

BIOLOGIC RESPONSES OF LIGAMENT AND TENDON GRAFT-DERIVED
FIBROBLASTS TO CLINICAL LEVELS OF CYCLIC STRAIN

A Dissertation
presented to
the Faculty of the Graduate School
at the University of Missouri-Columbia

In Partial Fulfillment
of the Requirements for the Degree
Doctor of Philosophy

by
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MAY 2020

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The undersigned, appointed by the dean of the Graduate School, have examined the dissertation entitled

**BIOLOGIC RESPONSES OF LIGAMENT AND TENDON GRAFT-DERIVED
FIBROBLASTS TO CLINICAL LEVELS OF CYCLIC STRAIN**

presented by Sebastian Cardona-Ramirez,

a candidate for the degree of Doctor of Philosophy,

and hereby certify that, in their opinion, it is worthy of acceptance.

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Professor Aaron M. Stoker

Professor Richard Ma

Professor Cristi Cook

Professor Trent Guess

DEDICATION

To all students from developing countries trying to pursue a career in science. Keep pushing, the journey is long, but the reward vastly outweighs the effort

“We must try to expand the boundaries of human wisdom, empathy and perception, and there is no way of doing that except through education.”

- J. William Fulbright

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LIST OF ABBREVIATIONS
(IN ALPHABETICAL ORDER)

2D: Two-dimensional

3D: Three-dimensional

ACL: Anterior Cruciate Ligament

ACLR: Anterior Cruciate Ligament Reconstruction

ACUC: Animal Care and Use Committee

AM: Antero-Medial

bFGF: Basic Fibroblastic Growth Factor

BPTB: Bone-Patellar Tendon-Bone

COL: Collagen

COX: Ciclo-Oxygenase

CrCL: Cranial Cruciate Ligament

DHT: Dihydrotestosterone

DMEM: Dulbecco's Modified Eagle's Medium

ECM: Extra Cellular Matrix

GAG: Glycosaminoglycan

HT: Hamstring Tendon

IL: Interleukin

IQR: Interquartile Range

KC: Keratinocyte Chemoattractant

MCP-1: Monocyte Chemoattractant Protein -1

MIP-1 β : Macrophage Inflammatory Protein-1 β

MMP: Matrix Metalloproteinase
MMPACT: MMP Activity
MSC: Mesenchymal Stem Cell
N: Newton
OA: Osteoarthritis
PCL: Posterior Cruciate Ligament
PDGF: Platelet Derived Growth Factor
PG: Proteoglycan
PGE: Prostaglandin E
PICP: Procollagen-I Carboxi-terminal Propeptide
PL: Postero-lateral
PRP: Platelet Rich Plasma
PT: Patellar Tendon
PTOA: Post Traumatic Osteoarthritis
QT: Quadriceps Tendon
ROM: Range of Motion
SYN: Synovium/Synoviocytes
TGF- β -1: Transforming Growth Factor β -1
TNF: Tumor Necrosis Factor
VEGF: Vascular Endothelial Growth factor

LIST OF PRODUCTS

(In Alphabetical order)

- 1,9-Dimethyl-Methylene Blue zinc chloride double salt: Sigma-Aldrich, St. Louis, MO, USA
- Fetal Bovine Serum: Gibco, Thermo Fisher Scientific, Waltham, MA, USA
- Insulin-Transferrin-Selenium: Gibco, Thermo Fisher Scientific, Waltham, MA, USA
- L-Ascorbic acid: Gibco, Thermo Fisher Scientific, Waltham, MA, USA
- L-Glutamine: Gibco, Thermo Fisher Scientific, Waltham, MA, USA
- Modified Eagle's medium nonessential amino acids: Gibco, Thermo Fisher Scientific, Waltham, MA, USA
- Olympus BX51 microscope: Center Valley, PA, USA
- Penicillin-streptomycin-amphotericin B: Antibiotic-Antimycotic, Gibco, Thermo Fisher Scientific, Waltham, MA, USA
- Resazurin sodium salt: Sigma-Aldrich, St. Louis, MO, USA Sodium chloride (0.9% NaCl): Hospira, Inc., Lake Forest, IL
- Sodium Pyruvate: Gibco, Thermo Fisher Scientific, Waltham, MA, USA
- Spectramax iD5 Multi-mode Microplate Reader, Molecular Devices, San Jose, CA, USA
- Trypan Blue Stain (0.4%): Gibco, Thermo Fisher Scientific, Waltham, MA, USA
- TrypLE™ Express Enzyme: Gibco, Thermo Fisher Scientific, Waltham, MA, USA

**BIOLOGIC RESPONSES OF LIGAMENT AND TENDON GRAFT -DERIVED
FIBROBLASTS TO CLINICAL LEVELS OF CYCLIC STRAIN**

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ABSTRACT

Anterior Cruciate Ligament (ACL) is one of the major ligaments in the knee. ACL injury is the partial or complete tear of the ACL usually as a consequence of a traumatic sports-related injury. ACL injury commonly affects athletes in the late adolescence and are usually more common in females than males. Factors such as metabolic response, cellular crosstalk among tissues and sex differences are not fully understood. Therefore, an increased comprehension of the biology of ACL injury and healing is required.

Management of ACL injuries is typically achieved by surgical stabilization of the knee. For this, arthroscopic ACL reconstruction with different tendon autografts is the gold standard technique. Additionally, ligament repair by suturing the torn ends of the ACL is an alternative procedure. Although current surgical treatment, is generally successful, ACL graft and repair healing biology needs to be further explored to decrease failure rates. Moreover, whether a specific activity level would in turn affect the healing process is still not known.

Several *in vitro* and *in vivo* preclinical models have been developed to explore current ACL injury and healing biology. Among these, large animals offer the advantage to be

similar to the human. The preferred model is the spontaneous onset of ACL injury, commonly found in dogs. In both species, extracellular matrix (ECM) remodeling driven by cellular responses to load can influence disease, as well as response to treatment.

However, most *in vitro* models do not include the multiple cell types in the joint or the mechanical environment that modulates their responses.

Consequently, we aimed to evaluate the metabolic responses of fibroblasts obtained from canine intraarticular tissues potentially involved in ACL injury as well as tendon grafts commonly used for ACL reconstruction subjected to varying levels of mechanical stress in a monolayer culture. We hypothesized that fibroblasts from these tissues would produce significantly different levels of inflammatory and remodeling biomarkers in response to a spectrum of cyclic strains *in vitro*.

We were able to demonstrate that ACL surrounding tissues are an important source of inflammatory and degradative biomarkers, and that metabolic responses of tendon graft fibroblasts are strain and tissue dependent. Moreover, male canine tendon fibroblasts seem to increase degradative responses while females tend to increase inflammatory responses when stimulated with different strain magnitudes. Also, synoviocytes are an important source of inflammation and human intraarticular tissue fibroblasts and tendon graft-derived fibroblasts have variable responses to stress but is not differentially affected by sex. Consequently, further studies are required to elucidate complex regulation mechanisms in both canines and humans.

CHAPTER 1

INTRODUCTION

Anterior Cruciate Ligament (ACL) injuries affect around 1.7 billion people worldwide and 1 in 3000 persons in the united states.¹ Female athletes are 2-8 times more prone to ACL tears than males.² Suturing the torn ends of the ACL was the first described technique to correct knee instability after ACL injury.³ However, an unreliable healing response has been associated with primary ACL repair.⁴ This has been attributed to several factors, including a hostile intra-synovial environment, specific post-inflammatory responses, and intrinsic cell deficiencies.^{5; 6} Cellular factors play an important role in ACL injury and healing. ACL rupture initiates a cascade of cytokines and catabolic enzymes.^{7; 8} Current work has shown that inflammatory and degradative molecules present after ACL injury can affect intraarticular tissues such as ligaments, tendon grafts and cartilage.⁸⁻¹¹ Therefore, the understanding of the early healing phase characterized by cellular release of molecules that regulate adequate balance between extracellular matrix (ECM) degradation and proliferation is crucial.

Due to the increased rate of failure with ACL repair techniques, ligament reconstruction with a tendon graft was adopted as the standard of care.¹² More than 100,000 ACL reconstruction surgeries are performed each year in the US. Although generally successful, 10% of those surgeries fail for a variety of reasons including inappropriate surgical technique, inadequate graft selection, improper graft fixation and incorporation and inappropriate postoperative rehabilitation.¹³ There is considerable debate regarding

the ideal autograft choice for anterior cruciate ligament (ACL) reconstruction. Common sources for ACL autografts include patellar tendon (PT), hamstring tendon (HT), and quadriceps tendon (QT). These different grafts have been associated with disparate outcomes in patients. PT, for instance, has been associated with increased prevalence of osteoarthritis (OA) after reconstruction, whereas HT has been associated with possible higher revision rates when compared to PT.^{14; 15} While material properties of various autograft tissues are known, there is little information on the biologic differences among these commonly-used ACL autografts. Additionally, the interactions between the biomechanical environment in the post-surgical healing stage with various autografts are not fully understood. Once an autograft is placed for ACL reconstruction, an inflammatory cascade ensues to begin the initial stages of graft ligamentization. The roles of the existing autograft fibroblasts in this early process are unknown.

In addition to the native ACL and the grafting tissue, it appears that surrounding tissues are also major contributors of healing molecules within the knee joint. It has been shown that synovium plays an important role in regulating the joint homeostasis by maintaining or amplifying the injury signal.¹⁶ Elevated levels of cytokines and growth factors within the knee intraarticular environment are often considered to be the contribution of synovium.¹⁷ Furthermore, the cross talk between synoviocytes and intraarticular ligaments has been shown to influence the healing response when fibroblasts are subjected to mechanical deformation.¹⁸

To evaluate the biologic events of healing in tendons and ligaments, *in vitro* models have been developed. However, traditional models often do not replicate the native environment within the knee joint due to the lack of mechanical strain, specifically axial

tension for cruciate ligaments and tendon grafts that occur with knee motion, that in turn modify cellular properties. Consequently, recent 2D and 3D models have been designed to assess the effect of mechanical loading in ligament and tendon cells.¹⁹ The Flexcell® tension is the most popular system to stress cells in monolayer culture. It has been widely used to evaluate specific cellular responses to tension loads in ligament and tendon fibroblasts.²⁰⁻²³ It permits to evaluate cells in monolayer or in co-culture as well as the addition of different molecular stimulus such as hormones, cytokines and growth factors.²⁴⁻²⁶

Hence, we were interested in elucidating main responses to varying levels of clinically relevant cyclic strains of cells obtained from ligaments and tendons that are commonly involved in either ACL healing, repair or reconstruction with a tendon graft. The objective of this work was to compare the pro-inflammatory and metabolic responses of fibroblasts obtained from intraarticular tissues and tendon graft-derived fibroblasts to varying levels of mechanical strain forces. Additionally, we aimed to compare the response of male and female fibroblasts to tension load and to evaluate if tendon graft-derived fibroblasts co-cultured with synoviocytes would significantly modify the production of inflammatory and metabolism related biomarkers. Our central hypothesis was that fibroblasts from these tissues would exhibit differential responses via production of varying levels of biomarkers to a spectrum of cyclic strains *in vitro*.

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CHAPTER 2

LITERATURE REVIEW

Biology of the anterior cruciate ligament:

The Anterior Cruciate Ligament (ACL) is an intraarticular ligament within the knee joint. Although it is intraarticular in nature, the ACL is extrasynovial, being isolated by the synovial sheet that is wrapped around the cruciate ligaments.¹ The ACL follows the typical organization of connective tissues. It is covered by a vascular layer called epiligament, that merges with the periosteum at the insertion with the bone.² The ACL possesses a characteristic hierarchical organization that comprises groups of parallel fibers' fascicles, surrounded by connective tissue. The fascicle can be in turn subdivided into subfasciculi (100-250µm diameter) covered by the epiligament. The epiligament is rich in cellular component and nerves, both proprioceptive and sensory.³ The subfascicular units are composed of collagen fibrils (20-155nm).⁴ Furthermore, the ACL inserts into the bone through the enthesis.

The enthesis is the region where a tendon or ligament inserts to the bone in a typical transition form, via fibrocartilage or mineralized cartilage.⁵⁻⁸ Fibrous entheses are more common where muscles attach to diaphyseal bones and are composed of dense bands of collagen (Sharpey's fibers) that merge with the periosteum and penetrates the cortical bone. Fibrocartilaginous entheses on the other hand, contain a zone of fibrocartilage at the area of attachment of the tendon or ligament and form a typical transition zone between the collagen fibers and the bone. There is no periosteum present at the fibrocartilaginous entheses. The fibrocartilaginous zone has both mineralized and non-mineralized regions separated by a characteristic tidemark. Furthermore, fibrocartilaginous entheses are more

common at ligament origins and insertions and at apophyseal or metaphyseal attachments of tendons.⁵

The specific organization of the entheses reflects the adaptation design to distribute stresses that are applied at the interface between structures with different strength and elasticity patterns.⁹ Moreover, fibrocartilaginous entheses must maintain integrity and function depending on directionality, tensile, compressive and shear stresses. Importantly, surgical reattachment of tendons or ligaments results in the formation of a fibrous enthesis that cannot adequately resist the complex loading patterns of fibrocartilaginous entheses.¹⁰

The cellular component of the ACL is composed by fibroblasts. They belong to the family of connective tissue cells and specialize in the production of extracellular matrix (ECM) components such as collagen and proteoglycans and provide mechanical strength to the tissue by offering a supporting framework to the ECM. Fibroblasts play a key role in the inflammatory and immune-mediated response to injury as well as homeostasis of musculoskeletal tissues.¹¹ Additionally, the ECM -the ground substance where fibroblasts are submerged in- provides the environment required for mechanical and biological functions.³

The ACL has a typical distribution of fibroblasts. Myofibroblasts exhibit contractile properties and are important in ligament healing.^{3; 12; 13} The antero-medial (AM) bundle, is highly cellular, especially near the proximal end with typical round or ovoid morphology.¹³ The midsubstance appears to be less cellular. Cells adopt a parallel orientation with fusiform and spindle-shaped morphology.¹⁴ Distally, spheroid cells are predominant, with a lower cell density and shorter crimp formation.^{3; 13} They also have abundant cellular organelles that indicate a high cellular activity.³ Additionally, near the enthesis,

chondrocytes can be found in the fibrocartilage and mineralized fibrocartilage zones that anchors the ligament to the bone.³

Composition of Tendons and Ligaments:

Tendons are dense bands of collagen-rich fibers that connect muscle to bone. Tendons are intended primarily to transfer forces after muscular contraction.^{15; 16} Similarly, ligaments are connective tissue bands that originate and insert on bones. They typically contribute to joint stability and to restrain excessive joint motion. Tendons and ligaments share similar cellular and extracellular biochemical composition.^{17; 18} However, these small differences in ECM structure and composition provide a wide difference in function.

Ligaments have lower collagen and increased water content compared to tendons. Importantly, tendon and ligament function and mechanical properties are highly correlated with density and morphology of collagen fibers. Moreover, collagen fiber integrity and stability are highly dependent on proper formation of covalent collagen cross-links.¹⁹ The lack of covalent cross links results in weaker mechanical and structural properties.²⁰ Amiel *et al.*, (1983) reported four to five times more collagen cross-links in the rabbit ACL compared to the patellar tendon.²¹ Additionally, Ng *et al.*, (1996) demonstrated that collagen cross-links increase in patellar tendon autograft three years after ACL reconstruction in a goat model.²² Similarly, a normally higher content of elastin in ligaments (1-15%) compared to tendons (1-5%) is responsible for supporting up to 30% of tensile stress under uniaxial strain. Consequently, a shift in elastin content would facilitate higher loads in the reconstructed ACL graft.

Both tissues are composed of cells and fibers embedded in a three-dimensional extracellular matrix. This amorphous ground substance, commonly produced by the

fibroblasts, is a complex mixture of type I collagen fibers with smaller quantities of types II, III, V, VI, IX and XI.²³ Proteoglycans, proteins, growth factors and glycoproteins are also important components of the ligament and tendon ECM.

Within the musculoskeletal system, tissue cells are named depending on the tissue they inhabit (i.e. tenocytes within tendon, ligamentocytes within ligament), each of them having specific morphology and phenotype. Tenocytes and ligamentocytes are aligned with the collagen fibers and interconnected through long cytoplasmic extensions. Other types of cells are also seen in the connective tissue including macrophages and leukocytes.²³

Different variations and adaptations in the composition and organization of the extracellular matrix provide specific mechanical properties.^{24; 25} In the case of tendons and ligaments, they are primarily intended to withstand the action of tension loads.

Collagen:

Collagen tertiary structure consists of a triple helix with three separate polypeptide molecules called alpha chain. Collagen fibrils are designed to resist tension forces. Fibrils are organized into parallel dense structures organized in a wave pattern, the crimp.³ The dominating type of collagen in ligaments and tendons is collagen type I, responsible for the tensile strength of the tissue. Type II collagen can be found in the fibrocartilaginous regions and in the insertion, sites being an indicator for applied pressure and shear stress.³

Type III collagen is found between collagen I fibers and is more abundant near the insertion sites.²⁶ It is also an indicator of remodeling after anterior cruciate ligament reconstruction.²⁷

Type IV collagen is usually associated with the basal lamina of the intra and periligamentous vessels which can serve as a vascularization indicator.^{3; 26} Type VI collagen is found in the distal zones of the ACL and serves as a gliding component between

functional fibrillar units and is associated with greater resistance to tension forces found in attachment sites compared to the mid-substance region.³

Mechanical tensile properties of collagen are determined by the collagen orientation, packing and fibril diameter. Additionally, hierarchical structure of collagen permits the generation of different fibrillar configurations that are specifically adapted for each specific tissue. Moreover, tendons and ligaments are subjected primarily to unidirectional tensile loads due to the parallel arrangement and dense packing of collagen fibers.²³ Larger diameter fibers are present in tendons and ligaments that experience greater loads. Smaller diameter fibers produce greater surface area and control viscoelastic properties through variations in proteoglycan content. They also show a typical viscoelastic response being affected by the rate of loading.²³ Typically, lower diameter collagen fibrils are common after tissue injury and repair and are associated with decreased mechanical properties of the scar tissue.²⁸

Glycosaminoglycans:

Glycosaminoglycans (GAGs) are molecules (carbohydrate conjugates of unbranched polysaccharides containing amino sugars) found in combination with proteoglycans (glycoproteins). GAGs possess an hexosamine unit that is highly sulfated. On the contrary, Hyaluronic acid, is a non-peptide, conjugated non-sulfated GAG consisting of D-glucuronic acid and D-glucosamine.^{29; 30}

Proteoglycans (PGs) consist primarily of a core protein with covalently attached glycosaminoglycans sidechains. PGs are highly present in the interfibrillar matrix and are associated with the surfaces of collagen fibrils. Main PGs include biglycan, decorin, fibromodulin and lumican. Biosynthesis of PGs include the addition and sulfation of GAG

sidechains within the endoplasmic reticulum and packaging within the Golgi apparatus for secretion.³¹ Large aggregating proteoglycans, namely aggrecan and versican, consist of a backbone of hyaluronic acid with multiple non-covalently linked proteoglycans. These aggregating PGs are highly anionic assemblies that have a high affinity for water. This leads to a high level of hydration and turgidity, important for compressive properties of the tissue.^{23; 29} PGs degradation involves the proteolytic cleavage of the core or link proteins at specific cleavage sites by specific matrix metalloproteinases (MMP) and aggrecanases.³² There are different types of degradative ECM enzymes. MMPs are zinc dependent endopeptidases that include collagenases, gelatinases and stromelysins that degrade a wide range of ECM proteins. Aggrecanases are proteolytic enzymes that cleave the core protein of aggrecan. Musculoskeletal pathology is commonly driven by an excessive degradative enzymatic activity and the inability of the tissues to assemble an effective reparative response that in turn will lead to altered composition and mechanical properties of the ECM.²³

Knee Kinematics and Biomechanics of the ACL

The knee is a complex joint with six degrees-of-freedom, three rotational (abduction/adduction, flexion/extension and internal/external rotation) and three translational (anterior/posterior, medial/lateral, and compression/distraction). Due to the lack of bony geometric constraint, joint stability is achieved mainly by soft tissue. Here, the ACL becomes the main restraint of the knee in anterior tibial translation, causing fibers to become taut.³³

According to Skelley *et al.*, (2015) microstructural properties in the human ACL differed between the anteromedial (AM) and posterolateral (PL) bundles.³⁴ The AM has higher

tissue modulus and failure stress under load compared to the PL bundle, probably due to differences in anatomical features in the femoral and tibial entheses.³⁵ Woo *et al.*, (1991), showed that the ACL tensile strength is around 2160 N and stiffness around 242N/mm.³⁶ ACL non-linear stiffness properties are demonstrated when initial small loads are required to elongate the ligament (toe region). There is a continuous fiber recruitment which in turn increases the stiffness until fibers are fully loaded. Furthermore, a typical linear relationship between increasing loads and elongation is seen. When the yield load is reached, structural non-reversible damage occurs to the tissue becoming non-linear at maximum failure loads when complete structural integrity is altered affecting tendon and ligament cross-links. When the ligaments are kept at a constant load over a period of time, the ligaments lengthen until a steady state is obtained, called creep.^{3; 36} This is achieved by the interaction of cross-links, collagen fibers and cellular components to avoid excessive tensional stress on the ligament under repetitive or continuous exercise.³

The fibers of the ACL undergo non-isometric length changes during physiological knee motion.³ It provides varying degrees of restraint to anterior tibial translation. However, this would depend on the activation of knee-related musculature and other soft tissues. *In vivo* analysis has found that quadriceps activation resulted in ACL extension of around 4%.³⁷ Additionally, at 90° flexion, the ACL did not show changes in length demonstrating the dominating function of the ACL near extension.³⁸ Similarly, authors have shown that simultaneous activation of hamstring and quadriceps muscles, significantly decreased load on the ACL and reduced anterior tibial translation and internal rotation.^{3; 39-41}

In situ forces indicate that under a 110N anterior tibial load, the ACL experienced a 103N at 15° flexion and 59N at 90° flexion. The PL bundle carried the highest load at extension

(67N) while the AM bundle experienced higher forces near flexion (90N at 60° flexion)⁴². This situation may have important implications for reconstruction when the knee is either hyperextended or hyperflexed, leading to strains up to 8% in the reconstructed ACL graft^{40; 43}.

Anterior Cruciate Ligament Injury

Partial or complete tear or rupture of the ACL is commonly associated with traumatic sports events. ACL tears affect more than 2000,000 people in the United States each year.⁴⁴ Most of ACL injuries occur under a non-contact mechanism, with no direct contact to the knee when the ACL is injured. Usually 72% of ACL injuries occurred with non-contact mechanism during sudden deceleration or landing maneuvers.^{45; 46} Main features include rapid lower extremity deceleration resulting in forceful quadriceps contraction and change of direction or landing with a slightly hyperextended knee and typically internal rotation and flexion. It is estimated that the cost of treatment of ACL injuries is more than U\$1.7 billion annually.⁴⁷

In addition to biomechanical and structural aspects, cellular factors also play an important role in ACL injury and healing. ACL rupture initiates a cascade of cytokine and catabolic enzyme activity.^{48; 49} Current work has shown that among molecules present after ACL injury, cytokines such as IL-1 could stimulate the production of matrix metalloproteinases (MMPs) which in turn could degrade collagen and reduce proteoglycan content, affecting both, ligament and cartilage. In this process downregulation of proteoglycan synthesis and upregulation of MMPs is also common. From these, it seems that IL-1 α , IL-1 β , IL-6, IL-8, TNF α and MMP-13 are commonly found after ACL injury.⁴⁹⁻⁵²

Since the production of different molecules will greatly depend on the stage of the disease, individual variation of these biomarkers would produce a differential response to surgical reconstruction.^{49; 50}

ACL Risk factors:

Risk factors could be classified into external and internal. External factors comprise type of competition, shoe-surface interface, knee bracing and weather.⁴⁵ Internal factors include femoral intercondylar notch size, lower limb alignment, posterior tibial plateau slope, intrinsic ACL material properties, patella tendon-tibia shaft angle, ACL elevation angle, hormonal variation and neuromuscular control, many of these are also specific for females.^{45; 53} Young age is also a risk factor, with adolescents from 16 to 18 years being at greater risk.⁵⁴

ACL injury tends to be two to ten times more common in female athletes than males depending on the activity.^{47; 55; 56} Additionally, several anatomical and neuromuscular factors (mentioned earlier) are involved.⁵³ Increased female injuries could be explained by specific motion patterns and greater forces and torques exerted in athletic activities such as smaller flexion angles, greater hip internal rotation angles, lower gluteus maximus activation and greater quadriceps-to-hamstring ratio activation resulting in increased loading at the ACL. Studies have shown that increased anterior shear forces and knee internal rotation moment could significantly increase ACL strain.⁵⁷

Diagnosis:

Diagnosis of ACL tears relies on clinical history, physical exam and specific diagnostic tests. The history usually involves twisting, landing or valgus blow to the knee.⁵⁸ Also, swelling could be present after a few hours. Some patients could hear a pop sound but not

always. Pain is also variable being severe and persistent or, mild and transient.⁵⁸ Chronic ACL tears could involve history of instability that occur during pivoting activities.

Physical exam include different diagnostic tests. Hey Groves, identified the pivot shift phenomenon when described the antero-lateral subluxation of the tibia in the ACL deficient knee.⁵⁹ Consequently, this finding led to the discovery of the “pivot-shift test”, a useful diagnostic assessment tool to identify ACL deficiency.⁶⁰ The pivot shift test is a specific but insensitive test for ACL tears.⁵⁸ However, it is the gold standard to rule out complete ACL tear in the anesthetized patient.⁵⁸ The examination under anesthesia dramatically increases pivot shift sensitivity.^{61; 62}

The Lachman test is the most reliable exam for ACL tears.^{58; 63} However, it might be inconclusive in acute injury, especially when trying to differentiate between partial or complete tears. The Lachman test should be considered definitive only if its negative with a firm endpoint. The Lachman test can also lead to false positive results if there is medial collateral ligament insufficiency with consequent valgus laxity. In these cases, tibial rotation must be controlled by the examiner. Moreover, the KT-1000 is a highly accurate method for definitive ACL tear diagnosis. A 4 mm or higher side-to-side difference is commonly associated with definitive ACL tear. Partial tears usually have 2-3mm laxity. Furthermore, after ACL reconstruction, 4-5mm laxity is commonly seen.⁶⁴

Radiographic diagnosis is usually not conclusive unless there is tibial avulsion fracture in skeletally immature patients. Magnetic resonance imaging (MRI) is more commonly associated with increased sensitivity being around 80-95%.^{65; 66} Additionally, high-field 1.5 tesla produces more accurate results when compared to low-field MRI.⁶⁴

Treatment:

ACL repair

Primary ACL repair with suture was the first technique described to correct knee instability due to ACL injury. Don O'Donoghue popularized routinely ACL repair after acute injury in the 1950s and 1960s.⁶⁷ Nevertheless, long term results of ACL repair showed high rates of knee instability leading to the abandonment of this technique.⁶⁸

Poor healing ability of the ACL has been demonstrated.^{68; 69} Reasons for increased failure include intrinsic cellular differences.^{70; 71} *In vitro* studies have shown that ACL and the medial collateral ligament (MCL) have comparable rates of proliferation, similar revascularization patterns and comparable collagen production after rupture.⁷²⁻⁷⁴ However, the ACL lacks a proper clot formation in the wound site.⁷² This clot acts as a provisional scaffold rich in growth factors that are necessary for successful healing.⁷⁵⁻⁷⁷ Consequently, poor healing response in the ACL could be due to the exposure of the epiligamentous tissue to degradative enzymes present in the synovial fluid such as MMPs, leading to the removal of the provisional scaffold between the two ends of the injured ACL as well as a disorganized collagen configuration and increased type III-collagen.^{12; 78}

Steadman et al., (2006) developed a technique in which micro holes were created within the femur near the ruptured ACL to produce a blood clot and hematoma formation leading to an improved healing response.⁷⁹ The hematoma is a carrier of growth factors assisting in wound healing, fracture repair and in this case ligament repair.^{80; 81} This technique has been shown to be successful in patients with proximal ACL tears from a middle age cohort of patients.⁸²

Based on this, Martha Murray group started to investigate ACL repair healing potential after augmentation. First, they described different stages of healing after rupture (inflammation, epiligamentous regeneration, proliferation and remodeling).^{73;83}. Inflammation is initiated by hemarthrosis. During the inflammatory phase, Murray *et al.*, (2000) found that the ends of the ACL were swollen and edematous. There was a blood clot covering part of the ligament remnants without bridging femoral and tibial ends. Additionally, cell population consisted of fibroblasts, polymorphonuclear neutrophils, lymphocytes and macrophages seen actively phagocytizing cell and tissue debris. Also, there were dilated arterioles with proliferation of smooth muscle cells and congested capillaries and thrombus formation.⁷³

During the epiligamentous repair phase, between three and eight weeks after rupture, the number of inflammatory cells decrease, and fibroblasts became the predominant cell type. The blood vessels show normal morphology and neo-vascularization within the ligament fascicles. Thickening of the epiligament and fibroblastic proliferation in the synovial tissue occur. The ends of the ruptured ligament are covered due to the action of myofibroblast-like cells containing α -smooth muscle actin with contractile properties responsible for the retraction of the ligament.¹³

In the proliferation phase (weeks 8 to 20), remnant tissue is not visible and is usually adhered distally to the periligamentous tissue of the PCL. Increase in cell number and blood vessel density among collagen fascicles is observed. Fibroblasts showed a disorganized arrangement. Also, immature capillaries forming a diffuse network are present. Additionally, a continuous layer of synovial tissue is seen with abundant α -smooth muscle actin-containing cells.^{13; 73}

Lastly, in the remodeling and maturation phase, between one and two years after rupture, ligament ends are dense and there is no connection between remnants. There is decreased blood vessel density and a persistent synovial sheet is seen. Cell number density decreased to those seen in normal ACL. However, it seems that the ACL does not form a bridging scar after rupture probably due to proteolytic substances within the synovial fluid.⁸⁴

Consequently, treatment options based on regenerative medicine have arisen, including single growth factor studies^{85; 86}, platelet rich plasma (PRP)^{87; 88}, mesenchymal stem cells (MSC)^{89; 90} and bioscaffolds^{91; 92}. Furthermore, combination of these methods has led to the introduction of augmented repair being considerably more effective than each method alone^{93; 94}.

Currently, bioenhanced ACL repair using a collagen bioscaffold seeded with platelet concentrate has shown similar biomechanical results in a large animal model when compared to traditional ACL reconstruction techniques.⁹⁴ Moreover, these promising results have led to the implementation of this technique in a human clinical trial.⁹⁵ Nevertheless, although ACL repair has shown good functional outcomes after two years, long term follow up has failed to demonstrate good results, hence, emphasizing the necessity for augmentation.⁹⁵⁻⁹⁷

ACL reconstruction:

Due to the increased rate of failure with ACL repair techniques, patients needed a different solution, leading to the idea that the ACL should be reconstructed with some kind of graft material rather than a repair. Paul Wagner in 1913 suggested the use of fascia for reconstructing complete ACL tears. The first complete ACL reconstruction was performed by Ernest William Hey Groves who utilized the entire fascia lata detached from the tibial

insertion threaded through the femur and tibial tunnels and sown onto the periosteum while leaving the tendon attached to its origin.⁶⁷ Also, Alwyn Smith modified Hey Groves technique using a strip of the fascia lata detached from the proximal muscle belly. Consequently, Groves and Smith had set the bases for ACL reconstruction.

Arthroscopic ACL reconstruction using a biologic autograft has shown significant improvement in the stability and function of the knee. Factors that affect ACLR outcomes include individual choice of the graft, bone tunnel placement, graft pre-tensioning, graft fixation technique, and initial graft fixation strength.⁹⁸

The Graft Choice for ACL Reconstruction

Graft choices for ACLR typically consist of autografts or allografts. Common autografts include patellar tendon-bone graft (PT), semitendinosus/gracilis (Hamstring tendon) -HT, or the quadriceps tendon (QT).⁹⁸ Often, graft choice is associated with intrinsic patient characteristics, surgeon preference and patient demographics.⁹⁹⁻¹⁰¹ However, independent of the graft choices, no graft has shown ultimate failure load or stiffness comparable to the ACL⁹⁸. Graft harvest, artificial fixation and biological process of healing and ligamentization will further decrease both, ultimate strength and stiffness.^{98; 102-105} Consequently, PT autografts should be used for young active athletes associated with early return to high-level activity, while HT autografts are preferred when a large skin incision or anterior knee pain is to be avoided. QT grafts are primarily used for revision surgery due to their difficulty in harvesting and compromise of knee extensor mechanism.^{98; 106}

Bone patellar tendon bone (BPTB) graft:

It was not until 1963 when Kenneth Jones suggested a new surgical technique utilizing the central third of the patellar tendon leaving the distal end attached to the bone and also

removing the proximal part with a small block of bone and passing it through a femoral tunnel placed in the intercondylar notch.¹⁰⁷ However, 50% of his patients sustained a positive drawer test. Nevertheless, this technique gained popularity and patellar tendon grafting became known as Jones technique (reviewed in ⁶⁷). Bruckner suggested the use of a free central strip of bone-patellar tendon bone-graft (BPTB). This technique showed 90% of his patients regaining normal stability.¹⁰⁸ Kurt Frank helped popularize this technique when the first publication was available in a large patient cohort showing long-term results utilizing a free graft of the central third of the patellar tendon. This led to the adoption of the BPTB as one of the most popular graft sources.⁶⁷

It is thought that BPTB grafts have a superior healing and integration capacity due to the presence of bone plugs at the end of each graft. This allows the graft to have a good fit within the bone tunnel when using either interference screw or suspensory fixation.¹⁰⁹ Benner and Shelbourne., (2018) state that the presence of bone plugs at the end of the BPTB graft permits early biologic graft incorporation and healing at around 6 weeks after implantation.¹⁰⁹ They also state that BPTB allows for aggressive early rehabilitation, including immediate range of motion exercises (ROM), such as full hyperextension on the day of surgery.¹¹⁰ According to the authors this strategy allows early full weight bearing and graft failure is rare. However, BPTB grafts do have disadvantages. The harvest technique from the extensor mechanism leaves weak points that are vulnerable for traumatic injury such as patella fracture and patellar tendon injuries.¹¹¹ In addition, postoperative complications such as anterior knee pain, quadriceps weakness and difficulty with kneeling are also present.¹¹²⁻¹¹⁴ Although literature reports are sometimes conflicting^{113; 115}, some references favor BPTB graft for lower revision rates and mora

stable knee kinematics.¹¹⁶⁻¹¹⁹ Importantly, BPTB have been more frequently associated with postoperative OA.^{114; 120; 121}

Quadriceps tendon graft:

since harvesting of the patellar tendon autograft was not free from harm, surgeons started to experiment with different tendon autografts including the central section of the quadriceps tendon. Blauth and Fulkerson in the 80s tried to promote the use of the quad tendon for ACL reconstruction considering it superior.¹²² However, results were inferior compared to BPTB graft. Therefore, it never gained as much attention as the BPTB technique.

Nevertheless, in the 90s and 2000s, interest was revived after some authors reported comparable results to both, BPTB and hamstring autografts using a bone-QT graft.¹²³⁻¹²⁷ Interestingly, main finding included less surgical morbidity when using QT compared to BPTB graft. Recently, QT autograft has gained popularity being from 2.5% to 10.6% for ACL reconstruction.^{128; 129} Additionally, QT graft has an increased collagen content and higher fibroblast density when compared to BPTB.¹³⁰ Moreover, authors have also shown that the QT is significantly stronger than the PT.^{131; 132}

Hamstring tendon graft:

First reports of ACLR using hamstring tendon grafts included the use of semitendinosus or gracilis either severed at its muscular junction or proximally attached through an intercondylar notch bone tunnel respectively, regaining normal knee function after two years.¹³³ Furthermore, Marc Friedman pioneered the use of a four-strand hamstring autograft with arthroscopically assistance and set the standard for ACL reconstruction with

hamstrings.¹³⁴ The four-strand HT autograft has equal or superior stability rates compared to BPTB.¹³⁵⁻¹³⁸ However, reports require thorough analysis.^{139; 140}

HT graft has similar or superior strength compared to BPTB graft.^{141; 142} In fact, it is the strongest autograft among all.¹⁴³ Although HT graft has a higher incidence for deep infection after harvest when compared to BPTB grafts¹⁴⁴, donor site morbidity and anterior knee pain are lower when compared to BPTB and QT grafts. Moreover, anterior knee pain has been reported when using either PT or QT grafts, making HT grafts attractive for patients whose daily activity involve frequent kneeling, squatting, stair climbing or jumping.¹⁴⁵ Prodromos (2018), only reports HT weakness, that can be corrected with compensatory semimembranosus hypertrophy and semitendinosus regrowth and reattachment on the proximal tibia, usually one year after the surgical intervention.¹⁴⁵ However, some authors have shown than in few cases, HT does not fully regrow, and a knee flexion deficit may persist.¹⁴⁶

Intra-articular scarring and stiffness appears to be increased when using PT grafts compared to HT probably due to the intraarticular nature of the PT.¹⁴⁵ This has been corrected with accelerated rehabilitation programs and brings the question of how mechanical deprivation or overload affects the reconstructed ACL and healing process.¹⁴⁷

It seems that HT autografts are the preferred choice for reconstruction.^{101; 148} However, this is highly variable if surgeon location and nationality is considered.^{99; 101} Additionally, although information is conflicting, there are reports showing that HT grafts tend to fail more frequent when compared to BPTB grafts, especially in young athletes.¹⁴⁹⁻¹⁵¹

Allografts:

Since autografts possess an increased risk of complications due to the nature of the tissue harvest, new alternatives are required. Tendon allografts have been widely used for ACL reconstruction and involve 42.4% of primary ACL reconstructions and 78% of revisions.¹⁵²⁻¹⁵⁴ They avoid donor site morbidity and are readily available for most tissue banks. Additionally, they include a shorter operative time, improved cosmetic appearance, multiple sizes and grafts and decreased cost.¹⁵⁵⁻¹⁵⁷ Nevertheless, they carry their own risk including disease transmission, increased risk of contamination, decreased healing and integration, and it is believed that they are not as strong as autografts.¹⁵⁸⁻¹⁶⁰ However, with the advent of new technology, these weaknesses can be avoided.

It has been shown that allografts have decreased tendon-to-bone healing and decreased osteoinductive and osteoconductive properties mostly related to the sterilization process, such as the use of gamma radiation and chemical sterilization that will result in inflammatory reaction, delayed remodeling and decreased mechanical strength.¹⁶¹⁻¹⁶⁴ Fortunately, techniques for augmenting integration and the use specific low dose gamma irradiation (1.2Mrad) allow to obtain similar results between autografts and allografts in terms of biomechanical outcomes.¹⁶⁵⁻¹⁶⁹ Additionally, a decellularization method proposed by Dong & colleagues (2015) to increase compatibility and reduce antigenicity might lead to better outcomes.¹⁷⁰ Although the use of allografts has great potential more research is needed to be able to replace autograft techniques.

ACL Graft Positioning and Pre-tension:

The physiologic roll-glide mechanism of the femorotibial joint is the key to restore proper knee function and avoid anterior tibial translation and altered rotation patterns. Hence, graft positioning arises as one of the most important factors in ACLR.^{98; 171; 172} Similarly, authors

have shown that bone tunnel malposition is the most common technical mistake that could lead to graft failure due to a change in the biomechanical environment of the graft as a result of increased loads and strains.^{106; 148; 173; 174} Therefore, non-physiological strain patterns throughout the functional range will increase the likelihood of ACLR failure.⁹⁸

Authors have shown that when the femoral tunnel is placed anterior to the anatomic ACL footprint, results in excessive graft tension during knee flexion and correlates with poor functional outcomes.¹⁷⁵ Consequently, anatomic graft placement results in kinematics similar to the normal knee.¹⁷¹

Another factor that could influence success rates in ACLR is the tension applied to the graft. Low graft tension will lead to decreased joint stability and excessive initial graft tension will lead to a restraint in range of motion leaving the graft susceptible to failure. *In vitro* and *in vivo* animal studies have investigated this aspect and have shown that grafts tend to lose their initial tension when cyclically loaded.^{176; 177} However, precise pretension magnitude in clinical setting is lacking.

ACL Graft Fixation

It is known that the fixation site is the weakest link during the early postoperative stage.¹⁷⁸ Most fixation devices links the graft material with the bone tunnel affecting healing and mechanical properties of the reconstructed ACL. Generally, fixation devices are anchored far away from the joint, leading to higher cyclic loading magnitudes. Consequently, there is increased longitudinal and transverse graft motion within the bone tunnel leading to delayed tendon-to-bone integration.¹⁷⁹ Fauno *et al.*, (2005) showed that the position of the fixation sites and the fixation device significantly affects tunnel widening after ACLR.¹⁸⁰ Similarly, Fu *et al.*, (1999) showed that placing the fixation device close to the tunnel

entrance increase the stiffness.¹⁷⁹ Additionally, authors have shown that interference screw fixation led to superior ultimate strength when using a patellar tendon-bone graft compared to sutures and staples in the tibia. However, metal or biodegradable interference screws had inferior strength when compared to washerplates or sutures in the tibial attachment for quadrupled hamstring tendons.¹⁸¹ Interestingly, femoral fixation devices have not shown significant differential strengths among each other.^{98; 182; 183} Furthermore, not only fixation strength is necessary but good biologic tendon-to-bone integration properties is required for successful healing.

ACL Reconstruction Healing and Tendon Graft Ligamentization

Successful intra-articular graft remodeling and intra-tunnel graft incorporation depend on the complex interplay among different factors including individual choice of graft material, graft pretensioning, and tunnel placement.⁹⁸ Various animal models have been used to strengthen our understanding of the ACL ligamentization process. Traditionally, ACL graft ligamentization has been divided into three stages. The early healing phase starts from the time of surgical intervention up to six months in animal models and up to twelve months in human studies^{27; 184; 185}. This stage is characterized by an initial release of growth factors such as bFGF, TGF- β 1, PDGF and cytokines such as TNF α , IL1 β and IL6.²⁷

A second stage known as the proliferation stage can last from 4 weeks up to 12 weeks postoperatively. This stage is characterized by an increased cellular activity usually surpassing that of the intact ACL. There is also a particular increase in bFGF, TGF- β -1, PDGF between the third and the sixth week postoperatively and decreases by twelve weeks after surgery.¹⁸⁶ Revascularization of the new graft starts around the fourth week postop, beginning with the release of VEGF and lasts until the twelfth week postop, just at the end

of the proliferation phase. An increase in vascular density is found around six months postop and might correlate with the decrease in mechanical properties of the graft in which the collagen fibril density is at its lower levels.¹⁸⁴ This remodeling stage appears to last up to two years in human studies and appears to have increased collagen synthesis shown by increased expression of COL-III.²⁷

The third stage is known as the ligamentization/maturation phase and involves the transformation of an ACL tendon graft into a ligament-like tissue that acquires these characteristics only until six months postop. In this stage, concentration of type III collagen decreases over time, but it is still higher compared to the intact ACL.²⁷ Additionally, mechanical properties of the reconstructed graft seem to improve in the ligamentization phase and keeps increasing up to one year postop. Maturation of the graft can start as early as nine months and lasts up to 48 months or even more.¹⁸⁷ Although animal models have been used for deepening the understanding of ACL reconstruction, they possess several differences when compared to human studies. First, animal studies have shown extensive graft necrosis during the graft healing process, which do not seem to reach the same extent in humans (not to exceeding 30%) based on grafts biopsies.²⁷ An additional feature of animal models includes a different duration of each healing stage depending on the animal model being evaluated. The effect of the particular animal species on graft healing is not known. Scheffler *et al.*, (2008) describe the early healing and remodeling phases to last around three months in animal models.²⁷ The proliferation phase in animals seems to drastically differ with human studies. The ligamentization phase seems to be similar in both human and animal studies.^{184; 188; 189} Overall healing and integration in animal models

appear to last from 6 to 12 months, which is much shorter compared to humans which can last up to 3 years.¹⁸⁷

Despite extensive work in animals, it is still unknown when and whether a reconstructed ACL can ever reach the same biomechanical strength and stiffness of the native uninjured ACL. No animal study has shown that the reconstructed ACL reaches similar structural properties compared to the native ACL. The ‘best case scenario’ only reaches 50-60% of the intact ACL. It seems that one of the main features in the remodeling phase included a lower extent of neovascularization in human studies compared to animal models. Scheffler *et al* (2008) describes the differences in ligamentization in animal models as a process of adaptation instead of a full restoration of native ACL properties.²⁷ Moreover, opposite to what has been shown in humans, early healing and proliferation phase in animals exhibits a greater extent of degeneration, leading to decreased mechanical properties.

Perioperative Management and Physical therapy

The objective of rehabilitation of patients after ACLR is to decrease swelling, pain, and lack of range of motion, and to provide muscle strength and dynamic stability to the lower extremity without interfering with the healing process of the graft.¹⁹⁰ The ACL graft and the graft fixation site are significantly weaker the first few months after surgery and can be injured with less force. The graft undergoes ligamentization and is particularly weak during this stage.

Depending on the graft choice, rehabilitation protocols should be modified. Soft tissue grafts, such as hamstring tendons, are thought to be less strong than bone-tendon-bone grafts such as the patellar tendon because they require a different graft fixation. Rodeo *et al.*, (1993) suggested that soft tissue graft incorporation at the insertion site may take from

8 to 12 weeks.¹⁹¹ On the contrary, a bone-patellar tendon-bone autograft integration requires around six weeks. Consequently, tensile load to the hamstring graft should be minimized the first weeks after surgery compared to the patellar tendon graft. Additionally, harvest sites are particularly injured early after surgery, thus, avoiding high load to the quadriceps when using a QT or PT graft or, hamstring loads when using an HT graft is advised. Therefore, early healing of the graft should start with exercises that minimize the load applied to the ACL graft while maintaining a proper homeostatic stress the induces healing and integration. Exercises with the knee flexed (from 50° to 100°) are recommended using both weight bearing and non-weight bearing techniques.¹⁹⁰

Understanding how the ACL is loaded during rehabilitation can help clinicians to adjust training and exercise regimens in a safe manner. Studies indicate that ACL loading is greater in non-weight bearing exercises compared to weight-bearing exercises.¹⁹⁰ Regardless of the activity, the ACL is loaded to a greater extent when the knee is near to full extension (10°-50°flexion). Additionally, loads on the ACL change depending on the exercise technique. Lower ACL forces are found in exercises such as squatting and lunging with a forward trunk tilt, seated knee extension. On the contrary, other activities such as squatting with excessive forward movement of the knees beyond the toes and with the heels off the ground produce greater forces on the ACL.¹⁹⁰ Consequently, rehabilitation exercises during early healing following ACL reconstruction could in turn produce excessive loads to the graft and its fixation.³

In the past, rehabilitation programs after ACLR included knee immobilization for around six weeks in order to avoid motion while inflammation decreased, and tendon graft starts to heal.¹⁹² However, *in vitro* and animal studies have shown the adverse effects of

immobilization on cartilage, ligaments, tendons and knee associated structures.¹⁹³ Several authors have shown that immobilization would lead to decreased total weight of tendons, stiffness and tensile strength.^{194; 195} Also, complete removal of load alters the structure of the healing tendon and insertion and decreases mechanical properties of the tendon.¹⁹⁶ However, some studies have shown that the early application of load resulted in decreased load-to-failure values as well as greater macrophages and osteoclasts accumulation along the graft-tunnel interface associated with a higher inflammatory profile and a lower bone production.¹⁹⁷⁻¹⁹⁹ Similarly, Thomopoulos *et al.*, (2003) showed superior structural, compositional and biomechanical properties in immobilized shoulder joints compared to post-operative activity in a rat model.²⁰⁰

Consequently, the adoption of postoperative programs that include brace-controlled motion or continuous passive motion have been advocated.^{201; 202} Some other studies have shown that early mobilization with full weight bearing activities is possible without increasing risk of healing failure.^{147; 203} Unfortunately, how much force to the graft is too small to provide inadequate stimulus for enhanced healing, or too high to potentially injure the graft is still not known.

In vitro models of Ligament and Tendon Mechanobiology

Due to the functional importance of ligaments and tendons, mechanical evaluation is essential to understand their role in physiological and pathological conditions. Although several research has been conducted in this area, how tendons and ligaments maintain tissue integrity while subjected to cyclic loading is still unclear. Physiological loading range for tendons and ligaments usually fall under 13% of their ultimate strength. Authors have shown that physiological load to ligament and tendons is around 2-6% strain.²⁰⁴

Similarly, supraphysiological load is usually above 8% strain. Tendon and ligament cells attached to collagen fibers can sense changes in the mechanical environment. When load is applied, cells respond through the deformation of their cytoskeleton and alteration of the cell-matrix interaction.²⁰⁵ It is known that mechanical stimulation plays a key role in tendon and ligament development, maturation, and degradation.¹⁹⁵

Musculoskeletal tissue cell type is of mesodermal origin. The main cell type is the fibroblast that can be differentiated according to each specific tissue type. One of the main features of connective tissue cells is mechanosensitivity. The process by which cells elaborate a biologic response to mechanical stimuli is called mechanotransduction. And encompasses many features of growth and physiological adaptation.²⁰⁶ Adaptation to load refers to the ability of tissues and cells to maintain specific properties such as strength and stiffness, required for the function and integrity in response to load transfer, producing distribution of stress and strain. Depending on the nature of the forces involved and the specific anatomic conditions of the tissue, the response of the tissue to stress and strain could be either physiological or pathologic. For most tissues, physiologic conditions mechanical loads may be considered trophic for musculoskeletal tissues. Under specific mechanic conditions, the cellular response involves a change in ECM composition resulting in functional adaptations in strength and stiffness. Intracellular signal that depends upon mechanical activation is not fully understood.

Forces are usually transferred through the ECM and cellular response to such deformations is the base of tissue adaptation. In ligament and tendon tissue, cells are regularly distributed throughout the ECM and are interconnected through cytoplasmic extensions and gap junctions.^{207; 208} This particular organization is thought to be responsible for the ability of

cells to create an adaptive tissue response to mechanical or biological stimuli.²⁰⁹ However, normal healing after injury in tendon and ligaments is not fully restored due to fibrosis and failed connectivity among scar fibroblasts. Therefore, leading to inferior mechanical properties and lack of adaptability of the scar compared to native tissue.²¹⁰

To assess the effect of mechanical loading in ligament and tendon cells adequate *In vitro* models are required. Various models of ligament and tendon mechanobiology have been developed. These include *ex vivo* loading, monolayer-2D and 3D *in vitro* loading. Successful models require a physiological approach for simulating *in vivo* conditions. Experiments performed in cells from tissues subjected to mechanical stress have revealed that cell structure and tissue organization can respond to varying amounts of forces including tension, compression and shear stress. Common methods for membrane deformation include vacuum, suction, indentation or pulling.²¹¹ Flexcell® tension is the most popular system and include monolayer models, either uniaxial (Uniflex®), or biaxial (Bioflex®), as well as 3D culture systems (TissueTrain®).

Uniaxial tension transferred through collagen bundles is the predominant force in both, tendon and ligament. However, secondary transverse load and shear and compression forces are also present *in vivo*. Additionally, Wang *et al.*, (2018) suggested that an appropriate load range to investigate physiological mechanobiology on monolayer *in vitro* systems should be between 4% and 6%.²¹² Similarly, authors also suggest that 10% strain on tenocytes represents over 13% strain overall tendon tissue and might represent an adequate overuse injury model.²¹²

Ex vivo loading models are achieved by applying mechanical stress to isolated tendons or tendon fascicles within a culture environment using a bioreactor.²¹² Different *ex vivo*

loading models have been evaluated commonly ranging from 3-10% uniaxial tension and 0.25-1Hz.²¹³⁻²¹⁸ Additionally, it has been shown that physiological loading maintains structural integrity and have a therapeutic effect compared to mechanical deprivation and overloading.^{213; 214; 219} Loading within physiological range can induce enhanced collagen production in *ex-vivo* loading models.^{214; 220} Similarly, mechanical deprivation and overstretch are found to be catabolic for tendons mediated by degradative enzymes and structural damage respectively.²¹²

Two-dimensional *in vitro* loading models have been developed to stress cell cultures in monolayer. Advantages of the 2D model include homogenous strain between the culture substrate, and the cells and provides accurate and controllable loading.²¹² However, it does not allow for evaluation of cell-matrix interaction since the ECM is not present in 2D cultures.²¹² Cells can be stretched either uniaxial or biaxial. Uniaxial loading is achieved by applying single direction tension to the substrate.²¹² It is a more physiological model for replicating tendon stresses. Nevertheless, since cruciate ligaments are twisted around each other biaxial and shear forces are also present. Physiological stress in uniaxial models have shown an increased anabolic response through collagen I production, and a decreased degradative response through lower COX-2, PGE-2 and MMPs production. Similarly, uniaxial overloading tends to increase inflammatory and catabolic factors.²²¹⁻²²⁴

Biaxial loading provides multidirectional stretch to the substrate in both, longitudinal and transverse directions. Low magnitude loading using Bioflex system has shown an increased expression of inflammatory genes as well as increased ECM degradation.^{225; 226} Additionally, physiological loading has shown enhanced expression of ECM synthesis

without increasing degradation.^{227; 228} Similarly, inflammatory mediators and pain modulators in tenocytes have been produced in response to supraphysiological loads.²²⁹⁻²³¹ Typically, the 3D model consists of a construct seeded with tenocytes and stretched in a bioreactor. Therefore, strains transferred throughout the construct depend on the material that is being made of. 3D hydrogels are commonly used because strain can be transferred homogenously across the structure allowing a more controlled environment.²¹² Interesting, Patel *et al.*, (2017) showed that physiological strain increases degradative markers in a 3D model. Moreover, authors used a more complicated model to further replicate tendon structure including longitudinal stress as well as shear stresses.²³²

Lastly, although several differences are found among different *in vitro* loading models, challenges remain when trying to find a good model that replicates *in vivo* scenarios. Hence, physiological relevance is only one of the many factors that should be considered when choosing a loading model. Furthermore, ease of access and manipulation arise as complementary characteristics to consider. Nevertheless, we must be aware that no model can perfectly replicate *in vivo* conditions and more research is needed in order to translate *in vitro* findings to *in vivo* models.

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Figures

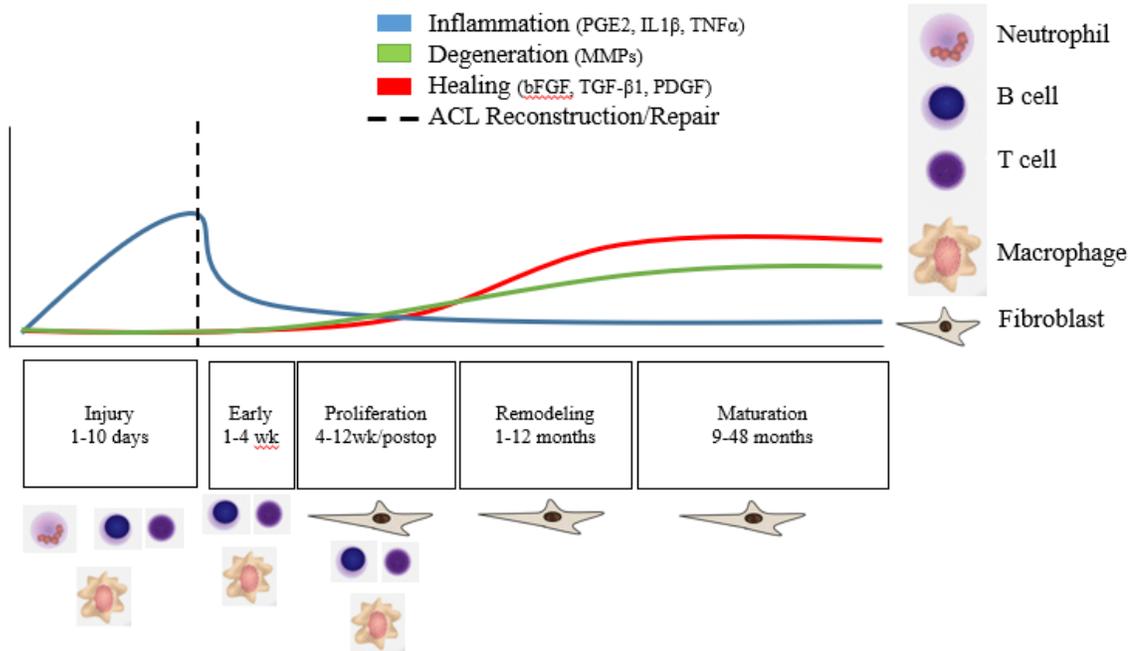


Figure 2.1. Main events in ACL healing and repair.

CHAPTER 3

EFFECT OF THREE DIFFERENT CYCLIC STRAIN PROTOCOLS ON NORMAL CANINE ACL TENDON GRAFTS CELLULAR METABOLIC RESPONSES

Introduction:

Anterior cruciate ligament (ACL) injury is the main cause of knee instability and a leading cause of post-traumatic osteoarthritis (OA).¹ Unfortunately, the ACL does not heal spontaneously after rupture, often requiring operative management in individuals who cannot function effectively with an ACL-deficient knee.² Although generally successful, failure rates after ACL reconstruction can be as high as 20%. According to Middleton *et al.*,(2014)³, 5.3 % of ACL reconstructions fail because of poor graft healing and incorporation.³

The current standard operative technique for ACL reconstruction involves the harvest and implantation of a tendon autograft or allograft for ligament reconstruction to stabilize the injured knee. Most common autograft choices include patellar tendon (PT), semitendinosus/gracilis tendon (HT), or quadriceps tendon (QT). Although the initial strength of the graft is comparable to the native ACL, graft harvest, fixation, and the subsequent ligamentization of the tendon graft significantly decrease ultimate strength and stiffness.⁴ In recent studies, tendon graft choice has been associated with different outcomes including variable risk of graft failure, early onset osteoarthritis (OA), and kneeling pain.⁵

Additionally, success with restoration of joint stability and return to sport are associated to specific biomechanical factors within the tissue. For instance, stress deprivation or

overuse have been associated with decreased structural properties of the grafts and increased inflammatory profiles respectively, while controlled loading has proven beneficial for postoperative healing and repair.⁶⁻⁹ ACL reconstruction with certain tissues, such as PT, are more related to development of lateral OA, increased production of inflammatory mediators and failure, possibly due to overloading of fibroblasts.⁶ While some authors have shown that PT grafts have lower revision rates, it has also been associated with increased donor site morbidity.^{10; 11} Similarly, although soft tissue grafts have yielded less stability, they have similar strength compared to PT with comparable functional scores and excellent graft ligamentization properties.¹²⁻¹⁵ Consequently, there is a lack of consensus regarding the optimal biomechanical environment for healing and function for each specific graft choice.

Successful healing of the tendon graft partly involves the understanding of the biological processes that occur during graft remodeling, maturation and incorporation. The early graft healing phase is characterized by the cellular release of molecules that regulate adequate balance between ECM degradation and synthesis. Main fibroblastic products involved in healing include cell signaling cytokines, inflammatory mediators, and degradative enzymes, as well as ECM structural components involved in maintaining the mechanical properties of the tissues.¹⁶ It is also important to recognize that these tendon graft cells are subjected to mechanical stress during rehabilitation after surgery, which can affect subsequent graft remodeling and tissue organization. Given that tendon graft fibroblasts respond to mechanical stress by altering the ECM of the tissue, it is important to understand tissue-specific differences in the biologic responses to mechanical stimulus in order to guide appropriate activity level after surgery until normal function is restored.

The goal of this work is to evaluate the metabolic responses of common tendon graft fibroblasts used for ACL reconstruction to clinically relevant mechanical stresses in order to characterize graft types by their healing potential and their ability to respond to various biomechanical environments. We hypothesize that tendon graft fibroblasts will respond in significantly different ways with respect to inflammation, ECM degradation and metabolism in response to strain levels that simulate different physiologic conditions. These data will inform clinical practice in terms of graft choice, surgical fixation technique, and postoperative physical therapy protocols towards reducing risk for failure and improving outcomes.

Materials and Methods:

Tissue processing and culture

With ACUC approval (ACUC#9163, 9164), tendon grafts (12 QT, 12 PT and 9 HT) were harvested from skeletally mature female dogs (n=16) euthanized for studies unrelated to the current work and free of any orthopedic disease based on orthopedic examination and radiographs. Tissues were aseptically minced into 0.5–1cm² pieces and digested overnight in Type 1A *Clostridium histolyticum* collagenase solution (Sigma-Aldrich, St. Louis, MO, USA) at a concentration of 0.5 mg/mL. The aliquot was then suspended with nutritional media (Gibco DMEM, Thermo Fisher Scientific, Waltham, MA, USA) containing 10% FBS, 0.002% Penicillin, 100 µg/mL Streptomycin, 25 µg/mL Amphotericin B, 0.002% L-Ascorbate and 0.01% L-glutamine (Sigma Chemical Co, St. Louis, MO), cultured in T75 flasks (TPP Techno Plastic Products AG, Trasadingen, Switzerland) and incubated at 5% CO₂, 37°C, and 95% humidity.

Biaxial mechanical stress

Once confluent, passage one cells were exposed to TrypLE express (Invitrogen, Carlsbad, CA) for five minutes, then resuspended and seeded (1×10^5 cells/well) in Collagen Type I-coated BioFlex® plates (Flexcell International, NC, USA). Cells were incubated for an additional 48h before strain was applied. Then, nutritional culture media was replaced with 0.5% FBS media for the remaining five days of culture. Fibroblasts were subjected to continuous mechanical stimulation (2-s strain and 10-s relaxation at a 0.5 Hz frequency) with a biaxial sinusoidal waveform with three different elongation strains (mechanical strain deprivation-0%, physiologic strain-4%, or supraphysiologic strain-10%)¹⁷ for 5 days using the Flexcell FX-4000T strain system (Flexcell International, NC, USA) (Fig. 3-1). Media was changed every 24h and stored at -20°C until analysis.

Biomarker assays

Media from 24 and 120 hours of culture were assessed for various biomarkers.

Proteoglycan (GAG) was assessed using the DMMB assay as previously described.¹⁸ The concentration of prostaglandin E₂ (PGE₂) (Cayman Chemical, Ann Arbor, MI, USA), cytokines (IL-6, IL-8, KC and MCP-1) (Millipore, Billerica, MA, USA), total matrix metalloproteinase activity (MMPACT) (Sensolyte 520 generic MMP assay, Anaspec, Inc., Fremont, CA), and MMP production (MMP-1, MMP-2 and MMP-3) (R&D Systems, Minneapolis, MN, USA), were all assessed using commercially available assays according to the manufacturer's protocol. At the end of the strain protocol (120h), cell viability analysis was performed using the resazurin assay (Sigma Aldrich, Saint Louis, MO).

Statistical Analysis

For statistical analysis, normal distribution was assessed by Shapiro-Wilk test. Data were not normally distributed so non-parametric analyses were used to determine significant differences between groups. Results were reported as median± interquartile range (IQR). Comparisons among strains and tissue types were performed using Kruskal-Wallis test and Dunn's test posthoc (R Core Team (2019), R version 3.6.2 Vienna, Austria), with significance set at $p < 0.05$.

Results:

Mechanical strain elicited significantly different biological responses from common ACL graft fibroblasts. The metabolic activity level decreased for HT fibroblasts as strain level increased. PT demonstrated no significant changes and QT demonstrated an increase in metabolic activity at supraphysiological strain relative to mechanical strain deprivation. Moreover, the metabolic activity of the QT was significantly lower than the other graft fibroblasts with strain deprivation (Fig. 3.2). Additionally, HT and PT metabolic activity was significantly higher than QT fibroblasts when stress deprived but there was not a significant difference between cell types at physiologic and supraphysiologic strain levels (Fig. 3.13).

Inflammation related responses

There was a significantly decreased production of PGE₂ when all graft fibroblasts were subjected to no strain (Fig. 3.3). Supraphysiological strain significantly overregulated the production of PGE₂ across all grafts. Physiological strain, stimulated a balanced production of PGE₂, being significantly different from abnormal loads, no strain and 10% strain. QT fibroblasts had a significantly lower production of PGE₂ at physiological

stress compared to HT at both time points (Fig. 3.14). Interestingly, HT significantly decreased the production of PGE2 only when subjected to supraphysiological strain. The production of IL-6 was consistently low in all graft fibroblasts regardless of the strain (Fig. 3.4). However, PT fibroblasts produced significantly more IL-6 at physiological strain relative to abnormal loads. Moreover, HT fibroblasts in the stress deprivation group produced significantly more IL-6 compared to HT fibroblasts in the physiological and supraphysiological strain groups after 120h of culture. IL-6 production was also significantly higher in HT when compared to PT fibroblasts at no strain at 120h of culture (Fig. 3.15).

The production of IL-8 by QT fibroblasts was significantly lower in the stress deprivation group compared to the supraphysiological strain group at 24h but was significantly higher stress deprivation group compared to the supraphysiological strain group after 120h of culture (Fig. 3.5). Similarly, PT consistently showed significantly lower levels of both KC at 24h and MCP-1 at 24h and 120h of culture in the strain deprivation group compared to the supraphysiological stress (Fig. 3.6 and 3.7). Whereas QT and HT fibroblasts did not show significant differences in KC production at any strain at any timepoint. Additionally, QT fibroblasts subjected to physiological strain exhibited a significantly increased production of MCP-1 compared to no strain at 24h of culture. Moreover, HT fibroblasts showed significantly increased production of MCP-1 at supraphysiological strain relative to both, no strain and 4% strain at 24h of culture. When comparing differences in cytokine production between tissue types, the production of IL-8 was significantly higher in the QT and PT fibroblasts compared to the HT fibroblasts in the physiological strain group at 24h, but not at 120h of culture (Fig. 3.16).

Moreover, there was a consistent decrease in IL-8 and KC from HT compared to QT fibroblasts after 120h of culture in both abnormal load groups, no strain and 10% strain (Fig. 3.16 – 17). Lastly, production of MCP-1 in the supraphysiological strain group was significantly higher in the HT fibroblasts compared to the QT and PT fibroblasts at 24h of culture (Fig. 3.18).

GAG and MMP activity

Although statistical differences in GAG production were not significant, there was a trend towards higher production of GAG at the 24h time point in the physiological and supraphysiological strain groups, which tended to decrease over time in the QT and HT fibroblast groups (Fig. 3.8). The production of GAG by the PT fibroblast was significantly higher than the QT and HT fibroblasts in the supraphysiological strain group at 120h of culture (Fig. 3.19).

Overall, general MMP activity was consistently increased in QT fibroblasts at physiological strain compared to both no strain and 10% strain at 24h and 120h of culture. Similarly, PT fibroblasts exhibited increased MMPACT at physiological strain relative to supraphysiological strain at 24h and 120h. In addition, HT fibroblasts showed significantly higher MMPACT at 4% strain at 24h but not at 120h of culture (Fig. 3.9). Interestingly, MMP activity levels produced by HT fibroblasts at physiological strain was significantly higher than the QT fibroblasts at 24h but significantly lower in the HT fibroblasts compared to the QT fibroblasts at 120h (Fig. 3.20).

Specific MMP Production

The production of MMP-1 by QT fibroblasts in the strain deprivation group was significantly higher than the physiological strain group at 120h of culture (Fig. 3.10).

Moreover, production of MMP-2 by QT fibroblasts in the strain deprivation group was significantly higher than the supraphysiological strain group at 120h of culture (Fig. 3.11). The production of MMP-3 was low by all fibroblasts at all strain levels, but production was significantly lower in HT fibroblasts compared to QT and PT fibroblasts in the strain deprived group at 24h and 120h of culture, respectively (Fig. 3.12). The production of MMP-1 and MMP-3 by PT fibroblasts were significantly lower than QT fibroblasts in the physiological strain group at 24h of culture (Fig. 3.21-23). Moreover, the production of MMP-2 by HT fibroblasts was significantly higher than the QT fibroblasts in the supraphysiological strain group at 120h of culture (Fig. 3.22).

Discussion

Tendon grafts can respond to different cyclic strains due to the mechanosensitive nature of their cellular component. The results of this work support the hypothesis that inflammation, degradative enzyme metabolism, and ECM metabolism are different in QT, PT and HT fibroblasts in response to different clinically relevant tension loads. Additionally, degradative enzyme metabolism tended to be higher at supraphysiological strain, while inflammation tended to be higher when stress deprived. However, the dynamics of the cellular responses to strain were variable and dependent on tissue type. The results of the present study suggest that fibroblasts from different autograft tendons exhibit different early biological responses to mechanical stress. These biological differences may explain certain clinical phenomenon associated with specific graft types. Metabolic activity decreased and MMP activity increased in HT fibroblasts in response to increasing strain levels relative to other tendon graft fibroblasts, which may result in an overall degradative environment and initial weakening of the graft. This may provide

some biological rationale for data that suggest HT grafts are associated with an increased risk for graft laxity relative to PT grafts.^{19; 20}

Inflammation is an adaptive response that is triggered by harmful stimuli such as tissue injury. It has the purpose of enabling effective repair responses and to re-establish tissue and organ homeostasis. However, if this balance is not reached within normal timeframes, a chronic malfunction would lead to tissue degeneration.²¹

The inflammatory profile of the tendon fibroblasts was evaluated by measuring PGE2, cytokines and chemokines. Mechanical strain deprivation did not induce production of PGE2 in any of the graft tenocytes. However, tendon fibroblasts increased the production of PGE2 as strain was applied to the cells in a magnitude dependent manner. Further, graft fibroblasts subjected to supraphysiological strain levels had the highest level of PGE2 production. This finding is in agreement with a previous study by Wang *et al.*, (2003), which found that human PT fibroblasts increased production of PGE2 when stretched at either 8% or 12% compared to non-stretched controls.²²

These findings suggest that abnormal mechanical loads, either stress deprivation or supraphysiological strain, might be harmful for effective healing due to a lack of inflammatory response with stress deprivation or an excessive inflammatory response with excessive strain that promotes further joint degradation and pain sensitization.^{23-25 26}

Sauerschnig *et al.*, (2018) found that selective COX-2 inhibitors caused impaired tendon-to-bone healing and weakened the mechanical stability of the graft in a rabbit model of ACL reconstruction using a semitendinosus tendon autograft.²⁷ In addition, Cilli *et al.*, (2004) determined that the presence of PGE2 equal to or greater than 10 ng/mL decreased collagen production and cell proliferation compared with the control group in human PT

fibroblasts.²⁸ Furthermore, physiological loads seem to maintain a balanced level of PGE2 required for physiological purposes, especially in QT and PT graft fibroblasts.²⁹ Cytokines are proteins secreted by many cell types in response to different antigenic responses and stimulate diverse responses of cells involved in immunity and inflammation.³⁰ IL-6 is a cytokine produced by fibroblasts and numerous other cell types in response to injury or stimulation with other cytokines such as IL-1 β and TNF- α .^{30, 31; 32} IL-8 is a cytokine that induces neutrophil chemotaxis and promotes angiogenesis.^{33, 34} Interestingly, IL-6 and IL-8 have been identified in the synovial fluid of dogs with natural and experimental cranial cruciate ligament injury.^{32; 34; 35} Similar studies in humans detected increased levels of both IL-6 and IL-8 after ACL injury and one month after ACL reconstruction but lower levels just before the surgery.³⁶ Similarly, Larsson *et al* (2017) found higher concentration of IL-6 and IL-8 at 4 and 8 months after ACL reconstruction immediately after ACL injury compared to delayed reconstruction possibly being the result of a second trauma induced by the reconstruction procedure itself.³⁷

Our results did not show IL-6 being produced by graft tenocytes at a large scale, probably due to the lack of stimulation by other cell types including macrophages and lymphocytes.³⁸ Additionally, since IL-6 has a short life span, it would not be possible to detect it in large amounts in tenocyte cultures even at 24 hours; therefore, earlier evaluation timepoints are required.^{39; 40} On the other hand, it seems that graft tenocytes can produce IL-8 in big amounts *In vitro* and such concentration is affected by cell type and magnitude of mechanical stimulus. Interestingly, it seems that PT and HT had a similar initial response to increasing tensile loads by overregulating IL8 production.

However, QT fibroblasts exhibited a more erratic response and completely shifted after 120h.

KC (Keratinocyte Chemoattractant) also known as GRO α or CXCL1 is a chemokine expressed by macrophages, neutrophils and epithelial cells characterized by neutrophil chemoattractant activity, mitogenic and angiogenic properties, and inflammatory attributes.³⁰ KC and IL-8 are members of the CXC cytokine family. They have been found in the synovial fluid of dogs with naturally occurring cranial cruciate ligament injury.⁴¹ Since in our study, lower levels of these cytokines were found in QT at physiological strain, it is possible that this graft fibroblastic type is involved in less inflammatory and degradative activation compared to PT and HT.⁴²

Similarly, Monocyte Chemoattractant Protein (MCP-1), also known as CCL2, is a chemokine responsible for the chemotaxis of mononuclear cells that increases the expression of MMP-3 and also inhibits the synthesis of proteoglycans inducing their release from chondrocytes *in vitro*.⁴¹ Interestingly, some authors have shown that MCP1 stability is affected by GAG content suggesting an interaction with the ECM.^{43; 44}

Our results indicate that chemokines are differentially produced in different graft fibroblasts and this production depend on the magnitude of the strain applied and the duration of the mechanical stimulus. It seems that PT fibroblasts are more reactive to changes in tension load shown by the significantly increased production of IL-8, KC and MCP1 relative to mechanical deprivation. Interestingly, KC production did not change significantly among tendon fibroblasts when physiologically stretched.

On the other hand, it seems that MCP1 production was significantly affected by supraphysiological strain in PT fibroblasts and was maintained after 120h. However,

tension load did not affect QT and HT fibroblasts at 120h. This might suggest that PT fibroblasts possess a higher chemotactic ability and that inflammatory regulation in tendons depend on a more complex pathway activation process.⁴⁵⁻⁴⁷

GAGs are negatively charged molecules responsible for the structural integrity of the ECM and influence the viscoelastic properties of the tissues.⁴⁸ Although GAG content is not as high in tendon as in other connective tissues, tendon GAGs are still critical to function and are important indicators of tendon injury.⁴⁹⁻⁵¹ They are produced by fibroblasts in response to external factors such as mechanical and biological stimuli in an attempt to maintain ECM homeostasis during health, after injury, or in disease states.^{51; 52} In fact, they are being constantly degraded by common MMPs also found in the synovial fluid of the healthy knee and after ACL rupture and reconstruction.⁵³⁻⁵⁶

Early immobilization after ACL reconstruction has been associated with increased fibrosis and reduced structural and mechanical properties.^{57; 58} This is consistent with our results that showed increased GAG content when QT and HT graft fibroblasts were stress deprived. Interestingly, GAG production was significantly increased when PT fibroblasts were supraphysiologically stretched compared to both, QT and HT. This reaction could occur as an attempt to withstand high loads by creating more ECM components.

However, since GAGs are not responsible for tensile strength, it is expected that this response is detrimental for appropriate healing of the graft. Additionally, when the femoral tunnel is placed anteriorly to the ACL footprint, the tension in the graft increases above the physiological level, resulting in higher tibio-femoral joint contact forces and altering the biomechanical environment of the joint.^{59; 60} Our findings suggest that

abnormal stresses produce either, insufficient or excessive GAG content that might hamper an adequate healing response.

The synovial fluid within the knee joint has a high activity of MMPs under normal conditions.^{61; 62} However, such activity depends on successful proteolytic activation.⁶³ In addition, active MMPs are being regulated by other molecules such as TIMPS.⁶⁴ This might be the reason why MMP activity was mostly detected only at physiological strain in QT and HT fibroblasts at 24h, and in QT and PT at 120h. Moreover, it seems that 4% strain successfully maintains adequate degradative levels for homeostatic remodeling and avoids deposition of scar tissue, fibrosis and adherences.^{56; 63; 65-67} It is also possible that abnormal loads upregulate MMP inhibition with TIMPS leading to a low MMPACT.

When analyzing specific MMPs production we found an initial increase in MMP1 that mitigated with time. On the contrary, MMP-2 increased after 120h, particularly when QT fibroblasts were stress deprived relative to supraphysiological stress. Interestingly, although concentration of MMP-3 concentration was consistently low, it was significantly higher when QT and PT were stress deprived at 24 and 120 hours, respectively. This might suggest that MMP-1 and 2 have specific roles in tendon fibroblastic responses to stress. Wang *et al.*, (2017) reported a significant increase in MMP-2 gene and protein expression four and eight weeks after ACL reconstruction with a semitendinosus autograft in a rabbit model.⁶⁸ Since MMP-2 can degrade denatured collagen as well as other types of collagen including I and III, it is believed that MMP-2 plays a key role after ACL reconstruction.^{56; 69} Similarly, it has been shown that MMPs 1,2 and 13 are overexpressed in healthy human hamstring tenocytes subjected to high

frequency loading (10 Hz).⁷⁰ Nevertheless, levels of MMPs after ACL reconstruction remains to be unknown.

There are several limitations to this work. First, the *in vitro* model utilized here is simple and does not account for interaction with other cell types. Additionally, cellular responses in monolayer cultures can greatly vary when compared with 3D cultures or whole explant models.^{17; 71} Moreover, equibiaxial load is rarely seen *in vivo* and other mechanical forces such as shear stress and torsion are not easily replicated *in vitro*. Finally, future research might include a more complete analysis on regulatory pathways including collagen metabolism, MMPs regulation through TIMPs and specific GAGs production including proteoglycans and sulfated and non-sulfated GAGs

Results shown in this work provide a framework for future investigation regarding *in vitro* analysis for ACL reconstruction. The exact roles for the proteins assessed in this study, presence of other molecules, and sequence and control of events in ACL graft healing are areas of ongoing work in our laboratory aimed at further characterizing the ACL ligamentization process.

There are a variety of clinical scenarios in which biomechanical environment of the tendon graft can be altered, including graft size, position in bone tunnels/sockets, graft fixation and physical rehabilitation protocol after surgery.^{3; 72-75} Similarly, mechanical deprivation has also led to undesirable results including significant muscle atrophy, arthrofibrosis and decreased mechanical properties of the graft.^{76; 77} Since the production of inflammatory mediators such as cytokines and PGE2 is key for mounting a successful healing response, the transformation of an ACL tendon graft into a ligament-like tissue will greatly depend on early postsurgical events.¹⁶ ACL graft ligamentization starts from

the time of surgical intervention up to six months in animal models and, up to twelve months in human studies.^{16; 78; 79} While the remodeling stage does not initiate until the first month postoperatively, we showed that ECM composition changes as early as five days after tendon fibroblasts are subjected to loading and those responses are greatly influenced by the magnitude of the tensile load applied.

The early events in graft healing are likely modulated by cellular release of molecules that regulate the balance between the early inflammatory cascade and the subsequent ECM degradation and synthesis processes. Hence, we can conclude that the mechanical environment greatly affects the early healing response

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Figures

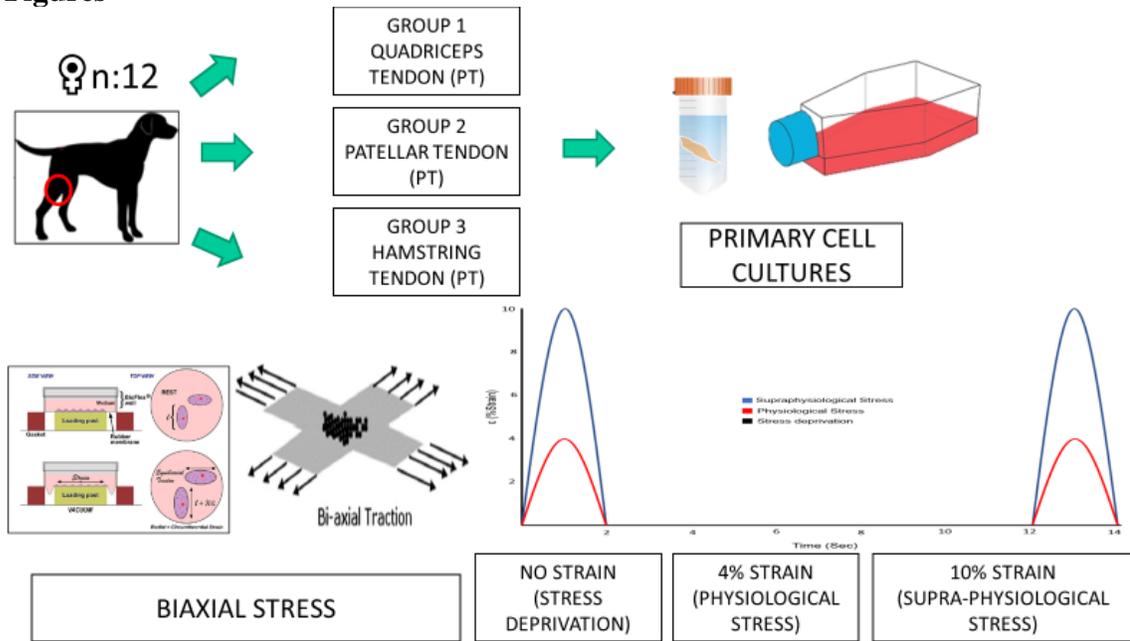


Figure 3.1. Experimental design.

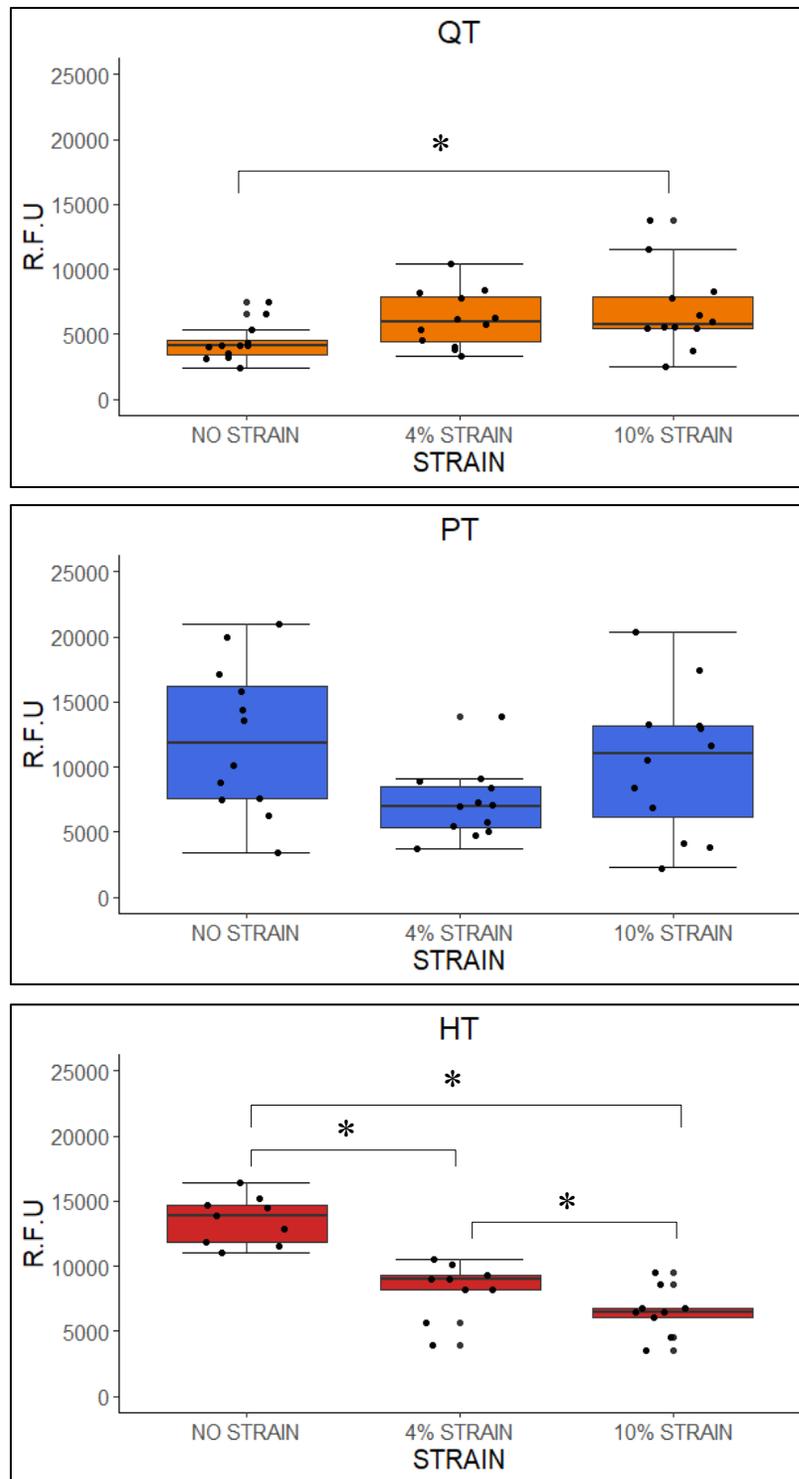


Figure 3.2. Metabolic activity after five days of culture. (*) Significant difference between strains. RFU: Resazurin Fluorescent Units QT: Quadriceps tendon; PT: Patellar tendon, HT: Hamstring tendon

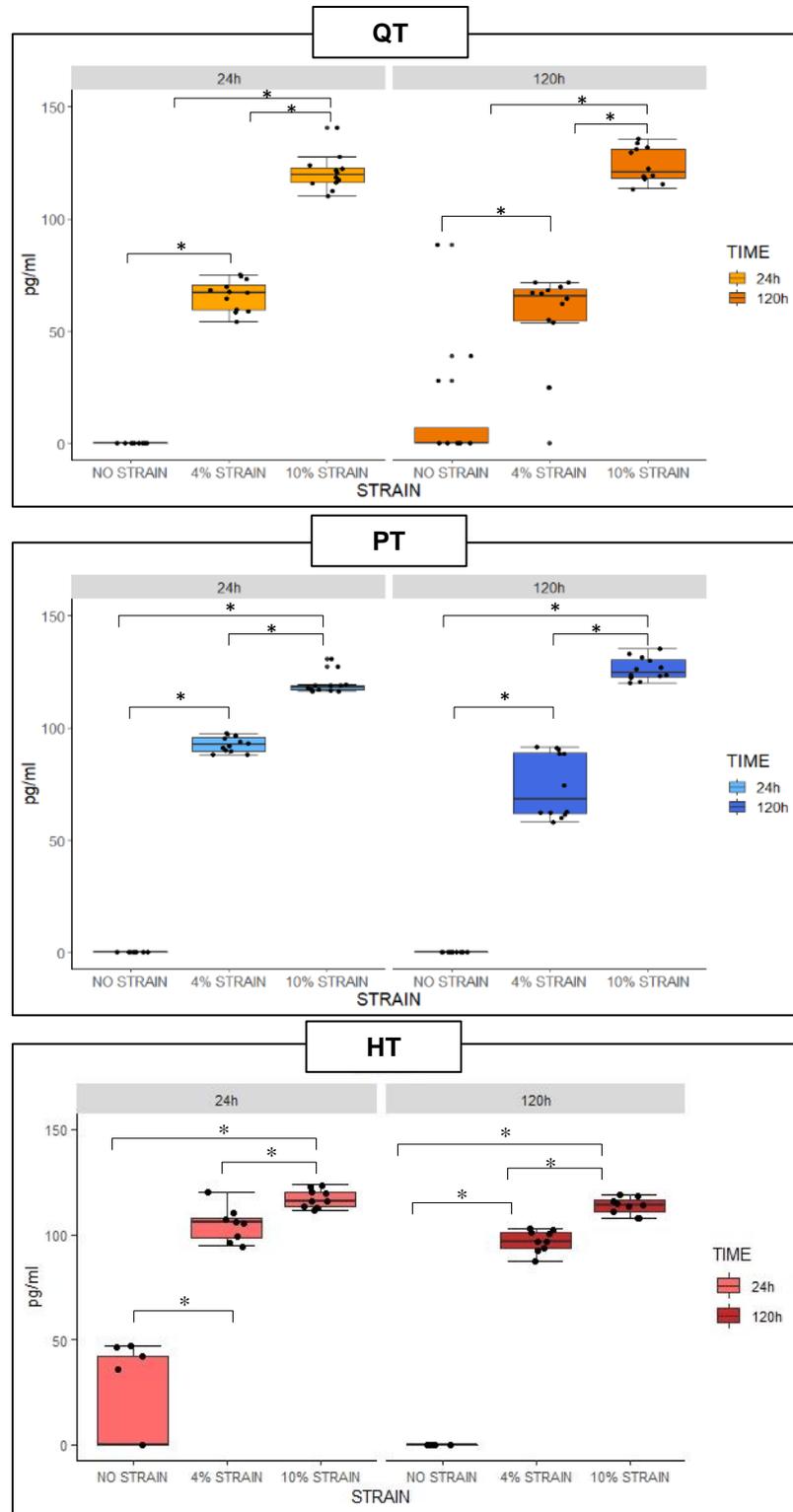


Figure 3.3. Concentration of PGE-2 released to the media. * significant difference between strains at 24 and 120 hours. QT: Quadriceps tendon; PT: Patellar tendon, HT: Hamstring tendon

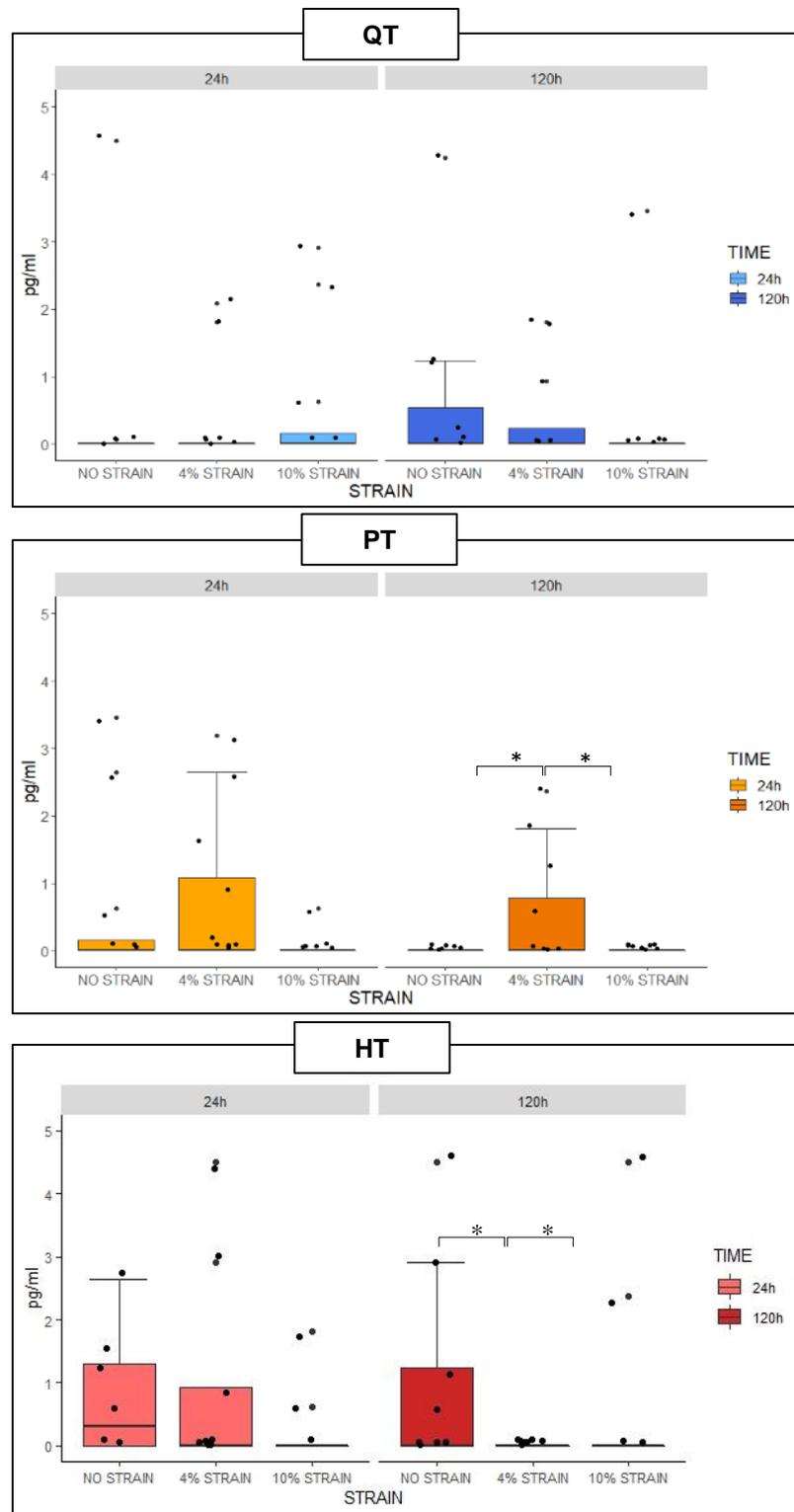


Figure 3.4. Concentration of IL-6 released to the media. * significant difference between strains at 24 and 120 hours. QT: Quadriceps tendon; PT: Patellar tendon, HT: Hamstring tendon

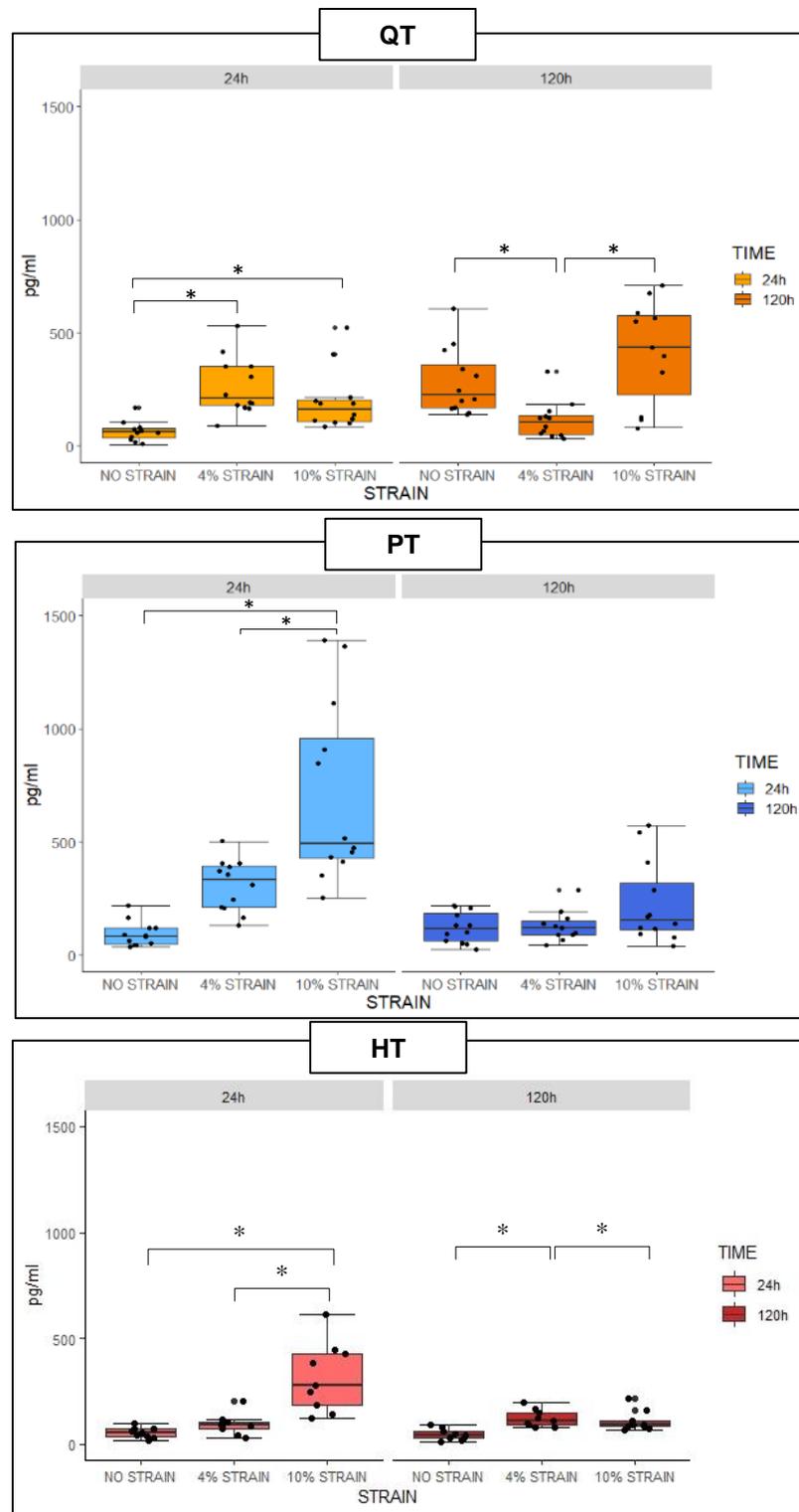


Figure 3.5. Concentration of IL-8 released to the media. * significant difference between strains at 24 and 120 hours. QT: Quadriceps tendon; PT: Patellar tendon, HT: Hamstring tendon

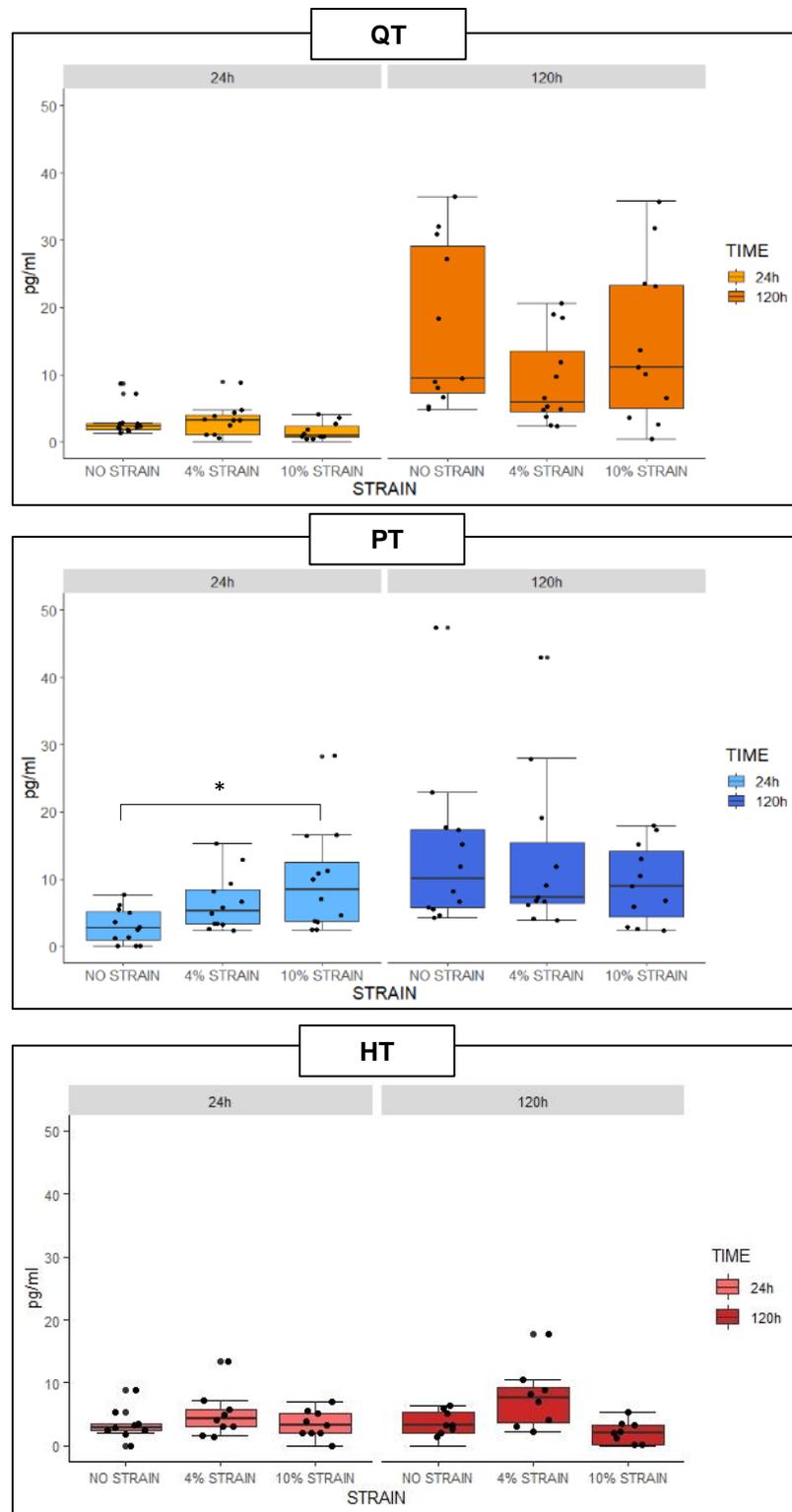


Figure 3.6. Concentration of KC released to the media. * significant difference between strains at 24 and 120 hours. QT: Quadriceps tendon; PT: Patellar tendon, HT: Hamstring tendon

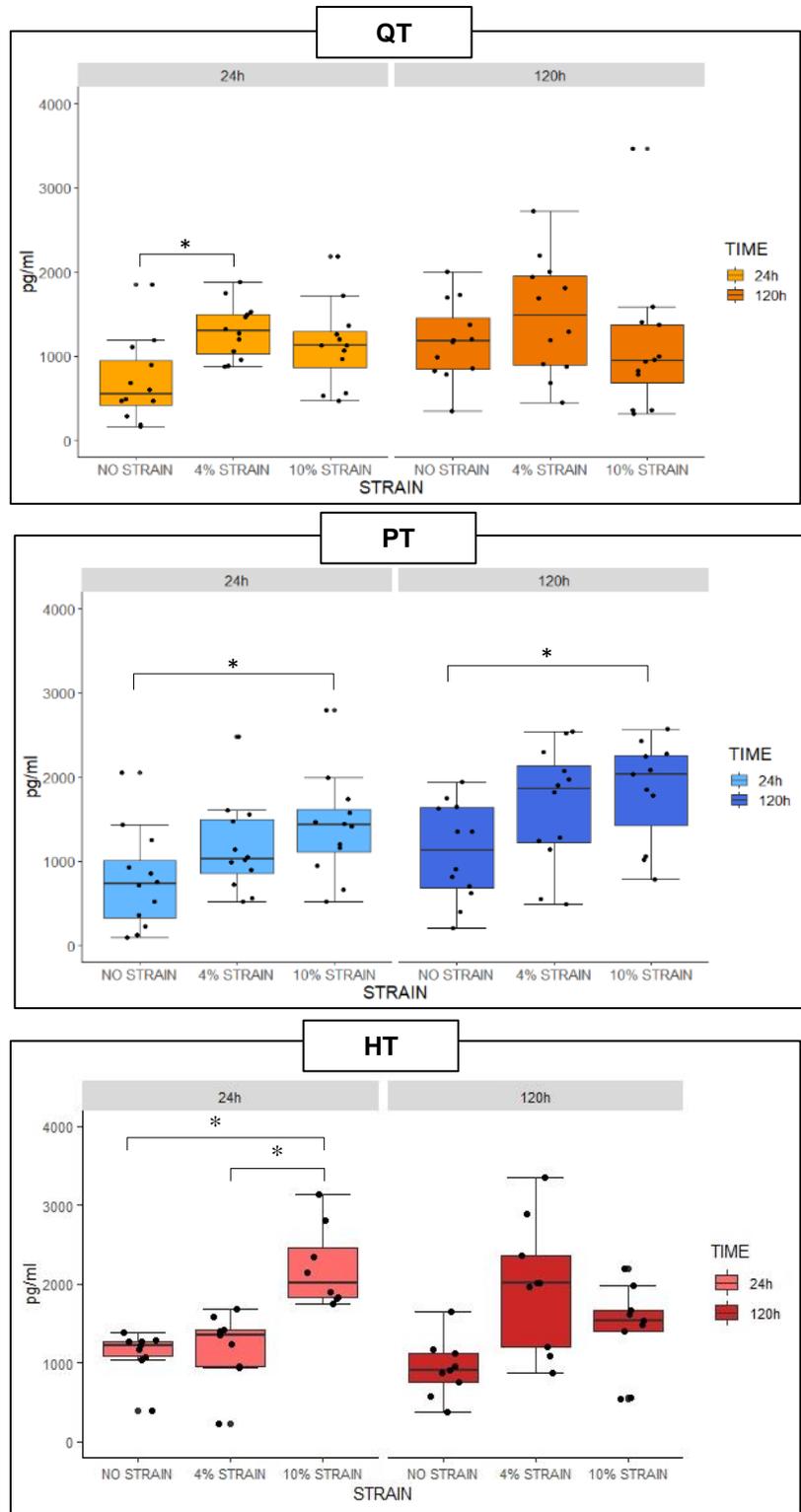


Figure 3.7. Concentration of MCP-1 released to the media. * significant difference between strains at 24 and 120 hours. QT: Quadriceps tendon; PT: Patellar tendon, HT: Hamstring tendon

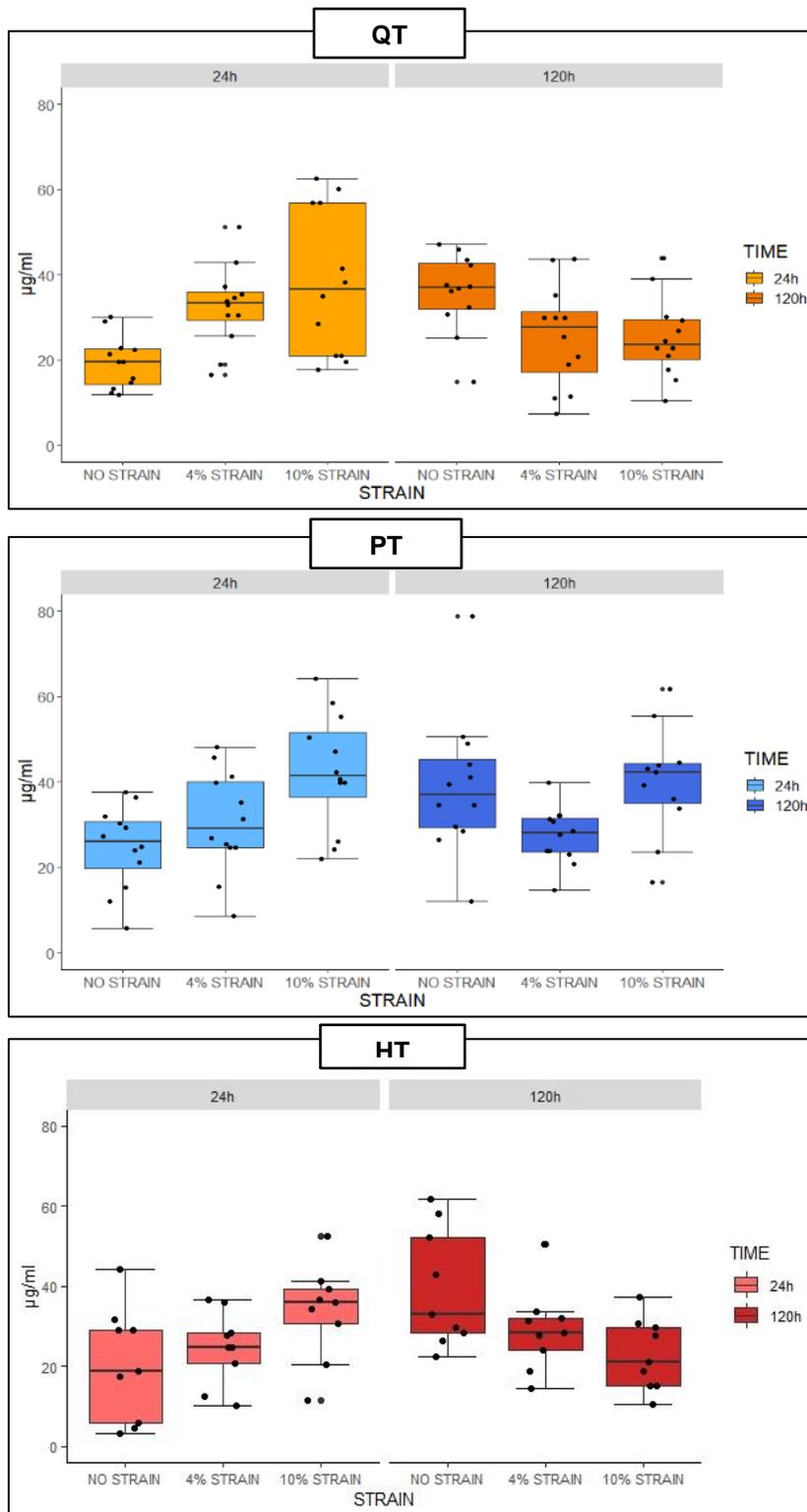


Figure 3.8. Concentration of Glycosaminoglycans (GAG) released to the media. * significant difference between strains at 24 and 120 hours. QT: Quadriceps tendon; PT: Patellar tendon, HT: Hamstring tendon

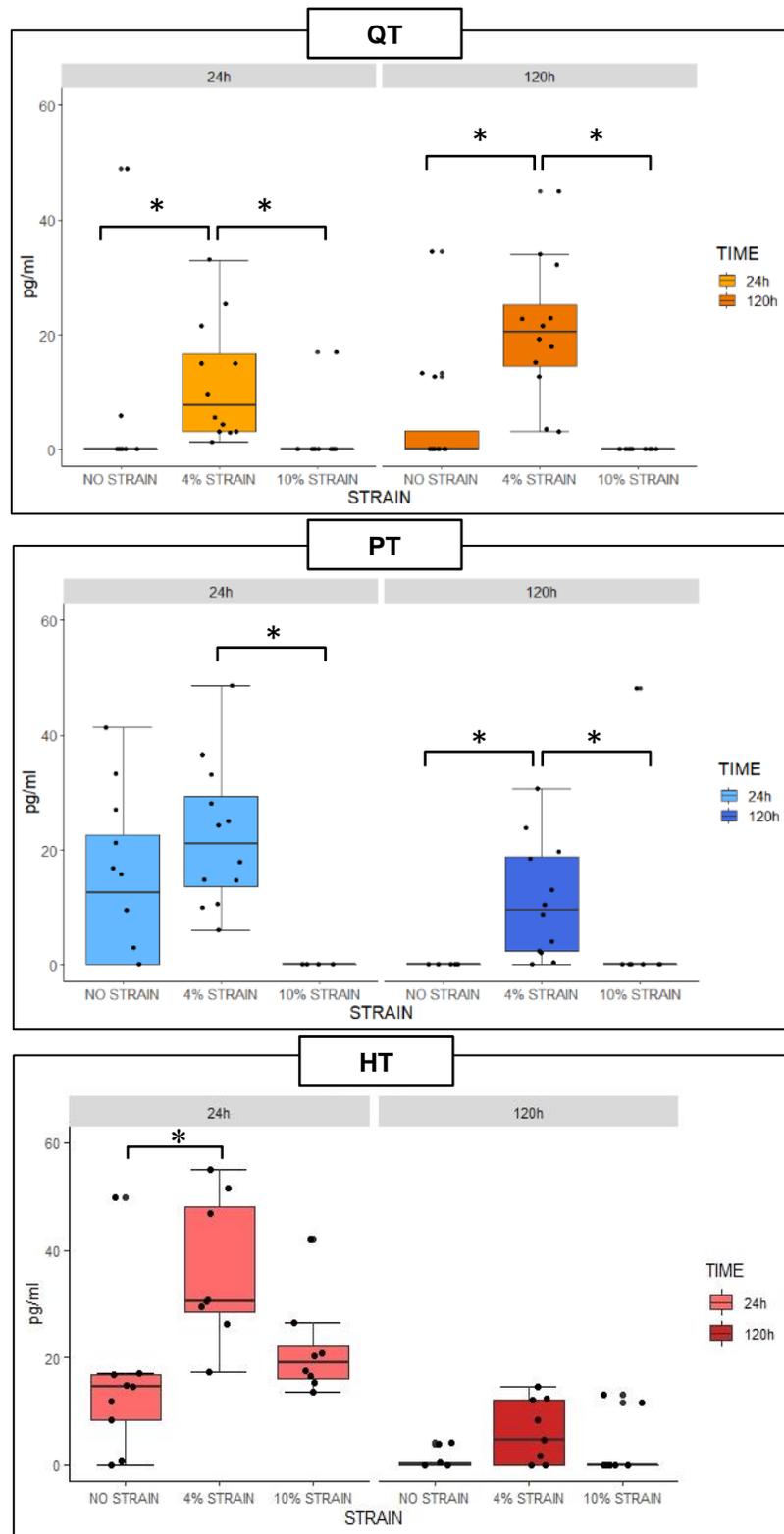


Figure 3.9. Concentration of MMP ACTIVITY (MMPACT) released to the media. * significant difference between strains at 24 and 120 hours. QT: Quadriceps tendon; PT: Patellar tendon, HT: Hamstring tendon

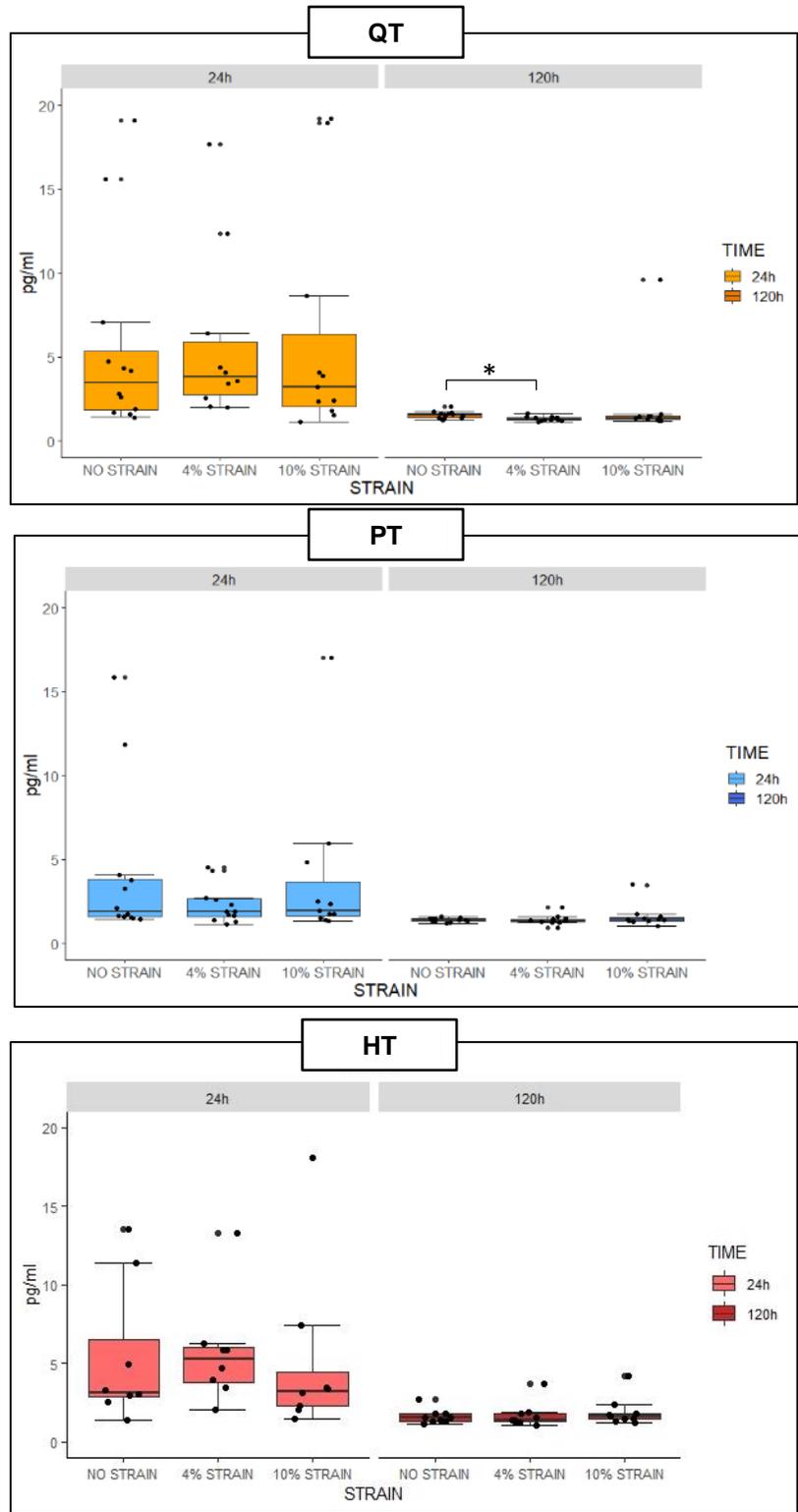


Figure 3.10. Concentration of MMP-1 released to the media. * significant difference between strains at 24 and 120 hours. QT: Quadriceps tendon; PT: Patellar tendon, HT: Hamstring tendon

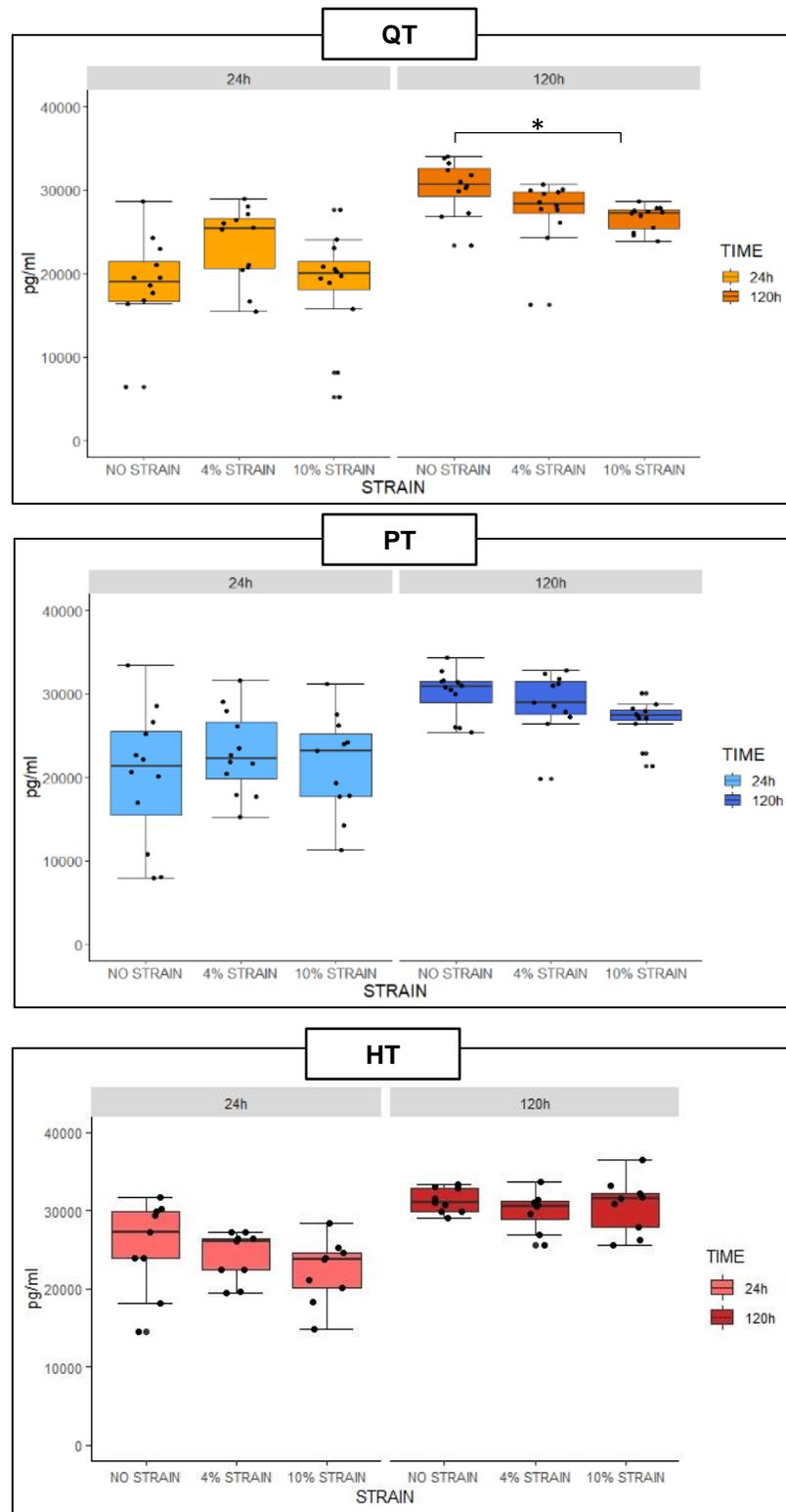


Figure 3.11. Concentration of MMP-2 released to the media. * significant difference between strains at 24 and 120 hours. QT: Quadriceps tendon; PT: Patellar tendon, HT: Hamstring tendon

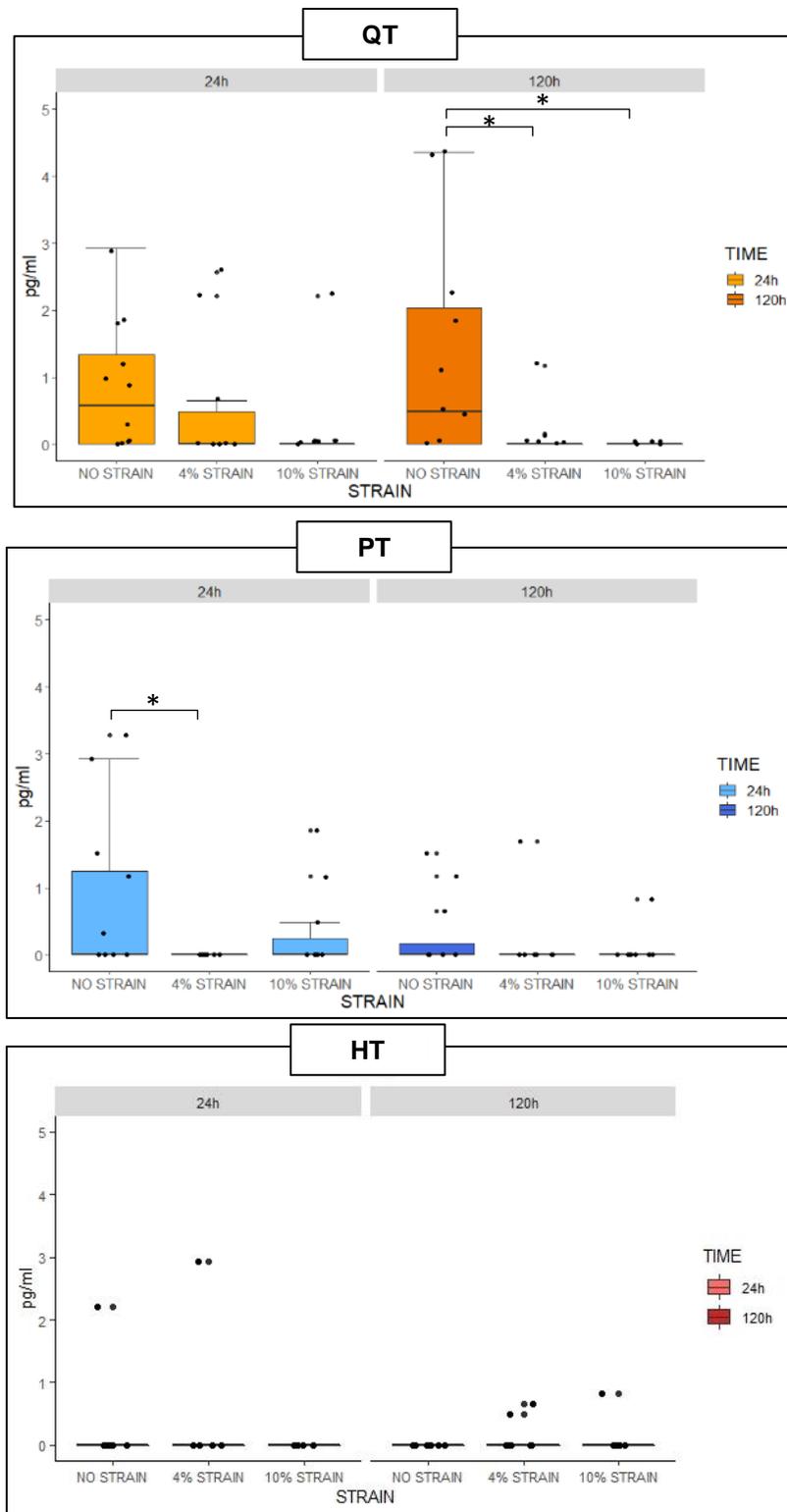


Figure 3.12. Concentration of MMP-3 released to the media. * significant difference between strains at 24 and 120 hours. QT: Quadriceps tendon; PT: Patellar tendon, HT: Hamstring tendon

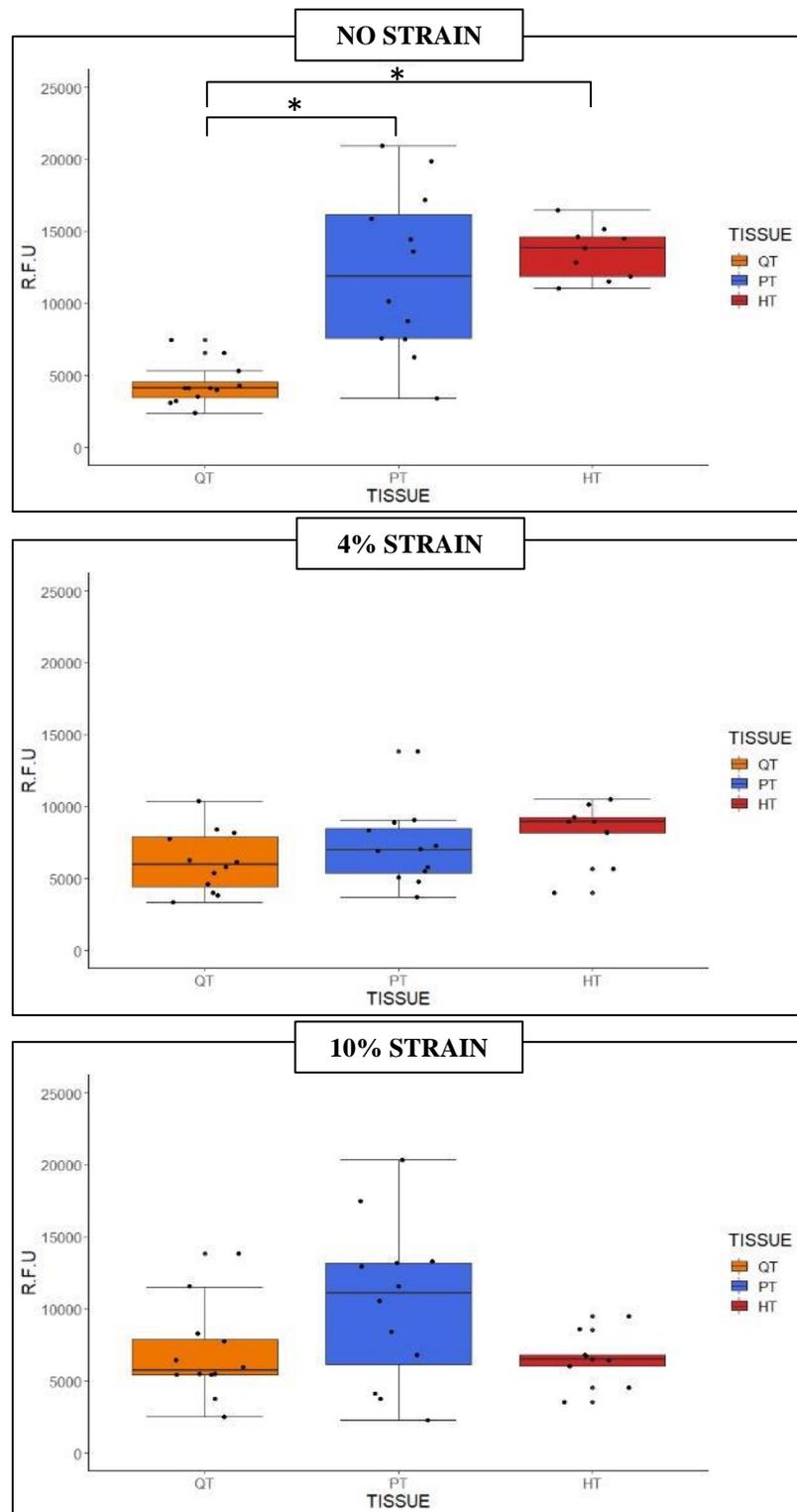


Figure 3.13. Metabolic activity after five days of culture. (*) significant difference between tissues. RFU: Resazurin Fluorescent Units QT: Quadriceps tendon; PT: Patellar tendon, HT: Hamstring tendon

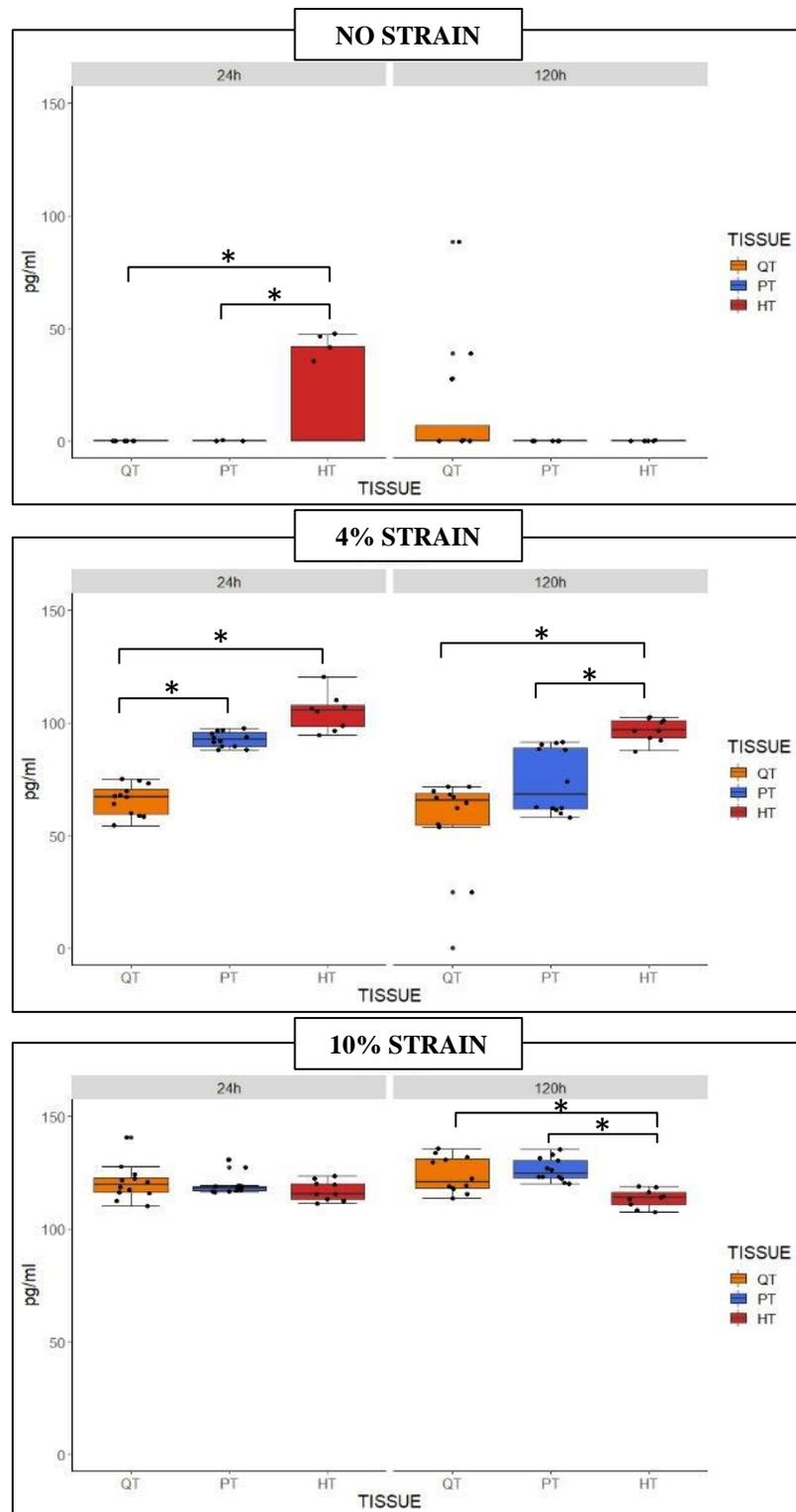


Figure 3.14. Concentration of PGE-2 released to the media. * significant difference between tissues at 24 and 120 hours. QT: Quadriceps tendon; PT: Patellar tendon, HT: Hamstring tendon

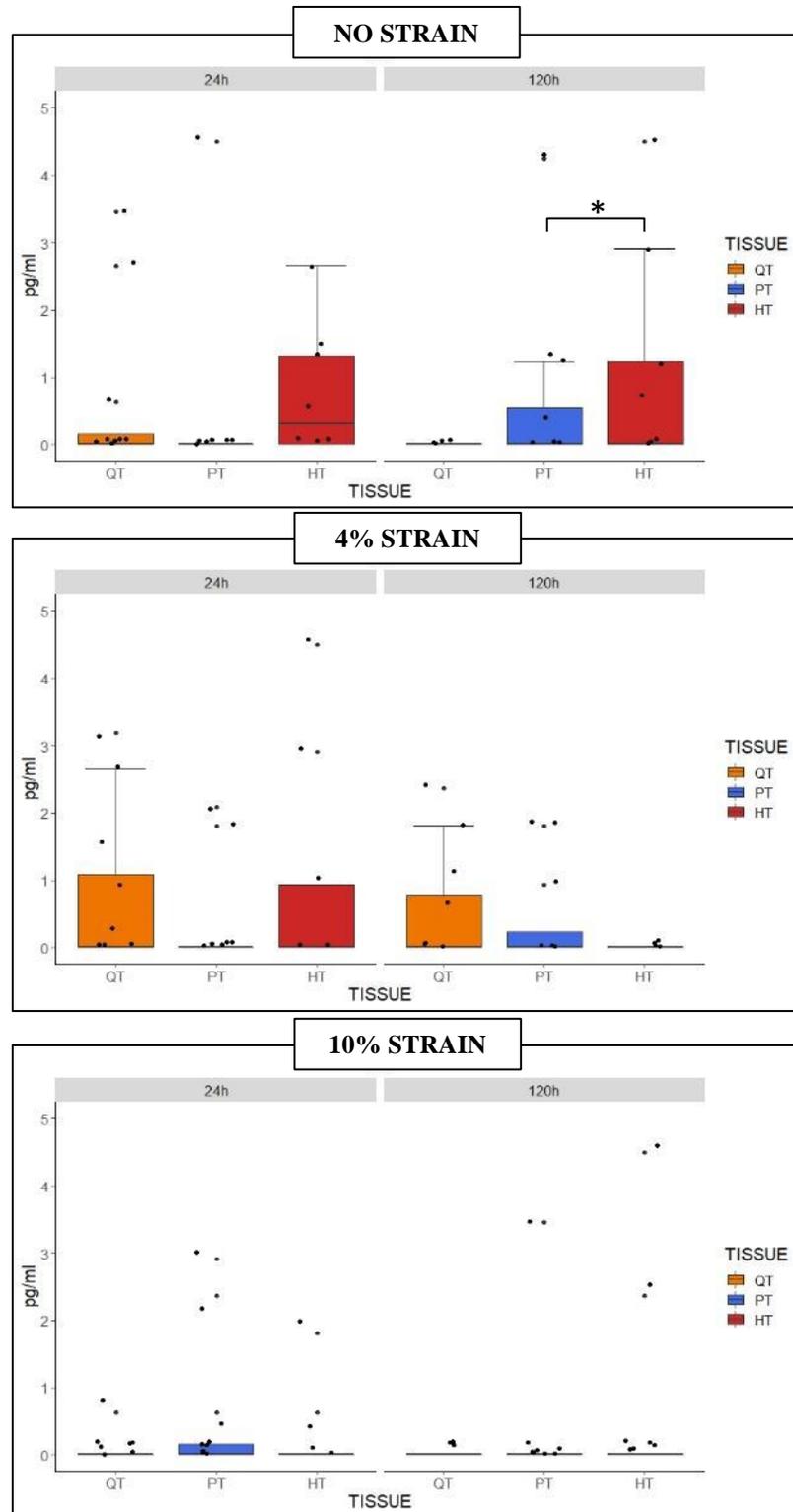


Figure 3.15. Concentration of IL-6 released to the media. * significant difference between tissues at 24 and 120 hours. QT: Quadriceps tendon; PT: Patellar tendon, HT: Hamstring tendon

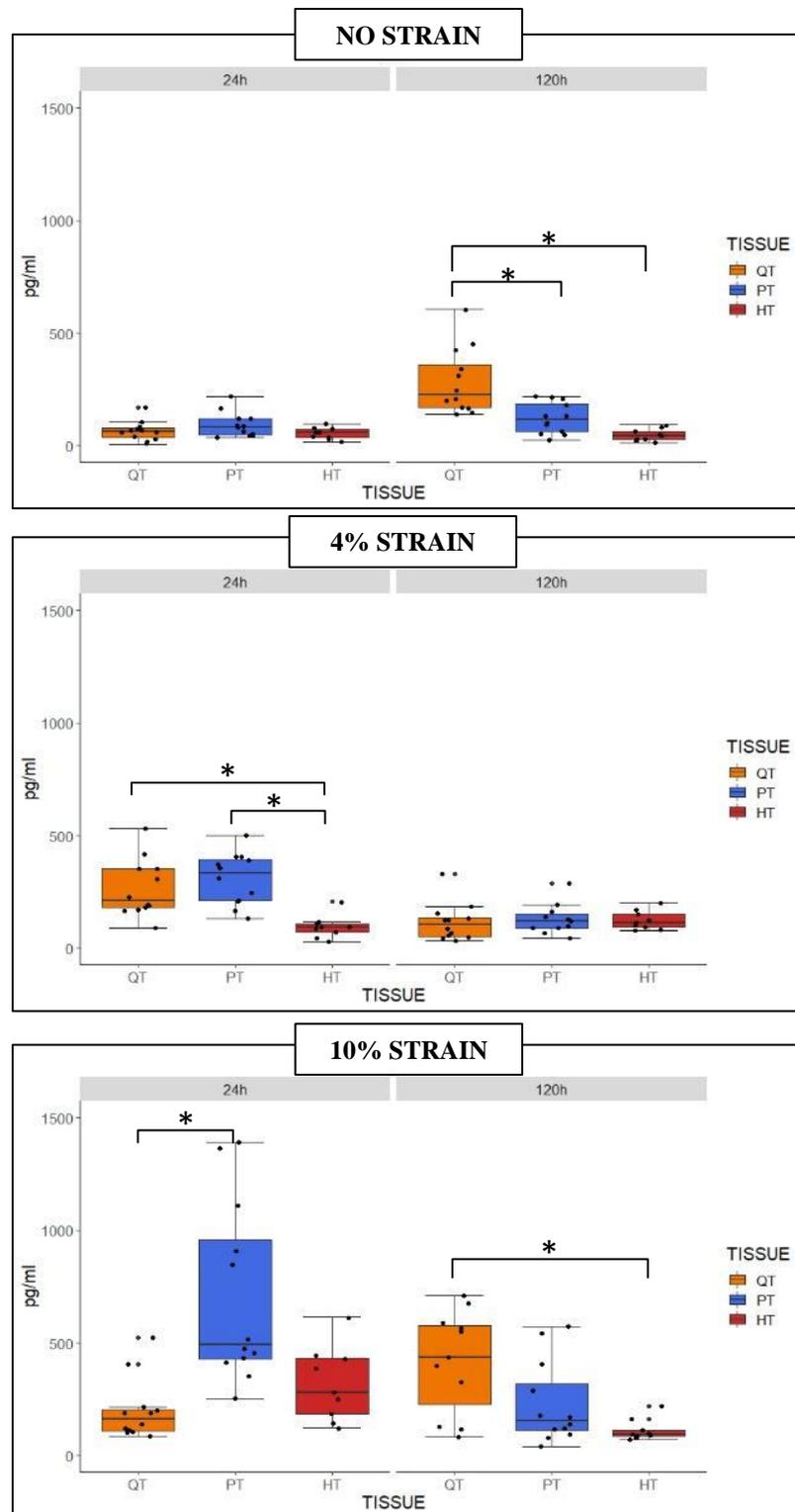


Figure 3.16. Concentration of IL-8 released to the media. * significant difference between tissues at 24 and 120 hours. QT: Quadriceps tendon; PT: Patellar tendon, HT: Hamstring tendon

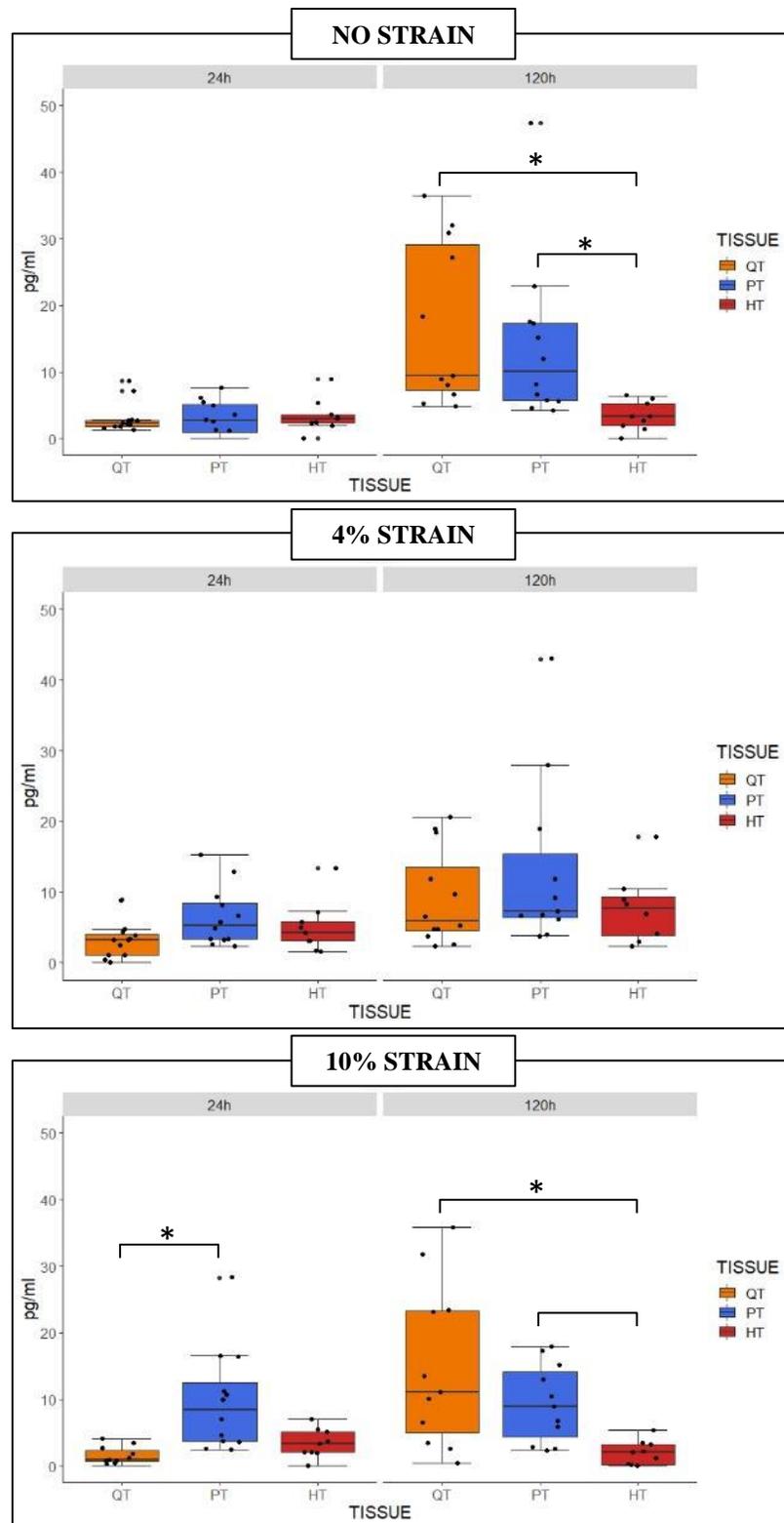


Figure 3.17. Concentration of KC released to the media. * significant difference between tissues at 24 and 120 hours. QT: Quadriceps tendon; PT: Patellar tendon, HT: Hamstring tendon

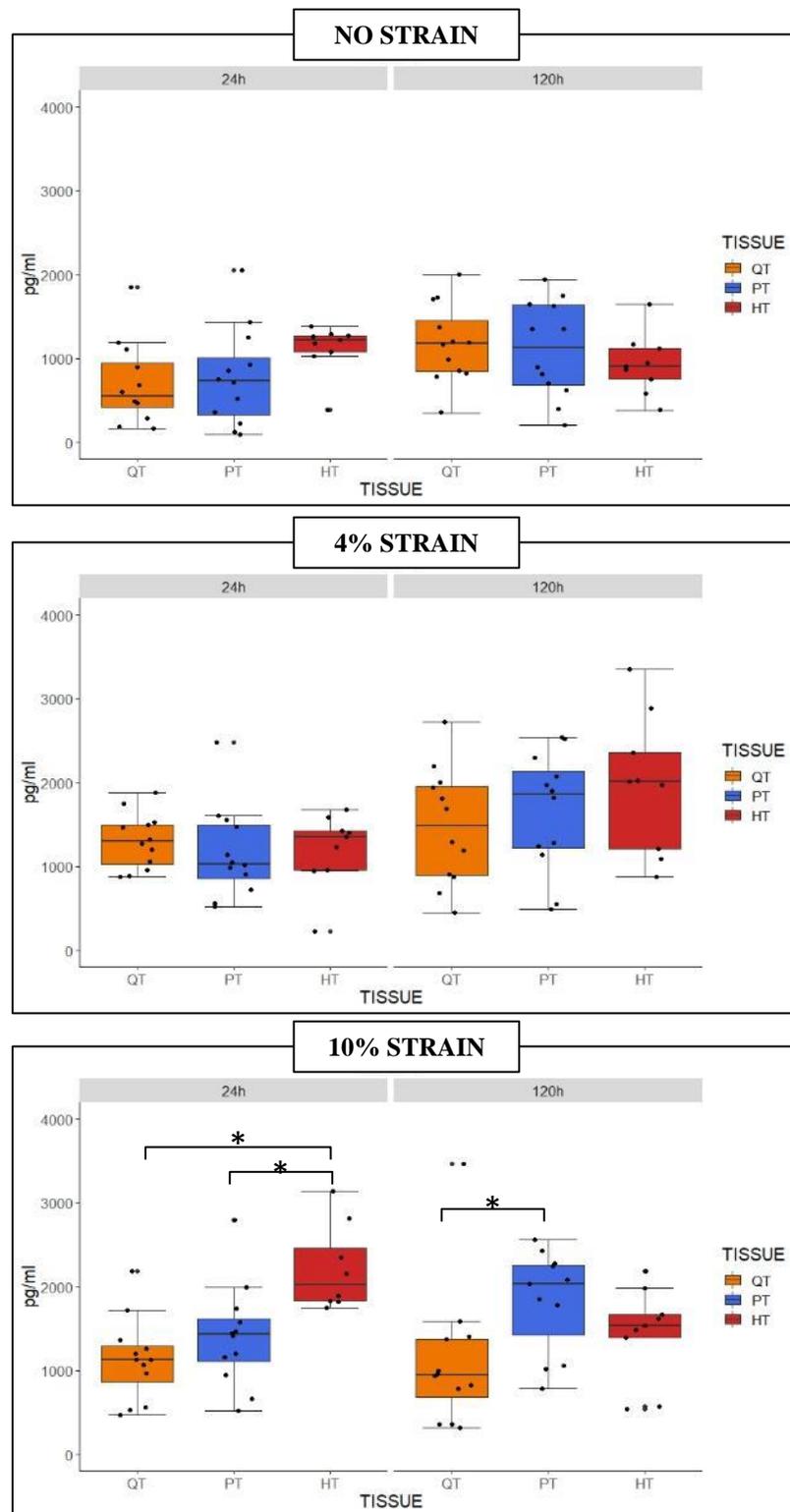


Figure 3.18. Concentration of MCP-1 released to the media. * significant difference between tissues at 24 and 120 hours. QT: Quadriceps tendon; PT: Patellar tendon, HT: Hamstring tendon

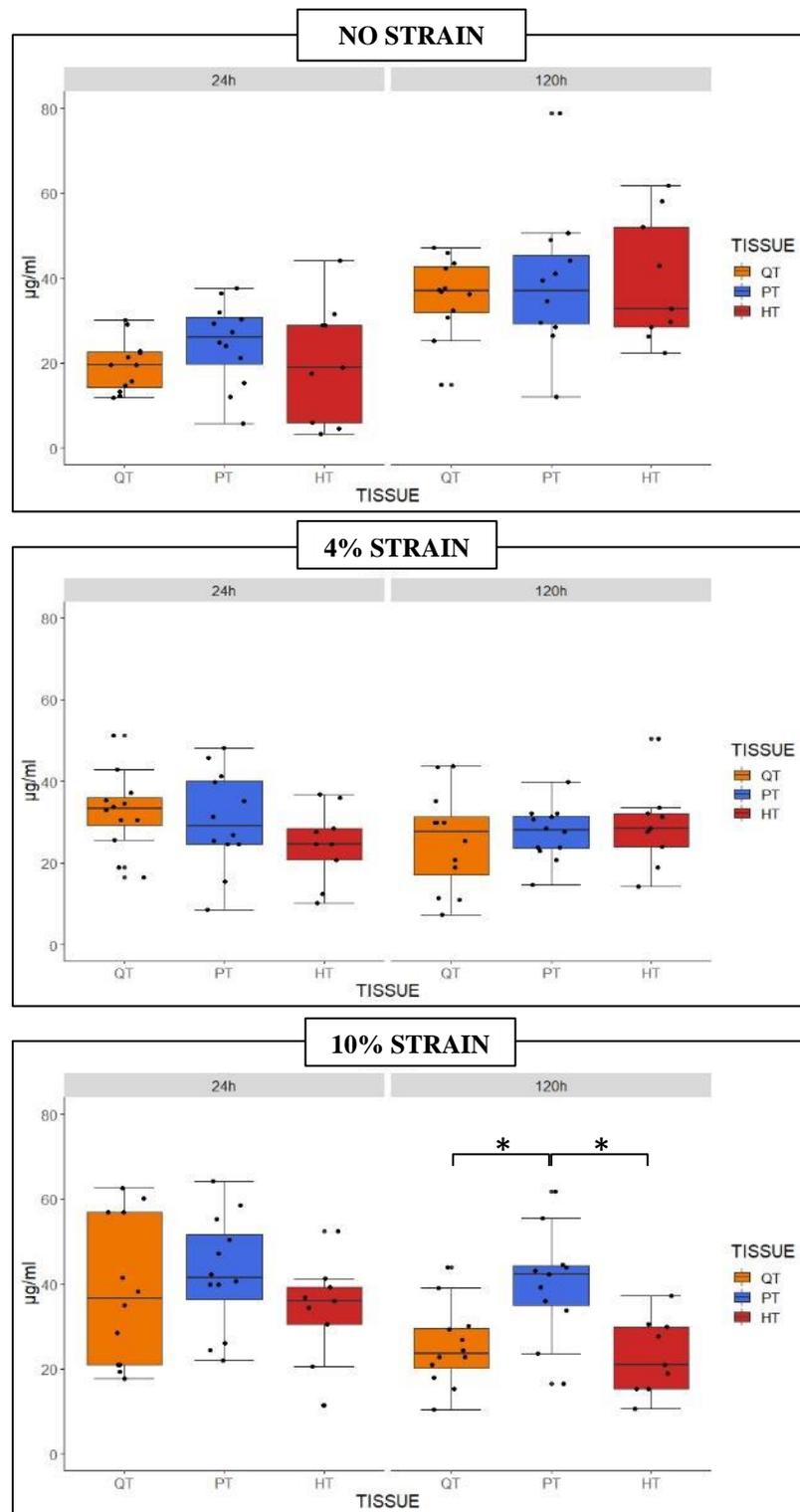


Figure 3.19. Concentration of GAG released to the media. * significant difference between tissues at 24 and 120 hours. QT: Quadriceps tendon; PT: Patellar tendon, HT: Hamstring tendon

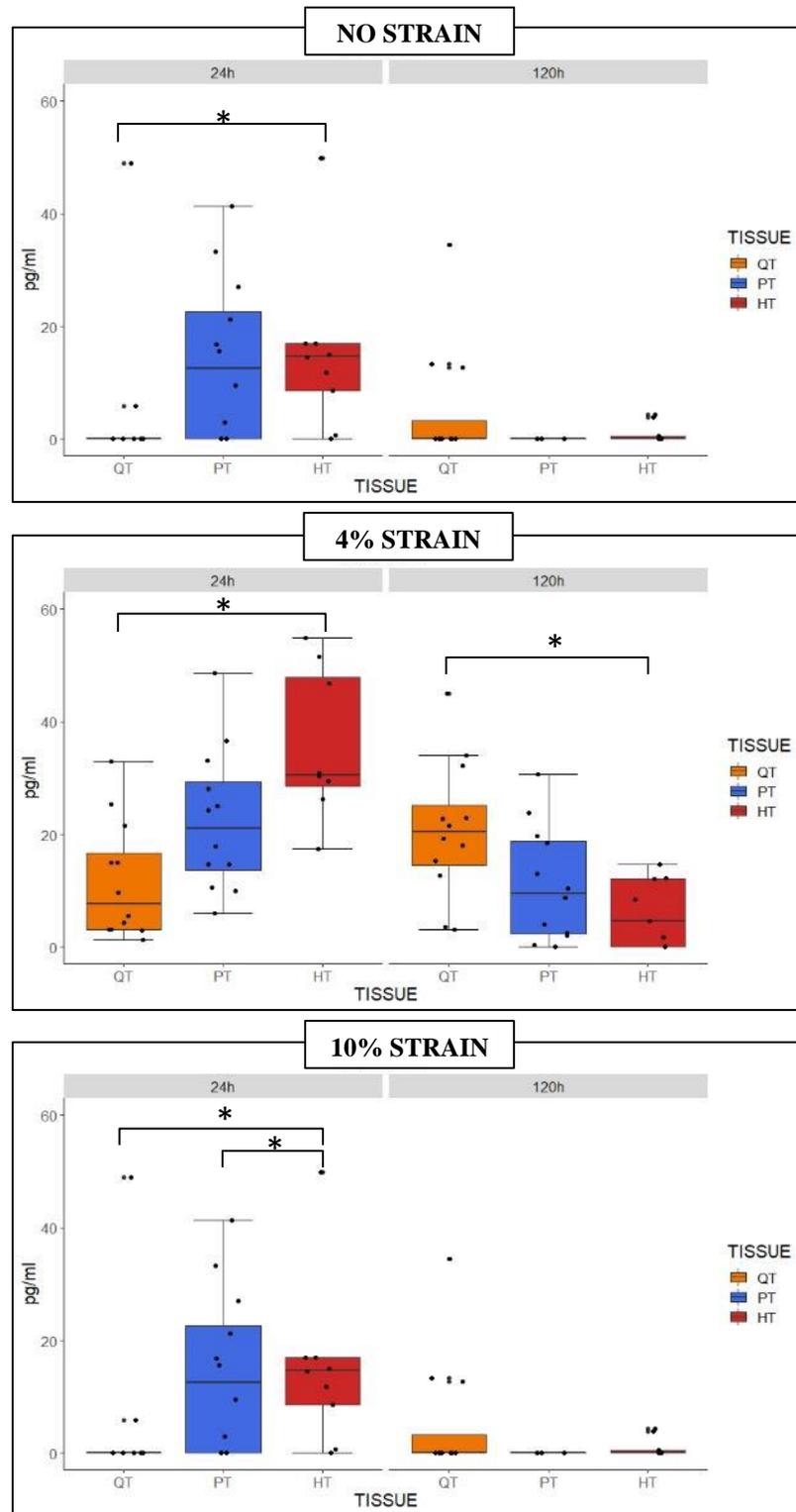


Figure 3.20. Concentration of MMP Activity (MMPACT) in the media. * significant difference between tissues at 24 and 120 hours. QT: Quadriceps tendon; PT: Patellar tendon, HT: Hamstring tendon

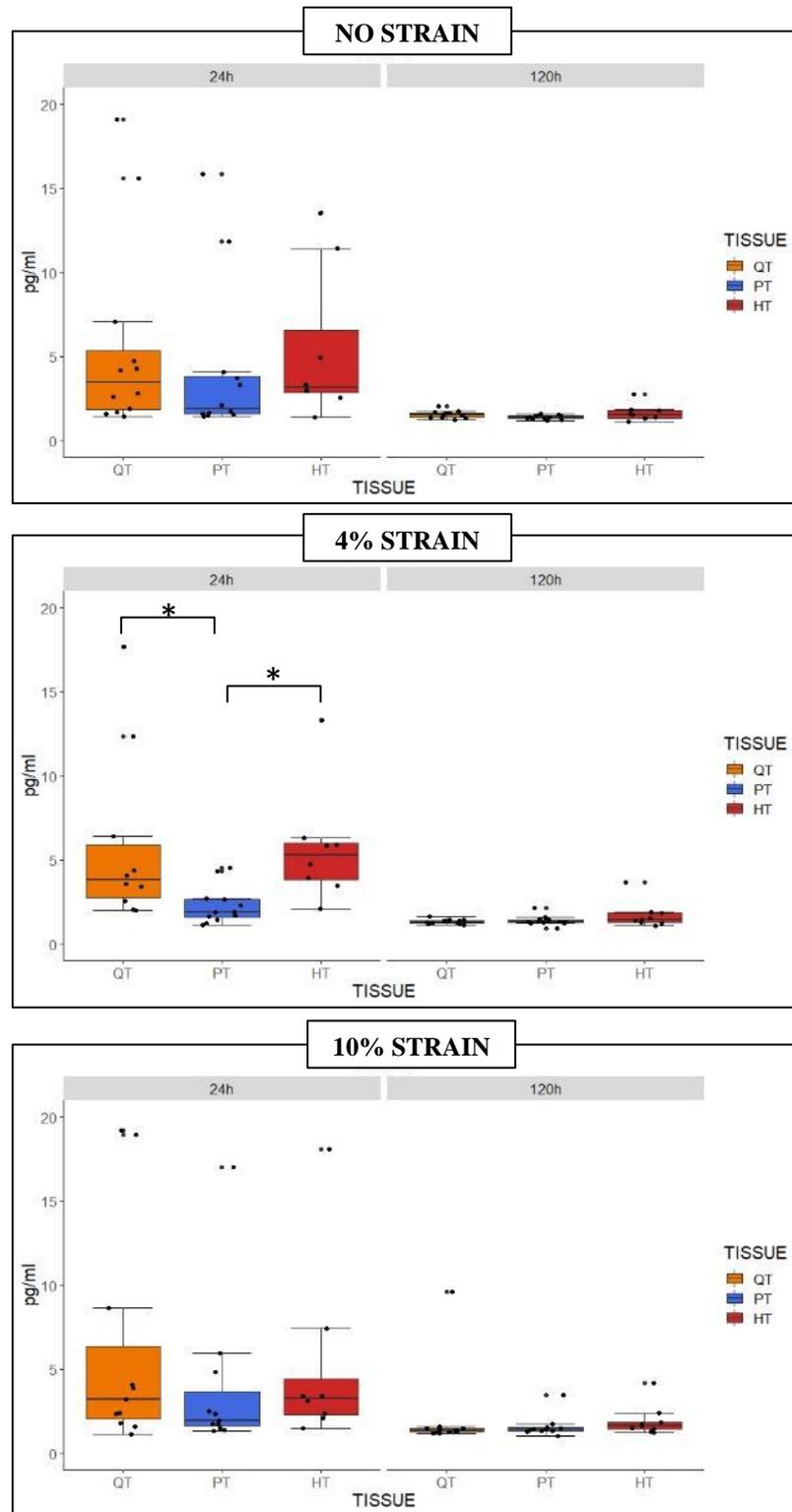


Figure 3.21. Concentration of MMP-1 in the media. * significant difference between tissues at 24 and 120 hours. QT: Quadriceps tendon; PT: Patellar tendon, HT: Hamstring tendon

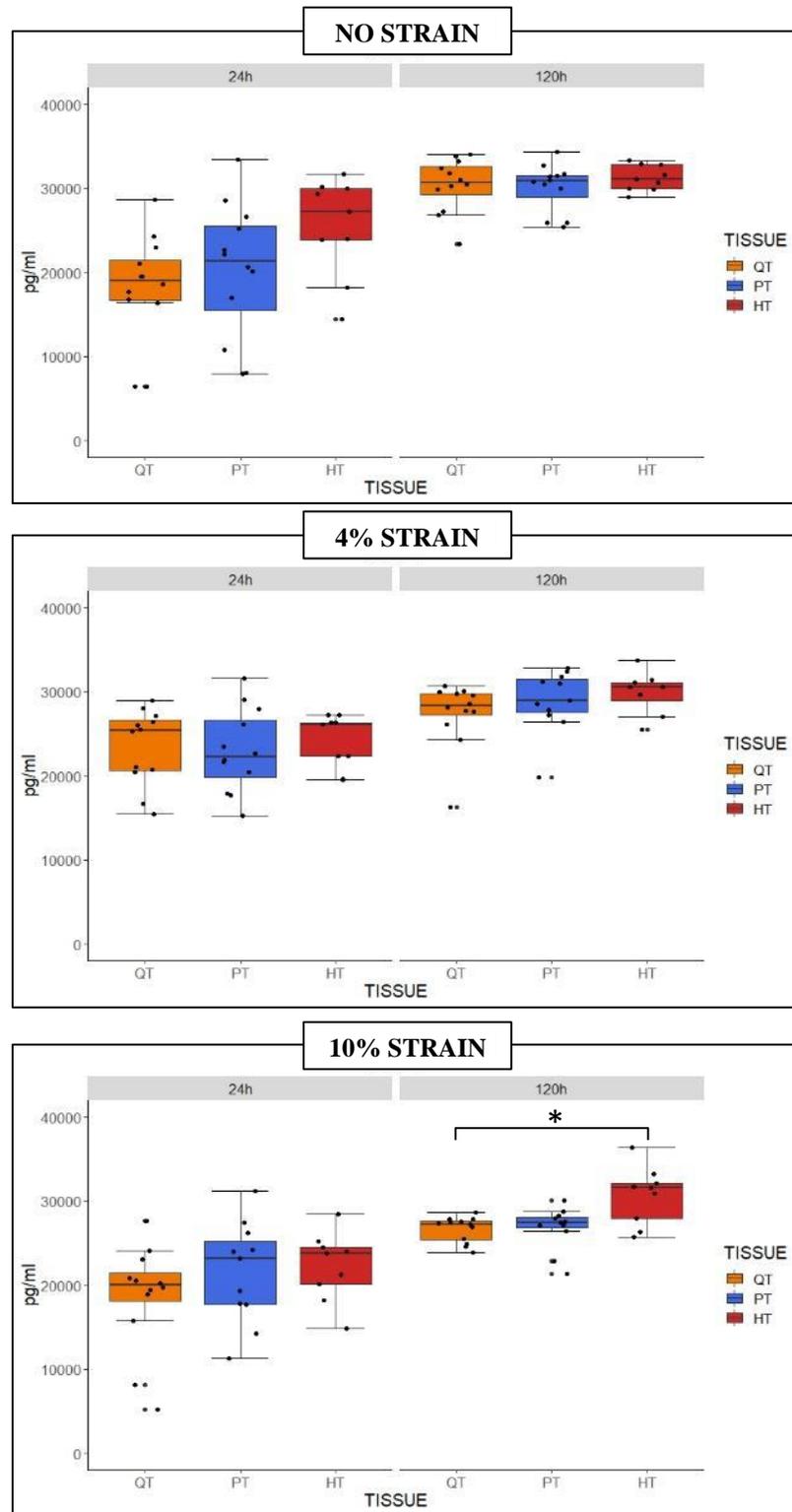


Figure 3.22. Concentration of MMP-2 in the media. * significant difference between tissues at 24 and 120 hours. QT: Quadriceps tendon; PT: Patellar tendon, HT: Hamstring tendon

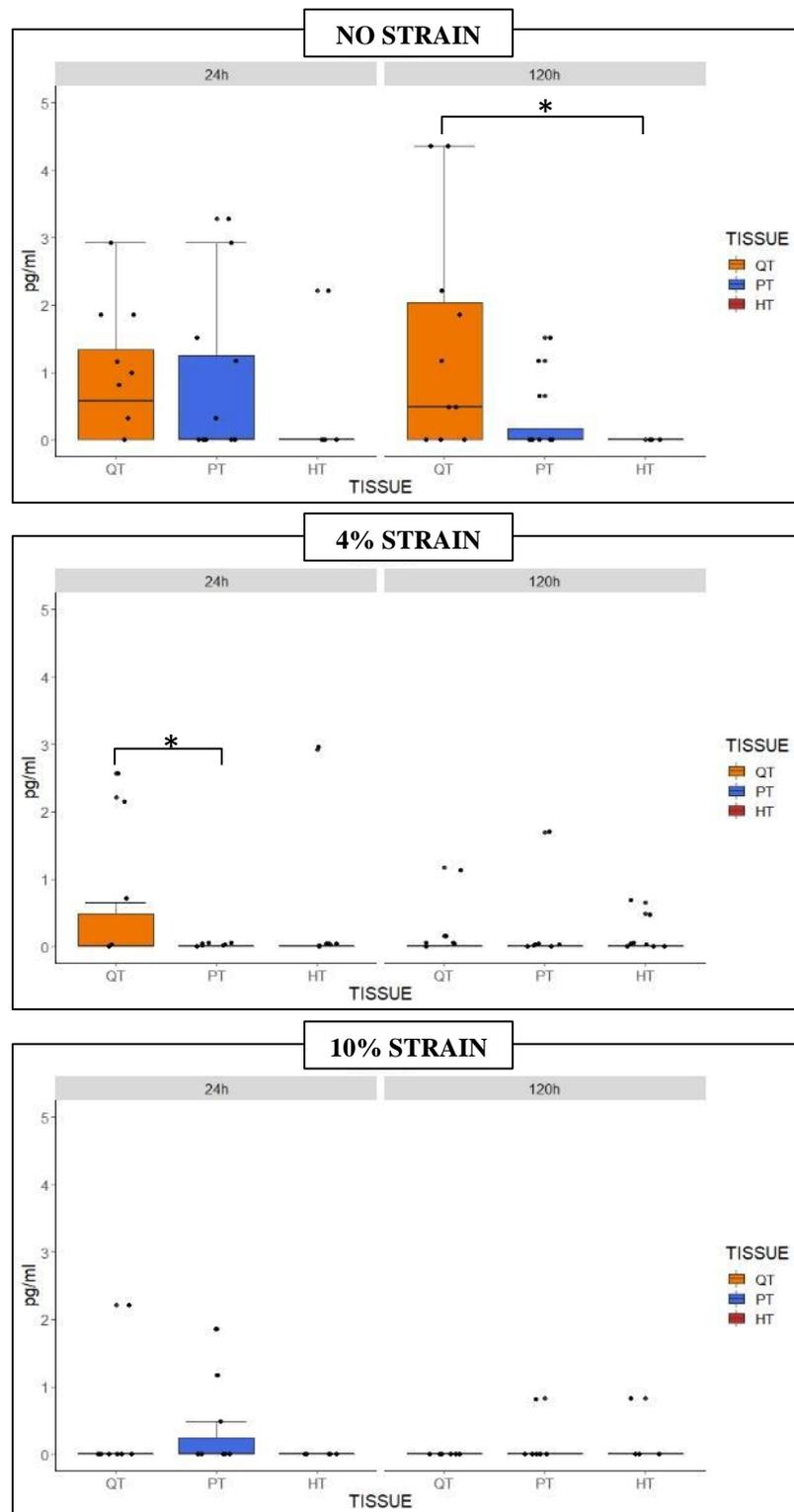


Figure 3.23. Concentration of MMP-3 in the media. * significant difference between tissues at 24 and 120 hours. QT: Quadriceps tendon; PT: Patellar tendon, HT: Hamstring tendon

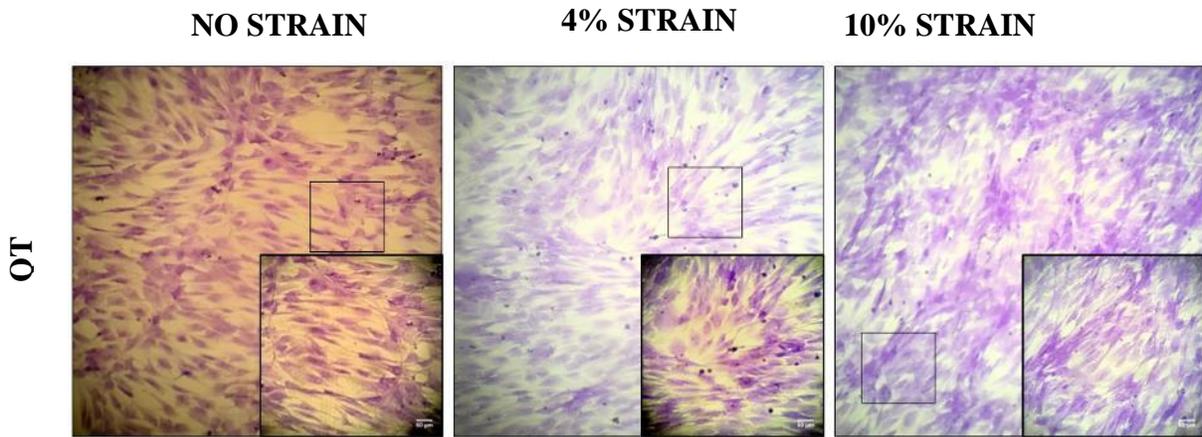


Figure 3.24. Quadriceps tendon (QT) fibroblasts after being stretched for 120 hours.

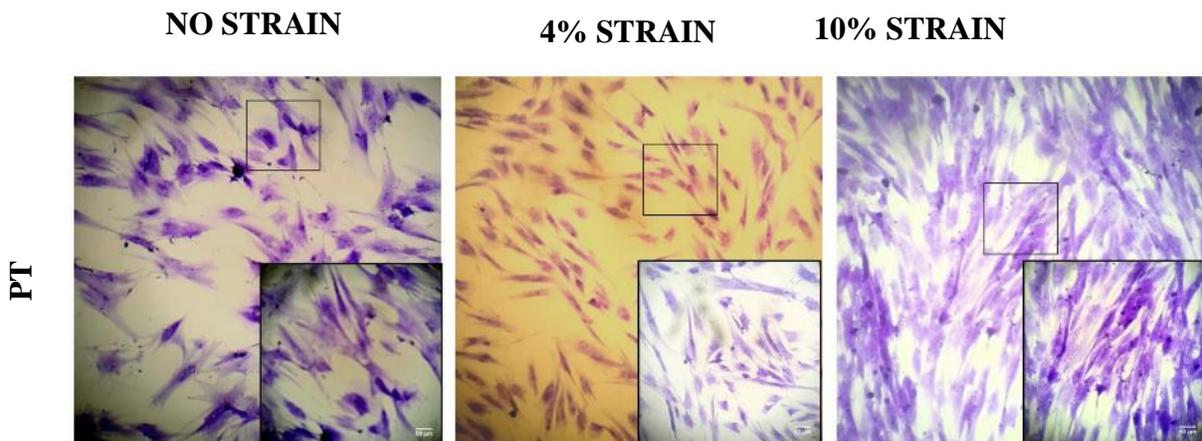


Figure 3.25. Patellar tendon (PT) fibroblasts after being stretched for 120 hours.

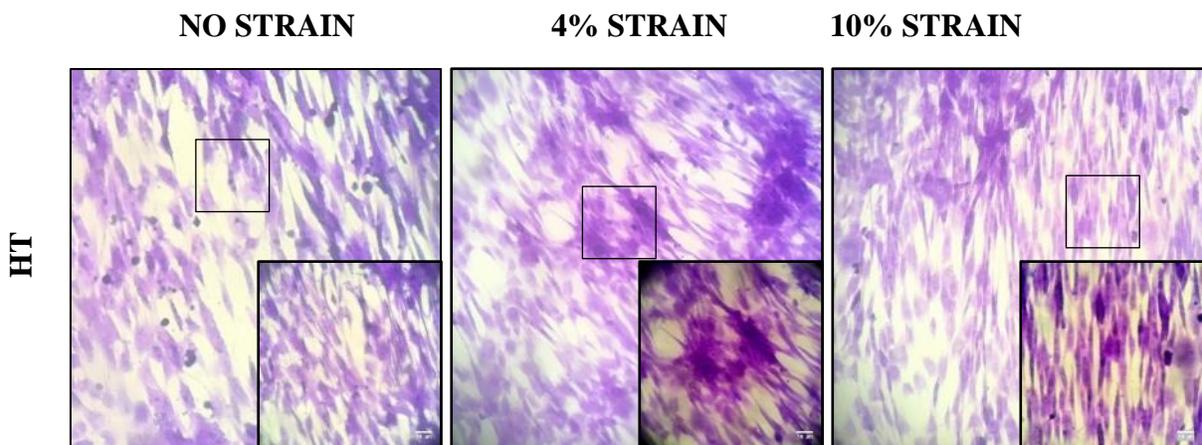


Figure 3.26. Hamstring tendon (HT) fibroblasts after being stretched for 120 hours.

CHAPTER 4

NORMAL CANINE ACL, PCL, SYN CELLULAR RESPONSE TO STRETCH

Introduction:

Cranial cruciate ligament (CrCL) deficiency is the most common cause of hindlimb lameness in the dog. The analogous human disorder, anterior cruciate ligament (ACL) deficiency, is the primary cause of knee instability in humans.^{1;2} In both species, extracellular matrix (ECM) remodeling which can be modulated by cellular responses to mechanical stress can influence the overall injury and healing response.^{3;4} Importantly, the joint is a complex organ with various intraarticular tissues playing vital roles in the joint injury response. However, the interplay between various tissue types in the knee are not fully understood.

ACL reconstruction using a tendon graft is the standard-of-care for surgical management of ACL tears in humans. However, morbidity associated with ACL reconstructive surgery include increased risk of failure in certain patients and the inability to consistently mitigate the onset of post-traumatic osteoarthritis (PTOA).^{5;6} There is also an ongoing debate if a reconstructed ACL recapitulates all of the properties of a native ACL including its proprioceptive or reflex responses.⁷ Therefore, a renewed interest in primary ACL repair has developed. While attractive, an unreliable healing response has been associated with primary ACL repair. This has been attributed to several factors, including a hostile intra-synovial environment, specific post-inflammatory responses, and intrinsic cell deficiencies.^{8;9} Therefore, understanding the early healing phase characterized by cellular release of molecules that regulate adequate balance between ECM degradation and

proliferation may be crucial for case selection in ACL repair and to optimize the rehabilitation protocols after surgery.

Experiments performed in cells from tissues subjected to mechanical stresses have revealed that cell structure and tissue organization can respond to varying amounts of forces including tension, compression and shear stress. Common methods for membrane deformation include vacuum, suction, indentation or pulling.¹⁰ The Flexercell® System has been developed to stress cell cultures in monoculture and promote morphological and biochemical changes. Hence, this tension system allows the measurement of cellular responses to mechanical stress, including ECM interactions.

The Flexercell system has been widely used in ACL research.¹¹⁻¹⁵ Due to the multiscale structure of ligaments and tendons, biomechanical research at each hierarchical scale should be conducted in order to gain a broad understanding on tendon/ligament pathology and healing. Authors have investigated this phenomenon in both tendons and ligaments.¹⁶⁻¹⁹ However, few studies have included the effect of mechanical stress in ACL repair.^{12; 20-22}

The objective of this work was to compare the pro-inflammatory and remodeling responses of fibroblasts obtained from canine intraarticular tissues of the stifle joint that may be involved in primary ACL repair (ACL, synovium (SYN), posterior cruciate ligament (PCL) when subjected to varying levels of mechanical strain forces. We hypothesized that fibroblasts from these tissues would produce significantly different levels of inflammatory and remodeling biomarkers in response to a spectrum of cyclic strains *in vitro*.

Materials and methods:

Tissue processing and culture

With ACUC approval (ACUC#9163, 9164), tissues (9 ACL, 12 PCL and 12 SYN) were harvested from skeletally mature female dogs (n=15) euthanized for studies unrelated to the current work. Tissues were minced into 0.5–1cm² pieces and digested in 0.5% Type 1A *Clostridium histolyticum* collagenase solution (Sigma-Aldrich, St. Louis, MO, USA). The aliquots were then suspended with nutritional media containing 10% FBS and cultured at 5% CO₂, 37°C, and 95% humidity. Once confluent, the cells were resuspended and seeded in Collagen Type I-coated BioFlex® plates (1 × 10⁵ cells/well). Cells were incubated for an additional 48h before strain was applied. Then, nutritional culture media was replaced with 0.5% FBS media for the remaining five days of culture. Fibroblasts were subjected to continuous mechanical stimulation (2-s strain and 10-s relaxation at a 0.5 Hz frequency) with a biaxial sinusoidal waveform with three different elongation strains (mechanical stress deprivation-0%, physiologic strain-4%, and high strain-10%)²³ for 5 days (120h) using the Flexcell FX-4000T strain system (Flexcell International, NC, USA) (Fig. 4.1). Media was changed every 24h and stored at -20°C until used for biomarker analysis.

Biomarker assays

Media was changed every 24h and stored at -20°C until analysis. Media from 24 and 120 hours of culture were assessed for various biomarkers. Proteoglycan (GAG) was assessed using the DMMB assay as previously described.²⁴ The concentration of prostaglandin E₂ (PGE₂) (Cayman Chemical, Ann Arbor, MI, USA), cytokines (IL-6, IL-8, KC and MCP-1) (Millipore, Billerica, MA, USA), total matrix metalloproteinase (MMP) activity (SensoLyte 520 generic MMP assay, Anaspec, Inc., Fremont, CA), and MMP production (MMP-1, MMP-2 and MMP-3) (R&D Systems, Minneapolis, MN, USA), were all

assessed using commercially available assays according to the manufacturer's protocol. At the end of the strain protocol (120h), cell viability analysis was performed using the resazurin assay (Sigma Aldrich, Saint Louis, MO).

Statistical Analysis

For statistical analysis, normal distribution was assessed by Shapiro-Wilk test. Because data were not normally distributed, non-parametric analyses were used. Significance levels were set at $p < 0.05$. Results were reported as median \pm interquartile range (IQR). Comparisons among strains and tissue types were performed using Kruskal-Wallis test and Dunn's test posthoc (R Core Team (2019), R version 3.6.2 Vienna, Austria).

Results

Metabolic activity was significantly affected by strain magnitude in all intraarticular fibroblasts evaluated. SYN fibroblasts exhibited a significant decrease in metabolic activity at supraphysiological strain (10%) compared to mechanical strain deprivation (no strain). Moreover, in ACL derived fibroblasts, physiological strain (4%) elicited an increase in metabolic activity compared to the no strain group. Interestingly, in PCL fibroblasts, no strain produced a significant increase in metabolic activity compared to 4% strain and 10% strain (Fig.4.2). Additionally, comparisons among tissue types showed that at no strain, fibroblasts derived from the PCL had a significantly higher metabolic activity compared to SYN and ACL fibroblasts. Interestingly, physiological strain (4%) did not elicit a differential metabolic activity among the different cell types. Moreover, supraphysiological strain (10%) produced an increased metabolic activity from PCL compared to ACL fibroblasts (Fig. 4.13).

Inflammatory responses were also significantly affected by both strain and tissue type. At 24h, PGE2 production was significantly increased in SYN and ACL fibroblasts subjected to supraphysiological strain compared to the no strain and 4% strain groups at 24h. Further, the production of PGE2 by ACL fibroblasts was significantly higher in the 10% strain group compared to the no strain and 4% strain groups at 120h. Moreover, PGE2 production was significantly increased in PCL fibroblasts at supraphysiological strain compared to 4% strain (Fig 4.3).

Tissue type differences showed that at no strain, PCL fibroblasts tended to exhibit a significantly increased production of PGE2 compared to both SYN and ACL at 24h. Also, at 120h, stress deprivation elicited a significantly decreased production of PGE2 in ACL relative to SYN and PCL fibroblasts. Moreover, at 24h of physiological strain, SYN fibroblasts showed a significantly decreased production of PGE2 relative to ACL and PCL but no significant differences at 120h. Additionally, at 24h of supraphysiological strain, PCL showed a significant decrease in PGE2 production compared to SYN and ACL and no difference among tissue types at 120h (Fig. 4.14).

The production of IL-6 by all fibroblasts was significantly higher at physiological strain compared to supraphysiological strain at 24h of culture. SYN fibroblasts significantly increased IL-6 production at no strain and physiological strain compared to 10% strain at 24h. Similarly, ACL production of IL-6 was also higher at 4% strain compared to 10% strain at 24h. Moreover, PCL production of IL-6 was significantly higher at 4% strain compared to 10% strain at 24h and at no strain compared to 4% strain at 120h (Fig.4.4).

Additionally, PCL fibroblasts exhibited a consistent increase in IL-6 compared to ACL fibroblasts at 24h and 120h of mechanical strain deprivation. Furthermore, physiological

strain was not significantly different among tissues at 24h but changed after 120h with ACL producing significantly lower levels of IL-6 compared to both SYN and PCL. Moreover, at 10% strain, PCL fibroblasts showed a significant increase in IL-6 production relative to SYN and ACL fibroblasts at 24h and 120h (Fig.4.15).

The production of IL-8 was significantly increased in SYN fibroblasts at both physiological and supraphysiological strains compared to no strain at 24h (Fig. 4.5). Moreover, ACL fibroblasts exhibited significantly higher levels of IL-8 at both, physiological and supraphysiological strain compared to no strain at 24h. PCL fibroblasts showed increased levels of IL-8 at 4% strain relative to both, no strain and 10% strain, which in turn was significantly higher than no strain at 24h. However, at 120h, mechanical strain deprivation elicited a significant increase of IL-8 from PCL fibroblasts when stress deprived compared to 4% strain.

Tissue differences showed that at 120h of mechanical strain deprivation, SYN and PCL fibroblasts produced significantly more IL-8 than ACL fibroblasts. Moreover, at 4% strain, PCL fibroblasts produced significantly more IL-8 than SYN and ACL fibroblasts at 24h. Similarly, after 120h, production of IL-8 from ACL fibroblasts was significantly lower than SYN and PCL fibroblasts. Also, at 10% strain, ACL fibroblasts produced significantly more IL-8 than SYN fibroblasts at 24h. However, at 120h, SYN and PCL exhibited a significantly greater production of IL-8 compared to ACL fibroblasts (Fig. 4.16).

Production of the chemokine KC significantly increased in ACL and PCL fibroblasts at 24h of physiological strain compared to no strain and supraphysiological strain. Moreover, no differences were seen in SYN at any strain or timepoint (Fig.4.6).

Tissue differences showed that at no strain, KC production significantly increased in PCL fibroblasts compared to ACL fibroblasts at 120h. Moreover, at 4% strain, KC production was significantly higher in PCL fibroblasts relative to SYN and ACL fibroblasts at 24h. Further, at 10% strain, ACL production of KC was significantly lower than both SYN and PCL fibroblasts at 120h (Fig. 4.18).

The chemokine MCP-1 was not significantly different in SYN fibroblasts at any strain or timepoint. However, in ACL fibroblasts, MCP-1 production was significantly increased at 4% strain relative to no strain at 24h. Similarly, PCL fibroblasts exhibited a significant increase in MCP-1 production after 24h of physiological strain compared to no strain and supraphysiological strain (Fig. 4.7).

Tissue source showed a significant increase in MCP-1 in PCL compared to ACL fibroblasts after 120h of mechanical strain deprivation. Moreover, at 4% strain, PCL fibroblasts produced significantly more MCP-1 than SYN and ACL fibroblasts at 24h. Furthermore, at 10% strain, ACL produced significantly less MCP-1 than both SYN and PCL fibroblasts at 120h (Fig. 4.18).

Increased production of GAG was observed in SYN and PCL fibroblasts at 24h with escalating strain magnitudes. No strain produced significantly less GAG than 4% and 10% strain at 24h. However, at 120h, 10% strain produced significantly less GAG than no strain and 4% strain in SYN fibroblasts. ACL fibroblasts exhibited significantly more GAG at no strain compared to 4% strain and 10% strain at 120h. Moreover, PCL fibroblasts showed significantly less GAG at no strain compared to 4% strain and 10% strain at 24h (Fig. 4.8).

Tissue source comparisons showed that the production of GAG by ACL fibroblasts was significantly lower than the production by the SYN and PCL fibroblasts at no strain and

4% strain at 120h. However, at 10% strain, GAG production was significantly higher in PCL fibroblasts compared to SYN and ACL after 120h of culture (Fig. 4.19).

The level of total MMPACT was not significantly different in SYN fibroblasts at any strain or timepoint. However, ACL fibroblasts exhibited significantly higher concentration of MMPACT at physiological strain compared to both, stress deprived and supraphysiological strain groups at 24h and 120h. Moreover, MMPACT increased in PCL fibroblasts at supraphysiological strain relative to 4% strain at 24h. Conversely, 4% strain showed significantly decreased levels of MMPACT after 120h of supraphysiological strain compared to no strain at 120h (Fig. 4.9).

Cell type comparisons indicated that at mechanical strain deprivation, PCL fibroblasts produced significantly increased levels of MMPACT compared to SYN and ACL fibroblasts at 24h and 120h. Interestingly, physiological strain did not produce considerable MMPACT at any timepoint in any of the fibroblast types. Moreover, at 10% strain, MMPACT production was significantly higher in PCL fibroblasts compared to both SYN and ACL fibroblasts at 24h and 120h (Fig. 4.20).

The production of MMP-1, MMP-2 and MMP-3 by SYN fibroblasts was significantly higher at physiological levels compared to supraphysiological strain at 24h (Fig. 4.10-4.11 and 4.12). The production of MMP-2 by ACL fibroblasts was significantly higher at physiological strain levels compared to supraphysiological strain levels at 120h. There was not a significant difference between strain groups for the production of MMPs by PCL fibroblasts at both timepoints. Interestingly, PCL exhibited a consistent increase in MMP-1 and MMP-3 when compared to ACL fibroblasts regardless of the strain at 24h and 120h (Fig. 4.21 and 4.23). Moreover, mechanical strain deprivation elicited a significant increase

in MMP-2 production compared to SYN and PCL fibroblasts at 120h. Similarly, at 4% strain, MMP-2 production was significantly higher in ACL compared to PCL fibroblasts at 120h. Moreover, at 10% strain, MMP-2 production was significantly lower in SYN compared to ACL and PCL fibroblasts (Fig. 4.22).

Discussion

In this study, we demonstrated that intraarticular fibroblasts from the ACL, PCL, and synovium produce significantly different levels of inflammatory and remodeling biomarkers in response to a spectrum of cyclic strains *in vitro*. This work supported the hypothesis that fibroblasts from intraarticular tissues involved in ACL injury and repair produce significantly different levels of inflammatory and remodeling biomarkers in response to cyclic strains *in vitro*.

Fibroblasts are found across all connective tissues including skin, tendons and ligaments and are the primary source of ECM components including collagens, proteoglycans and growth factors.²⁵ Studies have shown that fibroblasts from the anterior cruciate ligament, posterior cruciate ligament, and synovium are sensitive to mechanical strain variations.²⁶⁻³⁰ Moreover, abnormal loads applied to the tissue can contribute to the development and progression of osteoporosis, osteoarthritis and tendinopathy.³¹

In vivo, the PCL is a primary restraint to posterior tibial translation and internal rotation³². Some authors have reported the increase in the corresponding *in situ* ligament force when sectioning either the ACL or the PCL.^{33;34} In this work we believe that either, ACL or PCL injury will lead to excessive forces in the adjacent ligament. We assume that this force would be higher than 5% strain in cases of hyperextension, which is supraphysiological for any ligament and will lead to further cartilage degradation and a hostile environment.³⁵

Additionally, the alteration of the structural properties such as ligament stiffness or initial tautness of the cruciate ligaments could modify the mechanical role of both cruciate ligaments.³⁵ Moglo & Shirazi-Adl (2005) showed that the mechanical contribution of a cruciate ligament was strongly dependent on the force applied in the other ligament.³⁵ The authors suggest that alterations in ligament stiffness after ligament reconstruction surgery would alter the mechanical role not only of the treated ligament but the untreated one as well.³⁵ Moreover, these same effects could stimulate a different metabolic response due to the variation in mechanical loads in other intraarticular structures such as the meniscus.³⁶ In addition to the intraarticular ligaments, the synovial membrane also plays a key role in delivering nutrition and blood to both, the PCL and the ACL. The synovium also clears intra-articular debris from the joint and modulates the inflammatory response of the joint.³⁷ ³⁸ After cruciate ligament injury there is an increase in the concentration of cytokines and growth factors in the synovial fluid, which may originate from the synovium.³⁷ Tang *et al.*, (2009) showed an increased production of MMP-2 in synovium explants relative to intraarticular tissues including ACL and PCL.³⁷ Our findings demonstrate that in response to increased strain levels, the synovium produces significantly higher levels of IL-6 and IL-8 compared to the cruciate ligament fibroblasts during culture.

The poor healing ability of the ACL has been attributed to specific cellular cues and biochemical composition of the intrasynovial environment that may impair the initiation of the healing cascade.^{8; 39-42} Authors have demonstrated that a fine balance between understimulation and overloading must be met for effective ACL healing.⁴³⁻⁴⁵

Certain conditions must be met in order to elicit an optimal primary healing response. Close continuity of the torn ends of the ligament and a controlled functional motion may enhance

the healing process.⁴⁶ These conditions are rarely met in ACL tears to permit *in situ* healing without surgical intervention. Therefore, for ACL repair a reapproximation of the two ends of the ruptured ligaments has been attempted.⁴⁷ However, this technique has largely failed when using suture alone.⁴⁸ One possible cause for the failure of the repair is the inability of the tissue to maintain a bridging clot after repair. It is possible that the hematoma that develops is removed by enzymes present in the synovial fluid, which impairs the ability of the hematoma to deliver of growth factors that assist in wound healing and ligament repair.

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Murray et al., (2013) investigated the use of a bioactive scaffold containing a collagen-platelet composite to enhance suture repair of the torn ACL in an animal model.^{52; 53} Augmented repair showed promising results and led to similar outcomes when compared to ACL reconstruction.⁵⁴ Additionally, the authors have shown that a six-week delay on ACL bioenhanced repair has a negative effect on functional outcomes on the porcine ACL.⁵⁵ Similarly, authors also showed that sex plays a critical role on the biomechanical outcomes of bridge-enhanced ACL repair in this model.⁵⁶ Consequently, the promising findings from the Murray *et al's* Bridge-Enhanced Anterior Cruciate Ligament Repair (BEAR) technique have led to the translation of this methodology into encouraging human clinical trials.⁵⁷ Given the ability of this technique to salvage an individual's native ACL tissue, primary ACL repair may circumvent some of the disadvantages of ACL reconstruction including PTOA, the associated morbidity with tissue recovery from harvest when using an autograft, and delayed return to sport due to the length of time needed for the graft ligamentization after ACL reconstruction.⁸⁻⁶⁵

Additionally, impaired healing after primary repair may be the consequence of the mechanical environment on surrounding tissues. After ACL injury, neighboring tissues must bear supraphysiological loads that act as a cue for catabolic factors such as inflammatory molecules and degradative enzymes.⁶⁶ Our study shows that variable inflammatory responses from different intraarticular fibroblasts occur based on mechanical strain, which is relevant for ACL healing in the setting of injury and primary repair. Zhou *et al.*, (2005) found a differentially increased expression of MMP-2 in overstretched ACL fibroblasts at 12 hours stretch.⁶⁷ Conversely, our data shows that MMP-2 production was similar across all stretching protocols. One reason for these conflicting findings may be that authors investigated the release of MMP-2 to the culture media of these tissues after ACL injury while we investigated the fibroblastic response of normal tissues.

Unlike the PCL, the ACL fibroblasts were relatively quiescent in our experiments when subjected to different strain levels. This relative lack of biologic response has implications when considering ACL repair. Our results might explain the high rates of failure associated with ACL repair, particularly midsubstance repairs, as ACL fibroblasts appear to have dampened responses to mechanical strain for inflammatory and matrix production pathways. Bio-enhancement techniques therefore may be necessary after primary ACL repair.^{47; 68}

This study has potential limitations that need to be considered for interpretation. First, samples were obtained from healthy dogs without knee conditions. This may restrict the application of some of our results for ACL repair. Consequently, our lab is also focusing on evaluating metabolic responses from fibroblasts obtained from human patients that sustained ACL injury. Second, we did not see a high production of markers that may be

involved in ACL injury in vivo such as IL-6 and MMP-1 and 3. This could be related to a lack of cross-talking between different cell types and the absence of a proper extra cellular matrix that will expand further molecular interactions. Lastly, the system used to apply mechanical strain only involves biaxial strain, which is rarely found in vivo and other mechanical forces such as shear stress and rotation may play a role in fibroblastic responses to strain. Hence, additional investigation utilizing 3D cultures or ex vivo explant cultures with a proper bioreactor is highly recommended.

In summary, our results show that surrounding intraarticular tissues, such as the SYN and PCL, are important sources of biologic mediators depending on strain level. Moreover, findings from this work suggest that surrounding intraarticular tissues play important roles in modulating the joint biochemical environment in response to mechanical strain, which may have important implications in a post-primary ACL repair setting. Since physiological strain produced the lowest levels of inflammatory mediators by both PCL and SYN fibroblasts, early passive range of motion may be advantageous in a post-repair joint environment. These findings may have implications for joint injury and in the setting of primary intraarticular ligament repair. Future work in our laboratory is focused on elucidating the complex interplay between these tissues and their responses to mechanical stimulus using co-culture models in order to optimize treatment strategies in cruciate ligament repair.

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Figures

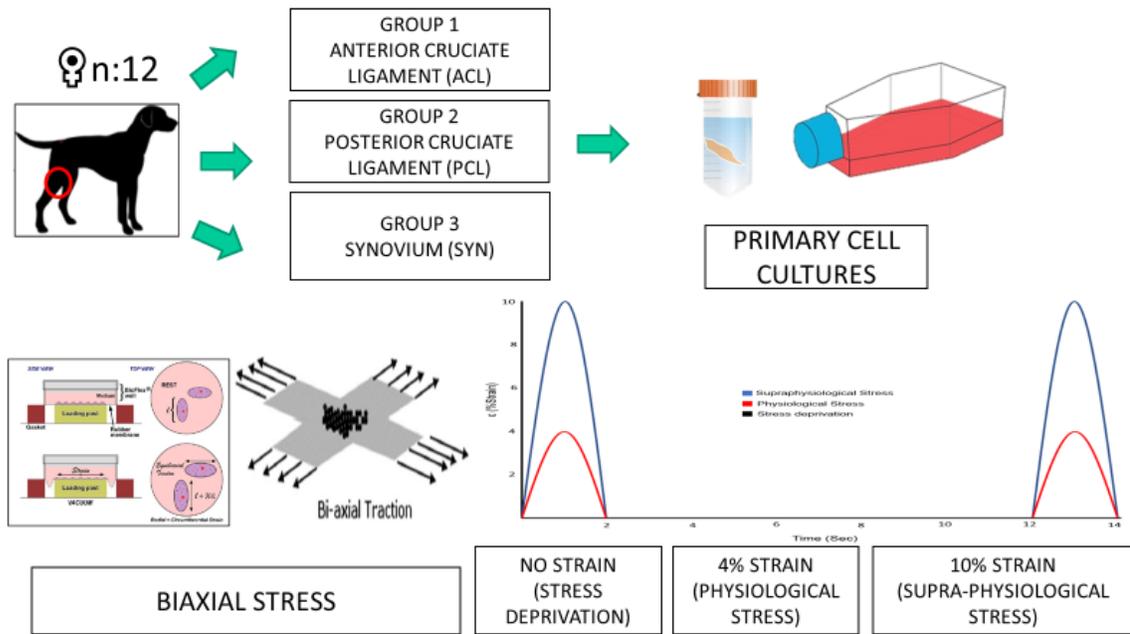


Figure 4.1. Experimental Design

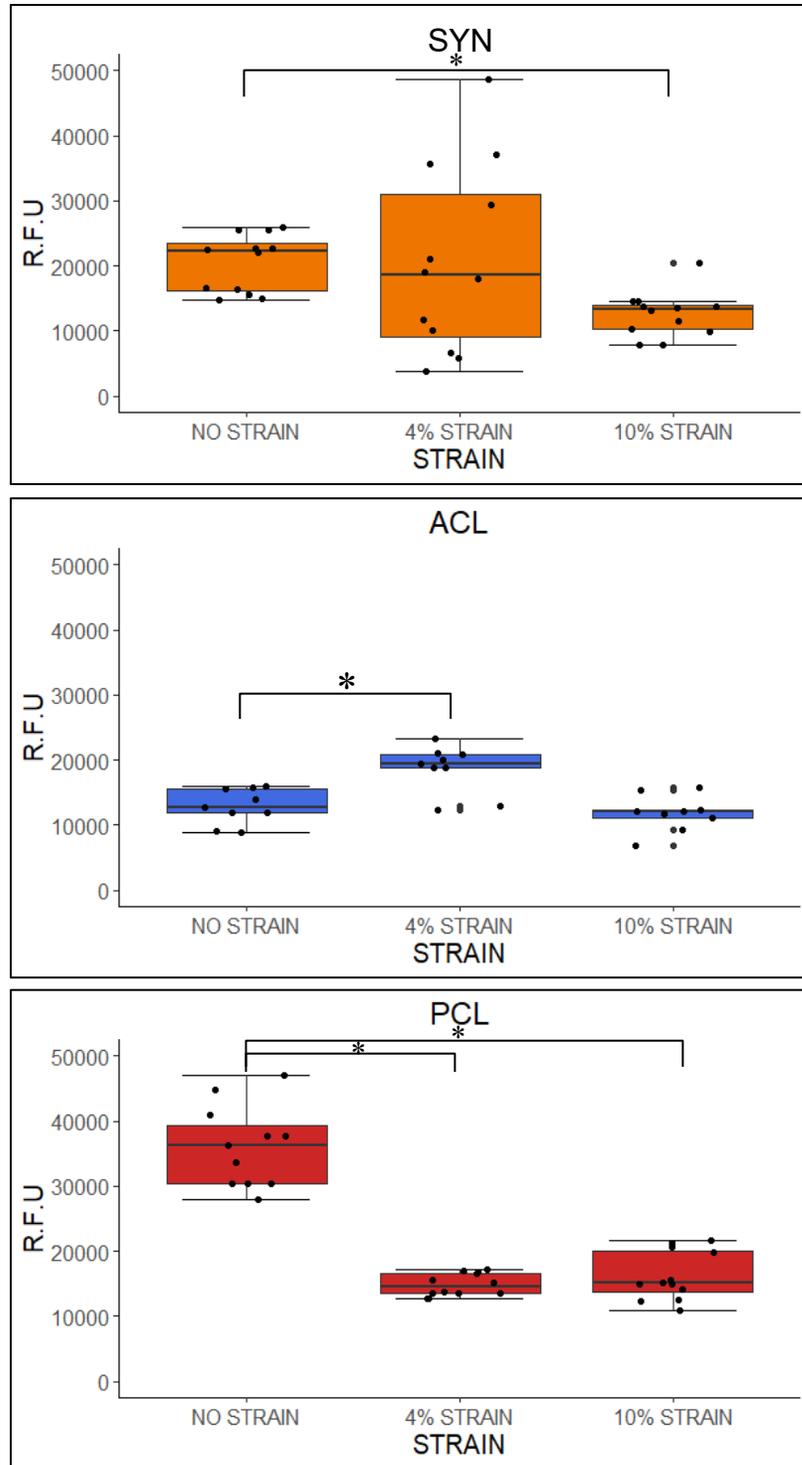


Figure 4.2. Metabolic Activity of fibroblasts. * significant difference between strains at 120 hours of culture. SYN: Synovium; ACL: Anterior cruciate ligament, PCL: Posterior cruciate ligament. R.F.U: Resazurin Fluorescent Units

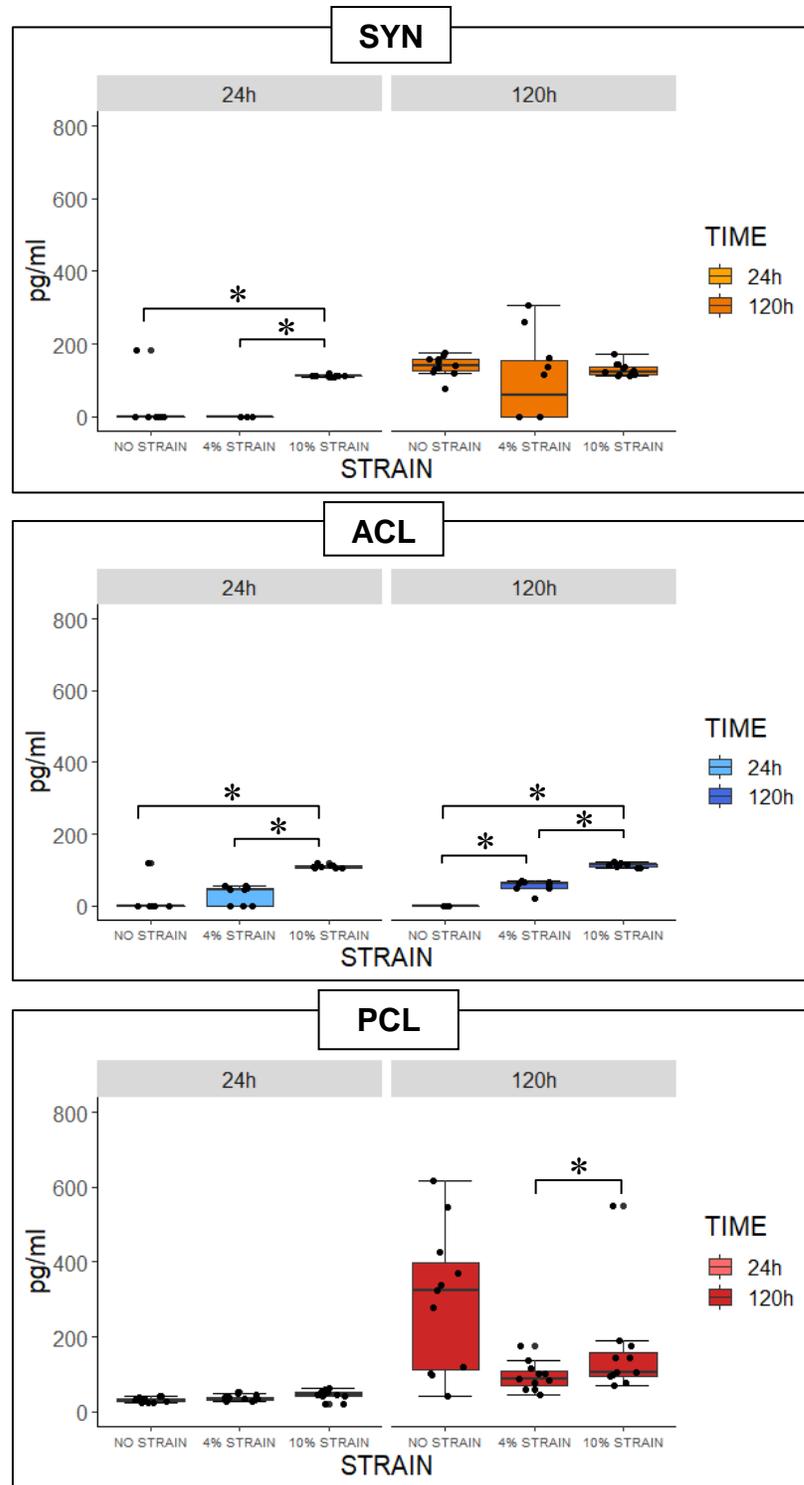


Figure 4.3. Concentration of PGE2 released to the media. * significant difference between strains at 24 and 120 hours of culture. SYN: Synovium; ACL: Anterior cruciate ligament, PCL: Posterior cruciate ligament

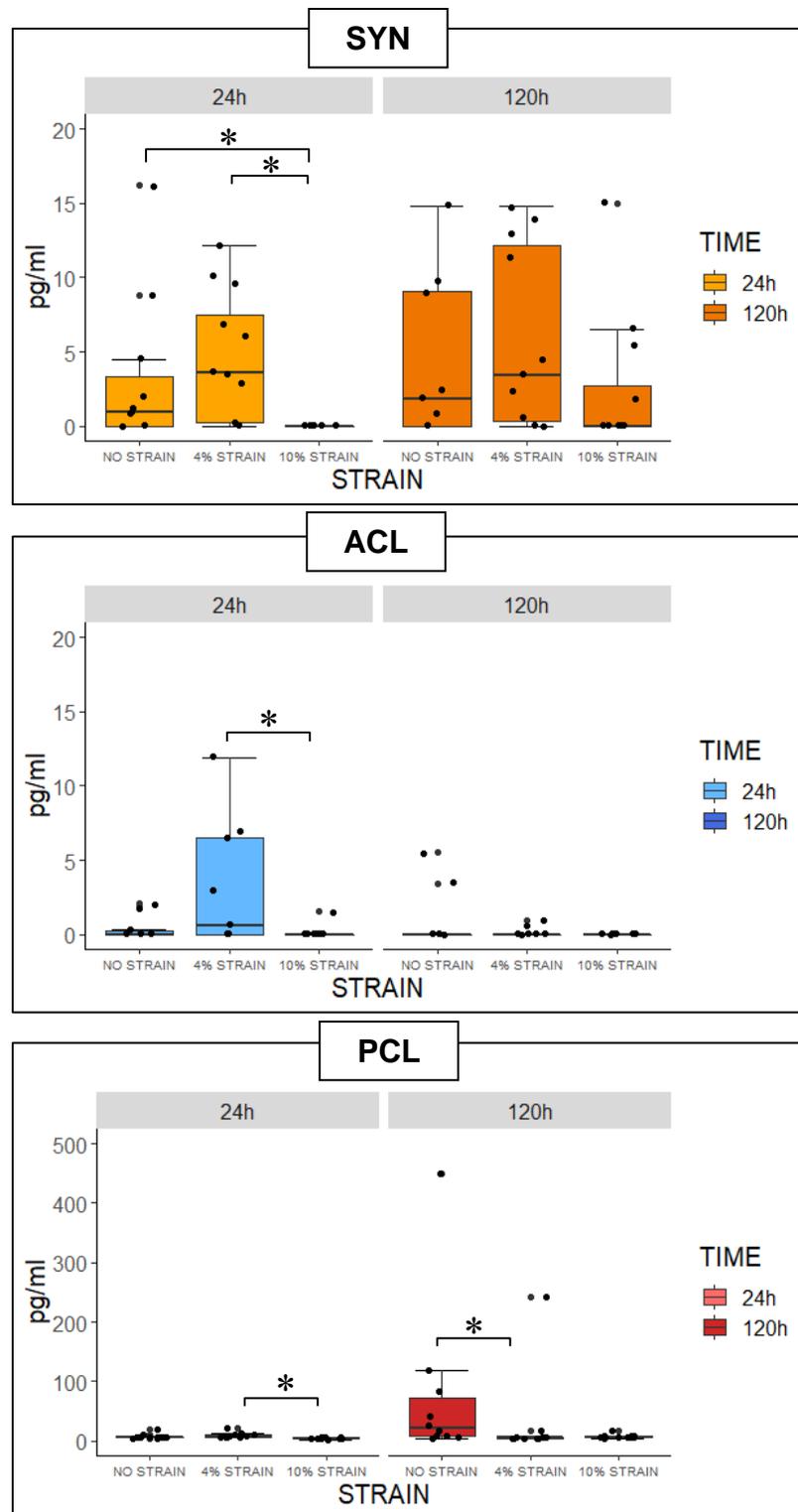


Figure 4.4. Concentration of IL-6 released to the media. * significant difference between strains at 24 and 120 hours of culture. SYN: Synovium; ACL: Anterior cruciate ligament, PCL: Posterior cruciate ligament

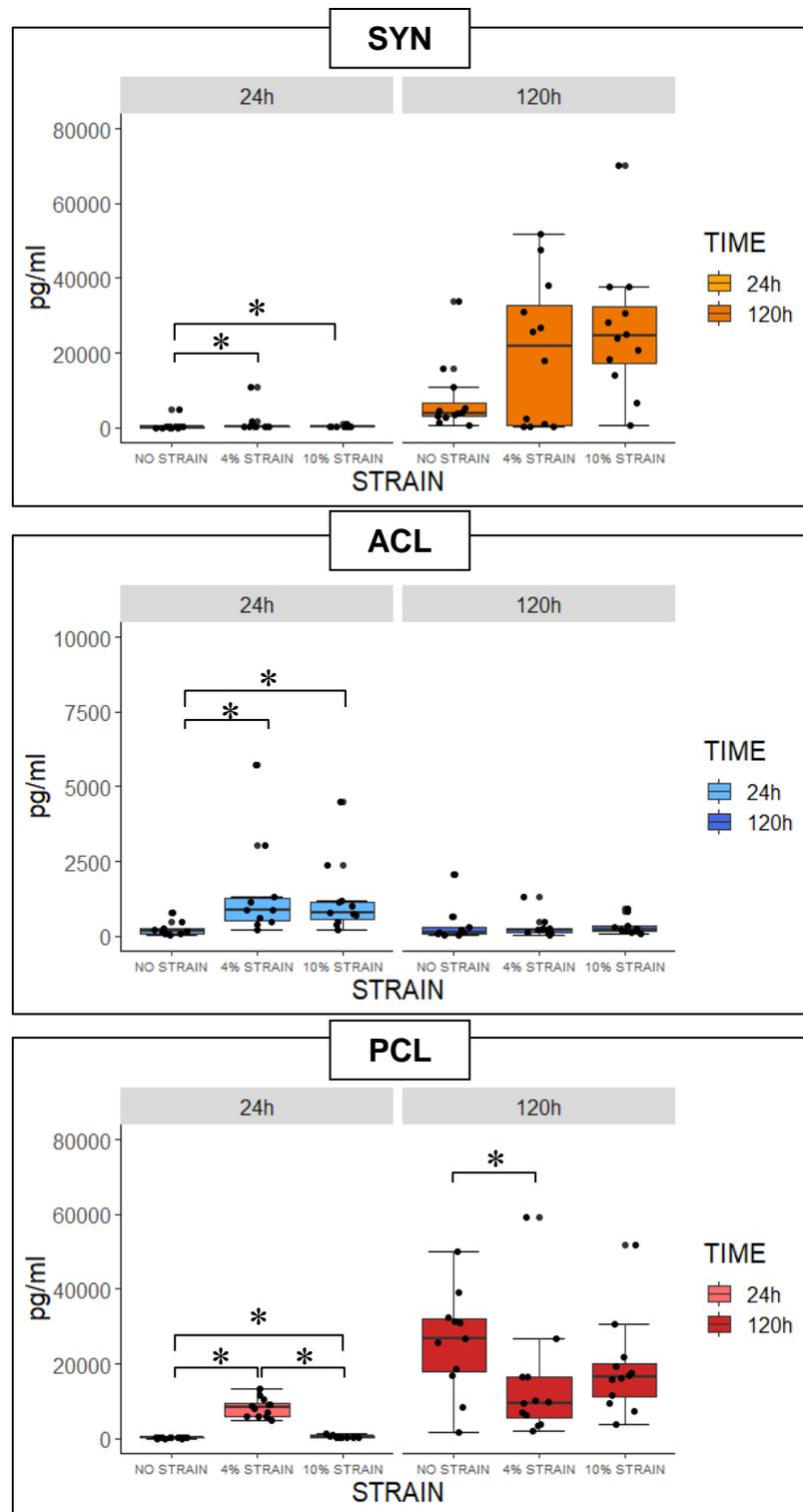


Figure 4.5. Concentration of IL-8 released to the media. * significant difference between strains at 24 and 120 hours of culture. SYN: Synovium; ACL: Anterior cruciate ligament, PCL: Posterior cruciate ligament

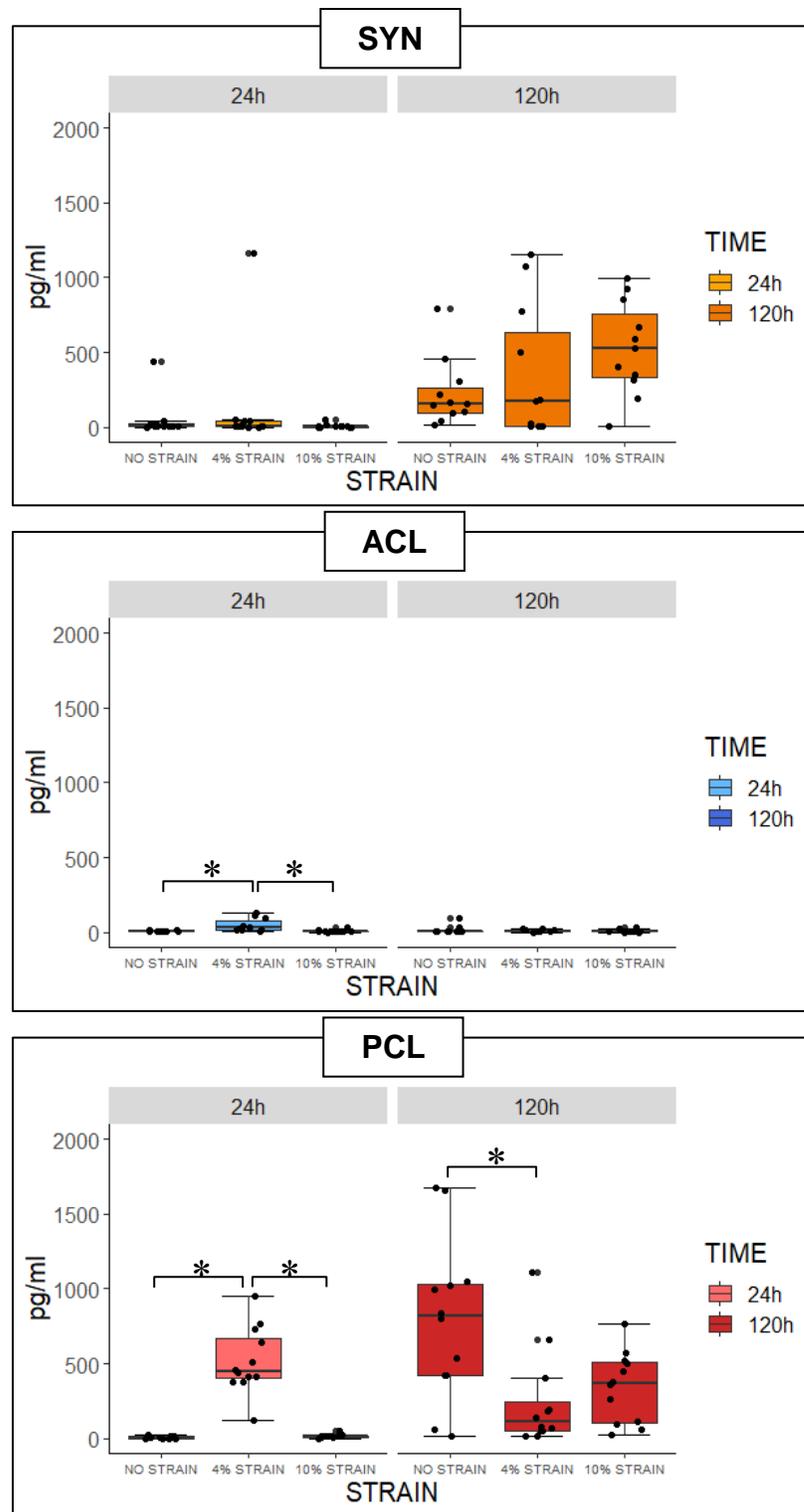


Figure 4.6. Concentration of KC released to the media. * significant difference between strains at 24 and 120 hours of culture. SYN: Synovium; ACL: Anterior cruciate ligament, PCL: Posterior cruciate ligament

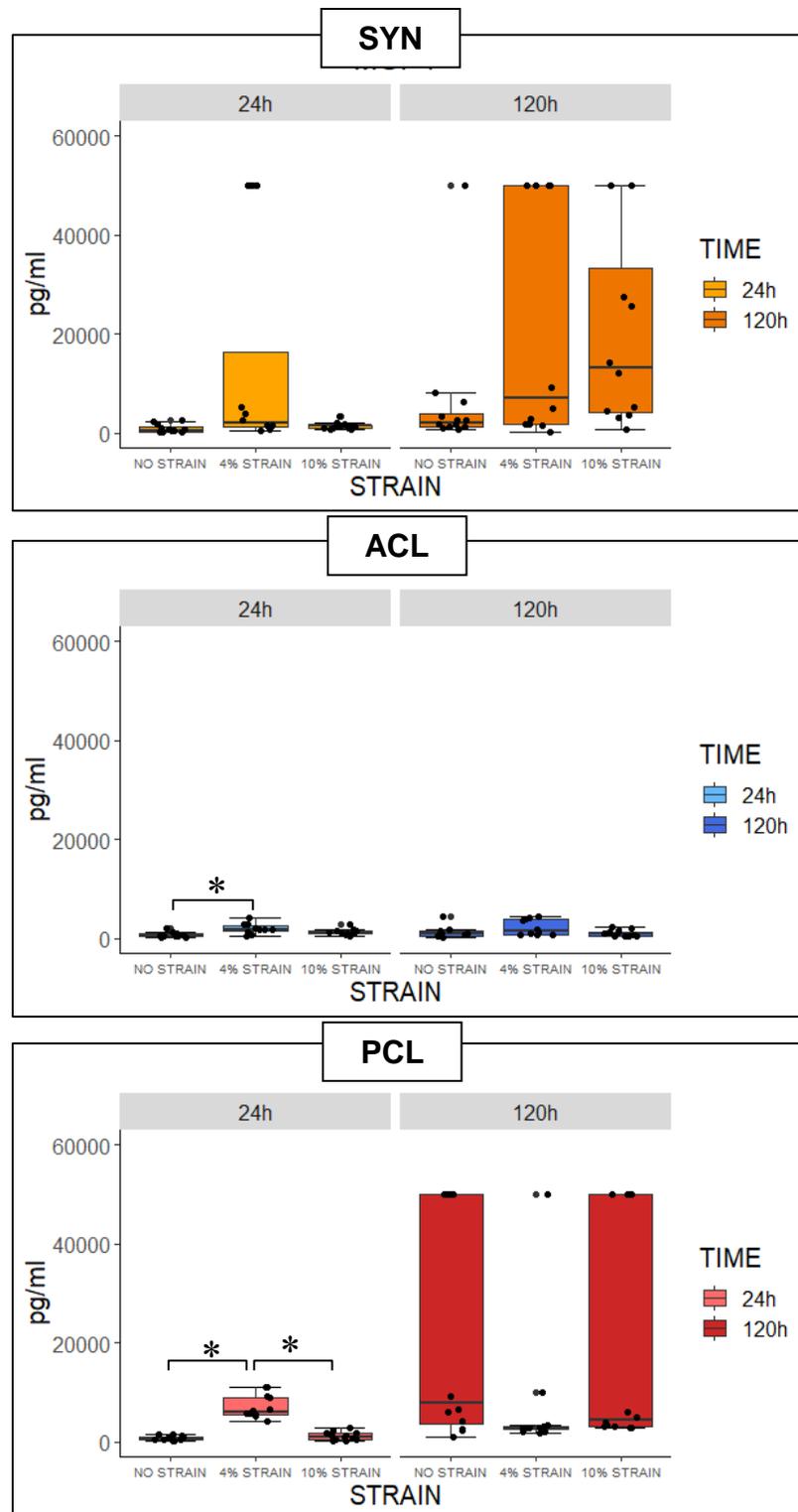


Figure 4.7. Concentration of MCP-1 released to the media. * significant difference between strains at 24 and 120 hours of culture. SYN: Synovium; ACL: Anterior cruciate ligament, PCL: Posterior cruciate ligament

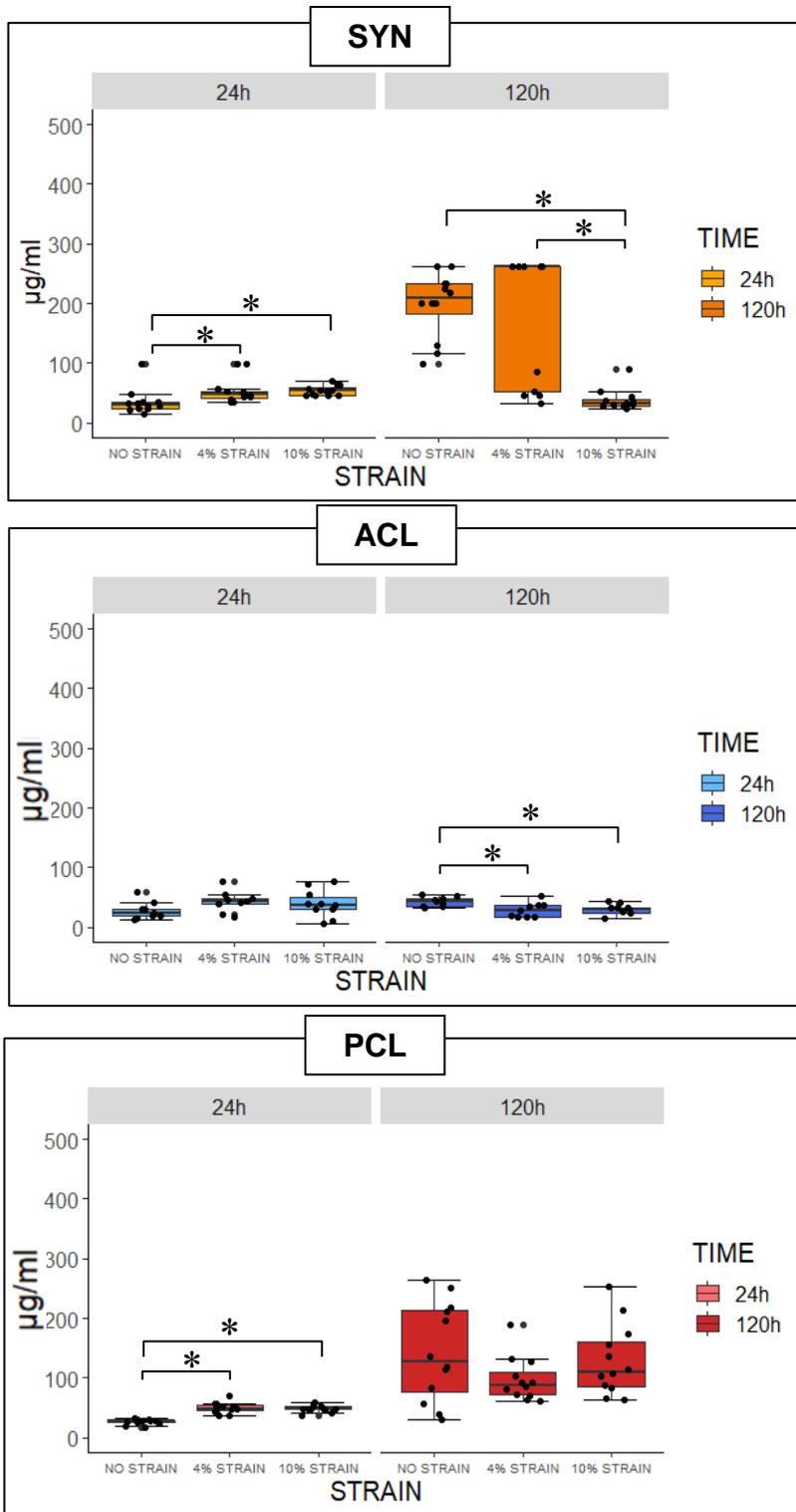


Figure 4.8. Concentration of Glycosaminoglycans (GAG) released to the media. * significant difference between strains at 24 and 120 hours of culture. SYN: Synovium; ACL: Anterior cruciate ligament, PCL: Posterior cruciate ligament

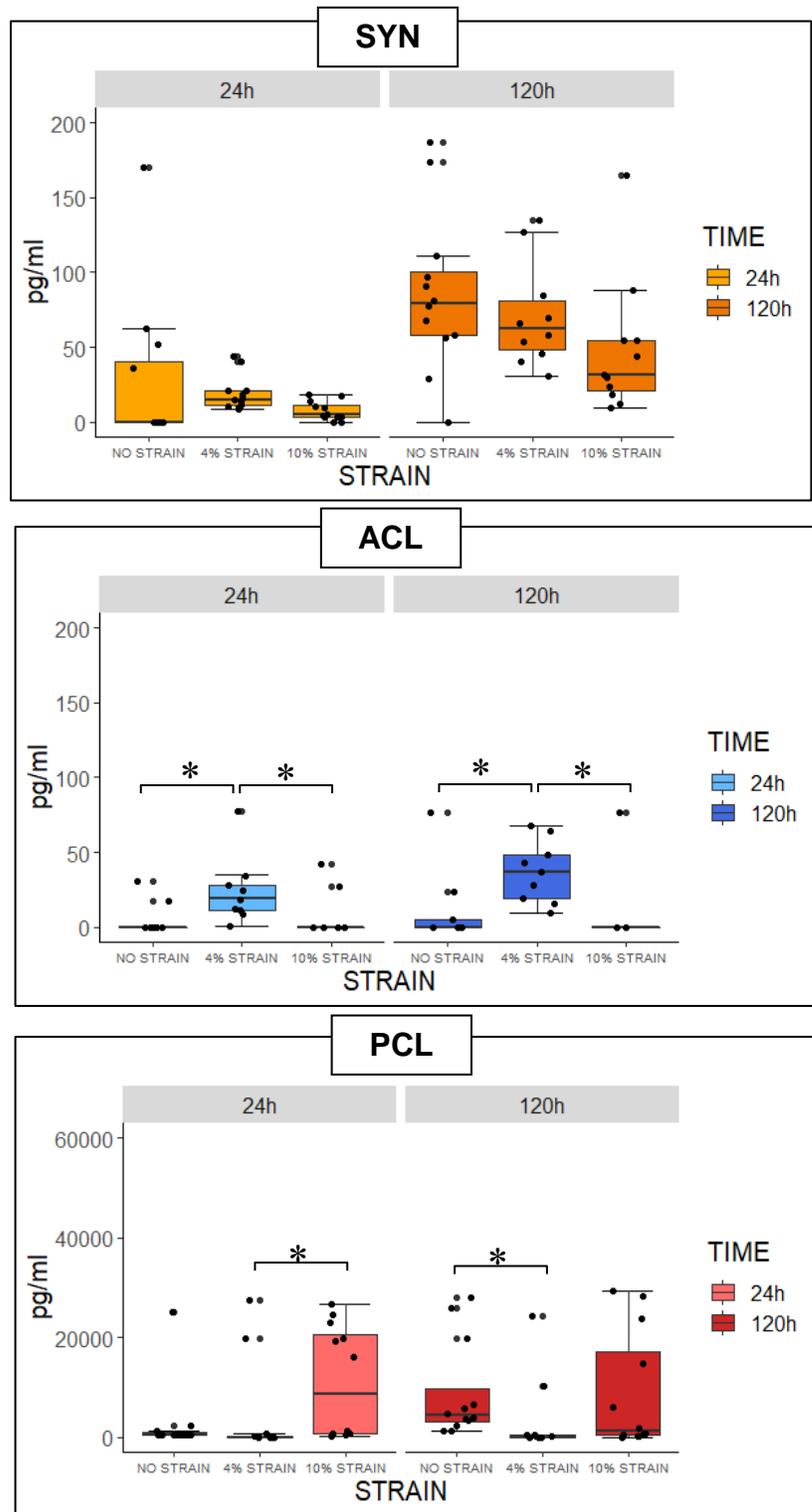


Figure 4.9. Concentration of total MMP activity (MMPACT) released to the media. * significant difference between strains at 24 and 120 hours of culture. SYN: Synovium; ACL: Anterior cruciate ligament, PCL: Posterior cruciate ligament

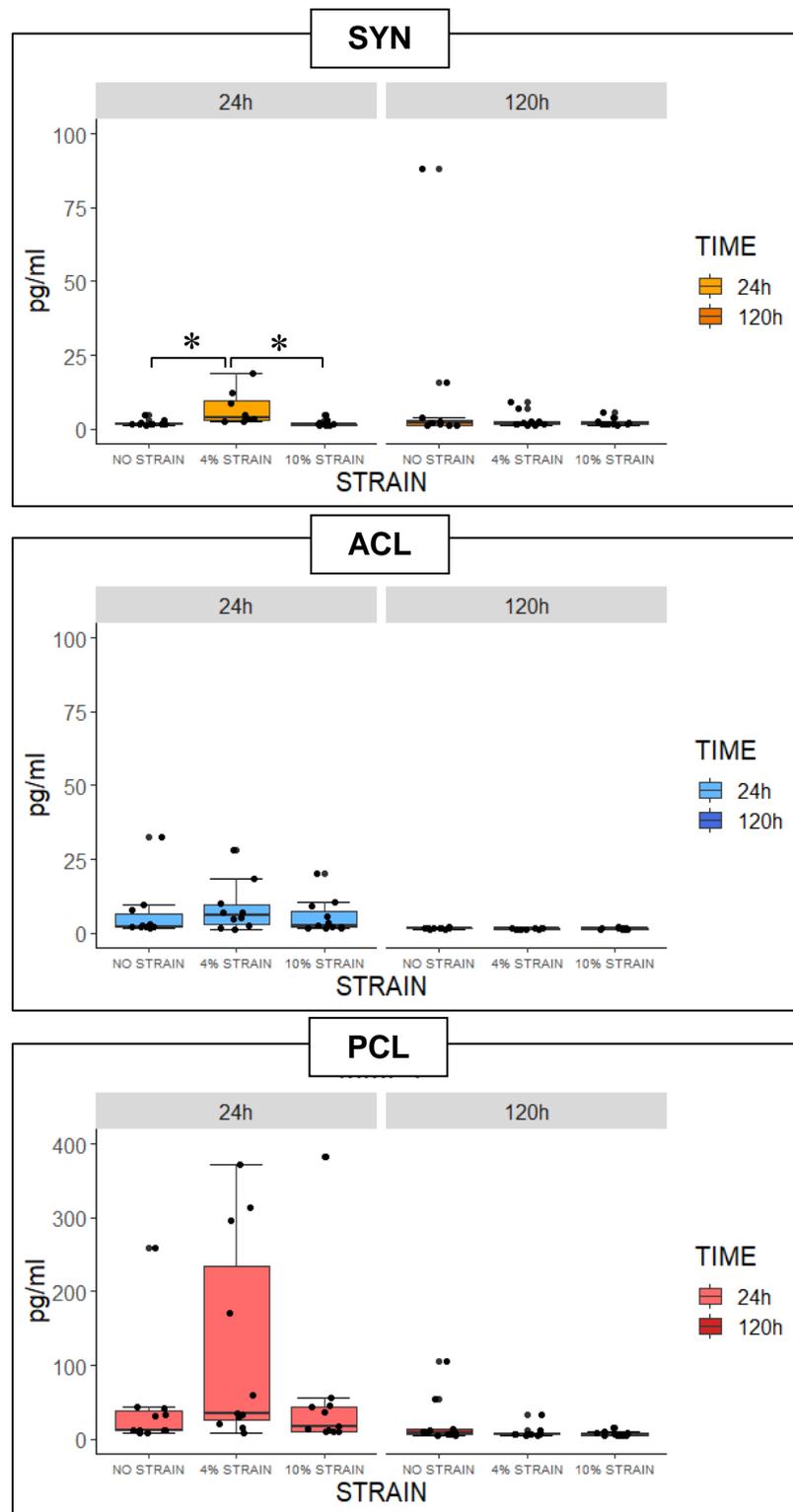


Figure 4.10. Concentration of matrix MMP-1 released to the media. * significant difference between strains at 24 and 120 hours of culture. SYN: Synovium; ACL: Anterior cruciate ligament, PCL: Posterior cruciate ligament

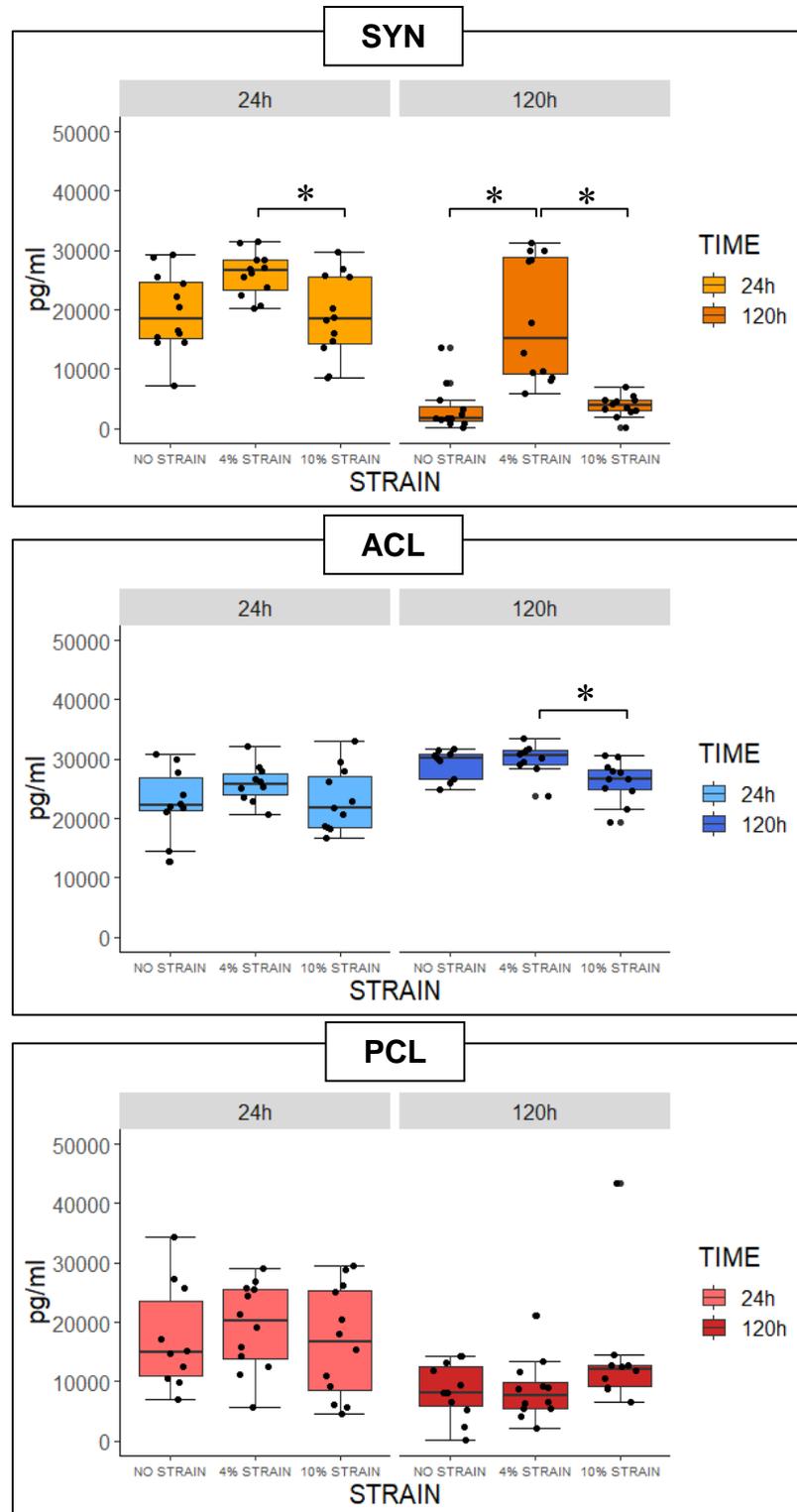


Figure 4.11. Concentration of matrix MMP-2 released to the media. * significant difference between strains at 24 and 120 hours of culture. SYN: Synovium; ACL: Anterior cruciate ligament, PCL: Posterior cruciate ligament

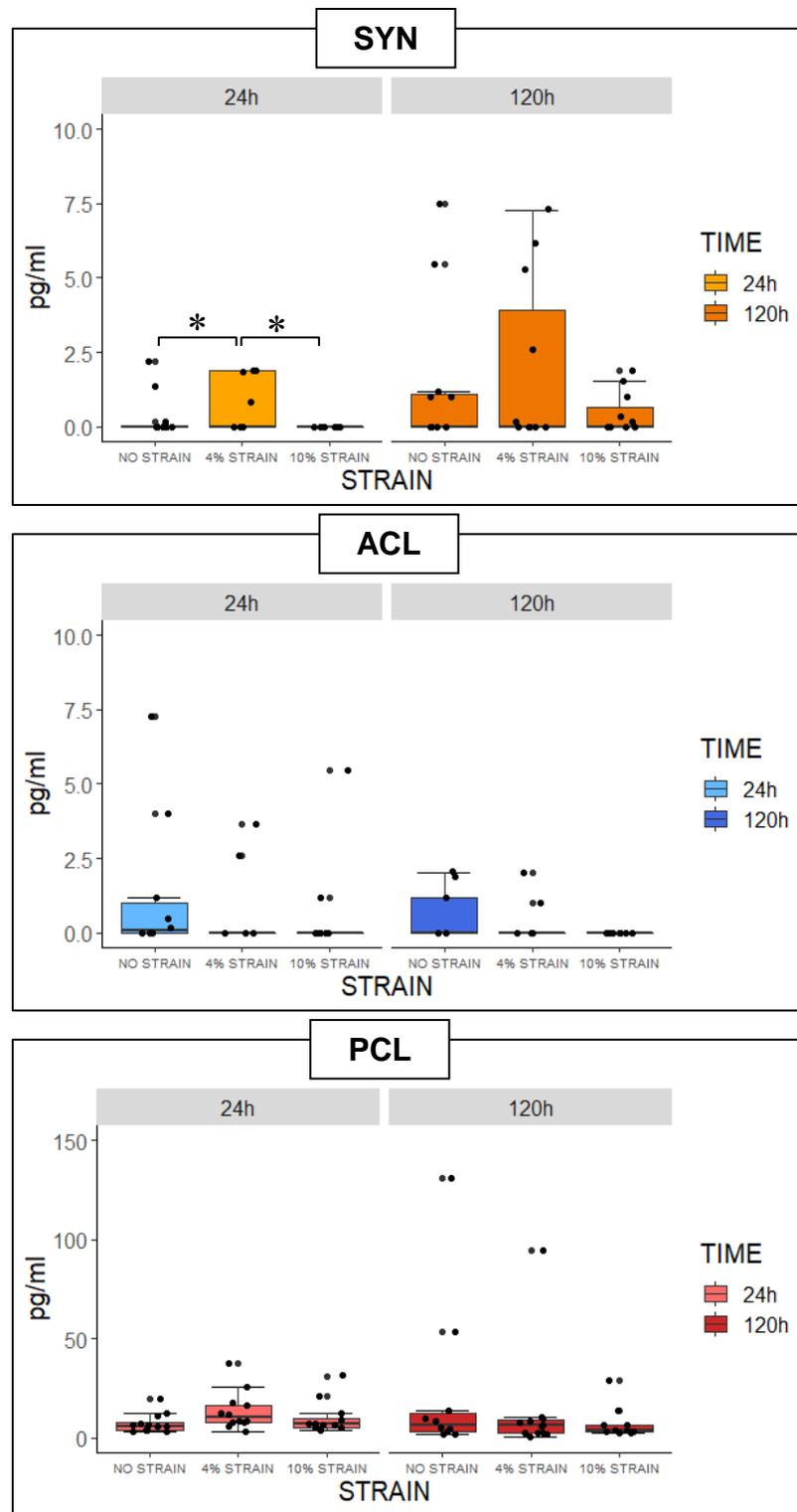


Figure 4.12. Concentration of matrix MMP-3 released to the media. * significant difference between strains at 24 and 120 hours of culture. SYN: Synovium; ACL: Anterior cruciate ligament, PCL: Posterior cruciate ligament

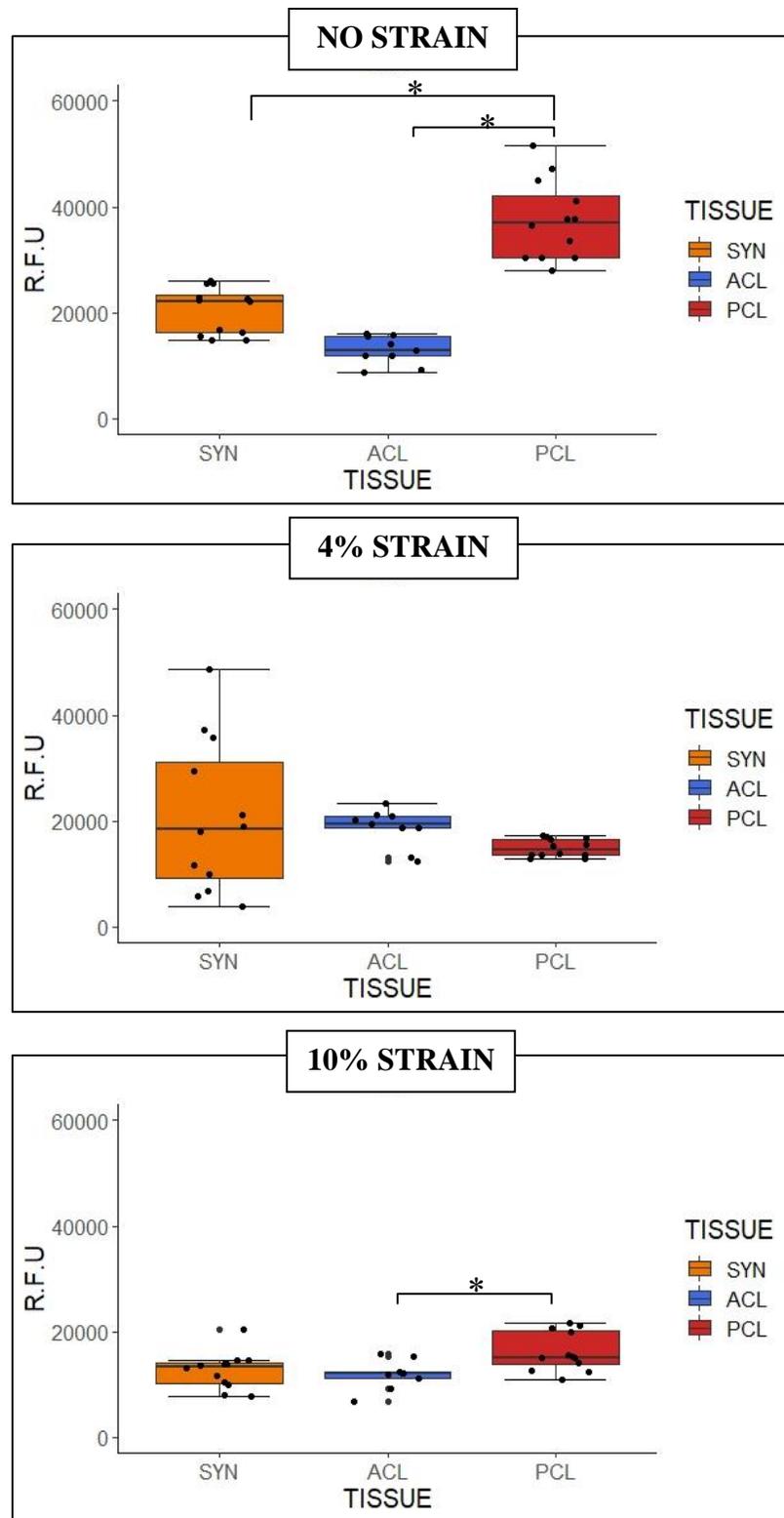


Figure 4.13. Metabolic Activity of fibroblasts. * significant difference between tissues at 120 hours of culture. SYN: Synovium; ACL: Anterior cruciate ligament, PCL: Posterior cruciate ligament. R.F.U: Resazurin Fluorescent Units

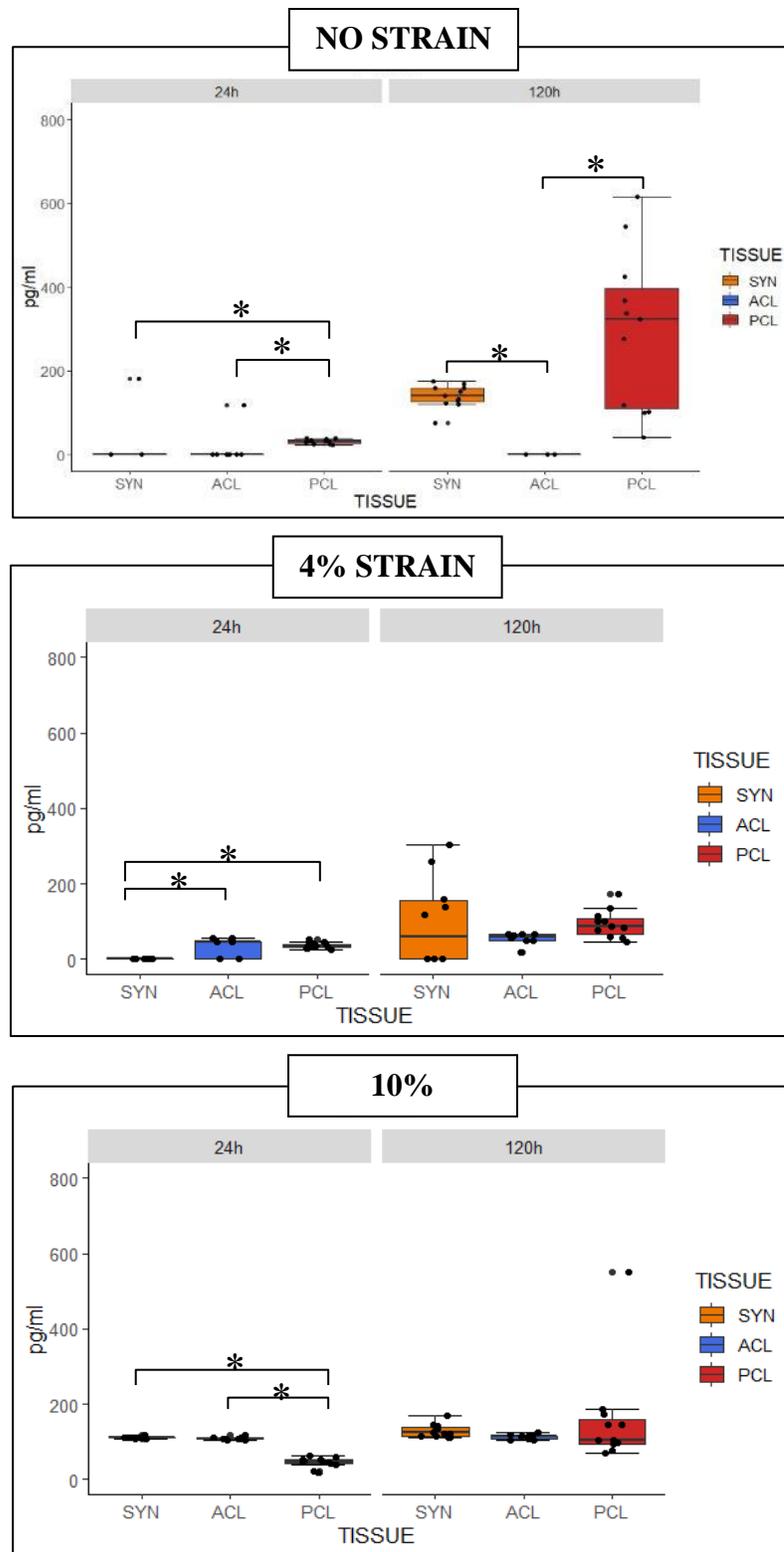


Figure 4.14. Concentration of PGE2 released to the media. * significant difference between tissues at 24 and 120 hours of culture. SYN: Synovium; ACL: Anterior cruciate ligament, PCL: Posterior cruciate ligament

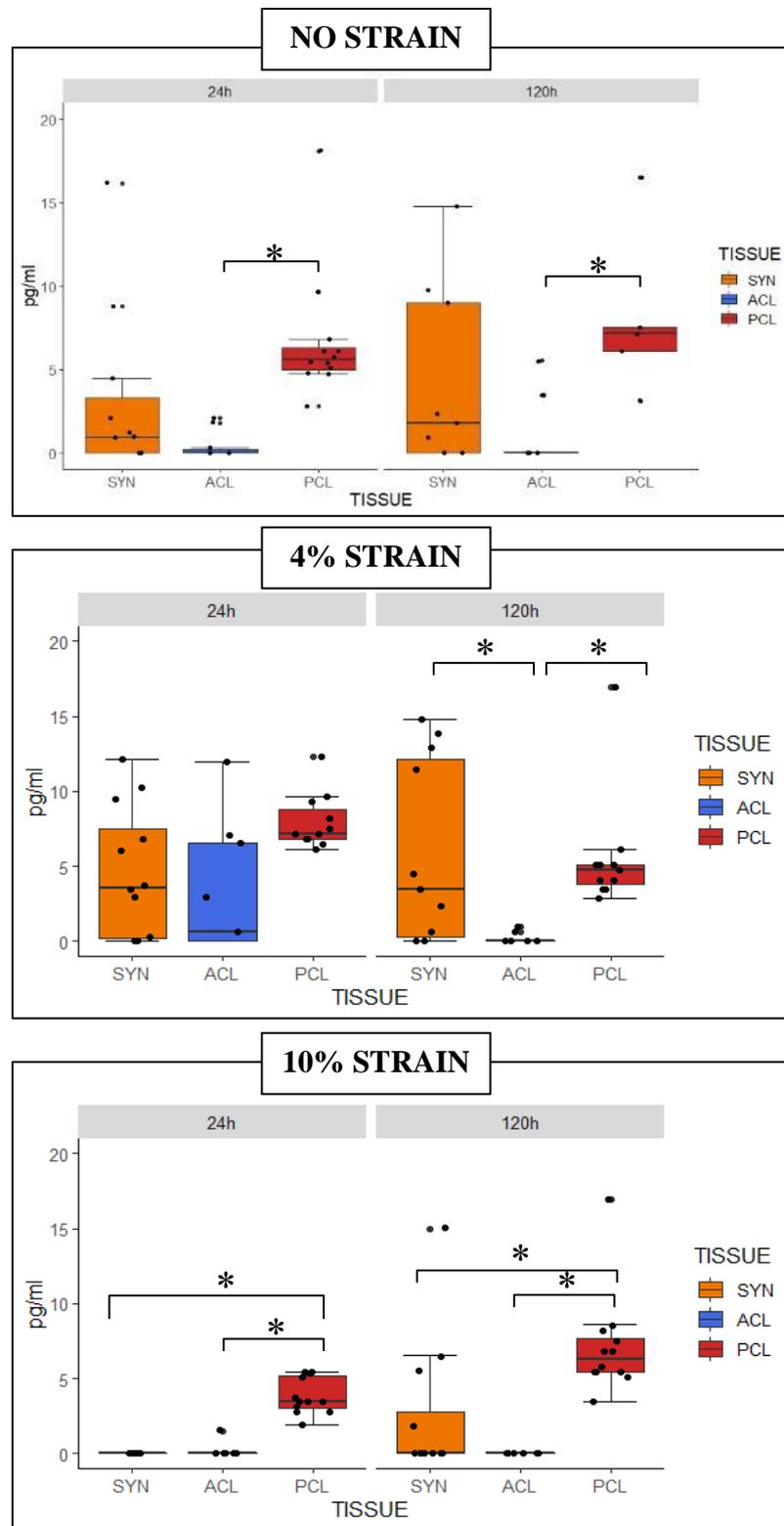


Figure 4.15. Concentration of IL-6 released to the media. * significant difference between tissues at 24 and 120 hours of culture. SYN: Synovium; ACL: Anterior cruciate ligament, PCL: Posterior cruciate ligament

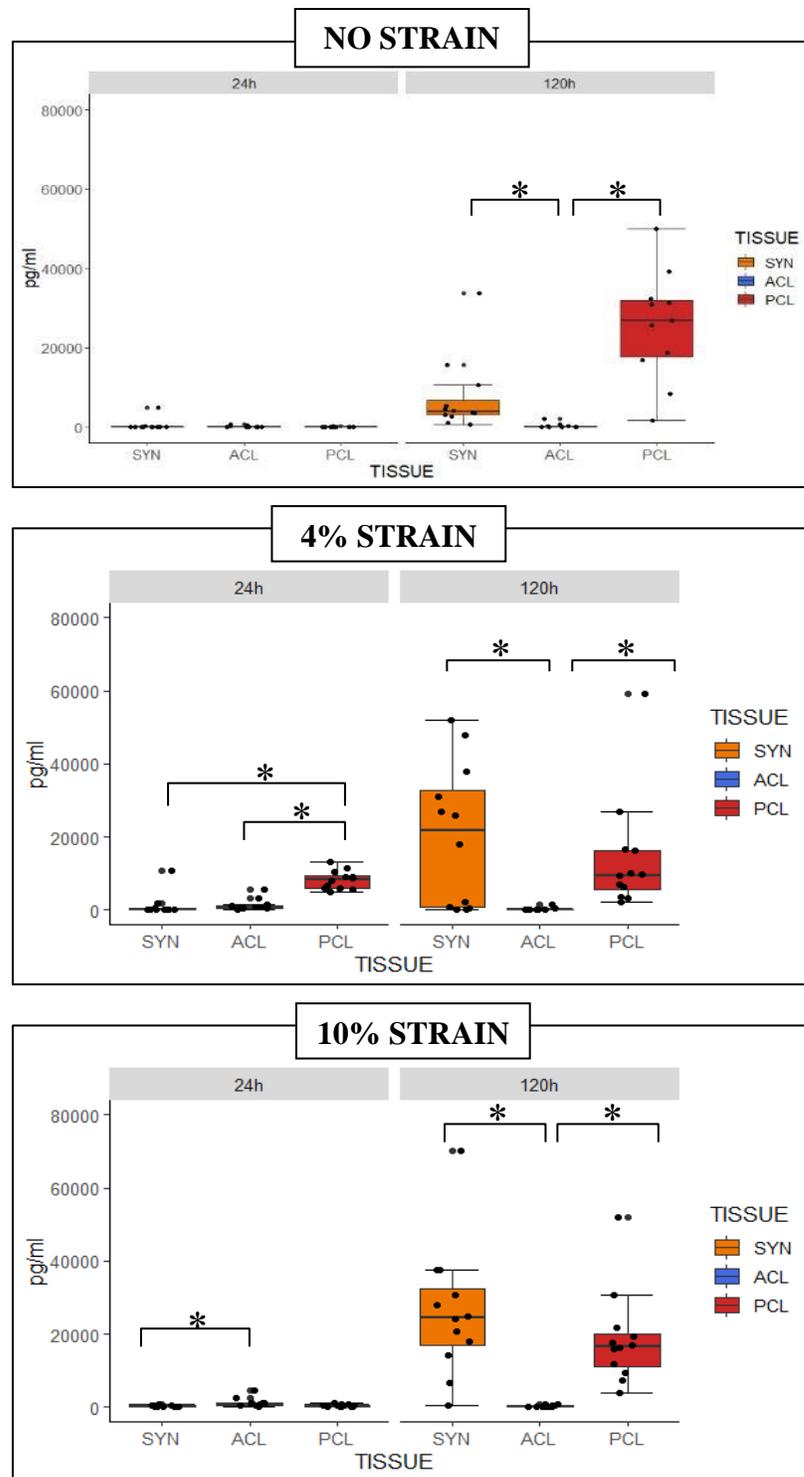


Figure 4.16. Concentration of IL-8 released to the media. * significant difference between tissues at 24 and 120 hours of culture. SYN: Synovium; ACL: Anterior cruciate ligament, PCL: Posterior cruciate ligament

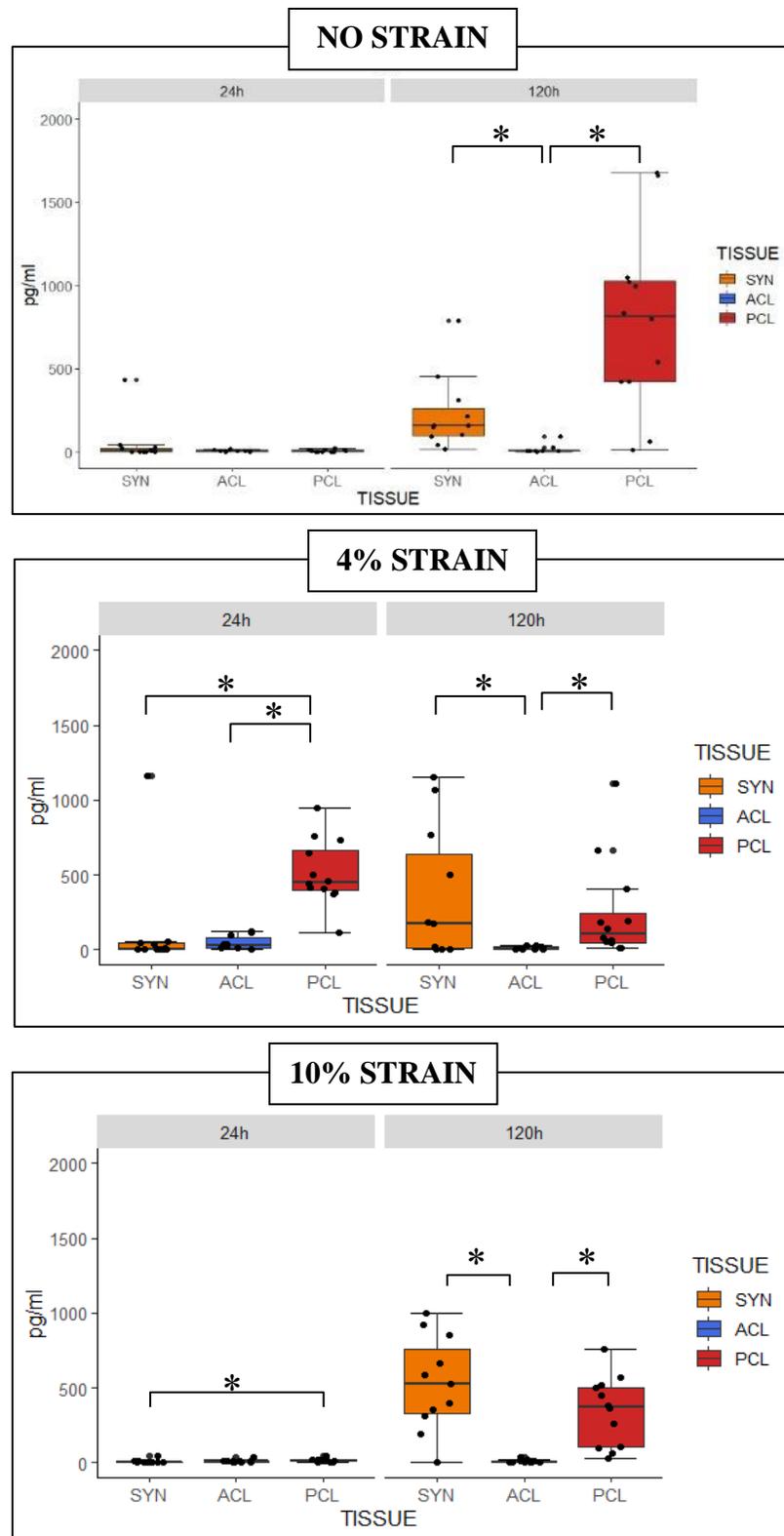


Figure 4.17. Concentration of KC released to the media. * significant difference between tissues at 24 and 120 hours of culture. SYN: Synovium; ACL: Anterior cruciate ligament, PCL: Posterior cruciate ligament

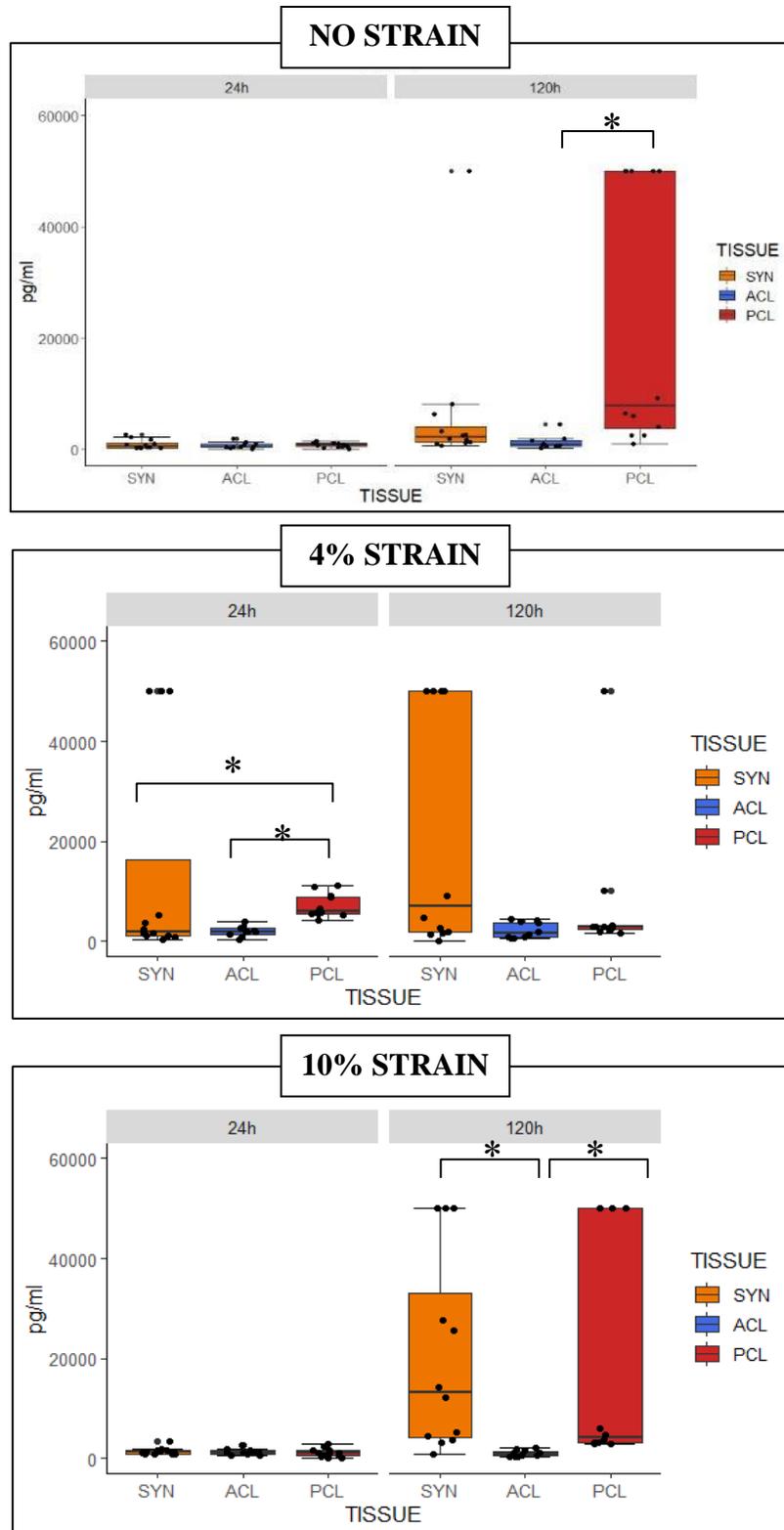


Figure 4.18. Concentration of MCP1 released to the media. * significant difference between tissues at 24 and 120 hours of culture. SYN: Synovium; ACL: Anterior cruciate ligament, PCL: Posterior cruciate ligament

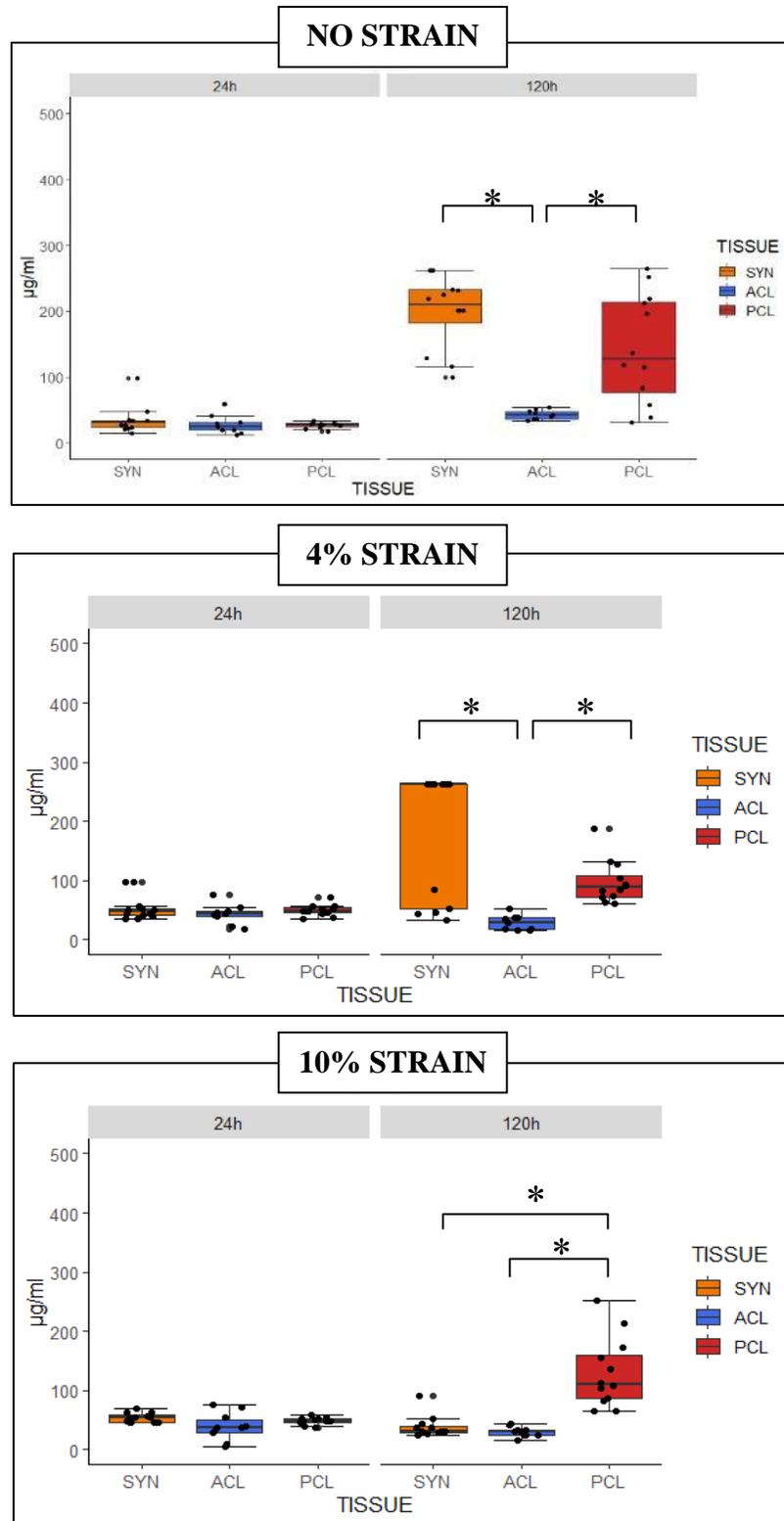


Figure 4.19. Concentration of GAG released to the media. * significant difference between tissues at 24 and 120 hours of culture. SYN: Synovium; ACL: Anterior cruciate ligament, PCL: Posterior cruciate ligament

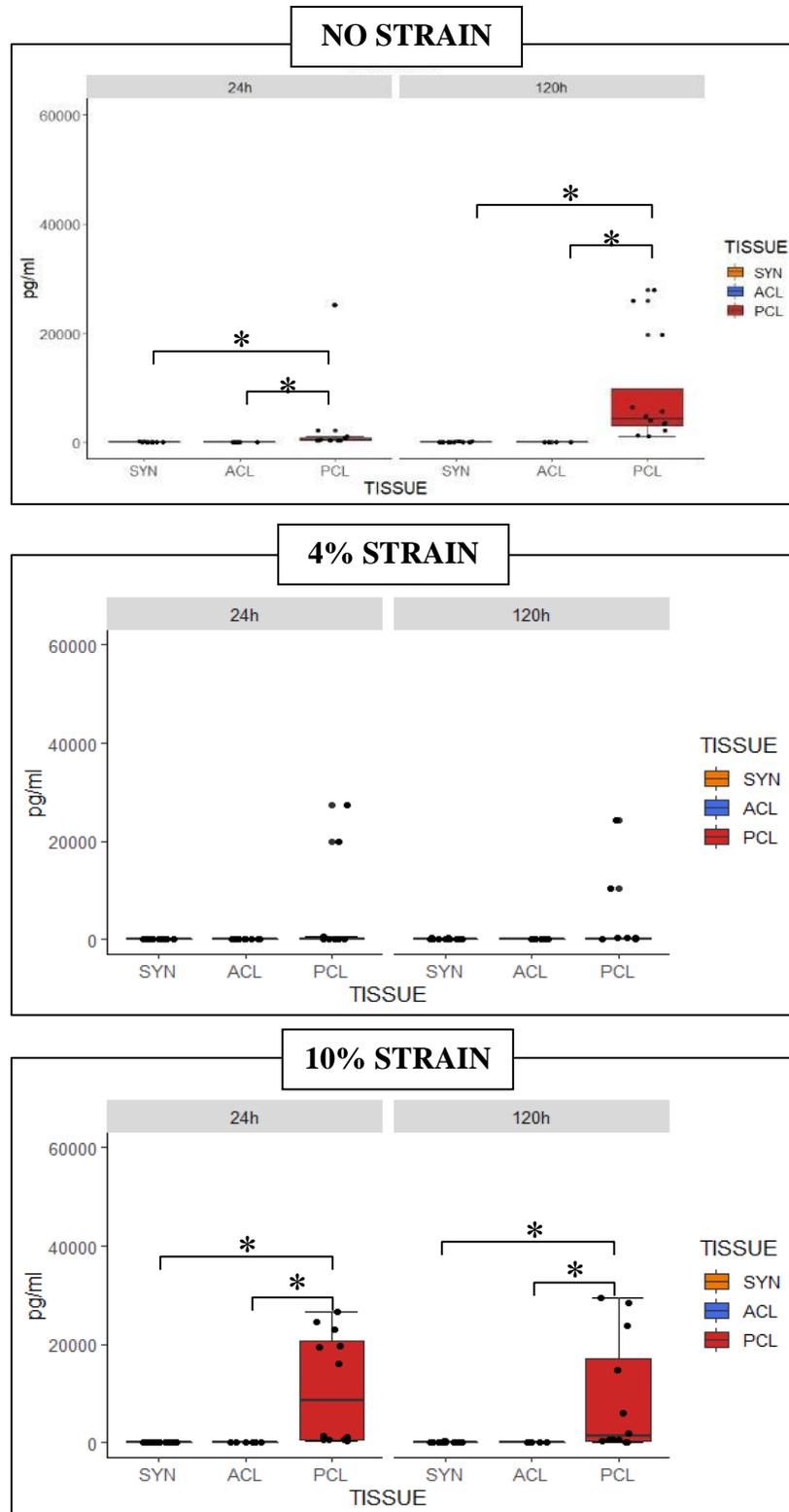


Figure 4.20. Concentration of MMP Activity (MMPACT) in the media. * significant difference between tissues at 24 and 120 hours of culture. SYN: Synovium; ACL: Anterior cruciate ligament, PCL: Posterior cruciate ligament

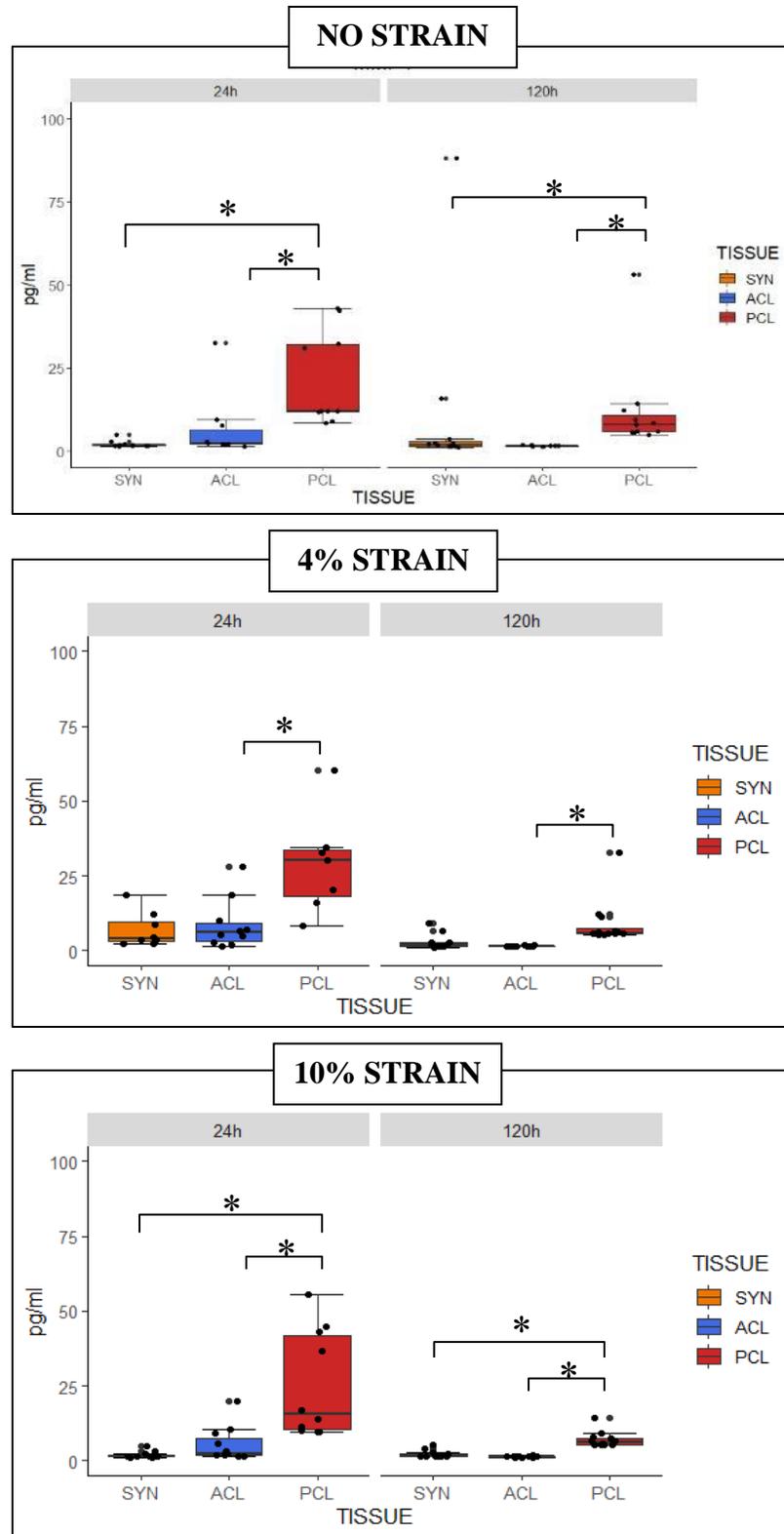


Figure 4.21. Concentration of MMP-1 released to the media. * significant difference between tissues at 24 and 120 hours of culture. SYN: Synovium; ACL: Anterior cruciate ligament, PCL: Posterior cruciate ligament

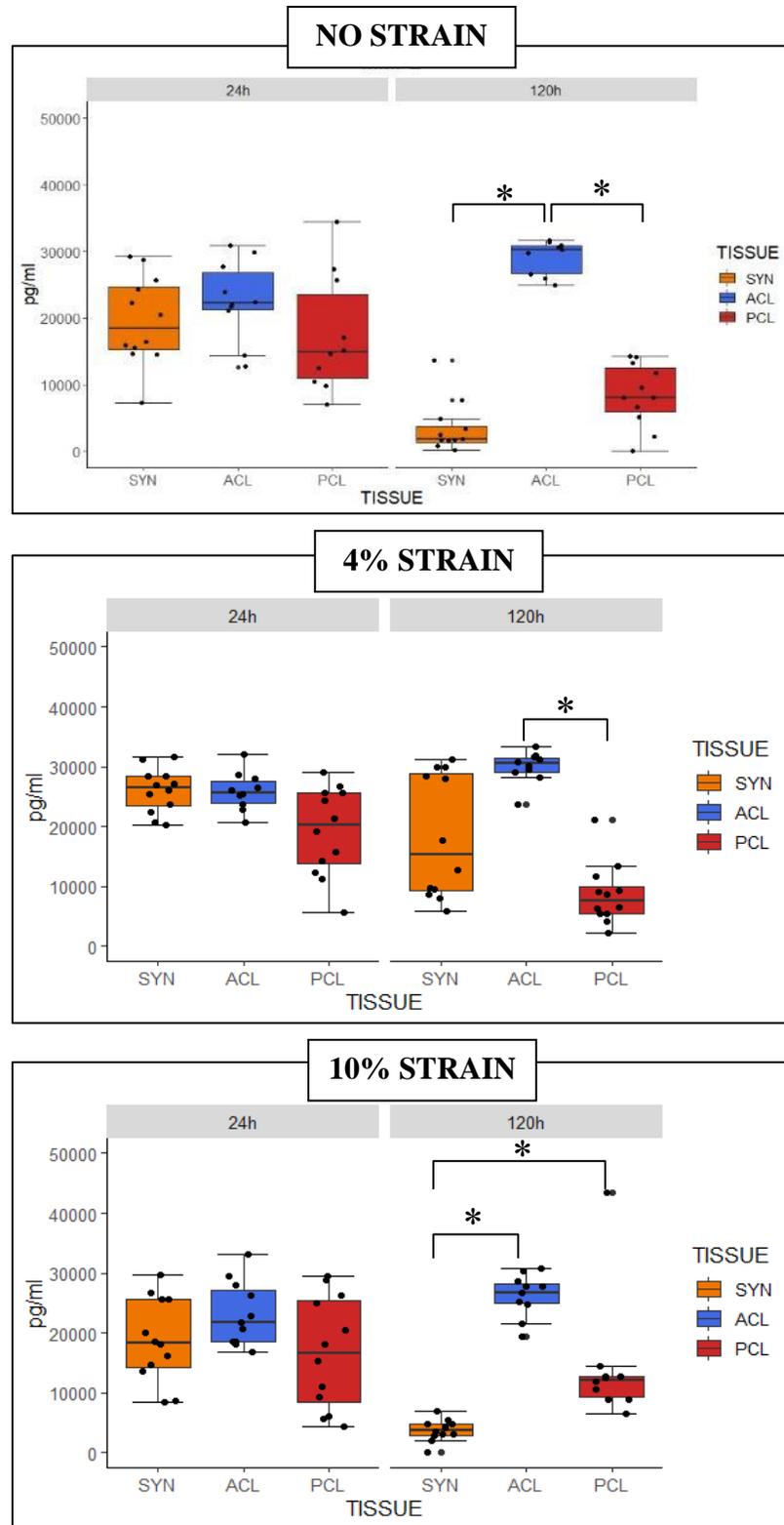


Figure 4.22. Concentration of MMP-2 released to the media. * significant difference between tissues at 24 and 120 hours of culture. SYN: Synovium; ACL: Anterior cruciate ligament, PCL: Posterior cruciate ligament

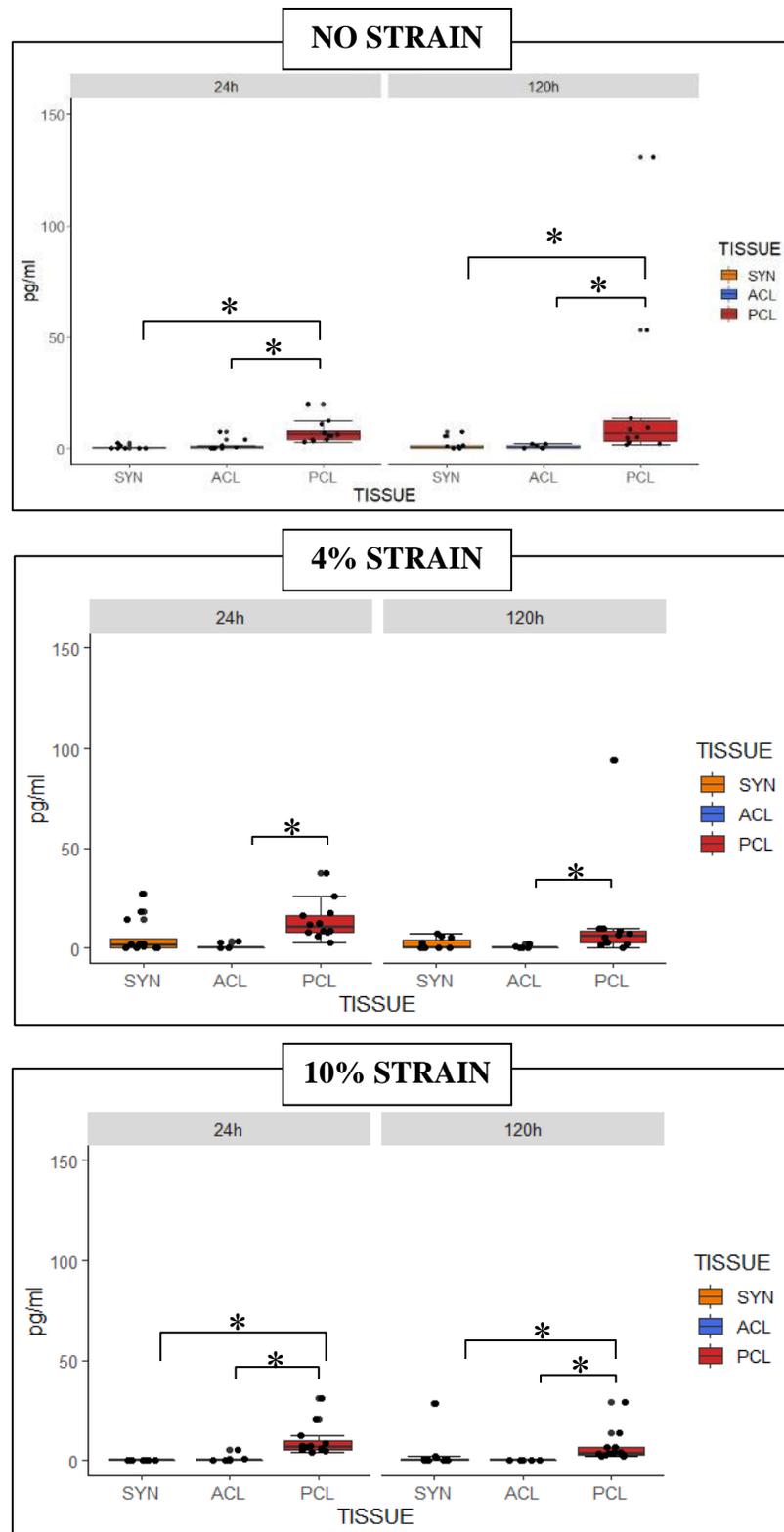


Figure 4.23. Concentration of MMP-3 released to the media. * significant difference between tissues at 24 and 120 hours of culture. SYN: Synovium; ACL: Anterior cruciate ligament, PCL: Posterior cruciate ligament

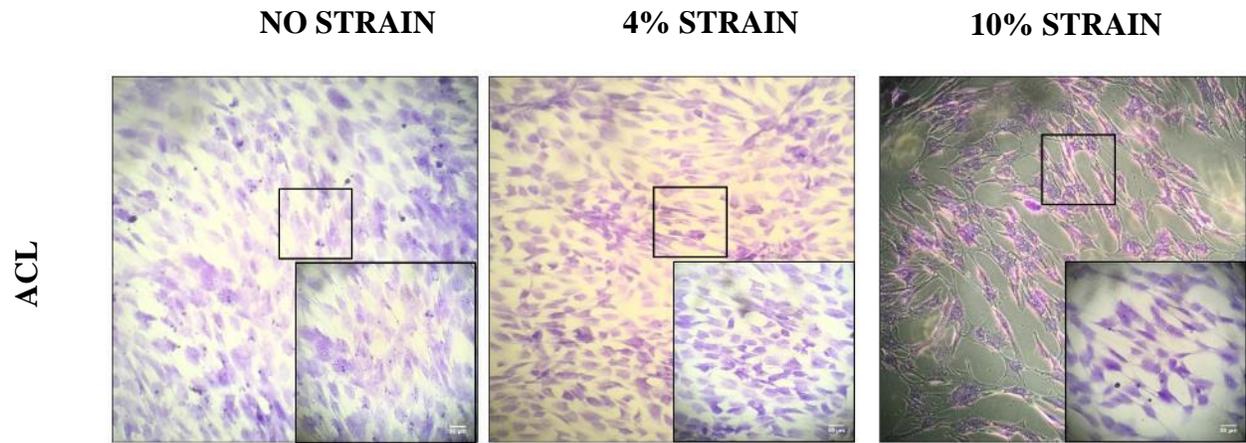


Figure 4.24. Anterior cruciate Ligament (ACL) fibroblasts after being stretched for 120 hours.

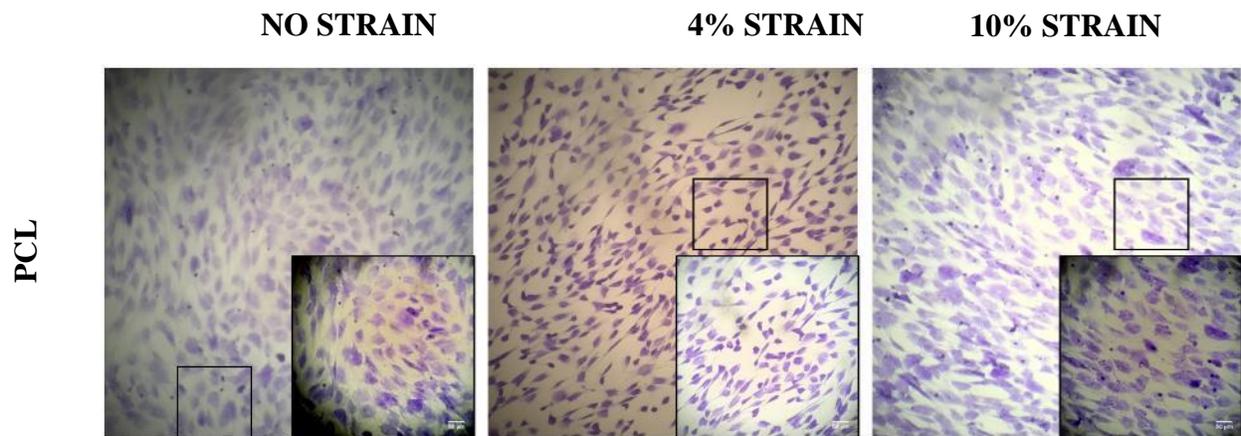


Figure 4.25. Posterior cruciate Ligament (PCL) fibroblasts after being stretched for 120 hours.

CHAPTER 5

SEX DIFFERENCES IN ANTERIOR CRUCIATE LIGAMENT TENDON GRAFT RESPONSE TO MECHANICAL STRAIN

Introduction:

Graft choice is one of the main topics of discussion between knee surgeons in regards of anterior cruciate ligament (ACL) reconstruction.¹ The ideal graft must possess good strength to allow accelerated rehabilitation, have no donor site morbidity, be readily available and heals and incorporates quickly into the bone tunnel². Several factors have been associated with poor outcomes or re-injury after ACL reconstruction including poor surgical technique, (inappropriate position of the bone tunnel, inadequate graft pre-tension, improper graft fixation), non-adequate biomechanical factors, failure of graft incorporation, and re-injury due to trauma.^{1; 3-5} However, the effect of sex on ACL reconstruction surgery is unknown.

There are known sex disparities in ACL injury and healing after surgery. ACL injury is two to five times more common in female athletes than in their male counterparts.^{6;7} Main reasons for increased female ACL rupture rates include increased femoral intercondylar notch height and plateau slope, decreased condylar width and notch angle, altered kinematic patterns and neuromuscular control showing lower limb alignment, smaller flexion angles, greater hip internal rotation angles, lower gluteus maximus activation and greater quadriceps-to-hamstring ratio activation in females compared to males.⁸⁻¹³ Similarly, females also show differences in intrinsic ACL material properties compared to males including decreased length, area and volume which in turn results in lower ultimate stress and stiffness.^{8; 14} Additionally, the effect of female hormones and hormonal variation

has been suggested as a possible cause for increased laxity and consequent rupture of the ACL in females.¹⁵⁻¹⁷ There is also ongoing debate whether sex is an adverse risk factor for patient outcomes after ACL surgery. While Thompson *et al.*, (2016) showed increased ACL graft rupture risk associated to male sex, Tan *et al.*, (2015) showed increased relative risk of revision surgery in females.^{18; 19} Therefore, the effect of sex on the probability of graft failure in ACL reconstruction is an active research topic.

In addition to sex, graft choice may impact success after ACL surgery. The biological differences of various tendon autografts used for ACL reconstruction are not well understood. Early ACL graft healing and remodeling require a fine balance between extracellular matrix (ECM) degradation and synthesis. A precise inflammatory response is also likely necessary to drive an effective healing response.²⁰⁻²² Evidence provided by human and animal studies on ACL healing indicates that the early biological environment likely play an important role in ACL graft maturation, and that the metabolism of the graft is further modified by biomechanical cues.^{21; 23} Tensile forces can alter responses of ligament fibroblasts to various stimuli, including sex hormone regulation.²⁴ However, it is not known if the sex of the patient is associated with significant differences in the cell's response to tensile forces. Further, the complex interplay between the mechanical environment of specific tissues used for ACL reconstruction and sex are still poorly understood. The objective of the current study is to determine if there are sex related differences in the biological responses of fibroblasts obtained from common ACL autografts to varying levels of mechanical strain. We aimed to evaluate the differences between male and female tendon graft-derived fibroblasts to different levels of cyclic strains that simulate clinical conditions. We hypothesized that male ACL graft fibroblasts

from quadriceps tendon (QT), patellar tendon (PT), and hamstring tendon (HT) will exhibit significantly lower degradative and inflammatory levels compared to female autograft fibroblasts.

Materials and methods:

Tissue processing and culture

Male (7 QT, 9 PT, 5 HT) and female (12QT, 12PT, 9HT) tissues were harvested from skeletally mature dogs (9 and 12 respectively) euthanatized for studies unrelated to the current work. Tissues were aseptically minced into 0.5–1cm² pieces and digested overnight in Type 1A *Clostridium histolyticum* collagenase solution (Sigma-Aldrich, St. Louis, MO, USA) at a concentration of 0.5 mg/mL. The aliquot was then suspended with nutritional media (Gibco DMEM, Thermo Fisher Scientific, Waltham, MA, USA) containing 10% FBS, 0.002% Penicillin, 100 µg/mL Streptomycin, 25 µg/mL Amphotericin B, 0.002% L-Ascorbate and 0.01% L-glutamine (Sigma Chemical Co, St. Louis, MO), cultured in T75 flasks (TPP Techno Plastic Products AG, Trasadingen, Switzerland) and incubated at 5% CO₂, 37°C, and 95% humidity.

Biaxial mechanical stress

Once confluent, passage one cells were exposed to TrypLE express (Invitrogen, Carlsbad, CA) for five minutes, then resuspended and seeded (1×10^5 cells/well) in Collagen Type I-coated BioFlex® plates (Flexcell International, NC, USA). Cells were incubated for an additional 48h before strain was applied. Then, nutritional culture media was replaced with 0.5% FBS media for the remaining five days of culture. Fibroblasts were subjected to continuous mechanical stimulation (2-s strain and 10-s relaxation at a 0.5 Hz frequency) with a biaxial sinusoidal waveform with three different elongation strains (mechanical

strain deprivation-0%, physiologic strain-4%, or supraphysiological strain-10%)²⁵ for 5 days using the Flexcell FX-4000T strain system (Flexcell International, NC, USA) (Fig. 5.1).

Biomarker assays

Media was changed every 24h and stored at -20°C until analysis. Media from 24 and 120 hours of culture were assessed for various biomarkers. Proteoglycan (GAG) was assessed using the DMMB assay as previously described.²⁶ The concentration of prostaglandin E₂ (PGE₂) (Cayman Chemical, Ann Arbor, MI, USA), cytokines (IL-6, IL-8, KC and MCP-1) (Millipore, Billerica, MA, USA), total matrix metalloproteinase (MMP) activity (SensoLyte 520 generic MMP assay, Anaspec, Inc., Fremont, CA), and MMP production (MMP-1, MMP-2 and MMP-3) (R&D Systems, Minneapolis, MN, USA), were all assessed using commercially available assays according to the manufacturer's protocol. At the end of the strain protocol (120h), cell viability analysis was performed using the resazurin assay (Sigma Aldrich, Saint Louis, MO).

Statistical Analysis

For statistical analysis, normal distribution was assessed by Shapiro-Wilk test. Because data were not normally distributed, non-parametric tests were used. Differences between male and female dogs were assessed with Mann-Whitney Test at 24 and 120 hours (R Core Team (2019), R version 3.6.2 Vienna, Austria). Comparisons among strains and tissue types were performed using Kruskal-Wallis test and Dunn's test posthoc. Results were reported as median± interquartile range (IQR). Significance levels were set at $p < 0.05$.

Results

Concentration of PGE₂ was significantly increased in all female tendon fibroblasts when mechanical strain was applied but decreased when subjected to mechanical strain deprivation at 24h in QT and PT fibroblasts. Additionally, at 120h of supraphysiological strain, production of PGE₂ significantly increased in female QT and PT fibroblasts.

Similarly, mechanical strain deprivation elicited an increased production of PGE in male PT and HT fibroblasts at 120h (Fig. 5.2).

Cytokine concentration in the culture media tended to significantly increase in female QT and PT fibroblasts at 4% strain and 10% strain groups at 24h. Also, female HT fibroblasts exhibited significantly more production of IL-8 than males at 24h of no strain and 10% strain. Additionally, only female QT stretched at supraphysiological strain produced significantly higher levels of IL-8 at 120h compared to male QT fibroblasts (Fig. 5.3).

The production of the chemokine KC was consistently increased in female QT fibroblasts compared to male fibroblasts at all strains and timepoints. Moreover, female PT also showed an increased KC production compared to male fibroblasts at all strain at 24h. Interestingly, female and male HT fibroblastic production of KC was not significantly different between males and females in response to strain with the exception of mechanical strain deprivation at 24h showing increased levels of KC in female HT compared to male fibroblasts. Moreover, persistent increase in KC after 120h was only observed in female QT fibroblasts at all strain levels (Fig. 5.4).

MCP-1 production significantly increased in female QT fibroblasts compared to males at all strain levels at 24h of culture. Moreover, female PT fibroblasts exhibited a significant increase in MCP-1 production at 4% strain and 10% strain at 24h. Furthermore, female

HT fibroblasts showed significantly increased production of MCP-1 at mechanical strain deprivation at 24h. Additionally, only female QT fibroblasts exhibited a sustained increase in MCP-1 production after 120h of culture when fibroblasts were loaded at 4% and 10% strain. Moreover, HT and PT did not show significant differences between male and females at 120h of culture (Fig. 5.5).

When evaluating production of extracellular matrix related markers, we found that GAG concentration increased in the culture media of female QT and PT fibroblasts at all strain levels at 24h. Female HT fibroblasts only increased the production of GAG when subjected to supraphysiological strain at 24h. Moreover, only female QT fibroblasts stretched at 10% strain maintained increased GAG production compared to males after 120h of culture (Fig. 5.6).

MMP activity (MMPACT) was found higher in male QT fibroblasts in no strain and 10% strain groups at 24h compared to females. Male PT fibroblasts showed significantly higher levels of MMPACT compared to females in the 10% strain group at 24h. Moreover, male HT fibroblasts showed a significantly higher production of MMPACT at mechanical strain deprivation but decreased at physiological strain relative to females at 24h. Interestingly, male QT fibroblasts maintained an increase in MMPACT at all strains, after 120h of culture. Additionally, male PT and HT fibroblasts showed a significantly higher level of MMPACT compared to females at abnormal loads, no strain and 10% strain groups at 120h of culture (Fig. 5.7).

MMP-2 production increased in male QT fibroblasts compared to females at supraphysiological strain at 24h. Additionally, at 120h, male QT fibroblasts showed a significant increase in MMP-2 production compared to females at all strain levels.

Interestingly, while male QT fibroblasts increased MMP-2 levels with respect to females at all strains, PT and HT did not exhibit significant differences between male and females at any timepoint (Fig. 5.8).

Strain differences in male dogs:

Metabolic activity was significantly higher in male QT fibroblasts at 10% strain compared to 4% strain. However, male PT and HT fibroblasts did not exhibit significant differences between strains (Fig. 5.9).

The production of the inflammatory biomarker PGE₂ was significantly increased in male QT and HT fibroblasts at no strain compared to 4% strain at 24h. However, all male graft fibroblasts did not show differences in PGE-2 production in response to strain at 120h (Fig. 5.10). Additionally, cytokine and chemokine production were not significantly affected by strain in male fibroblasts (Fig. 5.11-5.13).

The extracellular matrix related biomarker GAG did not exhibit significant changes with respect to strain level in any of the fibroblast types at 24h. However, significantly lower GAG content was seen in male QT fibroblasts at supraphysiological strain levels compared to mechanical strain deprivation at 120h of culture. PT and HT fibroblasts obtained from male dogs did not show significant differences in GAG content in response to strain at 120h (Fig. 5.14). Moreover, neither MMPACT nor MMP-2 production were affected by strain level in any of the tendon fibroblasts at any timepoint (Fig. 5.15-5.16).

When comparing differences among male fibroblastic tissue sources, fibroblasts did not differ among each other in any of the biomarkers evaluated at any strain or timepoint (5.17-5.24). However, there was a trend towards decreased production of MMP-2 arising from PT fibroblasts at 120h of culture (Fig. 5.24).

Discussion:

While it is generally accepted that there are sex differences in ACL injury risk, the effect of sex on outcomes after surgery is less certain. Although the hormonal effects of estrogen on native ACL properties have been widely investigated, information on effect of hormone on ACL tendon grafts is lacking.²⁷ Previous studies have indicated that women's tendons tend to have a lower structural quality, less dry mass, express higher level of type III collagen, and have decreased tendon stiffness and resistance to deformation under loading.²⁸ Additionally, estrogen has an inhibiting effect on tendon and ligament collagen synthesis.^{17; 29} In this study, we demonstrated that tendon graft fibroblasts exhibited different biologic responses as a function of sex, tissue source, and level of mechanical stress, independent of hormonal stimulation.

Magnusson *et al.*, (2007) showed that men and women have different adaptability to tendon loading.³⁰ Specifically, women have a decreased tendon hypertrophy response to habitual training, decreased tendon collagen synthesis rate following exercise, a diminished rate of tendon collagen synthesis due to higher levels of estradiol stimulation, and lower mechanical properties.³⁰ Moreover, authors also showed that collagen fascicles from patellar tendons obtained from men showed greater ultimate stress than those from women. Circi *et al.*, (2009) showed that the inflammatory response is greater in the presence of endogenous estrogen.³¹ Our results indicate that female fibroblasts may also have an innate higher inflammatory response that is independent of estrogen stimulation, at least for PT fibroblasts. In this study female PT fibroblasts produce higher concentrations of PGE₂, cytokines (IL-6, IL-8), and chemokines (KC and MCP-1) than males in a strain dependent manner. Female tendon fibroblasts showed decrease metabolic activity when compared to

male dogs regardless of the strain levels. This dampened cellular metabolism among female graft cells might explain the decrease in mechanical properties of female tendon grafts. Interestingly, tendon fibroblasts from male dogs tended to show a significant increase in MMP activity compared to females. Further, these responses seem to be tendon specific. Male QT fibroblasts seem to be more sensitive to strain as indicated by an increased production of MMP-2 compared to females, while PT and HT fibroblasts did not show significant differences in MMP-2 production.

Another factor that could influence poor outcomes after ACL reconstruction in females is the tendon to bone integration. It has been shown that the withdrawal of estrogen can lead to decreased bone mineral density and osteoporosis.³⁶⁻³⁸ Moreover, male sex hormone dihydrotestosterone (DHT) has been found to stimulate bone formation via increased alkaline phosphatase (AP) activity and PICP production. Additionally, DHT may enhance anchorage of osteoblastic cells when subjected to cyclic strain through increased expression of fibronectin.³⁹ Anchorage of bone cells to the extracellular matrix is a prerequisite for mechanotransduction. Any disruption in the continuity in the bone-tendon junction could lead to impaired healing and higher risk of failure after ACL reconstruction. Our findings support this theory because of the increased MMP activity and MMP-2 production seen in males compared to females, which may be indicative of a greater remodeling process with male tendon grafts. We believe that the increased remodeling activity in male tendons could potentially lead to better bone to tendon integration within the bone tunnel. Further work to evaluate bone turnover markers *in vitro* would be necessary in order to establish this relationship.

Previous studies have indicated that inflammatory responses are attenuated in females during pregnancy, and that cell proliferation and inflammatory responses are greater in the presence of endogenous estrogen.^{31; 40; 41} Our findings suggest that ECM remodeling may be increased in male autografts as indicated by the increased level of MMP activity during culture. Additionally, male graft fibroblasts were more metabolically active than females indicating that application of tension load tend to increase cell density in male tenocytes. Furthermore, female autograft fibroblasts tend to exhibit higher GAG production in high strain conditions, which may be indicative of an injury response. Female autograft fibroblasts also produced higher levels of inflammatory mediators under all strain conditions. This may explain the clinical phenomenon that females tend to report higher pain levels and lower patient-reported outcomes relative to males following ACL surgery.^{19; 42} Our collective findings suggest that there are likely sex differences in graft ligamentization after reconstruction which may contribute to differences seen in outcomes after ACL surgery. Moreover, Clatworthy *et al.*, (1999) found that tunnel widening is increased when hamstring autografts are used, which could be the consequence of the release of cytokines that may lead to increased osteolysis.⁴³ Hence, HT might have delayed healing and osteointegration in females due their increased cytokine production (IL-8, KC, MCP-1) compared to males at 24h when subjected to mechanical strain deprivation.

Some limitations to this work were identified. First, fibroblasts in culture did not produce enough collagen levels to be detected by the hydroxyproline assay. Consequently, interpretation of ECM remodeling and healing need to be based only on the results obtained from MMPs and GAGs, missing an important piece of information for accurate healing analysis. Second, the monolayer-equibiaxial model utilized in this work, may not reflect

motion *in vivo* due to the lack of interaction of forces such as shear stress and torsion. Additionally, our experiment only measured biomarker production for five days, therefore, biological processes that happen at a later timepoint were omitted. Hence, the production of biomarkers may be altered, especially ECM remodeling. Consequently, measuring collagen production using more sensitive assays is recommended. Moreover, the use of bioreactors that can apply rotational and shear stress along with tension load to whole tendon explants is suggested in order to replicate *in vivo* structures. Lastly, later timepoints that allow the assessment of metabolic responses after the initial inflammation period has occurred, may be useful to truly evaluate the effect of mechanical strain on healing responses of tendon grafts.

In summary, the data from this study provides evidence that biological properties of fibroblasts from common tendons used for ACL reconstruction vary significantly between males and females. Furthermore, graft tissue choice and level of mechanical stress may play a significant role in healing of ACL grafts in both, males and females based on the differential production of healing-related molecules such as MMPs and inflammatory mediators in response to tension load *in vitro*. Understanding how sex and mechanical stress affect the metabolism of the tendon graft may help optimize surgical outcomes by optimizing level of activity and ACL post-operative rehabilitation based on patient sex and ACL graft tissue choice.

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Figures

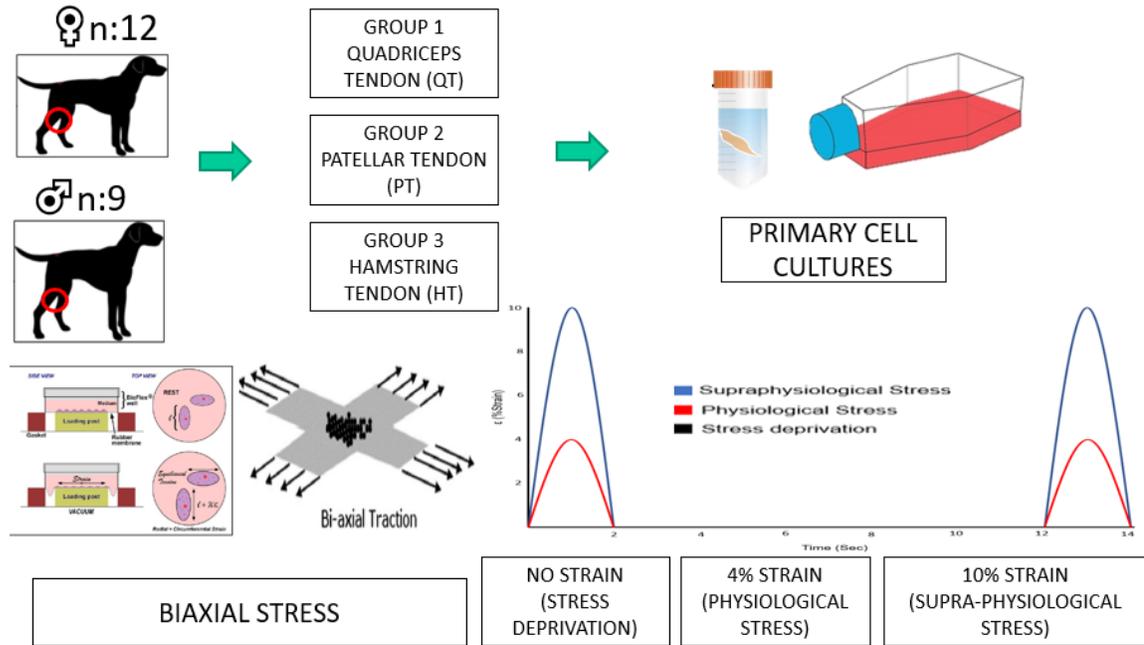


Figure 5.1. Experimental Design

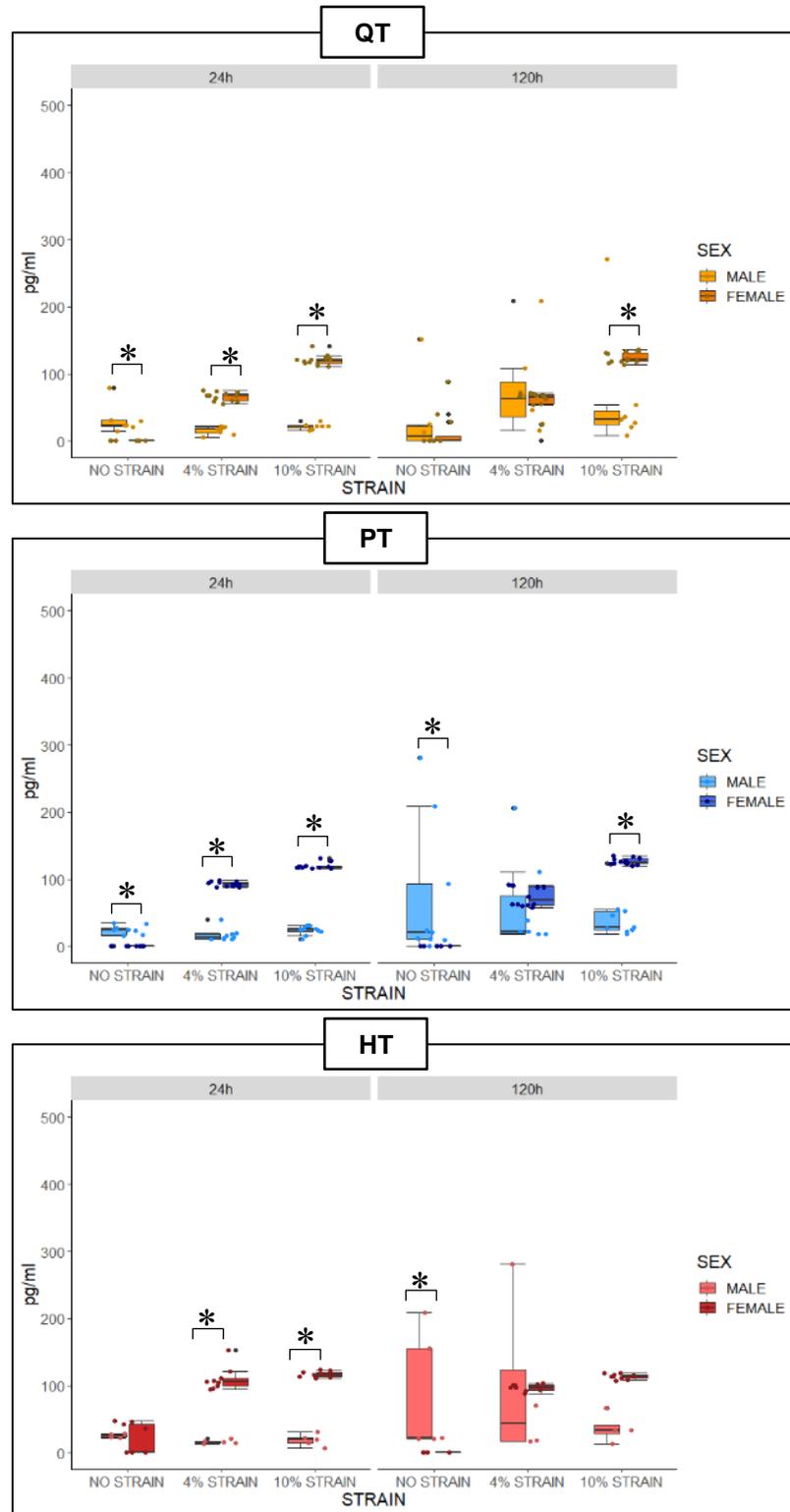


Figure 5.2. Concentration of prostaglandin E2 (PGE2) released to the media. * significant difference between males and females at 24 and 120 hours (Mann-Whitney test). QT: Quadriceps tendon; PT: Patellar tendon, HT: Hamstring tendon

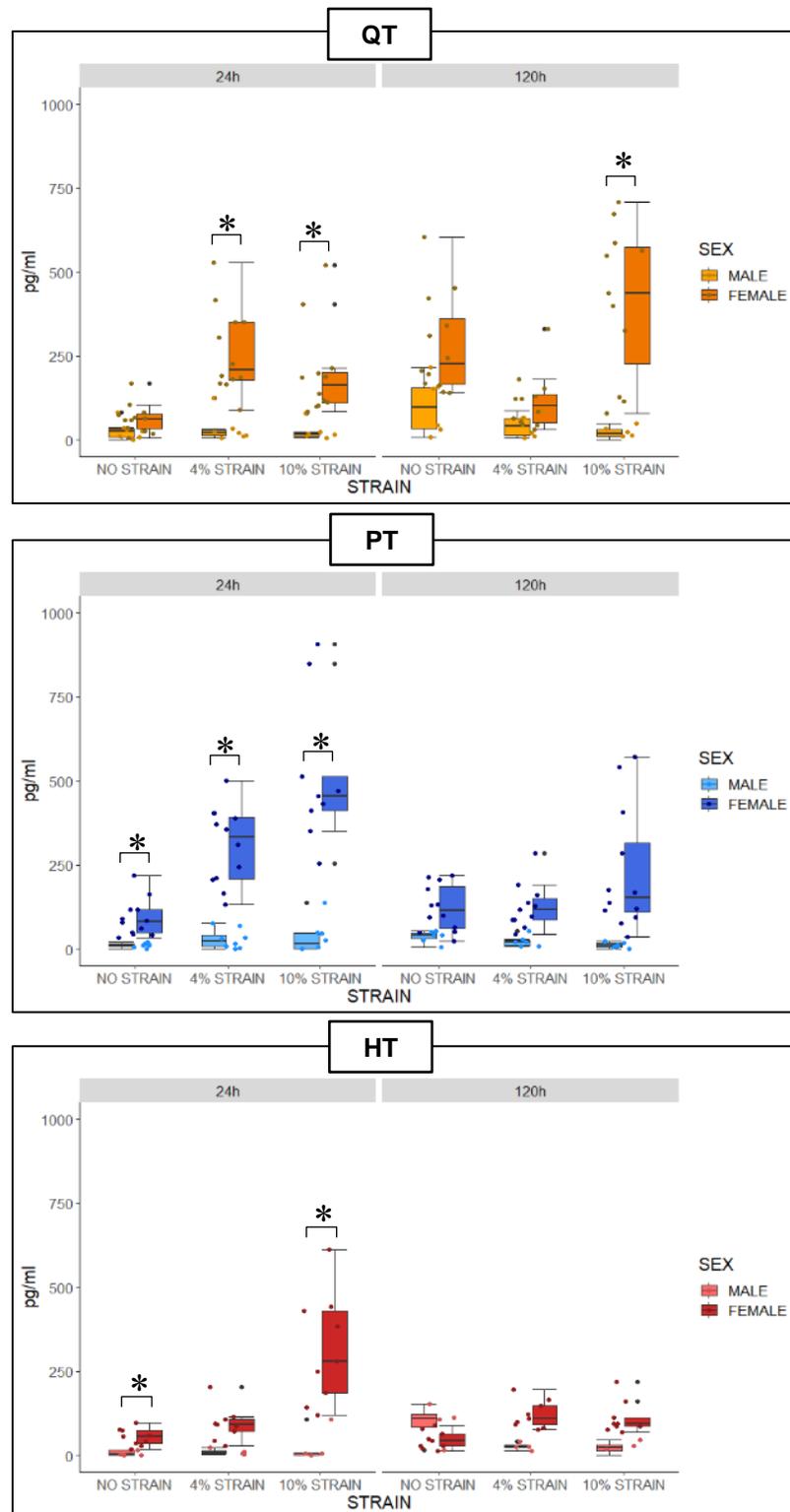


Figure 5.3. Concentration of interleukin-8 (IL-8) released to the media. * significant difference between males and females at 24 and 120 hours (Mann-Whitney test). QT: Quadriceps tendon; PT: Patellar tendon, HT: Hamstring tendon

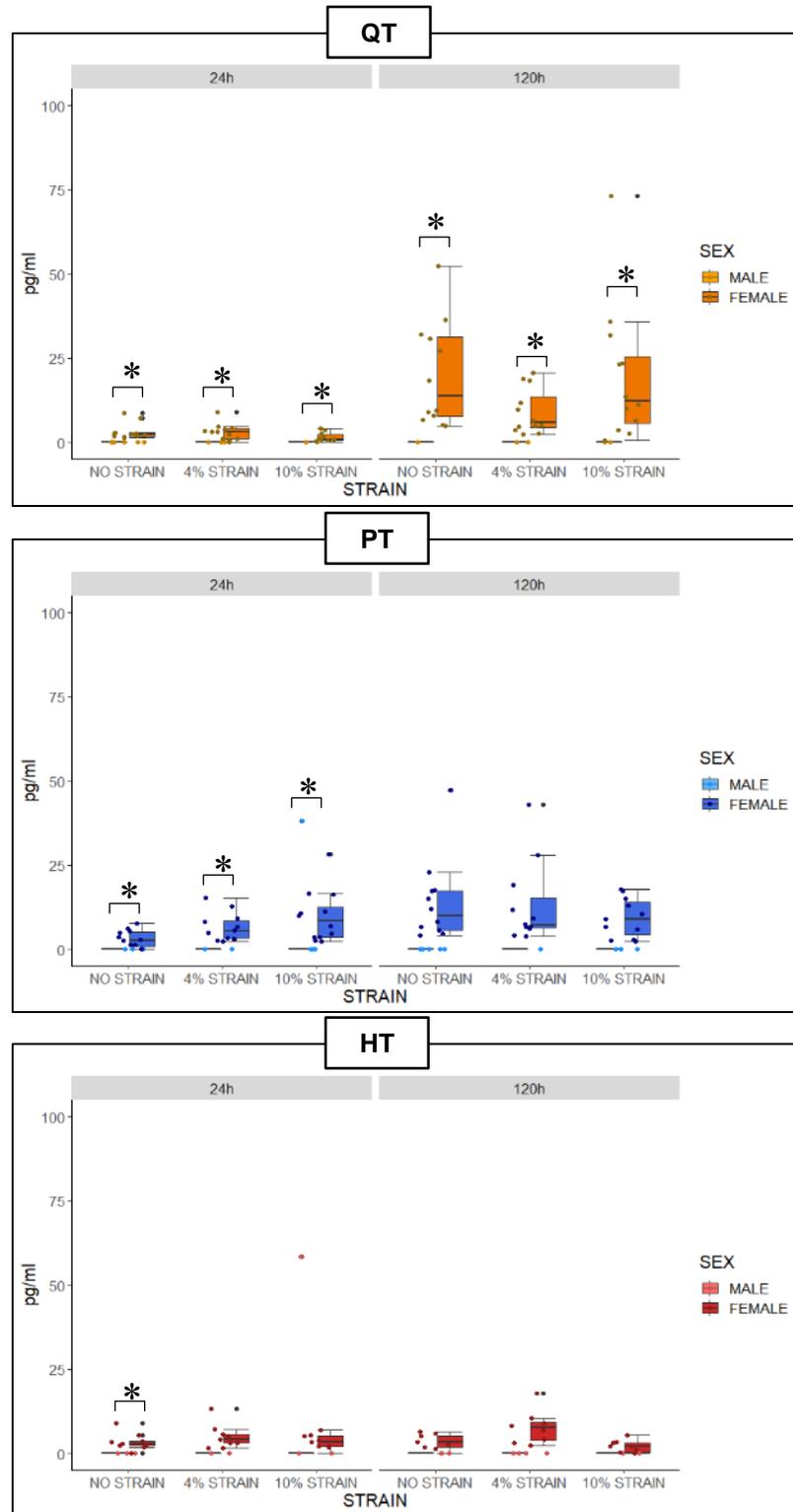


Figure 5.4. Concentration of keratinocyte chemoattractant protein (KC) released to the media. * significant difference between males and females at 24 and 120 hours (Mann-Whitney test). QT: Quadriceps tendon; PT: Patellar tendon, HT: Hamstring tendon

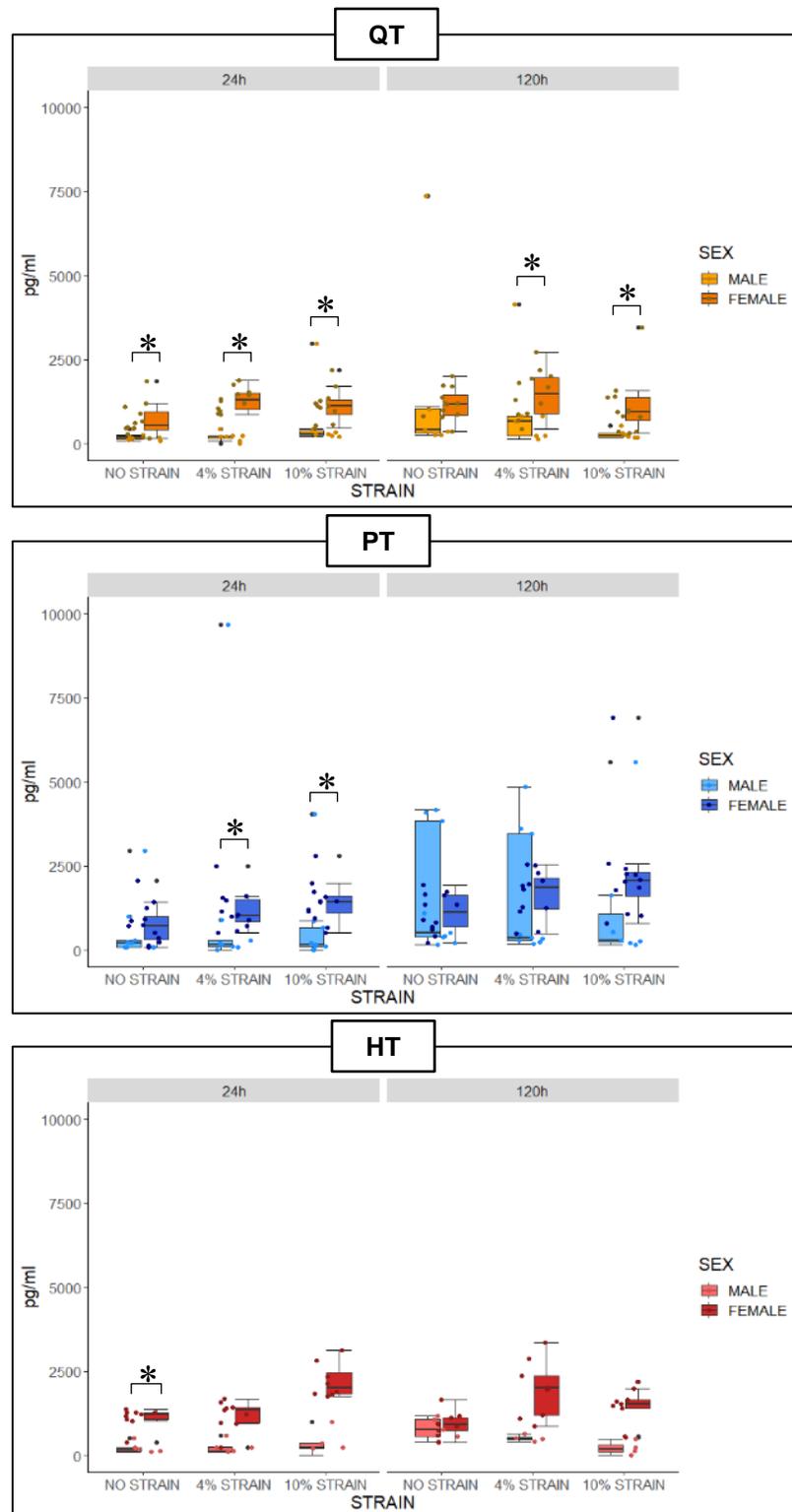


Figure 5.5. Concentration of monocyte chemoattractant protein-1 (MCP-1) released to the media.
 * significant difference between males and females at 24 and 120 hours (Mann-Whitney test).
 QT: Quadriceps tendon; PT: Patellar tendon, HT: Hamstring tendon

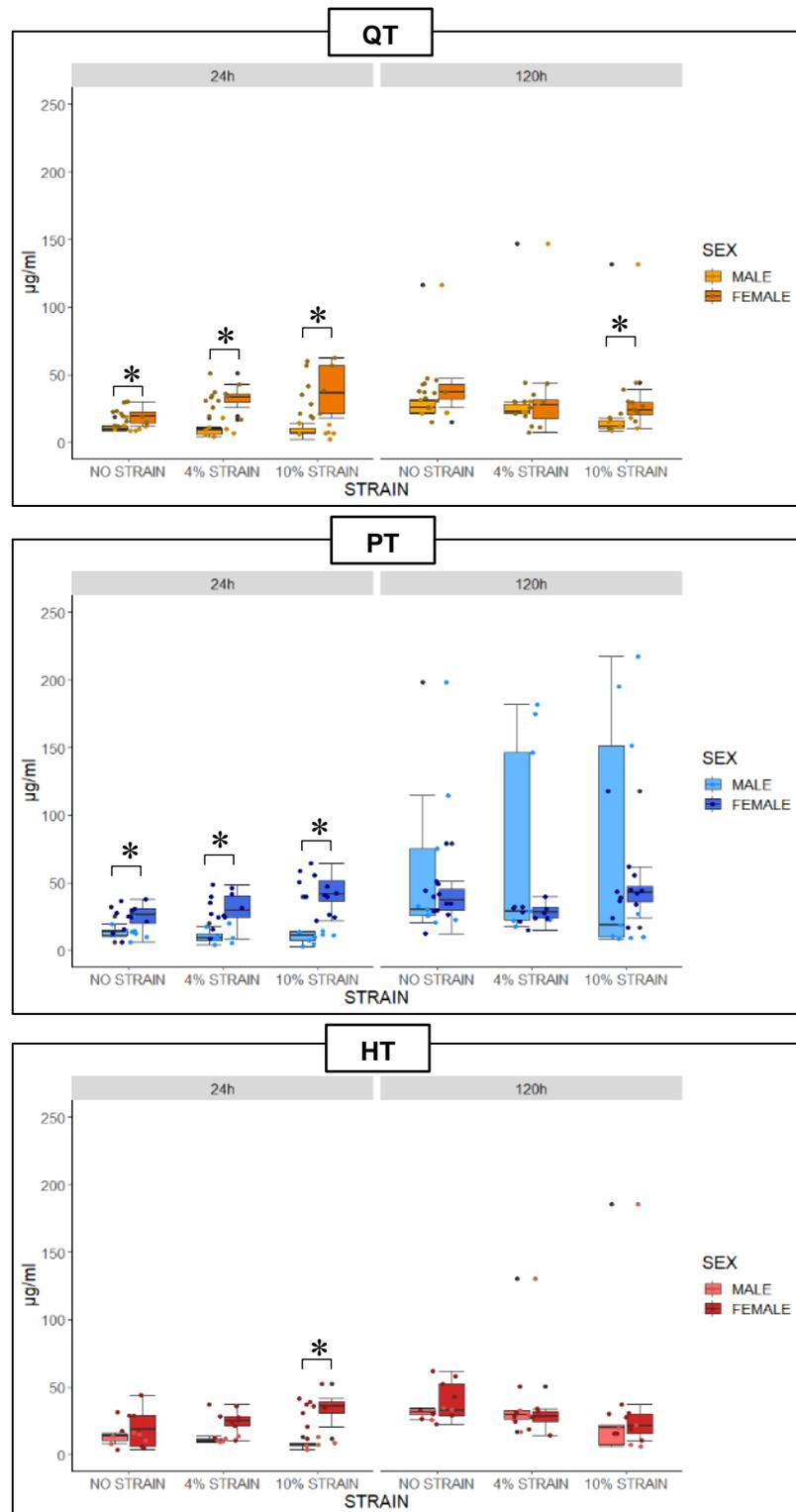


Figure 5.6. Concentration of Glycosaminoglycans (GAG) released to the media. * significant difference between males and females at 24 and 120 hours (Mann-Whitney test). QT: Quadriceps tendon; PT: Patellar tendon, HT: Hamstring tendon

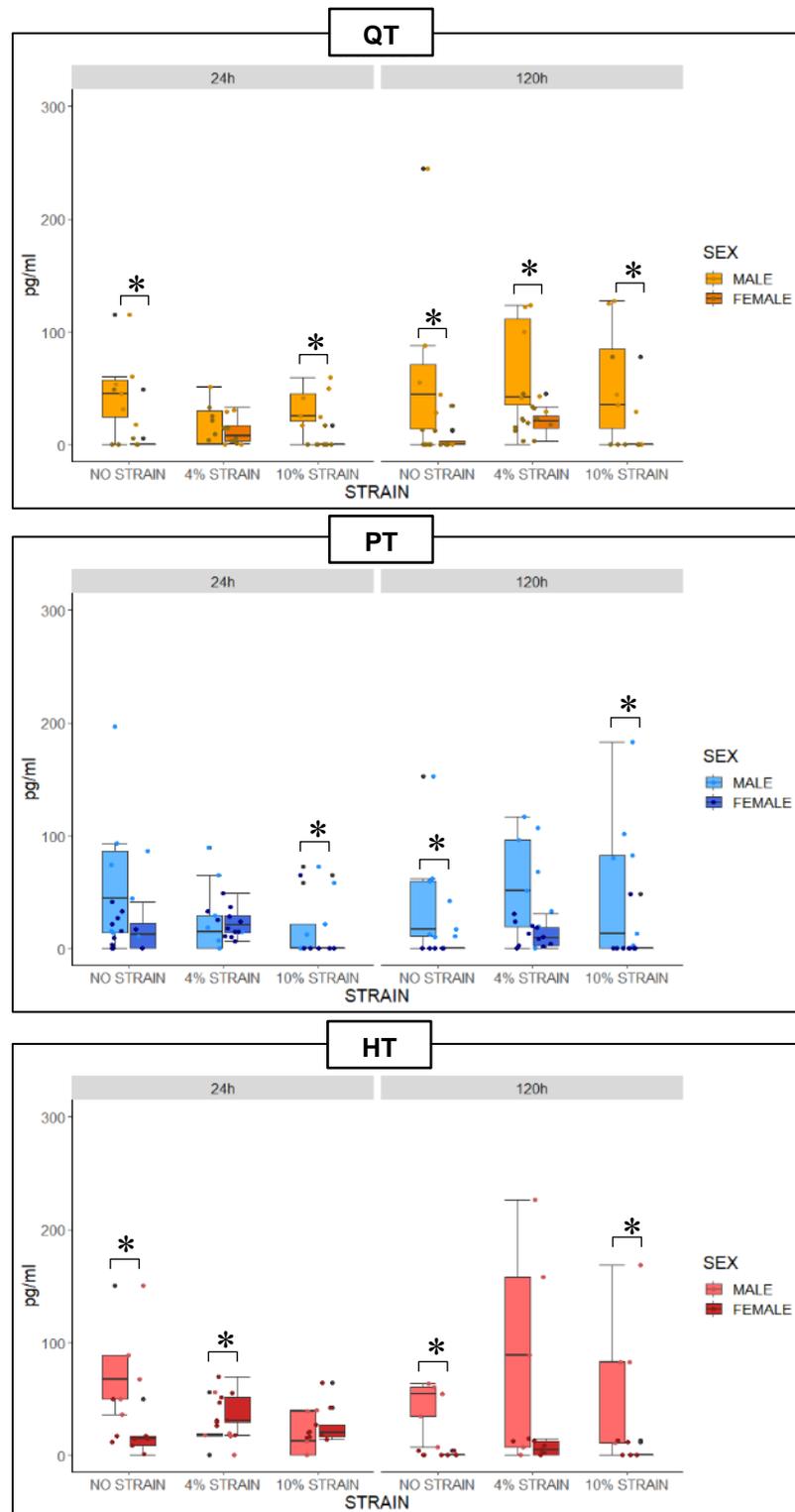


Figure 5.7. Concentration of total MMP activity (MMPACT) released to the media. * significant difference between males and females at 24 and 120 hours (Mann-Whitney test). QT: Quadriceps tendon; PT: Patellar tendon, HT: Hamstring tendon

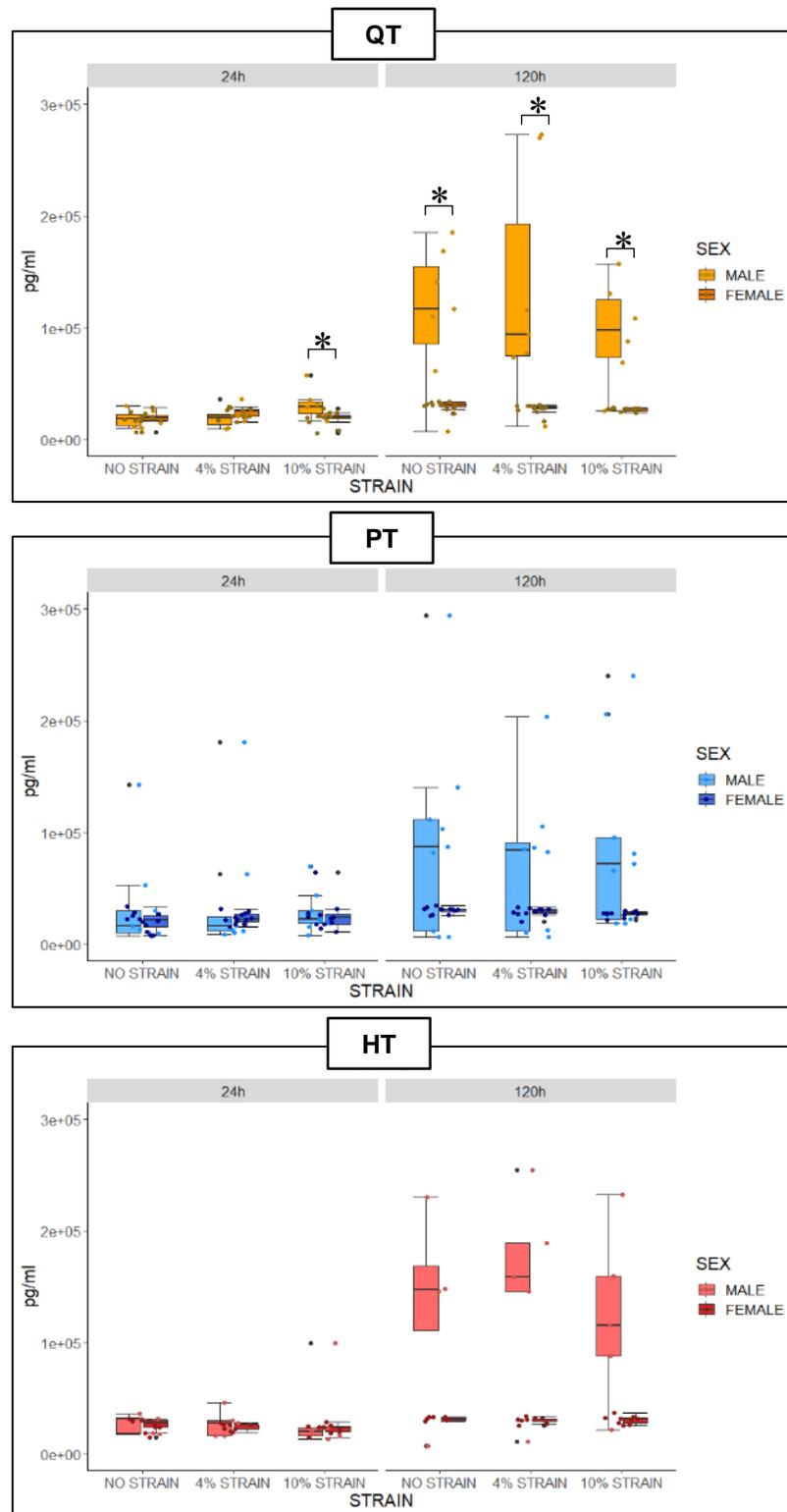


Figure 5.8. Concentration of matrix metalloproteinase 2 (MMP-2) released to the media. * significant difference between males and females at 24 and 120 hours (Mann-Whitney test). QT: Quadriceps tendon; PT: Patellar tendon, HT: Hamstring tendon

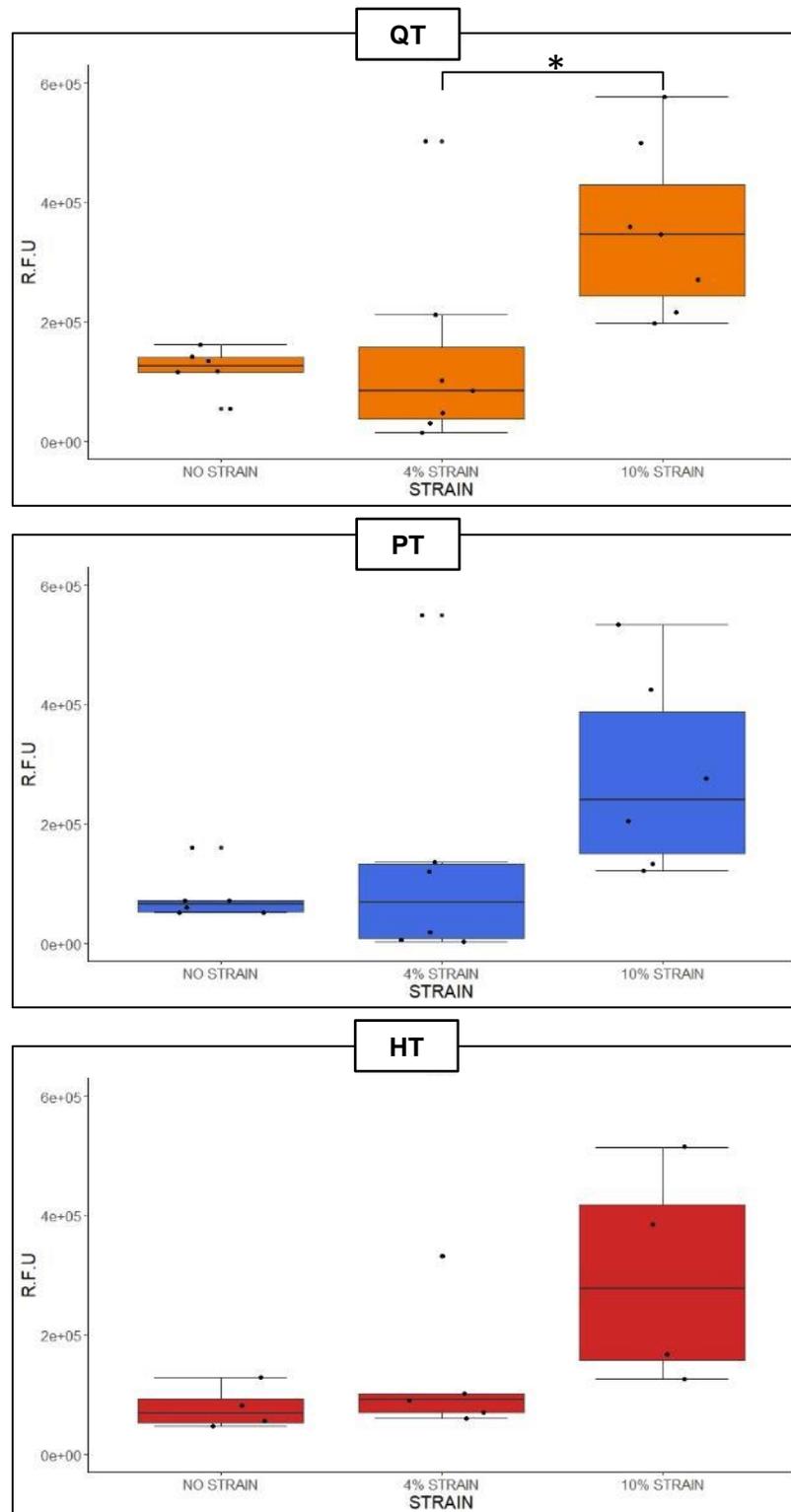


Figure 5.9. Cellular Metabolic Activity in male fibroblasts after 120 hours. * significant difference between tissues. QT: Quadriceps tendon; PT: Patellar tendon, HT: Hamstring tendon; RFU: Resazurin Fluorescent Units

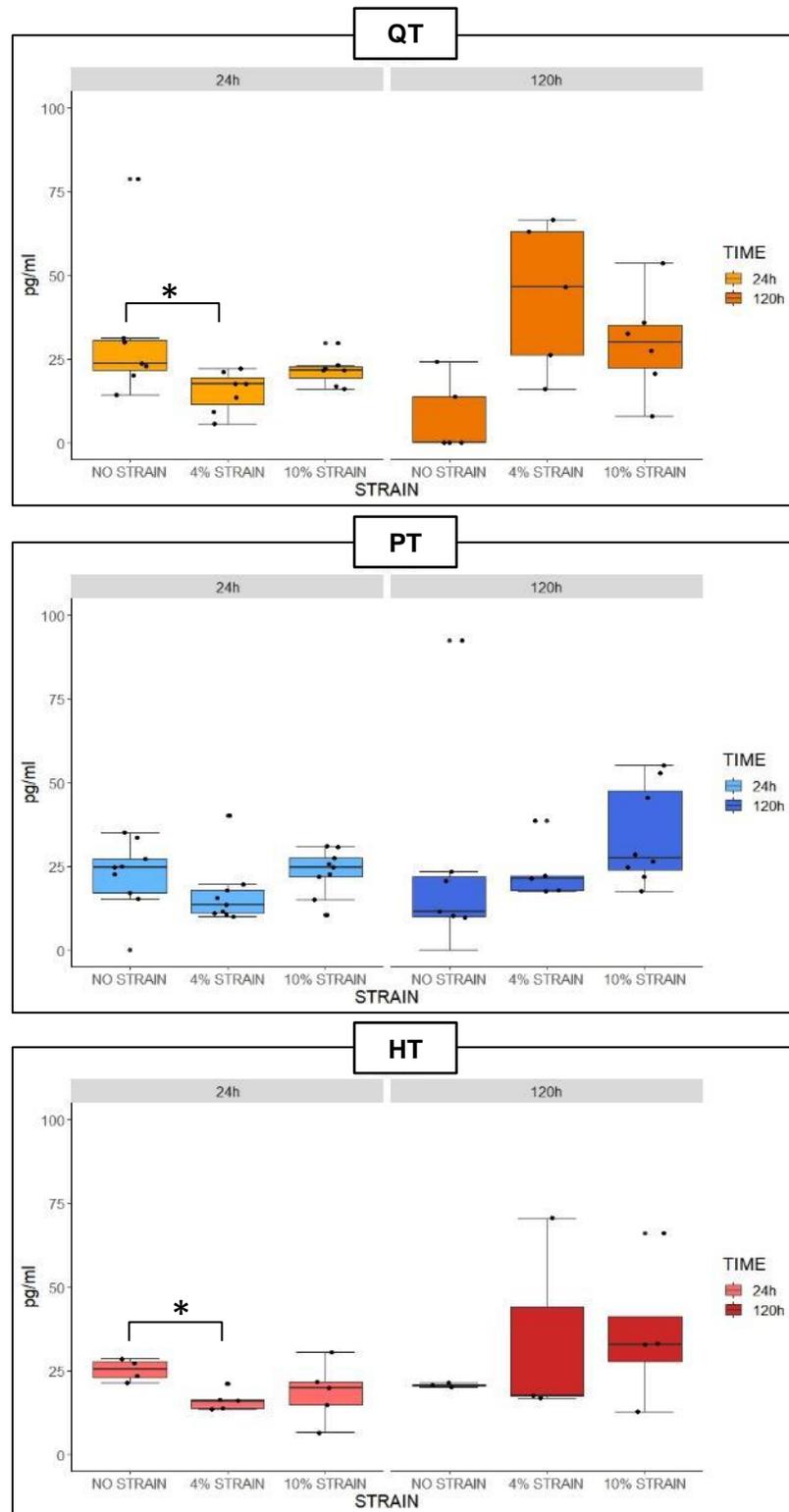


Figure 5.10. Concentration of PGE2 released to the media in males. * significant difference between graft type at 24 and 120 hours (Kruskal-Wallis test). QT: Quadriceps tendon; PT: Patellar tendon, HT: Hamstring tendon

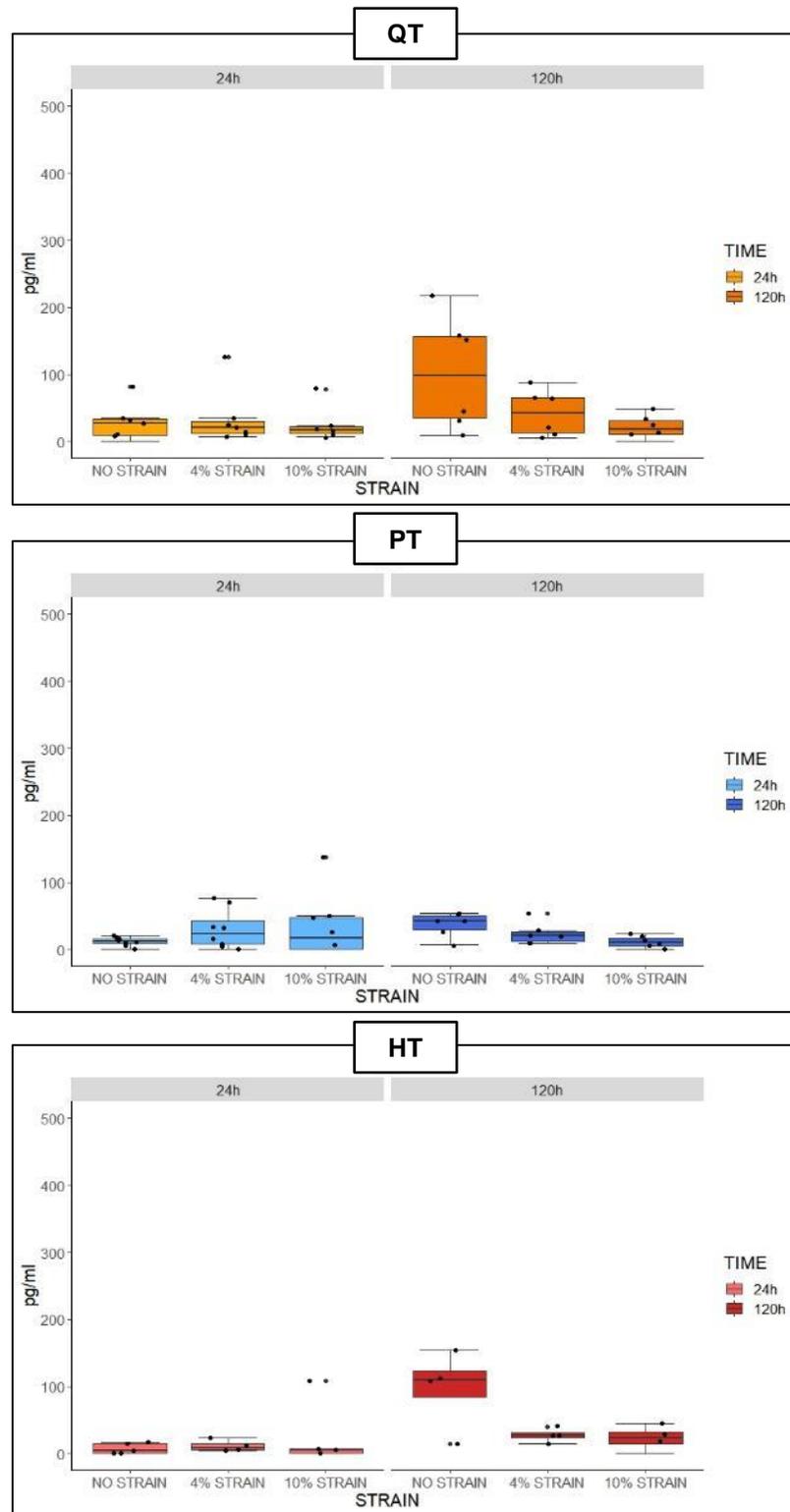


Figure 5.11. Concentration of IL-8 released to the media in males. * significant difference between graft type at 24 and 120 hours (Kruskal-Wallis test). QT: Quadriceps tendon; PT: Patellar tendon, HT: Hamstring tendon

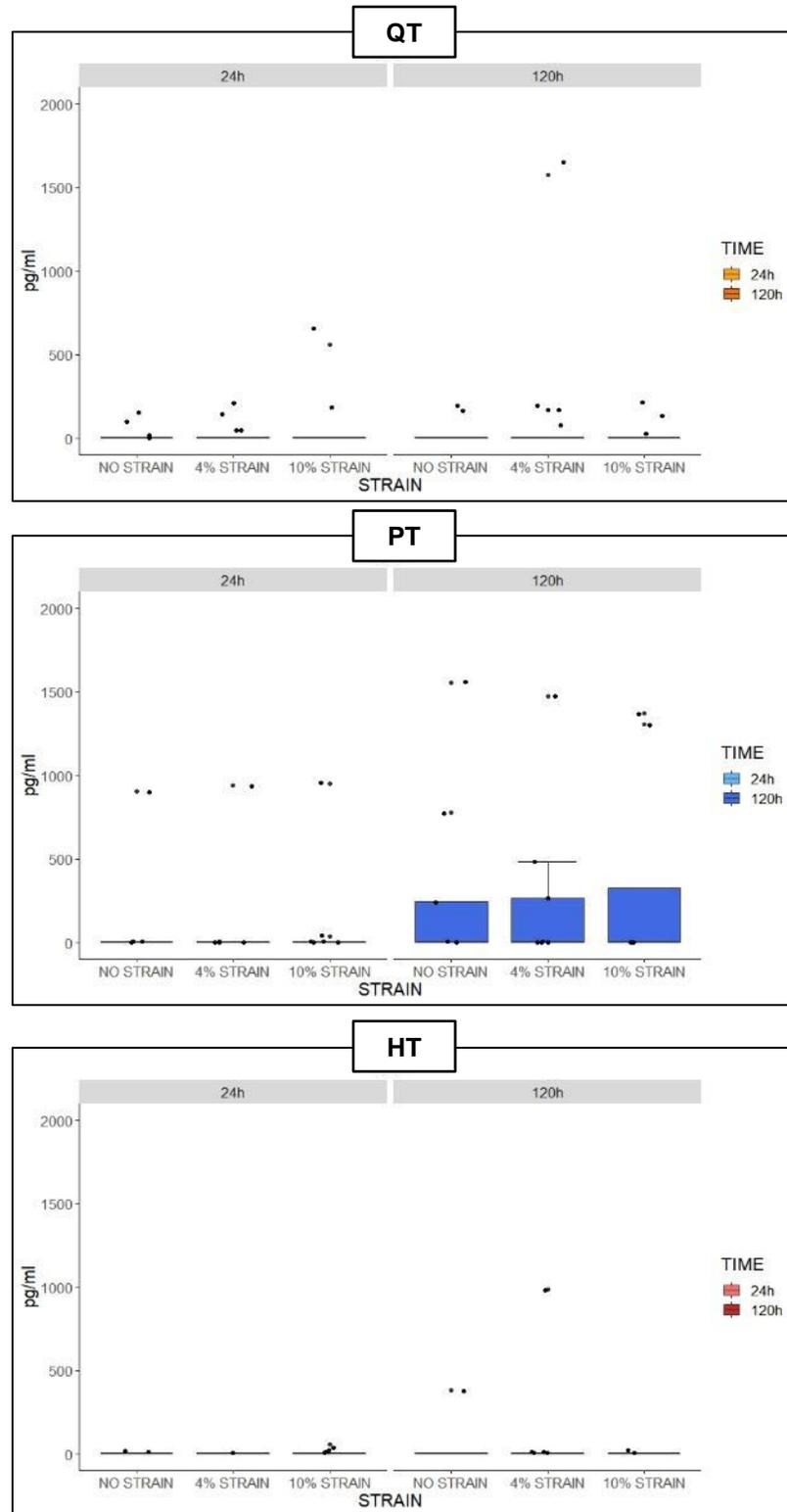


Figure 5.12. Concentration of KC released to the media in males. * significant difference between graft type at 24 and 120 hours (Kruskal-Wallis test). QT: Quadriceps tendon; PT: Patellar tendon, HT: Hamstring tendon

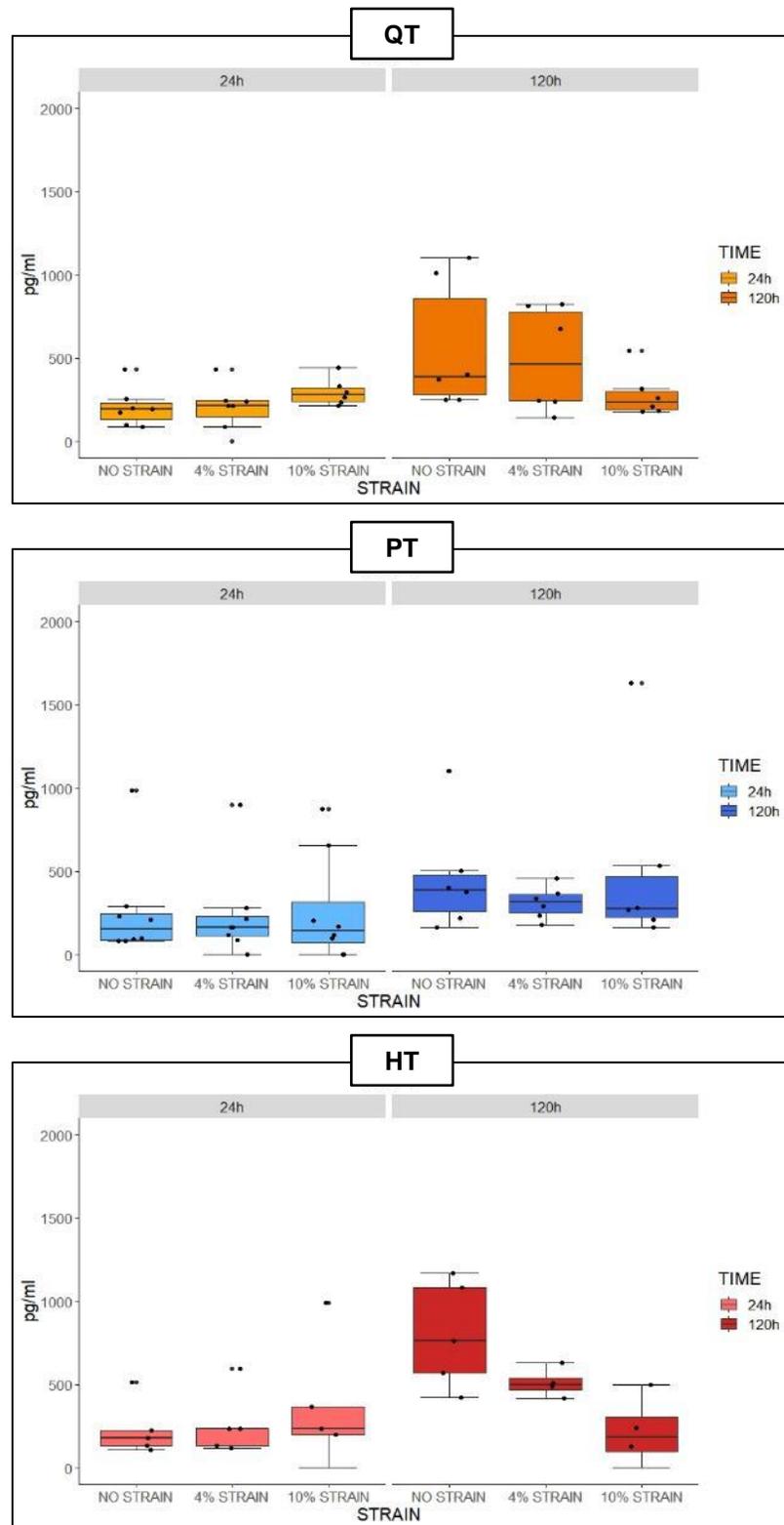


Figure 5.13. Concentration of MCP-1 released to the media in males. * significant difference between graft type at 24 and 120 hours (Kruskal-Wallis test). QT: Quadriceps tendon; PT: Patellar tendon, HT: Hamstring tendon

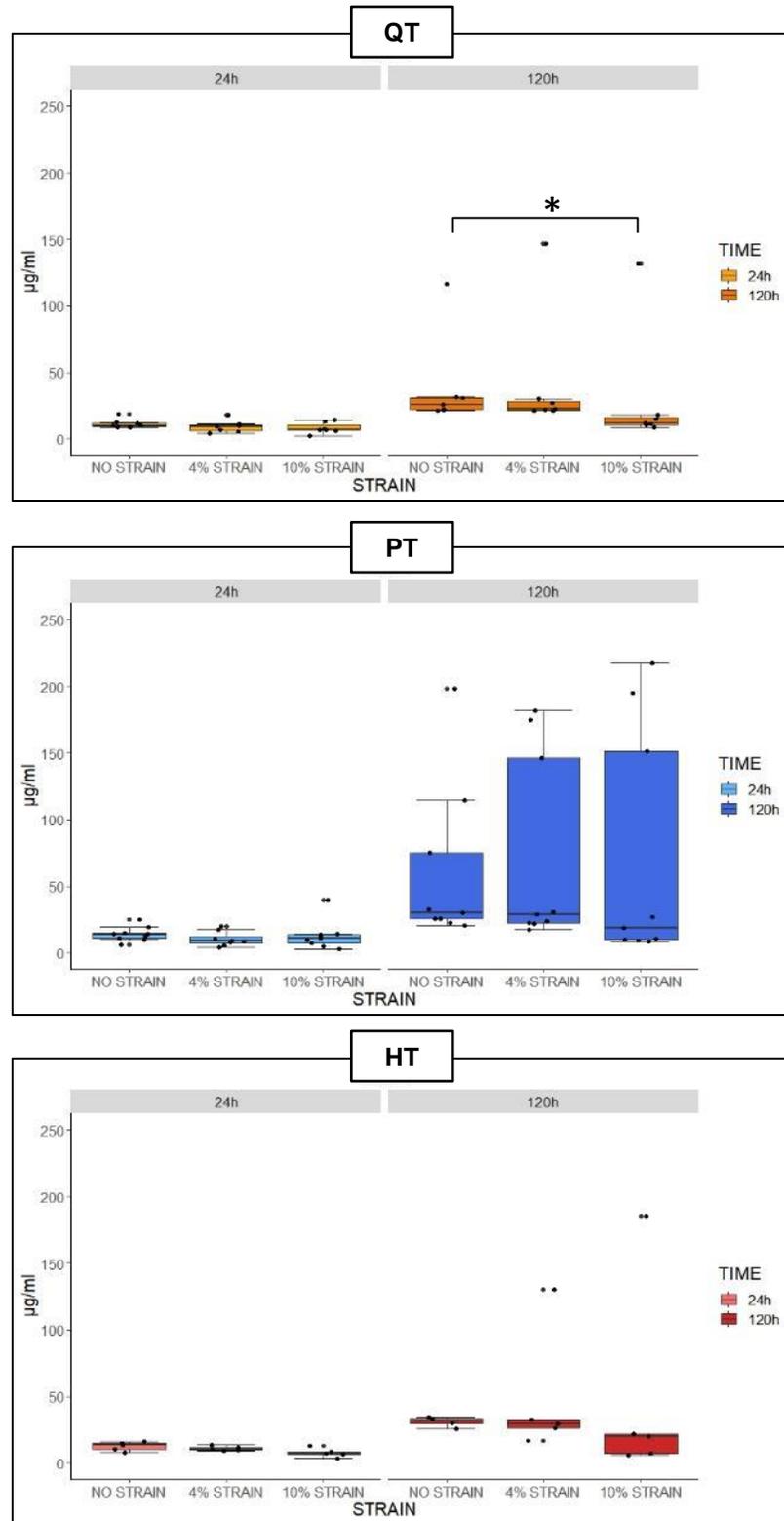


Figure 5.14. Concentration of GAG released to the media in males. * significant difference between graft type at 24 and 120 hours (Kruskal-Wallis test). QT: Quadriceps tendon; PT: Patellar tendon, HT: Hamstring tendon

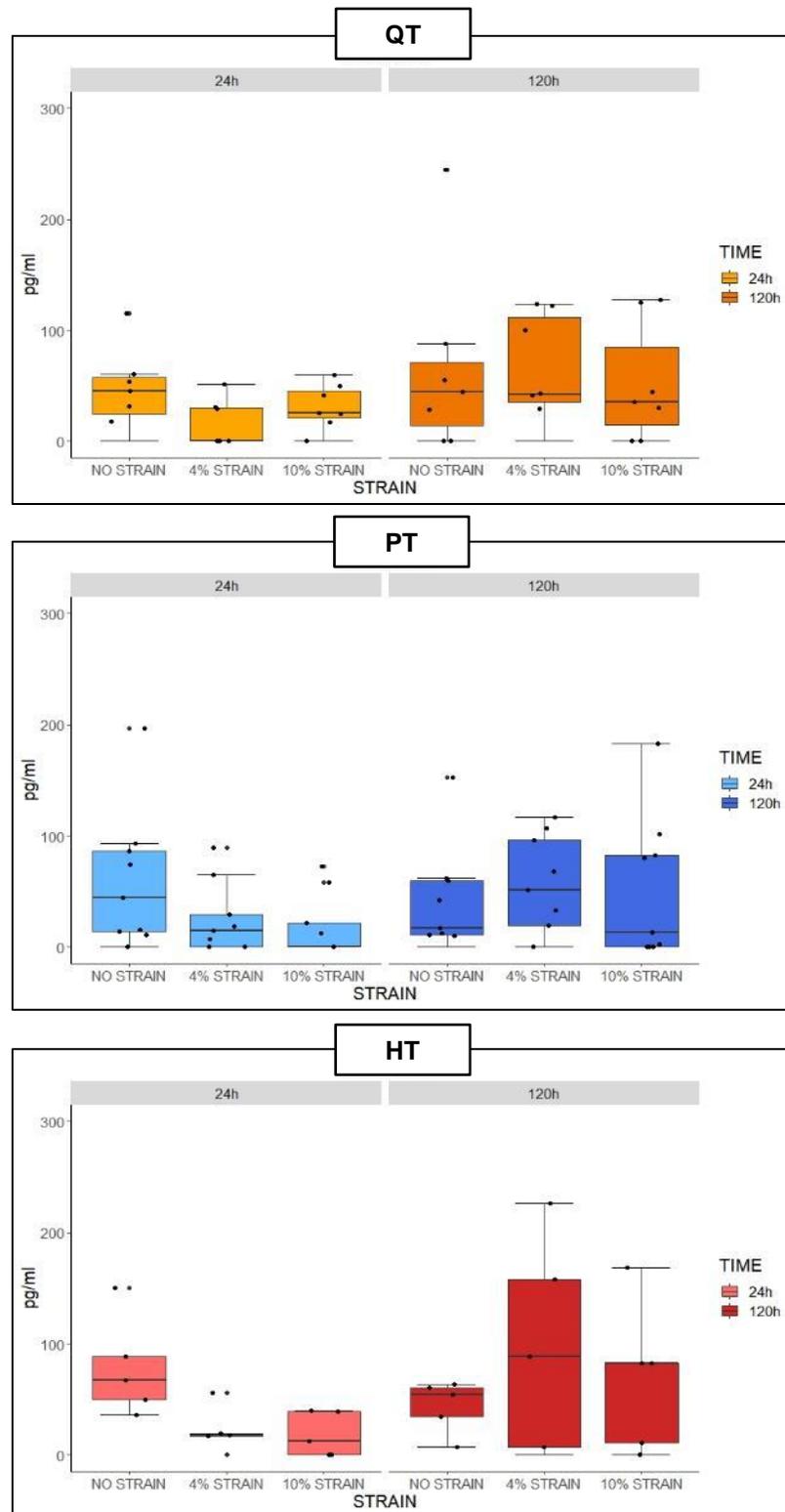


Figure 5.15. Concentration of MMPACT released to the media in males. * significant difference between graft type at 24 and 120 hours (Kruskal-Wallis test). QT: Quadriceps tendon; PT: Patellar tendon, HT: Hamstring tendon

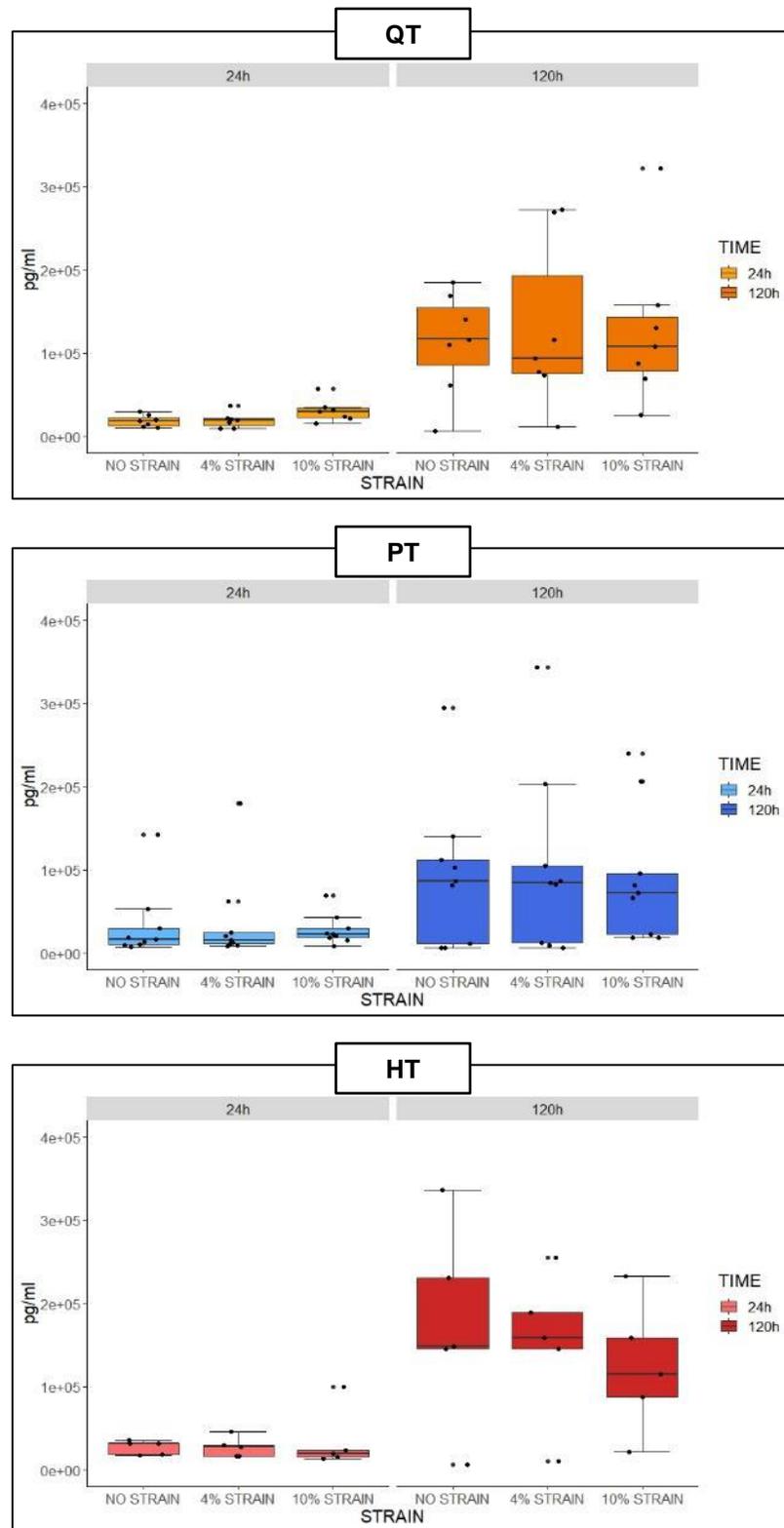


Figure 5.16. Concentration of MMP-2 released to the media in males. * significant difference between graft type at 24 and 120 hours (Kruskal-Wallis test). QT: Quadriceps tendon; PT: Patellar tendon, HT: Hamstring tendon

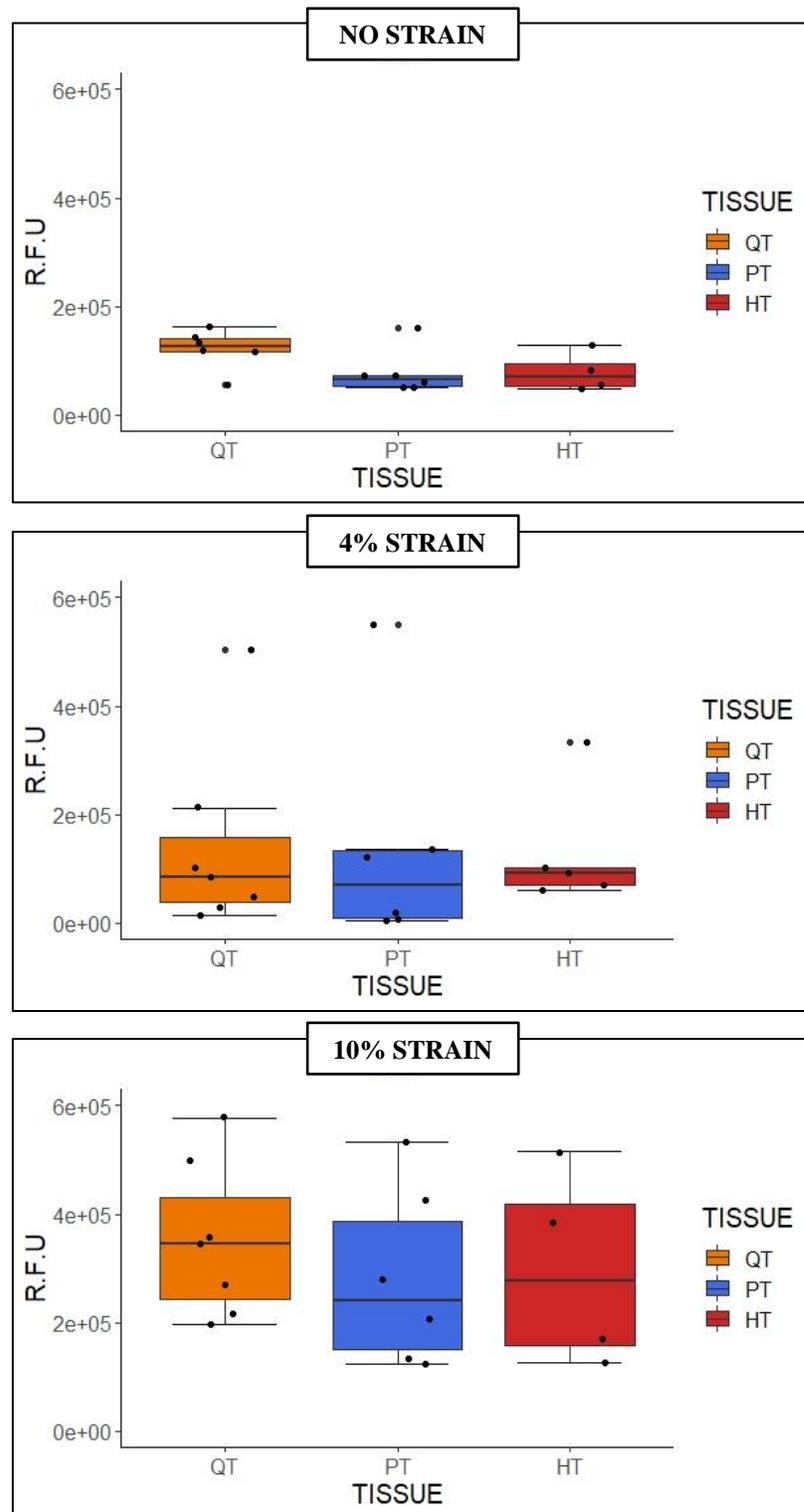


Figure 5.17. Cellular Metabolic Activity in males after 120 hours. * significant difference between strains. QT: Quadriceps tendon; PT: Patellar tendon, HT: Hamstring tendon: RFU: Resazurin Fluorescent Units

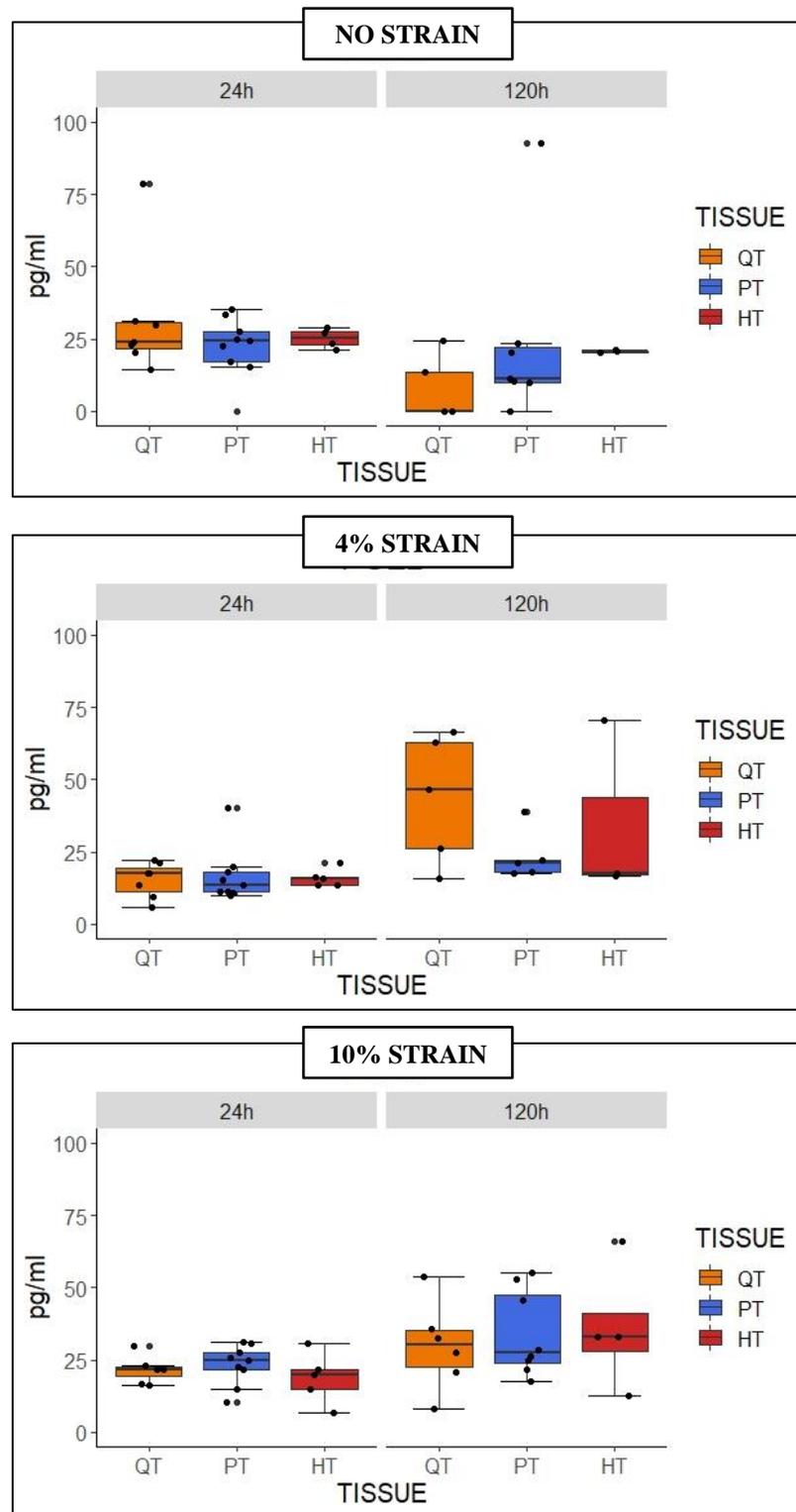


Figure 5.18. Concentration of PGE2 released to the media in males. * significant difference between TISSUES at 24 and 120 hours (Kruskal-Wallis test). QT: Quadriceps tendon; PT: Patellar tendon, HT: Hamstring tendon

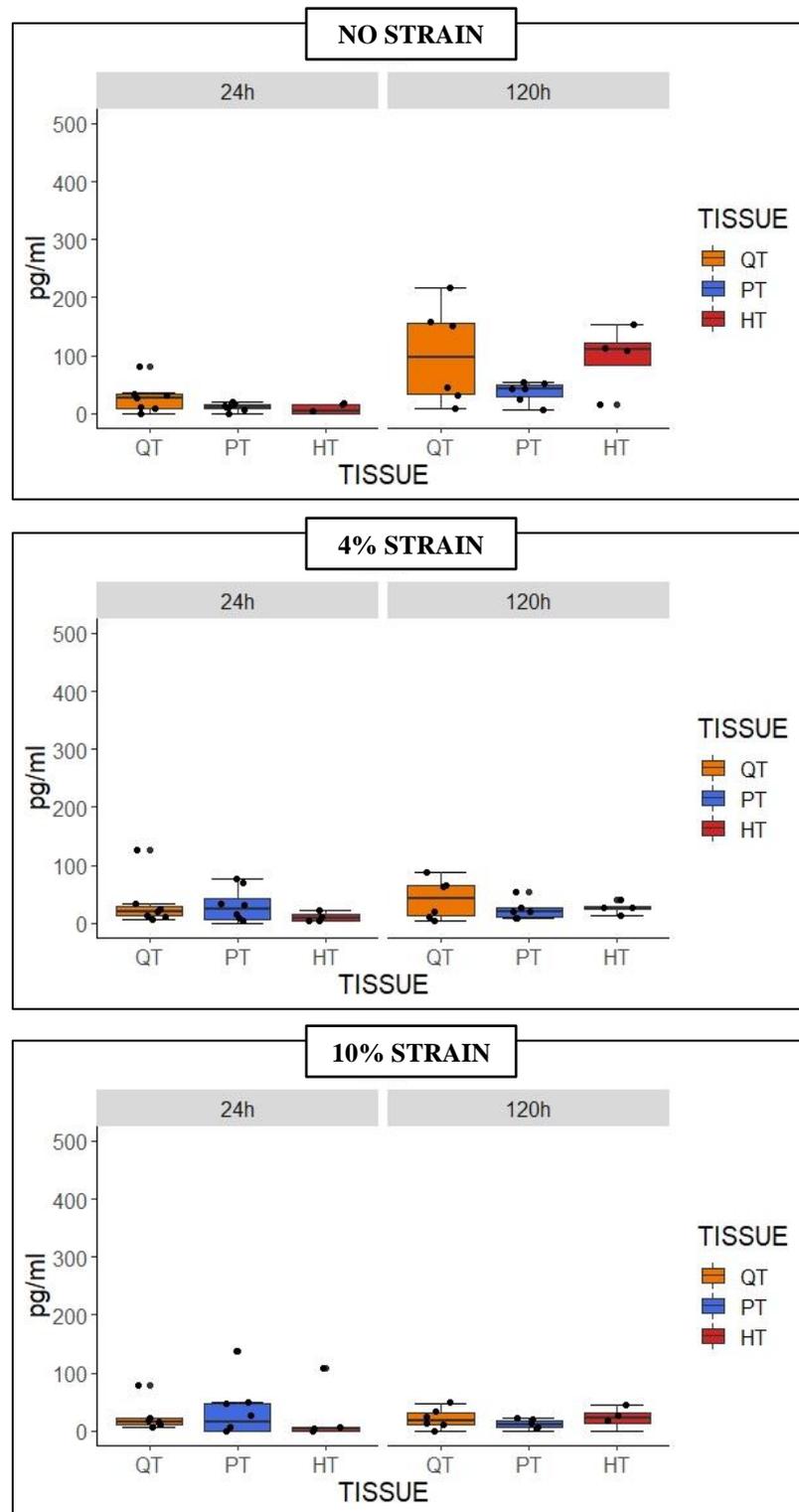


Figure 5.19. Concentration of IL-8 released to the media in males. * significant difference between TISSUES at 24 and 120 hours (Kruskal-Wallis test). QT: Quadriceps tendon; PT: Patellar tendon, HT: Hamstring tendon

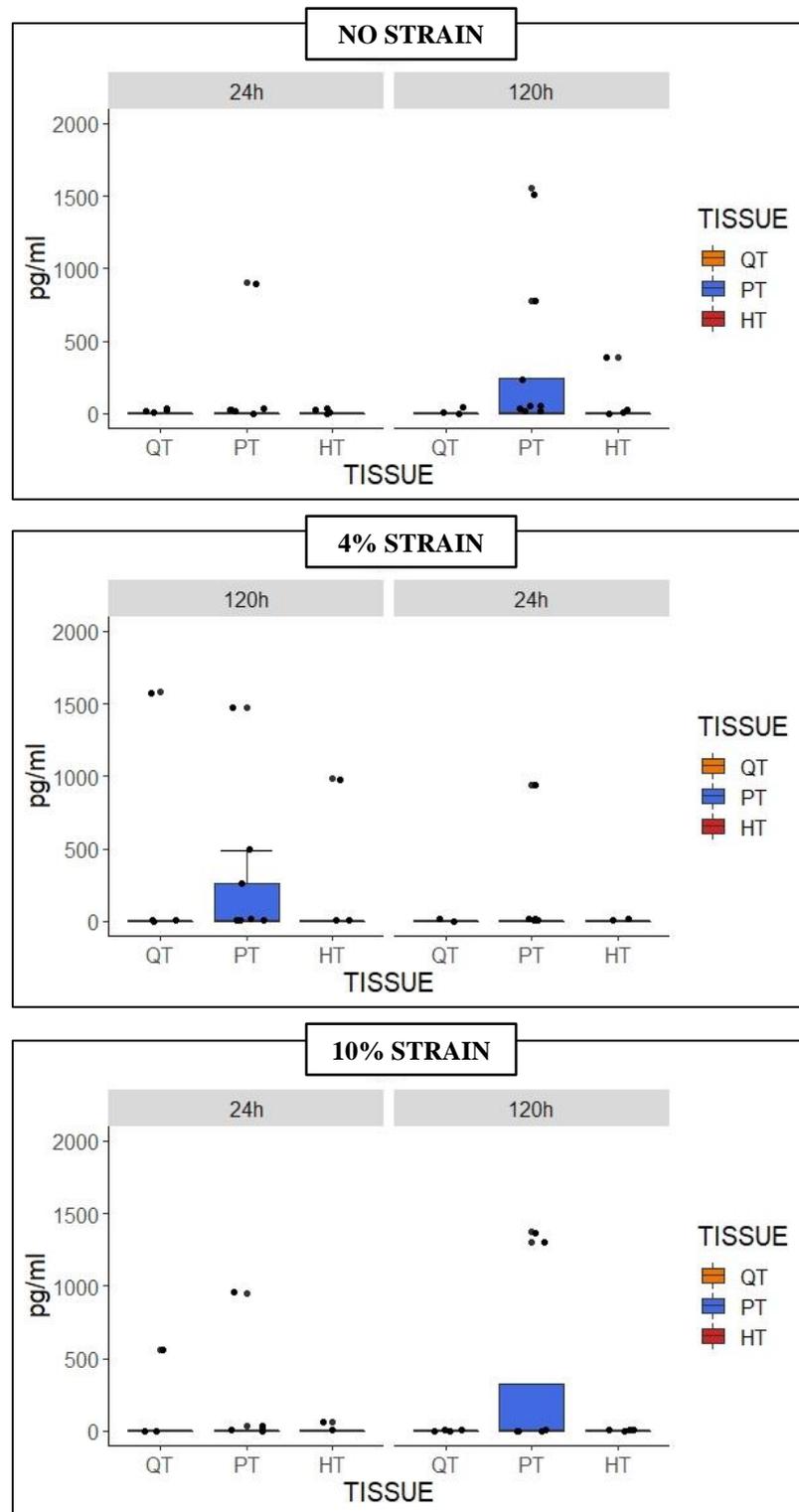


Figure 5.20. Concentration of KC released to the media in males. * significant difference between TISSUES at 24 and 120 hours (Kruskal-Wallis test). QT: Quadriceps tendon; PT: Patellar tendon, HT: Hamstring tendon

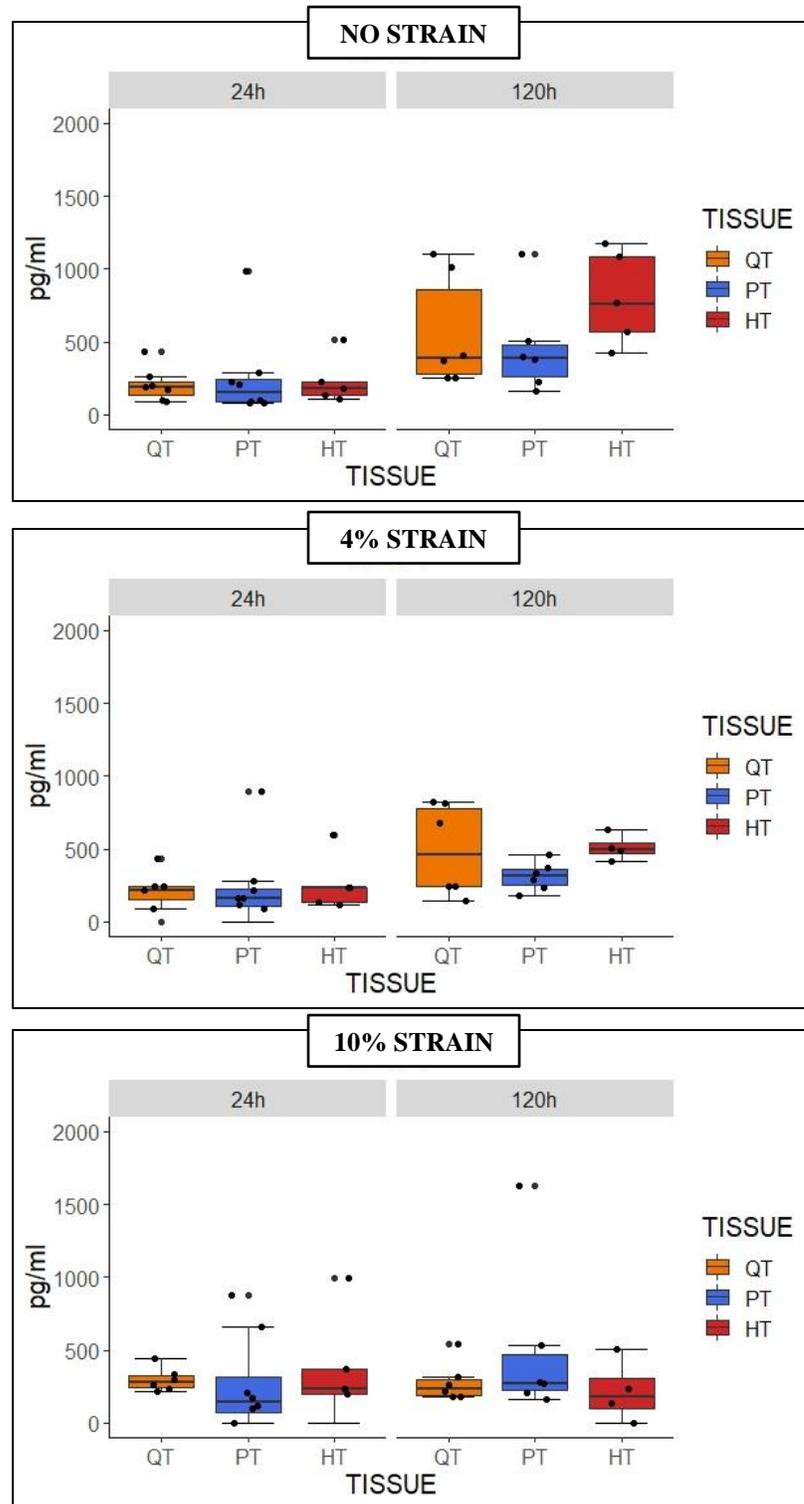


Figure 5.21. Concentration of MCP1 released to the media in males. * significant difference between TISSUES at 24 and 120 hours (Kruskal-Wallis test). QT: Quadriceps tendon; PT: Patellar tendon, HT: Hamstring tendon

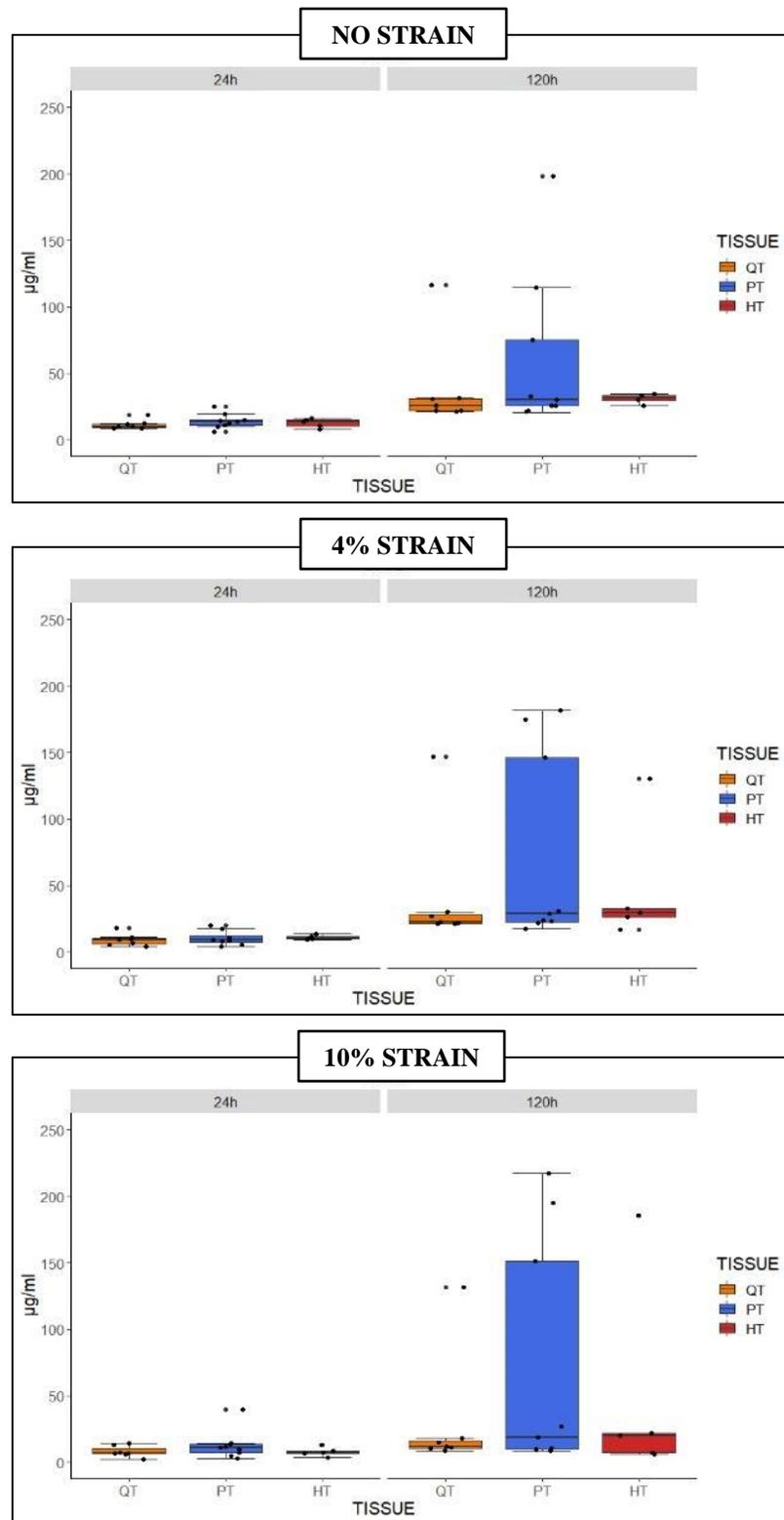


Figure 5.22. Concentration of GAG released to the media in males. * significant difference between TISSUES at 24 and 120 hours (Kruskal-Wallis test). QT: Quadriceps tendon; PT: Patellar tendon, HT: Hamstring tendon

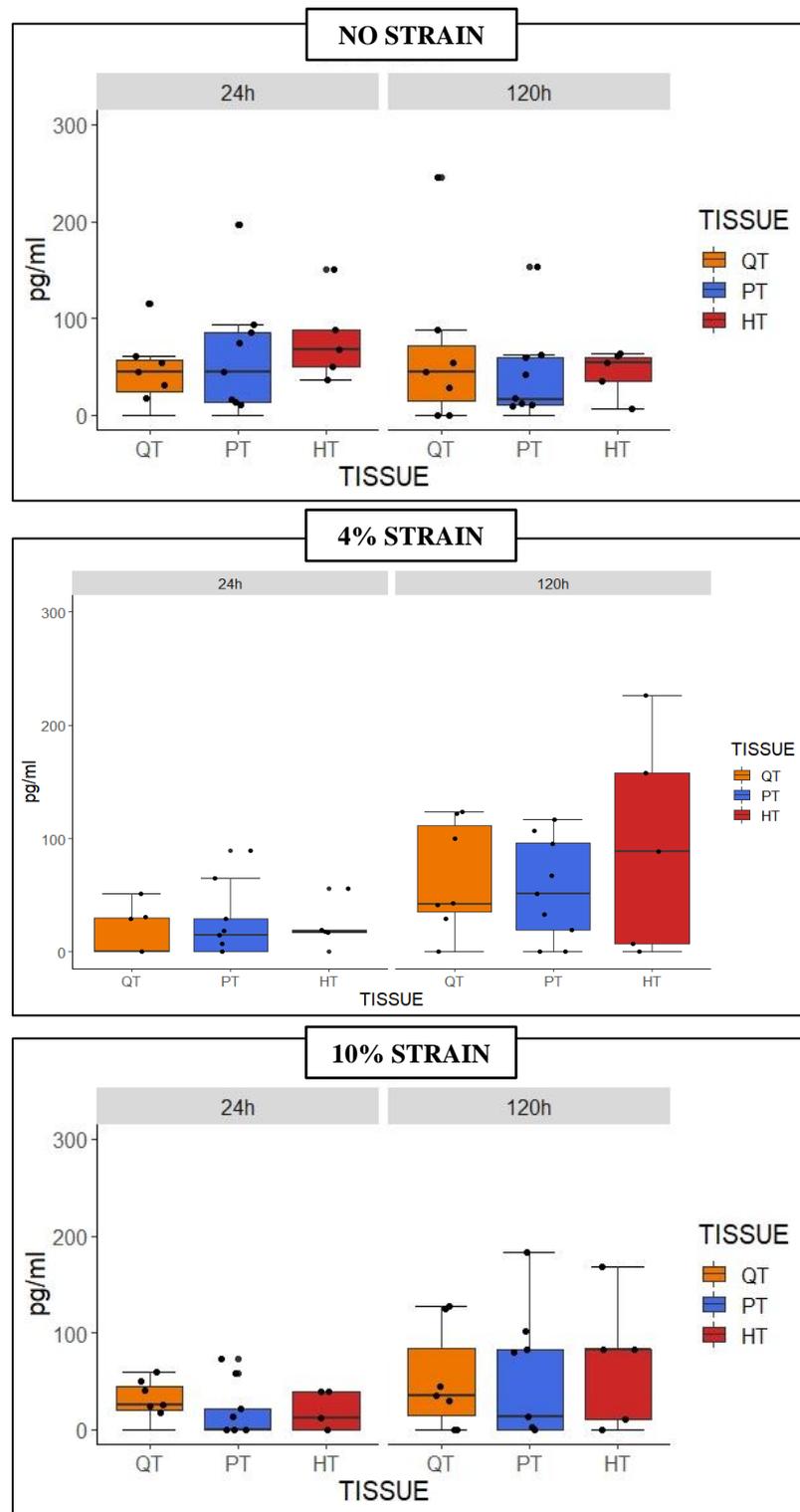


Figure 5.23. Concentration of MMPACT released to the media in males. * significant difference between TISSUES at 24 and 120 hours (Kruskal-Wallis test). QT: Quadriceps tendon; PT: Patellar tendon, HT: Hamstring tendon

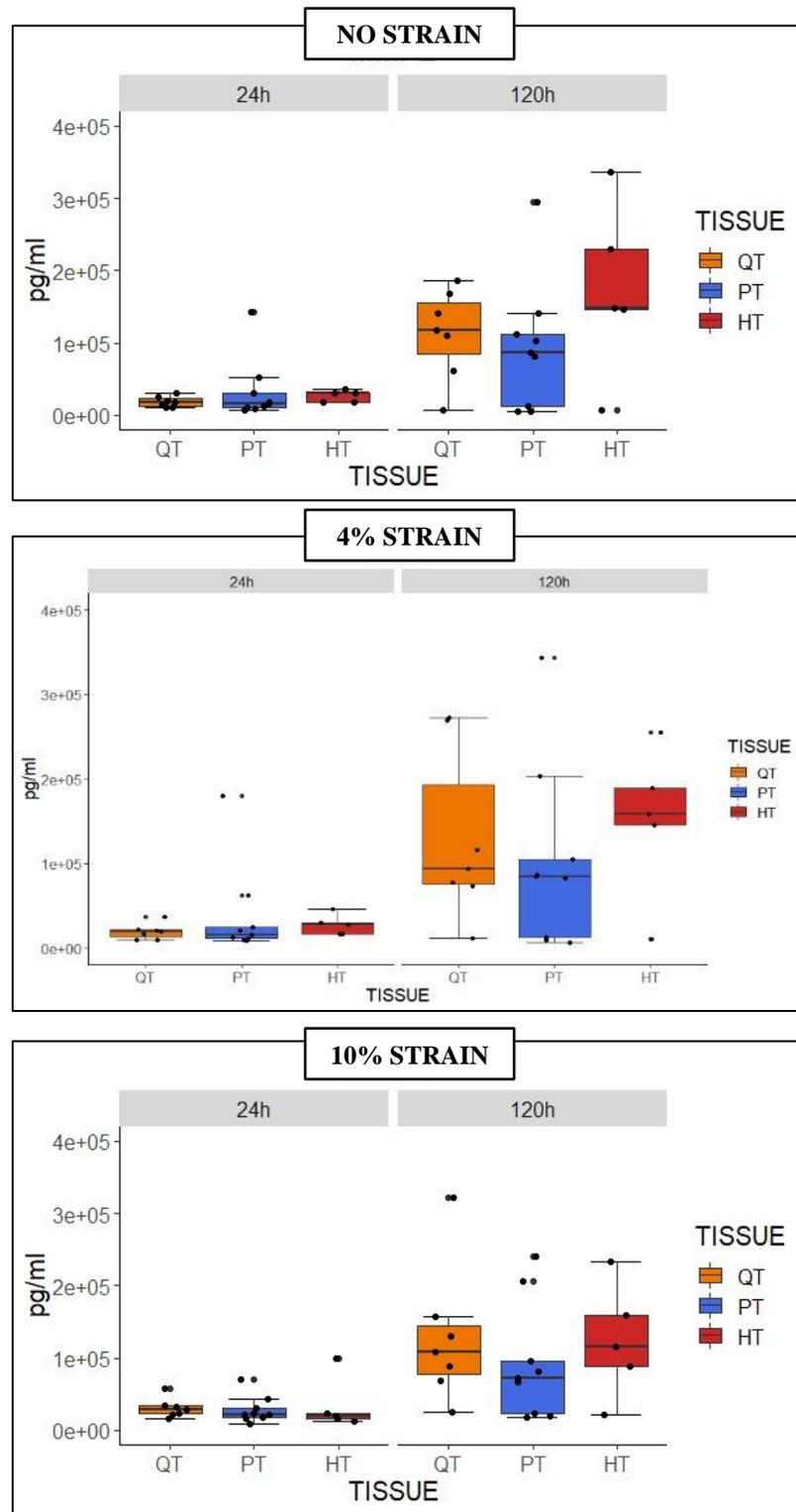


Figure 5.24. Concentration of MMP-2 released to the media in males. * significant difference between TISSUES at 24 and 120 hours (Kruskal-Wallis test). QT: Quadriceps tendon; PT: Patellar tendon, HT: Hamstring tendon

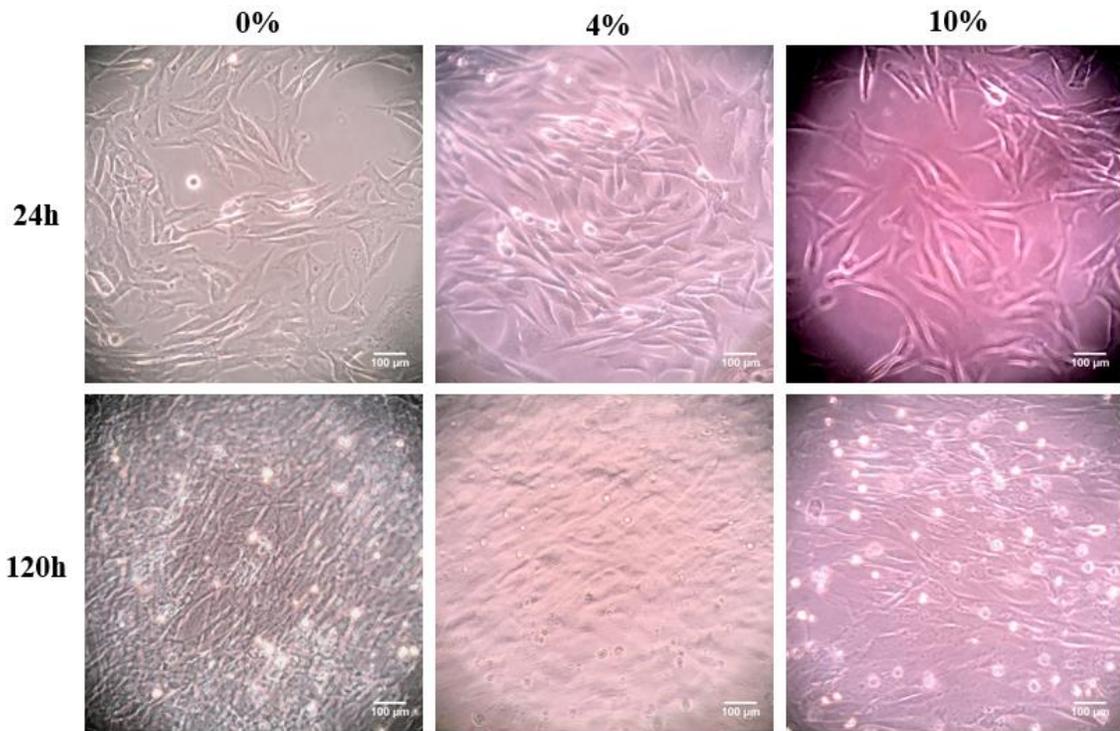


Figure 5.25. Quadriceps tendon (QT) fibroblasts stretched after 24 and 120 hours at three different strain magnitudes.

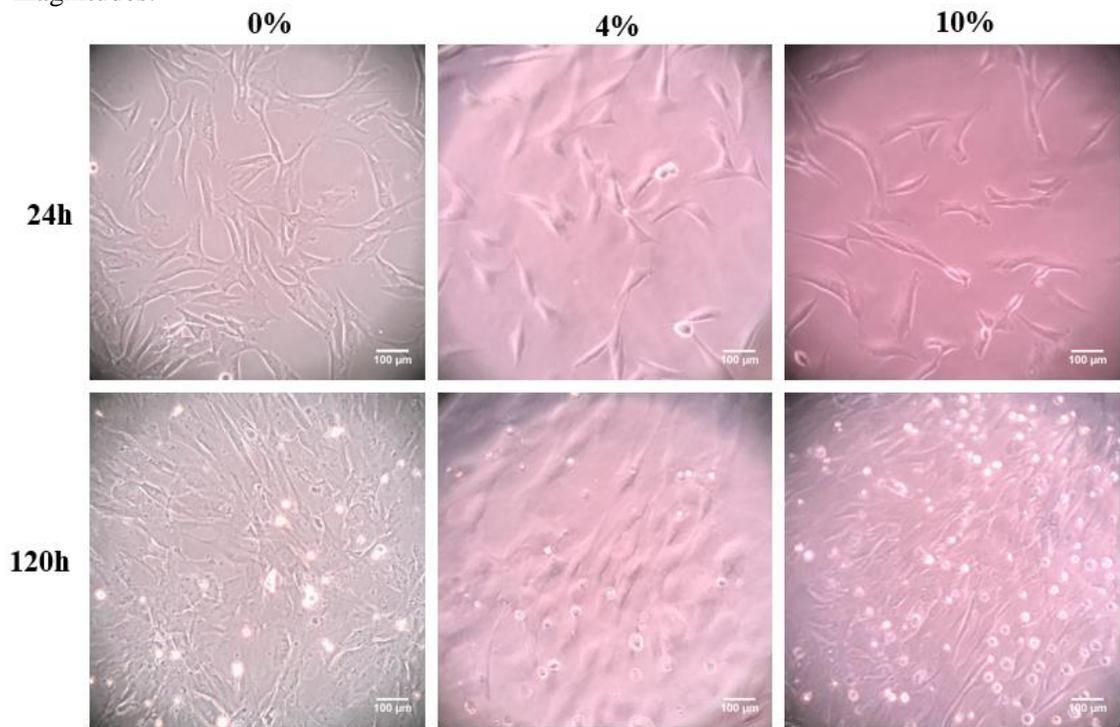


Figure 5.26. Patellar Tendon (PT) fibroblasts stretched after 24 and 120 hours at three different strain magnitudes.

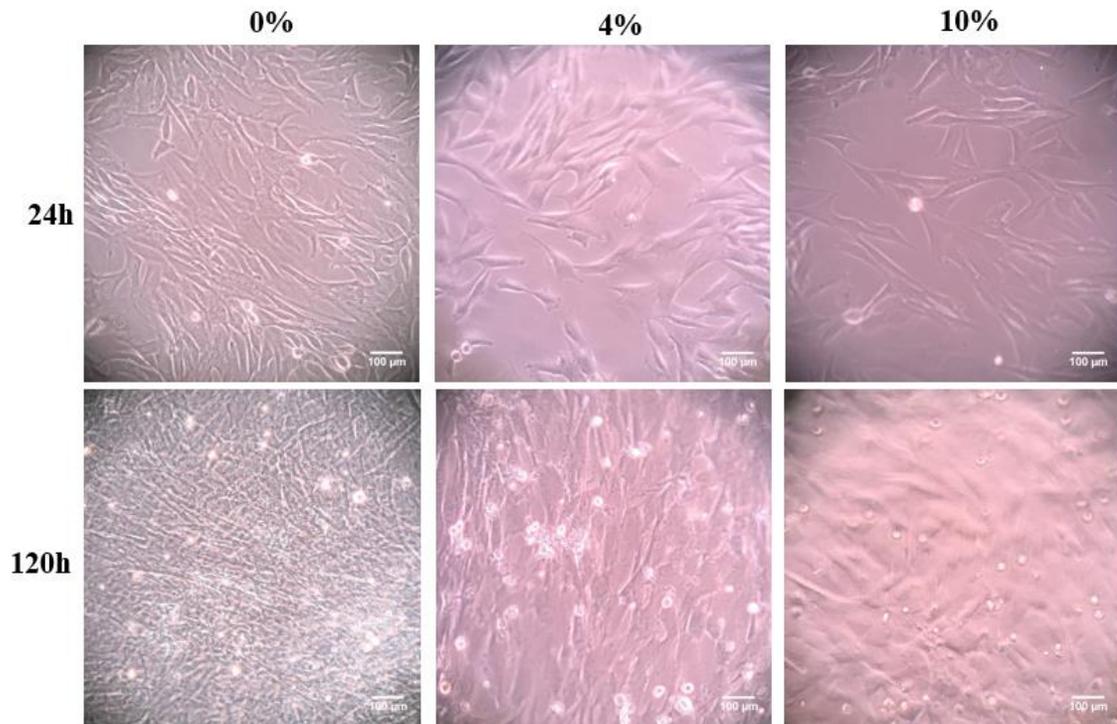


Figure 5.27. Hamstring Tendon (HT) fibroblasts stretched after 24 and 120 hours at three different strain magnitudes.

CHAPTER 6

NORMAL CANINE QT, PT, HT CELLS CO-CULTURED WITH SYNOVIOCYTES AND EXPOSED TO MECHANICAL STRAIN

Introduction

Anterior cruciate ligament (ACL) injury is one of the most common musculoskeletal injuries, compromising knee stability and function and places individuals at risk for further injuries and post-traumatic osteoarthritis (PTOA). Symptomatic ACL injuries are often surgically treated with reconstruction using a tendon autograft or allograft. Common tendon grafts used in ACL reconstruction include the quadriceps tendon (QT), patellar tendon (PT), and the hamstring tendon (HT). Regardless of the graft used, healing of the ACL graft involves the remodeling of the extracellular matrix (ECM) and ligamentization of the tendon graft. The healing of the ACL graft is influenced by the intraarticular environment, and the ligamentization process requires a biological compositional shift from tendon to ligament-like tissue resulting in changes in its biomechanical properties.¹ Hence, the evaluation of the metabolic responses in mechanosensitive tissues such as the tendon grafts, constitutes a key part in the understanding of the intraarticular healing responses.

The synovium may play a role in graft ligamentization after ACL reconstruction. While the native ACL is intraarticular in location, it is extrasynovial and is encased by a synovial sheath. The synovial sheath may play an important role in various tendon pathologies such as carpal tunnel syndrome (CTS).² Early tenosynovial changes seem to correlate with CTS symptoms. Marked ECM remodeling in the tenosynovium is an important characteristic of tendon healing and degradation. The synovial membrane that

line the intraarticular knee joint is also vital for regulating the joint homeostasis by maintaining or amplifying the inflammatory signals.³ Elevated levels of cytokines and growth factors within the knee intraarticular environment are often considered to be the contribution of synovium.⁴ Furthermore, the cross talk between synoviocytes and intraarticular ligaments has been shown to influence the healing response when fibroblasts are subjected to mechanical deformation.⁵ There is, however, no information on the role of the synoviocytes in ACL graft healing when subjected to mechanical strain levels commonly found in perioperative loads.

The biomechanical environment that the tendon graft experiences plays an important role in the graft ligamentization process after ACL reconstruction.⁶⁻¹¹ Successful intraarticular graft remodeling and intra-tunnel graft incorporation depend on the interplay of many factors. Depending on the mechanical stimulus that the ACL graft experiences, these forces can modulate bone formation and the tendon-to-bone tunnel healing process.¹² This could be explained by the ability of the tenocytes to remodel the ECM in response to different biomechanical environments.^{13; 14}

In vitro systems for modeling the ACL and its remodeling usually evaluate cellular responses in a static environment on isolated fibroblasts in monoculture.¹⁵⁻¹⁷ However, these static monoculture systems does not consider the interplay between various tissues within a complex biologic ecosystem such as the knee joint. Since the intraarticular environment of the knee is constantly subjected to mechanical strain, cellular responses of multiple tissues might be affected by the magnitude of strain that is applied, and in turn, affect one another. Given the importance of the synovium and its central role in modulating the intraarticular knee environment, we aimed to evaluate the effects that synoviocytes

have on common ACL graft fibroblasts after mechanical stress is applied in a dynamic co-culture system. We hypothesized that graft tendon fibroblasts co-cultured with synoviocytes will significantly increase the metabolic activity, and the production of pro-inflammatory biomarkers and remodeling biomarkers of common graft tendon fibroblast cells under mechanical stress when compared to monocultured graft fibroblasts.

Materials and methods

Tissue processing and culture

With ACUC approval (ACUC#9163, 9164), QT, PT, HT and synovial (SYN) (13 PT, 10 HT, 12 QT and 1 SYN) tissues were harvested from skeletally mature dogs (n=13) euthanatized for studies unrelated to the current work and free of any orthopedic disease based on orthopedic examination and radiographs. Tissues were aseptically minced into 0.5–1.0 cm² pieces and digested overnight in Type 1A *Clostridium histolyticum* collagenase solution (Sigma-Aldrich, St. Louis, MO, USA) at a concentration of 0.5 mg/mL. The aliquot was then suspended with nutritional media (Gibco DMEM, Thermo Fisher Scientific, Waltham, MA, USA) containing 10% FBS, 0.002% Penicillin, 100 µg/mL Streptomycin, 25 µg/mL Amphotericin B, 0.002% L-Ascorbate and 0.01% L-glutamine (Sigma Chemical Co, St. Louis, MO), cultured in T75 flasks (TPP Techno Plastic Products AG, Trasadingen, Switzerland) and incubated at 5% CO₂, 37°C, and 95% humidity.

Biaxial mechanical stress

Once confluent, graft fibroblasts were resuspended and seeded in Collagen Type I-coated BioFlex® plates (1 × 10⁵ cells/well). Polyethylene terephthalate (PET) coated co-culture inserts (Corning©) were seeded with SYN (5 × 10⁴ cells/well) and placed in the Bioflex®

plates using a Flexcell® transwell holder. Cells were incubated with 10% FBS media for an additional 48h before strain was applied. Then, nutritional culture media was replaced with 0.5% FBS media for the remaining five days of culture. Fibroblasts were subjected to continuous mechanical stimulation (2-s strain and 10-s relaxation at a 0.5 Hz frequency) with a biaxial sinusoidal waveform with three different elongation strains chosen to represent three physiologic conditions (mechanical stress deprivation-0%, physiologic strain-4%, and supraphysiologic strain-10%)¹⁸ for 5 days using the Flexcell FX-4000T strain system (Flexcell International, NC, USA) (Fig. 6.1). Culture media were changed every 24h and stored at -20°C until analysis. At the end of the strain protocol (120h), cell viability was assessed using the resazurin assay.

Cell viability assay

The resazurin dye added to the culture media is a cell permeable redox indicator used to monitor viable cells. Viable cells with active metabolism can reduce resazurin into the highly fluorescent metabolite resorufin. The quantity of resorufin produced is proportional to the number of viable cells in culture.¹⁹ Briefly, 10% volume of resazurin (Sigma-Aldrich, Saint Louis, MO) stock solution (0.1 mg/mL) was added to the media at the end of the culture period (120h) and incubated at 37°C for two hours. Then, fluorescence excitation and emission were measured at 540/580 nm.

Biomarker assays

Media from 24 and 120 hours of culture were assessed for various biomarkers. Proteoglycan (GAG) was assessed using the DMMB assay as previously described.²⁰ The concentration of prostaglandin E₂ (PGE₂) (Cayman Chemical, Ann Arbor, MI, USA), cytokines (IL-6, IL-8, KC and MCP-1) (Millipore, Billerica, MA, USA), total matrix

metalloproteinase (MMP) activity (SensoLyte 520 generic MMP assay, Anaspec, Inc., Fremont, CA), and MMP production (MMP-1, MMP-2 and MMP-3) (R&D Systems, Minneapolis, MN, USA), were all assessed using commercially available assays according to the manufacturer's protocol.

Statistical Analysis

For statistical analysis, normal distribution was assessed by Shapiro-Wilk test. Data were not normally distributed so non-parametric analyses were used. Comparisons between monoculture and co-culture at either 24 or 120 hours of culture were performed using Mann-Whitney U test. Additionally, comparisons among strains and tissue types were performed using Kruskal-Wallis test and Dunn's test posthoc (R Core Team (2019), R version 3.6.2 Vienna, Austria). Results were reported as median \pm interquartile range (IQR). Significance levels were set at $p < 0.05$.

Results

Differences between monoculture and co-culture

Co-culture with synoviocytes resulted in significantly higher metabolic activity in QT and PT fibroblasts co-cultured with SYN compared to monocultured fibroblasts regardless of the strain. Similarly, HT fibroblasts exhibited increased metabolic activity at no strain and 10% strain when cocultured with SYN compared to monoculture (Fig. 6.2).

Inflammatory mediator PGE₂, significantly increased at 24h in QT fibroblasts when co-cultured with SYN relative to monoculture at no strain. Moreover, at 120h, PGE₂ production significantly increased in cocultured QT fibroblasts relative to monoculture regardless of the strain. Similarly, PT fibroblasts cocultured with SYN, increased the production of PGE₂ at 24h and 120h of mechanical strain deprivation compared to

monoculture. Moreover, HT fibroblasts in cocultured showed an increased production of PGE2 at no strain 120h of culture. Interestingly, physiological strain elicited an increased production of PGE2 in monocultured QT fibroblasts compared to coculture at 24h. Similarly, PT and HT fibroblasts in monoculture, exhibited an increased production of PGE2 at 24h and 120h when subjected to physiological strain. Additionally, at 120h, PT fibroblasts in monoculture also showed higher production of PGE2 compared to cocultured fibroblasts (Fig. 6.3).

IL-6 production was not significantly different in cocultured when compared to monoculture in any of the tendon fibroblast types evaluated at any timepoint (Fig. 6.4). However, all the other cytokines were significantly increased in coculture.

The production of IL-8 was significantly increased in QT fibroblasts cocultured with SYN at 24h in the supraphysiological strain group. Moreover, at 120h, cocultured QT fibroblasts exhibited a significant increase in IL-8 production relative to monoculture in all strain groups. Similarly, cocultured PT fibroblasts, also exhibited a significantly higher production of IL-8 compared to monoculture at 120h in all strain groups. Interestingly, cocultured HT fibroblasts exhibited a significant decrease in IL-8 production relative to monoculture when subjected to 4% strain at 24h and 120h. Nevertheless, cocultured HT fibroblasts showed a significantly increased production of IL-8 compared to monoculture at 120h (Fig. 6.5).

The production of the chemokine KC was significantly higher in monocultured QT fibroblasts compared to co-culture in the no strain and 10% strain groups at 24h. However, at 120h, cocultured QT fibroblasts exhibited a significantly increased production of KC compared to monoculture regardless of the strain applied. Moreover, physiological strain

elicited an increased production of KC in monocultured PT and HT fibroblasts relative to coculture at 24h and 120h. However, at 120h, cocultured PT and HT fibroblasts exhibited significantly higher production of KC compared to monoculture at no strain (HT) and 10% strain (PT and HT) (Fig. 6.6).

The production of MCP-1 in cocultured QT fibroblasts was significantly higher than monoculture at 24h and 120h of culture regardless of the strain. Moreover, at 24h, cocultured PT fibroblasts exhibited increased production of MCP-1 compared to monoculture at no strain and 10% strain. At 120h, cocultured PT fibroblasts showed significantly higher levels of MCP-1 compared to monoculture in all strain groups. Additionally, cocultured HT fibroblasts exhibited significantly higher levels of MCP-1 compared to monoculture at 24h in all strain groups. At 120h, cocultured HT fibroblasts showed significantly higher production of MCP-1 compared to monoculture in the no strain and 10% strain groups (Fig. 6.7).

GAG production in co-cultured QT fibroblasts was significantly higher than monoculture in all strain groups at 24h of culture. Moreover, at 120h, GAG production was significantly higher in cocultured QT fibroblasts subjected to mechanical strain deprivation compared to monoculture. Similarly, cocultured PT fibroblasts showed significantly higher production of GAG than monoculture in the 10% strain group at 24h. However, at 120h, cocultured PT fibroblasts exhibited significantly lower production of GAG compared to monoculture when subjected to mechanical strain deprivation but significantly increased in cocultured PT fibroblasts at 4% strain relative to monoculture. Moreover, cocultured HT fibroblasts significantly increased production of GAG at 24h in the no strain and 4% strain

groups. Similarly, at 120h, cocultured HT fibroblasts showed significantly higher levels of GAG content when subjected to physiological strain compared to monoculture (Fig. 6.8). The level of MMPACT was consistently higher in co-cultured QT fibroblasts compared to monoculture at 24h in the no strain and 10% strain, but significantly lower in the 4% strain group. Similarly, at 120h, cocultured QT fibroblasts exhibited significantly decreased levels of MMPACT compared to monoculture in the 4% strain group. On the other hand, cocultured PT fibroblasts showed a significant increase in MMPACT compared to monoculture in the 4% strain and 10% strain groups at 24h. Also, at 120h, 4% strain showed a significant increase in MMPACT relative to monocultured PT fibroblasts. Moreover, cocultured HT fibroblasts showed significantly higher levels of MMPACT compared to monoculture in the 10% strain group at 24h. MMPACT was also significantly higher at 120h of supraphysiological strain in cocultured HT fibroblasts relative to monoculture (Fig. 6.9).

Co-culture conditions typically resulted in significantly decreased MMP-1 production in QT and PT fibroblasts compared to monoculture at all strains at both 24h and 120h. Similarly, cocultured HT fibroblasts produced significantly less MMP-2 in the no strain and 4% strain groups at 24h and in all strain groups at 120h (Fig. 6.10). On the other hand, production of MMP-2 was significantly increased in cocultured QT fibroblasts relative to monoculture in all strain groups at 24h of culture. However, at 120h, cocultured QT fibroblasts exhibited significantly less MMP-2 production compared to monoculture in the no strain group. Moreover, at 24h, there were no significant differences between cocultured and monocultured PT and HT fibroblasts. Interestingly, at 120h, MMP-2 production

significantly increased in monocultured PT fibroblasts compared to coculture but decreased in HT co-culture compared to monoculture at physiological strain (Fig. 6.11).

The production of MMP-3 was significantly higher in monocultured QT fibroblasts relative to monoculture in the no strain group at 24h and 120h. Moreover, MMP-3 production was significantly lower in monocultured PT fibroblasts relative to coculture in the no strain and 10% strain groups at 24h. No significant differences between cocultured and monocultured HT fibroblasts were seen in any strain group at 24h and 120h (Fig. 6.12).

Differences between strain loads within fibroblast cell type during co-culture

When evaluating strain responses in cocultured grafts it was observed that co-cultured QT fibroblasts subjected to mechanical strain deprivation had a significantly increased metabolic activity when compared to physiological and supraphysiological strain groups. Moreover, cocultured PT fibroblasts exhibited a significantly decreased metabolic activity supraphysiological strain relative to mechanical strain deprivation and physiological strain (Fig.6.13). Additionally, with mechanical deprivation, co-cultured QT fibroblasts had significantly higher metabolic activity compared to co-cultured PT and HT fibroblasts. 4% and 10% strain did not elicit significant differences in metabolic activity in any of the tissues (Fig. 6.21).

While the production of PGE2 in QT fibroblasts co-cultured with synoviocytes was not significantly different among strains, it typically resulted in significant increases in PGE2 from PT fibroblasts at no strain relative to 4% and 10% strain groups at 120h of culture. Moreover, the production of PGE2 by co-cultured HT fibroblasts was significantly lower at physiological stress compared to mechanical strain deprivation and supraphysiological strain at 24h. Further, at 120h, the production of PGE2 by co-cultured HT fibroblasts at

physiological stress was significantly lower than the production by co-cultured HT fibroblasts at mechanical strain deprivation and supraphysiological strain (Fig. 6.14).

The production of IL-8 production was consistently low in co-cultured QT fibroblasts subjected to mechanical strain deprivation relative to supraphysiological strain at 24h and 120h of culture. Cocultured PT fibroblasts exhibited a significantly decreased production of IL-8 after 120h of physiological strain relative to no strain and 10% strain groups. Moreover, cocultured HT fibroblasts exhibited significantly lower levels of IL-8 at 4% strain relative to 10% strain at 24h of culture. Additionally, at 120h, cocultured HT fibroblasts produced significantly lower levels of IL-8 in the physiological strain group compared to the mechanical strain deprivation and supraphysiological strain groups (Fig. 6.15).

KC production significantly increased by co-cultured tendon fibroblasts in the supraphysiologic strain groups compared to the physiological strain groups at 120h of culture. Cocultured QT fibroblasts showed significantly increased levels of KC after 120h of supraphysiological strain relative to no strain and 4% strain groups. Moreover, cocultured PT fibroblasts exhibited a significantly higher production of KC in the 10% strain group relative to the 4% strain group at 120h of culture. Additionally, co-cultured HT fibroblasts showed significantly lower levels of KC after 24h of physiological strain relative to 10% strain. Similarly, at 120h, cocultured HT fibroblast showed significantly lower production of KC in the 4% strain group relative to no strain and 10% strain groups (Fig. 6.16).

Further, co-cultured QT fibroblasts significantly lower production of MCP-1 at no strain compared to 4% and 10% strain at 24h. Similarly, at 120h, cocultured QT fibroblasts at no

strain produced significantly less MCP-1 than at 10% strain. Moreover, cocultured PT and HT fibroblasts produced significantly less MCP-1 at 4% strain compared to no strain and 10% strain at 24h and 120h of culture (Fig. 6.17).

The production of GAG in co-cultured QT fibroblasts was significantly lower in the supraphysiological strain group compared to the no strain and 10% strain groups at 24h of culture. Moreover, cocultured PT fibroblasts showed significantly higher levels of GAG after 120h of physiological strain relative to mechanical strain deprivation. Additionally, cocultured HT fibroblasts did not show significant changes among strain groups at any time point (Fig. 6.18).

The level of MMPACT was significantly lower in co-cultured QT fibroblasts at physiological strain compared to no strain and 10% strain at 24h. Moreover, co-cultured QT fibroblasts showed a significant increase in MMPACT when cells were subjected to mechanical strain deprivation compared to 4% and 10% strain at 120h of culture. Additionally, co-cultured PT fibroblasts showed a significant decrease in MMPACT in the no strain group relative to 4% and 10% strain at 24h of culture. Also, at 120h, cocultured PT fibroblasts showed a significantly higher production of MMPACT at 4% strain relative to no strain and 10% strain groups. In cocultured HT fibroblasts, MMPACT was significantly higher at 10% strain compared to 4% strain at 24h (Fig. 6.19)

The production of MMP-2 was significantly lower in cocultured QT fibroblasts subjected to physiological strain compared to no strain and 10% strain groups at 24h. Moreover, at 120h, supraphysiological strain elicited a significant increase in MMP2 production compared to no strain and 4% strain. Additionally, cocultured PT fibroblasts did not show significant differences in the production of MMP-2 at any strain level in any timepoint.

However, cocultured HT fibroblasts showed significantly increased levels of MMP-2 when subjected to 4% strain relative to no strain and 10% strain at 120h of culture (Fig. 6.20)

Differences between fibroblast types at each strain load during co-culture

PGE2 production was significantly different among tissue type fibroblasts. During mechanical deprivation, co-cultured PT and HT fibroblasts produced significantly higher levels of PGE2 compared to co-cultured QT fibroblasts at 24h of culture. Moreover, 4% strain elicited a significantly increased production of PGE2 in cocultured QT fibroblasts relative to PT and HT at 120h. PGE2 production was also significantly higher in cocultured QT fibroblasts relative to cocultured PT fibroblasts when subjected to supraphysiological strain at 24h and 120h (Fig. 6.22).

Mechanical strain deprivation did not elicit significant differences in IL-8 production among tendon fibroblasts at any timepoint. However, 4% and 10% strain elicited a significant increase in IL-8 production in cocultured QT fibroblasts relative to cocultured PT and HT fibroblasts at 120h (Fig. 6.23).

The production of KC was significantly higher in cocultured HT fibroblasts relative to QT after 120h of mechanical strain deprivation. Moreover, at 4% strain, cocultured QT fibroblasts exhibited a significantly higher production of KC relative to PT and HT fibroblasts at 120h of culture. Additionally, at 10% strain, cocultured HT fibroblasts showed a significantly increased production of KC relative to QT fibroblasts at 24h (Fig. 6.24).

MCP-1 production was significantly lower in cocultured QT fibroblasts compared to HT fibroblasts at 24h of mechanical strain deprivation. Similarly, after 120h of mechanical strain deprivation, a significant decrease in MCP-1 production was seen in cocultured QT

fibroblasts relative to PT and HT cocultured fibroblasts. Moreover, physiological strain elicited a significant increase in MCP-1 production in cocultured QT fibroblasts relative to PT and HT fibroblasts at 24h and 120h, respectively. No significant differences among tissues were seen at 10% strain in any of the tissue types at any time point (Fig. 6.25).

The production of GAG was significantly lower in cocultured PT fibroblasts relative to QT and HT fibroblasts at 120h of mechanical deprivation. Also, at 4% strain, cocultured PT fibroblasts exhibited a significant increase in GAG production relative to cocultured QT fibroblasts at 120h. There were no significant differences in GAG production among the different tissue fibroblasts at any timepoint (Fig. 6.26).

The level of MMPACT was not significantly different between tissue types at any time point in the mechanical strain deprivation and supraphysiological strain groups. However, during physiological strain, the level of MMPACT in cocultured PT fibroblasts was significantly higher than QT fibroblasts at 24h of culture. Also, the production of MMPACT was significantly lower in co-cultured QT fibroblasts compared to PT and HT fibroblasts at 120h of culture (Fig. 6.27).

The production of MMP-2 was not significantly different among tissue fibroblast types at mechanical strain deprivation at any time point. However, at 4% strain, cocultured QT fibroblasts produced significantly more MMP-2 than PT and HT fibroblast at 24h of culture. Moreover, at 120h, 4% strain elicited a significant increase in MMP-2 production in cocultured HT fibroblasts relative to QT and PT fibroblasts. At supraphysiological strain, the production of MMP-2 by cocultured QT fibroblasts was significantly higher than PT fibroblasts at 24h of culture (Fig. 6.28).

Discussion

The synovial membrane that surrounds the cruciate ligaments plays key roles in their nutrition and blood supply.^{4; 21} Synoviocytes are also responsible for the production of synovial fluid constituents and maintenance of joint homeostasis by regulating blood/synovial exchange, phagocytic signaling, and cell-cell and cell-matrix interactions.²²

The synovial membrane is disrupted when ACL is ruptured, which renders the ACL intra-synovial such that the internal epiligament is exposed to breakdown products and proteolytic enzymes that affect healing.^{23; 24}

Different tissues within the knee joint contribute to the production of metalloproteinases, after ACL injury. Tang *et al.*, (2009) showed that synovium from ACL-injured rats had a significantly higher concentration of MMP-2 when compared to normal non-injured rats.⁴ Synovial tissue had the greatest ability to convert pro-MMP-2 to the activated isoform of MMP-2 and release MMP-2 to synovial fluid.⁴ Further, it has been reported that release of MMP-2 is increased after type B synoviocytes are overstretched in an equi-biaxial bioreactor.⁴ Our results show that MMP-2 production is increased when QT graft fibroblasts are co-cultured with synoviocytes. Moreover, at supraphysiological strain levels (10%) the production of MMP-2 by co-cultured QT fibroblasts was significantly higher than mono-cultured QT fibroblasts at 24h of culture. Interestingly, the production of MMP-2 was significantly higher in mono-cultured PT fibroblasts compared to co-cultured PT-fibroblasts at physiological strain but not at mechanical strain deprivation or supraphysiological strain, suggesting that the regulatory function exerted by synoviocytes is tissue-dependent and that graft fibroblastic degradative responses are highly dependent on strain magnitude.

Intraarticular fibroblasts are involved in extracellular matrix (ECM) production including different types of collagen, proteoglycans and hyaluronic acid that are essential components in healing after injury or surgical repair.²⁵⁻²⁹ ECM turnover is also affected by cross-talk among tissues and differentially regulated by strain magnitude.^{30; 31} Our results suggest that ACL tendon grafts in co-culture with synoviocytes were associated with increased production of GAG and decreased MMP activity after five days of supraphysiological strain.

Animal models have shown that cytokine concentrations (IL-1 β , IL-6 and TNF- α) increased in the synovial fluid within three days after ACL injury.⁴ Authors suggest that synovial tissue might be the main producer of cytokines which in turn activate MMP production during an attempted healing response.⁴ Similarly, other authors have reported IL-6 overproduction in inflammatory and degenerative conditions such as osteoarthritis and ligament disease.³² In the present study, production of IL-6 was not detected. Other studies have reported that measurable IL-6 production from fibroblasts requires stimulation with a pro-inflammatory mediator.^{33; 34} Importantly, the inability to detect IL-6 with mechanical insults alone in the present study highlights the limitations of *in vitro* models in not fully mimicking *in vivo* conditions.^{32; 33; 35; 36}

The roles of cytokines on synovial fibroblasts under mechanical stress in monoculture have been demonstrated previously.³⁷ Cytokines and chemokines in the present study were produced at significantly higher levels when graft fibroblasts were co-cultured with synoviocytes. Moreover, non-physiological strain, either mechanical deprivation or supraphysiological strain conditions, induced increased production of cytokines and chemokines (IL-8, KC and MCP-1) in co-cultured fibroblasts compared to those in

monoculture. Interestingly, physiological stress decreased KC and IL-8 production levels by HT fibroblasts co-cultured with synoviocytes.

Co-cultured PT fibroblasts subjected to mechanical deprivation showed the highest increase in PGE2 relative to physiological strain. This might suggest that the reported increased risk of developing OA after BPTB reconstruction could be related to decreased fibroblastic stimulation and inadequate levels of postoperative activity or physical therapy³⁸⁻⁴⁰. Moreover, Hirata et al., (2004) showed that synovial fibroblasts subjected to a 20% strain deformation increased production of both VEGF and PGE2 after 6 hours.² Consequently, authors suggest that PGE2 might be functionally linked to VEGF and may contribute to tenosynovial pathology in carpal tunnel syndrome (CTS) when subjected to supraphysiological stresses.²

Xie et al., (2018) showed that PCL fibroblasts co-cultured with synoviocytes and subjected to supraphysiological strain (12% strain), expressed more MMP-2 than the same cell types in mono-culture and that MMP-2 activity increased with higher synoviocyte density in co-culture.⁴¹ In our experiment, MMP-1 and MMP-3 production was relatively low and not affected by culture condition, cell type, or strain level. Since MMP production responds to both, pro-inflammatory and growth factors stimulation, the low production of these MMPs in this study may relate to the lack of inflammatory stimulation in the model to stimulate the production of these degradative enzymes by the cells during *in vitro* culture.^{37; 42}

Huisman *et al.*, (2016), showed that human HT fibroblasts subjected to 10% strain exhibited higher MMP-2 gene expression compared to low strain (2%).⁴³ Additionally, Jiang *et al.*, (2012) showed that strain deprivation elicited a decreased collagen I synthesis in human PT fibroblasts compared to 4% and 8% strain.⁴⁴ Moreover, Demirag *et al.*, (2005)

demonstrated that blockage of MMPs with α_2 -Macroglobulin in a rabbit model of ACL reconstruction with semitendinosus graft, had higher ultimate load to failure and denser connective tissue around the bone tunnel compared to the control group (no α_2 -macroglobulin).⁴⁵ In this work, the production of MMP-2 was variable. There was decreased MMP-2 production in cocultured QT fibroblasts compared to monoculture after 120h of mechanical strain deprivation. Additionally, at 4% strain, while PT fibroblasts in coculture exhibited significantly lower production of MMP-2 than monoculture, cocultured HT fibroblasts showed higher MMP-2 production than in monoculture conditions. These findings demonstrate that MMP-2 production is both, strain and tissue dependent and that activity level should be matched appropriately to the type of graft.⁴⁶

In the present study, physiological load decreased the production of GAG in co-cultured fibroblasts, and increased levels of IL-8 and KC in mono-culture fibroblasts after 24 hours of culture. It is possible that synoviocytes protect graft fibroblasts from initial excessive ECM remodeling. However, graft type appears to play key roles in ECM production shown by the different GAG production profiles of each graft. Moreover, supraphysiological stress (10%), led to an initial (24h) increase in GAG and MMP-2 production by QT and HT fibroblasts when co-cultured, as well as a late (120h) increases in IL-8 and PGE2 production, showing the differential adaptability of each graft type to load.

This study demonstrates important interactions between ACL graft tenocytes and synoviocytes, and the importance of synoviocytes in regulating inflammatory and metabolic responses when graft fibroblasts are subjected to clinically relevant strain loads. Our findings suggest that there is an increased ECM remodeling during co-culture as indicated by the increased production of GAG from graft fibroblasts, especially at non-

physiological loads. This could be related to the decreased organizational structure (scar tissue) provoked by mechanical deprivation or microdamage due to overloading fatigue.⁸ Moreover, our results indicate that both graft type and mechanical strain are important factors to consider before selecting therapeutic protocols after ACL reconstruction. These data suggest that current perioperative activity protocols should be adjusted to each specific graft type in order to optimize the ligamentization of the tendon graft and potentially decrease failure rates after surgery.^{38; 47-51} The data from this study indicates that synoviocytes can affect the metabolism of tendon grafts after ACL reconstruction, and that the effects of synoviocytes are tendon cell type and strain magnitude dependent.

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Figures

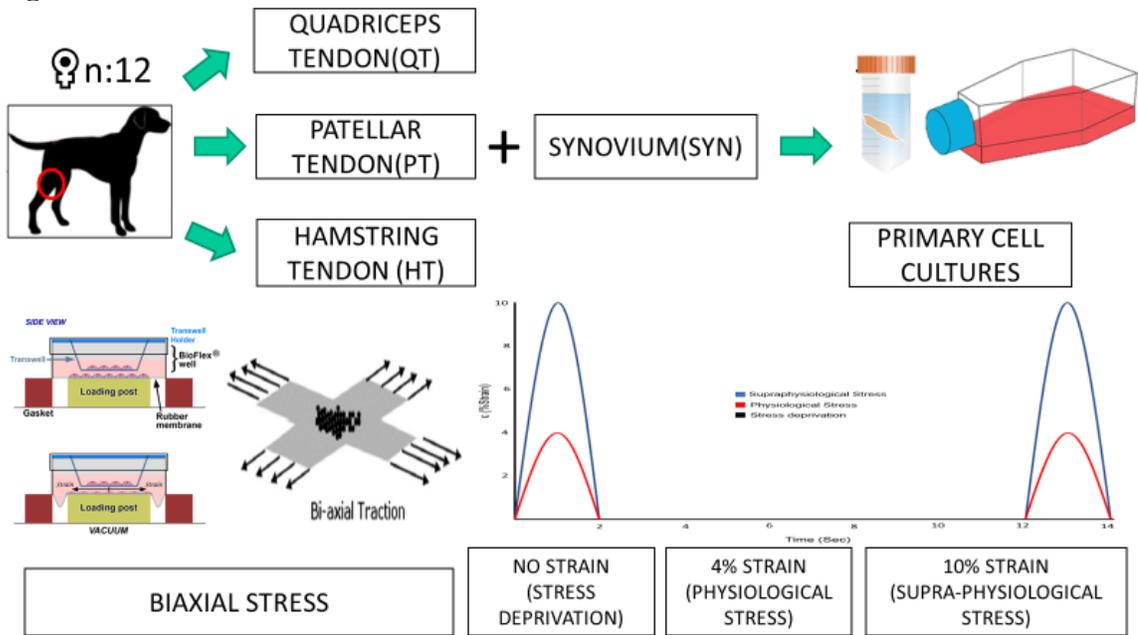


Figure 6.1. Experimental Design.

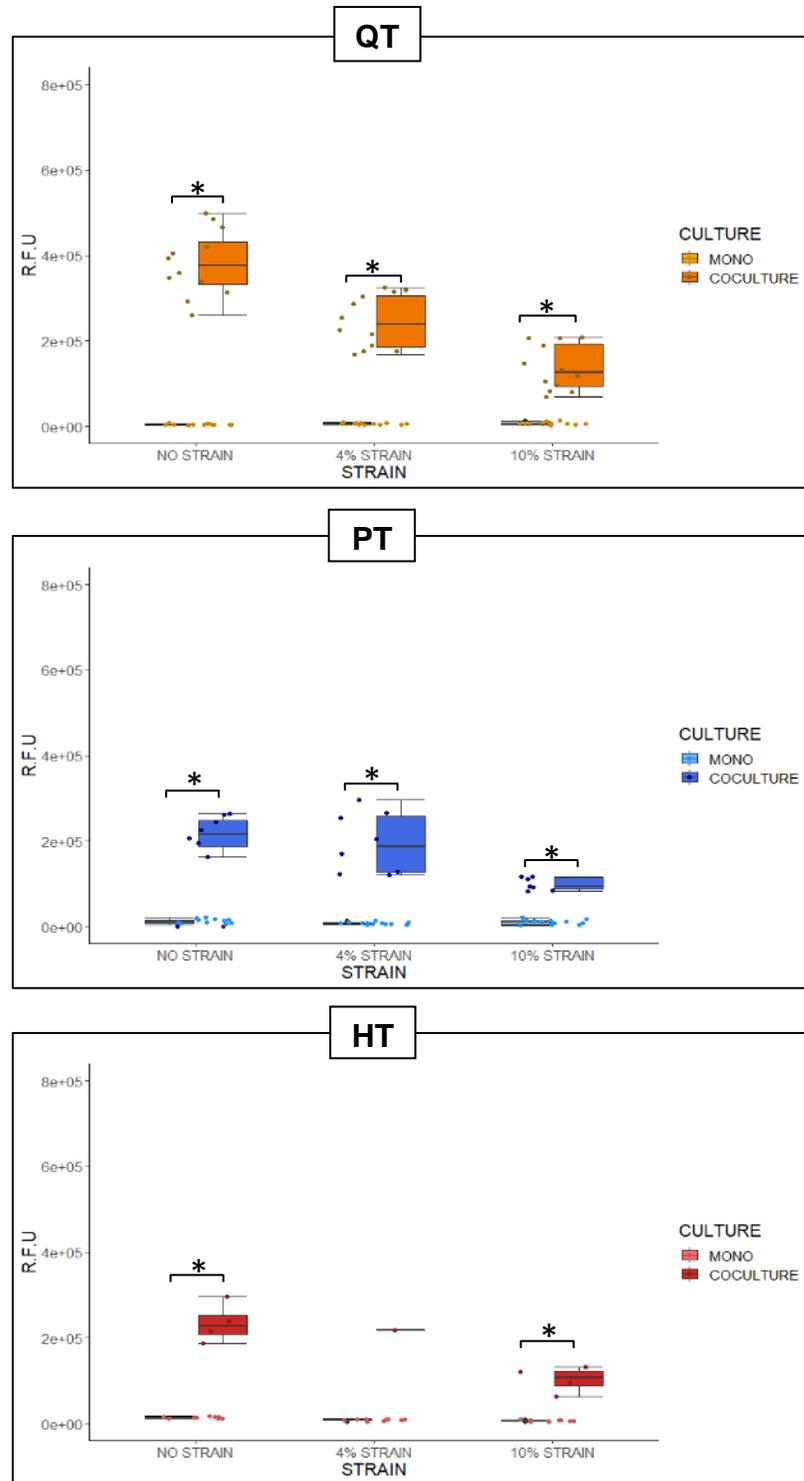


Figure 6.2. Metabolic Activity after 120h of culture. * significant difference between monoculture (MONO) and co-culture (Signed Rank test). QT: Quadriceps tendon; PT: Patellar tendon, HT: Hamstring tendon

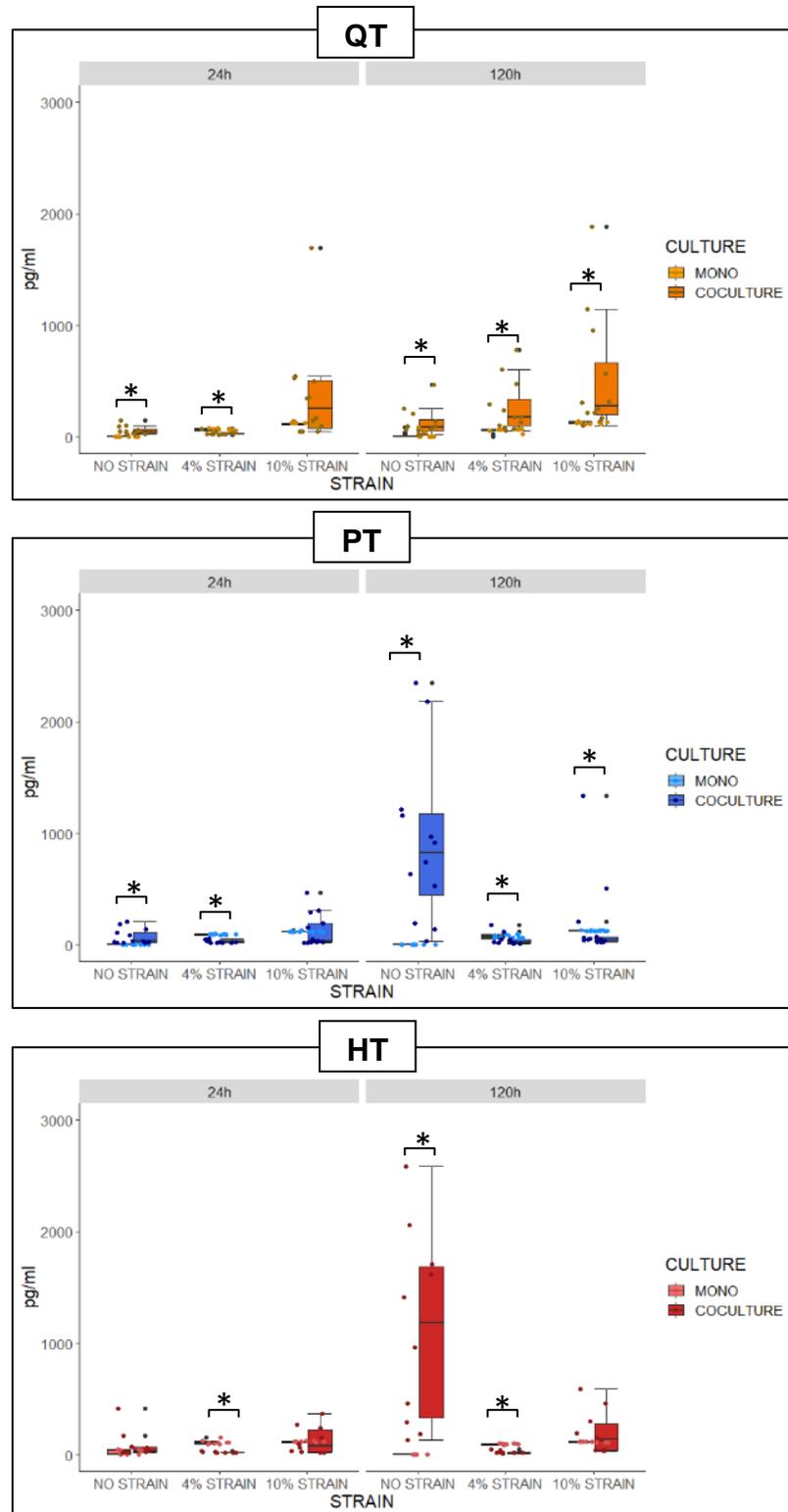


Figure 6.3. Concentration of prostaglandin E2 (PGE2) released to the media. * significant difference between monoculture (MONO) and co-culture at 24 and 120 hours (Signed Rank test); QT: Quadriceps tendon; PT: Patellar tendon, HT: Hamstring tendon

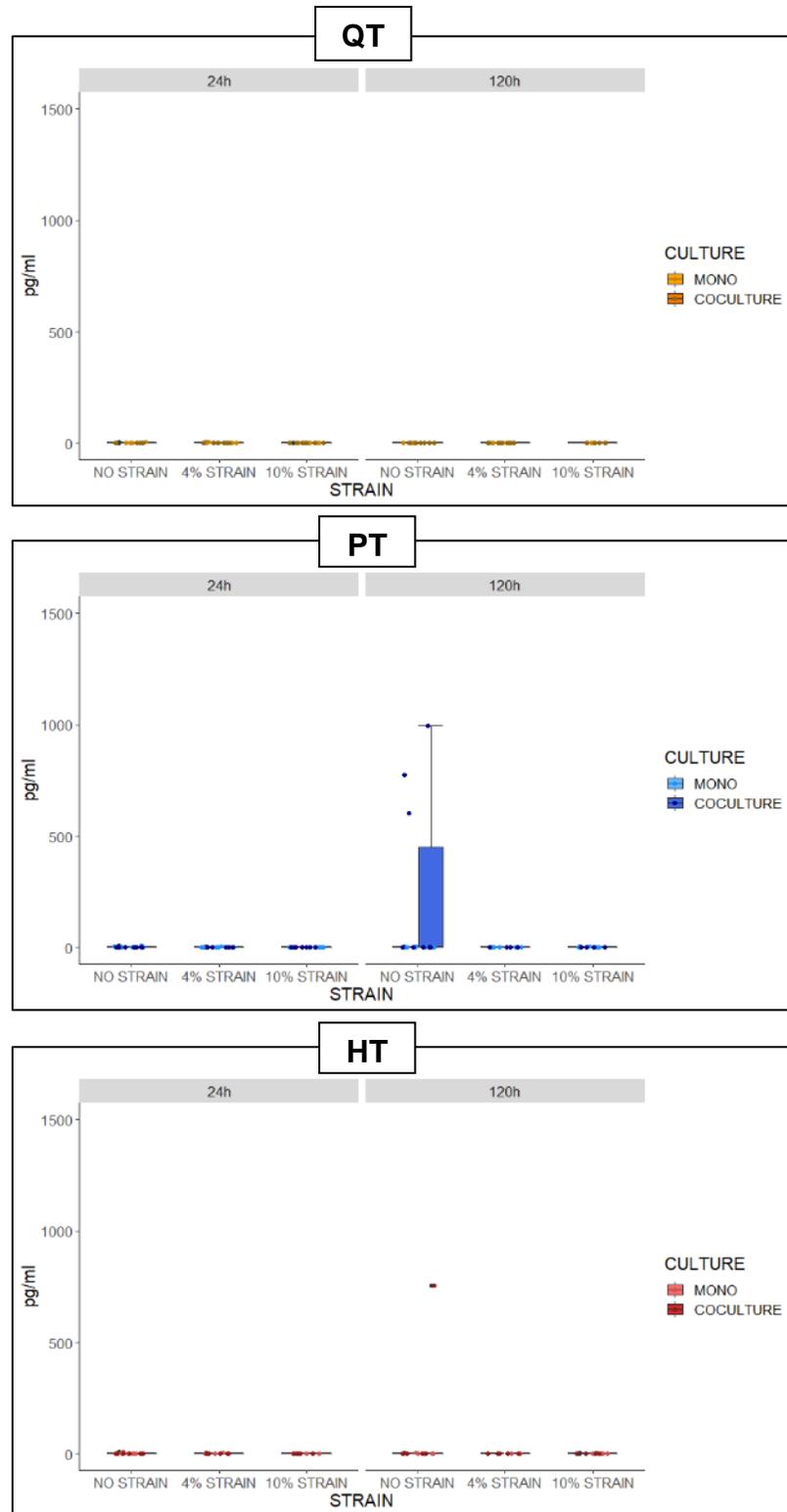


Figure 6.4. Concentration of interleukin-6 (IL-6) released to the media. * significant difference between monoculture (MONO) and co-culture (CO) at 24 and 120 hours (Signed Rank test). QT: Quadriceps tendon; PT: Patellar tendon, HT: Hamstring tendon

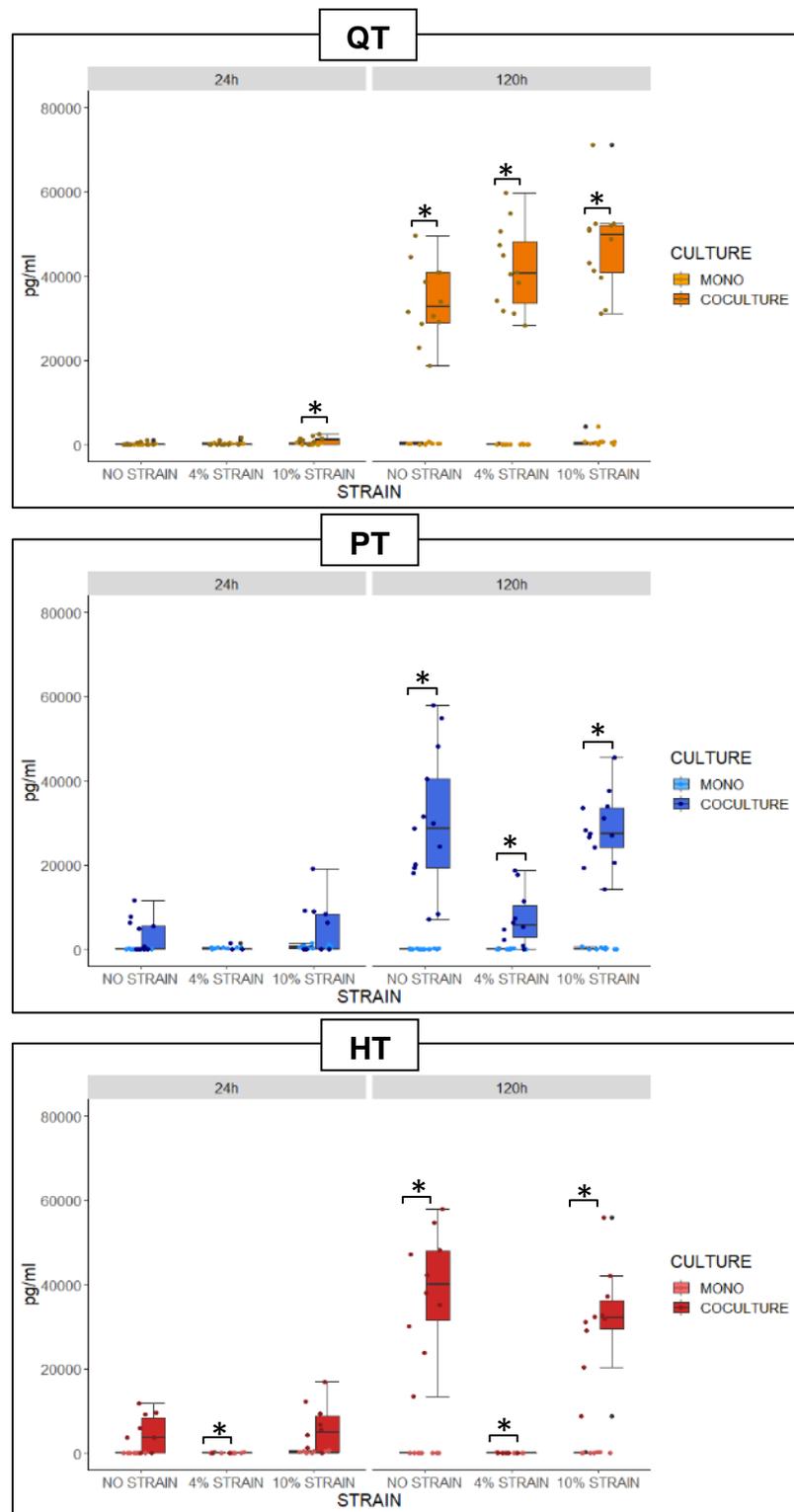


Figure 6.5. Concentration of interleukin-8 (IL-8), released to the media. * significant difference between monoculture (MONO) and co-culture at 24 and 120 hours (Signed Rank test). QT: Quadriceps tendon; PT: Patellar tendon, HT: Hamstring tendon

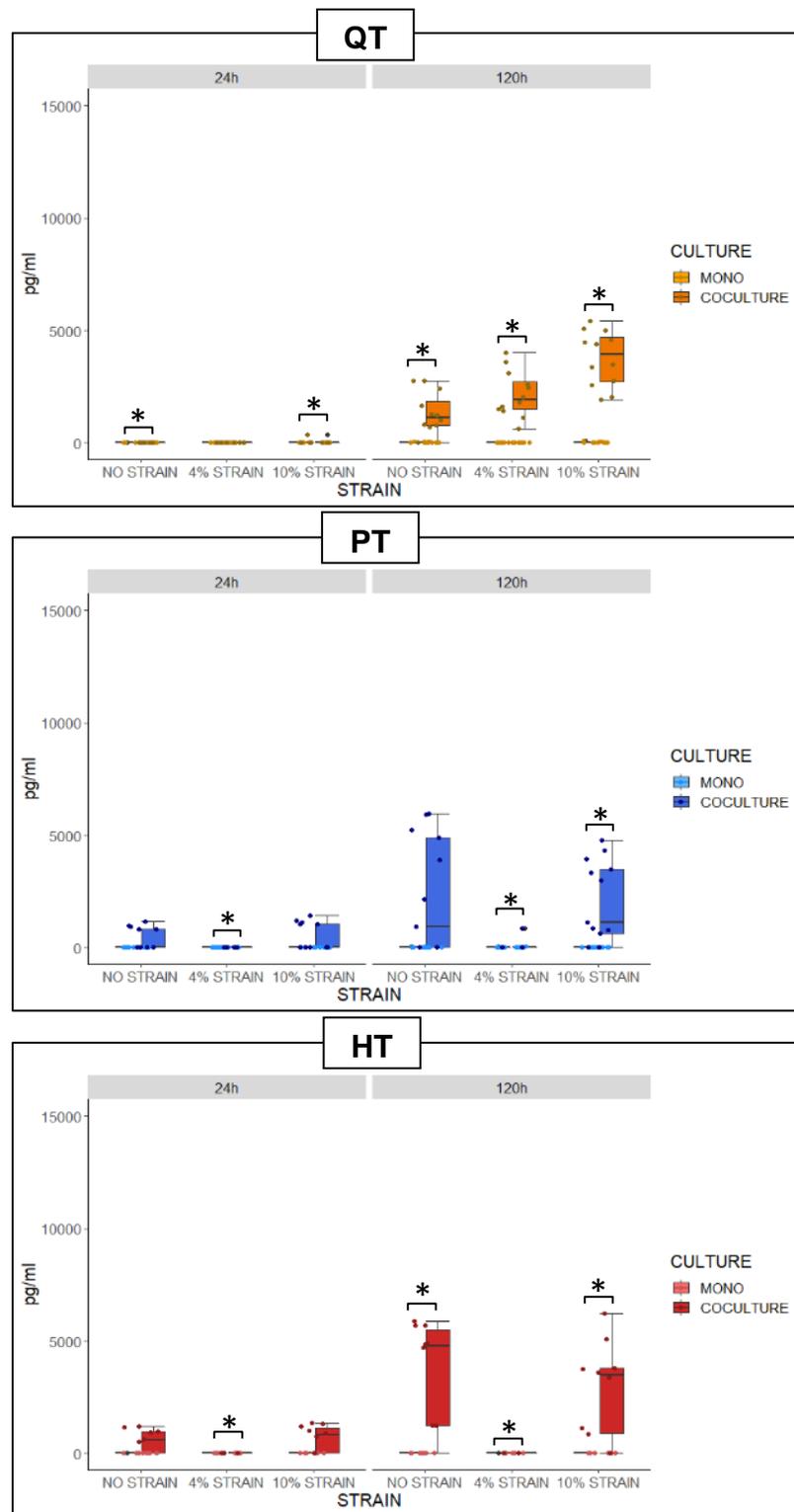


Figure 6.6. Concentration of keratinocyte chemoattractant protein (KC) released to the media. * significant difference between monoculture (MONO) and co-culture at 24 and 120 hours (Signed Rank test). QT: Quadriceps tendon; PT: Patellar tendon, HT: Hamstring tendon

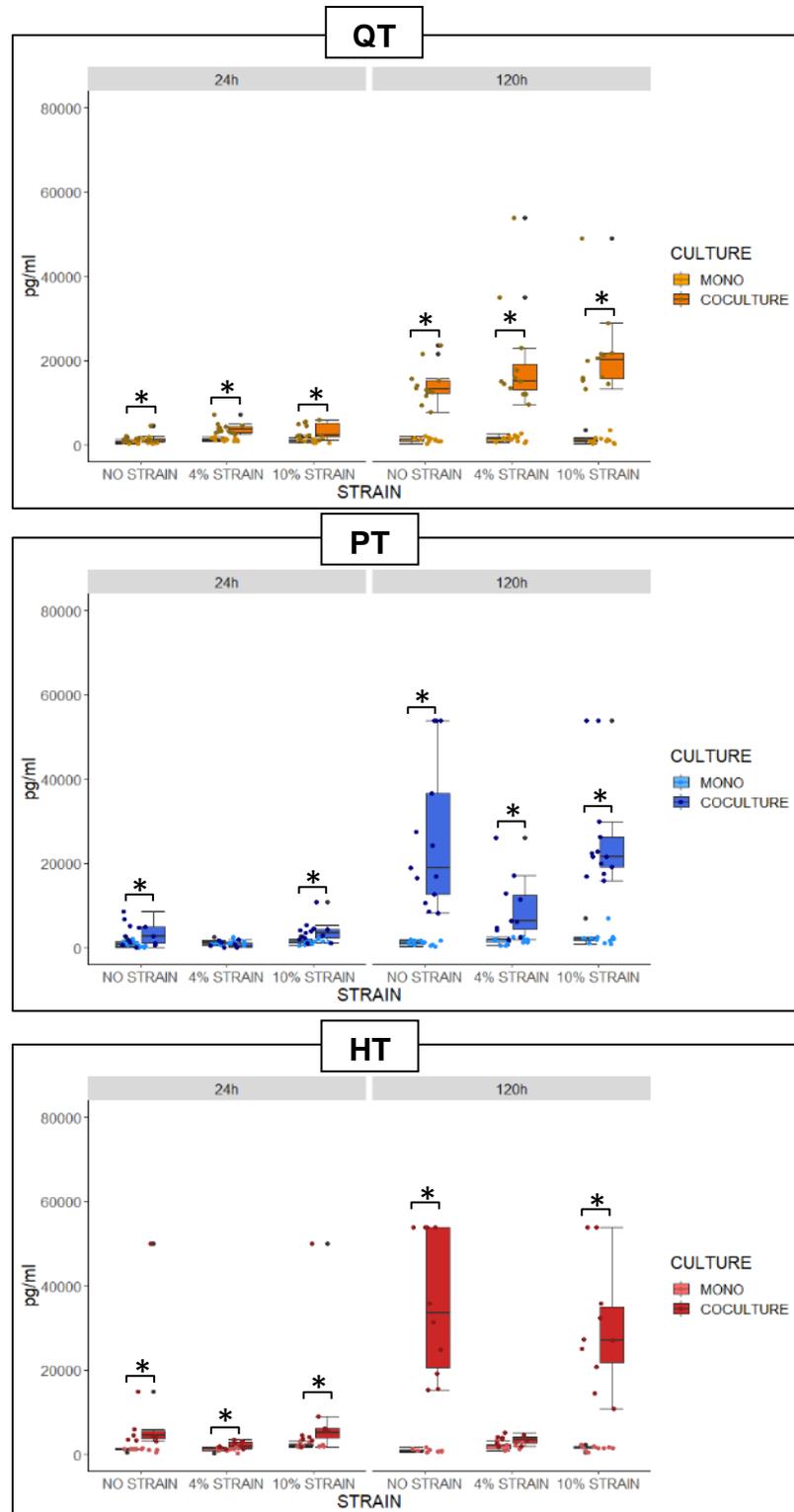


Figure 6.7. Concentration of monocyte chemoattractant protein-1 (MCP-1) released to the media. * significant difference between monoculture (MONO) and co-culture at 24 and 120 hours (Signed Rank test). QT: Quadriceps tendon; PT: Patellar tendon, HT: Hamstring tendon

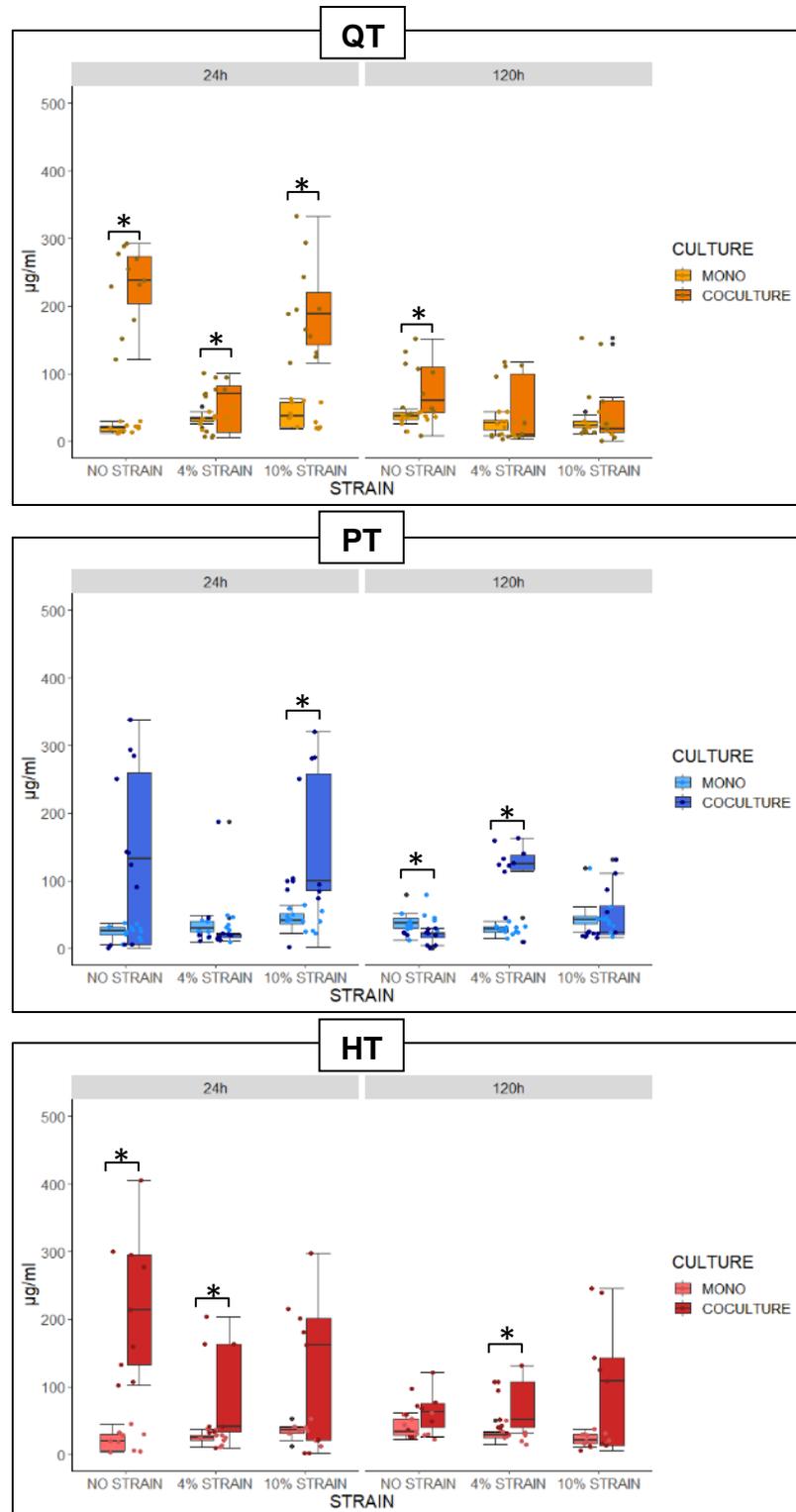


Figure 6.8. Concentration of Glycosaminoglycans (GAG) released to the media. * significant difference between monoculture (MONO) and co-culture at 24 and 120 hours (Signed Rank test). QT: Quadriceps tendon; PT: Patellar tendon, HT: Hamstring tendon

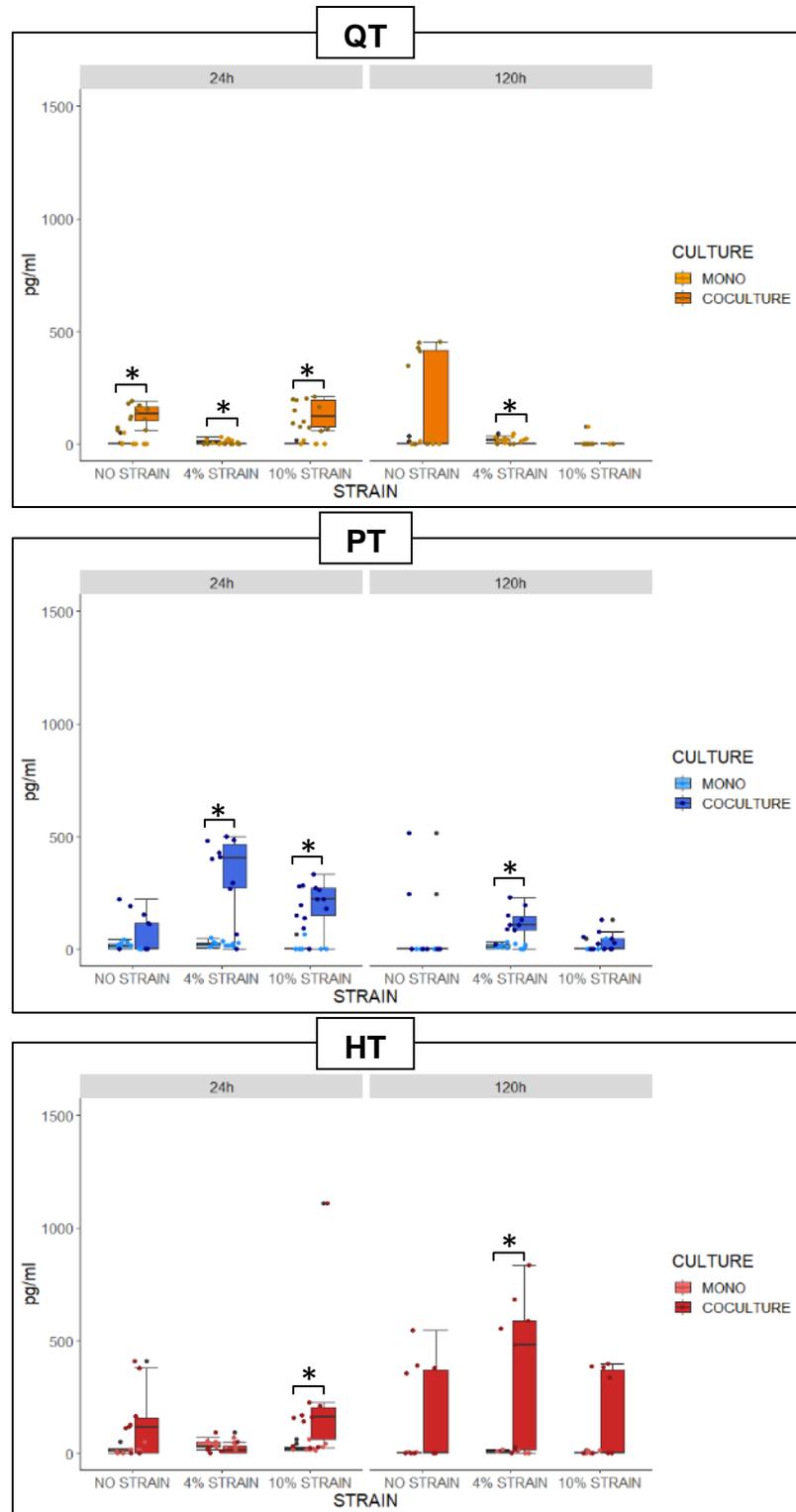


Figure 6.9. Concentration of total MMP activity (MMPACT) released to the media. * significant difference between monoculture (MONO) and co-culture at 24 and 120 hours (Signed Rank test). QT: Quadriceps tendon; PT: Patellar tendon, HT: Hamstring tendon

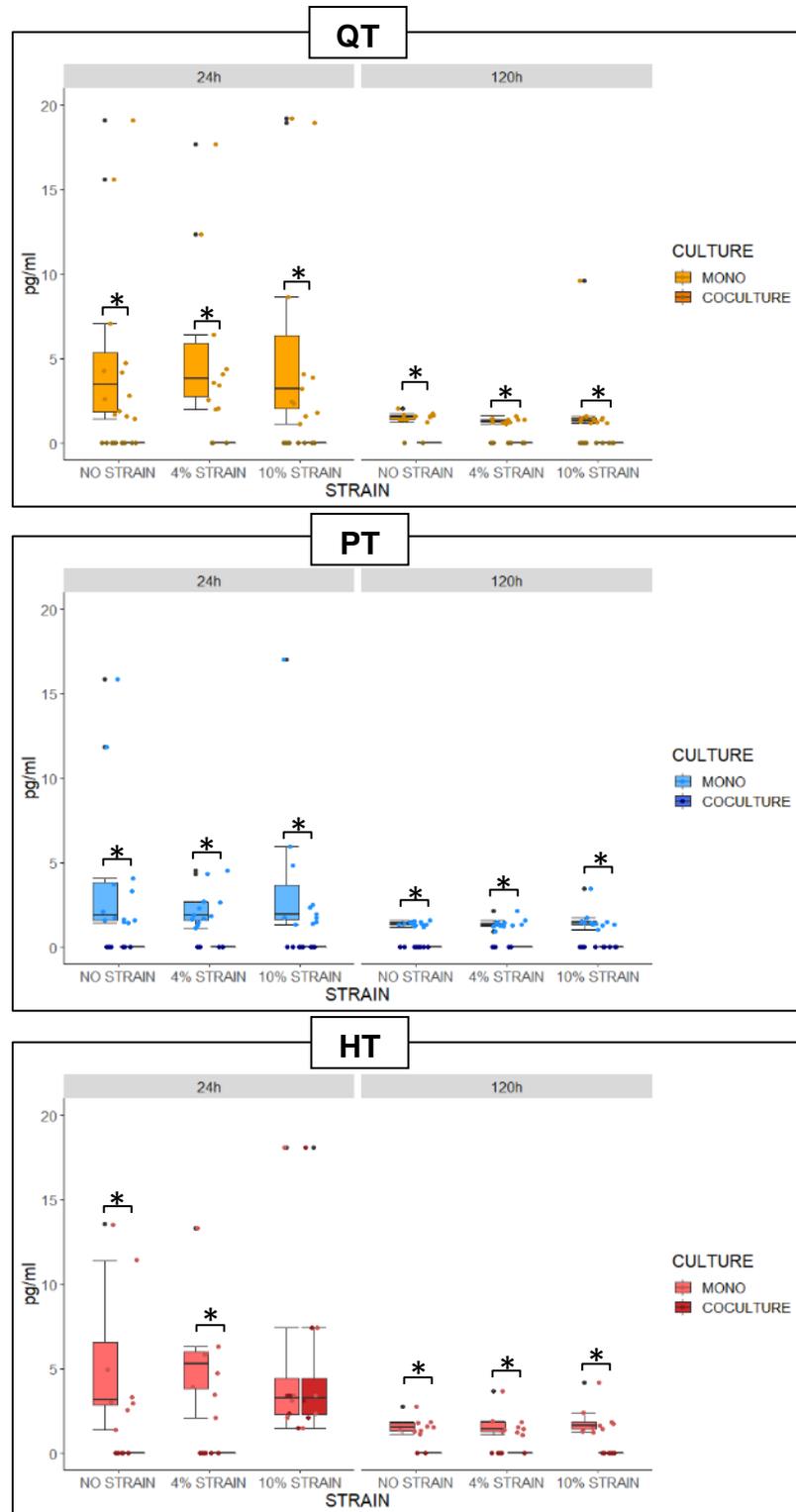


Figure 6.10. Concentration of MMP-1 released to the media. * significant difference between monoculture (MONO) and co-culture at 24 and 120 hours (Signed Rank test). QT: Quadriceps tendon; PT: Patellar tendon, HT: Hamstring tendon

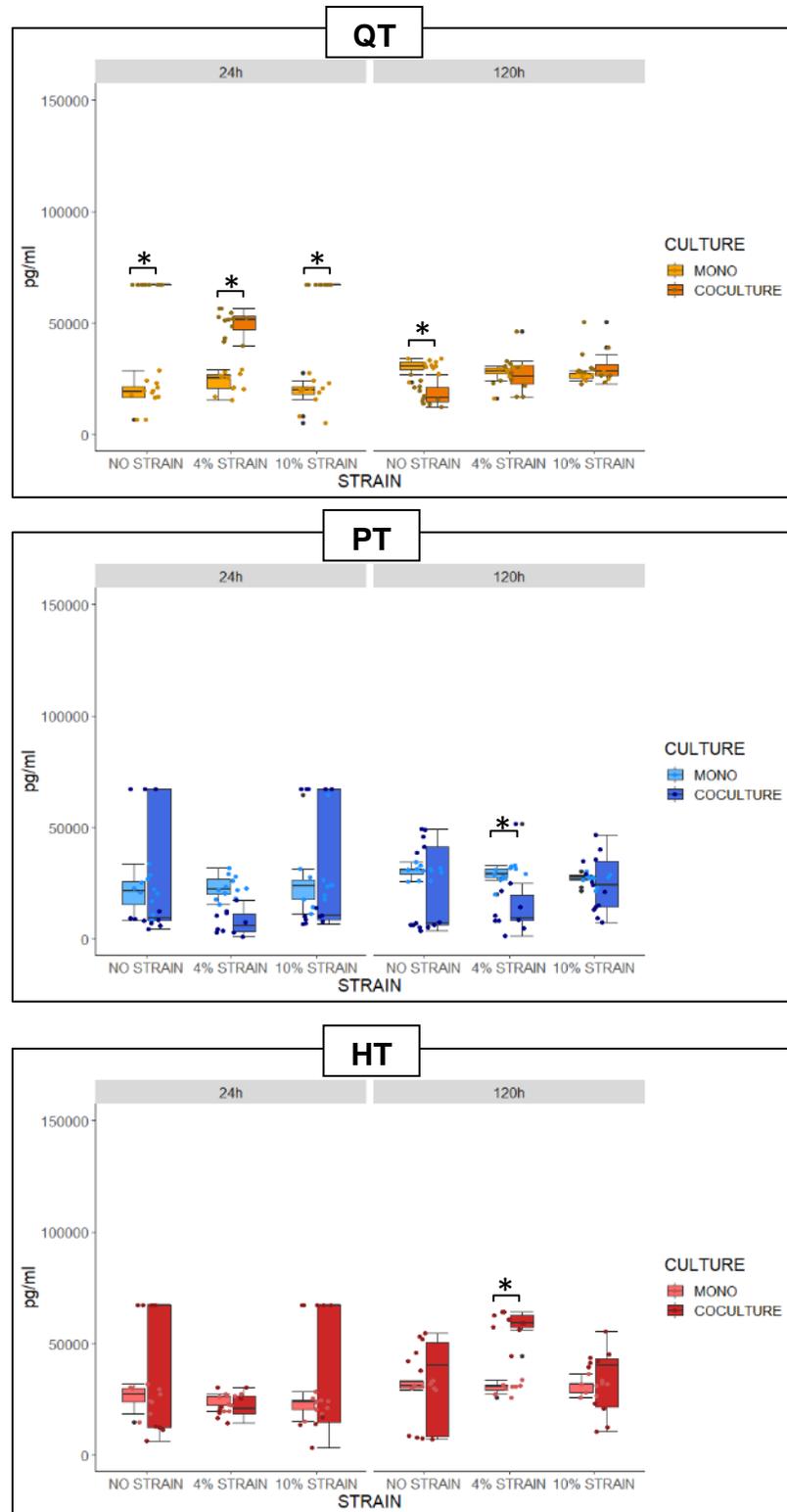


Figure 6.11. Concentration of MMP-2 released to the media. * significant difference between monoculture (MONO) and co-culture at 24 and 120 hours (Signed Rank test). QT: Quadriceps tendon; PT: Patellar tendon, HT: Hamstring tendon

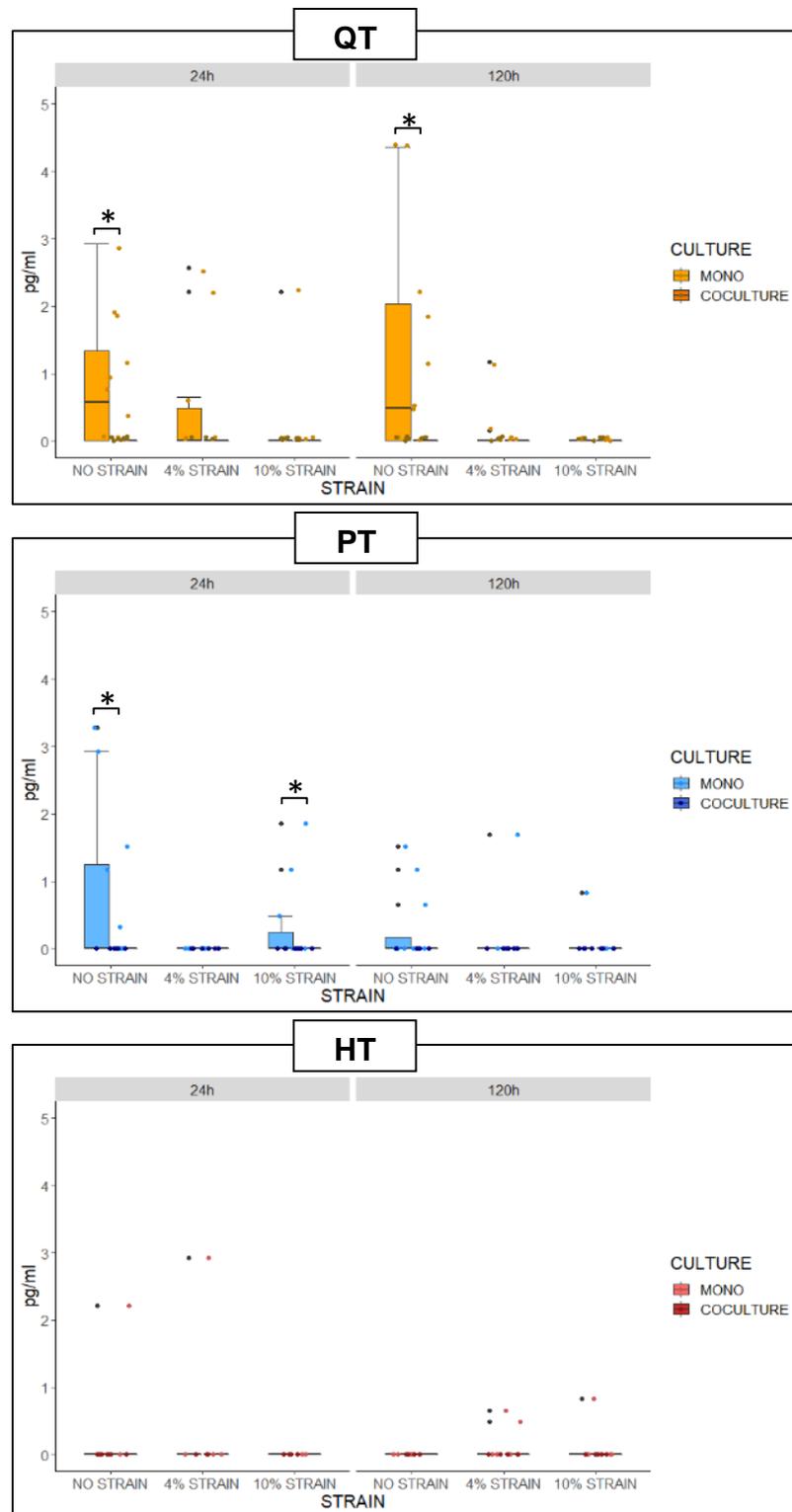


Figure 6.12. Concentration of MMP- 3 released to the media. * significant difference between monoculture (MONO) and co-culture at 24 and 120 hours (Signed Rank test). QT: Quadriceps tendon; PT: Patellar tendon, HT: Hamstring tendon

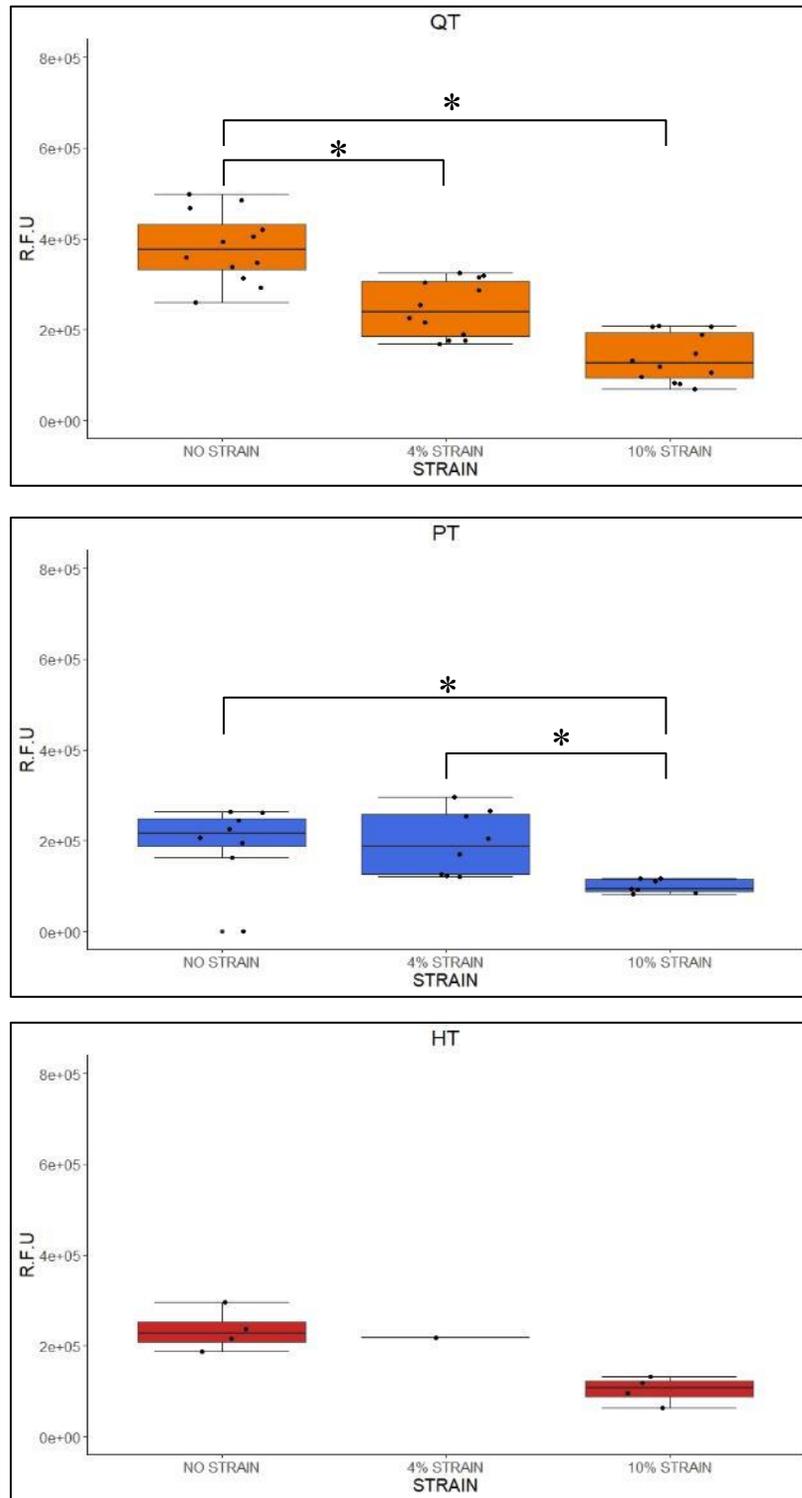


Figure 6.13. Metabolic Activity of graft fibroblasts. * significant difference between STRAINS at 120 hours (Kruskal-Wallis test). QT: Quadriceps tendon; PT: Patellar tendon, HT: Hamstring tendon.

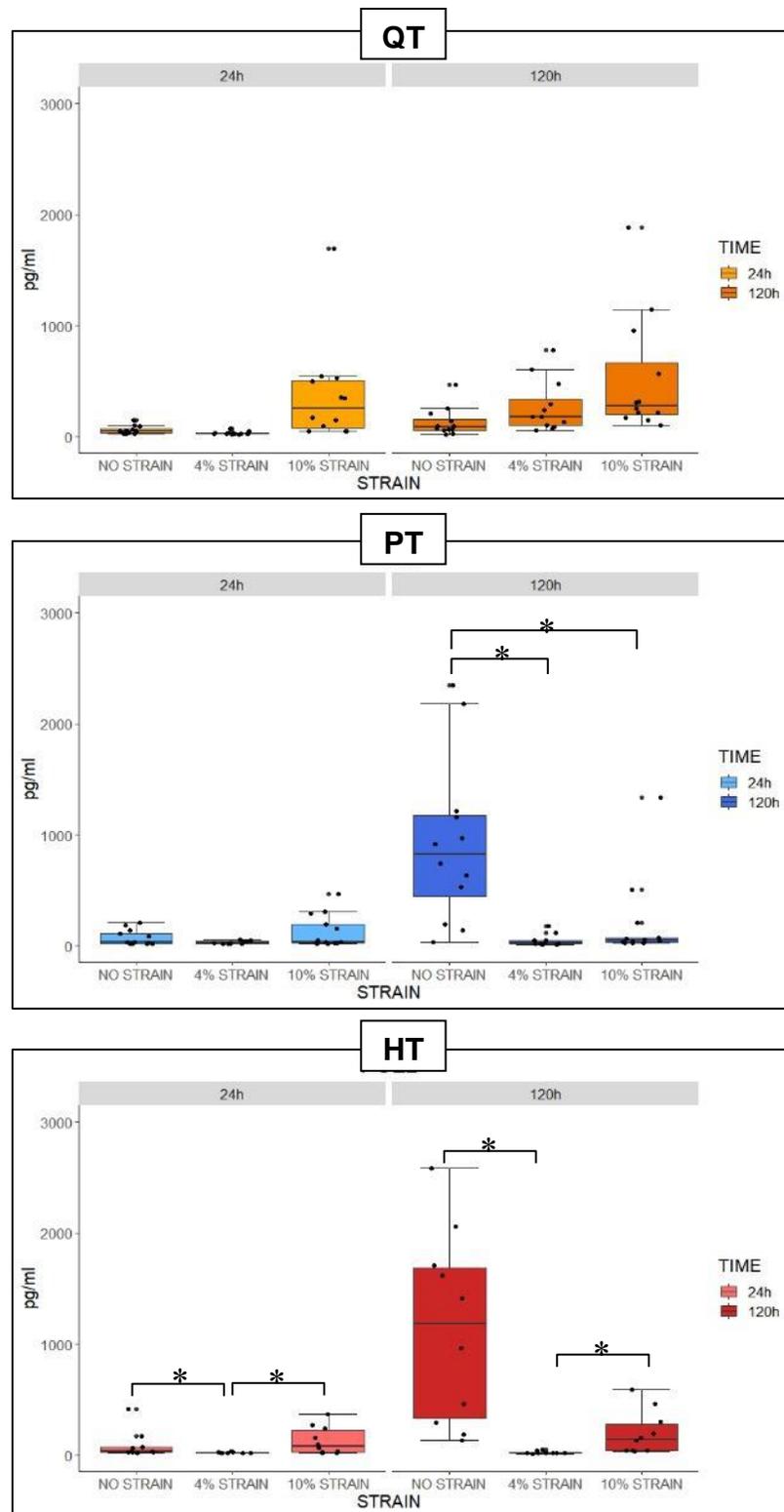


Figure 6.14. Concentration of PGE-2 released to the media when co-cultured with SYN. * significant difference between STRAINS at 24 and 120 hours (Kruskal-Wallis test). QT: Quadriceps tendon; PT: Patellar tendon, HT: Hamstring tendon

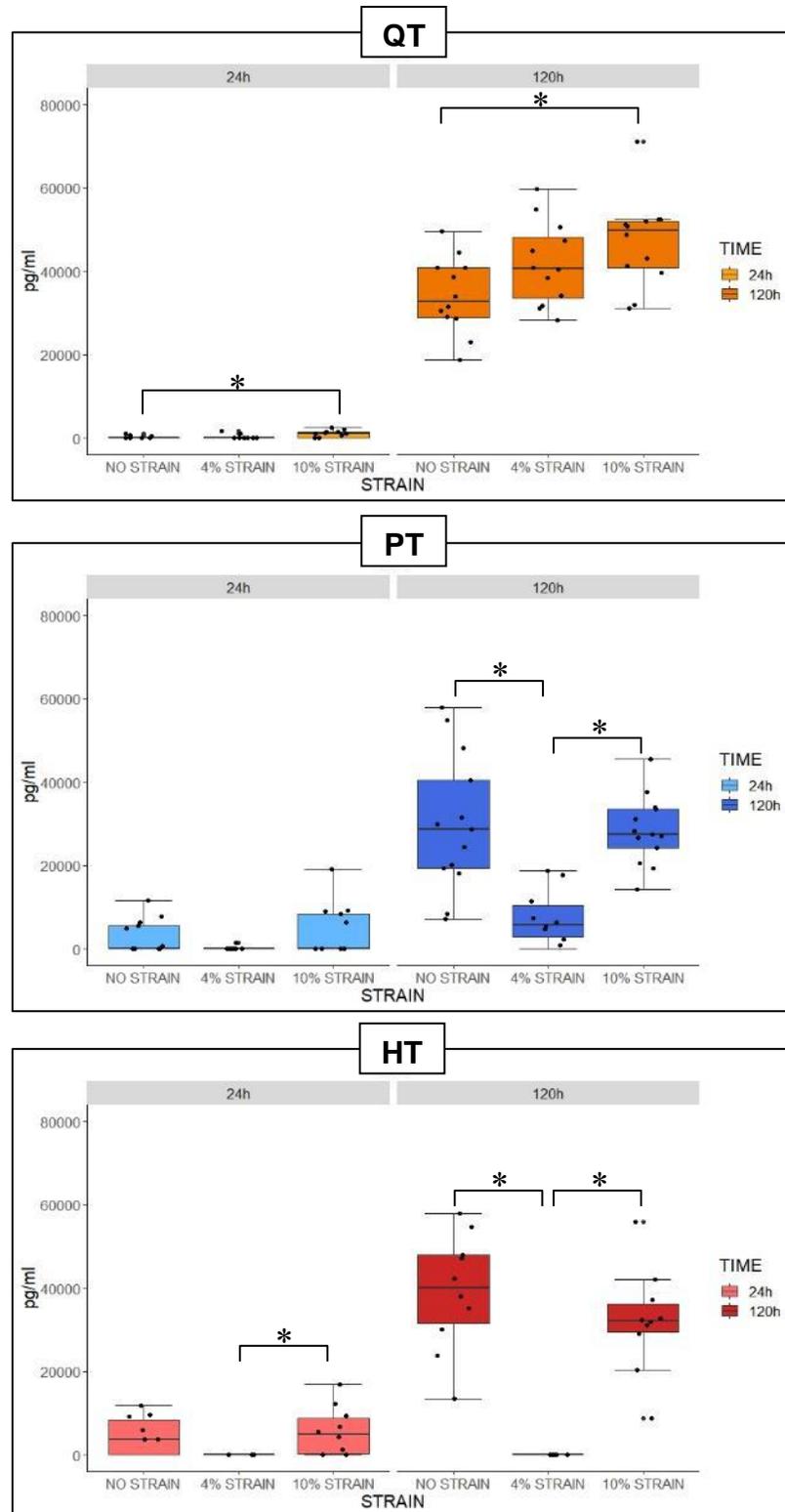


Figure 6.15. Concentration of IL-8 released to the media when co-cultured with SYN. * significant difference between STRAINS at 24 and 120 hours (Kruskal-Wallis test). QT: Quadriceps tendon; PT: Patellar tendon, HT: Hamstring tendon

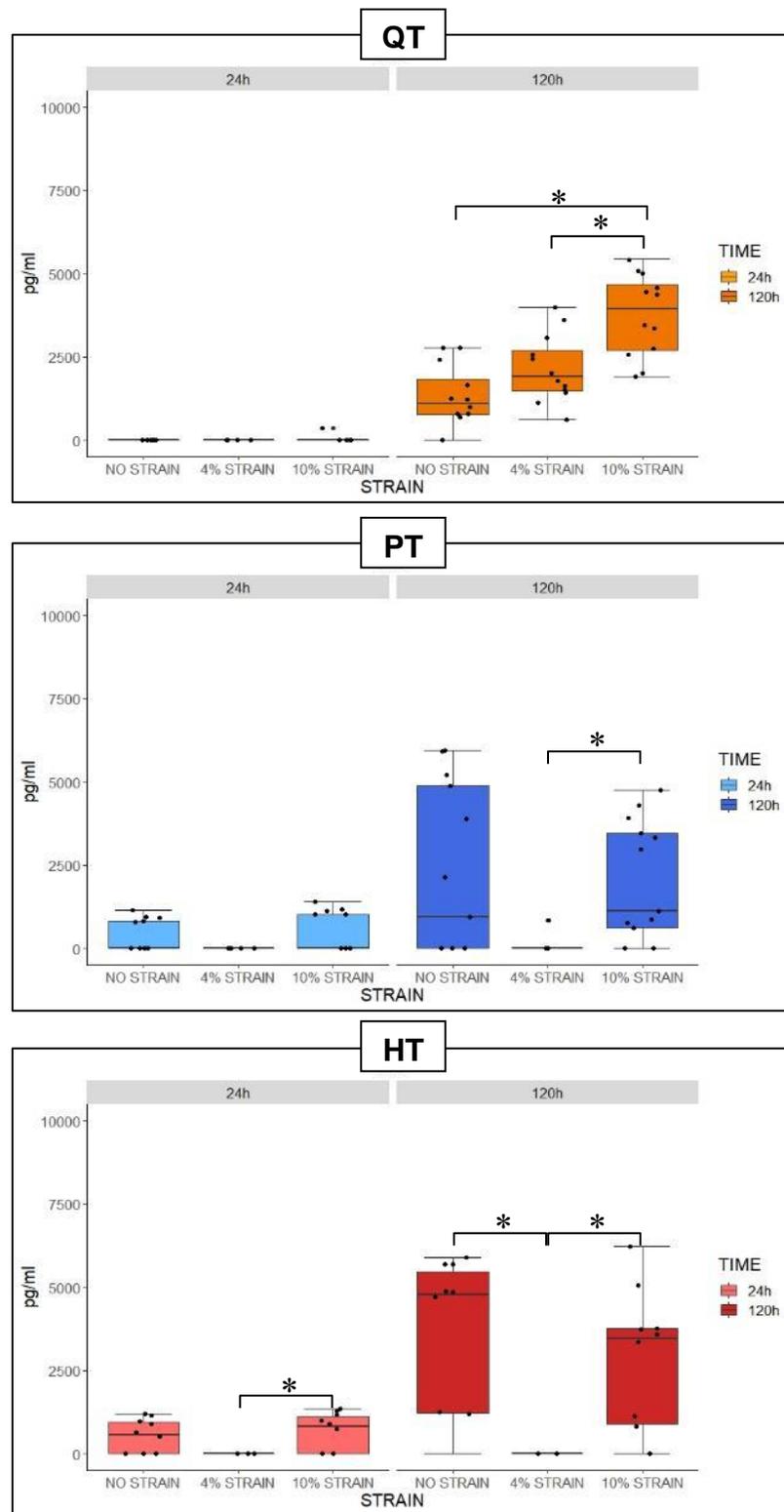


Figure 6.16. Concentration of KC released to the media when co-cultured with SYN. * significant difference between STRAINS at 24 and 120 hours (Kruskal-Wallis test). QT: Quadriceps tendon; PT: Patellar tendon, HT: Hamstring tendon

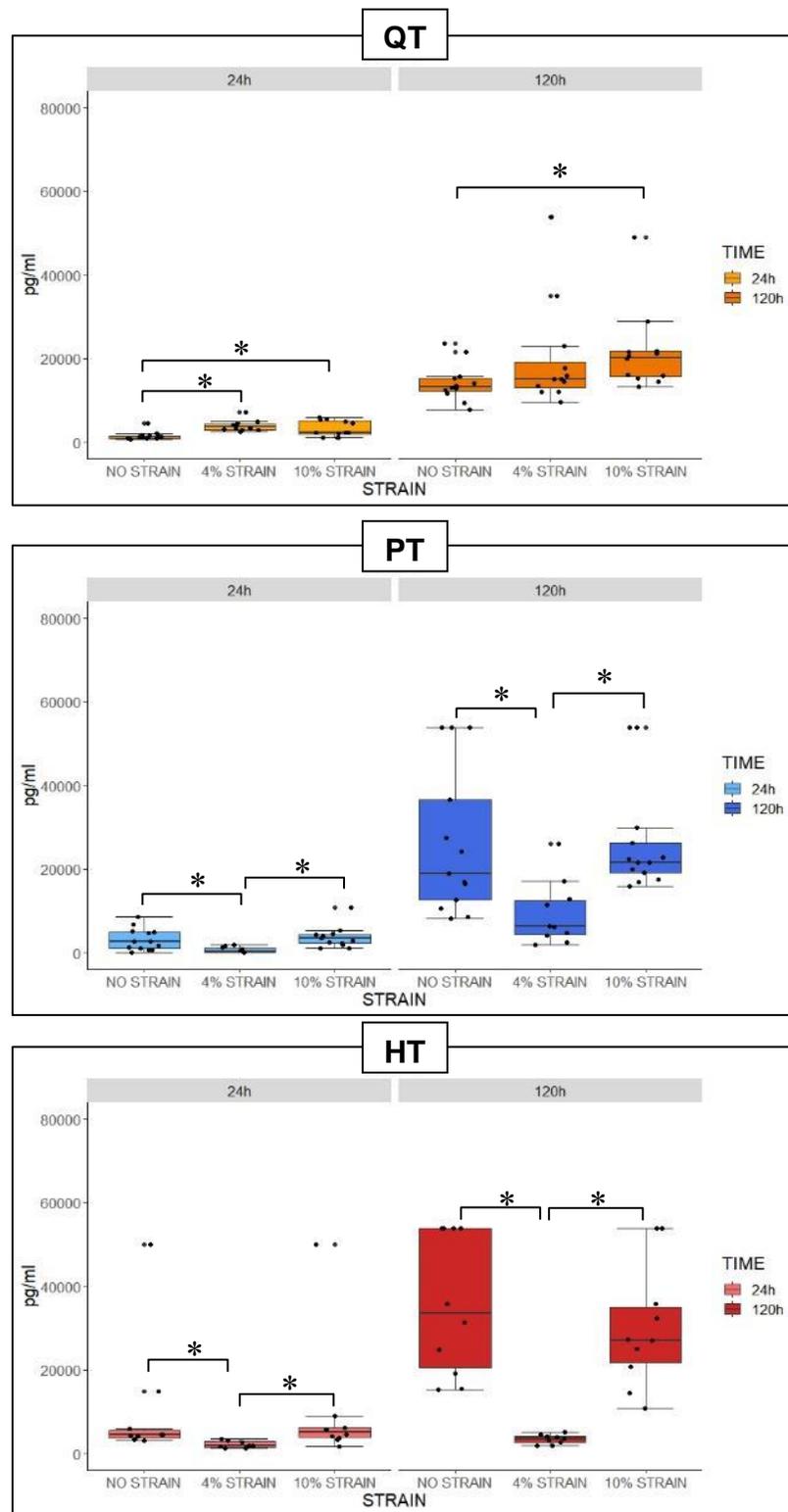


Figure 6.17. Concentration of MCP1 released to the media when co-cultured with SYN. * significant difference between STRAINS at 24 and 120 hours (Kruskal-Wallis test). QT: Quadriceps tendon; PT: Patellar tendon, HT: Hamstring tendon

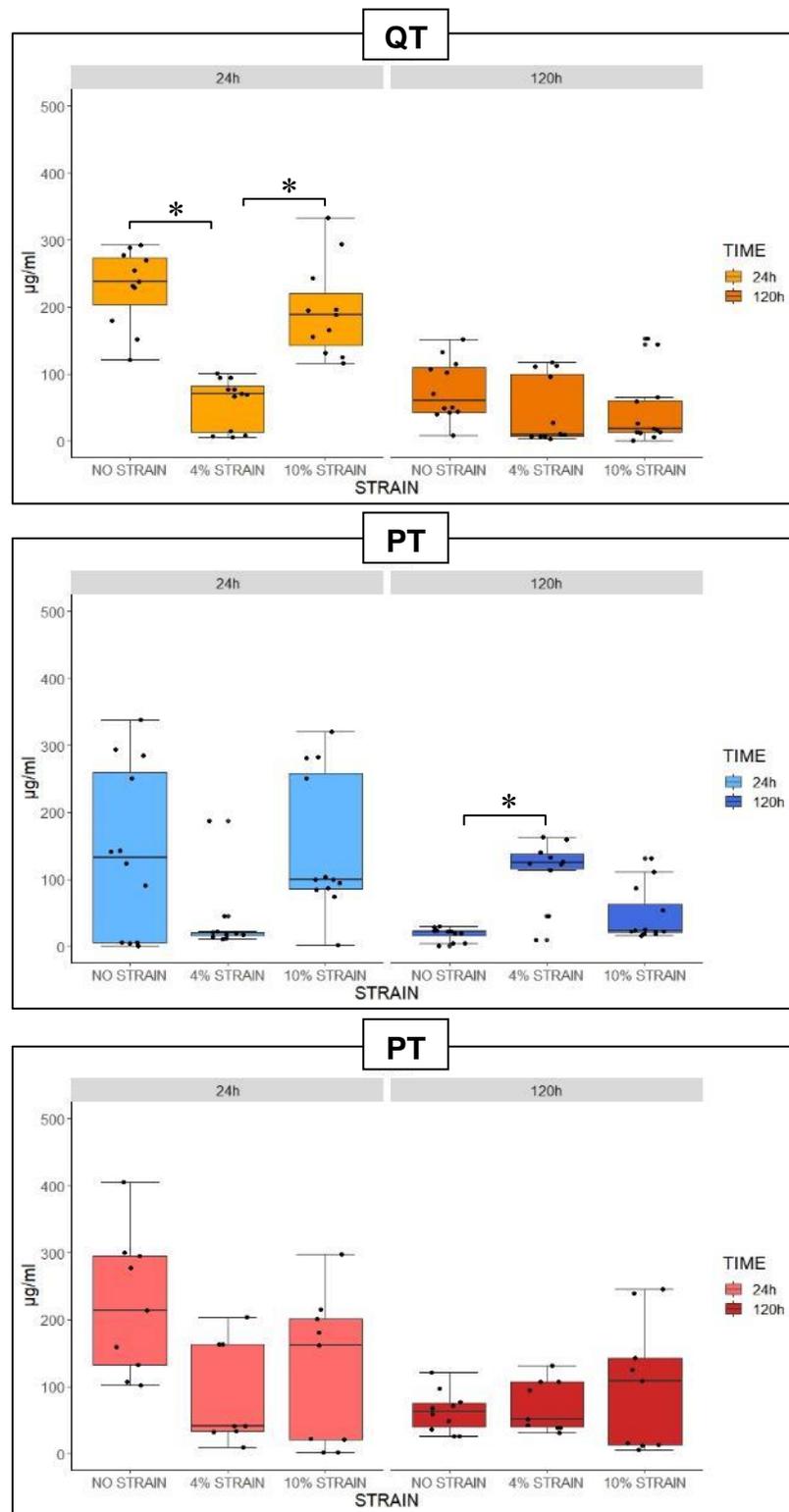


Figure 6.18. Concentration of GAG released to the media when co-cultured with SYN. * significant difference between STRAINS at 24 and 120 hours (Kruskal-Wallis test). QT: Quadriceps tendon; PT: Patellar tendon, HT: Hamstring tendon

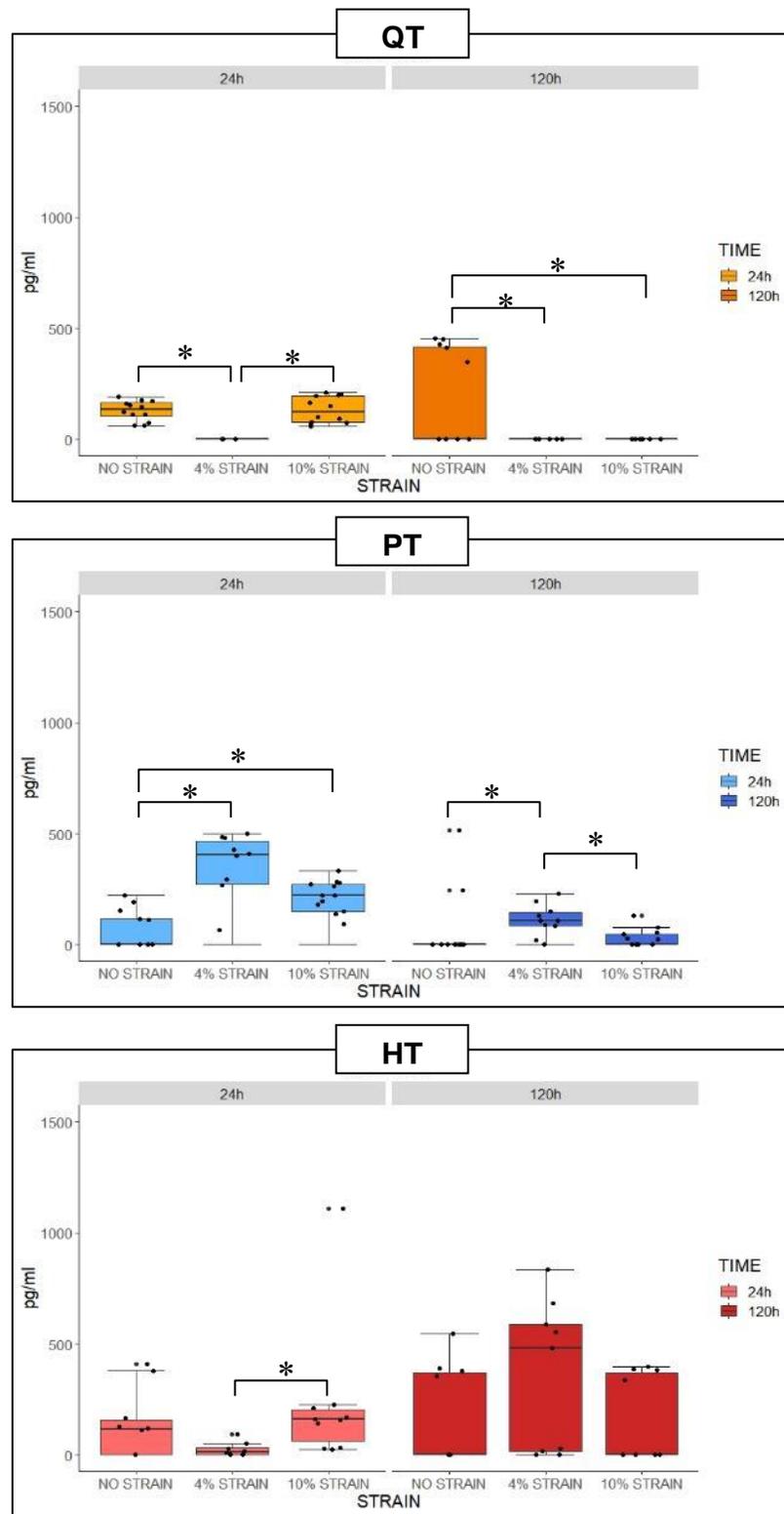


Figure 6.19. Concentration of MMPACT released to the media when co-cultured with SYN. * significant difference between STRAINS at 24 and 120 hours (Kruskal-Wallis test). QT: Quadriceps tendon; PT: Patellar tendon, HT: Hamstring tendon

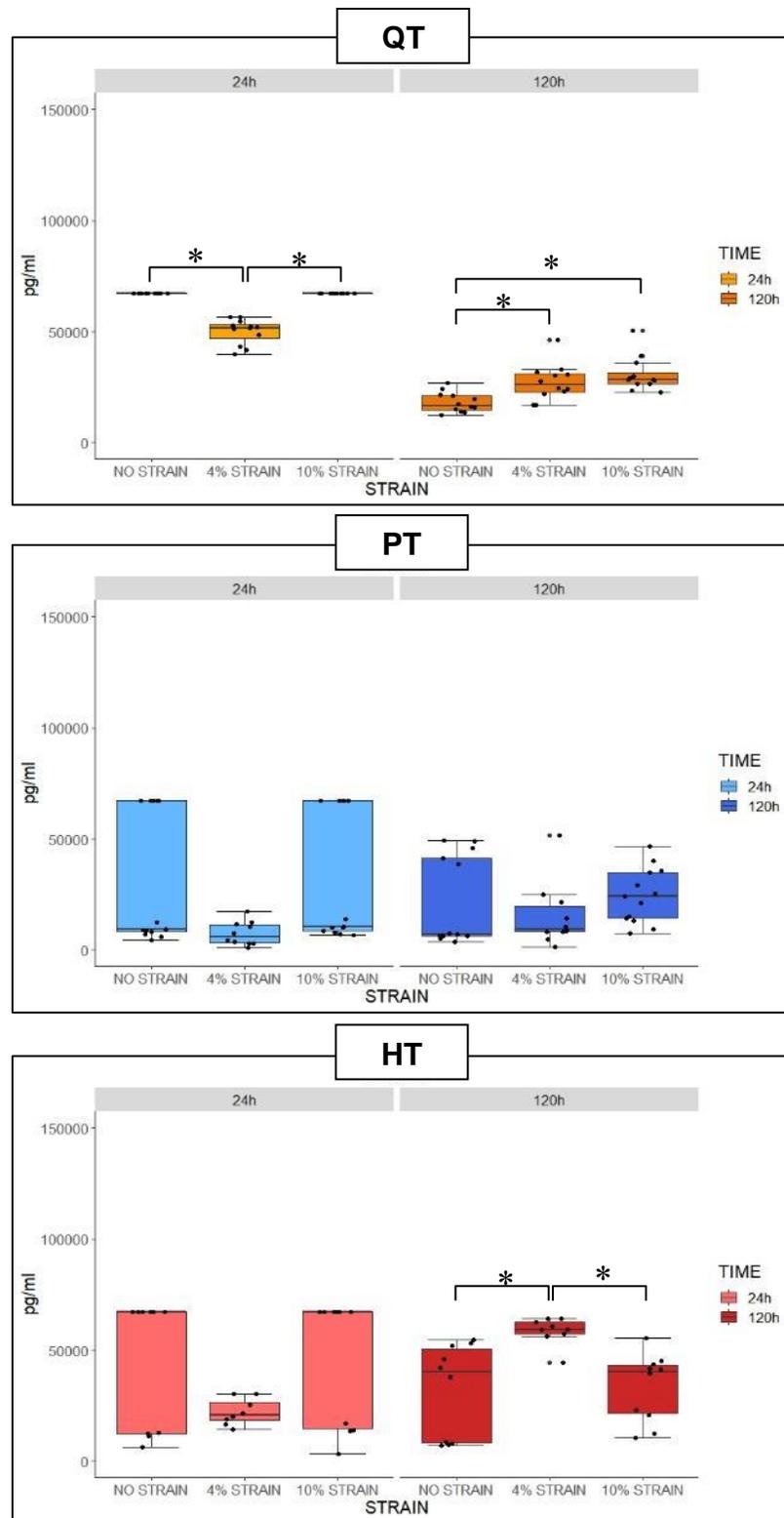


Figure 6.20. Concentration of MMP-2 released to the media when co-cultured with SYN. * significant difference between STRAINS at 24 and 120 hours (Kruskal-Wallis test). QT: Quadriceps tendon; PT: Patellar tendon, HT: Hamstring tendon

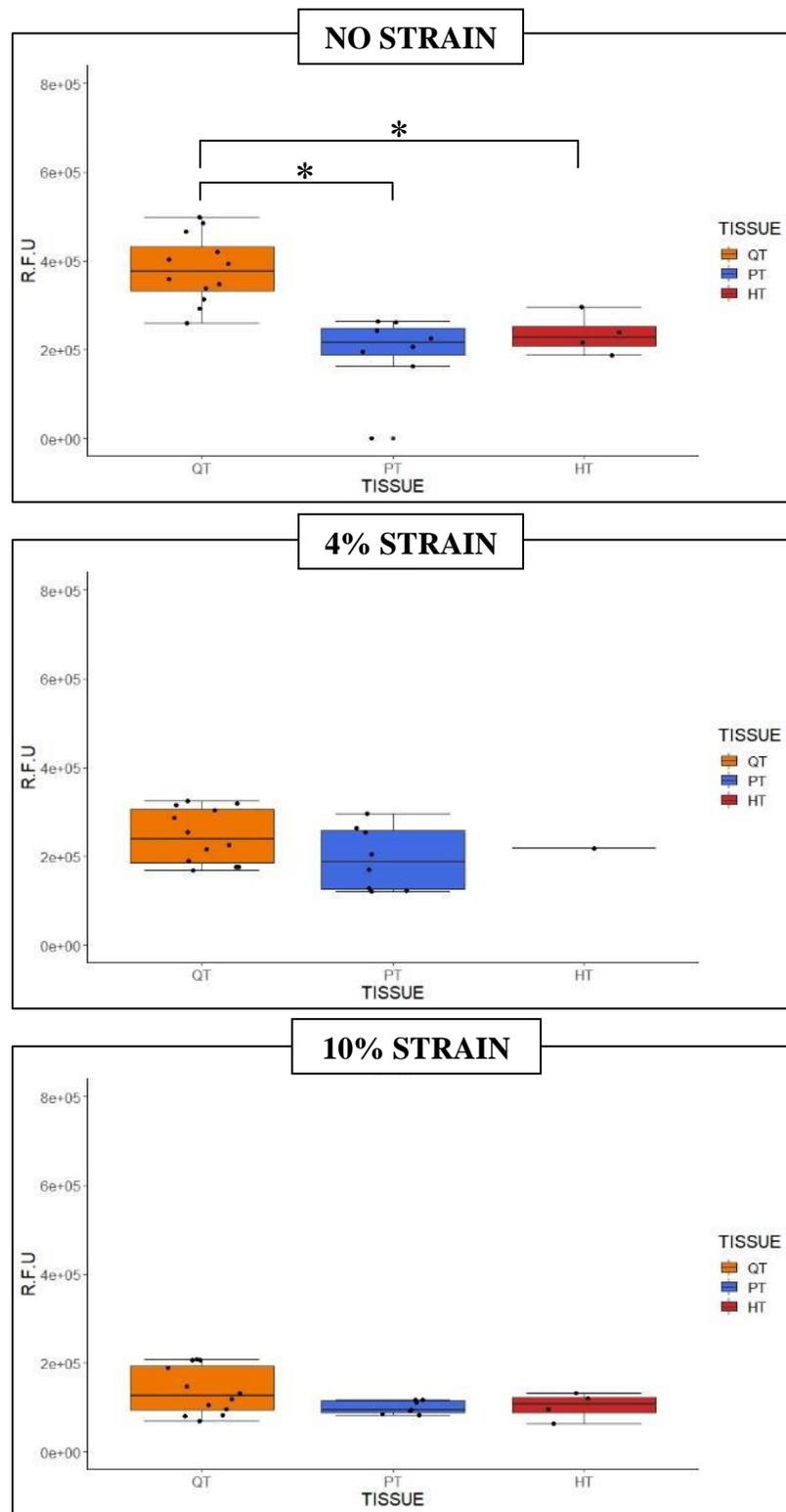


Figure 6.21. Metabolic Activity of graft fibroblasts. * significant difference between TISSUES at 120 hours (Kruskal-Wallis test). QT: Quadriceps tendon; PT: Patellar tendon, HT: Hamstring tendon.

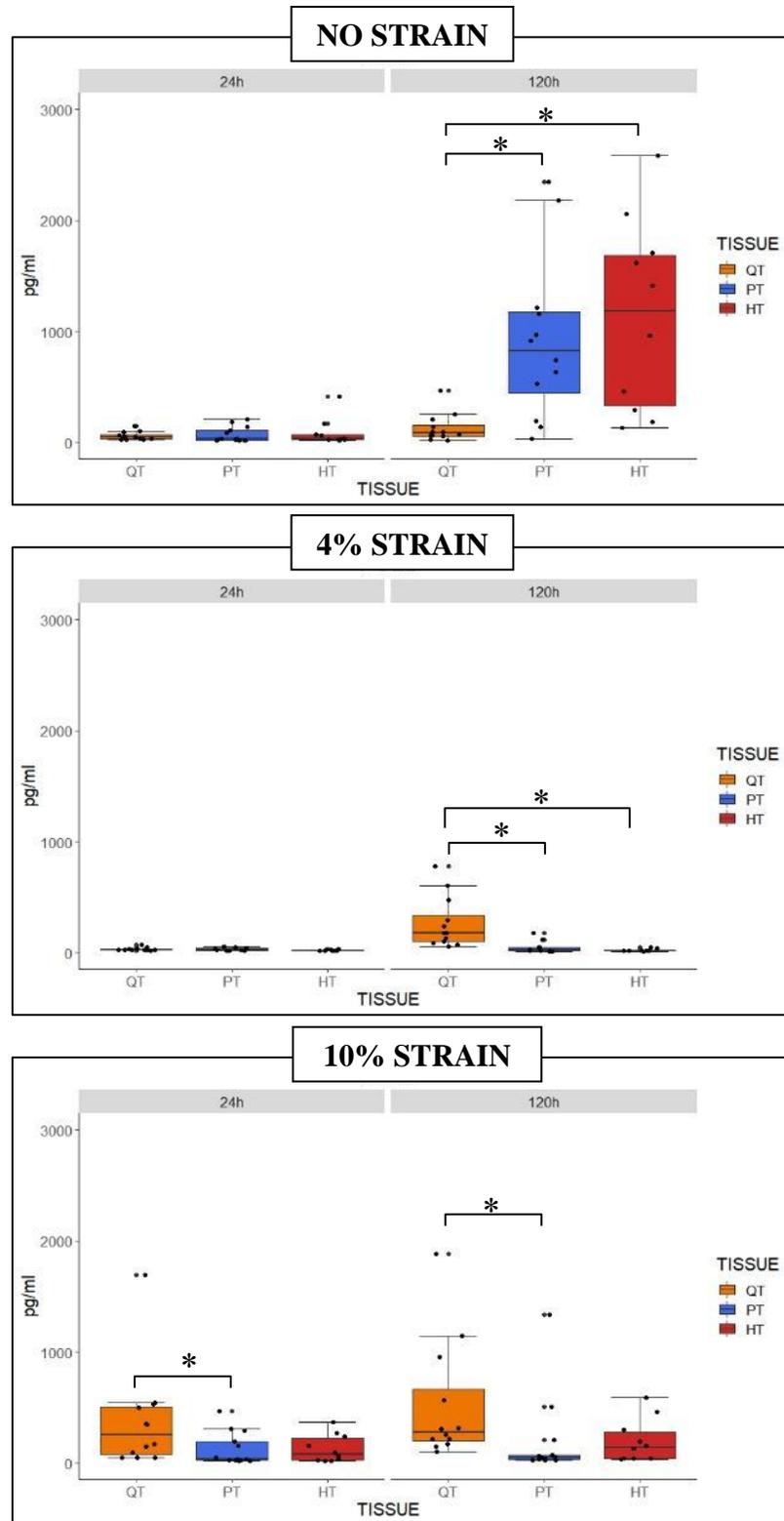


Figure 6.22. Concentration of PGE2 released to the media when co-cultured with SYN. * significant difference between TISSUES at 24 and 120 hours (Kruskal-Wallis test). QT: Quadriceps tendon; PT: Patellar tendon, HT: Hamstring tendon

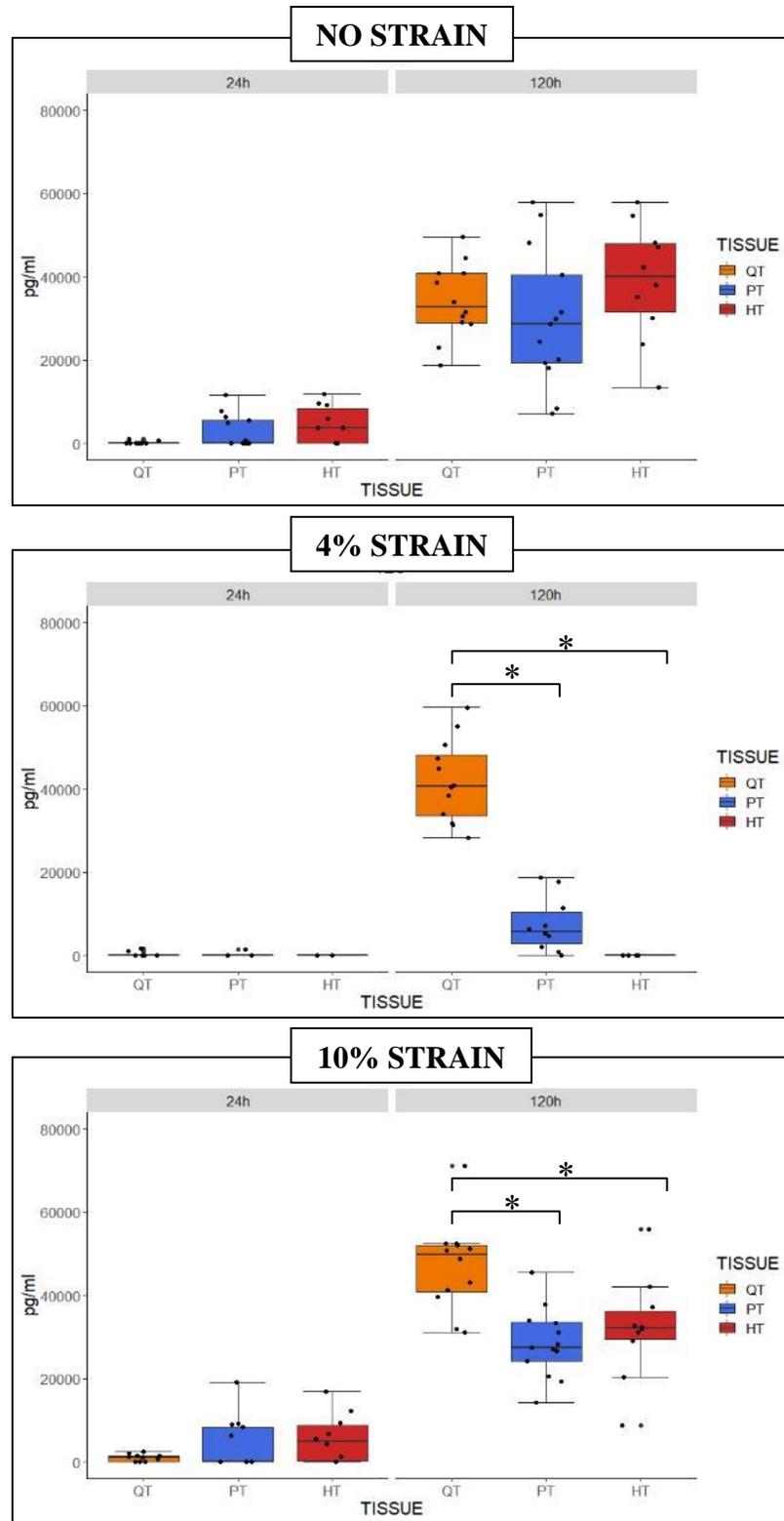


Figure 6.23. Concentration of IL-8 released to the media when co-cultured with SYN. * significant difference between TISSUES at 24 and 120 hours (Kruskal-Wallis test). QT: Quadriceps tendon; PT: Patellar tendon, HT: Hamstring tendon

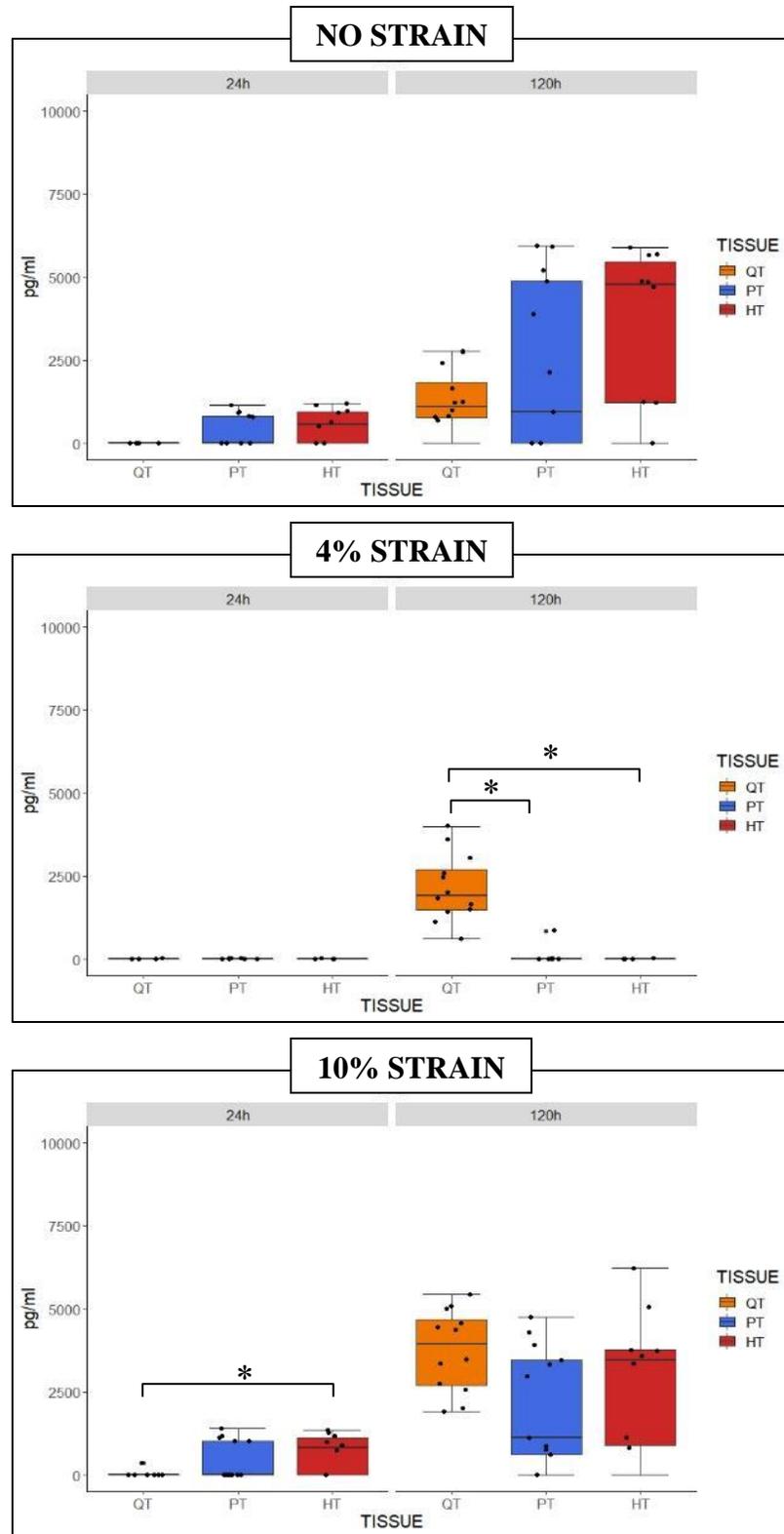


Figure 6.24. Concentration of KC released to the media when co-cultured with SYN. * significant difference between TISSUES at 24 and 120 hours (Kruskal-Wallis test). QT: Quadriceps tendon; PT: Patellar tendon, HT: Hamstring tendon

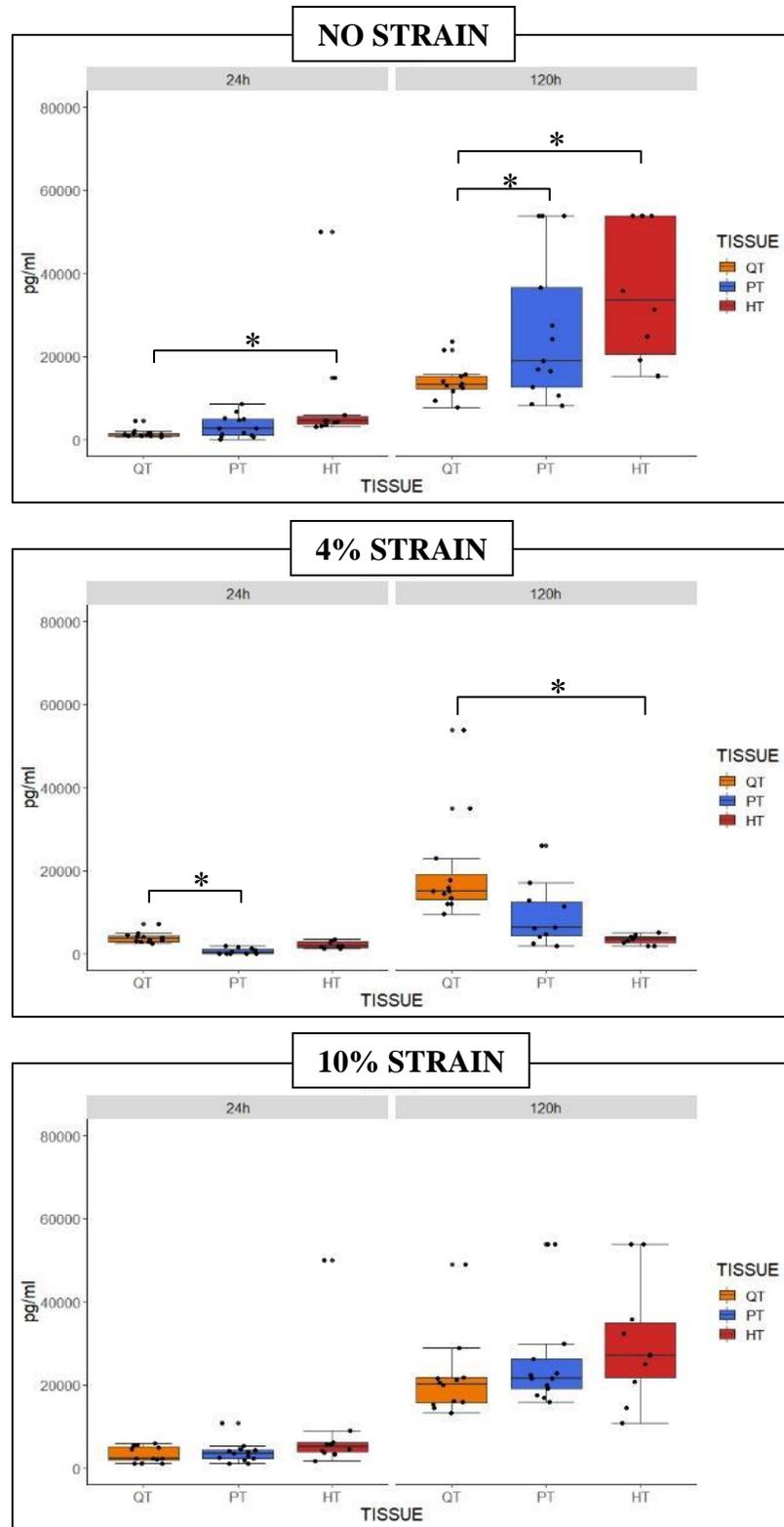


Figure 6.25. Concentration of MCP1 released to the media when co-cultured with SYN. * significant difference between TISSUES at 24 and 120 hours (Kruskal-Wallis test). QT: Quadriceps tendon; PT: Patellar tendon, HT: Hamstring tendon

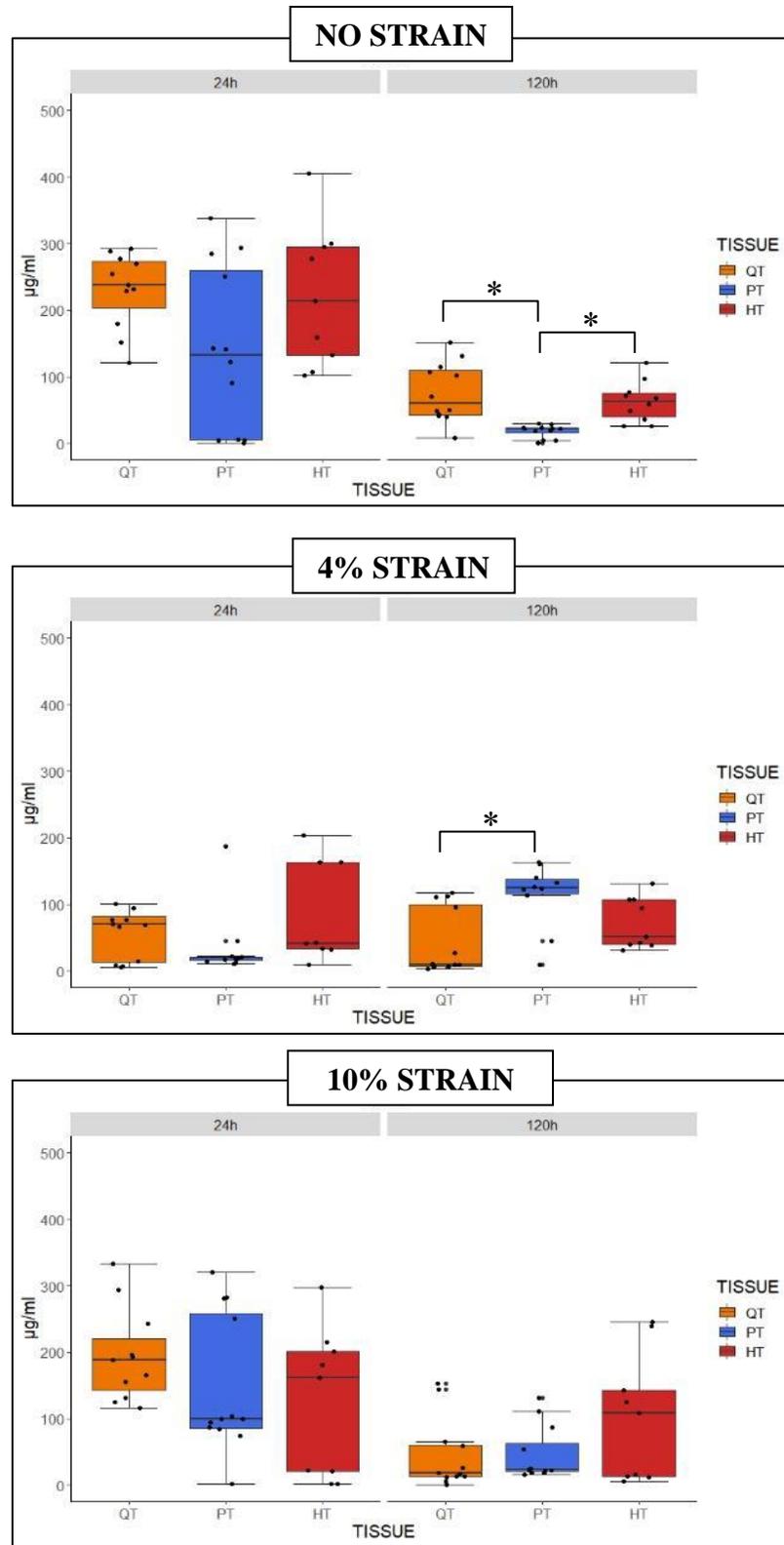


Figure 6.26. Concentration of GAG released to the media when co-cultured with SYN. * significant difference between TISSUES at 24 and 120 hours (Kruskal-Wallis test). QT: Quadriceps tendon; PT: Patellar tendon, HT: Hamstring tendon

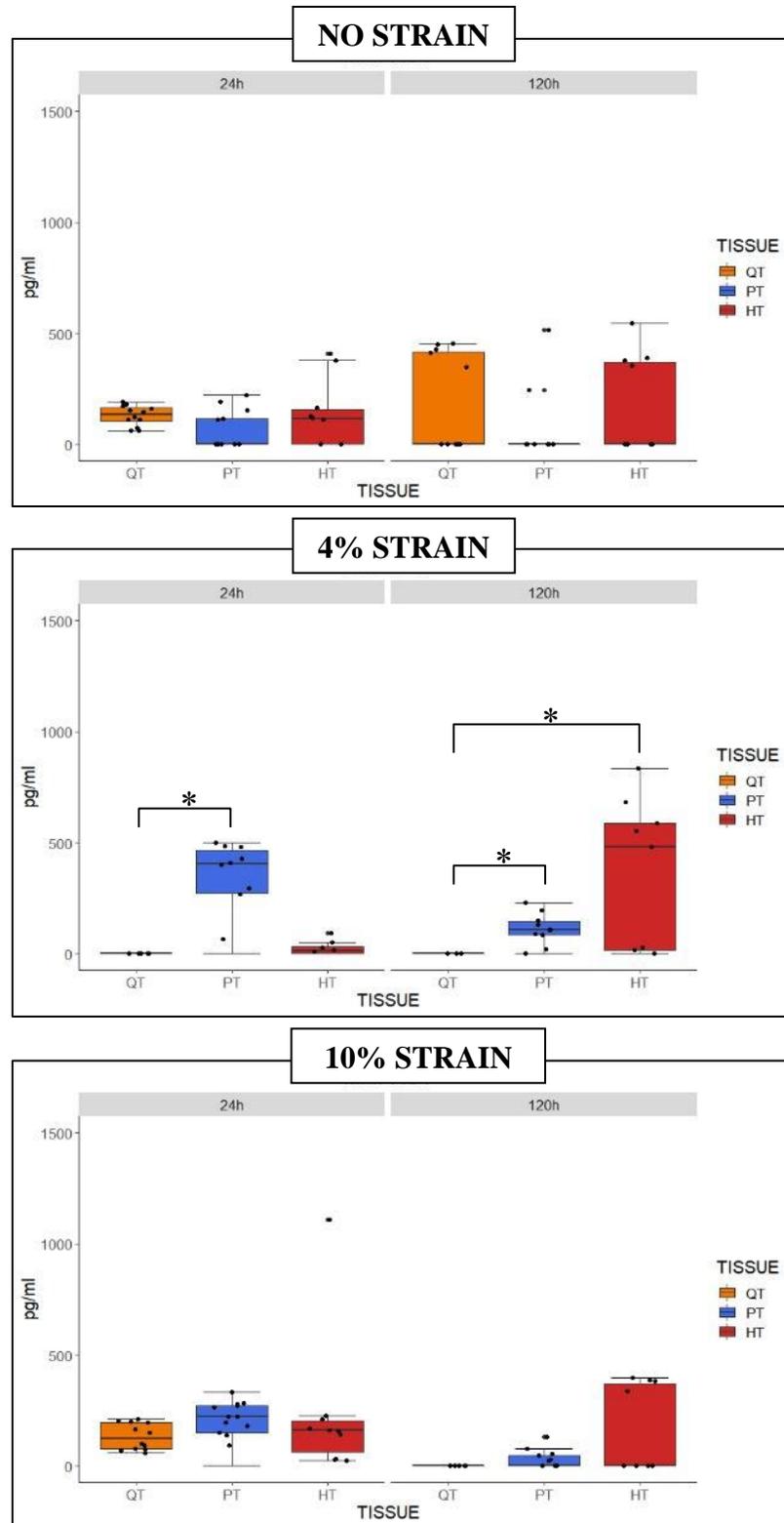


Figure 6.27. Concentration of MMPACT released to the media when co-cultured with SYN. * significant difference between TISSUES at 24 and 120 hours (Kruskal-Wallis test). QT: Quadriceps tendon; PT: Patellar tendon, HT: Hamstring tendon

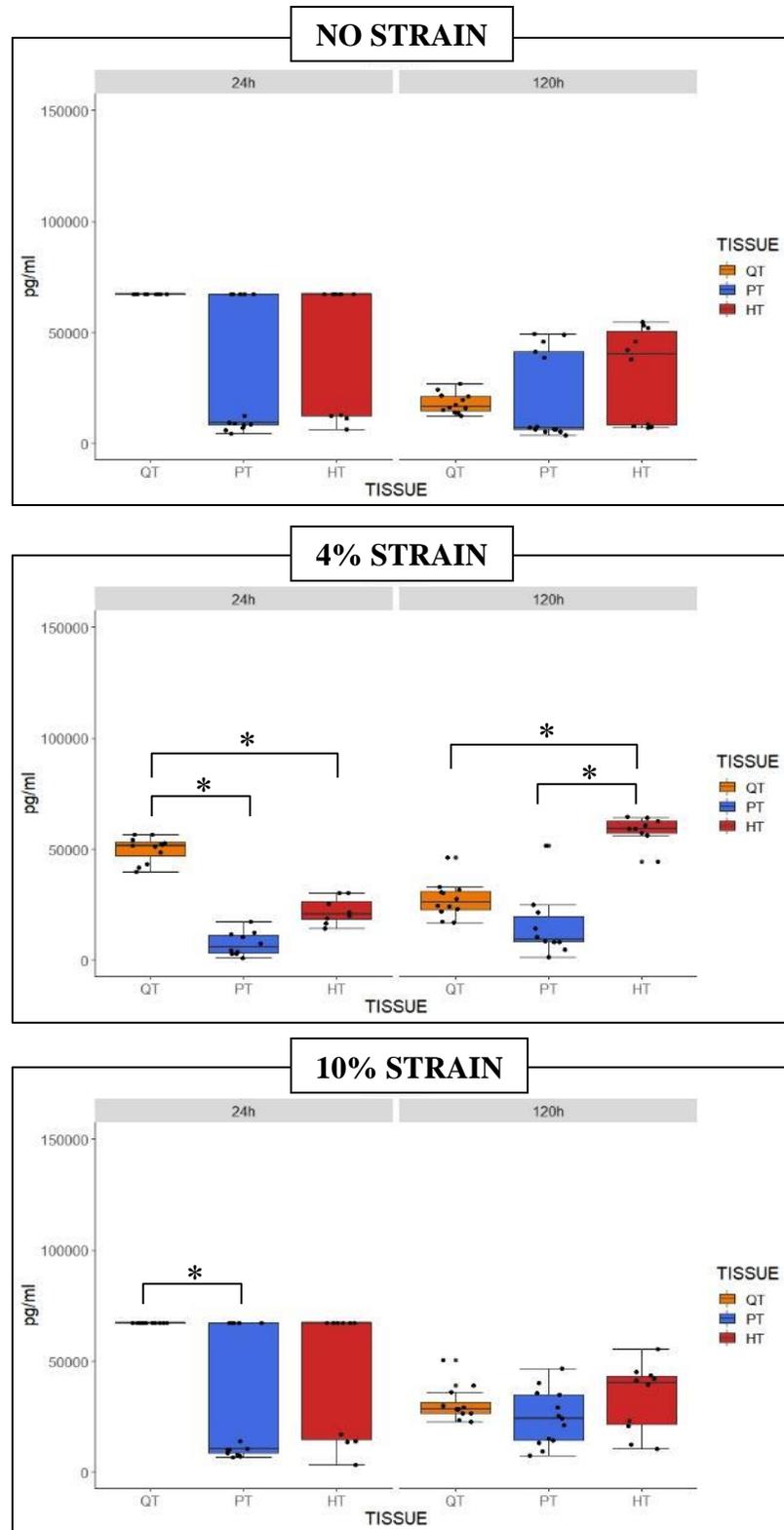


Figure 6.28. Concentration of MMP-2 released to the media when co-cultured with SYN. * significant difference between TISSUES at 24 and 120 hours (Kruskal-Wallis test). QT: Quadriceps tendon; PT: Patellar tendon, HT: Hamstring tendon

CHAPTER 7

EFFECTS OF MECHANICAL STRAIN AND SEX ON METABOLIC RESPONSES OF HUMAN ANTERIOR CRUCIATE LIGAMENT AND SYNOVIUM

Introduction

There are more than 100,000 anterior cruciate ligament (ACL) injuries in the United States every year.^{1; 2} The knee joint is now considered an organ with all of its constituent tissues (i.e. cartilage, meniscus, ligament, synovium) playing important roles during homeostasis and during injury or disease states. These tissue responses are driven by specific molecules released by the varying cell types found within the knee joint.

The synovium is a principal constituent involved in regulating knee joint homeostasis by modulating injury signals through the release of molecules such as cytokines, chemokines and adipokines, and plays important roles in determining downstream effects on articular cartilage.³⁻⁶ The intraarticular environment may also negatively impact the healing process after ACL tears, preventing primary healing.⁷⁻¹²

The individual's sex is known to contribute to risk of ACL injury.¹³⁻¹⁵ Females are noted to have a 2-10 fold increase risk of ACL injury when compared to their male counterparts.^{13; 16; 17} Possible causes for the female risk in ACL injury include hormonal variations, decreased structural and biochemical quality of female tendons and ligaments and differences in neuromuscular control and specific sex-related anatomical variants.^{14; 18-22} Additional reasons for increased risk of ACL rupture in females include anatomic factors such as intercondylar notch size, posterior tibial plateau slope and ACL elevation

angle among others.^{21; 22} However, sex differences in metabolic responses of relevant tissues after ACL tears are not fully elucidated.

Characterizing metabolic responses of the various tissues in the knee is important for understanding the injury as well as healing after treatment for ACL injuries. ACL reconstruction using a tendon graft has been the standard-of-care for surgical management of ACL tears in humans. However, there are long-recognized limitations with surgical reconstruction for ACL injuries. Morbidity from ACL reconstructive surgery including risk for graft laxity and clinical failure and inability to consistently mitigate onset of post-traumatic osteoarthritis (PTOA) despite successful reconstruction are notable limitations.^{23;}
²⁴ There is also an ongoing debate if a reconstructed ACL recapitulates all of the properties of a native ACL including its proprioceptive or reflex responses²⁵. Therefore, a renewed interest in primary ACL repair has developed.

While attractive, an unreliable healing response has been associated with primary ACL repair. This has been attributed to several factors, including a hostile intra-synovial environment, specific post-inflammatory responses, and intrinsic cell deficiencies.^{26; 27} The effects of sex in the injury response and the subsequent healing process of the injured ACL tissue are also unknown. Furthermore, given that ACL cells are likely sensitive to mechanical stimulus, sex-dependent differences in response to external mechanical stimuli need to be more fully characterized. Therefore, understanding the early healing phase characterized by cellular release of molecules that regulate adequate balance between extracellular matrix (ECM) degradation and proliferation, as well as how these processes are impacted by external mechanical stimulus and patient sex, may be crucial to case selection for ACL repair and optimizing rehabilitation after that surgery.

One method to evaluate cellular interactions involves *in vitro* models, which also permit evaluating cell responses to mechanical stress.²⁸ The Flexcell system has been used widely in both monolayer culture and 3D systems for this purpose.²⁹ The bi-axial loading system permits investigators to reliably model ligament-tendon fibroblastic load conditions while allowing for easy manipulation and accessibility.²⁸

Since the metabolic responses of the cellular component of knee intraarticular tissues are likely not only interdependent but also susceptible to mechanical strain, we aimed to evaluate the responses of ACL and SYN to three different biaxial mechanical loads that simulate strains associated with perioperative rehabilitation after ACL repair.^{28; 30; 31} Additionally, we aimed to assess the responses of ACL and SYN after rupture to the conditions described above. We hypothesized that SYN will significantly increase the production of inflammatory and degradative markers compared to ACL fibroblasts in a strain dependent manner.

Materials and Methods:

Tissue processing and culture

With IRB approval (IRB#2009879), explants from injured intraarticular tissues (20 ACL, 19 SYN) that would otherwise be discarded were collected at the time of cruciate ligament reconstruction (Fig.7.1). Explants were aseptically minced into 0.5–1.0 cm² pieces and digested for two hours in Type 1A *Clostridium histolyticum* collagenase solution (Sigma-Aldrich, St. Louis, MO, USA) at a concentration of 0.2 mg/mL. The aliquot was then suspended with nutritional media (Gibco DMEM, Thermo Fisher Scientific, Waltham, MA, USA) containing 10% FBS, 0.002% Penicillin, 100 µg/mL Streptomycin, 25 µg/mL Amphotericin B, 0.002% L-Ascorbate and 0.01% L-glutamine

(Sigma Chemical Co, St. Louis, MO), cultured in T25 flasks (passage zero) and T75 flasks (passage one) (TPP Techno Plastic Products AG, Trasadingen, Switzerland) and incubated at 5% CO₂, 37°C, and 95% humidity. Once confluent, passage one cells were stored at -80°C until used for culture.

Biaxial mechanical stress

Passage one cells were re-expanded in T75 flasks and when confluent, exposed to TrypLE express (Invitrogen, Carlsbad, CA) for five minutes, then resuspended and seeded (1×10^5 cells/well) in Collagen Type I-coated BioFlex® plates (Flexcell International, NC, USA). Cells were incubated for an additional 48h in 10% FBS nutritional media before strain was applied. Before fibroblasts were subjected to continuous mechanical stimulation media was changed to 2% FBS for the duration of the loaded culture (6 days).³² Strain loads (2-s strain and 10-s relaxation at a 0.5 Hz frequency) with a biaxial sinusoidal waveform with three different elongation strains that replicate 3 physiologic conditions (rest or mechanical stress deprivation-0%, physiologic strain-4%, and injury or high strain-10%)²⁸ was applied for 6 days using the Flexcell FX-4000T strain system (Flexcell International, NC, USA). Media were changed at day three and day six and stored at -20°C until analysis.

Biomarker assays

Media was changed every three days and stored at -20°C until analysis. Media from three (3d) and six (6d) days of culture were assessed for various biomarkers. Proteoglycan (GAG) was assessed using the DMMB assay as previously described.³³ The concentration of prostaglandin E₂ (PGE₂) (Cayman Chemical, Ann Arbor, MI, USA), cytokines (IL-6, IL-8, KC, MCP-1 and VEGF) (Millipore, Billerica, MA, USA), total

matrix metalloproteinase (MMP) activity (SensoLyte 520 generic MMP assay, Anaspec, Inc., Fremont, CA), and MMPs and TIMPs production (MMP-1, MMP-2, MMP-3, TIMP 1 and TIMP 2) (R&D Systems, Minneapolis, MN, USA), were all assessed using commercially available assays according to the manufacturer's protocol. At the end of the strain protocol (6d), cell viability analysis was performed using resazurin assay (Sigma Aldrich, Saint Louis, MO).

Statistical Analysis

For statistical analysis, normal distribution was assessed by Shapiro-Wilk test. Because data were not normally distributed, non-parametric analyses were used. Comparisons among strains and tissue types were performed using Kruskal-Wallis test and Dunn's test posthoc. Additionally, comparisons between males and females were performed using Mann-Whitney U test (R Core Team (2019), R version 3.6.2 Vienna, Austria). Results were reported as median± interquartile range (IQR). Significance levels were set at $p < 0.05$.

Results

Differences in ACL and Synovial Fibroblast to Mechanical Strain

Metabolic activity was not statistically affected by any strain in SYN fibroblasts.

However, the metabolic activity of ACL fibroblasts at supraphysiological strain was significantly lower than ACL fibroblasts at physiological strain (Fig. 7.2).

PGE2 concentration in SYN fibroblasts was significantly increased at supraphysiological strain relative to mechanical strain deprivation at six days of culture. The production of PGE2 by ACL fibroblasts increased significantly with increasing mechanical load.

Supraphysiological stress was significantly higher than both, mechanical deprivation and

physiological stress at three days of culture. Also, at 3d, ACL derived fibroblasts subjected to 4% strain produced significantly more PGE2 than no strain. Moreover, at six days, with stress deprivation there was a significant decrease in PGE2 concentration compared to 4% and 10% strain in ACL cultures (Fig.7.3)

The production of IL-6 by SYN fibroblasts was significantly higher at physiological stress compared to supraphysiological stress at day 6 of culture. Further, the production of IL-6 by ACL fibroblast at supraphysiological strain levels was significantly lower than at physiological and mechanical deprivation strain levels after six days of culture. While the production of IL-8 was not significantly affected by strain in SYN fibroblasts at any timepoint, the production of IL-8 by ACL fibroblasts at supraphysiological strain levels was significantly lower than at physiological strain levels at day 3 of culture (Fig. 7.4).

The production of KC was significantly decreased by ACL fibroblasts subjected to supraphysiological strain compared to physiological strain through 6 days of culture. Moreover, MCP-1 production was significantly decreased by ACL fibroblasts subjected to supraphysiological strain compared to stress deprivation at day 3 of culture. KC and MCP1 did not show a significant change at any strain and timepoint in SYN fibroblasts (Fig. 7.5).

The production of VEGF was not significantly different among strains in SYN fibroblasts at any timepoint. However, VEGF production was significantly increased by ACL fibroblasts at physiological strain compared to supraphysiological strain at three days of culture. Additionally, MIP-1 β production was significantly decreased by SYN fibroblasts at supraphysiological strain levels compared to both stress deprivation and physiological strain level at day 3 of culture. Similarly, ACL fibroblasts produced significantly less

MP1 β at 10% strain compared to no strain at day 3 of culture. Also, at day 6, supraphysiological strain elicited a significant decrease in MP1 β production compared to mechanical strain deprivation and physiological strain (Fig. 7.6).

Physiologic stress significantly increased the production of GAG by ACL fibroblasts compared to stress deprivation and supraphysiologic strain levels at days 3 and 6 of culture. There were no significant differences between groups for GAG production by SYN fibroblasts (Fig. 7.7). Further there were no significant differences between ACL and SYN fibroblast groups for MMP (Fig.7.8) or TIMP (Fig. 7.9) production at any timepoint.

Differences between ACL and Synovial Fibroblast Response to Mechanical Strain

Metabolic activity was not significantly different between SYN and ACL fibroblasts at low strains (no strain and 4% strain). However, at supraphysiological strain levels, the metabolic activity of ACL fibroblasts was significantly lower than SYN fibroblasts (Fig. 7.10).

PGE2 production was significantly lower in ACL compared to SYN fibroblasts when subjected to mechanical strain deprivation at three and six days of culture. Conversely, PGE2 production was significantly increased in ACL compared to SYN fibroblasts when subjected to physiological strain at day three and six of culture. No differences between ACL and SYN fibroblasts were seen at supraphysiological strain at any timepoints (Fig. 7.11).

The production of IL-6 and KC by SYN fibroblasts was significantly higher than ACL fibroblasts at day 3 of culture regardless of the strain applied (Fig. 7.12 and 7.14 respectively). Additionally, the production of IL-8 by SYN fibroblasts at

supraphysiological strain levels was significantly higher than ACL fibroblasts at day 3, but not day 6, of culture (Fig. 7.13).

The production of MCP-1 and VEGF by SYN fibroblasts was significantly higher than ACL fibroblasts at supraphysiological strain levels at day three of culture (Fig. 7.15 and 7.16). The production of MIP-1 β was significantly higher in ACL fibroblasts compared to SYN fibroblasts only when stress deprived at day six of culture. No significant differences in MIP-1 β production were seen between SYN and ACL fibroblasts at 4% and 10% strain at any timepoints (Fig. 7.17).

The production of GAG was significantly higher by ACL fibroblasts compared to SYN fibroblasts at stress deprivation and physiological stress levels at day 3 of culture.

Moreover, at day 6 of culture, the production of GAG by SYN fibroblasts was significantly higher than ACL fibroblasts during stress deprivation. No significant differences between SYN and ACL fibroblasts were seen at supraphysiological strain at any time point (Fig. 7.18).

The production of MMPs and TIMPS by the ACL and SYN fibroblasts were similar at most strain levels and time points tested. No significant differences were seen in MMP-1, MMP-2, MMP-3 and TIMP-1 between SYN and ACL fibroblasts at any strain level at any timepoint (Fig. 7.19, 7.20, 7.21, 7.22). Only the production of TIMP-2 by ACL fibroblasts was significantly higher than SYN fibroblasts when stress deprived after day 3 of culture (Fig. 7.23).

Males vs Females Differences in ACL and Synovial Fibroblast Response to

Mechanical Strain

There were few significant differences between male and female ACL or SYN fibroblasts regardless of strain or day of culture (Fig. 7.24-7.28). The metabolic activity of male SYN fibroblasts was significantly higher than female SYN fibroblasts at 4% strain (Fig. 7.24). The production of MIP-1 β by male SYN fibroblast was significantly higher than female SYN fibroblasts after six days of supraphysiological stress (Fig.7.27).

Discussion

This work was able to demonstrate that SYN and ACL fibroblasts modulate the production of inflammatory and metabolic markers in a strain dependent manner.

Moreover, we were able to show that such responses are significantly different between intraarticular tissue fibroblasts such as synovium and ACL. However, it appears that molecular response to load is not significantly different between males and females.

The inflammatory marker PGE2, seems to be increased with increasing loads.

Interestingly, lower cytokine production, was found at higher strain levels. Moreover, cytokines such as IL-6 and KC in SYN fibroblasts appear to consistently increase in response to raising strains while other inflammatory markers such as IL-8, MCP-1 and VEGF had a more erratic reaction.

This behavior may be explained by the direct reaction of cellular component of the ACL as a response to injury after ACL rupture^{12; 13; 3435}. The synovial lining of the knee joint is also a major player in knee injury responses and the intraarticular environment after an injury^{5; 36}. Therefore, understanding the time at which degradative and inflammatory molecules are produced by each tissue is important for understanding how healing may occur after surgery.

Some authors have suggested that ACL reconstruction should be delayed at least three weeks after injury in order to optimize the outcome in reconstruction scenarios.³⁷ In contrast, delay in surgery for primary ACL repair may not be advantageous. Magarian *et al* (2010) demonstrated that a two- or six-weeks delay may decrease functional outcomes after primary ACL repair in a pre-clinical model.³⁸ Authors suggest that although late repair may significantly decrease synovitis, the structural composition of the injured ACL and its retracted torn ends make the repair more difficult. This could be due in part from a decrease in ligament composition such as reduced GAG content when the ACL is stress deprived for 120h.

Clearly, early molecular events after ACL injury are likely important although not fully understood. Moreover, how the biomechanical environment affects the ACL healing capacity is not fully understood. Therefore, we aimed to evaluate how fibroblasts from injured ACL and synovium respond to mechanical stresses and how these individual tissue's biologic response may contribute to the intraarticular knee environment after ACL injury.

Ligaments are mechanosensitive tissues. Due to their multiscale hierarchical structure, loads applied to tissues can be sensed and transduced by fibroblasts into biochemical responses, transforming their extracellular matrix and ultrastructure.³⁹⁻⁴¹ Mechanical stress applied to cells also play an important role in inflammation.⁴²⁻⁴⁴ This work demonstrates that ACL fibroblasts may be more sensitive to extreme mechanical environments, either immobilization or high strain loads, responding with increased inflammatory production. In contrast, physiological strains led to increased release of cytokines and degradative molecules from synovial fibroblasts. This may be related to the

fact that in this work, synovial fibroblasts were isolated from the ECM and their activity may need to be regulated by other cell types.^{32; 45-48}

Differential fibroblastic responses to load seen between ACL and SYN may also play a role in ACL injury and repair.^{3; 32; 36} Since matrix and inflammatory biomarkers exhibit an opposite trend, higher GAG and TIMP-2 and lower cytokine production in ACL compared to SYN fibroblasts, it appears that mechanobiological regulation of healing is tissue dependent and, once again, providing the right amount of biomechanical stress is a key factor for proper healing and decreased injuries.

Sex differences in ACL injury has been the focus of a vast amount of research. This has led to many possible assumptions including anatomical variants, neuromuscular control differences, hormonal levels and variations.^{21; 22; 49} Recently, rather than the hormonal levels themselves, hormonal variation in females has been suggested as a main factor for increased rates of ACL injury in females. It seems that the cyclical periodic variation in hormone levels, estrogen and prostaglandin play a significant role in ACL injury.⁵⁰

Moreover, conflicting data has been found whether exogenous hormonal therapies are protective or not against ACL injury in female athletes.^{18; 51-53} Surprisingly, our current study did not find many biologic differences between male and female ACL and synovial fibroblasts in response to same levels of strain. The absence of differences may suggest that the main risk factors for increased ACL injuries in females do not lie in the cellular ability of injured tissues to withstand load rather than external factors.⁵⁴

It is advantageous that this analysis included human tissues from injured specimens. This helps better reflect the current condition of the tissues present in the ACL injured knee. We also included a reasonable sample size that permitted comparisons between males

and females. However, inclusion of more males in the study is strongly suggested. Additionally, because we included a wide range of patients, age and ethnicity are expected to play a role in diverse biological responses of intraarticular tissues. Hence, a greater study that allows to control for age and ethnicity seems an interesting option. There were also limitations associated with this experiment. First, this study was only performed for six days. While it is a long in vitro study using stressed cells in culture, it does not reflect typical conditions of a native tissue, disregarding many events in the healing process.^{10; 12; 55} Second, we did not measure collagen production or degradation. Since many of the markers evaluated directly affect collagen structure, it would have been interesting to analyze how fibroblasts would respond in terms of collagen synthesis/degradation. Lastly, the lack of other molecular techniques such as gene expression or fluorescent imaging prevents us for making a “healing targeted” approach where we could see how biological processes and how they are regulated in terms of inflammation/degradation resolution.

We conclude that inflammatory markers are being significantly produced in response to a variety of tension loads in the cellular environment of the intraarticular tissues. Moreover, SYN appears to contribute to some of the inflammatory markers in response to varying mechanical stresses. It seems that degradative enzymes are not being significantly affected by strain, at least at this point, and that remodeling will take place in future stages of the healing process. Lastly, it appears that metabolic response to stretch is not different between males and females. Future directions include the assessment of a dynamic co-culture using both SYN and ACL fibroblasts to understand the cross talk that

is taking place in the injured knee. Moreover, knowing the source of molecules involved in injury and healing is important for successfully preventing OA after surgery⁵⁶.

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Tables

Tissue source (n)	Sex (n)	Age Range
SYN (19)	Males (4)	17-35
	Females (15)	14-62
ACL (20)	Males (4)	17-22
	Females (16)	14-62

Table 7.1. Patient sex and age distribution by tissue source

Figures:

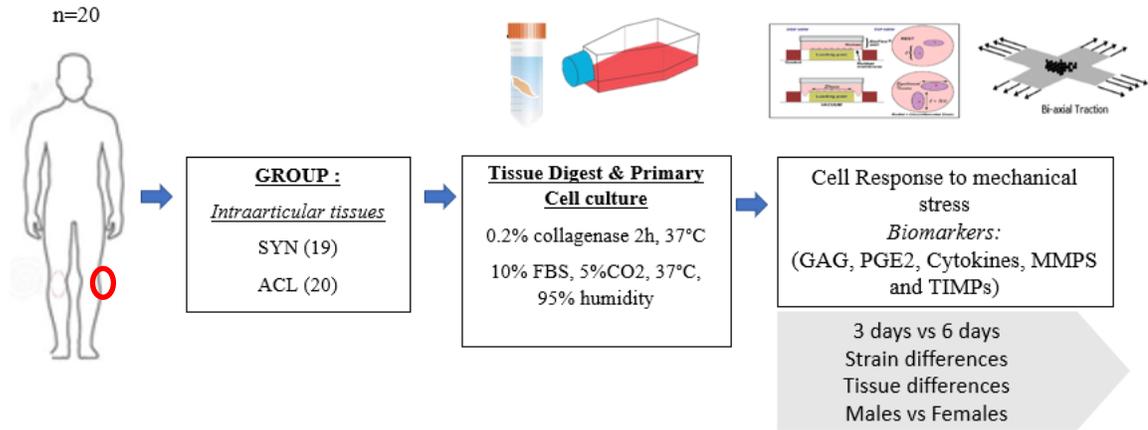


Figure 7.1: Experimental design

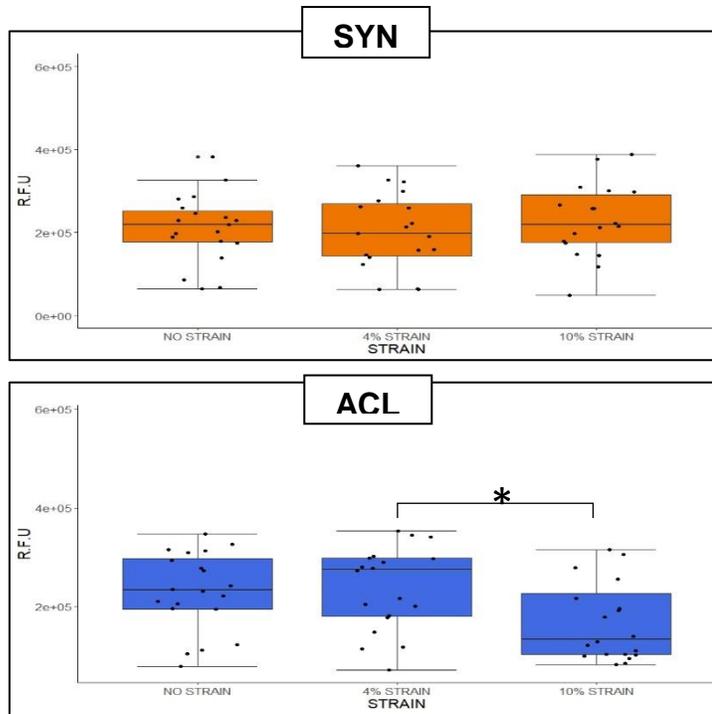


Figure 7.2. Metabolic Activity of intraarticular fibroblasts. * significant difference between STRAINS after 120 hours of culture (Kruskal-Wallis test). SYN: Synovium; ACL: Anterior Cruciate Ligament; R.F.U: Resazurin Fluorescent Units

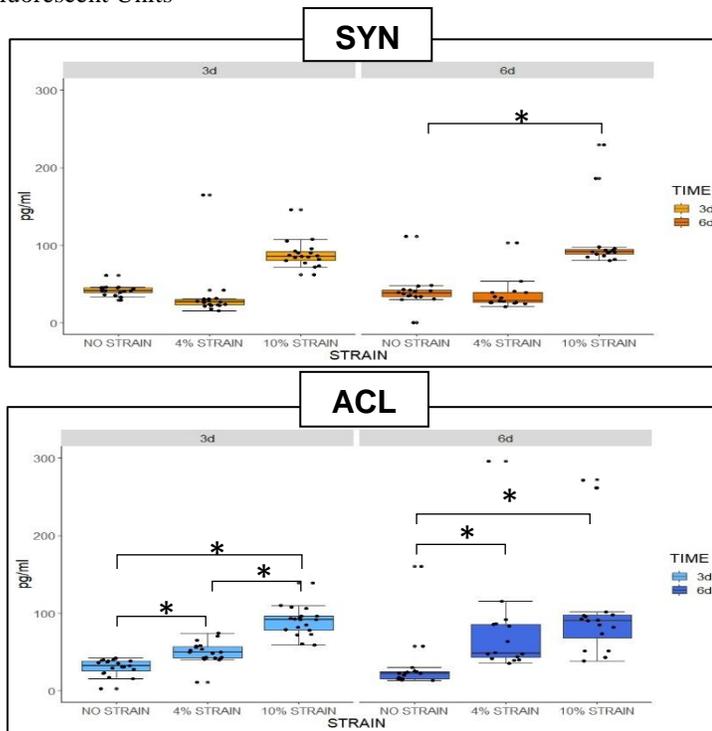


Figure 7.3. Concentration of PGE2 released to the media. * significant difference between STRAINS at 24 and 120 hours (Kruskal-Wallis test). SYN: Synovium; ACL: Anterior Cruciate Ligament

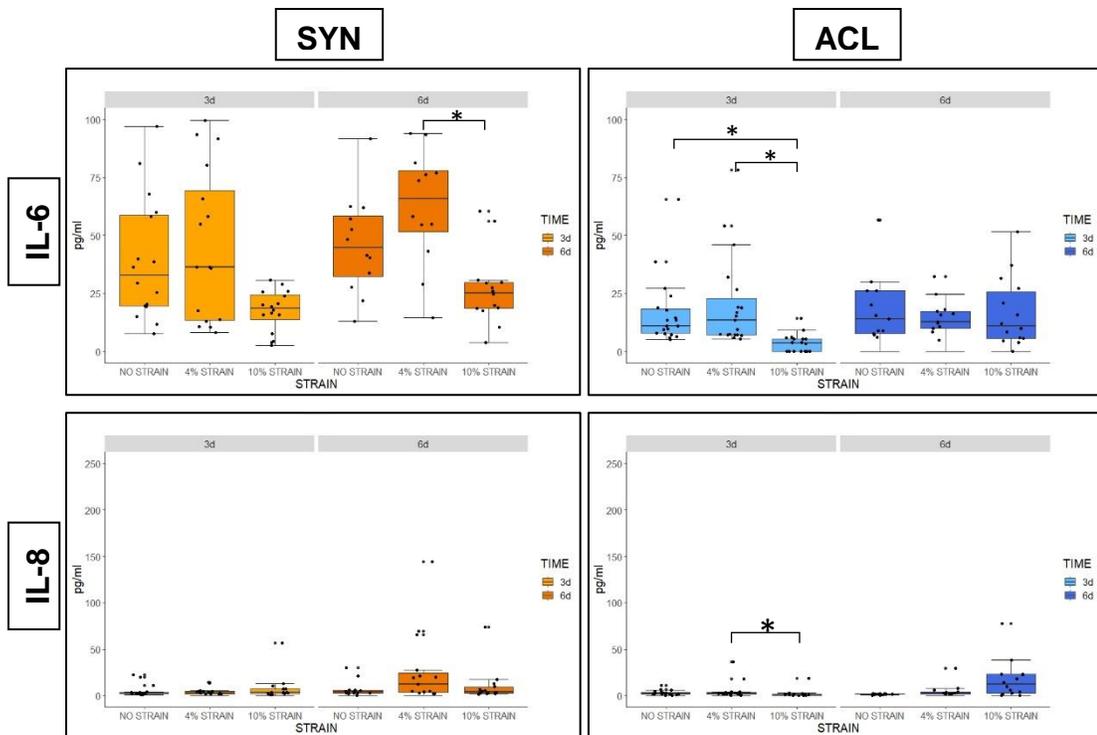


Figure 7.4. Concentration of interleukins 6 (IL-6) and 8 (IL-8) released to the media. * significant difference between STRAINS at 24 and 120 hours (Kruskal-Wallis test). SYN: Synovium; ACL: Anterior Cruciate Ligament

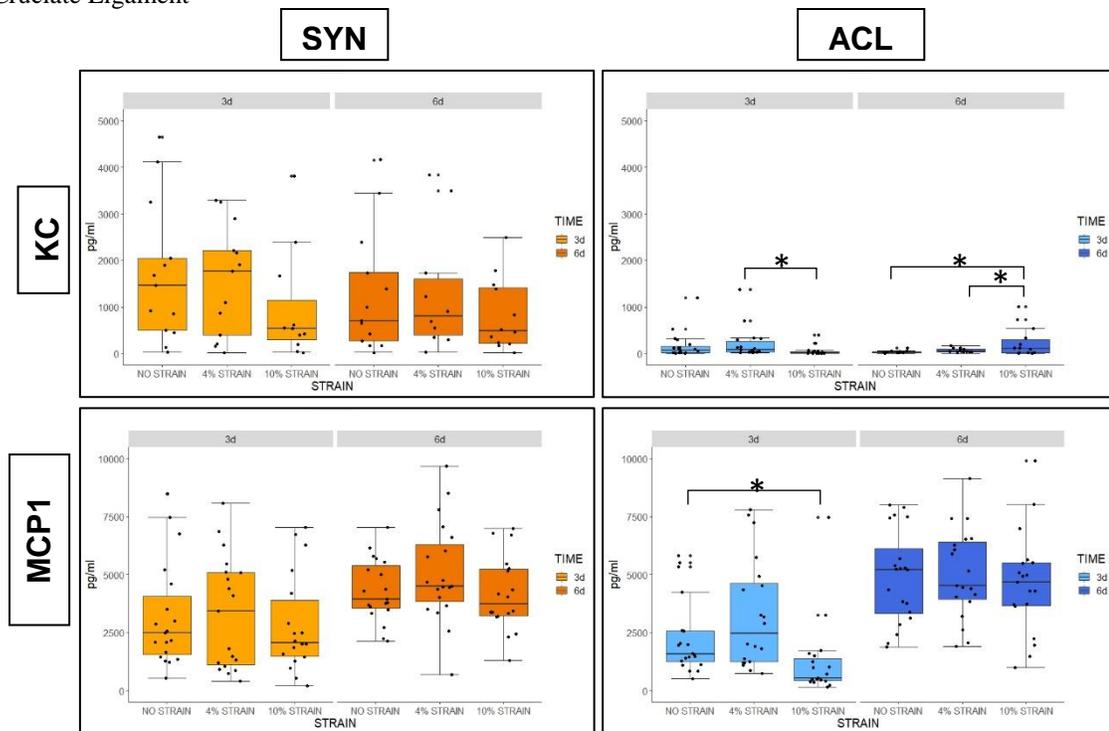


Figure 7.5. Concentration of chemokines (KC and MCP1) released to the media. * significant difference between STRAINS at 24 and 120 hours (Kruskal-Wallis test). SYN: Synovium; ACL: Anterior Cruciate Ligament

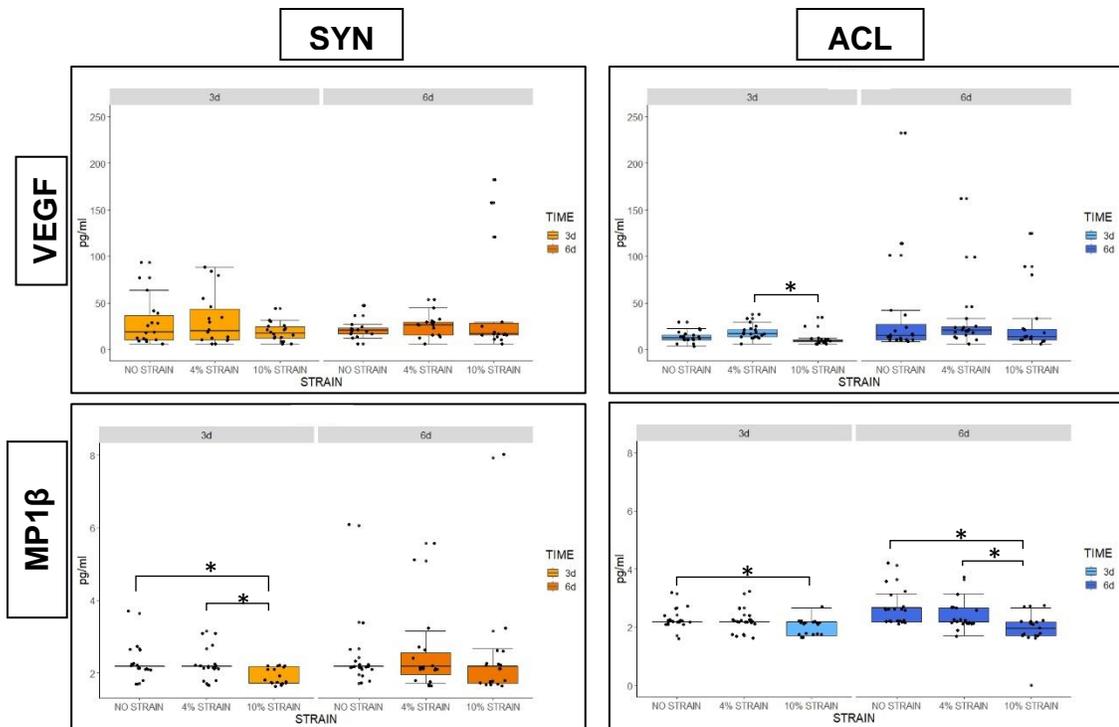


Figure 7.6. Concentration of Vascular Endothelial Growth Factor (VEGF) and macrophage inflammatory protein 1 β (MP1 β) released to the media. * significant difference between STRAINS at 24 and 120 hours (Kruskal-Wallis test). SYN: Synovium; ACL: Anterior Cruciate Ligament

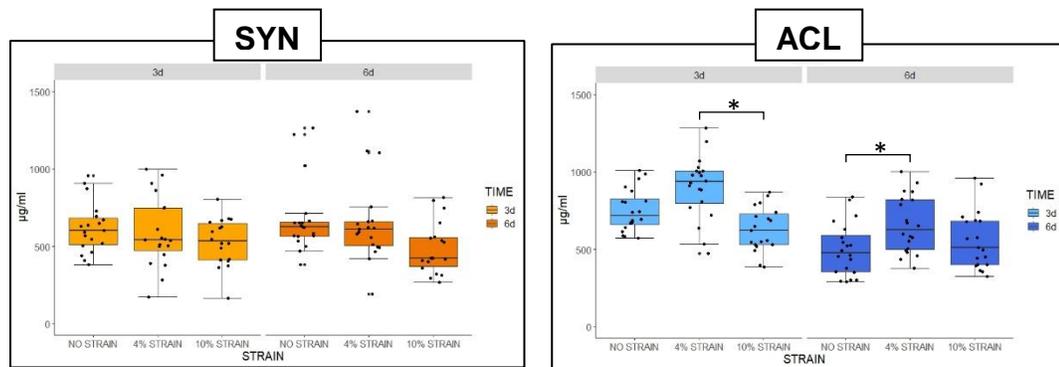


Figure 7.7. Concentration of GAG content released to the media. * significant difference between STRAINS at 24 and 120 hours (Kruskal-Wallis test). SYN: Synovium; ACL: Anterior Cruciate Ligament

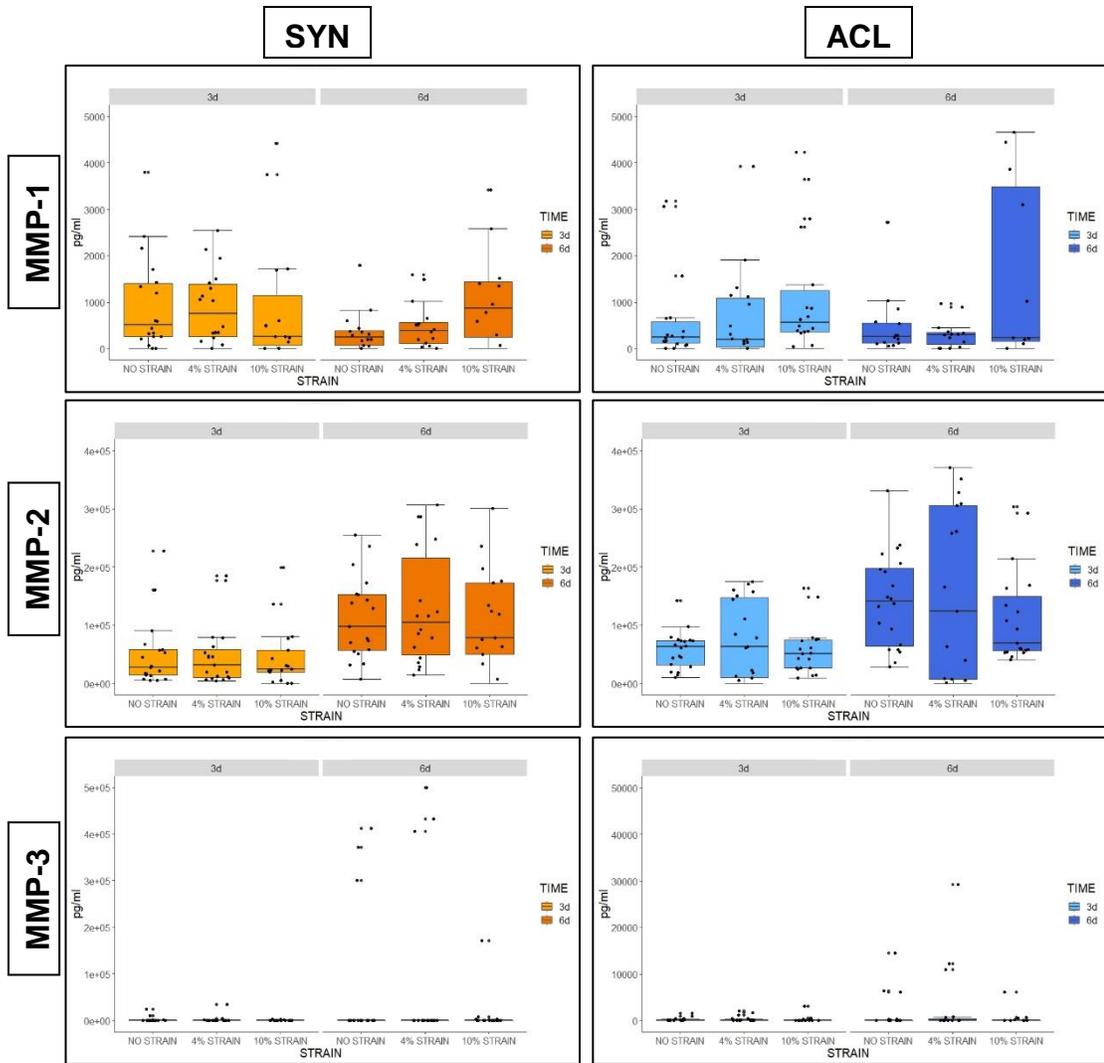


Figure 7.8. Concentration of matrix metalloproteinases (MMP) 1,2 and 3 released to the media. * significant difference between STRAINS at 24 and 120 hours (Kruskal-Wallis test). SYN: Synovium; ACL: Anterior Cruciate Ligament

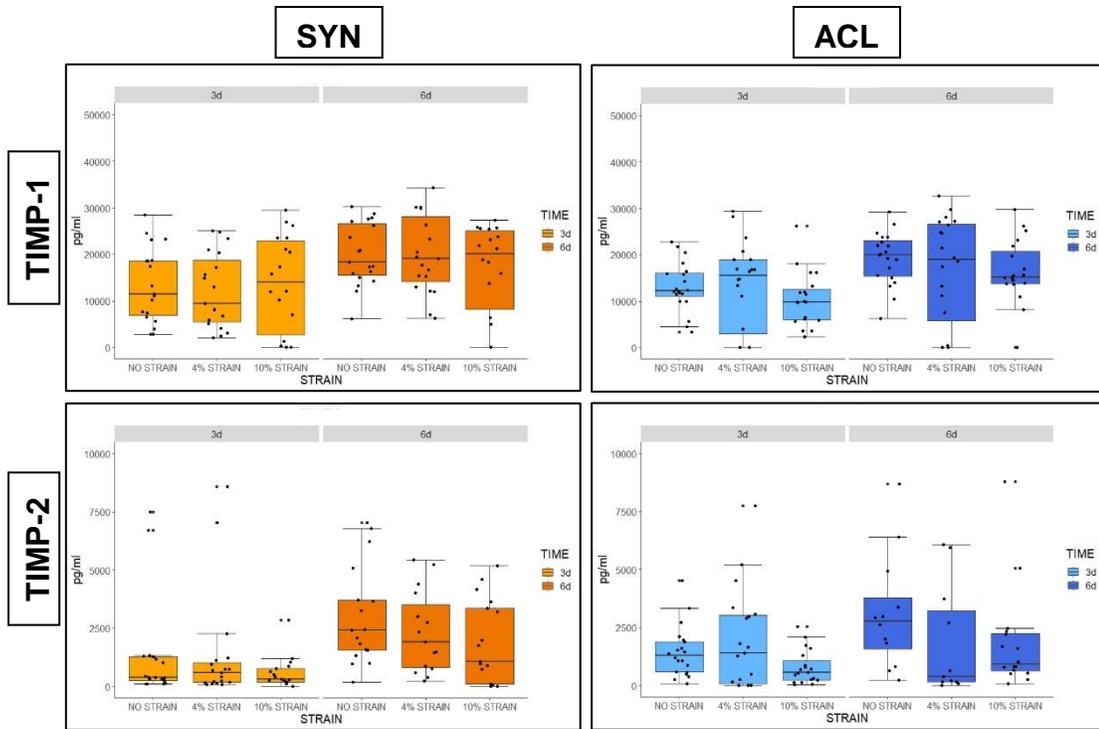


Figure 7.9. Concentration of tissue inhibitor of matrix metalloproteinases (TIMPs) 1 and 2 released to the media. * significant difference between STRAINS at 24 and 120 hours (Kruskal-Wallis test). SYN: Synovium; ACL: Anterior Cruciate Ligament

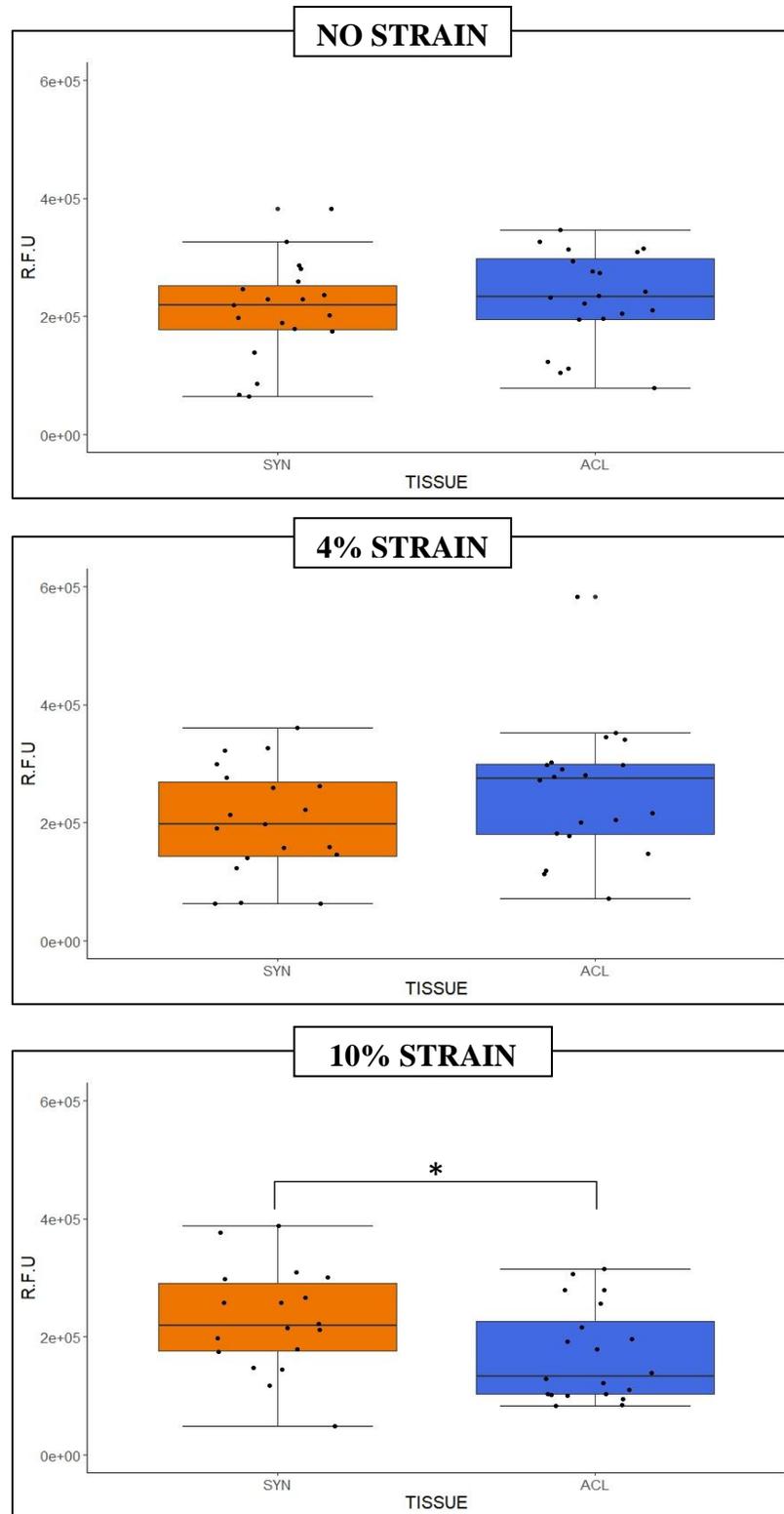


Figure 7.10. Metabolic Activity of fibroblasts after 120h of culture. * significant difference between TISSUES. SYN: Synovium; ACL: Anterior Cruciate Ligament; R.F.U: Resazurin Fluorescent Units.

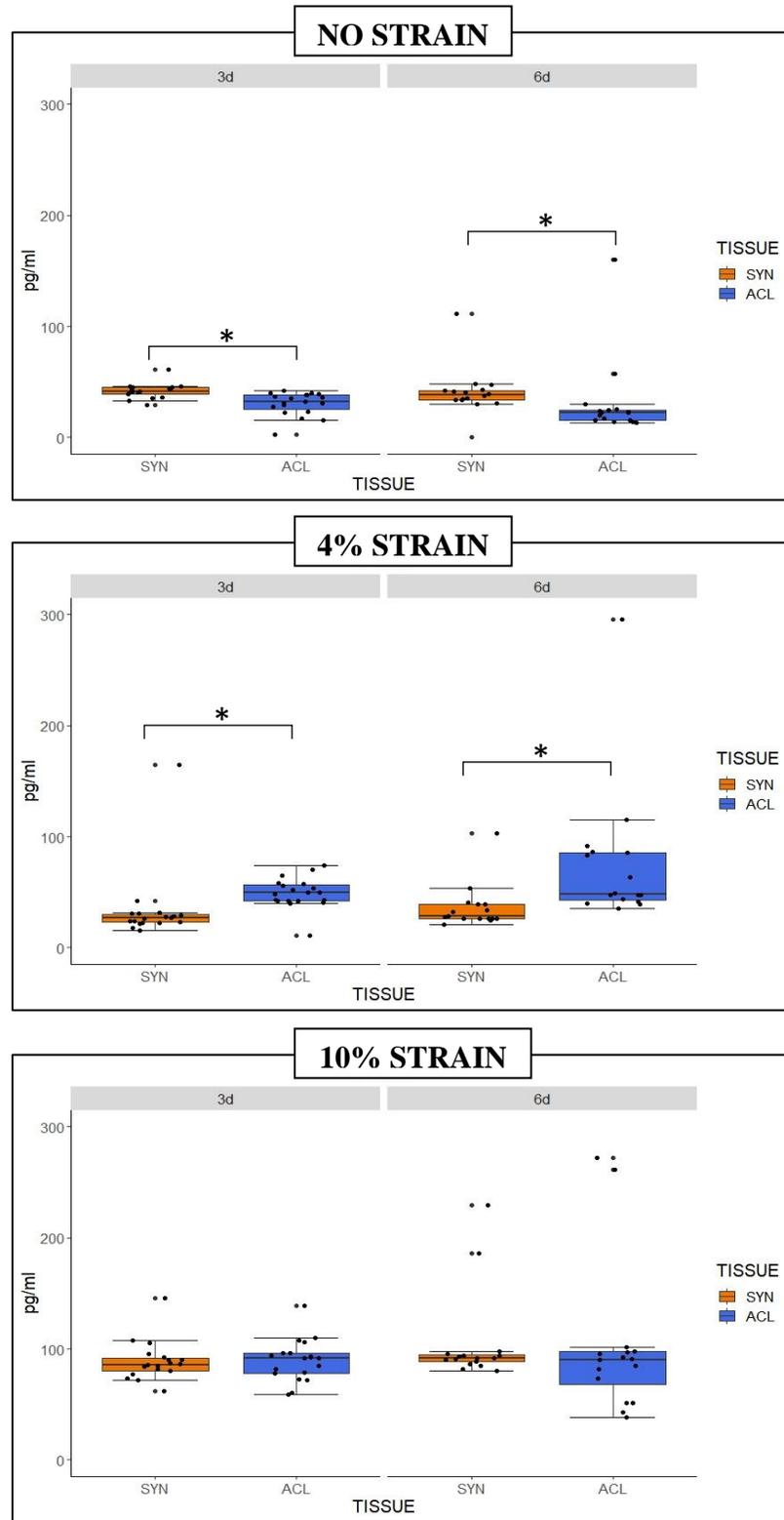


Figure 7.11. Concentration of PGE2 released to the media. * significant difference between TISSUES at 24 and 120 hours (Mann-Whitney test). SYN: Synovium; ACL: Anterior Cruciate Ligament.

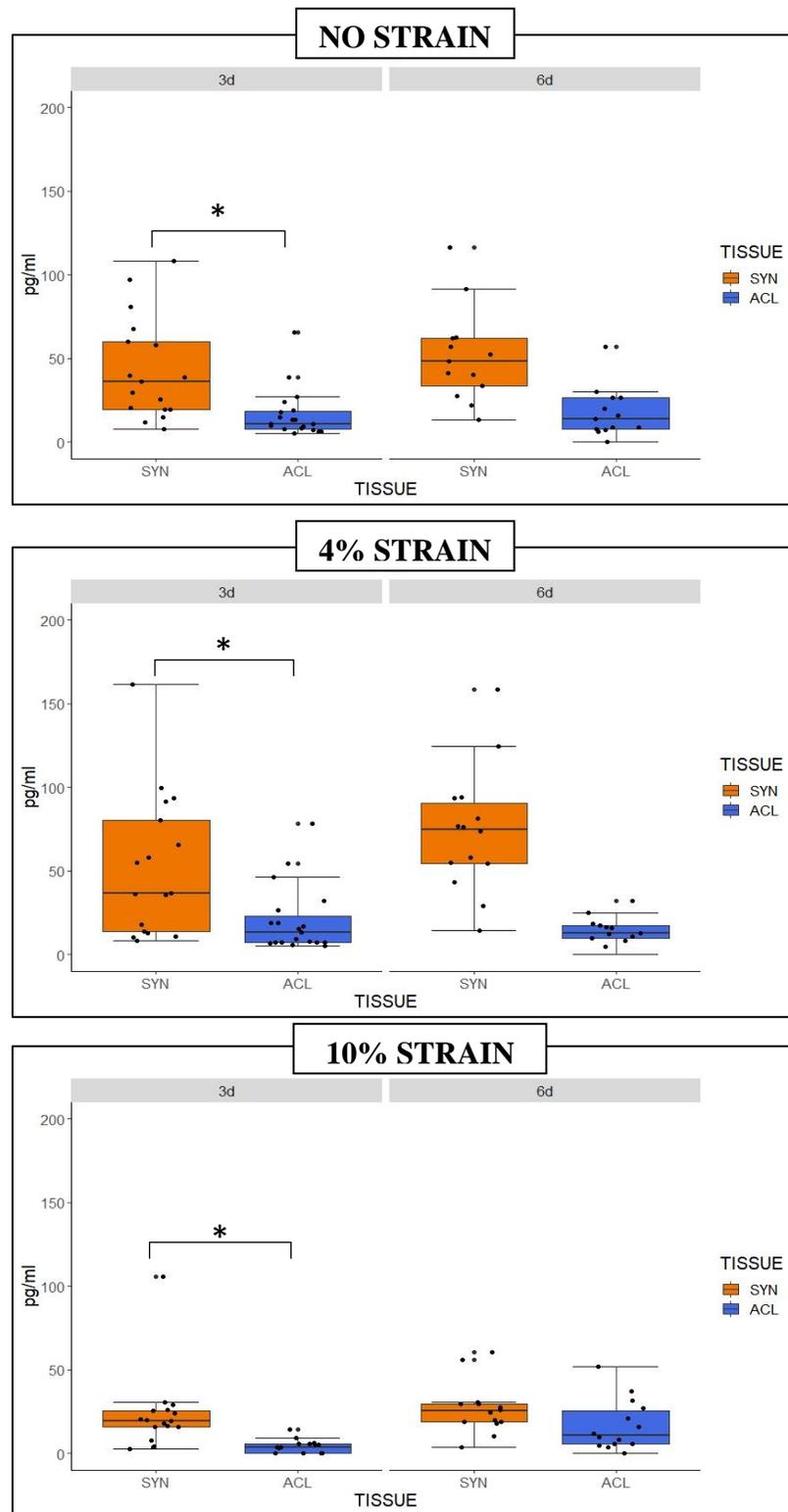


Figure 7.12. Concentration of IL-6 released to the media. * significant difference between TISSUES at 24 and 120 hours (Mann-Whitney test). SYN: Synovium; ACL: Anterior Cruciate Ligament.

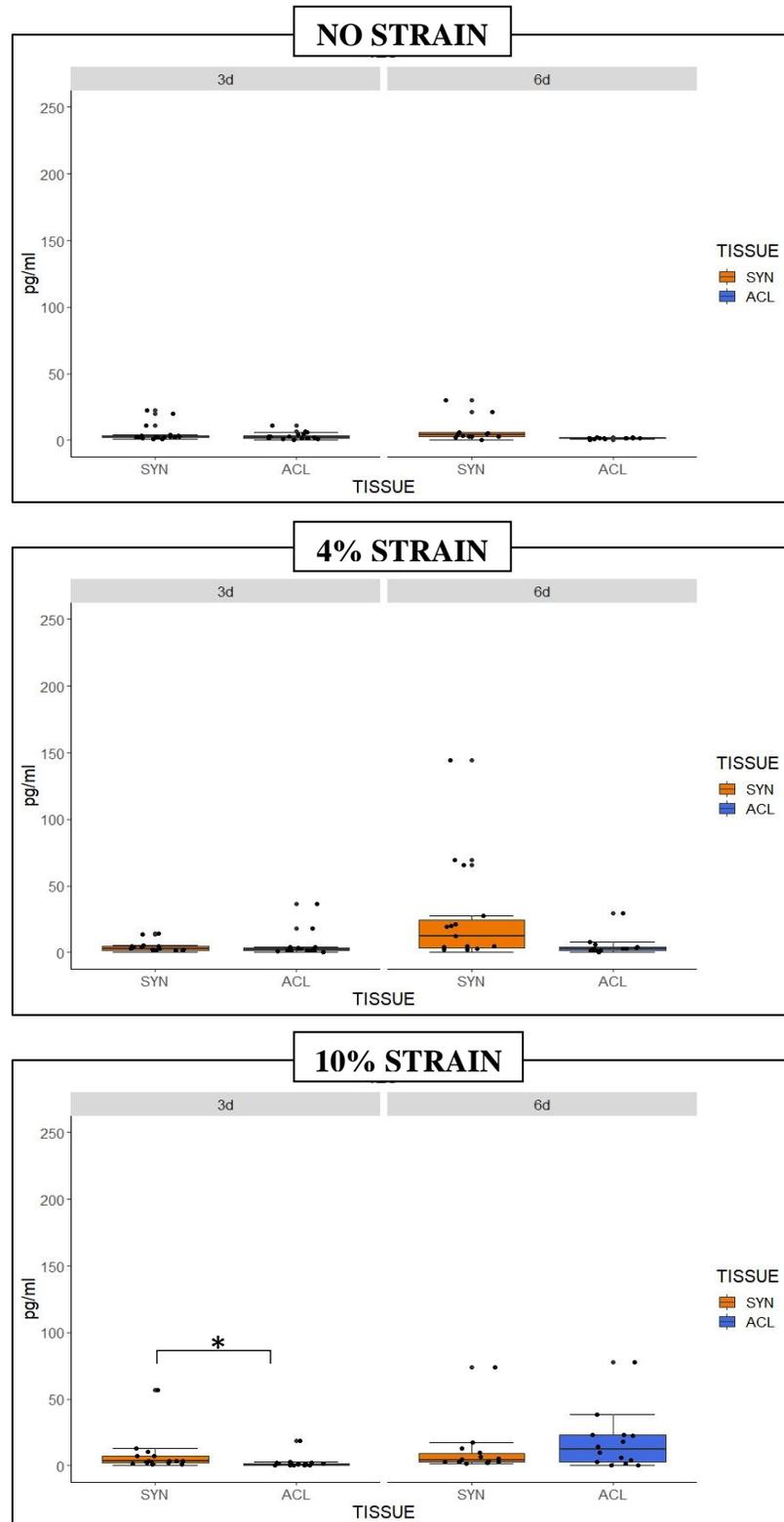


Figure 7.13. Concentration of IL-8 released to the media. * significant difference between TISSUES at 24 and 120 hours (Mann-Whitney test). SYN: Synovium; ACL: Anterior Cruciate Ligament.

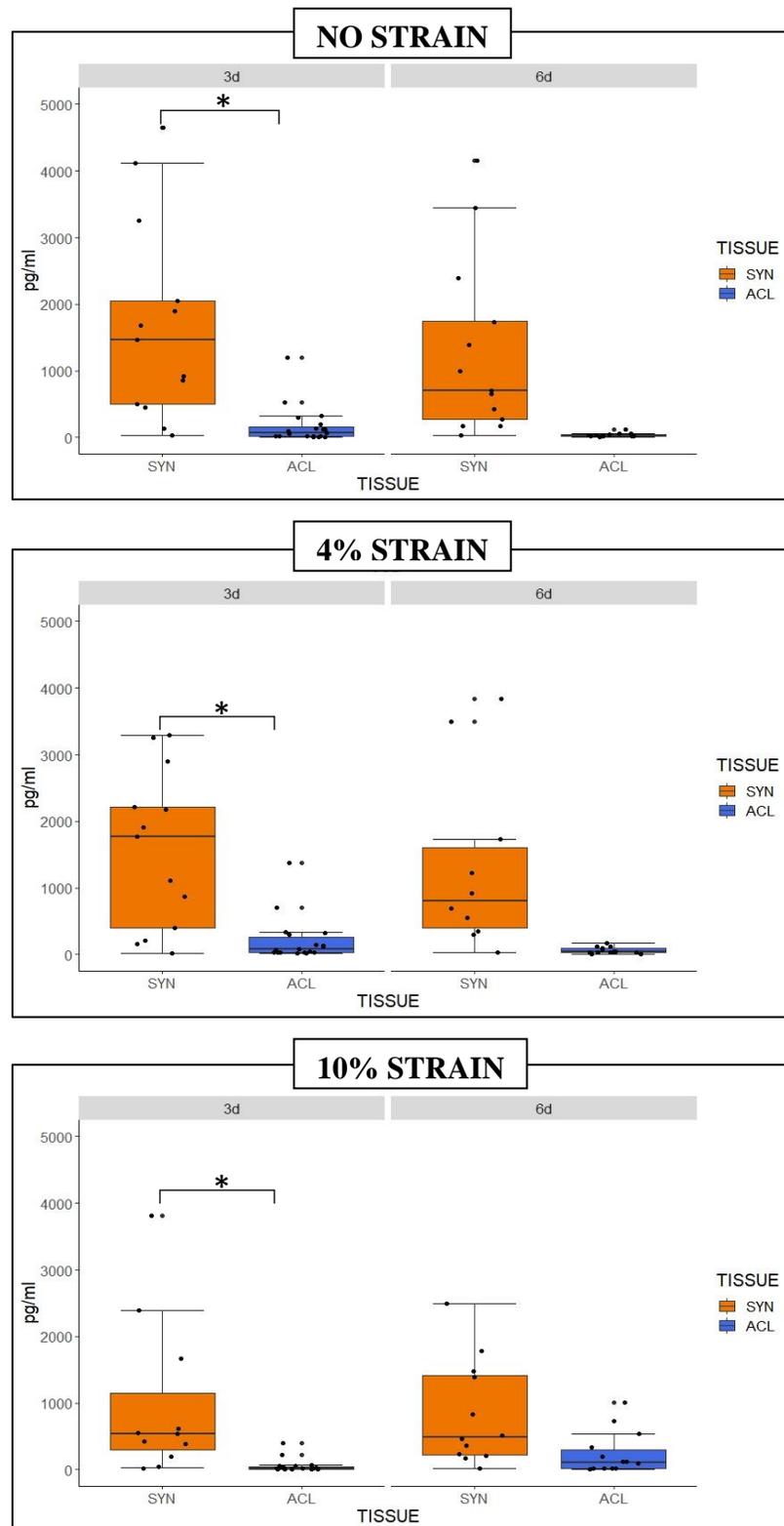


Figure 7.14. Concentration of KC released to the media. * significant difference between TISSUES at 24 and 120 hours (Mann-Whitney test). SYN: Synovium; ACL: Anterior Cruciate Ligament.

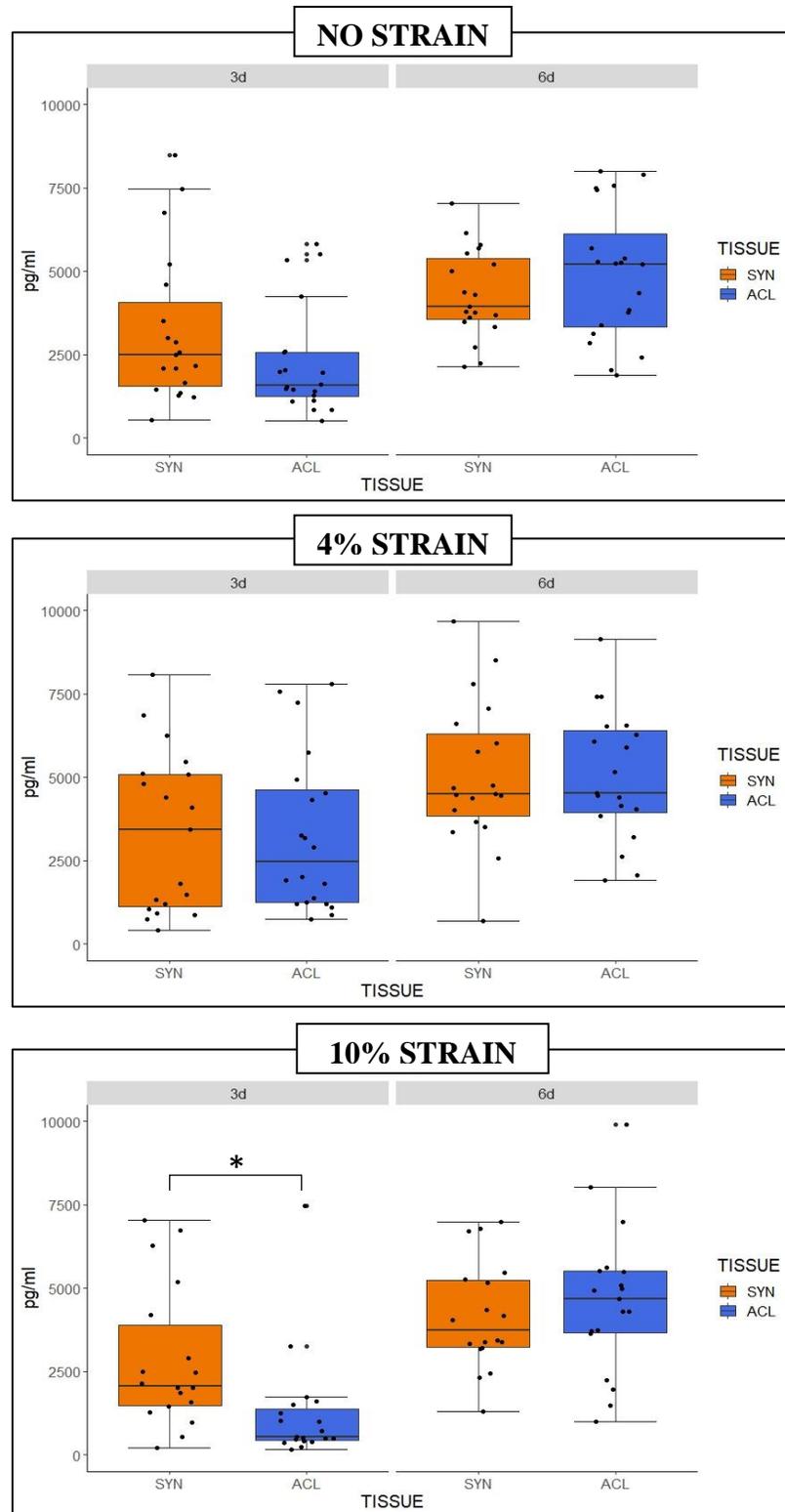


Figure 7.15. Concentration of MCP1 released to the media. * significant difference between TISSUES at 24 and 120 hours (Mann-Whitney test). SYN: Synovium; ACL: Anterior Cruciate Ligament.

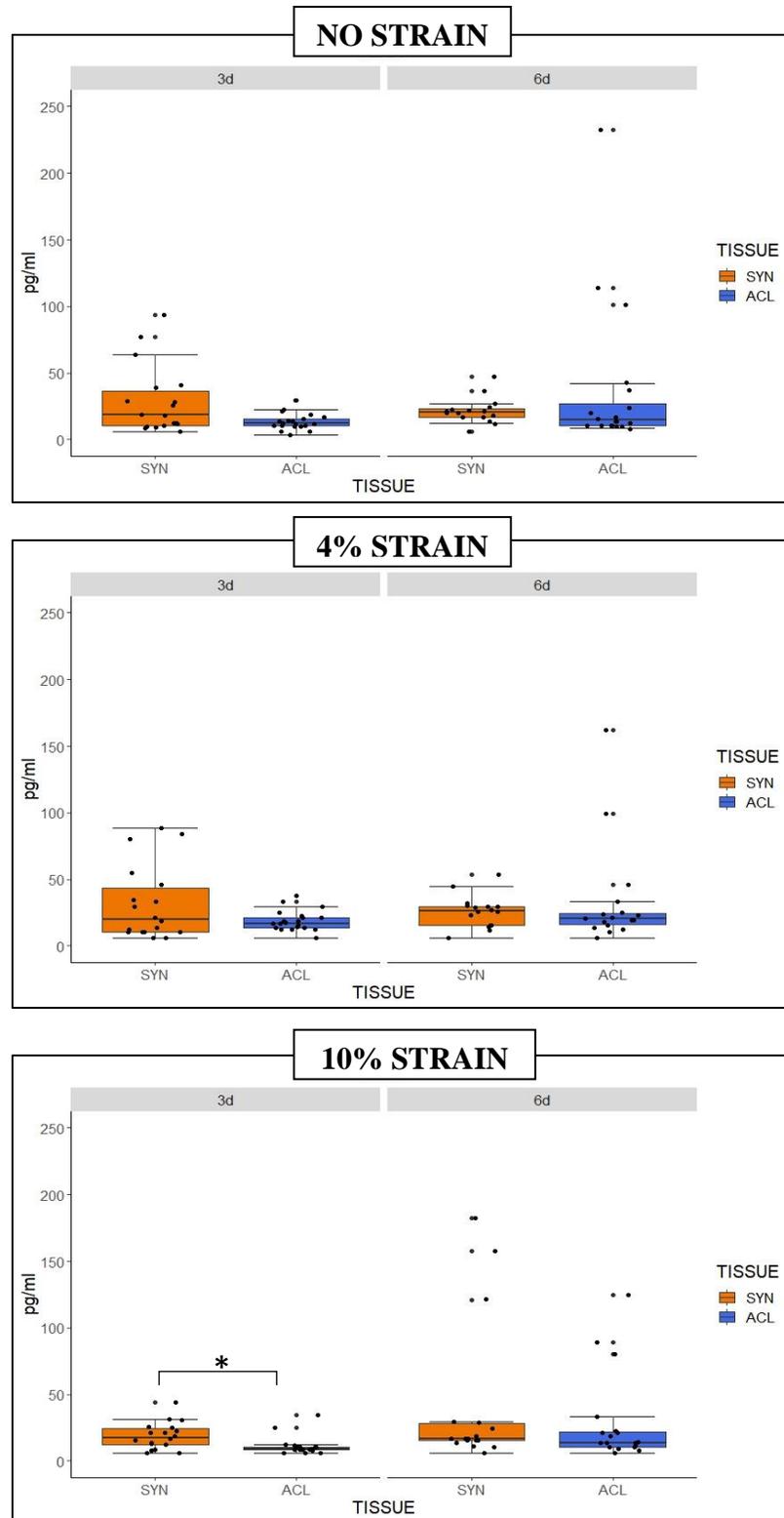


Figure 7.16. Concentration of VEGF released to the media. * significant difference between TISSUES at 24 and 120 hours (Mann-Whitney test). SYN: Synovium; ACL: Anterior Cruciate Ligament.

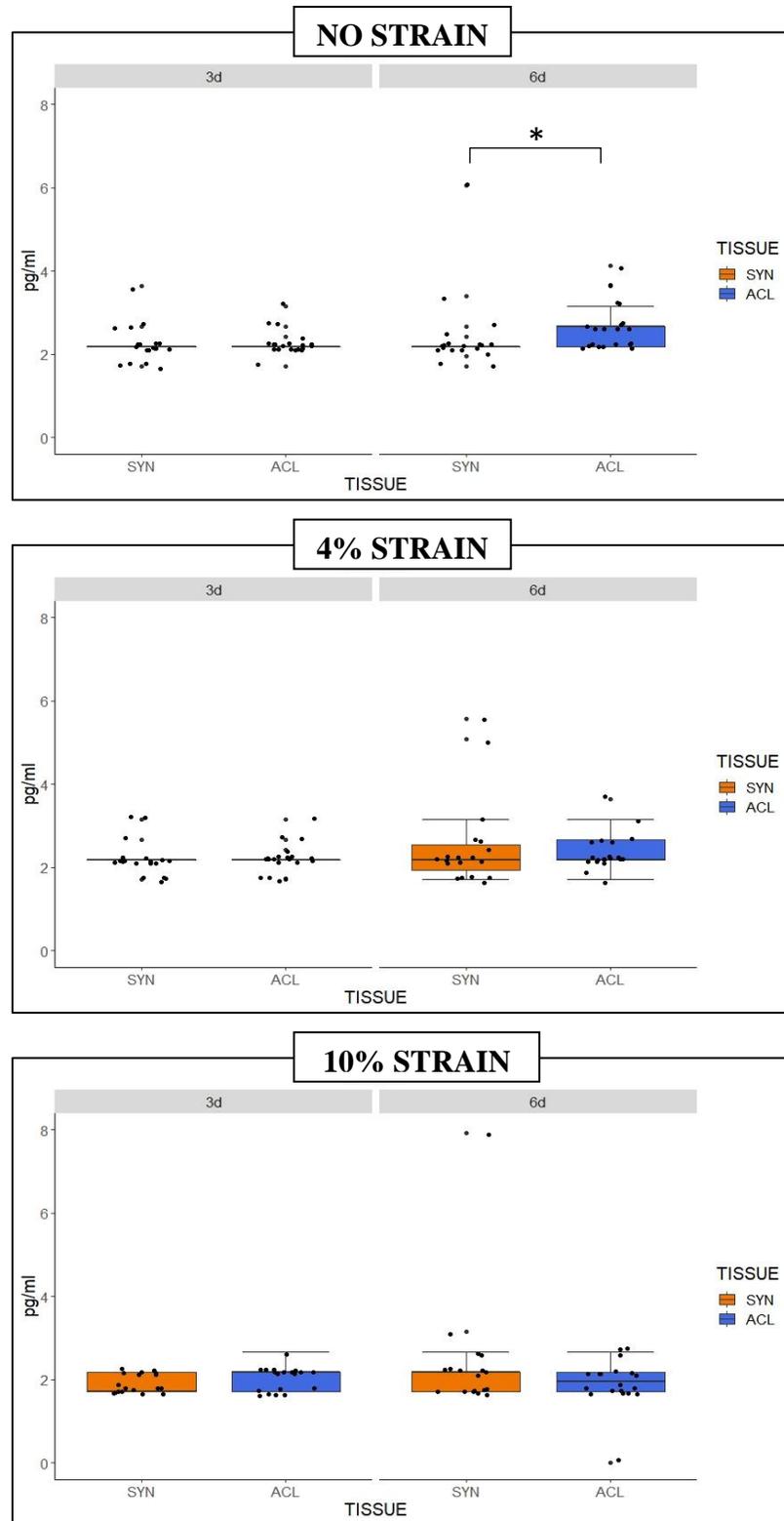


Figure 7.17. Concentration of MP1 β released to the media. * significant difference between TISSUES at 24 and 120 hours (Mann-Whitney test). SYN: Synovium; ACL: Anterior Cruciate Ligament.

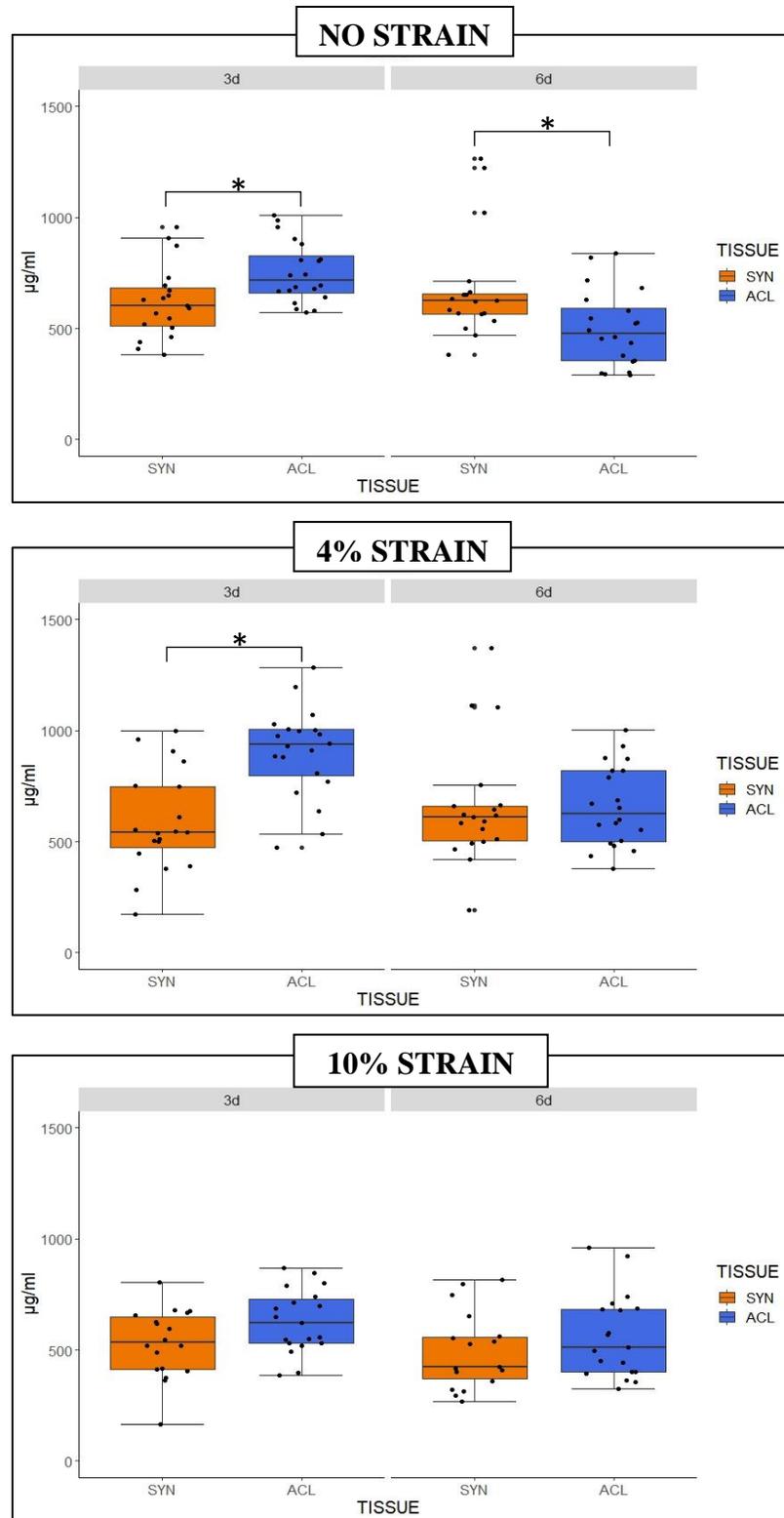


Figure 7.18. Concentration of GAG released to the media. * significant difference between TISSUES at 24 and 120 hours (Mann-Whitney test). SYN: Synovium; ACL: Anterior Cruciate Ligament.

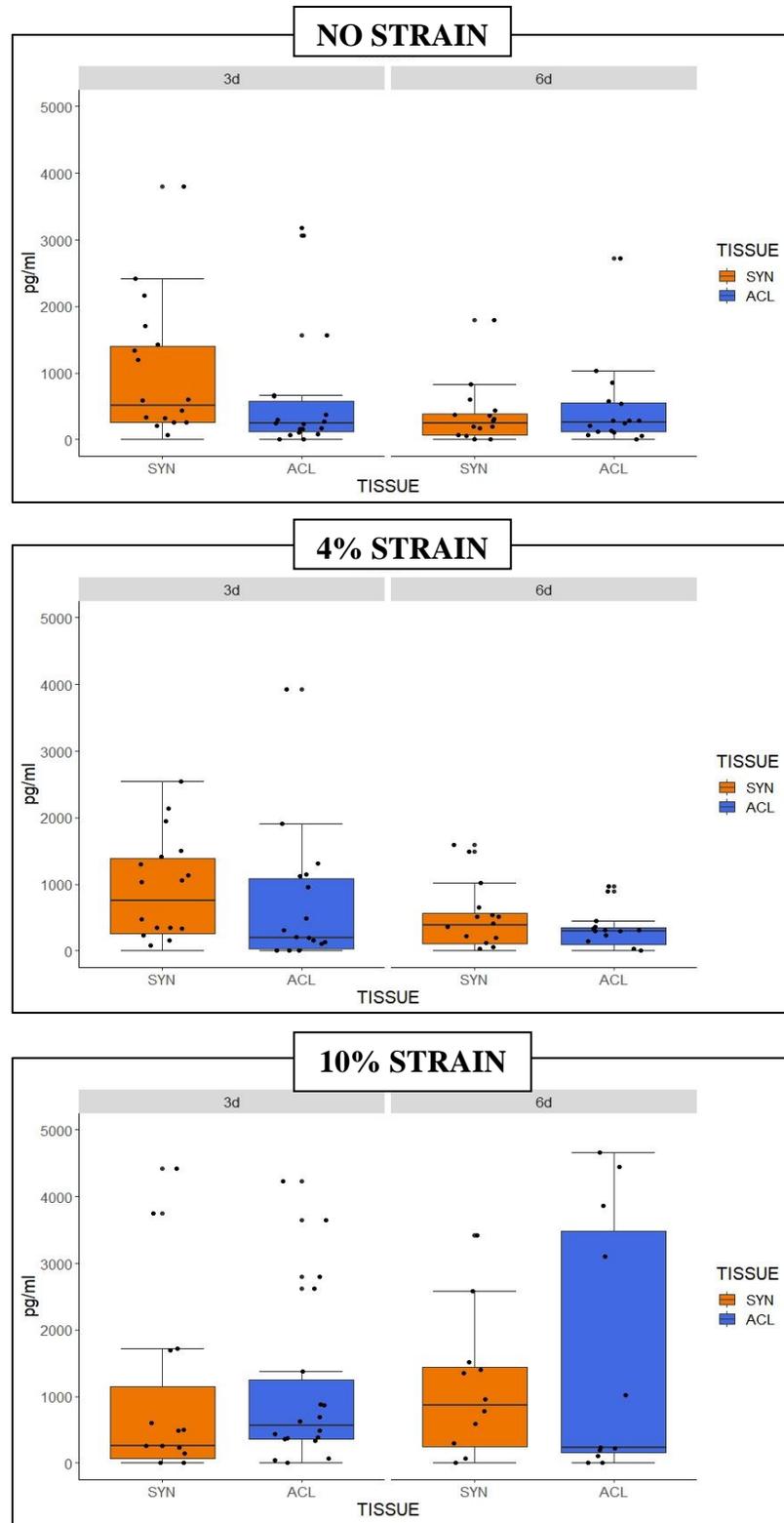


Figure 7.19. Concentration of MMP-1 released to the media. * significant difference between TISSUES at 24 and 120 hours (Mann-Whitney test). SYN: Synovium; ACL: Anterior Cruciate Ligament.

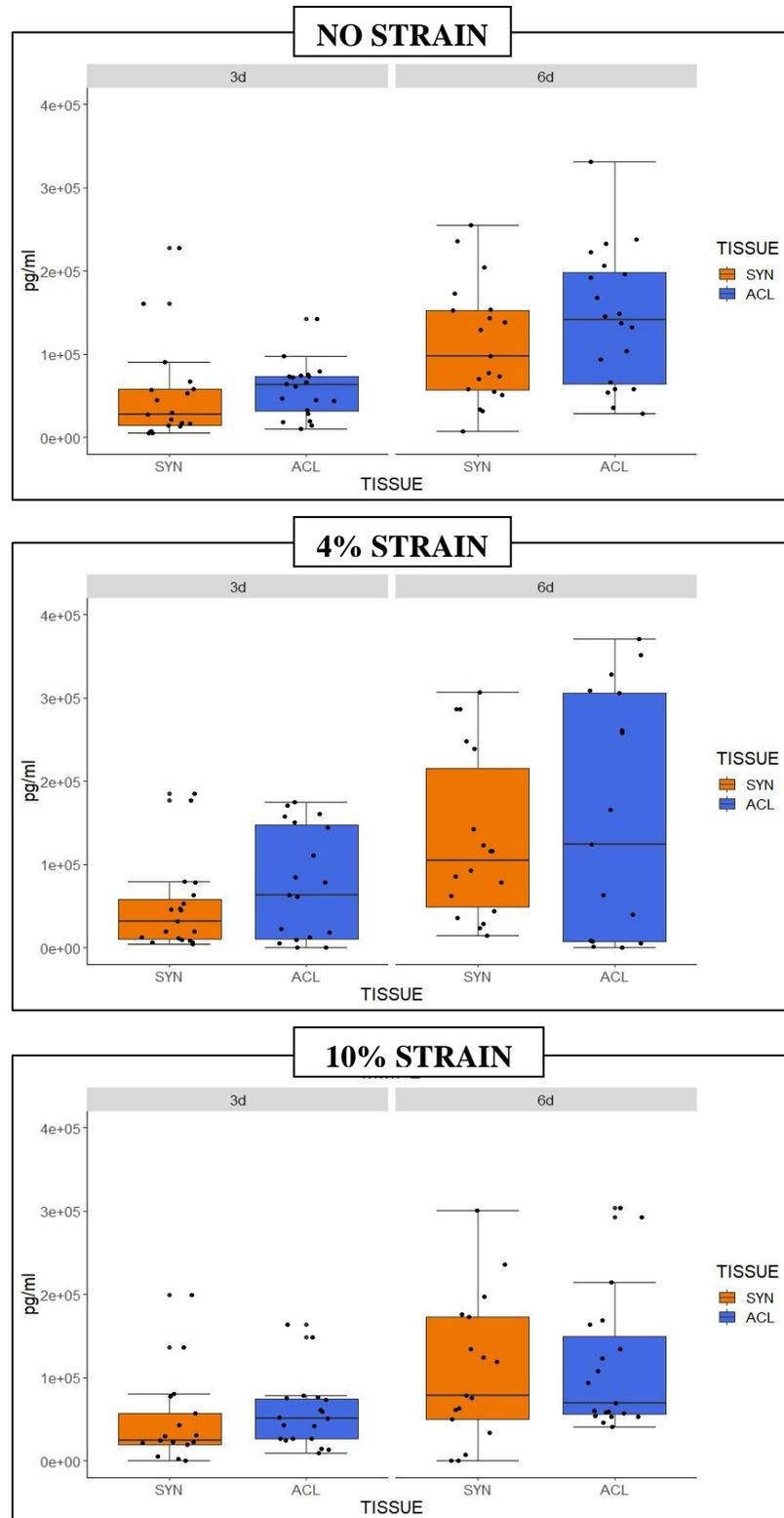


Figure 7.20. Concentration of MMP-2 released to the media. * significant difference between TISSUES at 24 and 120 hours (Mann-Whitney test). SYN: Synovium; ACL: Anterior Cruciate Ligament.

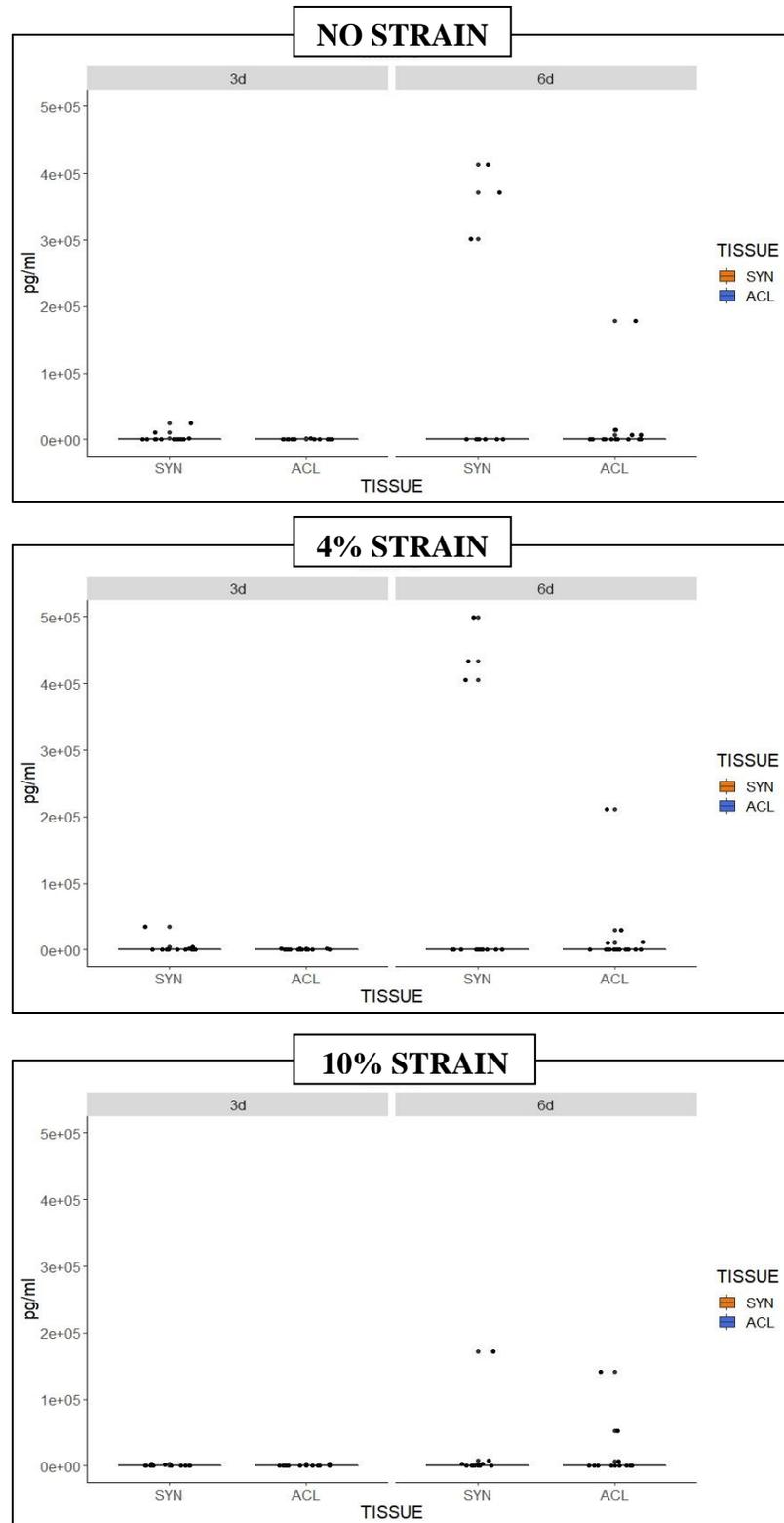


Figure 7.21. Concentration of MMP-3 released to the media. * significant difference between TISSUES at 24 and 120 hours (Mann-Whitney test). SYN: Synovium; ACL: Anterior Cruciate Ligament.

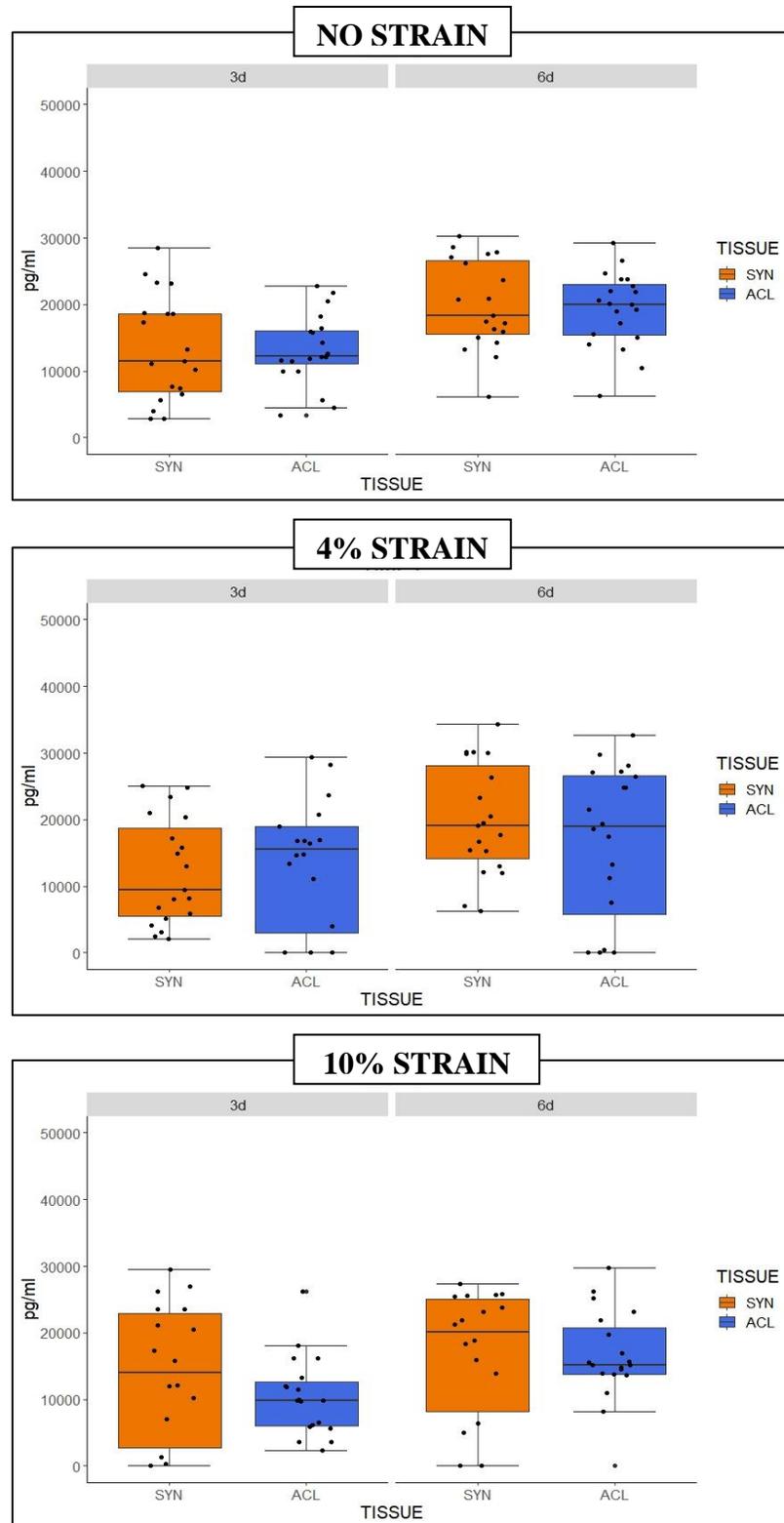


Figure 7.22. Concentration of TIMP-1 released to the media. * significant difference between TISSUES at 24 and 120 hours (Mann-Whitney test). SYN: Synovium; ACL: Anterior Cruciate Ligament.

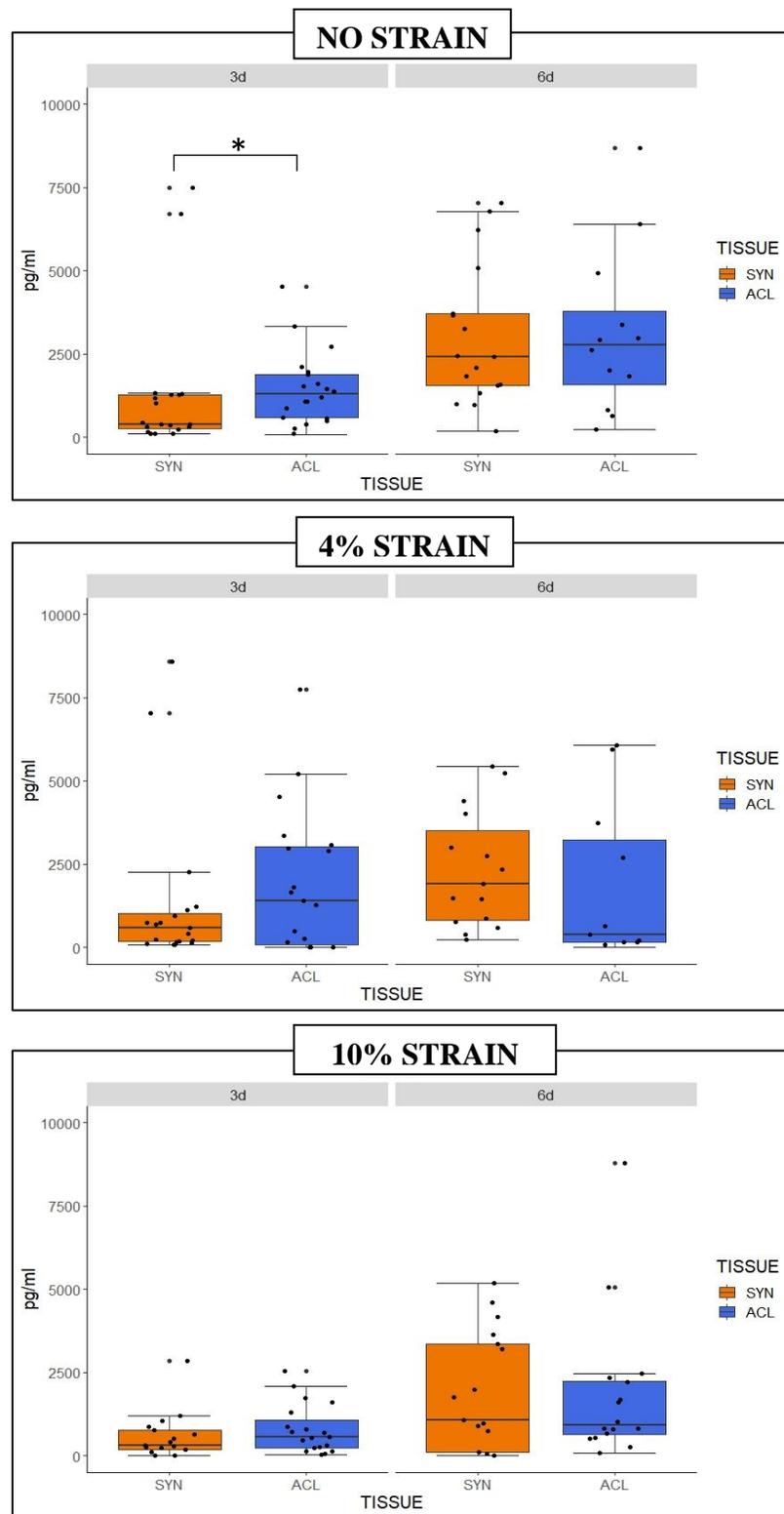


Figure 7.23. Concentration of TIMP-2 released to the media. * significant difference between TISSUES at 24 and 120 hours (Mann-Whitney test). SYN: Synovium; ACL: Anterior Cruciate Ligament.

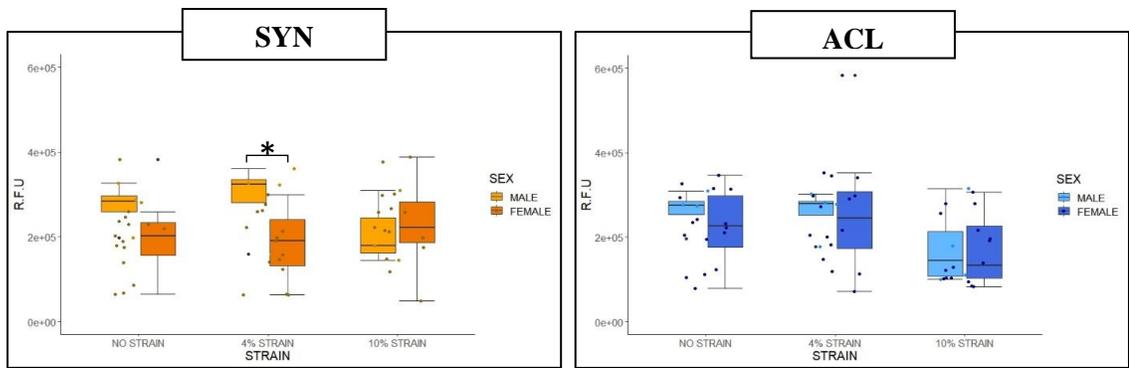


Figure 7.24. Metabolic activity of fibroblasts after 120h in culture. * significant difference between males and females (Mann-Whitney test). SYN: Synovium; ACL: Anterior Cruciate Ligament; R.F.U: Resazurin Fluorescent Units.

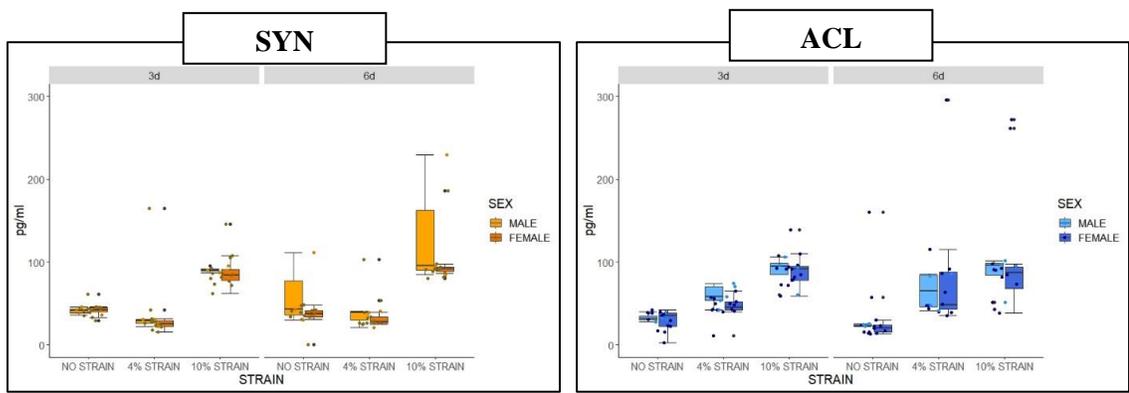


Figure 7.25. Concentration of PGE2 released to the media. * significant difference between males and females at 24 and 120 hours (Mann-Whitney test). SYN: Synovium; ACL: Anterior Cruciate Ligament

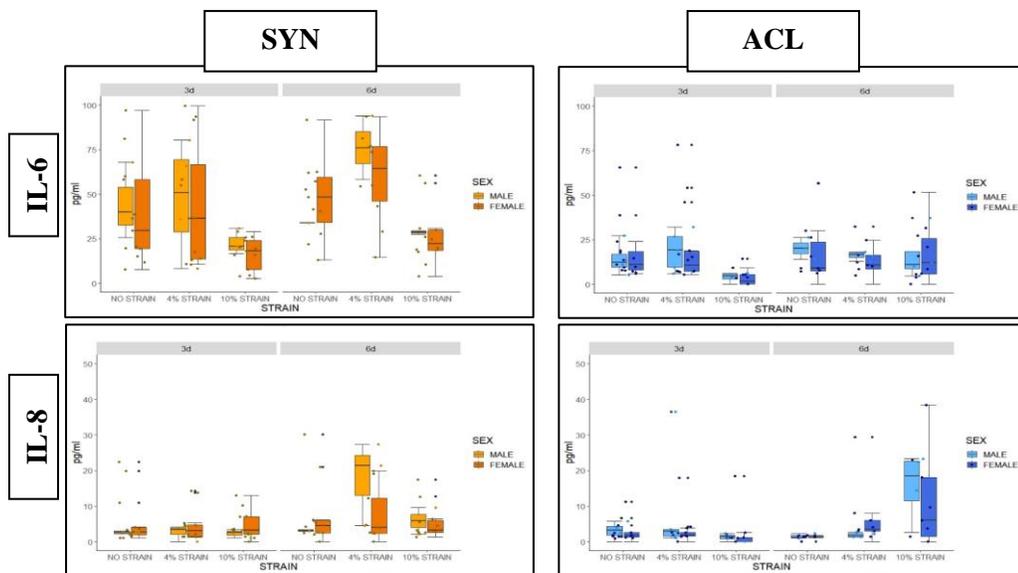


Figure 7.26. Concentration of Interleukins 6 (IL-6) and (IL-8) released to the media. * significant difference between males and females at 24 and 120 hours (Mann-Whitney test). SYN: Synovium; ACL: Anterior Cruciate Ligament

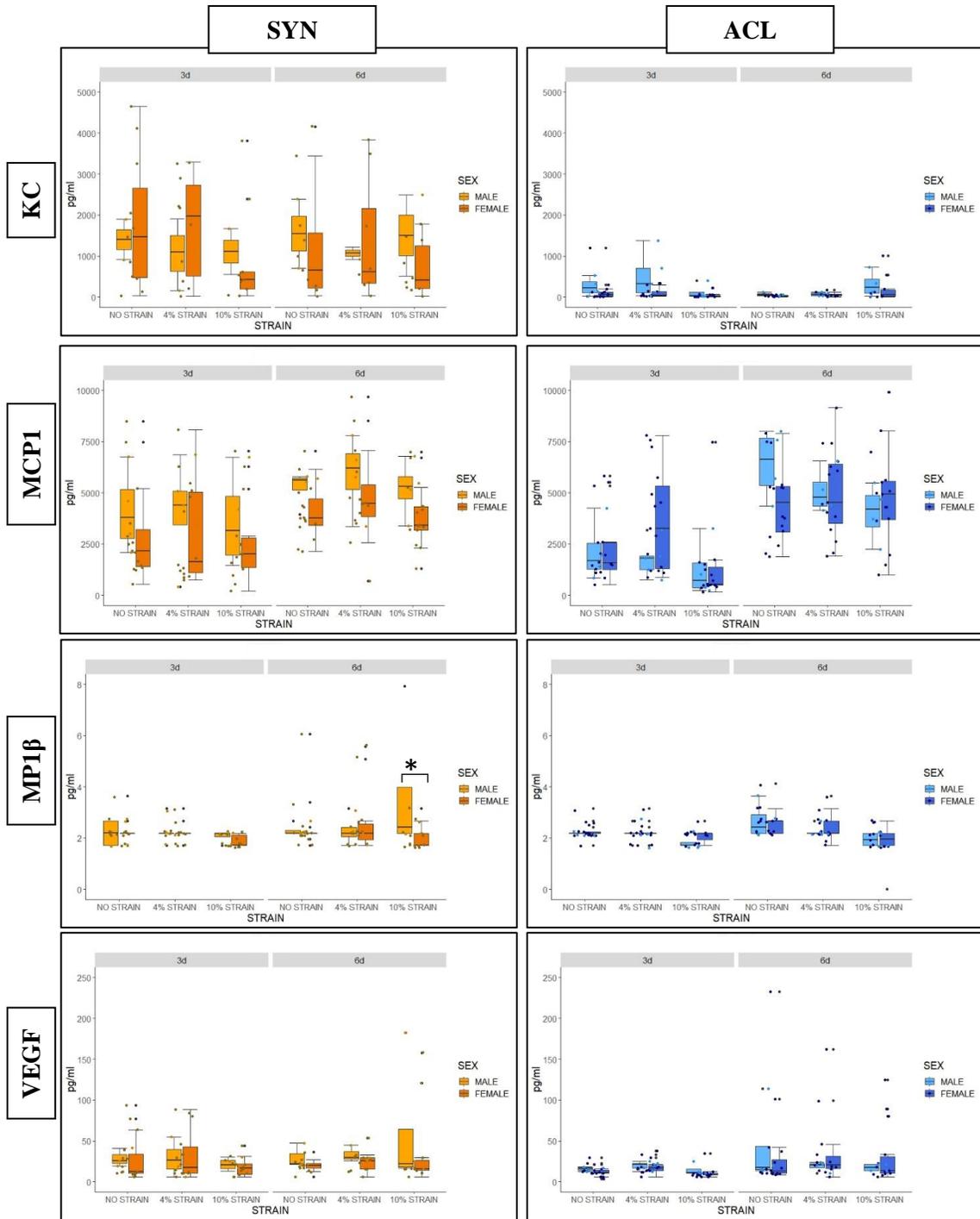


Figure 7.27. Concentration of chemokines (KC, MCP1, MP1B) and Vascular Endothelial Growth Factor (VEGF) released to the media. * significant difference between males and females at 24 and 120 hours (Mann-Whitney test). SYN: Synovium; ACL: Anterior Cruciate Ligament

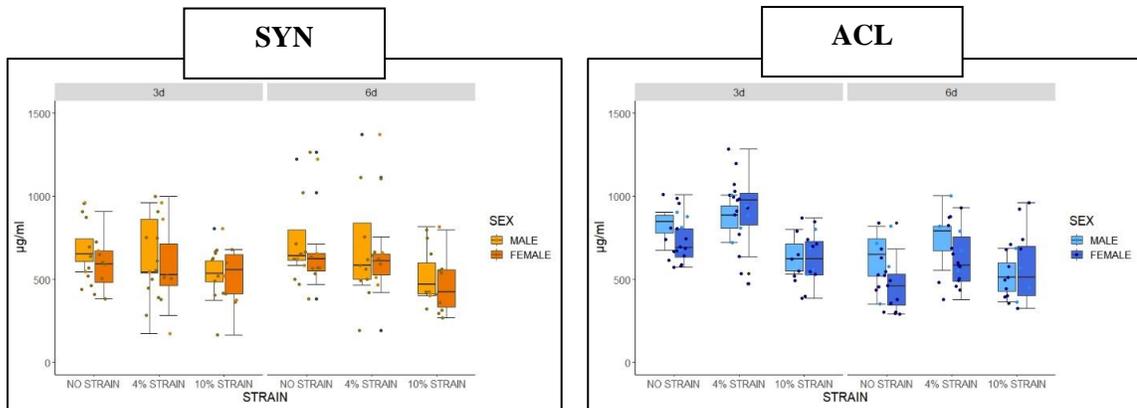


Figure 7.28. Concentration of GAG content released to the media. * significant difference between males and females at 24 and 120 hours (Mann-Whitney test). SYN: Synovium; ACL: Anterior Cruciate Ligament

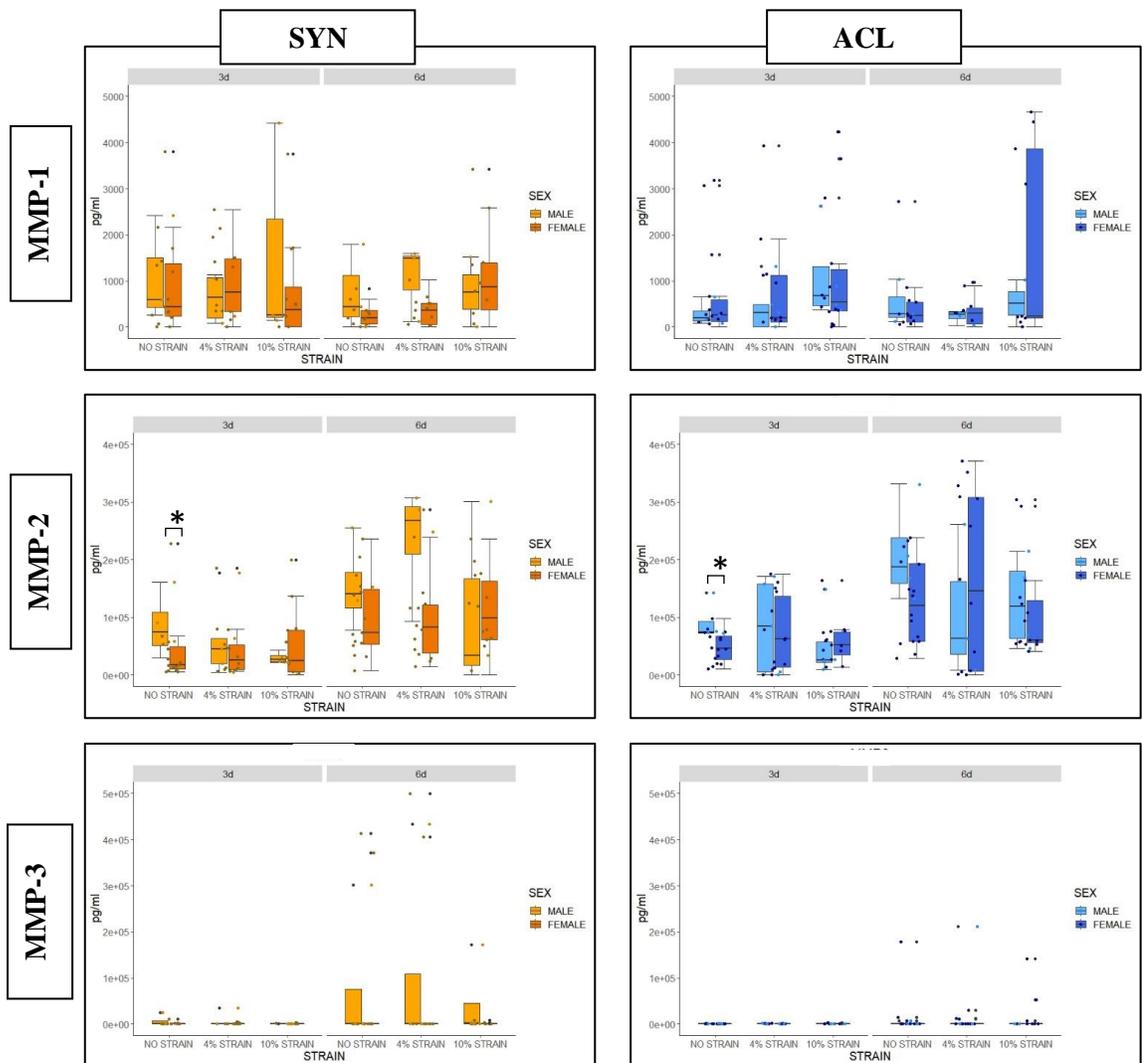


Figure 7.29. Concentration of Matrix Metalloproteinases (MMP-1, 2 and 3) released to the media. * significant difference between males and females at 24 and 120 hours (Mann-Whitney test). SYN: Synovium; ACL: Anterior Cruciate Ligament

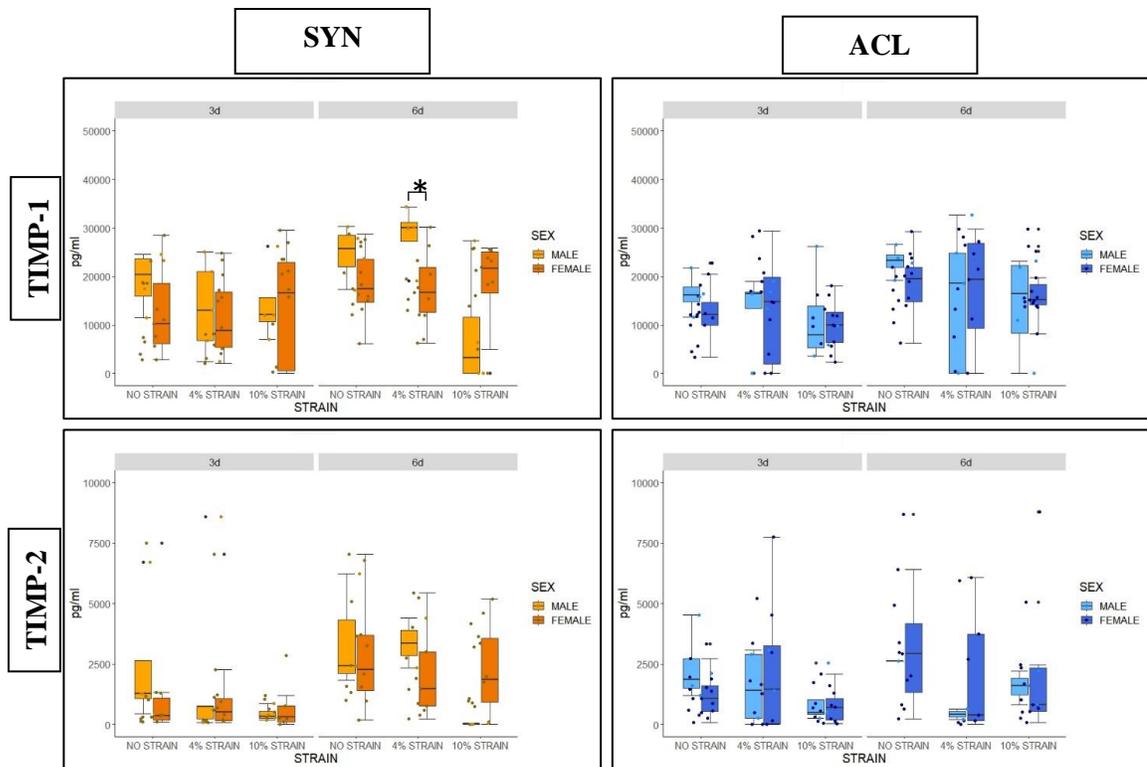


Figure 7.30. Concentration of Tissue Inhibitor of Matrix Metalloproteinases (TIMP-1 and 2) released to the media. * significant difference between males and females at 24 and 120 hours (Mann-Whitney test). SYN: Synovium; ACL: Anterior Cruciate Ligament

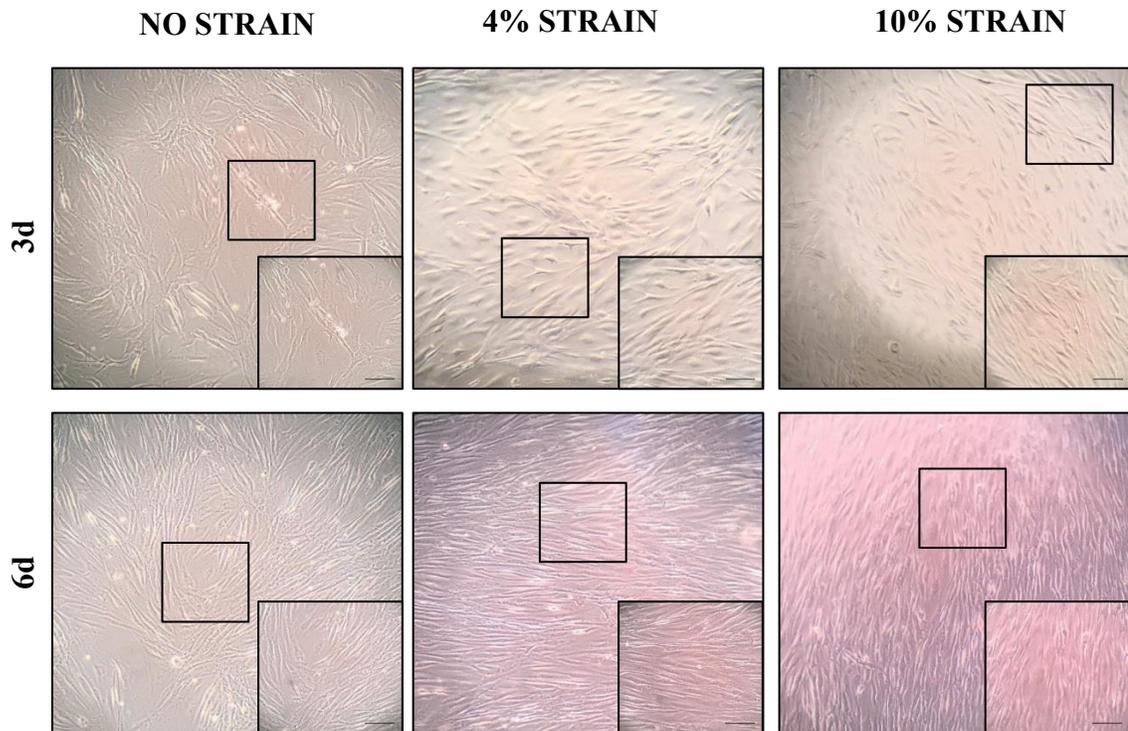


Figure 7.31. Synovium (SYN) fibroblasts stretched after three days and six days at three different strain magnitudes. Scale Bar:100 μ m

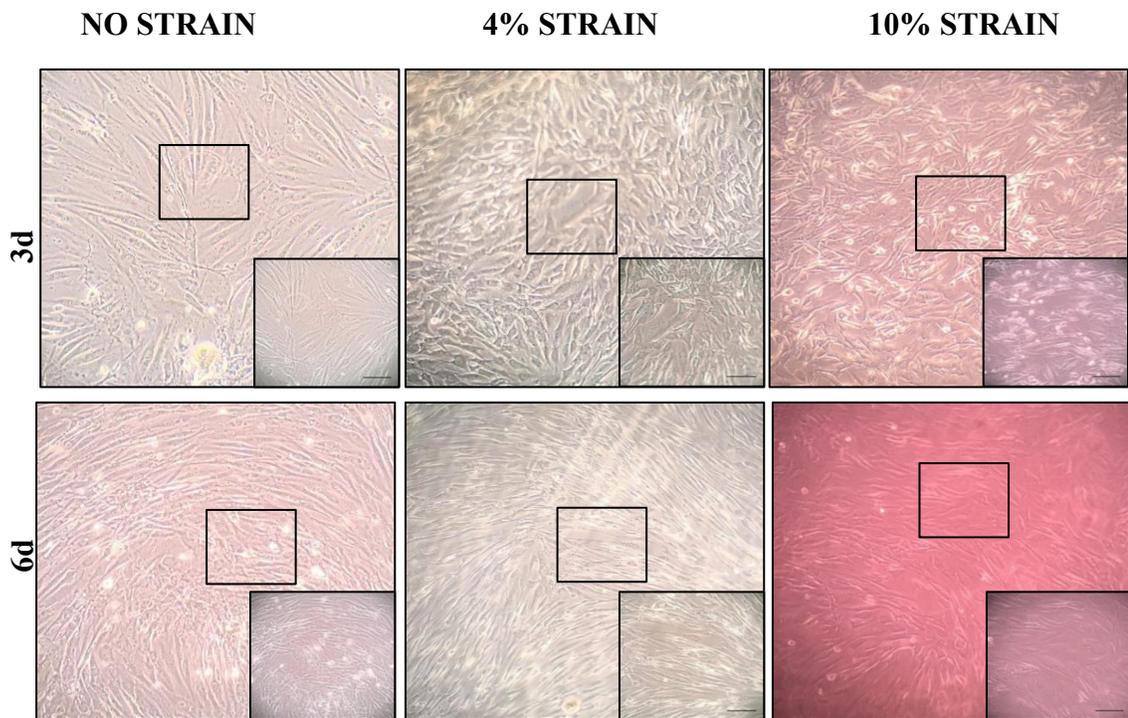


Figure 7.32. Anterior Cruciate Ligament (ACL) fibroblasts stretched after three days and six days at three different strain magnitudes. Scale Bar:100 μ m

CHAPTER 8

NORMAL HUMAN TENDON DERIVED FIBROBLASTS EXPOSED TO MECHANICAL STRAIN

Introduction

Anterior cruciate ligament (ACL) reconstruction with tendon autografts is the “gold standard” for surgical treatment of ACL injuries.¹ Common tendon graft choices include patellar tendon (PT), semitendinosus/gracilis tendon (HT), or quadriceps tendon (QT). In recent studies, the choice of which tendon graft used for ACL reconstruction has been associated with different outcomes including variable risk of developing recurrent knee instability or re-injury, osteoarthritis (OA), and harvest site morbidity such as kneeling pain.^{2,3,4} As an example, ACL reconstruction with PT have been associated with greater risk of develop post-surgical OA. This may be related to altered patellofemoral kinematics following harvest of the PT tissue. Hamstring tendon graft harvest, in comparison, have associated persistent knee flexion weakness. There are also concerns that hamstring autograft ACL reconstructions may have greater risk of laxity or re-rupture after surgery versus PT autograft in certain patient populations.⁵ Furthermore, while QT autografts appear to have better structural qualities, it has not been widely used and more studies are needed.⁶ Consequently, there is a lack of consensus regarding the most adequate biomechanical environment for any specific graft choice.

The healing of the ACL graft after reconstruction is unique since the tissue is subject to mechanical stresses during post-operative rehabilitation that play an important role in proper graft maturation after reconstruction. Dynamic cell culture *in vitro* models have

been developed to investigate the effects of mechanical forces on relevant cell populations, which may have particular relevance in the study of ACL graft tissues and tissue specific responses to the mechanical environment.⁷ Specifically, cyclic stretching of cells can affect its extracellular matrix (ECM) synthesis and remodeling⁸, potentially modifying tissue structure, composition, and function.⁹ Abnormal mechanical loading can result in injury, altering tissue homeostasis, and leading to pathological changes and potential tissue ECM failure. It is not known if there is an equivalence in biologic response of common tendon grafts if its cells were exposed to the same level of mechanical stress.

This study was designed to determine if there are differences in metabolic and inflammatory responses to mechanical stress between common tendon fibroblasts used for ACL reconstruction. Additionally, we aimed to evaluate the differences between male and female tendon fibroblasts under these tension loads. We hypothesized that strain and tendon source of ACL-graft fibroblast would significantly influence cellular metabolic responses to mechanical stress. Moreover, we hypothesized that female tendon fibroblasts will exhibit increased inflammatory and altered metabolism in response to load.

Furthermore, extracellular matrix (ECM) remodeling driven by cellular responses to load can influence disease, as well as response to treatment. The rationale for this proposal is based on previous reports and preliminary observations on canine fibroblasts, showing that an optimal level of mechanical load facilitates healing while decreasing scar tissue formation. We believe that cellular response correlates with healing status and can determine the amount of ECM produced.

Materials and Methods:

Tissue processing and culture

With IRB approval (IRB#2009879), tendon grafts (9 QT, 7 PT and 6 HT) were harvested from patients (11 males and 11 females, age range 14-43) at the time of anterior cruciate ligament reconstruction (Table 8.1, Fig.8.1). This tissue is typically removed and discarded following the surgical procedure. Tissues were aseptically minced into 0.5–1.0 cm² pieces and digested for two hours in Type 1A *Clostridium histolyticum* collagenase solution (Sigma-Aldrich, St. Louis, MO, USA) at a concentration of 0.2 mg/mL. The aliquot was then suspended with nutritional media (Gibco DMEM, Thermo Fisher Scientific, Waltham, MA, USA) containing 10% FBS, 0.002% Penicillin, 100 µg/mL Streptomycin, 25 µg/mL Amphotericin B, 0.002% L-Ascorbate and 0.01% L-glutamine (Sigma Chemical Co, St. Louis, MO), cultured in T25 flasks (passage zero) and T75 flasks (passage one) (TPP Techno Plastic Products AG, Trasadingen, Switzerland) and incubated at 5% CO₂, 37°C, and 95% humidity. Once confluent, passage one cells were stored at -80°C until analysis.

Biaxial mechanical stress

Passage one cells were re-expanded in T75 flasks in media described above and when confluent, exposed to TrypLE express (Invitrogen, Carlsbad, CA) for five minutes, then seeded (1×10^5 cells/well) on Collagen Type I-coated BioFlex® plates (Flexcell International, NC, USA). Cells were incubated for an additional 48h in 10% FBS nutritional media described before strain was applied. Before fibroblasts were subjected to continuous mechanical stimulation media was changed to 2% FBS to keep cells alive for six days (120h).¹⁰ A tension load (2-s strain and 10-s relaxation at a 0.5 Hz frequency) with a biaxial sinusoidal waveform with three different elongation strains was applied for

6 days using the Flexcell FX-4000T strain system (Flexcell International, NC, USA). The strain magnitude were chosen to approximate 3 different strain levels that may be experienced by the graft tissue during the post-operative healing process after ACL reconstruction, immobilization or mechanical stress deprivation-0%, physiologic strain-4%, and supraphysiologic strain-10%.⁷ Media was changed at day three and day six and stored at -20°C until analysis.

Biomarker assays

Media was changed every three days and stored at -20°C until analysis. Media from three (3d) and six (6d) days of culture were assessed for various biomarkers. Proteoglycan (GAG) was assessed using the DMMB assay as previously described.¹¹ The concentration of prostaglandin E₂ (PGE₂) (Cayman Chemical, Ann Arbor, MI, USA), cytokines (IL-6, IL-8, KC, MCP-1 and VEGF) (Millipore, Billerica, MA, USA), total matrix metalloproteinase (MMP) activity (SensoLyte 520 generic MMP assay, Anaspec, Inc., Fremont, CA), and MMPs and TIMPs production (MMP-1, MMP-2, MMP-3, TIMP 1 and TIMP 2) (R&D Systems, Minneapolis, MN, USA), were all assessed using commercially available assays according to the manufacturer's protocol. At the end of the strain protocol (6d), cell viability analysis was performed using resazurin assay (Sigma Aldrich, Saint Louis, MO).

Statistical Analysis

For statistical analysis, normal distribution was assessed by Shapiro-Wilk test. Because data were not normally distributed, non-parametric analyses were used. Comparisons among strains and tissue types were performed using Kruskal-Wallis test and Dunn's test posthoc. Additionally, comparisons between males and females were performed using

Mann-Whitney U test (R Core Team (2019), R version 3.6.2 Vienna, Austria). Results were reported as median± interquartile range (IQR). Significance levels will be set at $p < 0.05$.

Results

Differences in Graft Tendon Fibroblasts to Mechanical Strain

The metabolic activity was significantly higher in HT fibroblasts exposed to physiological strain compared to supraphysiological strain at day 6 of culture. There were no significant differences in metabolism of QT or PT fibroblasts in response to strain level applied at any timepoint (Fig.8.2).

Differences in inflammatory mediators released to the media were also seen as a function of strain level and tissue source. The production of PGE2 by QT and PT fibroblasts was significantly higher during mechanical strain deprivation compared to physiological strain at day 3 of culture. In contrast, the production of PGE2 by HT fibroblasts was significantly higher at supraphysiological strain levels relative to physiological strain levels at day 6 of culture (Fig. 8.3).

The production of IL-6 (Fig. 8.4) and IL-8 (Fig. 8.5) by PT fibroblasts was significantly lower when subjected to supraphysiological strain levels compared to physiological strain and mechanical strain deprivation at day 3 of culture. Significant differences in IL-6 and IL-8 production were not observed for HT or QT fibroblasts based on strain level at either time point.

Cellular chemoattractant levels also fluctuated depending on tissue type and strain levels. The production of KC by QT fibroblasts was significantly increased when subjected to physiological strains compared to supraphysiological strain at day 3 of culture (Fig. 8.6).

The production of MCP-1 did not show differences among different strain magnitudes in any of the tissue fibroblasts at any timepoint (Fig. 8.7). The production of MIP-1 β was significantly higher in HT fibroblasts subjected to mechanical strain deprivation compared to supraphysiological strain at day 3 of culture (Fig. 8.8). Moreover, VEGF was not statistically different in any tendon fibroblasts at any strain at either 3d or 6d (Fig. 8.9).

The production of GAG (Fig.8.10) and MMP-1 (Fig. 8.11) by the tendon fibroblasts were not significantly different in response to strain in any of the tendon fibroblasts at any timepoint. However, the production of MMP-2 by HT fibroblasts was significantly lower at supraphysiological strain compared to both, mechanical strain deprivation and physiological strain levels (Fig. 8. 12). The production of MMP-3 by PT fibroblasts was significantly higher during mechanical strain deprivation compared to supraphysiological strain at day 3, but not at day 6 of culture (Fig. 8.13). Moreover, the production of TIMP-1 by PT fibroblasts was significantly lower at supraphysiological strain levels compared to mechanical strain deprivation and physiological strain at day 3 of culture (Fig.8.14). Further, the production of TIMP-2 by PT fibroblasts was significantly lower at supraphysiological strain levels compared to mechanical strain deprivation and physiological strain levels at day 3 and day 6 of culture, respectively (Fig. 8.15).

Differences between QT, PT and HT Fibroblast Response to Mechanical Strain

The level of metabolic activity for HT fibroblasts was significantly higher than QT fibroblasts at mechanical stress deprivation and physiological strain levels, but not at supraphysiological strain. Additionally, the level of metabolic activity for HT fibroblasts

was significantly higher than PT fibroblasts in the mechanical strain deprivation group (Fig. 8.16).

The production of PGE2 by HT fibroblasts was significantly lower than PT and QT fibroblasts at all strain levels and time points tested (Fig. 8.17). Moreover, the production of IL-6 by HT fibroblasts was significantly higher than PT fibroblasts at supraphysiological strain on day 3 of culture (Fig. 8.18). Additionally, the production of IL-8 by PT fibroblasts was significantly lower than QT and HT fibroblasts at supraphysiological strain levels on day 3 of culture (Fig.8.19). The production of KC by HT fibroblasts was significantly lower than PT and QT fibroblasts at day 6 of culture at all strain levels. Further, the production of KC by HT fibroblasts was significantly lower than QT fibroblasts at day 3 of culture at physiological and supraphysiological strain levels (Fig.8.20). The production of MIP-1 β by HT fibroblasts was significantly lower than PT and QT fibroblasts at all strain levels and timepoints at days 3 and 6 of culture (Fig.8.21). The production of MCP-1 and VEGF were not significantly different between tissue types at all strain levels and days of culture analyzed (Fig.8.22 and 8.23 respectively).

The production of GAG by HT fibroblasts was significantly higher than PT and QT fibroblasts in the mechanical strain deprivation group on day 6 of culture (Fig.8.24). Moreover, the production of MMP-1 by QT fibroblasts was significantly higher than PT and HT fibroblasts at mechanical strain deprivation and physiological strain levels on day 3 of culture (Fig.8.25). The production of MMP-2 was not significantly different between tissue types at all strain levels and days of culture analyzed (Fig.8.26). Additionally, the production of MMP-3 by PT fibroblasts was significantly lower than QT fibroblasts at

physiological and supraphysiological strain levels on day 3 of culture (Fig.8.27). The production of TIMP-1 by PT fibroblasts was significantly higher than fibroblasts in the mechanical strain deprivation group on day 3 of culture. However, the production of TIMP-1 was not significantly different between tissue types at physiological and supraphysiological strain levels for any of the days of culture analyzed (Fig. 8.28). Additionally, the production of TIMP-2 was not significantly different between tissue types at all strain levels and days of culture analyzed (Fig.8.29).

When evaluating sex differences, we found that the metabolic activity of female QT fibroblasts was significantly higher than male QT fibroblasts at supraphysiological strain (Fig. 8.30). However, there were no other statistically significant differences between male and female fibroblasts at any strain level or timepoint tested in this study (Fig.8.31-8.43). There were some trends toward a difference between male and female PT fibroblasts for IL-6, MMP-1 and MMP-2 production, but these differences were not statistically different (Fig. 8.32, 8.39 and 8.40 respectively).

Discussion

It is known that mechanical stimulation plays a key role in normal or altered tendon and ligament development and maturation.¹² In the present study, we were able to demonstrate that tendon fibroblasts from grafting tissue respond differentially to varying levels of biaxial cyclic strain. Moreover, these differences were unique dependent on fibroblast tissue source. PT fibroblasts were more sensitive to changes in strain levels as demonstrated by more frequent differences within fibroblast from this tendon, especially in response to extremes loads such as mechanical stress deprivation and high strain levels.

Successful healing of the tendon graft after ACL reconstruction involves the biological processes that occur during graft necrosis, remodeling, maturation, and incorporation. Fibroblasts are dispersed within the tendon extracellular matrix and are the main resident cells of common tendons including patellar, quadriceps, and hamstring tendons.^{13; 14} While these cells are integral to production of extracellular matrix, particularly during the response to injury and repair, their role in the ligamentization process after reconstruction is largely not known. There is limited information on metabolism and biosynthesis differences between fibroblasts of various tendon tissues when subjected to mechanical strain.

For this study *in vitro* strains related to clinical scenarios commonly found in ACL reconstruction and tendon pathology were analyzed. It is known that normal tendon and ligament strain are in the order of 2-4%.^{15; 16} Moreover, strains higher than 8% are associated with microscopic fiber damage and altered material properties.^{7; 15; 16} Since the amount of strain applied to the graft after ACL reconstruction *in vivo* is still not fully understood, current approach is valid to evaluate main responses to strain.

In our study, we demonstrated that fibroblasts from different tendons, not only differed in their production of extracellular matrix, remodeling and inflammatory mediators, but this difference was dependent on level of strain experienced by the cell. Since the cellular density is also vastly different among grafts¹⁷, a relation between graft fibroblasts and the success of biological graft healing is likely present as these differences may be amplified with differences in density of cells within a given tissue.

Since PGE2 significantly changed within different tendon fibroblasts in response to changing strain levels in our experiment, it appears that PGE2 may be an important

mechanosensitive marker. The concentration of PGE2 was consistently increased at strain levels outside of physiologic levels. It is possible that extreme strain levels stimulate an inflammatory cascade and that PGE2 overproduction is an early event for tissue repair.¹⁸ Likewise, the significant increase of interleukins in PT fibroblasts at subnormal strain levels, may indicate that this type of cell requires a minimum of cytokine production, given by physiological strain, and that abnormal strains may facilitate inflammatory signaling in this type of graft.

Decreased MMP-2 concentration correlates with improved mechanical properties of the reconstructed graft.¹⁹ In this work, MMP-2 production was significantly lower in HT fibroblasts after 3 days of supraphysiological strain compared to mechanical strain deprivation and physiological strain. Similarly, MMP-3 production was significantly lower in PT fibroblasts after 3 days of supraphysiological strain compared to mechanical strain deprivation. Clinically, knee immobilization when using soft tissue grafts for ACL reconstruction intended to restrict motion will supposedly protect the recently operated knee from high strains.²⁰ However, our results indicate that mechanical strain deprivation may increase MMP production when using PT and HT grafts with potentially increased risk of graft weakness and failure.

The application of tension load to the reconstructed graft seem to increase tensile strength and to avoid adhesions formation.¹⁵ However, supraphysiological strain is believed to be detrimental for tendon-to-bone healing¹². Interestingly, our results showed a consistent decrease in cytokine (IL-6, IL-8) and TIMPs production (TIMP-1 and TIMP-2) in PT fibroblasts at day 3 of culture with supraphysiological strain. These findings might suggest that PT graft could indeed benefit from early mobilization at high strains as

reported *in vivo* and that that motion applied to the reconstructed knee may be more beneficial than immobilization.²⁰⁻²²

Differences between male and female fibroblasts were also evaluated in this work.

Although there were not many sex differences exhibited by tendon fibroblasts in response to strain, there was an increased metabolic activity of female QT fibroblasts compared to males. Since this measure is directly related to the number of cells in culture, it is possible that female QT grafts tend to increase cellularity when high loads are applied. Moreover, considering that cell necrosis of the graft is a main event in early postoperative period, we believe that this may be neutralized by adjusting the proper postoperative physical therapy to each specific graft.¹⁹

Curiously, the lack of differential responses between male and female fibroblasts to any type of strain may be an example of the simplicity of our *in vitro* model. Since many of the sex differences in ACL injury are due to neuromuscular control and specific anatomical features, it is not surprising that significant differences between fibroblasts based on sex were not observed during *in vitro* monolayer culture without these other factors included.^{23; 24} Additionally, the low sample size and high variability in this study may be responsible for the trends observed in different biomarkers such as IL-6, MMP-1 and MMP-2, especially in QT and PT fibroblasts. Hence, including a larger sample size may allow for the identification of significant differences in the metabolism of male and female fibroblast metabolism with and without applied strain.

Taken together, results of present study deepen the understanding of the effect of different clinical scenarios where strain applied to the reconstructed ACL graft drives essential biochemical changes. Moreover, this work highlights specific needs in the graft

healing and ligamentization process such as standardization of physical therapy for each specific graft type. Since many of our results are consistent with literature reports such as increased failure in HT grafts and increased OA in PT grafts, we could potentially use this work to understand biological processes that have an evident gap in knowledge. Importantly, the lack of consensus about the perioperative activity recommended for the patient that undergoes ACL reconstruction should draw the attention of physician-scientists to explore the mechanobiology of tendon grafts to diminish ACL reconstruction failures and revisions.

Since, fibroblasts within the tendon graft respond to mechanical stress by altering the ECM that surrounds them, we believe that modifying the mechanical environment by applying an adequate biaxial stress to the cellular component of the graft, will result in improved biological and structural properties of the tissues after ACL reconstruction. Once again, PT showed decreased production of TIMP-1 and TIMP-2 at high strains. Moreover, an increased production of TIMP-2 at physiological levels of load in PT after 6 days of stretch suggest that controlled motion to the graft is required to regulate excessive remodeling within the knee after ACL reconstruction.²⁵

As with any study there are inherent limitations to consider when interpreting the data. This was an *in vitro* study utilizing monolayer cell culture without the other biological and mechanical factors that are present *in vivo*. Additionally, since the *in vitro* system utilized in this work only allows for equi-biaxial strain, *in vivo* replication of hetero-biaxial strain (more tension to the longitudinal axis than the transverse axis) cannot be evaluated. Furthermore, 3D cultures that allow for a longer time period of evaluation will be interesting to see if biomarker production is maintained across time. Finally, the

samples size for this study was low, and could be responsible of the lack of significant differences seen when analyzing for sex differences. It could be interesting to see if sex plays a role in cellular metabolic responses to strain by increasing sample size.

In conclusion, this study demonstrated that human tendon fibroblasts that are used for ACL reconstruction respond differently to changing mechanical environment. These findings may have applicability given outcomes of ACL reconstruction in certain patient populations seems to be impacted by graft source and selection. This study suggests that early biological differences in fibroblast cellular protein expression between graft source may be one explanation for fundamental differences in clinical outcomes as it relates to graft selection in ACL reconstruction. Therefore, the clinician may need to consider graft type and sex to adjust physical therapy to reduce risk of failure for athletes. In this work we have provided an initial approach to evaluate specific cellular responses within a specific clinical context. Future work should include the evaluation of graft fibroblasts along with other cell types (co-culture) including synovium and cruciate ligamentocytes to replicate the *in vivo* multi-tissue environment. Ongoing studies in our laboratory are aimed at evaluating the metabolic response of graft fibroblasts co-cultured with various cell types and assess whole-tissue culture systems to better replicate the *in vivo* environment that the tenocyte experiences.

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Tables

Graft source		Sex	Age Range	
QT	9	Males	4	17-38
		Females	5	14-25
PT	7	Males	4	17-35
		Females	3	15-21
HT	6	Males	3	18-25
		Females	3	17-43

Table 8.1. Patient sex and age distribution by graft source

Figures

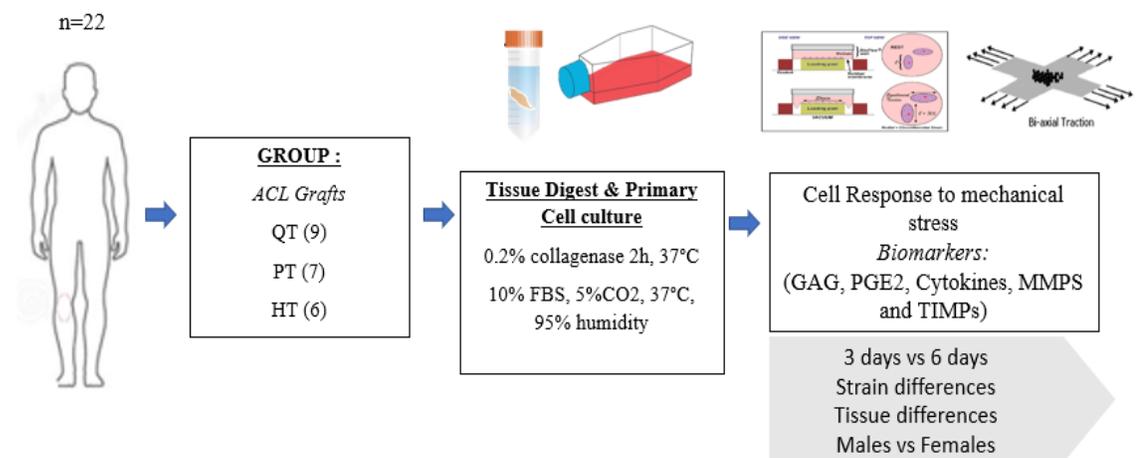


Figure 8.1. Experimental design

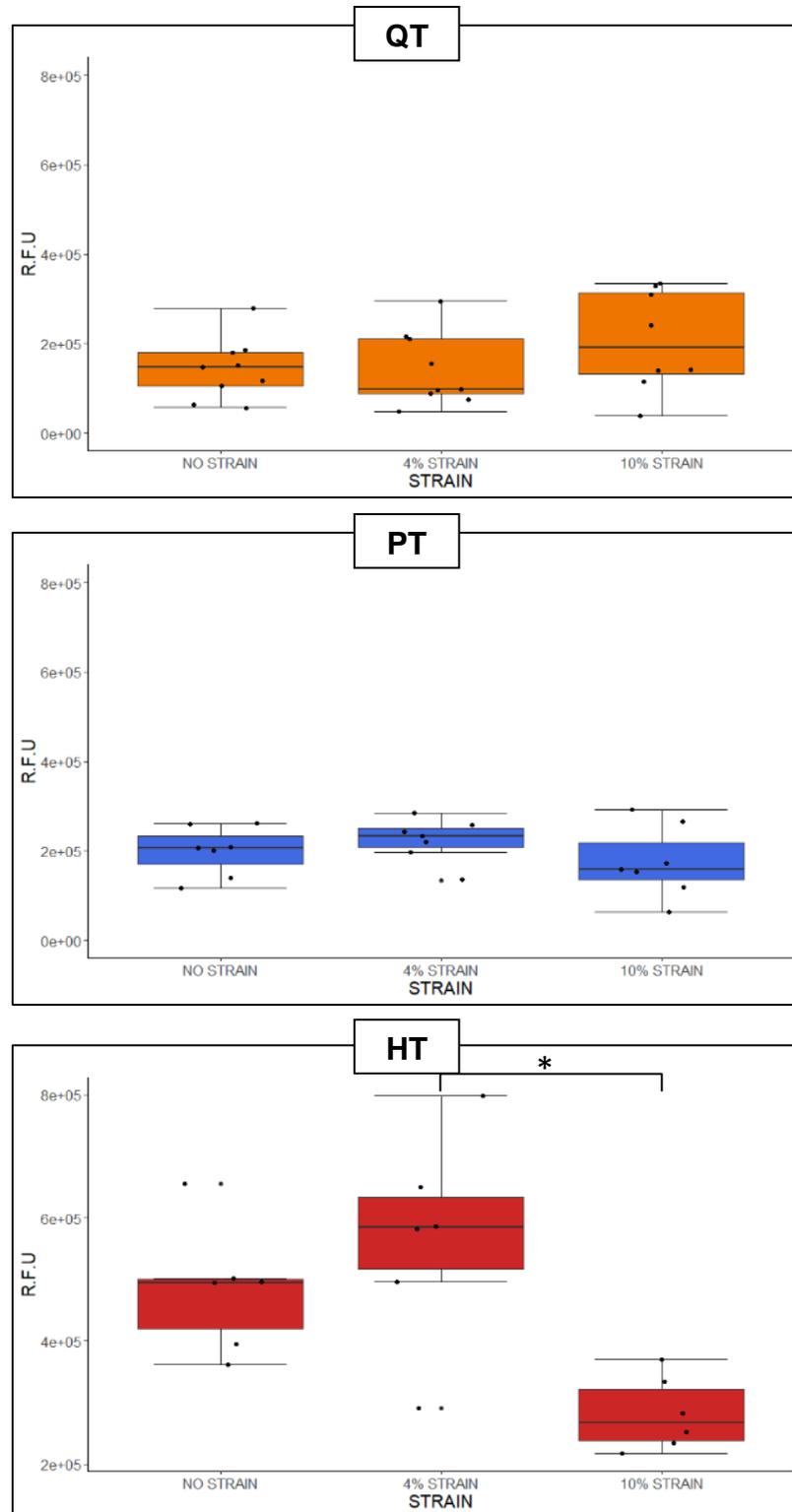


Figure 8.2. Metabolic activity of fibroblasts after six days of culture. * significant difference between STRAINS. QT: Quadriceps tendon; PT: Patellar tendon; HT: Hamstring tendon. R.F.U: Resazurin Fluorescent Units

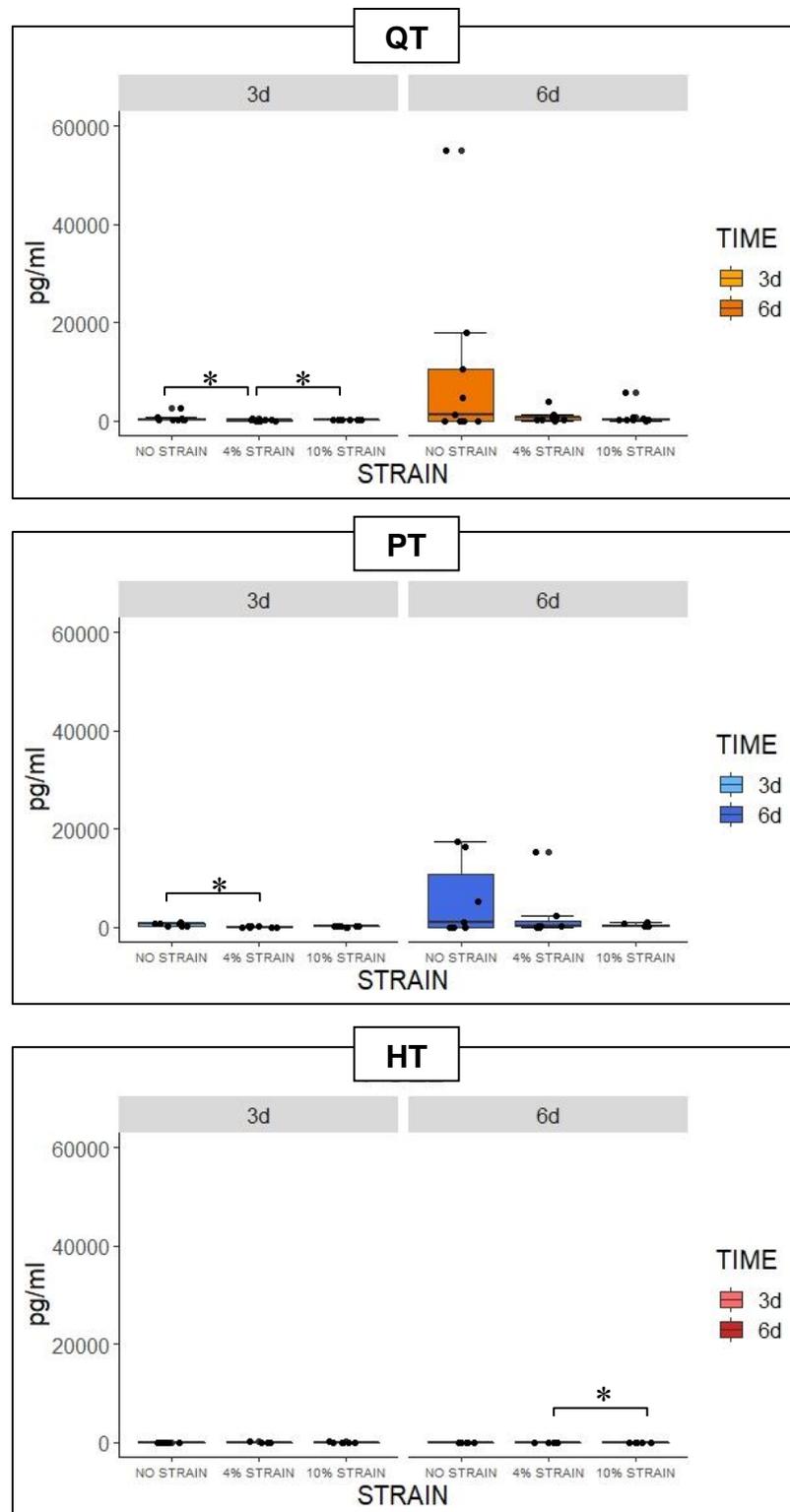


Figure 8.3. Concentration of PGE2 released to the media. * significant difference between STRAINS at three (3d) and six (6d) days of culture. QT: Quadriceps tendon; PT: Patellar tendon; HT: Hamstring tendon.

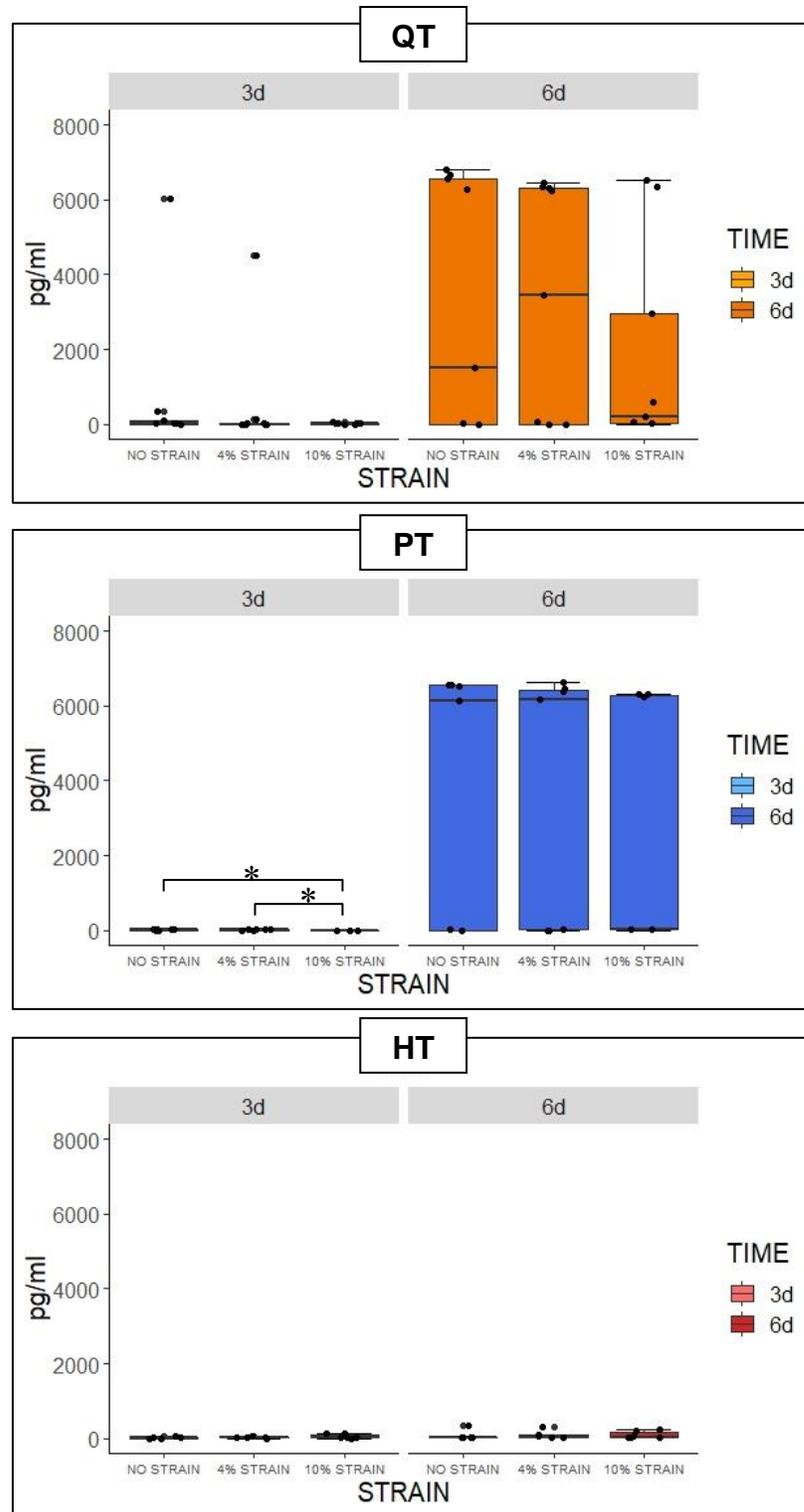


Figure 8.4. Concentration of IL-6 released to the media. * significant difference between STRAINS at three (3d) and six (6d) days of culture. QT: Quadriceps tendon; PT: Patellar tendon; HT: Hamstring tendon.

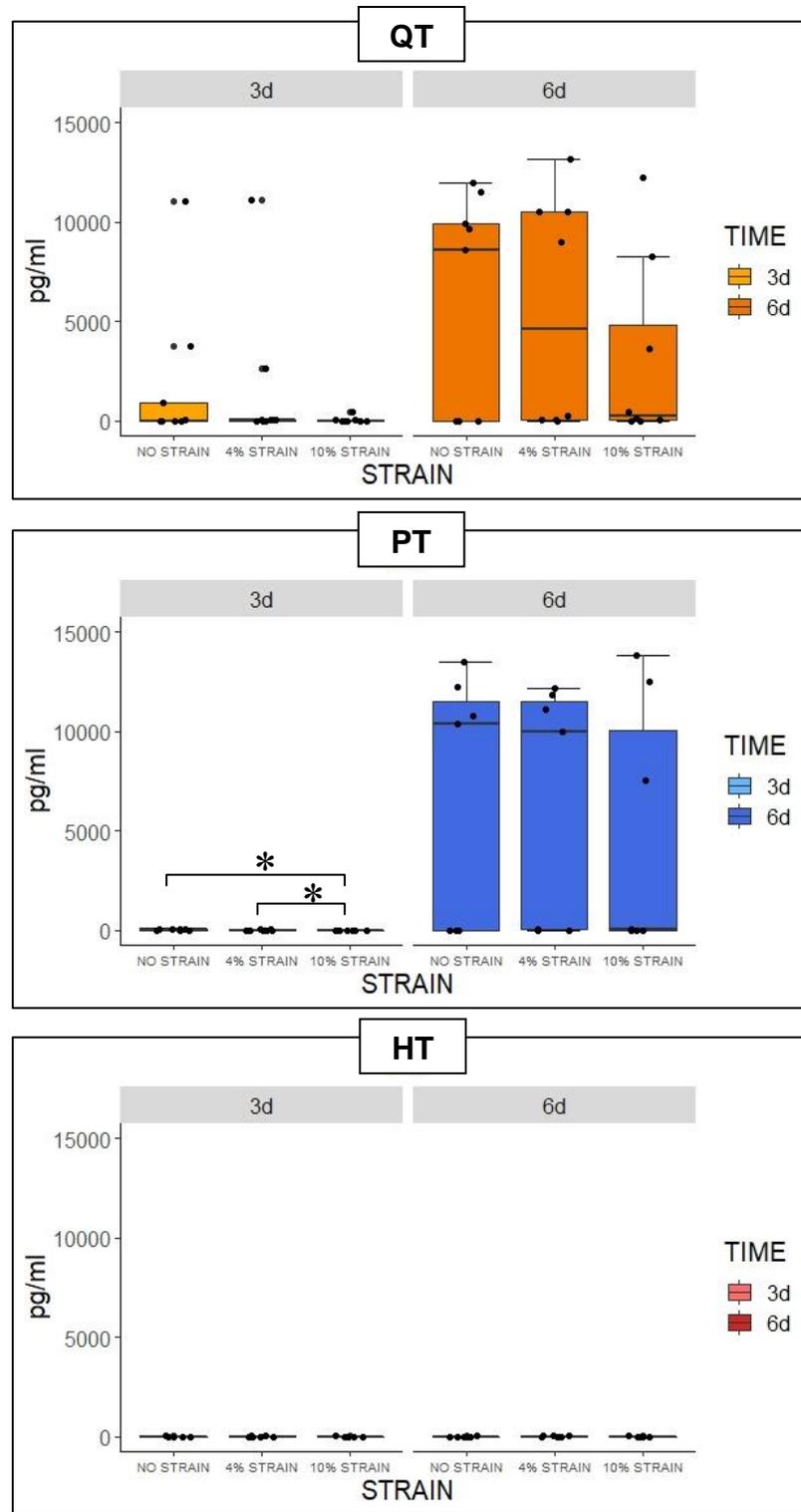


Figure 8.5. Concentration of IL-8 released to the media. * significant difference between STRAINS at three (3d) and six (6d) days of culture. QT: Quadriceps tendon; PT: Patellar tendon; HT: Hamstring tendon.

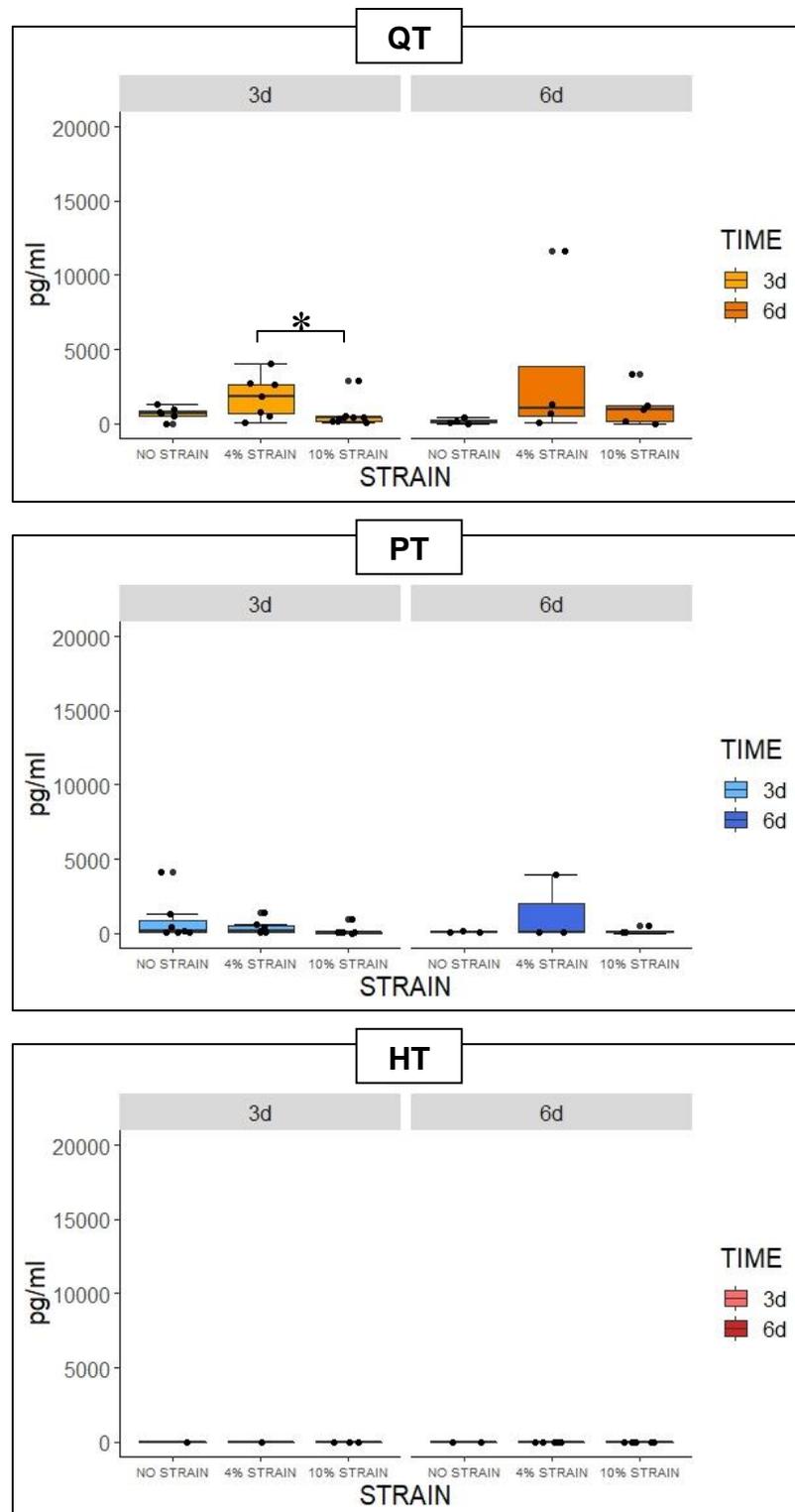


Figure 8.6. Concentration of KC released to the media. * significant difference between STRAINS at three (3d) and six (6d) days of culture. QT: Quadriceps tendon; PT: Patellar tendon; HT: Hamstring tendon.

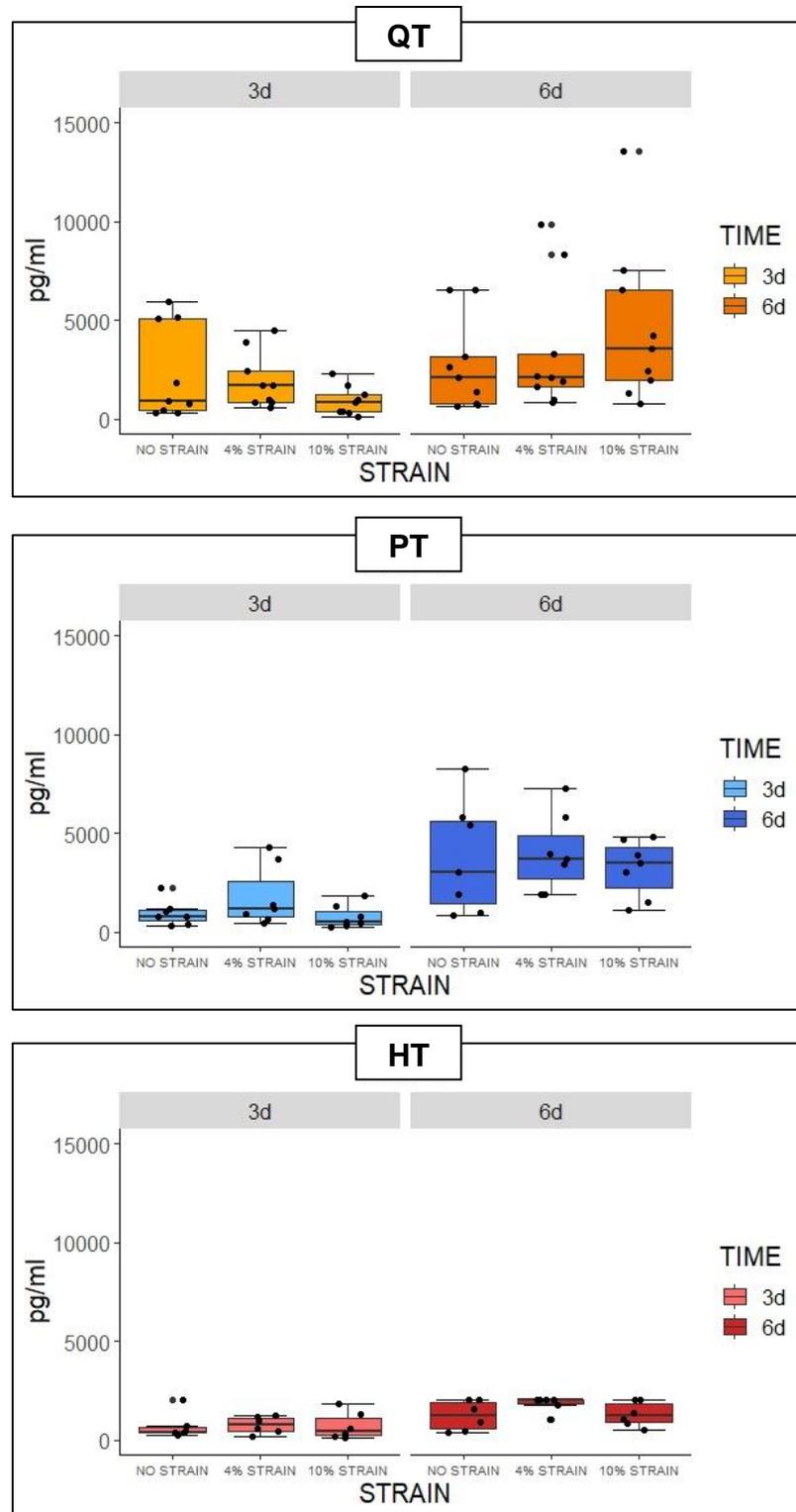


Figure 8.7. Concentration of MCP1 released to the media. * significant difference between STRAINS at three (3d) and six (6d) days of culture. QT: Quadriceps tendon; PT: Patellar tendon; HT: Hamstring tendon.

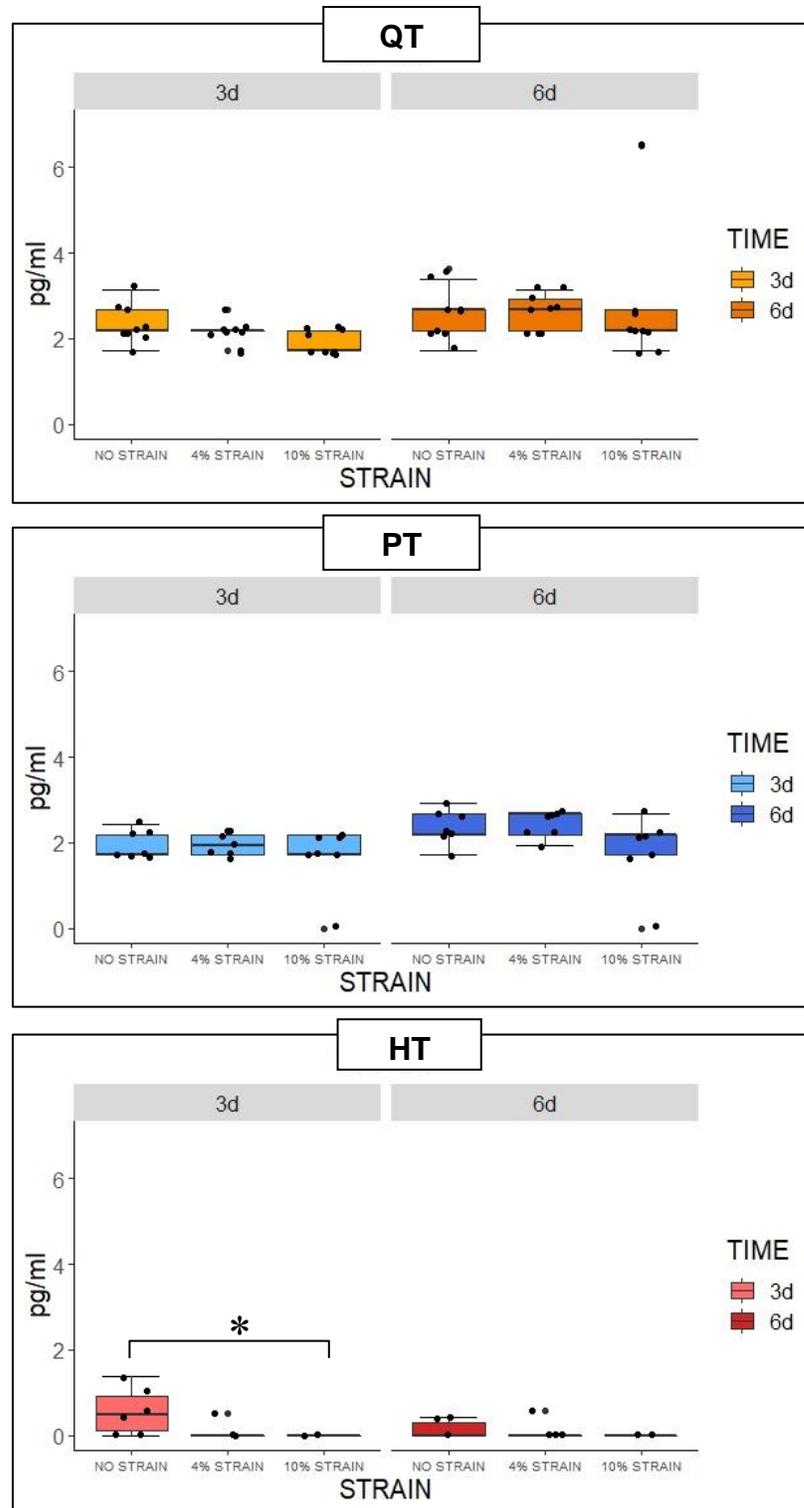


Figure 8.8. Concentration of MP1 β released to the media. * significant difference between STRAINS at three (3d) and six (6d) days of culture. QT: Quadriceps tendon; PT: Patellar tendon; HT: Hamstring tendon.

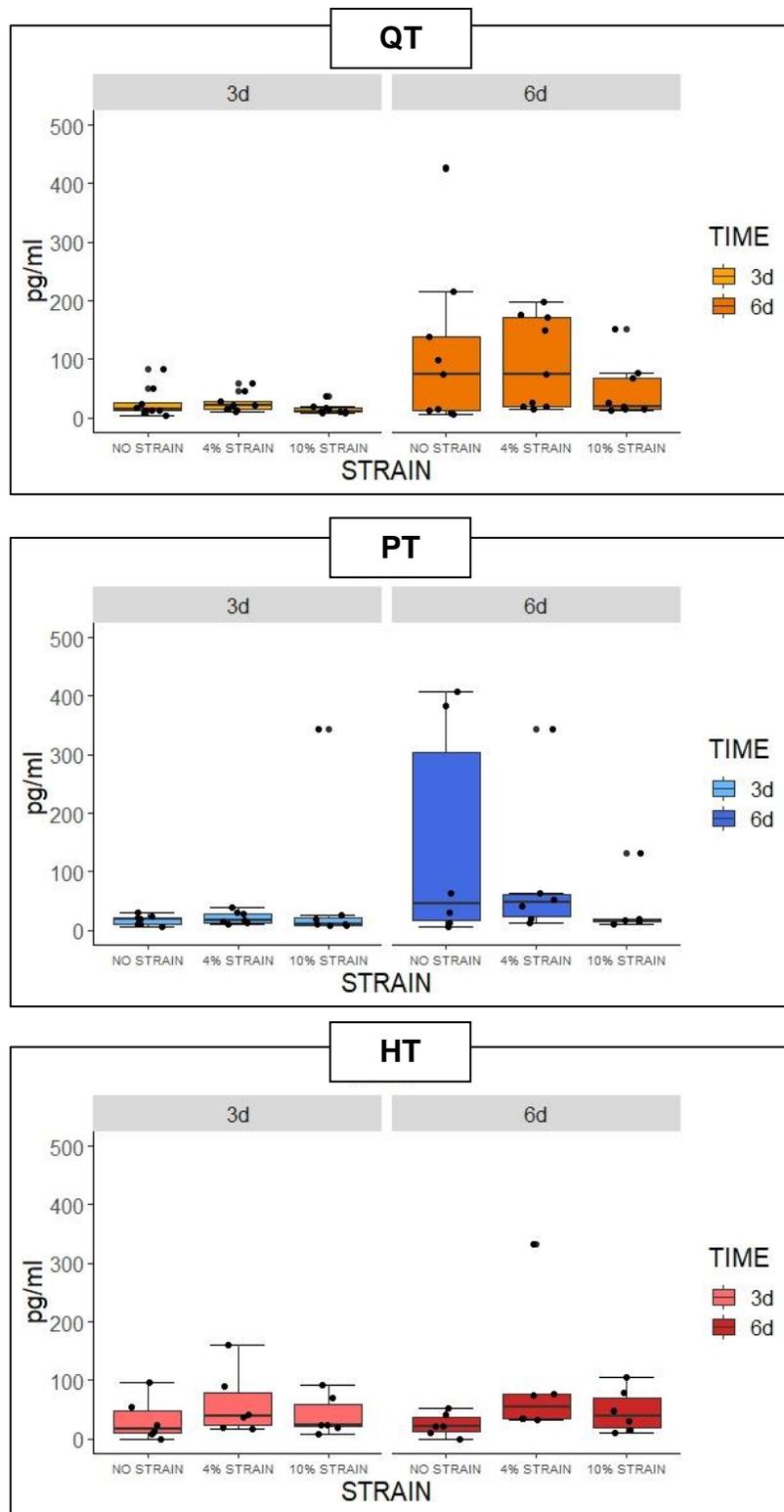


Figure 8.9. Concentration of VEGF released to the media. * significant difference between STRAINS at three (3d) and six (6d) days of culture. QT: Quadriceps tendon; PT: Patellar tendon; HT: Hamstring tendon.

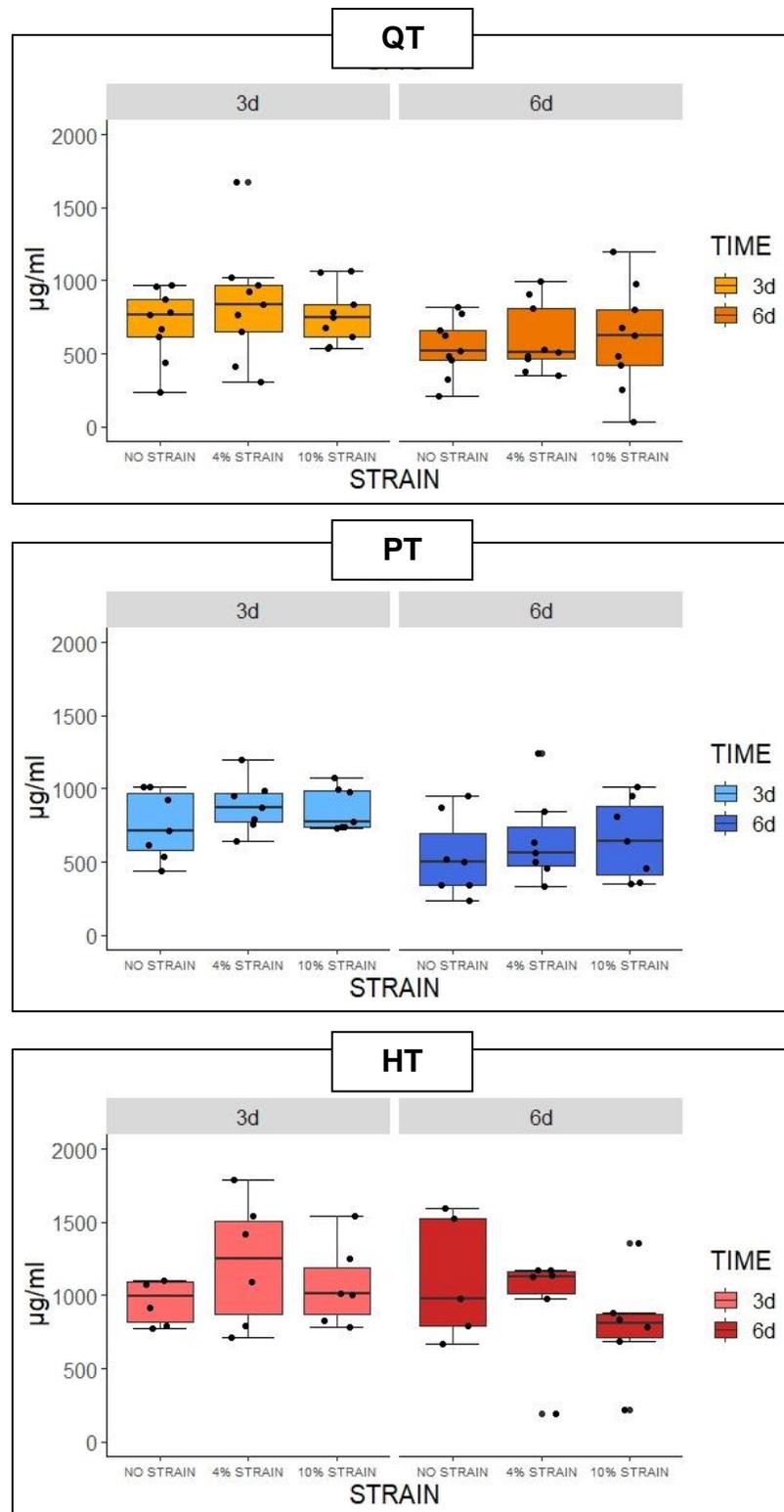


Figure 8.10. Concentration of GAG released to the media. * significant difference between STRAINS at three (3d) and six (6d) days of culture. QT: Quadriceps tendon; PT: Patellar tendon; HT: Hamstring tendon.

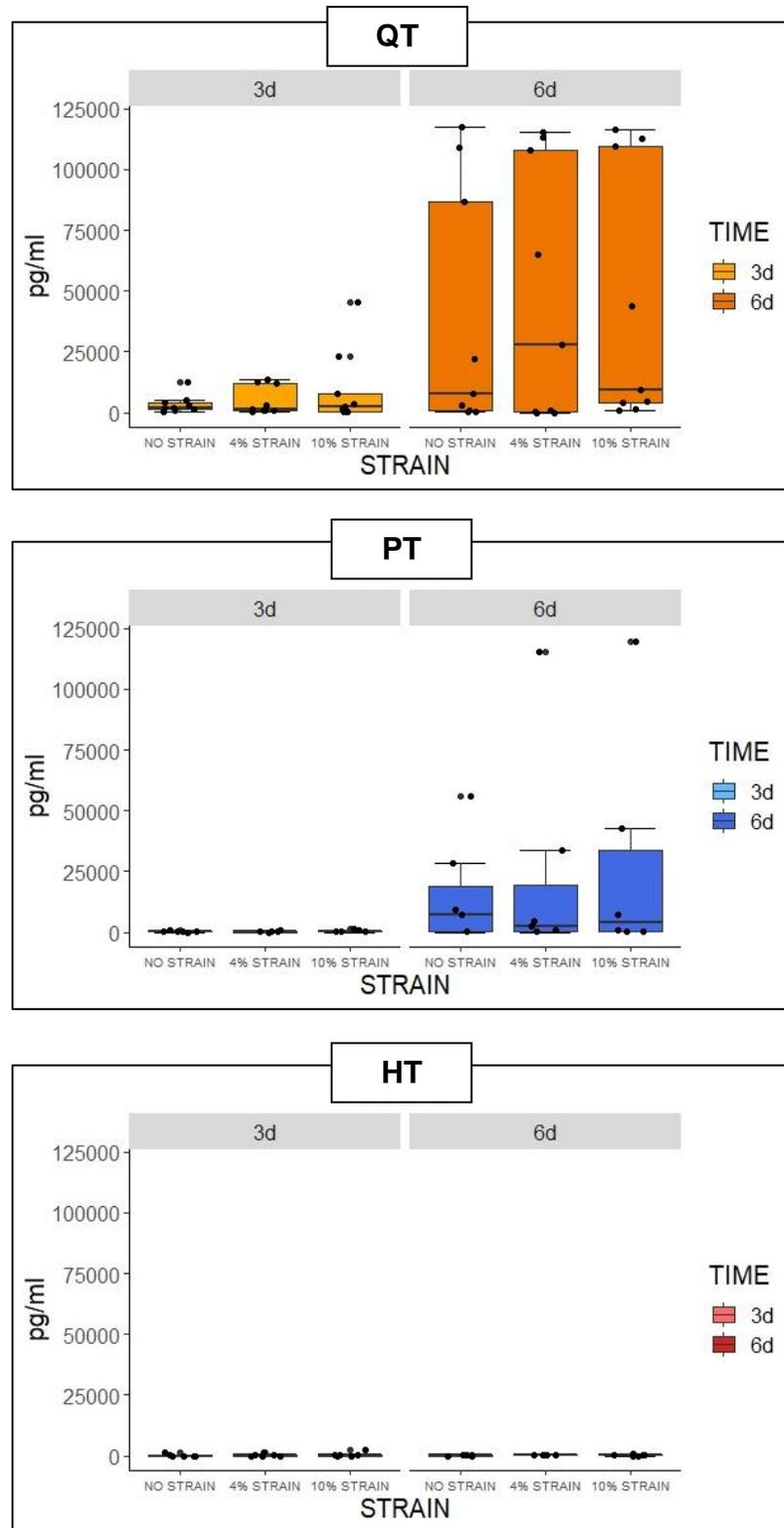


Figure 8.11. Concentration of MMP-1 released to the media. * significant difference between STRAINS at three (3d) and six (6d) days of culture. QT: Quadriceps tendon; PT: Patellar tendon; HT: Hamstring tendon.

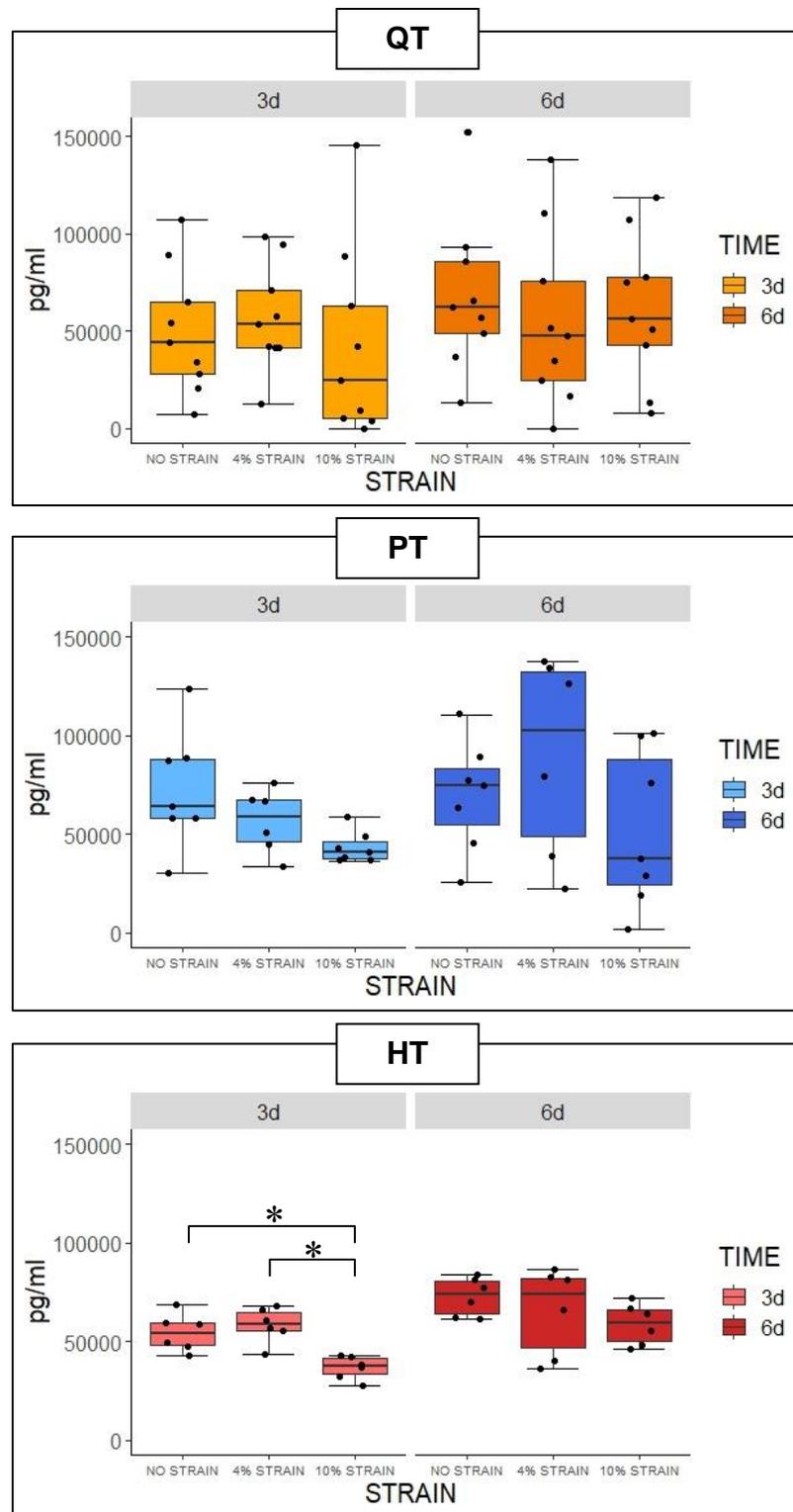


Figure 8.12. Concentration of MMP-2 released to the media. * significant difference between STRAINS at three (3d) and six (6d) days of culture. QT: Quadriceps tendon; PT: Patellar tendon; HT: Hamstring tendon.

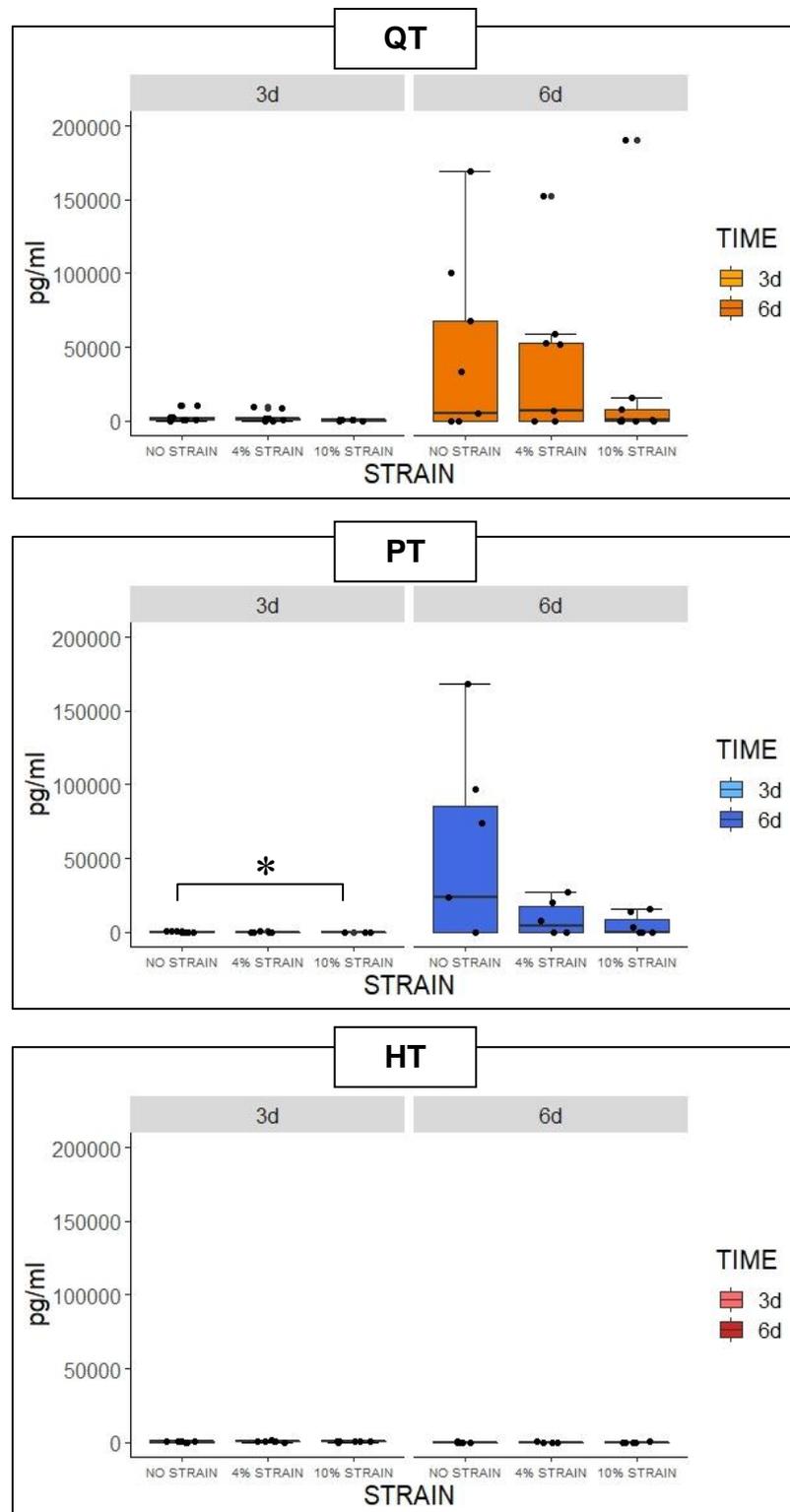


Figure 8.13. Concentration of MMP-3 released to the media. * significant difference between STRAINS at three (3d) and six (6d) days of culture. QT: Quadriceps tendon; PT: Patellar tendon; HT: Hamstring tendon.

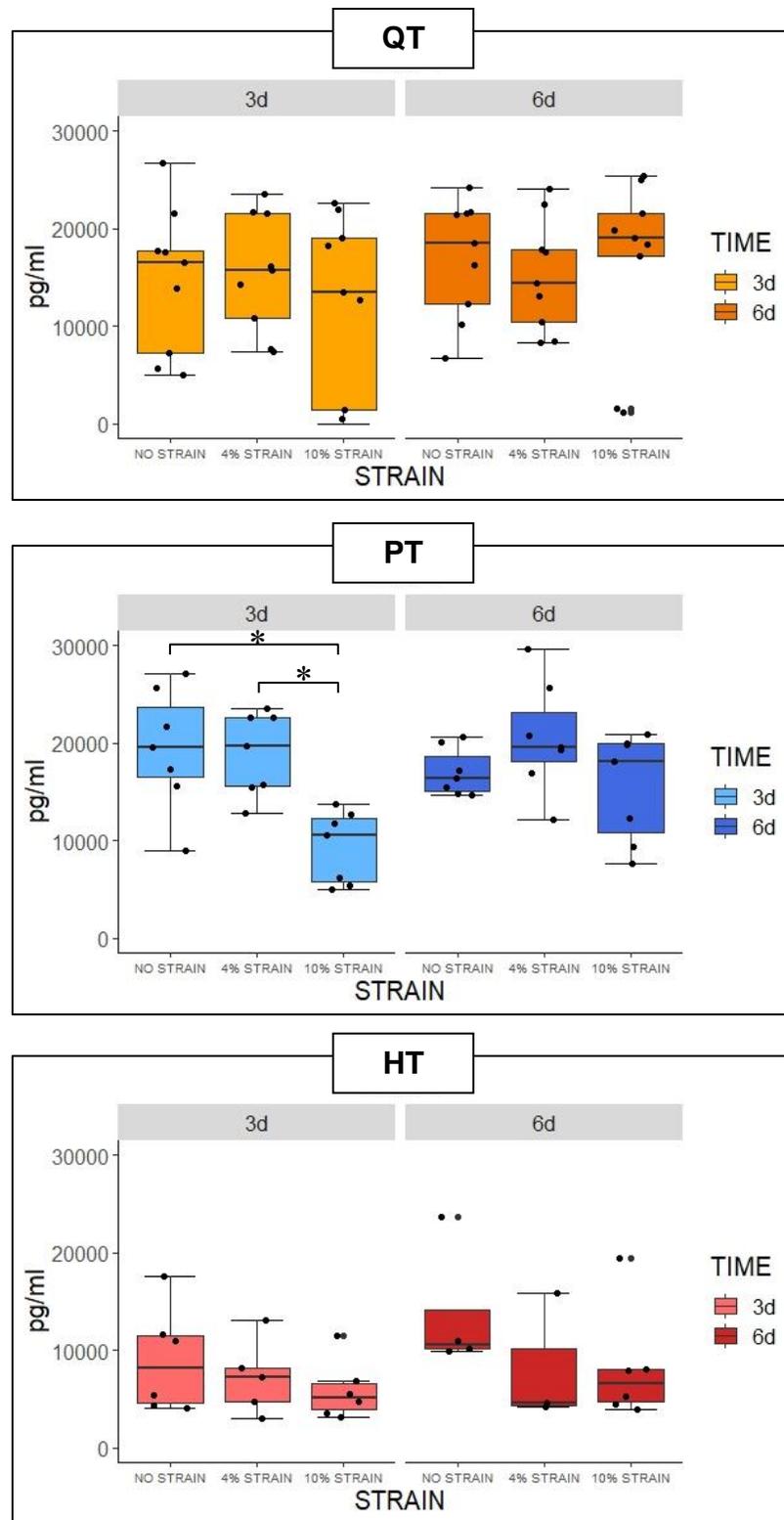


Figure 8.14. Concentration of Timp-1 released to the media. * significant difference between STRAINS at three (3d) and six (6d) days of culture. QT: Quadriceps tendon; PT: Patellar tendon; HT: Hamstring tendon.

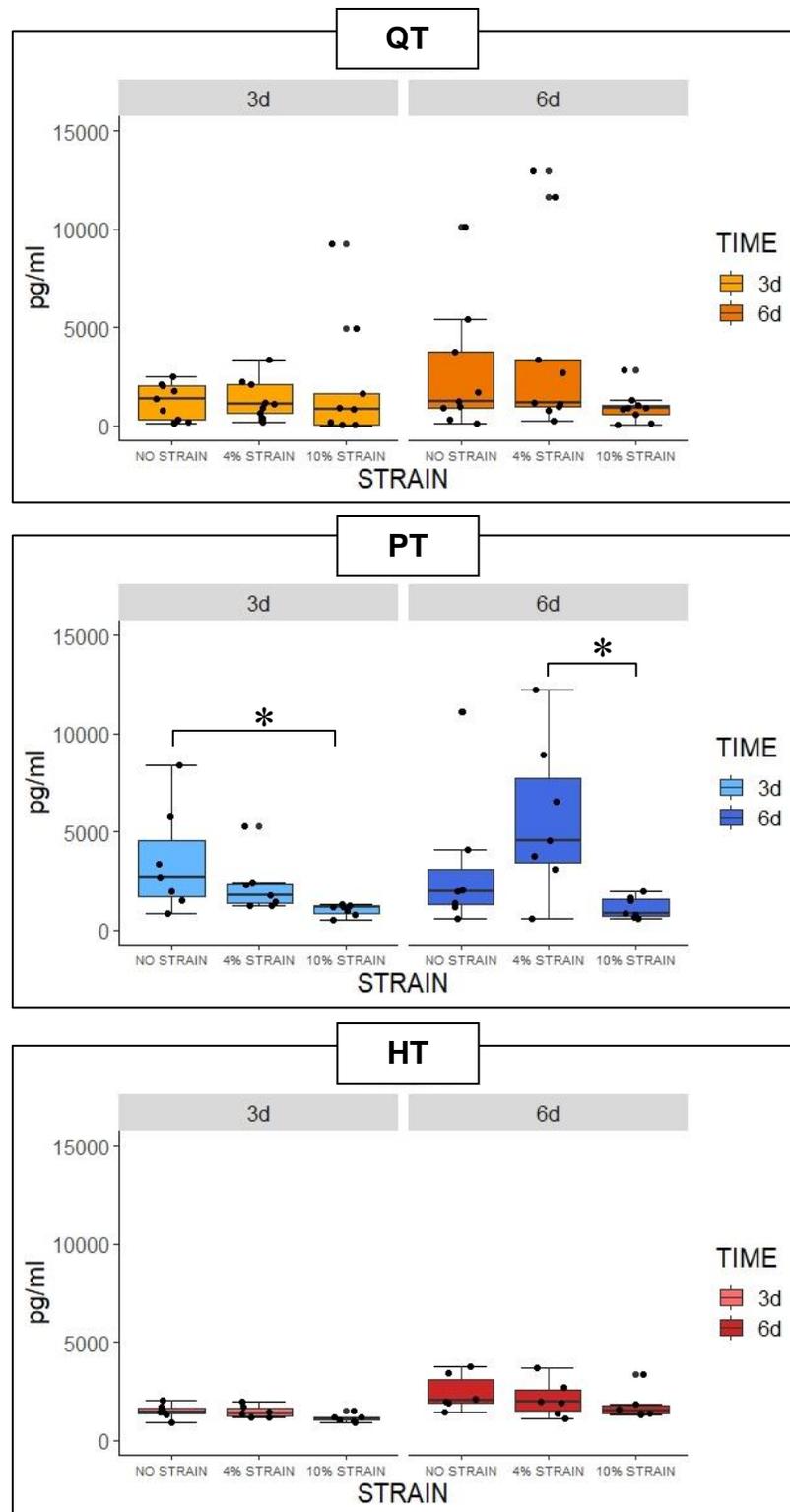


Figure 8.15. Concentration of TIMP-2 released to the media. * significant difference between STRAINS at three (3d) and six (6d) days of culture. QT: Quadriceps tendon; PT: Patellar tendon; HT: Hamstring tendon.

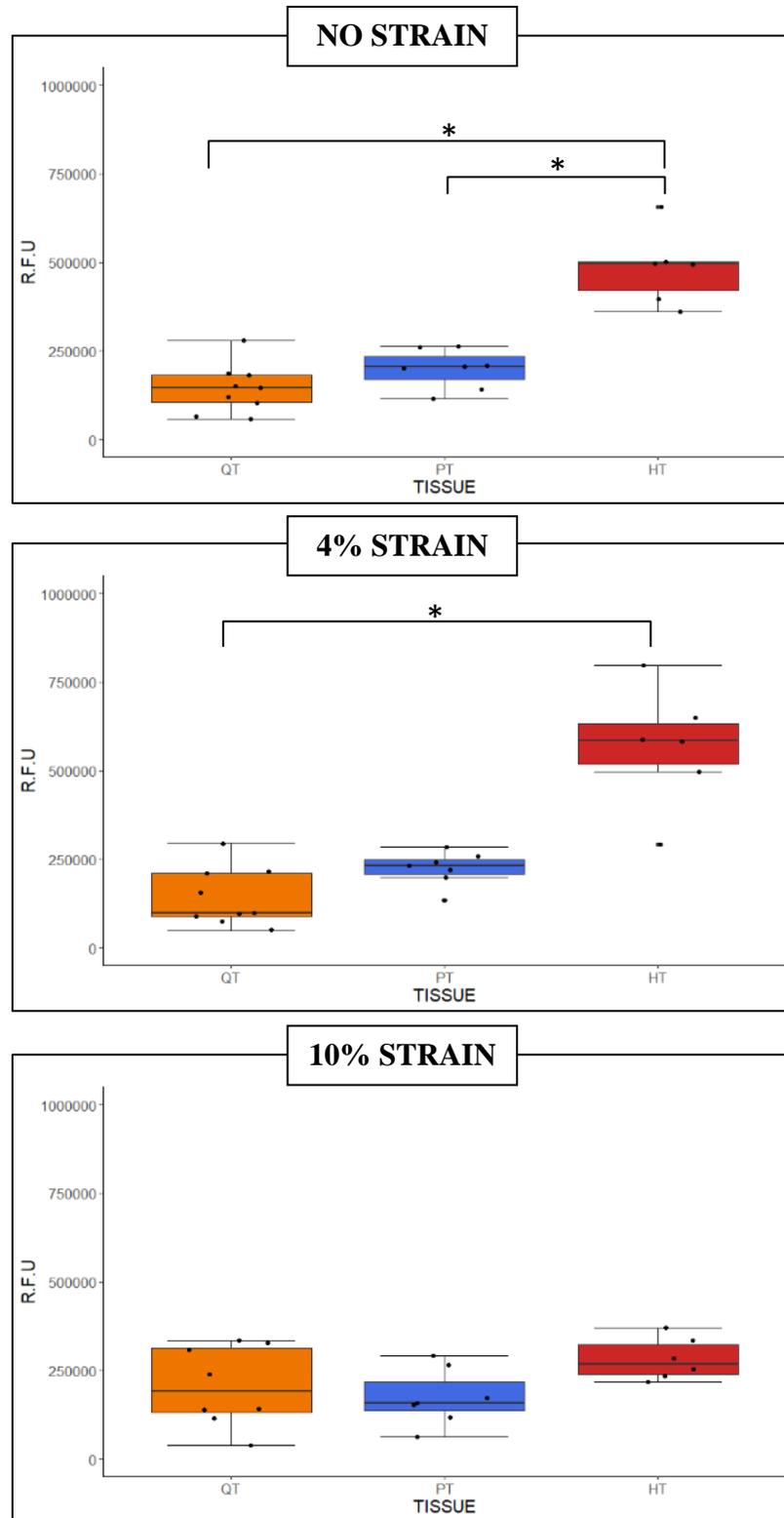


Figure 8.16. Metabolic activity of fibroblasts after six days of culture. * significant difference between TISSUES. QT: Quadriceps tendon; PT: Patellar tendon; HT: Hamstring tendon. R.F.U: Resazurin Fluorescent Units

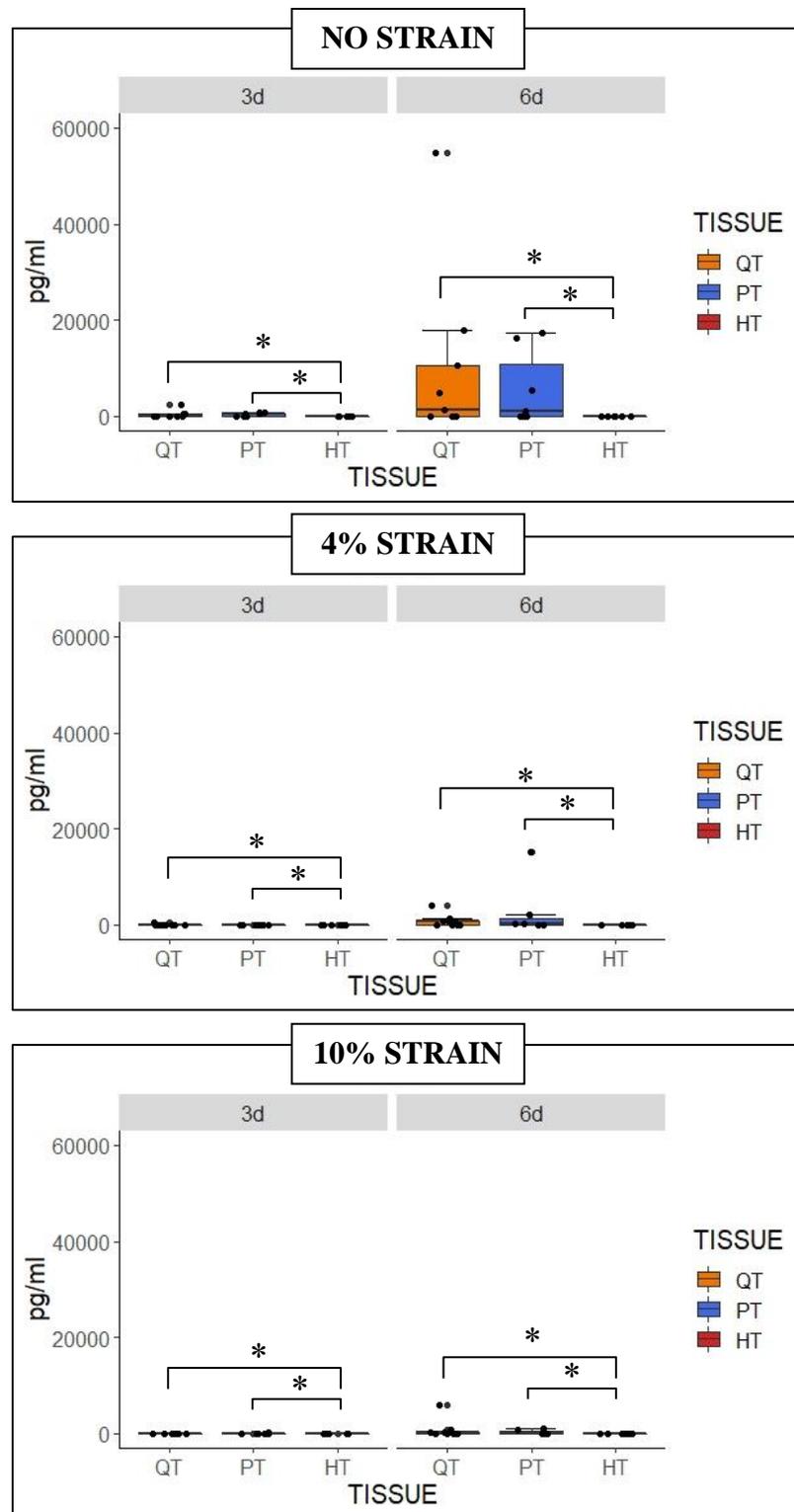


Figure 8.17. Concentration of PGE2 released to the media. * significant difference between TISSUES at three (3d) and six (6d) days of culture. QT: Quadriceps tendon; PT: Patellar tendon; HT: Hamstring tendon.

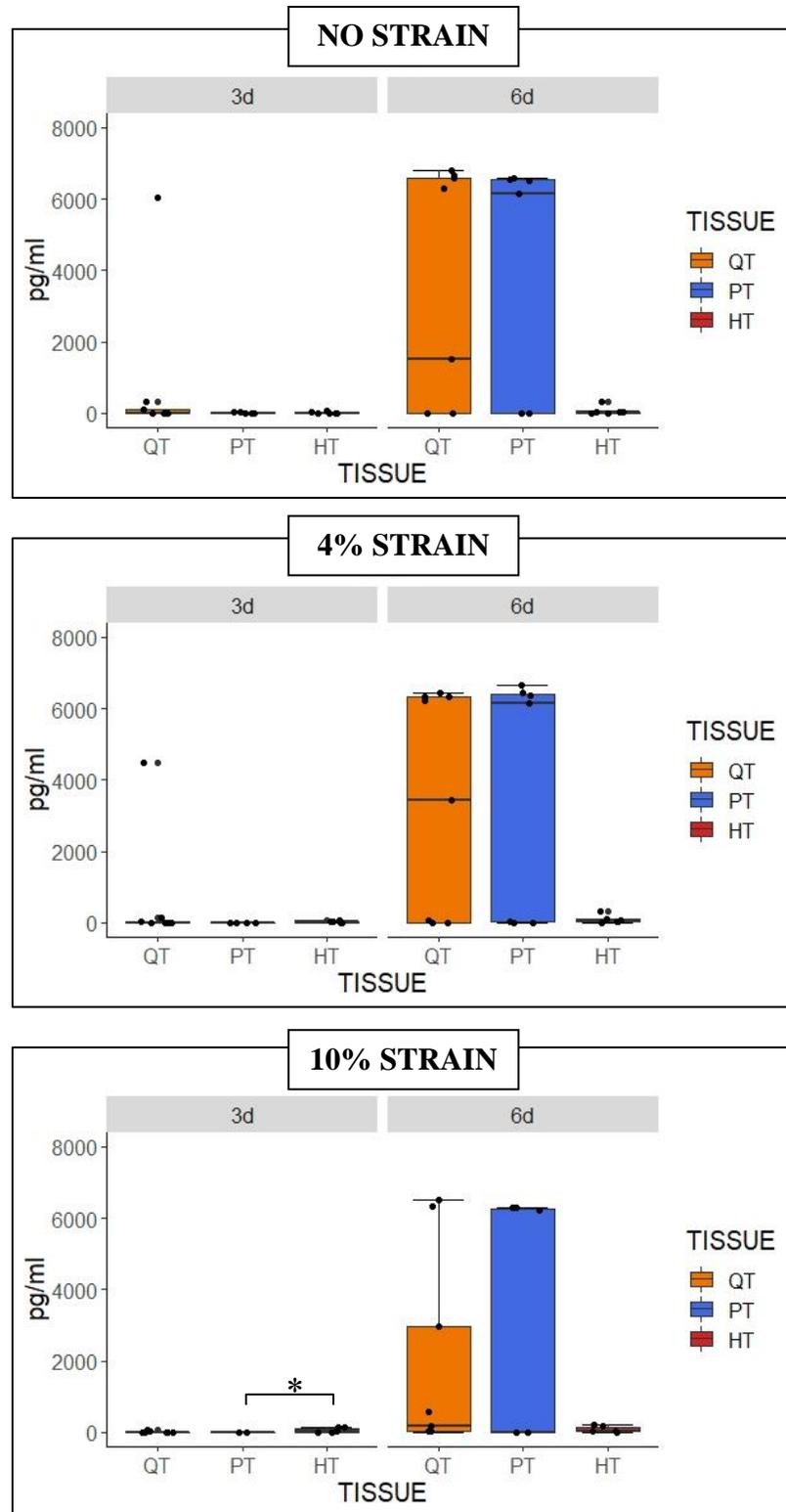


Figure 8.18. Concentration of IL-6 released to the media. * significant difference between TISSUES at three (3d) and six (6d) days of culture. QT: Quadriceps tendon; PT: Patellar tendon; HT: Hamstring tendon.

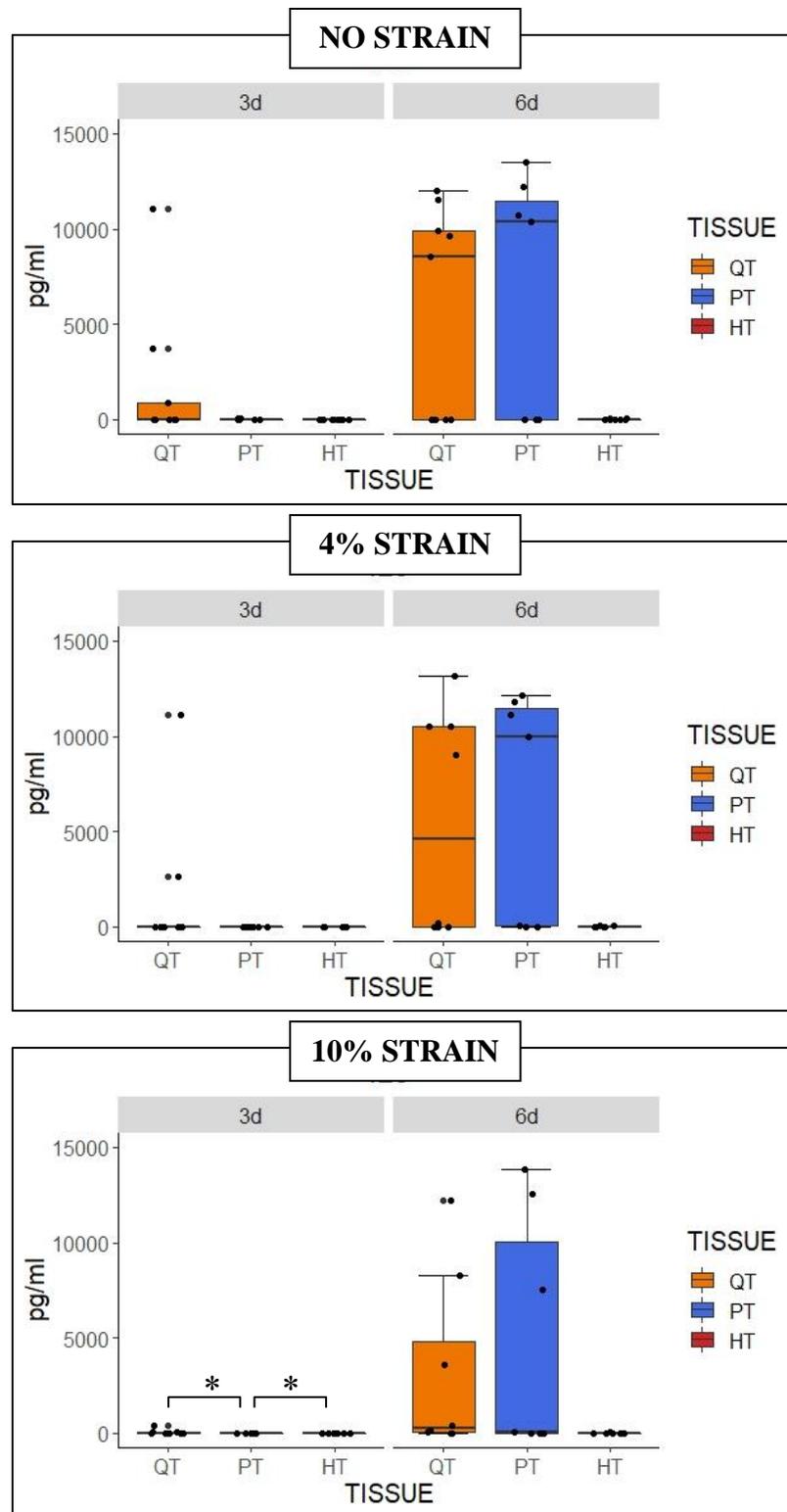


Figure 8.19. Concentration of IL-8 released to the media. * significant difference between TISSUES at three (3d) and six (6d) days of culture. QT: Quadriceps tendon; PT: Patellar tendon; HT: Hamstring tendon.

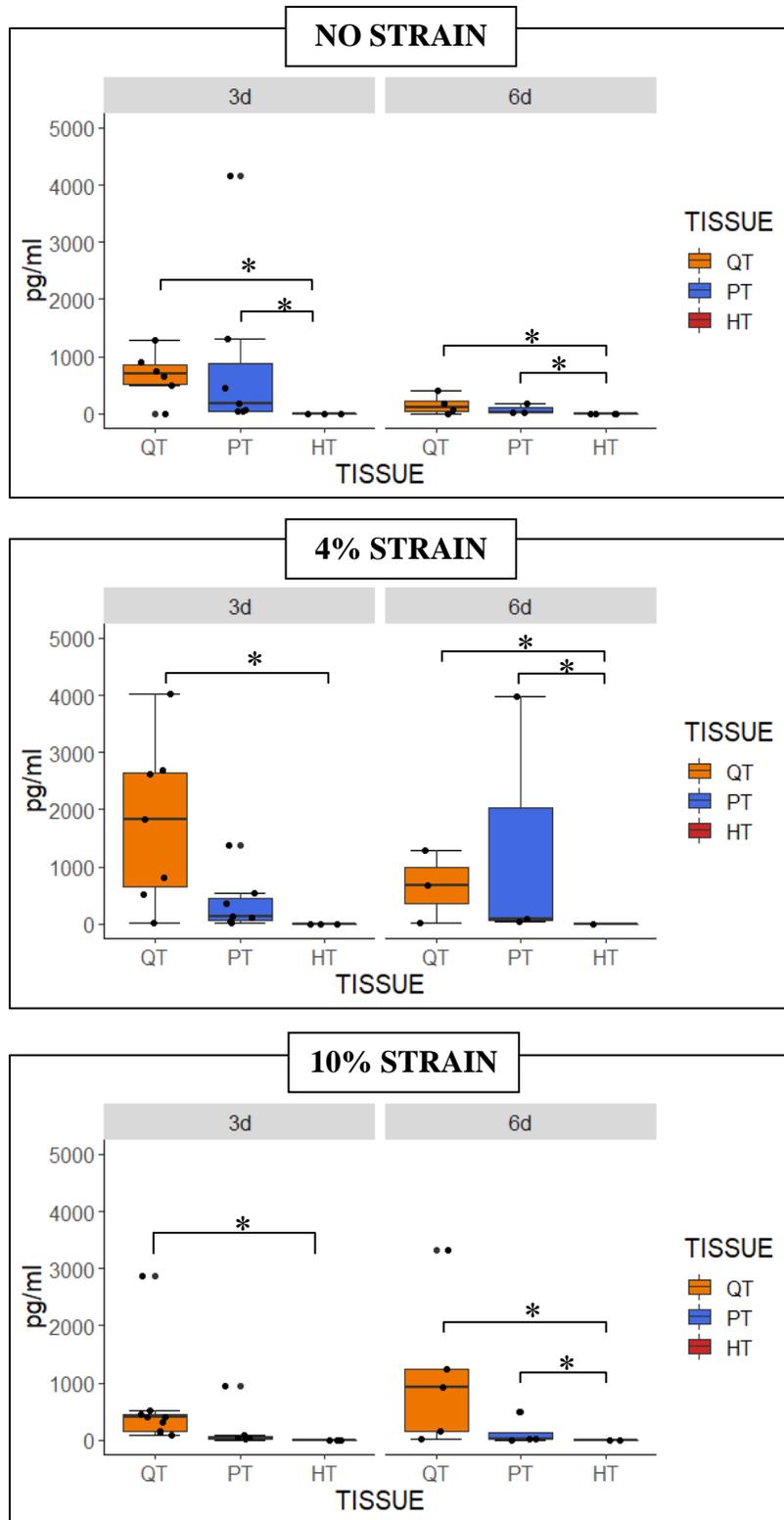


Figure 8.20. Concentration of KC released to the media. * significant difference between TISSUES at three (3d) and six (6d) days of culture. QT: Quadriceps tendon; PT: Patellar tendon; HT: Hamstring tendon.

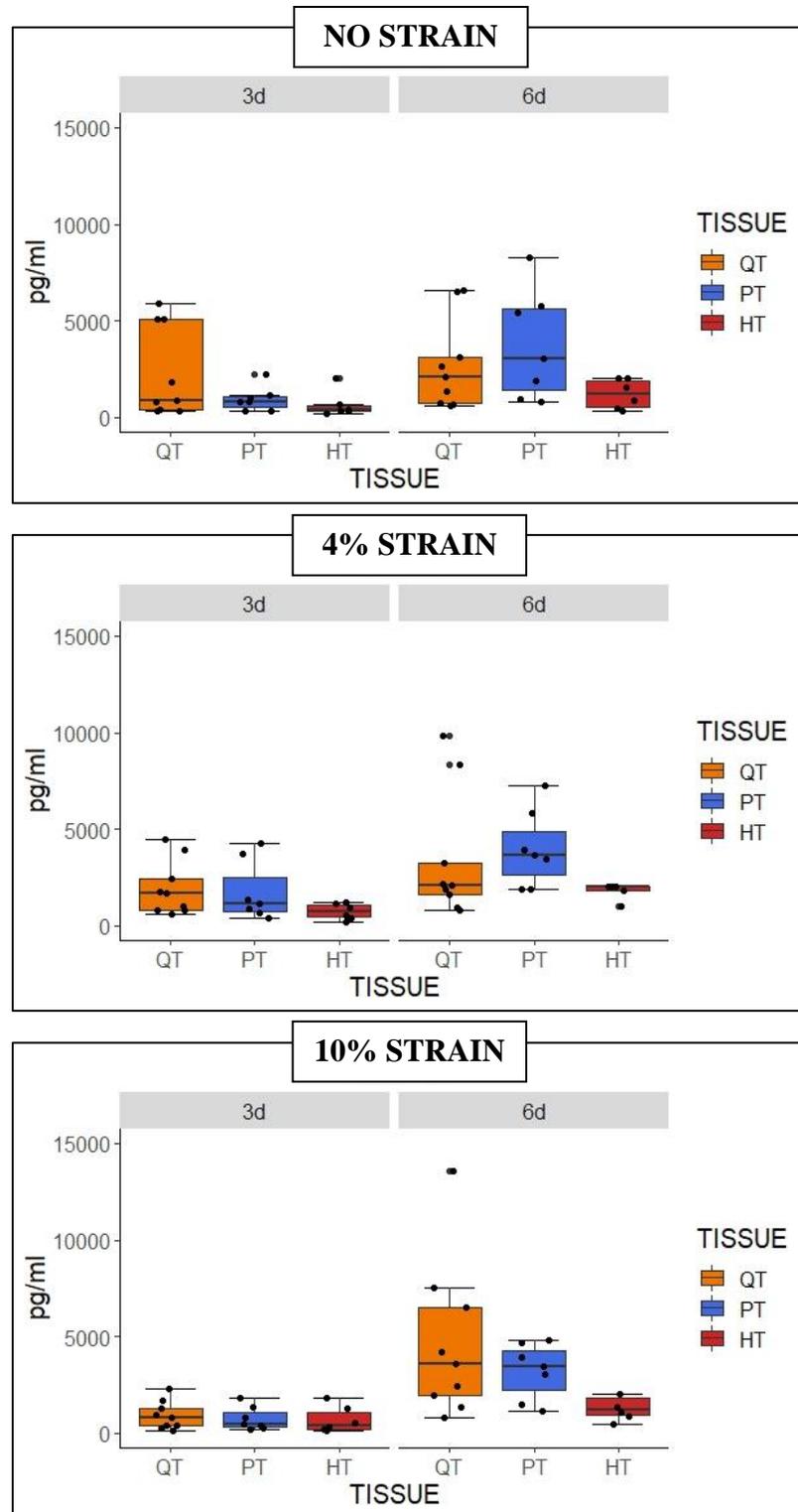


Figure 8.21. Concentration of MCP1 released to the media. * significant difference between TISSUES at three (3d) and six (6d) days of culture. QT: Quadriceps tendon; PT: Patellar tendon; HT: Hamstring tendon.

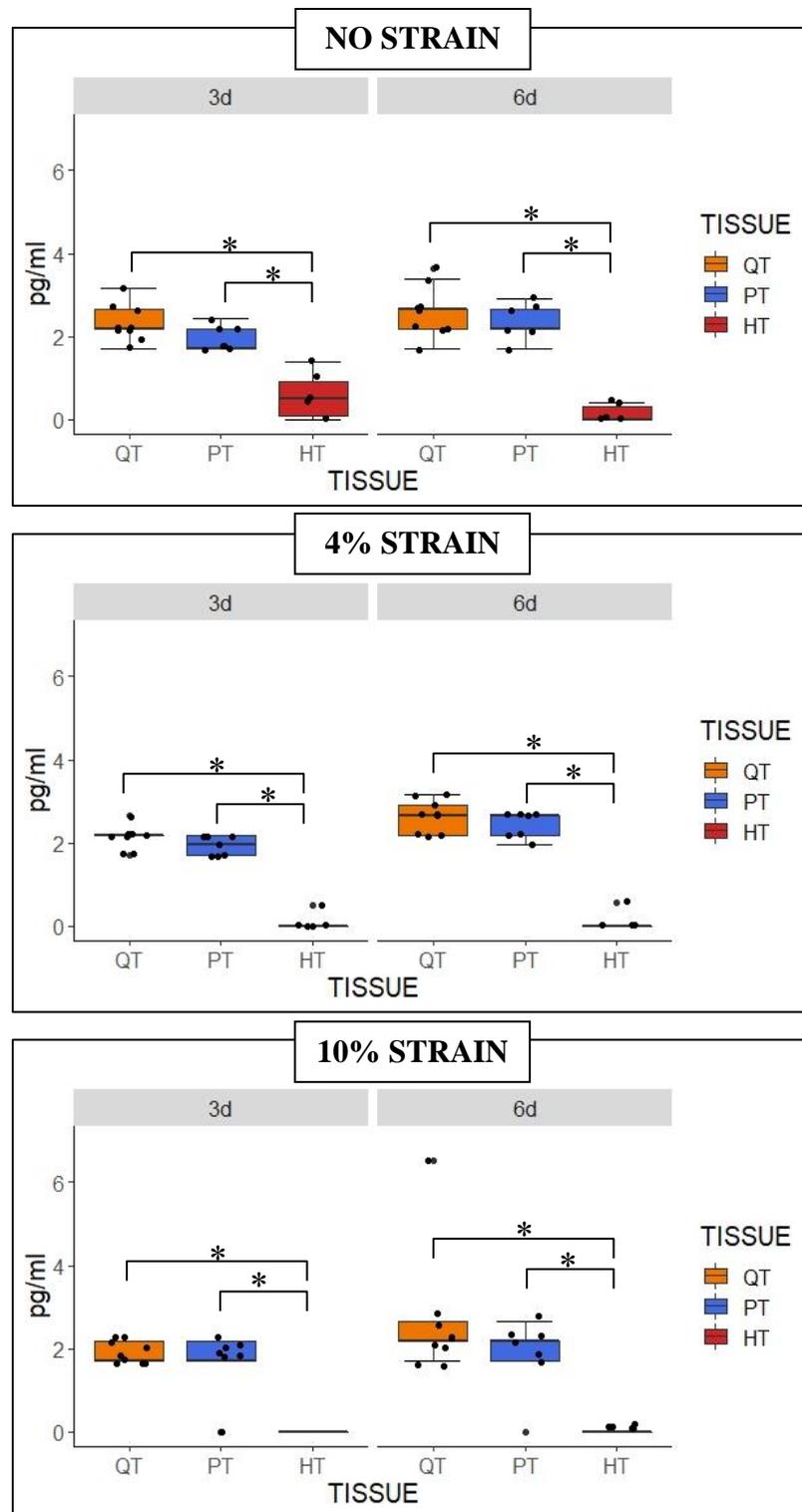


Figure 8.22. Concentration of MP1 β released to the media. * significant difference between TISSUES at three (3d) and six (6d) days of culture. QT: Quadriceps tendon; PT: Patellar tendon; HT: Hamstring tendon.

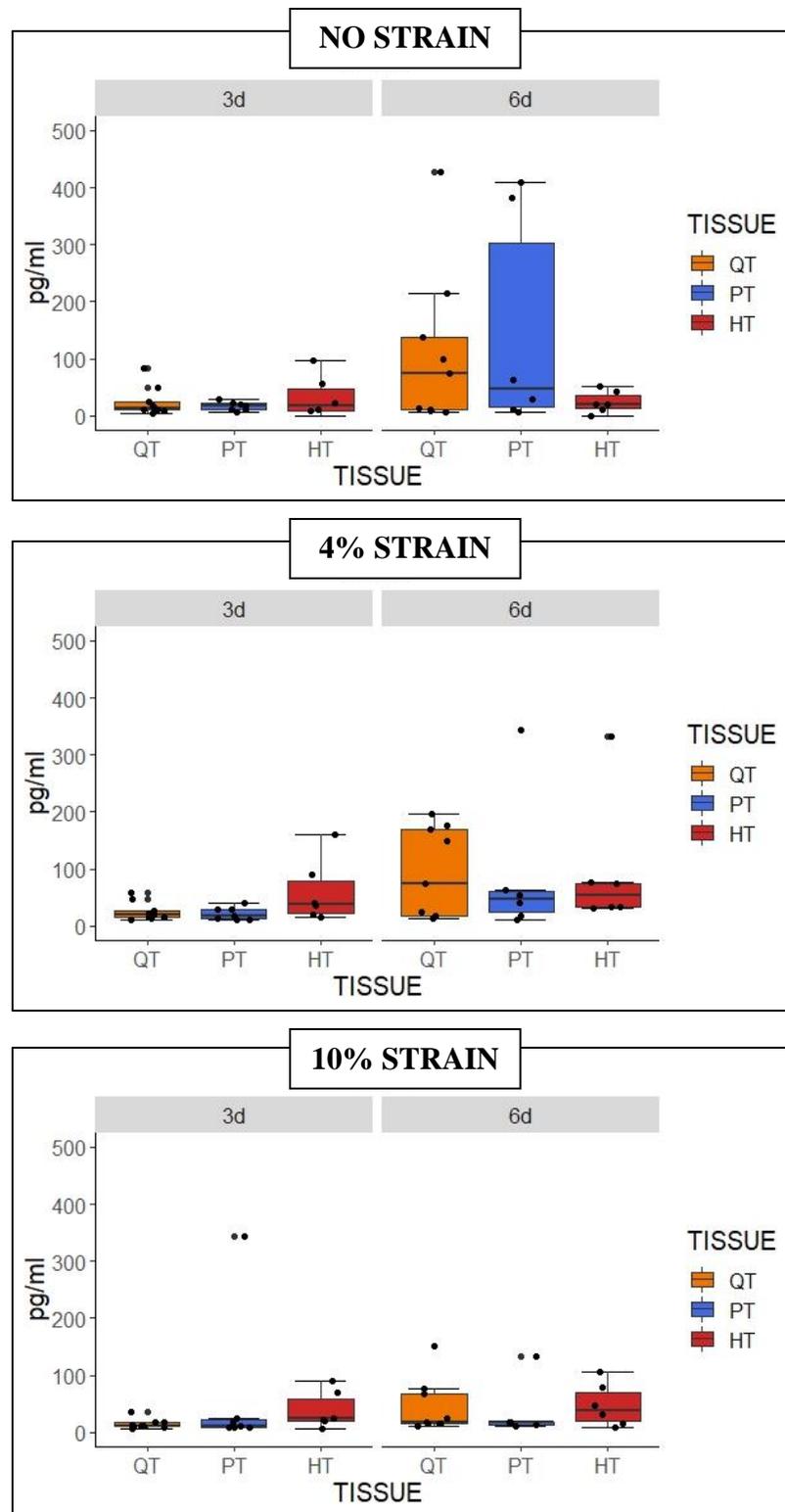


Figure 8.23. Concentration of VEGF released to the media. * significant difference between TISSUES at three (3d) and six (6d) days of culture. QT: Quadriceps tendon; PT: Patellar tendon; HT: Hamstring tendon.

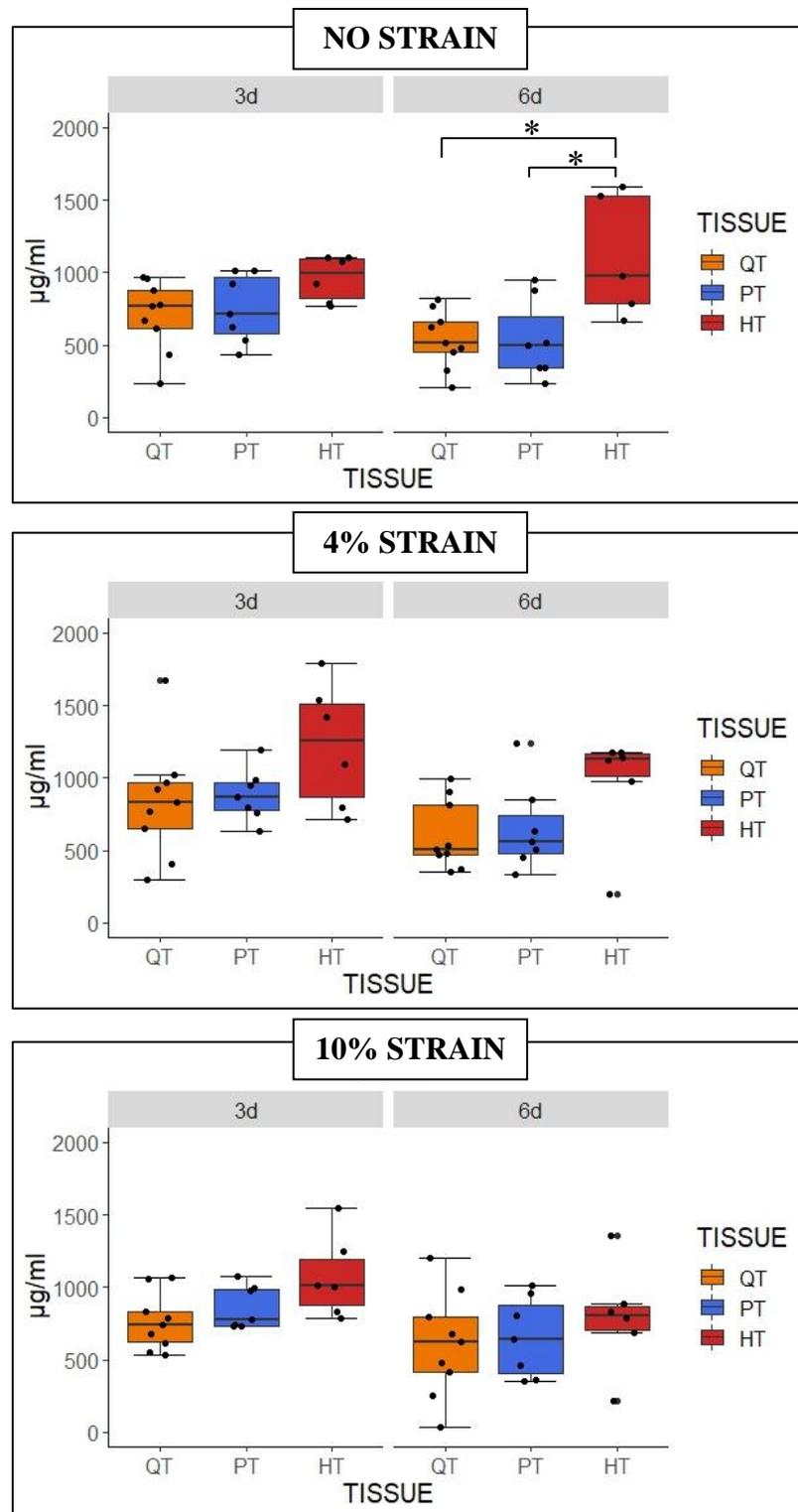


Figure 8.24. Concentration of GAG released to the media. * significant difference between TISSUES at three (3d) and six (6d) days of culture. QT: Quadriceps tendon; PT: Patellar tendon; HT: Hamstring tendon.

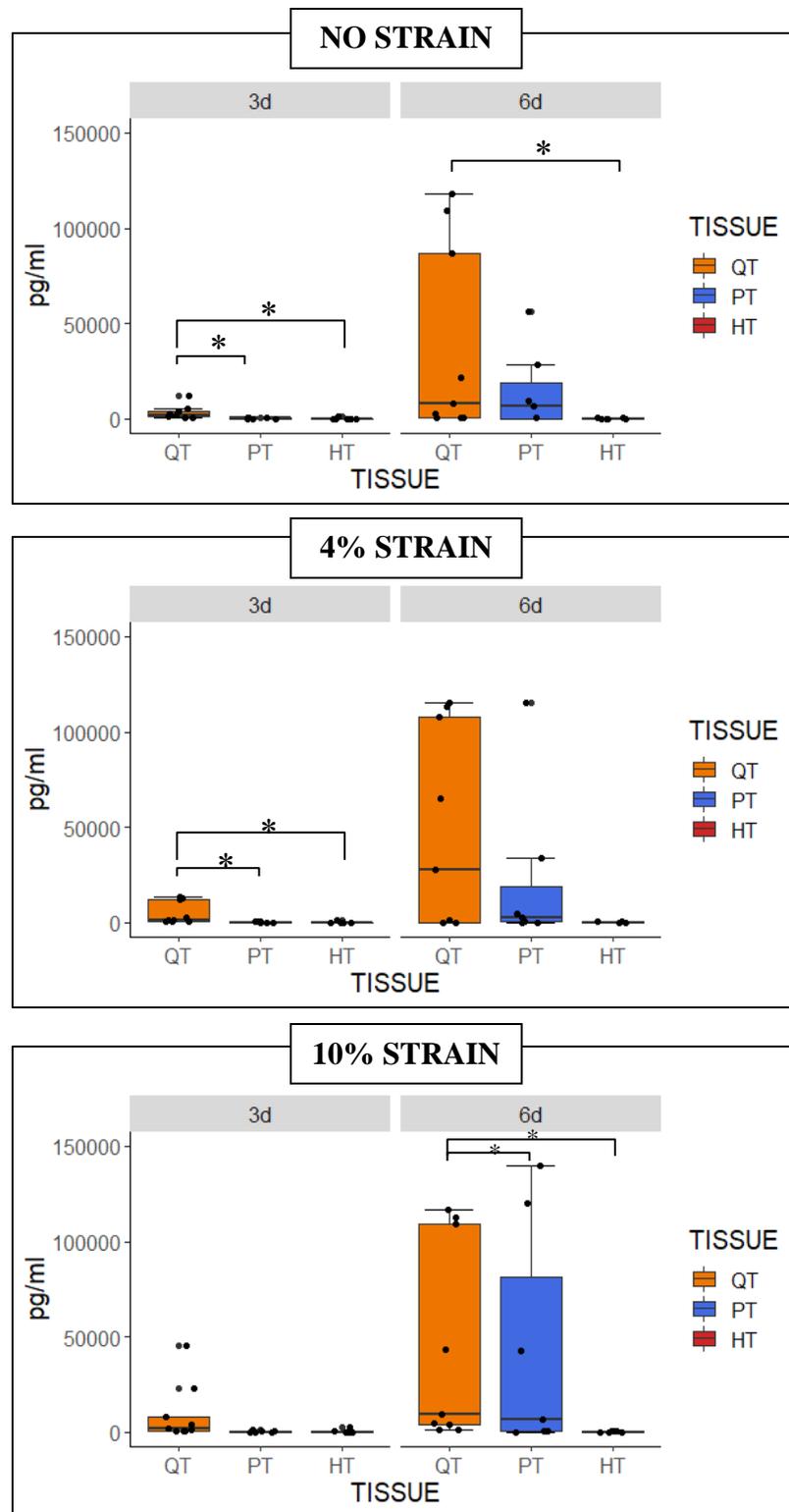


Figure 8.25. Concentration of MMP-1 released to the media. * significant difference between TISSUES at three (3d) and six (6d) days of culture. QT: Quadriceps tendon; PT: Patellar tendon; HT: Hamstring tendon.

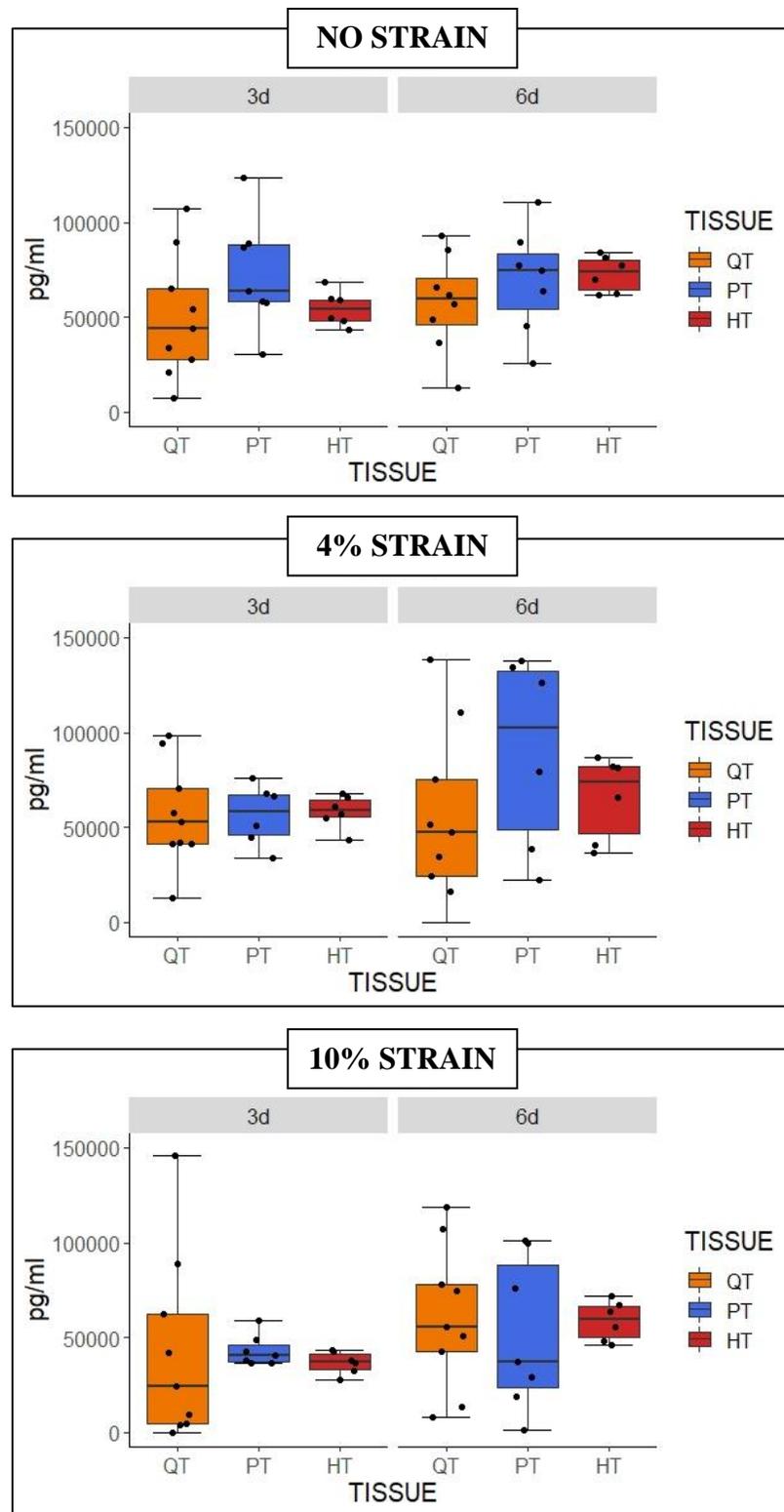


Figure 8.26. Concentration of MMP-2 released to the media. * significant difference between TISSUES at three (3d) and six (6d) days of culture. QT: Quadriceps tendon; PT: Patellar tendon; HT: Hamstring tendon.

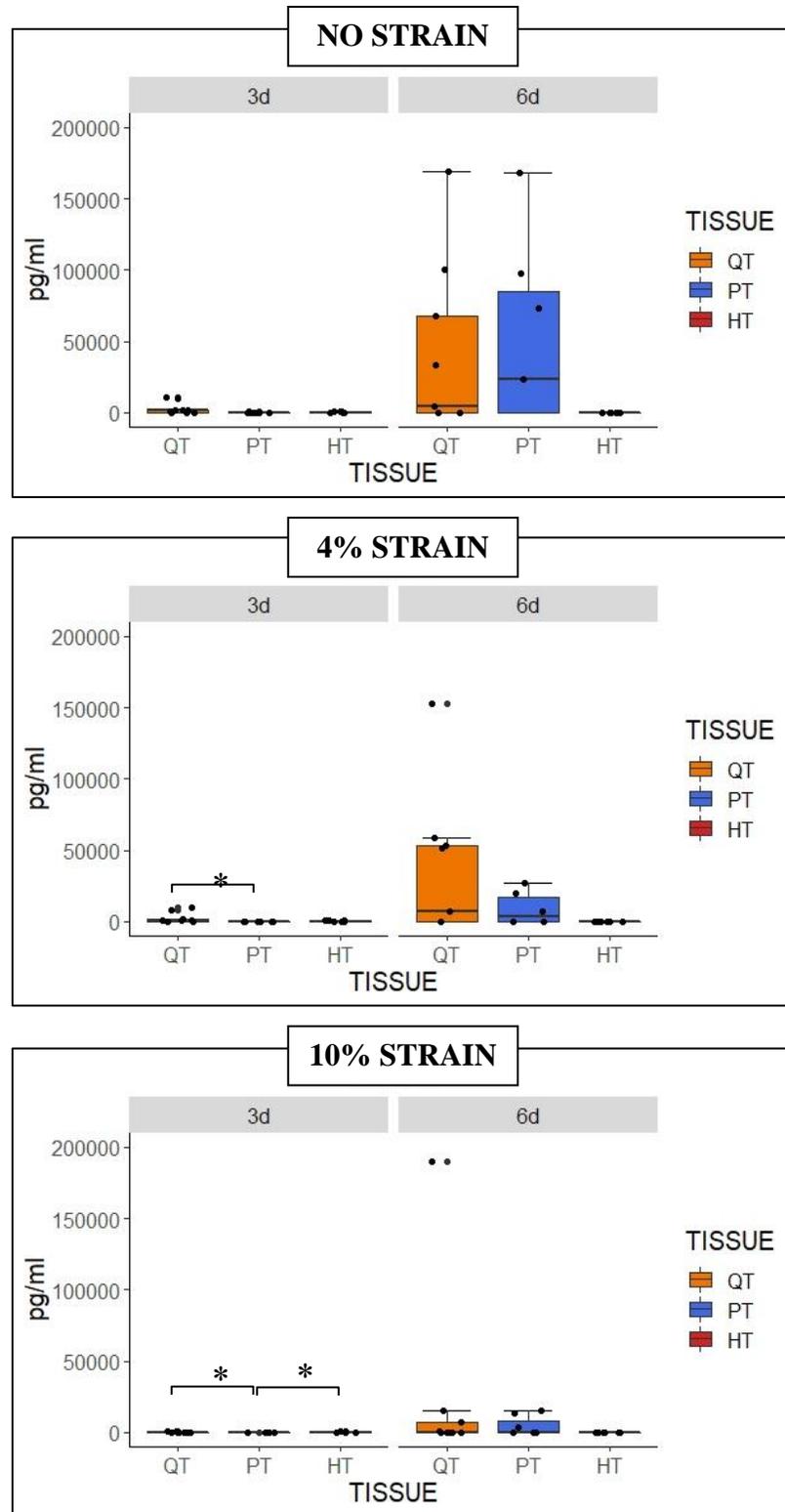


Figure 8.27. Concentration of MMP-3 released to the media. * significant difference between TISSUES at three (3d) and six (6d) days of culture. QT: Quadriceps tendon; PT: Patellar tendon; HT: Hamstring tendon.

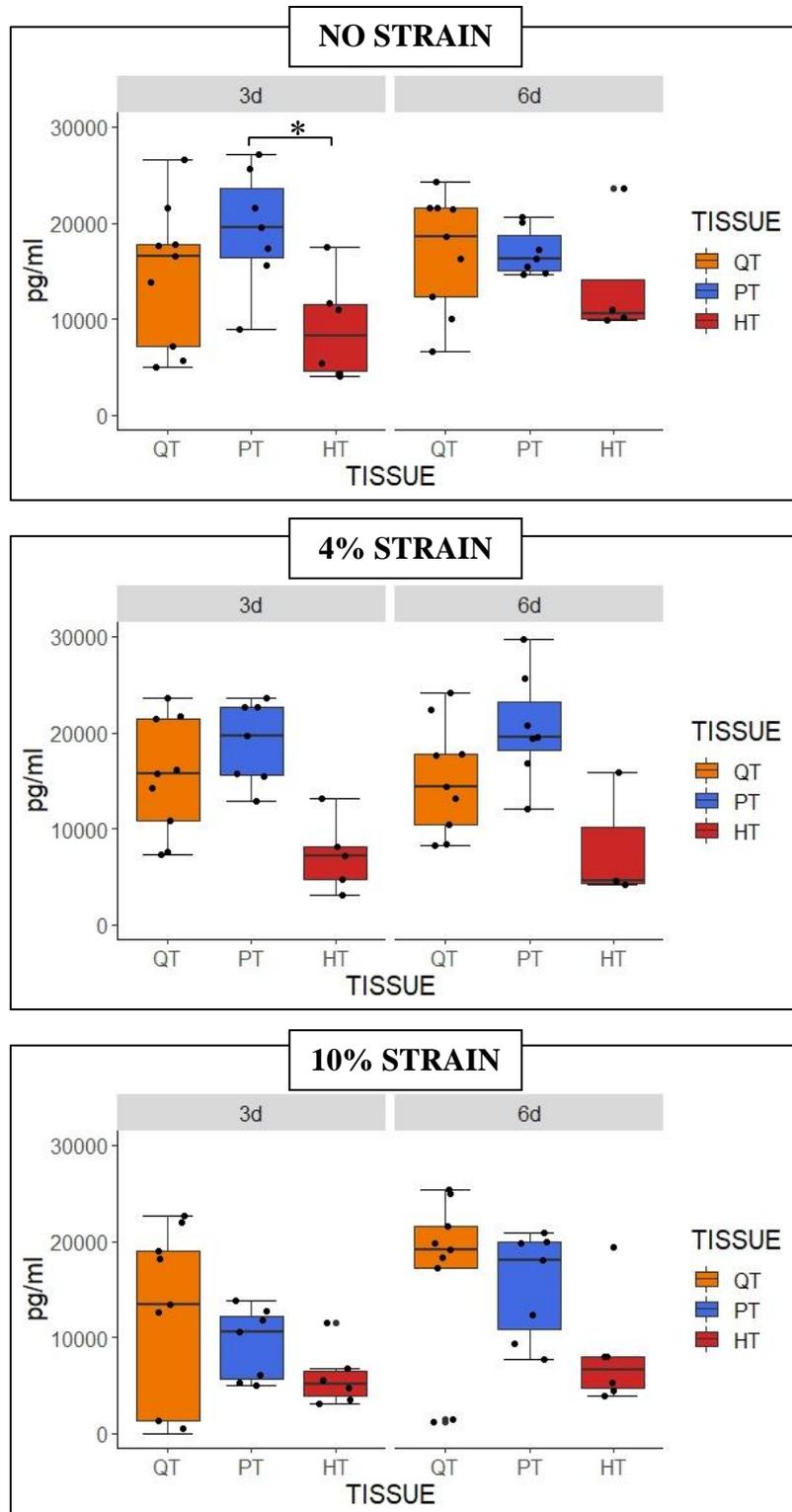


Figure 8.28. Concentration of TIMP-1 released to the media. * significant difference between TISSUES at three (3d) and six (6d) days of culture. QT: Quadriceps tendon; PT: Patellar tendon; HT: Hamstring tendon.

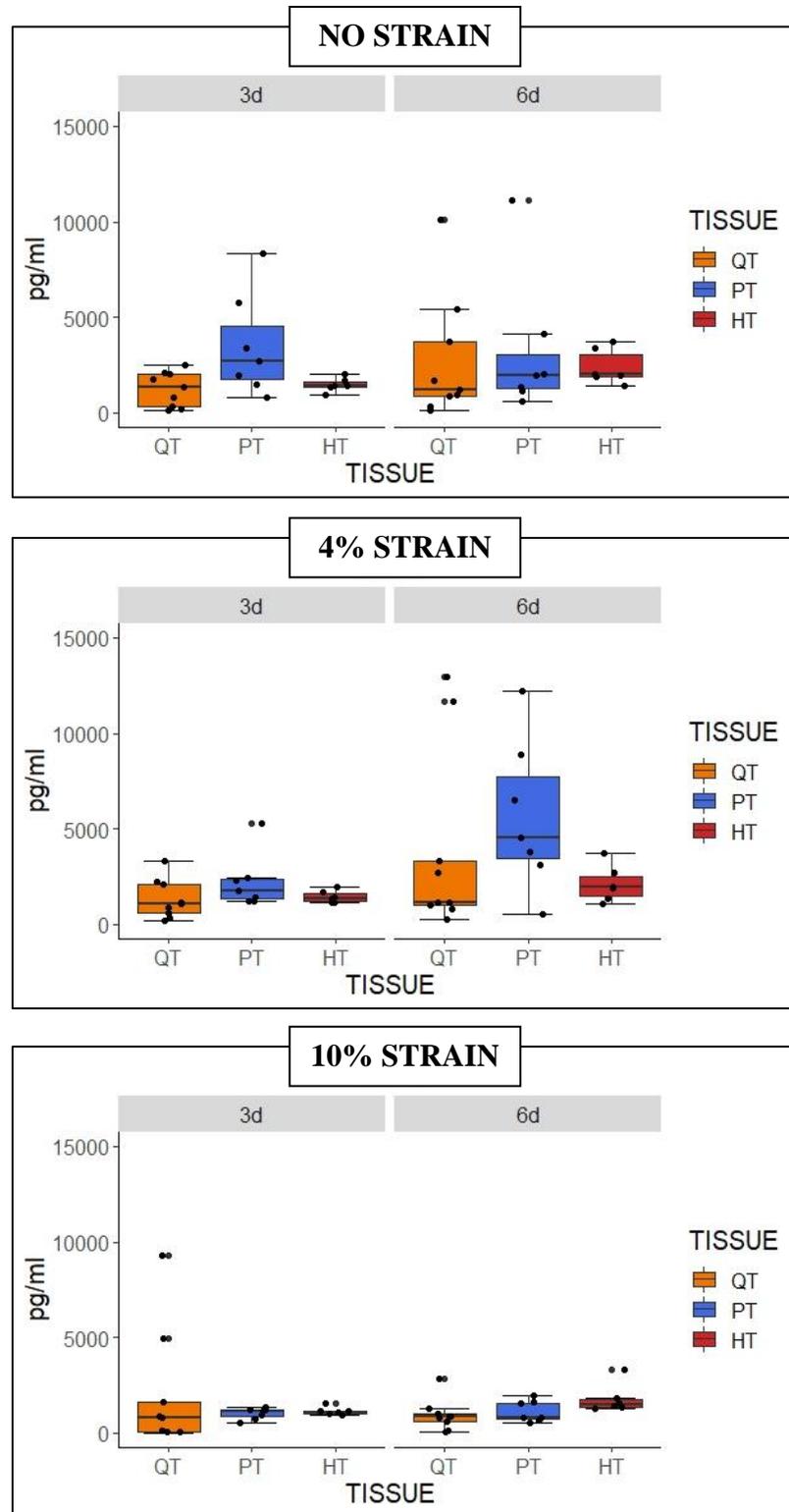


Figure 8.29. Concentration of TIMP-2 released to the media. * significant difference between TISSUES at three (3d) and six (6d) days of culture. QT: Quadriceps tendon; PT: Patellar tendon; HT: Hamstring tendon.

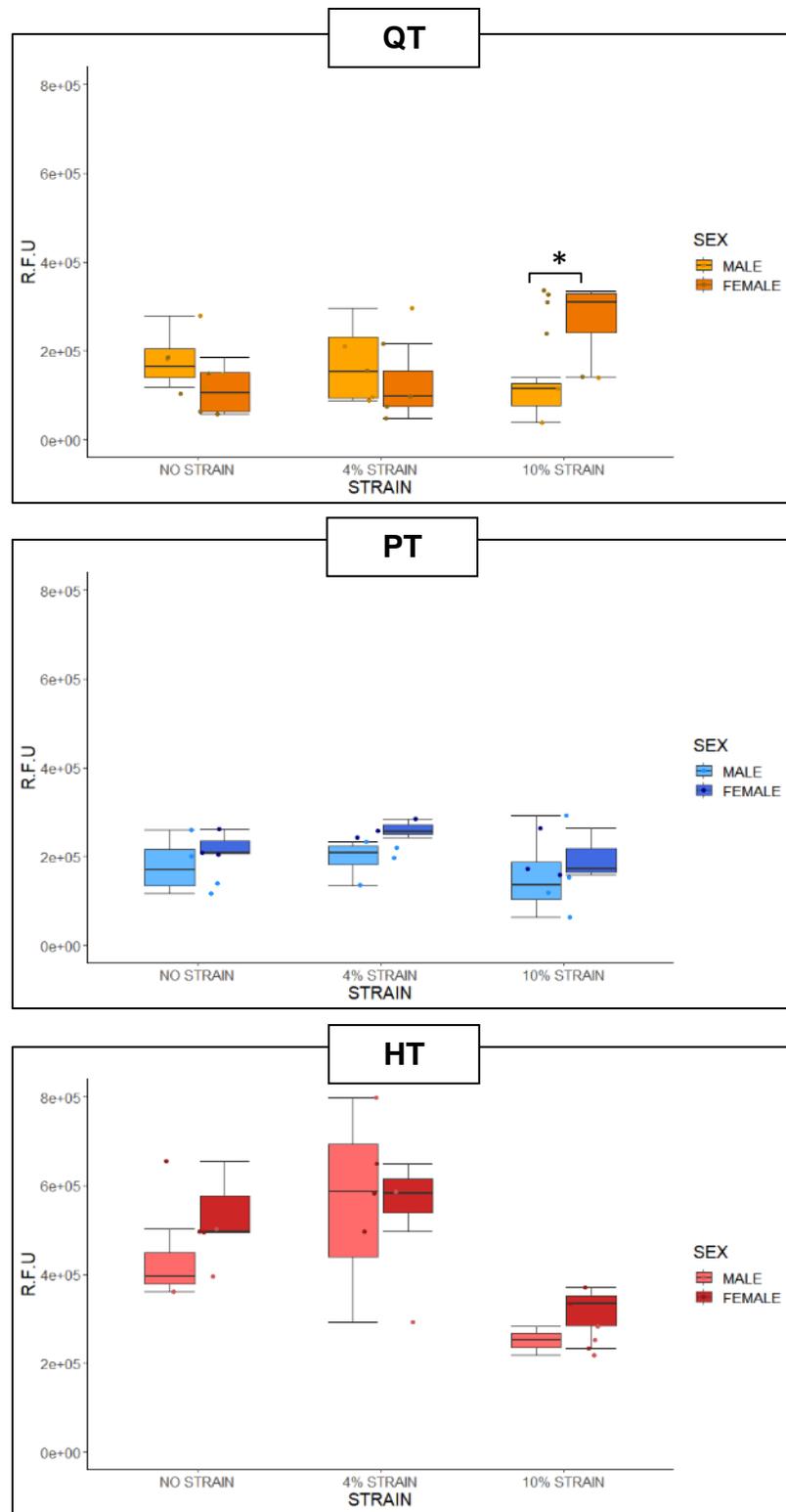


Figure 8.30. Metabolic Activity of fibroblasts after 120h of culture. * significant difference between males and females (Signed Rank test). QT: Quadriceps tendon; PT: Patellar tendon, HT: Hamstring tendon

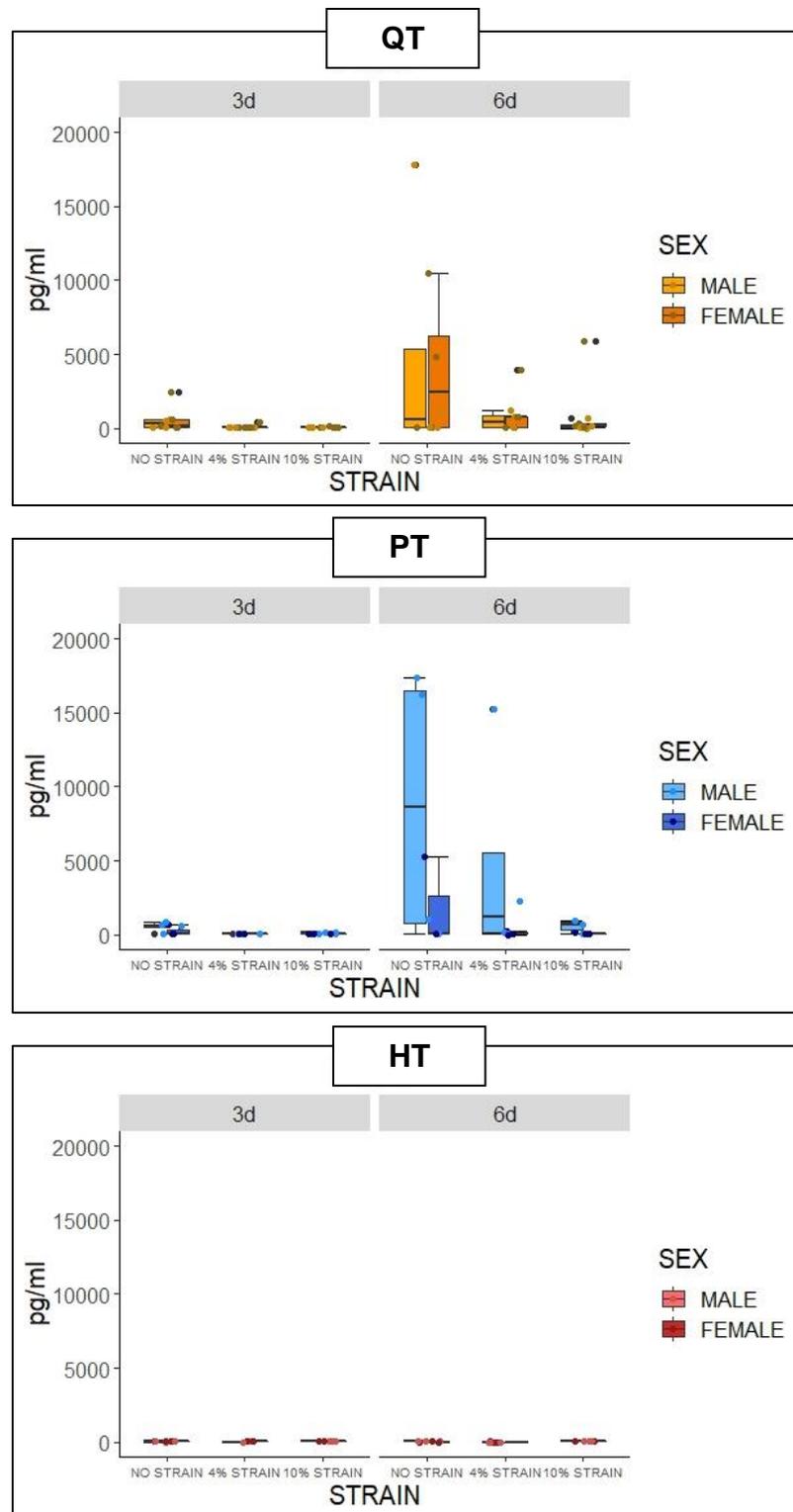


Figure 8.31. Concentration of PGE2 released to the media. * significant difference between males and females at 24 and 120 hours (Signed Rank test). QT: Quadriceps tendon; PT: Patellar tendon, HT: Hamstring tendon

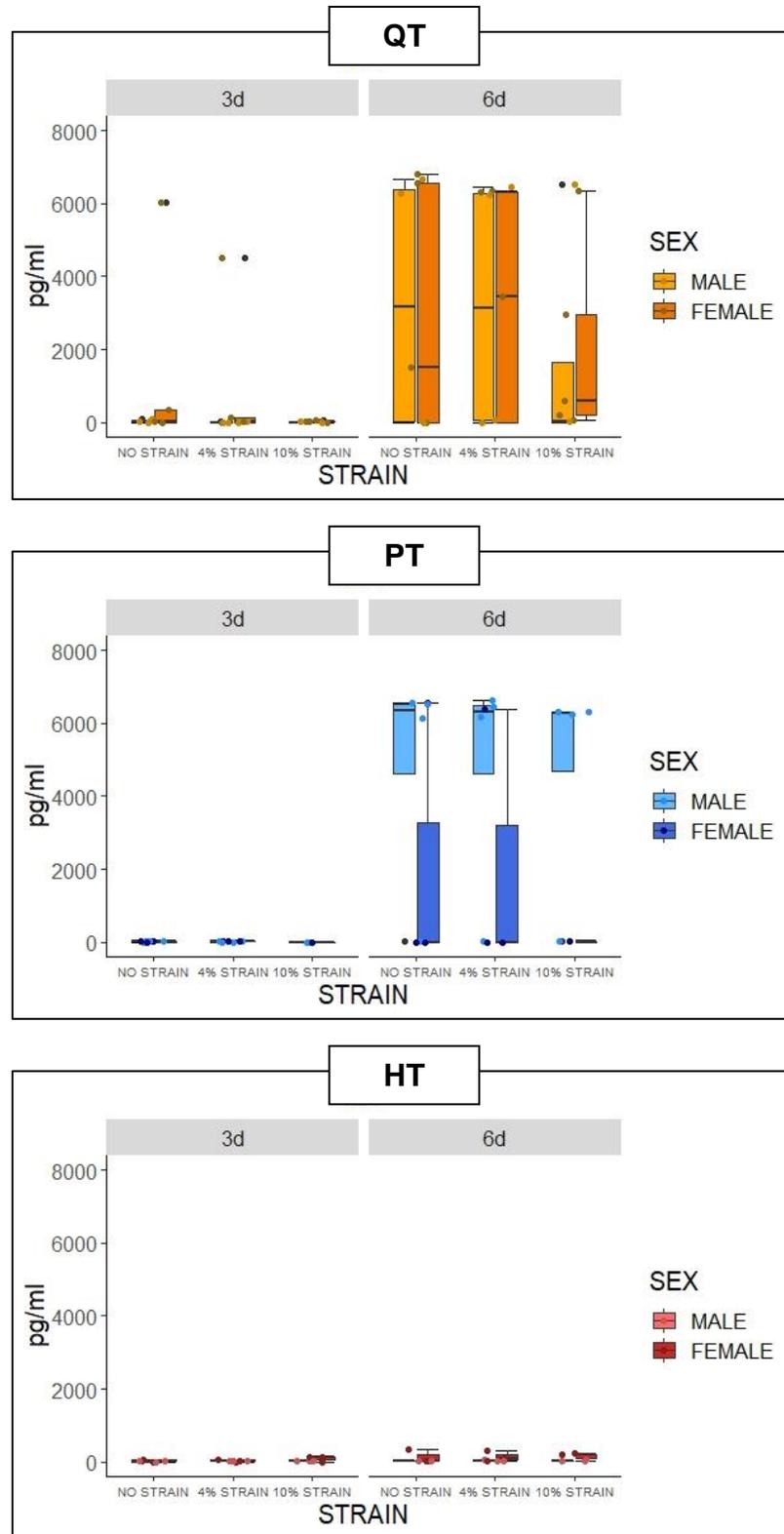


Figure 8.32. Concentration of IL-6 released to the media. * significant difference males and females at 24 and 120 hours (Signed Rank test). QT: Quadriceps tendon; PT: Patellar tendon, HT: Hamstring tendon

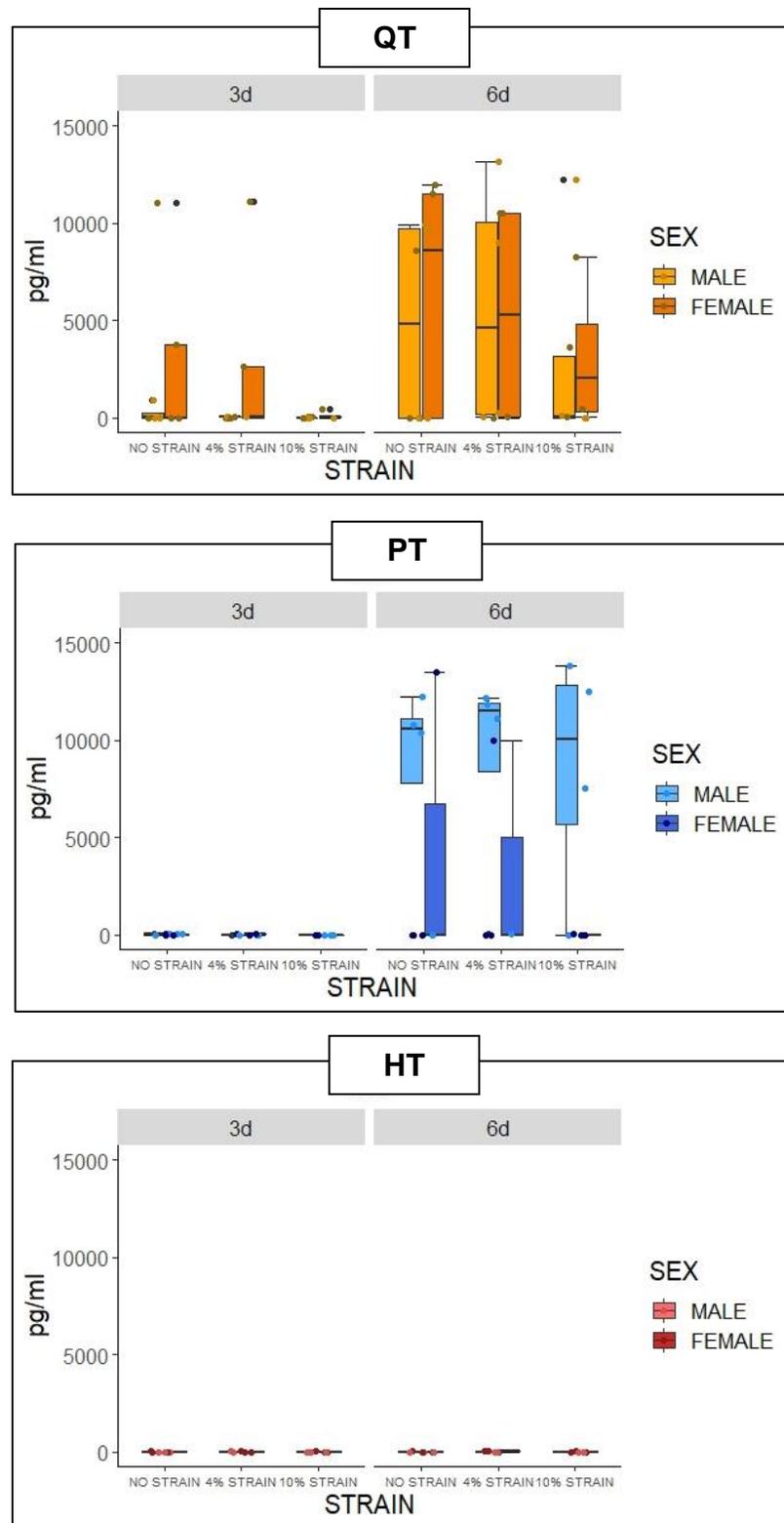


Figure 8.33. Concentration of IL-8 released to the media. * significant difference males and females at 24 and 120 hours (Signed Rank test). QT: Quadriceps tendon; PT: Patellar tendon, HT: Hamstring tendon

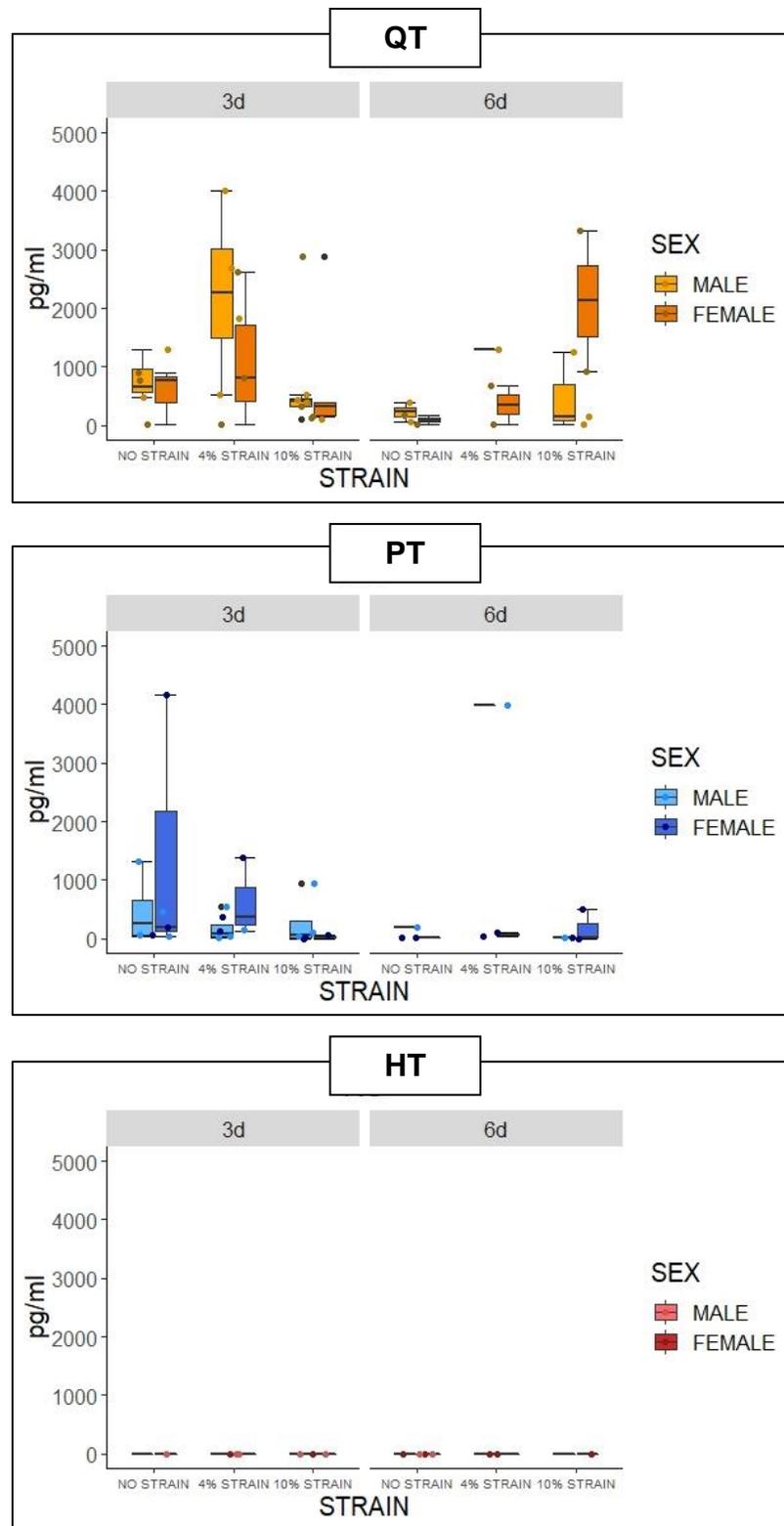


Figure 8.34. Concentration of KC released to the media. * significant difference between males and females at 24 and 120 hours (Signed Rank test). QT: Quadriceps tendon; PT: Patellar tendon, HT: Hamstring tendon

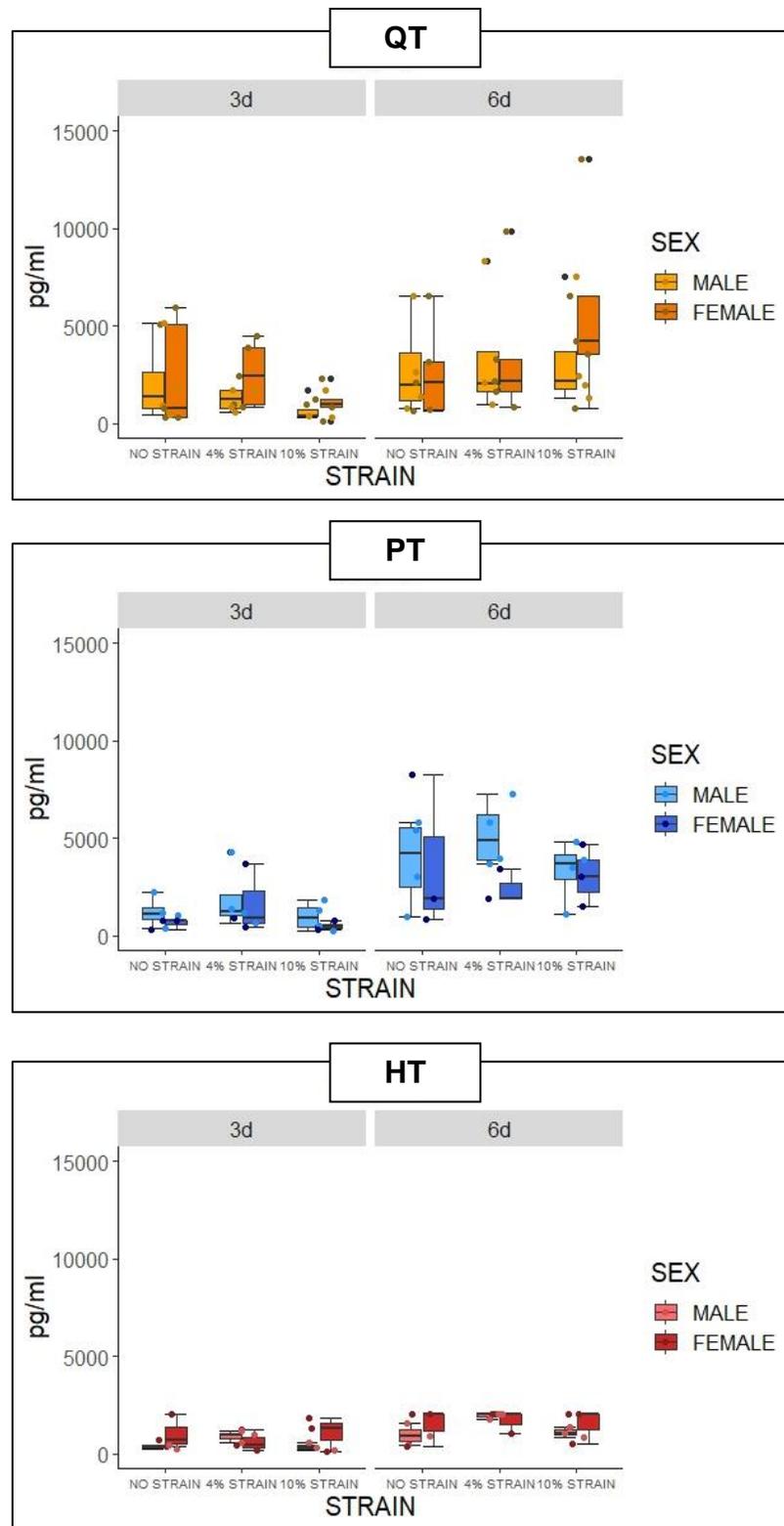


Figure 8.35. Concentration of MCP1 released to the media. * significant difference between males and females at 24 and 120 hours (Signed Rank test). QT: Quadriceps tendon; PT: Patellar tendon, HT: Hamstring tendon

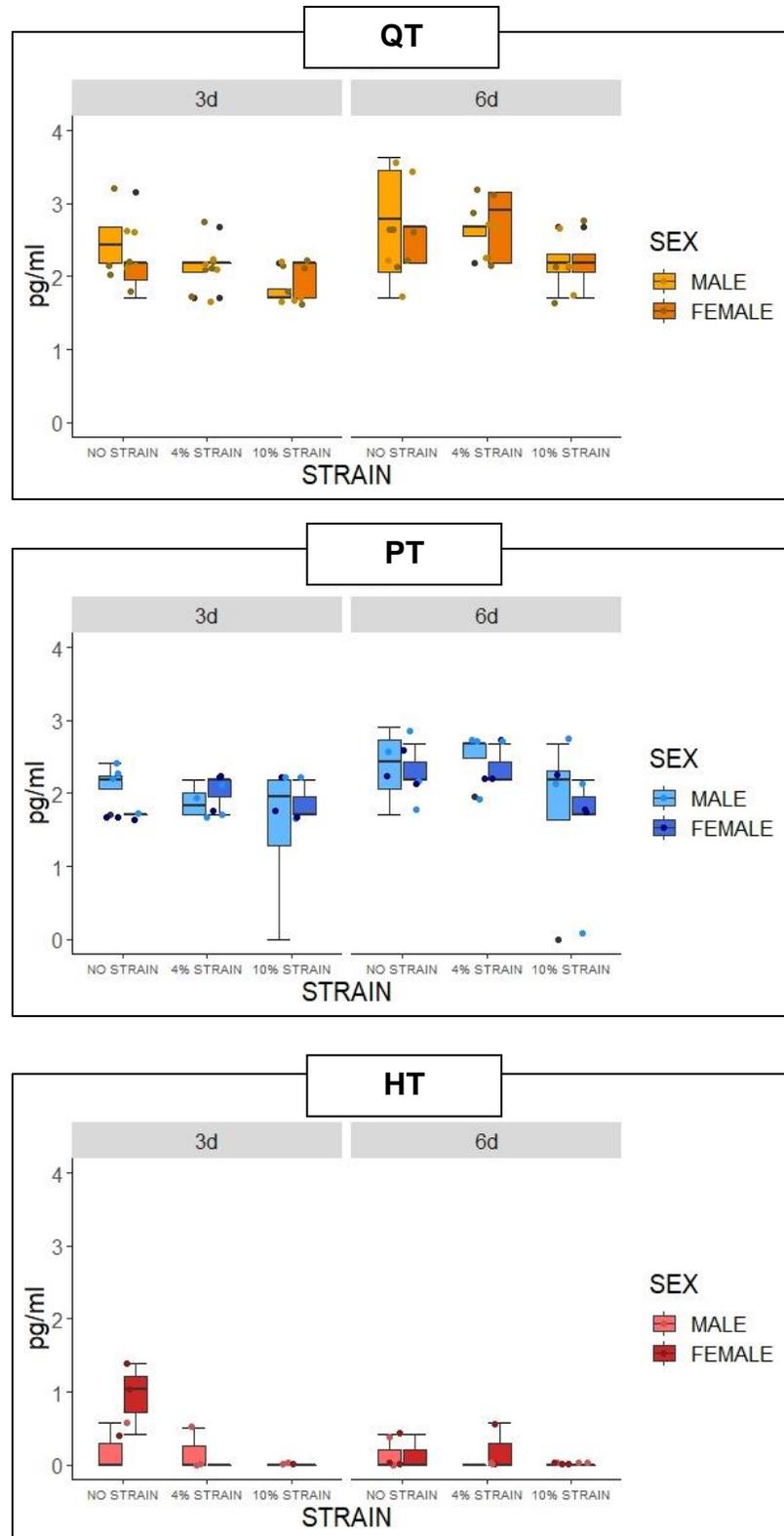


Figure 8.36. Concentration of MP1 β released to the media. * significant difference between males and females at 24 and 120 hours (Signed Rank test). QT: Quadriceps tendon; PT: Patellar tendon, HT: Hamstring tendon

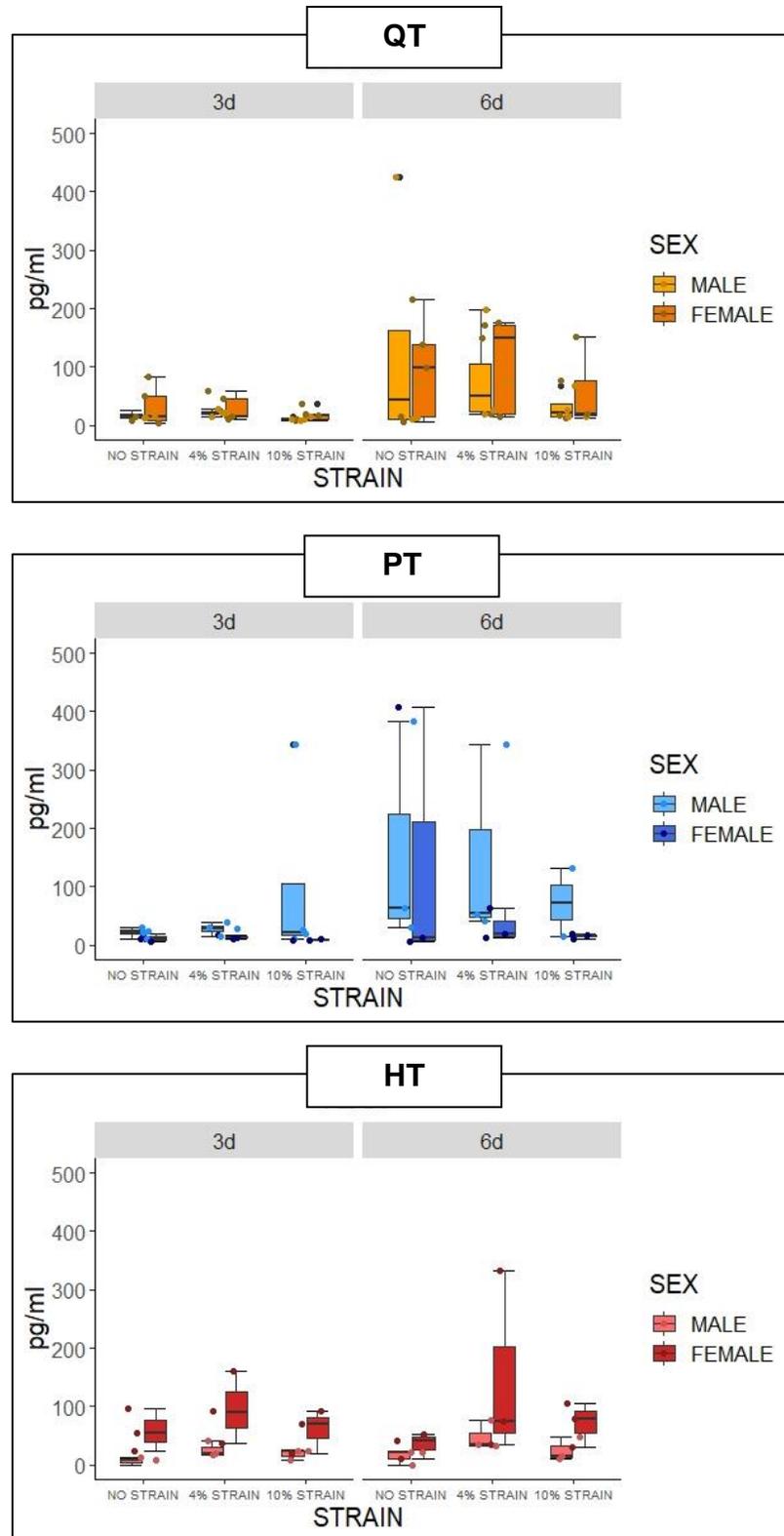


Figure 8.37. Concentration of VEGF released to the media. * significant difference between males and females at 24 and 120 hours (Signed Rank test). QT: Quadriceps tendon; PT: Patellar tendon, HT: Hamstring tendon

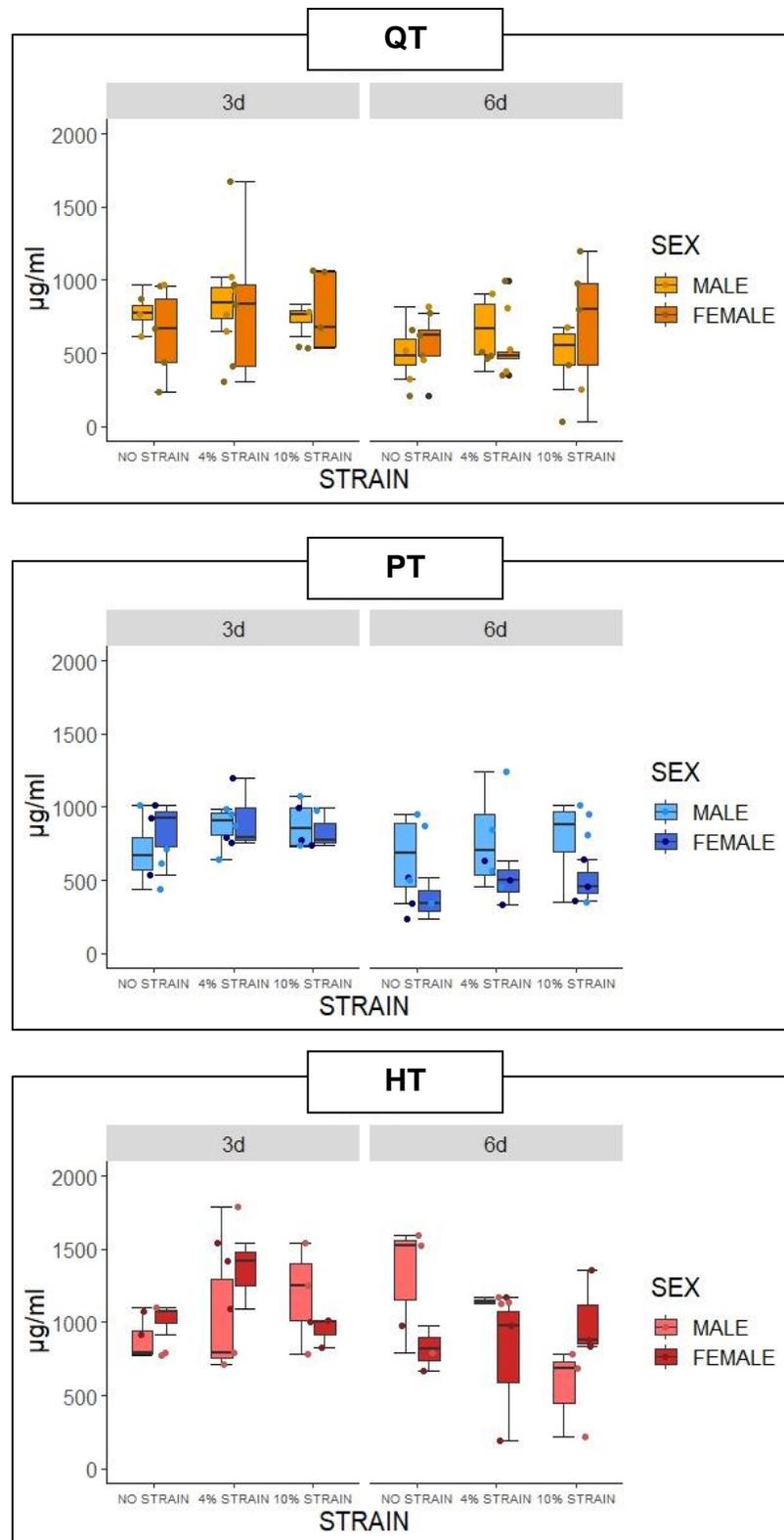


Figure 8.38. Concentration of GAG released to the media. * significant difference between males and females at 24 and 120 hours (Signed Rank test). QT: Quadriceps tendon; PT: Patellar tendon, HT: Hamstring tendon

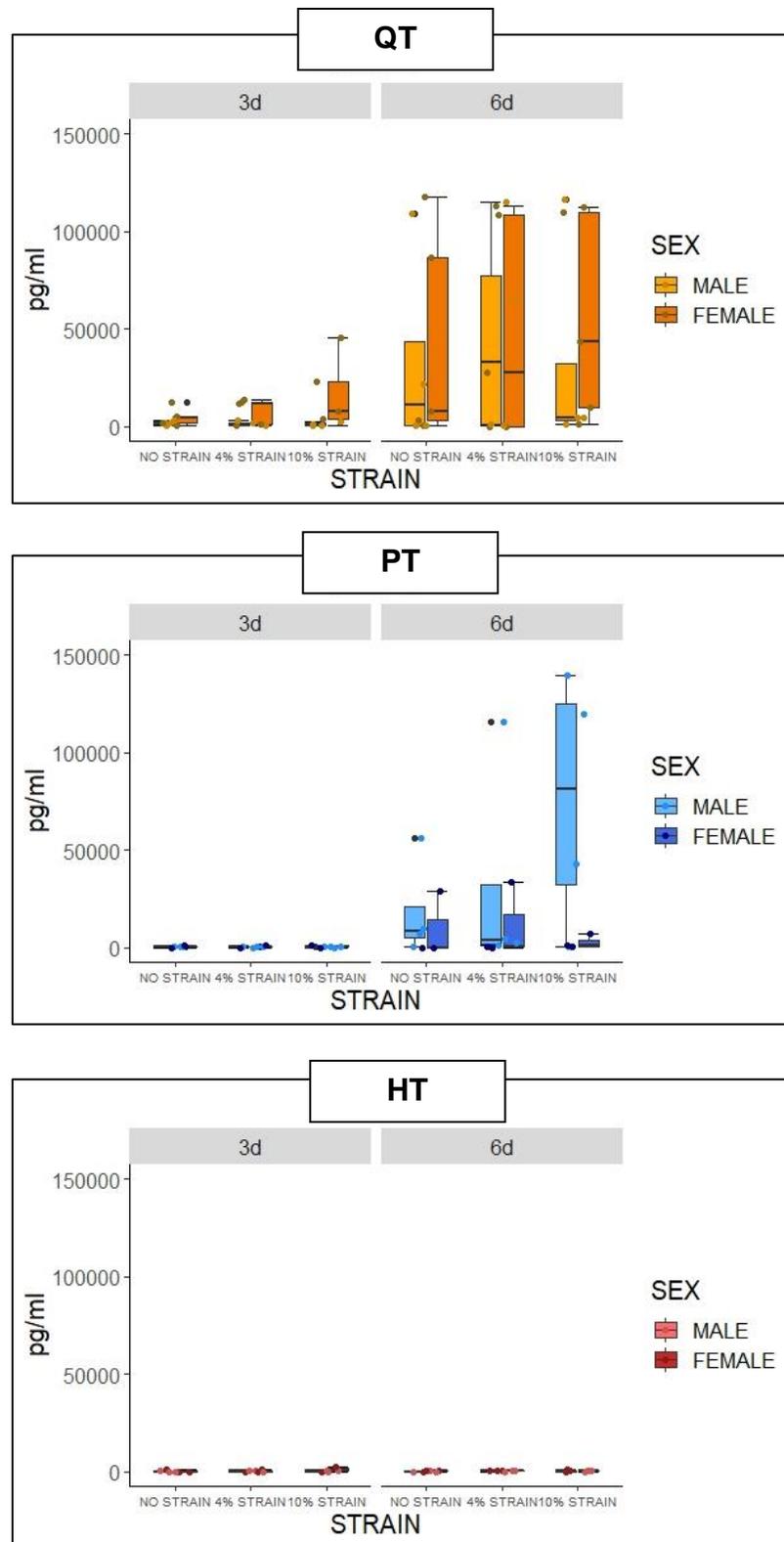


Figure 8.39. Concentration of MMP-1 released to the media. * significant difference males and females at 24 and 120 hours (Signed Rank test). QT: Quadriceps tendon; PT: Patellar tendon, HT: Hamstring tendon

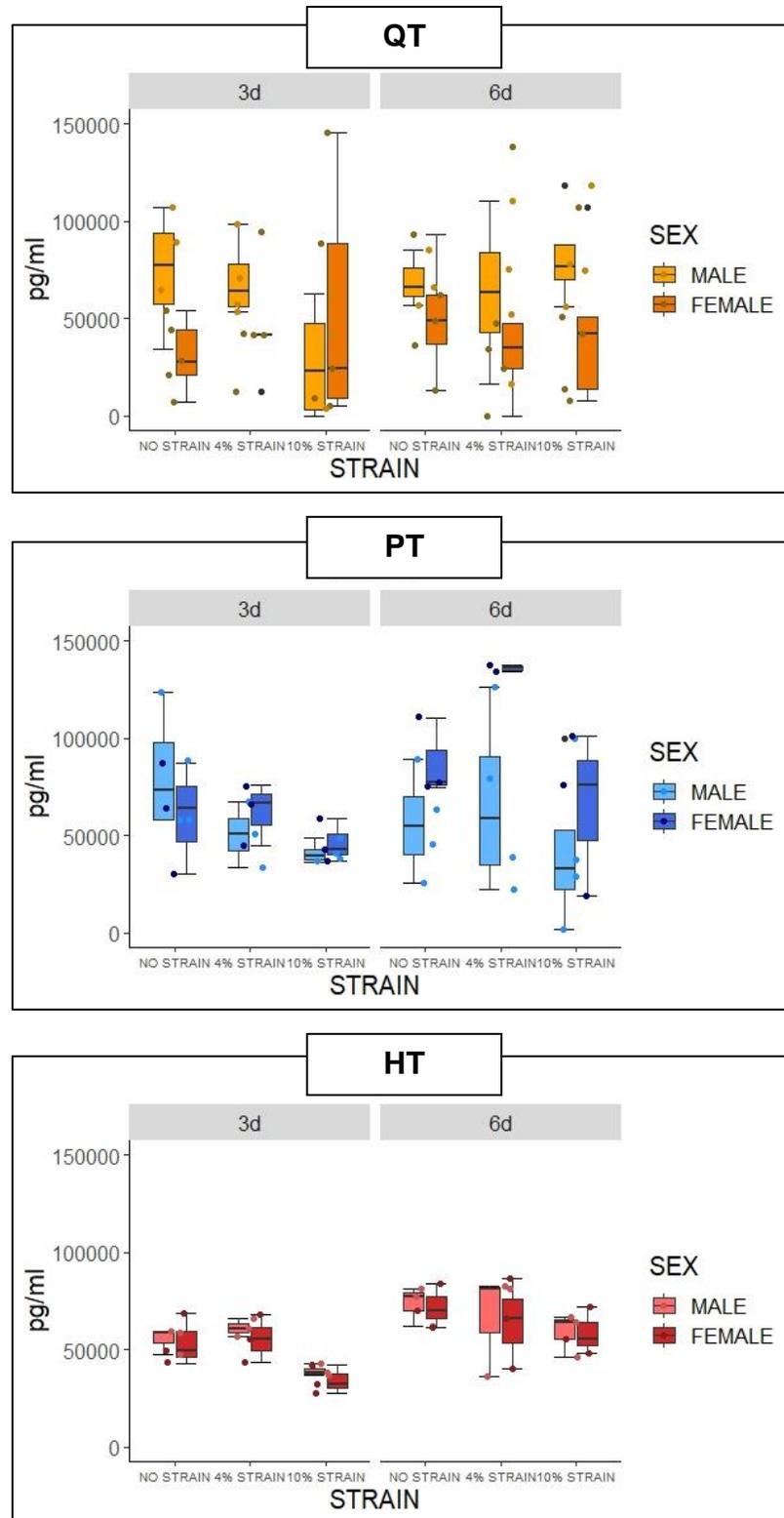


Figure 8.40. Concentration of MMP-2 released to the media. * significant difference between males and females at 24 and 120 hours (Signed Rank test). QT: Quadriceps tendon; PT: Patellar tendon, HT: Hamstring tendon

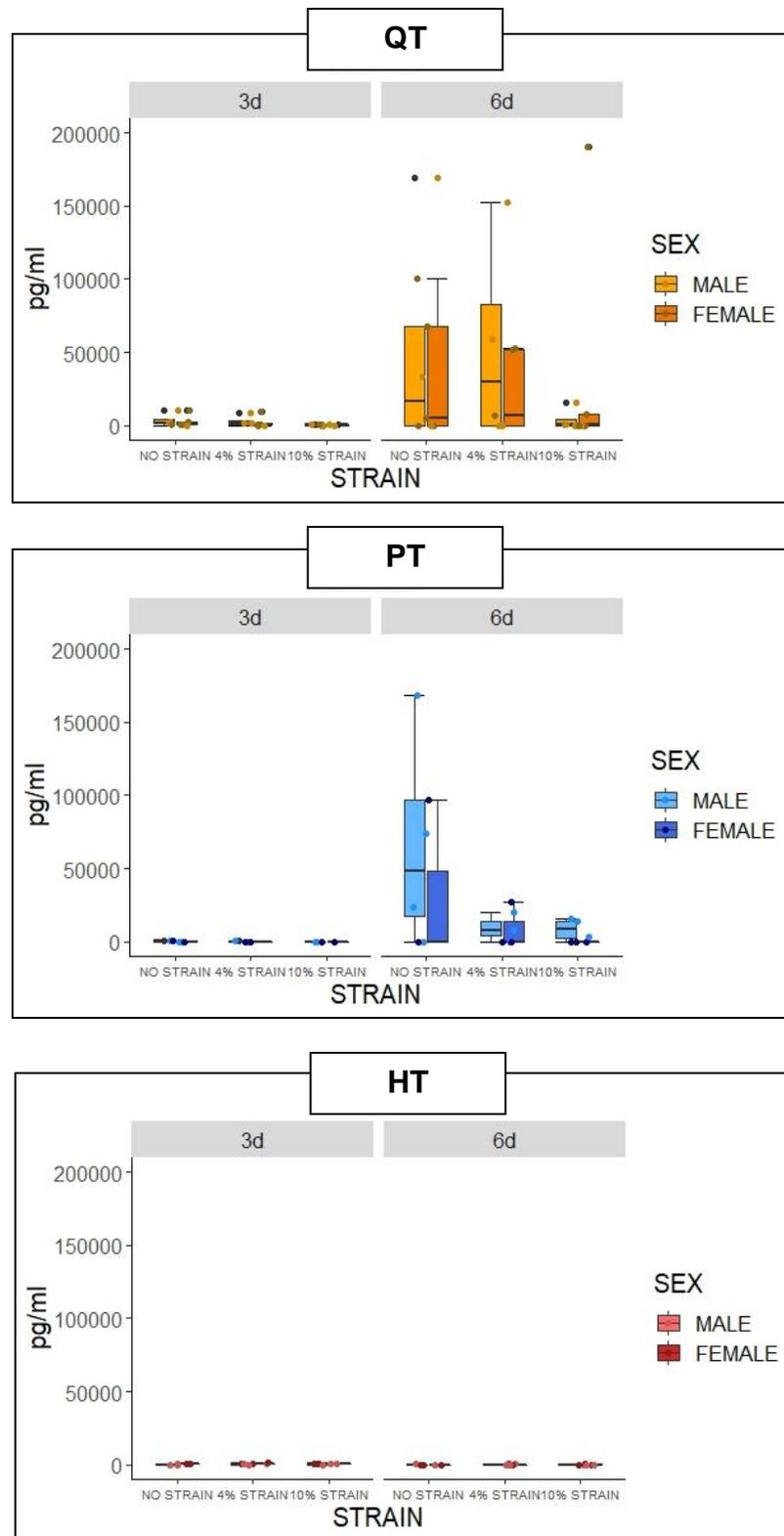


Figure 8.41. Concentration of MMP-3 released to the media. * significant difference males and females at 24 and 120 hours (Signed Rank test). QT: Quadriceps tendon; PT: Patellar tendon, HT: Hamstring tendon

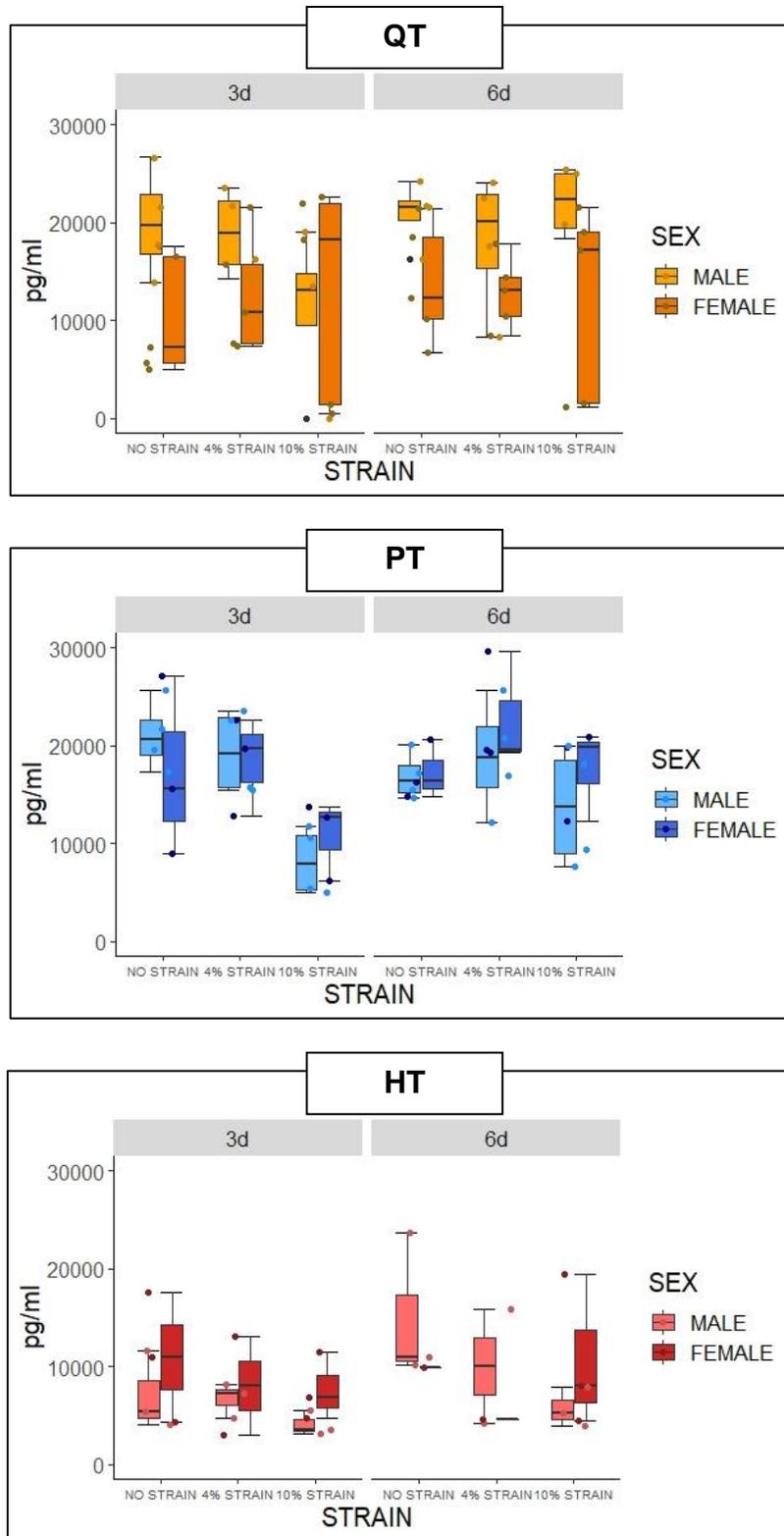


Figure 8.42. Concentration of TIMP-1 released to the media. * significant difference between males and females at 24 and 120 hours (Signed Rank test). QT: Quadriceps tendon; PT: Patellar tendon, HT: Hamstring tendon

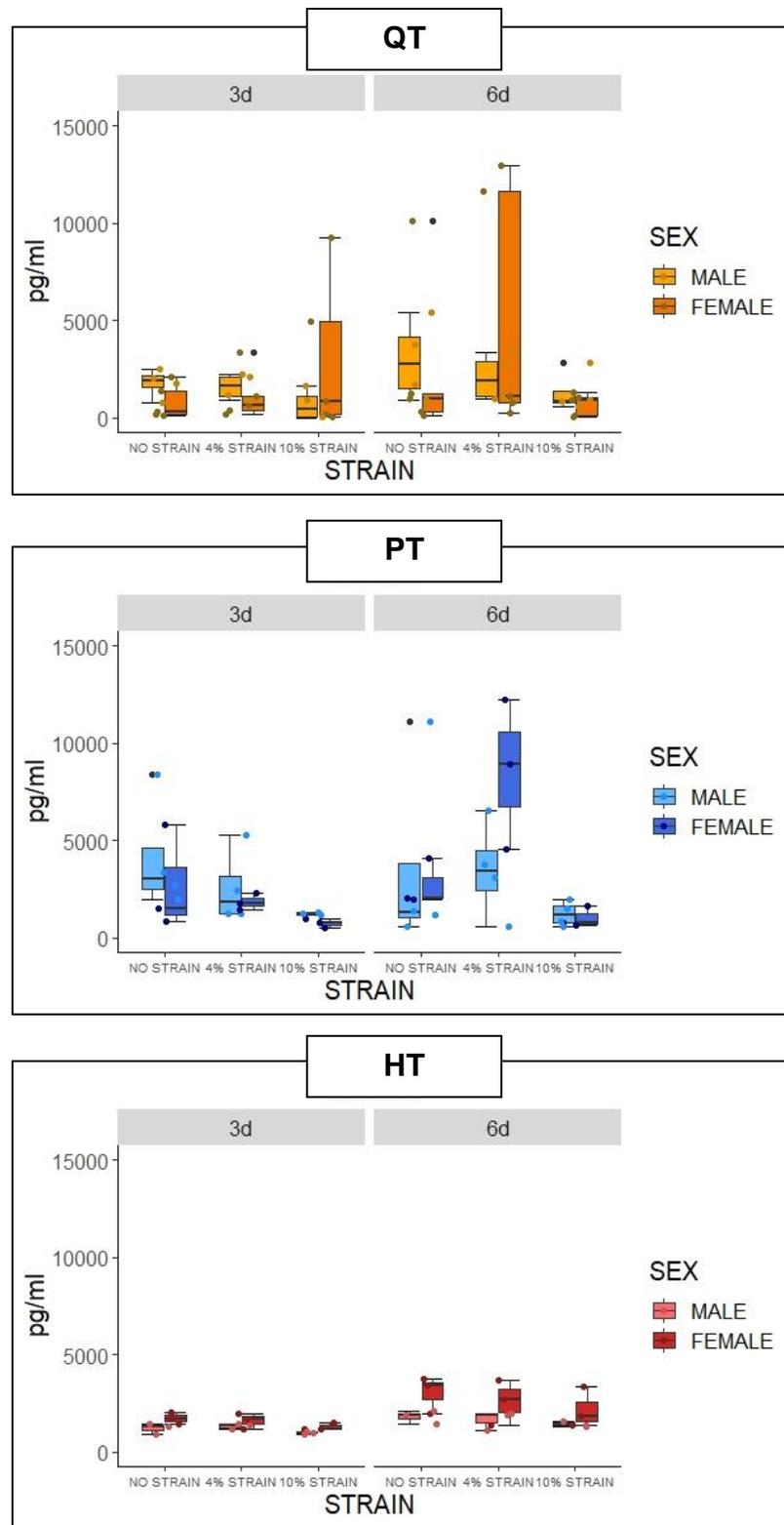


Figure 8.43. Concentration of TIMP-2 released to the media. * significant difference between males and females at 24 and 120 hours (Signed Rank test). QT: Quadriceps tendon; PT: Patellar tendon, HT: Hamstring tendon

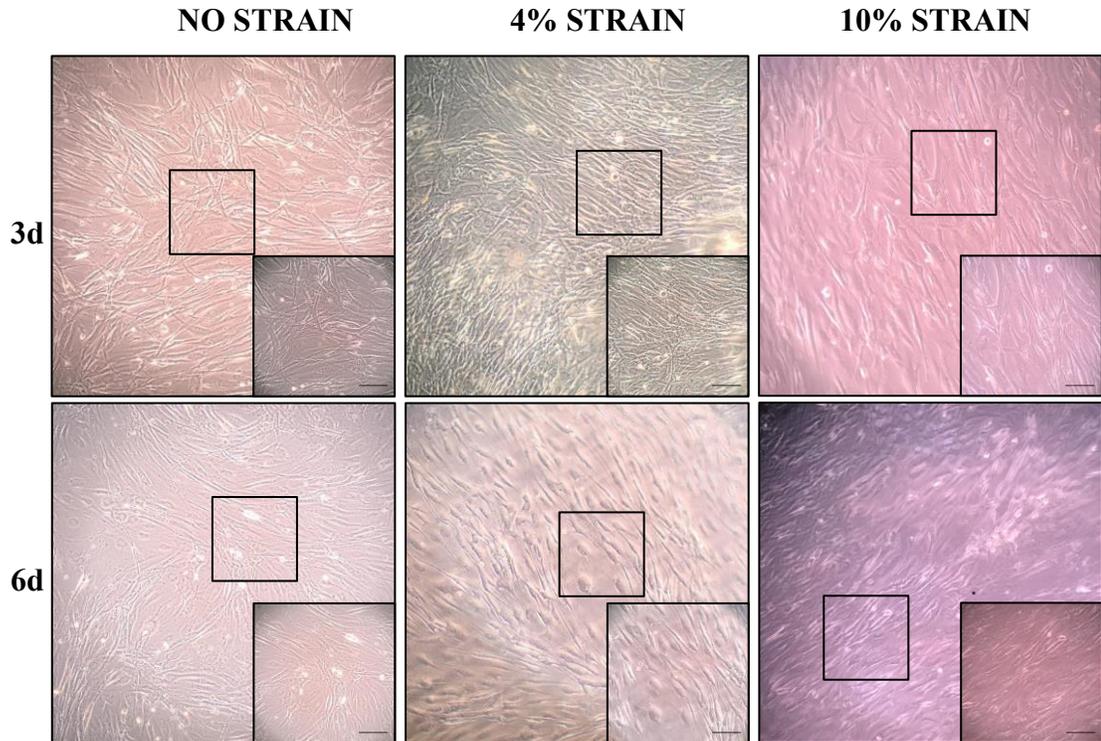


Figure 8.44. Quadriceps Tendon (QT) fibroblasts stretched after three days and six days at three different strain magnitudes. Scale Bar:50 μ m

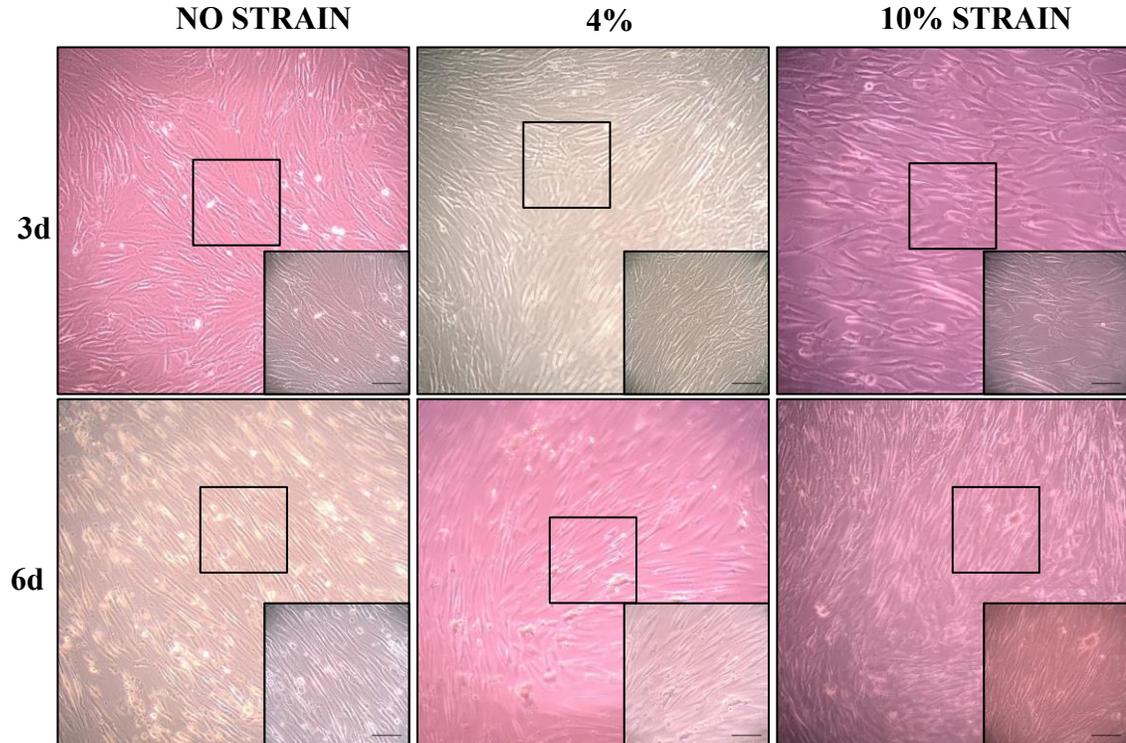


Figure 8.45. Patellar Tendon (PT) fibroblasts stretched after three days and six days at three different strain magnitudes. Scale Bar:50 μ m

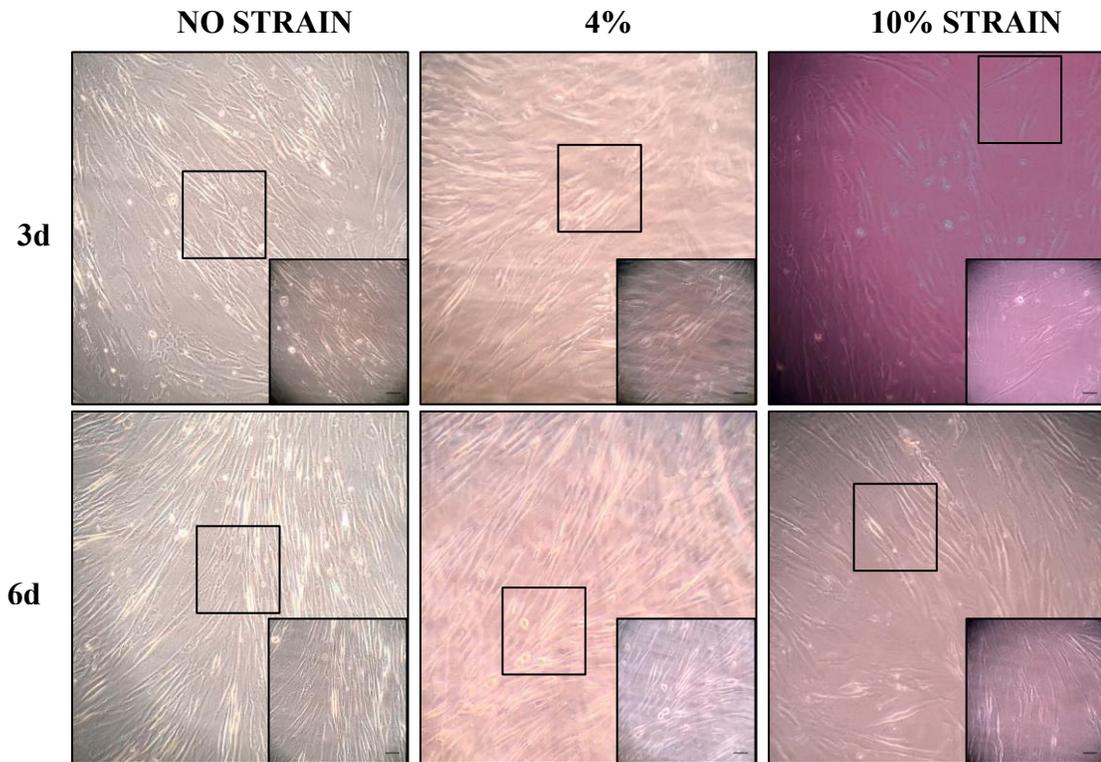


Figure 8.46. Hamstring Tendon (HT) fibroblasts stretched after three days and six days at three different strain magnitudes. Scale Bar:50 μ m

CONCLUSIONS AND FUTURE DIRECTIONS

This work uses a novel approach to understand cellular biology of the knee intraarticular tissues and tendon grafts. The use of a bioreactor to stress fibroblasts in monolayer, successfully stimulated cells to produce different metabolic biomarkers in response to varying levels of clinically relevant strains. Moreover, these responses are also tissue dependent. We have also demonstrated that fibroblasts obtained from different tissue sources are a prominent supply of metabolic markers in response to cyclic strain.

Important relations were noted in response to strain such as the influence of sex and culture conditions. Moreover, our study demonstrated similar patterns in both, dogs and humans, showing that the canine model is suitable for investigating metabolic responses in stressed monolayer tendon and ligament fibroblastic cultures. Subsequent studies will aim to evaluate specific variables such as bone-tendon and cartilage-tendon responses to strain. Further, research is needed to establish if metabolic responses *in vitro* are also found *in vivo*. Our lab is currently working on evaluating metabolic markers in synovial fluid and urine from patients undergoing ACL reconstruction. We are interested in evaluating how physical therapy and level of activity will affect ACL graft healing after surgery. Therefore, we recommend a thorough analysis to match the patient and the tendon graft based on sex and level of activity of the individual.

VITA

Dr. Sebastian Cardona-Ramirez was born in Manizales, Colombia. He attended Universidad de Caldas where he earned a Bachelor in Veterinary and Animal Sciences degree. He then continued to pursue postgraduate education in his alma mater and enrolled in a Master in Veterinary Sciences degree in 2013 being awarded *Cum Laude* thesis defense for his work on serum biomarkers of osteoarthritis in dogs with hip dysplasia. He was simultaneously working as a part-time professor in the Universidad del Tolima where he taught different courses including Small Animal Internal Medicine, Semiology and Diagnostic Imaging. He was also working as a veterinary clinician in private practice. In May 2016 he was awarded a Fulbright-COLCIENCIAS scholarship for funding Doctoral studies in the United States. He joined Dr. Cook's team in August 2016 in the Thompson Laboratory for Regenerative Orthopaedics (TLRO) as a full-time graduate student. He has been awarded several research and travel awards including Phi Zeta Grant, John D. Bies travel award and the TLRO Graduate Excellence in Research Award in 2019. He will pursue a postdoctoral fellowship in the TLRO to continue his investigation on *In Vitro* models for Orthopaedic Research.