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The Effect of Cytokines on Tendons and Ligaments: An In Vitro Study

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The Effect of Cytokines on Ligaments and Tendons:

An In Vitro Study

by

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A THESIS

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Abstract

Tendon and ligament explants are utilized in ACL reconstruction. After ACL reconstructive surgery, or ACL injury, there is an up-regulation of inflammation, which has been implicated in causing cellular death. The objective of this study is to examine the effect of cytokines on cells and tissue of ligaments and tendons. The mRNA expression of inflammatory and apoptotic markers were measured in primary cells after treatment with cytokines and cell death was measured with fluorescent dyes. Next ACL and PT explants were analyzed for mRNA expression. Furthermore, Annexin V and DNA laddering techniques were used to evaluate for apoptosis. Apoptosis does not seem to be a direct effect of cytokine treatment. In almost all treatment groups, SNP was the only reagent that caused significant amounts of apoptosis. Results showed evidence of a connection between IL1B, iNOS, and SNP. Additionally, tissues with more blade cuts had significantly higher amounts of cell death.

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Abbreviations

ACL – Anterior Cruciate Ligament

BMP – Bone Morphogenic Protein

DISC – Death Inducing Signalling Complex

DMEM – Dulbecco's Modified Eagle Medium

ECM- Extracellular Matrix

FADD – Fas Associated Protein with Death Domain

FasL- Fas Ligand

FasR- Fas Receptor

HIF-1A – Hypoxic Inducing Factor Type 1A

IL-Interleukin

iNOS – Inducible Nitric Oxide Synthase

MMP – Matrix Metalloproteinase

MPT – Mitochondial Permeability Transit

OA – Osteoarthritis

NO – Nitric Oxide

PCR – Polymerase Chain Reaction

PT- Patellar Tendon

PGE₂ – Prostaglandin E2

TIMP- Tissue Inhibitor Matrix Metalloproteinase

TGF β - Transforming Growth Factor Beta

TNF- Tumor Necrosis Factor

TNFR1 – Tumor Necrosis Factor Receptor Type 1

TRADD – Tumor Necrosis Factor Receptor Type 1-Associated Death Domain Protein

Chapter One: Introduction

1.1: Introduction

In a report in 2011, the Arthritis Alliance of Canada stated that currently there are 1 in 8 Canadians living with osteoarthritis, with this ratio estimated to double to 1 in 4 within 30 years (Arthritis Alliance of Canada, 2011). The authors of this report also estimated that the current burden of osteoarthritis (OA) in Canada is \$33 billion per annum. Presently, there exists no cure for osteoarthritis (The Arthritis Society, 2011) and this most likely stems from the fact that we do not fully understand the mechanism(s) of disease pathology. Although there has been extensive research within different areas of osteoarthritis, due to the complexity of the disease, the holistic picture of disease onset and progression has not been developed yet.

Anterior Cruciate Ligament (ACL) injury is one of the most common risk factors for osteoarthritis (Roos et al., 1995, Lotz, 2010, Streich et al. 2011, van Porat et al. 2004) as more than 60% of patients who have an ACL injury go on to develop osteoarthritis. Injury can involve a partial tear of the ligament or complete rupture. A common treatment for ACL injury is surgical reconstruction in which a graft is used to replace the ACL (Legnani et al. 2010). Patellar Tendon (PT) is one of the most common graft materials used for this procedure (Legnani et al. 2010, Fu et al. 2011). Although such reconstruction is common, its efficacy at preventing osteoarthritis has not been proven. For example, one cohort study comparing patients who underwent only physiotherapy or only surgical reconstruction after ACL injury showed similar functional scores as well as no difference in the prevalence of osteoarthritis (60% at 14 year follow-up) (Streich et al. 2011). Further evidence demonstrating the connection between ACL injury and OA is that one of the fastest ways of inducing OA in animal models is through ACL transection – simulating the biomechanical and biological changes of ACL rupture. It is common for animal models to be used to explore changes after injury and throughout the progression of OA. However, one animal model has shown that even with the use of an ‘ideal’ graft (the native ACL in its original anatomic position), early osteoarthritic-like changes are visible 2 weeks post-surgery (Heard et al. 2011). However, most studies postulate that a

combination of biological and biomechanical abnormalities is responsible for the degradative changes that occur after injury.

In terms of biology, it is apparent that there are significant biochemical changes occurring in the joint after ACL injury. These include an acute inflammatory response (which causes the up-regulation of various cytokines: IL-1 β , TNF- α , IL-6, IL-13) (Irie et al. 2003, Murray et al. 2000), the up-regulation of matrix metalloproteinases, collagen type I and type II production, collagenase production, proteoglycan production/presence, glycosaminoglycan production/presence, and prostaglandin E2 (PGE₂) production (Tetlow et al. 2001, Nelson et al. 2006, Lopez-Armada et al., 2006). Cytokines and matrix metalloproteinases have been shown to have a degradative effect on joint tissues (Mutsuzaki et al. 2010, Uchida et al. 2005).

ACL injury involves the rupture or tearing of the ligament tissue. This injury process may dispose torn ends to further vulnerability to any destructive substances. In conjunction with the fact that an inflammatory influx is observed after injury, the way in which the injured ligament tissue responds to the infiltration of inflammatory cytokines is essential to understanding the mechanism of the pathology associated with the joint after injury.

Another unknown effect of the post-trauma biochemical changes is whether or not the initial inflammatory response causes death of ligament cells. Cultured fibroblasts have been shown to undergo apoptosis in response to the introduction of TNF- α and IL-1 β : however this effect has not been studied with the cells in their extracellular matrix (ECM) (i.e. in tissue or explant form). The ECM and fibroblasts found in both tendon and ligament cells have a dynamic relationship consisting of protein signaling, cell to cell communications, and mechanically induced gene expression (Kjaer, 2004). Changes in tissue loading or injury cause expression of matrix metalloproteinases (Heard et al., 2011) which induce the destruction of the matrix. Cytokines have been shown to cause cell death in cultured human, osteoarthritic, and normal fibroblasts (Murakami et al. 2006, Shuerwegh et al. 2003, Mutsuzaki et al. 2010). However, whether or not cytokines cause cell death in ligament or tendon explants has not been studied. It follows that inflammation may be involved the mechanisms of cell death. Specifically, if injury causes the expression of matrix metalloproteinases and cell death and injury also induces

an influx of inflammation, it could be speculated that there may be a link between these two processes.

In brief, this study was designed to (1) evaluate whether or not cytokines can cause cell death in ligament and tendon explants; (2) characterize if cell death is necrotic or apoptotic; (3) determine if ruptured or injured ends of ligament or tendon are more vulnerable to cytokine effects.

The subsequent chapters will discuss the current evidence and knowledge regarding inflammation, apoptosis, cell death, injury, and reconstruction as it pertains to ACL and PT tissue. The methods utilized to explore whether or not cytokines cause cell death in tendon and ligament explants, the effect ruptured ends have on cell vulnerability, and whether or not apoptosis is occurring will be described. The results of the subsequent experiments will be presented, after which, a discussion of the results will lead to final conclusions.

Chapter Two: Review of Current Literature

2.1 Inflammation in the ACL

There have been four histologically observable stages of cell activity described for the human anterior cruciate ligament (ACL) cells after ACL injury (Murray et al. 2000). The first of these phases is the inflammatory stage in which inflammatory proteins and cells (specifically cytokines and or mononuclear cells) infiltrate the ACL cells for 3-12 weeks after the injury (Espregueira-Mendes et al. 1998). Using basic macro-level examination, Murray and colleagues (2000) identified inflammation, during the proposed 'inflammatory stage', as the ligament tissue being edematous and swollen. Furthermore, microscopic evaluation showed neutrophil, macrophage, and lymphocyte presence with the highest concentrations of these inflammatory cells being found around blood vessels. Although extensively studied, the mechanism of inflammation, subsequent poor healing and the development of other joint diseases after ACL injury is not well understood.

Irie et al. (2003) have demonstrated that certain cytokines, TNF α and Interleukins: 1 β , 6, 8, 1ra, 10 are present at the site of rupture (of the ACL) within 24 hours of the injury. Studies agree that after the inflammation period, there is a subsequent decrease in cytokine levels but individual cytokines display different patterns of concentration reduction. In one study with rabbits (n=20), cytokine levels remained higher (in the cells of the patella-tendon graft) than the controls (contralateral ACL cells) for 12 weeks post ACL reconstruction (Espregueira-Mendes et al. 1998).

In a study evaluating human subjects (n=34) with acute ACL tears, results showed that that cytokine levels started to decrease in the intra-articular fluid of subjects after 1 week (Irie et al 2003). In another human study (n=23), inflammation was observed in ACL cells (post rupture) for up to 3 weeks (Mutsuzaki et al. 2007). With respect to the levels of cytokines, it has also been demonstrated that 24 hr post ACL rupture, cytokine levels were higher compared to normal and arthritic patients. After this 24 hr time period (post rupture), Irie et al. (2003) reported that cytokine levels do not return to normal but rather decrease to the level seen in patients with chronic arthritis.

Cytokine levels in human synovial fluid have been assessed intra-operatively and post-operatively during ACL reconstruction, and results demonstrated that while

collagenase, TIMP-1 (tissue inhibitor of matrix metalloproteinases-1), and $\text{TNF}\alpha$ were all elevated post-operatively compared to intra-operative levels, only TIMP levels were significantly higher post-operatively (Akesen et al. 2009). The most prevalent cytokines that have been observed to be present after ACL injury are $\text{TNF}\alpha$ and Interleukins: 1β , 6, 8, 1ra, 10 (Irie et al. 2003). These cytokines increase catabolism of chondrocytes present at the ACL tibial insertion and can lead to cartilage degeneration (Irie et al. 2003).

It should also be noted that matrix metalloproteinases (MMPs 1,2, 3, 8 and 9), collagen type I and type II production, collagenase production, proteoglycan production/presence, glycosaminoglycan production/presence, and prostaglandin E₂ (PGE_2) have also been observed after ACL injury and have been suggested as possible mechanisms for cartilage damage and deterioration (Tetlow et al. 2001, Nelson et al. 2006, Arnold et al. 1983, Lopez-Armada. 2006).

2.2 Inflammation effects in tendon cells

In vitro analysis shows that tendons also respond to common cytokines found at the site of ACL injury. Specifically, when cultured tendon cells were exposed to $\text{IL-1}\beta$ (10,000x concentration found at site of ACL injury) for 16 hr, quantitative PCR analysis revealed that $\text{IL-1}\beta$ induced a cascade which included expression of various matrix metalloproteinases (MMP-1, 3, 13), aggrecanase-1, $\text{IL-1}\beta$, IL-6 and COX-2^a (Tsuzaki et al. 2003). Similar to findings within ligaments, mechanical stretch (5% elongation at 0.33 Hz, 6 hrs) increases the expression of matrix metalloproteinases when tendon cells (rabbit) are in the presence of $\text{IL-1}\beta$ compared to cells in unstretched tissue in the presence of $\text{IL-1}\beta$ (Archambault et al. 2006). Also, similar to the mechanisms within ligaments, in vivo analysis in rat tissue shows that there are various cytokines present at the site of exercise-induced tendon injury. These include IL-18 , IL-15 , IL-6 and $\text{TNF}\alpha$ (Millar et al. 2009). Interestingly, this latter study also looked at the connection between the up-regulation of these cytokines and apoptotic cell death. Results showed that at the site of tendon injury, there was an increase in the expression of caspases, 3 and 8 (Millar et al. 2009). Millar and colleagues (2009) concluded that due to the correlated expression of apoptotic pathways and cytokine molecules, cytokines are key modulators in the apoptotic process. However, it is unclear as to whether or not cytokines are released to

down-regulate or up-regulate apoptosis and therefore further work should be done to determine if cytokines are harmful or beneficial for tendon cells.

2.3 Cytokines associated with joint injury and Osteoarthritis

Of the various studies on cytokines, joint injury, and osteoarthritis, two cytokines are often present in elevated concentrations. Upon initiation of an inflammatory cascade, TNF α is secreted by fibroblasts (the main cell type in tendons and ligaments) and it also stimulates fibroblasts to produce adhesion molecules (Choy and Panayi 2001). TNF α is produced within the cell, inserted into the cell membrane and released into the extracellular environment after a serine metalloproteinase completes the cleavage of its membrane-anchoring domain. TNF α also has the ability to induce production of various other cytokines (IL-1, IL-6, IL-8, and granulocyte–monocyte colony-stimulating factor). Besides being present at the site of injury, inflammation and osteoarthritis, TNF α has documented effects on the structures within the joint. Another powerful action of TNF α is its ability to induce fibroblasts to produce matrix metalloproteinases (MMPs) (Choy and Panayi, 2001) which have been implicated as a cause of joint degradation (Heard et al. 2011). Similarly, IL-1 β also has the ability to induce fibroblast production of MMPs (Shingu et al. 1993). In contrast to TNF α , interleukin-1 has two cell membrane receptors, of which only type I delivers intracellular signals (Choy and Panayi, 2001). Type II interleukin receptors simply bind IL-1 molecules but do not deliver intracellular signals. Cytokine pathways have been extensively studied with regard to rheumatoid arthritis and osteoarthritis (Choy and Panayi, 2001, Pelletier et al. 2001); however, these studies were focused on the response/effect associated with chondrocytes and synovial fibroblasts. There has been no published study showing the effects of these cytokines. Given the prevalence of osteoarthritis found among people who have had previous ACL injury and the fact that fibroblasts are directly involved in the inflammatory process, ligament fibroblasts should be a target of investigation. Additionally, as tendons are the main graft material for ACL reconstruction and their cell population is predominantly fibroblasts, joint inflammation effects on tendon cells should be investigated.

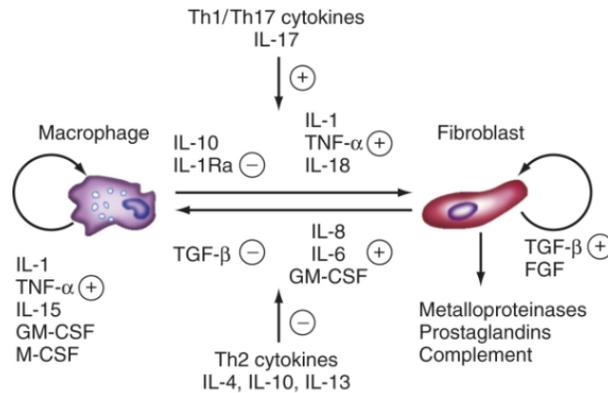


Figure 1. Illustration of the role of fibroblasts in producing and perpetuating inflammatory reaction (Firestein, 2008).

2.4 Cell death: definitions and pathways

There are two mechanisms of cell death: necrosis and apoptosis. Apoptosis is described as programmed cell death which includes a process that is genetically predetermined and results in the deletion of cells (Elmore, 2007). Apoptosis is essential for homeostasis and acts during development, remodelling and in concert with immune system reactions (Elmore, 2007). This form of cell death also usually requires an energy dependent process that involves the cleavage of cysteine substances called caspases. In contrast, necrosis is not dependent on energy stores and the cell participates in a passive series of events which are brought on by external or internal stimuli where the cell is rendered non-functional. There are 3 main pathways associated with apoptosis, the extrinsic, intrinsic and granzyme pathways. The extrinsic pathway is activated by transmembrane receptors from the tumor necrosis receptor family. These receptors have a cysteine rich domain referred to as the death domain. The most common ligands associated with these receptors in this extrinsic pathway are the FasL/FasR, TNF α /TNFR1 ligands. After binding, adapter proteins (FADD for Fas, TRADD for TNF), then go on to stimulate the action of procaspase-8 concluding the amalgamation of the death inducing signaling complex (DISC). This results in the auto-catalyzed activation of procaspase-8. As caspase-8 is activated, the mechanism of apoptotic death is triggered. In contrast to the specificity of the activation of the extrinsic pathway, the intrinsic pathway is initiated by various signals such as toxins, hypoxia, radiation, etc. which then go on to

produce a complex network of intracellular signaling. These signals work at the site of the mitochondrial membrane causing it to open the mitochondrial permeability transition (MPT) pore allowing substances (cytochrome C, serine proteases) to escape into the cytosol which activate the caspase pathway leading to apoptosis. The granzyme pathway can work in combination with the intrinsic pathway or can be initiated via cytotoxic T cells which work with the transmembrane protein perforin. Perforin allows the release of proteases and degradative molecules into the cytosol of the target cell.

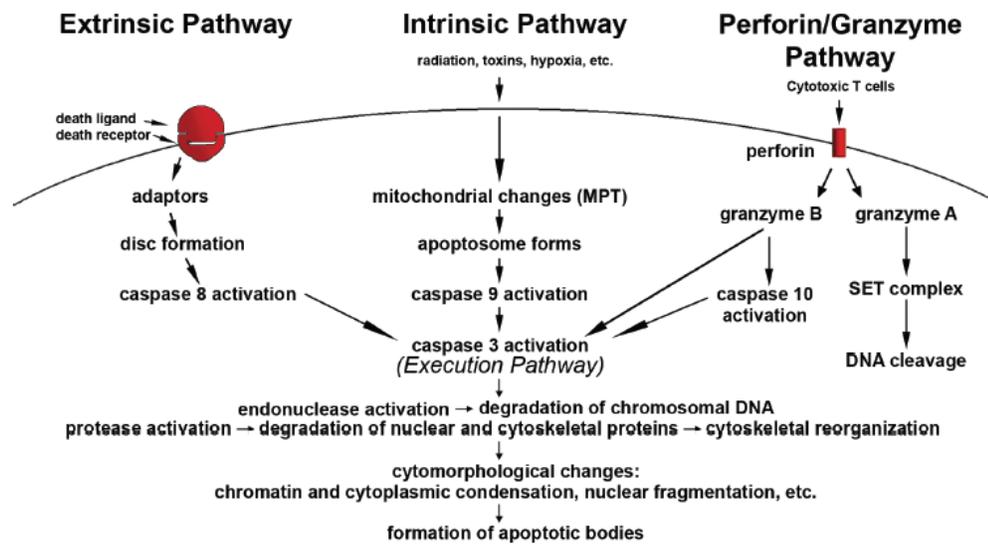


Figure 2. Illustration by Elmore (2007) showing the various pathways of apoptosis.

2.5 Inflammation and Apoptosis: The effect on cells within the ACL and PT

Recent studies have shown the distinctive nature of the fibroblasts in the ACL (with or without injury). For example, there has been much research into the differences in healing capabilities of the medial collateral ligament (MCL), an extra-articular ligament and the ACL, an intra-articular ligament. Although the decreased healing capacity of the ACL (versus the MCL), has been attributed to factors such as slower synovial cell proliferation to injury (in the ACL), decreased vascularisation of ACL (compared to the MCL), and the fact that the ACL is covered by the synovial lining (whereas the MCL is enveloped by strong, soft tissue outside the synovial lining), other research shows differences in the fibroblasts between these two structures (Fu et al. 1999).

Fibroblasts in the ACL exhibit slower cell migration and proliferation in response to injury versus the fibroblasts of the MCL (Nagineeni et al. 1992). Additionally, when exposed to inflammatory factors such as TNF α , Complement C5a, and lipopolysaccharide, healing times (migration of cells into empty area) of both ACL and MCL human cultured fibroblasts were significantly decreased when compared to normal controls (Witkowski et al. 1997). However, the ACL fibroblasts healed significantly more slowly than even the decreased rate exhibited by the MCL fibroblasts under inflammatory conditions. In order to get a better understanding of these decreased healing capabilities associated with the ACL, Wang and colleagues (2011) mimicked the intra-articular environment post ACL injury through the addition of cytokines (TNF α , IL-1 β), metalloproteinases (MMP 1, 2), and a hypoxic environment, utilizing hypoxia inducing factor (HIF-1a). The results showed that 100 (ng/ml) of TNF α increased MMP-2 level by 59% compared to the control. Additionally, 10 (ng/ml) of IL-1 β caused an increase of 54% MMP-2 activity, whereas 100 (ng/ml) increased MMP-2 production by 75%. This provides evidence that fibroblasts may be more sensitive to IL-1 β compared to TNF α , as a concentration of IL-1 β (10 ng/ml) 10x smaller than the concentration of TNF α (100 ng/ml) produced a similar increase in MMP-2 production. Furthermore, the combination of TNF α (10 ng/ml) + IL-1 β (10 ng/ml) increased MMP-2 activity significantly more than either TNF α or IL-1 β alone (Wang et al. 2011). These changes increased over time, up to 48 hrs. Also of note was that when these fibroblasts were exposed to mechanical stress (12% stress in equi-biaxial stretch chamber) TNF- α increased MMP-2 production by 200%, whereas IL-1 β increased MMP-2 production by 240%. However, when fibroblasts were exposed to both cytokines in combination, MMP-2 production increased by 321%, which was significant relative to TNF α but not IL-1 β .

Another mechanism involved in cytokine up-regulation is stress deprivation (Uchida et al. 2005). Forty rats were divided into 2 groups: a sham operation group, and a group which had their patellar tendon slackened. Stress deprived tendons showed a significantly higher ratio of cytokines (IL-1 β , TNF α and TGF-b) compared to the sham group. Conversely, mechanical stretch increased MMP-2 production in ACL fibroblasts (Wang et al. 2011). Injured ACLs have been described as a model of stress deprivation

(Mutsuzaki et al. 2010), and therefore, the process of injury itself, and the slack state of the torn ligament thereafter may be enough to increase MMP-2 production alone.

2.6 Link between Apoptosis and Inflammation

Rapid degeneration of ACL cells has been described post injury (Amiel et al. 1999). Schuerwegh et al. (2003) reported that pro-inflammatory cytokines are able to induce apoptosis in bovine chondrocytes. Additionally, apoptosis has been shown to occur in chondrocytes of the ACL tibial insertion (Mutsuzaki 2007, 2010) and on the articular surface of the knee joint (Nelson et al. 2006) post ACL rupture. Murakami (2006) showed that in vivo evaluation of ACL fibroblasts post rupture showed significant rates of apoptosis (TUNEL assay). Additionally, Mutsuzaki et Al. (2007) extracted 35 samples of the tibial insertions of the ACLs of patients undergoing ACL reconstructions and demonstrated that chondrocyte number (in the cartilage layer of the ACL tibial insertion) decreased over time. TUNEL assays confirmed that the most likely mechanism for the reduction in cell number was apoptosis. Results showed that 42.0% of chondrocytes were TUNEL positive chondrocytes after ACL rupture (Mutsuzaki et al. 2007). This apoptotic process continued from 19-206 days post rupture. That study also described that ACL rupture induces apoptosis in the cartilage layer of the tibial insertion, causing degenerative cartilage changes over time; potentially linked to OA. As previously mentioned, cytokine-induced apoptosis has been described by Lopez-Armada and colleagues (2006) using IL-1 β and TNF α as the cytokines in human cultured chondrocytes. While these studies seem to suggest cytokines are directly causing apoptosis in these cells, other studies have demonstrated that cytokines can have an indirect effect on apoptosis in fibroblastic and chondrocytic cells.

2.7 Other causes of apoptosis

Rik et al. (2003) showed that cultured fibroblast-like synoviocytes from human synovium underwent apoptosis in response to bone morphogenic protein 2 (BMP-2), with the apoptosis being enhanced by IL-1 β and TNF α (but not by interferon γ). Murakami et al. (2006) demonstrated that cytokine induced nitric oxide production caused apoptosis in rabbit ACL fibroblast cells. Interestingly in this latter study, while TNF α , tumor growth

factor-beta (TGF β), and IL-1 β were used to test the effect on cultured rabbit ACL fibroblasts, only IL-1 β decreased cell viability. Additionally, only IL-1 β up-regulated inducible nitric oxide synthase and NO (nitric oxide) production which then led to apoptosis in the cultured rabbit ACL fibroblasts (Murakami et al. 2005). These findings are consistent with those of Wang et al. (2011), showing IL-1 β to be the predominant cytokine causing biochemical change. Shuerwegh et al. (2003) also reported cytokine-induced (IL-1, TNF α) NO production led to apoptosis. Also, murine fibroblasts exhibited apoptosis triggered by cytokines and involved high levels of NO (Xie et al. 2003). Differential regulation of NO by IL-1 β and TNF α is consistent with the trends seen in the chondrocytes in the ACL tibial insertion where IL-1 β and TNF α differentially regulate the rate of apoptosis through the production of caspases (Armada- Lopez et al. 2003). In addition to nitric oxide, cultured human fibroblasts have been shown to release a reactive oxygen species in response to IL-1 β and TNF α (Meier et al. 1989). Although these studies agree that endogenous nitric oxide is a key player in cytokine induced apoptosis, there are some inconsistencies with other modulators of apoptosis in connective tissue.

For example, there are differing reports on the effect that matrix MMPs have on ACL cells in association with apoptosis. It has been suggested that chondrocyte degeneration after ACL injury has been caused by MMPs. Specifically, these proteins (MMP 1 and 2) were hypothesized as the cause of auto-degradation for fibroblasts in the ACL post rupture. However after gene expression, mRNA expression, and immunohistochemical analysis (in situ), it was shown that there was a lack of expression of matrix metalloproteinases in fibroblasts post ACL rupture (Spindler et al. 1996). Conversely, more recent studies suggest that MMPs are present at the time of ACL rupture and are linked to degenerative changes. For example, Tang et al. (2008) used a rat model of ACL rupture to show that MMP-2 production is significantly increased in synovial fluid post ACL rupture and various tissues in the intra-articular environment are responsible for its increase (posterior cruciate ligament, meniscus, and synovium). Although not directly linked to cell death in ACL cells, as of yet, MMPs are responsible for cartilage degeneration, and could be linked indirectly to apoptosis in chondrocytes as they are interconnected with cytokines which have been reported to be associated with apoptosis (Sabatini et al. 2000, Tetlow et al. 2001).

Lastly, one additional cause of ACL cell apoptosis maybe exposure to blood. Hooiveld and colleagues (2003) noted a threefold increase in chondrocyte apoptosis in human cultured cartilage cells after a short term (4-day) exposure to whole blood. There was also a significant inhibition of proteoglycan synthesis which was induced by the whole blood – this inhibition was proposed as the main mechanism for the increase in chondrocyte apoptosis. In agreement with this study, in a review on the causes of post-traumatic osteoarthritis, Lotz (2004) reported that exposure of the intra-articular cartilage to whole blood in vitro caused chondrocyte apoptosis. Additionally, this study linked inflammation and the presence of blood to cartilage degeneration in finding that mononuclear cells cause reversible glycosaminoglycan (GAG) and collagen suppression, whereas in the presence of red blood cells this process becomes irreversible (Lotz, 2004).

2.8 ACL Injury and Osteoarthritis

There is an important relationship between the ACL tibial insertion and the articular cartilage of the knee joint. The ACL tibial insertion is classified as a direct insertion which includes an interfacial ligamentous structure and two cartilage layers, a calcified and uncalcified layer, beyond which the ligament inserts into bone (Mutsuzaki et al. 2007). Due to their interconnected nature, it is not surprising that after ACL damage articular cartilage becomes vulnerable to further damage (Lotz, 2010).

Many studies have shown a high prevalence of the occurrence of osteoarthritis years after ACL damage (Nelson et al. 2006) and ACL graft surgery (Streich et al. 2011, van Porat et al. 2004). The healing capabilities of the ACL are very limited and therefore surgical intervention is often required post rupture (Mutsuzaki et al. 2007). Due to the high incidence of OA post ACL injury, many studies have assessed the benefits of repair on subsequent OA development. Recent studies have demonstrated that ACL reconstruction may be no better at decreasing OA after injury. In a study which evaluated two groups, one that underwent autologous patellar tendon graft reconstruction and another group which participated in physiotherapy (Streich et al. 2011), the 14 year follow-up demonstrated that the surgical group showed radiological incidence of OA of 62.5% whereas the physiotherapy group showed and OA incidence of 55%. This difference was found not to be significant.

2.9 Treatment of Inflammation/Apoptosis Post ACL Injury/Repair

Pain after ACL reconstruction is prevalent, as is poor healing, and therefore many studies have evaluated the effectiveness of certain treatment methods to reduce pain and increase healing capabilities post ACL repair (Beck et al. 2004). It has been shown that oral corticosteroids taken after ACL reconstruction resulted in a return to normal range of flexion in the knee joints in 78% of patients studied (John-Paul et al. 2008). Another study (n=62) compared two groups of human subjects undergoing ACL repair; the experimental group received intra-operative and post-operative treatment of corticosteroids (dexamethasone) and the control group received no corticosteroids. The experimental group used 50% less analgesics during their hospital stay, had a 59% shorter hospital stay, and ambulated 38% more quickly than the control group (Vargus and Ross, 1989). These studies suggest that corticosteroids have a beneficial effect on patient outcomes after ACL injury.

Other studies have cited work done in shoulder tendonitis in relation to ACL injury, as tendons affected by tendonitis show similarities with respect to apoptosis and ACL ligament injury (Mutsuzaki et al. 2010, Murray et al. 2000). Specifically, it is proposed that inflammation is a key player in the mechanism of injury for tendonitis, and studies show significant rates of apoptosis in shoulder tendonitis (Mutsuzaki, 2010). One study testing the effect of glucocorticosteroids on shoulder pain (in patients with shoulder tendonitis) on a tri-weekly schedule (one injection every 3 weeks), showed that one injection significantly reduced pain, but the effectiveness of 2-3 injections decreased with time (McIsaac, 2010). This is consistent with the findings that inflammation is time sensitive (Murray et al., 2000), and does decrease with time (i.e., Murray et al. (2000) noted that after phase one, (week 4, post ACL injury), there was a significant decrease in inflammatory cells). However, since patients continue to have pain and dysfunction, there must be other mechanisms at work. If apoptosis is linked to inflammation, apoptosis could be involved in the similar mechanisms that cause pain as apoptosis causes the death of cells.

There has been much more micro-analysis with regards to cytokine and apoptotic inhibition post ACL injury compared to ACL reconstruction or repair. In a rabbit model,

an intra-articular injection of a broad nitric oxide synthase inhibitor showed a significant inhibition of NO production (this NO production was up-regulated by IL-1 β) and a subsequent decrease in the rate of apoptosis (Murakami, 2006). Additionally, this study demonstrated that cultured rabbit ACL cells pretreated with a kinase inhibitor (p38 MAP) and then exposed to sodium nitroprusside (a known apoptotic inducer), showed a significant decrease in apoptosis.

Inhibition of other molecules associated with cartilage degeneration after ACL repair have also been studied. Human cultured fibroblasts showed a significant inhibition of MMP-2 production after being treated with a nF-KB inhibitor¹ (Wang et al. 2011). Furthermore, in addition to fact that chondrocytes are present in ACL tibial insertions, ACL injury and repair has been linked to the development of osteoarthritis. In human cultured osteoarthritic chondrocytes, a caspase 8 inhibitor significantly reduced TNF α /actinomycin induced cell death (Lopez-Armada et al., 2006). Also, IL-1 β -induced expression of PGE₂ protected human osteoarthritic chondrocytes from apoptosis. Conversely, in a horse model of osteoarthritis, exposure of an IL-1 receptor antagonist (adenoviral transfer) to the intra-articular space significantly reduced clinical observations of pain, and increased cartilage preservation (Frisbie et al. 2002). Therefore as some other inhibitors (i.e., caspase, NO, and NF-KB inhibitors) show a decrease in apoptosis while increases in PGE₂ also decrease apoptosis, it may be beneficial to test the effects of using multiple agents such as the above mentioned inhibitors and anti-inflammatories to better understand the disease process.

2.10 Surgical Reconstruction of the Anterior Cruciate Ligament

Two main types of graft are available for the surgical reconstruction of the anterior cruciate ligament: allografts which are tissues obtained from a donor and autografts which utilize tissues taken from the patient undergoing the operation. Within the autograft option, 3 tissues are commonly used. These include: bone-patellar-bone, semitendinosis and gracilis tendons (quadrupled hamstring tendon), or the quadriceps tendon (MacAulay et al. 2012). With regard to the exact location of ACL tears, a

¹ The nF-KB family are a group of molecules that induce transcription factors which regulate certain inflammatory cascades (Yamamoto and Gaynor, 2001). These molecules have been shown to exert a protective function on cells, inhibiting the process of apoptosis.

standardized description is difficult to ascertain as the region of rupture depends on multiple factors including the type of injury, the type of other joint structure damage, etc. (Noyes et al. 1989, Zantop et al. 2007). Noyes and colleagues (1989) studied partial tears within human ACLs and categorized the tears by percent damage (i.e. 25% tear, 50% tear, 75% tear) and the location of the tear (i.e. proximal, distal, midsubstance, and combinations of these three). Their results showed that 8/32 patients showed less than a 25% tear, 9/32 patients showed a 25% tear, 8/32 showed a 50% tear, and 7/32 showed a 75% tear. The locations of these tears varied but overall, anterior medial (13/32), anterior only (5/32), anterior proximal (4/32), and anterior distal (4/32) were the most commonly reported. Alternatively, Zantop et al. (2007) stated that rupture could be divided into the damage with respect to the anterior medial bundle (AM) and the posterior lateral bundle (PL). Of the 121 patients studied in this report, 75% experienced a complete rupture of the AM and PL bundles. The remaining patients experienced a rupture in one of the two bundles. Within the group of patients who endured a rupture of both bundles, 56% had an apparent tear at the femoral insertion (proximal tear). In all cases, even those where only the PL was torn, the AM bundle showed some signs of damage.

2.11 Gaps in Knowledge

Although there have been many studies on inflammation post ACL transection and post ACL reconstruction, many studies have focused on a variety of different cell types, such as chondrocytes in the tibial insertion, fibroblasts in the ACL, and synovium. Furthermore the majority of research has been concentrated on primary cell lines and cell culture versus explant studies (wherein cells are maintained in their extracellular matrix). Given the relationship between matrix and cells and the effect that matrix changes have on cellular gene expression, it is crucial to explore biological changes within explant models. Due to the variety of cell types and experimental models, cytokine and apoptotic changes are hard to compare between studies. Additionally, given the focus on cells (rather than matrix and cell models), current observations within cell models may not even be applicable in explant models or in vivo. Although the same may be stated with explant versus in vivo studies in that results are hard to compare, explant studies are essential to improving current reconstruction methods as types explants are actually used

for ACL reconstruction. Further limitations include the fact that inflammation has been defined in many ways including: histochemical (neutrophil, macrophage, lymphocyte presence) and immunochemical, such as using markers and biochemical assays. This also makes comparing studies and drawing conclusions difficult. Despite having an abundance of studies assessing the effect of injury or transection on the ACL, there are only a few studies on immunohistochemical changes after ACL reconstruction. Most studies that have reported on ACL reconstruction do so at a macro level including patient outcomes rather than at a micro level including cytokine and biochemical composition. To date, there have been no studies that specifically measure the effect of cytokines on the cells of the ACL and PT in an explant model. Given the abundance of studies showing that cytokines are key factors in the destruction of joint tissue and possible players in osteoarthritis, a comprehensive examination of the effects of these cytokines on the tissue must be explored to understand the response of the cells in the tissue. Furthermore, a greater understanding of the effects that cytokines have on cells and their matrix will give information that can be used to develop strategies to prevent destruction, improve reconstruction, and possibly enhance healing.

2.12 Hypothesis and Specific Aims

Based on this literature review, an explant model utilizing both PT and ACL tissue, will be used to explore the effects of cytokines on PT and ACL cell death. This study will include an examination of the hypothesis that:

IL-1 β and TNF α will cause cell death in ligament and tendon explants.

This hypothesis will be tested through the following specific aims:

- (1) Establish a method for standardized cutting of explants
- (2) Establish optimal methodology for assessing cell death and apoptosis in ligament and tendon explants
- (3) Determine if IL-1 β and TNF α change the regulation of apoptosis markers
- (4) Measure cell death in ligament and tendon explants
- (5) Determine if cell death is apoptotic

Chapter Three: Methodology

3.1 Overview of methods

My experimental methods are divided into two parts: (1) Optimization Methods and (2) Hypothesis experiments. Optimization experiments refer to experiments that contributed to optimizing, standardizing, and perfecting experimental conditions needed to answer the questions related to my hypothesis. Hypothesis experiments were those experiments that directly contributed to producing evidence relevant to my main hypothesis.

Optimization experiments included: Histological Analysis of 2 Groups of Sheep Tissue (3.4.1), Cytokine Preparation (3.4.2), Jig Creation and Testing (3.4.3), and the Assessment of Cell Density in Ligament and Tendon Regions. Hypothesis Experiments included: Cytokine Penetration and Downstream Effects in Primary Cells, Cell Death Rates in Primary Cells treated with Cytokines, Cytokine penetration and downstream effects in explants, Live/Dead Assay of PT&ACL explants in various experimental conditions, Annexin V Assay, and DNA Laddering.

3.1.2 Overview of Optimization Methods

Due to the fact that our lab conducts sheep ACL reconstruction surgeries frequently, upon initiation of my study, there was rapid access to histological sections of normal and surgically altered ACL tissue. It was thought that it would be logical to proceed with an examination of the histological sections to see if there was first-hand evidence (in addition to the evidence found in the literature) of differences between normal and surgically altered tissue. Additionally, this was done to gain a better understanding of the tissue as seen in histological section form. Furthermore, the sheep model utilized in our lab (Heard et al., 2011) was a perfect fit for this initial examination for many reasons. Firstly, the animal model described by Heard et al. (2011) utilizes sheep due to their similarities in joint loading to humans. Secondly, Heard et al. (2011), have noticed an up-regulation of inflammation (or cytokines) after their ACL reconstruction surgery. To ensure a sound basis for my hypothesis, first I examined whether or not any histological differences between normal and surgically altered sheep

(n=6). Specifically, I wanted to examine whether differences in cell type, cell number, blood vessel number, intracellular space or any other differences existed between normal tissue and tissue that had been subjected to an up-regulation of cytokines. My hypothesis involves testing explants: therefore, to test my hypothesis, I had to develop a jig or procedure in which to cut the explants of PT and ACL so that they would be the same size and results would be comparable (specific aim 1). Therefore, I developed a protocol to ensure a standardized explant size from both the PT and the ACL. To ensure accurate and optimal results during hypothesis testing, I then carried out a pilot sheep explant culture to test the jig, cytokine concentrations, histological methods, confocal microscopy methods, and harvest methods. This was done to understand if any improvements in methodology could be implemented. Using the jig, I carried out a preliminary explant cytokine culture to ensure standardized protocol, after which, the imaging procedure, cytokine concentrations, explant cutting, and histological procedures were optimized. Based on the results from the explant culture (major differences in total cell counts between treatment groups), the next step was to conduct an assessment of whether or not differences in fibroblast densities differed in regions of the PT and the ACL before cytokine treatment, or if the differences were due to cytokine treatment or human error. Therefore I conducted a study that explored differences in cell numbers in varying regions of the PT and the ACL. Additionally, I tested whether or not different media induced differences in a measure of swelling between PT and ACL in normal tissue . I investigated swelling as it could distort the field of view under the microscope and thereby change the cell numbers between tissues. To understand whether or not counting differences caused the major differences in cell number I carried out a study that tested the computerized counting program.

3.1.3 Overview of Hypothesis Methods

Given that the methodology for testing the hypothesis was now optimized, the first step in hypothesis testing was to test my hypothesis in primary cells before moving on to an explant model. This was performed specifically to test whether or not cytokines were penetrating and affecting the down-stream measures associated with apoptosis in primary cells before moving to explants. I needed to measure whether or not I could see

cytokine effects in cell lines before moving to tissue. This was also done to ensure that the hypothesis was aligned with my own evidence and to anticipate which markers would be most important in the explant testing (for the hypothesis). For this, I used primary cell lines of PT and ACL fibroblasts and tested mRNA expression after treatment with various cytokines. The percentage of cell death after treatment with the same cytokines was then determined in the primary cell lines of the ACL and PT. Given that the cell line experiments showed effects due to cytokine treatment, I moved on to testing the main hypothesis in explants. Specifically, PT and ACL explants were subjected to cytokines after which mRNA expression and cell death were measured. After measuring apoptotic death and inflammatory markers in mRNA expression, I next had to test whether there were similar results in the number of dead cells in explants. Therefore, lastly, I tested the percentage of apoptosis present in dead cells. For this, I utilized Annexin V fluorescence imaging and a DNA laddering procedure. Two measures of apoptosis testing were utilized to ensure similar results between procedures.

3.2 Optimization Experimental Methodology

3.2.1 Utilization of different Animal species

All animals used in the experiments were obtained from the University of Calgary Health Science Animal Resource Centre after euthanasia. All protocols were carried out in accordance with secondary tissue use protocols from the University of Calgary Ethics Board. The first exploration study utilized sheep tissue. This was due to the fact that our lab has developed a specialized protocol for ACL reconstruction that returns the native ACL to its anatomic position with disturbance of the femoral insertion only, thus providing the best structure in terms of biomechanics and immunity compared to the normal ACL structure. This is referred to as an 'ideal' graft ACL reconstruction (Heard et al., 2011). At the time of this preliminary study, our lab had sufficient histological sections from both normal and surgically altered ACLs. For the assessment of fibroblast density and jig creation and testing, I utilized pigs, as they were more readily available for experiments and the sizes of the PT and ACL in pigs are similar to the sizes of the PT and ACL in sheep. I continued with the use of sheep for the cytokine explant experiments

in order to reference the histological differences from the first experiment. Based on the fact that it seemed as if cutting was causing further cell death, all further experiments utilized rat tissue based on animal availability and size of the PT and ACL, as it was now important to have small tissues as we decided to use the intact PT and ACL as our explants.

3.2.3 PCR analysis of mRNA expression

This method was used for both cell line and explant experiments of PCR mRNA analysis. Specifically, this method was adapted to our specific cell conditions from the original methodology described by Reno et al. (1997). Briefly, at the end of each cytokine treatment, tissue was frozen to -70 degrees Celsius. Tissue was then powdered and all RNA was extracted from cells using a Qiagen RNeasy kit (Qiagen Sciences, Germantown, MD). The tissue was processed according to the manufacturer's specifications, including DNase I treatment and eluting with RNase free H₂O. One microgram of total RNA was then incubated (one hour, 37 degrees) with reverse transcriptase to generate single stranded cDNA using Qiagen Omniscript RT kit (Qiagen Sciences). The real time polymerase chain reaction used primers identified by the Gene Tissue Bank as specific to our animal populations and tissue of interest. Before use in experimental conditions, each primer was tested for efficiency and specificity and optimization occurred where necessary (Table 2). Each real time PCR reaction included the following mixture of solutions: BIO-RAD iQ SYBR Green Supermix (12.5 ul) (BIO-RAD, Hercules, CA, cat: 970-8882), distilled water (3.5ul), 7.5 ul of reverse transcriptase, and a forward and reverse primer (0.75 ml each). A BIORAD CFX96 real time PCR Detection System was used for amplification and detection and was used for the evaluation and optimization of primers. mRNA expression was normalized to the 18S ribosomal housekeeping gene. Quantification of the resulting mRNA expression in the experimental treatment groups was performed with Nanovue (GE Health Sciences, 28-9569-58)

3.2.3 Cytokine Preparation

Peprtech Human IL-1 β (cat: 100-0113) was converted from its lyophilized (10 mM) form to a stock solution of 5 mg/ml. This was further diluted with DMEM to a concentration of 5ng/ml, and 5 pg/ml for experiments. Peprtech Recombinant RAT TNF α (cat: 0700473) was converted from its lyophilized (0.5 mM) form to a stock solution of 10 mg/ml. This was further diluted with DMEM to concentrations of 10 ng/ml and 10 pg/ml for experiments.

3.2.4 Sodium Nitroprusside (SNP) Preparation

Sodium nitroprusside crystals (Sigma, cat: 71778) were dissolved in DMEM to make a 1 M stock solution. After a 1/1000 dilution, a working solution was divided into 1.5 ml aliquots for experimental conditions.

3.2.5 Histological analysis of Surgical ACL sheep and Control ACL sheep

Eighteen Suffolk Cross sheep were divided into 3 groups: Surgical (n=9), Sham (n=3), and Normal (n=6). The Surgical group underwent ‘ideal’ ACL reconstruction as described by Heard et al. (2011). Briefly, ‘ideal’ ACL reconstruction refers to the procedure in which the ACL is cored and freed from its femoral insertion and then replaced back into its original anatomical position. ‘Ideal’ ACL reconstruction is referenced, as in this model the biomechanics are immediately restored after injury (Heard et al. 2011). After 2 weeks, animals were euthanized and the ACLs were harvested. The ACLs were fixed in formalin overnight and then processed in a Leica 400 dewaxing/parafinizing machine. Ten micrometer sections were cut and stained with Safranin O. The numbers of cells per 10000 μm^2 were counted per (n=20 sections) per each group. The hypothesis for this study was: histological sections from surgically altered ACLs in sheep would be the same histologically as histological sections from control sheep.

3.2.6 Jig Creation and Testing

In order to standardize the size of explant pieces, a jig for cutting each explant was created. Testing of the jig was performed to ensure standardization among the dimensions of each explant. The jig was made out of two metal surgical blades (VWR Industrial

Razor Blades, surgical carbon steel, single edge #9, cat: 55911030) secured together with glue. A glass spacer was inserted between the metal blades to allow for necessary sterilization. The final dimensions of the metal jig were: length= 5 mm, width=1 mm. Four skeletally mature pigs were obtained from the University of Calgary Health Science Animal Resource Centre (HSARC). ACL, MCL, and PT tissues were harvested and placed in Dulbecco's Modified Eagle Medium (DMEM) for transport from the Animal Resource Centre to the tissue preparation room (10 mins). The metal jig and surgical scalpel were used to cut 18 explants from each structure (i.e. ACL, MCL, PT). Twelve explants per structure were measured (length, width, depth), and 6 explants per structure were weighed.

3.2.7 Explant cytokine culture

Three Suffolk Cross sheep were obtained from the University of Calgary Health Science Animal Resource Centre after euthanasia. The following tissues were harvested for explant culture: ACL, MCL, PT. After harvest, tissues were placed in DMEM for transport from the HSARC to the tissue preparation room (10 mins). Tissues were cut with the metal jig into 5x1x1 mm (and an additional metal jig made with the same materials, with different dimensions, 3x4x1 mm) explants and were put into two different concentrations of two different cytokines. The cytokines used in this experiment were tumor necrosis factor alpha (TNF α) and interleukin one beta (IL-1 β). Dosage concentrations used were those that have been most often published in explant and cell studies of ACL, MCL, and PT (10 ng/ml) (Carames et al., 2008) and the concentration of cytokines found at the site of ACL injury (10 ug/ml) (Irie et al, 2003). An injury concentration was chosen as its effects may provide further information on cellular behaviour after joint injury. A Max (published) concentration was used for two reasons (1) currently there are no studies that report on the effect of differing concentrations of cytokines (2) current studies only cite concentrations (for cytokine experiments) that are used on cell culture.

Table 1. Cytokine concentrations for Injury and Max doses (Carames et al., 2008).

Dose	TNFα	IL-1β
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Injury	10 pg/ml	5 pg/ml
Max	10 ng/ml	5 ng/ml

This resulted in the formation of four groups for each of the tissue explants: MAX (10ng/ml – IL-1 β , 100ng/ml –TNF α), Injury (10pg/ml- IL-1 β , 10pg/ml-TNF α). Each group was then subjected to incubation (37 degrees Celsius) for different time points: 0, 24, or 48 hours. Due to the lack of studies with PT and ACL explants, there was uncertainty as to the time needed for penetration of cytokines into the explants, therefore various time points were used to obtain the optimal time for penetration and for visualization of the effects of the cytokines. After the completion of each time point, medium was removed and replaced with 250 ul SytoX (Invitrogen #S11368) and Syto 13 dyes (Invitrogen #S7575) (1 hour, room temperature), dyes for live/dead cell analysis. Cells were imaged with a Zeiss Axiovert 100M Confocal Microscope. Specifically, cells were imaged in a 956 um x 956 um frame, 10X objective. Images were counted with cell imaging software called Pixcavator 5.3 (Intelligent Perception Co., West Virginia, 2013). The hypothesis of this experiment was: (1) Differing cytokine concentrations will have no effect on the number of dead cells present after cytokine treatment, (2) Differing jig sizes (increased tissue cutting) will not effect the amount of cell death found in explants.

3.2.8 Assessment of cell density differences between regions of ACL and PT

Murray and Spector (1999) noted three distinct regions of fibroblast distribution within the ACL. These consisted of (anterior to posterior) 1) Fusiform Cell Zone, 2) Ovoid Cell Zone, and 3) Spheroid Cell Zone. To assess differences in fibroblast numbers, the following experiment was carried out using the zones identified by Murray and Spector (1999) (Figure 6). Six skeletally mature pigs were obtained from the University of Calgary Health Science Animal Resource Centre after euthanasia. PT and ACL tissues were harvested and placed in DMEM. For the assessment of differences in osmolality between media and native synovial fluid, synovial fluid was extracted (at tissue harvest), via syringe, from the joint and tested on an Osmette A Automatic Osmometer to determine osmolality of synovial fluid versus the media. For the assessment of fibroblast differences between different regions of both PT and ACL, each tissue piece was cut into

3 pieces (Figure 3) and labeled A (Fusiform region), B (Ovoid region), or C (spheroid region). Samples were then submersed in 3 different media (DMEM, 10% sucrose¹, and XVIVO-10) for one hour (37°C). Tissue explants were weighed and their wet weights were recorded. The change in weight from before to after submersion in medium was used as a measure of swelling. Samples were then processed into paraffin sections, stained with H&E (Hematoxylin and Eosin) and analyzed through confocal microscopy (Zeiss Axiovert 100M Confocal Microscope). Cell counts were performed with Pixcavator version 5.3. A random sample of 50 sections was selected for manual counting and compared to computerized cell counting. *The hypothesis of this experiment was: All regions of the PT and ACL explants have the same number of cells.*

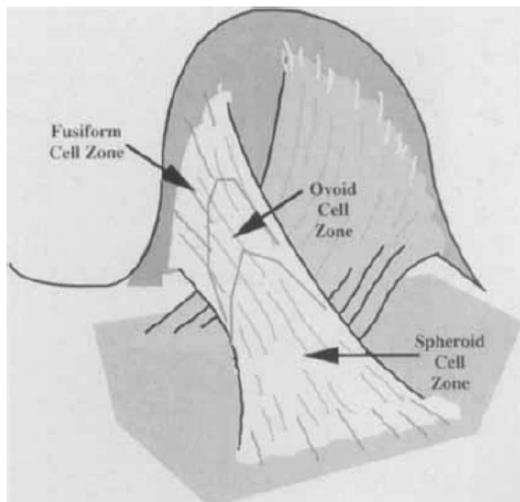


Figure 3. Fibroblast distribution varies among three distinct regions (Murray and Spector 1999).

3.2.9 Weight differences between ACL and PT

Six Sprague Dawley rats were obtained from University of Calgary Health Science Animal Resource Centre after euthanasia. ACL and PT structures were harvested and placed in Dulbecco's Modified Eagle Medium. Structures were weighed on scale and measurements were recorded and analyzed. The hypothesis of this experiment was: *There is no difference in weight between PT and ACL explants of the similar dimensions.*

3.3 Hypothesis Experimental Methodology

3.3.1 Cytokine penetration and downstream effects in primary cells

The ACL and PT of both legs were harvested, placed in DMEM medium supplemented with 1% FBS and were incubated at 37 degrees Celsius for 24 hours to equilibrate to new environmental conditions. The ACL and PT structures were cut into smaller pieces, and placed into a culture dish: cells were allowed to grow out of the tissue structures for ± 1.5 weeks. Cells were passaged through passage 2, with passage 3 cells being used for all experimental conditions. After the cells were grown to confluency, cells were divided into two groups: IL-1 β and TNF α . For both PT and ACL cells, IL-1 β cells were treated with two different concentrations of the cytokine. An Injury (5 pg/ml) concentration of IL-1 β was used to correspond to the concentration of IL-1 β at the site of ACL injury, and a Max (5 ng/ml) was used to coincide with recent studies of the same nature (Irie et al., 2002, Carames et al., 2008). Similarly for TNF α , the injury concentration used was 10pg/ml and the Max concentration was 10ng/ml (Irie et al., 2002, Carames et al., 2008). Cell groups were incubated with cytokines for 2 and 24 hours at 37°C. In preparation for my main hypothesis testing, I wanted to investigate if IL-1 β and TNF α could cause downstream effects in apoptotic and inflammatory markers to (1) provide better foundation for hypothesis testing (2) demonstrate whether or not IL-1 β and TNF α were getting into the tendon and ligament cells. Therefore, Reverse Transcriptase PCR was then carried out in all cell groups to evaluate the mRNA expression of IL-1 β , IL-6, TNF α , inducible nitric oxide synthase (iNOS), Caspase-3, and COX-2. *The hypothesis for this experiment was: IL-1 β treated cells and TNF-a treated cells will not change the regulation (in comparison to 18S) of IL-1 β , IL-6, TNF α , iNOS, Caspase-3, and COX-2 mRNA expression.*

3.3.2 Cell death rates in primary cells treated with cytokines

Six Sprague Dawgley rats were obtained from the HSARC. Both legs were utilized therefore 12 ACL and PT structures were harvested, placed in DMEM and incubated at 37 degrees Celsius for 24 hours to equilibrate to new environmental conditions. The structures were cut into smaller pieces, and placed into a culture dish and allowed to grow

for ± 1.5 weeks. Cells were passaged through passage 2: passage 3 cells were used for all experiments. After the cells were grown to confluency, they were divided into two groups based on their experimental treatment: IL-1 β and TNF α . Consistent with our previous experiments, an Injury (5 pg/ml for IL-1 β , 10 pg/ml for TNF α) concentration, and a MAX (5 ng/ml for IL-1 β , 10 ng/ml for TNF α) concentration were used for experimental treatments. Cells were incubated with Injury and Max cytokine concentrations for 2 and 24 hours. Cells were washed with DMEM, and were then incubated with SytoX, 5 mM SytoX Orange (Invitrogen #S11368) and Syto13 dyes (Invitrogen #S7575) for 30 minutes, at room temperature. Cells were imaged with a Leica DFC420 microscope and Leica Firecam and were subsequently counted with Pixcavator 5.3 (Intelligent Perception Co., West Virginia, 2013). The hypothesis of this experiment was: *Cells subjected to differing concentrations of IL-1 β and TNF α will display the same percentage of cell death compared to control cells.*

3.3.3 Cytokine effects in explants

Seven Sprague Dawgley rats were obtained from the HSARC. The ACL and PT (14 samples) were harvested, placed in DMEM and incubated at 37 degrees Celsius for 24 hours to equilibrate to new environmental conditions. The PT and ACL explants were divided among the following experimental groups: DMEM(1) (DMEM media+1%FBS), DMEM(10) (DMEM+10%FBS), IL-1 β (Interleukin 1 beta (5 ng/ml)), TNF α (Tumor necrosis factor alpha (10 ng/ml)), SNP (sodium nitroprusside (1 mM)). Reverse Transcriptase PCR was then carried out on all cell groups to evaluate the mRNA expression of IL-1 β , iNOS, and Caspase-3. Time Zero, DMEM(1), and DMEM(10) were potential control groups. DMEM(1) was chosen as the control group (the group to which all other treatment groups were standardized to) because DMEM(1) was the substance used to prepare SNP and the cytokines for use in their treatment groups. Therefore, DMEM(1) was the best control compared to DMEM(10) and Time Zero. Specifically, DMEM(10) has a higher percentage of fetal bovine serum (FBS) which may influence cytokine penetration. Additionally, Time Zero explants would only have experienced a brief amount of time outside of the joint compared to the DMEM(1) group. The hypothesis of this experiment was: *Explants treated with IL-1 β , TNF α , DMEM(1),*

DMEM(10), and SNP will not change the regulation of IL-1 β , iNOS, and Caspase-3 mRNA expression.

3.3.4 Live/Dead Assay of PT&ACL explants in various experimental conditions

Twenty-one Sprague Dawgley rats were obtained from HSARC. Forty-two explant samples of each of the ACL and PT structures were harvested, placed in DMEM and allowed to equilibrate for 24 hours before initiation of the experiment. These PT and ACL explants were divided among the following experimental groups: DMEM(1) (DMEM media+1%FBS), DMEM(10) (DMEM+10%FBS), IL-1 β (Interleukin 1 beta (5 ng/ml)), TNF α (Tumor necrosis alpha (10 ng/ml)), SNP (sodium nitroprusside (1 mM)). Experimental groups were incubated with respective reagents for 18 hours at 37°C. After incubation, media and reagents were removed and replaced with 5 uM SytoX Orange (Invitrogen #S11368) and 5 um Syto13 dyes (Invitrogen #S7575) for one hour at room temperature. Explants were imaged with a Zeiss Axiovert 100M Confocal Microscope in a 950 um x 950 um frame, at 10X objective. Imaging consisted of separate photos taken at specific areas on each explant (Figure 4) to ensure that perimeter areas and centre areas could be differentiated for analysis. Additionally, this pattern was carried out at 3 different depths (300 um apart) within the PT and ACL explants to determine live/dead cell distribution throughout the explants. All photos were imaged with Confocal microscopy, with no additional cuts being made to the explants. The hypothesis of this experiment was: *Explants treated with IL-1 β , TNF α , DMEM(1), DMEM(10), and SNP will show the same number of dead cells after treatment.*



Figure 4. Graphical depiction of photography pattern for confocal microscopy. The blue rectangle represents the ACL or PT explant and the small squares indicate regions where photographs were taken. Red squares represent photos which were considered 'perimeter' regions and the green square represents photos which were considered 'middle' regions.

3.3.6 Annexin V Assay

Six Sprague Dawgley rats were obtained from the HSARC. The ACL and PT were harvested from both legs, placed in DMEM and incubated at 37 degrees Celsius for 24 hours to equilibrate to new environmental conditions. The PT and ACL explants were divided up among the following experimental groups: DMEM(1) (DMEM media+1%FBS), DMEM(10) (DMEM+10%FBS), IL-1 β (Interleukin 1 beta (5 ng/ml)), TNF α (Tumor necrosis alpha (10 ng/ml)), SNP (sodium nitroprusside (1 mM)). Experimental groups were incubated with respective reagents for 18 hours at 37°C. After incubation, media and reagents were removed and replaced with media alone. An Annexin V-Cy3 Apoptosis Detection Kit (Abcam #ab14142) was prepared to the manufacturer's instructions. Explants were imaged with a Zeiss Axiovert 100M Confocal Microscope on a 950 μ m x 950 μ m frame, with a 10X objective. Photos were analysed with Pixcavator 5.3. Annexin V dye was red whereas live cells stained green (SYTO13). Apoptotic cells were counted per treatment group. Final apoptotic percentage was calculated as the number of apoptotic cells (from the annexin V assay) divided by the total number of cells counted in the live/dead assay, n(ACL)=169, n(PT)=248. The hypothesis if this experiment was: *Explants treated with IL-1 β , TNF α , DMEM(10), DMEM(1), and SNP will show the same number of apoptotic controls compared to each other and control explants.*

3.3.7 DNA Laddering

Six Sprague Dawgley rats were obtained from the HSARC. Their ACL and PT's were harvested, placed in DMEM and incubated at 37 degrees Celsius for 24 hours to equilibrate to new environmental conditions. The PT and ACL explants were divided up among the following experimental groups: DMEM(1) (DMEM media+1%FBS), DMEM(10)(DMEM+10%FBS), IL-1 β (Interleukin 1 beta (5 ng/ml)), TNF α (Tumor

necrosis alpha (10 ng/ml)), SNP (sodium nitroprusside (1 mM)). Experimental groups were incubated with respective reagents for 18 hours at 37°C. Protocol for the DNA laddering procedure was adapted to the specific study conditions from the original work done by Wang and Storm (2006). Briefly, explants were incubated with a digestion buffer (400 ug/ml Proteinase K, Invitrogen Life Technologies, cat: 2550-015) at 55 degrees Celsius overnight. The explant was then washed with ethanol and centrifuged (16000xg) for 30 minutes. The pellets were then dissolved in the TE buffer (10 mM Tris-HCl, 0.2 mM NaEDTA, pH 7.5) (50 ul for ACL, 100 ul for PT) in a speed vac dry process. Pellets were electrophoresed on 2% agarose gel. Quantification was done with Nanovue (GE Health Sciences, 28-9569-58). The hypothesis for this experiment was: *All experimental groups (IL-1 β , TNF α , DMEM(10), DMEM(1), SNP) will show the same DNA fragmentation compared to each other and to control samples.*

Table 2. Primers utilized in the RT PCR quantification. Primers were obtained from the Gene Tissue Bank. Optimal temperatures and PCR efficiency are shown below.

Protein	Sequence	TB#	Temp	PCR Efficiency Rate
IL6	F: TCC TAC CCC AAC TTC CAA TGC TC R: TTG GAT GGT CTT GGT CCT TAG CC	E02522	63.3	104.6
TNFα	F: AAA TGG GCT CCC TCT ATC AGT TC R: TCT GCT TGG TGG TTT GCT ACG AC	66539	58.9	104.7
iNOS	F: GCT ACA CTT CCA ACG CAA CA R: ACA ATC CAC AAC TCG CTC CA	* Chun et al. 2012)	61.4	106.1
Casp-3	F: CTG GAC TGC GGT ATT GAG AC R: CCG GGT GCB GTA GAG TAA GCC	A09515	55	106.1
COX-2	F: CCT TCC TCC TGT GGC TGA TGA C R: ACA CCT CTC CAC CGA TGA CC	1103389	58.9	97.7
18S	F: TGG TCG CTC GCT CCT CTC C R: CGC CTG CTG CCT TCC TTG G	X03205	58.9	96.6

3.4 Statistical Methods

All data were initially tested for normality with the Shapiro-Wilk test. Non-normally distributed data were then tested with the Kolmogorov-Smirnov test. If data tested normal for the Kolmogorov-Smirnov test or Shapiro-Wilk tests (i.e. $p > 0.05$), parametric analysis was conducted. To ensure reliability, data that tested normal with only Kolmogorov-Smirnov and not with Shapiro-Wilk were then subjected to Welch's test for homogeneity of means. Normally distributed data were subjected to parametric analysis including independent t-tests and one way ANOVAs. Non-normal data were subjected to non-parametric analysis including the Kruskal-Wallis test and the Mann-Whitney U test. All data were analysed with IBM SPSS Statistics version 21. Data were considered significantly different if $p < 0.05$.

3.5 Cell counting

All cell counting with the exception of the first cytokine explant culture was performed with Pixcavator 4.3 (Student Edition). The first computer input allows the user to choose the picture for analysis and the 'channel' (red, green, blue). Next the program allows adjustment of analysis settings. Options for input are size (number of pixels), maximal contrast (of image of interest compared to background), border contrast, average contrast, intensity (light and dark). Changing these settings changes what the program will consider 'a cell' (Figures 5,6). The program allows the user to isolate the objects it has counted as the object of interest and can display a photograph that colours the objects of interest to ensure that the analysis output has counted only the cells of interest. For cell counting, size varied between 10-15 pixels depending on the image, maximal contrast=10, and border contrast varied between 1-3 depending on the image.

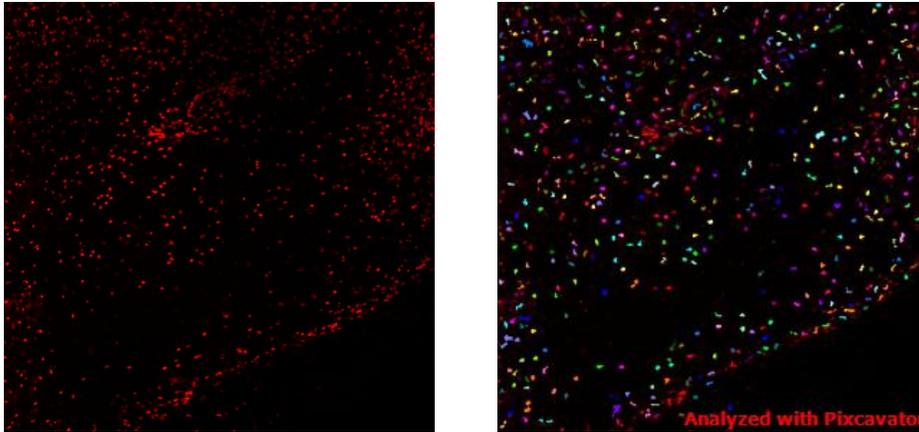


Figure 5. Confocal image of PT explant after SYTOX staining 960um x960 um (10x). Image on left shows regular confocal microscopy photograph (red dye stained cells). Image on right shows Pixcavator image (coloured circles represent cells counted by program). These are the same image.

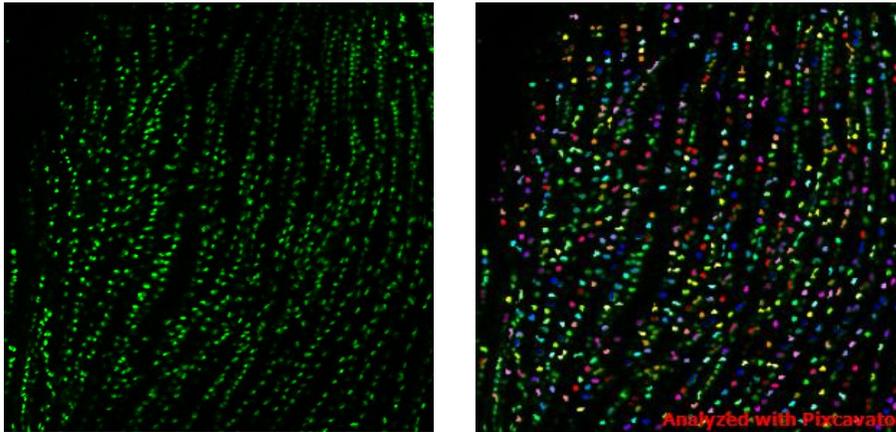


Figure 6. Confocal image of ACL explant after SYTOX staining 960um x960 um (10x). Image on left shows regular confocal microscopy photograph (red dye stained cells). Image on right shows Pixcavator image (coloured circles represent cells counted by program). These are the same image.

Chapter Four: Results

4.1 Overview of Results

Results will be presented in chronological order starting first with optimization experiment (3.4) results followed by hypothesis experiment (3.4) results.

4.1.2 Overview of Optimization Experiment Results

Examination of whether or not histological differences existed between the surgically altered and normal ACL (section 3.4) showed that the surgically altered ACL had a significantly lower mean percentage of cells compared to the normal ACL. Results of the jig creation and testing (section 3.5) showed that we successfully created a working jig that standardized the size of both PT and ACL explants (Section 4.3). After the preliminary cytokine explant culture (3.8), large differences were noted in total cell counts between treatment groups, increased cell death was seen near cut ends, and decreased cell death was observed with increased cytokine concentration. After the assessment of total cell counts (3.9), no significant differences were found in cell density between the A, B and C regions in both the PT and ACL, no difference in osmolality between media and synovial fluid was observed, no difference in swelling between media, and no difference in cell counts between manual and computerized counting.

4.1.3 Overview of Hypothesis Experiment Results

After measuring mRNA expression in primary ACL and PT cells (3.11), results showed evidence that cytokines were penetrating the cells through up-regulation of cytokines and other inflammatory and apoptotic markers. Measurement of mRNA expression in explants (3.13) did show significantly different results between treatment groups, particularly with, IL-1 β , SNP, and iNOS. Specific results are presented below. Results of measuring the percentage of dead cells in PT and ACL cells after cytokine treatment (3.12) showed no significant differences between cytokine-treated and media-treated groups. In contrast, the percentage of cell death in explants (3.14) treated with the same reagents as the primary cells was much higher. However, similar to the trend seen in the cells, most explant treatment groups did not show significantly higher percentages

of cell death compared to the media controls. Only our positive control, SNP, showed significant cell death rates. With respect to rates of apoptosis (3.15), ACL explants showed no difference between treatment groups with the exception of the apoptosis inducer SNP. Conversely, PT explants did show differences, particularly with cytokine-treated groups. Specific differences are presented below. DNA laddering (3.16) results supported the findings of the Annexin V assay (3.15) with ACL explants showing no significant difference to the SNP while PT explants treated with cytokines did show some significant differences compared to SNP.

4.2 Histological Analysis of 2 Groups of Sheep Tissue

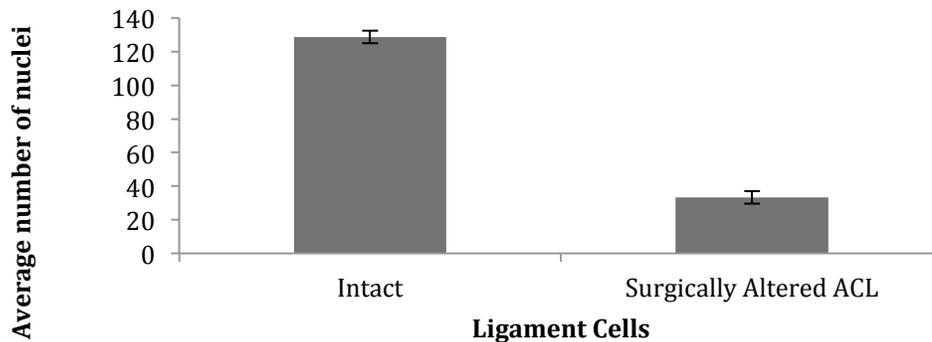


Figure 7. Mean number of nuclei per 6000 x 4000 μm , 70 x 0.50 (n=20). Evidence significant of hypocellularity is shown in surgically altered histological sections.

Cell counts between intact ACL and surgically altered histological sections were not normally distributed as assessed by Shapiro-Wilk's test and Kolmogorov-Smirnov's test ($p > 0.05$) (Figure 7). The mean number of nuclei counted in the normal group of histological slides was 128.7 versus the mean number of nuclei counted in the surgically altered group of histological slides, 33.2 (Figure 7). The surgically altered group had significantly lower cell count (75% less) compared to the normal group (n=20, $U = < 0.001$, $p = < 0.001$) (Figure 7). The null hypothesis for this study that histological sections from surgically altered ACLs in sheep would be the same histologically as histological sections

from control sheep (section 3.4, page 21) was therefore rejected in favour of the alternate hypothesis.

4.3 Jig Creation and Testing

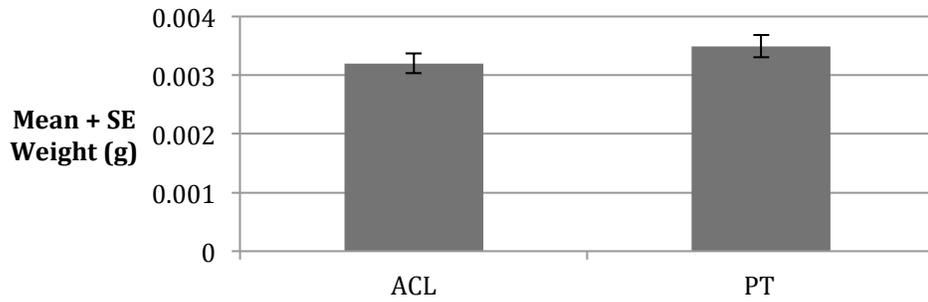


Figure 8. Mean \pm SE weight of PT (n=8), and ACL (n=8) explants after cutting with the metal jig. Evidence of standardization among explants is shown by the lack of variability in weight measurements. The coefficient of variation for ACL weight was 0.28 and 0.43 for PT weight.

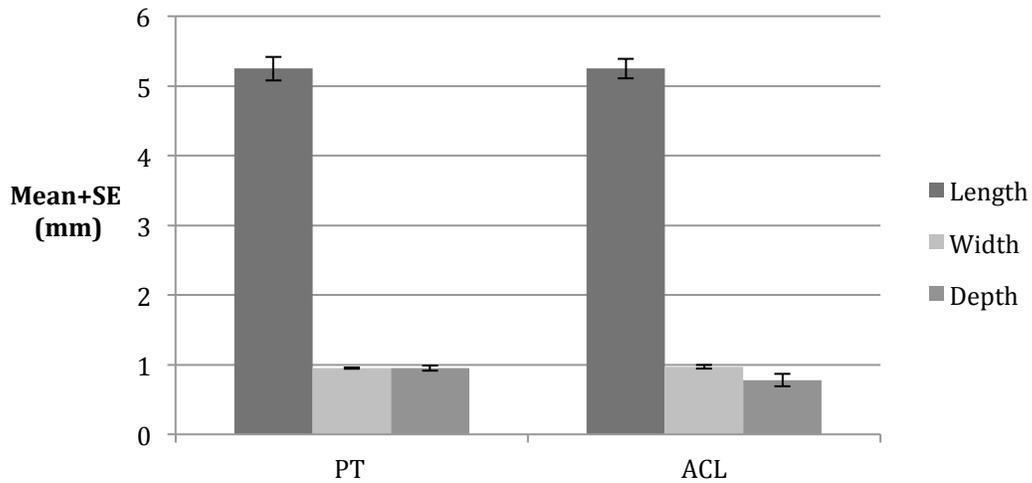


Figure 9. Mean \pm SE dimension measurements of explants: PT (n=12), ACL (n=12), after metal jig cutting.

The mean length of the PT explants was 5.30 mm, compared to 5.29 mm for the mean length of the ACL explants (Figure 9). The coefficient of variation for PT length was

0.07 and 0.06 for ACL length. The average width for ACL explants was 0.97 mm, whereas for PT explants the average width was 0.95 mm. The coefficient of variation for PT width was 0.10 and 0.07 for ACL width. In terms of depth dimensions, the mean PT explants measured 0.93 mm versus 0.97 mm for ACL explants. The coefficient of variation for PT depth was 0.12 and 0.25 for ACL depth. There were no significant differences between PT and ACL explants in all dimensions of length, width, and depth ($p=0.939$, $p=0.455$, $p=0.586$, respectively). The hypothesis for this experiment that a metal jig can standardize ACL and PT explants (section 3.5, page 21) was therefore accepted.

4.4 Explant Cytokine Culture

PT data showed a non-normal distribution (Shapiro-Wilk, Kolmogorov-Smirnov, $p<0.05$) (Table 3). Therefore, PT cell data were tested with Kruskal Wallis and subsequent Mann-Whitney U tests to determine specific differences. Kruskal-Wallis determined that there were significant differences in the percentages of cell death between Control, Injury, and Max groups. Specifically, the percentage of cell death found in the Control group was significantly less (91.5%) than the percentage of cell death found in the Injury cell group (94.3%) ($n=22$, $p<0.001$) (Table 3). In contrast, the Control group had a significantly higher percentage of cell death (91.5%) compared to the Max group (84.8%) ($n=22$, $p<0.001$). With regard to the cytokine-treated groups, the Injury group had a significantly higher percentage of cell death compared to the Max group (84.8%) ($n=22$, $p<0.001$). ACL cell data were assessed by the Kolmogorov-Smirnov test to have a normal distribution (Table 3). A one-way ANOVA was carried out showing a significant difference between treatment (Control, Injury, Published) groups ($p<0.001$, $df=2$, $n=60$). Similarly to the PT cells, the highest percentage of dead ACL cells was found in the control group. The lowest percentage of dead ACL cells was found in the group which was exposed to the highest concentration of TNF α . Specifically, control cells had a significantly higher percentage of cell death (77.3%) compared to those ACL cells treated with 10 ng/ml (MAX group) of tumor necrosis factor alpha (42.9%) ($n=60$, $df=2$, $p<0.001$) (Table 3). Furthermore, the injury concentration group

had a significantly higher percentage of dead cells (68%) versus the MAX concentration group, which had 42.9% dead cells after treatment (n=60, df=2, p=<0.001).

Table 3. Percentage of live versus dead cells in various joint structures. Concentrations refer to exposure to tumor necrosis factor alpha (TNF α) for 24 hours before analysis. 10 pg/ml was used to simulate concentration of cytokines found at the site of joint injury and 10 ng/ml was used in reference to current publications in the field of ligament biology (n=3 ACL, n=3 PT).

PT	Total Cells	% Dead	% Live
Control	1801.9	91.5	7.8
10 pg/ml (Injury)	3670.1	94.3	5.4
10 ng/ml (Max)	12826.0	84.8	14.2

ACL	Total Cells	% Dead	% Live
Control	12689.9	77.3	22.7
10 pg/ml (Injury)	1350.4	68.0	32.0
10 ng/ml (MAX)	2656.9	42.9	57.1

Table 4. Mean + SE percentage of dead cells with of multiple cut (5x1 mm) versus single cut (3x4 mm) and intact structure after jig cutting (n=8).

Area	5 (mm²) (Multiple Cut)	12 (mm²) (Single Cut)	Intact
Mean	84.3	67.4	42.3
SE	1.0	1.6	3.7

Data showed a normal distribution as assessed by Shapiro & Wilk test (n=69, p=0.122). A one way ANOVA showed significant differences between all groups. Specifically, the percentage of dead cells in multiple cut explants was significantly higher than both single cut explants and the intact structure (n=69, df=2, p<0.001). Additionally, the single cut explant had a significantly higher percentage of cell death compared to the intact structure (n=69, df=2, p<0.001). The hypothesis that differing jig sizes (increased tissue

cutting) will not effect the amount of cell death found in explants (Section 3.8, page 22) was rejected.

4.5 Assessment of differences in fibroblast distribution between regions of ACL and PT

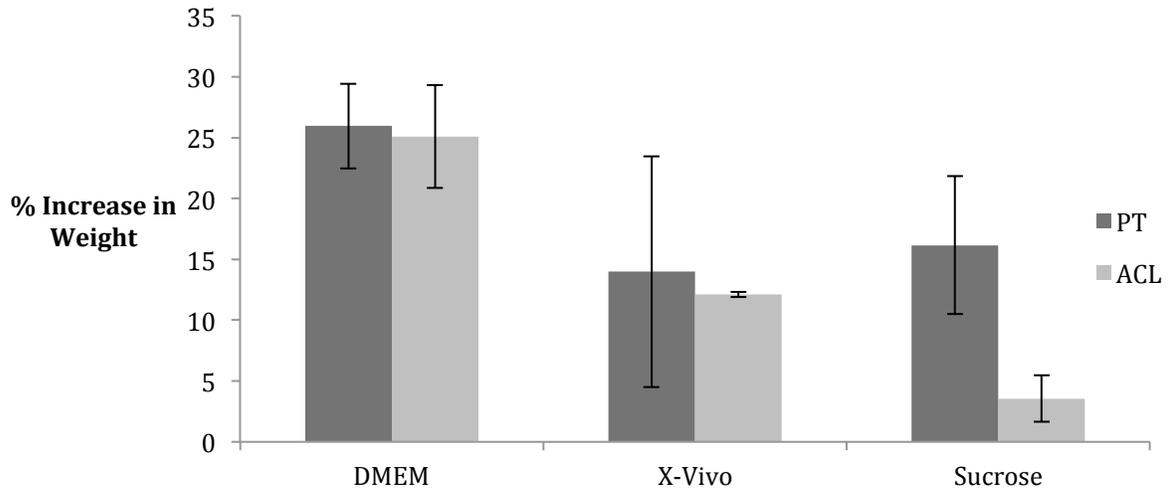


Figure 10. Mean \pm SE percentage increase in weight after 1 hour of submersion of PT and ACL tissues in different media (n=12).

The mean percentage change in weight for ACL explants submerged in DMEM was 25.9 (Figure 10). This was similar to the mean percentage change in weight for PT explants in DMEM, 25.6. Appropriately, the percentage change in weight after submersion in DMEM was not significantly different between PT and ACL explants after an independent t-test (n=6, df=4, p=0.880) (Figure 10). The mean percentage weight change for PT explants in XVIVO was 14.0 compared to 12.1 for ACL explants. Similar to the DMEM treatment, there was no significant difference in percent change in weight between PT and ACL explants after submersion in X-VIVO (n=6, df=4, p=0.439). For the sucrose treatment, mean percent change was 3.5 for PT explants and 16.2 for ACL explants. Analogous to the two other treatments, there was no significant difference between PT and ACL explants after submersion in Sucrose. It should be noted that despite finding no significant differences in means, the standard error in the XVIVO and Sucrose treatments was larger between PT and ACL explants. Specifically, for XVIVO, the standard error for PT was 10.8 and for ACL, 7.9; for Sucrose, 5.6 (PT) and 1.9 (ACL); for DMEM 3.4(PT) and 4.3(ACL). Mean \pm SE number of cells

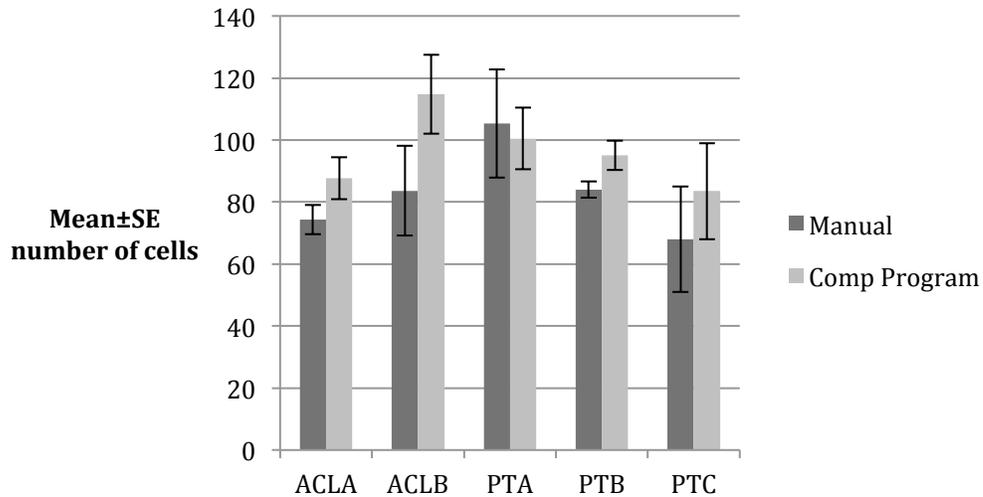


Figure 11. Mean \pm SE cell counts (40X Leica Microscope, field: 1000x1000um) per regions A, B, C (section 3.9, figure 4).

The mean numbers of cells for the ACL A and B parts were 84 and 102, respectively (Figure 11). The mean number of cells for the PT A, B, and C, parts were 103, 90 and 56. There were no significant differences found between the manual and the computerized counting of the pixcavator program: ACLA (n=6, df=4, p=0.188), ACLB (n=7, df=5, p=0.193), PTA (n=8, df=6, p=0.823), PTB (n=11, df=9, p=0.059), PTC (n=6, df=4, p=0.770). There was also no significant difference in cell numbers between ACLA and ACLB (n=13, df=11, p=0.137). Additionally, PTA, PTB, and PTC did not exhibit significant differences with respect to cell count (n=24, df=2, p=0.072). So, the hypothesis that all regions of the PT and ACL explants have the same number of cells (Section 3.9, Pgs 23-24) was accepted.

4.6 Weight differences between intact ACL and PT

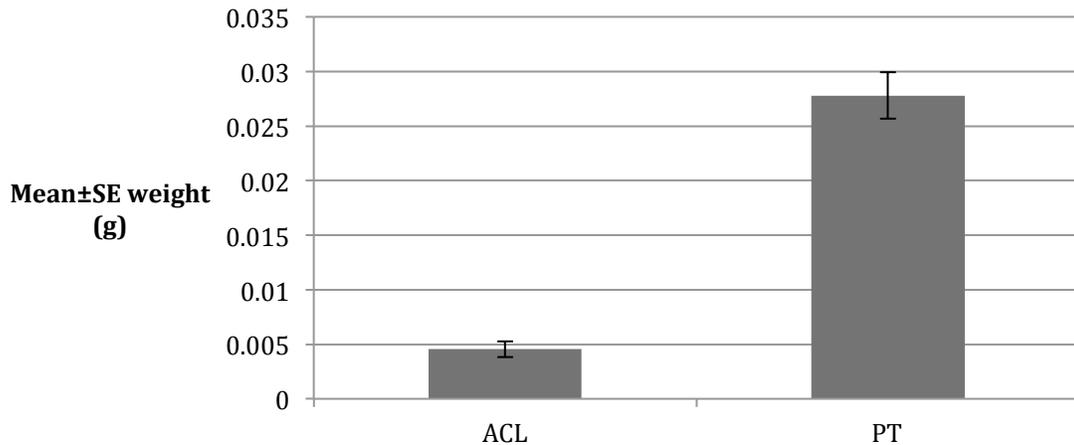


Figure 12. Mean ± SE ACL and PT weight (grams) (n=7 ACL, n=7 PT) (section 3.10).

The mean weight for intact ACL was 0.0045 g compared to 0.027 g for intact PT. After an independent sample t-test, the mean weight of PT was significantly larger than the ACL (n=23, df=21, p=<0.001).

4.7 Cytokine penetration and downstream effects in primary cells

Table 5. Summary of cytokine penetration and downstream effects by group and cytokine (n=2 ACL cell cultures/experimental group, n=2 PT cell cultures/experimental group).

The first column refers to up-regulation of the marker greater than the value seen in the control at 2 hours. The second column represents higher up-regulation in comparison to the 2 hour time point.

TNF α Injury Group

Marker	Up-regulation > Control 2 Hrs	Further Up-regulation > 24 Hrs
IL-6	(Y) 2.5	(Y) 2.4
TNFα	(N) 0.11	(Y) 0.10
iNOS	(N) 0.001	(Y) 0.01
Caspase-3	(N) 0.4	(Y) 0.9
COX-2	(Y) 14.4	(Y) 43.7

TNF α Max Group

Marker	Up-regulation > Control 2 Hrs	Up-regulation > Control 24 Hrs
IL-6	(Y) 13.1	(N) 1.2
TNFα	(Y) 2.2	(N) 0.01
iNOS	(N) 0.06	(N) 0.002
Caspase-3	(N) 0.4	(Y) 0.6
COX-2	(Y) 114.6	(Y) 13.9

IL1 β Injury Group

Marker	Up-regulation > Control 2 Hrs	Further Up-regulation > 24 Hrs
IL-6	(Y) 2.6	(Y) 52.8
IL-1β	(N) 0.03	(Y) 0.05
iNOS	(N) 0.002	(Y) 2.2
Caspase-3	(N) 0.2	(Y) 0.5
COX-2	(Y) 17.8	(Y) 956

IL1 β Max Group

Marker	Up-regulation > Control 2 Hrs	Further Up-regulation > 24 Hrs
IL-6	(Y) 4.9	(Y) 92.6
IL-1β	(N) 0	(Y) 0.06
iNOS	(N) 0.03	(Y) 4.4
Caspase-3	(N) 0.07	(Y) 0.7
COX-2	(Y) 121	(Y) 11

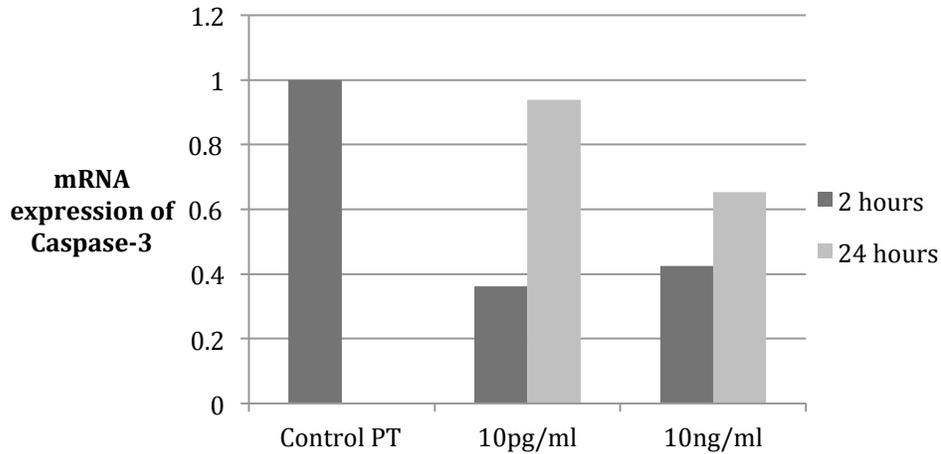


Figure 13. mRNA expression (normalized to 18s) of Caspase-3 of primary patellar tendon cells (passage 3) after 24 hr treatment with tumor necrosis factor alpha (n=2). Sample sizes were too small to perform statistics or generate standard error. PT control cell data are standardized to 1. All other data were then standardized to PT control cell in terms of fold expression.

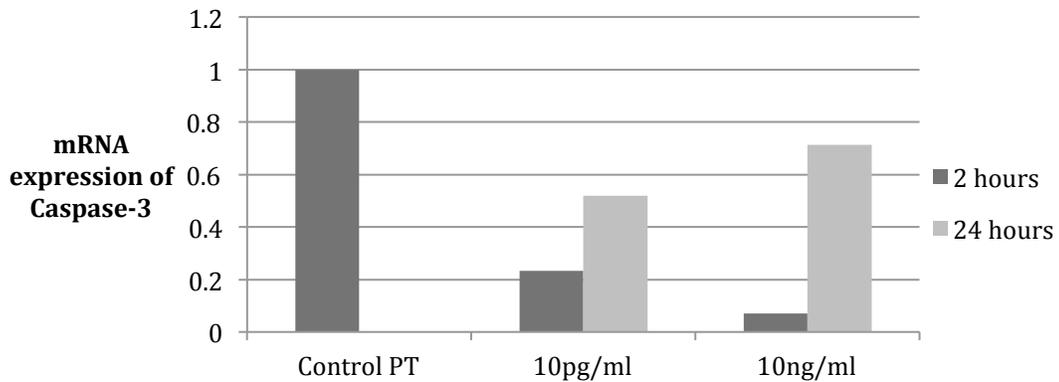


Figure 14. mRNA expression (normalized to 18S) of Caspase-3 of primary patellar tendon cells (passage 3) after 24 hr treatment of interleukin one beta (n=2). Sample sizes were too small to perform statistics or generate standard error. PT control cell data are standardized to 1. All other data were then standardized to PT control cell in terms of fold expression.

Similar trends with respect to mRNA expression of Caspase-3 were seen in both TNF α and IL1 β treated cells. Firstly, the highest mRNA expression of Caspase-3 was seen in control patellar tendon cells. Compared to the control, both the Injury and Max groups saw no up-regulation of the expression of Caspase-3. Despite being lower than the controls, expression did exceed zero in the treatment groups. For TNF α treated cells, the Injury group noted an increase in Caspase-3 of 0.36 (2 hours) and 0.94 (24 hours). For the Max group, mRNA expression of Caspase-3 increased to 0.43 (2 hours) and 0.65 (24 hours). Similarly to TNF α treated cells, IL1 β treated cells showed an mRNA expression (C-3) increase of 0.23 (2 hours) and 0.52 (24 hours) within the Injury. Within the Max group, mRNA expression of Caspase-3 at 2 hours was 0.071 and ten times higher at 24 hours (0.71).

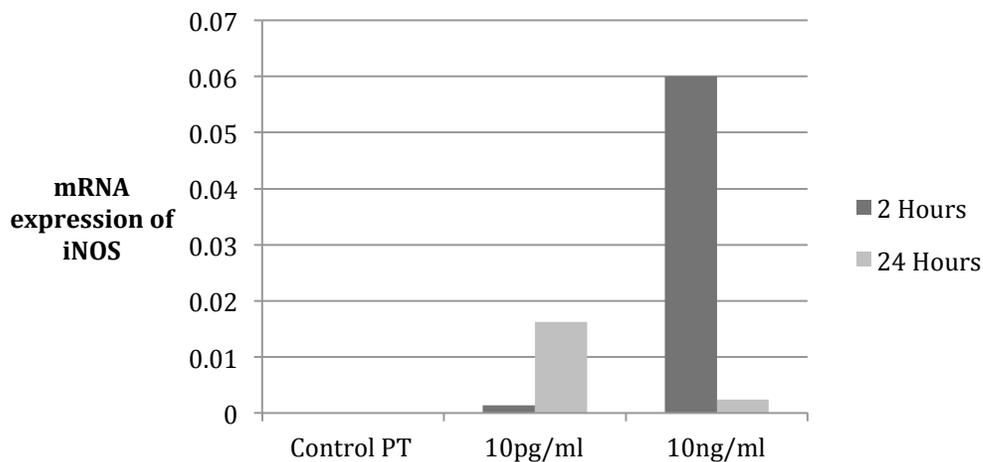


Figure 15. mRNA expression (normalized to 18S) of iNOS of primary patellar tendon cells (passage 3) after 24 hr treatment of tumor necrosis factor (n=2). Sample sizes were too small to perform statistics or generate standard error. In contrast to other datasets in this study group (cytokine penetration/downstream effects in primary cells), the control group showed 0 expression, therefore, data could not be standardized to control.

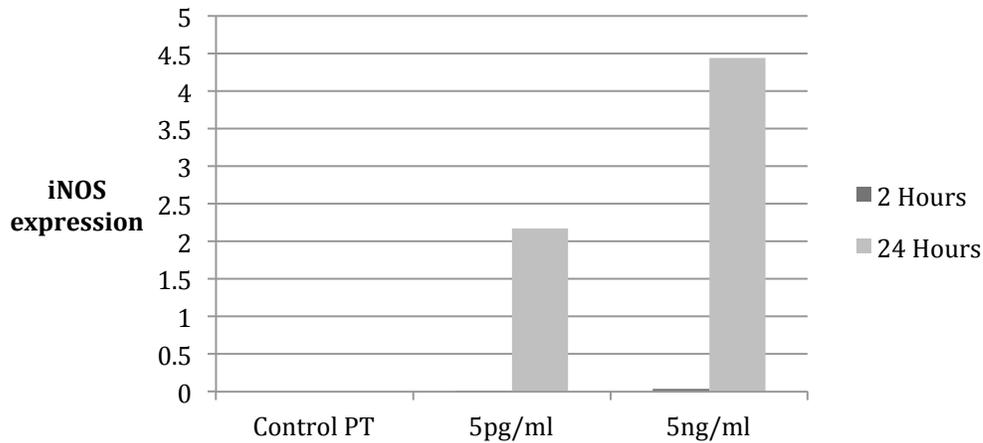


Figure 16. mRNA expression (normalized to 18S) of iNOS of primary patellar tendon cells (passage 3) after 24 hr treatment of interleukin one beta (n=2). Sample sizes were too small to perform statistics or generate standard error. In contrast to other datasets in this study group (cytokine penetration/downstream effects in primary cells), the control group showed 0 expression, therefore, data could not be standardized to control.

Compared to the Control group, TNF α treated cells showed a 10 fold increase expression of iNOS at 24 hours (0.01) compared to 2 hours (0.001) within the Injury concentration group. TNF α treated cells within the Max concentration group showed the opposite trend having the greatest mRNA expression of iNOS at 2 hours (0.06) compared to 24 hours (0.002). Despite numerically marginal differences with respect to control and treatment groups, mRNA expression of iNOS does seem to be affected by cytokine treatment. Switching to IL1 β treated cells and mRNA expression of iNOS, results showed that 24 hours produced a greater fold expression compared to 2 hours. Specifically, for both the Injury and Max groups, mRNA expression of iNOS was higher at 24 hours (2.2, 4.4, respectively) than at 2 hours (0.002, 0.04, respectively).

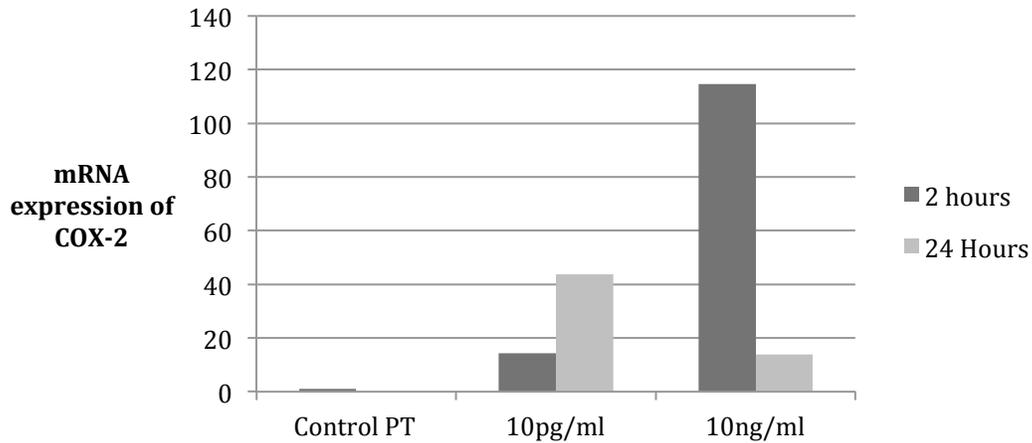


Figure 17. mRNA expression (normalized to 18S) of COX-2 of primary patellar tendon cells (passage 3) after 24 hr treatment of tumor necrosis factor alpha (n=2). Sample sizes were too small to perform statistics or generate standard error. PT control cell data are standardized to 1. All other data were then standardized to PT control cell in terms of fold expression

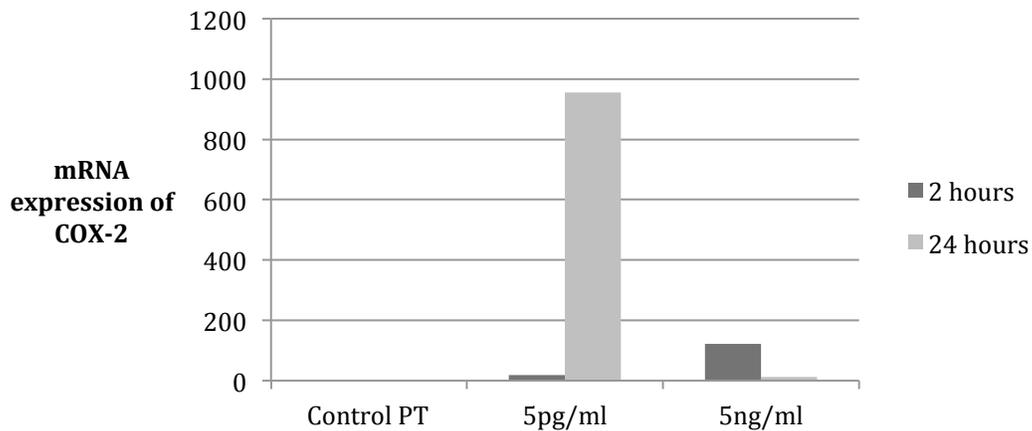


Figure 18. mRNA expression (normalized to 18S) of COX-2 of primary patellar tendon cells (passage 3) after 24 hr treatment of interleukin one beta (n=2). Sample sizes were too small to perform statistics or generate standard error. PT control cell data are standardized to 1. All other data were then standardized to PT control cell in terms of fold expression

Compared to the standardized value of 1 for control cells, both TNF α and IL1 β treated cells showed very high levels of mRNA expression of COX-2. Specifically for TNF α treated cells, even the Injury group at the 2 hour time point showed 14 times the expression of the control group. Additionally, the Injury Group's expression at 2 hours tripled at the 24 hour time point (43). The Max group of TNF α treated cells showed an inverse trend to the Injury Group in that the highest mRNA expression level of COX-2 was shown at 2 hours (114). Despite being lower than the 2 hour time point, mRNA expression of COX-2 in TNF α treated cells at 24 hours still showed an expression level 13 times that of the control cells. Turning to the other cytokine group, IL1 β treated cells showed great differences compared to control cells that also mirrored the trends seen in TNF α treated cells. Specifically, at 2 hours, the Injury group showed an mRNA expression fold increase of 17.8. At 24 hours, this value was almost 50 times the value seen at 2 hours and almost 1000 (956) times the expression seen in the control cells. For the Max Group of IL1 β treated cells, the 2 hour time point showed an expression level 121 times greater than the controls. At 24 hours, the expression level seen at 2 hours decreased by 11 times, but was still 11 times greater than the expression level seen in the control cells.

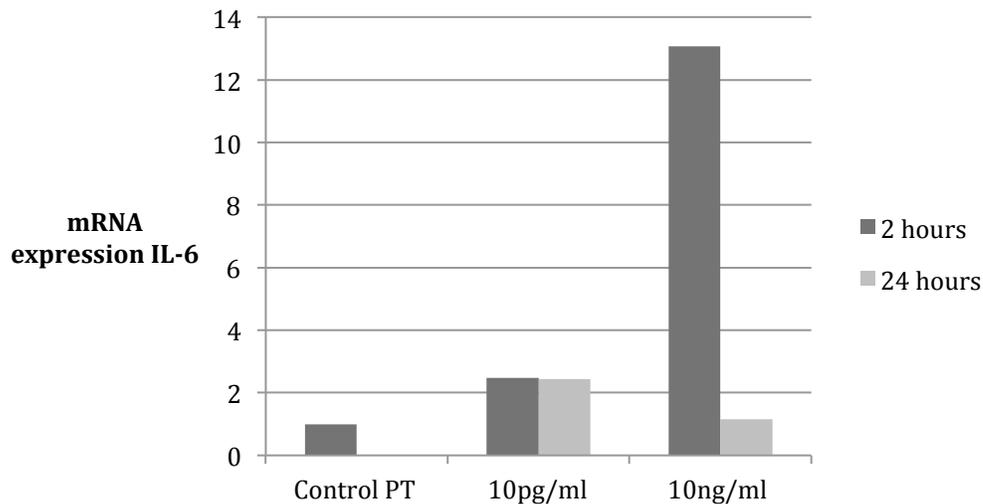


Figure 19. mRNA expression (normalized to 18S) of IL-6 of primary patellar tendon cells (passage 3) after 24 hr tumor necrosis factor alpha (n=2). Sample sizes were too

small to perform statistics or generate standard error. PT control cell data are standardized to 1. All other data were then standardized to PT control cell in terms of fold expression

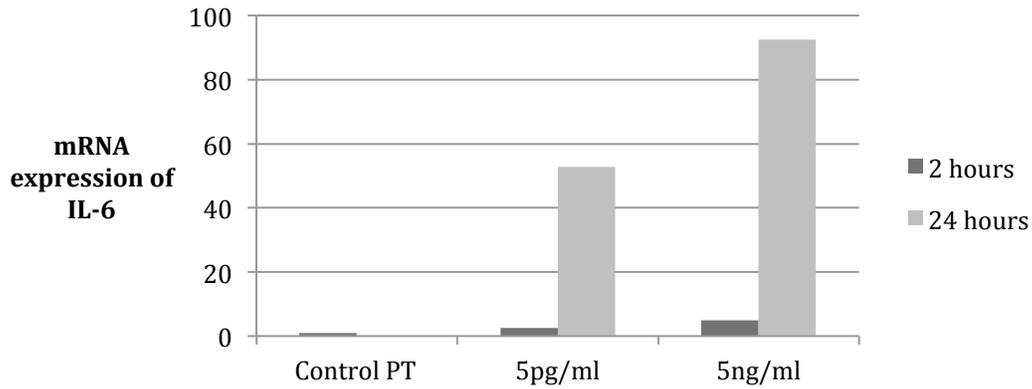


Figure 30. mRNA expression (normalized to 18S) of IL-6 of primary patellar tendon cells (passage 3) after 24 hr treatment of interleukin one beta (n=2). Sample sizes were too small to perform statistics or generate standard error. PT control cell data are standardized to 1. All other data were then standardized to PT control cell in terms of fold expression

TNF α and IL1 β showed different effects in PT primary cells in terms of IL-6 mRNA expression at 2 and 24 hours. To be specific, the Injury Group of TNF α treated cells showed very similar expression levels at both 2 (2.43) and 24 (2.48) hours. In contrast, the Max Group in TNF α treated cells, at 2 hours showed an expression level 13 times the control cells while at 24 hours, showed an mRNA expression level of COX-2 similar to control cells (1.3).

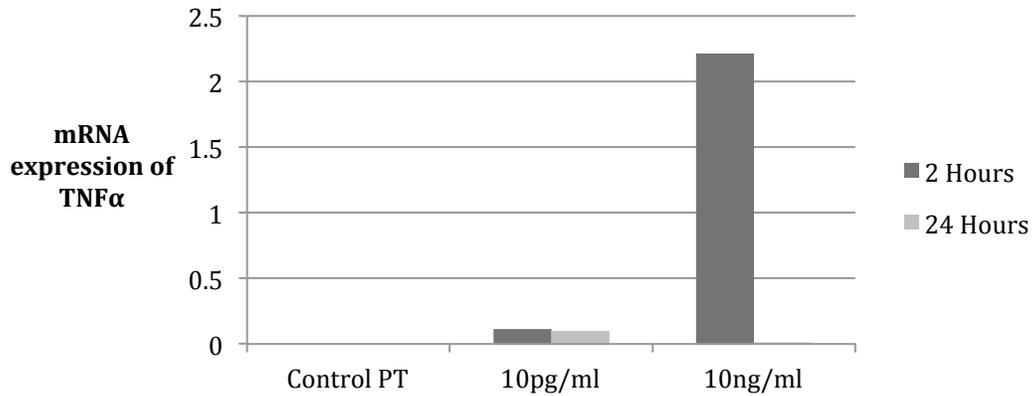


Figure 21. mRNA expression (normalized to 18S) of TNF α of primary patellar tendon cells (passage 3) after 24 hr treatment with TNF α (n=2). Sample sizes were too small to perform statistics or generate standard error. In contrast to other datasets in this study group (cytokine penetration/downstream effects in primary cells), the control group showed 0 expression, therefore, data could not be standardized to control.

A trend in expression levels indicates that Control group levels of mRNA expression were lower compared to treatment groups. The highest expression of TNF α was seen at 2 hours in the Max group (2.21). At 24 hours, the mRNA fold expression was much lower with a value of (0.01). In the Injury group, the 2 and 24 hour time points showed similar levels of expression, 0.11 and 0.10, respectively. The control group showed no expression (0 fold expression increase) of TNF α .

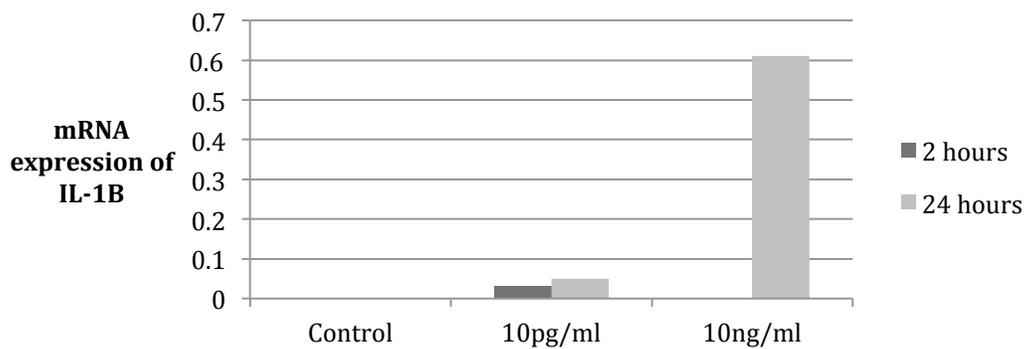


Figure 22. mRNA expression (normalized to 18S) of IL-1 β of primary patellar tendon cells (passage 3) after 24 hr treatment of interleukin one beta (n=2). Sample sizes were too small to perform statistics or generate standard error. In contrast to other datasets in

this study group (cytokine penetration/downstream effects in primary cells), the control group showed 0 expression, therefore, data could not be standardized to control.

Control patellar tendon cells showed no expression of IL-1 β . Injury and Max groups did show expression. Specifically, the Injury group showed similar levels of mRNA expression at both 2 (0.03) and 24 (0.05) hours with the 24-hour time point showing a higher expression level. Interestingly, at 2 hours, the Max group showed no expression but after 24 hours showed the highest expression seen in all groups, 0.6, which was approximately 15 times higher than the levels seen in the Injury group.

4.8 Cell death rates in primary cells treated with cytokines

Table 6. Percentage of dead cells after cytokine treatment in primary ACL cells (n=12 intact ACL).

Treatment	Injury (%)	Max(%)
Control	9	9
TNFα	3	9
IL1B	7	12

Similar trends with respect to the effects of both IL-1 β and TNF α were seen in ACL primary cells after 24 hours in cytokine treatment. Specifically for IL-1 β , the DMEM group showed that 9% of all cells were dead after 24 hours, the Injury group showed a cell death rate of 7%, while the Max group was observed to have 12% dead cells. In terms of TNF α , DMEM showed 9% cell death compared to 3% for the Injury group and 9% for the Max group. A one-way ANOVA showed no difference in cell death between DMEM, Injury, and Max groups in IL1B (n=15, df=2, p=0.584) and TNF treated cells(n=15, df=2, p=0.975) .

Table 7. Percentage of dead cells after cytokine treatment in primary PT cells

Treatment	Injury (%)	Max(%)
Control	43	43
TNFα	37	16
IL1B	11	3

Regarding the effect of TNF α , the percentage of dead cells for PT cells significantly decreased as the concentration of TNF α increased. Besides having the highest percentage of dead cells in all groups, the DMEM group (control) had a significantly higher percentage of dead cells (43%) compared to both the IL-1 β treated (10 pg/ml) (37%) and the Max group (10 ng/ml) (16%) (n=25, df=2, p=<0.001). Additionally, the Injury group had a significantly higher percentage of dead cells versus the Max group (n=25, df=2, p=0.003). With respect to the effect of IL-1 β , a similar trend to TNF α is observed. The control group also showed the highest percentage of dead cells (43%) and it was significantly higher than the percentage of cell death found in the Injury (11%) and Max groups (3%) (p=25, df=2, p=<0.001).

4.9 Cytokine penetration and downstream effects in explants

Table 8. Summary of highlights regarding differences in cytokine penetration and downstream effects in PT and ACL explants (n=14 ACL, n=14 PT).

	ACL	PT
iNOS	<ul style="list-style-type: none"> • SNP up-regulated iNOS expression 41 fold (p=0.004) • IL1B upregulated iNOS 9.9 fold (p=0.037) 	<ul style="list-style-type: none"> • SNP did not up-regulate iNOS expression (p=0.245) • IL1B up-regulated iNOS 5.6 fold (p=0.005)
Caspase-3	No significant differences (p=0.138)	Significant difference between T0 and SNP (p=0.002)
IL1B	<ul style="list-style-type: none"> • SNP up-regulated IL1B expression 157,142 fold (p=0.03) 	<ul style="list-style-type: none"> • SNP up-regulated IL1B expression 107 fold (p=0.013)

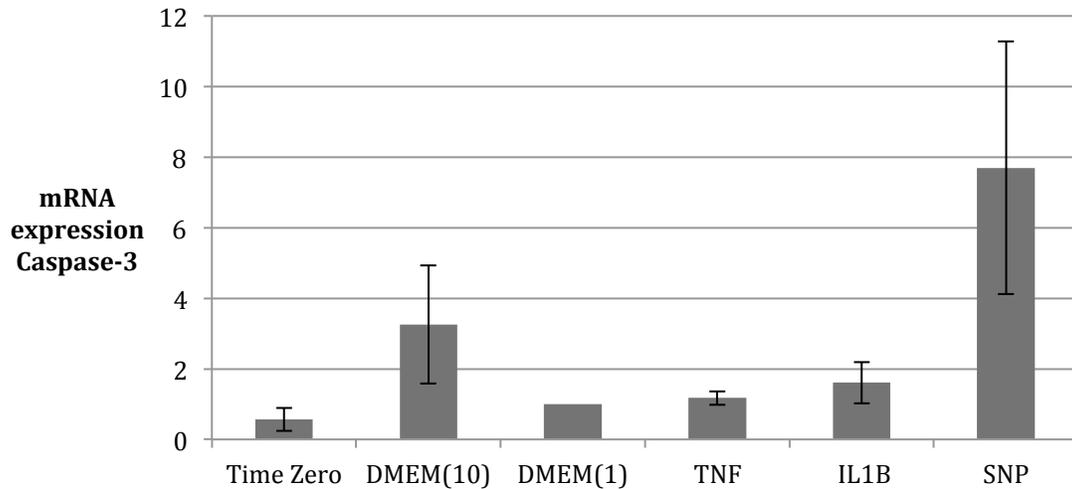


Figure 23. Mean \pm SE mRNA expression (normalized to 18S) of caspase-3 in ACL explants after various treatment conditions for 18 hours (n=14). Time zero refers to explant that was analysed immediately following harvest. DMEM(10) refers to Dulbecco's Modified Eagle Medium with 10% FBS. DMEM(1) refers to Dulbecco's Modified Eagle Medium with 1 % FBS and was utilized as the control. The DMEM(1) group was standardized to a value of 1 and all other groups were standardized to the DMEM(1) value. TNF refers to tumor necrosis factor alpha (10 ng/ml). IL-1 β refers to interleukin one beta (5 ng/ml). SNP refers to sodium nitroprusside.

The greatest fold increase in mRNA expression of Caspase-3 was found in SNP treated explants with a value of 7.7. However, the standard error was also very high in this group with a value of 3.4. The second greatest fold increase of Caspase-3 was found in DMEM(10) treated cells. This group showed a 3.3 fold increase. The groups of TNF α , and IL-1 β showed similar fold increases of 1.2 and 1.6, respectively. The lowest expression of Caspase-3 was found in the Time Zero explants, showing a fold increase slightly over one (0.6). Data were found to have a non-normal distribution as assessed by Kolmogorov-Smirnov ($p < 0.05$). A Kruskal-Wallis test revealed that there were no significant differences between any treatment groups (n=38, df=4, $p = 0.138$). Lack of significant differences were likely due to large standard error found in groups.

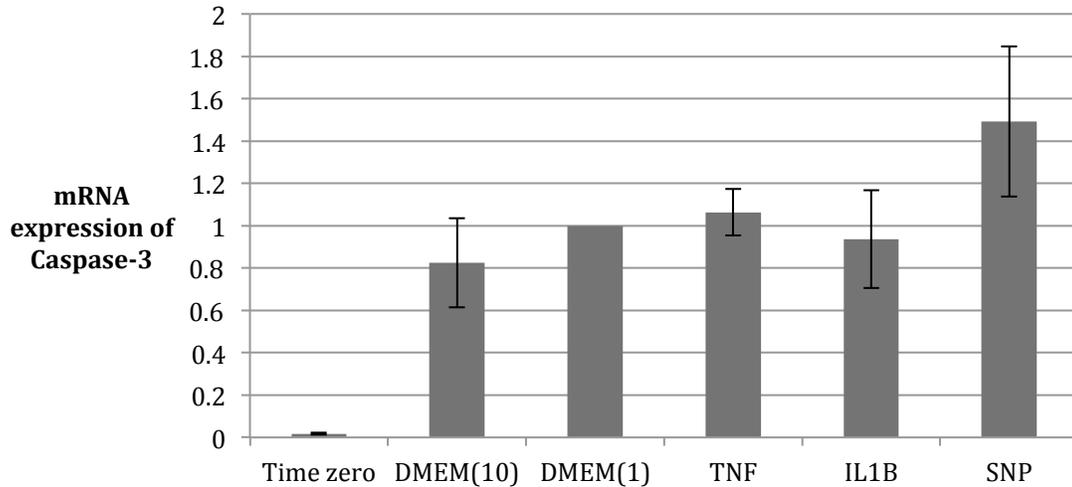


Figure 24. Mean \pm SE mRNA expression (normalized to 18S) of caspase-3 in PT explants after various treatment conditions for 18 hours (n=14). Time zero refers to explant that was analysed immediately following harvest. DMEM(10) refers to Dulbecco's Modified Eagle Medium with 10% FBS. DMEM(1) refers to Dulbecco's Modified Eagle Medium with 1 % FBS and was utilized as the control. The DMEM(1) group was standardized to a value of 1 and all other groups were standardized to the DMEM(1) value. TNF refers to tumor necrosis factor alpha (10 ng/ml). IL-1 β refers to interleukin one beta (5 ng/ml). SNP refers to sodium nitroprusside.

Caspase-3 mRNA expression showed a 1.5 fold increase in PT explants after 18 hour treatment in SNP. Conversely, Time Zero explants only showed a 0.0.17 fold increase in Caspase-3 expression. Other treatment groups were similar in expression levels: DMEM(10) showed a 0.83 fold increase, TNF α showed a 1.1 fold increase, and IL-1 β showed a 0.9 fold increase. A Kolmogorov-Smirnov test revealed normal distribution and an ANOVA test showed a significant difference between treatment groups (n=45, df=5, p=0.007). However, post-hoc tests showed that the only significant difference in Caspase-3 expression was between the Time Zero explant group and the SNP group (n=45, df=5, p=0.002).

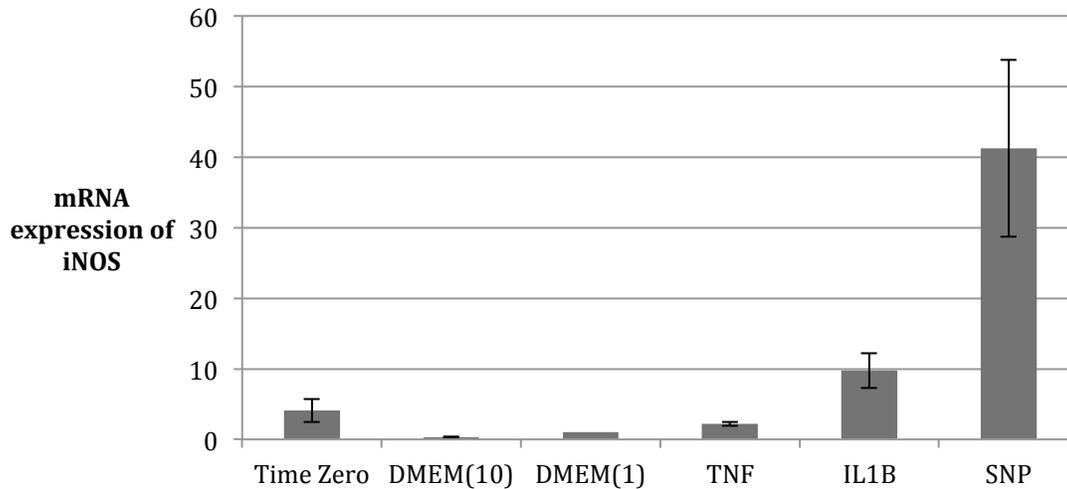


Figure 25. Mean \pm SE mRNA expression (normalized to 18S) of iNOS in ACL explants after various treatment conditions for 18 hours (n=14). Time zero refers to explant that was analysed immediately following harvest. DMEM(10) refers to Dulbecco’s Modified Eagle Medium with 10% FBS. DMEM(1) refers to Dulbecco’s Modified Eagle Medium with 1 % FBS and was utilized as the control. The DMEM(1) group was standardized to a value of 1 and all other groups were standardized to the DMEM(1) value.. TNF refers to tumor necrosis factor alpha (10 ng/ml). IL-1 β refers to interleukin one beta (5 ng/ml). SNP refers to sodium nitroprusside.

Explants treated with SNP showed the highest fold increase (41.3) in mRNA expression of iNOS. This was a significantly higher expression compared to Time Zero (4.1) (n=39, df=5, p=0.047), DMEM(10) (0.30) (n=39, df=5, p=.004), IL-1 β (9.7) (n=39, df=5, p=0.027), and TNF α (2.2) (n=39, df=5, p0.005). The mRNA expression of iNOS increased 9.7 fold in IL-1 β treated explants and this was significantly higher than iNOS mRNA expression in Time Zero Explants (n=20, df=5, p=0.037). Despite TNF α showing a fold increase of 2.2, it was not significantly different from Time Zero explants. DMEM(10) revealed a significantly lower mRNA expression of iNOS compared to TNF (n=39, df=5, p=0.004) and IL-1 β (n=39, df=5, p=0.002). The D1 was used as the control and was standardized to a value of 1. This was not significantly different than TNF α (p=0.568), IL-1 β (p=0.564), nor SNP explant groups (p=0.360). While mRNA expression of iNOS was not significantly different between the IL-1 β group or the TNF α

group ($p=0.554$), both the IL-1 β ($p=0.027$) group and TNF α ($p=0.005$) groups had significantly lower expression of iNOS compared to the SNP group.

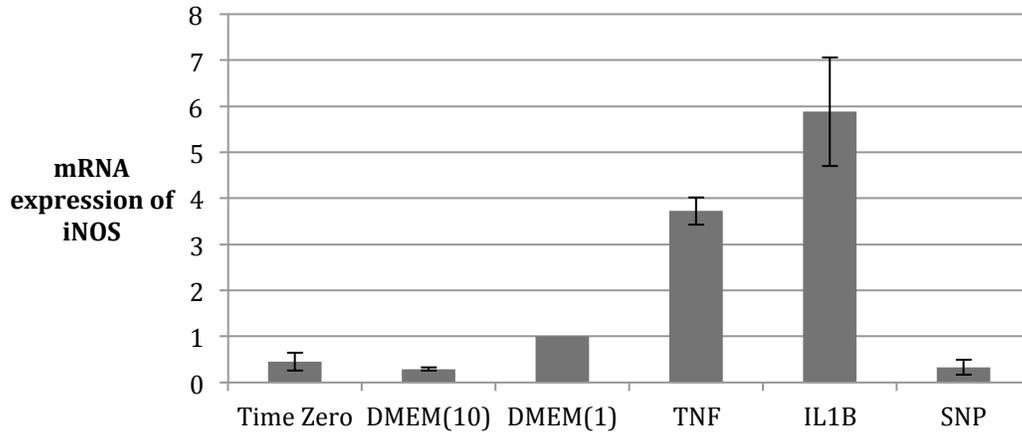


Figure 26. Mean \pm SE mRNA expression (normalized to 18S) of iNOS in PT explants after various treatment conditions for 18 hours ($n=14$). Time zero refers to explant that was analysed immediately following harvest. DMEM(10) refers to Dulbecco's Modified Eagle Medium with 10% FBS. DMEM(1) refers to Dulbecco's Modified Eagle Medium with 1 % FBS and was utilized as the control. The DMEM(1) group was standardized to a value of 1 and all other groups were standardized to the DMEM(1) value. TNF refers to tumor necrosis factor alpha (10 ng/ml). IL-1 β refers to interleukin one beta (5 ng/ml). SNP refers to sodium nitroprusside.

The evaluation of mRNA expression of iNOS in PT explants showed a non-normal distribution ($p=0.001$) by the Kolmogorov-Smirnov test. Subsequent Kruskal-Wallis testing revealed a significant difference in mRNA expression between treatment groups ($n=43$, $df=5$, $p<0.001$). Time Zero explants showed a mean iNOS expression fold increase of 0.45. The medium group D10 showed a mean mRNA expression fold increase of 0.30. The mean fold increases in iNOS expression seen in the cytokine groups of IL-1 β and TNF α were 5.9 and 0.4, respectively. The fold increase in mRNA expression of iNOS was 0.33 in the SNP group. Time Zero explants showed no significant difference in mRNA expression compared to the D10 group ($n=14$, $df=5$, $p=0.197$), the D1 group ($n=14$, $df=5$, $p=0.197$), and the SNP group ($n=14$, $df=5$, $p=0.245$).

In contrast, IL-1 β had a significantly greater fold increase in iNOS expression compared to Time Zero (n=14, df=5, p=0.005) as did TNF α versus Time Zero (n=14, df=5, p=<0.001). In comparison to Time Zero, a similar trend is seen with the D10 group. There were no significant differences found between D10 and D1 (n=14, df=5, p=0.210) and D10 and SNP (n=14, df=5, p=0.046). However, the TNF α group had a significantly higher fold increase in iNOS compared to D10 (n=14, df=5, p=0.002) as did IL-1 β (n=14, df=5, p=0.002). The control group (D1) was not significantly different than the IL-1 β group (n=14, df=5, p=0.083) and the SNP group (n=14, df=5, p=0.066). However, the D1 group showed a slightly significant difference with TNF α group (n=14, df=5, p=0.041). While the TNF α group and IL-1 β did not show significant differences in mRNA expression of iNOS, both TNF α (n=14, df=5, p=0.003) and IL-1 β (n=14, df=5, p=0.001) had a significantly higher expression of iNOS compared to the SNP group.

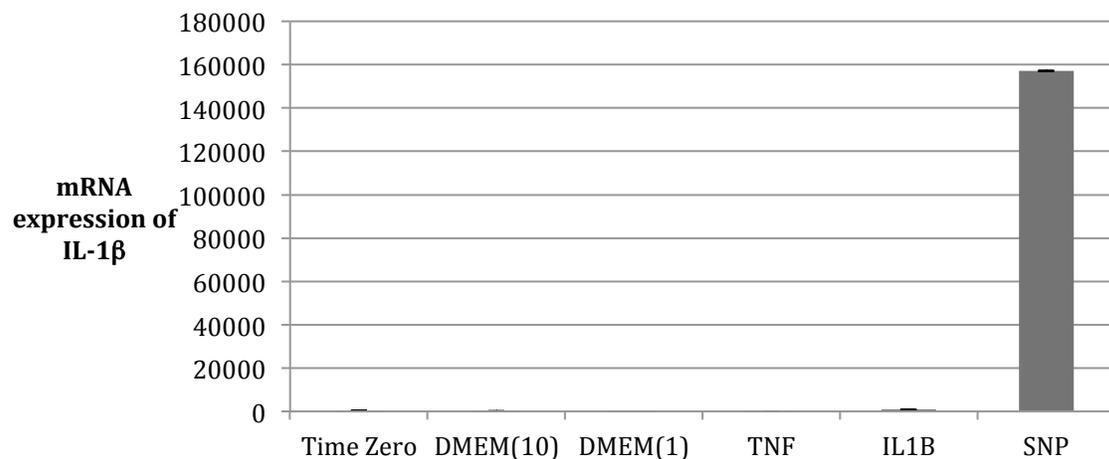


Figure 27. Mean \pm SE mRNA expression (normalized to 18S) of IL-1 β in ACL explants after various treatment conditions for 18 hours (n=14). Time zero refers to explant that was analysed immediately following harvest. DMEM(10) refers to Dulbecco's Modified Eagle Medium with 10% FBS. DMEM(1) refers to Dulbecco's Modified Eagle Medium with 1 % FBS and was utilized as the control. The DMEM(1) group was standardized to a value of 1 and all other groups were standardized to the DMEM(1) value. TNF refers to tumor necrosis factor alpha (10 ng/ml). IL-1 β refers to interleukin one beta (5 ng/ml). SNP refers to sodium nitroprusside.

Table 9. Fold expression values of mRNA expression of IL-1 β in ACL explants (n=14). DMEM(1) was used as the control group and standardized to 1. All other groups were standardized to the DMEM value (0.0014).

Time Zero	DMEM(10)	DMEM(1)	TNF	IL1B	SNP
307.1	114.3	1	24.3	928.7	157142.9

After testing the dataset of mRNA expression of IL-1 β in ACL explants, a Kolmogorov-Smirnov and Shapiro-Wilk test showed a non-normal distribution ($p > 0.05$). A Kruskal-Wallis test showed significant differences between treatment groups ($n=30$, $df=5$, $p=0.014$). The mRNA expression of IL-1 β increased by a mean 157,142.9 fold in the SNP group. This was a significantly higher fold increase compared to the D10 group ($n=8$, $p=0.036$) with a value of 114.3, the TNF α group ($n=10$, $p=0.017$) with a value of 24.3, and the IL-1 β ($n=13$, $p=0.01$) with a value of 928.7. Interestingly, both cytokine groups showed significant differences with two different controls. The IL-1 β group also showed a significantly higher iNOS mRNA expression compared to the D1 group ($n=13$, $p=0.014$). In contrast, the TNF α group had a significantly lower mRNA expression of iNOS compared the Time Zero group ($n=9$, $p=0.04$). Despite the high fold increase of iNOS mRNA expression found in the SNP group, it was not significantly different than the Time Zero Group ($n=5$, $p=0.083$). In terms of control groups, there was no significant differences found between the Time Zero and the D10 group ($n=7$, $p=0.245$), the D1 group ($n=5$, $p=0.20$), the IL-1 β group ($n=12$, $p=0.485$). For media groups, there was no significant difference between D10 and D1 ($n=8$, $p=0.881$), nor was there a significant difference between D10 and both cytokine groups, TNF α ($n=12$, $p=0.639$) and IL-1 β ($n=15$, $p=0.178$). Additionally, there was no difference found between the media group D1 and TNF α ($n=10$, $p=0.053$) and D1 and SNP ($n=6$, $p=0.50$). Also, there was no significant difference between the cytokine groups, IL-1 β versus TNF α ($n=17$, $p=0.626$).

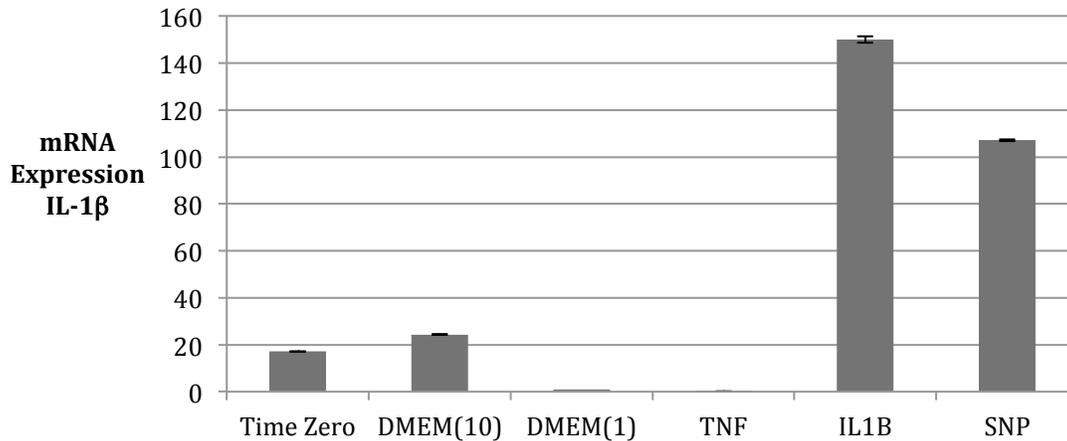


Figure 28. Mean \pm SE mRNA expression (normalized to 18S) of IL-1 β in PT explants after various treatment conditions for 18 hours (n=14). Time zero refers to explant that was analysed immediately following harvest. DMEM(10) refers to Dulbecco's Modified Eagle Medium with 10% FBS. DMEM(1) refers to Dulbecco's Modified Eagle Medium with 1 % FBS and was utilized as the control. The DMEM(1) group was standardized to a value of 1 and all other groups were standardized to the DMEM(1) value. TNF refers to tumor necrosis factor alpha (10 ng/ml). IL-1 β refers to interleukin one beta (5 ng/ml). SNP refers to sodium nitroprusside.

A Kruskal-Wallis test showed significant differences between groups (n=44, df=5, p<0.001). The highest mRNA expression of IL-1 β was found in IL-1 β treated explants with a fold increase of 150. The second greatest expression of IL-1 β was seen in the SNP group (107). All other treatment groups showed a fold increase of mRNA expression that was less than one: Time Zero (17.1), DMEM(10) (24.3), and TNF α (0.07). There were no significant differences found between Time Zero and D10 (n=15, p=0.856) and Time Zero and IL-1 β (n=16, p=0.77). Conversely, Time Zero did have a significantly higher expression of IL-1 β compared to the D1 group (n=13, p=0.013) and a significantly lower expression of IL-1 β compared to SNP (n=13, p=0.028). For the media groups, there were no significant differences between D10 and D1 (n=18, p=0.055) or D10 and IL-1 β (n=21, p=0.725). In contrast, D10 had a significantly higher expression of IL-1 β compared to TNF α (n=15, p=0.003) and a significantly lower expression of IL-1 β compared to SNP

(n=15, p=0.20). The D1 group had a significantly higher IL-1 β fold expression increase compared to TNF α (n=13, p=0.033), and a significantly lower expression of IL-1 β compared to both IL-1 β (n=19, p=0.015) and SNP (n=13, p=0.002). With respect to cytokine groups, the TNF α group had a significantly lower expression of IL-1 β compared to both IL-1 β (n=16, p=0.003) and SNP (n=10, p=0.009).

4.10 Live/Dead Assay of PT&ACL explants in various experimental conditions

Table 10. Percentage of dead cells in ‘Cut’ and ‘Non-Cut’ regions of ACL explants after experimental treatment (n=21).

	Time Zero	DMEM(1)	DMEM(10)	IL1B	TNFα	SNP
Cut (%)	35	55	56	40	35	88
Not Cut (%)	35	45	40	34	53	81

Significant difference between SNP and T0, D(1), D(10), IL1B and TNF (p=<0.001) In ACL explants, there was no significant difference found between cut and non cut regions (p=0.217).

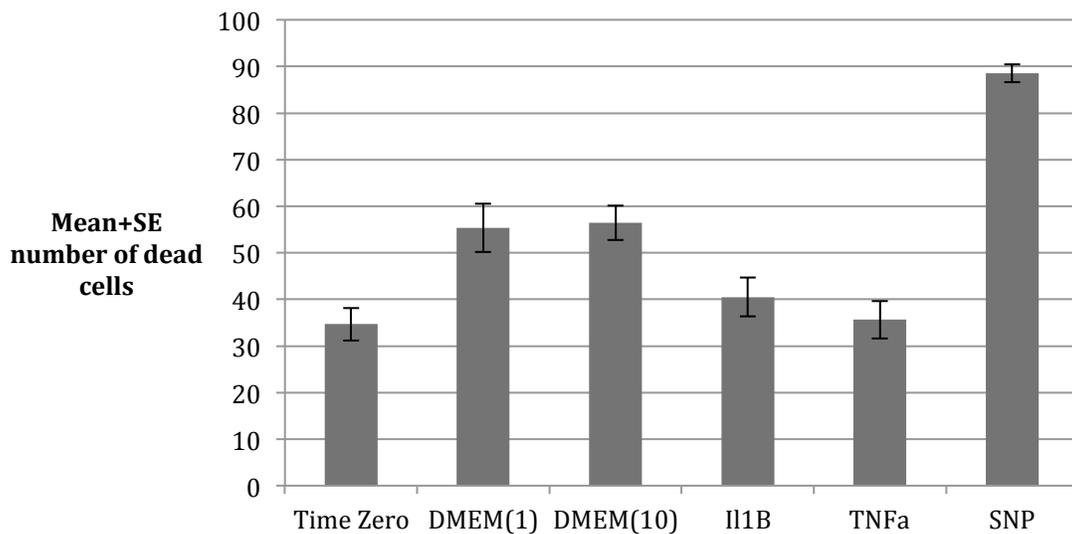


Figure 29. Mean percentage \pm Standard Error of live versus dead cells in ACL explants (n=21) per various treatment conditions. This graph only shows cells that were on the periphery of the explant. Time zero refers to explant that was analysed immediately following harvest. DMEM(10) refers to Dulbecco’s Modified Eagle Medium with 10%

FBS. DMEM(1) refers to Dulbecco's Modified Eagle Medium with 1 % FBS. TNF α refers to tumor necrosis factor alpha (10 ng/ml). IL-1 β refers to interleukin one beta (5 ng/ml). SNP refers to sodium nitroprusside

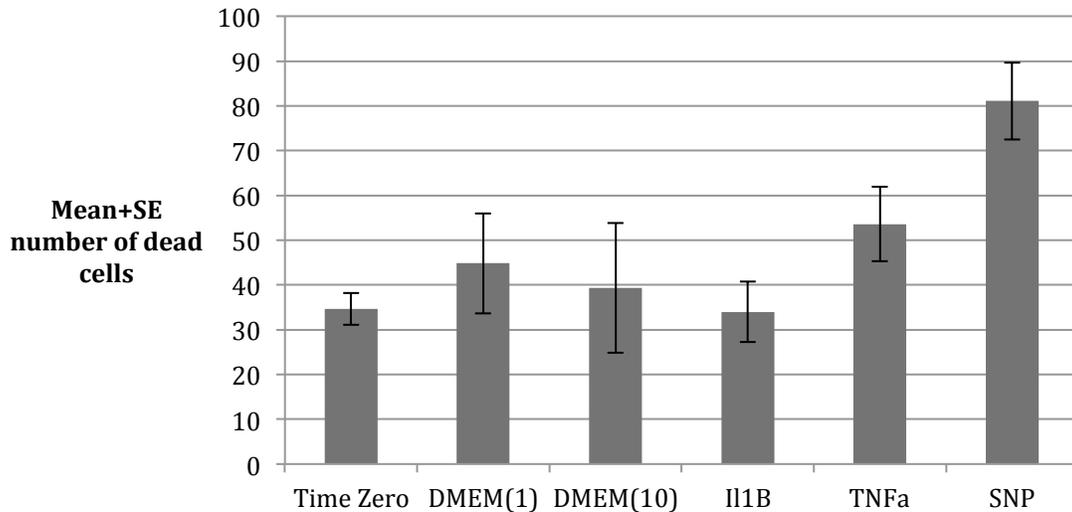


Figure 30. Mean percentage \pm Standard Error of live versus dead cells in ACL explants (n=21) per various treatment conditions. This graph only shows cells that were in the middle of the explant. Time zero refers to explant that was analyzed immediately following harvest. DMEM(10) refers to Dulbecco's Modified Eagle Medium with 10% FBS. DMEM(1) refers to Dulbecco's Modified Eagle Medium with 1 % FBS. TNF α refers to tumor necrosis factor alpha (10 ng/ml). IL-1 β refers to interleukin one beta (5 ng/ml). SNP refers to sodium nitroprusside.

Significant differences were only found between the SNP group and all other treatment groups. Specifically, SNP, with a mean percent cell death of 88% in cut regions and 81% in non-cut regions of the ACL explants had a significantly higher amount of cell death when compared to Time Zero, DMEM(1), DMEM(10), IL-1 β , and TNF α (n=172, df=5, p<0.001). Additionally, there was no significant difference between the percentages of cell death found in cut and non-cut regions of the ACL explants (n=172, df=169, p=0.217). Besides the SNP group, percentages of cell death were similar among all groups and among cut and non-cut regions. Specifically, the percentage of cell death found in the Time Zero group were, Cut: 34.6% and Non-cut: 34.6%, for DMEM(1) were,

Cut: 55.3% and Non-cut: 44.8%, DMEM(10), Cut: 56% and Non-cut: 39%, IL-1 β , Cut: 40% and Non-cut: 34%, and TNF α , Cut: 35% and Non-cut: 53%.

Table 10. Percentage of dead cells in ‘Cut’ and ‘Non-Cut’ regions of PT explants after experimental treatment (n=21).

	Time Zero	DMEM(1)	DMEM(10)	IL1B	TNFa	SNP
Cut (%)	57	61	56	47	57	79
Not Cut (%)	57	57	43	33	43	72

Significant differences were found between SNP and T0, D(1), D(10), IL1B and TNF (p=<0.006). In contrast to ACL explants, there was a significant difference in the percentage of Cut and Non-Cut regions of PT explants (p=0.041).

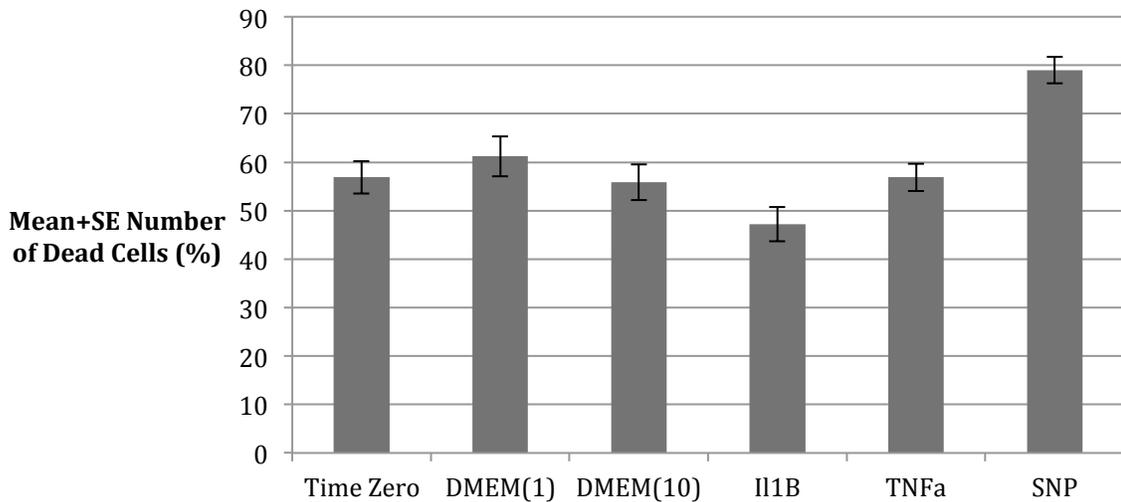


Figure 31. Mean percentage \pm Standard Error of live versus dead cells in PT explants (n=21) per various treatment conditions. This graph only shows cells that were on the periphery of the explant. Time zero refers to explant that was analysed immediately following harvest. DMEM(10) refers to Dulbecco’s Modified Eagle Medium with 10% FBS. DMEM(1) refers to Dulbecco’s Modified Eagle Medium with 1 % FBS. TNF α refers to tumor necrosis factor alpha (10 ng/ml). IL-1 β refers to interleukin one beta (5 ng/ml). SNP refers to sodium nitroprusside .

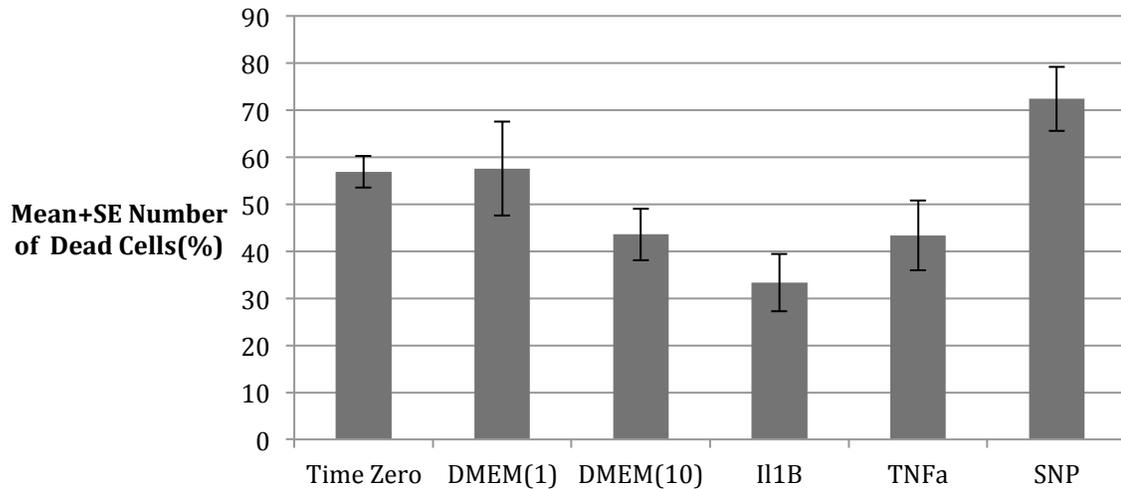


Figure 32. Mean percentage \pm Standard Error of live versus dead cells in PT explants (n=21) per various treatment conditions. This graph only shows cells that were in the middle of the explant. Time zero refers to explant that was analysed immediately following harvest. DMEM(10) refers to Dulbecco's Modified Eagle Medium with 10% FBS. DMEM(1) refers to Dulbecco's Modified Eagle Medium with 1 % FBS. TNF α refers to tumor necrosis factor alpha (10 ng/ml). IL-1 β refers to interleukin one beta (5 ng/ml). SNP refers to sodium nitroprusside.

An ANOVA test revealed that the only significant differences in percentage of cell death were between SNP and all other treatments (T0, D1, D10, IL-1 β , and TNF α) and between IL-1 β and Time Zero. Specifically, SNP with a percentage of 79% (Cut regions) and 72% (Non-cut regions) of cell death, was significantly higher compared to all other treatment groups (n=268, df=5, p=<0.006). The percentages of cell death in cut regions of the explants were significantly higher than in non-cut regions (n=268, df=266, p=0.041). Cell death percentages were recorded as follows: T0: Cut: 56.9% and Non-Cut: 56.9%, DMEM(10): Cut: 55.8% and Non-Cut: 43.4%, DMEM(1): Cut: 61% and Non-Cut: 57%, IL-1 β : Cut:47% and Non-Cut: 33%, TNF α : Cut: 56.8% and Non-Cut: 43%, and SNP: Cut 79% and Non-Cut:72%.

4.11 Annexin V Assay

Table 11. Summary of percentage of apoptotic cells after Annexin V assay.

Treatment Condition	Percentage(%) of Apoptotic Cells
ACL Explant DMEM(10)	0
ACL Explant DMEM(1)	0.23
PT Explants DMEM(1)	0.03
ACL Explant TNF α	0.10
PT Explant TNF α	0.82
ACL Explant IL-1 β	0.50
PT Explant IL-1 β	0.003
ACL Explant SNP	7.84
PT Explant SNP	9.20

For the ACL explants, the only significant differences in the percentage of apoptotic cells were found between the SNP treated explants and all other treatment groups. Particularly, the SNP treated explants had a significantly higher percentage of apoptosis versus Time Zero DMEM (1) (n=8, p=0.036), DMEM(10) (n=9, p=0.016), TNF α (n=10, p=0.008), and IL-1 β (n=9, p=0.013).

For the PT explants, the TNF α group had a significantly higher percentage of apoptotic cells versus both the IL-1 β group (n=11, p=0.01) and the D1 group (n=9, p=0.016). Additionally, there was no significant difference in percentage of apoptosis between SNP and IL-1 β (n=12, p=0.180), SNP and TNF (n=10, p=0.08), SNP and D1 (n=11, p=0.44).

4.12 DNA Laddering

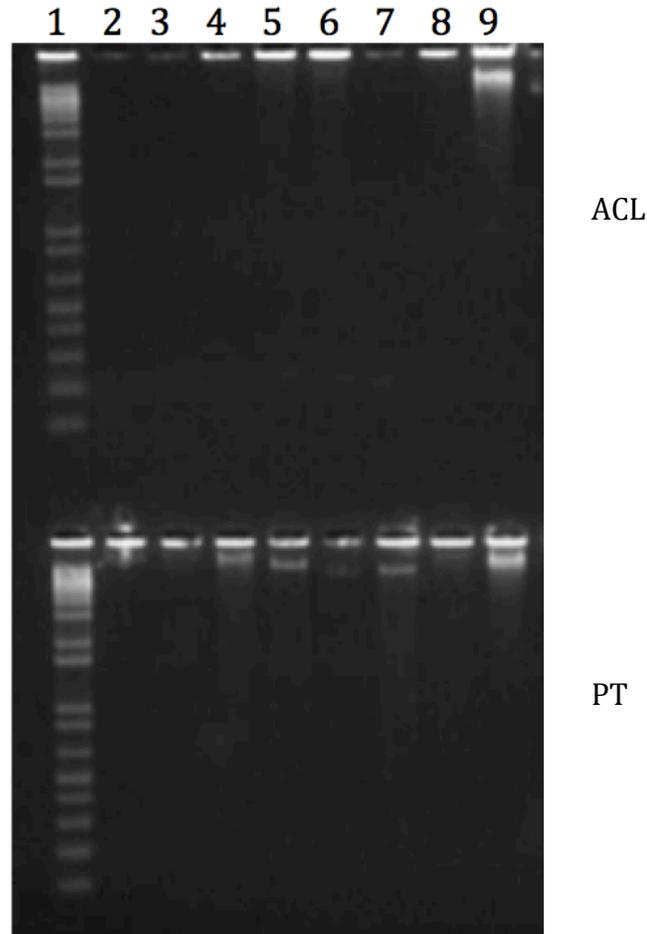


Figure 31. DNA laddering among various experimental conditions. 1. Positive control (DNase I), 2. DMEM(1), 3. DMEM(10), 4. IL-1 β , 5. IL-1 β , 6. TNF α , 7. TNF α , 8. SNP, 9. SNP. Top dataset contains ACL and bottom dataset contains PT.

A stronger signal corresponds to increased fragmentation of DNA. The first line on both ACL and PT datasets shows clear fragmentation as it has been treated with DNase I. There seems to be more fragmentation in PT tissue compared to ACL tissue across all groups. The last group, treated with SNP, shows the most fragmentation of all groups. In the ACL dataset, there seems to be no clear evidence of fragmentation with the exception of the SNP group. In the PT, there seems to be evidence of more fragmentation in the cytokine treated groups, but the strongest signal is in the SNP group.

Chapter Five: Discussion

5.1 Overview of Discussion

Experimental results will be discussed in the order of the procedures. Specifically, Optimization results will be discussed, following a discussion of hypothesis experiment results. Starting with optimization experimental results, I will discuss possible reasons for differences in cell numbers between normal and surgically altered ACLs. Next, a discussion of how the creation and testing of the jig and the assessment of fibroblast density (5.5) experiments aided in the optimization of later experiments. Our first explant cytokine culture also aided in the optimization of later experiments that included enhanced imaging techniques, non-jig cutting for explants, and a realization of the need for a positive control. Specifics will be discussed in section (5.4). Next, I will discuss hypothesis experiment results. The discussion of cytokine penetration in primary cells involves two parts, first, how the results of these experiments helped determine the time point for future experiments (section 5.6.1), and second, the overall results of these experiments (section 5.6.2). Then, I will discuss the relationship between cell death (both apoptotic and necrotic) and the observed effects in mRNA expression in PT and ACL explants. Lastly, I will provide insight into the limitations of this study.

Discussion of Optimization Results

5.1.2 Histological Analysis of 2 Groups of Sheep Tissue

It has long been reported that the anterior cruciate ligament displays poor healing capabilities after injury or rupture (Arnockzy et al., 1979, Murray et al., 2000, Cabaud et al., 1979). Murray et al. suggest 4 phases of ligaments healing: inflammation, epiligamentous regeneration, proliferation, and remodeling. Within the remodeling phase, they report a decrease in cell density as seen in Figure 7. However, the remodeling stage is stated to happen 1-2 years after rupture whereas the tissues assessed in my experiment were 20 weeks after rupture. Additionally, Murray et al. (2000) report that cell density in

the remodeling stage resembles cell density in an intact ligament whereas my data show significant differences in comparison to intact ligaments. In fact, in terms of the Murray et al. (2000) stages, the tissues analyzed would have been in the ‘proliferation stage’ in which cell density increases. These results are in contrast to the significantly lower cell density found in my histological analysis of sheep tissue. Murray et al.’s work was done with human samples whereas I used an animal model, which may account for some differences. Additionally, Murray et al.’s model only used ruptured ligaments, whereas the model utilized in my experiments were ligaments that were re-inserted into their original insertion after rupture. This provides some evidence to support a difference in cell density between ruptured ligaments and surgically altered ligaments. A possible explanation for decreased cell density found in Figure 7 could be due to cell death that seems to occur after an alteration in the ligament’s normal environment (Figs. 32,33).

5.1.3 Jig Creation and Testing

It was imperative to the success of my experiments to ensure that patellar tendon and anterior cruciate ligament explants were standardized for size and shape. Studies using ACL and PT explants are lacking in the literature. Spindler et al. (1996) evaluated growth factors in PT and ACL explants, but failed to describe specific methodology for harvesting the explants. Given that the PT is approximately 6.2 times larger (Figure 12.) than the ACL, the jig that was created was needed to standardize, length, width, and depth for each tissue. After our jig testing experiments, our jig successfully standardized weight between both ACL and PT explants (Figure 8). Additionally, the jig standardized dimensions of explants between PT and ACL tissues (Figure 9). However, data also showed a significantly higher percentage of dead cells in explants that experienced more cutting compared to intact tissues (Table 4). Given that the objective of this study was to measure cell death, an explant harvest procedure that causes cell death should not be used. The conclusion of this experiment was to utilize intact PT and ACL tissue in further experiments aimed at assessing cell death.

5.1.4 Assessment of differences in fibroblast distribution between regions of ACL and PT

After removing the ACL and PT from the native joint and replacing them in a tensionless environment of media, they swell (Thornton et al., 2001). I investigated whether or not medium type may be playing a role in the differences in total cells seen in the explant cytokine culture. There were no significant differences found in the percentage change in weight after submersion in DMEM, X-VIVO, or sucrose. However, variability in the percentage change in weight was much higher in X-VIVO and Sucrose. This is an interesting finding as X-VIVO is a medium specifically formulated for use in in vitro experiments (Lonza, Walkersville Inc., 2013). Additionally, sucrose has been cited as an optimal medium for ligament tissues as ligament tissues exhibit decreased swelling in sucrose compared to other media (Thornton et al., 2001). We also examined the differences in osmolality of different media as compared to synovial fluid as the ACL and PT would be exposed to synovial fluid in normal conditions. However, as shown in Table 5, osmolality of synovial fluid and various media was similar. Given that the purpose of this experiment was to evaluate the optimal medium, for comparison of ACL and PT, we concluded that further experimentation would utilize DMEM as the optimal medium as the variability between ACL and PT in DMEM was the most homogenous compared to sucrose and XVIVO.

Because no significant differences in media or swelling were found, an evaluation of differences in cell density by location was necessary in both the PT and the ACL. Murray and Spector (1999) describe 3 specific regions in the ACL, namely, the fusiform, ovoid, and spheroid zones. Our data support this categorization, as a trend similar to that described by Murray and Spector (1999) was found in that the A and B zones or fusiform and ovoid zones have a similar density of cells. Though we collected data on the spheroid zone (or ACL C) the number of observations was too small for analysis. For the PT explants, there were no significant differences by region in terms of cell numbers. We also investigated the reliability of the computer program Pixcavator versus manual counting. No significant differences were found between manual and computer counting. I believe this is due to enhanced computer capabilities in comparison to earlier experiments with ImageJ. The Pixcavator program allows the user to pinpoint the type of image needed to be counted. The conclusion of this experiment was to use DMEM for all medium uses in subsequent experiments and to use Pixcavator for cell counting.

5.1.5 Cytokine penetration and downstream effects in primary cells (Optimization)

In terms of the optimization for further experiments, results from the cytokine penetration in primary cells (4.7) gave some insight into the appropriate time points needed for penetration experiments in explants, cell death assessment in explants, and the Annexin V assay. With the exception of the Max group of TNF α treated PT cells, all other treatments saw an increase in fold mRNA expression after 24 hours (Table 5). Therefore, this helped in choosing the time point of 18 hours to accommodate for the fact that most proteins showed increased expression at 24 hours wherein also accommodating for TNF α expression which seemed to happen earlier than 24 hours.

Discussion of Hypothesis Results

5.2 Explant Cytokine Culture

To ensure the correct context for result discussion, reasons for differences in total cell numbers will be presented first. Results show no significant differences in fibroblast density, or media effect on swelling (Section 4.5, Figure 10). Differences in total cell numbers must stem from the fact that ACL and PT cells were not the only cells counted. Harvest of PT and ACL tissues always involves the removal of surrounding structures such as cartilage and fat. While these structures may look completely removed to the naked eye, further microscopic evaluation can show portions of other structures. In this case, I believe there were remnants of the fat pad (which includes densely populated cells compared to sparsely populated cells of PT and ACL) and these cells of the fat pad were counted along with PT and ACL cells. This would explain large differences in cell numbers in standardized ligament and tendon pieces. Specifically, some explants would have had fat pad remnants and others would not. This explains that while no significant differences exist in PT and ACL populations, the addition or removal of fat cells can significantly change the overall cell count. Future experiments utilized a more educated eye for correct cell counting and a more robust cell counting program. Despite major differences in cell death, the percentage of cell death (i.e. standardized over the entire population) may still provide some insight into the behavior of cytokines. The general trend seen after the first cytokine explant culture within the ACL group was that the

percentage of cell death decreased as the concentration of cytokine increased (Table 3). The results were similar for the PT group in that the lowest percentage of cell death was seen in the Max group, which had the highest concentration of cytokine (Table 3). This provokes the thought of cytokines exhibiting a protective effect on ligament and tendon explants. Other data in my thesis work agrees with this conclusion. Results in Tables 6 and 7 show that higher percentages of cell death were found in those cells without TNF α and IL-1 β treatment compared to those with TNF α and IL-1 β . In my explant studies (Sections 4.9, 4.10), no significant differences were found between treatment groups, however, in both ACL and PT explants, TNF α and IL-1 β treated explants displayed numerically lower values for percentage of cell death compared to the media groups (no cytokines). Furthermore, PT explants treated with TNF α and IL-1 β showed lower percentages of death than time zero explants and ACL explants showed similar values to time zero explants. Although not ever shown in tendon and ligament cells, IL-1 β and TNF α have been shown to induce a protective effect against cell killing induced the combination of TNF α and cycloheximide (Wong and Goeddel, 1988). Wong and Goeddel reported this protective effect in A549 cells (carcinoma lung cell line) in noting that both IL-1 β and TNF α induced the expression of MnSOD (Magnesium-Zinc Super Oxide Dismutase), which acts to destroy superoxide oxygen radicals. Interestingly, Actinomycin D, a known apoptotic inducer, blocked this induction by IL-1 β and TNF α . This work done by Wong and Goeddel (1988) is supported and further explained by later work by Barger et al., (1995). Specifically, Barger et al., (1995) explored the role of TNF α pretreatment to neuron cells before the formation of the neurotoxic 'A beta' plaque. These plaques are toxic due to the subsequent formation of reactive oxygen species, which decreases neuronal cell viability. Pretreatment with TNF α protected neuronal cells against this toxicity and the authors suggested this was evidence that TNF α induces anti-oxidant pathways. As mentioned, protective effects against cell death have never been reported within tendon and ligament tissues. My studies in both cell and explant culture provide evidence of this phenomenon. Future studies should investigate the specific mechanisms as those shown by Goeddel and Wong (1988) and Barger and colleagues (1995) involved in cytokine protection against cell death in order to confirm the

mechanisms that may be at work here.

5.2.2 Cytokine penetration and downstream effects in primary cells

There are many proteins that have been cited as being important mediators in the apoptotic pathway in cells. These include caspases, iNOS, COX-2 and other inflammatory mediators such as cytokines (IL-6, IL-1 β , TNF α) (Wang et al., 2011, Uchida et al., 2006, Mutsuzaki et al., 2010). As previously mentioned, Caspases are proteins that are involved in initiation of the apoptotic pathway (Millar et al., 2009). Carames and colleagues (2008) showed that TNF α regulated apoptosis in cultured human chondrocytes. Specifically, TNF α did induce activation of caspases 3, 7 and 8, and apoptosis, whereas IL-1 β did not. Conversely, we observed the highest expression of Caspase-3 in normal cells compared to cytokine treated groups (Figure 13, 14). Additionally, with respect to cell death, we also saw no significant differences between Control and cytokine-treated groups (Tables 6&7). There is one major differentiator between my study and the studies done by Carames and colleagues (2008); my study investigated mRNA expression of Caspase-3 while Carames and colleagues (2008) investigated the role of protein expression of Caspase-3. In general, mRNA expression is a widely accepted measure of cell behavior in response to stimuli (Greenbaum et al., 2003). However, one review reports that while correlations can be drawn between mRNA expression and protein expression, causation can only come from measuring actual protein expression (Greenbaum et al., 2003). This discussion will focus on meaningful correlations that might serve as a foundation for future work that should explore protein expression in a cell death context.

Some studies have attributed cell apoptosis to increased nitric oxide production (Murakami et al, 2005, Murakami et al. 2006, Hashimoto et al., 1998). In my primary cell experiments, control cells did not show any expression of iNOS. TNF α treated cells did not show large fold expression increase in mRNA expression of iNOS. However, IL-1 β treated cells did see a 2.2 fold increase in the Injury group and a 4.4 fold increase in the Max group after 24 hours, supporting the results found by Murakami et al. (2006) and providing further evidence of a relationship between IL-1 β and iNOS.

In addition to iNOS, COX-2 has been reported as having a role in apoptosis. Jeffrey and Aspden (2007) studied articular cartilage explants in humans, finding that COX-2 inhibitors reduced the percentage of apoptotic cells in vitro. Harris et al., (2002) describe COX-2's role in apoptosis in that COX-2 initiates prostaglandin activation that induces apoptosis. In my study, mRNA expression of COX-2 showed the highest fold increases in mRNA expression of all apoptosis markers used in our cell line experiments. Both TNF α and IL1 β induced major changes in mRNA expression of COX-2 (Figure 17, 18). TNF α treated cells showed mRNA expression levels 114 times larger (at its largest increase) than the control cells. IL-1 β induced a fold increase 956 times larger than the controls in terms of COX-2 expression (Table 5). Later results show that apoptosis is not occurring in response to cytokines and therefore these effects are correlations with the large role that COX-2 plays in inflammation regulation (Williams et al., 1999). Specifically, in response to TNF α and IL1 β , fibroblasts will readily induce expression of COX-2 (Williams et al., 1999).

Other high fold increases were seen with the IL-6 marker. Specifically, TNF α treated cells induced a fold increase 13 times the control cells (at its largest increase). With respect to IL-1 β treated cells, results showed fold increases 53 times greater than the control in the Injury group and 93 times larger than the control in the Max group. In terms of these IL-1 β results, they do agree with results found by Tsuzaki et al., (2003) in which they found that IL-1 β up-regulated mRNA expression of IL-6 in human tendon cells.

The other outcomes in the primary cell studies were done mainly to ensure the penetration of the cytokines into the tissues. In detail, all markers (IL-6, TNF α , iNOS, COX-2, and IL-1 β) show fold increases numerically greater than the control cells except caspase-3. Caspase-3 is the only marker (in the group tested in my studies) that is only involved in apoptosis and not inflammation. All other markers have been shown to be involved in both apoptosis and inflammation in various cell, explant, and animal studies (Murakami et al, 2006, Uchida et al., 2005, Lopez-Armada et al., 1998) . The finding that TNF α and IL-1 β did not induce Caspase-3 mRNA expression in cells agrees with the results I found regarding apoptosis in explants; TNF α and IL-1 β do not cause apoptosis

in explants. This explains why other markers would show increases in fold expression in response to cytokines, as they are involved in inflammation. Additionally, this explains why Caspase-3 would not respond to a cytokine stimulus as Caspase-3 only involved in apoptosis.

With reference to cell death in IL-1 β and TNF α treated ACL cells, there were no significant differences in cell death between control and treatment cells (Section 4.8). This in contrast to current literature on cell culture, but in accordance with the results in this study regarding explant cell death Section (4.10). With respect to current literature and cell culture, as previously mentioned, some studies show that COX-2 inhibitors decrease apoptotic rates (Jeffrey and Aspden, 2007) wherein my study, the same cytokine treatment that increased COX-2 mRNA expression by 956 times (Table 5) did not affect cell death rates. This suggests that other molecules associated with COX-2 instead of COX-2 itself, as reported in Jeffrey and Aspden's (2007) findings may be associated with apoptotic activity and or cell death. Other studies state that IL-1 β and TNF α can directly induce apoptosis in bovine chondrocytes (Shuerwegh et al., 2003). These results are not supported in my primary cell studies (Sections 4.7, 4.8) or explant studies (Sections, 4.9, 4.10). The simple fact that Shuerwegh and colleagues (2003) reported on chondrocytes whereas my study investigated ACL and PT cells could account for differences in response to cytokines. However, another study with chondrocytes found results that support the findings in my primary cell studies. Lopez- Armada et al., (2006) found that IL-1 β and TNF α alone, could not induce apoptosis in cultured osteoarthritic chondrocytes. A combination TNF α and Actinomycin-D (an apoptosis inducer) did cause apoptosis. In response to Jeffrey and Aspden's (2007) results, it's possible that while the COX-2 inhibitor reduced apoptosis in tendon explants, it was the inhibition of COX-2 effects (i.e. prostaglandin activation) rather than COX-2 activity itself.

5.2.3 Cytokine Penetration in Explants and Live/Dead Assay in Explants

Pelletier et al. (2000) studied the effect of the inhibition of nitric oxide on Caspase production and subsequent apoptosis in varying populations of dogs (normal, surgical, and OA). Results revealed elevated levels of caspase-3 and chondrocyte apoptosis in OA

dogs as well as a positive correlation between caspase-3 and chondrocyte apoptosis. In agreement with Pelletier's study, the highest expression of caspase-3 was found in the SNP treated cells, our positive control (Figures 26, 27) and this was significantly different than our control. Despite finding no significant differences in cell death between caspase-3 and controls, the second highest expression of caspase-3 was found in IL-1 β treated explants. Murakami et al. 2006, evaluated ACL explants (after injury) and their susceptibility to iNOS induced apoptosis, finding that ACL explants were more susceptible to apoptosis in the presence of iNOS and that IL-1 β increased the NO-induced apoptosis in the ACL explants. Interestingly, our study also found a link between IL-1 β and iNOS in ACL explants. For the evaluation of mRNA expression of iNOS, after SNP, IL-1 β had the highest fold increase compared to all other treatment groups (Figure 25, 29). In the PT assessment of iNOS expression, IL-1 β displayed a higher fold increase of iNOS expression compared to the positive control, SNP. This supports Murakami's findings regarding the possibility that iNOS production and IL-1 β are linked. However, the role of IL-1 β is not supported with cell death. In both, PT and ACL explants, SNP shows the highest percentage of cell death and the highest rates of apoptosis. IL-1 β showed 0.5% apoptotic cells in ACL explants and 0.003% in PT explants. Additionally, the IL-1 β group, in both PT and ACL showed similar percentages of cell death to the media controls (Figures 32-35). Lastly on the subject of iNOS, apoptosis and IL-1 β , there was an interesting observation in the mRNA expression of IL-1 β in the SNP group. Specifically, SNP induced a 220 fold expression of IL-1 β . This provides further evidence that IL-1 β and iNOS are linked as SNP causes cell death via the iNOS pathway. Particularly, the above evidence demonstrates that IL-1 β is involved in the iNOS pathway: however, IL-1 β does not have a direct effect in causing cell death. Further studies should investigate the specifics of this pathway to understand where inhibitors or pharmaceuticals may intervene for treatment of disease.

Another interesting observation regarding cell death is the amount of death that is found in the control (Time Zero) and media groups (DMEM(1), DMEM(10)) (Figure 29-35). Immediately after harvest from the joint, approximately 40-50% of cells were found to be dead (Time Zero Group). Uchida et al. (2006) used patellar tendons to investigate the effect of stress deprivation on the tissue. This study found an increased expression of

cytokines IL-1 β and TNF α on slackened patellar tendons compared to control tendons. Another study from the same group utilized a stress shielding method to alleviate all tension from the patellar tendon of rabbits and noted deterioration of the patellar tendon after 3 weeks (Yamamoto et al. 1993). Therefore, our findings may suggest that the lack of tension after harvest from the joint may stimulate inflammatory reactions that indirectly affect cell death pathways as seen with IL-1 β and Caspase-3 and IL-1 β and iNOS. Alternatively, the harvest from the joint could disrupt cell-signaling which causes cells to activate death pathways. In a review about the importance of ligament loading, Benhardt and Cosgriff-Hernandez (2009) describe the extracellular matrix (ECM) in connective tissue as the essential organizer of all connective tissue cells. Cell-to-cell signaling in connective tissue involves the integrins and adhesion molecules on the surface of the ECM. In response to injury, fibroblasts send signals to the ECM to up-regulate protein synthesis (Benhardt and Cosgriff-Hernandez, 2009). Specifically, Chiquet (1999) noted that certain ECM proteins (tenascin-C and collagen XII) are up-regulated when the ECM is experiencing tension and down-regulated when the ECM is relaxed. Furthermore, this reaction is immediate and can be seen at the mRNA level. This could be an alternate explanation to the high percentage of cell death found in control and media groups. Particularly that the lack of tension causes a unique cascade of cell-signaling that causes the cells to up-regulate degradative and death pathways. Although many studies have assessed mechanical stress on ligament and tendon tissues, few have done so in a cell death context. Further investigation should include ECM changes, protein expression, mRNA expression, and their effect on cell death.

Besides gene expression changes, cell death was increased in tissues that experienced more cutting (Table 4, Figure 32). This provides clinical implications for surgeons in which grafts are cut from existing structures and utilized for repair in injured tissues for reconstruction surgeries. To date, no study has measured the impact that physical cutting has on tissues with respect to cell death. Currently, healing rates and normal function after ACL reconstruction are not optimal. As previously mentioned at least 60% of patients go on to develop osteoarthritis after ACL reconstruction (Nelson et al. 2006). Furthermore, a study found that physiotherapy interventions were no better than surgical reconstruction interventions after ACL injury with respect to functional and

pain outcomes (Streich et al. 2011, van Porat et al. 2004). Therefore, techniques or surgical procedures could be improved. My study suggests that the initial cutting of the tissue causes increased cell death. This is supported by evidence of lower overall cell numbers in tissue that has been surgically altered (Figure 7). It is possible that with increased death due to cutting, healing is delayed or more difficult. Additionally, if after healing there are less cells, biomechanical and biochemical homeostasis cannot be maintained to that of a normal ACL which could account for the prevalence of future injuries, pathogenesis, and disease.

While presenting strong evidence that one of these factors (lack of tension, cell signaling, cutting), is responsible for the high death rate associated with tissue removal from the joint, environmental factors should be discussed. Particularly, an obvious difference between the intra-articular joint environment and lab environment after harvest is the presence of O₂. One early study measured gas tensions within the knee joint comparing normal and pathological patients. Savage and Taylor (1939), described a variety of case studies concluding that the O₂ tension inside normal joints is higher compared to osteoarthritic joints and lower than joints affected by rheumatoid arthritis. The values presented for intra-articular O₂ tension were on average 34 mmHg for normal patients, 9 mmHg for osteoarthritic patients, and 57 mmHg for rheumatoid patients. Average sea-level pressure (comparable to what would be experienced in the lab during experimental procedures) is 760 mmHg. Numerically, these seem like large differences and therefore perhaps the increased O₂ tension tissues experience when being removed from the joint act to induce cell death. Alternatively, the combination of cutting, increased O₂ tension, cell signaling, and lack of tension could be working collectively to induce cell death. Results from my initial pilot study titled histological analysis of 2 groups of sheep tissue (Figure 7) seem to point to the fact that lack of tension may lead to a reduction in cells within the tissue as after repositioning the ACL a reduction in cells was observed (Figure 7) However, the difference between the histological repositioning analysis and explant studies is that the repositioning a measure of the in vivo cell behavior where my explant studies were performed in vitro. To that point, there is evidence that O₂ tension effects in vivo blood flow. Gronlund et al., (2008) found that increasing the O₂ tension to 75 mmHg significantly reduces joint blood flow. Future

studies should explore O₂ tension, lack of physical tension, cutting, and extracellular matrix signaling to understand the exact mechanisms at work.

Continuing with the discussion of cell death in explants, another interesting finding in my study is the differences in percentages of cell death between explants and primary cells. ACL primary cells showed a range of cell death percentages from 3-12% while in ACL explants cell death ranged from 35-88% (Table 6). Similarly, primary PT cells showed a range of cell death from 3-43%, while PT explants displayed a range of cell death from 33-79% (Table 7). This evidence identifies the trend that cell culture and explant culture are very different. Cell culture lacks the presence of the ECM. As previously mentioned, the ECM and fibroblasts found in both tendon and ligament cells have a dynamic relationship consisting of protein signaling, cell to cell communications, and mechanically induced gene expression (Kjaer, 2004). This could account for differences between cell culture and explant culture.

Besides differences between cell culture and explant experiments, there seem to be apparent differences between ACL and PT in both of my primary cell and explant studies. It seems that in most cases in both primary cell studies (Section 4.9) and explant studies (Section 4.11) that patellar tendon cells and tissue experience higher percentages of cell death. For example, primary PT cells showed 43% cell death in control cells compared to the 9% seen in ACL cells. In explants, every PT experimental treatment group (in the Cut group and almost every experimental treatment group in the Non Cut group), showed a higher percentage of cell death compared to its ACL counterpart (Tables 9, 10). In general, tendon tissues bear a higher degree of tensile strength than ligament tissues (Noyes et al., 1984). Additionally, tendons contain more blood vessels than ligament tissues (Ralphs and Benjamin, 1997). Perhaps due to the fact that the PT experiences more tension on a regular basis than the lack of tension (from cutting/removal from joint) exerts a larger effect on the tendon and that is the reason for the higher percentage of cell death found in PT compared to ACL. Additionally, the PT may be more reliant on blood supply for homeostasis and lack of this blood supply may hinder cell viability wherein the ACL is less vascularized and might not depend on blood supply for cell viability.

5.3 Limitations of this study

The limitations of the histological analysis of sheep tissue include the fact that only 20 week samples were examined whereas representative samples from more acute stages (i.e. 24 hours) would be applicable to the other results in this study. In terms of the first cytokine explant culture, it should be noted that I was inexperienced in histology cutting and confocal imaging and while attention to precision was practiced, results should be viewed as preliminary as there was a higher chance for possible error. In the assessment of fibroblast distribution, in the testing of media differences, small sample sizes were used which may place limitations on the results. However, sample sizes of at least $n=4$ were used which should provide sufficient evidence for optimization experiments. Furthermore on the topic of sample sizes, an $n=2$ was used for the mRNA expression in primary cells which may limit interpretation of the results. However, as discussed in section 5.6.1, our results do agree with current knowledge, which strengthens our findings. While these results were not robust enough to run statistics, these experiments served their purpose in providing partial evidence for cytokine penetration. Lastly, this study utilized multiple animal species for the experimental procedures which could make comparisons between experiments difficult. However, most differences in animal species were utilized in experiments which were completed in order to optimize later experiments. Additionally, results in which conclusions were drawn in the discussion utilized all of the same species (rat).

Chapter Six: Conclusion

6.1 Conclusion

Based on the results presented from my studies, I can conclude that IL-1 β and TNF α do not cause significantly different percentages of apoptosis or necrosis after 18-hour treatment compared to their controls. However, apoptotic processes may regulate inflammatory reactions. In detail, the finding that SNP (an apoptosis inducer, via the iNOS pathway), caused a 220 fold increase in IL-1 β production, points to the possibility that apoptosis causes an up-regulation of cytokines via an iNOS mechanism. Despite the fact that cytokines are not causing cell death, upon harvest from the joint, another mechanism is causing cell death. There is strong evidence pointing to physical cutting of the tissue in that PT explants showed higher percentages of cell death in Cut regions compared to Non- Cut regions. However, it is possible that these levels of cell death, in combination with cutting, are due to oxygen presence, cell-signaling, and/or lack of tension. Besides ACL explants showing no difference in the percentage of cell death, there were other differences between ACL and PT explants. Most notably were the differences between up-regulation of mRNA expression of IL-1 β and iNOS. Specifically that ACL explants showed large fold increases in expression where PT showed much lower levels of mRNA expression. In addition to explants, cell behavior between ACL and PT was different. Furthermore, in response to the same stimuli, under the same experimental conditions, PT and ACL cells responded differently than their explant counterparts.

6.2 Future Directions

While finding significant results and answering my hypothesis, my study generates many new hypotheses regarding ligament and tendon behavior. Future work should be focused on determining the specific mechanisms that produced the results presented in this thesis work. For example, new studies could investigate the time dependency on cell death; does cell death increase with time or is there an initial peak of death after which there is a plateau? Understanding the role of time could help improve

ACL reconstruction surgery. Specifically, if death is time dependent, and intervention to protect from death might be useful in reversing the effects seen in my study. Other future work should examine if there is a difference in the rates of cell death between a transected ACL that remains in the body and an ACL explant. In this type of experiment, the lack of tension would be constant but exposure to environmental conditions would be variable including O₂ tension. Other experiments could assess if percentages of cell death change when explants regain tension. For example, after removal from the joint, studies joint explore if putting the explants back in a tension system decreases cell death. Following up with the results seen in this thesis should include an assessment of the relationship between the mRNA expression shown in my cell and explant studies and the actual protein expression in similar studies. Protein or mRNA analysis on different locations in explants would give clearer insight into Cut versus Not Cut regions. For example, testing protein expression on Cut and Not Cut regions may reveal an up-regulation of death signals which would make a stronger case for cutting being the main reason behind cell death.

While this study has implications for ACL reconstruction in that explants are a part of the procedure and therefore biological and biochemical changes with respect to cytokines could be useful in the improvement of current methods, future studies should take a similar approach (as this study) *in vivo*. Specifically, future *in vivo* studies should focus on measuring the variety of cytokines present after ACL transection and their subsequent effect on cell death. *In vivo* studies would observe the natural up-regulation of inflammation and the natural response to cell death. Alternatively, there is still further information needed from explant studies. For example, different time points, besides the 18 hour time point used in this study would give a further understanding of differences in apoptotic death. This study can only comment on apoptosis at 18 hours. Future studies should examine how frequent apoptotic changes can be over 24-72 hours to gain a further understanding and improve future methodology. Implications for ACL reconstruction should also be noted as cutting of tissue causes increased cell death. Further studies should use this new information to find ways to protect cells from cell death or reduce cell death for use in surgical procedures.

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Appendix A

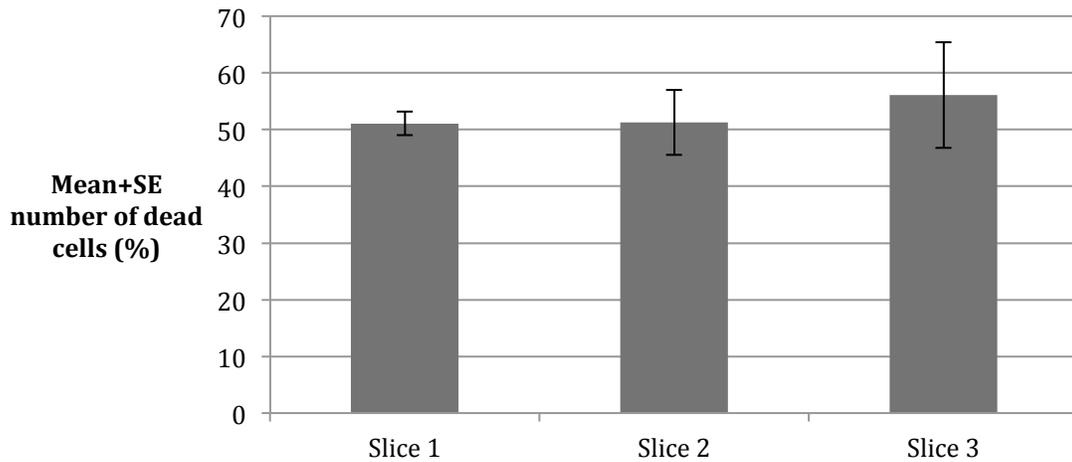


Figure 1. Comparison of explant depths and associated cell death in ACL explants (n=21 explants). Visual Slice 1 is the surface of the explant, Slice 2 is the middle of the explant, Slice 3 is bottom of the explant. Slices were photographed at a standardized 250um apart. All experimental groups were included in this analysis (i.e. T0, D(1), D(10), IL-1 β , TNF α , and SNP). No significant differences were found between any slice groups (n=176, df=2, p=0.74).

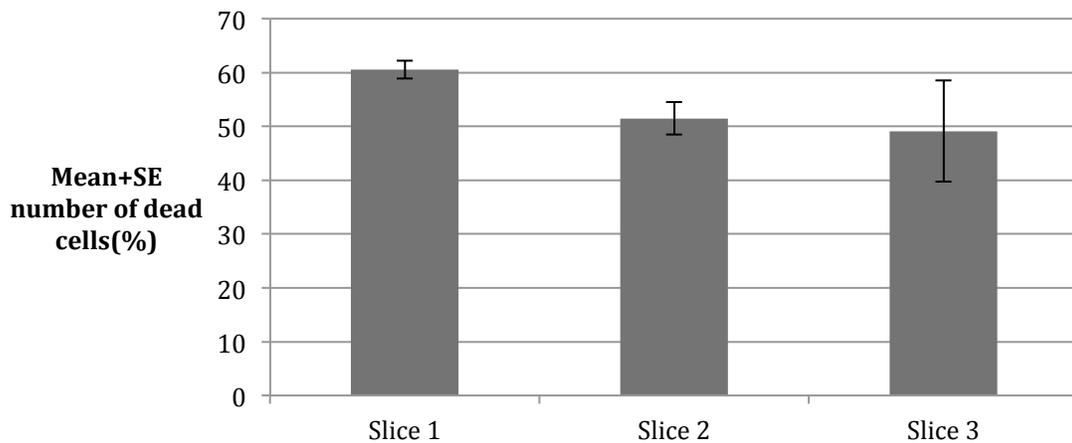


Figure 1. Comparison of explant depths and associated cell death in PT explants (n=21 explants). Visual Slice 1 is the surface of the explant, Slice 2 is the middle of the explant, Slice 3 is bottom of the explant. Slices were photographed at a standardized 250um apart. All experimental groups were included in this analysis (i.e. T0, D(1), D(10), IL-1 β , TNF α , and SNP). There only significant difference between groups was found between Slice 1 and Slice 2 (n=258, df=2, p=0.002)