricin following joint injury are not fully understood. A prior study revealed increased PRG4 synovial membrane gene expression in horses with naturally occurring carpal osteoarthritis, suggesting that lubricin upregulation may be a protective response to joint injury[16]. Another possibility is that the

increased lubricin detected in synovial fluid may be lubricin that is degraded, sheared off the articular cartilage surface, or otherwise non-functional; however, synovial fluid lubricating function was not impaired in horses with elevated lubricin following experimental carpal fragmentation[17]. Data suggests that synovial fluid lubricin concentrations may correlate with measures of joint instability, such as anterior-posterior laxity in humans with knee injury[32], and *in vitro* studies show that both compression and increased dynamic shear stress can increase lubricin production[33–35]. Therefore, increased lubricin could be secondary to altered mechanical loading or a result of inflammation induced by joint trauma.

Based on our evaluation of healthy equine joints, lubricin concentrations were conserved amongst the sampled high-motion joints. In healthy joints, neither age, joint, nor synovial fluid total protein were predictive of lubricin concentrations. Elevated lubricin expression has been previously associated with fibrillated cartilage and cartilaginous deposits in subchondral bone adjacent to regions of exposed articular bone in human OA[36], suggesting the possible involvement of lubricin in the reparative process of damaged joints[10,16]. Further investigation of lubricin in healthy joints and those with subclinical joint disease is needed to determine the utility of lubricin as a sensitive biomarker for early cartilage injury or subclinical joint disease. The absence of variation as a function of age or joint might represent an advantage of synovial fluid lubricin as a potential biomarker for joint disease as compared to HA.

Marked losses of HA were noted in both the osteochondral fragmentation and cartilage impact models in the immediate postoperative period. This dramatic and acute response may be related to rapid degradation of HA secondary to acute joint injury[18]. However, arthroscopic lavage alone is not benign, and mechanical washout of HA may instead be the major factor underpinning this finding in the early postoperative period[37–39]. While HA concentrations after osteochondral fragmentation increased back to pre-injury levels within the study timeframe (<70 days), HA losses were still evident 168 days post-injury in the cartilage impact model. In our multi-joint comparison, synovial fluid from healthy tarsocrural joints had the lowest concentrations of HA amongst all sampled high-motion joints, demonstrating an inherent variation between joints, which could explain the disparity in HA rebound timeframes. This variation in response could also be a specific finding secondary to the focal cartilage impact injury inducing a more sustained loss of HA.

In contrast to the osteochondral fragmentation and cartilage impact injury models, HA depletion was not detected following the creation of full-thickness cartilage defects in the femoropatellar joint. However, the first sampling time point (day 84) was nearly 3 months after the initial joint injury. As prior studies have shown normalization of HA concentrations 3 months after experimental cartilage injury and repair in the equine stifle[18] and no disparity between HA concentration in healthy joints and those with naturally-occurring OA greater than 3 weeks duration[10], the timing of synovial fluid collection in this particular model limits our ability to draw significant conclusions about HA activity following creation of cartilage defects. Additional studies with earlier and more frequent sampling would be useful to further investigate this.

Variations in relative proportions of HA MW were noted for the MW categories of >6.1 MDa and 1.5 – 3.1 MDa, but only in relation to medial femorotibial joints. As the medial femorotibial joint has a smaller sample size as compared to other joints, it is unclear whether this relationship would hold true with increased sample sizes. Additionally, given the weak correlations between relative proportions of HA MW and HA concentration, these differences could also be related to the relative insensitivity of gel electrophoresis to detect lower MW HA. In humans with OA or rheumatoid arthritis, HA molecules can be degraded or cleaved early in production such that there are increased relative concentrations of low MW HA[40]. The biologic activity of low MW HA is under question; however, there is a large body of evidence to suggest that hyaluronan fragments induce inflammatory cytokines, further perpetuating the cycle of joint inflammation post-injury[41,42]. More sensitive methods for detection of HA size are being evaluated[43] and may provide additional insight into HA MW distribution following various types of joint injury.

In this study, banked synovial fluid samples obtained from three distinct joint injury models were evaluated to determine whether lubricin was increased, decreased or unchanged from baseline values prior to induction of injury. Each joint injury model involved repeated arthrocentesis through the respective study time periods to monitor serial alterations in lubricin and hyaluronan. While frequent arthrocentesis alone can induce an inflammatory response, sampling without administration of any concurrent medications at the relative frequency of ≥ 1 week performed here has not been shown to induce clinically significant synovial fluid alterations[44] and is consistent with recommendations of spacing serial arthrocenteses ≥ 1 week apart to avoid this as a confounding factor[45]. A limitation of the study is that all injury models were not performed concurrently, and banked synovial fluid samples were stored at −80°C for different durations of time. However, all synovial fluid samples were similarly obtained via direct aspiration, processed to remove cellular debris, and stored at −80°C until processing. In addition, horses in each joint injury model were confirmed to be free of clinical joint disease based on initial subjective lameness exam and radiographs of the joint of interest. Evidence of induced joint disease was confirmed on post-mortem examination of the injured joint and the contralateral joint[16, 27, 28]. Joint samples collected from horses for comparison of high-motion joints were evaluated, and the average lubricin concentrations (64.9 ± 4.18 ug/ml) and the average HA concentrations (0.47 ± 0.03 mg/ml) were within published ranges for healthy equine joints[16,46].

An additional limitation of this study is that the lubricin sandwich ELISA is not able to discern whether the lubricin detected is intact, as both mAb 9G3 and PNA react against epitopes within the mucin-rich domain of lubricin[47,48]. While this is a limitation of several prior studies, it would be ideal to be able to detect both the N- and C-termini of lubricin using a sandwich ELISA. Unfortunately, we are unaware of any N-terminal antibodies that cross-react with equine lubricin. In order to address this potential limitation, we performed immunoblotting using mAb 9G3, PNA and a C-terminal antibody, PA3–118. Given that the C-terminal antibody immunoblot revealed an ~ 220 kDa band that did not correspond to any reactivity with the mucin-domain reagents, it is possible that this lower MW band could represent a non- or under-glycosylated variant of lubricin or a non-O-glycosylated lubricin cleavage product. However, we can conclude, based on the absence of any mAb 9G3 or PNA

signal at this ~ 220 kDa band, that this is not a cleavage product that was being detected with our lubricin sandwich ELISA.

The response to joint trauma is influenced by a combination of factors, including the type of injury, the anatomy of the joint, and the biomechanical forces acting upon that joint. The three joints evaluated in this study are all considered high-motion joints, but do vary anatomically and biomechanically. The equine femoropatellar joint is structurally similar to humans in that the patella lies in the intertrochlear groove and glides between the lateral and medial trochlear ridges during flexion and extension of the knee. The equine tarsocrural joint has been described as having primarily helical motion or screw action during flexion and extension, with the distal aspect of the tibia rotating around the talus[49]. While the femoropatellar and tarsocrural joints share biomechanical similarities, the femoropatellar joint is a non-weight bearing joint and the tarsocrural joint is a weight-bearing joint. The middle carpal joint is a weight-bearing joint and functions in a hinge fashion with only approximately 45 degrees of articulation[50]. The variations in the underlying biomechanics of the joints evaluated here may have had some impact on the response to joint trauma; however, the increased lubricin concentrations observed in response to joint injury were conserved across all joints.

This study evaluated serial concentrations of lubricin, HA, and relative HA MW distributions across three distinct equine joint injury models. Despite conflicting literature about lubricin in the PTOA joint, here we demonstrate that synovial fluid lubricin is increased from baseline pre-injury values in three distinct models of equine PTOA, including intra-articular fracture, cartilage impact injury, and full-thickness cartilage defects. Increases in synovial fluid lubricin in response to PTOA spanned three separate high-motion joints, including the carpus (wrist), tarsus (ankle) and femoropatellar joint (knee). Whereas lubricin concentrations were increased in all three models, synovial fluid HA was decreased in both intra-articular fracture and cartilage impact injury. Lubricin may be elevated post-injury to protect joints from inflammation and to maintain joint lubrication by compensating for loss of HA[17]. Further investigation into the mechanisms regulating synovial fluid lubricin concentrations following joint injury is warranted, including whether lubricin has potential value as a biomarker for PTOA.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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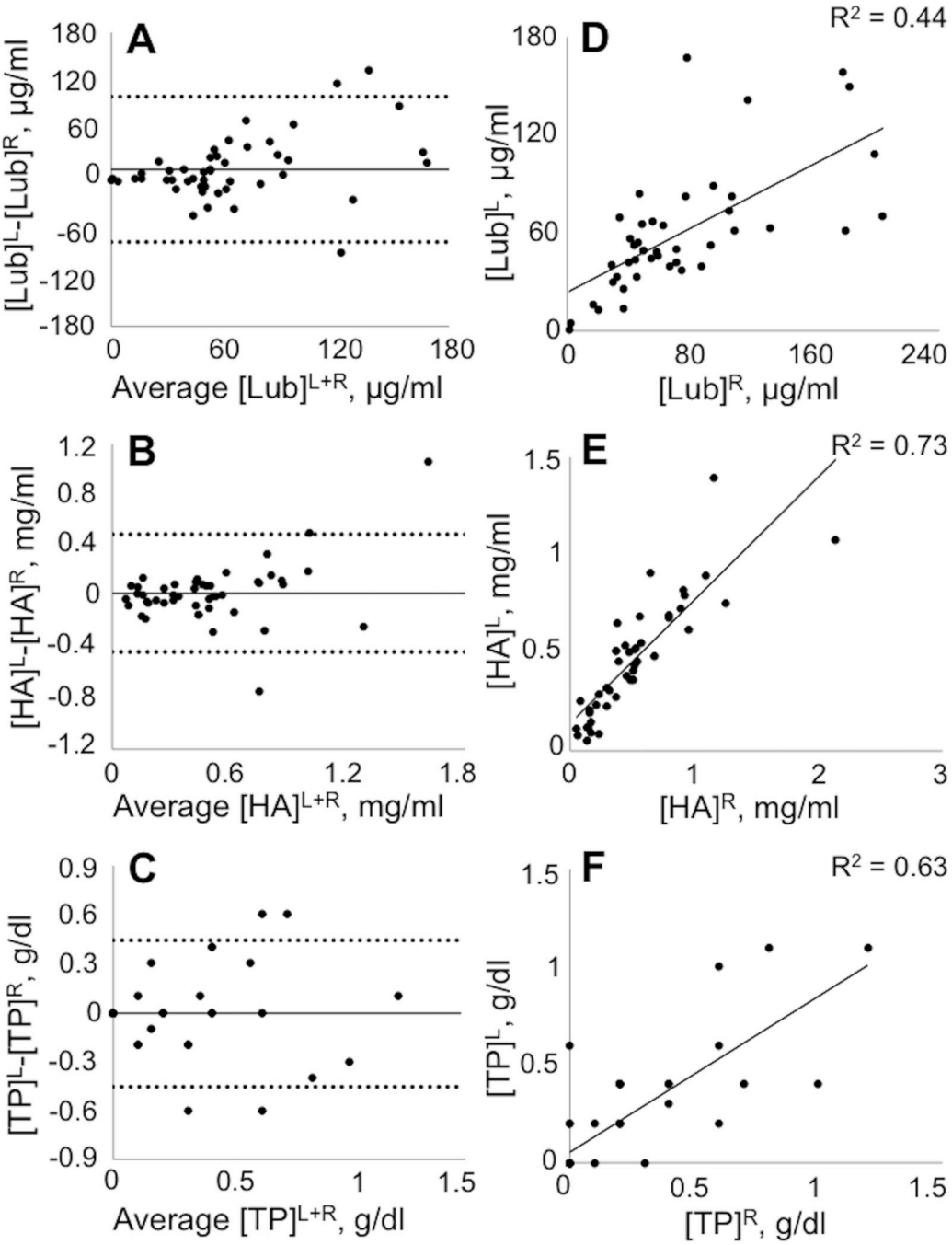

Figure 1.

Graphical representation of similarity of synovial fluid parameters between left versus right healthy, high-motion equine joints. Bland-Altman plots of individual differences between left and right joints for lubricin (Lub) (**A**), hyaluronan (HA) (**B**), and total protein (TP) (**C**) concentrations plotted against their mean. The solid line represents the mean difference, and the dashed lines represent ±2 standard deviations of the individual difference. Data for left (*y axis*) and right (*x axis*) joints plotted for lubricin (**D**), hyaluronan (**E**), and total protein (**F**) concentrations. n = 54–59 joints.

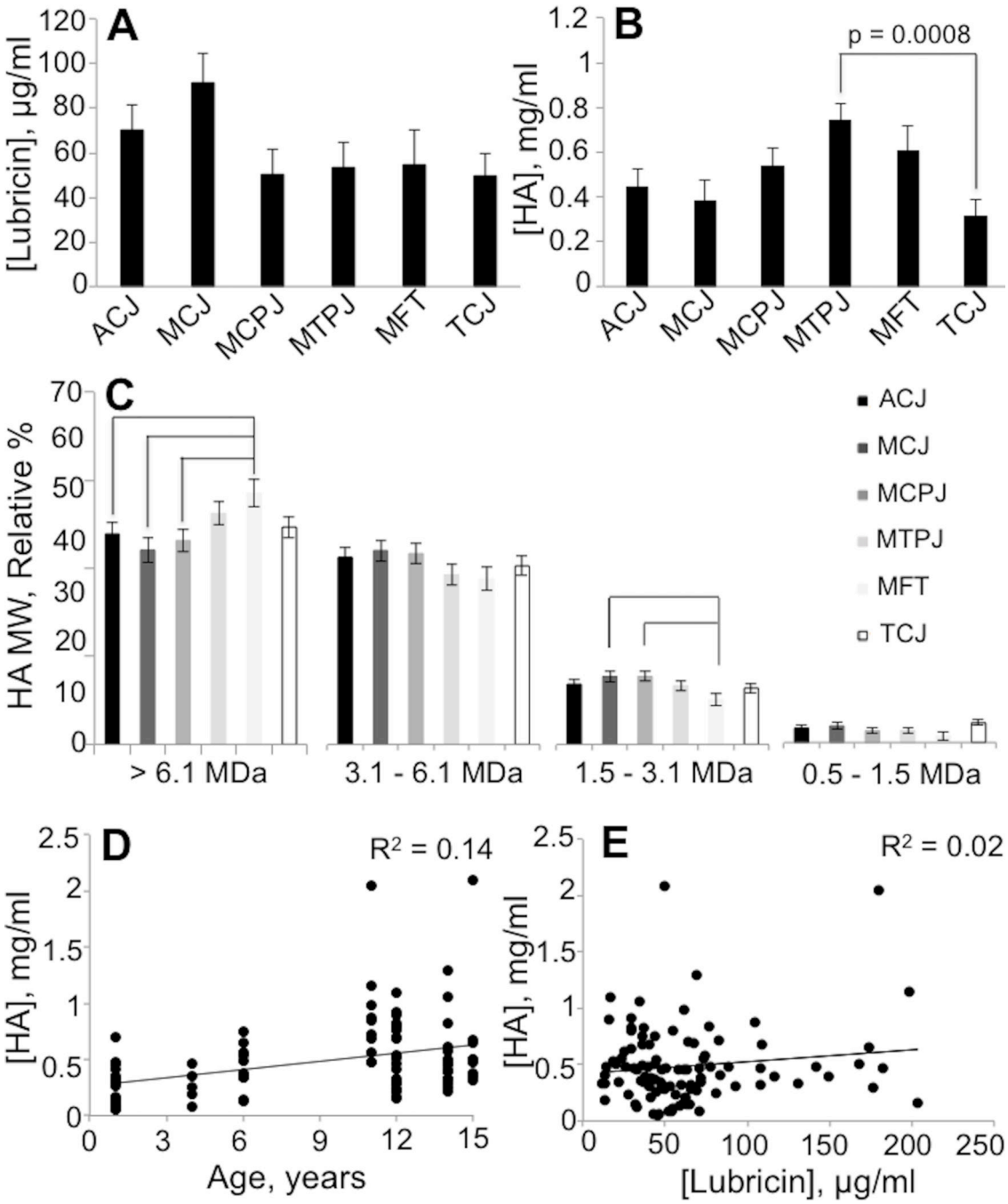

Figure 2.

Graphical representation of similarity of lubricin concentration (**A**), hyaluronan concentration (**B**), and relative proportions of hyaluronan molecular weight (**C**) in synovial fluid from various healthy, high-motion equine joints. Least square means ± standard error of the mean are plotted. Lines indicate a significant difference between joints. Scatterplots of hyaluronan versus age (**D**) and lubricin (**E**).

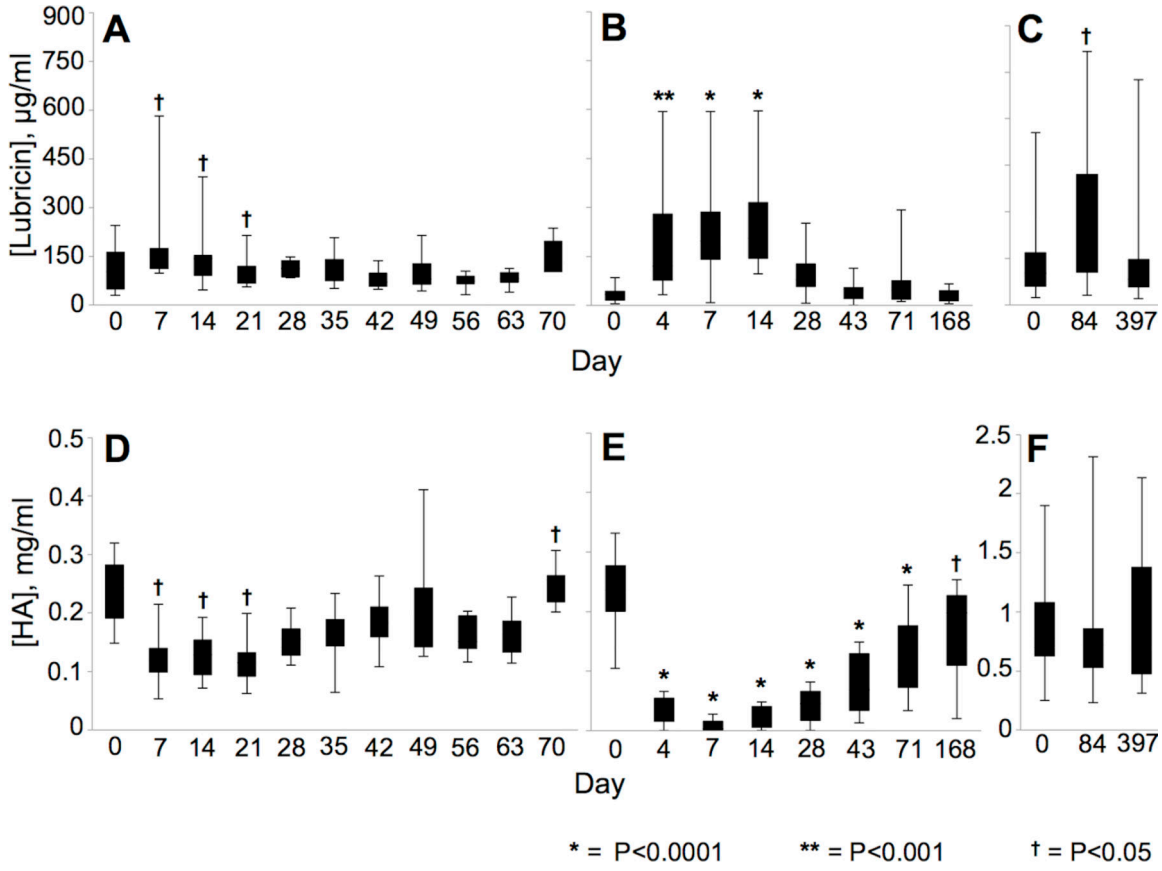

Figure 3.

Effect of time post-surgery on lubricin and hyaluronan concentrations in each post-traumatic osteoarthritis model. Observed means ± standard error of the means are plotted. Symbols (***** = P<0.0001; ****** = P<0.001; † = P<0.05) indicate significant differences from pre-surgery (day 0). Lubricin concentrations increased following carpal osteochondral fragmentation (**A**), talar cartilage impact (**B**), and trochlear ridge full-thickness cartilage defect creation (**C**). Hyaluronan depletion is shown following carpal osteochondral fragmentation (**D**), and talar cartilage impact (**E**); however, loss of hyaluronan was not detected following creation of full-thickness cartilage defects in the trochlear ridge (**F**).

Table 1.

Synovial fluid lubricin concentrations, hyaluronic acid concentrations, and relative percentages of hyaluronic acid molecular weight categories in sampled high-motion equine joints. Data are presented as least square means ± standard error of the mean.

Joint	[Lubricin], μg/ml	[HA], mg/ml	>6.1 MDa	3.1 – 6.1 MDa	1.5 – 1.3 MDa	0.5 – 1.5 MDa	[TP], g/dl
ACJ (n=17)	72.2 ± 9.7	0.46 ± 0.06	48.3 ± 2.1	37.1 ± 1.4	11.8 ± 0.9	2.82 ± 0.6	0.24 ± 0.06
MCJ (n=18)	99.4 ± 13.5	0.48 ± 0.06	44.4 ± 2.1	38.7 ± 1.5	13.8 ± 0.7	3.14 ± 0.6	0.53 ± 0.05
MCPJ (n=18)	49.4 ± 8.8	0.50 ± 0.06	47.7 ± 1.9	36.8 ± 1.6	13.0 ± 0.7	2.53 ± 0.3	0.02 ± 0.05
MTPJ (n=18)	52.2 ± 9.9	0.72 ± 0.11	54.0 ± 1.5	32.3 ± 1.2	11.2 ± 0.5	2.6 ± 0.3	0.04 ± 0.05
MFT (n=9)	55.1 ± 8.1	0.59 ± 0.20	54.1 ± 1.8	34.7 ± 1.5	10.0 ± 0.7	1.13 ± 0.2	0.37 ± 0.08
TCJ (n=22)	48.2 ± 4.5	0.28 ± 0.03	50.4 ± 2.3	34.6 ± 2.1	10.9 ± 0.9	4.13 ± 0.8	0.11 ± 0.05

Table 2.

Linear, least square regression models for log-transformed synovial fluid parameters (lubricin and hyaluronic acid concentrations) in high-motion equine joints. Intercept omitted for clarity.

	Log [Lubricin] (n=102)			Log [HA] (n=102)		
Predictors	**Estimate**	**Sth. Error**	**P-value**	**Estimate**	**Std. Error**	**P-value**
Joint			0.13			< 0.01
ACJ	0.20	0.16		−0.10	0.15	
MCJ	0.45	0.19		−0.21	0.17	
MCPJ	−0.25	0.17		0.11	0.15	
MTPJ	−0.22	0.16		0.47	0.15	
MFT	−0.03	0.21		0.18	0.19	
Age (years)	0.01	0.01	0.61	0.06	0.01	< 0.01
Sex (female)	0.01	0.08	0.86	−0.01	0.07	0.90
TP (g/dl)	0.18	0.28	0.52	0.62	0.24	0.01
R^2 (Adj. R^2)	0.75 (0.73)				0.87 (0.87)	

Table 3.

Linear, least square regression models for synovial fluid parameters (lubricin and hyaluronic acid concentrations) in each post-traumatic osteoarthritis model: A. osteochondral fragmentation in the equine carpus; B. cartilage impact injury in the equine tarsus; C. full-thickness cartilage defect in the equine femoropatellar joint. Variations in samples size can be accounted for via exhaustion of banked synovial fluid samples at various time-points. Intercept omitted for clarity.

	A. Osteochondral fragmentation					
	Lubricin (n=160)			HA (n=168)		
Predictors	Estimate	Std. Error	P-value	Estimate	Std. Error	P-value
Day			0.001			<0.0001
0	−69.18	28.34		0.031	0.015	
7	75.66	26.09		−0.080	0.015	
14	50.04	27.40		−0.064	0.015	
21	44.87	24.29		−0.054	0.014	
28	30.89	24.31		−0.027	0.014	
35	−18.66	23.51		−0.004	0.014	
42	0.24	24.29		0.031	0.014	
49	−23.39	23.51		0.052	0.014	
56	−59.73	24.31		0.010	0.014	
63	−46.37	23.51		0.006	0.014	
R^2 (Adj. R^2)		0.52 (0.45)			0.68 (0.63)	
	B. Cartilage impact					
	Lubricin (n=106)			HA (n=108)		
Predictors	Estimate	Std. Error	P-value	Estimate	Std. Error	P-value
Day			<0.0001			<0.0001
0	−81.48	25.07		0.15	0.01	
4	75.69	25.58		−0.06	0.01	
7	104.84	26.56		−0.08	0.01	
14	112.59	29.97		−0.08	0.01	
28	−27.25	29.12		−0.05	0.01	
43	−72.43	27.28		−0.01	0.01	
71	−37.74	31.73		0.04	0.01	
TP (g/dl)	9.60	9.29	0.3	0.00	0.00	0.08
R^2 (Adj. R^2)		0.58 (0.51)			0.90 (0.88)	
	C. Cartilage defect					
	Lubricin (n=64)			HA (n=65)		
Predictors	Estimate	Std. Error	P-value	Estimate	Std. Error	P-value
Day			0.03			0.6

0	−41.44	26.49	0.00	0.07
84	74.90	26.90	−0.06	0.07
R^2 (Adj. R^2)		0.49 (0.48)		0.45 (0.43)