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A Clinically Relevant Relevant Post-Traumatic Osteoarthritis Mouse Model

Chantel P. Arce, *The University of Western Ontario*

Supervisor: Dr Dianne Bryant, *The University of Western Ontario*

A thesis submitted in partial fulfillment of the requirements for the Master of Science degree in Kinesiology

A CLINICALLY RELEVANT POST-TRAUMATIC OSTEOARTHRITIS MOUSE MODEL

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by

Chantel Pauline Arce

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The School of Graduate and Postdoctoral Studies
The University of Western Ontario
London, Ontario, Canada

Abstract

Osteoarthritis affects 13-20% of Canadians with the majority being under 65years of age. Post-traumatic osteoarthritis (PTOA) is of great concern in young athletes following knee injury. Current research attempts at modeling the disease fall short. This study aimed to incorporate two important aspects of injury, the nature of the injury and the post-injury standard of care in humans, to a model of PTOA in mice. The study validated a non-invasive protocol to elicit an anterior cruciate ligament (ACL) injury at varying loading speeds addressing the closed capsule nature of an ACL injury that occurs in humans. Secondly, we proposed a stabilization surgery implemented after an ACL transection event addressing the post-injury standard of care often ignored in animal models. This procedure provided protection in mice at ten weeks following the injury. Future research should incorporate the two protocols and create a better model that is more clinically relevant to the field PTOA.

Keywords

Keywords: mouse model, osteoarthritis, post-traumatic osteoarthritis, axial loading, surgical stabilization, ACL injury

Co-Authorship Statement

Myself, Dr. Dianne Bryant, Dr. Alan Getgood and Dr. Frank Beier contributed to the development of my model of post-traumatic osteoarthritis. Dr. Ian Welch helped develop the knee stabilization surgery used. Dr. Alan Getgood and Dr. Alexander El-Warrak rated the severity of knee injuries on microCT. PhD candidate Paxton Moon rated the severity of knee injuries on histological slides. PhD candidates Michael Pest and Anusha Ratneswaran scored osteoarthritis severity on histological slides. Chris Norley at the Robarts Research Institute Imaging Lab processed microCT scans. Animal handling, sample collection, sample preparation, histology, sample randomization, data acquisition and data analysis were my responsibility. I wrote and edited the original copy of this manuscript and Dr. Bryant, Dr. Beier and Dr. Getgood provided suggestions and edits for the final copy.

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Chapter 1

1 Introduction

The prevalence of osteoarthritis (OA) in Canada and around the world is around 20% in females and 10% in males [1, 2] This progressive degenerative disease is characterized by the break down of the cartilage on our articulating joints. Importantly, OA can limit mobility, increase pain and lead to further degeneration of the bone and surrounding tissue.

OA can be divided into two categories: primary and secondary. The cause of primary OA is unknown and often occurs in older individuals. Secondary OA is due to an underlying medical condition or traumatic event thus affecting individuals of any age. A specific type of secondary OA is post-traumatic osteoarthritis (PTOA). PTOA is observed in approximately half of individuals 10-20 years after an anterior cruciate ligament (ACL) injury and or meniscal damage [3]. Therefore, a disease typically associated with old age is manifesting itself in younger individuals and in younger athletes.

Significant understanding and research in the field of musculoskeletal disease, especially OA, has come from animal models. Particularly small rodent models are used because they are easily tractable, have rapid disease progression, require small doses of drugs and are economical [4]. Mice have been used to understand the genetic components of OA, injury pathways, mechanisms of injury, and possible treatments. Unfortunately, most mouse models lack translatability to the human form of the disease.

In PTOA research there are two major limitations to the models proposed. The first being the type of injury used to model a traumatic event. In human ACL injuries there is some mechanism that creates an internal injury however, in mouse models these injuries tend to be invasive. The second limitation is the post injury protocol followed in animals. Typically, humans undergo some reconstructive event following an ACL injury. This intervention is used to decrease further damage that an unstable knee may incur yet, a surgical stabilization is not performed on animals modeling PTOA. Therefore, the goal

of this pilot study was to validate a non-invasive loading protocol to create internal ACL injuries and to propose a surgical intervention that would stabilize an ACL deficient knee.

Chapter 2

2 Literature Review

2.1 OA

OA is a degenerative synovial joint disease. The disease is characterized by focal articular cartilage loss, formation of osteophytes, subchondral bone changes and narrowing of the joint space. Patient reported symptoms such as pain and joint stiffness accompany OA.

The incidence and distribution of OA are wide spread throughout the Canadian population and affect many aspects of the Canadian health care sector. OA affects one in ten Canadians. From the Canadian Community Health Survey of 2010 to 2011, 20% of women and 13% of men reported having arthritis and 56% of those affected were less than 65 years of age [1]. World estimates show a similar trend towards higher prevalence of OA in females, 18.0%, than in men, 9.6% [2]. The economic impact, health care costs and loss of productivity costs, resulting from arthritis are estimated at $33 billion per year and are projected to double in the next 20 years [5].

Some of the factors thought to contribute to the development of OA are systemic such as age, sex, ethnicity and genetic background. Specifically, the incidence of OA increases with age and may be caused by general wear and tear, and the body's decreased ability to repair itself. In addition, females have a greater incidence of OA and are reported to have more severe OA [6]. This trend may be a result of the role estrogen and other hormonal fluctuations play in bone and cartilage health. Ethnic differences in the prevalence of OA are more commonly cited in the hip joint and may be related to anatomical differences in the femoral head and acetabulum. These ethnic specific prevalence rates have not been studied in the knee joint [6]. Additionally, a mutation in the growth/differentiation factors family of genes has been associated with chondrodysplasia, abnormal growth of cartilage in the long bones, and OA [7, 8]. Furthermore, mutations to the collagen X gene have been associated with osteochondral dysplasias which affect the developmental skeleton and

can result in conditions such as dwarfism that eventually develop OA secondary to more severe diseases [9].

More localized risk factors for OA include obesity, anatomical alignment, occupation, physical activity and injury. Specifically, obesity affects the weight bearing joints such as the knee by increasing the forces through these joints. Furthermore, the alignment of the knee joint is important for proper force distribution. If the knee is in varus alignment (bow legged) this can result in medial compartment OA. Conversely if the knee is in valgus alignment (knock kneed), then it can result in lateral compartment OA. Occupation and physical activity can cause repetitive overuse injuries and this damage over time can lead to OA.

2.2 PTOA

PTOA is a form of secondary OA resulting from an injury to the joint. Traumatic injury to the articular joint surfaces is believed to start a cascade of events that leads to the degeneration of the articular cartilage and further deterioration of the subchondral bone. This compromised cartilage becomes stiffer and under tensile forces the cartilage draws more water into the cartilage compromising its shock absorbent properties [10]. These changes in physical properties cascade to further cartilage damage as mechanical stresses are magnified and fibrillations, small vertical erosions, begin to form at the surface of the cartilage [2].

Unlike other forms of OA, PTOA most often affects young athletes; occurring in 50% of individuals diagnosed with an ACL injury and or meniscal damage 10-20 years after the injury [3]. Bone bruises or initial impact injuries to the cartilage leads to atypical mechanical loading and cell death. Following mechanical injury, chondrocytes, the cells that create cartilage, have higher levels of glycosaminoglycan release and apoptotic markers that are consistent with matrix degeneration and OA [11].

2.2.1 Human ACL Injuries

The knee is made up of structures within the region of the distal femur and the proximal tibia. The knee primarily works in a hinge-like fashion to allow extension, flexion and

some rotation in the lower limb. The knee joint is a synovial joint with a synovial membrane that separates the intracapsular and extracapsular components. Intracapsular components include the synovial fluid, meniscus, ACL, and posterior cruciate ligament (PCL). Extracapsular components include the medial collateral ligament, lateral collateral ligament, patellar ligament, oblique and arcuate popliteal ligaments, bursae, patella and muscular dynamic stabilizers. All components of the knee work together to lubricate, stabilize and mobilize the knee. Specifically, the ACL is primarily responsible for the limiting anterior tibial translation.

Injury to the ACL can result from several different mechanisms. The most common mechanism of injury involves a moment of deceleration combined with a change in direction and results in the disruption of the ligamentous structure. This motion is the same as the cutting or pivoting motion seen in soccer, football and basketball. A second mechanism of injury requires an individual to land on uneven surfaces or land with the foot in inversion. This movement is typical in jumping sports such as basketball and volleyball. The third, and least likely, mechanism of injury is force or contact dependent. An anterior, lateral or medial force can result in hyperextension of the knee, valgus collapse or varus collapse respectively and lead to ACL failure [12]. This is common in sports that have impact components such as football and rugby. The ACL is particularly vulnerable at 30 degrees of knee flexion, in valgus alignment and with the tibia in external rotation [13]. Thus any movement that puts the knee into this alignment can result in injury to the ACL.

In addition to the instability in the knee following ACL rupture an initial impact event or bony bruise can also be characterized. The mechanisms of injury for ACL injuries often occur in conjunction with bone contusions [14, 15, 16]. Given that cartilage acts as a shock absorptive component of the knee, damage to the cartilage from bone on bone contact can impair its function leading to disease progression [10]. It has been postulated that an initial impact to the cartilage may lead to PTOA. Conversely, an ACL rupture followed by subsequent bone on bone injury has also been postulated to lead to PTOA. This uncertainty makes the treatment of PTOA more difficult.

2.2.2 Animal ACL Injuries

In quadruped animals the disruption of the ACL is a rare injury. However, quadrupeds can engage in specific movements that make their ACLs susceptible to injury. Reported mechanisms of injury include excessive loading of the hind limb, such as in jumping movements for example; excessive tibial internal rotation, when the toes are pointing inward, and in instances of traumatic hyperextension of the hind limb often seen when leaping off of high elevations and extending the hind limbs [17].

2.2.3 ACL Reconstruction

Surgical reconstruction of the ACL is the most common treatment choice following ACL rupture. Although there are several approaches to surgical reconstruction, the underlying methodology is similar. ACL reconstruction cannot prevent the development of OA, however, it can provide stability to an ACL deficient knee, thus protecting the knee from any further damage to the cartilage and meniscus. This is currently the best option when considering preventative treatment for OA, especially in regards to PTOA.

2.3 Animal Models

Understanding and progress in the field of OA has come in large part from laboratory research. In particular, the development of OA has been studied in small animal models. Animal models are used for their translatability to humans. Although there are obvious difference between animals and humans, researchers use data gathered from animal experiments to provide evidence for new concepts, treatments and theories. Similar to humans, canines develop OA after ACL damage [17, 18] . Although OA can occur spontaneously [19, 20] in mice and large animals, the progression is slow and does not have a 100 percent incidence rate [21]. In OA research, mice and rats are often used as animal models because of the tractability of the disease; the low cost to purchase and house the animals; the rapid disease onset if intervened; the ability to genetically modify mice and the low quantity of drug required for investigative purposes.

2.3.1 Differences and Similarities Between Animals and Humans

The biomechanical features that exist between quadrupeds and bipeds are different. The most notable difference is how humans and mice move around. Humans are bipedal animals that concentrate all their weight on the legs and walk upright. Mice are quadrupeds therefore, they walk on all four limbs and have their weight distributed to all four limbs. More specific differences exist at the knee joint level. Typically, the quadruped knee is loaded in a flexed configuration with the small contact area between the femoral condyles and the tibia. Conversely, in humans functional loading occurs in a fully extended configuration of the knee. This leads to a greater contact area between the femoral condyles and the tibial condyles in bipeds. Mice also have sesamoid fabella bones on the lateral sides of the knee that are only present in 10-30% of humans [22]. These bones are not directly involved in the articulation of the knee however these bones are important in animal stabilization protocols.

There are several similarities that allow researchers to extrapolate findings from animal studies and predict the impact on humans. Although differences in locomotion exist, both humans and mice are plantigrade walkers so they use the plantar surface of the foot. Furthermore, there are similarities in the anatomy and the function of the knee between quadrupeds and bipeds. The knee, in humans, and the stifle, in animals, are used for the flexion, extension and rotation of the lower hind limb.

Of particular interest is the ACL, which is analogous to the cranial cruciate ligament (CrCL). The CrCL has similar functional features to the ACL in that it limits the medial or internal rotation of the tibia with relation to the femur and limits anterior or cranial translation of the tibia with relation to the femur. The CrCL and the ACL are both composed of two bundles that contribute to the different axes of constraint and stability that the ligament provides in both quadrupeds and bipeds. In the human, the ACL is comprised of the anteromedial and the posterolateral bundles whereas in the quadruped, the CrCL is composed of a craniomedial and a caudolateral bundle.

Furthermore, ligaments have high levels of type I collagen. The ligamentous zone in both animals and humans are composed of elongated fibroblasts and parallel collagen

fibrils. In addition, humans and animals have a transition region from the ligamentous midsheath to the boney origins and insertions that contribute to overall ligamentous properties.

The use of animal models is important in studying diseases that affect humans. As such, researchers must choose models that best fit with the criteria of the disease and data collection means. Results and conclusions from animal models should be critically evaluated due to the intrinsic differences between humans and the model subjects.

2.3.2 Mice Models of OA

There are several validated procedures that model OA in mice. These methods range from transgenic manipulations, behavioural modifications, intraarticular injections, surgical interventions and impact dependent models.

2.3.2.1 Transgenic Mice

Transgenic mice are used for OA research to detail the progression of the disease and to identify key genes and proteins that participate in the etiology of the disease. Mice can have genes knocked-out or knocked-in to make the genes nonfunctional or more functional. More complex genetic manipulations can temporally and spatially affect genes so that the genes can be expressed at specific time points in development or expressed only in certain areas of the body. This has allowed researchers to manipulate how much of a gene is expressed, when it is expressed, and where in the organism the manipulation takes place.

For example, Helminen et al. created a line of mice that caused joint degeneration and osteoarthritic changes in knee cartilage [23]. They bread seven generations of mice and selected for one normal gene and one defective gene in the mouse line to create heterozygotes. The mice had an internal deletion of human COL2A1 gene. Collagen II production was not hindered however, the gene product was unable to fold into its functional form and was degraded. This experiment showed the link between collagen degradation and articular cartilage degradation. Other studies looking at collagen IX deficient mouse models have shown similar results in mice with a loss of the gene

yielding osteoarthritic changes in the joint of the animals [24].

In 1996, Glasson et al. characterized a line of "blotchy" mice [25]. These mice developed OA as a result of poor cross-links between the collagen fibrils in the cartilage leading to more collagen breakdown by the enzyme collagenase [25]. These mice developed cartilage defects and Glasson et al. correlated the degradation of collagen to enzymatic processes. The loss of cross linkages changed the composition of the extracellular matrix and this initial disruption can start the cascade of further degeneration seen in OA.

With further analysis into the increased expression of catabolic substrates with cartilage damage, other genetic profiles have been tested. Clements et al. in 2003 explored the role of interleukin-1β, interleukin-1β converting enzyme, stromelysin 1 and inducible nitric oxide synthase [26]. These genes encode catabolic factors that degrade cartilage. These genes were deleted in male mice and were compared to wild type male mice. All mice underwent a partial medial meniscectomy to induce OA. The knees were histologically observed to have developed earlier and more severe cartilage lesions in the knocked-out mice compared to the wild type mice. Since cartilage degeneration was present even in the absence of a catabolic gene, it suggested that although catabolic in nature, the corresponding gene products of the deleted genes were necessary in suppressing other catabolic responses. As such, it can be difficult to create genetic models of an epigenetic disease like OA.

In addition to these genes, the role of interleukin-6 has been studied. De Hooge et al. created a knock-out strain of mice that were deficient in the interleukin-6 gene [27]. Researchers compared the knock-outs to wild type mice. Furthermore, the mice were either housed for 18-23 months to test the aging effects of interleukin-6 or had collagenase injections to induce OA. The aged mice comparison showed an increase in spontaneous OA development in the knock-out group compared to matched wild type controls. The induced OA group comparison showed no difference in the severity of the disease. Thus, de Hooge et al. concluded that interleukin-6 could have a protective effect on OA with aging.

Another target of genetically manipulated mice models is a disintegrin and

metalloproteinase with thrombospondin motifs (ADAMTS) gene. This family of genes has several functions, however, pertinent to OA is its role in aggrecan, a component of cartilage, cleavage. Glasson et al. in 2005, created an ADAMTS 5 knock-out strain of mice and compared it to wild type mice after creating instability in both groups through destabilization of the medial meniscus (DMM) [28]. The knock-out mice had decreased cartilage degeneration when compared to the wild type mice. Glasson et al. concluded that ADAMTS 5 was highly responsible for aggrecan cleavage.

Postulations that OA has an inflammatory component lead to genetic mutations of $CD4^+$ cells. These cells are involved in the immune system and induce further inflammatory responses. Research by Shen et al. attempted to define the role of the CD4 gene by creating a knock-out strain of mice [29]. The investigators compared CD4 knock-outs to wild type mice after ACL transection. The knock-out mice had lower levels of macrophage inflammatory protein, a cytokine associated with OA, and slower cartilage degeneration as compared to a wild type group. Thus the loss of CD4 is protective to the knee after destabilization and the signaling properties of the $CD4^+$ cells were important in OA.

Low-density lipoprotein receptor-related protein 5 (LRP5) mutations in humans have shown a link to osteoporotic and osteoarthritic changes. As such, Lodewyckx et al. tested the effect of the gene in knock-out mice [30]. The LRP5 deficient mice and a wild type group of mice were then exposed to two methods of OA induction, papain and collagenase injection or DMM. The LRP5 deficient mice had low bone mineral density, weighed less and had more cartilage degeneration than their wild type counterparts. The authors concluded that the gene played a definite role in embryonic joint and bone development. As such, the osteoarthritic changes observed were likely due to the developmental role that LRP5 plays and OA is a subsequent product of the bone and joint formation abnormalities.

Most recently peroxisome proliferator-activated receptor gamma (PPARγ) has been used to study the signaling pathway of cartilage homeostasis. Vasheghani et al., used a cartilage specific knock-out of PPARγ to characterize the progression of the disease after

DMM [31]. These mice were compared to wild type mice with functional PPARγ genes. The loss of PPARγ lead to more severe OA changes, chondrocyte cell death and increased inflammatory markers. Thus, the PPARγ gene product and subsequent signaling pathways were important in regulating articular cartilage health.

Although transgenic mice have allowed the development of drugs that target particular molecules in the disease pathway, these models often characterize systemic disease manifestations that are atypical of OA in humans. For example, besides OA Helminen's mice had manifestations of chondrodysplasia and delayed mineralization. Similarly, the "blotchy" mice also had complications with emphysema and aortic aneurysms and the LRP5 deficient mouse developed bones with low bone mineral density. Furthermore, many transgenic mice studies are useful in understanding the intricate details of the disease progression but prove inefficient for human OA modeling.

2.3.2.2 Behavioural Modification Models

Behavioural studies on mice have also led to some interesting models of OA. For example, the immobilization of limbs in animals can lead to articular cartilage degeneration since the maintenance of healthy cartilage requires mechanical loading and muscular activation. The immobilization of a joint decreases the normal loading mechanics and causes nutritional deficits and atrophy of the tissue. These experiments have been performed on rabbits, dogs and rats [32, 14].

Another behavioural modification addresses the correlative relationship between obesity and OA. Griffin et al. used the following three groups of mice: a control or normal diet group, a low fat content diet group and a high fat content diet group [33]. Mice on the high fat diet developed metabolic conditions such as hyperglycemia, hyperinsulinemia, hypertension, and central adiposity all of which are associated with obesity in humans. Furthermore, mice on the high fat diet developed OA and the control group did not.

Although behavioural studies can provide information about how the entire system reacts to an intervention, it becomes difficult to decipher true causative relationships from multifactorial processes and subsequent epigenetic changes observed in the animals.

Thus, the use of behavioural models is limited to systemic diseases and makes it difficult to detect cause and effect relationships.

2.3.2.3 Intraarticular Injection Models

Intraarticular injection models include injecting the joint with substrates known to create instability such as: papain, a proteoglycan degrader; iodoacetate, a glycolysis inhibitor; collagenase, an extracellular degrader; and zymosan, an inducer of degrading enzymes [34, 35, 36, 37, 38]. For example, iodoacetic acid is an inhibitor of glycolysis that can cause the death of chondrocytes, the cells that maintain the cartilage, when injected into the joint. Guingamp et al. worked on rats with varying concentrations of mono-iodoacetate to assess the degenerative changes of cartilage [39]. Rats that were treated were given 0.01mg, 0.03mg, 0.1mg, or 0.3mg of mono-acetate. One group acted as a control and had saline injected instead of mono-acetate. A total of 8 rats per group were used. Rats were assessed 30 days after injection and degeneration of the cartilage was present. The injection decreased the presence of proteoglycans and changed the matrix in the joint. Agents such as cytokines, transforming growth factor and enzymes that target collagen and hyaluronic acid all have a similar effect on the degeneration of the extracellular matrix and articular cartilage.

Intraarticular injections are useful for the identification of important molecules that protect or harm the joint. Further research in the use of injections also shows that the injection of a substrate to induce OA also affects the cruciate ligaments leading to further unintentional damage [40]. Nonetheless, injection models are not representative of the true etiology for human disease progression and introduce the possibility of infection caused by the injection itself.

2.3.2.4 Surgical Interventions

Surgical interventions to mimic destabilization of knee are also used to induce OA in animals. A common procedure in mice models for OA is the ACL transection, which involves a lateral incision from the distal patella to the tibial plateau, medial patellar dislocation to isolate the ACL and then transection of the ACL. Glasson et al. compared this procedure to a ‘no surgery’ and a sham surgery group that left the ACL intact. The

mice that underwent ACL transection had significantly more severe OA scores at four and eight weeks after the surgery [41].

Another method, previously introduced, is inducing instability of the knee through a DMM. This surgery involves a medial incision from the distal patella to the tibial plateau and dissection of the medial meniscotibial ligament. The instability produced is sufficient to observe the progression towards OA. Glasson et al. compared the DMM group to a no surgery and a sham surgery that exposed the joint but did not disrupt the medial meniscotibial ligament. The DMM group was observed to develop OA at four and eight week time points however the progression towards OA was slower than in ACL transection models. These two procedures provide a plethora of information with relevancy in the progression of OA post meniscal injury and post ACL injury. Nevertheless, the invasive nature of exposing the synovial capsule may occlude true changes seen in humans who have meniscal injuries and or ACL injuries without invasive joint capsule opening.

2.3.2.5 Impact Modeling

PTOA, which affects a younger demographic than is typically seen with OA, is poorly understood and only recently have models attempted to account for this subcategory of OA. Impact modeling involves using force to create an injury to a specific joint in a uniform and reproducible way. The impact modeling of OA attempts to mimic the actual mechanism of injury in PTOA. Injury to the knee often causes damages to the subchondral bone in humans. Furman et al. proposed a mouse model to address said bone changes in the development of OA [42]. The model created a closed tibial plateau fracture using an indenter to apply 55 N of force at a rate of 20 N/s. Although this mouse model creates an appropriately injured knee with non-invasive surgical intervention, it falls short of mimicking the human etiology. The progression towards OA or specifically PTOA may not be due to a single blunt impact to the tibial plateau rather, it may be the result of a biomechanical instability which creates subchondral and cartilage damage because of the abnormal loading patterns incurred.

Poulet et al. developed a model that used multiple impact forces to achieve OA

progression [43]. Mice were placed into an apparatus that loaded the knees while in flexion. The mice underwent one of five different protocols that varied from one loading event to 15 loading events with varying time points from one day to 5 weeks. One loading event consisted of 40 cycles of mechanical force. The use of multiple bouts of force was non-invasive and made the study pragmatic for human translational purposes. This method of OA induction is more representative of long-term degenerative changes seen with overuse or aging and not PTOA.

To control for extraneous changes due to open surgery Christiansen et al. developed an axial loading apparatus that recreated a subluxation event resulting in an ACL deficient knee [44]. The mice were laid prone in a contraption with their knee flexed to 90 degrees and their ankles lined up directly over the knee and flexed at 30 degrees. A single bout of axial load was applied and mice were returned to regular housing and activity. Subsequent osteoarthritic changes were observed at six time intervals spanning from one day to eight weeks post injury. A follow up study by Lockwood et al. investigated the effect of varying speeds and the resulting injury [45]. Specifically, they compared a low rate, 1 mm/s, and a high rate, 500 mm/s, injury model and concluded that low rates resulted in avulsions while the high rate loading resulted in midsheath ACL rupture. These non-invasive, single bout models more closely resemble ACL injuries in humans and addresses the subchondral and cartilage changes incurred from a compromised knee that were not considered in the Furman et al. model. Although the mechanism of injury, axial load while in 60 degrees of knee flexion and 30 degrees of ankle flexion, is not the same as humans there are several similar features including a subluxation event, possible bone injury and a reduction that leaves the joint capsule intact that are relevant in the study of PTOA.

2.3.3 Treatment of OA in Animals and Relevance of Models

To properly assess the potential effect of new interventions in preventing PTOA following ACL rupture using an animal model, it is not only important that the mechanism of initial injury be comparable to the mechanism of injury observed in human injury, but that the model also factor in the common practice of reconstructing the ligament surgically. It is possible that the effect of some interventions is mediated by the

stability of the joint and subsequent avoidance of secondary injuries all of which contribute to PTOA. Even the most relevant models like impact models may be under representing a treatment's true effect by not first stabilizing the joint.

In canines there are surgical options to correct for CrCL deficiencies comparable to ACL surgical options in humans. Canines that display severe tibial plateau angulation can undergo tibial osteotomies to correct the tibial plateau angle [17]. This procedure takes a portion of the proximal tibia and rotates it caudally. A CrCL deficient joint that undergoes a tibial plateau leveling osteotomy will have a decreased cranial thrust and stabilization restored. Another tibial osteotomy technique advances the tibial tuberosity to decrease cranial thrust in an unstable knee. Knee stability can be achieved through CrCL reconstruction with intracapsular stabilization techniques using ligamentous structures or synthetics. Extracapsular stabilization can also achieve stability in CrCL deficient knees in canines. The lateral fabella is used as an anchorage site, then the suture is passed under the patella ligament and through the tibial tuberosity from the medial to the lateral side. The suture is tensioned in flexion and fastened. The extracapsular technique achieves short-term stabilization that allows the ruptured CrCL to tact down on itself and this provides long-term stabilization [17].

Novel interventions are currently in development and may provide answers on how to prophylactically inhibit PTOA with application at the time of initial injury. Molecules such as P188 surfactant, bone morphogenetic protein OP-1 [15] and fibroblast growth factor-18 have shown promise for cartilage repair and protection in both in-vitro and in-vivo models. An impact model that includes return of joint stability in the mouse that develops OA quickly may be an important contribution toward the investigation of these types of intervention as a first step in demonstrating efficacy prior to human trials.

2.4 Summary

The knee joint is composed of articulating bones that achieve stability through both static and dynamic soft tissue systems. These controls are required for normal joint loading and normal articulation. There are differences in the locomotion and function of structures when comparing biped and quadruped animals nevertheless, analogous

structures allow scientists to interpret data and connect the two systems. In particular, the use of animal models is crucial in medical advancement in the field of OA.

Murine models of OA are created to address the gaps in scientific knowledge. Genetically modified mice provide information about particular genes and protein interactions that affect important anabolic or catabolic pathways. Behavioural modifications simulate systemic and overuse models of OA that are helpful for the understanding of idiopathic or primary OA. Invasive interventions using injections and surgical transections are used for traumatic models of the disease. Unfortunately, the invasive nature of any surgery calls into question what degree of the disease progression is a result of surgery or the intended instability. A recent axial loading model addressed the need for non-invasive procedures to mimic a ligamentous injury in humans.

A shortcoming in all animal models has been the lack of intervention after establishing an injury. In humans, reconstructive surgery after a ligamentous injury adds stability back to the disrupted knee. Comparable surgical options exist for canine and other quadrupeds. Yet, this reconstructive element is not observed in the scientific models of OA, specifically PTOA. This clinically relevant procedure may provide a better understanding for how and why certain promising treatments do not reach clinical trial status.

Chapter 3

3 Objectives

3.1 Axial Loading Validation

The primary objective of this project was to validate a non-invasive model of PTOA using the Instron® materials testing machine. Secondary objectives included analyzing the effects of speed on the injury inflicted and assessing the agreement between microCT ligament findings and histology findings.

3.2 Surgical Stabilization Model

The primary objective of this project was to develop a surgical intervention that could introduce stability to the mouse knee following ACL transection. Secondary objectives included determining the effects of the stabilization at five and ten week time points in histological findings and gait parameters.

Chapter 4

4 Axial Loading Validation

4.1 Introduction

OA is a degenerative disease that affects 20% of Canadian women and 13% of Canadian men [1]. Of these Canadians, 56% are under the age of 65. Unlike other forms of OA previously studied, PTOA is a form secondary OA that affects a younger demographic. Thus, increasing research in the field of musculoskeletal health has attempted to study and treat this progressive disease.

Animal models have long been used to model human disease. In the field of OA many different rodent models have been used. Models in transgenic manipulations [23, 25, 26, 27, 29, 30, 31], behavioural modifications [32, 14, 33], injectables [34, 35, 36, 37, 38], and surgical interventions [41] have dominated the field of OA animal research. Recently, work done by Christensen et al. [44] and Lockwood et al. [45] has provided the field of OA with a new model to characterize PTOA specifically.

The purpose of this study was to validate the protocol proposed by Christensen et al. in 2012, and Lockwood et al. in 2013. Additionally, we tested the agreement between raters and between micro-computer tomography (microCT) and histology.

4.2 Methods

C57BL/6 Mice

Male C57BL/6 mice were obtained from Charles River (MA, USA). Mice were obtained at 46-64 days of age. Mice were housed in traditional shoebox cages with one to four mice per cage and given the usual house and bedding for enrichment. C57BL/6 mice were used because their genome is sequenced, they are genetically stable, and are a general purpose strain allowing for comparison with other studies that use the same animal. Sample sizes were based on the minimum number of animals deemed ethically appropriate by the Animal Care and Veterinary Services Research Ethics Board at the

University of Western Ontario and the minimum number of animals required to set up a pilot study.

Axial Loading

Twelve mice were anaesthetized using isoflurane and placed in the axial loading device, the Instron® materials testing machine (Instron®, Norwood, MA; model: 8874), with continued administration of anaesthesia through a tube mounted onto the device. The mice were placed prone with three of four limbs supported by a platform and the fourth limb, the right hind limb, supported by the knee cup. The right hind limb was flexed to approximately 60 degrees of knee flexion and ankle was positioned directly above the knee with approximately 30 degrees of dorsiflexion and positioned into the device (Figure 1A). This orientation in addition to the axial load caused the tibia to subluxate anterior to the femur (Figure 1B). The loading cell was preset to 2N and then the computer software Wavematrix(TM) (Norwood, MA) applied 12-14N of force at a rate of 1 mm/s, 250 mm/s and 500 mm/s. Total vertical displacement of 2 mm was achieved.

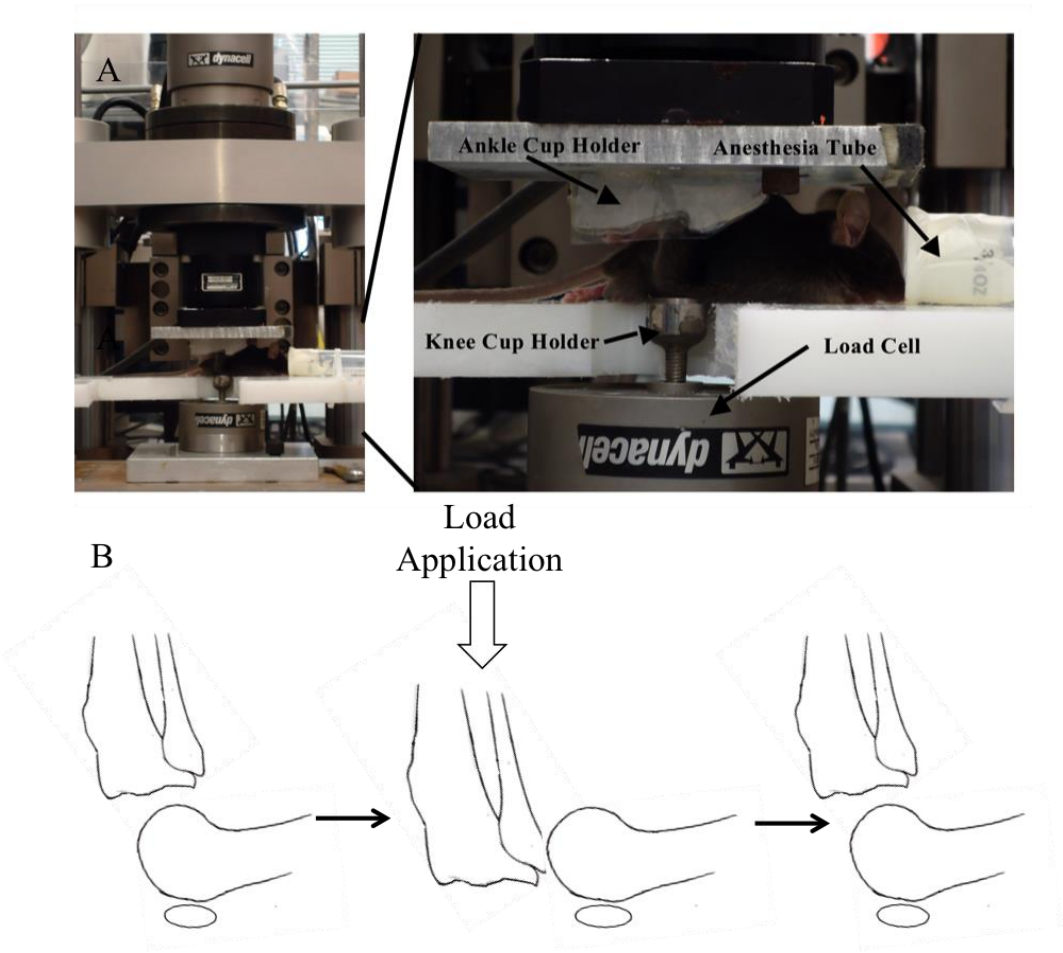

Figure 1: Loading Set-Up. The Instron material testing machine ® was used to create ACL injury in the mice (A). The mice were placed in a prone position. In a close up, the mouse can be seen with its right hind limb placed into the knee and the ankle holders. A schematic representation of the mouse knee before being loaded, during axial loading when the tibia is subluxed and after the load was removed (B).

Five mice underwent the 1 mm/s protocol, four mice underwent the 250 mm/s protocol and four mice underwent the 500 mm/s protocol. A single loading procedure took less than a minute. Mice were closely monitored for signs of discomfort (limping, hunching, failure to groom) and given 0.05 mL buprenorphine for pain at the time of injury and one day post loading. Mice were euthanized one day after the injury using CO_2.

The preset loaded parameters of this experiment were reviewed to ensure accuracy. Forces measured during the loading were equivalent to those of previous studies [45, 44].

The speed of the machine was important in determining the causative relationship between the injury type and the different preset values. Analysis of the initial vertical displacement to time output given by the software measured the accurate speed of the machine. Trials set at 1 mm/s ran on average at 1.00 mm/s, trials set at 250 mm/s ran on average at 299.19 mm/s and trials set at 500 mm/s ran on average at 356.99 mm/s.

Dissections

Mice knees were dissected and harvested for tissue processing. The knee was exposed using a lateral incision to allow photographs of the knee using the Leica DFC295 camera and Leica Application Suite software version 3.8.0 (Leica Microsystems, Richmond Hill, ON, Canada). Knees were dissected in phosphate buffered saline (PBS). The femur was cut proximal to the hip and the tibia was cut proximal to the ankle. Excess muscle tissue was removed without disturbing the knee capsule. Knees were fixed in 4% paraformaldehyde (PFA) for 24 hours before further tissue enhancement for microCT scanning.

Tissue Enhancement

Knees underwent serial dehydrations in 30%, 50% and 70% ethanol before being soaked in a 2.5% solution of phosphotungstic acid (PTA) dissolved in 70% ethanol [46]. Knees were soaked for four days with the PTA solution and the solution was refreshed on day two. This protocol was used to enhance the contrast imaged in the microCT scans and optimized to ensure sufficient solution penetration into the synovial capsule. PTA in solution is preferentially taken up by soft tissue and binds to collagen that is present in ligaments [46]. PTA allows the ligaments to absorb the x-rays making the soft, poorly imaged ligament appear brighter and detectable under microCT since microCT is a reconstruction of multiple x-rays.

Tissue Processing

Following the contrast enhancement and microCT scanning the knees were placed in ethylenediaminetetraacetic acid (EDTA) on a rocker at room temperature for decalcification. EDTA solution was changed every two days a total of four times.

Decalcification was determined by physical end-point testing. Once tissue was sufficiently decalcified samples were placed in 70% ethanol and refrigerated before processing. The right knees of all axial loaded mice were collected as well as three random contralateral knees.

All harvested knees were sent for processing at the Molecular Pathology Laboratory at Robarts Research Institute (London, ON, Canada). Knees were dehydrated, cleared, and infiltrated with paraffin wax. Knees were embedded on the sagittal plane in paraffin wax for microtome sectioning and histological sectioning.

Histology

Axial load applied on the mouse stifle has shown ACL disruption and subsequent OA development in mice [41, 42]. Lockwood et al. demonstrated that the severity of the ligament disruption was a function of the speed that the load was applied. The fast, 500 mm/s, impact model demonstrated midsheath ruptures while the slow, 1 mm/s, impact model resulted in ligament disruptions and avulsions on the tibia. This was confirmed through microCT and histological findings.

Knees embedded in paraffin wax were sectioned at Robarts Research Institute (London, ON, Canada). Serial sections 5µm thick were collected onto slides and dehydrated overnight in an oven at 40 degrees Celsius. Slides were dewaxed using two five-minute xylene solution washes. Slides were then rehydrated using 100%, 95%, and 70% ethanol solutions followed by tap water rising. After dewaxing, the slides were stained with Safranin-O and Fast Green.

Slides were dipped in 50% hematoxylin for 15 to 30 seconds and immediately rinsed with tap water until water flowing off was clear and then dipped in tap water for five minutes. Slides were placed in 0.02% Fast Green solution for 30 minutes for back ground staining. Slides were dipped in 1% Glacial Acetic Acid for no more than 15 seconds. Slides were exposed to 1.5% Safranin-O solution for three and a half minutes then subsequently dipped twice in distilled water to remove excess stain. Slides were dipped three to five times in 70%, 95%, 100% and another 100% ethanol solution and then in two xylene

solutions to dehydrate the samples. Slides were allowed to air dry for five to ten minutes before mounting a coverslip with a xylene based mounting medium. Cartilage and proteoglycans stain red and other tissue such as matrix and bone stain green.

Knees were imaged with a Leica DFC295 camera, Leica DM1000 microscope and Leica Application Suite software version 3.8.0 (Leica Microsystems, Richmond Hill, ON, Canada). An experienced rater was given randomized sagittal sections of 16 mice knees to rate as ACL intact, PCL intact, avulsed or normal. A non-rater reordered the slides and created new mouse ID labels on glass slides to randomize. A randomization key was kept separate from slides at all times. The rater was blinded to the number of knees per group and to the expected results of the experiment.

MicroCT

Knees were embedded in 1% agarose gel to stabilize for scanning protocol. Knees were scanned on the GE Locus RS microCT (GE Healthcare, London, Ontario, Canada). Samples were scanned for six and a half hours using a 450 μA tube current, 80kVp x-ray spectrum, with 900 views, 0.4° increment angle per view and 4500 ms exposure time. Images were reconstructed with 20μm isotropic voxels with five frames averaged per view. The software used to analyze the images was the MicroView software (version ABA 2.2, GE Healthcare, London, Ontario).

Two clinicians, a veterinarian and an orthopaedic surgeon, rated the degree of knee injury on the knees based on the micro CT scans. Raters assessed randomized scans and rated them as ACL intact, PCL intact, avulsed or normal. Raters were blinded to the number of animals in each group. MicroCT images were reordered, relabeled and cropped so that only one knee was visible at a time.

Statistics

All statistical analysis was performed using SPSS (IBM, V.22.0, Armonk, New York, USA). MicroCT data was analyzed for agreement between the independent raters using Cohen's kappa. Agreement between microCT and histological findings was assessed

using a Fleiss kappa for agreement among three raters. Values reported included kappas, p values and 95% confidence intervals (CI).

All of the statistical analysis was underpowered because of our small sample sizes. Thus, we suggest that the CI and general trends should be used to interpret the findings.

4.3 Results

Histology

Sagittal slides were used to assess ACL tears. The ACL was torn at all three loading speeds (Figure 2). Arrows on Figure 2 indicate the ends of the torn ACL or the region where the ACL is torn. To assess the integrity of the PCL we compared it to normal PCLs of other knees (Figure 3 A-C). Figure 3A is a normal knee with a line superimposed to characterize the shape of a normal PCL. Figure 3B is the PCL with a superimposed line to characterize the common buckling effect on the PCL when the knee is ACL deficient (Figure 3 C-F). The PCL was disrupted in one knee at 1 mm/s, two knees at 250 mm/s and two knees at 500 mm/s (Figure 3 D-F). Avulsions were reported in four knees that underwent 1 mm/s, two knees that underwent 250 mm/s and four knees that underwent 500 mm/s. Normal knees or contralateral knees were correctly identified as having no ligament disruptions and no avulsions.

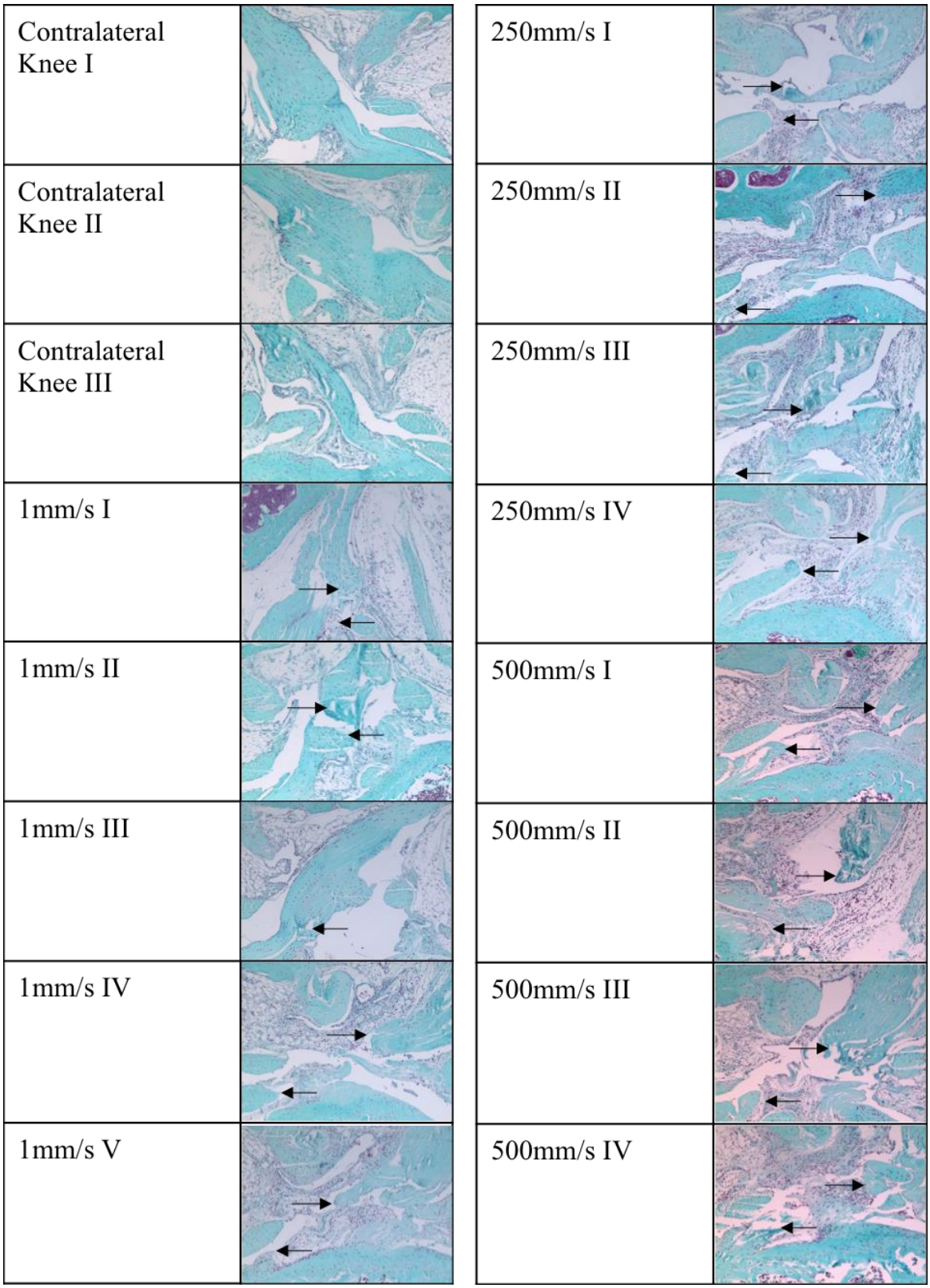

Figure 2: ACL Disruptions. Histological slides were stained with Safranin-O and Fast Green. Intact and torn ACLs were visualized in the sagittal plane with arrows indicate the ends of the torn ligaments.

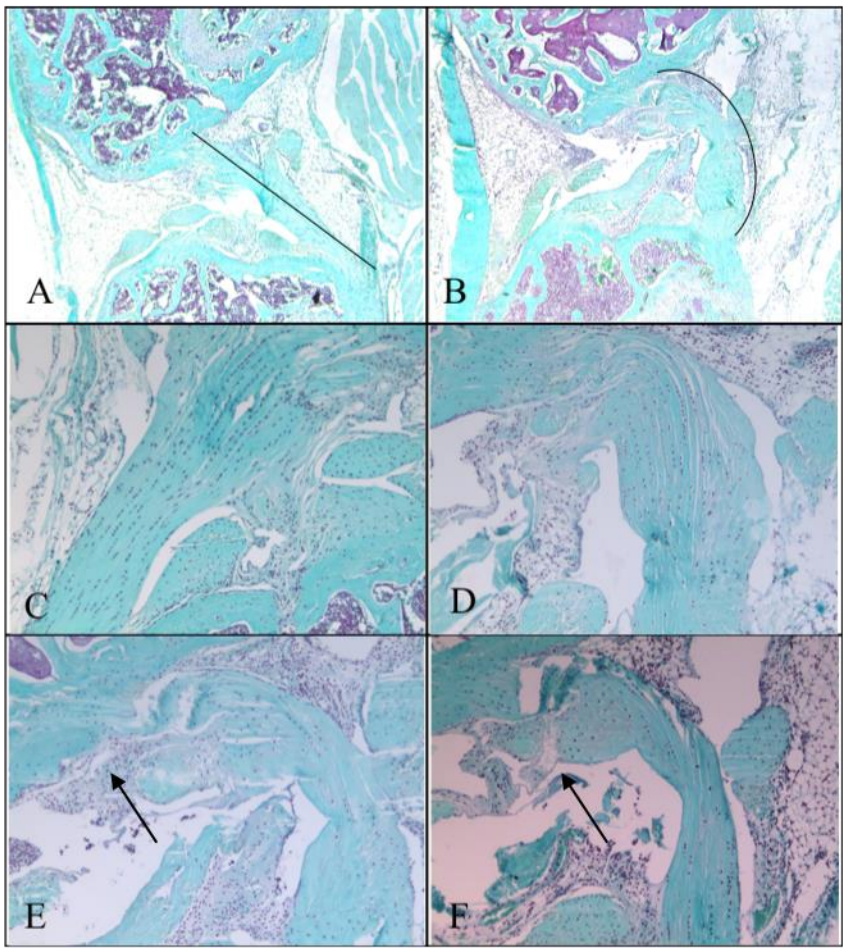

Figure 3: PCL Loading Injuries. Histological slides were stained with Safranin-O and Fast green. Knees were sectioned on the sagittal plane to visualize the ligaments. For comparison a normal knee with a superimposed line to visualize its orientation (A) and a typical ACL deficient knee with a "buckling" PCL with a superimposed line (B) are shown. A non-loaded knee (C) and knees loaded at 1 mm/s, 250 mm/s, and 500 mm/s respectively (D-F) were rated for damage with arrows indicating areas of PCL damage.

Micro CT

Randomized scanned knees were graded for injury type. The absence of the ACL, the absence of the PCL, the presence of an avulsion, and normal knee identification were used to grade the knees. The agreement between the raters in identifying a ruptured ACL

(Table 1), a ruptured PCL (Table 2), an avulsion (Table 3) and a normal knee (Table 4) were the following: 11/15, 12/15, 6/15 and 7/15 respectively. All tables had observed ACL ruptures for micro CT and histology grading of knee loading protocol and the respective agreements between raters and between two objective measures. Values are shown as total observed divided by total samples per group.

Table 1: ACL Absolute Agreement

	Micro CT			Histology
	Rater A	Rater B	Agreement	
0 mm/s	1/2	1/2	2/2	0/3
1 mm/s	2/5	3/5	3/5	5/5
250 mm/s	3/4	4/4	3/4	4/4
500 mm/s	3/4	3/4	2/4	4/4

Table 2: PCL Absolute Agreement

	Micro CT			Histology
	Rater A	Rater B	Agreement	
0 mm/s	0/2	0/2	2/2	0/3
1 mm/s	0/5	1/5	4/5	1/5
250 mm/s	1/4	0/4	3/4	2/4
500 mm/s	1/4	0/4	3/4	2/4

Table 3: Avulsion Absolute Agreement

	Micro CT			Histology
	Rater A	Rater B	Agreement	
0 mm/s	0/2	2/2	0/2	0/3
1 mm/s	0/5	3/5	2/5	4/5
250 mm/s	2/4	4/4	1/4	1/4
500 mm/s	1/4	2/4	3/4	4/4

Table 4: Normal Knee Absolute Agreement

	Micro CT			Histology
	Rater A	Rater B	Agreement	
0 mm/s	1/2	1/2	1/2	3/3
1 mm/s	3/5	4/5	4/5	0/5
250 mm/s	1/4	4/4	1/4	0/4
500 mm/s	1/4	4/4	1/4	0/4

Agreement

The raters agreement on ACL ruptures on microCT had a Cohen's kappa=0.41 (p=0.10), 95% CI (-0.05, 0.88). The raters agreement on PCL ruptures on microCT had a Cohen's Kappa=-0.10 (p=0.69), 95% CI (-0.24, 0.04). The raters agreement on the presence of an avulsion had a Cohen's kappa=0.17 (p=0.24), 95% CI (-0.05, 0.39). Agreement between microCT observations and histological observations had a Fleiss kappa=0.20, 95% CI (-0.09,0.50) for ACL rupture observations. PCL disruption agreement had a Fleiss kappa=-0.06, 95% CI (-0.36, 0.23). Finally, the avulsion agreement had a Fleiss kappa=-0.07, 95% CI (-0.36, 0.22).

Subgroup analysis of rater A and histology observations yielded a Cohen's kappa=0.06 (p=0.76), 95% CI (-0.35, 0.47) for ACL rupture agreement, a Cohen's kappa=0.12 (p=0.59), 95% CI (-0.35, 0.58) for PCL rupture agreement, and a Cohen's kappa=0.00 (p=1.00), 95% CI (-0.32, 0.32) for avulsion identification agreement.

Subgroup analysis of rater B and histology observations yielded a Cohen's kappa=0.19 (p=0.42), 95% CI (-0.34, 0.718) for ACL rupture agreement, a Cohen's kappa=0.13 (p=0.46), 95% CI (-0.35, 0.10) for PCL rupture agreement, and a Cohen's kappa=-0.11 (p=0.68), 95% CI (-0.58, 0.37) for avulsion identification agreement.

4.4 Discussion

The first objective of our work was to validate the loading protocol and determine how three different speeds affected the type of knee injury incurred. Previous work had demonstrated the use of an axial load to create ACL injuries. Furthermore, speed determined the type of injury created where faster speeds of 500 mm/s created midsheath tears and slower speed of 1 mm/s created ligament disruptions with avulsions present [41, 42]. We hypothesized that although high speeds were necessary for midsheath disruptions an intermediate speed would lead to similar outcomes in injury as the fastest set speed.

Thus, we employed three speeds to determine how 1 mm/s, 250 mm/s and 500 mm/s affected the knee joint. We expected that the 1 mm/s protocol would result in avulsions and ACL disruptions. Our results determined that all knees had ACL tears and the 1

mm/s group had avulsions in four out of five knees. At 250 mm/s we hypothesized that the ACL would have midsheath ruptures and no avulsions. When assessed all knees had ACL ruptures and half the knees had avulsions. The fastest speed was predicted to have ACL tears and no avulsions present. Our results showed that all knees in this group did have ACL disruptions but all the knees also had avulsions present. Although the loading protocol did produce ACL damage, the presence of an avulsion was not consistent with previous findings. In fact, the fastest and slowest speeds resulted in avulsions. With the intermediate speed leading to two of four knees having an avulsion.

Assessment of the PCL was also determined. Previous work on this model did not report damage of the PCL however, upon assessing the PCL in our project we determined that the PCL was also damaged. There was one knee in the 1 mm/s cohort, and two knees each on the 250 mm/s and 500 mm/s cohort that sustained PCL damage. This additional damage could result in non-uniform injuries and inconsistent injury patterns.

Our second objective was to compare the use of microCT to histological observations of the same knee joints to assess the practicality of using microCT in determining knee integrity following an injury. To assess agreement between observers we performed a Cohen's kappa. In all cases the kappas were non-significant and had poor (kappa >0.2) or fair agreement (kappa=0.21-0.4) between rater A and rater B. In addition, a negative kappa was obtained when comparing PCL disruption agreement, suggesting that there was no agreement or that agreement occurred less often than expected by chance. As such, this kappa suggests that the microCT is not appropriate for identifying injury to this ligament under the given protocol used. This could explain why the PCL damage was not reported in previous work since the microCT is a poor tool for this characterization.

To compare the agreement between the microCT and the histology results we calculated Fleiss kappa followed by a subgroup analysis to determine if either microCT rater had a better agreement with the histology observations than the other rater. Differences in agreement could be due to level of experience and or exposure in the field of animal x-rays. Fleiss kappa values were non-significant and there were two negative values indicating that the identification of a PCL disruption and an avulsion had ineffective

agreement and the use of microCT compared to histology is poor for assessing these injuries. Our subgroup analysis on the individual raters compared to the histology observations also resulted in non-significant p values and poor kappa agreements. Additionally, negative kappas were obtained for rater two when compared to the histological observations. There were no statistically significant differences between the two microCT raters implying that the agreement was poor and although contrast enhanced, the microCT may be a poor modality for the identification of ligamentous injury post axial loading in the mouse knee.

Limitations

Our small sample size was a limitation to interpreting our results. In animal trials sample sizes tend to be small which can decrease our ability to detect differences and change. Thus an increase in sample size could make our statistical findings more robust and increase our confidence in the trends we observed thus far.

Another limitation encountered was the need to optimize the staining protocol for the microCT imaging. This resulted in the knees being stained twice and scanned twice. Although the knees were fixed prior to the staining this additional time and agitation of the specimens could have lead to unexpected damage and unexpected changes which could account for the differences we observed in our experiment and previous work.

Lastly, previous studies looking at the axial loading of mouse knees to create an ACL injury used the Bose Electro- Force 3200 machine. This machine operates with electromagnetic components while the Instron machine operates through hydraulics. As stated previously, we did not reach the maximal speeds we hoped to achieve with this loading protocol. The hydraulics in the Instron may have contributed to a slower than expected speeds that were achieved. Ultimately the change in the machine type and model may have other repercussions on the validation of this axial loading protocol.

Future directions

This study demonstrated some unexpected outcomes associated with the axial loading of the mouse knee. As such, a larger sample size could be used to determine whether the

PCL and avulsion trends are true trends that have not been reported previously. In addition, work to compare the loading in the Instron to the Bose loading machines could also clarify whether our findings can be compared to those in previously published work. If the machines are comparable then our results would add more support to the work in this area of injury modeling.

Further investigation into the longitudinal effects of such an injury is important for a comprehensive understanding of our model. Given the differences we observed between our model and previous models it would be beneficial to follow the mice out to appropriate time points to observe any differences that could be attributed to the additional ligament damage observed. Particularly, disease specific pathways and changes in behaviour following the injury would benefit the field of musculoskeletal research and lead to better animal modeling of human OA.

The cause of PTOA has been linked to the instability in the knee after an injury as well as bony bruises or an initial bony impact at the time of the injury. This axial loading protocol is conducive to creating bony bruises as the tibia subluxes relative to the femur. Thus, future studies of this loading model should visualize the articular surface to see if there are bony bruises similar to those seen in humans. This additional piece of information could make the model even more translatable to the human disease.

Application of this model for knee destabilization should also be used for the further understanding of post-traumatic osteoarthritis. This model can be used to investigate the inflammatory pathways already studied to better understand the human disease progression of patients who have ligament damage. In addition, it would be of interest to investigate different genetically protective mouse lines to quantify the protective nature of these genes in a more pragmatic model of injury such as this one.

Chapter 5

5 Surgical Stabilization Model

5.1 Introduction

The use of animal models in translational research is common. These models have provided insight into the development, progression and treatment of human diseases. As such the creation and modification of these models is essential to create more robust and accurate models. OA is a musculoskeletal disease that affects 13% to 20% of Canadians [1]. Of particular interest is PTOA that affects up to 50% of individuals diagnosed with an ACL injury and or meniscal damage 10-20 years after the injury [3]. Use of animals has long been employed in the field of OA however, current rodent models lack an intervention that resembles restored stability like that seen in humans.

We proposed a surgical intervention to restore stability in the mouse knee following ACL transection. Furthermore, we assessed differences on histological slides and gait parameters on mice at five and ten weeks post intervention.

5.2 Methods

C57BL/6 Mice

Male C57BL/6 mice were obtained from Charles River (MA, USA). Mice were obtained at 46-64 days of age. Mice were housed in traditional shoebox cages with one to four mice per cage. Mice were given the usual house and bedding for enrichment. C57BL/6 mice were used because their genome is sequenced, they are genetically stable, and are a general purpose strain allowing for comparison with other studies that use the same animal. Sample sizes were based on the minimum number of animals deemed ethically appropriate by the Animal Care and Veterinary Services Research Ethics Board at the University of Western Ontario and the minimum number of animals required to set up a pilot study.

Surgical Destabilization

Ten C57 black mice underwent surgical transection of the ACL under general isoflurane anaesthesia. Sterile technique was used to make a skin incision over the lateral side of the right knee. The subcutaneous tissue was dissected and the deep fascia was separated exposing the lateral side of the joint. The patella was moved medially to further expose the joint capsule and the ACL. Both the joint capsule and the ACL were cut under microscope and anteroposterior instability was confirmed (Figure 4 A-D). Four mice were left as non-stabilized controls and the joint was flushed with warm physiological saline and the deep fascia was imbricated and closed with a 5-0 prolene suture. The subcutaneous layer was closed with 5-0 vicryl and 3M Vetbond (no.1469 SB) tissue adhesive. Anaesthesia was turned off and the mouse was allowed to recover after an injection of ampicillin and buprenorphine subcutaneously. Mice were closely monitored the day of surgery and provided with a second dose of analgesic, buprenorphine, 24 hours postoperatively. Mice were checked daily for signs of discomfort.

Surgical Stabilization

The remaining six mice underwent ACL stabilization. The surgical intervention was chosen based on canine ACL repair techniques. A 5-0 prolene suture was used to stabilize the stifle joint using an extracapsular technique. A lateral approach was used to identify the lateral fabella and a non-absorbable (5-0 prolene) suture was placed around the fabellofemoral ligament. The suture was run under the patellar tendon to the medial side of the tibia. A 30-gauge needle was used to drill a hole in the tibial crest, proximal to the patellar ligament. The suture was passed from the medial side of the tibial crest hole to the lateral side. The knee was flexed to 90^{o} and the suture was tied down and tensioned on the lateral side. The joint was flushed with warm physiological saline and the deep fascia was closed with a 5-0 prolene suture. The subcutaneous layer was imbricated and closed with 5-0 vicryl and 3M Vetbond (no.1469 SB) tissue adhesive (Figure 4 E-H). Anaesthesia was turned off and the mouse was allowed to recover after an injection of 0.333 μL ampicillin and 0.05mL buprenorphine subcutaneously. Mice were closely monitored the day of surgery and provided with a second dose of analgesic,

buprenorphine, 24 hours postoperatively. Mice were checked daily for signs of discomfort. Three mice were euthanized at five weeks and three at ten weeks postoperatively using CO_2. The remaining four mice were euthanized at five weeks and at ten weeks postoperatively using CO_2.

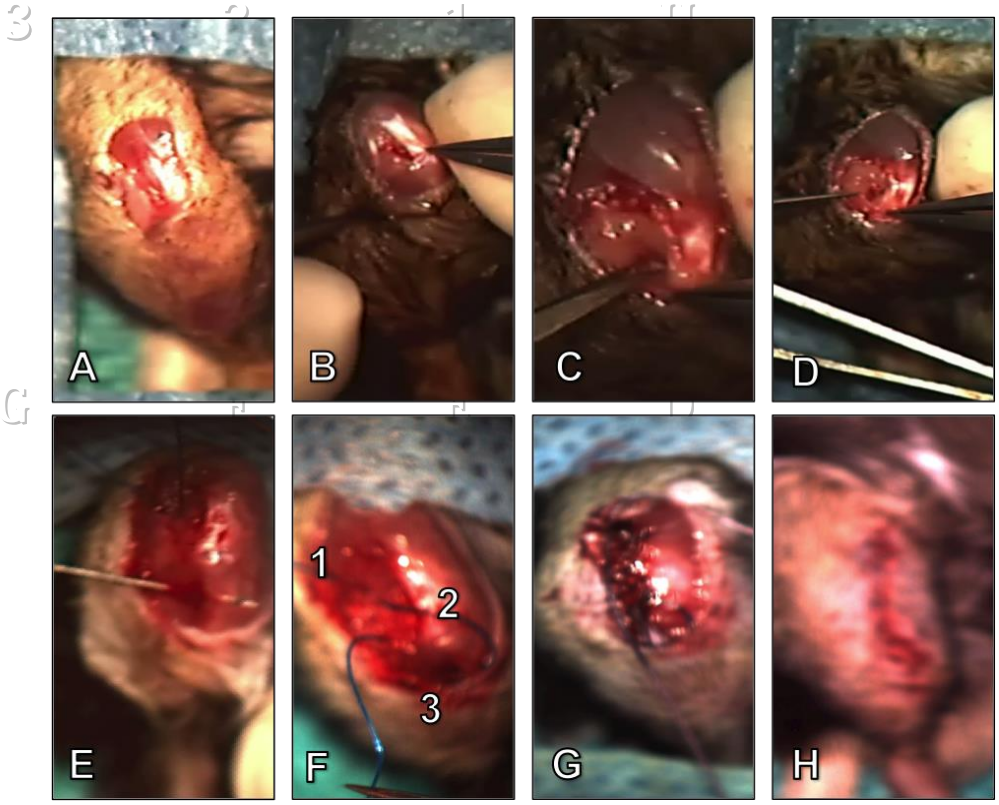

Figure 4: Surgical Intervention. ACL transection was used to destabilize the mouse knee (A-D). (A) Initial lateral incision; (B) incision to expose the joint at the lateral side of the patella; (C) scissors pointing to the ACL prior to transection; (D) view of the joint post-transection. Stabilization of ACL transected knees (E-H). (E) The tibial tunnel created with a 30 gauge needle proximal to the patellar tendon insertion; (F) the suture, 5-0 prolene, is seen under the fabella (1), under the patella (2), then from medial to lateral through the drilled tibial tunnel (3); (G) the imbrication of the fascia post stabilization; (H) closure of the dermis.

Dissections

Mice knees were dissected and harvested for tissue processing. A lateral incision was used to expose the knee to allow photographs of the knee using the Leica DFC295 camera and Leica Application Suite software version 3.8.0 (Leica Microsystems, Richmond Hill, ON, Canada). Knees were dissected in PBS.

The femur was cut proximal to the hip and the tibia was cut proximal to the ankle. Excess muscle tissue was removed with out disturbing the knee capsule. Knees were fixed in 4% PFA for 24 hours before decalcification. Knees were placed in EDTA on a rocker at room temperature. EDTA solution was changed every two days a total of four times. Decalcification was determined by physical end-point testing. Once tissue was sufficiently decalcified samples were placed in 70% ethanol and refrigerated before processing. Both the left and right knees of the surgically stabilized mice were collected.

Tissue Processing

All harvested knees were sent for processing at the Molecular Pathology Laboratory at Robarts Research Institute (London, ON, Canada). Knees were dehydrated, cleared, and infiltrated with paraffin wax. Surgically stabilized knees were oriented frontally and embedded in paraffin wax for histological sectioning.

Histology

Knees embedded in paraffin wax were sectioned at Robarts Research Institute (London, ON, Canada). Serial sections 5μm thick were collected onto slides and dehydrated overnight in an oven at 40 degrees Celsius. Slides were dewaxed using two five-minute xylene solution washes. Slides were then rehydrated using 100%, 95%, and 70% ethanol solutions followed by tap water rising. After dewaxing half the slides were stained with Safranin-O or Toluidine blue.

Half of the slides were dipped in 50% hematoxylin for 15 to 30 seconds and immediately rinsed with tap water until water flowing off was clear and then dipped in tap water for five minutes. Slides were placed in 0.02% Fast Green solution for 30 minutes for back

ground staining. Slides were dipped in 1% Glacial Acetic Acid for no more than 15 seconds. Slides were exposed to 1.5% Safranin-O solution for three and a half minutes then subsequently dipped twice in distilled water to remove excess stain. Slides were dipped three to five times in 70%, 95%, 100% and another 100% ethanol solution and then in two xylene solutions to dehydrate the samples. Slides were allowed to air dry for five to ten minutes before mounting a coverslip with a xylene based mounting medium. Cartilage and proteoglycans stain red and other tissue such as matrix and bone stain green.

The remaining slides were stained with 0.04% Toluidine blue for 5 minutes and rinsed for one minute in tap water. Slides were placed in a 37^{o}C oven to air dry for 20 minutes. Slides were placed in a xylene solution for five minutes. Slides were allowed to air dry before mounting a coverslip with a xylene based mounting medium. Cartilage matrix and nuclei stain deep violet and cytoplasm and other tissue elements stain as various shades of light blue.

Outcome measures

Gait Analysis

Gait was analyzed using the CatWalk system (Noldus Information Technology, VA) at the Robarts Research Institute Behavioural Facility to identify any differences in gait parameters between intervention groups and between time points (London, ON, Canada). Mice were acclimatized to the room and allowed to run the length of the CatWalk before recording. Mice were recorded for 20 seconds a minimum of 5 times. The CatWalk was calibrated to contrast level 3990, brightness level 160, pixel threshold level 40, pixel number 5, aperture level -1.4 and a focus level of -0.1. Runs were valid if mice maintained a uniform speed across the recording region of the CatWalk. Paws were labeled using the CatWalk 7.1 software and a pre-processing threshold of 75 (Noldus Information Technology, VA). A minimum of five runs per mouse were averaged and used to statistically analyze stride length (mm), paw intensity, duty cycle and regularity index.

Stride length was assessed to determine whether the constraint placed on the knee joint from the surgical stabilization event could have lead to observed differences in gait pattern. A difference in stride length amongst the mice could explain further differences we observed in other gait parameters.

Paw intensity is measured as light reflection intensity. This measure has been correlated with load cell outputs [47] where less intensity captured can be interpreted as less force being applied. A difference between the groups could be a result of the animal not weight-bearing on that limb due to pain or discomfort [48].

Duty cycle is the ratio between the stance duration and the complete step-cycle, from paw print to paw print, duration. Different rodent models of OA have shown statistically significant differences in duty cycle between groups with higher percentages indicating worse OA symptoms [49].

The regulatory index (RI) is a ratio measurement of the number of normal step sequences to the number of paw placements. Previous studies have established correlations between differences observed in the RI between groups and coordination deficits in movement [50].

OARSI Scoring

The Osteoarthritis Research Society International (OARSI) grading system is an established semi-quantitative analysis that is useful in assessing OA status and development in the knee joint of mice following an injury or intervention [51]. Two experienced and blinded observers scored both Safranin-O and Toluidine blue stained sections of ACL transected mice with and without surgical stabilization at five and ten week time points. The medial femoral condyle (MFC), lateral femoral condyle (LFC), medial tibial plateau (MTP) and the lateral tibial plateau (LTP) of each section were assessed and averaged for each animal. Total knee OARSI scores were also obtained by summing the four quadrants. Knees were imaged with a Leica DFC295 camera, Leica DM1000 microscope and Leica Application Suite software version 3.8.0 (Leica Microsystems, Richmond Hill, ON, Canada). Two experienced raters were given randomized frontal slides of ACL transected mice +/- stabilization at 5 and 10 weeks.

Raters were blinded to the number of animals in each group and the time point of the intervention. A non-rater reordered the individual slides and relabeled glass slides. A randomization key was kept separate from slides at all times.

Statistics

All statistical analysis was performed using SPSS (IBM, V.22.0, Armonk, New York, USA). All graphs were constructed using mean values and 95% CIs with GraphPad Prism software version 6. Stride length was assessed using a one-way analysis of variance (ANOVA). Paw intensity, duty cycle, and RI using a two-way ANOVA followed by pair-wise comparisons when appropriate. Parameters were chosen a priori. OA scoring was analyzed using a two-way ANOVA followed by pair-wise comparisons and Tukey's HSD post-hoc test when appropriate.

All of the statistical analysis was underpowered because of our small samples sizes. Thus, we suggest that the CI and general trends should be used to interpret the findings.

5.3 Results

Gait

Analysis of stride length on the left and right hind limb showed no difference between the stabilized and non-stabilized groups (Table 5) (Figure 5). There were no significant differences noted when analyzing the effect of intervention and time point (Table 5) on paw intensity (Figure 6), duty cycle (Figure 7) and RI (Figure 8). The Levene test for homogeneity of variance was significant only in the stride length right hind limb and stride length left hind limb outcome data but the Shapiro Wilks test for normality were all also non-significant.

Table 5: Summary Table of Gait Parameters

Gait Parameter	Intervention	Mean	Standard Deviation	Minimum	Maximum
Stride Length Right Hind Limb (mm)	Non-stabilized 5weeks	59.66	0.14	59.56	59.76
	Stabilized 5weeks	66.02	4.48	62.30	70.99
	Non-stabilized 10weeks	52.05	8.26	46.21	57.90
	Stabilized 10weeks	55.52	3.91	51.16	58.70
Stride Length Left Hind Limb (mm)	Non-stabilized 5weeks	60.27	0.14	60.18	60.37
	Stabilized 5weeks	67.42	10.79	59.59	79.73
	Non-stabilized 10weeks	52.53	8.75	46.34	58.72
	Stabilized 10weeks	57.59	1.98	55.81	59.72
Paw Intensity Right Hind Limb (0-255)	Non-stabilized 5weeks	130.45	5.97	126.23	134.67
	Stabilized 5weeks	132.96	3.57	128.86	135.36
	Non-stabilized 10weeks	132.76	0.98	132.07	133.46
	Stabilized 10weeks	128.72	8.15	119.95	136.06
Paw Intensity Left Hind Limb (0-255)	Non-stabilized 5weeks	135.36	3.11	133.16	137.56
	Stabilized 5weeks	127.68	4.68	122.52	131.65
	Non-stabilized 10weeks	136.63	3.45	134.19	139.07
	Stabilized 10weeks	131.92	9.58	122.96	142.01

Duty Cycle Right Hind Limb (%)	Non-stabilized 5weeks	52.96	0.20	52.10	52.82
	Stabilized 5weeks	53.29	4.75	48.22	57.65
	Non-stabilized 10weeks	57.79	1.06	57.05	58.54
	Stabilized 10weeks	55.90	2.40	53.42	58.22
Duty Cycle Left Hind Limb (%)	Non-stabilized 5weeks	55.80	1.20	54.95	56.65
	Stabilized 5weeks	57.67	4.74	52.23	60.87
	Non-stabilized 10weeks	64.91	6.75	60.13	69.68
	Stabilized 10weeks	59.38	3.76	55.60	63.13
Regularity Index (%)	Non-stabilized 5weeks	99.41	0.83	98.82	100.00
	Stabilized 5weeks	97.61	2.11	96.00	100.00
	Non-stabilized 10weeks	99.52	0.67	99.05	100.00
	Stabilized 10weeks	97.60	3.37	93.75	100.00

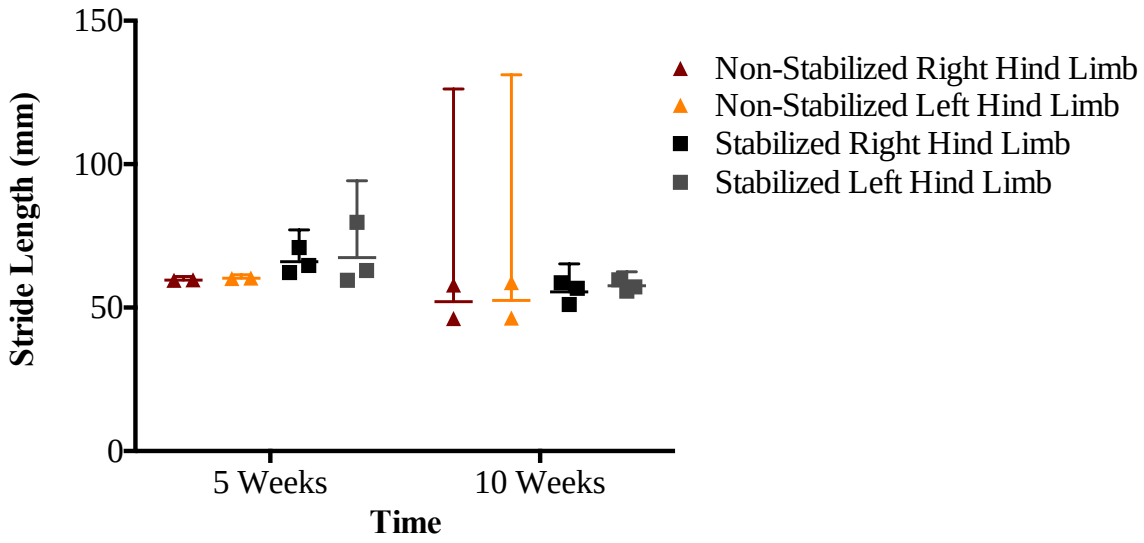

Figure 5: Stride Length. Following ACL transection +/- stabilization the mice were walked on the Nodulus CatWalk system at 5 weeks and 10 weeks. Stride length was captured and values shown are the individual data points with 95% CI.

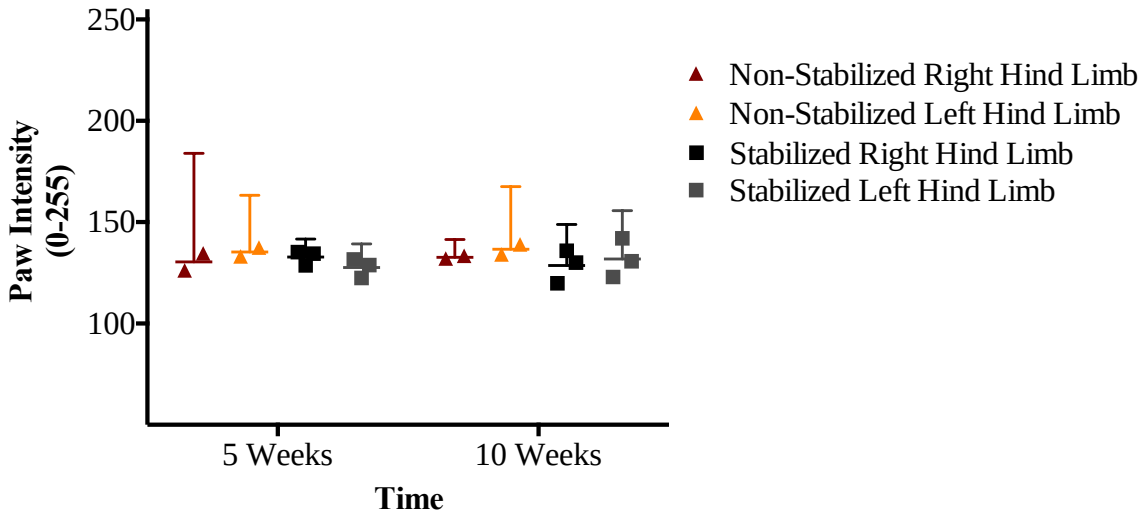

Figure 6: Paw Intensity. Following ACL transection +/- stabilization the mice were walked on the Nodulus CatWalk system at 5 weeks and 10 weeks. Paw intensity was captured and values shown are the individual data points with 95% CI.

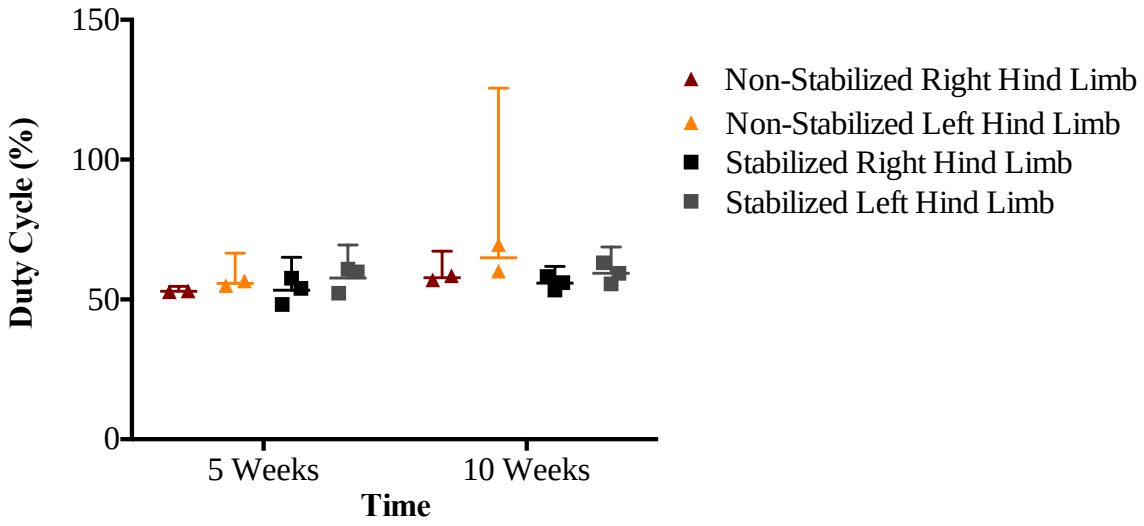

Figure 7: Duty Cycle. Following ACL transection +/- stabilization the mice were walked on the Nodulus CatWalk system at 5 weeks and 10 weeks. Duty cycle was captured and values shown are the individual data points with 95% CI.

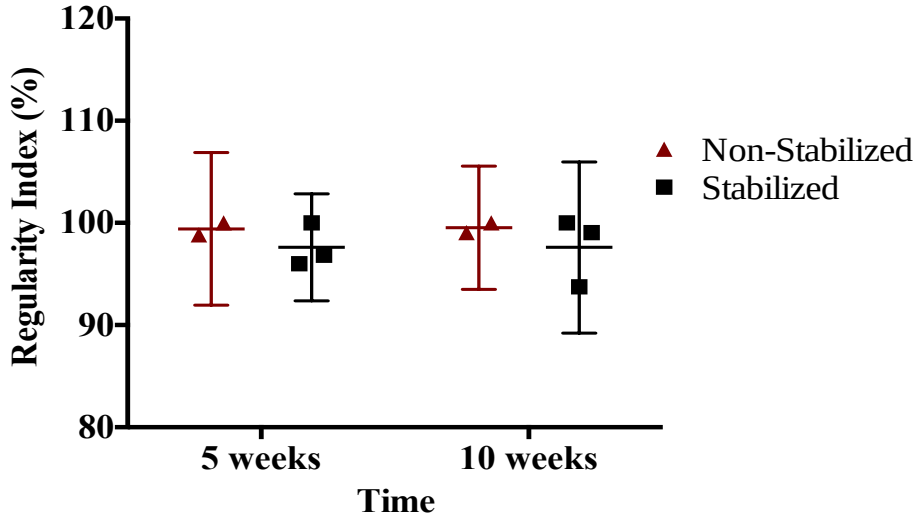

Figure 8: Regularity Index. Following ACL transection +/- stabilization the mice were walked on the Nodulus CatWalk system at 5 weeks and 10 weeks. Regularity index was captured and values shown are the individual data points with 95% CI.

Histology

There were no significant differences between groups for five week data (Figure 9). Data was normally distributed and had homogeneous variance as assessed with the Shapiro Wilks test and the Levene test respectively. Additionally, we used a one-way ANOVA to test for statistical differences between groups because we were unable to score ten week data. The one-way ANOVA test was not significant with a non-stabilized group mean score of 0.89, 95% CI (0.08, 1.70) and a stabilized group mean score of 0.51, 95% CI (0.21, 0.82). The total knee and each knee quadrant was scored and graphed with 95% CI (Figure 9).

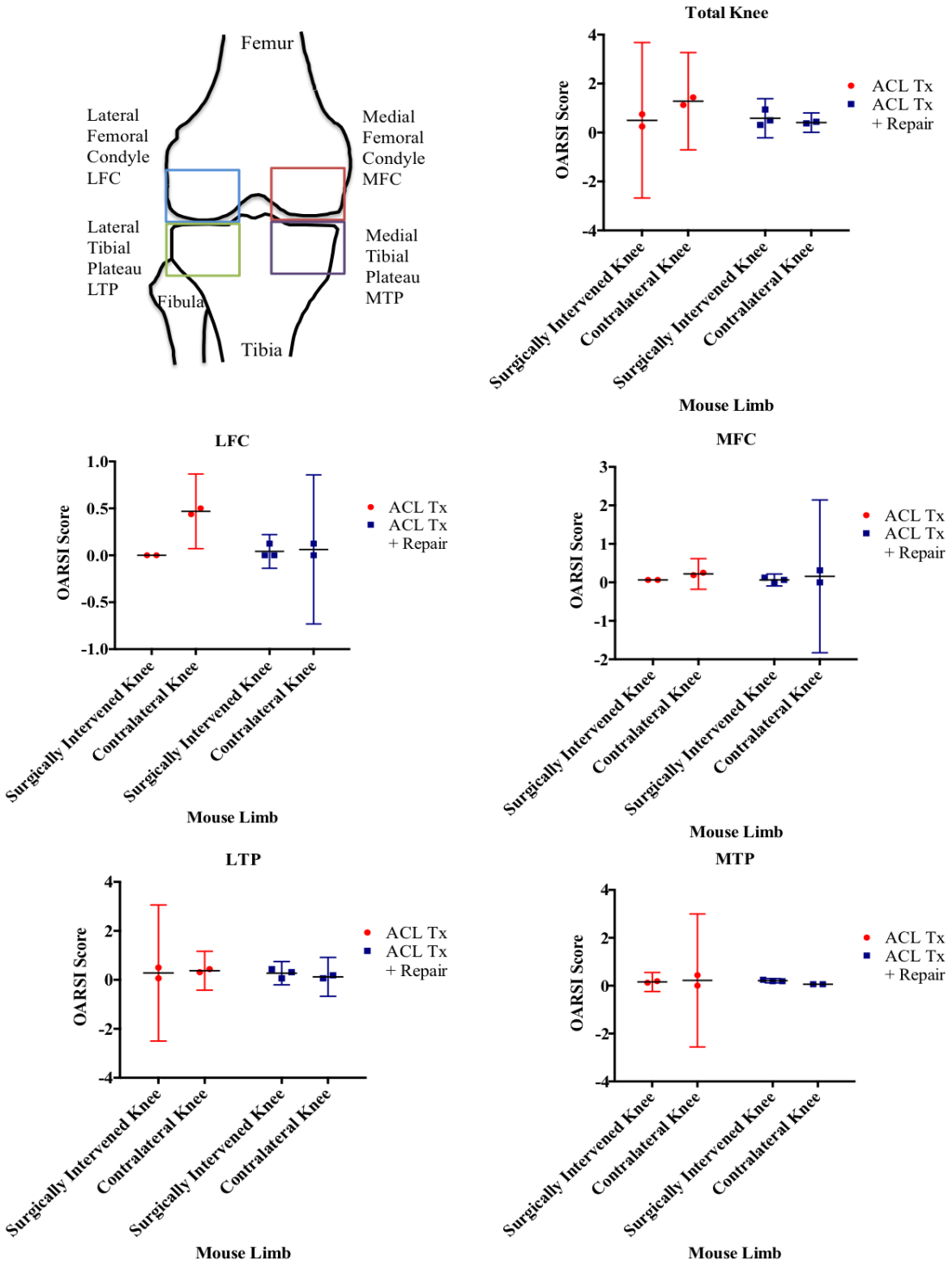

Figure 9: OARSI Scores. Semi-quantitative analysis of OA grading showed no statistically significant differences between the groups at 5 weeks based off of histological slide. Values shown are the individual data points with 95% CI.

We were unable to score ten week data given the scale used. All knees observed were too severe for the zero to six histological scale. Nevertheless, we noted qualitative differences. Knees with an ACL transection and no surgical intervention had severe damage to the articulating surface, surrounding subchondral bone and synovial space. In addition, extracapsular fibrous growth on the lateral side was more extensive in this group (Figure 10). These changes were observed and noted as too extensive to grade or characterize as OA. Contralateral knees were also noted to have injuries to the cartilage and articulating surface however these knees were not as damaged at the right limbs. There was a noted difference in the extent of damage to the articulating surface and cartilage of the ACL transected and surgical stabilized group. However the synovial space and extracapsular space were not. The stabilized group's contralateral limbs had damage to the articulating surface and cartilage however, these contralateral knees were not as damaged as the intervention knee from a qualitative perspective (Figure 11).

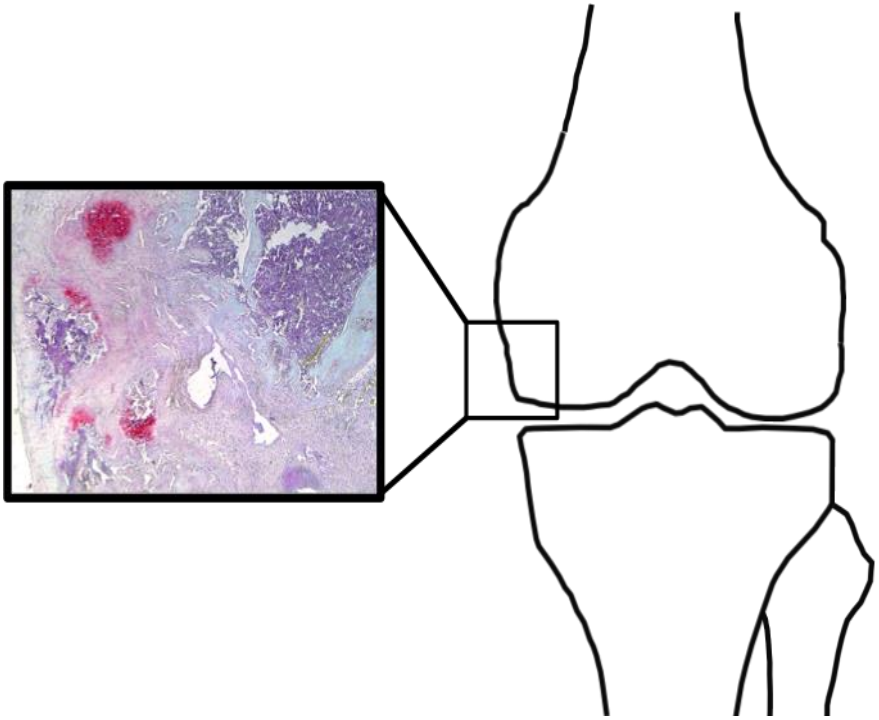

Figure 10: Extracapsular Fibrous Growth. Histological slides were stained with Safranin-O and Fast Green. Fibrous tissue was observed on the medial side of the knee in the ACL transected without stabilization group.

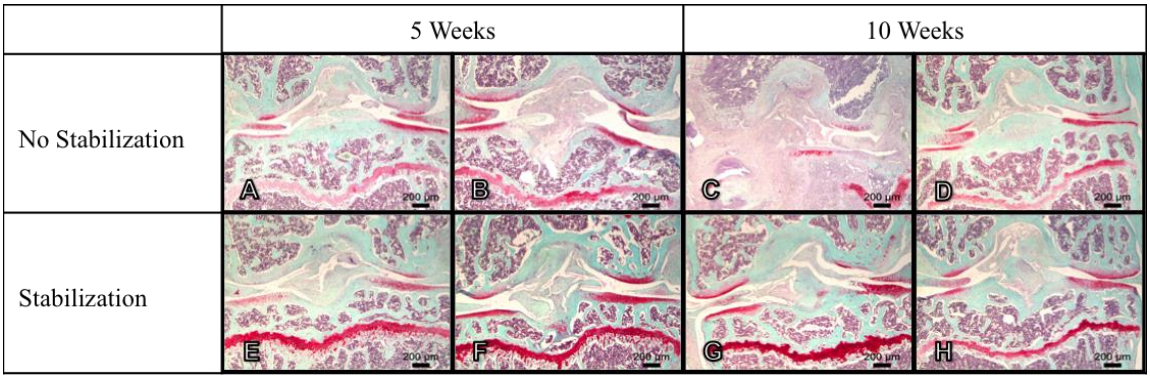

Figure 11: Representative Histological Images of Five and Ten Week Knees. Histological slides stained with Safranin O and Fast Green. (A, C, E, G). Right mouse knees that had an ACL transection +/- stabilization. (B, D, F, H). Left mouse knees used as contralateral controls.

5.4 Discussion

Gait

We used stride length as a proxy for limb angle constriction. If the angle of flexion about the knee was too restrictive, the limb would not have full range of motion. This limitation could result in differences in stride length and subsequent differences observed in other gait parameters. Fortunately, we found no significant differences stride length for the left and right hind limb, which implies that all mice had a similar range of motion. However, our confidence intervals were quite wide for the data around ten weeks for non-stabilized mice, which can be a result of small sample size, and the variability inherent with a non-stable knee.

Paw intensity is a direct measure of contact area and weight bearing capabilities. Differences between groups would indicate weight-bearing changes resulting from pain or discomfort. We found no statistically significant difference in paw intensities between groups, which may mean that weight-bearing was similar between groups. However, we also report wide confidence intervals in five week non-stabilized mice. This is likely a result of small sample size and increased variability between mice because of the

destabilization of the joint. Since there was no difference between the groups we can assume that the stabilization is not hindering the mice's weight bearing ability.

To quantify OA progression in the different groups we measured duty cycle. Higher duty cycle percentages would indicate more severe OA in the mouse. There were no significant differences observed in the duty cycle of our animals. Ten week non-stabilized left hind limb had the highest mean with the largest CI. This variability could be a result of small sample sizes. Alternatively, the animals in this group could be attempting to compensate as needed by spending more time in the stance phase or shortening its step-cycle to use their uninjured hind limb preferentially. If the latter is true then at ten weeks the stabilized mice do not have to compensate during their gait cycle.

Differences in RI were assessed to quantify changes in coordination. Difference in the RI could be due to the development of OA. There were no statistically significant differences observed between the stabilized and non-stabilized groups and all limbs had fairly wide CIs. Since there was no difference the mice did not appear affected by their OA development even in the severely damaged ten week non-stabilized group. The mice did not demonstrate gross changes to their housing cage behaviour either thus minute gait differences may be undetectable in this resilient animal.

Histology

We found no difference between groups at five weeks for the intervention or time point. This result may be explained by the small sample size or there may be no benefit from the stabilization at five weeks post injury. Ten week data could not be scored using the conventional OARSI grading system. Thus, an observational approach was taken as opposed to the semi-quantitative scoring. As a result our original two-way ANOVA plan for analysis was ad hoc switched for a one-way ANOVA. Nonetheless, there were marked differences in the ten week data which can be attributed to the stabilization technique employed. Since the mice were not restricted from movement, the ten week mice would have had more time to sustain secondary injuries which the stabilized mice may have been protected against. Contralateral limb cartilage in the non-stabilized group

had more cartilage loss than in the stabilized group. This was postulated to be a result of the mice trying to compensate or alter their gait because of the osteoarthritic changes. Thus any protective effects from the knee stabilization are important to consider when modeling arthritis and more specifically PTOA following ACL surgery in humans.

Limitations

Our trial was a pilot study to determine whether a stabilization event following an injury was protective to the mouse knee. Our experimental conclusions were based on a small sample size and our work could benefit from more animals to increase our certainty. To better understand the extent of the stabilization achieved with our extra-articular procedure we could have measured the total anteroposterior translation before and after the stabilization. Although there was some assessment done we were hesitant to aggressively test this parameter in case the knee was damaged or the tibial tunnel was excessively stressed. Additionally, our study lacked a sham and no treatment group. The addition of a sham group would provide a group that had surgery to expose the knee and dislocate the patella. This could provide insight into the fibrous growth and the OA development in the contralateral limb that we observed in our ACL transected mice without stabilization. If similar results were observed then the fibrous growth and contralateral OA development may have resulted from exposing the joint. A group of mice that received no treatment would consist of mice of the same age as intervention mice and would be followed to the same time points to observe the natural aging of the joint and compare that to our other groups. The addition of these groups could provide further support and establish conclusive relationships between the intervention and the outcome measures.

Future Directions

Given that we found a marked protective difference in the ten week data we would be interested in conducting a larger trial with more mice and two additional groups to be used as controls. These two groups would be: a sham surgery group that receives a lateral incision and knee manipulations and is closed up; and a no-intervention group that gets anesthetic but have no incision made.

Additionally, this stabilization technique showed benefits after ACL transection. We would be interested in the application of this surgical intervention that minimizes damage post injury in other models of OA. Given the invasive nature of the ACL transection our next step would be to use a less invasive OA model such as a loading protocol (Chapter 4) that creates instability in the knee through ACL disruption.

The reason for translational research is to apply laboratory concepts and results to medicine. Therefore, there is a need to develop clinically relevant animal models. This surgical intervention was developed to better represent the true events that a human may undergo following an injury to the ACL. As such, drugs aimed at modifying PTOA may benefit from testing their effects in our proposed animal model that more closely resembles human interventions.

Chapter 6

6 Summary

The purpose of the axial loading project was three fold. The primary objective was to validate an existing model of ACL injury to create an unstable knee in a mouse. Our work provided evidence that the loading protocol and the machine set up did create ACL ruptures. This created an unstable knee. The second objective was to establish a relationship between the speed of an impact and the type of injury produced. It was hypothesized that the faster speeds would lead to mid-sheath ruptures of the ACL and slower speeds would result in avulsion of the ACL. When analyzed the fastest and the slowest speeds both produced more avulsion injuries than the intermediate speed. Furthermore, the fastest speed also produced more extensive injuries involving the PCL that was not documented as injured in previous studies. Finally the last objective was to assess the agreement between raters and modalities in correctly identifying injuries produced from the loading protocol. Based on the literature in microCT scanning, we used PTA to contrast our samples. Although the PTA solution did enhance the microCT images, the agreement between the raters and the agreement between the microCT and the histology slides was poor. This poor consensus provided support that even when optimized the microCT did not provide the raters with enough information to identify the status of the mouse knee post injury.

The purpose of the surgical stabilization project was two fold. The first objective was to develop a surgery in mice that could introduce stability to the knee joint following ACL transection. A surgical intervention based on canine ACL surgery was used to stabilize the knee extra-articularly. There were no statistical differences between the knees that were stabilized, the contralateral limbs or the unstable knees. The second objective of this study was to assess any differences in histology and gait of the animals due to the stabilization surgery or to the time from the intervention. Five week histology data was assessed and no differences were observed between the groups. Ten week data was assessed qualitatively because the damage inflicted on the knees were not quantifiable on the semi-quantitative scoring system used with the five week mice. Notable differences

were observed between the ACL transected knees and the ACL transected and stabilized knees. The stabilization event provided some protection to the knee from what we concluded were secondary injuries. When we analyzed the differences in gait parameters we did not find significant differences between the groups at five or ten weeks post intervention.

Future work should look at combining both projects to create a more robust clinically relevant model of OA in mice that can be used to better the understanding, diagnosis and treatment of PTOA in humans.

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Appendices

Appendix A: Ethics Approval

From: eSiriusWebServer [mailto:esiriusadmin@uwo.ca]
Sent: August 5, 2014 2:53 PM
To: tbeier@uwo.ca
Cc: auspc@uwo.ca; auspc@uwo.ca
Subject: eSirius Notification - Annual Protocol Renewal APPROVED by the AUS 2007-045-06::7

Western

2007-045-06::7:

AUP Number: 2007-045-06
AUP Title: Regulation of Endochondral Bone Growth by Hormones

Yearly Renewal Date: 08/01/2014

The YEARLY RENEWAL to Animal Use Protocol (AUP) 2007-045-06 has been approved, and will be approved for one year following the above review date.

1. This AUP number must be indicated when ordering animals for this project.
2. Animals for other projects may not be ordered under this AUP number.
3. Purchases of animals other than through this system must be cleared through the ACVS office. Health certificates will be required.

REQUIREMENTS/COMMENTS
Please ensure that individual(s) performing procedures on live animals, as described in this protocol, are familiar with the contents of this document.

The holder of this Animal Use Protocol is responsible to ensure that all associated safety components (biosafety, radiation safety, general laboratory safety) comply with institutional safety standards and have received all necessary approvals. Please consult directly with your institutional safety officers.

Submitted by: Kinchlea, Will D
on behalf of the Animal Use Subcommittee

Appendix B: Abbreviations

OA	Osteoarthritis
PTOA	Post-traumatic osteoarthritis
ACL	Anterior cruciate ligament
PCL	Posterior cruciate ligament
ADAMTS	A disintegrin and metalloproteinase with thrombospondin motifs
DMM	Destabilization of medial meniscus
CrCL	Cranial cruciate ligament
LRP5	Low-density lipoprotein receptor-related protein 5
microCT	Micro computed-tomography
PBS	Phosphate buffered saline
PTA	Phosphotungstic acid
EDTA	Ethylenediaminetetraacetic acid
RI	Regularity index
OARSI	Osteoarthritis research society international
MFC	Medial femoral condyle
LFC	Lateral femoral condyle
MTP	Medial tibial plateau
LTP	Lateral tibial plateau
CI	Confidence interval
ANOVA	Analysis of variance

Curriculum Vitae

Name:	Chantel Pauline Arce
Post-secondary Education and Degrees:	McMaster University Hamilton, Ontario, Canada 2008-2012 Hons.B.Sc.
Honours and Awards:	McMaster Entrance Scholarship 2008
	Marauder Scholars Award 2010-2011
	Western Graduate Research Scholarship 2013-2014, 2014-2015
	CIHR Graduate Fellow in Musculoskeletal Health Research in the Joint Motion Program (JuMP) 2014-2015
Related Work Experience	Research Student Toronto Medical Discover Tower 2012-2013
	Teaching Assistant The University of Western Ontario 2013-2015
Conferences:	Yasufuku, K., Arce, C., Azad, Sassan, S., Cyril, F., Niall, W.D., Thomas F.K., Keshavjee, S. Ten Years Experience with Extra Corporeal Life Support for Adult Respiratory Failure: Evolution of Indications, Techniques and Outcomes. Presented at the American Society of Clinical Oncology meeting 2014
	Arce, C., Welch, I., Beier, F., Bryant, D., Getgood, A."Stabilization of the Mouse ACL Deficient Knee: A Clinically Relevant Model of Post Traumatic Osteoarthritis". Faculty of Health Sciences Symposium at The University of Western Ontario (March 2015)

Arce, C., Welch, I., Beier, F., Bryant, D., Getgood, A. A novel mouse stifle stabilization model: bridging the gap from lab bench to bedside in the study of post-traumatic osteoarthritis. Poster presented at the International Cartilage Repair Society (May 2015)

Publications:

Reck dos Santos,P.A., Sakamoto,J., Chen, M., Linacre, V., Arce, C., Hwang, D., de Perrot, M., Liu, M., Leighl, N.B., Keshavjee, S., Waddell, T.K., Cypel,M.(2014) Modified isolated lung perfusion technique for allowance of prolonged perfusion without acute lung injury: A preclinical study with doxorubicin. Journal of Clinical Oncology, 32:5s.

Cypel, M., Paraghamian, A., de Perrot, M., Pierre, A., Yasufuku, K., Arce, C., Azad, S., Serrick, C., Ferguson, N.D., Waddell, T.K., Fan, E., Keshavjee, S. (2013) Ten Years Experience with Extra Corporeal Life Support for Adult Respiratory Failure: Evolution of Indications, Techniques and Outcomes. Abstract approved at the American Association for Thoracic Surgery.