EVALUATION OF IMPACT INJURY AS A MODEL OF EXPERIMENTALLY INDUCED POST-TRAUMATIC OSTEOARTHRITIS IN THE EQUINE METACARPOPHALANGEAL JOINT

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ABSTRACT

EVALUATION OF IMPACT INJURY AS A MODEL OF EXPERIMENTALLY INDUCED POST-TRAUMATIC OSTEOARTHRITIS IN THE EQUINE METACARPOPHALANGEAL JOINT

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The purpose of this study was to develop a model of post-traumatic osteoarthritis in the palmar metacarpal condyle and to evaluate the timing of the early events following impact trauma on subchondral bone and articular cartilage.

In each of 12 skeletally mature horses, an impact injury was created on the palmar metacarpal condyle of one randomly chosen limb, under arthroscopic and fluoroscopic guidance. A low to moderate level of forced exercise was instituted; and horses were evaluated clinically via lameness examinations, synovial fluid analysis, and radiographs. Macroscopic examination, micro-computed tomography, and sample collection were performed following euthanasia at one month (3 horses), 4 months (4 horses), and 8-10 months (5 horses) after impact injury.

There was variability in impact-lesion location, depth, and area on macroscopic inspection; histologic evaluation revealed more consistent cartilage defects due to impact injury. Cartilage degeneration, in terms of color and clarity, was observed in impacted
joints. The mean sulfated glycosaminoglycan (sGAG) concentration from cartilage at the impact site was significantly lower than for a similar site in control limbs. Higher concentrations of cartilage oligomeric matrix protein (COMP) were observed in synovial fluid from impacted joints. Bone viability, as evaluated by the Alamar blue assay, was significantly decreased in impact specimens versus control specimens one month after impact injury.

This impact injury model caused lesions consistent with mild focal osteoarthritis in the palmar metacarpophalangeal joint, involving cartilage more than subchondral bone. Further development is required to create a reliable and consistent model of naturally occurring post-traumatic osteoarthritis at this site.
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DECLARATION OF WORK PERFORMED

I declare that all work reported in this thesis was performed by Ellen Rickey, with the exception of selected assays (synovial fluid cytology, sGAG assay, COMP assay, histological and immunohistochemistry staining) performed by Michelle Beaudoin and by Mike Brown (India ink analysis), and statistical analysis performed by Gabrielle Monteith.
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1.0 – INTRODUCTION

Osteoarthritis is the most common cause of equine lameness, costing the horse industry close to $1 billion annually (US figures). One form of the disease, post-traumatic osteoarthritis in the palmar metacarpal condyle affects many Thoroughbred and Standardbred racehorses. Pathological changes in subchondral bone are thought to play an important role in the disease mechanism, but it is uncertain whether cartilage or subchondral bone injury is the inciting cause. In addition, little is currently known about early changes that occur before osteoarthritis becomes clinically detectable.

The purpose of this study was to evaluate the timing of the early events following impact trauma on subchondral bone and cartilage. A previously described impact model in our laboratory (Bolam, Hurtig, Cruz, et al 2006), which produced osteoarthritis in the equine stifle joint, was applied to the palmar aspect of the metacarpal condyle, a naturally occurring site of subchondral bone disease and osteoarthritis. Understanding the sequence of events, mechanisms, and interrelation of articular cartilage and subchondral bone damage in early arthritis will provide insight into its pathogenesis and will help to elucidate the timing of irreversible osteoarthritic changes. This knowledge should facilitate development of new methods for early diagnosis and treatment of this devastating disease.
1.1 – GOALS AND HYPOTHESIS

The ultimate goal of this research was to gain insight into the pathogenesis of early osteoarthritis by achieving a deeper understanding of the sequence of events, mechanisms, and interrelation of articular cartilage and subchondral bone. The purpose of this study was to develop a model of post-traumatic osteoarthritis in the palmar metacarpal condyle and to evaluate the timing of the early events following impact trauma on subchondral bone and cartilage. Our hypothesis was that impact trauma would lead to simultaneous degeneration of articular cartilage and subchondral bone remodeling events.
1.2 – LITERATURE REVIEW

1.2.1 – Osteoarthritis

Osteoarthritis is the most common cause of lameness in the horse (McIlwraith 1996). The definition of osteoarthritis is progressive, permanent deterioration of articular cartilage resulting in loss of function, and associated changes in the bone and soft tissues of the joint (McIlwraith 1996). Osteoarthritis can be associated with normal loads on abnormal cartilage or with abnormal loads on normal cartilage (Riggs 2006; Goldring & Goldring 2007). Articular cartilage damage can occur due to direct mechanical injury, locally produced catabolic enzymes, a change in the mechanical environment (e.g. subchondral bone sclerosis), chondrocyte injury/death, or altered matrix molecule synthesis (Radin & Rose 1986; McIlwraith 1996; Aigner, Kurz, Naoshi, et al 2002; Thibault, Poole & Buschmann 2002; Riggs 2006).

Extensive work has investigated the effects of this disease on articular cartilage. Many components of articular cartilage, including chondrocytes, extracellular matrix, and collagen, which are altered by osteoarthritis, have been studied. Chondrocyte cloning appears to be an attempt at localized tissue regeneration and repair (Goldring & Goldring 2007). Apoptosis of chondrocytes is increased in osteoarthritis, starting in the superficial zone and progressing to the deeper layers (Goldring & Goldring 2007); and this chondrocyte apoptosis has been correlated with histopathological evidence of extracellular matrix degeneration (Kim, Taylor, Moore, et al 2003). A well-documented characteristic of osteoarthritis is proteoglycan loss (Goldring & Goldring 2007), specifically a decrease in the glycosaminoglycan content of articular cartilage (van der
Harst, DeGroot, Kiers, *et al* 2005). Pro-inflammatory cytokines, such as interleukin 1 (IL-1) and tumor necrosis factor alpha (TNFα), initiate extracellular matrix degradation by inducing various proteinases, such as the matrix metalloproteinases, which degrade collagen and proteoglycans (McIlwraith 1996). Another well-documented characteristic of osteoarthritis is collagen type II cleavage (Goldring & Goldring 2007). The presence of type X collagen, a hypertrophic chondrocyte marker, indicates recapitulation of developmental processes (Goldring & Goldring 2007).

Additional changes within the articular cartilage also occur. Angiogenesis takes place in the osteochondral junction, at the tidemark in the zone of calcified cartilage; and correlates with cartilage changes as well as with the course of clinical disease (Goldring & Goldring 2007). Wear lines observed on macroscopic examination are consistent with death of chondrocytes, matrix proteoglycan loss, and superficial zone collagen fiber disruption (Pool 1996; Riggs 2006). In addition, synovial inflammation contributes to the pathogenesis of osteoarthritis by producing activated lymphocytes and proinflammatory mediators (Goldring & Goldring 2007). Traditionally osteoarthritis has been considered a disease of articular cartilage, but inflammation of the synovium and changes in the subchondral bone are also contributors (Goldring & Goldring 2007).

Articular cartilage is generally considered to be incapable of repair, but this is not quite true. If the injury is not overwhelming, the labile chondrocyte population does have some reproductive capability, which is described as intrinsic repair (McIlwraith 1996; Frisbie, Bowman, Colhoun, *et al* 2008; Bramlage 2009). Tissue repair and restoration of homeostasis can be achieved by cellular recovery and synthetic processes in some cases of mild injury (Riggs 2006). The capacity exists to create collagen and replace
proteoglycan, but the arching configuration of the collagen architecture, which is vitally important in anchoring the articular cartilage to underlying bone, cannot be recreated (Vachon, Bramlage, Gabel, *et al* 1986; Shamis, Bramlage, Gabel, *et al* 1989; Frisbie, Morisset, Ho, *et al* 2006; Bramlage 2009). Any injury to adult articular cartilage, other than very mild damage, can only be repaired by fibrocartilage, which does not have the same structural, biochemical, and functional characteristics as hyaline cartilage. The vital importance of intact articular cartilage is illustrated by the observation that joint dysfunction and disability are mainly caused by loss of articular cartilage (Riggs 2006), although subchondral bone damage plays an important role in the clinical manifestation of disease in the equine palmar metacarpophalangeal joint (Cruz & Hurtig 2008).

Much less is known about the role of the subchondral bone in osteoarthritis, which is a focus of the current study. In contrast to articular cartilage, bone has an excellent capacity for repair and for adaptation to specific load conditions. The normal response of subchondral bone to cyclic loading is appositional bone deposition and modeling/remodeling in bone density and strength (Pool 1996; Kawcak, McIlwraith, Norrdin, *et al* 2001). In osteoarthritic subchondral bone, modeling/remodeling is initiated by apoptosis of osteocytes due to damage to the canaliculi from microcracks, also known as microfissures, which continue into the calcified cartilage (Burr 2004). The resulting sclerosis causes the subchondral bone to become stiff and to partially lose its shock absorption ability, resulting in transfer of a greater proportion of ground reaction forces onto the articular cartilage (Pool 1996). The volume of subchondral bone increases in osteoarthritic joints, which is visible radiographically as sclerosis; however, this bone may be undermineralized, which decreases its stiffness (Burr 2004). This loss in stiffness
from undermineralization necessitates a corresponding increase in density (bone volume fraction) to maintain a normal level of overall bone stiffness (Burr 2004). Therefore, the affected bone may be more or less stiff than normal, depending upon the stage of osteoarthritis.

The connections between the pathophysiological events of osteoarthritis in articular cartilage and subchondral bone are still unknown. Both of these components play a role in the disease, but the timing of their involvement and the intricacies of their interactions remain to be determined. In fact, there is considerable debate about whether the initiating event of osteoarthritis occurs in articular cartilage or subchondral bone, although there is no doubt that both tissues become involved at some stage of the disease (Santschi 2008, Kawcak et al 2001). Under normal conditions, it appears that intact subchondral bone protects articular cartilage from damage by serving as a shock absorber and by anchoring the deep layers of cartilage, thus limiting the amount of cartilage deformation (Radin 1999; Lewis, Deloria, Oyen-Tiesma, et al 2003; Riggs 2006; Natoli, Scott & Athanasiou 2008). Although the primary function of cartilage is to provide a deformable, near frictionless surface, one study found that removal of articular cartilage increased the peak load by approximately 8%; indicating that articular cartilage is roughly 10 times more effective than an equivalent amount of subchondral bone in reducing peak loads received by bovine osteochondral specimens (Radin & Paul 1971). Despite this contribution of articular cartilage to the absorption of shock, subchondral bone provides the majority of shock absorption. Therefore, if there are changes in the material characteristics of subchondral bone due to disease, this can affect the strain placed on the overlying articular cartilage (Riggs 2006). The metabolic activity of
articular cartilage could also be affected by altered metabolic activity in subchondral bone (Riggs 2006). It has been suggested that the process of subchondral bone thickening has more influence on cartilage disease development than does the actual thickness of the bone (Riggs 2006). Although, models that simulate osteoarthritis by creating joint instability may initially result in articular cartilage changes, models using impulse loading initially cause subchondral bone damage (Kawcak et al 2001). It may be that the former is due to static strain, while the later is the result of dynamic strain, which causes changes in subchondral bone earlier in the disease course (Kawcak et al 2001). In addition, instability models lead to disuse osteoporosis and thus resorption of bone, while impact models stimulate formation of bone due to advancement of the tidemark (Radin, Martin, Burr, et al 1984; Riggs 2006). Therefore, a goal of the current study was to investigate the timing and interrelation of osteoarthritic changes in both articular cartilage and subchondral bone using an impulse loading model.

1.2.2 – Early Osteoarthritis

Although established osteoarthritis has been relatively well characterized, there is still considerable debate about the changes and their timing in the early stages of the disease. One study evaluated naturally occurring mild osteoarthritis in the metacarpophalangeal joints of 29 Warmblood horses (van der Harst et al 2005). The region adjacent to the proximodorsal articular margin of the first phalanx, which is intermittently loaded and is the first site to be affected by osteoarthritis, and the area at the central fovea of the first phalanx, which is continuously loaded and is the last site to be affected, were evaluated. There were subtle but statistically significant biochemical
differences in the articular cartilage, subchondral bone, and trabecular bone, with both the
dorsal and central sites being affected in the articular cartilage and subchondral bone, but
only the central region affected in the trabecular bone. Osteoarthritic changes involved
cartilage glycosaminoglycan and the collagen of the subchondral and trabecular bone,
with only minimal changes in mineral content (van der Harst et al 2005). In this study of
naturally occurring disease (van der Harst et al 2005), it was impossible to know the
exact timing of these changes.

The early events of osteoarthritis in bone and calcified cartilage include
subchondral bone sclerosis, death of osteocytes, and debris filling canaliculi (Santschi
2008). As the disease progresses, these events lead to loss of perfusion and osteocyte
communication, the significance of which is unknown at this time (Santschi 2008). Then
small microcracks form, become larger, and eventually branch to involve the calcified
cartilage (Santschi 2008). When the bone modeling and remodeling become
maladaptive, continued loading causes collapse of the bone and cartilage, with “traumatic
osteochondrosis” as the end result of the process (Santschi 2008).

1.2.3 – Post-Traumatic Osteoarthritis

Post-traumatic osteoarthritis develops due to joint injuries, such as ligamentous
injuries or intra-articular fractures, as opposed to idiopathic osteoarthritis, which occurs
with age (Borrelli & Ricci 2004; Buckwalter & Brown 2004). The causative trauma can
be a single episode or repetitive injury (Riggs 2006). In humans, post-traumatic
osteoarthritis affects young and middle-aged adults (Buckwalter & Brown 2004). The 3
theories for the etiology of post-traumatic osteoarthritis include direct impact damage,
articulart incongruity, and articular instability (Borrelli & Ricci 2004; McKinley, Rudert, Koos, et al 2004). The latter 2 etiologies are important clinically as considerations for intra articular fracture repair. Joint tissues are damaged more by impact loads than by similar magnitude loads applied at a slower rate of loading (Radin, Parker, Pugh, et al 1973; Riggs 2006). One study demonstrated collagen network weakening, increased hydraulic permeability, and increased denatured type II collagen due to cyclical loading at high physiological strain rates (Thibault et al 2002; Riggs 2006). The fluid pressure within the articular cartilage matrix can be increased to such an extent due to high strain rates from impact loads, that tensile failure of the collagen network by exceeding its restraining capacity results (Morel & Quinn 2004; Riggs 2006).

1.2.4 – Bone Response to Exercise

In order to discuss the etiology of post-traumatic osteoarthritis, it is important to understand the response of bone to load during exercise. Loading causes bone deformation, or strain, which results in modeling and remodeling, in a process currently known as high-strain cyclic fatigue (Nunamaker 1996; Davidson & Ross 2003). Basically, repetitive small injuries over time lead to accumulation of microdamage in bone (Santschi 2008), which is greater at high speed than at low speed (Stover 2003). Bone responds to the increased strain by increasing its inertial properties, which it accomplishes by modeling, the formation of new bone, and by remodeling, the repair of microdamage and replacement of biomechanically inferior bone (Davidson & Ross 2003). Removal of bone by osteoclasts occurs rapidly in days to 2 weeks, but in the horse, there can be uncoupling of bone resorption and formation. Therefore, bone
replacement may take months, which may result in a focal stress riser and long bone fragility (Stover 2003). Continuing high speed exercise results in accumulation of damage and the relatively constant rate of repair is not able to keep up (Stover 2003). When bone resorption exceeds bone replacement, it can lead to microfractures (Davidson & Ross 2003). The removal of bone at a speed greater than the capacity to replace bone is termed maladaptive bone disease (Davidson & Ross 2003). This incomplete remodeling response is often observed prior to a stress or fatigue fracture (Davidson & Ross 2003). Maladaptive bone disease is the beginning of a pathological continuum due to stress-related bone injury, which involves cortical and subchondral bone changes that cause lameness (Davidson & Ross 2003). Changes along this continuum include sclerosis, subchondral lucency, periosteal callus, incomplete (stress or fatigue) and complete fracture, and eventually development of osteoarthritis (Davidson & Ross 2003). However, when adaptive remodeling is successful, it improves bone’s ability to tolerate a specific level of exercise.

Dynamic loading within the physiological range causes a positive response by chondrocytes, including increased proteoglycan and collagen synthesis as well as an enhanced response to insulin-like growth factor I (Bonnasar, Grodzinsky, Frank, et al 2001; Riggs 2006). However, the chronic repetitive loading experienced by racehorses often exceeds this physiological range and the chondrocytes’ ability to respond is often affected by changes in the underlying subchondral bone. The articular cartilage overlying sclerotic subchondral bone responds by thinning, specifically in the calcified cartilage layer, becoming fragmented, and eventually collapsing into the subchondral bone to cause synovitis (Norrdin, Kawcak, Capwell, et al 1998; Davidson & Ross 2003).
In addition, this high-speed cyclic loading does not allow sufficient time for the lag phase of cartilage after loading, during which time the cartilage relaxes and redistributes matrix fluid (Mow & Hung 2003; Riggs 2006).

1.2.5 – Post-Traumatic Osteoarthritis in Horses

The horse is an ideal model for post-traumatic osteoarthritis because this condition occurs commonly in the palmar metacarpal condyle of athletic horses. In general, the greatest number of distinct traumatic and degenerative lesions of racehorse joints occurs in the metacarpophalangeal joint (Pool 1996). The fetlock joint’s comparatively small surface area, large range of motion, and responsibility for transferring the entire body weight to the ground during one phase of the gallop may cause this joint to be more vulnerable to injury (Pool 1996). The types of lesions observed clinically include traumatic synovitis of the dorsal joint capsule, remodeling and chip fractures of the proximodorsal joint margin of the first phalanx, supracondylar lysis of the palmar cortex of the distal cannon bone, “traumatic osteochondrosis” of the palmar/plantar surface of the condyle of the cannon bone, and transverse ridge arthrosis (Pool 1996). The area of interest in this study is traumatic osteochondrosis because it is one of the most common, but least understood, of these conditions.

Traumatic osteochondrosis, fetlock arthrosis, and palmar/plantar osteochondral disease are various terms used to describe post-traumatic osteoarthritis in this region (Pool 1996; Davidson & Ross 2003; Riggs 2006; Santschi 2008; Barr, Pinchbeck, Clegg, et al 2009; Bramlage 2009). All of these terms refer to a condition caused by repetitive high-speed loading experienced by racehorses. Cartilage damage, subchondral bone
sclerosis, and subchondral bone necrosis are observed in fetlock arthrosis, which is caused by chronic mechanical overload (Riggs, Whitehouse, Boyde, et al. 1999b; Stover 2003; Stepnik, Radtke, Scollay, et al. 2004; Norrdin & Stover 2006; Easton & Kawcak 2007; Santschi 2008). In a recent study, the prevalence of palmar osteochondral disease in Thoroughbred racehorses was 67%, but most of the lesions were not severe (Barr et al. 2009).

1.2.6 – Post-Traumatic Osteoarthritis in the Palmar Metacarpus

Subchondral bone changes in the palmar fetlock joint occur commonly in Thoroughbred and Standardbred racehorses and can result in sclerosis, “traumatic osteochondrosis”, or catastrophic fracture of the metacarpal condyle. The predilection site for these changes is the palmar aspect of the third metacarpal condyle (Norrdin & Stover 2006; Young, Samii, Mattoon, et al. 2007). “Traumatic osteochondrosis” causes 2 to 4 mm oval lesions, which are located 5 to 8 mm proximal to the transverse ridge and 3 to 15 mm medial or lateral to the sagittal ridge (Pool 1996). Macroscopic lesions in the palmar/plantar distal metacarpus/metatarsus range from mild, such as blue discoloration of the subchondral bone evident through the articular cartilage, to severe, such as osteochondral ulcers (Riggs 2006; Barr et al. 2009). Interestingly, the palmar/plantar subchondral bone is denser than the dorsal subchondral bone in normal racehorse fetlocks, due to the different load applied to these areas (Pool 1996). The medial metacarpal condyle is affected more often than the lateral condyle (Norrdin & Stover 2006; Young et al. 2007; Bramlage 2009), which is attributed to the horse’s eccentric center of gravity in comparison to the limb axis (Smith, Bertone, Weisbrode, et al. 2006).
1.2.7 – Clinical Signs

Clinical signs of stress-related bone injury can be subtle and inconclusive in early disease. Epidemiologically, affected Thoroughbreds were an average of 3.3 years old and affected Standardbreds were an average of 3.1 years old (Arthur & Constantinide 1995; Ehrlich, Dohoo & O’Callaghan 1999; Davidson & Ross 2003). Lameness is characteristically acute and severe after racing or training, and improves with rest, although signs of poor performance or intermittent lameness can linger for weeks to months (Davidson & Ross 2003). Lameness can vary from absent to severe, with the majority of horses displaying mild to moderate lameness (Davidson & Ross 2003; Ross 2003). Degree of lameness does not correlate well with macroscopic findings (Riggs 2006). On physical examination, findings such as swelling or pain on palpation are frequently minimal or not present (Davidson & Ross 2003). Response to flexion is often inconclusive (Davidson & Ross 2003). Early in the disease, joint effusion is rare due to minimal involvement of the overlying articular cartilage, but effusion can develop along with progression of cartilage damage (Arthur, Ross, Moloney, et al 2003; Davidson & Ross 2003; Pilsworth 2003; Richardson 2003; Ross 2003). In a study of Thoroughbred racehorses, lameness, a positive response to lower limb flexion, or obvious joint effusion was observed in 71% of affected horses (Martinelli, Chambers, Baker, et al 1994; Davidson & Ross 2003). It is important to regard this disease as a progression of clinical signs and pathological changes. For example, acute collapse of an osteonecrotic ulcer can cause severe lameness, joint pain, and obvious radiographic abnormalities (Riggs 2006).
1.2.8 – Clinical Diagnosis

Post-traumatic osteoarthritis of the fetlock joint is diagnosed clinically via diagnostic analgesia, radiographs, and nuclear scintigraphy. Intra-articular analgesia does not commonly resolve the lameness, especially early in the course of disease, but a response can often be seen to a low palmar/plantar nerve block or a low palmar/plantar metacarpal/metatarsal nerve block (Davidson & Ross 2003; Ross 2003). A complete radiographic study should include dorsal-30° proximal-45° “down-angled” dorsolateral palmaro/plantaromedial oblique and dorsomedial palmaro/plantarolateral oblique views to highlight the palmar/plantar aspect of the distal metacarpus/metatarsus without overlap from the proximal sesamoid bones or proximal phalanx; as well as either a flexed-dorsopalmar or a 125°-dorsodistal palmaroproximal view (Hornof & O’Brien 1980; Davidson & Ross 2003). In order for radiographic changes to be observable, there must be a 30-50% loss of bone or a lesion of 1-1.5 cm in diameter (O’Callaghan 1991; Davidson & Ross 2003). Therefore, lesions may take 2-3 weeks or more after injury to become radiographically evident (O’Callaghan 1991; Davidson & Ross 2003).

Nuclear scintigraphy is more sensitive and can identify lesions of subchondral bone injury earlier than radiographs (Davidson & Ross 2003; Richardson 2003). Physiologically based scintigraphic images may also differentiate active lesions from resolved changes, as opposed to anatomically/structurally based radiographic images (Davidson & Ross 2003). Interpretation of scintigraphy can be equivocal due to the bone turnover expected with age or occupation, unless there is marked asymmetry in the uptake (Santschi 2008). Increased radiopharmaceutical uptake in the distal
palmar/plantar third metacarpus/metatarsus was observed in 21% of forelimbs and 19% of hind limbs in Standardbred racehorses, and in 50% of forelimbs and 28% of hind limbs in Thoroughbred racehorses. This region was the most common site of increased radiopharmaceutical uptake in Thoroughbreds (Arthur & Constantinide 1995; Ehrlich et al 1999; Davidson & Ross 2003). In several studies of horses with increased radiopharmaceutical uptake in the fetlock joint, 46%, 49%, and 20% had no radiographic abnormalities (Martinelli et al 1994; Ross 1998; Ehrlich et al 1999; Davidson & Ross 2003). Bilateral increased radiopharmaceutical uptake was observed in 62% of forelimbs and 69% of hind limbs of Thoroughbreds, and in 73% of forelimbs and 76% of hind limbs in Standardbreds (Arthur & Constantinide 1995; Ehrlich et al 1999; Davidson & Ross 2003). All 4 fetlock joints revealed increased radiopharmaceutical uptake in 28% of racehorses (Martinelli et al 1994; Davidson & Ross 2003). A flexed lateral scintigraphic view is useful in differentiating increased radiopharmaceutical uptake in the palmar/plantar metacarpus/metatarsus from uptake in the proximal sesamoid bones (Davidson & Ross 2003). There is potential for magnetic resonance imaging to provide additional diagnostic information, and this technique had been useful in several cases (Martinelli, Baker, Clarkson, et al 1996; Zubrod, Schneider, Tucker, et al 2004; Riggs 2006; Dyson & Murray 2007; Santschi 2008).

1.2.9 – Postmortem Findings

Macroscopic examination and analysis has also been utilized to characterize post-traumatic osteoarthritis. In one survey, approximately 1/3 of 2- and 3-year-old Thoroughbreds had metacarpophalangeal joint osteoarthritis (Neundorf, Lowerison, Cruz,
et al 2010). Additional evaluation of articular cartilage from the palmar metacarpus of horses with fetlock osteoarthritis revealed molecular changes consistent with a catabolic profile, evidenced by decreased gene expression for protein biosynthesis, anti-apoptotic function, and extracellular proteoglycan matrix formation (Smith et al 2006). Evidence of osteoarthritis in the subchondral bone of the palmar metacarpus has also been identified via quantitative computed tomography, which correlated well with histologic changes in bone and articular cartilage (Young et al 2007). Evaluation of metacarpal condyles from racehorses via electron microscopy revealed increased lamellar bone and microcracks, with osteoclastic activity indicative of remodeling in the sclerotic region adjacent to the zone of calcified cartilage (Norrdin & Stover 2006). The smallest, and presumably earliest, microcracks occurred within 1-3 mm of calcified cartilage in the center of the area of sclerosis, and then extended parallel to the surface with minor branching (Norrdin & Stover 2006). When the microcracks became enlarged, with fragmentation and gaps, they were then considered microfractures (Norrdin & Stover 2006). In instances of failure, osteoclastic erosion indicated active remodeling (Norrdin & Stover 2006). Microfractures with chronically rounded edges, which were observed in a horse that was still racing, may indicate devitalized bone (Norrdin & Stover 2006). The study by Norrdin and Stover (2006) provided an excellent description of the microcracks and microfractures that can form in the subchondral bone of racehorses, but there was no correlation with clinically applicable diagnostic modalities other than radiographs.

In a study of racing Thoroughbreds, microcracks most commonly occurred in the condylar groove and were oriented at an angle of 45° towards the condyle (Muir, Peterson, Sample, et al 2008). A proposed explanation of this predilection site was the
decreased width of the calcified cartilage in the condylar groove, which may increase the load at this location (Muir et al 2008). In a study evaluating condylar fractures using scanning electron microscopy, it appeared that the initiation of a microcrack most likely occurred at or below the level of the collagen type I fiber (Stepnik et al 2004). Bone adaptation to exercise increases the ability to form microcracks that do not coalesce, which is theoretically an advantage due to enhanced energy absorption and decreased chances of forming a fracture (Stepnik et al 2004). Bone collagen orientation changes, new cement lines, and new bone lamellae mineralization have all been hypothesized to be contributors to the enhanced ability to form microcracks (Stepnik et al 2004).

1.2.10 – Experimental Models of Post-Traumatic Osteoarthritis

Post-traumatic osteoarthritis has been investigated by both in-vitro and in-vivo studies. Impaction of isolated articular cartilage explants produced chondrocyte death, cartilage mechanical disruption, and initiation of the degradation cascade, including a decrease in proteoglycan synthesis and an increase in water content (Patwari, Fay, Cook, et al 2001; Borrelli & Ricci 2004). When the underlying bone was left attached to the cartilage explants, there was less damage to the articular cartilage at the same impact forces because the bone sustained the damage in its role as a shock absorber (Patwari et al 2001; Borrelli & Ricci 2004).

In-vivo impact models used blunt trauma applied at a rapid rate to closed dog or rabbit joints, resulting in increased chondrocyte clones, increased vascularization, increased water content, decreased proteoglycan content, chondrocyte apoptosis, and surface fibrillation of the articular cartilage (Borrelli & Ricci 2004). Chondrocyte
apoptosis appears to play a role in both osteoarthritis and traumatic injury because these chondrocytes are unable to maintain the extracellular matrix, resulting in cartilage degeneration (Vrahas, Mithoefer, Joseph, et al 2004). Although the amount of apoptosis correlates well with the severity of osteoarthritis, it is still unclear whether the impact induces apoptosis by damaging the chondrocytes directly or by damaging the extracellular matrix (D’Lima, Hashimoto, Chen, et al 2001; Vrahas et al 2004). However, all of these studies focused on the effects of post-traumatic osteoarthritis on articular cartilage, rather than subchondral bone.

Multiple impact injury models have been developed in other species. Various models create injury via joint instability, a single load, or cyclic loading. Post-traumatic osteoarthritis has most often been investigated in-vivo via the canine anterior cruciate ligament transection model (Patwari et al 2001), which causes repeated impact injury due to joint instability and subluxation. In the horse, an instability model has been created by transecting the lateral collateral ligament and lateral collateral sesamoidean ligament in the equine metacarpophalangeal joint (Simmons, Bertone & Weisbrode 1999). Drop towers have been used to cause single impact injury to bovine (Natoli et al 2008) and equine (Huser & Davies 2006) cadaver specimens. A drop tower has also been used to apply an impact load to the patella in a closed joint model in dogs (Mrosek, Lahm, Erggelet, et al 2006) and rabbits (Ewers, Weaver & Haut 2002). Single impact injury to canine cadaver specimens has been created in a similar manner using a mechanical testing system (Vener, Thompson, Lewis, et al 1992). Cyclic loading models have been described using a mechanical testing and loading apparatus in bovine osteochondral disks
(D’Lima et al 2001; Thibault et al 2002). An in-vivo model of daily cyclic loading involving the entire limb has been described in rabbits (Radin et al 1973).

Previous work in our laboratory developed a technique for experimentally inducing post-traumatic osteoarthritis in the ovine (Hurtig, Chubinskaya, Dickey, et al 2009) and equine (Bolam et al 2006) medial femorotibial joint. In the ovine study, a spring-loaded impact device with a 6.0-mm-diameter aluminum tip was used to create 2 adjacent impact injuries of 30 MPa on the middle weight-bearing third of the medial femoral condyle (Hurtig et al 2009). Post-traumatic osteoarthritis consisting of articular cartilage degeneration and mild subchondral bone changes was observed, with slow progression from 3 to 6 to 9 months and severe end-stage osteoarthritis evident at 2 years postoperatively (Hurtig et al 2009). In the equine study, a handheld impactor device with a 6.5-mm-diameter tip was used to create 4 adjacent, but not overlapping, impact injuries of 60 MPa on the axial weight-bearing nonmeniscus-covered articular cartilage of the medial femoral condyle (Bolam et al 2006). Evaluation of this impact site at 84 and 180 days revealed articular cartilage changes consistent with progressive osteoarthritis; but minimal evaluation of the subchondral bone was performed, consisting of radiographs and histological evaluation (Bolam et al 2006). This study showed that a single traumatic episode can produce progressive irreversible osteoarthritis at 3 and 6 months (Bolam et al 2006).

1.2.11 – Treatment

The current treatment for subchondral bone injury in the metacarpal/metatarsal condyle is conservative, consisting of rest and rehabilitation, controlling inflammation,
shoeing changes, and intra-articular injections (Richardson 2003; Ross 2003; Santschi 2008). The bisphosphonate tiludronate, a potent inhibitor of bone resorption, is effective in improving horses with osteoarthritis of the thoracolumbar vertebral column (Coudry, Thibaud, Riccio, et al 2007) and the distal tarsal joints (Gough, Thibaud & Smith 2010) and shows promise for treatment of subchondral bone injury. Horses with pain localized to the distal metacarpus/metatarsus were successfully treated with paddock rest, rather than stall rest, in order to allow the bone to recover (Bramlage 2009; Tull 2009). If not treated by rest, the maladaptive response to exercise stress can progress to palmar articular degeneration, subchondral bone collapse, and eventually basal sesamoid fracture (Bramlage 2009). Strategies for prevention of all degrees of musculoskeletal injury include shoeing without toe grabs, decreasing the amount of high-speed exercise, and early intervention, since mild injury precedes most overt musculoskeletal injuries (Stover 2003; Santschi 2008). Modifying training techniques from a single long distance high-speed work to several shorter distance high-speed exercise periods can decrease the amount of microdamage accumulation, and therefore the incidence of injuries (Stover 2003). Surgical options include joint lavage to remove cytokines and cartilage debris, as well as removal or internal fixation of osteochondral fragments or fractures (Santschi 2008). The prognosis for return to racing is guarded to fair due to the progression of osteoarthritis (Richardson 2003; Ross 2003).

A proposed diagnostic and treatment modality for post-traumatic osteoarthritis is arthroscopic surgery. On routine arthroscopic examination of the metacarpo/metatarsophalangeal joint, visualization of the distal palmar/plantar aspect of the condyle has been described as “physically impossible” (Barr et al 2009). However,
one Standardbred treated with arthroscopic debridement returned to racing with
significantly improved race times (Byron & Goetz 2007). An arthroscopic approach to
the palmarodistal aspect of the equine fetlock joint has been described in a case of
naturally occurring subchondral bone disease, involving a focal radiolucency with a
sclerotic rim in the palmar third metacarpus (Byron & Goetz 2007). Placement of a
70-degree forward viewing arthroscope in the proximopalmar joint pouch allowed
adequate visualization for debridement of the affected articular cartilage and bone, via an
instrument portal placed through the most distal portion of the medial collateral
sesamoidean ligament (Byron & Goetz 2007). There were no adverse effects from the
incision through the collateral sesamoidean ligament; but since this report only involved
one case, further investigation is necessary to confirm the safety of this technique (Byron
& Goetz 2007). Preliminary work in our laboratory demonstrated that with some
adjustments to portal placement, this arthroscopic approach could be adapted for
performing contusive impacts on the palmar metacarpal condyle.

1.2.12 – Conclusion

Investigation of the pathogenesis of post-traumatic osteoarthritis, improvement of early
diagnosis, and further development of arthroscopic techniques in this region will be
clinically relevant for affected racehorses. The authors hoped to accomplish this goal by
studying the early events of post-traumatic osteoarthritis in a naturally occurring site,
with an emphasis on the interactions between subchondral bone and articular cartilage.
In addition, we hoped to unravel the mystery surrounding the relationship of articular
cartilage and subchondral bone during early stages of post-traumatic osteoarthritis, with
the aim to detect time points at which inflammatory changes may precipitate degenerative changes.
2.0 – EVALUATION OF IMPACT INJURY AS A MODEL OF
EXPERIMENTALLY INDUCED POST-TRAUMATIC OSTEOARTHRITIS IN
THE EQUINE METACARPOPHALANGEAL JOINT

(To be submitted to the Am J Vet Res)

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2.1 – Abstract

**Objective:** The purpose of this study was to develop a model of post-traumatic osteoarthritis due to impact trauma on the palmar metacarpal condyle and to evaluate the timing of the early events following impact trauma on subchondral bone and articular cartilage.

**Animals:** 12 horses.

**Procedures:** In each horse, an impact injury was created on the palmar metacarpal condyle of one randomly chosen limb, under arthroscopic and fluoroscopic guidance. A low to moderate level of forced exercise was instituted; and horses were evaluated clinically via lameness examinations, synovial fluid analysis, and radiographs. Macroscopic examination, micro-computed tomography, and sample collection were performed following euthanasia at 1 month (3 horses), 4 months (4 horses), and 8-10 months (5 horses) post-impaction.

**Results:** There was variability in impact lesion location, depth, and area on macroscopic inspection; histologic evaluation revealed more consistent cartilage defects due to impact injury. Cartilage degeneration, in terms of color and clarity, was observed in impacted joints. The mean sulfated glycosaminoglycan (sGAG) concentration from cartilage at the impact site was significantly lower than for a similar site in control limbs. Higher concentrations of cartilage oligomeric matrix protein (COMP) were observed in synovial fluid from impacted joints. Bone viability, as evaluated by the Alamar blue assay, was significantly decreased in impact specimens versus control specimens at one month post-impaction.
Conclusions and Clinical Relevance: This impact injury model caused mild focal osteoarthritic lesions in the palmar metacarpophalangeal joint, involving cartilage more than subchondral bone. Further development is required to create a reliable and consistent model of naturally occurring post-traumatic osteoarthritis at this site.
2.2 – Introduction

The metacarpophalangeal joint has the greatest number of traumatic and degenerative lesions of all joints in racehorses.\(^1\) A common result of injury in this region is post-traumatic osteoarthritis, which predominantly affects the palmar metacarpal condyle. Post-traumatic osteoarthritis has also been termed traumatic osteochondrosis, fetlock arthrosis, and palmar/plantar osteochondral disease.\(^1\)\(^{-3}\) It is believed to be associated with the repetitive loading of exercise, which is often greater than the bone’s ability to respond; and thus leads to maladaptation rather than accommodation.\(^4\) This failure of the remodeling process to successfully repair bone may be due to ongoing trauma, alterations in blood supply, and/or the severity of damage.\(^3\)\(^,5\) Early in the course of this disease, clinical signs such as lameness, joint effusion, and response to flexion, are seldom obvious.\(^6\)\(^{-11}\) Lameness is often observed acutely after racing or training, and then improves with rest; although poor performance or intermittent lameness may continue for weeks to months.\(^7\) Radiographs usually reveal subchondral sclerosis or lucency in the palmar metacarpal condyle only after the disease is relatively advanced, so earlier diagnosis is often based on diagnostic analgesia and nuclear scintigraphy.\(^7\) As the disease progresses, so do the clinical signs and imaging findings.\(^7\) Postmortem evidence of osteoarthritis in the subchondral bone of the palmar metacarpus has been identified via quantitative computed tomography, which correlated well with histologic changes in bone and articular cartilage.\(^12\) Evaluation of metacarpal condyles from racehorses via electron microscopy revealed increased lamellar bone and microcracks, with osteoclastic
remodeling adjacent to the zone of calcified cartilage. When these microcracks enlarge and develop fragmentation and gaps, they become microfractures.\textsuperscript{13}

Stress-related injury of subchondral bone eventually leads to osteoarthritis.\textsuperscript{7} Extensive work has investigated the effects of osteoarthritis on articular cartilage, but much less is known about the role of subchondral bone. Mechanical loading causes bone strain, which induces adaptation of the subchondral bone’s density and strength through modeling and remodeling.\textsuperscript{14} In osteoarthritic subchondral bone, damage from the microcracks described above initiates bone remodeling via apoptosis of osteocytes.\textsuperscript{15} The volume, or apparent density, of subchondral bone increases in osteoarthritic joints, which is visible radiographically as sclerosis. However, this bone may be undermineralized, which decreases its material density and therefore its stiffness. This loss in stiffness from undermineralization necessitates a corresponding increase in bone volume to maintain a normal level of overall bone stiffness.\textsuperscript{15}

The connections between the pathogenesis of osteoarthritis in articular cartilage and subchondral bone are still unknown. Models that simulate osteoarthritis by creating joint instability may initially result in articular cartilage changes, but models using impulsive loading initially cause subchondral bone damage.\textsuperscript{14} It may be that the former is due to static strain, while the latter is the result of dynamic strain.\textsuperscript{14}

Previous work in our laboratory developed a technique for experimentally inducing post-traumatic osteoarthritis in the equine medial femorotibial joint.\textsuperscript{16} A handheld spring-driven impactor device with a 6.5-mm-diameter tip was used to create impact injuries on the medial femoral condyle via arthroscopic guidance.\textsuperscript{16} Evaluation of this impact site at 84 and 180 days revealed articular cartilage changes consistent with
osteoarthritis, but minimal evaluation of the subchondral bone was performed.\textsuperscript{16} The present study aimed to evaluate subchondral bone and articular cartilage after using this impactor device in a location of naturally occurring subchondral bone disease, the palmar metacarpal condyle. The purpose of this study was to develop a model of post-traumatic osteoarthritis in the palmar metacarpal condyle and to evaluate the timing of the early events following impact trauma on subchondral bone and cartilage. Our hypothesis was that impact trauma would lead to simultaneous degeneration of articular cartilage and subchondral bone remodeling events.

2.3 – Materials and Methods

Twelve skeletally mature horses, with no clinical signs of musculoskeletal disease, were included in the study. These horses (8 Standardbreds, 2 Thoroughbreds, 1 Quarter Horse, 1 mixed breed; 8 mares, 4 geldings) ranged from 3 to 10 years in age (mean ± SD = 5.3 ± 2.1 years, median 5 years) and from 382 to 558 kg in weight (466 ± 59 kg, median 475 kg). Horses were screened for evidence of musculoskeletal disease via detailed lameness examination by 2 examiners (EJR, AMC), radiographic evaluation of the metacarpophalangeal joints, and cytological analysis of synovial fluid from the metacarpophalangeal joints. Horses were allocated randomly to study endpoints at 1 month (3 horses), 4 months (4 horses), 8 months (3 horses), or 10 months (2 horses). This study was approved by the University of Guelph Animal Care Committee, in accordance with Canadian Council of Animal Care guidelines.
Operative Technique – In a pilot study, using cadaveric equine forelimbs, the reported arthroscopic approach to the palmar aspect of the metacarpal condyle\textsuperscript{17} was modified for application of the impactor device.

In the main study, each horse received preoperative antimicrobial (penicillin-G sodium, 22,000 IU/kg IV) and anti-inflammatory (phenylbutazone, 4.4 mg/kg IV) therapy, and was premedicated with xylazine (1 mg/kg IV). Induction of general anesthesia was performed using diazepam (0.2 mg/kg IV) and ketamine (2 mg/kg IV), followed by maintenance with isoflurane in 100% oxygen.\textsuperscript{18} The horse was placed in lateral recumbency with the control limb, as determined by randomization, uppermost. The treatment limb was always operated first. Each fetlock region was prepared & draped for aseptic surgery. An arthroscopic portal was created in the medial palmaroproximal aspect of the metacarpophalangeal joint. Arthroscopic evaluation of the palmar pouch was performed to confirm absence of clinically significant cartilage abnormalities. A needle was introduced into the joint distal to the abaxial border of the medial proximal sesamoid bone to determine the site for the instrument portal, which was placed in a routine manner.

A custom-designed aiming device (Figure 1) was clamped into the medial and lateral epicondylar fossae to guide the placement of the impactor tip. The aiming device consisted of a modified condylar clamp, to secure the device to the limb via attachment to the epicondylar fossae; and a jig with adjustable rotation and medial to lateral location, which could be locked so that the impactor tip would be stabilized when placed through the hole in the jig (Figure 1). A handheld spring-driven impactor with a 6.5-mm-diameter nonporous tip\textsuperscript{16} was placed through the aiming device. Four impact
injuries of 80 MPa each were created in a cloverleaf pattern at the midpoint of the palmarodistal aspect of the medial metacarpal condyle as close as possible to the site of naturally occurring disease, which required full extension of the metacarpophalangeal joint. During prior work in our laboratory, using fresh cadaver limbs impacted with forces of 30, 50, 70, 80, and 90 MPa via a drop tower, we had determined that a minimum impact force of 80 MPa was required to produce chondrocyte death extending deeper than the superficial zone without macroscopic cartilage damage (data not shown). Arthroscopic guidance was used to confirm contact with the articular surface; then the fetlock was maximally extended while the impactor was slid along the articular surface, which caused movement of the impactor distally. The impactor tip was positioned perpendicular to the articular surface, using fluoroscopic guidance to visualize the final impactor placement site. Bupivacaine (0.02 mg/kg) and morphine (0.1 mg/kg) were injected into the joint prior to closure of the skin with #0 polypropylene suture in a simple interrupted or cruciate pattern. A sham procedure was performed on the control limb in the same manner, including introduction of the impactor tip into the joint, but without creating an impact injury. Both distal forelimbs were bandaged prior to unassisted recovery from anesthesia.

**Postoperative Care** – Sodium penicillin (22,000 IU/kg IV q8h) and phenylbutazone (4.4 mg/kg IV or PO q24h) were administered for 3 and 5 days, respectively. Additional analgesia (butorphanol, 0.05-0.1 mg/kg IV) was available if required. The distal limbs were kept bandaged for 10 days and sutures were removed 12-14 days after surgery. Horses were confined to stall rest for 10 days, prior to pasture
turnout. Forced exercise (lunging in both directions on a dirt or grass surface) was initiated 2 weeks after surgery, starting at 5 minutes daily for 5 days per week. During each subsequent week, daily exercise was increased by 5 minutes, to a maximum of 30 minutes per day. Forced exercise was discontinued 5½ to 6 months after surgery due to constraints of weather conditions. Horses were allocated to groups with endpoints at 1, 4, 8, and 10 months post impact injury, at which time they were humanely euthanized (xylazine, 300 mg IV; followed by sodium pentobarbital, 40.8 g IV).

**Clinical Assessment** – Lameness examinations by 2 examiners (EJR, & AMC or alternate) were performed weekly for 5 months, and then every other week for the remainder of the study. At a walk and trot in a straight line, lameness was graded as: 0 = normal, 1 = mild head nod at the trot, but not at the walk, 2 = obvious head nod at the trot, mild at the walk, 3 = obvious head nod with every step at the walk and trot, 4 = partial weight bearing, or 5 = non-weight bearing. Half grades were assigned if indicated. This grading scale was developed to provide more descriptive degrees of lameness at the trot in a straight line than the AAEP lameness scale.

Radiographic examinations performed prior to entering the study and immediately prior to euthanasia consisted of dorsopalmar, lateromedial, flexed lateromedial, dorsomedial palmarolateral oblique, dorsolateral palmaromedial oblique, and 125-degree dorsodistal palmaroproximal (“Hornof”) projections of both metacarpophalangeal joints. Radiographs were evaluated by a board-certified radiologist (SGN) blinded to treatment group and were scored for evidence of osteoarthritis (0 = normal, 1 = minimal, 2 = mild, 3 = moderate, 4 = severe). Additional abnormalities, such as osteochondral fragments
and proximal sesamoid bone sclerosis, determined not to be contributing to osteoarthritis were noted.

**Synovial Fluid Parameters** – Synovial fluid was collected from both metacarpophalangeal joints of each horse prior to entering the study and at 1, 2, 3, 4, 6, 8, and 10 months. Synovial fluid analysis consisting of total protein level, white blood cell count, and differential leukocyte count was performed immediately, and aliquots were frozen for later analysis of sulfated glycosaminoglycan (sGAG) and cartilage oligomeric matrix protein (COMP) levels.

**Synovial Fluid Sulfated Glycosaminoglycan (sGAG)** – Synovial fluid samples were analyzed for sGAG concentration as previously described. Briefly, frozen synovial fluid samples were thawed and digested in an equal volume of papain in digest buffer at 65°C overnight. Digested synovial fluid samples were analyzed in triplicate via a microplate, according to the 1,9-dimethylmethylene blue dye assay; and results were expressed as a mean of 3 readings and reported as chondroitin sulfate C (CSC) micrograms per microliter of synovial fluid.

**Cartilage Oligomeric Matrix Protein (COMP)** – COMP was measured on previously frozen synovial fluid aliquots, using a commercially available EIA kit designed for human synovial fluid and serum. The assay was performed on all impact samples at all time points, on all control samples at baseline (12 horses), 180 days (5 horses), and 240 days (5 horses), and on 4 randomly selected control samples at all other time points.
**Postmortem Evaluations** – Postmortem evaluation consisted of assessment of macroscopic appearance, cartilage, and bone. The metacarpophalangeal joints were macroscopically scored (0 = normal, 1 = mild, 2 = moderate, 3 = severe) for effusion, subcutaneous thickening, joint capsule fibrosis, enlargement of the entire joint, inflammation, cartilage color, cartilage clarity, cartilage surface roughness, osteophytes, synovial membrane hypertrophy, and synovial membrane darkening (0 = white, 1 = amber, 2 = orange, 3 = red). An estimate of the percentage of abnormal cartilage area was performed in each region, including the medial metacarpal condyle, lateral metacarpal condyle, sagittal ridge, sagittal groove, medial first phalanx, lateral first phalanx, medial proximal sesamoid bone, and lateral proximal sesamoid bone. Abnormalities were drawn on a diagram of the joint and digital photodocumentation was performed prior to and following India ink staining.

The joint surfaces were stained with India ink to delineate areas of disruption in the cartilage surface; and these areas were traced onto acetate sheets, then scanned into image analysis software. After manual calibration and thresholding, this software was used to measure the amount of damaged cartilage with India ink uptake compared to the total amount of cartilage in each of the following regions: medial metacarpal condyle, lateral metacarpal condyle, medial proximal sesamoid bone, lateral proximal sesamoid bone, medial proximal phalanx, and lateral proximal phalanx. Results were expressed as the percent damaged area in each region.

**Cartilage Assessment** – Full-thickness cartilage samples were collected, using a #15 scalpel blade, from the lesion site and from a site distant to the lesion on the palmar
medial metacarpal condyle. These full-thickness cartilage samples were used for paravital staining, methylthiazolyldiphenyl-tetrazolium bromide (MTT) cell viability assay, and sulfated glycosaminoglycan analysis. Osteochondral sections for decalcified histology were collected from the medial metacarpal condyle at the lesion site, the medial first phalanx, and the medial proximal sesamoid bone. Lactated Ringer’s solution was applied as necessary to prevent drying of the cartilage surface during processing.

**Paravital Staining of Cartilage** – Fresh cartilage was sectioned perpendicular to the articular surface into 100-micron slices on a vibratome, and stained with SYTO-13 green fluorescent nucleic acid stain (1:20 solution with lactated Ringer’s solution) and ethidium bromide (1:20 solution with distilled water) in lactated Ringer’s solution. The sections were viewed on a microscope with ultraviolet incidence light, using a wide band-pass filter, and digital images were captured. The superficial, middle, and deep cartilage zones were defined as 9%, 47%, and 44% of average cartilage thickness, respectively, using image analysis software. Live (fluorescent green) and dead (fluorescent red) cells were counted, using a semi-automated method after manual thresholding of the image. Percent viability was calculated from the proportion of SYTO-13 and ethidium bromide stained cells.

**MTT Assay for Cartilage Metabolism** – Fresh cartilage for the methylthiazolyldiphenyl-tetrazolium bromide (MTT) cell viability assay was weighed and incubated with MTT (50 µl of 3-[4,5-Dimethyl-2-thiazolyl]-2,5-diphenyl-2H-tetrazolium bromide) and media (500 µl) for 3 hours at room temperature on an Adams nutator. Then the MTT was removed and the cartilage was incubated with extraction buffer (500 µl of 96% isopropanol and 4% 1 M hydrochloric acid) for an additional 30
minutes at room temperature on an Adams nutator. The resulting solution was read on a plate reader in triplicate (100 µl per well) at an absorbance of 530 nm. The 3 optical density readings were averaged and divided by the wet weight of the cartilage in milligrams to yield the average MTT per mg of cartilage.

**Cartilage Sulfated Glycosaminoglycan (sGAG)** – Cartilage samples were analyzed for sGAG concentration as described above for synovial fluid samples. Briefly, cartilage samples were weighed and digested in 1 ml papain in digest buffer per 10 mg of cartilage at 65°C overnight, prior to freezing at -80°C. The 1,9-dimethylmethylene blue dye assay was performed as previously described and results were expressed as a mean of 3 readings and reported as chondroitin sulfate C (CSC) micrograms per milligram of cartilage.

**Histology, Histological Scoring and Immunostaining** – Osteochondral sections for histologic evaluation were fixed in neutral buffered 10% formalin, decalcified in 20% citric acid and 40% formic acid mixed 1:1 before use, and embedded in paraffin; then cut into 5-micron-thick sections, which were routinely stained with hematoxylin and eosin, safranin O, and picrosirius red for collagen orientation. Sections were scored independently by 3 blinded observers (EJR, MBH, BJM) using the OARSI scoring system.\(^\text{19}\) The OARSI score is calculated from the mathematical product of grade, which assesses the depth of cartilage damage indicating the severity of osteoarthritis, and stage, which assesses the area of cartilage affected. Stage was determined by manual measurements performed on digital images of the histology slides and expressed as a percentage of affected cartilage, which was converted to a scoring system (0 to 4) as previously described.\(^\text{19}\)
Immunohistochemistry was performed on the impacted medial metacarpal condyle sections for collagen 2-3/4C_short and TUNEL staining for apoptosis, as previously described. Briefly, osteochondral sections were deparaffinized using xylene and ethanol. Pretreatment of TUNEL slides consisted of testicular hyaluronidase and proteinase K, followed by staining with a commercially available kit. A positive control, consisting of mammary gland tissue from a normal female rat taken 3-5 days after weaning rat pups, and a negative control, consisting of normal equine articular cartilage, were stained with TUNEL at the same time. COL2-3/4C_short slides were pretreated with 0.5% hydrogen peroxide in methanol, digested using chondroitinase, and then treated with a protein blocking agent. Slides were incubated at 37°C with the COL2-3/4C_short antibody, incubated at room temperature with the biotinylated secondary antibody, and treated with streptavidin-horseradish peroxidase conjugate and diaminobenzidine substrate. Immunohistochemistry slides were scored according to the degree of staining (mild, moderate, marked) by 2 independent observers (EJR, MGB).

**Bone Assessment** – After evaluation of the intact third metacarpal bone using micro-computed tomography (microCT), bone sections were collected from the medial metacarpal condyle for undecalcified histology to assess fluorochrome uptake (lesion site) and for osteocyte viability, via Alamar blue assay (lesion site & distant site).

**MicroCT** – The distal epiphysis of each third metacarpal bone was dissected free of soft tissues and microCT images were acquired at 80 kV and 450 µA with a 45-micron isotropic voxel resolution. A calibration phantom present during scanning was used to convert CT values into Hounsfield units, as previously described. Images were
reconstructed into a 3-dimensional format and imported into analysis software\(^k\). The spatial location of the impact site was measured on digital photographs taken at postmortem; and these measurements were used to locate the impact site on the 3-dimensional microCT image, with the mirror image coordinates used to identify a control site on the contralateral limb of the same horse. A 3 mm by 3 mm by 1 mm deep region of interest was created at the bone surface of the condyle at the measured impact site, which was identified as the superficial region of the subchondral bone plate. An identical region of interest was placed 1 mm deep to the first region and was designated the deep region. Bone mineral density (BMD, mg/cc), total mineral density (TMD, mg/cc), and bone volume fraction (BVF) were calculated for each region of interest by the analysis software after automatic thresholding of the image, as previously described.\(^{20}\)

**Alamar Blue Assay for Bone Viability** – Bone viability was evaluated using the Alamar blue assay, which has been used extensively for viability determination of cultured cells; and its use for bovine bone has been described.\(^{21}\) Fresh bone sections, approximately 10 mm by 5 mm by 3 mm in size, were incubated overnight (12-16 hours) in phosphate-buffered saline at 37°C. Then, the phosphate-buffered saline was removed and 6 ml of 5% Alamar blue in phosphate buffered saline was added to each bone section. Incubation at 37°C in the dark was continued and readings were taken every hour from 3 to up to 12 hours after initiation of the assay. Samples (200 µl) in triplicate were read on the fluorescence plate reader\(^1\) with excitation at 550 nm and emission at 590 nm. Readings were averaged and the blank (5% Alamar blue without bone incubated simultaneously) was subtracted to yield a value in relative fluorescence units.
Evaluation of Bone Remodeling – A fluorochrome bone label was administered (oxytetracycline, 10 mg/kg IV) to all remaining horses at 3 months post-impact injury. Calcein green (5 mg/kg IV) was administered as a second bone label to the 4-month endpoint horses at 24 hours prior to euthanasia, and to the 8- and 10-month endpoint horses at 6 months post-impact injury. A second dose of oxytetracycline (10 mg/kg IV) was also administered to the 8- and 10-month endpoint horses at 24 hours prior to euthanasia. Undecalcified sections for fluorochrome analysis were fixed in 10% neutral-buffered formalin for 48 hours and then stored in 70% ethanol prior to infiltration with methyl methacrylate and embedding in benzoyl peroxide and methyl methacrylate. Fluorochrome sections were evaluated under ultraviolet incidence light and the number of remodeling osteons per high power field (200x) was counted sequentially for the full length of the articular surface.

Statistical Methods – A generalized linear mixed-model was employed to analyze MTT, cartilage sGAG, microCT data, percent abnormal cartilage, effusion, subcutaneous thickening, scar, joint enlargement, inflammation, synovial darkening, synovial hypertrophy, India ink data, paravital staining, and fluorochrome data. Factors included in the model were treatment, lesion site vs. distant site, and endpoint, as well as their interactions. The random effect of horse and limb were taken into account. For data that was measured repeatedly over time (Alamar blue, synovial fluid sGAG & COMP, synovial fluid analysis data, lameness score), the AKAIKE information criterion (AIC)m was used to determine an error structure for the auto-regression. The assumptions of the ANOVA were assessed by comprehensive residual analyses. A Shapiro-Wilk test, a
Kolmogorov-Smirnov test, a Cramer-von Mises test, and an Anderson-Darling test were conducted to assess overall normality. Residuals were plotted against predicted values and explanatory variables (treatment, site, endpoint, lesion) to look for patterns in the data that suggest outliers, unequal variance, or other problems. If residual analyses suggested a need for data transformation or data was presented as a ratio, analyses were done on a log scale. If the overall f test was significant, a Dunnett’s test for comparison to baseline within a treatment or a Tukey test between treatments and sites at each time was applied. To test for agreement between observers on scored data, such as lameness scores and histological OARSI scores, a weighted kappa for more than 2 categories and a simple kappa for 2 categories were used with an exact p value. To test for differences between the treatment groups for binary scored data (clarity, color, surface roughness, osteophytes) a McNemar’s test was applied. Wilcoxon Mann Whitney test was used to compare the mean scores (1 to 4 for clarity, color, surface roughness, osteophytes) for impact and control at each endpoint. Radiographic scores were analyzed with a Wilcoxon signed rank test on the difference pre vs. post within a treatment and on the difference between treatments. The data from the 8- and 10-month endpoints were pooled for all tests and all analysis was blocked for horse. A 2-sided p value of less than 0.05 was considered significant.

2.4 – Results

**Surgical Outcome** – The surgical technique was successfully completed in all 12 horses. Arthroscopic and fluoroscopic guidance were used to determine placement of the impact injuries, but the area of interest was not completely visible at full extension with
either of these techniques. One horse (horse 6) experienced a complication related to the surgical procedure in the control limb, when a #15 scalpel blade was broken while making an instrument portal and the blade fragment could not be retrieved from the tissues distal to the medial proximal sesamoid bone. This horse showed moderate (maximum grade 3.5) lameness of the control limb postoperatively, although the cause of lameness could not be definitively attributed to the blade fragment. No horses required rescue analgesia postoperatively.

**Clinical Assessment** – Lameness examinations revealed lameness in the impact limb, the control limb, or both (Figure 2). The most severe lameness was grade 3 for an impact limb and grade 3.5 for a control limb, as described above. On average, lameness was observed in the impact limb for 6 ± 6.5 weeks (mean ± SD, range 0-21 weeks). Intermittent lameness in either the impact or control limb was present in several horses. However, there was no significant difference in lameness between impact and control limbs. The timepoint of the lameness examination in weeks was statistically significant for both observers (p < 0.01). Lameness scores for both impact and control limbs combined were significantly higher than baseline during weeks 1-5 for both observers (EJR, & AMC or alternate) and during weeks 6-9 for one observer (EJR). Agreement between observers was good with a weighted kappa of 0.72 (p < 0.01).

Radiography did not consistently demonstrate progression of the injury during the study period. Two control limbs had increased radiographic scores at endpoint, while grades increased in 6 impact limbs (Table 1). The radiographic changes were subtle, as indicated by the largest endpoint grade being the same as the largest baseline grade
(grade 2 = mild). Although initial assessment by a board-certified radiologist (SGN) found all baseline radiographs to be free of evidence of osteoarthritis, blinded scoring by the same radiologist at the end of the study found mild osteoarthritis in one limb and minimal osteoarthritis in 4 limbs. Radiographic scores for impact limbs were significantly higher at endpoint than baseline (p < 0.05), while no significant difference was noted for control limbs (p = 0.50). However, pairwise comparison of impact and control limbs in the same horse also showed no significant difference (p = 0.13).

**Synovial Fluid Parameters:**  

**Cytology** – Results of synovial fluid analysis were within normal limits for all horses at all timepoints (Table 2). Although no treatment effect was observed for synovial fluid analysis overall, there was a trend of decreased total white blood cell count in synovial fluid from impact joints (p = 0.10).

**Synovial Fluid sGAG and Cartilage Oligomeric Matrix Protein** – Biomarkers of osteoarthritis in synovial fluid only showed a mild effect from impact trauma on the joint. Synovial fluid sGAG concentrations were not statistically significant for treatment (p = 0.48) or collection time in weeks (p = 0.71). Synovial fluid COMP concentrations showed a significant effect for the combination of treatment and synovial fluid collection time in weeks (p < 0.05), with higher concentrations in impact joints. In week 33, there was a significant difference in COMP levels between impact joints and control joints (p < 0.01). Among impact joints, there was a significant difference between week 9 and week 24 (p < 0.01) (Figure 3).
**Postmortem Evaluations** – At postmortem examination, impact lesions were located in a variety of areas throughout the palmar medial metacarpal condyle. There was a wide variation in the extent and depth of the lesions, with some being quite focal while others were more diffuse. It appeared that in some joints the impactor had glanced off the cartilage, creating a shear injury, while in others the cartilage defect suggested a more ideal perpendicular impact. Independent evaluation of the postmortem images (Figure 4) by the authors (EJR, AMC, MBH, BJM) categorized the lesions in terms of severity as mild (horses 2, 3, 4), moderate (horses 1, 6, 9, 10), or severe (horses 5, 7, 8, 11, 12). All 4 observers agreed on the categorization of 6 horses (horses 3 & 4 mild, horses 6 & 10 moderate, horses 8 & 11 severe), and no difference between observers was greater than one grade. When these categories were evaluated by endpoint, there were 1 moderate and 2 severe at 1 month, 1 mild and 3 moderate at 4 months, 1 mild and 2 severe at 8 months, and 1 mild and 1 severe at 10 months.

**Macroscopic Assessment** – Macroscopic changes associated with the experimental injuries included statistically significant abnormalities in color and clarity of articular cartilage, effusion, joint enlargement, and percentage of abnormal cartilage. There were no significant differences in cartilage surface roughness, osteophytes, synovial membrane hypertrophy or darkening, subcutaneous thickening, joint capsule fibrosis, or inflammation. Postmortem scores were significantly worse in impact joints than in control joints for clarity at one month and for color at the combined 8- and 10-month endpoint (p < 0.05). When endpoints were compared within treatment groups, there was a significant difference for effusion in impact joints and for enlargement of both the impact and control joints (p < 0.05). There was a significant difference for the interaction
of site and endpoint (p < 0.01) for the percentage of abnormal cartilage. Specifically, the percentage of abnormal cartilage on the medial metacarpal condyle of impact and control limbs combined was significantly greater than: the lateral proximal phalanx at 1 month (p < 0.01); the sagittal groove at 1 month, 4 months, and 8-10 months (p < 0.01); the sagittal ridge at 1 month and 8-10 months (p < 0.01); the medial proximal sesamoid bone at 4 months (p < 0.05); and the lateral proximal sesamoid bone at 4 months (p < 0.01).

Disruption of the articular surface occurred in a larger area on the medial metacarpal condyle than at other sites. The percent damaged area of articular cartilage, as evidenced by India ink uptake, had a significant site effect (p < 0.01), but no treatment effect (p = 0.90). Specifically, the percentage of damaged area of the medial metacarpal condyle was significantly different from: the medial proximal phalanx (p < 0.01), the lateral proximal phalanx (p < 0.01), the medial proximal sesamoid bone (p < 0.05), and the lateral proximal sesamoid bone (p < 0.01).

**Cartilage Assessment: Paravital Staining** – Cell death was restricted to the superficial zone in most cases. Specifically, in only 2 horses was there decreased cell viability through all 3 zones (superficial, middle, deep) of impacted cartilage (horse 1, no live or dead cells; and horse 5). Most horses had decreased cell viability in the superficial zone (24-72% viable), but good viability in the middle (89-99% viable) and deep (89-99% viable) zones. In the superficial zone, cell viability was significantly decreased at the combined 8- and 10-month endpoint compared to the 1-month and 4-month endpoints (p < 0.01) (Table 3). There was also a significant decrease in viability in the superficial zone compared to the middle zone and the deep zone at 4 months and at
8-10 months (p < 0.01). The highest cell viability was observed in the middle and deep zones at 4 months, but this difference was not significant. Among all zones, mean cell viability at the lesion site in impact joints (39.8%) was lower than at the distant site in impact joints (53.9%) and at the “lesion” (54.5%) and distant (67.4%) sites in control joints, but these differences were also not statistically significant (p = 0.26).

**MTT Assay for Cartilage Metabolism** – Cell proliferation and viability in cartilage, as assessed via the MTT assay, declined progressively with time after impact injury (p = 0.05). At the lesion site, the mean ± standard deviation MTT value decreased from 14.2 ± 3.1 Avg MTT/mg cartilage at 1 month, to 12.2 ± 1.3 Avg MTT/mg cartilage at 4 months, to 11.1 ± 3.1 Avg MTT/mg cartilage at 8-10 months. The mean MTT value at the distant site decreased from 12.2 ± 2.5 Avg MTT/mg cartilage at 1 month to 11.6 ± 2.5 Avg MTT/mg cartilage at 8-10 months, but with a slight increase to 13.6 ± 2.4 Avg MTT/mg cartilage at 4 months.

**Cartilage Sulfated Glycosaminoglycan (sGAG)** – Depletion of sGAG from cartilage was observed due to impact injury. The mean sGAG concentration from cartilage at the impact joint lesion site (44.1 µg/mg) was significantly (p < 0.05) lower than at the control joint lesion site (53.3 µg/mg), impact joint distant site (52.4 µg/mg), and control joint distant site (50.0 µg/mg).

**Histology, Histological Scoring and Immunostaining** – Cartilage defects were observed on histologic evaluation at all sites of impact injury. Histologic evaluation, expressed as the mean of OARSI scores by 3 observers, revealed a significant effect for treatment (p < 0.05) and site (p < 0.01). OARSI scores for control joints (mean 0.5) were lower than for impact joints (mean 1.0). There was a significant difference in average
OARSI score for combined impact and control joints between all comparisons of the 3 sites (p < 0.01). The highest average OARSI score was observed at the medial metacarpal condyle (mean 6.7), with much lower scores at the medial proximal sesamoid bone (mean 1.7) and medial proximal phalanx (mean 0.9). The inter-observer agreement for OARSI scoring was almost perfect, with a coefficient of concordance of 0.81 to 0.99 for the 3 observers at all 3 sites. However, there was evidence of bias between 2 observers (EJR, MBH) at the medial proximal phalanx (p < 0.01).

Immunohistochemistry revealed mild evidence of apoptosis and mild to moderate collagen 2-3/4C\textsubscript{short} expression. TUNEL immunohistochemistry staining to indicate apoptosis did not reveal a significant effect of endpoint (p = 0.42) in the impacted medial metacarpal condyle sections. Inter-observer agreement could not be evaluated due to lack of equal use of the scoring categories by both observers. Immunohistochemistry, using the COL2-3/4C\textsubscript{short} antibody as evidence of osteoarthritis progression, had a significant endpoint effect (p < 0.05). There was a significant difference between the 1-month and 4-month endpoints (p < 0.05) and a nearly significant difference between the 4-month and 8-10 month endpoints (p = 0.05), but there was no difference between 1 month and 8-10 months (p = 0.73). The impacted medial metacarpal sections had the highest average score at 4 months (mean 9.9), with the lowest average score at 1 month (mean 4.2), and an intermediate average score at 8-10 months (mean 5.2). There was good inter-observer agreement for COL2-3/4C\textsubscript{short} (weighted kappa = 0.74, p < 0.01). Since immunohistochemistry was not performed on control sections, a treatment effect could not be evaluated for apoptosis or COL2-3/4C\textsubscript{short} expression.
**Bone Assessment:** *MicroCT* – Due to variability in the site of impact injury, microCT analysis of the lesion site also occurred at different anatomical locations. MicroCT analysis for bone mineral density (BMD) revealed a significant site and endpoint interaction ($p < 0.05$); but no treatment effect, so impact and control values were combined. Mean BMD values were highest at 1 month, decreased by 4 months, and then increased again by 8-10 months (Figure 5). MicroCT analysis for total mineral density (TMD) revealed a significant site effect ($p < 0.05$) and endpoint effect ($p < 0.01$), as well as a trend for a site and endpoint interaction ($p = 0.07$). Mean TMD values, consisting of combined impact and control values due to lack of treatment effect, were significantly higher for the superficial site (815.0 mg/cc) than the deep location (799.6 mg/cc) ($p < 0.05$). Similar to BMD values, TMD values were highest at 1 month, decreased at 4 months, and increased again at 8-10 months (Figure 6). MicroCT analysis for bone volume fraction (BVF) did not reveal any significant differences, although there was a trend for a site effect ($p = 0.08$) and a site by endpoint interaction ($p = 0.06$).

**Alamar Blue Assay for Bone Viability** – Alamar blue results showed a significant interaction between treatment, endpoint, and reading time of the assay ($p < 0.01$). Significant differences in Alamar blue readings between impact and control joints were observed at the 1-month endpoint at the 8-hour reading ($p < 0.05$) and the 10-hour reading ($p < 0.01$), which indicated decreased viability in the impact samples (Figure 7).

**Evaluation of Bone Remodeling** – The fluorochrome calcein green was distinct in areas of remodeling, but oxytetracycline uptake was not discernible. Therefore, measurement of the amount of remodeling occurring between fluorochrome injections could not be performed. Histological evaluation of sections for fluorochrome deposition
revealed a significant endpoint effect, with a significant difference between sections harvested at 4 months and those collected at 8-10 months (p < 0.01). There was more evidence of remodeling at 4 months (mean 6.2 remodeling units per field) than at 8-10 months (mean 3.2 remodeling units per field).

### 2.5 – Discussion

This impact injury model succeeded in creating mild changes in some outcome parameters consistent with the development of osteoarthritis, specifically: synovial fluid COMP levels, cartilage color and clarity, cartilage sGAG concentration, histologic evaluation via OARSI scoring, and bone viability as evaluated by the Alamar blue assay. The lack of consistent evidence of osteoarthritis in all outcome parameters can be attributed to the variability in the impact lesions created, inadequate severity of the model, or insufficient sensitivity of the outcome parameters.

The major limitation of the study was the variability in location (site), depth, and area of the impact lesions, which was most likely due to an inability to directly observe lesion creation. The small joint volume and thick periarticular soft tissue of the palmarodistal metacarpophalangeal joint severely interfered with viewing and positioning of the impactor tip. Variation in lesion location became a complicating factor in many outcome parameters. The inconsistent injury site complicated interpretation of results because of the highly site-specific variation in structural and functional properties observed in the metacarpal condyles. Due to the curved geometry of the condyles, small alterations in the impact site may have also altered contact between the impactor tip and the articular surface, resulting in a shear rather than a perpendicular force. This
less-than-90° angle of impact may have caused the impacter tip to slide along the curved articular surface and thus disperse the force applied to the underlying osteochondral unit, which may have had more influence on the outcome parameters than the differences in lesion location. Macroscopic evidence of this motion was observed on postmortem examination. Inadequate visualization also contributed to placement of the 4 impact injuries in overlapping sites in some cases, rather than in a cloverleaf pattern. Although this overlap increased the force at that site, it decreased the area of the lesion below that of a critically-sized defect. The impact injuries created appeared sufficient to create a focal response, but were not severe enough to cause global osteoarthritis in the joint. A more significant injury, by using repetitive impacts or higher impact energy, may be required to cause joint dysfunction and continuing lameness. The impact injury, by itself, may not be sufficient to cause progression of osteoarthritis, but additional trauma in the form of high-speed exercise on a hard surface, such as a treadmill, may also be required.

All of the outcome parameters, other than the Alamar blue assay, used in this study have been previously described as indicators of osteoarthritis, although in more severe models. Since this model produced lesions of various sizes, sampling of tissue from the less affected periphery of the impact site was a source of error. Sampling error may have contributed more to the error than the sensitivity of the measurements.

Clinically, all horses showed mild signs of osteoarthritis. Postoperative lameness was mild to moderate and decreased with time after approximately 1-2 months, which suggests that the lameness was more likely due to soft tissue damage at surgery than to progression of osteoarthritis. Some horses also had intermittent control limb lameness, which further supports the suspicion that soft tissue injury during surgery contributed to
the lameness. Although unlikely, an occult pre-existing lameness that was not initially detected cannot be ruled out. Radiographic scores for evidence of osteoarthritis in impact limbs were significantly worse at endpoint than at baseline. However, this difference was not consistent enough to withstand pairwise comparison of impact and control limbs in the same horse, possibly due to a variable degree of pre-existing osteoarthritis. Radiographs often demonstrate little about cartilage integrity until joint space narrowing and collapse are present.\textsuperscript{23,24} Of the 6 horses in this study with radiographic progression of osteoarthritis in the impact limb, 2 were categorized as mild, 2 moderate, and 2 severe during macroscopic assessment at postmortem. In our research horses, as in naturally occurring cases, it was difficult to detect and predict the clinical progression of osteoarthritis.\textsuperscript{25} This unpredictability in the pathological consequences of joint injuries may be due, in part, to the variability in involvement of the different tissues in the joint.\textsuperscript{25}

Synovial fluid cytologic analysis and sGAG concentration did not reveal any treatment effect. The lack of synovial fluid sGAG changes was unusual, because proteoglycan fragments usually increase in synovial fluid during early osteoarthritis.\textsuperscript{25,26} However, the small focal lesions created in this study may not have liberated enough proteoglycan fragments to be detectable. Higher concentrations of the articular cartilage component COMP were present in synovial fluid from impact joints than from control joints, which indicated more cartilage destruction due to the catabolic effects of osteoarthritis.

Macroscopic evaluation of the metacarpophalangeal joints revealed some osteoarthritic changes associated with the impact injuries, although not all parameters were consistently affected. There were abnormalities in the color and clarity of articular
cartilage, effusion, joint enlargement, and a larger percentage of abnormal cartilage in impact joints. Articular cartilage clarity changes consisted of early loss of clarity, while color abnormalities were observed late in the progression of osteoarthritis in this study, indicating the accumulation of non-enzymatic glycation products.  

Effusion increased with time as synovial fluid production reflected the development of osteoarthritis, while joint enlargement decreased as surrounding soft tissues remodeled postoperatively.

Disruption in the articular cartilage surface allows uptake of India ink applied to that surface. Although there was more India ink uptake on the medial metacarpal condyle than at other sites, there was no difference between impact and control joints, which raises concerns about the validity of the model, the normalcy of the horses at baseline, or the specimen preparation. The focal nature of the impact injuries and their limited progression may not have been sufficient to influence India ink results for the entire condyle. A study involving quantification of India ink uptake found a 10% error in the staining of nondegenerated cartilage in equine proximal phalanx specimens. This nonspecific uptake may have occurred in our study as well. In addition, India ink staining at sites of pre-existing disease or incidental cartilage damage may have obscured differences due to the impact injury alone. Some pre-existing disease would have been likely in this cohort of horses, according to a recently published survey of Thoroughbred racehorses.

Cell viability, as determined by paravital staining of cartilage, was lowest in the superficial zone, with a significant decrease at the 8-10 month endpoint. This decrease in viability over time could be considered indicative of progressive osteoarthritis, but this is unlikely due to the lack of a significant difference between treatment groups. There was
only mild damage to the middle and deep cartilage zones, where severe damage would be more consistent with severe osteoarthritis. Interestingly, cell viability was highest in the middle and deep zones at 4 months, which may indicate some degree of cell recovery; but could also be explained by variability in impact injury or pre-existing osteoarthritis.

Similarly, the amount of metabolically active cells, as assessed by the MTT assay, showed a strong trend to decrease over time. When combined, the paravital staining and MTT assay results indicate that a mild amount of progressive superficial cartilage damage was occurring, although there was no statistically significant effect of impact injury.

The decreased concentration of sGAG in cartilage from the impact joint lesion site indicated a local effect of focal osteoarthritis due to the impact injury. It is interesting that there was a local effect of sGAG depletion in the cartilage at the impact joint lesion site, but there was no change in the sGAG concentration in impact joint synovial fluid. This finding indicated that the local effect was not significant enough to have an effect on the entire joint. However, in the experience of the authors, focal changes in the palmar metacarpal condyle cause lameness, even without widespread osteoarthritis involving the entire joint. Thus, clinical disease may be caused by focal osteoarthritis in this region.

Higher OARSI scores for impact sections than control sections indicated that there was more histologic evidence of osteoarthritis in impact joints. The scores for impacted medial metacarpal condyles were relatively consistent, with grades ranging from 4.5 to 5.5 by all observers in 10 of the 12 horses (lower scores in horses 3 & 4). This uniformity made it difficult to categorize the horses according to lesion severity, as
had been possible with macroscopic evaluation. The excellent agreement between observers indicated that the OARSI scoring system was consistent and repeatable.

Immunohistochemical evidence of apoptosis was mild (mean score 1.5), with no difference between endpoints. Our model may not have been severe enough to cause progression of apoptosis over time. Interestingly, COL2-3/4C\textsubscript{short} expression was significantly higher at 4 months than at the other endpoints. There may have been some progression of osteoarthritis from 1 month to 4 months, but it would be unlikely for osteoarthritis to improve by 8-10 months. It could be concluded that the 4-month joints may have sustained more severe impact injuries (i.e. the ideal perpendicular impact, rather than a glancing shear impact). However, this conclusion was not supported by our macroscopic classification of the 4-month samples as mild in one horse and moderate in 3 horses. Another explanation for the elevated COL2-3/4C\textsubscript{short} expression at 4 months could be that these horses had more pre-existing disease, although this theory was not supported by baseline lameness or radiographic findings. Alternately, osteoarthritis progression can be phasic; and without ongoing injury from high-speed training, collagen metabolism may have been normalizing after the 4-month time point.

MicroCT data was difficult to normalize due to the wide variety of lesion locations, which made it hard to differentiate between true sclerosis and a normal increase in bone density due to the anatomy of the site. The variability in density of the distal third metacarpal bone according to anatomical location has been well documented.\textsuperscript{30-32} The BMD and TMD values from the medial metacarpal condyle in our study were lower than those reported by Rubio-Martinez \textit{et al}\textsuperscript{20} for Thoroughbred racehorses with mild to severe subchondral bone disease. However, sections analyzed in
that study were located slightly closer to the sagittal ridge than sections in the current study. Alternatively, the lower values in our study may have been due to a lower level of exercise. BMD values in our study were also lower than those for the palmaromedial metacarpal condyle reported by Olive, D’Angou, Alexander et al.\textsuperscript{33} in Thoroughbreds and Standardbreds with a variable degree of osteoarthritis lesions. However, sections in that study were located 1 mm from the osteochondral junction. It is interesting that both BMD and TMD values were highest at 1 month, decreased at 4 months, and increased slightly again at 8-10 months. This finding suggests that initial sclerosis was undergoing remodeling at 4 months and returned to normal at 8-10 months, which would be consistent with the aforementioned COL2-3/4C\textsubscript{short} immunohistochemical data. Although the exact timing of the peak of remodeling after subchondral-bone injury is still unclear, our results would be expected to represent delayed or prolonged remodeling due to exercise or repeated injury, but there is no other evidence of this occurring.

Alternatively, the palmar metacarpal condyle may have a different remodeling pattern than has been observed in other locations in the horse, or in other species. The degree of bone injury may have been small enough that it was completely healed by 8 months, due to the lack of repetitive injury. The highest number of significant differences was observed with BMD, while no significant differences were seen with BVF; suggesting that BMD is the most sensitive indicator of subtle changes in the bone, or that there was a beta error associated with BVF. A confounding factor was that a macroscopic defect in the bone was identifiable in only one horse (horse 2); so as previously mentioned, correlating the lesion location between the 2-dimensional postmortem photographs and the 3-dimensional microCT images was a source of error. In future studies, a radio-
opaque marker could be placed over the site of the cartilage defect in order to accurately identify the impact site, due to the lack of visible evidence of impact on microCT images.

A decrease in Alamar blue values, indicating decreased bone viability, would be expected if the impact lesion affected the bone, but a treatment effect was observed only at the 1-month endpoint at 2 of the later reading times for the assay. A possible explanation for this limited treatment effect may be that the impact injury to the bone was minimal and not ongoing, so the bone could respond completely prior to 4 months. Since the relative fluorescence unit readings did not peak as expected during the assay, additional work may be required to further adapt this technique for assessing viability in equine bone. Although a similar dose of oxytetracycline was used in this study as previously reported, uptake of this fluorochrome was not evident. Explanations for this lack of uptake may be that the fluorescence of oxytetracycline was overpowered by the calcein green, since the fluorescence of these 2 chemicals is similar in color; or that the dose or formulation used was not sufficient in our horses. The difference between our dose of 10 mg/kg and the previously reported dose of 25 mg/kg may have been significant. The number of remodeling osteons would be expected to increase with time as the bone responded to the impact injury; the opposite effect was observed. This finding supports the Alamar blue results, which suggest that the bone only required minimal time to respond to the impact injury. Together these results indicate that the impact injury in this study was insufficient to cause long-term effects in the bone. Additionally, these results suggest that the impact model used in this study caused cartilage damage, rather than bone damage, as the initiating event of osteoarthritis.
The major limitation of our model was the inability to adequately visualize the impactor tip on the cartilage surface. Although the technique described by Byron and Goetz\textsuperscript{17} allowed instrument access to the site of interest on the palmarodistal metacarpal condyle, it was unlikely that a 70-degree arthroscope would allow visualization of this location during hyperextension of the joint, as is required when placing the impactor tip perpendicular to the articular cartilage. An alternative method of creating impact injury to the palmar metacarpal condyle could be to use the proximal sesamoid bones as the mechanism for delivering the impact force, in a similar manner to impacting the patella against the distal femur in other species.\textsuperscript{35}

In conclusion, our impact injury model created mild evidence of focal osteoarthritis in the palmar medial metacarpal condyle. There was some cartilage damage inflicted, but minimal bone damage occurred; so there was limited evidence for progression of osteoarthritis over time. Therefore, additional work is required to develop a more reliable and consistent model for palmar metacarpal disease in horses.
2.6 – Footnotes


b. Alpco Diagnostics Cat# 13-COMP-200, Salem, NH.

c. Northern Eclipse, Empix Imaging Inc Version 6.0, Mississauga, ON, Canada.

d. Vibratome series 1000, Bannockburn, IL.

e. Molecular Probes Cat# S-7575, Eugene, OR.

f. Sigma Cat# E-8751, Oakville, ON, Canada.

g. Sigma Cat# M-2128, Oakville, ON, Canada.

h. Dulbecco’s Modified Eagle Medium, Invitrogen Cat#31600-034, Burlington, ON, Canada.

i. 6.0N, Fisher Scientific Cat# LC153702, Nepean, ON, Canada.

j. eXplore Locus micro-computed tomography scanner, GE Medical Systems, London, ON, Canada.


l. Perkin Elmer, Victor 3 1420, Woodbridge, ON, Canada.

2.7 – References


2.8 – Figures

Figure 1 – The custom-designed aiming device, consisting of the adjustable jig (black arrows) mounted on the modified condylar clamp (black arrowheads), and the impactor tip (white arrows) in place for making an impact injury at surgery. Note that the body of the spring-driven impaction device must be attached to the impactor tip in order to create the impact injury.
Figure 2 – Average lameness score for impact and control limbs of all horses by postoperative week. Error bars represent standard error, with impact error bars in solid black and control error bars in a dotted line. Error bars extending below zero are not shown. Note that all 12 horses are included for weeks 1-4, while 9 horses are included for weeks 5-16, 5 horses are included for weeks 17-33, and 2 horses are included for weeks 36-40.
Figure 3 – Average cartilage oligomeric matrix protein (COMP) levels in synovial fluid from impact (black bars) and control (grey bars) joints over time. Note that the assay was performed on all impact samples at all time points, on all control samples at baseline (12 horses), 180 days (5 horses), and 240 days (5 horses), and on 4 randomly selected control samples at all other time points. Error bars represent standard error. Letters (a-b) indicate significant differences (p < 0.05).
Figure 4 – Photographs of palmar metacarpal condyles at postmortem, demonstrating the range of impact injuries. Horse identification, endpoint, and macroscopic classification are indicated as follows:

A – Horse 1, 1 month, moderate  
B – Horse 2, 4 months, mild  
C – Horse 3, 10 months, mild  
D – Horse 4, 8 months, mild  
E – Horse 5, 8 months, severe  
F – Horse 6, 4 months, moderate  
G – Horse 7, 8 months, severe  
H – Horse 8, 10 months, severe  
I – Horse 9, 4 months, moderate  
J – Horse 10, 4 months, moderate  
K – Horse 11, 1 month, severe  
L – Horse 12, 1 month, severe
Figure 5 – Average bone mineral density (BMD) for combined impact and control limbs at superficial (hatched bars) and deep (solid bars) sites on the medial metacarpal condyle, and at endpoints of 1, 4, and 8-10 months. Error bars represent standard error. Letters (a-e) indicate significant differences (p < 0.05).
Figure 6 – Average total mineral density (TMD) for combined impact and control limbs at superficial (hatched bars) and deep (solid bars) sites on the medial metacarpal condyle, and at endpoints of 1, 4, and 8-10 months. Error bars represent standard error. Letters (a-d) indicate significant differences (p < 0.05).
Figure 7 – Average relative fluorescence unit readings for Alamar blue assay for combined impact (black bars) and control (grey bars) samples from the medial metacarpal condyle. Readings were taken hourly from 3 to 10 hours. A higher relative fluorescence unit reading indicates more viable cells present in the bone. Error bars represent standard error. Star (*) indicates significant differences (p < 0.05) between impact and control.
### 2.9 – Tables

<table>
<thead>
<tr>
<th>Horse</th>
<th>Endpoint (months)</th>
<th>Radiographic score</th>
<th>Additional abnormalities</th>
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<tr>
<td></td>
<td></td>
<td>Impact joint</td>
<td>Control joint</td>
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<tr>
<td></td>
<td></td>
<td>Baseline</td>
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<tr>
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</table>

Table 1 – Radiographic scores of the metacarpophalangeal joints prior to entering the study (baseline) and at endpoint prior to euthanasia. Scoring for evidence of osteoarthritis was as follows: 0 = normal, 1 = minimal, 2 = mild, 3 = moderate, 4 = severe. Osteochondral fragments appeared inactive and were deemed not to be contributing to osteoarthritis. Osteochondral fragments and proximal sesamoid bone sclerosis were similar in appearance at baseline and endpoint.
<table>
<thead>
<tr>
<th></th>
<th>Mean ± SD</th>
<th>Range</th>
<th>Median</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>WBC Count</strong></td>
<td>0.39 ± 1.73 x 10⁹ cells/L</td>
<td>0 - 18.65 x 10⁹ cells/L</td>
<td>0.08 x 10⁹ cells/L</td>
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<tr>
<td><strong>TP</strong></td>
<td>20.2 ± 1.4 g/L</td>
<td>&lt;20 - 34 g/L</td>
<td>20 g/L</td>
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<td><strong>% Lymphocytes</strong></td>
<td>71.2 ± 20.1%</td>
<td>12.5 - 100%</td>
<td>76.0%</td>
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<tr>
<td><strong>% Monocytes</strong></td>
<td>18.7 ± 15.3%</td>
<td>0 - 64.7%</td>
<td>13.9%</td>
</tr>
<tr>
<td><strong>% Seg Neutrophils</strong></td>
<td>7.4 ± 12.1%</td>
<td>0 - 23.1%</td>
<td>2.1%</td>
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</tbody>
</table>

Table 2 – Synovial fluid cytologic analysis results for all horses at all timepoints, including mean ± standard deviation, range, and median. SD = standard deviation, WBC = white blood cell, TP = total protein, Seg = segmented.
Table 3 – Average percent cell viability of impacted cartilage from the medial metacarpal condyle, as assessed by evaluation of paravital staining data, listed by cartilage zone and endpoint. Letters (a-f) indicate significant differences (p < 0.05).
3.0 – LIMITATIONS AND FUTURE AREAS OF STUDY

This study had one major limitation. The physical limitations of the metacarpophalangeal joint in hyperextension, which was required for placement of the impactor tip on the distal aspect of the palmar metacarpal condyle, prevented adequate visualization via arthroscopy or fluoroscopy during impaction. This lack of visualization resulted in significant variability in lesion location and severity due to the inability to confirm placement of the impactor tip exactly perpendicular to the articular cartilage. This variability in impact lesions was the major limitation of this technique, due to the complicating factor of variability in articular cartilage and subchondral bone structure and function based upon location.

Several minor limitations were also present in this study. The unexpected lack of a visible defect in the bone at the site of impact injury on microCT images made accurate identification of the impact site difficult. Measurements were taken from postmortem images in an attempt to overcome this limitation, but there are inherent inaccuracies in transferring measurements from a 2-dimensional image to a 3-dimensional image. This limitation could be easily overcome by placing a radio-opaque marker over the site of the impact injury prior to performing the microCT scan. Another limitation may be that it was not feasible to subject the horses to a high level of exercise consistent with race training. Other impact injury models have not required exercise to make the impact damage progress to osteoarthritis. Lack of documentation regarding performance history prior to entering the study may have influenced the results, since some of the horses may
have raced or trained. Additionally, some of the outcome parameters may have been limited in their ability to detect the minor changes associated with early osteoarthritis.

In future studies, impacting the proximal sesamoid bones against the palmar metacarpal condyles in a closed joint system may prove to be a more reliable and consistent model of post-traumatic osteoarthritis. Theoretically, such a model would more accurately recreate the impact of the proximal sesamoid bones on the palmar metacarpal condyles, which occurs during hyperextension while racing. The experimental impaction process would likely need to be repeated multiple times in order to mimic naturally-occurring disease.
3.1 – GENERAL CONCLUSIONS

The purpose of this study was to create a model of post-traumatic osteoarthritis in the palmar metacarpophalangeal joint using impact injury. Our impact injury model was successful in creating lesions consistent with mild focal osteoarthritis. The majority of the changes associated with osteoarthritis occurred in the articular cartilage, with minimal changes in the subchondral bone. In addition, there were some changes observed in synovial fluid, as a result of articular cartilage degeneration. These findings suggest that osteoarthritis develops first in articular cartilage, rather than subchondral bone, in this impact injury model. This conclusion is in contrast to other studies, which have found that impulse-loading models cause subchondral bone changes first; while joint instability models initially result in articular cartilage damage. Despite placing the instrument portal through the distal sesamoidean ligaments, there should be no reason for joint instability in our model. Therefore, this study calls into question the previous hypothesis that specific types of joint conditions result in articular cartilage damage or subchondral bone damage, which becomes the initial event in osteoarthritis progression. However, since our model did not consistently produce classically defined osteoarthritis affecting the entire joint, we could not entirely refute this hypothesis. Further work is required to answer this intriguing question.
4.0 – MASTER REFERENCE LIST


